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## Article

# Cystathionine Gamma Lyase Is Regulated by Flow and Controls Smooth Muscle Migration in Human Saphenous Vein

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**Abstract:** The saphenous vein is the conduit of choice for bypass grafting. Unfortunately, the hemodynamic stress associated with the arterial environment of the bypass vein graft leads to the development of intimal hyperplasia (IH), an excessive cellular growth and collagen deposition that results in restenosis and secondary graft occlusion. Hydrogen sulfide (H<sub>2</sub>S) is a ubiquitous redox-modifying gasotransmitter that inhibits IH. H<sub>2</sub>S is produced via the reverse trans-sulfuration pathway by 3 enzymes: cystathionine  $\gamma$ -lyase (CSE), cystathionine  $\beta$ -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST). However, the expression and regulation of these enzymes in the human vasculature remains unclear. Here, we investigated the expression of CSE, CBS and 3-MST in segments of native human saphenous vein and large arteries. Furthermore, we evaluated the regulation of these enzymes in vein segments cultured under static, venous (7 mmHg pressure) or arterial (100 mmHg pressure) pressure. CSE was expressed in the media, neointima and intima of the vessels and was negatively regulated by arterial shear stress. Adenoviral-mediated CSE overexpression or RNA interference-mediated CSE knock-down revealed that CSE inhibited primary human VSMC migration but not proliferation. We propose that high shear stress in arteriovenous bypass grafts inhibits CSE expression in both the media and endothelium, which may contribute to increased VSMC migration in the context of IH.

**Keywords:** hydrogen sulfide; cystathionine- $\gamma$ -lyase; CSE; H<sub>2</sub>S; intimal hyperplasia; venous bypass

## 1. Introduction

Arterio-venous bypass surgery is one of the main approaches for revascularization of chronic limb-threatening ischemia (CLTI) patients. However, veins are not designed to support arterial pressure and undergo significant vascular remodeling to adapt to the arterial environment. This remodeling is accompanied by the development of intimal hyperplasia (IH), i.e., the formation of a collagen-rich neointima layer between the media and the innermost layer (intima/endothelium) of the vein. IH is due to a cascade of cellular events leading to the differentiation, proliferation, and migration of vascular smooth muscle cells (VSMC) from the vessel wall into the intima [1]. This excessive cell growth and collagen deposition eventually lead to reduced blood flow (restenosis) or occlusion of the bypass. 30 to 50% of the saphenous grafts fail 1–18 months after the implantation [2].

Hydrogen sulfide (H<sub>2</sub>S) contributes to the homeostasis of a wide range of systems, including the cardiovascular systems [3]. Notably, endogenous H<sub>2</sub>S bioavailability is attenuated in patients with CLTI and in patients with diabetes-related vascular inflammation [4]. Circulating H<sub>2</sub>S is also reduced

in humans suffering from vascular occlusive disease [5, 6], and patients undergoing surgical revascularization with lower H<sub>2</sub>S production capacity have higher postoperative mortality rates [7].

H<sub>2</sub>S is produced in mammalian cells through the reverse transsulfuration pathway by two pyridoxal 5'-phosphate dependent enzymes, cystathionine  $\gamma$ -lyase (CSE) and cystathionine  $\beta$ -synthase (CBS), and by a combination of two additional enzymes, 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (CAT). Mice lacking Cse display increased IH in a model of carotid artery ligation [8, 9]. On the contrary, Cse overexpression decreases IH formation in a murine model of vein graft by carotid-interposition cuff technique [10]. In addition, we and others demonstrated that systemic treatment using diverse H<sub>2</sub>S donors inhibit IH *in vivo* in various models in rats [11], rabbits [12] and mice [8, 9, 13]. We also showed that several H<sub>2</sub>S donors inhibit IH *ex vivo* in human vein segments [9, 13, 14]. The study of Cse<sup>-/-</sup> mice supports that CSE expression in endothelial cells (EC) is the main source of endogenous H<sub>2</sub>S production in vessels [15-18]. However, CSE expression has also been found in VSMC, and may contribute to VSMC proliferation and migration, vascular remodeling and IH [8, 19]. CBS is also found in the cardiovascular system, but its role and distribution in vessels is unclear [3]. Other reports suggest a key role of 3-MST in H<sub>2</sub>S production in the vascular endothelium [20]. It was recently demonstrated that Cse expression is negatively regulated by shear stress *in vitro* [21]. This is in line with a previous study showing that only disturbed flow regions show discernible CSE protein expression after carotid artery ligation in the mouse [22]. However, the expression of CSE in human vessels remains poorly characterized. In this study, we studied the expression and regulation of CSE, CBS and 3MST in segments from healthy human saphenous vein and artery. We observed that CSE is expressed both in the endothelium and media of large vessels, while CBS expression is detectable only in the media, and 3-MST expression is mainly restricted to the endothelium of small vessels. Our data confirms that CSE expression in vein is negatively regulated by shear stress and, as a result, upregulated in absence of flow and downregulated in vein segments placed under arterial perfusion. We further confirm that CSE is involved in primary human VSMC migration, but not proliferation.

## 2. Materials and Methods

For details on materials and reagents please see the Supplementary Table S1 and S2.

### 2.1. Human vessels culture

Healthy human artery segments were obtained from patients undergoing vascular reconstruction as part of our biobank. 9 artery segments were used in this study. Healthy human saphenous vein segments were surplus segments of non-varicose veins from donors who underwent lower limb bypass surgery. Static vein culture was performed as described [13, 14, 23]. Briefly, segments of great saphenous vein were cut in 5 mm segments randomly distributed between conditions. One segment (D0) was immediately preserved in formalin or flash frozen in liquid nitrogen and the others were maintained in culture for 7 days in RPMI-1640 Glutamax I supplemented with 10 % FBS and 1% antibiotic solution (10,000 U/mL penicillin G, 10,000 U/mL streptomycin sulphate) in cell culture incubator at 37°C, 5% CO<sub>2</sub> and 21% O<sub>2</sub>.

Pulsatile vein culture using an *ex vivo* vein perfusion system was performed as previously described [24-27]. Upon harvest, veins were stored at 4 °C in a RPMI-1640 Glutamax medium, supplemented with 12.5% fetal calf serum (Gibco). Within 1 h after the surgery, the segment with an external diameter of 2.5–4 mm were divided in two equal parts. One part was fixed in either formalin for immunohistochemistry or rapidly frozen in liquid nitrogen for molecular analyses. A second part was perfused in the EVPS for 7 days to a pulsatile biphasic flow of 60 pulses/min under either low (LP = 7 mmHg; systolic/diastolic pressure = 8  $\pm$  1/6  $\pm$  1 mmHg) or high perfusion pressure (HP = 100 mmHg; systolic/diastolic pressure = 120  $\pm$  5/90  $\pm$  5 mmHg). Upon completion of the perfusion, the 5 mm proximal and distal ends, which attached the vein to the equipment, were discarded. A central, 5 mm-thick ring was cut from the remaining segment and fixed in formalin for morphometry. The remaining fragments were frozen and reduced into powder for RT-PCR and Western blot analysis. The veins segments in the EVPS were maintained in RPMI-

1640, supplemented with Glutamax, 12.5% fetal calf serum (Gibco), 8% 70 kDa dextran (Sigma), and 1% antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin sulphate, 25 mg/ml amphotericin B, and 0.5 µg/ml gentamycin). This medium was changed every 2 days. In this study, eight veins obtained from randomly selected patients who underwent lower limb bypass surgery for critical ischemia were used.

## 2.2. Cell culture

Human VSMCs were prepared from human saphenous vein segments as previously described [28, 29]. Vein explants were plated on the dry surface of a cell culture plate coated with 1% Gelatine type B (Sigma-Aldrich). Explants were maintained in RPMI, 10% FBS medium in a cell culture incubator at 37°C, 5% CO<sub>2</sub>, 5% O<sub>2</sub> environment. 9 different veins/patients were used in this study to generate VSMC.

## 2.3. siRNA-mediated knock-down and adenoviral-mediated overexpression

CSE knockdown was performed using human siRNA targeting CTH (Ambion-Life Technologies, ID: s3710 and s3712). The control siRNA (siCtrl) was the AllStars Negative Control siRNA (Qiagen, SI03650318). VSMC grown at 70% confluence were transfected overnight with 30 nM siRNA using lipofectamin RNAiMax (Invitrogen, 13778-075). After washing, cells were maintained in full media for 48h prior to assessment.

CSE overexpression was achieved using a replication-deficient recombinant adenoviral (DE1/E3) vector. Adenoviral infection was achieved overnight in complete medium using AdCTH (kindly provided by James R. Mitchell [30], produced and purified by Vector Biolabs, Philadelphia, PA, U.S.A.), or the negative control virus Ad-eGFP (Vector Biolabs, Cat. No: 1060). After washing, cells were maintained in full media for 48h prior to assessment.

## 2.3. Histology

After 7 days in culture, or immediately upon vein isolation (D0), human vessel segments were fixed in buffered formalin, embedded in paraffin, cut into 5 µm sections, and stained with VGEL as previously described [14]. Slides were scanned using a ZEISS Axioscan 7 Microscope Slide Scanner. Polychrome Herovici staining was performed on paraffin sections as described [31]. Young collagen was stained blue, while mature collagen was pink. Cytoplasm was counterstained yellow. Hematoxylin was used to counterstain nuclei blue to black. For intimal and medial thickness, 96 (4 measurements/photos and 4 photos per cross section on six cross sections) measurements were performed [26, 27]. Two independent researchers blinded to the conditions did the morphometric measurements using the *Olympus Stream Start 2.3* software (Olympus, Switzerland) [13, 14, 23].

CSE, CBS and 3-MST immunohistochemistry were performed on paraffin sections [32]. After rehydration and antigen retrieval (TRIS-EDTA buffer, pH 9, 1 min in an electric pressure cooker autocuise Instant Pot duo 60 under high pressure), immunostaining was performed on human vein or artery sections using the EnVision® Dual Link System-HRP (DAB+) according to manufacturer's instructions. Slides were further counterstained with hematoxylin. The positive immunostaining area was quantified using the *Fiji (ImageJ 1.53t)* software and normalized to the total area of the tissue by two independent observers blinded to the conditions.

## 2.4. Western blotting

Vessels were flash-frozen in liquid nitrogen, grinded to power and resuspended in SDS lysis buffer (62.5 mM TRIS pH6.8, 5% SDS, 10 mM EDTA). Protein concentration was determined by DC protein assay (Bio-Rad Laboratories, Reinach, Switzerland). 10 to 20 µg of protein were loaded per well. Primary cells were washed once with ice-cold PBS and directly lysed with Laemmli buffer as previously described [14, 28]. Lysates were resolved by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore AG, Switzerland). Immunoblot analyses were performed as previously described [28] using a CSE antibodies described in **Supplementary Table S1**. Membranes



were revealed by enhanced chemiluminescence (Immobilon, Millipore) using the Azure 280 device (Azure Biosystems) and analyzed using the Fiji (*ImageJ* 1.53t) software. Protein abundance was normalized to total protein using Pierce™ Reversible Protein Stain Kit for PVDF Membranes (cat 24585; Thermo Fisher Scientific).

#### 2.5. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Flash frozen vessels powder was homogenized in Tripure Isolation Reagent (Roche, Switzerland), and total RNA was extracted according to the manufacturer's instructions. After RNA Reverse transcription (Prime Script RT reagent, Takara), cDNA levels were measured by qPCR Fast SYBR™ Green Master Mix (Ref: 4385618, Applied Biosystems, ThermoFischer Scientific AG, Switzerland) in a Quant Studio 5 Real-Time PCR System (Applied Biosystems, ThermoFischer Scientific AG, Switzerland), using the following primers: MPST Fw: 5'-CCGAGACGGCATTGAACCT-3'; MPST Rv: 5'-CCTGGCTCAGGAAGTCTGTG-3'; CTH Fw: 5'-CCAGCACTCGGGTTTTGAAT-3'; CTH Rv: 5'-TACTTAGCCCCATCCAGTGC-3'; CBS Fw: 5'-GCAGATCCAGTACCACAGCA-3'; CBS Rv: 5'-GCCACGAAGTTCAGCAAGTC-3'.

#### 2.6. Lead acetate (CSE activity assay)

Flash frozen vessels powder was homogenized in passive lysis buffer (Promega) and protein content was determined using a BCA protein assay (ThermoFischer). Using a 96 well plate, 300mg of proteins were diluted into 100µl of PBS supplemented with 10mM Cysteine and 1mM pyridoxal phosphate as substrate and cofactor for CSE. The plate was then covered with Whatman paper impregnated with 20mM lead acetate and incubated at 37°C for 5h. Lead sulfide precipitate on the Whatman paper were scanned using a high-resolution scanner (HP) and quantified using Fiji (*ImageJ* 1.53t) software.

#### 2.7. H<sub>2</sub>S and persulfidation measurement

Free H<sub>2</sub>S was measured in cells using the SF<sub>7</sub>-AM fluorescent probe [33] (Sigma-Aldrich). The probe was dissolved in anhydrous DMF at 5 mM and used at 5 µM in serum-free RPMI. Live-cell image acquisition was performed using a Nikon Ti2 spinning disk confocal microscope. Global protein persulfidation was assessed on VSMC grown on glass coverslips as previously described [9]. Cells were incubated for 20 minutes with 1mM 4-Chloro-7-nitrobenzofurazan (NBF-Cl, Sigma-Aldrich) diluted in PBS. Then, cells were washed with PBS and fixed for 10 minutes in ice-cold methanol. Coverslips were rehydrated in PBS and incubated with 1mM NBF-Cl for 1h at 37°C. Cells were further incubated at 37°C for 1h in Daz2-Cy5.5 solution. Finally, coverslips were washed 3 times in methanol and 2 times in PBS, mounted in Vectashield mounting medium with DAPI, and visualized with a 90i Nikon fluorescence microscope. Persulfidation was measured as the ratio of Daz2-Cy5.5 over NBF-Cl signal per cell by two independent experimenter blinded to the conditions using the Fiji (*ImageJ* 1.53t) software.

#### 2.8. BrdU assay

VSMCs were grown at 80% confluence (5·10<sup>3</sup> cells per well) on glass coverslips in a 24-well plate and starved overnight in serum-free medium. Then, VSMC were either treated or not (ctrl) with the drug of choice for 24 hours in full medium (RPMI 10% FBS) in presence of 10µM BrdU. All conditions were tested in parallel. All cells were fixed in ice-cold methanol 100% after 24 hours of incubation and immunostained for BrdU. Images were acquired using a Nikon Eclipse 90i microscope. BrdU-positive nuclei and total DAPI-positive nuclei were automatically detected using the Fiji (*ImageJ* 1.53t) software [29].

#### 2.9. Wound healing assay

VSMCs were grown at confluence (10<sup>4</sup> cells per well) in a 12-well plate and starved overnight in serum-free medium. Then, a scratch wound was created using a sterile p200 pipette tip and medium

was changed to full medium (RPMI 10% FBS) in presence of 0.5µg/mL mitomycin C to block proliferation. Repopulation of the wounded areas was recorded by phase-contrast microscopy in a Nikon Ti2-E live cell microscope. All conditions were tested in parallel. The area of the denuded area was measured automatically using the macro Wound\_healing\_size\_tool\_updated.ijm [34] in the Fiji (*ImageJ 1.53t*) software. Data were expressed as a percentage of the wound closure. Morphometric measurement of cell geometry was performed manually by two independent experimenters blinded to the conditions using the shape descriptors in measurements in Fiji (*ImageJ 1.53t*) software

#### 2.10. Statistical analyses

All experiments adhered to the ARRIVE guidelines and followed strict randomization. All experiments and data analysis were conducted in a blind manner using coded tags rather than actual group name. All experiments were analyzed using GraphPad Prism 9. Normal distribution of the data was assessed using Kolmogorov-Smirnov tests. All data with normal distribution were analyzed by unpaired bilateral Student's t-tests or Mixed-effects model (REML) followed by post-hoc t-tests with the appropriate correction for multiple comparisons when comparing more than 2 groups. For non-normal distributed data, Kruskal-Wallis non-parametric ranking tests were performed, followed by Dunn's multiple comparisons test to calculate adjusted p values. Unless otherwise specified, p-values are reported according to the APA 7<sup>th</sup> edition and New England Journal of Medicine statistical guidelines. \*p<.033, \*\*p<.002, \*\*\*p<0.001.

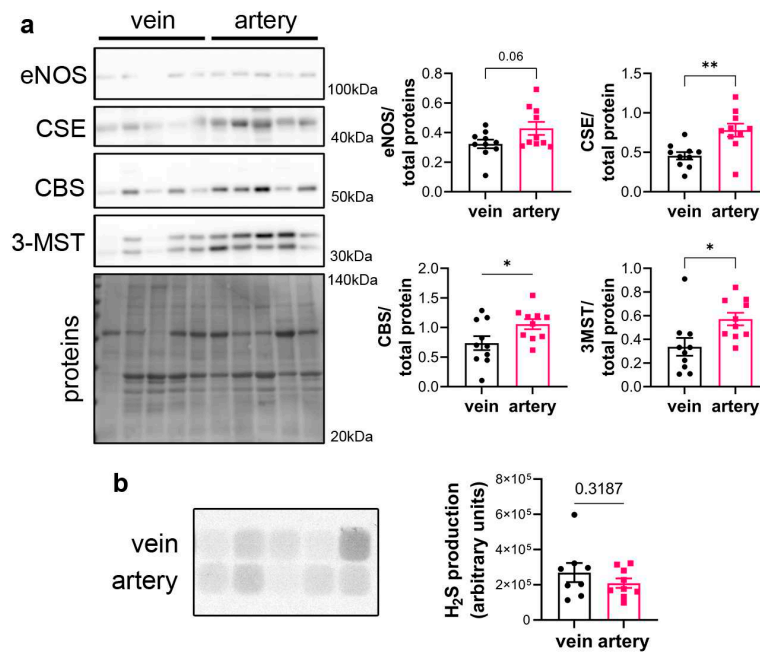
#### 2.11. Ethics Statement

Human vein and artery segments were obtained from donors who underwent vascular surgery at the Lausanne University Hospital. Written, informed consent was obtained from all donors. The study protocols for organ collection and use were reviewed and approved by the Lausanne University Hospital (CHUV) and the Cantonal Human Research Ethics Committee (<http://www.cer-vd.ch/>, no IRB number, Protocol Number 170/02), and are in accordance with the principles outlined in the Declaration of Helsinki of 1975, as revised in 1983 for the use of human tissues.

### 3. Results

#### 3.1. CSE is expressed in the media and intima of human saphenous vein and artery segments

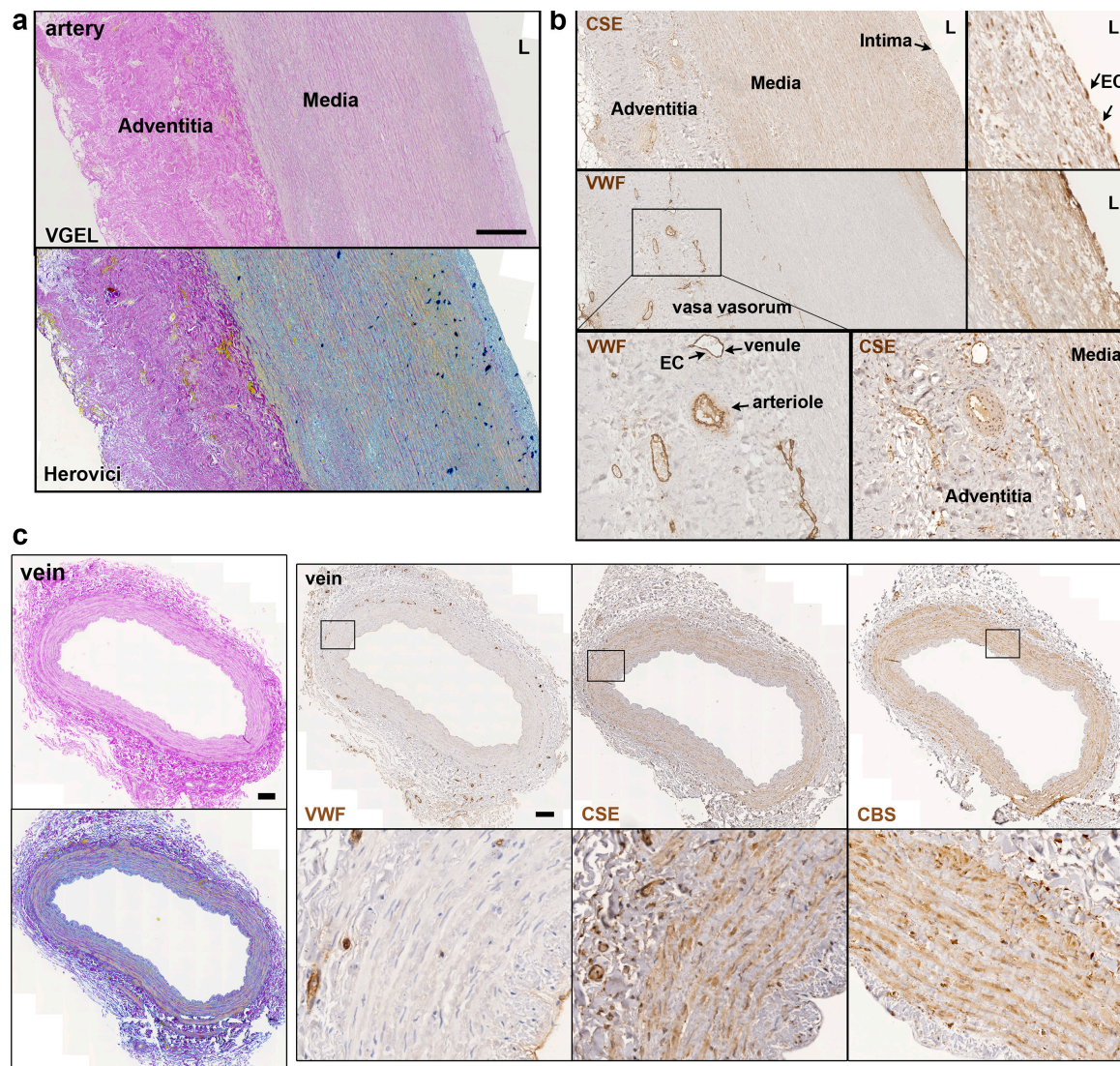
CSE protein was assessed in segments of healthy human vein and artery. Artery segments expressed higher levels of CSE, eNOS, CBS and 3MST than vein segments as measured by WB analysis (**Figure 1a**). However, this did not translate in higher H<sub>2</sub>S production capacity as assessed by led acetate assay (**Figure 1b**).



**Figure 1. CSE is expressed in human artery and saphenous vein segments.** a) Representative Western blot and quantitative assessment of eNOS, CSE, CBS and 3-MST from freshly isolated human arteries and saphenous veins. Data are mean±SEM of 10 arteries and 9 veins. b) Lead acetate assay in freshly isolated human arteries and saphenous veins. Data are mean±SEM of 9 arteries and 8 veins. \* $p < .033$ , \*\* $p < .002$ , as determined by bilateral paired t-test.

Further CSE immunohistology show that CSE was expressed both in the media and intima layers of the artery segments (**Figure 2a, b**). CSE was also expressed in the endothelium of the small vessels of the vasa vasorum of arteries, easily identified using a Von Willebrand factor (VWF) immunostaining of EC (**Figure 2a, b**). In human veins, CSE was mostly detectable in the media layer, but not so much in the endothelium (**Figure 2c**). CSE was also expressed in the endothelium of the small vessels of the vasa vasorum (**Figure 2c**). In veins, CBS was expressed mostly in the media layer (**Figure 2c**). In artery segments, 3-MST was detectable in the media, but was mostly expressed by the EC of the intima and smaller vessels of the vasa vasorum. Interestingly, some arteries featured benign intimal hyperplasia and 3-MST was not expressed in this neointima layer (**Figure S1**). In contrast, CSE was similarly expressed throughout the media and neointima layers. In the saphenous vein, 3MST was not expressed in the media layer, mainly detected in the vasa vasorum vessels of the media and adventitia layers, and seldom detected in the EC from the intima (**Figure S1**).



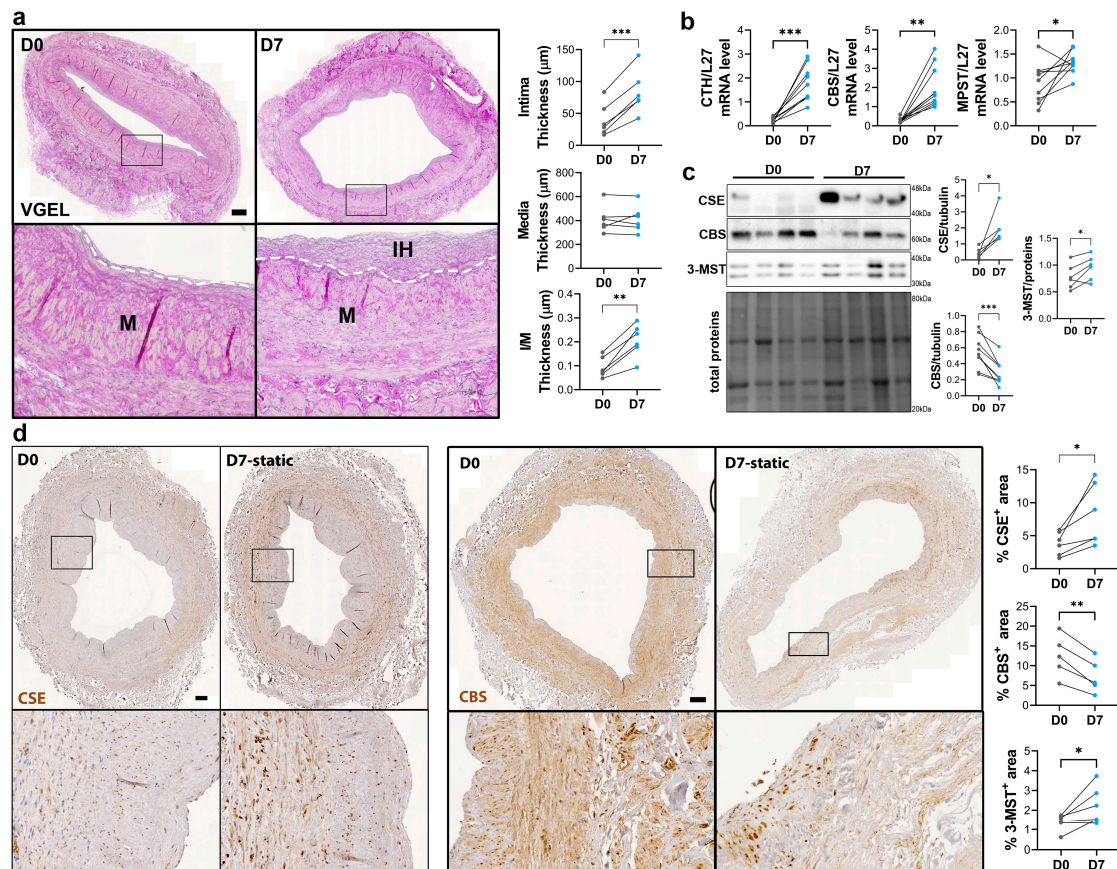


**Figure 2. CSE is expressed in the media and intima of human artery and saphenous vein.** Representative, VGEL, Herovici, CSE, CBS and VWF staining as indicated in section of a human artery (a, b) and a saphenous vein (c). Images are representative of 8 arteries and 10 saphenous veins. Scale bar=100 $\mu$ m.

### 3.2. CSE expression is regulated by flow

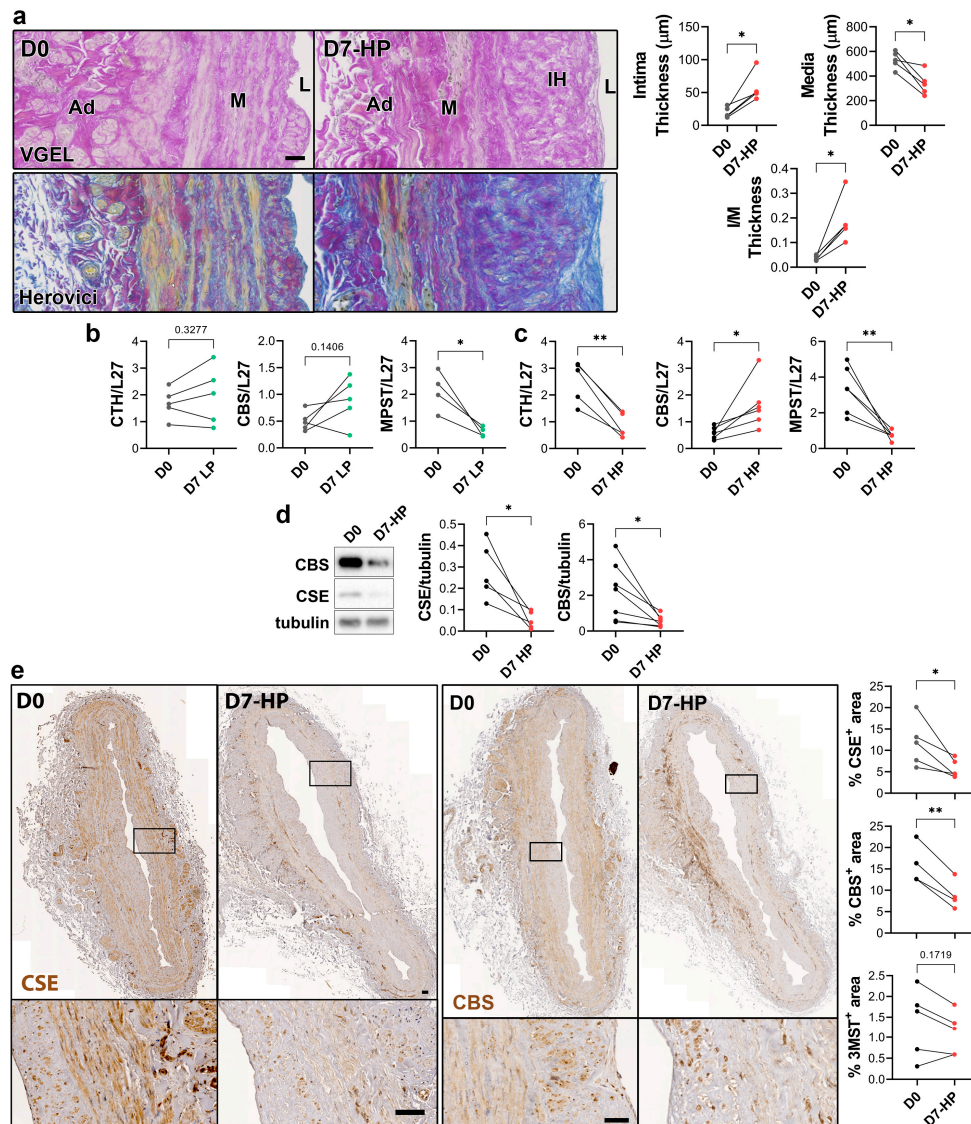
To study the regulation of CSE expression in response to flow, we next investigated CSE expression in a model of static human vein culture. Static vein culture leads to formation of IH as previously described [9, 13, 14], as assessed by VGEL staining (Figure 3a). qPCR analysis revealed that CSE mRNA expression was increased 4-fold after 7 days in static culture (Figure 3b). CBS mRNA expression was also increased about 4-fold, while MPST mRNA expression increased about 2-fold (Figure 3b). WB analysis confirmed CSE 4-fold protein overexpression in static culture, whereas CBS protein levels were decreased by about 30%, and 3-MST was increased about 1.5-fold (Figure 3c). The increase in CSE expression was accompanied by an increase in H<sub>2</sub>S production, as measured by lead acetate (Figure S2). IHC analyses confirmed that CSE expression was increased in the media layer of human veins after static culture (Figure 3d). Of note, CSE expression seemed reduced in the endothelium after 7 days in static culture (Figure 3d). CBS expression was reduced and restricted to fewer cells expressing higher CBS levels in the media and CBS expression was not detectable in the endothelium after 7 days in static culture (Figure 3d). 3-MST expression was lost in the EC of the intima, still detectable in the EC of the vasa vasorum, as well as in the media layer (Figure S3a).





**Figure 3. Static culture significantly increases CSE expression in human vein segments.** **a)** Representative histological VGEL staining and morphometric measurements of intima thickness, media thickness and intima over media ratio of freshly isolated human vein segments (D0) or after 7 days (D7) in static culture. Scale bar 100  $\mu$ m. **b-d)** normalized CSE (CTH), CBS and 3-MST (MPST) mRNA (**b**) and protein expression as assessed by Western blotting (**c**) or immunohistochemistry (**d**) in freshly isolated human vein segments (D0) or after 7 days (D7) in static culture. Scale bar 100  $\mu$ m. 5 to 8 different veins/patients. \* $p < .033$ , \*\* $p < .002$ , \*\*\* $p < .001$ , as determined by bilateral paired t-test.

To selectively evaluate the role of pressure and shear stress on CSE expression, we further assessed CSE, 3-MST and CBS expression in human saphenous veins segments perfused under pulsatile low pressure (LP; venous regimen mean pressure=7mmHg) or high pressure (HP; arterial regimen mean pressure=100mmHg). LP perfusion did not induce the development of IH (data not shown) as previously described [26, 27], while arterial (HP) perfusion stimulated thinning of the media layer and the formation of IH, as assessed by VGEL and Herovici staining (**Figure 4a**). After 7 days in LP conditions, CTH and CBS mRNA expression remained unchanged, while MPST expression was reduced 4-fold (**Figure 4b**). After 7 days in HP condition, MPST was further reduced and CTH expression was reduced 3-fold, while CBS mRNA expression increased 2-fold (**Figure 4c**). We further looked at CSE and CBS protein expression in human vein segments perfused at high pressure (HP). Western blot analysis revealed that both CSE and CBS protein levels were reduced in those conditions compared to the native vein (**Figure 4d**). IHC staining confirmed that CSE expression was decreased by HP throughout the media and intima layers (**Figure 4e**). Similar to what we observed in static vein culture (**Figure 3d**), the CBS protein expression was reduced and restricted to fewer cells expressing higher CBS levels in the media. CBS was not detectable in the endothelium (**Figure 4e**). In contrast with static vein culture, 3-MST expression remained undetectable in the media layer. 3-MST was still detectable in the EC of the small vessels of the vasa vasorum (**Figure S3b**).

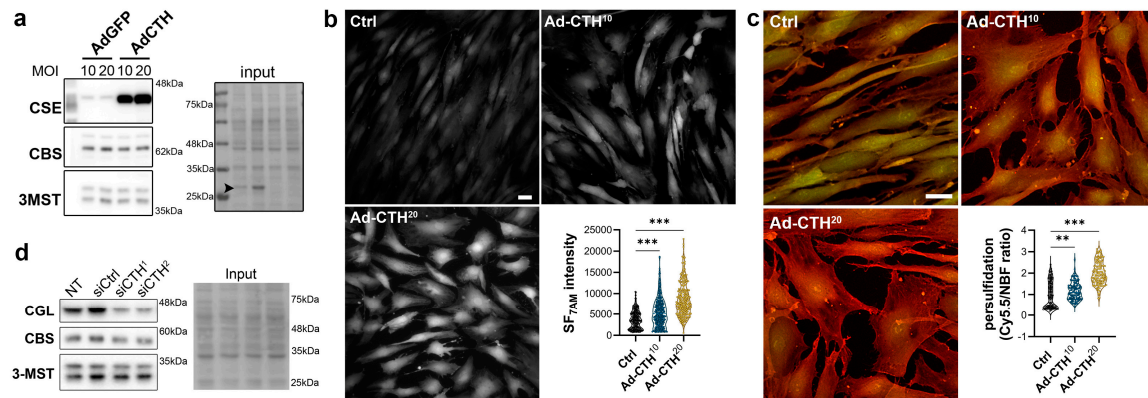


**Figure 4. High pressure perfusion decreases CSE expression in human vein segments.** a) Representative histological sections (left panels) stained for elastin (VGEL) and collagen (Herovici) and morphometric measurements (right panels) of intima thickness, media thickness and intima over media ratio of freshly isolated human vein segments (D0) or in veins exposed to pulsatile high pressure (D7-HP; mean=100 mmHg) perfusion for 7 days. Scale bar 50  $\mu$ m. b-e) Normalized CSE, CBS and 3-MST (MPST) mRNA (b-c) and protein expression as assessed by Western blotting (d) or immunohistochemistry (e) in freshly isolated human vein segments (D0) or in veins exposed to pulsatile low pressure (D7-LP; mean=7 mmHg) or high pressure (D7-HP; mean=100 mmHg) perfusion for 7 days. Scale bar 100  $\mu$ m. 4 to 5 different veins/patients. \* $p$ <.033, \*\* $p$ <.002, \*\*\* $p$ <.001, as determined by bilateral paired t-test.

### 3.3. CSE regulates human VSMC migration

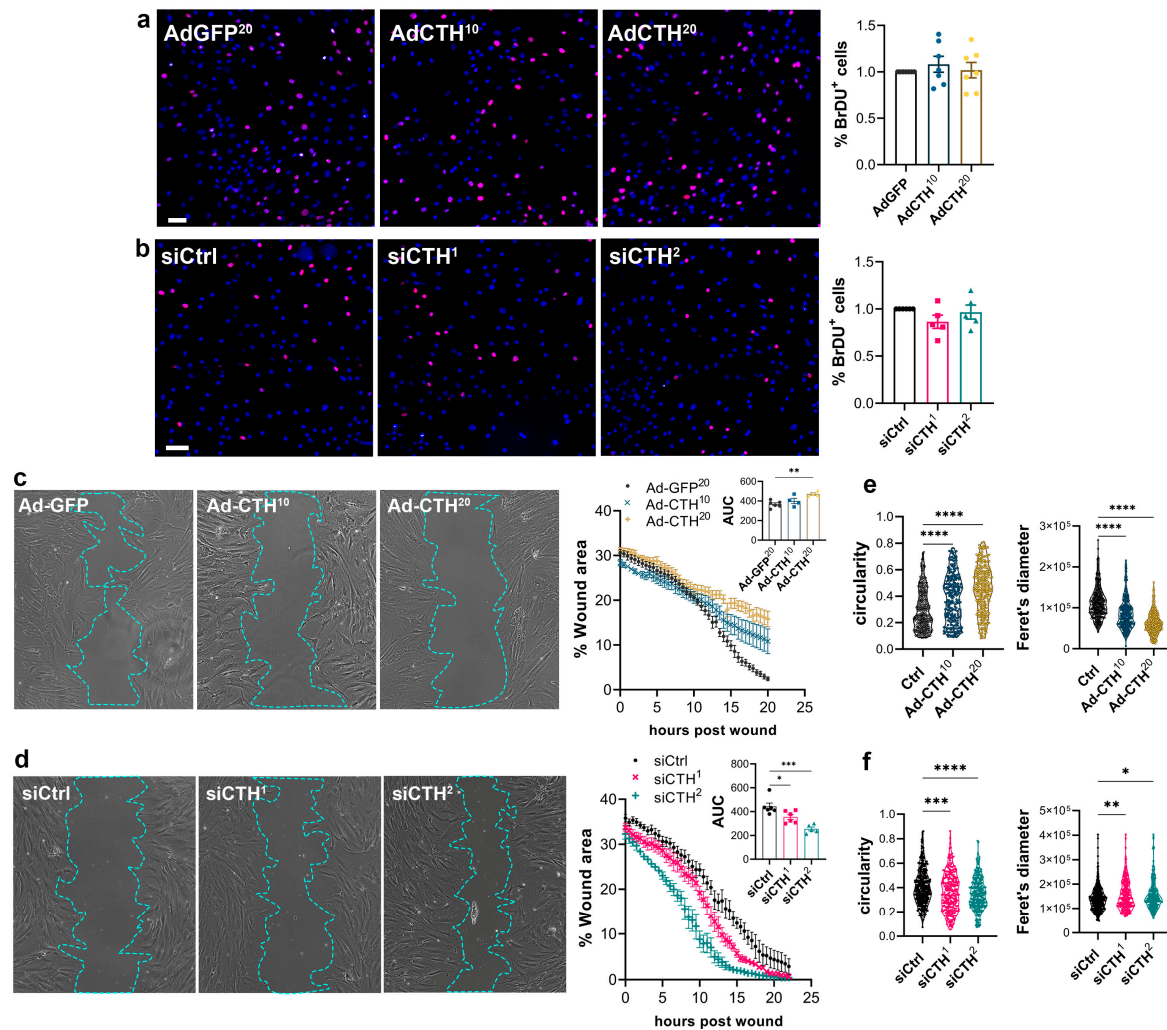
CSE expression in the media of large human vein was decreased under pulsatile arterial high pressure (Figures 3-4). We then tested the role of CSE in VSMC proliferation and migration in primary human VSMC derived from great saphenous vein segments. Adenoviral-mediated CSE overexpression increased CSE protein expression in primary VSMC but did no impact CBS and 3MST expression (Figure 5a). As expected, CSE overexpression increased H<sub>2</sub>S production (SF<sub>7AM</sub>; Figure 5b) and protein persulfidation (Figure 5c). In contrast, siRNA-mediated CSE silencing reduced CSE protein level by 60-70% (siCTH<sup>1</sup>=0.37 $\pm$ 0.09,  $p$ =.008; siCTH<sup>2</sup>=0.45 $\pm$ 0.05,  $p$ =.02 by mixed effect model with Šidák's multiple comparisons test), without impacting CBS or 3MST protein levels (Figure 5d).





**Figure 5. CSE overexpression in human VSMC increases H<sub>2</sub>S production and protein persulfidation.** **a)** CSE, CBS and 3-MST protein expression 48 hours post VSMC infection with an adenovirus encoding GFP (AdGFP) or CSE (AdCTH) at MOI 10 or 20, as indicated. Arrowhead in input indicate GFP expression upon Ad-GFP infection. Data are representative of 6 independent experiments. **b)** Live-cell imaging of H<sub>2</sub>S production using the SF<sub>7AM</sub> probe in VSMC infected, or not (Ctrl), with an adenovirus encoding CSE at MOI 10 or 20, as indicated. Images are representative of 5 independent experiments. Quantitative assessment (violin plots) of SF<sub>7AM</sub> fluorescent in individual cells across 5 experiments. **c)** In situ labelling of intracellular protein persulfidation assessed by DAz-2: Cy5.5 (red), normalized to NBF-adducts fluorescence (green), in VSMC infected, or not (Ctrl), with an adenovirus encoding CSE at MOI 10 or 20, as indicated. Data are representative of 5 independent experiments. Representative images of 5 independent experiments. Violin plots of DAz-2: Cy5.5 over NBF fluorescence in individual cells across 5 experiments. **b-c)** Scale bar 20  $\mu$ m. \*\* $p < .002$ , \*\*\* $p < .001$  report adjusted p-values as determined by Kruskal-Wallis non-parametric ranking followed by Dunn's multiple comparisons tests. **d)** Western blot analyses of CSE, CBS and 3-MST expression 48 hours post VSMC transfection with a control siRNA (siCtrl) or two distinct CTH siRNA (siCTH<sup>1</sup> and siCTH<sup>2</sup>), as indicated. Data are representative of 6 independent experiments.

CSE overexpression or knock-down had no impact on VSMC proliferation as assessed by BrdU incorporation (**Figure 6a-b**). In contrast, CSE overexpression slowed-down migration in a wound healing assay (**Figure 6c**), whereas CSE knock-down accelerated wound healing (**Figure 6d**). Of note, CSE overexpression also increased the circularity and reduced the feret diameter of the VSMC, whereas CSE knock-down reduced circularity and increased the feret diameter, indicating that CSE modulation had an impact on the geometry of the cells associated with migration (**Figure 6 e, f**). CSE overexpression caused the most prominent changes, characterized by reduced spindle shape morphology due to a more static phenotype. In contrast, CSE knocked-down cells were more mobile and more elongated (decreased circularity and increased feret diameter).



**Figure 6. CSE inhibits VSMC migration, but not proliferation *in vitro*.** **a)** VSMC proliferation (BrdU incorporation) in VSMC infected with an adenovirus encoding GFP (AdGFP) or CSE (AdCTH) at MOI 10 or 20, as indicated. **b)** VSMC proliferation (BrdU incorporation) in VSMC transfected with a control siRNA (siCtrl) or two distinct CTH siRNA (siCTH 1 and 2), as indicated. **a-b)** Data are % of BrdU positive nuclei (pink) over DAPI positive nuclei. Scale bar: 50  $\mu$ m. Data shown as mean  $\pm$  SEM of 5 to 6 independent experiments. No statistical differences as determined by repeated measures one-way ANOVA with Dunnett's multiple comparisons tests. **c)** VSMC migration (wound healing) in VSMC infected with an adenovirus encoding GFP (AdGFP) or CSE (AdCTH) at MOI 10 or 20, as indicated. **d)** VSMC migration (wound healing) in VSMC transfected with a control siRNA (siCtrl) or two distinct CTH siRNA (siCTH 1 and 2), as indicated. **c-d)** Bright field images of VSMC 12 h post wound. Scale bar: 100  $\mu$ m. Data are mean  $\pm$  SEM of the percentage of wound closure in 5 to 6 independent experiments. Insets show area under the curve (AUC) of wound healing. \* $p < .033$ , \*\* $p < .002$ , \*\*\* $p < .001$ , report adjusted p-values as determined by one-way ANOVA followed by Dunnett's multiple comparisons tests. **e-f)** Cell morphology during wound healing is expressed as the circularity index and the Feret's diameter shown as violin plots of individual cells across 5 independent experiments. \* $p < .033$ , \*\* $p < .002$ , \*\*\* $p < .001$  report adjusted p-values as determined by Kruskal-Wallis non-parametric ranking followed by Dunn's multiple comparisons tests.

CSE and H<sub>2</sub>S have been proposed to regulate cell proliferation and migration via the ERK1,2 kinase and the mTOR pathways in VSMC [13, 36]. Here, CSE overexpression or CSE knock-down had no effect on P-ERK and P-S6RP levels in VSMC in our experimental settings (**Figure S4**).

#### 4. Discussion



Endogenous H<sub>2</sub>S production in mammals results from the oxidation of the sulfur-containing amino acids cysteine and homocysteine via the reverse "trans-sulfuration" pathway mainly via CSE and CBS, and 3-MST. Although the enzymes and pathways responsible for H<sub>2</sub>S production are well described, little is known about their regulation in pathophysiologic conditions. In this study, we investigated the expression and regulation of CSE, CBS and 3-MST in segments of human saphenous vein and artery. CBS was mainly detected in the media layer of human vessels. In contrast, 3-MST was mainly detected in the endothelium and in the EC of small vessels of the vasa vasorum in both arteries and veins. This suggests a more prominent role of 3-MST in EC of small caliber vessels and capillaries, which is consistent with previous studies suggesting a key role for 3-MST in EC [20]. 3-MST is also expressed in the media of arteries, but not the media of veins, indicating differential regulation of 3-MST in arterial and venous vessels. Rodent studies suggest that CSE is mainly expressed in EC in the cardiovascular system and that the endothelium is the main source of H<sub>2</sub>S in blood vessels [15-18, 37]. That said, CSE has also been described in VSMC and proposed to be a functional, albeit minor source of H<sub>2</sub>S [8, 19, 38]. Here, CSE was expressed in the endothelium of large vessels, and in small vessels of the vasa vasorum in native human artery and vein. CSE was also abundant in the media layer.

We then investigated the regulation of the three enzymes in vein segments placed in *ex vivo* culture. CBS protein expression was reduced in vein segments placed in pathological culture conditions *ex vivo*, both in the absence of flow and in high pressure flow. This indicates that shear stress does not regulate CBS expression in VSMC. Interestingly, while CBS protein levels were lower, CBS mRNA levels were increased in *ex vivo* culture, suggesting a differential regulation of mRNA and protein expression and a possible effect of *ex vivo* culture on protein stability. However, histological analysis suggested a more complex regulation, as CBS was no longer detected in most cells, while it was overexpressed in a few cells present in the media and neointimal layer. Further studies are required to determine which cells overexpress CBS when most cells in the media and neointima appear to down-regulate CBS expression. This is of particular interest as adult VSMC are highly plastic cells [39], and the switch from a quiescent 'contractile' phenotype to a proliferative 'synthetic' phenotype plays a major role in the context of IH [40]. Recent VSMC lineage tracing studies in mice using *in vivo* cell fate tracing with SMC-specific genetic reporter tools suggest that a small subset of VSMCs expand after injury to form clonal patches of neointimal cells [41-43]. Further studies are required to elucidate the identity of this small subset of VSMCs in human tissue, and whether CBS is expressed or not in this subset in the context of IH.

3-MST mRNA and protein expression were severely reduced by *ex vivo* perfusion of vein segments, independent of flow and shear stress. This is not surprising as our model of *ex vivo* culture results in endothelial dysfunction and rapid loss of endothelial-specific markers [26, 27, 44]. However, static *ex vivo* culture tended to stimulate 3-MST mRNA and protein expression despite severe endothelial dysfunction. This suggests that 3-MST may be negatively regulated by shear stress in a similar way to CSE. 3-MST was largely undetectable in the media layer of veins, but it was detectable in the media of arteries, suggesting that 3-MST plays a role in arterial VSMC, but that high shear stress per se does not negatively regulate 3-MST expression. 3-MST was overexpressed in VSMC in veins in static condition and in cultured VSMC *in vitro*, suggesting that 3-MST could be involved in VSMC reprogramming in the context of IH. However, arterial perfusion prevented 3-MST expression, so it is unlikely that 3-MST play a main role in IH *in vivo*. In a recent study, 3-MST was found to be expressed in VSMC and cardiomyocytes, and 3-Mst<sup>-/-</sup> mice were protected against myocardial ischemia-reperfusion injury [45]. Further studies are required to better characterize the role and regulation of 3-MST in VSMC.

About CSE, our data confirm that CSE is negatively regulated by shear stress. Using our model of *ex vivo* vein perfusion, we observed that high pressure inhibits CSE expression both in the media and in the endothelium. In contrast, static *ex vivo* vein culture stimulates CSE expression. This is in line with previous evidence showing that CSE expression is downregulated by high shear stress and is predominantly found in regions of disturbed flow.[21, 22]. However, we did not observe higher levels of CSE in native human vein segments compared to aortic segments, despite the high shear

stress in arteries. In fact, CSE, CBS, and 3MST levels were higher in arteries than in veins. However, this could be due to a higher cell content in arterial tissue compared to venous tissue, which contains more connective tissue than arteries. Consistent with this hypothesis, higher CSE levels did not translate into higher CSE activity as measured by the lead acetate assay. Interestingly, this regulation by shear stress is not unique to EC and was also observed in VSMC. Further studies are needed to better understand the regulation of CSE expression in vein and artery, specifically in VSMC and EC.

Given that CSE is highly expressed in VSMC and downregulated by high-pressure perfusion in veins, we further investigated the role of CSE in human primary venous VSMC. We document that CSE is a specific modulator of VSMC function independent of EC or EC-derived H<sub>2</sub>S production, and that CSE controls the migration of primary human VSMC. This is in agreement with studies showing that VSMCs isolated from Cse<sup>-/-</sup> mice are more motile than their WT counterparts, and that blocking Cse activity with PAG in VSMC increases cell migration [8, 19]. We and others reported that H<sub>2</sub>S donors inhibit VSMC proliferation [12, 14, 36]. Here, we did not observe a significant effect of CSE on cell proliferation. This contrasts with a previous study showing that Cse overexpression decreased proliferation and even induced VSMC apoptosis. This discrepancy probably results from the level of Cse overexpression, which was much higher in the previous study by Yang et al. [36]. In this study, the authors used arterial VSMC, whereas we used venous VSMC. Venous and arterial VSMC are functionally different so the venous versus arterial origin of VSMC may also contribute to the discrepancies in our results. Nevertheless, it would not be surprising if higher CSE expression and activity, leading to greater H<sub>2</sub>S production, resulted in cell apoptosis. Indeed, elevated levels of H<sub>2</sub>S are known to induce cell cycle arrest and apoptosis [46]. Note that evidence suggests that the threshold for the pro-apoptotic effect of CSE and H<sub>2</sub>S may differ depending on the cell type [8, 19], which may reflect cell specific sulfur metabolism and H<sub>2</sub>S detoxifying capacities.

The mechanisms whereby H<sub>2</sub>S affect VSMC proliferation and/or migration are not fully understood. Cytotoxic CSE overexpression or exogenous H<sub>2</sub>S supplementation induces VSMC cycle arrest and apoptosis by stimulating ERK1/2, p38 MAPK, and p21 Cip [36]. Exogenous H<sub>2</sub>S donor treatment in VSMC has also been shown to inhibit the MAPK pathway, especially ERK1/2, and the mTOR pathways, which correlates with reduced VSMC proliferation and migration [13]. Here, no effects on ERK and mTOR signaling were observed upon CSE manipulation. This probably reflects the more subtle CSE variation in our experimental design compared with exogenous H<sub>2</sub>S supply or high Cse overexpression in previous studies. Rather than a physiological response to Cse-derived H<sub>2</sub>S, the effect of Cse overexpression on VSMC proliferation may be due to cell cycle arrest associated with a cytotoxic effect of H<sub>2</sub>S. In accordance with this hypothesis, it was previously shown that inhibition of ERK did not prevent the effect of NaHS on VSMC migration [8], while ERK inhibition was instrumental in the effect of Cse and H<sub>2</sub>S on VSMC proliferation [36]. Of note, several studies in EC also reported that Cse regulates cell migration, but not proliferation [30, 47]. We propose that low levels of H<sub>2</sub>S affect VSMC migration without affecting their proliferation. Our main finding is that CSE variations reshape VSMC, suggesting an effect on the cytoskeleton and interaction with the ECM. This is consistent with our previous finding that H<sub>2</sub>S donors inhibit microtubule polymerization in VSMC [9], and the findings that CSE deficiency in mouse VSMC results in increased expressions of  $\beta$ 1-integrin and increased migration [8]. In EC, it was recently shown that integrins are extensively sulfhydrated, and that  $\beta$ 3 integrin S-sulfhydration promotes adhesion and is required for EC alignment with flow [48]. In this study, they further demonstrated that Cse deficiency in EC leads to overactivation of RhoA, a major hub regulating cell migration and adhesion. Interestingly,  $\beta$ 3-integrin is also expressed in VSMC and  $\beta$ 3-integrin signaling is instrumental for enhanced VSMC proliferation and migration in vascular disease [49, 50]. We hypothesize that CSE-mediated integrin sulfhydration promotes VSMC adhesion, thereby limiting migration. Further studies are needed to determine the exact role and balance between  $\beta$ 1- and  $\beta$ 3-integrins and RhoA signaling in the regulation of cell migration by CSE in VSMC.

The fact that high pressure inhibits CSE expression in vein segments suggests that CSE downregulation occurs *in vivo* in arterio-venous bypass grafts, which contributes to VSMC migration and graft IH.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Table S1: Antibodies, Supplementary Table S2: reagents Figure S1: 3MST is mainly expressed in endothelial cells in large artery and vein segments, Figure S2. Static vein culture promotes CSE-derived H<sub>2</sub>S production, Figure S3. Static vein culture promotes 3-MST expression in the media of saphenous veins, Figure S4. CSE modulation does not regulate P-ERK or P-S6RP levels.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, reviewed and approved by the Lausanne University hospital (CHUV) and the Cantonal Human Research Ethics Committee (<http://www.cer-vd.ch/>, no IRB number, Protocol Number 170/02).

**Informed Consent Statement:** Human vessels were obtained from donors who underwent surgery in the Lausanne University Hospital. Written, informed consent was obtained from all donors.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

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