Université de Strasbourg

École Doctorale des Sciences de la Vie et de la Santé

Médecine CardioVasculaire Translationnelle UR3074

THÈSE présentée par **Ali MROUEH**

Soutenue le **06 Juin 2024**

Pour obtenir le grade de **Docteur de l' Université de Strasbourg**

Discipline/Spécialité **Pharmacologie et Thérapeutique**

Etudes pré-cliniques et translationnelles de l'expression et du rôle des co-transporteurs sodium-glucose SGLT1 et SGLT2 dans les artères et le cœur en condition physiologique et physiopathologique

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ACKNOWLEDGMENT

I would like to sincerely thank Pr. Valerie B. **Schini-Kerth** for hosting me in her team and giving me the opportunity to accomplish this work.

I would like to extend my gratitude to Pr. Olivier **Morel** for co-supervising this work.

I would like to express my deepest recognition to the respectful members of the jury Pr. Agnes **Görlach**, Pr. Carol Ann **Remme**, Pr. Faiez **Zannad**, Pr. François **Roubille** and Pr. Jean **Sibilia** for taking the time to evaluate this work.

My appreciation reaches out to my lab mates of the previous and current team, particularly Dr. **Walaa Fakih** with whom I had the special occasion to work alongside and share successful achievements back home and abroad as well.

My thoughts are as well with every kind and humble teacher, professor, mentor and supervisor I have ever had since day one in life and up to this moment.

Finally, this work is dedicated to my **Dad** and **Mom** to whom I am eternally indebted and to **my two pretty little sisters**.

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SCIENTIFIC CONTRIBUTIONS

ORIGINAL ARTICLES

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3- Ola Al-Assi*, Rana Ghali*, **Ali Mroueh***, Abdullah Kaplan, Nahed Mougharbil, Ali H Eid, Fouad A Zouein, Ahmed F El-Yazbi. "Cardiac Autonomic Neuropathy as a Result of Mild Hypercaloric Challenge in Absence of Signs of Diabetes: Modulation by Antidiabetic Drugs," *Oxid Med Cell Longev*, 2018.

Contributing Author:

4- Walaa Fakih, **Ali Mroueh**, Dal-Seong Gong, Michael Paul Pieper, Michel Kindo, Patrick Ohlmann, Olivier Morel, Valérie Schini-Kerth, Laurence Jesel, "FXa stimulates atrial endothelial cells and tissues to promote remodeling responses

through AT1R/NADPH oxidases/SGLT1/2". *Cardiovascular Research,* 2024; MS # CVR-2023-0959, In press.

5- Nada J Habeichi, Rana Ghali*, **Ali Mroueh***, Abdullah Kaplan, Cynthia Tannous, Abdo Jurjus, Ghadir Amin, Mathias Mericskay, George W Booz, Ahmed El-Yazbi, Fouad A Zouein. "Sexual dimorphism in acute myocardial infarction-induced acute kidney injury: cardiorenal deteriorating effects of ovariectomy in premenopausal female mice," *Clin Sci (Lond)*, **2023**; 137:47-63.

6- Christophe Bruckert , Kensuke Matsushita, **Ali Mroueh**, Said Amissi, Cyril Auger, Ursula Houngue, Lamia Remila, Ahmed Bey Chaker, Sin-Hee Park, Paola Algara-Suarez, Eugenia Belcastro, Laurence Jesel, Patrick Ohlmann, Olivier Morel, Valérie B Schini-Kerth. "Empagliflozin prevents angiotensin II-induced hypertension related micro and macrovascular endothelial cell activation and diastolic dysfunction in rats despite persistent hypertension: Role of endothelial SGLT1 and 2," *Vascul Pharmacol*, **2022**; 146.

7- Haneen S Dwaib, Ghina Ajouz, Ibrahim AlZaim, Rim Rafeh, **Ali Mroueh**, Nahed Mougharbil, Marie-Elizabeth Ragi, Marwan Refaat, Omar Obeid, Ahmed F El-Yazbi. "Phosphorus Supplementation Mitigates Perivascular Adipose Inflammation-Induced Cardiovascular Consequences in Early Metabolic Impairment," *J Am Heart Assoc*, **2021**; 10.

8- Nour-Mounira Z Bakkar, Nahed Mougharbil, **Ali Mroueh**, Abdullah Kaplan, Ali H Eid, Souha Fares, Fouad A Zouein, Ahmed F El-Yazbi. "Worsening baroreflex sensitivity on progression to type 2 diabetes: localized vs. systemic inflammation and role of antidiabetic therapy," *Am J Physiol Endocrinol Metab*, **2020**; 319:E835-E851.

9- Nada J Habeichi, **Ali Mroueh**, Abdullah Kaplan, Rana Ghali, Hiam Al-Awassi, Cynthia Tannous, Ahmad Husari, Abdo Jurjus, Raffaele Altara, George W Booz, Ahmed El-Yazbi, Fouad A Zouein. "Sex-based differences in myocardial infarction-induced kidney damage following cigarette smoking exposure: more renal protection in premenopausal female mice," *Biosci Rep*, **2020**; 40.

10- Walaa Fakih, **Ali Mroueh**, Houssein Salah, Ali H Eid, Makram Obeid, Firas Kobeissy, Hala Darwish, Ahmed F El-Yazbi. "Dysfunctional cerebrovascular tone

contributes to cognitive impairment in a non-obese rat model of prediabetic challenge: Role of suppression of autophagy and modulation by anti-diabetic drugs," *Biochem Pharmacol*, **2020**; 178.

11- Rana Alaaeddine, Mohammed AW Elkhatib, **Ali Mroueh**, Hosny Fouad, Evan I Saad, Marwan E El-Sabban, Frances Plane, Ahmed F El-Yazbi. "Impaired endothelium-dependent hyperpolarization underlies endothelial dysfunction during early metabolic challenge: Increased ROS generation and possible interference with NO function," *J Pharmacol Exp Ther*, **2019**; 371:567-582.

Under Revision:

1- **Ali Mroueh**, Paola Algara-Suarez, Walaa Fakih, Dal-Seong Gong, Kensuke Matsushita, Sin-Hee Park, Said Amissi, Cyril Auger, Gilles Kauffenstein, Nicolas Meyer, Patrick Ohlmann, Laurence Jesel, Michael Paul Pieper, Benjamin Marchandot, Olivier Morel, MD, Jean-Philippe Mazzucotelli, Valérie B. Schini-Kerth, "SGLT2 expression in human vasculature and heart correlates with low-grade inflammation and causes eNOS-NO/ROS imbalance" *Cardiovascular Research*, 2024, MS # CVR-2024-0355.

REVIEWS ARTICLES

1- Rana Alaaeddine, **Ali Mroueh** Stephen Gust, Ali H Eid, Frances Plane, Ahmed F El-Yazbi. "Impaired cross-talk between NO and hyperpolarization in myoendothelial feedback: a novel therapeutic target in early endothelial dysfunction of metabolic disease, " *Curr Opin Pharmacol*, **2019**; 45:33-41.

ORAL COMMUNICATION

1- **A. Mroueh**, W. Fakih, A. Carmona, A. Trimaille, K. Matsushita, B. Marchandot, AW. Qureshi, DS. Gong, C. Auger, L. Sattler, A. Reydel, S. Hess, W. Oulehri, O. Vollmer, JM. Lessinger, N. Meyer, MP. Pieper, L. Jesel, M. Bäck, V. Schini-Kerth, O. Morel "COVID-19 promotes acute and long-term endothelial dysfunction and thrombogenicity: Role of the pro-inflammatory cytokines/SGLT2 pro-oxidant pathway" **ESC 2022, Barcelona.**

POSTER PRESENTATIONS

1 **A. Mroueh**, W. Fakih, DS. Gong, S. Amissi, C. Auger, MP. Pieper, O. Morel, JP. Mazzucotelli, V. Schini-Kerth "SGLT2 expression in the left ventricle of cardiac patients is correlated with low-grade inflammation involving the pro-oxidant AT1R/NADPH oxidases/SGLT2 crosstalk: Potential role in heart failure" **ESC 2023, Amsterdam.**

2 **A. Mroueh**, W. Fakih, DS Gong, C. Auger, K. Matsushita, O. Morel, JP Mazzucotelli, V. Schini-Kerth "SGLT2 expression in the coronary microvessel endothelium and cardiomyocytes of cardiac patients: Determinant role of low-grade inflammation and induction of endothelial dysfunction" **AHA 2022, Chicago.**

List of Abbreviations

ACE: Angiotensin converting enzyme

ADAMTS13: ADAM metallopeptidase with thrombospondin type 1 motif 13

AF: Atrial fibrillation

AKI: Acute kidney injury

Akt: Protein kinase B

AMPK: Adenosine monophosphate-activated protein kinase

Ang II: Angiotensin II

ARB: AT1R blockers

ASCVD: Atherosclerotic CVD

AT1R: Ang II type I Receptor

ATPase: Adenosinetriphosphatase

AV node: Atrioventricular node

BNP: B-type natriuretic peptide

CA: Coronary artery

Ca^{2+}: Calcium ion

CABG: Coronary artery bypass surgery

CAD: Coronary artery disease

CAM: Cell adhesion molecule

CAMKII: Calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CEC: Cardiac EC

cGK: cGMP-dependent kinase

cGMP: Cyclic 3',5'-guanosine monophosphate

CKD: Chronic kidney disease

CM: Cardiomyocyte

COPD: Chronic obstructive pulmonary disease

CV: Cardiovascular

CVD: CV disease

CVS: CV system

EC: Endothelial cell

ED: Endothelial dysfunction

EF: Ejection fraction

eGFR: Estimated glomerular filtration rate

EMA: European medicines agency

eNOS: Endothelial NO synthase

ERK: Extracellular signal-regulated kinase

e-Selectin: Endothelial selectin

FDA: Food and drug administration

GLUT: Glucose transporter

GTP: Guanosine-5' triphosphate

H_2O_2: Hydrogen peroxide

HbA1c: Glycated hemoglobin

HF: Heart failure

HFmrEF: HF with mildly reduced EF

HFpEF: HF with preserved EF

HFrEF: HF with reduced EF

ICAM-1: Intercellular CAM-1

IL-1β: Interleukin-1 beta

IL-6: Interleukin-6

I_{Na+}: Sodium current

ITA: Internal thoracic artery

I-κB: Inhibitory-kappa-B

JNK: Jun N-terminal kinase

K^+: Potassium ion

LA: Left atrium

LDL: Low-density apolipoproteins

LV: Left ventricle

LVEF: Left ventricular ejection fraction

MACE: Major adverse CV events

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemotactic protein-1

MI: Myocardial infarction

MLC: Myosin light chain

MLCP: Myosin light chain phosphatase

MMP: Metalloproteinase

Na^+: Sodium ion

Na+/K+ ATPase: sodium–potassium pump

NADPH: Nicotinamide-adenine dinucleotide phosphate

Nav: Voltage gated sodium channel

NCX: Na^+/Ca^{2+} exchanger

NET: Neutrophil extracellular traps

NF-κB: Nuclear-factor-kappa-B

NHE: NA+/H+ exchanger

NLRP3: Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3

NO: Nitric oxide

NOXA1: NOX activator 1

NOXO1: NOX organizer 1

Nrf2: Nuclear factor erythroid 2-related factor 2

NSTEMI: Non-ST-elevated MI

NT-pro-BNP: N-terminal pro-BNP

O_2: Oxygen

O^{2-}: Oxygen anion

PAI-1: Plasminogen-activator-1

PCI: Percutaneous coronary intervention

PECAM-1: Platelet-endothelial CAM-1

PGI_2: Prostaglandin I2, Prostacyclin

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PKG: Protein kinase G

PLC: Phospholipase C

p-Selectin: Platelet selectin

RA: Right atrium

RAAS: Renin angiotensin aldosterone system

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INTRODUCTION

I. Endothelium in Health and Disease

I.I: Anatomy of the Endothelium

i. Vascular Endothelium

Vascular endothelium is a thin monolayer of endothelial cells (ECs) than lines the inner surface of vasculature -arteries, veins and capillaries- throughout the body. At the origin of its discovery, the endothelium was thought to solely form a physical barrier separating blood components at its luminal side from the underlying muscular layer of the vasculature located basally. However, years later, scientific discoveries proved that the endothelium possesses pivotal regulatory roles on the surrounding environment including vascular function and blood homeostasis **(1)**.

Due to their localization, luminal and basal proteome of ECs differs contributing to their polarization **(2)**. At the basal side, ECs are layered over a sheet-like structure known as the basal lamina. The basal lamina itself is composed of structural and specialized proteins/glycoproteins such as collagen, elastin, fibronectin and laminin among other extracellular matrix proteins, which are synthesized, released and anchored to ECs membrane by ECs themselves **(2)**. On the other hand, the apical surface of ECs is surrounded by the so-called glycocalyx. The glycocalyx is a carbohydrate-rich negatively charged luminal mesh composed of several glycolipids, glycoproteins and proteoglycans.

Owing to its complex structure and negative charge, the glycocalyx forms a mechanical and electrical barrier that repels and prevents contact of circulating blood cells, proteins and/or extracellular vesicles with ECs as well as their leakage. Additional roles of the glycocalyx include contributing to vasomotor tone adjustment through glycocalyx framework changes in addition to aiding ECs in sensing mechanical stress through the mechano-sensitive characteristics of certain intracellular domains of glycocalyx proteins known as mechanoreceptors **(3)**.

Taking into consideration the heterogeneity of the vascular bed, ECs along the vascular tree, despite performing similar common functions, are required to endure different physiological conditions and possess site-specific characteristics. For instance, elasticity of ECs and sensitivity to vasomotor changes differ in large conducting vessel from small resistant ones. In fact, ECs in large arteries are required to handle elevated pressure as compared to smaller arteries or even veins. Further, the continuity and permeability of the endothelial layer among the vascular bed is not identical. In areas where exchange requirements are limited to oxygen and smaller nutrients as glucose such as the brain, the endothelial layer tends to be continuous, held together and anchored to the basal lamina by increased proportion of self-synthesized tight junctions. On the other hand, in areas of increased requirement for exchange such as renal glomeruli, hepatic vessels and spleen, the

endothelium layer tends to be discontinuous and porous. In addition, abundance and types of gap junctions expressed by ECs vary among the vascular tree depending on the rate and types of exchange required in different body areas. As such, ECs throughout the vasculature exhibit heterogeneous structural morphology (size and thickness), cell surface proteome (glycocalyx components) and secretome (extracellular matrix, basal lamina composition) **(4,5)**.

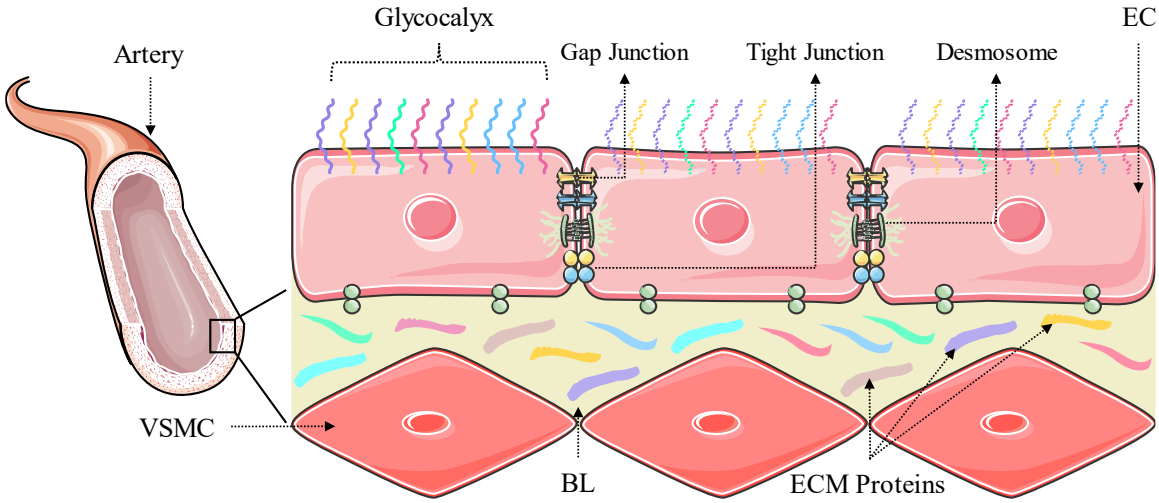

Figure 1: Illustration of vascular endothelium structure and histology. BL, basal lamina; EC, endothelial cell; ECM, extracellular matrix; VSMC, vascular smooth muscle cell. (Image was adjusted by the author using illustrations provided by smart.servier.com).

ii. Cardiac Endothelium

Similar to vascular ECs (VECs), cardiac ECs (CECs) constitute an active physical barrier -known as the blood-heart barrier- between blood circulating in the internal cavity of the heart and underlying cardiomyocytes. Further, CECs form a vital endocrine tissue that helps maintaining cardiomyocytes functional and structural integrity. Additionally, during embryonic heart development, CECs arrange in a cushion-like structures to later shape independent intra-cardiac structures including valves and septum membranes **(6)**.

Being ECs in structure and nature, CECs and VECs share several common features including common endocrine messengers and surface markers. However, despite evolving at once during embryonic development, CECs and VECs originate from different embryonic ancestors. This hints the formation of sub-categorized ECs each of which owns specific traits allowing them to meet the functional and structural requirement of their allocated destination. Morphologically, CECs are larger than VECs. Structurally, CECs have less cytoskeletal actin filament and more developed intracellular organelles, particularly Golgi apparatus, as compared to VECs. Functionally, CECs synthesize and produce higher amount of endothelial mediators **(6,7)**.

Another important characteristic of CECs is the higher abundance of complex gap junctions connecting CECs as compared to VECs. CECs,

and in respect to their location, are required to transmit electrical signals in the form of ions and secondary messengers in a highly efficient and organized matter. This is assured by the distinctly developed, ion channels-rich gap junctions network, which connects CECs to one another ensuring that CECs act as individual entity. Additionally, such network contributes to the passive trans-cellular transfer of ions between the blood and the cardiomyocytes interstitial space **(6,7)**.

I.II: Controllers of Endothelial Function

i. Endothelial Surface Markers

Being an active and dynamic tissue, ECs perform several vital functions to help maintaining general homeostasis. ECs major roles include but are not limited to controlling hemostasis through regulating leukocyte trafficking, platelet/leukocyte adhesion, thrombosis and fibrinolysis, as well as crucial implication in flow-sensing, modulation of vascular tone, angiogenesis in addition to performing other metabolic activities **(8)**. In order to fulfill all the diverse functions, ECs depend on specific cell surface proteins and endothelium-derived released factors. A summary of different ECs functions and the corresponding contribution of surface markers and soluble factors are summarized in **Table 1**.

Several biomechanical variables including shear stress and biochemical variables such as O_2, hormones, pH, cytokines, autacoids and growth factors, influence endothelial structural and functional development. Such variables are indeed inconsistent through different body organs and vessel types. Hence, this leads to the maturation of specific endothelial subsets that are able to fulfill the requirements of different specific organs and tissues. This was evident when differences in populations of certain surface markers between ECs derived from

different organs were revealed **(9)**. Yet, and despite the heterogeneity, ECs all across the body do express specific surface markers that allows their identification as ECs in the first place in addition to contributing to the major common functions of ECs.

(1) vWF: ECs markers can be either constitutively expressed or induced following a stimulus. An example of a constitutively expressed marker of ECs is von Willebrand factor (vWF). vWF is a multimeric protein that is produced by endothelial cells to be either secreted or stored intra-cellularly. vWF serves as a platelet binding site hence promoting platelet adhesion and marking the first step of platelet aggregation. Additional role of vWF is regulating angiogenesis as vWF deficient animal models showed increased abrupt vascularization **(9-11)**.

(2) CD31: Also known as platelet-endothelial cell adhesion molecule-1 (PECAM-1) is another example of a constitutively expressed ECs marker. CD31 is considered as the hallmark marker for ECs identification. The expression of CD31 on the surface of ECs ensures proper intercellular junction and maintenance of the endothelium layer integrity. In addition, CD31 can act as a mechano-transducer hence aiding ECs in sensing shear stress and ensuring proper blood flow **(9,11)**.

(3) ACE 1: Angiotensin converting enzyme 1 (CD143) is a luminally expressed enzyme on the surface of ECs. ACE 1 catalyzes the conversion of the angiotensin I into the potent vasoactive angiotensin II (Ang II). ACE 1 expression levels can increase depending on different physiological and pathophysiological conditions including circulating inflammatory cytokines **(9,12)**.

(4) TF: Tissue factor is an example of induced ECs surface protein. Although TF is abundant in vascular tissue extracellular matrix, healthy ECs do not express TF on their surface. TF binds circulating coagulation factor VII to mark the initiation of the coagulation cascade during injury. Following vascular injury, ECs produce and extensively express TF on their surface which plays a crucial role in the activation and propagation of the coagulation cascade and hence hemostasis **(9-11)**.

(5) MCP-1: Monocyte chemotactic protein-1 is another example of induced ECs proteins. Under inflammatory conditions, ECs overexpress MCP-1 to be mainly secreted. MCP-1 attracts blood monocytes and tissue macrophages to the site of injury or inflammation hence propagating the inflammatory signal. As such, elevated levels of circulating MCP-1 can be considered as an indicator of endothelial inflammation **(9,13)**.

(6) Cell Adhesion Molecules (CAMs): Under normal physiological conditions, healthy ECs express low to absent levels of

specific adhesion molecules. Yet, in response to inflammation or other stimuli and injuries, ECs tend to substantially increase the expression of notably vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and other selectins (e-Selectin, p-Selectin). Expression of such molecules promotes binding of leukocytes to ECs and hence infiltration of inflammatory cells into the underlying tissue **(9,11)**.

ii. Endothelium-Derived Factors

ECs regulate blood flow and vascular tone through the release of several vasoactive molecules. Vasoactive compounds can either be dilators or constrictors. Endothelium-derived relaxing factors include nitric oxide (NO) and prostacyclin PGI_2. In addition to the endothelium-derived relaxing factors, ECs can promote vasodilation through what is known as endothelium-derived hyperpolarization. Endothelium-derived hyperpolarization occurs when hyperpolarizing signals are transmitted from ECs to underneath vascular smooth muscle cells (VSMCs) through myo-endothelial gap junctions leading to the efflux of potassium from VSMCs and hence hyperpolarization of VSMCs and vascular relaxation. In contrast, examples of endothelium derived contracting factors include Ang II, endothelin-1, and thromboxane A_2 **(12)**.

(1) NO: NO was the first endothelium-derived relaxing factor to be described and identified. It is an unstable gas radical with high bio-reactivity. In ECs, NO is produced by endothelial NO synthase (eNOS, an enzyme with dual activity: N-terminal oxygenase and C-terminal reductase) through NADPH-dependent hydroxylation of L-Arginine. Induction of NO formation by eNOS can be triggered either in response to agonists such as acetylcholine and bradykinin, or in response to shear stress. In the absence of any induction, eNOS is found bound to and inhibited by caveolin-1. Agonist activation increases intracellular Ca^{2+} leading to the binding of calmodulin to eNOS and its simultaneous release from caveolin-1. Released eNOS forms homodimers around a zinc core and become active Additionally, configurational change of calmodulin by intracellular Ca^{2+} promotes calmodulin-dependent kinase II (CAMKII) activation which in turn, phosphorylates eNOS at Ser1179 promoting its activation. On the other hand, shear stress induces a dual phase production of NO. The first phase includes shear stress activating surface channels leading to intracellular Ca^{2+} accumulation, eNOS dissociation from caveolin-1 and binding to calmodulin, activation of protein kinase A (PKA), eNOS phosphorylation at Ser1177, an activation site, and hence a rapid transient burst in NO production. The second phase includes further phosphorylation of eNOS by PKA at Ser633, which in turn attenuates

eNOS activity leading to minimal yet sustained production of NO at basal Ca^{2+} levels persistent with shear stress **(12,14)**.

Once generated, NO diffuses from ECs into adjacent VSMCs and interact with soluble guanylyl cyclase through its heme core. This induces guanylyl cyclase activation and enhanced conversion of guanosine-5' triphosphate (GTP) into cyclic 3',5'-guanosine monophosphate (cGMP) which acts as a secondary messenger. cGMP prevalence in turn activates cGMP-dependent kinases (cGK) notably cGK-I. In VSMCs, cGK-I activation leads to decreased intracellular calcium release and accumulation in addition to reduced sensitivity of contractile filament to Ca^{2+}. An example of the latter includes the activation of myosin light chain (MLC) phosphatase (MLCP) by cGK-I leading to MLC dephosphorylation and desensitization to calcium **(12,14)**.

Similarly, in the cardiac tissue, NO diffuses into cardiomyocytes (CMs) and leads to the activation of protein kinase G (PKG) by cGMP. PKG in turn phosphorylates myofilaments and calcium handling proteins hence contributes to the regulation of CMs contractility **(15)**.

Additional importance of NO resides in its antithrombotic effects. For instance, NO induces redox regulation of sarcoplasmic reticulum ATPase (SERCA) in platelet cells leading to increased Ca^{2+} uptake. This reduces intracellular Ca^{2+} and as such prevents platelet

aggregation. Further, NO was shown to prevent VSMC proliferation and migration **(12,16)**.

(2) Ang II: Ang II is an octapeptide produced by ECs using ACE 1 enzymes expressed on their surface. Ang II promotes vasoconstriction through binding to its predominant surface receptor Ang II type 1 receptor (AT1R) on VSMC surface. AT1R is a G-protein coupled receptor, specifically Gq coupled whose activation promotes phospholipase C (PLC) downstream signaling. In turn, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol triphosphate leading to protein kinase C (PKC) activation. PKC phosphorylates MLC and hence inducing VSMC contraction. Further, activation of AT1R activates Rho-A/Rho-A kinase pathway which inhibits MLCP and hence sustains VSMC contraction **(17)**.

In addition to its potent vasoconstrictor effect, Ang II through its AT1R-PLC-PKC signaling pathway in ECs can over-boost endothelial NADPH oxidases activation. This leads to increased formation of reactive oxygen species (ROS) and intracellular oxidative stress which hinders endothelium dependent relaxation. Further, Ang II was found to induce endothelial expression of several CAMs, thus facilitating leukocyte adhesion and infiltration **(18)**.

Table 1: Summary of endothelial mediated functions and corresponding attributors (9-13).

Function	Effect	Marker
Blood Flow	Vasodilation	NO, PGI_2, EDH
	Vasoconstriction	Ang II, thromboxane A_2, endothelin-1
Platelets	Adhesion	vWF
	Inhibition	NO, PGI_2, ectonucleotidases, ADAMTS13
Coagulation	Activation	TF, FVIII
	Inhibition	Thrombomodulin, TFPI, EPCR
Fibrinolysis		t-PA, u-PA
Angiogenesis		VEGFR, angiopoietin/TIE receptor, endoglin (a TGF-β Receptor)
VSMC migration	Inhibition	VE-Statin
Leukocyte trafficking		CAMs (CD31, ICAM-1, VCAM-1), selectins (e-selectin, p-selectin)
Vascular permeability		CD31, VE-Cadherin

Abbreviations: ADAMTS13, ADAM metallopeptidase with thrombospondin type 1 motif 13; Ang II, Angiotensin II; EDH, Endothelium-derived hyperpolarization; EPCR, Endothelial protein C receptor; e-selectin, Endothelial selectin; FVIII, coagulation factor VIII; ICAM-1, Intercellular cell adhesion molecule-1; NO, Nitric oxide; PGI_2, Prostaglandin I2/Prostacyclin; p-selectin, Platelet selectin; TF, Tissue factor; TGF-β, Transforming growth factor β, TFPI, Tissue factor pathway inhibitor; t-PA, Tissue plasminogen activator; u-PA,

Urokinase plasminogen activator; VEGFR, Vascular endothelial growth factor receptor; VE-Cadherin, Vascular endothelial cadherin; VE-Statin, Vascular endothelial statin; VCAM-1, Vascular cell adhesion molecule-1; VSMC, Vascular smooth muscle cell; vWF, von Willebrand factor.

I.III: Inducers of Endothelial Dysfunction

Situated at the interface between circulating blood and underlying tissue, ECs are the first to be affected by any trigger leading to endothelial dysfunction (ED). ED can be characterized and induced by 3 main factors including inflammation, elevated ROS levels and decreased levels of NO bioavailability. In consequence, ECs lose partially or completely their regulatory roles over vascular tone and hemostasis **(8,19)**.

i. Oxidative Stress and NADPH Oxidases:

Oxidative stress is defined as elevated levels of intracellular ROS, free oxygen radicals in the form of H_2O_2 or $O_2^{\cdot -}$ mainly. ROS basal formation and generation at physiologic conditions contribute to various cellular defense mechanisms and normal cell physiology. However, accumulation of intracellular ROS is damaging and toxic. ROS accumulation can be a result of either its reduced clearance or increased production by anti-oxidant or pro-oxidant enzymes respectively. In ECs the anti-oxidant machinery is composed of several enzymes including the following: superoxide dismutase, glutathione peroxidase, thioredoxin and catalase. On the other hand, the pro-oxidant machinery is mainly composed of an enzyme family known as

NADPH oxidases. For instance, in ECs and vasculature, NADPH oxidases are the main source of ROS generation **(20)**.

NADPH oxidases are multi-subunit enzymes consisting of membrane bound subunits NOX and $p22^{phox}$ in addition to cytoplasmic subunits $p40^{phox}$, $p47^{phox}$ (and its homologue NOX organizer 1, NOXO1), and $p67^{phox}$ (and its homologue NOX activator 1, NOXA1). NOX exist in 7 different isoforms of which NOX1, 2, and 4 are the most studied in endothelial function and dysfunction. NADPH oxidases becomes functional when the two membrane-bound sub-units come together (one NOX isoform and $p22^{phox}$), followed by the recruitment of the regulatory cytoplasmic phox subunits in response to $p47^{phox}$ phosphorylation or NOXO1/NOXA1 in response to NOXO1 phosphorylation **(21)**.

Several agonists can bind to ECs surface and activate NADPH oxidases. One of the most potent and identified agonists is Ang II. For instance, Ang II leads through AT1R downstream signaling to the activation of downstream PKC, a kinase that is well known to phosphorylate $p47^{phox}$, hence leading to NADPH oxidases activation **(22)**. In healthy ECs and vasculature, basal activity of NADPH oxidases has been identified contributing to mainly NOX4 mediated physiologic production of ROS. On the other hand, several conditions have been described to induce NADPH oxidases over-activity, particularly NOX2 mediated, such as diabetes, dyslipidemia and

hypertension **(23)**. The resulting NADPH oxidases mediated overproduction of ROS is well described to induce ED through several mechanisms including DNA damage, eNOS uncoupling through inhibiting tetrahydrobiopterin regeneration leading to reduced NO bioavailability, activation of redox sensitive kinases (p38 mitogen-activated protein kinase, p38 MAPK; Jun N-terminal kinase/stress-activated protein kinases, JNK/SAPK; extracellular signal-regulated kinase, ERK1/2), over-expression of CAMS and pro-thrombotic proteins as TF, and induction of inflammation **(23-25)**.

ii. Inflammation and NF-κB:

Endothelial inflammation is a major trigger of ED. ECs inflammation occurs primarily in response to pro-inflammatory cytokine activation of respective ECs membrane receptors **(26)**. Examples of pro-inflammatory ligands include but are not limited to interleukin- (IL)-1β and tumor necrosis factor- (TNF)-α where they bind to their respective receptors IL-1R and TNFR. The hallmark response of such binding is achieved through the activation of the nuclear factor kappa-B (NF-κB). NF-κB is considered the major pro-inflammatory transcription factor. It exists in different homo- or heterodimer formation of which p50/p65 hetero-dimer is the highest abundant and most studied. Under normal physiological conditions,

NF-κB is found in the cytoplasm where it is bound to and inhibited by the inhibitory-κB (I-κB). Following an activation signal, I-κB becomes phosphorylated under the action of an active (phosphorylated) I-κB kinase leading to its detachment from NF-κB. Liberated NF-κB then becomes accessible to its corresponding kinases (such as I-κB kinase kinase) which phosphorylate the p65 subunit, a step that leads to NF-κB activation and translocation into the nucleus **(27)**.

Active NF-κB induces the transcription of several downstream genes including inducible CAMs such as VCAM-1 and ICAM-1, secreted chemokines such as MCP-1 and pro-thrombotic protein TF **(28)**. Additionally, NF-κB activation was shown through potentiating p53 to induce endothelial senescence, an endothelial pathological state in which ECs lose their ability to perform their essential roles such as NO formation, inhibition of coagulation and control of immune cell infiltration **(29)**.

Besides being activated through ligand-receptor binding (TNF-α/TNFR), NF-κB can be induced in response to oxidative stress. Oxidative stress is known to induce the phosphoinositide 3-kinase/protein kinase B (PI3k/Akt) signaling pathway through preventing the activity of its negative-regulator. In turn Akt phosphorylates and activates I-κB kinase leading to subsequent NF-κB activation. In contrast to ROS, NO was shown to inhibit I-κB kinase and hence prevent NF-κB activation. On the other hand, NF-κB

activation can in turn lead to elevated ROS production particularly through inducing NOX2 subunit of NAPDH oxidase **(30-32)**.

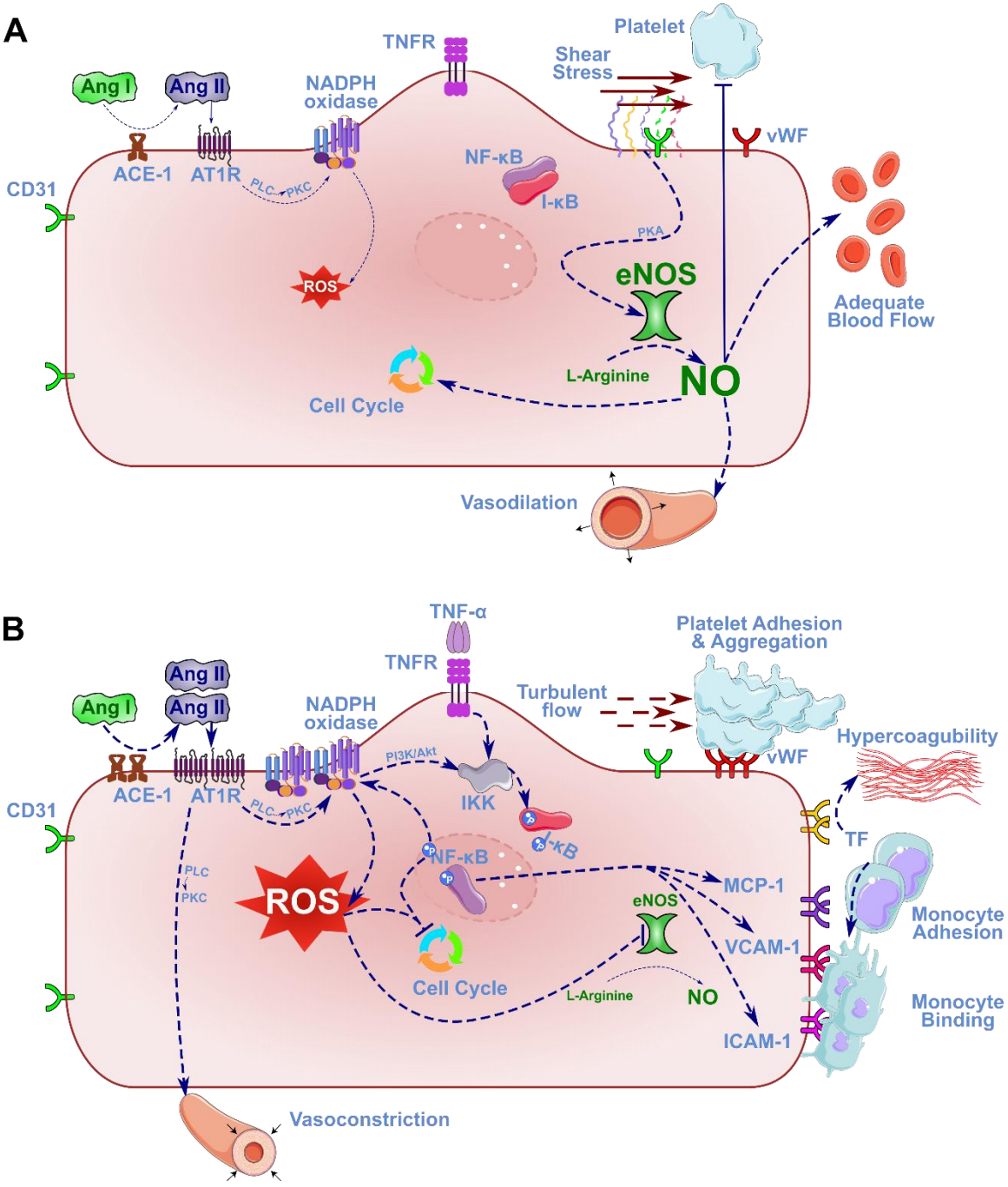

Figure 2: Endothelium function in health (A) and disease (B). ACE 1, angiotensin converting enzyme 1; Ang I, angiotensin I; Ang II, angiotensin II; AT1R, angiotensin II type I receptor; eNOS, endothelial

nitric oxide synthase; ICAM-1, intercellular adhesion molecule 1; I-κB, inhibitory-kappa B; IKK, I-κB kinase; MCP-1, monocyte chemoattractant protein 1; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TNF-α, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; VCAM-1, vascular cell adhesion molecule 1; vWF, von Willebrand factor. (Image was adjusted by the author using illustrations provided by smart.servier.com).

INTRODUCTION

II. Cardiac Physiology and Pathology

II.I: Cardiac Structure and Function

i. Cardiac Histology:

Histologically, the heart can be divided into three layers: external layer or the epicardium, intermediate layer or the myocardium and the internal layer or the endocardium.

(1) The Epicardium: The heart is surrounded by a layer called the pericardium which is divided into two different parts; the internal or visceral part known as the epicardium and an external transparent layer which acts a protective sac enclosing the heart. The two parts of the pericardium are separated by what is known as the pericardial cavity. The epicardium itself is single layer of epithelial cells known as the mesothelium which is connected to the myocardium through epicardial fat and connective tissue **(33)**.

(2) The Myocardium: The myocardium is the thickest of the cardiac layers and the part which is responsible for the contractile function of the heart **(34)**. The myocardium is mainly composed of CMs which possess distinctive characteristics and features as compared to other muscular fibers, allowing them to have their unique contractile function. CMs are striated and distinguished through their intercalated discs, which are areas rich in gap junctions. The junctions play a

pivotal role in facilitating CM-CM connection and allowing rapid and organized spread of the electrical current in the form of ions. Depolarization signals from pace-making sinoatrial (SA) node reach the myocardium through gap junctions inducing the opening of voltage-gated Na^+ channels (Nav). This leads to the primary depolarization of CMs. Depolarized CMs trigger the opening of voltage gated K^+ and Ca^{2+} channels allowing partial exit of K^+ and intracellular accumulation of Ca^{2+}. Internalization of Ca^{2+} promotes Ca^{2+} release from sarcoplasmic reticulum in a phenomenon known as calcium induced calcium release. Calcium induced calcium release is a distinguished feature of CMs to which they owe their prolonged depolarization periods. Thereafter, intracellular Ca^{2+} binds to troponin C which frees myosin binding site of actin. This is followed by the actin-myosin binding and CMs contraction. Finally, SERCA restores Ca^{2+} into sarcoplasmic reticulum leading to drop of intracellular Ca^{2+} levels concomitantly with exit of K^+ marking the repolarization and hence relaxation of CMs. Physiologically, contraction and relaxation of the myocardium compose what is known as systole and diastole respectively **(35)**.

In addition to CMs, other cell-types are found in the myocardium, including VSMCs, ECs, fibroblasts and astrocytes. These cells are derived from the coronary vasculature, autonomic innervation and lymphatic system which supply the heart at the myocardium **(36,37)**.

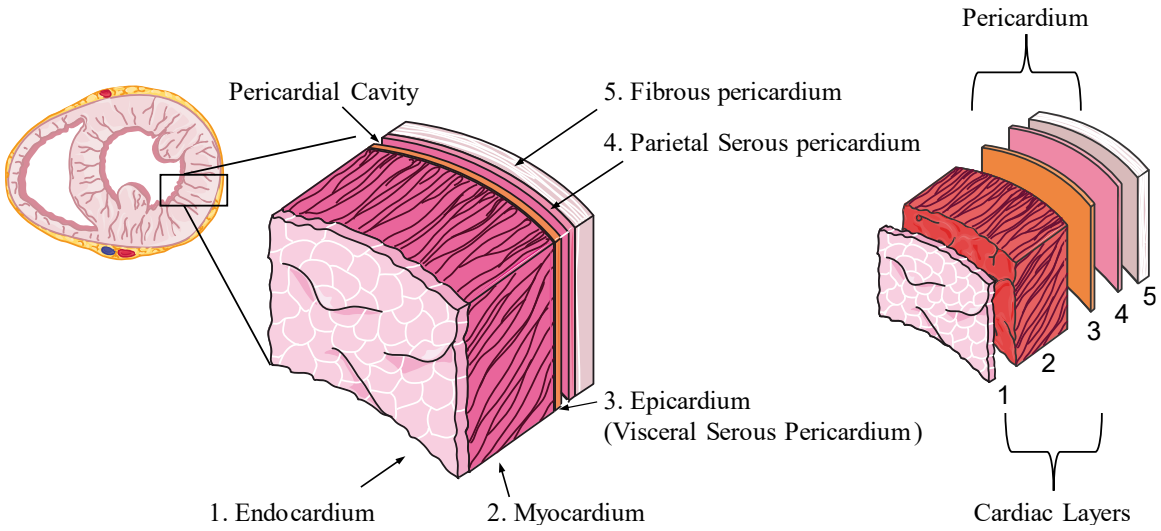

Figure 3: Cardiac layers (Image was adjusted by the author using illustrations provided by smart.servier.com).

(3) The Endocardium: The endocardium is the innermost layer of the heart; it lines all the internal cavities and structures as valves hence forming a barrier between the circulating blood and underneath myocardium **(38)**. The endocardium is mainly composed of a layer of CECs and sub-endothelial connective tissue. CECs regulate myocardial function through a variety of released factors. For instance, NO generated from CECs diffuses into CMs and lead to the activation of PKG by cGMP. PKG in turn phosphorylates titin among other myofilaments and reduces calcium sensitivity of troponin C and hence regulates CMs contractility and reduce CMs stiffness. Additional CECs generated factors that contribute to its control over CMs include but are not limited PGI_2, endothelin-1, Ang II and connective tissue growth factor **(39,40)**.

Besides controlling CMs contractility, healthy CECs prevent abrupt thrombosis formation. For instance, in cardiac cavity areas of regular shear stress, which is a major contributor to healthy endothelial function, formation of thrombi is not well documented. In contrast, cardiac cavities with turbulent blood flow, such as atrial appendages, are characterized with ED leading to thrombus formation **(41)**.

The endocardium and myocardium are separated by the sub-endocardium. This layer has specialized cardiac cells that conduct electrical impulses across the heart generating an impulse conducting system. The impulse conducting system is a depolarization which spontaneously initiates at the SA node located at the interface of superior vena cava and right atrium. From there, the depolarizing signal traverse into the atrioventricular (AV) node situated at the interatrial septum. Thereafter, it descends through interventricular septum forming the bundle of His after which they divide into the ventricles. At the ventricles, the bundles branch into the Purkinjie fibers which spread electrical impulses to ventricular myocardium and activate right and left ventricles. As such, the above-described impulse conducting system plays a pivotal role in maintaining an in-rhythm heart beating and thus an effective cardiac function **(42)**.

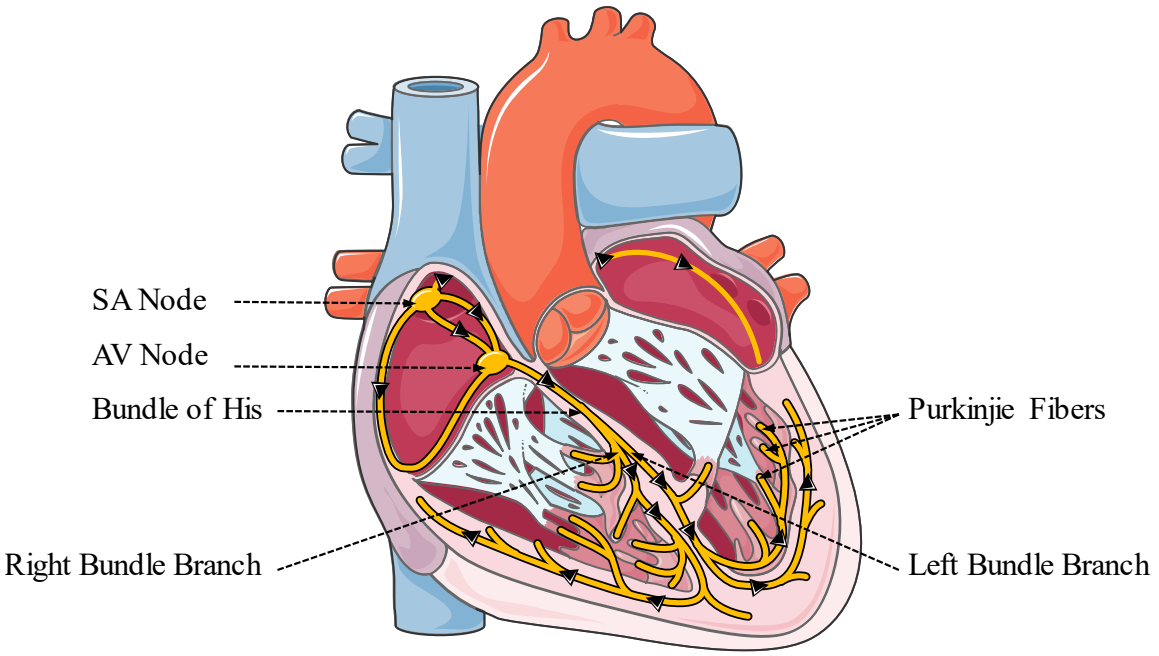

Figure 4: Cardiac conduction system. AV, atrioventricular; SA, sinoatrial. (Image was adjusted by the author using illustrations provided by smart.servier.com).

ii. Cardiac Compartments

The heart serves as a blood pump whose main function is to assure oxygen and nutrient delivery to the distinct body parts by pumping sufficient and effective amount of blood. It is divided into four main compartments called right atrium (RA), right ventricle (RV), left atrium (LA) and left ventricle (LV). RV and LV are separated by the interventricular septum while the atria are separated by the interatrial septum. On the other hand, RA and RV are separated by the tricuspid valve, while LA and LV are separated by the mitral valve. The major role of these valves is to prevent blood back-flow by ensuring its unidirectional flow from the atrium into the ventricle. The

cardiac cycle starts when RA receives blood through venous return from the vena cava (superior and inferior) and pumps it to the RV through the tricuspid valve. In turn, RV pumps the blood into the pulmonary artery through the pulmonary valve. At this stage, the blood passes through the pulmonary circulation in order to be oxygenated. From there, the heart receives back the blood through the pulmonary vein into the LA. Thereafter, the LA passes the blood into the LV through the mitral valve in a step known as LV filling, which is the major part of the diastole. Once the left ventricle is filled, the heart goes into systole, which is when the blood is pumped from LV through the aortic valve into the aorta. The aorta in turn is responsible of the delivery of the blood into the whole body **(43,44)**.

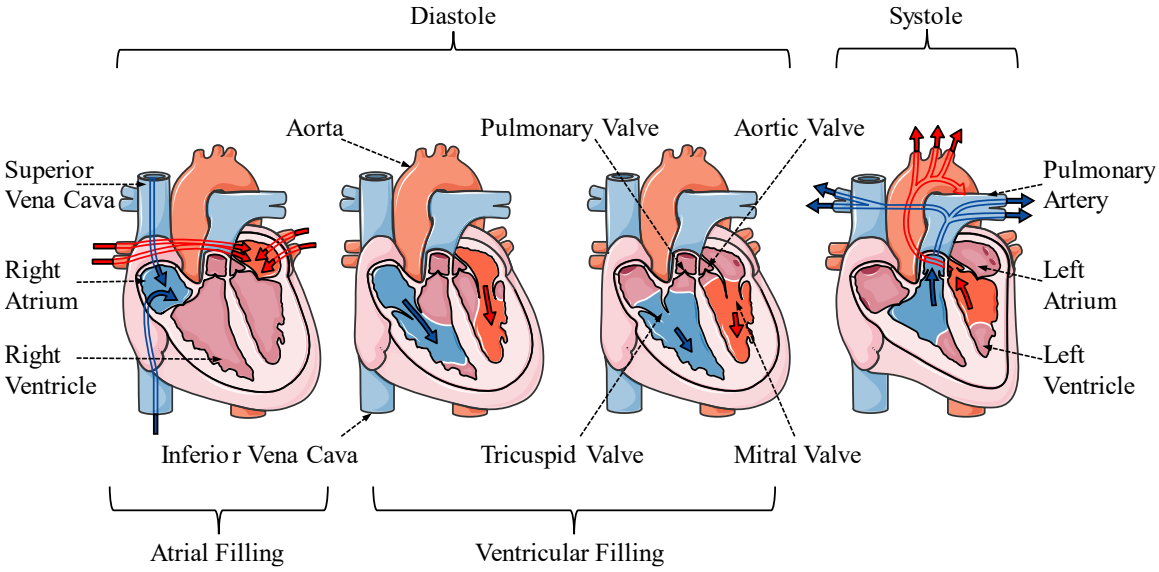

Figure 5: Cardiac cycle (Image was adjusted by the author using illustrations provided by smart.servier.com).

iii. Cardiac Output

The efficacy of the cardiac function can be mainly evaluated through cardiac output. Cardiac output is the amount of blood pumped by heart in a minute. In healthy resting humans, cardiac output is around 5 L/min and can reach 35 L/min while exercising. Changes in cardiac output are mandatory to cope with O_2 and nutrients demands of vital organs. Cardiac output can be measured as the multiplication of heart rate with the stroke volume. In a broader description, cardiac output is the result of complex coordination between autonomic nervous system, endocrine/paracrine signaling, myocardial capacity and vascular health **(44,45)**.

(1) Heart Rate: Heart rate is the number of heart beats per minute; it is determined mainly by the SA firing rate. Intrinsic SA depolarization is estimated to be between 60 and 100 per minute, yet, this can be affected by the autonomic activation leading to changes in heart rate. Generally, increased heart rate leads to improved cardiac output. However, this is only valid within limits. For instance, permanently elevated heart rate, such as in patients with tachycardia, might lead to CMs fatigue and hence significant decrease in their effectiveness and subsequently cardiac output **(44-46)**.

(2) Stroke Volume: The second parameter affecting the cardiac output is stroke volume. Stroke volume is the amount of blood pumped during each beat or, in other words, the volume of blood that

leaves the LV during systole. It is calculated by subtracting the end systolic volume (the volume of blood remaining in the LV after systole) from the end diastolic volume (the volume of blood in LV at the end of diastole). Stroke volume is mainly dependent on three main factors known as the preload, CMs contractility and afterload. Preload is the tension exerted on the LV wall during diastole, the more CMs are stretched the higher the force generated by CMs and hence stroke volume. Preload as such is dependent on end diastolic volume. End diastolic volume in turn can be dependent on a complexity of factors including but not limited to venous return, arrhythmia, LA performance and LA to LV pressure gradient, mitral valve compliance, myocardial elasticity and LV filling time, the latter being affected by heart rate. On the other hand, myocardial contractility is the force generated by CMs during systole, the higher the force the higher the stroke volume. Finally, afterload is the force exerted on the LV during systole, this is mainly dependent on systemic blood pressure and aortic valve condition. As such, the higher the afterload the lower the SV **(44,45)**.

In addition to cardiac output, another way to measure systolic cardiac function is through measuring ejection fraction (EF). EF is the measurement of the percentage of stroke volume to end diastolic volume. In healthy individuals, EF ranges between 50-70%, indicating that during systole, only 50-70 % of the end diastolic volume is ejected while the remaining composes the end systolic volume **(47)**.

II.II: Coronary Circulation

Similar to any other functional organ or tissue, the myocardium needs to be supplied by sufficient amount of O_2 and nutrients to maintain its proper function and health. As such, well-organized blood supply system ensures myocardial nourishing and is known as the coronary circulation.

i. Coronary Arteries:

Coronary arteries (CAs) originate at the base of the aorta where they split into two parts, right and left CAs. Right CA supplies the RA and RV including SA and AV nodes. Supplying the right half of the heart, right CA plunges into multiple branches including the posterior descending artery. The posterior descending artery supplies blood to the posterior part of the interventricular septum. On the other hand, left CA supplies blood to LA and LV. It normally has two branches known as the left anterior descending and the left circumflex. While the left circumflex supplies blood to the LA and lateral-posterior LV, left anterior descending nourishes the anterior part of LV including the anterior interventricular septum. Noteworthy, CA are filled mainly during the diastole at which the myocardium is maximally relaxed. Interestingly, anastomosis (connection or opening) among different

coronary arteries exist as well, particularly between the posterior descending artery and left anterior descending artery from one side and right CA and left circumflex on the other side **(48)**.

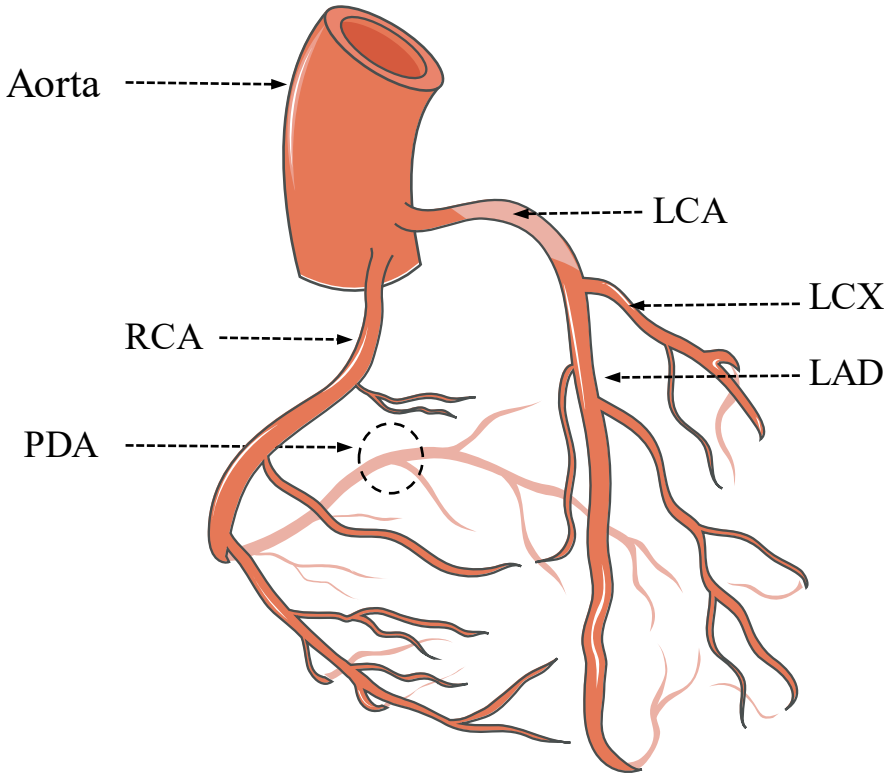

Figure 6: Coronary circulation. LAD, left anterior descending; LCA, left coronary artery; LCX, left circumflex; PDA, posterior descending artery; RCA, right coronary artery. (Image was adjusted by the author using illustrations provided by smart.servier.com).

Structurally, CA system is composed of large conducting arteries (> 400 μm), pre-arterioles (100-400 μm), arterioles (< 100 μm) and capillaries (< 10 μm). Large conducting arteries are part of the epicardium and include right CA, left CA and their major branches.

Plunging deep into the myocardium, conducting arteries tighten and branch into pre-arterioles and subsequently arterioles. Pre-arterioles and arterioles in turn are resistant arteries and characterized by their vasomotor activity. Their diameter changes in response to myocardial need for oxygenation and they are highly innervated with autonomic connections **(49)**. Similar to peripheral arteries and arterioles, CA and coronary arterioles are composed of three different layers namely tunica externa or adventitia, tunica media and tunica intima. The adventitia is the outermost part and provides structural support to the arteries. It is a collagen-rich connective tissue whose main cellular components are fibroblasts and myo-fibroblasts. The adventitia is surrounded by a layer of perivascular adipose tissue; an active endocrine tissue which contributes to proper vascular physiology. On the other hand, the tunica media is the middle layer and it is composed mainly of VSMCs. Tunica media is the thickest part of the vessel and it is responsible of vascular contraction. As for the tunica intima, it is the innermost layer and it is composed of a single layer of ECs including its glycocalyx and underlying basal lamina. It is worth mentioning that external and internal elastic laminas (thin layers composed of elastic fibers) separate the adventitia from the tunica media and the tunica media from the intima respectively **(50)**.

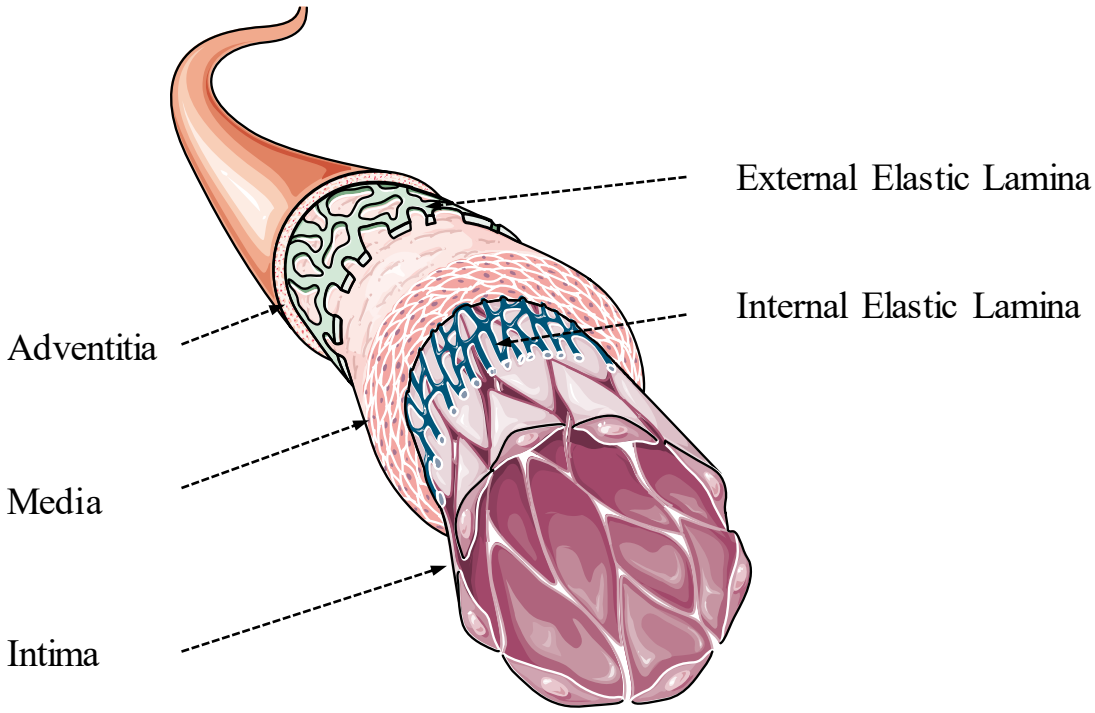

Figure 7: Arterial layers. (Image was adjusted by the author using illustrations provided by smart.servier.com).

Finally, coronary capillaries are the site at which exchange between blood and the myocardium takes place. They lack any muscular layer and are composed of thin single layer of ECs surrounded by a thin protective layer **(48-50)**.

ii. Coronary Veins:

Coronary venules drain the myocardium from deoxygenated blood. Thereafter, venules merge intro coronary veins which can be divided into two types greater and smaller cardiac veins. All cardiac veins eventually evacuate into the coronary sinus, the biggest coronary vein. In turn, coronary sinus empties directly into the right atrium. It is note mentioning that coronary veins are composed of the same layers as CA, yet the muscular and connective tissue parts are thinner owing to the fact that blood pressure in the veins is lower **(51)**.

II.III: Cardiovascular Disease

Cardiovascular diseases (CVDs) are the leading cause of mortality worldwide accounting for more than 30% of global deaths. Several risk factors increase the occurrence of CVDs including hypertension, dyslipidemia, obesity, smoking, physical inactivity, kidney and lung diseases among others **(52)**. Several forms of CVDs exist, including but not limited to heart attack, heart failure (HF), stroke, arrhythmias, valvular disorders, congenital heart diseases and others **(Table 2)**. Due to their wide array and heterogeneous phenotypes, symptoms of CVDs differ although some of them are common to most CVDs such as dyspnea, fatigue and chest discomfort. CVDs can have acute onset such as in case of Takotsubo cardiomyopathy or chronic onset as in HF and arrhythmias. Yet, and despite their short-term onset, acute CVDs can still cause lifelong morbidities. Such effects can be due to the underlying low-grade inflammation accompanied with most of the CVDs. For instance, inflammatory infiltrates and biomarkers have been widely observed in several CVDs hence pointing out the role of low-grade inflammation in initiating and propagating CVDs. Such observations included coronary artery disease (CAD) and HF two of the most common and most serious CVDs **(53,54)**.

i. Coronary Artery Disease (CAD)

CAD is the most common CVD and the leading cause of death worldwide. Its prevalence is dependent on several factors including genetics, lifestyle (smoking, physical inactivity, obesity, stress) and co-occurrence of other diseases such as diabetes, dyslipidemia and hypertension. CAD occurs when inflammatory atherosclerotic plaques develop in the coronary arteries. This leads to reduced blood flow to the myocardium and hence imbalance between O_2 supply and demand. Arising symptoms to reduced O_2 supply include chest pain (angina) and heaviness, shortness of breath (dyspnea), nausea and pressure-like feeling radiating to the jaws, arms and back **(55)**.

The etiology of CAD originates at the endothelial layer of CA. Inflamed CA endothelium, particularly at higher risk areas such as bifurcations, favors lipoprotein deposition over the intima. Typically, water-insoluble lipids circulate in the blood bound to water-soluble carriers known as apolipoproteins. Elevated concentration of circulating apolipoproteins (a form of dyslipidemia) particularly low-density apolipoproteins (LDL) allows their penetration of the disrupted CA endothelium. Accumulation of LDL leads to its oxidation, a reaction that triggers local inflammatory activation. This is further supported by increased expression of CAMs on the surface of pathological ECs hence enhancing leukocyte adhesion and infiltration. ECs activation and inflammation is associated with increased secretion

of various mediators and enzymes (such as matrix metalloproteinases; MMPs) that trigger VSMCs proliferation, migration and deposition of excessive amounts of extracellular matrix. Such changes in VSMCs activity are marked by the formation of fibrous plaque overlying a highly thrombogenic lipid-rich core. The formed fibrous plaques surround the lumen of CA leading to its tightening, reduced elasticity and thus lower perfusion to the myocardium. In advanced cases, either rupture or erosion of the plaque occurs leading to thrombus formation in epicardial CA, thrombus migration into smaller CAs and consequently total occlusion of blood flow **(55-57)**.

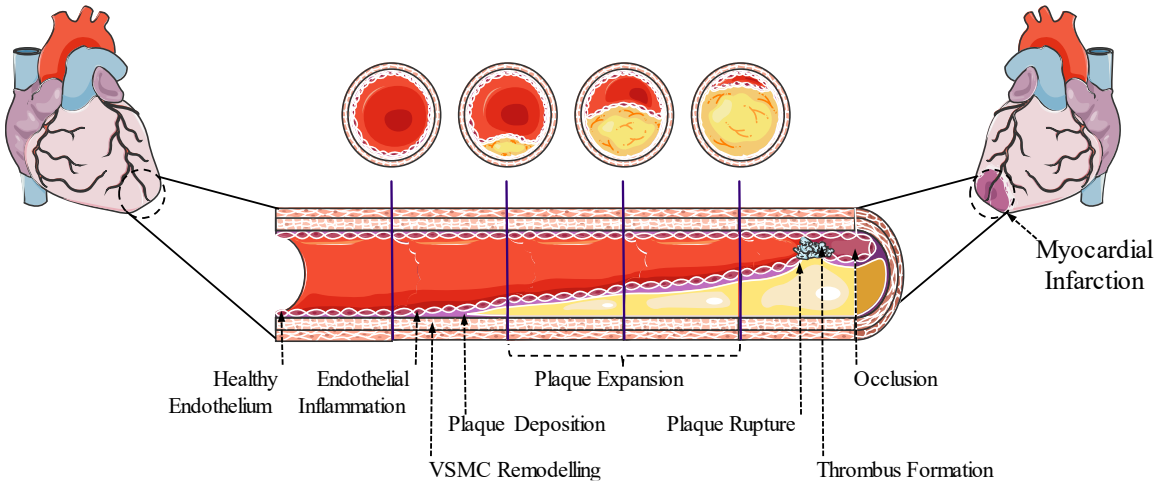

Figure 8: Progression of plaque formation to thrombus and occlusion. VSMC: vascular smooth muscle cell. (Image was adjusted by the author using illustrations provided by smart.servier.com).

Depending on the afore-mentioned manifestations of CAD, CAD can be categorized into the following: stable angina (occurs only during over-activity), unstable angina (occurs at rest and follows irregular pattern) and myocardial infarction (MI, or heart attack). Depending on the severity of the occlusion and the duration of attack before treatment, electrocardiography results can vary leading to the categorization of MI into non-ST-elevated MI (NSTEMI) or ST-elevated MI (STEMI), the latter being the most severe. Both atherothrombosis-induced forms of MI (STEMI, NSTEMI) are known as type 1 MI **(55-57)**. While STEMI is always a type 1 MI, NSTEMI can be triggered by any mismatch in O_2 demand and supply even in the absence of atherothrombotic events. Such form of MI is called type 2 MI or non-obstructive MI. Type 2 MI can be induced by uncompensated reduction in O_2 supply such as in cases of CA endothelial dysfunction, CA spasm, CA embolism (as a result of atrial fibrillation AF, LV systolic dysfunction, and valvulopathy), systemic hypotension (as in cases of shock), bradyarrhythmia and severe anemia. On the other hand, type 2 MI can be also a result of unmatched increase in O_2 demands such as in cases of tachyarrhythmia and severe hypertension. Due to the big diversity in MI etiologies, MI can be defined as any event leading to changes in blood levels of markers of cardiac damage (Troponin I or T) along with clinical evidence for MI diagnosis **(57-59)**.

Depending on the severity and etiology of CAD, treatment options for CAD include non-invasive and/or invasive regimens. Non-invasive treatment options or conservative management of CAD includes lifestyle modifications such as diet changes, smoking quitting and exercise in addition to medical therapy. Medications include anti-ischemic drugs such as β-blockers, Ca^{2+} channel blockers and nitrates, anti-platelet therapy to prevent atherothrombotic events in addition to control of other concomitant risk factors such as hypertension (mainly through inhibitors of the renin angiotensin aldosterone system; RAAS), dyslipidemia (mainly statins) and diabetes. On the other hand, invasive regimens include mainly non-surgical percutaneous coronary intervention (PCI, or formerly known as coronary angioplasty) and coronary artery bypass surgery (or coronary artery bypass graft, CABG). The choice of medication and/or invasive intervention depends on the individual situation of the patient **(55-57)**.

ii. Heart Failure (HF)

HF is a complex disease characterized by functional and structural malfunctions of the heart. Major HF risk factors include CAD and hypertension, in addition to other underlying mechanisms such as valvulopathy, arrhythmias, diabetes, renal disease and metabolic disorder. Common clinical symptoms of HF include dyspnea (at rest, during exercise, lying down), chest discomfort and edema of the lower limbs in addition to wheezing and persistent cough in other cases. HF is the leading cause of hospitalization worldwide in patients above 65 years and is associated with elevated mortality rates ranging from 20 % of diagnosed patients within 1 year of diagnosis reaching up to 50% of the patients within 5 years **(60)**. Reduction in LVEF is a form of HF which depending on the reduction amplitude it can be categorized into HF with mildly reduced EF (HFmrEF; 41% ≤ LVEF < 50%) or with reduced EF (HFrEF; LVEF ≤ 40%). On the other hand, another form of HF exists known as HF with preserved EF (HFpEF) in which LVEF remains ≥ 50 **(61,62)**. The different forms of HF differ in their risk factors, etiology and underlying mechanisms, yet can still be interconnected and one can lead to the other.

(1) HFrEF: HFrEF is a systolic dysfunction resulting from the inability of the heart to function properly during the systole phase. It accounts for almost 50% of HF cases and is mainly due to the imbalance between O_2 supply and demand to CMs. In other terms, ischemic damage to CMs such as in case of MI leads to CM death and reduced contractility. As such stroke volume drops leading to decrease in cardiac output. Reduced cardiac output is perceived by the body as reduced blood volume which activates baroreceptors that in turn increase sympathetic firing. Activated sympathetic nervous system increases heart rate as a way of increasing cardiac output, yet from a failing heart, which is another reason for CMs overload. Activation of the sympathetic system leads as well to vasoconstriction and increases systemic blood pressure, which is an increase in afterload and hence places an extra overload on CMs. Further, vasoconstriction along with reduced cardiac output triggers renal hypo-perfusion, which stimulates the kidneys to increase water and salt retention particularly through activating RAAS. RAAS activation leads to further arterial constriction in addition to volume overload due to water and salt retention. Volume overload induces *de novo* synthesis and alignment of sarcomeres in a series manner thus increasing CMs length in an attempt to increase contractility. This is known as maladaptive eccentric remodeling of CMs. Noteworthy, eccentric remodeling of CMs, which is not matched by increased vascularization, favors oxidative damage and further death

of CMs. This leads to LV dilation, a signature phenotype of HFrEF **(63-66)**.

In response to increased volume overload and CMs stretch, remodeling CMs during HFrEF highly produce pro-BNP (pro- B-type natriuretic peptide). Pro-BNP is cleaved into NT-pro-BNP (N-terminal pro-BNP) and BNP, the latter being the active part. As such NT-pro-BNP and BNP serve as crucial plasma markers used to diagnose HFrEF. Further, since HFrEF is associated with injury and/or loss of CMs, cardiac Troponin T plasma levels were as well found to be elevated. As such, high sensitivity cardiac Troponin T can serve as an additional biomarker in HFrEF **(67)**. Besides MI, myocarditis and valvular diseases (such as aortic valve regurgitation) can as well induce CMs ischemic injury and subsequently HFrEF **(68)**. Noteworthy, HFrEF is also characterized by concomitant diastolic impairment which is a result of several factors including pro-inflammatory cytokines (particularly IL-6 and TNF-α)-mediated induction of systemic and coronary endothelial dysfunction **(69)**.

Depending on the stage of the disease and the particular case of each patient, treatment of HFrEF can include implantable devices such as pacemakers in addition to pharmacological treatment. Medications used in HFrEF include drugs that counteract the neuro-hormonal induced modifications such as β-blockers and most importantly RAAS inhibitors. Further, recent clinical discoveries have favored an

additional family of drugs named Sodium-Glucose co-transporter (SGLT) -2 (SGLT2) inhibitors (SGLT2i) in the treatment regimen of HFrEF **(70,71)**. Interestingly, HFmrEF responds successfully to HFrEF medications and as such both HF sub-categories can be considered to share common pathophysiology. However, HFmrEF has better prognosis as compared to HFrEF **(72)**.

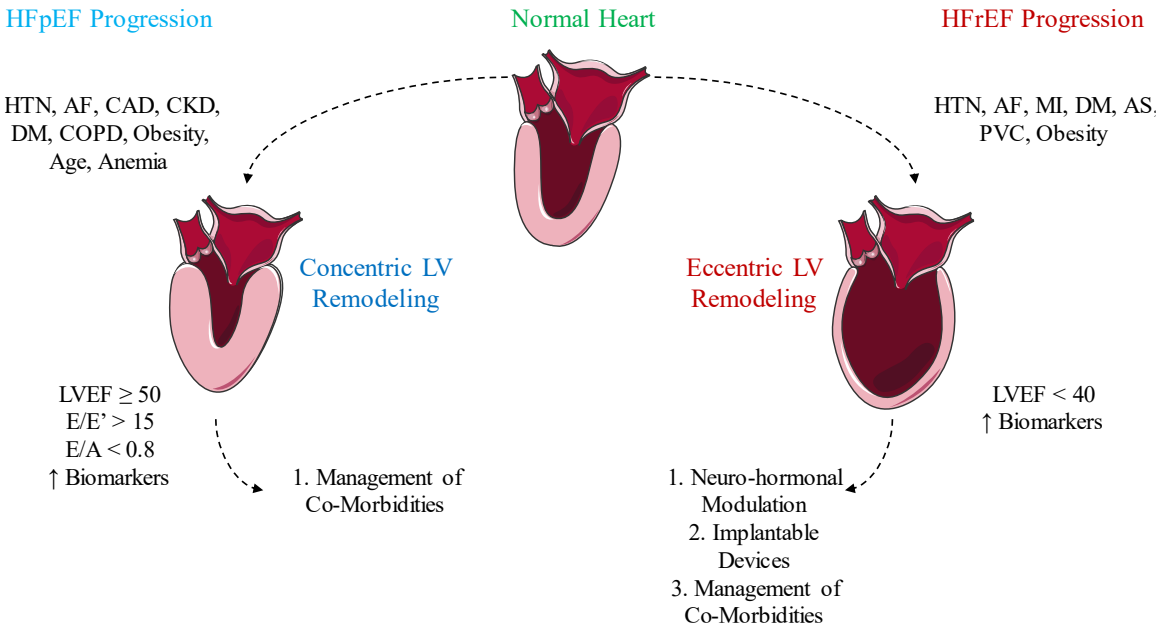

Figure 9: Progression of HFpEF and HFrEF. AF: Atrial Fibrillation, AS: Aortic Stenosis, CAD: Coronary Artery Disease, CKD: Chronic Kidney Disease, COPD: Chronic Obstructive Pulmonary Disease, DM: Diabetes Mellitus, HTN: Hypertension, LVEF: Left Ventricular Ejection Fraction, PVC: Premature Ventricular Contraction. (Image was adjusted by the author using illustrations provided by smart.servier.com).

(2) HFpEF: HFpEF accounts for almost 40% of total HF cases. It is a disease characterized by diastolic dysfunction in the absence of systolic involvement. HFpEF usually occurs in older patient cohort as compared to HFrEF and affects females more than males, which is the opposite in HFrEF. HFpEF usually co-exist with other risk factors including hypertension, diabetes, chronic kidney disease (CKD) and chronic obstructive pulmonary disease (COPD) **(64-66)**. Notably, all the aforementioned risk factors are characterized by systemic low-grade inflammation which serves as the most common underlying etiology for HFpEF. For instance, low-grade inflammation induces systemic endothelial dysfunction notably affecting the endocardium and coronary micro-vessels leading to coronary microvascular dysfunction. Inflammatory stimulation of the endothelium leads to activation of NADPH oxidases and increased intracellular oxidative stress. In turn, ROS accumulation favors eNOS uncoupling and hence a drop in NO bioavailability. Absence of sufficient NO levels deteriorate ECs-CMs crosstalk particularly through reduced cGMP-PKG signaling. This is conveyed as increased CMs stiffness and thus impaired LV relaxation and hence LV filling. In addition to elevated oxidative stress, inflamed ECs over-express CAMs (such as VCAM-1, ICAM-1 and e-Selectin) hence favoring the infiltration of activated macrophages into the myocardium. Subsequently, this induces the release of pro-fibrotic markers such as transforming growth factor beta (TGF-β) which is a

potent inducer of cardiac fibroblast transformation into myofibroblasts and concomitant production of extracellular matrix. Such trans-differentiation and elevated extracellular matrix production leads to concentric CMs remodeling and increased LV thickness and stiffness **(73-75)**.

Concentric CMs remodeling in HFpEF is also trigerred by hypertension, which is the leading cause of HFpEF. For instance, persistent hypertension induces what is known as hypertensive heart disease. Briefly, hypertension and associated vascular stiffness induces pressure overload on LV which is the major form of afterload. To maintain the stroke volume (which is inversely proportional to afterload) and hence cardiac output and EF, additional layers of CMs is added which leads to increased LV hypertrophy, a major sign of hypertensive heart disease. LV hypertrophy in hypertension is mediated by several inducers particularly the over-activation of the RAAS, which is the leading cause for hypertension **(76)**. Ang II for instance is a potent inducer of fibroblast trans-differentiation through induction of TGF-β and its downstream signaling. Additionally, Ang II potentiates the activation of MAPK such as ERK1/2 and JNK which serve as another pathway for fibrotic transformation. Further, RAAS activation is associated with elevated pro-inflammatory cytokines (IL-1β, IL-6) and AT1R downstream signaling includes the activation of NADPH oxidases, subsequent ROS accumulation and endothelial dysfunction.

All these modifications enhance CMs concentric hypertrophy through deposition of *de novo* sarcomeres in a parallel manner in addition to extracellular matrix expansion particularly through deposition of collagen fibers (type-1a and type-3a). This in turn favors LV stiffness and diastolic dysfunction **(77-80)**. In addition to hypertension, aortic stenosis is a major cardiovascular disease that is associated with increased afterload, as such pressure overload on LV, and thus considered as one of the leading risk factors for HFpEF development **(68)**.

Despite differences in etiology and underlying mechanisms, HFpEF and HFrEF can coexist in certain patients just as type-I and type-II diabetes do. As a simple example, HFmrEF shares similar comorbidities as HFpEF (obesity, diabetes, old age, female sex) yet is as well marked by incidence of MI as in HFrEF. As such, HFmrEF population can be viewed as either recovering HFrEF or in other cases as HFpEF patients with CAD **(81)**.

Besides its unique etiology, HFpEF can as well be characterized by a set of biomarkers. For instance, NT-BNP and high sensitivity cardiac Troponin T were identified as possible markers of transmission from LV hypertrophy into HFpEF. In fact, both biomarkers were found to be elevated in HFpEF conditions yet to significantly lower levels as compared to HFrEF. On the other hand, high sensitivity C-reactive protein levels were found to be significantly higher in HFpEF as

compared to HFrEF **(67)**. In contrast, more specific biomarkers of HFpEF have been recently identified and can be linked to the mechanism of HFpEF progression. Such markers include: tissue inhibitor of metalloproteinases (plays a central role in extracellular matrix deposition during concentric hypertrophy), Galectin-3 (a lectin produced by macrophages and participates in macrophage induced inflammation and fibrosis), sST2 (the soluble/decoy form of ST2, a Toll-like receptor that possess anti-apoptotic/hypertrophic/fibrotic actions) and growth differentiation factor 15 (a member of the TGF-β cytokine family) **(82)**.

Owing to its complex mechanism, not many treatment options are available for HFpEF. In fact, until recently, treatment regimens for HFpEF were based on management of co-existing comorbidities. These treatments included RAAS inhibitors (ACE 1 inhibitors, AT1R blockers or ARBs, and mineralocorticoid receptor antagonist) to manage hypertension and/or CKD, in addition to management of other co-existing risk factors such as diabetes, obesity, dyslipidemia (through statins) and AF. However, recently, SGLT2i demonstrated improved outcome in patients with HFpEF independent of other comorbidities and were as such included as part of the treatment regimen **(83-85)**.

Table 2: Summary of Most Common Cardiovascular Diseases, their Symptoms, Risk Factors and Complications (86-99).

Condition	Common Forms	Prevalence x 10^6	Annual Incidence x 10^6	Symptoms	Risk Factor	Complications
CAD	Stable angina ACS STEMI NSTEMI Unstable angina Vasospastic angina Spontaneous CA dissection	~200	~1.5 MI	angina, dyspnea, fatigue. In case of MI, angina spreading into the shoulders/arms/ back /neck/jaw, cold sweat, dyspnea, heartburn, sudden dizziness	age, sex, family history, smoking, HTN, dyslipidemia, diabetes, obesity, CKD, physical inactivity, alcohol	HF, arrhythmia, VHD, cardiomyopathy, CS
HF	HFpEF HFmrEF HFrEF	~64		dyspnea, fatigue, exercise intolerance, lower limb edema, cough, wheezing, decreased alertness, loss of appetite	MI, CAD, HTN, VHD, myocarditis, CHD, arrhythmia, diabetes, age, smoking, alcohol, sleep apnea	kidney failure, liver damage, VHD, arrhythmia, CS, SCD
Arrhythmias	AF			dyspnea, angina,		

	Atrial Flutter SVT VT Vfib Brugada syndrome Long QT syndrome Conduction block Sick sinus Syndrome	~40		chest fluttering, slow/racing heartbeat, anxiety, fatigue	MI, CAD, VHD, HF, cardiac surgery, CHD, HTN, thyroid disease, diabetes, sleep apnea, electrolyte imbalance, stress, stimulants (caffeine)	HF, stroke, blood clots, SCD
VHD	MR AS AR MS TR	~35		dyspnea, fatigue, angina, dizziness, arrhythmia, lower limb edema	RHD, IE, age, MI, HTN, dyslipidemia, diabetes	HF, stroke, arrhythmia, blood clots
Cardio-myopathy	Dilated Hypertrophic Arrhythmo-genic RV Restrictive Peripartum Cardiac Amyloidosis Cardiac Sarcoidosis	~16		dyspnea, angina, lower limb edema, cough, fatigue, rapid/pounding heartbeat, dizziness	CAD, MI, HTN, tachycardia, VHD, diabetes, obesity, thyroid disease, amyloidosis, sarcoidoisis, hemochromatosis, alcohol, smoking, drug abuse, chemotherapy, stress, anxiety	HF, blood clots, VHD, CS, SCD

	Takotsubo syndrome					
Pericarditis	Acute Constrictive		~2	cough, fatigue, low-grade fever, palpitations, dyspnea, lower limb edema	AID, cardiac surgery, MI, kidney failure, infection, cardiac or chest injury	pericardial effusion, cardiac tamponade
Myocarditis	Acute Fulminant Chronic		~1.5	fever, joint aches, muscle aches, sore throat, dyspnea, arrhythmia, fatigue, angina, lower limb edema	infections (viruses, bacteria, fungi, parasites), chemotherapy and certain antibiotics, AID, chemicals, radiation	HF, MI, CS, stroke, arrhythmia, SCD
Infective Endocarditis			~0.5	fever, joint/ muscle aches, dyspnea, angina, fatigue, nocturnal sweats, murmurs, lower limb edema, hematuria, petechiae	artificial valves, implanted heart devices, damaged valves due to previous IE/rheumatic fever, CHD, age, poor dental health	VHD, HF, stroke, MI, CS, PE, kidney damage, splenomegaly, abscesses in heart/ lung/brain

Cerebro-vascular disease	Ischaemic stroke Hemorhagic stroke Cerebral aneurysm	~100	~15	severe headache, blurred/blackened vision, unilateral numbness/ weakness/ paralysis, confusion, discoordination, imbalance	PAD, HTN, diabetes, dyslipidemia, sleep apnea, HF, CHD, heart infection, obesity, AF, family history, age, hormone therapy (estrogen) physical inactivity, alcohol, drug abuse	paralysis, dementia, depression, trouble of talking/thinking/ understanding
Aortic disease	Aortic aneurysm Aortic dissection Takayasu arteritis Marfan syndrome	~35	~ 0.1-0.3 (Aortic dissection)	abdominal/lower back pain, pulsating feeling in the belly. In case of rupture, sudden severe leg/sotmach/chest/ upperback pain and stroke -like symptoms	age, HTN, atherosclerosis, aortic valve defects, connective tissue disorders, high-intensity resistance training	aortic dissection leading to, internal bleeding, kidney failure, intestinal damage, AR, stroke, cardiac tamponade
PAD	Lower extremity artery disease Carotid artery disease			claudication, lower limb muscles cramps, coldness/numbness in the legs, changes in skin color of the legs	atherosclerosis, age, dyslipidemia, HTN, diabetes, smoking, obesity, elevated levels of homocysteine amino acid	critical limb ischemia, stroke, MI

	Renal artery stenosis			(shiny), hair loss in the legs		
Venous disease	DVT PE Varicose vein		~8	leg swelling, leg pain/cramps, local sensation of heat, red/purple areas in the legs. In case of PE, sudden shortness of breath, angina, tachypnea, tachycardia, excessive sweating, hemoptysis, fainting,	age, lack of movement, surgery, pregnancy, oral contraceptives and hormone replacement therapy, obesity, smoking, HF, IBD, family history of DVT	PE
Pulmonary hypertension	PAH CTEPH	~30		dyspnea, blue/gray skin, angina, palpitations, fatigue, dizziness/faintness, lower limb swelling	COPD, PE, blood clotting disorders, HF, VHD, cirrhosis, sleep apnea, exposure to high altitudes, family history of the disease, obesity, smoking, CHD	HF, AF, life threatening pulmonary clots or bleeding
CHD	Atrial septal defect Ventricular septal defect Patent ductus arteriosus	~12	~1 (1% of annual births)	arrhythmia, murmur, dyspnea, exercise intolerance, edema, cyanosis	gestational diabetes, genetics, gestational smoking or alcohol consumption, embryonic exposure to	arrhythmia, IE, stroke, pulmonary hypertension, HF,

	Ebstein anomaly Tetralogy of Fallot Pulmonary atresia				maternal drugs (lithium, anticonvulsants, tranquilizers, antiemetics, analgesics, external hormones, isotretinoin), infection during pregnancy (rubella)	

Abbreviations: ACS, Acute Coronary Syndrome; AF, Atrial Fibrillation; AID, Autoimmune Disease; AR, Aortic Regurgitation; AS, Aortic Stenosis; CA, Coronary Artery; CAD, Coronary Artery Disease; CHD, Congenital Heart Disease; CKD, Chronic Kidney Disease; COPD, Chronic Obstructive Pulmonary Disease; CS, Cardiogenic Shock; CTEPH, Chronic ThromboEmbolic Pulmonary Hypertension; DVT, Deep Venous Thrombosis; HF, Heart Failure; HFmrEF, Heart Failure with Mildly Reduced Ejection Fraction; HFpEF, Heart Failure with Preserved Ejection Fraction; HFrEF, Heart Failure with Reduced Ejection Fraction; HTN, Hypertension; IBD, Inflammatory Bowel Disease; IE, Infective Endocarditis; MI, Myocardial Infarction; MR, Mitral Regurgitation; MS, Mitral Stenosis; NSTEMI, non-ST-elevated MI; PAD, Peripheral Artery Disease; PAH, Pulmonary Artery Hypertension; PE, Pulmonary Embolism; RHD, Rheumatic Heart Disease; SCD, Sudden Cardiac Death; STEMI, ST-elevated MI; SVT, Supraventricular Tachycardia; TR, Tricuspid Regurgitation; Vfib, Ventricular Fibrillation; VHD, Valvular Heart Disease; VT, Ventricular Tachycardia.

INTRODUCTION

III. COVID-19

III.I: COVID-19 Pandemic and Associated Inflammation

COVID-19 is an acute respiratory distress syndrome that affected more than 700 million patients worldwide and claimed the lives of around 7 million victims **(100)**. Most common symptoms of COVID-19 in the order of their prevalence include fever, dry cough, fatigue, dyspnea, myalgia, sore throat, headache and chills. COVID-19 pandemic is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the Betacoronavirus family, which include SARS-CoV-1 and Middle East Respiratory Syndrome-Coronavirus among others. SARS-CoV-2 has a large genome of positive-sense single stranded RNA. While the first 2/3 of its genome encodes non-structural proteins, the last 1/3 part encodes mainly 4 structural proteins namely, S for spike, E for envelope, M for membrane and N for nucleocaspid. SARS-CoV-2 uses its spike protein S to gain entry into target cells which interacts with ACE 2 receptors through its receptor binding domain. The abundant expression of ACE 2 in the upper respiratory tract and lungs makes them the first target of the virus and hence explain the viral ways of transmission and associated respiratory symptoms. However, ACE 2 is known to be sparsely expressed throughout the body on a wide range of cellular subtypes hence rendering the characterization of SAR-CoV-2 tropism complicated. For instance, several studies have concluded that SARS-

CoV-2 was able to infect multiple organs including lungs, kidney, liver, heart and vasculature **(100)**.

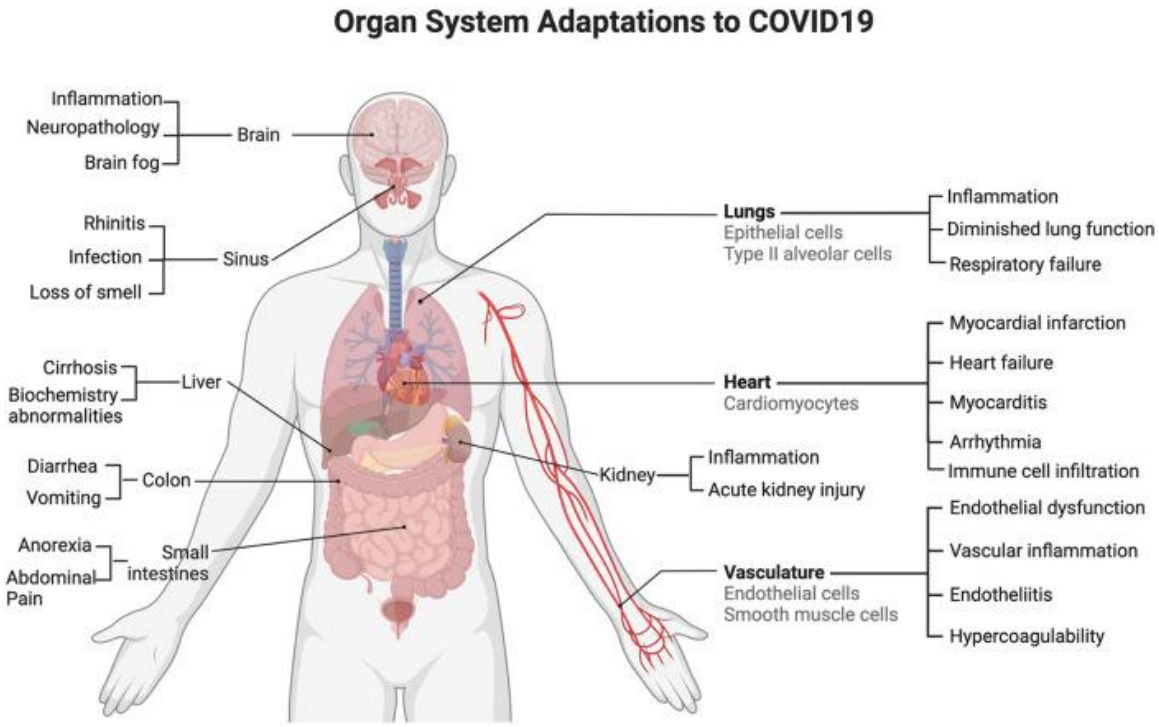

Figure 10: Organ adaptations to COVID-19 and associated symptoms **(101)**.

The binding of viral S protein to ACE 2 initiates viral endosomal internalization by the host in a Ca^{2+} dependent manner. Internalized SARS-CoV-2 drives cellular genomic machinery towards the amplification of viral genome and uses cellular surface for expressing viral proteins. This alters cellular physiology including repair mechanisms, transcription, replication and metabolism, all of which leads to inflammatory response. In fact, COVID-19 is well characterized by an NF-κB mediated inflammatory profile. Several mechanisms underlie such response, which starts with the binding of

viral protein S to ACE 2. Such binding inhibits the ability of ACE 2 to bind and inactivate bradykinin pro-inflammatory metabolite Des-Arg9-Bradykinin. In turn, the pro-inflammatory metabolite promotes NF-κB mediated TNF-α expression and macrophage adhesion into affected areas. Another SARS-CoV-2 mediated inducer of NF-κB includes the dysregulation of intracellular Ca^{2+} homeostasis. In fact, SARS-CoV-2 E protein act as virioporin, a transmembrane pore-forming protein that serves as ion channel particularly Ca^{2+} channels in this case. This enhances Ca^{2+} intracellular entry which besides favoring viral entry and replication, leads as well to increased PLC-PKC signaling, subsequent activation of NADPH oxidases, oxidative damage and activation of NF-κB. Additional implication of SARS-CoV-2 in the induction of NF-κB mediated inflammation is through the activation of Toll-like receptor (TLR) signaling through multiple mechanisms. For instance, endosomal internalization of the virus can potentially activate TLR4. In addition, TLR7 and TLR8 identify the viral single stranded RNA. Further, viral proteins act as pathogen associated molecular patterns, which are recognized by pattern recognition receptors and hence lead to activation of TLR2. Collectively, activation of TLRs in infected host leads to overexpression and enhanced activation of NF-κB and consequent overproduction of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. On the other hand, viral RNA recognition within the host initiates mitochondrial antiviral signaling which activates

downstream IKK leading to phosphorylation, translocation and activation of NF-κB. Adding on, SARS-CoV-2 was shown to enhance mitochondrial ROS production and induction of mitochondrial dysfunction, a phenotype that was shown to be associated with increased intracellular pro-inflammatory signaling. Furthermore, SARS-CoV-2 was demonstrated to repress the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor responsible of the expression of numerous antioxidant proteins. This halts the intracellular defensive mechanism against oxidative stress and triggers inflammation through multiple pathways including NF-κB, MAPKs (p38, ERK1/2, JNK) and NLRP3 (nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3) inflammasome **(100,101)**.

III.II: COVID-19 and Cardiovascular Diseases

COVID-19 and CVDs have a complex connection where the first leads to the latter and vice versa. Primarily, SARS-CoV-2 benefits from the inherent function of the CV system (CVS). For instance, CVS promotes delivery of the viral replicates into diverse ACE 2 expressing cells in a wide range of organs. Adding on, patients with CVD are more prone to COVID-19 infection and severe symptoms, where up to 25% of the COVID-19 affected population had at least one concomitant CVD. In fact, patients with underlying CVD have higher mortality risk in response to COVID-19. A possible explanation of this observation can be due to the fact that patients with CVDs express elevated levels of ACE 2, which increases viral infectious capacity **(102)**.

On the other hand, CV complications are common outcome of COVID-19. For instance, most common mortality reasons in response to COVID-19 are respiratory failure and cardiac complications. CVDs associated with COVID-19 vary greatly from patient to patient and can include endo/myo/pericarditis, arterial and venous thrombotic events, MI, HF, cardiomyopathy and arrhythmias. Interestingly, and despite the fact that most severe COVID-19 associated CV symptoms occur during the acute phase of the syndrome (< 4 weeks), CV issues also appear during the ongoing phase of the syndrome (4-12 weeks) or even in the

post-COVID-19 syndrome (> 12 weeks); the latter 2 being known as long-COVID **(103)**.

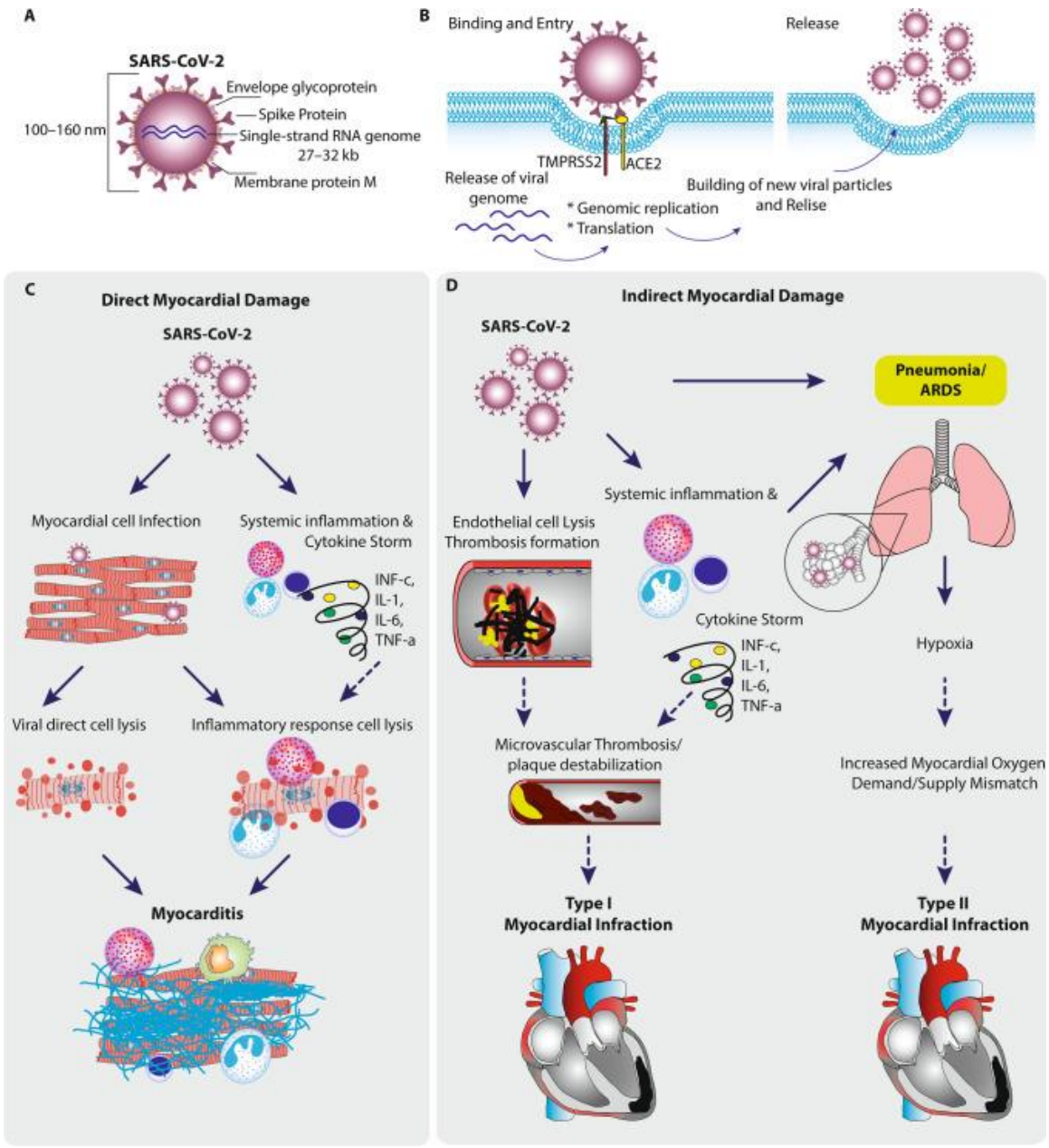

Figure 11: Direct and indirect COVID-19 associated myocardial injury **(103)**. INF-c, interferon c; IL-1, interleukin-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.

Several mechanisms have been suggested to explain CV complications in COVID-19. Direct myocardial injury due to viral infection is one of them **(104)**. In fact, multiple autopsy studies have demonstrated infiltration of SARS-CoV-2 into CMs. This is associated with decrease in structural and functional signature proteins as viral load increases, hence promoting imbalances in CMs Ca^{2+} handling and CMs inflammation or myocarditis. Further, imbalances of Ca^{2+} induced by COVID-19 could as well support cardiac dysfunction. For instance, lower serum Ca^{2+} levels have been observed in COVID-19 patients **(105, 106)**. Despite the direct mechanism, CV complications can occur in response to indirect ischemic effects either as a result of low O_2 prevalence due to lung pathology, or as a consequence of viral toxicity of micro- and macro-vascular beds. Interestingly, lung pathology leads to low O_2 saturation, which can be translated as an imbalance between CMs O_2 supply and demand. In turn, this leads to type-II MI **(105, 107)**. On the other hand, viral invasion of the vascular bed and particularly ECs can explain several mechanisms implicated in COVID-19 induced CV injury. In fact, viral infection of ECs has been demonstrated in different body organs including lungs, heart kidney and liver. This is mainly due to the high expression of ACE 2 on ECs **(105, 108)**.

SARS-CoV-2 binding to ACE 2 on ECs inhibits its enzymatic activity. Under normal conditions, ACE 2 plays a vital role in regulating vascular tone through catalyzing the breakdown of Ang II into Ang 1-7.

As such, loss of ACE 2 enzymatic activity is accompanied with increased levels of circulating Ang II and subsequently vasoconstriction, a form of ischemic trigger. Further, lack of Ang II clearance is associated with increased levels of plasminogen-activator-1 (PAI-1), a risk factor for MI **(105, 108)**.

In the same context, whether direct viral infection of ECs or the cytokine storm (elevated plasma levels of IL-1β, IL-6, TNF-α and MCP-1 among others) associated with COVID-19 both trigger severe endothelial inflammation and dysfunction. This leads to oxidative damage of ECs, which is mainly through induction of eNOS uncoupling and superoxide degradation of NO yielding peroxynitrites ($ONOO^{\cdot-}$). Peroxynitrites are in turn powerful nitrosylating agents which nitrosylates intracellular proteins leading to endothelial dysfunction. Additionally low NO bioavailability reduces VSMCs and CMs cGMP-PKG downstream signaling and as such increases vascular and cardiac stiffness. In coronary circulation, vascular stiffness is associated with reduced blood flow to the myocardium rendering it ischemic. On the other hand, low NO paracrine signaling into the myocardium is a major risk factor of HFpEF development **(108, 109)**.

Another impact of COVID-19 endotheliitis lies in the increased coagulopathy. Damage of the endothelial layer exposes the underlying BL, which is enriched by increased levels of vWF produced by inflamed ECs. This enhances platelet binding and aggregation. Adding

on, inflamed ECs express lower levels of ADAMTS13 (ADAM metallopeptidase with thrombospondin type 1 motif 13, a vWF cleaving metalloprotease) which leads to lower clearance of platelet-rich thrombosis. Interestingly, lower plasma levels of ADAMTS13 were detected in patients with severe COVID-19 and as such ADAMTS13 were founded to be potent predictors of COVID-19 outcomes. Similarly, endotheliitis is characterized by increased expression of CAMs (ICAM-1, VCAM-1) and selectins (e- and p-selectin) both of which favors leukocyte adhesion and particularly activated monocytes. In turn both inflamed ECs and infiltrated monocytes produce increased levels of TF, which boosts coagulation at the surface of inflamed ECs. Additionally, adhesion markers at the surface of activated ECs could induce NETosis, cell death dependent on the formation of neutrophil extracellular traps (NET, mainly composed of proteases and decondensed chromatin), particularly through the activation of the complement system. Consequently, this leads to the inhibition of tissue factor pathway inhibitor (TFPI), an inhibitory pathway that prevents TF mediated hyper-coagulation **(109-111)**.

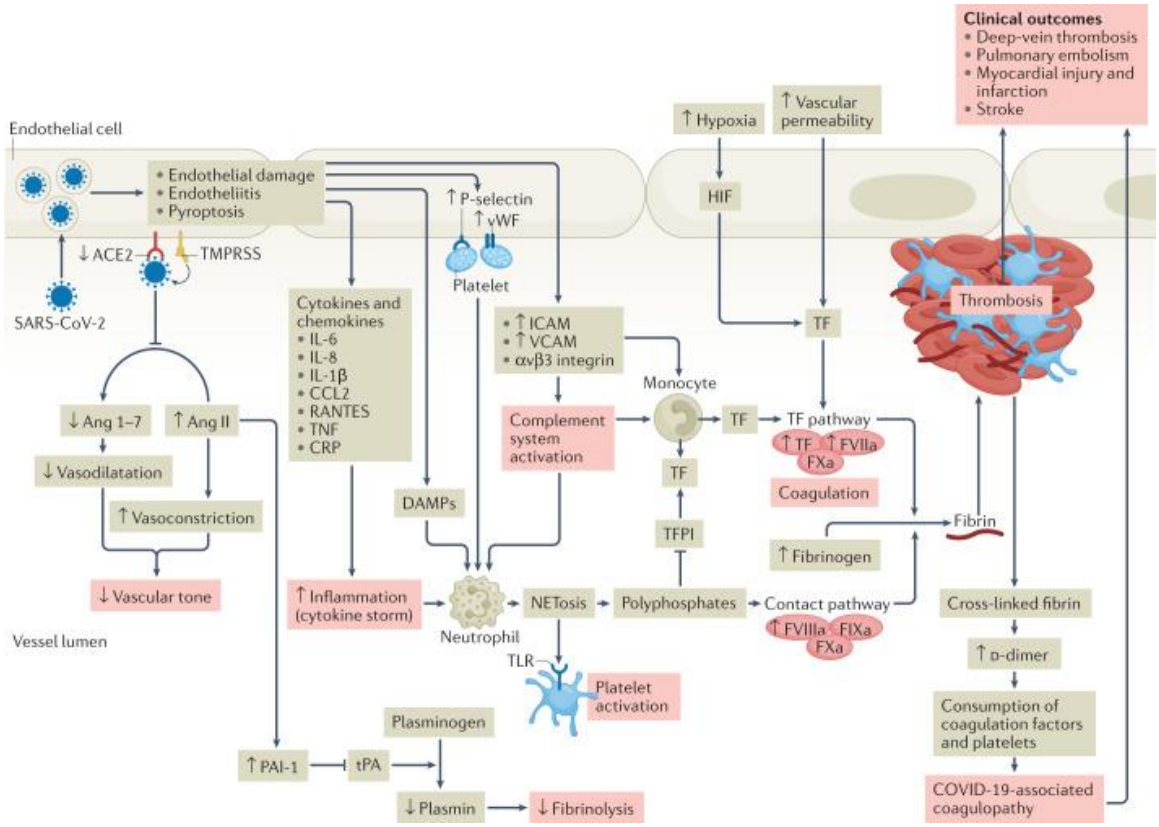

Figure 12: Endothelial mechanism of COVID-19 induced coagulopathy **(110)**. ACE2, angiotensin converting enzyme 2; Ang 1–7, angiotensin 1-7; Ang II, angiotensin II; CCL2, CC-motif chemokine 2; CRP, C-reactive protein; DAMP, damage-associated molecular pattern; FVIIa, activated coagulation factor VII; FVIIIa, activated coagulation factor VIII; FIXa, activated coagulation factor IX; FXa, activated coagulation factor X; HIF, hypoxia-inducible factor; ICAM, intercellular adhesion molecule; IL-1, interleukin 1; IL-6, interleukin 6; IL-8, interleukin 8; NETosis, neutrophil extracellular traps; PAI-1, plasminogen activator inhibitor 1; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TLR, toll-like receptor; TMPRSS, transmembrane serine protease 2; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; vWF, von Willebrand factor.

Moreover, inflamed ECs shed excessive amounts of phosphatidylserine-rich vesicles known as extracellular vesicles. Extracellular vesicles are known to be potent activators of several coagulation factors particularly factor X and prothrombin. Collectively, overexpression of vWF and TF accompanied with the concomitant down-regulation of ADAMTS13 and TFPI, in addition to extracellular vesicles shedding all could explain the increased micro- and macro-vascular arterial and venous thrombotic events observed in COVID-19 which contribute to cardiac ischemic events **(111, 112)**.

INTRODUCTION

IV. SGLTs, Gliflozins and Cardiovascular Disease

IV.I: Sodium-glucose co-Transporters: Structure, Function and Localization

Glucose homeostasis is of pivotal importance. In fact, glucose is the first source of energy in the brain and second only to free fatty acids (FFAs) in the heart. As such, glucose plays a major role in cellular metabolism and hence, adequate intracellular levels of glucose should be maintained to ensure proper cellular function. Cellular needs of glucose are received from circulating blood, and thus, blood glucose (glycemia) levels should be well preserved to avoid hypoglycemic seizures and hyperglycemic toxicities. Intracellular entry of glucose requires membrane transporters, which will allow the hydrophilic molecule to cross lipid plasma membranes. In humans, three different families of glucose transporters have been identified: GLUTs, SGLTs and SWEETs **(113)**.

SWEETs (Sugars Will Eventually be Exported Transporters) are the last of the three to be identified and not much about their function is known in animals as compared to their well described contributions in plants. In contrast, GLUTs (GLUcose Transporters) are the first of the three to be sequenced and studied. The GLUT family is expressed by SLC2 gene and is part of the major facilitator superfamily of membrane transporters. In humans, fourteen different GLUT proteins have been identified, which have different cellular localizations and different

affinities for different hexoses. While all the GLUTs are passive transporters, i.e., transport glucose down its concentration gradient, some of them are insulin dependent (GLUT4) while others are not (GLUT1, GLUT2) **(113, 114)**.

SGLTs (Sodium GLucose co-Transporters) on the other hand, are insulin-independent active glucose transporters. As a matter of fact, SGLTs mediate intracellular apical glucose transportation against its concentration gradient along with concomitant downhill transport of Na^+ in the same direction. The Na^+ gradient is in turn maintained through active Na^+ extracellular transport mainly by Na^+/K^+ ATPase. As such, while glucose transport through SGLTs is not dependent on insulin, it is indeed dependent on Na^+ homeostasis **(113, 115, 116)**.

SGLTs are 50-80 kDa transmembrane proteins and are encoded by the SLC5 gene family. While 12 members of the gene family are identified in humans, only 7 encode for SGLT proteins while the expression of the remaining produces co-transporters of sodium along with other solutes. The 7 members of the SGLT protein family (SGLT1-5, along with sodium myo-inositol co-transporters SMIT1-2) have variable tissue localizations and differ by their specificity, affinity and capacity of transport **(Table 3)**. Interestingly, SGLT3 is not a glucose transporter per se, but rather a glucose sensor. Among the 6 SGLT proteins, SGLT1 and SGLT2 are the major isoforms that have been widely studied and explored **(113, 115, 116)**.

Table 3: Distribution, Substrates, and Properties of SGLT (115-118).

Gene	Protein	Substrate	Sodium:Glucose Stoichioetry	Km (Major Substrate, mM)	Localization
SLC5A1	SGLT1	Glucose = Galactose	2:1	0.4	Intestine, Trachea, Kidney, Heart, Brain, Testis, Prostate
SLC5A2	SGLT2	Glucose	1:1	2	Kidney, Brain, Liver, Thyroid, Heart[#]
SLC5A4	SGLT3	Glucose[$]	N/A	20	Intestine, Brain, Uterus, Lung, Spleen, Kidney
SLC5A9	SGLT4	Mannose >> Glucose > Fructose > Galactose		0.15	Intestine, Kidney, Liver, Brain
SLC5A10	SGLT5	Mannose > Fructose >> Glucose > Galactose		0.45	Kidney cortex
SLC5A3	SMIT1	Myo-Inositol,		0.05	Heart, Brain, Kidney
SLC5A11	SMIT2 (SGLT6)	Myo-Inositol, Chiro-Inositol		0.1-0.3	Brain, Kidney, Liver, Intestine, Lung, Muscle

Abbreviations: Na^+, Sodium; SGLT, Sodium-Glucose co-transporter; SMIT, Sodium Myo-Inositol co-transporter. * SGLT1 acts as a uniporter for H_2O and urea as well; # SGLT2 expression in human heart is still controversial; $ SGLT3 in humans do not transport glucose, but rather acts as a glucose sensor (glucose-activated Na^+ channel).

i. SGLT1:

SGLT1 was the first of the 6 isoforms to be discovered. It was first introduced in 1960 in Prague at the Symposium on Membrane Transport and Metabolism. The introduction of SGLT1 came as an explanation of the active glucose transport through the luminal brush-border membrane of the enterocytes in the small intestine. Later, SGLT1 was proved to act as Na+ co-transporter for both glucose and galactose in addition to serving as uniporter for H_2O and urea. Besides the small intestine, which is the major site of SGLT1 expression, SGLT1 has as well been shown to be inherently expressed in several organs including kidney, heart, brain and others. SGLT1 functions as a high-affinity low-efficiency transporter of glucose. For instance, while it is the major transporter involved in the absorption of glucose across the intestinal membrane, SGLT1 activity corresponds to only less than 10% of glucose reabsorption in the kidney. SGLT1 expression in the intestine was shown to be only affected by luminal glucose levels but not glycemia. For instance, oral ingestion of carbohydrates-rich diet was shown to elevate SGLT1 expression in the intestine, which was not

the case following intravenous injection of glucose **(115-119)**. Additionally, SGLT1 expression was shown to follow a diurnal rhythm where higher SGLT1 levels are observed during waking hours. Post-translational regulation of SGLT1 by protein kinases has been studied as well. In fact, both PKA and PKC active sites have been found on SGLT1. PKA-dependent phosphorylation has been demonstrated to increase cytoplasmic SGLT1 localization into plasma membrane as well as increased SGLT1 activity in response to the activation of the cyclic adenosine monophosphate (cAMP) pathway. Similarly, PKC in humans has been shown to increase SGLT1 mediated transport capacity. PKC mediated effects are predicted to include activation of several redox-sensitive kinases including p38 MAPK, JNK and ERK1/2. On the other hand, studies have shown that while SGLT1 expression and function are not affected by the absence of leptin, significantly elevated leptin levels reduce SGLT1 intestinal expression and activity. In contrast, elevated leptin levels significantly increased SGLT1 expression in cardiac tissue. Within the same context, increased cardiac glucose uptake in response to insulin and leptin were both shown to be partially mediated by SGLT1 **(120, 121)**. Noteworthy, cardiac SGLT1 expression (CECs, CMs, and cardiac fibroblasts) was shown to be significantly elevated in several conditions including pressure/volume overload and diabetic/ischemic cardiomyopathy **(122)**.

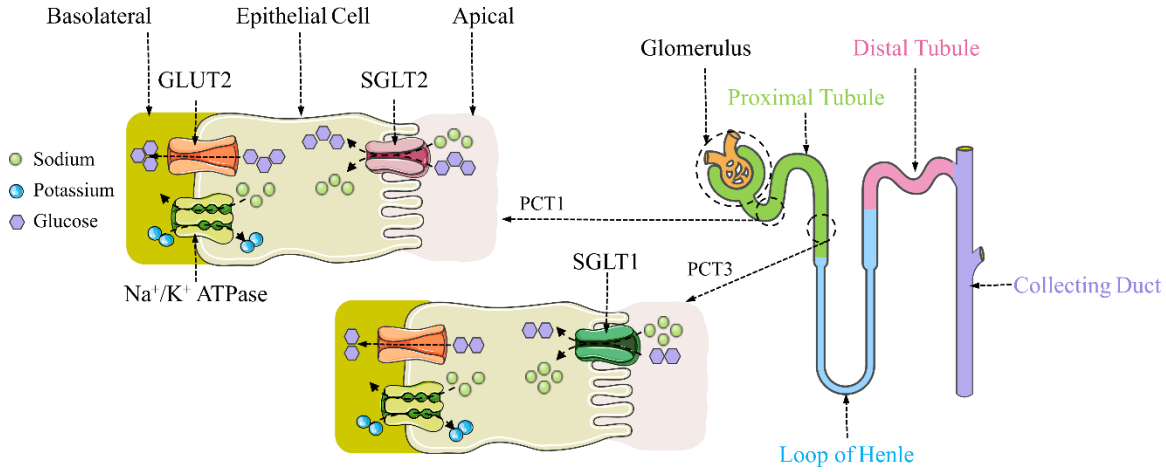

Figure 12: SGLT1/2 mediated glucose reabsorption in the kidney. (Image was adjusted by the author using illustrations provided by smart.servier.com).

ii. SGLT2

SGLT2 was first identified in the kidneys. In fact, human kidneys filter around 180 g of glucose daily of which less than 1 % is excreted into the urine. This highly efficient reabsorption of filtered glucose back into blood stream ensures adequate glycemia levels (4-8 mmol/L depending on fasting duration) and hence proper glucose supply for cellular use of diverse human organs. Interestingly, SGLT2 is responsible for almost 90% of total glucose reabsorption in the kidneys, while SGLT1 contributes to the remaining 10%. Briefly, SGLT2 is located mainly in the early proximal tubule segments S1 and S2 at the apical side of tubular epithelial cells in contrast to SGLT1 which is localized to S3. Filtered glucose by renal glomeruli is actively transported into tubular epithelial cells through SGLT2 (or SGLT1), to

be later exported downhill its concentration gradient from the basal side into the blood through GLUT2. SGLT2 works at a Na^+/glucose coupling ratio of 1:1 (1 Na^+ molecule is reabsorbed for each 1 glucose molecule) in contrast to SGLT1 that requires 2 Na^+ for each glucose molecule. As such and despite the lower affinity of SGLT2 as compared to SGLT1, SGLT2 is more efficient in glucose transport. Hence, SGLT2 is known as a low-affinity high-capacity transporter **(115-119)**. Extra-renal expression of SGLT2 is still debatable. While most of the data in literature strictly insist on specific kidney expression of SGLT2, only sparse number of studies have demonstrated limited SGLT2 mRNA levels in organs as liver, heart and brain. Nevertheless, proof of expression of functional SGLT2 protein in these extra-renal organs was not evidenced **(123)**.

SGLT2 expression in the renal proximal tubules is well maintained and controlled under normal conditions, however, elevated renal levels of the transporter are observed in response to hyperglycemia **(123)**. Such overexpression aims to increase the glucose reabsorption capacity, despite the fact that transporter saturation is reached in cases of persistent highly elevated glycemia and significant amount of glucose will be excreted in urine. Elevated levels of SGLT2 in diabetic conditions lead as well to increased reabsorption of Na^+ at the proximal tubule. Consequently, Na^+ levels at the downstream macula densa decrease, which induces diabetes-mediated renal hyper-filtration

through activation of RAAS. The long-term effect of such change is diabetic nephropathy, the major cause of end-stage kidney failure **(120, 121, 123)**.

IV.II: Discovery of SGLT inhibitors: Story of the gliflozins

Owing to the major contribution of SGLT to the renal glucose handling and subsequently glucose hemostasis and overall glucose homeostasis, these transporters have been widely studied in the field of diabetes. Of particular interest was SGLT2 as it was believed that inhibiting the transporter function would aid in reducing glycemic levels as a consequence of inhibiting filtered glucose reabsorption at the renal proximal tubules into bloodstream. This was further supported by the fact that SGLT2 expression and function were found to be elevated in diabetic situations leading to higher glucose reabsorption. As such, SGLT2 was regarded as a contributor to the overt hyperglycemia and the potential role of SGLT2 inhibitors (SGLT2is) in diabetic situations was examined **(124)**.

The first described SGLT inhibitor is phlorizin, a phloretin O-glucoside. Phlorizin is a naturally occurring polyphenol; it was isolated in 1835 and is highly abundant in the bark of apple, pear and cherry trees. The molecule was later shown to significantly induce glycosuria in an unknown mechanism which was a century later understood to be a consequence of inhibiting SGLT2-mediated glucose reabsorption in the kidneys. This discovery made phlorizin a tempting molecule to treat type 2 diabetes-mellitus (T2DM). However, phlorezin inhibition of SGLT2 was not specific, as the molecule induced potent inhibitory

effects on SGLT1 as well. The high abundance of SGLT1 in the intestinal brush led to phlorizin-induced multiple gastro-intestinal side effects which made the molecule unpleasant to use for curative purposes. Another obstacle faced the usage of phlorizin was its low oral bioavailability due to the susceptibility of O-glucoside to the intestinal β-glucosidases. Subsequently, usage of phlorizin and any of its O-glucoside derivative was not further investigated. In the early 21st century, C-glucoside derivatives of phlorizin were introduced and shown to escape β-glucosidases cleavage due to the C-C bond rather than O-C. These derivatives that are now known as gliflozins have higher oral bioavailability and improved SGLT2-to-SGLT1 specificity as compared to phlorizin **(125-127)**.

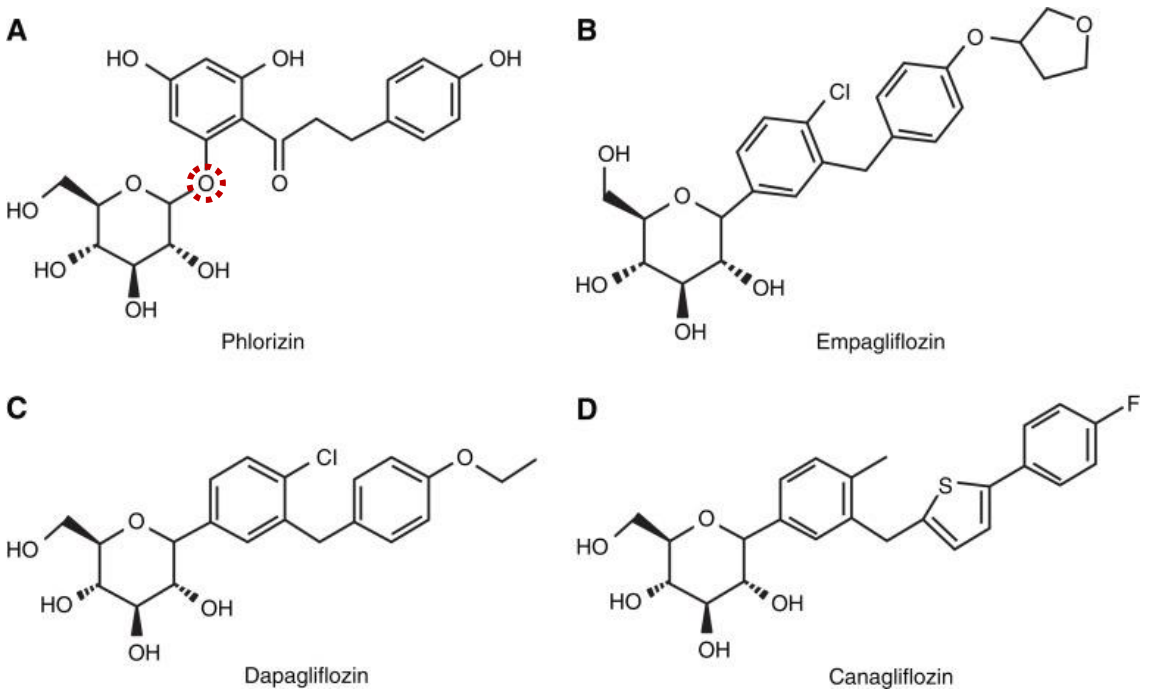

Figure 13: Chemical structure of phlorizin (A), empagliflozin (B), dapagliflozin (C) and canagliflozin (D) **(124)**.

In clinic, gliflozins were indeed well tolerated and demonstrated significant glucose-lowering effects in diabetic patients. Glycated hemoglobin (HbA1c) reduction following gliflozin therapy ranged between 0.5% and 1%. Interestingly, gliflozins significantly reduced systolic blood pressure (3-5 mmHg) as well as body weight (2-3 kg) in diabetic patients. To date, 6 members of the gliflozin family are approved by the American Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of T2DM. These drugs are canagliflozin, dapagliflozin, empagliflozin, ertugliflozin, bexagliflozin and sotagliflozin. While the first five are all considered as

selective SGLT2 inhibitors with selectivity to SGLT2 at least 100 folds greater than to SGLT1, sotagliflozin on the other hand falls into the category of SGLT1 and SGLT2 dual inhibitor. Of interest, EMA has as well approved sotagliflozin usage in type 1 diabetes as a supportive treatment to insulin while FDA raised concerns about associated diabetic ketoacidosis and thus reserved its approval for T2DM only. The different characteristics of the approved gliflozins are demonstrated in **Table 4 (128-131)**.

Table 4: Pharmacokinetic and Pharmacodynamic Characteristics of Gliflozins (128-131).

	Bexagliflozin	Canagliflozin	Dapagliflozin	Empagliflozin	Ertugliflozin	Sotagliflozin
FDA Approval	2023	2013	2014	2014	2017	2023
EMA Approval	N/A	2013	2012	2014	2018	2019
Daily Dosage (mg)	20	100-300	5-10	10-25	5-15	200-400
Oral Bioavailability (%)	78	65	78	70-90	~ 100	71
IC_{50} SGLT2 (nM)	2	2.7	1.2	3.1	0.87	1.8
IC_{50} SGLT1 (nM)	5600	710	1400	8300	1960	36
SGLT2 Selectivity (fold)	2800	263	1166	2677	2252	20
Indication	T2DM	T2DM, CKD*, HF*	T2DM, CKD, HF	T2DM, CKD, HF	T2DM	T2DM, CKD, HF, T1D#

Abbreviations: EMA, European Medicines Agency; FDA, Food and Drug Administration; CKD, Chronic Kidney Disease; HF, Heart Failure; T1D, Type 1 Diabetes; T2DM, Type 2 Diabetes Mellitus. * Only in patients with T2DM. # Approval only by EMA.

IV.III: Cardiovascular and Renal Outcomes of Gliflozins

T2DM is a common metabolic disease and a major risk factor for CVD. Additionally, presence of T2DM is often accompanied with other CV risk factors such as dyslipidemia, hypertension and others. As such, patients receiving treatment for T2DM might as well be receiving other medications for other CV risk factors or even CVD, hence treating T2DM should take into consideration the overall situation of the patient and particularly cardiac protection. As a matter of fact, vigorous glycemic control, which is the major target of T2DM treatment does not always lead to CV protection, in contrast, it might be deleterious in certain situations. Collectively, and as a consequence of all the aforementioned, the FDA in 2008 followed by EMA in 2012 have recommended that novel anti-diabetic drugs undergo additional clinical evaluation of their potential to reduce major adverse CV events (MACE) in patients with diabetes **(132, 133)**.

i. Gliflozins in Atherosclerotic CVD

The first anti-diabetic drug and SGLT2i clinical outcome to demonstrate reduced MACE was EMPA-REG OUTCOME trial which was held back in 2015 **(134)**. The trial showed that patients with concomitant T2DM and overt CVD who received empagliflozin had

38% and 35 % relative risk reduction in CV death and HF hospitalization respectively. While the EMPA-REG outcome trial included only T2DM patients with established atherosclerotic CVD (ASCVD), other SGLT2i OUTCOME trials such as DECLARE-TIMI 58 and CANVAS for dapagliflozin and canagliflozin respectively included T2DM patients with either ASCVD or risk factors for ASCVD **(135, 136)**. Interestingly, these studies demonstrated significant MACE reduction in both subpopulations receiving either of the SGLT2i as compared to placebo. However, a randomized meta-analysis of cardiovascular outcome trials on patients with T2DM concluded that SGLT2i prevented atherosclerotic MACE (MI, CV death, stroke) only in patients with ASCVD (ischemic heart disease, stroke) while such beneficial effects were limited in the absence of overt ASCVD **(137)**. Of importance, clinical data from the EMMY trial showed that post-MI treatment with empagliflozin significantly reduced NT-pro-BNP levels as compared to placebo independent of T2DM **(138)**. On the other hand, a multicenter international registry (SGLT2-I AMI PROTECT Registry) demonstrated that usage of SGLT2i in diabetic patients with acute MI significantly decreased MACE as compared to other anti-diabetic drugs **(139)**. In the same context, a recent meta-analysis came to a conclusion that SGLT2i-mediated MACE reduction (HF, AF, stroke and MI) in patients with T2DM was

independent of the SGLT2i used, the comparison included empagliflozin, dapagliflozin and canagliflozin **(140)**.

ii. Gliflozins in HF

The SGLT2i-mediated reduction in HF hospitalization of diabetic patients revealed by EMPA-REG trial was of high importance and thus was further delineated. Two consequent randomized placebo controlled clinical trials were as such designed as follow up studies in conditions of HFrEF and HFpEF named EMPEROR-Reduced and EMPEROR-Preserved respectively. Interestingly, the trials evaluated the effect of empagliflozin on HF among patients with and without T2DM. Both studies showed significant reduction in the combined risk of CV death or HF hospitalization by 25% and 21% respectively **(141, 142)**. Further, similar outcomes were as well revealed in the randomized placebo controlled DAPA-HF and DELIVER clinical trials, which investigated the effect of dapagliflozin on diabetic and non-diabetic patients with either HFrEF or HFpEF, respectively. The trials with dapagliflozin revealed significant reduction in worsening HF events or CV death by 26% and 18 % respectively **(143, 144)**. Interestingly, all the four trials showed similar cardio-protection regardless of the presence or absence of T2DM. Therefore, these results indicate that the class-effect benefit of SGLT2i in HF covers the whole

ejection fraction spectrum and is independent of the glycemic condition of the patient.

iii. Gliflozin in Renal Disease

One of the major complications of uncontrolled diabetes is diabetic nephropathy, the leading cause of end-stage kidney failure. Despite the fact that the intended design of the major CV outcome trials (EMPA-REG OUTCOME, DECLARE TIMI 58 and CANVAS) was not to evaluate renal outcome of SGLT2i in T2DM **(134-136)**, yet, post-hoc and secondary analyses of these studies revealed that SGLT2i significantly decreased composite kidney outcome which was specified as doubling of serum creatinine or 40% decrease in estimated glomerular filtration rate (eGFR), end-stage kidney disease or mortality due to renal complications. The overall risk reduction was evaluated to be > 30% **(145-147)**. Similarly, pooled analysis of EMPEROR-Reduced and EMPROR-Preserved revealed that annual eGFR decrease was significantly higher in placebo group as compared to empagliflozin group. Interestingly, the results revealed that empagliflozin has more prominent reno-protective effects in HFpEF group as compared to HFrEF, yet the effects were indifferent between diabetic and non-diabetic groups **(148)**. Consistently, in the EMPA-Kidney trial, empagliflozin significantly reduced the 2 years progression of kidney

disease in patients with CKD independent of T2DM and throughout eGFR spectrum ranging from 20 to 90 mL/min/1.73 m^2 **(149)**. Another demonstration of the effect of SGLT2i in patients with CKD was through the clinical trial DAPA-CKD. The trial included patients with CKD and with or without T2DM. The primary outcome of the trial was sustained decrease of at least 50% in eGFR, end-stage kidney disease or death from renal/CV reasons. The DAPA-CKD trial concluded that dapagliflozin significantly reduced the incidence of the afore-mentioned composite kidney outcomes by 40% as compared to placebo **(150)**. Similar results were observed in the randomized double blinded placebo controlled clinical trial CREDENCE. The reported outcomes concluded that canagliflozin reduced by 34% the renal composite (end-stage kidney disease, doubling of creatinine levels or mortality from renal disease) in patients with T2DM and CKD. Of interest, the outcomes of CREDENCE study were independent of RAAS as all the patients were on RAAS inhibitors **(151)**. Adding on, gathered information from recent meta-analyses of clinical data showed that SGLT2i could decrease the risk of acute kidney injury (AKI) by > 40% as compared to placebo and by 20-30% as compared to other anti-diabetic drugs **(152-154)**.

IV.IV: Mechanisms of Gliflozin-Mediated Cardiovascular Protection

The exact mechanism by which SGLT2i exert their cardio-protective effects is not fully understood, yet several mechanisms have been proposed to explain the class-effect of SGLT2i. These mechanisms include indirect systemic effects of SGLT2i and direct cardiac effects.

i. Systemic Cardio-Protective Effects of SGLT2i

Systemic cardio-protective effects of SGLT2i can be summarized through their contribution to the improvement of renal function, vascular physiology or overall homeostasis.

(1) Glycemic Control: SGLT2i are indeed associated with significant decrease of blood glucose levels in patients with T2DM. Taking into consideration that hyperglycemia is a major risk factor for CVD, glycemic control is in favor of reducing MACE. However, SGLT2i cardio-protective effects were prominent in patients with and without diabetes. Additionally, several anti-diabetic drugs with superior reduction in HbA1c levels as compared to SGLT2i failed to reduce MACE in clinical trials. As such, while glycemic control might indeed

reduce MACE risk, it cannot be the only explanation of SGLT2i effects **(155, 156)**.

(2) Diuresis and Natriuresis: SGLT2i promote osmotic diuresis through natriuresis and glycosuria. This is associated with reduction in volume overload, and as such reduced CM eccentric remodeling and improved CM contractility. On the other hand, clinical studies with other diuretics, such as hydrochlorothiazide or loop diuretics did not show reduction in HF incidence. Comparison studies revealed that SGLT2i diuretic effect might lead to different outcomes as compared to other diuretics including hemo-concentration and reduced plasma volume particularly in the interstitial space as compared to luminal plasma. Such differential diuretic effect can indeed reduce neuro-hormonal reflex stimulation that usually occurs in response to intravascular volume reduction observed with other diuretics, however account only for partial explanation of SGLT2i protective effects **(155)**.

(3) Reduced Hyperuricemia: Elevated levels of uric acid in the blood were shown to be associated with worse outcomes in patients with HF. On the other hand, SGLT2i in the clinic showed modest decrease in uric acid levels as a consequence of their increased glycosuria. Yet, such observations cannot fully address a causative role in SGLT2i cardio-protection rather than being just another marker of HF improvement **(155)**.

(4) Weight Loss: SGLT2i mediated glycosuria leads to reduced glucose availability for cellular metabolism, and instead, FFAs from adipose stores are used. This is indeed translated into the weight loss observed in patients receiving SGLT2i. However, weight loss strategies have always failed to demonstrate improved HF outcomes as the ones seen with SGLT2i, and hence such phenotype cannot be the sole mechanism to which SGLT2i cardio-protective effects are addressed **(155)**.

(5) Lower Blood Pressure: Hypertension is the precipitating factor for hypertensive heart disease and one of the major risk factors for HF. Surprisingly, SGLT2is in clinic were associated with significant decrease in blood pressure which was explained by several mechanisms including natriuresis, reduced RAAS activation, decreased vascular stiffness, improved vascular tone and enhanced vascular function. Such blood lowering effect is indeed a decrease in cardiac after-load and hence can partially explain cardio-protection observed with SGLT2i **(155)**.

(6) Increased Hematocrit: DAPA-HF trial has shown that dapagliflozin treatment in patients with or without diabetes had increased hematocrit. Further, another study demonstrated that one month treatment with empagliflozin was associated with increased renal production of erythropoietin in patients with T2DM **(155)**.

Collectively, these results suggest that SGLT2i treatment might lead to increased O_2 supply to CMs **(155)**.

(7) Reduced sympathetic nervous system firing: SGLT2i in preclinical animal models of both diabetes and obesity have shown inhibitory effect on the sympathetic nervous system. These data could explain the clinical observation that SGLT2i reduce blood pressure in the absence of increased HR **(155)**.

(8) Improved Endothelial Function: Endothelial dysfunction is a major component of multiple CV diseases. Indeed, ECs appear to be the first to be affected in several aspects especially as a response to the low-grade inflammation associated with CVD. Additionally, ED is reported to be the priming factor leading to ASCVD and HFpEF. Interestingly, SGLT2i in clinical practice have been reported to enhance flow-mediated dilation in T2DM patients with ASCVD and HF (**155, 157)**. Different preclinical studies in different animal models have as well documented the role of empagliflozin on endothelium-mediated vaso-relaxation particularly through protecting the NO component. *In vitro* studies on human and animal cultured cells have as well reported that SGLT2i decrease intracellular oxidative stress in ECs particularly through reducing the expression and/or function of different subunits of NADPH oxidases. Additionally, SGLT2is in culture were able to prevent TNF-α induced ROS formation, NF-κB activation as well as CAMs expression (**155, 157)**.

As such, all of the afore-mentioned results in addition to others do give a reasonable hint about a potential role of SGLT2is in preventing ED.

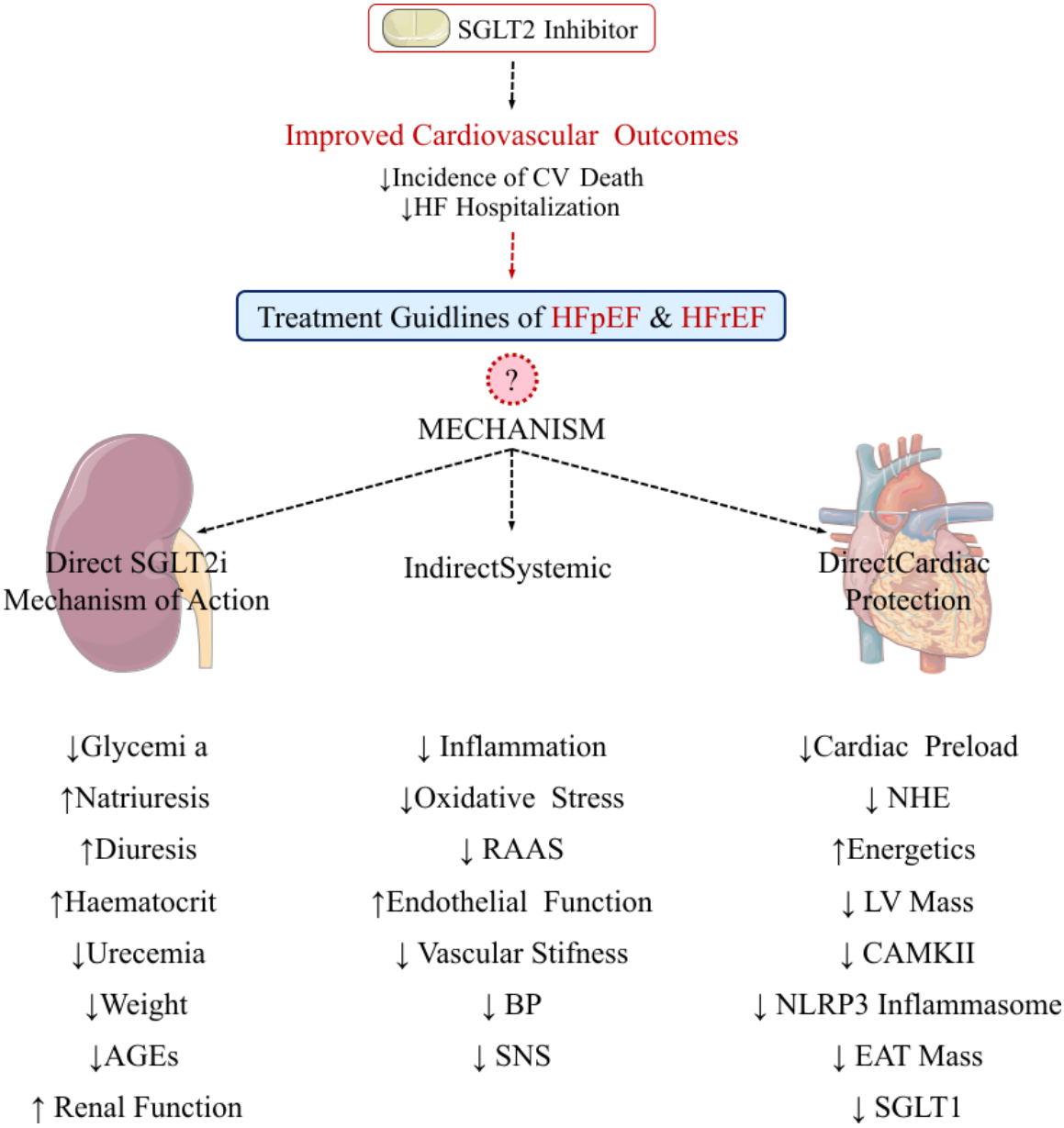

Figure 13: Mechanisms of SGLT2-mediated cardiovascular protection. AGE, advanced glycated end-product; BP, blood pressure; CAMKII, calmodulin kinase II; EAT, epicardial adipose tissue; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LV, left ventricle; NHE, sodium-hydrogen exchanger; RAAS, renin angiotensin aldosterone system; SNS, sympathetic nervous system; SGLT, sodium glucose co-transporter; SGLT2i, SGLT2 inhibitor. (Image was adjusted by the author using illustrations provided by smart.servier.com).

ii. Direct Cardio-Protective Effects of SGLT2i

In the absence of robust data supporting SGLT2 expression in the heart, direct cardiac protection of SGLT2i have been doubted until several off-target mechanisms were proposed.

(1) Maintaining Intracellular Na^+ Homoeostasis: Na^+ homeostasis is essential for cardiac proper functioning. Several membrane channels contribute to Na^+ trafficking including sodium-hydrogen exchanger (NA^+/H^+, NHE) which exchanges 1 extracellular Na^+ for 1 intracellular H^+, Na^+/Ca^{2+} exchanger (NCX) which exchanges 3 extracellular Na^+ for 1 intracellular Ca^{2+} and Na^+/K^+ ATPase which exchanges 3 intracellular Na^+ for 2 extracellular K^+. While the first drives influx of Na^+ along its concentration gradient, NA^+/K^+ ATPase maintains Na^+ gradient through active efflux of Na^+. As such, improper function of any of these channels could affect the function of the other and hence perturbate Na^+, K^+ and Ca^{2+} homeostasis. Interestingly, in

HF, NHE expression is increased leading to elevated intracellular concentrations of Na^+ ($[Na^+]_i$). This leads to reduction in NCX mediated Na^+ influx and hence Ca^{2+} efflux which is the major efflux route for Ca^{2+}, and hence leading to increased $[Ca^{2+}]_i$ as well. Subsequently, such alterations underline electrical imbalance in CMs and contribute to cardiac stiffness and abnormal contractility both of which precipitate and exaggerate HF. Surprisingly, docking simulations revealed that SGLT2i have a high affinity for the extracellular Na^+ binding site of NHE. Additionally, preclinical studies have demonstrated that SGLT2is were able to inhibit NHE mediated Na^+ influx to levels similar to cariporide (selective NHE inhibitor). Such inhibition was associated with improved cardiac function in animal models of HF **(155, 157, 158)**. Another contributor to Na^+ homeostasis is the voltage gated sodium channel (Nav). In the heart, Nav1.5 is the major isoform expressed. The cardiac action potential is characterized by a late sodium current (I_{Na+}) which occurs at the action potential plateau as a result of Na^+ influx via Nav1.5. This influx prolongs cardiac action potential and enhances Ca^{2+} influx. Several reports have underlined the pathophysiological role of increased late-I_{Na+} in various CV diseases including HF, MI and AF. Such increases in the amplitude of late-I_{Na+} are often accompanied with elevated $[Ca^{2+}]_i$, impaired ventricular relaxation and contractility, arrhythmias and disturbed myocardial energetics **(159)**. Interestingly, *in silico* analysis have

revealed that SGLT2is bind to Nav1.5 at the same region as ranolazine (selective inhibitor of Nav1.5). Further, electrophysiological studies on cardiomyocytes from rodent models of diabetes and HF have revealed that SGLT2is attenuate late-I_{Na+}, accelerate its inactivation process and reduce spontaneous Ca^{2+} transients in response to late-I_{Na+} activation **(157)**.

(2) Inhibiting Cardiac SGLT1: While SGLT2 expression is not well proven in human heart, SGLT1 is natively expressed. As such, and despite the low specificity of SGLT2i to SGLT1, yet direct cardiac actions of SGLT2is could be at least partially attributed to their inhibition of cardiac SGLT1. Subsequently, binding to and inhibition of SGLT1 by a SGLT2i would result in reduced glucose influx into CMs and hence decreased ROS formation, mitochondrial dysfunction and enhance cardiac energetics **(155, 157, 158)**. However, such explanation remains uncertain, especially with the absence of major differences in cardiovascular outcomes among various SGLT2i regardless the variability in their SGLT2 specificity, in addition to the non-superiority of the SGLT1-SGLT2 dual inhibitor Sotagliflozin in preventing MACE as compared to selective SGLT2is in clinic **(160)**.

(3) Improving Cardiac Remodeling: Adverse cardiac remodeling subsequent to cardiac injury and/or remodeling signals contribute to worsening HF outcome through induction of cardiac fibrosis, hypertrophy inflammation and death of CMs. Of interest,

SGLT2i in clinic demonstrated lower LV mass following 6 months of treatment in patients with T2DM and history of CAD, however, the underlying mechanism was not clarified. Similar results were also observed in preclinical studies where SGLT2i in post-MI rodent models were associated with lower collagen deposition and extracellular matrix expansion and anti-fibrotic effects. Subsequently, cardiac remodeling was improved, LV wall stress was reduced and overall cardiac function was enhanced. Worth mentioning, lower activation of RAAS observed with SGLT2is can as well partially explain the improved cardiac remodeling as Ang II through AT1R plays a detrimental role in adverse remodeling **(155, 157, 158)**.

(4) Enhancing Cardiac Energetics: HF progression as accompanied with drastic change in cardiac metabolism and decrease in mitochondrial oxidative phosphorylation **(161)**. As such, failing heart relies more and more on glycolysis for energy production. Yet, in condition of HF, glucose oxidation rates drop leading to uncoupling of glucose oxidation to glycolysis, which is associated with reduced cardiac work /O_2 consumption and as such decreased cardiac efficiency. On the other hand, SGLT2is mobilize free fatty acids in adipose stores and thus increased plasma levels of free fatty acids in patients with T2DM were observed following SGLT2i treatment **(155, 157)**. As a possible consequence, availability of free fatty acids to CMs would increase and a shift in cardiac mitochondrial metabolism occurs

favoring fatty acid oxidation at the expense of glucose oxidation. Such observations were confirmed in a non-diabetic post-MI porcine model. Additionally, SGLT2i could promote hepatic ketogenesis in response to reduced glucose availability. This increases circulating ketone bodies which was observed in patients with and without T2DM following SGLT2i treatment regimens **(155, 157)**. Availability of ketone bodies and induction of ketone oxidation serves as an additional fuel supply for the failing heart. Further, ketone oxidation yields higher amounts of ATP molecules for the same amount of O_2 consumed as compared to glucose and thus leading to oxygen sparing **(155, 157)**. For instance, HF patients who were perfused with ketones demonstrated improved cardiac contractility **(162)**. Interestingly, empagliflozin enhanced ketone, free fatty acids and branched amino acids cardiac metabolism in a porcine model of MI. Further, empagliflozin treatment in patients with T2DM was associated with increased ratio of phosphocreatine/ATP which is considered as a hall-mark improvement in cardiac energetic state **(155, 157)**. Another implication of SGLT2i on cardiac energy could be consequent to the reduction of cardiac glucose uptake. This signals a fasting state to the heart which activates silent information regulator transcript-1 (SIRT1) and concomitant adenosine monophosphate-activated protein kinase (AMPK) pathway to increase fatty acid β-oxidation, ketogenesis and improved energy generation by mitochondria **(163, 164)**.

(5) Reducing Cardiac Inflammation and Oxidative Stress: Elevated levels of pro-inflammatory markers have been observed in patients with HF, whether HFpEF or HFrEF, and their levels positively correlate with the worse outcomes of the disease. Such chronic-low grade inflammation can initiate and propagate HF. Additionally, increased levels of cardiac oxidative stress have as well been observed in conditions of HF, which contributes to subsequent inflammatory activation. Of particular interest is the NLRP3 inflammasome. For instance, activation of NLRP3 inflammasome worsens HF severity due to chronic cardiac inflammation. *In vivo* studies of diverse rodent models revealed that SGLT2i significantly reduced cardiac oxidative stress and NLRP3 inflammasome activation. Interestingly, the reduction was consistent among HFpEF and HFrEF models **(155, 157)**. Additionally, in human cardiomyocytes and cardiac tissue, SGLT2i were associated with lower levels of NLRP3 inflammasome mediated inflammation. Noteworthy, SGLT2i in patients with T2DM decreased NLRP3 activation in human macrophages. The mechanism through which SGLT2i repress NLRP3 inflammasome is not fully understood, yet elevated ketone bodies observed with SGLT2i can be an explanation as the ketone β-hydroxybutyrate is a potent inhibitor of NLRP-mediated inflammation. On top of that, SGLT2i could improve cardiac autophagy which is impaired in conditions of diabetes and HF **(155, 157, 158)**. Such

observation can as well be a secondary explanation of the cardio-protective effects associated with SGLT2i treatment.

(6) Alleviating Ischemia-Reperfusion Injury: Ischemia-reperfusion cardiac injury is a pertinent cause of CM death and HF. Ischemia-reperfusion injury is mainly a result of disturbed intracellular Ca^{2+} load which is associated with increased activity of CAMKII during ischemic periods and to a higher extent during early reperfusion. Such activation leads to alterations in CM stiffness, contractility and noteworthy arrhythmias. Notably, in preclinical models of diabetic and non-diabetic rats, usage of SGLT2i was associated with significant reduction in CAMKII activity and subsequent enhancement in Ca^{2+} flux from sarcoplasmic reticulum and CM contractility; all of which underlined the reduced ischemia-reperfusion cardiac injury observed in the treated rats **(155, 157, 158)**. However, a clinical effect of SGLT2i in ischemia-reperfusion cardiac injury is still not well affirmed.

(7) Decreasing Epicardial Fat Mass: Several reports have associated increased epicardial adipose tissue mass with increased risk of several CV events including HF and AF. In fact, epicardial adipose tissue is a highly active metabolic tissue that secretes a variety of bioactive molecules which affect myocardial function through paracrine signaling. In cases of hypertrophy epicardial adipose tissue produces increased levels of cytokines such as TNF-α and thrombotic molecules such as PAI-I both of which have detrimental effects on

proper cardiac function. Interestingly, SGLT2i in patients with diabetes have been associated with reduced epicardial adipose tissue mass and inflammation, which can alleviate adverse cardiac remodeling signals **(155)**.

OBJECTIVES

PART I: Despite the advances in pharmacological and clinical research, CVDs remain a major cause of morbidity and the leading cause of mortality worldwide. Unfortunately, with the increased abundance of unhealthy lifestyle and the aging of the population, the prevalence of CVDs is expected to increase including that of HF. Several treatment regimens have been applied to treat HFrEF, however, no treatment showed beneficial effects in HFpEF until the discovery of SGLT2is. Interestingly, SGLT2is in clinic were associated with reduced HF hospitalization and MACE despite patients' EF and independent of diabetes, CAD or previous incidence of MI. Further, SGLT2is have recently demonstrated improved CV outcomes following acute MI and reduced recurrency of AF in diabetic patients following ablation. However, the underlying mechanism for the afore-mentioned cardio-protective effects are still not fully elucidated.

On the other hand, low-grade inflammation is now widely described as an underlying trigger for the incidence and progression of multiple CVDs, and targeting low-grade inflammation remains under investigation with the aim of reducing the burden of major CVDs. Even though several mechanisms have been proposed for the cardio-protective effects observed with SGLT2is, a major obstacle remains the lack of robust scientific evidence describing SGLT2 expression pattern in human CV system. In fact, until recently extra-renal SGLT2 expression was still a doubt and hence, clarifying the role of SGLT2 in

the CV system is important to uncover the reality behind the clinical effects of SGLT2i and could as well pave the way for more effective and safer application of these drugs. As such, the objective of the first part of the current work was to: 1) evaluate SGLT2 expression in human vascular conduits and determine the potential trigger; 2) establish, if any, an expression pattern of SGLT2 in human cardiac tissue and find out its connection to low-grade inflammation and/or oxidative stress; 3) localize SGLT2 expression in cellular subtypes of human CV system; 4) clarify whether direct inflammatory stimulant could induce SGLT2 expression in ECs and discover the underlying pathway; 5) line out the difference between the role of SGLT1 and SGLT2 in ED; 6) figure out the potential protective effect of SGLT2i on inflamed ECs.

PART II: COVID-19 pandemic was associated with major acute and long-term CV complications including increased risk of arrhythmias, HF, ischemic heart disease, stroke and thromboembolic events. Such manifestations were remarked even in the absence of previous CV risk yet were dependent on the severity of the infection. Several mechanisms have been proposed to explain the observed outcomes one of which includes endotheliopathy as a consequence of the cytokine storm. Then again, with the major protective effects observed with SGLT2i in CVDs characterized by low-grade inflammation and ED, the objective of the second part of the current work was to: 1) examine whether plasma from COVID-19 patients could induce ED using a translational approach; 2) determine the role of single pro-inflammatory cytokine or combination of cytokines present in the plasma of COVID-19 patients in promoting ED, 3) investigate the implication of the AT1R/NADPH oxidases pro-oxidant pathway in mediating ED in response to stimulation with plasma of COVID-19 patients, 4) evaluate the contribution of SGLT2 to the propagation and amplification of the late pro-oxidant signal consequently to the initial insult with plasma of COVID-19 patients; 5) uncover the endothelial thrombogenic phenotype following induction with plasma of COVID-19 patients; 6) figure out the potential anti-thrombogenic effects of SGLT2i on dysfunctional ECs.

RESULTS

PART I

SGLT2 Expression in Human Vasculature and Heart Correlates with Low-Grade Inflammation and Causes eNOS-NO/ROS Imbalance

SGLT2is are the first class of drugs that eliminates glucose from human body, and hence the primary goal for their development was to treat T2DM. Surprisingly, CV outcome trials of these drugs demonstrated robust cardiorenal and CV protective effects in patients with or without T2DM. The effects included reduced risk for HF hospitalization, post-MI complications and overall CV mortality. As such, these results led to the inclusion of SGLT2i in the treatment regimen of symptomatic HF throughout the whole ejection fraction.

Several mechanisms have been proposed to explain the CV protection observed with SGLT2is including improved cardiac energetics, enhanced cardiac remodeling, diminished ischemia-reperfusion injury and reduced CV and endothelial inflammation and oxidative stress. However, all these mechanisms did not consider SGLT2 expression in the CV system as the main target of the inhibitors. In fact, the observed effects were rather annotated to be either due to off target cardiac receptors of SGLT2is or as a consequence of their systemic effects such as reduced glycemia, uricemia and blood pressure, weight loss,

improved natriuresis, diuresis and hematocrit in addition to enhanced renal function.

Therefore, in the current work, we investigated the expression pattern of SGLT2 in human vasculature (using 70 internal thoracic arteries) and cardiac biospecimens (using 20 left ventricle biopsies) and evaluated whether such pattern correlates to common CV risk factors including inflammation and oxidative stress. Additionally, we aimed to establish a direct effect of pro-inflammatory cytokines on SGLT2 expression in endothelial cells (ECs) and examined the consequences and protective effects of SGLT2 inhibition.

L’expression des SGLT2 dans le système vasculaire et le cœur humains est en corrélation avec une inflammation de bas grade et provoque un déséquilibre eNOS-NO/ROS

Les inhibiteurs des cotransporteurs 2 sodium-glucose (SGLT2is) sont la première classe de médicaments éliminant le glucose dans le corps humain ; le premier objectif pour leur développement fut ainsi le traitement du diabète de type 2 (T2DM). Étonnement, les essais cliniques cardiovasculaires (CV) de ces médicaments ont montré de forts effets protecteurs au niveau cardio-rénal et cardiovasculaire chez des patients atteints ou non atteints de T2DM. Les effets comprenaient un risque réduit d’hospitalisation pour insuffisance cardiaque (IC), de complications suite à un infarctus du myocarde (IM) et de la mortalité CV en général. Ces résultats en tant que tels conduisaient à l’inclusion de SGLT2i dans le schéma thérapeutique de l'IC symptomatique pendant toute la fraction d’éjection.

Plusieurs mécanismes ont été proposés afin d’expliquer la protection CV observée avec SGLT2is, y compris une amélioration de l’énergétique cardiaque, un meilleur remodelage cardiaque, une lésion d’ischémie-reperfusion moindre ainsi qu’une réduction de l’inflammation et du stress oxydatif au niveaux CV et endothélial. Cependant, tous ces mécanismes ne considéraient pas l’expression des

SGLT2 dans le système CV comme principale cible de ces inhibiteurs. Ainsi, les effets observés ont plutôt été commentés comme reposant soit sur des récepteurs cardiaques non ciblés des SGLT2is ou comme étant une conséquence de leurs effets systémiques tels qu'une diminution de la glycémie, de l'uricémie et de la tension artérielle, une perte de poids, une meilleure natriurie, diurèse et valeur d'hématocrite en complément d'une fonction rénale améliorée.

Par conséquent, dans le cadre de nos travaux actuels, nous avons étudié le modèle d'expression des SGLT2 dans des échantillons biologiques humains issus du système vasculaire (utilisant 70 artères thoraciques internes) et du cœur (utilisant 20 biopsies du ventricule gauche) et avons évalué si de tels modèles sont en corrélation avec les facteurs de risque CV courants, comprenant inflammation et stress oxydatif. En complément, nous avons essayé d'établir un lien d'effet direct des cytokines pro-inflammatoires sur l'expression des SGLT2 dans les cellules endothéliales (CEs) et avons étudié les conséquences et les effets protecteurs de l'inhibition des SGLT2.

SGLT2 expression in human vasculature and heart correlates with low-grade inflammation and causes eNOS-NO/ROS imbalance

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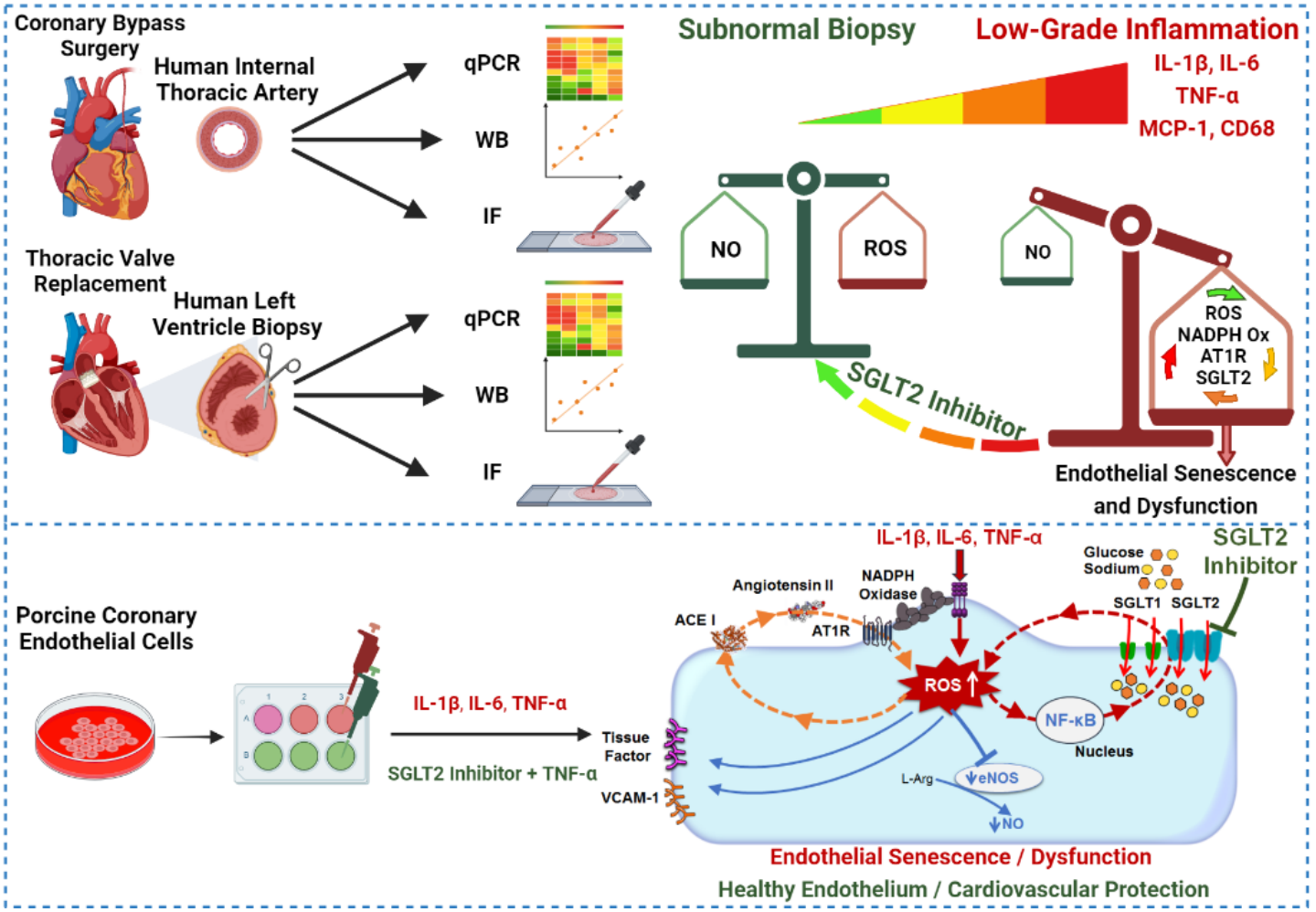
Category of the Manuscript: Original Article

Word Count: (7348 words)


Coronary Bypass Surgery
Human Internal Thoracic Artery
qPCR
WB
IF
Thoracic Valve Replacement
Human Left Ventricle Biopsy
qPCR
WB
IF
Subnormal Biopsy
Low-Grade Inflammation
IL-1β, IL-6
TNF-α
MCP-1, CD68
NO
ROS
NO
ROS
NADPH Ox
AT1R
SGLT2
SGLT2 Inhibitor
Endothelial Senescence and Dysfunction
Porcine Coronary Endothelial Cells
IL-1β, IL-6, TNF-α
SGLT2 Inhibitor + TNF-α
Tissue Factor
VCAM-1
IL-1β, IL-6, TNF-α
Angiotensin II
NADPH Oxidase
ACE I
AT1R
ROS ↑
Glucose
Sodium
SGLT1
SGLT2
SGLT2 Inhibitor
NF-κB
Nucleus
L-Arg
eNOS
NO
Endothelial Senescence / Dysfunction
Healthy Endothelium / Cardiovascular Protection

ABSTRACT

Background Sodium-glucose co-transporter2 inhibitors (SGLT2i) show a cardioprotective effect in heart failure and myocardial infarction, pathologies often associated with low-grade inflammation.
Objectives This cross-sectional study aims to investigate whether low-grade inflammation regulates SGLT2 expression and function in human vasculature, heart, and endothelial cells (ECs).
Methods Human internal thoracic artery (ITA), left ventricle (LV) specimens and cultured porcine coronary artery ECs were used. Expression of target molecules was assessed using RT-qPCR, Western blot analysis, and immunofluorescence staining, and the generation of reactive oxygen species (ROS) and nitric oxide (NO) using fluorescent probes. The function of SGLT2 was investigated using empagliflozin and SGLT1 or 2 siRNA.
Results SGLT2 mRNA and protein levels in ITA and LV specimens were correlated with the level of low-grade inflammation, markers of the angiotensin system, and EC activation. SGLT2 staining was observed in the ITA endothelium and smooth muscle, the coronary microcirculation, and cardiomyocytes. Elevated ROS formation in high SGLT2-expressing specimens was reduced by inhibition of the angiotensin system, SGLT2, and TNF-α. Exposure of ECs to IL-1ß, IL-6, and TNF-α led to an increase in SGLT1 and SGLT2 mRNA and protein expression, upregulation of components of the angiotensin system, enhanced ROS and decreased NO formation, and activation of NF-κB. The stimulatory effect of TNF-α was prevented by N-acetylcysteine and inhibition of the angiotensin system, SGLT2 but not SGLT1, and NF-κB.
Conclusions Low-grade inflammation is closely associated with SGLT2 expression in human vasculature and heart, and this response contributes to a feedforward mechanism with the AT1R/NADPH oxidases pathway to cause eNOS-NO/ROS imbalance.

KEY WORDS: Cardiovascular disease, SGLT2, pro-inflammatory cytokines, local angiotensin system, oxidative stress, endothelial dysfunction

TRANSLATIONAL PERSPECTIVE: In this study, we showed SGLT2 expression in the human endothelium and vascular smooth muscle of ITA, and in the human coronary microcirculation and cardiomyocytes in LV areas affected by macrophage infiltration and low-grade inflammation, and contributed to the pro-oxidant activator signal. Moreover, we showed that pro-inflammatory cytokines upregulated SGLT2 expression in endothelial cells to perpetuate oxidative stress leading to endothelial dysfunction and pro-inflammatory responses. As such, the understanding of expression pattern of SGLT2 in human vascular and heart affected by inflammation contributes to a better understanding of the role of SGLT2 in the pathophysiology of cardiovascular disease and may lead to optimize personalized medicine and suggest a potential role of SGLT2i in inflammatory disease.

ABBREVIATIONS AND ACRONYMS

Ang II = angiotensin II
ACE1 = angiotensin-converting enzyme1
AT1R = angiotensin II type 1 receptor
ECs = endothelial cells
eNOS = endothelial NO synthase
HF = heart failure
ICAM-1 = intercellular cell adhesion molecule-1
IL-1β = interleukin-1β
IL-6 = interleukin-6
ITA = internal thoracic artery
LV = left ventricle
NF-κB = nuclear factor-κB
NO = nitric oxide
ROS = reactive oxygen species
RT-qPCR = real-time quantitative polymerase chain reaction
SGLT1 = sodium-glucose co-transporter1
SGLT2 = sodium-glucose co-transporter2
T2DM = type 2 diabetes mellitus
TNF-α = tumor necrosis factor-α
VCAM-1 = vascular cell adhesion molecule-1

INTRODUCTION

Sodium-glucose co-transporter 2 inhibitors (SGLT2i) originally intended for use in diabetes, show an unexpected, pronounced cardiorenal benefit in patients at high cardiovascular risk and were recently approved for use as foundational therapy for symptomatic heart failure (HF) across the entire spectrum of ejection fraction and regardless of diabetes status. The mechanisms underlying the cardiovascular benefit of SGLT2i still remain incompletely understood. SGLT2 inhibition has been linked to the attenuation of oxidative stress, the improvement in cardiac energetics, the promotion of mitochondrial health and renewal, and the reduction of systemic inflammation and pro-apoptotic responses.[1] In patients with type 2 diabetes mellitus (T2DM) chronic inflammation and oxidative stress have been closely linked to the onset and progression of cardiorenal dysfunction and diabetic atherosclerosis.[2] Increased SGLT2 mRNA and protein levels together with markers of oxidative stress and inflammation were observed in asymptomatic atherosclerotic plaques of diabetic patients undergoing carotid endarterectomy compared to non-diabetic patients, and to current users of SGLT2i.[3] *In vitro* studies further indicated that high glucose levels can directly upregulate SGLT2 mRNA and protein levels in AC16 cardiomyocytes, leading to enhanced glucose uptake, oxidative stress and apoptosis,[4] and in ECs, resulting in pro-oxidant, pro-inflammatory, and pro-thrombotic responses and the induction of endothelial senescence and dysfunction.[3,5] Notably, similar effects were also observed in ECs in response to angiotensin II (Ang II) and H_2O_2 under normoglucose conditions.[5,6] The characterization of the stimulatory effect of SGLT2 inducers indicated that SGLT2 expression is mediated by the AT1R/NADPH oxidases pro-oxidant pathway and contributed sustaining the oxidative stress activator signal.[5,6] *In vivo* studies showed that increased vascular SGLT2 levels contributed to oxidative stress, inflammation, and endothelial dysfunction in Ang II-induced hypertensive rats, particularly at arterial sites with varying flow rates, promoting atherosclerosis by stimulating pro-inflammatory cytokine expression.[6-8]

Altogether these observations support the notion that SGLT2 expression is associated with the transition from cardiovascular health to disease characterized by oxidative stress, inflammation, and endothelial dysfunction.[9,10] Nonetheless, there is limited information available regarding the expression of SGLT2, besides in atherosclerotic plaques, in the heart and vasculature of patients with cardiovascular disease. A critical analysis of the mechanisms underlying the cardiovascular benefits of SGLT2 inhibitors from an inflammatory perspective, as well as an evaluation of the inflammatory signaling/pro-oxidant activation hypothesis, remains unexplored.

As of now, it appears that when SGLT2 is upregulated, it plays a pivotal role in sustaining oxidative stress, thus actively promoting the progression of the disease. To assess the translational importance of the inflammatory signaling/pro-oxidant activation hypothesis of SGLT2, we aimed to evaluate SGLT2 expression in human vasculature (internal thoracic artery [ITA] specimens from patients undergoing bypass surgery) and heart (left ventricle [LV] specimens of patients undergoing aortic valve surgery) and to determine its relationship with pro-oxidant and EC activation responses and with the level of low-grade inflammation. In addition, the possible direct effect of pro-inflammatory cytokines on SGLT2 expression in ECs and its functional consequences were investigated.

METHODS

STUDY POPULATION

PATIENTS AND TISSUE HARVESTING

The cross-sectional study protocol complied with the principles of the Declaration of Helsinki and patients gave informed written consent before enrollment in accordance with the local Ethics Committee from Strasbourg University Hospital. Based on the translational research group experience, 70 patients with coronary artery disease undergoing bypass surgery (median age 69.5 [64.75-75.0] years old) and 20 patients with aortic stenosis requiring left ventricular outflow tract enlargement by sub-aortic myomectomy followed by aortic valve replacement (median age 69.5 [65.5-73.0] years old) were enrolled at the University Hospital of Strasbourg, France. Patients with a history of chronic inflammatory disorders or atrial fibrillation were excluded. The extent of coronary artery disease was characterized by coronary angiography, and aortic stenosis by echocardiography and computed tomography scan, respectively. Clinical characteristics of patients are presented in **Tables 1, 2 and 3**. Human ITA segments and LV specimens were processed as described previously[11] (see Supplementary material online).

ENDOTHELIAL CELL CULTURE

Freshly harvested pig hearts were obtained from a local slaughterhouse, and ECs were isolated from right coronary artery using collagenase (type I) and cultured in MCDB 131 medium supplemented with fungizone (2.5 µg/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), and 15% fetal calf serum as described previously.[6] All experiments were performed with ECs at passage 1 to avoid the induction of replicative senescence, and cells were exposed to serum-free culture medium for 2 h before treatment.

RNA ISOLATION AND REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

RNA was purified using TRIZOL® and processed for cDNA generation and gene expression analysis as previously described[6] (see Supplementary material online).

WESTERN BLOT ANALYSIS

Western immunoblotting was performed on ITA and LV homogenates, and ECs lysates as previously described[6] (see Supplementary material online).

IMMUNOFLUORESCENCE STAINING OF PROTEINS AND NF-κB NUCLEAR TRANSLOCATION

Target proteins in human ITA and LV cryosections, and in ECs were detected using immunofluorescence staining and fluorescence microscopy as described previously[6] (see Supplementary material online).

TISSUE AND CELLULAR LEVELS OF OXIDATIVE STRESS

The *in situ* generation of reactive oxygen species (ROS) in ITA and LV cryosections, and ECs was determined using dihydroethidium and fluorescence microscopy as described previously[6] (see Supplementary material online). The formation of superoxide anion in ECs was assessed by electron paramagnetic resonance (EPR) using the CMH probe (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpytrolidine HI) and the E-scan spectrometer as described previously[12] (see Supplementary material online).

ENDOTHELIAL FORMATION OF NITRIC OXIDE

NO formation in ECs was determined using the NO probe, DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluororescein diacetate) as described previously[6,11] (see Supplementary material online).

ENDOTHELIAL GLUCOSE UPTAKE BY FLOW CYTOMETRY

Glucose uptake was determined using the fluorescent probe 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBD-Glucose) as described previously[5] (see Supplementary material online).

SGLT1 AND SGLT2 siRNA TRANSFECTION STUDIES

SGLT1 siRNA, SGLT2 siRNA, and negative control siRNA (Eurogentec) were used to knock down the expression of SGLT1 and SGLT2 in ECs using Lipofectamine RNAiMAX (# 13778-150, Invitrogen) as per manufacturer's instructions (see Supplementary material online).

STATISTICAL ANALYSIS

For human studies, to assess the independent effect of SGLT2 expressions on the expression of pro-inflammatory cytokines in ITA and LV, we performed multiple regression analyses, where SGLT1 and SGLT2 were used as a dependent variable and IL-1ß, IL-6, TNF-α, age, male sex, hypertension, dyslipidemia, diabetes, smoking status, and medications including statins, beta-blockers, angiotensin-converting enzyme (ACE) inhibitor/ angiotensin II type 1 receptor (AT1R) blockers, and anti-platelet therapy as independent variables. The standardized beta values are presented for each covariate.

For *in vitro* studies, values are expressed as means ± standard error of the mean. Kolmogorov-Smirnov test was used to check out normality and statistical analysis was assessed by one-way analysis of variance followed by Tukey's multiple comparison post-hoc test using GraphPad

Prism (Version 9). The differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

SGLT2 EXPRESSION AND FUNCTION ARE CLOSELY CORRELATED WITH LOW-GRADE INFLAMMATION IN HUMAN INTERNAL THORACIC ARTERY SPECIMENS

Using real-time quantitative polymerase chain reaction (RT-qPCR), we analyzed 30 ITA specimens from patients undergoing bypass surgery. The findings revealed substantial variations in the expression of SGLT2 mRNA and SGLT1 mRNA among patients, as seen in **Figures 1A and 1B**, and Supplemental Figures 1A and 1B. The highest increases in expression levels were around 30-fold for SGLT1 mRNA and 35-fold for SGLT2 mRNA compared to their respective minimums. The levels of SGLT1 mRNA in ITA were 7 times higher than those of SGLT2 mRNA, with a strong positive correlation (r^2 = 0.9496, P < 0.0001; **Figure 1B).** Furthermore, analysis revealed a negative correlation between SGLT2 mRNA expression levels and those of eNOS in ITA, as well as a positive correlation with markers of vascular inflammation (IL-1ß, IL-6, TNF-α, and the macrophage marker CD68), the angiotensin system (AT1R and ACE1), the p47phox NADPH oxidase subunit, pro-thrombotic and pro-inflammatory responses (tissue factor, VCAM-1, ICAM-1, e-selectin, and MCP-1), and of senescence (p53, p21 and p16). These findings are illustrated in **Figure 1B** and Supplemental Figure 1B.

Next, a multivariate regression analysis (log-transformed values) was performed to determine whether SGLT2 gene expression level was associated with each of the markers independent of the baseline characteristics. SGLT2 expression was positively related to the pro-inflammatory cytokines IL-1ß (stand.ß: 0.87, *P* < 0.001), IL-6 (stand.ß: 1.06, *P* < 0.001), and TNF-α (stand.ß: 1.33, *P* < 0.001) independent of age, male sex, hypertension, dyslipidemia, diabetes, smoking, and also of medication treatment with the exception of statin treatment for IL-1ß (stand.ß: -4.88, *P* < 0.02), and male sex (stand.ß: -2.56, *P* < 0.04) and anti-platelet therapy (stand.ß: -5.36, *P* <

0.049) for TNF-α (Supplemental Table 1). A multivariate linear regression analysis also revealed that SGLT1 expression was positively related to IL-1ß (stand.ß: 0.73, $P < 0.001$), IL-6 (stand.ß: 0.94, $P < 0.001$), and TNF-α (stand.ß: 1.15, $P < 0.001$) independent of age, male sex, hypertension, dyslipidemia, diabetes, smoking and also of medication treatment with the exception of statin treatment for IL-1ß (stand.ß: -4.17, $P < 0.048$) (Supplemental Table 1).

Western blot analysis of 30 ITA specimens confirmed the heterogenous expression levels of SGLT2 and SGLT1 proteins with a 19-fold and 16-fold difference, respectively between specimens with the corresponding lowest and highest signal intensity (**Figure 2A**). SGLT1 and SGLT2 protein levels were strongly correlated (r^2=0.5604, $P < 0.0001$) and associated with the level of p-p65 NF-κB (SGLT1 vs p-p65 NF-κB: r^2=0.4874, $P < 0.0001$; SGLT2 vs p-p65 NF-κB: r^2=0.8471, $P < 0.0001$; **Figure 2A**). No such association was observed with VCAM-1 and AT1R protein levels (**Figure 2A**). Immunofluorescence analysis of ITA samples expressing SGLT2 above median value (high SGLT2 expression) as assessed by qPCR and Western blot analysis revealed a robust staining of SGLT2, CD68, TNF-α and VCAM-1 in the endothelium and the media (**Figure 2B**). In addition, SGLT1 and SGLT2 immunofluorescence signals were observed in the intimal thickening of 7 out of 13 and 4 out of 13 ITA specimens, and in the media of 17 out of 22 and 14 out of 22, respectively (Supplemental Figure 2).

Since the AT1R/NADPH oxidases/SGLT2 pathway participated in sustaining the pro-oxidant activator signal of Ang II-activated ECs,[6] the level of ROS was examined in ITA. The analysis of ITA cryosections revealed that oxidative stress levels in sections with high SGLT2 expression levels were about 3 times higher than in sections with low SGLT2 expression levels (**Figure 2C**). Notably, the pro-oxidant signals were observed in both the endothelium and the media (**Figure 2C**). The evaluation of oxidative stress levels in ITA with high SGLT2 expression levels showed a significant decrease with the use of various treatments. These included the antioxidant N-acetylcysteine, inhibitors of the Ang II/NADPH oxidases pathway

(the NADPH oxidases inhibitor VAS-2870, the ACE inhibitor perindoprilat, and the AT1R receptor blocker losartan), the dual SGLT1 and SGLT2 inhibitor sotagliflozin, the SGLT2 inhibitor empagliflozin, and the TNF-α inhibitor infliximab. These results suggest the involvement of an inflammatory process and a pro-oxidant pathway involving the AT1R/NADPH oxidases/SGLT2 pathway.

SGLT2 EXPRESSION AND FUNCTION ARE CORRELATED WITH LOW-GRADE INFLAMMATION IN HUMAN HEART SPECIMENS

In total, 18 of 20 LV biopsy specimens from patients undergoing aortic valve replacement were analyzed using RT-qPCR; the remaining two specimens containing an insufficient amount of tissue were used only for WB. Consistently, high SGLT1 and SGLT2 mRNA levels were observed in 13 and 5 of the specimens, respectively (**Figure 3A**, Supplemental Figure 3). However, no significant correlation was observed between SGLT1 and SGLT2 mRNA levels (**Figure 3A**, Supplemental Figure 3). The levels of SGLT2 mRNA expression showed a positive correlation with markers of vascular inflammation (IL-1ß, IL-6, TNF-α, and CD68), AT1R, and pro-inflammatory responses (MCP-1 and VCAM-1) (**Figure 3B**). A multivariate linear regression analysis indicated that SGLT2 mRNA levels were positively related to IL-1ß (stand.ß: 0.83, $P = 0.02$), IL-6 (stand.ß: 0.65, $P = 0.02$), and TNF-α levels (stand.ß: 0.72, $P = 0.001$) independent of age, male sex, hypertension, dyslipidemia, diabetes, smoking, and medication treatment (Supplemental Table 2). SGLT1 mRNA levels were related neither with cardiovascular risk factors nor with medication treatment (Supplemental Table 2).

Western blot analysis confirmed the heterogenous SGLT2 protein expression level in heart specimens with a difference of approximately 3.5-fold in signal intensity between the specimens with the highest and lowest levels of expression (**Figure 4A**). The expression levels of SGLT2 protein showed a positive correlation with markers of inflammation (p-p65 NF-κB, macrophage

M1 marker CD86/M2 marker CD163 and COX-2/COX-1 ratios), oxidative stress (AT1R, NOX-1, nitrotyrosine), and of EC activation (VCAM-1), and a negative correlation with levels of eNOS (**Figure 4A**). Immunofluorescence analysis of LV cryosections expressing high levels of SGLT2 showed SGLT2 staining in the endothelium of the coronary microcirculation, as indicated by its co-localization with the endothelial cell marker CD31, and also in the cardiomyocytes, as indicated by its co-localization with troponin T (**Figure 4B**). SGLT2 signals were observed in areas showing signals for TNF-α, p-p65 NF-κB, CD68 and VCAM-1 suggesting that SGLT2 expression is localized in LV specimens exhibiting inflammation (**Figure 4B**). Analysis of the extent of oxidative stress indicated that LV specimens with high SGLT2 protein levels showed substantial ROS formation, which was inhibited by N-acetylcysteine, VAS-2870, perindoprilat, losartan, sotagliflozin, and empagliflozin as well as infliximab, suggesting the involvement of a low-grade inflammatory process and the AT1R/NADPH oxidases/SGLT2 pro-oxidant pathway (**Figure 4C**).

PRO-INFLAMMATORY CYTOKINES UPREGULATE SGLT2 EXPRESSION IN ENDOTHELIAL CELLS TO PROMOTE ENDOTHELIAL DYSFUNCTION: ROLE OF THE AT1R/NADPH OXIDASES/NF-κB PATHWAY

Since the presence of SGLT 2 expression was detected in human vasculature and heart specimens affected by low-level inflammation, the direct effect of key pro-inflammatory cytokines was studied. A concentration-dependent increase in the levels of SGLT2 and SGLT1 protein expression was observed in ECs after exposure of ECs to IL-1ß, IL-6 or TNF-α (**Figure 5A**, Supplemental Figures 5A and 5B). The stimulatory effect of pro-inflammatory cytokines was accompanied by a down-regulation of eNOS protein levels and an upregulation of VCAM-1, ACE1 and AT1R, indicating the induction of endothelial dysfunction and activation of the local angiotensin system (**Figure 5A**, Supplemental Figures 5A and 5B).

To further assure the TNF-α induced overexpression of SGLT1/2 in ECs, ECs were stimulated with TNF-α and fluorescent 2-NBD-Glucose absorption was assessed in the presence or absence of SGLT1 and/or SGLT2 inhibitors. **Figure 5B** shows that TNF-α induced significant increase of 2-NBD-Glucose fluorescent signal, which was significantly reduced in the presence of sotagliflozin, empagliflozin or phloretin (a GLUT inhibitor). TNF-α treatment also led to a sustained pro-oxidant response that persisted for at least 24 h (**Figure 5C**). Next, the role of the local angiotensin system in the stimulatory effect of TNF-α was examined. Exposure of ECs to N-acetylcysteine, VAS-2870, losartan, and perindoprilat inhibited the TNF-α-induced pro-oxidant response and prevented the upregulation of SGLT1, SGLT2, and VCAM-1 proteins and the down-regulation of eNOS protein observed after 24 h (**Figures 5C and 5D**). Thus, the AT1R/NADPH oxidases pro-oxidant pathway mediates oxidative stress promoting SGLT1/2 expression in response to TNF-α. To determine whether SGLT1 and SGLT2 contribute to the deleterious effects of TNF-α on ECs, the effect of sotagliflozin, empagliflozin and the depletion of either SGLT1 or SGLT2 using a siRNA approach was examined. Both sotagliflozin and empagliflozin prevented the TNF-α-induced sustained pro-oxidant response, down-regulation of eNOS, and upregulation of SGLT1, SGLT2, VCAM-1, ACE1 and AT1R protein levels (**Figures 6A, 6C and 6D**). A similar effect was also observed following the depletion of SGLT2 in ECs, whereas depletion of SGLT1 had no such effect (**Figure 6B**). Notably, depletion of SGLT2 in ECs also reduced the TNF-α-induced upregulation of SGLT1 protein level, whereas depletion of SGLT1 did not affect the SGLT2 protein level (**Figure 6B**). Thus, these observations indicate that SGLT2 is a key SGLT isoform mediating the pathological activation of ECs in response to TNF-α. Additionally, the inhibitory effect of sotagliflozin and empagliflozin on the TNF-α-induced formation of ROS including superoxide anions was associated with preserved formation of NO in response to bradykinin, whereas the low basal formation of NO was not affected (**Figure 6E**).

Next, the role of NF-κB, a redox-sensitive transcription factor with putative binding sites in the SGLT2 promoter,[17] was studied. Exposure of ECs to TNF-α for 24 h caused NF-κB activation, as indicated by a concentration-dependent increase in the levels of the p-p65 NF-κB subunit and the nuclear translocation of NF-kB (**Figures 7A and 7B**). The TNF-α-induced activation of NF-κB was prevented by N-acetylcysteine, VAS-2870, losartan, perindoprilat, sotagliflozin and empagliflozin indicating the involvement of the AT1R/NADPH oxidases/SGLT2 pro-oxidant pathway (**Figures 7A and 7B**). NF-κB inhibitor Bay 11-7082 effectively prevented the increase of SGLT2 mRNA expression and decrease in eNOS mRNA expression triggered by TNF-α and IL-1ß in ECs, highlighting the crucial role of NF-κB in transmitting the pro-oxidant activating signal to the SGLT2 and eNOS genes (**Figure 7C**). Next, the role of redox-sensitive protein kinases in the signal transduction pathway mediating the pro-oxidant signal to target genes in response to TNF-α was investigated. TNF-α initiated, within 1 h, the phosphorylation of Akt and the mitogen-activated protein kinases (MAPKs) p-38 MAPK, JNK and ERK1/2 (Supplemental Figure 5C). This effect was blocked by N-acetylcysteine demonstrating redox-sensitive activation (Supplemental Figure 5). The inhibition of Akt, p-38 MAPK or JNK prevented the TNF-α-induced nuclear translocation of NF-κB and upregulation of SGLT2 protein levels in ECs, whereas the inhibition of ERK1/2 led to a small but statistically insignificant inhibitory effect (**Figures 7D and 7E**). These findings indicate the involvement of Akt and p-38 MAPK and JNK.

DISCUSSION

In a translational approach, we delineate the orchestrated interplay between low-grade inflammation and SGLT2 expression throughout the human cardiovascular system. Recently, low-grade inflammation has been recognized as a pivotal contributor to cardiovascular disease, including atherosclerosis and HF, as well as to the "inflamm-aging" of age-dependent cardiovascular disease.[10,14] Several clinical trials have investigated various anti-inflammatory agents in ischemic heart disease (*e.g.*, COLCOT trial, CANTOS trial) and HF (e.g. CORONA trial). Indeed, multiple biochemical pathways contribute to inflammation-driven heart injury and can be targeted at various levels.[15] Recently, five landmark trials (DELIVER trial, SOLOIST-WHF trial, EMPEROR-Preserved trial, EMPEROR-Reduced trial, and DAPA-HF trial) have revolutionized the management of HF, demonstrating an impressive 23% reduction in the risk of cardiovascular death or hospitalization for HF (0.77 [0.72-0.82]).[16] Unprecedentedly, the manifestation of benefits for hard endpoints in clinical trials occurred prior to the understanding of the basic and essential explanations for the benefits of gliflozins in cardiovascular disease.[1] Our research offers novel insights into the direct induction of SGLT2 expression by low-grade inflammation in the human cardiovascular system. This induction leads to enhanced oxidative stress, pro-inflammatory signaling, endothelial senescence and dysfunction, highlighting the pivotal role of the AT1R/NADPH oxidases/SGLT2 pro-oxidant pathway.

The major finding of the present study is an unexpected non-uniform distribution of elevated SGLT2 mRNA and protein expression levels among human vascular and LV specimens. Considering that all ITA specimens were acquired from patients with atherosclerotic cardiovascular disease and all LV biospecimens were obtained from patients with cardiac valvular disease, the elevated levels of SGLT2 may be attributed to intrinsic characteristics of the human tissues. This elevation could potentially result from the presence of SGLT2 inducers

originating from the systemic circulation and/or produced within the pathological tissue itself. Chronic low-grade inflammation-induced oxidative stress is now widely acknowledged as a significant driver in the development and progression of cardiovascular disease and associated comorbidities.[10,14] Furthermore, oxidative stress is a potent inducer of SGLT2 expression in ECs,[5,17] prompting investigations into the role of inflammation. SGLT2 expression in vascular and LV specimens was closely correlated with those of infiltrated type M1 macrophages, pro-inflammatory cytokines such as TNF-α, IL-1ß, and IL-6, and the activation of the master pro-inflammatory transcription factor NF-κB. Hence, persistent local low-grade inflammation emerges as a critical event that contributes to elevated SGLT2 levels in human vasculature and LV biopsies.

Characterization of human ITA specimens revealed a close association between the expression levels of SGLT2 mRNA and protein and those of SGLT1. Interestingly, both were dependent on the local inflammatory status. However, SGLT1 expression was neither related to SGLT2 levels nor to inflammatory signals in human LV biopsies, suggesting the involvement of diverse regulatory mechanisms that warrant further exploration. The unique regulatory mechanisms governing SGLT1 and SGLT2 expression may account for the abundant SGLT1 expression observed in atrial and LV tissues in various human HF pathologies, in contrast to the low levels of SGLT2.[18,19]

In vascular specimens exhibiting pro-inflammatory signals, SGLT2 expression was observed in the endothelium and vascular smooth muscle of the media, in the neointima, in inflamed LV tissues, and in the endothelium of the coronary microcirculation and cardiomyocytes. These findings indicate that SGLT2 expression is a common response in all major cell types of the inflamed cardiovascular system. Thus, SGLT2 expression appears to accompany the transition from a healthy to a disease state associated with inflammatory responses in the cardiovascular system.

SGLT2 EXPRESSION MEDIATES THE PRO-OXIDANT ACTIVATOR SIGNAL OF PATHOLOGICAL RESPONSES IN INFLAMED HUMAN VASCULATURE AND HEART

Vascular and LV specimens with elevated SGLT2 expression levels showed increased levels of oxidative stress involving the local AT1R/NADPH oxidases pro-oxidant pathway and inflammatory signaling. These findings underscore the pivotal role of SGLT2 in the pro-oxidant state. Indeed, SGLT2i decreased the level of oxidative stress in the inflamed LV and normalized it in the vasculature to a similar level to that observed in the non-inflamed vasculature. Previous *in vitro* studies with ECs indicated that SGLT2 inhibition abrogated TNF-α-induced oxidative stress in microvascular ECs leading to preserved NO formation with subsequent normal cardiomyocyte contractility.[20] Additionally, Ang II increased the level of oxidative stress through the AT1R/NADPH oxidases pathway and led to the upregulation of SGLT2 expression.[6] Once SGLT2 was expressed, the pro-oxidant response to Ang II was strongly inhibited by SGLT2i and dependent on extracellular glucose and Na^+.[6]

Noteworthy, elevated SGLT2 expression levels in the human vasculature correlated with components of the local angiotensin system (ACE1, AT1R and the NADPH oxidase subunit p47phox) at the mRNA level, and in the human LV biopsies to AT1R at the mRNA and protein level. Altogether, these findings suggest that the AT1R/NADPH oxidases/SGLT2 pro-oxidant pathway emerges as a pivotal contributor to the pathological activation of the inflamed cardiovascular system. Elevated SGLT2 expression levels in inflamed human vasculature and heart tissues were indeed tightly related to blunted endothelial protective signals as indicated by reduced levels of eNOS and the elevated appearance of nitrotyrosine levels. This finding indicates the degradation of NO, and the occurrence of pro-inflammatory and pro-thrombotic responses such as increased expression levels of MCP-1, VCAM-1, ICAM-1, e-selectin and tissue factor as well as markers of senescence such as p53/p21 and p16.

PRO-INFLAMMATORY CYTOKINES ARE DIRECT INDUCERS OF SGLT2 EXPRESSION IN ECs PROMOTING PREMATURE SENESCENCE AND DYSFUNCTION

In vitro studies using coronary ECs indicated that pro-inflammatory cytokines including IL-1ß, IL-6 and TNF-α can directly stimulate the expression of SGLT2 mRNA and protein expression levels to impair the eNOS-NO/ROS balance. The characterization of the expression of SGLT2 indicated a pivotal role of oxidative stress mediated by the AT1R/NADPH oxidases/SGLT2 pro-oxidant pathway. Based on the concomitant upregulation of ACE1 and AT1R expression alongside SGLT2, it appears that such an inducible pro-oxidant stimulatory pathway is perfectly suited to enhance and perpetuate the pro-oxidant activator signal, promoting the development and progression from cardiovascular health to disease characterized by low-grade inflammation. The characterization of the signal transduction pathway mediating the pro-oxidant activator signal to SGLT2 expression indicated a major role of redox-sensitive kinases of the PI-3kinase/Akt and p38 MAPK and JNK pathways leading to the activation of the transcription factor NF-κB. In addition to SGLT2, TNF-α also upregulated SGLT1 expression in ECs. However, the TNF-α induced activation of ECs, as indicated by reduced levels of eNOS and enhanced levels of VCAM-1, was averted with the knockdown of SGLT2 but not with the knockdown of SGLT1. This finding suggests that SGLT2 modulates the pathological activation of ECs. Previous studies with renal tubular epithelial cells indicated that NF-κB mediated insulin-induced SGLT2 expression and identified a functional p65 binding site in the promoter of SGT2.[13]

Furthermore, our results provide evidence for a spatial heterogeneity of SGLT2 expression mediated, at least in part, by the local low-grade inflammation. This evidence may identify a specific “target site” in the vasculature and heart with an important role in the development of cardiac dysfunction, remodeling, hypertrophy, and endothelial dysfunction. In clinical studies,

SGLT2i reduced inflammatory and oxidative stress levels in patients with HFpEF, leading to improvement in endothelial function and a reduction in pathological cardiomyocyte stiffness,[21] improved LV hypertrophy in patients with T2DM, along with lower levels of high-sensitivity C-reactive protein,[22] and mitigated systemic inflammatory profile and oxidative stress in patients with concomitant T2DM and coronary artery disease.[23] Moreover, these studies indicated an enhanced myocardial flow reserve in patients with T2DM, possibly due to an improvement of the coronary microvascular function.[24] Additionally, a study identified differentially expressed proteins in HF patients treated with SGLT2i that were associated with autophagic flux, blunted oxidative stress, reduced inflammation and fibrosis, and enhanced mitochondrial health and energy, repair, and regenerative capacity.[25] SGLT2i also demonstrated reduced inflammatory and oxidative status in various pre-clinical models, leading to slow the progression of atherosclerosis.[26]

Of interest, recently a translational study has shown that plasma samples from patients affected by Covid-19 during the acute, post-acute and long Covid-19 phase with elevated levels of pro-inflammatory cytokines (IL-1ß, IL-6 and TNF-α) upregulated SGLT2 and caused the subsequent induction of persistent oxidative stress in coronary ECs.[27] The stimulatory effect involved pro-inflammatory cytokines and resulted in EC activation with pro-inflammatory, pro-adhesive and pro-coagulant responses in addition to endothelial dysfunction. Thus, both systemic and tissular inflammation can upregulate SGLT2 in the cardiovascular system, and that, once expressed, SGLT2 appears to have a pivotal role in promoting the deleterious impact of inflammation in the vasculature and heart.

STUDY LIMITATIONS

Our study evidenced elevated levels of SGLT2 at sites of low-grade inflammation in human vascular and LV specimens. We provided direct evidence for a stimulatory effect of pro-

inflammatory cytokines on SGLT2 expression in ECs, resulting in impaired endothelial function, and a protective effect of empagliflozin. Our investigation focused on the human ITA, an arterial conduit used for myocardial revascularization during coronary artery bypass surgery and known to be protected from the development of atherosclerosis. It is worth mentioning that we did not evaluate SGLT2 expression and function in other disease-prone or resistant arterial conduits. Furthermore, we could not rule out the potential involvement of additional mediators (apart from TNF-α, IL-6, and IL-1β) of inflamed cardiac and vasculature tissues or systemic low-grade inflammation, which stand as significant contributors to cardiovascular disease, in regulating SGLT2 expression.

We demonstrated as well that increased SGLT2 levels in cardiomyocytes and the microvascular endothelium at sites of inflammation contributed to elevated levels of oxidative stress in patients with cardiac valvulopathy. Further investigations are necessary to assess the contribution of SGLT2-mediated pro-oxidant responses in HF patients across the spectrum of LVEF. Additionally, it is important to determine whether the bioavailability of endothelial NO is preserved, as it plays a crucial role in endothelial control of cardiomyocyte contractile properties, coronary microvascular perfusion, and prevention of heart remodeling and fibrosis.

CONCLUSIONS

Our study highlights the pivotal role of low-grade inflammation in upregulating SGLT2 expression in human vasculature and heart, leading to impaired eNOS-NO/ROS balance and promoting pro-inflammatory and pro-thrombotic responses. Furthermore, our investigation provides new insights into the beneficial effects of SGLT2i on the cardiovascular system observed in different patients such as those affected by HFrEF, HFpEF, T2DM, chronic kidney disease, and also recently suggested in rheumatic diseases or long Covid-19, all pathologies

characterized by ongoing inflammation. They further suggest that patients with low-grade inflammation may particularly benefit of SGLT2 inhibition.

Funding support: This study was supported by Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany to V.S.-K. and J.-P.M., and by Groupe pour l'Enseignement, la prévention et la Recherche **Cardiologique** en Alsace (GERCA).

Author Contribution: V.S.K. contributed to the design of the work. A.M., P.A-S., W.F., D-S.G., K.M., S-H. P. and S. A. contributed to the acquisition, analysis and/or interpretation of data for the work. A.M., C.A., G.K., N.M., P.O., L.J. B.M., O.M., J-P. M. and V.S-K. contributed to the drafting of the work. M-P.P., O.M, J-P.M. and V.S-K. gave the final approval of the version to be published.

Author disclosures: V.S.-K. declared grants from Boehringer Ingelheim, Nugerontix Limited, and Servier and O.M. from AstraZeneca, Medtronic and Boehringer Ingelheim. M.-P.P. is an employee of Boehringer Ingelheim. The other authors have nothing to disclose.

HIGHLIGHTS

• Although SGLT2i showed beneficial cardiovascular effect in heart failure and myocardial ischemia, the expression of SGLT2 and its role in the vasculature and heart remain poorly studied.

• In human internal mammary artery and heart biopsies, SGLT2 expression was observed in the endothelium, vascular smooth muscle, coronary microcirculation and cardiomyocytes in areas affected by inflammation and contributed to oxidative stress. Moreover, pro-inflammatory cytokines upregulated SGLT2 expression in endothelial cells to perpetuate via the AT1R/NADPH oxidases pro-oxidant pathway the redox-sensitive NF-κB-mediated expression of SGLT2 leading to endothelial dysfunction.

• SGLT2 expression appears to be a key mediator of the deleterious impact of low-grade inflammation on the vasculature and heart and suggests that targeting SGLT2 may help to optimize treatment of patients with low-grade inflammatory cardiovascular disease and potentially also inflammatory disease.

REFERENCES

1. Packer M. SGLT2 inhibitors: role in protective reprogramming of cardiac nutrient transport and metabolism. *Nat Rev Cardiol*. 2023 Jul;**20**(7):443-462.
2. Luc K, Schramm-Luc A, Guzik TJ, Mikolajczyk TP. Oxidative stress and inflammatory markers in prediabetes and diabetes. *J Physiol Pharmacol*. 2019;**70**(6).
3. D'Onofrio L, Pieralice S, Maddaloni E, Mignogna C, Sterpetti S, Coraggio L, Luordi C, Guarisco G, Leto G, Leonetti F, Manfrini S, Buzzetti R. "Effects of the COVID-19 lockdown on glycaemic control in subjects with type 2 diabetes: the glycalock study". *Diabetes Obes Metab*. 2021;**23**(7):1624-1630.
4. Marfella R, Sardu C, D'Onofrio N, Prattichizzo F, Scisciola L, Messina V, La Grotta R, Balestrieri ML, Maggi P, Napoli C, Ceriello A, Paolisso G. "Glycaemic control is associated with SARS-CoV-2 breakthrough infections in vaccinated patients with type 2 diabetes". *Nat Commun*. 2022;**13**(1):2318.
5. Khemais-Benkhiat S, Belcastro E, Idris-Khodja N, Park SH, Amoura L, Abbas M, Auger C, Kessler L, Mayoux E, Toti F, Schini-Kerth VB. "Angiotensin II-induced redox-sensitive SGLT1 and 2 expression promotes high glucose-induced endothelial cell senescence". *J Cell Mol Med*. 2020;**24**(3):2109-2122.
6. Park SH, Belcastro E, Hasan H, Matsushita K, Marchandot B, Abbas M, Toti F, Auger C, Jesel L, Ohlmann P, Morel O, Schini-Kerth VB. "Angiotensin II-induced upregulation of SGLT1 and 2 contributes to human microparticle-stimulated endothelial senescence and dysfunction: protective effect of gliflozins". *Cardiovasc Diabetol*. 2021;**20**(1):65.
7. Terasaki M, Hiromura M, Mori Y, Kohashi K, Nagashima M, Kushima H, Watanabe T, Hirano T. "Amelioration of Hyperglycemia with a Sodium-Glucose Cotransporter 2 Inhibitor Prevents Macrophage-Driven Atherosclerosis through Macrophage Foam Cell Formation Suppression in Type 1 and Type 2 Diabetic Mice". *PLoS One*. 2015;**10**(11):e0143396.
8. Bruckert C, Matsushita K, Mroueh A, Amissi S, Auger C, Houngue U, Remila L, Chaker AB, Park SH, Algara-Suarez P, Belcastro E, Jesel L, Ohlmann P, Morel O, Schini-Kerth VB. "Empagliflozin prevents angiotensin II-induced hypertension related micro and macrovascular endothelial cell activation and diastolic dysfunction in rats despite persistent hypertension: Role of endothelial SGLT1 and 2". *Vascul Pharmacol*. 2022;**146**:107095.
9. Zelniker TA, Braunwald E. "Mechanisms of Cardiorenal Effects of Sodium-Glucose Cotransporter 2 Inhibitors: JACC State-of-the-Art Review". *J Am Coll Cardiol*. 2020;**75**(4):422-434
10. Liberale L, Badimon L, Montecucco F, Lüscher TF, Libby P, Camici GG. "Inflammation, Aging, and Cardiovascular Disease: JACC Review Topic of the Week". *J Am Coll Cardiol*. 2022;**79**(8):837-847.
11. Abbas M, Jesel L, Auger C, Amoura L, Messas N, Manin G, Rumig C, León-González AJ, Ribeiro TP, Silva GC, Abou-Merhi R, Hamade E, Hecker M, Georg Y, Chakfe N, Ohlmann P, Schini-Kerth VB, Toti F, Morel O. "Endothelial Microparticles From Acute Coronary Syndrome Patients Induce Premature Coronary Artery Endothelial Cell Aging

and Thrombogenicity: Role of the Ang II/AT1 Receptor/NADPH Oxidase-Mediated Activation of MAPKs and PI3-Kinase Pathways". *Circulation*. 2017;**135**(3):280-296.
12. Amissi S, Boisramé-Helms J, Burban M, Rashid SK, León-González AJ, Auger C, Toti F, Meziani F, Schini-Kerth VB. Lipid Emulsions Containing Medium Chain Triacylglycerols Blunt Bradykinin-Induced Endothelium-Dependent Relaxation in Porcine Coronary Artery Rings. *Lipids*. 2017;**52**(3):235-243.
13. Fu M, Yu J, Chen Z, Tang Y, Dong R, Yang Y, Luo J, Hu S, Tu L, Xu X. "Epoxyeicosatrienoic acids improve glucose homeostasis by preventing NF-κB-mediated transcription of SGLT2 in renal tubular epithelial cells". *Mol Cell Endocrinol*. 2021;**523**:111149.
14. Libby P. "The changing landscape of atherosclerosis". *Nature*. 2021;**592**(7855):524-533.
15. Pugliese NR, Pellicori P, Filidei F, De Biase N, Maffia P, Guzik TJ, Masi S, Taddei S, Cleland JGF. "Inflammatory pathways in heart failure with preserved left ventricular ejection fraction: implications for future interventions". *Cardiovasc Res*. 2023;**118**(18):3536-3555.
16. Vaduganathan M, Docherty KF, Claggett BL, Jhund PS, de Boer RA, Hernandez AF, Inzucchi SE, Kosiborod MN, Lam CSP, Martinez F, Shah SJ, Desai AS, McMurray JJV, Solomon SD. "SGLT-2 inhibitors in patients with heart failure: a comprehensive meta-analysis of five randomised controlled trials". *Lancet*. 2022;**400**(10354):757-767.
17. Packer M, Anker SD, Butler J, Filippatos G, Pocock SJ, Carson P, Januzzi J, Verma S, Tsutsui H, Brueckmann M, Jamal W, Kimura K, Schnee J, Zeller C, Cotton D, Bocchi E, Böhm M, Choi DJ, Chopra V, Chuquiure E, Giannetti N, Janssens S, Zhang J, Gonzalez Juanatey JR, Kaul S, Brunner-La Rocca HP, Merkely B, Nicholls SJ, Perrone S, Pina I, Ponikowski P, Sattar N, Senni M, Seronde MF, Spinar J, Squire I, Taddei S, Wanner C, Zannad F; EMPEROR-Reduced Trial Investigators. "Cardiovascular and Renal Outcomes with Empagliflozin in Heart Failure". *N Engl J Med*. 2020;**383**(15):1413-1424.
18. Sayour AA, Ruppert M, Oláh A, Benke K, Barta BA, Zsáry E, Merkely B, Radovits T. "Effects of SGLT2 Inhibitors beyond Glycemic Control-Focus on Myocardial SGLT1". *Int J Mol Sci*. 2021;**22**(18):9852.
19. Kondo H, Akoumianakis I, Badi I, Akawi N, Kotanidis CP, Polkinghorne M, Stadiotti I, Sommariva E, Antonopoulos AS, Carena MC, Oikonomou EK, Reus EM, Sayeed R, Krasopoulos G, Srivastava V, Farid S, Chuaiphichai S, Shirodaria C, Channon KM, Casadei B, Antoniades C. "Effects of canagliflozin on human myocardial redox signalling: clinical implications". *Eur Heart J*. 2021;**42**(48):4947-4960.
20. Juni RP, Kuster DWD, Goebel M, Helmes M, Musters RJP, van der Velden J, Koolwijk P, Paulus WJ, van Hinsbergh VWM. "Cardiac Microvascular Endothelial Enhancement of Cardiomyocyte Function Is Impaired by Inflammation and Restored by Empagliflozin". *JACC Basic Transl Sci*. 2019;**4**(5):575-591.
21. Kolijn D, Pabel S, Tian Y, Lódi M, Herwig M, Carrizzo A, Zhazykbayeva S, Kovács Á, Fülöp GÁ, Falcão-Pires I, Reusch PH, Linthout SV, Papp Z, van Heerebeek L, Vecchione C, Maier LS, Ciccarelli M, Tschöpe C, Mügge A, Bagi Z, Sossalla S, Hamdani N. "Empagliflozin improves endothelial and cardiomyocyte function in human heart failure with preserved ejection fraction via reduced pro-inflammatory-oxidative pathways and protein kinase Gα oxidation". *Cardiovasc Res*. 2021;**117**(2):495-507.

22. Brown E, Wilding JPH, Alam U, Barber TM, Karalliedde J, Cuthbertson DJ. “The expanding role of SGLT2 inhibitors beyond glucose-lowering to cardiorenal protection”. *Ann Med*. 2021;**53**(1):2072-2089.
23. Gohari S, Reshadmanesh T, Khodabandehloo H, Karbalaee-Hasani A, Ahangar H, Arsang-Jang S, Ismail-Beigi F, Dadashi M, Ghanbari S, Taheri H, Fathi M, Muhammadi MJ, Mahmoodian R, Asgari A, Tayaranian M, Moharrami M, Mahjani M, Ghobadian B, Chiti H, Gohari S. “The effect of EMPAgliflozin on markers of inflammation in patients with concomitant type 2 diabetes mellitus and Coronary ARtery Disease: the EMPA-CARD randomized controlled trial”. *Diabetol Metab Syndr*. 2022;**14**(1):170.
24. Leccisotti L, Cinti F, Sorice GP, D'Amario D, Lorusso M, Guzzardi MA, Mezza T, Gugliandolo S, Cocchi C, Capece U, Indovina L, Ferraro PM, Iozzo P, Crea F, Giordano A, Giaccari A. “Dapagliflozin improves myocardial flow reserve in patients with type 2 diabetes: the DAPAHEART Trial: a preliminary report”. *Cardiovasc Diabetol*. 2022;**21**(1):173.
25. Zannad F, Ferreira JP, Butler J, Filippatos G, Januzzi JL, Sumin M, Zwick M, Saadati M, Pocock SJ, Sattar N, Anker SD, Packer M. “Effect of empagliflozin on circulating proteomics in heart failure: mechanistic insights into the EMPEROR programme”. *Eur Heart J*. 2022;**43**(48):4991-5002.
26. Scisciola L, Cataldo V, Taktaz F, Fontanella RA, Pesapane A, Ghosh P, Franzese M, Puocci A, De Angelis A, Sportiello L, Marfella R, Barbieri M. “Anti-inflammatory role of SGLT2 inhibitors as part of their anti-atherosclerotic activity: Data from basic science and clinical trials”. *Front Cardiovasc Med*. 2022;**9**:1008922.
27. Mroueh A, Fakih W, Carmona A, Trimaille A, Matsushita K, Marchandot B, Qureshi AW, Gong DS, Auger C, Sattler L, Reydel A, Hess S, Oulehri W, Vollmer O, Lessinger JM, Meyer N, Pieper MP, Jesel L, Bäck M, Schini-Kerth V, Morel O. COVID-19 promotes endothelial dysfunction and thrombogenicity: role of proinflammatory cytokines/SGLT2 prooxidant pathway. *J Thromb Haemost*. 2024;**22**(1):286-299.

FIGURE LEGENDS

FIGURE 1 SGLT2 mRNA Expression in Human Internal Thoracic Artery

Heatmap of target genes in ITA segments from 30 patients undergoing bypass surgery as assessed by RT-qPCR (A). The correlation of SGLT2 mRNA levels with those of different markers was established using Pearson's correlation and shown (B).

FIGURE 2 SGLT2 Protein Expression Related to Low-grade Inflammation in Human Artery

(A) Representative Western blot analysis of segments of ITA from 10 different patients (top), and correlation of SGLT1 and SGLT2 protein levels and with those of p-p65 NF-κB (bottom). (B) Representative immunofluorescence staining of SGLT2, macrophages infiltration (CD68), TNF-α, and VCAM-1 in cryosections of ITA with high SGLT2 expression levels. (C) Increased levels of oxidative stress in high compared to low SGLT2 expressing ITA are reduced by N-acetylcysteine (NAC), inhibitors of the angiotensin system (the NADPH oxidases inhibitor VAS-2870 (VAS), the ACE inhibitor perindopril (Per), the AT1R receptor antagonist losartan (Los), the dual SGLT1 and SGLT2 inhibitor sotagliflozin (Sota), the selective SGLT2 inhibitor empagliflozin (Empa) and the TNF-α inhibitor infliximab (Inf). Data are presented as mean ± SEM of n = 30 (A) and 5 (B and C). $^{*}P < 0.05$ vs low SGLT2 expressing ITA, $^{\#}P < 0.05$ versus high SGLT2 expressing ITA using one-way ANOVA followed by Tukey's multiple comparison test.

FIGURE 3 SGLT2 mRNA Expression in Human Heart

Heatmap of target genes in left ventricle (LV) biopsies of 18 patients undergoing valve surgery as assessed by RT-qPCR (A). The correlation of SGLT2 mRNA levels with those of different markers was established using Pearson's correlation and shown (B).

FIGURE 4 SGLT2 Protein Expression Related to Low-grade Inflammation in Human Heart

(A) Representative Western blot analysis of human LV biopsies from 10 different patients, and correlation of SGLT2 protein levels with those of different markers of endothelial and LV tissue activation. (B) Representative immunofluorescence staining of SGLT2, macrophage infiltration (CD68), TNF-α, p-p65 NF-κB, and VCAM-1 in high SGLT2 expressing LV biopsies (top panel). Representative immunofluorescence dual staining of LV biopsies for SGLT2 (in red) and endothelial cells using CD31 (in yellow, middle panel), and cardiomyocytes using troponin T (in yellow, bottom panel). Dapi was used to stain nuclei. (C) Characterization of oxidative stress in high expressing SGLT2 LV biopsies. Data are presented as mean ± SEM of n = 19 (A), 4 (B) and 6 (C). $*P < 0.05$ vs high SGLT2 expressing heart using one-way ANOVA followed by Tukey's multiple comparison test. Abbreviations as in **Figure 2**.

FIGURE 5 TNF-α Induces SGLT2 expression in Endothelial Cells

(A) ECs were exposed to TNF-α for 24 h before determination of the expression level of target proteins by Western blot analysis. Left panels show representative Western blot analysis and right panels cumulative data. (B) TNF-α-induced increased SGLT2 expression is associated with increased 2-NBD-Glucose absorption in ECs. Left panel shows representative flow cytometry curves and right panel cumulative data. (C) TNF-α-induced formation of ROS as assessed using dihydroethidium. Top panels show representative photomigraphs and bottom panels cumulative data. (D) Characterization of TNF-α-induced upregulation of SGLT2 protein expression levels in ECs. Left panels show representative Western blot analysis and right panels cumulative data. Data are presented as mean ± SEM of n = 4-5. $*P < 0.05$ vs control (C), $^{\#}P < 0.05$ versus respective TNF-α treatment using one-way ANOVA followed by Tukey's multiple comparison test. Abbreviations as in **Figure 2**.

FIGURE 6 SGLT2 Mediates the TNF-α-induced Endothelial Dysfunction

(A) Characterization of TNF-α-induced protein expression levels of SGLT1 and 2, markers of endothelial cell activation, and components of the angiotensin system. Left panels show representative Western blot analysis and right panels cumulative data. (B) Depletion of SGLT2 but not SGLT1 in ECs using a siRNA approach prevented the TNF-α-induced expression of SGLT1, SGLT2 and VCAM-1 and the down-regulation of eNOS. Left panels show representative Western blot analysis and right panels cumulative data. (C,E) Sotagliflozin and empagliflozin prevented the TNF-α-induced formation of ROS and the blunted formation of NO in response to bradykinin in ECs as assessed using DAF-FM. Top panels show representative photomigraphs and bottom panels cumulative data. (D) Empagliflozin and MnTMPyP significantly reduced TNF-α-induced formation of superoxide anions. Graph shows cumulative resonance data. Data are presented as mean ± SEM of n = 3-6. $^{*}P < 0.05$ vs control (C), $^{\#}P < 0.05$ versus TNF-α (A-C, E) or versus bradykinin treatment (D), $^{\$}P < 0.05$ versus TNF-α plus bradykinin treatment using one-way ANOVA followed by Tukey's multiple comparison test. Abbreviations as in **Figure 2**.

FIGURE 7 NF-κB and Redox-sensitive Protein Kinases Mediate TNF-α-induced Expression of SGLT2

(A) TNF-α-induced phosphorylation of p65 NF-κB is prevented by inhibitors of the angiotensin system, NAC and by sotagliflozin and empagliflozin. (B) Characterization of TNF-α-induced nuclear translocation of NF-κB. Left panel shows representative photomicrographs and right panel cumulative data. (C) The NF-κB inhibitor (Bay 11-7082) prevented IL-1ß- and TNF-α-induced upregulation of SGLT2 mRNA and down-regulation of eNOS mRNA. (D,E) The nuclear translocation of NF-κB and the upregulation of SGLT2 protein levels in TNF-α-treated

ECs were abolished by inhibitors of either Akt (Akt-I, wortmannin), p38 MAPK kinases (p38-I, SB 2023580), or JNK (JNK-I, SP 600125) and insignificantly reduced by an inhibitor of ERK1/2 (ERK-I, PD 98059). Upper panels show representative immunofluorescence photomicrographs (D) or Western blot analysis (E), and bottom panels cumulative data. Data are presented as mean ± SEM of n = 4-5. *$P < 0.05$ vs control (C), $^{\#}P < 0.05$ versus respective cytokine treatment using one-way ANOVA followed by Tukey's multiple comparison test. Abbreviations as in **Figure 2**.

Table 1 Characteristics of Study Patients of Internal Thoracic Artery Specimens

	n = 70
Age (median in years)	69.50 [64.75-75.00]
Sex (male)	53 (75.71%)
Hypertension	55 (78.57%)
Dyslipidemia	42 (60%)
Diabetes	23 (32.85%)
Smoking	29 (41.42%)
Family History of coronary artery disease	5 (7.14%)
Body Mass Index (Kg/m^2)	27.70 [23.53-30.47]
History of percutaneous coronary intervention	17
Hb (g/L)	13.77 [13-14.83]
Creatinine clearance (mL/min)	97.84 [71.75-98]
eGFR (mL/min/1.73 m^2)	75.41 [63.93-88.50]
Statins	52 (74.28%)
Beta-blockers	50 (71.42%)
ACE/AT1R inhibitors	48 (68.57%)
Anti-diabetic agents	17 (24.28%)
Calcium channel blockers	13 (18.57%)
Anti-platelet therapy	63 (90%)
Diuretics	21 (30%)

Values are presented as (%) or median [25th-75th Percentile]. Hb, hemoglobin; eGFR, estimated glomerular filtration rate.

Table 2 Characteristics of Study Patients of Left Ventricle Specimens

	n = 20
Age (median in years)	69.5[65.50-73.0]
Sex (male)	11 (55%)
Hypertension	13 (65%)
Dyslipidemia	11 (55%)
Diabetes	6 (30%)
Smoking	6 (30%)
Family history of coronary artery disease	1 (5%)
Body Mass Index (kg/m^2)	26.05 [23.68-31.03]
History of percutaneous coronary intervention	1 (5%)
Hb (g/L)	14.2 [13.13-14.88]
Creatinine clearance (mL/min)	72.5 [62.5-88.75]
eGFR (mL/min/1.73 m^2)	86 [73-93]
Statins	11 (55%)
Beta-blockers	7 (35%)
ACE/AT1R inhibitors	8 (40%)
Anti-diabetic agents	5 (25%)
Calcium channel blockers	5 (25%)
Anti-platelet therapy	9 (45%)

Values are presented as (%) or median [25th-75th Percentile]. Hb, hemoglobin; eGFR, estimated glomerular filtration rate.

Table 3 Echocardiographic Parameters of Study Patients of Left Ventricle Specimens

	n = 20
AVA (cm^2)	**0.8550 [0.7-0.9275]**
E/A ratio	**0.8 [0.6-1]**
EWDT (ms)	**208.5 [186-272.3]**
IVST (mm)	**12 [9.25-13]**
LAVI (ml/m^2)	**28 [23-31]**
LVEDD (mm)	**43.5 [40.25-48.75]**
LVEF (%)	**64.5 [59.25-68]**
LVMI (mg/m^2)	**89.5 [72.25-111]**
LVWT (mm)	**10 [7.25-12]**
MATVG (mmHg)	**45 [42.5-52.5]**

Values are presented as median [25th-75th Percentile]. AVA, aortic valve area; E/A ratio, ratio between E-wave and A-wave; EWDT, E wave deceleration time; IVST**,** interventricular septum thickness; LAVI, left atrial volume index; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass index; LVWT, left ventricular wall thickness; MATVG, Mean aortic transvalvular gradient.

Figure 1

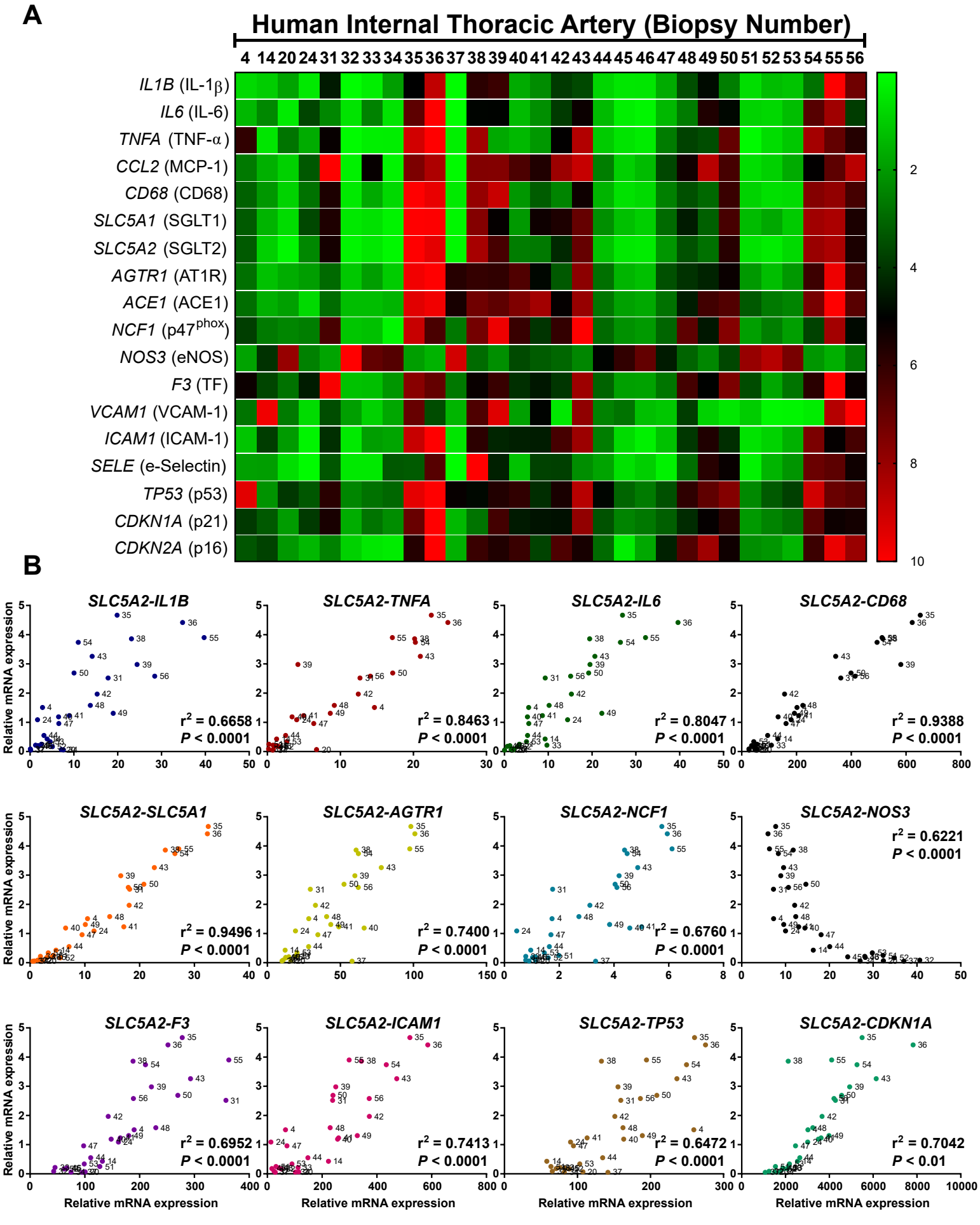
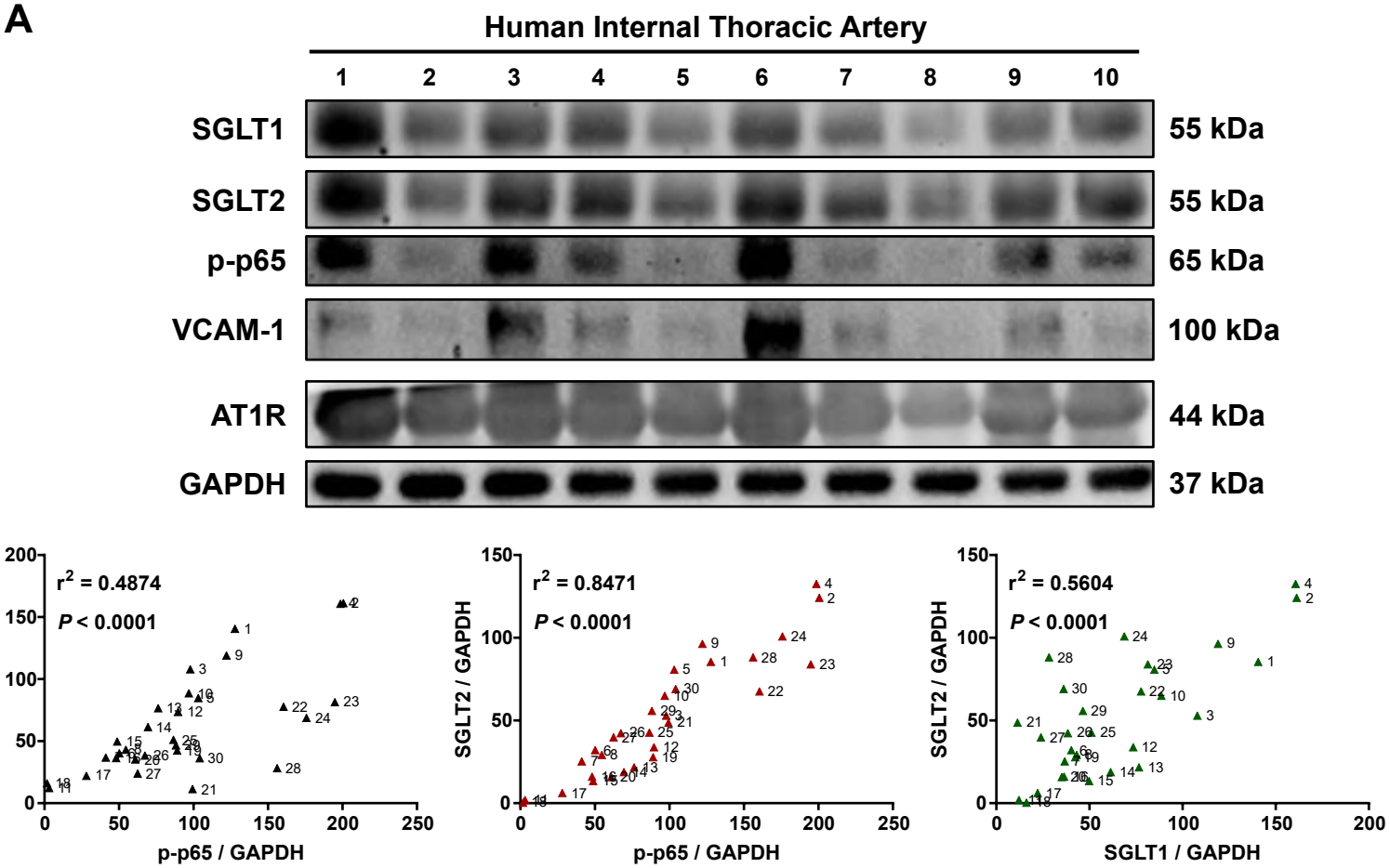
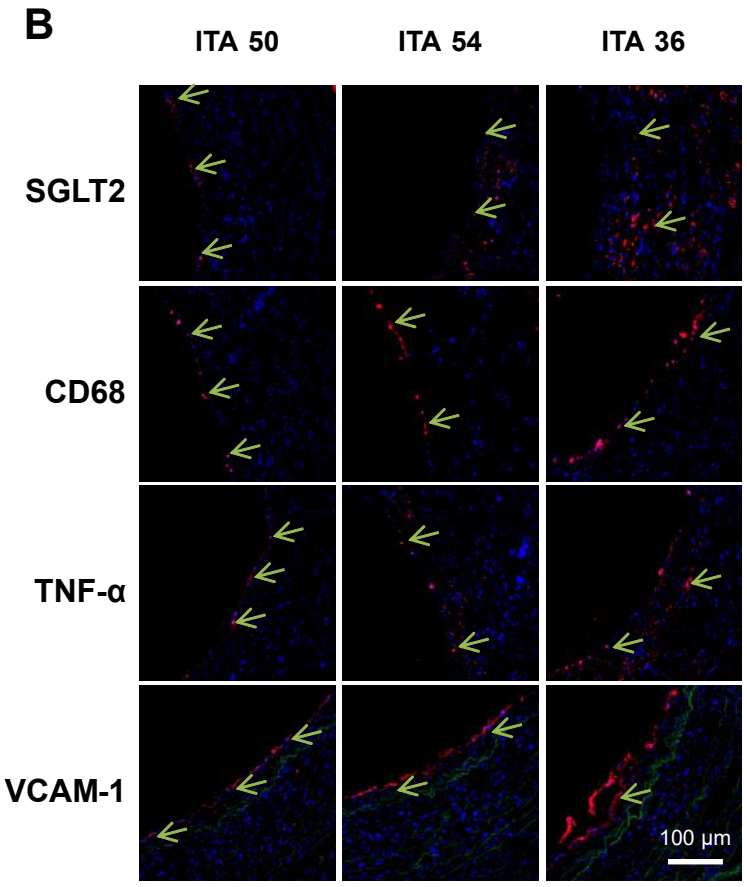

A
Human Internal Thoracic Artery (Biopsy Number)
4 14 20 24 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
IL1B (IL-1β)
IL6 (IL-6)
TNFA (TNF-α)
CCL2 (MCP-1)
CD68 (CD68)
SLC5A1 (SGLT1)
SLC5A2 (SGLT2)
AGTR1 (AT1R)
ACE1 (ACE1)
NCF1 (p47phox)
NOS3 (eNOS)
F3 (TF)
VCAM1 (VCAM-1)
ICAM1 (ICAM-1)
SELE (e-Selectin)
TP53 (p53)
CDKN1A (p21)
CDKN2A (p16)
2
4
6
8
10
B
SLC5A2-IL1B
r² = 0.6658
P < 0.0001
SLC5A2-TNFA
r² = 0.8463
P < 0.0001
SLC5A2-IL6
r² = 0.8047
P < 0.0001
SLC5A2-CD68
r² = 0.9388
P < 0.0001
SLC5A2-SLC5A1
r² = 0.9496
P < 0.0001
SLC5A2-AGTR1
r² = 0.7400
P < 0.0001
SLC5A2-NCF1
r² = 0.6760
P < 0.0001
SLC5A2-NOS3
r² = 0.6221
P < 0.0001
SLC5A2-F3
r² = 0.6952
P < 0.0001
SLC5A2-ICAM1
r² = 0.7413
P < 0.0001
SLC5A2-TP53
r² = 0.6472
P < 0.0001
SLC5A2-CDKN1A
r² = 0.7042
P < 0.01
Relative mRNA expression
Relative mRNA expression
Relative mRNA expression
Relative mRNA expression

Figure 2


A
Human Internal Thoracic Artery
1 2 3 4 5 6 7 8 9 10
SGLT1
SGLT2
p-p65
VCAM-1
AT1R
GAPDH
55 kDa
55 kDa
65 kDa
100 kDa
44 kDa
37 kDa
r² = 0.4874
P < 0.0001
SGLT1 / GAPDH
p-p65 / GAPDH
r² = 0.8471
P < 0.0001
SGLT2 / GAPDH
p-p65 / GAPDH
r² = 0.5604
P < 0.0001
SGLT2 / GAPDH
SGLT1 / GAPDH


B
ITA 50
ITA 54
ITA 36
SGLT2
CD68
TNF-α
VCAM-1
100 μm

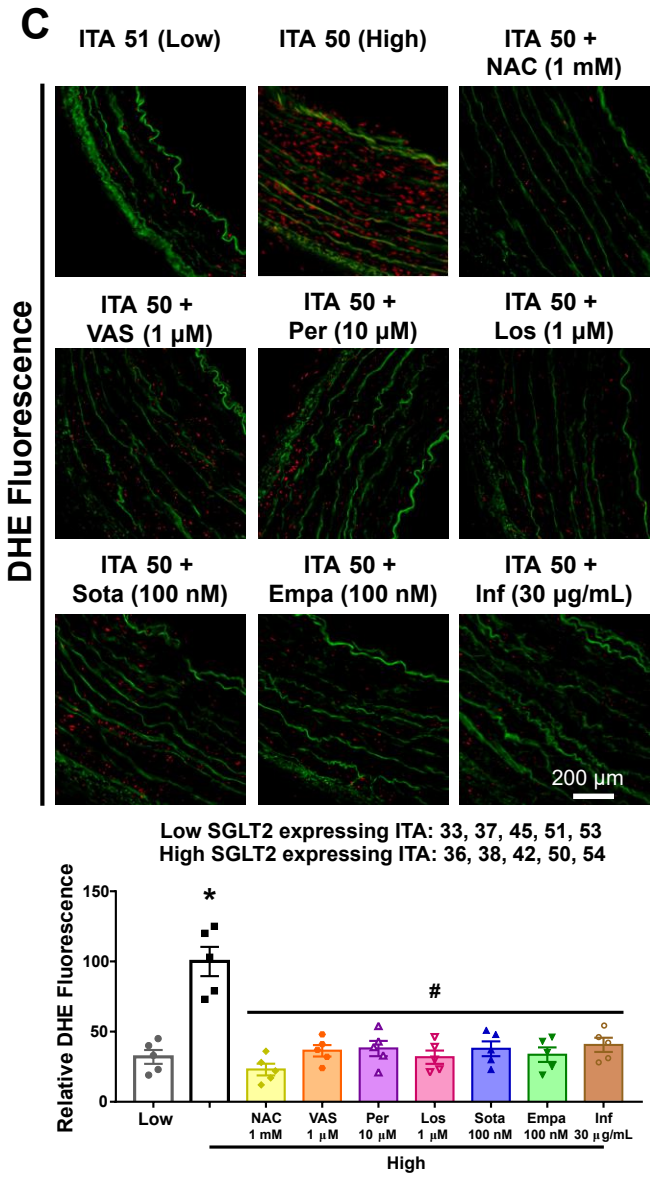
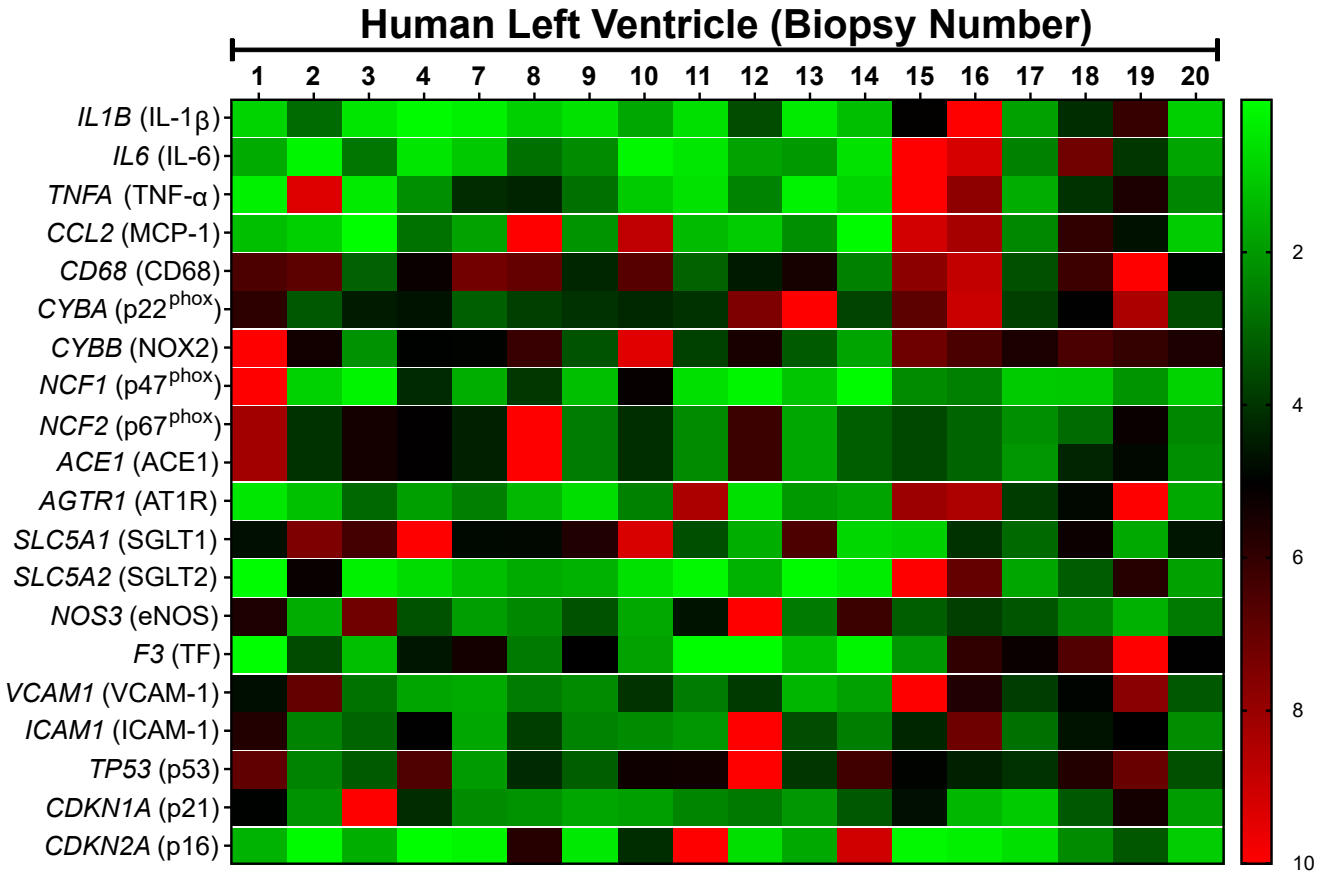

C
ITA 51 (Low)
ITA 50 (High)
ITA 50 + NAC (1 mM)
ITA 50 + VAS (1 μM)
ITA 50 + Per (10 μM)
ITA 50 + Los (1 μM)
ITA 50 + Sota (100 nM)
ITA 50 + Empa (100 nM)
ITA 50 + Inf (30 μg/mL)
DHE Fluorescence
200 μm
Low SGLT2 expressing ITA: 33, 37, 45, 51, 53
High SGLT2 expressing ITA: 36, 38, 42, 50, 54
Relative DHE Fluorescence
Low
NAC 1 mM
VAS 1 μM
Per 10 μM
Los 1 μM
Sota 100 nM
Empa 100 nM
Inf 30 μg/mL
High

Figure 3

A


Human Left Ventricle (Biopsy Number)
1 2 3 4 7 8 9 10 11 12 13 14 15 16 17 18 19 20
IL1B (IL-1β)
IL6 (IL-6)
TNFA (TNF-α)
CCL2 (MCP-1)
CD68 (CD68)
CYBA (p22phox)
CYBB (NOX2)
NCF1 (p47phox)
NCF2 (p67phox)
ACE1 (ACE1)
AGTR1 (AT1R)
SLC5A1 (SGLT1)
SLC5A2 (SGLT2)
NOS3 (eNOS)
F3 (TF)
VCAM1 (VCAM-1)
ICAM1 (ICAM-1)
TP53 (p53)
CDKN1A (p21)
CDKN2A (p16)
2
4
6
8
10

B

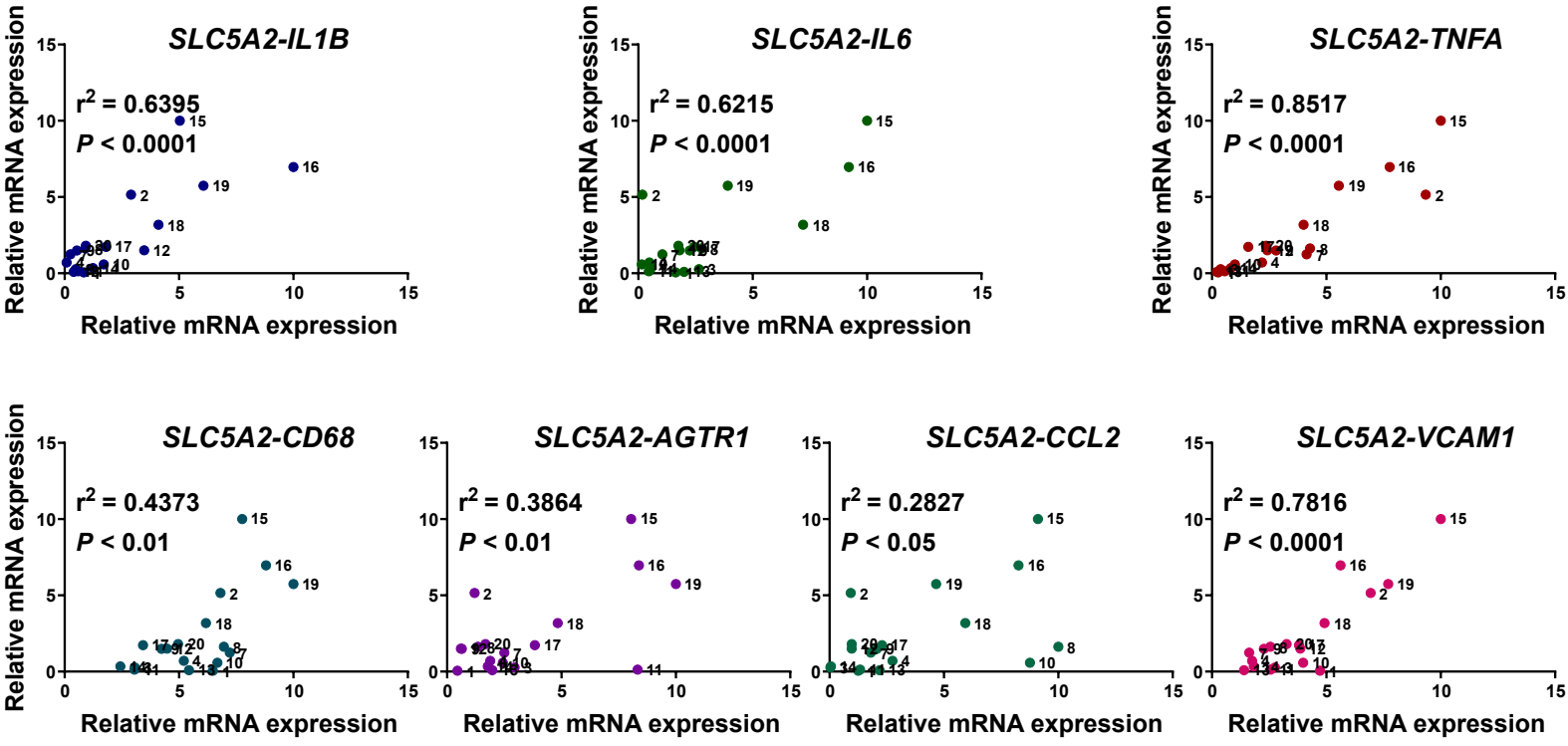

SLC5A2-IL1B
r2 = 0.6395
P < 0.0001
SLC5A2-IL6
r2 = 0.6215
P < 0.0001
SLC5A2-TNFA
r2 = 0.8517
P < 0.0001
SLC5A2-CD68
r2 = 0.4373
P < 0.01
SLC5A2-AGTR1
r2 = 0.3864
P < 0.01
SLC5A2-CCL2
r2 = 0.2827
P < 0.05
SLC5A2-VCAM1
r2 = 0.7816
P < 0.0001
Relative mRNA expression
Relative mRNA expression

Figure 4

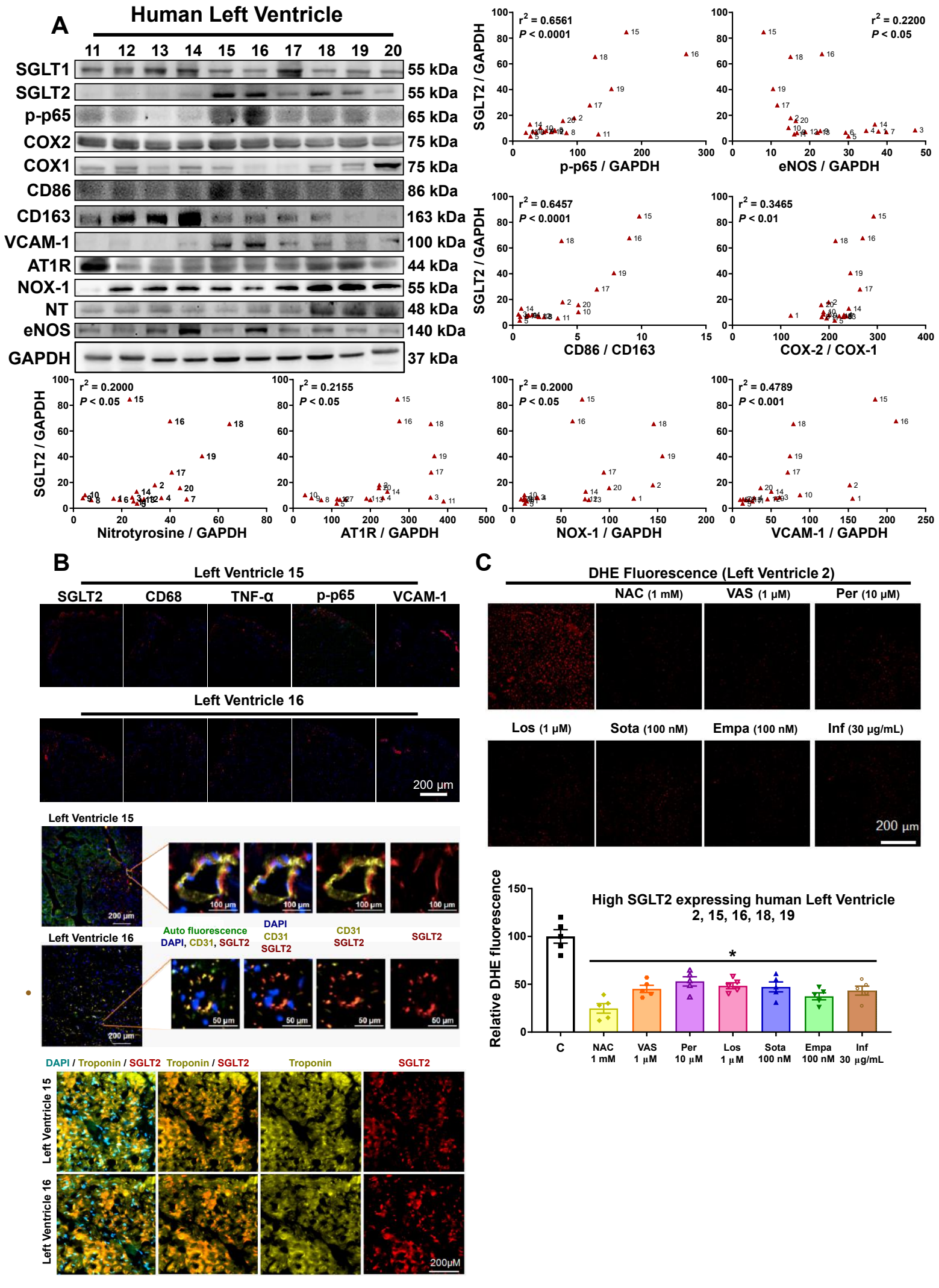

A
Human Left Ventricle
11 12 13 14 15 16 17 18 19 20
SGLT1 55 kDa
SGLT2 55 kDa
p-p65 65 kDa
COX2 75 kDa
COX1 75 kDa
CD86 86 kDa
CD163 163 kDa
VCAM-1 100 kDa
AT1R 44 kDa
NOX-1 55 kDa
NT 48 kDa
eNOS 140 kDa
GAPDH 37 kDa
SGLT2 / GAPDH
r² = 0.6561 P < 0.0001
p-p65 / GAPDH
r² = 0.2200 P < 0.05
eNOS / GAPDH
r² = 0.6457 P < 0.0001
CD86 / CD163
r² = 0.3465 P < 0.01
COX-2 / COX-1
r² = 0.2000 P < 0.05
Nitrotyrosine / GAPDH
r² = 0.2155 P < 0.05
AT1R / GAPDH
r² = 0.2000 P < 0.05
NOX-1 / GAPDH
r² = 0.4789 P < 0.001
VCAM-1 / GAPDH
B
Left Ventricle 15
SGLT2 CD68 TNF-α p-p65 VCAM-1
Left Ventricle 16
200 μm
Left Ventricle 15
Left Ventricle 16
Auto fluorescence
DAPI, CD31, SGLT2
DAPI
CD31
SGLT2
CD31
SGLT2
SGLT2
DAPI / Troponin / SGLT2
Troponin / SGLT2
Troponin
SGLT2
Left Ventricle 15
Left Ventricle 16
200μM
C
DHE Fluorescence (Left Ventricle 2)
NAC (1 mM) VAS (1 μM) Per (10 μM)
Los (1 μM) Sota (100 nM) Empa (100 nM) Inf (30 μg/mL)
200 μm
High SGLT2 expressing human Left Ventricle
2, 15, 16, 18, 19
Relative DHE fluorescence
C
NAC 1 mM
VAS 1 μM
Per 10 μM
Los 1 μM
Sota 100 nM
Empa 100 nM
Inf 30 μg/mL

Figure 5

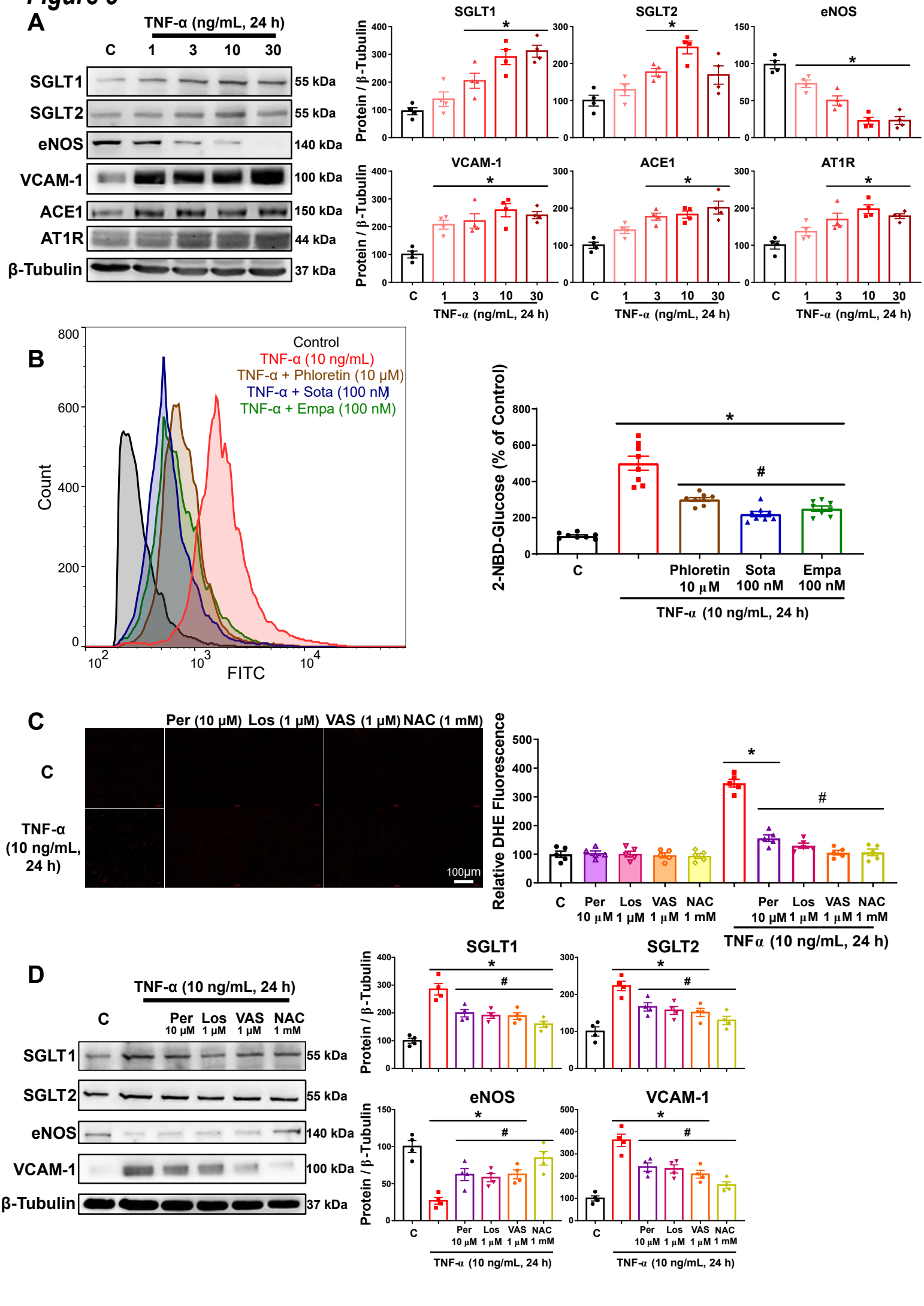

A
TNF-α (ng/mL, 24 h)
C
1
3
10
30
SGLT1
SGLT2
eNOS
VCAM-1
ACE1
AT1R
β-Tubulin
55 kDa
55 kDa
140 kDa
100 kDa
150 kDa
44 kDa
37 kDa
Protein / β-Tubulin
TNF-α (ng/mL, 24 h)
B
Control
TNF-α (10 ng/mL)
TNF-α + Phloretin (10 μM)
TNF-α + Sota (100 nM)
TNF-α + Empa (100 nM)
Count
FITC
2-NBD-Glucose (% of Control)
Phloretin 10 μM
Sota 100 nM
Empa 100 nM
TNF-α (10 ng/mL, 24 h)
C
Per (10 μM)
Los (1 μM)
VAS (1 μM)
NAC (1 mM)
TNF-α (10 ng/mL, 24 h)
100μm
Relative DHE Fluorescence
D
TNF-α (10 ng/mL, 24 h)
Per 10 μM
Los 1 μM
VAS 1 μM
NAC 1 mM

Figure 6

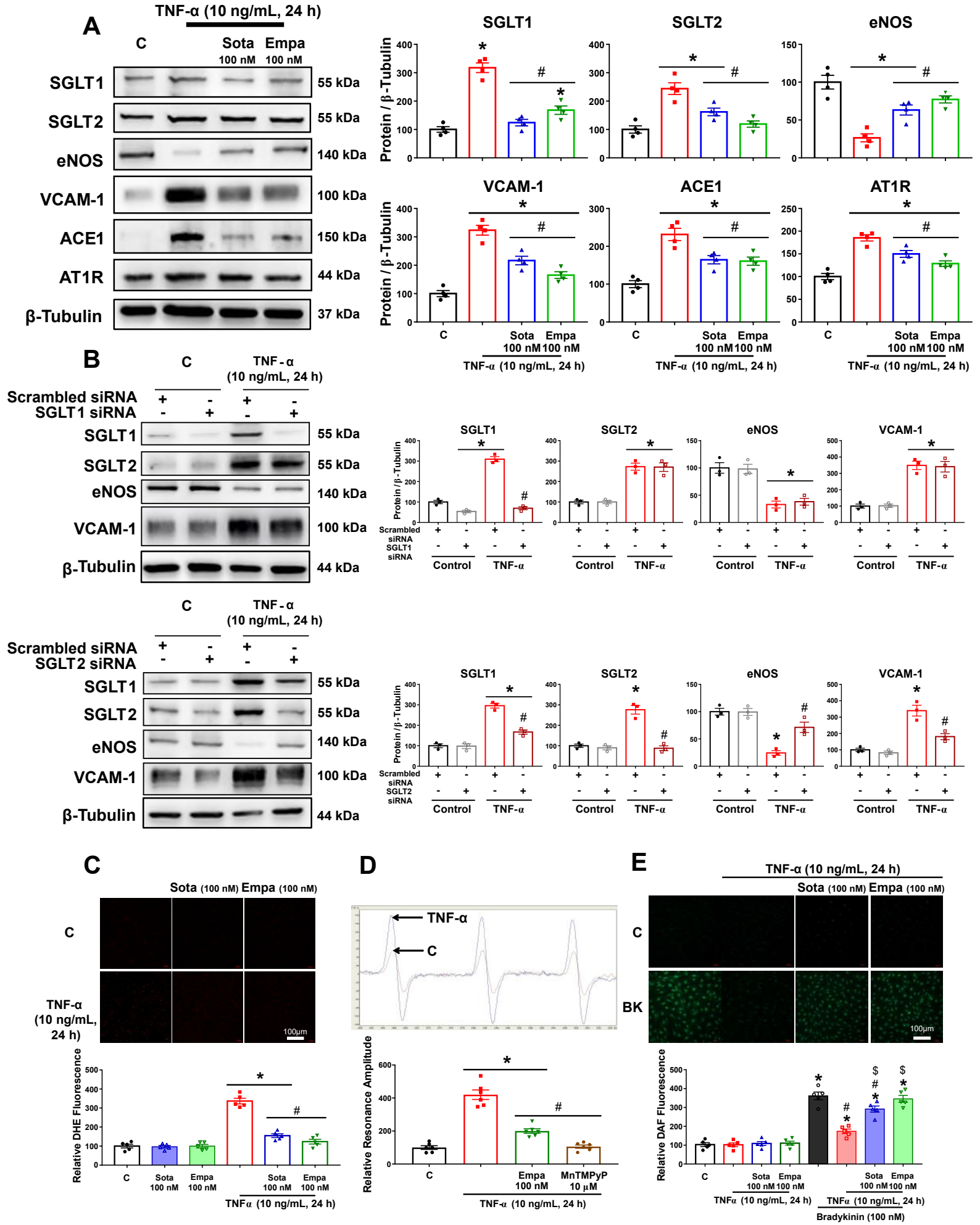
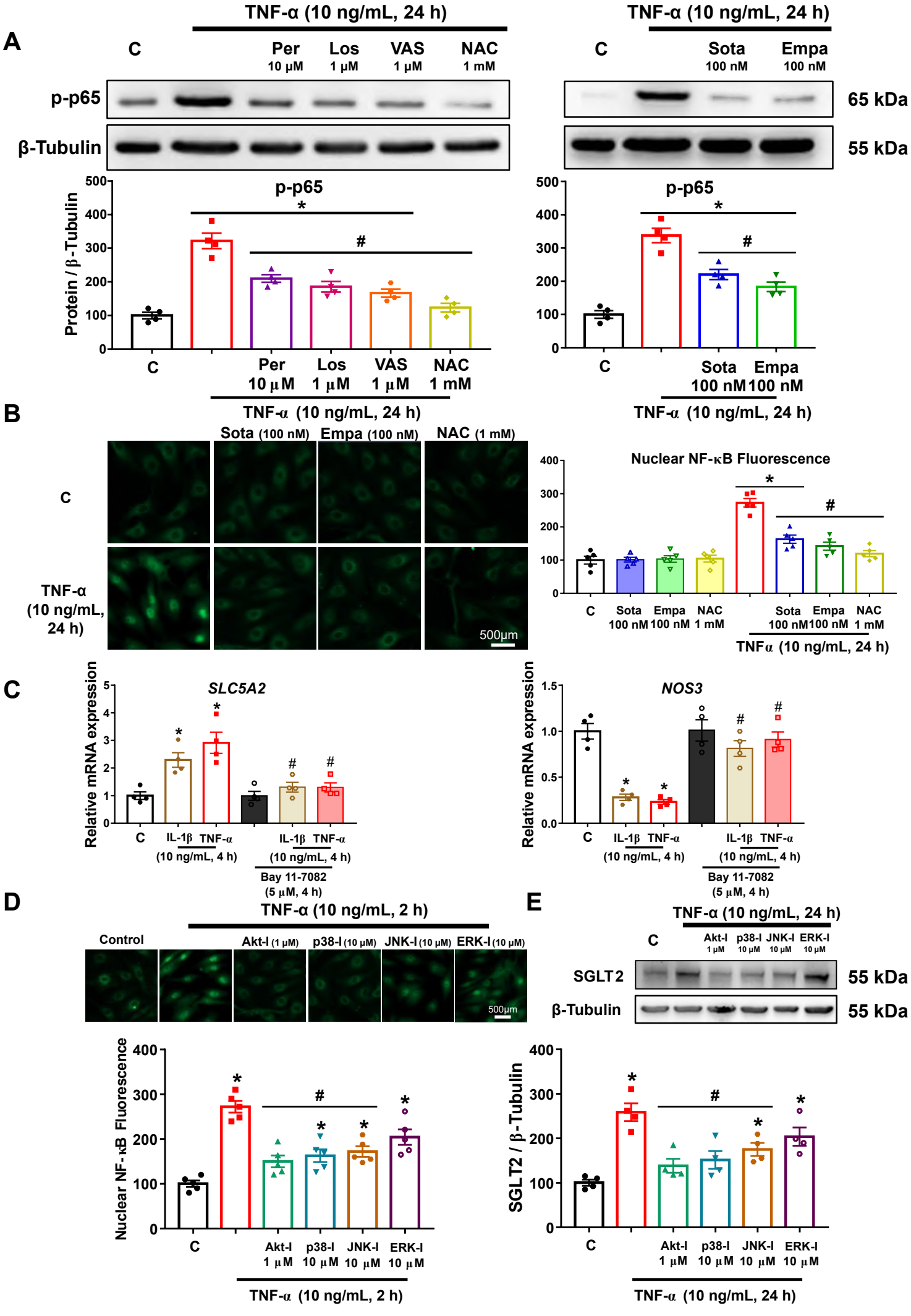

A
TNF-α (10 ng/mL, 24 h)
C
Sota 100 nM
Empa 100 nM
SGLT1
SGLT2
eNOS
VCAM-1
ACE1
AT1R
β-Tubulin
55 kDa
55 kDa
140 kDa
100 kDa
150 kDa
44 kDa
37 kDa
Protein / β-Tubulin
TNF-α (10 ng/mL, 24 h)
B
Scrambled siRNA
SGLT1 siRNA
SGLT2 siRNA
Control
TNF-α
C
D
E
TNF-α
Relative DHE Fluorescence
Relative Resonance Amplitude
Relative DAF Fluorescence
MnTMPyP 10 μM
Bradykinin (100 nM)
100μm

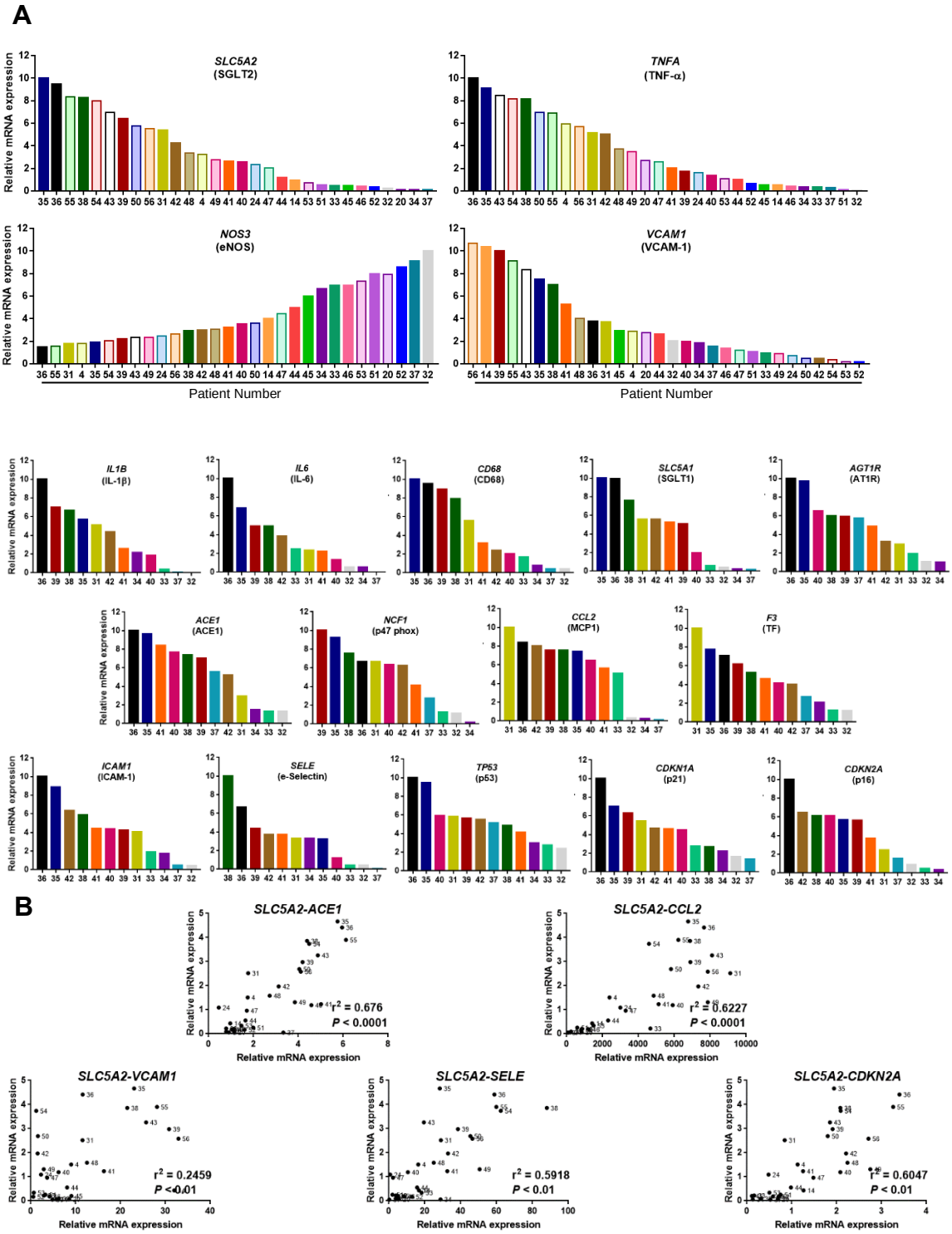
Figure 7


A
TNF-α (10 ng/mL, 24 h)
C
Per 10 μM
Los 1 μM
VAS 1 μM
NAC 1 mM
Sota 100 nM
Empa 100 nM
p-p65
β-Tubulin
65 kDa
55 kDa
Protein / β-Tubulin
p-p65
B
Sota (100 nM)
Empa (100 nM)
NAC (1 mM)
TNF-α (10 ng/mL, 24 h)
500μm
Nuclear NF-κB Fluorescence
C
Sota 100 nM
Empa 100 nM
NAC 1 mM
TNFα (10 ng/mL, 24 h)
C
SLC5A2
NOS3
Relative mRNA expression
IL-1β (10 ng/mL, 4 h)
TNF-α (10 ng/mL, 4 h)
Bay 11-7082 (5 μM, 4 h)
D
TNF-α (10 ng/mL, 2 h)
Control
Akt-I (1 μM)
p38-I (10 μM)
JNK-I (10 μM)
ERK-I (10 μM)
500μm
Nuclear NF-κB Fluorescence
E
TNF-α (10 ng/mL, 24 h)
SGLT2
β-Tubulin
55 kDa
55 kDa
SGLT2 / β-Tubulin

SGLT2 expression in human vasculature and heart correlates with low-grade inflammation and causes eNOS-NO/ROS imbalance

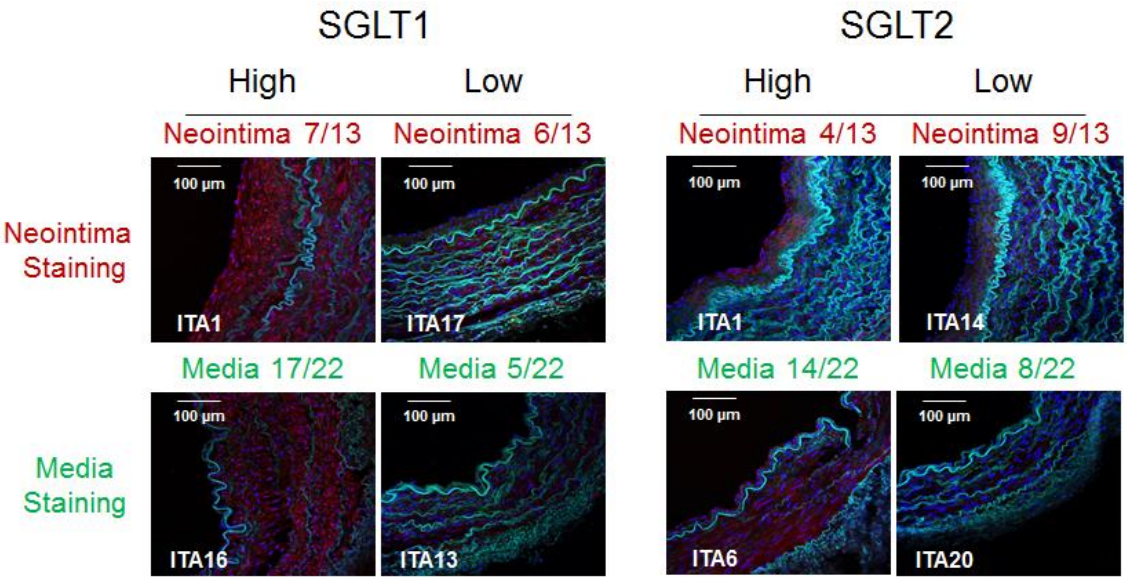
Supplemental Figures

Supplemental Figure 1 SGLT2 mRNA expression in human internal thoracic artery



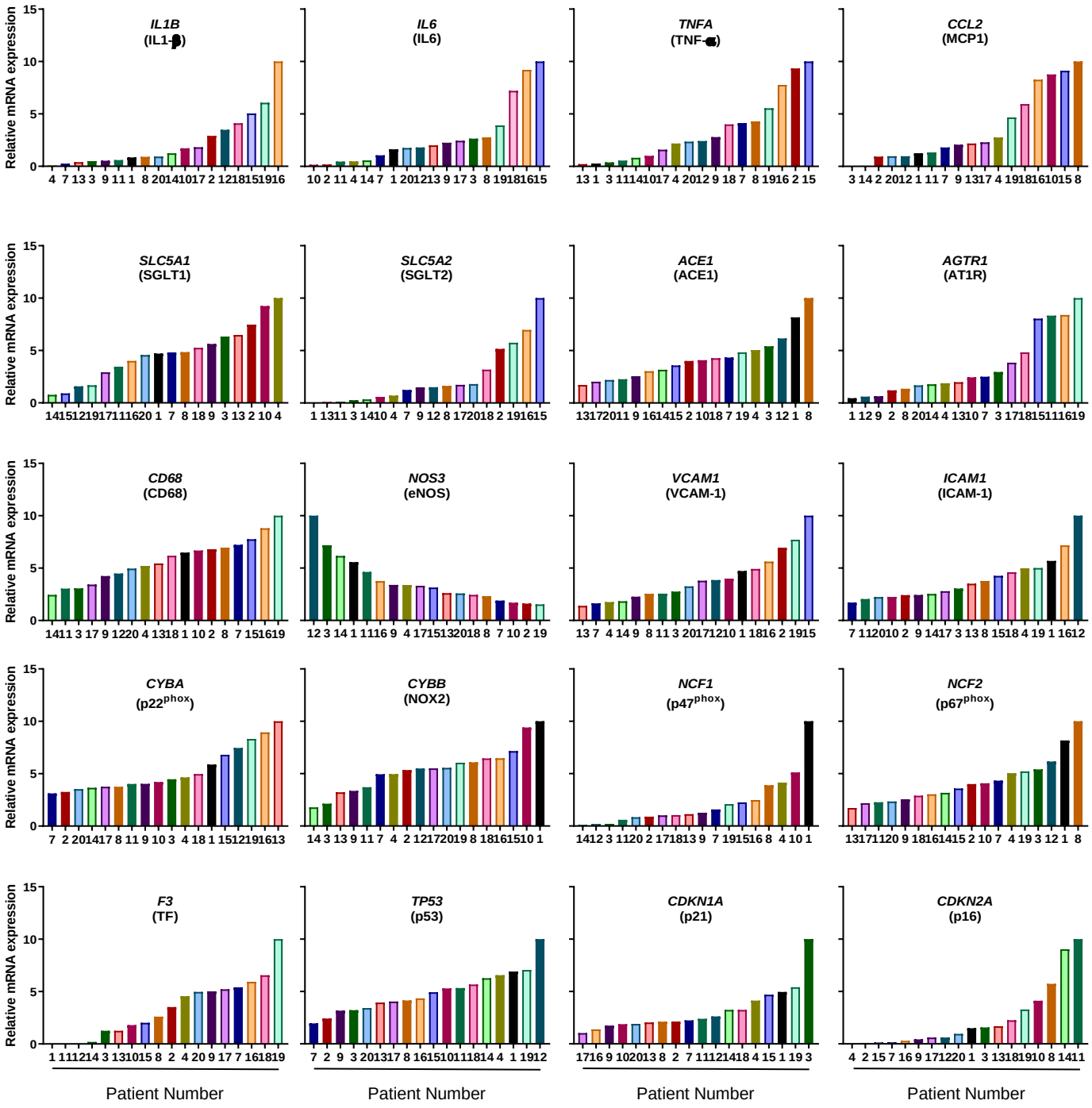
Expression levels of target genes in ITA segments from 12 or 30 patients undergoing bypass surgery as assessed by RT-qPCR (A). The correlation of SGLT2 mRNA levels with those of different markers was established using Pearson's correlation and shown (B).

Supplemental Figure 2 SGLT1 and SGLT2 are expressed in the media and neointima of human ITA



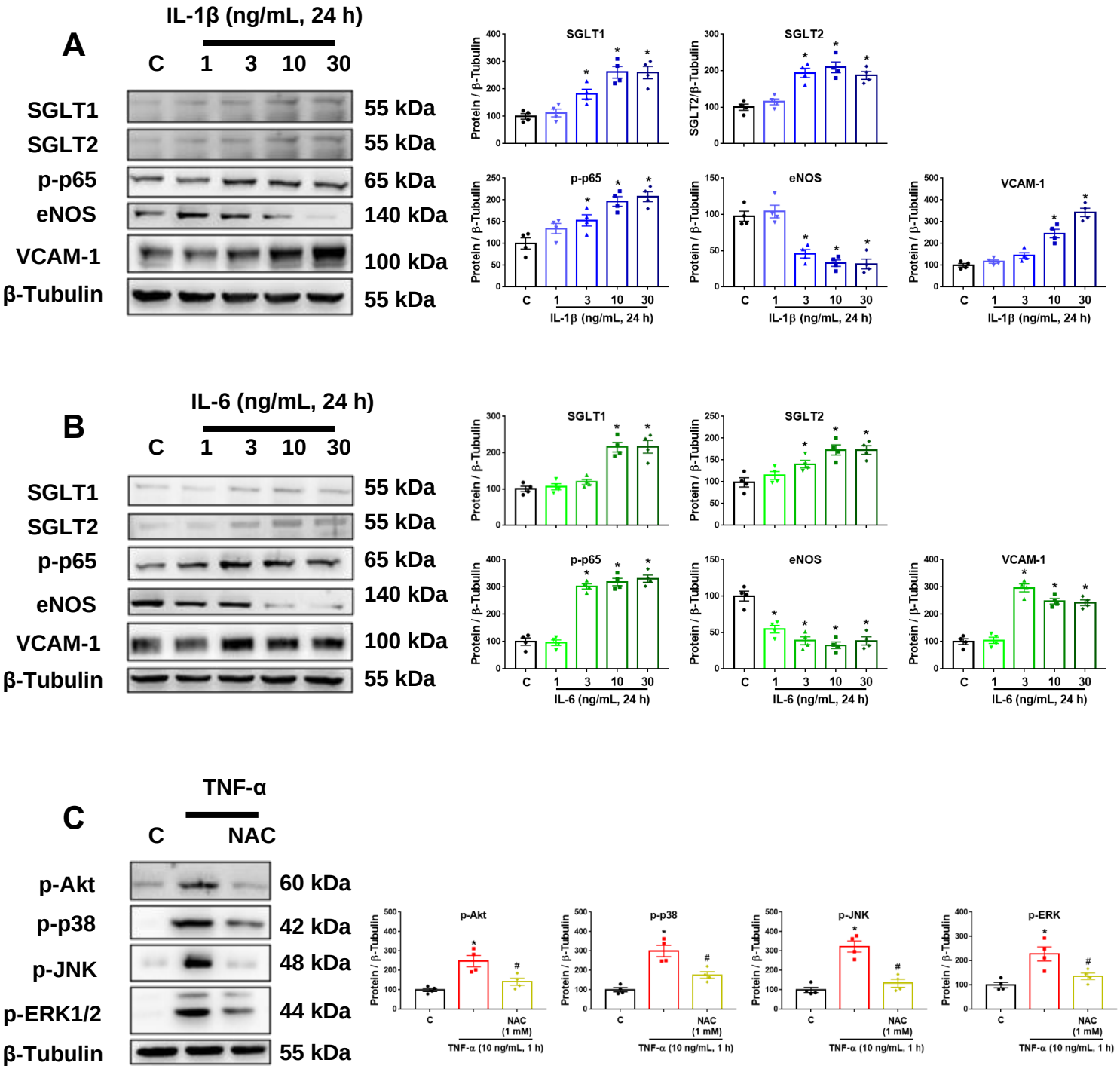
Representative immunofluorescence staining of SGLT1 and SGLT2 in cryosections of ITA in the neointima and the media.

Supplemental Figure 3 SGLT2 mRNA expression in human LV



Expression levels of target genes in left ventricle biopsies of 18 patients undergoing aortic valve surgery as assessed by RT-qPCR.

Supplemental Figure 4 Pro-inflammatory cytokines induce SGLT2 expression in endothelial cells



(A, B) ECs were exposed to a pro-inflammatory cytokine for 24 h before the determination of the expression level of target proteins by Western blot analysis. Left panels show representative Western blot analysis and right panels cumulative data. (C) TNF-α caused the redox-sensitive phosphorylation of Akt, p38 MAPK, JNK and ERK1/2.

Data are presented as mean ± SEM of n=4 $*P < 0.05$ vs control (C), $^{\#}P < 0.05$ versus TNF-α treatment using one-way ANOVA followed by Tukey's multiple comparison test.

Supplemental Tables

Supplemental Table 1 Simple and multiple linear regression analyses to assess the independent effect of SGLT2 and SGLT1 expression on the expression of pro-inflammatory cytokines in 30 ITA specimens

Simple and multiple regression analyses were performed where SGLT2 or SGLT1 was used as a dependent variable and IL-1ß, IL-6, TNF-α, age, male sex, hypertension, dyslipidemia, diabetes, smoking status, and medications including statins, beta-blockers, ACE/AT1R inhibitors, and anti-platelet therapy as independent variables. The standardized beta values are presented for each covariate.

Table S1.1: Simple and multiple linear regression analyses of SGLT2 in 30 ITA specimens (Model with IL-1β)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-1β	0.92	0.12	<0.001	0.87	0.13	<0.001
Age (years)	0.04	0.26	0.86	0.34	0.17	0.07
Male sex	-1.63	2.81	0.57	1.30	1.73	0.46
Hypertension	-0.06	3.04	0.98	-2.27	1.89	0.25
Dyslipidemia	0.10	2.33	0.97	2.80	1.55	0.09
Diabetes	3.47	2.75	0.22	2.60	1.60	0.12
Smoking	-1.29	2.32	0.58	-1.06	1.52	0.50
Statins	-1.55	2.75	0.58	-4.88	1.97	0.02
Beta-blockers	3.83	2.88	0.20	2.38	1.82	0.21
ACE/AT1R inhibitors	-7.17	2.24	0.003	-2.33	1.84	0.22
Anti-platelet therapy	1.71	6.16	0.78	0.95	3.69	0.80

Table S1.2: Simple and multiple linear regression analyses of SGLT2 in 30 ITA specimens (Model with IL-6)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-6	1.04	0.10	<0.001	1.06	0.13	<0.001
Age (years)	0.04	0.26	0.86	0.02	0.16	0.92
Male sex	-1.63	2.81	0.57	0.42	1.46	0.78
Hypertension	-0.06	3.04	0.98	-0.81	1.66	0.63
Dyslipidemia	0.10	2.33	0.97	1.91	1.33	0.17
Diabetes	3.47	2.75	0.22	0.62	1.44	0.67
Smoking	-1.29	2.32	0.58	-0.89	1.31	0.51
Statins	-1.55	2.75	0.58	-3.05	1.69	0.09
Beta-blockers	3.83	2.88	0.20	-0.52	1.58	0.74
ACE/AT1R inhibitors	-7.17	2.24	0.003	-0.83	1.67	0.63
Anti-platelet therapy	1.71	6.16	0.78	-0.52	3.17	0.87

Table S1.3: Simple and multiple linear regression analyses of SGLT2 in 30 ITA specimens (Model with TNF-α)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of TNF-α	1.38	0.11	<0.001	1.33	0.12	<0.001
Age (years)	0.04	0.26	0.86	-0.07	0.13	0.58
Male sex	-1.63	2.81	0.57	-2.56	1.14	0.04
Hypertension	-0.06	3.04	0.98	-1.15	1.30	0.39
Dyslipidemia	0.10	2.33	0.97	-0.33	1.07	0.76
Diabetes	3.47	2.75	0.22	1.94	1.10	0.10
Smoking	-1.29	2.32	0.58	0.45	1.02	0.67
Statins	-1.55	2.75	0.58	2.59	1.44	0.09
Beta-blockers	3.83	2.88	0.20	0.86	1.24	0.50
ACE/AT1R inhibitors	-7.17	2.24	0.003	-2.19	1.23	0.09
Anti-platelet therapy	1.71	6.16	0.78	-5.36	2.53	0.049

Table S1.4: Simple and multiple linear regression analyses of SGLT1 in 30 ITA specimens (Model with IL-1β)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-1β	0.73	0.11	<0.001	0.73	0.13	<0.001
Age (years)	0.05	0.21	0.82	0.28	0.17	0.12
Male sex	-1.25	2.32	0.59	1.46	1.51	0.41
Hypertension	0.24	2.50	0.93	-0.94	1.88	0.62
Dyslipidemia	0.11	1.91	0.96	2.04	1.54	0.20
Diabetes	2.66	2.27	0.25	1.87	1.59	0.26
Smoking	-0.57	1.91	0.77	-0.57	1.51	0.71
Statins	-1.21	2.27	0.60	-4.17	1.96	0.048
Beta-blockers	2.79	2.39	0.25	2.01	1.81	0.28
ACE/AT1R inhibitors	-5.16	1.92	0.01	-0.82	1.83	0.66
Anti-platelet therapy	3.13	5.04	0.54	3.23	3.67	0.39

Table S1.5: Simple and multiple linear regression analyses of SGLT1 in 30 ITA specimens (Model with IL-6)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-6	0.85	0.08	<0.001	0.94	0.11	<0.001
Age (years)	0.05	0.21	0.82	-0.01	0.13	0.92
Male sex	-1.25	2.32	0.59	0.85	1.23	0.50
Hypertension	0.24	2.50	0.93	0.43	1.39	0.76
Dyslipidemia	0.11	1.91	0.96	1.31	1.12	0.26
Diabetes	2.66	2.27	0.25	0.01	1.21	0.99
Smoking	-0.57	1.91	0.77	-0.52	1.10	0.64
Statins	-1.21	2.27	0.60	-2.62	1.42	0.08
Beta-blockers	2.79	2.39	0.25	-0.51	1.33	0.71
ACE/AT1R inhibitors	-5.16	1.92	0.01	0.88	1.41	0.54
Anti-platelet therapy	3.13	5.04	0.54	2.07	2.67	0.45

Table S1.6: Simple and multiple linear regression analyses of SGLT1 in 30 ITA specimens (Model with TNF-α)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of TNF-α	1.14	0.09	<0.001	1.15	0.12	<0.001
Age (years)	0.05	0.21	0.82	-0.08	0.13	0.55
Male sex	-1.25	2.32	0.59	-1.80	1.14	0.13
Hypertension	0.24	2.50	0.93	0.06	1.30	0.96
Dyslipidemia	0.11	1.91	0.96	-0.64	1.07	0.56
Diabetes	2.66	2.27	0.25	1.26	1.10	0.27
Smoking	-0.57	1.91	0.77	0.68	1.02	0.51
Statins	-1.21	2.27	0.60	2.25	1.44	0.14
Beta-blockers	2.79	2.39	0.25	0.73	1.24	0.56
ACE/AT1R inhibitors	-5.16	1.92	0.01	-0.52	1.24	0.68
Anti-platelet therapy	3.13	5.04	0.54	-2.15	2.53	0.41

Supplemental Table 2 Simple and multiple regression analyses to assess the independent effect of SGLT2 and SGLT1 expression on the expression of pro-inflammatory cytokines in 18 LV specimens

Simple and multiple regression analyses were performed where SGLT2 or SGLT1 was used as a dependent variable and IL-1ß, IL-6, TNF-α, age, male sex, hypertension, dyslipidemia, diabetes, smoking status and medications including statins, beta-blockers, ACE/AT1R inhibitors, and anti-platelet therapy as independent variables. The standardized beta values are presented for each covariate.

Table S2.1: Simple and multiple linear regression analyses of SGLT2 in 18 LV specimens (Model with IL-1β)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-1β	0.86	0.16	<0.001	0.83	0.27	0.02
Age (years)	-0.01	0.10	0.93	0.01	0.09	0.91
Male sex	0.36	0.67	0.60	0.45	0.59	0.47
Hypertension	-0.63	0.68	0.37	-0.54	0.86	0.55
Dyslipidemia	0.62	0.68	0.37	-0.60	0.87	0.52
Diabetes	0.81	0.73	0.29	0.54	0.65	0.43
Smoking	0.25	0.76	0.74	1.00	0.81	0.26
Statins	-0.43	0.67	0.53	0.53	0.63	0.43
Beta-blockers	0.40	0.71	0.58	0.01	0.72	0.99
ACE/AT1R inhibitors	0.11	0.72	0.88	0.56	0.94	0.57
Anti-platelet therapy	-1.10	0.64	0.10	-1.09	0.94	0.29

Table S2.2: Simple and multiple linear regression analyses of SGLT2 in 18 LV specimens (Model with IL-6)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-6	0.74	0.14	<0.001	0.65	0.21	0.02
Age (years)	-0.01	0.10	0.93	-0.05	0.09	0.60
Male sex	0.36	0.67	0.60	-0.24	0.63	0.71
Hypertension	-0.63	0.68	0.37	-0.64	0.87	0.49
Dyslipidemia	0.62	0.68	0.37	0.20	0.77	0.80
Diabetes	0.81	0.73	0.29	0.81	0.64	0.25
Smoking	0.25	0.76	0.74	0.60	0.86	0.52
Statins	-0.43	0.67	0.53	-0.17	0.58	0.78
Beta-blockers	0.40	0.71	0.58	-0.35	0.71	0.64
ACE/AT1R inhibitors	0.11	0.72	0.88	0.17	0.93	0.86
Anti-platelet therapy	-1.10	0.64	0.10	-1.06	0.95	0.31

Table S2.3: Simple and multiple linear regression analyses of SGLT2 in 18 LV specimens (Model with TNF-α)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of TNF-α	0.84	0.09	<0.001	0.72	0.12	0.001
Age (years)	-0.01	0.10	0.93	-0.01	0.05	0.89
Male sex	0.36	0.67	0.60	0.05	0.36	0.90
Hypertension	-0.63	0.68	0.37	-0.07	0.53	0.90
Dyslipidemia	0.62	0.68	0.37	0.18	0.46	0.71
Diabetes	0.81	0.73	0.29	0.68	0.39	0.13
Smoking	0.25	0.76	0.74	0.66	0.50	0.24
Statins	-0.43	0.67	0.53	-0.24	0.35	0.52
Beta-blockers	0.40	0.71	0.58	0.13	0.44	0.78
ACE/AT1R inhibitors	0.11	0.72	0.88	-0.23	0.55	0.69
Anti-platelet therapy	-1.10	0.64	0.10	-0.68	0.59	0.29

Table S2.4: Simple and multiple linear regression analyses of SGLT1 in 18 LV specimens (Model with IL-1β)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-1β	-0.38	0.23	0.13	-0.48	0.42	0.30
Age (years)	-0.09	0.09	0.34	-0.15	0.14	0.34
Male sex	-0.11	0.64	0.87	0.05	0.93	0.96
Hypertension	-0.42	0.65	0.53	-0.02	1.37	0.99
Dyslipidemia	-1.02	0.60	0.11	0.70	1.37	0.63
Diabetes	-1.67	0.58	0.01	-1.43	1.03	0.21
Smoking	-0.15	0.71	0.84	0.99	1.28	0.47
Statins	-0.31	0.63	0.63	-0.04	1.00	0.97
Beta-blockers	0.14	0.68	0.83	-0.37	1.14	0.76
ACE/AT1R inhibitors	-0.23	0.67	0.74	-1.03	1.50	0.52
Anti-platelet therapy	-0.49	0.64	0.46	-1.09	1.48	0.49

Table S2.5: Simple and multiple linear regression analyses of SGLT1 in 18 LV specimens (Model with IL-6)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of IL-6	-0.34	0.20	0.12	-0.50	0.31	0.15
Age (years)	-0.09	0.09	0.34	-0.11	0.13	0.41
Male sex	-0.11	0.64	0.87	0.57	0.90	0.55
Hypertension	-0.42	0.65	0.53	0.03	1.25	0.98
Dyslipidemia	-1.02	0.60	0.11	0.35	1.10	0.76
Diabetes	-1.67	0.58	0.01	-1.54	0.92	0.14
Smoking	-0.15	0.71	0.84	1.43	1.24	0.29
Statins	-0.31	0.63	0.63	0.35	0.83	0.69
Beta-blockers	0.14	0.68	0.83	-0.18	1.02	0.87
ACE/AT1R inhibitors	-0.23	0.67	0.74	-0.90	1.33	0.52
Anti-platelet therapy	-0.49	0.64	0.46	-1.35	1.37	0.36

Table S2.6: Simple and multiple linear regression analyses of SGLT1 in 18 LV specimens (Model with TNF-α)

	Simple regression			Multiple regression		
	β	SE	*P* value	β	SE	*P* value
Gene expression of TNF-α	-0.16	0.21	0.45	-0.23	0.33	0.51
Age (years)	-0.09	0.09	0.34	-0.13	0.15	0.42
Male sex	-0.11	0.64	0.87	0.20	1.00	0.85
Hypertension	-0.42	0.65	0.53	-0.14	1.47	0.93
Dyslipidemia	-1.02	0.60	0.11	0.08	1.27	0.95
Diabetes	-1.67	0.58	0.01	-1.60	1.07	0.18
Smoking	-0.15	0.71	0.84	0.93	1.39	0.57
Statins	-0.31	0.63	0.63	0.41	0.96	0.68
Beta-blockers	0.14	0.68	0.83	-0.30	1.21	0.81
ACE/AT1R inhibitors	-0.23	0.67	0.74	-0.56	1.52	0.72
Anti-platelet therapy	-0.49	0.64	0.46	-0.93	1.61	0.59

Supplemental Material

Supplemental methods

Endothelial cell culture

Porcine hearts were collected from the local slaughterhouse (SOCOPA, Holtzheim, France). ECs were isolated from the right coronary artery as described previously.[6] Briefly, the coronary artery was dissected and cleaned of surrounding connective tissues. After washing with calcium-free PBS to remove remaining blood, ECs were isolated by type I collagenase (Gibco, #17100-017, USA) treatment at 1 mg/mL (250 U/mg) for 20 min at 37 °C and cultured in a T25 flask containing MCDB 131 medium supplemented with 15 % fetal calf serum (Dominique Dutcher), fungizone (2.5 µg/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM, all from PAN Biotech, Germany) and grown to 70-80 % confluence for 48-72 h. ECs at passage 1 were exposed to serum-free culture medium for 2 h before the addition of a single cytokine (IL-1β, IL-6, TNF-α). In some experiments, ECs were pretreated with a pharmacological modulator for at least 30 min before the cytokine stimulation.

RNA isolation and quantitative real time-polymerase chain reaction

Cells stimulated with TNF-α (10 ng/mL, 4 h) were washed 3 times with PBS then scraped using 1 mL of QIAzol **R** from Qiagen. In some experiments, cells were treated with an NF-κB inhibitor (Bay 11-7082, 5 µM) for 1 h prior to stimulation with TNF-α. On the other hand, frozen ITA were homogenized using liquid nitrogen then added into 1 mL of QIAzol**R**. RNA was quantified using Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, USA) and a total of 1000 ng of RNA was reverse transcribed using Maxima first strand cDNA synthesis kit with dsDNase k1672 (Thermo Fischer Scientific) in My iQ 576BR0703 icycler (Biorad, USA) to produce cDNA. Quantitative PCR was then performed using CFX connect 788BR2133 (Biorad). Amplifications were carried out in 10 µL reaction solutions containing 5 µL 2x PowerUp SYBR Green master mix A25742 (Applied Biosystems), 10 ng cDNA and 300 nM of each specific forward and reverse primer. The used primers are listed

in the Table below. PCR conditions were 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min. The specificity of each pair of primers was checked by melting curve analysis (95 °C for 15 s, 60 °C for 30 s and a continuous raise in temperature to 95 °C at 0.5 °C/s ramp rate followed by 95 °C for 15 s). The mean of three different house-keeping genes were used for normalization and quantification was performed using traditional 2^-ΔΔCt formula.

PORCINE			
Gene	**Protein**	**Forward Primer Sequence**	**Reverse Primer Sequence**
18S	18S ribosomal RNA	GCCCTCGGTCGAGTTGTC	CTTGCAGGGCGGTGACAG
Gusb	Glucuronidase beta (GUSB)	GGTGTGGTATGAACGGGAGG	CATTCACCCACACAATGGCG
Hprt	Hypoxanthine phosphoribosyltransferase 1 (HPRT)	CCCAGCGTCGTGATTAGTGA	GCCGTTCAGTCCTGTCCATA
Nos3	Endothelial nitric oxide synthase (eNOS)	GCATCGCCAGAAAGAAGACG	GAATTGACGCCTTCACTCGC
Slc5a2	Sodium-glucose co-transporter2 (SGLT2)	CATCACCATGATCTACACTGTGAC	GGTCTGCACCGTATCCGT

HUMAN			
Gene	**Proteine**	**Forward Primer Sequence**	**Reverse Primer Sequence**
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
GUSB	Glucuronidase beta (GUSB)	CGCCCTGCCTATCTGTATTC	TCCCCACAGGGAGTGTGTAG
ACTB	β-Actin	GCCAGGGCTTACCTGTACACT	CATTTTTAAGGTGTGCACTTTTATTC
ACE	Angiotensin-converting enzyme1 (ACE1)	AACAGGTGCTGTTCCAGAGC	CAGCTCCTTGGCCTTCTGG
AGTR1	Angiotensin II receptor type 1 (AT1R)	ATTTTGTGAAAGAAGGAGCAAGA	TGCTCATTTGGTAGTGAAGTGC
CCL2	Monocyte chemoattractant protein-1	CACCTTCATTCCCCAAGGGC	ACACTTGCTGCTGGTGATTCT

	(MCP-1)		
CD68	Cluster of differentiation (CD68)	TCTTTCACCAGCTGTCCACC	CACTGGGGCAGGAGAAACTT
CDKN1A	Cyclin dependent kinase-interacting protein 1A (p21)	GGCAGACCAGCATGACAGATT	AGGGCTTCCTCTTGGAGAAGAT
CDKN2A	Cyclin dependent kinase inhibitor 2A (p16)	CGCGATGTCGCACGGTA	TCTATGCGGGCATGGTTACT
CYBA	Human neutrophil cytochrome b light chain ($p22^{Phox}$)	CGAGCGGCATCTACCTACTG	GCTTGATGGTGCCTCCGAT
CYBB	NADPH oxidase 2 (NOX2)	CCACCAATCTGAAGCTCAAAA	AAACCACTCAAAGGCATGTGT
F3	Tissue factor	GGGAACCCAAACCCGTCAAT	GTCGGTGAGGTCACACTCTG
ICAM1	Intercellular adhesion molecule-1 (ICAM-1)	CAACCTCAGCCTCGCTATGG	CGGGGCAGGATGACTTTTGA
IL1B	Interleukin 1 beta (IL-1β)	GCAGAAGTACCTGAGCTCGC	AAGTCATCCTCATTGCCACTGT
IL6	Interleukin 6 (IL-6)	CCACCGGGAACGAAAGAGAA	GAGAAGGCAACTGGACCGAA
NCF1	Phagocyte oxidase ($p47^{Phox}$)	CCCAGCCAGCACTATGTGTA	GATCGCCCCTGCCTCAATAG
NCF2	Neutrophil cytosolic factor-2 ($p67^{Phox}$)	GTGGCATCTGTGGTGGATCA	CTCTGGGGTTTTCGGTCTGG
NOS3	Endothelial nitric oxide synthase (eNOS)	GACCCTCACCGCTACAACAT	CCGGGTATCCAGGTCCAT
SELE	Endothelial selectin (e-Selectin)	ACACAGCTGCCTGTACCAAT	CCAGGGCTGTACAGTTCACA
SLC5A1	Sodium-glucose co-transporter1 (SGLT1)	GTTTGCTTATGGAACCGGGAG	TGGCGAAGAGGATAATGGCA

SLC5A2	Sodium-glucose co-transporter2 (SGLT2)	CTCTCTCTTCGCCAGCAACA	CAGTAGCAGCACCACGAAGA
TNFA	Tumor necrosis factor alpha (TNF-α)	CTGCACTTTGGAGTGATCGG	CTCGGGGTTCGAGAAGATGA
TP53	Tumor suppressor protein (p53)	AGTCACAGCACATGACGGAG	ACCATCGCTATCTGAGCAGC
VCAM1	Vascular cell adesion molecule-1 (VCAM-1)	CCTGGGAAGATGGTCGTGAT	GATTCTGGGGTGGTCTCGAT

Western blot analysis

After stimulation, ECs washed with cold PBS were homogenized and lysed in RIPA extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5), NaCl 150, Na_3VO_4 1, $Na_4P_2O_7$ 10, NaF 20, N-ethylmaleimide 20 mM, 0.1% sodium dodecyl sulfate, 1 % Triton X-100 and protease inhibitor cocktail (Complete Mini, Roche)). In some experiments, cells were treated with perindoprilat (10 μM), losartan (1 μM), VAS-2870 (1 μM), sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min, NAC (1 mM) for 2 h or PI3k/Akt inhibitor (Wortmannin, 1 μM; TOCRIS, #1945-26-7), p38-MAPK inhibitor (SB203580, 10 μM; TOCRIS #152121-47-6), JNK inhibitor (SP600125, 10 μM; TOCRIS, #29-56-6) or ERK1/2 inhibitor (PD98058, 10 μM; TOCRIS, #67869-21-8) for 1 h prior to stimulation with TNF-α (10 ng/mL).

Frozen ITA and LV biopsies were homogenized using liquid nitrogen, lysed in RIPA extraction buffer, subjected to sonication (10 s) prior to centrifugation (15000 rpm, 30 min, 4 °C) and supernatant collection. Total proteins (ECs: 10 μg, tissue lysate: 20 μg) were separated on 8 % SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose membrane (Biorad) using Trans-Blot Turbo transfer system (Biorad). After blocking with 5 % bovine serum albumin in Tris-buffered saline (TBS) containing 0.1 % Tween 20 for 1 h at room temperature, membranes were incubated with 1:1000 dilution of a primary antibody of either mouse monoclonal anti-eNOS (Abcam, AB76198), rabbit monoclonal anti-VCAM-1 (Abcam, ab134047), mouse monoclonal anti-ICAM-1 (Abcam; ab171123), rabbit polyclonal anti-SGLT1 (Alomone lab; agt-031), rabbit polyclonal anti-SGLT2 (Alomone Labs, AGT-032), rabbit monoclonal anti-p-p65 (3033S; Cell Signaling), rabbit polyclonal anti-angiotensin-converting enzyme (ACE, LifeSpan Biosciences; C353970), rabbit polyclonal anti-angiotensin type 1 receptor (AT1R, Abcam; ab124505), rabbit monoclonal anti-COX1 (Abcam; ab109025), rabbit polyclonal anti-COX2 (Abcam; ab15191), mouse monoclonal anti-nitrotyrosine (EMD-Millipore; 05-233), mouse monoclonal anti-GAPDH (Abcam; ab8245) or mouse monoclonal anti-β-actin (1:10,000, Sigma Aldrich, T7816) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin G, 1:10,000, Invitrogen, #31466, #31450, respectively) for 1 h

at room temperature. The immunoreactive bands were developed by enhanced chemiluminescence (Clarity Western ECL substrate, Biorad) and analyzed using ImageJ software.

Immunofluorescence staining of proteins and NF-κB nuclear translocation

For immunofluorescence, ECs were cultured on 8-well Lab-Tek® chambers and stimulated with TNF-α (10 ng/mL) in serum-free culture medium for 24 h. In some experiments, ECs were pretreated with sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min, NAC (1 mM) for 2 h or PI3k/Akt inhibitor (Wortmannin, 1 μM; TOCRIS, #1945-26-7), p38-MAPK inhibitor (SB203580, 10 μM; TOCRIS #152121-47-6), JNK inhibitor (SP600125, 10 μM; TOCRIS, #29-56-6) or ERK1/2 inhibitor (PD98058, 10 μM; TOCRIS, #67869-21-8) for 1 h prior to stimulation with TNF-α (10 ng/mL). Regarding tissue staining, frozen sections were cut at a 10 μm thickness and mounted on slides. Thereafter, samples (cells/tissue sections) were washed twice in PBS, fixed during 30 min with 4 % (w/v) paraformaldehyde, washed thoroughly and then incubated with blocking/permeabilizing buffer (PBS containing 1 % BSA (w/v) and 0.5 % Triton X-100 (w/v)) for 30 min at room temperature. After buffer removal, cells/tissue sections were incubated with 1:100 dilution of an Ab directed against either of the following: p65 NF-κB (SC-8008, Santa Cruz), mouse monoclonal anti-CD31 (Abcam; ab281583), mouse monoclonal anti-CD68 (Invitrogen; 14-0688-82), mouse monoclonal anti-TNF-α (Santacruz; SC-52746), rabbit monoclonal anti-p-p65 (3033S; Cell Signaling), rabbit polyclonal anti-SGLT1 (Alomone lab; agt-031), rabbit polyclonal anti-SGLT2 (AGT-032, Alomone Lab), rabbit monoclonal anti-VCAM-1 (Abcam, ab134047) overnight at 4 °C. After washing 3 times with PBS, they were further incubated with a 1:250 dilution of either Alexa Fluor™ 633 goat anti-rabbit immunoglobulin G (A21071, Invitrogen) or Alexa Fluor™ 488 goat anti-mouse immunoglobulin G (A11029, Invitrogen) for 1 h at room temperature in the dark. For negative controls, primary antibodies were omitted. After washing 2 times with PBS, cells/tissue sections were incubated with 1 μg/mL 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, #62248, Thermo Fisher Scientific) during 3 min at room temperature, in order to counterstain nuclei. After disassembling, slides were mounted with

fluorescent mounting medium. Images were acquired using a Zeiss epi-fluorescence microscope and quantification was performed using ImageJ software (Version 1.53c for Windows, NIH).

Tissue and cellular formation of ROS

ECs were cultured on 8-well Lab-Tek® chambers and stimulated with TNF-α (10 ng/mL) in serum-free culture medium for 24 h. In some experiments, ECs were pretreated with perindoprilat (10 μM), losartan (1 μM), VAS-2870 (1 μM), sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min, or NAC (1 mM) for 2 h prior to stimulation with TNF-α (10 ng/mL). As for *in situ* ROS formation in ITA and LV, frozen tissue sections were cut at a 15 μm thickness and mounted on slides. In some experiments, tissue sections were pretreated with perindoprilat (10 μM), losartan (1 μM), VAS-2870 (1 μM), sotagliflozin (100 nM), empagliflozin (100 nM) or infliximab (30 μg/mL, Thermofisher) for 30 min, or NAC (1 mM) for 2 h prior to incubation with the dihydroethidium probe. Thereafter, cells/tissue sections were exposed to dihydroethidium (5 μM), a redox-sensitive fluorescent dye for 30 min at 37 °C in the dark. After washing 3 times with PBS, cells/tissue sections were mounted with fluorescent mounting medium (fluorescence editing medium, Dako, Agilent Technologies France, Les Ulis, France) and cover-slipped before being examined by fluorescent microscopy (epi-fluorescent microscope, Zeiss). Quantification of the ROS signal was performed by using ImageJ software (Version 1.53c for Windows, NIH).

Measurement of endothelial superoxide anion formation by electron paramagnetic resonance

ECs were seeded at a density of 50 000 cells per well in a 24 well plate and incubated overnight. Then, ECs were incubated in serum-free medium for 2 h before being stimulated with TNF-α (10 ng/mL) for 24 h. In some experiments, ECs were pretreated with either empagliflozin (100 nM) for 30 min or MnTMPyP (10 μM) for 1 h prior to stimulation with TNF-α. Cells were then washed with PBS and trypsinized before being centrifuged at 500 g for 5 min at room temperature. Thereafter, ECs were solubilized in Krebs-Henseleit buffer (final density of 5 x 10^5 cell / mL) and added into a 96-well plate. CMH probe (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpytrolidine HI) was then added to a final concentration of 200 μM and incubated for 30 min at 37 °C.

Finally, ECs were placed on ice and 40 µL of the mixture obtained was introduced into a glass EPR capillary tube (Noxygen Science Transfer & Diagnostics, Elzach, Germany) and placed inside the cavity of the e-scan spectrometer (E-scan, Bruker-Biospin, Rheinstetten, Germany). The EPR signal obtained is proportional to the superoxide anion quantity in ECs.

Endothelial formation of NO

ECs were cultured on 8-well Lab-Tek® chambers then stimulated with TNF-α (10 ng/mL) in serum-free culture medium for 24 h. In some experiments, ECs were pretreated with sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min prior to stimulation with TNF-α. Thereafter, ECs were exposed to DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluororescein diacetate, 1 µM), a NO-sensitive fluorescent dye, for 20 min at 37 °C in the dark. The formation of NO was induced by the exposure of ECs to bradykinin (100 nM) for 15 min. After washing 3 times with PBS, cells were mounted with fluorescent mounting medium. Images were acquired using a Zeiss epi-fluorescence microscope. Quantification of the NO signal was performed by using ImageJ software (Version 1.53c for Windows, NIH).

SGLT1 and 2 siRNA transfection studies

ECs were transfected with siRNA (50 nM) targeting SGLT1 (Eurogentec; SGLT1 siRNA sense GUAUCUAGAUUGUCCUAGA, antisense UCUAGGACAAUCUAGAUAC), SGLT2 (Eurogentec; SGLT2 siRNA sense GCCUCAAUCUUUAACAGCA, antisense UGCUGUUAAAGAUUGAGGC) or negative control (Scrambled) siRNA (sense AUUCUAAUCCGUGAUGUAG, antisense CUACAUCACGGAUUAGAAU) in OPTI-MEM™ (# 31985070, Gibco) culture medium for 48-72 h to knock down SGLT1 or SGLT2 using Lipofectamine RNAiMAX (# 13778-150, Invitrogen). Thereafter, ECs were stimulated with TNF-α (10 ng/mL) for 24 h.

Endothelial glucose uptake by flow cytometry

ECs were seeded at a density of 50 000 cells per well in a 24 well plate and incubated overnight. Then, ECs were incubated in reduced glucose (1 g/L) serum-free medium for 6 h before being stimulated with TNF-α (10 ng/mL) for 24 h. In some experiments, ECs were pretreated with sotagliflozin (100 nM) or empagliflozin (100 nM) for 30 min or phloretin (10 μM) for 1 h prior to stimulation with TNF-α. Cells were then washed once with PBS and incubated with 100 μM of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBD-glucose, Life Technologies, SAS) for 1 h at 37°C in glucose-free Krebs-Henseleit buffer. Thereafter, ECs were trypsinized before being centrifuged at 500 g for 5 min at room temperature and washed once with PBS. Cell pellets were resuspended in 300 μl PBS supplemented with 10 % FBS and the 2-NBD-glucose fluorescence was determined in the FITC channel (FL-1) using a flow cytometer (FACScan). Mean fluorescence intensity of 2-NBD-glucose was used to measure glucose uptake by ECs. Unstained control was used to optimize FACS settings.

Concluding Remarks of Part I

In the current work we demonstrated that:

- SGLT2 exhibits variable expression and function in human internal thoracic artery which is correlated to low-grade inflammation.
- SGLT2 expression and function are variable in human heart specimens and correlate to low-grade inflammation.
- SGLT2 expression in endothelial cells is upregulated following pro-inflammatory stimulation to promote oxidative-induced endothelial dysfunction through the activation of the AT1R/NADPH oxidases/NF-κB pathway.

Remarques finales

Dans le cadre des travaux actuels nous avons démontré que:

- les SGLT2 se caractérisent par une expression et une fonction variables dans l'artère thoracique interne humaine en corrélation avec l'inflammation de bas grade.
- l'expression et la fonction des SGLT2 varient dans les prélèvements cardiaques humains et sont en corrélation avec l'inflammation de bas grade.
- l'expression des SGLT2 dans les cellules endothéliales est activée à la suite d'une stimulation pro-inflammatoire favorisant un dysfonctionnement endothélial induit par l'oxydation à travers l'activation de la voie AT1R/NADPH oxydases/NF-κB.

RESULTS

PART II

COVID-19 Promotes Endothelial Dysfunction and Thrombogenicity: Role of pro-Inflammatory Cytokines/SGLT2 pro-Oxidant Pathway

Coronavirus disease 2019 (COVID-19) is an acute respiratory distress syndrome which was shown to be associated with severe cardiovascular (CV) complications. In fact, death from CV reasons is only second to respiratory failure following COVID-19. Several forms of COVID-19 associated CV complications have been described including arrhythmias, HF, ischemic heart disease, stroke and thromboembolic events, all of which were dependent on the severity of the infection despite the presence or absence of previous CV risk. Different mechanisms have been proposed and studied to explain COVID-19 associated CV complications including direct cardiotoxicity, manifestations of respiratory complications and endothelial dysfunction secondary to pro-inflammatory cytokine storm, a remarkable signature of COVID-19. Endotheliopathy is indeed a common risk factor that underlies several CV diseases and is often a consequence of chronic low-grade inflammation and oxidative stress. On the other hand, sodium-glucose co-transporter 2 inhibitors (SGLT2is), originally a class of anti-diabetic drugs, demonstrated significant CV protection in clinic including reduced HF hospitalization and CV death. Further, preclinical experiments demonstrated that SGLT2is improved endothelial function whether in animal models of diabetes and hypertension or in response to *in vitro* stimuli. However, possible CV protection of SGLT2is in the context of COVID-19 is still a topic under investigation. Therefore, in the current work we examined the plasma of 100 COVID-19 patients and evaluated

whether the pro-inflammatory profile of the plasma samples was associated with increased endothelial dysfunction in a translational approach. Further, using the same translational approach we studied the pro-oxidant, pro-inflammatory and pro-thrombotic role of SGLT2 following stimulation of ECs with COVID-19 plasma.

Le COVID-19 favorise le dysfonctionnement endothélial et la thrombogénèse: Rôle des cytokines pro-inflammatoires/de la voie pro-oxydante SGLT2

La maladie à coronavirus 2019 (COVID-19) est un syndrome de détresse respiratoire aiguë dont l'association avec de graves complications cardiovasculaires (CV) a été montrée. En effet, les raisons CV ne représentent que la deuxième cause de décès comparés à l'arrêt respiratoire à la suite d'une infection au COVID-19. Plusieurs formes de complications CV associées avec le COVID-19 ont été décrites, y compris arythmies, IC, cardiopathie ischémique, accident vasculaire-cérébral et évènements thromboemboliques, tous dépendants de la gravité de l'infection malgré la présence ou absence d'un risque CV préexistant. Différents mécanismes ont été proposés et étudiés afin d'expliquer les complications CV associées au COVID-19, incluant la cardiotoxicité directe, des évènements de complications respiratoires et un dysfonctionnement endothélial suite à une tempête de cytokines pro-inflammatoires, caractéristique marquant du COVID-19. L'endothéliopathie est en effet un facteur de risque courant de plusieurs maladies CV et est souvent la conséquence d'une inflammation chronique de bas grade et de stress oxydatif. D'autre part, les inhibiteurs des cotransporteurs sodium-glucose 2 (SGLT2is), initialement une classe de médicaments antidiabétiques, montraient une protection CV significative dans l'utilisation clinique, comprenant la réduction du nombre d'hospitalisations dues à l'IC et de mort CV. De plus, des expériences précliniques ont démontré que SGLT2is améliore la fonction endothéliale

aussi bien dans les modèles animaux de diabète et d'hypertension qu'en réponse à des stimuli *in vitro*. La possible protection CV des SGLT2is dans le contexte du COVID-19 est cependant encore un sujet en cours d'investigation. C'est la raison pour laquelle nous avons étudié le plasma de 100 patients atteints du COVID-19 dans les travaux actuels et avons évalué dans une approche translationnelle si le profil pro-inflammatoire des échantillons de plasma était associé avec un dysfonctionnement endothélial plus élevé. Se servant de la même approche translationnelle, nous avons aussi étudié le rôle pro-oxydant, pro-inflammatoire et pro-thrombotique des SGLT2 suite à la stimulation des CEs avec du plasma COVID-19.

COVID-19 promotes endothelial dysfunction and thrombogenicity: Role of pro-inflammatory cytokines/SGLT2 pro-oxidant pathway

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Preprint server: No

Text word count: 4543 words (main body of the text)

Abstract word count: 246 words

Reference Numbers: 43

Number of illustrations (Figures): 5

Number of Tables: 2

Supplementary Appendix Methods, Figures S1, S2, S3, S4, S5 Tables S1, S2, S3

Healthy volunteers

Healthy

(N = 25)

Patients with cardiovascular risk factors

COVID-

(N = 50)

COVID-19 positive patients

COVID+

(N = 100)

IL-1β

IL-6

TNF-α

MCP-1

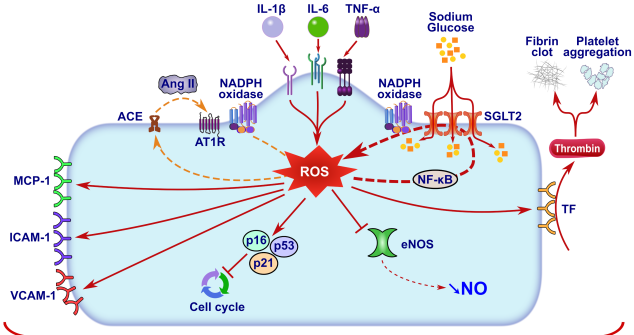
sVCAM-1

sICAM-1

COVID+ plasma upper quartile for IL-1β, IL-6, TNF-α

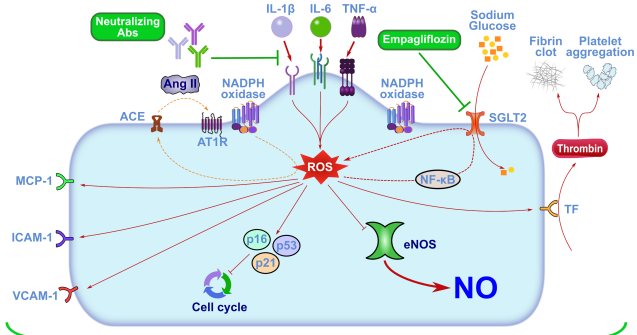
(N = 9)

Porcine coronary endothelial cells



Endothelial senescence and dysfunction

Cardiovascular risk



Improved endothelial health

Cardiovascular protection

Essentials

- The exact mechanisms of COVID-19 related endothelium damage are not completely understood
- Plasma from COVID-19 patients were used to stimulate porcine coronary artery endothelial cells
- COVID-19 plasma induced endothelial dysfunction through redox-sensitive upregulation of SGLT2
- SGLT2 inhibition attenuated these effects and may represent a possible strategy in COVID-19

Abstract

Background. COVID-19 is associated with increased risk of cardiovascular complications. Although cytokines have a predominant role in endothelium damage, the precise molecular mechanisms are far from being elucidated.

Objectives. The present study hypothesizes that inflammation in COVID-19 patients contributes to endothelial dysfunction through redox sensitive SGLT2 overexpression and investigates the protective effect of SGLT2 inhibition by empagliflozin.

Methods. Human plasma samples were collected from acute, sub-acute and long COVID-19 patients (n=100), non-COVID-19 patients with cardiovascular risk factors (n=50), and healthy volunteers (n=25). Porcine coronary artery endothelial cells (ECs) were incubated with plasma (10%). Expression levels of proteins were determined using Western blot analyses and immunofluorescence staining, mRNA expression by RT-qPCR and the level of oxidative stress by dihydroethidium staining. Platelet adhesion and aggregation, and thrombin generation were determined.

Results. Increased plasma levels of IL-1β, IL-6, TNF-α, MCP-1 and sICAM-1 were observed in COVID-19 patients. Exposure of ECs to COVID-19 plasma with high cytokines levels induced redox-sensitive upregulation of SGLT2 expression via pro-inflammatory cytokines IL-1β, IL-6 and TNF-α which, in turn, fueled endothelial dysfunction, senescence, NF-κB activation, inflammation, platelet adhesion and aggregation, vWF secretion and thrombin generation. The stimulatory effect of COVID-19 plasma was blunted by neutralizing antibodies against pro-inflammatory cytokines, and by empagliflozin.

Conclusions. In COVID-19 patients, pro-inflammatory cytokines induced a redox-sensitive up-regulation of SGLT2 expression in ECs, which in turn promoted endothelial injury, senescence,

platelet adhesion, aggregation, and thrombin generation. SGLT2 inhibition with empagliflozin, appeared as an attractive strategy to restore vascular homeostasis in COVID-19.

Key words: COVID-19, endothelium, inflammation, SGLT2, thrombosis.

Introduction

Mounting evidence from basic science, histopathological reports and clinical observations has revealed that cardiovascular manifestations of coronavirus disease 2019 (COVID-19) result from a profound endothelial dysfunction [1, 2]. The latter could be triggered by immune activation, a cytokine storm, hypoxemia, altered shear stress, or the local activation of the renin-angiotensin-aldosterone system (RAAS) [3-9]. Beyond the acute phase, a recent analysis has demonstrated an increased risk of incident cardiovascular disease regardless of previous cardiovascular risk factors [10]. Existing evidence now suggests that patients with the most severe COVID-19 cases (e.g., intensive care) are at increased risk of post-COVID long-term complications [10]. Overall, these findings highlight that COVID-19 substantially modifies the vasculature not only at the acute phase, but also at the chronic phase (>12 weeks), and suggest that the long-term risk of COVID-19 may depend on the magnitude of the initial vascular injury. Additional reports have indicated that COVID-19 prompts late endothelial dysfunction [11,12], arterial stiffness, chronic sub-clinical inflammation [13], elevation of coagulation markers [14] and circulating endothelial cells [15] and hypothesized persistent endotheliopathy as an optimal nidus for the development of long-term cardiovascular complications [14,16,17].

To date, the molecular mechanisms contributing to cardiovascular complications after COVID-19 are far from being elucidated. Beyond this, such mechanisms could involve the downregulation of angiotensin-converting enzyme (ACE2) activity [8,18], viral activation of the RAAS [18,19], endothelin-mediated vascular injury [20], elevated levels of pro-inflammatory cytokines [13, 21], activation of transforming growth factor (TGF-β)-mediated pro-fibrotic pathway [22], shedding of procoagulant microparticles [7, 23, 24], and continued activation of the immune-inflammatory-procoagulant cascade. Several studies have emphasized that severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) triggers and

fuels a self-amplifying cycle of excessive inflammation and dysregulation of ACE2 [8,25] contributing to endothelial dysfunction. Due to the dramatic burden of cardiovascular events, exploring COVID-19 mediated endotheliitis is compulsory to understand underlying mechanisms and facilitate novel advances in therapeutic strategies targeting the preservation and/or restoration of endothelial function [4].

The present study hypothesizes that high levels of cytokines detected within the plasma of COVID-19 patients promote oxidative stress and contribute to endothelial dysfunction. Through an *in vitro* approach, we investigated whether exposure of coronary endothelial cells (ECs) to plasma from COVID-19 patients induced a pro-oxidant response leading to endothelial dysfunction and determined the role of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, and the angiotensin system. Our previous studies indicated that oxidative stress upregulated sodium-glucose cotransporter2 (SGLT2) expression in ECs to perpetuate the pro-oxidant activator signal as part of a vicious auto-amplification loop [26, 27]. The latter blunted endothelial formation of nitric oxide (NO) and anti-platelet aggregatory activity and promoted pro-inflammatory and pro-coagulant responses. Therefore, we studied the role of SGLT2 on the activation of ECs by plasma of COVID-19 patients using empagliflozin, a selective SGLT2 inhibitor, (SGLTi) and by silencing SGLT2.

Methods

This prospective study (PRI 2020/7829) recruited 100 COVID-19 patients (COVID+) admitted to Strasbourg University Hospital, France, from March 2020 to June 2021. Patients were confirmed to have SARS-CoV-2 infection through positive reverse transcription-polymerase chain reaction (RT-PCR). The criteria for hospital admission required inpatient care because of the severity of illness based on laboratory and radiological parameters, and clinical findings. Patient enrollment and blood sampling were performed at the convalescent phase. Additionally, 25 healthy volunteers and 50 non-COVID patients (COVID-) referred to the catheterization lab for coronary angiography, and at least two cardiovascular risk factors from the following among active smoking, history of arterial hypertension, dyslipidemia, diabetes mellitus, obesity or a familial history of cardiovascular disease, were enrolled for comparison. Patients under SGLTi treatment were excluded. Ethical approval for this study was obtained from Comité de Protection des Personnes Sud Méditerranée 03/06/2020 (ID RCB 2020-A01500-39) and written informed consent was obtained from all participants according to the principles of the declaration of Helsinki.

Cell culture and biological responses

ECs were isolated from right coronary artery of freshly collected pig hearts from the local slaughterhouse using collagenase (type I) and cultured in MCDB131 supplemented with fungizone (2.5 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and 15% fetal calf serum as described previously (22). All experiments were performed with ECs at passage 1 exposed to a serum-free culture medium for 2 h before the addition of a treatment. In some experiments, ECs were exposed to a pharmacological modulator for at least 30 min before the addition of plasma.

Plasma levels of pro-inflammatory markers (interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1)) and secretion of von Willebrand factor (vWF) by ECs were measured using commercial ELISA kits, levels of oxidative stress in ECs by fluorescence microscopy using the redox-sensitive probe dihydroethidium, NO levels by microscopy using the NO probe DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluororescein diacetate), levels of mRNA expression by RT-qPCR, proteins by Western blot analyses and immunofluorescence staining, nuclear translocation of NF-κB by immunofluorescence staining, tissue factor (TF) activity by the generation of thrombin on the surface of ECs using the chromogenic substrate of thrombin, (ß-Ala-Gly-Arg *p*-nitroanilide diacetate), platelet adhesion using fluorometric adhesion assay and platelet aggregation using an aggregometer. For transfection studies, SGLT2 siRNA (sense GCCUCAAUCUUUAACAGCA, antisense UGCUGUUAAAGAUUGAGGC) and negative control siRNA (sense AUUCUAAUCCGUGAUGUAG, antisense CUACAUCACGGAUUAGAAU) were used to knock down SGLT2 in ECs using lipofectamine transfection kit as recommended by the supplier.

Statistical analysis

For patient characteristics, categorical variables are represented as frequencies and percentages, and continuous variables are expressed as median and inter-quartile values. Fischer exact tests or χ^2 tests were used to analyze categorical variables. Continuous variable comparisons were performed using Wilcoxon test (for two groups) or by Kruskal-Wallis test (for three groups). For *in vitro* experiments, normality of data was assessed using the Kolmogorov-Smirnov test. Non-normally distributed variables are presented as median and inter-quartile values, whereas

normally distributed variables are presented as mean ± standard error of the mean. To compare normally distributed parameters, either one or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison were used. When data were not normally distributed, non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison was used. Bilateral Correlations were assessed using Spearman test. JMP 13 software® (SAS Institute, Cary, NC) and GraphPad Prism software (Version 7 for Windows, GraphPad Software Inc., San Diego, CA, USA) were used to perform all statistical analysis. Differences were considered significant at $P < 0.05$.

Results

Patients' characteristics

The baseline characteristics are summarized in *Table 1*. COVID-19 patients were included in the acute phase (≤4 weeks) (n = 41), sub-acute phase (4-12 weeks), (n = 5) or in the long-COVID phase (> 12 weeks, n = 54). The median time from positive PCR testing to enrollment in the study and blood sampling was 115 days (IQR 6 to 156). Comorbidities were more frequently observed in COVID- patients with cardiovascular risk factors than in COVID-19 patients.

The clinical characteristics of the COVID-19 patients are displayed in *Table S1*. Severe (30%) and critical (33%) lung pulmonary injuries were evidenced in a large proportion of the cohort. The incidence of venous thromboembolism in hospitalized patients was 20%, despite the use of a prophylactic dose of low-molecular-weight heparin or unfractionated heparin (LMWH/UFH) in 13%, a reinforced dose in 42%, and a therapeutic dose in 34%.

Characterization of pro-inflammatory cytokines and endothelial damage in COVID-19 patients

Distribution of cytokines, cytoadhesins and DHE values among acute, sub-acute, long COVID-19, COVID- patients with cardiovascular risk factors and HV are represented in *Tables S2* and *Table 2*. Compared to values measured in both healthy volunteers and COVID-patients, higher levels of IL-1β, TNF-α and MCP-1 and sICAM-1 were evidenced in COVID-19 patients (*Figure 1 and Table 2*). To ascertain that the pro-inflammatory response persists beyond the acute phase, the patients enrolled at the acute and sub-acute phase (< 12 weeks days; n = 46)

were excluded, and the remaining 54 patients were analyzed *(Table 2*). Elevated levels of IL-1β, IL-6, TNF-α, MCP-1, sICAM-1 and DHE were still evidenced in long COVID-19 patients compared to healthy volunteers, indicating a persistent pro-inflammatory and pro-oxidative response (*Table 2*).

Pro-oxidant state of COVID-19 plasma treated coronary endothelial cells: Role of pro-inflammatory cytokines, the angiotensin system and SGLT2

Since pro-inflammatory cytokines promoted oxidative stress in ECs [27], we used an *in vitro* approach to study the ability of COVID-19 plasma (n = 100) to induce oxidative stress in coronary ECs. Controls were constituted by 9 subjects of each group (non-COVID-19 patients with cardiovascular risk factors or healthy volunteers) with the highest cytokines values. *Figure 2A* shows that the exposure of ECs to long COVID-19 plasma (10% v/v) induced significant increase in long-term (24 h) pro-oxidant responses in ECs as compared to the plasma of healthy volunteers. On the other hand, pro-oxidant response in ECs induced by long COVID-19 plasma was significantly lower than that induced by acute COVID-19 plasma (Supplementary material online, *Figure S1B*). For the subsequent experiments, only COVID-19 plasma samples that fit into the top quartile of IL-1β, IL-6 and TNF-α (n = 9) were used. In this cohort, the exposure of ECs to COVID-19 plasma (10% v/v) induced greater short-term (30 min) and long-term (24 h) pro-oxidant responses than plasma of COVID- patients (*Figures 2B and C*). Additionally, the plasma of healthy volunteers was inactive (*Figures 2B and C*). Pro-oxidant response of ECs induced by COVID-19 plasma decreased over time (Table S2) and was significantly correlated to the levels of pro-inflammatory cytokines IL-1β ($r = 0.4877$, $P < 0.0001$), IL-6 ($r = 0.5555$, $P < 0.0001$), TNF-α ($r = 0.6403$, $P < 0.0001$), MCP-1 ($r = 0.1971$, $P < 0.05$) (*Figures 2F, G and H*) and to a lesser extent to a marker of endothelial activation sVCAM-1 ($r = 0.2187$, $P < 0.05$)

(Supplementary material online, *Figure S1C, D*). When the analysis was restricted to the long COVID-19 phase, (n = 54), pro-oxidant response of ECs was still correlated with IL-1β (r =0.422; *P* = 0.001) and TNF-α (r = 0.913; *P* < 0.001).

The long-term pro-oxidant effect of COVID-19 plasma persisted after removal of microparticles by centrifugation indicating the involvement of soluble mediators (Supplementary material online, *Figure S1A*). Then, extensive characterization of plasma-induced endothelial damage was conducted using 9 subjects of each group with the highest cytokines values. Baseline clinical and biological characteristics of COVID-19 patients with the highest cytokine levels as defined by concentrations of IL-1β, IL-6 and TNF-α in the upper quartile (n = 9) are compared with the subset of patients with lower cytokines levels (n = 91) are displayed in Supplementary material online, *Table S3.*

Both short- and long-term pro-oxidant responses induced by COVID-19 plasma on ECs were partially prevented by a single neutralizing antibody (Ab) directed against either IL-1β, IL-6 or TNF-α, and to a greater extent by combining the three neutralizing Abs (*Figures 2D and E*). This process indicated a determinant role of the three cytokines in triggering the pro-oxidant signal. In contrast to cytokines, empagliflozin strongly prevented solely the long-term pro-oxidant response, suggesting that SGLT2 was involved in perpetuating the pro-oxidant signal (*Figures 2D and E*). Further, the long-term pro-oxidant response of COVID-19 plasma was also partially prevented by the inhibition of the angiotensin system by either perindoprilat (ACE1 inhibitor) or losartan (AT1R antagonist). On the other hand, a more pronounced inhibitory effect was observed with the NADPH oxidase inhibitor VAS-2870 (*Figure 2I*). Moreover, the sustained pro-oxidant response induced by the exposure of ECs to COVID-19 plasma for 24 h was reduced by the subsequent addition of either VAS-2870 or empagliflozin to ECs, whereas the combination of neutralizing Abs targeting IL-1β, IL-6 and TNF-α,

perindoprilat or losartan were inactive, implying a major role of NADPH oxidase and SGLT2 in perpetuating the pro-oxidant signal (*Figure 2J*).

Pro-inflammatory genes expression profile of COVID-19 plasma treated endothelial cells: Role of pro-inflammatory cytokines and SGLT2

After establishing a pro-oxidant signal of COVID-19 plasma in ECs, we explored its downstream effects on the expression profile of redox-sensitive genes that are involved in EC activation [27] (*Figure 3*). Exposure of ECs to COVID-19 plasma for 6 h resulted in a down-regulation of the level of *NOS3* mRNA (eNOS), a non-significant decrease of *ACE2* mRNA by 39%, and an upregulation of the mRNA levels of senescence markers *TP53, CDKN1A, CDKN2A* (p53, p21 and p16), cytoadhesins *VCAM1, ICAM1, SELE, SELP* (VCAM-1, ICAM-1, E-selectin and P-selectin), modulators of thrombotic responses *F3, TFPI, THBD, SERPINE1* (TF, TFPI, thrombomodulin, PAI-1), components of the Ang II II/NADPH oxidase pathway *ACE1, AGTR1, CYBA, NCF1* (ACE1, AT1R, $p22^{phox}$, $p47^{phox}$), pro-inflammatory cytokines *IL1B, IL6, TNFA* (IL-1β, IL-6, TNF-α), and *SLC5A2* (SGLT2) (*Supplementary material online, Figure S2A*). In contrast, the plasma from COVID- patients with cardiovascular risk factors somewhat increased the mRNA expression levels of some but not all factors (*VCAM1, ICAM1, ACE1, AGTR1, CYBA, NCF1 and IL6*), whereas the plasma from healthy volunteers was inactive (Supplementary material online, *Figure S2A*). The stimulatory effect of the COVID-19 plasma on gene expression was prevented by a combination of neutralizing Abs directed against IL-1β, IL-6 and TNF-α with the exception of *NOS3, TFPI* and *SERPINE1*, and by empagliflozin except for *TFPI* and *SERPINE1* (Supplementary material online, *Figure S2B*). Additionally, a single neutralizing Ab directed against either IL-1β, IL-6 or TNF-α affected

either only slightly or not at all the stimulatory effect of COVID-19 plasma (Supplementary material online, *Figure S2C*).

Feedforward upregulation of SGLT2 expression in COVID-19 plasma treated endothelial cells: Role of pro-inflammatory cytokines and redox-sensitive NF-κB

In contrast to the short-term response, the long-term pro-oxidant response of ECs to COVID-19 plasma was strongly inhibited by empagliflozin. Thus, we explored the effect of COVID-19 on the expression level of SGLT2 as assessed by immunofluorescence staining. The exposure of ECs to COVID-19 plasma for 24 h resulted in a concentration-dependent upregulation of SGLT2 protein (*Figure 4A*). A significant stimulatory effect was observed at concentrations of plasma as low as 3% v/v (*Figure 4A*). The increased SGLT2 signal was prevented to some extent by losartan and to a greater extent by VAS-2870, the NF-κB inhibitor Bay 11-7082, and by empagliflozin, suggesting the involvement of a redox-sensitive NF-κB-mediated feedforward amplifying loop (*Figure 4B*). In line with this result, COVID-19 plasma caused the nuclear translocation of NF-κB and this response was partially inhibited by losartan and to a greater extent by the combination of the three neutralizing Abs directed against IL-1β, IL-6 and TNF-α, VAS-2870, empagliflozin and Bay 11-7082 (*Figure 4C*). Additionally, a single pro-inflammatory cytokine, including IL-1β, IL-6 or TNF-α at 3 ng/ml increased SGLT2 staining in ECs and the combination of the three cytokines tested at a lower concentration (1 ng/ml), which were inactive alone (*Figure 4D*).

To confirm that COVID-19 plasma stimulates SGLT2 expression and causes EC activation, we analyzed protein expression levels in ECs using Western blot analysis. COVID-19 plasma increased the protein expression levels of SGLT2, VCAM-1 and TF and down-regulated that of eNOS (*Figure 4E*). The stimulatory effect of COVID-19 plasma was prevented by the pre-

treatment with either combination of neutralizing Abs directed against IL-1β, IL-6 and TNF-α, or empagliflozin and following knock down of the expression of SGLT2 in ECs using siRNA, whereas scrambled siRNA was inactive (*Figure 4E;* Supplementary material online, *Figure S4*). Similarly, stimulatory effect of COVID-19 plasma was prevented by the simultaneous treatment with empagliflozin (Supplementary material online, *Figure S5*)

Increased thrombogenicity in COVID-19 plasma treated endothelial cells: Role of pro-inflammatory cytokines and SGLT2

To strengthen the link between endothelial dysfunction triggered by COVID-19 plasma and increased thrombogenicity, we investigated platelet aggregation, platelet adhesion and thrombin generation at the ECs' surface in addition to vWF secretion by ECs. Overall, the COVID-19 plasma reduced both basal and bradykinin-induced formation of NO in ECs (*Figure 5A*). These effects were prevented by using the combination of the three neutralizing Abs directed against IL-1β, IL-6 and TNF-α, and empagliflozin (Figure 5A). Similarly, COVID-19 plasma reduced the anti-aggregatory effect of ECs in response to bradykinin, and this effect was prevented by the combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α, and empagliflozin (*Figure 5B*). Beyond this, *Figure 5B* highlights the effect of N^G-nitro L-arginine (NO synthase inhibitor) on the anti-aggregatory effect of ECs. The results show that the inhibitory effect of endothelial cells on platelet aggregation is lost once NO synthase is inhibited, hence suggesting a key role of NO in the demonstrated protective effect. Adding on, COVID-19 plasma increased platelet adhesion to ECs surface which was associated with increased secretion of vWF by ECs, both of which were significantly prevented by the combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α, and empagliflozin (*Figure 5C, D*). Considering the high prevalence of thrombotic events in

COVID-19 patients, we investigated whether COVID-19 plasma could generate thrombin at the ECs' surface (*Figure 5E*). These findings represent the first evidence that COVID-19 plasma enhanced thrombin generation at the ECs' surface when exposed to recalcified plasma from healthy volunteers. This effect was prevented by the combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α, empagliflozin, dabigatran (FIIa inhibitor), and a TF neutralizing Ab.

Discussion

The major findings of this study indicate that in COVID-19 patients, inflammation causes a redox-sensitive up-regulation of SGLT2 expression in coronary ECs. In turn, this process fuels endothelial dysfunction, senescence, inflammation, platelet adhesion and aggregation and thrombin generation. Furthermore, these results indicate that empagliflozin, a potent SGLT2i, improves the deleterious impact of COVID-19 plasma on ECs suggesting that SGLT2i might be a novel therapeutic option to restore vascular homeostasis.

Inflammatory response in COVID-19 patients

At the acute phase, early reports have emphasized that the cytokine storm with IL-1β and IL-6 release, together with endothelial dysfunction, contributed in the vasculature to enhanced cell activation, TF expression, procoagulant microparticles release and thrombin generation. These effects paved the way to for both micro- and macrovascular thrombosis [7, 23].

At the convalescent phase, recent studies have emphasized an ongoing sustained inflammatory response [13,16] following even mild to moderate acute COVID-19 infection, which was not evidenced in other coronarovirus infections [21]. The mediators of this activation remain undetermined but could include the persistence of antigen or viral particles, autoimmunity driven by antigen cross-reactivity, and impact of damage repair or overactivation of monocytes/macrophages [18]. Beyond these immunity-driven mechanisms, the persistent imbalance of the RAAS system, at the expense of ACE2, could favor the stimulation of the pro-inflammatory ACE1/Ang II/AT1R axis and trigger a detrimental vascular response, including a cytokine release [18,21]. Consistent with previous reports [13,21], COVID-19 patients in our

study displayed sustained release of IL-1β, TNF-α, and to a lesser extent, IL-6, which persisted beyond the acute phase.

As a possible consequence of ongoing inflammation, endothelial dysfunction has been hypothesized to be responsible for long-term cardiovascular complications. Microvascular thrombosis, capillary leakage, and reduced oxygen extraction were described in the course of COVID-19 as possible consequences of endothelial damage [16,29]. The present study revealed a link between ongoing inflammation and endothelial damage. Plasma from COVID-19 patients with the highest cytokine values induced a pronounced endothelial dysfunction. The latter was characterized by enhanced short- and long-term oxidative stress, blunted formation of NO, and the overexpression of TF, PAI-1, thrombomodulin, selectins (E-selectin, P-selectin), cytoadhesins (VCAM-1, ICAM-1) and cytokines (IL-1β, IL-6, TNF-α). Importantly, this effect was also observed with the plasma of COVID-19 patients enrolled far beyond the acute phase (>12 weeks). The stimulatory effect of the COVID-19 plasma was particularly intense, much more than the one observed with the plasma of COVID- patients with cardiovascular risk factors, whereas the plasma of healthy volunteers remained inactive. The involvement of pro-inflammatory cytokines, as key noxious components conveying the stimulatory effect of plasma of COVID-19 patients on ECs, was evidenced by the fact that neutralizing Abs targeting either IL-1β, IL-6 or TNF-α used individually inhibited only partially ECs' activation whereas the combination of the three neutralizing Abs was significantly more active. This effect suggested that these three cytokines could act synergistically. Moreover, the stimulatory effect of COVID-19 plasma on ECs was reproduced by authentic cytokines at clinically relevant concentrations [28]. Another significant phenotypical shift induced by COVID-19 plasma in ECs included the modulation of the Ang II/NADPH oxidase pathway, as witnessed by an upregulation of ACE1, AT1R, $p22^{phox}$ and $p47^{phox}$ and a trend towards a downregulation of ACE2. Another study has stressed that SARS-CoV-2 can mediate myocardial inflammation and myocardial damage due

to the down-regulation of the myocardial ACE2 system. [30] In this study, the involvement of the Ang II/AT1R/NADPH oxidase pathway in generating endothelial damage in response to COVID-19 plasma was stressed by the ability of perindoprilat and losartan to partially inhibit sustained oxidative stress whilst drastic inhibition was observed with the potent NADPH oxidase inhibitor VAS-2870.

Beyond the stimulation of the Ang II/ATIR/NADPH oxidase pathway, we established a significant shift in the endothelial physiology towards a pro-senescence pattern as witnessed by the enhanced mRNA expression of p53, p21 and p16 genes. Previous studies have underlined that ECs senescence acts as a key early signal promoting endothelial dysfunction [27,31]. Other reports have highlighted a correlation between the degree of 'biological' aging and the severity of the acute phase [16,32,33]. Furthermore, a reduced expression of ACE2 has been suggested to accelerate the epigenetic 'clock' resulting in accelerated biological aging as determined by telomere length or senescence-associated secretory phenotype in COVID-19 survivors [16,22]. Together, these data clearly emphasize that in COVID-19 patients, cytokines contribute to trigger endothelial dysfunction and senescence by activating the Ang II/AT1R/NADPH oxidase pathway.

To combat the induction of endothelial dysfunction by cytokines, we investigated the benefits of empagliflozin, a SGLT2i. Recent clinical trials have highlighted that SGLT2is have remarkable beneficial cardiovascular effects [33]. In COVID- patients with cardiovascular risk factors who were hospitalized with COVID-19, treatment with the SGLT2i dapagliflozin was associated with lower rates of organ dysfunction or death without reaching statistical significance [34]. To date, the pathophysiological mechanisms underlying the cardiac protective effects of SGLT2i have not been fully described but could include decreased oxidative stress and inflammation [35] and the regulation of immune signaling pathways [36]. Until recently, the presence of SGLT2 within the cardiovascular system was questioned [35]. However, the

pioneering work of Li has described the significant upregulation of SGLT2 in SARS-CoV-2-infected cardiomyocytes coexisting with the down-regulation of antioxidant mechanisms including superoxide dismutase and catalase, and the upregulation of profibrotic genes and pro-inflammatory cytokines [37]. In the vasculature, we have recently established that Ang II and microparticles from blood of patients with coronary artery disease, possible mediators of endothelial damage during COVID-19 [7,23-25], act as potent inducers of SGLT1 and SGLT2 expression in ECs and *in vivo* in the arterial wall to promote endothelial damage [27,38]. In this study, we provided evidence that in COVID-19 patients cytokines act as potent inducers of SGLT2 expression in coronary ECs through sustained oxidative stress. Furthermore, increased SGLT2 levels have a determinant role in the induction of endothelial senescence, endothelial dysfunction and thrombogenicity in COVID-19 plasma as indicated by the pronounced protective effect of empagliflozin. Such beneficial effect is most likely attributable to the high effectiveness of empagliflozin to impede the sustained pro-oxidant activator signal. The characterization of the pro-oxidant response of COVID-19 plasma has indicated a major role of pro-inflammatory cytokines in initiating the early pro-oxidant signal whereas the sustained signal involved the cytokines/NADPH oxidase/SGLT2 pathway. The particular importance of the late pro-oxidant signal to drive the persistent activation of ECs likely relies on a redox-sensitive NF-κB-mediated feedforward amplifying loop since the NADPH oxidase inhibitor, the NF-κB inhibitor, and empagliflozin strongly inhibited the sustained activation of NF-κB and the expression of SGLT2. To maintain vascular homeostasis, we demonstrated that empagliflozin significantly alleviated the deleterious impact of pro-inflammatory cytokines on both basal and stimulated endothelial formation of NO, the activation of the Ang II/AT1R/NADPH oxidase pathway, inflammation, cellular adhesion and thrombogenicity. Additionally, the vascular protective effect of empagliflozin was higher than that provided by a single neutralizing cytokine Ab and at least as effective as the combination of the three

neutralizing Abs. Our data extend previous works demonstrating the potent anti-inflammatory properties of SGLT2 inhibitors in the vasculature [35,39]. One major issue when treating the vascular injury is that treatment is usually administered after the acute inflammatory insult, limiting the potential benefit of these therapeutic interventions [40]. We demonstrated that the sustained pro-oxidant response within the ECs was alleviated by the subsequent addition of empagliflozin, whereas the combination of neutralizing Abs targeting IL-1β, IL-6 and TNF-α, perindoprilat or losartan remained ineffective. Together, these data emphasized how SGLT2 inhibition could restore endothelial integrity even after the initial insult.

Throughout COVID-19 infections, numerous reports have stressed the importance of both early [41] and late thrombotic events [10], and the importance of platelets, leukocytes and endothelial interactions [7]. Potential therapeutic approaches, such as non-specific anti-inflammatory agents (i.e., colchicine or statins), endothelin antagonism [20] or antithrombotic therapies including anticoagulants or antiplatelet therapies were proposed to reduce these risks [5]. Besides common mechanisms involved in thrombus formation such as phosphatidylserine exposure, microparticles release [7], NETose, ADAMTS13 loss of function [22] complement activation [9], or vWF release [42], we have established that cytokines promote a prothrombotic phenotype characterized by enhanced expression of TF, PAI-1, selectins, cytoadhesins together with reduced endothelial NO synthase expression. The functional relevance of these mechanisms was demonstrated by the reduced inhibitory effect of ECs towards platelet adhesion and aggregation, and the increased generation of thrombin at the ECs' surface, with a major thrombotic impact mediated by pro-inflammatory cytokines. Beyond the inhibitory effect of the combination of neutralizing antibodies directed against IL-1β, IL-6 and TNF-α, an anti-TF neutralizing Ab or dabigatran, a direct thrombin inhibitor, this study demonstrated a potent antithrombotic effect of SGLT2 inhibition by empagliflozin. Thus, although these data should

be confirmed *in vivo*, they provide some rationale to investigate the benefit of SGLT2i to prevent thrombotic burden in patients exposed to inflammatory processes.

Study limitations

First, data on the full characterization of endothelial cell activation status mediated by the plasma of COVID-19 patients were based on a small sample size (n =9) with important heterogeneity concerning the timing of blood sampling after infection onset. Nevertheless, the results appeared consistent among the cohort of 9 patients used for *in vitro* experiments whether enrolled before 30 days (n = 7) and after (n = 2). Moreover, significant correlation between cytokines levels and DHE, as a marker of endothelial oxidative stress could be established even when the analysis was restricted to the long COVID-19 phase. There are several challenges in designing studies for COVID-19, such as the timing of inclusion. At the time of inclusion for this study (March 2020 to June 2021), the natural history, clinical course, and consequences of long COVID were unknown, or at best, the scientific community was only beginning to gain insights into this lingering disorder. Thus, variability in inclusion time after positive RT-PCR testing was clearly in conflict with the current standards and definitions regarding the post-SARS-CoV-2 viral period with a new dichotomy between a post-acute period (4 to 12 weeks) and a long COVID-19 phase (> 12 weeks). Second, the noxious effects of cytokines and the protective effect of SGLT2 inhibition could be demonstrated only *in vitro*. Third, systematic Sars-Cov-2 RT-PCR analysis was not performed at the time of blood sampling. Finally, although these results depicted a wide range of endothelial dysfunction mediated by cytokines, these data are only the benchmark to clinical rationale supporting the need for randomized clinical trials.

Conclusions

In COVID-19 patients, sub-clinical inflammation induces a redox-sensitive up-regulation of SGLT2 expression in coronary endothelial cells. In turn this process fuels endothelial dysfunction, senescence, inflammation, platelet adhesion and aggregation and thrombin generation. These data indicate *in vitro* that the potent SGLT2i empagliflozin, appears as an attractive novel strategy to restore vascular homeostasis in COVID-19 patients with ongoing inflammation. Our data provide a benchmark for clinical rationale supporting the need of clinical randomized trials aiming to restore endothelium function and prevent cardiovascular complications using SGLT2i in COVID-19 patients [43].

Author's contribution

A.M. substantially contributed to the acquisition, analysis, and interpretation of data for the work, performed RNA and protein extractions, and PCR analysis. W.F. substantially contributed to the acquisition, analysis, and interpretation of data for the work, performed RNA and protein extractions, and PCR analysis. A.C. substantially contributed to the conception and design of the study, patient enrolment and follow-up, analysis, and interpretation of data for the work and made critical revisions. A.T. substantially contributed to the conception and design of the study, patient enrolment and follow-up, analysis, and interpretation of data for the work and made critical revisions. K.M. substantially contributed to the conception and design of the study, statistical analysis, and interpretation of data for the work, reviewed the manuscript and made critical revisions. B.M. substantially contributed to the conception and design of the study, interpretation of data for the work, reviewed the manuscript and made critical revisions. A.W.Q. substantially contributed to the acquisition, analysis, and interpretation of data for the work, performed RNA and protein extractions, and PCR analysis. D-S.G. substantially

contributed to the acquisition, analysis, and interpretation of data for the work, performed RNA and protein extractions, and PCR analysis. C.A. substantially contributed to the acquisition, analysis, and interpretation of data for the work. L.S. substantially contributed to the acquisition, analysis, and interpretation of data for the work. A.R. substantially contributed to the conception and design of the study, patient enrolment and follow-up, analysis, and interpretation of data for the work and made critical revisions. S.H. substantially contributed to the conception and design of the study, patient enrolment and follow-up, analysis, and interpretation of data for the work and made critical revisions. W.O. substantially contributed to the conception and design of the study, patient enrolment and follow-up, analysis, and interpretation of data for the work and made critical revisions. O.V. substantially contributed to the conception and design of the study, patient enrolment and follow-up, analysis, and interpretation of data for the work and made critical revisions. J-M.M. substantially contributed to the conception and design of the study, and interpretation of data for the work and made critical revisions. N.M. substantially contributed to the conception and design of the study, statistical analysis, and interpretation of data for the work and made critical revisions. M-P.P. substantially contributed to the conception and design of the study, and interpretation of data for the work and made critical revisions. L.J. contributed to the conception and design of the study, patient enrolment and follow-up, analysis, and interpretation of data for the work and made critical revisions. M.B. assisted with interpretation of the findings, reviewed the manuscript, and made critical revisions. V.S.K. substantially contributed to the conception and design of the work, supervised the experiments, interpreted data, and wrote the manuscript. O.M. substantially contributed to the conception and design of the work, supervised the experiments, interpreted data, and wrote the manuscript.

Acknowledgements

The authors would like to formally acknowledge the commitment of all front-line healthcare workers during the COVID-19 crisis in the Cardiology Department as they commit themselves to difficult work and also put themselves at risk of infection. Strasbourg's Cardiology Department is proud of the commitment of all nurses, including newly graduated nurses, medical students, health support staff and volunteers. We thank Mrs Dessislava Ivanova for her help in patient enrollment and follow-up.

Conflict of interest

O.M. declared grants from AstraZeneca, Medtronic and Boehringer Ingelheim and V.S.-K. from Boehringer Ingelheim, Nugerontix Limited, and Servier. M.-P.P. is an employee of Boehringer Ingelheim. The other authors have nothing to disclose.

Funding

This study was supported by the Fondation Cœur et Recherche (Grant 2020, France) to O.M., Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany to V.S.-K. and O.M., and by Groupe pour l'Enseignement, la prévention et la Recherche Cardiologique en Alsace (GERCA), France.

References

[1] Choi D, Waksman O, Shaik A, Mar P, Chen Q, Cho DJ, Kim H, Smith RL, Goonewardena SN, Rosenson RS. Association of blood viscosity with mortality among patients hospitalized with COVID-19. *J Am Coll Cardiol*. 2022;80:316-328

[2] Gluckman TJ, Bhave NM, Allen LA, Chung EH, Spatz ES, Ammirati E, Baggish AL, Bozkurt B, Cornwell WK, 3rd, Harmon KG, Kim JH, Lala A, Levine BD, Martinez MW, Onuma O, Phelan D, Puntmann VO, Rajpal S, Taub PR, Verma AK. 2022 acc expert consensus decision pathway on cardiovascular sequelae of COVID-19 in adults: Myocarditis and other myocardial involvement, post-acute sequelae of sars-cov-2 infection, and return to play: A report of the american college of cardiology solution set oversight committee. *J Am Coll Cardiol*. 2022;79:1717-1756

[3] Libby P, Luscher T. COVID-19 is, in the end, an endothelial disease. *Eur Heart J*. 2020;41:3038-3044

[4] Evans PC, Rainger GE, Mason JC, Guzik TJ, Osto E, Stamataki Z, Neil D, Hoefer IE, Fragiadaki M, Waltenberger J, Weber C, Bochaton-Piallat ML, Back M. Endothelial dysfunction in COVID-19: A position paper of the esc working group for atherosclerosis and vascular biology, and the esc council of basic cardiovascular science. *Cardiovasc Res*. 2020;116:2177-2184

[5] Siddiqi HK, Libby P, Ridker PM. COVID-19 - a vascular disease. *Trends Cardiovasc Med*. 2021;31:1-5

[6] Marchandot B, Sattler L, Jesel L, Matsushita K, Schini-Kerth V, Grunebaum L, Morel O. COVID-19 related coagulopathy: A distinct entity? *J Clin Med*. 2020;9

[7] Canzano P, Brambilla M, Porro B, Cosentino N, Tortorici E, Vicini S, Poggio P, Cascella A, Pengo MF, Veglia F, Fiorelli S, Bonomi A, Cavalca V, Trabattoni D, Andreini D, Omodeo Sale E, Parati G, Tremoli E, Camera M. Platelet and endothelial activation as potential mechanisms behind the thrombotic complications of COVID-19 patients. *JACC Basic Transl Sci*. 2021;6:202-218

[8] Wang K, Gheblawi M, Nikhanj A, Munan M, MacIntyre E, O'Neil C, Poglitsch M, Colombo D, Del Nonno F, Kassiri Z, Sligl W, Oudit GY. Dysregulation of ace (angiotensin-converting

enzyme)-2 and renin-angiotensin peptides in sars-cov-2 mediated mortality and end-organ injuries. *Hypertension*. 2022;79:365-378

[9] Bonaventura A, Vecchie A, Dagna L, Martinod K, Dixon DL, Van Tassell BW, Dentali F, Montecucco F, Massberg S, Levi M, Abbate A. Endothelial dysfunction and immunothrombosis as key pathogenic mechanisms in COVID-19. *Nat Rev Immunol*. 2021;21:319-329

[10] Xie Y, Xu E, Bowe B, Al-Aly Z. Long-term cardiovascular outcomes of COVID-19. *Nat Med*. 2022;28:583-590

[11] Ambrosino P, Calcaterra I, Molino A, Moretta P, Lupoli R, Spedicato GA, Papa A, Motta A, Maniscalco M, Di Minno MND. Persistent endothelial dysfunction in post-acute COVID-19 syndrome: A case-control study. *Biomedicines*. 2021;9

[12] Jud P, Gressenberger P, Muster V, Avian A, Meinitzer A, Strohmaier H, Sourij H, Raggam RB, Stradner MH, Demel U, Kessler HH, Eller K, Brodmann M. Evaluation of endothelial dysfunction and inflammatory vasculopathy after sars-cov-2 infection-a cross-sectional study. *Front Cardiovasc Med*. 2021;8:750887

[13] Schultheiss C, Willscher E, Paschold L, Gottschick C, Klee B, Henkes SS, Bosurgi L, Dutzmann J, Sedding D, Frese T, Girndt M, Holl JI, Gekle M, Mikolajczyk R, Binder M. The il-1beta, il-6, and tnf cytokine triad is associated with post-acute sequelae of COVID-19. *Cell Rep Med*. 2022;3:100663

[14] Fogarty H, Townsend L, Morrin H, Ahmad A, Comerford C, Karampini E, Englert H, Byrne M, Bergin C, O'Sullivan JM, Martin-Loeches I, Nadarajan P, Bannan C, Mallon PW, Curley GF, Preston RJS, Rehill AM, McGonagle D, Ni Cheallaigh C, Baker RI, Renne T, Ward SE, O'Donnell JS, Irish C-VSi. Persistent endotheliopathy in the pathogenesis of long covid syndrome. *J Thromb Haemost*. 2021;19:2546-2553

[15] Chioh FW, Fong SW, Young BE, Wu KX, Siau A, Krishnan S, Chan YH, Carissimo G, Teo LL, Gao F, Tan RS, Zhong L, Koh AS, Tan SY, Tambyah PA, Renia L, Ng LF, Lye DC, Cheung C. Convalescent COVID-19 patients are susceptible to endothelial dysfunction due to persistent immune activation. *Elife*. 2021;10

[16] Gyongyosi M, Alcaide P, Asselbergs FW, Brundel B, Camici GG, da Costa Martins P, Ferdinandy P, Fontana M, Girao H, Gnecchi M, Gollmann-Tepekoylu C, Kleinbongard P, Krieg T, Madonna R, Paillard M, Pantazis A, Perrino C, Pesce M, Schiattarella GG, Sluijter JPG, Steffens S, Tschope C, Van Linthout S, Davidson SM. Long covid and the cardiovascular system - elucidating causes and cellular mechanisms in order to develop targeted diagnostic and therapeutic strategies: A joint scientific statement of the esc working groups on cellular biology of the heart and myocardial & pericardial diseases. *Cardiovasc Res*. 2022

[17] Kim YE, Huh K, Park YJ, Peck KR, Jung J. Association between vaccination and acute myocardial infarction and ischemic stroke after COVID-19 infection. *JAMA*. 2022

[18] Khazaal S, Harb J, Rima M, Annweiler C, Wu Y, Cao Z, Abi Khattar Z, Legros C, Kovacic H, Fajloun Z, Sabatier JM. The pathophysiology of long covid throughout the renin-angiotensin system. *Molecules*. 2022;27

[19] Vaduganathan M, Vardeny O, Michel T, McMurray JJV, Pfeffer MA, Solomon SD. Renin-angiotensin-aldosterone system inhibitors in patients with COVID-19. *N Engl J Med*. 2020;382:1653-1659

[20] Fisk M, Althage M, Moosmang S, Greasley PJ, Cope AP, Jayne DR, Galloway J, Hall F, Wilkinson IB, Ambery P, Cheriyan J. Endothelin antagonism and sodium glucose Co-transporter 2 inhibition. A potential combination therapeutic strategy for COVID-19. Pulm *Pharmacol Ther*. 2021 Aug;69:102035

[21] Phetsouphanh C, Darley DR, Wilson DB, Howe A, Munier CML, Patel SK, Juno JA, Burrell LM, Kent SJ, Dore GJ, Kelleher AD, Matthews GV. Immunological dysfunction persists for 8 months following initial mild-to-moderate sars-cov-2 infection. *Nat Immunol*. 2022;23:210-216

[22] Roh JD, Kitchen RR, Guseh JS, McNeill JN, Aid M, Martinot AJ, Yu A, Platt C, Rhee J, Weber B, Trager LE, Hastings MH, Ducat S, Xia P, Castro C, Singh A, Atlason B, Churchill TW, Di Carli MF, Ellinor PT, Barouch DH, Ho JE, Rosenzweig A. Plasma proteomics of COVID-19-associated cardiovascular complications: Implications for pathophysiology and therapeutics. *JACC Basic Transl Sci*. 2022;7:425-441

[23] Morel O, Marchandot B, Jesel L, Sattler L, Trimaille A, Curtiaud A, Ohana M, Fafi-Kremer S, Schini-Kerth V, Grunebaum L, Freyssinet JM. Microparticles in COVID-19 as a link between lung injury extension and thrombosis. *ERJ Open Res*. 2021;7

[24] Abbas M, Jesel L, Auger C, Amoura L, Messas N, Manin G, Rumig C, León-González AJ, Ribeiro TP, Silva GC, Abou-Merhi R, Hamade E, Hecker M, Georg Y, Chakfe N, Ohlmann P, Schini-Kerth VB, Toti F, Morel O. Endothelial microparticles from acute coronary syndrome patients induce premature coronary artery endothelial cell aging and thrombogenicity: Role of the ang ii/at1 receptor/nadph oxidase-mediated activation of mapks and pi3-kinase pathways. *Circulation*. 2017;135:280-296

[25] Camargo RL, Bombassaro B, Monfort-Pires M, Mansour E, Palma AC, Ribeiro LC, Ulaf RG, Bernardes AF, Nunes TA, Agrela MV, Dertkigil RP, Dertkigil SS, Araujo EP, Nadruz W, Moretti ML, Velloso LA, Sposito AC. Plasma angiotensin ii is increased in critical coronavirus disease 2019. *Front Cardiovasc Med*. 2022;9:847809

[26] Khemais-Benkhiat S, Belcastro E, Idris-Khodja N, Park SH, Amoura L, Abbas M, Auger C, Kessler L, Mayoux E, Toti F, Schini-Kerth VB. Angiotensin ii-induced redox-sensitive sglt1 and 2 expression promotes high glucose-induced endothelial cell senescence. *J Cell Mol Med*. 2020;24:2109-2122

[27] Park SH, Belcastro E, Hasan H, Matsushita K, Marchandot B, Abbas M, Toti F, Auger C, Jesel L, Ohlmann P, Morel O, Schini-Kerth VB. Angiotensin ii-induced upregulation of sglt1 and 2 contributes to human microparticle-stimulated endothelial senescence and dysfunction: Protective effect of gliflozins. *Cardiovasc Diab*. 2021;20:65

[28] Kang S, Kishimoto T. Interplay between interleukin-6 signaling and the vascular endothelium in cytokine storms. *Exp Mol Med*. 2021;53:1116-1123

[29] Ostergaard L. Sars cov-2 related microvascular damage and symptoms during and after COVID-19: Consequences of capillary transit-time changes, tissue hypoxia and inflammation. *Physiol Rep*. 2021;9:e14726

[30] Oudit GY, Kassiri Z, Jiang C, Liu PP, Poutanen SM, Penninger JM, Butany J. SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS. *Eur J Clin Invest*. 2009 Jul;39(7):618-25.

[31] Kumar A, Kim CS, Hoffman TA, Naqvi A, Dericco J, Jung SB, Lin Z, Jain MK, Irani K. P53 impairs endothelial function by transcriptionally repressing kruppel-like factor 2. *Arterioscler Thromb Vasc Biol*. 2011;31:133-141

[32] Froidure A, Mahieu M, Hoton D, Laterre PF, Yombi JC, Koenig S, Ghaye B, Defour JP, Decottignies A. Short telomeres increase the risk of severe COVID-19. *Aging*. 2020;12:19911-19922

[33] Sanchez-Vazquez R, Guio-Carrion A, Zapatero-Gaviria A, Martinez P, Blasco MA. Shorter telomere lengths in patients with severe COVID-19 disease. *Aging*. 2021;13:1-15

[34] Kosiborod MN, Esterline R, Furtado RHM, Oscarsson J, Gasparyan SB, Koch GG, Martinez F, Mukhtar O, Verma S, Chopra V, Buenconsejo J, Langkilde AM, Ambery P, Tang F, Gosch K, Windsor SL, Akin EE, Soares RVP, Moia DDF, Aboudara M, Hoffmann Filho CR, Feitosa ADM, Fonseca A, Garla V, Gordon RA, Javaheri A, Jaeger CP, Leaes PE, Nassif M, Pursley M, Silveira FS, Barroso WKS, Lazcano Soto JR, Nigro Maia L, Berwanger O. Dapagliflozin in patients with cardiometabolic risk factors hospitalised with COVID-19 (dare-19): A randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Diabetes Endocrinol*. 2021;9:586-594

[35] Lopaschuk GD, Verma S. Mechanisms of cardiovascular benefits of sodium glucose co-transporter 2 (sglt2) inhibitors: A state-of-the-art review. *JACC Basic Trans Science*. 2020;5:632-644

[36] Xie L, Xiao Y, Tai S, Yang H, Zhou S, Zhou Z. Emerging roles of sodium glucose cotransporter 2 (sglt-2) inhibitors in diabetic cardiovascular diseases: Focusing on immunity, inflammation and metabolism. *Front Pharmacol*. 2022;13:836849

[37] Li XT, Zhang MW, Zhang ZZ, Cao YD, Liu XY, Miao R, Xu Y, Song XF, Song JW, Liu Y, Xu YL, Li J, Dong Y, Zhong JC. Abnormal apelin-ace2 and sglt2 signaling contribute to adverse cardiorenal injury in patients with COVID-19. *Int J Cardiol*. 2021;336:123-129

[38] Bruckert C, Matsushita K, Mroueh A, Amissi S, Auger C, Hounge U, Remila L, Bey Chaker A, Park S-H, Algara-Suarez P, Belcastro E, Jesel L, Ohlmann P, Morel O, Schini-Kerth V. Empaglifozin prevents angiotensin ii induced hypertesnsion related micro and macrovascular endothelial cell activation and diastolic dysfunction in rats despite persistent hypertension: Role of endothelial sglt1 and 2. *Vascul Pharmacol*. 2022

[39] Kang Y, Zhan F, He M, Liu Z, Song X. Anti-inflammatory effects of sodium-glucose co-transporter 2 inhibitors on atherosclerosis. *Vascul Pharmacol*. 2020;133-134:106779

[40] Levy JH, Iba T, Connors JM. Editorial commentary: Vascular injury in acute infections and COVID-19: Everything old is new again. *Trends Cardiovasc Med*. 2021;31:6-7

[41] Dupont A, Rauch A, Staessens S, Moussa M, Rosa M, Corseaux D, Jeanpierre E, Goutay J, Caplan M, Varlet P, Lefevre G, Lassalle F, Bauters A, Faure K, Lambert M, Duhamel A, Labreuche J, Garrigue D, De Meyer SF, Staels B, Vincent F, Rousse N, Kipnis E, Lenting P, Poissy J, Susen S, Lille Covid Research N. Vascular endothelial damage in the pathogenesis of organ injury in severe COVID-19. *Arterioscler Thromb Vasc Biol*. 2021;41:1760-1773

[42] Karampini E, Fogarty H, Elliott S, Morrin H, Bergin C, O'Sullivan JM, Byrne M, Martin-Loeches I, Mallon PW, Curley GF, Glavey S, Baker RI, Lavin M, Preston RJS, Cheallaigh CN, Ward SE, O'Donnell JS. Endothelial cell activation, Weibel-Palade body secretion, and enhanced angiogenesis in severe COVID-19. *Res Pract Thromb Haemost.* 2023 Feb;7(2):100085

[43] Fernandez-Fernandez B, D'Marco L, Gorriz JL, Jacobs-Cacha C, Kanbay M, Luis-Lima S, Porrini E, Sarafidis P, Soler MJ, Ortiz A. Exploring sodium glucose co-transporter-2 (sglt2) inhibitors for organ protection in COVID-19. *J Clin Med*. 2020;9

Figures legends

Figure 1 Plasma levels of pro-inflammatory cytokines (IL-1 β, IL-6, (TNF-α, monocyte chemoattractant protein-1 (MCP-1)), and cytoadhesins (soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble intercellular adhesion molecule-1 (sICAM-1)) in healthy volunteers (n = 25), COVID- patients with cardiovascular risk factors (n = 50) and COVID+ patients (n = 100). Data are presented as median [25^{th} - 75^{th}] percentile. *$P < 0.05$ vs. Healthy volunteers, $^{\#}P < 0.05$ vs. COVID- patients using Kruskal-Wallis with Dunn's multiple comparison test.

Figure 2 Pro-oxidant state of COVID-19 plasma treated coronary endothelial cells: Role of pro-inflammatory cytokines, the angiotensin system and SGLT2. Long-COVID-19 plasma (10% v/v) induced significant increase in long-term pro-oxidant response in ECs (A). COVID-19 plasma (10% v/v) of the highest quartile of IL-1β, IL-6, and TNF-α induced greater short-term (30 min, B) and long-term (24 h, C) pro-oxidant responses in ECs than that of COVID- patients with cardiovascular risk factors whereas plasma of healthy volunteers was inactive. (D, E) Both the short-term and the long-term pro-oxidant responses of ECs to COVID-19 plasma are inhibited partially by a single neutralizing Abs directed against either IL-1β, IL-6 or TNF-α, and to a greater extent by the combination of the 3 neutralizing Abs and by the antioxidant N-acetylcysteine (NAC) whereas empagliflozin (Empa) inhibited strongly only the sustained response. (F-H) Long-term pro-oxidant response of ECs to COVID-19 plasma is correlated to the levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in plasma samples. (I) The sustained pro-oxidant response to COVID-19 plasma was also prevented partially by inhibition of the angiotensin system by either perindoprilat (Per, ACE1 inhibitor) or losartan (Los, AT1R antagonist) and to a greater extent by the NADPH oxidase inhibitor VAS-2870 (VAS). Once

established, the sustained pro-oxidant response of ECs to COVID-19 plasma after a 24 h incubation period was reduced by the subsequent addition of either VAS-2870 or empagliflozin for 30 min whereas the combination of neutralizing Abs targeting IL-1β, IL-6 and TNF-α, perindoprilat or losartan were inactive (J). Data are presented as mean ± SEM (n = 5-54) or (F-H) as correlation graphs (n = 100). (B, C) Scale bars on micrographies represent 100 µm. *$P <$ 0.05 vs. control ECs, $^{\#}P < 0.05$ vs. healthy volunteers, $^{\$}P < 0.05$ vs. COVID- patients, $^{\dagger}P < 0.05$ vs. COVID+ patients, $^{\S}P < 0.05$ vs. the combination of neutralizing Abs directed against IL-1β, IL-6 and TNF-α, $^{\ddagger}P < 0.05$ vs. empagliflozin using one-way ANOVA followed by Tukey's multiple comparison test or using Spearman correlation test (F-H).

Figure 3 Heat map of gene expression variations in each group. Exposure of ECs to COVID-19 plasma for 6 h is associated with a downregulation of the expression levels of *NOS3* and *TFPI* mRNA, and an upregulation of mRNA levels of markers of senescence (*TP53*, *CDKN1A* and *CDKN2A*), cytoadhesins (*VCAM1*, *ICAM1*, *SELE* and *SELP*), modulators of thrombotic responses (*F3*, *THBD*, *SERPINE1*), the angiotensin/NADPH oxidase pathway (*ACE1*, *AGTR1*, *CYBA*, *NCF1*), pro-inflammatory cytokines (*IL1B*, *IL6*, *TNFA*) and *SLC5A2*. The stimulatory effect of COVID-19 plasma is prevented by the combination of neutralizing Abs directed against IL-1β, IL-6 and TNF-α except for *NOS3*, *TFPI* and *SERPINE1*, and also by empagliflozin except for *TFPI* and *SERPINE1*.

Figure 4 Feedforward upregulation of SGLT2 expression in COVID-19 plasma treated endothelial cells and role of pro-inflammatory cytokines and redox-sensitive NF-κB. (A) Exposure of ECs to COVID-19 plasma for 24 h is associated with a concentration-dependent upregulation of SGLT2 protein levels as assessed by immunofluorescence. (B) The stimulatory

effect of COVID-19 plasma is partially prevented by losartan, and to a greater extent by the NADPH oxidase inhibitor VAS-2870 (VAS), the NF-κB inhibitor Bay 11-7082 (BAY), and by empagliflozin (Empa). (C) COVID-19 plasma stimulates the nuclear translocation of NF-κB in ECs. This response is inhibited by either losartan, VAS-2870, empagliflozin or Bay 11-7082 and unaffected by perindoprilat (Per). (D) Increased levels of SGLT2 staining are observed in ECs in response to pro-inflammatory cytokines (IL-1β, IL-6, TNF-α; cytokines mix is a combination of the three cytokines). (E) COVID-19 plasma promotes in ECs increased protein levels of SGLT2, VCAM-1 and TF, and a down-regulation of that of eNOS as assessed by Western blot analysis. The stimulatory effect of COVID-19 plasma is prevented by the combination of neutralizing Abs directed against IL-1β, IL-6 and TNF-α, by empagliflozin and also following knock down of SGLT2 using siRNA whereas scrambled (Scrbl) siRNA is inactive. Data are presented as mean ± SEM (n = 9). Scale bars on micrographies represent (A) 100 µm and (C) 200 µm. $^{*}P < 0.05$ vs. control ECs, $^{\dagger}P < 0.05$ vs. COVID+ plasma, $^{\ddagger}P < 0.05$ vs. empagliflozin using one-way ANOVA followed by Tukey's multiple comparison test.

Figure 5 Increased thrombogenicity in COVID-19 plasma treated endothelial cells: Role of pro-inflammatory cytokines and SGLT2. (A) COVID-19 plasma reduced the basal and the bradykinin-induced formation of NO in ECs, and this effect was prevented by the combination of the three neutralizing Abs directed against IL-1β, IL-6 and TNF-α, and also by empagliflozin. (B) COVID-19 plasma reduced the anti-aggregatory effect of ECs in response to bradykinin, and this effect was prevented by the combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α, and by empagliflozin. The effect of N^{G}-nitro L-arginine (LNA, added 30 min post the 24 h stimulation with COVID-19 plasma) on the anti-aggregatory effect of ECs is also shown. COVID-19 plasma increased platelet adhesion to ECs surface (C) and vWF secretion by ECs (D), and these effects were prevented by the combination of three neutralizing

Abs directed against IL-1β, IL-6 and TNF-α, and by empagliflozin. (E) Exposure of ECs to COVID-19 plasma is associated with an enhanced ability of the cell surface to generate thrombin following exposure to recalcified plasma of healthy volunteers. This effect is prevented by the combination of three neutralizing Ab directed against IL-1β, IL-6 and TNF-α, and by empagliflozin. It is also acutely inhibited by a neutralizing Ab directed against TF and by the thrombin inhibitor, dabigatran, whereas the factor Xa inhibitor, rivaroxaban, is inactive. Data are presented as mean ± SEM (n = 9). (A) Scale bars on micrographies represent 100 µm. $^{*}P < 0.05$ vs. control ECs, $^{\#}P < 0.05$ vs. bradykinin-stimulated ECs, $^{\dagger}P < 0.05$ vs. COVID+ plasma-treated ECs, $^{\ddagger}P < 0.05$ vs. bradykinin-stimulated COVID+ plasma-treated ECs, $^{¤}P < 0.05$ for within-group comparison (w/wo bradykinin) using one-way (A, B) or two-way (C) ANOVA followed by Tukey's multiple comparison test.

Supplementary Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Methods S1 Supplementary methods.

Figure S1 Role of microparticles, MCP-1 and sVCAM-1 in COVID-19 plasma induced pro-oxidant response of endothelial cells.

Figure S2 Pro-inflammatory genes expression profile of COVID-19 plasma treated endothelial cells: Role of pro-inflammatory cytokines and SGLT2 .

Figure S3 COVID-19 plasma, siRNA mediated SGLT2 knockdown and empagliflozin has no effect on ECs viability.

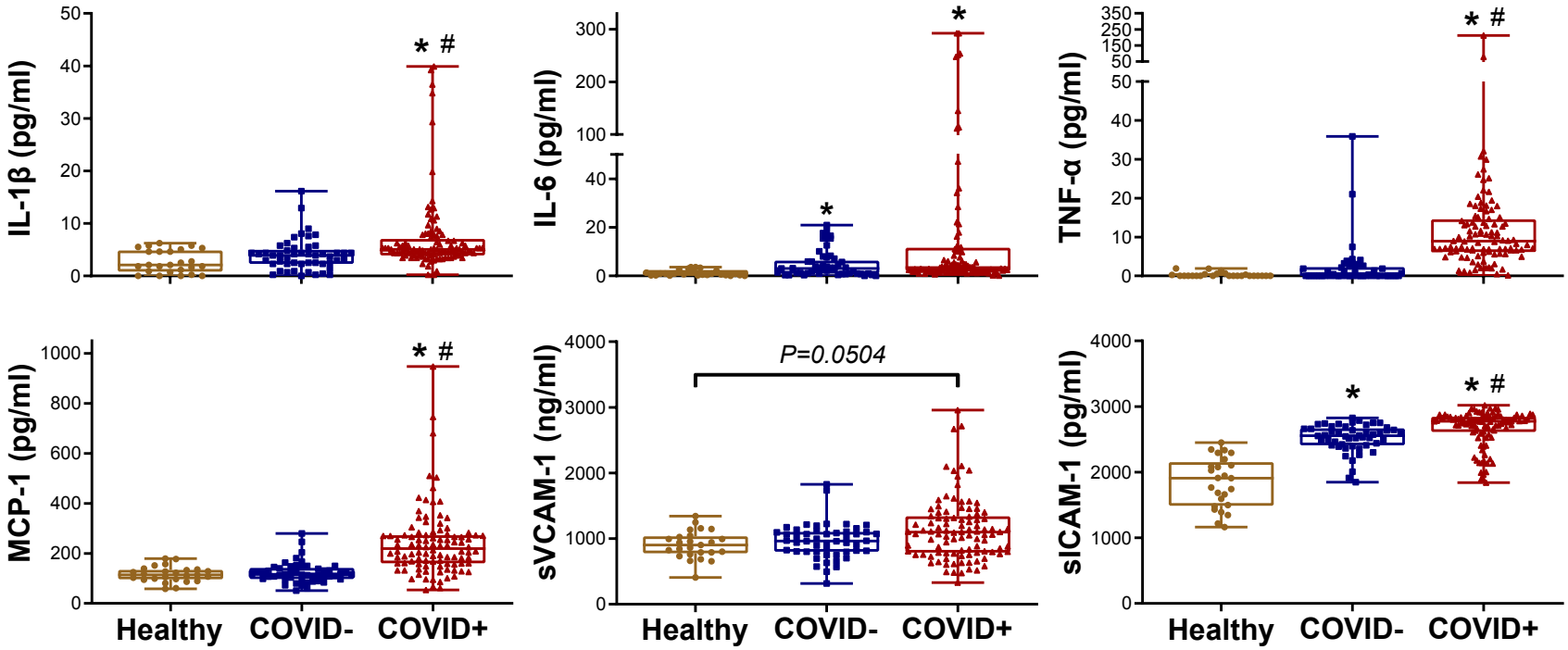
Figure S4 Upregulation of SGLT2 protein by COVID-19 plasma in endothelial cells is prevented by knock down of SGLT2 by siRNA whereas scrambled siRNA was inactive.

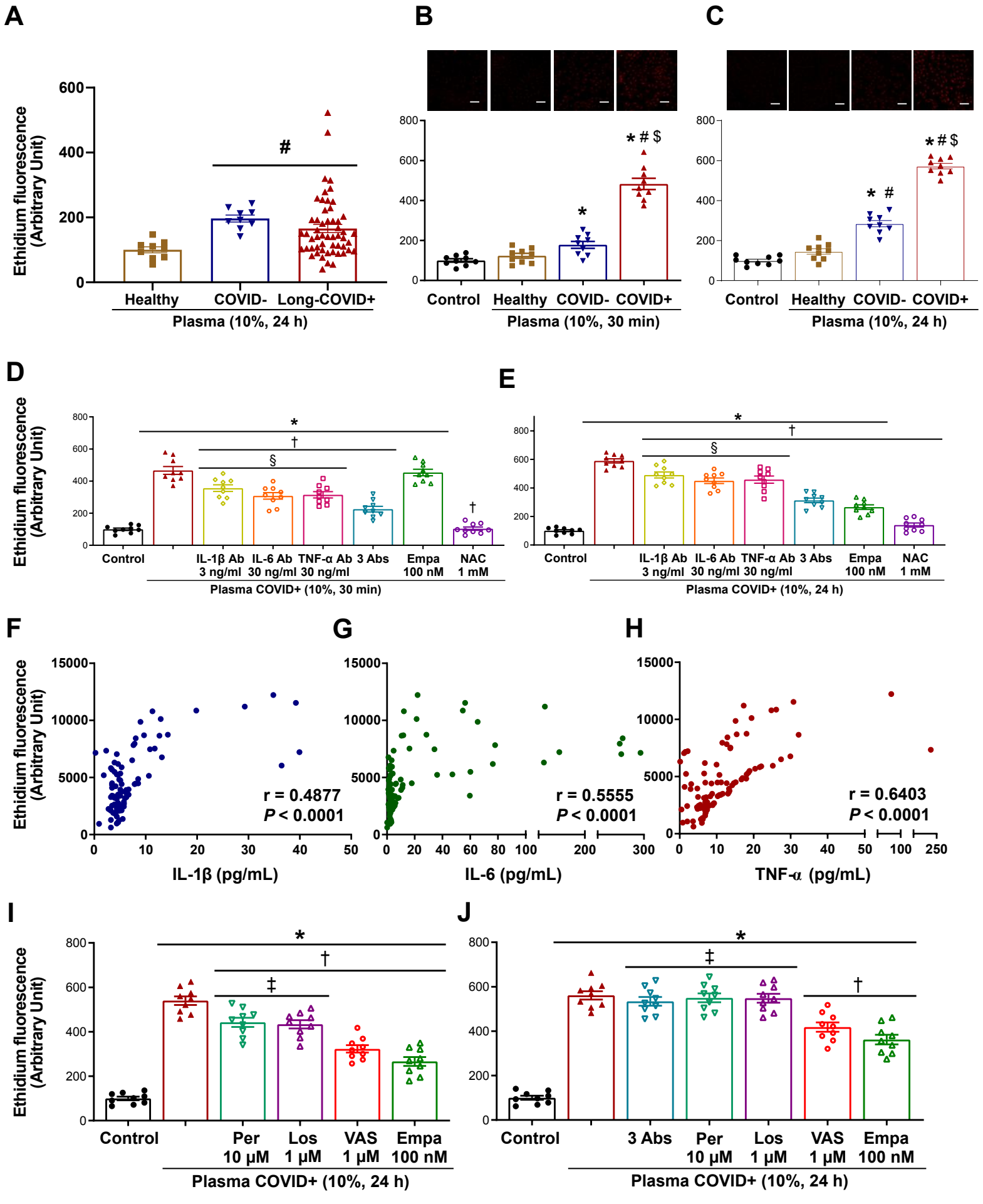
Figures S5 Upregulation of SGLT2 and TF proteins by COVID-19 plasma in endothelial cells is prevented by simultaneous addition of empagliflozin.

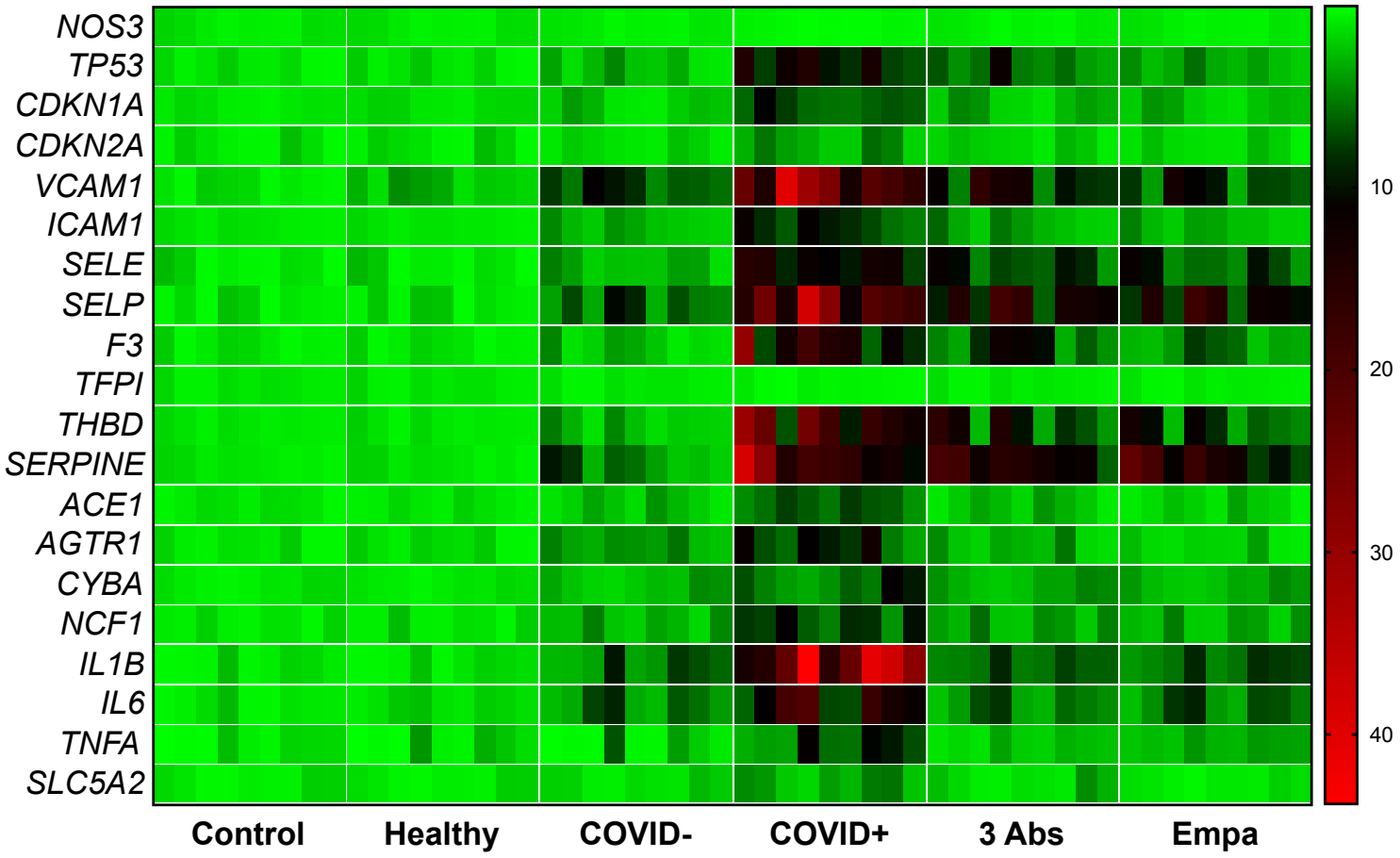
Table S1 Clinical characteristics of COVID-19 patients .

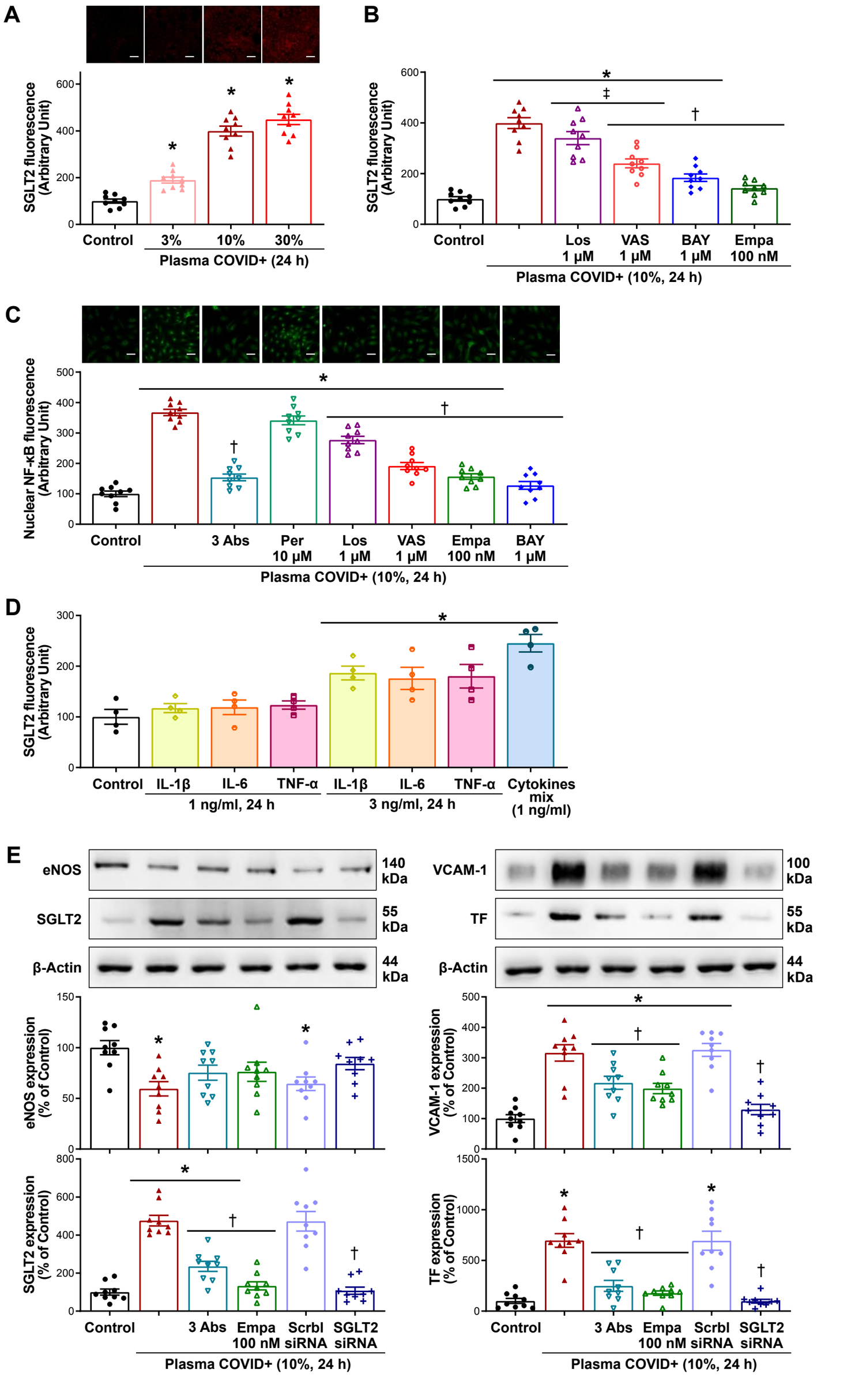
Table S2 Circulating cytokines, cytoadhesins and DHE values in COVID-19 patients stratified in acute, sub-acute and long COVID-19.

Table S3 Clinical characteristics of COVID-19 patients with low and high cytokine levels .


IL-1β (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
MCP-1 (pg/ml)
sVCAM-1 (ng/ml)
sICAM-1 (pg/ml)
P=0.0504
Healthy
COVID-
COVID+


A
B
C
Ethidium fluorescence (Arbitrary Unit)
Healthy
COVID-
Long-COVID+
Plasma (10%, 24 h)
Control
Healthy
COVID-
COVID+
Plasma (10%, 30 min)
Plasma (10%, 24 h)
D
E
IL-1β Ab 3 ng/ml
IL-6 Ab 30 ng/ml
TNF-α Ab 30 ng/ml
3 Abs
Empa 100 nM
NAC 1 mM
Plasma COVID+ (10%, 30 min)
Plasma COVID+ (10%, 24 h)
F
G
H
r = 0.4877
P < 0.0001
r = 0.5555
P < 0.0001
r = 0.6403
P < 0.0001
IL-1β (pg/mL)
IL-6 (pg/mL)
TNF-α (pg/mL)
I
J
Per 10 μM
Los 1 μM
VAS 1 μM
Empa 100 nM
Plasma COVID+ (10%, 24 h)


NOS3
TP53
CDKN1A
CDKN2A
VCAM1
ICAM1
SELE
SELP
F3
TFPI
THBD
SERPINE
ACE1
AGTR1
CYBA
NCF1
IL1B
IL6
TNFA
SLC5A2
10
20
30
40
Control
Healthy
COVID-
COVID+
3 Abs
Empa


A
SGLT2 fluorescence (Arbitrary Unit)
Control
3%
10%
30%
Plasma COVID+ (24 h)
B
SGLT2 fluorescence (Arbitrary Unit)
Control
Los 1 μM
VAS 1 μM
BAY 1 μM
Empa 100 nM
Plasma COVID+ (10%, 24 h)
C
Nuclear NF-κB fluorescence (Arbitrary Unit)
Control
3 Abs
Per 10 μM
Los 1 μM
VAS 1 μM
Empa 100 nM
BAY 1 μM
Plasma COVID+ (10%, 24 h)
D
SGLT2 fluorescence (Arbitrary Unit)
Control
IL-1β
IL-6
TNF-α
1 ng/ml, 24 h
IL-1β
IL-6
TNF-α
3 ng/ml, 24 h
Cytokines mix (1 ng/ml)
E
eNOS
SGLT2
β-Actin
140 kDa
55 kDa
44 kDa
VCAM-1
TF
β-Actin
100 kDa
55 kDa
44 kDa
eNOS expression (% of Control)
SGLT2 expression (% of Control)
VCAM-1 expression (% of Control)
TF expression (% of Control)
Control
3 Abs
Empa 100 nM
Scrbl siRNA
SGLT2 siRNA
Plasma COVID+ (10%, 24 h)

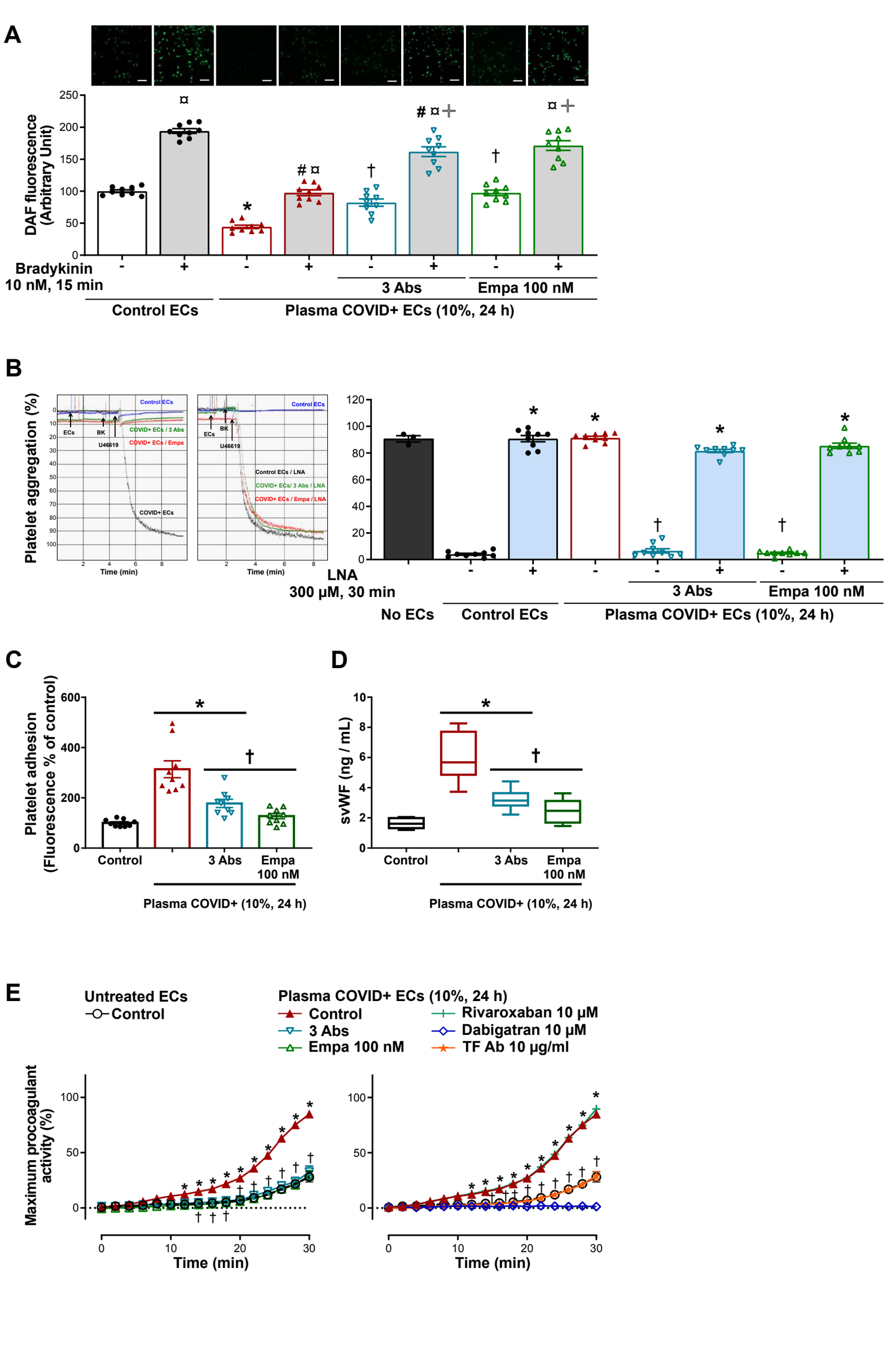

A
DAF fluorescence (Arbitrary Unit)
Bradykinin 10 nM, 15 min
3 Abs
Empa 100 nM
Control ECs
Plasma COVID+ ECs (10%, 24 h)
B
Platelet aggregation (%)
Control ECs
COVID+ ECs / 3 Abs
COVID+ ECs / Empa
COVID+ ECs
Control ECs / LNA
COVID+ ECs/ 3 Abs / LNA
COVID+ ECs / Empa / LNA
ECs
BK
U46619
Time (min)
LNA 300 µM, 30 min
No ECs
C
Platelet adhesion (Fluorescence % of control)
Control
3 Abs
Empa 100 nM
Plasma COVID+ (10%, 24 h)
D
svWF (ng / mL)
E
Untreated ECs
Control
Plasma COVID+ ECs (10%, 24 h)
Rivaroxaban 10 µM
Dabigatran 10 µM
TF Ab 10 µg/ml
Maximum procoagulant activity (%)
Time (min)

Table 1. Baseline characteristics of participating subjects.

Variable	Healthy volunteers (n = 25)	COVID- (n = 50)	COVID+ (n = 100)	*P* value
Age, years	39 (29 - 56)	70 (64 - 78)[§]	62 (53 - 70)[†, ‡]	<0.001
Male	9 (36)	29 (58)	64 (64)[†]	0.04
BMI, kg/m^2	22.0 (20.8 - 24.2)	27.9 (24.2 - 33.1)[§]	26.8 (24.5 - 30.1)[†]	0.002
Hypertension	0 (0)	39 (78)[§]	42 (42)[†, ‡]	<0.001
Diabetes mellitus	0 (0)	21 (42)[§]	16 (16)[†, ‡]	<0.001
Dyslipidemia	0 (0)	38 (76)[§]	23 (23)[†, ‡]	<0.001
Obesity (BMI ≥ 30kg/m^2)	0 (0)	20 (40)[§]	26 (26)[†]	0.001
Current smoker	0 (0)	14 (28)[§]	9 (9)[‡]	<0.001
Family history of CAD	0 (0)	11 (22)[§]	0 (0)[‡]	<0.001
CKD (Cr > 150 μM)	0 (0)	5 (10)	5 (5)	0.19
COPD	0 (0)	6 (12)	9 (9)	0.21
Stroke	0 (0)	4 (8)	3 (3)	0.18
Atrial fibrillation	0 (0)	8 (16)[§]	3 (3)[‡]	0.003
CAD	0 (0)	25 (50)[§]	12 (12)[‡]	<0.001
Heart failure	0 (0)	4 (8)	4 (4)	0.27
Peripheral artery disease	0 (0)	13 (26)[§]	2 (2)[‡]	<0.001
Previous PE	0 (0)	0 (0)	2 (2)	0.47
Previous DVT	0 (0)	3 (6)	2 (2)	0.25
History of cancer	0 (0)	6 (12)	8 (8)	0.20
Medications at baseline				
Antiplatelet therapy	0 (0)	36 (72)[§]	15 (15)[†, ‡]	<0.001
Anticoagulants	0 (0)	7 (14)	0 (0)[‡]	<0.001
ACE inhibitors	0 (0)	19 (38)[§]	12 (12)[‡]	<0.001
ARBs	0 (0)	12 (24)[§]	14 (14)	0.02
MRAs	0 (0)	6 (12)	2 (2)[‡]	0.01
Beta-blockers	0 (0)	25 (50)[§]	21 (21)[†, ‡]	<0.001
Statins	0 (0)	33 (66)[§]	18 (18)[†, ‡]	<0.001
Ca-blockers	0 (0)	17 (34)[§]	15 (15)[†, ‡]	<0.001
Diuretics	0 (0)	14 (28)[§]	6 (6)[‡]	<0.001
Metformin	0 (0)	12 (24)[§]	7 (7)[‡]	0.001

Values are presented as n (%) or median (interquartile range). ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; BMI, body mass index; CAD, coronary artery disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; Cr, creatinine; DVT, deep vein thrombosis; MRA, mineralocorticoid receptor antagonist; PE, pulmonary embolism. $^{\dagger}P < 0.05$, COVID+ vs. Healthy volunteers; $^{\ddagger}P < 0.05$, COVID+ vs. COVID-; $^{\S}P < 0.05$, COVID- vs. Healthy volunteers.

Table 2. Circulating cytokines, cytoadhesins and DHE staining in long COVID-19 as compared to COVID- patients with cardiovascular risk factors and healthy volunteers.

Variable	Long COVID-19 (n=54)	COVID- (n=50)	Healthy volunteers (n=25)	*P* long COVID vs COVID-	*P* long COVID vs HV
IL-1β, pg/mL	5 (4-6)	4 (2.5)	2 (1-5)	0.013	0.002
IL-6, pg/mL	3 (2-4)	3 (1-6.2)	1 (0-2.5)	0.406	0.019
TNF-α, pg/mL	9.5 (7-14.25)	0 (0 - 2.25)	0 (0 - 0.0	< 0.001	0.001
MCP-1, pg/mL	201 (159-262)	116 (97-141)	114 (96-134)	< 0.001	<0.001
sVCAM-1, ng/mL	877 (719-1151)	964 (800-1101)	902 (776-1034)	0.957	0.454
sICAM-1, pg/mL	3284 (2319-4501)	2556 (2410-2665)	1909 (1489-2151)	0.137	<0.001
DHE staining (AU)	3284 (2319-4501)	4500 (4059-5221)	2415 (1771-2771)	0.281	0.035

Values are presented as median (interquartile range). COVID, coronavirus disease; IL, interleukin; MCP, monocyte chemoattractant protein; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; TNF, tumor necrosis factor; DHE, dihydroethidium.

Supplementary Material

I. Supplementary Methods

Materials

Empagliflozin was provided by Boehringer Ingelheim Pharma GmbH & Co KG (Biberach an der Riss, Germany). Other chemicals were from Sigma-Aldrich (Sigma-Aldrich Chemie SARL, St Quentin Fallavier, France) or as indicated.

Population study and ethics statement

The Institutional Review Board approved the study (number 2020-A01500-39) and all participants gave written informed consent. One hundred COVID-19 patients (COVID+) admitted to 3 specific COVID-19 units (two general wards and one intensive care unit) were prospectively enrolled at Strasbourg University Hospital from March 2020 to June 2021. The diagnosis of COVID-19 was made with a positive result of a reverse transcriptase-polymerase chain reaction (RT-PCR) assay of a specimen collected on a nasopharyngeal swab. From March 2020 to May 2021, only patients with a positive result for lupus anticoagulant (LAC) were included. From May 2021, a protocol amendment allowed us to include all COVID-19 patients regardless of LAC status. Medical management was left at the discretion of the treating physicians, in accordance with current medical practice and guidelines at the time of hospitalization. Information was collected during a 3- and 6-month follow-up period, and this included a physical examination and documentation of specified outcomes. Blood samples were collected at inclusion and during follow-up visits.

Control groups included 50 non-COVID-19 patients (COVID-) with at least two cardiovascular risk factors, and 25 healthy volunteers. Demographic information, cardiovascular risk factors and medications are presented in *Table 1*.

Blood sample preparation

Blood samples collected by venous puncture into tubes containing 129 mM sodium citrate were centrifuged at 500 *g* for 15 min at room temperature to isolate the plasma. Platelet-poor plasma samples were prepared by centrifugation of plasma (13,000 *g* for 3 min) at room temperature, aliquoted and immediately stored at -80 °C until use. In some experiments, platelet-poor plasma samples were depleted of circulating microparticles by centrifugation at 14,000 *g* for 1.5 h at 4 °C and, thereafter, the supernatant was collected, aliquoted and stored at -80 °C.

Plasma level of pro-inflammatory mediators

IL-1β, IL-6, TNF-α, MCP-1, sVCAM-1 and sICAM-1 concentrations were determined using Quantikine ELISA kits as recommended (R&D Systems, Minneapolis, MN, USA).

Cell culture

Porcine hearts were collected from the local slaughterhouse (SOCOPA, Holtzheim, France). ECs were isolated from the right coronary artery as described previously (21). Briefly, the coronary artery was dissected and cleaned of surrounding connective tissues. After washing with calcium-free PBS to remove remaining blood, ECs were isolated by type I collagenase (Gibco, #17100-017, USA) treatment at 1 mg/mL (250 U/mg) for 20 min at 37 °C and cultured in a T25 flask containing MCDB 131 medium supplemented with 15 % fetal calf serum (Dominique Dutcher), fungizone (2.5 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM, all from PAN Biotech, Germany) and grown to 70-80 % confluence for 48-72 h. ECs at passage 1 were exposed to serum-free culture medium for 2 h before the addition of either human plasma, a single cytokine or a combination of cytokines. In some experiments, ECs were pretreated with a pharmacological modulator for at least 30 min before the addition of human plasma or as indicated.

Cellular level of oxidative stress

ECs were cultured on 8-well Lab-Tek® chambers before the addition of human plasma for either 30 min or 24 h. In some experiments, ECs were pretreated with either an antioxidant (N-acetylcysteine, 1 mM) for 2 h, an IL-1β neutralizing Ab (3 ng/ml, R&D Systems, USA), an IL-6 neutralizing Ab (30 ng/ml, R&D Systems, USA), a TNF-α neutralizing Ab (infliximab, 30 ng/ml, # Y0002047, Sigma Aldrich), or a combination of the 3 neutralizing Abs for 1 h, perindoprilat (10 µM), losartan (1 µM), VAS-2870 (1 µM) or empagliflozin (100 nM) for 30 min before the addition of human plasma. Thereafter, cells were exposed to dihydroethidium (5 µM), a redox-sensitive fluorescent dye for 30 min at 37 °C in the dark. After washing 3 times with PBS, cells were mounted with fluorescent mounting medium (fluorescence editing medium, Dako, Agilent Technologies France, Les Ulis, France) and cover-slipped before being examined by fluorescent microscopy (epi-fluorescent microscope, Zeiss). Quantification of the reactive oxygen species signal was performed by using ImageJ software (Version 1.53c for Windows, NIH) and mean intensities were expressed as arbitrary densitometry units.

Immunofluorescence staining of SGLT2 and NF-κB nuclear translocation

For immunofluorescence studies, ECs were cultured on 8-well Lab-Tek® chambers and exposed to human plasma, a single cytokine (IL-1β, IL-6, or TNF-α) or a combination of the three cytokines in serum-free culture medium for 24 h. The cells were washed twice in sterile PBS, fixed during 30 min with 4 % (w/v) paraformaldehyde, washed thoroughly and then incubated with blocking/permeabilizing buffer (PBS containing 1 % BSA (w/v) and 0.5 % Triton X-100 (w/v)) for 30 min at room temperature. After buffer removal, cells were incubated with 1:100 dilution of either an Ab directed against SGLT2 (AGT-032, Alomone Lab) or p65 NF-κB (SC-8008, Santa Cruz) overnight at 4 °C. After washing 3 times with PBS, they were further incubated with a 1:250 dilution of either Alexa Fluor™ 633 goat anti-rabbit immunoglobulin G (A21071, Invitrogen) or Alexa Fluor™ 488 goat anti-mouse immunoglobulin G (A11029, Invitrogen) for 1 h at room temperature in the dark. For negative controls, primary antibodies were omitted. After washing 2 times with PBS, cells were incubated with

1 µg/ml 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, #62248, Thermo Fisher Scientific) during 3 min at room temperature, in order to counterstain nuclei. After disassembling, slides were mounted with fluorescent mounting medium. Images were acquired using a Zeiss epi-fluorescence microscope.

SGLT2 siRNA transfection studies

ECs were transfected with siRNA (50 nM) targeting SGLT2 (Eurogentec; SGLT2 siRNA sense GCCUCAAUCUUUAACAGCA, antisense UGCUGUUAAAGAUUGAGGC), negative control (Scrambled) siRNA (sense AUUCUAAUCCGUGAUGUAG, antisense CUACAUCACGGAUUAGAAU) in OPTI-MEM™ (# 31985070, Gibco) culture medium for 48 h to knock down SGLT2 using Lipofectamine RNAiMAX (# 13778-150, Invitrogen). Thereafter, ECs were stimulated with human plasma for 24 h.

RNA isolation and quantitative real time-polymerase chain reaction (RT-PCR)

After treatment, total RNA was isolated from ECs using TRIzol®. RNA was quantified using Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, USA) and a total of 1000 ng of RNA was reverse transcribed using Maxima first strand cDNA synthesis kit with dsDNase k1672 (Thermo Fischer Scientific) in My iQ 576BR0703 icycler (Biorad, USA) to produce cDNA. Quantitative PCR was then performed using CFX connect 788BR2133 (Biorad). Amplifications were carried out in 10 µl reaction solutions containing 5 µl 2x PowerUp SYBR Green master mix A25742 (Applied biosystems), 10 ng cDNA and 300 nM of each specific forward and reverse primer. The used primers are listed in the Table below. PCR conditions were 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min. The specificity of each pair of primers was checked by melting curve analysis (95 °C for 15 s, 60 °C for 30 s and a continuous raise in temperature to 95 °C at 0.5 °C/s ramp rate followed by 95 °C for 15 s). The mean of three different house-keeping genes were used for normalization (18S, GusB, Hprt) and quantification was performed using traditional 2^-ΔΔCt formula.

Gene	Protein	Forward Primer Sequence	Reverse Primer Sequence
18S	18S ribosomal RNA	GCCCTCGGTCGAGTTGTC	CTTGCAGGGCGGTGACAG
Gusb	Glucuronidase beta (GUSB)	GGTGTGGTATGAACGGGAGG	CATTCACCCACACAATGGCG
Hprt	Hypoxanthine phosphoribosyltransferase 1 (HPRT)	CCCAGCGTCGTGATTAGTGA	GCCGTTCAGTCCTGTCCATA
Ace1	Angiotensin-converting enzyme 1 (ACE1)	CGCCAACAGCACTTGTCTTC	CAGCTCCTCATAGCTTCGGG
Ace2	Angiotensin-converting enzyme 2 (ACE2)	GAAAAGTGGCGGTGGATGGT	CATCATGGGGCAGAGGTTCC
Agtr1	Angiotensin II type 1 receptor (AT1R)	GTCCCGAGTGCGGATTTGAT	CAGACACTACGCCAAATGCAC
Cdkn1a	Cyclin-dependent kinase-interacting protein 1 (p21)	CACCCTTGTGCCTCACTCC	AGAAGATCAGCCGGCGTTTG
Cdkn2a	Cyclin-dependent kinase inhibitor 2A (p16)	ACCCCAGGGTGTTTCATTCG	CCTCCACGCGATCTTACAGG
Cyba	Human neutrophil cytochrome b light chain ($p22^{phox}$)	CATGGGACAGATCGAGTGGG	AGGTACCACTGCGTGAACTG
F3	Tissue factor (TF) coagulation factor III	AGTCCCGAAAGTCGCATTGA	GGAACAGTTCTCGGGACACA
Icam1	Intercellular adhesion molecule 1	GCCATGCAGTATGTCAGGGA	CGGGAACCAGTATACGGTGAG

	(ICAM-1)		
Il1b	Interleukin-1 beta (IL-1β)	GACTCAAGCCAGAGAAGCAAG	CTGGTCAGGGGAAGTATCCTC
Il6	Interleukin-6 (IL-6)	CACCTCTCCGGACAAAACTGA	GCCAGTACCTCCTTGCTGTT
Mas1	MAS1 proto-oncogene, G protein-coupled receptor	CTCCCAGATACGCTAAGGCG	GTCCGACATGTGTGTAGGCA
Ncf1	Phagocyte NADPH oxidase organizer ($p47^{phox}$)	CCTGTCAAGATCTCCCGCTG	GGCCATCAGGTATGTCTCGG
Nos3	Endothelial nitric oxide synthase (eNOS)	GCATCGCCAGAAAGAAGACG	GAATTGACGCCTTCACTCGC
Sele	Endothelial selectin (E-Selectin)	CTCCGAATGCCTTCAACCCAA	GAAGAGCCAGAGACCTTAGCAG
Selp	Platelet selectin (P-Selectin)	GCATTAGTTGGGCCAGAGGT	TCGAAGACGGGAAGCAATCC
Serpine1	Plasminogen activator inhibitor (PAI-1)	CCCATGATGGCTCAGACCAA	AGGGCAATTCCAGGATGTCG
Slc5a2	Sodium-glucose co-transporter2 (SGLT2)	CATCACCATGATCTACACTGTGAC	GGTCTGCACCGTATCCGT
TFPI	Tissue factor pathway inhibitor (TFPI)	CCTACCAAAGCACCCAGCTT	GCGGCATTTCCCAATGACTG
THBD	Thrombomodulin	CGTCGAGCATGACTGCTTTG	TCAAGTGGCCCTGTAGATGC
TNFA	Tumor necrosis factor-alpha (TNF-α)	TTGTCGCTACATCGCTGAAC	CCAGTAGGGCGGTTACAGAC
TP53	Tumor protein P53 (p53)	TCCTGCATTCTGGAACAGCC	GCTTATTGAGGGCAGGGGAG
VCAM1	Vascular cell adhesion molecule-1 (VCAM-1)	TTTACGTGTGCGAGGGAGTT	TCCCTGGGAGCAACTTGAAC

Western blot analysis

After treatment, ECs washed with cold PBS were homogenized and lysed in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5), NaCl 150, Na_3VO_4 1, $Na_4P_2O_7$ 10, NaF 20, N-ethylmaleimide 20 mM, 0.1% sodium dodecyl sulfate, 1 % Triton X-100 and protease inhibitor cocktail (Complete Mini, Roche)). Total proteins (10 µg) were separated on 8 % SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose membrane (Biorad) using Trans-Blot Turbo transfer system (Biorad). After blocking with 5 % bovine serum albumin in Tris-buffered saline (TBS) containing 0.1 % Tween 20 for 1 h at room temperature, membranes were incubated with a primary antibody of either mouse monoclonal anti-eNOS (1:1,000, Abcam, AB76198), rabbit monoclonal anti-VCAM-1 (1:5,000, Abcam, ab134047), rabbit polyclonal anti-SGLT2 (1:1,000, Alomone Labs, AGT-032), mouse monoclonal anti-tissue factor (TF, 1:100, Merck Millipore, 612161), or mouse monoclonal anti-β-actin (1:10,000, Sigma Aldrich, T7816) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin G, 1:10,000, Invitrogen, #31466, #31450, respectively) for 1 h at room temperature. The immunoreactive bands were developed by enhanced chemiluminescence (Clarity Western ECL substrate, Biorad) and analyzed using ImageJ software.

Endothelial formation of NO

ECs were cultured on 8-well Lab-Tek® chambers before the addition of human plasma in serum-free culture medium for 24 h. In some wells, a combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α or empagliflozin (100 nM) was added for 1 h and 30 min respectively before the addition of human plasma. Thereafter, ECs were exposed to DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluororescein diacetate, 1 µM), a NO-sensitive fluorescent dye, for 20 min at 37 °C in the dark. The formation of NO was induced by the exposure of ECs to bradykinin (100 nM) for 15

min. After washing 3 times with PBS, cells were mounted with fluorescent mounting medium. Images were acquired using a Zeiss epi-fluorescence microscope.

Determination of the platelet anti-aggregatory effect of ECs

Platelets isolated and suspended in Tyrode buffer at 310,000 platelets/µl from blood of healthy donors were obtained from the Etablissement Français du Sang-Alsace (Strasbourg, France). Suspensions of platelets (450 µl) were incubated into a cuvette with stirring at 37 °C in an aggregometer (Chronolog 490, Diagnostica Stago SAS, Asnière sur Seine, France). ECs were cultured on Cytodex 3 microcarrier beads before the addition of human plasma in serum-free culture medium for 24 h. In some experiments, ECs were pretreated with either a combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α or empagliflozin (100 nM) for 1 h or 30 min respectively before the addition of human plasma. ECs on Cytodex 3 beads (about 500 cells) were added to suspensions of platelets for 1 min before the addition of bradykinin (100 nM) to stimulate the endothelial formation of NO for 1 min. In some experiments, ECs were pretreated with N^G-nitro-L-arginine (NLA, 300 µM) for 30 min following the 24 h stimulation with the COVID-19 plasma. Thereafter, a thromboxane A_2 analog (U46619) was added to stimulate platelet aggregation to about 70-90%.

Determination of platelet adhesion to ECs

Platelets isolated, stained using Oregon green and suspended in Tyrode buffer at 310,000 platelets/µl from blood of healthy donors were obtained from the Etablissement Français du Sang-Alsace (Strasbourg, France). ECs were cultured on 96-well culture plates before the addition of human plasma in serum-free culture medium for 24 h. In some experiments, ECs were pretreated with either a combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α or empagliflozin (100 nM) for 1 h or 30 min respectively before the addition of human plasma. ECs were then washed with Tyrode buffer for 3X and then 100 µl of platelet solution was added and allowed to adhere for 30 min

at 37 °C in a cell-culture hood supplemented with 5 % CO_2. The plate was then read using plate reader (excitation: 498 nm; emission: 526 nm) to evaluate total fluorescence, thereafter washed using Tyrode buffer and then read again to determine adhesion percentage.

Determination of vWF secretion by ECs

ECs were cultured on 48-well culture plates before the addition of human plasma in serum-free culture medium for 24 h. In some experiments, ECs were pretreated with either a combination of three neutralizing Abs directed against IL-1β, IL-6 and TNF-α or empagliflozin (100 nM) for 1 h or 30 min respectively before the addition of human plasma. Thereafter, the supernatant was collected and assayed using vWF ELISA Kit (Cusabio; # CSB-E09395p) as per supplier recommendation.

Determination of generation of thrombin on the ECs surface

ECs were cultured on 24-well plates before being exposed to human plasma in serum-free culture medium for 24 h. In some wells, ECs were exposed to either a combination of neutralizing Abs directed against IL-1β, IL-6 and TNF-α or empagliflozin (100 nM) for 1 h or 30 min respectively before the addition of human plasma. Thereafter, ECs were washed with calcium free HEPES buffered solution before being incubated with HEPES buffered solution containing 20% of sodium citrate-treated pooled plasma of healthy volunteers at 37 °C. The generation of thrombin in the incubation medium of ECs was initiated by the addition of calcium (16.7 mM). To determine the role of tissue factor, a neutralizing Ab directed against TF (10 µg/ml, Merck Millipore, 612161) was added to the incubation medium of ECs before the addition of calcium. Thereafter, aliquots were taken and the generation of thrombin was determined using the chromogenic substrate (ß-Ala-Gly-Arg *p*-nitroanilide diacetate; Sigma-Aldrich) as recommended. The role of the serine proteases thrombin and factor Xa was assessed using dabigatran (10 µM) and rivaroxaban (10 µM), respectively.

Determination of ECs viability

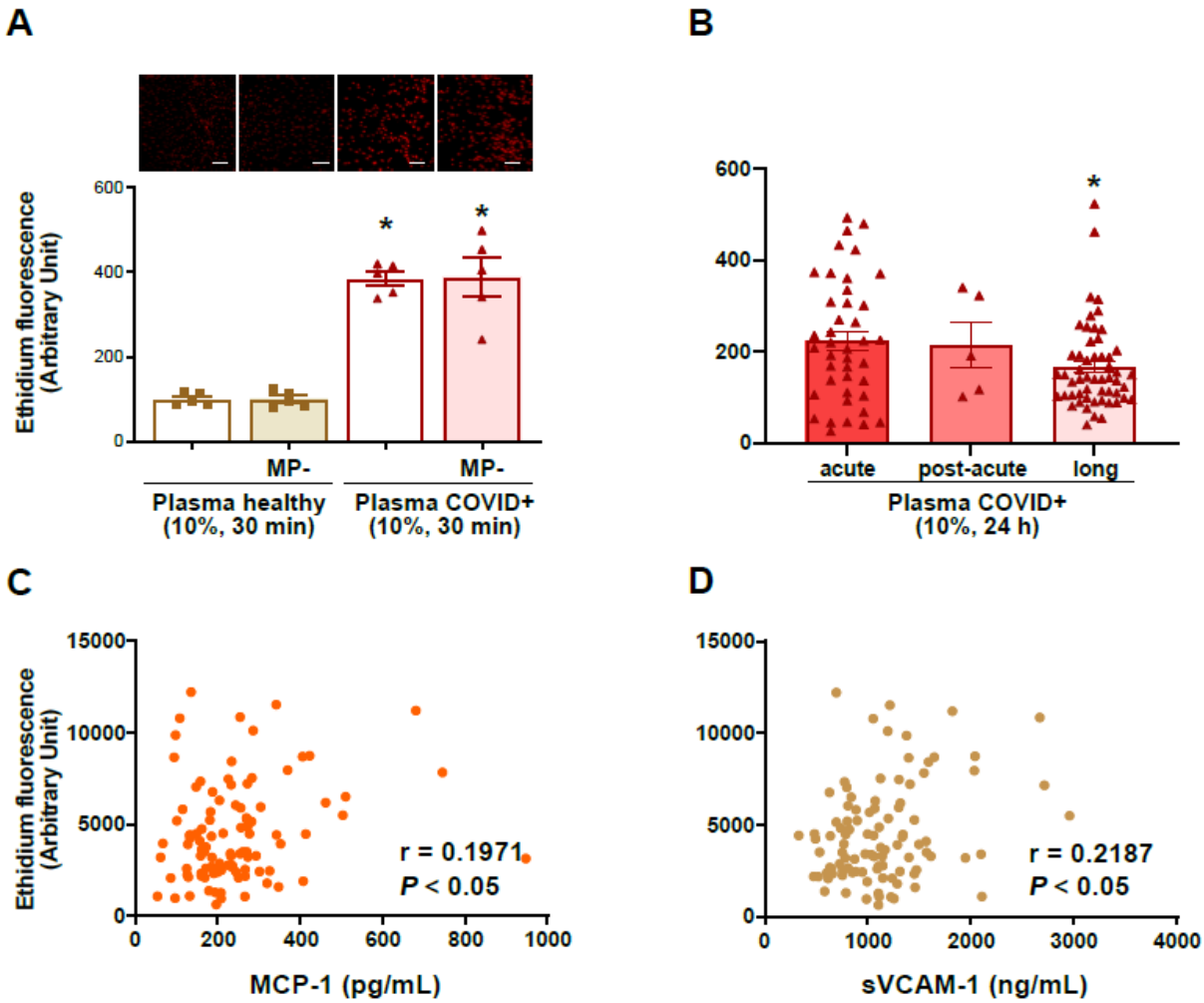
ECs were cultured on 96-well culture plates before the addition of human plasma in serum-free culture medium for 24 h. In some wells, SGLT2 specific siRNA or empagliflozin (100 nM) was added for 48 h and 30 min respectively before the addition of human plasma. Thereafter, cell viability was assayed using MTT assay kit (abcam, ab211091) as recommended by the supplier.

Statistical analysis

For patient characteristics, categorical variables are represented as frequencies and percentages, while continuous variables are expressed as median and inter-quartile values. Fischer exact or χ^2 tests were used to analyze categorical variables. Continuous variable comparisons were performed using Wilcoxon test (for two groups) or by Kruskal-Wallis test (for three groups). For *in vitro* experiments, normality of data was assessed using the Kolmogorov-Smirnov test. Non-normally distributed variables are presented as median and inter-quartile values, whereas normally distributed variables are presented as mean $\pm$ standard error of the mean. To compare normally distributed parameters, either one or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison were used. When data were not normally distributed, non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison was used. JMP 13 software® (SAS Institute, Cary, NC) and GraphPad Prism software (Version 7 for Windows, GraphPad Software Inc., San Diego, CA, USA) were used to perform all statistical analysis. Differences were considered significant at $P < 0.05$.

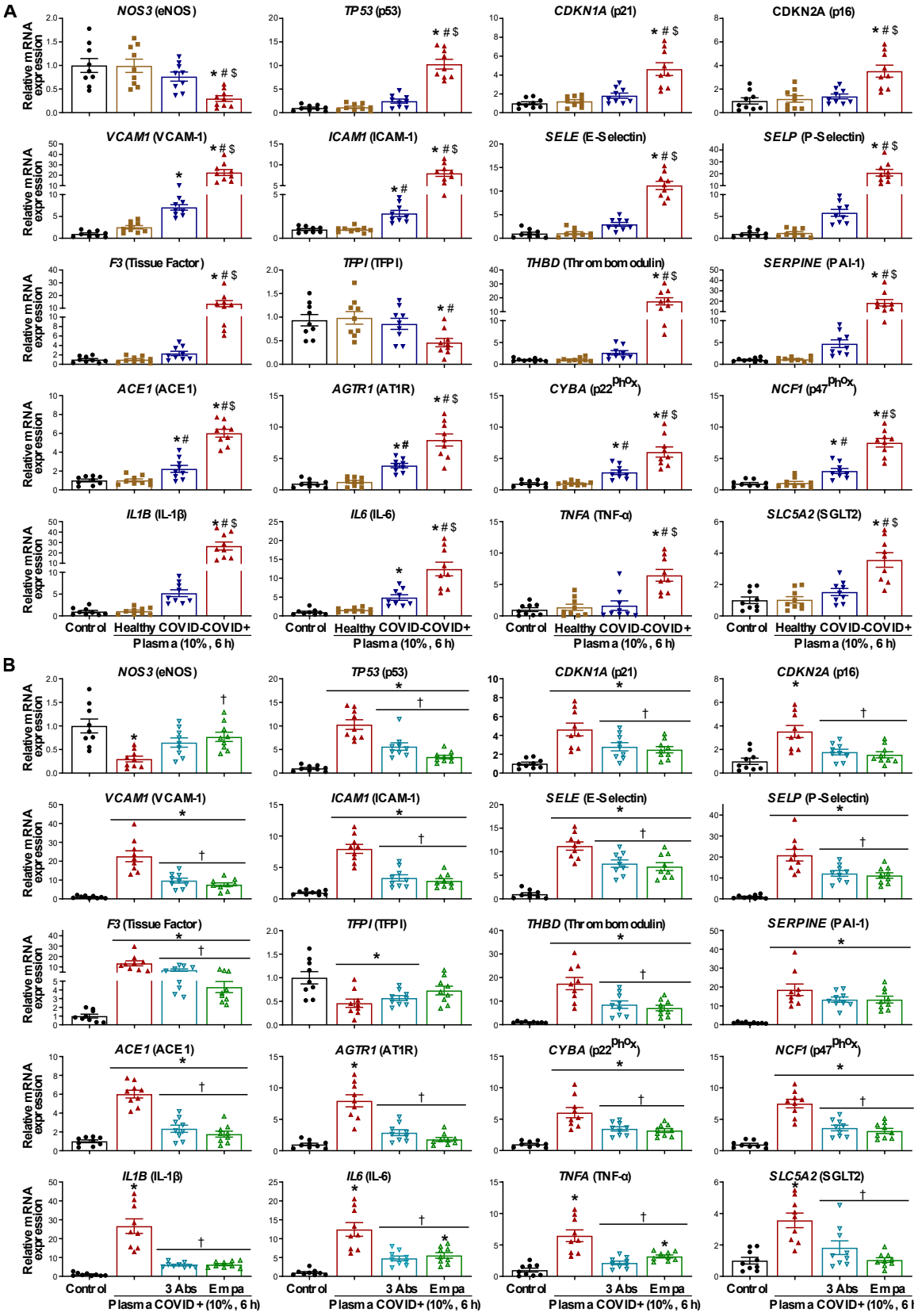
II. Supplementary Figures and Tables

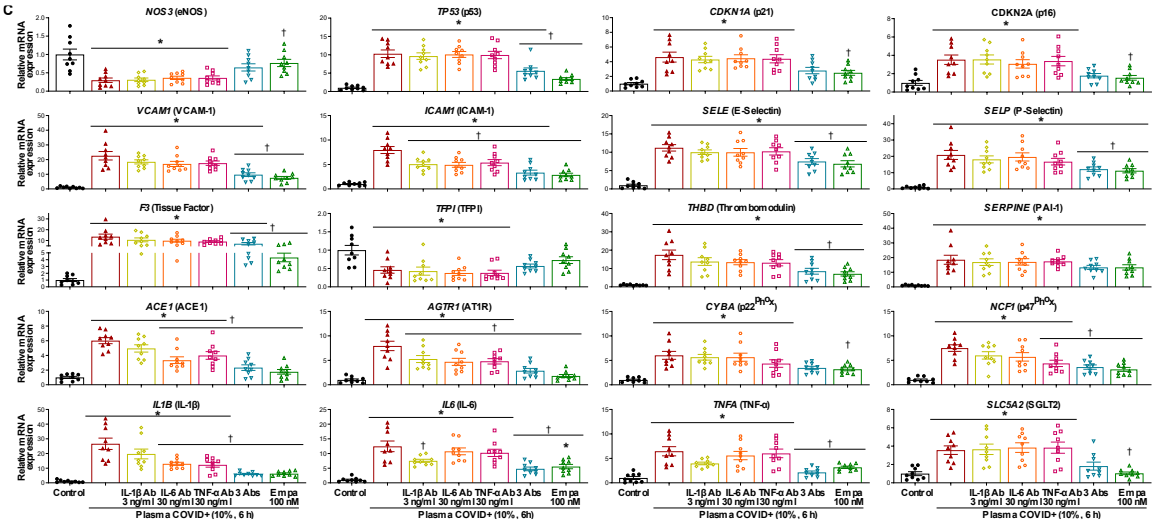
Figure S1 Role of microparticles, plasma sampling time, MCP-1 and sVCAM-1 in COVID-19 plasma induced pro-oxidant response of endothelial cells.



Data are presented as (A, B) mean ± SEM (n = 5-54) or (C, D) correlation graphs (n = 100). *$P < 0.05$ vs. Healthy volunteers plasma (A) or acute COVID+ plasma (B) treated ECs using one-way ANOVA followed by Tukey's multiple comparison test (A, B) or using Spearman correlation test (C, D). MP, microparticles.

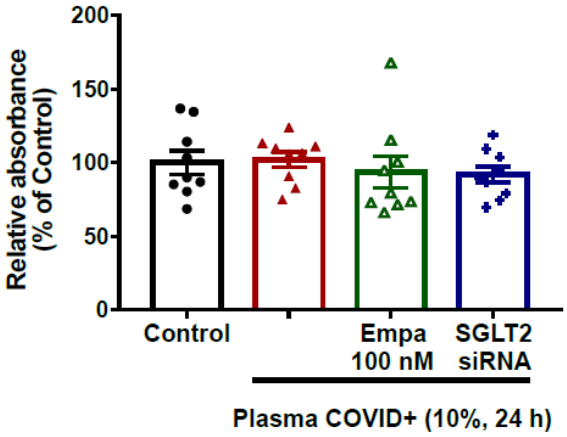
Figure S2 Pro-inflammatory genes expression profile of COVID-19 plasma treated endothelial cells: Role of pro-inflammatory cytokines and SGLT2





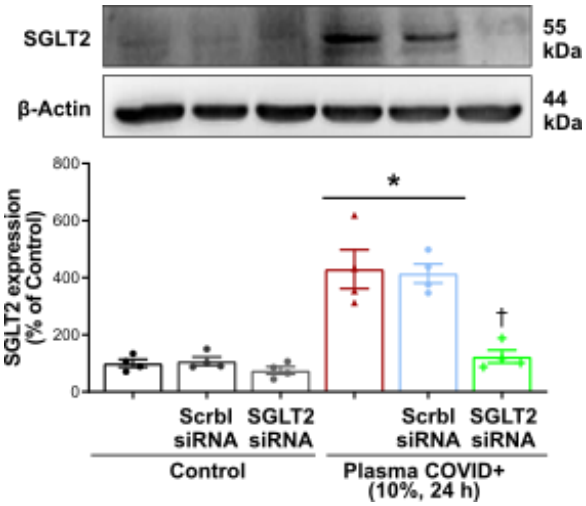
Data are presented as mean ± SEM (n = 9). *$P < 0.05$ vs. control ECs, $^{\#}P < 0.05$ vs. Healthy volunteers, $^{\$}P < 0.05$ vs. COVID- patients, $^{\dagger}P < 0.05$ vs. COVID+ patients using one-way ANOVA followed by Tukey's multiple comparison test. Three Abs, combination of IL-1β Ab, IL-6 Ab and TNF-α Ab; Empa, empagliflozin.

Figure S3 COVID-19 plasma, siRNA mediated SGLT2 knockdown and empagliflozin have no effect on ECs viability**.**



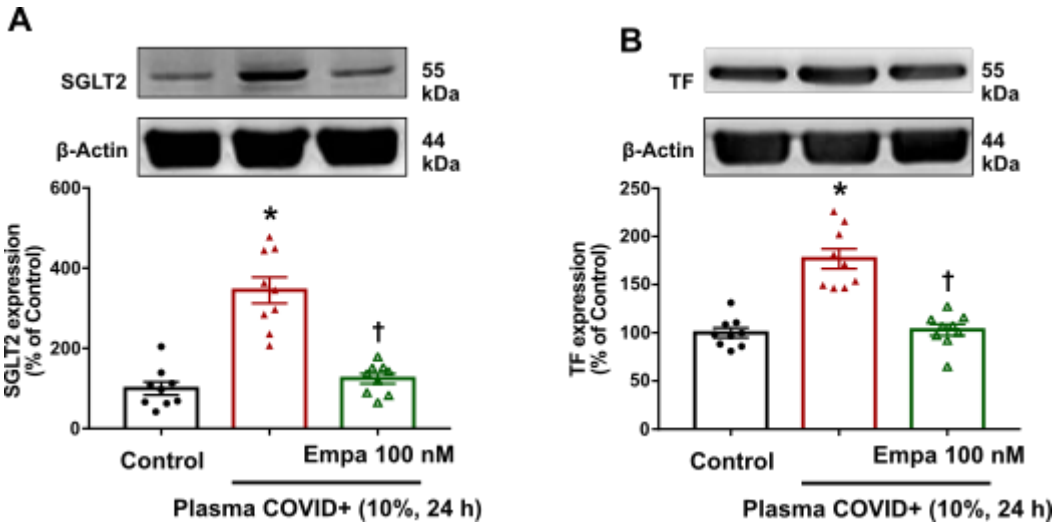
Data are presented as mean ± SEM (n = 9).

Figure S4 Upregulation of SGLT2 protein by COVID-19 plasma in endothelial cells is prevented by knock down of SGLT2 by siRNA whereas scrambled siRNA was inactive.



Data are presented as mean ± SEM (n = 4). *$P < 0.05$ vs. control ECs, $^{\dagger}P < 0.05$ vs. COVID+ plasma using one-way ANOVA followed by Tukey's multiple comparison test.

Figure S5 Upregulation of SGLT2 and TF proteins by COVID-19 plasma in endothelial cells is prevented by simultaneous addition of empagliflozin.



Data are presented as mean ± SEM (n = 9). *$P < 0.05$ vs. control ECs, $^{\dagger}P < 0.05$ vs. COVID+ plasma using one-way ANOVA followed by Tukey's multiple comparison test.

Table S1 Clinical characteristics of COVID-19 patients

Variable	COVID+ (n = 100)
Clinical symptoms	
Fever	90 (90)
Chest pain	8 (8)
Myalgia	41 (41)
Dyspnea	94 (94)
Cough	68 (68)
Anosmia / Ageusia	11 (11)
Digestive symptoms	27 (27)
Positive RT-PCR for SARS-CoV-2	100 (100)
CT severity (n = 92)	
Minimal (<10%)	2/92 (2)
Moderate (10 to 25%)	32/92 (35)
Severe (25 to 50%)	28/92 (30)
Critical (>50%)	30/92 (33)
Laboratory tests	
White blood cell, 10^3 /mm^3	6.9 (5.5 - 8.3)
Lymphocyte, 10^3 /mm^3	1.9 (1.4 - 2.5)
Hemoglobin, g/dl	13.2 (11.9 - 14.6)
Platelet, 10^3 /mm^3	265 (213 - 318)
Creatinine, µmol/l	70.3 (60.2 - 85.2)
eGFR, mL/min/1.73m^2	93 (72 - 106)
CRP, mg/l	4.0 (4.0 - 9.6)
PT-INR	1.06 (1.01 - 1.12)
APTT, sec	34.0 (34.0 - 37.4)
Fibrinogen, g/l	3.8 (3.1 - 4.7)
D-dimer, µg/l	370 (270 - 620)
von Willebrand factor antigen, %	175 (121 - 258)
Treatments	
Antiviral drugs	30 (30)
Antibiotics	67 (67)
Corticosteroids	45 (45)
Oxygen	99 (99)
Anticoagulant therapy	
No anticoagulation	11 (11)
Preventive anticoagulation	13 (13)
Reinforced anticoagulation	42 (42)
Therapeutic anticoagulation	34 (34)

In-hospital events	
Venous thromboembolism	20 (20)
PE	16 (16)
DVT	7 (7)
ICU transfer	61 (61)
Mechanical ventilation	48 (48)
Death	2 (2)
Length of hospital stay, days	20 (9 - 37)
Symptoms at follow-up (n = 87)*	
Long COVID (patients with symptoms)	48/87 (55)
Dyspnea	31/87 (36)
Chest pain	0/87 (0)
Palpitation	0/87 (0)
Fatigue	22/87 (25)
Cough	2/87 (2)
Anosmia / Ageusia	2/87 (2)
Neurologic symptoms	11/87 (13)
Digestive symptoms	0/87 (0)
Fever	1/87 (1)

Values are presented as n (%), n/N (%) or median (interquartile range). APTT, activated partial thromboplastin time; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; CT, computed tomography; DVT, deep vein thrombosis; eGFR, estimated glomerular filtration rate; ICU, intensive care unit; PE, pulmonary embolism; PT-INR, prothrombin time-international normalized ratio; RT-PCR, reverse transcriptase-polymerase chain reaction; SARS-CoV, severe acute respiratory syndrome coronavirus. *Median follow-up of 166 days.

Table S2 Circulating cytokines, cytoadhesins and DHE values in COVID-19 patients stratified in acute, sub-acute and long COVID-19.

Variable	Acute (n=41)	Sub-acute (n=5)	Long COVID-19 (n=54)	*P* (Long vs acute)	*P* (Long vs sub-acute)
IL-1β, pg/mL	5 (4-9)	8 (5.5-10)	5 (4-6)	0.173	0.649
IL-6, pg/mL	11 (2.5-60)	7 (2-132)	3 (2-4)	<0.001	0.037
TNF-α, pg/mL	7 (2.5-16)	7 (6.5-13)	9.5 (7-14.25)	0.165	0.500
MCP-1, pg/mL	233 (152-284)	283 (217-392)	201 (159-262)	0.397	0.259
sVCAM-1, ng/mL	1255 (1007-1555)	1123 (854-1688)	877 (719-1151)	<0.001	0.176
sICAM-1, pg/mL	2789 (2718-2848)	2673 (2205-2736)	3284 (2319-4501)	0.007	0.460
DHE staining (AU)	4871 (2515-7206)	4467 (2557-7743)	3284 (2319-4501)	0.014	0.335

Values are presented as median interquartile range. COVID, coronavirus disease; IL, interleukin; MCP, monocyte chemoattractant protein; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; TNF, tumor necrosis factor; DHE, dihydroethidium.

Table S3 Clinical characteristics of COVID-19 patients with low and high cytokine levels.

Variable	Low cytokines (n = 91)	High cytokines (n = 9)	*P* value
Age, years	61 (53 - 70)	63 (47 - 75)	0.79
Male	57 (63)	7 (78)	0.48
Body mass index, kg/m^2	26.4 (24.1 - 30.2)	27.1 (26.2 - 33.2)	0.30
Hypertension	39 (43)	3 (33)	0.73
Diabetes mellitus	13 (14)	3 (33)	0.15
Dyslipidemia	22 (24)	1 (11)	0.68
Obesity (BMI ≥ 30kg/m^2)	24 (26)	2 (22)	1.00
Current smoker	8 (9)	1 (11)	0.59
Family history of CAD	0 (0)	0 (0)	-
CKD (Cr > 150μmol/L)	5 (5)	0 (0)	1.00
COPD	7 (8)	2 (22)	0.18
Stroke	3 (3)	0 (0)	1.00
Atrial fibrillation	2 (2)	1 (11)	0.25
CAD	9 (10)	3 (33)	0.07
Heart failure	4 (4)	0 (0)	1.00
Peripheral artery disease	2 (2)	0 (0)	1.00
Previous PE	2 (2)	0 (0)	1.00
Previous DVT	2 (2)	0 (0)	1.00
History of cancer	7 (8)	1 (11)	0.54
Medications at baseline			
Antiplatelet therapy	13 (14)	2 (22)	0.62
Anticoagulants	0 (0)	0 (0)	-
ACE inhibitors	11 (12)	1 (11)	1.00
ARBs	13 (14)	1 (11)	1.00
MRAs	2 (2)	0 (0)	1.00
Beta-blockers	18 (20)	3 (33)	0.39
Statins	17 (19)	1 (11)	1.00
Ca-blockers	13 (14)	2 (22)	0.62
Diuretics	5 (5)	1 (11)	0.44
Metformin	5 (5)	2 (22)	0.12
Clinical symptoms			
Fever	82 (90)	8 (89)	1.00
Chest pain	7 (8)	1 (11)	0.54
Myalgia	39 (43)	2 (22)	0.30
Dyspnea	85 (93)	9 (100)	1.00
Cough	65 (71)	3 (33)	0.03
Anosmia / Ageusia	10 (11)	1 (11)	1.00

Digestive symptoms	24 (26)	3 (33)	0.70
Positive RT-PCR for SARS-CoV-2	91 (100)	9 (100)	-
CT severity (n = 92)			0.47
Minimal (<10%)	2/83 (2)	0/9 (0)	
Moderate (10 to 25%)	28/83 (34)	4/9 (44)	
Severe (25 to 50%)	24/83 (29)	4/9 (44)	
Critical (>50%)	29/83 (35)	1/9 (11)	
Laboratory tests			
White blood cell, 10^3 /mm^3	6.9 (5.5 - 8.3)	6.2 (5.2 - 8.2)	0.48
Lymphocyte, 10^3 /mm^3	2.0 (1.5 - 2.5)	1.7 (1.0 - 2.1)	0.09
Hemoglobin, g/dl	13.2 (11.9 - 14.5)	13.7 (12.2 - 15.1)	0.60
Platelet, 10^3 /mm^3	265 (216 - 316)	251 (182 - 328)	0.67
Creatinine, µmol/l	71.6 (60.8 - 85.3)	69.5 (51.9 - 85.2)	0.96
eGFR, mL/min/1.73m^2	92 (71 - 106)	94 (71 - 112)	0.93
CRP, mg/l	4.0 (4.0 - 9.3)	7.6 (4.0 - 35.3)	0.20
PT-INR	1.06 (1.01 - 1.11)	1.08 (1.00 - 1.15)	0.82
APTT, sec	34.0 (34.0 - 37.4)	34.0 (34.0 - 40.8)	0.87
Fibrinogen, g/l	3.8 (3.1 - 4.7)	3.8 (3.4 - 5.9)	0.46
D-dimer, µg/l	345 (270 - 605)	510 (415 - 750)	0.11
von Willebrand factor antigen, %	158 (121 - 231)	305 (200 - 406)	0.01
IL-1β, pg/ml	4.8 (3.9 - 6.0)	14.3 (10.5 - 32.1)	<0.001
IL-6, pg/ml	3.0 (1.9 - 6.6)	28.4 (16.8 - 60.8)	<0.001
TNF-α, pg/ml	8.0 (5.7 - 13.1)	19.4 (16.4 - 28.4)	<0.001
MCP-1, pg/ml	208.6 (160.4 - 271.0)	285.7 (121.8 - 414.4)	0.28
sVCAM-1, ng/ml	1068.4 (780.8 - 1312.2)	1375.9 (1123.2 - 1931.1)	0.02
sICAM-1, pg/ml	2777.4 (2610.6 - 2846.6)	2713.6 (2514.9 - 2799.5)	0.39
Time from positive RT-PCR to bleed sampling, days	125 (7 - 165)	12 (5 - 85)	0.16
Treatments			
Antiviral drugs	29 (32)	1 (11)	0.27
Antibiotics	63 (69)	4 (44)	0.15
Corticosteroids	38 (42)	7 (78)	0.07
Oxygen	90 (99)	9 (100)	1.00
Anticoagulant therapy			
No anticoagulation	10 (11)	1 (11)	1.00
Preventive anticoagulation	13 (14)	0 (0)	0.60
Reinforced anticoagulation	35 (38)	7 (78)	0.03
Therapeutic anticoagulation	33 (36)	1 (11)	0.16
In-hospital events			
Venous thromboembolism	19 (21)	1 (11)	0.68
PE	15 (16)	1 (11)	1.00

DVT	7 (0)	0 (0)	1.00
ICU transfer	57 (63)	4 (44)	0.31
Mechanical ventilation	45 (49)	3 (33)	0.49
Death	2 (2)	0 (0)	1.00
Length of hospital stay, days	23 (9 - 37)	12 (6 - 19)	0.045
Symptoms at follow-up (n = 87)*			
Long COVID (patients with symptoms)	44/81 (54)	4/6 (67)	0.69
Dyspnea	27/81 (33)	4/6 (67)	0.18
Chest pain	0/81 (0)	0/6 (0)	-
Palpitation	0/81 (0)	0/6 (0)	-
Fatigue	20/81 (25)	2/6 (33)	0.64
Cough	2/81 (2)	0/6 (0)	1.00
Anosmia / Ageusia	2/81 (2)	0/6 (0)	1.00
Neurologic symptoms	11/81 (14)	0/6 (0)	1.00
Digestive symptoms	0/81 (0)	0/6 (0)	-
Fever	1/81 (1)	0/6 (0)	1.00

Values are presented as n (%), n/N (%) or median (interquartile range). ACE, angiotensin-converting enzyme; APTT, activated partial thromboplastin time; ARB, angiotensin II receptor blocker; BMI, body mass index; CAD, coronary artery disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; Cr, creatinine; CRP, C-reactive protein; CT, computed tomography; DVT, deep vein thrombosis; eGFR, estimated glomerular filtration rate; ICU, intensive care unit; IL, interleukin; MCP, monocyte chemoattractant protein; MRA, mineralocorticoid receptor antagonist; PE, pulmonary embolism; PT-INR, prothrombin time-international normalized ratio; RT-PCR, reverse transcriptase-polymerase chain reaction; SARS-CoV, severe acute respiratory syndrome coronavirus; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; TNF, tumor necrosis factor. *Median follow-up of 166 days.

Concluding Remarks of Part II

In the current work we showed that:

- Plasma from COVID-19 patients promote variable levels of endothelial oxidative stress depending on the pro-inflammatory cytokine profile.
- Stimulation of ECs with COVID-19 plasma containing elevated levels of pro-inflammatory cytokines induce endothelial expression of pro-inflammatory, pro-oxidant and pro-thrombotic markers in addition to elevated levels of SGLT2.
- Inhibition of SGLT2 prevents COVID-19 plasma induced endothelial dysfunction and thrombogenicity evident by restored NO formation, lower levels of vWF secretion, reduced platelet adhesion and blunted TF-dependent thrombin generation at the surface of ECs.

Remarques finales

Dans le cadre des travaux actuels nous avons démontré que:

- le plasma de patients atteints du COVID-19 favorise des niveaux de stress oxydatif endothélial variables selon le profil des cytokines pro-inflammatoires.
- la stimulation des CEs avec du plasma COVID-19 contenant de niveaux élevés de cytokines pro-inflammatoires entraîne l'expression endothélial de marqueurs pro-inflammatoires, pro-oxydants et pro-thrombotiques en complément de niveaux élevés de SGLT2.
- l'inhibition des SGLT2 prévient le dysfonctionnement endothélial provoqué par le plasma COVID-19 ainsi que la thrombogénèse par une production de NO restaurée, de niveaux de sécrétion de vWF diminués, une adhérence et agrégation plaquettaires diminuées ainsi qu'une production de thrombine TF-dépendante atténuée à la surface des CE.

DISCUSSION

I. SGLT2 Expression in Human Vasculature Correlates with the in situ Inflammatory and Oxidative State

CAD is one of the leading causes of mortality worldwide and a major risk factor for several other CVDs including HF. Treatment regimens applied in CAD depends on the severity of the disease and include lifestyle modifications, medical treatments and eventually clinical interventions. While PCI, a non-invasive clinical intervention, is being more widely used for CAD management particularly in patients with elevated risk for surgical intervention, CABG, the invasive surgical intervention for CAD, remains a relevant approach **(165)**. In fact, CABG is indicated over PCI in several conditions including but not limited to > 50% stenosis of the left main CA, > 70% stenosis of two CAs if one of which is left anterior descending or > 70% stenosis of three CAs. Additionally, CABG is the target interventional approach in case PCI failed to alleviate myocardial ischemia **(165,166)**.

Several vascular conduits are used in CABG including internal thoracic artery (ITA), saphenous vein, radial artery, gastroepiploic artery, ulnar artery, splenic artery and inferior epigastric artery. ITA is one of the most used and considered as the best conduit **(167)**. The attraction of ITA resides in its higher resistance to the development of

atherosclerotic lesions as compared to other used conduits **(168)**. However, and despite absence of phenotypic clinical changes in ITA, the subclinical condition and inter-individual variabilities of these vital conduits have not been well studied. In our current work, we analyzed 70 ITA specimens obtained from patients with atherosclerotic CAD undergoing CABG.

Importantly, and despite the homogeneity of the patient population (all having coronary atherosclerosis), our gene expression analyses of ITA did not homogenous results. In fact, inflammatory markers including genes of pro-inflammatory cytokines (*IL1B*, *IL6*, *TNFA*), macrophage surface marker (*CD68*) and monocyte chemoattractant protein (*CCL2*) showed pertinent variabilities reaching > 50-fold differences for certain markers. At the protein level, similar results were observed where the activation of the pro-inflammatory transcription factor NF-κB evident by the phosphorylation of the p65 subunit demonstrated > 100-fold differences between ITA specimens obtained from different patients. These observations are of particular interest since atherosclerosis itself is considered as an inflammatory disease underlying endothelial dysfunction and lesions **(169)**. Similar to the pro-inflammatory profile, gene expression pattern of both SGLT isoforms SGLT1 and SGLT2 (*SLC5A1* and *SLC5A2*) showed increases reaching > 30-fold differences, and expression amplitude of both isoforms were correlated at both mRNA and protein levels. Interestingly, mRNA levels of

SGLT2 showed significant positive correlation with levels of the afore-mentioned pro-inflammatory cytokines, markers of oxidative stress, RAAS activation, and endothelial senescence while being inversely correlated with that of eNOS (*NOS3*); all of which hint to elevated SGLT2 mRNA expression in condition of endothelial inflammation dysfunction. Additionally, protein expression of SGLT2 in ITA showed significant positive correlation with that of p-p65 and localized to areas of inflammation and macrophage infiltration as evident by the immunostaining of TNF-α and CD68 respectively. Further, SGLT2 was shown to be expressed in both vascular ECs and SMCs. Moreover, elevated protein levels of SGLT2 were associated with elevated *in situ* formation of ROS which was driven by the activation of local inflammatory machinery (TNF-α) and components of the AT1R/NADPH oxidases pro-oxidant pathway. While all the previously mentioned observations do not conclude a cause-effect relationship between local inflammation, SGLT2 expression and vascular endothelial dysfunction, yet these observations remain of importance since they demonstrate that the subclinical condition of ITA can vary depending on *in situ* activation of diverse cellular mechanisms. Additionally, they prove that SGLT2 can be expressed in human vasculature which is in line with the observations demonstrating presence of SGLT2 in inflamed human plaques **(170)**. However, the novelty of our results resides in the identification of SGLT2 expression

pattern in sub-clinical pathological conditions and in the absence of atherosclerosis since ITA is thought to be resilient to atherosclerotic plaque formation **(171)**. Furthermore, they hint out a possible role of SGLT2 in promoting oxidative damage in human vasculature as evident by the significant decrease of ROS formation following the inhibition of SGLT2.

II. In situ Low-Grade Inflammation Dictates the Expression of SGLT2 in Human Heart Promoting Hyper-Oxidative Conditions

Chronic low-grade inflammation has been widely described to be an initiating and or precipitating factor in several CVDs including ASCVD and HF **(172,173)**. The latter being a major risk for mortality in elderly, is as such widely studied in attempts to reduce its health burden. Anti-inflammatory approaches including colchicine (COLCOT and LODCOLT trials) and IL-1β inhibitor canakinumab (CANTOS trial) were shown to decrease cardiovascular mortality and hospitalization for HF respectively **(174-176)**. However, these studies demonstrated cardioprotective effects only in patients with prior MI. On the other hand, gliflozins (DAPA-HF, DELIVER, EMPEROR-Reduced, EMPEROR-Preserved, CANVAS) demonstrated significant

decrease in HF hospitalization and death of CVDs in wide array of patients including those with or without diabetes and in the presence or absence of previous MI incidence **(136, 141-144)**. The revolutionary cardiovascular outcomes with gliflozins covered the entire EF spectrum with an average reduction of > 20 % in HF hospitalization regardless of the studied molecule **(134-136,141-144,177-179)**. Additionally, there was no significant difference between different gliflozins in the prevention risk of MI, AF and stroke development in patients with T2DM **(140)**. These results point out a cardiovascular class effect of the gliflozin family yet without a clear mechanism explaining them.

Considering the absence of robust data supporting SGLT2 expression in human cardiac tissues, the class-effect of gliflozins was rather affiliated to either systemic or off-target cardiac effects of the drug **(180)**. In contrast, our results prove otherwise. In our current study we analyzed 20 human LV biopsies from patients undergoing aortic valve surgery due to aortic stenosis, a major risk factor for HF development **(181)**. Surprisingly, SGLT2 mRNA expression was detected in all the 20 specimens however with huge variability reaching > 20-fold increase among the studied biopsies. Of importance, SGLT2 mRNA levels correlated positively and significantly with those of pro-inflammatory cytokines, macrophage surface markers, components of the AT1R/NADPH oxidases pro-oxidant pathway and endothelial activation and senescence. These gene expression patterns were similar

to those observed at the protein level where SGLT2 protein expression was significantly correlated to inflammatory pathways (p-p65, COX2/COX1), pro-inflammatory macrophage polarization (M1/M2), AT1R and NOX1 of the AT1R/NADPH oxidases pro-oxidant pathway and VCAM-1. Additionally, SGLT2 was shown to be elevated in specimens demonstrating markers of blunted NO-mediated endothelial protection as evident by increased nitrotyrosine and reduced eNOS levels. These particular specimens as compared to others generated higher levels of ROS which was sensitive to the inhibition of TNF-α, AT1R, NADPH oxidases and SGLT2. Such results suggest that SGLT2 expression in human cardiac tissue despite being questionable during the recent years, plays a pivotal role in oxidative stress propagation and possibly CVDs development in response to inflammatory stimulation. Additionally, they could provide an explanation to the reduced inflammatory and oxidative stress levels observed in human myocardium following SGLT2i treatment as SGLT2 expression in these tissues would underlie elevated inflammatory and oxidative activation as shown by our results **(182)**. However, whether SGLT2 induction is due to local inflammatory activation, systemic chronic low-grade inflammation or both cannot be addressed since plasma samples of those patients were not studied. Further, the concomitant expression of SGLT2 with components of the RAAS could as well point out the contribution of the co-transporter to the propagation of the

remodeling and hypertrophic changes induced by this system, where local activation of the AT1R is one of the initiating signals for cardiac hypertrophy **(183)**. Of interest, our data also showed that SGLT2 expression in LV biopsies localized to areas of inflammation and macrophage infiltration as evident by immunostaining, which as well revealed SGLT2 expression in microvascular endothelium and CMs. These results support the recently published data demonstrating SGLT2 expression in human CMs of transplanted heart biopsies in patients with T2DM **(184)**.

Alternatively, our data from human LV biopsies came different to those observed in ITA with regards to SGLT1 expression. For instance, SGLT1 expression in LV specimens showed a relatively uniform distribution, which is in line to the inherent expression of SGLT1 in cardiac tissue reported in the literature **(185)**. However, SGLT1 expression did not show significant correlation to that of SGTL2 or any of the afore-mentioned markers. These observations could be explained by the fact that SGLT1 is a low-capacity transporter and as such its overexpression in a highly energy-demanding tissue as the heart would not necessarily lead to relevant glucose supply. In addition, with the absence of selective SGLT1 inhibitors in the market and the non-significant difference in reducing all-cause mortality, CV death and HF hospitalization between the dual SGLT1 and SGLT2 inhibitor sotagliflozin and the more selective SGLT2 inhibitors dapagliflozin and

empagliflozin **(160)**, these preliminary observations would as well question to which extent the cardioprotective effects of the dual inhibitor are SGLT1 and/or SGLT2 dependent.

III. Pro-Inflammatory Cytokines Induce Redox-Sensitive Expression of SGLT2 in ECs Leading To ED

Inflammation-induced ED is described extensively in the literature. In fact, endothelial structure, localization and multifunctional role make it prone to inflammatory stimulants which promote changes in the properties of endothelial tissue **(186, 187)**. On the other hand, ED is now well perceived as an underlying risk factor for multiple CVDs including ASCVD, HF, AF and others **(188-191)**. In our *in vitro* model we induced ED using three different pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). The evidence of ED was through changes in ECs marker expression including reduced eNOS expression level **(192, 193)**. Interestingly, the inflammatory-induced endothelial transformation from health to disease was associated with increased expression levels of both SGLT1 and SGLT2 and as such, this work might be the first to report direct induction of SGLT1 and SGLT2 by pro-inflammatory cytokines in ECs. Additionally, pro-inflammatory cytokines induced the expression of both ACE1 and AT1R, both of

which have pro-oxidant characteristics as reported by several other studies **(194, 195)**. The endothelial transformation induced by the pro-inflammatory cytokines, particularly TNF-α, included as well significant increases in intracellular oxidative stress, one of the hall marks of ED **(194-196)**. Interestingly, the oxidative stress was sensitive to inhibitors of the AT1R/NADPH oxidases pathway, which could also prevent SGLT1 and SGLT2 over-expression in response to TNF-α. These results further support previous studies of our team in which it was shown that SGLT1 and SGLT2 are both redox-sensitive and could be induced by the activation of any of the players of the AT1R/NADPH oxidases pro-oxidant pathway **(197-199)**.

The over-expression of SGLT1 and SGLT2 by ECs was in turn associated with several functional and structural changes. For instance, inhibiting either SGLT2 or both SGLT1 and SGLT2 using empagliflozin and sotagliflozin respectively could reduce inflammation-induced oxidative stress. These reductions in intracellular endothelial oxidative levels could be a result of the reduced glucose uptake demonstrated by both inhibitors in response to TNF-α, where increased intracellular glucose is indeed a potent inducer of oxidative stress **(197, 200)**. Additionally, the inhibitors could as well reduce TNF-α induced overexpression of the components of the AT1R/NADPH oxidases pro-oxidant pathway which is in line with the anti-oxidant effects seen with SGLT2i and could in turn provide and

additional explanation of the protective effects demonstrated by the inhibitors **(199, 201-203)**. Such observations could also explain the reduction of LV hypertrophy in patients with T2DM receiving SGLT2i treatment **(204, 205)**. In fact, and in addition to the pro-oxidant role of the AT1R/NADPH oxidases pathway, activation of the local angiotensin system is a potent inducer of remodeling signals including the promotion of TGF-β mediated cardiac hypertrophic phenotypes **(183)**.

Importantly, both sotagliflozin and empagliflozin prevented TNF-α induced overexpression of SGLT1 and SGLT2 which points out that the co-transporters could regulate their own redox-sensitive expression. To delineate the effects of SGLT1 from that of SGLT2, we used siRNA targeting either of the co-transporters. Of particular interest, knock-down of SGLT2 but not SGLT1 could prevent TNF-α induced endothelial dysfunction as evident by the reduction of TNF-α mediated over expression of VCAM-1 and downregulation of eNOS. As such, these results could be the first to differentiate between the role of SGLT1 and SGLT2 in ECs. Additionally, and based on the above-mentioned results we could point out the role of SGLT2 in particular as a key component of an AT1R/NADPH oxidases/SGLT2 pro-oxidant pathway, which once induced leads to a vicious cycle of intracellular oxidative stress that could promote the development and progression of inflammatory-induced CVDs **(206-208)**.

Besides oxidative stress, inhibition of SGLT2 could as well prevent TNF-α induced blunted NO formation by ECs. This observation is key since NO is well known to act in a paracrine way to enhance cardiomyocyte relaxation and improve myocardial blood supply through promoting coronary dilation **(209-211)**. As such these observations could be an explanation to the reported pre-clinical and clinical observations demonstrating improved diastolic dysfunction following SGLT2is treatment which is normally a consequence of reduced myocardial hypertrophy and improved cardiomyocyte stiffness **(199, 204, 212-214)**. In addition, they could also underlie the hypothesis that SGLT2i reduced cardiovascular events in patients with T2DM and CAD is due to improved coronary microvascular function as suggested by other studies **(215-217)**.

Further, the endothelial transformation mentioned above included the redox-sensitive activation of the pro-inflammatory transcription factor NF-κB as evident by the increased phosphorylation of the p65 subunit and the subsequent nuclear translocation of NF-κB. Such activation was indeed mitigated by inhibiting the components of the AT1R/NADPH oxidases/SGLT2 pathway to reduce endothelial inflammation. These results could support observations in several pre-clinical models. For instance, dapagliflozin showed inhibitory effects on NF-κB activation in a model of lipopolysaccharide-activated human umbilical vein endothelial cells **(218)**. Similarly, in rodent models of

diabetes and/or atherosclerosis, administration of SGLT2i was associated with reduced markers of cardiac inflammation. Additionally, administration of SGLT2i was associated with improved inflammatory profile in human myocardial tissue **(208, 219)**. On the other hand, inhibition of NF-κB nuclear translocation was associated with significant reduction in TNF-α induced elevation of SGLT2 mRNA expression. These data are to our knowledge the first to demonstrate the downstream regulation of SGLT2 by NF-κB in cardiac ECs and are in line with a previous study which showed that SGLT2 promoter in renal tubular epithelial cells contains a functional p65 binding site **(220)**.

Moreover, our results have shown that NF-κB-induced SGLT2 overexpression in ECs passes through the activation of the redox-sensitive protein kinases including PI3K/Akt, p38 MAPK and SAPK/JNK pathways where direct inhibition of any of those kinases reduced TNF-α induced SGLT2 over-expression in ECs. Previous data from our lab have demonstrated similar inductions of SGLT2 by PI3K/Akt and p38 MAPK following stimulation of ECs with microparticles isolated from CAD patients' blood **(221)**. These observations reinforce the role of SGLT2 at the interface of multiple dysfunctional endothelial activation pathways. Further, they identify SGLT2 as a primary consequence of inflammatory induction of endothelial oxidative stress from one side and as a major contributor to the propagation of the initial insult from another side.

IV. Cytokine Storm of Plasma from Acute, Sub-Acute and Long COVID-19 Patients Promotes Endotheliitis in an SGLT2-Dependent Manner

COVID-19 and despite being an acute respiratory distress syndrome, is as well associated with elevated incidence of CV complications and death due to CV reasons **(100-102,104)**. Several mechanisms have been proposed as an explanation of the observed clinical manifestations one of which is COVID-19 induced endotheliopathy. Such endothelial dysfunction could be promoted by multiple plasma derived factors including microparticles, viral antigens and indeed pro-inflammatory cytokines **(109, 222)**. For instance, COVID-19 is associated with a signature increase in several pro-inflammatory mediators including IL-1β and IL-6 **(223, 224)**. Similarly, our results from 100 plasma samples taken from patients at the acute (< 4 weeks), sub-acute (4-12 weeks) and chronic (> 12 weeks) phase of COVID-19 showed that the levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, chemokine MCP-1 and cell adhesion molecule ICAM-1 were all significantly elevated as compared to their levels in the plasma of patients with CVD or healthy donors. Interestingly, the elevated pro-inflammatory signal persisted beyond the acute phase which is consistent with previous studies **(224, 225)**. These results are in line with the observations demonstrating that

COVID-19 and in contrast to other previous coronavirus infections, is associated with prolonged inflammatory manifestations. This could explain the long-term increased risk of incident CVD in post-COVID-19 particularly in patients with the most severe infection even in the absence of previous CV manifestations **(224-228)**.

Alternatively, the incubation of ECs with the 100 plasma samples taken from COVID-19 patients was associated with elevated levels of intracellular ROS. These elevations were indeed correlated to the level of pro-inflammatory cytokines observed in the plasma and to lesser extent secreted cyto-adhesions. The inflammatory induction of endothelial oxidative stress is well perceived as a principle and is as such extensively investigated in COVID-19 **(229, 230)**. Similarly, when the samples with the highest pro-inflammatory score (samples that fit into the top quartile of IL-1β, IL-6 and TNF-α) were used to stimulate ECs, elevations in ROS generation exceeded that induced by the plasma taken from patients with CVD. The elevated oxidative stress was observed following both short- and long-term stimulation. Interestingly, while the short-term production of ROS was sensitive to inhibiting any of the three cytokines, long-term ROS generation could be partially prevented only by the concomitant inhibition of all the three. As such, these results point out the individual and synergistic role played by these cytokines to promote oxidative changes in ECs. Additionally, these results are in line with other study showing that

serum from diseased COVID-19 patients induced endothelial oxidative stress that was partially sensitive to the inhibition of TNFR in human endothelial cells **(231)**.

Besides the sensitivity of endothelial oxidative stress to the inflammatory signal, activation of the AT1R/NADPH oxidases axis was as well involved in both short- and long-term generation of ROS. The involvement of this pathway has indeed been described by our team in other models of endothelial dysfunction, such as H_2O_2 or high glucose induced **(197)**. In contrast, inhibition of SGLT2 was only effective following long-term stimulation of ECs with plasma of COVID-19 patients. This particular observation proves that the effect of the SGLT2i empagliflozin is not mediated through direct antioxidant activity but through inhibiting the activity of SGLT2 following its induction by the oxidative signal. Further, the inhibition of SGLT2i even subsequent to long-term induction of the pro-oxidant signal could rescue ECs from the sustained ROS generation, which was not the case with the use of the combination of the neutralizing antibodies or even inhibitors of the RAAS. Such result points out the central role played by SGLT2 in promoting endothelial injury in response to late pro-oxidant signal, which is a potent precipitating factor of ED **(229-232)**.

Besides oxidative stress, stimulation of ECs with the plasma from COVID-19 patients induced changes in the expression pattern of several genes that fall into specific categories. For instance,

upregulation of the ACE1/AT1R axis accompanied by NADPH oxidases subunits at the expense of reduced ACE2 although the latter did not reach significance. This diversion in the RAS system toward the pro-oxidant and pro-inflammatory ACE1 axis has been well described in the context of COVID-19. In fact, reports have suggested that ACE2 downregulation in CMs is associated with myocardial inflammation and damage. Further, another work concluded that restoring ACE/ACE2 balance could prevent COVID-19 associated morbidity and mortality **(233-238)**. In addition to promoting ACE1/AT1R activation, stimulation of ECs with COVID-19 plasma induced endothelial senescence phenotype as evident by the increased expression of the genes corresponding to p16, p21 and p53 proteins. Importantly, endothelial senescence has been widely studied in CVDs. For instance, endothelial senescence is thought to be a major inducer of aging related cardiovascular diseases **(172, 239)**.

Similarly, the inflammatory-induced changes in endothelial gene expression included the over-expression of CAMs (VCAM-1, ICAM-1) and selectins (e-Selectin, p-Selectin) similar to observation reported by other teams **(240)**. These cell surface markers play a pivotal role in modulating the endothelial layer permeability and affinity toward leukocytes. In fact, elevated plasma levels of cyto-adhesions were reported in patients with turbulent coronary flow **(241, 242)**. Cyto-adhesions have also showed pertinent role in promoting endothelial

inflammation during the development of atherosclerotic lesion **(243, 244)**.

Endothelial inflammation in turn was also an outcome of stimulating ECs with COVID-19 plasma as evident by the increased expression of mRNA corresponding to IL-1β, IL-6 and TNF-α. In line with that, a recent study reported elevated IL-1β production by ECs following stimulation with exosomes from COVID-19 patients **(245)**. Interestingly, this phenotypic change was as well associated with modulation of mRNA expression profile of several genes responsible of thrombotic responses including the up-regulation of TF and PAI-1 concomitant with down-regulation of TFPI. All the above-mentioned changes could explain the chronic long-term modifications promoted by COVID-19 leading to CV manifestations and possibly serve as an explanation for the observed fibrinogenesis-fibrinolysis imbalance in COVID-19 patients **(246, 247)**. The role of pro-inflammatory cytokines in driving these changes was tested as well through the usage of neutralizing antibodies directed against either IL-1β, IL-6 or TNF-α, or the joint inhibition of all 3 using a combination of the neutralizing antibodies. Importantly, the protective effect of single neutralizing antibody was much less than that observed with all 3 used together. Such results mimic the synergistic inhibitory effect observed on oxidative stress and point out the detrimental role of inflammatory stimulation in promoting oxidative-dependent endothelial dysfunction.

Additionally, they reinforce the role of the early inflammatory response associated with COVID-19 in priming endothelial changes that would later underlie the increased CV risk **(231-233, 248)**.

The role of SGLT2 in the context of the endothelial dysfunction induced by COVID-19 plasma was also investigated. In fact, mRNA expression of SGLT2 in ECs was significantly increased following stimulation with COVID-19 plasma and interestingly this increase was reduced by the combination of the 3 neutralizing antibodies and SGLT2i empagliflozin. Additionally, inhibiting SGLT2 did as well prevent the major modifications induced by COVID-19 plasma on ECs gene expression to a similar extent, if not exceeding in certain cases, as did the combination of the neutralizing antibodies. The reliance of SGLT2 expression on the inflammatory induction was also evident by the concentration response increases in SGLT2 protein expression in ECs by COVID-19 plasma. Similar observations were as well reported in another study showing that SGLT2 expression in cardiomyocytes increased following SARS-CoV-2 infection concomitant with increased pro-inflammatory cytokines **(249)**. Further, at the protein level, COVID-19 plasma promoted the activation of the pro-inflammatory transcription factor NF-κB, which was prevented by SGLT2i and the combination of the neutralizing antibodies as well. These data are in line with the anti-inflammatory effects of SGLT2i observed in vasculature **(208, 219, 250)**. Further, increased expression of VCAM-1

and TF proteins as well as reduced eNOS levels were observed as a consequence of ECs stimulation with COVID-19 plasma, all of which were prevented by the combination of the neutralizing antibodies and SGLT2i. Interestingly, the effects of the SGLT2i on the expression of the afore-mentioned proteins was mimicked by the usage of SGLT2 specific siRNA, These results provide a clear evidence of the detrimental role played by SGLT2 in promoting ED and strongly suggest that reported endothelial protective effects of SGLT2i could be at least in part due to direct inhibition of SGLT2 rather that off-target effects of the drugs **(251)**.

V. COVID-19-Induced Endotheliopathy is Associated with SGLT2-Mediated Increased Thrombogenicity

Several reports have pointed out the increased thrombotic events following COVID-19. The mechanism for increased thrombotic events is not fully clear yet could hold several underlying triggers including elevation in coagulation markers, chronic low-grade inflammation and indeed endotheliopathy **(252-255)**. One of the major endothelial released factors that controls blood fluidity is NO. In fact, reduced NO prevalence increases thrombotic formation at EC surface and has as well been reported in several CVDs **(256)**. In our work, we

demonstrated that COVID-19 induced endothelial dysfunction was associated with blunted NO formation, which was successfully attenuated with either the combination of the neutralizing antibodies or by inhibiting SGLT2. Similarly, studies have demonstrated increased vWF in COVID-19 patients, which serves as the anchor protein for primary platelet adhesion **(257-259)**. Our results came similar and showed that the blunted NO formation was accompanied by increased ECs secretion of vWF following stimulation with COVID-19 plasma. Importantly, this phenotypic change led to increased platelet adhesion to ECs surface, both of which were inhibited by either the combination of the neutralizing antibodies or by inhibiting SGLT2. Another consequence of reduced NO production by ECs was the loss of the anti-aggregatory effect following stimulation with COVID-19 plasma. This phenotype was as well reduced by the combination of the neutralizing antibodies or by inhibiting SGLT2, which could be of interest knowing that patients with COVID-19 had increased platelet activity **(260)**. On the other hand, SGLT2i has been indeed shown to reduce atherothrombotic events in patients with T2DM **(261, 262)**, however, their anti-thrombotic characteristics are poorly addressed in the context of COVID-19. In our study, we demonstrated that plasma from COVID-19 patients increased thrombin generation at the surface of ECs as a consequence of promoting increased TF expression and activity. Interestingly, relieving the initial consult using the

combination of the neutralizing antibodies or preventing the amplifying mediator through inhibiting SGLT2 both significantly reduced thrombin production.

CONCLUSION

In this study, we highlighted the pivotal role of low-grade inflammation in regulating SGLT2 expression in the human endothelium and vascular smooth muscle of ITA and demonstrated SGLT2 expression in the human coronary microcirculation and cardiomyocytes in LV areas affected by macrophage infiltration and low-grade inflammation, which in turn perpetuated a pro-oxidant activator signal leading to eNOS-NO/ROS imbalance. Additionally, we showed that pro-inflammatory cytokines and subclinical inflammation in patients with COVID-19, both induced a redox-sensitive upregulation of SGLT2 expression in coronary ECs. In turn, this process fueled endothelial inflammation, senescence, dysfunction, platelet adhesion and aggregation, and thrombin generation.

As such, our investigation contributes to a better understanding of the role of SGLT2 in the pathophysiology of CVDs and provides new insights into the beneficial effects of SGLT2is on the CVS observed in different patients such as those affected by HFrEF, HFpEF, MI, AF, T2DM and CKD, all pathologies characterized by ongoing inflammation. They further suggest that patients with low-grade inflammation may particularly benefit of SGLT2 inhibition in an attempt to optimize personalized medicine. Furthermore, with the

elevated athero-thrombotic events seen in COVID-19 patients and the prevalence of non-desired adverse effects accompanying anticoagulant therapies, the improved thrombotic responses demonstrated following the inhibition of SGLT2 could possibly point out an additional attractive novel strategy to restore endothelial homeostasis and prevent CV complications in patients with ongoing COVID-19 inflammation.

PERSPECTIVE

Beyond the protective role of SGLT2is in various CVDs, recent study has demonstrated reduced MACE and incident kidney disease in patients with systemic lupus erythematosus **(263)**. On the other hand, several clinical observational studies supported the usage of SGLT2is in cardio-oncology as these drugs demonstrated reduced cardiac toxicity evident by lower HF hospitalization, cardiomyopathy and cardiac arrhythmias in patients with cancer including lymphomas, hematomas, breast and gastrointestinal cancer **(264)**. As such, these observations pave the door to further investigate the underlying mechanisms responsible for the aforementioned outcomes and why not identifying notable contribution of SGLT2 in autoimmune diseases and tumorigenesis.

List of References

1- Krüger-Genge A, Blocki A, Franke RP, Jung F. Vascular Endothelial Cell Biology: An Update. *Int J Mol Sci*. **2019**;20(18):4411.

2- Cai Z, Gong Z, Li Z, Li L, Kong W. Vascular Extracellular Matrix Remodeling and Hypertension. *Antioxid Redox Signal*. **2021**;34(10):765-783.

3- Fu BM, Tarbell JM. Mechano-sensing and transduction by endothelial surface glycocalyx: composition, structure, and function. *Wiley Interdiscip Rev Syst Biol Med*. **2013**;5(3):381-90.

4- Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res*. **2007**;100(2):158-73.

5- Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circ Res*. **2007**;100(2):174-90.

6- Segers VFM, Brutsaert DL, De Keulenaer GW. Cardiac Remodeling: Endothelial Cells Have More to Say Than Just NO. *Front Physiol*. **2018**;9:382.

7- Smiljic S. The clinical significance of endocardial endothelial dysfunction. *Medicina (Kaunas)*. **2017**;53(5):295-302.

8- Boulanger CM. Endothelium. *Arterioscler Thromb Vasc Biol*. **2016**;36(4):e26-31.

9- Ribatti D, Tamma R, Ruggieri S, Annese T, Crivellato E. Surface markers: An identity card of endothelial cells. *Microcirculation*. **2020**;27(1):e12587.

10- Yau JW, Teoh H, Verma S. Endothelial cell control of thrombosis. *BMC Cardiovasc Disord*. **2015**;15:130.

11- Félétou M. The Endothelium: Part 1: Multiple Functions of the Endothelial Cells—Focus on Endothelium-Derived Vasoactive Mediators. *San Rafael (CA): Morgan & Claypool Life Sciences*; **2011**. Chapter 2, Multiple Functions of the Endothelial Cells.

12- Félétou M. The Endothelium: Part 1: Multiple Functions of the Endothelial Cells—Focus on Endothelium-Derived Vasoactive Mediators. *San Rafael (CA): Morgan & Claypool Life Sciences*; **2011**. Chapter 4, Endothelium-Dependent Regulation of Vascular Tone.

13- Goncharov NV, Popova PI, Avdonin PP, Kudryavtsev IV, Serebryakova MK, Korf EA, Avdonin PV. Markers of Endothelial Cells in Normal and Pathological Conditions. *Biochem (Mosc) Suppl Ser A Membr Cell Biol*. **2020**;14(3):167-183.

14- Janaszak-Jasiecka A, Płoska A, Wierońska JM, Dobrucki LW, Kalinowski L. Endothelial dysfunction due to eNOS uncoupling: molecular mechanisms as potential therapeutic targets. *Cell Mol Biol Lett*. **2023**;28(1):21.

15- Mollace R, Scarano F, Bava I, Carresi C, Maiuolo J, Tavernese A, Gliozzi M, Musolino V, Muscoli S, Palma E, Muscoli C, Salvemini D, Federici M, Macrì R, Mollace V. Modulation of the nitric oxide/cGMP pathway in cardiac contraction and relaxation: Potential role in heart failure treatment. *Pharmacol Res*. **2023**;196:106931.

16- Eelen G, de Zeeuw P, Treps L, Harjes U, Wong BW, Carmeliet P. Endothelial Cell Metabolism. *Physiol Rev*. **2018**;98(1):3-58.

17- Wynne BM, Chiao CW, Webb RC. Vascular Smooth Muscle Cell Signaling Mechanisms for Contraction to Angiotensin II and Endothelin-1. *J Am Soc Hypertens*. **2009**;3(2):84-95.

18- Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM. Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. *Antioxid Redox Signal*. **2013**;19(10):1110-20.

19- Godo S, Shimokawa H. Endothelial Functions. *Arterioscler Thromb Vasc Biol*. **2017**;37(9):e108-e114.

20- Scioli MG, Storti G, D'Amico F, Rodríguez Guzmán R, Centofanti F, Doldo E, Céspedes Miranda EM, Orlandi A. Oxidative Stress and New Pathogenetic Mechanisms in Endothelial Dysfunction: Potential Diagnostic Biomarkers and Therapeutic Targets. *J Clin Med*. **2020**;9(6):1995.

21- Panday A, Sahoo MK, Osorio D, Batra S. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cell Mol Immunol*. **2015**;12(1):5-23.

22- El-Benna J, Dang PM, Gougerot-Pocidalo MA, Marie JC, Braut-Boucher F. p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. *Exp Mol Med*. **2009**;41(4):217-25.

23- Poznyak AV, Grechko AV, Orekhova VA, Khotina V, Ivanova EA, Orekhov AN. NADPH Oxidases and Their Role in Atherosclerosis. *Biomedicines*. **2020**;8(7):206.
24- Tang Y, Long J, Liu J. Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging. Vol. 1: Molecular Mechanisms. *Academic Press*. **2014**. Chapter 8, Hyperglycemia-Associated Oxidative Stress Induces Autophagy: Involvement of the ROS-ERK/JNK-p53 Pathway
25- Gräb J, Rybniker J. The Expanding Role of p38 Mitogen-Activated Protein Kinase in Programmed Host Cell Death. *Microbiol Insights*. **2019** 24;12:1178636119864594.
26- Sun HJ, Wu ZY, Nie XW, Bian JS. Role of Endothelial Dysfunction in Cardiovascular Diseases: The Link Between Inflammation and Hydrogen Sulfide. *Front Pharmacol*. **2020**;10:1568.
27- Giridharan S, Srinivasan M. Mechanisms of NF-κB p65 and strategies for therapeutic manipulation. *J Inflamm Res*. **2018**;11:407-419.
28- Gimbrone MA Jr, García-Cardeña G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circ Res*. **2016**;118(4):620-36.
29- Han Y, Kim SY. Endothelial senescence in vascular diseases: current understanding and future opportunities in senotherapeutics. *Exp Mol Med*. **2023**;55(1):1-12.
30- Lingappan K. NF-κB in Oxidative Stress. *Curr Opin Toxicol*. **2018**;7:81-86.
31- Koundouros N, Poulogiannis G. Phosphoinositide 3-Kinase/Akt Signaling and Redox Metabolism in Cancer. *Front Oncol*. **2018**;8:160.
32- Cyr AR, Huckaby LV, Shiva SS, Zuckerbraun BS. Nitric Oxide and Endothelial Dysfunction. *Crit Care Clin*. **2020**;36(2):307-321.
33- Rodriguez ER, Tan CD. Structure and Anatomy of the Human Pericardium. Prog *Cardiovasc Dis*. **2017**;59(4):327-340.
34- Dusturia N, Choi SW, Song KS, Lim KM. Effect of myocardial heterogeneity on ventricular electro-mechanical responses: a computational study. *Biomed Eng Online*. **2019**;18(1):23.
35- Eisner DA, Caldwell JL, Kistamás K, Trafford AW. Calcium and Excitation-Contraction Coupling in the Heart. *Circ Res*. **2017**;121(2):181-195.

36- Zhang P, Su J, Mende U. Cross talk between cardiac myocytes and fibroblasts: from multiscale investigative approaches to mechanisms and functional consequences. *Am J Physiol Heart Circ Physiol*. **2012**;303(12):H1385-96.

37- Pfenniger A, Arora R. Cardiac regulation by the autonomic nervous system: A fine balance. *J Cardiovasc Electrophysiol*. **2019** May;30(5):747-748.

38- Harris IS, Black BL. Development of the endocardium. *Pediatr Cardiol*. **2010**;31(3):391-9.

39- Talman V, Kivelä R. Cardiomyocyte-Endothelial Cell Interactions in Cardiac Remodeling and Regeneration. *Front Cardiovasc Med*. **2018**;5:101.

40- Koser F, Loescher C, Linke WA. Posttranslational modifications of titin from cardiac muscle: how, where, and what for? *FEBS J*. **2019**;286(12):2240-2260.

41- Lyaker MR, Tulman DB, Dimitrova GT, Pin RH, Papadimos TJ. Arterial embolism. *Int J Crit Illn Inj Sci*. **2013** Jan;3(1):77-87.

42- Padala SK, Cabrera JA, Ellenbogen KA. Anatomy of the cardiac conduction system. *Pacing Clin Electrophysiol*. **2021** Jan;44(1):15-25.

43- Shinelle Whiteman, Yusuf Alimi, Mark Carrasco, Jerzy Gielecki, Anna Zurada, Marios Loukas. Anatomy of the cardiac chambers: A review of the left ventricle. *Translational Research in Anatomy*.**2021**;23:100095.

44- Katja Anttila, Anthony P. Farrell. Physiology of cardiac pumping. *Reference Module in Life Sciences*.**2022**.

45- Vincent JL. Understanding cardiac output. *Crit Care*. **2008**;12(4):174.

46- Tiwari R, Kumar R, Malik S, Raj T, Kumar P. Analysis of Heart Rate Variability and Implication of Different Factors on Heart Rate Variability. *Curr Cardiol Rev*. **2021**;17(5):e160721189770.

47- Marwick TH. Ejection Fraction Pros and Cons: JACC State-of-the-Art Review. *J Am Coll Cardiol*. **2018**;72(19):2360-2379.

48- Goodwill AG, Dick GM, Kiel AM, Tune JD. Regulation of Coronary Blood Flow. *Compr Physiol*. **2017**;7(2):321-382.

49- Harry J. Carpenter, Alireza Gholipour, Mergen H. Ghayesh, Anthony C. Zander, Peter J. Psaltis. A review on the biomechanics of coronary arteries. *International*

Journal of Engineering Science.**2020**;147:103201.

50- Camici PG, Crea F. Coronary microvascular dysfunction. *N Engl J Med*. **2007**;356(8):830-40.

51- Mohammad W. Kassem, Sasha Lake, Wallisa Roberts, Sonja Salandy, Marios Loukas. Cardiac veins, an anatomical review. *Translational Research in Anatomy*. **2021**;23:100096.

52- Townsend N, Kazakiewicz D, Lucy Wright F, Timmis A, Huculeci R, Torbica A, Gale CP, Achenbach S, Weidinger F, Vardas P. Epidemiology of cardiovascular disease in Europe. *Nat Rev Cardiol*. **2022**;19(2):133-143.

53- Matsumori A. Targeting Inflammation in the Diagnosis, Management, and Prevention of Cardiovascular Diseases. *Glob Heart*. **2022**;17(1):80.

54- Battineni G, Sagaro GG, Chintalapudi N, Amenta F, Tomassoni D, Tayebati SK. Impact of Obesity-Induced Inflammation on Cardiovascular Diseases (CVD). *Int J Mol Sci*. **2021**;22(9):4798.

55- Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. *N Engl J Med*. **2013**;368(21):2004-13.

56- Malakar AK, Choudhury D, Halder B, Paul P, Uddin A, Chakraborty S. A review on coronary artery disease, its risk factors, and therapeutics. *J Cell Physiol*. **2019**;234(10):16812-16823.

57- Saleh M, Ambrose JA. Understanding myocardial infarction. *F1000Res*. **2018**;7:F1000 Faculty Rev-1378.

58- Sandoval Y, Jaffe AS. Type 2 Myocardial Infarction: JACC Review Topic of the Week. *J Am Coll Cardiol*. **2019**;73(14):1846-1860.

59- Reynolds HR, Smilowitz NR. Myocardial Infarction with Nonobstructive Coronary Arteries. *Annu Rev Med*. **2023**;74:171-188.

60- Savarese G, Lund LH. Global Public Health Burden of Heart Failure. *Card Fail Rev*. **2017**;3(1):7-11.

61- Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, Falk V, González-Juanatey JR, Harjola VP, Jankowska EA, Jessup M, Linde C, Nihoyannopoulos P, Parissis JT, Pieske B, Riley JP, Rosano GMC, Ruilope LM, Ruschitzka F, Rutten FH, van der Meer P; ESC Scientific Document Group. 2016 ESC Guidelines for the diagnosis and treatment of acute and

chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J. **2016**;37(27):2129-2200.

62- WRITING COMMITTEE MEMBERS; Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE Jr, Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJ, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson LW, Tang WH, Tsai EJ, Wilkoff BL; American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation*. **2013**;128(16):e240-327.

63- Chiorescu RM, Lazar RD, Buksa SB, Mocan M, Blendea D. Biomarkers of Volume Overload and Edema in Heart Failure With Reduced Ejection Fraction. *Front Cardiovasc Med*. **2022**;9:910100.

64- Borlaug BA, Redfield MM. Diastolic and systolic heart failure are distinct phenotypes within the heart failure spectrum. *Circulation*. **2011**;123(18):2006-13.

65- Lee MP, Glynn RJ, Schneeweiss S, Lin KJ, Patorno E, Barberio J, Levin R, Evers T, Wang SV, Desai RJ. Risk Factors for Heart Failure with Preserved or Reduced Ejection Fraction Among Medicare Beneficiaries: Application of Competing Risks Analysis and Gradient Boosted Model. *Clin Epidemiol*. **2020**;12:607-616.

66- Simmonds SJ, Cuijpers I, Heymans S, Jones EAV. Cellular and Molecular Differences between HFpEF and HFrEF: A Step Ahead in an Improved Pathological Understanding. *Cells*. **2020**;9(1):242.

67- Takvorian KS, Wang D, Courchesne P, Vasan RS, Benjamin EJ, Cheng S, Larson MG, Levy D, Ho JE. The Association of Protein Biomarkers With Incident Heart Failure With Preserved and Reduced Ejection Fraction. *Circ Heart Fail*. **2023**;16(1):e009446.

68- Zhang S, Liu C, Zhang Y, Wu Z, Feng K, Lai Y, Pei J, Guan T.

Different heart failure phenotypes of valvular heart disease: the role of mitochondrial dysfunction. *Front Cardiovasc Med*. **2023**;10:1135938.

69- Paulus WJ, Dal Canto E. Distinct Myocardial Targets for Diabetes Therapy in Heart Failure With Preserved or Reduced Ejection Fraction. *JACC Heart Fail*. **2018**;6(1):1-7.

70- Naraen A, Duvva D, Rao A. Heart Failure and Cardiac Device Therapy: A Review of Current National Institute of Health and Care Excellence and European Society of Cardiology Guidelines. *Arrhythm Electrophysiol Rev*. **2023**;12:e21.

71- Pagnesi M, Baldetti L, Aimo A, Inciardi RM, Tomasoni D, Vizzardi E, Vergaro G, Emdin M, Lombardi CM. Prognostic Benefit of New Drugs for HFrEF: A Systematic Review and Network Meta-Analysis. *J Clin Med*. **2022**;11(2):348.

72- Chioncel O, Lainscak M, Seferovic PM, Anker SD, Crespo-Leiro MG, Harjola VP, Parissis J, Laroche C, Piepoli MF, Fonseca C, Mebazaa A, Lund L, Ambrosio GA, Coats AJ, Ferrari R, Ruschitzka F, Maggioni AP, Filippatos G. Epidemiology and one-year outcomes in patients with chronic heart failure and preserved, mid-range and reduced ejection fraction: an analysis of the ESC Heart Failure Long-Term Registry. *Eur J Heart Fail*. **2017**;19(12):1574-1585.

73- Harper AR, Patel HC, Lyon AR. Heart failure with preserved ejection fraction. *Clin Med (Lond)*. **2018**;18(Suppl 2):s24-s29.

74- Cornuault L, Rouault P, Duplàa C, Couffinhal T, Renault MA. Endothelial Dysfunction in Heart Failure With Preserved Ejection Fraction: What are the Experimental Proofs? *Front Physiol*. **2022**;13:906272.

75- Mesquita T, Lin YN, Ibrahim A. Chronic low-grade inflammation in heart failure with preserved ejection fraction. *Aging Cell*. **2021**;20(9):e13453.

76- Ekström M, Hellman A, Hasselström J, Hage C, Kahan T, Ugander M, Wallén H, Persson H, Linde C. The transition from hypertension to hypertensive heart disease and heart failure: the PREFERS Hypertension study. *ESC Heart Fail*. **2020**;7(2):737-746.

77- Saheera S, Krishnamurthy P. Cardiovascular Changes Associated with Hypertensive Heart Disease

and Aging. *Cell Transplant*. **2020**;29:963689720920830.

78- Pacurari M, Kafoury R, Tchounwou PB, Ndebele K. The Renin-Angiotensin-aldosterone system in vascular inflammation and remodeling. *Int J Inflam*. **2014**;2014:689360.

79- Paz Ocaranza M, Riquelme JA, García L, Jalil JE, Chiong M, Santos RAS, Lavandero S. Counter-regulatory renin-angiotensin system in cardiovascular disease. *Nat Rev Cardiol*. **2020**;17(2):116-129.

80- Laghlam D, Jozwiak M, Nguyen LS. Renin-Angiotensin-Aldosterone System and Immunomodulation: A State-of-the-Art Review. *Cells*. **2021**;10(7):1767.

81- Schiattarella GG, Tong D, Hill JA. Can HFpEF and HFrEF Coexist? *Circulation*. **2020**;141(9):709-711.

82- Morfino P, Aimo A, Castiglione V, Vergaro G, Emdin M, Clerico A. Biomarkers of HFpEF: Natriuretic Peptides, High-Sensitivity Troponins and Beyond. *J Cardiovasc Dev Dis*. **2022**;9(8):256.

83- Zafeiropoulos S, Farmakis IT, Milioglou I, Doundoulakis I, Gorodeski EZ, Konstantinides SV, Cooper L, Zanos S, Stavrakis S, Giamouzis G, Butler J, Giannakoulas G. Pharmacological Treatments in Heart Failure With Mildly Reduced and Preserved Ejection Fraction: Systematic Review and Network Meta-Analysis. *JACC Heart Fail*.:S2213-1779(23)00410-9.

84- Khan MS, Fonarow GC, Khan H, Greene SJ, Anker SD, Gheorghiade M, Butler J. Renin-angiotensin blockade in heart failure with preserved ejection fraction: a systematic review and meta-analysis. *ESC Heart Fail*. **2017**;4(4):402-408.

85- Kaur G, Jones M, Howes L, Hattingh HL. Systematic review and meta-analysis of the association between all-cause mortality and statin therapy in patients with preserved ejection fraction heart failure (HFpEF). *Int J Cardiol*. **2023**;372:63-70.

86- Manoharan MP, Raja R, Jamil A, Csendes D, Gutlapalli SD, Prakash K, Swarnakari KM, Bai M, Desai DM, Desai A, Penumetcha SS. Obesity and Coronary Artery Disease: An Updated Systematic Review 2022. *Cureus*. **2022**;14(9):e29480.

87- Savarese G, Becher PM, Lund LH, Seferovic P, Rosano GMC, Coats AJS. Global burden of heart failure:

a comprehensive and updated review of epidemiology. *Cardiovasc Res*. **2023**;118(17):3272-3287.

88- Kingma J, Simard C, Drolet B. Overview of Cardiac Arrhythmias and Treatment Strategies. *Pharmaceuticals (Basel)*. **2023**;16(6):844.

89- Eleid MF, Nkomo VT, Pislaru SV, Gersh BJ. Valvular Heart Disease: New Concepts in Pathophysiology and Therapeutic Approaches. *Annu Rev Med*. **2023**;74:155-170.

90- Ciarambino T, Menna G, Sansone G, Giordano M. Cardiomyopathies: An Overview. *Int J Mol Sci*. **2021**;22(14):7722.

91- Lazarou E, Tsioufis P, Vlachopoulos C, Tsioufis C, Lazaros G. Acute Pericarditis: Update. *Curr Cardiol Rep*. **2022**;24(8):905-913.

92- Basso C. Myocarditis. *N Engl J Med*. **2022**;387(16):1488-1500.

93- Rajani R, Klein JL. Infective endocarditis: A contemporary update. *Clin Med (Lond)*. **2020**;20(1):31-35.

94- Kuriakose D, Xiao Z. Pathophysiology and Treatment of Stroke: Present Status and Future Perspectives. *Int J Mol Sci*. **2020**;21(20):7609.

95- Clift PF, Cervi E. A review of thoracic aortic aneurysm disease. *Echo Res Pract*. **2019**;7(1):R1-R10.

96- Shamaki GR, Markson F, Soji-Ayoade D, Agwuegbo CC, Bamgbose MO, Tamunoinemi BM. Peripheral Artery Disease: A Comprehensive Updated Review. *Curr Probl Cardiol*. **2022**;47(11):101082.

97- Chopard R, Albertsen IE, Piazza G. Diagnosis and Treatment of Lower Extremity Venous Thromboembolism: A Review. *JAMA*. **2020**;324(17):1765-1776.

98- Mocumbi A, Humbert M, Saxena A, Jing ZC, Sliwa K, Thienemann F, Archer SL, Stewart S. Pulmonary hypertension. *Nat Rev Dis Primers*. **2024**;10(1):1.

99- Yasuhara J, Garg V. Genetics of congenital heart disease: a narrative review of recent advances and clinical implications. *Transl Pediatr*. **2021** Sep;10(9):2366-2386.

100- Jamison DA Jr, Anand Narayanan S, Trovão NS, Guarnieri JW, Topper MJ, Moraes-Vieira PM, Zaksas V, Singh KK, Wurtele ES, Beheshti A. A comprehensive SARS-CoV-2 and COVID-19 review, Part 1: Intracellular overdrive for SARS-CoV-

2 infection. *Eur J Hum Genet*. **2022**;30(8):889-898.

101- Narayanan SA, Jamison DA Jr, Guarnieri JW, Zaksas V, Topper M, Koutnik AP, Park J, Clark KB, Enguita FJ, Leitão AL, Das S, Moraes-Vieira PM, Galeano D, Mason CE, Trovão NS, Schwartz RE, Schisler JC, Coelho-Dos-Reis JGA, Wurtele ES, Beheshti A. A comprehensive SARS-CoV-2 and COVID-19 review, Part 2: host extracellular to systemic effects of SARS-CoV-2 infection. *Eur J Hum Genet*. **2024**;32(1):10-20.

102- Vosko I, Zirlik A, Bugger H. Impact of COVID-19 on Cardiovascular Disease. *Viruses*. **2023**;15(2):508.

103- Kole C, Stefanou E, Karvelas N, Schizas D, Toutouzas KP. Acute and Post-Acute COVID-19 Cardiovascular Complications: A Comprehensive Review. *Cardiovasc Drugs Ther*. **2023**:1–16.

104- Zhu H, Rhee JW, Cheng P, Waliany S, Chang A, Witteles RM, Maecker H, Davis MM, Nguyen PK, Wu SM. Cardiovascular Complications in Patients with COVID-19: Consequences of Viral Toxicities and Host Immune Response. *Curr Cardiol Rep*. **2020**;22(5):32.

105- Chung MK, Zidar DA, Bristow MR, Cameron SJ, Chan T, Harding CV 3rd, Kwon DH, Singh T, Tilton JC, Tsai EJ, Tucker NR, Barnard J, Loscalzo J. COVID-19 and Cardiovascular Disease: From Bench to Bedside. *Circ Res*. **2021**;128(8):1214-1236.

106- di Filippo L, Doga M, Frara S, Giustina A. Hypocalcemia in COVID-19: Prevalence, clinical significance and therapeutic implications. *Rev Endocr Metab Disord*. **2022**;23(2):299-308.

107- Del Prete A, Conway F, Della Rocca DG, Biondi-Zoccai G, De Felice F, Musto C, Picichè M, Martuscelli E, Natale A, Versaci F. COVID-19, Acute Myocardial Injury, and Infarction. *Card Electrophysiol Clin*. **2022**;14(1):29-39.

108- Nishiga M, Wang DW, Han Y, Lewis DB, Wu JC. COVID-19 and cardiovascular disease: from basic mechanisms to clinical perspectives. *Nat Rev Cardiol*. **2020**;17(9):543-558.

109- Xu SW, Ilyas I, Weng JP. Endothelial dysfunction in COVID-19: an overview of evidence, biomarkers, mechanisms and potential therapies. *Acta Pharmacol Sin*. **2023**;44(4):695-709.

110- Gorog DA, Storey RF, Gurbel PA, Tantry US, Berger JS, Chan MY, Duerschmied D, Smyth SS, Parker WAE, Ajjan RA, Vilahur G, Badimon

L, Berg JMT, Cate HT, Peyvandi F, Wang TT, Becker RC. Current and novel biomarkers of thrombotic risk in COVID-19: a Consensus Statement from the International COVID-19 Thrombosis Biomarkers Colloquium. *Nat Rev Cardiol*. **2022**;19(7):475-495.

111- Jing H, Wu X, Xiang M, Liu L, Novakovic VA, Shi J. Pathophysiological mechanisms of thrombosis in acute and long COVID-19. *Front Immunol*. **2022**;13:992384.

112- Liu H, Hu T, Zhang C, Chen X, Zhang S, Li M, Jing H, Wang C, Hu T, Shi J. Mechanisms of COVID-19 thrombosis in an inflammatory environment and new anticoagulant targets. *Am J Transl Res*. **2021**;13(5):3925-3941.

113- Deng D, Yan N. GLUT, SGLT, and SWEET: Structural and mechanistic investigations of the glucose transporters. *Protein Sci*. **2016**;25(3):546-58.

114- Thorens B, Mueckler M. Glucose transporters in the 21st Century. *Am J Physiol Endocrinol Metab*. **2010**;298(2):E141-5.

115- Wright EM, Loo DD, Hirayama BA. Biology of human sodium glucose transporters. *Physiol Rev*. **2011**;91(2):733-94.

116- Wright EM, Ghezzi C, Loo DDF. Novel and Unexpected Functions of SGLTs. Physiology (Bethesda). 2017;32(6):435-443.

117- Gyimesi G, Pujol-Giménez J, Kanai Y, Hediger MA. Sodium-coupled glucose transport, the SLC5 family, and therapeutically relevant inhibitors: from molecular discovery to clinical application. *Pflugers Arch*. **2020**;472(9):1177-1206.

118- Carbó R, Rodríguez E. Relevance of Sugar Transport across the Cell Membrane. *Int J Mol Sci*. **2023**;24(7):6085.

119- Song P, Onishi A, Koepsell H, Vallon V. Sodium glucose cotransporter SGLT1 as a therapeutic target in diabetes mellitus. *Expert Opin Ther Targets*. **2016**;20(9):1109-25.

120- Poulsen SB, Fenton RA, Rieg T. Sodium-glucose cotransport. *Curr Opin Nephrol Hypertens*. **2015**;24(5):463-9.

121- Sano R, Shinozaki Y, Ohta T. Sodium-glucose cotransporters: Functional properties and pharmaceutical potential. *J Diabetes Investig*. **2020**;11(4):770-782.

122- Banerjee SK, McGaffin KR, Pastor-Soler NM, Ahmad F. SGLT1 is a novel cardiac glucose transporter that is perturbed in disease states. *Cardiovasc Res*. **2009**;84(1):111-8.

123- Ghezzi C, Loo DDF, Wright EM. Physiology of renal glucose

handling via SGLT1, SGLT2 and GLUT2. *Diabetologia*. **2018**;61(10):2087-2097.

124- Wright EM. SGLT2 Inhibitors: Physiology and Pharmacology. *Kidney360*. **2021**;2(12):2027-2037.

125- Larson GL. The synthesis of gliflozins. *Oligos Peptides*. **2015**;33(2):37-40.

126- Cai W, Jiang L, Xie Y, Liu Y, Liu W, Zhao G. Design of SGLT2 Inhibitors for the Treatment of Type 2 Diabetes: A History Driven by Biology to Chemistry. *Med Chem*. **2015**;11(4):317-28.

127- Guo C, Hu M, DeOrazio RJ, Usyatinsky A, Fitzpatrick K, Zhang Z, Maeng JH, Kitchen DB, Tom S, Luche M, Khmelnitsky Y, Mhyre AJ, Guzzo PR, Liu S. The design and synthesis of novel SGLT2 inhibitors: C-glycosides with benzyltriazolopyridinone and phenylhydantoin as the aglycone moieties. *Bioorg Med Chem*. **2014**;22(13):3414-22.

128- Cowart K, Coon S, Carris NW. A Review of the Safety and Efficacy of Bexagliflozin for the Management of Type 2 Diabetes. *Ann Pharmacother*. **2023**:10600280231190443.

129- Aggarwal R, Bhatt DL, Szarek M, Cannon CP, McGuire DK, Inzucchi SE, Lopes RD, Davies MJ, Banks P, Pitt B, Steg PG. Efficacy of Sotagliflozin in Adults With Type 2 Diabetes in Relation to Baseline Hemoglobin A1c. *J Am Coll Cardiol*. **2023**;82(19):1842-1851.

130- Young, C.F.; Farnoudi, N.; Chen, J.; Shubrook, J.H. Exploring SGLT-2 Inhibitors: Benefits beyond the Glucose-Lowering Effect—What Is New in 2023? *Endocrines*. **2023**;4(3):630-655.

131- Zelniker TA, Braunwald E. Cardiac and Renal Effects of Sodium-Glucose Co-Transporter 2 Inhibitors in Diabetes: JACC State-of-the-Art Review. *J Am Coll Cardiol*. **2018**;72(15):1845-1855.

132- Ferro EG, Michos ED, Bhatt DL, Lincoff AM, Elshazly MB. New Decade, New FDA Guidance for Diabetes Drug Development: Lessons Learned and Future Directions. *J Am Coll Cardiol*. **2020**;76(21):2522-2526.

133- Sharma A, Pagidipati NJ, Califf RM, McGuire DK, Green JB, Demets D, George JT, Gerstein HC, Hobbs T, Holman RR, Lawson FC, Leiter LA, Pfeffer MA, Reusch J, Riesmeyer JS, Roe MT, Rosenberg Y, Temple R, Wiviott S, McMurray J, Granger C. Impact of Regulatory Guidance on Evaluating Cardiovascular Risk of New Glucose-Lowering

Therapies to Treat Type 2 Diabetes Mellitus: Lessons Learned and Future Directions. *Circulation*. **2020**;141(10):843-862.

134- Zinman B, Wanner C, Lachin JM, Fitchett D, Bluhmki E, Hantel S, Mattheus M, Devins T, Johansen OE, Woerle HJ, Broedl UC, Inzucchi SE; EMPA-REG OUTCOME Investigators. Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *N Engl J Med*. **2015**;373(22):2117-28.

135- Wiviott SD, Raz I, Bonaca MP, Mosenzon O, Kato ET, Cahn A, Silverman MG, Zelniker TA, Kuder JF, Murphy SA, Bhatt DL, Leiter LA, McGuire DK, Wilding JPH, Ruff CT, Gause-Nilsson IAM, Fredriksson M, Johansson PA, Langkilde AM, Sabatine MS; DECLARE–TIMI 58 Investigators. Dapagliflozin and Cardiovascular Outcomes in Type 2 Diabetes. *N Engl J Med*. **2019**;380(4):347-357.

136- Neal B, Perkovic V, Mahaffey KW, de Zeeuw D, Fulcher G, Erondu N, Shaw W, Law G, Desai M, Matthews DR; CANVAS Program Collaborative Group. Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes. *N Engl J Med*. **2017** Aug 17;377(7):644-657. doi: 10.1056/NEJMoa1611925. Epub 2017 Jun 12. PMID: 28605608.

137- Zelniker TA, Wiviott SD, Raz I, Im K, Goodrich EL, Bonaca MP, Mosenzon O, Kato ET, Cahn A, Furtado RHM, Bhatt DL, Leiter LA, McGuire DK, Wilding JPH, Sabatine MS. SGLT2 inhibitors for primary and secondary prevention of cardiovascular and renal outcomes in type 2 diabetes: a systematic review and meta-analysis of cardiovascular outcome trials. *Lancet*. **2019**;393(10166):31-39.

138- von Lewinski D, Kolesnik E, Tripolt NJ, Pferschy PN, Benedikt M, Wallner M, Alber H, Berger R, Lichtenauer M, Saely CH, Moertl D, Auersperg P, Reiter C, Rieder T, Siller-Matula JM, Gager GM, Hasun M, Weidinger F, Pieber TR, Zechner PM, Herrmann M, Zirlik A, Holman RR, Oulhaj A, Sourij H. Empagliflozin in acute myocardial infarction: the EMMY trial. *Eur Heart J*. **2022**;43(41):4421-4432.

139- Paolisso P, Bergamaschi L, Gragnano F, Gallinoro E, Cesaro A, Sardu C, Mileva N, Foà A, Armillotta M, Sansonetti A, Amicone S, Impellizzeri A, Esposito G, Morici N, Andrea OJ, Casella G, Mauro C, Vassilev D, Galie N, Santulli G, Marfella R, Calabrò P, Pizzi C, Barbato E. Outcomes in diabetic patients treated

with SGLT2-Inhibitors with acute myocardial infarction undergoing PCI: The SGLT2-I AMI PROTECT Registry. *Pharmacol Res*. **2023**;187:106597.

140- Suzuki Y, Kaneko H, Okada A, Itoh H, Matsuoka S, Fujiu K, Michihata N, Jo T, Takeda N, Morita H, Kamiya K, Matsunaga A, Ako J, Node K, Yasunaga H, Komuro I. Comparison of cardiovascular outcomes between SGLT2 inhibitors in diabetes mellitus. *Cardiovasc Diabetol*. **2022**;21(1):67.

141- Packer M, Anker SD, Butler J, Filippatos G, Pocock SJ, Carson P, Januzzi J, Verma S, Tsutsui H, Brueckmann M, Jamal W, Kimura K, Schnee J, Zeller C, Cotton D, Bocchi E, Böhm M, Choi DJ, Chopra V, Chuquiure E, Giannetti N, Janssens S, Zhang J, Gonzalez Juanatey JR, Kaul S, Brunner-La Rocca HP, Merkely B, Nicholls SJ, Perrone S, Pina I, Ponikowski P, Sattar N, Senni M, Seronde MF, Spinar J, Squire I, Taddei S, Wanner C, Zannad F; EMPEROR-Reduced Trial Investigators. Cardiovascular and Renal Outcomes with Empagliflozin in Heart Failure. *N Engl J Med*. **2020**;383(15):1413-1424.

142- Anker SD, Butler J, Filippatos G, Ferreira JP, Bocchi E, Böhm M, Brunner-La Rocca HP, Choi DJ, Chopra V, Chuquiure-Valenzuela E, Giannetti N, Gomez-Mesa JE, Janssens S, Januzzi JL, Gonzalez-Juanatey JR, Merkely B, Nicholls SJ, Perrone SV, Piña IL, Ponikowski P, Senni M, Sim D, Spinar J, Squire I, Taddei S, Tsutsui H, Verma S, Vinereanu D, Zhang J, Carson P, Lam CSP, Marx N, Zeller C, Sattar N, Jamal W, Schnaidt S, Schnee JM, Brueckmann M, Pocock SJ, Zannad F, Packer M; EMPEROR-Preserved Trial Investigators. Empagliflozin in Heart Failure with a Preserved Ejection Fraction. *N Engl J Med*. **2021**;385(16):1451-1461.

143- McMurray JJV, Solomon SD, Inzucchi SE, Køber L, Kosiborod MN, Martinez FA, Ponikowski P, Sabatine MS, Anand IS, Bělohlávek J, Böhm M, Chiang CE, Chopra VK, de Boer RA, Desai AS, Diez M, Drozdz J, Dukát A, Ge J, Howlett JG, Katova T, Kitakaze M, Ljungman CEA, Merkely B, Nicolau JC, O'Meara E, Petrie MC, Vinh PN, Schou M, Tereshchenko S, Verma S, Held C, DeMets DL, Docherty KF, Jhund PS, Bengtsson O, Sjöstrand M, Langkilde AM; DAPA-HF Trial Committees and Investigators. Dapagliflozin in Patients with Heart Failure and Reduced Ejection Fraction. *N Engl J Med*. **2019**;381(21):1995-2008.

144- Solomon SD, McMurray JJV, Claggett B, de Boer RA, DeMets D, Hernandez AF, Inzucchi SE, Kosiborod MN, Lam CSP, Martinez F, Shah SJ, Desai AS, Jhund PS, Belohlavek J, Chiang CE, Borleffs CJW, Comin-Colet J, Dobreanu D, Drozdz J, Fang JC, Alcocer-Gamba MA, Al Habeeb W, Han Y, Cabrera Honorio JW, Janssens SP, Katova T, Kitakaze M, Merkely B, O'Meara E, Saraiva JFK, Tereshchenko SN, Thierer J, Vaduganathan M, Vardeny O, Verma S, Pham VN, Wilderäng U, Zaozerska N, Bachus E, Lindholm D, Petersson M, Langkilde AM; DELIVER Trial Committees and Investigators. Dapagliflozin in Heart Failure with Mildly Reduced or Preserved Ejection Fraction. *N Engl J Med*. **2022**;387(12):1089-1098.

145- Yau K, Dharia A, Alrowiyti I, Cherney DZI. Prescribing SGLT2 Inhibitors in Patients With CKD: Expanding Indications and Practical Considerations. *Kidney Int Rep*. **2022**;7(7):1463-1476.

146- Giorgino F, Vora J, Fenici P, Solini A. Renoprotection with SGLT2 inhibitors in type 2 diabetes over a spectrum of cardiovascular and renal risk. *Cardiovasc Diabetol*. **2020**;19(1):196.

147- Kansara A, Mubeen F, Shakil J. SGLT2 Inhibitors in Patients with Chronic Kidney Disease and Heart Disease: A Literature Review. *Methodist Debakey Cardiovasc J*. **2022**;18(4):62-72.

148- Packer M, Butler J, Zannad F, Pocock SJ, Filippatos G, Ferreira JP, Brueckmann M, Jamal W, Zeller C, Wanner C, Anker SD; EMPEROR Study Group. Empagliflozin and Major Renal Outcomes in Heart Failure. *N Engl J Med*. **2021**;385(16):1531-1533.

149- The EMPA-KIDNEY Collaborative Group; Herrington WG, Staplin N, Wanner C, Green JB, Hauske SJ, Emberson JR, Preiss D, Judge P, Mayne KJ, Ng SYA, Sammons E, Zhu D, Hill M, Stevens W, Wallendszus K, Brenner S, Cheung AK, Liu ZH, Li J, Hooi LS, Liu W, Kadowaki T, Nangaku M, Levin A, Cherney D, Maggioni AP, Pontremoli R, Deo R, Goto S, Rossello X, Tuttle KR, Steubl D, Petrini M, Massey D, Eilbracht J, Brueckmann M, Landray MJ, Baigent C, Haynes R. Empagliflozin in Patients with Chronic Kidney Disease. *N Engl J Med*. **2023**;388(2):117-127.

150- Heerspink HJL, Stefánsson BV, Correa-Rotter R, Chertow GM, Greene T, Hou FF, Mann JFE, McMurray JJV, Lindberg M, Rossing P, Sjöström CD, Toto RD, Langkilde AM,

Wheeler DC; DAPA-CKD Trial Committees and Investigators. Dapagliflozin in Patients with Chronic Kidney Disease. *N Engl J Med*. **2020**;383(15):1436-1446.

151- Perkovic V, Jardine MJ, Neal B, Bompoint S, Heerspink HJL, Charytan DM, Edwards R, Agarwal R, Bakris G, Bull S, Cannon CP, Capuano G, Chu PL, de Zeeuw D, Greene T, Levin A, Pollock C, Wheeler DC, Yavin Y, Zhang H, Zinman B, Meininger G, Brenner BM, Mahaffey KW; CREDENCE Trial Investigators. Canagliflozin and Renal Outcomes in Type 2 Diabetes and Nephropathy. *N Engl J Med*. **2019**;380(24):2295-2306.

152- Alkabbani W, Zongo A, Minhas-Sandhu JK, Eurich DT, Shah BR, Alsabbagh MW, Gamble JM. Renal effectiveness and safety of the sodium-glucose cotransporter-2 inhibitors: a population-based cohort study. *BMJ Open Diabetes Res Care*. **2021**;9(2):e002496.

153- Zhuo M, Paik JM, Wexler DJ, Bonventre JV, Kim SC, Patorno E. SGLT2 Inhibitors and the Risk of Acute Kidney Injury in Older Adults With Type 2 Diabetes. *Am J Kidney Dis*. **2021**;79(6):858-867.

154- Klen J, Dolžan V. SGLT2 Inhibitors in the Treatment of Diabetic Kidney Disease: More than Just Glucose Regulation. *Pharmaceutics*. **2023**;15(7):1995.

155- Lopaschuk GD, Verma S. Mechanisms of Cardiovascular Benefits of Sodium Glucose Co-Transporter 2 (SGLT2) Inhibitors: A State-of-the-Art Review. *JACC Basic Transl Sci*. **2020**;5(6):632-644.

156- Xu B, Li S, Kang B, Zhou J. The current role of sodium-glucose cotransporter 2 inhibitors in type 2 diabetes mellitus management. Cardiovasc Diabetol. 2022;21(1):83.

157- Dyck JRB, Sossalla S, Hamdani N, Coronel R, Weber NC, Light PE, Zuurbier CJ. Cardiac mechanisms of the beneficial effects of SGLT2 inhibitors in heart failure: Evidence for potential off-target effects. *J Mol Cell Cardiol*. **2022**;167:17-31.

158- Uthman L, Baartscheer A, Schumacher CA, Fiolet JWT, Kuschma MC, Hollmann MW, Coronel R, Weber NC, Zuurbier CJ. Direct Cardiac Actions of Sodium Glucose Cotransporter 2 Inhibitors Target Pathogenic Mechanisms Underlying Heart Failure in Diabetic Patients. *Front Physiol*. **2018**;9:1575.

159- Horvath B, Bers DM. The late sodium current in heart failure: pathophysiology and clinical relevance. *ESC Heart Fail*. **2014**;1(1):26-40.

160- Chen HB, Yang YL, Meng RS, Liu XW. Indirect comparison of SGLT2 inhibitors in patients with established heart failure: evidence based on Bayesian methods. *ESC Heart Fail*. **2023**;10(2):1231-1241.

161- Packer M. Role of Deranged Energy Deprivation Signaling in the Pathogenesis of Cardiac and Renal Disease in States of Perceived Nutrient Overabundance. *Circulation*. **2020**;141(25):2095-2105.

162- Selvaraj S, Kelly DP, Margulies KB. Implications of Altered Ketone Metabolism and Therapeutic Ketosis in Heart Failure. *Circulation*. **2020**;141(22):1800-1812.

163- Gao YM, Feng ST, Wen Y, Tang TT, Wang B, Liu BC. Cardiorenal protection of SGLT2 inhibitors-Perspectives from metabolic reprogramming. *EBioMedicine*. **2022**;83:104215.

164- Gao YM, Feng ST, Wen Y, Tang TT, Wang B, Liu BC. Cardiorenal protection of SGLT2 inhibitors-Perspectives from metabolic reprogramming. *EBioMedicine*. **2022**;83:104215.

165- Welt FGP. CABG versus PCI - End of the Debate? *N Engl J Med*. **2022**;386(2):185-187.

166- Hillis LD, Smith PK, Anderson JL, Bittl JA, Bridges CR, Byrne JG, Cigarroa JE, Disesa VJ, Hiratzka LF, Hutter AM Jr, Jessen ME, Keeley EC, Lahey SJ, Lange RA, London MJ, Mack MJ, Patel MR, Puskas JD, Sabik JF, Selnes O, Shahian DM, Trost JC, Winniford MD. 2011 ACCF/AHA Guideline for Coronary Artery Bypass Graft Surgery: executive summary: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation*. **2011**;124(23):2610-42.

167- Gaudino M, Hameed I, Robinson NB, Ruan Y, Rahouma M, Naik A, Weidenmann V, Demetres M, Y Tam D, Hare DL, Girardi LN, Biondi-Zoccai G, E Fremes S. Angiographic Patency of Coronary Artery Bypass Conduits: A Network Meta-Analysis of Randomized Trials. *J Am Heart Assoc*. **2021**;10(6):e019206.

168- Corona S, Pernot M, Modine TE. The Pursuit of a Perfect Conduit. *JACC Basic Transl Sci*. **2023**;8(1):35-36.

169- Soehnlein O, Libby P. Targeting inflammation in atherosclerosis - from experimental insights to the clinic. *Nat Rev Drug Discov*. **2021**;20(8):589-610.

170- D'Onofrio N, Sardu C, Trotta MC, Scisciola L, Turriziani F,

Ferraraccio F, Panarese I, Petrella L, Fanelli M, Modugno P, Massetti M, Marfella LV, Sasso FC, Rizzo MR, Barbieri M, Furbatto F, Minicucci F, Mauro C, Federici M, Balestrieri ML, Paolisso G, Marfella R. Sodium-glucose co-transporter2 expression and inflammatory activity in diabetic atherosclerotic plaques: Effects of sodium-glucose co-transporter2 inhibitor treatment. *Mol Metab*. **2021**;54:101337.

171- Kraler S, Libby P, Evans PC, Akhmedov A, Schmiady MO, Reinehr M, Camici GG, Lüscher TF. Resilience of the Internal Mammary Artery to Atherogenesis: Shifting From Risk to Resistance to Address Unmet Needs. *Arterioscler Thromb Vasc Biol*. **2021**;41(8):2237-2251.

172- Liberale L, Badimon L, Montecucco F, Lüscher TF, Libby P, Camici GG. Inflammation, Aging, and Cardiovascular Disease: JACC Review Topic of the Week. *J Am Coll Cardiol*. **2022**;79(8):837-847.

173- Koenig W. Low-Grade Inflammation Modifies Cardiovascular Risk Even at Very Low LDL-C Levels: Are We Aiming for a Dual Target Concept? *Circulation*. **2018**;138(2):150-153. doi: 10.1161/CIRCULATIONAHA.118.035107. PMID: 29986958.

174- Tardif JC, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, Pinto FJ, Ibrahim R, Gamra H, Kiwan GS, Berry C, López-Sendón J, Ostadal P, Koenig W, Angoulvant D, Grégoire JC, Lavoie MA, Dubé MP, Rhainds D, Provencher M, Blondeau L, Orfanos A, L'Allier PL, Guertin MC, Roubille F. Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. *N Engl J Med*. **2019**;381(26):2497-2505.

175- Tardif JC, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, Pinto FJ, Ibrahim R, Gamra H, Kiwan GS, Berry C, López-Sendón J, Ostadal P, Koenig W, Angoulvant D, Grégoire JC, Lavoie MA, Dubé MP, Rhainds D, Provencher M, Blondeau L, Orfanos A, L'Allier PL, Guertin MC, Roubille F. Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. N Engl J Med. 2019;381(26):2497-2505.

176- Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ; CANTOS Trial Group.

Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med*. **2017**;377(12):1119-1131.

177- He G, Yang G, Huang X, Luo D, Tang C, Zhang Z. SGLT2 inhibitors for prevention of primary and secondary cardiovascular outcomes: A meta-analysis of randomized controlled trials. *Heart Lung*. **2023**;59:109-116.

178- Wagdy K. The EMPEROR-Reduced trial: SGLT2 inhibitors for heart failure get more support. *Glob Cardiol Sci Pract*. **2020**;2020(3):e202031.

179- Kwon O, Myong JP, Lee Y, Choi YJ, Yi JE, Seo SM, Jang SW, Kim PJ, Lee JM. Sodium-Glucose Cotransporter-2 Inhibitors After Acute Myocardial Infarction in Patients With Type 2 Diabetes: A Population-Based Investigation. *J Am Heart Assoc*. **2023**;12(14):e027824.

180- Packer M. SGLT2 inhibitors: role in protective reprogramming of cardiac nutrient transport and metabolism. *Nat Rev Cardiol*. **2023**;20(7):443-462.

181- Okumus N, Abraham S, Puri R, Tang WHW. Aortic Valve Disease, Transcatheter Aortic Valve Replacement, and the Heart Failure Patient: A State-of-the-Art Review. *JACC Heart Fail*. **2023**;11(8 Pt 2):1070-1083. doi: 10.1016/j.jchf.2023.07.003. PMID: 37611989.

182- Chen S, Coronel R, Hollmann MW, Weber NC, Zuurbier CJ. Direct cardiac effects of SGLT2 inhibitors. *Cardiovasc Diabetol*. **2022**;21(1):45.

183- Jia G, Aroor AR, Hill MA, Sowers JR. Role of Renin-Angiotensin-Aldosterone System Activation in Promoting Cardiovascular Fibrosis and Stiffness. *Hypertension*. **2018**;72(3):537-548.

184- Marfella R, Scisciola L, D'Onofrio N, Maiello C, Trotta MC, Sardu C, Panarese I, Ferraraccio F, Capuano A, Barbieri M, Balestrieri ML, Napoli C, Paolisso G. Sodium-glucose cotransporter-2 (SGLT2) expression in diabetic and non-diabetic failing human cardiomyocytes. *Pharmacol Res*. **2022**;184:106448.

185- Sayour AA, Oláh A, Ruppert M, Barta BA, Horváth EM, Benke K, Pólos M, Hartyánszky I, Merkely B, Radovits T. Characterization of left ventricular myocardial sodium-glucose cotransporter 1 expression in patients with end-stage heart failure. Cardiovasc Diabetol. 2020;19(1):159.

186- Theofilis P, Sagris M, Oikonomou E, Antonopoulos AS, Siasos G, Tsioufis C, Tousoulis D.

Inflammatory Mechanisms Contributing to Endothelial Dysfunction. *Biomedicines*. **2021**;9(7):781.

187- Dri E, Lampas E, Lazaros G, Lazarou E, Theofilis P, Tsioufis C, Tousoulis D. Inflammatory Mediators of Endothelial Dysfunction. *Life (Basel)*. **2023**;13(6):1420.

188- Bockus L, Kim F. Coronary endothelial dysfunction: from pathogenesis to clinical implications. *Open Heart* **2022**:e002200.

189- Little PJ, Askew CD, Xu S, Kamato D. Endothelial Dysfunction and Cardiovascular Disease: History and Analysis of the Clinical Utility of the Relationship. *Biomedicines*. **2021**;9(6):699.

190- Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol*. **2003**;23(2):168-75.

191- Widlansky ME, Gokce N, Keaney JF Jr, Vita JA. The clinical implications of endothelial dysfunction. *J Am Coll Cardiol*. **2003**;42(7):1149-60.

192- Förstermann U, Münzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation*. **2006**;113(13):1708-14.

193- Heiss C, Rodriguez-Mateos A, Kelm M. Central role of eNOS in the maintenance of endothelial homeostasis. *Antioxid Redox Signal*. **2015**;22(14):1230-42.

194- Chrissobolis S, Banfi B, Sobey CG, Faraci FM. Role of Nox isoforms in angiotensin II-induced oxidative stress and endothelial dysfunction in brain. J Appl Physiol (1985). 2012;113(2):184-91.

195- Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*. **2000**;87(10):840-4.

196- Higashi Y. Roles of Oxidative Stress and Inflammation in Vascular Endothelial Dysfunction-Related Disease. Antioxidants (Basel). 2022;11(10):1958.

197- Khemais-Benkhiat S, Belcastro E, Idris-Khodja N, Park SH, Amoura L, Abbas M, Auger C, Kessler L, Mayoux E, Toti F, Schini-Kerth VB. Angiotensin II-induced redox-sensitive SGLT1 and 2 expression promotes high glucose-induced endothelial cell senescence. *J Cell Mol Med*. **2020**;24(3):2109-2122.

198- Park SH, Belcastro E, Hasan H, Matsushita K, Marchandot B, Abbas M, Toti F, Auger C, Jesel L, Ohlmann P, Morel O, Schini-Kerth VB.

Angiotensin II-induced upregulation of SGLT1 and 2 contributes to human microparticle-stimulated endothelial senescence and dysfunction: protective effect of gliflozins. *Cardiovasc Diabetol*. **2021**;20(1):65.

199- Bruckert C, Matsushita K, Mroueh A, Amissi S, Auger C, Houngue U, Remila L, Chaker AB, Park SH, Algara-Suarez P, Belcastro E, Jesel L, Ohlmann P, Morel O, Schini-Kerth VB. Empagliflozin prevents angiotensin II-induced hypertension related micro and macrovascular endothelial cell activation and diastolic dysfunction in rats despite persistent hypertension: Role of endothelial SGLT1 and 2. *Vascul Pharmacol*. **2022**;146:107095.

200- An Y, Xu BT, Wan SR, Ma XM, Long Y, Xu Y, Jiang ZZ. The role of oxidative stress in diabetes mellitus-induced vascular endothelial dysfunction. *Cardiovasc Diabetol*. **2023**;22(1):237.

201- Tsai KF, Chen YL, Chiou TT, Chu TH, Li LC, Ng HY, Lee WC, Lee CT. Emergence of SGLT2 Inhibitors as Powerful Antioxidants in Human Diseases. Antioxidants (Basel). 2021;10(8):1166.

202- Li C, Zhang J, Xue M, Li X, Han F, Liu X, Xu L, Lu Y, Cheng Y, Li T, Yu X, Sun B, Chen L. SGLT2 inhibition with empagliflozin attenuates myocardial oxidative stress and fibrosis in diabetic mice heart. *Cardiovasc Diabetol*. **2019** Feb 2;18(1):15.

203- Li X, Flynn ER, do Carmo JM, Wang Z, da Silva AA, Mouton AJ, Omoto ACM, Hall ME, Hall JE. Direct Cardiac Actions of Sodium-Glucose Cotransporter 2 Inhibition Improve Mitochondrial Function and Attenuate Oxidative Stress in Pressure Overload-Induced Heart Failure. *Front Cardiovasc Med*. **2022**;9:859253.

204- Verma S, Mazer CD, Yan AT, Mason T, Garg V, Teoh H, Zuo F, Quan A, Farkouh ME, Fitchett DH, Goodman SG, Goldenberg RM, Al-Omran M, Gilbert RE, Bhatt DL, Leiter LA, Jüni P, Zinman B, Connelly KA. Effect of Empagliflozin on Left Ventricular Mass in Patients With Type 2 Diabetes Mellitus and Coronary Artery Disease: The EMPA-HEART CardioLink-6 Randomized Clinical Trial. *Circulation*. **2019**;140(21):1693-1702.

205- Wang Y, Zhong Y, Zhang Z, Yang S, Zhang Q, Chu B, Hu X. Effect of sodium-glucose cotransporter protein-2 inhibitors on left ventricular hypertrophy in patients with type 2 diabetes: A systematic review and meta-analysis. *Front Endocrinol (Lausanne)*. **2023**;13:1088820.

206- Dubois-Deruy E, Peugnet V, Turkieh A, Pinet F. Oxidative Stress in Cardiovascular Diseases. *Antioxidants (Basel)*. **2020**;9(9):864.

207- Yan Q, Liu S, Sun Y, Chen C, Yang S, Lin M, Long J, Yao J, Lin Y, Yi F, Meng L, Tan Y, Ai Q, Chen N, Yang Y. Targeting oxidative stress as a preventive and therapeutic approach for cardiovascular disease. *J Transl Med*. **2023**;21(1):519.

208- Schönberger E, Mihaljević V, Steiner K, Šarić S, Kurevija T, Majnarić LT, Bilić Ćurčić I, Canecki-Varžić S. Immunomodulatory Effects of SGLT2 Inhibitors-Targeting Inflammation and Oxidative Stress in Aging. *Int J Environ Res Public Health*. **2023**;20(17):6671.

209- Mayourian J, Ceholski DK, Gonzalez DM, Cashman TJ, Sahoo S, Hajjar RJ, Costa KD. Physiologic, Pathologic, and Therapeutic Paracrine Modulation of Cardiac Excitation-Contraction Coupling. *Circ Res*. **2018**;122(1):167-183.

210- Chen H, Chen C, Spanos M, Li G, Lu R, Bei Y, Xiao J. Exercise training maintains cardiovascular health: signaling pathways involved and potential therapeutics. *Signal Transduct Target Ther*. **2022**;7(1):306.

211- Talman V, Kivelä R. Cardiomyocyte-Endothelial Cell Interactions in Cardiac Remodeling and Regeneration. *Front Cardiovasc Med*. **2018**;5:101.

212- Brown AJM, Gandy S, McCrimmon R, Houston JG, Struthers AD, Lang CC. A randomized controlled trial of dapagliflozin on left ventricular hypertrophy in people with type two diabetes: the DAPA-LVH trial. *Eur Heart J*. **2020**;41(36):3421-3432.

213- Matsutani D, Sakamoto M, Kayama Y, Takeda N, Horiuchi R, Utsunomiya K. Effect of canagliflozin on left ventricular diastolic function in patients with type 2 diabetes. *Cardiovasc Diabetol*. **2018**;17(1):73.

214- Shim CY, Seo J, Cho I, Lee CJ, Cho IJ, Lhagvasuren P, Kang SM, Ha JW, Han G, Jang Y, Hong GR. Randomized, Controlled Trial to Evaluate the Effect of Dapagliflozin on Left Ventricular Diastolic Function in Patients With Type 2 Diabetes Mellitus: The IDDIA Trial. *Circulation*. **2021**;143(5):510-512.

215- Adingupu DD, Göpel SO, Grönros J, Behrendt M, Sotak M, Miliotis T, Dahlqvist U, Gan LM, Jönsson-Rylander AC. SGLT2 inhibition with empagliflozin improves coronary microvascular function and cardiac contractility in prediabetic ob/ob-/- mice. *Cardiovasc Diabetol*. **2019**;18(1):16.

216- Liu Y, Wu M, Xu J, Xu B, Kang L. Empagliflozin prevents from early cardiac injury post myocardial infarction in non-diabetic mice. *Eur J Pharm Sci*. **2021**;161:105788.

217- Udell JA, Jones WS, Petrie MC, Harrington J, Anker SD, Bhatt DL, Hernandez AF, Butler J. Sodium Glucose Cotransporter-2 Inhibition for Acute Myocardial Infarction: JACC Review Topic of the Week. *J Am Coll Cardiol*. **2022**;79(20):2058-2068.

218- Abdollahi E, Keyhanfar F, Delbandi AA, Falak R, Hajimiresmaiel SJ, Shafiei M. Dapagliflozin exerts anti-inflammatory effects via inhibition of LPS-induced TLR-4 overexpression and NF-κB activation in human endothelial cells and differentiated macrophages. Eur J Pharmacol. 2022 Mar 5;918:174715.

219- Scisciola L, Cataldo V, Taktaz F, Fontanella RA, Pesapane A, Ghosh P, Franzese M, Puocci A, De Angelis A, Sportiello L, Marfella R, Barbieri M. Anti-inflammatory role of SGLT2 inhibitors as part of their anti-atherosclerotic activity: Data from basic science and clinical trials. *Front Cardiovasc Med*. **2022**;9:1008922.

220- Fu M, Yu J, Chen Z, Tang Y, Dong R, Yang Y, Luo J, Hu S, Tu L, Xu X. Epoxyeicosatrienoic acids improve glucose homeostasis by preventing NF-κB-mediated transcription of SGLT2 in renal tubular epithelial cells. *Mol Cell Endocrinol*. **2021**;523:111149.

221- Abbas M, Jesel L, Auger C, Amoura L, Messas N, Manin G, Rumig C, León-González AJ, Ribeiro TP, Silva GC, Abou-Merhi R, Hamade E, Hecker M, Georg Y, Chakfe N, Ohlmann P, Schini-Kerth VB, Toti F, Morel O. Endothelial Microparticles From Acute Coronary Syndrome Patients Induce Premature Coronary Artery Endothelial Cell Aging and Thrombogenicity: Role of the Ang II/AT1 Receptor/NADPH Oxidase-Mediated Activation of MAPKs and PI3-Kinase Pathways. *Circulation*. **2017**;135(3):280-296.

222- Six I, Guillaume N, Jacob V, Mentaverri R, Kamel S, Boullier A, Slama M. The Endothelium and COVID-19: An Increasingly Clear Link Brief Title: Endotheliopathy in COVID-19. *Int J Mol Sci*. **2022**;23(11):6196.

223- Hawerkamp HC, Dyer AH, Patil ND, McElheron M, O'Dowd N, O'Doherty L, Mhaonaigh AU, George AM, O'Halloran AM, Reddy C, Kenny RA, Little MA, Martin-Loeches I, Bergin C, Kennelly SP, Donnelly SC, Bourke NM, Long A, Sui J, Doherty DG, Conlon N, Cheallaigh CN, Fallon PG. Characterisation of the pro-inflammatory cytokine signature in

severe COVID-19. Front Immunol. 2023;14:1170012.

224- Schultheiß C, Willscher E, Paschold L, Gottschick C, Klee B, Henkes SS, Bosurgi L, Dutzmann J, Sedding D, Frese T, Girndt M, Höll JI, Gekle M, Mikolajczyk R, Binder M. The IL-1β, IL-6, and TNF cytokine triad is associated with post-acute sequelae of COVID-19. *Cell Rep Med*. **2022**;3(6):100663.

225- Ong SWX, Fong SW, Young BE, Chan YH, Lee B, Amrun SN, Chee RS, Yeo NK, Tambyah P, Pada S, Tan SY, Ding Y, Renia L, Leo YS, Ng LFP, Lye DC. Persistent Symptoms and Association With Inflammatory Cytokine Signatures in Recovered Coronavirus Disease 2019 Patients. *Open Forum Infect Dis*. **2021**;8(6):ofab156.

226- Weatherhead JE, Clark E, Vogel TP, Atmar RL, Kulkarni PA. Inflammatory syndromes associated with SARS-CoV-2 infection: dysregulation of the immune response across the age spectrum. *J Clin Invest*. **2020**;130(12):6194-6197.

227- Davis HE, McCorkell L, Vogel JM, Topol EJ. Long COVID: major findings, mechanisms and recommendations. *Nat Rev Microbiol*. **2023**;21(3):133-146

228- Vivaldi G, Pfeffer PE, Talaei M, Basera TJ, Shaheen SO, Martineau AR. Long-term symptom profiles after COVID-19 vs other acute respiratory infections: an analysis of data from the COVIDENCE UK study. *EClinicalMedicine*. **2023**;65:102251.

229- Fodor A, Tiperciuc B, Login C, Orasan OH, Lazar AL, Buchman C, Hanghicel P, Sitar-Taut A, Suharoschi R, Vulturar R, Cozma A. Endothelial Dysfunction, Inflammation, and Oxidative Stress in COVID-19-Mechanisms and Therapeutic Targets. *Oxid Med Cell Longev*. **2021**;2021:8671713.

230- Ciacci P, Paraninfi A, Orlando F, Rella S, Maggio E, Oliva A, Cangemi R, Carnevale R, Bartimoccia S, Cammisotto V, D'Amico A, Magna A, Nocella C, Mastroianni CM, Pignatelli P, Violi F, Loffredo L. Endothelial dysfunction, oxidative stress and low-grade endotoxemia in COVID-19 patients hospitalised in medical wards. *Microvasc Res*. **2023**;149:104557.

231- Jankauskas SS, Kansakar U, Sardu C, Varzideh F, Avvisato R, Wang X, Matarese A, Marfella R, Ziosi M, Gambardella J, Santulli G. COVID-19 Causes Ferroptosis and Oxidative Stress in Human Endothelial Cells. *Antioxidants (Basel)*. **2023**;12(2):326.

232- Montiel V, Lobysheva I, Gérard L, Vermeersch M, Perez-Morga D, Castelein T, Mesland JB, Hantson P, Collienne C, Gruson D, van Dievoet MA, Persu A, Beauloye C, Dechamps M, Belkhir L, Robert A, Derive M, Laterre PF, Danser AHJ, Wittebole X, Balligand JL. Oxidative stress-induced endothelial dysfunction and decreased vascular nitric oxide in COVID-19 patients. *EBioMedicine*. **2022**;77:103893.

233- Gul R, Kim UH, Alfadda AA. Renin-angiotensin system at the interface of COVID-19 infection. *Eur J Pharmacol*. **2021**;890:173656.

234- Khazaal S, Harb J, Rima M, Annweiler C, Wu Y, Cao Z, Abi Khattar Z, Legros C, Kovacic H, Fajloun Z, Sabatier JM. The Pathophysiology of Long COVID throughout the Renin-Angiotensin System. *Molecules*. **2022**;27(9):2903.

235- Ramos SG, Rattis BADC, Ottaviani G, Celes MRN, Dias EP. ACE2 Down-Regulation May Act as a Transient Molecular Disease Causing RAAS Dysregulation and Tissue Damage in the Microcirculatory Environment Among COVID-19 Patients. *Am J Pathol*. **2021**;191(7):1154-1164.

236- Vaduganathan M, Vardeny O, Michel T, McMurray JJV, Pfeffer MA, Solomon SD. Renin-Angiotensin-Aldosterone System Inhibitors in Patients with Covid-19. *N Engl J Med*. **2020**;382(17):1653-1659.

237- Ni W, Yang X, Yang D, Bao J, Li R, Xiao Y, Hou C, Wang H, Liu J, Yang D, Xu Y, Cao Z, Gao Z. Role of angiotensin-converting enzyme 2 (ACE2) in COVID-19. *Crit Care*. **2020**;24(1):422.

238- Oudit GY, Kassiri Z, Jiang C, Liu PP, Poutanen SM, Penninger JM, Butany J. SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS. *Eur J Clin Invest*. **2009**;39(7):618-25.

239- Han Y, Kim SY. Endothelial senescence in vascular diseases: current understanding and future opportunities in senotherapeutics. Exp Mol Med. 2023;55(1):1-12.

240- Beltrán-Camacho L, Eslava-Alcón S, Rojas-Torres M, Sánchez-Morillo D, Martinez-Nicolás MP, Martín-Bermejo V, de la Torre IG, Berrocoso E, Moreno JA, Moreno-Luna R, Durán-Ruiz MC. The serum of COVID-19 asymptomatic patients up-regulates proteins related to endothelial dysfunction and viral response in circulating angiogenic cells ex-vivo. *Mol Med*. **2022**;28(1):40.

241- Gencer S, Evans BR, van der Vorst EPC, Döring Y, Weber C. Inflammatory Chemokines in Atherosclerosis. *Cells*. **2021**;10(2):226.

242- Turhan H, Erbay AR, Yasar AS, Aksoy Y, Bicer A, Yetkin G, Yetkin E. Plasma soluble adhesion molecules; intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin levels in patients with isolated coronary artery ectasia. Coron Artery Dis. 2005;16(1):45-50.

243- Kong P, Cui ZY, Huang XF, Zhang DD, Guo RJ, Han M. Inflammation and atherosclerosis: signaling pathways and therapeutic intervention. *Signal Transduct Target Ther*. **2022**;7(1):131.

244- Richards GHC, Hong KL, Henein MY, Hanratty C, Boles U. Coronary Artery Ectasia: Review of the Non-Atherosclerotic Molecular and Pathophysiologic Concepts. *Int J Mol Sci*. **2022**;23(9):5195.

245- Sur S, Steele R, Isbell TS, Ray R, Ray RB. Circulatory Exosomes from COVID-19 Patients Trigger NLRP3 Inflammasome in Endothelial Cells. mBio. 2022;13(3):e0095122.

246- Kwaan HC, Lindholm PF. The Central Role of Fibrinolytic Response in COVID-19-A Hematologist's Perspective. *Int J Mol Sci*. **2021**;22(3):1283.

247- Meizoso JP, Moore HB, Moore EE. Fibrinolysis Shutdown in COVID-19: Clinical Manifestations, Molecular Mechanisms, and Therapeutic Implications. *J Am Coll Surg*. **2021**;232(6):995-1003.

248- Xie Y, Xu E, Bowe B, Al-Aly Z. Long-term cardiovascular outcomes of COVID-19. *Nat Med*. **2022**;28(3):583-590.

249- Li XT, Zhang MW, Zhang ZZ, Cao YD, Liu XY, Miao R, Xu Y, Song XF, Song JW, Liu Y, Xu YL, Li J, Dong Y, Zhong JC. Abnormal apelin-ACE2 and SGLT2 signaling contribute to adverse cardiorenal injury in patients with COVID-19. *Int J Cardiol*. **2021**;336:123-129.

250- Elrakaybi A, Laubner K, Zhou Q, Hug MJ, Seufert J. Cardiovascular protection by SGLT2 inhibitors - Do anti-inflammatory mechanisms play a role? *Mol Metab*. **2022**;64:101549.

251- Alshnbari AS, Millar SA, O'Sullivan SE, Idris I. Effect of Sodium-Glucose Cotransporter-2 Inhibitors on Endothelial Function: A Systematic Review of Preclinical Studies. *Diabetes Ther*. **2020**;11(9):1947-1963.

252- Jing H, Wu X, Xiang M, Liu L, Novakovic VA, Shi J. Pathophysiological mechanisms of thrombosis in acute and long COVID-19. *Front Immunol*. **2022**;13:992384.

253- Jud P, Gressenberger P, Muster V, Avian A, Meinitzer A, Strohmaier H, Sourij H, Raggam RB, Stradner MH, Demel U, Kessler HH, Eller K, Brodmann M. Evaluation of Endothelial Dysfunction and Inflammatory Vasculopathy After SARS-CoV-2 Infection-A Cross-Sectional Study. *Front Cardiovasc Med*. **2021**;8:750887.

254- Fogarty H, Townsend L, Morrin H, Ahmad A, Comerford C, Karampini E, Englert H, Byrne M, Bergin C, O'Sullivan JM, Martin-Loeches I, Nadarajan P, Bannan C, Mallon PW, Curley GF, Preston RJS, Rehill AM, McGonagle D, Ni Cheallaigh C, Baker RI, Renné T, Ward SE, O'Donnell JS; Irish COVID-19 Vasculopathy Study (iCVS) investigators. Persistent endotheliopathy in the pathogenesis of long COVID syndrome. *J Thromb Haemost*. **2021**;19(10):2546-2553.

255- Bonaventura A, Vecchié A, Dagna L, Martinod K, Dixon DL, Van Tassell BW, Dentali F, Montecucco F, Massberg S, Levi M, Abbate A. Endothelial dysfunction and immunothrombosis as key pathogenic mechanisms in COVID-19. *Nat Rev Immunol*. **2021**;21(5):319-329.

256- Stark K, Massberg S. Interplay between inflammation and thrombosis in cardiovascular pathology. *Nat Rev Cardiol*. **2021**;18(9):666-682.

257- Ladikou EE, Sivaloganathan H, Milne KM, Arter WE, Ramasamy R, Saad R, Stoneham SM, Philips B, Eziefula AC, Chevassut T. Von Willebrand factor (vWF): marker of endothelial damage and thrombotic risk in COVID-19? *Clin Med (Lond)*. **2020**;20(5):e178-e182.

258- Rostami M, Mansouritorghabeh H, Parsa-Kondelaji M. High levels of Von Willebrand factor markers in COVID-19: a systematic review and meta-analysis. *Clin Exp Med*. **2022**;22(3):347-357.

259- Wibowo A, Pranata R, Lim MA, Akbara MR, Martha JW. Endotheliopathy marked by high von Willebrand factor (vWF) antigen in COVID-19 is associated with poor outcome: a systematic review and meta-analysis. *Int J Infect Dis*. **2022**;117:267-273.

260- Srihirun S, Sriwantana T, Srichatrapimuk S, Vivithanaporn P, Kirdlarp S, Sungkanuparph S, Phusanti S, Nanthatanti N, Suwannalert P, Sibmooh N. Increased platelet

activation and lower platelet-monocyte aggregates in COVID-19 patients with severe pneumonia. *PLoS One*. **2023**;18(3):e0282785.

261- Patti G, Cavallari I, Andreotti F, Calabrò P, Cirillo P, Denas G, Galli M, Golia E, Maddaloni E, Marcucci R, Parato VM, Pengo V, Prisco D, Ricottini E, Renda G, Santilli F, Simeone P, De Caterina R; Working Group on Thrombosis of the Italian Society of Cardiology. Prevention of atherothrombotic events in patients with diabetes mellitus: from antithrombotic therapies to new-generation glucose-lowering drugs. *Nat Rev Cardiol.* **2019**;16(2):113-130.

262- Marfella R, Sardu C, D'Onofrio N, Fumagalli C, Scisciola L, Sasso FC, Siniscalchi M, Marfella LV, D'Andrea D, Minicucci F, Signoriello G, Cesaro A, Trotta MC, Frigé C, Prattichizzo F, Balestrieri ML, Ceriello A, Calabrò P, Mauro C, Del Viscovo L, Paolisso G. SGLT-2 inhibitors and in-stent restenosis-related events after acute myocardial infarction: an observational study in patients with type 2 diabetes. *BMC Med*. **2023**;21(1):71.

263- Jorge A, Zhou B, McCormick N, Yokose C, Zhang Y, Choi H. Sodium-Glucose Co-transporter-2 Inhibitors and the Risk of Cardiac and Renal Outcomes in Systemic Lupus Erythematosus [abstract]. Arthritis Rheumatol. 2023; 75 (suppl 9). https://acrabstracts.org/abstract/sodium-glucose-co-transporter-2-inhibitors-and-the-risk-of-cardiac-and-renal-outcomes-in-systemic-lupus-erythematosus/. Accessed April 18, 2024.

264- Dabour, M, George, M, Daniel, M. et al. The Cardioprotective and Anticancer Effects of SGLT2 Inhibitors: JACC: CardioOncology State-of-the-Art Review. J Am Coll Cardiol CardioOnc. 2024 Apr, 6 (2) 159–182.

Ali MROUEH

Etudes pré-cliniques et translationnelles de l'expression et du rôle des co-transporteurs sodium-glucose SGLT1 et SGLT2 dans les artères et le cœur en condition physiologique et physiopathologique

Résumé de Thèse

Contexte : Les maladies cardiovasculaires (MCVs) touchent environ 620 millions de patients dans le monde et sont responsables de presque 18 millions de morts par an, étant ainsi la première cause de mortalité dans le monde. La pathophysiologie des divers MCVs repose sur de multiples étiologies, comprenant l'inflammation chronique de bas grade et le dysfonctionnement endothélial (DE). En outre, la pandémie de COVID-19, un syndrome de détresse respiratoire, fut associée avec un haut risque de MCV, comprenant insuffisance cardiaque (IC), arythmies et évènements athéro-thrombotiques en plus de la mortalité cardiovasculaire. La pandémie a également été caractérisée par une tempête de cytokines aiguë pouvant promouvoir des changements pathophysiologiques chroniques tels que le DE entraînant les

complications CV à long terme. Le mécanisme moléculaire précis de la transition endothéliale de l'état sain vers l'état malade n'est toutefois pas bien élucidé. D'autre part, les inhibiteurs des cotransporteurs sodium-glucose de type 2 (iSGLT2s) sont une classe de médicaments anti-diabétiques qui montraient des effets cardio-protecteurs pertinents dans leurs essais cliniques cardiovasculaires correspondants, comprenant une diminution du nombre d'hospitalisations pour insuffisance cardiaque (IC), de complications de l'infarctus du myocarde (IM) ainsi que de la mortalité cardiovasculaire en général. Cependant, le profil d'expression des SGLT2 dans le système CV humain est, malgré les effets précités, toujours discutable. Le mécanisme de la protection CV véhiculée par les iSGLT2s en tant que tel reste un sujet en cours d'investigation.

Objectifs : Actuellement, nous essayons d'étudier le profil d'expression inflammatoire des SGLT2 dans des cellules vasculaires, cardiaques et endothéliales humaines (CEs) et de déterminer sa contribution fonctionnelle. En outre, nous avons cherché à savoir si la tempête de cytokines inflammatoire chez les patients atteints du COVID-19 allait promouvoir le DE par une surexpression, sensible à l'oxydoréduction, des SGLT2 et nous avons étudié l'effet protecteur de l'inhibition des SGLT2.

Méthodes : Des prélèvements d'artère thoracique interne (ATI, n = 70) et du ventricule gauche (VG, n = 20) humains ainsi que des

échantillons de plasma de patients atteint du COVID-19 aigu, subaigu et long (n = 100), de patients non affectés par le COVID-19 ayant des facteurs de risque cardiovasculaire (n = 50) ainsi que de volontaires en bonne santé (n = 25) ont été collectés à l'hôpital universitaire. Des cellules endothéliales (CE) porcines d'artère coronaire ont été utilisées et stimulées soit avec d'authentiques cytokines pro-inflammatoires ou du plasma humain (10%). Les concentrations circulantes de protéines cibles dans le plasma humain ont été examinées par ELISA, les degrés d'expression des protéines cibles dans les tissus humains et les cellules cultivées ont été évalués à travers une analyse de Western blot ainsi qu'une coloration par immunofluorescence, les niveaux d'expression de l'ARN messager ont été évalués à travers une réaction de polymérase en chaîne quantitative avec transcription inverse et la production d'espèces réactives de l'oxygène (ROS) tandis que l'absorption d'oxyde nitrique (ON) et de glucose a été évaluée avec l'aide de sondes fluorescentes. L'adhésion et l'agrégation plaquettaires ainsi que la production de thrombine ont été déterminées à l'aide d'essais correspondants et la fonction des SGLT2 a été étudiée en utilisant l'empagliflozine ainsi que SGLT1 ou SGLT2 ARNsi.

Résultats : L'ARNm des SGLT2 et les niveaux de protéines dans les prélèvements d'ATI et de VG ont été mis en corrélation avec le degré d'inflammation de bas grade, les marqueurs du système angiotensine, du stress oxydatif et de l'activation des CE. Une coloration des SGLT2

a été observée dans l'endothélium et le muscle lisse d'ATI, dans la microcirculation coronaire et les cardiomyocytes et a été localisée dans des régions avec une infiltration de macrophages élevée. L'élévation de la formation de ROS dans les prélèvements avec une forte expression de SGLT2 a été réduite par l'inhibition du système angiotensine, l'oxydase NADPH, SGLT2 et TNF-α. L'exposition des CEs à l'interleukine (IL)-1β, IL-6 ou le facteur de nécrose tumoral (TNF)-α a entraîné une surexpression de l'ARNm des SGLT1 et SGLT2 et des niveaux de protéines, une absorption de glucose médiée par SGLT2 augmentée, une activation des composants du système angiotensine et des sous-unités de la NADPH oxydase, une élévation de la formation de ROS intracellulaire, une activation de NF-κB et une réduction de la production de NO. L'effet stimulant de TNF-α a été empêché par la N-acétylcystéine et l'inhibition du système angiotensine, la NADPH oxydase, SGLT2 mais pas SGLT1 et NF-κB.

De plus, des niveaux plasmatiques élevés d'IL-1β, IL-6, TNF-α, de la protéine chimio-attractive des monocytes et de la molécule d'adhésion intercellulaire soluble 1 ont été observés dans des patients atteints du COVID-19. L'exposition des CE à du plasma COVID-19 avec de niveaux de cytokines élevés a induit une activation sensible à l'oxydoréduction de l'expression des SGLT2 à travers les cytokines pro-inflammatoires IL-1β, IL-6 et TNF-α qui à leur tour alimentent l'activation de NF-κB, l'inflammation endothéliale, la sénescence et le

dysfonctionnement menant à une sécrétion élevée du facteur von Willebrand par les CEs, une adhérence et agrégation plaquettaire élevée sur la surface des CEs en plus d'une production exacerbée de thrombine dépendant du facteur tissulaire sur leur surface. L'effet stimulateur du plasma COVID-19 a été atténué par la combinaison d'anticorps neutralisants contre les cytokines pro-inflammatoire précités et l'inhibition des SGLT2.

Conclusion : L'inflammation de bas grade contribue à l'activation de l'expression des SGLT2 dans le système vasculaire et le cœur humains menant à une détérioration de l'équilibre eNOS-NO/ROS et favorisant des réponses pro-inflammatoires et pro-thrombotiques à travers l'activation de la voie AT1R/NADPH oxydases/SGLT2 pro-oxydant. De la même façon, dans des patients atteints de COVID-19, les cytokines pro-inflammatoires induisent une activation sensible à l'oxydoréduction de l'expression des SGLT2 dans les CEs, qui à leur tour favorisent les lésions endothéliales, la sénescence, l'adhérence et l'agrégation plaquettaires en complément de la production de thrombine. L'inhibition des SGLT2 avec l'empagliflozine semble être une stratégie intéressante afin de restaurer l'homéostasie cardiovasculaire en réponse à des stimulations inflammatoires de bas grade comme dans le cas du COVID-19.

Ali MROUEH

Pre-clinical and translational studies of the expression and role of sodium glucose co-transporters SGLT1 and SGLT2 in arteries and hearts under physiological and pathophysiological conditions

Thesis Abstract

Background: Cardiovascular diseases (CVDs) affect around 620 million patients worldwide and are responsible for almost 18 million annual deaths, placing them as the leading cause of mortality worldwide. Several etiologies underlie the pathophysiology of the wide array of CVDs, including chronic low-grade inflammation and endothelial dysfunction (ED). Alternatively, COVID-19 pandemic, a respiratory distress syndrome, was associated with elevated risk for CVD including heart failure (HF), arrhythmias and athero-thrombotic events in addition to cardiovascular mortality. The pandemic was as well characterized by acute cytokine storm that could promote chronic pathophysiological changes such as ED leading to the long-term CV complications. However, the precise molecular mechanism of endothelial transition from health to disease is not well elucidated. On

the other hand, sodium glucose co-transporter 2 inhibitors (SGLT2is) are a class of anti-diabetic drugs that showed pertinent cardiovascular protective effects in their corresponding clinical cardiovascular outcome trials. This included reduction in hospitalization for heart failure (HF), complications of myocardial infarction (MI) and overall cardiovascular mortality. Yet, and despite the afore-mentioned effects, SGLT2 expression pattern in human CV system is still debatable. As such, the mechanism of SGLT2is-mediated CV protection remains a topic under investigation.

Objectives: In the current work we aimed to investigate the inflammatory-mediated expression pattern of SGLT2 in human vasculature, heart, and endothelial cells (ECs) and to determine its functional contribution. In addition, we examined whether the inflammatory cytokine storm in patients with COVID-19 promoted ED through redox-sensitive SGLT2 overexpression and investigated the protective effect of SGLT2 inhibition.

Methods: Human internal thoracic artery (ITA, n = 70), left ventricle (LV, n = 20) specimens and plasma samples from patients with acute, subacute, and long COVID-19 (n = 100), patients with non-COVID-19 and cardiovascular risk factors (n = 50), and healthy volunteers (n = 25) were collected at the university hospital. Porcine coronary artery endothelial cells (ECs) were used and stimulated with either authentic pro-inflammatory cytokines or human plasma (10%). Circulating

concentrations of target proteins in human plasma were examined using ELISA, expression levels of target proteins in human tissues and cultured cells were assessed using Western blot analysis and immunofluorescence staining, mRNA expression levels were assessed by quantitative reverse transcription-polymerase chain reaction and the generation of reactive oxygen species (ROS) and nitric oxide (NO) and glucose uptake were assessed using fluorescent probes. Platelet adhesion, aggregation, and thrombin generation were determined using corresponding assays and the function of SGLT2 was investigated using empagliflozin and SGLT1 or SGLT2 siRNA.

Results: SGLT2 mRNA and protein levels in ITA and LV specimens were correlated with the level of low-grade inflammation, markers of the angiotensin system, oxidative stress and EC activation. SGLT2 staining was observed in the ITA endothelium and smooth muscle, the coronary microcirculation, and cardiomyocytes and was localized to the areas of enriched macrophage infiltration. Elevated ROS formation in high SGLT2-expressing specimens was reduced by inhibition of the angiotensin system, NADPH oxidase, SGLT2 and TNF-α. Exposure of ECs to interleukin (IL)-1β, IL-6, or tumor necrosis factor (TNF)-α led to overexpression of SGLT1 and SGLT2 mRNA and protein levels, increased SGLT2-mediated glucose uptake, upregulation of components of the angiotensin system and NADPH oxidase subunits, elevated intracellular ROS formation, activation of NF-κB and

decreased NO production. The stimulatory effect of TNF-α was prevented by N-acetylcysteine and inhibition of the angiotensin system, NADPH oxidase, SGLT2 but not SGLT1, and NF-κB.

Alternatively, increased plasma levels of IL-1β, IL-6, TNF-α, monocyte chemoattractant protein-1, and soluble intercellular adhesion molecule-1 were observed in patients with COVID-19. Exposure of ECs to COVID-19 plasma with high cytokines levels induced redox-sensitive upregulation of SGLT2 expression via pro-inflammatory cytokines IL-1β, IL-6 and TNF-α which, in turn, fueled NF-κB activation, endothelial inflammation, senescence and dysfunction leading to increased von Willebrand factor secretion by ECs, elevated platelet adhesion and aggregation at the surface of ECs in addition to exacerbated tissue factor-dependent thrombin generation at their surface. The stimulatory effect of COVID-19 plasma was blunted by combination of neutralizing antibodies against the afore-mentioned pro-inflammatory cytokines and inhibition of SGLT2.

Conclusion: Low-grade inflammation contributes to the upregulation of SGLT2 expression in human vasculature and heart, leading to impaired eNOS-NO/ROS balance and promoting pro-inflammatory and pro-thrombotic responses via the activation of the AT1R/NADPH oxidases/SGLT2 pro-oxidant pathway. Similarly, in patients with COVID-19, pro-inflammatory cytokines induced a redox-sensitive upregulation of SGLT2 expression in ECs, which in turn promoted

endothelial injury, senescence, platelet adhesion and aggregation in addition to thrombin generation. SGLT2 inhibition with empagliflozin appeared as an attractive strategy to restore cardiovascular homeostasis in response to low-grade inflammatory stimulations such as in the case of COVID-19.