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SGLT2i and aortic valve stenosis: impact on valve calcification

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SGLT2i and aortic valve stenosis: impact on valve calcification

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UC Dissertação - DECLARAÇÃO DE TRANSPARÊNCIA RELATIVAMENTE À UTILIZAÇÃO DE FERRAMENTAS DE CHATBOT GENERATIVO BASEADAS EM LARGE LANGUAGE MODELS

Eu, *Pedro Emanuel Resende Gonçalves*, abaixo assinado, nº mecanográfico *201904932*, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro que:

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ABSTRACT

Background: Aortic valve stenosis (AVS) is a cardiovascular disease characterized by a continuous process of fibrosis and calcification mainly led by the activation of valvular interstitial cells (VICs). Despite its high prevalence, there is still no effective pharmacological therapies to prevent or to treat this disease. Sodium-glucose co-transporter 2 inhibitors (SGLT2i), such as empagliflozin (EMPA), have shown cardiovascular benefits, but their direct effects on VICs remain unclear. This study aimed to investigate the effects of SGLT2i on VICs'-mediated calcification.

Methods: Human VICs were isolated from aortic valves of six patients (three men and three women) undergoing surgical valve replacement for severe AVS. Cells were isolated, expanded and cultured under either baseline conditions (VIC-GM) or pro-osteogenic medium (OM), with increasing concentrations of EMPA. After 21 days, cell viability was assessed using a Resazurin Assay, and calcification was quantified by Alizarin Red S staining, followed by image analysis.

Results: VICs maintained viability above 70% at all EMPA concentrations tested, up to 100 µM, indicating good drug tolerance. However, significant reductions in viability were detected from 10 µM onward in men. Male-derived VICs exhibited significantly higher baseline calcification compared to female-derived VICs (66.4% vs. 38.0%, p=0.0003), suggesting intrinsic sex-related differences in osteogenic potential. Under osteogenic stimulation, EMPA reduced calcification in male VICs at 10 µM (11.6%, p=0.0392). In contrast, female VICs showed no significant reduction in calcification. These findings suggest a sex-specific cellular response to EMPA.

Conclusions: EMPA may attenuate VIC-mediated calcification in a sex-specific manner, reducing calcium deposition in male-derived VICs but not in those from females. These findings suggest that SGLT2i could represent a novel pharmacological strategy for men with AVS and reinforce the importance of considering sex differences in the development of novel therapies.

Keywords: Aortic Valve Stenosis; Valvular Interstitial Cells; Sodium-Glucose Cotransporter 2 Inhibitors; Calcification.

1. INTRODUCTION

1.1 Aortic Stenosis

1.1.1 Epidemiology

Aortic valve stenosis (AVS) is the most common heart valve disorder in developed countries and one of the most frequent cardiovascular diseases, after systemic arterial hypertension and coronary artery disease. This disease can develop in both normal tricuspid and congenital bicuspid aortic valves (1,2). The latter affects 1-2% of the general population and leads to the development of valve calcification a few decades earlier (3). It has been reported by several population-based studies in the United States and Europe that the prevalence of AVS increases with age (4,5). It is estimated that almost 50% of people aged over 85 years have AVS. This prevalence is expected to increase worldwide over the next years owing to the lack of an effective preventive strategy and population aging (6).

The most common etiology of AVS is degenerative, but other possible etiologies include systemic inflammatory diseases (including rheumatic heart disease), endocarditis, congenital valve defects (including bicuspid valves), and many other conditions (7). In developing countries, the most common causes are inflammatory/rheumatic diseases, while in Europe and North America, the degenerative cause is more prevalent (8). Several factors are also associated with an increased risk of developing degenerative AVS, such as dyslipidemia, hypertension, increased body mass index, smoking, a sedentary lifestyle, chronic kidney disease (CKD), and diabetes mellitus (9–11). Some studies also found a higher prevalence of the disease among males rather than females (12). Also, hereditary factors are believed to have an important role in AVS prevalence. Genetic studies have demonstrated the polygenic nature of this disease, focusing more on aspects related to lipid metabolism, inflammation, fibrosis, and calcification (3). Some of the genes implicated in this process include NOTCH1, SMAD6, ROBO4, vascular endothelial growth factor-A (VEGF-A) or even ADAMTS19 (13).

AVS leads to increased mortality and morbidity if untreated. It may be responsible for myocardial infarction, embolic stroke, or even sudden cardiac death (14,15). Therefore, there is a need to invest in the management of AVS patients to relieve the burden on healthcare.

1.1.2 Anatomy and pathophysiology

The anatomical bridge between the ascending aorta and the left ventricle is called the aortic root. It is composed of aortic valve leaflets and inter-leaflet triangles. These leaflets have semilunar attachments, extending from the sinotubular junction to the left ventricular outflow tract. The sinuses and leaflets are named as left, right, and non-coronary, according to the origins of the coronary arteries (16). The root is also considered to be a functional bridge, since its distal and proximal components can resist changes in arterial and ventricular pressures.

A healthy aortic valve is an avascular tissue supplied with nutrients and oxygen via diffusion from circulating blood (17,18). It is usually composed of three distinct leaflets, referred to as cusps, which are <1 mm thick. Those leaflets are attached to the aortic wall and to the supporting ventricular structures, being continuous with the aortic leaflet of the mitral valve, forming part of the subaortic curtain. The leaflets are endocardial folds with a tenuous central fibrous core (19). Each one presents three different layers, the *ventricularis*, the *spongiosa*, and the *fibrosa*, with different functions and compositions. The *lamina fibrosa*, facing the aorta, provides tensile strength and resists the high circumferential stress during systole, maintaining the valve's structural integrity and ensuring proper coaptation during diastole. The *lamina ventricularis*, facing the left ventricular outflow tract, allows the valve to accommodate cyclic loading and unloading by facilitating stretch and recoil during valve opening and closure, contributing to its flexibility and durability. The *lamina spongiosa*, located between the fibrosa and *ventricularis*, functions as a shock absorber, distributing mechanical stresses between the other layers. It also facilitates smooth motion by reducing friction and providing a lubricating effect during valve leaflet movement (20). These layers have a different matrix. While the *lamina fibrosa* is richer in type 1 collagen, the *lamina ventricularis* is richer in elastin, and the *lamina spongiosa* in proteoglycans (21). Valvular interstitial cells (VICs) are the most prevalent cell population, being found in all three layers. These cells can present different phenotypes, defining their molecular functions. Embryonic VICs (eVICs) are responsible for valvulogenesis, promoting the formation of the valves during fetal development (22). Quiescent VICs (qVICs) maintain the valve matrix throughout adult life, which remain mostly inactive during their lifetime (23). They sense mechanical forces and, upon

increased pressure and stress, they differentiate into activated VICs (aVICs), adopting a myofibroblastic phenotype and producing extracellular matrix (ECM) proteins and α-smooth muscle actin. Usually, collagen produced by VICs serves as a scaffold, but in pathological conditions like AVS, overproduction and disorganization of collagen ensue (24). Progenitor VICs (pVICs) express surface markers of progenitor cells and have a role that is still not well understood (25). Osteoblastic VICs (obVICs) are the cells responsible for the calcification process, expressing osteoblastic markers like runt-related transcription factor 2 (RUNX2) and alkaline phosphatase (ALP). They mostly arise from qVICs with valve aging (26). In *lamina fibrosa*, VICs tend to present an ellipsoidal morphology due to the higher collagen I and III density. In *lamina ventricularis* and *lamina spongiosa*, VICs tend to have a polygonal morphology (27). Other less numerous cell populations of the valve include endothelial cells (VECs) and smooth muscle cells (SMCs).

VECs mostly sit on the surface of the valve, having a crucial role in maintaining tissue homeostasis through their paracrine actions, forming a physical barrier to the valve, and sensing environmental changes. Injuries on the endothelial layer promote the uptake of lipids, proteins, immune cells, and red blood cells, initiating a pro-inflammatory state, which can lead to the activation of VICs (28–30).

AVS can be described as having two distinct but overlapping phases. The first phase, also called the initiation phase, is characterized by valve injury, lipid deposition, and an underlying inflammatory response. Clinically, this stage is defined as aortic valve sclerosis. Mechanical stress plays a crucial role in the initiation of AVS. The aortic valve is subjected to constant mechanical forces, including shear stress from blood flow and tensile stress during valve opening and closing. These forces can lead to endothelial damage, disrupting the protective barrier of the valve and facilitating the infiltration of lipids and inflammatory cells into the valvular interstitium (31). Inflammation is driven by some factors such as low-density lipoprotein (LDL), lipoprotein (a) (Lp(a)), oxidized phospholipids, and interleukins-1β and 6 (IL-1β/ IL-6). Accumulating lipids can oxidize and activate toll-like receptors (TLRs), expressed by VICs, which then activate the NF-κB pathway, leading to osteogenic differentiation. aVICs show increased expression of bone morphogenic protein-2 (BMP-2) that contribute to valve mineralization (7,32,33). During aortic valve sclerosis, an imbalance between M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages towards M1 is also observed in association

with the inflammatory response (34). VECs with antioxidant properties typically release nitric oxide (NO), inhibiting the osteogenic differentiation of VICs. However, under oxidative stress, NO production is significantly reduced, thereby failing to prevent VIC osteogenic differentiation and contributing to the progression of sclerosis (35). At this point, the NOTCH pathway is activated, increasing transforming growth factor-β (TGF-β) levels, an important protein involved in valve fibrosis, stimulating the production of ECM (7). In summary, the initiation or sclerotic phase involves inflammatory cell infiltration and early calcific deposits, which leads to focal leaflet thickening without significant obstruction to blood flow (36,37).

The second phase, called the propagation phase, is marked by extensive valve fibrosis and calcification (38–41). This phase is, essentially, characterized by osteogenic differentiation of VICs, which are the major contributors to valve calcification (42,43). At this point, there is an imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). MMPs are enzymes responsible for the degradation of ECM components, facilitating tissue remodeling. In the context of AVS, certain MMPs, such as MMP-10, are overexpressed and contribute to pathological remodeling by promoting inflammatory, fibrotic, and osteogenic processes within the valve tissue (44). Conversely, TIMPs regulate MMP activity to maintain ECM integrity. Studies have shown that calcified valves often exhibit reduced levels of TIMP-2, disrupting the MMP/TIMP balance and contributing to the progression of the disease (45). The infiltration of macrophages and mast cells in the aortic valve may promote local angiotensin-II production, which enhances the myofibroblastic activation of VICs via angiotensin-II type 1 receptor. This pathway exerts pro-fibrotic effects, such as through increased type I collagen synthesis, contributing to aortic valve thickening (46,47). Further valve degeneration is the result of complex calcification processes. Some studies show an association between cadherin-11, TGF-β3, BMP-2, BMP-4, BMP-7 and Runx2 levels with an enhanced osteogenic response (48–51). Key pathways involved in valve calcification include OPG/RANKL/RANK, Wnt/β-catenin and NOTCH signaling pathways (8). Signals in the HIF-2 axis from the NF-κB signaling pathway are necessary for calcification in the valve (52). At this point, reduced NO expression and upregulation of the renin–angiotensin system also contribute to the disease's development (53). IL-6 expression also correlates with AVS severity, inducing VICs osteoblastic activation (54). aVICs produce pro-angiogenic factors, such as

VEGF-A, stimulating neovessel formation. Myofibroblasts and endothelial cells are also responsible for increased angiogenic activity, expressing vascular endothelial growth factor receptor-2 (VEGFR-2), Tie-2 and smooth muscle a-actin (a-SMA) (55–57). This leads to the formation of intrinsic micro-vasculature, speeding up the uptake of lipoproteins and immune cells from the blood, contributing to the inflammatory process and subsequent exacerbation of valve calcification (46,58).

Besides all those mechanisms, the dysregulation of calcium-phosphate metabolism also seems to be involved in aortic valve calcification. Some diseases, such as CKD or osteoporosis, may contribute to structural alteration of phosphates, shifting it from its organic form (biologically active) to an inorganic form, leading to cellular phosphate extravasation, and promoting mineralization throughout the valvular ECM (59,60).

In summary, this disease has a very complex progression, with many unanswered questions, not only about the sequence of events driving fibrocalcific remodeling but also about the optimal therapeutic strategies and molecular targets to combat the disease.

1.1.3 Symptoms and Signs

AVS is usually characterized by having an asymptomatic latent period, where atrial augmentation of preload and left ventricular hypertrophy compensate for the increase in afterload (61). This stage usually occurs during the aortic valve sclerosis phase, progressing to valve obstruction (stenosis) in approximately 10-15% of patients, over a period of 2 to 5 years (62). At this point, mortality is comparable to patients without aortic valve disease. Patients tend to remain asymptomatic until the valve orifice is narrowed to less than 1 cm^2 (63). However, when symptoms appear, survival rapidly decreases. The symptoms occur essentially due to left ventricular dysfunction, reduction in stroke volume, and reduction in cardiac output, caused by progressive valve obstruction (64,65). When symptomatic, it normally presents a progressive clinical syndrome of cardiorespiratory symptoms with rapid deterioration. The cardinal symptoms of AVS include angina pectoris, syncope, and dyspnea. Also, due to structural changes in the heart, the development of paroxysmal or recurrent arrhythmias, marked by palpitations, is common. A decrease in exercise tolerance, orthopnea, paroxysmal nocturnal dyspnea and pulmonary edema may also be present (66). Some other non-

cardiac features may also be present occasionally, such as Heyde syndrome, iron-deficiency anemia or even cognitive impairment (67–69).

During physical examination, it is common to find, by auscultation, a harsh, late-peaking systolic *crescendo-decrescendo* murmur, which is usually loudest over the second right intercostal space with carotid or apex irradiation. The Valsalva maneuver may help to differentiate between AVS and hypertrophic obstructive cardiomyopathy, reducing preload and end-diastolic volume, resulting in accentuated murmurs (70). However, this finding may also be absent or diminished, especially in older patients. Pulsus *parvus et tardus* may be palpated as the disease progresses, usually considered as one of the pathognomonic signs of this disease (71).

AVS should usually be considered if any cardinal symptoms and a systolic murmur are present, but if these conditions are not met, AVS should not be excluded either. The only finding that can exclude severe AVS is a normally split-second heart sound (72).

1.1.4 Diagnosis

It is extremely important to perform a good physical examination on every patient with any of the symptoms previously mentioned, because some findings may be suggestive of AVS (73), especially the auscultatory findings. Later in the disease course, some palpatory findings may also become more prominent, including a laterally displaced apical impulse or a double apical impulse due to left ventricle (LV) hypertrophy (74).

In the initial diagnosis of AVS, chest X-ray may have a role in symptomatic undiagnosed patients, particularly when there are already compensatory mechanisms of the disease, but does not give a definitive diagnosis (75). Electrocardiography can also be useful to detect comorbidities like coronary heart disease, and inspect LV hypertrophy (76), but again it does not give a definitive diagnosis. According to the 2021 European Society of Cardiology and the European Association for Cardio-Thoracic Surgery (ESC/EACTS) guidelines, echocardiography is the gold standard to confirm the diagnosis and to evaluate the severity of AVS (77). Also, According to the 2020 American College of Cardiology/American Heart Association (ACC/AHA)

Guideline for the Management of Patients With Valvular Heart Disease, with a Clinical Outcome Relevance (COR) of 1 and Level of Evidence (LOE) of A, it is recommended to perform Transthoracic Echocardiography (TTE) to any patient with signs and symptoms of AVS for accurate diagnosis of the disease, determination of prognosis and timing of possible valve intervention (78).

Echocardiography provides very important information for evaluating the disease, including valve area, peak transvalvular velocity, and mean pressure gradient. The classification of AVS severity is easier when these criteria are concordant, however, in discordant cases, it is also important to evaluate other parameters, such as Doppler velocity index (DVI), the degree of valve calcification, LV function, and the degree of LV hypertrophy or stroke volume (79,80). It can also help detect other valve or aortic diseases, such as aortic regurgitation, and provides prognostic information. Stress imaging can also be helpful to define severity, when this is uncertain. The classification of the severity of AVS can then be defined according to the ESC/EACTS algorithm (Table 1). When peak transvalvular velocity is above 4.0 m/s, mean pressure gradient is above 40 mmHg and aortic valve area under 1.0 cm^2, it is suggestive of severe stenosis. In cases of discordance between these parameters, one should look into DVI (81). When DVI is < 0.25, it suggests that severe AVS is highly likely. With cardiac computed tomography, it is possible to accurately determine the calcium score, predicting disease progression and clinical events (82,83).

Table 1: Classification of Aortic Valve Stenosis Severity (According to ESC 2021 Guidelines).

		Mild stenosis	Moderate stenosis	Severe stenosis
Peak transvalvular velocity (m/s)		<3.0	3.0 – 4-0	≥4.0
Mean pressure gradient (mmHg)		<20	20 -39	≥40
Aortic valve area (cm^2)		>1.5	1.0 – 1.5	≤1.0
Doppler velocity index (DVI)		>0.50	0.25 – 0.50	<0.25
Calcium score (Agatson)	**Men**	<1600	1600 – 2000	>2000
	Women	<800	800 - 1200	>1200

1.1.5 Monitoring, Treatment, and Prognosis

Currently, there is no pharmacological therapy capable of preventing disease progression and altering the natural history of the disease. In previous clinical trials (ASTRONOMER, SEAS, SALTIRE), statins failed to revert or halt the progression of AVS (84–86). Thus, statins are not part of the guideline-directed medical therapy for AVS (87,88). Thus, aortic valve replacement (AVR) is currently the only effective treatment for AVS. Surgical aortic valve replacement (SAVR) and transcatheter aortic valve implantation (TAVI) are two distinct approaches to treat AVS. SAVR is a technique characterized by an open-heart surgery, where the chest is opened to access the heart, and the diseased aortic valve is surgically removed and replaced with either a mechanical or bioprosthetic valve. Meanwhile, TAVI is a minimally invasive alternative where, instead of open surgery, a catheter is typically inserted through the femoral artery through the groin or through a small incision in the chest. The replacement valve is delivered via the catheter and deployed within the diseased valve without removing it (89,90).

In symptomatic severe AVS, early intervention is usually recommended, unless the patient's life expectancy is less than one year or the intervention is unlikely to improve survival or quality of life. Severe AVS is usually associated with a poor prognosis, but AVR has shown survival benefit compared to conservative management (91,92). In asymptomatic severe AVS, intervention is recommended for patients with impaired LV function of no other cause and for those who develop symptoms during exercise testing. In other cases, intervention is controversial, and it is important to carefully monitor the patient. Normally, if adverse prognostic features are not present, watchful waiting is the recommendation (93). Patients with heart failure (HF) who are waiting or are not suitable for TAVI or SAVR should be treated according to ESC heart failure guidelines, including evidence-based medical therapy (angiotensin-converting enzyme inhibitors (ACEI)/ angiotensin receptor blockers (ARB), beta-blockers, aldosterone antagonists, SGLT2 inhibitors (SGLT2i)), tight blood volume control, and strategies to improve perfusion and reduce cardiac burden (94).

SAVR is usually the preferred treatment option in younger patients (<75 years) due to prosthetic heart valve durability. In contrast, in older patients (≥75 years) or those with high surgery risk, TAVI is preferred. In some cases, balloon aortic valvuloplasty (BAV) can be considered as a bridge to SAVR or TAVI in patients with

severe AVS who require urgent non-cardiac surgery or in patients with myocardial decompensation. BAV is based on positioning and inflating a balloon in the aortic valve, increasing the valvular area, and immediately improving the gradient (95). However, it is extremely important that the Heart Team make a proper decision based on the individual characteristics of the patients (83). The Heart Team can be summarily described as a multidisciplinary group of cardiovascular specialists that may include cardiologists, cardiac surgeons, imaging experts, and nurses, who collaborate to determine the most appropriate treatment strategies for patients with complex heart conditions (96).

AVS leads to significant morbidity and mortality and there are very limited treatment options beyond SAVR and TAVI. Developing pharmacological strategies could slow disease progression, reduce the need for invasive interventions, and improve patient outcomes. Over the last few years, several trials have been carried out to test the potential of different promising therapeutic modalities, although these ultimately did not provide robust evidence of efficacy. Some examples include: ataciguat, a guanylyl cyclase activator, which may be useful in reducing blood pressure, and, thus, reducing overall pressure afterload (97); ARBs, which may contribute to slow AVS progression and LV remodeling (98); eplerenone, a selective aldosterone receptor antagonist, which may improve cardiac hemodynamics after TAVR (99); folic acid (Clinicaltrials.gov ID: NCT05861648)(100) and menaquinone (vitamin K2) (Clinicaltrials.gov ID: NCT04429035)(101) which may also slow the progression of valve calcification.

It was also shown that it is important to control diabetes mellitus, as it is considered one of the risk factors for the development and worsening of AVS, probably due to the associated inflammatory status (102–104).

Serum biomarkers play an important role in monitoring patients with AVS. For now, guidelines have only approved N-terminal pro-B-type natriuretic peptide (NT-proBNP) and B-type natriuretic peptide (BNP) serum concentrations as prognostic biomarkers (105). BNP can be used as a prognostic marker in asymptomatic patients with severe AVS, and its concentration can help clinicians determine when AVR is needed. BNP and NT-proBNP combined with other biomarkers could possibly enhance diagnostic and therapeutic accuracy in AVS patients, but additional research is required to validate their clinical utility and

establish standardized thresholds (106). Moreover, many studies are trying to find other biomarkers that could possibly be used to help monitor AVS progression and to help assess disease prognosis. Examples include high-sensitivity troponin T, Lp(a) (107), blood total cholesterol/high-density lipoprotein cholesterol (HDL-C) ratio (108), C-reactive protein (CRP) (109), growth differentiation factor-15 (GDF-15) (110), procalcitonin (PCT) (111), among others.

Current clinical recommendations provide specific guidance regarding the monitoring and management of patients with AVS. According to the 2021 ESC/EACTS guidelines for the management of valvular heart disease, patients with mild to moderate AVS and significant valve calcification should be followed every 12 months. Young patients with mild AVS and without significant valve calcification should be followed up every 2-3 years. Patients with severe AVS who remain asymptomatic should be followed up every 6 months with exercise testing and echocardiography, while also considering the possibility of BNP analysis (77).

1.1.6 Sex Differences in Aortic Stenosis

It is well-established that AVS presents sex-specific features with relevant impact on pathophysiology, outcomes, and management of the disease (112).

Regarding the anatomy, women tend to have smaller aortic annuli, shorter distances between the annulus and the coronary ostia, and smaller hearts in general (with subsequently smaller LV mass). Women have a lower prevalence of congenital bicuspid aortic valve, leading to a lower incidence of AVS in younger patients (113–117).

A major difference between men and women lies in the extent of valve calcification and fibrosis. Some studies showed that, for a similar hemodynamic AVS severity, men tend to have higher calcification levels but lower levels of fibrosis (118,119). Simard *et al*. characterized patients with AVS 3 months before aortic valve replacement, by Doppler echocardiography and multidetector computed tomography to evaluate possible sex differences. They found that the median aortic valve calcification in women (1279 (882–1915) Agatston units) was lower than men (2741 (1839–3858) Agatston units) ($p<0.0001$), and that mean valve weight in

women (1.87±0.58g) was also lower than men (2.66±1.07g) ($p<0.0001$). It also shown that, after adjustment, female sex was an independent risk factor for higher fibrosis score in AVS valves (P=0.003) (120–123).

Women have greater odds of presenting nuanced symptoms when compared to men, typically presenting nonspecific symptoms like shortness of breath and dizziness. They also have a shorter duration of exercise and a lower anaerobic threshold but are less likely to suffer from angina when compared to men. These differences may also be responsible for delaying the diagnosis and treatment in women (114,116,124), which leads to an exacerbation of LV remodeling. In fact, women are referred for SAVR later than men (125), not only due to discordance in echocardiographic parameters but also because they often need concomitant procedures, such as aorta repair or replacement, or coronary artery bypass grafting (112). Women are, in fact, more likely to undergo TAVR, not only because they are diagnosed or develop the disease at a more advanced age, but also because they may benefit substantially more from this procedure than men (116,126–128). Still, women seem to have a higher incidence of major bleeding and postprocedural vascular complications after TAVR than men (128–130).

From the cyto-histological point of view, male VICs tend to produce greater levels of myofibroblastic markers, and higher collagenolytic, and gelatinolytic activity, when compared to females. On the other hand, women's cells show greater metabolic activity and collagen production (131). The molecular mechanisms responsible for these differences between the sexes are not yet fully understood; however, it is believed that they may be related to the differences in inflammation and lipoprotein profile, matrix remodeling, and the hormonal status (testosterone levels) (8,123).

Sex-specific differences in AVS affect its presentation, progression, and management, highlighting the complexity of the disease. Thus, further research is needed to fully understand the underlying mechanisms, and treatment strategies should be optimized and personalized for both sexes.

1.2 The promise of sodium-glucose cotransporter-2 inhibitors

1.2.1 Definition and mechanisms of action

Sodium-glucose cotransporter-2 (SGLT2) is a key transporter for glucose reabsorption in the kidneys. It is essentially located in the proximal portion of the renal tubule, mainly in the S1 and S2 segments. It is a high-affinity, low-capacity transporter capable of moving glucose against its concentration gradient from the nephron lumen to the tubular cells. It uses the energy generated from sodium influx down the concentration gradient, the latter created by the basolateral Na^+/K^+ ATPase. It contrasts with the glucose transporter 2 (GLUT2), which only allows passive glucose transport towards the plasma (132). SGLT2 and GLUT2 account for almost 90% of renal glucose retention, while the other 10% is recovered by the sodium-glucose cotransporter-1 (SGLT1), located more distally in the tubule (133).

SGLT2i, also known as gliflozins, are a class of drugs originally designed to reduce the rate of glucose reabsorption in the proximal tubule by inhibiting this co-transporter. The administration of SGLT2i results in an increased excretion of glucose in urine. It was shown that, in monotherapy, SGLT2i can reduce glycated hemoglobin A1c (A1c) by between 0.5%-1.0% in patients with type 2 diabetes mellitus (T2DM), also causing weight loss and decreasing blood pressure (134).

1.2.2 Pharmacokinetics

SGLT2i are absorbed through the gastrointestinal tract. There is no significant effect of food on SGLT2 absorption; however, they should be taken before the meal to avoid postprandial plasma glucose elevation. SGLT2i have high plasma protein binding capacity and extensive tissue distribution. These drugs undergo glucuronidation by uridine 5'-diphosphate-glucuronosyltransferases (UGTs) in the liver and kidneys (135), while cytochrome P450 has minimal effect on SGLT2i. SGLT2i are filtered from plasma at the glomeruli and bind to the SGLT2 at the early segments of the nephron. They have a long elimination half-life, allowing a once-daily administration (136). SGLT2i and their metabolites tend to be largely excreted in the urine, with a portion of the dose also eliminated through feces (137).

1.2.3 Systemic effects

Despite being initially designed as glucose-lowering drugs, gliflozins show many beneficial effects on the whole body, translating into cardiovascular benefits. This includes a reduction in blood pressure, a decrease in body weight, a decrease in uric acid, and a decrease in albuminuria (138,139). Gliflozins also show hemodynamic effects, such as an increase in osmotic diuresis, increased natriuresis, decreased fluid overload, decreased arterial stiffness, and improved endothelial function. SGLT2i also show endocrine effects, namely increased β-cell function, decreased insulin resistance, increased glucagon levels, decreased sympathetic tone, and increased angiotensin 1–7 levels. At the myocardial level, gliflozins decrease myocardial stretch, oxidative stress, inflammation, sodium-hydrogen antiporter 1 levels, while increasing ketone levels (138,139). For now, the specific effects on the aortic valve, particularly in the context of AVS, remain largely unexplored and not yet well understood.

1.2.4 Adverse Effects and Drug Interactions

Some common adverse effects of SGLT2i include an increased risk of hypoglycemia, urinary tract infection events (including mycotic infections), volume depletion and hypotension (140). Other rarer effects (less than 1% of cases) include diabetic ketoacidosis, lower limb amputation, and, in the most serious cases, Fournier gangrene (141). The risk of these major adverse effects is higher in patients aged ≥65 years, patients using loop-diuretics, and patients who present an estimated glomerular filtration rate (eGFR) <60mL/min/1.73m^2 (138).

There are some known interactions between SGLT2i and other drugs. When SGLT2i are combined with an insulin secretagogue, such as a sulfonylurea, or insulin itself, there is an increased risk of hypoglycemia; therefore, it is important to adjust the doses to avoid it. The use of an SGLT2i may reduce serum lithium levels when these are administered. UGT enzyme inducers, like rifampicin, may reduce the effectiveness of some SGLT2i, such as canagliflozin; therefore, it is important to consider increasing the dose of the SGLT2i when those are used concomitantly. Also, there are other known interactions with other drugs, such as non-selective beta blockers or digoxin (138).

1.2.5 Indications

At this moment, four gliflozins are approved by the Food Drug Administration (FDA) for their use in adults (canagliflozin, empagliflozin (EMPA), dapagliflozin and ertugliflozin). Their indications vary per agent; however, they are all approved in T2DM to help control blood glucose (142). Other FDA-approved indications include the improvement of cardiovascular health and the reduction of the risk of death in patients with heart failure (143,144), reduction of the risk of eGFR decline and progression of CKD (145), and reduction of major cardiovascular events in patients with T2DM and cardiovascular disease (146). These drugs can also be used off-label in some circumstances, such as in the management of obesity (in combination with glucagon-like peptide-1 receptor agonists) (147) or in the management of non-alcoholic fatty liver disease (148).

In Europe, the panorama is similar. According to the ESC Guidelines 2021, SGLT2i are also recommended to reduce the risk of heart attack and stroke in all patients with diabetes and CVD (77). They are also recommended for patients with diabetes and CKD, to reduce the risk of CVD and kidney failure. In the most recent update (2023), EMPA and dapagliflozin are now Class I level A recommendation for patients with Heart Failure with mildly reduced Ejection Fraction (HFmrEF) and Heart Failure with preserved Ejection Fraction (HFpEF), being the only treatment with Class I level A recommendation in patients with HFpEF (149). However, despite the cardiovascular benefits, there is no specific recommendation for valvular heart disease, particularly AVS.

1.3 Valve interstitial cells: the main disease drivers in aortic valve stenosis

VICs are a highly relevant model for studying AVS, due to their direct involvement in the disease's pathological processes, such as calcification and fibrosis. These cells, which constitute the primary cellular population of the aortic valve, play critical roles in extracellular matrix remodeling, osteogenic differentiation, and fibrotic responses, key features of AVS progression (150).

Unlike animal models or other cell types, human VICs provide a more accurate reflection of the human-specific molecular and cellular mechanisms underlying AVS. For instance, while porcine and murine

models are commonly used due to their morphological similarities to human valves, significant interspecies differences exist in cellular signaling pathways, such as those involved in calcification and inflammation (151,152). These discrepancies can limit the translational applicability of findings derived from animal cells. Additionally, one of the main drawbacks of animal VICs is that experiments must later be replicated in human VICs, in order to draw meaningful conclusions that can be translated to the clinical setting.

The ideal source of VICs to model this disease would be a healthy adult human heart valve, since most of the cells are still quiescent (153). However, these are rarely available (except on the occasion of heart transplantation) and impose relevant ethical constraints. Obviously, it is also possible to use animal-derived cells. Animal VICs may be more readily accessible, with fewer ethical restrictions concerning their acquisition and handling compared to human VICs. Also, animals with a native genotype do not naturally develop aortic valve calcification, allowing the direct induction of valve mineralization without interference of comorbidities, which are relevant confounding factors. Furthermore, animal cells tend to show very accelerated growth in culture, saving time and laboratory resources (154). The main animal models used nowadays are the swine and ovine. Pigs are by far the most used animal for obtaining VICs, because they are easily obtained and because porcine VICs grow easily in vitro (155). Sheep are a promising alternative to study the process of valve calcification, since sheep exhibit a higher calcium metabolism (156). In any case, the use of human VICs avoids the need for cross-species extrapolation, which can introduce variability and limit the clinical relevance of results. Human-derived VICs have consistently demonstrated their value in modeling AVS-specific mechanisms. For example, Bogdanova et al. (157) successfully used human VICs to investigate fibrosis and calcification pathways, highlighting their utility in identifying potential therapeutic targets. Similarly, Rutkovskiy et al. (43) emphasized the phenotypic plasticity of VICs and their relevance in osteogenic and myofibroblastic differentiation, critical processes in AVS pathophysiology. VICs have also been used for screening potential pharmacological inhibitors, particularly through standardized *in vitro* models, enabling reproducible assessment of anti-calcific effects (150,158).

To study the process of valve calcification and fibrosis, VICs can be maintained in a pro-osteogenic or pro-fibrotic medium, respectively. Pro-osteogenic media induce VICs osteogenic differentiation by providing

factors that stimulate the expression of osteoblast-specific genes and proteins. These media typically contain components such as dexamethasone, ascorbic acid, and β-glycerophosphate, which collectively promote the differentiation of VICs into osteoblast-like cells. β-glycerophosphate donates the phosphate groups for the formation of calcium phosphate crystals (159). Ascorbic acid promotes osteogenic differentiation by stimulating the production of type I collagen, essential for the formation of the bone extracellular matrix. Dexamethasone enhances osteogenic differentiation by increasing the expression of markers such as alkaline phosphatase and osteocalcin, promoting mineralization (160). This process is characterized by increased expression of osteogenic markers like osteocalcin and alkaline phosphatase (154). To assess calcification at the histological level, alizarin red (1.2-dihydroxyanthraquinone) staining is one of the most common methods, being optimal in terms of cost, simplicity, and time consumption (150). To induce fibrosis, VICs medium should be supplemented with specific pro-fibrotic factors that promote ECM deposition and myofibroblastic differentiation, such as transforming growth factor-beta 1 (TGF-β1), a cytokine that strongly induces fibrosis by stimulating collagen production and facilitating the transition of VICs into an activated, myofibroblast-like state (161). For fibrosis assessment, the most common method is to quantify biomarkers such as collagen type I or to use ECM-specific stains such as Picro-Sirius Red (162).

1.4 Do SGLT2i affect the pathways involved in the development and progression of AVS?

The progression of AVS involves multiple molecular pathways that contribute to increased valve fibrosis and calcification. Key pathways associated with valve calcification include the NOTCH pathway, the WNT–β-catenin pathway, the TGF-β1/BMP pathway, and the OPG/RANKL/RANK pathway (163–165). Additionally, calcific AVS is characterized by significant increases in non-collagenous matrix proteins (e.g. osteopontin and osteocalcin), Runx2, BMP2, and TLR-2/4 (164). Proteins of the NLRP3 inflammasome also seem to play a significant role in the calcification process, being increased in AVS as well (166).

Evidence suggests that SGLT2i can modulate these pathways in distinct contexts, being associated with the inhibition of the WNT–β-catenin, PI3K/AKT/GSK-3β/mTOR (in HepG2 cells, a widely used human liver

cancer cell line) (167) and NLRP3 inflammasome pathways (in human and rat vascular SMCs) (168). In some studies, they also seem to reduce the expression of TLR-2/4 (169), Runx2 and BMP2 molecules (168,170). In addition, SGLT2i are associated with an enhanced expression and activity of Sirtuin 1 (Sirt1), a class III deacetylase, which has been shown to reduce inflammation (171,172). The TGF-β1/Smad pathway, critical in the fibrotic response, also seems to be affected by SGLT2i. Santos-Gallego et al. (173) showed reduced levels and activity of TGF-β in the myocardium of EMPA-treated swines, resulting in reduced interstitial myocardial fibrosis (103,173). The fact that the drug interferes with signaling pathways implicated in AVS in other cells and tissues suggests that it may also hold promise for the treatment of AVS.

In the context of AVS, only one research group has focused on the effects of EMPA in the disease, specifically on VECs. Hmadeh et al. (174) evaluated the impact of EMPA on oxidative stress and endothelial dysfunction, using healthy porcine VECs. The results demonstrated that EMPA markedly reduced oxidative stress, decreasing the production of ROS by approximately 30%. Furthermore, EMPA significantly reduced the pro-oxidant response. This SGLT2i also minimized the protein levels of $p67^{phox}$ (a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit) and ACE. It also prevented p65 NF-κB phosphorylation. These effects were accompanied by an approximately 40% reduction in THP1 cell adhesion (174). These findings underline the efficacy of EMPA in mitigating oxidative stress, inflammation, and thrombogenicity in porcine VECs, providing a strong rationale for investigating the therapeutic potential of SGLT2i in human AVS. Yet, the effects of EMPA in VICs remain elusive, and such experiments could provide more relevant insights into the therapeutic potential of SGLT2i in AVS, since they are the main disease drivers, while VECs are mostly implicated in disease initiation. As mentioned previously, unlike VECs, which primarily regulate the endothelial barrier and thrombogenicity, VICs directly drive the calcification and fibrosis that characterize the disease. By targeting VICs, one can elucidate whether SGLT2i influence the pathological remodeling of the aortic valve and generate preclinical evidence for a drug repurposing trial to treat AVS (174).

Main goal

Given the significant morbidity and mortality associated with AVS, the absence of effective pharmacological therapies underscores the critical need for the development of innovative treatments. Since fibrosis and calcification are the main pathological hallmarks of AVS, these treatments should prioritize tackling these deleterious processes.

Our main objective is to investigate whether there is a direct effect of SGLT2i on the calcification process, one of the key pathological hallmarks of AVS. To this end, this project aimed to collect aortic valves from male and female patients with severe AVS, implement a protocol for the isolation and expansion of VICs (175), and subsequently induce calcification using a combination of β-glycerophosphate, L-ascorbic acid 2-phosphate, and dexamethasone to model the disease in vitro. Finally, the project aims to assess if VICs cultured under pro-osteogenic conditions and treated with SGLT2i would exhibit reduced calcification, thus providing preclinical evidence to support potential drug repurpose. A schematic representation of our study is shown in Figure 1.

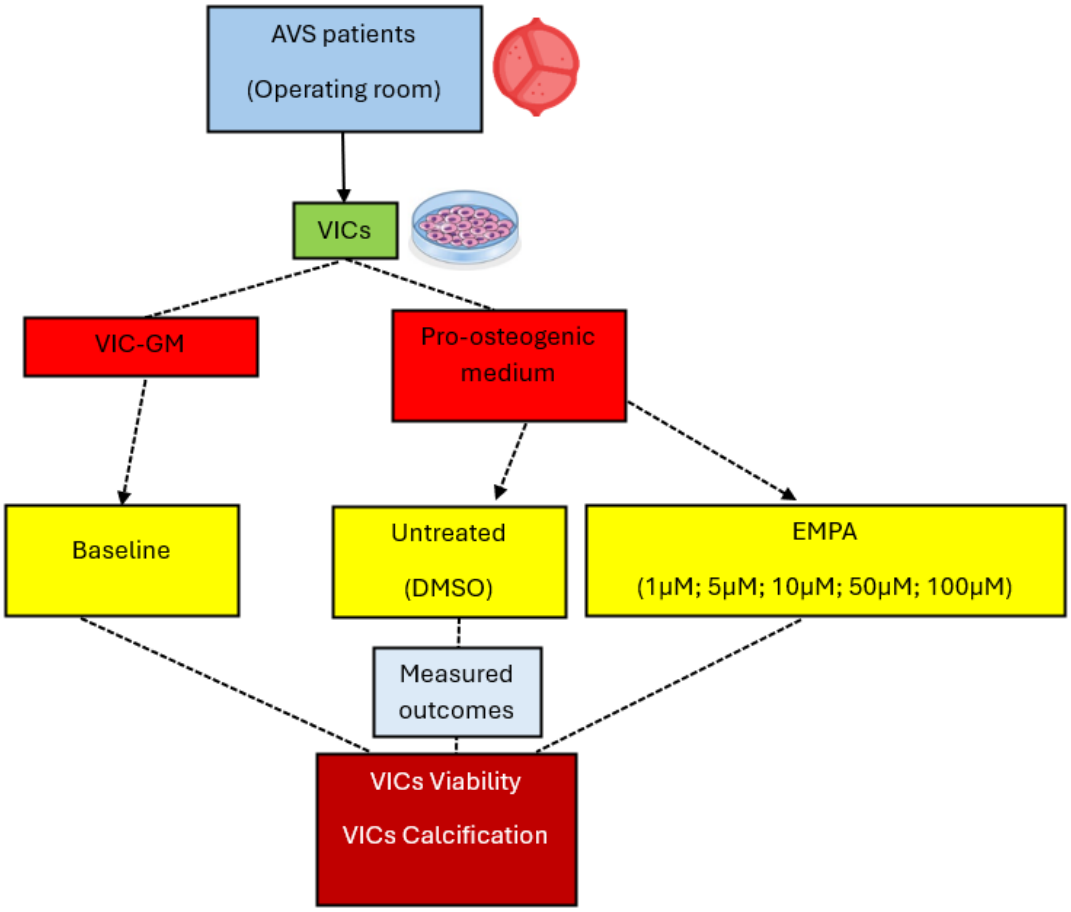

Fig 1: Schematic representation of the experimental strategy. Aortic valve tissues were obtained from patients undergoing SAVR due to severe AVS. VICs were isolated, expanded in standard growth medium (VIC-GM), and cryopreserved until use. For the experiments, cells were seeded and cultured under either standard conditions (VIC-GM) or pro-osteogenic medium, with increasing concentrations of EMPA. After 21 days, outcomes were assessed: cell viability was measured using a resazurin-based assay, and calcification was evaluated by Alizarin Red S staining followed by image analysis. SAVR = surgical aortic valve replacement; AVS = aortic valve stenosis; VICs = valvular interstitial cells; VIC-GM = VIC growth medium; EMPA = empagliflozin.

2. METHODS

2.1 Patient recruitment and sample collection

Between April and November of 2024, tricuspid aortic valves were obtained from six patients with severe AVS undergoing SAVR at the Cardiovascular Surgery Department of *Unidade Local de Saúde de São João, Porto, Portugal*. Clinical data, including sex, age, comorbidities, and medication, were collected from medical records. Disease severity was determined by transthoracic Doppler echocardiography. Severe AVS was defined by i) a mean transvalvular pressure gradient >40 mmHg; ii) an indexed aortic valve area <0.6 cm^2/m^2; iii) the need for surgical valve replacement. Three male and three female patients were included

after providing written informed consent. Patients with congenital heart valve malformations (including bicuspid valves) or rheumatic aortic valve disease were excluded from the study. For protocol optimization, one valve from a patient with aortic valve insufficiency was included. This investigation is in accordance with the 1964 Declaration of Helsinki and its later amendments and was approved by the institutional review board (ethics committee of *Unidade Local de Saúde de São João*, ref CEC109-2020).

Immediately after surgical excision, the aortic valves were stored in a cardioplegic solution (Custodiol) containing 1% of an antibiotic-antimycotic solution (Antibiotic-Antimycotic, Gibco, Cat#15240-062) to minimize microbial growth. Samples were kept at 4°C until delivery to the laboratory for further processing.

2.2 VICs isolation and expansion

A modified protocol based on Cuevas et al. (176) was followed for VICs isolation. Under a biosafety cabinet, the valve was transferred to a pre-weighed sterile 10-cm dish, weighed, and photographed. The valve was washed thoroughly with Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, Cat# 14190-094) supplemented with 2% AA, and non-fibrotic and non-calcified sections of the leaflets were excised using sterilized scissors and forceps. The gross fragments were washed again in a 50 mL conical tube filled with DPBS enriched with 2% AA solution by inverting several times for about two minutes.

The tissue fragments were subsequently transferred to a 60-mm dish and incubated in 7 mL of a 1%-collagenase solution for 10 min at 37°C, rocking the tissue every 2 minutes to facilitate dissociation of the endothelium. VECs were then removed from both sides (aortic and ventricular) through mechanical swabbing, and the remaining tissue was further digested overnight for about 18h. The VICs were released by pipetting with a sterile Pasteur pipette and the resultant cell suspension was passed through a 0.70µm filter. To maximize cell yield, 7 mL of VICs growth medium [VIC-GM: Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Cat# 11965-092), 10% fetal bovine serum (FBS) (Gibco, Cat# 10270-106), and 1% penicillin-streptomycin (Gibco, Cat# 15140-122)] was added to the cell strainer. The suspension was centrifuged at 200×*g* for 5 min at 20°C, and the pellet was resuspended in VIC-GM. Viable cells were determined by Trypan-blue exclusion using an automated cell counter (Countess, ThermoFisher Scientific).

The isolated VICs were seeded at around 1 million cells/75 cm^2 in type I collagen-precoated T75 flasks. Essentially, a 0.1% collagen solution (0.1M acetic acid) was diluted 10x with sterile water, and 4mL was added to each flask. The flasks were then incubated under the hood for 4h at RT plus 1h with the UV light on, and the remnant solution discarded. Finally, the flasks were fully dried overnight in the cell incubator. Cells media was replaced every 2-3 days, and cells were passaged upon reaching 90% confluence. To split cells, these were treated with 4mL of pre-warmed dissociated reagent (a Low-Endotoxin Trypsin (TrypLE) solution, Gibco, Cat# 12605-010) for 10 minutes at 37°C. After incubation, 8mL of VIC-GM 2%AA was added to each flask to neutralize trypsin, and the cells recovered by centrifugation at 200xg for 5min. Then, the supernatant was removed, the pellet was resuspended in pre-warmed VIC-GM 2%AA and the number of viable cells was determined. After the initial expansion, cells were seeded at ~0.5 million viable cells for T75 flask (passage 0, P0) and incubated at 37ºC. When cells reached a 90% confluency, these were trypsinized and cryopreserved in cryopreservation medium (Hyclone Hycryo-STEM) (Cat# SR30002.02). In this work, cells were used until passage 2.

2.3 Experimental conditions and treatment protocols

For the assessment of calcification, VICs at passage 2 were seeded (30000 cells/well) in 48-well plates, and for the viability assays, VICs at the same passage were seeded (10000 cells/well) in 96-well plates. Cells were initially cultured in VIC-GM until 100% confluence was reached. Subsequently, the medium was replaced with osteogenic medium (OM), consisting of DMEM supplemented with 5% FBS, 10mM β-glycerophosphate, 50µg/mL vitamin C, and 10nM dexamethasone.

To evaluate the effects of EMPA on VICs viability and calcification, increasing concentrations of EMPA (0 µM, 1 µM, 5 µM, 10 µM, 50 µM, and 100 µM) were added to OM. Dimethyl sulfoxide (DMSO) was used as a vehicle control. Media were replaced every 2-3 days, and cells were incubated for a total of 21 days.

2.3.1 Cell viability assay

To assess cell viability in response to EMPA treatment, a resazurin reduction assay was performed in 96-well plates. After 21 days of culture, the medium was removed, and the cells were incubated with 50µM resazurin in OM. The cells were incubated for 4 hours, and the absorbance of the reduced compound (resorubin) was read at every hour. Absorbance was measured at 570nm and at 600nm for correction, using a TECAN Spark microplate reader. Each condition was triplicated. To assess the effect of EMPA on cell viability, the absorbance in wells treated with 0µM EMPA was used as reference (100%) and the ratio of absorbance was calculated for each drug concentration.

2.3.2 Calcification assessment

To evaluate calcification, an initial experiment was conducted to assess the baseline calcification potential of the VICs. For this purpose, a plate with three replicates of each cell line was prepared and maintained in standard VIC-GM for 24h, without osteogenic stimulation or drug treatment. Subsequently, for the main calcification assays under osteogenic conditions and EMPA treatment, cells were cultured in 48-well plates and calcium crystals were stained with Alizarin Red S solution. Briefly, the culture medium was carefully removed, and the cells were washed twice with DPBS before being fixed with cold neutral buffered formalin for 30 minutes. Subsequently, the formalin was removed, the cells were washed twice with distilled water and stained by incubating with 250 µL/well of Alizarin Red S solution (2%, Sigma-Aldrich, Cat# A5533, St. Louis, MO, USA), for 30 minutes in the dark. Following the incubation, cells were washed twice with 300 µL/well deionized water. Once the cells were completely dried, they were photographed under a digital, transmitted light inverted microscope (EVOS^{TM} XL Core Imaging System, Invitrogen) at a 100x magnification (4 images per well; 1 image per quadrant). Additional settings were brightness level 55, TIF format, and 3MP resolution. The images were analyzed using the ImageJ Pro Plus program. Calcified areas were delimited and calculated and normalized to the total area covered by cells.

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 10.4.1, with a significance threshold set at $p < 0.05$. Continuous clinical variables were expressed as mean ± standard deviation (SD), while categorical variables were reported as absolute frequencies and their respective percentages. For the cellular experiments, results were presented as fold-change values relative to the respective baseline or control condition, depending on the specific experimental context. To assess normality, Shapiro-Wilk tests were performed.

For clinical data, comparisons of continuous variables between female and male patients were conducted using unpaired *t*-tests when normality was confirmed, or Mann–Whitney *U* tests when normality was not met. Categorical variables were compared using Fisher's exact test.

For the cellular assays, differences across multiple EMPA concentrations within the same cell group were analyzed using one-way or two-way ANOVA for normally distributed data, followed by Tukey's multiple comparisons tests. When normality was not met, a Kruskal–Wallis test was applied, followed by Dunn's post hoc test. Comparisons between female and male VICs at each concentration were performed using unpaired *t*-tests or Mann–Whitney *U* tests, depending on data distribution.

3. RESULTS

The main objective of this study was to investigate whether SGLT2i, namely EMPA, modulates the process of valve calcification, one of the main pathological mechanisms of AVS. The following sections present the results of each experimental stage, providing a stepwise analysis of cell behavior.

3.1 Characterization of the patients and of the excised valves

First, we evaluated the clinical characteristics of the donor patients and the main features of their aortic valves. This characterization is crucial to contextualize the *in vitro* findings and to explore potential correlations between patient-specific features and VICs' behavior.

The baseline characteristics of the 6 patients enrolled in the present study are listed in Table 2.

Table 2: Clinical and echocardiographic features of the patients

	Female	Male	p value
Demographics:			
Age, mean ± SD (years)	73 ± 3.6	68.3 ± 0.6	0.20
BMI, mean ± SD (kg/m2)	32.5 ± 6.3	28.6 ± 4.2	0.43
Smokin (former or current) (%)	0	66.7	0.40
Diabetes (%)	33.3	33.3	>0.99
Hypertension (%)	100	66.7	>0.99
Dyslipidemia (%)	66.7	33.3	>0.99
Extracardiac arteriopathy (%)	0	66.7	0.40
Disease severity:			
Aortic valve mean gradient, mean ± SD (mmHg)	48.0 ± 2	72.0 ± 16.5	0.06
Aortic valve max gradient, mean ± SD (mmHg)	72.3 ± 9.1	116.3 ± 25.0	**0.048**
Aortic valve area, mean ± SD (cm^2)	0.87 ± 0.1	0.73 ± 0.1	0.06
Ejection fraction, mean ± SD (%)	58 [a]	59.7 ± 3.3	[a]
Usual medication:			
Statins (%)	100	33.3	0.40
ACEI (%)	66.7	0	0.40
ARBs (%)	33.3	66.7	>0.99
Diuretics (%)	100	66.7	>0.99
Antiplatelets (%)	66.7	33.3	>0.99
SGLT2i (%)	33.3	33.3	>0.99

The comparison was made between male and female patients, using parametric t-tests for variables with a normal distribution or non-parametric t-tests (Mann-Whitney test) for variables with a non-normal distribution. For categorical variables, Fisher's Exact Test was used. [a] - due to the lack of information from one of the donors, it was only possible to calculate the average of 2 female patients (n=2), so statistical significance was not possible to assess for this variable. SD = standard deviation; BMI = body mass index; ACEI = angiotensin-converting enzyme inhibitor; ARBs = angiotensin receptor blockers; SGLT2i = Sodium-Glucose Co-Transporter 2 Inhibitor.

Among the collected clinical parameters, the only statistically significant difference between sexes was observed in the maximum aortic valve gradient, which was higher in the male group. Although not statistically significant, probably due to the limited sample size, male patients tended to present higher mean

gradients and smaller valve areas. Preoperative ejection fraction was missing for one female patient, hindering group comparison.

Representative photographs of each of the human stenotic aortic valves excised prior to VIC isolation can be seen in Figure 2. Notably, male valves, particularly valves 3 and 4, exhibited a more prominent vascularized appearance, which may reflect advanced pathological remodeling and neovascularization, features often associated with severe calcific aortic valve disease. In fact, valve 4 displayed the most irregular and heterogeneous surface, with visible fibrotic thickening and vascular structures, likely contributing to its lower cell viability observed during the isolation phase.

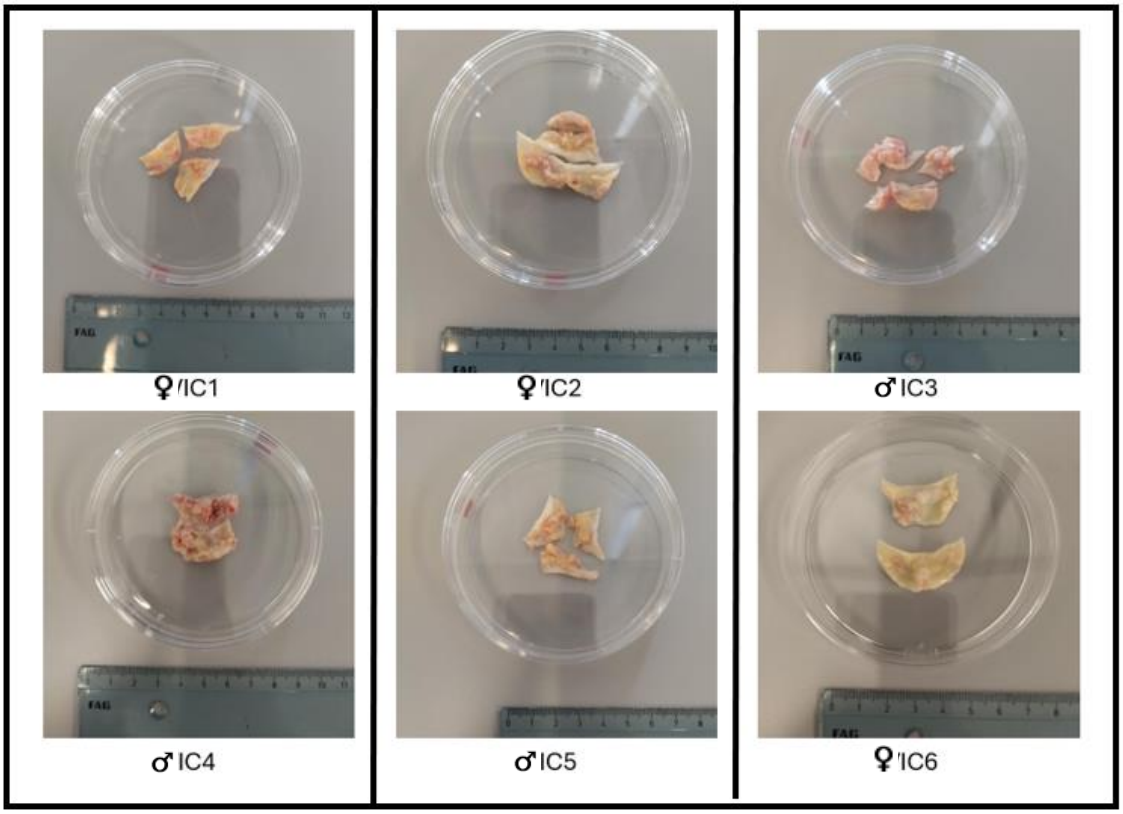

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Fig 2: Representative photographs of the human stenotic aortic valves. A ruler was included as reference. The photographs were taken before the isolation of VICs.

3.2 Isolation and expansion of VICs

Human VICs were isolated from stenotic aortic valves in order to obtain a relevant and representative cellular model of the disease. First, VICs were isolated and expanded to establish viable cell cultures that were sufficiently numerous for subsequent assays.

Of note, a valve from a patient with aortic valve insufficiency was used initially to optimize the isolation and culture techniques. Although not included in experimental analyses, it provided valuable insights on the efficacy of trypsinization time, plate coating importance, and measures to control microbiological contamination. This approach contributed to improved cell yield and viability across the remaining VIC lines. Meanwhile, VICS1 and VICS2 (porcine valvular interstitial cell lines) were obtained from 2 healthy castrated male pigs, both 5-months old and both submitted to extracorporeal circulation. They allowed the comparison of VICs' isolation and expansion between healthy tissues and those derived from calcified valves.

The initial isolation of VICs demonstrated high efficiency, with cell viabilities above 80% in most cases, except for VIC4 (69%). Female VICs showed an average yield of 2.42×10^6 cells with an average viability of 87.7%, reaching 90% confluence in 12 days. Male VICs showed an average yield of 1.59×10^6 cells, with an average viability of 82.3%, reaching 90% confluence in 10 days, although with greater variability between samples. No significant differences in cell yield were found between sexes ($p > 0.05$). These initial differences between groups are graphically represented in Figures 3 and 4, illustrating the initial cell yield and viability of human VICs after isolation, respectively. More detailed data about VICs' isolation and expansion are available in the Supplementary Materials.

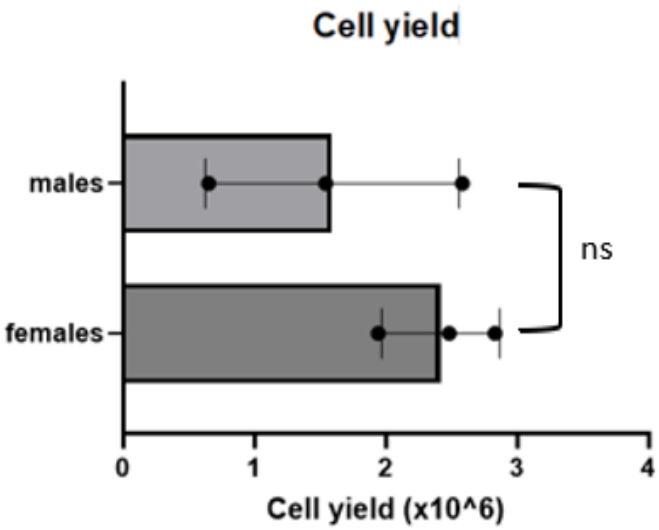


Fig 3: Initial yield of human VICs after isolation (males and females). Bar chart comparing the initial yield of VICs isolated from male and female aortic valves. Each bar represents the average yield (total number of viable cells obtained immediately after enzymatic digestion and filtering) per valve. ns = non-significant. Comparisons were performed by unpaired t tests.

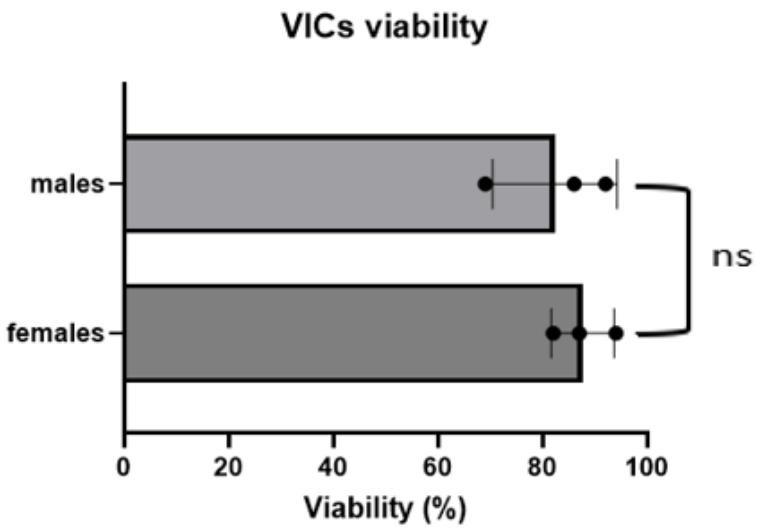


Fig 4: Human VICs' initial cell viability. Bar chart showing the initial viability (%) of VICs immediately after isolation, comparing male and female samples. Viability was assessed using the Trypan Blue Exclusion method and automated cell counting. ns = non-significant. Comparisons were performed by unpaired t tests.

Porcine valves provided better yields (6.93 × 10^6 cells on average) (p=0.008) (Figure 5), viabilities above 85%, and reached 90% confluence in only 4-5 days.

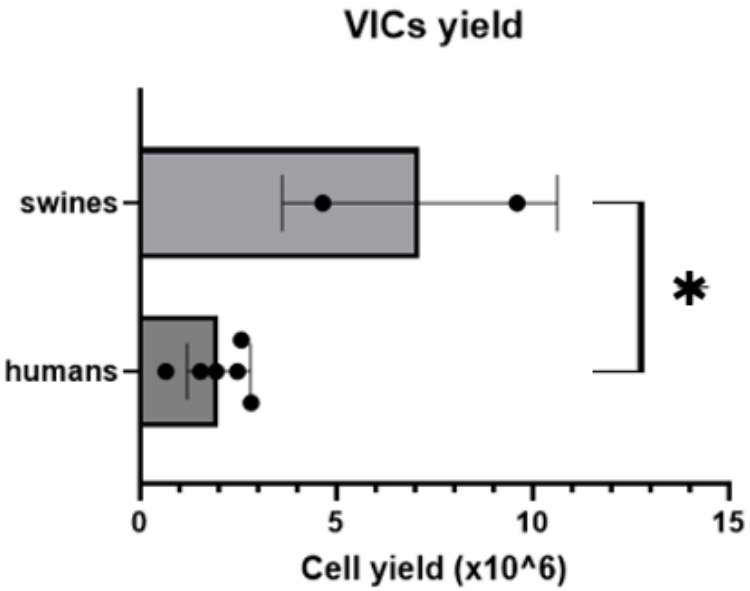


Fig 5: VICs' initial cell yield (humans vs swines). Bar chart comparing the initial yield of VICs obtained from human versus porcine aortic valves. (*) p<0.05. Comparisons were performed by unpaired t tests, showing significant differences between species (p=0.0080).

During subsequent passages, all VICs maintained high viabilities, with 90% confluence being reached between 7-14 days at P0, 6-9 days at P1, and 7-8 days at P2, reflecting a good proliferative capacity.

3.3 VICs' tolerance to EMPA

To evaluate the therapeutic potential of EMPA, it was first essential to confirm that the drug did not exhibit significant cytotoxicity toward VICs. For this purpose, a cell viability assay was performed for all 6 VIC lines, in 96-well plates, using increasing concentrations of EMPA, aiming to assess its impact on cell survival following a prolonged exposure period of 21 days.

Since the time needed to reduce Resaruzin depends on the specific cell metabolic activity, VICs were incubated with this compound for a total of 4 hours, and the absorbance was read every hour. An incubation time of 2 hours provided the best discrimination and was selected for comparisons. All VIC lines showed consistent results across the three independent experiments performed, both in terms of numerical values and macroscopic colorimetric progression, except for VIC1. Although VIC1 results were consistent throughout the 3 experiments, they systematically diverged from the results of all other cell lines. The absorbance values for VIC1 wells, specifically for the 0 µM EMPA wells, were consistently and significantly lower than those observed for the other cell lines, thereby skewing the overall viability calculations. This pattern suggests that VIC1 behaved as an outlier rather than exhibiting random variability. A possible explanation could be an edge effect or another technical artifact specific to that well position. As can be seen in Figure 6, the color of the VIC1-0 µM well is very similar to the color of the control well with no cells. This occurred in all three independent experiments, suggesting a systematic error occurring with these cells. Therefore, appearing to be an outlier, we decided to exclude the VIC1 readings from the statistical analysis, so as not to bias the results.

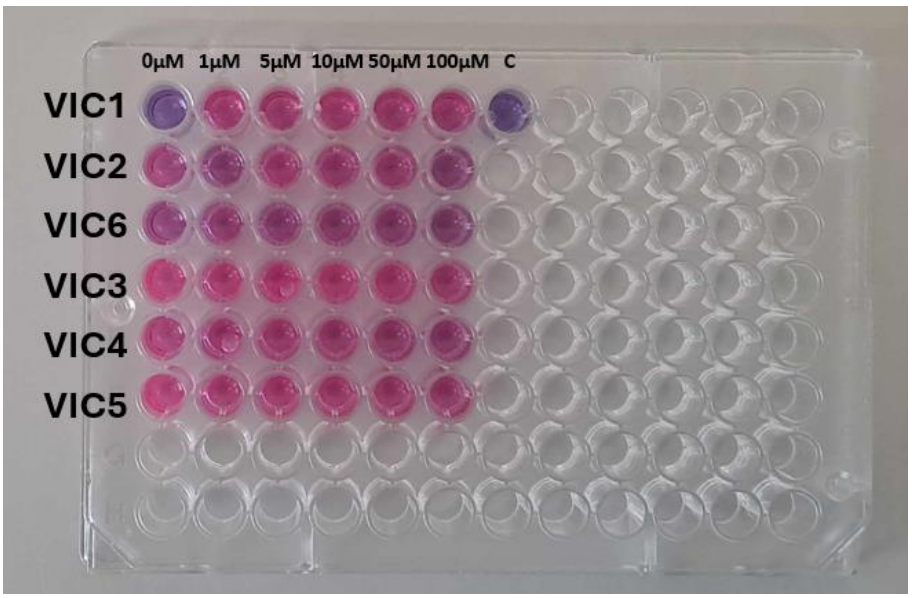


Fig 6: Representative photograph of the cell viability assay using Resazurin, taken after 2 hours of incubation. After 21 days of treatment with different EMPA concentrations, cells were exposed to Resazurin solution to assess metabolic activity. Viable VICs reduced Resazurin to Resorufin, producing a visible color shift.

The viability of each VIC cell line in response to different doses of EMPA can be seen in Figure 7. From this analysis, no major differences are observed across the different EMPA doses, with a general trend of gradual viability reduction as the concentration increases. Additionally, cell viability appears to be consistently higher in female VIC lines.

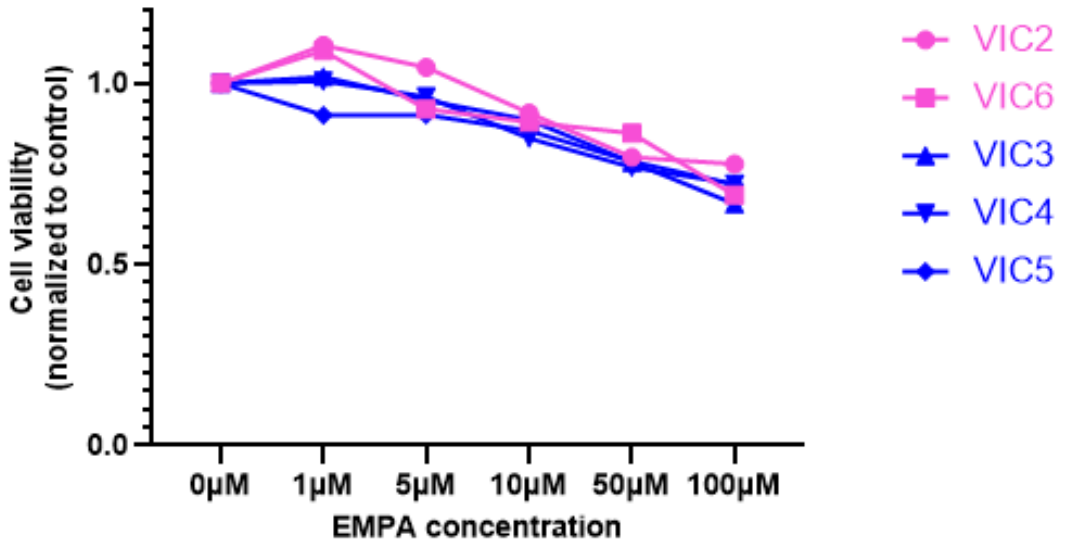
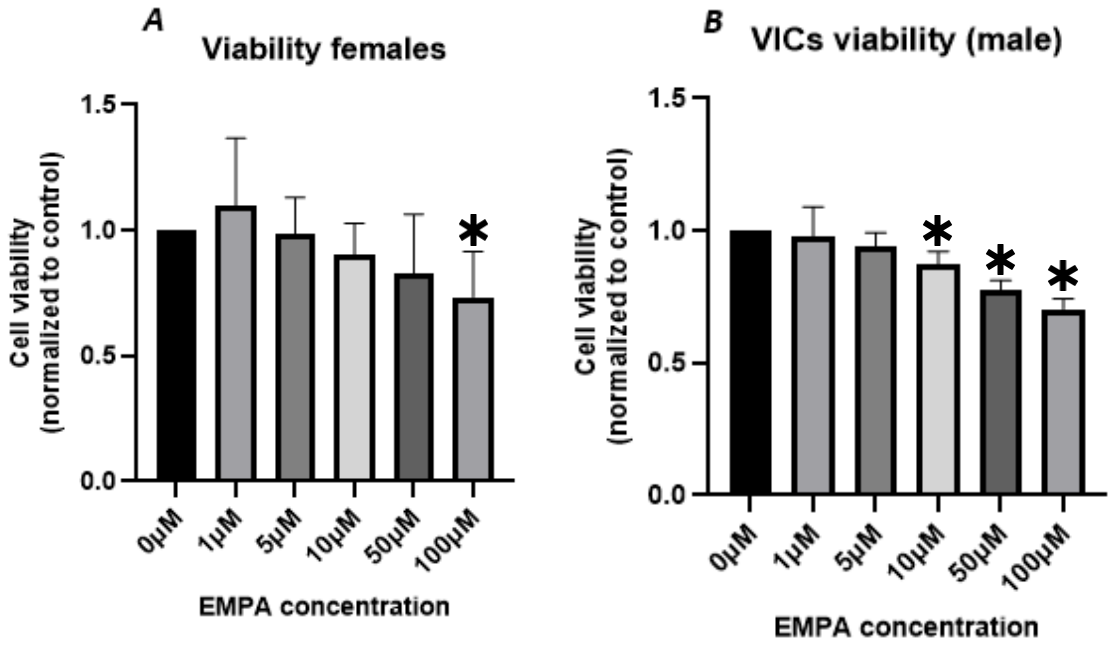


Fig 7: Cell viability of each VIC cell line, under EMPA treatment, assessed after 21 days of treatment with increasing concentrations of EMPA. Viability was determined using a Resazurin assay, with absorbance values indicating metabolic activity.

The Resazurin assay results show a gradual reduction in viability as the EMPA dose increases. Male VICs showed a significant reduction in viability starting at 10 µM EMPA concentration (Figure 8B), while in female VICs viability significantly decreased at 100 µM EMPA only (Figure 8A). In both sexes, the average viability in each group never decreased below 70%, not even at the highest doses (100 µM) of the drug, demonstrating a good safety profile of EMPA.



Figures 8A and 8B: VICs' viability in female (8A) and male (8B) donors, respectively, after 21 days of EMPA treatment. Bar charts showing the viability of human VICs from female and male donors after 21 days of treatment with increasing concentrations of EMPA. (*) $p<0.05$. Statistical comparisons were performed using parametric tests (One-way ANOVA followed by Tukey's multiple comparisons test). In female VICs, a significant reduction in viability was observed only at the highest concentration tested (100 µM), whereas in male VICs, viability significantly decreased starting at 10 µM, with a progressive decline at higher concentrations.

In summary, the Resazurin assay revealed a dose-dependent decrease in cell viability, with higher concentrations of EMPA progressively reducing cell survival. Although low concentrations did not significantly affect viability, cytotoxic effects became increasingly evident at higher doses. Importantly, this pattern was not consistent across both male and female derived VICs.

3.4 Impact of EMPA on VICs' Calcification

Finally, we quantified the effect of EMPA using Alizarin Red S staining and digital image analysis. This step aimed to verify whether EMPA treatment would be able to attenuate the valve mineralization process, and whether there would be differences in response between VICs derived from male and female individuals.

To evaluate whether EMPA can limit the calcification of human VICs under osteogenic stimulation, cells were cultured in 48-well plates with pro-osteogenic media and treated with increasing concentrations of EMPA for 21 days. After the incubation period, calcification was assessed using Alizarin Red S staining. Following staining, macroscopic photographs of the 48-well plates were taken to document overall calcification patterns, and only then were high-resolution microscopic images acquired from each well. These images were subsequently analyzed using ImageJ software to quantify the percentage of calcified area relative to total tissue area. This method enabled a detailed comparison of calcium deposition across conditions and between VICs from male and female donors, aiming to explore potential sex-specific responses to EMPA.

Before the drug assay, the proclivity to calcification was tested in all cell lines, maintained under pro-osteogenic conditions. It was found that male cell lines presented a significantly higher extent of calcification (66.39 ± 0.20%) than female cell lines (37.95 ± 0.01%) (p=0.0003, Figure 9).

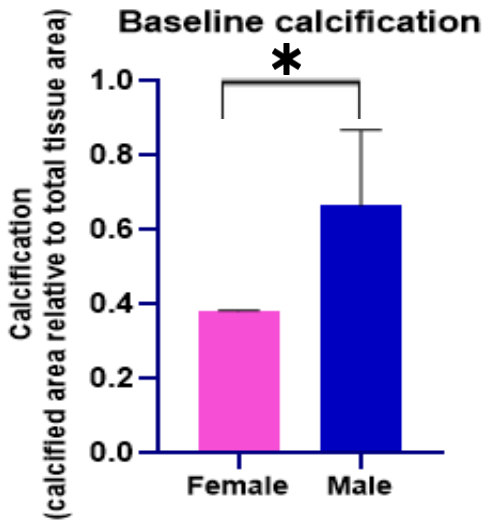
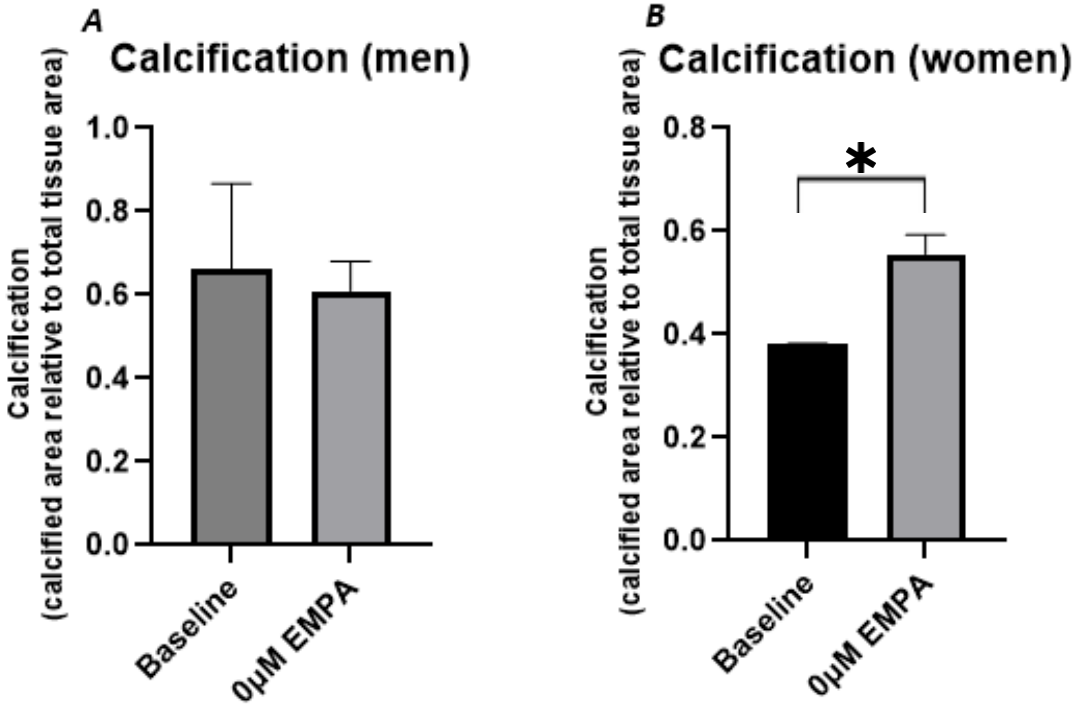


Fig 9 VICs' calcification (Baseline). Bar chart showing the the relative calcification levels in male and female VICs under baseline conditions. Quantification of the calcified area, after Alizarin Red S staining, using ImageJ software. (*) $p<0.05$. Statistical comparison was performed using a Mann–Whitney U test.

The comparison of the calcification extent between unstimulated (baseline) and stimulated conditions (osteogenic media) showed sex-specificity, with no differences observed for male cells (Figure 10A), and with a significantly higher calcification in female cells under pro-osteogenic conditions ($p < 0.0001$, Figure 10B).



Figures 10A and 10B: Comparison between baseline and osteogenic-induced calcification in male (10A) and female (10B) VICs, respectively. Bar charts comparing calcification levels in human VICs under baseline conditions and after 21 days of osteogenic stimulation without EMPA treatment (0 µM), for male (10A) and female (10B) donors. Quantification of the calcified area was performed after Alizarin Red S staining, using ImageJ software. (*) $p<0.05$. Statistical comparisons were performed using a Mann–Whitney U test. A significant increase in calcification was observed in female VICs under osteogenic conditions ($p < 0.0001$), while no significant difference was detected in male VICs.

Figure 11 shows how calcification varied with treatments with different doses of the SGLT2i in each of the VIC lines used in the study. Overall, it seems that there are no major differences between sexes at lower EMPA doses. At intermediate concentrations (5–10 µM), calcification appears to slightly increase in female VICs while decreasing in those from males. At higher doses (50–100 µM), the levels seem to stabilize in both sexes, with no clear difference from the control condition.

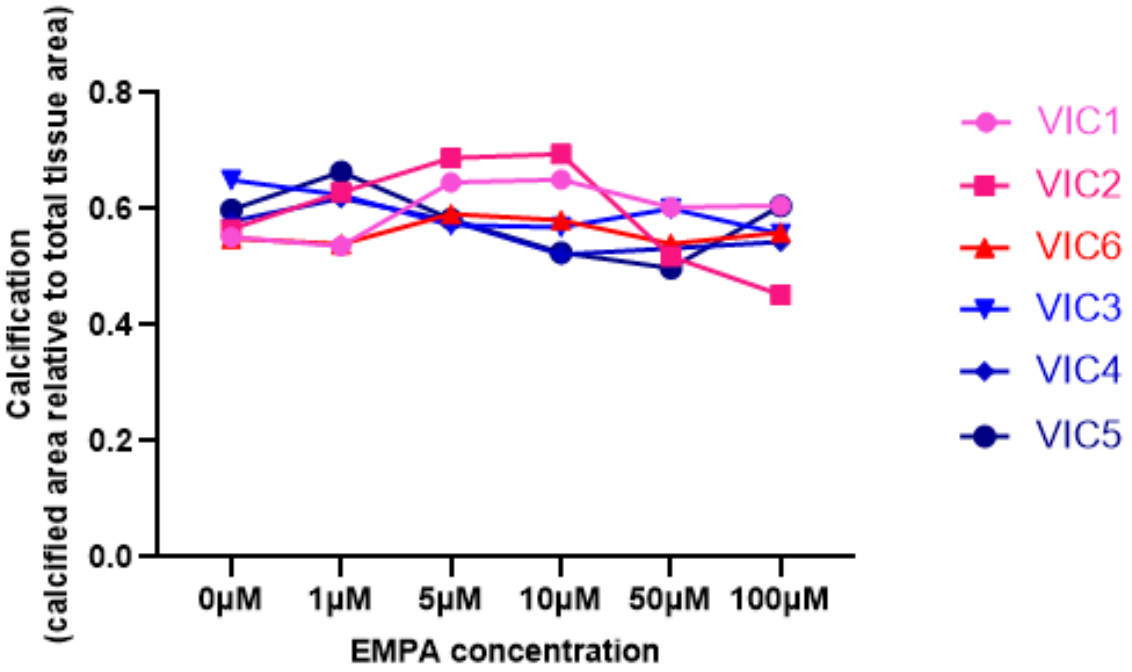


Fig 11: Extent of calcification (ratio of total tissue area) in each VIC line, under increasing EMPA concentrations. Quantification of the calcified area was performed after 21 days under EMPA treatment, after Alizarin Red S staining, using ImageJ software.

Figure 12 presents representative microscopic images detailing calcification and cellular morphology after the 21-day assay. When analyzing the effects of EMPA on VIC calcification in a sex-specific manner, distinct patterns emerged. In the case of women (Figure 13A), there is no significant alteration in calcification, comparing the 0 µM condition to any other concentration of EMPA. As for men (Figure 13B), there was in fact a significant reduction in the calcification of VICs, at a dose of 10 µM EMPA ($p=0.0392$), reducing about 11.6% of calcification. At all other concentrations, the differences relative to the condition with 0 µM EMPA were not significant.

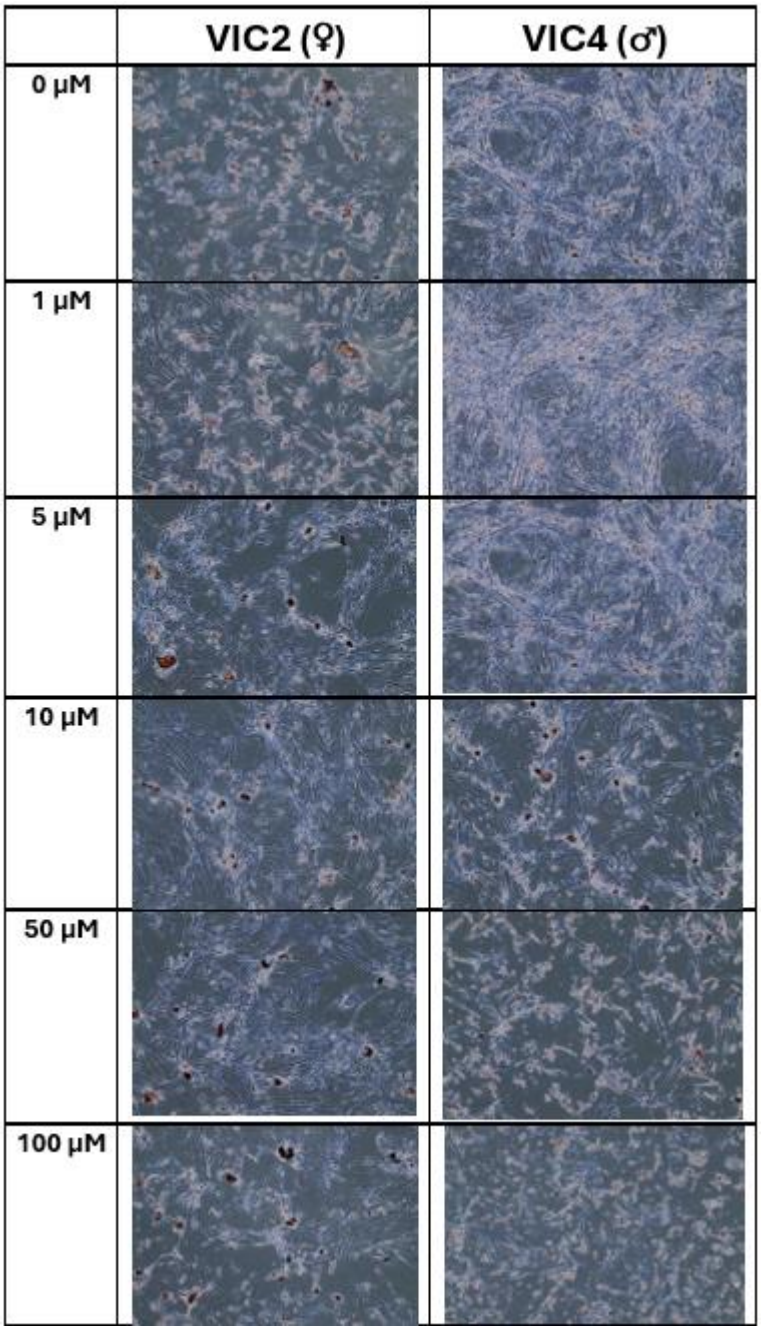
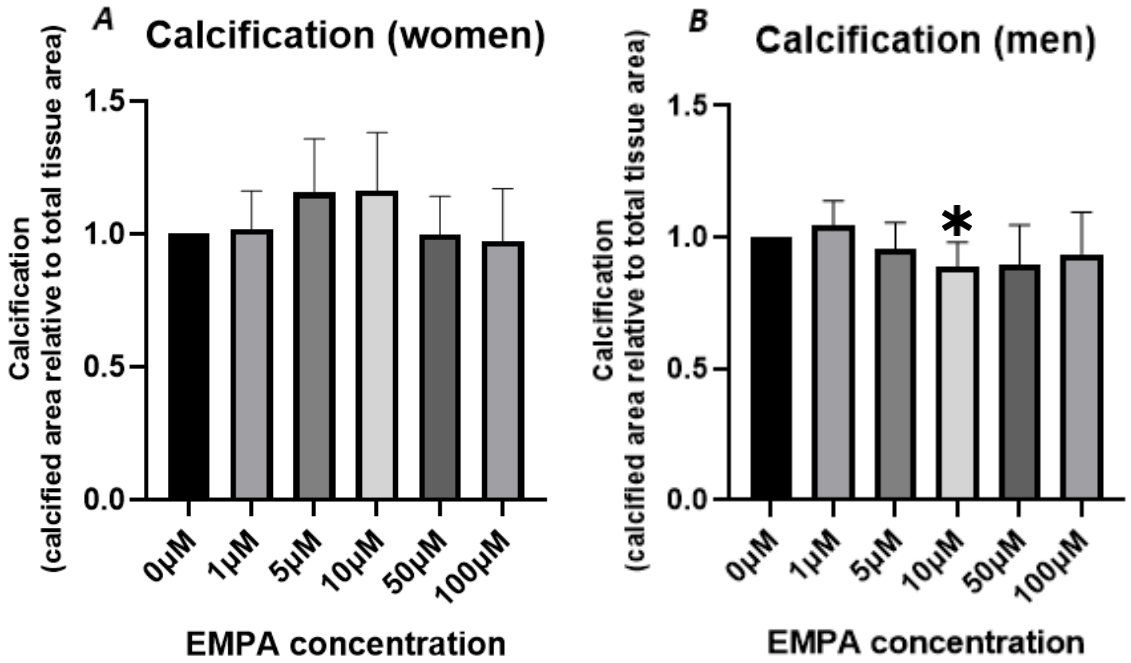


Fig 12: Representative microscopic photographs of wells after 21 days of the calcification protocol, illustrating a male cell line (VIC4) and a female cell line (VIC2). Photographs were taken under a digital, transmitted light inverted microscope (EVOSTM XL Core Imaging System, Invitrogen) at 100x magnification. Additional settings: brightness level 55; TIF format; 3MP resolution.



Figures 13A and 13B: Relative calcification values of VICs from female (13A) and male (13B) patients, across different EMPA treatment conditions. Quantification of the calcified area, after Alizarin Red S staining, using ImageJ software. All values were normalized to the 0 μM EMPA condition. (*) $p<0.05$. For male cell lines, which exhibited a normal distribution, statistical comparisons were performed using One-way ANOVA, followed by Tukey's multiple comparisons test. Conversely, for female cell lines, given their non-normal distribution, statistical comparisons were performed using Kruskal–Wallis, followed by Dunn's multiple comparisons test.

In summary, the calcification assays showed that male VICs exhibited a significantly higher baseline calcification level compared to female VICs, which may explain why male cells showed no significant calcification change following osteogenic induction, while female cells, with their lower intrinsic calcification, displayed a marked increase under osteogenic stimuli. EMPA also seems to exert a dose-dependent influence on VICs calcification, with a concentration of 10 μM reducing calcification in male VICs, but paradoxically increasing calcification in female VICs.

4. DISCUSSION

AVS is a disease characterized by progressive thickening and calcification of the aortic valve, leading to the narrowing of the valve orifice and consequent hemodynamic overload in the heart. This process is strongly associated with changes in VICs, which, under normal conditions, play a crucial role in maintaining the valvular ECM. However, under inflammatory, mechanical, and metabolic stimuli, these cells acquire an

activated phenotype, contributing to abnormal matrix deposition and the development of calcification. Furthermore, apoptosis of VICs can amplify this process, since the release of apoptotic vesicles favors the nucleation of calcium crystals. SGLT2i have emerged as potential modulators of inflammation and cellular metabolism in several cardiovascular diseases, and their impact on VICs is still an area open for exploration. Evidence suggests that SGLT2i may exert antifibrotic and anti-inflammatory effects and even interfere with the apoptosis pathway, which could influence the progression of valve calcification (177).

The main goal of this study was to investigate the effects of SGLT2i on calcification of the aortic valve, responsible for the pathological valve stenosis, in order to provide valuable preclinical data and new insights into the therapeutic potential of this drug class in AVS. We also aimed to evaluate the sexual differences that these drugs could have in this disease, comparing male cells with female cells, considering the differences already studied and described between sexes.

For this project, we chose to use biological material from patients with previous AVS pathology. In addition to presenting far fewer ethical restrictions, there is also much greater availability of valves from patients with previous valve disease than from people without previous valve pathology. When using these valves, isolated VICs will have a greater tendency to present a more pro-calcific phenotype, since they have already experienced disease stressors for a long time and have endured significant pathophysiological remodeling for years/decades. These valves should present a population richer in aVICs and obVICs, and not so many qVICs, presenting a greater intrinsic tendency for calcification. On the one hand, this may be limiting in the sense that it is not possible to have a clear idea of whether the introduction of SGLT2i can prevent the progression of qVICs to aVICs and obVICs, thus having an active role in the prevention of the pathology. On the other hand, it allows us to appraise whether it may reverse the disease process or, at least, prevent the calcium production activity of these cells, responsible for the progression of the disease.

Analyzing the clinical data of the patients whose valves were used in this study, some differences between sexes were noted. In the case of aortic valve max gradient, these differences were significant, with men presenting much higher maximum gradients, around 116.3 mmHg, compared to women, who were around 72.3 mmHg. In addition to this feature, other clinical characteristics, such as aortic valve mean

gradient and aortic valve area, appear to show some tendency towards differences between sexes, with p values between 0.05 and 0.10. Men appear to present higher mean gradients (avg. 72 mmHg) than women (avg. 48 mmHg), while women appear to present higher functional areas. These findings are not incidental and are consistent with current evidence. Previous studies show that, over time, women tend to have slower flow and a lower valve pressure gradient than men, as seen in this sample (178,179). These differences can be explained by several physiological, anatomical, and hemodynamic factors. Women generally have a smaller aortic root and left ventricle, which influences the hemodynamics of the aortic valve and can modify the pressure gradients. They also have smaller ventricular mass, generating lower gradients as aortic stenosis progresses. Hormonal factors, such as the influence of estrogen on arterial compliance, can impact the hemodynamic response to pressure overload, making the valve dynamics distinct between the sexes (180). Regarding age, women's age (avg. 73 years) was slightly higher than men's (avg. 68.3 years). While this difference was not significant, it aligns with existing knowledge that women tend to undergo surgery for aortic valve stenosis at a later age (116). It is worth noting that one male and one female patient were already taking SGLT2i. While this might suggest a bias in this study's findings, the lack of information on the duration of treatment and the specific indication makes it difficult to draw definitive conclusions.

A key observation during the initial phases of our study concerned the isolation and expansion of VICs, where no significant differences were noted between sexes. However, regarding cell yield, we can see that female valves (avg. 2.42×10^6 cells) were more fruitful than those of males (avg. 1.59×10^6 cells). Therefore, possibly, with a larger sample size, these differences could eventually become significant. This can be explained by a combination of biological, histological, and molecular factors. The fact that women predominantly manifest fibrosis favors the preservation of the extracellular matrix and facilitates cellular dissociation during isolation (181). Furthermore, sex hormones, such as estrogen, exert protective effects on female valve tissue, reducing inflammation, oxidative stress, and apoptosis, contributing to greater cell viability (182). These differences are also reflected in distinct levels of gene expression and metabolic activity in VICs, with female cells demonstrating greater phenotypic plasticity and ability to adapt to the culture environment (131,183).

The porcine VICs appear to behave differently from human cells. Despite the small sample size, some differences are clearly visible. VICs from swines showed quite higher cell yields (avg. $6.93x10^6$ cells), compared to the human VICs (avg. $2.0x10^6$ cells). These observed differences likely stem not only from inter-species differences but also from the health status of the tissue donors. The human VICs were obtained from patients with severe AVS submitted to SAVR, so these cells were previously affected by cellular stress, inflammation, and structural damage. On the other hand, porcine VICs were isolated from healthy, young pig valves (around 5 months old), thereby minimizing the influence of both cellular senescence and advanced pathology

The process of isolation and expansion took place without major issues. It is important to highlight the fact that these tissues were not sterile when they were manipulated in the lab. This is because we are using primary cell lines from human samples, which inherently contain endogenous microbiota. Furthermore, despite the aseptic conditions of surgery, the patient is not sterile, even with antibiotic prophylaxis. Yet, contamination was well-controlled with the use of antibiotics. No significant growth of these microorganisms was observed throughout the experiments, and, with each passage, their quantity decreased as assessed by microscopic visualization (400x magnification), which indicates that careful handling of the cells under the biosafety cabinet and abidance to general cell culture aseptic practices is enough to guarantee safe use of VICs, for up to 21 days, without major contamination risks. The existence of these microorganisms was consistent across all human cell lines, so this should not be interpreted as translating some type of bias in the final evaluation of the results.

Assessing cell viability is crucial to determine the potential toxicity of any therapeutic agent and to establish the range of concentrations that can be tolerated by cells. Therefore, we first started to perform cell viability assays using resazurin. The results were consistent across all cell lines tested. The exception to this was the VIC1 line, which consistently showed discrepant values. A probable explanation for this erratic behavior was a double-edge effect, particularly since it was always observed in the same well: A1. This may have led to an artifactual decrease of cell viability in the control condition that jeopardized the comparison with the other drug conditions. In all other cases, a gradual reduction in cell viability was observed as the pharmacological dose increased, being consistent with previous studies investigating the effects of SGLT2i on

other cell lines. Our data show that EMPA induced reductions in cell viability in a concentration-dependent manner. For male VICs, this effect became evident from 10 µM, a threshold that aligns with findings from previous studies where significant effects of SGLT2i typically occur around this concentration. Studies such as Dasari et al. (184), using cardiomyocytes as cellular model, and Cristovão et al. (185), using pancreatic tumor cell lines as cellular model, reported a significant reduction in cell viability with Canagliflozin at concentrations approximating 10 µM. Conversely, in female VICs, significant reductions in viability were only observed at 100 µM. This higher threshold for significance in female cells might potentially be influenced by sample size (only 2 cell lines, after VIC1 exclusion) or inherent sex-specific cellular responses. Despite these variations in specific thresholds, the overall pattern remains consistent, showing a gradual concentration-dependent decrease in viability, suggesting a degree of convergence in the effects of SGLT2i across different cell types and compounds. Importantly, in all conditions tested, viability levels remained above 70%, which, according to International Organisation for Standardisation (ISO) 10993-5 guidelines, indicates that EMPA does not exert major cytotoxic effects in this cellular model at these concentrations (186). Furthermore, although the differences between men and women, at each EMPA concentration, were not statistically significant, the viability was consistently higher in women. With a larger sample size, it is likely that these differences would have become significant, which would agree with known data, as previous studies reported higher metabolic activity and viability in female VICs (187,188). It is important to emphasize that the analysis was carried out across all available human VIC lines, rather than limiting the comparison to a single male and female donor. This approach enhances the robustness and generalizability of the findings, accounting for inter-individual biological variability, despite the sample size still being relatively small.

Given that calcification and fibrosis are key pathological hallmarks of AVS, understanding the influence of EMPA on these processes is crucial for evaluating its therapeutic potential. However, due to temporal and logistical constraints, this study focused primarily on the calcification aspect.

Before delving into EMPA effect on calcification, the baseline differences in calcification were compared between sexes and different cell lines. This is because primary cell lines were used in this work, carrying an intrinsic heterogeneity that reflects disease severity and exposure to different comorbidities,

pharmacological therapies, etc. Male cell lines showed a significantly higher mean calcification than that observed in female cells. The observation of such high basal calcification levels in male cells may explain the fact that no significant differences were found between the baseline and after osteogenic induction, since these cells appear to have, intrinsically, a greater propensity for calcification. On the other hand, in female cells, whose natural tendency seems to be more towards fibrosis than mineralization, the osteogenic stimulus induced a significant response in relation to their baseline. Thus, this baseline analysis reinforces the importance of considering biological sex as a fundamental variable in pharmacological studies to tackle valve calcification.

Regarding the calcification assays performed, it is important to note some technical challenges encountered during the imaging phase. Unfortunately, after fixing the cells with Alizarin, cells in some wells accumulated in certain regions, making it impossible to capture photographs in 4 different points of the well as initially planned. In these specific cases, between 1 and 3 photographs were taken, depending on the tissue configuration within the well. However, this technical limitation does not appear to have significantly altered the overall observed trends in calcification. The results showed that, in general, exposure to SGLT2i did not lead to a consistent reduction in mineralization under all conditions tested. In female VICs, the calcification assays did not show any significant diferences across the different concentrations of EMPA tested. This absence of significant alteration in calcification is consistent with current understanding of AVS pathophysiology in women, where calcification is not always the predominant feature. Instead, fibrotic processes are often highlighted as a more significant aspect in the progression of AVS in this population (131,189,190). On the other hand, in male VICs, there was a significant reduction in calcification at 10 µM dose, standing out as one of the most promising findings of this study. This effect suggests that, at certain concentrations, SGLT2i may positively interfere in pathways associated with valvular calcification, possibly by modulating oxidative stress, reducing inflammation, or interfering with osteogenic pathways, preventing/reversing the phenotypic transition of VICs to an osteoblastic state. Thus, this data reinforces the therapeutic potential of SGLT2i in the valvular context, particularly in male individuals.

SGLT2i, like EMPA, have a significant potential in mitigating calcification in male VICs when subject to pro-calcific conditions. This is promising for the future development of effective strategies to combat AVS, based on a therapeutic scheme that includes drugs from this pharmacological class. EMPA may affect VICs' differentiation and activity through the modulation of key signaling pathways involved in their activation. As we previously mentioned, pathways like the Wnt/β-catenin or the PI3K/AKT/GSK-3β pathways play critical roles in promoting osteoblastic differentiation of VICs. Once activated, they lead to the upregulation of osteogenic markers, such as Runx2 or BMP-2, which are essential for calcium deposition. In distinct cellular models, SGLT2i have been shown to inhibit some of those pathways and to reduce the expression of the osteogenic markers, suppressing, thus, the mineralization process. Chronic inflammation is a known driver for this disease, where the activation of TLR-2/4 and elevated levels of IL6 seem to contribute to VICs' activation. But the existing evidence shows that SGLT2i has anti-inflammatory properties, downregulating TLR expression, inhibiting the NLRP3 inflammasome pathway and enhancing SIRT1 activity, leading to a decrease of inflammatory markers, like IL-6 (191). Oxidative stress also seems to play an important role in VICs activation and differentiation. In this regard, these drugs also appear to have antioxidant effects, as evidenced by decreased ROS production and downregulation of NADPH oxidase components in previous studies (174). The study of these pathways is undoubtedly important; however, our study primarily focused on the final phenotype. We are planning to investigate these pathways and their effectors, such as alkaline phosphatase, in the near future, to gain a deeper understanding of the mechanisms underlying EMPA-induced mitigation of calcification.

Considering that the current treatment options for AVS are primarily surgical and the lack of approved pharmacological therapies capable of halting or reversing disease progression, SGLT2i emerge as a potential new pharmacological approach. These drugs are not new, and their safety and usage profiles have already been extensively studied in recent years. This further facilitates the legislation and approval of clinical trials to repurpose these drugs to this pathology. Notably, our findings suggest that SGLT2i, specifically EMPA, demonstrated a potential to mitigate calcification predominantly in male VICs. Future research should also prioritize sex-specific analyses when evaluating the efficacy of pharmacological interventions for AVS, to

develop more personalized and effective therapies, accounting for the unique pathophysiological characteristics of each sex.

The discovery of an effective pharmacological treatment capable of halting or slowing the progression of AVS can positively impact patient outcomes and clinical practice, providing the potential for early intervention. Currently, there are no established screening programs for AVS, mostly because no treatment can alter the disease's natural progression. However, if an effective treatment is found, it could justify the implementation of well-defined screening programs. These screening programs should be carried out in people with risk factors for developing the pathology, such as advanced age, bicuspid aortic valve, metabolic syndrome, among other cardiovascular risk factors. It could possibly delay the disease's progression even before the onset of symptoms and appearance of hemodynamic imbalances. Early detection of aortic sclerosis or mild aortic stenosis through transthoracic ultrasound allows a timely therapeutic intervention (192,193), which, in the future, may be materialized through administration of SGLT2i, at least in men.

Due to time and logistics constraints, we were unable to assess the influence of these drugs on the fibrotic processes promoted by VICs. However, we are planning to move forward with these studies soon, employing a methodology that, while distinct in its triggers (TGF-β) and shorter incubation times (typically 3 to 7 days), will also allow us to assess fibrosis, using Picro Sirius Red staining. This is particularly promising, considering that fibrosis is a predominant hallmark in female AVS. Consequently, SGLT2i might still offer considerable benefits in reducing fibrosis in women, even if its effect on calcification in female valves appears limited.

To summarize, the results of this study point to a possible dose-dependent effect of EMPA in modulating the valve calcification process, with a distinct response profile between sexes. The observation of a reduction in calcification in male cells treated with 10µM EMPA reinforces the hypothesis that SGLT2i may positively interfere in the molecular mechanisms associated with the osteogenic differentiation of VICs. On the other hand, the absence of significant effects on calcification in female cells at the tested doses highlights the importance of a careful analysis of the biological effects of SGLT2i in different cellular contexts and sexes. This also underscores the need to find and test alternative, potentially more beneficial, drugs for women,

such as anti-fibrotic agents, given the predominant role of fibrosis in female AVS. These findings highlight the relevance of a personalized approach and reinforce the need for future research focused on *in vivo* validation, long-term efficacy, and safety studies, with larger sample sizes and additional evaluation of molecular markers, facilitating the translation of this therapeutic approach into clinical practice. If confirmed, these discoveries could really revolutionize disease management, justifying the implementation of population screening programs for high-risk groups, enabling early pharmacological intervention and rising the potential for personalized treatment strategies. This could help reduce the need for SAVR, alleviating the burden on healthcare systems and, ultimately, decreasing AVS-related morbidity and mortality.

5. LIMITATIONS

Despite the promising findings of this study, some limitations are evident. Our study had a limited number of aortic valve samples (6 patients), which can limit our conclusions about the effectiveness of the use of SGLT2i on the prevention and treatment of calcific AVS and restrict the generalizability of the findings due to patient-specific factors. Larger studies might be necessary in the future to be able to evaluate this interaction more accurately.

This study was conducted in a laboratory setting with isolated VICs, which may not fully replicate the complex physiological conditions present *in vivo*. The behavior of VICs may differ in a three-dimensional tissue context, due to the interactions with the extracellular matrix, the endothelial cells, and other biomechanical factors.

Also, this study used aortic valves from patients with already established severe AVS, limiting the possibility of studying and evaluating the effects of EMPA in preventing the development and progression of the disease, considering the possible altered state of the patient's cells. For ethical reasons, it was not possible to acquire valves from healthy people. The closest sample we were able to acquire was 2 valves from healthy pigs, which we intend to continue studying in the upcoming months.

The use of Alizarin red staining for calcification quantification also has some drawbacks. This technique demonstrates only moderate sensitivity, making it more challenging to detect slight but significant changes. To address this, we might consider using a pro-calcific medium supplemented with calcium phosphate offers greater sensitivity, compared to the osteogenic medium used (194).

To date, there are no other previous studies that have evaluated the role of SGLT2i on VICs to investigate their effects on AVS. Therefore, it is not possible to compare our findings directly with previous research. Some previous studies have explored the cardioprotective effects of SGLT2i in different contexts, but none have specifically evaluated their impact on VICs. This highlights the novelty of our approach and underscores the importance of further research to elucidate these mechanisms.

Furthermore, our study did not focus on the evaluation of gene expression or biomolecular markers associated with the effect of EMPA on calcification. So, a more in-depth study of gene expression changes, protein-level alterations, as well as the expression of relevant biomarkers (ALP; collagen I; inflammatory cytokines; …), could help elucidate the precise molecular mechanisms involved in this therapy.

6. CONCLUSIONS

This study explored the effects of SGLT2i, precisely empagliflozin, on the calcification of human VICs obtained from patients with AVS. Cell viability assays demonstrated that EMPA was generally well-tolerated, with viability consistently remaining above 70% even at the highest concentration tested (100 µM), supporting its safety across the tested range. Regarding calcification, a reduction was observed in male cell models when treated with EMPA at the concentration of 10 µM, suggesting that SGLT2i may exert a protective effect against pathological mineralization in this subgroup. In contrast, a significant reduction in calcification was not observed in female VICs across the tested concentrations. These data contribute to the phenotypic understanding of the interaction between SGLT2i and valvular calcification, also highlighting the importance of considering biological differences between sexes in this context.

By paving the way for a potential pharmacological modulation of processes until now considered irreversible, these results may transform the therapeutic approach to aortic stenosis, currently limited to invasive interventions.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Consent for publication

Not applicable.

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References

1. Mubarik A, Sharma S, Law MA. Bicuspid Aortic Valve. 2024.

2. Roos-Hesselink JW, Schölzel BE, Heijdra RJ, Spitaels SEC, Meijboom FJ, Boersma E, et al. Aortic valve and aortic arch pathology after coarctation repair. Heart. 2003 Sep;89(9):1074–7.

3. Summerhill VI, Moschetta D, Orekhov AN, Poggio P, Myasoedova VA. Sex-Specific Features of Calcific Aortic Valve Disease. Int J Mol Sci. 2020 Aug 6;21(16).

4. Rajput FA, Zeltser R. Aortic Valve Replacement. 2024.

5. Iung B, Baron G, Butchart EG, Delahaye F, Gohlke-Bärwolf C, Levang OW, et al. A prospective survey of patients with valvular heart disease in Europe: The Euro Heart Survey on Valvular Heart Disease. Eur Heart J. 2003 Jul;24(13):1231–43.

6. Lindman BR, Clavel MA, Mathieu P, Iung B, Lancellotti P, Otto CM, et al. Calcific aortic stenosis. Nat Rev Dis Primers. 2016 Mar 3;2(1):16006.

7. Akahori H, Tsujino T, Masuyama T, Ishihara M. Mechanisms of aortic stenosis. J Cardiol. 2018 Mar;71(3):215–20.

8. Lindman BR, Clavel MA, Mathieu P, Iung B, Lancellotti P, Otto CM, et al. Calcific aortic stenosis. Nat Rev Dis Primers. 2016 Mar 3;2(1):16006.

9. Mohler ER, Sheridan MJ, Nichols R, Harvey WP, Waller BF. Development and progression of aortic valve stenosis: atherosclerosis risk factors--a causal relationship? A clinical morphologic study. Clin Cardiol. 1991 Dec;14(12):995–9.

10. Stewart BF, Siscovick D, Lind BK, Gardin JM, Gottdiener JS, Smith VE, et al. Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. J Am Coll Cardiol. 1997 Mar 1;29(3):630–4.

11. Novaro GM, Katz R, Aviles RJ, Gottdiener JS, Cushman M, Psaty BM, et al. Clinical factors, but not C-reactive protein, predict progression of calcific aortic-valve disease: the Cardiovascular Health Study. J Am Coll Cardiol. 2007 Nov 13;50(20):1992–8.

12. Yi B, Zeng W, Lv L, Hua P. Changing epidemiology of calcific aortic valve disease: 30-year trends of incidence, prevalence, and deaths across 204 countries and territories. Aging. 2021 May 11;13(9):12710–32.

13. Yasuhara J, Schultz K, Bigelow AM, Garg V. Congenital aortic valve stenosis: from pathophysiology to molecular genetics and the need for novel therapeutics. Front Cardiovasc Med. 2023;10:1142707.

14. Abraham B, Farina JM, Fath A, Abdou M, Elbanna M, Suppah M, et al. The impact of moderate aortic stenosis in acute myocardial infarction: A multicenter retrospective study. Catheter Cardiovasc Interv. 2023 Jul;102(1):159–65.

15. Taniguchi T, Morimoto T, Shiomi H, Ando K, Kanamori N, Murata K, et al. Sudden Death in Patients With Severe Aortic Stenosis: Observations From the CURRENT AS Registry. J Am Heart Assoc. 2018 May 18;7(11).

16. Loukas M, Bilinsky E, Bilinsky S, Blaak C, Tubbs RS, Anderson RH. The anatomy of the aortic root. Clin Anat. 2014 Jul;27(5):748–56.

17. Katsi V, Magkas N, Antonopoulos A, Trantalis G, Toutouzas K, Tousoulis D. Aortic valve: anatomy and structure and the role of vasculature in the degenerative process. Acta Cardiol. 2021 Jun;76(4):335–48.

18. Charest A, Pépin A, Shetty R, Côté C, Voisine P, Dagenais F, et al. Distribution of SPARC during neovascularisation of degenerative aortic stenosis. Heart. 2006 Dec;92(12):1844–9.

19. Ho SY, McCarthy KP, Faletra FF. Anatomy of the left atrium for interventional echocardiography. Eur J Echocardiogr. 2011 Oct;12(10):i11-5.

20. Sacks MS, Yoganathan AP. Heart valve function: a biomechanical perspective. Philos Trans R Soc Lond B Biol Sci. 2007 Aug 29;362(1484):1369–91.

21. Aikawa E, Libby P. A Rock and a Hard Place: Chiseling Away at the Multiple Mechanisms of Aortic Stenosis. Circulation. 2017 May 16;135(20):1951–5.

22. Butcher JT, Markwald RR. Valvulogenesis: the moving target. Philos Trans R Soc Lond B Biol Sci. 2007 Aug 29;362(1484):1489–503.

23. Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, et al. Human semilunar cardiac valve remodeling by activated cells from fetus to adult: implications for postnatal adaptation, pathology, and tissue engineering. Circulation. 2006 Mar 14;113(10):1344–52.

24. Dutta P, James JF, Kazik H, Lincoln J. Genetic and Developmental Contributors to Aortic Stenosis. Circ Res. 2021 Apr 30;128(9):1330–43.

25. Wang H, Sridhar B, Leinwand LA, Anseth KS. Characterization of cell subpopulations expressing progenitor cell markers in porcine cardiac valves. PLoS One. 2013;8(7):e69667.

26. Liu AC, Joag VR, Gotlieb AI. The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology. Am J Pathol. 2007 Nov;171(5):1407–18.

27. Straka F, Schornik D, Masin J, Filova E, Mirejovsky T, Burdikova Z, et al. A human pericardium biopolymeric scaffold for autologous heart valve tissue engineering: cellular and extracellular matrix structure and biomechanical properties in comparison with a normal aortic heart valve. J Biomater Sci Polym Ed. 2018 Apr;29(6):599–634.

28. Kraler S, Blaser MC, Aikawa E, Camici GG, Lüscher TF. Calcific aortic valve disease: from molecular and cellular mechanisms to medical therapy. Eur Heart J. 2022 Feb 12;43(7):683–97.

29. Morvan M, Arangalage D, Franck G, Perez F, Cattan-Levy L, Codogno I, et al. Relationship of Iron Deposition to Calcium Deposition in Human Aortic Valve Leaflets. J Am Coll Cardiol. 2019 Mar;73(9):1043–54.

30. Bosse K, Hans CP, Zhao N, Koenig SN, Huang N, Guggilam A, et al. Endothelial nitric oxide signaling regulates Notch1 in aortic valve disease. J Mol Cell Cardiol. 2013 Jul;60:27–35.

31. Balachandran K, Sucosky P, Yoganathan AP. Hemodynamics and mechanobiology of aortic valve inflammation and calcification. Int J Inflam. 2011;2011:263870.

32. Abdelbaky A, Corsini E, Figueroa AL, Subramanian S, Fontanez S, Emami H, et al. Early aortic valve inflammation precedes calcification: A longitudinal FDG-PET/CT study. Atherosclerosis. 2015 Feb;238(2):165–72.

33. O'Brien KD, Reichenbach DD, Marcovina SM, Kuusisto J, Alpers CE, Otto CM. Apolipoproteins B, (a), and E Accumulate in the Morphologically Early Lesion of 'Degenerative' Valvular Aortic Stenosis. Arterioscler Thromb Vasc Biol. 1996 Apr;16(4):523–32.

34. Raddatz MA, Madhur MS, Merryman WD. Adaptive immune cells in calcific aortic valve disease. Am J Physiol Heart Circ Physiol. 2019 Jul 1;317(1):H141–55.

35. Alushi B, Curini L, Christopher MR, Grubitzch H, Landmesser U, Amedei A, et al. Calcific Aortic Valve Disease-Natural History and Future Therapeutic Strategies. Front Pharmacol. 2020;11:685.

36. Ntusi NAB, Piechnik SK, Francis JM, Ferreira VM, Matthews PM, Robson MD, et al. Diffuse Myocardial Fibrosis and Inflammation in Rheumatoid Arthritis: Insights From CMR T1 Mapping. JACC Cardiovasc Imaging. 2015 May;8(5):526–36.

37. Coté N, Mahmut A, Bosse Y, Couture C, Pagé S, Trahan S, et al. Inflammation is associated with the remodeling of calcific aortic valve disease. Inflammation. 2013 Jun;36(3):573–81.

38. Goody PR, Hosen MR, Christmann D, Niepmann ST, Zietzer A, Adam M, et al. Aortic Valve Stenosis: From Basic Mechanisms to Novel Therapeutic Targets. Arterioscler Thromb Vasc Biol. 2020 Apr;40(4):885–900.

39. Aikawa E, Nahrendorf M, Figueiredo JL, Swirski FK, Shtatland T, Kohler RH, et al. Osteogenesis Associates With Inflammation in Early-Stage Atherosclerosis Evaluated by Molecular Imaging In Vivo. Circulation. 2007 Dec 11;116(24):2841–50.

40. Pawade TA, Newby DE, Dweck MR. Calcification in Aortic Stenosis. J Am Coll Cardiol. 2015 Aug;66(5):561–77.

41. New SEP, Aikawa E. Molecular Imaging Insights Into Early Inflammatory Stages of Arterial and Aortic Valve Calcification. Circ Res. 2011 May 27;108(11):1381–91.

42. Caira FC, Stock SR, Gleason TG, McGee EC, Huang J, Bonow RO, et al. Human Degenerative Valve Disease Is Associated With Up-Regulation of Low-Density Lipoprotein Receptor-Related Protein 5 Receptor-Mediated Bone Formation. J Am Coll Cardiol. 2006 Apr;47(8):1707–12.

43. Rutkovskiy A, Malashicheva A, Sullivan G, Bogdanova M, Kostareva A, Stensløkken KO, et al. Valve Interstitial Cells: The Key to Understanding the Pathophysiology of Heart Valve Calcification. J Am Heart Assoc. 2017 Sep 14;6(9).

44. Matilla L, Roncal C, Ibarrola J, Arrieta V, García-Peña A, Fernández-Celis A, et al. A Role for MMP-10 (Matrix Metalloproteinase-10) in Calcific Aortic Valve Stenosis. Arterioscler Thromb Vasc Biol. 2020 May;40(5):1370–82.

45. Satta J, Oiva J, Salo T, Eriksen H, Ohtonen P, Biancari F, et al. Evidence for an altered balance between matrix metalloproteinase-9 and its inhibitors in calcific aortic stenosis. Ann Thorac Surg. 2003 Sep;76(3):681–8; discussion 688.

46. Helske S, Lindstedt KA, Laine M, Mäyränpää M, Werkkala K, Lommi J, et al. Induction of local angiotensin II-producing systems in stenotic aortic valves. J Am Coll Cardiol. 2004 Nov 2;44(9):1859–66.

47. Fujisaka S, Usui I, Ikutani M, Aminuddin A, Takikawa A, Tsuneyama K, et al. Adipose tissue hypoxia induces inflammatory M1 polarity of macrophages in an HIF-1α-dependent and HIF-1α-independent manner in obese mice. Diabetologia. 2013 Jun;56(6):1403–12.

48. Yang X, Fullerton DA, Su X, Ao L, Cleveland JC, Meng X. Pro-Osteogenic Phenotype of Human Aortic Valve Interstitial Cells Is Associated With Higher Levels of Toll-Like Receptors 2 and 4 and Enhanced Expression of Bone Morphogenetic Protein 2. J Am Coll Cardiol. 2009 Feb;53(6):491–500.

49. Osman L, Yacoub MH, Latif N, Amrani M, Chester AH. Role of Human Valve Interstitial Cells in Valve Calcification and Their Response to Atorvastatin. Circulation. 2006 Jul 4;114(1_supplement).

50. Zhou J, Bowen C, Lu G, Knapp III C, Recknagel A, Norris RA, et al. Cadherin-11 Expression Patterns in Heart Valves Associate with Key Functions during Embryonic Cushion Formation, Valve Maturation and Calcification. Cells Tissues Organs. 2013;198(4):300–10.

51. Hutcheson JD, Chen J, Sewell-Loftin MK, Ryzhova LM, Fisher CI, Su YR, et al. Cadherin-11 Regulates Cell–Cell Tension Necessary for Calcific Nodule Formation by Valvular Myofibroblasts. Arterioscler Thromb Vasc Biol. 2013 Jan;33(1):114–20.

52. Parhami F, Morrow AD, Balucan J, Leitinger N, Watson AD, Tintut Y, et al. Lipid Oxidation Products Have Opposite Effects on Calcifying Vascular Cell and Bone Cell Differentiation. Arterioscler Thromb Vasc Biol. 1997 Apr;17(4):680–7.

53. Peeters FECM, Meex SJR, Dweck MR, Aikawa E, Crijns HJGM, Schurgers LJ, et al. Calcific aortic valve stenosis: hard disease in the heart: A biomolecular approach towards diagnosis and treatment. Eur Heart J. 2018 Jul 21;39(28):2618–24.

54. Côté N, Mahmut A, Fournier D, Boulanger MC, Couture C, Després JP, et al. Angiotensin receptor blockers are associated with reduced fibrosis and interleukin-6 expression in calcific aortic valve disease. Pathobiology. 2014;81(1):15–24.

55. Syväranta S, Helske S, Laine M, Lappalainen J, Kupari M, Mäyränpää MI, et al. Vascular endothelial growth factor-secreting mast cells and myofibroblasts: a novel self-perpetuating angiogenic pathway in aortic valve stenosis. Arterioscler Thromb Vasc Biol. 2010 Jun;30(6):1220–7.

56. Durbin AD, Gotlieb AI. Advances towards understanding heart valve response to injury. Cardiovasc Pathol. 2002;11(2):69–77.

57. Chalajour F, Treede H, Gehling UM, Ebrahimnejad A, Boehm DH, Riemer RK, et al. Identification and characterization of cells with high angiogenic potential and transitional phenotype in calcific aortic valve. Exp Cell Res. 2007 Jul 1;313(11):2326–35.

58. Fujisaka T, Hoshiga M, Hotchi J, Takeda Y, Jin D, Takai S, et al. Angiotensin II promotes aortic valve thickening independent of elevated blood pressure in apolipoprotein-E deficient mice. Atherosclerosis. 2013 Jan;226(1):82–7.

59. Vavilis G, Bäck M, Occhino G, Trevisan M, Bellocco R, Evans M, et al. Kidney Dysfunction and the Risk of Developing Aortic Stenosis. J Am Coll Cardiol. 2019 Jan 29;73(3):305–14.

60. Bäck M, Michel JB. From organic and inorganic phosphates to valvular and vascular calcifications. Cardiovasc Res. 2021 Jul 27;117(9):2016–29.

61. Lee HJ, Kim HK. Natural History Data in Symptomatic Severe Aortic Stenosis Alerts Cardiologists to the Dangers of No Action. Korean Circ J. 2019 Feb;49(2):170–2.

62. Owens DS, Katz R, Takasu J, Kronmal R, Budoff MJ, O'Brien KD. Incidence and progression of aortic valve calcium in the Multi-ethnic Study of Atherosclerosis (MESA). Am J Cardiol. 2010 Mar 1;105(5):701–8.

63. Roberts WC, Ko JM. Frequency by decades of unicuspid, bicuspid, and tricuspid aortic valves in adults having isolated aortic valve replacement for aortic stenosis, with or without associated aortic regurgitation. Circulation. 2005 Feb 22;111(7):920–5.

64. Kennedy JW, Doces J, Stewart DK. Left ventricular function before and following aortic valve replacement. Circulation. 1977 Dec;56(6):944–50.

65. Kanwar A, Thaden JJ, Nkomo VT. Management of Patients With Aortic Valve Stenosis. Mayo Clin Proc. 2018 Apr;93(4):488–508.

66. Pujari SH, Agasthi P. Aortic Stenosis. 2024.

67. Tsuchiya S, Matsumoto Y, Suzuki H, Takanami K, Kikuchi Y, Takahashi J, et al. Transcatheter aortic valve implantation and cognitive function in elderly patients with severe aortic stenosis. EuroIntervention. 2020 Apr 3;15(18):e1580–7.

68. Kvaslerud AB, Hussain AI, Auensen A, Ueland T, Michelsen AE, Pettersen KI, et al. Prevalence and prognostic implication of iron deficiency and anaemia in patients with severe aortic stenosis. Open Heart. 2018;5(2):e000901.

69. Lourdusamy D, Mupparaju VK, Sharif NF, Ibebuogu UN. Aortic stenosis and Heyde's syndrome: A comprehensive review. World J Clin Cases. 2021 Sep 6;9(25):7319–29.

70. Srivastav S, Jamil RT, Zeltser R. Valsalva Maneuver. 2024.

71. Kurtz CE, Otto CM. Aortic stenosis: clinical aspects of diagnosis and management, with 10 illustrative case reports from a 25-year experience. Medicine. 2010 Nov;89(6):349–79.

72. Grimard BH, Safford RE, Burns EL. Aortic Stenosis: Diagnosis and Treatment. Am Fam Physician. 2016 Mar 1;93(5):371–8.

73. Jain H, Goyal A, Khan ATMA, Khan NU, Jain J, Chopra S, et al. Insights into calcific aortic valve stenosis: a comprehensive overview of the disease and advancing treatment strategies. Ann Med Surg (Lond). 2024 Jun;86(6):3577–90.

74. Kurtz CE, Otto CM. Aortic stenosis: clinical aspects of diagnosis and management, with 10 illustrative case reports from a 25-year experience. Medicine. 2010 Nov;89(6):349–79.

75. Rana M. Aortic Valve Stenosis: Diagnostic Approaches and Recommendations of the 2021 ESC/EACTS Guidelines for the Management of Valvular Heart Disease -A Review of the Literature. Cardiol Cardiovasc Med. 2022 Jun;6(3):315–24.

76. Mino T, Kimura S, Kitaura A, Iwamoto T, Yuasa H, Chiba Y, et al. Can left ventricular hypertrophy on electrocardiography detect severe aortic valve stenosis? PLoS One. 2020;15(11):e0241591.

77. Vahanian A, Beyersdorf F, Praz F, Milojevic M, Baldus S, Bauersachs J, et al. 2021 ESC/EACTS Guidelines for the management of valvular heart disease. Eur Heart J. 2022 Feb 12;43(7):561–632.

78. Otto CM, Nishimura RA, Bonow RO, Carabello BA, Erwin JP, Gentile F, et al. 2020 ACC/AHA Guideline for the Management of Patients With Valvular Heart Disease: Executive Summary: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. Circulation. 2021 Feb 2;143(5):e35–71.

79. Prihadi EA, Vollema EM, Ng ACT, Ajmone Marsan N, Bax JJ, Delgado V. Determinants and prognostic implications of left ventricular mechanical dispersion in aortic stenosis. Eur Heart J Cardiovasc Imaging. 2019 Jul 1;20(7):740–8.

80. Tastet L, Tribouilloy C, Maréchaux S, Vollema EM, Delgado V, Salaun E, et al. Staging Cardiac Damage in Patients With Asymptomatic Aortic Valve Stenosis. J Am Coll Cardiol. 2019 Jul 30;74(4):550–63.

81. Rusinaru D, Malaquin D, Maréchaux S, Debry N, Tribouilloy C. Relation of Dimensionless Index to Long-Term Outcome in Aortic Stenosis With Preserved LVEF. JACC Cardiovasc Imaging. 2015 Jul;8(7):766–75.

82. Pawade T, Clavel MA, Tribouilloy C, Dreyfus J, Mathieu T, Tastet L, et al. Computed Tomography Aortic Valve Calcium Scoring in Patients With Aortic Stenosis. Circ Cardiovasc Imaging. 2018 Mar;11(3):e007146.

83. Vahanian A, Beyersdorf F, Praz F, Milojevic M, Baldus S, Bauersachs J, et al. 2021 ESC/EACTS Guidelines for the management of valvular heart disease: Developed by the Task Force for the management of valvular heart disease of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS). Revista Española de Cardiología (English Edition). 2022 Jun;75(6):524.

84. Cowell SJ, Newby DE, Prescott RJ, Bloomfield P, Reid J, Northridge DB, et al. A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis. N Engl J Med. 2005 Jun 9;352(23):2389–97.

85. Carabello BA. The SEAS Trial. Curr Cardiol Rep. 2010 Mar;12(2):122–4.

86. Chan KL, Teo K, Dumesnil JG, Ni A, Tam J, ASTRONOMER Investigators. Effect of Lipid lowering with rosuvastatin on progression of aortic stenosis: results of the aortic stenosis progression observation: measuring effects of rosuvastatin (ASTRONOMER) trial. Circulation. 2010 Jan 19;121(2):306–14.

87. Donato M, Ferri N, Lupo MG, Faggin E, Rattazzi M. Current Evidence and Future Perspectives on Pharmacological Treatment of Calcific Aortic Valve Stenosis. Int J Mol Sci. 2020 Nov 4;21(21).

88. Rossebø AB, Pedersen TR, Boman K, Brudi P, Chambers JB, Egstrup K, et al. Intensive lipid lowering with simvastatin and ezetimibe in aortic stenosis. N Engl J Med. 2008 Sep 25;359(13):1343–56.

89. Park DY, An S, Kassab K, Jolly N, Attanasio S, Sawaqed R, et al. Chronological comparison of TAVI and SAVR stratified to surgical risk: a systematic review, meta-analysis, and meta-regression. Acta Cardiol. 2023 Sep;78(7):778–89.

90. Webb JG, Blanke P, Meier D, Sathananthan J, Lauck S, Chatfield AG, et al. TAVI in 2022: Remaining issues and future direction. Arch Cardiovasc Dis. 2022 Apr;115(4):235–42.

91. Tribouilloy C, Lévy F, Rusinaru D, Guéret P, Petit-Eisenmann H, Baleynaud S, et al. Outcome After Aortic Valve Replacement for Low-Flow/Low-Gradient Aortic Stenosis Without Contractile Reserve on Dobutamine Stress Echocardiography. J Am Coll Cardiol. 2009 May;53(20):1865–73.

92. Sharma N, Sachedina AK, Kumar S. Low-flow, Low-gradient Severe Aortic Stenosis: A Review. Heart Int. 2023;17(1):8.

93. Di Pietro E, Frittitta V, Motta S, Strazzieri O, Valvo R, Reddavid C, et al. Treatment in patients with severe asymptomatic aortic stenosis: is it best not to wait? Eur Heart J Suppl. 2022 Nov;24(Suppl I):I170–4.

94. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. Eur Heart J. 2016 Jul 14;37(27):2129–200.

95. Samaja GA. Balloon Aortic Valvuloplasty in the Transcatheter Aortic Valve Implantation Era. Heart Int. 2023;17(1):13–8.

96. Holmes DR, Mack M. The Truly Functional Heart Team: The Devil Is in the Details. J Am Heart Assoc. 2020 Apr 21;9(8):e05035.

97. Zhang B, Enriquez-Sarano M, Schaff H V, Michelena HI, Roos CM, Hagler MA, et al. Reactivation of Oxidized Soluble Guanylate Cyclase as a Novel Treatment Strategy to Slow Progression of Calcific Aortic Valve Stenosis: Preclinical and Randomized Clinical Trials to Assess Safety and Efficacy. Circulation. 2025 Feb 24;

98. ClinicalTrials.gov. U.S. National Library of Medicine. 2021 [cited 2025 Jan 3]. A Prospective Randomized Study to Assess the Efficacy of Valsartan in Retarding the Progression of Aortic Stenosis (VALVE Trial). Available from: https://clinicaltrials.gov/ct2/show/NCT04913870

99. ClinicalTrials.gov. U.S. National Library of Medicine. 2019 [cited 2025 Jan 3]. Pressure Assessment to Improve Outcomes After TAVR: a Registry. Available from: https://clinicaltrials.gov/ct2/show/NCT03923530

100. ClinicalTrials.gov. U.S. National Library of Medicine. 2023 [cited 2025 Jan 3]. Folic Acid Supplementation in Calcific Aortic Valve Disease. Available from: https://clinicaltrials.gov/ct2/show/NCT05861648

101. ClinicalTrials.gov. U.S. National Library of Medicine. 2020 [cited 2025 Jan 3]. Vitamin K and Vitamin K2 and Menaquinone in Aortic Valve Calcification. Available from: https://clinicaltrials.gov/ct2/show/NCT04429035

102. Czestkowska E, Rożanowska A, Długosz D, Bolt K, Świerszcz J, Kruszelnicka O, et al. Depressed systemic arterial compliance and impaired left ventricular midwall performance in aortic stenosis with concomitant type 2 diabetes: a retrospective cross-sectional study. Cardiovasc Diabetol. 2019 Dec 17;18(1):92.

103. Theofilis P, Sagris M, Oikonomou E, Antonopoulos AS, Siasos G, Tsioufis K, et al. The impact of SGLT2 inhibitors on inflammation: A systematic review and meta-analysis of studies in rodents. Int Immunopharmacol. 2022 Oct;111:109080.

104. Pahud de Mortanges A, Salvador Jr. D, Laimer M, Muka T, Wilhelm M, Bano A. The Role of SGLT2 Inhibitors in Atherosclerosis: A Narrative Mini-Review. Front Pharmacol. 2021 Nov 5;12.

105. Otto CM, Nishimura RA, Bonow RO, Carabello BA, Erwin JP, Gentile F, et al. 2020 ACC/AHA Guideline for the Management of Patients With Valvular Heart Disease: Executive Summary: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. Circulation. 2021 Feb 2;143(5).

106. Cavalcante PN, Kanhouche G, Rosa VEE, Campos CM, Lopes MP, Lopes MAAA de M, et al. B-type natriuretic peptide and N-terminal Pro-B-type natriuretic peptide in severe aortic stenosis: a comprehensive literature review. Front Cardiovasc Med. 2023;10:1182530.

107. Thanassoulis G. Lipoprotein (a) in calcific aortic valve disease: from genomics to novel drug target for aortic stenosis. J Lipid Res. 2016 Jun;57(6):917–24.

108. Olgun Küçük H, Küçük U, Demirtaş C, Özdemir M. Role of serum high density lipoprotein levels and functions in calcific aortic valve stenosis progression. Int J Clin Exp Med. 2015;8(12):22543–9.

109. Zieliński K, Kalińczuk Ł, Chmielak Z, Mintz GS, Dąbrowski M, Pręgowski J, et al. Additive Value of High-Density Lipoprotein Cholesterol and C-Reactive Protein Level Assessment for Prediction of 2-year Mortality After Transcatheter Aortic Valve Implantation. Am J Cardiol. 2020 Jul;126:66–72.

110. Redfors B, Furer A, Lindman BR, Burkhoff D, Marquis-Gravel G, Francese DP, et al. Biomarkers in Aortic Stenosis: A Systematic Review. Structural Heart. 2017 Jan;1(1–2):18–30.

111. Keranov S, Kim WK, Arsalan M, Renker M, Keller T, Bauer T, et al. Predictive value of preprocedural procalcitonin for short- and long-term mortality after transfemoral transcatheter aortic valve implantation. Heart Vessels. 2019 Dec 7;34(12):1993–2001.

112. Bienjonetti-Boudreau D, Fleury MA, Voisine M, Paquin A, Chouinard I, Tailleur M, et al. Impact of sex on the management and outcome of aortic stenosis patients. Eur Heart J. 2021 Jul 15;42(27):2683–91.

113. Springhetti P, Abdoun K, Clavel MA. Sex Differences in Aortic Stenosis: From the Pathophysiology to the Intervention, Current Challenges, and Future Perspectives. J Clin Med. 2024 Jul 19;13(14).

114. Côté N, Clavel MA. Sex Differences in the Pathophysiology, Diagnosis, and Management of Aortic Stenosis. Cardiol Clin. 2020 Feb;38(1):129–38.

115. Lang RM, Badano LP, Mor-Avi V, Afilalo J, Armstrong A, Ernande L, et al. Recommendations for cardiac chamber quantification by echocardiography in adults: an update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. J Am Soc Echocardiogr. 2015 Jan;28(1):1-39.e14.

116. Shan Y, Pellikka PA. Aortic stenosis in women. Heart. 2020 Jul;106(13):970–6.

117. Singh A, Chan DCS, Greenwood JP, Dawson DK, Sonecki P, Hogrefe K, et al. Symptom Onset in Aortic Stenosis: Relation to Sex Differences in Left Ventricular Remodeling. JACC Cardiovasc Imaging. 2019 Jan;12(1):96–105.

118. Thaden JJ, Nkomo VT, Suri RM, Maleszewski JJ, Soderberg DJ, Clavel MA, et al. Sex-related differences in calcific aortic stenosis: correlating clinical and echocardiographic characteristics and computed tomography aortic valve calcium score to excised aortic valve weight. Eur Heart J. 2016 Feb 21;37(8):693–9.

119. Voisine M, Hervault M, Shen M, Boilard AJ, Filion B, Rosa M, et al. Age, Sex, and Valve Phenotype Differences in Fibro-Calcific Remodeling of Calcified Aortic Valve. J Am Heart Assoc. 2020 May 18;9(10):e015610.

120. Simard L, Côté N, Dagenais F, Mathieu P, Couture C, Trahan S, et al. Sex-Related Discordance Between Aortic Valve Calcification and Hemodynamic Severity of Aortic Stenosis: Is Valvular Fibrosis the Explanation? Circ Res. 2017 Feb 17;120(4):681–91.

121. Cramariuc D, Rieck AE, Staal EM, Wachtell K, Eriksen E, Rossebø AB, et al. Factors influencing left ventricular structure and stress-corrected systolic function in men and women with asymptomatic aortic valve stenosis (a SEAS Substudy). Am J Cardiol. 2008 Feb 15;101(4):510–5.

122. Capoulade R, Clavel MA, Le Ven F, Dahou A, Thébault C, Tastet L, et al. Impact of left ventricular remodelling patterns on outcomes in patients with aortic stenosis. Eur Heart J Cardiovasc Imaging. 2017 Dec 1;18(12):1378–87.

123. McCoy CM, Nicholas DQ, Masters KS. Sex-related differences in gene expression by porcine aortic valvular interstitial cells. PLoS One. 2012;7(7):e39980.

124. Larsson SC, Wolk A, Håkansson N, Bäck M. Overall and abdominal obesity and incident aortic valve stenosis: two prospective cohort studies. Eur Heart J. 2017 Jul 21;38(28):2192–7.

125. Tribouilloy C, Bohbot Y, Rusinaru D, Belkhir K, Diouf M, Altes A, et al. Excess Mortality and Undertreatment of Women With Severe Aortic Stenosis. J Am Heart Assoc. 2021 Jan 5;10(1):e018816.

126. Katz M, Carlos Bacelar Nunes Filho A, Caixeta A, Antonio Carvalho L, Sarmento-Leite R, Alves Lemos Neto P, et al. Gender-related differences on short- and long-term outcomes of patients undergoing transcatheter aortic valve implantation. Catheter Cardiovasc Interv. 2017 Feb 15;89(3):429–36.

127. Czarnecki A, Qiu F, Koh M, Prasad TJ, Cantor WJ, Cheema AN, et al. Clinical outcomes after trans-catheter aortic valve replacement in men and women in Ontario, Canada. Catheter Cardiovasc Interv. 2017 Sep 1;90(3):486–94.

128. Kodali S, Williams MR, Doshi D, Hahn RT, Humphries KH, Nkomo VT, et al. Sex-Specific Differences at Presentation and Outcomes Among Patients Undergoing Transcatheter Aortic Valve Replacement: A Cohort Study. Ann Intern Med. 2016 Mar 15;164(6):377–84.

129. Conrotto F, D'Ascenzo F, Presbitero P, Humphries KH, Webb JG, O'Connor SA, et al. Effect of gender after transcatheter aortic valve implantation: a meta-analysis. Ann Thorac Surg. 2015 Mar;99(3):809–16.

130. Brown JM, O'Brien SM, Wu C, Sikora JAH, Griffith BP, Gammie JS. Isolated aortic valve replacement in North America comprising 108,687 patients in 10 years: changes in risks, valve types, and outcomes in the Society of Thoracic Surgeons National Database. J Thorac Cardiovasc Surg. 2009 Jan;137(1):82–90.

131. Simon LR, Scott AJ, Figueroa Rios L, Zembles J, Masters KS. Cellular-scale sex differences in extracellular matrix remodeling by valvular interstitial cells. Heart Vessels. 2023 Jan;38(1):122–30.

132. Ghezzi C, Loo DDF, Wright EM. Physiology of renal glucose handling via SGLT1, SGLT2 and GLUT2. Diabetologia. 2018 Oct;61(10):2087–97.

133. Wright EM, Loo DDF, Hirayama BA. Biology of human sodium glucose transporters. Physiol Rev. 2011 Apr;91(2):733–94.

134. Zelniker TA, Braunwald E. Mechanisms of Cardiorenal Effects of Sodium-Glucose Cotransporter 2 Inhibitors. J Am Coll Cardiol. 2020 Feb;75(4):422–34.

135. Sanchez-Dominguez C, Gallardo-Blanco H, Salinas-Santander M, Ortiz-Lopez R. Uridine 5'-diphospho-glucronosyltrasferase: Its role in pharmacogenomics and human disease (Review). Exp Ther Med. 2018 May 18;

136. Padda IS, Mahtani AU, Parmar M. Sodium-Glucose Transport Protein 2 (SGLT2) Inhibitors. 2024.

137. Dhillon S. Dapagliflozin: A Review in Type 2 Diabetes. Drugs. 2019 Jul;79(10):1135–46.

138. Scheen AJ. Sodium–glucose cotransporter type 2 inhibitors for the treatment of type 2 diabetes mellitus. Nat Rev Endocrinol. 2020 Oct 27;16(10):556–77.

139. Empagliflozin. Aust Prescr. 2015 Apr;38(2):64–6.

140. Liu Y, An C, Liu P, Yang F, Zhao Q. Comparative safety of sodium-glucose co-transporter 2 inhibitors in elderly patients with type 2 diabetes mellitus and diabetic kidney disease: a systematic review and meta-analysis. Ren Fail. 2023 Dec;45(1):2217287.

141. Suciu IM, Greluș A, Cozlac AR, Suciu BS, Stoica S, Luca S, et al. Fournier's Gangrene as an Adverse Event Following Treatment with Sodium Glucose Cotransporter 2 Inhibitors. Medicina (Kaunas). 2024 May 20;60(5).

142. American Diabetes Association Professional Practice Committee. 9. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes-2022. Diabetes Care. 2022 Jan 1;45(Suppl 1):S125–43.

143. Monzo L, Ferrari I, Cicogna F, Tota C, Calò L. Sodium-glucose co-transporter-2 inhibitors eligibility in patients with heart failure with reduced ejection fraction. Int J Cardiol. 2021 Oct 15;341:56–9.

144. Pabel S, Hamdani N, Singh J, Sossalla S. Potential Mechanisms of SGLT2 Inhibitors for the Treatment of Heart Failure With Preserved Ejection Fraction. Front Physiol. 2021;12:752370.

145. Kidney Disease: Improving Global Outcomes (KDIGO) Diabetes Work Group. KDIGO 2022 Clinical Practice Guideline for Diabetes Management in Chronic Kidney Disease. Kidney Int. 2022 Nov;102(5S):S1–127.

146. Zelniker TA, Wiviott SD, Raz I, Im K, Goodrich EL, Bonaca MP, et al. 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 Jan 5;393(10166):31–9.

147. Pereira MJ, Eriksson JW. Emerging Role of SGLT-2 Inhibitors for the Treatment of Obesity. Drugs. 2019 Feb;79(3):219–30.

148. Cusi K, Isaacs S, Barb D, Basu R, Caprio S, Garvey WT, et al. American Association of Clinical Endocrinology Clinical Practice Guideline for the Diagnosis and Management of Nonalcoholic Fatty Liver Disease in Primary Care and Endocrinology Clinical Settings: Co-Sponsored by the American Association for the Study of Liver Diseases (AASLD). Endocr Pract. 2022 May;28(5):528–62.

149. Marx N, Federici M, Schütt K, Müller-Wieland D, Ajjan RA, Antunes MJ, et al. 2023 ESC Guidelines for the management of cardiovascular disease in patients with diabetes. Eur Heart J. 2023 Oct 14;44(39):4043–140.

150. Bogdanova M, Zabirnyk A, Malashicheva A, Semenova D, Kvitting JPE, Kaljusto ML, et al. Models and Techniques to Study Aortic Valve Calcification in Vitro, ex Vivo and in Vivo. An Overview. Front Pharmacol. 2022;13:835825.

151. Bond WS, Roberts EL, Warnock JN. Evaluation of porcine aortic valve interstitial cell activity using different serum types in two- and three-dimensional culture. Tissue Eng. 2007 Feb;13(2):343–9.

152. El Husseini D, Boulanger MC, Mahmut A, Bouchareb R, Laflamme MH, Fournier D, et al. P2Y2 receptor represses IL-6 expression by valve interstitial cells through Akt: implication for calcific aortic valve disease. J Mol Cell Cardiol. 2014 Jul;72:146–56.

153. Xue Y, St Hilaire C, Hortells L, Phillippi JA, Sant V, Sant S. Shape-Specific Nanoceria Mitigate Oxidative Stress-Induced Calcification in Primary Human Valvular Interstitial Cell Culture. Cell Mol Bioeng. 2017 Oct;10(5):483–500.

154. Rutkovskiy A, Malashicheva A, Sullivan G, Bogdanova M, Kostareva A, Stensløkken K, et al. Valve Interstitial Cells: The Key to Understanding the Pathophysiology of Heart Valve Calcification. J Am Heart Assoc. 2017 Sep 22;6(9).

155. Ground M, Callon K, Walker R, Milsom P, Cornish J. Perspective Chapter: Valvular Interstitial Cells – Physiology, Isolation, and Culture. In 2024.

156. Tsang HG, Clark EL, Markby GR, Bush SJ, Hume DA, Corcoran BM, et al. Expression of Calcification and Extracellular Matrix Genes in the Cardiovascular System of the Healthy Domestic Sheep (Ovis aries). Front Genet. 2020;11:919.

157. Bogdanova M, Zabirnyk A, Malashicheva A, Semenova D, Kvitting JPE, Kaljusto ML, et al. Models and Techniques to Study Aortic Valve Calcification in Vitro, ex Vivo and in Vivo. An Overview. Front Pharmacol. 2022;13:835825.

158. Goto S, Rogers MA, Blaser MC, Higashi H, Lee LH, Schlotter F, et al. Standardization of Human Calcific Aortic Valve Disease in vitro Modeling Reveals Passage-Dependent Calcification. Front Cardiovasc Med. 2019;6:49.

159. Babu AN, Meng X, Zou N, Yang X, Wang M, Song Y, et al. Lipopolysaccharide stimulation of human aortic valve interstitial cells activates inflammation and osteogenesis. Ann Thorac Surg. 2008 Jul;86(1):71–6.

160. Langenbach F, Handschel J. Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Ther. 2013;4(5):117.

161. Iqbal F, Schlotter F, Becker-Greene D, Lupieri A, Goettsch C, Hutcheson JD, et al. Sortilin enhances fibrosis and calcification in aortic valve disease by inducing interstitial cell heterogeneity. Eur Heart J. 2023 Mar 7;44(10):885–98.

162. Purushothaman KR, Purushothaman M, Turnbull IC, Adams DH, Anyanwu A, Krishnan P, et al. Association of altered collagen content and lysyl oxidase expression in degenerative mitral valve disease. Cardiovasc Pathol. 2017;29:11–8.

163. Moncla LHM, Briend M, Bossé Y, Mathieu P. Calcific aortic valve disease: mechanisms, prevention and treatment. Nat Rev Cardiol. 2023 Aug;20(8):546–59.

164. Falcão-Pires I, Gavina C, Leite-Moreira AF. Understanding the molecular and cellular changes behind aortic valve stenosis. Curr Pharm Biotechnol. 2012 Oct;13(13):2485–96.

165. Tian J, Zhang M, Suo M, Liu D, Wang X, Liu M, et al. Dapagliflozin alleviates cardiac fibrosis through suppressing EndMT and fibroblast activation via AMPKα/TGF-β/Smad signalling in type 2 diabetic rats. J Cell Mol Med. 2021 Aug 25;25(16):7642–59.

166. Lu J, Xie S, Deng Y, Xie X, Liu Y. Blocking the NLRP3 inflammasome reduces osteogenic calcification and M1 macrophage polarization in a mouse model of calcified aortic valve stenosis. Atherosclerosis. 2022 Apr;347:28–38.

167. Abdel-Rafei MK, Thabet NM, Rashed LA, Moustafa EM. Canagliflozin, a SGLT-2 inhibitor, relieves ER stress, modulates autophagy and induces apoptosis in irradiated HepG2 cells: Signal transduction between PI3K/AKT/GSK-3β/mTOR and Wnt/β-catenin pathways; in vitro. J Cancer Res Ther. 2021;17(6):1404–18.

168. Chen A, Lan Z, Li L, Xie L, Liu X, Yang X, et al. Sodium-glucose cotransporter 2 inhibitor canagliflozin alleviates vascular calcification through suppression of nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 inflammasome. Cardiovasc Res. 2023 Oct 24;119(13):2368–81.

169. Ahmed AS, Mona MM, Abdel-Kareem MA, Elsisy RA. SGLT2 inhibitor empagliflozin monotherapy alleviates renal oxidative stress in albino Wistar diabetic rats after myocardial infarction induction. Biomed Pharmacother. 2021 Jul;139:111624.

170. Scisciola L, Paolisso P, Belmonte M, Gallinoro E, Delrue L, Taktaz F, et al. Myocardial sodium-glucose cotransporter 2 expression and cardiac remodelling in patients with severe aortic stenosis: The BIO-AS study. Eur J Heart Fail. 2024 Feb;26(2):471–82.

171. Carter S, Miard S, Roy-Bellavance C, Boivin L, Li Z, Pibarot P, et al. Sirt1 inhibits resistin expression in aortic stenosis. PLoS One. 2012;7(4):e35110.

172. Packer M. Critical Reanalysis of the Mechanisms Underlying the Cardiorenal Benefits of SGLT2 Inhibitors and Reaffirmation of the Nutrient Deprivation Signaling/Autophagy Hypothesis. Circulation. 2022 Nov;146(18):1383–405.

173. Santos-Gallego CG, Requena-Ibanez JA, San Antonio R, Garcia-Ropero A, Ishikawa K, Watanabe S, et al. Empagliflozin Ameliorates Diastolic Dysfunction and Left Ventricular Fibrosis/Stiffness in Nondiabetic Heart Failure: A Multimodality Study. JACC Cardiovasc Imaging. 2021 Feb;14(2):393–407.

174. Hmadeh S, Trimaille A, Matsushita K, Marchandot B, Carmona A, Zobairi F, et al. Human Aortic Stenotic Valve-Derived Extracellular Vesicles Induce Endothelial Dysfunction and Thrombogenicity Through AT1R/NADPH Oxidases/SGLT2 Pro-Oxidant Pathway. JACC Basic Transl Sci. 2024 Jul;9(7):845–64.

175. Cuevas RA, Chu CC, Moorhead WJ, Wong R, Sultan I, St Hilaire C. Isolation of Human Primary Valve Cells for In vitro Disease Modeling. J Vis Exp. 2021 Apr 16;(170).

176. Cuevas RA, Chu CC, Moorhead WJ, Wong R, Sultan I, St Hilaire C. Isolation of Human Primary Valve Cells for In vitro Disease Modeling. J Vis Exp. 2021 Apr 16;(170).

177. Shu L, Yuan Z, Li F, Cai Z. Oxidative stress and valvular endothelial cells in aortic valve calcification. Biomed Pharmacother. 2023 Jul;163:114775.

178. Pellikka PA, Kato N. Aortic Stenosis Risk and Management: Sex Disparities or Just Differences? JACC Cardiovasc Imaging. 2024 Jan;17(1):13–5.

179. Hariri EH, El Halabi J, Kassis N, Al Hammoud MM, Badwan OZ, Layoun H, et al. Sex Differences in the Progression and Long-Term Outcomes of Native Mild to Moderate Aortic Stenosis. JACC Cardiovasc Imaging. 2024 Jan;17(1):1–12.

180. Ogola BO, Zimmerman MA, Clark GL, Abshire CM, Gentry KM, Miller KS, et al. New insights into arterial stiffening: does sex matter? Am J Physiol Heart Circ Physiol. 2018 Nov 1;315(5):H1073–87.

181. Matilla L, Garaikoetxea M, Arrieta V, García-Peña A, Fernández-Celis A, Navarro A, et al. Sex-Differences in Aortic Stenosis: Mechanistic Insights and Clinical Implications. Front Cardiovasc Med. 2022;9:818371.

182. Fliegner D, Ellieva A, Angelov A, Petrov G, Regitz-Zagrosek V. Sex differences and estrogen effects in cardiac mitochondria in human aortic stenosis and in the mouse heart. Front Endocrinol (Lausanne). 2023;14:1181044.

183. McCoy CM, Nicholas DQ, Masters KS. Sex-related differences in gene expression by porcine aortic valvular interstitial cells. PLoS One. 2012;7(7):e39980.

184. Cristovão A, Andrade N, Martel F, Silva C. Effect of Sodium-Glucose Co-Transporter 2 Inhibitors Combined with Metformin on Pancreatic Cancer Cell Lines. Int J Mol Sci. 2024 Sep 14;25(18).

185. Dasari D, Bhat A, Mangali S, Ghatage T, Lahane GP, Sriram D, et al. Canagliflozin and Dapagliflozin Attenuate Glucolipotoxicity-Induced Oxidative Stress and Apoptosis in Cardiomyocytes via Inhibition of Sodium-Glucose Cotransporter-1. ACS Pharmacol Transl Sci. 2022 Apr 8;5(4):216–25.

186. International Organization for Standardization. ISO 10993-5:2009 – Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity. Geneva; 2009.

187. Côté N, El Husseini D, Pépin A, Guauque-Olarte S, Ducharme V, Bouchard-Cannon P, et al. ATP acts as a survival signal and prevents the mineralization of aortic valve. J Mol Cell Cardiol. 2012 May;52(5):1191–202.

188. Simon LR, Scott AJ, Figueroa Rios L, Zembles J, Masters KS. Cellular-scale sex differences in extracellular matrix remodeling by valvular interstitial cells. Heart Vessels. 2023 Jan;38(1):122–30.

189. Dalfino G, Simone S, Porreca S, Cosola C, Balestra C, Manno C, et al. Bone morphogenetic protein-2 may represent the molecular link between oxidative stress and vascular stiffness in chronic kidney disease. Atherosclerosis. 2010 Aug;211(2):418–23.

190. Chen Q, Wang ZY, Chen LY, Hu HY. Roles of High Mobility Group Box 1 in Cardiovascular Calcification. Cell Physiol Biochem. 2017;42(2):427–40.

191. Rykova EY, Klimontov V V, Shmakova E, Korbut AI, Merkulova TI, Kzhyshkowska J. Anti-Inflammatory Effects of SGLT2 Inhibitors: Focus on Macrophages. Int J Mol Sci. 2025 Feb 15;26(4).

192. Narula S, Shameer K, Salem Omar AM, Dudley JT, Sengupta PP. Machine-Learning Algorithms to Automate Morphological and Functional Assessments in 2D Echocardiography. J Am Coll Cardiol. 2016 Nov 29;68(21):2287–95.

193. Thoenes M, Bramlage P, Zamorano P, Messika-Zeitoun D, Wendt D, Kasel M, et al. Patient screening for early detection of aortic stenosis (AS)-review of current practice and future perspectives. J Thorac Dis. 2018 Sep;10(9):5584–94.

194. Bernar A, Gebetsberger JV, Bauer M, Streif W, Schirmer M. Optimization of the Alizarin Red S Assay by Enhancing Mineralization of Osteoblasts. Int J Mol Sci. 2022 Dec 31;24(1).

SUPPLEMENTARY MATERIALS:

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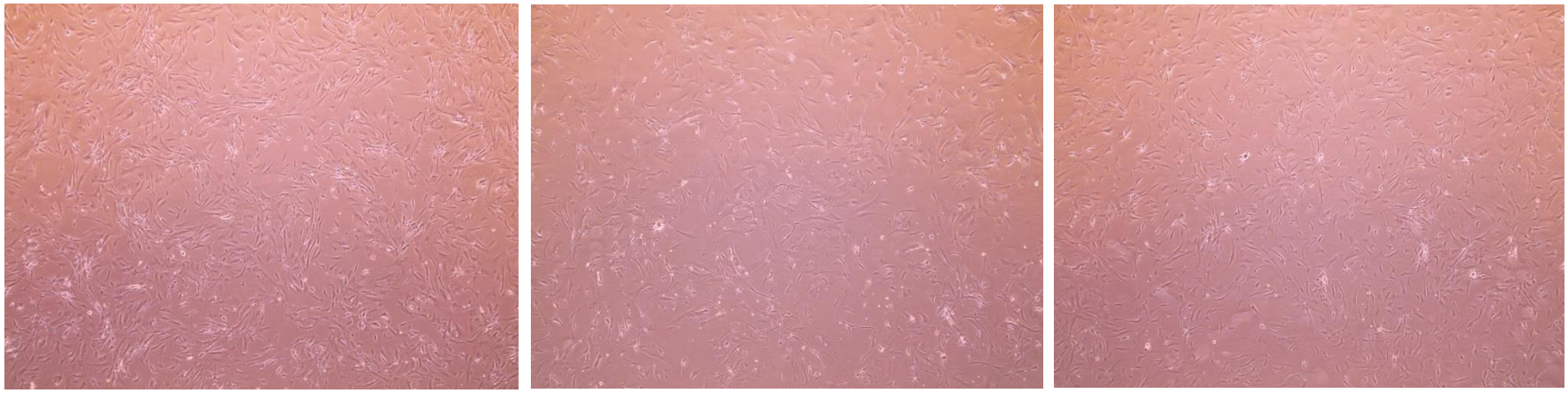

Figure S2 – Representative microscopic photographs of some plates during cell' expansion. They were photographed under a digital, transmitted light inverted microscope (EVOS^{TM} XL Core Imaging System, Invitrogen) at a 100x magnification.

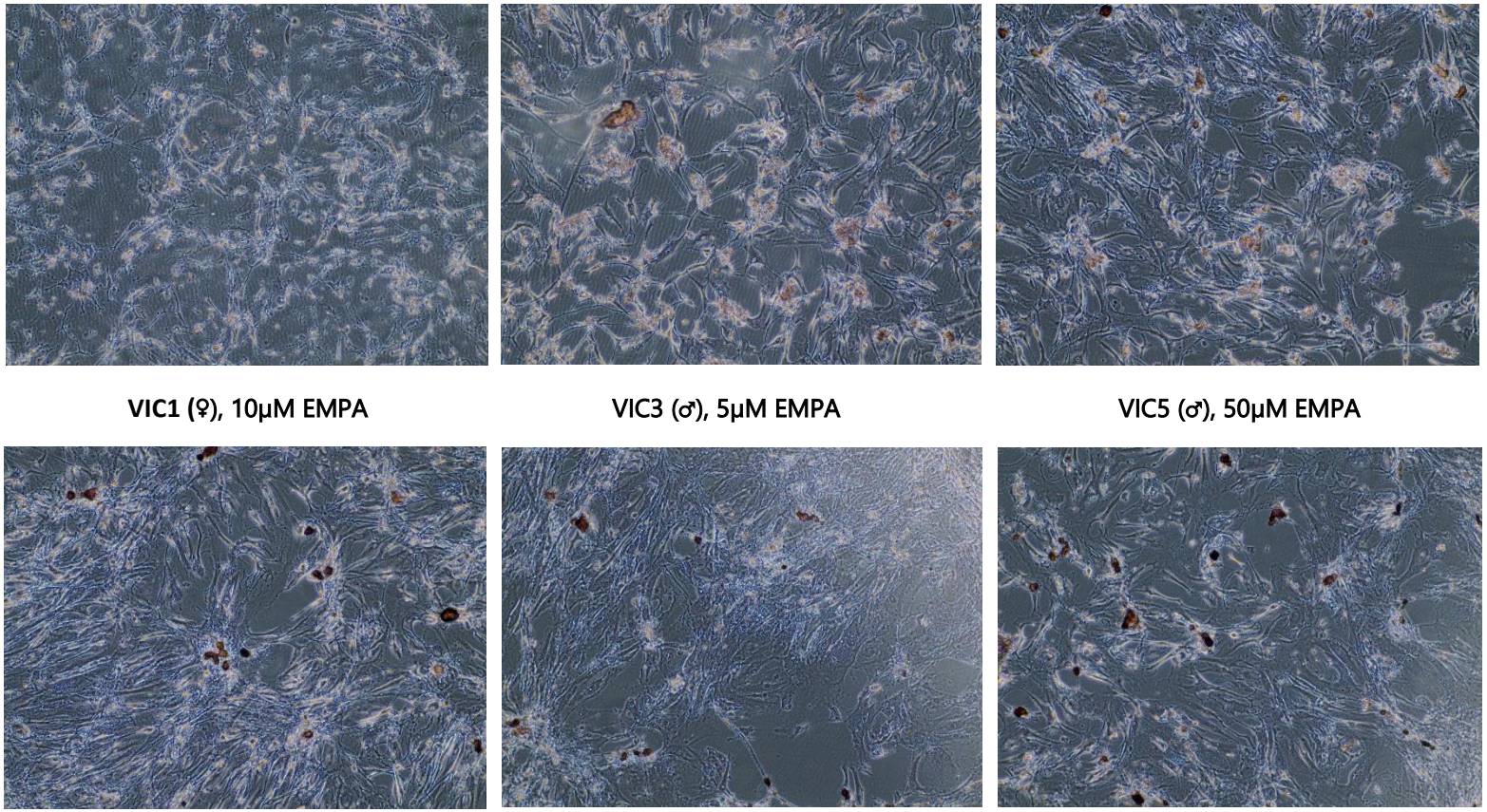

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Figure S1 – Representative microscopic photographs of some wells after 21 days of the calcification protocol. They were photographed under a digital, transmitted light inverted microscope (EVOS^{TM} XL Core Imaging System, Invitrogen) at a 100x magnification. Additional settings: brightness level 55; TIF format; 3MP resolution.

Table S1 – Clinical information of human VICs' donors.

	VIC0	VIC1	VIC3	VIC4	VIC5	VIC6	VIC2
Clinical information provided	Severe aortic regurgitation (bicuspid valve) + aortic root dilation	Severe and symptomatic aortic stenosis (elective surgery)	Severe aortic stenosis + acute myocardial infarction + atrial fibrillation (Cardiac catheterization: no significant coronary artery disease; moderate ostial and mid-segment disease in the descending artery)	Severe and symptomatic aortic stenosis (urgent surgery)	Severe and symptomatic aortic stenosis (elective surgery)	Severe and symptomatic aortic stenosis (elective surgery)	Severe aortic stenosis + aortic valve repair + annular enlargement + myectomy
Birth date (month/year)	12/1949	03/1950	10/1966	09/1956	10/1955	10/1955	06/1948
Gender	Male	Female	Male	Male	Male	Female	Female
Weight (kg)	65	58	98	68	72	103	76
Height (cm)	163	143	173	167	158	167	161
Smoking status	Current smoker	Never smoked	Current smoker	Current smoker	Never smoked	Never smoked	Never-smoked
Diabetes	no	Yes, treated	no	no	Yes, tretead	no	no
Hypertension	Yes, treated	Yes, treated	Yes, treated	no	Yes, treated	Yes, treated	Yes, treated
Dyslipidemia	no	yes	no	yes	no	no	yes
Extracardiac arteriopathy	Cerebrovascular disease	no	no	Cerebrovascular disease	Cerebrovascular disease	no	no
Type PAD	no	no	no	no	no	no	no
Type cerebrovascular disease	CVA	no	no	carotid stenosis > 50% oclusion	CVA	no	no
Pre-op heart rhythm	Sinus rhythm	Sinus rhythm	AF new	Sinus rhythm	Sinus rhythm	Sinus rhythm	Sinus rhythm
Previous AF	no	no	yes	no	no	no	no
Previos echocardiogram	yes	yes	yes	yes	yes	yes	yes
Ejection fraction	normal	normal	normal	normal	normal	normal	normal
Ejection fraction (%)	Unknown	62	62	55	62	54	Unknown
Aortic valve	bicuspid (RC/LC)	tricuspid	tricuspid	tricuspid	tricuspid	tricuspid	tricuspid

Aortic valve pathology	regurgitation	regurgitation + stenosis	Regurgitation + stenosis	Regurgitation + stenosis	Regurgitation + stenosis	stenosis	stenosis
Aortic regurgitation	severe	mild	moderate	mild-to-moderate	mild	absent	mild
Aortic valve stenosis	absent	severe	severe	severe	severe	severe	severe
Aortic valve mean gradient (mmHg)	26	48	63	91	62	50	46
Aortic valve max gradient (mmHg)	Unknown	71	105	145	99	82	64
Aortic valve area (cm2)	1,57	0,8	0,8	0,6	0,8	0,75	1
Urgency	elective	elective	urgent	urgent	elective	elective	elective
Main reason for surgery	valve dysfunction	valve dysfunction	valve dysfunction	valve dysfunction	valve dysfunction	valve dysfunction	valve dysfunction
Usual Medication	Felodipine 10 mg id, Indapamide 1.5 mg id, Ramipril 5 mg id, Atorvastatin 20 mg id, Edistride (Dapagliflozin) 10 mg id, Clonazepam (Rivotril) 0.5 mg id, Escitalopram 10 mg id, Quetiapine 50 mg id, Sodium bicarbonate 1000 mg id, Patiromer (Veltassa) 8.4 g id	Perindopril / Amlodipine 5/5 mg id, Linagliptin 5 mg id, Rosuvastatin / Ezetimibe 20/10 mg id, Aspirin 100 mg id, Metformin / Dapagliflozin 1000/5 mg id, Furosemide 40 mg id	Losartan / Hydrochlorothiazide 100/25 mg ½ tablet id	Levothyroxine 0.1 mg id	Dapagliflozin (Forxiga) 10 mg id, Insulin Glargine12 U id, Valsartan / Hydrochlorothiazide 320/12.5 mg id, Pantoprazole 20 mg id, Rosuvastatin 20 mg id, Amlodipine 5 mg id, Aspirin 100 mg id	Olanzapine 5 mg id, Risperidone 1 mg id, Quetiapine 100 mg id, Memantine 5 mg id, Sertraline 50 mg id, Aspirin / Atorvastatin / Ramipril 100/20/5 mg id, Furosemide 40 mg id	Losartan / Hydrochlorothiazide 100/12.5 mg id, Atorvastatin 10 mg id, Levothyroxine (Eutirox) id

Abbreviations: RC – right coronary; LC – left coronary; CVA - cerebrovascular accident; U – units; AF – atrial fibrillation; PAD - peripheral artery disease; id – individual dose. Some clinical data were not provided for all donors and are indicated as “Unknown”.

Table S2 — Valves and cell isolation data

	Cell Line	Collection date	Indication for surgery	Valve weight	Photo	Custodiol	Time elapse_collection-isolation	Collagenase REF	Collagenase LOT	Incubation time collagenase	Cell yield	Cell Viability	Frozen cells (total)	Frozen cells per aliquot	Number of aliquots	Volume of each aliquot
		Date		**g**						**h**	**x10^6**	**%**	**x10^6**	**x10^6**		**mL**
♀	**VIC0**	30/04/2024	AV insufficiency	Missing data	Yes	No	< 2h	17101-015	2202404	17	1.36	86	0	0	0	-
♀	**VIC1**	04/07/2024	AV stenosis	1.72	Yes	Yes – no AA	< 2h	17101-015	2202404	18	1.94	82	0	0	0	
♀	**VIC2**	10/07/2024	AV stenosis	2.21	Yes	Yes – AA	< 6h	17101-015	2202404	18	2.48	87	0	0	0	
♂	**VIC3**	16/07/2024	AV stenosis	2.42	Yes	Yes – AA	< 23h	17101-015	2202404	18	1.54	86	0	0	0	
♂	**VIC4**	01/10/2024	AV stenosis	3.97	Yes	Yes – no AA	< 22h	17101-015	2202404	18	0.65	69	0	0	0	
♂	**VIC5**	08/10/2024	AV stenosis	1.29	Yes	Yes – AA	< 23h	17101-015	2202404	18	2.58	92	0	0	0	
♀	**VIC6**	21/11/2024	AV stenosis	2.95	Yes	Yes – AA	< 2h	17101-015	2202404	18	2.83*	94	0	0	0	
Swine model	**VICS1**	13/01/2025	Pig sacrifice	0.36	No	Yes – AA	< 18h	17101-015	2202404	20.5	4.25	87	0	0	0	
Swine model	**VICS2**	22/01/2025	Pig sacrifice	0.33	Yes	Yes – AA	< 3h	17101-015	2202404	16	9.60	96	0	0	0	

* Unreliable cell count (Quantification seems to have been influenced by Trypan Blue).

Clinical and laboratory parameters of human and porcine VICs isolations, including clinical background, elapsed time until processing, enzymatic digestion conditions, cell yield and viability. Abbreviations: AV – aortic valve; AA – antibiotic-antimycotic; VIC – valvular interstitial cell; VIC-S – swine-derived VIC; REF - reagent reference number; LOT – lot number.

Table S3 — Primary cell culture characteristics: VICs isolation (P0)

Cell Line	Cells per Plate distribution	Days to 90% confluency	DMEM REF	DMEM LOT	FBS REF	FBS LOT	AM REF	AM LOT	Trypsin REF	Trypsin LOT	Cell yield	Cell viability	Frozen cells (total)	Frozen cells per aliquot	Number of aliquots	Volume of each aliquot
											x10^6	%	x10^6	x10^6		mL
VIC0	1.36 / (2x10 cm^2)	12	41966-029	2661123	10270-106	2437711	15240-062	2441427	25300-062	2698732	2.18	90	0	0	0	0
VIC1	1.94 / (2x75 cm^2)	11	41966-029	2661123	10270-106	2437711	15240-062	2441427	12605-028	2682317	1,60	78	0	0	0	0
VIC2	2.48 / (3x75 cm^2)	12	41966-029	2661123 2850893	10270-106	2437711	15240-062	2441427	12605-028	2682317	2.33	88	0	0	0	0
VIC3 *	1.54 / (1x75 cm^2)	8	41966-029	2661123	10270-106	2437711	15240-062	2441427	12605-028	2682317	2.16	88	0	0	0	0
VIC4	0.65 / (1x10 cm^2)	14	41966-029	2850893	10270-106	2437711	15240-062	2441427	12605-028	2682317	1.15	91	0	0	0	0
VIC5	2.58 / (3x75 cm^2)	7	41966-029	2850893	10270-106	2437711	15240-062	2441427	12605-028	2682317	1.81	90	0	0	0	0
VIC6	2.13 / (1x75 cm^2)	12	41966-029	2962893	10270-106	2437711	15240-062	2441427	12605-028	2682317	1.00	81	0	0	0	0
VICS1	4.25 / (4x75 cm^2)	5	41966-029	3023253	A5256701	B2695494RP	15240-062	2441427	12605-028	2682317	23.00	99	22	2.0	11	1
VICS2	4.00 / (4x75 cm^2)	4	41966-029	3023253	A5256701	B2695494RP	15240-062	2441427	12605-028	2682317	24.00	98	22	2.0	11	1

* P0' [0.71(1x75 cm^2)]

Culture parameters of human and porcine VICs at passage 0 (P0), including time to confluence, reagent reference and batch numbers, and cell yields. DMEM – Dulbecco's Modified Eagle Medium; FBS – fetal bovine serum; AM – antibiotic-antimycotic; P0 – passage 0 (first passage); VIC-S – swine-derived VIC; REF – reagent reference number; LOT – lot number

Table S4 — Primary cell culture characteristics: initial expansion (P0)

Cell Line	Cells per Plate distribution	Days to 90% confluency	DMEM REF	DMEM LOT	FBS REF	FBS LOT	AM REF	AM LOT	Trypsin REF	Trypsin LOT	Cell yield	Cell viability	Frozen cells (total)	Frozen cell per aliquot	Number of aliquots	Volume of each aliquot
											$x10^6$	%	$x10^6$	$x10^6$		mL
VIC0	0.5 / (1x75 cm^2)	8									1.3	77	1	0.5	2	
VIC1	1.6 / (3x75 cm^2)	9	41966-029	2858893 2661123	10270-106	2437711	15240-062	2441427	12605-028	2682317	2.4	86	2.4	1.2	2	1
VIC2	2.33 / (5x75 cm^2)	6	41966-029	2858893	10270-106	2437711	15240-062	2441427	12605-028	2682317	4.5	81	4.5	1.14	4	1
VIC3	1.16 / (3x75 cm^2)	7	41966-029	2858893	10270-106	2437711	15240-062	2441427	12605-028	2682317	5.1	94	5.1	1.02	5	1
VIC4	1.15 / (2x75 cm^2)	7	41966-029	2902893	10270-106	2437711	15240-062	2441427	12605-028	2682317	2.7	96	2.7	2x1 + 1x0.6	3	2x1 + 1x0.6
VIC5	1.81 / (4x75 cm^2)	6	41966-029	2902893	10270-106	2437711	15240-062	2441427	12605-028	2682317	6.1	96	6.1	1.02	6	1
VIC6	1.00 / (2x75 cm^2)	7	41966-029	3023253	10270-106	2437711	15240-062	2441427	12605-028	2682317	1.0	80	2.0	1.0	2	1
VICS1	1.00 / (2x75 cm^2)	5	41966-029	3023253	A5256701	B2695494RP	15240-062	2913064	12605-028	2682317	13.6	100	13.6	1.94	7	1
VICS2	1.40 / (2x75 cm^2)	4	41966-029	3023253	A5256701	B2695494RP	15240-062	2913064	12605-028	2682317	23.6	99	23.6	2.0	12	1

Culture parameters of human and porcine VICs at passage 1 (P1), including seeding distribution, reagents, viability, and cell yields. P1 – second passage; DMEM – Dulbecco's Modified Eagle Medium; FBS – fetal bovine serum; AM – antibiotic-antimycotic; VIC-S – swine-derived VIC; REF – reagent reference number; LOT – lot number.

Table S5 — Primary cell culture characteristics: expansion (P1)

Cell Line	Cells per Plate distribution	Days to 90%	DMEM REF	DMEM LOT	FBS REF	FBS LOT	AM REF	AM LOT	Trypsin REF	Trypsin LOT	Cell yield	Cell viability	Frozen cells (total)	Frozen cells per aliquot	Number of aliquots	Volume per aliquot
											x10^6	**%**	**x10^6**	**x10^6**		**mL**
VIC1	1.2 / (6x75 cm^2)	7	41966-029	3023253	10270-106	2437711*	15140-122	2585627	25300-062	269873	3.44	81	0	0	0	
VIC2	1.14 / (6x75 cm^2)	6	41966-029	2858893	10270-106	2437711	15140-122	2585627	25300-062	269873	23.38	92	0	0	0	
VIC3	1.02 / (5x75 cm^2)	6	41966-029	2858893	10270-106	2437711	15140-122	2585627	25300-062	269873	2.33	89	0	0	0	
VIC4	1.00 / (4x75 cm^2)	13	41966-029	3023253	10270-106	2437711*	15140-122	2585627	25300-062	269873	6.36	77	3.0	1.0	0	1.0 mL →P1'
VIC5	1.02 / (6x75 cm^2)	9	41966-029	3023253	10270-106	2437711*	15140-122	2585627	25300-062	269873	3.50	89	0	0	0	
VIC6	Directly to P2															

* FBS A5256701
B2695494RP

Seeding distribution and culture conditions of human VICs at P1. VIC6 was excluded from this phase and directly advanced to P2. P1 – second passage; VIC – valvular interstitial cell; DMEM – Dulbecco's Modified Eagle Medium; FBS – fetal bovine serum; AM – antibiotic-antimycotic; REF – reagent reference number; LOT – lot number.

Table S6 — Primary cell culture characteristics: expansion (P2)

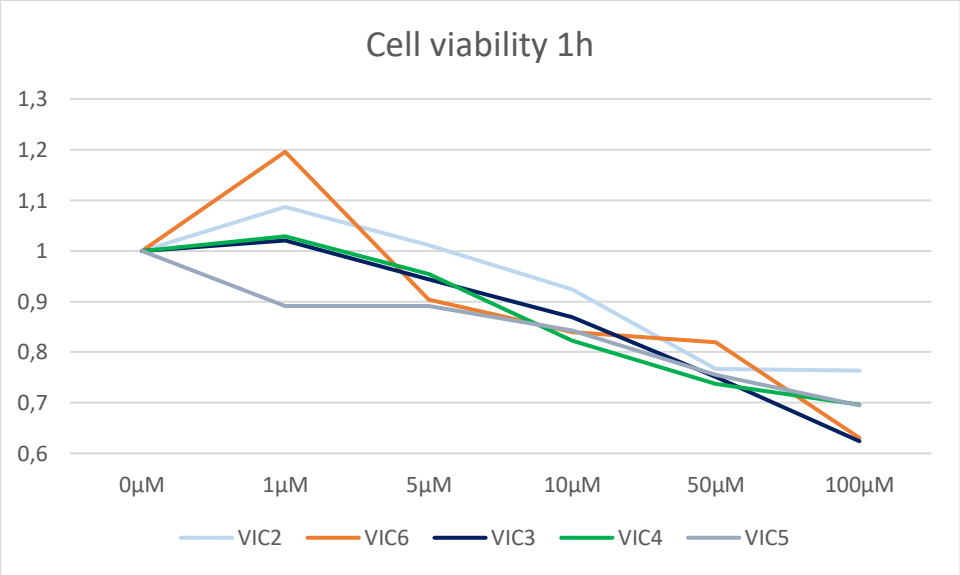
Date P0 → P1	Cell Line	Cells per Plate distribution	Days to 90% confluency	DMEM REF	DMEM LOT	FBS REF	FBS LOT	AM REF	AM LOT	Trypsin REF	Trypsin LOT	Cell yield	Cell viability	Frozen cells (total)	Frozen cells per aliquot	Number of aliquots	Volume of each aliquot
												$x10^6$	**%**	**$x10^6$**	**$x10^6$**		**mL**
10/12/2024	**VIC1**	3.44 / (6x75 cm^2)	8	41966-029	3023253	A5256701	B2695494RP	15140-122	2585627	25300-062	269873	17.8*	81*	17.8	1.6	11	1
25/11/2024	**VIC2**	3.38 / (6x75 cm^2)	7	41966-029	2858893	10270-106	2437711	15140-122	2585627	25300-062	269873	12.8	96	12.8	1.42	9	1
25/11/2024	**VIC3**	2.33 / (5x75 cm^2)	7	41966-029	2858893	10270-106	2437711	15140-122	2585627	25300-062	269873	12.0	95	12.0	1.5	8	1
16/12/2024	**VIC4**	3.36 / (4x75 cm^2)	7	41966-029	2858893	10270-106	2437711	15140-122	2585627	25300-062	269873	3.50	93	3.5	0.87	4	1
15/01/2025	**VIC5**	3.50 / (6x75 cm^2)	7	41966-029	2858893	A5256701	B2695494RP	15140-122	2585627	25300-062	269873	6.80	95	6.8	0.97 ≅ 1	7	1
11/12/2024	**VIC6**	2.00 / (4x75 cm^2)	8	41966-029	3023253	A5256701	B2695494RP	15140-122	2585627	25300-062	269873	6.30	95	6.3	1.0	6	1

1590 * Unreliable cell count (Quantification seems to have been influenced by Trypan Blue).

Culture data of human VICs at passage 2 (P2), including confluence times, reagents, cell viability, and yields. P2 – third passage; DMEM – Dulbecco's Modified Eagle Medium; FBS – fetal bovine serum; AM – antibiotic-antimycotic; VIC – valvular interstitial cell; REF – reagent reference number; LOT – lot number.

Table S7 - VICs' viability 1h

Viability 1h						
	0µM	**1µM**	**5µM**	**10µM**	**50µM**	**100µM**
VIC1	1	13,46179	18,34629	18,22586	17,47267	13,24277
VIC2	1	1,08686	1,01115	0,92425	0,76626	0,76356
VIC6	1	1,19576	0,90331	0,83969	0,81933	0,63048
VIC3	1	1,02053	0,94343	0,86863	0,75134	0,62399
VIC4	1	1,02876	0,95465	0,82293	0,73719	0,69632
VIC5	1	0,89131	0,89161	0,84253	0,75510	0,69482



1600

Figure S3 – VICs' viability 1h

Table S8 - VICs' viability 2h

Viability 2h						
	0µM	**1µM**	**5µM**	**10µM**	**50µM**	**100µM**
VIC1	1	11,85012	17,86263	17,59711	16,93730	12,47227
VIC2	1	1,10516	1,04409	0,91811	0,79683	0,77725
VIC6	1	1,09019	0,92984	0,89383	0,86258	0,69155
VIC3	1	1,01849	0,95399	0,89950	0,78239	0,66740
VIC4	1	1,00659	0,96314	0,84817	0,76893	0,72277
VIC5	1	0,91292	0,91338	0,86973	0,78449	0,72120

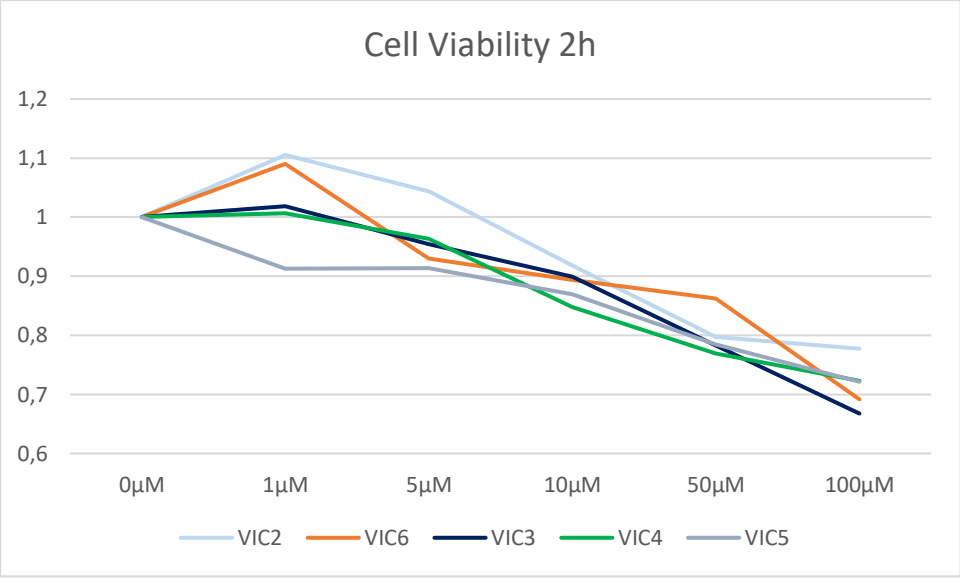


Figure S4 - VICs' viability 2h

Table S9 - VICs' viability 3h

Viability 3h						
	0µM	**1µM**	**5µM**	**10µM**	**50µM**	**100µM**
VIC1	1	8,14011	14,10613	13,80698	13,40639	9,47998
VIC2	1	1,10034	1,04289	0,93622	0,82035	0,79263
VIC6	1	1,08916	0,94715	0,91791	0,88529	0,72677
VIC3	1	1,02776	0,98002	0,93810	0,85072	0,74239
VIC4	1	0,94242	0,97065	0,87292	0,79819	0,75200
VIC5	1	0,93653	0,93640	0,90315	0,82540	0,76875

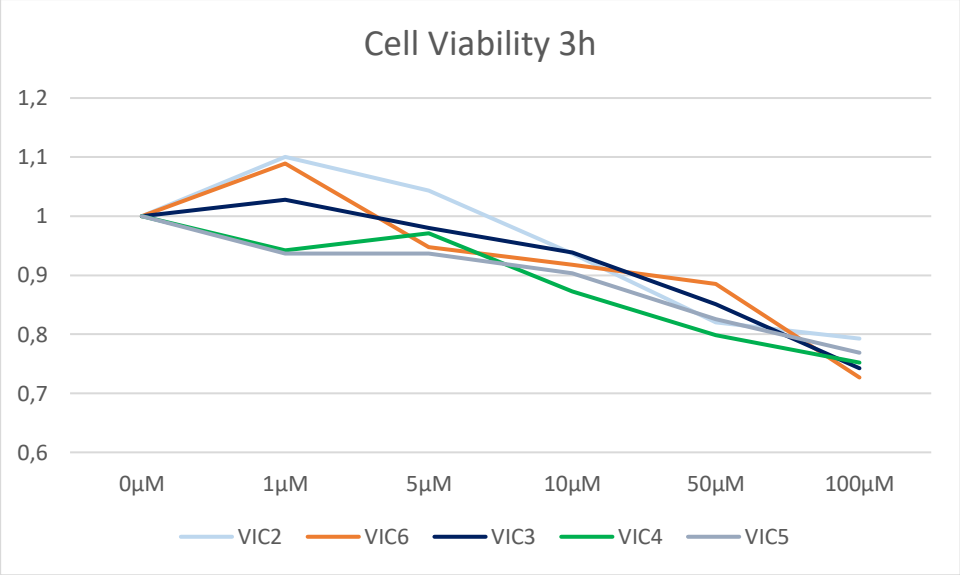


Figure S5 - VICs' viability 3h

Table S10 - VICs' viability 4h

Viabilidade 4h						
	0µM	**1µM**	**5µM**	**10µM**	**50µM**	**100µM**
VIC1	1	7,07949	13,11870	12,95945	12,64959	8,95483
VIC2	1	1,07491	1,03829	0,94086	0,83671	0,80741
VIC6	1	1,08384	0,95706	0,93206	0,90030	0,75173
VIC3	1	1,03622	1,03118	0,98433	0,93881	0,85056
VIC4	1	1,00839	0,98695	0,89523	0,82636	0,78286
VIC5	1	0,96101	0,96332	0,93825	0,87372	0,82862

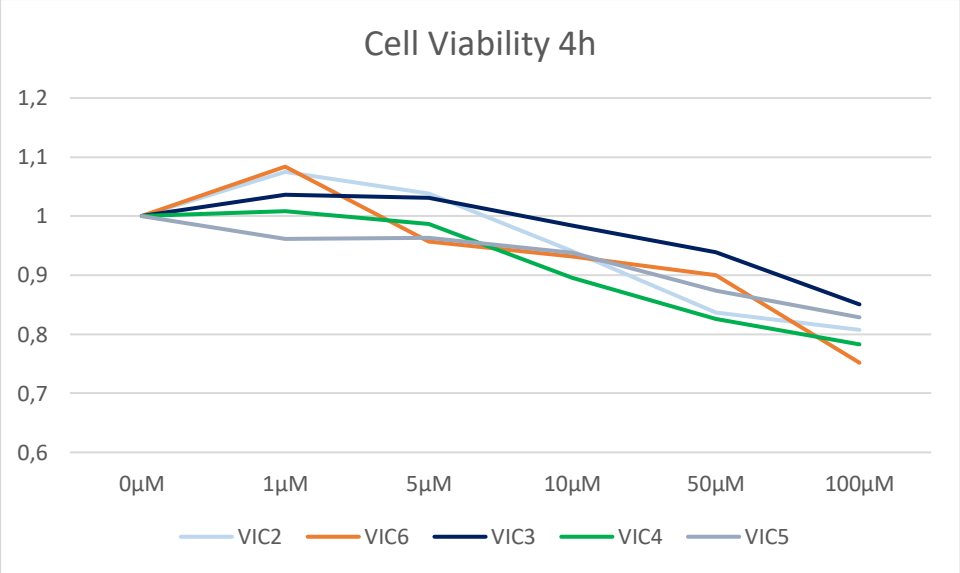


Figure S6 - VICs' viability 4h

(Tabels S7 - S10; Figures S3-S6): Relative cell viability of human VICs after exposure to increasing concentrations of empagliflozin (0–100 µM) for 1h, 2h, 3h, and 4h, respectively. Cells were incubated with a resazurin-based viability reagent, and absorbance was measured. Data were normalized to the 0 µM EMPA control condition. VIC1 was not included in the graphical analysis due to highly discrepant values (possible outlier). VIC – valvular interstitial cell.

Table S11 – Baseline calcification quantification

Baseline calcification			
	Experiment 1	**Experiment 2**	**Experiment 3**
VIC1	0,32770	0,40535	0,39171
VIC2	0,41590	0,35935	0,39981
VIC6	0,39453	0,38275	0,33828
VIC3	0,33417	0,71806	0,83324
VIC4	0,55100	0,79410	0,81145
VIC5	0,41352	0,71256	0,80675

Quantification of baseline calcification in human VICs cultured under standard growth conditions (VIC-GM), without osteogenic stimulation or any specific treatment. After 21 days in culture, Alizarin Red S staining was performed to assess calcium deposition. For each well, up to four microscopic images were acquired, and the ratio of calcified area was determined for each image with ImageJ. The values presented correspond to the mean ratio of calcification to total tissue area.

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Table S12 – VICs' calcification under osteogenic stimulation with EMPA

VICs' calcification							
		0µM	**1µM**	**5µM**	**10µM**	**50µM**	**100µM**
VIC1	**Plate 1**	0,50132	0,40482	0,64344	0,67219	0,66575	0,68887
	Plate 2	0,59223	0,66653	0,64323	0,63479	0,58845	0,59658
	Plate 3	0,56162	0,53800	0,65192	0,64567	0,55397	0,53441
VIC2	**Plate 1**	0,53009	0,54731	0,69195	0,68385	0,53108	0,36714
	Plate 2	0,61347	0,60806	0,52834	0,52978	0,55202	0,58198
	Plate 3	0,55356	0,73247	0,84435	0,87204	0,47152	0,40762
VIC6	**Plate 1**	0,56033	0,54484	0,66950	0,54588	0,58675	0,54065
	Plate 2	0,57955	0,59970	0,60841	0,57932	0,58675	0,62633
	Plate3	0,50920	0,47412	0,47488	0,61796	0,44039	0,51300
VIC3	**Plate 1**	0,54035	0,51660	0,49507	0,49975	0,55818	0,58787
	Plate 2	0,69308	0,67524	0,67825	0,65066	0,68572	0,58234
	Plate 3	0,71847	0,68130	0,54449	0,55710	0,55914	0,72583
VIC4	**Plate 1**	0,51978	0,60207	0,56256	0,53264	0,54980	0,50375
	Plate 2	0,61770	0,60207	0,63979	0,53370	0,55551	0,66933
	Plate 3	0,59971	0,57280	0,53797	0,49939	0,49183	0,45887
VIC5	**Plate 1**	0,52573	0,54076	0,55517	0,48715	0,47159	0,48491
	Plate 2	0,64566	0,76721	0,62992	0,62296	0,65355	0,72583
	Plate 3	0,62543	0,68503	0,56390	0,46476	0,37009	---

Quantification of calcification in human VICs cultured under pro-osteogenic conditions (OM) with increasing concentrations of EMPA. After 21 days, Alizarin Red S staining was performed to evaluate calcium deposition. For each well, up to four microscopic images were analyzed, and the percentage of calcified area was determined per image. The values presented correspond to the mean ratio of calcification to total tissue area.

Note:

As suggested by the course faculty, I used the **ARRIVE reporting guidelines and checklist** in an adapted manner for this study, in order to fulfill this dissertation requirement. Given that these guidelines are primarily designed for *in vivo* animal research, their direct application to our study was not entirely appropriate. Therefore, their completion was based on an adaptation of the presented criteria to align with our study's methodology.

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	Page 25: "Aortic valve tissues were obtained from patients undergoing SAVR due to severe AVS. VICs were isolated, expanded in standard growth medium (VIC-GM), and cryopreserved until use. For the experiments, cells were seeded and cultured under either standard conditions (VIC-GM) or pro-osteogenic medium, with increasing concentrations of EMPA. After 21 days, outcomes were assessed: cell viability was measured using a resazurin-based assay, and calcification was evaluated by Alizarin Red S staining followed by image analysis."
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	Page 25: "Between April and November of 2024, tricuspid aortic valves were obtained from six patients with severe AVS undergoing SAVR at the Cardiovascular Surgery Department of Unidade Local de Saúde de São João, Porto, Portugal. [...] Three male and three female patients were included after providing written informed consent."
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	Page 25: "Three male and three female patients were included after providing written informed consent. Patients with congenital heart valve malformations (including bicuspid valves) or rheumatic aortic valve disease were excluded from the study." Inclusion criteria → Patients with severe AVS undergoing SAVR at Centro Hospitalar Universitário São João. Exclusion criteria → Patients with bicuspid aortic valves or rheumatic disease. All VIC isolations that met the inclusion criteria were included in the final analysis. No data points or samples were excluded.
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	No randomisation was performed, as VICs were obtained from individual patients and were cultured in vitro under predefined experimental conditions. Therefore, allocation to experimental groups was determined by design, not by randomization.

Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Blinding was not applied. Given the *in vitro* nature of the study and the use of VICs from identifiable individual patients, experimenters were aware of group allocations throughout.
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	**Primary Outcome** → Degree of Calcification, assessed via Alizarin Red S staining and quantified using image analysis. **Secondary Outcomes** → VICs proliferation and viability at different stages.
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Page 29: "Statistical analysis was performed using GraphPad Prism 10.4.1, with a significance threshold set at $p < 0.05$. Continuous clinical variables were expressed as mean ± standard deviation (SD), while categorical variables were reported as absolute frequencies and their respective percentages. For the cellular experiments, results were presented as fold-change values relative to the respective baseline or control condition, depending on the specific experimental context. To assess normality, Shapiro-Wilk tests were performed. For clinical data, comparisons of continuous variables between female and male patients were conducted using unpaired t-tests when normality was confirmed, or Mann–Whitney U tests when normality was not met. Categorical variables were compared using Fisher's exact test. For the cellular assays, differences across multiple EMPA concentrations within the same cell group were analyzed using one-way or two-way ANOVA for normally distributed data, followed by Tukey's multiple comparisons tests. When normality was not met, a Kruskal–Wallis test was applied, followed by Dunn's post hoc test. Comparisons between female and male VICs at each concentration were performed using unpaired t-tests or Mann–Whitney U tests, depending on data distribution."
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Page 30: Table 2 Human VICs were used. The donor profile was as follows: **VIC1**: Female, 74 years old, BMI 28.4 kg/m² **VIC2**: Female, 76 years old, BMI 29.3 kg/m² **VIC3**: Male, 68 years old, BMI 32.7 kg/m² **VIC4**: Male, 68 years old, BMI 24.4 kg/m² **VIC5**: Male, 69 years old, BMI 28.8 kg/m² **VIC6**: Female, 69 years old, BMI 39.7 kg/m²
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation	Page 26: "A modified protocol based on Cuevas et al. was followed for VICs isolation. Under a biosafety cabinet, the valve was transferred to a pre-weighed sterile 10-cm dish, weighed, and photographed. The valve was washed thoroughly with Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 2%

periods). d. Why (provide rationale for procedures).	AA, and non-fibrotic and non-calcified sections of the leaflets were excised using sterilized scissors and forceps. [...] The tissue fragments were subsequently transferred to a 60-mm dish and incubated in 7 mL of a 1%-collagenase solution for 10 min at 37°C, rocking the tissue every 2 minutes to facilitate dissociation of the endothelium. [...] The remaining tissue was further digested overnight for about 18h. The VICs were released by pipetting with a sterile Pasteur pipette and the resultant cell suspension was passed through a 0.70µm filter. [...] The suspension was centrifuged at 200×g for 5 min at 20°C, and the pellet was resuspended in VIC-GM. Viable cells were determined by Trypan-blue exclusion using an automated cell counter (Countess, ThermoFisher Scientific). [...] After the initial expansion, cells were seeded at ~0.5 million viable cells per T75 flask (passage 0, P0) and incubated at 37ºC. When cells reached 90% confluency, these were trypsinized and cryopreserved in cryopreservation medium (Hyclone Hycryo-STEM). In this work, cells were used until passage 2. […] For the assessment of calcification, VICs at passage 2 were seeded (30,000 cells/well) in 48-well plates, and for the viability assays, VICs at the same passage were seeded (10,000 cells/well) in 96-well plates. Cells were initially cultured in VIC-GM until 100% confluence was reached. Subsequently, the medium was replaced with osteogenic medium (OM), consisting of DMEM supplemented with 5% FBS, 10 mM β-glycerophosphate, 50 µg/mL vitamin C, and 10 nM dexamethasone. […] To evaluate the effects of EMPA on VIC viability and calcification, increasing concentrations of EMPA (0 µM, 1 µM, 5 µM, 10 µM, 50 µM, and 100 µM) were added to OM. Dimethyl sulfoxide (DMSO) was used as a vehicle control. Media were replaced every 2–3 days, and cells were incubated for a total of 21 days. […] To assess cell viability, a resazurin reduction assay was performed. After 21 days of culture, the medium was removed, and the cells were incubated with 50 µM resazurin in OM. The cells were incubated for 4 hours, and the absorbance of the reduced compound (resorufin) was read at every hour. Absorbance was measured at 570 nm and at 600 nm for correction, using a TECAN Spark microplate reader. […] To evaluate calcification, [...] cells were cultured in 48-well plates and calcium crystals were stained with Alizarin Red S solution. [...] Cells were fixed with cold neutral buffered formalin for 30 minutes, then stained with 2% Alizarin Red S solution for 30 minutes in

			the dark. The stained wells were photographed with an inverted microscope, and the images were analyzed using ImageJ software to quantify the calcified area relative to total tissue area."
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	Page 36: "The Resazurin assay results show a gradual reduction in viability as the EMPA dose increases. Male VICs showed a significant reduction in viability starting at 10 µM EMPA concentration (Figure 8B), while in female VICs viability significantly decreased at 100 µM EMPA only (Figure 8A). In both sexes, the average viability in each group never decreased below 70%, not even at the highest doses (100 µM) of the drug, demonstrating a good safety profile of EMPA. […] When analyzing the effects of EMPA on VIC calcification in a sex-specific manner, distinct patterns emerged. In the case of women (Figure 13A), there is no significant alteration in calcification, comparing the 0 µM condition to any other concentration of EMPA. As for men (Figure 13B), there was in fact a significant reduction in the calcification of VICs, at a dose of 10 µM EMPA (p=0.0392), reducing about 11.6% of calcification. At all other concentrations, the differences relative to the condition with 0 µM EMPA were not significant."

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	Our study aimed to evaluate the effects of SGLT2i (in this case EMPA) on calcification mediated by VICs in the context of AVS. VICs were isolated from human aortic valves collected during surgical replacement and cultured under osteogenic conditions with increasing concentrations of EMPA. Cell viability and calcification were assessed through Resazurin assay and Alizarin Red S staining, respectively. Results showed that EMPA reduced calcification in VICs derived from male patients at 10µM, while, in female-derived VICs, no differences were found, revealing a sex-specific cellular response. These findings suggest that SGLT2i may have therapeutic potential in AVS, particularly in male patients, highlighting the importance of considering sex differences in future pharmacological research.
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study and explain the experimental approach. b. Explain how the animal species and models used address the scientific objectives and, where appropriate, the relevance to human biology.	Page 6: "Aortic valve stenosis (AVS) is a cardiovascular disease characterized by a continuous process of fibrosis and calcification mainly led by the activation of valvular interstitial cells (VICs). Despite its high prevalence, there is still no effective pharmacological therapies to prevent or to treat this disease. Sodium-glucose co-transporter 2 inhibitors (SGLT2i), such as empagliflozin (EMPA), have shown cardiovascular benefits, but their direct effects on VICs remain unclear. This study aimed to investigate the effects of SGLT2i on VICs'-mediated calcification."
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	Page 24: "Our main objective is to investigate whether there is a direct effect of SGLT2i on the calcification process, one of the key pathological hallmarks of AVS. To this end, this project aimed to collect aortic valves from male and female patients with severe AVS, implement a protocol for the isolation and expansion of VICs (175), and subsequently induce calcification using a combination of β-glycerophosphate, L-ascorbic acid 2-phosphate, and dexamethasone to model the disease in vitro. Finally, the project aims to assess if VICs cultured under pro-osteogenic conditions and treated with SGLT2i would exhibit reduced calcification, thus providing preclinical evidence to support potential drug repurposing. A schematic representation of our study is shown in Figure 1."

			Research question → Does EMPA reduce calcification in AVS and how do sex-related differences influence its effects? The primary goal of this study was to determine whether EMPA can reduce calcification in VICs cultured under pro-osteogenic conditions. A secondary aim was to explore whether there are sex-specific differences in response to EMPA, given the known disparities in valve calcification patterns between males and females.
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant license or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	Pages 25a and 26: "Three male and three female patients were included after providing written informed consent. […] This investigation is in accordance with the 1964 Declaration of Helsinki and its later amendments and was approved by the institutional review board (ethics committee of Unidade Local de Saúde de São João, ref CEC109-2020)."
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	**Not applicable**, as this is an in vitro study.
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	**Not applicable** (despite the use of 2 swine aortic valves, these animals were sacrificed following another protocol from another research team, and the animals' aortic valves were reused for this study)
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	Page 6: "EMPA may attenuate VIC-mediated calcification in a sex-specific manner, reducing calcium deposition in male-derived VICs but not in those from females. These findings suggest that SGLT2i could represent a novel pharmacological strategy for men with AVS and reinforce the importance of considering sex differences in the development of novel therapies."
Generalizability / translation	18	Comment on whether, and how, the findings of this study are likely to generalize to other species or experimental conditions, including any relevance to human biology (where appropriate).	Although this study was conducted entirely *in vitro*, the results provide a relevant starting point for translational research. The identification of sex-specific responses to EMPA supports the need for personalized approaches in future clinical trials targeting AVS.
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	No formal protocol registration was completed prior to the start of the experiments, but the experimental design, including the definition of groups, treatment concentrations, and outcome measures, was established in advance. Data

			analysis plans and criteria for interpreting results were also determined before the experiments were initiated.
Data access	20	Provide a statement describing if and where study data are available.	The manuscript does not include a formal data availability statement.
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	Page 51: "The authors have no conflicts of interest to declare." Page 51: "This work was supported by national funds through FCT (Fundação para a Ciência e a Tecnologia, I.P.), within the scope of the Cardiovascular R&D Center (UIDB/00051/2020, UIDP/00051/2020) and RISE (LA/P/0053/2020)."

(The underlined points are the aspects that were adapted to our study.)

PLOS ONE Submission guidelines

For this study, we followed the PLOS ONE publication guidelines to ensure clarity, transparency, and scientific rigor. The specific instructions for authors that guided the structure and formatting of this manuscript can be found below.

Style and Format

File format

Manuscript files can be in the following formats: DOC, DOCX, or RTF. Microsoft Word documents should not be locked or protected.

LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.

Length

Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information.

We encourage you to present and discuss your findings concisely.

Font

Use a standard font size and any standard font, except for the font named “Symbol”. To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.

Headings

Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.

Layout and spacing

Manuscript text should be double-spaced.

Do not format text in multiple columns.

Page and line numbers

Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).

Footnotes

Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).

Language

Manuscripts must be submitted in English.

You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.

Abbreviations

Define abbreviations upon first appearance in the text.

Do not use non-standard abbreviations unless they appear at least three times in the text.

Keep abbreviations to a minimum.

Reference style

PLOS uses "Vancouver" style, as outlined in the ICMJE sample references.

See reference formatting examples and additional instructions below.

Equations

We recommend using MathType for display and inline equations, as it will provide the most reliable outcome.

If this is not possible, Equation Editor or Microsoft's Insert→Equation function is acceptable.

Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., "$a^2 + b^2 = c^2$"), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values.

Do not use MathType, Equation Editor, or the Insert→Equation function for only a portion of an equation. Rather, ensure that the entire equation is included. Equations should not contain a mix of different equation

tools. Avoid "hybrid" inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.

Nomenclature

Use correct and established nomenclature wherever possible.

Units of measurement

Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.

Drugs

Provide the Recommended International Non-Proprietary Name (rINN).

Species names

Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).

Genes, mutations, genotypes, and alleles

Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HGNC for human genes; we strongly recommend using this tool to check against previously approved names). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).

Allergens

The systematic allergen nomenclature of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee should be used for manuscripts that include the description or use of allergenic proteins. For manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-

Committee prior to manuscript publication. Examples of the systematic allergen nomenclature can be found at the WHO/IUIS Allergen Nomenclature site.

Manuscript Organization

Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section

The following elements are required, in order:

- Title page: List title, authors, and affiliations as first page of the manuscript;
- Abstract;
- Introduction.

Middle section

The following elements can be renamed as needed and presented in any order:

- Materials and Methods;
- Results;
- Discussion;
- Conclusions (optional).

Ending section

The following elements are required, in order:

- Acknowledgments;
- References;
- Supporting information captions (if applicable).

Other elements

- Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately;
- Tables are inserted immediately after the first paragraph in which they are cited;
- Supporting information files are uploaded separately.

Title

Include a full title and a short title for the manuscript.

Full title

Length

250 characters.

Guidelines

Specific, descriptive, concise, and comprehensible to readers outside the field.

Examples

Impact of cigarette smoke exposure on innate immunity: A *Caenorhabditis elegans* model.

Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial.

Short title

Length

100 characters.

Guidelines

State the topic of the study.

Examples

Cigarette smoke exposure and innate immunity

SODIS and childhood diarrhoea.

Author list

Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

- First name (or initials, if used)
- Middle name (or initials, if used)
- Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled “current address.” At a minimum, the address must include the author’s current institution, city, and country.

If an author has multiple affiliations, enter all affiliations on the title page only. In the submission system, enter only the preferred or primary affiliation. Author affiliations will be listed in the typeset PDF article in the same order that authors are listed in the submission.

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature

- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

Supporting reproducibility with protocols

To enhance the reproducibility of your results, we recommend and encourage you to make your protocols public. There are several options:

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Protocol documents may be uploaded as Supporting Information or linked from the Methods section of the article. For laboratory protocols, we recommend protocols.io. Include the DOI link in the Methods section of your manuscript using the following format: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]. This allows editors and reviewers to consult the detailed step-by-step protocol when evaluating your manuscript. You can choose to keep the protocol private on the protocols.io platform until your article is published—at which time it will be published automatically.

Protocols published in their own right

PLOS One offers two options for publishing stand-alone protocol articles: Lab Protocols that describe reusable methodologies and Study Protocols that describe detailed plans and proposals for research projects. Specific guidelines apply to the submission of Lab Protocol and Study Protocol manuscripts. Read the detailed instructions for submitting Lab Protocols and Study Protocols.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS One editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS One* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

- Submitted research should not rely upon retracted research. You should avoid citing retracted articles unless you need to discuss retracted work to provide historical context for your submitted research. If it is necessary to discuss retracted work, state the article's retracted status in your article's text and reference list.

Ensure that your reference list includes full and current bibliography details for every cited work at the time of your article's submission (and publication, if accepted). If cited work is corrected, retracted, or marked with an expression of concern before your article is published, and if you feel it is appropriate to cite the work even in light of the post-publication notice, include in your manuscript citations and full references for both the affected article and the post-publication notice. Email the journal office if you have questions.

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., "We used the techniques developed by our colleagues [19] to analyze the data"). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Figures and tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order at first appearance in the manuscript file.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and "Figure" abbreviated to "Fig" (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of "Fig 1" must refer to a figure file named "Fig1.tif").
- A concise, descriptive title

The caption may also include a legend as needed.

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Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., "Table 1") and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Statistical reporting

Manuscripts submitted to *PLOS One* are expected to report statistical methods in sufficient detail for others to replicate the analysis performed. Ensure that results are rigorously reported in accordance with community standards and that statistical methods employed are appropriate for the study design.

Reporting of statistical methods

In the methods, include a section on statistical analysis that reports a detailed description of the statistical methods. In this section:

- List the name and version of any software package used, alongside any relevant references
- Describe technical details or procedures required to reproduce the analysis
- Provide the repository identifier for any code used in the analysis (See our code-sharing policy).

Statistical reporting guidelines:

- Identify research design and independent variables as being between- or within-subjects
- For pre-processed data:
    - Describe any analysis carried out to confirm the data meets the assumptions of the analysis performed (e.g. linearity, co-linearity, normality of the distribution).
    - If data were transformed include this information, with a reason for doing so and a description of the transformation performed
- Provide details of how outliers were treated and your analysis, both with the full dataset and with the outliers removed
- If relevantav describe how missing/excluded data were handled
- Define the threshold for significance (alpha)
- If appropriate, provide sample sizes, along with a description of how they were determined. If a sample size calculation was performed, specify the inputs for power, effect size and alpha. Where relevant, report the number of independent replications for each experiment.
- For analyses of variance (ANOVAs), detail any post hoc tests that were performed
- Include details of any corrections applied to account for multiple comparisons. If corrections were not applied, include a justification for not doing so
- Describe all options for statistical procedures. For example, if t-tests were performed, state whether these were one- or two-tailed. Include details of the type of t-test conducted (e.g. one sample, within-/between-subjects).

- For step-wise multiple regression analyses:
    - Report the alpha level used
    - Discuss whether the variables were assessed for collinearity and interaction
    - Describe the variable selection process by which the final model was developed (e.g., forward-stepwise; best subset). See SAMPL guidelines.
- For Bayesian analysis explain the choice of prior trial probabilities and how they were selected. Markov chain Monte Carlo settings should be reported.

Reporting of statistical results

Results must be rigorously and appropriately reported, in keeping with community standards.

- **Units of measurement.** Clearly define measurement units in all tables and figures.
- **Properties of distribution.** It should be clear from the text which measures of variance (standard deviation, standard error of the mean, confidence intervals) and central tendency (mean, median) are being presented.
- **Regression analyses.** Include the full results of any regression analysis performed as a supplementary file. Include all estimated regression coefficients, their standard error, p-values, and confidence intervals, as well as the measures of goodness of fit.
- **Reporting parameters.** Test statistics (F/t/r) and associated degrees of freedom should be provided. Effect sizes and confidence intervals should be reported where appropriate. If percentages are provided, the numerator and denominator should also be given.
- **P-values.** Report exact p-values for all values greater than or equal to 0.001. P-values less than 0.001 may be expressed as $p < 0.001$, or as exponentials in studies of genetic associations.
- **Displaying data in plots.** Format plots so that they accurately depict the sample distribution. 3D effects in plots can bias and hinder interpretation of values, so avoid them in cases where regular plots are sufficient to display the data.

- **Open data.** As explained in PLOS's Data Policy, be sure to make individual data points, underlying graphs and summary statistics available at the time of publication. Data can be deposited in a repository or included within the Supporting Information files.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

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- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

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- A reference to the published article that first described the cell line; AND/OR
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Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

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