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Genetic variants in calcium calmodulin pathway in association with cardiovascular disease: focus on the potential role of CaMKK1 in heart and vessels

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TABLE OF CONTENT

ABSTRACT

Cardiovascular disease is the leading cause of death all over the world and affects annually an increasing number of people. Numerous risk factors are involved in the etiology of this complex disease. In addition to an unhealthy lifestyle, environmental factors, and other comorbidity, it is important consider also genetics.

The study of genetic variants in association with cardiovascular disease is nowadays one of the main topics of interest, especially for the identification of genetic biomarkers for the prevention, prediction and follow up of patients affected by cardiovascular pathologies.

Among the several pathways that regulate the physiology and molecular biology of the heart, the regulation of calcium through calmodulin is one of the main important. Indeed, calmodulin (CaM) binds calcium and regulates calcium channels, playing a crucial role in different process as the mechanism of excitation-contraction coupling.

Considering that several works have shown how some genetic variants, as polymorphism, can be considered predisposing factors to complex diseases, it can be assumed that the study and characterization of polymorphic variants in the CaM pathway could be important for investigate how genetic traits can influence the predisposition to specific cardiovascular disease.

In this project, attention is focused on genetic variants in some common proteins involved in the pathway of three different isoforms of CaM (CaM 1, 2, 3): NOS (nitric oxide synthase protein); CaMK (calcium/calmodulin dependent protein kinase), and CaMKK (calcium/calmodulin dependent protein kinase kinase).

Among these proteins, the main focus of interest has been the in-depth study of the potential role of CaMKK1 in the heart and vessels, through *in vitro* studies on human vascular smooth muscle cells, used as model for different cardiovascular diseases as atherosclerosis.

Introduction

1. Cardiovascular diseases epidemiology

Cardiovascular diseases are considered the main cause of death all over the world. According to the recent statistics of the World Health Association, 17,9 million of people die each year for this type of disorders [1], more than for any other disease.

The Global Burden of Diseases, *Injuries, and Risk Factors Study 2019*[2], a multinational collaboration study that analyzed the global disease burden in order to collect and provide consistent data of health from 1990 to 2019, showed that the number of deaths caused by cardiovascular diseases increased from 12.1 million in 1990 to around 18.6 million in 2019. This study collected data of population health in 204 countries and territories through analysis and studies on different available population-level data such as case fatality, mortality, incidence, and prevalence[2]. According to this study in 2019, the main causes of CVD deaths globally were ischemic heart disease and stroke; China, India, Russia, United States of America, and Indonesia were the most affected countries[2].

The latest statistics from the European Society of Cardiology (2019)3 showed that cardiovascular diseases are the most common cause of death in Europe.

Data from Eurostat (2018) reported that in Europe, for the disease of the circulatory system, the 50-60 % of all deaths are accounted in the Baltic Member States and Romania, while this percentage reached close to two thirds (65,8 %) of all deaths in Bulgaria. On the contrary less than one quarter of all deaths were caused by cardiovascular disease in Denmark (22,6%), France (24,3%, 2016 data) and the Netherlands (25%).

Even though the death rate in 2020 and 2021 was significantly increased by the COVID-19 pandemic, cardiovascular diseases remain one of the first causes of death worldwide. IO

According to the Health System Tracker, in the U.S. only in December 2020 the COVID-19 mortality rate has overcome that of heart disease, remaining the first cause of death until March 2021.

With the rapid spread of vaccinations, COVID-19 deaths decreased significantly, and again cardiovascular disease came back to be the first cause of death in US[4].

2. Types of cardiovascular diseases

Cardiovascular diseases generally refer to every type of condition that affects blood vessels and heart. They are a heterogeneous group of heart and vessel diseases which includes arrhythmia, valve disease, coronary artery disease, heart failure, peripheral artery disease, aortic disease, congenital heart disease, aneurysm, pericardial disease, cerebrovascular diseases, and other types of conditions.

More information about the basis and the mechanism of these diseases can be found in the appendix, which reports the principles of the anatomy and physiology of the heart.

2.1 Coronary artery disease [5,6]

Among CVDs, coronary artery disease is the most widespread all over the world.

This type of disease consists in the narrowing or blockage of the coronary arteries, usually caused by the build-up of a plaque inside the inner wall of the vessels. This event is called atherosclerosis, a serious condition that can bring to the acute event of heart attack. The plaque deposition can be of different etiology, such as waste products, calcium, clot-making substance fibrin and mainly cholesterol.

The growth and the rupture of the plaque is a multistage disease, that starts with inflammation. Usually, the plaques are stable if the inflammation cascade culminates in a return to the state of equilibrium. The speed of the plaque formation differs from one person to another, and it can start since childhood. As soon as the deposit of the plaque begins, the system response delivers white blood cells to attack the cholesterol, enhancing the inflammation. This mechanism triggers other cells in the artery wall to form a soft cap over the plaque.

Different causes, such as blood pressure, can break and/or damage the thin cap over the plaque. At this point platelets stick to the site of the injury and cause the formation of a clot. A blood clot can break apart on its own or block blood flow through the artery, depriving the heart of oxygen. It might happen that the blood clot breaks apart naturally.

The clog or the damage of the arteries due to the plaque limits or, even worse, completely blocks the blood flow to the heart muscle. If the heart doesn’t receive oxygenated blood and nutrients, it can’t work properly. This condition is called ischemia. The lack of blood flow to the heart muscle can lead to angina, a type of chest pain and a sign of heart attack or stroke risk.

2.2 Diseases of the aorta and arterial pathologies[5,6]

The wall of the aorta is made up of different layers: a thin layer called the inner tunic (endothelium), a thick layer called the media tunic (elastic fibers, smooth muscle fibers, collagen) and a relatively thin outer layer called the adventitia (collagen). The smooth muscle of the vessels is found in the media, and its main function is to maintain the vascular tone, which is regulated through the stimuli received by the endothelium and through the autonomic nervous system. The contraction of smooth muscle depends on the entry into the cytoplasm of extracellular calcium, which allows the interaction between actin and myosin, in the same way as in striated and cardiac muscles.

2.2.1 Aneurysm [5,6]

Aneurysm is another vascular disease very common in the worldwide population. It consists in an abnormal bulge and enlargement in the wall of a blood vessel, and it occurs usually in the aorta (aorta aneurysm).

It is important to differentiate the true aortic aneurysm, which causes the dilation of the three layers of the wall, from the pseudoaneurysm or false aneurysm, which is the rupture of the intimate and media tunic, that remain enveloped by the adventitia.

The thoracic aortic aneurysms may occur in the descending arch or in the descending aorta.

Atherosclerosis is the most common cause of aneurysm of the arch and descending aorta. As for ascending aortic aneurysms, the most frequent cause is cystic degeneration of the media.

Thoracic aneurysms are generally accompanied by generalized atherosclerosis, especially of the renal, cerebral, and coronary arteries. In most cases, patients are asymptomatic but symptoms such as deep pain, dysphagia, dyspnea, etc. may appear in the course of the history of aneurism.

The most common type of aortic aneurysm occurs in the abdominal aorta. This type of aneurysm mostly has a fusiform morphology, like those of the ascending aorta.

Atherosclerosis is usually the main cause, leading to the rupture of the elastic fibers and the weakening of the wall, with consequent dilation. High blood pressure and family predisposition are other causes. Atherosclerosis is generally a condition that causes fewer symptoms than thoracic aneurysms. The suspect often arises from an abdominal x-ray or from a physical examination. The condition must be suspected in case of sensation of fullness and epigastric pain.

The main risk factor for an abdominal aneurysm rupture is its diameter: with more than 6 cm, rupture is the most frequent complication.

2.2.2 Valvular heart disease[5,6]

Valvular heart disease occurs when any valve in the heart shows a damage. There are several causes of valve disease, depending on which one is affected. The atria are related to the ventricles through the atrioventricular valves.

The right tricuspid or atrioventricular valve consists of three cusps. This valve allows the blood to flow from the right atrium to the right ventricle. The left mitral or atrioventricular valve (bicuspid valve) has two cusps and allows blood to flow from the left atrium to the left ventricle. In addition to the atrioventricular valves, there are the semilunar valves: the aortic valve and the pulmonary valve, composed by three semilunar cusps. The first one allows the blood to flow from the left ventricle to the aorta, the second one allows the blood to flow from the right ventricle to the pulmonary artery.

The valves open and close to regulate the blood flowing both into the heart and then away from it. The mitral valve has only two leaflets, while the other three valves are composed of three leaflets that work together in order to control the blood flow.

Only healthy heart valve leaflets can fully open and close the valve during the heartbeat, while diseased valves might not perform properly. Any valve in the heart can become diseased at any time, but the aortic valve is the most commonly affected. Sometimes the diseased valves can become leaky if they don't close completely; this is called *regurgitation*. Once this happens, blood leaks back and not enough blood can be pushed forward through the heart.

Another common type of heart valve condition is when the opening of the valve is narrowed and stiff, and it cannot open fully when the blood is trying to transit; this is called *stenosis*. Sometimes the valve may be missing a leaflet, especially the aortic valve.

With diseased valves, the heart cannot effectively pump blood throughout the body and needs to work harder, either while the blood is leaking back into the chamber or against a narrowed opening. This condition can lead to heart failure, sudden cardiac arrest (when the heart stops beating), and death.

2.2.3 Aortic stenosis[5,6]

Aortic stenosis is considered one of the main and most serious valve diseases. It consists in a narrowing of the aortic valve opening, which is reduced to an area of less than 2 cm^2. This event restricts the blood flow from the left ventricle to the aorta and may also affect the pressure in the left atrium. The obstruction can be localized at different levels: above the valve, in Williams

syndrome (supravalvular); below the valve, i.e., at the level of the outflow tract from the left ventricle (sub-valvular), in hypertrophic cardiomyopathy or at the level of the valve itself, the latter being the most frequent site.

The most frequent etiology in the general population is degenerative (calcification), which generally affects elderly patients, and which has the same risk factors as atherosclerosis. In younger patients, the most common cause is a congenital degeneration of a bicuspid aortic valve.

The symptoms of aortic stenosis might cause a patient to feel faint, weak, or lethargic. Moreover, the left ventricle must work harder in order to pump blood through the narrow valve opening into the aorta, hence it might show muscular thickening.

The thickened wall requires more space inside the lower heart chamber, which consequently allows less room for an adequate amount of blood for the whole body. This condition may lead to heart failure. The progress of this disease can be reversed or slowed down with appropriate treatment.

3. Vascular smooth muscle cells

The main component of blood vessel are human vascular smooth muscle cells (VSMCs), the most abundant cell types in arterial vessel wall[7,8].

Blood vessels are composed by three different parts: tunica intima, tunica media and tunica adventitia. VSMCs are located around the vascular lumen in the tunica media (Figure 1). Usually, the medium vessels have around forty layers of vascular smooth muscle cells, while the large vessels have around 60 layers [5,9]. On the other hand, the tunica intima is composed by endothelial cells and constitutes the luminal part of a blood vessel. The external part of the vessel is called tunica adventitia and is mainly composed by fibroblast and nerve. In the adventitia of big vessels there are also small blood vessels called vasa vasorum.

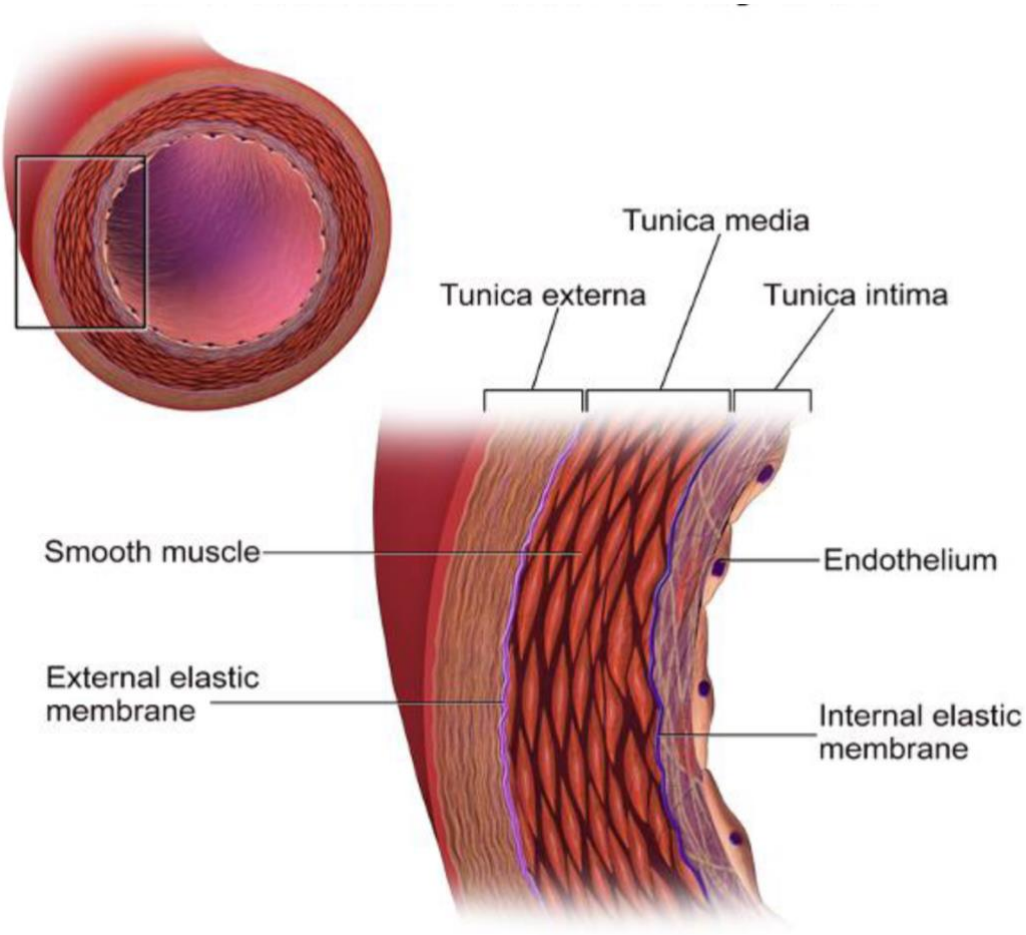


Figure 1: The structure of an artery wall. Vascular smooth muscle cells are in the tunica media. (from Tyson et al., 2020)

VSMCs play crucial roles in maintaining the structure and function of the vessel [8,9]. Moreover, thanks to their higher degree of plasticity, hVSMCs are very important in the pathophysiology of blood vessels. In physiological condition in an adult and health organism, VSMCs are in a quiescent contractile phenotype (Figure 2). This phenotype makes sure that the blood vessels contract and relax to regulate the blood circulation, ensuring vasoconstriction, vasodilatation, and other functions, such as synthesis of extracellular matrix [7,10–12]. Contractile VSMCs are composed by contractile fibers and proteins, such as alpha actin, calponin and SM1 and SM2 myosin heavy chain.

During the onset and development of vascular diseases, vascular smooth muscle cells can change their phenotype in a process called phenotype switching (Figure 2). In fact, under pathological conditions or biological stress, vascular smooth muscle cells switch their phenotype: they lose their contractility and switch to a synthetic phenotype [7,11,13,14]. This phenotype expresses protein involved in proliferation and migration and this characterizes VSMCs to be active in migration and growth, leading to intimal thickening, formation of atherosclerotic plaques, stenosis of vascular lumen and thickening during hypertension of the blood vessel wall [10,12,14,15].

The capability of VSMCs to undergo phenotype switching is also influenced by other factors such as the origin, the location in the vascular system, the age, the gender, and species[14]. However, the quiescent and non-proliferative population of VSMCs in vessels of healthy adults is heterogenous, composed of primarily contractile cells and of contractile cells specialized in the production of extracellular matrix.

Phenotype switching in hVSMCs is a process that can be reproduced *in vitro* when the cells are under specific conditions[14]. The synthetic phenotype can be induced by platelet growth factors (PDGF)[16–18], protease-activated receptors (PARs) and tumor necrosis factor-alpha (TNF-alpha) [16,17,19,20]. Synthetic VSMCs begin vessel repair and can switch back to the contractile phenotype, driven by factors such as heparin, laminin, or low concentration of FBS in the condition media [13,14].

In vitro, contractile VSMCs are elongated and show spindle-shape morphology. On the other hand, synthetic VSMCs are rhomboidal and display cobblestone morphology[21,22].

Synthetic VSMCs express lower levels of proteins involved in contraction: α-SMA (α-smooth muscle actin), SMMHC (SM myosin heavy chain), SM22α (smooth muscle 22α), CNN (SM-calponin), and smoothelin-B[23,24]. Moreover, synthetic VSMCs are characterized by increased proliferation and migration[16,23].

VSMCs can also give rise to other cells in the vessel wall, such as osteo/chondrogenic-like and macrophage-like cells, as it has been reviewed by others[10,11,17,20] and described in the next paragraph.

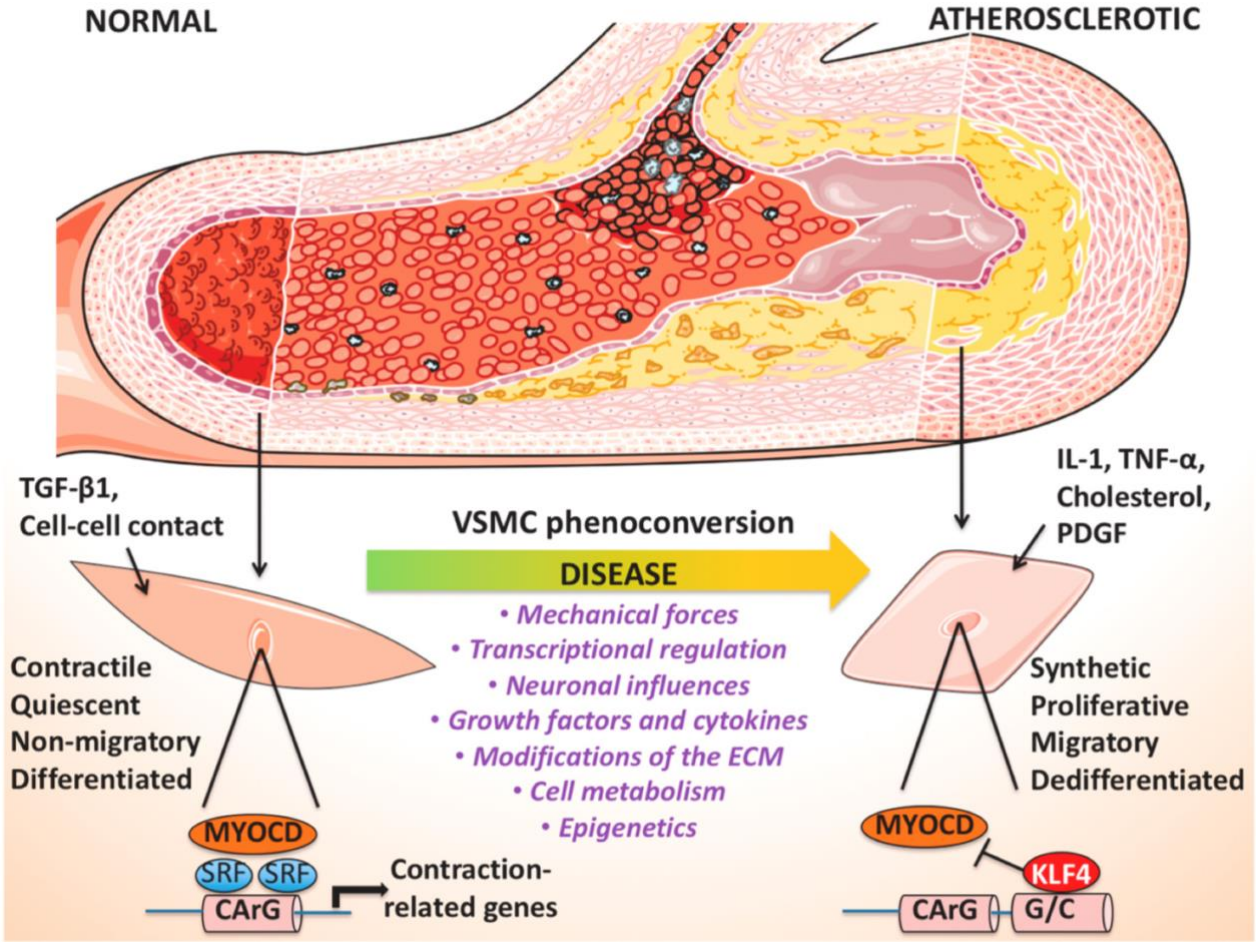


Figure 2: Schematic representation of the main mechanism of vascular smooth muscle cell phenotype switching, from contractile to synthetic. ECM: extracellular matrix; IL-1: interleukin-1; KLF4: Krüppel- like factor 4; MYOCD: myocardin; PDGF: platelet-derived growth factor; TGF-β1: transforming growth factor beta 1; TNF-α: Tumor necrosis factor alpha; SRF: serum response factor. (from U. Kansakar et al., 2021)

3.1 Vascular smooth muscle cells in vascular pathology

VSMCs are involved in several vascular diseases, such as atherosclerosis, hypertension, and aneurysm formation [7,12,14,15]. The onset of this type of pathologies is due to a mechanical or biochemical damage to the endothelium cell layer that becomes permeable, thrombogenic and immunogenic [11,12]. In this condition, the endothelium is also subject to inflammatory activation, increasing the expression in the endothelial cells of adhesion molecules of selectin and immunoglobulin families, the intercellular adhesion molecule-1 (ICAM-1), the endothelial-leucocyte adhesion molecule-1 (ELAM-1) and the vascular cell adhesion molecule-1 (VCAM-1)[25]. All these molecules are located on the membrane of endothelial cells and bind monocytes, macrophages, mast cells, lymphocytes and leucocytes, all cells of the immune system.

In the damaged vascular wall, VSMCs start their phenotypic modulation, migrating from the tunica media to the tunica intima, where they start to proliferate. This important proliferation can determine a full erasing of the vascular lumen and lead to stenosis[11,12,14,20]. In fact, during phenotype switching and modulation, VSMCs lose the differentiation markers, while the markers of proliferation and migration persist. Moreover, it is important to consider that hVSMCs are an *in vitro* model for the study of calcification, an event that is involved in many vascular diseases as atherosclerosis and aneurysm.

Vascular calcification is an active process and consists in the deposition of minerals in the vascular system. This event can occur in the intimal or medial layers of the vessel wall and can also affect the valves of the heart [7,11,12,20,21,24,26]. During atherosclerosis, calcification occurs when the intima becomes inflamed and thickened. The calcification of the coronary arteries leads to atherosclerotic plaque burden and rupture.[27]

Compared to the intimal layer of vessel, where the calcification has a diffuse localization, the calcification of the media is along the elastic lamina. Usually, the medial calcification causes stiffening of the artery wall and thus it is associated with the increase of blood pressure. The calcification of the media is mightily connected with the phenotypic switching of VSMCs. During this event due to the increase of calcium and phosphate levels, VSMCs switch their phenotype into the osteogenic one, where the cells have the characteristic of chondrocytes and osteoblasts[17,28,29]. The osteo/chondrogenic VSMCs express bone-specific proteins, that regulate the extracellular matrix (ECM) mineralization, and show an increased expression of osteogenic markers, such as alkaline phosphatase, BMP-2 and runt-related transcription factor 2 (Runx2) [11,30,31].

Elevated calcium levels cause at VSMC an overload of intracellular calcium that may lead to microcalcification and consequentially to macrocalcification, which induce vascular stiffness and compromise the structural integrity and function of the vessel wall [11,14,32].

Moreover, osteogenic VSMCs release extracellular vesicles that promote vascular calcification. Indeed, EVs derived from VSMCs have similar features with EVs from osteoblasts, as the capability to bind calcium and to produce osteoblast-like ECM [14,33,34].

4. Biomarkers in cardiovascular disease

Cardiovascular disease, in addition to being the leading cause of death worldwide, carries a high economic toll on national health systems. Therefore, it is necessary to give importance to the screening and prediction of these types of diseases[35–37].

The prevention of cardiovascular diseases needs to be a mix of strategies that complement each other and involve different aspects. Beside a reduction of the bad lifestyle habits, it is important to highlight the role of biomarkers. Studies in biomarker research related to CVD have led to more accurate screening methods, improved clinical outcomes and sensitivity in the detection and diagnosis. The use of biomarkers in CVD is still an important area of research since their role can be used for different purpose[38,39]. The definition of biomarker was given in 2001 during the National Institute of Health Consortium and it refers to a *"characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"*[40]. This definition is very interesting because it does not refer only to the common blood tests, which are the most frequent meaning of biomarkers in cardiovascular disease, but it includes all the representations of a biological process: circulating molecules, genetic and cellular markers, results of imaging or physical examinations. A few years later, in 2009, the American Heart Association highlighted the extensive criteria for how newer biomarkers should be evaluated in a standardized fashion before recommending their clinical use[41].

Traditionally, biomarkers are classified accordingly to their intended use as screening, diagnostic or prognostic. Instead, from a precision medicine perspective, they could be classified as prognostic, pharmacodynamic or predictive biomarkers. Prognostic biomarkers provide information on the course of the illness in an untreated individual or in an individual who's treated with conventional therapies. Predictive biomarkers identify those patients who are most likely to respond to a given therapy and distinguish candidates who can be considered for specific targeted therapies. Pharmacodynamic biomarkers usually measure the effect of a therapy on the disease state itself[38].

Biomarkers generally represent a biochemical change in a tissue or a body organ. They are associated to biologic or pathologic processes but the clinical outcomes in terms of biomarkers as disease indicators might be different. For example, troponin is one of the most important biomarkers used in the field of CVD, as its elevation up to a certain level occur in congestive heart failure, pulmonary embolism and in acute myocardial ischemia/infarction.

Compared to the traditional risk factors, nowadays genetic biomarkers represent the main field of interest for their benefits and for their potential utility in relations with the CVD. In this contest it is important to consider that the genetic biomarkers arise at birth or even prior birth, as opposed to the circulating or imagining biomarkers.

Recent genetic studies showed that some loci or gene are associated both with a higher risk to develop a specific CVD and with several CVD risk factors. Moreover, both DNA and epigenetic changes, that results in variation in gene expression and phenotypes, were also associated with CVD traits and disease risk.[42]

Through studies of gene expression, it was possible to identify patterns of different forms of CVD disorders, as heart failure, myocardial infarction, hypertrophy[43–46]. Usually, the classical approach to analyze the link between genetic markers and disease outcomes include on one hand the linkage approach and on the other one association studies. Linkage studies map single gene disorders with large genetic effects. Usually, this type of approach is a family-based study that identify large segments of genome with millions of DNA bases, which are similar among patients with the disease of interest within families. Subsequently, a more detailed mapping identifies a single gene in those large segments of genome, to link it with a specific disease.

Association studies can assess more complex diseases with modest genetic effect. For example, the GWAS (Genome Wild Association Study) surveys the whole genome to create single nucleotide polymorphism (SNP) maps and databases. This approach identifies clear genetic markers associated with CVD[47–50]. Furthermore, it is also important to consider that the analysis of protein expression is important both to discover new potential biomarker and to confirm the potential impact of a SNP identified previously as genetic biomarkers through other analysis.

There are large scale databases of cardiac proteins that play some pivotal roles, as for example the identification of exact physiologic pathways that may help the assessment of CVD risk[51], allowing further progress in drug discovery and therapeutic approaches, and allowing the characterization of changes in protein expression, to associate them with specific phenotypes [52–54].

5. Genetics in cardiovascular diseases

The inheritance that characterizes CVDs doesn't follow a clear Mendelian pattern, since this group of pathologies is considered complex because multifactorial[55–61]. This means that different factors coexist together increasing the risk to develop a specific disorder.

In the context of the heart and vessels, modifiable and non-modifiable factors can be identified. The first group is mainly characterized by lifestyle habits (smoke, high-fat diets, sedentary life), by environmental factors (smog, UV) and by comorbidities, such as hypertension, diabetes, cholesterolemia[55,62,63]. The second group (non-modifiable factors) includes factors that characterize the individual as age, sex, ethnicity and obviously also genetics. In fact, many cardiovascular disorders, as arrhythmias, cardiomyopathy, coronary artery diseases, can run in families, indicating an inherited genetic risk.

Genetics can affect the risk for cardiovascular disease in numerous ways, for example genetic variants might change the activity of protein involved in the control of cholesterol, mis-regulating processes that might increase the probability of blocked arteries.

Cardiovascular system is controlled by genes in every aspect, from the way cells in the heart communicate to the strength of the blood vessels [57,64,65].

It is important to consider that genetic variants are generally passed from parents to children and can modify the probability of developing heart disease. In this context is important to mention that "genetic variation" is a broad concept which includes both mutations and polymorphisms. In general, "genetic variation" is considered as any change in a DNA sequence away from "normal". It implies the presence of an allele considered "normal" and prevalent in the population. The main difference is that the mutation is a rare and abnormal variant. In contrast, a polymorphism is a DNA sequence variation that is common in the population, with a frequency that is higher than the 1%52,140,145.

Polymorphisms are characterized by different types: single nucleotide polymorphism (SNP), variable number tandem repeat (VNTR), short tandem repeat (STR), restriction fragment length polymorphisms (RFLP)[61].

The idea that the risk of CAD is heritable has been supported by numerous clinical observations and studies started during the 1950s[68,69]. As an example, the Framingham Heart Study, one of the main population-based observational cohort studies, promoted by the United States Public Health Service in 1948, investigated risk factors and epidemiology for cardiovascular disease[70–72].

All these studies aimed to understand the full spectrum of common and rare genetic variations that contributes to the higher risk to develop a specific cardiovascular disease, with the goal to discover a novel biological approach and to translate these results into clinical practice.

The sequencing of the human genome, the reduced costs of genotyping and the share of information through data bank or multinational collaborations, have played a key role in discovering the genetic drivers of CAD[61,73]. Studies on families with a predisposition to early-onset CAD, generally performed via linkage analysis, offered opportunities to look deeply into monogenic drivers of CAD[69]. Moreover, genotyping chips designed to capture most of the common inter-individual genetic variation allowed to understand the basics for common variant association studies (CVAS), also termed genome-wide association studies (GWAS). Common variants (polymorphisms) occur very often, so it is easy to analyze each variant individually by comparing its frequency both in disease cases and disease-free controls[48–50].

Several studies highlighted that there is a significant enrichment of variants in regulatory regions with an important impact of CAD risk variants on the alteration of gene expression. Despite this, it is also important to consider that the genome wide association studies can also identify the variants located outside the protein-coding regions, whereas their functional interpretation is demanding and is increasingly an object of interest. Indeed, human genome non-coding regions can play a pivotal role in human traits and complex diseases, as cardiovascular diseases[38].

6. Genetic in calcium calmodulin pathway

Considering the important role of calmodulin pathway in cardiovascular diseases, in a previous review we studied the genetic variants present in literature in the genes involved in the calcium calmodulin pathway, associated with a higher risk to develop cardiovascular diseases[67].

The association of some polymorphisms of the CaM pathway with cardiovascular pathology might be relevant both clinically, to understand the severity of coronary pathology, and epidemiologically, for evaluating the risk of pathology in the general population[67,74–78].

We considered each pathway that involves the three different isoforms of CaM (CaM1; CaM2 and CaM3), focusing only some common genes involved in the three pathways. We analysed and studied some genetic variants associated with CVDs in the three different isoforms of calmodulin (CaM 1, 2, 3) and three related protein superfamilies: NOS (nitric oxide synthases); PPP3C (protein phosphatase catalytic subunits) and CaMK (calcium/calmodulin dependent protein kinase)[67]. We also decided to focus on the family protein of the calcium calmodulin-dependent protein kinase kinases, which are upstream to the CaMK family proteins.

We considered three different types of polymorphisms: those in exon regions, those in the intron region and those in the untranslated regions ((5'-UTR and 3'-UTR). Although the genetic variants in the intron region did not affect the folding and the function of proteins, they could influence transcription levels.

Before describing in detail this part regarding the genetic variants in calcium calmodulin pathway, it is important to understand the crucial role of calcium and its regulation in the heart.

7. Calcium

Calcium is a divalent ion (Ca^{2+}), fundamental element in the life of all species and has an important role as second messenger in mammal cells: variations in its concentration regulate contractility in the muscles and heart [79–81].

Since Ca^{2+} is highly reactive, it is important that its concentration remains low in the cell. There are many ionic pumps which transport calcium and, thanks to their spatial distribution and temporal kinetic, a different calcium concentration is present in different cell.

Ca^{2+} is also released by intracellular stores via inositol trisphosphate receptors or ryanodine receptors located in the membrane of endoplasmic and sarcoplasmic reticulum. Spatial and temporal profiles of Ca^{2+} signal depend on the specific expression pattern of Ca^{2+} release molecules [82–86].

Calcium is considered one of the main important elements in the regulation of the physiology and pathology of the heart and vessels, even if only 1% is found in the blood and soft tissue and the other 99% is found in bones and teeth.

Inside the cardiac cells, the concentration of calcium is heterogeneously distributed due to different microdomains in the cytosol or in the spaces between intracellular organelles and different membrane delimitated compartments, such as the sarcoplasmic reticulum, mitochondria, and lysosomes [87,88].

One of the main roles of calcium in the context of the heart is to be involved in the excito-contraction coupling (ECC), an event in which the electrical contraction is linked with the mechanical activity [79–81,89–92]. During this event, both the extracellular and intracellular calcium concentrations have a key role in cell membrane potential, fundamental in the ECC of cardiac and smooth muscle cells[90,92].

The concentrations of calcium ions in the extracellular fluid are sensed by the presence of calcium-sensing receptors, as for example calmodulin, one of the first sensors of calcium, that activates a series of downstream pathways regulating different cell functions[93]. The simplest Ca^{2+} signaling elements involve enzymes that are regulated by cytosolic Ca^{2+}, such as Ca^{2+}-calmodulin-dependent kinase (CaMKII), and NO synthases, both proteins downstream to calmodulin[67,94].

Calcium is also a co-factor for many enzymes, and it is involved in blood coagulation[95,96]. In fact, calcium ions activate platelet and other several coagulation factors, including coagulation Factor XIII (FXIII) responsible of the maintaining of the clot architecture and strength[96,97]. In addition, Ca^{2+} plays an important role in the heart in the regulation of long-term processes, such as turning

genes on or off in order to change protein expression, and it has a vital role not only in development but in adaptive processes, such as hypertrophy, which might be either beneficial or harmful[80]. Moreover, calcium is involved in the event of calcification inside the arterial vessel and/or valve, a severe pathological condition that predicts numerous cardiovascular disorders such as atherosclerosis, aneurysm, and vascular stiffness[27].

During this active process where crystals of calcium deposit within the vessel, hVSCMCs play a key role in regulating the remodeling processes of the vessel wall[12,20,98]. In fact, hVSCMCs are considered an important *in vitro* model to study calcification, thanks to their plasticity and capability to switch phenotype (contractile, non-contractile, osteogenic)[24,98]. The intracellular Ca^{2+} signaling is significantly different between the two phenotypes due to the non-identical expression of Ca^{2+} transport proteins and so to the several pathways that regulate the entry of calcium[98].

Contractile VSMCs express Ca^{2+} transporters, such as voltage-gated L-type Ca^{2+} channels and SERCA2a pump, which can maintain low resting cytosolic Ca^{2+} and allow dynamic changes of Ca^{2+} in the spatial and temporal domain; on the other hand, non-contractile VSMCs have significantly reduced voltage dependence of Ca^{2+} entry [98,99].

These alterations associated with phenotypic switching are caused by changes in gene expression programs, where the expression of phenotype-specific proteins and other proteins is suppressed. Moreover, it is important to underline that different type of Ca^{2+} signaling are involved in controlling VSMC phenotype, activating different types of Ca^{2+} -sensitive transcription factors [91,98,100], such as serum response factor (SRF)[101], cAMP response element–binding protein (CREB)[102] and nuclear factor of activated T lymphocytes (NFAT)[103].

8. Calmodulin

One of the most important sensors of intracellular calcium levels in eukaryotic cells, both in biological and pathological conditions, is calmodulin (CaM)[67,104]. It is characterized by a conserved domain called EF-hands, composed by four non identical sites, through which CaM binds four ions of calcium. By binding 4 ions of calcium, calmodulin becomes saturated, and its structure undergoes a conformational change that allows to interact and activate downstream proteins[105,106] (Figure 3).

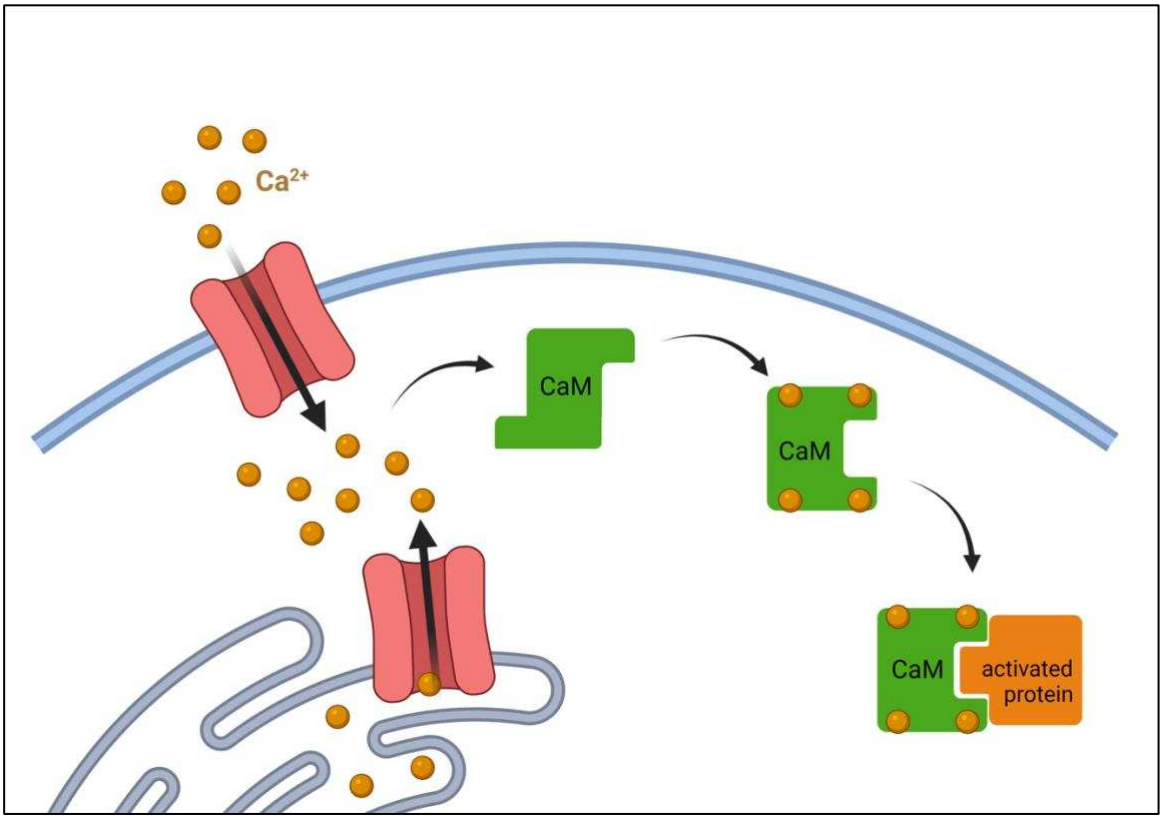


Figure 3: Schematic representation of calmodulin pathway. Adapted from "Calcium signalling in heart and vessels Role of calmodulin and downstream Ca2+-CaM dependent protein kinases", (from Beghi et.al, 2021- to be submitted).

The EF-hands has a helix-loop-helix structure domain, characterized by two alpha-helix regions linked together by a loop region of around 12 amino acids[105,106].

Calmodulin is a low-molecular-weight protein highly conserved in the eukaryotes and it is involved in many processes thanks to its capability to interact with more than 300 different proteins[93,107,108].

For this functional versatility, due to the diversity among its 4 calcium binding sites, CaM could affect many different cellular functions, as muscle contraction, metabolism, inflammation,

apoptosis, immune response, short-term and long-term memory[67,107,109]. Through the mechanism called “wrap-around”, the C- and the N- termini of calmodulin bind to the same regions of target proteins.

There are three different isoforms of calmodulin in humans (CaM1, CaM2, CaM3). The three genes are located respectively on chromosome 14, 2 and 19 and encode three highly conserved proteins that differ at the nucleotide level (Figure 4). Compared to CaM1 and CaM3, CaM2 have also 48 additional amino acids. Moreover, in the CaM2 isoform there is a lysine instead of a methionine (CaM1 and CaM3) as the first amino acid of the alignment [67].

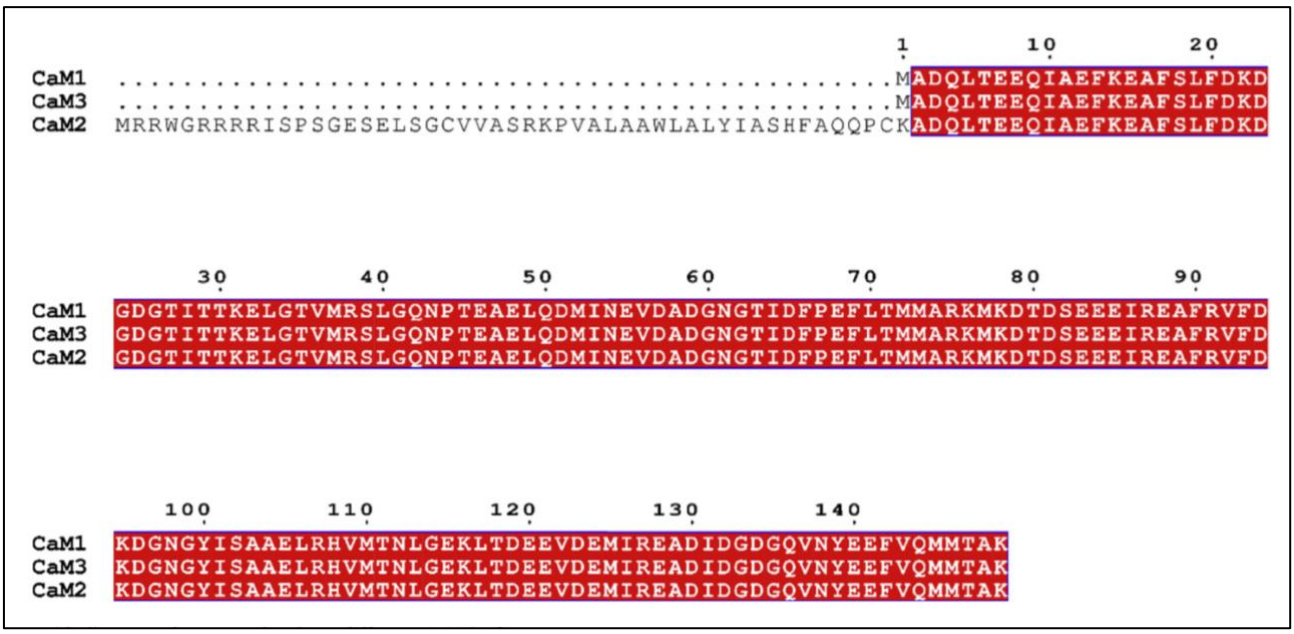


Figure 4: Amino acid alignment between the three different CaM isoforms (from Beghi et al., 2020)

This high level of conservation underlines the important role of calmodulin[110,111]. In fact, mutations in these proteins are deleterious since early infancy, giving rise to pathological cardiovascular disorders. Indeed, defects in CaM structure and function affect important calcium signaling events in the heart[78,112–118].

Calmodulinopathy[77,119] is considered a wide spectrum of clinical manifestation in association to CaM mutations as long QT syndrome (LQTS), catecholaminergic polymorphic ventricular tachycardia (CPVT), catecholaminergic polymorphic ventricular tachycardia mutations or idiopathic ventricular fibrillation (IVF). CaM mutations have been also identified in autopsy-negative sudden unexplained deaths (SUD) in young individuals[115,116].

As first sensor of the calcium signaling, CaM plays different important roles in the heart. One of its main functions is the modulation of the action potential[104]. During this event CaM regulates

different channels (the voltage-gated sodium channels67, the voltage gated potassium channels68 and the voltage-gated calcium channels) which cause the rapid contraction of a different group of cardiac cells[122].

CaM is also involved in regulating the sarcoplasmic reticulum Ca^{2+} release channel (RyR2), the main source of intracellular calcium to cause a contraction with each heartbeat69,70. Moreover, CaM is linked with secondary pathway effectors of cardiomyocyte contraction, such as the beta-adrenergic pathway49,71 and the cyclic nucleotide signaling[104]. Plus, CaM interacts with a lot of downstream enzymes, such as the calcium calmodulin kinase 2 (CaMK2) and nitric oxide synthase family protein (NOS), leading and supporting calcium to recycle enough cardiomyocytes to maintain calcium homeostasis in preparation for a new excitation event[67,104].

8.1 Calmodulin polymorphisms

The three isoforms of CaM are characterized by a high level of conservation that reflects the important role of calmodulin[106,110,111,126]. For this reason, we can find more mutation than polymorphisms affecting calmodulin gene sequence in relation to CVD.

It has been showed previously that deleterious variants in CaM genes (*CaM1*, *CaM2*, and *CaM3*) disrupt important calcium signalling events in the heart[77]. On the other hand, few studies have analysed and studied the association between CaM polymorphisms and cardiovascular diseases (Table 1).

Among these studies, two interesting polymorphisms were found in the 3'-UTR of the CaM1 gene. The polymorphism rs3179089 has been associated with ischemic stroke in a Han Chinese population[74]. The polymorphism rs3814843 was correlated with an increased risk for stroke[127,128]. This polymorphism was also reported by Liu et al. to be associated with a higher risk for sudden cardiac death (SCD) in a Chinese population[129].

A new polymorphism rs7259810 (-34T > A) has been recently identified in the promoter region of the human calmodulin III gene (CaM3), where the T allele affects the level of CaM3 transcript. It has been showed that patients with familiar hypertrophic cardiomyopathy (FHC) have a higher frequency of the TT-genotype compared to controls; it could be a modifier gene for FHC[130].

In this project thesis, the attention has been focused on the genetic variants rs7259810 and rs10113 in CaM3 in association with cardiovascular diseases.

Rs10113 is a single nucleotide polymorphism in the 3' prime UTR region of CaM3. It is a nucleotide change from T to C and, according to the 1000 Genome Browser, the minor frequent allele in the population is the T (MAF=0.50).

The polymorphism rs7259810 is a single nucleotide polymorphism that causes a nucleotide change from T to C in a non-coding transcript variant in CaM3. According to the 100 Genome Browser[61], the less frequently allele is the T allele with a MAF of 0.33.

Table 1: Human Calmodulin gene (CaM1 and CaM3) polymorphisms and associations with cardiovascular disease (rearranged from Beghi et al., 2020)

Calmodulin 1 (CaM1)					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Year [Refs.]**
Promoter Region					
rs3179089	C/G	3'-UTR	Ischemic stroke	Han Chinese	2018[74]
rs3814843	A/C/T	3'-UTR	Ischemic stroke	Unspecified	2009[127]
			Sudden Cardiac Death	Han Chinese	2015[129]
Calmodulin 3 (CaM3					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Refs**
Promoter Region					
	T>A	-34	Familial Hypertrophic Cardiomyopathy	Caucasian	2009[129]

9. Calcium calmodulin kinases

The transduction and integration of calcium signaling sensed by CaM results in regulation (activation or deactivation) of downstream proteins involved in phosphorylation, since calmodulin has not enzymatic activity[67,94,104,123].

Among the different downstream targets of CaM, the calmodulin-dependent kinases (CaM-kinases) family protein is one of the most important and well characterized[94]. This class of enzyme is involved in different cellular functions, such as cell death, cell survival, learning, memory, gene transcription and cytoskeletal reorganization.

The main role of the kinases is to catalyze flow of phosphate from the gamma position of ATP to the hydroxyl group of Ser, Thr or Tyr within protein substrates[67,93,94]. Comparisons of different protein kinases sequence showed a conserved region of around 250 amino acids. These amino acids create the structural core of all known kinases: a bi-lobed catalytic core with a small ATP binding domain.

The general domain structure of the CaM-kinases is also characterized by a regulatory domain composed by an autoinhibitory domain and a CaM-binding domain[94] (Figure 5).

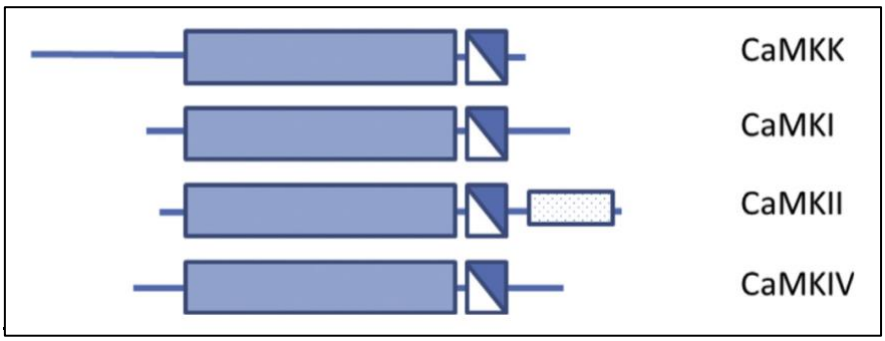


Figure 5: Schematic representation of domain structure of CaM-kinases. The n N-terminal catalytic domain is in light blue, followed by a regulatory region that contains overlapping autoinhibitory (WHITE) and CaM-binding domains (BLUE). The C-terminal association domain of CaMKII is shown as white with black dots. (from Beghi et al., 2020).

At basal Ca^{2+} levels, CaM-kinases are held in an inactive state due to the interactions between the catalytic and the autoinhibitory domain. As intracellular calcium concentrations rise, CaM binds four ions of calcium and become sutured, undergoing modifications necessary to bind the cam-binding domain on CaM-kinases[94,109,131].

In order to be activated, some CaM-kinases require both the binding of the calcium but also additional modifications such as phosphorylation. Moreover, some kinases (CAMK4 and CaMK2)

can be independent of CaM, allowing them to function beyond the duration of a transient elevation in Ca^{2+}.

The calcium calmodulin dependent kinase family can be divided in two groups. The first one is characterized by multifunctional kinases with multiple downstream targets, and it include CaMKK1, CAMKK2, CaMK1, CaMK2 and CaMK4. The second group of CaM-kinases, including CaMK3, phosphorylase kinase and myosin light chain kinase, is substrate-specific having only one downstream target. Within the first group of kinases the CAMKKs subfamily protein, CaMK1 and CaMK4 share the same signaling pathway called calcium calmodulin dependent kinase cascade[67,94].

CaMK2 itself is involved in different important processes in the heart and vessel: first of all, it has a key role in the mechanism of the excitation contraction coupling[132–136].

9.1 Ca^{2+}-CaM-dependent kinase cascade

9.1.1 CaMKKs family protein

The CaM- dependent kinase cascade is involved in different processes, such as stem cell militance, apoptosis immune cell function, cell proliferation and glucose homeostasis[131].

Dysregulation in these kinases can affect numerous types of diseases, such as obesity, diabetes, cancer, neuronal and cardiovascular disorders[137,138].

The first kinases upstream in this cascade are called calcium calmodulin kinase kinase proteins (CaMKKs)[139,140]. This group of kinases is the first to be activated by calmodulin and is necessary to transduce the calcium signaling to the downstream kinases CaMK1 and CaMK4 (Figure 6).

There are two isoforms of CaMKKs, CaMKKα and CaMKKβ, encoded by the genes *CAMKK1* and *CAMKK2* respectively51,95. These kinases have a high sequence homology and a common domain structure described above, shared by all the other kinases. The only difference is that CaMKK1 is held in a dormant state until calmodulin binds to its target and relieves the mechanism of autoinhibition. CaMKK2 shows-a partially autonomous activity in the absence of Ca^{2+}-CaM[94,140].

On the other hand, the phosphorylation by cAMP-dependent protein kinase-A (PKA)[141–143] at different Ser or Thr residues within the CaM kinase binding domain (S458 and S475 in CaMKK1, T482, S495 and S511 in CaMKK2) and within the ATP binding region in the catalytic domain (S24, S52, S74 and S108 in CaMKK1; S100, S129, S133, S137 and T145 in CaMKK2)., causes the partial inhibition of CaMKK1 and CaMKK2[143]. Moreover, both CAMMK1 and CAMKK2 have one site of autophosphorylation, respectively on S24 and on T482. Specifically, the T482 creates a partial

autonomous activity that does not depend on Ca^{2+}-CaM and disrupts the autoinhibitory mechanism of CaMKK2. This last one can respond to other stimuli of longer duration, being not dependent on rapid fluxes in intracellular calcium for basal activity[94,131,143].

CaMKK1 and CaMKK2 have some common downstream targets, as for example the phosphorylation and activation of CaMK1 and CaMK4, at residues Thr177 and Thr196, respectively (Figure 6). Two other important proteins activated by CaMKKs are AMP-activated protein kinase (AMPK), which has the main role of the cellular energy balance regulation, and AKT (also known as protein kinase B or PKB), an important oncology target[144,145].

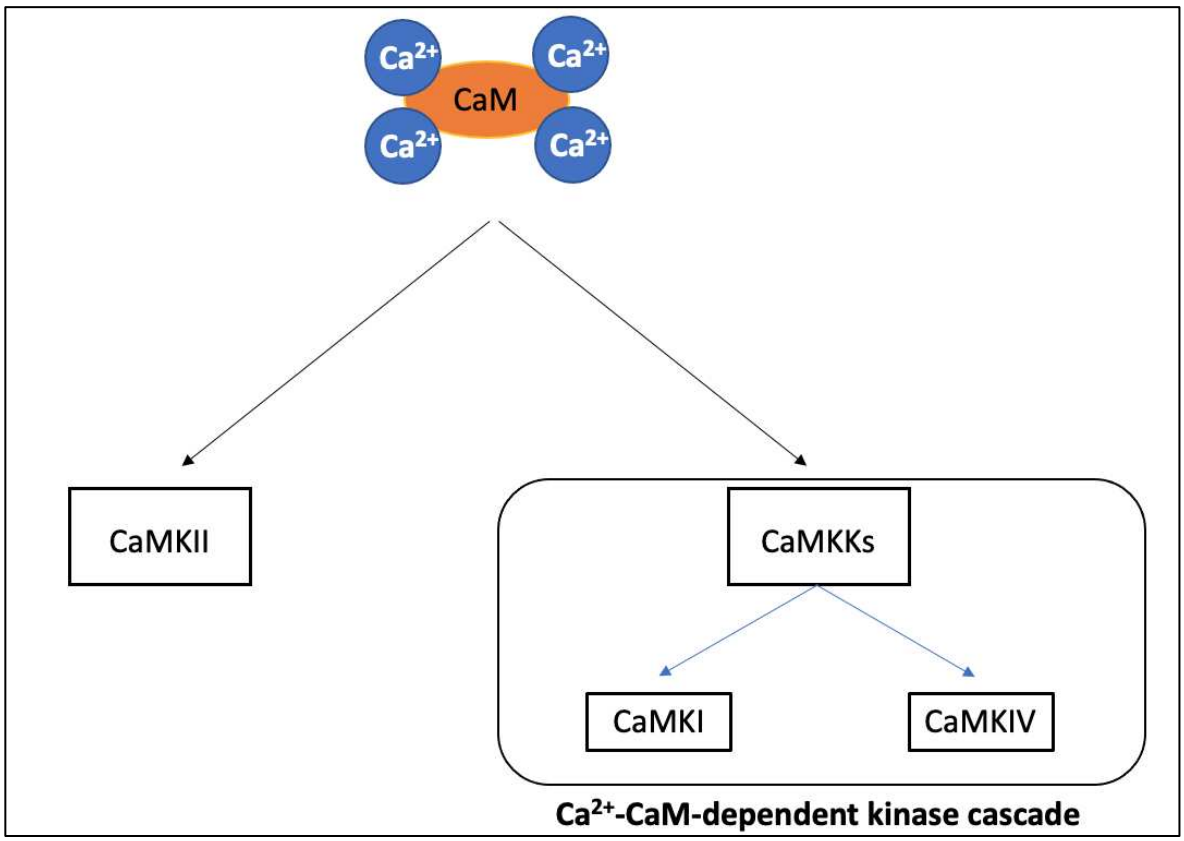


Figure 6: Schematic representation of the CaM-kinases pathways downstream to calmodulin: on the left the activation of CaMKII, on the right the activation of Ca2+-CaM-dependent kinase cascade (CaMKKs, CaMKI and CaMKIV) (rearranged from Beghi et.al, 2021- to be submitted).

9.1.1.1 CaMKK1

Differently from CaMKK2, CaMKK1 is fully dependent on calmodulin[94,140]. Recently, Santiago et al. (2018) determined the crystal structure of CaMKK1 with two ATP-competitive inhibitors[140]. The structures revealed some differences, especially in the ATP-binding sites, between CaMKK1 and CAMKK2, despite the high sequence identity (70% over the kinase domain).

Being a kinase involved in the transduction of calcium signaling, CaMKK1 has different important roles, specifically in neuro and cardiovascular biology[140,146,147]. A new potential role has been recently associated to CaMKK1, as regulator of the exosome released by mesenchymal stem cell in cardiovascular disease; specifically, it was showed to be a potential therapeutic target for infarcted tissue. Indeed, injections of conditioned media of MSC CaMKK1 overexpressed in rats heart showed improvement in cardiac function after AMI (acute myocardial infarction), with decreased scare tissue and increased vascular density[146]. Moreover, CaMKK1 has important functions in muscle mass and growth, protein content, protein synthesis, and mTORC1 signaling. The interaction between CaMKK1 and mTORC might be important also in the cardiovascular system due to the regulatory function of mTOR pathway in cardiovascular physiology and pathology[148].

Recently, CaMKK1 has been studied from a genetic perspective. Specifically, the focus is on the genetic variant rs7214723, a single nucleotide polymorphism (SNP) that causes an amino acid change from glutamic acid (E) to glycine (G) at position 375, inside the catalytic domain of CaMKK1. This SNP might have a high impact on the activity of CaMKK1 because these amino acid changes create a charge variation on the surface of the protein and influence the substrate specificity of CaMKK1, inhibiting downstream CaMK1 and CaMK4. In fact, rs7214723, previously associated with lung cancer[149,150], it is now studied in association with cardiovascular diseases[147].

9.1.1.2 CaMKK2

The role and the function of CaMKK2 has been more studied, compared to CaMKK1[140]. It is always involved in the transduction of the calcium signaling to the downstream proteins CaMK1 and CaMK4, but one of the main targets of CaMKK2 is AMPK, that it is involved in the regulation of different physiological processes[151–153]. In the context of the heart, it seems that the interaction CaMKK2-AMPK is important to produce cardiac energy in order to protect the heart against Ca^{2+} overload caused by sustained high blood pressure [152,153].

It has been showed that during cardiac hypertrophy the AMPK signaling is repressed and cardiac-specific knockout of AMPK cause a stress-induced cardiac hypertrophy. Indirectly, the inactivation

of CaMKK2 might also result in the development of metabolic dysfunction and cardiac hypertrophy[154].

Another important pathway in which CaMKK2 is involved is the SOCE-CaMKK2-mTOR signaling[155,156]. This pathway plays a key role in the calcium-induced autophagy regulation and is an important homeostatic mechanism at basal levels in cells of cardiovascular origin as an important homeostatic mechanism. The activation of CaMKK2 and the deactivation of mTOR was associated with autophagy modulation[156].

9.1.1.3 CaMK1 and CaMK4

Calcium calmodulin kinases 1 and 4 are downstream targets to the CaMKKs family protein and are involved in calcium calmodulin dependent cascade[67,94,131]. Like the other kinases, CaMK1 and CaMK4 share the same structure, which is characterized by a conserved catalytic domain, an auto-inhibitory domains, and a CAM binding domain.

CaMK1 has four isoforms encoded by four different genes: CAMKIα, CAMKIβ/Pnck, CAMKIγ/CLICK3 and CAMKIδ/CKLiK[94,143,157]. CaMK4 has two splicing variants, the two isoforms CAMKIVα and CAMKIVβ [143].

CAMK1 is activated by two important events: phosphorylation by CaMKK on Thr177 and the binding of Ca^{2+}/calmodulin complex[158]. The binding of Ca^{2+}/calmodulin complex to the region near to the auto-inhibitory domain leads to the exposure of Thr177, so the auto-inhibitory domain detaches from the catalytic domain and the ATP and protein substrates bind CaMK1. CAMK1 is distributed in many different tissues, and it performs different activities, such as the transcription factor activity, hormones production, cell cycle, differentiation, and actin filaments organization. The activation of CaMK4 depends on CaMKK1 phosphorylation on Thr196, but CamK4 can have also a Ca^{2+}/CaM-independent activity thanks to auto-phosphorylation of Ser/Thr at N-terminus. The auto-phosphorylation of Ser^{332}allows an additional level of self-regulation[94,159].

About the two isoforms of CAMKIV, they are mainly expressed in brain, immune cells, testes, and ovaries[143]. In these districts, CAMKIV is involved in the regulation of cyclic AMP element binding protein (CREB), cell proliferation and in many regulatory processes such as neurite outgrowth, fear memory, homeostatic plasticity, inflammatory response, and immune response[94,143].

CaMK4 has recently received attention for its role in cardiovascular pathophysiology; in fact, many studied showed an association of the genetic variant rs10491334 in CaMK4 with increased

diastolic blood pressure. Furthermore, rs10491334 was also related with a reduction in the expression levels of CaMK4 in hypertensive patients[160,161]. Moreover, CaMKK/CaMK4 seems to have an endogenous protective role in ischemia and plays a key function as regulator of blood-brain barrier integrity.

In the context of the vessels, the interaction CaMKK/CaMK4 is important in the regulation of CREB (cAMP response element-binding protein) during phenotype switching of vascular smooth muscle cells (VSMC)[98]. The events that occur during the phenotype switching in hVSMCs depend on the activity and form of different calcium dependent transcription factors, such as CREB, and changes in gene transcription[102]. The regulation of VSMC phenotype appears to be under the control of the Ca^{2+}-CaM kinase pathway. Nuclear CaMK4 plays a vital role in the activation of CREB whereas CaMKII seems to inhibit CREB phosphorylation[91,162]. Increased intracellular calcium leads to a nuclear translocation of Ca^{2+}-CaMKII and activates CaMK4, which subsequently phosphorylates and activates CREB.

9.2 Ca^{2+} - CaM kinases polymorphisms

Among the calcium calmodulin kinases, CaMK2 plays a key role in the heart, specifically in the mechanism of excitation-contraction coupling; due to this important function in the heart, studies have been conducted in order to assess the potential involvement of polymorphisms in this class of proteins associated with cardio- vascular pathologies[135,136] (Table 2).

Even if the clinical research in cardiovascular diseases usually analyses genetic variants in protein coding regions, recently the mutations located within the non-coding regions are being considered, in connection to the pathogenesis of complex human disease[163–165]. For example, Gong et al. analysed 366 potential CAD-associated super enhancer SNPs in 67b loci[164]. This study highlighted the potential function of super enhancer SNPs in association with coronary artery disease. Burgner et al. (2009) identified in a Caucasian population three intronic variants in CAMK2D (rs17531554; rs4834340 and rs11728021), that resulted significantly associated with Kawasaki disease (a paediatric vasculitis that damages the coronary arteries, especially in young children)[47].

Important analysis of single nucleotide polymorphism in association with cardiovascular disorders were conducted on CAMK4, a calcium calmodulin dependent protein downstream to the CAMKKs family protein (Table 3).

A genome wide association study identified the genetic variant rs10491334 in association with the elevated diastolic blood pressure, suggesting the potential role of CAMK4 in its control[71]. The study of Santulli et al. (2012) confirmed this result, identifying a significant correlation between rs10491334 and diastolic pressure in hypertensive patients[160]. The association between this SNP and hypertension resulted also in the Uygur population in China[161].

Considering the important role of kinases in the heart and vessels, in this project we also focused the attention on the upstream kinases activated by calmodulin: the CaMKKs. We analysed the genetic variant rs7214723 in CaMKK1 in association with the higher risk to develop a specific type of cardiovascular diseases[147] (Table 4). It has been shown how the rs7214723 in CaMMK1 has a completely different distribution of the genotypic and allelic frequencies between a cohort of 300 cardiopathic subject and the European reference group from the Browser 1000 Genomes[61]. Specifically, it was observed an enrichment of the C allele in a stratification group of no coronary patients with aortic stenosis.

This SNP was previously studied in association with lung cancer[149,150]. It is a single nucleotide polymorphism that causes an amino acid change from glutamic acid to glycine at the position 375 within the kinase domain. This missense variant possibly decreases the activating ability of CaMKK1, and this might also influence the activation of the downstream protein CAMK1 and CAMK4.

Table 2: Human CAMK2D gene polymorphisms and associations with cardiovascular disease (rearranged from Beghi et al., 2020)

Calcium calmodulin dependent kinase 2 D (CAMK2D)					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Year [Refs.]**
Intron Region					
rs11728021	A/G	-	Kawasaki disease	Caucasian	2009 [166]
rs4834340	G/A	-	Kawasaki disease	Caucasian Chinese	2009 [166] 2015[167]
rs17531554	C/T	-	Kawasaki disease	Caucasian	2009 [166]

Table 3: Human CAMK4 gene polymorphisms and associations with cardiovascular disease (rearranged from Beghi et al., 2020)

Calcium calmodulin dependent kinase 4 (CAMK4)					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Year [Refs.]**
Intron Region					
rs10491334	C/T	-	Diastolic blood pressure and arterial stiffness Diastolic blood pressure Hypertension	American Italian Chinese*	2007 [71] 2012[160] 2016 [161]

Table 4: Human CAMKK1 gene polymorphisms and associations with cardiovascular disease (rearranged from Beghi et al., 2020)

Calcium calmodulin dependent kinase kinase 1 (CAMKK1)					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Year [Refs.]**
Exon Region					
rs7214723	E375G	Exon 12	Cardiovascular diseases	Italian	2021 147

10. Nitric oxide synthase family protein

Nitric oxide synthase is a protein family composed by three different isoforms: neuronal nitric oxide synthase (nNOS or NOS1), inducible nitric oxide synthase (iNOS or NOS2) and endothelial nitric oxide synthase (eNOS or NOS3)[67,168].

NOS1 is generally expressed in brain and neuronal tissue, where it influences long-term potentiation (LTP), an important process in the context of synaptic plasticity, and it is involved in neurotransmission. Moreover, in physiological conditions it is expressed in heart.

NOS2 is an inducible isoform, and in the context of the heart, it is usually expressed in pathological conditions[67]. Anyway, its role is yet to be understood in the cardiovascular system.

NOS3 is expressed in vascular endothelium, and it is important in the physiology of the heart.

The activity of the neuronal and endothelial isoforms is controlled by CaM, by binding a Ca^{2+} concentration dependent manner. In contrast, the inducible isoform binds CaM permanently as an additional subunit. This means that iNOS is under transcriptional control [168–170] and it is not regulated by CaM binding.

The three isoforms, eNOS, nNOS, and iNOS, are self-sufficient enzymes and share the same structure with two major functional domains merged into a single polypeptide. The N-terminal catalytic domain binds the heme prosthetic group and the redox cofactor, tetrahydrobiopterin (H4B). The C-terminal reductase domain has the binding sites for FMN, FAD, and NADPH[169,170].

NOS proteins are large enzyme ranging in size from 135 to 160 kDa but the monomeric form is inactive. The formation of a tight dimer through the haem domain (HD) is required for activity. The union of a calmodulin dimer with a NOS dimer determines its activation, leading NOS to catalyze the production of L-citruline from L-arginine and producing nitric oxide (NO) in different tissues. In physiologic and pathologic conditions, NO influences some cardiac functions such as systole, diastole, and chronotropic functions. Moreover, NO is involved in nytrosilation of the thiol-groups of tyrosine and cysteine of the proteins involved in cGMP (cyclic guanosine monophosphate) pathway, which is important in order to regulate the EC-coupling and heart function[169,171–175].

10.1 NOS1

NOS1 or nNOS gene was identified the first time in nitrergic nerves and brain tissue, and it encodes the neuronal nitric oxide synthase isoform. The human NOS1 gene is located on

chromosome 12 and it is composed by 29 exons and 28 introns. It encodes a 160 kDa protein including a region of 1434 bp.

The structure of NOS1 is the same of all the other isoforms of NOS and it is characterized by two domains: a reductase domain (C-terminal) and an oxygenase domain (N-terminal. The two domains can be departed by a calmodulin binding motif.

Despite NOS1 was identified firstly in nitrergic nerves and brain tissue, in which it is the main source of nitric oxide in the central nervous system, it plays an import role also in the heart to produce nitric oxide[67,176]. Moreover, it plays a pivotal role in the heart contractility, influencing different cardiac proteins involved in the excito-contraction coupling, such as L-type Ca2+ channels (LTCCs), ryanodine receptor (RyR2) and sarco-endoplasmic reticulum calcium ATPase (SERCA)[172,173,177,178]. For these characteristics, an imbalance in NOS1 function might be implicated in pathological heart conditions.

The presence of genetic variants in the NOS1 sequence can alter the function of NOS1 and, thus, the production of nitric oxide[67]. Several genetic variants have been identified and associated with the higher risk to develop a specific cardiovascular disease.

10.2 NOS2

NOS2 or iNOS encodes the inducible isoform of NOS protein. The human NOS1 gene is located on chromosome 17 and it is composed by 26 exons and 25 introns. It encodes a 131 kDa protein including a region of 1153 amino acids-.

Despite to the activities of NOS1 and NO3 that are controlled by intracellular calcium/calmodulin, numerous different phosphorylation mechanisms, and by binding of the molecular chaperone heat shock protein 90 (HSP90), the activity of NOS2 is controlled at the level of gene transcription[179].

NOS2 plays a key role in several physiological and pathophysiological conditions, such blood pressure regulation, inflammation, infection, and the onset and progression of malignant diseases. iNOS has been studied both as a marker and a therapeutic target in these situations[180].

Moreover, contrary to the other isoforms, NOS2 is not expressed in healthy myocardium, but its upregulation was showed to occur in heart failure patients. Its expression in myocardial cells increases the production of nitric oxide, leading to a disturbance in Ca2+ cycling, which later impairs E-C coupling[67,181]. In fact, several genetic variants in NOS2 have been identified and associated with the higher risk to develop a specific cardiovascular disease, due to a potential alteration in the function of NOS2 and, thus, in the production of nitric oxide.

10.3 NOS3

Among the three main isoforms of nitric oxide synthase, NOS3 is the most studied and is the most involved in NO production in the cardiovascular system. In this context, NO plays a key role in vascular homeostasis[175,181,182]. Its gene is located on chromosome 7 and it is composed by 25 introns and 26 exons. It encodes a 135 kDa protein including 1203 amino acids. The structure of the NOS3 is the same of other NOSs, characterized by an oxygenase and a reductase domain[170,183,184].

NOS3 is activated by two different mechanisms: the first one is allowed by calmodulin and the heat shock protein 90 (Hsp90), which binds to NOS3 and leads to its dissociation from caveolin, relieving inhibition. The second mechanism is induced by some stimuli, such as insulin, stress, and isometric vessel contraction, which activate NOS3 through phosphorylation[67,185].

NOS3 is active only in dimeric form and heme is fundamental for dimerization. In fact, in absence of heme, NOS3 appears in the monomeric form producing superoxide anion instead of NO, influencing the development of cardiovascular diseases[168,182]. A reduction in the NO production promotes the atherosclerosis onset, and this happens because NO plays a central role in atheroprotective function (Figure 7). It regulates blood flow and blood pressure, modulates vascular homeostasis, and promotes the protection from thrombosis by inhibiting platelet and leukocytes adhesion[169,171,175,182,185]. Furthermore, NO plays an indirect role on muscular relaxation: it stimulates the production of cGMP through the activation of the soluble guanylate cyclase (sGC) enzyme, which later activates the protein kinase G-1 (PKG) leading to phosphorylation of many downstream proteins in vascular smooth muscle cells and promoting the muscular relaxation[183,184,186].

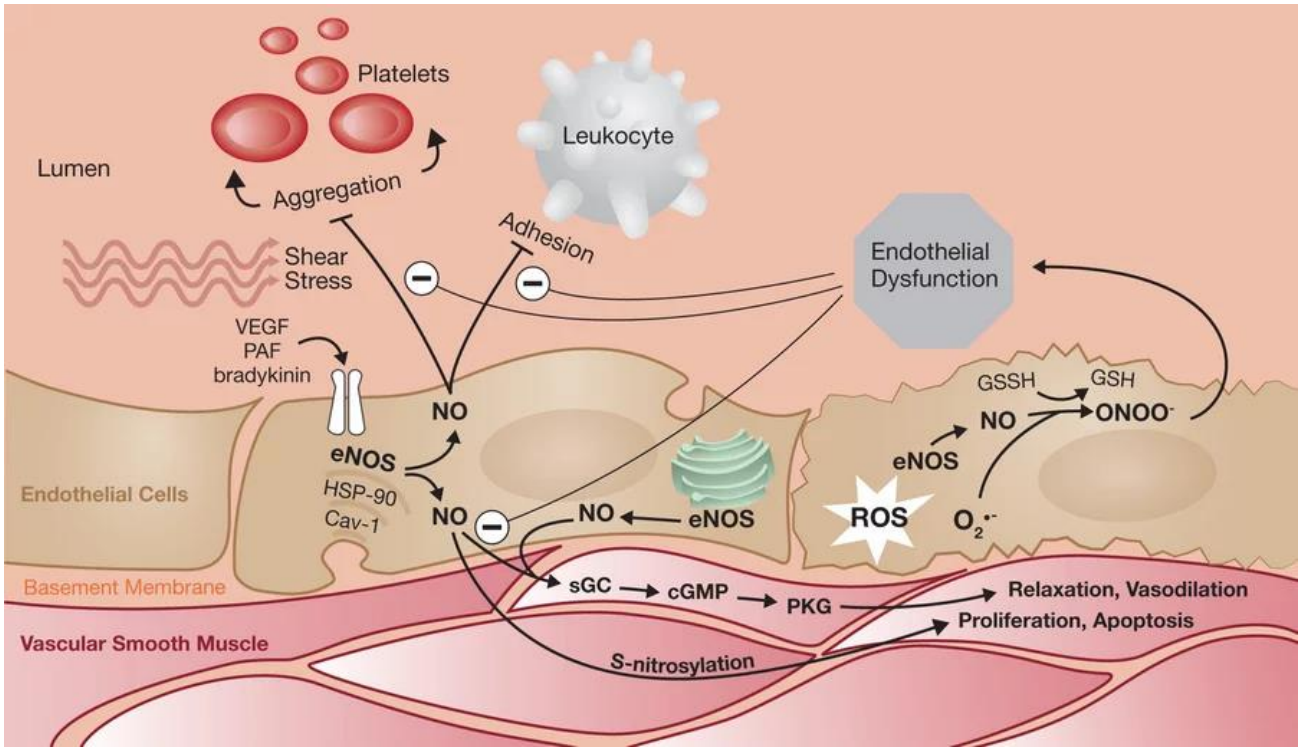


Figure 7: Representation of Endothelial Nitric Oxide Signaling (from Olivia May, 2012)

Moreover, cGMP binds to cyclic-GMP-dependent protein kinases, which then promote the phosphorylation of Ca^{2+} L-type channels (LTCCs). Thus, the inhibition of NOS3 lowers cellular Ca^{2+} concentrations and promotes vascular relaxation[184]. In addition, cGMP activates a cAMP phosphodiesterase 2, which then converts cAMP (cyclic adenosine monophosphate) in AMP (adenosine mono- phosphate). In caveolae, eNOS is also compartmentalized with β- adrenergic receptors (β-ARs) which stimulate contraction[187]. The β-agonists increase LTCC function through different ways: by stimulation the adenylyl cyclase, by formation of cAMP and by activation of protein kinase A (PKA). This latter protein, PKA, alongside the protein kinase Akt, stimulates the phosphorylation and the activation of eNOS [188]. As a result, NOS3 decreases the functional response to β-AR stimulation [184] by inducing a negative feedback effect [187,189].

Many studies highlighted that any variation inside the gene sequence and/or changes in the protein structure of eNOS are associated with a dysregulation of the activity and production of NO, leading to the onset and development of cardiovascular diseases and disorders[67].
Several genetic variants of eNOS were discovered and studied, three of them have been analyzed in correlation with the predisposition to CVDs, they are rs1799983, rs2070744 and rs61722009.

10.4 Nitric oxide synthase polymorphisms

Considering the crucial role played by NOS in cardiovascular diseases through an altered production of nitric oxide, an important modulator of different biological and physiological process in the heart, it is important to consider that variations in the gene sequence can compromise its bioavailability. Indeed, genetic variant can change its activity and/or the expression of NOS enzymes[181,190].

Since NOS enzymes are of particular interest in relation to cardiovascular diseases, efforts have been made to study the potential involvement of polymorphisms in this class of proteins in association to cardiovascular pathologies[67]. Manso et al. (2012) identified in a Portugese population seven SNPs in nNOS associated with ischemic stroke: rs2293050, rs2139733, rs7309163, rs11068445, rs547954, rs7308402, rs14837 [191]. Dai et al. (2013) analysed four SNPs, out of the seven analysed by Manso et al. (2012), in a Han Chinese population of Northern China in association with ischemic stroke [192]. In contrast to the results obtained in the Portuguese populations, the genotype and allele frequency in case and control groups in the Han Chinese population were not different. Moreover, in this study there was a different allelic frequency distribution of the SNP rs7308402 and this fact suggested that this SNP could be a protective factor.

In order to clarify the contribution of NOS1 polymorphisms in cardiovascular diseases, we performed some meta-analyses based on the available studies[67]. Only three NOS1 polymorphisms were studied for the same pathology, in more than two independent studies. Taking into consideration these papers, the association between NOS1 rs2293050, rs2139733, rs1483757 and IS (ischemic stroke) was evaluated under four different genetic models[191–194]. Further information about how the meta-analyses were performed and its results are reported in the chapters of Material and Methods and Results.

Other SNPs of nNOS have been studied and associated with other type of cardiovascular disorders. For example, Levinsson et al. (2014) have identified and associated two SNPs in nNOS, rs3782218 and rs2682826, with the higher risk to develop coronary heart disease (CHD)[195]. The SNP rs3782218 has been associated also with hypertension.

Several genetic variants associated with the higher risk to develop a specific cardiovascular disease have been identified also in NOS2. Gleen et al. (1993) performed the first association study on iNOS gene polymorphisms in association with hypertension in an Australian Anglo-Caucasian population, identifying variations in the number of repeats for two polymorphisms of NOS2,

localized 0.7-kb upstream and 2.6-kb upstream of the NOS2 gene, the AAAT/AAAAT (insertion or deletion of 1 repeat unit: "+" allele and "– "allele, respectively) and the (CCTTT)n repeats, respectively [196].

In another study, two SNPs in iNOS (rs2779249 and rs2297518) were associated with hypertension in a Finnish population[197]. The SNP rs2297518 caused an amino acid change from Ser to Leu that led to an increase of the NOS2 protein activity, and thus an increase of NO production.

A higher level of nitric oxide was also associated with pregnancy- related CVDs, especially with hypertensive disorders (HPD), such as pre-eclampsia (PE) and gestational hypertension (GH), which are common pregnancy complications and may induce maternal/foetal morbidity and mortality[197–200].

Considering the important role of NOS3 in cardiovascular field discussed previously, there are many studies regarding the presence of numerous genetic variants in the promoter, intron, and exon regions in correlation with cardiovascular diseases [67].

Among these variants, there are three main polymorphisms in NOS3 gene that have been genotyped in different populations and analysed for the predisposition to cardiovascular diseases.

The single nucleotide polymorphism rs1799983 is in the exon7. It causes an amino acid change from Glu to Asp at the residue 298, affecting the susceptibility of NOS3 to enzymatic cleavage: only the protein with aspartate can be cleaved generating N-terminal 35-kDa and C-terminal 100-kDa fragments.

The SNP rs1799983 was studied by Hingorani et al. (1999), in correlation with coronary artery disease and myocardial infarction on an East Anglias population. Patients with CAD and MI (myocardial infarction) presented a greater number homozygous (TT) with Asp at the position 298, compared to the control group, and thus a strong correlation with the higher risk to develop CAD and MI[201]. The same results were found in a Pakistani population, where patients TT had a higher risk to develop coronary artery disease[202]. On the contrary, Rossi et al. (2003) didn't find any correlation between the same missense variant rs1799983 and CAD in a Caucasian population[203].

Many other studies focused the attention on this variant in correlation with CVD and the results were different and sometimes contradictory, but this depends on the population and the cardiovascular disease analyzed[204–209].

Other polymorphisms in NOS3 found in correlation with CVDs are mainly in the intronic regions.

One of the main interesting one is the variable number of tandem repeat (VNTR) of a 27-bp consensus sequence repeat in intron 4.

This VNTR was previously associated with a reduction in nitric oxide levels in the plasma and to the inhibition of NOS3 mRNA expression in the production of a small RNA, which has been showed to interfere with its expression[210,211]. The number of the 27-bp sequence repetitions made a distinction between the predominant 4a (repeated 4 times) and 4b alleles (repeated five times), although rarer alleles have also been reported (allele 4c and allele 4y, with six or two copies of the 27-bp DNA fragment, respectively)[212]. According to the study of Zhang et al., cells with five repetitions (4b allele) of the 27 bp sequence caused lower levels of NOS3 mRNA, compared to cells with four repetitions (4a allele)[213].

This VNTR, rs61722009, has been studied in correlation with different types of cardiovascular disorders. For example, a statistically significant association between this polymorphism and the higher risk to develop ischemic stroke in a Han Chinese population hasn't been found[214]. On the contrary, in another study Hou et al. (2001) found a correlation between the A allele with four repeats and ischemic stroke in a Chinese population[215].

No associations were found between the VNTR and coronary artery disease in a Caucasia and Tunisian populations[204,216], instead the 4a/4b polymorphism was associated with CAD in a Korean population[217].

Within the promoter regions of NOS3 it has been identified different allelic variants.

For example, the polymorphism rs2070744 is located in the promoter region at the position 786 and it is an allelic substitution from T allele to C. This SNP has been significantly associated with coronary artery disease in a Caucasian population, as well as in Iranian, Korean, and Chinese populations[203,205,218,219].

Different authors have analysed both SNP rs2070744 and rs1799983 through a linkage disequilibrium study. Colombo et al. (2003) found a significant linkage disequilibrium between these two polymorphisms and the higher risk to develop CAD[220]. Moreover, numerous meta-analyses reported the correlation between NOS3 SNPs and different type of cardiovascular diseases. For example, rs177983 resulted to be a predisposing factor for hypertension, coronary artery disease and thrombotic disease.

Meta-analysis studies regarding the polymorphism rs2070744 in association with CAD found a positive correlation in different genetic models, in particular amongst Middle Easterners[221–224].

The 27 bp VNTR was positively associated with ischemic stroke. Two meta-analyses regarding the association between this genetic variant and CAD resulted statistically significant, mainly in Asian population compared to European, African, Middle Eastern and Asian-Indian population[214,223–225]. Since the results from the meta-analysis regarding the three SNPs suggest that all of them are predisposing factors for CVDs, in this research project we decided to analyse these genetic variants in correlation with the higher risk to develop a specific cardiovascular disorder. We also included the analysis of the SNP rs1549758, a missense variant that causes an allelic change from the C to the T allele at the position 774.

Some other variants present in literature in NOS1, NOS2 and NOS3 are reported in Table 5, 6, and 7.

Table 5: Human NOS1 gene polymorphisms and associations with cardiovascular disease (rearranged from Beghi et al., 2020)

Nitric oxide synthase 1 (NOS1)					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Year [Refs.]**
Exon Region					
rs1047735	G-A	His902His	Ischemic stroke	Korean	2016[194]
rs2293044	G>C,T	Val1353Val	Ischemic stroke	Korean	2016[194]
rs2293054	A>G,T	Ile734Ile	Ischemic stroke	Korean	2016[194]
Promoter Region					
rs2682826	G-A	3'-UTR	Chronic Heart Disease	Sweden	2014 [195]
			Ischemic Stroke	Korean	2016[194]
Intron Region					
rs2293050	C>A,G,T	117281017	Ischemic stroke	Portuguese	2012 [191]
				Han Chinese	2013 [192]
				Han Chinese	2014 [226]
			LAA-Caused IS Risk	Chinese	2014 [226]
rs2139733	T-A	117288937*	Ischemic stroke	Portuguese	2012 [191]
				Han Chinese	2013 [192]
				Han Chinese	2014 [226]
			LAA-Caused IS Risk	Han Chinese	2014 [226]
rs7309163	C-T	117291469*	Ischemic stroke	Portuguese	2012 [191]
rs11068445	G-A	117307124*	Ischemic stroke	Portuguese	2012 [191]
rs547954	A-G	117316701*	Ischemic stroke	Portuguese	2012 [191]
rs7308402	G-A	117321642*	Ischemic stroke	Portuguese	2012 [191]
				Han Chinese	2013 [192]
rs1483757	A>G,T	117323735*	Ischemic stroke	Portuguese	2012[191]
				Han Chinese	2013 [192]
				Han Chinese	2014[226]
				Chinese	2015 [193]
			LAA-Caused IS Risk	Han Chinese	2014 [226]
rs3782218	C-T	117333706*	Chronic Heart Disease	Sweden	2014 [195]

*(GRCh38.p12)

Table 6: Human NOS2 gene polymorphisms and associations with cardiovascular disease (rearranged from Beghi et al., 2020)

Nitric oxide synthase 2 (NOS2)					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Year [Refs.]**
Exon Region					
rs2297518	G-A	Exon 6	Hypertension	Finnish	2015 [197]
				Unspecified	2012 [200]
Promoter region					
-	4bp(+/-) AAAT/AAAAT	0,7kb upstream	Hypertension	Anglo-Caucasians	1999 [196]
			Coronary artery disease	Caucasian	2001 [227]
				Caucasian	2005 [228]
-	VNTR (CCTTT)n	2,6kb upstream	Hypertension	Anglo-Caucasians	1999 [196]
				Unspecified	2012 [200]
			Coronary artery disease	Caucasian	2005 [228]
			Cardiovascular events	Spanish	2009 [229]
			Atrial fibrillation	Taiwanese	2017 [230]
rs2779249	C-A	-1026C/A	Hypertension	Finnish	2015 [197]
				Han Chinese	2009 [198]
				Unspecified	2012 [200]
			Gestational Hypertension	Unspecified	2012 [199]
			Pre-Eclampsia	Unspecified	2012 [199]

Table 7: Human NOS3 gene polymorphisms and associations with cardiovascular disease (rearranged from Beghi et al., 2020)

Nitric oxide synthase 3 (NOS3)					
SNPs	**Genetic Variant**	**Location**	**Disease evaluated**	**Population**	**Year [Refs.]**
Exon Region					
rs1799983	G/T	Exon 7 (Glu298Asp)	Coronary artery disease	East Anglian	1999 [231]
				Han Chinese	2016 [232]
				Caucasian	2003 [233]
				Tunisian	2015 [204]
				Pakistan	2014 [234]
				Australian	1999 [235]
				Korean	2007 [236]
				Korean	2000 [237]
			Premature CAD	Egyptian	2013[238]
			Myocardial infarction	East Anglian	1999 [201]
				Australian	1999 [208]
			Hypertension	Sudan	2017 [239]
				Japanase	1998 [240]
				Caucasian	1998 [241]
				Han Chinese	2015 [242]
Promoter Region					
rs2070744	T-786C		Coronary artery disease	Caucasian	2003 [233]
				Han Chinese	2016[205]
				Korean	2007 [218]
				Iranian	2012[219]
Intron Region					
rs61722009	VNTR (27bp) [4a/4b]	Intron 4	Ischemic Stroke	Han Chinese	2013 [214]
				Chinese	2001 [215]
				Singapore	2008[243]
			Coronary artery disease	Tunisian	2015 [204]
				Caucasian	2000 [204]
				Korean	2007 [218]
				Korean	2000[209]
			Hypetension	Japanase	1998 [206]
-	VNTR (CA)n	Intron 13	Coronary artery disease	Caucasian	2000 [244]
			Hypertension	Japanese	1997[245]
				Caucasian	1995 [246]

-	A-27C	Intron 18	Hypertension	Caucasian	1995 [246]
				Japanase	1998[206]
				Caucasian	1998 [207]
-	G-10T	Intron 23	Hypertension	Caucasian	1995[246]
				Japanase	1998 [206]
				Caucasian	1998 [207]
			Coronary artery disease	Korean	2000[209]

Aim of the research

Cardiovascular diseases are the leading cause of death all over the world, and for this reason every year they represent a significant source of interest in scientific research.

Although the pathophysiological spectrum of most cardiovascular diseases is well known, they are considered complex disease because a lot of factors coexist together increasing the risk of their development. Behind modifiable factors as an unhealthy lifestyle, other comorbidity, and environmental factors, it is important to consider the non-modifiable factors as the genetic background. It is becoming increasingly realistic to believe that genetic factors could contribute to determine the individual's risk. Specifically, recent genetic studies have shown how genetic variants can be independently associated with CVD risk factors and with higher risk of CVD.

The study of polymorphism is increasingly at the forefront in identifying potential biomarkers for the prediction of risk factors associated with the onset of cardiovascular disease or for monitoring patient follow-up. Genetic biomarkers, compared to the traditional ones, can be ascertained even prior to birth. Moreover, for this characteristic, they are not influenced by environmental factors, that are often responsible for increasing the risk of developing disease states.

Mapping and identification of single gene disorders with large genetic effects are significant but limited to identifying links for polygenic diseases of multifactorial etiology. For this important aspect, the interest of this study was to analyze several genetic variants involved in the calcium calmodulin pathway in order to have a panel of candidate gene to analyze in association with cardiovascular disease; hypothesizing that the identification of polymorphic variants of genes involved in the calmodulin (CaM) pathway could be important for understanding how genetic traits can influence predisposition to CVD.

Calmodulin is highly conserved in eukaryotes, and it is the first sensor of calcium signaling, which play a crucial role in the heart, as for example in the mechanism of excito-contraction coupling. This research project considered each pathway of the three different isoforms of calmodulin (CaM1; CaM2; CaM3) and focuses on some common proteins involved in the three pathways. The genetic variants analyzed were: rs10113 and rs7259810 in CaM3; rs7214723 in CaMKK1; rs1799983, rs2070744, rs1549758 and rs61722009 in NOS3. All these variants are single nucleotide polymorphisms, except for rs61727009 that is a variable number of tandem repeats of a 27-bp consensus sequence

In order to study the risk of a subject to be more susceptible to a specific cardiovascular disease, each genetic variant, considered in this study, was analyzed in a cohort of 300 cardiopathic subjects and compared to the European population of 1000 Genomes, used as reference group.

Thanks to the approach of the RFLP-PCR, it was possible to discriminate the genotype of each patient for each genetic variants in order to calculate their genotypic and allelic frequencies and observe if existed a different distribution compared to the reference group. Among all the data collected, the genetic variant in CaMKK1 resulted to be very interesting for its different trend in the distribution of the genotypic and allelic frequencies in the cardiopathic population, object of study, compared to the European one.

The genetic variant rs7214723 in CaMKK1 was also analyzed in a cohort of about 70 Dutch patients with different types of cardiovascular disease, confirming the genotypic analyzes conducted on the Italian population. For this reason, the second aim of the project, conducted at the University of Maastricht, was to study the potential role of CaMKK1 in cardiovascular disease, considering that it plays a crucial role in the cascade of intracellular calcium signaling and that, there is few information about this protein in the field of cardiovascular diseases. This part of the study was conducted *in vitro* on human vascular smooth muscle cells (VSMCs) because they are involved in many vascular diseases, such as atherosclerosis and aneurysm formation, and are used as model to study different process as calcification, an event that affect different CVDs. Moreover, this cell line has the capability to switch its phenotype. In physiological condition in an adult and healthy organism, VSCMs are in a quiescent contractile phenotype. This phenotype ensures that the blood vessel contract and relax in order to regulate the blood circulation, guaranteeing vasoconstriction, vasodilatation and other function as synthesis of extracellular matrix.

During the onset and development of vascular disease, VSMCs have the capability to change its phenotype through a process called phenotype switching, in which they lose their contractility and switch in a synthetic phenotype, leading to intimal thickening, formation of atherosclerotic plaques and stenosis of vascular lumen.

The first goal was to understand how CaMKK1 could influence phenotype switching and its possible role. After the analysis of CaMKK1 gene and protein expression in hVSMC treated for phenotype switching, we silenced the protein with a siRNA-CaMKK1 and analyzed the expression of different markers specific for phenotype switching. Starting from the conditioned media of VSMC siRNA-CaMKK1 treated for phenotype switching, extra vesicles (EVs) analysis was performed to check if the knock down of the protein of interest affected the amount of released EVs. In addition, to better understand the pathway in which CaMKK1 is involved and the interactions with other proteins, we performed on VSMC sirna-CaMKK1 treated for phenotype switching a kinase assay with PamChip technology, that is based on measuring peptide phosphorylation by protein

kinases. Furthermore, some ongoing experiments are focusing on identifying the role of CaMKK1 in hVSMC cells treated with higher concentrations of calcium and / or phosphate, to mimic the calcification event.

Materials and methods

1. Population study

The study protocol (study number 97/2017) has been approved by the Ethical Committees of the Università degli Studi dell'Insubria (Varese, Italy). Informed consent has been obtained in accordance with the principles outlined in the Declaration of Helsinki.

The study involved 300 cardiopathic subjects requiring cardiac surgery recruited at the Cardiac Surgery of Varese Hospital (Italy). Among the 300 patients, the percentage of non-Italian subjects was below 5%.

All patients were subjected to clinical (presence/absence of chest pain, comorbidity, and comorbility) and instrumental diagnosis. All patients underwent a series of tests routinely performed before any cardiac operation, including electrocardiogram (ECG), echocardiogram (echo), and coronary angiography. The patients were subsequently divided in two subgroups. The first group included 150 patients with coronary artery disease (CAD group), while the second included 150 patients without CADs (NOCAD group). Among all the patients, 107 had AS, and 71 of them belonged to the NOCAD group (Figure 1).

The population studied in Maastricht (in agreement with the Dutch Code for Proper Secondary Use of Human Tissue; http://www.fmwv.nl) is instead composed by 79 patients with different type of cardiovascular diseases, mainly aortic aneurysm, and aortic stenosis.

2. Sample collection

Regarding the study protocol number 97/2017 samples were collected from September 2017 to March 2019 by the Cardiac surgery of Varese. The amount of blood collected from all patients via peripheral venous puncture was approximately 5 cc. An aliquot of 125 µl was later spotted on Whatman FTA cards for subsequent genetic analysis. FTA cards are characterized by a special matrix containing chemicals with specific roles: they can lyze cells, denature proteins and protect nucleic acids from nucleases, oxidation, and UV damage. This allows to a long-term room temperature storage of the nucleic acids, which remain entrapped among the fibers of the matrix.

Regarding the study in Maastricht, pieces of aorta tissue samples from patients that underwent to a cardiac surgery, were stocked in RNA later (Sigma-Aldrich) at -20°C.

3.DNA isolation

Harris Micro Punch (Whatman) was used to isolate DNA from FTA cards (Whatman). It is a specific tool used for cutting and removing from FTA cards samples with a diameter of 1.2mm. Two disks, derived from each sample, were inserted in 200 µl PCR (Polymerase Chain Reaction) tube. Between one sample and another, Harris Micro Punch is cleaned with 96% ethanol. Then, in order to have a better DNA quality, 200µl of FTA purification (Whattman) reagent are added in every PCR tube. The FTA purification reagent is a specific buffer which promotes the removal of PCR inhibitors. After 5 minutes of incubation, the reagent was removed from PCR tube and the passages were repeated for a total of 3 times. Subsequently, 200 µl of buffer TE at pH 8 (Tris-HCl 10mM pH8, EDTA 1mM pH8) were added to PCR tube for 5 minutes: this buffer solubilizes the DNA and protects it by degradation. This procedure was repeated for a total of 2 times. Finally, PCR tubes were put in a thermoblock (Thermoblock, Falc) for 10 minutes at 56 °C and, subsequently, the disks were dried in PCR tubes. DNA was then ready for the PCR reaction.

The isolation of DNA from tissue was performed using the DNeasy® Blood & Tissue Kit (QIAGEN). Small pieces of tissue were cut, minced and place in a 1,5 ml tube. The protocol of the kit was used.

4. Primer design

Primers forward (FR) and reverse (RV) for the PCR reactions were identified in literature or designed in order to amplify the region of interest with the specific SNP and subsequently synthesized by Eurofins. The FASTA sequence with indicate the position of the SNP was downloaded from the dbSNP database of NCBI.

The following rules were used to design the primers:

- Length between the 18 and the 24 bases.
- 40-60% G/C content.
- Melting temperature (Tm) of 50-60°C.
- Primer pairs should have a Tm within 5°C of each other.
- Start and end with 1-2 G/C pairs (when possible)

Moreover, it was necessary to design the primers considering also to have a different length from the starting of each primer to the position of the SNP, in order to discriminate the different

genotype with a specific restriction enzyme, thanks to different fragment lengths. Regarding the VNTR in NOS3, the discrimination of the genotypes depends on the copies of 27 bp repeats (5 copies for the wildtype, 4 copies for the mutant).

PRIMERS for the SNP rs7214723 (T → C) in CAMKK1:

FW_CAMKK1: 5' **–** AACAGCACCGCCACCTTCATA – 3'

RV_CAMKK1**:** 5' – GGTCCTTCTCATGTAATGGGAGC – 3'

Amplicon length: 326 bp

PRIMERS for the SNP rs1799983 (G → T) in NOS3:

FW_NOS3: 5' - CATGAGGCTCAGCCCCAGAAC - 3'

RV_NOS3: 5' - AGTCAATCCCTTTGGTGCTCACG – 3'

Amplicon length: 206 bp

PRIMERS for the SNP rs2070744 (C→ T) in NOS3:

FW_NOS3: 5'-ATGCTCCCACCAGGGCATCA-3'

RV_NOS3: 5'-GTCCTTGAGTCTGACATTAGGG-3'

Amplicon length: 237 bp

PRIMERS for the SNP rs1549758 (T→ C) in NOS3:

FW_NOS3: 5'- GAAATGTTCACCTACATCTGC- 3'

RV_NOS3: 5'- ATCCTTTATCTCACCGAGGCT -3'

Amplicon length: 406 bp

PRIMERS for the VNTR rs61722009 in NOS3:

FW_NOS3: 5'- AGGCCCTATGGTAGTGCCTTT -3'

RV_NOS3: 5'- TCT CTT AGT GCT GTG GTC AC -3'

Amplicon length with 5 copies of the sequence: 420 bp

Amplicon length with 4 copies of the sequence: 393 bp

PRIMERS for the SNP rs10113 (C→ T) in CalM3:

FW_CALM3: 5'- ACAAAAGATTTGTCCCAAGC -3'

RV_CALM3: 5'- AATCACAGGGCTGGTTGC -3'

Amplicon length: 347 bp

PRIMERS for the SNP rs7259810 (C→ T) in CalM3:

FW_CALM3: 5' - TTAGGATTAAAGGCATGAGC - 3'

RV_CALM3**:** 5' – TGAAACTTTGTTTATAGGAGC - 3'

Amplicon length: 398 bp

5. RFLP-PCR

The SNPs rs10113 and rs7259810 in CaM3, the SNPs rs179983, rs2070744 and rs1549758, and the SNP rs7214723 in *CaMKK1* gene were genotyped by PCR-restriction fragment length polymorphism (RFLP) strategy.

The polymorphic region of gene of interest was amplified via PCR in a thermal cycler 2720 (Applied Biosystems) using two primers previously designed to amplify the region containing the single nucleotide polymorphism (SNP) of interest. PCR was performed in a reaction volume of 25 µl using the DNA Dream Taq Polymerase (#EP0705, Thermo Fischer Scientific) with the following cycling conditions: denaturation, annealing, extension etc. After the amplification, the PCR product fragment was detected using an UV transilluminator. PCR products were then digested using a specific restriction enzyme, which had been previously identified with RestrictionMapper version 3 (http://www.restrictionmapper.org), in a total reaction volume of 25 µl with the aim to discriminate the genotype for each SNP.

Enzymatic digestions were performed at 37°C for 1 h. The fragments were run on 3% agarose gel for approximately 50 min at 150 V and the bands were visualized on a UV transilluminator.

The 27 bp-VNTR in NOS3 were detach in one step through a PCR.

For the SNP **rs7214723** (T → C; T1282C; Glu375Gly) in exon 12 of CaMKK1, the restriction enzyme BseRI (5'- GAGGAG$(N)_{10}$ - 3') (NEB) was used.

The enzyme recognizes and cuts the sequence in the presence of the T allele, generating two fragments of 212 bp and 114 bp respectively. When there is the C allele, the sequence will not be recognized by the BseRI enzyme, and will produce an intact fragment of 326 bp.

Thus, the three genotype have been discriminated on the electrophoresis gel thanks to the presence of different bands: two bands for the homozygous TT; one band for the homozygous CC; three bands for the heterozygous TC.

For the SNP **rs1799983** (G → T; G894T; Glu298Asp) in exon 7 of NOS3, was used the restriction enzyme MboI (5'- GATC - 3') (NEB) was used.
The enzyme recognizes and cuts the sequence in the presence of the T allele, generating two fragments of 119 bp and 87 bp respectively. When there is the G allele, the sequence will not be recognized by the MboI enzyme, and will produce an intact fragment of 206 bp.
Thus, the three genotype have been discriminated on the electrophoresis gel thanks to the presence of different bands: two bands for the homozygous TT; one band for the homozygous GG; three bands for the heterozygous TG.

For the SNP **rs2070744** (C→ T; -786T/C) located in the 5' flanking region of NOS3, the restriction enzyme NaeI (5'- GCCGGC - 3') (Thermo Scientific) was used.
The enzyme recognizes and cuts the sequence in the presence of the C allele, generating two fragments of 202 bp and 35 bp respectively. When there is the T allele, the sequence will not be recognized by the NaeI enzyme, and will produce an intact fragment of 237 bp.
Thus, the three genotypes have been discriminated on the electrophoresis gel thanks to the presence of different bands. Considering that the fragment of 35bp can't be seen on gel due to the small size, the presence of only the band of 202 bp indicates the homozygous CC; one band of 237 bp indicates the homozygous TT; the presence of two bands indicates the heterozygous TG.

For the SNP **rs1549758** (T→ C; C774T; Asp258Asp) in exon 6 in NOS3, the restriction enzyme FokI (5'- GGATG$(N)_9$ - 3') (Thermo Scientific) was used.
The enzyme recognizes and cuts the sequence in the presence of the T allele, generating two fragments of 259 bp and 147 bp respectively. When there is the C allele, the sequence will not be recognized by the FokI enzyme, and will produce an intact fragment of 406 bp.
Thus, the three genotype have been discriminated on the electrophoresis gel thanks to the presence of different bands: two bands for the homozygous TT; one band for the homozygous CC; three bands for the heterozygous TC.

For the VNTR **rs61722009** (T→ C) in intron 4 of NOS3 it was not used a restriction enzyme, since the discrimination of the genotypes depend on the number of copies of the 27bp fragment. Thus, the 27-VNTR polymorphism in intron 4 was determined by standard PCR amplification using the primers reported in the previoous paragraph. The number of the 27-bp sequence is repeated four (allele 4a) or five (allele 4b) times. In presence of five copies of 27 bp repeats, it is generated a 420 bp band, in presence of four copies of 27 bp repeats, it is generated a 393 bp band.

For the SNP **rs10113** (T→ C) located in the 3' UTR region in CaM3, the restriction enzyme BmgBI (5'- CACGTC - 3') (Thermo Scientific) was used.
The enzyme recognizes and cuts the sequence in the presence of the C allele, generating two fragments of 104 bp and 243 bp respectively. When there is the T allele, the sequence will not be recognized by the BmgBI enzyme, and will produce an intact fragment of 347 bp.
Thus, the three genotype have been discriminate on the electrophoresis gel thanks to the presence of different bands: two bands for the homozygous CC; one band for the homozygous TT; three bands for the heterozygous TC.

For the SNP **rs7259810** (T→ C) located in a non-coding transcript exon variant in CaM3, the restriction enzyme SduI (5'- GDGCHC - 3') (Thermo Scientific) was used.
Considering the site recognized by the restriction enzyme is present more times inside the amplicon, the discrimination of the three genotypes depends on the number of fragments generated by the enzyme cleavage. Four fragments of 18 bp, 29 bp, 152 bp and 199 bp for the homozygous TT; five fragments of 18 bp, 29 bp, 70 bp, 129 bp and 152 bp for the homozygous CC and six fragments can be seen respectively of 18 bp, 29 bp, 70 bp, 129 bp, 152 bp and 199 bp for the heterozygous.
Considering that the fragment of 18 and 29 bp can't be seen on gel for the small size, at the end two bands indicate the genotype TT; three bands indicate the genotype CC and 4 bands indicate the genotype TC.

6. Cell Culture and treatments

Human primary VSMCs were derived from tissue explants as described before[247] and cultured for in Medium 199 (Gibco) supplemented with 1% Penicillin Streptomycin antibiotics (Gibco) and 20% FBS (Gibco). hVSMC were passaged when 80%-90% confluent and washed with PBS before

treatments. hVSMCs in passages 5 to 9 were used. Human aortic samples were obtained from patients undergoing open aortic surgery at Maastricht University Medical Centre. Collection, storage, and use of tissue and patient data were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue (http://www.fmwv.nl). This study complies with the Declaration of Helsinki.

For phenotype switching hVSMCs were treated with heparin (200 U/ml) in M199 with 2,5% FBS and/or low FBS (0,5%) for the contractile phenotype and PDGF-BB (platelet derived growth factor subunit B, 10 μg/ml) in 2,5% FBS for the synthetic phenotype. The cells were treated 1 day after the seeding, media were refreshed on day 4 after treatment, and cells were analyzed or harvested on day 6 in order to isolate DNA, RNA and proteins.

For the osteogenic differentiation, hVSMCs were treated with high calcium (3.6 mmol/L CaCl2) medium, phosphate (2.5 mmol/L NaH2PO4) and with calcium and phosphate (2.7 mmol/L CaCl2 and 2,5 mM/L NaH2PO4), all in M199 (Gibco) with 2,5% FBS (Gibco).

7. Microarray analysis of mRNA

Microarray mRNA analysis was performed as described before[248] VSMCs were fasted overnight and stimulated with heparin (200 U/ml), PDGF (20 ng/ml) or control media for 4 hours. Data is shown as fold change compared to control.

8. SiRNA transfections

Transfection of hVSMCs at passage 6 with CaMKK1 SiRNA (L-004912-00-0005, Horizon) and non-targeting control SiRNA pool (D-001810-10-05, Horizon) was performed using the Neon™ Transfection System (Invitrogen) according to the manufacturer's protocol. Briefly, a total of 6x105 divided in two 1.5 ml tubes (3x105 cells in each) were centrifuged 5 minutes at 200 x g at 4°C. Subsequently the pellet was washed with 1 ml of PBS and centrifuged again with same setting. The pellet was resuspended with 100 μl buffer R (Resuspension Buffer-Invitrogen) and 20 μl of 20 μM siRNA were added into each tube. 100 μl of this cell suspension was electroporated in the Neon® system at 1475 V for 20 ms, two pulses. Then the transfected cells were pipetted into a 12 well plate, according to the conditions of the experiment.

9. Immunoblotting

hVSMC were lysed with 50-100 µl RIPA buffer (Thermo Scientific) with MS-SAFE (Sigma) and a DC Protein Assay (BioRad) was performed according to the manufacturer's protocol. A total of 20 µg of proteins was loaded in 10% SDS gel (Biorad) and immunoblotting was performed as previously described.[249] The following commercially available antibodies were used for Western blots: rabbit anti-CamKK1 (1:1000; Ab NBP1-42683 NOVUS Biologicals), rabbit anti-SM22α (Abcam, ab14106, 1:1000), mouse anti-α-SM-actin, mouse anti-pMLC (Cell Signalling, 36755, 1:500), rabbit anti-CNN1 (Abcam, ab46794, 1:5000), rabbit anti-S100A4 (Dako, A5114, 1:1000), rabbit anti-osteocalcin (Santa Cruz, sc-30044, 1:400). Rabbit anti-VCL (1:10000, ab19002, Abcam) was used as loading control. The following secondary antibodies were used: goat anti mouse HRP (p0447, Dako, 1:3000), goat anti-rabbit (7074S, Cell Signalling, 1:3000). Bands were detected using chemiluminescence (SuperSignal West Dura Extended Duration Substrate ECL, ThermoFisher) with the Invitrogen iBright Imaging System (Thermo Scientific). The signal was subsequently quantified with Image Studio Lite (Li-Cor).

10. Kinase activity profiling

Serine-Threonine kinase profiles were determined using the PamChip® Ser/Thr Kinase assay (STK; PamGene International, ´s-Hertogenbosch, The Netherlands). Each STK-PamChip® array contains 144 individual phospho-site(s) that are peptide sequences derived from substrates for Ser/Thr kinases. Cells transfected with siRNA and treated for phenotype switching with 3 biological replicates per condition, were washed once in ice-cold PBS after respective treatments and lysed for 15 min on ice using M-PER Mammalian Extraction Buffer containing Halt Phosphatase Inhibitor and EDTA-free Halt Protease Inhibitor Cocktail (1:100 each; Thermo Fischer Scientific). Lysates were centrifuged for 15 min at 16.000 x g at 4°C. Protein quantification was performed with Pierce™ Coomassie Plus (Bradford) Assay according to the manufacturer's instructions.

For the STK assay, 1 µg of protein and 400 µM ATP were applied per array (N=3 per condition) together with an antibody mix to detect the phosphorylated Ser/Thr. After incubation for an hour (30oC) where the sample is pumped back and forth through the porous material to maximize binding kinetics and minimize assay time, a secondary FITC-conjugated antibody is used to detect the phosphorylation signal. Imaging was done using a LED imaging system and the spot intensity at each time point was quantified (and corrected for local background) using the BioNavigator software version 6.3 (PamGene International, 's-Hertogenbosch, The Netherlands).

11. EV Quantification

Conditioned media from hVSMCs treated for phenotype switching and transfected with siRNA were collected in 1,5 ml tubes and centrifuged for 8 minutes at 6000 x g. Medium 199 (Gibco) EVs free supplemented with 1% Penicillin Streptomycin antibiotics (Gibco) and 2,5% FBS (Gibco) was used. The samples were loaded directly in the Zeta View NTA (nanoparticle tracking analysis) for the quantification of EVs secreted in the cell culture media. Each sample was diluted 1:40 and 1 ml were loaded in the machine with a syringe at 21°C. The data collected were normalized to the protein concentrations (mg/ml). Pattern parameters, such as intensity fluctuations, surface geometry and shape of the particles as well as particle concentration are documented at each recording and can be used to distinguish sub-populations.

12. Statistical analysis

The browser Ensembl and the 1000 Genomes data bank were used in order to evaluate the allelic and genotypic distribution of rs7214723, rs1799983, rs2070744, rs1549759, rs61722009, rs10113 and rs7259810 polymorphisms in the European population, which was used as reference group in the study.

Differences in genotypic and allelic frequencies between the two populations were examined by means of a χ 2 test and Hardy-Weinberg equilibrium (HWE) for CaMKK1 genotype distributions was tested by a goodness-of-fit χ2 test. In order to evaluate distribution differences in the groups, a Chi-square Test was applied between the two subgroups of CAD and NO CAD to evaluate statistically significance of age, sex, diabetes, BMI and hypertension distributions. A multiple comparison method was here applied using a simple Bonferroni correction technique (*P*=0.0071).

The statistical tests are performed with Microsoft Excel and STATA software. The level of statistical significance was set at $P<0.05$.

Regarding the *in vitro* part, data are shown as mean ± SD (standard deviation) with individual data points and were obtained in 3 or more independent experiments. Each experiment was repeated in hVSMCs from at least 3 different donors in triplicate. Normality of data was tested using Shapiro-Wilks test or ascertained based on previous literature reports. All the data was deemed normally distributed, therefore statistical significance was tested with t-test and/or 1-way ANOVA. The exact test used for each data set is mentioned in figure legends. Statistical analysis was performed using GraphPad Prism 8.2.0. *$P<0.05$. Representative images for figures, which best reflected the data, were selected manually.

Upstream Kinase Analysis (UKA, PMID: 30610604), a functional scoring method (PamGene) was used to rank kinases based on combined specificity scores (based on peptides linked to a kinase, derived from 6 databases) and sensitivity scores (based on treatment-control differences). The red/blue reflects the Median Kinase Statistic, where positive (red) means upregulation and negative (blue) means downregulation. For the Mean Finale Score, 1.2 is the cut-off value that defines an upregulation.

Results

1. Characteristics of the cardiopathic population

For this study, 300 patients with different cardiovascular pathologies, all requiring cardiac surgery, were recruited (total cardiopathic population). Among the different type of cardiovascular disorder, 150 subjects were suffering from coronary artery diseases (CAD patients), the other 150 were patients without coronary artery disease (NOCAD patients). In this subgroup there are some patients with valve disease, as mitral pathology.

The distributions of some characteristics, as gender, age, incidence of diabetes, dyslipidemia, hypercholesterolemia, previous history of neoplasia and BMI, were analyzed in the total cardiopathic population, considering the subdivision in the CAD and NOCAD patients (Table 1). CAD and NOCAD resulted to have statistically different distributions in gender (*P*=0.000), incidence of diabetes (*P*=0.000) and dyslipidemia (*P*=0.000) (Table 1). Instead, the two subgroups did not differ significantly in the distribution of age (*P*=0.033), hypertension (*P*=0.101), BMI (*P*=0.026) and previous history of neoplasia (*P*=0.558).

Table 1: Number and frequency distribution (in brackets) of selected characteristics of study subjects, divided in CAD and NOCAD groups (from Beghi et al., 2021)

Variable	CAD	NOCAD	*P*-value
Age (years)			0.033
<60	19 (12.7%)	33 (22%)	
≥60	131 (87.3%)	117 (78%)	
Gender			0.000
Male	116 (77.3%)	76 (50.6%)	
Female	34 (22.7%)	74 (49.3%)	
Hypertension			0.101
Yes	120 (80.5%)	108 (72.5%)	
No	29 (19.5%)	41 (27.5%)	
Diabetes			0.000
Yes	55 (36.9%)	12 (8%)	
No	94 (63.1%)	137 (92%)	
BMI			0.026
<25%	44 (33.9%)	63 (47.3%)	
≥25%	86 (66.1%)	70 (52.7%)	
Prev. neoplasia			0.558
Yes	16 (10.8%)	13 (8.8%)	
No	133 (89.2°%)	136 (91.2%)	
Dyslipidemia			0.000
Yes	93 (62.4%)	54 (36.2%)	
No	56 (37.6%)	95 (63.8%)	

Abbreviation: Prev. neoplasia, previous history of neoplasia.

Several polymorphisms were analyzed: rs10113 and rs7259810 in CaM3, rs7214723 in CaMKK1, rs1799983, rs2070744, rs1549758 and rs61722009 in NOS3.

The genotype distributions in the total cardiopathic population for the polymorphisms rs7259810 in CaM3, rs7214723 in CaMKK1, rs1549758 and rs61722009 in NOS3 were not in Hardy Weinberg Equilibrium (HWE); rs2070744 in NOS3 and rs10113 in CaM3 resulted to be slightly out from the HWE. On the contrary the genotype distributions for rs1799983 in NOS3 followed the HWE.
Regarding the population analyzed in Maastricht, no specific information as gender, age, BMI and other comorbidity are known since the tissue aorta samples, from which the DNA it has been isolated. This group was composed of 79 subjects that required a cardiac surgery, mainly affected by aneurysm without coronary artery disease.

2. Reference group

The reference group was identified on the 1000 genomes browser (via Ensembl), the European population was chosen as the best informative one.
The European reference group included in the 1000 Genomes data set; it is composed by 503 subjects from five different groups:

- CEU: population residing in Utah (U.S.A.) but with ancestors from Northern and Eastern Europe, made up 99 subjects
- FIN: Finnish population (Finland), made up 99 subjects;
- GBR: British population in England and Scotland (United Kingdom), made up 91 subjects;
- IBS: Iberian population (Spain), made up 107 subjects;
- TSI: Tuscan population (Italy) made up 107 subjects.

In Table 2 are reported, the analysis of the genotype relating to each polymorphism, divided by population groups, with the numbers and the frequencies of alleles and genotypes.

Table 2: Analysis of the genotype relating to the polymorphism rs7259810, rs10113, rs1799983, rs2070744, rs1549758 and rs7214723 from databank 1000 Genomes. The gene frequencies both of alleles, on the right, and of genotypes on the left in the European population is reported. Respectively, the total number of the allele and genotype is shown in brackets.

Population	Allele: frequency (count)		Genotype: frequency (count)		
rs7259810 in CaM3					
European (EUR)	T: 0.557 (560)	C: 0.443 (446)	T|T: 0.318 (160)	C|C: 0.205 (103)	C|T: 0.447 (240)
rs10113 in CaM3					
European (EUR)	T: 0.520 (523)	C: 0.480 (483)	T|T: 0.264 (133)	C|C: 0.225 (113)	C|T: 0.511 (257)
rs1799983 in NOS3					
European (EUR)	G: 0.656 (660)	T: 0.344 (346)	G|G: 0.443 (223)	T|T: 0.131 (66)	G|T: 0.425 (214)
rs2070744 in NOS3					
European (EUR)	C: 0.438 (441)	T: 0.562 (565)	C|C: 0.183 (92)	T|T: 0.306 (154)	C|T: 0.511 (257)
rs1549758 in NOS3					
European (EUR)	C: 0.659 (663)	T: 0.341 (343)	C|C: 0.451 (227)	T|T: 0.133 (67)	C|T: 0.416 (209)
rs7214723 in CaMKK1					
European (EUR)	T: 0.557 (560)	C: 0.443 (446)	T|T: 0.318 (160)	C|C: 0.205 (103)	C|T: 0.477 (240)

3. Genotypic and allelic frequencies

The genotypic and allelic frequencies of each genetic variant were analyzed.

The first comparison was made in order to evaluate the genetic frequencies between the total cardiopathic population and the European reference population. Subsequently, several other comparisons were made and analyzed including the groups of subjects with different cardiovascular diseases. Indeed, the total cardiopathic population was subdivided in four groups on the basis of them presenting CAD and AS (Figure 1). The 150 patients in the CAD group were

compared to other 150 patients in the NOCAD group. Among these 300 patients, there were 107 subjects with AS, 71 belonging to the NOCAD group and 36 to the CAD group, and 193 patients without AS, 79 belonging to the NOCAD group and 114 to the CAD group. The comparisons were performed between the CAD and NOCAD groups; between the AS and NOAS groups.

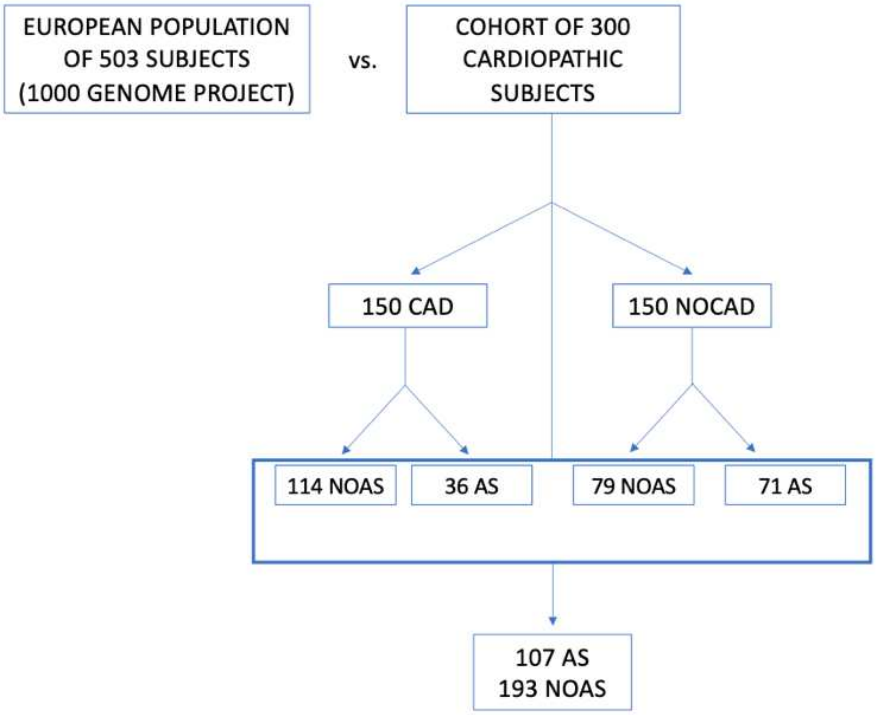


Figure 1: Schematic representation of the populations object of study (from Beghi et al., 2021)

Moreover, the distributions of the allelic frequencies of each polymorphism among the groups were analyzed. In this contest is important consider that within a population, the minor allelic frequency (MAF) is defined as the ratio between the frequency of the rarest variant and the most common variant of a specific SNP.

The comparisons of the allelic distributions were performed between the reference European group and the total cardiopathic population, between the CAD and NOCAD groups; and between the AS and NOAS groups.

Subsequently, the group of NOCAD was further stratified in two subgroups of NOCAD with AS and NOCAD without AS, in order to exclude CAD patient from the AS group. Since AS has the same pathophysiological basis as coronary heart disease, the NOCAD stratification can provide more

power in testing for the phenotypic association with each SNP involved in this study. In this stratification, both the genotypic and allelic frequencies were analyzed. Among the 150 non coronary patients, 79 without AS (NOAS) and 71 with AS.

The results of each polymorphism are reported in the next paragraphs.

4. Polymorphism analyzed in CaM3

4.1 SNP rs7259810

4.1.1 Genotypic frequencies

In the comparison between the total cardiopathic population and the European reference population, the frequency distribution of all the genotypes is the same: TC>CC>TT, but it is important to highlight that on one hand there is a higher frequency of the homozygous TT in the cardiopathic population compared to the European reference group (EUR TT= 18%; Cardiopathic population TT= 24%) and on the other hand a lower frequency of the homozygous CC in the cardiopathic population compared to the European reference group (EUR CC= 33%; Cardiopathic population CC= 29%) (Figure 2A).

Anyway, chi square test did not reveal any statistical significance in the genotype distribution between the two groups (*P*=0.12).

Regarding the comparisons among the subgroups, the NOCAD and the NOAS group have the same frequency distribution trend (CT>CC>TT) of the total cardiopathic population, instead the CAD and AS subgroups show different frequencies in the homozygous genotype. In the CAD subgroup the frequency of the TT and CC is the same (TT= 27%, CC=27%), instead, in the AS subgroup it is important observe that the frequency of the CC allele is the highest one compared to all the other groups (Figure 2B).

Anyway, chi-square test performed between CAD and NOCAD groups and between AS and NOAS groups did not reveal any statistical difference regarding the distribution of genotypes with P=0,34, and P=0,49, respectively.

4.1.2 Allelic frequencies

The polymorphism rs7259810 appears to be not very common in the population: the 1000 Genomes database reports a MAF index T=0.3293 (1649/5008, 1000G) and it always consider the T allele as the ancestral allele.

In the reference populations group (European), in the total cardiopathic population and in the CVDs subgroups, the frequency of the C allele is higher compared to T allele, except for the CAD subgroups in which the two alleles have the same frequency (Figure 2C). Moreover, it is interesting observe two aspects: the first one is that the allelic frequencies of the NOCAD subgroup are the more similar to the European reference group (EUR T=42% C= 58%; Cardiopathic population T=44% C=56%), the second aspect is that the allelic frequency are the same among the cardiopathic population, the AS and NOAS subgroups.

Chi square test performed between EUR and the Cardiopathic population resulted on the border of significance (P=0,06) in the allelic distribution. On the contrary, the comparison between CAD and NOCAD (P=0,14) and between AS and NOAS (P=0,96) did not reveal any statistical difference in the allelic distribution.

4.1.3 NOCAD stratification

In the stratification inside the group of NOCAD, chi-square test performed between NOCAD with AS and NOCAD with AS revealed no statistical difference regarding the distribution of the genotypes (P=0,97) and alleles (P=0,99) (Figure 2D-E).

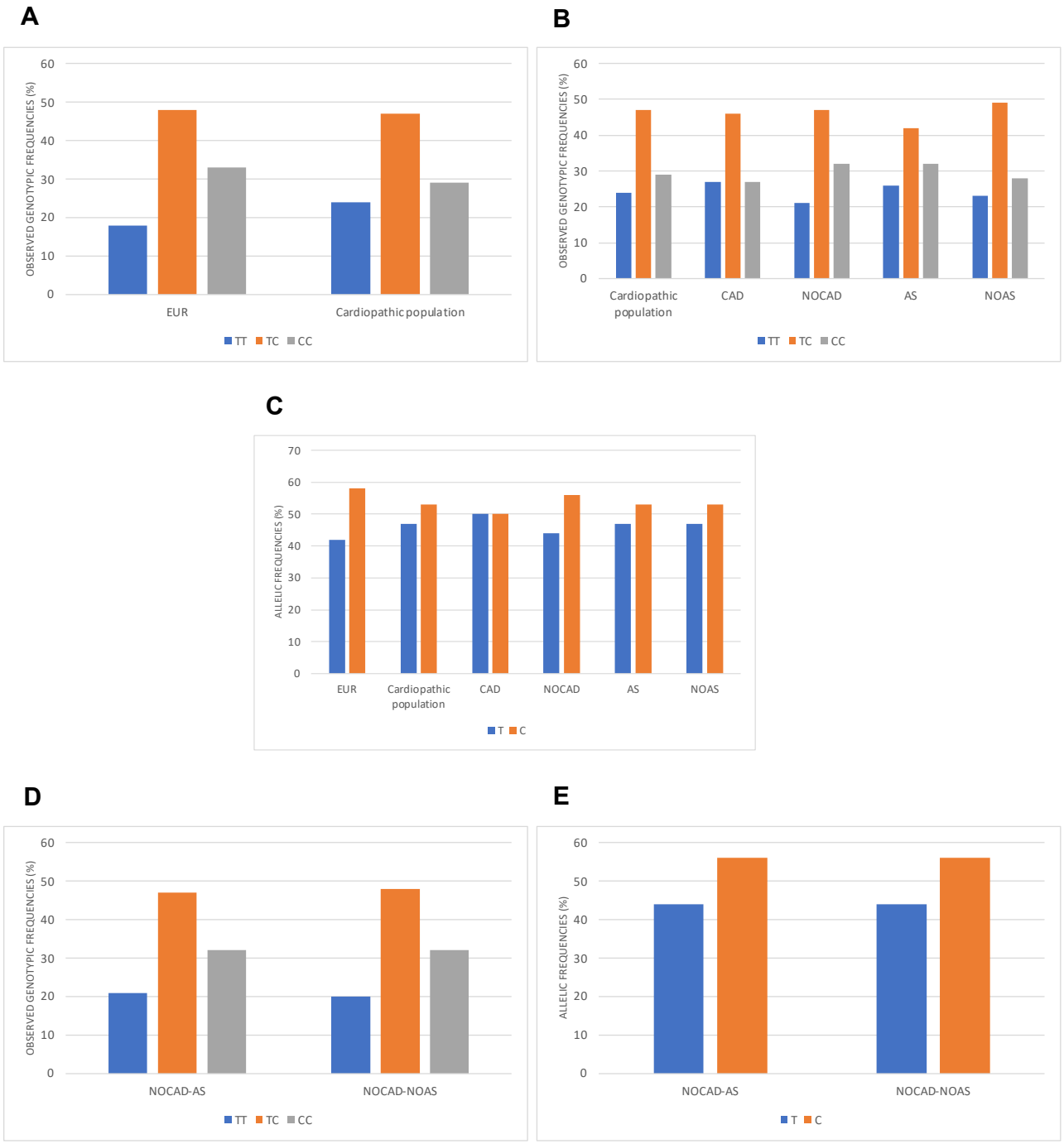


Figure 2: Histogram representation of the genotypic and allelic frequencies of the SNP rs7259810 in CaM3 among the different group of population considered in this study. A) Observed genotypic frequency's distribution in the European population and the total cardiopathic population; **B)** Observed genotypic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **C)** Allelic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **D-E)** Observed genotypic and allelic frequency's distribution in the stratification of NOCAD-AS and NOCAD-NOAS.

4.2 SNP rs10113

4.2.1 Genotypic frequencies

In the analysis of the genotypic frequency's distribution between the reference group and the cardiopathic population, it is observed a similar trend with a higher number of the heterozygous TC, followed by the homozygous TT and by the homozygous CC (Figure 3A).

If in the European population the difference between the two homozygous is not so high (TT=26%; CC=23%), on the contrary in the cardiopathic population there are a higher number of the homozygous TT compared to the CC (TT=29%; CC=16%).

Chi square test performed between EUR and the Cardiopathic population resulted on the border of significance (P=0,08) for the genotypic distribution.

In the comparisons between the subgroups (CAD, NOCAD, AS and NOAS), it is interesting to observe that the CAD group has the lower frequency of the genotype CC, even if the genotypic distribution trend is the same among all the subgroups (TC>TT>CC) (Figure 3B).

The NOCAD and the NOAS group have the same frequency distribution trend (CT>CC>TT) of the total cardiopathic population, instead the CAD and AS subgroups show different frequencies in the that the frequency of the CC allele is the highest one compared to all the other groups.

Anyway, chi-square test performed between CAD and NOCAD groups and between AS and NOAS groups did not reveal any statistical difference regarding the distribution of genotypes with P=0,37, and P=0,44, respectively.

4.2.2 Allelic frequencies

The polymorphism rs10113 appears to be not very common in the population: the 1000 Genomes database reports a MAF index T=0.4986 (2497/5008, 1000G)) and it consider always the T allele as the ancestral allele.

Regarding the allelic frequencies, in the reference group the T and C alleles results to have almost the same frequency, on the contrary in the subgroups it is more frequent the T allele compared to the C (Figure 3C).

Chi square test performed between EUR and the Cardiopathic population resulted on the border of significance (P=0,09) in the allelic distribution. On the contrary, the comparison between CAD and NOCAD (P=0,62) and between AS and NOAS (P=0,55) did not reveal any statistical difference in the allelic distribution.

4.2.3 NOCAD stratification

In the stratification inside the group of NOCAD, chi-square test performed between NOCAD with AS and NOCAD with AS revealed no statistical difference regarding the distribution of the genotypes (P=0,73) and alleles (P=0,57) (Figure 3D-E).

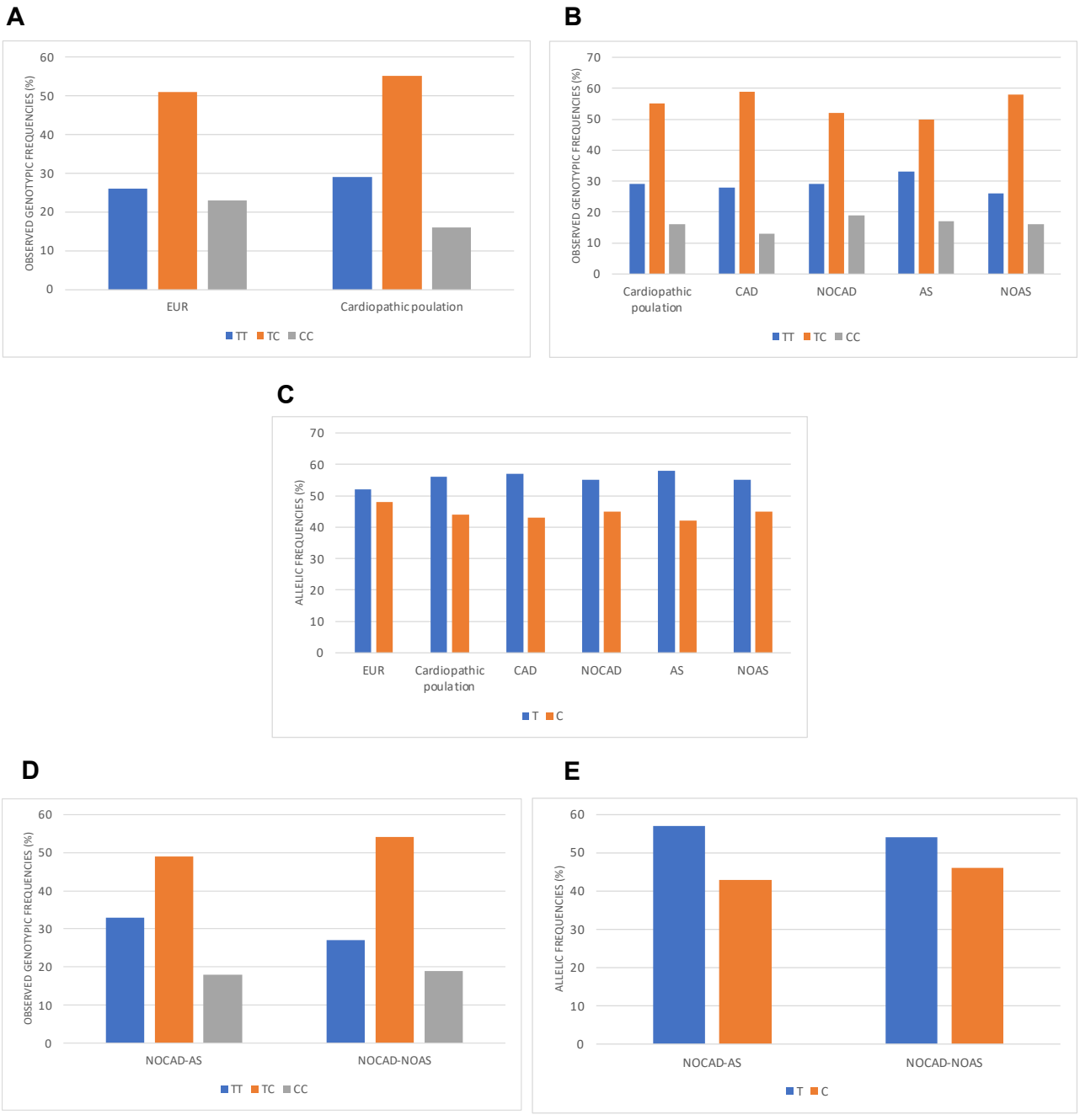


Figure 3: Histogram representation of the genotypic and allelic frequencies of the SNP rs10113 in CaM3 among the different group of population considered in this study. A) Observed genotypic frequency's distribution in the European population and the total cardiopathic population; **B)** Observed genotypic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **C)** Allelic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **D-E)** Observed genotypic and allelic frequency's distribution in the stratification of NOCAD-AS and NOCAD-NOAS.

5. Polymorphism analyzed in NOS3

5.1 SNP rs1799983

5.1.1 Genotypic frequencies

In the analysis of the genotypic frequencies between the total cardiopathic population and the European reference population, it is interesting to observe that the genotypic distributions are the same between the cardiopathic population and the European reference group (Cardiopathic population GG = 43%; TT= 12%; GT= 45%; European group GG = 44%; TT= 13%; GT= 43%) (Figure 4A).

No statistically genotypic differences were detected.

Also, the chi-square test performed between CAD and NOCAD groups and AS and NOAS groups did not reveal any statistical difference regarding the distribution of genotypes (Figure 4B).

5.1.2 Allelic frequencies

The polymorphism rs1799983, appears to be not very common in the population: the 1000 Genomes database reports a MAF index T = 0.1763/5008 and the G allele as the ancestral allele.

The comparison of the allelic frequencies shows the same distribution of the G and T allele between the entire cardiopathic population and the European reference population (Figure 4C).

Also, in all the subgroups (CAD; NOCAD; AS; NOAS) alleles were present with the same frequencies: the T allele remain the minor frequent allele.

Any statistical difference was found in the distribution of the allele between the different group. (EUR vs Cardiopathic population: P=0,85; CAD vs NOCAD P=0,1; AS vs. NOAS P=0,79).

5.1.3 NOCAD stratification

In the stratification inside the group of NOCAD, the Chi-square test performed between NOCAD with AS and NOCAD with AS did not reveal statistical difference regarding the genotype (P=0,76) and allele (P=0,79) distribution (Figure 4D-E).

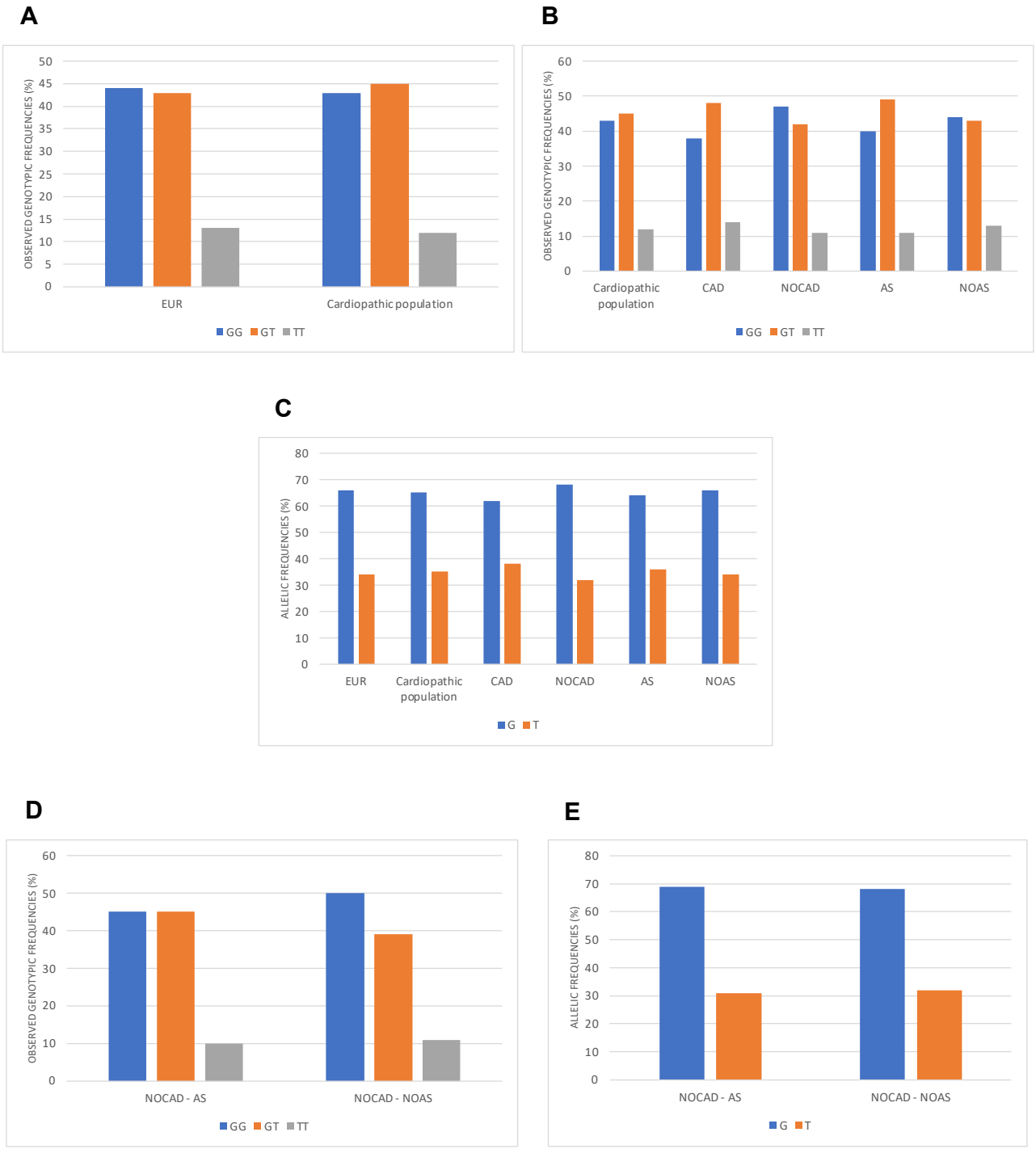


Figure 4: Histogram representation of the genotypic and allelic frequencies of the SNP rs1799983 in NOS3 among the different group of population considered in this study. A) Observed genotypic frequency's distribution in the European population and the total cardiopathic population; **B)** Observed genotypic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **C)** Allelic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **D-E)** Observed genotypic and allelic frequency's distribution in the stratification of NOCAD-AS and NOCAD-NOAS.

5.2 SNP rs2070744

5.2.1 Genotypic frequencies

The genotypic frequencies compared between the total cardiopathic population, and the European reference population did not result to be statistically different (P=0,34). Indeed, it is possible observe that distributions of the genotype are the same between the cardiopathic population and the European reference group (Cardiopathic population CC = 14%; TT= 32%; CT= 54%; European group CC = 18%; TT= 31%; GT= 51%) (Figure 5A).

Chi-square test performed between CAD and NOCAD resulted to be slightly statistically significant (P=0,046) in the distribution of the genotypic frequency, chi-square test between AS and NOAS groups did not reveal any statistical difference regarding the distribution of genotypes (P=0,88). Anyway, it is interesting observe that in the NOCAD group there is the higher percentage of heterozygous. (NOCAD CT: 60%; CAD CT: 47%; AS CT: 54%; NOAS: %54) (Figure 5B).

5.2.2 Allelic frequencies

The polymorphism rs2070744, appears to be not very common in the population: the 1000 Genomes database reports a MAF index C = 0.2344 (1174/5008) and always the C allele is reported as the ancestral one.

Allelic frequencies comparison confirms the genotype frequencies with the same trend for the C and T allele between the entire cardiopathic population and the European reference population (Figure 5C).

Also, in all the subgroups (CAD; NOCAD; AS; NOAS), alleles were present at the same frequencies: the C allele remains the minor frequent allele. No statistical difference was found among the groups. Any statistical difference was found in the distribution of the allele between the different group.(EUR vs Cardiopathic population: P=0,32; CAD vs NOCAD P=0,86; AS vs. NOAS P=0,79).

5.2.3 NOCAD stratification

In the stratification inside the group of NOCAD, chi-square test performed between NOCAD with AS and NOCAD with NOAS revealed no statistical difference regarding the distribution of the genotypes (P=0,29) and alleles (P=0,85) (Figure 5D-E).

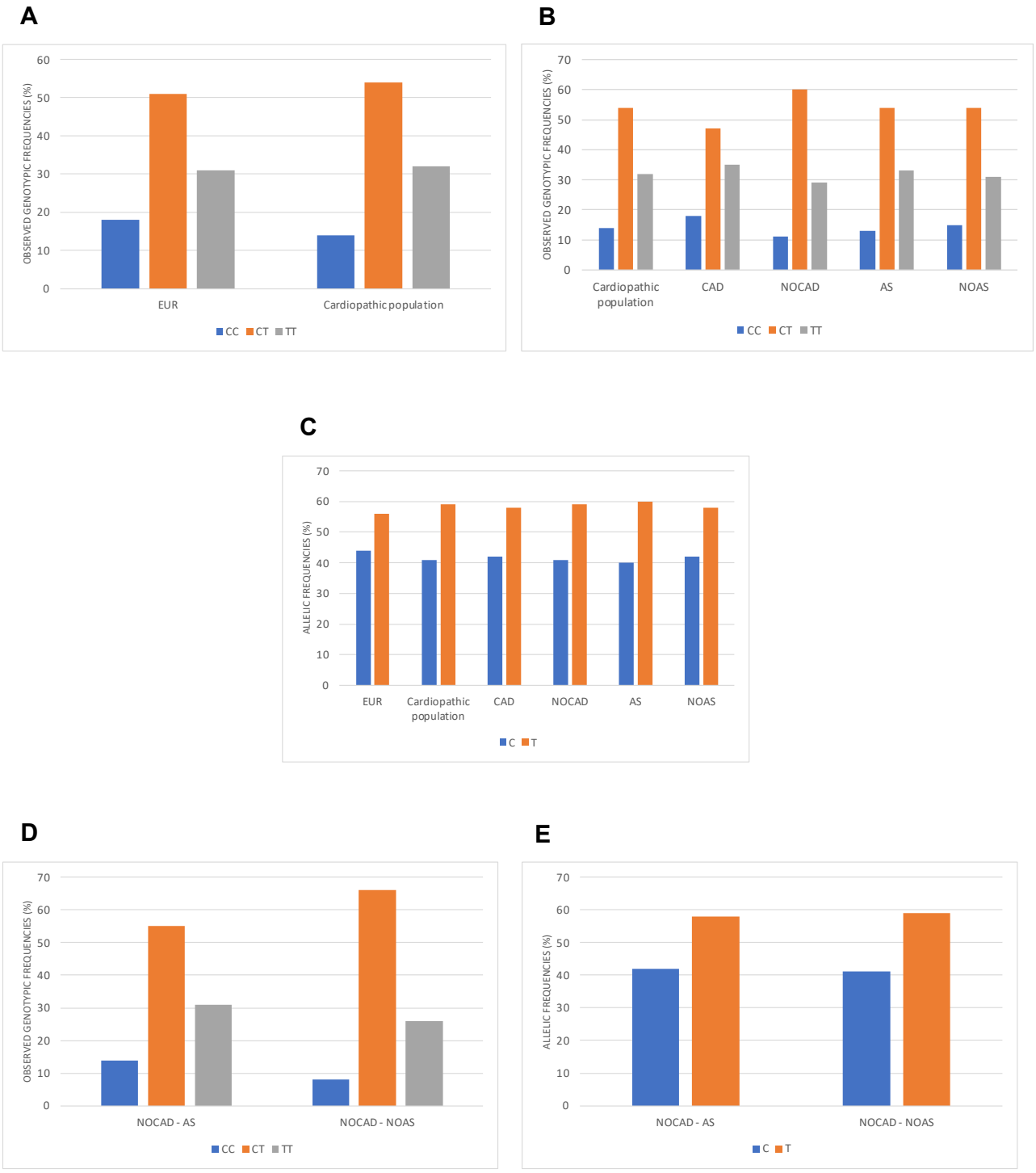


Figure 5: Histogram representation of the genotypic and allelic frequencies of the SNP rs2070744 in NOS3 among the different group of population considered in this study. A) Observed genotypic frequency's distribution in the European population and the total cardiopathic population; **B)** Observed genotypic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **C)** Allelic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **D-E)** Observed genotypic and allelic frequency's distribution in the stratification of NOCAD-AS and NOCAD-NOAS.

5.3 SNP rs1549758

5.3.1 Genotypic frequencies

In the genotypic frequencies comparison between the total cardiopathic population and the European reference population, it is interesting observe that the heterozygotes were more common in the total cardiopathic group than in the reference group (Cardiopathic population TC = 60%; European TC = 42%;) (Figure 6A).

Moreover, in the reference European population, the frequency of the two homozygous was higher compared to the cardiopathic population (EUR: CC= 45%; TT = 14%, Cardiopathic population: CC= 36%; TT= 4%)

It is clearly interesting observe that in the Cardiopathic population the frequency of the TT is almost absent.

A significant difference in the genotype distribution between the two groups was detected (P<0.000).

Regarding the comparison among the subgroups, both CAD, NOCAD, AS and NOAS have the same frequency distribution trend (CT>CC>TT) of the total cardiopathic population, but it is important observe that in the NOCAD subgroups the TT genotype disappears (Figure 6B).

Chi-square test performed between CAD and NOCAD groups resulted to be statistical different (P=0,0001). On the contrary chi square test did not reveal any statistical difference regarding the distribution of genotypes between the AS and NOAS groups (P=0,4). It is interestingly observed that the distributions of all the three genotype is similar between the NOCAD and NOAS group and between the CAD and AS group.

5.3.2 Allelic frequencies

The polymorphism rs1549758, appears to be not very common in the population: the 1000 Genomes database reports a MAF index T = 0.1823 (913/5008) and the C allele as the ancestral allele.

Allelic frequencies comparison confirms the genotype frequencies, with the same trend in the C and T allele between the entire cardiopathic population and the European reference population (Figure 6C).

Also, in all the subgroups (CAD; NOCAD; AS; NOAS) the T allele remain the minor frequent allele, with the minor frequency in the NOCAD subgroup (T= 31%) and the higher one in the CAD subgroup (T=37%).

Chi square test performed between EUR and the Cardiopathic population (P=0,96) and between AS and NOAS (P=0,44) did not reveal any statistical difference in the allelic distribution. On the other hand, the comparison between CAD and NOCAD resulted on the border of significance (P=0,08).

5.3.3 NOCAD stratification

In the stratification inside the group of NOCAD, chi-square test performed between NOCAD with AS and NOCAD with AS revealed no statistical difference regarding the distribution of the genotypes (P=0,13) and alleles (P=0,26) (Figure 6D-E).

Anyway, it is interesting observe that in this stratification inside the NOCAD group are not present patient with the genotype.

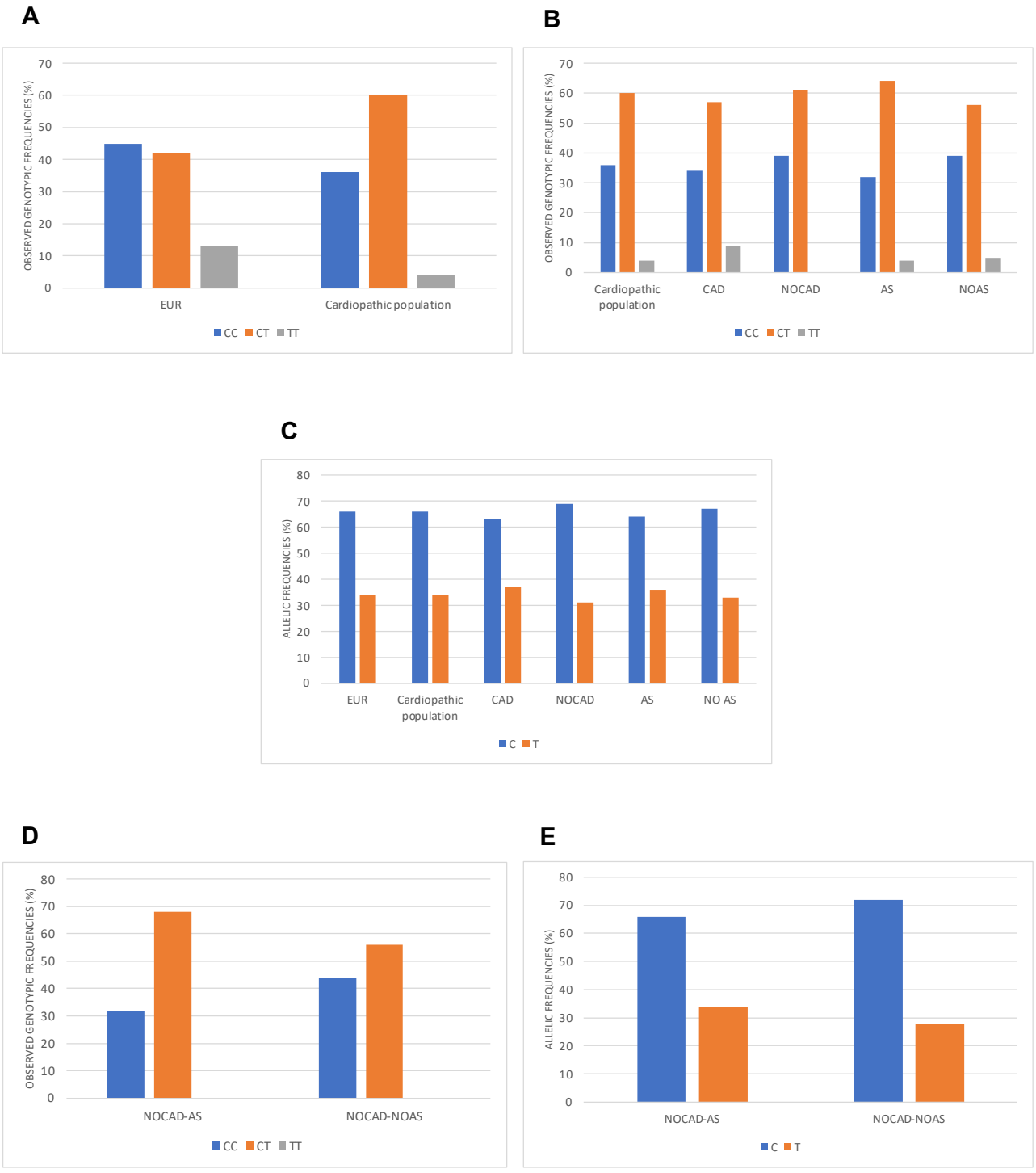


Figure 6: Histogram representation of the genotypic and allelic frequencies of the SNP rs1549758 in NOS3 among the different group of population considered in this study. A) Observed genotypic frequency's distribution in the European population and the total cardiopathic population; **B)** Observed genotypic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **C)** Allelic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **D-E)** Observed genotypic and allelic frequency's distribution in the stratification of NOCAD-AS and NOCAD-NOAS.

5.4 VNTR rs61722009

5.4.1 Genotypic frequencies

For this genetic variant, it was not possible compare the genotypic frequencies of the total cardiopathic population with a reference group, since the genotypic frequencies for the VNTR rs61722009 for the European population were not included in the 1000 Genomes Browser.

Considering this aspect, the comparison was performed between the CAD and NOCAD groups; and between the AS and NOAS groups (Figure 7A).

Chi-square test performed between CAD and NOCAD resulted statistical difference regarding the distribution of genotypes (P=0,03); on the contrary any statistical difference was found between AS and NOAS groups (P=0,25).

5.4.2 Allelic frequencies

In absence of the European reference group, the allelic frequencies were calculated in the CVDs subgroups (CAD; NOCAD; AS; NOAS).

Chi-square did not find any statistical difference in the allelic distribution between CAD and NOCAD groups (P= 0,39) and between AS and NOAS groups (P=0,98) (Figure 7B).

Anyway, it is interesting to observe that in all the four subgroups the 4b allele was more common compared to the 4a allele. Moreover, the percentage of the two allele 4b and 4a has the same distribution in the AS and NOAS (AS 4b: 63%; 4a: 37%; NOAS 4b: 63%; 4a: 37%).

5.4.3 NOCAD stratification

In the stratification inside the group of NOCAD, chi-square test performed between NOCAD with AS and NOCAD with AS did not reveal any statistical difference regarding the distribution of the genotypes (P=0,8) and alleles (P=0,73) (Figure 7C-D).

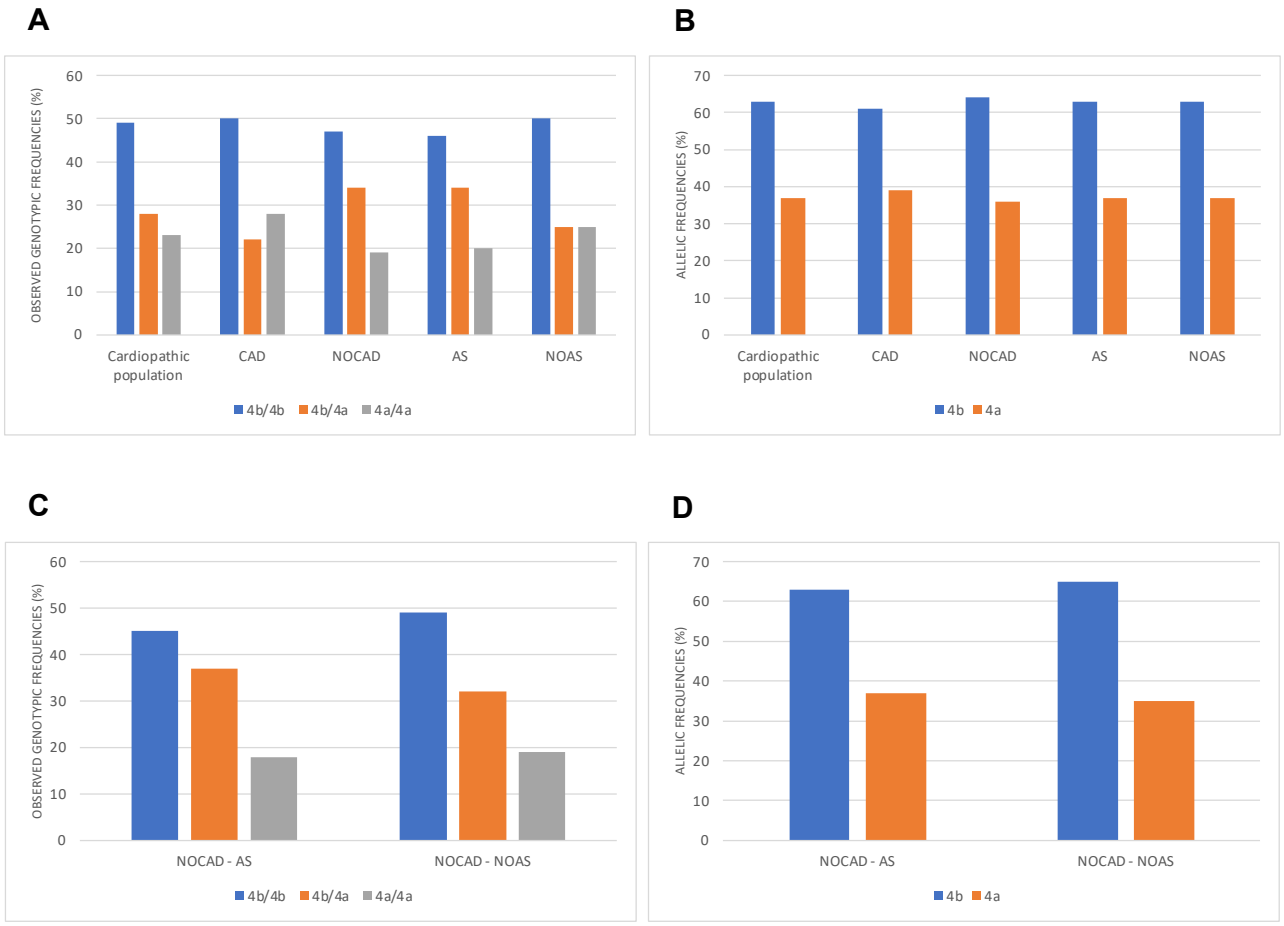


Figure 7: Histogram representation of the genotypic and allelic frequencies of the VNTR rs61722009 in NOS3 among the different group of population considered in this study. **A)** Observed genotypic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **B)** Allelic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **C-D)** Observed genotypic and allelic frequency's distribution in the stratification of NOCAD-AS and NOCAD-NOAS.

6. Polymorphism analyzed in CaMKK1

6.1 SNP rs7214723

6.1.1 Genotypic frequencies

In the analysis of the genotypic frequencies between the total cardiopathic population and the European reference population (Figure 8A), it is interesting observe that the heterozygotes were more common in the cardiopathic than the reference group (Cardiopathic population TC = 64%; European TC = 48%;).

Moreover, in the reference European population, the frequency of the ancestral TT genotype was higher than the CC genotype (TT = 32%; CC = 20%), on the contrary in the total cardiopathic population was observed a lower percentage of TT and a higher percentage of CC (TT = 11%; CC = 25%) .A significant difference in the distribution of the genotypes between the two groups was detected (*P*=0.000).

The comparisons among the subgroups did not revealed particularly difference in the distribution of the genotype.The Chi-square test performed between CAD and NOCAD groups and AS and no aortic stenosis (NOAS) groups did not reveal any statistical difference regarding the distribution of genotypes (CAD vs NOCAD, *P*=0.225; AS vs NOAS, *P*=0.08), but it interesting to observe that the distributions of all the three genotypes are more similar between the NOCAD and NOAS group and between the CAD and AS group (Figure 8B).

For the SNP rs7214723 in CaMKK1 a further comparison was performed.

Genotypic frequencies between the cardiopathic group of Maastricht were analyzed and subsequently compared with the total cardiopathic group of 300 subjects and the European reference group (Figure 8D). The Maastricht group presents genotypic trend a similar to that the total cardiopathic group, with a higher percentage of the heterozygous TC, followed by the homozygous CC and TT (TC= 50%; CC= 29%; TT= 21%).Chi square test performed between EUR and Maastricht population for the genotypic distribution resulted on the border of significance (P=0,09), probably this is due the poor number of patients in the Maastricht group.

6.1.2 Allelic frequencies

The polymorphism rs7214723, appears to be common in the population: the 1000 Genomes database reports a MAF index C = 0.3954/1980 and the T allele as the ancestral allele.

The allelic frequencies were therefore calculated both in the reference populations group (European), in the total cardiopathic population and in the CVDs subgroups.

Allelic frequencies comparison confirms the genotype frequencies with an opposite trend in the T and C allele between the entire cardiopathic population sample and the European reference population (Figure 8C).

Chi-square test performed between the reference group and the total cardiopathic population reveal a statistical difference regarding the distribution of the allele (P=0,000).

In the reference European population, the ancestral T allele was the predominant one, while in the total cardiopathic population, and in all the other subgroups (CAD; NOCAD; AS; NOAS) the C allele was the most frequent, especially in the non-CAD and non-AS subgroups.

Chi-square test performed between CAD and NOCAD groups did not reveal any statistical difference regarding the distribution of the alleles (P=0,21). On the other hand, the comparison between AS and NOAS groups resulted on the border of significance (P=0,06).

Regarding the Maastricht populations, the allelic frequencies showed the same trend of the total cardiopathic population with a higher percentage of the C allele compared to the T (T=46%; C=54%), thus again an opposite trend from the European reference group (T=55%; C=45%). Chi square test revealed statistical significance in the allelic distribution between the Maastricht population and the European reference group (P=0,02) (Figure 8E).

6.1.3 NOCAD stratification

In the NOCAD stratification, through a Chi-square test, the distribution resulted statistical different both for the genotypic (*P*=0.02) and allelic (*P*=0.05) distributions, the C allele and CC homozygotes were more frequent in patients NOCAD with no AS compared with patients NOCAD with AS (Figure 8F-G).

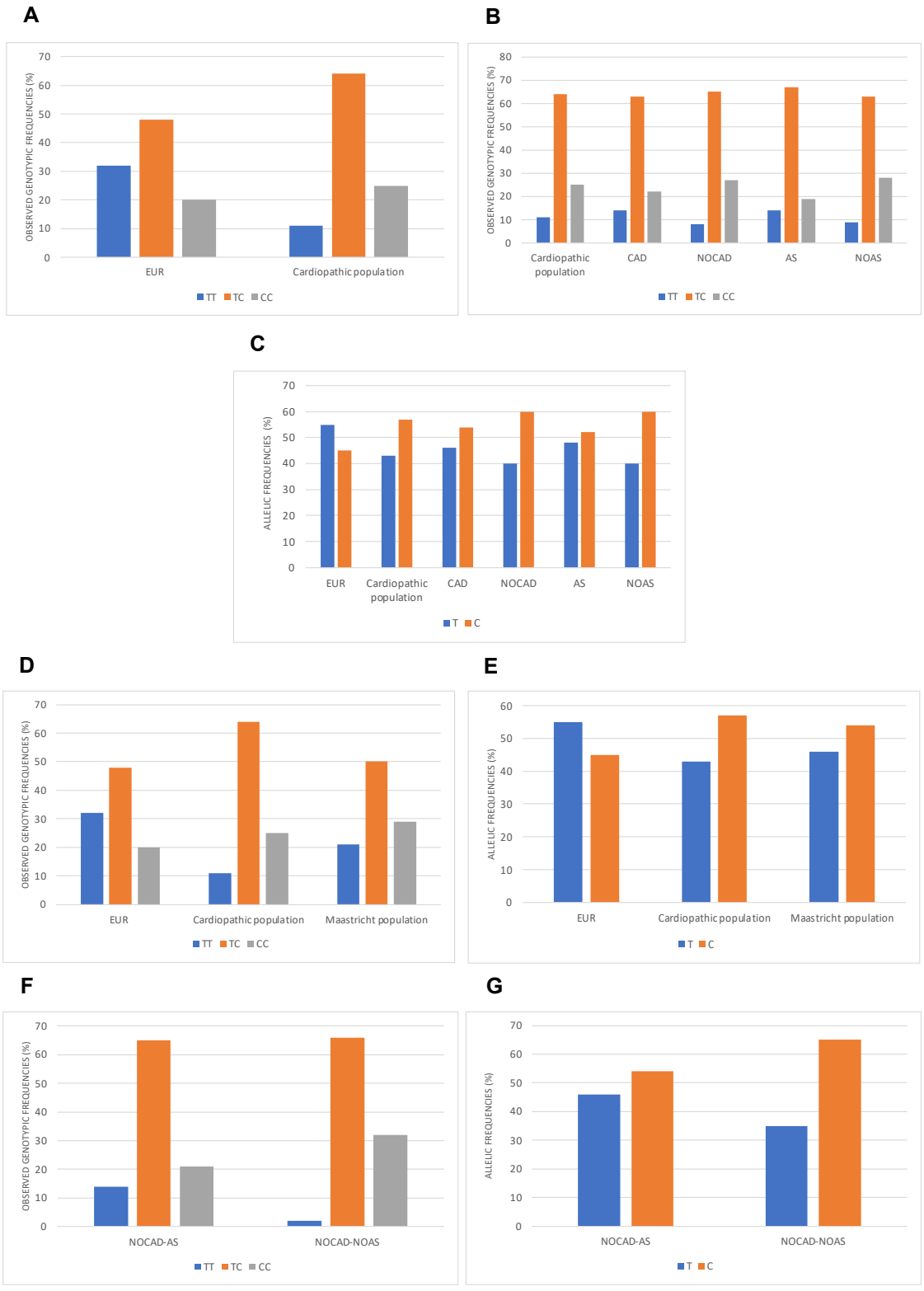


Figure 8: Histogram representation of the genotypic and allelic frequencies of the SNP rs7214723 in CaMKK1 among the different group of population considered in this study. A) Observed genotypic frequency's distribution in the European population and the total cardiopathic population; **B)** Observed genotypic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **C)** Allelic frequency's distribution in the total cardiopathic population and in the 4 subgroups (CAD; NOCAD; AS; NOAS); **D-E)** Observed genotypic and allelic frequency's distribution in the European population, in the total cardiopathic Italian population and in the Maastricht population; **F-G)** Observed genotypic and allelic frequency's distribution in the stratification of NOCAD-AS and NOCAD-NOAS.

Considering the interesting results obtained of the polymorphism rs7214723 in CaMKK1, it was also performed a logistic regression analysis, in order to test a possible direct association between the SNP and the higher risk to develop coronary artery disease (CAD vs NOCAD) and aortic stenosis (AS vs. NOAS).

Logistic regression analyzes simultaneously the influence of independent variables (genotype, sex, age, diabetes, hypertension, BMI, and previous history of neoplasia) on dependent variable (the risk of developing CAD and AS). The tables, present in the paper of Beghi et al. (2021)[147] in the appendix section, show the frequencies of the genotypes for rs7214723 polymorphism for CAD/NOCAD and AS/NOAS.

Three different genetic models were analyzed: additive model (TT; TC; CC), dominant model (CC+TC versus TT), and recessive model (CC versus TC+TT), considering C as the minor allele (C) in accordance with the frequency reported in the 1000 Genomes Database.

The logistic regression analysis reported not a significant association between the rs7214723 polymorphism and the risk of CAD and AS independently of the model taken in consideration (additive, recessive, and dominant)[147].

On the other hand, the CaMKK1 rs7214723 polymorphism in male participants resulted positively associated with the increased risk of CAD in all the three models, additive: OR = 4.018 (2.210–7.305), *P*=0.000); recessive: OR = 3.73 (2.074–6.717), *P*=0.000; dominant: OR = 4.019 (2.212–7.305), *P*=0.000). Similarly, non-diabetic patients and patients under 60 years showed lower risk to develop CAD irrespective of the model taken in consideration, but no associations were found for hypertension, BMI, and previous history of neoplasia[147]. Male participants showed positive association when the rs7214723 polymorphism was tested for association with the risk of AS. On the other hand, none of the other variables showed significant results.

7. Synthetic phenotype in hVSMCs is associated with increased expression of CaMKK1

First, we set out to evaluate whether CaMKK1 (calcium calmodulin kinase kinase 1) expression changes due to VSMC phenotype switching *in vitro*. hVSMCs were treated with heparin or medium with low concentration of FBS to induce a contractile phenotype and with PDGF-BB to induce a synthetic phenotype, an established model of VSMC phenotype switching *in vitro*[249]. Expression was analysed by real-time PCR, RNAseq and western blotting for CaMKK1 as well as for markers specific for the contractile VSMC phenotype, i.e CNN-1 (calponin 1), MYH11 (myosin heavy chain 11), SMTN (smoothelin), α-SMA (α-smooth muscle actin), SM22-α (smooth muscle protein 22α), pMLC (phosphorylated myosin light chain) and for the synthetic VSMC phenotype, i.e S100A4 (S100 calcium binding protein A4). was analyzed.

While qPCR results show no changes in gene expression (Supplementary Figure 1A-E), RNAseq data showed decreased expression of SM22α and MLC in both PDGF- and heparin-treated cells (Figure 9A-B), as well as higher expression of CaMKK1 in these conditions (Figure 9C). However, no changes in MYH11 and αSMA expression were observed (Supplementary Figure 1F-G).

Western blotting results confirmed phenotype switching of VSMCs treated with heparin and PDGF (Figure 9D-L). Indeed, synthetic VSMCs showed decreased expression of contractile markers CNN-1, α-SMA, SM22-α, and pMLC and increased expression in synthetic marker S100A4 compared with contractile VSMCs. Expression of CaMKK1 was higher in synthetic VSMCs compared to untreated and contractile cells, but no difference was observed between contractile and untreated VSMCs. These results suggest that CaMKK1 either plays a role in regulating phenotype switching or is affected by VSMC phenotype switching.

Since we have shown before that polymorphism rs7214723 in CaMKK1 in a cohort of 300 cardiopathic subjects is associated with a higher susceptibility to cardiovascular disease, we hypothesized that the genotype of CaMKK1 could play a role in regulating VSMC phenotype. Therefore, we genotyped the SNP rs72142723 in the CaMKK1 gene in VSMCs isolated from 7 different donors used for these experiments (Figure 9E-L, see color coding legend). Among this group, 3 were homozygous TT, 2 homozygous CC and 3 heterozygous TC. Interestingly, the patients with the genotype CC are the only VSMCs among the three genotype to have a higher expression of CaMKK1 in contractile cells, suggesting that the genotype variation might be important for regulating CaMKK1 expression under phenotypic switching conditions. However, a

statistical test could not be performed to confirm this association due to the low donor number. Any differences between genotypes were not apparent in VSMC phenotype markers analyzed.

We also examined CaMKK1 expression under calcifying conditions, which is a more extreme form of phenotype switching and found no differences, while expression of osteocalcin (OCN) was increased, indicative of osteogenic differentiation (Supplementary Figure 2A-B). This suggests that CaMKK1 is not involved in osteogenic differentiation of VSMCs.

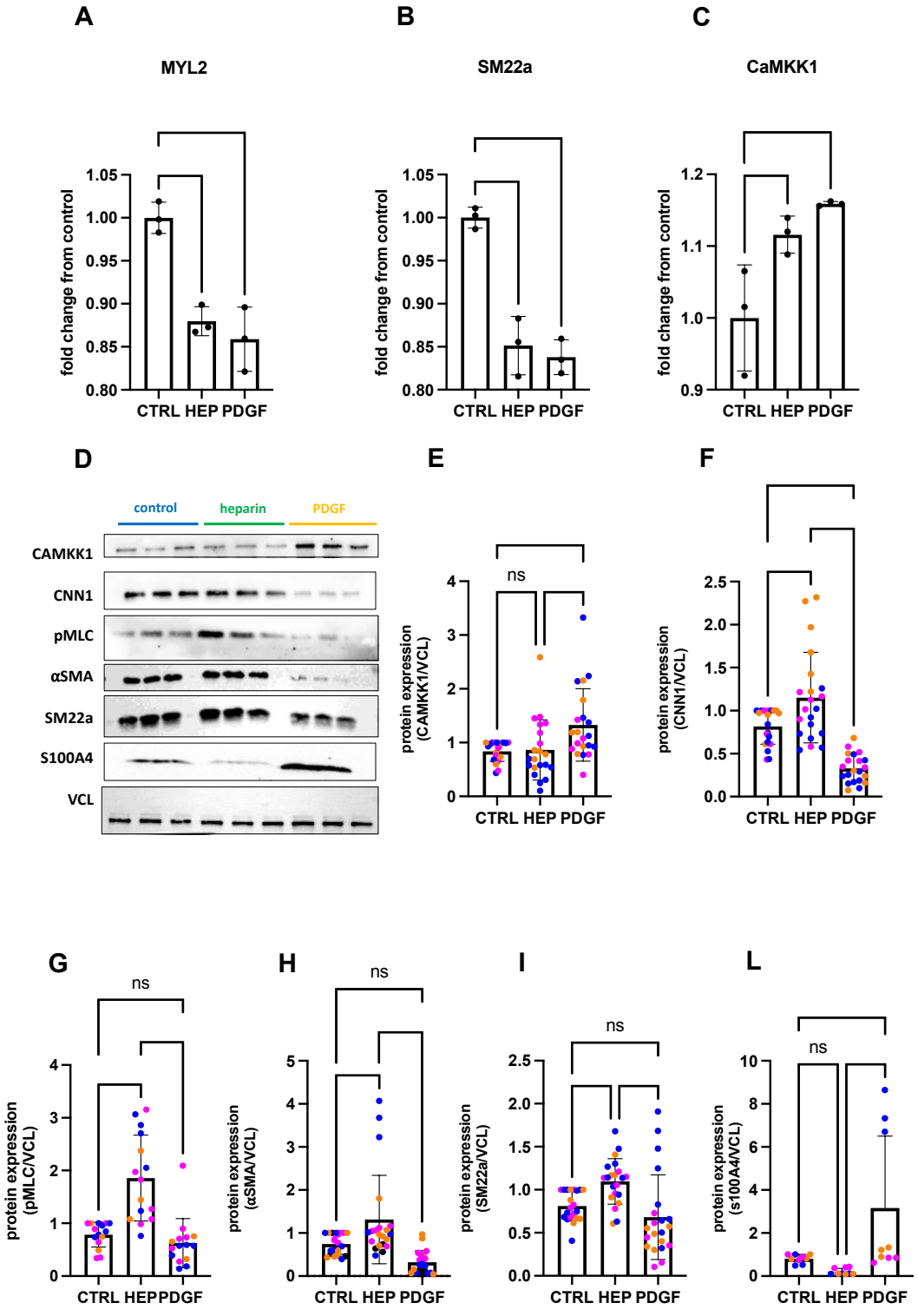


Figure 9: Phenotypic switching of hVSMCs is associated with changes in contractile gene and CaMKK1 expression. hVSMCS were treated with PDGF-BB (10 µg/ml) and heparin (200 U/ml) in M199 with 2.5% FBS for seven days. **A-B)** Contractile markers MYL2 (MLC) , SM22a and **C)** CAMKK1 expression analysed in an RNA microarray. **D)** Western blot and **E-L)** quantification of CaMKK1, contractile proteins (CNN1, pMLC, α-SM-actin, SM22α) and synthetic marker S100A4. The results confirm phenotypic switching of hVSMC and show higher expression of CaMKK1 in hVSMC treated with PDGF. Graphs show pooled data from 3 to 7 independent experiments performed in triplicate. Colored dots indicate the genotypes associated with the polymorphism rs7214723 in CaMKK1: in blue the homozygous TT, in pink the homozygous CC, in orange the heterozygous TC. Statistical analysis was performed using GraphPad Prism 9. Statistical significance was tested using One-Way ordinary ANOVA; ns - not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

8. CaMKK1 promotes the synthetic phenotype of VSMCs

To further elucidate the link between CaMKK1 and VSMC phenotype we performed siRNA knockdown of CaMKK1 (Figure 10A-G, Supplementary Figure 3A contains uncropped blots). Western blotting confirmed that siRNA knockdown of CaMKK1 was successful in cells treated with heparin, low FBS and PDGF. CAMKK1 knockdown caused an increase in CNN1, αSMA and SM22α at baseline (Figure 10A-G), but not in heparin-, low FBS- or PDGF- treated cells. CaMKK1 knockdown had no effect on the expression of pMLC and synthetic marker S100A4. This suggests that CaMKK1 promotes a synthetic VSMC phenotype, as absence of CaMKK1 increased contractile marker expression.

Changes in EV release has been reported in VSMCs undergoing phenotype switching. Synthetic VSMCs secrete more whereas contractile VSMCs secrete fewer EVs than a mixed population of untreated cells[250]. Therefore, we quantified EVs secreted by hVSMC treated for phenotype switching (control; low FBS; PDGF) in the context of siRNA knockdown of CaMKK1. Our data show that CaMKK1 knockdown results in a trend of lowering EV release in low FBS- and PDGF-treated VSMCs, which did not reach statistical significance (Figure 10H). This is consistent with the western blot findings, in which absence of CaMKK1 decreased EV release, a phenomenon associated with the contractile VSMC phenotype.

Changes in intracellular calcium are another hallmark of hVSMC phenotype switching[249]. Therefore, we analyzed intracellular calcium in a set of experiments. Intracellular calcium measurement showed a trend of higher intracellular calcium in VSMCs knockdown for CaMKK1 (Supplementary Figure 3B).

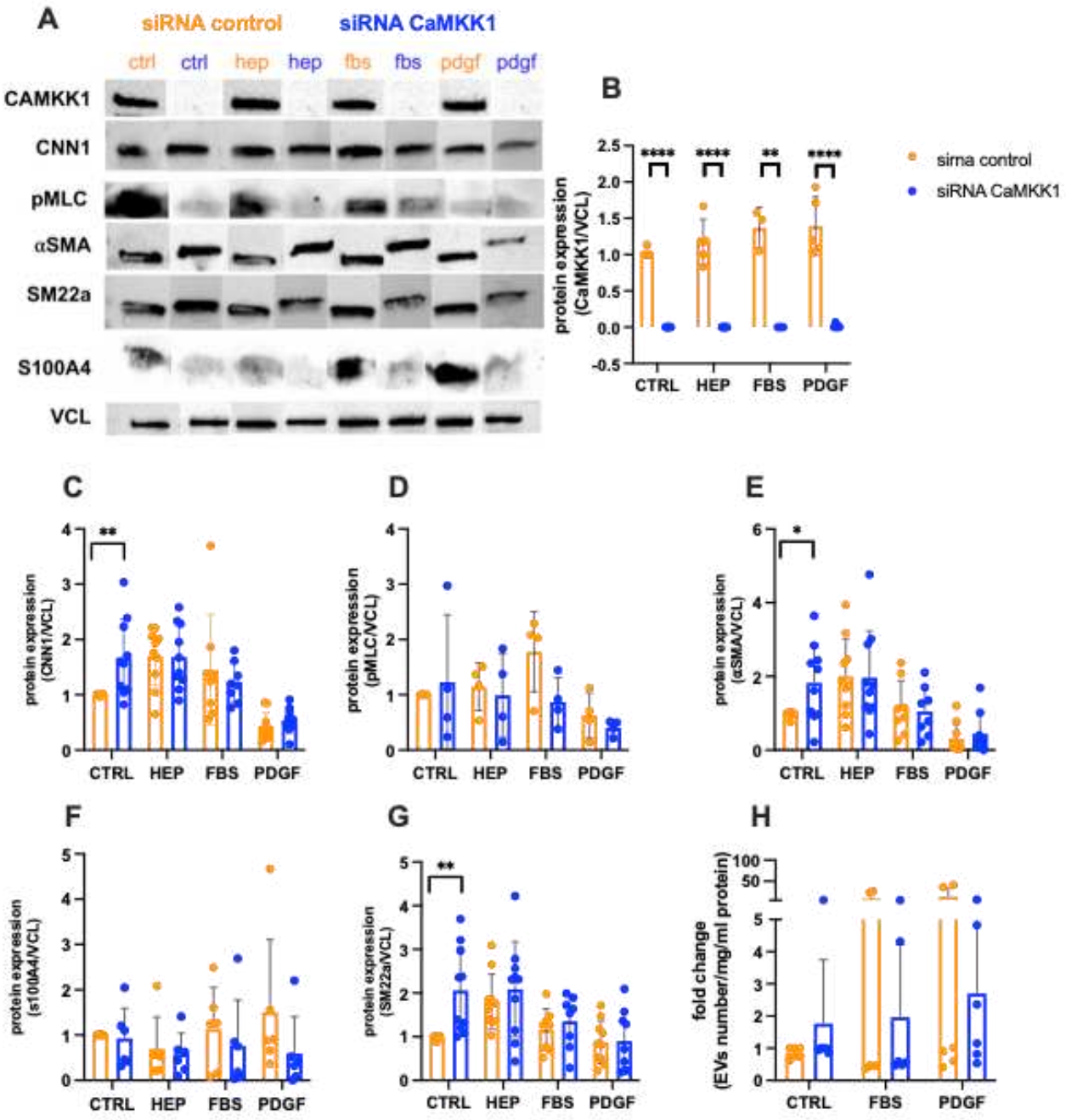


Figure 10: CaMKK1 regulates hVSCMC contractile marker expression. hVSMCS were transfected with siRNA non targeting pool control and siRNA CaMKK1, respectively. After 24 hours hVSMC were treated with PDGF-BB (10 μg/ml), 0.5% FBS (low FBS), heparin (200 U/ml) in M199 with 2.5% FBS for seven days. **A)** Western blot and **B-G)** quantification of CaMKK1, contractile proteins (CNN1, pMLC, *α*-SM-actin, SM22*α*) and synthetic marker S100A4. The results confirm both successful.

9. CAMKK1 regulates activity of kinases involved in hVSMC phenotype switching

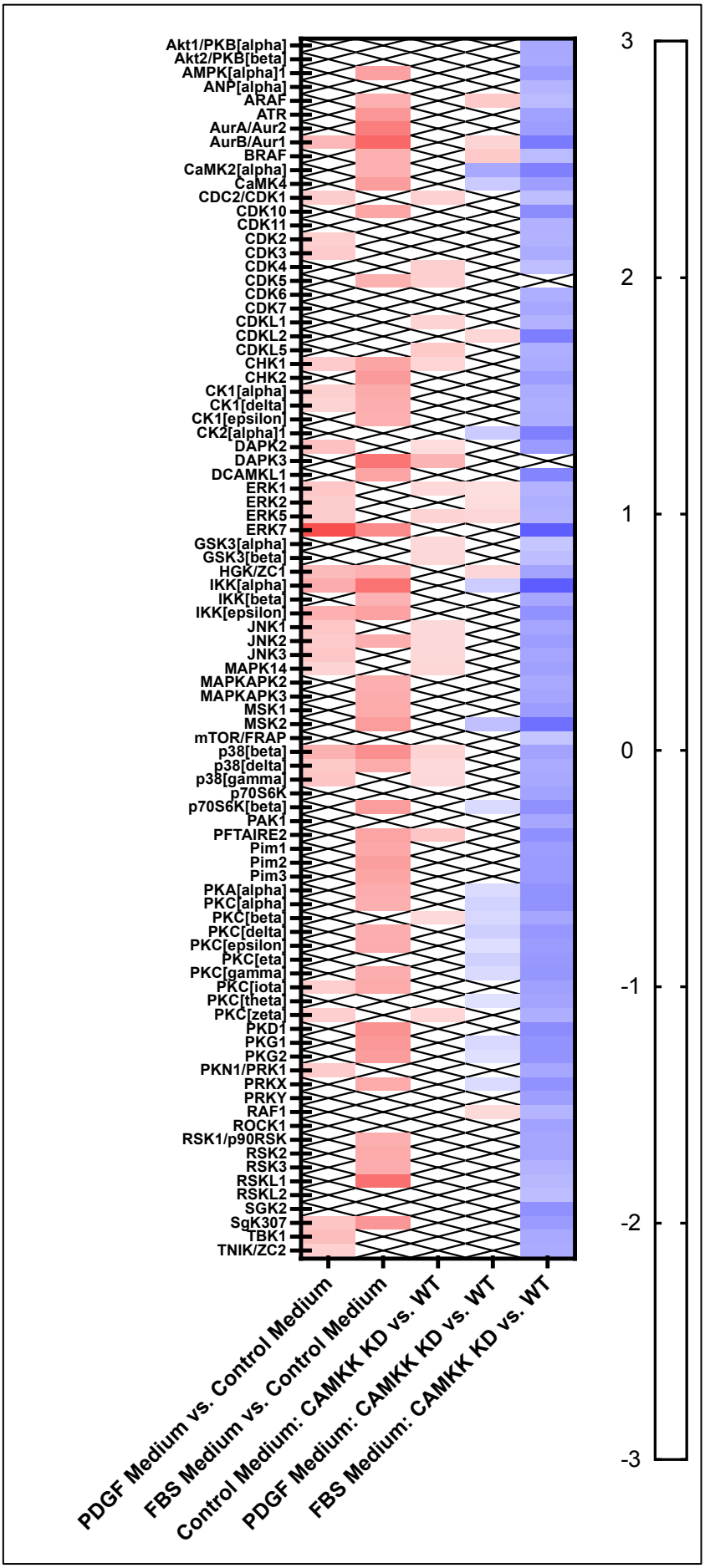
Considering the lack of information regarding CaMKK1 in literature and the interesting results of its possible role in phenotype switching in VSMC, the next goal was trying to understand in which signaling pathways CaMKK1 is involved. Specifically, we were interested in kinase activity profiling, which is based on measuring peptide phosphorylation by protein kinases (Figure 11, Supplemental Files 1-5). Pairwise comparisons were made between VSMCs treated with control, low FBS or PDGF-BB, in the absence and presence of CAMKK1 siRNA.

We found that ERK family kinases (ERK1, ERK2, ERK5, ERK7), belonging to the CMGC family, have a higher activity in PDGF-treated cells compared to control, and specifically ERK7 stands out as one with the highest activity (Figure 11, Supplemental File 1). Further to that, kinases mostly belonging to the CAMK and AGC family were more active in VSMC treated with low FBS compared to control (Figure 33, Supplemental File 2; e.g., ERK7; AurA/Aur2; AurB/Aur1; DAPK3; IKK; RSK2). Interesting to note, ERK1, ERK2 and ERK5 activity showed no difference. These results confirm that VSMC phenotype switching is associated with changes in kinase activity.

Next, we investigated effects of control siRNA to CaMKK1 siRNA in control, PDGF- and low FBS-treated VSMCs. In control medium, multiple kinases showed moderately increased activity with CaMKK1 knockdown, mainly in kinases belonging to the CMGC family (Figure 11, Supplemental File 1; e.g., CDK4, CDK5, CDKL5). In PDGF-BB with CaMKK1 knockdown treated VSMCs, some kinases showed moderately increased activity (e.g., ARAF, BRAF) while some others showed a downregulation, mainly kinases belonging to the CaMK family (e.g. CaMK2, CaMK4, MSK2) and kinases belonging to the AGC family (e.g. PKAα, PKAβ, PKG1, PKG2; Supplemental Figure 3, File 4). Interestingly, in low FBS- treated VSMCs, all kinases present in the microarray are downregulated by CaMKK1 knockdown, with the exception of CDK5 and DAPK3, whose activity did not change. This downregulation highlighted several kinases in the CMGC, CAMK, AGC families. Other kinases that were affected were in the CK1, STE and TKL families (Figure 11, Supplemental File 5). Taken together these results show that the knockdown of CaMKK1 is associated with changes in kinase activity during phenotype switching of VSMCs. Among the kinases with lower activity after CaMKK1 knockdown in low FBS-treated VSMCs there are AurB/Aur1, CaMK2, CDK10, CDKL2, CK2α1, ERK7, IKKα and MSK2.

Focusing the attention on kinases downstream of CaMKK1, we analysed CaMK4 activity. Interestingly there were no differences in CaMK4 activity in PDGF-treated cells compared to

control (Figure 11). However, CaMK4 showed higher activity in the low FBS-treated cells compared to control. Moreover, CAMKK1 knockdown induced downregulation of activity of CaMK4 both in the PDGF- and low FBS-treated VSMCs. CaMK2α, a calcium calmodulin dependent kinase not downstream to CaMKK1 and involved in different processes in the vessel, such as the regulation of contraction of VSMC, shows the same pattern of regulation in conditions described above (Figure 11). This suggests that the knockdown of CaMKK1 affects the activity of both CaMK4 and CaMK2α.


Akt1/PKB[alpha]
Akt2/PKB[beta]
AMPK[alpha]1
ANP[alpha]
ARAF
ATR
AurA/Aur2
AurB/Aur1
BRAF
CaMK2[alpha]
CaMK4
CDC2/CDK1
CDK10
CDK11
CDK2
CDK3
CDK4
CDK5
CDK6
CDK7
CDKL1
CDKL2
CDKL5
CHK1
CHK2
CK1[alpha]
CK1[delta]
CK1[epsilon]
CK2[alpha]1
DAPK2
DAPK3
DCAMKL1
ERK1
ERK2
ERK5
ERK7
GSK3[alpha]
GSK3[beta]
HGK/ZC1
IKK[alpha]
IKK[beta]
IKK[epsilon]
JNK1
JNK2
JNK3
MAPK14
MAPKAPK2
MAPKAPK3
MSK1
MSK2
mTOR/FRAP
p38[beta]
p38[delta]
p38[gamma]
p70S6K
p70S6K[beta]
PAK1
PFTAIRE2
Pim1
Pim2
Pim3
PKA[alpha]
PKC[alpha]
PKC[beta]
PKC[delta]
PKC[epsilon]
PKC[eta]
PKC[gamma]
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PKC[theta]
PKC[zeta]
PKD1
PKG1
PKG2
PKN1/PRK1
PRKX
PRKY
RAF1
ROCK1
RSK1/p90RSK
RSK2
RSK3
RSKL1
RSKL2
SGK2
SgK307
TBK1
TNIK/ZC2
PDGF Medium vs. Control Medium
FBS Medium vs. Control Medium
Control Medium: CAMKK KD vs. WT
PDGF Medium: CAMKK KD vs. WT
FBS Medium: CAMKK KD vs. WT
3
2
1
0
-1
-2
-3

Figure 11: CaMKK1 and phenotype switching affect the activity of a panel of kinases in hVSMCs. hVSMCS were transfected with siRNA non targeting pool control and siRNA CaMKK1, respectively. After 24 hours hVSMC were treated with PDGF-BB (10 μg/ml), 0.5% FBS (low FBS), heparin (200 U/ml) in M199 with 2.5% FBS for seven days. The heatmap shows the activity of 88 Ser/Thr kinases of a total of 144 present on the Pamchip microarray. These 88 kinases were active (the cut-off for the Mean Finale Score is 1.2) in at least one of the three conditions used for phenotype switching (CTRL, low FBS, PDGF) in hVSMC transfected for siRNA CaMKK1 and siRNA control. The red and blue reflect the median kinase statistic: the red shows relative high activity and blue represents relative lower activity. Black crosses indicate that in that specific comparison the kinase activity is not changed. In the comparisons, the second condition is always used as reference.

Discussion

Cardiovascular disease is now the leading cause of death worldwide, even in this last year of the pandemic (2020) in which the death rate has significantly increased due to COVID19. Despite the improvement of the therapy and knowledge to contrast CVD are rising every day, it has not yet halted the epidemic of cardiovascular disease and their impact on the health system care[251].

For this, further to the medical therapy or assistance, prevention needs to be the first treatment to contrast this type of disease. In fact, over the last few years it is giving more and more importance to individual genetic variants as potential predictor to CVD[57–59,62,65,67].

Genetic variants, specifically polymorphisms, are defined as a difference in DNA sequence among individuals, groups, or population with an occurrence greater than 1%. In the contest of cardiovascular disease, polymorphisms might play a crucial role as biomarkers for the diagnosis and prognosis of health, diseases, epidemiology and pharmacology[38,39].

The study of genetic variants can be very important to underline the complex genetic mechanism that regulate multifactorial disease, as CVD. Moreover, their identification can be very useful because they can cooperate with the canonical markers, such as troponin, in the diagnosis, prognosis and risk prediction of CVDs.

In this study we analysed different polymorphisms, known in literature, in the calcium calmodulin pathway, specifically in CaM3 and in gene downstream to calmodulin: NOS3 and CaMKK1[67].

This pathway is involved in the reception and transduction of the calcium signaling and plays different important roles in heart and vessels as the regulation of the mechanism of excitation contraction coupling.

In this study the distribution of allelic and genotypic frequencies has been analysed, related to six SNP (rs10113, rs7259810 in CaM3, rs1799983, rs2070744, rs1549758 in NOS3 and rs7214723 in CaMKK1) and to one VNTR in NOS3 (rs61722009), in a cardiopathic population composed of 300 subjects. The results of each of the six SNPs was respectively compared with an European population taken from the 1000 Genomes database.

Regarding the two SNPs analysed in CaM3, any statistical difference was found in the comparison between the total cardiopathic population and the European reference group, and also in the comparison among the different subgroups (CAD vs NOCAS, AS vs NOAS, NOCAD-AS vs NOCAD-NOAS). These results are probably due to the higher evolutionary conservation that characterize the calmodulin family protein. Indeed, in most human calmodulins the onset of mutations is deleterious and associated with life-threatening conditions in early childhood[77,110,111].

An analysis to identify the concomitant presence of the two SNPs in CaM3 among the 300 cardiopathic patients was performed. It was interesting to observe that 22 subjects, 10 NOCAD-NOAS, showed, in homozygosity, both the SNPs with the non-ancestral allele.

Table 1: P values from chi-square test performed among the different comparison, for each genetic variant, are reported. The level of statistical significance was set at P<0.05.

SNPs		**European reference group vs. Cardiopathic population**		**CAD vs. NOCAD**	**AS vs. NOAS**	**NOCAD-AS vs. NOCAD-NOAS**
rs7259810 (CaM3)	Genotypic freq. Allelic freq.	**P=** 0.13 **P=** 0.06		**P=** 0.35 **P=** 0.14	**P=** 0.49 **P=** 0.96	**P=** 0.98 **P=** 0.99
rs10113 (CaM3)	Genotypic freq. Allelic freq.	**P=** 0.09 **P=** 0.09		**P=** 0.37 **P=** 0.62	**P=** 0.42 **P=** 0.55	**P=** 0.73 **P=** 0.57
rs1799983 (NOS3)	Genotypic freq. Allelic freq.	**P=** 0.79 **P=** 0.86		**P=** 0.24 **P=** 0.1	**P=** 0.64 **P=** 0.79	**P=** 0.76 **P=** 0.8
rs2070744 (NOS3)	Genotypic freq. Allelic freq.	**P=** 0.34 **P=** 0.33		**P=** 0.05 **P=** 0.87	**P=** 0.88 **P=** 0.67	**P=** 0.30 **P=** 0.85
rs1549758 (NOS3)	Genotypic freq. Allelic freq.	**P<** 0.00 **P=** 0.96		**P=** 0.001 **P=** 0.08	**P=** 0.40 **P=** 0.44	**P=** 0.13 **P=** 0.3
rs61202009 (NOS3)	Genotypic freq. Allelic freq.			**P=** 0.03 **P=** 0.39	**P=** 0.25 **P=** 0.98	**P=** 0.80 **P=** 0.73
rs7214723 (CaMKK1)	Genotypic freq. Allelic freq.	**P<** 0.00 **P=** 0.00	**(EUR. vs. Maastricht)** **P=** 0.09 **P=** 0.03	**P=** 0.22 **P=** 0.21	**P=** 0.08 **P=** 0.27	**P=** 0.02 **P=** 0.05

Analysis of genetic variants in NOS3 identified two polymorphisms with significantly different frequencies in the populations studied, the SNP rs1549758 and the VNTR rs61722009.The SNP rs1549758 is an exon variant that create the nucleotide change C774T. It produces a synonymous mutation (Asp258Asp), that doesn't affect the structure of the protein. Considering the interesting genotypic and allelic frequencies in the total cardiopathic population and in the subgroups of CAD-NOCAD and considering the that the SNP rs1549758 doesn't affect the structure of the protein, it was necessary performed further analysis to better understand the potential impact of this SNP on the higher risk to develop coronary artery disease. Through detailed literature and database

analysis, it is possible to assume that the impact of this SNP might be at the transduction level in codon-anticodon recognition. Indeed, in eucaryote the frequency of tRNA with the anticodon AUC (that recognise the codon sequence GAT) is very low, compared to the tRNA with the anticodon GUC for the sequence with the C allele (codon GAC). This means that the SNP rs1549758 might decrease the efficiency of the transduction of NOS3. Consequently, this might reduce the production of NO by NOS3, that is an essential molecule for maintaining the vascular tone, preventing vasoconstriction. This might explain the interesting results of the genotypic frequencies in our cardiopathic population and its subgroups. Although SNP rs1549758 appears not to be very common in the population with a low MAF index of the T allele (T = 0.1823 (913/508), the genotypic frequency of the total cardiopathic population is statistically different from that of the European reference group. This significant difference is present also between the CAD and NOCAD subgroups, in which it is very interesting observe that in the NOCAD the genotype TT completely disappears.

As regards the VNTR-4a / 4b in intron 4 of NOS3, it is characterized by the presence of 5 repeat copies of 27bp (4b / 4b) or by the presence of 4 repeat copies of 27bp It has been observed in literature that this VNTR is associated with a lower bioavailability of NO, specifically it has been showed that the five 27 bp repetition produced a higher level of siRNA which interferes with the NOS3 mRNA, reducing its level.

Among the stratification of the total cardiopathic population, it was interesting observe that the genotypic frequencies between the CAD and the NOCAD groups resulted statistically different distributed. In the CAD groups there is a higher frequency of the genotype 4b/4b and 4a/4a and a lower frequency of the heterozygous 4a/4b, compared to the NOCAD subgroups.

In absence of the European reference group, it was possible to perform further comparison with other CAD and NOCAD groups from literature[214,252–256]. The CAD subgroup of the present study has the same genotypic distribution of the Iranian CAD group [255] with a higher percentage of the 4b/4b genotype compared to the 4a/4a, and opposite to the Cypriot Turkish[256]. The NOCAD subgroup of the present study has the same genotypic frequencies distribution with the NOCAD Cypriot Turkish[256] , with a higher percentage of the allele 4b/4b. Regarding the allelic frequencies recorded in other studies (CAD and NO CAD subgroups), the trend is the same as the one showed in the present study, with a higher percentage of the 4b allele compared to the 4a, except for the allelic frequencies in the CAD group in the Cypriot Turkish[256].Considering the important potential impact of rs1549758 and rs61202009variants in NOS3, , it was also performed an analysis to

observe the coexistence of the two SNPs among the 300 cardiopathic patients, concomitantly. Only 3 subjects, CAD-AS, showed both the genetic variant with the homozygous non wildtype (ancestral) genotype. This data suggested absence of a strong coexistence of the two genetic variants. Further analysis will be performed to better understand the impact and association that different SNPs might have on the pathology, if inherited simultaneously for the non-ancestral homozygous genotype. Moreover, it will be important in future to enlarge the number of subjects in the cohort population to better discriminate polymorphism in CaM3 and NOS3 genes in association with CVD risk.

Among the seven genetic variants, one of the main interesting single nucleotide polymorphisms analyzed in this research project, in association with the higher risk to develop cardiovascular disease, is rs7214723 in CaMKK1 that create an amino acid change, from Glutamate to Glycine, at the position 375 inside the kinase domain of the protein[147]. This missense variant might be involved in a conformational change and could decrease the activity of CaMKK1, contributing to modulate the calcium signaling pathway and the higher susceptibility to CVDs. The total cardiopathic population resulted statistical different compared to the European reference group, in which there is a higher frequency of the homozygous TT compared to the CC. On the contrary, in the total cardiopathic population there was a higher frequency of the homozygous CC, compared to the TT, and also of the heterozygous (Cardiopathic population TC = 64%; European TC = 48%).

Among the subgroups it was observed the same genotypic trend, but specifically in the NOCAD and NOAS subgroups the percentage of the CC was the highest one.

These results suggested to perform a further stratification inside the NOCAD subgroups, excluding from the AS and NOAS subgroups the patients with CAD. The exclusion of the CAD patients from the AS group is clinically appropriate since AS has the same pathophysiological basis as coronary heart disease. The stratification showed that subjects NOCAD-NOAS have a higher enrichment of the C allele compared to NOCAD-AS.

In general, it is interesting observe that in the cardiopathic population, a selected and specific population group, the results showed an enrichment of the non-ancestral genotype CC, specifically in subject NOCAD-NOAS. The increase of a specific genotype (CC) in a selected population might suggest a higher risk for the subjects carrying this genotype to develop CVD. The enrichment specifically observed in the NOCAD and NOAS subgroups might suggest a major difficulty in bearing the specific damage caused to the tissue by no CAD (as valve diseases) or AS[147].

The enrichment of the CC genotype was also observed in a cohort of 79 Dutch patients from Maastricht, subjected to cardiac surgery, mainly for aneurysm. As the Italian total cardiopathic population, the Maastricht population showed a higher frequency of the homozygous CC, compared to the European population. Also, the allelic frequencies showed an opposite trend between the Maastricht population and the European one, confirmed by a chi square test that resulted statistical different.

This study confirms and strengthens the potential role of the SNP rs7214723 in association with the higher risk and fragility to develop cardiovascular diseases[147].

We hypothesize that the amino acid change (E375G) within the kinase domain of CaMKK1 might have a decisive role in the kinase activity of the protein and can influence the downstream pathways of calcium signaling, contributing to develop heart or vessels problems, and increasing the predisposition to this kind of disease. Since the results obtained in this study are very promising, we decide to better understand the molecular and biological role of the *CAMMK1* gene in CVDs, considering the lack of information present, about this protein, in literature.

In the *in vitro* study we identified CaMKK1 as a new regulator of VSMC phenotype.

In this study it we identified CaMKK1 as a new regulator of VSMC phenotype. We observed that the expression of CaMKK1 increased when hVSMCs were treated with PDGF to induce the synthetic phenotype. We also show that CaMKK1 knockdown caused an increase in contractile marker expression at baseline and trend towards decreased EV release in PDGF and low FBS treatment (Figure 2H).

To the best of our knowledge, this is the first study showing increased expression of CaMKK1 in synthetic hVSMCs. These results suggest that the conditions favoring vascular remodeling activate at least part of the CaM cascade. This is in line with literature showing that CaMKKs are involved in the regulation of phenotype switching in human vascular smooth muscle cells, through the activation of CaMK4 and subsequently of CREB (cAMP response element-binding protein)[98].

CaMKK1 knockdown caused an increase in some, but not all of the studied hVSMC contractile markers at baseline. This suggests that CaMKK1 exerts an inhibitory effect on the expression of CNN1, αSMA and SM22α, implying that its activation promotes the synthetic phenotype. CaMKK1 had no effect on the expression of pMLC and synthetic marker S100A4, suggesting that CaMKK1 regulates expression programs responsible for hVSMC phenotype switching partially, not comprehensively. These effects of CaMKK1 knockdown were not observed in synthetic or

contractile hVSMCs, suggesting that perhaps in the presence of other stimuli the absence of CaMKK1 is compensated for by other pathways regulating hVSMCs phenotype marker expression.
Elevated EV release is an important hallmark of hVSMC phenotype switching[249,250]. Here we show that hVSMCs with downregulated CaMKK1 and treated for phenotype switching showed a trend toward decreased EV release in both contractile and synthetic hVSMCs, and no change at baseline. This suggests that CaMKK1 stimulates EV release, further supporting its role in promoting the synthetic phenotype. This data is supported by literature, which has shown that CaMKK1 increased EV release in rat mesenchymal stem cells[146].

In this work we also showed that both hVSMC phenotype switching and knockdown of CaMKK1 affect the Ser-Thr kinase activity in hVSMCs. The most changes were seen in the CMGC (which includes ERKs) and CaMK families. Specifically, several ERK kinases (ERK7 in particular) had a higher activity in hVSMC treated with PDGF compared to the control. This confirms that PDGF stimulation leads to the initiation of ERK signaling, contributing hVSMC proliferation,[257–260] which is a hallmark of the synthetic phenotype. Additionally, several other kinases from the CMGC family (p38, JNK), are involved in the ERK signaling and regulate cell proliferation and migration, characteristic of the synthetic phenotype induced by PDGF[259–262].
Some of them (ERK7, p38 α, β, γ, δ, JNK2,RSK) were activated also in the low FBS condition and this might be because ERK MAP kinases also regulate the contraction pathways, that characterize the contractile phenotype induced by low FBS.. [263–265]

In this study we identify several other kinases involved in hVSMC phenotype switching, some of them (Aur, IKK, DAPK) with more activity compared to other. The lack of information of their role in hVSMCs suggests the identification of many new potential regulators of hVSMC phenotype switching.
Moreover, the knockdown of CaMKK1 affected the downregulation of the CMGC protein family more in the low FBS treatment than in the PDGF, suggesting that in this last one there might be other signaling at play that partially compensates for the lack of CaMKK1.
Importantly, in the hVSMC treated with low FBS, there was an upregulation of kinases from the AGC family. Among this group, some are involved in regulating contraction (PKA, PKC, p70S6K)[266,267] and others (e.g. PKG)[268] are involved in the inhibition of hVSMC proliferation and migration, consistent with this treatment promoting the contractile phenotype. However, the knockdown of CaMKK1 in the low FBS condition downregulated the activity of these pro-

contractile AGC family members, suggesting that CaMKK1 might have disparate effects on signaling relevant to hVSMC phenotype.

Additionaly,For example, regarding the CaMK family, the results in this study show that both the phenotype changing treatments and CAMKK1 knockdown affect the activity of the CaMK family. The fact that PDGF and low heparin regulate activity of the CaMK family further confirms that the CaM cascade is involved in hVSMC phenotype switching. Specifically, it is interesting observe that the knockdown of CaMKK1 induced a general downregulation of all the kinases present in the Pamchip microarray, both in the PDGF and low FBS treatment, in this last one ERK7 was the most downregulated (confirming again its role described previously).

Focusing the attention on CaMK4, a downstream target of CaMKK1 (CaMK1 was not present in the array), it is interesting observe also how change its activity in the different treatment. Indeed, in hVSMC knockdown for CaMKK1, CaMK4 resulted to be downregulated both in the low FBS and PDGF treatment. On the contrary, it was upregulated in hVSMC treated with low FBS, compared to the control. These results confirm the involvement of this pathway in hVSMC phenotype switching. We hypothesize that this pathway can be involved in the activation of CREB (cAMP response element-binding protein), an important transcription factor that regulates expression contractile and synthetic marker genes during hVSMC phenotype switching when it is activated by CaMK4[98]. Due to the lack information in literature, it will be necessary to perform further experiment to better investigate the specific role of this pathway in hVSMC phenotype switching.

Moreover, our results highlight the potential role of single nucleotide polymorphism (SNP) rs7214723 in CaMKK1 in association with the higher risk to develop cardiovascular disease.[147] Previously an enrichment of the C allele was identified in a cohort of 300 cardiopathic subjects and specifically, the subgroup without coronary artery disease and aortic stenosis (NOCAD-NOAS) had higher frequency of the CC genotype. In this study, we show that in the treatment with heparin, hVSMC isolated from patients with the genotype CC for the SNP in CaMKK1 showed the higher expression of CaMKK1. While this change was not statistically significant, it is tempting to speculate that CaMKK1 genotype could have an effect of the response of hVSMCs to phenotype-altering stimuli.

This study has other several limitations. First, it is important consider that in some experiments the standard deviation is high and statistical significance could not be reached. This is due to the inherent variability of cells isolated from several different human donors, on which their experiments were performed on. Additionally, due to a low donor number we could not perform

statistical analysis to verify the association between the polymorphism in CaMKK1 and phenotype switching.

This in vitro part has shown that CaMKK1 has a new role in the regulation of phenotype switching in human

hVSMCs, influencing the activity of several Ser-Thr kinases and expression of phenotype markers. These findings are important to cardiovascular diseases that involve vascular remodeling such as atherosclerosis, calcification, hypertension and aneurysm, as they provide a new potential pathway to investigate possible novel therapeutic targets. In conclusion, this research highlights the potential role of CaMKK1 as a new biomarker in the prediction, prognosis and follow up of patient in cardiovascular diseases. Despite the association of only one genetic variant with a complex disease is not consistent, the results obtained in this study are very interesting and promising, leading to hypothesize a new pivotal and crucial role of CaMKK1 in heart and vessel, suggesting also further analysis on the genes involved in the calcium calmodulin pathway.

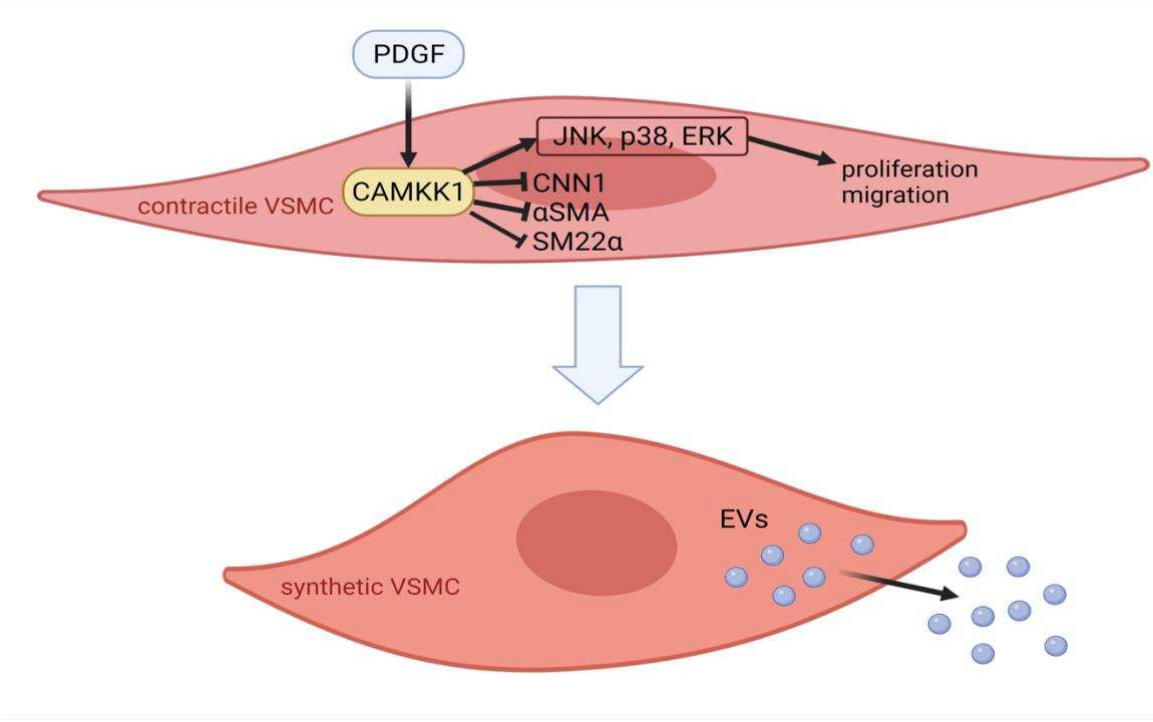

Figure 1: CaMKK1 regulates VSMC phenotype switching. PDGF treatment increases CaMKK1 expression. This leads to inhibition of contractile marker CNN1, αSMA and SM22α expression and increase in activity of kinases known to promote mirgration and proliferation. All of these changes stimulate synthetic differentiation of hVSMCs, which is associated with increased EV release (from Beghi et al., 2021- to be submitted)

Societal impact

Cardiovascular disease (CVD) still represents one of the major challenges for public health, being the number one cause of death world-wide. The remarkable success of several therapeutical and clinical approaches introduced toward the end of the 20th century have not yet halted the epidemic of CVD. Instead, the burden of ischemic cardiovascular conditions has risen to become a top cause of morbidity, and mortality worldwide. CVD is characterized by a high complexity due to its multifactorial character: different factors coexist increasing the risk to develop and suffer of CVD. Further an unhealthy lifestyle, environmental factors and other comorbidity, genetic play a key role in the etiology of CVD. Vascular remodelling, i.e. hypertension, vascular stiffness and calcification, have become the main public health problem in the Western world. Currently, vascular remodelling and calcification is understood to be an actively regulated process involving cellular and humoral contributions that may offer novel targets for diagnosis and intervention. Moreover, the identification of genetic variant associated with the higher risk and susceptibility to develop a specific CVD, provides an important cue for the prevention, prediction and follow up of CVD patients.

In my thesis, I specifically focused on the identification of genetic variants, specifically certain polymorphisms, in genes involved in the calcium-calmodulin pathway in association with the risk of developing CVD. My aim was to search new potential biomarkers that are present already at birth and can cooperate with canonical markers developing during disease (e.g., troponin), ultimately resuling in better prognosis, diagnosis, and risk prediction of CVD. Applying such combined “nature and nurture” approach will contribute to improving health status of people across countries and might reduce the economic impact that CVD has on health care systems.

The research described in section of the results on the genetic variant in the genes CaM3, NOS3 and CaMKK1 involved in the calcium-calmodulin pathway, led to the identification of the crucial and potential role and highlights the importance of intracellular calcium signaling and need for more research. Among the different genetic variants analyzed in our study, specifically we discover a new potential role in calcium calmodulin kinase kinase (CaMKK1). Our research on unraveling the role of the genetic as well as the bio-molecular level of CaMKK1 might result in novel treatment avenues for CVD.

In the results section, analysing a cohort of 300 cardiopathic subjects, we identified that the single nucleotide polymorphism rs7214723 was associated with a higher risk to develop CVD. Specifically, we found a higher susceptibility and risk for patients with the polymorphism

rs7214723 CC genotype. Since the calcium calmodulin pathway is key for the transduction of the calcium signaling both in the heart and vessels, we further studied in depth the role of CaMKK1 in human vascular smooth muscle cells (hVSMCs). We found that CaMKK1 is a novel regulator of phenotypic switching of hVSMC towards synthetic VSMCs, thereby providing CaMKK1 as a new therapeutic target to reduce vascular remodeling.

In conclusion, my research described in this thesis lays the foundation for a novel role of this genetic variant in the calcium-calmodulin pathway, providing a new biomarker for the diagnosis and prevention/ treatment of CVD. Early diagnosis and preventive treatment might be a cost-effective strategy to reduce the high number of CVD morbidity and mortality, and its associated high economic burden. The association of only one single genetic variant with a complex disease such as CVD needs further research to unravel the precise role of CaMKK1. Yet our results are promising and might lead to novel theragnostic strategies targeting CaMKK1 in heart and vessel. Finally, we propose further analysis of genes involved in the calcium calmodulin pathway to specific CVD, such as hypertension, arterial stiffness, vascular calcification and heart disease.

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APPENDIX 1

Principles of Cardiac Anatomy and Physiology

1. Principles of Cardiac Anatomy and Physiology

Knowing cardiac anatomy and physiology is essential to understand ischemic cardiomyopathy and its clinical consequences.

1.1 Heart and Pericardium [5,6]

The heart is a hollow, muscular organ located inside the chest, between the lungs and above the diaphragm; it is surrounded by a fibrous sac called pericardium, which separates the heart from other contiguous organs.

The heart has an inverted cone shape with the apex pointing down-left, approximately large as a fist, grossly projected on the anterior chest in the area between the right margin sternal line and the left hemi-clavear lines and between the 3rd and the 5th ribs. A frontal view, the heart appears to have two margins, a base, an apex and two faces (anterior and posterior, see Figure 1). A transverse line, the atrio-ventricular sulcus, separates the atria from the ventricles while an interventricular sulcus separates on the surface of the heart the left ventricle from the right one. The heart is composed of two upper chambers, namely the atria, and two lower chambers called ventricles. Atria and ventricular chambers communicate through atrio-ventricular orifices (left and right) both characterized by the presence of atrio-ventricular valves, which control blood flow direction and, when open, plunge to the ventricular chamber.

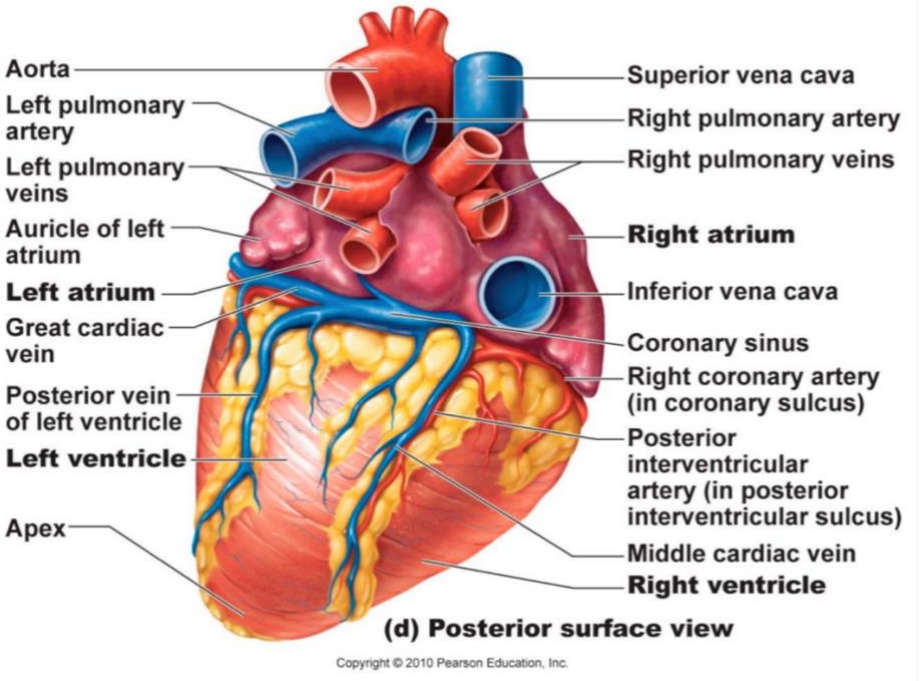


Figure 1: Heart Diagram and chart. Copyright 2010 Pearson Education, Inc.

The internal surface of the heart is irregular, given the presence of numerous muscular bundles forming pillars that entangle in various shapes. Among these muscular structures, papillary muscles appear as the most relevant ones from a functional point of view, since from the tips of these muscles in the left and right ventricles take off the tendinous chords that connect the papillary muscle to the atrio-ventricular valves. Other trabeculae are instead entirely laying on the inner surface of the heart. Both atria lack of papillary muscles and muscular pillars are smaller and less evident when compared to the ventricular ones. In the ventricles these pillars become particularly evident toward the apex where they entangle reciprocally, forming the cavernous portion of the ventricles.

1.2 Coronary anatomy

1.2.1 Arterial Circulation [5,6]

The heart is perfused by two coronary arteries, left and right, which alone retain nearly 5% of the entire cardiac output. The coronary arteries and its branches lay on the surface of the heart, covered by a thin layer called epicardium, along atrioventricular and interventricular sulci, frequently surrounded by small amounts of adipose tissue (Figure 2).

Occasionally small segments of coronary arteries can lay under narrow muscular bundles called muscular bridges. When those bridges are thicker and involve a longer segment of coronary artery, this can represent a functional/dynamic stenosis and cause ischemia. Smaller branches instead dive into the muscular wall of the heart perfusing the coronary capillary bed which is spread into the interstitial connective tissue down to the subendocardial layers.

Coronary blood perfusion happens predominantly during cardiac diastolic phase, because during the systolic phase the squeeze induced by the cardiac muscular contraction functionally compresses the small coronary branches laying inside the cardiac walls, limiting an adequate perfusion.

Coronary arteries origin from the first tract of the thoracic aorta, the aortic root, and precisely at the level of Valsalva sinuses named after the of coronary arteries that origin there, as left coronary and right coronary sinuses.

The average diameter of proximal coronary arteries is around 3-4 mm with the left coronary artery (LCA) slightly larger than the right coronary artery (RCA). However, independently from coronary size, the dominance of a coronary on the other only depends on which of them gives origin to the

posterior descending artery, laying on the posterior wall of the heart in the interventricular sulcus. Right dominance occurs in nearly 90% of patients, while in 10% a left dominance is found; moreover, in less than 1% of patients a balanced circulation between the two arteries is found, with two posterior descending arteries one from the left and one from the right coronary system.

Coronary circulation is a terminal circulation, although a limited amount of collateral homochromatic or heterochromatic circulation can be found through natural anastomoses, which are generally thin vessel, incapable of compensating an acute coronary occlusion but progressively developing in case of chronic ischemic coronary disease. Most natural anastomoses are through the interventricular septum, the apex, and the anterior face of the atria.

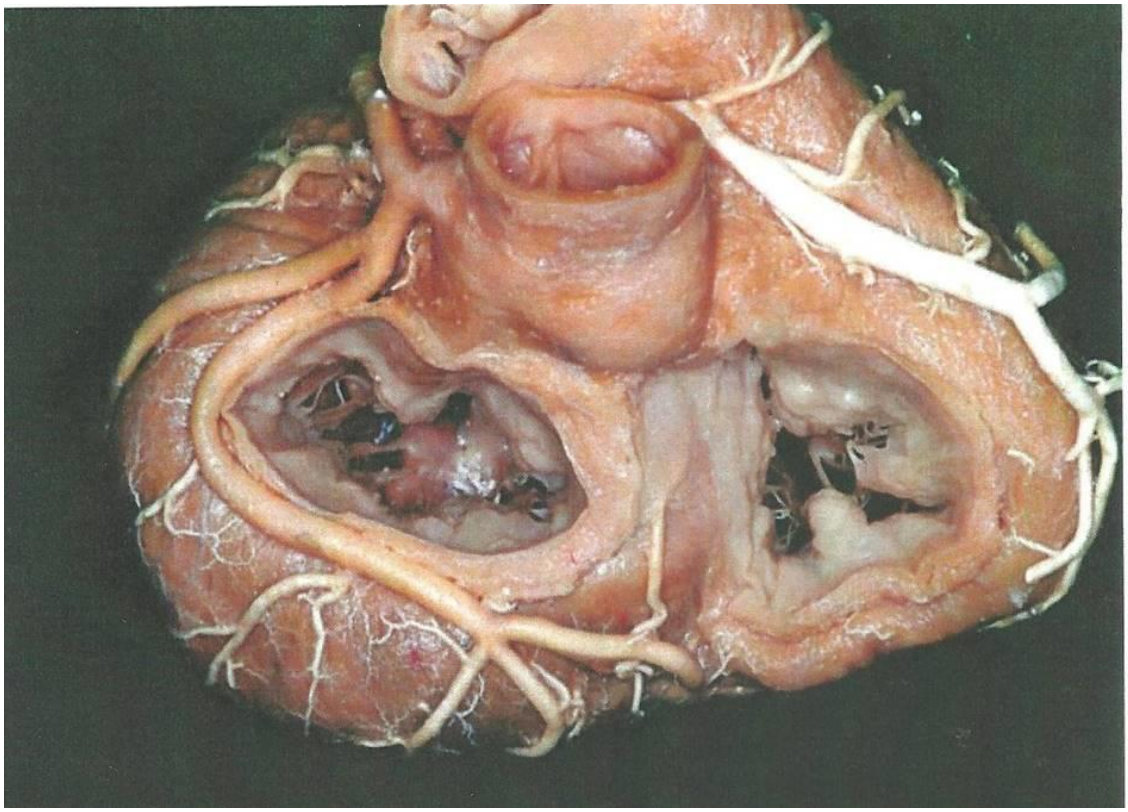


Figure 2: Anatomical detail of superior vision of the coronary system (from "Operative Anatomy of the Heart", Ed. Springer).

1.2.2 Right coronary artery [5]

The right coronary artery originates from the right Valsalva sinus and points downward to the right in the anterior part of the atrio-ventricular sulcus, between the right atrial appendage and the sternocostal face of the right ventricle. After passing over the acute margin of the heart, the RCA continues into the posterior atrio-ventricular sulcus reaching the crux cordis. In 90% of patients at this point makes a 90° turn and from the atrio-ventricular sulcus enters the posterior interventricular sulcus, originating the posterior descending artery (PDA). The RCA along origins also the following branches: sino-atrial node artery; infundibular artery; atrial branches; ventricular branches (in particular acute margin arteries); posterior descending artery

1.2.3 Left coronary artery [5]

The left coronary artery originates from the left coronary Valsalva sinus and runs in the space between the aorta and the pulmonary artery. This first segment, called left main stem, reaches the coronary sulcus at the level of the left atrial appendage, and bifurcates into the left anterior descending artery (LAD) and the circumflex artery (LCX). The LAD is the most important coronary branch of the left ventricle providing nearly 50% of coronary perfusion in particular on the anterolateral wall of the left ventricle and the anterior part of the interventricular septum. Most relevant division branches of the LAD are diagonal (branches). The circumflex artery runs in the atrioventricular sulcus along the obtuse margin of the heart giving branches for the lateral wall of the left ventricle called obtuse marginal branches. In 30% of patient a third branch between LAD and LCX is present; it is called intermediate branch, and essentially behaves as a large diagonal branch of the LAD, running on the antero-lateral surface of the left ventricle.

2. Cardiac Physiology

2.1 General principles [5,6,269]

Cardiac musculature, namely myocardium, represents the prevailing component of the heart, and behaves functionally as a pump that pushes forward the blood through the systemic and pulmonary circulation.

The myocardial tissue is predominantly composed by cardiomyocytes which microscopically present transverse lines due to the presence of myofibrilla organized in sarcomeres, the basic structures of contractility. These structures are composed of actin and myosin filaments which alternate inside the myofibrilla and can slide one on the other provoking muscular contraction, as it happens in the skeletal muscle.

Muscular bundles are composed by multiple muscular cells in series: cell membranes are fused forming tight junctions that allow the passage of electrolytes from a cell to the next. This is called a functional syncytium that allows the conduction of electric impulses, namely an action potential from a cell to the next one, even laterally allowing depolarization and repolarization of the muscular tissue.

The heart is composed by two separate functional syncytia: the atrial syncytium and the ventricular syncytium. Despite these two syncytia are separated by the fibrous tissue constituting the fibrous skeleton of the heart (i.e., trigona and annular structures of the atrio-ventricular orifices), a specific conduction system called atrio-ventricular bundle crosses the fibrous skeleton of the heart and connects the two syncytia allowing the sequential contraction of atria first and subsequently ventricles.

The conduction system of the heart allows a nearly simultaneous contraction of all the myocytes composing a syncytium, achieving macroscopically atrial and then ventricular systole. Another property of the conduction system is the intrinsic automaticity to trigger a depolarization wave at a certain specific frequency which functions as natural pacemaker: this happens in specific segments of the conduction system where a particular type of myocyte (called His-Purkinje cells) is found, particularly in the sinoatrial node, in the atrioventricular node in the His bundle and in its branches. These special myocytes have peculiar histologic characteristic, and their function can be better understood by recording electric potential through intracellular microelectrodes.

2.2 Myocardial cell excitability and excitation-contraction coupling [269]

The cell membrane of myocardial cells has a resting electrical potential of -85 to -95 mV, while Purkinje cells within the conduction system of the heart have a resting potential of -90 to -100 mV. When the membrane electrical potential becomes slightly positive around +20 mV, an action potential is triggered; after the initial spike, the cell membrane remains depolarized for around 0,2 seconds at the level of atrial musculature and 0,3 seconds for the ventricular musculature, generating a plateau at the end of which the membrane returns repolarized. Looking at the depolarization diagram of myocardial cells (Figure 3), it seems that the hyperpolarization is not immediately followed by a repolarization, while the plateau allows a more persistent muscular contraction up to 15 times of the skeletal muscle fibers.

There might be two explanations for such a long action potential. First, in myocardiocytes the action potential is due to the opening of two different transmembrane channels that are voltage-triggered: the fast sodium channels and another series of channels, called slow calcium channels or calcium-sodium channels. These last one can open slowly and remain there for quite a long period (few tenths of seconds and not milliseconds as the fast sodium channels).

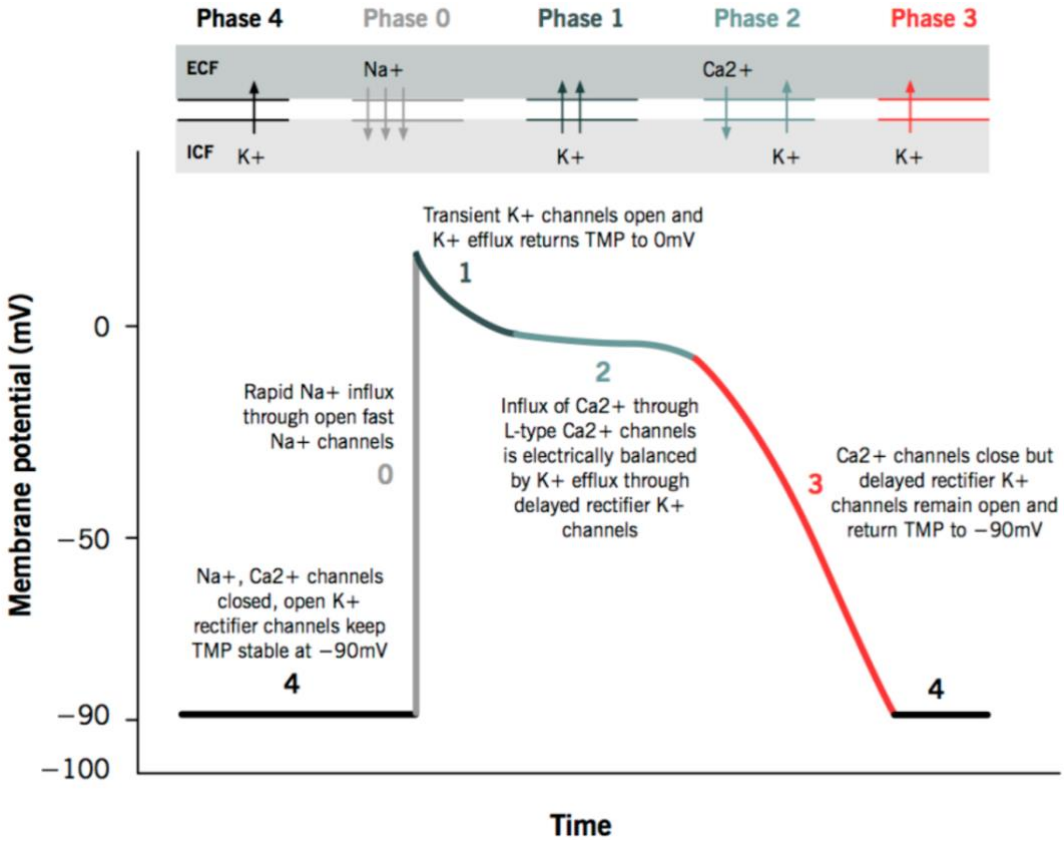


Figure 3: Action potential of cardiac muscles. (from Eric Wong, 2013)

Once fast sodium channels are open, sodium enters quickly inside the cell causing a prompt increase of membrane potential; subsequently slow channels let calcium and sodium cross the cardiac cell membrane sustaining a more prolonged plateau of depolarization. Calcium ions have also a role in the contraction process, as it will be discussed in the next paragraph.

The second mechanism that explains the depolarization plateau is the five-fold decrease of cell membrane reduction of permeability toward potassium ions. Once calcium and sodium channels get closed, the permeability to potassium ions suddenly increases back to normal causing membrane repolarization.

The mechanism of excitation-contraction coupling is described in figure 4. As in the skeletal muscular cells, the action potential is transmitted not only along the membrane but also through the muscular fibers, passing through the so-called T transverse tubes.

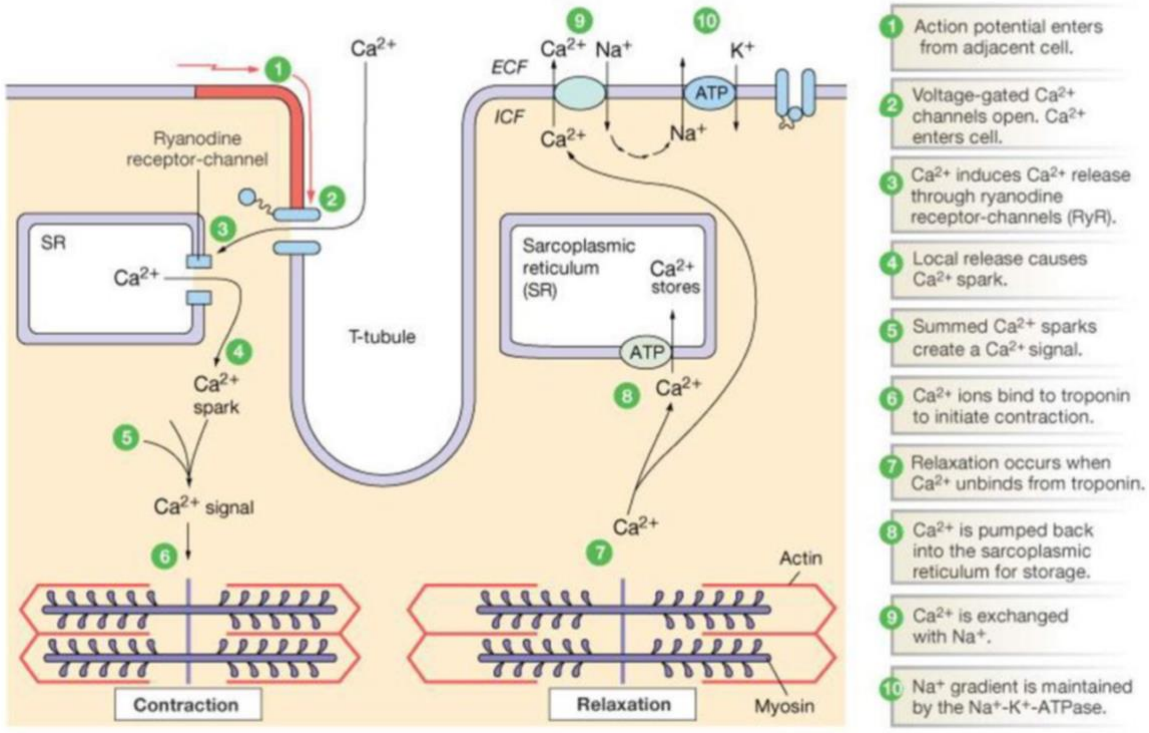


Figure 4: Excitation-contraction coupling and relaxation in cardiac muscle(Copyright © 2004 Pearson Education, Inc., publishing as Benjamin Cummings)

Subsequently the action potential involves the membranes of longitudinal sarcoplasmic tubes, that cause the release of calcium ions in the sarcoplasm, from the sarcoplasmic reticulum. Therefore, in few milliseconds' calcium spreads through myofibrils inducing the sliding of actin

filaments over myosin filaments, that induce contraction. In order to achieve muscular contraction, the bond between calcium and troponin has a crucial role. The action potential not only causes calcium release from the sarcoplasmic reticule but also calcium release from T tube that are in direct connection with the outside of myocardial fibers. In fact, myocardial strength of contraction depends on the calcium concentration in the extracellular medium. When the plateau phase is about to end, the calcium flow toward the cell promptly decreases while the calcium present in the sarcoplasm gets pumped back in the sarcoplasmic reticule and inside the t-tubes, interrupting the muscular contraction.

APPENDIX 2

Supplemental materials

1. Supplemental methods

RNA extraction and cDNA synthesis

Total RNA was extracted from hVMSC using a TRI Reagent (Sgma) according to the manufactirer's protocol. RNA concentrations were quantified spectrophotometrically at 260 nm using a Nanodrop ND1000 (ThermoFisher). RNA integrity was evaluated using denaturing agarose gel electrophoresis. 250 ng of total RNA was treated with DNase I (Promega, Leiden The Netherlands). The purified RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) for 1 hour at 37°C, in the presence of RNAse Out, dNTPs, dithiothreitol (all from Invitrogen), and an oligo(dT) primer (Eurogentec, Maastricht, The Netherlands).

Quantitative real-time PCR

Gene expression levels were quantified by real-time quantitative PCR (qPCR) in a LightCycler 480 (Roche Applied Science). Amplification reactions were carried out in a volume of 10 µl, containing 100 ng of total cDNA, 5 µl QuantiTect SYBR Green PCR Kit (Qiagen) and 0.5 µM of 5' and 3' primers (Eurogentec). An initial denaturation step (15 minutes at 95°C) was followed by 50 cycles of amplification (denaturation: 15 seconds at 95°C, annealing: 30 seconds at 57°C, extension: 45 seconds at 72°C). The specificity of amplification was controlled by melt curve analysis. Fluorescence curves were analyzed with LightCycler 480 Software (Version 1.5) and relative quantification was performed with the $2-\Delta\Delta Ct$ method. All samples were assayed in triplicate.

Intracellular calcium measurement

hVSMC were plated in a black, clear-bottom 96 well plate in quadruplicate for each condition. The cells were transfected with siRNA in Medium 199 (Gibco) supplemented with 1% Penicillin Streptomycin antibiotics (Gibco) and 2,5% FBS (Gibco). Treatment with 3.6 mM CaCl2 and 1 mM ionomycin (Sigma) was used as a positive control and hVSMCs not loaded with Fluo-4 (Invitrogen) as the negative control. 24 h after the siRNA transfection cells (excepted the negative control), were loaded with 50 µl/well Fluo-4-AM (Invitrogen) mix: M199 (Gibco) +20% FBS (Gibco)+ 1% Penicillin Streptomycin antibiotics (Gibco) + Fluo-4 (5µM final concentration; Invitrogen) + Pluronic F-127 (Sigma-Aldrich, 1:500 of 200 µg/ml stock) + 1 mg/ml Hoechst (Invitrogen). After 30 minutes, all the wells were washed 2 times with PBS. The treatments were applied in KRPG (Krebs-Ringer phosphate glucose buffer) + BackDrop Background Suppressor (Invitrogen, 1:50). The intracellular calcium measurement was performed using the Cytation 3 (Biotek) imaging plate reader.

Quantification was performed from pictures using the signal of GFP from the total area of the wells.

2. Supplemental figures

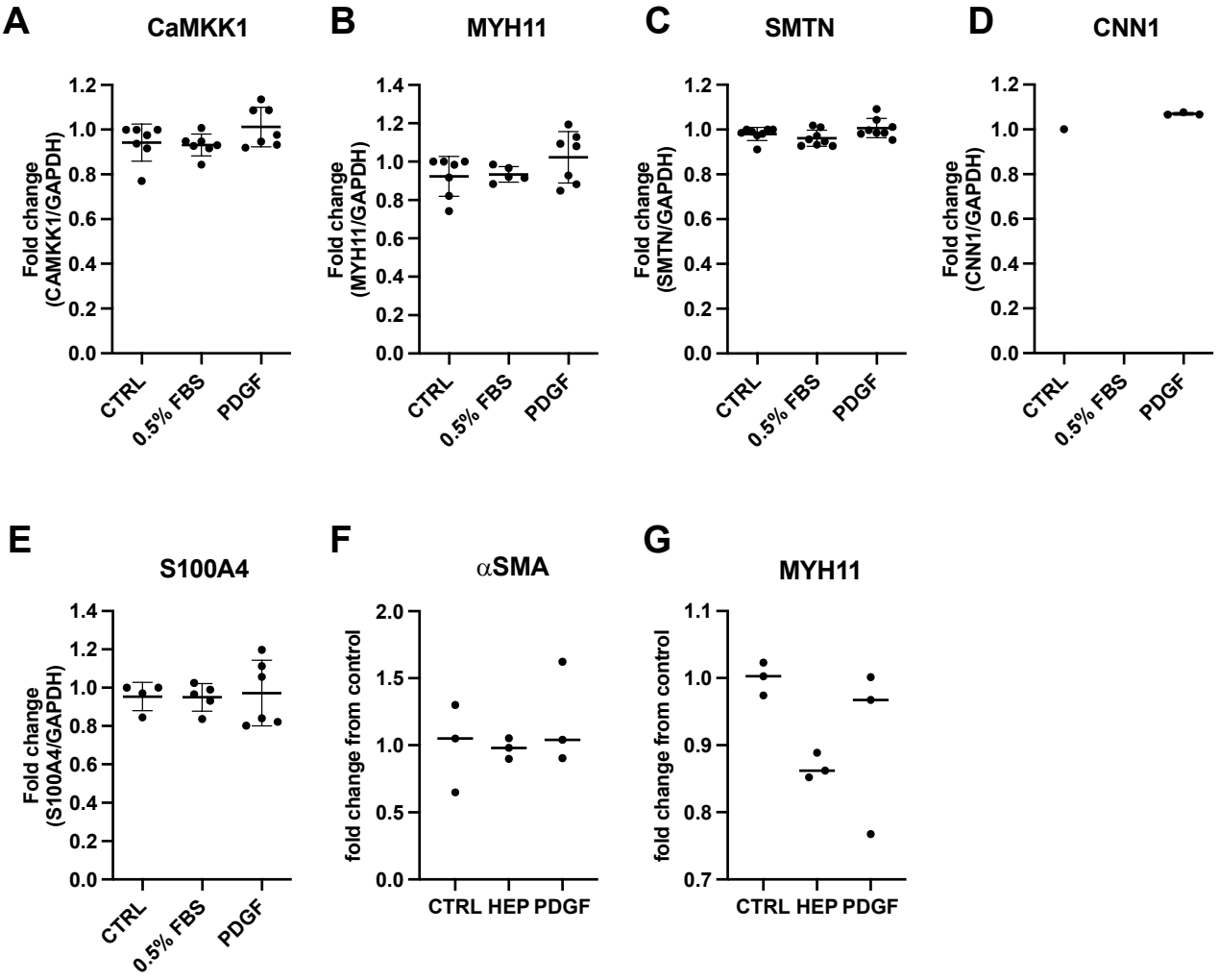


Figure 1. Phenotype switching in hVSMCs. Cells were treated with PDGF-BB (10 μg/ml), heparin (200 U/ml), 0.5% FBS (low FBS) in M199 with 2.5% FBS for seven days. **A-E)** Expression of CAMKK1, contractile markers MYH11, SMTN and CNN1, as well as synthetic marker S100A4 measured by qPCR did not change. **F-G**) Expression of contractile markers MYH11 and αSMA measured by RNA array did not change significantly. All graphs show pooled data from 2 to 3 independent experiments, each treatment (control, HEP, low FBS, PDGF) performed in duplicate for the qPCR and in triplicate for the RNA-seq. Statistical analysis was performed using GraphPad Prism 9. Statistical significance was tested using One-Way ordinary Anova. ns not significant, *P<0.05, **P<0.01, ***P<0.001.

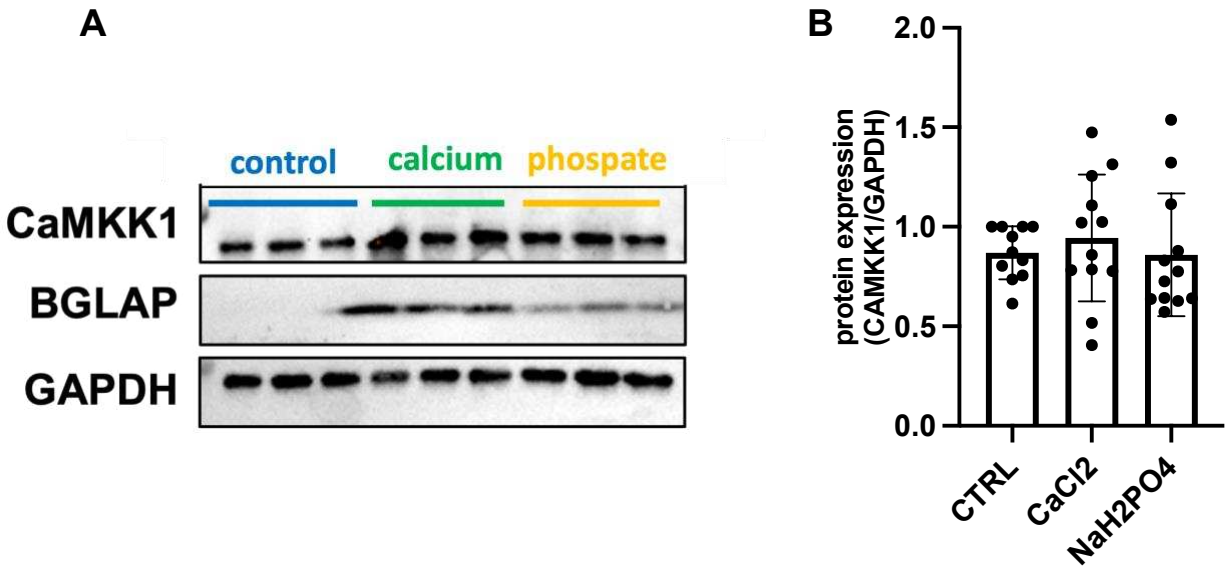


Figure 2. Osteogenic differentiation does not change CaMKK1 expression in hVSMCs. Cells were treated with high concentrations of calcium (3.6 mmol/L $CaCl^2$) and phosphate (2.5 mmol/L NaH^2PO^4) to induce osteogenic differentiation. **A)** Western blotting, and **B)** quantification of CaMKK1 and osteocalcin (BGLAP). Graphs show that the concentrations of CaMKK1 does not change significantly between high concentrations of calcium (3.6 mmol/L $CaCl^2$) and phosphate (2.5 mmol/L NaH^2PO^4). The BGLAP bands in calcium (3.6 mmol/L $CaCl^2$) and phosphate (2.5 mmol/L NaH^2PO^4) confirm the osteogenic differentiation (A). Statistical analysis was performed using GraphPad Prism 9. Statistical significance was tested using One-Way ordinary Anova. No statistical significance was found.

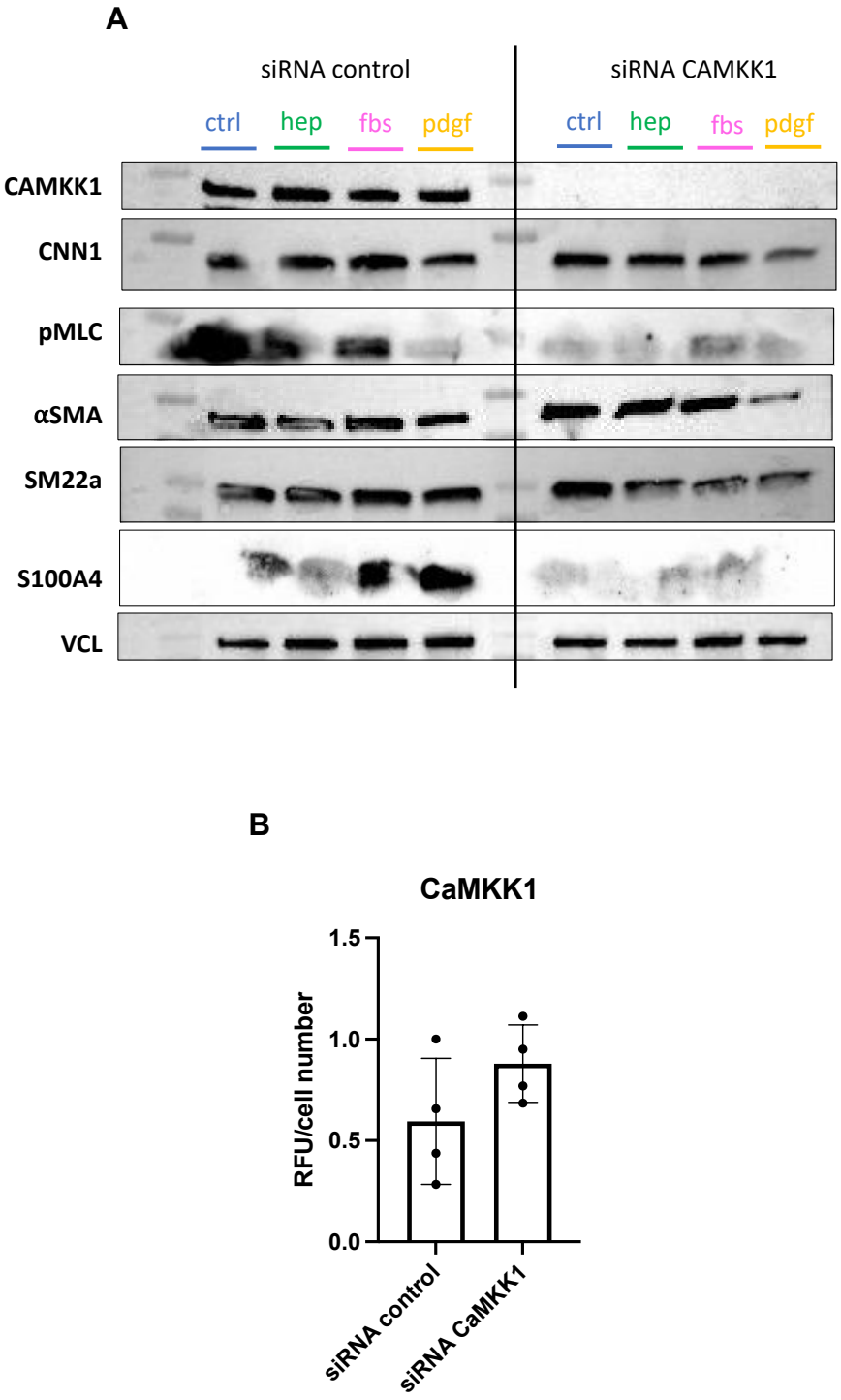
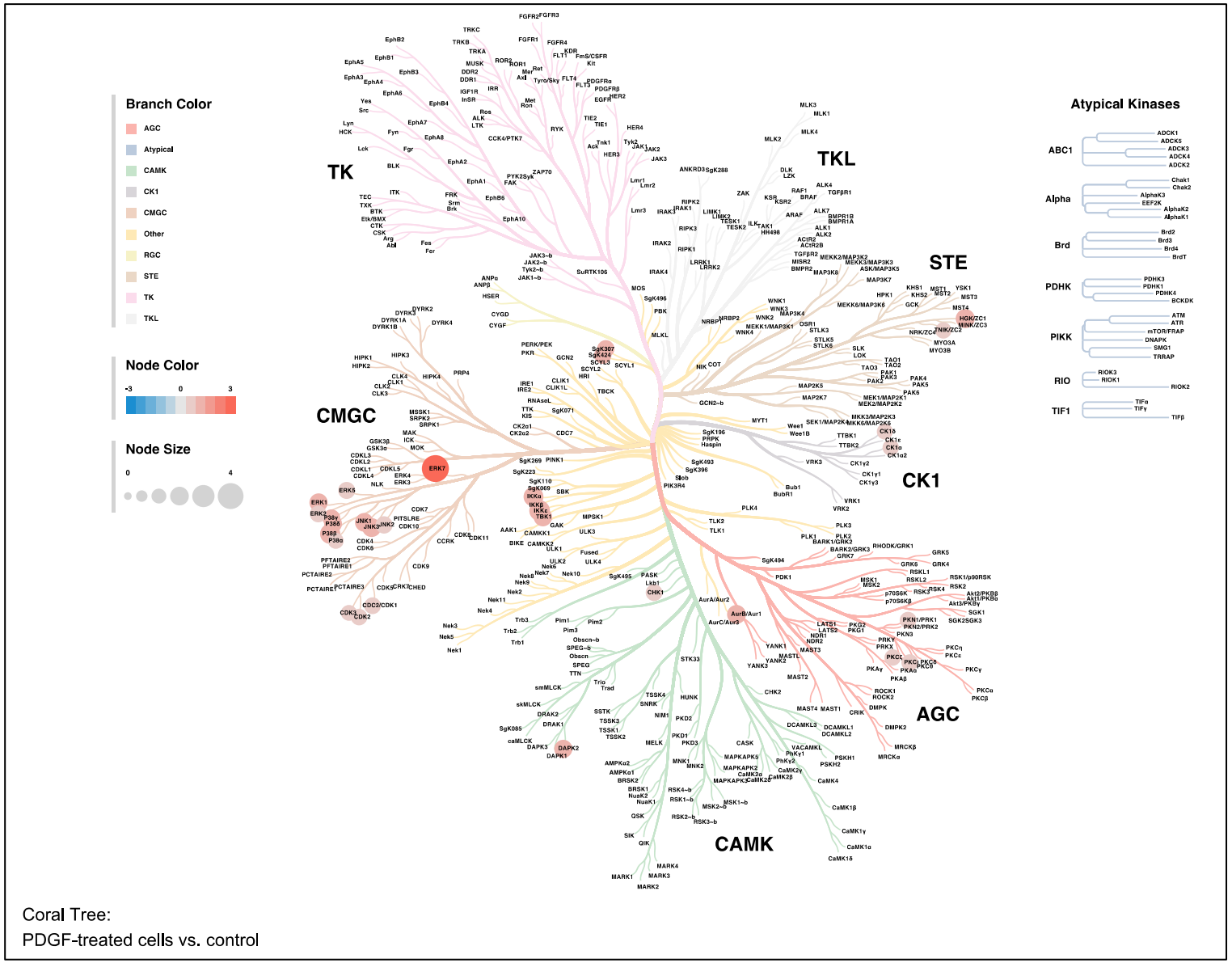
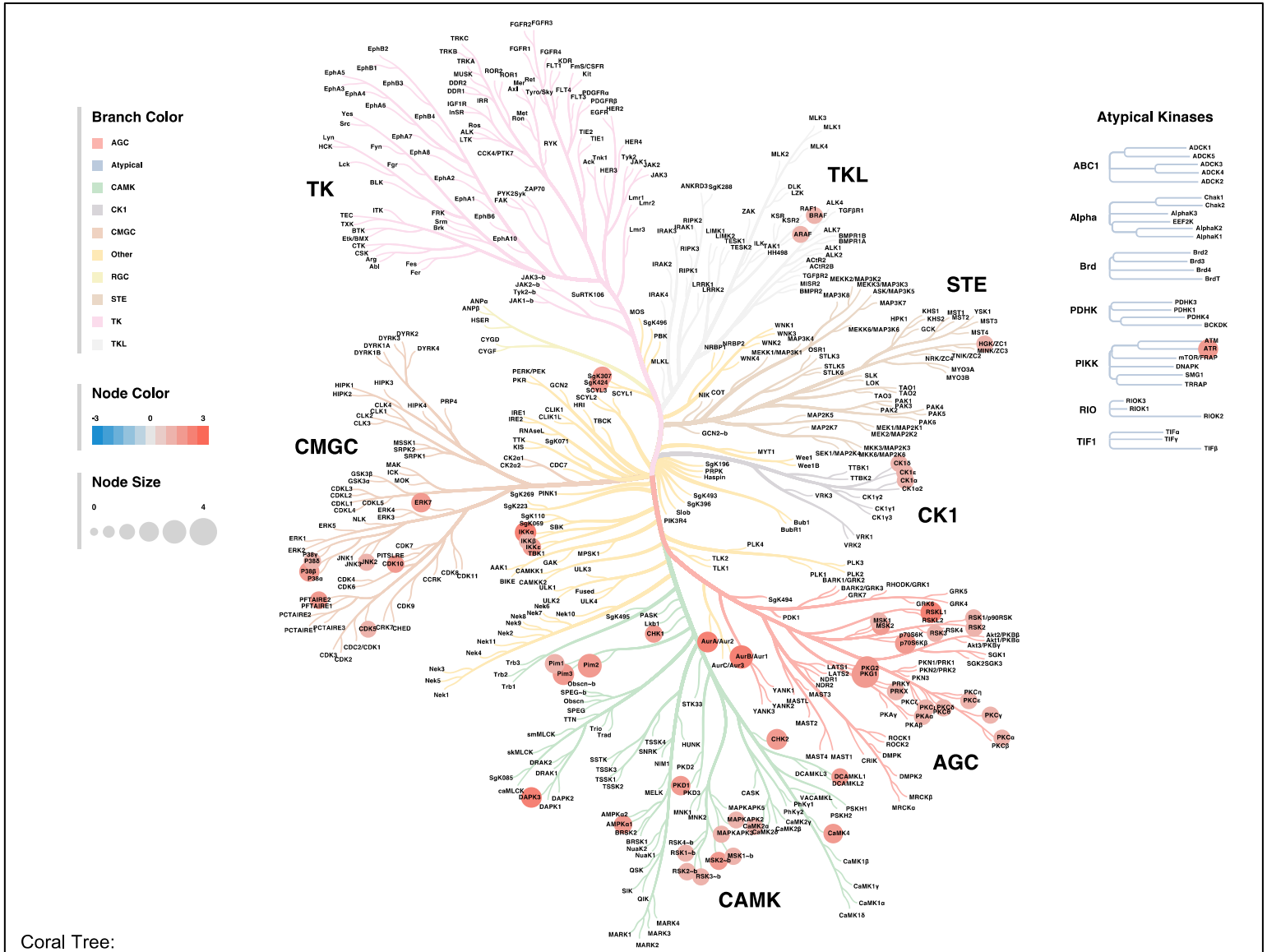


Figure 3. SiRNA knockdown of CaMKK1 in hVSMCs. hVSMCS were transfected with siRNA non targeting pool control and siRNA CaMKK1, respectively. **A)** After 24 hours hVSMC were treated with PDGF-BB (10 μg/ml), 0.5% FBS (low FBS), heparin (200 U/ml) in M199 with 2.5% FBS for seven days Uncropped western blots from Figure 2. **B)** Intracellular calcium was measured using Fluo-4. CaMMK1 knockdown did not affect the intracellular calcium levels. This graph shows data obtained from a single intracellular calcium measurement.

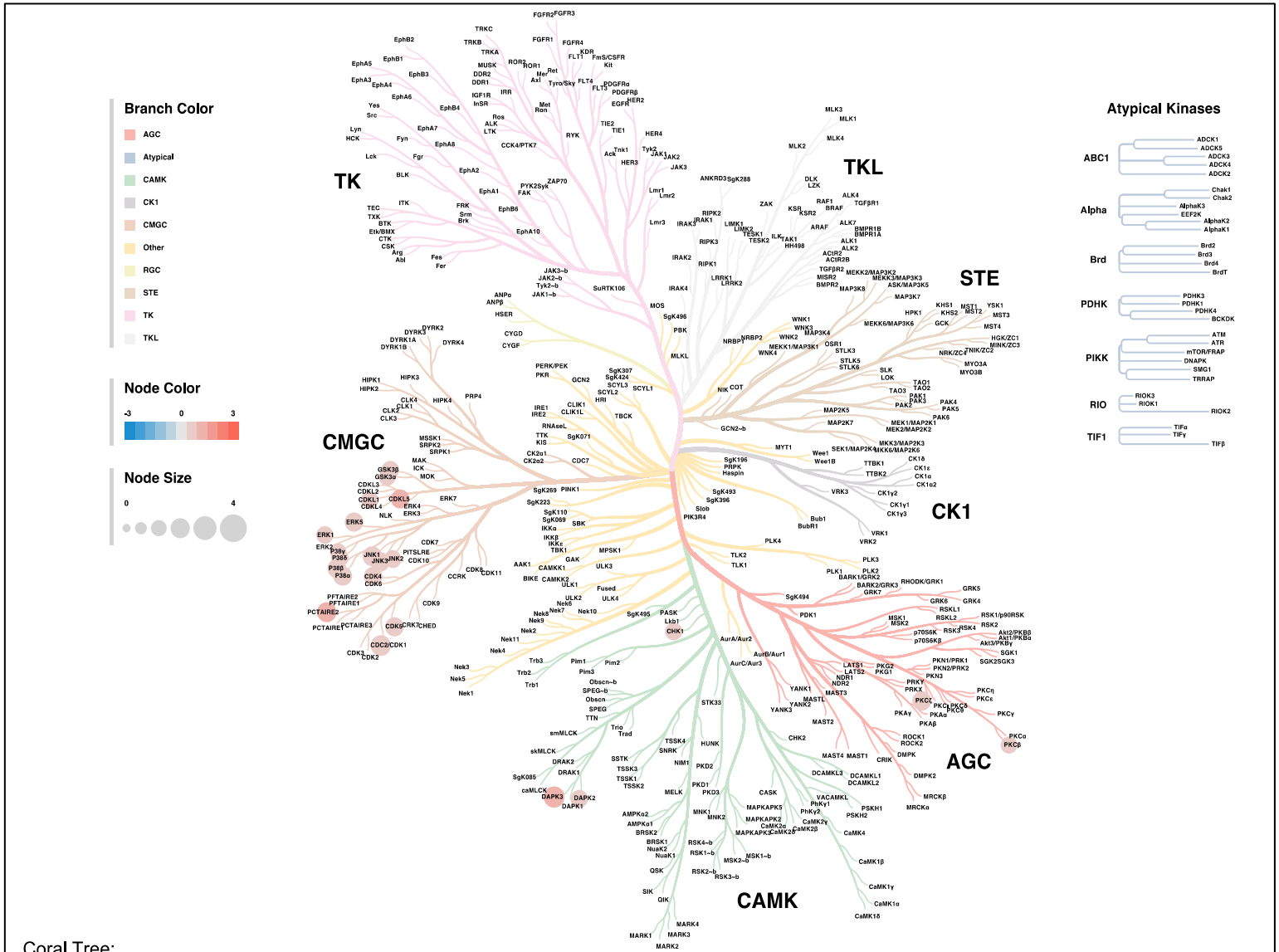
3. Supplemental files



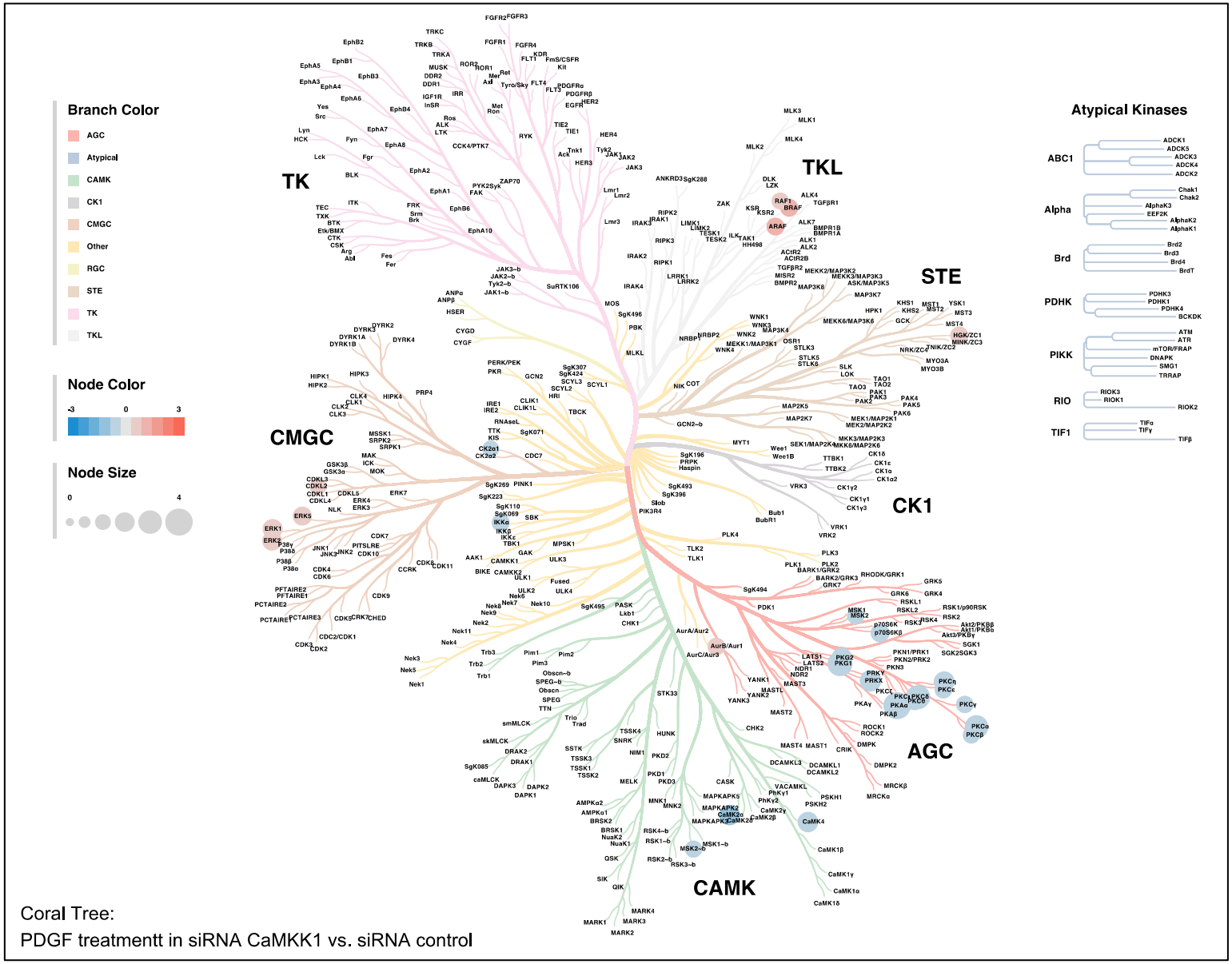
Coral Tree:
PDGF-treated cells vs. control



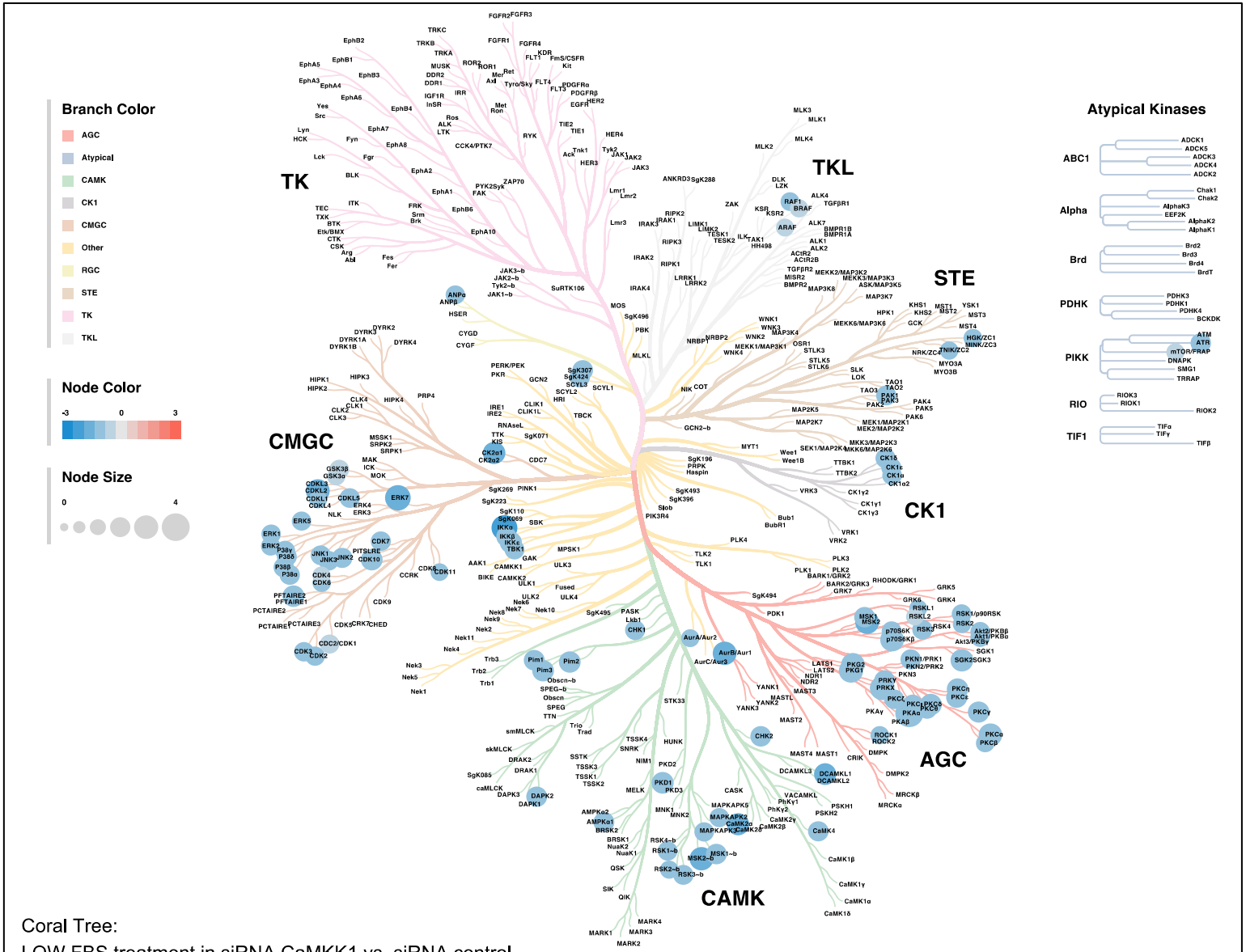
Coral Tree:
LOW FBS-treated cells vs. control



Coral Tree:
CONTROL treatment in siRNA CaMKK1 vs. siRNA control



Coral Tree:
PDGF treatmentt in siRNA CaMKK1 vs. siRNA control



Coral Tree:
LOW FBS treatment in siRNA CaMKK1 vs. siRNA control

APPENDIX 3

Papers

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Review

Gene polymorphisms in calcium-calmodulin pathway: Focus on cardiovascular disease

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ABSTRACT

Cardiovascular disease is the leading cause of death in industrialized countries and affects an increasing number of people. Several risk factors play an important role in the etiology of this disease, such as an unhealthy lifestyle. It is increasingly clear that genetic factors influencing the molecular basis of excitation-contraction mechanisms in the heart could contribute to modify the individual's risk.

Thanks to the progress that has been made in understanding calcium signaling in the heart, it is assumed that calmodulin can play a crucial role in the excitation-contraction coupling. In fact, calmodulin (CaM) binds calcium and consequently regulates calcium channels. Several works show how some polymorphic variants can be considered predisposing factors to complex pathologies. Therefore, we hypothesize that the identification of polymorphic variants of proteins involved in the CaM pathway could be important for understanding how genetic traits can influence predisposition to myocardial infarction. This review considers each pathway of the three different isoforms of calmodulin (CaM1; CaM2; CaM3) and focuses on some common proteins involved in the three pathways, with the aim of analyzing the polymorphisms studied in the literature and understanding if they are associated with cardiovascular disease.

Contents

1. Introduction

Cardiovascular disease (CVD) is one of the main causes of death and disability in the world: more people die annually from this type of disease than from any other causes [1,2]. CVD refers to various conditions that can affect heart function and blood vessels. The leading cause of death in industrialized countries is specifically myocardial infarction (MI). In Europe, MI is one of the main causes of death and it affects an increasing number of people, while in the United States MI is ranked fifth among the causes of death, killing about 133,000 people every year [3].

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1383-5742/

Today, chronic heart failure is one of the most serious health problems, as it has a significant impact on society. Patients with chronic heart failure often have a significantly reduced quality of life: about 30 % of diagnosed individuals (i.e., 1.5 million in the United States) have difficulty breathing and show physical limitations in daily activities. This forced sedentary lifestyle leads inevitably to further physical and mental disturbances [4,5].

Although the pathophysiological spectrum extending from ischemia to mechanical complications of the infarct is well known, the atherosclerotic pathogenesis is multifactorial and is continually being studied [1,2].

In addition to tobacco usage, air pollution, an unhealthy diet, alcohol abuse and physical inactivity, it is increasingly suggested that genetic factors influencing heart excitation-contraction mechanisms play an important role in modifying an individual's risk for different CVDs. [4,6,7].

Several publications illustrate the enormous progress that has been made in understanding calcium signaling in the heart and excitation-contraction (E-C) coupling [8–10].

Inside the complex mechanism of E-C coupling, Ca2+ is crucial to convert electric stimulation into mechanic output: calcium release and reuptake accompanies each heartbeat [11].

The entrance of a low amount of extracellular Ca2+ is allowed by an electrical signal, called action potential, which depolarizes the plasma membrane of cardiac myocytes. This extracellular Ca2+ in turn induces high Ca2+ release from the sarcoplasmic reticulum (SR) [11]. Cytosolic Ca2+ binds to myofilaments and activates contractile machinery [12,13]. This mechanism of "Ca2+ induced and Ca2+ released" allows the functional coupling between plasma membrane and SR. Thus, calcium firstly plays a role as second messenger: it undergoes a transient increase in the myoplasm and triggers the mechanochemical reaction of contraction. The transient increase in myoplasmic free calcium concentration is the result of a flux of calcium release from the sarcoplasmic reticulum (SR) [14]. Ca2+ has also the role of agonist for opening the Ca channels of the SR. The increase in [Ca2+]j in the vicinity of the SR release channels constitutes the normal mechanism of triggering release in the heart (Ca-induced Ca release) [15,16]. Moreover calcium in the E-C coupling seems to have another important role at the "prejunctional" level in the T-tubular membrane of the T-SR junction [14].

The events that occur in E–C coupling depend not only on changes in intracellular calcium concentration ($[Ca2+]_i$) or the combination of properties of Ca channels and transporters, but also on the proteins that regulate Ca channels [10,17,18].

Among all the proteins involved in this complex mechanism it is assumed that calmodulin could be an important regulator of excitation-contraction coupling because of its ability to bind

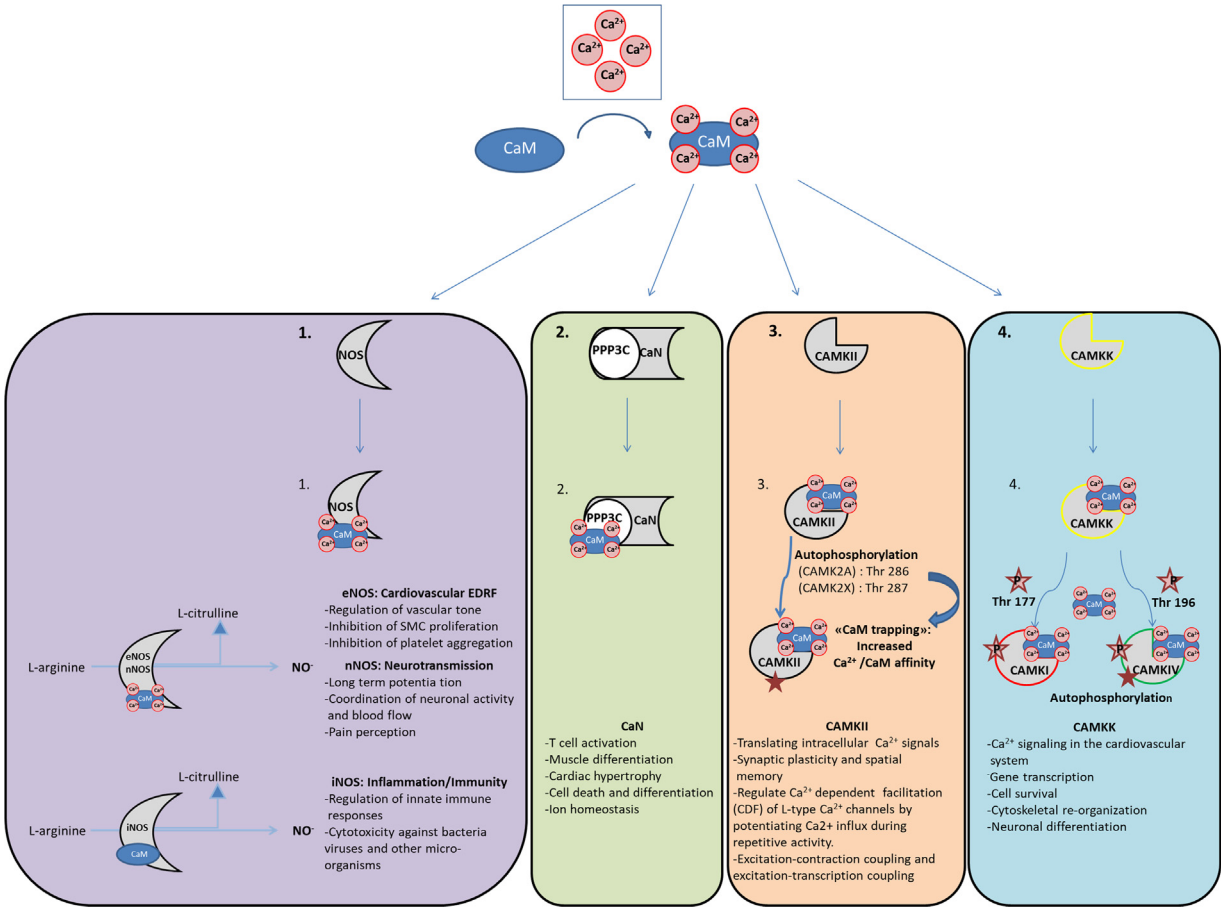

Fig. 1. Interactions between CaM and NOS, PPP3C, CaMK, CaMKK and description of the principal functions in which each complex is involved.
Calmodulin (CaM) is an intracellular target of the secondary messenger Ca2 + . In order to be activated, CaM needs to bind four ions of Ca2 + . Once bound to Ca2+, CaM acts as part of a calcium signal transduction pathway by modifying its interactions with various target proteins. Thus, the binding of CaM is necessary for the activation of all protein family members: NOS, PPP3C, CaMK and CaMKK.
1) The interaction between Ca2+/CaM and NOS family proteins is depicted. Unlike eNOS and nNOS, iNOS activity is independent of the level of calcium in the cell; however its activity is dependent on the binding of CaM. 2) The activation of CaN after the binding of Ca2+/CaM to PPP3C, the catalytic subunit of CaN is depicted. 3) The interaction of Ca2 +/CaM with CAMKII family proteins is depicted. 4) The interaction between Ca2+/CaM and CAMKK family protein and the subsequently downstream phosphorylation pathway is depicted.

calcium and its modulation of Ca channels, even though its precise role has not been defined yet [19,20].

Because several studies show how some polymorphic variants can be considered predisposing factors to complex pathologies [6,21], we hypothesize that the identification of some polymorphic variants of proteins involved in the CaM pathway might be important for understanding whether CVDs have genetic bases.

We started by considering each pathway involving the three different isoforms of CaM (CaM1; CaM2; CaM3) and decided to focus on some common proteins involved in the three pathways.

Based upon initial results, we focused our attention on polymorphisms associated with CVDs: three different isoforms of calmodulin (CaM 1, 2, 3) and three related protein superfamilies: NOS (nitric oxide synthases); PPP3C (protein phosphatase catalytic subunits) and CaMK (calcium/calmodulin dependent protein kinase).

Since this last family CaMK seems to have a central role in the heart [22,23], we also decided to focus on the family of proteins that activates it: calcium calmodulin-dependent protein kinase kinase (CaMKK).

The association of some polymorphisms of the CaM pathway with cardiovascular pathology could be relevant both clinically, for interpreting the severity of coronary pathology, and epidemiologically, for understanding the risk of pathology in the general population.

Among genes considered in this review, we decided to conduct preliminary meta-analyses of polymorphisms investigated in two or more studies, for the same pathology. This choice was made in order to collect enough controls and cases for a robust statistical analysis. In the literature, there are a large number of studies on the polymorphisms of *NOS3*, which have already been the subject of meta-analysis. Thus, to avoid duplicate analysis, we focused our attention on other genes. Only three polymorphisms belonging to *NOS1* met our criteria, having been the subject of three different studies that considered their association with the risk of ischemic stroke (IS). No meta-analyses have been published in relation to these three polymorphism and cardiovascular disease so far. Thus, our approach represents the first attempt to better clarify their role in the increased risk of IS.

2. The three pathways of calmodulin in cardiovascular disease

In this review, we have taken into consideration three different classes of polymorphisms: those in the intron region, those in the untranslated regions (5'-UTR and 3'-UTR) and those in exon regions. Although variations in the intron region could not affect the folding and, thus, the function of proteins, they could influence transcription levels. For this reason, variations localized in regulatory regions should be examined for a possible genetic relationship with disease.

2.1. Calmodulin

Intracellular Ca2+ is an important signal for cardiac contraction and plays a central role in cascade signalling mediated by the protein calmodulin (CaM). CaM binds Ca2+ through four non-identical sites (Fig. 1) creating a conserved domain, called EF-hands (helix-loop-helix structure domain), consisting of two α-helix regions bound together through a loop region of generally 12 amino acids [24]. CaM is a highly-conserved protein and it is involved in many processes because it is able to interact with more than 300 different proteins, with diversity among its four Ca2+-binding sites contributing to its functional versatility [24,25]. Given the important role played by Ca2+ as a secondary messenger and the versatility of CaM, CaM could affect many different cellular functions, such as inflammation, metabolism, apoptosis, muscle contraction, intracellular movement, short-term and long-term memory, nerve growth and the immune response. The canonical mechanism of binding with the other target proteins is the so-called "wrap-around" mechanism, in which the C- and the N-termini of CaM bind to the same regions of the target proteins. In human, there are three different isoforms of calmodulin (CaM1, CaM2, CaM3). The respective genes are dispersed throughout the entire genome (chromosome 14, 2 and 19, respectively), and they encode three highly conserved proteins (differing only at the nucleotide level). The only two variations between them are the presence of 48 additional amino acids in the N-terminal of the CaM2 protein, and the occurrence of a lysine (CaM2) instead of a methionine (CaM1 and CaM3) as the first amino acid of the alignment (Fig. 2).

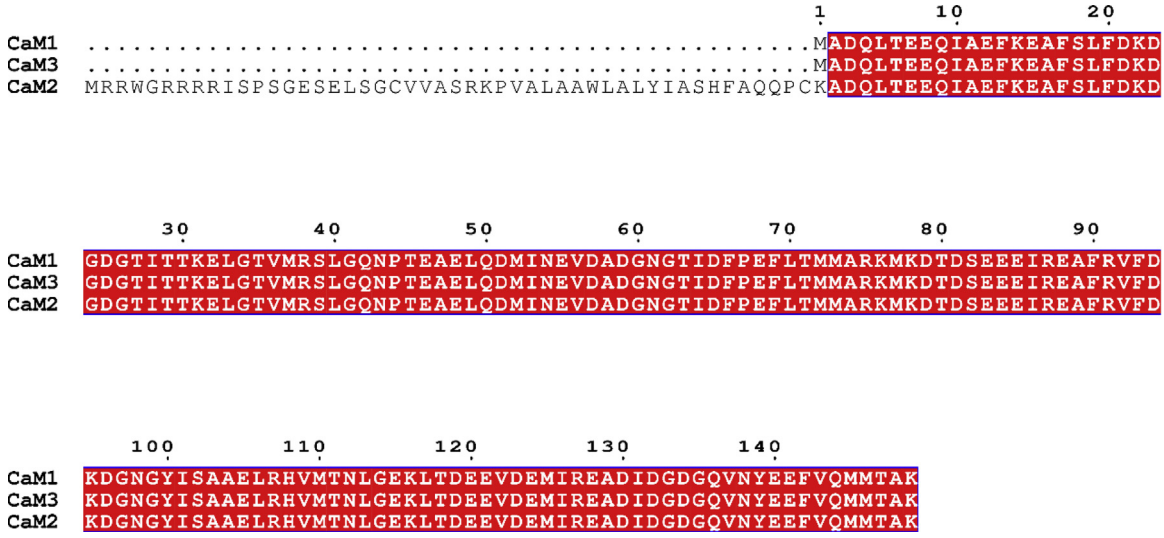

Fig. 2. Amino acid alignment between the three different CaM isoforms.
There is a high level of conservation between the three different CaM isoforms. The only differences are localized in the N-terminal region. In fact, the CaM2 isoform has 48 additional amino acids compared to CaM1 and CaM3. Moreover, the first amino acid of the alignment is a lysine in the CaM2 isoform, instead of a methionine in the CaM1 and CaM3 isoforms. This visualization of alignment was generated using the ClustalX program and ESPript 3.0 utility.

The highly level of conservation among these proteins reflects the important role of calmodulin. In fact, mutations in this class of proteins are related to pathological effects and serious diseases of the heart, like catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden cardiac death (SCD), ventricular fibrillation (VF) and sudden death in adolescence and childhood, due to congenital arrhythmia susceptibility [26–29].

Polymorphisms in CaM gene isoforms have been correlated with different pathologies, like osteoarthritis, adolescent idiopathic scoliosis, etc. [30,31]. However, few studies have attempted to link CaM polymorphisms to cardiovascular disease (Table 1). The polymorphism rs3179089 in the 3'-UTR of the CaM1 gene has been statistically correlated with IS in a Han Chinese population [32]. The polymorphism rs3814843 (also in the 3'-UTR) has been associated with an increased risk for stroke [33,34]. This latter polymorphism was also annotated as conferring a greater risk for sudden cardiac death (SCD) in a Chinese population by Liu et al. [35].

Recently, a new polymorphism (-34T > A) has been identified in the promoter region of the human calmodulin III gene (CaM3), in which the T allele affects the level of CaM3 transcript. It has been reported that patients with familiar hypertrophic cardiomyopathy (FHC) have a higher frequency of the TT-genotype compared to controls and, thus, it could be a modifier gene for FHC [36].

3. The nitric oxide synthase (NOS) pathway

There are three different isoforms of nitric oxide synthase (NOS), the neuronal *NOS1* (nNOS), the endothelial *NOS3* (eNOS), and the inducible *NOS2* (iNOS). These three isoforms have 50–57 % homology. Although named as "neuronal," "endothelial" and "inducible," they can be expressed in a variety of cell types. Thus, the name reflects only the tissue where their cDNA was first isolated [37]. In fact, nNOS and eNOS are constitutively expressed in the cardiomyocyte, whereas the iNOS isoform is normally not expressed in the healthy heart, but inflammation or cardiac damage can induce its expression. The NOS enzymes are activated by the CaM, which they bind, forming a tetramer composed of two monomers of NOSs and two CaMs [38]. After their activation, NOS enzymes catalyse the conversion of L-arginine into L-citrulline, producing nitric oxide (NO) [39], which is an important modulator of myocardial function [40]. In fact, NO· mediates systolic, diastolic, and chronotropic cardiac functions, both in the normal and pathological conditions. NO is important for the correct E-C coupling, because it is involved in the cGMP (cyclic guanosine monophosphate) pathway and in the direct nitrosylation of tyrosine and cysteine thiol-groups of key proteins that regulates E-C coupling and, therefore, heart function [41]. Thus, when the bioavailability of NO is compromised, due to the changes in the activity and/or in the expression of NOS enzymes, or due to its degradation induced by binding with reactive oxygen species (ROS), some cardiovascular pathologies may occur [42].

Based on the important role of NO in cardiac function, NOS enzymes are of particular interest in relation to cardiovascular diseases. Efforts have been made to study the possible involvement of polymorphisms in this class of proteins to cardiovascular pathologies, which are reviewed in this paper.

3.1. NOS1 polymorphism

The human *NOS1* gene (located 12q24.22) consists of 29 exons and 28 introns, with the locus dispersed over a region of 240 kb. This gene encodes the neuronal nitric oxide synthase isoform (nNOS), that was first identified in nitrergic nerves and brain tissue [43]. However, in 1999 Xu et al. [44] identified nNOS expression in cardiac sarcoplasmic reticulum (SR) of cardiac myocytes [45]. Since that time, nNOS has been considered the major endogenous source of myocardial nitric oxide (NO) [45]. NO production by nNOS is fundamental for the regulation of myocardial contraction and relaxation in both the healthy and the diseased heart. nNOS leads to variable effects on heart contractility, mainly influencing four important cardiac proteins: L-type Ca2+ channels (LTCCs), xanthine oxidoreductase (XOR), ryanodine receptor (RyR2) and sarco-endoplasmic reticulum calcium ATPase (SERCA). All of these proteins are involved in the fine regulation of E-C coupling.

In more detail, when LTCC is in the active conformation, there is a release of Ca2+ in the cell. This Ca2+ influx stimulates the opening of RyR2, located in the sarco-plasmatic reticulum, further increasing Ca2+ levels in the cell. Ca2+ binds to troponin C allowing the conformational changes needed for the contraction.

Conversely, relaxation occurs when Ca2+ concentration decreases, and this process is mediated by three proteins: SERCA, that reuptakes Ca2+ into the lumen of SR, and by Na+/Ca2+ exchanger (NCX1) and, to a lesser extent, the plasmalemma Ca2+ ATPase (PMCA), which allow Ca2+ leakage outside the cell [46].

nNOS is located in the SR in close proximity to XOR, a superoxide-generating enzyme, and RyR2. When nNOS is expressed, the NO production exerts an inhibitory effect on myocardial XOR activity, ensuring low doses of superoxide anion and, therefore, the correct nitrosylation of RyR2 cysteines. S-nitrosylation of RyR2 stabilizes the RyR2 open conformation and the release of Ca2+ in the cell that is fundamental for heart contractility through troponin C [41,47].

Moreover, nNOS is also implicated in the relaxation cardiac phase through its influence on SERCA and LTCC proteins. In fact, nNOS-derived NO raises phospholamban (PLB) phosphorylation, a protein closely associated with SERCA, inducing an increase in Ca2 + reuptake into the sarcoplasmic reticulum (SR) [48]. nNOS-

Table 1
Human Calmodulin gene (CaM1 and CaM3) polymorphisms and associations with cardiovascular disease.

Calmodulin 1 (CaM1)

SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases/controls	Model: OR (95 % CI)	Year [Refs.]
Promoter Region							
rs3179089	C/G	3'-UTR	Ischemic stroke	Han Chinese	549/546	Dominant: 1.24 (0.96, 1.61)†	2018 [32]
rs3814843	A/C/T	3'-UTR	Ischemic stroke	Unspecified	3849/—#	Hazard ratio (90 % CI): 1.31 (1.02–1.68)	2009 [34]
			Sudden Cardiac Death	Han Chinese	1429/—#	Recessive - Hazard ratio (95 % CI): 5.54 (2.05 14.95)†	2015 [35]

Calmodulin 3 (CaM3)

SNPs	Genetic Variant	Location	Disease evaluated	Population	Statistical Association	Model: OR (95 % CI)	Refs
Promoter Region							
	T > A	−34	Familial Hypertrophic Cardiomyopathy	Caucasian	429/134	1.19 (0.75−1.90)†	2009 [36]

† when multiple genetic models and/or adjusted OR have been analysed, we reported the most significant one in terms of OR value.
# No case/control study.

derived NO also modulates L-type Ca2+ channels (LTCCs) in the plasma membrane and, consequently, reduces intracellular Ca2+ handling through a S-nitrosylation- or a cGMP-dependent mechanism that induces LTCC inhibition [45] (Figs. 1 and 3).

Thus, given the important role carried out by nNOS, it follows that an imbalance in nNOS function could be implicated in pathological heart conditions. In order to assess a possible relationship between genetic variations and cardiovascular disease, several allelic variations in *NOS1* have been identified and evaluated (Table 2).

The relationship between seven *NOS1* SNPs (rs2293050, rs2139733, rs7309163, rs11068445, rs547954, rs7308402, rs1483757) and IS has been analysed by Manso et al. [49] in a Portuguese population. All of those SNPs seem to be associated with IS under a log-additive model after adjusting for significant covariates, but only four (rs2293050, rs2139733, rs7308402 and rs1483757) of the seven SNPs remained associated significantly with IS after correcting for conducting multiple statistical tests. Moreover, the authors have found that two of them (rs2293050 and rs2139733) are in almost complete linkage-disequilibrium (r2 $\approx$ 0.97). Haplotype analyses showed eight SNPs are associated with stroke, four of which remained significant after correction for multiple comparisons [49].

Dai et al. [50] have investigated the correlation between the same four polymorphisms genotyped by Manso et al. (rs2293050, rs2139733, rs7308402, and rs1483757) of the *NOS1* gene and IS in a Han Chinese population of Northern China. Contrary to the results obtained in the Portuguese populations, the genotype and allele frequency in case and control groups in the Han Chinese population were not different. However, the AG genotype and A allele frequency of rs7308402 were significantly less frequent in IS patients compared to controls and, thus, they have hypothesized that this polymorphism could be a protective factor.

He et al. [51] genotyped three of the four nNOS polymorphisms (rs1483757, rs2293050, rs2139733) in a Northern China Han population, to study the relationship with IS. Because different initial triggers can cause IS, the authors focused their attention on the small artery occlusion (SAO) and left atrial appendage (LAA), which are the main causes of IS. No statistical difference between patients and controls was found in genotypic and allelic analyses when the authors directly assessed the relationship with IS, in agreement with the study Dai et al. (2013) carried out in the same ethnic population. However, when the relationship between the polymorphisms and LAA-caused IS was analysed, significant differences were found in the frequency of T allele (rs2293050) and A allele (rs2139733). Specifically, these polymorphisms were more prevalent in the LAA-caused IS cases. Moreover, in a gender-specific analysis, a marginally significant association with LAA-caused IS was found for the recessive model for both rs2293050 and rs2139733. No statistically significant association was reported for the rs1483757 polymorphism [51].

Pooled genetic SNP data from more than seven hundred blood patient samples was analysed in a genome-wide association study (GWAS) conducted by Zhang et al. [52], with the aim to identify the significant gene variants that are associated with IS in the Chinese population. Variants in eight different genes identified through high throughput sequencing were found strongly associated with IS. Among them, the rs1483757 nNOS polymorphism was associated with IS, contrary to what was reported for Chinese populations in the studies of Dai et al. [50] and He et al. [51], but in agreement with the study of Manso et al. [49] in a Portuguese population.

Levinsson et al. [53] genotyped 58 *NOS* genes between subjects with coronary heart disease (CHD), hypertension patients and case controls. They genotyped 2791 controls and 560 known CHD patients from a Swedish population. Two unreported

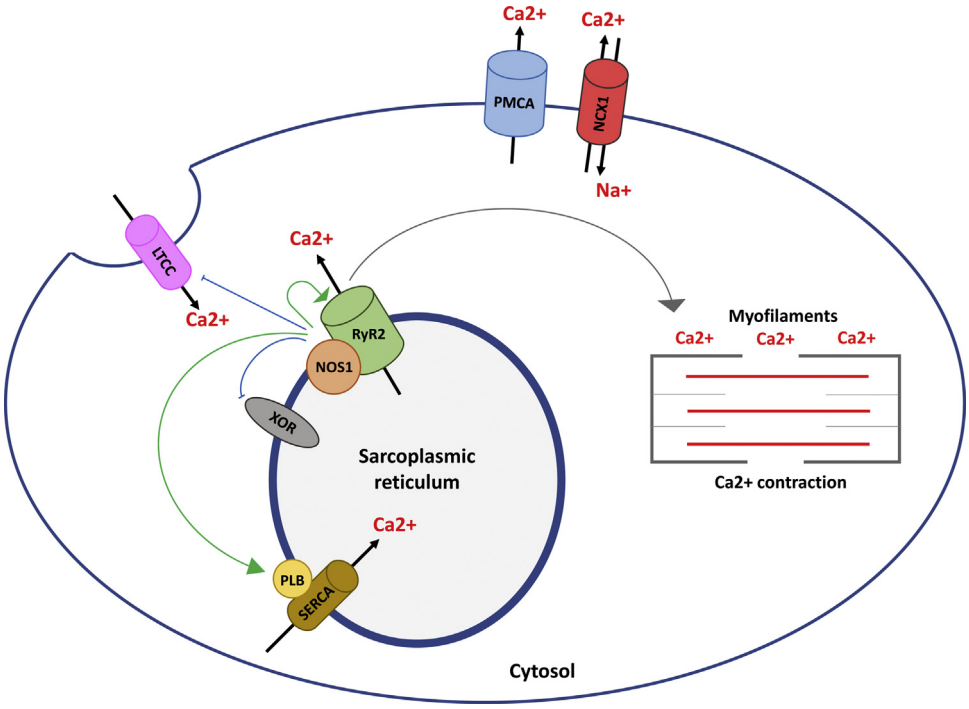

Fig. 3. Positive and negative ionotropic effects of nNOS.
The 'neuronal' NOS isoform (nNOS) is predominantly localized to the sarcoplasmic reticulum (SR) where it is tightly associated with both RyR2 and xanthine oxidoreductase (XOR). When Ca2+ levels increase in the cell through the opening of L-type Ca2+ channel (LTCC), it stimulates RyR2, which releases Ca2+ from SR inside the cytosol, activating the contraction of myofilaments. NOS1 has dual functions in the heart. 1) It is involved in the heart contraction and it inhibits XOR, thus low levels of superoxide anion are present. This allows the correct nitrosylation of RyR2, which is needed for maintaining the receptor in the open conformation. 2) nNOS is also involved in relation because it decreases intracellular Ca2+ through the inhibition of LTCC and the activation of the phospholamban (PLB)-SERCA complex, which reuptakes Ca2+ into the SR.

Table 2
Human NOS1 gene polymorphisms and associations with cardiovascular disease.

Nitric oxide synthase 1 (NOS1)							
SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases/controls	Model: OR (95 % CI)[†]	Year [Refs.]
Exon Region							
rs1047735	G-A	His902His	Ischemic stroke	Korean	120/314	Recessive: 1.29 (0.77–2.17)[†]	2016 [54]
rs2293044	G > C,T	Val1353Val	Ischemic stroke	Korean	120/314	A allele: 0.72 (0.35–1.49)[†]	2016 [54]
rs2293054	A > G,T	Ile734Ile	Ischemic stroke	Korean	120/314	Codominant2: 1.37 (0.52–3.56)[†]	2016 [54]
Promoter Region							
rs2682826	G-A	3'-UTR	Chronic Heart Disease	Sweden	521/2719	Recessive: 1.73 (1.07–2.82)[†]	2014 [53]
			Ischemic Stroke	Korean	120/314	Codominant2: 1.35 (0.63–2.90)[†]	2016 [54]
Intron Region							
rs2293050	C > A,G,T	117281017	Ischemic stroke	Portuguese	472/481	Log-additive: 0.76 (0.63–0.92)	2012 [49]
				Han Chinese	477/413	Recessive: 1.07 (0.73–1.58)[†]	2013 [50]
				Han Chinese	381/366	Recessive: 1.19 (0.80–1.76)[†]	2014 [51]
			LAA-Caused IS Risk	Chinese	ND	ND	2014 [51]
rs2139733	T-A	117288937[*]	Ischemic stroke	Portuguese	447/455	Log-additive: 0.76 (0.62–0.92)	2012 [49]
				Han Chinese	477/413	T allele: 0.99 (0.77–1.29)[†]	2013 [50]
				Han Chinese	381/366	Recessive: 1.14 (0.77–1.69)[†]	2014 [51]
			LAA-Caused IS Risk	Han Chinese	ND	ND	2014 [51]
rs7309163	C-T	117291469[*]	Ischemic stroke	Portuguese	455/465	Log-additive: 1.23 (1.01–1.51)	2012 [49]
rs11068445	G-A	117307124[*]	Ischemic stroke	Portuguese	454/445	Log-additive: 0.72 (0.55–0.96)	2012 [49]
rs547954	A-G	117316701[*]	Ischemic stroke	Portuguese	471/478	Log-additive: 1.29 (1.03–1.61)	2012 [49]
rs7308402	G-A	117321642[*]	Ischemic stroke	Portuguese	472/481	Log-additive: 0.74 (0.60–0.91)	2012 [49]
				Han Chinese	477/413	A allele: 0.61 (0.38–0.99)[†]	2013 [50]
rs1483757	A > G,T	117323735[*]	Ischemic stroke	Portuguese	473/482	Log-additive: 0.77 (0.64–0.93)	2012 [49]
				Han Chinese	477/413	G allele: 1.14 (0.95–1.38)[†]	2013 [50]
				Han Chinese	381/366	Additive: 1.12 (0.73–1.70)[†]	2014 [51]
				Chinese	753/—[#]	GWAS[#]	2015 [52]
			LAA-Caused IS Risk	Han Chinese	ND	ND	2014 [51]
rs3782218	C-T	117333706[*]	Chronic Heart Disease	Sweden	446/2614	Additive: 0.59 (0.44–0.80)	2014 [53]

[*] (GRCh38.p12).
[†] when multiple genetic models and/or adjusted OR have been analysed, we reported the most significant one in terms of OR value.
[#] No case/control study.

polymorphisms in the *NOS1* gene (rs3782218, located in an intronic region, and rs2682826, locate in the 3'-UTR region) and one SNP in the *NOS3* gene (rs1549758, a synonymous polymorphism at the amino acid position of 258) have been significantly associated with CHD through a logistic regression approach. The rs3782218 (*NOS1*) polymorphism (T allele) is strongly associated with both CHD and hypertension. Moreover, Levinsson et al. [53] conducted haplotype analyses and found that the haplotype GT (rs2682826 - rs3782218, related to the two polymorphisms in the *NOS1* gene mentioned previously) confers a protective effect against CHD, with an odds ratio of 0.5.

No statistically significant correlation was found between four single nucleotide polymorphisms of the *NOS1* gene (rs2293054, rs1047735, rs2293044, rs2682826) in a Korean population and IS by Yoo et al. [54]. However, in clinical phenotype analyses, a statistically significant correlation was found between rs2293054 and rs2682826 and the NIHSS (National Institute of Health Stroke Scale) scores of IS patients. Moreover, in allele frequency analyses, Yoo et. al. also reported that T alleles of rs2293054 are associated with lower NIHSS scores ($p = 0.007$) and rs2293054 has a significant association with the Modified Barthel Index (MBI) scores for ISs.

To clarify better the contribution of *NOS1* polymorphisms in cardiovascular diseases, we performed some meta-analyses based on the available studies. Only three *NOS1* polymorphisms have been studied for the same pathology in more than two independent studies. Taking in consideration these papers, the association between *NOS1* rs2293050, rs2139733, rs1483757 and IS was evaluated under four different genetic models. Detailed information about how the meta-analyses were performed and its results are presented in Supplementary Material.

For all the analysed polymorphisms, a total of three articles were retrieved representing case-control studies involving patients with IS belonging to Han Chinese and Portuguese populations. None of the meta-analyses performed showed significant results. From our pooled analysis, the rs1483757 polymorphism does not appear to induce an increased risk for IS since all genetic models showed a lower probability ratio and close to unity (Figs. S2 and S3 and Table S1 of Supplementary material).

On the other hand, our meta-analyses, although not significant, suggest a possible negative association between *NOS1* rs2293050 and rs2139733 polymorphisms and the risk of IS in homozygote, heterozygote and dominant genetic models, but not in a recessive model (Supplementary Material, Fig. S1, Fig. S2 and Table S1), which could be evaluated on a larger number of subjects.

3.2. NOS2 polymorphisms

The human *NOS2* gene is located on chromosome 17, where it encodes the inducible isoform of NOS protein (iNOS). Normally, it is not expressed in healthy myocardium. However, iNOS upregulation has been reported to occur in heart failure patients. The exact role of iNOS in MI is not yet understood, but its expression in myocardial cell increases NO production (Fig. 1), leading to a disturbance in Ca2+ cycling that impairs E-C coupling [41].

Based on a previous study, a locus in proximity to the *iNOS* gene on chromosome 17 has been identified as a relevant region for predisposition to hypertension. Thus, Glenn et al. [55] conducted the first association study of *iNOS* gene polymorphisms and hypertension in an Australian Anglo-Caucasian population. In particular, they assessed variation in the number of repeats for two

polymorphism of *NOS2*, the AAAT/AAAAT (insertion or deletion of 1 repeat unit: "+" allele and "– "allele, respectively) and the $(CCTTT)_n$ repeats, localized 0.7-kb upstream and 2.6-kb upstream of the *NOS2* gene, respectively. The genotypes and allele frequencies were not statistically significant for either variant although, when analyses considered different age groups, homozygosity for the "+" allele was associated with hypertension in older patients.

Previous studies reported that there is an upregulation of *iNOS* in hypertension patients, suggesting a possible role in CVD [56]. Based on this knowledge, Nikkari et al. [57] genotyped two different polymorphisms of the *iNOS* gene in a Finnish population: rs2779249 (C/A) in the promoter region that leads to an overexpression of *iNOS*, and rs2297518 (G/A) in the exon 16, which encodes a Ser/Leu amino acid change, leading to an increased NOS2 protein activity and NO production. The A-allele of rs2297249 was significantly associated with hypertension in 35, 40, and 50 year-old patients as compared to the CC-genotype. On the contrary, no statistical differences were found for G/A rs2297518. In order to study the haplotype effects on hypertension, three different haplotypes were analysed by the authors, and the risk of hypertension was two times higher in the CA-GA haplotype group compared with the CC-GG haplotype group, after adjusting regression analysis for Body Mass Index (BMI) [57].

The rs2779249 polymorphism was also assessed in a Chinese Han population by Fu et al. [58], specifically investigating its correlation with hypertension. Contrary to the results of Nikkari et al. [57] a significantly higher frequency of the CC genotype was found in affected patients after adjusting for age, gender and BMI. Moreover, the AA genotype was mainly identified in the normotensive group, and inversely associated with hypertension, highlighting a possible protective effect of the AA genotype.

High levels of NO have also been correlated with pregnancy-related CVDs, in particular with hypertensive disorders (HPD), such as pre-eclampsia (PE) and gestational hypertension (GH), which are common pregnancy complications that may induce maternal and foetal morbidity and mortality. Thus, Amaral et al. [59] have analysed the correlation between PE or GH and the same polymorphisms genotyped by Nikkari et al. [57]. No significant differences in the allele and genotype frequencies were found between rs2779249 and either of the HPD. However, the A allele frequency of rs2297518 was higher in PE patients compared with the healthy controls. Moreover, taking into account both these polymorphisms, no haplotype differences were found.

The 4 basepair (bp) insertion/deletion (+/-) polymorphism located 0.7 kb upstream to the *iNOS* gene was also analysed by Morris et al. [60] to investigate the correlation between this polymorphism and coronary artery disease (CAD). No correlation was found for either genotype in a Caucasian population. However, a significant association with the plasma glucose concentration and waist/hip ratio was detected in male patients only [60].

The promoter region of the *NOS2* gene has a polymorphism related to the number of repeats of a CCTTT sequence, which is correlated with levels of iNOS expression [61].

Muntwyler et al. [61] genotyped the AAAT/AAAAT (-/+) polymorphism and the CCTTT repeat polymorphisms located in the promoter region of *NOS2* gene to analyse its correlation with CAD. None of the polymorphisms was statistically different between cases and controls in a Caucasian population.

The number of CCTTT repeats was also analysed by Gonzalez-Gay et al. [62] to investigate their association with cardiovascular events (CV) in rheumatoid arthritis (RA) patients, because the accelerated atherosclerosis induced by CV constitutes the main cause of mortality in RA patients. However, no statistical differences were found between RA patient with and without CV, even when the cohort was stratified into those with fewer (8–11) and greater (12–16) numbers of repeats.

In a study that included 200 atrial fibrillation (AF) patients and 240 controls, a number of repeats greater than 12 was significantly associated with AF in a Taiwanese population [63].

A polymorphism in the *iNOS* promoter region, rs2779249 (C/A), the number of CCTTT repeats, and the rs2297518 (G/A) polymorphism in exon 16 were also analysed by Oliveira-Paula et al. [64], without finding a statistically significant association with hypertension. However, when the GA and AA variants of rs2297518 were considered together, a significantly association was found with hypertension. In haplotype analyses using these three variants of *NOS2*, only the SCA haplotype [S group: the number of $(CCTTT)_n$ comprised between 8–11; C for the C/A rs277249 SNP; A for the rs2297518 polymorphism] was positively associated with increased risk of hypertension [64].

Kunnas et al. [65] investigated the association between the AAAT/AAAT repeat polymorphism with indices of stenosis and

Table 3
Human NOS2 gene polymorphisms and associations with cardiovascular disease.

Nitric oxide synthase 2 (NOS2)							
SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases/controls	Model: OR (95 % CI)	Year [Refs.]
Exon Region							
rs2297518	G-A	Exon 6	Hypertension	Finnish	320/439	1.44 (0.51–4.09)†	2015 [57]
				Unspecified	197/113	OR (CI): 2.05 (1.16–3.75)†	2012 [64]
Promoter Region							
–	4bp(+/-) AAAT/AAAAT	0,7 kb upstream	Hypertension	Anglo-Caucasians	ND#	ND#	1999 [55]
			Coronary artery disease	Caucasian	856/—#	ND#	2001 [60]
				Caucasian	260/238	1.10 (0.77–1.58)	2005 [61]
–	VNTR (CCTTT)n	2,6 kb upstream	Hypertension	Anglo-Caucasians	ND#	ND#	1999 [55]
				Unspecified	197/113	OR (CI): 1.22 (0.4–3.41)†	2012 [64]
			Coronary artery disease	Caucasian	260/238	1.02 (0.70–1.48)	2005 [61]
			Cardiovascular events	Spanish	182/—#	1.52 (0.65–3.54)#	2009 [62]
			Atrial fibrillation	Taiwanese	200/240	1.87 (1.10–3.17)	2017 [63]
rs2779249	C-A	−1026C/A	Hypertension	Finnish	320/439	3.83 (1.20–12.27)†	2015 [57]
				Han Chinese	463/432	3.01 (2.27–4.00)†	2009 [58]
				Unspecified	197/113	OR (CI): 0.93 (0.37–2.39)†	2012 [64]
			Gestational Hypertension	Unspecified	166/212	1.10 (0.57–2.13)†	2012 [66]
			Pre-Eclampsia	Unspecified	187/212	1.29 (0.84–1.97)†	2012 [66]

† when multiple genetic models and/or adjusted OR have been analysed, we reported the most significant one in terms of OR value.
# no case/control study.

atherosclerosis of the left anterior descending artery (LAD), right coronary artery (RCA) and left circumflex artery (LCX) in an autopsy study using data from Finnish men. They found a significant correlation between the genotype frequencies only for the subgroup of men aged > 55 years and LAD.

A summary of the results that relate *NOS2* to CVDs is provided in Table 3.

3.3. NOS3 polymorphism

Endothelial NOS (eNOS), also known as nitric oxide synthase 3 (NOS3), is the primary enzyme responsible for the generation of NO in the vascular endothelium, where NO plays an important role in vascular homeostasis. eNOS lacks a PDZ domain, a short and modular amino acid sequence involved in anchoring the protein in the cell membrane. Co-translational and post-translational modifications, like myristoylation and palmitoylation enable eNOS association mainly with the Golgi complex and plasma membrane caveolae, where it is tightly associated with caveolin, which inhibits eNOS. The activation of eNOS is induced by two different mechanisms. The first one involves calmodulin and the heat shock protein 90 (Hsp90), which binds to eNOS and causes its dissociation from caveolin, relieving inhibition. The second mechanism is induced by stimuli, such as stress, insulin, and isometric vessel contraction, which activate eNOS through phosphorylation [67]. Recent studies have shown that nNOS and eNOS have different effects on cardiomyocytes [68]. eNOS has a negative ionotropic effect. Conversely, as reported in Section *3.1*, nNOS has a positive ionotropic effect.

Once activated in endothelial cells, eNOS stimulates the production of cGMP through the activation of the enzyme, soluble guanylate cyclase (sGC). cGMP binds to cyclic-GMP-dependent protein kinases that promote the phosphorylation of L-type Ca2+ channels (LTCCs). Thus, eNOS inhibition lowers cellular Ca2+ concentrations and promotes vascular relaxation. Moreover, cGMP activates a cAMP phosphodiesterase 2, which converts cAMP (cyclic adenosine monophosphate) in AMP (adenosine monophosphate). In caveolae, nNOS is also compartmentalized with β-adrenergic receptors (β-ARs) that stimulate contraction [69]. In fact, β-agonists increase LTCC function by stimulation of adenylyl cyclase, formation of cAMP and activation of protein kinase A (PKA). This latter protein, PKA, in conjunction with the protein kinase Akt, stimulates the phosphorylation and the activation of nNOS [70]. As a consequence, NOS3 decreases the functional response to β-AR stimulation [68] inducing a negative feedback effect [71].

Of the three main isoforms of nitric oxide synthase, NOS3 is the most studied. There is a lot of evidence regarding its correlation with CVDs. In fact, different variants in the promoter, intron and exon regions have been investigated in a large number of populations, but the results remain inconclusive. This paper includes a representative review of this work to illustrate the state of understanding regarding such polymorphisms in cardiac pathologies.

The eNOS gene locating on chromosome 7q35–36 contains 26 exons and encodes a 135 kD protein including 1203 amino acids.

Three main polymorphisms in the *NOS3* gene have been genotyped and analysed for the predisposition to CVDs (Figs. 1 and 4 and Table 4).

One of these polymorphisms is in a coding region and was widely studied in different populations. The rs1799983 polymorphism (G/T) is located at the exon 7 of the *NOS3* gene and encodes an amino acid substitution at the residue 298 of eNOS protein (Glu^{298}→Asp^{298}). The variation of glutamate acid for aspartate

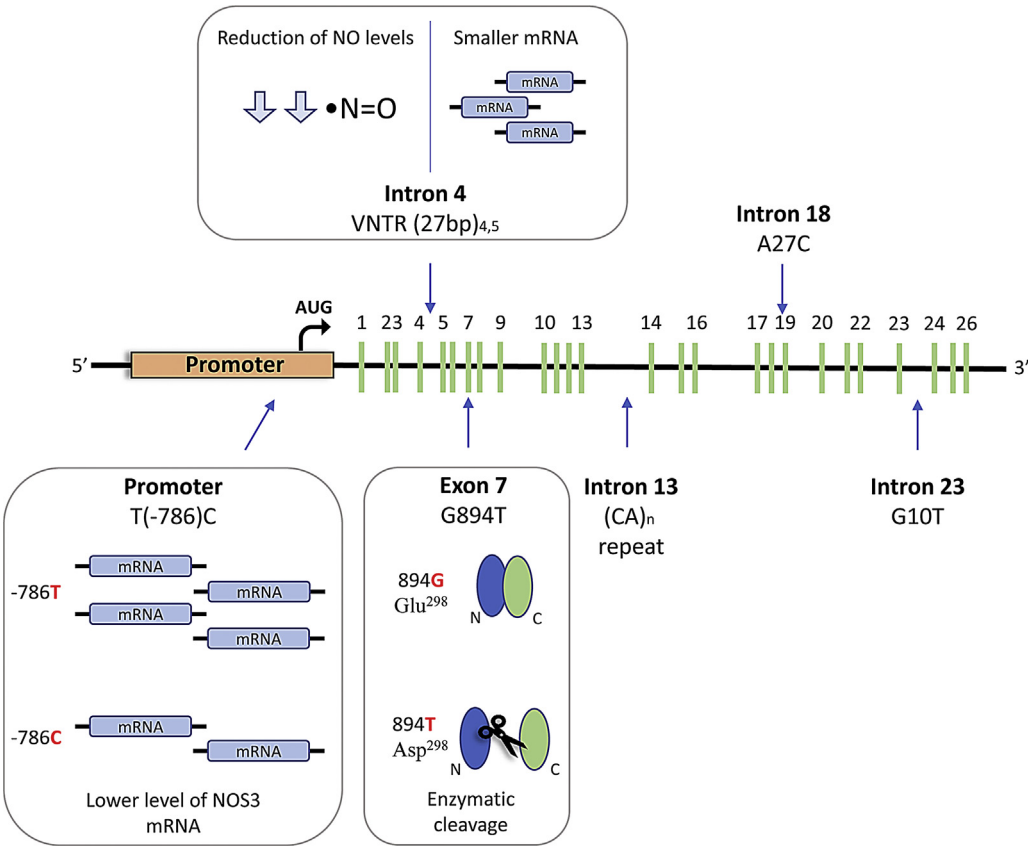

Fig. 4. The polymorphisms in the *NOS3* gene most relevant for CVD and their known pre- and post-transcriptional effects.

Table 4
Human NOS3 gene polymorphisms and associations with cardiovascular disease.

Nitric oxide synthase 3 (NOS3)							
SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases/controls	Model: OR (95 % CI)	Year [Refs.]
Exon Region							
rs1799983	G/T	Exon 7 (Glu298Asp)	Coronary artery disease	East Anglian	298/138	4.2 (2.3–7.9)	1999 [73]
				Han Chinese	208/217	1.649 (1.41−2.382)	2016 [92]
				Caucasian	1106/119	ND	2003 [75]
				Tunisian	332/368	Dominant: 2.84 (2.09–3.86)†	2015 [85]
				Pakistan	198/178	5.717 (3.586−9.115)	2014 [74]
				Australian	763/—#	ND#	1999 [100]
				Korean	147/222	1.12 (0.65–1.92)	2007 [86]
				Korean	110/128	ND	2000 [101]
			Premature CAD	Egyptian	116/119	2.6 (1.2–5.7)	2013 [102]
			Myocardial infarction	East Anglian	249/183	2.5 (1.3–4.2)	1999 [73]
				Australian	763/—#	ND#	1999 [100]
			Hypertension	Sudan	147/82	T allele: 0.80 (0.47–1.35)†	2017 [103]
				Japanase	218/240	2.3 (1.4–3.9)	1998 [90]
				Caucasian	311/128	ND	1998 [91]
				Han Chinese	888/637	1.16 (0.90–1.51)†	2015 [76]
Promoter Region							
rs2070744	T-786C		Coronary artery disease	Caucasian	1106/119	1.672 (1.062–2.527)	2003 [75]
				Han Chinese	208/217	1.490 (1.031−2.153)	2016 [92]
				Korean	147/222	1.61 (0.97–2.69)	2007 [86]
				Iranian	241/261	2.1 (1.5–3)	2012 [93]
Intron Region							
rs61722009	VNTR (27bp) [4a/4b]	Intron 4	Ischemic Stroke	Han Chinese	457/457	1.19 (0.84–1.70)	2013 [82]
				Chinese	364/517	2.44 (1.60–3.71)†	2001 [83]
				Singapore	120/207	ND	2008 [104]
			Coronary artery disease	Tunisian	332/368	1.01 (0.75–1.37)†	2015 [85]
				Caucasian	625/413	ND	2000 [84]
				Korean	147/222	1.44 (0.87–2.39)	2007 [86]
				Korean	110/128	ND	2000 [101]
			Hypetension	Japanase	218/240	1.1 (0.8–1.6)	1998 [90]
–	VNTR (CA)n	Intron 13	Coronary artery disease	Caucasian	1000/1000	1.94 (1.31–2.86)†	2000 [87]
			Hypertension	Japanese	200/246	3.7 (ND)	1997 [88]
				Caucasian	198/106	ND	1995 [89]
–	A-27C	Intron 18	Hypertension	Caucasian	198/106	0.88 (0.60–1.27)	1995 [89]
				Japanase	218/240	1.4 (0.6–3.3)	1998 [90]
				Caucasian	311/128	ND	1998 [91]
–	G-10T	Intron 23	Hypertension	Caucasian	198/106	0.86 (0.60–1.24)	1995 [89]
				Japanase	218/240	1.1 (0.8–1.7)	1998 [90]
				Caucasian	311/128	ND	1998 [91]
			Coronary artery disease	Korean	110/128	ND	2000 [101]

† when multiple genetic models and/or adjusted OR have been analysed, we reported the most significant one in terms of OR value.
# no case/control study.

affects the susceptibility of eNOS to enzymatic cleavage. Specifically, only the protein with aspartate can be cleaved, generating N-terminal 35-kDa and C-terminal 100-kDa fragments [72].

One of the first studies in which *NOS3* polymorphisms were associated with CVD was conducted by Hingorani et al. [73], who analysed in two independent studies (CHAOS and CHAOS II) the correlation between the rs1799983 polymorphism located in the exon 7 of eNOS and two different CVDs [atherosclerotic coronary artery disease (CAD) and the MI] on an East Anglia population. They found a greater number of Asp298 homozygotes, both in patients with CAD and MI compared to the control cohort, and a stronger correlation with CAD than MI.

Similarly to those results, a very strong association was found between the TT polymorphism (Asp298) and CAD in a Pakistani population, with the polymorphism accounting for a 5.717 times increased risk [74].

However, the results of association studies related to the G894 T polymorphism and CVDs are contradictory. The same polymorphism was analysed for a correlation with CAD by Rossi et al. [75] on a Caucasian population, where they did not detect any effect of the Glu298Asp missense variant on CAD.

To clarify a possible role of rs1799983 in CVDs, similar studies were conducted on different population cohorts, the results differed depending on the population, and the cardiovascular pathologies analysed. For example, Liu et al. [76] highlighted how this polymorphism was associated with CAD for a Northern but not for a Southern Chinese population. In this review, we summarized the most impactful reports related to this polymorphism, with additional studies presented in Table 4 and/or Wattanapitayakul et al. [77].

Other relevant polymorphisms found in CVDs are those present in intronic regions. Of particular interest is the variable number of tandem repeats (VNTR) of a 27-bp consensus sequence repeat in intron 4, which have been associated with the reduction of NO levels in the plasma and to the inhibition of *NOS3* mRNA expression through the production of a small RNA that seems to interfere with its expression [78,79].

The number of the 27-bp sequence repetitions distinguishes the predominant 4*a* (repeated 4 times) and 4*b alleles* (repeated five times), although rarer alleles have been reported (*allele 4c* and *allele 4y*, with six and two copies of the 27-bp DNA fragment, respectively) [80]. Zhang et al. [81] reported that cells containing five repetitions (4b allele) of the 27-bp sequence produced higher

levels of siRNA and, thus, lower levels of *NOS3* mRNA than cells with four repetitions (4a allele).

Many studies have examined the rs1799983 polymorphism. However, a clear correlation with CVDs has not been found. The results, including some in the same specific cardiovascular pathology, appear to be contradictory. For example, no statistical significance has been found for this polymorphism and IS in a Han Chinese population [82]. On the contrary, Hou et al. [83] reported a statistically significant association between the *a allele* (allele with four repeats) and IS in a Chinese population.

Although Sigusch et al. [84] and Ben Ali et al. [85] found no association between the 27-bp tandem repeat polymorphism and CAD in Caucasian and Tunisian populations, the 4a/4b polymorphism was associated with CAD in a Korean population after adjustment for cardiovascular risk factors [86]. Thus, the role and contributions of NOS3 intron variations remain unclear.

A second VNTR has been identified within intron 13 of the *NOS3* gene. Some studies have attempted to relate this polymorphism with cardiovascular pathologies. For example, Caucasian subjects who possess a number of CA bases repeats greater than or equal to 38 could be at increased risk of developing CAD [87].

Other studies have focused on hypertension and a statistical association between hypertension and the CA microsatellite polymorphism was found in a Japanese population [88]. Conversely, another study found the number of CA repeats to be similar in hypertensive versus normotensive subjects in a Caucasian population [89].

Two other polymorphisms in intron regions were analysed for a predisposition to hypertension disease, specifically the nucleotide substitution A27 to C in intron 18 and the G10 to T polymorphism in intron 23. However, neither of the two polymorphisms were implicated in the predisposition to hypertension, because no statistically significant differences were found between patient and control cohorts [89–91].

The third type of allelic variation that has been identified is within promoter regions. For example, the −786 T > C polymorphism, located in the promoter region of the *NOS3* gene markedly decreases the levels of the *NOS3* transcript. Generally, it has been found that the incidence of this polymorphism is greater in CAD patients than controls, and a strong association has been reported in different studies analysing different populations.

In fact, Rossi et al. [69] have found that the -786 T→C polymorphism was significantly associated with CAD in a Caucasian population, as well as in Chinese, Korean and Iranian populations [75,86,92,93].

Other authors have also analysed the linkage disequilibrium between two or more *NOS3* polymorphisms. For example, Colombo et al. considered the Glu^{298}→Asp and T^{786}→C variants in an Italian

Table 5
Human PPP3CA, PPP3CB, PPP3CC gene polymorphisms and associations with cardiovascular diseases.

Protein Phosphatase 3 catalytic subunit alpha (PPP3CA)

SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases /controls	OR (95 % CI)	Year [Refs.]
Exon Region							
–	G/A A83A	Exon 2	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]
–	G/C L365L	Exon 10	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]
Promoter Region							
–	G193T	5' region	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]
–	CGG repeat (from one copy up to four repeats)	5' UTR	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]
Intron Region							
–	T/C	Intron 1	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]
–	T/G	Intron 1	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]

Protein Phosphatase 3 catalytic subunit beta (PPP3CB)

SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases /controls	OR (95 % CI)	Year [Refs.]
Promoter Region							
–	G/A	3' UTR	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]
Intron Region							
–	G/C	Intron 7	Cardiac hypertrophy Dilated cardiomyopathy	European	95/—[#]	ND	2003 [111]
rs3763679	C/T	Intron 3	Cardiac phenotype (i.e resting HR) at the pre-training state	Chinese	102/—[#]	ND	2010 [112]

Protein Phosphatase 3 catalytic subunit gamma (PPP3CC)

SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases /controls	OR (95 % CI)	Year [Refs.]
Promoter Region							
rs7430	C/G	3' UTR	Training responsiveness of cardiac phenotypes after a cycle-ergometry exercise bout	Chinese	102/—[#]	ND	2010 [112]
Intron Region							
rs1879793	C/T	Intron 1	Training responsiveness of cardiac phenotypes after a cycle-ergometry exercise bout	Chinese	102/—[#]	ND	2010 [112]
rs10108011	A/G	Intron 1	Training responsiveness of cardiac phenotypes after a cycle-ergometry exercise bout	Chinese	102/—[#]	ND	2010 [112]
rs1075534	A/G	Intron 6	Training responsiveness of cardiac phenotypes after a cycle-ergometry exercise bout	Chinese	102/—[#]	ND	2010 [112]
rs2661483	C/T	Intron 10	Training responsiveness of cardiac phenotypes after a cycle-ergometry exercise bout	Chinese	102/—[#]	ND	2010 [112]

[#] NO case/control study.

population. Significant linkage disequilibrium was found between these two polymorphisms and CAD; in particular, individuals with Asp/Asp genotype and at least one C^{786} allele had an higher risk of CAD compared with individuals with only one of the two polymorphisms [94].

Several meta-analyses and systematic reviews have considered the correlation between *NOS3* polymorphisms and different kinds of CVDs. Polymorphism G894 T seems to be a predisposing factor for different CVDs, such as essential hypertension (ES) [76], thrombotic disease [95] and CAD (especially amongst the Middle Easterners) [96,97]. Similar results have been obtained for 27bp-VNTR, which was positively associated with IS (IS) [82]. However, controversial results have been obtained from three meta-analysis studies for predisposition to CAD [96–98]. Two of them reported a significant positive association with CAD [96,98], which was greater for in Asian population than European, Middle Eastern, Asian-Indian and African population [96]. Another study did not find any association [97]. Nevertheless, it should be mentioned that this latter study took into consideration a limited number of papers and subjects compared to the first two. Meta-analyses studies were also performed on the -786 T > C polymorphism and CAD, finding a positive association in different genetic models [99] and in particular amongst Middle Easterners [96]. Thus, the 786 T > C polymorphism seems to confer a lower CAD risk compared with the polymorphism G894 T [96]. Taken together, meta-analyses results suggest all three polymorphisms arepredisposing factors for CVDs.

4. PPP3C pathway and polymorphisms

PPP3CA, PPP3CB, PPP3CC, protein phosphatase 3 catalytic subunit alpha, beta and gamma are three isoforms encoded by separate genes on chromosome 4, 10 and 8 respectively. They compose Calcineurin A (CaNA), the catalytic subunit of calcineurin. Calcineurin (CaN) is a serine/threonine phosphatase comprised of two subunits: the catalytic subunit A (CaNA) described previously, which contains the phosphatase domain, and the regulatory subunit B (CaNB), which binds calcium and the A subunit [105,106].

This complex plays an essential role in the transduction of intracellular Ca^{2+}-mediated signals. Specifically, in response to growth stimuli, cardiomyocytes increase their cytosolic Ca^{2+} level, which in turn activates calcineurin [107,108]. At low calcium concentration, CaN is in an inactive state with the catalytic subunit CaNA that binds the regulatory subunit CaNB; in which only two high-affinity binding sites are occupied by Ca^{2+}. Upon an increase in calcium concentration, there is a conformational change and the subsequent binding of Ca2+-bound CaM activates the phosphatase (CaNA) (Fig. 1) [106].

Among the different pathways in which CaN is involved in multiple pathways, including T-cell activation, muscle differentiation, ion homeostasis, development and function of the central nervous system. Molkentin et al. [109] reported that calcineurin has an important function in the development of cardiac hypertrophy, through activation by dephosphorylation of the nucleic factor of activated T cells (NFATC4). Furthermore, the CaN pathway plays a critical role in cardiac remodeling, development of chamber dilatation and progression of heart failure. Components of the CaN pathway are involved in myocardium hypertrophy regulation, angiogenesis and apoptosis [109,110].

Given the potential importance of the calcineurin pathway in cardiac hypertrophy in humans, it is important to understand the genetic variability within its components, such as *PPP3CA, PPP3CB* and *PPP3CC*, which are the three isoforms of CaNA.

Poirier et al. [111] designed a study where they screened genes encoding different major components of the heart CaN pathway, including *PPP3CA* and *PPP3CB*, in 95 individuals and identified 27 polymorphisms [111].

Among the observed variants, six were located in the *PPP3CA* gene and two in the *PPP3CB* gene, but none of them was associated with cardiac phenotype. The position and the type of these polymorphisms are described in Table 5. The only variants associated with indexes of cardiac hypertrophy were found in *NFATC4*, a gene involved in the CaN pathway [111]. It might be interesting in the future to investigate these polymorphisms of the cardiac calcineurin pathway in patients with other types of heart disease and in situations where they could act as response modifiers of cardiac phenotypes.

In another study, He et al. [112] analyzed the association of 55 polymorphisms in genes coding for *CaNA* (PPP3CA, PPP3CB, PPP3CC) and calcineurin B (PPP3R1, PPP3R2), by specifically analyzing both the pre-training levels and responsiveness to endurance training in echocardiographic variables in healthy young Han Chinese men.Focusing only on the calcineurin genes involved in CaM pathway, among the 55 polymorphisms considered, 34 were located in PPP3CA, 3 in PPP3CB, and 12 in PPP3CC. The main association they found was between the polymorphisms rs1879793, rs1075534, rs7430, rs2461483 and rs10108011 in PPP3CC and the training responsiveness of cardiac phenotypes after a cycle-ergometry exercise bout. Only one polymorphism rs3763679 in the PPP3CB gene influenced an important cardiac phenotype (i.e., resting heart rate) at the pre-training state. Polymorphism rs3763679 in the *PPP3CB* and polymorphisms rs1879793, rs1075534, rs2461483, rs10108011 in *PPP3CC* are all intron variants, whereas polymorphism rs7430 is located in the 3' UTR (see Table 5).He et al. [112] found that the remaining several SNPs are associated with trainability of cardiac phenotypes but they are located in intron genomic regions and so it is uncertain whether these intronic SNPs are functional variants. Because these polymorphisms are associated with the training responsiveness of cardiac phenotypes, it might be interesting to study whether these polymorphisms are also associated with some specific CVD.

5. CaMK pathway and polymorphisms

Among the downstream targets of CaM proteins studied in this review, Ca^{2+}/calmodulin dependent kinase (CaMK) is one of the best characterized groups of proteins. This category of enzymes catalyzes the transfer of phosphate from the gamma position of ATP to the hydroxyl group of Ser, Thr or Tyr within protein substrates [113–115].

CaM protein kinases have similar protein structures (see Fig. 5), with a conserved catalytic domain and a regulatory domain, containing an autoinhibitory domain and a CaM-binding domain [116]. CaMK is an important class of proteins, involved in different processes and cellular functions, including membrane excitability, gene expression, cell cycle activity and ion channels regulation [115,117,118]. Specifically, in the context of the heart, protein kinase activity regulates heart function through different signal

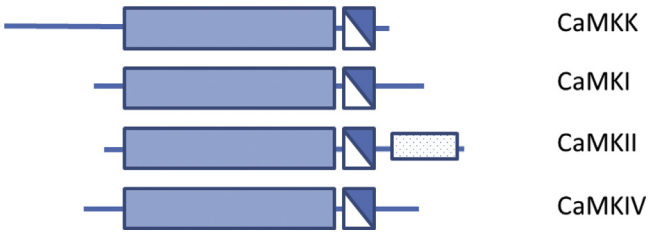

Fig. 5. Domain structures of CaM-kinases.
The general domain structure and organization of the CaM-kinases is shown, with an N-terminal catalytic domain (LIGHT BLUE) followed by a regulatory region that contains overlapping autoinhibitory (WHITE) and CaM-binding domains (BLUE). The C-terminal association domain of CaMKII is shown as white with black dots.

transduction cascades, both in pathologic and physiologic conditions [114,115].

Among the various kinases, Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) is one of the best-studied members. CaMKII is a serine-threonine kinase with unique macromolecular structure assembled into homo- or hetero-multimeric holoenzymes, composed of 12 subunits with two hexameric rings stacked one on top of the other [115,119]. Each CaMKII monomer consists of three subunits: a catalytic, a regulatory and an associated subunit [120,121].

There are four different isoforms of CaMKII (α, β, γ, δ) in mammals and each of them is encoded by a separate gene (*CAMK2A, CAMK2B, CAMK2G, CAMK2D*) [122]. *CAMK2A, CAMK2B*, and *CAMK2D* are involved in pathways impacted by CaM1, CaM2, CaM3, respectively.

CaMKII is held in an inactive state by its autoinhibitory domain [113]. Upon elevation of intracellular calcium concentration, CaM can bind Ca2+ and activate each subunit of the CaMKII holoenzyme. Subsequently to the Ca2+-CaM binding, every subunit can then be autophosphorylated (at Thr286 in CaMKII alpha or Thr287 in the other isoforms) by a neighboring activated CaMKII subunit (Fig. 1). This phosphorylation generates Ca2+/CaM-independent or autonomous activity and, in addition, increases the affinity of CaMKII for Ca2+/CaM (an event known as "CaM-trapping") [113,123]. Together, CaM-trapping and autonomous activity keep the kinase activated much longer than the transient timeframe Ca2 + took to initially switch on the molecule.

Among the different pathways in which *CAMK2* is involved (e.g. translation of intracellular Ca2+ signals; synaptic plasticity and spatial memory), in the heart it plays a key role in different signaling cascades and, specifically, it is critical for cellular processes such as sarcoplasmic reticulum Ca^{2+} uptake and release through RyRs excitation–contraction coupling (ECC) and excitation–transcription coupling (ETC) [115,124]. It was shown that high level and activity of CaMKII was increased consistently in myocardial tissues from patients with heart disease [125,126]. Also, expression of CaMKII in patients with advanced and end-stage HF was significantly increased [127,128]. In heart disease, CaMKII may be involved in cell death and inflammation [129].

Considering the important role of *CAMK2* in cardiac function, efforts have been made to study the possible involvement of polymorphisms in this class of proteins associated with cardiovascular pathologies, because genetic variants may alter the structure and function of a protein [130].

Clinical research in CAD usually studies and analyzes the genetic variants in protein-coding regions, but recently mutations located within non-coding regions are being considered in relation to the pathogenesis of complex human disease. For example SNPs located in super enhancers may have an essential role in disease metabolism [122,130,131]. Gong et al. [130] identified 366 potential CAD-associated super enhancer SNPs in 67 loci, highlighting the potential functional importance of CAD-associated super enhancer SNPs [130].

Among these loci, they found that four CAD-associated super enhancer SNPs in *CAMK2G* were clustered into the same or neighboring super enhancers [120]. These SNPs could be interesting to study because *CAMK2* has been linked to electrical remodeling following MI, as well as atrial and ventricular arrhythmias [122,132]. Also, it appears to act as a critical regulator of cardiac function and to have a key role in cardiac hypertrophy [133] and heart failure [127]

Through GWAS of a Caucasian population, Burgner et al. [134] identified novel and functionally related variants associated with Kawasaki disease, a pediatric vasculitis that damages the coronary arteries predominantly in young children. Among the 40 SNPs analyzed in different genes, they found three significantly associated intronic variants in *CAMK2D*: rs17531554; rs4834340 and rs11728021 (see Table 6) [134]. In this study Burgner et al. [134]. hypothesized *CAMK2D,* along with four other genes, is involved in a single putative functional network of interacting genes in Kawasaki disease. Moreover, *CAMK2D* itself seems to have a central role in this network [124].

In a systematic confirmatory GWAS, Lou et al. [135] analyzed the association between the genetic variant rs4834340 in *CAMK2D* and the onset of Kawasaki disease in a Chinese population, but they did not find an association. This result is inconsistent with the previous study conducted by Burgner et al. [134] and this might be attributed to the ethnic difference in study populations, Chinese versus Caucasians.

Therefore, future detailed studies of these variants in *CAMK2D* may ultimately lead to novel diagnostics and treatment for Kawasaki disease.

Because some consistent evidence exists, showing that a wide range of genetic factors strongly influences the risk of CVD [136], it would be interesting to understand whether there are other genetic variants of *CAMK2* isoforms that are predisposing factors for a particular CVD [130].

Among CaMKs, it is also important to mention CaMKI and CaMKIV, respectively calcium/calmodulin-dependent protein kinase I and IV. There is little information available on the role of CaMKI in the heart. Although for CaMKIV was previously considered important only for the nervous system [137,138], it has recently become a focus of attention in relation to cardiovascular pathophysiology.

In a genome-wide analysis, Levy et al. [137] showed an association between rs10491334 of *CAMK4* and elevated diastolic blood pressure (DBP), suggesting that the kinase may have a role in the control of blood pressure. Santulli et al. [140] confirmed this result by performing an association analysis and finding a significant correlation between the levels of rs10491334 and diastolic pressure in hypertensive patients. Furthermore, this study showed that this polymorphism was associated with a reduction in

Table 6
Human CAMK2D gene polymorphisms and associations with cardiovascular diseases.

Calcium calmodulin dependent kinase 2 D (CAMK2D)							
SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases /controls	OR (95 % CI)	Year [Refs.]
Intron Region							
rs11728021	A/G	–	Kawasaki disease	Caucasian	#	1.23(0.98–1.52)□	2009 [134]
rs4834340	G/A	–	Kawasaki disease	Caucasian	#	0.86(0.72–1.02) □	2009 [134]
				Chinese	428 / 493	1.17 (0.07–18.85)□	2015 [134]
rs17531554	C/T	–	Kawasaki disease	Caucasian	#	2.1(1.3–3.3) □	2009 [134]

□ When multiple genetic models and/or adjusted OR have been analysed, we reported the most significant one in terms of OR value.
GWAS study was performed on 119 KD cases and 135 healthy control, followed by replication in an independent cohort and subsequent fine-mapping, for a total of 893 KD cases plus population and family controls.

Table 7
Human CAMK4 gene polymorphisms and associations with cardiovascular diseases.

Calcium calmodulin dependent kinase 4 (CAMK4)							
SNPs	Genetic Variant	Location	Disease evaluated	Population	Cases / controls	OR (95 % CI)	Year [Refs.]
Intron Region							
rs10491334	C/T	–	Diastolic blood pressure and arterial stiffness	American	#	ND	2007 [137]
			Diastolic blood pressure	Italian	730 / 457	ND	2012 [139]
			Hypertension	Chinese*	1124 / 967	2.200 (1.473–3.285)	2016 [138]

The Framingham Offspring Cohort of The Framingham Heart Study.

the expression levels of CaMKIV. A strong association between the rs10491334 SNP of *CAMK4* and hyperthension was also showed in the Uygur population in China [138].

Data on rs10491334 polymorphism in *CAMK4* are reported in Table 7.

Both the CaMK proteins, CaMKI and CaMKIV are downstream targets of the CaM-kinase cascade (Fig. 1), and therefore their activation requires both the binding of Ca2+/CaM and the subsequent phosphorylation of calcium calmodulin kinase kinase (CaMKK), respectively at Thr 177 and Thr 196 [113]. Unlike CaMKI, once CaMKK is phosphorylated, CaMKIV can undergo an intra-subunit autophosphorylation of its Ser/Thr-rich N-terminus. This autophosphorylation generates an activity independent of Ca2+/CaM and this allows CaMKIV to maintain its functionality beyond the transitory period of increase in the concentration of Ca2+ [113]. In cardiac myocytes and muscle cells, during contraction, the calcium concentration increases and Ca2+ binds CaM [125,127,130]. Calcium-bound calmodulin activates CaMKK, which in turn activates CaMK. Because of the important role of this protein family, this review summarizes the literature on the CaMKK superfamily.

There are two isoforms of CaMKK, CaMKK1 and CaMKK2, each expressed by a separate gene. The domains of both CaMKK1 and CaMKK2 are similar to other CaM-kinases with autoinhibitory, catalytic and CaM binding domains (see Fig. 5) [113,140]. This class of enzymes is the most upstream element in the CaM-kinase cascade. CaMKK1 specifically activates CaMKI and CaMKIV by phosphorylation of a Thr residue in the activation loop [141–143].

Currently, there are no studies regarding a potential association between CVD and polymorphisms in *CAMKK1* or *CAMKK2*. However, Erlinge et al. [144] conducted a large genetic association study and found that a variant in intron 8 of *CAMKK2* (rs2686342) was associated with a decreased risk of IS.

Despite it being hypothesized that the CaMKK family is involved in Ca2+ signaling in the cardiovascular system [145], the precise role and function of *CAMKK1* in the heart has not yet been defined. Nevertheless, recent studies show that over-expression of *CAMKK1* in mesenchymal stem cells (MSC) induces the release of paracrine factors in the secretome, resulting in improved cardiac function after infarction [146]. This new discovery of *CAMKK1* function suggests research is needed on genetic variants in this protein family.

CaMKK1 is a transferase of the Ser/Thr protein kinase family, which belongs to the Ca2+ calmodulin-dependent protein kinase subfamily. The CaMKK pathway has different cellular functions and influences numerous processes such as gene transcription, cell survival, apoptosis, cytoskeletal re-organization, and neuronal differentiation [147].

CAMKK1 is also involved in the regulation of calcium-triggered signaling in cellular processes. Specifically, it is an upstream regulator of the kinase cascade pathway in the major cells, of calcium in the heart, and is basic for the electromechanical activity of the heart. Increasingly, it is believed that the proteins involved in the excito-contraction mechanisms of the heart, such as CaMKK proteins, could be decisive for CVDs, like heart failure.

CAMKK1 presents polymorphic variants in the population of clinical interest. For example, the rs7214723 polymorphism, which causes an amino acid change from glutamic acid (E) to glycine (G) at the amino acid position 375, has been associated with an increased risk of lung cancer [147,148].

Zhang et al. [147] proposed that as an amino acid change in the kinase domain (E375 G) possibly decreases the activating ability of *CAMKK1* and represses cell proliferation. Studying the same polymorphism, Chen et al. [136] suggested that this polymorphism might influence the substrate specificity of CaMKK1 and inhibit specific downstream protein kinases, such as CaMKIV and CaMKI. This kinase cascade inhibition may promote cell cycle progression, thereby accelerating the proliferation of cells in cancer. For these reasons, it could be interesting to investigate whether this known polymorphism (rs7214723), studied in lung cancer, might contribute also to the risk of some CVD, because this polymorphism appears to be highly represented in the population (MAF index: C = 0.3954 / 1980–1000 Genomes [149]).

In fact, it is known that some polymorphic variants can be considered predisposing factors to complex pathologies.

6. Conclusion

Some polymorphic variants are considered predisposing factors for complex pathologies (i.e. CVDs) and could be used as biomarkers to identify individual risks, in order to implement alternative strategies for preventing the onset of the pathology. In addition, polymorphisms can cooperate with common biomarkers in the diagnosis of patients to identify individual risk. The identification and the assessment of cardiac biomarkers might play a crucial role in preventing the onset of diseases and in monitoring patients during follow up. For this reason, the object of our review was the analysis of literature on the polymorphisms of proteins involved in the calcium calmodulin pathway, which is very important in cardiac activity. We focused on four protein families, as well as CaM, NOS, PPP3C and CaMK.

Our literature exploration showed that for some proteins there are a greater number of papers reporting results of correlation between CVD and polymorphisms. The super family of NOS, in particular *NOS1* and *NOS3*, has been studied extensively. When multiple studies addressing the correlation between a polymorphism and a specific pathology are available, a meta-analysis study can be performed. The combination of results from multiple studies allows to create a very large pooled population that enhances the statistical power and yields more informative results about the contribution of a polymorphism to the predisposition for disease.

In the literature, a large number of meta-analysis have been published regarding the role of *NOS3* polymorphisms in different CVDs, showing that the polymorphisms G894 T, 27bp-VNTR and -786 T > C could be predisposing factors for different CVDs [76,82,95–99].

Less information is available regarding the other proteins involved in the calcium pathway. For example, we noticed that only a few studies presented correlations between polymorphisms in

the PPPRC or CaMK families and CVD. Only the *NOS1* gene was examined in more than two studies for the same CVD. In an attempt to better clarify the contribution of existing polymorphisms of genes involved in the calcium-calmodulin pathway and CVDs, we performed a preliminary meta-analysis for the evaluation of *NOS1* gene polymorphisms in susceptibility to IS. Only three reports considering several *NOS1* polymorphisms (rs2293050, rs2139733 and rs1483757) and their association with IS were found in the literature. Two independent publications conducted in Han Chinese showed that these three polymorphisms were not associated with IS. Conversely, another study on a Portuguese population reported that these genetic variants were significantly associated with stroke susceptibility after adjusting for demographic, clinical and life-style risk factors. Our meta-analysis reported a negative association with the predisposition to IS for all the three polymorphisms (especially for rs2293050 and rs2139733 genetic variations), although odds ratio values were only weakly lower than 1.0. Thus, none of the three polymorphisms have resulted as a predisposition factor for IS. However, further studies should be conducted, particularly on rs2293050 and rs2139733 polymorphisms, to better understand their involvement in this cardiovascular disease in different ethnic groups and to increase the number of case and control samples. Moreover, linkage disequilibrium (LD) in association analyses with these three polymorphisms may be interesting and conducting meta-analyses using such additional studies would likely be informative.

Regarding the other proteins considered in this review, few reports have been published regarding the involvement of their polymorphisms in CVDs. Particularly, since CaM has a central role in different processes; polymorphisms in its exon regions are very rare. However, some genetic variants have been reported for the regulatory and promoter regions. Among these studies, the genetic variation rs3814843 in the 3'-UTR seems to have a strong correlation with sudden cardiac death (HR: 5.54; 95 % CI: 2.05 14.95) [35]. Thus, this specific variant requires future investigation.

Our literature review demonstrated that NOS2 polymorphisms show a weak positive association with different CVDs. Considering the reported odds ratio values, the most significant genetic variants in the *NOS2* gene (rs2297518 and rs2779249) seem to be associated with hypertension disease [57,58,64].

After an accurate study of the literature and a detailed analysis of data from the 1000 Genomes Project (https://www.internationalgenome.org/1000-genomes-browsers/) [150], we found few polymorphisms studied in PPPRC or CaMK superfamilies associated with increased risk of developing CVD. The only associations (OR not reported) of *PPP3C* polymorphisms to CVD were studied by He et al. [112]. Five polymorphisms (rs1879793, rs1075534, rs7430, rs2461483 and rs10108011) in *PPP3CC* were associated with the training responsiveness of cardiac phenotypes after a cycle-ergometry exercise, and one polymorphism (rs3763679) in *PPP3CB* to an important cardiac phenotype (i.e., resting heart rate) at the pre-training state.

Regarding *CAMK2*, Burgner et al. [134] reported in a GWAS study interesting data on three polymorphisms (rs17531554; rs4834340 and rs11728021) in *CAMK2D* and their associations with Kawasaki disease. Among these polymorphisms, the genetic variation rs17531554 seems to have a stronger correlation with Kawasaki disease (OR: 2.1; 95 % CI: 1.3–3.3).

The availability of more data regarding the genetic variants in PPP3C and CAMK2 could allow us to understand their role in CVDs

On the other hand, we identified some interesting data in studies on *CAMK4*, even if it is not directly linked with the three CaM isoforms. Polymorphism rs10491334 in *CAMK4* was studied in three different populations (American, Italian and Chinese) and associated with diastolic blood pressure and hypertension [139–141]. Despite these three studies, the total numbers of cases was not sufficient to perform a meta-analysis. For this reason, additional studies are needed to collect more data about rs10491334 in CAMK4, to better understand the association with this kind of this polymorphism with CVD and to provide an interesting epidemiology view of the same disease in different ethnicities.

Because some consistent evidence exists showing that a wide range of genetic factors strongly influence the risk of CVDs [136], we conclude that additional studies addressing other polymorphisms in the genes involved in the calcium-calmodulin pathway should be conducted. Moreover, analyzing allelic frequency distributions in healthy and affected populations might be very important for understanding the impact of genetic variations on CVD predisposition, addressing the research area in the context of epidemiology.

Authors' contributions

All authors contributed equally to this work.

Declaration of Competing Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mrrev.2020.108325.

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SUPPLEMENTARY MATERIAL

Materials and Methods

Statistical Analysis

Association between NOS1 rs2293050, rs2139733, rs1483757 and ischemic stroke was evaluated by odds ratio (OR) and 95% confidence interval. Four different genetic models were considered in our analysis: homozygote model (BB versus AA), heterozygote model (BA versus AA), dominant model (BB+BA versus AA) and recessive model (BB versus BA+AA), considering the minor allele (B) versus the wild-type (A) one according to the reported frequency allele in 1000Genomes Database [1]. Heterogeneity between studies was assessed using p value of Q test and I^2 values. Heterogeneity between studies could be assessed using statistical method based on Chi-squared distribution, such as the Cochrane's Q test. It reports a measure of the variation across studies, thus when large, it indicates a large heterogeneity. However, a limitation of Cochran's Q test is that it has low power when small sample size or few studies have been included in the meta-analysis. Thus, although a p value threshold of 0.05 is conventional used, in these cases a P value of 0.10 is employed for statistical significance. Conversely, the test has excessive power when many studies have been included, especially when those studies includes large population samples. Moreover, since systematic review and meta-analysis bring together studies that could be diverse, heterogeneity is inevitable [2]. Thus, an alternative method to quantify the effect of heterogeneity is the measurement of the degree of inconsistency in the study's results using the I^2 value. Although I^2 values should not be appropriate for all circumstance, the authors assign adjectives of low, moderate and high heterogeneity to I^2 value of 0-25% (a fixed effect models can be used), 25-50% (the appropriate model should be chosen based on the Q test value, too) and 50-75% (a random effect models should be used) [2–4]. Thus, to account for observed significant heterogeneity ($I^2 > 50\%$ and $p < 0.10$) among studies, a random-effects model was used. However, in case of no heterogeneity ($I^2 = 0\text{-}25\%$ and $p > 0.10$) a fixed-effects model was applied. Since the few number of included studies in our meta-analysis (three in total), the examination of publication bias was not statistical relevant and, thus, it was not included in the analysis. All these statistical analyses were performed by ProMeta software v 3.0 (Internovi, Cesena FC, Italy).

Results

Meta-analysis results

Relationship between rs2293050 and ischemic stroke

A total of three articles were retrieved representing case-control studies. Taking together these studies involved 1260 healthy controls and 1330 patients with IS belonging to Han Chinese and Portuguese population. Four different genetic models were used for this meta-analysis. Homogeneity between studies was only observed in recessive (TT vs TC+CC) genetic model and a fixed-effects model was used. On the contrary, high inconsistency between studies was observed for the other three genetic models considered, although the homozygote (TT vs CC) genetic model showed value near to the moderate inconsistency range with an $I^2 = 54.29$ ($I^2 > 50\%$) and $p = 0.112$ ($p > 0.1$) (Table S1). The pooled analyses indicated a mild negative association, not significant, of *NOS1* rs2293050 polymorphism with the risk of IS in homozygote, heterozygote, dominant and recessive genetic models (Fig. S1 and Table S1).

Relationship between rs2139733 and ischemic stroke

A total of three articles were retrieved representing case-control studies. Taking together these studies involved 1234 healthy controls and 1305 patients with IS belonging to Han Chinese and Portuguese population. Four different genetic models were used for this meta-analysis. Homogeneity between studies was observed in homozygote (AA vs TT) and recessive (AA vs AT+TT) genetic models and a fixed-effects model was used. On the contrary, high inconsistency between studies was observed for the other two genetic models considered. From our pooled analysis, the polymorphism rs2139733 seems to be not an increased risk for ischemic stroke in all the genetic models analysed (Figure S2 and Table S1).

Relationship between rs1483757 and ischemic stroke

A total of three articles were retrieved representing case-control studies. Taking together these studies involved 1261 healthy controls and 1331 patients with IS belonging to Han Chinese and Portuguese population. Homogeneity between studies was only observed in recessive (GG vs GA+AA) genetic model and a fixed-effects model was used. On the contrary, high inconsistency between studies was observed for the other three genetic models considered and a random-effects model was employed. The polymorphism *NOS1* rs1483757 A>G seems to be not an increased factor for ischemic stroke since the calculated OR are lower than and close to the unit for all the genetic model analysed (Fig. S3 and Table S1).

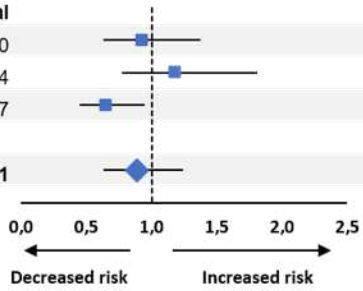
Table S1:
Summarised meta-analysis results of *NOS1* polymorphisms and Ischemic Stroke (IS) risk

Polymorphism	Study number	Genetic model	Type of Model	Heterogeneity		p value	OR	95% CI
				I^2 (%)	p			
rs2293050 C>T								
	3	TT vs CC	Random	54,29	0,112	0,48	0,89	0,63 - 1,24
	3	TC vs CC	Random	70,99	0,032	0,40	0,87	0,63 - 1,21
	3	TT+TC vs CC	Random	71,50	0,03	0,40	0,88	0,64 - 1,19
	3	TT vs TC+CC	Fixed	31,99	0,23	0,62	0,95	0,78 - 1,16
rs2139733 T>A								
	3	AA vs TT	Fixed	48,39	0,144	0,25	0,87	0,69 - 1,10
	3	AT vs TT	Random	71,82	0,029	0,36	0,86	0,61 - 1,20
	3	AA+AT vs TT	Random	72,23	0,027	0,37	0,86	0,63 - 1,19
	3	AA vs AT+TT	Fixed	3,34	0,355	0,68	0,96	0,78 - 1,17
rs1483757 A>G								
	3	GG vs AA	Random	70,61	0,033	0,70	0,92	0,61 - 1,39
	3	GA vs AA	Random	69,79	0,037	0,77	0,95	0,67 - 1,34
	3	GG+GA vs AA	Random	75,91	0,013	0,74	0,94	0,65 - 1,36
	3	GG vs GA+AA	Fixed	23,97	0,268	0,58	0,95	0,79 - 1,14

(a) TT vs CC

Study	Cases Events	Cases Total	Controls Events	Controls Total	Odds ratio	95% CI	Weight
Dai et al., 2013	82	225	80	210	0,93	0,63 - 1,37	33,81%
He et al., 2014	74	196	59	174	1,18	0,77 - 1,81	30,99%
Manso et al., 2012	78	266	92	237	0,65	0,45 - 0,95	35,20%
Overall (random-effects model)		**687**		**621**	0,89	0,63 - 1,24	100%
Prediction interval							

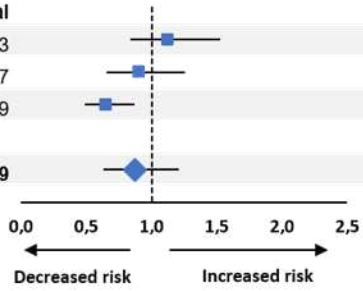
Heterogeneity: I^2 = 54,29%; p = 0,11; Q = 4,38



(b) TC vs CC

Study	Cases Events	Cases Total	Controls Events	Controls Total	Odds ratio	95% CI	Weight
Dai et al., 2013	252	395	203	333	1,13	0,84 - 1,52	33,51%
He et al., 2014	185	307	192	307	0,91	0,66 - 1,26	31,98%
Manso et al., 2012	206	394	244	389	0,65	0,49 - 0,87	34,52%
Overall (random-effects model)		**1096**		**1029**	0,87	0,63 - 1,21	100%
Prediction interval							

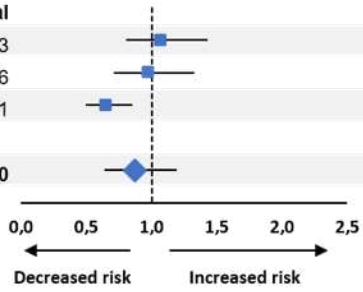
Heterogeneity: I^2 = 70,99%; p = 0,0322; Q = 6,89



(c) TC+TT vs CC

Study	Cases Events	Cases Total	Controls Events	Controls Total	Odds ratio	95% CI	Weight
Dai et al., 2013	334	477	283	413	1,07	0,81 - 1,43	33,45%
He et al., 2014	259	381	251	366	0,97	0,71 - 1,32	31,95%
Manso et al., 2012	284	472	336	481	0,65	0,50 - 0,85	34,60%
Overall (random-effects model)		**1330**		**1260**	0,88	0,64 - 1,19	100%
Prediction interval							

Heterogeneity: I^2 = 71,50%; p = 0,03; Q = 7,02



(d) TT vs TC+CC

Study	Cases Events	Cases Total	Controls Events	Controls Total	Odds ratio	95% CI	Weight
Dai et al., 2013	82	477	80	413	0,86	0,61 - 1,21	34,45%
He et al., 2014	74	381	59	366	1,25	0,86 - 1,83	29,97%
Manso et al., 2012	78	472	92	481	0,84	0,60 - 1,17	35,58%
Overall (fixed-effects model)		**1330**		**1260**	0,95	0,78 - 1,16	100%
Prediction interval							

Heterogeneity: I^2 = 31,99%; p = 0,23; Q = 2,94

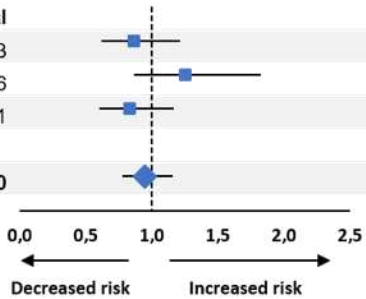


Figure S1: Forest plots for the association between rs2293050 polymorphism and ischemic stroke under the (a) homozygote, (b) heterozygote, (c) dominant and (d) recessive mode of inheritance. Black boxes represent the value of odds ratio (OR). Horizontal line is the 95% Confidence Interval (CI) of OR. The summary OR is represented by the diamond, where the center of the diamond indicates the OR.

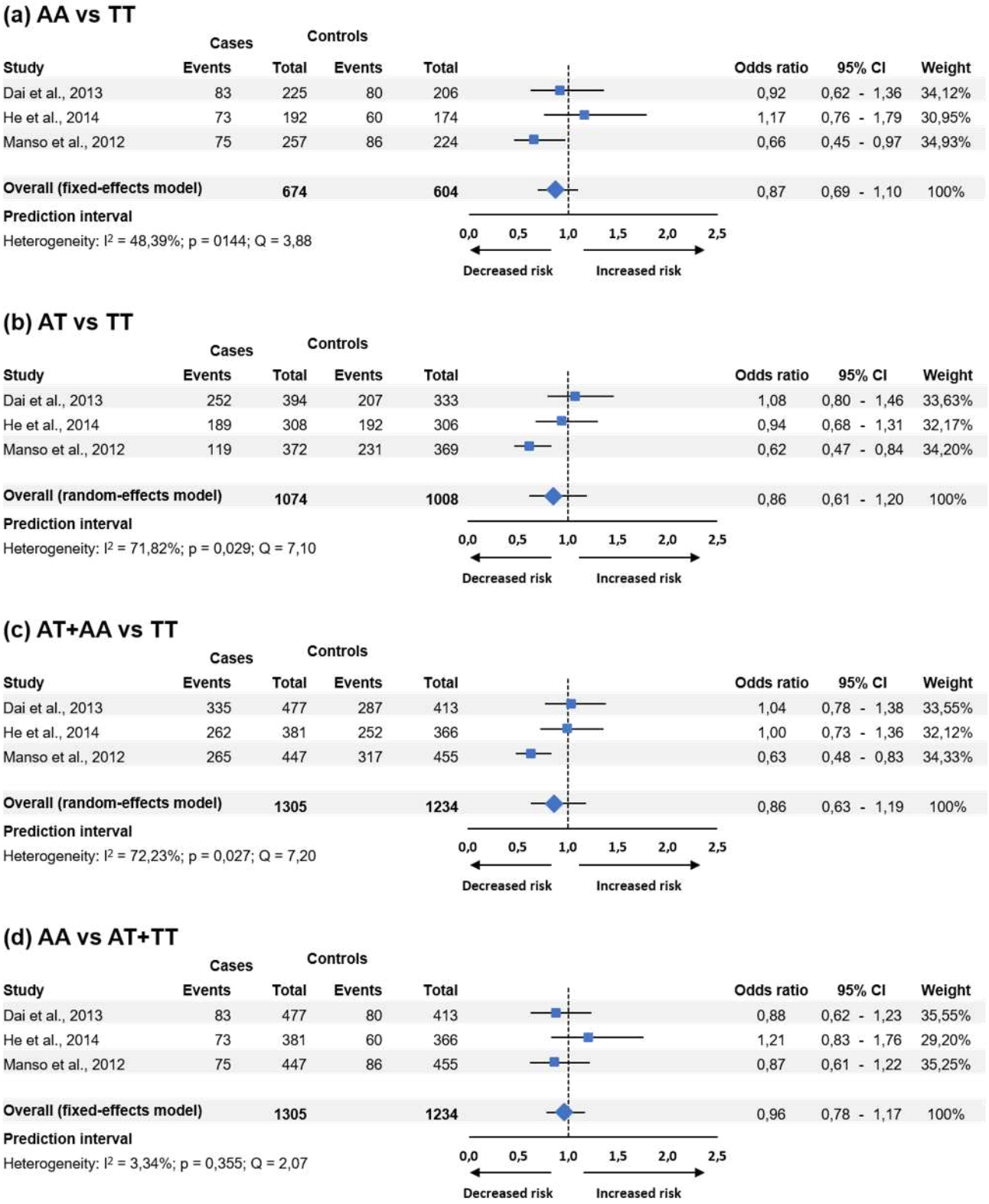


Figure S2: Forest plots for the association between rs2139733 polymorphism and ischemic stroke under the (a) homozygote, (b) heterozygote, (c) dominant and (d) recessive mode of inheritance. Black boxes represent the value of odds ratio (OR). Horizontal line is the 95% Confidence Interval (CI) of OR. The summary OR is represented by the diamond, where the center of the diamond indicates the OR.

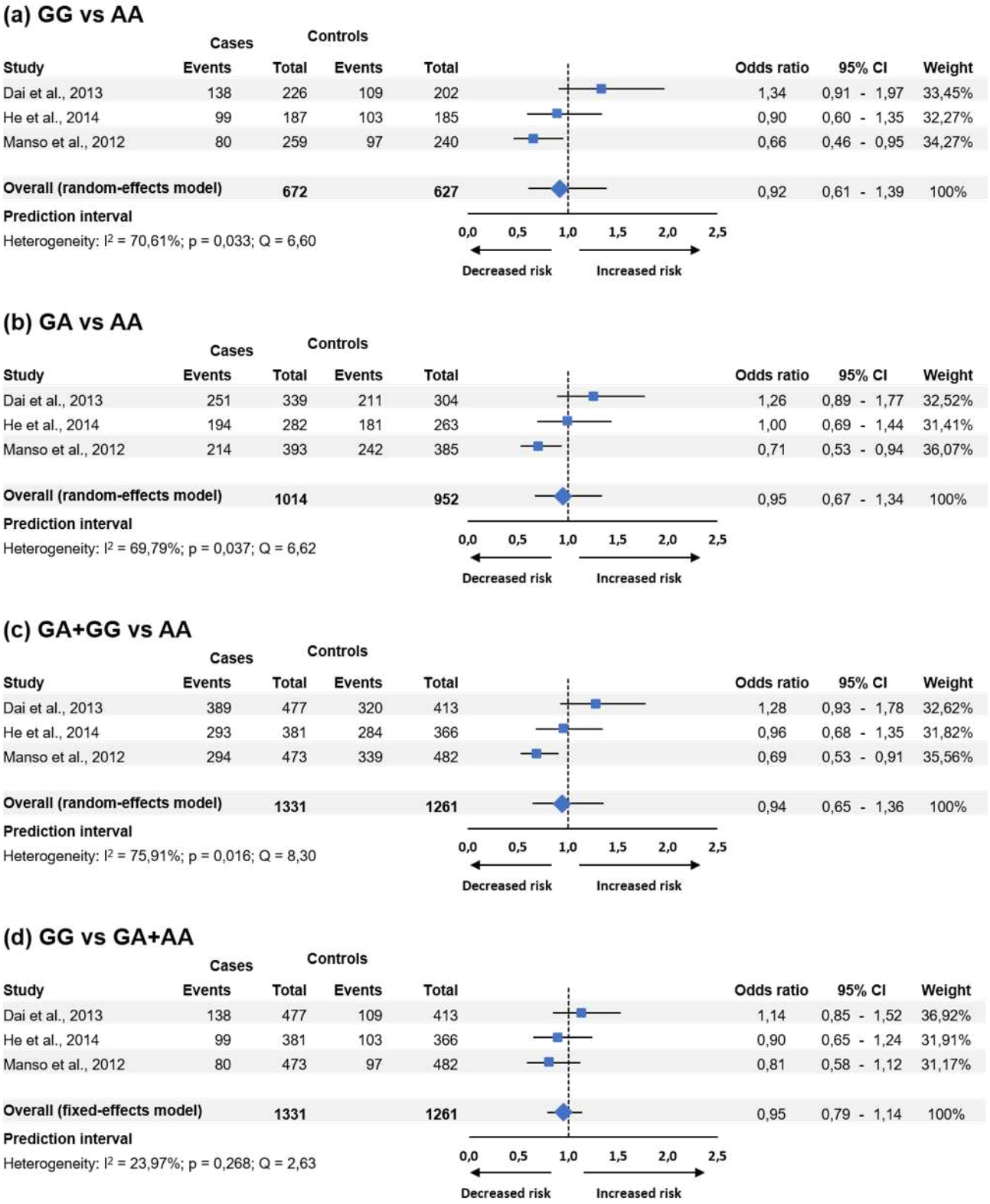


Figure S3: Forest plots for the association between rs1483757 polymorphism and ischemic stroke under the (a) homozygote, (b) heterozygote, (c) dominant and (d) recessive mode of inheritance. Black boxes represent the value of odds ratio (OR). Horizontal line is the 95% Confidence Interval (CI) of OR. The summary OR is represented by the diamond, where the center of the diamond indicates the OR.

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Research Article

Polymorphism rs7214723 in CAMKK1: a new genetic variant associated with cardiovascular diseases

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Cardiovascular diseases (CVDs) are the leading cause of deaths worldwide. CVDs have a complex etiology due to the several factors underlying its development including environment, lifestyle, and genetics. Given the role of calcium signal transduction in several CVDs, we investigated via PCR-restriction fragment length polymorphism (RFLP) the single nucleotide polymorphism (SNP) rs7214723 within the calcium/calmodulin-dependent kinase kinase 1 (*CAMKK1*) gene coding for the Ca^{2+}/calmodulin-dependent protein kinase kinase I. The variant rs7214723 causes E375G substitution within the kinase domain of CAMKK1. A cross-sectional study was conducted on 300 cardiac patients. RFLP-PCR technique was applied, and statistical analysis was performed to evaluate genotypic and allelic frequencies and to identify an association between SNP and risk of developing specific CVD. Genotype and allele frequencies for rs7214723 were statistically different between cardiopathic and several European reference populations. A logistic regression analysis adjusted for gender, age, diabetes, hypertension, BMI and previous history of malignancy was applied on cardiopathic genotypic data and no association was found between rs7214723 polymorphism and risk of developing specific coronary artery disease (CAD) and aortic stenosis (AS). These results suggest the potential role of rs7214723 in CVD susceptibility as a possible genetic biomarker.

Introduction

Cardiovascular diseases (CVDs) are the leading cause of deaths in industrialized countries affecting increasing number of people every year [1]. CVDs refer to a wide range of conditions affecting heart function and blood vessels, two of the most frequent being coronary artery disease (CAD) and aortic stenosis (AS).

CAD results from progressive stenosis of coronary arteries caused by the atherosclerosis process. Clinical syndromes are caused by an imbalance between oxygen supply and demand, resulting in a myocardial perfusion inadequate to meet metabolic demand (ischemia). Plaque rupture with superimposed thrombosis is responsible for most acute coronary syndromes and different types of myocardial infarction [2]. AS results from complex and non-completely understood pathophysiologic mechanisms, causing thickening, calcification, and/or fusion of the aortic valve leaflets (LV), determining a variable degree of stenosis in the left ventricle outflow tract [2]

Even if the pathophysiology in CVDs is well known and there are multiple approaches to treat patients, it is important to consider that this kind of conditions are complex because multifactorial [1,3]. In addition to lifestyle habits (tobacco usage, unhealthy diet, alcohol abuse, physical inactivity), it is important to stress that CVDs represent a complex trait with multiple genetic and environmental components. Since complex diseases do not follow a clear pattern of Mendelian inheritance, the study of genetic variants in candidate genes is expected to provide insights into the genetic basis of not only for disease predisposition but also for responses to drugs and aging [4–6].

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In recent years, different studies have been focusing on genetic association as an approach for assessing the contribution of specific gene variants to disease risk [4,5,7].

The identification of polymorphisms as prognostic and predictive biomarkers in CVDs could be really important for both preventive screenings and monitoring of patients with specific CVDs [4,5]. Among the several genes involved in the physiology and anatomy of the heart, genetic factors involved in the calcium/calmodulin pathway might play a key role in modifying the individual risk of developing CVDs. The calcium signaling pathway in particular plays a significant role in heart and vessels [8–11], being involved in different processes as for example the regulation of the excitation–contraction mechanism [10,12,13].

In fact, it has been shown that Ca^{2+} regulates the rhythmic contractions of the heart via diverse signaling pathways, creating an interconnected network involved different specific functions in different regions of the heart [10,14].

Within the different calcium signaling pathways, calmodulin has a central role: its function is to integrate the calcium signal and to transduce it to other downstream enzymes, such as NOS, PPP3C as well as the proteins of the CAMK subfamily. In a previous review [14] we focused our attention on the role played by genetic variants in these family of proteins in the development of CVDs [15–21]. Here we focus on CAMKK1, a calcium/calmodulin-dependent kinase kinase 1, functionally located upstream to others CAMK kinases involved in the transduction of calcium signaling.

CAMKK1 has been recently assigned a novel role as regulator of the mesenchimal stem cell (MSC) secretome, specifically of the exosome. It was also demonstrated that direct overexpression of CAMKK1 in infarcted cardiac tissue has therapeutic beneficial effects, improving ejection fraction and decreasing infarct size after acute myocardial infarction [22].

CAMKK1 is a transferase of the Ser/Thr protein kinase family and it belongs to the $Ca^{(2+)}$/calmodulin-dependent protein kinase subfamily [23]. This protein is the most upstream element of CaM-kinase cascade, and it is maintained in a dormant state when calcium levels are basal. When calcium levels increase, calmodulin binds four ions of calcium and activates CAMKK1 for the phosphorylation of downstream proteins [23].

The activity of CAMKK1 is important for different processes, including neuronal differentiation, stress resistance in skeletal muscle, mitochondrial morphology, cell proliferation and neurodegenerative disorders [23–25].

It is supposed that alteration in the *CAMKK1* gene sequence might have an impact on the protein structure and its activity. The T to C variation at rs7214723 in CAMKK1 causes the change in the amino acid glutamate into glycine at the position 375 and has been associated with lung cancer [25,26]. Since this amino acid variation is located in the kinase domain, there is the possibility that such a change could decrease the activity of CAMKK1 and inhibit selected downstream protein kinases, causing specific disorders [25,26].

In the present study, we investigated the polymorphism rs7214723 in CAMKK1 for its potential association with CVDs, with the aim of finding new biomarkers related to an increased risk of developing these diseases, highlighting the role of CAMKK1 in CVDs and providing insights in the physiology and molecular biology of CVDs.

Methods

Population study

The study protocol (study number 97/2017) was approved by the Ethical Committees of the Università degli Studi dell'Insubria (Varese, Italy). Informed consent was obtained in accordance with the principles outlined in the Declaration of Helsinki.

The study involved 300 cardiopathic subjects requiring cardiac surgery recruited at the Cardiac Surgery of Varese Hospital (Italy). Of the 300 patients, the percentage of non-Italian subjects was below 5%.

All patients were subjected to clinical (presence/absence of chest pain, comorbidity, and comorbility) and instrumental diagnosis. Patients underwent a series of tests routinely performed before any cardiac operation, including electrocardiogram (ECG), echocardiogram (echo), and coronary angiography. These tests allowed the subdivision of the patients in two subgroups. The first group comprised 150 patients with coronary artery disease (CAD group), while the second included 150 patients without CADs (NOCAD group). Among all the patients, 107 had AS, of which 71 belonged to the NOCAD group (Figure 1).

Sample collection

Samples were collected from September 2017 to March 2019. Approximately 5 cc of whole blood was collected from all patients via peripheral venous puncture. An aliquot of 125 μl was then spotted on Whatman FTA cards for subsequent genetic analysis.

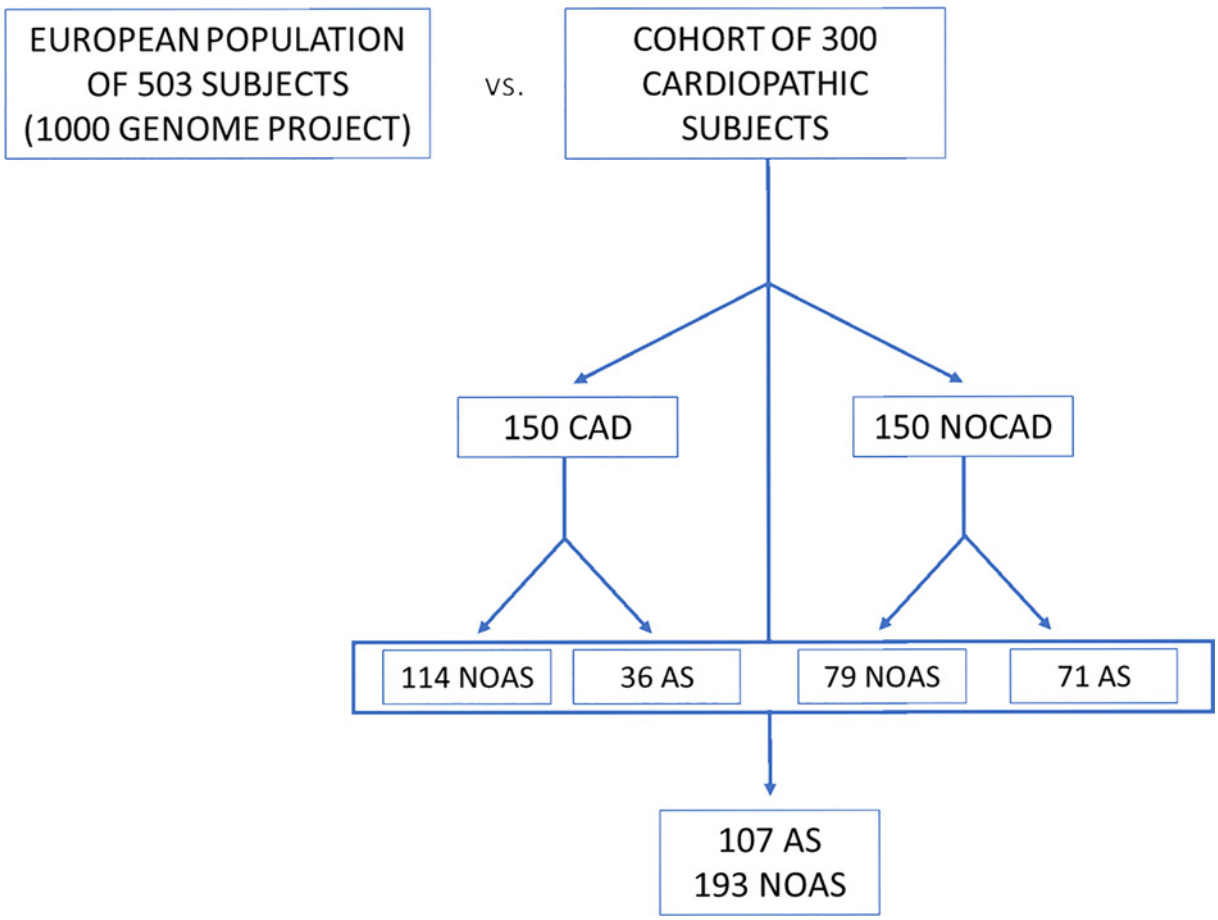

Figure 1. Scheme of the populations object of study

European population (reference group) compared with the cardiopathic population. It is also possible to observe how it was designed, the stratification inside the cohort of 300 cardiopathic subjects.

DNA extraction and CAMKK1 genotyping

The polymorphism rs7214723 of *CaMKK 1* gene was genotyped by PCR-restriction fragment length polymorphism (RFLP) strategy. DNA was extracted from blood samples spotted on FTA cards following a specific protocol optimized in our laboratory. Briefly, the DNA of each patient was isolated from two 1.2-mm diameter disks of FTA picked up through Harris Micro Punch and subsequently inserted in a PCR tube. Each sample was incubated for 5 min with 200 µl of FTA Purification Reagent. We repeated this step three times, to ensure greater DNA quality for PCR analysis by removing inhibitors and other contaminants. Each sample was then incubated twice for 5 min with 200 µl of TE buffer at pH = 8.0 (Tris-HCl 10 mm pH 8.0; EDTA 1 mm pH 8.0). Each sample was finally left for 10 min at 56°C before being used for the PCR.

The polymorphic region of the CAMKK1 was amplified via PCR in a thermal cycler 2720 (Applied Biosystems) using two primers (synthesized by Eurofins) previously designed to amplify the region of 326 bp containing the single nucleotide polymorphism (SNP) of interest: forward primer: 5′-AACAGCACCGCCACCTTCATA-3′; reverse primer: 5′-GGTCCTTCTCATGTAATGGGAGC-3′. PCR was performed in a reaction volume of 25 µl using the DNA Dream Taq Polymerase (#EP0705, Thermo Fischer Scientific) using the following cycling conditions: denaturation, annealing, extension etc. After the amplification, the amplified PCR product of 326-bp fragment was detected using an UV transilluminator. PCR products were then digested using the restriction enzyme BseRI (#R0581L, NEB) in a total reaction volume of 25 µl. The rs7214723 polymorphic site is within the restriction site recognised by BseRI allowing the discrimination of the different genotypes. In presence of allele T, BseRI cuts the PCR products into two fragments of 114 and 212 bp; in presence of allele C, an intact fragment of 326 bp was obtained (Figure 2). Enzymatic

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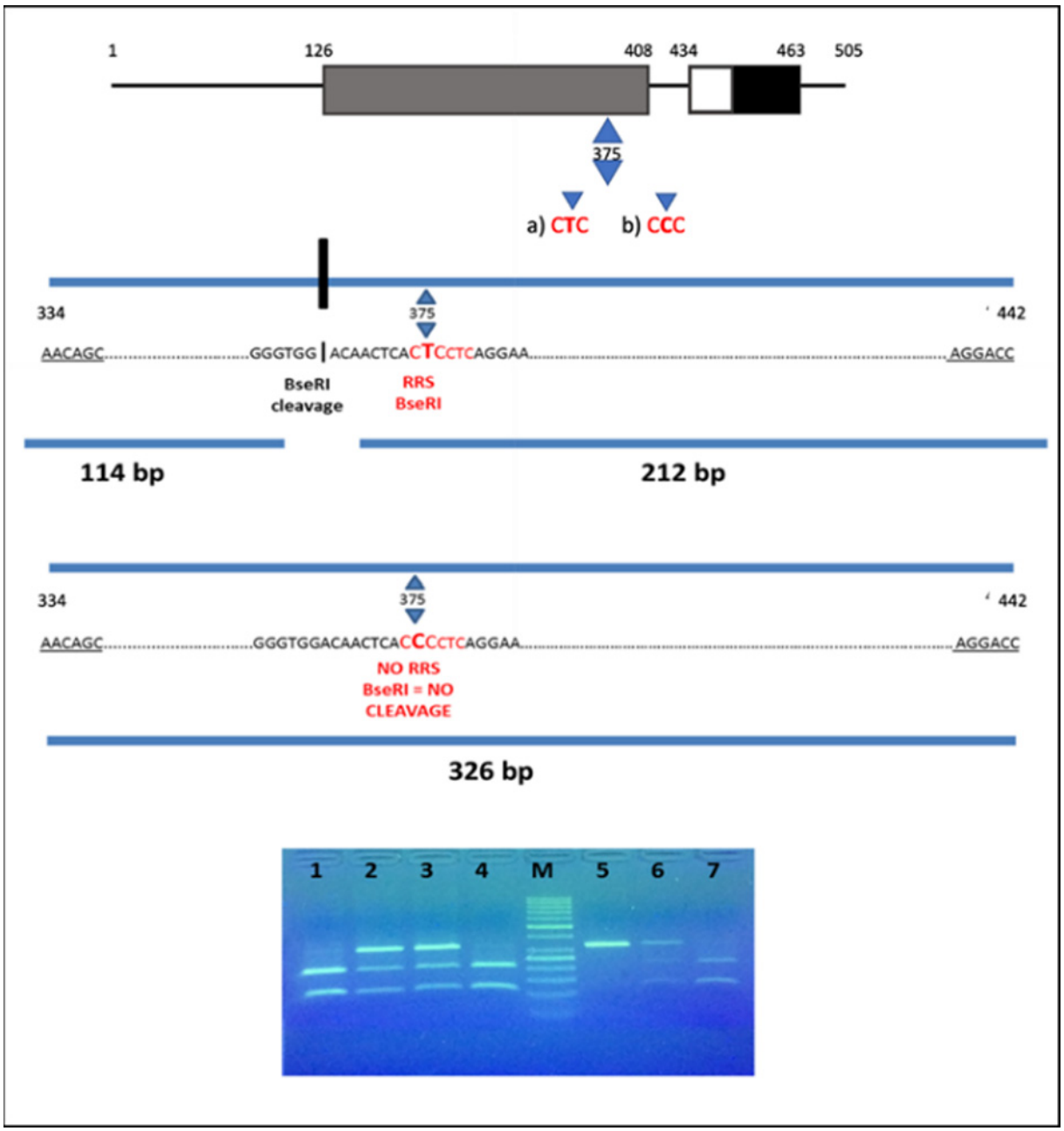

Figure 2. Identification of the E375G polymorphism (Glu^{375}Gly variant) in CAMKK1, detected by BseRI restriction enzyme

Top: schematic presentation of CAMKK1 domain structure. The conserved CaM-kinase domain is shown in gray (catalytic domain), white (autoinhibitory domain) and black (CaM-binding domains). The little triangles show the amino acid position of E375G polymorphism (CTC→CCC) in the kinase domain of CAMKK1. In (a) it is shown that the T allele generates a restriction recognition site (RRS) for BseRI. Its cleavage creates two fragments of 114 and 212 bp. In (b) it is shown that the C allele does not generate an RRS for BseRI, thus it can not cleave. The result is a fragment of 326 bp. Bottom: Electrophoresis on 3% agarose gel of BseRI restriction products: Lanes 1, 4 and 7, TT genotype (212 and 114 bp); s 2, 3 and 6, TC genotype (326, 212 and 114 bp); lane 5, CC genotype (326 bp); lane M, DNA ladder.

digestions were carried out at 37°C for 1 h. The fragments were run on 3% agarose gel for approx. 50 min at 150 V and the bands were visualized on a UV transilluminator.

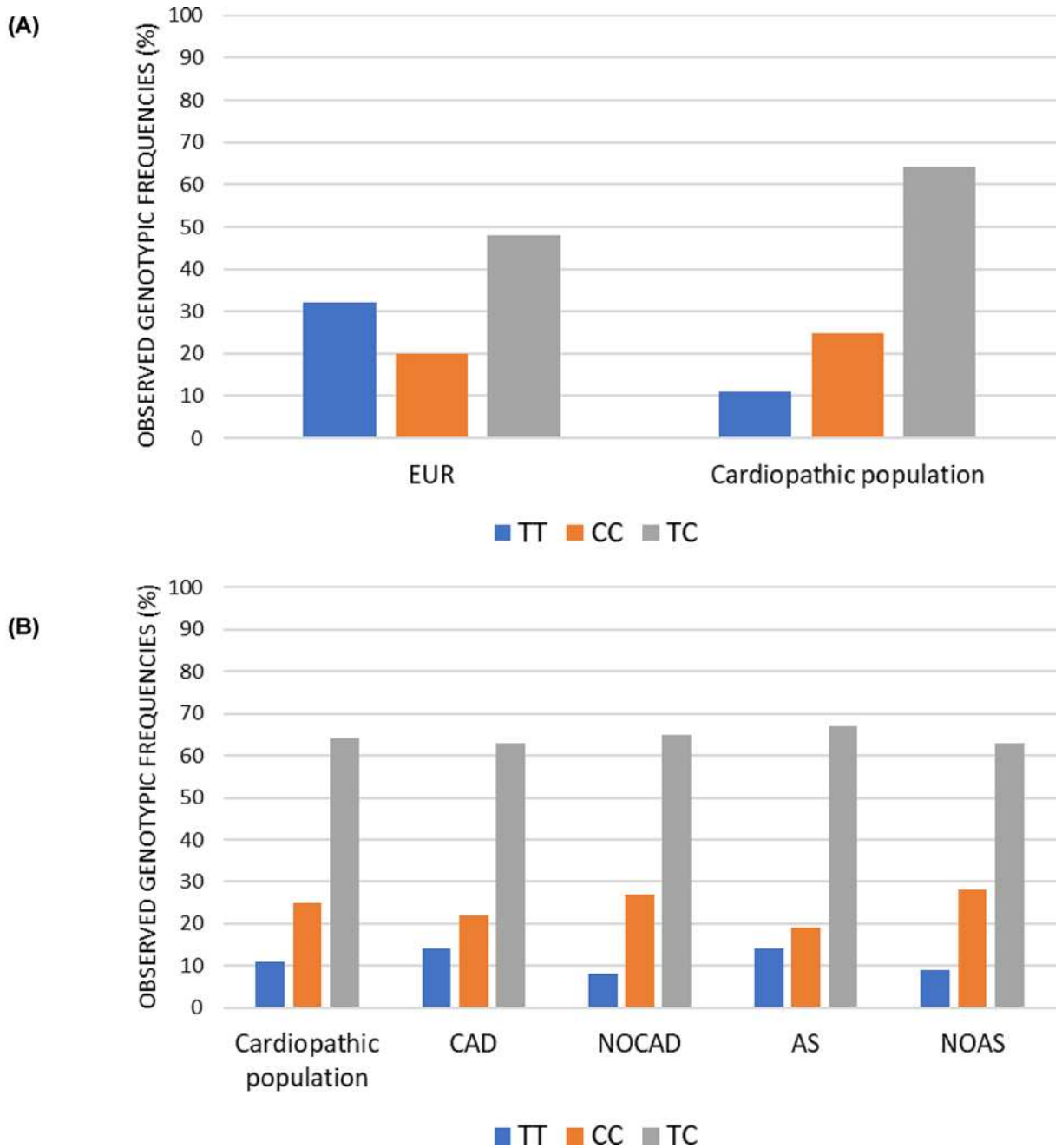

Figure 3. Observed genotypic frequencies

(**A**) Histogram representation of the genotypic distribution frequencies relating to rs7214723 polymorphism of *CAMKK1* gene. All the frequencies are expressed in percentage. Cardiopathic population represents the total of 300 patients. The reference group used belongs to the European population (EUR). (**B**) Histogram representation of frequencies of the genotypic distribution relating to rs7214723 polymorphism of *CAMKK1* gene. All the frequencies are expressed in percentage. Focus on the pathology's subdivision of CAD, NOCAD, AS and NOAS groups.

Statistical analysis

As the subjects included in the present study were all cardiopathic patients, we referred to the 1000 Genomes SNP data (https://www.internationalgenome.org/1000-genomes-browsers/) [27], and we chose reference group: the European population.

Bioscience Reports (2021) **41** BSR20210326
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Table 1 Number and frequency distribution (in brackets) of selected characteristics of study subjects, divided in CAD and NOCAD groups

Variable	CAD	NOCAD	*P*-value
Age (years)			0.033
<60	19 (12.7%)	33 (22%)	
≥60	131 (87.3%)	117 (78%)	
Gender			0.000
Male	116 (77.3%)	76 (50.6%)	
Female	34 (22.7%)	74 (49.3%)	
Hypertension			0.101
Yes	120 (80.5%)	108 (72.5%)	
No	29 (19.5%)	41 (27.5%)	
Diabetes			0.000
Yes	55 (36.9%)	12 (8%)	
No	94 (63.1%)	137 (92%)	
BMI			0.026
<25%	44 (33.9%)	63 (47.3%)	
≥25%	86 (66.1%)	70 (52.7%)	
Prev. neoplasia			0.558
Yes	16 (10.8%)	13 (8.8%)	
No	133 (89.2◦%)	136 (91.2%)	
Dyslipidemia			0.000
Yes	93 (62.4%)	54 (36.2%)	
No	56 (37.6%)	95 (63.8%)	

Abbreviation: Prev. neoplasia, previous history of neoplasia.

Differences in genotypic and allelic frequencies between the two populations were examined by means of a χ^2 test. Hardy–Weinberg equilibrium (HWE) for CAMKK1 genotype distributions was tested by a goodness-of-fit χ^2 test.

The χ^2 test was also applied to analyze the differences in the distributions of age, sex, diabetes, hypertension, body mass index (BMI), previous history of neoplasia and dyslipidemia between CAD and NOCAD groups. A multiple comparison method was here applied using a simple Bonferroni correction technique (P=0.0071).

To estimate the association between the CAMKK1 polymorphism and the risk of CAD, odds ratio (OR) and 95% confidence intervals (95% CIs) were estimated using unconditional logistic regression, once controlled for age, sex, hypertension, diabetes, BMI, and previous history of neoplasia.

The STATA 15 software was used for all statistical analyses. The level of statistical significance was set at $P<0.05$.

Results

Characteristics of the cardiopathic population

The cardiopathic population was composed of subjects with different pathologies all requiring cardiac surgery. The most frequent condition was CAD, which was present in 150 subjects (CAD patients). Across CAD and NOCAD patients, 107 subjects presented AS. A little group, belonging to the NOCAD subpopulation, was composed of patients with mitral pathology.

CaMKK1 genotype distributions for rs7214723 polymorphism in the total cardiopathic population were not in HWE.

The characteristics of the total population study were analyzed considering the subdivision in CAD and NOCAD patients (Table 1). CAD and NOCAD resulted to have statistically different distributions in gender (P=0.000), incidence of diabetes (P=0.000) and dyslipidemia (P=0.000) (Table 1). This latter result further confirms the strong association between diabetes and coronary atherosclerotic disease. Conversely, the two subgroups did not differ significantly in age (P=0.033), hypertension (P=0.101), BMI (P=0.026) and previous history of neoplasia (P=0.558).

Reference population

We retrieved the allele frequencies of the European population included in the 1000 Genomes Project for the rs7214723 polymorphism via Ensembl [27].

Table 2 Analysis of the genotype relating to the polymorphism rs7214723 from databank 1000 Genomes

Population	Allele: frequency (count)		Genotype: frequency (count)		
European (EUR)	T: 0.557 (560)	C: 0.443 (446)	T|T: 0.318 (160)	C|C: 0.205 (103)	C|T: 0.477 (240)
-CEU	T: 0.520 (103)	C: 0.480 (95)	T|T: 0.273 (27)	C|C: 0.232 (23)	C|T: 0.495 (49)
-FIN	T: 0.631 (125)	C: 0.369 (73)	T|T: 0.404 (40)	C|C: 0.141 (14)	C|T: 0.455 (45)
-GBR	T: 0.560 (102)	C: 0.440 (80)	T|T: 0.286 (26)	C|C: 0.165 (15)	C|T: 0.549 (50)
-IBS	T: 0.509 (109)	C: 0.491 (105)	T|T: 0.290 (31)	C|C: 0.271 (29)	C|T: 0.439 (47)
-TSI	T: 0.565 (121)	C: 0.435 (93)	T|T: 0.336 (36)	C|C: 0.206 (22)	C|T: 0.458 (49)

It is reported: the gene frequencies both of alleles, on the right, and of genotypes on the left in the European population. Respectively, the total number of the allele and genotype is shown in brackets. Population description according to 1000 Genomes: CEU, Utah residents (CEPH) with Northern and Western European ancestry; FIN, Finnish in Finland; GBR, British in England and Scotland; IBS, Iberian population in Spain; TSI, Tuscan Italy.

The European population includes 503 subjects from five different groups:

- CEU: population residing in Utah (U.S.A.) but with ancestors from Northern and Eastern Europe, made up 99 subjects
- FIN: Finnish population (Finland), made up 99 subjects;
- GBR: British population in England and Scotland (United Kingdom), made up 91 subjects;
- IBS: Iberian population (Spain), made up 107 subjects;
- TSI: Tuscan population (Italy) made up 107 subjects.

No other information about the distribution of sex, age, and other parameters are reported.

In Table 2, for each population group, the analysis of the genotype relating to the polymorphism rs7214723 is reported. The number and the frequencies of alleles and genotypes are also indicated.

Genotypic frequencies

Genotypic frequencies were first compared between the total heart disease population (cardiopathic population) and the European reference population. Subsequently, several comparisons were made considering the groups of subjects with different heart diseases. (Figure 1).

Heterozygotes were more common in the cardiopathic than the reference group (Cardiopathic population TC = 64%; European TC = 48%;).

In the reference European population, the frequency of the ancestral TT genotype was higher than the CC genotype (TT = 32%; CC = 20%), while the opposite was observed in the total cardiopathic population (TT = 11%; CC = 25%) (Figure 3A).

A significant genotype difference distribution between the two groups was detected (P=0.000).

We then focused on patients and subdivided them in four groups on the basis of them presenting CAD and AS (Figure 3B). The CAD group was composed of 150 patients, and it was compared with the NOCAD group composed of 150 patients. Among these patients, there were 107 subjects with AS, of which 71 belonging to the NOCAD group and 36 to the CAD group, and 193 patients without AS, of which 79 belonged to the NOCAD group and 114 belonged to the CAD group. The Chi-square test performed between CAD and NOCAD groups and AS and no aortic stenosis (NOAS) groups did not reveal any statistical difference when the distribution of genotypes was considered (CAD vs NOCAD, P=0.225; AS vs NOAS, P=0.08).

Allelic frequencies

In polymorphisms' analysis, it is important to consider allelic frequencies. Inside a population, the minor allelic frequency (MAF) can be determined, and it is defined as the ratio between the frequency of the rarest variant and the most common variant of a specific SNP.

Allelic frequencies were first compared between the entire cardiopathic population sample and the European reference population. Subsequently, several comparisons were made between the groups of subjects with different heart diseases, as done for the genotypic analysis (Figure 1).

The polymorphism studied in the present paper, rs7214723, appears to be common in the population: the 1000 Genomes database reports a MAF index C = 0.3954/1980 and the T allele as the ancestral allele.

Bioscience Reports (2021) **41** BSR20210326
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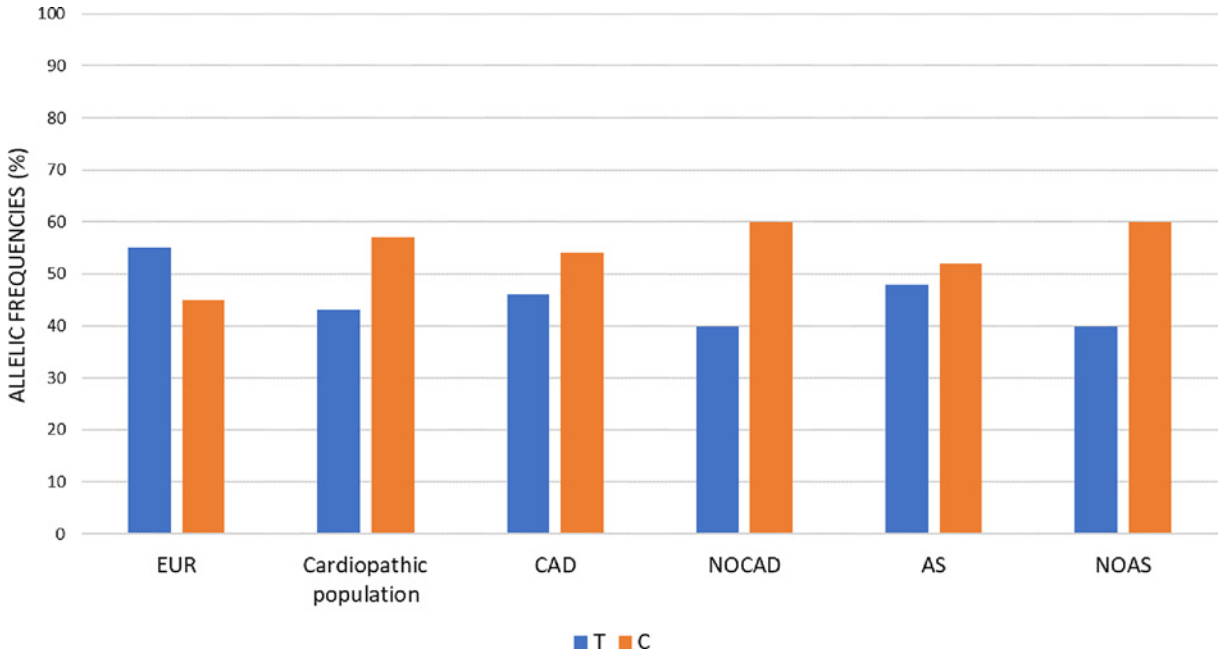

Figure 4. Allelic frequencies

Histogram representation of allelic distribution frequencies relating to rs7214723 polymorphism of *CAMKK1* gene, both in reference European (EUR) group and the study groups of CAD, NOCAD, AS and NOAS. All the frequencies are expressed in percentage.

The allelic frequencies were therefore calculated both in the reference populations group (European) and in the CVDs cohort here are studied. As for the genotypic frequencies, alleles were present at different frequencies in the two groups. In the reference European population, the ancestral allele T was the predominant one, while in the total cardiopathic population, and in all the other subgroups (CAD; NOCAD; AS; NOAS) the C allele was the most frequent, especially in the non-CAD and non-AS subgroups (Figure 4).

NOCAD stratification

Considering that AS patients comprised individuals with coronary condition patients and that AS has the same pathophysiological basis as coronary heart disease [2], we decided to further stratify the subgroup of non-coronary patients. The exclusion of the CAD patients from the AS group is clinically appropriate and can provides more power in testing for the phenotypic association with the SNP rs7214723.

Of the 150 non coronary patients, 79 had no AS (Figure 1).

The Chi-square test performed revealed statistical differences both for the genotypic (P=0.02) and allelic (P=0.05) distributions, the C allele and CC homozygotes more common in patients NOCAD with no AS compared with patients NOCAD with AS (Figure 5A,B).

Logistic regression analysis

We then tested a possible direct association between polymorphism rs7214723 in CAMKK1 and the risk of developing a specific CVD. Two models were estimated: the first concerned the risk of developing CAD, while the second one focused on AS.

Logistic regression analysis was applied to analyze simultaneously the influence of independent variables (genotype, sex, age, diabetes, hypertension, BMI and previous history of neoplasia) on the risk of developing CAD and AS (Tables 3 and 4, respectively). In both tables, the frequencies of the genotypes for rs7214723 polymorphism for CAD/NOCAD and AS/NOAS are shown.

Three different genetic models were considered in our analysis: additive model (TT; TC; CC), dominant model (CC+TC versus TT), and recessive model (CC versus TC+TT), considering C as the minor allele (C) in accordance with the frequency reported in the 1000 Genomes Database [27].

Not a significant association between the rs7214723 polymorphism and the risk of CAD was reported independently of the model taken in consideration (additive, recessive, and dominant; Table 3).

Table 3 Analysis of association between CAMKK1 rs7214723 and risk of CAD, through an unconditional logistic regression analysis adjusted for sex, age, diabetes, hypertension, BMI, previous history of neoplasia

rs7214723	CAD no.(%)	NOCAD no. (%)	OR (95% CI)	Robust Std. Err.	*P*> |Z|
Additive model					
Genotype					
TT	21 (14%)	12 (8%)	1.000 (reference)		
TC	95 (63.4%)	98 (65.4%)	0.496 (0.199–1.235)	0.230	0.132
CC	34 (22.6%)	40 (26.6%)	0.516 (0.185–1.437)	0.269	0.206
Age					
<60	19 (12.7%)	33 (22%)	0.468 (0.225–0.974)	0.175	0.043
≥60	131 (87.3%)	117 (78%)	1.000 (reference)		
Sex					
M	116 (77.3%)	76 (50.6%)	4.018 (2.210–7.305)	1.225	0.000
F	34 (22.7%)	74 (49.3%)	1.000 (reference)		
Hypertension					
YES	120 (80.5%)	108 (72.5%)	1.077 (0.566–2.049)	0.353	0.820
NO	29 (19.5%)	41 (27.5%)	1.000 (reference)		
Diabetes					
YES	55 (36.9%)	12 (8%)	5.540 (2.524–12.160)	2.222	0.000
NO	94 (63.1%)	137 (92%)	1.000 (reference)		
Pre. neoplasia					
YES	16 (10.8%)	13 (8.8%)	0.848 (0.299–2.397)	0.449	0.756
NO	133 (89.2%)	136 (91.2%)	1.000 (reference)		
BMI					
≥25%	86 (66.1%)	70 (52.7%)	1.220 (0.684–2.177)	0.360	0.499
<25%	44 (33.9%)	63 (47.3%)	1.000 (reference)		
Recessive model					
Genotype					
TT+TC	116 (77.3%)	110 (73.4%)	1.000 (reference)		
CC	34 (22.7%)	40 (26.6%)	0.949 (0.504–1.786)	0.306	0.872
Age					
<60	19 (12.7%)	33 (22%)	0.445 (0.215–0.922)	0.165	0.029
≥60	131 (87.3%)	117 (78%)	1.000 (reference)		
Sex					
M	116 (77.3%)	76 (50.6%)	3.732 (2.074–6.717)	1.119	0.000
F	34 (22.7%)	74 (49.3%)	1.000 (reference)		
Hypertension					
YES	120 (80.5%)	108 (72.5%)	1.069 (0.568–2.013)	0.345	0.835
NO	29 (19.5%)	41 (27.5%)	1.000 (reference)		
Diabetes					
YES	55 (36.9%)	12 (8%)	5.762 (2.640–12.573)	2.293	0.000
NO	94 (63.1%)	137 (92%)	1.000 (reference)		
Pre. neoplasia					
YES	16 (10.8%)	13 (8.8%)	0.843 (0.301–2.359)	0.442	0.746
NO	133 (89.2%)	136 (91.2%)	1.000 (reference)		
BMI					
≥25%	86 (66.1%)	70 (52.7%)	1.240 (0.698–2.200)	0.362	0.462
<25%	44 (33.9%)	63 (47.3%)	1.000 (reference)		
Dominant model					
Genotype					
TT	21 (14%)	12 (8%)	0.501 (0.204–1.231)		
CC+TC	129 (86%)	138 (92%)	1.000 (reference)	0.204	0.132
Age					
<60	19 (12.7%)	33 (22%)	0.469 (0.226–0.974)	0.174	0.042
≥60	131 (87.3%)	117 (78%)	1.000 (reference)		
Sex					
M	116 (77.3%)	76 (50.6%)	4.019 (2.212–7.305)	1.225	0.000
F	34 (22.7%)	74 (49.3%)	1.000 (reference)		

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Bioscience Reports (2021) **41** BSR20210326
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Table 3 Analysis of association between CAMKK1 rs7214723 and risk of CAD, through an unconditional logistic regression analysis adjusted for sex, age, diabetes, hypertension, BMI, previous history of neoplasia (Continued)

rs7214723	CAD no.(%)	NOCAD no. (%)	OR (95% CI)	Robust Std. Err.	*P*> |Z|
Hypertension					
YES	120 (80.5%)	108 (72.5%)	1.077 (0.566–2.048)	0.353	0.821
NO	29 (19.5%)	41 (27.5%)	1.000 (reference)		
Diabetes					
YES	55 (36.9%)	12 (8%)	5.528 (2.519–12.133)	2.217	0.000
NO	94 (63.1%)	137 (92%)	1.000 (reference)		
Pre. neoplasia					
YES	16 (10.8%)	13 (8.8%)	0.852 (0.302–2.396)	0.449	0.761
NO	133 (89.2%)	136 (91.2%)	1.000 (reference)		
BMI					
≥25%	86 (66.1%)	70 (52.7%)	1.220 (0.684–2.176)	0.360	0.499
<25%	44 (33.9%)	63 (47.3%)	1.000 (reference)		

Prev. neoplasia, previous history of neoplasia; *P*> |Z|, *P*-value from Z score; Robust Std. Err., robust standard error.
Additive model
Log pseudolikelihood = −151.48372
Prob > χ^2 = 0.0000
Recessive model
Log pseudolikelihood = −152.62122
Prob > χ^2 = 0.0000
Dominant model
Log pseudolikelihood = −151.49063
Prob > χ^2 = 0.0000

On the contrary, in male participants the CAMKK1 rs7214723 polymorphism resulted positively associated with the increased risk of CAD in all the three models, additive: OR = 4.018 (2.210–7.305), *P*=0.000); recessive: OR = 3.73 (2.074–6.717), *P*=0.000; dominant: OR = 4.019 (2.212–7.305), *P*=0.000).

Similarly, patients under 60 years and non-diabetic patients appear to have a lower risk to develop CAD irrespective of the model taken in consideration, but no association was found for hypertension, BMI, and previous history of neoplasia (Table 3).

When the rs7214723 polymorphism was tested for association with the risk of AS, male participants showed positive association. On the contrary, none of the other variables provided a significant result (Table 4).

Discussion

Over the last few years, genetic variants have raised interest for their relevance in different contexts. Polymorphisms have in fact been shown to have important roles as biomarkers for the prognosis and diagnosis of diseases, health prevention, epidemiology, and pharmacology [28].

The identification and study of genetic variants involved in multifactorial diseases is particularly important given the complexity of their underlying genetic architecture.

Within this context, CVDs are extremely important to investigate as represent one of the main causes of death in industrialized countries. For example, the search for specific genetic and epigenetic biomarkers that can cooperate with the canonical markers (e.g. troponin) in the diagnosis, prognosis, and risk prediction of CVDs might significantly contribute in improving the health status of industrialized societies [29].

In this context, it is really important to consider also the epidemiology of the CVDs and the role played by non-genetic factors as the environment, climate change, historical heritage and different lifestyle habits.

In a previous review, we discussed the several SNPs associated with CVDs in genes involved in the calcium/calmodulin pathway [14–21]. In particular, this study focused on some downstream genes to calmodulin, the first calcium sensor and signal transducer. In fact, this pathway is involved in the reception and transduction of the calcium signaling, that plays a key role in the heart being involved in different processes. This review highlights the high number of studies regarding nitric oxide synthase gene (*NOS*), especially the endothelial isoform (eNOS). Different studies have suggested the important role of eNOS in CVDs and how genetic variants might affect its activity and increase the higher risk to develop CVDs. In fact, the principal role of eNOS is the synthesis of nitric oxide, that

Table 4 Analysis of association between CAMKK1 rs7214723 and risk of AS, through an unconditional logistic regression analysis adjusted for sex, age, diabetes, hypertension, BMI and previous history of neoplasia

rs7214723	AS no. (%)	NOAS no. (%)	OR (95% CI)	Robust Std. Err.	P> |Z|
Additive model					
Genotype					
TT	16 (15%)	17 (8.8%)	1.000 (reference)		
TC	71 (66.4%)	122 (63.2%)	0.668 (0.303–1.475)	0.270	0.319
CC	20 (18.6%)	54 (28%)	0.466 (0.185–1.177	0.220	0.107
Age					
<60	10 (9.3%)	42 (21.8%)	0.518 (0.223–1.204)	0.222	0.127
≥60	97 (90.7%)	151 (78.2%)	1.000 (reference)		
Sex					
M	56 (52.3%)	136 (70.5%)	0.445 (0.256–0.774)	0.125	0.004
F	51 (47.7%)	57 (29.5%)	1.000 (reference)		
Hypertension					
YES	88 (82.2%)	140 (73.3%)	1.297 (0.657–2.559)	0.449	0.453
NO	19 17.8%)	51 (26.7%)	1.000 (reference)		
Diabetes					
YES	23 (21.5%)	44 (23%)	0.789 (0.411–1.513)	0.262	0.476
NO	84 (78.5%)	147 (77%)	1.000 (reference)		
Pre. neoplasia					
YES	15 (14%)	14 (7.3%)	1.780 (0.704–4.499)	0.842	0.223
NO	92 (86%)	177 (92.7%)	1.000 (reference)		
BMI					
≥25%	52 (53.6%)	112 (62.2%)	1.183 (0.682–2.053)	0.332	0.549
<25%	45 (46.4%)	68 (37.8%)	1.000 (reference)		
Recessive model					
Genotype					
TT+TC	87 (81.3%)	139 (72%)	1.000 (reference)		
CC	20 (18.7%)	54 (28%)	0.657 (0.349–1.236)	0.211	0.193
Age					
<60	10 (9.3%)	42 (21.8%)	0.500 (0.215–1.159)	0.214	0.106
≥60	97 (90.7%)	151 (78.2%)	1.000 (reference)		
Sex					
M	56 (52.3%)	136 (70.5%)	0.433 (0.250–0.752)	0.121	0.003
F	51 (47.7%)	57 (29.5%)	1.000 (reference)		
Hypertension					
YES	88 (82.2%)	140 (73.3%)	1.299 (0.655–2.575)	0.453	0.453
NO	19 17.8%)	51 (26.7%)	1.000 (reference)		
Diabetes					
YES	23 (21.5%)	44 (23%)	0.823 (0.435–1.557)	0.267	0.550
NO	84 (78.5%)	147 (77%)	1.000 (reference)		
Pre. neoplasia					
YES	15 (14%)	14 (7.3%)	1.797 (0.705–4.580)	0.857	0.219
NO	92 (86%)	177 (92.7%)	1.000 (reference)		
BMI					
≥25%	52 (53.6%)	112 (62.2%)	1.193 (0.687–2.069)	0.335	0.530
<25%	45 (46.4%)	68 (37.8%)	1.000 (reference)		
Dominant model					
Genotype					
TT	16 (15%)	17 (8.8%)	0.613 (0.282–1.334)	0.243	0.218
CC+TC	91 (85%)	176 (91.1%)	1.000 (reference)		
Age					
< 60	10 (9.3%)	42 (21.8%)	0.509 (0.219–1.184)	0.218	0.117
≥ 60	97 (90.7%)	151 (78.2%)	1.000 (reference)		
Sex					
M	56 (52.3%)	136 (70.5%)	0.445 (0.258–0.769)	0.124	0.004
F	51 (47.7%)	57 (29.5%)	1.000 (reference)		

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Bioscience Reports (2021) **41** BSR20210326
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Table 4 Analysis of association between CAMKK1 rs7214723 and risk of AS, through an unconditional logistic regression analysis adjusted for sex, age, diabetes, hypertension, BMI and previous history of neoplasia (Continued)

rs7214723	AS no. (%)	NOAS no. (%)	OR (95% CI)	Robust Std. Err.	P> |Z|
Hypertension					
YES	88 (82.2%)	140 (73.3%)	1.291 (0.657–2.535)	0.444	0.458
NO	19 17.8%)	51 (26.7%)	1.000 (reference)		
Diabetes					
YES	23 (21.5%)	44 (23%)	0.803 (0.421–1.533)	0.264	0.508
NO	84 (78.5%)	147 (77%)	1.000 (reference)		
Pre. neoplasia					
YES	15 (14%)	14 (7.3%)	1.718 (0.694–4.253)	0.794	0.242
NO	92 (86%)	177 (92.7%)	1.000 (reference)		
BMI					
≥25%	52 (53.6%)	112 (62.2%)	1.186 (0.684–2.056)	0.332	0.541
<25%	45 (46.4%)	68 (37.8%)	1.000 (reference)		

Abbreviations: Prev. neoplasia, previous history of neoplasia; *P*> |Z|, *P*-value from Z score; Robust Std. Err., robust standard error.
Additive model
Log pseudolikelihood = −162.35768
Prob > χ^2 = 0.0177
Recessive model
Log pseudolikelihood = −162.83012
Prob > χ^2 = 0.0112
Dominant model
Log pseudolikelihood = −162.95108
Prob > χ^2 = 0.0171

plays a crucial role in improving vascular density and maintaining cardiac performance [30,31]. Genetic variants in eNOS have been associated with low nitric oxide concentrations and vascular density [32–35].

Among these genes, the calcium/calmodulin kinase family proteins represent a group of genes particularly associated with CVDs [24,36,37]. Among them, CAMK2 is the most studied in the contest of the physiology of the heart [38–40]. CAMK2 has a key role in the excitation contraction coupling [41–43] because of its phosphorylation activity on several Ca^{2+} handling proteins, including *sarcoplasmic reticulum* (SR) Ca^{2+} release channels or ryanodine receptors (RyRs) [44], phospholamban (PLB) [45], and L-type Ca^{2+} channels [41]. Only a few genetic variants within this gene have been tested for their association with disease metabolism and CAD.

In the present study we focused on another member of the calcium/calmodulin kinase family, CAMKK1, in particular testing for the potential association between the SNP rs7214723 and a higher risk to develop CVDs. CAMKK1 is a calcium calmodulin dependent kinase kinase 1, a protein of the calcium calmodulin pathway.

The C to T variants lead to an amino acid change inside the kinase domain of CAMKK1 (E375G) which might create a conformational change and decrease it. This might contribute to modulate the calcium signaling pathway and thus increase the susceptibility to CVDs.

In the present study, we have analyzed the distribution of the genetic frequencies, related to rs7214723, in a cardiopathic population composed of 300 subjects and in a European population taken from the 1000 Genomes database [27].

The data obtained in the different genotypic distribution among European and cardiopathic study population resulted statistically significant: first, it was observed an increase of the homozygous frequency (TC) in a cardiopathic study group (Cardiopathic population TC = 64%; European TC = 48%). Moreover, it was possible to highlight the opposite trend of the homozygous genotype (TT and CC), between the two groups.

In the reference population groups, the frequency of TT was higher than that CC. On the other hand, in the population study, the trend was exactly the opposite: the frequency of CC was higher than that TT.

The same trend was observed also for the subgroups (CAD, NOCAD, AS, NOAS) (Figure 3 and Supplementary Table S1). Specifically, in the NOCAD and NOAS, the percentage of CC was the higher (NOCAD: CC = 27%; NOAS: CC = 28%, CAD: CC = 22%; AS: CC = 19%).

Considering that the study population group was a selected and specific group of cardiopathic subjects requiring cardiac surgery, it was interesting to observe the enrichment for the non-ancestral genotype CC, particularly in subjects with NOCAD and NOAS.

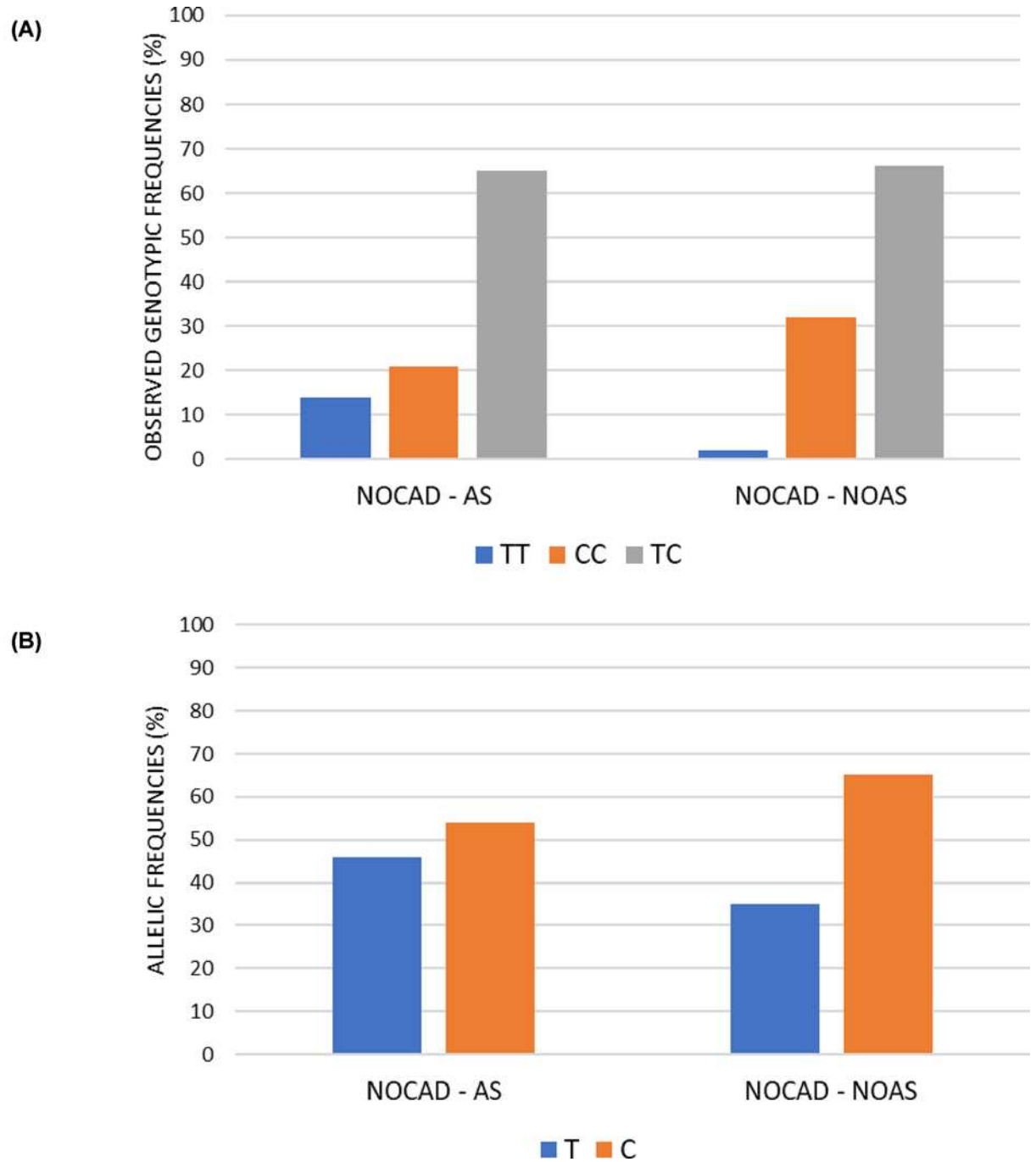

Figure 5. Focus on the pathology's subdivision of AS and NOAS in NOCAD group

(**A**) Histogram representation of frequencies of the genotypic distribution relating to rs7214723 polymorphism of *CAMKK1* gene. All the frequencies are expressed in percentage. (**B**) Histogram representation of allelic distribution frequencies relating to rs7214723 polymorphism of *CAMKK1* gene. All the frequencies are expressed in percentage.

The increase in a population specifically selected might suggest a higher risk for the subjects with the CC genotype to develop CVD. The enrichment specifically observed in the NOCAD and NOAS subgroups might suggest a major difficulty in bearing the specific damage caused to the tissue by no CAD (as valve diseases) or AS.

In view of the interesting data of the NOCAD and NOAS subgroups, the no coronary subgroup was further stratified to exclude from the AS and NOAS subgroups the patients with CAD, since these two pathologies have a similar

Bioscience Reports (2021) **41** BSR20210326
https://doi.org/10.1042/BSR20210326

pathophysiological basis. This stratification highlighted that no-coronary patients with no-AS have an higher enrichment of the C allele compared with no-coronary patients with AS (NOCAD/NOAS C = 65%, NOCAD/AS C = 54%).

The logistic regression analysis adjusted for sex, age, diabetes, hypertension, BMI, and previous history of neoplasia showed no association between polymorphism rs7214723 in CAMMK1 and the risk to develop CAD or AS was not found.

However, for male participants, CAMKK1 rs7214723 polymorphism showed a positive correlation with the increased risk of both CAD and AS.

The significance of the logistic regression analysis performed in the subdivision CAD/NOCAD held true only for diabetics and patients older than 60 years a higher risk to develop CAD. There was no significant association in the analysis of hypertension, BMI, and previous history of neoplasia.

The results obtained from the logistic regression analysis of AS/NOAS showed no significant association with the analysis of age, diabetes, hypertension, BMI, and previous history of neoplasia.

In summary, the results obtained in the present study suggest the need to better understanding the molecular and biological role of the *CAMMK1* gene in CVDs. The amino acid change (E375G) within the kinase domain of CAMKK1 might have a decisive role in the kinase activity of the protein influencing the downstream pathways of calcium signaling. This event might contribute to develop heart problems, increasing the predisposition to this kind of disease.

Several limitations exist in the present study. First, the small sample size of the population could have limited the statistical power to detect additional significant associations for each of the pathological types. Second, internal controls may need to be undertaken. Furthermore, considering that CVDs are multifactorial diseases, the comparison of the genetic data of the study population with the data relating to the database of 1000 genomes did not allow the evaluation of concomitant genetic and environmental factors in cases and controls. Considering this, it will be important in the future to perform gene association studies in order to investigate whether the combination of all polymorphisms of interest in the calcium calmodulin pathway, each with small effect, it could affect the development of the disease, more than a single variant itself. Further prospective population-based studies and biochemical experiments are needed to verify the conclusion.

Some evidence indicates that in addition to a possible role of polymorphic variants in the CAMKK1 protein, epigenetic modifications associated with the modulating activity of this protein could have an impact on cardiovascular risk. In this context, future studies will also be aimed at investigating the impact on the epigenome of both the different genotypes identified and of different concentrations of the protein.

Data Availability

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Sofia Beghi: Conceptualization, Data curation, Formal analysis, Writing—original draft, Writing—review and editing. **Francesca Cavaliere:** Conceptualization, Investigation, Methodology, Writing—review and editing. **Matteo Manfredini:** Data curation, Formal analysis, Validation, Writing—review and editing. **Sandro Ferrarese:** Data curation, Investigation, Writing—review and editing. **Claudio Corazzari:** Data curation, Investigation, Writing—review and editing. **Cesare Beghi:** Conceptualization, Resources, Supervision, Validation, Writing—review and editing. **Annamaria Buschini:** Conceptualization, Supervision, Funding acquisition, Validation, Writing—original draft, Project administration, Writing—review and editing.

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Abbreviations

AS, aortic stenosis; BMI, body mass index; CAD, coronary artery disease; CAMK, calcium/calmodulin- dependent kinase; CAMKK1, calcium/calmodulin-dependent kinase kinase 1; CVD, cardiovascular disease; eNOS, endothelial isoform of nitric oxide synthase; HWE, Hardy–Weinberg equilibrium; LV, leaflet valve; MAF, minor allelic frequency; MSC, mesenchimal stem cell; NOAS, no aortic stenosis; PPP3C, protein phosphatase 3 catalytic subunit; SNP, single nucleotide polymorphism.

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SUPPLEMENTARY MATERIAL

Polymorphism rs7214723 in CAMKK1: a new genetic variant associated with cardiovascular diseases.

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Table S1 - Number of subjects, allelic and genotypic frequency distributions of the populations under study. CAD (coronary artery disease); NOCAD (no coronary artery disease); AS (aortic stenosis); NOAS (no aortic stenosis).

Population (n. subjects)	Allelic frequency (count)	Genotypic frequency (count)	p value (genotypic frequency)
European population (503)	T: 55% (560) C: 45% (446)	TT: 32% (160) CC: 20% (103) CT: 48% (240)	EUR vs Cardiopathic population p=0.000
Cardiopathic population (300)	T: 43% (259) C: 57% (341)	TT: 11% (33) CC: 25% (74) CT: 64% (193)	
CAD (150)	T: 46% (137) C: 54% (163)	TT: 14% (21) CC: 22% (34) CT: 63% (95)	CAD vs NOCAD p= 0.225
NOCAD (150)	T: 40% (122) C: 60% (178)	TT: 8% (12) CC: 27% (40) CT: 65% (98)	
AS (107)	T: 49% (103) C: 51% (111)	TT: 14% (16) CC: 19% (20) CT: 67% (71)	AS vs. NOAS p=0.08
NOAS (193)	T: 40% (156) C: 60% (230)	TT: 9% (17) CC: 28% (54) CT: 63% (122)	

Calcium signalling in heart and vessels

Role of calmodulin and downstream Ca^{2+}-CaM dependent protein kinases

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Abstract

Worldwide cardiovascular disease is the leading cause of death. The remarkable success of medication and other preventive measures introduced toward the end of the 20th century have not yet halted the epidemic of cardiovascular disease. Instead, the burden of ischemic cardiovascular conditions has risen to become a top cause of morbidity, and mortality worldwide. Even though the pathophysiology and molecular biology of the heart and vessels have been extensively studied, it did not halt the progression of these conditions. Here we reviewed the regulation of calcium signalling as an important new research direction in cardiovascular disease.

Calcium plays several roles in the cardiovascular system. An important role is its involvement in the mechanism of excitation-contraction coupling that regulates a series of events ranging from regulating the action potential (electrical impulse), to the contraction of the heart and human vascular smooth muscle cell contraction, for the regulation of vasculature tone.

Both in the heart and vessels the rise of intracellular calcium is sensed by calmodulin. Calmodulin is a highly conserved protein in eukaryotes that regulates and activates a series of downstream kinases involved in the regulation of calcium signalling. Among them, the calcium calmodulin kinase family is particularly interesting because they are involved in the regulation of cardiovascular functions, such as electrophysiology and cardiac cell hypertrophy.

In this review we focus on the potential role of calcium calmodulin pathways in heart and vessels, specifically on calcium calmodulin dependent kinases downstream of calmodulin, with the aim to summarize the information regarding this topic present in literature and to identify missing aspects for future investigation.

Keywords

Cardiovascular disease, calcium signalling, calmodulin, calcium calmodulin dependent protein kinases

INTRODUCTION

Cardiovascular disease (CVD) is a complex disease and many factors and events are involved in the regulation of the physiology, mechanobiology and molecular biology of heart and vessels. Indeed, CVD is multifactorial because different factors coexist and cooperate together increasing the risk to progress CVD.[1–3] CVD is the leading cause of death all over the world and more people die annually from this type of disease than from any other cause. According to the World Health Organization, every year globally 17 million people die of CVD. Also in 2020, in which the death rate increased due to COVID-19, CVD still remained the principal cause of death.[4]

The term CVD refers generally to all conditions affecting heart and blood vessels. Among these conditions, the most common is atherosclerosis, which is associated with the growth and build-up of lipid rich plaques within the vessel wall. This event can block blood flow to the heart completely, causing an acute ischemic stroke. Atherosclerosis, as well as aortic aneurysm can be aggravated by vascular calcification, a pathological condition caused by calcium-phosphate crystals deposition leading to vascular stiffness. [5,6] Vascular calcification is an active process with a key role for human vascular smooth muscle cells (hVSMCs), the major cells type in the medial layer of arteries. hVSMCs are involved in regulating vascular tone and mediate remodelling processes in the vessel wall upon injury. [5–7] Calcification can also be deposited in the aortic valve causing aortic valve insufficiency. This event narrows the opening of the valve, reducing blood flow and is defined as aortic valve stenosis. [8–10]

Current knowledge of CVD and the underlying pathological mechanisms is insufficient to decrease the burden of disease. The aim of our review is to summarize the current state-of-the-art on the role of calcium (Ca^{2+}) signalling, with a focus on calmodulin (CaM) and its downstream calcium kinases.

The role of calcium in the cardiovascular system

Calcium is an abundant element in nature and the most abundant mineral in the human body. Some 99% of calcium in the body is found in bones and teeth, while the other 1% is found in blood and soft tissues. Calcium plays a crucial role in heart and vessels, both under physiological and pathological conditions.[11–15]

Cells in heart and blood vessels need calcium to contract and to perform their function. The concentration of calcium needed within the cell is some 100 nM. The calcium concentration outside the cell is 2 mM, thus a factor of 20,000 times higher. Therefore, calcium influx needs to be tightly regulated. The presence

of calcium-sensing receptors, such as calmodulin, is essential to sense calcium ions in the extracellular fluid and to activate a series of downstream pathways that directly regulate cellular functions [11,12]. The concentration of calcium inside cardiac cells has a heterogeneous distribution thanks to the presence of different microdomains in the cytosol, spaces between intracellular organelles and delimited compartments, and the sarcoplasmic reticulum. The main function of calcium in the heart is the regulation of excitation-contraction coupling (ECC). [15] ECC is a number of events starting at the production of the action potential (electrical impulse) to the contraction of heart muscles. Additionally, calcium is a co-factor for many enzymes, including proteins involved in blood coagulation. Platelets and several coagulation factors are activated by calcium ions, responsible for maintaining blood clot architecture and strength [16,17]. Calcium is also involved in the regulation of long term processes, such as gene-regulation in order to change protein expression. Moreover, calcium has a central role in adaptive tissue regulation, such as hypertrophy.[14]

Calcium also plays a crucial role in the physiology of hVSMCs, responsible for regulating blood flow and vascular luminal diameter. Calcium induces contraction of hVSMCs by creating a complex with the ubiquitous calcium binding protein calmodulin and increasing the activity of myosin light-chain kinase. [18] hVSMCs are characterized by a high degree of plasticity, having the capability to switch phenotype. Under physiological conditions, hVSMCs are in a quiescent contractile phenotype. However, upon biological or mechanical stress hVSMCs switch phenotype towards synthetic hVSMCs, characterized by a higher degree of migration and proliferation.[6,19,20] In both hVSMC phenotypes, intracellular calcium plays a different but pivotal role. The influx of calcium in contractile hVSMCs is regulated mainly via voltage-dependent L-type calcium channels, responsible for excitation-contraction coupling. In synthetic hVSMC, calcium entry is less dependent on voltage-dependent L-type calcium channels. [18] In synthetic hVSMCs, calcium entry is in part mediated via extracellular vesicles and may contribute to vascular calcification.[20] Intracellular calcium levels in hVSMCs are very dynamic, especially in contractile hVSMC where it changes both in spatial and temporal domains. The oscillations of intracellular calcium are more pronounced in contractile hVSMCs compared to synthetic. The higher oscillation frequencies in contractile hVSMC can increase kinase activity in a frequency-dependent manner through trapping phenomenon. [18]

Below we describe calmodulin and its signalling pathways in more detail in relation to CVD.

2. Calmodulin

CaM is involved in the regulation of calcium metabolism and transduction of calcium signalling. CaM is an important primary sensor of intracellular calcium levels in eukaryotic cells, playing a pivotal role in the transduction and deciphering of calcium signalling. [21,22] CaM is a low-molecular-weight protein of 16 kDa, composed of 149 amino acid residues. There are three different human genes (*CaM1, CaM2, CaM3*) that encode for three identical proteins. [23–26] CaM is an α-helical protein composed of N- and C- terminal lobes, each containing two calcium binding EF-hands. Thus, a total of four calcium ions can be bound per CaM (Figure 1). Due to intra and inter lobe cooperativity, CaM has evolved a high sensitivity to changes in intracellular calcium levels. [21–23] Calcium binding to CaM induces a conformational change, which is unequivocal for activation and modulation of second messenger downstream proteins such as adenylate cyclase, Serine/Threonine (Ser/Thr) kinases, nitric oxide synthase and (Ser/Thr) protein phosphatases (i.e. calcineurin), all involved in the transduction of the calcium signalling. CaM also regulates calcium transport via plasma membrane ATPase, a high-affinity calcium pump that contributes to the maintenance of intracellular calcium homeostasis.[27] CaM also binds to several structural proteins such as spectrin, neuromodulin, and caldesmon.[26,28] These proteins have been shown to play important roles in maintenance of plasma membrane integrity and cytoskeletal structure and in the regulation of smooth muscle and non-muscle contraction, respectively. As a primary sensor of intracellular calcium levels, CaM plays a key role in a multitude of process having a subcellular localization, such as the regulation of the cell cycle, fertilization, intracellular signalling, differentiation, cell death and cell contraction .[22,29]

The main function of CaM in the heart is the modulation of the action potential, leading to rapid contraction of a distinct group of cardiac cells through the regulation of different channels, [14,21] such as voltage-gated Na^+ channels, [30] voltage gated K^+ channels[31] and voltage-gated Ca^{2+} channels.[32] CaM is also involved in regulation of the sarcoplasmic reticulum Ca^{2+} release channel (RyR2), the main source of intracellular calcium necessary to trigger contraction with each heartbeat. [33,34] Moreover, CaM interacts with secondary pathway effectors of cardiomyocyte contraction, such as the beta-adrenergic pathway [35,36] and cyclic nucleotide signaling.[21] Finally, CaM interacts with downstream calcium CaM dependent kinases, supporting calcium recycling in cardiomyocytes necessary to maintain calcium homeostasis in preparation for a new excitation event. [21]

During evolution, the number of functions for CaM has increased steadily, also increasing pressure for sequence conservation. [37] In fact, CaM is highly conserved and most of human calmodulin mutations are deleterious and associated with life-threatening conditions in early infancy. Defects in CaM function

disrupt important calcium signalling events, resulting in fatal heart disease [27,28,37–39] (Table 1). Indeed, deleterious variants in CaM genes (*CaM1*, *CaM2*, and *CaM3*) cause calmodulinopathy, a rare life-threatening arrhythmia syndrome. [37,39] The term specifically refers to a wide spectrum of clinical manifestations associated with CaM mutations,[39] such as long QT syndrome (LQTS),[37,40–43] catecholaminergic polymorphic ventricular tachycardia (CPVT)[44] or idiopathic ventricular fibrillation (IVF).[45] CaM mutations were also identified in autopsy-negative sudden unexplained deaths (SUD) in young individuals.[39,46] Three heterozygous *de novo* mutations in either *CaM1* or *CaM2* were identified that caused an alteration of the residues near the calcium binding loops in the calmodulin carboxyl-terminal domain. These alterations were observed in infants who exhibited life-threatening ventricular arrhythmias combined variably with epilepsy and delayed neurodevelopment.[37] Moreover, the identification of a common mutation in a highly conserved residue (Phe90) in CaM1 showed underlying IVF manifestation in childhood and adolescence.[45] Further, a novel missense mutation in CaM1 was identified in a Moroccan family with a history of ventricular tachycardia and sudden death. This missense mutation was associated with exercise-induced QT prolongation, ventricular tachycardia and sudden cardiac death during childhood.[47]

In hVSMCs CaM is a critical calcium sensor, which regulates different downstream proteins. The Ca^{2+}-CaM complex is necessary to activate myosin light chain (MLC) subsequently leading to MLC phosphorylation, actin-myosin interaction and finally hVSMC contraction.[48–50] Calcium binding also commands the interaction between alpha-smooth muscle actin (αSMA) and myosin, affecting hVSMC elasticity and adhesion processes.[51] Another important target of Ca^{2+}-CaM in hVSMCs is the Ser/Thr phosphatase calcineurin and the family of calcium CaM dependent protein kinases, both involved in the regulation of cell cycle progression.[48] The role of CaM in hVSMC pathology and specifically vascular disease (atherosclerosis, aneurysm, calcification) has not been studied in detail thus far.

Taken together these findings highlight the role and function of CaM in the cardiovascular system. Indeed, CaM is one of the primary regulators of intracellular calcium. In fact, CaM transduces calcium signalling via adapting activity of ion channels and other second target proteins contributing significantly to physiological functions of electrically excitable tissues such as heart, blood vessels and brain. [21,37,39]

3. Calcium calmodulin kinases

The transduction and amplification of intracellular calcium sensed by CaM results in regulation, activation or deactivation, of downstream effectors involved in phosphorylation. Specifically, proteins involved in this pathway are of the calcium calmodulin kinase protein family (CaMK): a category of enzymes all classified as Ser/Thr kinases (Figure 2). CaMKs catalyse the transfer of phosphate from the gamma position of ATP to the hydroxyl group of protein bound Ser or Thr.[22,29,52] CaMKs can be divided in two different groups. The first consists of CaMKK1, CAMKK2, CaMKI, CaMKII and CaMKIV which are multifunctional. The second group includes substrate-specific kinases with only one downstream target: CaMKIII, phosphorylase kinase, and myosin light chain kinase.[52] All CaMKs share a common structure with a bi-lobed catalytic domain followed by a regulatory domain containing both an autoinhibitory as well as a CaM-binding site (Figure 3). [52,53] In case of basal levels of intracellular calcium, CaMKs are in an inactivate state due to the autoinhibitory domain that interacts and blocks the CaM binding or catalytic site. When intracellular calcium levels rise, CaM binds 4 calcium ions and becomes saturated, creating a conformational change. This conformational change results in interaction with the CaM binding domain on CaMKs, resulting in activation. The activation of some CaMKs, i.e., CaMKI, requires both binding of calcium as well as an additional modification, such as phosphorylation. Moreover, the activity of some kinases (i.e., CaMKIV and CaMKII) can be independent of CaM, allowing them to be functional beyond the duration of a transient elevation in calcium. [54–56] These differences in regulation are important for the control of many cellular functions of CaM and downstream signalling that affects many pathways.

The CaMK family controls several different functions.[53] CaMKI is implicated in control of long term potentiation, aldosterone synthase expression, bone resorption and proliferation, synapsin in nerve terminals and axon/dendritic outgrowth and growth cone motility. [57–62]

CaMKII family kinases are present in every tissue and therefore regulate a variety of functions: neuronal functions (neurotransmitter synthesis and release, neurite extension, and synaptic plasticity),[63–66] excitotoxicity/ischaemic-induced cell death, apoptosis, cell proliferation, osteogenic differentiation, regulation of fertilisation, maintenance of vascular tone, and cardiac function.[58,67–72] CaMKIV has shown to be involved in the regulation of cyclic AMP element binding protein (CREB), cell proliferation, fear memory, neurite outgrowth, immune and inflammatory responses.[73–77] Moreover, all of them control a broad spectrum of cancer-related functions, such as proliferation, migration, invasion and survival, in several tumour types. [53] In the following sections we describe in more detail the role of various CAMKs in heart and vasculature (patho)physiology.

3.1 CAMKII

CaMKII is the most important and abundant calcium calmodulin kinase in the heart.[13,78,79] There are four different isoforms, namely CAMKII α, β, γ, and δ, encoded by four different genes. All four are involved in cellular functions such as calcium homeostasis, membrane excitement, cell cycle, cytoskeletal organization, cell contraction, learning, memory, and gene expression and secretion.[52,56,79] It is important to note that the four isoforms of CaMKII have a different tissue distribution.[80] CaMKII γ and δ are mainly present in cardiac tissue whereas CaMKII α and β are mainly expressed in the neuronal system. [13,36,72] Specifically, CaMKII δ is the predominant isoform in the heart and plays a crucial role in the regulation of different cardiac functions. CaMKIIδ is involved in the integration of calcium signalling through a variety of pathways to maintain cardiac homeostasis as it is predominantly involved in the excitation contraction coupling (ECC) in the heart.[21,56,72,78,81]

The effect of CaMKII on ECC is crucial It phosphorylates several calcium handling proteins including sarcoplasmic reticulum (SR) calcium release channels, ryanodine receptors (RyR)[82], phospholamban (PLB)[78], and L-type calcium channels.[83] These downstream proteins are responsible for the regulation of cellular calcium influx, calcium release from the SR, as well as calcium uptake into the SR.[13] The phosphorylation of myofilament proteins (cardiac myosin binding protein-C) and myosin regulatory light chain 2 by CaMKII, contributes to myofilament calcium sensitization and interactions, putting forward its important role in ECC.[84,85] Additionally, CaMKII targets cardiac Na^+ [86,87] and K^+ channels, [34,88–90] which are also involved in the regulation of calcium homeostasis.

The activation of CaMKII depends on CaM activity. Upon elevation of intracellular calcium, Ca^{2+}-CaM binds CaMKII and subsequently activates all twelve subunits in separate holoenzymes forming the dodecameric structure of CaMKII. [91] CaMKII can also function independently from CaM. Here, Ca^{2+}-CaM bound to CaMKII ensures that one subunit is auto-phosphorylated at Thr286 by a neighbouring activated CaMKII subunit. This event generates autonomous activity, increasing the affinity of CaMKII for Ca^{2+}-CaM some 1000-fold, an event known as “CaM-trapping”. [52,55,56]

This autonomous activity of CaMKII can also be mediated by other posttranslational modifications such as oxidation, nitrosylation and glycosylation . [92] The altered hyperactivate regulation of CaMKII has been shown to be increased in myocardial disease, contributing to apoptosis, arrhythmias, [93] defective ECC and excitation-transcription coupling (ETC)[33,94,95] favouring pathological hypertrophy[80] and contractile

dysfunction specifically during heart failure (HF). [34,80,94,95] Since CaMKII is a pro-arrhythmogenic protein that is increased during CVD, i.e. myocardial injury, [96] atrial fibrillation, [97] cardiac hypertrophy, ischemia/reperfusion injury, [98] and heart failures, it has been put forward that inhibition of CaMKII might be a treatment option for cardiac pathologies. [13,99,100]

Also in blood vessels CaMKII plays a crucial role. In hVSMC CaMKII is activated by Ca^{2+}-CaM to regulate the activation of myosin light chain kinase (MLCK) by phosphorylation.[48] In hVSMCs, CaMKII has also been shown to inhibit CREB through different phosphorylation sites. CREB is an important transcription factor regulating expression of contractile and synthetic genes during hVSMC phenotype switching. The rise of intracellular Ca^{2} in hVSMCs promotes the nuclear translocation of Ca^{2+}-CaMKII, which then activates CaMKIV phosphorylation of CREB.[18] Moreover, it has been shown that Ca^{2+}-CaMKII and Erk1/2 activation play a crucial role in the regulation of hVSMC proliferation by agents such as s-adrenergic receptor agonists.[101] Finally, in hVSMCs, it was recently shown that CAMKIIδ and calponin 3 play critical roles in circRNA CDR1as/miR-7-5p-induced human pulmonary hVSMC calcification in hypoxic conditions.[102]

3.2 Ca^{2+}-CaM-Dependent kinase cascade

3.2.1 CaMKK family

The Ca^{2+}-CaM-dependent kinase cascade (Figure 2) consists of Ca^{2+}-CaM kinase-kinase family (CaMKK1 and CaMKK2), CaMKI and CaMKIV. This cascade is involved in different processes, such as cell proliferation, apoptosis, immune cell function, stem cell maintenance as well as glucose homeostasis.[13] Dysregulation of kinases in the Ca^{2+}-CaM-dependent kinase cascade is associated with a variety of diseases such as cancer, obesity, diabetes, neuronal and CVD.[103,104]

CaMKKs support transduction of calcium to other downstream kinases such as CaMKI and CaMKIV.[33,63] They are present both in the cytoplasm as well as in the nucleus of the cell. There are two isoforms of CaMKKs encoded by the genes *CAMKK1* and *CAMKK2.*[22,52,105] Both CaMKKs share a high sequence homology and the same common domain structure typical for all kinase proteins (Figure 3).[105,106] The main difference between them is that CaMKK1 is kept in an inactive state until binding of Ca^{2+}-CaM relieves the autoinhibitory mechanism, while CaMKK2 is characterized by a partially autonomous activity in the absence of Ca^{2+}-CaM.[52,53,106] Both kinases can be partially inhibited by cAMP-dependent protein kinase-A (PKA) that phosphorylates CaMKKs at different Ser or Thr residues within the CaM kinase binding domain and within the ATP binding region in the catalytic domain. Specifically, when PKA phosphorylates CaMKK1

at Ser458 it prevents binding to Ca^{2+}-CaM thereby inhibiting CaMKK from responding to increased intracellular calcium levels.[53,107] Moreover, the principal site of autophosphorylation for CaMKK2 is Thr482, which generates a partial autonomous activity independent of Ca^{2+}-CaM, disrupting the autoinhibitory mechanism of CaMKK2. As CaMKK2 is not dependent on rapid fluxes of intracellular calcium for basal activity, it can respond to other stimuli for longer duration.[13,52,53] The temporal sequence of these two distinct signalling events, PKA phosphorylation on CaMKK and its ability to block Ca^{2+}-CaM binding, plays an important role in CaMKK-dependent signalling.

A common characteristic of both CaMKKs is that they phosphorylate CaMKI and CaMKIV at residues Thr177 and Thr196, respectively.[22,52,53] In addition, both CAMKKs activate AMP-activated protein kinase (AMPK), a key regulator of cellular energy balance[108] (Figure 2). Sallé-Lefort *et al*. showed that the CaMKK/AMPK complex is involved in the activation of hypoxia-inducible factor-1α (HIF-1α). HIF-1α controls Malat1 (Metastasis-associated lung adenocarcinoma transcript 1), enhancing its transcription upon low oxygen conditions. Malat1 expression is upregulated under hypoxic conditions by the CaMKK/AMPK/HIF-1α axis.[109] Another downstream substrate of CaMKKs is AKT (also known as protein kinase B or PKB), an important oncology target. Activated AKT promotes phosphorylation of cellular substrates, thereby inducing cell proliferation, metabolism, cell growth and survival[53,110] (Figure 2).

Below we discuss the roles of CaMKK1, CaMKK2, CaMKI, and CaMKIV in CVD in more detail.

3.2.1.1 CaMKK1

The main role of CaMKK1 is phosphorylation of the downstream CaMKI and CaMKIV. It is important to note that the activation of CaMKK1 fully depends on CaM.[22,52,53,106] Recently, the crystal structure of CaMKK1 was revealed using two ATP-competitive inhibitors. The structural differences between CaMKK1 and CaMKK2 therefore can lead to the generation of CaMKK specific inhibitors.[106] Research supports the importance of CaMKKs in cardiovascular biology,[106,111] in the nervous system[106,111–114], and in skeletal muscle hypertrophy via mTOR.[115] Thus, CaMKK1 might have an important role in the cardiovascular system considering that the mTOR pathway plays a key regulatory function in cardiovascular physiology and pathology (Figure 2). [116] More recently it was shown that CaMKK1 is involved in the regulation of the mesenchymal stem cell (MSC) secretome.[117] In rats, injections in the heart of conditioned media of MSC overexpression CaMKK1 showed improvement in cardiac function after acute myocardial infarction, with increased vascular density and decreased scar tissue.[117] The direct overexpression of CaMKK1 in infarcted tissue using a CaMKK1-encoding plasmid significantly improved ejection fraction and decreased infarct

size after acute myocardial infarction. These data put forward a novel role of CaMKK1 as a regulator of the MSC secretome, indicating a potential therapeutic target for infarcted tissue. [117]

Recently, the genetic variant rs7214723 of CaMKK1 was shown to be associated with a higher risk of developing CVD.[118] This genetic variant, which was earlier associated with lung cancer risk,[119,120] is a single nucleotide polymorphism (SNP) that causes an amino acid change from glutamic acid (E) to glycine (G) at position 375 inside the catalytic domain of CaMKK1. This variation creates a charge change on the surface of the protein influencing substrate specificity, thereby inhibiting the downstream targets CaMKI and CaMKIV.[22,119,120] This polymorphism appears to be highly represented in the population (MAF index: C = 0.3954 / 19–0 - 1000 Genome). [121] Results from a cross-sectional study conducted on 300 cardiac patients showed a statistical difference between cardiopathic and several European reference populations between the genotype and allele frequencies for rs7214723. These data suggest a potential role of rs7214723 in CVD susceptibility that could be used as a possible genetic biomarker.[118]

While it is evident that interest in the function of CaMKK1 in the heart is growing, there is still a lack of information in literature regarding the role of CaMKK1 in vasculature and related vascular diseases.

3.2.2.2 CAMKK2

More research has been published on CAMKK2 compared to CAMKK1. Specifically, the interaction between CaMKK2 and AMPK has been well studied, as it regulates many physiological processes such as hypothalamic control of feeding behaviour, hepatic gluconeogenesis, adipocyte differentiation and macro-autophagy.[108,110,122] Since AMPK activity depends on CaMKK2, CaMKK2 has a possible role in cardiac energy production to protect the heart against calcium overload induced by sustained pressure load.[21] Moreover, AMPK signalling is repressed during cardiac hypertrophy, and cardiac-specific knockout of AMPK promotes stress-induced cardiac hypertrophy.[21,123] Thus, the inactivation of CaMKK2 might indirectly result in the development of metabolic dysfunction and cardiac hypertrophy (Figure 2). Histone demethylase JMJD1C, an important epigenetic factor, represses the activation of AMPK during cardiac hypertrophy through the reduction of CaMKK2 expression. This confirms that AMPK-signalling is involved in JMJD1C-mediated function in pathological cardiac hypertrophy.[124] Inhibition of kinase activity of CaMKK2 in mice resulted in increased left ventricular dilatation and dysfunction and subsequently mortality. This demonstrates that CaMKK2 can exert beneficial effects against pressure-overload-induced heart failure, thereby providing a therapeutic target for treatment of heart failure.[125] Additionally, CaMKK2 plays an important role in GLUT4 translocation through AMPK activation in cardiomyocytes. The

pan-CaMKK inhibitor STO-609 as well as overexpression of the dominant-negative form of CaMKK2 in cardiomyocytes inhibited H_2O_2-mediated translocation of GLUT4[124–126] (Figure 2). It has also been shown that CaMKK2 is involved in regulating mechanosensitive CaMKK2-AMPK-VASP/MLC signalling for migration of cells and for assembly of contractile actin stress fibers.[127] Moreover, recently it was demonstrated that this process is promoted by myosin-18. [128]

Diabetes is an important risk factor for the development of CVD. In type 2 diabetic mice, endocrine hormone fibroblast growth factor 21 (FGF21) has been shown to activate CaMKK2/AMPKα, thereby suppressing oxidative stress and enhancing endothelial nitric oxide synthase (eNOS) signalling, improving vessel relaxation.[129] This suggests the potential therapeutic use of FGF21 to activate CaMKK2/AMPKα for the treatment of endothelial dysfunction in diabetes. CaMKK2 is also involved in SOCE-CAMKK2-mTOR signalling, which is important for calcium-induced autophagy regulation.[130] This pathway is active at basal levels in cells of cardiovascular origin as an important homeostatic mechanism.[131] CaMKK2 activation and mTOR deactivation is associated with autophagy modulation (Figure 2). Here, a novel signalling pathway of SOCE-CAMKK2 in the regulation of autophagy was identified, offering new insights in maintaining proliferation and survival capability of endothelial progenitor cells (EPCs). This offers beneficial ways to improve EPCs transplantation efficacy to enhance vascular re-endothelialization in patients with hypercholesterolemia. [130] Moreover, tetrahydrobiopterin (BH4), a multifunctional cofactor implicated in regulation of nervous, immune, and cardiovascular systems, is a new potential endogenous activator of CaMKK2. BH4 targets CaMKK2 and promotes recovery of mitochondria in diabetic cardiomyopathy.[132]

AMPK, downstream to CaMKK2, is a master sensor of cellular energy status involved in progression of vascular calcification.[133] It is known that AMPK activators are associated with reduced calcification deposits[133], indicating a potential therapeutic role of AMPK in vascular calcification.[134] A recent study in mice showed that exogenous omentin-1 attenuates osteoblastic differentiation of hVSMCs through the activation of AMPK/Akt signalling pathway.[135] On the contrary, the inhibition of AMPK and Akt signalling reverses the anti-calcific effect induced by omentin-1 both *in vitro* and *in vivo*. [135] Moreover, Lai and colleagues demonstrated that during calcification KMUP-3 (xanthine derivative 7-[2-[4-(4-nitrobenzene)-piperazinyl]ethyl]−1,3-dimethylxanthine) inhibits both mTOR, downstream to CaMKK2, and β-catenin upregulation, essential for hVSMC phenotypic switching, as well as enhancing AMPK activation inhibiting hVSMC phenotypic switching.[136]

3.2.2 CaMKI and CaMKIV

CaMKI and CaMKIV are two downstream targets of CaMKKs (Figure 2). These kinases share the same common kinase-structure (Figure 3) and their activation requires both binding of Ca^{2+}-CaM and subsequent phosphorylation of Ca^{2+}-CaM kinase kinase proteins (CaMKKs) at Thr 177 and Thr 196, respectively.[13,52,53.] The difference in activation between CaMKI and CaMKIV is that CaMKI remains entirely Ca^{2+}-CaM-dependent whereas CaMKIV can undergo an intra-subunit autophosphorylation of the Ser/Thr-rich N-terminus generating Ca^{2+}-CaM independent activity. This allows CaMKIV to maintain its functionality beyond an increase in the concentration of intracellular calcium.[22,52] CaMKI is a monomeric kinase that is expressed by three different genes encoding α, β, and γ isoforms.[137,138] CaMKI is a cytosolic protein, which serves a diverse set of functions including transcription activator activity, cell cycle control, hormone production, cell differentiation, actin filament organization and neurite outgrowth (Figure 2). However, the precise role of CaMKI in CVD is not completely understood. [22]

CaMKIV is a monomeric kinase consisting of two isoforms (α and β) encoded by a single gene [77] and CaMKIV is considered important specifically in the nervous system, [139,140] but recently received attention for its role in cardiovascular pathophysiology.[141] Several studies showed an association of the genetic variant rs10491334 in CaMKIV with elevated diastolic blood pressure[139–141] (Figure 2). Furthermore, rs10491334 was also associated with a reduction in the expression levels of CaMKIV in hypertensive patients.[141] Moreover, CaMKK/CaMKIV has been shown to be a key endogenous protective pathway in ischemia and an important regulator of blood-brain barrier integrity. [142] The deletion of CaMKK or CaMKIV in mice exacerbates stroke outcome, such as infarct volume, edema formation and behavioural deficits. This data suggests that CaMKK signalling plays a protective role in stroke development. [143]

In the vasculature, the interaction between CaMKK and CaMKIV is important in the regulation of CREB during phenotype switching of hVSMCs [18] (Figure 2). hVSMC phenotype depends on the activity and form of different calcium-dependent transcription factors, such as CREB.[18,144] The regulation of hVSMC phenotype is thus partly governed by the Ca^{2+}-CaM kinase pathway. Nuclear CaMKIV plays a crucial role in the activation of CREB whereas CaMKII inhibits CREB phosphorylation. [144,145]

CONCLUSIONS

In this review we have summarised the current state of literature on Ca^{2+}-CaM -dependent kinase cascade (CAMKKs, CAMKI, CAMKIV) functions, with a focus on the cardiovascular system (Table 2). Our review clearly points towards an important role for CaM and Ca^{2+} kinase proteins in CVD, as all discussed kinases are directly or indirectly involved in heart tissue and vessel wall . CaMKKs are involved in the connection between calcium signalling cardiac metabolism resulting in calcium-induced autophagy, an important homeostatic mechanism active at basal levels in cells of cardiovascular origin.[130,131] Moreover, the phosphorylation cascade CaMKK/CaMKIV has been shown to be a key endogenous protective mechanism in ischemia and an important regulator of blood-brain barrier integrity. [142] In addition, genetic variants in several Ca^{2+}-CaM dependent kinases were associated with CVD.[22,118,139,141] Further, it is important to underline the role of Ca^{2+}-CaM -dependent kinases in hVSMC integrity. While it is known that the interaction of CaMKK2/CaMKIV is involved in phenotype switching of hVSMCs,[18] limited literature reports on the role of CaM and CAMKK1 in vessel wall homeostasis. As the Ca^{2+}-CaM -dependent kinase cascade regulates many cellular processes in CVD, further investigation of this cascade is necessary to understand this process and to open-up novel avenues that can be used to treat CVD.

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Table 1: List of the main human CaMs gene polymorphisms. CaM polymorphisms are associated with different type of arrhythmic phenotypes. Adapted from Kotta et al.[39]

Gene	Nucleotide change	Amino acid change	Associated phenotype	Refs
CALM1	c.389A>G	p.D130G	LQTS	37,39
CALM1	c.426C>G	p.F142L	LQTS	37,39
CALM1	c.161A>T	p.N54I	CPVT	39,44
CALM1	c.293A>G	p.N98S	LQTS, CPVT	39,44
CALM1	c.268T>C	p.F90L	IVF, SUD	45,47
CALM1	c.395A>T	p.D132V	LQTS	42
CALM2	c.293A>G	p.N98S	LQTS, SUD	39,40,46
CALM2	c.287A>T	p.D96V	LQTS	37,39
CALM2	c.293A>T	p.N98I	LQTS	39,40
CALM2	c.400G>C	p.D134H	LQTS	39, 40
CALM2	c.389A>G	p.D130G	LQTS	39
CALM2	c.396T>G	p.D132E	LQTS, CPVT	39,40
CALM2	c.394G>C	p.D132H	LQTS	42
CALM2	c.407A>C	p.Q136P	LQTS, CPVT	40
CALM3	c.389A>G	p.D130G	LQTS	39,41
CALM3	c.308C>T	p.A103V	CPVT	39
CALM3	c.286G>C	p.D96H	LQTS	39,43
CALM3	c.426T>G	p.F142L	LQTS	39,43

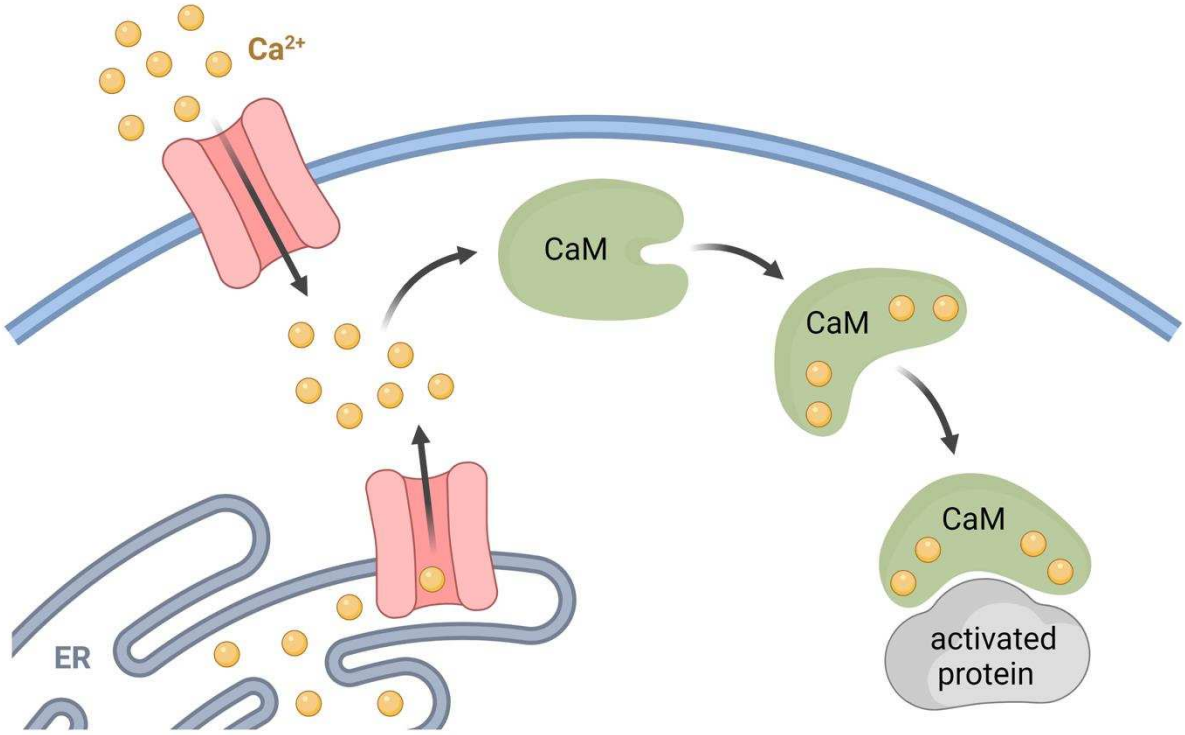

Figure 1: Schematic representation of Ca^{2+}-CaM activation. After binding 4 ions of Ca^{2+}, calmodulin undergoes a conformational changes that lead to activation of downstream proteins.

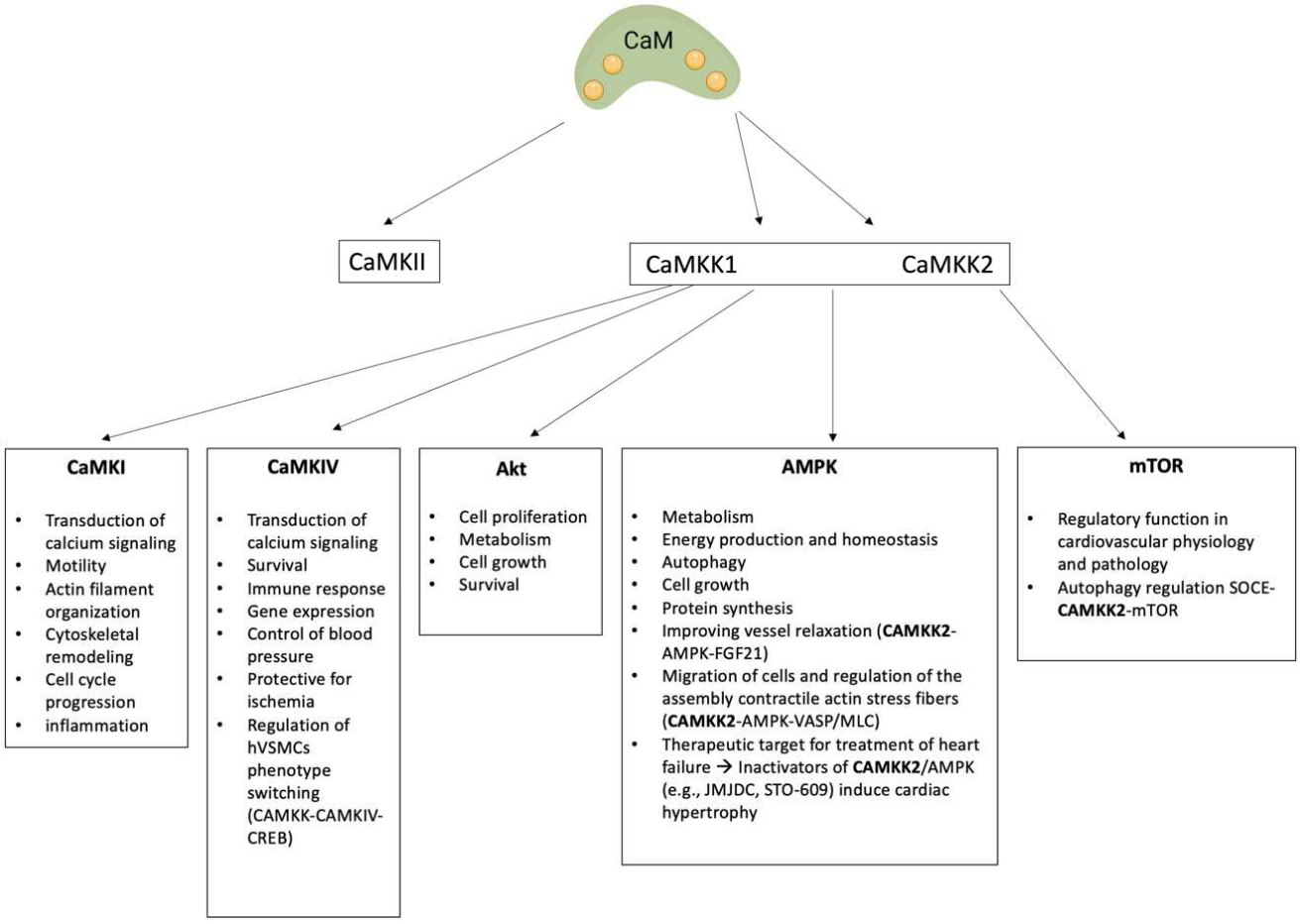

Figure 2: Ca^{2+}-CaM kinases downstream of calmodulin. Binding of calcium to calmodulin leads to activation of the Ca^{2+}-CaM dependent kinase cascade, consisting of CaMKK1 and CaMKK2 , CaMKI and CaMKIV, as well as activation of CaMKII. The activation of CaMKKs regulates crucial cellular functions. Calcium calmodulin mediates downstream signalling of CaMKI; CaMKIV; AMPK; Akt and mTOR.

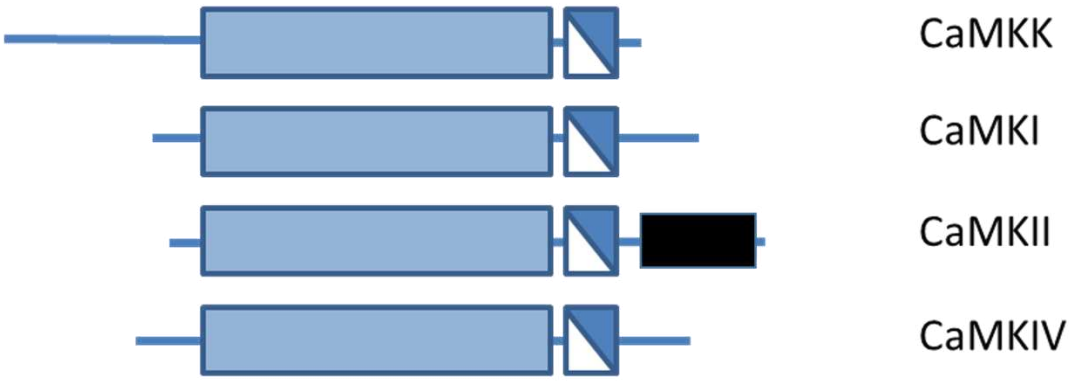

Figure 3: The general structure shared by the CaM-kinases. The N-terminal catalytic domain is represented in light blue, followed by a regulatory region containing in white the autoinhibitory domain and in dark blue the CaM-binding domain. The black block shows the C-terminal association domain of CaMKII. Adapted from Beghi S, et al. 2020.[22]

Table 2. Summary of the involvement of the CaM cascade in cardiovascular disease.

Kinase	In cardiac pathology	In vasculature pathology
CaM	Polymorphisms linked to calmodulinopathy, arrhythmia. [27,28,37–47]	Unknown
CaMKII	Implicated in myocardial injury, [96] atrial fibrillation,[97] cardiac hypertrophy, ischemia/reperfusion injury,[98] heart failures, contributing to apoptosis, arrhythmias, [93] defective ECC and ETC,[33,94,95] pathological hypertrophy[80] and contractile dysfunction during heart failure. [34,80,94,95.]	Regulation of VSMCs phenotype switching through the inhibition of CREB.[18]
CaMKK1	Polymorphism linked to the higher risk to develop CVD.[118]	Regulation of VSMCs phenotype switching (CaMKK-CaMKIV-CREB). [18]
CaMKK2	Inactivation of CaMKK2 indirectly results in the development of metabolic dysfunction and cardiac hypertrophy. [124]	Regulation of VSMCs phenotype switching (CaMKK-CaMKIV-CREB). [18]
CAMKI	Unknown	Unknown
CAMKIV	Polymorphisms linked to elevated diastolic blood pressure. [139–141]	Regulation of VSMCs phenotype switching (CaMKK-CaMKIV-CREB). [18]

CaMKK1 as a novel regulator of phenotype switching in human VSMCs

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Abstract

Cardiovascular diseases are the main cause of death all over the world: every year more people die for this type of disease than from any other causes. Ischemic heart disease and stroke are the world's biggest killers, with diseases of the vasculature (atherosclerosis, hypertension and vascular calcification) being the common underlying pathology.

Vascular smooth muscle cells (VSMCs) are the main cells in the arterial wall and play a crucial role in the development of vascular pathology. In physiological conditions, they exist as contractile cells, regulating vascular tone. In response to injury VSMCs lose expression of contractility-related genes, proliferate, migrate secrete extracellular matrix, and secrete increased amounts of extracellular vesicles (EVs) in order to repair the damaged vessel. In all vascular pathologies the loss of contractile phenotype is the initial event, however the molecular determinants of phenotype switching are not fully understood and was the aim of this study. We investigated the role of calcium calmodulin kinase kinase 1 (CaMKKK1), a member of the calcium calmodulin pathway in VSMC phenotype switching.

Using an in vitro human primary VSMC phenotype switching model we show that CaMKK1 expression was upregulated in synthetic VSMCs. We also show that siRNA knockdown of CaMKK1 induced an increase of contractile markers CNN1, αSMA and SM22α and decreased EV release, suggesting that CaMKK1 promotes changes associated with the synthetic phenotype. Additionally, we show that phenotype switching of VSMCs is associated with changes in the activity of kinases in the CaMK, CMGC and AGC families. CaMKK1 knockdown affects the activity of these kinases, which further supports the role of CaMKK1 in VSMC phenotype regulation.

In conclusion, we show that CaMKK1 is a novel regulator of phenotype switching in human VSMCs. Our results pave the way for new therapies for cardiovascular diseases that involve vascular remodeling such as atherosclerosis, calcification, hypertension and aneurysm.

Keywords

Cardiovascular disease, human vascular smooth muscle cells, calcium signalling, calcium calmodulin dependent protein kinases

Introduction

According to the World Health Organization, more people die annually from cardiovascular disease than from any other cause. Ischemic heart disease and stroke are the world's biggest killers, accounting for a combined 15 million deaths in 2015. These diseases have remained the leading causes of death globally in the last 15 years.[1] Diseases of the vasculature are the common pathology underlying heart disease and stroke, with atherosclerosis being the leading cause of vascular disease worldwide.[2] Others include hypertension and vascular calcification.[3] These statistics emphasize the importance of studying novel molecular mechanisms underlying vascular pathology, as existing therapies are not fully effective. With statins reducing the risk of coronary events by 20%[4] and antihypertensive therapies reducing the risk of stroke by 40%, myocardial infarction by 25% and heart failure by 50%[5] there is clearly room for improvement.

Human vascular smooth muscle cells (hVSMCs), the main cells building the arterial wall, are known to be crucial in the development of vascular pathologies. hVSMCs do not terminally differentiate but show phenotypic plasticity.[6–8] In physiological conditions they exist as contractile cells, regulating vascular tone. In response to injury VSMCs lose expression of contractility-related genes (SM22α, calponin (CNN1), myosin light chain (MLC), smoothelin (SMTN), α-Smooth muscle actin (αSMA)[9–11]), proliferate, migrate and secrete extracellular matrix (ECM)-related proteins in order to repair the damaged vessel.[12] Synthetic hVSMCs are also known to secrete increased amounts of extracellular vesicles (EVs)[13] and express synthetic markers S100A4 and Nox5. [14,15] In atherosclerosis, proliferation and migration of hVSMCs is key in the formation of a fibrous cap over the plaque[6] and hVSMCs have been shown to transdifferentiate into macrophages.[16] Dysregulated migration and proliferation of hVSMCs are known to contribute to hypertension[17] as well as formation of aortic aneurysms.[18] Finally, in vascular calcification hVSMCs assume characteristics of osteo/ chondrogenic cells.[19] In all these pathologies the loss of contractile phenotype is the initial event, however the molecular determinants of phenotype switching are not fully understood.

Among the different pathways involved in cardiovascular homeostasis, regulation of calcium signaling plays a crucial role, as it is involved in excitation contraction coupling.[20–24] The rise of intracellular calcium (Ca^{2+}) in cardiomyocytes and hVSMCs is sensed first by calmodulin (CaM), a multifunctional intermediate target of the secondary messenger Ca^{2+}.[25–27] Once CaM binds 4 ions of calcium, it is active, and under goes a conformational change that leads to binding and activation of downstream effectors: the calcium calmodulin kinase family (CaMKs) , including CaMKK1, CAMKK2, CaMK1, CaMK2 and CaMK4 which are multifunctional, and substrate-specific kinases, such as

CaMK3, phosphorylase kinase, and myosin light chain kinase.[28–30] This signaling pathway is called the Ca2+/CaM-dependent kinase cascade[27,31] and it is involved in many other cellular processes, such as glucose homeostasis, apoptosis, hematopoietic stem cell maintenance, normal immune cell function and cell proliferation.

Recently a single nucleotide polymorphism rs7214723 in CaMKK1 has been shown to be associated with higher risk of developing cardiovascular disease in a cohort of 300 cardiopathic subjects[32]. The polymorphism causes an amino acid change from glutamic acid (E) to glycine (G) at position 375 inside the catalytic domain of CaMKK1. This variation creates a charge change on the surface of the protein, influencing substrate specificity, thereby inhibiting activation of CaMK1 and CaMK4, which are downstream of CaMKK1.[26,32–34] CaMKK1 has been shown to be involved in nervous system pathology[28,35–38] skeletal muscle hypertrophy,[39] and in the regulation of the mesenchymal stem cell secretome.[40] However, its role in hVSMC phenotype switching and cardiovascular disease is unknown and was the aim of this research.

Materials and Methods

Cell Culture and treatments

Human primary VSMCs were derived from tissue explants as described before [41] and cultured for in Medium 199 (Gibco) supplemented with 1% Penicillin Streptomycin antibiotics (Gibco) and 20% FBS (Gibco). hVSMC were passaged when 80%-90% confluent and washed with PBS before treatments. hVSMCs in passages 5 to 9 were used. Human aortic samples were obtained from patients undergoing open aortic surgery at Maastricht University Medical Centre. Collection, storage, and use of tissue and patient data were performed in agreement with the Dutch Code for Proper Secondary Use of Human Tissue (http://www.fmwv.nl). This study complies with the Declaration of Helsinki.

For phenotype switching hVSMCs were treated with heparin (200 U/ml) in M199 with 2,5% FBS and/or low FBS (0,5%) for the contractile phenotype and PDGF-BB (platelet derived growth factor subunit B, 10 μg/ml) in 2,5% FBS for the synthetic phenotype. The cells were treated 1 day after the seeding, media were refreshed on day 4 after treatment, and cells were analyzed or harvested on day 6 in order to isolate DNA, RNA and proteins.

For the osteogenic differentiation, hVSMCs were treated with high calcium (3.6 mmol/L $CaCl_2$) medium, phosphate (2.5 mmol/L NaH_2PO_4) and with calcium and phosphate (2.7 mmol/L $CaCl_2$ and 2,5 mM/L NaH_2PO_4), all in M199 (Gibco) with 2,5% FBS (Gibco).

Microarray analysis of mRNA

Microarray mRNA analysis was performed as described before[42] hVSMCs were fasted overnight and stimulated with heparin (200 U/ml), PDGF (20 ng/ml) or control media for 4 hours. Data is shown as fold change compared to control.

SiRNA transfections

Transfection of hVSMCs at passage 6 with CaMKK1 SiRNA (L-004912-00-0005, Horizon) and non-targeting control SiRNA pool (D-001810-10-05, Horizon) was performed using the Neon™ Transfection System (Invitrogen) according to the manufacturer's protocol. Briefly, a total of $6x10^5$ divided in two 1.5 ml tubes ($3x10^5$ cells in each) were centrifuged 5 minutes at 200 x g at 4°C. Subsequently the pellet was washed with 1 ml of PBS and centrifuged again with same setting. The pellet was resuspended with 100 μl buffer R (Resuspension Buffer-Invitrogen) and 20 μl of 20 μM siRNA were added into each tube. 100 μl of this cell suspension was electroporated in the Neon® system at 1475 V for 20 ms, two pulses. Then the transfected cells were pipetted into a 12 well plate, according to the conditions of the experiment.

Immunoblotting

hVSMC were lysed with 50-100 μl RIPA buffer (Thermo Scientific) with MS-SAFE (Sigma) and a DC Protein Assay (BioRad) was performed according to the manufacturer's protocol. A total of 20 μg of proteins were loaded in 10% SDS gel (Biorad) and immunoblotting was performed as previously described[15] . The following commercially available antibodies were used for Western blots: rabbit anti-CamKK1 (1:1000; Ab NBP1-42683 NOVUS Biologicals), rabbit anti-SM22α (Abcam, ab14106, 1:1000), mouse anti-*α*-SM-actin, mouse anti-pMLC (Cell Signalling, 36755, 1:500), rabbit anti-CNN1 (Abcam, ab46794, 1:5000), rabbit anti-S100A4 (Dako, A5114, 1:1000), rabbit anti-osteocalcin (Santa Cruz, sc-30044, 1:400). Rabbit anti-VCL (1:10000, ab19002, Abcam) was used as loading control. The following secondary antibodies were used: goat anti mouse HRP (p0447, Dako, 1:3000), goat anti-rabbit (7074S, Cell Signalling, 1:3000). Bands were detected using chemiluminescence (SuperSignal

West Dura Extended Duration Substrate ECL, ThermoFisher) with the Invitrogen iBright Imaging System (Thermo Scientific). The signal was subsequently quantified with Image Studio Lite (Li-Cor).

Kinase activity profiling

Serine-Threonine kinase profiles were determined using the PamChip® Ser/Thr Kinase assay (STK; PamGene International, ´s-Hertogenbosch, The Netherlands). Each STK-PamChip® array contains 144 individual phospho-site(s) that are peptide sequences derived from substrates for Ser/Thr kinases. Cells transfected with siRNA and treated for phenotype switching with 3 biological replicates per condition, were washed once in ice-cold PBS after respective treatments and lysed for 15 min on ice using M-PER Mammalian Extraction Buffer containing Halt Phosphatase Inhibitor and EDTA-free Halt Protease Inhibitor Cocktail (1:100 each; Thermo Fischer Scientific). Lysates were centrifuged for 15 min at 16.000 x g at 4°C. Protein quantification was performed with Pierce™ Coomassie Plus (Bradford) Assay according to the manufacturer's instructions.

For the STK assay, 1 µg of protein and 400 µM ATP were applied per array (N=3 per condition) together with an antibody mix to detect the phosphorylated Ser/Thr. After incubation for an hour (30^{o}C) where the sample is pumped back and forth through the porous material to maximize binding kinetics and minimize assay time, a secondary FITC-conjugated antibody is used to detect the phosphorylation signal. Imaging was done using a LED imaging system and the spot intensity at each time point was quantified (and corrected for local background) using the BioNavigator software version 6.3 (PamGene International, 's-Hertogenbosch, The Netherlands).

EV Quantification

Conditioned media from hVSMCs treated for phenotype switching and transfected with siRNA were collected in 1,5 ml tubes and centrifuged for 8 minutes at 6000 x g. Medium 199 (Gibco) EVs free supplemented with 1% Penicillin Streptomycin antibiotics (Gibco) and 2,5% FBS (Gibco) was used. The samples were loaded directly in the Zeta View NTA (nanoparticle tracking analysis) for the quantification of EVs secreted in the cell culture media. Each sample was diluted 1:40 and 1 ml were loaded in the machine with a syringe at 21°C. The data collected were normalized to the protein concentrations (mg/ml). Pattern parameters, such as intensity fluctuations, surface geometry and shape of the particles as well as particle concentration are documented at each recording and can be used to distinguish sub-populations.

Statistical analysis

Data are shown as mean ± SD (standard deviation) with individual data points and were obtained in 3 or more independent experiments. Each experiment was repeated in hVSMCs from at least 3 different donors in triplicate. Normality of data was tested using Shapiro-Wilks test or ascertained based on previous literature reports. All the data was deemed normally distributed, therefore statistical significance was tested with t-test and/or 1-way ANOVA. The exact test used for each data set is mentioned in figure legends. Statistical analysis was performed using GraphPad Prism 8.2.0. *P<0.05. Representative images for figures, which best reflected the data, were selected manually.

Upstream Kinase Analysis (UKA, PMID: 30610604), a functional scoring method (PamGene) was used to rank kinases based on combined specificity scores (based on peptides linked to a kinase, derived from 6 databases) and sensitivity scores (based on treatment-control differences). The red/blue reflects the Median Kinase Statistic, where positive (red) means upregulation and negative (blue) means downregulation. For the Mean Finale Score, 1.2 is the cut-off value that defines an upregulation.

Results

Synthetic phenotype in hVSMCs is associated with increased expression of CaMKK1

First, we set out to evaluate whether CaMKK1 (calcium calmodulin kinase kinase 1) expression changes due to hVSMC phenotype switching *in vitro*. hVSMCs were treated with heparin or medium with low concentration of FBS to induce a contractile phenotype and with PDGF-BB to induce a synthetic phenotype, an established model of hVSMC phenotype switching *in vitro*.[15] Expression was analysed by real-time PCR, microarray analysis of mRNA and western blotting for CaMKK1 as well as for markers specific for the contractile hVSMC phenotype, i.e CNN-1 (calponin 1), MYH11 (myosin heavy chain 11), SMTN (smoothelin), α-SMA (α-smooth muscle actin), SM22-α (smooth muscle protein 22α), pMLC (phosphorylated myosin light chain) and for the synthetic hVSMC phenotype, i.e S100A4 (S100 calcium binding protein A4). was analyzed.

While qPCR results show no changes in gene expression (Supplementary Figure 1A-E), mRNA microarray data showed decreased expression of SM22α and MLC in both PDGF- and heparin-treated cells (Figure 1A-B), as well as higher expression of CaMKK1 in these conditions (Figure 1C). However, no changes in MYH11 and αSMA expression were observed (Supplementary Figure 1F-G).

Western blotting results confirmed phenotype switching of hVSMCs treated with heparin and PDGF (Figure 1D-L). Indeed, synthetic hVSMCs showed decreased expression of contractile markers CNN-1, α-SMA, SM22-α, and pMLC and increased expression in synthetic marker S100A4 compared with contractile hVSMCs. Expression of CaMKK1 was higher in synthetic hVSMCs compared to untreated and contractile cells, but no difference was observed between contractile and untreated hVSMCs. These results suggest that CaMKK1 either plays a role in regulating phenotype switching or is affected by hVSMC phenotype switching.

Since we have shown before that polymorphism rs7214723 in CaMKK1 in a cohort of 300 cardiopathic subjects is associated with a higher susceptibility to cardiovascular disease, we hypothesized that the genotype of CaMKK1 could play a role in regulating hVSMC phenotype. Therefore, we genotyped the SNP rs72142723 in the CaMKK1 gene in hVSMCs isolated from 7 different donors used for these experiments (Figure 1E-L, see color coding legend). Among this group, 3 were homozygous TT, 2 homozygous CC and 3 heterozygous TC. Interestingly, the patients with the genotype CC are the only hVSMCs among the three genotype to have a higher expression of CaMKK1 in contractile cells, suggesting that the genotype variation might be important for regulating CaMKK1 expression under phenotypic switching conditions. However, a statistical test could not be performed to confirm this association due to the low donor number. Any differences between genotypes were not apparent in hVSMC phenotype markers analyzed.

We also examined CaMKK1 expression under calcifying conditions, which is a more extreme form of phenotype switching and found no differences, while expression of osteocalcin (OCN) was increased, indicative of osteogenic differentiation (Supplementary Figure 2A-B). This suggests that CaMKK1 is not involved in osteogenic differentiation of hVSMCs.

CaMKK1 promotes the synthetic phenotype of hVSMCs

To further elucidate the link between CaMKK1 and hVSMC phenotype we performed siRNA knockdown of CaMKK1 (Figure 2A-G, Supplementary Figure 3A contains uncropped blots). Western blotting confirmed that siRNA knockdown of CaMKK1 was successful in cells treated with heparin, low FBS and PDGF. CaMKK1 knockdown caused an increase in CNN1, αSMA and SM22α at baseline (Figure 2A-G), but not in heparin-, low FBS- or PDGF- treated cells. CaMKK1 knockdown had no effect on the expression of pMLC and synthetic marker S100A4. This suggests that CaMKK1 promotes a synthetic hVSMC phenotype, as absence of CaMKK1 increased contractile marker expression.

Changes in EV release has been reported in hVSMCs undergoing phenotype switching. Synthetic hVSMCs secrete more whereas contractile hVSMCs secrete fewer EVs than a mixed population of untreated cells.[13] Therefore, we quantified EVs secreted by hVSMC treated for phenotype switching (control; low FBS; PDGF) in the context of siRNA knockdown of CaMKK1. Our data show that CaMKK1 knockdown results in a trend of lowering EV release in low FBS- and PDGF-treated hVSMCs, which did not reach statistical significance (Figure 3). This is consistent with the western blot findings, in which absence of CaMKK1 decreased EV release, a phenomenon associated with the contractile hVSMC phenotype.

Changes in intracellular calcium are another hallmark of hVSMC phenotype switching.[15] Therefore, we analyzed intracellular calcium in a set of experiments. Intracellular calcium measurement showed a trend of higher intracellular calcium in hVSMCs knockdown for CaMKK1 (Supplementary Figure 3B).

CaMKK1 regulates activity of kinases involved in hVSMC phenotype switching

Considering the lack of information regarding CaMKK1 in literature and the interesting results of its possible role in phenotype switching in hVSMC, the next goal was trying to understand in which signaling pathways CaMKK1 is involved. Specifically, we were interested in kinase activity profiling, which is based on measuring peptide phosphorylation by protein kinases (Figure 4, Supplemental Files 1-5). Pairwise comparisons were made between hVSMCs treated with control, low FBS or PDGF-BB, in the absence and presence of CaMKK1 siRNA.

We found that ERK family kinases (ERK1, ERK2, ERK5, ERK7), belonging to the CMGC family, have a higher activity in PDGF-treated cells compared to control, and specifically ERK7 stands out as one with the highest activity (Figure 4, Supplemental File 1). Further to that, kinases mostly belonging to the CAMK and AGC family were more active in hVSMC treated with low FBS compared to control (Figure 4, Supplemental File 2; e.g., ERK7; AurA/Aur2; AurB/Aur1; DAPK3; IKK; RSK2). Interesting to note, ERK1, ERK2 and ERK5 activity showed no difference. These results confirm that hVSMC phenotype switching is associated with changes in kinase activity.

Next, we investigated effects of control siRNA to CaMKK1 siRNA in control, PDGF- and low FBS-treated hVSMCs. In control medium, multiple kinases showed moderately increased activity with CaMKK1 knockdown, mainly in kinases belonging to the CMGC family (Figure 4, Supplemental File 1; e.g., CDK4, CDK5, CDKL5). In PDGF-BB with CaMKK1 knockdown treated hVSMCs, some kinases

showed moderately increased activity (e.g., ARAF, BRAF) while some others showed a downregulation, mainly kinases belonging to the CaMK family (e.g. CaMK2, CaMK4, MSK2) and kinases belonging to the AGC family (e.g. PKAα, PKAβ, PKG1, PKG2; Supplemental Figure 4,File 4). Interestingly, in low FBS- treated hVSMCs, all kinases present in the microarray are downregulated by CaMKK1 knockdown, with the exception of CDK5 and DAPK3, whose activity did not change. This downregulation highlighted several kinases in the CMGC, CAMK, AGC families. Other kinases that were affected were in the CK1, STE and TKL families (Figure 4, Supplemental File 5). Taken together these results show that the knockdown of CaMKK1 is associated with changes in kinase activity during phenotype switching of VSMCs. Among the kinases with lower activity after CaMKK1 knockdown in low FBS-treated VSMCs there are AurB/Aur1, CaMK2, CDK10, CDKL2, CK2α1, ERK7, IKKα and MSK2.

Focusing the attention on kinases downstream of CaMKK1, we analysed CaMK4 activity. Interestingly there were no differences in CaMK4 activity in PDGF-treated cells compared to control (Figure 4). However, CaMK4 showed higher activity in the low FBS-treated cells compared to control. Moreover, CaMKK1 knockdown induced downregulation of activity of CaMK4 both in the PDGF- and low FBS-treated VSMCs. CaMK2α, a calcium calmodulin dependent kinase not downstream to CaMKK1 and involved in different processes in the vessel, such as the regulation of contraction of hVSMC, shows the same pattern of regulation in conditions described above (Figure 4). This suggests that the knockdown of CaMKK1 affects the activity of both CaMK4 and CaMK2α.

DISCUSSION

In this study it we identified CaMKK1 as a new regulator of VSMC phenotype. We observed that the expression of CaMKK1 increased when hVSMCs were treated with PDGF to induce the synthetic phenotype. We also show that CaMKK1 knockdown caused an increase in contractile marker expression at baseline and trend towards decreased EV release in PDGF and low FBS treatment (Figure 2H).

To the best of our knowledge, this is the first study showing increased expression of CaMKK1 in synthetic hVSMCs. These results suggest that the conditions favoring vascular remodeling activate at least part of the CaM cascade. This is in line with literature showing that CaMKKs are involved in the regulation of phenotype switching in human vascular smooth muscle cells, through the activation of CaMK4 and subsequently of CREB (cAMP response element-binding protein). [43]

CaMKK1 knockdown caused an increase in some, but not all of the studied hVSMC contractile markers at baseline. This suggests that CaMKK1 exerts an inhibitory effect on the expression of CNN1, αSMA and SM22α, implying that its activation promotes the synthetic phenotype. CaMKK1 had no effect on the expression of pMLC and synthetic marker S100A4, suggesting that CaMKK1 regulates expression programs responsible for hVSMC phenotype switching partially, not comprehensively. These effects of CaMKK1 knockdown were not observed in synthetic or contractile hVSMCs, suggesting that perhaps in the presence of other stimuli the absence of CaMKK1 is compensated for by other pathways regulating hVSMCs phenotype marker expression.

Elevated EV release is an important hallmark of hVSMC phenotype switching.[13,44] Here we show that hVSMCs with downregulated CaMKK1 and treated for phenotype switching showed a trend toward decreased EV release in both contractile and synthetic hVSMCs, and no change at baseline. This suggests that CaMKK1 stimulates EV release, further supporting its role in promoting the synthetic phenotype. This data is supported by literature, which has shown that CaMKK1 increased EV release in rat mesenchymal stem cells.[40]

In this work we also showed that both hVSMC phenotype switching and knockdown of CaMKK1 affect the Ser-Thr kinase activity in hVSMCs. The most changes were seen in the CMGC (which includes ERKs) and CaMK families. Specifically, several ERK kinases (ERK7 in particular) had a higher activity in hVSMC treated with PDGF compared to the control. This confirms that PDGF stimulation leads to the initiation of ERK signaling, contributing hVSMC proliferation,[45–48] which is a hallmark of the synthetic phenotype. Additionally, several other kinases from the CMGC family (p38, JNK), are involved in the ERK signaling and regulate cell proliferation and migration, characteristic of the synthetic phenotype induced by PDGF.[47–50]

Some of them (ERK7, p38 α, β, γ, δ, JNK2,RSK) were activated also in the low FBS condition and this might be because ERK MAP kinases also regulate the contraction pathways, that characterize the contractile phenotype induced by low FBS.. [51–53]

In this study we identify several other kinases involved in hVSMC phenotype switching, some of them (Aur, IKK, DAPK) with more activity compared to other. The lack of information of their role in hVSMCs suggests the identification of many new potential regulators of hVSMC phenotype switching.

Moreover, the knockdown of CaMKK1 affected the downregulation of the CMGC protein family more in the low FBS treatment than in the PDGF, suggesting that in this last one there might be other signaling at play that partially compensates for the lack of CaMKK1.

Importantly, in the hVSMC treated with low FBS, there was an upregulation of kinases from the AGC family. Among this group, some are involved in regulating contraction (PKA, PKC, p70S6K)[54,55] and others (e.g. PKG)[56] are involved in the inhibition of hVSMC proliferation and migration, consistent with this treatment promoting the contractile phenotype. However, the knockdown of CaMKK1 in the low FBS condition downregulated the activity of these pro-contractile AGC family members, suggesting that CaMKK1 might have disparate effects on signaling relevant to hVSMC phenotype.
Additionaly,For example, regarding the CaMK family, the results in this study show that both the phenotype changing treatments and CAMKK1 knockdown affect the activity of the CaMK family. The fact that PDGF and low heparin regulate activity of the CaMK family further confirms that the CaM cascade is involved in hVSMC phenotype switching. Specifically, it is interesting observe that the knockdown of CaMKK1 induced a general downregulation of all the kinases present in the Pamchip microarray, both in the PDGF and low FBS treatment, in this last one ERK7 was the most downregulated (confirming again its role described previously).

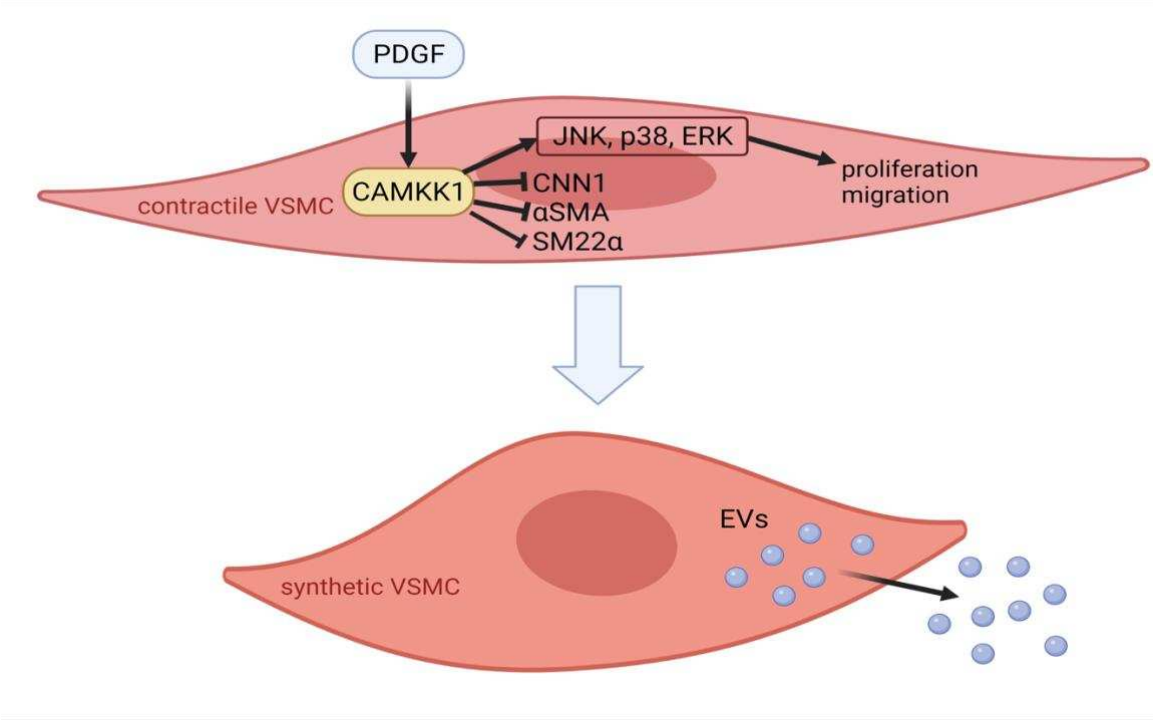
Focusing the attention on CaMK4, a downstream target of CaMKK1 (CaMK1 was not present in the array), it is interesting observe also how change its activity in the different treatment. Indeed, in hVSMC knockdown for CaMKK1, CaMK4 resulted to be downregulated both in the low FBS and PDGF treatment. On the contrary, it was upregulated in hVSMC treated with low FBS, compared to the control. These results confirm the involvement of this pathway in hVSMC phenotype switching. We hypothesize that this pathway can be involved in the activation of CREB (cAMP response element-binding protein), an important transcription factor that regulates expression contractile and synthetic marker genes during hVSMC phenotype switching when it is activated by CaMK4[43]. Due to the lack information in literature, it will be necessary to perform further experiment to better investigate the specific role of this pathway in hVSMC phenotype switching.

Moreover, our results highlight the potential role of single nucleotide polymorphism (SNP) rs7214723 in CaMKK1 in association with the higher risk to develop cardiovascular disease.[32] Previously an enrichment of the C allele was identified in a cohort of 300 cardiopathic subjects and specifically, the subgroup without coronary artery disease and aortic stenosis (NOCAD-NOAS) had higher frequency of the CC genotype. In this study, we show that in the treatment with heparin, hVSMC isolated from patients with the genotype CC for the SNP in CaMKK1 showed the higher expression of CaMKK1. While this change was not statistically significant, it is tempting to speculate that CaMKK1 genotype could have an effect of the response of hVSMCs to phenotype-altering stimuli.

This study has other several limitations. First, it is important consider that in some experiments the standard deviation is high and statistical significance could not be reached. This is due to the inherent variability of cells isolated from several different human donors, on which their experiments were performed on. Additionally, due to a low donor number we could not perform statistical analysis to verify the association between the polymorphism in CaMKK1 and phenotype switching.

In conclusion, we show that CaMKK1 has a new role in the regulation of phenotype switching in human h

hVSMCs, influencing the activity of several Ser-Thr kinases and expression of phenotype markers. These findings are important to cardiovascular diseases that involve vascular remodeling such as atherosclerosis, calcification, hypertension and aneurysm, as they provide a new potential pathway to investigate possible novel therapeutic targets.



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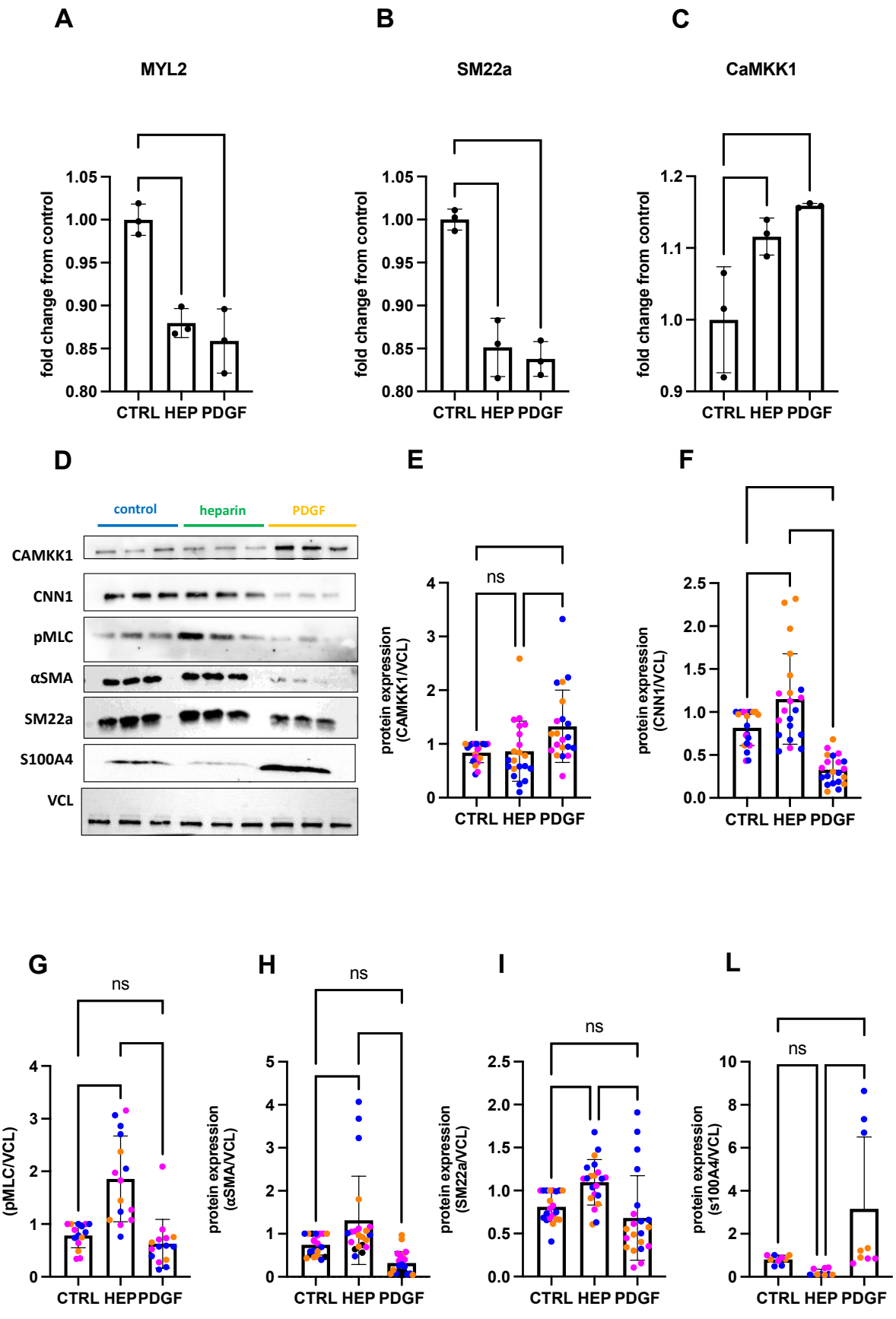
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1.00
0.95
0.90
0.85
0.80
CTRL HEP PDGF
B
SM22a
fold change from control
1.05
1.00
0.95
0.90
0.85
0.80
CTRL HEP PDGF
C
CaMKK1
fold change from control
1.2
1.1
1.0
0.9
CTRL HEP PDGF
D
control
heparin
PDGF
CAMKK1
CNN1
pMLC
αSMA
SM22a
S100A4
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E
ns
protein expression (CAMKK1/VCL)
4
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CTRL HEP PDGF
F
protein expression (CNN1/VCL)
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protein expression (pMLC/VCL)
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protein expression (αSMA/VCL)
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protein expression (SM22a/VCL)
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Figure 1: Phenotypic switching of hVSMCs is associated with changes in contractile gene and CaMKK1 expression. hVSMCS were treated with PDGF-BB (10 μg/ml) and heparin (200 U/ml) in M199 with 2.5% FBS for seven days. **A-B)** Contractile markers MYL2 (MLC) , SM22a and **C)** CaMKK1 expression analysed in an RNA microarray. **D)** Western blot and **E-L)** quantification of CaMKK1, contractile proteins (CNN1, pMLC, α-SM-actin, SM22α) and synthetic marker S100A4. The results confirm phenotypic switching of hVSMC and show higher expression of CaMKK1 in hVSMC treated with PDGF. Graphs show pooled data from 3 to 7 independent experiments performed in triplicate. Colored dots indicate the genotypes associated with the polymorphism rs7214723 in CaMKK1: in blue the homozygous TT, in pink the homozygous CC, in orange the heterozygous TC. Statistical analysis was performed using GraphPad Prism 9. Statistical significance was tested using One-Way ordinary ANOVA; ns - not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

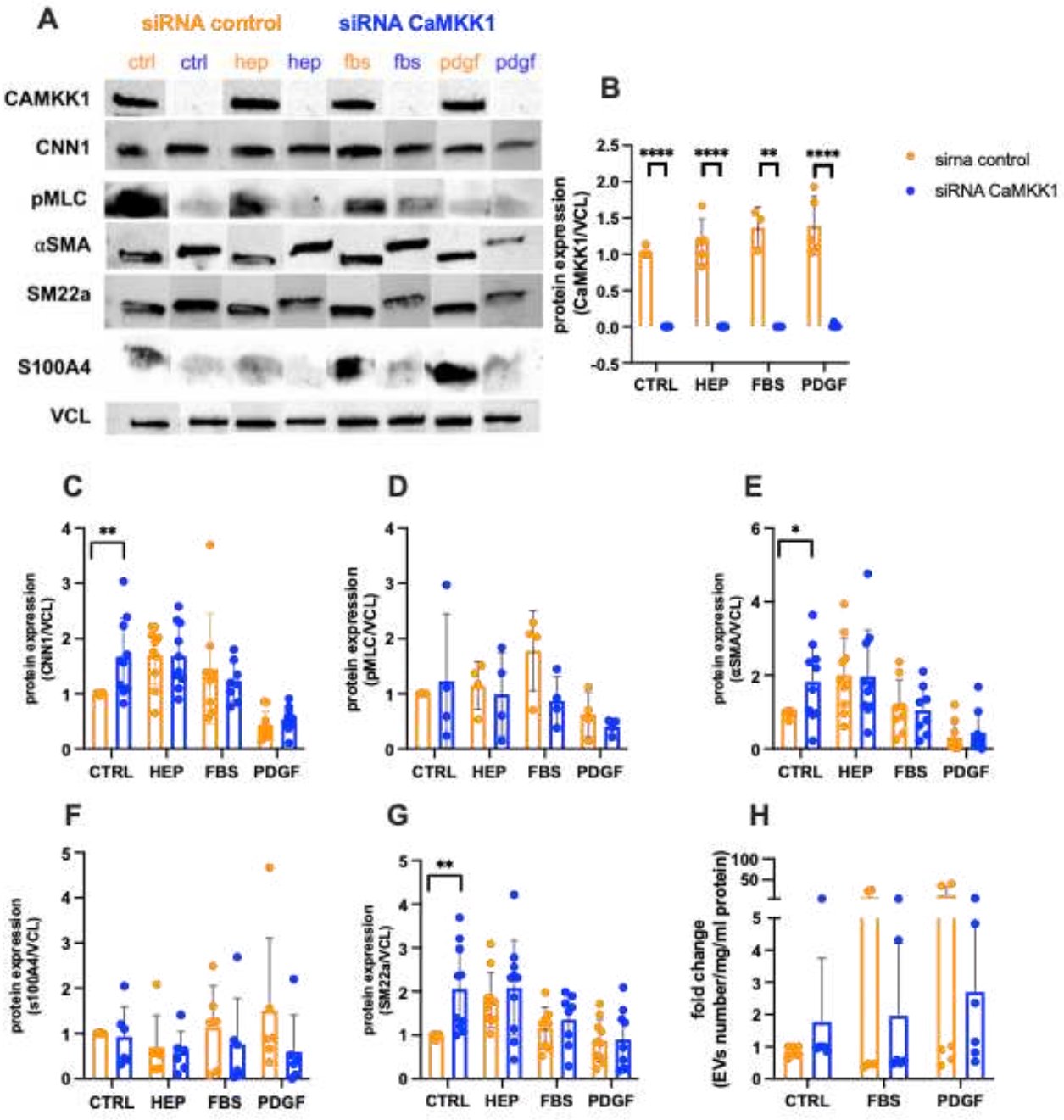
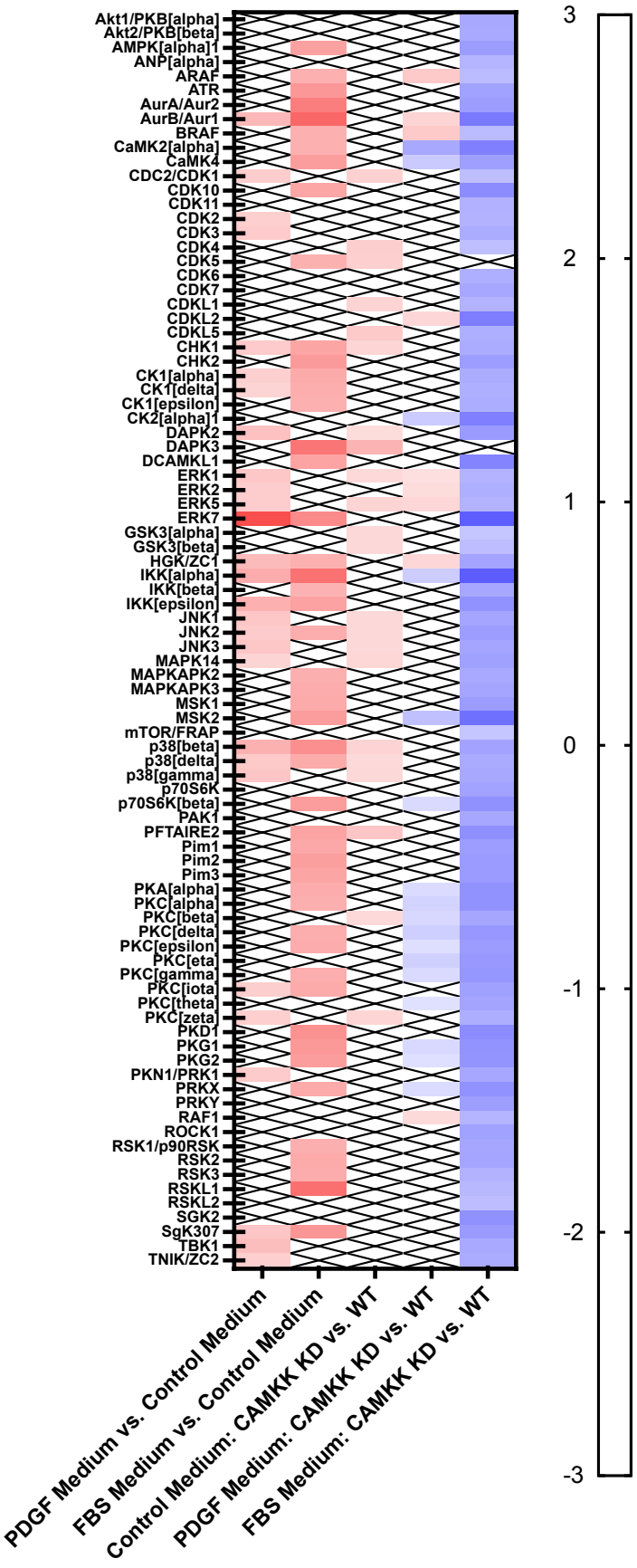


Figure 2: CaMKK1 regulates hVSCMC contractile marker expression. hVSMCS were transfected with siRNA non targeting pool control and siRNA CaMKK1, respectively. After 24 hours hVSMC were treated with PDGF-BB (10 μg/ml), 0.5% FBS (low FBS), heparin (200 U/ml) in M199 with 2.5% FBS for seven days. **A)** Western blot and **B-G)** quantification of CaMKK1, contractile proteins (CNN1, pMLC, *α*-SM-actin, SM22*α*) and synthetic marker S100A4. The results confirm both successful knockdown of CaMKK1 in and significant differences in CTRL hVSMC between siRNA control and siRNA CaMKK1 in CNN1, αSMA and SM22a. **H).** Quantification of extracellular vesicles isolated by ultracentrifugation using nanoparticle tracking analysis. All graphs show pooled data from 2 to 5

independent experiments performed in duplicate. Statistical analysis was performed using GraphPad Prism 9. Statistical significance was tested using a t test; ns - not significant, *P<0.05, **P<0.01.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BRAF
CaMK2[alpha]
CaMK4
CDC2/CDK1
CDK10
CDK11
CDK2
CDK3
CDK4
CDK5
CDK6
CDK7
CDKL1
CDKL2
CDKL5
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CK1[delta]
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CK2[alpha]1
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DAPK3
DCAMKL1
ERK1
ERK2
ERK5
ERK7
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IKK[epsilon]
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JNK3
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RAF1
ROCK1
RSK1/p90RSK
RSK2
RSK3
RSKL1
RSKL2
SGK2
SgK307
TBK1
TNIK/ZC2
PDGF Medium vs. Control Medium
FBS Medium vs. Control Medium
Control Medium: CAMKK KD vs. WT
PDGF Medium: CAMKK KD vs. WT
FBS Medium: CAMKK KD vs. WT
3
2
1
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-1
-2
-3

Figure 3: CaMKK1 and phenotype switching affect the activity of a panel of kinases in hVSMCs. hVSMCS were transfected with siRNA non targeting pool control and siRNA CaMKK1, respectively. After 24 hours hVSMC were treated with PDGF-BB (10 μg/ml), 0.5% FBS (low FBS), heparin (200 U/ml) in M199 with 2.5% FBS for seven days. The heatmap shows the activity of 88 Ser/Thr kinases of a total of 144 present on the Pamchip microarray. These 88 kinases were active (the cut-off for the Mean Finale Score is 1.2) in at least one of the three conditions used for phenotype switching (CTRL, low FBS, PDGF) in hVSMC transfected for siRNA CaMKK1 and siRNA control. The red and blue reflect the median kinase statistic: the red shows relative high activity and blue represents relative lower activity. Black crosses indicate that in that specific comparison the kinase activity is not changed. In the comparisons, the second condition is always used as reference.

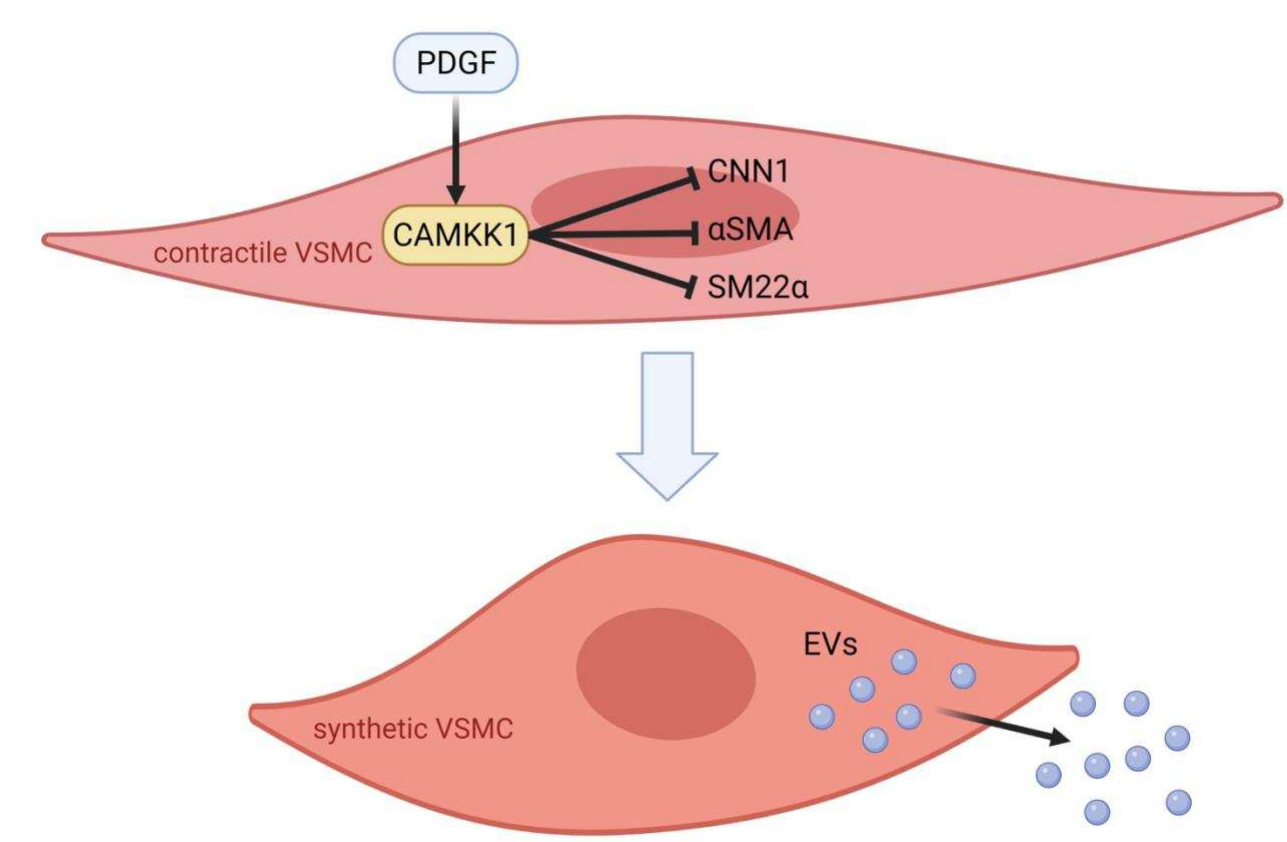

Figure 5. CaMKK1 regulates VSMC phenotype switching. PDGF treatment increases CaMKK1 expression. This leads to inhibition of contractile marker CNN1, αSMA and SM22α expression and increase in activity of kinases known to promote mirgration and proliferation. All of these changes stimulate synthetic differentiation of hVSMCs, which is associated with increased EV release.

SUPPLEMENTARY MATERIALS

Supplemental methods

RNA extraction and cDNA synthesis

Total RNA was extracted from hVMSC using a TRI Reagent (Sgma) according to the manufactirer's protocol. RNA concentrations were quantified spectrophotometrically at 260 nm using a Nanodrop ND1000 (ThermoFisher). RNA integrity was evaluated using denaturing agarose gel electrophoresis. 250 ng of total RNA was treated with DNase I (Promega, Leiden The Netherlands). The purified RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) for 1 hour at 37°C, in the presence of RNAse Out, dNTPs, dithiothreitol (all from Invitrogen), and an oligo(dT) primer (Eurogentec, Maastricht, The Netherlands).

Quantitative real-time PCR

Gene expression levels were quantified by real-time quantitative PCR (qPCR) in a LightCycler 480 (Roche Applied Science). Amplification reactions were carried out in a volume of 10 µl, containing 100 ng of total cDNA, 5 µl QuantiTect SYBR Green PCR Kit (Qiagen) and 0.5 µM of 5' and 3' primers (Eurogentec). An initial denaturation step (15 minutes at 95°C) was followed by 50 cycles of amplification (denaturation: 15 seconds at 95°C, annealing: 30 seconds at 57°C, extension: 45 seconds at 72°C). The specificity of amplification was controlled by melt curve analysis. Fluorescence curves were analyzed with LightCycler 480 Software (Version 1.5) and relative quantification was performed with the $2^{-\Delta\Delta Ct}$ method. All samples were assayed in triplicate.

Intracellular calcium measurment

hVSMC were plated in a black, clear-bottom 96 well plate in quadruplicate for each condition. The cells were transfected with siRNA in Medium 199 (Gibco) supplemented with 1% Penicillin Streptomycin antibiotics (Gibco) and 2,5% FBS (Gibco) . Treatment with 3.6 mM $CaCl_2$ and 1 mM ionomycin (Sigma) was used as a positive control and hVSMCs not loaded with Fluo-4 (Invitrogen) as the negative control. 24 h after the siRNA transfection cells (excepted the negative control), were loaded with 50 µl/well Fluo-4-AM (Invitrogen) mix: M199 (Gibco) +20% FBS (Gibco)+ 1% Penicillin Streptomycin antibiotics (Gibco) + Fluo-4 (5µM final concentration; Invitrogen) + Pluronic F-127 (Sigma-Aldrich, 1:500 of 200 µg/ml stock) + 1 mg/ml Hoechst (Invitrogen). After 30 minutes, all the wells were washed 2 times with PBS. The treatments were applied in KRPG (Krebs-Ringer phosphate glucose buffer) + BackDrop Background Suppressor (Invitrogen, 1:50). The intracellular calcium

measurement was performed using the Cytation 3 (Biotek) imaging plate reader. Quantification was performed from pictures using the signal of GFP from the total area of the wells.

Supplementary Figures

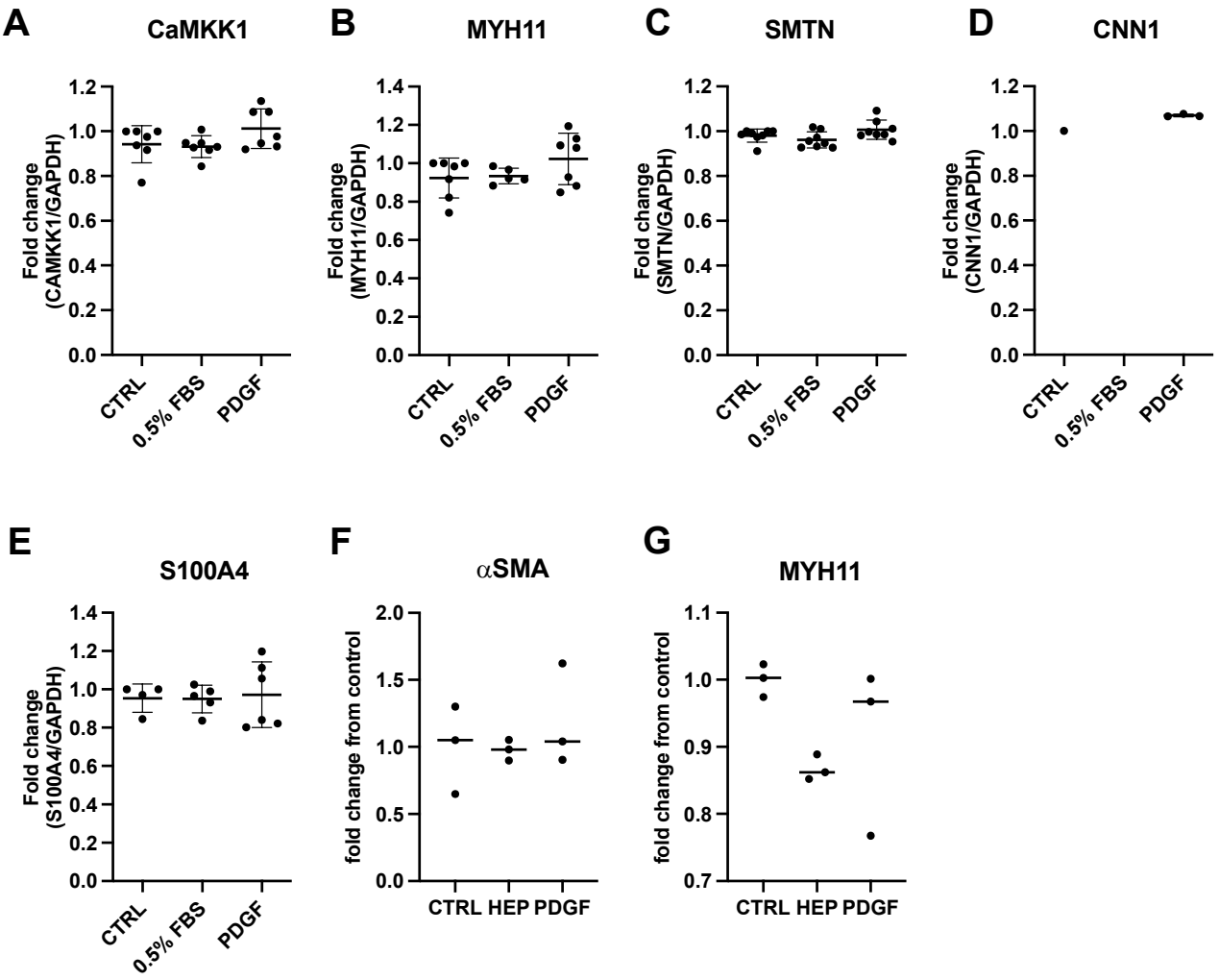

Figure 1. Phenotype switching in hVSMCs. Cells were treated with PDGF-BB (10 μg/ml), heparin (200 U/ml), 0.5% FBS (low FBS) in M199 with 2.5% FBS for seven days. **A-E)** Expression of CAMKK1, contractile markers MYH11, SMTN and CNN1, as well as synthetic marker S100A4 measured by qPCR did not change. **F-G**) Expression of contractile markers MYH11 and αSMA measured by mRNA array did not change significantly. All graphs show pooled data from 2 to 3 independent experiments, each treatment (control, HEP, low FBS, PDGF) performed in duplicate for the qPCR and in triplicate for the mRNA array. Statistical analysis was performed using GraphPad Prism 9. Statistical significance was tested using One-Way ordinary Anova. ns not significant, *P<0.05, **P<0.01, ***P<0.001.

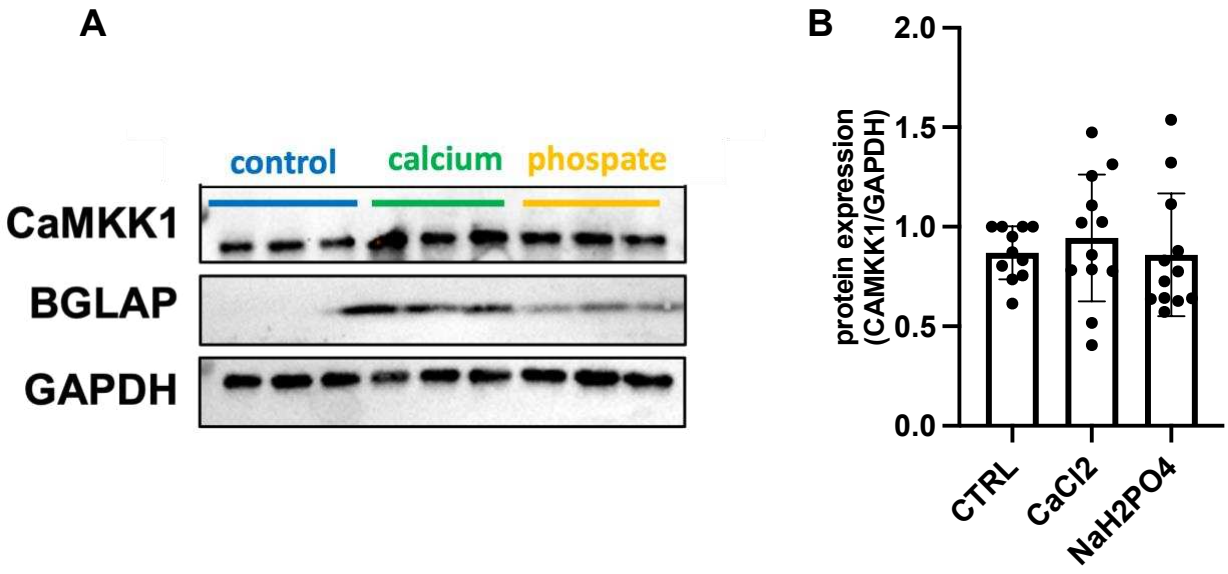

Figure 2. Osteogenic differentiation does not change CaMKK1 expression in hVSMCs. Cells were treated with high concentrations of calcium (3.6 mmol/L $CaCl^2$) and phosphate (2.5 mmol/L NaH^2PO^4) to induce osteogenic differentiation. **A)** Western blotting, and **B)** quantification of CaMKK1 and osteocalcin (BGLAP). Graphs show that the concentrations of CaMKK1 does not change significantly between high concentrations of calcium (3.6 mmol/L $CaCl^2$) and phosphate (2.5 mmol/L NaH^2PO^4). The BGLAP bands in calcium (3.6 mmol/L $CaCl^2$) and phosphate (2.5 mmol/L NaH^2PO^4) confirm the osteogenic differentiation (A).
Statistical analysis was performed using GraphPad Prism 9. Statistical significance was tested using One-Way ordinary Anova. No statistical significance was found.

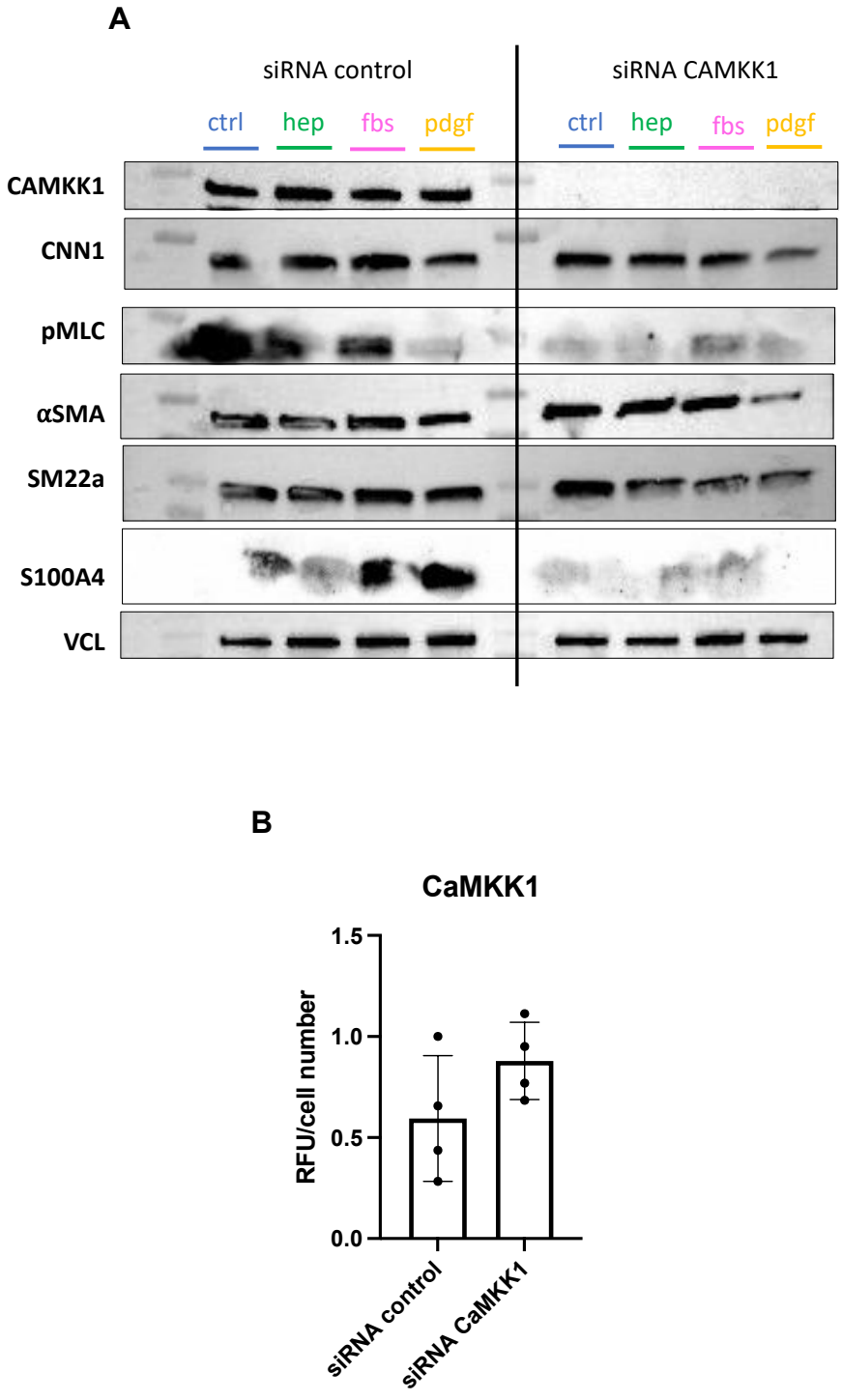

Figure 3. SiRNA knockdown of CaMKK1 in hVSMCs. hVSMCS were transfected with siRNA non targeting pool control and siRNA CaMKK1, respectively. **A)** After 24 hours hVSMC were treated with PDGF-BB (10 ?g/ml), 0.5% FBS (low FBS), heparin (200 U/ml) in M199 with 2.5% FBS for seven days Uncropped western blots from Figure 2. **B)** Intracellular calcium was measured using Fluo-4. CaMMK1 knockdown did not affect the intracellular calcium levels. This graph shows data obtained from a single intracellular calcium measurement.

Thank you to everyone I met during my journey, who sent me the love, the good vibes and the strength to pursue my goals it in the best way.
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To Angela.
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To my grandparents: Liliana, Brando, Lina e Giorgio.
To my best and beautiful friends: Giulia, Giorgia and Natalia
To my everything Francesco.

I will never stop to thank you all.