

**Federico II University of Naples**  
**Department of Molecular Medicine and Medical Biotechnology**  
**(formerly Department of Molecular and Cellular Biology and Pathology)**

**Doctorate School in Molecular Medicine**  
**Doctorate Program in Molecular**  
**Pathology and Physiopathology**  
**XXV cycle 2009-2012**



**“Study of Calcium-Calmodulin dependent kinases  
in the pathogenesis of the atherosclerotic plaque”**

Coordinator  
Prof. Vittorio Enrico Avvedimento

Candidate  
Angela Serena Maione

Tutor  
Dott. Maddalena Illario

Rare sono le persone che usano la mente,  
poche coloro che usano il cuore  
e uniche coloro che usano entrambi.

*Rita Levi Montalcini*

# TABLE OF CONTENTS

	Page
ABSTRACT	1
1. INTRODUCTION	2
1.1 The Calcium/Calmodulin dependent Kinases family (CaMKs)	2
1.2 Arteriosclerosis and Ca <sup>2+</sup> signalling	7
1.3 Plaque stability and instability: a balance between inflammation and repair	10
2. AIMS OF STUDY	17
3. RESULTS	18
3.1 Expression of CaMKs mRNA transcripts in human atherosclerotic plaques	18
3.2 Expression of CaMKs in human atherosclerotic plaques	19
3.3 CaMKII activity in human atherosclerotic plaques	20
3.4 Cellular localization of CaMKII in human atherosclerotic plaques	21
3.5 Macrophage conditioned medium modified CaMKs expression and activation in VSMCs	22
3.6 Macrophage conditioned medium reduce VSMCs proliferation	23
4. DISCUSSION AND CONCLUSIONS	24
5. MATERIALS AND METHODS	27
5.1 Cell culture	27
5.2 Western blot and immunoprecipitation procedures	27
5.3 CaMKII activity assay	28
5.4 Cell proliferation and [ <sup>3</sup> H]thymidine incorporation	28
5.5 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)	29
5.6 Immunofluorescence	29
5.7 Statistical analysis	30
6. ACKNOWLEDGEMENTS	31
7. REFERENCES	32
8. LIST OF PUBLICATIONS	40

## ABSTRACT

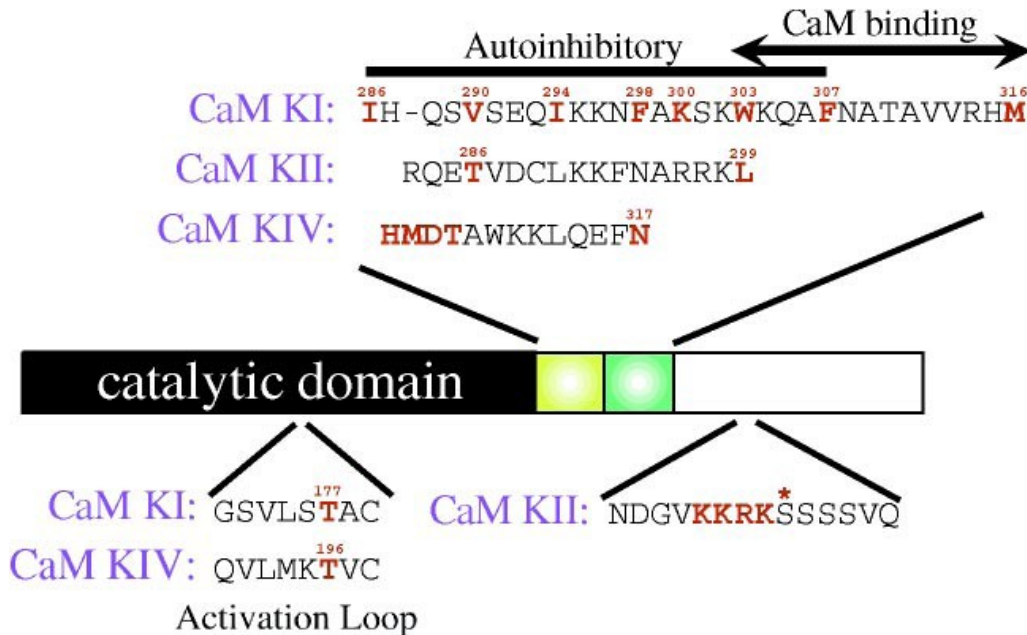
Arteriosclerosis is a degenerative process that is physiological in aging but can be accelerated by number of risk factors, such as hypertension, diabetes, obesity, smoking, familial history. Healthy vessel walls harbour vascular smooth muscle cells, fibroblasts, endothelial cells, whereas in arteriosclerotic vessels inflammatory cells such as activated macrophages are also present. The biology of all these cells is tuned by calcium, that regulates both acute and adaptive, chronic events. Calcium signalling plays an important role in the regulation of cell proliferation through the crosstalk between multifunctional Calcium-Calmodulin dependent kinases (CaMKs) and a number of other signalling pathways, such as Erk. Nevertheless, how these cellular pathways interact with each other has yet to be investigated. Our recent studies demonstrate that a murine model lacking CaMKIV presents early onset arteriosclerosis, as well as increased CaMKII activity in selected cell types, thus implying a role for calcium signaling in the process. The recent genome-wide analysis of the Framingham Heart Study 100K Project shows an association between elevated diastolic blood pressure and the rs10491334 T/C Single Nucleotide Polymorphism of human CaMKIV gene. This finding suggests for this kinase, whose expression was once thought to be confined to the nervous tissue, a yet undisclosed role in vascular responses. We also obtained evidences of the existence of a crosstalk between CaMKII and CaMKIV, involved in the regulation of cell cycle progression. These results indicate a novel interplay between CaMKs, that has never been investigated in the modulation of cell proliferative responses, and might have tremendous implications in arteriosclerosis.

# 1. INTRODUCTION

## 1.1 The Calcium/Calmodulin dependent Kinases family (CaMKs)

Calcium ( $\text{Ca}^{2+}$ ) is an ubiquitous intracellular second messenger that regulates cell processes including proliferation, development, motility, secretion, learning and memory. Several stimuli, such as hormones, growth factors, cytokines, and neurotransmitters induce changes in the intracellular levels of  $\text{Ca}^{2+}$ . A resting cell typically has a cytosolic  $\text{Ca}^{2+}$  concentration of 100 nM, a concentration approximately 20,000-fold lower than that of extracellular  $\text{Ca}^{2+}$  (Hook and Means, 2001). Cells control cytoplasmic  $\text{Ca}^{2+}$  levels through ATP-dependent  $\text{Ca}^{2+}$  pumps that transport  $\text{Ca}^{2+}$  into the two major  $\text{Ca}^{2+}$  stores: the endoplasmic reticulum (ER) and the extracellular space. Different signals stimulate either waves or spikes of increased intracellular  $\text{Ca}^{2+}$  and the origin of  $\text{Ca}^{2+}$  depends upon the stimulus. Receptor tyrosine kinases and G-protein-coupled receptors classically increase  $\text{Ca}^{2+}$  levels by producing IP<sub>3</sub>, which induces  $\text{Ca}^{2+}$  release from the ER via the IP<sub>3</sub> receptor (Berridge, 1993; Berridge and Irvine, 1989). Similarly, cyclic ADP ribose can release  $\text{Ca}^{2+}$  from intracellular stores. Ligand gated ion channels and voltage-dependent ion channels in the plasma membrane, however, initiate  $\text{Ca}^{2+}$  entry via extracellular stores. Subsets of each of these types of channels have also been shown to cause  $\text{Ca}^{2+}$  release from intracellular stores via either the IP<sub>3</sub> receptor or the ryanodine receptor. The most ubiquitous and abundant protein that serves as a receptor to sense changes in  $\text{Ca}^{2+}$  concentrations is Calmodulin (CaM), thus mediating the role as second messenger of this ion. CaM is a 148-amino acid protein (16,680 daltons) comprised of 4 helix-loop-helix protein folding motifs called EF hands, with two making up the N-terminal domain and two comprising the C-terminal domain (Tjandra et al., 1995). Each of the four EF hands binds one  $\text{Ca}^{2+}$  ion. The binding affinity to each site is approximately 1  $\mu\text{M}$ , although the pair of EF-hands in the N terminus is of slightly lower affinity than the pair in the C terminus. When the four binding sites are filled, CaM undergoes a conformational change exposing a flexible eight-turn  $\alpha$ -helix, which separates the hydrophobic pockets that form in each of the globular ends of the protein. Calmodulin thus becomes “loaded” to interact with one of its many target proteins in the cell. This target protein interaction, while usually of high affinity, is rapidly reversible upon a decline in  $\text{Ca}^{2+}$  concentration (Means, 2000). Among the many  $\text{Ca}^{2+}$ /CaM binding proteins activated by CaM, there is a family of serine/threonine protein kinases called  $\text{Ca}^{2+}$ /CaM-dependent protein kinases. This family includes kinases such as phosphorylase kinase, myosin–light chain kinase (MLCK), and CaM kinases I, II, III, and IV (Means, 2000). These kinases are grouped according to whether they are dedicated kinases having a single substrate (phosphorylase kinase, CaM KIII, and MLCK) or whether they are multifunctional

(CaM KI, II, and IV) and have several substrates. The general structure of CaMKs (figure 1.1) includes an N-terminal kinase domain, an autoregulatory domain, an overlapping CaM-binding domain and, in phosphorylase kinase and CaMKII, a C-terminal association domain that is essential for multimerization and targeting.



**Figure 1.1 | Schematic diagram of the CaM kinases highlighting their domain structure.**

The sequences of the overlapping autoinhibitory and CaM-binding domains of CaM KI and homologous regions of CaM KII and IV are shown. Within the catalytic domain of CaM KI and IV is the activation loop containing the T residues phosphorylated by CaM KK. The C-terminal NLS of CaM KII is shown with the S phosphorylated by CaM KI and IV specified with an asterisk. (Hook and Means, 2001)

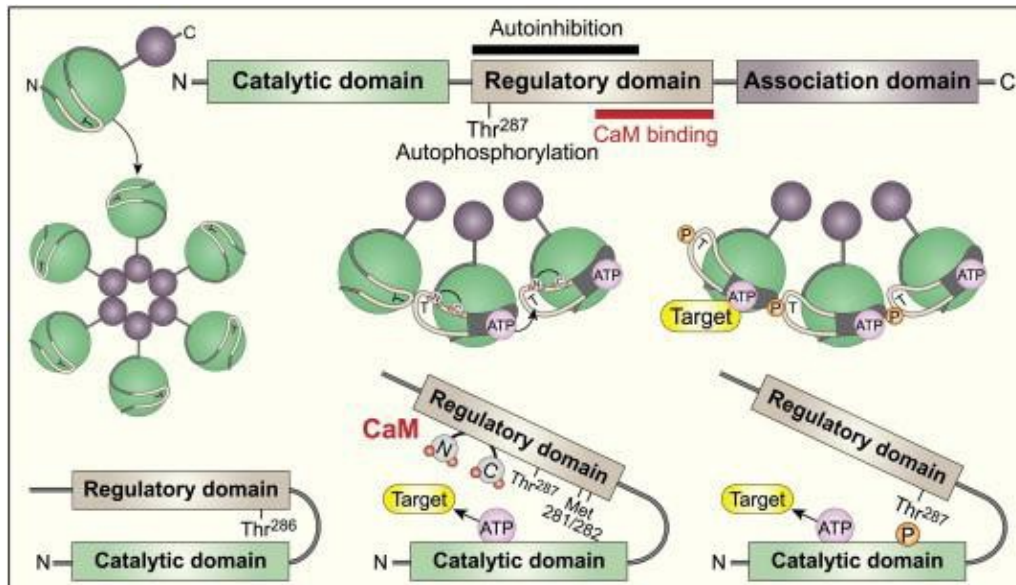
The activation process involves a conformational change of the target protein upon binding of calcium/CaM to a short region known as the CaM binding domain. In the case of CaM-dependent kinases such as myosin-light chain kinase or the multifunctional CaM kinases I, II and IV, an autoinhibitory domain, interacts with the catalytic domain keeping the enzyme inactive at resting calcium levels. According to this 'intrasteric inhibition' model (Cruzalegui et al., 1992), calcium/CaM binding to a region adjacent to the autoinhibitory domain causes a change in conformation that permits access of peptide substrates and adenosine triphosphate (ATP) to the catalytic domain. In this manner, CaM-dependent kinases can rapidly sense elevations of intracellular calcium becoming active as

calcium-bound CaM binds to them. In addition, some of these enzymes are also equipped with mechanisms that enable them to prolong their activity after calcium has returned to basal levels. This form of 'molecular memory' is best documented for CaM kinase II, a multimeric enzyme composed of at least 10 subunits. Each one of the subunits is a 50–60-kDa polypeptide containing an N-terminal catalytic domain, a central autoinhibitory and CaM binding region and a C-terminal domain responsible for multimerization and intracellular localization. The unique multimeric structure of CaM kinase II allows very rapid trans-phosphorylation of subunits within the complex. As a consequence of trans-phosphorylation, a subunit acquires  $\text{Ca}^{2+}$ /CaM-independent activity (Miller and Kennedy, 1986). In addition, the affinity of the autophosphorylated subunits for CaM increases, making it more sensitive to further calcium elevations (De Koninck and Schulman, 1998). These properties of CaM kinase II allow the enzyme to remain active for some time after calcium levels have dropped below activation threshold, which explains why CaM kinase II activity is stimulated not only by sustained increases in calcium concentration but also by calcium oscillations (De Koninck and Schulman, 1998). The activity of CaM kinases I and IV, which act as monomeric enzymes, is also modulated by phosphorylation. However, in contrast to CaM kinase II, the regulatory phosphorylation events are catalysed by a distinct group of kinases termed CaM kinase kinases (CaMKK). Two CaMKK isoforms, a and b, have been cloned, and were found to be expressed in many tissues (including the nervous system) (Edelman et al., 1996; Tokumitsu et al., 1995). CaMKKs themselves are calcium/CaM-dependent enzymes. They phosphorylate calcium/CaM-bound CaM kinase I and IV on a threonine residue situated within the activation loop (threonine residue 177 of the human CaM kinase I (Haribabu et al., 1995); threonine residue 200 and 196 of the human and rat CaM kinase IV, respectively (Anderson et al., 1998; Selbert et al., 1995). These phosphorylation events cause the activity of CaM kinase I and IV to increase several folds. Similar to CaM kinase II, CaM kinase IV can autophosphorylate on serines 12 and 13, which is required for the enzyme to be active. While CaM kinase IV is predominantly nuclear (Jensen et al., 1991) and CaM kinase I appears to be a cytosolic enzyme (Picciotto et al., 1995), the subcellular distribution of CaM kinase II can vary. Four types of subunits of CaM kinase II have been identified (a, b, g, d) that are encoded by different genes with differing tissue-specific expression. Alternative splicing within the C-terminal sequence of each gene produces further isoforms. Although the biochemical characteristics of CaM kinase II purified from many tissues are practically identical, the subunit composition, that is dependent on the source, seems to determine the subcellular localization of the complex. The regulatory domain of CaMKII is just distal to its catalytic domain and is bipartite: N-terminal sequences (aa 282-300) are believed to interact with the catalytic domain to block both ATP (Colbran et al., 1989; Smith et al., 1992) and substrate

(Mukherji and Soderling, 1995) sites, whereas the C-terminal (aa 293-310) end binds  $\text{Ca}^{2+}/\text{CaM}$ . The overlap of these subdomains is no accident. Binding of  $\text{Ca}^{2+}/\text{CaM}$  is the primary signal for release of autoinhibition. Current models of activation posit that the binding of  $\text{Ca}^{2+}/\text{CaM}$  serves to disrupt the interactions of specific residues within the autoinhibitory domain with the catalytic domain (Smith et al., 1992). In addition to relieving autoinhibition, binding of  $\text{Ca}^{2+}/\text{CaM}$  also initiates autophosphorylation of CaMKII, providing additional layers of regulation (figure 1.2). The first autophosphorylation site to be identified in the rat  $\alpha\text{CaMKII}$  was Thr286. Phosphorylation of this site occurs as an inter-subunit reaction in the holoenzyme and requires  $\text{Ca}^{2+}/\text{CaM}$  binding to both the “kinase” and “substrate” subunits (Hanson et al., 1994). This site is associated with the development by the enzyme of  $\text{Ca}^{2+}/\text{CaM}$ -independent activity (Miller et al., 1988), that never reaches the level attainable by the fully  $\text{Ca}^{2+}/\text{CaM}$ -stimulated enzyme, but can be substantial and is important for synaptic and cell plasticity. Phosphorylation of Thr286 alters the interaction of the regulatory domain with the catalytic core, but it also alters the interaction of the kinase with  $\text{Ca}^{2+}/\text{CaM}$ , causing its off-rate to fall by over three orders of magnitude (Meyer et al., 1992). This results in a phenomenon that has been termed “CaM trapping.” Peptide models of trapping suggested that autophosphorylation induces a local conformational change that allows formation of additional, stabilizing interactions between CaM and Phe293 and Asn294 of CaMKII (Putkey and Waxham, 1996). Studies using CaMKII holoenzyme confirmed this model and showed that these residues interact with specific side chains in the C-terminal domain of CaM (Singla et al., 2001). This ability of CaMKII to hang onto CaM long after calcium levels have fallen is integral to its ability to act as a neuronal frequency detector (Eshete and Fields, 2001). Indeed, if calcium levels are low for long enough,  $\text{Ca}^{2+}/\text{CaM}$  will dissociate from the kinase. Dissociation makes additional autophosphorylation sites in the regulatory domain available. Thr305 and Thr306, which are protected by bound  $\text{Ca}^{2+}/\text{CaM}$  (Meador et al., 1993), can now undergo autophosphorylation if the kinase is still in its active, pThr286, state. Phosphorylation of these additional sites prevents rebinding of  $\text{Ca}^{2+}/\text{CaM}$  and maximal activation of the kinase. Thr305/Thr306 have been called “inhibitory” sites, but, for the kinase as studied in vitro, this is somewhat of a misnomer because only kinase that is constitutively active as a result of Thr286 phosphorylation can become phosphorylated at these sites at a high rate. Slow basal phosphorylation of Thr306 occurs in the absence of kinase activation, presumably because of the proximity of this residue to the catalytic site (Colbran, 1993) producing an enzyme that cannot be activated. As will be discussed below, there is an additional mechanism involving a CaMKII-binding protein that can rapidly mediate phosphorylation of these sites, in the absence of pThr286, to



produce an inhibited kinase that requires phosphatase to regain its ability to be stimulated by  $\text{Ca}^{2+}/\text{CaM}$ .



**Figure 1.2 | CaMKII structural domains and regulation.**

CaMKII monomers consist of an N terminal catalytic domain and a C terminal association domain that bound a regulatory domain (top). The association domains (maroon circles) are required for assembly of the CaMKII monomers into the holoenzyme (middle panels). Under resting conditions the catalytic domain is constrained by the regulatory domain (left middle and bottom panels). After intracellular  $\text{Ca}^{2+}$  rises and complexes with calmodulin (CaM) the  $\text{Ca}^{2+}/\text{CaM}$  binds to the C terminal portion of the CaMKII regulatory domain (mid portion of the top, middle and bottom panels) to prevent autoinhibition of the regulatory domain on the catalytic domain, activating CaMKII. With sustained  $\text{Ca}^{2+}/\text{CaM}$  or increased oxidation, CaMKII transitions into a  $\text{Ca}^{2+}/\text{CaM}$ -autonomous active enzyme after autophosphorylation (at Thr 287) or oxidation (at Met281/282) of amino acids in the regulatory domain. (Anderson et al.)

Because CaM kinases I, II, and IV have quite similar substrate specificity determinants, it is not completely surprising that they sometimes phosphorylate the same proteins. One substrate for all three of these kinases is the cAMP-response element binding protein, CREB. CREB is a ubiquitous transcription factor that binds the cAMP-response element (CRE) that was named owing to its original description as a target of cAMP-dependent protein kinase (PKA) (Shaywitz and Greenberg, 1999). The transcriptional activity of CREB is regulated by phosphorylation of S133, which promotes recruitment of the transcriptional co-activator CBP, a protein that displays acetyltransferase activity towards both histone and other transcriptional regulatory proteins. The phospho-CREB–CBP complex then interacts with the basal transcriptional apparatus to initiate RNA synthesis. Subsequent studies showed that CREB can be phosphorylated by a

variety of kinases including PKA, RSK, RSK 1–3, MSK, MAPKAPK2 and p38MAPK. CaMKI and IV can phosphorylate the activating S133 of CREB (Enslen et al., 1994; Sheng et al., 1991) and consequently, can markedly stimulate CRE-mediated transcription. The literature reveals that CaMKI is predominantly cytoplasmic (Picciotto et al., 1995) and the question as to whether it directly or indirectly phosphorylates CREB remains controversial. In contrast, endogenous CaMKIV is present in the nucleus, and T cell experiments indicate that it could be a relevant CREB kinase for interleukin-2 (IL-2) production (Anderson et al., 1997). In addition to S133, CaMKII also phosphorylates S142, a modification that inhibits the transcriptional activity of CREB. Indeed, phosphorylation of Ser-142 was not only inhibitory, but this modification was also dominant and could reverse the activation of CREB resulting from its phosphorylation on Ser-133 by PKA. This phosphorylation seems to be destabilizing for the association between CREB and CBP21. Interestingly, the nature of the effect of CaMKII on transcription is both cell and promoter dependent.

## **1.2 Arteriosclerosis and $\text{Ca}^{2+}$ signalling**

The physiological process of aging determines the replacement of the collagen in the vessel walls, thus modifying their thickness and elasticity. These changes determine medio-intimal hyperplasia, and are responsible for the isolated increase of systolic blood pressure above 140 mmHg in the elderly, in the presence of normal diastolic pressure (<90 mmHg). This physiological process can be sped up by risk factors such as hypertension, smoke, diabetes, dyslipidemia, and generate different clinical and morphological conditions. The histological phenomenon that precipitates arteriosclerosis the most is atherosclerosis, that is characterized by the lipid stuffing of the vessel walls, usually housed inside the inflammatory cells, as well as by the proliferation of the vascular smooth muscle cells, with the consequent lumen narrowing. Conventionally, the atherosclerotic plaque is defined when the thickness of the vessel wall is above 1.3 mm. By definition, arteriosclerosis is an evolving condition, therefore without the removal of the risk factors sustaining it, the atherosclerotic process progresses. Different stages can co-exist at different stages of the vascular tree. The primum movens is the activation of the endothelium, once considered just an internal lining of the vessel, and characterized by a terrific endocrine and paracrine potential. Endothelial receptors allow this structure to be affected by micro-environmental variations, and change its phenotype: indeed, the endothelium can be either in a quiescent or in an activated state; the presence of risk factors induces its activation, and the secretion of inflammatory cytokines. The endothelium then exposes adhesion molecules allowing the adhesion and localization of circulating monocytes; gaps open and the tight junctions are reduced, to facilitate the outgrowth of the

monocytes from the blood into the vessel's wall. This event leads to the transformation of monocytes into macrophages and dendritic cells, that on turn sustain the inflammation of the wall. Inflammatory cytokines (such as TNF, IL1, MCP-1), vaso-constrictor factors (Angiotensin, Endothelin, Cathecolamines), and pro-angiogenetic factors (VEGF, FGF) are released into the parietal space, and support both VSMC proliferation, and the metalloproteases that digest the collagen of the vessel's wall. Therefore, the atherosclerotic plaque increases its volume due to the self-sustaining of the inflammatory events especially when the risk factors are not removed. The ongoing inflammation of the vessel's wall leads to degenerative events in the plaque, with the development of necrosis in the plaque's core. On the contrary, when the risk factors are removed, the inflammation reduces, and regenerative events prevail, thus leading to VSMC proliferation and calcium deposition in the intercellular spaces. Histologically, the plaque can evolve either towards a stable plaque, or an unstable plaque. The prevalence of inflammatory and necrotic events leads to instability, and the plaque ulcers, generating blood clots. When the atherosclerosis involves the coronary arteries the case history of this condition corresponds to unstable angina and acute myocardial infarction, recently joined as acute coronary syndrome, whose symptoms are not induced by stress, and uprise at rest, spontaneously, due to its thrombogenic nature. On the contrary, a stable plaque is less prone to ulcer, as it is made up by muscle cells, that are lined with newly forming endothelium, with a higher calcium component. In this case, the symptoms are due to the discrepancy between the capacity of the coronary flow to adequately perfuse the myocardium, and its need, especially when the metabolic demand increases (stress, hypertensive or tachycardia attack).  $\text{Ca}^{2+}$  is involved in the emergence and progression of arteriosclerosis at different extents. Basic  $\text{Ca}^{2+}$  phosphate deposition underlies the development of arterial calcification, a frequent component of atherosclerosis. The body mass index, waist circumference, and visceral adipose tissue are associated with subclinical cardiovascular disease, as well as with abdominal aortic calcification (Fox et al., 2009), and coronary artery calcium predicts coronary artery disease events (Wong et al., 2009). Of note, inflammatory macrophages co-localize with  $\text{Ca}^{2+}$ -phosphate deposits in developing atherosclerotic lesions, and can promote calcification through the release of TNFalpha. Pathological calcification then is not merely a passive consequence of chronic inflammatory disease, but may lead to a positive feedback loop of calcification and inflammation driving disease progression.  $\text{Ca}^{2+}$  is a key signal transduction ion, and as such it is involved in the pathogenesis of arteriosclerosis. Indeed,  $\text{Ca}^{2+}$  channel blockers have the ability to retard or even reverse early atherosclerotic process (Ikeda et al., 2009), and its role concerns all cell types at the basis of the process. Chronic inflammation is the pathogenetic feature of atherosclerosis and cardiovascular disease mediated by angiotensin II, proinflammatory cytokines, free fatty acids,

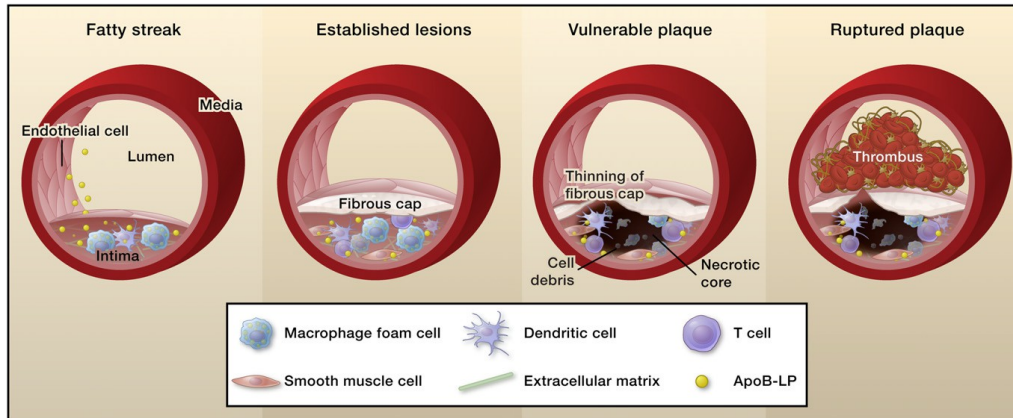
that promote the generation of reactive oxygen species (ROS) in vascular smooth muscle cells and endothelial cells, and mediate injury through several mechanisms involving  $\text{Ca}^{2+}$  signaling.  $\text{Ca}^{2+}$  is a pivotal second messenger controlling the activation of several immune cell types. Among the early critical events taking place in arteriosclerosis, there is monocyte transendothelial extravasation, that is mediated by cell adhesion molecules and chemoattractants signaling inflammation or injury (Imhof and Aurrand-Lions, 2004). The recruited mononuclear phagocytes accumulate at the inflammatory sites and terminally differentiate into macrophages and dendritic cells (DC) (Murdoch et al., 2004). Local hypoxia regulates the expression profile of the genes expressed by monocytes at various differentiation stages, and promotes the switch from a pro-inflammatory to a migratory phenotype. The distinct steps of the transendothelial migration are mediated by intracellular signals in the endothelium, that include changes in the intraendothelial free  $\text{Ca}^{2+}$  concentration. Indeed, the increase of the intraendothelial  $\text{Ca}^{2+}$  favours monocytes extravasation (Kielbassa-Schnepp et al., 2001). CaMKs are involved in the regulation of monocyte function and survival to different extent: CaMKIV regulates survival and differentiation of DC by preventing accumulation of pCREB (Illario et al., 2008), whereas CaMKII is involved in TNF $\alpha$ -induced CD44 expression, that plays a pivotal role in the migration of immune cells (Mishra et al., 2005). Furthermore, the CaMKs could be involved in the regulation of monocytes through the tuning of the extracellular-signal-regulated kinase (ERK) pathway, that is not only involved in the proliferative events of arteriosclerosis. ERK inhibition indeed significantly reduced Monocyte chemotactic protein-1 (MCP-1)-activated ROS generation, where MCP-1 is a potent chemoattractant for monocytes. Transfection of an active mutant of MEK1 (ERK 1/2 kinase) markedly increased superoxide production in rat aortic smooth muscle cells, suggesting that ERK 1/2 activation stimulates ROS generation (Lo et al., 2005). Among the cytokines involved in monocyte migration is IL (interleukin)-6: ERK1/2 activation is crucial for IL-6-dependent expression of MCP-1, that plays a major role in atherosclerosis (Sobota et al., 2008), and ERK could be on turn regulated by the CaMKs pathway. A relevant consequence of monocyte transmigration into the endothelial layer is the stimulation of endothelial proliferation, that is another feature of arteriosclerosis. Monocyte-induced endothelial cell proliferation is accompanied by prolonged ERK activation and is inhibited by the specific ERK inhibitor PD98059. The contact-mediated effect of monocytes is specific to endothelial cells and does not occur with vascular smooth muscle cells (Schubert et al., 2008). In the atherosclerotic process typical of arteriosclerosis, the normally quiescent VSMC of the artery proliferate into the intima, and specific genetic changes take place, that enhance atherosclerosis (Komatsu et al., 1998). This phenomenon is referred to as neointimal hyperplasia, and is induced by agonists secreted by immune cells, platelets, VSMC themselves.

CaMKs have never been taken into account while looking at mechanisms of atherosclerosis. It has recently been demonstrated that CaMKs regulate biological processes that are relevant to the process of atherosclerosis, by affecting the biology of vascular wall cells and infiltrating macrophages. In particular, CaMKIV plays a pivotal role in blood pressure regulation through the control of endothelial nitric oxide synthase activity in endothelial cells (Berridge et al., 2000). Furthermore, a critical role for CaMKII has been demonstrated in VSMC proliferation (Braun and Schulman, 1995; Carafoli, 2002). Finally, CaMKs actively regulates chemiotaxis, diapedesis, chemokine production in macrophages and the survival program of dendritic cells (Hook and Means, 2001).

### **1.3 Plaque stability and instability: a balance between inflammation and repair**

Atherosclerosis is currently the leading cause of death and disease in developed countries, soon to become the pre-eminent health problem worldwide (Murray and Lopez, 1997). It is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries and constitutes the single most important contributor to the growing burden of cardiovascular disease.

Atherosclerosis first involves a decades-long expansion of the arterial intima, a normally small area between the endothelium and the underlying smooth muscle cells of the media, with lipids, cells, and extracellular matrix. Atherogenesis begins with the subendothelial retention of apolipoprotein B-containing lipoproteins in focal areas of the arterial subendothelium. These lipoproteins, following oxidative and other types of modification, then trigger a series of maladaptive inflammatory responses. Key among these responses is the attraction of blood-borne monocytes to activated endothelial cells (ECs) overlying areas of lipoprotein retention, followed by monocyte differentiation into macrophages. The macrophages internalize the retained lipoproteins, leading to foam cell formation, and the macrophages become activated and inflammatory (Figure 1.3). Dendritic cells, T cells, mast cells, B cells, and possibly neutrophils also enter the growing lesion and contribute to the inflammatory response to retained lipoproteins. As lesions develop, the inflammatory response amplifies and does not resolve, which is likely a result of the continuing and even accelerated process of lipoprotein retention. As such, the subendothelial space expands with cells and extracellular matrix. Although this process itself rarely leads to major symptoms due to preservation of the arterial lumen, a few of these lesions undergo necrotic breakdown, which precipitates acute, occlusive luminal thrombosis and its consequences: myocardial infarction, unstable angina (accelerating chest pain due to ongoing heart muscle ischemia), sudden cardiac death, and stroke (Virmani et al., 2002).



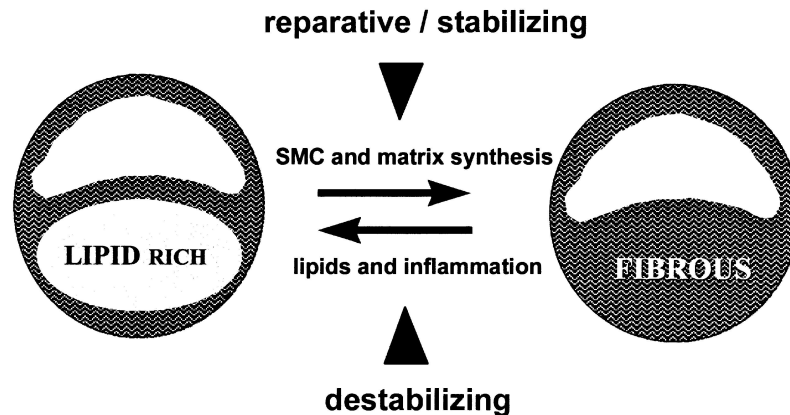
**Figure 1.3 | Progression of an Atherosclerotic Lesion**

Early fatty streak lesions are characterized by the accumulation of apolipoprotein B-containing lipoproteins (apoB-LPs) in the subendothelial space, which incites the recruitment of dendritic cells and macrophages. As the atherosclerotic lesion progresses, smooth muscle and T cells also infiltrate the intima, and apoB-LP retention is amplified. Vulnerable plaques are characterized by the accumulation of apoptotic cells and defective phagocytic clearance (efferocytosis), resulting in the lipid-filled necrotic core. A thinning fibrous cap decreases lesion stability, making these atherosclerotic plaques susceptible to rupture and the formation of a thrombus. (Moore and Tabas)

A principal feature of inflammation is the accumulation of leukocytes; in longlasting chronic inflammatory processes these leukocytes are macrophages, lymphocytes and mast cells. Basically the leukocytes have a protective function and serve in host defense by eliminating injurious agents, but their secretory products may also augment injury by damaging surrounding tissue components. During inflammation another series of events is initiated that must lead to the healing of injured tissue, either by regeneration or by scarring (fibrosis). Several studies pointed out that both inflammation and repair are key events in the natural course of atherosclerosis (Ross, 1993), and there is a great deal of evidence that inflammatory cells and their secretory products have profound effects on the integrity of the connective tissue meshwork of plaques (Libby, 1995). Most of the extracellular matrix components of a plaque, including collagens, elastin and various types of proteoglycans are products of smooth muscle cells. Transforming growth factor beta (TGF- $\beta$ ) is one of the most potent stimulators of connective tissue production by smooth muscle cells. Large amounts of this growth factor are detected in restenosis lesions, and it also participates in the repair process after natural plaque disruption. Thrombin generated during episodes of local thrombosis is another stimulator of smooth muscle cell growth. TGF- $\beta$  and other growth factors, including platelet derived growth factor (PDGF) and basic fibroblast

growth factor (bFGF), play an important role in wound healing and the reparative stage of many chronic inflammatory diseases. In the atherosclerotic plaque these growth factors are produced by 'injured' endothelial cells and macrophages or released from thrombus (Ross, 1993). Smooth muscle proliferation and matrix synthesis implies a mechanism of slowly progressive growth of plaques; it serves to encapsulate the soft atheroma and organizes episodes of thrombus formation, either spontaneously or artificially induced. The result is a reparative and stabilizing effect on the plaque structure. Activated T-lymphocytes and macrophages produce several mediators that promote destabilizing effects in plaques. The T-cell cytokine IFN- $\gamma$  appears to play an important role in this process, by inhibiting the proliferation of smooth muscle cells, as well as decreasing their synthesis of collagen fibrils. Apoptosis, an intrinsically programmed mode of cell death, can be activated by inflammatory mediators, and is recognized as a mechanism of foam cell death in plaques. Cell death leads to the spill of lipids and, hence, the enlargement of the soft lipid core (Bjorkerud and Bjorkerud, 1996). Apoptosis of cells has been observed in atherosclerotic plaques and in restenosis lesions. In early and restenosis lesions, smooth muscle cell apoptosis can have beneficial effects and promote regression, but in the fibrous cap of advanced lesions it introduces another potential of plaque destabilization through the loss of repair cells. In addition, during inflammation an even more powerful pathway of plaque desintegration is initiated by the extracellular matrix degrading metalloproteinases interstitial collagenase (MMP1), stromelysin (MMP3) and the gelatinases MMP2 and MMP9 (Galis et al., 1994). Once activated by plasmin or mast cell products, they initiate a cascade of proteolytic activities with a very broad substrate specificity, including all the extracellular matrix components of the fibrous cap. An observation of particular interest is that synthesis as well as lytic activity of these enzymes is most abundant in the lipid laden macrophages and in the extracellular space around lipid cores of plaques. There are several arguments in support of a correlation between lipids and inflammation. First, during activation of the scavenging pathway for phagocytosis of oxidized lipoproteins, macrophages exert a number of secretory functions with detrimental effect on the plaque tissue. In addition, specific T cell mediated immune responses appear to be involved in atherogenesis, and there is increasing evidence that a direct link may exist between accumulation of cholesterol in the vessel wall and activation of T cells, possibly by autoimmune responses to modified lipoproteins (Mitchinson et al., 1990). Therefore, during the ongoing process of lesion formation, and also in mature clinically relevant plaques, two major tissue remodeling forces may be operative. Smooth muscle cells increase the structural strength by producing the connective tissue matrix of a plaque. On the other hand, lipid associated inflammation introduces tissue degrading effects.

Whether a plaque tends to stability or instability will depend on which mechanism dominates the course of plaque formation in a given period of time (figure 1.4).



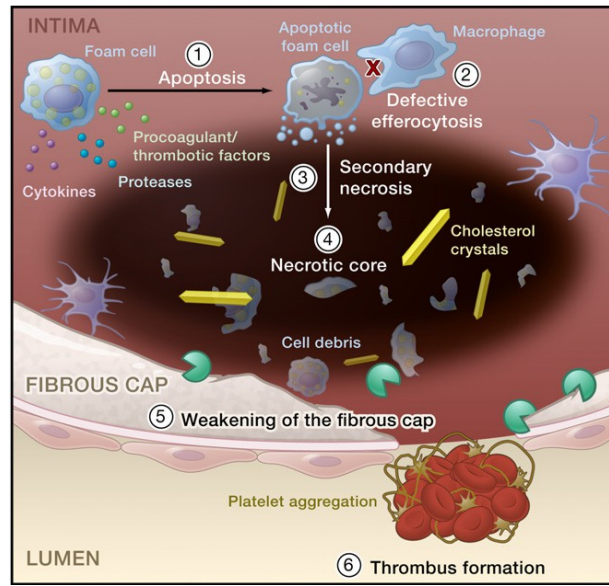
**Figure 1.4 | Schematic view of the two major tissue remodeling forces in atherosclerotic plaques**

On the left side is a lipid-rich plaque. On the right side is a fibrous plaque. Arrow to the left indicates the destabilizing effects of lipids and inflammation, thus creating a vulnerable lipid-rich plaque. Arrow to the right indicates the reparative effects of smooth muscle cells, leading to the formation of a stable fibrous plaque (Allard C. van der Wal, 1998).

Any process that decreases the synthesis of fibrous cap collagen by intimal fibromyoblast-like smooth muscle cells (SMCs) and/or contributes to cap collagen degradation would be expected to promote the formation of plaques prone to rupture (Figure 4). Vulnerable plaques show evidence of SMC death and decreased numbers of SMCs, and in vitro data show that macrophages can trigger apoptosis in SMCs by activating their Fas apoptotic pathway and by secreting proapoptotic TNF $\alpha$  and nitric oxide (Boyle et al., 2003). Macrophages may also decrease collagen synthesis in intimal SMCs without actually killing the cells. For example, in regions of vulnerable plaques that have defective clearance of apoptotic cells, macrophage secretion of TGF $\beta$  may be decreased, which would deprive neighbouring SMCs of this important inducer of collagen biosynthesis. Macrophage-derived matrix metalloproteinases (MMPs) may also be involved in thinning of the fibrous cap (figure 1.5). The regulation of MMPs by athero-relevant factors is complex, and it is influenced by transcriptional inducers and repressors and by protease activators and inhibitors. Moreover, once activated, certain MMPs can activate other ones. Studies showing a temporal and spatial correlation between the presence of macrophages in rupture-prone shoulder regions of plaques, thinning of the fibrous cap in these regions, and local accumulation of activated MMPs, especially MMP-2 and MMP-9, stimulated great



interest in the potential role of MMPs in plaque rupture (Galis et al., 1994). Observational data in vulnerable human plaques and the known biology of MMPs generated plausible hypotheses regarding the role of certain MMPs in fibrous cap thinning and plaque rupture.

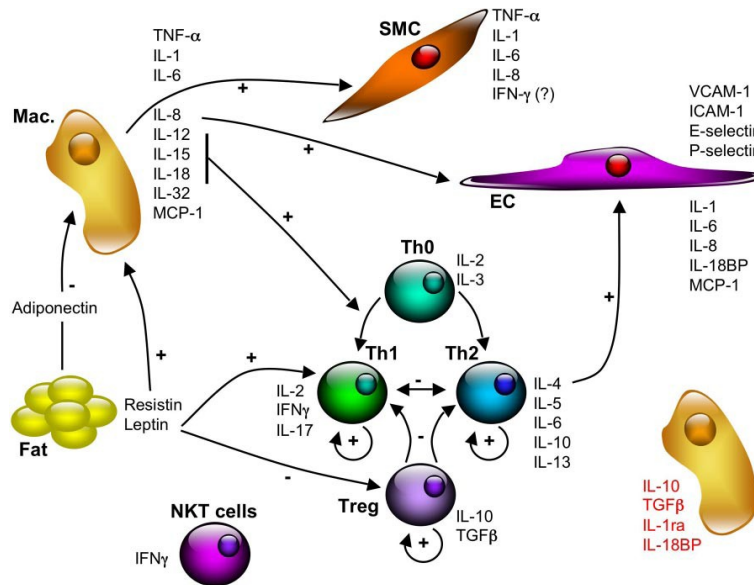


**Figure 1.5 | Macrophages in Advanced Atherosclerotic Lesions**

(1) Macrophage foam cells undergo apoptosis as a result of prolonged endoplasmic reticulum (ER) stress and other stimuli. (2) These apoptotic cells are not effectively cleared by macrophages (defective efferocytosis) in advanced lesions, (3) leading to secondary cellular necrosis. (4) Secondary necrosis, over time, contributes to the formation of a necrotic core. (5) Smooth muscle cell death and protease degradation of the extracellular matrix weakens the fibrous cap, making it susceptible to rupture. (6) Exposure of the thrombogenic material in the lesion causes platelet aggregation and thrombus formation. Also note that living macrophages in advanced plaques can contribute to vulnerable plaque formation through the secretion of cytokines, proteases, and procoagulant/thrombotic factors (Moore and Tabas).

A second critical feature of dangerous plaques is their necrotic core (Figure 4), which contributes to inflammation, thrombosis, proteolytic plaque breakdown, and physical stress on the fibrous cap (Virmani et al., 2002). Necrotic cores arise from the combination of apoptosis of advanced lesional macrophages and defective phagocytic clearance (or efferocytosis) of the apoptotic macrophages in advanced plaques (Tabas). This combination is critical: macrophage death also occurs in early atherosclerotic lesions, where efferocytic clearance is efficient, leading to decreases in lesion cellularity, inflammation, and plaque progression rather than an increase in plaque necrosis. A number of processes in advanced lesions may trigger macrophage death, and it is almost certain that a combination of factors and

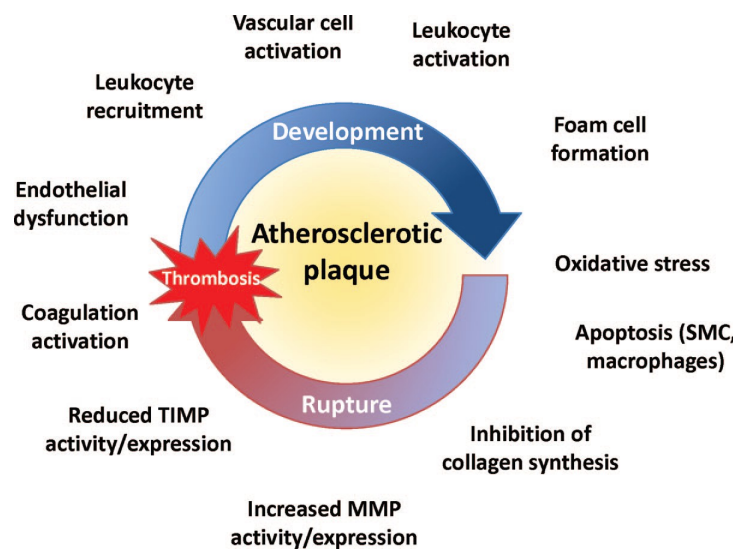
processes plays a role in vivo. Examples include growth factor deprivation, oxidative stress, and death receptor activation by ligands that exist in advanced atheroma. Human and animal studies established that atherosclerosis is driven by a chronic inflammatory process within the arterial wall initiated mainly in response to endogenously modified structures, particularly oxidized lipoproteins that stimulate both innate and adaptive immune responses (Tedgui and Mallat, 2006). The innate response is instigated by the activation of both vascular cells and monocytes/macrophages. Subsequently, an adaptive immune response develops against an array of potential antigens presented to effector T lymphocytes by antigen-presenting cells. Vascular cells, endothelial cells (EC), and smooth muscle cells (SMC) participate in the development of the disease by mediating leukocyte recruitment and vascular remodeling, as well as feeding back to promote perpetuation of inflammation through the release of proinflammatory cytokines and chemokines (figure 1.6).



**Figure 1.6 | Cytokines involved in atherogenesis.**

Cytokines are produced by several cell types, including inflammatory and vascular cells, as well as adipocytes. IL-12 and IL-18 produced by macrophages are potent inducers of IFN- and promote the differentiation of naive T cells into proatherogenic Th1 cells. Macrophage or macrophage-derived cytokines also activate smooth muscle cells (SMC) and endothelial cells (EC) to produce an array of proinflammatory mediators. On the other hand, the anti-inflammatory cytokines IL-10 and TGF-, also produced by macrophages, promote antiatherogenic Treg cell differentiation. Other anti-inflammatory mediators with potent antiatherogenic properties include IL-1 receptor antagonist (IL-1ra) and IL-18 binding protein (IL-18BP). Interestingly, IL-4, the prototype of Th2 cells, has proinflammatory properties on EC. Adipocytes produce both pro- and anti-inflammatory mediators. Leptin activates Th1 cells but inhibits Treg cell function. Adiponectin has been shown to dampen macrophage activation (Tedgui and Mallat, 2006).

The biological effects of proinflammatory cytokines that may account for their proatherogenic activity are multiple. In the early stages of atherosclerosis, cytokines can alter endothelial functions. Cytokines also induce the expression of chemokines and adhesion molecules on the vascular endothelium, thus favoring the recruitment, adherence, and migration of lymphocytes and monocytes into the inflamed vessel wall. Once in the intima, leukocytes can be permanently activated by locally generated cytokines, which can accelerate the transformation of macrophages into foam cells by stimulating the expression of scavenger receptors and enhancing cell-mediated oxidation. At a more advanced stage of the disease (figure 1.7), proinflammatory cytokines destabilize atherosclerotic plaques by promoting cell apoptosis and matrix degradation. Macrophage apoptosis results in the formation of cell debris, which contributes to enlargement of the lipid core. Plaque SMC apoptosis leads to thinning in the fibrous cap, favoring its rupture (Clarke et al., 2006; Mallat and Tedgui, 2001; Tabas, 2005).



**Figure 1.7 | Effects of cytokines on atherosclerosis development and plaque destabilization.**

## 2. AIMS OF STUDY

Atherosclerosis is a progressive, multifactorial chronic disease. The initial pro-atherogenic process implicates upregulation of adhesion molecules on endothelial cells, binding of low density lipoproteins to endothelium, activation of macrophages and proliferation of vascular smooth muscle cells. It has recently been demonstrated that CaMKs regulate biological processes relevant to atherosclerosis, by affecting the biology of vascular cell walls and infiltrating macrophages. In particular, CaMKIV plays a pivotal role in blood pressure regulation through the control of endothelial nitric oxide synthase activity (Santulli et al, 2012). A critical role for CaMKII has been demonstrated in VSMC proliferation (Cipolletta et al, 2010; Abraham, 1997), and CaMKs actively regulate macrophage functions, as well as the survival program of dendritic cells (Illario et al, Blood 2008).

**Aim of this doctorate thesis is to define the role of CaMKs in the atheroma mediated inflammation, and investigate whether CaMKII and CaMKIV represent a suitable novel therapeutic target to prevent the emergence and progression of arteriosclerosis.**

To this purpose I analyzed:

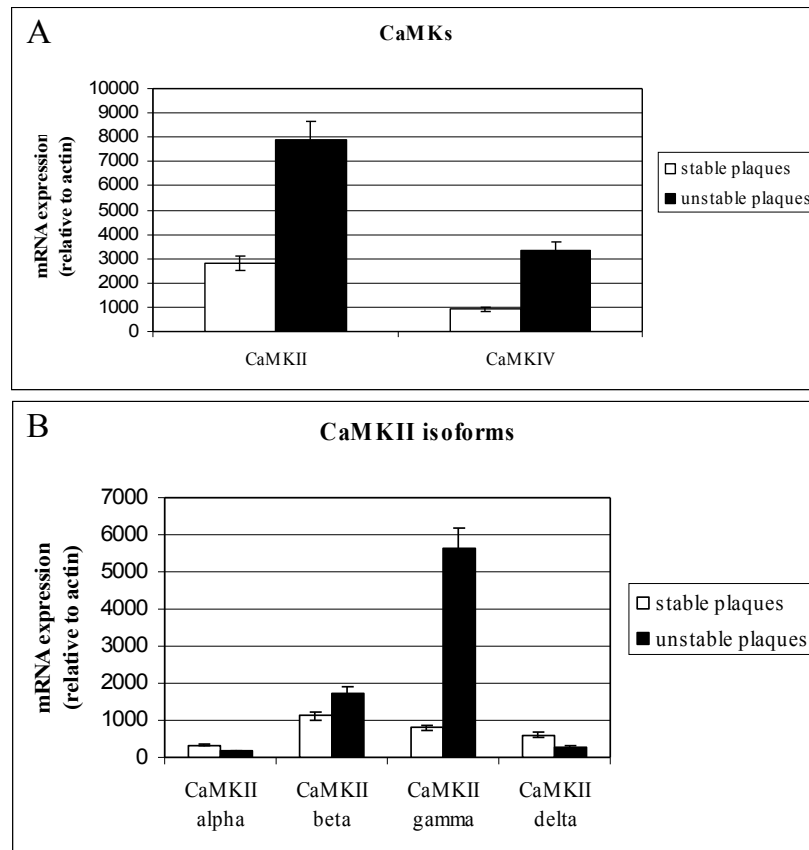
Molecular characterization of stable and unstable plaques:

1. Patterns of CaMKs expression and activation to define a possible role in plaque formation and progression;
2. Differential role of CaMKs in resident and infiltrating vascular wall cell types: effects on proliferation and differentiation.

### 3. RESULTS

#### 3.1 Expression of CaMKs mRNA transcripts in human atherosclerotic plaques

To determine the expression and quantity of CaMKs mRNA in human atherosclerotic plaques, a SYBER Green real-time PCR was performed on 6 unstable atherosclerotic plaques and 6 stable atherosclerotic plaques. The data indicated a low expression of CaMKIV mRNA compared to CaMKII mRNA (A); in particular unstable plaques predominantly express CaMKII $\gamma$  isoform while stable plaques express mainly CaMKII $\beta$  (B).

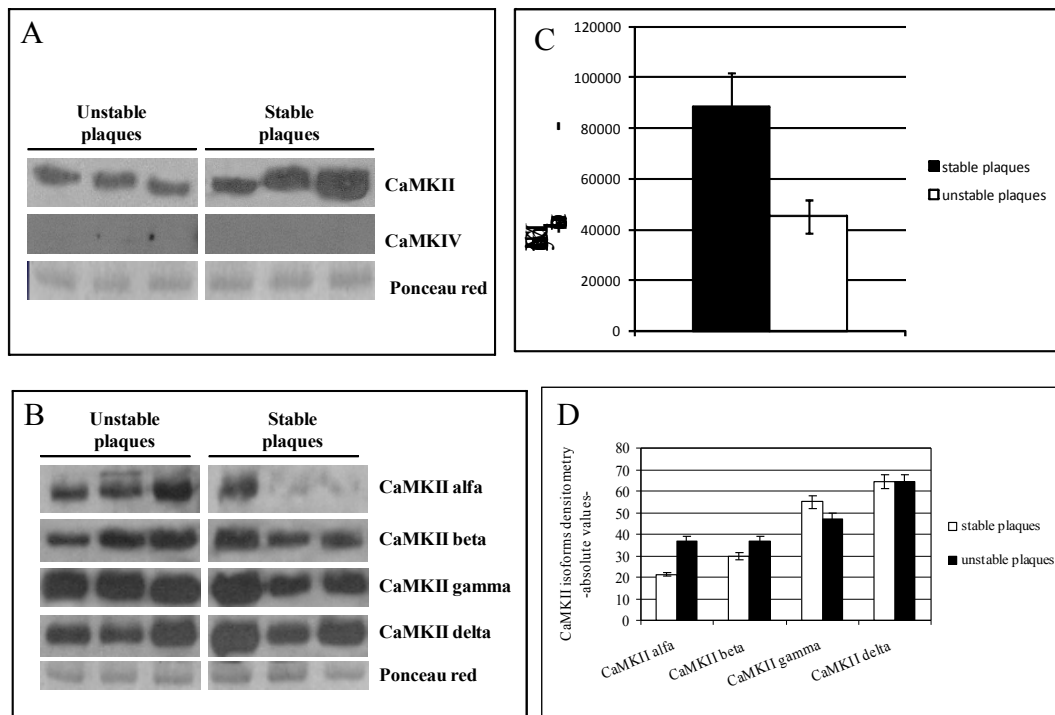


**Figure 3.2 | Expression of CaMKs mRNA transcripts in human atherosclerotic plaques**

SYBER Green real-time PCR analysis of CaMKs (A) and CaMKII isoforms (B) mRNA expression in unstable stable atherosclerotic plaques. Total RNA were extracted from human atherosclerotic plaques and reverse transcribed. The mRNA were analyzed by SYBER Green real-time PCR with specific primers to CaMKs (CaMKII, CaMKIV) and CaMKII isoforms (CaMKII $\alpha$ , CaMKII $\beta$ , CaMKII $\gamma$ , CaMKII $\delta$ )

### 3.2 Expression of CaMKs in human atherosclerotic plaques

Western blot experiments were performed on protein extracts from 10 unstable atherosclerotic plaques and 10 stable atherosclerotic plaques. CaMKII protein was highly expressed in all atherosclerotic plaques while there was no expression of CaMKV protein (A). In particular CaMKII is highly expressed in stable atherosclerotic plaques as attested the densitometric analysis (B). Furthermore, both plaque groups expressed high levels of CaMKII $\gamma$  and CaMKII $\delta$  isoforms (B and D)

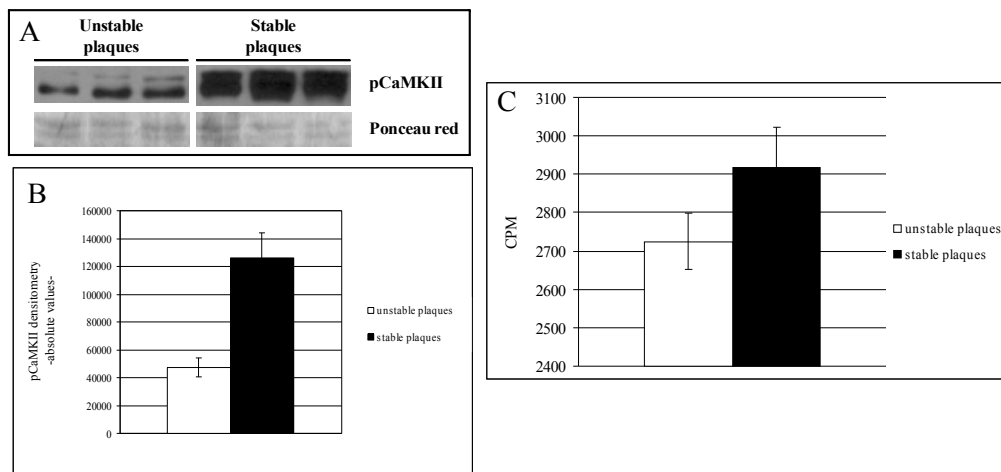


**Figure 3.1 | Expression of CaMKs in human atherosclerotic plaques**

Representative western blot showing expression of CaMKs in human atherosclerotic plaques (A). Significant expression of CaMKII was found in atherosclerotic plaques, in particular in the stable group (C). All atherosclerotic plaques expressed CaMKII gamma and delta isoforms (C and D) Ponceau red staining is included to show total protein load.

### 3.3 CaMKII activity in human atherosclerotic plaques

In order to define whether CaMKII was active in human atherosclerotic plaques a western blot experiment was performed. The western blot experiment showed high levels of CaMKII phosphorylation in stable plaques compared to unstable plaques (A and B). To strongly confirm CaMKII activity in human stable plaques an activity assay was performed. The activity assay confirmed that CaMKII is more active in human stable plaques (C).

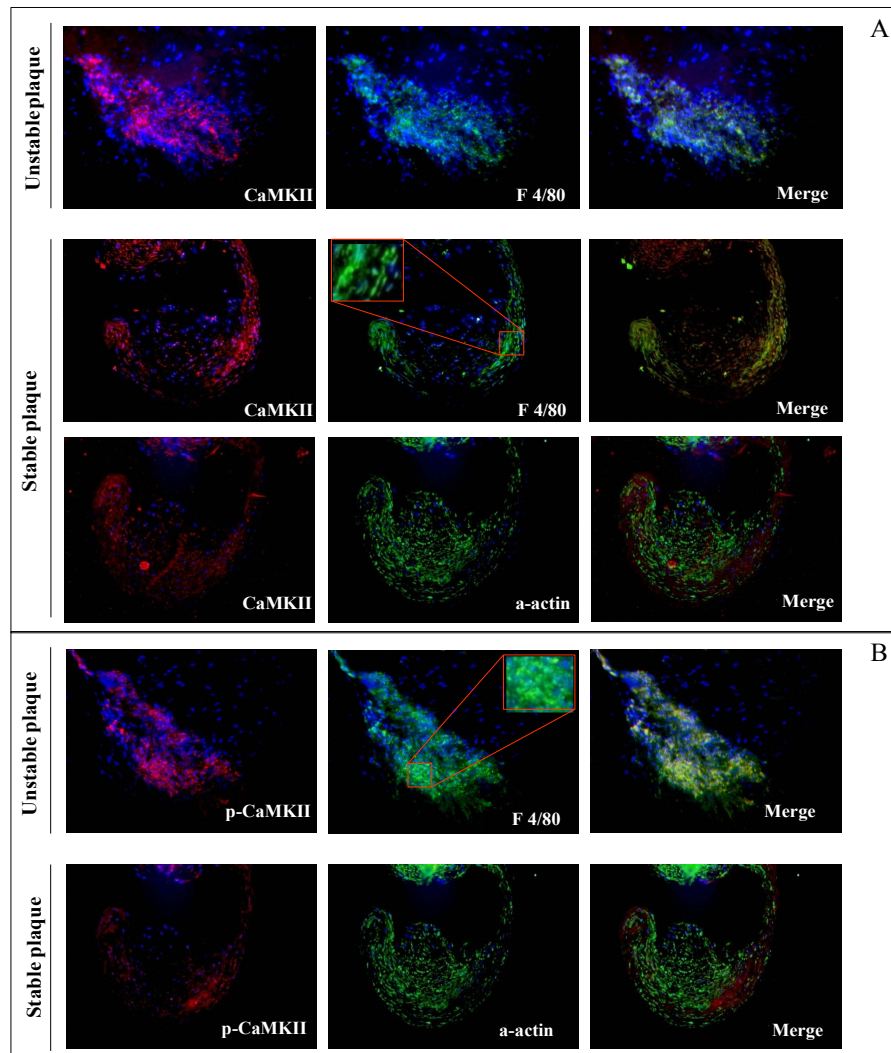


**Figure 3.3 | CaMKII activity in human atherosclerotic plaques**

Representative western blot showing activation of CaMKII in human atherosclerotic plaques (A). Significant activation of CaMKII was found in atherosclerotic plaques, in particular in the stable group (B). This activation was confirmed by CaMKII activity assay (C) Ponceau red staining is included to show total protein load.

### 3.4 Cellular localization of CaMKII in human atherosclerotic plaques

To determine the cell localization of CaMKII in human atherosclerotic plaques, immunofluorescence studies were performed on 6 human atherosclerotic plaques. As shown in Figure 3.4 (A), CaMKII was expressed mainly in F4/80 positive cells, a marker for macrophages, while it is a very low expression in VSMCs. In addition, CaMKII is activated in atherosclerotic plaque macrophages (B).



**Figure 3.3 | Cell localization of CaMKII in human atherosclerotic plaques**

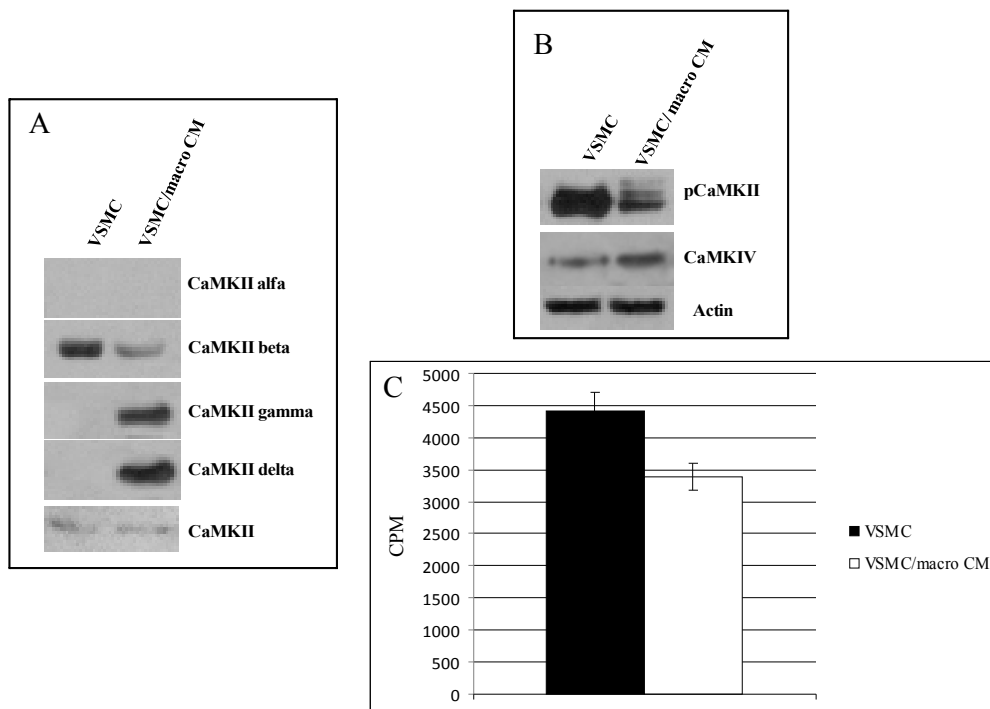
Representative immunofluorescence showing CaMKII expression and activation in human atherosclerotic plaques. Macrophages were identified by anti-F4/80 antibody while VSMCs were identified by anti-  $\alpha$ -actin antibody.



### 3.5 Macrophage conditioned medium modified CaMKs expression and activation in VSMCs

VSMCs were treated for 24h with cellular surnatant of cultured macrophage extracted from human atherosclerotic plaques. Macrophages conditioned medium treatment induced a reduction of CaMKII $\beta$  isoform, an ex-novo expression of CaMKII $\gamma$  and CaMKII $\delta$  isoforms, while CaMKII $\alpha$  isoform was undetectable; the treatment had no effect on CaMKII (A). CaMKII activation is reduced by macrophages conditioned medium treatment while CaMKIV expression is increased following treatment (B).

Treatment of VSMC with macrophages conditioned medium induced a reduction of CaMKII activity (C).



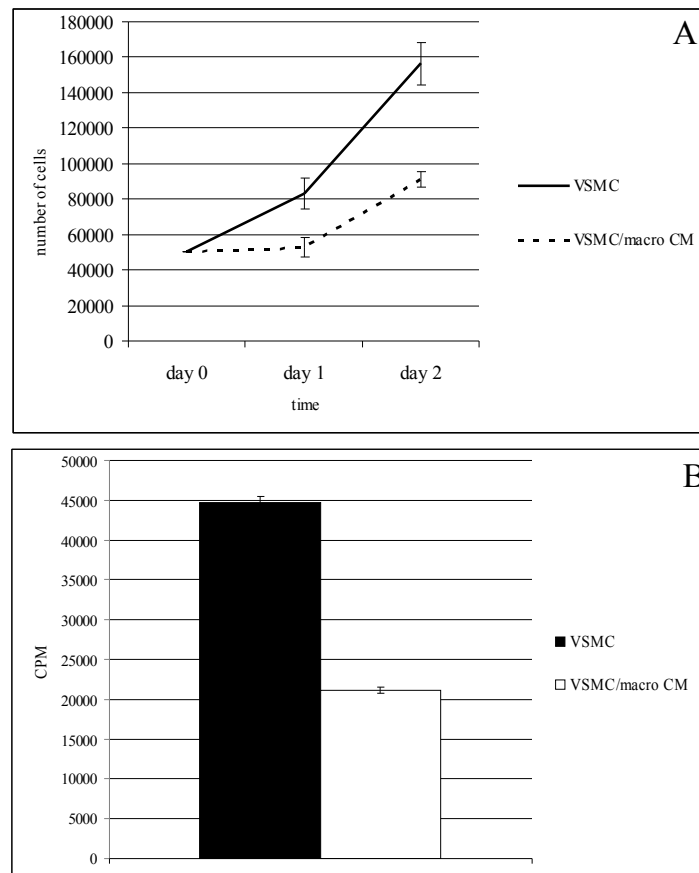
**Figure 3.5 | Macrophage conditioned medium modified CaMKs expression and activation in VSMCs**

Representative western blot showing CaMKs expression and activation in VSMCs following the treatment with macrophage conditioned medium. The treatment modified CaMKs expression and activation (A and B). The result was supported by the results of CaMKII activity assay (C).

### 3.6 Macrophage conditioned medium reduce VSMCs proliferation

It was demonstrated that CaMKII has a role on VSMC proliferation (Cipolletta et al., 2010).

In order to define the effect of macrophages conditioned medium on VSMC proliferation, [ $^3\text{H}$ ] thymidine incorporation and proliferation assays were performed. VSMCs proliferation and [ $^3\text{H}$ ] thymidine incorporation were strongly reduced by conditioned medium treatment.



**Figure 3.6 | Macrophage conditioned medium reduce VSMCs proliferation**

VSMCs were plated in 6-well plates and treated with cellular supernatant of cultured macrophage extracted from human atherosclerotic plaques for 24 hours. Following treatment, the medium was removed, cells detached from the plates, and counted (A). [ $^3\text{H}$ ] thymidine incorporation was used to measure DNA synthesis in VSMC cells treated with supernatant of cultured macrophage for 24 h (B).

## 4. DISCUSSION AND CONCLUSIONS

I investigated the role of CaMKs in the inflammation process supporting atheroma, to determine whether CaMKs represent a suitable novel therapeutic target to prevent the formation and progression of human atherosclerotic plaques.

Atherosclerosis is an inflammatory disease: identification of critical regulatory pathways is important to improve the knowledge of its emergence and progression, to identify novel therapeutic pathways and to pave the way for novel therapeutic strategies to reduce its associated mortality.

Two major cell types are present in the atherosclerotic plaque: activated macrophages and VSMCs. The prevalence of one cell types over the other participate in the fate of the atherosclerotic plaque towards stability or instability. This study allows for the first time to better identify the intracellular signalling in the progression of atherosclerosis towards instability.

Several intracellular signalling pathways are involved in atherosclerosis development and progression. Among these cascades,  $\text{Ca}^{2+}$ -mediated ones play a major role. CaMKs are important transducers of changes in intracellular  $\text{Ca}^{2+}$ , and they regulate biological processes relevant to atherosclerosis, by affecting the biology of vascular cell walls and of infiltrating macrophages.

In the first part of my study I analyzed the patterns of CaMKs expression and activation in human atherosclerotic plaques, to define their possible role in plaque formation and progression. The plaques samples were obtained from carotid endarterectomy procedures, and were classified into stable plaques group and unstable plaques group.

In the present thesis I generated data demonstrating that CaMKII is highly expressed in atherosclerotic stable plaques compared to atherosclerotic unstable plaques, whereas CaMKIV is undetectable. Furthermore, all atherosclerotic plaques mainly expressed CaMKII gamma and delta isoforms.

I subsequently demonstrated by western blot analysis that CaMKII is highly phosphorylated in human atherosclerotic plaques. Indeed, as shown in the *in vivo* kinase assay, CaMKII is mainly active in the stable plaques group.

In the early stages of atherosclerosis, cytokines alter endothelial functions, resulting in restructuring of the intercellular junctions, and leading to loss of barrier function, thus facilitating leukocyte transmigration. At a more advanced stage of the disease, proinflammatory cytokines destabilize atherosclerotic plaques by promoting cell apoptosis and matrix degradation. Macrophage apoptosis results in the formation of cell debris, which contribute to enlargement of the lipid core. Plaque SMC apoptosis leads to thinning of the fibrous cap, favouring its rupture (Ait-Oufella et al.). Therefore, living macrophages in advanced plaques can contribute to vulnerable plaque formation through the secretion of cytokines,

proteases, and procoagulant/thrombotic factors. Indeed, the prevalence of inflammatory and necrotic events leads to instability, and a role for CaMKII activation in VSMC was demonstrated (Cipolletta et al, 2010).

Therefore, in the second part of my study, I investigated the differential role of CaMKs in resident and infiltrating vascular wall cell types and the effects on proliferation and differentiation.

First of all, I confirm that stable and unstable plaques express different cellular types. In particular prevalence of macrophages is observed in unstable plaques. This observation must be taken into consideration when comparing CaMKII and CaMKII isoforms expression and activity between the two kind of lesions.

I investigated CaMKII cellular localization in human atherosclerotic plaques. The immunofluorescence analysis showed that CaMKII is concentrated and activated in macrophages population, while it is very poorly expressed in the vascular component of the atherosclerotic plaque.

Secondly, to define the effect of the inflammatory system on CaMKs expression and activation, a conditioned-medium model system was set-up. Treatment with macrophages conditioned medium modified CaMKs expression and phosphorylation in cultured VSMCs. The treatment induced a reduction of CaMKII activity and of VSMCs proliferation, as shown in the proliferation assays. This finding is well in agreement with the observation of increased CaMKIV expression, which is only present in VSMCs. Indeed, we show that CaMKIV expression increases in the unstable atherosclerotic lesion as well as in VSMCs exposed to conditioned medium. These two findings together suggest that CaMKII and CaMKIV are counterregulated in proliferative cells. Although this has to be confirmed in other cell types.

Cardiovascular diseases represent a leading cause of morbidity and mortality, with an ever increasing burden on health care systems. Acute events recognize atherosclerosis as the common pathogenesis. There are many evidences in the literature on the cell based mechanisms and in vivo processes involved in atherosclerosis, that have been stimulated by the consideration that it is the leading cause of death in the industrialized world. The investigation of unstable plaque biology will help to identify novel therapeutic targets: so far, CaMKs have never been taken into account while looking at mechanisms of atherosclerosis. It has recently been demonstrated that CaMKs regulate biological processes that are relevant to the process of atherosclerosis, by affecting the biology of vascular wall cells and infiltrating macrophages (Santulli et al, 2012).

In summary with this elaborate I show:

- 1) Different expression of CaMKII in VSMCs and macrophages in atherosclerotic lesions**
- 2) Abundance of macrophages in unstable atherosclerotic lesions causes reduced cell proliferation of VSMCs**

**3) This latter phenomenon is accompanied by changes in CaMKII and CaMKIV expression in VSMCs**

The characterization of the role of the CaMKs in the pathogenesis of arteriosclerosis, especially in the inflammatory and proliferative events determining its evolution, could provide a terrific opportunity to understand the molecular mechanism underpinning atherosclerotic plaque progression. My data pose the bases of new therapeutic strategies based on the cell type specific inhibition of CaMKII in macrophages for stabilization of atherosclerotic lesions

## **5. MATERIALS AND METHODS**

### **5.1 Cell culture**

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by U.S. National Institutes of Health and approved by the Ethics Committee of the Federico II University. Primary cultures of VSMCs were obtained from thoracic aortas of WKY rats as described previously (Muthalif et al., 1996). VSMC were cultured in Dulbecco's minimal essential medium (BioWhittaker, Virviers, Belgium), containing 10% fetal calf serum (GIBCO, Grand Island, NY, USA), 200 mM L-glutamine, penicillin (100 mg/ml) and streptomycin (100 mg/ml). The plates were incubated at 37°C, in the presence of 5% CO<sub>2</sub>.

Human atherosclerotic plaque samples of carotid were obtained from patients undergoing surgery for carotid stenosis.

To isolate macrophages from human atherosclerotic plaques were used magnetic beads bound to anti-CD14 antibody. The fresh tissue samples were transferred to a test tube containing sterile Hanks' balanced salts without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Seromed, Biochrom KG). The tissues were washed from contaminating red blood cells and then dissected from adventitia. The intima-media was cut into 1-mm<sup>3</sup> pieces and then incubated in a proteolytic solution containing 450 U/mL collagenase, 1 mg/mL trypsin inhibitor, and 4.8 mg/mL HEPES in Hanks' balanced salts in a siliconized bottle with a magnetic bar stirring slowly at 37°C. Free cells were collected every 10 to 15 minutes for up to 2 hours. Large fibers such as collagen and elastin were taken away by passing through a 100-µm mesh nylon filter and by incubating with 30 NL/mL uncoated magnetic beads for 10 minutes at 4°C. After washing, the cells were resuspended in RPMI 1640 supplemented with 1% BSA and incubated with 30 NL/mL CD14 beads (0.9 mg/mL) for 30 minutes at 4°C. CD14-positive cells were collected with an magnetic particle concentrator. Unbound cells were washed away with Dulbecco's PBS containing 0.1% BSA.

### **5.2 Western blot and immunoprecipitation procedures**

The human atherosclerotic plaques and the VSMC were lysed on ice in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 2 mM PMSF, 5 µg/mL leupeptin, 5 µg/mL pepstatin). The lysate were quantified by Biorad DC protein assay. An equal amount of proteins from each sample was loaded with Laemmli buffer. Protein were resolved by SDS-PAGE and transferred to an Immobilon P membrane (Millipore Corporation, Bedford, MA). Membranes were blocked by incubation with PBS 0.2% tween, 5% nonfat dry milk for one

hour at room temperature. The membranes were then incubated overnight with primary antibodies at 4°C, washed for 40 minutes with PBS 0,2% tween and incubated for 1 hour with a horseradish peroxidase -conjugated secondary antibody. Finally, protein bands were detected by an enhanced chemiluminescence system (ECL, Amersham Bioscience). Computer-acquired images were quantified using ImageQuant software (Amersham Bio-sciences).

Commercial antibodies were diluted 1:1000 and purchased as follows: total CaMKII, actin, CaMKII $\alpha$ , CaMKII $\gamma$ , CaMKII $\delta$  (Santa Cruz); total CaMKII $\beta$  and phosphoCaMKII (Zymed, Invitrogen), total CaMKIV (BD).

For immunoprecipitation assay the human atherosclerotic plaques and the VSMC were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 10 mM NaF, 5 mM EGTA, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonylfluoride).

Primary antibodies vs protein of interest and Protein G plus/protein A agarose beads (Oncogene Science, Boston, MA) were used to immunoprecipitate corresponding proteins from 500  $\mu$ g of total lysate. Non immune rabbit IgG were used as control. Rabbit policlonal antibody to CaMKII were from Santa Cruz Biotechnology.

### **5.3 CaMKII activity assay**

In a first reaction step CaMKII was incubated for 30 minutes at 30 °C with 5 mM CaCl<sub>2</sub> and 5 $\mu$ M CaM in 50  $\mu$ l of reaction mixture consisting of 50 mM HEPES pH 7.5, 10 mM MgCl<sub>2</sub>, 0,5 mM dithiothreitol (DTT), 2 $\mu$ M CaM, 100 nM microcystin, 0,5mM cold ATP .A 10  $\mu$ l aliquot from the first reaction was than incubated with 25mM EGTA, 0,5 mM Autocamtide and 50  $\mu$ M ATP (1500 cpm/pmol [ $\gamma$ 32P]ATP) in order to determine CaMKII autonomous activity on its peptide substrate Autocamtide. The reaction was carried out for 30 minutes at 30 °C and 20  $\mu$ l aliquots of the reaction mixture were spotted onto p81 phosphocellulose filters (Upstate Biothechnology, Lake placid, NY) The level of [32P] incorporation into Autocamtide was determined by liquid scintillation counting. Autocamtide was provided by prof. P. Campiglia (University of Salerno).

### **5.4 Cell proliferation and [ $^3$ H]thymidine incorporation**

VSMCs were plated in 6-well plates (50,000 cells/well) and treated with cellular surnatant of cultured macrophage extracted from human atherosclerotic plaques for 24 hours. After treatment, the medium was removed, cells detached from the plates, and counted. To determine DNA synthesis, cells were plated in 6-well

plate, and treated as described previously. 0.5  $\mu$ Ci [ $^3$ H]thymidine were then added to the plates. After 24 h, the plates were gently washed with PBS, then with 10% trichloroacetic acid (TCA), and incubated 10 min with 20% TCA at 4°C. TCA was removed and cells were lysed with 0.2% SDS for 15 min at 4°C. The lysates were then resuspended in 5 ml scintillation fluid and counted in a  $\beta$ -counter (Beckton Dickinson).

### 5.5 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA were extracted from human atherosclerotic plaques with a RNeasy Lipid Tissue Mini Kit (Qiagen) and reverse transcribed using a High Capacity Reverse Transcriptase Kit (Applied Biosystems). QRT-PCR was performed using a Bio-Rad IC5 thermo cycler (Bio-Rad Laboratories, Hercules, CA) using specific primers:

CaMKIV 5'-GGC ACA GGC TGA GCT GAT G-3' forward  
 5'-CTA GTT CCA GGT CAG CCA CCT TT-3' reverse  
 CaMKII 5'-AAA CAG TCT CGT AAG CCC AG-3' forward  
 5'-ATC CCA TCT GTA GCG TTG TG-3' reverse  
 CaMKII $\alpha$  5'-GAA GAG CGA TGG TGT GAA GA-3' forward  
 5'-ACT TTG GTG TCT TCA TCC TCG -3' reverse  
 CaMKII $\beta$  5'-CTC TCG CCA CAA TGT CTT CA -3' forward  
 5'-CTG TCA GCC AGA GAT CAC CA -3' reverse  
 CaMKII $\gamma$  5'-TCC GAG CCT CAC GTT CTA GT -3' forward  
 5'-CCG ACG ACT ACC AGC TCT TC -3' reverse  
 CaMKII $\delta$  5'-GGC TGC TGA GAA ATT CCT TG -3' forward  
 5'-ACT ATC AAC CCT GCC AAA CG -3' reverse

Cycle threshold (Ct) values from 3 independent experiments were normalized to the internal  $\beta$ -actin control. The ratio of fold change was calculated using the Pfaffl method (Pfaffl 2001).

### 5.6 Immunofluorescence

Plaques obtained from human carotid endarterectomy were fixed in formaline, embedded in paraffin and sectioned at 5  $\mu$ m with a rotary microtome. Sections were dewaxed, rehydrated and incubated with anti-F4/80, CamKII, p-CamKII,  $\alpha$ -actin (SantaCruz) primary antibodies at a 1:50 dilution at room temperature for two hours. Specific fluorescent labeled secondary antibodies (Alexa Fluor, Invitrogen) at a 1:100 dilution were incubated at room temperature for 1 hour. Images were taken by using an Eclipse E1000 Fluorescence Microscope (Nikon) and acquired by using Sigma Scan Pro software (Jandel). Images were optimized for contrast in Adobe PhotoShop, but no further manipulations were made.



### **5.7 Statistical analysis**

Student's t-test was used to assess statistical significance and a p-value  $<0.05$  was deemed significant. Statistics were computed with GraphPad Prism software (San Diego, CA).

## 6. ACKNOWLEDGEMENTS

Thanks to prof. Vittorio Enrico Avvedimento, who allowed me to attend the doctorate program in Molecular Pathology and Physiopathology under his supervision.

Thanks to doctor Maddalena Illario for believing in me from the first day.

Thanks to prof. Guido Iaccarino for critical discussion of the data.

Thanks to all my colleagues of the Illario's Lab for their fundamental collaboration. I would like to thank doctor Maria Rosaria Rusciano for working with me side by side as always; doctor Maria Soprano to be a scrupulous scientist and a sincere person; doctor Catherine Crola and doctor Mario Neri for helping me and making funnier my hard days in the lab. Thanks to Simona Valente to be a diligent student and Francesca Iommelli for her curiosity.

Thanks to doctor Daniela Sorriento for providing essential expertise for this study.

Thanks to prof Francesco Beguinot and prof Pietro Formisano for supporting me during my doctorate.

Thanks to doctor Lucio Maresca, doctor Giuseppe Nobile and Luigi Giuliano from Monaldi Hospital for their essential contribution to this study.

Thanks to doctor Francesco Maria Pacifico for his suggestions day by day and Concetta Volpe to be careful to the needs of PhD students.

Thanks to prof. Vicente Andres Garcia for the opportunity he gave me to spend three months in his laboratory at Centro Nacional de Investigaciones Cardiovasculares and all my colleagues and friends from CNIC, who made my stay in Madrid unforgettable.

Thanks to my friends Carmela Passaro to be with me everyday and Eleonora Russo for making these three years much happier.

Thanks to my "foreign friends" Sara Monaco, Raffaele Teperino, Felicia Basilicata and Marcella Salzano to be anyway present in my life.

Thanks to my family that always believe in me. Their support and their love is my strength.

## **7. REFERENCES**

- Ait-Oufella, H., Taleb, S., Mallat, Z., and Tedgui, A. Recent advances on the role of cytokines in atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* 31, 969-979.**
- Anderson, K.A., Means, R.L., Huang, Q.H., Kemp, B.E., Goldstein, E.G., Selbert, M.A., Edelman, A.M., Freneau, R.T., and Means, A.R. (1998). Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase beta. *J Biol Chem* 273, 31880-31889.**
- Anderson, K.A., Ribar, T.J., Illario, M., and Means, A.R. (1997). Defective survival and activation of thymocytes in transgenic mice expressing a catalytically inactive form of Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV. *Mol Endocrinol* 11, 725-737.**
- Anderson, M.E., Brown, J.H., and Bers, D.M. CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol* 51, 468-473.**
- Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature* 361, 315-325.**
- Berridge, M.J., and Irvine, R.F. (1989). Inositol phosphates and cell signalling. *Nature* 341, 197-205.**
- Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1, 11-21.**
- Bjorkerud, S., and Bjorkerud, B. (1996). Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol* 149, 367-380.**

**Boyle, J.J., Weissberg, P.L., and Bennett, M.R. (2003). Tumor necrosis factor- $\alpha$  promotes macrophage-induced vascular smooth muscle cell apoptosis by direct and autocrine mechanisms. *Arteriosclerosis, thrombosis, and vascular biology* 23, 1553-1558.**

**Braun, A.P., and Schulman, H. (1995). The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu Rev Physiol* 57, 417-445.**

**Carafoli, E. (2002). Calcium signaling: a tale for all seasons. *Proc Natl Acad Sci U S A* 99, 1115-1122.**

**Cipolletta E., Monaco S., Maione A.S., Vitiello L., Campiglia P., Pastore L., Franchini C., Novellino E., Limongelli V., Bayer K.U., Means A.R., Rossi G., Trimarco B., Iaccarino G., Illario M. (2010). Calmodulin-dependent kinase II mediates vascular smooth muscle cell proliferation and is potentiated by extracellular signal regulated kinase. *Endocrinology* 151(6), 2747-59**

**Clarke, M.C., Figg, N., Maguire, J.J., Davenport, A.P., Goddard, M., Littlewood, T.D., and Bennett, M.R. (2006). Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nature medicine* 12, 1075-1080.**

**Colbran, R.J. (1993). Inactivation of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II by basal autophosphorylation. *J Biol Chem* 268, 7163-7170.**

**Colbran, R.J., Smith, M.K., Schworer, C.M., Fong, Y.L., and Soderling, T.R. (1989). Regulatory domain of calcium/calmodulin-dependent protein kinase II. Mechanism of inhibition and regulation by phosphorylation. *J Biol Chem* 264, 4800-4804.**

**Cruzalegui, F.H., Kapiloff, M.S., Morfin, J.P., Kemp, B.E., Rosenfeld, M.G., and Means, A.R. (1992). Regulation of intrasteric inhibition of the multifunctional calcium/calmodulin-dependent protein kinase. *Proc Natl Acad Sci U S A* 89, 12127-12131.**

De Koninck, P., and Schulman, H. (1998). Sensitivity of CaM kinase II to the frequency of Ca<sup>2+</sup> oscillations. *Science* 279, 227-230.

Edelman, A.M., Mitchelhill, K.I., Selbert, M.A., Anderson, K.A., Hook, S.S., Stapleton, D., Goldstein, E.G., Means, A.R., and Kemp, B.E. (1996). Multiple Ca(2+)-calmodulin-dependent protein kinase kinases from rat brain. Purification, regulation by Ca(2+)-calmodulin, and partial amino acid sequence. *J Biol Chem* 271, 10806-10810.

Enslen, H., Sun, P., Brickey, D., Soderling, S.H., Klamo, E., and Soderling, T.R. (1994). Characterization of Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. *J Biol Chem* 269, 15520-15527.

Eshete, F., and Fields, R.D. (2001). Spike frequency decoding and autonomous activation of Ca<sup>2+</sup>-calmodulin-dependent protein kinase II in dorsal root ganglion neurons. *J Neurosci* 21, 6694-6705.

Fox, C.S., Hwang, S.J., Massaro, J.M., Lieb, K., Vasan, R.S., O'Donnell, C.J., and Hoffmann, U. (2009). Relation of subcutaneous and visceral adipose tissue to coronary and abdominal aortic calcium (from the Framingham Heart Study). *Am J Cardiol* 104, 543-547.

Galis, Z.S., Sukhova, G.K., Lark, M.W., and Libby, P. (1994). Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 94, 2493-2503.

Hanson, P.I., Meyer, T., Stryer, L., and Schulman, H. (1994). Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. *Neuron* 12, 943-956.

Haribabu, B., Hook, S.S., Selbert, M.A., Goldstein, E.G., Tomhave, E.D., Edelman, A.M., Snyderman, R., and Means, A.R. (1995). Human calcium-calmodulin dependent protein kinase I: cDNA cloning, domain structure and activation by phosphorylation at threonine-177 by calcium-calmodulin dependent protein kinase I kinase. *Embo J* 14, 3679-3686.

Hook, S.S., and Means, A.R. (2001). Ca(2+)/CaM-dependent kinases: from activation to function. *Annu Rev Pharmacol Toxicol* 41, 471-505.

Ikeda, H., Minamikawa, J., Nakamura, Y., Honjo, S., Hamamoto, Y., Wada, Y., Nabe, K., and Koshiyama, H. (2009). Comparison of effects of amlodipine and angiotensin receptor blockers on the intima-media thickness of carotid arterial wall (AAA study: amlodipine vs. ARB in atherosclerosis study). *Diabetes Res Clin Pract* 83, 50-53.

Illario, M., Giardino-Torchia, M.L., Sankar, U., Ribar, T.J., Galgani, M., Vitiello, L., Masci, A.M., Bertani, F.R., Ciaglia, E., Astone, D., *et al.* (2008). Calmodulin-dependent kinase IV links Toll-like receptor 4 signaling with survival pathway of activated dendritic cells. *Blood* 111, 723-731.

Imhof, B.A., and Aurrand-Lions, M. (2004). Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol* 4, 432-444.

Jensen, K.F., Ohmstede, C.A., Fisher, R.S., and Sahyoun, N. (1991). Nuclear and axonal localization of Ca<sup>2+</sup>/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. *Proc Natl Acad Sci U S A* 88, 2850-2853.

Kielbassa-Schnepp, K., Strey, A., Janning, A., Missiaen, L., Nilius, B., and Gerke, V. (2001). Endothelial intracellular Ca<sup>2+</sup> release following monocyte adhesion is required for the transendothelial migration of monocytes. *Cell Calcium* 30, 29-40.

Komatsu, R., Ueda, M., Naruko, T., Kojima, A., and Becker, A.E. (1998). Neointimal tissue response at sites of coronary stenting in humans: macroscopic, histological, and immunohistochemical analyses. *Circulation* 98, 224-233.

Libby, P. (1995). Molecular bases of the acute coronary syndromes. *Circulation* 91, 2844-2850.

**Lo, I.C., Shih, J.M., and Jiang, M.J. (2005). Reactive oxygen species and ERK 1/2 mediate monocyte chemotactic protein-1-stimulated smooth muscle cell migration. J Biomed Sci 12, 377-388.**

**Mallat, Z., and Tedgui, A. (2001). Current perspective on the role of apoptosis in atherothrombotic disease. Circulation research 88, 998-1003.**

**Meador, W.E., Means, A.R., and Quirocho, F.A. (1993). Modulation of calmodulin plasticity in molecular recognition on the basis of x-ray structures. Science 262, 1718-1721.**

**Means, A.R. (2000). Regulatory cascades involving calmodulin-dependent protein kinases. Mol Endocrinol 14, 4-13.**

**Meyer, T., Hanson, P.I., Stryer, L., and Schulman, H. (1992). Calmodulin trapping by calcium-calmodulin-dependent protein kinase. Science 256, 1199-1202.**

**Miller, S.G., and Kennedy, M.B. (1986). Regulation of brain type II Ca<sup>2+</sup>/calmodulin-dependent protein kinase by autophosphorylation: a Ca<sup>2+</sup>-triggered molecular switch. Cell 44, 861-870.**

**Miller, S.G., Patton, B.L., and Kennedy, M.B. (1988). Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca<sup>2+</sup>(+)-independent activity. Neuron 1, 593-604.**

**Mishra, S., Mishra, J.P., Gee, K., McManus, D.C., LaCasse, E.C., and Kumar, A. (2005). Distinct role of calmodulin and calmodulin-dependent protein kinase-II in lipopolysaccharide and tumor necrosis factor- $\alpha$ -mediated suppression of apoptosis and antiapoptotic c-IAP2 gene expression in human monocytic cells. J Biol Chem 280, 37536-37546.**

**Mitchinson, M.J., Ball, R.Y., Carpenter, K.L., and Enright, J.H. (1990). Macrophages and ceroid in human atherosclerosis. Eur Heart J 11 Suppl E, 116-121.**

**Moore, K.J., and Tabas, I. Macrophages in the pathogenesis of atherosclerosis. *Cell* 145, 341-355.**

**Mukherji, S., and Soderling, T.R. (1995). Mutational analysis of Ca(2+)-independent autophosphorylation of calcium/calmodulin-dependent protein kinase II. *J Biol Chem* 270, 14062-14067.**

**Murdoch, C., Giannoudis, A., and Lewis, C.E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* 104, 2224-2234.**

**Murray, C.J., and Lopez, A.D. (1997). Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet* 349, 1436-1442.**

**Muthalif M.M., Benter I.F., Uddin M.R., Malik K.U. (1996). Calcium/calmodulin-dependent protein kinase II $\alpha$  mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells. *J Biol Chem*. 271(47), 30149-57.**

**Picciotto, M.R., Zoli, M., Bertuzzi, G., and Nairn, A.C. (1995). Immunochemical localization of calcium/calmodulin-dependent protein kinase I. *Synapse* 20, 75-84.**

**Putkey, J.A., and Waxham, M.N. (1996). A peptide model for calmodulin trapping by calcium/calmodulin-dependent protein kinase II. *J Biol Chem* 271, 29619-29623.**

**Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362, 801-809.**

**Santulli, G., Cipolletta, E., Sorriento, D., Del Giudice, C., Anastasio, A., Monaco, S., Maione, A.S., Condorelli, G., Puca, A., Trimarco, B., *et al.* CaMK4 Gene Deletion Induces Hypertension. *J Am Heart Assoc* 1, e001081.**



Schubert, S.Y., Benarroch, A., Ostvang, J., and Edelman, E.R. (2008). Regulation of endothelial cell proliferation by primary monocytes. *Arteriosclerosis, thrombosis, and vascular biology* 28, 97-104.

Selbert, M.A., Anderson, K.A., Huang, Q.H., Goldstein, E.G., Means, A.R., and Edelman, A.M. (1995). Phosphorylation and activation of Ca(2+)-calmodulin-dependent protein kinase IV by Ca(2+)-calmodulin-dependent protein kinase Ia kinase. Phosphorylation of threonine 196 is essential for activation. *J Biol Chem* 270, 17616-17621.

Shaywitz, A.J., and Greenberg, M.E. (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 68, 821-861.

Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* 252, 1427-1430.

Singla, S.I., Hudmon, A., Goldberg, J.M., Smith, J.L., and Schulman, H. (2001). Molecular characterization of calmodulin trapping by calcium/calmodulin-dependent protein kinase II. *J Biol Chem* 276, 29353-29360.

Smith, M.K., Colbran, R.J., Brickey, D.A., and Soderling, T.R. (1992). Functional determinants in the autoinhibitory domain of calcium/calmodulin-dependent protein kinase II. Role of His282 and multiple basic residues. *J Biol Chem* 267, 1761-1768.

Sobota, R.M., Muller, P.J., Heinrich, P.C., and Schaper, F. (2008). Prostaglandin E1 inhibits IL-6-induced MCP-1 expression by interfering specifically in IL-6-dependent ERK1/2, but not STAT3, activation. *Biochem J* 412, 65-72.

Tabas, I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* 10, 36-46.

**Tabas, I. (2005). Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arteriosclerosis, thrombosis, and vascular biology* 25, 2255-2264.**

**Tedgui, A., and Mallat, Z. (2006). Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiological reviews* 86, 515-581.**

**Tjandra, N., Kuboniwa, H., Ren, H., and Bax, A. (1995). Rotational dynamics of calcium-free calmodulin studied by <sup>15</sup>N-NMR relaxation measurements. *Eur J Biochem* 230, 1014-1024.**

**Tokumitsu, H., Enslen, H., and Soderling, T.R. (1995). Characterization of a Ca<sup>2+</sup>/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase. *J Biol Chem* 270, 19320-19324.**

**Virmani, R., Burke, A.P., Kolodgie, F.D., and Farb, A. (2002). Vulnerable plaque: the pathology of unstable coronary lesions. *J Interv Cardiol* 15, 439-446.**

**Wong, N.D., Gransar, H., Shaw, L., Polk, D., Moon, J.H., Miranda-Peats, R., Hayes, S.W., Thomson, L.E., Rozanski, A., Friedman, J.D., *et al.* (2009). Thoracic aortic calcium versus coronary artery calcium for the prediction of coronary heart disease and cardiovascular disease events. *JACC Cardiovasc Imaging* 2, 319-326.**

## 8. LIST OF PUBLICATIONS

1) *CaMKII mediates vascular smooth muscle cell proliferation and is potentiated by erk*

Ersilia Cipolletta, Sara Monaco , **Angela Serena Maione**, Laura Vitiello , Pietro Campiglia , Lucio Pastore, C. Franchini, Ettore Novellino, V. Limongelli , UK Bayer, A.R. Means, Guido Rossi , Bruno Trimarco , Guido Iaccarino, Maddalena Illario

Endocrinology 2010 Jun;151(6):2747-59. Epub 2010 Apr 14.

2) *Genome-Wide Association Study on Long-Living Individuals from Southern Italy Identifies rs10491334 in CAMK4 Regulating SIRT1 Activity*

Alberto Malovini, Maddalena Ilario, Guido Iaccarino, Francesco Villa, Anna Ferrario, Roberta Roncarati, Luca Simonelli, Chiara Viviani Anselmi, Valeria Novelli, Ersilia Cipolletta, Eleonora Leggiero, Stephanie J Brewster, Alessandro Orro, Maria Rosaria Rusciano, Luciano Milanese, **Angela Serena Maione**, Gianluigi Condorelli, Riccardo Bellazzi, Annibale A. Puca.

Rejuvenation Res. 2011 Jun;14(3):283-91. Epub 2011 May 25.

3) *Design, Synthesis, and Cytotoxic Evaluation of Acyl Derivatives of 3-Aminonaphtho[2,3-b]thiophene 4,9-dione, a Fully Aromatic Quinone DTNQ-Related System*

Pietro Campiglia, Claudio Aquino, Alessia Bertamino, Ilaria Granata, Alfonso Carotenuto, Diego Brancaccio, Paola Stiuso, Maria Rosaria Rusciano, **Angela Serena Maione**, Paolo Grieco, Bruno Maresca, Isabel Gomez-Monterrey, Ettore Novellino.

J Med Chem. 2011 Jun 23;54(12):4077-91. Epub 2011 May 23.

4) *Inhibition of autophagy enhances the effects of E1A defective oncolytic adenovirus dl922-947 against glioma cells in vitro and in vivo*

Ginevra Botta, Carmela Passaro, Silvana Libertini, Antonella Abagnale, Sara Barbato, **Angela Serena Maione**, Gunnel Hallden, Francesco Beguinot, Pietro Formisano and Giuseppe Portella

Hum Gene Ther. 2012 Jun;23(6):623-34. doi: 10.1089/hum.2011.120. Epub 2012 Jun 5.

5) *Sisters acts: converging signaling between CaMKII and CaMKIV, two members of the same family*

M.R. Rusciano, **A.S. Maione**, M. Illario

Translational Medicine @ UniSa - ISSN 2239-9747 2012, 4(8): 66-72.

6) *CaMK4 Gene Deletion Induces Hypertension*

Santulli G, Cipolletta E, Sorriento D, Del Giudice C, Anastasio A, Monaco S, **Maione AS**, Condorelli G, Puca A, Trimarco B, Illario M, Iaccarino G.

J Am Heart Assoc. 2012 Aug;1(4):e001081. doi: 10.1161/JAHA.112.001081.

Epub 2012 Aug 24.

7) *Characterization of a selective CaMKII peptide inhibitor*

Gomez-Monterrey I, Sala M, Rusciano MR, Monaco S, **Maione AS**, Iaccarino G, Tortorella P, D'Ursi AM, Scrima M, Carotenuto A, De Rosa G, Bertamino A, Vernieri E, Grieco P, Novellino E, Illario M, Campiglia P.

Eur J Med Chem. 2013 Jan 22;62C:425-434. doi: 10.1016/j.ejmech.2012.12.053.