

Vascular memory:
from old concepts to a new model:
Sustained down-regulation of alpha-Smooth
Muscle Actin induced by Angiotensin II

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Abbreviations list

α 1-sGC – soluble guanylyl cyclase α 1 subunit

α SMA – Alpha smooth muscle actin

Ac – Acetylation

ACCORD – Action to Control

Cardiovascular Risk in Diabetes trial

ACE – Angiotensin-converting enzyme

ACE2 – Angiotensin-converting enzyme 2

ACTA2 – Alpha smooth muscle actin (gene)

ADDITION – Anglo-Danish-Dutch study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care

ADVANCE – Action in Diabetes and Vascular Disease Preterax and Diamicon study

AGE – Advance glycation end products

AGT – Angiotensinogen

ALLHAT – Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial

ANBP2 – Second Australian National BP study

Ang I – Angiotensin I

Ang II – Angiotensin II

AP-1 – Activator protein 1

APOB – Apolipoprotein B

ASCOT – Anglo-Scandinavian Cardiac Outcomes Trial

ASCOT-LLA – Anglo-Scandinavian Cardiac Outcomes Trial Lipid Lowering Arm

AT₁R – Angiotensin type-1 receptor

AT₂R – Angiotensin type-2 receptor

BMI – Body mass index

BMP2 – Bone morphogenic protein 2

BP – Blood pressure

CAD – Coronary artery disease

CBP/P300 – CREB-binding protein

CHD – Coronary heart disease

circRNA – Circular RNA

CpG – 5'-cytosine-phosphate-guanine-3' dinucleotides

CV – Cardiovascular

CVD – Cardiovascular disease

DAS – Diabetes and Aging Study

DCCT – Diabetes Control and Complications Trial

DM – Diabetes mellitus

DNAme– DNA methylation

DNMT – DNA methyltransferases

EC – Endothelial cell

ECM – Extracellular matrix

ED – Endothelial dysfunction

EDIC – Epidemiology of Diabetes Intervention and Complication study

ERK – Extracellular signal regulated kinases

ESS – Endothelial shear stress

ET-1 – Endothelin 1

EZH2 – Enhancer of zest homolog 2

FDR – False discovery rate

FGF – Fibroblast growth factor

FHS – Framingham heart study

GCN5 – General control of amino acid synthesis protein 5-like 2

Gm13034 – SMARCA5 pseudogene

GPCR – G protein-coupled receptor

GSEA – Gene-set enrichment analysis

GUCY1B1 – Guanylate cyclase 1 soluble subunit beta 1

GWAS – Genome wide association study

H – Histone

H₂O₂ – Hydrogen peroxide

HAT – Histone acetyltransferases

HAT1 – Histone acetyltransferases 1

HB-EGF – Heparin-binding epidermal growth factor

Hb1Ac – Glycosylated hemoglobin

HDAC – Histone deacetylases

HDL – High density lipoprotein

HIF – Hypoxia inducible factor

HMT – Histone methyltransferase
 HOPE – Heart Outcomes Prevention Evaluation 3 trial
 HSP90AA1 – Heat shock protein 90 alpha family class a member 1
 HSPB1 – Heat shock protein family b (small) member 1

 ICAM-1 – Intercellular adhesion molecule 1
 IFN γ – Interferon gamma
 IGHG2B – Immunoglobulin heavy constant gamma 2B
 IL-1 – Interleukin 1
 IL-10 – Interleukin 10

 JAK – Janus kinases
 JNK – c-Jun N-terminal kinase

 K – Lysine
 KCNC4 – Potassium Voltage-Gated Channel Subfamily C Member 4
 KDM – Lysine demethylase
 KEGG – Kyoto Encyclopedia of Genes and Genomes
 KLF2 – Krüppel-like factor 2
 KLF4 – Krüppel-like factor 4

 LANCL3 – LanC like 3
 LDL – Low density lipoprotein
 LDLR – Low-density lipoprotein cholesterol receptor
 LIFE – Losartan Intervention For Endpoint reduction in hypertension
 LIPID – Long-term Intervention with Pravastatin in Ischaemic Disease trial
 lncRNA – Long noncoding RNA
 LOX – Lectin-like receptors
 LPS – Lipopolysaccharids
 LSD1 – Lysine demethylase 1
 LVW/BW – Left ventricular weight/body weight ratio
 LVW/TL – Left ventricular weight/tibial length ratio

 MAPK – Mitogen activated kinase

MCP-1 – Monocyte chemoattractant protein 1
 Me – Methylation
 MGP – Matrix Gla protein
 MI – Myocardial infarction
 miRNA – MicroRNA
 MLC – Myosin light chain
 MMP – Matrix metalloproteinases
 MTA – Methyl-thioadenosine
 MYH11 – Smooth muscle myosin heavy chain 11
 MYH6 – Myosin heavy chain 6
 MYH7 – Myosin heavy chain 7
 MYL9 – Myosin light chain 9
 MYOCD – Myocardin

 ncRNA – Noncoding RNA
 NF- κ B – Nuclear Factor-kappa B
 NO – Nitric oxide
 NOX – NADPH oxidase
 NPPA – Natriuretic peptide A
 NPPB – Natriuretic peptide B
 NRF2 – Nuclear factor erythroid 2

 •O $_2^-$ – Superoxide anion
 ONOO $^-$ – Peroxynitrite
 ORA – Over-representation analysis
 oxLDL – Oxidized low density lipoprotein

 PAI-1 – Plasminogen activator inhibitor
 PCSK9 – Proprotein convertase subtilisin/kexin type 9
 PDGF – Platelets derived growth factor
 PECAM-1 – Platelet endothelial cell adhesion molecule-1
 PFKFB3 – PFK-2/FBPase isozyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
 PFKP – Phosphofructokinase, platelet
 PGI2 – Prostacyclin
 Phox – Phagocyte oxidase
 PI3K – Phosphoinositide 3-kinase
 piRNA – piwi-interacting RNA
 PKC – Phosphokinase C
 PLA2 – Phospholipase A2

PLC – Phospholipase C
PLD – Phospholipase D

R – Arginine
RAAS– Renin angiotensin aldosterone system
RISC – RNA-induced silencing complex
RNA-seq – RNA sequencing
ROADMAP – Randomized Olmesartan And Diabetes MicroAlbuminuria Prevention trial
ROS – Reactive oxygen species
RT-qPCR – Real time polymerase chain reaction

S – Serine
SET – Set Domain Containing methyltransferase family
SHEP – Systolic Hypertension in the Elderly Program trial
SM22 α – Smooth muscle 22-alpha
SMARCA5 – SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 5
SRA – Scavenger receptor A
STAT – Signal transducer and activator of transcription proteins
STBD1 – Starch Binding Domain 1
SUV39H 1/2 – Suppressor Of Variegation 3-9 Homolog 1 or 2
SYNPO2 – Synaptopodin 2 (myopodin)

T – Threonine
TAGLN – Transgelin (SM22 α gene)
TDG – Thymine DNA glycosylase
TET – Teneleven translocation enzyme
TF – Tissue factor
TGF- β – Transforming growth factor β
TH/TL – Total heart/tibial length ratio
TIMPS – Tissue inhibitor of metalloproteinase
TLR – Toll-like receptor
TNF – Tumor necrosis factor
TRP53INP1 – Tumor Protein P53 Inducible Nuclear Protein 1

UKPDS – United Kingdom Prospective Diabetes Study

VADT – Veterans Affairs Diabetes Trial
VCAM-1 – Vascular cell adhesion molecule 1
VLDL – Very low density lipoprotein
VSMC – Vascular smooth muscle cells
vWF – vonWillebrand factor

WOSCOPS – West of Scotland Coronary Prevention Stud

Xk – X Linked KX blood group

5mC – 5-methylcytosine
5hmc – 5-hydroxymethyl-cytosine

1. Introduction-background

A. Cardiovascular diseases and cardiovascular risk factors

1. Cardiovascular diseases

Cardiovascular diseases (CVD) remain the leading cause of death worldwide, with 17.9 million lives lost each year, or approximatively 30% of all deaths¹. It has also a major impact in low- and middle-income countries, in which more than 75% of CVD deaths occur¹. Cardiovascular diseases affect both heart or arteries. Arterial cardiovascular diseases share specific features, i.e. cardiovascular risk factors as inducers, and atherosclerosis as pathophysiological expression. Decades might take place between atherosclerosis initial steps and consecutive clinical manifestations.

2. CV risk factors

It was only during the second part of the 20th century that research into the causes of CVD accelerated. The premature death in 1945 of the US President Franklin D. Roosevelt from hypertensive heart disease and stroke particularly stimulated this research in the USA^{2,3}. The Framingham heart study (FHS) was a national project set up in reaction to his death, in order to identify

factors that contribute to CVD. The little town of Framingham (Massachusetts, USA) was chosen due to its geographical proximity to Harvard Medical School ³. Researchers followed there the development of coronary heart disease (CHD) over a long period in a large group of participants who had not yet developed symptoms of CVD. Since 1948, three generations of Framingham citizens were successively included in this trial ³. FHS was responsible of the identification of major risk factors of CVD. Other studies have shown that the risk factors identified in this group apply universally ³. Moreover, the notion of risk factors led to the development of preventive strategies in clinical practice, on top of effective treatment of established cardiovascular disease. Framingham risk scores are now widely used in North America to assess cardiovascular diseases risk ².

There are many risk factors for CVD, and among them only some can be controlled (**Figure 1**). The risk factors that are modifiable include high blood pressure; high blood cholesterol levels; smoking; diabetes; overweight or obesity; lack of physical activity; pollution (microparticles); unhealthy diet and stress. Those that cannot be controlled are age (increase in age increases cardiovascular risk); sex (men are at greater risk than premenopausal women); family history/genetics; and ethnicity ³.

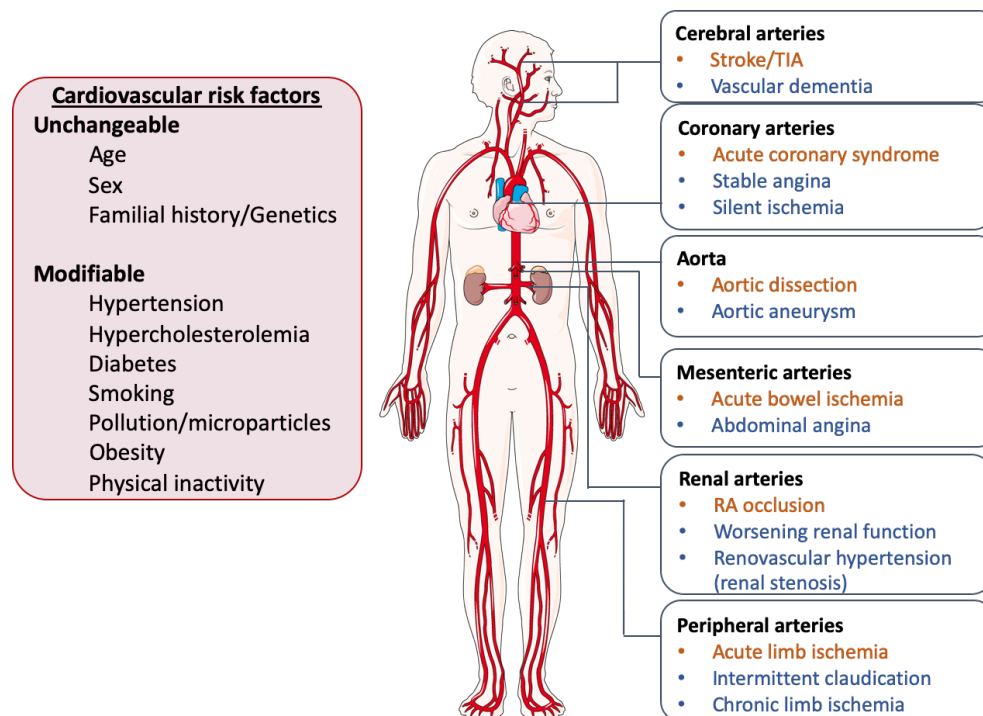


Figure 1. Cardiovascular diseases and cardiovascular risk factors. Right: Acute (**orange**) and chronic (**blue**) clinical presentation of arterial cardiovascular diseases, by arterial anatomical beds. Left: Known cardiovascular risk factors at the basis of cardiovascular diseases.

a. Hypertension

Hypertension is defined by European Society of Cardiology guidelines by systolic blood pressure above 139 mmHg and/or diastolic blood pressure above 89 mmHg. These cut-off values are based on evidence from clinical trials showing that treatment of patients with these BP values significantly lowers their risk ⁴. However, the relationship between blood pressure (BP) and CV events is continuous, with a risk of death from either coronary artery disease (CAD) or stroke increasing linearly from BP levels as low as 90 mmHg systolic and 75 mmHg diastolic upwards ⁵.

FHS study was the first to identify hypertension as risk factor for CVD. Physicians thought previously that high blood pressure was necessary to push blood through stiffened arteries in elderly persons and that it was an “adaptative” effect of aging. Essential hypertension was called benign and isolated systolic hypertension considered as harmless ³. FHS challenged this existing belief as investigators found an increased risk of CAD morbidity associated with rising baseline BP ⁶. It is now universally accepted that hypertension increases atherosclerotic CVD incidence. Hypertension is secondary in about 5 to 15% of cases. As such, secondary hypertension is due to an identifiable cause, which may be treatable with specific interventions ⁴, e.g in renovascular stenosis, endocrinopathy, obstructive sleep apnea, etc.

Some characteristics suggest patients in whom screening of causes should be considered, e.g. young age, resistance to treatment, or signs of hypertension mediated organ damage ⁴. In the other 85% of cases, hypertension is considered as primary.

b. Hypercholesterolemia

One other major risk factor of CVD is hypercholesterolemia, currently defined as total serum cholesterol level above 190 mg/dl or low-density lipoprotein (LDL-C) above 115 mg/dl.

Cholesterol is an essential component of cell membranes, a precursor of bile acids and steroid hormones. Due to its a hydrophobic nature, it is only minimally soluble in the blood. Cholesterol of both exogenous and endogenous origin is therefore transported in plasma in packaged lipoproteins with different densities and floatation characteristics. By order of increasing density, these include chylomicrons, containing exogenous triglycerides in post prandial periods; very-low-density lipoprotein (VLDL) containing endogenous triglycerides; intermediate-density lipoprotein (IDL) carrying cholesterol esters and triglycerides, low-density lipoprotein (LDL) containing cholesterol esters directed to cells; and high-density lipoprotein (HDL) containing phospholipids and cholesterol esters, directed

to liver. In most people, LDL particles constitute ~90% of circulating apoB-containing lipoproteins in fasting blood ⁷.

FHS study and others confirmed the association between cholesterol levels and CHD mortality, initially suggested by animal and clinical observation ³. Later on, it was discovered that one component of cholesterol-containing lipoproteins – LDL-C was directly associated with CVD ⁷, and current guidelines use LDL-C as target for cholesterol-modifying therapy ⁵. Actually, the relative reduction in CVD risk is proportional to the absolute size of the change in LDL-C, irrespective of the drug used to achieve such change ⁵. Interestingly, on the opposite, HDL-C is inversely associated with CVD risk (up to 70 mg/dl), but there is no evidence from interventional studies that raising plasma HDL-C reduces risk ⁵. Nevertheless, more recent mendelian randomization studies have highlighted a residual risk associated with elevated triglycerides-containing particles ⁸ and current trials are testing drug or molecular approaches to decrease triglycerides-rich fractions for their cardiovascular benefit ⁹.

c. Diabetes

Diabetes mellitus (DM) is a group of metabolic disorders characterized by chronic high blood sugar level. There are two main types of DM: type 1 diabetes, a consequence of autoimmune destruction of beta islet insulin

producing cells in pancreas, usually developing in childhood or adolescence; and type 2 diabetes, usually developing in adulthood, related to insulin resistance in obese, non-physically active or with unhealthy diet patients ¹⁰. In this situation, intermediates states of hyperglycemia (impaired glucose tolerance or prediabetes) have been defined, usually progressing to diabetes if no modification in weight or lifestyle is implemented.

The role of diabetes in the pathogenesis of CVD was unclear until the late seventies. In 1979, based once again on 20 year-surveillance of the Framingham cohort, a two to three-fold increased risk of clinical atherosclerotic disease associated with diabetes was reported ^{3, 11}.

Type 1 DM, type 2 DM, and prediabetes are now widely considered as independent risk factors for CVD, increasing risk by about two-fold, depending on the population and therapeutic control. Women with type 2 DM appear to have a particularly higher risk for stroke. Moreover, patients with type 2 DM are likely to have multiple other CV risk factors (including dyslipidemia and hypertension), each of which mediates an increase in risk ⁵.

d. Smoking

The Framingham study again played a major role in the identification of cigarette smoking as supplemental risk factor: in this cohort, smokers had an increased risk of myocardial infarction or sudden death dependent of the

number of cigarettes smoked each day ¹², and this was confirmed by other epidemiological studies ³. A lifetime smoker has a 50% probability of dying due to smoking, and on average will lose 10 years of life. The CVD risk in smokers <50 years of age is five-fold higher than in non-smokers. Prolonged smoking is more hazardous for women than for men. Second-hand smoke is also associated with an increase in CVD risk ⁵.

e. Obesity-adiposity

Over recent decades, body mass index (BMI)— weight (in kg) reported to squared valued of height (in m²)—has increased worldwide in adults and in children to epidemic proportions, with over 4 million people dying each year as a result of being overweight or obese in 2017 according to the Global Burden of Disease ¹⁰. Its association with CVD was first noted in FHS more than a half century ago ³. It is a metabolic condition also associated with other comorbidities such as type 2 DM, hypertension and sleep apnea syndrome ³. It has been also reported that high BMI during childhood is associated with an increased risk of CHD in adulthood ¹³. A meta-analysis of 58 prospective studies assessed that BMI (and waist circumference) is strongly and continuously associated with CVD ⁵.

f. Physical inactivity

The association of physical activity and CV risk was pointed for the first time in 1953, based on the observation of a cohort of London red bus drivers ¹⁴. Since then, this relationship was confirmed by other epidemiological studies. The relative risk of death from CHD for sedentary compared to active individual is 1,9 ¹⁵. Insufficient physical activity can be defined as less than 5 times 30 minutes of moderate activity per week, or less than 3 times 20 minutes of vigorous activity per week, or equivalent ¹⁰.

g. Non-modifiable CV risk factors

A tripling of the risk of CV or heart disease is associated with each decade of life ¹⁶. Age is associated with increase in cholesterol level ¹⁷, but also to reduced arterial compliance that might lead to artery and cardiac disease. Men are at greater risk than pre-menopausal women ¹⁶. Genetics plays also an important role in atherosclerosis development. Indeed a positive family history of early coronary heart disease has been recognized to be a risk factor for a long time ³. In familial hypercholesterolemia, monoallelic gene mutation in low-density lipoprotein cholesterol receptor (LDLR), apolipoprotein B (APOB) or proprotein convertase subtilisin/kexin type 9 (PCSK9) are causing significant reduction in liver LDL clearance, leading to severe hypercholesterolemia since childhood, and subsequent early atherosclerosis

development¹⁸. Another example of genetic risk factor is linked to nitric oxide signaling¹⁸. Within arteries, after stimulation with nitric oxide, soluble guanylyl cyclase generates cGMP, which induces vascular smooth muscle cell relaxation, vasodilation and inhibits platelet activation. Mutations leading to a loss of function in the $\alpha 1$ subunit of soluble guanylyl cyclase ($\alpha 1$ -sGC), the GUCY1A1 locus, was detected through next-generation sequencing in a family with several CAD affected individuals¹⁹. More recently, genome-wide association studies (GWAS) led to the identification of more than 200 loci or variants associated with CAD, associated to pathophysiological pathways such as plaque initiation or progression, platelet function; or unknown mechanisms¹⁸. Actually, the majority of heritable component of cardiovascular risk consist in combined effects of many genes (single nucleotide polymorphisms or mutations), each contributing in a small fraction to overall individual susceptibility²⁰. Based on high-throughput genotyping analysis, polygenic genetic risk scores have therefore been developed, with the potential to help to predict risk of CAD or early development of hypercholesterolemia^{20, 21} (*see box below*).

The concept of polygenic risk score (PRS). *With the help of artificial intelligence, a set of training data extracted from a large cohort of individuals, comprising both input parameters (genetic, epigenetic profiles, and/or individual environmental exposure) and output results (development of disease “X”, or absence of response to treatment “Y”), is subjected to an algorithm capable of autonomously learning the rules which translate the combinations of input into output result. Once the rules are established, the same algorithm can predict for a determined individual scores that quantify the relative risk of developing the disease “X” or the absence of response to treatment “Y” in question.*

The above-cited cardiovascular risk factors will cumulate during lifetime and determine the individual CVD risk. Guidelines were therefore developed and regularly updated to calculate this risk, in order to help physician in detection, management and prevention of CVD ⁵. As a consequence of aging and additional CV risk factors, atherosclerosis, the pathology underlying cardiovascular disease, develops silently over many years, and is usually advanced by the time symptoms of CVD occur.

B. Atherosclerosis: Vascular remodeling in response to pathological stimulus

1. Healthy artery

The arterial wall is made up of different cell types: endothelial cells (ECs), vascular smooth muscle cells (VSMCs), fibroblasts, pericytes; associated with extracellular matrix (ECM) components: collagen and elastin. The vessel wall is divided in three layers (**Figure 2**). The inner layer is the barrier between blood lumen and vessel wall, made of endothelial cells. Surrounding ECs is the tunica media, consisting of circumferentially orientated VSMCs, stacked between two interconnected elastic lamellae. VSMCs are responsible of vessel constriction and dilatation, controlling vessel tone. The outer layer contains fibroblasts and progenitor cells (e.g mesenchymal stem cell-like) and ECM. This layer is in charge of maintaining structural integrity under mechanical load. Interplay of all these components is needed to preserve homeostasis in the cardiovascular system, the disequilibrium of which contributes to the pathogenesis of atherosclerosis ^{22, 23}.

Atherosclerosis is characterized by the accumulation of fatty and/or fibrous material in the in the arterial wall, leading progressively to plaque formation ²⁴. The term atherosclerosis derives from the Greek word for ‘gruel’ or

‘porridge’, reflecting the appearance of a typical atherosclerotic plaque (called atheroma). With time, the plaque can become more inflamed, fibrous and accumulate calcium. Advanced atherosclerotic plaques can protrude into the arterial lumen, impeding blood flow and leading to chronic tissue ischemia. A plaque can also be disrupted and, by exposing the bare subintimal layers to circulating platelets, initiate the formation of a thrombus that can brutally occlude the lumen leading to acute ischemia ²⁵.

Throughout plaque development, reactive changes in the affected vessel wall known as vascular remodeling occur, in which the flow-limiting potential of the atherosclerotic lesion may be accentuated or attenuated. Arterial remodeling is orchestrated by various cell types, including endothelial cells, macrophages, and VSMCs ²⁴.

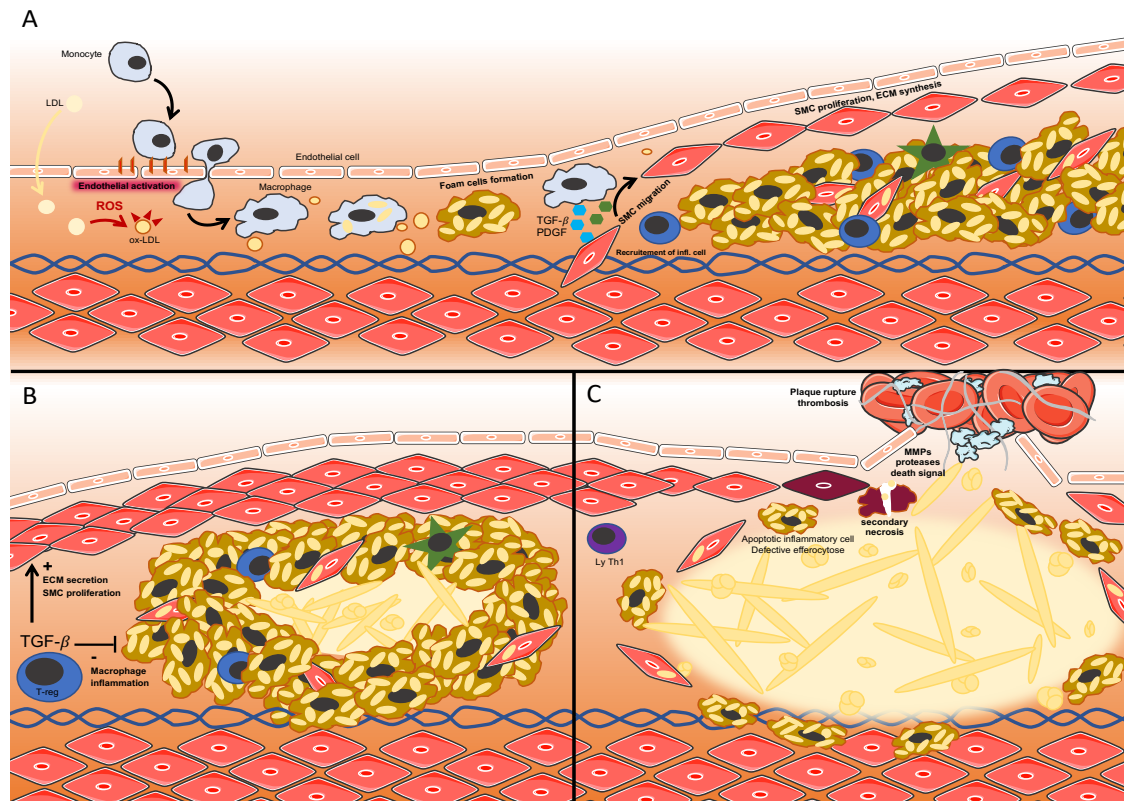


Figure 2. A. Initial steps: LDL retention and oxidization in the subendothelial space promotes in turn activation of endothelial cells. Endothelial cells increase expression of adhesion molecules (selectins, VCAM-1) and chemoattractants (MCP-1) leading to attachment and transmigration of monocytes into the intimal space. The monocytes differentiate into macrophages and express receptors mediating the internalization of oxLDL, to become foam cells. In addition, inflammatory signaling pathways are activated in foam cells promoting more inflammatory cell recruitment (dendritic cells, mast cells, regulatory T (T-reg) cells, and T helper 1 (Th-1) cells) and LDL modification. Foam cells and inflammatory cells produce chemoattractant and proliferation factors, including transforming growth factor- β (TGF- β), platelet-derived growth factor (PDGF) isoforms, matrix metalloproteinases, fibroblast growth factors (FGF), and

heparin-binding epidermal growth factor (HB-EGF), that recruit smooth muscle cells to the luminal side. They migrate, proliferate and generate an extracellular matrix network to form a barrier between intraplaque prothrombotic factors and blood, or express macrophage receptors to become foam cells (so called phenotypic switch). **B. Vascular remodeling:** intima volume increase. The thick fibrous cap of the plaque provides an effective barrier preventing exposure of lesion prothrombotic factors to blood, thereby limiting thrombus formation. Maintenance of a thick fibrous cap is enabled by regulation of the inflammatory status of the foam cell core of the lesion. Regulatory T (T-reg) cells produce transforming growth factor- β (TGF- β) and IL-

10, and in addition, inhibit antigen-specific activation of T helper 1 (Th-1) cell to produce interferon gamma (IFN γ). This, in turn, reduce the proinflammatory macrophage phenotype leading to reduced cell death, effective efferocytosis (phagocytosis of dead cells), and anti-inflammatory cytokine production (i.e. TGF- β , IL-10). The production of TGF- β by T-reg cells and macrophages maintains fibrous cap quality by being also a potent stimulator of collagen production by smooth muscle cells. **C. Vulnerable plaque** results from a heightened inflammatory status of the foam cell core. Antigen-specific activation of T helper 1 (Th-1) cells produces interferon gamma (IFN γ) resulting in a proinflammatory macrophage phenotype, with enhanced inflammatory cytokine secretion and apoptosis susceptibility. They secrete less of the anti-inflammatory cytokines, TGF- β and IL-10. In addition, proinflammatory macrophages have impaired cholesterol efflux and efferocytosis, resulting in secondary necrosis, leading to an enlarged necrotic core composed of leaked oxidative and inflammatory components. Increased smooth muscle cell death, enhanced extracellular matrix degradation and decreased extracellular matrix production cause thinning of the fibrous cap. Areas of thin fibrous cap are prone to rupture, exposing prothrombotic components to platelets and pro-coagulation factors leading to thrombus formation.

2. Early steps: endothelial dysfunction and activation

The endothelial lining is a source of various factors that can influence the behavior of other types of cells within the vessel wall (pericytes and smooth muscle cells) and in the circulating blood (platelets and leukocytes). The endothelium responds to humoral, neural, and hemodynamic stimuli; modulates vascular tone by synthesizing and releasing a number of vasoactive factors that may have vasodilatory effects, such as nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor; and vasoconstrictor effects, such as thromboxane A₂ and endothelin-1 (ET-1) ²⁶. As a result of damage to endothelium, the production of these factors is disrupted, with a decrease in NO synthesis and bioavailability, and an increase in vasoconstrictive molecules. The ensuing imbalance leads to endothelial dysfunction (ED), which is considered as an early step towards atherosclerosis ^{23, 27}. Numerous endothelial “aggressors” causing of ED have now been described, such as oxidized lipids (oxLDL) in hypercholesterolemia; Angiotensin II (Ang II) and reactive oxygen species (ROS) in hypertension, advanced glycation end products (AGE) in diabetes, or interleukin-1 (IL-1) and tumor necrosis factor (TNF) as proinflammatory agents ^{23, 27}. The hemodynamic environment also affects endothelial functions. The endothelial cell (EC) surface is equipped with

mechanoreceptors detecting and responding to endothelial shear stress (ESS) stimuli, such as integrins (e.g platelet endothelial cell adhesion molecule-1, PECAM1). When activated, these receptors trigger several pathways that, through GTPase and several serine kinases, converge to mitogen-activated kinase (MAPK) cascade. These pathways lead to activation of transcription factors, (e.g nuclear factor-kappa B (NF- κ B), nuclear factor erythroid 2 (Nrf-2) or Krüppel-like factor 2 (KLF2)), which bind positive or negative shear stress responsive elements at promoters of mechanosensitive genes that, *in fine*, modulate cellular function and morphology^{23,28}. In arterial regions with non-disturbed flow (laminar flow), where ESS varies within a physiologic range, the ECs express various atheroprotective genes and suppress several pro-atherogenic ones. In contrast, in regions with low and disturbed (oscillatory) flow where low ESS occurs, the atheroprotective genes are suppressed, whereas the pro-atherogenic genes are upregulated (through enhanced expression of NF κ B) thereby promoting the atherosclerotic process. Indeed, atherosclerotic plaques tend to form at sites of flow disturbance, whereas sites in the arterial tree where laminar shear stress predominates generally resist atheroma formation²⁵.

On top of endothelial dysfunction, the same stimuli can lead to endothelial activation, a coordinated program of genes expression within the endothelial

cell, characterized by cell surface adhesion molecules expression (e.g. Vascular Cell Adhesion Molecule-1 VCAM-1, InterCellular Adhesion Molecule-1 ICAM, or selectins), secreted and membrane associated chemokines (eg, Monocyte Chemotractant Protein-1 MCP-1), and prothrombotic mediators (plasminogen activator inhibitor PAI-1, Tissue Factor TF, and vonWillebrand factor vWF). Proinflammatory molecules such as IL-1 or TNF, oxidized lipoproteins, Ang II, AGE, adipokines or disturbed blood flow can indeed initiate the endothelial cell activation response²³. This will promote the recruitment of monocytes and T-lymphocytes, which will facilitate their invasion to the sub-endothelial space (**Figure 2**).

3. Further steps: fatty streaks, inflammation and foam cells

Changes in endothelial permeability promote the entry and retention of inflammatory cells and cholesterol-containing LDL (and other triglycerides-rich non-HDL) particles in the artery wall. Protected from plasma antioxidants, LDL undergo modifications such as metal ion catalysis (through the Fenton reaction) leading to their oxidation. This kind of modification renders them pro-inflammatory and immunogenic, and facilitates their uptake by inflammatory cells²⁵. On the other hand, once resident in the artery wall, monocytes — the most numerous white blood cells in plaques — differentiate into tissue macrophages. In the nascent atheroma, also known as fatty streaks,

these mononuclear phagocytes express scavenger receptors (such as CD36, scavenger receptor A— SRA, lectin-like receptors — LOX, and toll-like receptors — TLR4) that allow them to bind modified lipoprotein particles; upon overloading with the latter, phagocytes become foam cells, a term that reflects the microscopic appearance of these lipid-laden macrophages ²⁹. In addition, inflammatory signaling pathways are activated in macrophage foam cells leading to vicious circle of more inflammatory cell recruitment and LDL modification ³⁰. T lymphocytes, although numerically less abundant than monocytes in fatty streaks, also enter the intima, and regulate functions of the innate immune cells as well as the endothelial and smooth muscle cells function, e.g. endothelial activation and smooth muscle cells migration/proliferation ²⁵ (**Figure 2**). Experimental evidence in mice also suggests that certain common clonal mutations occurring in inflammatory cells precursors contribute to atherosclerosis. This “clonal hematopoiesis” refers to the expansion of an hematopoietic stem cell due to a mutation that gives rise to blood cell “clones”, abundant in the circulation. Clonal hematopoiesis is more frequent with aging. One explanation of its link with atherosclerosis, is that the clones, particularly monocytes and neutrophils, are more proinflammatory (e.g. teneleven translocation (TET) enzyme TET2

DNA demethylase clonal mutation which increases macrophage IL-6 expression); promoting lesion growth.²⁰

4. Vascular remodeling: progression of plaque

Fatty streaks do not result in clinical complications and can even undergo regression. However, once the lesions become more advanced, as a result of smooth muscle cells infiltration, regression is less likely to occur. Actually, small populations of VSMCs already present in the intima proliferate in response to growth factors produced by inflammatory macrophages. In addition, macrophage-derived chemo-attractants cause tunica media smooth muscle cells to migrate into the intima and proliferate also. Critical smooth muscle cell chemo-attractants and growth factors include platelet derived growth factor (PDGF) isoforms, matrix metalloproteinases (MMP), fibroblast growth factors (FGF), and heparin-binding epidermal growth factor (HB-EGF).³⁰

Migration and proliferation of VSMCs contribute to thickening of the arterial intima, a phenomenon called vascular remodeling. Unlike most cell types, VSMCs are not terminally differentiated and consequently display plasticity in their phenotypes ranging from contractile–quiescent to either migratory–proliferative–synthetic, osteogenic, or macrophage-like²⁴. This process of de-differentiation, induced by inflammatory cell cytokines or vascular injury,

is called “phenotypic switching”. It is considered as a key mechanism in arterial remodeling ³¹. One major characteristic of basal “normal” contractile phenotype in VSMC is a low proliferation rate, and the expression of the contractile protein machinery such as smooth muscle 22-alpha (SM22 α) also known as Transgelin (TAGLN), alpha smooth muscle actin (α SMA) and smooth muscle myosin heavy chain 11 (MYH11). On the opposite, synthetic VSMC will down regulate the expression of the contractile machinery proteins and increase the production of elastolytic enzymes (MMPs) which facilitate migration by detaching cells from the basement membrane and ECM. These cells will then migrate from the media to the intima, where they excessively proliferate and synthesize ECM components and promote lipid deposition, consequently facilitating arterial wall remodeling. A subset of smooth muscle cells will express macrophage receptors and internalize lipoproteins to become foam cells ³⁰. Osteogenic VSMCs are characterized by down regulation of mineralization inhibitory proteins, upregulation of alkaline phosphatase and release of matrix vesicles ³¹. Phenotypically modulated VSMCs within lesions can comprise about 30% of the total cell count as confirmed by lineage tracing experiments ²⁴. Intimal invasion by synthetic VSMC results in neointima formation (**Figure 2**).

5. Final steps: stabilization or plaque rupture

As lesions progress, substantial extracellular lipids accumulate in the core, in part due to large cholesteryl-rich particles arising from dead macrophage foam cells, and also as VSMCs and macrophages in advanced lesions have reduced lysosomal function, leading to trapping of cholesterol within their lysosomes; altogether contributing to the overall sterol accumulation in the lesion ³⁰.

Atherosclerotic plaques can either stabilize or rupture, the latter leading to acute thrombosis and sudden occlusion. As an example, rupture of atherosclerotic plaques is the most common trigger of acute thrombosis of coronary arteries that causes myocardial infarction ²⁵. A so-called stable plaque contains a thick fibrous cap composed of layers of smooth muscle cells stacked in a substantial extracellular matrix network that constitute an efficient barrier preventing plaque rupture and exposure of prothrombotic factors to the circulating blood. Maintenance of a thick fibrous cap is enabled by regulation of the inflammatory status of the foam cell core of the lesion: regulatory T cells produce transforming growth factor- β (TGF- β) and IL-10 and inhibit antigen-specific activation of T helper 1 (Th-1) cell to produce interferon gamma (IFN γ). Increased TGF- β and IL-10 and decreased IFN γ reduce the proinflammatory macrophage phenotype leading to reduced cell

death, effective efferocytosis (phagocytosis of dead cells), and anti-inflammatory cytokine production (i.e. TGF- β , IL-10). Thus, stable plaques have small necrotic cores containing macrophage debris and extracellular lipid resulting from secondary necrosis of apoptotic macrophage foam cells. The production of TGF- β by regulatory T cells and macrophages maintains fibrous cap quality by being a potent stimulator of collagen production by smooth muscle cells ³⁰.

On the opposite, vulnerable plaques result from unresolved inflammatory status of the lesional foam cell core in which activated Th-1 cells produce IFN γ and regulatory T cells secrete less of the anti-inflammatory cytokines TGF- β and IL-10, resulting in a proinflammatory macrophage phenotype. Those macrophages are characterized by enhanced inflammatory cytokine secretion, apoptosis susceptibility and have impaired cholesterol efflux and efferocytosis, resulting in secondary necrosis leading to an enlarged necrotic core (mainly composed of leaked oxidative and inflammatory components). Due to an increased smooth muscle cell death, enhanced extracellular matrix degradation and decreased extracellular matrix production, this unresolved inflammation causes progressive thinning of the fibrous cap. Areas of thin fibrous cap are prone to rupture, (e.g. under hemodynamic stress, such as increased blood pressure) exposing prothrombotic components to platelets

and pro-coagulation factors, finally leading to thrombus formation and clinical events ³⁰ (**Figure 2**).

C. Angiotensin and vascular injury

As detailed above, vascular injury (characterized by endothelial dysfunction, structural remodeling, inflammation and fibrosis), plays a major role in cardiovascular diseases development. Among many factors responsible for vascular injury is Ang II.

The renin-angiotensin-aldosterone system (RAAS) is involved in numerous physiological functions : regulation of vasoconstriction, of extracellular volume, cardiac output, cell growth, and vascular wall integrity, among others. Production of angiotensin peptides is first initiated by the synthesis and processing of pre-prorenin in juxtaglomerular cells neighboring the renal glomerulus with subsequent proteolytic cleavage of the signal peptide, intracellular sorting of prorenin to dense-core secretory vesicles, and cleavage of the pro-segment, producing catalytically active renin that is secreted in the systemic circulation. Classically, renin cleaves liver-derived angiotensinogen (AGT) into angiotensin I (AngI), a decapeptide [Ang(1–10)] which is then further processed by angiotensin-converting enzyme (ACE) into the octapeptide angiotensin II [Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, Ang(1–8)]³². Ang II mediates its actions through two receptors, both members of the G protein-coupled receptor (GPCR) superfamily. Ang II type I receptor (AT₁R) is responsible of most of the pathophysiological effects of Ang II, such as

vasoconstriction, inflammation, and cell growth. Ang II type 2 receptor (AT₂R;) is considered as counteracting many of the AT₁R mediated actions and associated with vasodilation or apoptosis ³³. AngII was initially considered as the major peptide of the RAAS, but the role of AngII derived small peptides has been identified. Ang-(1-7) is formed from Ang II or Ang I by several peptidases, or by angiotensin-converting enzyme 2 (ACE2). Its binding to a third receptor, Mas, counteract Ang II effects by mediating anti-inflammatory response, growth inhibition and vasodilation, amongst others ³³. This peptide can also interact with AT₁R, blocking AngII actions. This ACE2-Ang-(1-7) axis is considered as a counter-regulatory system to the classical ACE-AngII-AT₁R axis. **Figure 3.**

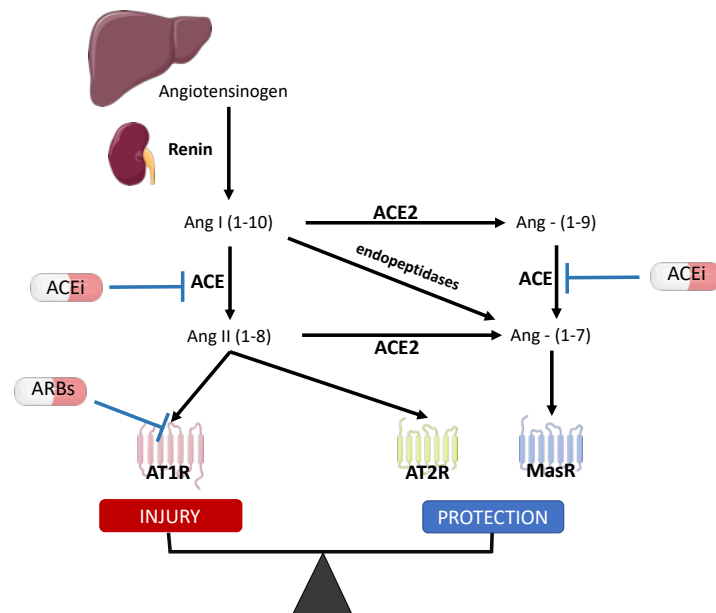


Figure 3. RAAS cascade, Angiotensin II and related peptides synthesis .

Ang II is of importance in physiological conditions: within seconds to minutes of binding to AT₁Rs, it activates signaling pathways leading to VSMC contraction, maintaining vascular tone, in order to maintain blood pressure and to perfuse vital organs. In addition to stimulating the synthesis and release of aldosterone and increasing renal Na⁺ absorption, Ang II's actions on the central nervous system are critical to maintain sympathetic outflow to the vasculature and to autoregulate cerebral blood flow. When the balance of the RAAS is perturbed (due to genetic, environmental, and lifestyle factors, usually under longer exposure), pathological effects of Ang II develop: these promote cell growth and migration, extracellular matrix deposition, and vascular remodeling ³⁴.

In addition to the systemic circulating endocrine RAAS, the importance of local generation and action of the RAAS has also been documented. Such local system, characterized by the presence of all RAAS components (AGT, renin, ACE, Ang I, Ang II and Ang II receptors), is present in heart tissue, vessels, kidney, adrenal gland, pancreas, central nervous system, reproductive system, lymphatic, eye and adipose tissue. Increased activation of tissue RAAS was demonstrated in atherosclerosis, myocardial infarction, cardiac failure and diabetes, independently of BP increase ³³.

1. Ang II major intracellular signaling pathways

As previously written, most deleterious effects of Ang II is driven by AT₁R pathways. Ang II binding to AT₁R results in coupling of G proteins (Gq/G11 and/or GiGo) to the C-terminus of the receptor and to stimulation of many signaling pathways, including phospholipase C (PLC), Ca²⁺ channels, phospholipase D (PLD), phospholipase A2 (PLA2), MAPK, Janus kinases (JAK) and signal transducer and activator of transcription proteins (STAT) pathways, and NADPH oxidase.

One major growth signaling pathways activated by Ang II is MAPK pathway. In VSMCs, Ang II activates extra-cellular signal regulated kinases (ERK1/2) which are key growth signaling kinases; p38MAP kinase and c-Jun N-Terminal kinases (JNK) which influence cell survival, apoptosis, differentiation and inflammation; and ERK5, involved in protein synthesis, cell cycle progression and cell growth³³. Ang II induces also phosphorylation of tyrosine kinases, such as c-Src and JAK kinases family. RhoA, a member of the Rho family of small GTPase binding proteins, is abundantly expressed in vascular smooth muscle cells and participates in vasoconstriction via phosphorylation of myosin light chain (MLC) and sensitization of contractile proteins to Ca²⁺. Activation of RhoA and its downstream target Rho-kinase

play an important role in Ang II-mediated vascular contraction and growth³³
(**Figure 4**).

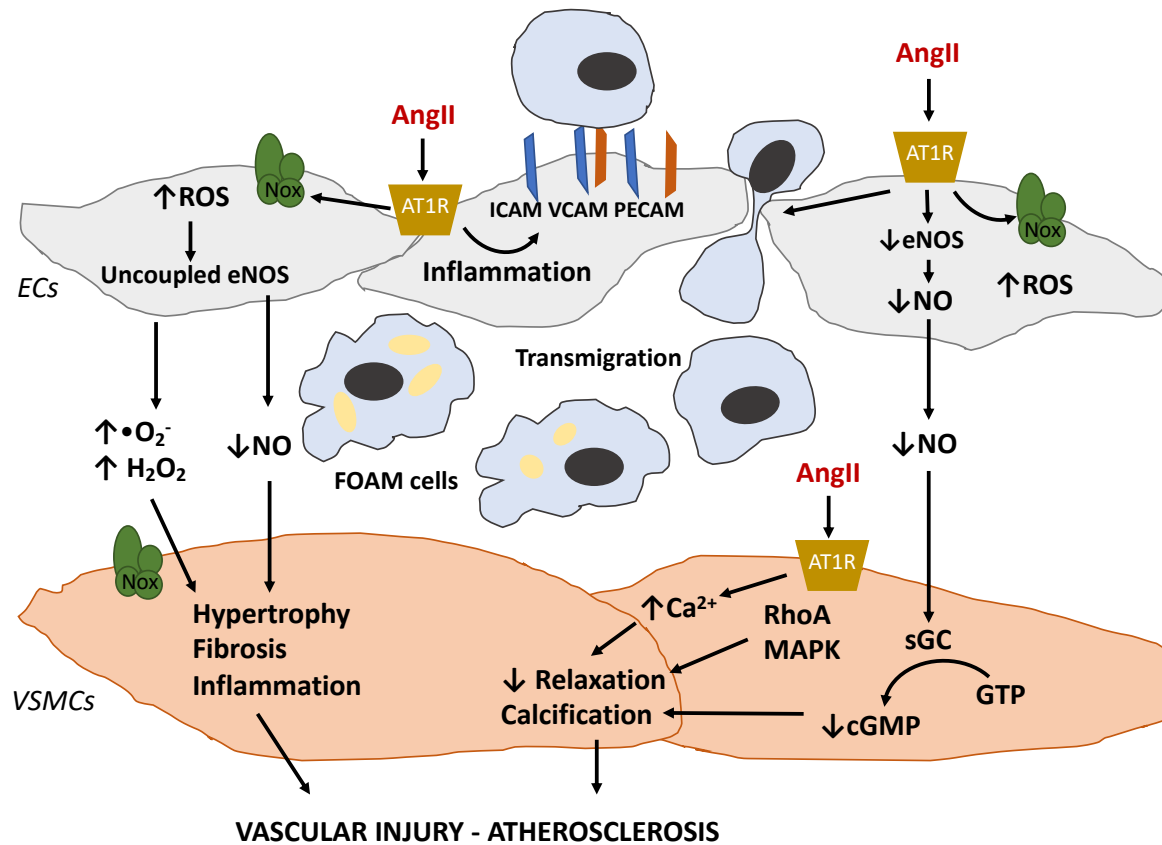


Figure 4. AngII AT₁R signaling pathways in vascular wall. (Inspired by Montezano³³). Binding of Ang II to its cell membrane receptor induces impaired NO synthesis by reducing eNOS activity and promoting NOS uncoupling. Ang II also activates NADPH oxidase (Nox), leading to increased generation of reactive oxygen species (ROS), that are proinflammatory, mitogenic, prothrombotic and pro-fibrotic. These events also contribute to atherogenesis, through macrophage infiltration and foam cell formation. Ang II activates signaling pathways through calcium mobilization, MAP kinase activation, TGFb synthesis and by inhibiting cyclic GMP (cGMP), leading to altered vascular

reactivity, growth, calcification and fibrosis. Together, these processes contribute to vascular injury that underlies cardiovascular diseases

2. Ang II and oxidative stress

Ang II mediates a number of its cellular effect by stimulating the production of reactive oxygen species (ROS), which are highly reactive and short-live molecules derived from reduction of molecular oxygen. In the cardiovascular system, of the many types of ROS generated, superoxide anion ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite (ONOO^-) are the main effectors. ROS are produced in endothelial, adventitial or VSMCs; generated mainly from NADPH oxidase and nitric oxide synthases. The former is a multi-subunit enzyme catalyzing the production of $\bullet\text{O}_2^-$ by the electron reduction of oxygen using NADPH as the electron donor. The prototypical NADPH oxidase (Nox) comprises five components: p47phox, p67phox, p40phox, p22phox and gp91phox (phox for PHagocyte OXidase) and the small G proteins Rac 1/2. The Nox family comprises seven isoforms, Nox1 to 5, Duox1 and Duox2. In arteries, Nox1, Nox4, and Nox5 are expressed by vascular smooth muscular cells, while Nox1, Nox2, Nox4 and Nox5 are present in the endothelium. Macrophages recruited to the subendothelial cells also express Nox2³⁵. Upon stimulation, catalytic subunits and other subunits of vascular oxidases (Nox1, Nox2, Nox5) will assemble and generate $\bullet\text{O}_2^-$ in a burst-like manner extracellularly³⁶. Nox4 is

distributed in the endoplasmic reticulum and is considered to be activated constantly, producing hydrogen peroxide in a predominant fashion ³⁷.

Ang II is a potent stimulator of vascular NADPH oxidase, as it can regulate all vascular Nox isoforms (Nox1, Nox2, Nox4 and Nox5) ³⁶. It induces activation of the enzyme, increases expression of NADPH oxidase subunits and stimulates ROS production in cultured VSMC and intact arteries. Mechanisms linking Ang II and upstream signaling molecules modulating vascular Nox activity include PLD, phosphokinase C (PKC), c-Src, phosphoinositide 3-kinase (PI3K) and Rac family. In turn superoxide anion and H₂O₂ activate multiple signaling molecules, including MAP kinases (p38MAP kinase, JNK, ERK-5 and ERK1/2), non-receptor tyrosine kinases (c-Src, JAK2, STAT, p21Ras, Pyk2 and Akt), receptor tyrosine kinases (EGFR, IGFR and PDGFR), protein tyrosine phosphatases, calcium channels and redox-sensitive transcription factors (NF- κ B, activator protein 1 (AP-1) and hypoxia-inducible factor 1 (HIF-1)). Moreover, endothelial oxidative stress generated by NADPH oxidase stimulation, lead to eNOS uncoupling, a change in eNOS activity from NO production to superoxide anion production. Activation of these molecules participates in cell growth, migration, expression of pro-inflammatory genes, production of extracellular matrix proteins and contraction ³³.

3. Ang II and vascular remodeling

Ang II, among others, is a recognized stimulus of endothelial dysfunction and vascular smooth muscle phenotypic switching³¹. Through AT₁R, it promotes, in VSMCs, a change from a contractile to a proliferative and synthetic phenotype, leading to modifications (e.g decreased expression) in the contractile machinery, vascular hypertrophy/proliferation and secretion of mitogenic, inflammatory and pro-fibrotic mediators. In this Ang II-mediated processes, changes in intracellular calcium and magnesium, MAP and Rho kinase signaling and ROS generation are all of importance.

Vascular fibrosis involves accumulation of extracellular matrix proteins, such as collagen, fibronectin and proteoglycans in the tunica media and is increased in vascular remodeling. Ang II is a known activator of TGF- β 1, which will increase the synthesis of ECM proteins, reduce collagenase production and stimulates expression of tissue inhibitor of metalloproteinases (TIMPS), resulting in excessive matrix accumulation. TGF- β signaling predominantly occurs through the cytoplasmic proteins Smads, which translocate to the nucleus and act as transcription factors.³⁸ Various genetic syndromes leading to aortic aneurysm susceptibility, such as Marfan disease, Loeys-Dietz syndrome or cutis laxa, are characterized by increased TGF- β signaling, this could be secondary to elevated RAS signaling. Indeed,

abnormal upregulation of ACE and subsequent elevated local Ang II-AT₁R signaling has been demonstrated in mice harboring a VSMC-specific knock-out of *Fbln4*, a mice model of cutis laxa, leading to ascending aortic aneurysms ³⁹.

Cytokines, chemokines and other pro-inflammatory mediators are also produced by circulating leukocytes, lymphocytes and by resident macrophages in the vessel wall in response to Ang II stimulation. Inflammation may also activate the RAAS, and thereby further contribute to vascular remodeling ³³.

Vascular calcification is a highly regulated process influenced also by Ang II, which induces upregulation of osteogenic proteins and signaling pathways. In particular, Ang II stimulates expression of bone morphogenetic protein 2 (BMP2) and of the osteoblast transcription factor Runx2/Cbfa, and it stimulates transcellular transport of cations such as Ca²⁺ and Mg²⁺. Ang II also inhibits matrix Gla protein (MGP), a physiological inhibitor of ectopic tissue calcification secreted by vascular smooth muscle cells ³³.

Expression of AT₁R is increased in atherosclerosis and experimental data have shown that inhibition of AT₁R has protective effects on the development of atherosclerosis and metabolic disorders. On the opposite, development of atherosclerosis is reduced in mice that have overexpression of AT₂R. Ang II

stimulates expression of adhesion molecules, chemokines and cytokines, and promotes endothelial dysfunction, oxidation and uptake of LDL and proliferation of VSMCs, many of these effects mediated through Nox-derived ROS. In advanced atherosclerosis, Ang II stimulates expression of MMPs and PAI-1, with resultant destabilization of atherosclerotic plaques ³³.

Moreover, Ang II provides a link between atherosclerotic risk factors such as hypercholesterolemia and hypertension, since high cholesterol levels have been shown to increase AGT and Ang. In apolipoprotein E-deficient mice, inhibition of AT₁Rs by AT₁R blocker losartan prevents lipid peroxidation, decreasing atherosclerotic lesion formation. On the opposite, in the same ApoE^{-/-} mice, Ang II infusion increases aortic atherosclerosis and aneurysm formation, independently of blood pressure increase. Male apoE/AT₁AR double knockout mice also have reduced atherosclerosis, indicating that alterations in AT₁R expression affect vascular pathology ³⁴.

D. Vascular memory: more than a theory

This part of the thesis was published as a narrative review entitled: “Legacy in cardiovascular risk factors control: from theory to future therapeutic strategies?” L. Pothen, JL Balligand. Antioxidants (Basel) 2021 Nov 22;10(11):1849.

As extensively explained above, atherosclerosis results from vascular wall exposure to pathological stimuli, such as oxLDL, hyperglycemia, or Ang II. Such remodeling participates to the pathophysiology of cardiovascular diseases. Optimized control of modifiable cardiovascular risk factors is therefore essential to reduce the incidence of atherosclerosis and CVD.

It was thought for a long time that atherosclerosis was evolving continuously and in proportion to risk factor exposure and that the efficient control of such risk factors was sufficient to stop the progression of the disease. Data from clinical trials have more recently demonstrated less efficacy in reducing vascular complications when such control of cardiovascular risk factors is delayed in time, or, on the opposite, a sustained advantage on CVD reduction after early initiation of intensive CV risk factors control. These observations led to a new concept in cardiovascular research: the “legacy effect”⁴⁰.

1. Definition of legacy

“Legacy” in English refers to what one generation passes to the next, or something that remains from an earlier time. In medicine, a “legacy effect” is defined as the sustained beneficial effect of a given treatment on disease outcomes or complications, even after cessation of the intervention.

In cardiovascular research, where “legacy effect” were mostly examined, the term was initially used in the context of diabetes when long-term follow-up results of two diabetes management trials—the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS)— were revealed ^{41, 42}. Both demonstrated the efficiency of intensive control of glycemia on cardiovascular outcome in type 1 and type 2 diabetic patients, but also that this favorable effect was maintained in passive long-term follow-up, despite loss of glycemic control. In parallel, similar lasting effect have been observed in lipid-lowering and blood pressure control trials ⁴³.

2. *Legacy effect in clinical trials*

a. *“Legacy effect” in diabetic patients*

As mentioned above, the concept of “legacy effect” emerged at the beginning of 21st century from two clinical studies conducted in diabetic patients (**Table 1**). The DCCT study was evaluating the effect of intensive control of glycemia compared to conventional therapy in a cohort of 1441 type 1 diabetic patients. After over 6 years of intervention, the development of microvascular complications was significantly diminished in the intensive treatment group, i.e. severe retinopathy (by 53%), clinical neuropathy (by 40%), and microalbuminuria (by 61%) ⁴¹. After the trial, patients were followed for 17 years in the Epidemiology of Diabetes Interventions and Complications (EDIC) study. Despite interruption of the intensive control on glycemia (7,9% of Hb1Ac in previously intensive treatment group; 7,8% in conventional group), the authors observed a persistent reduction in the incidence of cardiovascular events (nonfatal myocardial infarction (MI), stroke, or death from CVD; by 57%) and nephropathy (by 46%) in the former intensive treatment group ^{44, 45}.

Concordant results were observed later in UKPDS, a study conducted in 3867 newly diagnosed type 2 diabetic patients. In the intervention period, after a

three-month diet, patients were randomized either to intensive glucose-lowering treatment (sulfonylurea, insulin, metformin) or to conventional dietary management, and followed-up for ten years. Intensive glucose lowering was associated with a significant decrease in any diabetes-related endpoint (by 12%), any diabetes-related death (by 10%) and in microvascular endpoints (by 25%). At this point, there was no significant reduction in macrovascular endpoints ⁴². Another 10 years of follow-up of this cohort showed persistent benefit despite no difference in glycosylated hemoglobin (HbA1c) already after one year. In the former intensively-treated group, the relative risk reductions persisted for any diabetes-related endpoint (by 9%), for microvascular disease (by 24%), and were extended also for MI (by 15%) and for death from any cause (by 13%) ⁴⁶.

Subsequently, several other studies failed to reproduce the same “legacy effect”. The Veterans Affairs Diabetes Trial (VADT) performed in long-standing type 2 diabetic patients ⁴⁷⁻⁴⁹; the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial, with more than 10 thousands long lasting type 2 diabetic patients ^{50, 51}; and the Action in Diabetes and Vascular Disease Preterax and Diamicron (ADVANCE) study in 11 140 type 2 diabetic patients ^{52, 53}. Long-term follow-up did not reveal any long-term benefit of the

intervention period in all these cohorts. A common feature of these randomized trials is the inclusion of long-lasting diabetic patients (around 10 years of disease) and, in some studies, a high proportion of patients who had already presented one cardiovascular event at randomization (e.g. VADT).

On the other hand, additional real-life studies reinforced the “legacy effect” theory in diabetes control, such as The Fremantle Diabetes Study ⁵⁴, or the Anglo-Danish-Dutch study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care (ADDITION) ^{55, 56} and the Diabetes and Aging Study (DAS) ⁵⁷.

Taken together, the above-mentioned data suggest that to obtain a legacy effect, interventions on glycemic control need to be implemented early in the disease, certainly before any cardiovascular event, perhaps even within the first year. The accrued evidence then led to a new concept in diabetes clinical care: “The sooner, the better”.

Study Name Type	Patients characteristics (n int/n follow-up)	Effect of intervention? (study duration)	Legacy effect? (follow up duration)	Comments
DCCT ⁴¹ <i>RCT</i> EDIC ^{44, 45} <i>OFU</i>	T1DM Mean age 27 y (1441/1394)	(6,5y) (-) 53 % severe retinopathy (-) 40 % clinical neuropathy (-) 61 % microalbuminuria	(17y) Legacy effect of intervention (-) 57% nonfatal MI, stroke, or death from CVD (-) 46% nephropathy	In OFU Hb1Ac 7,9% intensive vs 7,8% conventional
UKPDS <i>RCT</i> ⁴² <i>OFU</i> ⁴⁶	Newly diagnosed T2DM Mean age 56,4 y (3867/3277)	(10y) (-) 12% diabetes-related endpoints (-) 10% diabetes-related death (-) 25% microvascular endpoints	(10y) Legacy effect of intervention (-) 9% diabetes related endpoints (-) 24% microvascular endpoints (-) 15% MI (-) 13% death from any cause	No diff in Hb1Ac in OFU
VADT ⁴⁷⁻⁴⁹ <i>RCT</i>	T2DM long duration (11,5y) poorly controlled Mean age 60,4y (1791/1391)	(5,6y) No difference in major CV outcomes, death or microvascular complications except for progression of albuminuria (13,1% vs 9,1% in int. treat. group)	(10y) Legacy effect of intervention (-) 17% major CV events (15y) No difference in major CV events or death	40% already had a CV event at inclusion HbA1c curves still separated at 10y f.u., no more at 15y
ACCORD ⁵⁰	T2DM long duration (10y) Mean age 62,2y (10251/8601)	(3,5y) No effect on primary composite endpoint (non-fatal MI, non-fatal stroke or death) (-) 24% MI in int. treat. group (+) 22% death from any cause and (+)36% death from CV causes in int. treat group	(9y) No effect on primary composite endpoint Trend to lower non-fatal MI (+) 20% death from CV causes in int. group	35% had had a previous CV event
ADVANCE ⁵² <i>RCT</i>	T2DM long duration (8y) ≥55y	(5y) (-) 10% combined major micro and macrovascular events	(6y) No differences in risk of death or major CV events	

ADVANCE- ON ⁵³ <i>OFU</i>	≥ 1 CV risk factor or history of major macro or microvascular disease (11140/8494)	(-) 21% nephropathy no effect on major macrovascular events, CV death or death from any cause	(-) 46% end stage renal disease but very few events	
ADDITION ^{55, 56} <i>Registered based nonRCT</i>	Newly diagnosed T2DM after screening compared to unscreened population, mean age 59,9y (registered 153107, diagnosed 1533)	(5y screening period) No comparison in intervention Diabetes detected 2,2y earlier in screened group	(10y) In screened group: lower mortality (HR 0,79) lower CV mortality (HR 0,80) lower diabetes-related mortality (HR 0,66) -lower CVD event (HR 0,84)	No effect at a national population level
DAS ⁵⁷ <i>Cohort study</i>	Newly diagnosed T2DM (34737) mean age 56,8y Stratified by mean Hb1Ac during first year, comparison to Hb1Ac <6,5%	NA	(10y) -Hb1Ac ≥ 6,5% within 1 st year: increased micro and macro CV events (HR 1,2) -Hb1Ac ≥ 7,0% within 1 st year: increased mortality (HR 1,29) - ≥ 8,0% for > 2y increased microvasc event and mortality risk	

Table 1: Randomized control trials evaluating legacy effect in diabetes. T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus ; CV : cardiovascular ; MI : myocardial infarction ; y : years ; RCT : randomized control trial ; CHD : coronary heart disease; CHF: congestive heart failure; HR: hazard ratio; RR: relative Risk; OR: Odd ratio; f.u: Follow-up; OFU: observational follow up

b. Legacy effect from optimization of lipid profile

As for diabetic patients and optimized glycemic control, lipid lowering therapies have also shown long-term benefit on cardiovascular outcomes (**Table 2**). In the Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) trial, pravastatin, given in secondary prevention for 6 years, reduced mortality and cardiovascular events in patients with previous acute coronary syndromes ⁵⁸. After a subsequent open label period of 2 years in which patients were offered pravastatin irrespective of their original assigned therapy, a sustained significant benefit of the initial intervention was observed on all-cause mortality, coronary heart disease mortality, myocardial infarction and stroke ⁵⁹.

In the Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lowering Arm (ASCOT-LLA) ten thousand hypercholesterolemic and hypertensive patients, with at least three other cardiovascular risk factors, were randomized to receive either atorvastatin or placebo, in primary prevention. The trial was stopped prematurely (median of 3,3 years of intervention) because of a 36% relative risk reduction in the primary outcome (consisting of non-fatal myocardial infarction and fatal coronary heart disease) in favor of atorvastatin; and a non-significant reduction in CV deaths (by 16%) and all-

cause mortality (by 13%)⁶⁰. In the long-term follow-up (median of 11 years), all-cause mortality appeared significantly lower in patients originally assigned atorvastatin (decreased by 14%). Fewer cardiovascular deaths were observed (11% less; not significant) as well as non-cardiovascular deaths (15%; reaching significance) in those formerly assigned atorvastatin, despite extensive crossover to statin treatment after the end of the initial trial. This reduction was intriguingly driven by a reduction in deaths due to infection and respiratory illness, raising still unanswered question of the underlying mechanisms⁶¹. In the West of Scotland Coronary Prevention Study (WOSCOPS), men were proposed pravastatin as primary prevention or placebo during 5 years. Once again, on-trial statin treatment produced a beneficial effect vs. placebo on nonfatal myocardial infarctions (by 31%), death from coronary heart disease (by 28 %); and death from all cardiovascular causes (by 32 %); despite low adherence at this point (only 38,7% of the intervention group were found to be on statins at 5 years)⁶². Over 20 years of follow-up revealed a sustained beneficial effect on all-cause mortality (by 13%), attributable mainly to a 21% decrease in cardiovascular death⁶³, again despite crossover to statins in the original placebo group after the trial. The above-mentioned study ACCORD included a “lipid” arm (ACCORD-Lipid), in which type 2 diabetic patients were randomized to

simvastatin plus fibrate or simvastatin plus placebo for 5 years. At the end of the intervention, there was no evidence of a beneficial effect of combined therapy compared to statins alone on cardiovascular outcomes and mortality⁶⁴. However, the follow-up study (ACCORDION) showed that allocation to the combined fibrate-statin treatment arm during the trial period had a beneficial legacy effect on all-cause mortality (35%), on top of decreased incidence rates of non-fatal myocardial infarction, congestive heart failure, major coronary heart disease and CVD mortality⁶⁵. The Heart Outcomes Prevention Evaluation (HOPE) 3 trial evaluated the benefit of lipid lowering therapy (using rosuvastatin) and/or blood pressure optimization (using candesartan plus hydrochlorothiazide) in 12 thousand intermediate-risk patients; e.g. patients with at least one cardiovascular risk factor, without any previous cardiovascular event. To note, inclusion in the trial did not mandate specific lipid or blood-pressure levels before randomization. Each intervention was compared to placebo (the study included also combined treatment) for a duration of almost 6 years. Treatment with rosuvastatin was associated with a significant decrease (by 24%) for the first coprimary outcome namely MACE-1 (composite of death from CV causes, non-fatal MI, non-fatal stroke), and consistently, with a significant decrease (by 25%) for the second outcome namely MACE-2 (composite of revascularization,

heart failure, and resuscitated cardiac arrest) ⁶⁶. In the recently published analysis of the long term follow up of 8,7 years, the benefit of rosuvastatin was maintained with 21 % reduction in risk of MACE-1 and 21 % reduction in risk of MACE-2 for the total follow up. To note, only 36% of patients initially randomized to rosuvastatin and 38% of those randomized to placebo were prescribed a statin after the active phase of the study ⁶⁷. A recent meta-analysis evaluated the “legacy” effect of lipid-lowering treatment by statins, analyzing 8 randomized studies, either in primary or secondary prevention. Globally, direct in-trial effects were greater than post-trial effects (likely attributable to crossover effects), regarding cardiovascular and all-cause mortality. There was no evidence of significant legacy effects on CVD mortality, but some evidence of legacy effects on all-cause mortality; a subgroup analysis of the 3 trials performed as primary prevention also demonstrated legacy effects on both CVD mortality (by 13%) and all-cause mortality (by 10%) ⁶⁸.

The Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) included a subgroup in which hypertensive patients with a least another cardiovascular risk factor received pravastatin compared to usual care. No effect of the intervention was observed in all-cause mortality

or coronary heart disease. This may be due to the modest differential in lipid profile between the intervention group and usual care group (16,7% lower LDL-C compared to control). Moreover, the study was not blinded, at a period of publication of a series of guidelines stimulating optimization of the lipid profile and statin use which contributed to treatment crossovers. Indeed, the trial follow-up showed a decrease in total cholesterol and LDL-C over time in both groups ⁶⁹. Consistently, long-term follow-up did not reveal any “legacy effect” of pravastatin in this trial ⁷⁰. The Second Australian National BP study (ANBP2) was an open-label study including older hypertensive patients and designed to compare two anti-hypertensive therapies. In a post-hoc analysis of the ANBP2 cohort, patients were stratified according to lipid lowering therapy at entry as primary prevention versus usual care. Outcomes were compared regardless of their randomized treatment. Lipid lowering treatment was significantly associated with long-term (11-years) decrease in all-cause and non-CVD mortality (by 22% and 30% respectively); however, no statistically significant association with short-term mortality (4 years) was observed ⁷¹. Beside the beneficial legacy effect of optimized control on lipid profile, long-term detrimental effects of a transient exposure to hypercholesterolemia have also been demonstrated. In a study in young adults born prematurely, endothelial function assessed by measurements of pulse

wave velocity was compared between those who received intravenous lipids at early stage of life as part of parenteral nutrition, and matched controls with equivalent perinatal characteristics, who did not receive such supplements. Perinatal hyperlipemia secondary to intravenous lipids administration was associated with a significantly altered endothelial function compared to controls in adult life ⁷².

Study Name Type	Patients characteristics (n int/n follow-up)	Effect of intervention? (study duration)	Legacy effect? (follow up duration)	Comments
LIPID ⁵⁸ RCT LIPID follow up ⁵⁹ OFU	Pravastatin vs placebo in recent MI or unstable angina Median age 62y Median cholesterol 218 mg/dl (9014/7680)	(6y) vs placebo (RR): (-) 24% death from CHD (-) 22% overall mortality (-) 29% MI (-)19% stroke	(2y) Open label period, cross over: 86% gr. placebo on pravastatin and 88% gr prava still on pravastatin, with level of cholesterol similar Legacy effect of intervention (RR) (-) 25% death from CHD (-) 19% overall mortality (-) 15% MI (-) 24% stroke	
ASCOT-LLA ⁶⁰ RCT UK ASCOT-LLA legacy ⁶¹ OFU	Atorvastatin vs placebo in hypertensive patients with at least 3 other CV risk factors in primary prevention Mean age 61,4y (4605/4432)	(3,3y) In favor of atorvastatin HR 0,64 for non fatal MI and fatal CHD HR 0,79 for CV events HR 0,71 for coronary events Trend to less death (HR 0,87, p=0,16)	(11y) Open label cross over (2/3 placebo on statin) Legacy effect in favor of atorvastatin: HR 0,86 all cause mortality HR 0,85 non CV death HR 0,89 CV death but not significant	
WOSCOPS ⁶² RCT WOSCOPS follow up ⁶³ OFU	Pravastatin vs placebo in primary prevention in men Mean age 55y High LDL (6596/6408)	(4,9y) Favor pravastatin (RR) : (-) 31% in non-fatal MI or death from CHD (-) 32% in death from CV causes	(18y) 38,7% (former prava) and 35,2% (former placebo) at 5 years on statin, no further data Legacy effect in favor of pravastatin (RR): (-) 13% mortality (-) 21% in death from CV causes	
ACCORD-LLA ⁶⁴	Combined therapy (simvastatin + fibrate vs	(5y)	(10y)	

<i>RCT</i> <i>ACCORDION</i> ⁶⁵ <i>OFU</i>	simva+ placebo) in T2D long duration with dyslipidemia Mean age 61,8y (940/765)	No effect of combined treatment on cardiovascular outcomes or mortality	Same level of lipid profile in both groups but legacy effect of combined treatment: HR 0,68 in all-cause mortality HR 0,63 in CVD mortality HR 0,66 in major CHD	
<i>HOPE-3</i> ⁶⁶ <i>RCT</i> <i>HOPE-3 FU</i> ⁶⁷ <i>OFU</i>	Rosuvastatin vs placebo, at least 1 CV risk factor, no CV disease mean age 65,7y (12705/9326)	(5,6) Favor rosuvastatin: HR 0,76 in MACE-1 (composite of death from CV causes, non-fatal MI, non-fatal stroke); HR 0,75 in MACE-2 (composite of revascularization, HF, resuscitated CA)	(3,1) Legacy effect of rosuvastatin treatment: HR 0,80 MACE-1, HR 0,83 MACE -2 Total FU: HR 0,79 MACE-1 HR 0,79 MACE -2	In OFU: 37% on statin (36% of former rosu, 38% of former placebo gr.)
<i>ALLHAT-LLT</i> ⁶⁹ <i>RCT</i> <i>ALLHAT-LLT</i> ⁷⁰ <i>OFU</i>	Pravastatin vs usual care in hypertensive patient + at least 1 CV risk factor mean age 66y (10355/1672)	(4,8y) No effect on all-cause mortality or CHD	(8-13y) No legacy effect	Only 16% of difference in LDL between groups at the end of intervention period

Table 2: Randomized control trials evaluating legacy effect in hypercholesterolemia treatment. T1DM: type 1 diabetes mellitus ; T2DM: type 2 diabetes mellitus ; CV : cardiovascular ; CA: cardiac arrest ; MI : myocardial infarction ; y : years ; RCT : randomized control trial ; CHD : coronary heart disease; CHF: congestive heart failure; HR: hazard ratio; RR: relative Risk; OR: Odd ratio; f.u: Follow-up; OFU: observational follow up.

c. *“Legacy effect” of optimized blood pressure control*

Similar legacy findings have been observed with blood pressure control (**Table 3**). A meta-analysis of 18 randomized clinical trials using blood pressure-lowering medications (including 132 854 patients in total) showed a lower mortality (by 16%) in the different intervention groups during the trial period. Mortality was also lower (by 15%) during the open-label follow-up phases, when all of the patients were advised to take the same therapy, and rates of receiving active therapy were similar in the 2 groups ⁷³. In the Systolic Hypertension in the Elderly Program (SHEP) trial, chlortalidone-based therapy resulted in a lower rate of cardiovascular events (strokes, MI and heart failure) at 4,5 years, but no significant effect on all-cause and cardiovascular mortality ⁷⁴. In the long term follow-up of 22 years, a significant life expectancy gain, free from CVD-related death was observed in the intervention group, corresponding to one day of life gained for each month of treatment ⁷⁵. In the Randomized Olmesartan And Diabetes MicroAlbuminuria Prevention (ROADMAP) trial, patients with type 2 diabetes with at least one other cardiovascular risk factor were assigned to receive either olmesartan or placebo for almost 3 years, with a principal favorable outcome as a significantly delayed microalbuminuria onset in the

intervention group ⁷⁶. In the observational follow-up of 3,3 years, despite an increase in mean systolic blood pressure in both groups, the incidence of diabetic retinopathy and congestive heart failure were significantly lower (OR 0,34 and 0,23 respectively) in the original intervention group. Moreover, patients who had developed microalbuminuria during the trial had a higher incidence of cardio– and cerebro-vascular events (OR 1,77) ⁷⁷. The above-mentioned ASCOT study also included one arm of blood pressure control, in which patients were assigned either to an amlodipine-based regimen (with perindopril if required) or to atenolol (with bendroflumethiazide if required). Although mean blood pressure reduction was similar in both groups, cardiovascular events and all-cause mortality was significantly lower in the amlodipine (plus perindopril) group suggesting that hypotensive medications are not equal in the prevention of CVD ⁷⁸. Long-term follow up to 16 years showed no overall difference in all-cause mortality but significantly fewer deaths from stroke (by 29%) in the amlodipine (plus perindopril) group ⁷⁹. The UKPDS trial also included a subgroup of newly diagnosed type 2 diabetic patients in which blood pressure control was optimized. Significant relative risk reductions were found during the trial for any diabetes-related end point, diabetes-related death, microvascular disease, and stroke in the group receiving tight, as compared with less tight blood-pressure control ⁸⁰.

However, this effect was not sustained during the post-trial follow-up. Nevertheless, a risk reduction for peripheral vascular disease associated with tight blood-pressure control remained significant after 10 years ⁸¹. As previously mentioned, the HOPE-3 study included an arm to compare candesartan plus hydrochlorothiazide in intermediate-risk patients. No effect on both composite outcomes (MACE-1 and MACE-2) emerged after the intervention period, except in a subgroup of patient with high systolic blood pressure ($> 143,5$ mmHg) with a significant decrease by 27 % and 24% respectively ⁸². Long term follow up analysis revealed a legacy effect of combined therapy in the same subgroup of patient ($> 143,5$ mmHg) with a significant decrease (by 24 %) in MACE-1 composite outcome ⁶⁷. Other randomized studies, however, failed to detect any legacy effect in blood pressure lowering. In the previously cited study ALLHAT, more than 30 000 patients were randomized to receive either chlorthalidone, amlodipine or lisinopril for at least 4 years (active phase) with an observational period of 8 to 13 years afterwards. No differential effect between these interventions was observed during the trial phase, explaining the absence of legacy effect ⁸³. As cited above, the Second Australian National BP study (ANBP2) compared in elderly patients two different blood pressure lowering regimens (perindopril vs diuretics). A benefit of ACE inhibition on cardiovascular events and deaths

was observed, particularly in men ⁸⁴. Long term observational post-hoc analysis failed to identify any legacy effect, when treatment “naïve” patients were compared with those treated at inclusion ⁸⁵. This result could be explained by one of the main limitations of this study, i.e. a non-randomized post-hoc analysis, and performed in a cohort of older patients (mean age 71 years). In a meta-analysis of three randomized studies including close to 5000 moderately hypertensive middle-aged patients, early initiation of antihypertensive treatment did not reduce cardiovascular morbidity or mortality compared to delayed initiation. No legacy effect was therefore observed in the long-term follow-up observational post-hoc analysis ⁸⁶.

Such discrepancies highlight the limitations in interpreting open-label extension studies, where many confounding factors may superimpose on any remaining treatment effect. In addition, any short-term effect of a specific molecular mechanism, as identified in experimental studies, may likely be “diluted” by superimposed phenomena in long-term clinical observations.

Nevertheless, one class of blood pressure lowering therapy emerged from clinical studies with a potential beneficial effect on cardiovascular outcomes beyond blood pressure lowering. In patients with heart failure resulting from myocardial infarction, a renin angiotensin aldosterone system (RAAS)

inhibitor led to prognosis improvement with only minimal (if any) effect on blood pressure ⁸⁷. Moreover, numerous studies in diabetic nephropathy demonstrated renal protection by RAAS inhibition independent from its blood pressure lowering effect. Further mechanistic studies led to the proposition that the reno-protection results from a decrease in angiotensin II-dependent glomerular efferent arteriolar tone, thereby reducing filtration pressure ⁸⁷. Finally, in the Losartan Intervention For Endpoint reduction in hypertension (LIFE) study, the angiotensin type-1 receptor (AT₁R) blocking, RAAS inhibitor prevented more cardiovascular morbidity and deaths compared to atenolol, despite similar blood pressure reduction ⁸⁸.

Study Name Type	Patients characteristics (n int/n follow-up)	Effect of intervention? (study duration)	Legacy effect? (follow up duration)	Comments
<i>SHEP</i> ⁷⁴ <i>RCT</i> <i>SHEP follow-up</i> ⁷⁵ <i>OFU</i>	Chlortalidone +/- atenolol vs placebo in isolated hypertensive patients Mean age 71,6y (4736/1885)	(4,5y) In favor of intervention HR 0,64 in stroke HR 0,73 in non-fatal MI + HR 0,46 LV failure	(22y) Legacy effect of intervention HR 0,89 CV death One day of life expectancy gained in intervention group per month of treatment	
<i>ROADMAP</i> ⁷⁶ <i>RCT</i> <i>ROADMAP</i> <i>OFU</i> ⁷⁷ <i>OFU</i>	Olmesartan vs placebo in T2D patients ≥ 1 CV risk factor Mean age 57,7y (4447/1758)	(3,2y) Increased time to onset of microalbuminuria (25%)	(6y) despite crossover and increase in BP, legacy effect of olmesartan: OR 0,34 in diabetic retinopathy OR 0,23 in CHF	! higher rate of fatal CV events in int.group in patient with preexisting CHD
<i>ASCOT</i> ⁷⁸ <i>RCT</i> <i>ASCOT legacy</i> ⁷⁹ <i>OFU</i>	Amlodipine (+/- perindopril) vs atenolol (+/- thiazide) hypertensive patients ≥ 3 other CV risk factors Mean age 63y (19257/8580)	(5,5y) In favor of amlodipine based regiment HR 0,77 stroke (fatal and non fatal) HR 0,84 in CV events HR 0,76 in CV mortality HR 0,70 in new onset of diabetes	(16y) Legacy effect of amlodipine- based regiment HR death from stroke 0,71	
<i>UKPDS</i> 38 ⁸¹ <i>RCT</i> Follow-up ⁸¹ <i>OFU</i>	Tight vs less tight blood pressure control T2DM patients (captopril and atenolol) Mean age 56,8y (1148/884)	(8,4y) In favor of tight control (-) 24% diabetes related endpoints (-) 32% deaths related to diabetes (-) 44% strokes (-) 37% microvascular endpoints	(10y) Legacy effect in favor of tight control (RR) (-) 50% in peripheral vascular disease	
<i>HOPE-3</i> ⁸² <i>HOPE-3 FU</i> ⁶⁷	Candesartan+HCTZ vs placebo	(5,6) No significative difference	(3,1) Legacy effect	2/3 in follow-up 1 $\geq$ BP-lowering

<i>OFU</i>	At least 1 CV risk factor No CV disease Mean age 65,7y (12705/9326)	Except in subgroup > 143.5 mmHg HR 0,73 MACE 1 HR 0,76 MACE 2	In subgroup > 143,5 mmHg HR 0,76 MACE 1	medication and 30% were on 2 $\geq$ BP- lowering medications (similar in both groups)
<i>ALLHAT</i> ⁸³ <i>RCT</i>	Hypertensive patients+ ≥ 1 CHD risk factor, chlortalidone vs amlodipine vs lisinopril Mean age 67y (32804/27755)	(4,9y) No difference (increase in HF for amlodipine, and increase in stroke mortality for lisinopril)	(8-13y) No legacy effect	
<i>ANBP2</i> ⁸⁴ <i>RCT</i> <i>ANBP2 follow-up</i> ⁸⁵ <i>OFU</i>	Hypertensive patients ACE inhibitor vs diuretics Mean age 72y (6083/5378)	(4,1y) in favor of ACE inhibition HR 0,89 CV event or death HR 0,68 nonfatal MI But HR 1,91 for fatal stroke	(10y) No legacy effect	Different comparison in OFU: treatment- naïve vs not naïve

Table 3: Randomized control trials evaluating legacy effect in hypertension treatment. T1DM: type 1 diabetes mellitus ; T2DM: type 2 diabetes mellitus ; CV : cardiovascular ; MI : myocardial infarction ; y : years ; RCT : randomized control trial ; CHD : coronary heart disease; CHF: congestive heart failure; HR: hazard ratio; RR: relative Risk; OR: Odd ratio; f.u: Follow-up; OFU: observational follow-up.

3. Mechanisms underlying “legacy”: a role for epigenetics ?

Epigenetics (including the Greek prefix *epi* that means “around or over”) is the study of stable, but reversible, heritable phenotypic changes that do not involve direct DNA (i.e. gene) sequence alterations ^{89, 90}. Epigenetic modifications can regulate gene expression, activity, or accessibility to transcription factors; may last despite cell divisions, for multiple generations, leading to differential expression of an organism’s genes. Therefore the epigenetic hypothesis naturally came into focus to explain “legacy” effects observed in cardiovascular trials.

One well-known example of epigenetic regulation is the process of cellular differentiation in mammalian development. Indeed, during morphogenesis, one fertilized totipotent cell (zygote) divides in pluripotent daughter cells, which in turn, will produce differentiated cell lines such as neurons, epithelia and others, through the activation of regulatory genes and inhibition of others via epigenetic precise regulation mechanisms ⁹¹.

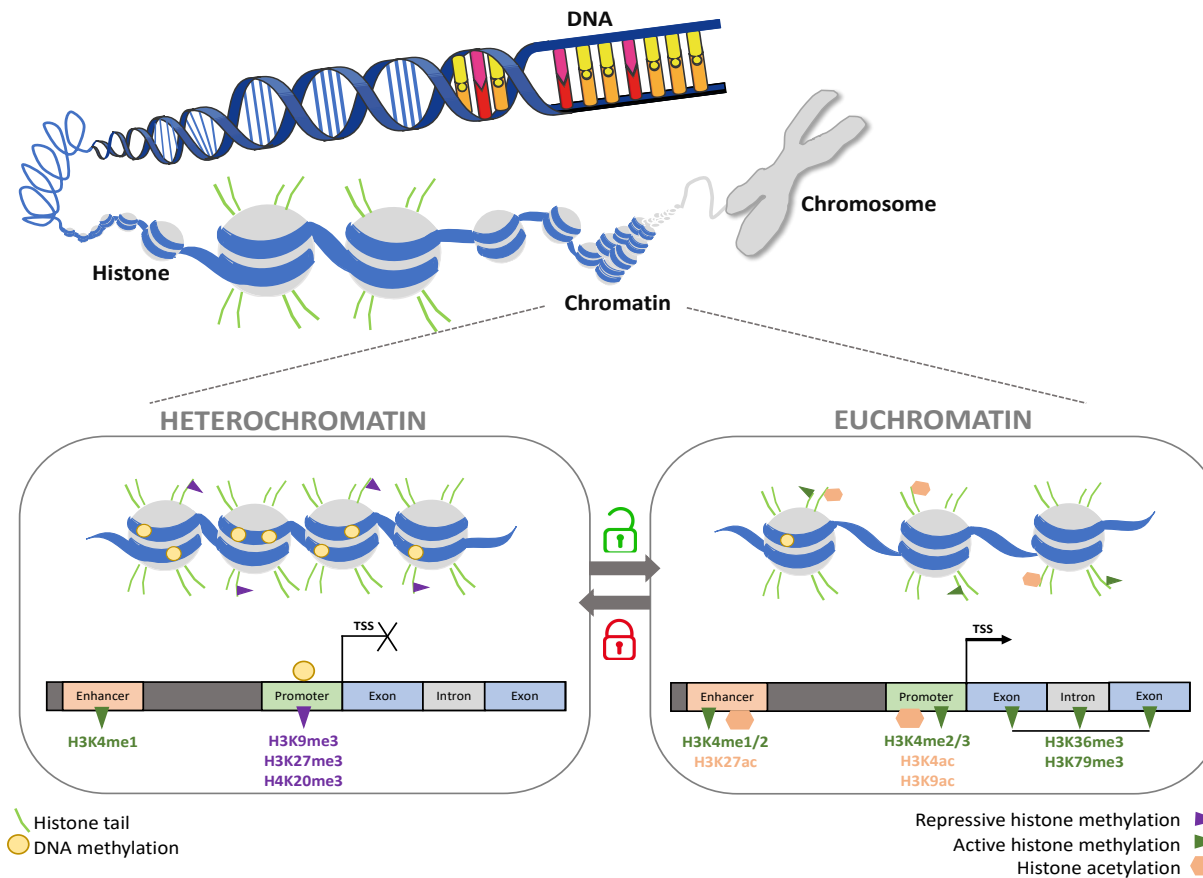
Epigenetics also underlie the link between environment and gene expression. As an example, genetically identical larvae following differential feeding with royal jelly will develop in fertile queens or sterile workers as alternative

forms of the adult female honeybee ⁹²; it also explains discordant phenotypes and disease susceptibility among genetically identical twins ⁹³.

a. General mechanisms of epigenetic regulation

General mechanisms of epigenetic regulation are usually classified as follows: histone posttranslational modifications (acetylation, methylation, ubiquitination, or phosphorylation); DNA methylation; and non-coding RNA-mediated translational control.

Within the eukaryotic cell nucleus, DNA is organized in a highly conserved structural polymer, named chromatin, which supports and controls functions of the genome ⁹⁴. The fundamental repeating unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of core histone proteins — H2A, H2B, H3, and H4, forming the characteristic “beads on a string” morphology. Linker histones of the H1 class associate with DNA between single nucleosomes establishing a higher level of organization, the so-called ‘solenoid’ helical fibers (30 nm fibers) (**Figure 5**). The accessibility of DNA to transcription machinery, and subsequent gene expression, is dependent of the dynamic of the chromatin structure: from euchromatin (“open”) to heterochromatin (“closed”).



repressive or facilitating transcription status of DNA.

Figure 5. DNA organization and chromatin. DNA is packaged inside microscopic nuclei with the help of histones, forming complexes called nucleosomes. Each nucleosome is composed of DNA wound 1.65 times around eight histone proteins.

Nucleosomes fold up to form chromatin fiber, which is tightly coiled into the chromatid of a chromosome. Within the same chromosome, chromatin can take two conformations: hetero (« closed ») and euchromatin (« open ») depending on DNA and histone tail modifications (methylation and acetylation), leading to a more

b. Histone covalent modification

Histones function as acceptors for a variety of post-translational modifications, altering chromatin packing, locally and globally. These include acetylation, methylation and ubiquitination of lysine (K) residues, phosphorylation of serine (S) and threonine (T) residues, and methylation of arginine (R) residues ⁹⁴.

Modifications can be also divided into those correlating with activation and those associated with repression of gene expression. Grossly, acetylation, methylation, phosphorylation, and ubiquitination have been implicated in activation whereas methylation, ubiquitination, sumoylation have been implicated in repression. However, any given modification has the potential to activate or repress under different conditions. For example, methylation at H3K36 has a positive effect when found on the coding region and a negative effect when in the promoter sequence ⁹⁵.

To summarize, at gene promoters, histone lysine (K) acetylation (Kac), (H3K9ac, H3K14ac, or H4K5ac) correlates with transcription activation, whereas de-acetylation is associated with gene repression. Histone K methylation (Kme) is either associated with gene activation or repression, depending on the residue modified and the degree of methylation: mono–

(Kme1), di- (Kme2) or tri- methylation (Kme3). In general, H3K4me1/2/3 and H3K26me2/3 are associated with transcriptionally active genomic regions, and H3K9me3, H3K27me3 and H4K20me3 with repressed domains^{95, 96}. Distinct patterns of specific histone modifications can distinguish key regulatory regions, including promoters, enhancers, and gene bodies. Indeed, transcriptionally active promoters are usually enriched with H3K9ac, H3K4me2 and H3K4me3 marks, and silent gene promoters enriched with repressive marks H3K9me3 and H3K27me3^{96, 97} (**Figure 5**).

Lysine acetylation is mediated by histone acetyl transferases (HAT) such as CREB-binding protein (CBP/P300), Histone Acetyl Transferase 1 (HAT1), or General Control Of Amino Acid Synthesis Protein 5-Like 2 (GCN5). Histones deacetylases (HDAC) include HDAC 1 to 11 and sirtuins. Lysine methylation is performed by histone lysine methyltransferases (HMTs) such as Suppressor Of Variegation 3-9 Homolog 1 or 2 (SUV39H1 or 2), Set Domain Containing methyltransferase family (SET1A, SET1B, SET2) or Enhancer of zeste homolog 2 (EZH2), and removed by lysine demethylases (KDMs) such as Lysine Demethylase 1A (LSD1) or Jumonji C domain-containing family^{95, 96}. Notably, histone modifying enzymes can also modify lysine residues on non-histone proteins, such as transcription factors⁹⁸.

c. DNA methylation

DNA methylation (DNAm) is the most well-established epigenetic mark. It usually takes place at 5' cytosines of 5'-cytosine-phosphate-guanine-3' (CpG) dinucleotides. However non-CpG methylation may also happen, but its function is currently unknown. Approximately half of the genes in vertebrate genomes contain short (1kb) CpG-enriched regions called CpG islands, and the rest of the genome is depleted in CpGs. De novo DNAm is mediated by DNA methyltransferases, DNMT3A and B, and maintenance is provided by DNMT1⁹⁹. Although DNAm is thought to be the most stable epigenetic mark, it can be dynamically removed by DNA demethylases such as ten-eleven translocation (TET) enzymes that catalyze the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethyl-cytosine (5hmC)^{99, 100}. TET enzymes further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). During active demethylation, these 5fC- and 5caC-modified cytosines are rapidly excised by thymine DNA glycosylase (TDG), after which they are replaced by unmodified cytosines through base excision repair mechanisms¹⁰⁰.

DNAm at promoter regions leads to gene repression; within gene bodies it might regulate alternative splicing and even stimulate transcription

elongation ⁹⁹. This epigenetic mark is also recognized by specific methyl binding protein that will recruit transcriptional co-repressors to alter gene expression ⁹⁹.

Notably, methylation of cytosine strongly increases the rate of C→T transition mutations and is thought to be responsible for about one-third of all disease-causing mutations in the germline. Several diseases, such as fragile X syndrome and immunodeficiency, centromere instability and facial anomalies syndrome, also have been linked to DNAm. ⁹⁹

d. Non-coding RNA mediated translational control

Recent improvement in high throughput RNA sequencing technique has revealed that at least 75% of the genome is transcribed, whereas only about 2% encode for proteins ¹⁰¹. Some noncoding RNA sequences (ncRNA) appeared to be epigenetic regulators that can modulate gene expression through numerous transcriptional and post-transcriptional mechanisms. ncRNAs include small ncRNAs such as miRNAs (about 22 nucleotides long), and lncRNAs, which are >200 nucleotides, up to 100 kb, in length. However, some ncRNAs with variable length might belong to two classifications at the same time, such as circular RNAs (circRNAs) ¹⁰².

Within the nucleus, lncRNA alter transcription of genes through interaction with transcription factors, chromatin-modifying complexes or heterogeneous ribonucleoprotein complexes (HnRNPs), a class of nuclear RNA-binding proteins that interact with precursor mRNAs¹⁰³. In the cytosol, lncRNA can control stability of target mRNAs¹⁰³. One of the best studied nuclear lncRNAs, Xist, is a 17 kb transcript from the inactive X chromosome, which mediates the silencing of the inactive X chromosome in human female cells¹⁰⁴.

Small non-coding RNAs are microRNA (miRNA), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). miRNAs are the most abundant class of small ncRNAs. They regulate gene expression in both cytoplasm and nucleus; mediating gene silencing at the post-transcriptional level. In the cytoplasm, miRNAs assemble into the RNA-induced silencing complex (RISC). RISC complexes that are loaded with miRNA (miRISC) bind to target mRNA by forming a bulge sequence in the middle that is not suitable for RNA cleavage, and repress translation by exploiting the translational machinery. Translationally repressed mRNA is either stored in cytoplasmic granules (called P-bodies) or enters the mRNA-decay pathway

for destruction ¹⁰⁵. To date, more than 40 thousand miRNA have been discovered in a wide range of species ¹⁰².

siRNAs are double-strand RNA molecules interfering with RNA pathways, mostly described in plants. In addition, siRNAs have been shown to induce heterochromatin formation via an RNA-induced transcriptional silencing (RITS) complex which when bound to siRNA promotes H3K9 methylation and chromatin condensation ¹⁰⁶. piRNA are animal specific ncRNA, forming silencing complexes through binding with PIWI proteins, which silence targets at transcriptional and post-transcriptional levels ¹⁰².

CircRNAs are ncRNA forming covalently closed loop structures. They have a wide range of sizes (from 100 nucleotides to over 10,000 nucleotides), while most of them in mammals and plants are hundreds of nucleotides. Similar to other regulatory ncRNAs, circRNAs play important roles in diverse biological processes, such as regulating alternative RNA splicing or transcription, acting as competing endogenous RNAs (ceRNAs) or miRNA sponges ¹⁰² (**Figure 6**).

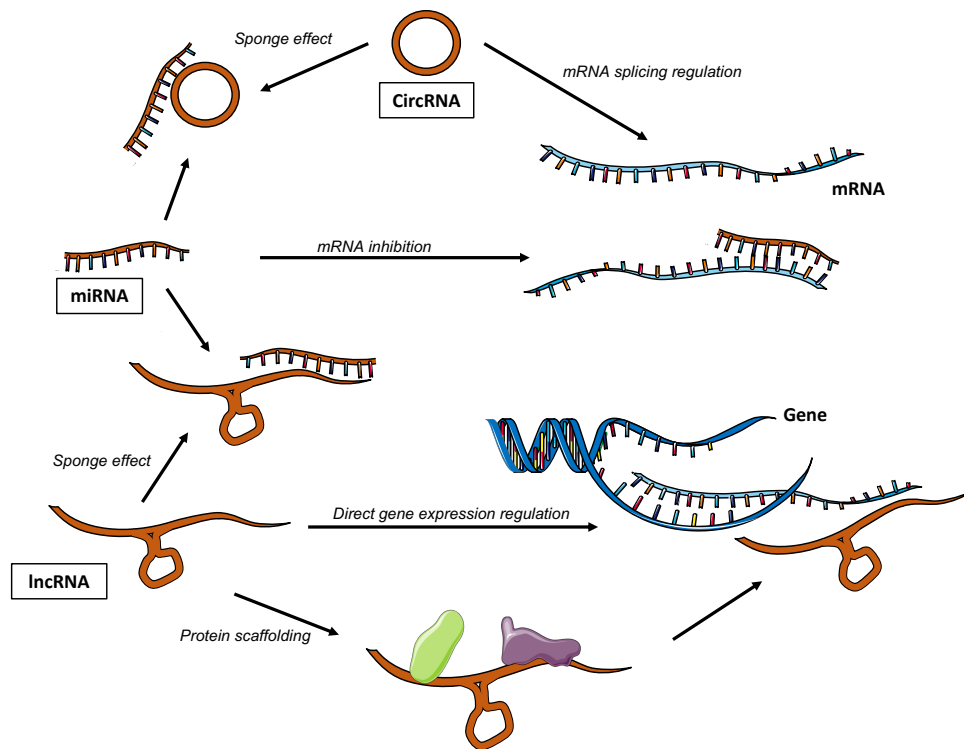


Figure 6. ncRNA regulatory mechanism's. ncRNA can regulate gene expression by directly binding to the mRNA (miRNA and lncRNA), to the gene nascent transcript (lncRNA), or to histone modifiers bringing them to the gene (lncRNA). They can also regulate each other (circRNA, lncRNA and miRNA) by the « sponge » effect. Finally they can regulate gene expression through mRNA splicing (circRNA)

In summary, epigenetics mechanisms (combining histone modifications, DNA methylation, and ncRNA mediated translational control) are controlling tissue and cell specific response to environmental changes. They are stable, albeit erasable, modifications, inducing sustained phenotypic changes. Based on the above considerations, epigenetics mechanisms naturally come into focus to explain “legacy” or “memory” effects observed in cardiovascular trials.

4. Metabolic memory and other memory phenomena in pre-clinical models

The mechanisms underlying clinical “legacy effects” were thoroughly examined in diabetic conditions, with pre-clinical models showing metabolic changes that lead to sustained oxidative stress and indeed epigenetic modifications; these, in turn, would induce a feed-forward phenomenon (or “vicious circle”) that perpetuates oxidative stress, inflammation and damage to the vascular wall ¹⁰⁷. The mechanisms for “trained immunity” by exposure to ox-LDL ¹⁰⁸ or “Ang II memory” ^{109, 110} are less well-characterized, but probably converge on, at least, some common effectors.

a. Metabolic memory: what is hidden behind the “legacy effect”.

The phenomenon of “metabolic memory” had already been described in animal models before the concept of “legacy effect” emerged from clinical studies. In 1987, in an dog model of diabetic retinopathy, Engerman et al observed that animals with late onset optimization of glycemic control had higher incidence in progression of this microvascular complication of diabetes ¹¹¹. A few years later, the word “memory” appeared in the scientific literature with the observation of lasting overexpression of fibronectin mRNA in several organs (e.g. kidney and heart) of streptozotocin-induced diabetic rats, despite restoration of euglycemia. The same authors observed overexpression of fibronectin and collagen IV in cultured endothelial cells exposed to hyperglycemia. This increased expression was sustained in time despite normalization of glucose concentration in culture media and subsequent cells passages ¹¹². The observation that early –but not late– islet transplantation, i.e. before retinal changes, prevented progression of retinopathy in sucrose-fed diabetic Cohen rats further substantiated the “memory” theory ¹¹³. Metabolic memory was further illustrated in several models in vitro and in vivo, including endothelial or vascular smooth muscle

cells in culture, but also in animal models of atherosclerosis, diabetic nephropathy and retinopathy ^{96, 114}.

These models allowed to propose some putative mechanisms, as follows: hyperglycemia induces an overproduction mitochondrial superoxide. The ensuing oxidative stress leads to the activation of 5 major pathways: increased formation of AGEs and expression of its receptor (RAGE), activation of protein kinase C isoforms, overactivity of the hexosamine pathway and polyol pathway that, in turn, activate inflammatory reactions. The same mediators also produce long-lasting epigenetic changes driving sustained expression of proinflammatory genes, despite restoration of glycemic control, leading to the observed “hyperglycemic memory” ¹¹⁵⁻¹¹⁸ (**Figure 6**). As an example, several histone lysine methylations have been described following transient high glucose levels, associated with persistent transcriptional induction of the RELA gene, coding for the p65 subunit of NF- κ B, despite subsequent incubation of cells with normal glucose concentrations; this led to transcriptional activation of target genes implicated in inflammation and endothelial dysfunction ¹¹⁹. Indeed, hyperglycemia-dependent ROS production induced mono-methylation of histone 3 at lysine 4 amino-acid residue (H3K4me) by the methyltransferase Set7/9, a posttranslational

modification favoring gene transcription in mammals; the ensuing modification of histone binding the proximal promoter region of the RELA gene resulted in upregulation of the NF-κB subunit p65 and persistent vascular inflammation ¹¹⁷. ROS production also induced CpG hypomethylation and increased H3 histone acetylation in the p66Shc promoter region; this mitochondrial adaptor, in turn, modulates the intracellular redox state, so that ROS-induced epigenetic modifications are associated with higher levels of p66Shc and with significant activation of PKC, therefore sustaining oxidative stress, endothelial dysfunction and vascular damages ^{120, 121}.

Moreover, upon epigenome profiling of white blood cells of patient from the DCCT trial, an enrichment of histone acetylation marks at promoters of inflammatory genes and other genes related to diabetic complications was observed in selected patients with progressive retinopathy and nephropathy compared with controls. This epigenetic mark also significantly correlated with the mean HbA1C during the trial ¹²².

b. Trained immunity: oxLDL “memory”

As for metabolic memory, several *in vitro* studies evaluated the effects of transient exposure to lipoproteins on the vascular wall or its components,

leading to the concept of “trained immunity”¹⁰⁸ resulting from the priming of different cell types with oxLDL (**Figure 6**). Upon pre-incubation with oxLDL *in vitro* and further stimulation by a TLR agonist such as lipopolysaccharids (LPS) or lipopeptide PAM3cys4, endothelial cells expressed ICAM-1, VCAM-1 and E-selectin, which are adhesion molecules controlling rolling, adhesion and transendothelial migration of leukocytes. In parallel, oxLDL priming also induced the enrichment of activating histone marks (H3K27ac and H3K4me3) on the ICAM-1 promoter¹²³. In human coronary smooth muscle cells *in vitro*, oxLDL similarly induced a proinflammatory priming effect with a significant increase in IL6, IL8 and MCP1 production following restimulation with TLR agonist. This increase was blocked by the inhibition of mTOR and TRL signaling pathways, and by the histone methyltransferase inhibitor methyl-thioadenosine (MTA)¹²⁴. Similar results were observed in isolated human monocytes pre-exposed to oxLDL *in vitro*: when primed by oxLDL (and not with non-oxidized LDL), subsequent stimulation with a TLR agonist such as LPS induced expression of several proatherogenic proteins (e.g IL-6, TNF- α , MCP-1, and MMP 2 and 9); enhanced foam cells formation; and increased H3K4me3 in promoter region of *TNF α* , *IL-6*, *IL-18*, *MCP-1* genes. Pretreatment of monocytes with the histone methyltransferase inhibitor MTA completely prevented the

oxLDL-induced proinflammatory phenotype ¹²⁵. Another pathway involved upon oxLDL priming in monocytes *in vitro* is the production of ROS through mTOR activation of NADPH oxidase: cytosolic and mitochondrial ROS production is induced by oxLDL priming in monocytes in culture, and this increase was blocked by pharmacological inhibition of mTOR; and the development of the trained immunity phenotype was blocked by antioxidant treatment ¹²⁶. Priming with oxLDL also has a major effect on metabolism in vascular wall cellular components, with increase in glycolysis and lactate production ^{123, 127}. In preclinical studies in patients with established atherosclerosis, circulating monocytes also exhibit a higher production of pro-inflammatory cytokines upon LPS stimulation than healthy controls, associated with epigenetic remodeling (lower H3K27me3 on TNF α promoter) and increased expression of rate limiting enzymes of the glycolysis pathway and the pentose phosphate pathway. Interestingly, this pro-inflammatory phenotype was present only in patients with severe symptomatic coronary atherosclerosis, and not in patients with mild asymptomatic carotid atherosclerosis ¹²⁸. More recently, a concomitant upregulation of glycolytic activity and oxygen consumption was observed in oxLDL-primed human primary monocytes. In healthy volunteers, the impact of genetic variation (SNPs) in glycolytic genes on the training capacity of

monocytes was evaluated: variants of genes encoding the inducible PFK-2/FBPase isozyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and phosphofructokinase (PFKP) were associated with the potentiation of TNF- α and IL-6 production upon priming with oxLDL. Subsequent functional validation with inhibitors of glycolytic metabolism revealed dose-dependent inhibition of trained immunity *in vitro*. *In vivo*, the administration of metformin, a modulator of glucose metabolism abrogated the ability of human monocytes to mount a trained response to oxLDL *ex vivo*

127.

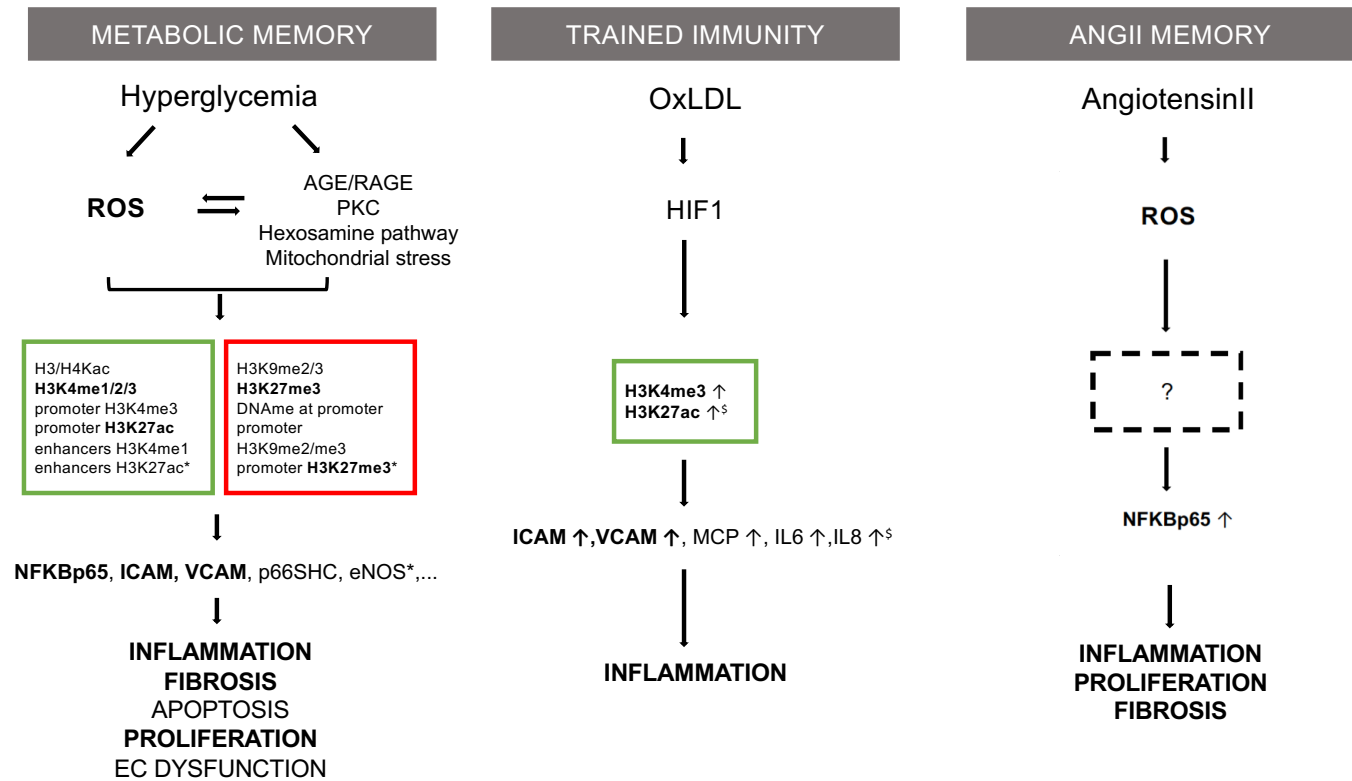


Figure 7. Mechanistic comparison of 3 « memory » theories in vascular research: metabolic memory, trained immunity and AngII memory. Common features are in bold type. Described epigenetic marks are listed in squares (red= repressive; green= permissive). Based on: *¹⁰⁷ and §¹²⁶

c. Ang II “memory”

Ang II, as previously written is the main product and effector of the RAAS stimulation and acts as a physiological regulator of blood pressure ^{32, 129}. Infusions of Ang II have been widely used experimentally to induce endothelial dysfunction or to mimic a hypertensive condition in vivo ^{130, 131}. Contrary to the “metabolic memory”, published evidence on a putative “memory effect” of Ang II is scarce, aside from indirect causality inferred from the clinical trials with RAAS blockers, as reviewed above. A residual effect of temporary Ang II infusion on blood pressure and insulin sensitivity was observed in a study on young rats. A month after the end of infusion of a hypertensive dose of Ang II, blood pressure remained higher and insulin sensitivity was decreased in previously treated rats compared to saline-infused controls. These residual effects were attenuated by the co-administration of tempol, a free radical scavenger, or of candesartan together with Ang II during the infusion period, and the effects of candesartan were not mimicked by hydralazine at a dose producing a similar decrease of blood pressure; the data suggested a link with oxidative stress and an Ang II receptor specificity for this effect ¹³². In another study on mice, a sustained vascular and heart injury was observed up to one week after withdrawal of an initial Ang II infusion with persistent activation of multiple signaling pathways

(JNK1/2, STAT3 and NF- κ B) and increase in ROS production; as the sustained effect was attenuated with apocynin, a NOX inhibitor, the data suggested a link with persistent NADPH oxidase activation (**Figure 7**). However, these studies did not investigate the upstream mechanism for the sustained oxidative stress, including through potential epigenetic regulation ^{109, 110}. In the abovementioned model, hypertension persisted during the week after Ang II withdrawal, introducing a confounding bias about its role in the persisting cardiac and vascular remodeling.

II. Hypothesis-Aims

As demonstrated above, delayed or sustained legacy effects have been reported after early (or despite late) optimized control of cardiovascular risk factors. The aim of this thesis project is to test the hypothesis of the existence of a “hypertension memory” of the vascular wall. Contrary to metabolic memory or oxLDL trained immunity, published evidence on a putative memory effect of hypertension induced by Ang II is scarce and those models included confounding bias in the interpretation of the observed phenotype. Keeping the same parallel with metabolic memory and oxLDL trained immunity, my aim is to examine the implication of a putative epigenetic imprinting, including common epigenetic effectors, at the basis of a hypertension “legacy effect”.

First, I developed an *in vivo* “Ang II memory model” in wild-type mice to assess long term effects of temporary exposure to Ang II, in terms of cardiac remodeling, vascular damage, and systemic oxidative stress. Second, I used an unbiased approach, whole tissue RNA sequencing (RNA-seq), to identify key genes and regulatory pathways involved in the observed memory phenotype in aortic tissue. From there, I identified potential regulators and epigenetic marks associated with this “Ang II memory,” and attempted to

reproduce the same “Ang II memory” phenomenon *in vitro*, in cultured human VSMCs.

III. New model of vascular memory: Angiotensin-II

Sustained down-regulation of vascular smooth muscle Acta2 after transient Angiotensin II infusion: a new model of “vascular memory.

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A. Abstract

Activation of the renin-angiotensin-aldosterone system (RAAS) plays a critical role in the development of hypertension. Published evidence on a putative “memory effect” of Ang II on vascular components is however scarce. **Aim:** to evaluate long-term effects of transient exposure to Ang II on mouse heart and arterial tissue. **Methods:** blood pressure, cardiovascular tissue damage and remodeling, and systemic oxidative stress were evaluated in C57/B6/J mice at the end of a 2-week Ang II infusion (*Ang II*); 2 and 3 weeks after the interruption of a 2-week Ang II treatment (*Ang II+2W* and *Ang II +3W*; so called “memory” conditions) and control littermate (*CTRL*). RNA-seq profiling of aortic tissues was used to identify potential key regulated genes accounting for legacy effects on the vascular phenotype. RNA-seq results were validated by RT qPCR and immunohistochemistry in a reproduction cohort of mice. Key findings were reproduced in a homotypic cell culture model. **Results:** 2 weeks Ang II infusion induced cardiac hypertrophy and aortic damage, that persisted beyond Ang II interruption and despite blood pressure normalization, with sustained vascular expression of

ICAM1, infiltration by CD45+ cells, and cell proliferation associated with systemic oxidative stress. RNA-seq profiling in aortic tissue identified robust *Acta2* down regulation at transcript and protein levels (α -Smooth Muscle Actin) that was maintained beyond interruption of Ang II treatment. Among regulators of *Acta2* expression, the transcription factor Myocardin (*Myocd*), exhibited a similar expression pattern. The sustained downregulation of *Acta2* and *Myocd* was associated with an increase in H3K27me3 in nuclei of aortic sections from mice in the “memory” conditions. A sustained downregulation of *ACTA2* and *MYOCD* was reproduced in cultured human aortic vascular smooth muscle cells upon transient exposure to Ang II. **Conclusion** A transient exposure to Ang II produces prolonged vascular remodeling with robust *ACTA2* downregulation, associated with an epigenetic imprinting supporting a “memory” effect despite stimulus withdrawal.

B. Introduction

Hypertension is a well-recognized cardiovascular risk factor, causing up to 7.6 million deaths per year worldwide (13.5% of total deaths)¹³³. It usually develops as a slow and gradual increase in blood pressure, with occasional acute hypertensive peaks. This silent disease can remain unrecognized until

potentially fatal complications occur, such as hypertrophic cardiomyopathy, strokes or ischemic heart disease.

Hypertension is conventionally associated with a neurohormonal activation from the sympathetic nervous and the RAA system ^{134, 135}. The RAAS is involved in numerous physiological functions, including vasoconstriction, fluid volume regulation, cardiac remodeling, cell growth, and vascular wall integrity. Angiotensin II, as the main product and effector of the RAAS, is a potent regulator of blood pressure ^{32, 129}. As such, it is also a key player in hypertension development, mainly through activation of the type 1 Ang II receptors, that triggers structural remodeling and inflammation in heart and vascular tissue ¹³⁶. In particular, cellular processes underlying vascular injury include, among others, impaired endothelial function and a phenotypic switch of VSMC, characterized by a reduced expression of myofibrillar proteins and contractility, evolving towards a more proliferative and synthetic state, with increased production, e.g. of proteoglycans ^{33, 137, 138}.

Activation of the RAAS usually accompanies the development of other cardiovascular risk factors than hypertension, such as diabetes or metabolic syndrome, including hypercholesterolemia. The resulting cardiovascular pathologies evolve with sustained deleterious effects despite the removal of

the pathogenic stimulus (e.g. hyperglycemia), a phenomenon that has been coined « metabolic memory »¹³⁹. In previous models of diabetes, temporary exposure to hyperglycemia leads to an epigenetic imprinting in endothelial cells, with sustained mitochondrial oxidative stress despite glycemic normalization^{114, 117}. Similar epigenetic mechanisms may explain the enduring increase in cardiovascular risk despite glycemic control in clinical studies, named « legacy effect »^{45,46}. The specific participation of the RAAS, particularly of Ang II, in these enduring effects is less clear. Intriguingly, other clinical trials suggest that treatment with a RAAS inhibitor, e.g. sartans that inhibit Ang II binding to the AT₁R, could prevent organ damage and reduce cardiovascular events through protective effects beyond blood pressure lowering in hypertensive patients⁸⁸. Published evidence on a putative “memory effect” of Ang II is scarce. One previous study observed a sustained vascular injury with persistent activation of multiple signaling pathways (JNK1/2, STAT3 and NF-κB) and increase in reactive oxygen species (ROS) production up to one week after withdrawal of an initial Ang II infusion in mice; the data suggested a link with persistent NADPH oxidase activation. However, the upstream mechanism for this sustained oxidative stress was not established^{109, 110}.

Surprisingly, while transcriptomic profiles of Ang II-treated organs/tissues are available in kidneys ¹⁴⁰, heart ¹⁴¹ or abdominal aortic aneurysm in ApoE^{-/-} mice ¹⁴², the Ang II treatment protocol varies widely between studies, and transcriptomic data on arterial tissue of WT mice were rarely reported ¹⁴³⁻¹⁴⁵, with no attempt to analyze any legacy effect.

In our study, we developed an Ang II « memory » model in which we examined long-term effects of temporary exposure to Ang II on heart and arterial tissue, including cardiovascular remodeling and VSMC phenotypic switch. Longitudinal transcriptomic profiling of aortic tissues after Ang II withdrawal identified uniquely regulated genes potentially involved in legacy effects on the vascular phenotype.

C. Results

The experimental design is illustrated in **Figure 1A**. Blood pressure was recorded longitudinally in each mouse by telemetry at four time-points: at baseline (*CTRL*); at the end of the 2-week treatment with Ang II (*Ang II*); and 2 and 3 weeks after the end of the Ang II infusion (*Ang II+2W* and *Ang II+3W* respectively); the last two composing the « memory » groups. As expected, we observed an increase in systolic and diastolic blood pressure during Ang

II infusion (**Figure 1B and C**). Importantly, blood pressure values reverted to normal levels after the end of the 2-week treatment, confirming treatment interruption and transient exposure to Ang II, as planned. We next evaluated specific parameters of cardiovascular remodeling classically affected by Ang II and their eventual persistence at longer time points.

1. Cardiac hypertrophy

As expected ^{32, 146}, we observed a significant increase in total heart/tibial length (TH/TL), left ventricular weight/tibial length (LVW/TL), and left ventricular weight/body weight (LVW/BW) ratios in the *Ang II* group (**Figure 1D, E and F**). This hypertrophic phenotype was sustained in time with ratios significantly increased in *Ang II+2W* and *Ang II+3W* groups (**Figure 1D, E and F**). This was reflected by concordant increases in cardiac myocyte transverse area in the *Ang II* group (**Figure 1G**), which remained significantly elevated in *Ang II+2W* and *Ang II+3W* groups.

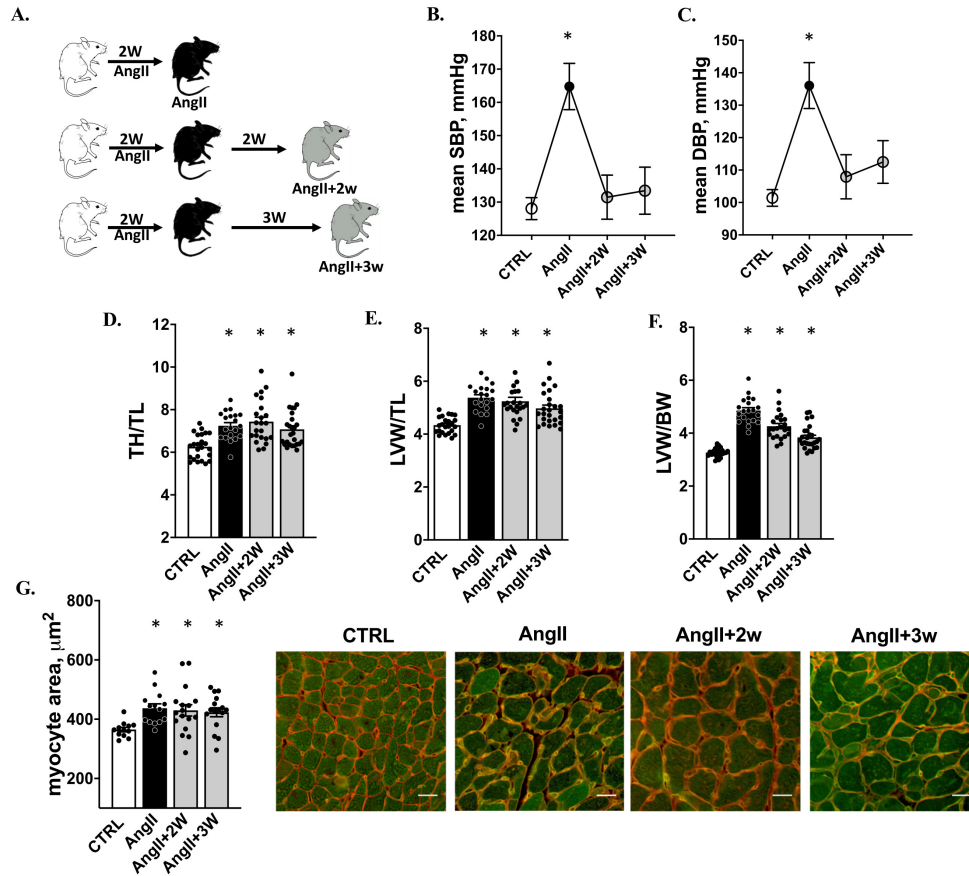


Figure 1. Experimental procedure and cardiac hypertrophy

Experimental procedure: A. C57Bl6 mice were implanted with osmotic minipump delivering 2.8mg/kg/d of Ang II during 2 weeks. One group was sacrificed at the end of the treatment (*Ang II*), 2 others were sacrificed respectively 2 weeks (*Ang II+2W*) and 3 weeks (*Ang II+3W*) after the end of the minipump, and were considered as « memory groups ». Control mice (*CTRL*) were littermate age-matched mice sacrificed at each time point (2 weeks of Ang II, 2 weeks of Ang II + 2 weeks, 2 weeks of Ang II + 3 weeks). **B. Systolic** and **C. Diastolic** blood pressure measured with implanted telemetry devices. n=19; * p< 0.05, One-way ANOVA for Repeated Measures, mixed-effect analysis followed by Tukey's multiple comparison test.

D-G. Cardiac Hypertrophy: **D.** Total Heart / Tibial length ratio (TH/TL); **E.** Left ventricular Weight / Tibial Length ratio (LVW/TL); and **F.** Left Ventricular Weight / Body Weight ratio (LVW/BW). n= 93 (initial+ replication cohorts); * p<0.05, One-way ANOVA followed by Holm-Sidak's multiple comparisons test. **G.** Quantification of myocyte size area by isolectin/WGA staining and representative pictures (white bar scale = 20μm). n= 59; * p< 0.05, One-way ANOVA followed by Dunett's multiple comparisons test.

2. Vascular remodeling

Similar analyses were performed on the vascular phenotype. Histological analysis of carotid sections in the *Ang II* group revealed an increased endothelial expression of ICAM-1, reflective of endothelial activation. ICAM-1 expression remained elevated in the *Ang II+2W* and *Ang II+3W* group (**Figure 2A**). Consistently, CD45 labelling was increased in aortic tissue of the *Ang II* group, reflective of inflammation that was also sustained in time in the *Ang II+2W* and *Ang II+3W* groups (**Figure 2B**). In line with the known proliferative effect of Ang II in vascular tissue ^{147, 148}, Ki67 labelling of aortic tissue was increased in the *Ang II* group and, again, maintained in the *Ang II+3W* group (**Figure 2C**).

3. Oxidative stress

Ang II stimulates tissue nicotinamide adenine dinucleotide phosphate (NADPH) oxidases in cardiac and vascular cells to produce superoxide anions and, upon dismutation with extracellular SOD, the secondary oxidizing product H₂O₂ ³⁴. Accordingly, plasma hydroperoxides were elevated in the *Ang II* group, as well as in the *Ang II+2W* group (**supplemental figure 1**), reflecting persistent systemic oxidant stress at least up to two weeks after Ang II removal in our model.

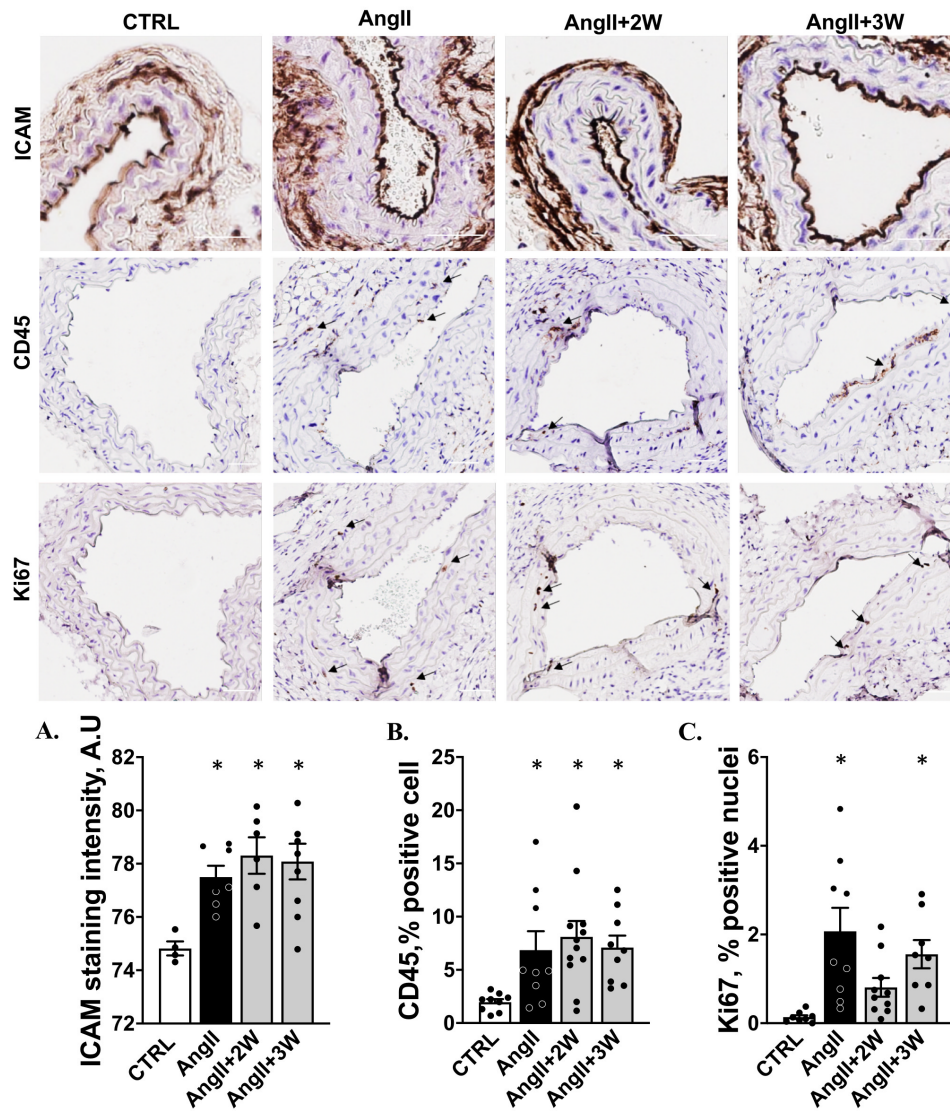


Figure 2. Memory effect on vascular endothelial activation, cell proliferation and inflammation. **A.** Quantification of endothelial staining for **ICAM-1** in paraffin embedded sections of carotid arteries, expressed in arbitrary units of staining intensity. $n=26$ **B.** quantification of staining for **CD45** in paraffin embedded sections of aortas, expressed as % of stained cell. **C.** quantification of staining for **Ki67** in paraffin embedded sections of aortas, expressed as % of stained cells. * $p < 0.05$; $n=39$ (B,C); One-way ANOVA followed by Dunett's multiple comparisons test. Arrows indicate specific staining for CD45 and Ki67, scale bar = 100 μm .

4. *Comparative transcriptomic profiling of Ang II vs Ang II “memory” conditions*

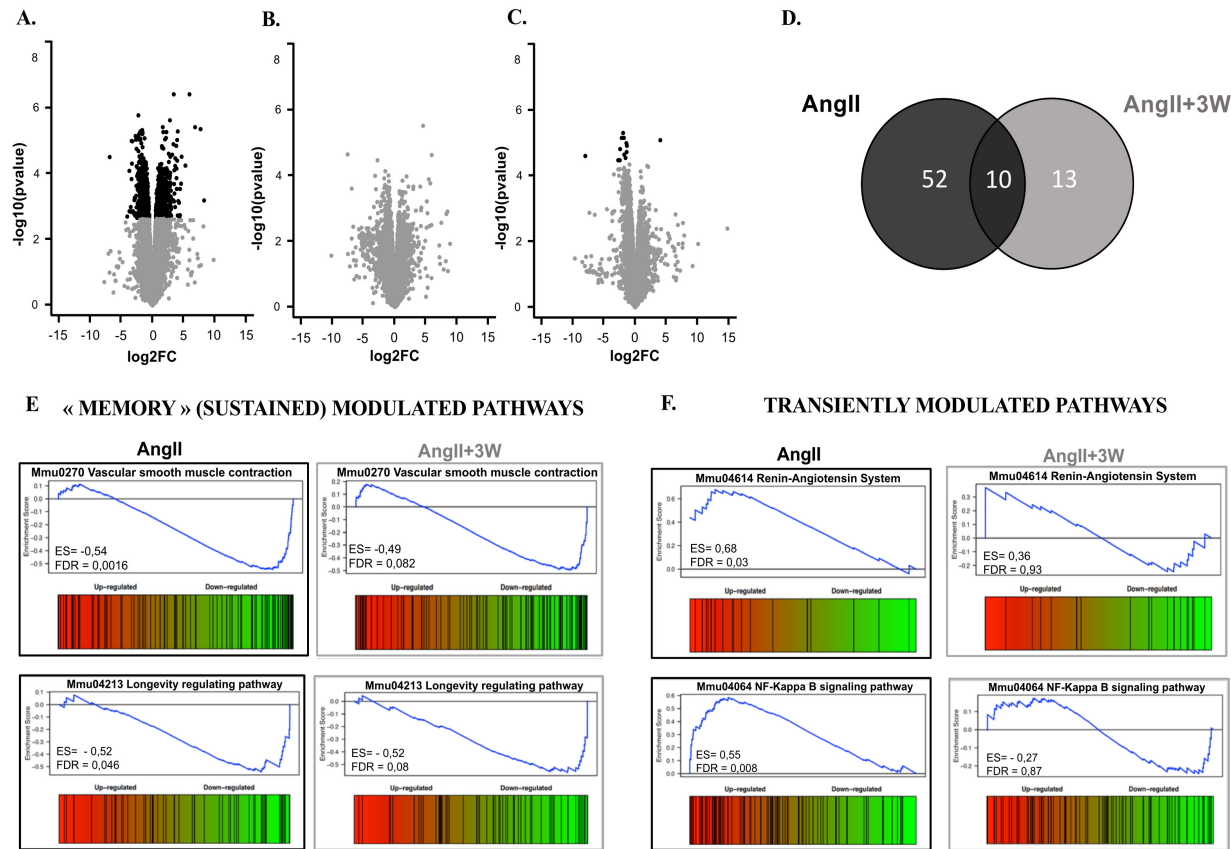
To gain further mechanistic insight into the sustained effect of Ang II on this vascular phenotype, we used an unbiased approach through whole aortic tissue RNA-sequencing. We first assessed the differential expression of genes and underlying signaling pathways in the *Ang II* group compared to control, untreated mice. Next, we performed a similar comparison between *Ang II+2W* and *Ang II+3W* groups (i.e. “memory” conditions) versus control, untreated mice. We also compared the resulting list of genes and pathways to identify transcripts similarly modulated in *Ang II* and “memory” groups, assuming that these sustained up and/or downregulated transcripts may be related to the observed “memory” phenotype.

Volcano plots in **Figure 3A-C** represent differential expression of gene data sets comparing each condition to *CTRL*, considering $FDR < 0.05$ as a cut-off. As shown in **Figure 3A and 3C**, we found 808 genes significantly differentially expressed in *Ang II* and 13 genes in *Ang II+3W* memory condition. Unlike the *Ang II* condition (**Figure 3A**) in which 55% of genes were up-regulated, we found a vast majority of significantly under-expressed genes in *Ang II+3W* memory condition (**Figure 3C**). As no significant

differentially expressed gene was found with this FDR cut-off in *Ang II+2W* group (**Figure 3B**), we focused on the *Ang II+3W* « memory » group compared with *Ang II* for further analysis.

Gene set enrichment analysis of the 2 data sets is summarized in **Figure 3D-F**. A total of 62 KEGG pathways were significantly differentially modulated in *Ang II* group, and 23 in *Ang II+3W* group, using FDR <0.1 as a cut-off. The Venn diagram in **Figure 3D** shows that 10 of them were commonly modulated pathways between the 2 groups. Among these 10 pathways, we next searched for those modulated in the same direction, e.g. up or down regulated. Four of the 10 commonly modulated pathways were similarly downregulated, and identified as: “dilated cardiomyopathy” (mmu05414); “hypertrophic cardiomyopathy” (mmu05410); “longevity regulating pathway” (mmu04213) and, notably, “vascular smooth muscle contraction” (mmu04270) (**Figure 3E** and **supplemental figure 2**). Conversely, other pathways known to be regulated by Ang II, such as “Renin angiotensin system” (mmu044614), “NF-KappaB signaling” (mmmu04064) and “cGMP signaling” (mmu04022) were, as expected, up– (for the first two) or down-regulated (for the latter) in the *Ang II* group, but returned to normal level of

enrichment in the *Ang II+3W* «memory» group (**Figure 3F and supplemental figure 2**).



condition, and the overlap for 10 of them (FDR value < 0.1) **E. Enrichment score diagram** illustrating the expression pattern of 2 of the 10 commonly regulated pathways (e.g. present in *Ang II* and in *Ang II+3W*, and modulated in the same direction) “vascular smooth muscle contraction” and “longevity regulating pathway”. **F. Enrichment score diagram** illustrating the expression pattern of 2 transiently modulated pathways by Angiotensin II infusion: “Renin angiotensin System” and “NF KappaB signalling pathway”

Figure 3. Transcriptomic profiling of aortic tissue from Ang II memory model. Volcano plots representing the analysis of differential expression of genes in **A.** *Ang II* vs *CTRL*; **B.** *Ang II+2W* vs *CTRL* and **C.** *Ang II+3W* vs *CTRL* data sets. Black points mark the genes with significantly ($FDR < 0.05$) increased or decreased expression compared to *CTRL*. The x-axis shows $\log_2$ fold-changes in expression and the y-axis the $-\log_{10}(pvalue)$. **D.** Gene set enrichment analysis (GSEA) from compiled KEGG pathways. Venn diagram showing the number of gene-sets differentially modulated in each

We next compared the gene lists to identify transcripts commonly regulated in both *Ang II* and *Ang II+3W* conditions. 13 genes were commonly differentially expressed in both conditions, i.e., 13 genes similarly and significantly modulated both in the “memory” group vs. control, untreated mice and in the *Ang II* group vs. control, untreated mice. **Figure 4A** represents a heat map with these 13 common transcripts, illustrating changes in expression level in each condition (*Ang II* and *Ang II+3W*) compared with control, untreated condition (see also **supplemental figure 3**).

Among these, our attention was drawn to *Acta2*. *Acta2* is a gene coding for alpha-smooth muscle actin (α SMA), a protein of the cytoskeleton mainly expressed in smooth muscle cells that is involved in vascular contractility and blood pressure homeostasis. Mutations in this gene cause a variety of vascular diseases, such as thoracic dilated aortic disease, coronary artery disease, stroke and Moyamoya disease ¹⁴⁹, as well as multisystemic smooth muscle dysfunction syndrome ¹⁵⁰. On the other hand, down regulation of α SMA expression induced by *Ang II* has been described in vascular SMC *in vitro* ^{151, 152}.

To better delineate the putative role of *Acta2* in the context of our pathway enrichment analysis, we drew a virtual protein-protein interaction network using the Cytoscape software (fed from the String database). We only included the list of genes that were differentially expressed in *Ang II+3W* memory condition (compared with *CTRL*), with enlarged FDR cut-off of < 0.1 for a more comprehensive picture (**Figure 4C**). Note that this interactome did not consider the direction of modulation (e.g. if a transcript was either up or down regulated, to the extent that protein abundance is modulated similarly). Interestingly, we observed that *Acta2* was located at a central hub of this protein-protein interaction network.

Based on the above observations, we focused on *Acta2* as a potential driver of our phenotype.

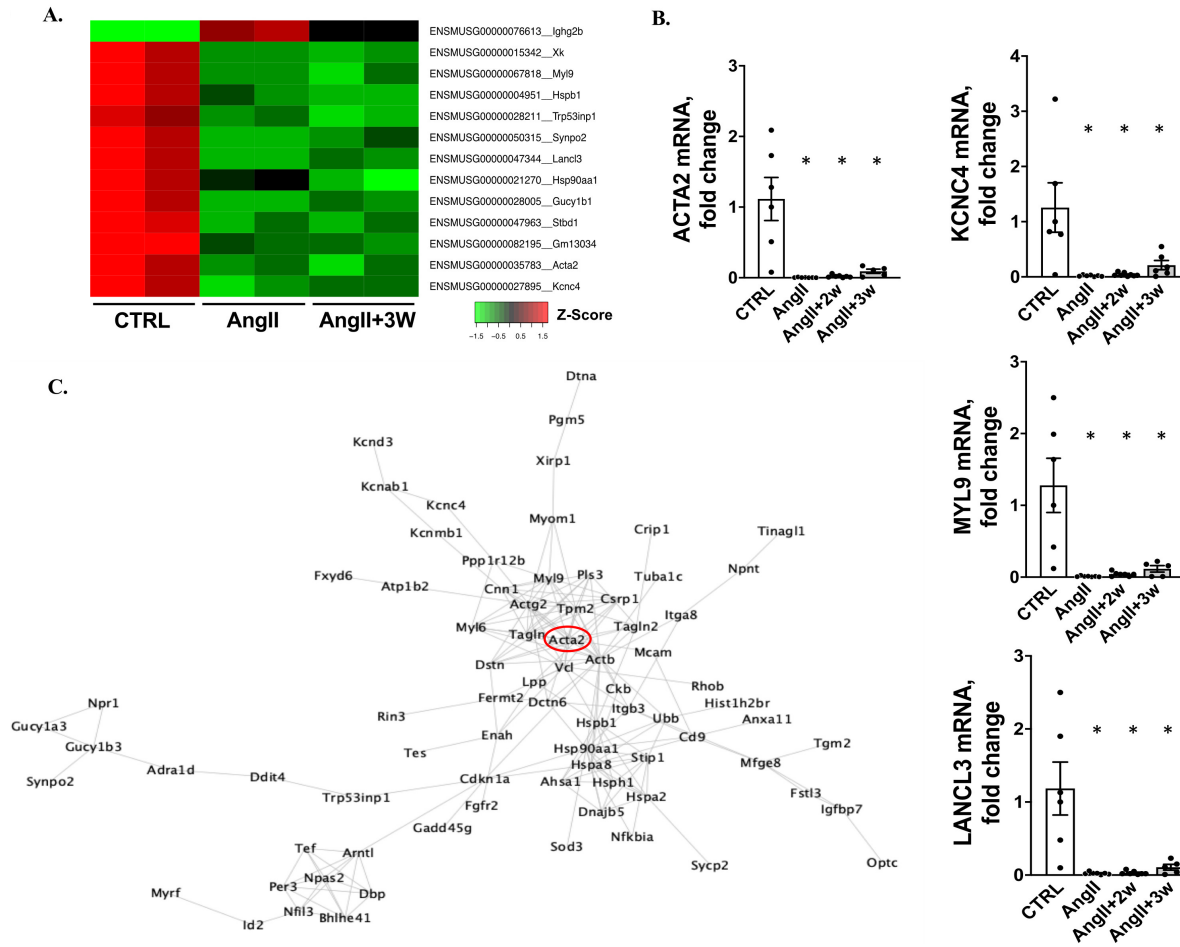


Figure 4. Common downregulation of specific genes in aortic tissue from Ang II and “memory model” and corresponding interactome mapping

A. HeatMap representing expression level (Z-score) of the 13 identified commonly modulated transcripts in Ang II+3W and Ang II versus CTRL conditions.

B. RT qPCR on a replication group of mice confirming RNA seq expression data on selected genes: *Lanc13*, *Kcnc4*, *Myl9*, and *Acta2*, $n=25$; * $p<0.05$, One Way ANOVA followed by Holm’s-Sidak comparison test.

C. Protein-protein interaction network corresponding to differentially expressed genes in aorta in the “memory” condition (*Ang II*+3w) vs. *CTRL*. To build a comprehensive picture, genes with FDR up to <0.1 were included. Based on Cytoscape String Network database.

5. Sustained down regulation of *Acta2* in a replication cohort: putative role of myocardin and histone methylation.

We confirmed our RNA-seq data in a replication cohort of identically treated mice, in which we found a downregulation of *Acta2* mRNA expression by RT-qPCR in *Ang II*, and both *Ang II+ 2W* and *Ang II+3W* groups, compared with *CTRL* (**Figure 4B**); among the 13 genes identified in the RNA-seq data, downregulation of *Myh9*, *Kcnc4* and *Lancl3* mRNA expression were also confirmed (**Figure 4B**). α SMA protein levels were also evaluated through quantitative immunostaining on aortic tissue. We confirmed a significant decrease in α SMA protein expression in the *Ang II* group, which was significantly maintained in both “memory” conditions, *Ang II+2W* and *Ang II+3W* group (**Figure 5A**). Remarkably, the cytoskeleton in smooth muscle cells of the arterial media in these two groups was profoundly altered, with important structural defects and disorganization (see representative pictures in **Figure 5**).

Among factors controlling α SMA expression are transcription factors Myocardin (*Myocd*) and Serum Response Factor (*SRF*). Altogether they form a complex which binds CArG (CCA/T_(rich)GG) sequence motif upstream *Acta2* (and other contractile genes), *SRF* serving as docking platform for

Myocd activity, leading to active contractile transcription machinery. In our RNA-seq data we observed that *Myocd* and *SRF* transcripts were significantly down-regulated under Ang II ($-2,07 \log_2\text{FC}$, FDR= 0,02), with the same trend in the memory condition for *Myocd* ($-1,42 \log_2\text{FC}$, FDR= 0,13). We decided to evaluate *Myocd* expression by RT qPCR in our replication group of mice. We observed that this transcription factor was robustly repressed under Ang II infusion and that this repression was sustained in time despite the end of the pharmacological stimulation (**Figure 5B**).

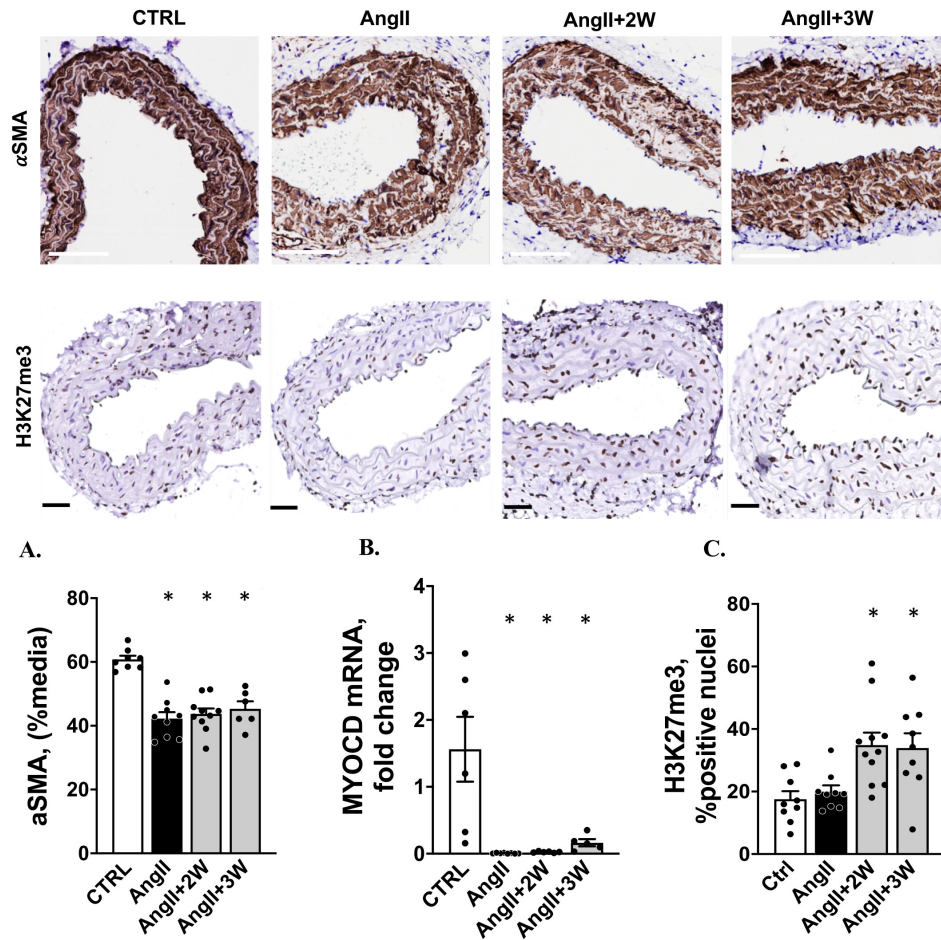


Figure 5. Coordinated regulation of Alpha-Smooth Muscle Actin, its transcription factor *Myocd* and histone trimethylation in aortic tissue from the Ang II “memory” model

A. Sustained down regulation of αSMA protein in aortic tissue from a replication cohort by immunostaining (white bar = 100 μm). n=33; * = p<0.05, One Way ANOVA followed by Dunett’s multiple comparison test.

B. *Myocd* mRNA expression by RTqPCR in mouse aortas from a replication cohort, n=26; * p<0.05, One Way ANOVA followed by Holm’s-Sidak comparison test. **C. H3K27me3 staining and quantification** in paraffin embedded sections of aortas, expressed as % of positive nuclei (black bar = 50 μm), n=39; * p<0.05, One Way ANOVA followed by Dunett’s multiple comparison test.

To gain further understanding of the sustained down regulation of *Acta2* and *Myocd*, we examined putative epigenetic regulatory mechanisms. Indeed, our pathway analysis using ORA or GSEA, identified significant enrichment of several epigenetic pathways under Ang II such as “HDAC’s deacetylase histone” (R-MMU-3214815), “HATs acetylate histone” (R-MMU-3214847), and also “PRC2 methylates histone and DNA”(R-MMU-21230) (see supplemental **table material**. Reactome T1 ORA, Reactome T1 GSEA). Interestingly, PRC2 is a protein complex that keeps transcriptionally silent genes in a repressed state by trimethylating histone H3 on lysine 27. We then evaluated the status of this epigenetic mark in our model, using immunostaining on aortic sections. Notably, this revealed a significant increase in H3K27me3, only in the 2 memory conditions, *Ang II+2W* and *Ang II+3W* (**Figure 5C**).

6. *In vitro* Ang II memory model in Human Aortic Vascular Smooth Muscle Cells

To verify these observations in a homotypic cell system of human origin, we developed an *in vitro* model of Ang II memory on cultured human aortic VSMCs. Cells were exposed to continuous Ang II at 1μM for 72 hours (*Ang II*); or to the same serum deprived control medium for 72h (*CTRL*), or 48 hours of Ang II, followed by 24 hours of control medium (*MemAng II*). First,

we observed the same down regulation of *ACTA2* under Ang II stimulation, as depicted by mRNA expression and protein levels (**Figure 6A and C**). Notably, this downregulation was also maintained in the “memory” condition. This was paralleled with a down regulation of mRNA expression of the transcription factor *MYOCD*, upon continuous Ang II, as well as in the “memory” condition (**Figure 6B**).

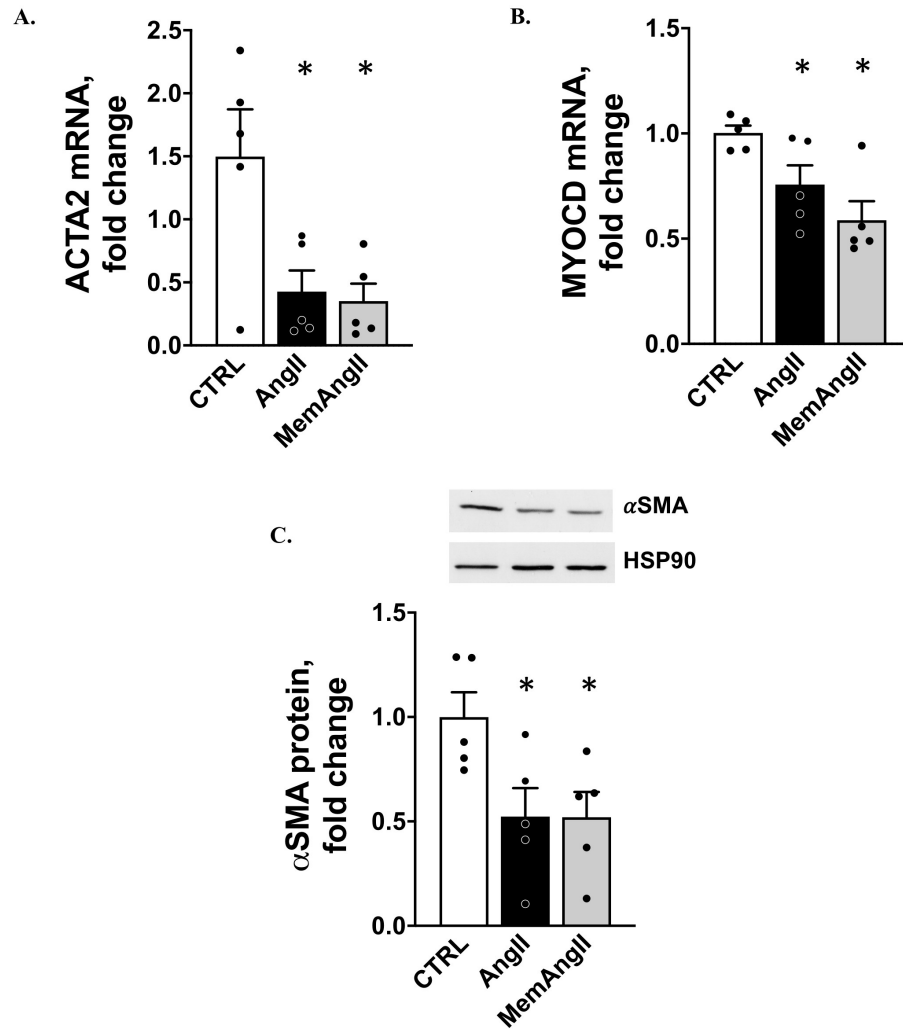


Figure 6. Downregulation of *ACTA2* and *MYOCD* in a homotypic cell culture model of human vascular smooth muscle cells reproducing the Ang II “memory” effect *in vitro*.

A. *ACTA2* mRNA expression (by RT qPCR) in HAVSMC exposed continuously (*Ang II*) or transiently (*MemAng II*) to Ang II compared to control cells maintained in serum deprived control media. **B.** *MYOCD* mRNA expression in the same *in vitro* model **C.** **αSMA protein level** in the same model, N=5 independent experiments, * $p < 0.05$ One Way ANOVA followed by Sidak’s multiple comparisons test.

D. Discussion

The main findings of our study can be summarized as follows; (i) an initial 2-week exposure to Ang II induces profound changes in cardiac and vascular remodeling, including endothelial activation, vascular inflammation and oxidant stress, all of which are maintained up to three weeks after Ang II withdrawal; notably, this phenotype is sustained despite early normalization of blood pressure after Ang II withdrawal, a proxy to a “legacy” or “memory” effect in this mouse model. (ii) comparison of the transcriptomic profiles at the end of the 2-week Ang II treatment (*Ang II* group) or 3 weeks after Ang II withdrawal (*Ang II+3 weeks* group) identified 13 commonly regulated transcripts (1 up and 12 down), and a set of 4 commonly modulated pathways by GSEA, some of which point to altered structural or contractile properties of the arterial wall. Conversely, many other transcripts classically associated with Ang II effects and (mostly) up-regulated in the *Ang II* group, are not persistently regulated in the *Ang II+3 weeks* group; this highlights the above 13 genes, corresponding to the sustained downregulated transcripts, as potential targets for a “memory” effect. (iii) among these, *Acta2* is a likely candidate, as first confirmed in a replication cohort, including at the protein level, but also from our observation of striking downregulation of Myocardin

(*Myocd*), its transcriptional co-regulator and from changes in histone methylation, as corresponding epigenetic repressive marks, in aortas of both memory groups, *Ang II+2W* and *Ang II+3W* groups.

Ours is one of the few studies examining the effect of Ang II on the full transcriptomic profile of mouse aortic or arterial tissues in “wild-type” (C57Bl6/J) mice. In a micro-array study on ApoE^{-/-} mice treated with Ang II, Rush et al identified genes overexpressed in mice that did not develop aneurysms under pharmacological Ang II stimulation, i.e. protective against abdominal aortic aneurysms ¹⁴². Consistent with their results, a majority of those transcripts were significantly down-regulated in our *Ang II* group of mice, such as *Sost*, *Dstn*, or *Hspa1a* (see supplemental data). Our profiles are also in line with results by Spin et al identifying genes decreased in prematurely ruptured aneurysm in Ang II-treated ApoE^{-/-} mice (e.g. *Bmp6*, *Ltbpl*, *Rock1*) ¹⁵³. Another transcriptomic analysis of 3 differential arterial beds of C57Bl6 mice treated with Ang II identified *Sphk1* as commonly modulated transcript in the 3 types of tissue. This transcript is also upregulated under Ang II in our RNA-seq data, albeit not significant with an FDR cutoff of 0.05. Other transcripts such as *Thy1* or *Htatip2*, described to be over-expressed in thoracic aorta, are similarly regulated in our *Ang II*

group. Notably, as we did, the same study identified a down regulation of *Gucyl1b3* transcripts (corresponding to the beta subunit of soluble guanylyl cyclase) in thoracic and abdominal aortic tissue, expected to be associated with a reduced vascular relaxation ¹⁴³. Finally, in a recent study in C57Bl6 mice treated for a longer time with lower dose of Ang II, Lv et al identified 773 genes differentially expressed in aortas of hypertensive mice ¹⁴⁴. As in our study, under Ang II, mostly were up-regulated genes ¹⁴⁴. Among identified transcripts, we confirmed the over expression of IGF1 in our RNA-seq data in Ang II infused mice.

Even fewer studies have attempted to develop an Ang II “memory” model ^{109, 110, 132}, and in none of them was a transcriptomic profiling performed. In concordance with the only 2 studies in mice found in the literature, we observed sustained macroscopic and microscopic cardiac hypertrophy ¹⁰⁹; ICAM-1 endothelial expression, inflammation in the vascular wall (e.g CD45 positive cell infiltration), and oxidative stress ¹¹⁰ that persisted after Ang II withdrawal. A major difference in the aforementioned study compared to ours is that blood pressure remained elevated one week after the end of Ang II infusion, the only “remote” time-point examined ¹¹⁰. This could simply be explained by the different Ang II dosage and timing for the “memory”

condition (one week *vs* 2 weeks after the end of Ang II infusion). Although the distinction between differential delays for phenotype reversal (e.g. slower for tissue remodeling, more rapid for blood pressure) versus “memory” effect could be argued, the present model shows the persistence of remote effects (up to three weeks) despite clear evidence of termination of the initial stimulus (normalization of blood pressure). At the very least, and contrary to the previous study, it excludes that the long-lasting effects on remodeling result from sustained high blood pressure.

Another strength of our study is the use of an unbiased approach to understand our phenotype through transcriptomic profiling. While differential transcript regulation did not reach the more stringent significance level in the *Ang II+2W* group probably because of insufficient statistical power, the genes differentially expressed in the *Ang II+3W* group at FDR<0.05 were similarly regulated in *Ang II+2W* (supplemental data), suggesting a continuum in their regulation.

Ang II, among other factors, promotes a switch in vascular smooth muscle cells from a contractile to “secretory” phenotype¹⁵⁴. Through AT₁R, Ang II stimulation produces changes in the contractile machinery, consisting in decreased expression of contractile markers such as α SMA (*ACTA2*),

SM22a (*TAGLN*) and SMMHC (*MYH11*)^{33, 155}. As others^{151, 156}, in parallel with vascular remodeling, we observed significant changes in contractile markers expression in aortas under Ang II infusion, with downregulation of “vascular smooth muscle contraction” pathway, and *ACTA2* expression, but also *TAGLN* and *MYH11* (see raw data). In addition, this phenotypic switch was maintained in time despite the end of Ang II stimulation, for the “vascular smooth muscle contraction pathway”, as well as for α SMA expression, both at mRNA and protein levels (**Figure 4B and 5A**). Among factors controlling VSMC phenotypic plasticity are transcription factors Myocardin (*Myocd*) and Serum Response Factor (*SRF*). Altogether they form a complex which binds CArG (CCA/T_(rich)GG) sequence motif upstream contractile genes, with SRF serving as docking platform for *Myocd* activity, leading to active contractile transcription machinery. Conversely, Kruppel-like Factor 4 (*KLF4*) and ETS domain containign protein-1 (*ELK1*) binding to G/C repressor element, inhibit *Myocd*/*SRF* complex, leading to decreased expression of SMC differentiation markers¹⁵⁵. In our RNA-seq data we observed that *Myocd* and *SRF* transcripts were significantly down-regulated under Ang II (– 2,07 log₂FC, FDR= 0,02), with the same trend in the *Ang II+3W* memory condition for (-1,42 log₂FC, FDR= 0,13). This was not the case for *KLF4* and *ELK1* (see raw data). We confirmed this downregulation of *Myocd* transcripts by

RT-qPCR, including in the 2 memory conditions (*Ang II+2W* and *Ang II+3W* group) (**Figure 5B**). Interestingly, we could reproduce same results in HAVSMC in culture, in which we observed a sustained down expression of *ACTA2* and protein α SMA induced by Ang II, associated with reduced expression of transcription factor *Myocd*; and despite removal of the pharmacologic stimulus. This makes *MYOCD* a likely candidate for upstream, sustained downregulation of *ACTA2* expression in Ang II “memory” context.

Although this aspect was less dissected in our study, epigenetic regulation may also account for the sustained alteration of the vascular phenotype in the “memory” groups *in vivo*. Indeed, Histones H3 and H4 associated with CArG containing regulatory elements of SM-MHCs (*MYH11*), SM22a (*TAGLN*), and α SMA (*ACTA2*) have been shown to be acetylated (a characteristic of chromatin accessibility) in contractile SMCs, facilitating SRF binding to the CArG box ¹⁵⁷. Increased histone acetyltransferase (HAT) activity stimulates SM22a expression whereas increased histone deacetylase (HDACs) prevents SM22a expression ¹⁵⁸. Consistently, our pathway analysis using ORA or GSEA, identified significant enrichment of several epigenetic pathways

under Ang II (such as “HDAC’s deacetylase histone” (R-MMU-3214815), “HATs acetylate histone” (R-MMU-3214847).

Other modalities of epigenetic regulation may also be at play. H3 histone dimethylation in lysine 4 (H3K4me2) is a marker of differentiated SMCs and is maintained even if SMCs undergo phenotypic modulation ¹⁵⁹. DNA demethylation by TET2 increases DNA accessibility to transcription factors resulting in increased SMC differentiation marker expression ¹⁶⁰. Of interest, the *ACTA2* promoter was shown to be hypermethylated (a usual mark of repressed expression) in genome-wide methylation studies in human atherosclerotic aortas ^{161, 162}. As we observed sustained downregulation of *Acta2* transcripts in our “memory” condition, an Ang II-induced methylation leading to repressive imprinting and phenotypic switch may well be involved. Again, our RNA-seq pathway analysis suggests the involvement of epigenetic regulators involved in methylation, such as “PRC2 methylates histone and DNA”(R-MMU-21230) (see supplemental data Reactome T1 ORA, Reactome T1 GSEA). In particular, PRC2 complex catalyzes trimethylation of histone H3 on lysine 27 (H3K27me3), a histone mark necessary for maintaining transcriptional repression during multicellular development. Cell type-specific patterns of H3K27me3 are crucial for preserving cell identity

¹⁶³. Consistent with this analysis, we observed a significant increase in H3K27me3 epigenetic mark in aortic tissue and, intriguingly, only in both memory conditions (**Figure 5C**). However, the signaling elements involved in this response to Ang II remain to be studied in more details.

Altogether, our observations strongly support a “memory” effect sustained beyond Ang II induced hypertension and leading to downregulation of specific gene expression, such as *Acta2*, and vascular injury. Future characterization of the underlying Ang II-dependent signaling may unveil new targets for its therapeutic modulation and reversal of this adverse legacy effect.

E. Acknowledgments

We thank M. De Beukelaer for its precious assistance throughout immunohistochemistry experiments.

F. Material and methods

1. Animal experimental protocol

11-week male C57B6/J mice were implanted with osmotic minipumps delivering of 2.8mg/kg/day of Ang II (Sigma, A9525) for 2 weeks. To

confirm the effect of the pharmacological treatment, blood pressure signals from aortic arch before (*CTRL*); under Ang II up to 2 weeks (*Ang II*); and 2 and 3 weeks after the interruption of Ang II (*Ang II+2W*; *Ang II+3w*, respectively) were recorded in selected, conscious, and unrestrained animals, with surgically implanted miniaturized telemetry devices (DSI, USA) as previously described ¹⁶⁴. Implants (DSI, USA) were surgically inserted as following: under anaesthesia with a mixture of ketamine and xylazine, the left common carotid artery was isolated and the tip of the catheter was retrogradely inserted into the aorta until the aortic arch. The catheter was connected to the body of the implant, placed in a subcutaneous pouch in the right flank. After 5 days of recovery, short- or long-term (24 hours) online recordings were digitized (range, 20 to 2000 Hz) and stored for further analysis. Recordings were also obtained during the resting period of the animals, under visual monitoring of their activity, for maximal reproducibility. Osmotic minipumps were implanted the day after BP basal (*CTRL*) recording.

Mice were sacrificed after anesthesia at the same time points and age-matched littermate served as control (**Figure 1A**). All investigations conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publications no.

8023, revised 2011) and were approved by the Institutional Animal Care and Research Advisory Committee of the Université Catholique de Louvain.

2. Histo-morphometric analysis of hearts

Morphometric and histologic measurements were obtained from hearts arrested in diastole in KCl solution, washed, subsequently fixed with 4% formaldehyde and paraffin embedded. To assess cardiac myocyte transverse area, tissue was co-stained with wheat germ agglutinin (WGA; for plasma membrane staining, rhodamine-conjugated) and isolectin B4 (GS-IB4; for endothelial staining, biotin-conjugated and revealed with fluorescein-conjugated streptavidin). Cell area from 150 to 200 cells per slide was determined using AxioVision software 4.8.2.0.

3. Plasmatic peroxides

Systemic oxidative status was evaluated by measurement of hydroperoxides plasmatic levels using a d-ROM kit based on Fenton's reaction (Diacron International, MC001). Briefly, in living animals under anesthesia with ketamine, blood was collected by retro-orbital puncture through heparinized glass pipet, centrifuged for red blood cells separation, and plasma was immediately frozen in liquid nitrogen. Thawed plasma was

used later according manufacturer's recommendations. The formation of reactive products was registered at 505 nm by SpectraMax i3 microplate automatic photometer (Molecular Devices, LLS, USA).

4. Immunostaining on carotid arteries and aortas

Vessels (carotid arteries and aortas in totality) were carefully dissected, washed in cold PBS, and divided in several pieces for further experiments. One piece was subsequently fixed with 4% formaldehyde and paraffin embedded. To assess endothelial activation, carotid sections were stained with ICAM-1 primary antibody (R&D #AF796, 1/1000). On aortic tissue, to evaluate inflammatory cells infiltration and proliferation, sections were stained with anti-CD45 primary antibody (BD Biosciences #55053P, 1/50) or rabbit anti-Ki67 primary antibody (CST #12202, 1/200), respectively. Smooth muscle actin and H3K27me3 stainings were performed using anti- α SMA primary antibody (CST #19245, 1/400) and anti-Tri-Methyl-Histone H3 antibody (CST #9733, 1/200). Primary antibodies were revealed with EnVision-HRP systems (Agilent) and DAB (Agilent). Nuclei were finally counterstained with hematoxylin (Agilent). Slides were digitized with a slide scanner (SCN400 Leica) and blindly analyzed with QuPath software (University of Edinburgh) ¹⁶⁵.

5. RNA-seq on aortic tissue

One third of aorta was immediately frozen in liquid nitrogen in dry Eppendorf. Because of low yield of RNA extracted in preliminary experiments, and to ensure sufficient amount and quality of RNA for sequencing, tissues from two animals were pooled in 1 ml of Tri-Reagent (TR118, MRC) for subsequent homogenization with the use of a Precellys Evolution homogenizer (Bertin Instrument, France). Total RNA was extracted with the PureLink™ RNA Micro Scale Kit (Invitrogen) according to the manufacturer's instructions, including a DNase step. In total, RNA from 8 samples, corresponding to 16 mice, were quantified by Qubit RNA BR assay kit (Thermo Fisher Scientific, Q10211) on a Qubit 4 Fluorometer (Thermo Fisher Scientific). RNA integrity was evaluated on the Agilent 2100 Bioanalyzer using the RNA 6000 nanokit (Agilent, 5067-1511). All samples had RNA integrity number values between 6.8 and 7.7.

Libraries were prepared starting from 150 ng of total RNA using the KAPA RNA HyperPrep Kit with RiboErase (HMR) (KAPA Biosystems, KK8560) following the manufacturer's recommendations (KR1351 – v1.16). Libraries were equimolarly pooled and sequenced on a single lane on an Illumina NovaSeq 6000 platform. All libraries were paired-end (2x100 bp reads)

sequenced and a minimum of 35 million paired-end reads were generated per sample.

All sequencing data were analyzed using the Automated Reproducible MODular workflow for preprocessing and differential analysis of RNA-seq data (ARMOR) pipeline v.1.2.0¹⁶⁶. In this pipeline, reads underwent a quality check using FastQC v0.11.7¹⁶⁷. Quantification and quality control results were summarized in a MultiQC report¹⁶⁸ before being mapped using Salmon¹⁶⁹ to the transcriptome index which was built using all Ensembl cDNA sequences obtained in the Mus_musculus.GRCm38.cdna.all.fa (release 101) file¹⁷⁰. Then, estimated transcript abundances from Salmon were imported into R using the tximeta (1.7.14) package^{171,172} and analyzed for differential gene expression with edgeR (3.31.4) package, in which p-value was adjusted using Benjamini-Hochberg method¹⁷³. Accordingly, each experimental group (*Ang II*, *Ang II+2W*, *Ang II+3W*) was compared against the control group (*CTRL*), thereby producing 3 lists of differentially expressed genes. Differential gene expression results from edgeR were used to conduct Over-Representation Analysis (ORA) and Gene Set Enrichment Analysis (GSEA) with the WebGestaltR (v.0.4.3) package¹⁷⁴. These analyses were conducted on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome

database. RNA-seq full data are available in the NCBI Gene Expression Omnibus (GEO) database under the study accession code GSE175588.

6. RT-qPCR on aortas in a replication cohort

Total RNA was extracted from one third of total aorta in a replication cohort of mice, with the same protocol and extraction procedure, i.e. freeze-drying, further homogenization in Trizol with Precellys Evolution homogenizer, and extraction with PureLink™ RNA Micro Scale Kit. Extracted RNA was reverse-transcribed and analyzed by quantitative polymerase chain reaction (qPCR) with *GAPDH* as housekeeping gene. The primer sequences used for qPCR are presented in Table I.

7. HVSMC culture and in vitro Ang II memory model

Human aortic vascular smooth muscle cells (HAVSMC) were purchased at ScienCell Research Laboratories. Cells were grown in full Smooth Muscle Cell medium (ScienCell #1101), in T75 flask coated with polylysine (2µg/cm²); medium was renewed every 24 hours as cells reached 80% confluence. After serum starvation (0.1% FBS for 18 hours) cells were treated with Ang II 1µM for 72 hours (*Ang II*), or 48 hours and then 24h in control, serum deprived medium (*MemAng II*); and compared to cells maintained 72h in control, serum deprived medium (*CTRL*). Cells between passage 4 and 8

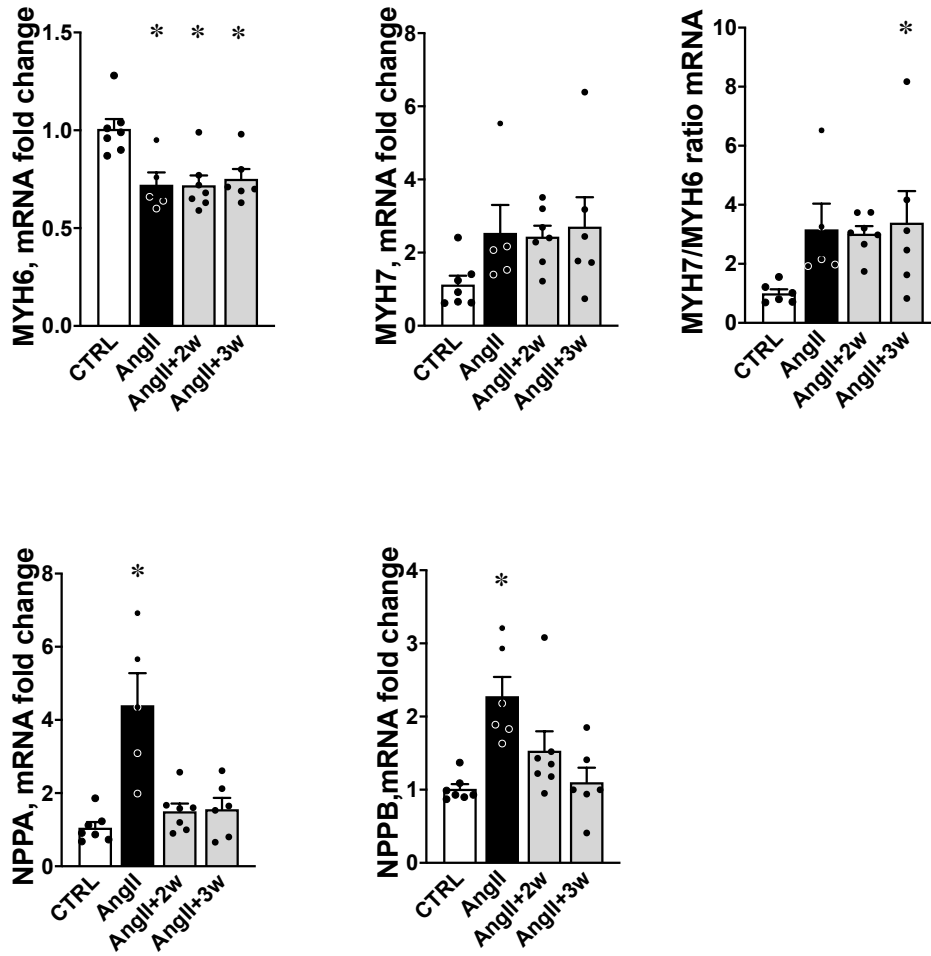
were used for experiments. Total RNA was extracted with Maxwell Kit (Promega, #AS1340). Extracted RNA was reverse-transcribed and analyzed by quantitative polymerase chain reaction (qPCR) with *GAPDH* as housekeeping gene. The primer sequences used for qPCR are presented in Table I in Supplemental material. For protein extraction, cells were scrapped in RIPA buffer containing proteinases and phosphates inhibitors. Denatured proteins (in Laemmli buffer) were separated by SDS-PAGE and transferred on PVDF membrane. Membranes were then blocked 1 hour in 5% non-fat dry milk in TBS-Tween and incubated overnight at 4°C in 1% milk Tween-TBS with primary antibodies. Antibodies were α SMA (CST #19245, 1/10000) and HSP90 (BD Biosciences #610419, 1/2500). Membranes were visualized by enhanced chemiluminescence on CL-Xposure film (Thermo Scientific).

8. Statistics

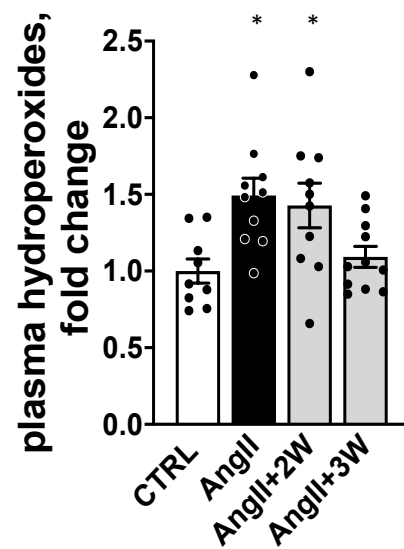
Statistical tests were performed using GraphPadPrism (GraphPad Software, Inc., San Diego, CA). Results are reported as mean and standard error of the mean. Statistical analysis was performed using parametric or nonparametric tests where appropriate after verifying normality of values

distribution. P value <0.05 is considered as significant with *
meaning P value <0.05 .

G. Supplemental materials

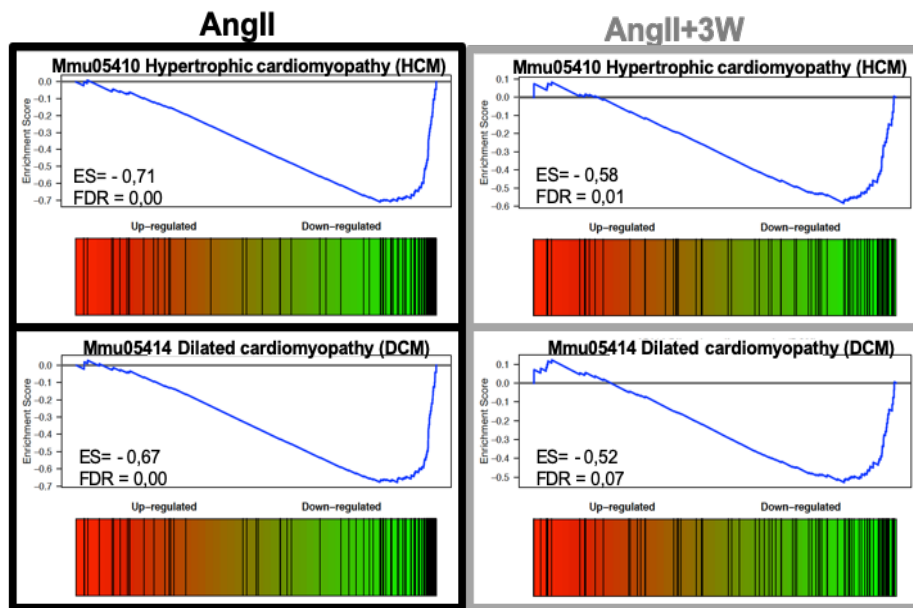


Supplemental figure 1. Hypertrophic markers expression in heart extracts in Ang II memory model. Quantification of MYH7/MYH6 ratio, NPPA and NPPB mRNA levels, expressed as fold change of ctrl *p < 0.05; One-way ANOVA followed by Dunett's multiple comparisons test, n = 35.



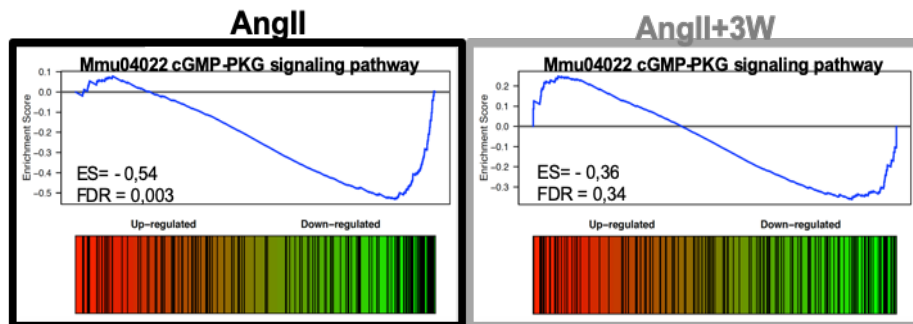
Supplemental figure 2. Oxidative stress measurement in Ang II memory model in vivo.
Quantification of plasma peroxides, expressed as fold change of ctrl mice * $p < 0.05$; One-way ANOVA followed by Dunett's multiple comparisons test, $n=40$.

A. SUSTAINED MODULATED PATHWAY



B.

TRANSIENTLY MODULATED PATHWAYS



Supplemental figure 3. Gene set enrichment analysis (GSEA) from compiled Kegg pathways. A. Enrichment score diagram of 2 supplemental commonly modulated pathway in *Ang II* and *Ang II+3W* groups: “dilated cardiomyopathy” and “hypertrophic cardiomyopathy”. B. Enrichment score diagram illustrating “cGMP-PKG signaling pathway” downregulation under Ang II that was not sustained after Ang II interruption.

	Ang II		Ang II+3W		Ang II+2W	
gene_name	Log ₂ FC	FDR	Log ₂ FC	FDR	Log ₂ FC	FDR
<i>Ighg2b</i>	5.94	0.003	4.03	0.036	4.53	0.053
<i>Xk</i>	-1.95	0.006	-1.95	0.036	-1.04	0.173
<i>Myl9</i>	-2.09	0.006	-2.17	0.036	-1.03	0.215
<i>Hspb1</i>	-1.44	0.012	-1.81	0.036	-0.986	0.173
<i>Trp53inp1</i>	-1.1	0.014	-1.42	0.036	-0.244	0.578
<i>Synpo2</i>	-2.15	0.006	-1.77	0.043	-0.944	0.244
<i>Lancl3</i>	-2.64	0.006	-2.42	0.039	-1.25	0.215
<i>Hsp90aa1</i>	-0.716	0.030	-1.33	0.036	-0.743	0.202
<i>Gucy1b1</i>	-1.62	0.006	-1.38	0.041	-0.921	0.173
<i>Stbd1</i>	-1.49	0.012	-1.55	0.045	-0.904	0.215
<i>Gm13034</i>	-6.87	0.011	-8.01	0.044	-7.58	0.137
<i>Acta2</i>	-2.18	0.013	-2.47	0.045	-1.35	0.244
<i>Kcnc4</i>	-3.34	0.0062	-2.74	0.045	-1.68	0.202

Supplemental figure 4. Common downregulation of specific genes in aortic tissue from Ang II memory model. Differential expression level (log₂FC) of the 13 identified commonly modulated transcripts in Ang II+3W and Ang II versus CTRL conditions, with FDR <0.05. Differential expression level (log₂FC) of the same 13 transcripts and respective FDR values in the other memory condition (Ang II+2W).

	Fw	Rv
Mm-ACTA2	ACTACTGCCGAGCGTGAGAT	CTTCTCCAGGGAGGAAGAGG
Hs-ACTA2	ACTGAGCGTGGCTATTCCTCC GTT	GCAGTGGCCATCTCATTTTCA
Mm-MYL9	TGTGGAGTTGTCTCAGCACC	CAGCCTTGTATTAAGAGACTGTCC
Mm- LANCL3	GAAACCGCAGTACCTGGACA	TGGCATCACGCTGTCTTCAT
Mm-KCNC4	GGGACAGCACCTACAGTGAC	TCCCTCCTCATCAGACAGCA
Mm-GAPDH	TGCACCACCACCTGCTTAGC	GGATGCAGGGATGATGTTCT
Hs-GAPDH	GGAGTCAACGGATTTGGTCGT A	GGCAACAATATCCACTTTAACAGA GT
Mm-MYOCD	CTGTGTGGAGTCCTCAGGTCA AACC	GATGTGCTGCGGGCTCTTCAG
Hs-MYOCD	AGGTGGGGCCAAAGTTTCA	AGGACAGCAGTTGGACTTCG
Mm-NPPA	TCGTCTTGGCCTTTTGGCT	TCCAGGTGGTCTAGCAGGTTCT
Mm-NPPB	GCCAGTCTCCAGAGCAATTC	AGCTGTCTCTGGGCCATT
Mm-MYH6	TGTGGTGCCTCGTTCCA	TTTCGGAGGTACTGGGCTG
Mm-MYH7	ACCAACCTGTCCAAGTTCCG	ACTCCTCATTAGGCCCTTG

Table I. List of primer

IV. General conclusions

In the first part of this manuscript we extensively reviewed clinical trials testing optimized control of cardiovascular risk factors in at-risk patients. We provided convincing evidence in favor of a “legacy effect”, despite heterogeneity in study populations, duration of in-trial and follow-up periods, and clinical outcomes. A “legacy effect” was firmly established in diabetic patients, but also discernible in hypercholesterolemic and hypertensive patients.

One common feature is that, to be effective and produce beneficial “legacy” effects, interventions on risk factors have to be implemented at early stages. The expression “the sooner the better” should therefore be extended from diabetes to the control of hypertension and hyperlipidemia. The mechanisms underlying such “legacy effect” were thoroughly examined in diabetic conditions, with pre-clinical models showing metabolic changes that lead to sustained oxidative stress and epigenetic modifications; these, in turn, would induce a feed-forward phenomenon (or “vicious circle”) that perpetuates oxidative stress, inflammation and damage to the vascular wall ¹⁰⁷. The mechanisms for “trained immunity” by exposure to oxLDL ¹⁰⁸ or “Ang II

memory”^{109, 110} were less well-characterized, but probably converge on, at least, some common effectors.

Even if pathophysiological mechanisms are not completely understood, the idea of an “organ memory” rather than memory specific to each risk factor is an attractive theory. Arterial beds, for example, can be considered as a unique organ, consisting of highly sensitive and interacting cell types, directly in contact with blood flow where the inner endothelial cell lining could perpetuate oxidative stress induced by an initial “aggressor”; as an example, oxLDL produce endothelial dysfunction and “prime” monocytes thereby promoting pro-inflammatory susceptibility^{117, 124}. As all “aggressors” converge on the same resulting phenotype (e.g. atherosclerosis) and as they cumulate their effects together —indeed hypertension, lipid disorders and diabetes often coexist within the same individual— one can imagine that despite differences in initial steps of the disease, all cardiovascular risk factors lead to the same “memory” behavior of the vascular wall, just as rivers meet in the same sea. Notably, epigenetic mechanisms appear at the center of all the above-mentioned memory effects. In metabolic memory, several epigenetic changes (histone methylation, histone and protein acetylation, CpG island methylation content, miRNA) have been involved in the sustained

oxidative stress in endothelial cells ^{107, 118}. In trained immunity, H3K4me3 and H3K27ac were identified as marks of priming by oxLDL in monocytes ¹⁰⁸ and endothelial cells ¹²³, associated with the subsequent pro-inflammatory phenotype. Despite the lack of congruency in these epigenetic changes, one can recognize that, albeit different, they share common features and lead to persistent activation of common effectors, consistent with this idea of an organized memory pattern of the arterial bed.

Based on this hypothesis of vascular “organ” memory, we developed a new model of vascular memory to hypertension induced by Ang II, and presented it in the second part of this manuscript. The main findings of our study are that exposure to Ang II induces profound changes in cardiac and vascular remodeling, including endothelial activation, vascular inflammation and oxidative stress, all of which are maintained up to three weeks after Ang II withdrawal and, notably, despite normalization of blood pressure after Ang II withdrawal. Comparison of the transcriptomic profiles at the end of Ang II treatment and in the memory group at 3 weeks identified among 13 commonly regulated transcripts *Acta2* as likely candidate. *Acta2* integrated with other VSMC contractile markers downregulation in the observed phenotype of sustained phenotypic switch. Finally, among potential

regulating mechanisms and factors, we pointed to the sustained down regulation of Myocardin (*Myocd*), an *Acta2* transcriptional co-regulator, paralleled with an increase of H3K27me, a repressive epigenetic mark. While the time-course was substantially different from the *in vivo* model, we also confirmed the sustain effect of short term exposition to Ang II on human Myocardin and *Acta2 in vitro*, comparable to “metabolic memory” models in endothelial cells ^{119, 175}.

This transcriptomic analysis on aortic tissue under Ang II infusion and in memory conditions brings novelty, identifying genes that were not reported to be linked with Ang II or hypertension, such as *Lancl3*, *Kcnc4*, *Ighg2b* or *Gm13034* (see section V. “Perspectives” below). Our data are also concordant with published evidence on *Acta2*. In human, heterozygous mutations in *Acta2* gene predispose to thoracic aortic aneurysms, acute aortic dissections, and occlusive vascular diseases, such as premature onset stroke and coronary artery disease and Moyamoya disease ¹⁷⁶. *Acta2*^{-/-} mice are prone to develop aortic arch enlargement compared to WT mice, despite hypotensive pattern; this enlargement is prevented by administration of the RAAS inhibitor losartan ¹⁷⁷. In this mutant mice, it has been demonstrated that disruption of α SMA filaments in SMCs increases cellular ROS levels, which increases nuclear factor NF- κ B signaling, leading to increased expression of *Agtr1a*.

These cellular changes result in increased Ang II signaling, which contributes to aortic root enlargement without increased Ang II levels ¹⁷⁷. Moreover, genome-wide DNA methylation studies in human atherosclerotic aorta identified hypermethylated regions (e.g. suppressive) at enhancer of *Acta2* and *Myocd* gene ¹⁶¹.

Our study has several limitations. Our “memory” phenotype was examined exclusively in male mice. As previously stated in the introduction, gender influences the cardiovascular risk factor *per se*. Cellular and molecular mechanisms underlying the differences between males and females regarding vascular diseases are still poorly understood ¹⁷⁸, despite emerging data from experimental animal studies ¹⁷⁹. Transposing the same experimental model in female mice could certainly be of interest. The observed cardiovascular phenotype may also vary according to the mouse genetic background. Indeed, it has been shown that compared to SV129, another commonly used background, C57Bl6 mice have the highest dynamic range for vasoreactivity and stiffness studies. On the opposite, SV129 mice are a poor choice for vasoreactivity and stiffness studies ¹⁸⁰. As such, the C57/Bl6 background in our experiments may not allow optimal expression of cardiovascular diseases which may be more prominently expressed in others, such as SV129. Another limitation of our study, as we used aortic tissue in totality, is that the

anatomical part of the aortic tissue analyzed in RNAseq was not taken into account. Indeed, region-specific transcriptomic profiles under AngII infusion have been described in thoracic versus abdominal aortas ¹⁴³. However, in this study, as in our data, *Gucy1b3*, was among the 200 RNA transcripts commonly down regulated by AngII-induced hypertension in aortic tissue from both origin. We also did not thoroughly examine the extent of fibrosis and the role of myofibroblasts in our model. This could be part of further characterization, e.g. by using Sirius Red staining or quantification of collagen on aortic sections. As plasma peroxides were elevated in our mice, and knowing the role of NOXs in Ang II pro-oxidant effects, the involvement of NADPH oxidases in our phenotype could have been examined, e.g. with the use of NOX inhibitors (Naxibs). However, as most of the Naxibs in development lack isoform specificity and, in some cases, have enzyme-independent antioxidant effects, such experiments would hardly be conclusive. Moreover, ROS also have important beneficial signaling functions ¹⁸¹, so that attempts to reverse our phenotype with Naxibs may be difficult to interpret. Finally, one could question the relatively short duration of AngII infusion (2 weeks) *in vivo* and its pathophysiologic relevance at human scale. As the expected lifetime of a mouse is around 2 years (104 weeks), a 2 week-treatment would be comparable to one year and a half of

severe and continuous hypertension in a human life of 80 years, although such comparison remains somewhat speculative.

Despite these limitations, altogether our data are aligned with and complete the above-mentioned pre-clinical vascular “memory” models, i.e. glycemic metabolic memory and ox-LDL trained immunity, and are consistent with our vascular organ “memory” theory.

V. Perspectives

However, several questions remain unanswered, which offer numerous perspectives for future research. Among them, I selected 3 principal directions: *Myocd* regulation, the involvement of PRC2 complex (including the role of EZH2) in Ang II memory, and *Gm13034* as potential new regulating ncRNA.

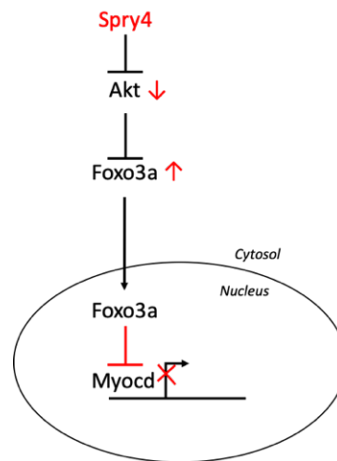
A. *ACTA2 and Myocardin regulation in Ang II memory model*

We observed a downregulation of *Acta2* expression (at transcript and protein levels) as marker of phenotypic switch in our Ang II memory models. Further investigations in an AngII memory model in *Acta2*^{+/-} (or mouse models bearing one of the dominant negative mutations of *Acta2*) could eventually show a more pronounced phenotype, or longer lasting “memory”, comforting our hypothesis of the important role of *Acta2* in our model.

The downregulation of *Acta2* was associated with a reduced expression of its transcription factor *Myocd*. However, the mechanism of regulation of *Myocd* remained unidentified. *Myocd* is a potent co-transcriptional activator of

Serum Response Factor (*SRF*), which modulates the expression of *SRF* target genes, and regulates the development and differentiation of smooth muscle cell lineage. While there is no tissue specificity for *SRF* expression, *Myocd* is expressed specifically in smooth and cardiac muscle¹⁸². Altogether they form a complex in which *SRF* binds a CArG (CCA/T_(rich)GG) sequence motif upstream target genes, serving as docking platform for *Myocd* activity. Down regulation of *Myocd* level is observed in vascular tissues in response to acute injuries, in atherosclerotic animal models and patients. Its expression is recognized as protective against VSMC phenotypic switch, VSMC proliferation, foam cell formation and inflammation¹⁸².

This transcription factor has several known negative regulators; such as KLF4 and PDGF-bb isoform which can bind an upstream repression region (URR) within the *Myocd* promoter, mediating transcriptional repression of *Myocd* expression¹⁸². Foxo3a, a downstream target of PI3K/Akt pathway, translocates into the nucleus and directly inhibits *Myocd* transcription activity. Spry4 by decreasing Akt activity, which subsequently abrogates the repression of Foxo3, inhibits *Myocd* transcription activity (see **Figure**).



Epigenetic regulation may also be at play; for example, miR-9 can regulate *Myocd* through direct target binding to the 3'UTR of *Myocd* mRNA ¹⁸². Exploring myocardin regulators in our Ang II memory models is therefore of interest.

B. Role of the PRC2 complex

As we were confirming a “memory” phenomenon induced by Ang II with sustained vascular damage, we immediately thought about epigenetic regulation of this phenotype. Especially since our RNA-seq pathway analysis pointed to several epigenetic pathways under Ang II infusion, such as “HDAC’s deacetylate histone”, “HATs acetylate histone” but also “PRC2

complex methylates histone and DNA”. Intriguingly, all these activated pathways were no longer activated in the “memory” conditions, suggesting their regulation solely under continuous Ang II infusion. As on the basis of our transcriptomics, expression was decreased for the vast majority of genes, we focused on potential repressive epigenetic regulators, such as the PRC2 complex, and its associated repressive mark, H3K27me3.

The core PRC2 complex comprises four components: EZH2, SUZ12, EED and RbAp46/48 (also known as RBBP7/4), in which EZH2 enzymatic subunits is responsible for the methylation (di- and tri-) of Lys 27 of histone H3 (H3K27me_{2/3})¹⁸³. EZH2 adopts an autoinhibited conformation when on its own; its catalytic activity and *in vivo* stability both require binding to EED and SUZ12. RbAp46/48 is also necessary for full H3K27 methyltransferase activity¹⁶³. Methylation of H3K27 is processive (H3K27me₃ results from mono-methylation of H3K27me₂), and H3K27me₃ is considered a stable mark¹⁸³. Interestingly PRC2 has high-affinity binding to its catalytic product, H3K27me₃. The ensuing contact triggers a stabilization of the active site and a robust increase in the catalytic rate of the complex towards new substrates. This stimulation mechanism suggests a model of preservation through chromosome replication and cell division of a given configuration of

H3K27me3, in which existing H3K27me3 marks would instruct the deposition of new marks on adjacent, newly incorporated nucleosomes ¹⁶³. This mechanism could be an explanation of why we observed the H3K27me3 mark only in the 2 memory conditions.

H3K27me3 regulates transcription indirectly, by sterically preventing proteins from binding to chromatin ¹⁸⁴. It also contributes to the recruitment of further regulatory factors ¹⁸³. The presence of PRC2 and PRC1 at chromatin also leads to chromatin compaction, reducing the accessibility both of transcription factors and ATP-dependent chromatin-remodeling machineries, such as SWI/SNF ¹⁸⁴.

It has been demonstrated that there is an almost-perfect overlap between PRC2-target genes and CpG islands, and studies have shown that GC-rich elements are sufficient to recruit PRC2. There are 3 theories explaining recruitment of PRC2 to CpG island. First, proteins that purify in almost stoichiometric levels with PRC2 show affinities for GC-rich stretches and for specific post-translational modifications of histones. For example, the proteins JARID2 and AEBP2 have an affinity for GC-rich stretches. Second, sequence-specific transcription factors have been proposed to regulate the recruitment of PRC2 to target genes, implicating SNAIL, REST, PLZF, PLZF-RAR α and PML-RAR ¹⁸⁴. Third, non-coding RNA have a role in PRC2

recruitment. Indeed, it has been shown PRC2 proteins and H3K27me3 accumulate on the inactive X chromosome during X inactivation and that the long noncoding Xist transcript can interact with PRC2. Another example of noncoding RNA, HOTAIR, has been reported to recruit PRC2 to the HOXD locus. Moreover, RNA-immunoprecipitation techniques have identified several thousand RNAs associated with PRC2 ¹⁸⁴.

PRC2, and in particular EZH2, have already been implicated in atherosclerosis. In apoE^{-/-} mice, overexpression of EZH2 promotes lipid accumulation in macrophages and accelerates the progression of atherosclerotic lesions. It induces recruitment and binding of DNA methyltransferase 1 (DNMT1) and methyl-CpG-binding protein-2 (MeCP2) to the ATP-binding cassette transporter A1 (ABCA1) promoter, leading to ABCA1 silencing and acceleration of foam cell formation, as ABCA1 is responsible of macrophage cholesterol efflux ¹⁸⁵. In the endothelium, dyslipidemia, hyper-homocysteinemia, hyperglycemia and oscillary shear stress block the expression of endothelial microRNAs that repress EZH2 translation under physiological conditions. For example, in human umbilical vein endothelial cells (HUVECs) treated with oxLDL, miR-200a expression is decreased, causing an upregulation of EZH2 ¹⁸⁶.

Moreover, a link between *Myocd* and EZH2 has been described. In lung mesothelium EZH2 is responsible of repression of *Myocd*, as well as the transcription factor Tbx18, with increase of H3K27me3 mark in *Myocd* promoter region, leading to repression of the smooth muscle differentiation program ¹⁸⁷.

Finally, another recent study showed that patients with Marfan syndrome and other types of thoracic aortic aneurysm had increased aortic expression of another epigenetic repressor histone deacetylase 9 (HDAC9), associated with destabilization of the actin cytoskeleton and decreased contractile protein expression in VSMC. In vitro experiments in VSMC indicated that HDAC9 forms a complex with the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and the chromatin-remodeling protein Brahma-related gene 1 (BRG1). In turn, the HDAC9-MALAT1-BRG1 complex is recruited to the promoters of VSMC-specific contractile genes leading to transcriptional repression and subsequent deleterious VSMC phenotype. In this study, the repression of contractile genes was associated with H3K27me3, via EZH2 recruitment by the HDAC9-MALAT1-BRG1 complex ¹⁸⁸. Increased co-association of the HDAC9-MALAT1-BRG1 complex was confirmed in aortic VSMCs from Marfan syndrome

Fbn1C1039G/+ mice, and deletion of either HDAC9 or MALAT1 resulted in an improved thoracic aortic phenotype. In a similar manner, Fbn1C1039G/+ mice treated with GSK343, a small-molecule inhibitor of EZH2, showed an improvement in aortic architecture and restoration of contractile protein expression in aortic VSMC ¹⁸⁹.

Investigation of PRC2 complex activity under Ang II, but also in the memory conditions, is therefore of great interest. For example, we could use CHIP-seq analysis to identify H3K27me3 mark close to either *Acta2* or *Myocd* promoter sequences. Functional assays (with gain or loss of function of EZH2) would be another interesting option to better understand the role of PRC2 in our model. Finally, GSK343 or several antagonists of EZH2 currently under investigation in oncological field could be used in our Ang II memory model, eventually to reverse memory ¹⁸⁶. Should these molecules prove to be efficient in our (and other) pre-clinical model(s) of vascular memory, their use would be worth considering in patients with vascular disease characterized by deficient alpha-SMA expression or function, such as patients bearing mutations of the ACTA2 gene ^{190, 191}.

C. Gm13034: a potential new ncRNA ?

In our RNA-seq data, among the 13 transcripts identified, we focused on *Acta2* as several of our investigations (interactome and pathway analysis) pointed to it. However, another candidate, *Gm13034*, could open a wide field of research. *Gm13034* is the transcript corresponding to the SMARCA5 pseudogene. As the transcript from a pseudogene, it shared some similarity (close to 95%) with the SMARCA5 gene transcript, and can be considered as non-coding RNA.

SMARCA 5 is SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 5; a member of SMARCA subgroup of genes belonging to the SWI1/SNF1 family, responsible for chromatin remodeling and repair. This type of function explains the relationship between SMARCA4 genetic deficiency, another member of this family, with several solid malignancies ¹⁹². Also known as SNF2H, SMARCA5, the fifth member, is less characterized. This gene encodes the ATPase subunit of the ATP-dependent chromatin assembly factor (ACF), a member of the ISWI (“imitation switch”) family of chromatin remodeling complexes ¹⁹³. Interestingly, independent reports have identified a circular isoform derived from exons 15 and 16 of the SMARCA5 gene (circBase ID

hsa_circ_0001445 (<http://www.circbase.org/>), also named circSMARCA5)¹⁹⁴. In a population study of 200 CAD patients, circSMARCA5 levels were associated with coronary atherosclerosis extent. Levels of circSMARCA5 in this study were inversely proportional to coronary atherosclerotic burden, even after comprehensive adjustment for cardiovascular risk factors, medications, and established biomarkers¹⁹⁴. In our RNA-seq data, *Gm13034* appeared to be also the most down regulated transcript, in both Ang II and memory conditions, from -7 to -8 FC in expression level compared to control mice.

As non-coding RNAs have a role in PRC2 regulation and recruitment, an enticing theory would be that *Gm13034* is a non-coding RNA regulating repressively PRC2. However, to be investigated, this hypothesis would need further experimentation on non-coding RNA functional characterization.

In conclusion, clinical trials on optimization of cardiovascular risk factors illustrated a “legacy effect” of interventions on glycemic, lipid profile or blood pressure control. The mechanisms underlying this organ “legacy” are not yet fully understood, however our data add additional arguments in favor of a vascular memory. Temporary hypertension induces sustained changes in cardiac and vascular tissue, with a persistent VSMC phenotypic switch and downregulation of Acta2 for the last, associated with sustained downregulation of a major VSMC fate transcription factor (Myocardin) and an increase in a repressive epigenetic mark (H3K27me3). Altogether these results open perspectives into the understanding of “legacy” phenomena in cardiovascular diseases, and, possibly, may lead to new therapeutic strategies to reverse them.

VI. Personal work

A. Personal contribution

The above-mentioned experiments were performed by myself with the appreciable help of the FATH technicians team. Roxane Verdoy performed RT-qPCR (*in vivo* and *in vitro* models), Delphine De Mulder and I developed the *in vitro* model, Hrag Esfahani was involved in the *in vivo* model, especially for the surgical implantation of the minipumps and telemetry devices. The majority of immunostaining of *ex vivo* tissues was performed at our IREC imaging platform (2IP), and I deeply thank Caroline Bouzin, Michèle De Beukelaere, et Aurélie Daumerie for their precious help and expertise.

RNA-seq was performed in collaboration with IREC's platform of the Centre de Technologies Moléculaires Appliquées (CTMA), with the contribution of Bertrand Bearzatto (pre-analytic and libraries generation), and Jérôme Ambroise (bioinformatic, statistics, data generation). I designed the protein-protein interaction network using Cytoscape software, and analyzed in detail (GeneCard, Pubmed,...) all candidates key genes extracted from the analysis. I also performed a lot of fine tuning or preliminary experiments (RNA-seq material extraction and quality optimization, WB and immunostaining with

various antibodies and tissues, *in vivo* and *in vitro* oxidative stress assessment, different cell types culture and models, Fourier transformation of BP variability spectra, blood HbNO concentrations by EPR spectroscopy measurements, LDL *ex vivo* oxidation,...) that for many of them were unfortunately unexploitable. As for many scientific experimental endeavors, this manuscript only contains the tip of a huge iceberg of work.

B. Articles

1. 1st author

Legacy in Cardiovascular Risk Factors Control: From Theory to Future Therapeutic Strategies? Pothen L, Balligand JL. 2021.

Antioxidants (Basel) 10 (11). doi:10.3390/antiox10111849

Sustained down-regulation of vascular smooth muscle Acta2 after transient Angiotensin II infusion: a new model of “vascular memory”.

Lucie Pothen, Roxane Verdoy, Delphine De Mulder, Hrag Esfahani, Charlotte Farah, Lauriane Michel, Flavia Dei Zotti, Bertrand Bearzatto, Jerome Ambroise, Caroline Bouzin, Chantal Dessy, Jean-Luc Balligand

Frontiers In Cardiovascular Research: 14 March 2022.
doi:10.3389/fcvm.2022.854361

2. Posters communications

ESC Basic Science Summer School Nice 14/06/15 **Metabolic memory of endothelial dysfunction: Persistent ROS production after transient exposure of endothelial cells to Angiotensin II.** Pothen L, Lobysheva I, Hermida N, Dessy C, Balligand JL

International Meeting Perspectives in vascular Biology Frankfurt 26/09/16

Stress memory of endothelial cell: persistent ROS production after transient exposure of endothelial cells to Angiotensin II Pothen L, Dei

Zotti F, Lobysheva I, Hermida N, Balligand JL

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