UNIVERSITY OF MILANO-BICOCCA
SCHOOL OF MEDICINE AND FACULTY
OF SCIENCE

PHD PROGRAM IN TRASLATIONAL AND MOLECULAR
MEDICINE - DIMET

Gene expression profiling of peripheral blood in patients with abdominal aortic aneurysm and caotid stenosis

Coordinator: Prof. Andrea Biondi
Tutor: Prof. Marialuisa Lavitrano

Dr. Laura Farina
Matr. No. 043416

XXII CYCLE – ACADEMIC YEAR 2008-2009

TABLE OF CONTENTS

INTRODUCTION

The history of cerebrovascular disease spans many centuries, although the observations that have influenced current practice were described within the last 45 years. Until then, some 55% of strokes were attributed to "vasospasm". Chiari in 1905 and a few years later Hunt, Moniz, and Hultquist, among others, described the possible association of CAD and stroke. In Prague, Chiari[1] found thrombus superimposed on carotid artery atherosclerotic plaques of 7 patients in a series of 400 consecutive autopsies. Four of these patients had suffered cerebral embolism, and he suggested that embolic material could arise from the carotid artery and affect the brain. This could be considered the first accurate description on the pathophysiology of cerebral embolism from the carotid artery. Hunt[2] n 1914 reported the clinical characteristics of 20 patients with hemiplegia but did not have autopsy data. He proposed that "the cerebral lesions in most stroke victims could be the effect and not the cause." In 1937, Moniz et al.[3] reported occlusion of the internal carotid artery in 4 patients diagnosed with angiography. Hultquist[4] in 1942 published the clinicopathologic correlation of patients with stroke in a series of 1400 autopsies. He performed examinations of the entire carotid circulation in 400 patients and found a 3% incidence of thrombosis. These important descriptions did not flourish, however, because corroboration of the ideas proposed was hindered by the absence of routine postmortem study of the carotids at a time when angiography was either not available (Chiari and Hunt) or considered too dangerous (Moniz and Hultquist). An important milestone in the history of carotid stroke occurred in 1951, when C. Miller Fisher reported the occurrence of TIAs in relation to CAD[5]. ; although Chiari, Hunt, Moniz, and

Hultquist have described the association of CAD and stroke, this received little attention until C.M.Fisher published his clinicoanatomic correlations in "Occlusion of the Carotid Arteries." The opportunity to prove that carotid occlusion was the cause of the symptoms arose when, during an autopsy, founded an occluded artery, which did not mean much in those years when the first diagnosis considered was "spasm." However, this finding caused great excitement in Dr Fisher, who had in front of him the first case in which an internal carotid artery occlusion, proved at autopsy, had been clinically suspected on the basis of characteristic premonitory phenomena of TMB and hemiplegia. All that was left was to define the mechanism by which occlusion caused the transient phenomena. At that time, Dr Fisher started collecting clinical data associating CAD and TIAs, calling attention to the variable frequency of episodes ranging from 1 to 500 in different cases. This was probably his most important contribution to the understanding of cerebrovascular disease. Also, establishing the sequential correlation of prodromal symptoms and stroke, Dr Fisher defined a therapeutic window for the administration of medications. He further speculated on the use of anticoagulation to arrest transient attacks. Although Dr Fisher referred to treatment as a "subject where there is little definite to state," he laid the foundations for what became the most important and controversial subject in CAD, carotid endarterectomy. ". . . [S]ome day vascular surgery will find a way to by-pass the occluded portion of the internal carotid artery during the period of ominous fleeting symptoms." With this statement, Dr Fisher predicted in 1951 the surgical treatment of CAD[5]. It required more

than 40 years thereafter to scientifically establish the proven benefits of carotid endarterectomy [6].

ATHEROSCLEROSIS

Despite of medical and surgical advances, atherosclerotic disease remains the leading cause of death in the Western world and it is the underlying cause of many different disease; among these, a large proportion of strokes. In the 19th century, there were two major hypotheses to explain the pathogenesis of atherosclerosis: the incrustation hypothesis, proposed by von Rokitansky in 1852, and the lipid hypothesis, proposed by Virchow in 1856 [7,8]. These hypotheses focused on fibrin deposition, lipid accumulation, and extracellular matrix formation. In addition, Virchow used for the first time the name endarteritis deformans, linking inflammation to the disease and forming the basis of the response-to-injury hypothesis of Ross more than a century later[9-11]. Today, atherogenesis is considered a complex interaction of risk factors including cells of the artery wall and the blood and molecular messages that they exchange; it also well known the prominent role of inflammation in the development of the pathology.

ATHEROGENESIS

Atherothrombosis is a systemic arterial disease originally involving mostly the intima of large- and medium-sized systemic arteries

including the carotid, aorta, coronary, and peripheral arteries. The main components of atherothrombotic plaques are[12–16]: 1) connective tissue extracellular matrix, including collagen, proteoglycans, and fibronectin elastic fibers; 2) crystalline cholesterol, cholesteryl esters, and phospholipids; 3) cells such as monocyte-derived macrophages, T-lymphocytes, and smooth-muscle cells; and 4) thrombotic material with platelets and fibrin deposition. Varying proportions of these components occur in different plaques, thus giving rise to a heterogeneity or spectrum of lesions. These components mainly affect the intima, but secondary changes also occur in the media and adventitia, including growth of vasa vasorum.

EARLY ATHEROTROMBOSIS

This is the initial phase of atherogenesisis; clinically, lesions are small, commonly seen in young people, and categorized into three types as follows (according to a simplified modification of the criteria previously set forth by the American Heart Association (AHA) Committee on Vascular Lesions[13], and more recently by Stary[17]): type I lesions, consisting of macrophage-derived foam cells that contain lipid droplets; type II lesions, consisting of both macrophages and smooth-muscle cells and mild extracellular lipid deposits; and type III lesions, consisting of smooth muscle cells surrounded by extracellular connective tissue, fibrils, and lipid deposits. As the lesion progress, calcification may then occur through mechanisms similar to those in bone formation. The molecular events characteristic of this phase are endothelial dysfunction, the lipoprotein transport and, at the end, the innate immune response to auto-antigens.

Endothelial dysfunction. The endothelium is a dynamic autocrine and paracrine organ that regulates antiinflammatory, mitogenic, and contractile activities of the vessel wall, as well as the hemostatic process within the vessel lumen[18]. A single molecule, nitric oxide (NO), is responsible for these regulatory processes[19]. A dysfunctional endothelium, characterized by decreased NO synthesis, facilitates vessel wall entry and oxidation of circulating lipoproteins, monocyte entry and internalization or inflammation, smooth cell proliferation and extracellular matrix deposition, vasoconstriction, as well as a prothrombotic state within the vessel lumen[20.21]. Endothelial dysfunction, traditionally known as the earliest manifestation of atherothrombosis, is often the result of a disturbance in the physiological pattern of blood flow (flow reversal or oscillating shear stress) at bending points and near bifurcations[22,23]. In addition to biomechanical shear forces enhanced by hypertension[24], the coexistence of other biohumoral risk factors such as hypercholesterolemia,advanced glycation end-products in diabetes and in elderly people, chemical irritants in tobacco smoke, circulating vasoactive amines, and immunocomplexes, have been associated with endothelial dysfunction[25-27]. Thus, as a response to reversal or oscillatory shear stress, endothelial cell activation is characterized by the expression of cell adhesive molecules (CAMs)from the selectin superfamily (E- and P-selectins). These proteins facilitate the homing (margination and adhesion) of the circulating monocytes to the activated endothelial cells and will facilitate the internalization of the adhered monocytes into the arterial wall, contributing to atherogenesis.

Lipoprotein transport and proteoglycans. Low-density lipoproteins (LDLs) infiltrate through the arterial endothelium into the intima[28]. This binding seems to relate to an ionic interaction of apolipoprotein B with matrix proteins including proteoglycans, collagen, and fibronectin. Proteoglycans are macromolecules located between the basement membrane of the endothelial cell and the internal elastic lamina (IEL), composed of a core protein and long-chain carbohydrates called glycosaminoglycans. The interactions between oxidized LDL and proteoglycans are crucial in early atherosclerosis, mostly related to lipoprotein retention, intravascular aggregation of LDL leading to chemical modification, and induction of inflammation[29].

Innate immune response to auto-antigens.

Development of atherosclerosis is influenced by innate and immune responses[30]. Innate immunity represents the first inflammatory response to microorganisms and pathogens, based on detection by macrophages and dendritic cells[31]. The most important receptors for innate immunity in atherothrombosis are the scavenger receptors and the toll-like receptors (TLRs)[32]. In the first line of innate immunity, the scavenger receptors SR-A and CD-36 are responsible for the uptake of oxidized LDL, transforming the macrophage into a foam cell[33,34]. Furthermore, this pathway activates the NFkappa-B nuclear transcriptional factor, triggering a potent chemoattractant cycle of monocyte migration and macrophage/foam cell formation (i.e., monocyte chemoattractant protein [MCP]-1, leukotriene LTB4, and

monocyte-colony stimulating factor [M-CSF])[35-38]. Macrophage/foam cells produce cytokines that activate neighboring smoothmuscle cells, resulting in extracellular formation and fibrosis. The second line of innate immunity, the TLRs, has gained significant recognition recently. For example, the receptor for bacterial lipopolysaccharides, TLR4, is known to recognize cellular fibronectin and heat shock proteins, endogenous peptides produced during tissue injury that may act as auto-antigens early in the disease[39-40]. The TLR4 co-localizes with fibroblasts and macrophages in the adventitia and the intima of human coronary atherothrombosis. Stimulation of TLR4-induced activation of NF-kappa-B and increased mRNAs of various cytokines[41]. Furthermore, adventitial TLR4 activation augmented neointima formation in a mouse model, suggesting a link between the immune receptor TLR4 and intimal lesion formation. More recently, TLR4 has been shown to be involved not only in the initiation but also in progression and expansive remodeling of atherothrombosis[41].

ADVANCED ATHEROTHROMBOSIS

Continuous exposure to the systemic, pro-atherogenic milieu will increase chemotaxis of monocytes leading to lipid accumulation, necrotic core, and fibrous cap formation, evolving into advanced atherosclerosis. Clinically, according to a simplified modification of the criteria previously set forth by the American Heart Association (AHA) Committee on Vascular Lesions, and more recently by Stary, advances atherotrombosis plaque can be subdivided into four relevant phases of plaque progression.

Phase 2. Lesions, although not necessarily stenotic, may be prone to rupture because of their high lipid content, increased inflammation, and thin fibrous cap. These plaques are categorized morphologically as one of two variants: type IV lesions, consisting of confluent cellular lesions with a great deal of extracellular lipid intermixed with normal intima, which may predominate as an outer layer or cap; or type Va lesions, possessing an extracellular lipid core covered by an acquired fibrous cap. Phase 2 plaques can evolve into the acute phases 3 and 4.

Phase 3. These lesions are characterized by acute complicated type VI lesions, originating from ruptured (type IV or Va) or eroded lesions, and leading to mural, non-obstructive thrombosis. This process is clinically silent, but occasionally may lead to the onset of angina[16].

Phase 4. These lesions are characterized by acute complicated type VI lesions, with fixed or repetitive occlusive thrombosis. This process becomes clinically apparent in the form of an acute coronary syndrome (ACS), although not infrequently it is silent[34,35]. About two-thirds of ACS are caused by occlusive thrombosis on a non-stenotic plaque, although in about one-third, the thrombus occurs on the surface of a stenotic plaque[13]. In phases 3 and 4, changes in the geometry of ruptured plaques, as well as organization of the occlusive or mural thrombus by connective tissue, can lead to the occlusive or significantly stenotic and fibrotic plaques.

Phase 5. These lesions are characterized by type Vb (calcific) or Vc (fibrotic) lesions that cause frequently stenosis or occlusion with associated ischemia.

The most important problem associated with the advanced atherothrobosis phase is that the lipid core expansion and the macrophage accumulation at the edge of the plaque lead to fibrous cap rupture and thrombotic complications.

Thrombotic complications

Rupture of a high-risk vulnerable plaque changes plaque geometry and triggers coronary thrombosis[13]. Such a rapid change in plaque geometry may result in acute occlusion or subocclusion with clinical manifestations of unstable angina or other ACS[42,43]. More frequently, however, the rapid changes seem to result in mural thrombus without evident clinical symptoms. Two factors—plaque-dependent thrombogenic substrate and systemic procoagulant activity—may influence the magnitude and stability of the resulting thrombus and thus, the severity of the coronary syndrome[44].

Plaque-dependent thrombogenic substrate.

Exposure of a thrombogenic substrate is a key factor in determining thrombogenicity at the local arterial site. Heterogeneity of plaque composition varies even within the same subject. Tissue factor, a small-molecular-weight glycoprotein, initiates the extrinsic clotting cascade and is believed to be a major regulator of coagulation, hemostasis, and thrombosis[45]. Tissue factor forms a high-affinity complex with coagulation factors VII/VIIa; TF/VIIa complex activates factors IX and X, which in turn leads to thrombin generation[46]. Co-localization analysis of coronary atherectomy specimens from patients with unstable angina showed a strong relation

between TF and macrophages[47]. This relation suggests a cell-mediated thrombogenicity in patients with unstable angina and ACS. Furthermore, TF is particularly present in apoptotic macrophages, highlighting the role of local TF in ACS[48]. In addition, specific inhibition of vascular TF by the use of r-tissue factor pathway inhibitor was associated with a significant reduction of acute thrombus formation in human lipid-rich plaques[49] and in pig injured plaques[50]. Such observations document the active role of TF in coronary thrombosis and open a new therapeutic strategy in the prevention of ACS[53].

Systemic procoagulant activity

The 30% of coronary thrombosis occurs at sites of superficial erosion of a fibrotic plaque. Thus, complicated thrombi in such cases may well be dependent on a hyper-thrombotic state triggered by systemic factors[8]. The proteins deeply involved in systemic procoagulant activity seems to be tissue factor(TF), C-reactive protein (CRP) and CD40L. Changes in lipid metabolism, cigarette smoking, hyperglycemia, hemostasis, and others are associated with increased blood thrombogenicity[59-62]. Elevated LDL cholesterol levels increase blood thrombogenicity and growth of thrombus[63,64]. Reducing LDL cholesterol levels using statins decreased thrombus growth by approximately 20%[64]. Smoking increases catecholamine release, potentiating platelet activation[65] and increasing fibrinogen levels[66]. Catecholamine-dependent effects may explain the increased incidence of sudden death and acute cardiovascular events after emotional and physical stress[67]. Patients with diabetes, especially those with poorly

controlled diabetes, have increased blood thrombogenicity[68-70]. Platelets from patients with diabetes have increased reactivity and hyper-aggregability and expose a variety of activation-dependent adhesion proteins[69-71]; such abnormal platelet function is reflected by increased platelet consumption and increased accumulation of platelets on the altered vessel wall[71-73]. Recent observations indicate that the thrombogenic state associated with high LDL cholesterol, cigarette smoking, and diabetes may share a common biological pathway. That is an activation of leukocyte-platelet interactions associated with release of TF and thrombin activation has been observed in these conditions[70], being more particularly studied in the diabetic population, Furthermore, reversal of such risk factors may alter such cell-cell interactions, being particularly studied with the statins[74-76]. Recent studies showed increased levels of circulating TF antigen in patients with cardiovascular disease[77] and coagulation disorders, such as disseminated intravascular coagulation[78-79]. Circulating TF antigen has been associated with increased blood thrombogenicity in patients with ACS[80] and chronic coronary artery disease[81]. Furthermore, Increased TF-positive procoagulant microparticles are present in the circulating blood of patients under pathophysiologic conditions[82]. Thus far, the cellular origin of TF-positive microparticles in the circulating blood has not been established. As described previously, atherosclerotic plaques have been shown to contain TF that is associated with macrophages within the lesion. Indeed, the predictive value of C-reactive protein (CRP) and CD40L may in part be a manifestation of such systemic phenomena; CRP, like fibrinogen, is a protein of the acute-phase

response and a sensitive marker of low-grade inflammation. It is produced in the liver as a result of mediators such as interleukin-6 generated by inflammation in the vessel wall (i.e., macrophages) or extravascularly (i.e., circulating monocytes)[83]. Increased levels of CRP have been reported to independently predict acute coronary events[84] even in people whose blood lipid values are below the median levels in the population[85-86]. Furthermore, statin therapy prevented coronary events in individuals with high CRP and relatively normal LDL cholesterol values. Of interest, the lowering effect of statin on CRP values was independent of its effect on lipid levels. Whether CRP reflects the inflammatory component of atherosclerotic plaques or of the circulating blood and whether it is a surrogate marker ora biologically active element in the process of plaque development or thrombus formation is not known[87]. However, recent studies support that CRP is an activator of blood monocyte and vessel wall endothelial cells[88-90]. This encourages further investigation into the effect of certain risk factors in the activation of inflammation of the vessel wall and circulating blood, probably leading toan active role of TF, CRP, and perhaps CD40[91,92] as local and systemic key factors in the process of atherothrombosis. Stroke is the most serious pathology related to thrombotic complications.

CAROTID ARTERY STENOSIS: CURRENT CLINICAL APPLICATION

SURGERY

Carotid endarterectomy (CEA)

Carotid endarterectomy (CEA) is a prophylactic operation. It is performed in patients who are at risk of stroke from emboli arising from atheromatous plaque at the carotid bifurcation. The indications for CEA in various circumstances were reviewed by the American Academy of Neurology[93]. There are two groups of patients to consider: symptomatic patients who have active plaque giving rise to emboli that enter the cerebral circulation and cause transient ischaemic attacks (TIAs) and reversible ischaemic neurological deficits, and asymptomatic patients who have demonstrable disease at the carotid bifurcation but no history of a recent neurological event attributable to this lesion. There is unequivocal evidence to support CEA in symptomatic patients with 70% carotid stenosis in the relevant carotid territory. For patients with a carotid stenosis of 70% or more there was absolute risk reduction for the combined outcome of perioperative death or subsequent stroke more than 5 yr of 16% yielding a number needed to treat of 6.3. The benefit in patients with 50– 69% stenosis was less marked with an absolute risk reduction of 4.6% more than 5 yr and a number needed to treat of 22. CEA was not beneficial to symptomatic patients with 30–49% stenosis or near carotid occlusion, and was harmful in symptomatic patients with 30% stenosis. Patients with asymptomatic carotid disease with 50%

stenosis are a different population to patients with 'active' plaque that is discharging emboli into the cerebral circulation. There are data to support CEA in asymptomatic patients but they are less robust than those supporting the operation for patients with symptoms. All these considerations are based particularly on two large studies, the North American Symptomatic Endarterectomy Trial (NASCET) and the European Carotid Surgery Trial (ECST), both of which compared surgery with best medical treatment[94]. Actually, CEA is performed only for symptomatic patients with stenosis of 70% or more.

Surgical procedure[95]

After careful surgical exposure, the external, internal, and common carotid arteries are cross-clamped so that the carotid bifurcation is isolated from the circulation. The artery is opened and the plaque removed. Most often this is done through a longitudinal incision and the artery is patched upon closure as this reduces the incidence of re-stenosis. Care must be taken to remove all of the debris from the intimal surface of the artery to prevent postoperative emboli occurring. The two most feared major perioperative complications of CEA are cerebrovascular accident and myocardial infarction.

Angioplasty and stanting (CAS)

Carotid artery stenting (CAS) has emerged as a promising minimal invasive treatment alternative to carotid arterectomy for patients with symptomatic and asymptomatic carotid artery stenosis. As was reported before, randomized trials have established the benefit of carotid endarterectomy (CEA) in reducing the risk for stroke in patients with moderate to severe (50%) symptomatic carotid artery

stenosis [1–3] and to a less extent in those with asymptomatic (60%) carotid stenosis[96,97]. CEA, however, is associated with short-term risks for stroke or death of about 3% in asymptomatic patients[98]and 5%in symptomatic patients[99]. As compared with CEA, carotid angioplasty and stenting. CAS is associated with the following benefits: it usually avoids the need for general anaesthesia and its complications; it avoids neck incision and subsequent cranial and cutaneous damage; it may be associated with lower costs by reducing recovery time and hospital stay; and it may be the only treatment option in patients who are at high risk after surgery because of co-morbidity. CAS however also carries risks for stroke and local complications: cardiovascular complication (vasovagal reaction 5-10%, vasodepressor reaction 5-10%, myocardial infarction), artery complication (dissection <1%, thrombosis <1%, perforation <1%, transient vasospasm 5-10%), neurological complication (stroke/TIA, intracranial haemorrhage <1%), general complication (access site injury 5%, contrast nephropathy 2%, contrast reaction 1%) .

Surgical procedure[100]

A preoperative imaging study is preferable for the assessment and planning for carotid stent placement.8 An arch aortogram and selective carotid arteriogram or a good quality magnetic resonance angiography with arch, cervical, and intracranial images may be used for evaluation of a patient's arch and carotid anatomy and CAS planning. This allows for proper selection of access catheters, sheaths, cerebral protection devices, and stents. The procedure involves the crushing of atherosclerotic plaque material against the vessel wall with a high-pressure balloon and the subsequent placement of a metal

mesh tube (stent) to hold this material back, prevent elastic recoil, and cover any dissection caused by the angioplasty procedure.

Coronary artery bypass surgery (CABS)

Surgical myocardial revascularisation by coronary artery bypass grafting (CABG), aims at restoring and normalising coronary blood flow in hypoperfused ischaemic territories by grafting arterial and venous conduits from the aorta to the diseased coronary artery beyond the stenosis. Surgical revascularisation is indicated in subjects with three- or two-vesselcoronary disease with involvement of the proximal left anterior descending (LAD) artery, left main coronary artery disease and depressed left ventricular function (left ventricle ejection fraction <45%) . About the choice of the grafts, typically the left internal thoracic artery (LITA)is grafted to the left anterior descending artery and a combination of other arteries and veins is used for other coronary arteries. The right internal thoracic artery (RITA), the great saphenous vein from the leg and the radial artery from the forearm are also frequently used[101].

PHARMACOLOGICAL THERAPY

Cholesterol-lowering drugs

Ever since the connection between cholesterol levels and risk for coronary artery desease (CAD) was established, the goal of lowering total cholesterol and low-density lipoprotein (LDL) cholesterol became a very important health issue.For this reason, a very important pharmacological class is represented by cholesterol-lowering drugs.

STATINS

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are principal therapeutic agents for the treatment of hypercholesterolemia. Several landmark clinical trials, such as Scandinavian Simvastatin Survival Study (4S)[102], Cholesterol and Recurrent Events (CARE)[103], Long-term Intervention with Pravastatin in Ischemia Disease (LIPID)[104], West of Scotland Coronary Prevention Study (WOSCOPS)[105], Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS)[106]and the Heart Protection Study (HPS)[107], have demonstrated the beneficial effects of statin therapy for primary and secondary prevention of cardiovascular disease. Because 60–70% of serum cholesterol is derived from hepatic synthesis and HMG-CoA reductase is the crucial, rate limiting enzyme in the cholesterol biosynthetic pathway, inhibition of this enzyme by statins results in a dramatic reduction in circulating low-density lipoprotein (LDL)-cholesterol. In addition, reduction of LDL-cholesterol leads to upregulation of the LDL receptor and increased LDL clearance. The lowering of serum cholesterol levels is therefore thought to be the primary mechanism underlying the therapeutic benefits of statin therapy in cardiovascular disease[102]. Statins, however, might also exert cholesterol independent or pleiotropic effects. Several studies in fact suggest that statins might be involved in immunomodulation, increasing in circulating endothelial progenitor cells, up-regulation of endothelial nitric oxide synthase (eNOS) and modulation of thrombosis and coagulation.

Immunomodulation

Major histocompatibility complex class II (MHC-II) molecules are directly involved in the activation of T lymphocytes and in the control of the immune response. Only a limited number of specialized cell types express MHC-II constitutively, numerous other cells become MHC-II positive upon induction by interferon gamma (INF-gamma). Statins act as direct inhibitors of the induction of MHC-II expression by IFN-gamma and thus as repressors of MHC-II-mediated T-cell activation. This effect of statins is due to inhibition of the inducible promoter IV of the transactivator CIITA and is observed in several cell types, including primary human endothelial cell (EC) and monocyte-macrophages. It is of note that this inhibition is specific for inducible MHC-II expression and does not concern constitutive expression of CIITA and MHC-II. MHC-II are required for antigen presentation and T-cell activation via the T-cell receptor (TCR). TCR activation may trigger both proliferation and differentiation of T cells, and influence their effector functions, such as the release of cytokines. Cytokines released by activated T cells induce further T-cell proliferation and differentiation, APC activation and B-cell antibody production. CD4+ helper T cells (TH cells) differentiate into two distinct effector cell populations. TH1 cells secrete pro-inflammatory cytokines such as IFN-γ and tumor necrosis factor. TH2 cells secrete anti-inflammatory cytokines (such as IL-4, IL-10, IL-13), but also factors that promote immediate-type hypersensitivity. A reduction of TH1 responses, a shift towards TH2-cell responses or both therefore seems desirable in diseases involving delayed-type hypersensitivity reactions, such as graft (transplant) atherosclerosis and other chronic inflammatory

pathologies[108]. Statins have the ability to do the shift from TH1 lymphocytes to TH2 lymphocytes[109].

Increase in circulating endothelial progenitor cells

Circulating bone marrow derived endothelial progenitor cells (EPCs) were shown to augment the neovascularization of ischemic tissue. In addition to lipid-lowering activity, statins reportedly promote the neovascularization of ischemic tissue in normocholesterolemic animals. A study that examined the effects of statins on the EPCs was reported recently: 15 patients with angiographically documented stable coronary artery disease (CAD)were prospectively treated with 40mg of atorvastatin daily for 4 weeks. Before and weekly after the initiation of statin therapy, EPCs were isolated from peripheral blood and counted. In addition, the number of hematopoietic precursor cells positive for CD34, CD133, and CD34/kinase insert domain receptors were analyzed. Statin treatment of patients with stable CAD was associated with a 1.5-fold increase in the number of circulating EPCs by 1 week after initiation of treatment. This was followed by sustained increased levels to 3-fold throughout the 4-week study period. Moreover, the number of CD34/kinase insert domain receptor-positive hematopoietic progenitor cells was significantly augmented after 4 weeks of therapy. Atorvastatin treatment increased further the functional activity of EPCs, as assessed by their migratory capacity. The results of this study define a novel mechanism of action of statin treatment in patients with stable CAD: the augmentation of circulating EPCs with enhanced functional activity. Given the well-established role of EPCs of participation in repair after ischemic injury, stimula-

tion of EPCs by statins may contribute to the clinical benefit of statin therapy in patients with CAD[110].

Up-regulation of endothelial nitric oxide synthase (eNOS)

It was found that in a time dependent manner, ox-LDL decreased eNOS mRNA and protein levels. It has been estabislished that statins upregulated eNOS expression and completely prevented its downregulation by ox-LDL[111]. Under inflammatory conditions, the Rho-Rho kinase pathway, which is a negative regulator of eNOS activity and mRNA stability becomes activated, resulting in reduced NO biosynthesis. Statin treatment reduces prenylation of endothelial Rho and causes a concomitant increase in eNOS activity. Statin mediated post-translational activation of eNOS has been attributed to activation of the PI3K-Akt pathway. This may also increase the binding affinity of eNOS for calmodulin, resulting in displacement of the inhibitory partner caveolin 1. Statins reduce caveolin 1 levels, decreasing its inhibitory effects on NO synthesis[112]. There is evidence that NO prevents leukocyte chemotaxis and down regulates adhesion pathways through an inhibition of adhesion molecules expression, which attenuate leukocytemigration. Inflammatory pathways such as the NFkB system as well as redox sensitive pathways and also pathways that involve vascular angiotensin converting enzyme activation—all play a crucial regulatory role in eNOS expression in the human endothelium.

Modulation of thrombosis and coagulation

The extrinsic coagulation pathway plays a central role in vivo in atherothrombosis progression. When factor VIIa encounters tissue factor (TF) exposed at the site of injury, they form extrinsic complexes on cell membranes that induce thrombosis. Impaired TF expression on cultured human macrophages, which is induced by statins, has been demonstrated in vitro and has been attributed to the inhibition of the TF gene induction. In a study that was done in 17 patients who had advanced coronary artery disease and high cholesterol, the tissue factor-initiated coagulation was assessed in blood samples collected every 30 s from bleeding-time wounds of these patients. It was found that statin treatment reduced blood clotting, and was associated with reduced rates of prothrombin activation, factor Va generation, fibrinogen cleavage, factor XIII activation, and an increased rate of factor Va inactivation. These effects are not related to cholesterol reduction and most probably are related to the beneficial effects of statins that are not related to lipid profile change[113]. Numerous experimental and clinical studies have shown that statins have beneficial effects on atherothrombosis by reducing the progression of the atheroma and the incidence of acute thrombosis-related vascular events. In particular, they increase the stability of the plaque whose rupture leads to thrombosis by exposing blood to the highly thrombogenic contents of its lipid core. Rather than reducing lipid levels (which reduces plaque size and modifies the physiochemical compositions of the lipid core), statins exert their beneficial effects by decreasing the infiltration and activity of macrophages and T lymphocytes within the plaque, and inhibiting proteolytic enzymes such as matrix metalloproteinases (MMPs),

which are thought to be responsible for the plaque rupture induced by the thinning, ulceration and fissuring of the fibrous cap.

FIBRATES

Fibrates have been in clinical use for more than 40 years. Known to be effective at reducing serum triglyceride levels and raising serum high-density lipoproteincholesterol (HDL-C) levels, their modest effect on lowering low-density lipoprotein-cholesterol (LDL-C) has relegated them to second-line agents behind statins in the CAD pharmacological treatments.

Mechanism of action[114]

Fibric acid derivatives activate the peroxisome proliferator- activated receptor-alpha (PPAR-alpha) subtype. This family of nuclear receptors, when bound to ligands, activates or represses the transcription of target genes. The alpha-subtype is predominantly expressed in liver, kidney, heart, and muscle tissue where high amounts of fatty acids are metabolized. Through activation of the PPAR-alpha genes, fibrates exert the following actions:

(1) stimulation of lipolysis;
(2) increase in hepatic fatty acid uptake and reduction of hepatic triglyceride production;
(3) increase in removal of LDL particles through the formation of particles with a higher affinity for the LDL receptor;
(4) reduction in neutral lipid exchange between very low-density lipoprotein (VLDL) and HDL;
(5) increase in HDL production and stimulation of ATP-binding cassette protein-A1-mediated reverse cholesterol transport,3,4

thereby increasing HDL-C levels approximately 15% to 25%.

Angiotensin-converting enzyme inhibitors

ACE inhibition it has been recently recognized as to influence outcomes in stable coronary artery disease (CAD), including total and cardiovascular mortality, fatal and nonfatal myocardial infarction, heart failure, revascularization and stroke. The renin-angiotensin system plays an important role in the regulation of blood pressure and it's well demonstrated that hypertension is one of the so called "risk factors" for atherosclerosis development. It evolved as a mechanism to preserve fluid volume in ancient times of limited salt supply and to maintain blood pressure and prevent ischemia under conditions of acute volume loss[115].However, excessive activation of the rennin-angiotensin system can have deleterious effects on cardiovascular and renal function. As a result, blockade of the renin-angiotensin system with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) is a key element of strategies to reduce hypertension and, consequently,cardiovascular risk[116]. Moreover, it was recently discovered that ACE system is be able to promote also the VSMCs proliferation and migration, the inflammatory state of the atherosclerotic lesion and in general to participate to the process responsible of the vulnerability of the plaque (Fig.1).

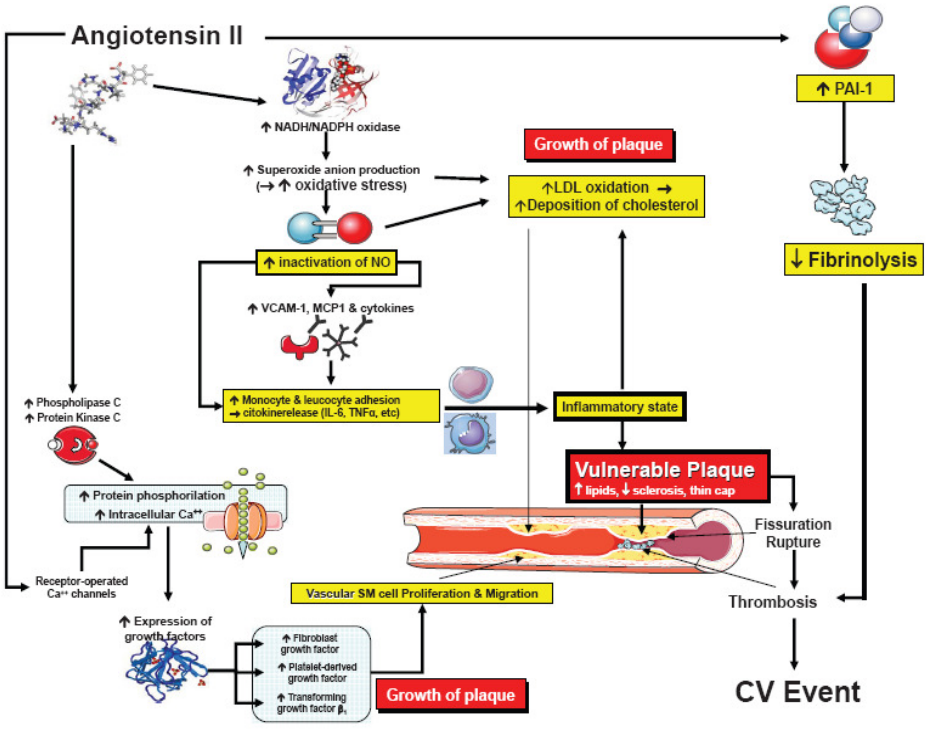


Fig1: Angoitensin participate to the process responsible of the vulnerability of the plaque

Vascular Health and Risk Management 2008:4(5) 971–981

As a result, blockade of the renin-angiotensin system with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) is a key element of strategies to reduce cardiovascular risk[116].

Anticoagulation and antiplatelet drugs

Because severe aortic plaque is associated with intravascular embolization of thrombus, the first attempts to prevent embolization in patients with severe aortic plaque involved treatment with anticoagulation. The most used drugs of this category are Eparin, an

inhibitor of trombin and Xa factor, and Aspirin, responsible for the thromboxane A2 inhibition.

Calcium channel blockers (CCB)

Drugs currently known as calcium channel blockers (CCB) were initially called calcium antagonists because of their ability to inhibit calcium-evoked contractions in depolarized smooth muscles. Blocking the entry of calcium reduces the active tone of vascular smooth muscle and produces vasodilatation. This pharmacological property has been the basis for the use of CCBs in the management of hypertension and coronary heart disease. Moreover, we know that CCB are be able to inhibit cellular proliferation and platelets aggregation by blocking L calcium channel, and expression of adhesion molecules and cytokines by blocking NF-kB transcription[117].

ACUTE ISCHEMIC STROKE

Ischemic stroke is the second most common cause of death and the leading cause of acquired disability in adults[118,119]. In western countries, stroke causes 10–12% of all deaths. The series of neurochemical processes that are unleashed by transient or permanent focal cerebral ischemia are referred to as the ischemic cascade. This is a complex series of events that evolve in time and space (Fig.2). It's a highly heterogeneous phenomenon which can be summarized as cellular bioenergetic failure due to focal cerebral hypoperfusion, followed by excitotoxicity, oxidative stress, blood–brain barrier dysfunction, microvascular injury, haemostatic activation, post-ischemic inflammation and finally cell death of neurons, glia and endothelial cells. The ischemic cascade usually goes on for hours but can last for days, even after restoration of blood circulation[120]. Although reperfusion of ischemic brain tissue is critical for restoring normal function, it can paradoxically result in secondary damage, called reperfusion injury.

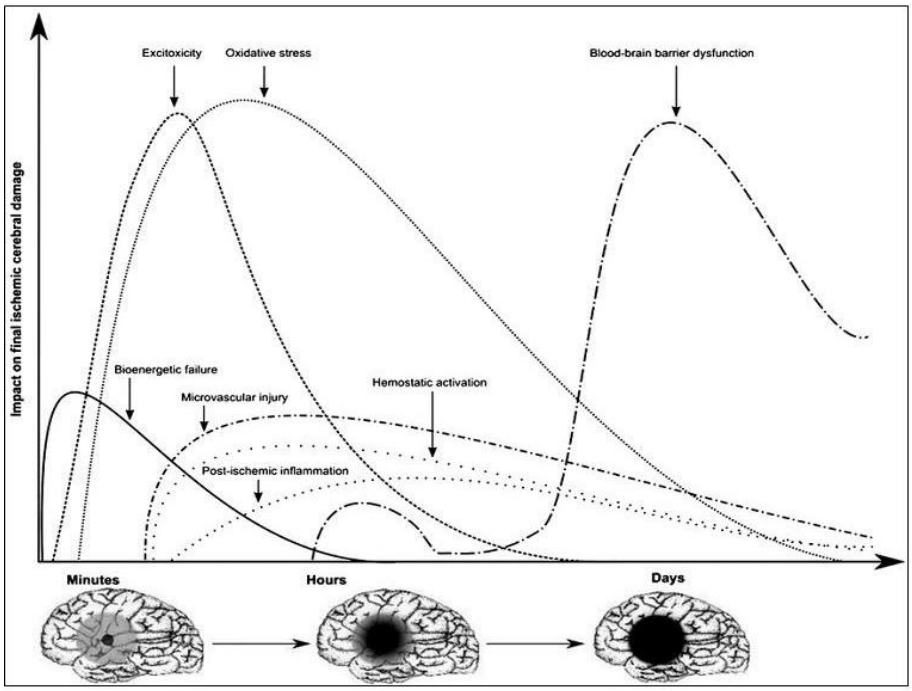


Fig. 2. Graph representing the temporal profile of the main pathophysiological mechanisms underlying acute focal cerebral ischemia and their impact on the final ischemicdamage. In absence of early reperfusion, cells in the ischemic penumbra (gray) subside due to ongoing ischemic injury, resulting in expansion of the infarcted core (black).

Clinical Neurology and Neurosurgery 111 (2009) 483–495

The ischemic core and the penumbra

Ischemic stroke begins with severe focal hypoperfusion, but cerebral injury continues over hours or even days. The amount of permanent damage will depend on two factors: the degree and the duration of ischemia. Regions of the brain with severely impaired blood flow, become rapidly and irreversibly injured and are referred to as the ischemic core[121-125]. Cells in the core are killed rapidly by lipolysis, proteolysis, disaggregation of microtubules, total bioenergetic failure and breakdown of ion homeostasis. According to the paradigm of the ischemic penumbra, a region of functionally impaired but structurally

intact tissue lies between the lethally damaged core and the normal brain (Fig. 2). The penumbra is an area of constrained blood flow with partially preserved energy metabolism[126-129]. It is reagion where the ischemic cascade with several deleterious mechanisms is triggered, resulting in ongoing cellular injury and infarct progression. However, the penumbra can be rescued by improving the blood flow and/or interfering with the ischemic cascade. Since salvage of this tissue is associated with neurological improvement and recovery, it is the target for acute stroke therapy[130,131].

MOLECULAR EVENTS IN ACUTE ISCHEMIC STROKE

Cellular bioenergetic failure

Brain tissue has a relatively high consumption of oxygen and glucose, and depends almost exclusively on oxidative phosphorylation for energy production. Focal hypoperfusion restricts the delivery of essential substrates and causes the brain cells' normal process for adenosine triphosphate (ATP) production for energy to fail. This quickly leads to dysfunction of energy-dependent ion transport pumps and depolarization of neurones and glia[130,131]. Consequently, somatodendritic as well as presynaptic voltage-dependent Ca^{2+} channels become activated and excitatory aminoacids are released into the extracellular space. At the same time, electrogenic transport of glutamate from depolarized astrocytes is induced and energy-dependent processes, among which presynaptic reuptake of excitatory amino acids, are impeded[132]. This further increases the accumulation of glutamate in the extracellular space . Additionally, reduced oxygen availability results in anaerobic glycolysis and accumulation of

lactate[133-135]. Elevated lactate levels are thought to not only to be a marker of anaerobic metabolism in stroke, but also a possible cause of secondary damage leading to infarct expansion and poor outcome, especially incortical infarction[136,137].

Excitotoxicity

Excitotoxicity refers to the secondary damage caused by pathological activation and calcium uptake by neurons due to abnormal release of excitatory neurotransmitters from dying cells[138,139]. As described above, excitatory neurotransmitters, especially glutamate, accumulate in the extracellular space. This event results in overstimulation of -amino-3-hydroxy-5-methyl-4 isoxazole propionic acid (AMPA), kainate and N-methyl-d-aspartic acid (NMDA)-type glutamate receptors on other neurons, with consequent influx of Na^+, Cl^- and Ca^{2+} ions through the channels gated by these receptors[140]. These neurons then become depolarized, causing more calcium influx and more glutamate release leading to local amplification of the initial ischemic insult. Additionally, water passively follows the ion influx, resulting in cytotoxic edema[140]and intracellular increase of the universal second messenger Ca2+ initiates a series of cytoplasmatic and nuclear events, among which generation of free radicals and activation of Ca2+-dependent enzymes. These include proteolytic enzymes that degrade cytoskeletal proteins[141]and extracellular matrix proteins[142], as well as phospholipase A2, calpain, endonucleases, adenosine triphosphatase, cyclooxygenase and nitric oxide synthase type I resulting in extensive cellular damage and generation of free radical species[143-145]. The production of free radicals and activation of

degradative enzymes leads to acute cell death through necrosis, but excitotoxic mechanisms can also initiate molecular events that lead to apoptosis. In addition, the intracellular signalling pathways activated during excitotoxicity trigger the expression of genes that initiate post-ischemic inflammation, another process that contributes to ischemic injury.

Oxidative stress

Oxidative stress occurs when the production of free radicals overpowers the endogenous scavenging capacity of cellular antioxidant defences. There is considerable evidence that reactive oxygen and nitrogen molecules are important mediators of tissue injury in acute ischemic stroke. Brain ischemia generates superoxide (O^{2-}), through xanthine oxidase and leakage from the mitochondrial electron transport chain. Superoxide is the primary radical from which hydrogen peroxide (H_2O_2) is formed. Hydrogen peroxide in its turn is the source of hydroxyl radical (OH)[146]. Nitric oxide is a water- and lipid-soluble free radical with a half-life of only a few seconds. It is produced from l-arginine by three types of nitric oxide synthases (NOS). NOS type I and III are Ca2+-dependent and constitutively expressed, respectively primarily in nerve tissue and endothelial cells. Upregulation of NOS type II (inducible enzyme) is mediated transcriptionally by a variety of cytokines[147]. Ischemia causes a surge in NOS type I and III activity in neurons and vascular endothelium, respectively. At a later stage elevated NOS type II activity occurs in a range of cells including glia and infiltrating neutrophils[148]. In the context of brain ischemia, NOS type I and II are deleterious, but

production of nitric oxide in blood vessels by NOS type III improves blood flow to the ischemic penumbra through vasodilation and inhibition of platelet adhesion, it scavenges oxygen radicals and has an anti-inflammatory effect through inhibition of leukocyte adhesion to the endothelial cell.

Inhibition of NOS by asymmetrical dimethylarginine may result in decreased nitric oxide bioavailability[149]which is associated with vasoconstriction[150,151]increased generation of free radicals[152], platelet aggregation and leukocyte adhesion on the endothelial surfaces[153], processes that in turn may aggravate cerebral ischemia. Interaction of oxygen radicals with other tissue components produces a variety of other radicals. Of particular importance is the formation of the highly toxic peroxynitrite from superoxide and nitric oxide. Peroxynitrite decomposes spontaneously to produce the hydroxyl radical. Hydrogen peroxide is lipid soluble and readily crosses cell membranes. Similarly, superoxide crosses the cell membrane via the anion channel. Therefore, remote effects of these two agents are possible. On the other hand, hydroxyl radical is themost reactive oxygen radical, probably causes the most tissue injury and is very short lived. Free radical exhibit a spectrum of cellular effects including inactivation of enzymes, release of calcium ions from intracellular stores, protein denaturation, lipid peroxidation, damage to the cytoskeleton and DNA and chemotaxis. Mitochondrial function is impaired by free radical-mediated disruption of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport, H+ extrusion and ATP production. Cytochrome *C* is released from mitochondria and provides a trigger for apoptosis.

Severe oxidative stress causes cell death through necrosis while moderate oxidation can trigger apoptosis[157-159]. Besides cerebral cellular damage, oxidative stress also increases blood–brain barrier permeability through activation of matrixmetalloproteinases (MMP), especially MMP-9[157,158], and through endothelial cell damage. Moreover, free radicals influence cerebral blood flow; they are strong cerebral vasodilators[159]and due to interaction between nitric oxide and superoxide, they alter vascular reactivity to CO_2 hereby inducing vasoconstriction instead of vasodilation. Additionally, oxygen radicals increase platelet aggregability.

Blood–brain barrier dysfunction

Blood–brain barrier (BBB) integrity protects the neuronal microenvironment. The reported incidence of BBB disruption in acute ischemic stroke varies considerably from15% to 66%, depending on stroke severity, the applied methodology and timing of the evaluation[160-163]. Several mechanisms contribute to ischemic damage of the BBB, that appears to be biphasic. Endothelial basal lamina dissolution starts as soon as 2 h after the onset of ischemia[164]and is rapidly followed by an increase in BBB permeability. Early reperfusion may temporarily alleviate BBB alterations, but the use of thrombolytic therapy and delayed reperfusion conversely may exacerbate the endothelial injury[165]. Loss of the blood–brain barrier may result from accumulation of bradykinin[166], vascular endothelial growth factor[167], thrombin[168], active matrix metalloproteinases and other protease activities. As described above, oxidative stress is an early stimulus for BBB injury and may trigger MMP-9 release by

neurons, glia and endothelial cells resulting in BBB damage through digestion of the endothelial basal lamina. After the early BBB opening, there is a second phase of severe BBB injury within 24–72 h after infarction. This phase is more complicated and results in greater tissue damage through leukocyte infiltration and marked MMP-9 release from neutrophils transmigrated to the ischemic brain. Disruption of the BBB allows leakage of blood components into the brain parenchyma. Extravasation of high molecular weight molecules is followed by water due to osmosis and leads to vasogenic edema, which may cause secondary damage through intracranial hypertension. Additionally, extravasation of red blood cells leads to hemorrhagic transformation of the infarcted area. Finally, the leaky BBB facilitates transmigration of inflammatory cells, promoting the post-ischemic inflammatory response[169].

Ischemia-induced microvascular injury

Ischemia also targets the microvasculature, which contributes to cerebral tissue damage by increased endothelial cell permeability, eukocyte-endothelial cell adhesion, matrix degradation and loss of autoregulation. Cerebrovascular autoregulation refers to the intrinsic ability of the cerebrovascular bed to maintain a constant perfusion in spite of changing blood pressure[170,171]. As cerebral perfusion pressure falls, arteriolar vasodilation reduces the vascular resistance in an attempt to maintain cerebral blood flow. Metabolic factors (hypoxia, adenosine, carbon dioxide and acidosis), myogenic processes (smooth muscle relaxation upon diminishing intravascular pressure) and endothelial mechanisms (nitric oxide, prostacyclin and endothelin-1)

can be responsible for the vasodilation[172,173]. The pathophysiology of impaired cerebrovascular autoregulation in acute ischemic stroke is still controversial, but ischemia-induced endothelial damage may play a role. Endothelial injury reduces the release of nitric oxide and prostacyclin, and may induce endothelin-1 production. These processes lead to increased vascular tone which may further impair blood flow in the area of the cerebral infarction and collateral vessels, hereby enhancing the ischemic injury. Additional decrease in nitric oxide bioavailability may be caused by inhibition of NOS by asymmetrical dimethylarginine. Endothelin-1 is a highly potent vasoconstrictor[174]to which cerebral microvessels show marked sensitivity. Plasma levels of endothelin-1 are elevated in ischemic stroke[175-176] and are associated with cerebral edema[177]. In addition to vasoconstriction, dysfunctional autoregulation leaves the vulnerable ischemic penumbra unprotected against potentially harmful blood pressure changes. Hypotension during acute stroke is known to be detrimental to tissue injury and hyper-tension may improve outcome after stroke in some patients, but may be harmful in others[178-182]. The cerebral microvasculature rapidly displays multiple dynamic responses to focal ischemia, among which presentation of leukocyte adhesion receptors on endothelial cells. This not only is an essential step in the post-ischemic inflammatory response, but also contributes to the "no-reflow" phenomenon, which is the obstruction of the downstream microvascular bed after reperfusion of the occluded supply arteries. It is attributed to extrinsic compression from edema, endothelial swelling and intravascular obstruction due to local activation of leukocytes, platelets and coagulation[183,184].

Haemostatic activation: hypercoagulable state and platelet activation

Endothelial cell injury results in exposure of tissue factor to blood. Subsequently, this substance acts in concert with Factor VIIa and phospholipid to convert Factor IX to IXa and Factor X to Xa. Factor Xa is the active catalytic component of the "prothrombinase" complex which converts prothrombin to thrombin[185]. Thrombin cleaves fibrinopeptides from fibrinogen, allowing the resultant fibrin monomers to polymerize, and converts factor XIII to XIIIa, which cross links the fibrin clot[186]. The fibrin molecules aggregate, trapping platelets, clotting factors and erythrocytes to form the clot. Activation of procarboxypeptidase U, also denoted thrombin activatable fibrinolysis inhibitor, by thrombin, plasmin or the thrombin/thrombomodulin complex results in carboxypeptidase U which attenuates fibrinolysis[187]. Marked decrease in procarboxypeptidase U activity occurs in the first 72 h after ischemic stroke[188] and in patients with poor response to thrombolytic therapy, probably reflecting stronger activation of the procarboxypeptidase U/carboxypeptidase U pathway and thrombus propagation[189]. Under conditions of ischemia and high shear stress, platelets are activated. Activated platelets accumulate in microvessels within 2 h of vascular occlusion. They release a variety of biochemical mediators, catalyse interactions between coagulation factors and contribute to the "no-reflow phenomenon"by adhering to both leukocytes and microvascular endothelial cells[190-193]. Additionally, platelets can cause temporary vasospasm by releasing thromboxane A2 and free radicals

and they may exacerbate the inflammatory cascade by releasing chemotactic mediators for leukocyte migration.Whether these mechanisms are important in ischemic stroke remains to be elucidated. In acute ischemic stroke, the endogenous fibrinolysis is usually outweighed by ongoing activation of the coagulation cascade and platelet activation[194]. This is reflected in elevated levels of hemostatic indicators among which D-dimer, fibrin monomer, thrombin–antithrombin III complex and fibrinopeptide1.2.

Post-ischemic inflammation

A strong inflammatory reaction follows focal cerebral ischemia. Several cell types contribute to post-ischemic inflammation. First of all, microglia and astrocytes are activated by reactive oxygen species. Astrocytes are capable of secreting inflammatory factors such as cytokines, chemokines and inducible nitric oxide synthase[195]. On the other hand, they express major histocompatibility complex and costimulatory molecules, which are associated with anti-inflammatory responses[196]. Microglia are the resident macrophages of the brain and play a critical role as resident immunocompetent and phagocytic cells in the central nervous system[197]. After activation by ischemia, microglia can transform into phagocytes and they can release of a variety of substances many of which are cytotoxic and/or cytoprotective. Within 4–6 h after ischemia onset, circulating leukocytes adhere to vessel walls and migrate into the brain with subsequent release of more pro-inflammatory mediators and secondary injury of potentially salvageable tissue in the penumbra. Neutrophils are the earliest leukocyte subtype to infiltrate areas of

brain ischemia[198]. Data on the role of lymphocytes are conflicting and infiltration of monocytes starts only after a delay of several days[199]. Adhesion molecules play a pivotal role in the infiltration of leukocytes into the brain parenchyma[200]. The interaction between leukocytes and the vascular endothelium is mediated by three main groups of cell adhesion molecules: selectins, the immunoglobulin superfamily and integrins. Selectins, especially E- and P-selectins are upregulated and mediate leukocyte rolling and recruitment during the early stages of ischemia[201]. Among all the immunoglobulin family members, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 have been the most extensively investigated in cerebral ischemia. Within hours after stroke onset, intercellular adhesion molecule-1 expression increases upon stimulation by cytokines[202]. The role of vascular cell adhesion molecule-1 in stroke is less clear[203].

Leukocyte integrins are expressed on the cell surface and activated by chemokines, cytokines, and other chemoattractants. Cytokines are important inflammatory mediators that are produced by immune cells and resident brain cells after ischemia[204]. The most studied cytokines related to inflammation in stroke are interleukin-1, tumor necrosis factor alpha, interleukin-6 and interleukin-10[205,206]. Interleukin-1 is the main proinflammatory cytokine, whereas tumor necrosis factor-alpha has pleiotropic functions and may influence apoptosis or survival through different pathways[207]. Interleukin-6 is largely thought of as a pro-inflammatory cytokine, but its role in ischemic stroke is more obscure. Increased production of pro-inflammatory cytokines and lower levels of the anti-inflammatory interleukin-10 are related to larger infarctions and poorer clinical outcome[208]. Chemokines are

important for cellular communication and inflammatory cell recruitment. Expression of chemokines such as monocyte chemoattractant protein-1, macrophage inflammatory protein-1 and fractalkine following focal ischemia is thought to have a deleterious effect by increasing leukocyte infiltration[209]. In addition to chemotactic properties, chemokines were found to directly affect blood–brain barrier permeability[210]. The inflammatory cascade also includes upregulation of several enzymes. The arachidonic acid cascade is initiated via the activation of phospholipase A2 secondary to elevated intracellular calcium levels[211]. This enzyme hydrolyses glycerophospholipids to release arachidonic acid, which is metabolised to prostaglandins or leukotrienes, respectively by cyclooxygenase or lipoxygenase. There are two forms of cyclooxygenase; type 1 is constitutively expressed during in many cell types, including microglia and leukocytes[212]. Cyclooxygenase type 2 is upregulated after ischemia and exerts toxic effects[213], mainly through prostaglandins rather than reactive oxygen radicals, even though it can generate both[214]. There is limited knowledge on the role of the lipoxygenase pathway in brain ischemia.

Leukotrienes are potent chemoattractants and are implicated in blood–brain barrier dysfunction, edema and neuronal death[215]. I have already described how nitric oxide synthases are upregulated in ischemia, especially in circulating leukocytes, microglia and astrocytes and causes damage through several mechanisms. Inflammatory cells also generate reactive oxygen species and produce matrix metalloproteinases inducing more damage to the ischemic brain.

Ischemia-induced cell death

Ischemic injury produces necrosis, a rapid form of cell death associated with failure of the plasma membrane and cytotoxic edema of both the cell and internal organelles[216]. If the cell dies through necrosis, it releases more glutamate and toxins into the environment, affecting surrounding neurons. In parallel, many brain cells undergo apoptosis, which is the consequence of a genetically regulated programme that allows cells to die with minimal inflammation or release of genetic material. Several factors determine which process predominates, including the local degree of ischemia, cell maturity, the concentration of intracellular free Ca^{2+} and the cellular microenvironment . Activation of glutamate receptors may promote apoptosis by inducing sufficient cellular injury to activate cellular sensors linked the apoptosis cascade[217-220]. In addition or alternatively, early mitochondrial production of reactive oxygen species, reduction of intracellular $K^{+,}$ and enhancement of toxic Zn^{2+} influx may trigger apoptosis[221-223]. Caspase-mediated apoptosis is initiated by release of cytochrome from mitochondria, through activation of the apoptosome complex, which in turn activates caspase 3[224]. Activated caspases are protein-cleaving enzymes that modify crucial homeostasis and repair proteins. Especially caspases 1 and 3 seem to play a pivotal role in ischemia-mediated apoptosis but other caspase-family members might be important in the late stages of cell death. Finally, caspase-independent programmed cell death appears to be a complex program that is distinct from the above mentioned necrosis and apoptosis. It also plays a significant role in delayed neuronal death following ischemic stroke through mitochondrial proteins such as apoptosis

inducing factor and Bcl-2/adenovirus E1B—interacting protein[225]. Necrosis is the predominant mechanism that follows acute, permanent vascular occlusion, whereas milder injury, particularly within the ischemic penumbra often results in apoptosis.

Cerebral reperfusion injury

Prompt restoration of the blood supply can reduce infarct size through salvation of the penumbra and can improve clinical outcome in patients with ischemic stroke[226]. Paradoxically, reperfusion may exacerbate the brain injury and produce a so called cerebral reperfusion injury[227]. This can be defined as a deterioration of ischemic but salvageable brain tissue after reperfusion and has a multifactorial etiology[228]. Early recanalisation in patients with severe ischemia also carries an increased risk of reperfusion related brain hemorrhage, which may be due to microvascular injury. Leukocytes appear to play a critical role in reperfusion injury through damaging of the endothelium, obstruction of the microcirculation, disruption the blood–brain barrier and infiltration in the brain tissue where they release cytokines and propagate inflammation[229]. Platelets play a synergistic role with leukocytes in reperfusion injury via the "no-reflow phenomenon" and release a variety of biochemical mediators that may lead to vasospasm and exacerbation of oxidative stress and the inflammatory cascade[230]. Further, experimental studies have shown that complement activation is an important component of reperfusion injury through the formation of several inflammatory mediators and the membrane attack complex[231]. Finally, breakdown of the blood–

brain barrier and post-ischemic hyperperfusion may cause vasogenic brain edema and hemorrhage[232].

CURRENT CLINICAL APPLICATIONS

The management of acute ischemic stroke has undergone a dramatic change since the introduction of stroke care units and treatment with intravenous rt-PA. The main two concepts behind these are neuroprotection and early recanalisation. Despite these developments, the actual therapeutic arsenal for acute ischemic stroke is highly limited and up to date, no new treatment has been proven to be efficacious and safe in randomised clinical trials[233]. Nevertheless, at least 25 clinical phase II and III trials are currently recruiting patients for the evaluation of newtherapeutics for acute ischemic stroke[234]. Most of them are based on key biochemical and molecular mechanisms underlying acute focal cerebral ischemia, including improvement of focal blood flow, optimising aerobic glycolysis, interventions in deleterious processes like excitotoxicity, oxidative stress, microvascular injury or inflammation, and finally prevention of cerebral cell death or support of brain regeneration. The diagnostic and prognostic value of numerous biochemical substances has been evaluated in animal models and in stroke patients and some biochemical markers was assessed in acute ischemic stroke patients (Brouns, De Deyn, 2009). Most of these biomarkers are believed to reflect a relevant pathophysiological process in acute focal cerebral ischemia, thereby contributing to a better understanding of the underlying disease mechanisms. But until now, no biomarker with

sufficient specificity and sensitivity has been identified to justify its use in routine clinical practise[235]. Ideally, biomarkers for this indication should be based on a blood test that easily can be obtained at the patient's bedside with limited cost. However, development of these biomarkers for ischemic stroke faces peculiar difficulties, including the blood–brain barrier which substantially slows their release into blood and the limited specificity since many potential blood markers of cerebral ischemia also are elevated in conditions that not rarely co-occur with stroke (for instance myocardial infarction) or mimic stroke (like brain infection). Nevertheless, a reliable biomarker or a set of biomarkers would improve the accuracy of acute stroke diagnosis, enable better patient selection for certain stroke treatments, serve as early indicators of therapeutic efficacy in clinical trials and allow more adequate prediction of long-term stroke outcome

ABDOMINAL AORTIC ANEURYSM

In addition to atherosclerosis, one of the major disease processes affecting the aorta are aortic aneurysms: this disease account for nearly 16,000 deaths annually. Aortic aneurysms tend to expand asymptomatically until a catastrophic event occurs such as aortic rupture or dissection, which is associated with a high degree of morbidity, mortality, and medical expenditure. The most common location for aneurysms is the infrarenal abdominal aorta, followed by the ascending thoracic aorta. Abdominal aortic aneurysm (AAA) is defined as a permanent segmental dilatation of the abdominal aorta. Diagnosis is typically performed by non-invasive imaging methods, and an abdominal aorta of 3 cm or larger in maximal diameter is generally considered to indicate aneurysm formation. Ultrasound screening studies suggest that the prevalence of AAA is 5% in the adult population over 60 years of age, and an increase in the detection of AAA will be observed in the next decade[236].

PATOPHYSIOLOGY OF AAA FORMATION

The pathological features of AAA are characterized by chronic aortic wall inflammation, destruction of the elastic media, neovascularization, and depletion of vascular smooth muscle cells (VSMC). Recent studies have revealed that a number of molecular mediators and extracellular matrix-degrading proteinases contribute to the pathological process of aortic wall degradation, and the

histological changes in the aneurysm wall are thought to result from complex interactions among these factors.

Inflammation in aortic wall

Chronic inflammation of the aortic wall plays an important role in the pathogenesis of AAA. Studies of human AAA tissue have shown extensive inflammatory infiltrates containing macrophages and lymphocytes in both the media and adventitia, and increasing aneurysm diameter was associated with a higher density of inflammatory cells in the adventitia[237].Activated macrophages are the main cells secreting various proteases, leading to the disruption of the orderly lamellar structure of the aortic media. The trigger of inflammation is not clear, but angiotensin II(Ang II) is considered to be one of the factors inducing aortic inflammation. Ang II is the main effector peptide in therenin–angiotensin system (RAS) and exerts pro-inflammatory actions through an increase in the expression of several mediators including leucocyte adhesion molecules and chemokines. Sustained infusion of Ang II leads to aneurysmal lesions in the atherosclerosis-prone ApoE-/- mouse, without the presence of systemic hypertension, and the initial identified event in AAA formation is the medial accumulation of macrophages[238,239].Therefore, Ang II has emerged as a central factor in the initiation and progression of AAA. Infiltration of lymphocytes is also associated with AAA formation. The dominant lymphocytes are Th2-restricted CD3+ lymphocytes expressing IL-4, -5, -8, and -10 and tumour necrosis factor (TNF)-a for the regulation of the local immune response.14 Infiltrated immune cells, especially Th2-type lymphocytes, release Fas

ligand and FAP-1 as well as cytokines, leading to the apoptosis of VSMC[240]. Recently, the accumulation of mast cells has been identified in the outer media and adventitia in the human aneurysm wall. Moreover, degranulated mast cells were increased in the aneurysm wall compared with atherosclerotic aorta[241]. Mast cells synthesize and release several proteases, pro-inflammatory cytokines, growth factors, and chemokines, such as chymase and cathepsin G. Numerous studies suggest that these mediators induce adventitial inflammation, apoptosis of VSMC, activation of matrix metalloproteinases (MMPs), and neovascularization in the arterial wall[242-244]. In addition, mast cells promote the activation of T lymphocytes and macrophages by releasing pro-inflammatory cytokines[245]. Indeed, mast cell-deficient mice showed resistance to aneurysm formation.Therefore, the accumulation of mast cells is thought to be an important factor in AAA formation.

Proteolysis of extracellular matrix proteins

Aneurysm development involves a complex remodelling process with an imbalance between the synthesis and degradation of connective tissue proteins. Various extracellular proteinases participate in the process of the destruction of the human aortic wall; in particular, MMPs are considered to be the predominant proteinases. Several MMPs have been focused on in AAA, including four that degrade elastic fibres (MMP-2, -7, -9, and -12), several that degrade interstitial collagen (MMP-1, -2, -8, -13, and -14), and others that degrade denatured collagen (MMP-2 and -9)[246-251]. Particularly, MMP-2 and MMP-9 have attracted interest in the process of AAA development.

Patients with AAA have elevated MMP-2 and MMP-9 protein levels in the vasculature remote from the aorta, and the increase in these proteins was correlated with aneurysm diameter[252-254].The activation of MMPs is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs), and mRNA levels of TIMPs were decreased in AAA tissue[255]. Other proteases are also reported to contribute to the initiation and progression of AAA. Upregulation of cysteine proteases is detected in the aneurysm wall as well as in atherosclerosis. Cathepsins are members of cysteine proteases and are regulated by the inhibitor cystatin C. Abisi et al. [256] reported that the activities of cathepsin B, H, L, and S were significantly higher, and the level of cystatin C was lower in the aneurysm wall than in the aortic wall of occlusive aortic disease. In addition, a clinical study of AAA demonstrated that increased AAA diameter correlated negatively with serum cystatin C level[257].

Production of extracellular matrix proteins

Elastin and collagens are the major structural components of the aortic wall. Collagens are responsible for tensile strength and prevent aneurysm rupture. The major fibrillar collagens in the aortic wall are type I and III. The turnover of type III collagen was enhanced in the serum and aneurysm wall tissue of patients with aneurysms.However, the newly synthesized collagen was mainly present in the media and resulted in impaired fibril formation of the aneurysm wall.In contrast, the production of pro-collagen type I was maintained at a low rate in serum and in the aneurysm wall[258,259]. Elastic fibres maintain the structure of the vascular wall against haemodynamic stress, resulting

in the prevention of aortic dilatation. In adults, elastin turnover is slow and its production is almost absent. To maintain a steady state of synthesis, various factors participate in the downregulation of elastin synthesis, but a recent study revealed that macrophages and VSMC begin to synthesize elastin in the human aneurysm wall[260].

Oxidative stress

Recent studies suggest an association of oxidative stress with the formation of AAA. Several stimuli enhance reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, leading to cell and tissue damage in many physiological conditions. In human studies, ROS and RNS were increased in the aneurysm wall compared with the normal aorta and adjacent non-aneurysmal aortic wall[261]. Infiltrated inflammatory cells are the main source of ROS production such as O2 and H2O2 through the upregulated activity of NADPH oxidase. In addition, pro-inflammatory cytokines,mechanical stretch, growth factors, and lipid mediators might upregulate NADPH oxidase in resident vascular cells, resulting in an increase in the production of ROS and lipid peroxidation products[262].Overexpressed ROS and NO increased the expression of MMPs through the activation of nuclear factor-kappaB (NFkB) and induced apoptosis of VSMC in the aneurysm wall.

CURRENT CLINICAL APPLICATIONS

SURGERY

Despite improvement in the surgical treatment and peri-operative care, AAA is among the 15 leading causes of death in the USA.The high mortality of AAA is mainly due to aneurysm rupture. Maximum diameter of AAA is thought to be the most important predictor of AAA rupture[263,264]. As patients with a large AAA, at least 5.5 cm in diameter, have an increased risk of rupture, elective surgical (OSR) or endovascular repair (EVAR) is performed in these patients to prevent rupture. In contrast, despite gradual expansion, AAAs with a diameter <5.5 cm, small AAAs, have a low risk of rupture, and there is currently no well defined treatment strategy for them. Although a large number of asymptomatic patients with AAA have been detected during routine abdominal screening, 90% of these patients have an aneurysm diameter of 5.5cm[265,266]. Interestingly, two large randomized trials reported that survival was not improved by elective surgical repair of small AAA[267,268].Therefore, these patients do not receive effective treatment for the early stage of AAA.

OSR has been considered the gold-standard for prevention of AAA rupture and death. However, it has the mortality risk of major vascular surgery with perioperative complication of about 32% including myocardial ischemia, respiratory failure, renal failure, ischemic colitis, spinal cord ischemia and prosthetic graft infection. During OSR, a vascular graft, comprised of a non-textile synthetic material or

a woven synthetic textile that is usually sealed with collagen, gelatine or albumin is sutured into the aorta.

EVAR(Endovascular repair) consists of the placement of a graft across the aneurysm and fixation to the normal aortic and iliac wall with stents at both ends. Endovascular access is via a transfemoral or transiliac artery approach with the aim of excluding the aneurysm by this graft. The advantages of an endovascular approach include decreased blood loss, length of hospitalization, and early morbidity and mortality. Endovascular therapy is ideally suited for the elderly, higher-risk patient and for those with prior aortic operations. Properly selected patients have a relatively low incidence of secondary problems including migration, endoleak, and sac expansion.

PHARMACOLOGICAL THERAPY

On the basis of increasing evidence of the molecular mechanisms in the process of AAA formation, numerous strategies have been proposed to prevent AAA development. Pharmacological therapy has also been regarded as an effective approach for treating AAA, and some agents have undergone clinical trials. Potential targets for AAA treatment are as follows:

- inhibition of proteolytic activity
- inhibition of inflammatory response
- suppression of oxidative stress
- upregulation of synthesis of extracellular matrix proteins.

STATINS

Despite the absence of a clear relationship between serum cholesterol level and AAA growth rate, statin therapy is expected to prevent AAA development, because the pleiotrophic effects of statins include an anti-inflammatory effect, anti-oxidative effect, and the reduction of MMP secretion. In experimental studies, simvastatin suppressed AAA progression in a mouse model, accompanied by a reduction of MMP-9 and an increase of TIMP-1, whereas inflammatory cell infiltration was not inhibited[269,270].In an ex vivo human organ culture system, the application of cerivastatin reduced the tissue level of MMP-9 in a concentration-dependent manner, accompanied by the inhibition of the activation of infiltrated inflammatory cells[271].These results suggest that, at least in part, distinct statins affect different signal transduction pathways to prevent AAA progression. In addition, several observational studies have shown beneficial effects of statins in patients with AAA[272,273].

ANGIOTENSIN-CONVERTING ENZYME-INHIBITHORS AND ANGII RECEPTOR BLOCKERS

Both angiotensin-converting enzyme (ACE)-inhibitors and Ang II receptor blockers (ARBs) are currently widely used in the treatment of cardiovascular disease, such as hypertension and chronic heart failure. In accordance with increasing evidence of an association between Ang II and AAA formation, several studies have been performed to examine the effect of blockade of RAS at several points on the formation of AAA. Liao et al.[274]reported that three different ACE-

inhibitors (captopril, lisinopril, and enalapril), but not an ARB (losartan), suppressed the development of elastase-induced AAA in rats. These therapeutic effects of ACE-inhibitors were observed independent of their lowering of arterial blood pressure and inflammatory response in the arterial wall. Moreover, recent clinical evidence have demonstrated that treatment with valsartan, an ARB, significantly prevented the progression of experimental AAA through the inhibition of NFkB activation, MMP expression, and infiltration of macrophages[275].These results suggest that ACE-inhibitors and ARBs might be useful for treating AAA.

NOVEL THERAPEUTIC STRATEGIES FOR TREATING AAA

On the basis of basic investigations, many researchers have searched for new molecular targets, and novel therapeutic strategies have been proposed to treat AAA.

Inhibitors of mast cell degranulation

The effects of mast cell stabilization on AAA formation were examined in an experimental study. Application of disodium cromoglycate, an inhibitor of mast cell degranulation, reduced aortic expansion by 40% in an elastase-induced mouse AAA model, accompanied by the inhibition of recruitment of mast cells and macrophages[276].Similarly, Tsuruda T et al.[277] demonstrated that treatment with tranilast attenuated AAA progression in a CaCl2-induced rat AAA model. These mast cell stabilizers have been used clinically to control allergic disorders, such as bronchial asthma. Therefore, the impact of mast cell stabilizers on AAA formation needs to be clarified in a clinical study.

c-Jun N terminal kinase inhibitor

Recently, pharmacological inhibition of c-Jun N terminal kinase (JNK) has been reported to regress AAA[278].The authors demonstrated that phosphorylated JNK was elevated in human AAA tissue, leading to the activation of MMP-9 and pro-inflammatory signalling in VSMC. In addition, selective inhibition of JNK using a specific inhibitor not only prevented AAA formation, but also caused the regression of established AAA in CaCl2-induced mouse AAA and AngII-induced ApoE-/- mouse AAA models. Importantly, in addition to the suppression of MMP activation and migration of inflammatory cells, the inhibition of JNK restored the architecture of aortic tissue. They demonstrated that the activation of JNK suppressed genes encoding extracellular matrix biosynthetic enzymes in cultured rat VSMC. This study showed an important role of the upregulation of extracellular matrix protein synthesis in the treatment of AAA. Thus, a clinical trial using a JNK inhibitor is eagerly anticipated, because a JNK inhibitor might regress AAA in humans as well as in an animal model.

AIM OF THE WORK

As we discussed, despite medical and surgical advances, atherosclerotic and aneurysmatic diseases remain the leading cause of death in the Western world. Presently, the factors responsible for the onset and the development of the atherosclerotic plaque and the aneurysmatic lesions are still unknown and only in some cases they are related to the so called risk factors (hypertension, diabetes, dyslipidemya, smoke, obesity, stress, inactivity, age, gender). Since the incomplete understanding of the etiopathogenesis of these pathologies, remains difficult to assess the optimal treatment according to the disease state. About atherosclerosis, in particular, the lesions with associated high risk of developing stroke are today still difficult to identify especially for asymptomatic patients.

In fact, here are several reasons why isn't possible to predict the behaviour of carotid atheroma in asymptomatic patients:

- Plaque rupture may remain an 'asymptomatic' phenomenon, either because systemic anti-coagulant factors minimise thrombus development or because thrombus embolises to a 'clinically silent' area of the brain.
- Multiple atheromatous plaques are commonly present in combination. Studies in the coronary circulation have demonstrated that patients often have more than one ruptured plaque in the artery supplying an infarcted myocardium, although usually only one site has evidence of thrombosis. Accordingly, any technique for identifying rupture prone plaque may have difficulty in identifying the most clinically important anatomical lesion.
- Any plaque features that are to be useful in identifying patients at high risk of stroke must be present for a sufficient time period before

the neurological event occurs in order to permit detection and treatment. Some of the pathological features linked with symptomatic plaques (macrophage accumulation and proteolytic enzyme release) are likely to be present only shortly before fibrous cap rupture.

In order to reduce the percentages of death and disability caused by rupture of the atherosclerotic or aneurysmatic lesions, would be useful the identification of susceptibility genes and the correlation between imaging data related to pathological factors (such as plaque echolucency, and molecular data) in order to assess a pre-symptomatic diagnosis and more specific treatments.

So, the aim of this work was to characterize patients gene profiles in order to understand the genetic factors involved in the pathologies and to identify new potential biomarkers and therapeutic target. We studied the expression levels of some genes related to lipid metabolism and inflammation. Our final goal was to try to develop a Clinical Decision Support system to support the decision process about the therapeutic strategy (surgical, pharmacological) by means of statistical models of the pathology based on the analysis of the heterogeneous data sources: molecular analysis, clinical analysis, clinical imaging, patient anamnesis.

METHODS

BLOOD SAMPLES COLLECTION

Blood samples were taken from patients right before the surgical procedure and collected in five BD Vacutainer® sterile tubes :

3 tubes with citrate buffer (blue cap)

1 tube with EDTA (violet cap)

1 tube with spray-coated silica and a polymer gel for serum separation (yellow cap)

Then, each sample was centrifuged:

samples contained in the violet and yellow cap tubes were centrifuged at 3000 rpm for 15' and the resulting supernatant was collected in 2ml tubes and stored for further analysis.

samples contained in the blue cap tubes were centrifuged at 1500 rpm for 15'(in order to preserve the cellular integrity), the resulting supernatant collected and the corpuscolate fraction used for PBMCs extraction.

Separation of blood leukocytes (PBMCs) by Ficoll gradient

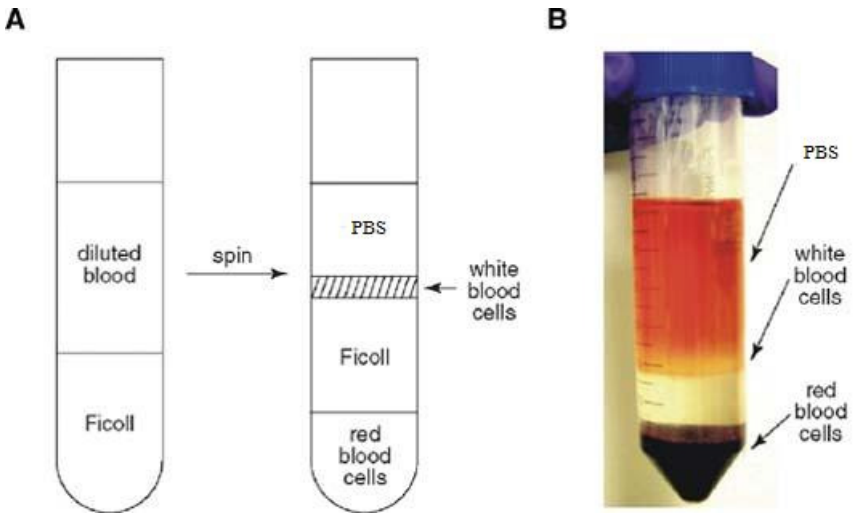
The corpuscolate fraction obtained from centrifugation was added with an equal volume of PBS, and the mix stratified on a Ficoll volume equivalent to 1/3 of the total mix volume. After centrifugation for 30' it's possible to see in the tube a separation of different phases:

red blood cells phase

Ficoll phase

White blood cells phase

PBS phase



The white blood cells ring was accurately collected and washed with PBS added with erythrocyte lysing solution, then the cellular suspension was centrifuged for 15' at 1500 rmp, and finally the resulting pellet was added with PBS for viability assays. Cell viability was evaluated adding 3ul of Trypan Blue to an aliquot of cell suspension, and cells was counted in a Burker Chamber.

Assessed the viability of the white blood cells, cellular suspension was washed once more time with PBS, the pellet was added with 2ml of FBS/DMSO (20%) solution and then stored in liquid nitrogen.

CLINICAL AND HAEMATOCHEMICAL DATA COLLECTION

In collaboration with the Vascular Surgery Unit - S.Gerardo Teaching Hospital, Monza, we have collected for each patient information about the anamnesis, the recent clinical history and the follow up after surgical treatment; we have so created a clinical database useful to accurately classify patients and also to define different experimental subgroups in order to perform the following analysis.

ANAGRAPHIC DATA

- First name and Second name, associated to a numeric code for privacy reasons
- Date of birth
- Date of surgery
- Age
- Gender
- Type of surgery

HAEMATOCHEMICAL DATA

- WBC
- RBC
- Platelet count
- total haemoglobin concentration
- creatinemia
- glycemia
- triglycerides

cholesterolemia

HDL

LDL

RISK FACTORS

hypertension

diabetes

dyslipidemia

tabagism

OTHER PATHOLOGY

CAD (Coronary Artery Disease): pathology characterized by insufficient coronary flow due to coronary atherosclerotic lesions.

PAD or PVD (Peripheral Artery/Vascular Disease): pathology caused by the obstruction of arms and legs large arteries due to atherosclerosis, inflammatory processes leading to stenosis and thrombi formation.

COPD (Chronic Obstructive Pulmonary Disease): generally refers to chronic bronchitis and emphysema, leads to a limitation of the pulmonary air flow and it's caused by a pulmonary chronic inflammation.

GSM VALUE (only for patients with carotid stenosis)

GRAY-SCALE MEDIAN (GSM) AND THE ECHOLUCENCY EVALUATION

ECHOLUCENCY PREDICT THE RISK OF STROKE

Stroke is defined as a nontransient, acute neurological injury resulting from disruption of blood flow to cerebral tissue. The disruption of blood flow is thought to be a result of embolization arising from degenerative breakdown or thrombotic occlusion of complex plaques in the extracranial vessels,areas that are readily accessible to ultrasound imaging. The spectrum of clinical presentation is probably related to the size of the embolus, its composition, its propensity to disaggregate, and the state of the collateral circulation. The European Carotid Surgery Trial 6 and North American Symptomatic Carotid Endarterectomy Trial 7 have shown the benefit of carotid endarterectomy in symptomatic patients with a high-grade stenosis (70%). Despite the relatively higher risk associated with high-grade stenoses, evaluated in absolute numbers most patients experiencing neurological symptoms have stenoses of 50%.

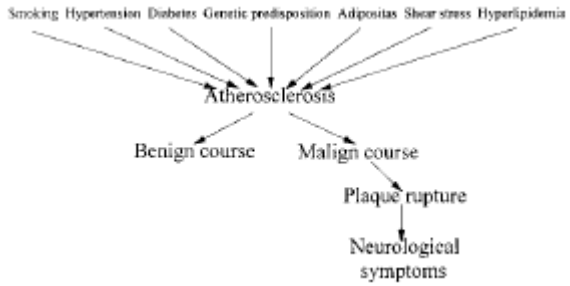


Fig.3: predisposing factors and sequelae of unstable atheromatous plaques

Marie-Louise M. Grønholdt Arterioscler Thromb Vasc Biol 1999;19;2-13

Recent studies support the notion that not only the degree of stenosis but also the morphology of the carotid artery plaque, as evaluated with ultrasound B-mode imaging, and surface characteristics like ulceration may be of pathogenetic importance. Concerning surface characteristics, ulceration is believed to be important because it causes exposure of the thrombogenic layers of the plaque, with the possibility of subsequent thrombus adhering to the plaque, leading to embolus formation and resultant neurological symptoms. Unfortunately, there is no standardized definition of ulceration. Ulceration can be classified from ultrasonic and angiographic findings or from macroscopic or microscopic pathology of the endarterectomy specimen. Ultrasonographically, an ulceration is an irregularity or break in the echoreflective surface of the plaque. Moore et al.[279]defined ulcer size on angiograms as the multiplication of length and width of a crater (in millimeters), and these authors divided ulceration into 3 groups according to size. Macroscopically, Comerata et al.[280]defined ulcers according to crater width and a depth of 1 mm or more, whereas O'Donnell et al.[281]defined them as a definite surface irregularity with a punched-out characteristic.Seen microscopically, ulceration according to the definitions varies from loss of surface endothelium to deep, undermining depressions in the plaque[282,283]. However, the ability to detect ulceration is affected by the degree of stenosis. With the use of B-mode ultrasound, the sensitivity for identification of ulceration was 77% in plaques with 50% stenosis or less but only 41% in plaques of . 50% stenosis (*P*50.03).The sensitivity for arteriography was similar: 77% and 48%, respectively.

These findings indicate that the presence of ulcers may be of prognostic relevance, but if one considers the fact that most patients undergoing surgery have severe lesions in which ulcers are not easily seen preoperatively, this parameter may be difficult to use as an indication for surgery.

Angiography merely yields information on the degree and location of the stenosis, whereas high-resolution B-mode imaging allows characterization of carotid plaques by echogenicity, defined as reflectance of the ultrasound signal. Echogenicity can be classified according to the criteria of Johnson et al[284]from 1985 (calcified, dense, and soft, Fig.4) or alternatively, the criteria of Gray-Weale et al.[285]from 1988 describing 4 plaque types, from dominantly echolucent with a thin, echogenic cap to dominantly echogenic with small areas of echolucency, through 2 types of mixed echogenicity.

Risk	Johnson[10]	Gray-Weale[23]	Reilly[24]	ECPS[36]	
High risk	Soft	Echolucent (thin, echorich cap)	Heterogeneous (mixed-level echoes)	Echolucent	Heterogeneous
		Dominantly echolucent (small, echorich area)			
	Dense			Intermediate	
		Dominantly echorich (<25% echolucent)			
Low risk	Calcified	Echorich	Homogeneous (medium- or high-level echoes)	Echorich	Homogeneous

Fig.4 Classification of plaque morphology in relation to risk of neurological symptoms

Marie-Louise M. Grønholdt Arterioscler Thromb Vasc Biol 1999;19;2-13

Most studies relating ultrasonic plaque morphology to clinical outcome agree that echolucent as well as ulcerated plaques carry a higher risk of neurological symptoms compared with echorich plaques. Summarizing, it appears clearly that patients with an echolucent and heterogeneous plaque evaluated by ultrasound B-mode have a higher risk of developing neurological events than do patients

with homogeneous, echorich plaques. The echolucent plaques were associated with a high content of lipid and hemorrhage, whereas echorich plaques contained more calcification (causing acoustic shadowing) and fibrous tissue (collagen rich). Ultrasound could not reliably distinguish between lipid and hemorrhage in the plaque; to characterize B-mode images of plaques more objectively, digital image processing or videodensitometric analysis, the GSM, has been introduced.

GRAY-SCALE MEDIAN (GSM) CALCULATION

Biasi et al.[286], assessed for the first time, a study (ICAROS study) in order to analyze the risk of stroke during carotid artery stenting (CAS) using a new computer-assisted highly reproducible index of echogenicity, namely the gray-scale median (GSM). Here is summarized the GSM calculation.

Images acquisition

With the patient in the supine position, the carotid bifurcation was imaged in longitudinal (anterolateral, lateral, and posterolateral) and transverse projections. The transducer was kept parallel to the vessel so that the near and far wall adventitia was imaged at right angles. The aim was to obtain a carotid plaque B-mode image with a relatively noiseless vessel lumen, with an echo-dense area of adventitia in the vicinity of the plaque and with an echoically informative plaque that can be outlined easily. The projection that fulfilled these criteria was chosen. Plaques included in an acoustic shadow were analyzed only if >50% of their area depicted real acoustic information, and only this

area was subjected to analysis (the number of pixels in the area of interest was at least half of the total number of pixels in the area of the plaque, as calculated by the Adobe Photoshop software).

B-Mode Image Normalization

B-mode images are digitized and transferred to a personal computer. With the use of the software Adobe Photoshop (version 3.0 or later) and the "histogram" facility, the gray scale median (GSM) of the 2 reference points (blood and adventitia) in the original B-mode image is defined. Algebraic (linear) scaling of the image is performed with the "curves" option of the software so that in the resultant image the GSM of blood equals 0 to 5 and that of the adventitia equals 185 to 195. In this way, the gray scale values of all the pixels in the image are adjusted according to the input and output values of the 2 reference points (blood: input value=measured GSM before linear scaling; output value=0 to 5; adventitia: input value=measured GSM before linear scaling; output value=185 to 195) (Fig.5). The GSM of the plaque (the median of the frequency distribution of the gray levels of the pixels in the plaque) in the normalized image (adjusted image using linear scaling) is used to quantify its echogenicity (Fig.6).

With this study it was been demostrated that quantification by means of GSM allows to use simple statistics to assess variability and to define easily the limits of experimental error. These findings may have significant implications in reducing the variability of plaque characterization and quantifying true changes (those outside the limits of experimental error) of plaque echogenicity in prospective multicenter studies.

They concluding that carotid plaque echolucency, as measured by GSM <25, increases the risk of stroke in CAS, and that the inclusion of echolucency measured as GSM in the planning of any endovascular procedure of carotid lesions allows stratification of patients at different risks of complications in CAS.

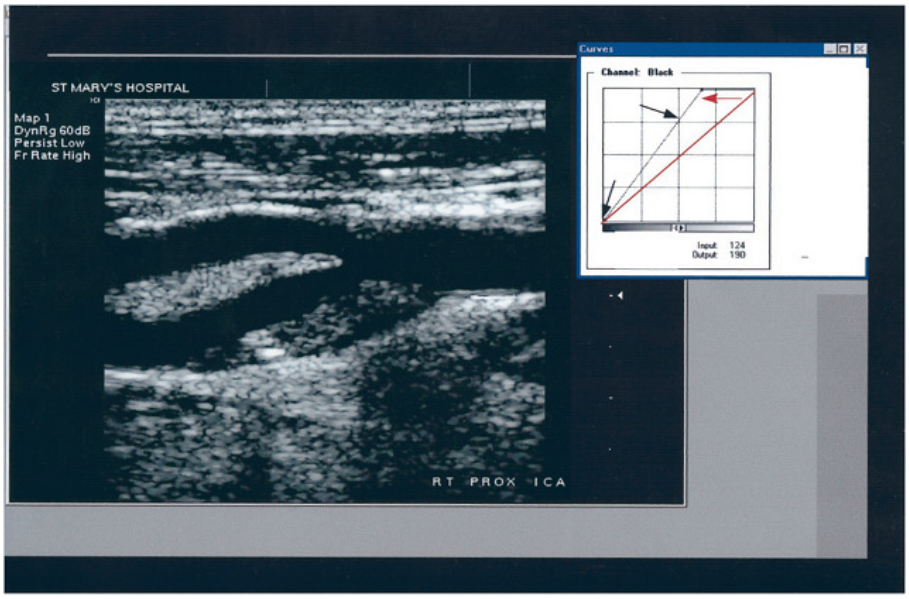


Fig.5 Normalization of the B-mode image using algebraic (linear) scaling. Pixels' gray scale value in the whole image is adjusted according to the input and output values of the reference echo structures (vessel lumen: input and output value50; adventitia: input value5124, output value5190) (black arrows).

Circulation. 2004 Aug 10;110(6):756-62

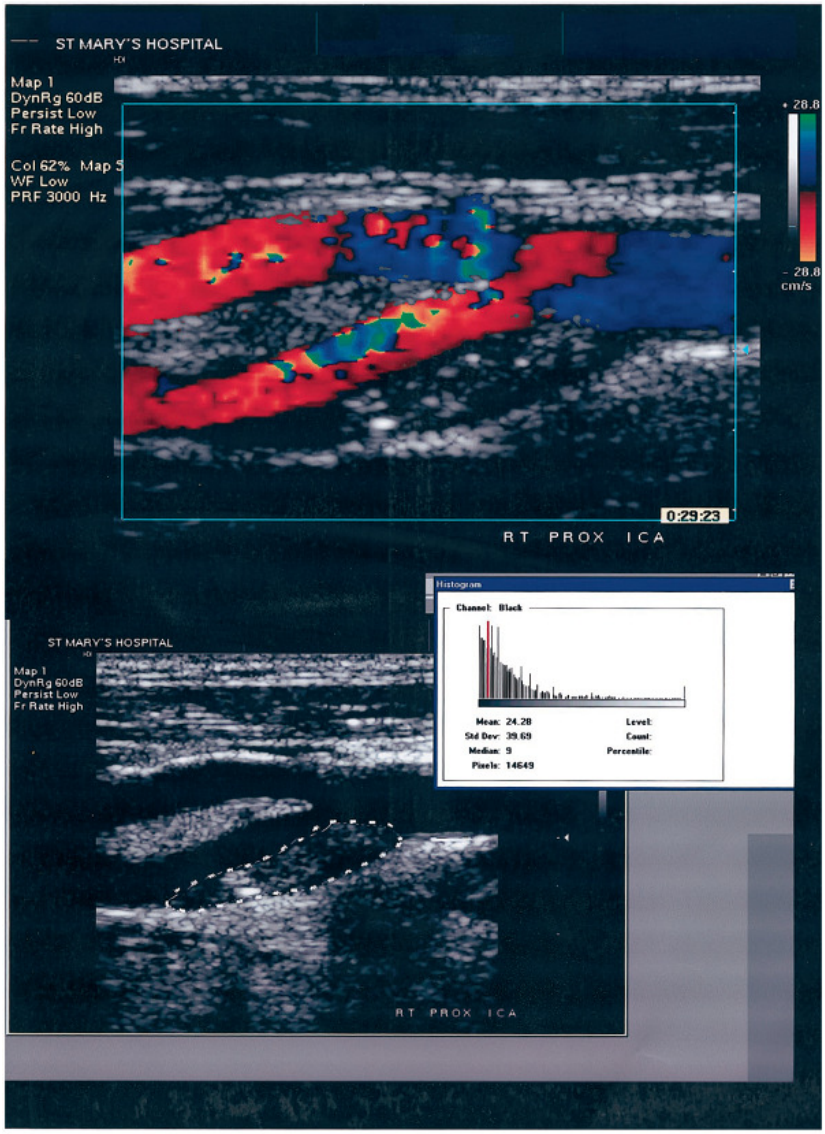


Fig.6. Hypoechoic plaque (GSM59) with the histogram of the gray tone frequency distribution of the pixels in the selected area (plaque) of the normalized image. The colored image is used as a guide to outline the area of interest.

Circulation. 2004 Aug 10;110(6):756-62

RNA EXTRACTION

For each patient, we have extracted RNA from PBMC's samples previously collected and stored. After the cell suspension thawing, we added immediately 10 ml of PBS in order to neutralize the toxicity of DMSO. Then, we have centrifuged for 15' at 1500 rpm, and the resulting pellet used for the RNA extraction by Quiagen RNeasy Mini Kit.This kit allows efficient purification of total RNA (up to 100 µg of RNA) from small amounts of starting material (1 x 10^7 cells); the elution volume is about 30–100 µl.

RNA EXTRACTION PROTOCOL

- after pelleting the appropriate number of cells(1 x 10^7 cells), in order to disrupt the cells we add 500µl of Buffer RLT previousy added with 10 µl of beta- mercaptoethanol (B-ME), pipetting to mix;
- then we add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting (without centrifuge the mix);
- we transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube; then we centrifuge for 15s at 10,000 rpm and discard the flow-through;
- in order to eliminate genomic DNA contamination, we perform a DNase digestion
- then, we add 700 µl of Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 10,000 rpm to wash the spin column membrane, discarding the flow-through; often is necessary to repeat this step one more time.

- At this point the extraction process is finish; in order to elute RNA, we place the RNeasy spin column in a new 1.5 ml collection tube and Add 30–50 µl of RNase-free water directly to the spin column membrane, centrifuging at least for 1 min at 10,000 rpm.

MICROARRAY TECHNOLOGY

cDNA microrarray experiments typically involve hybridizing two mRNA samples, each of which has been converted into cDNA and labelled with its own fluorophore, on a single glass slide that has been spotted with 10,000–20,000 cDNA probes. Data from such experiments provide information on the relative expression of the sample genes, which correspond to the probes.

cDNA microarray experiment is a competitive hybridization between a sample that is labelled with the red-fluorescent dye Cyanine 5 (Cy5) and a sample that is labelled with the green-fluorescent dye Cyanine 3 (Cy3).Unlike gene-expression data from nylon membranes (filter) or GeneChip (Affymetrix), cDNA microarray data are inherently comparative. This is because the filter or Affymetrix data measure gene expression levels for each sample separately, whereas, in the case of cDNA experiments, the pairing of target samples for hybridization leads to relative expression values and constrains the types of design that can be considered. So, each cDNA microarray experiment gives us the relative abundance of two sets of mRNA.

For ours experiments, we have selected 2 groups of patients, the AAA group (Abdominal Aortic Aneurysm patients, n=10) and a CTRL group (formed by healthy subjects, n=10), divided into subgroups of 5 patients each:

Group A = AAA patients, n=5
Group B = AAA patients, n=5
Group C = CTRL subjects, n=5
Group D = CTRL subjects, n=5

In the following table clinical data of subject included in the experimental groups are reported.

About the microarray technique, we have chosen to perform a Dye-swap experiment in which each hybridization is done twice, with the dye assignments reversed in the second hybridization. This type of experiments are useful for reducing systematic bias; in fact, most cDNA microarray experiments show systematic differences in the red and green intensities, which require correction at the normalization step. Using this strategy, we have compared the 4 experimental subgroups (group A Vs group C, group B Vs group D).

Arrays representing 14000 genes (70 mer oligonucleotides, AROS Human v1.1, Operon Technologies,CA, USA); for each microarray an internal normalization was performed, discarding low or abnormal signals by the use of the R package SMA ″Statistics for Microarray Analysis″(**http://www.stat.berkeley.edu/users/terry/zarray/Html/normspie.html**). Data analysis was performed using SAM "Significant Analysis of Microarray" technique, a statistical technique established in 2001 by Tusher, Tibshirani and Chu for determining whether changes in gene expression are statistically significant; delta value selected was 0.8, generating 91 differently expressed genes with FDR (false discovery rate) of 2.2%.

	Group A (n=5)	Group C (n=5)	Group B (n=5)	Group D (n=5)
Age	67 (60-81)	65 (52-85)	68 (62-82)	66 (54-83)
Gender (n°♂)	5 (100%)	5 (100%)	3 (60%)	3 (60%)
Tabagism	4 (80%)	1 (20%)	4 (80%)	1 (20%)
Diabetes	1 (20%)	0	0	0
Hypertension	3 (60%)	1 (20%)	4 (80%)	1 (20%)
Dislipidemia	4 (80%)	1 (20%)	3 (60%)	0
CAD	1 (20%)	0	0	0
CAS	1 (20%)	0	0	0
POAD	1 (20%)	0	0	0
COPD	3 (60%)	0	3 (60%)	0

Table 1. **Clinical data of subject included in the experimental groups** (CAD = Coronary Artery Disease; CAS = Carotid Artery Stenosis; POAD = Peripheral Obstructive Artery Disease; COPD = Chronic Obstructive Pulmonary Disease)

RNA RETROTRANSCRIPTION: RT-PCR

RT-PCR analysis was performed on the haematic samples of 3 patients groups (n=40 sbj each) in collaboration with the Microbiology Laboratory - University of Milano-Bicocca.

RNA VALIDATION

The first RNA validation was performed by UV spectrophotometric analysis, considering only samples with ratio 260nm/280nm ranged from 1.6 to 2.2. Then, RNA samples was analyzed with agarose gel electrophoresis (Fig.7), and we decided to use for each RT-PCR reaction 100ng of RNA (≈20ng cDNA obtained with following retrotrascription).

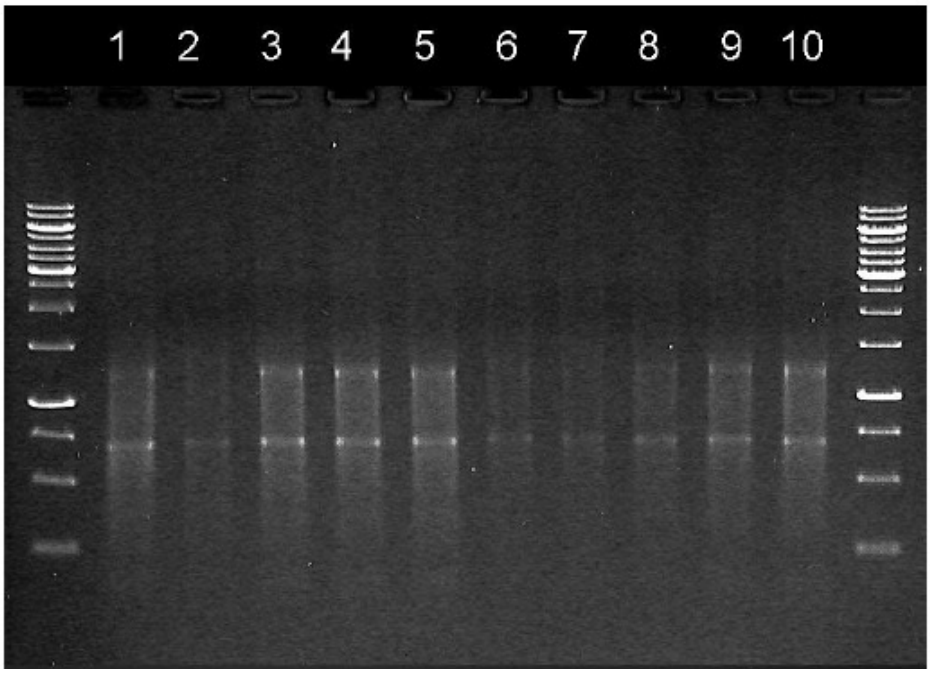


Fig.7 RNA analysis with agarose gelelectrophoresis

RNA RETRO-TRANSCRIPTION

The retro-transcription was performed accordingly to the standard protocol of SuperScript™ II RT, Invitrogen.

- in a nuclease-free microcentrifuge tube we added:

  1µl Oligo(dT) (concentration: 500 µg/ml)

  4-5µg total RNA

  12µl Milli-Q water

- we heated mixture to 65°C for 5 min and quick chilled on ice;
- then we added:

  4µl 5X First-Strand Buffer

  2µl 0.1 M DTT

  1µl 10mM dNTP Mix, pH 7.5

- after an incubation for 2' at 42°, we added 1µl (200 units)of SuperScript™ II RT and incubated at 42°C for 50'.
- after the incubation and the inactivation of the reaction by heating at 70°C for 15 min, in order to remove RNA complementary to the cDNA, we added 1 µl(2 units) of *E. coli* RNase H and incubate at 37°C for 20 min; at this point, the cDNA was ready to be used as a template for amplification in PCR.

REAL -TIME PCR

Real-time PCR is quantitative PCR method for the determination of cope number of PCR templates such as DNA or cDNA in a PCR reaction. There are two type of real-time PCR: probe-based and intercalator-based. Both methods require a special thermocycler equipped with a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction. Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers as regular PCR and an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. Intercalator-based method, also known as SYBR Green method, requires instead a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and gives fluorescence.

We performed a SYBR Green real-time PCR (Fig.8).

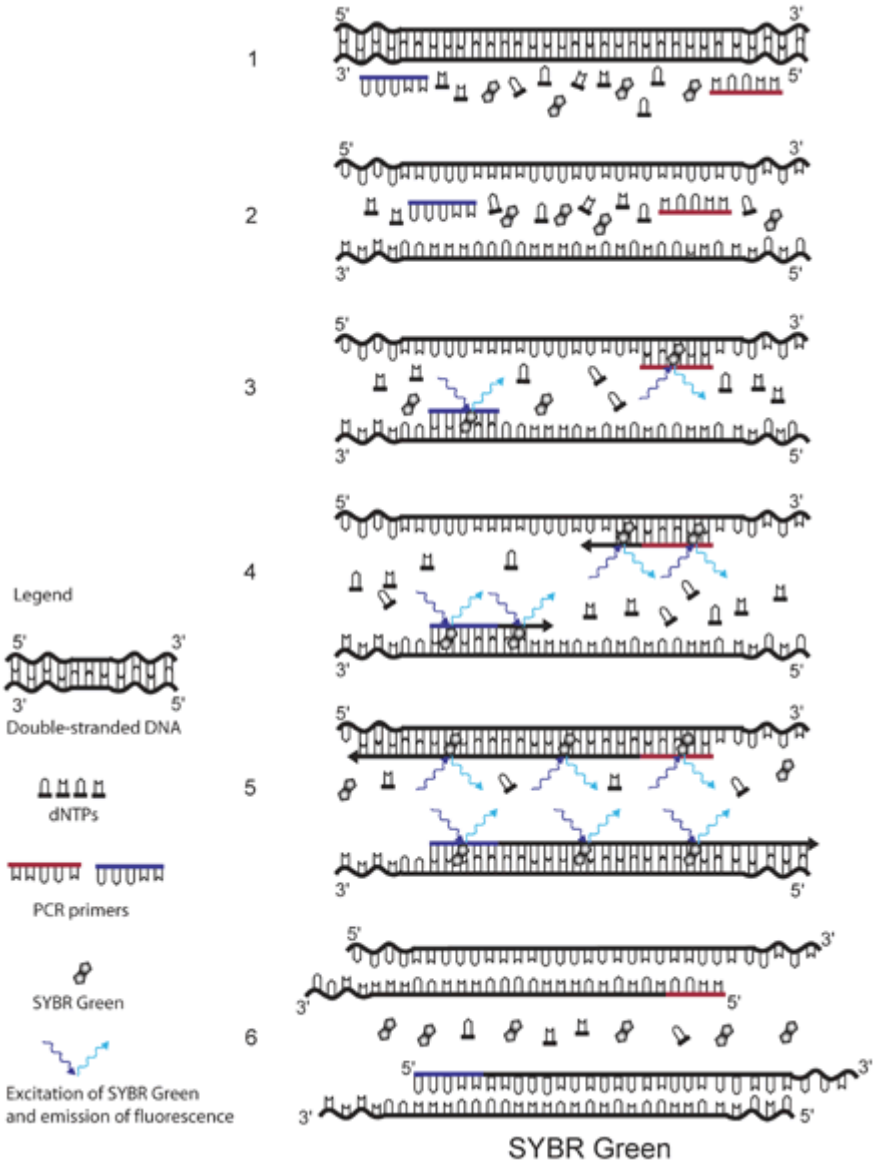


Fig.8 The principle of SYBR Green detection in real-time PCR . The fluorescent dye SYBR Green is added to the PCR mixture (1) SYBR Green is a DNA binding dye that fluoresces strongly when bound to double-stranded DNA. At the begin of the reaction, very little double stranded DNA is present, and so the fluorescent signal detected is low (3). As the reaction proceeds and PCR product accumulates, the amount of double-stranded DNA increases and with it the fluorescence signal (4-5). The signal is only detectable during annealing and extension, since the denaturation step contains predominantly single-stranded DNA (6).

QUANTIFICATION

The diagram below shows a typical reading from a single PCR cycle. The vertical axis represents copy number (arbitrary units) and the horizontal axis shows the PCR cycle number. The dotted threshold line is an arbitrary value, usually about 0.1 and is the "copy number" used to determine Ct. The lower a Ct value, the more copies are present in the specific sample. When plotted on a linear scale, as below, the curve has a sigmoidal course with an exponential phase and a platau phase. The platau phase is really determined by the amount of primer in the master mix rather than the nucleotide template. Theoretically, the amount of DNA doubles every cycle during the exponential phase, but this can affected by the efficiency of the primer used; for this reason, in addition to a negative control that is always performed with no template to show the lack of intrinsic fluorescence, a positive control using a housekeeping gene (a gene relatively abundant in all cell types) is performed to allow the comparisons between samples. Typical housekeeping genes include 18S rRNA, GAPDH and actin. The figure 9 shows a typical reading from a single PCR cycle in a real-time PCR machine

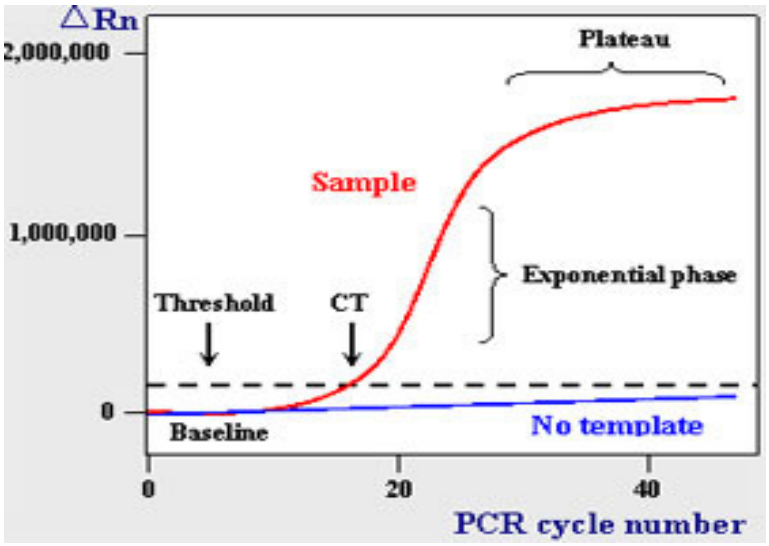
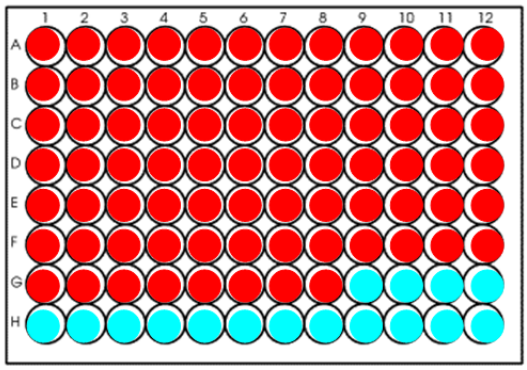


Fig.9 Typical reading from a single PCR cycle

In the diagram the three phases are evident: a)**Exponential =** the exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). b)**Linear =** (High Variability) The reaction components are being consumed, the reaction is slowing, and products are starting to degrade. c)**Plateau** (End-Point) = the reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade.

PLATE DESIGN

A **B**



MASTER MIX	25 µl
PCR Mix	12.5 µl
Primer 1 (FWD)	0.75 µl
Primer 2 (RWS)	0.75 µl
c-DNA	11 µl

The plate design is shown in the panel **A** of the picture. For each gene, we have used 40 samples taken from the 3 experimental group (AAA patients, TEA patients or CTRL subjects - red spots) *spotted* in *duplicate,* and the remaining 16 wells were used for positive and negative controls (blue spots). We have performed 30 real-time PCR experiments for each group, overall 120 experiments. In the panel **B** of the picture is reported the Master Mix composition of each well.

PRIMERS DESIGN

After the choice of candidate genes, we have performed the primers selection using the software Primer3 (**http://frodo.wi.mit.edu/primer3/**); all the information about the coding sequence of each gene were obtained by Ensembl database

(http://www.ensembl.org/index.html) and subsequently the primers were verified with Primerblast (**http://www.ncbi.nlm.nih.gov/tools/primer-blast/**).

The chosen primers anneal only on coding sequence in order to reduce genomic DNA contamination as much as possible.

MONOGLYCERIDE LIPASE - MGLL

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	AGACGCACAGGAAGCAAAGT	20	54.36	50.00%
Reverse primer	CGCTGCGATTCTCCACTACT	20	54.18	55.00%

Products on target templates

>NM_007283.5 Homo sapiens monoglyceride lipase (MGLL), transcript variant 1, mRNA

```
product length = 197
Forward primer  1    AGACGCACAGGAAGCAAAGT  20
Template        114  ....................  133

Reverse primer  1    CGCTGCGATTCTCCACTACT  20
Template        310  ....................  291
```

FREE FATTY ACID RECEPTOR 2 - FFAR2

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	GCCTGGTGCTCTTCTTCATC	20	52.88	55.00%
Reverse primer	AGGTGGGACACGTTGTAAGG	20	53.76	55.00%

Products on target templates

>NM_005306.2 Homo sapiens free fatty acid receptor 2 (FFAR2), mRNA

```
product length = 178
Forward primer  1    GCCTGGTGCTCTTCTTCATC  20
Template        551  ....................  570

Reverse primer  1    AGGTGGGACACGTTGTAAGG  20
Template        728  ....................  709
```

ADIPONECTIN RECEPTOR 1 – ADIPOR1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	CTTCTACTGCTCCCCACAGC	20	54.31	60.00%
Reverse primer	GACAAAGCCCTCAGCGATAG	20	52.73	55.00%

Products on target templates

>NM_001127687.1 Homo sapiens adiponectin receptor 1 (ADIPOR1), transcript variant 2, mRNA

```
product length = 196
Forward primer  1    CTTCTACTGCTCCCCACAGC  20
Template        940  ....................  959

Reverse primer  1    GACAAAGCCCTCAGCGATAG  20
Template        1135 ....................  1116
```

PHOSPHOLIPASE A2, (IB GROUP) – PLA2GIB

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	GTGCTAGCTGTGCTGCTCAC	20	55.96	60.00%
Reverse primer	GGCCTGGTCATAGCAGTTGT	20	54.17	55.00%

Products on target templates

>NM_000928.2 Homo sapiens phospholipase A2, group IB (pancreas) (PLA2G1B), mRNA

```
product length = 219
Forward primer  1    GTGCTAGCTGTGCTGCTCAC  20
Template        49   ....................  68

Reverse primer  1    GGCCTGGTCATAGCAGTTGT  20
Template        267  ....................  248
```

HYDROSTEROID (17-B) DEIDROGENASE 14 – HSD17B14

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	GCTGGAGCTGAACCTACTGG	20	54.31	60.00%
Reverse primer	AAGCTTTGGTCATGGCTGTT	20	52.41	45.00%

Products on target templates

>NM_016246.2 Homo sapiens hydroxysteroid (17-beta) dehydrogenase 14 (HSD17B14), mRNA

```
product length = 173
Forward primer  1    GCTGGAGCTGAACCTACTGG  20
Template        596  ....................  615

Reverse primer  1    AAGCTTTGGTCATGGCTGTT  20
Template        768  ....................  749
```

ACYL-COENZYME A DEHYDROGENASE - ACADS

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	GCGACTCATGGGTTCTGAAT	20	52.20	50.00%
Reverse primer	TGCGACAGTCCTCAAAGATG	20	52.19	50.00%

Products on target templates

>NM_000017.2 Homo sapiens acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain (ACADS), nuclear gene encoding mitochondrial protein, mRNA

```
product length = 222
Forward primer  1    GCGACTCATGGGTTCTGAAT  20
Template        639  ....................  658

Reverse primer  1    TGCGACAGTCCTCAAAGATG  20
Template        860  ....................  841
```

LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 5 – LRP5

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	CATCCTGGTGTTCCACTCCT	20	53.09	55.00%
Reverse primer	TGAACAGCAAGAAGGTGGTG	20	52.53	50.00%

Products on target templates

>NM_002335.2 Homo sapiens low density lipoprotein receptor-related protein 5 (LRP5), mRNA

```
product length = 179
Forward primer  1     CATCCTGGTGTTCCACTCCT  20
Template        2748  ....................  2767

Reverse primer  1     TGAACAGCAAGAAGGTGGTG  20
Template        2926  ....................  2907
```

TOLL-LIKE RECEPTOR ADAPTOR MOLECULE 1 – TICAM 1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	CAGGAGCCTGAGGAGATGAG	20	53.12	60.00%
Reverse primer	CTGGGTAGTTGGTGCTGGTT	20	54.00	55.00%

Products on target templates

>NM_182919.2 Homo sapiens toll-like receptor adaptor molecule 1 (TICAM1), mRNA

```
product length = 163
Forward primer  1     CAGGAGCCTGAGGAGATGAG  20
Template        993   ....................  1012

Reverse primer  1     CTGGGTAGTTGGTGCTGGTT  20
Template        1155  ....................  1136
```

INTERLEUKIN 1 RECEPTOR ACCESSORY PROTEIN-LIKE 1 – IL1RAPL1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	TGCCCTTGAAATCCTACCTG	20	51.31	50.00%
Reverse primer	CGAAGTCTGGTTTCCAGCTC	20	52.88	55.00%

Products on target templates

>NM_014271.3 Homo sapiens interleukin 1 receptor accessory protein-like 1 (IL1RAPL1), mRNA

```
product length = 204
Forward primer  1     TGCCCTTGAAATCCTACCTG  20
Template        1795  ....................  1814

Reverse primer  1     CGAAGTCTGGTTTCCAGCTC  20
Template        1998  ....................  1979
```

TUMOR NECROSIS FACTOR TYPE 1 RECEPTOR ASSOCIATED PROTEIN – TRAP1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	CTGCACCTTCGTGAGTTTGA	20	52.70	50.00%
Reverse primer	ACCCCAGCACATTTCTCATC	20	52.01	50.00%

Products on target templates

>NM_016292.2 Homo sapiens TNF receptor-associated protein 1 (TRAP1), mRNA

```
product length = 166
Forward primer  1     CTGCACCTTCGTGAGTTTGA  20
Template        1698  ....................  1717

Reverse primer  1     ACCCCAGCACATTTCTCATC  20
Template        1863  ....................  1844
```

GAPDH – Housekeeping gene

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	GAGTCAACGGATTTGGTCGT	20	52.50	50.00%
Reverse primer	TTGATTTTGGAGGGATCTCG	20	49.36	45.00%

Products on target templates

>NM_002046.3 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA

```
product length = 238
Forward primer  1    GAGTCAACGGATTTGGTCGT  20
Template        122  ....................  141

Reverse primer  1    TTGATTTTGGAGGGATCTCG  20
Template        359  ....................  340
```

STATISTICAL ANALYSES

The primary data obtained from Microarray analysis were normalized using the SMA software (Statistical Microarray Analysis – **www.stat.berkeley.edu/~terry/zarray/Software/smacode.html**); subsequently, the data were analyzed using SAM algorithm (Significant Analysis of Microarray). About real-time PCR data, we have used a two-tailed T Test.

RESULTS

SAMPLES COLLECTION AND DATABASE CREATION

During the period 2006-2009, 420 haematic samples were collected from patients of the Vascular Surgery Unit - S.Gerardo Teaching Hospital, Monza, subjected to surgical operation:

Abdominal Aortic Aneurysm (A.A.A.) surgery	77 pz
A.A.A. endovascular surgery	20 pz
Carotid Endoarterectomy (T.E.A.)	275 pz
Carotid stent surgery	39 pz
Iliac Artery Aneurysm endovascular surgery	3 pz
Thoracic Aortic Aneurysm (T.A.A.) surgery	2 pz
Splenic Artery Aneurysm (S.A.A.) surgery	1 pz
Popliteal Artery Aneurysm (P.A.A) surgery	1 pz
Artery Ostruction surgery	1 pz
Carotid stent + A.A.A. surgery	1 pz
TOT:	420 pz

For each patient we have collected also information about the anamnesis, the recent clinical history and the follow up after surgical treatment; a clinical database was created in order to accurately classify patients, defining different experimental subgroups in order to perform the following genetic analysis.

CLINIC DATABASE

Cardiovascular risk factors*:*

dislipidemia: 32%

tabagism: 55%

diabetes: 29%

hypertension: 46%

Co-morbidity association

CAD (coronary artery disease): 28%

POAD (peripheral obstructive artery disease): 17%

COPD (chronic obstructive pulmonary disease): 32%

GSM values

In patients affected by carotid stenosis, the mean GSM value was 22 and the percentage of stenosis 80.5% .

Patients anamnesis

MICROARRAY ANALYSES

	Group A (n=5) A.A.A. pz	Group C (n=5) CTRL	Group B (n=5) A.A.A. pz	Group D (n=5) CTRL
Age	67 (60-81)	65 (52-85)	68 (62-82)	66 (54-83)
Gender (n° ♂)	5 (100%)	5 (100%)	3 (60%)	3 (60%)
Tabagism	4 (80%)	1 (20%)	4 (80%)	1 (20%)
Diabetes	1 (20%)	0	0	0
Hypertension	3 (60%)	1 (20%)	4 (80%)	1 (20%)
Dislipidemia	4 (80%)	1 (20%)	3 (60%)	0
CAD	1 (20%)	0	0	0
CAS	1 (20%)	0	0	0
POAD	1 (20%)	0	0	0
COPD	3 (60%)	0	3 (60%)	0

REAL-TIME PCR ANALYSES

	A.A.A. pz (n=40)	CTRL (n=40)	T.E.A. pz (n=40)
Age (mean)	68	71	69
Gender (n° ♂)	35 (87.5%)	35(87.5%)	35(87.5%)
Tabagism	26 (65%)	15 (37,5%)	31 (77.5%)
Diabetes	3(7.5%)	0	2 (5%)
Hypertension	31 (77.5%)	10 (25%)	35 (87.5%)
Dislipidemia	28 (70%)	2 (5%)	23 (57%)
CAD	12 (30%)	0	11 (27.5%)
CAS	8 (20%)	0	8 (20%)
POAD	7 (17.5%)	0	5 (12.5%)
COPD	20 (50%)	0	18 (45%)

MICROARRAY ANALYSES

Of all 14000 transcripts represented on the arrays, 3981 resulted in a positive hybridization after the normalization procedure; with the analysis using SAM "Significant Analysis of Microarray" technique, 91 genes differentially expressed in the 2 A.A.A. patients groups (A and B) in comparison with the controls (C and D) was revealed, in particular 76 upregulated and 15 downregualted (d>1,6 and F.D.R = 2.2%). In the following tables the genes differentially expressed are reported.

UPREGULATED GENES

Gene's name	symbol	Accession n°	Gene ID	d
Hemoglobin, alpha 2	HBA2	V00488	3040	5.30
Monoglyceride lipase	MGLL	U67963	11343	4.79
Guanylate kinase 1	GUK1	L76200	2987	3.52
Hemoglobin, epsilon 1	HBE1	NM_005330	3046	3.49
Cell death-inducing DFFA-like effector a	CIDEA	AF041378	1149	3.34
ATPase, Na+/K+ transporting, alpha polypeptide-like 1	ATP12A	L42563	479	3.21
PDZ domain containing RING finger 4	PDZRN4	AL133067	29951	2.92
CGI-69 protein	CGI-69	NM_016016	51629	2.88
Hemoglobin, delta	HBD	V00505	3045	2.78
Defensin, alpha 1, myeloid-related sequence	DEFA1	M26602	1667	2.48
Hemoglobin, theta 1	HBQ1	NM_005331	3049	2.44
Zinc finger and BTB domain containing 39	ZBTB39	AB002350	9880	2.44
Immunoglobulin lambda joining 3	IGLJ3	X57812	28831	2.42
Makorin, ring finger protein, 1	MKRN1	NM_013446	23608	2.40
Cyclin B3	CCNB3	AL137550	85417	2.36
Zinc finger protein 193	ZNF193	U62392	148022	2.29
Toll-like receptor adaptor molecule 1	TICAM1	AF070530	7746	2.29
SEC24 (Saccharomyces cerevisiae) related gene family, member D	SEC24D	AB018298	9871	2.27
Serologically defined colon cancer antigen 1	SDCCAG1	NM_004713	9147	2.24

Ubiquitin-conjugating enzyme E2M (homologous to yeast UBC12)	UBE2M	AF075599	9040	2.19
Free fatty acid receptor 2	FFAR2	AF024690	2867	2.18
Glycophorin C (Gerbich blood group)	GYPC	NM_002101	2995	2.16
Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting1	PIN1	U49070	5300	2.14
Three prime repair exonuclease 2	TREX2	AF151107	11219	2.12
MTERF domain containing 2	MTERFD2	BX648184	130916	2.07
Flotillin 1	FLOT1	AF089750	10211	2.01
Interleukin 1receptor accessory protein-like1	IL1RAPL1	AJ243874	11141	2.00
Chorionic somatomammotropin hormone-like 1	CSHL1	BC029365	1428	1.99
Solute carrier family 25, member 37	SLC25A37	AF223466	1444	1.99
Glioma-associated oncogene homolog (zinc finger protein)	GLI1	X07384	2735	1.99
Crystallin, mu	CRYM	L02950	51312	1.99
Nucleoporin 88 kD	NUP88	Y08612	2039	1.93
Erythrocyte membrane protein band 4.9 (dematin)	EPB49	U28389	4927	1.93
Splicing factor, arginine/serine-rich (transformer 2 Drosophila homolog) 10	SFRS10	U68063	6434	1.91
ATG5 autophagy related 5 homolog (S. cerevisiae)	ATG5	Y11588	9474	1.90
Chromosome 20 open reading frame 29	C20orf29	AK002030	55317	1.90
S100 calcium-binding protein A6 (calcyclin)	S100A6	J02763	6277	1.90
Lipocalin 2 (oncogene 24p3)	LCN2	X99133	3934	1.89
Potassium channel tetramerisation domain containing 12	KCTD12	AF359381	115207	1.87
Small EDRK-rich factor 2	SERF2	NM_005770	10169	1.86
Platelet factor 4 variant 1	PF4V1	M26167	5197	1.85
Ribosomal protein L18	RPL18	L11566	6141	1.84
Erythrocyte membrane protein band 7.2 (stomatin)	STOM	U33931	2040	1.84
Paralemmin	PALM	Y16270	5064	1.80
Myelin transcription factor 2	MYT2	AF006822	8827	1.79
Adiponectin receptor 1	ADIPOR1	AK001484	51094	1.78
Interferon, alpha 13	IFNA13	BC069427	3447	1.78
SMC (mouse) homolog, X chromosome	SMCX	L25270	8242	1.77
Nuclear factor I/B	NFIB	U85193	4781	1.76
Similar to HSPC323	LOC284422	AF161441	284422	1.75
Phospholipase A2, group IB (pancreas)	PLA2G1B	M21054	5319	1.75

Zinc finger protein 145 (Kruppel-like, expressed in promyelocytic leucemia)	ZBTB16	Z19002	28904	1.73
Immunoglobulin kappa variable 1D-8	IGKV1D-8	M63438	7704	1.73
Potassium voltage-gated channel, shaker-related subfamily, member 3	KCNA3	M85217	51171	1.72
Hydroxysteroid (17-beta) dehydrogenase 14	HSD17B14	NM_016246	3738	1.72
Mitogen-activated protein kinase kinase 3	MAP2K3	D87116	509	1.71
ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	ATP5C1	D16562	5606	1.71
Translocase of inner mitochondrial membrane 8 (yeast) homolog A	TIMM8A	U66035	1678	1.71
HUS1 (Saccharomyces pombe) checkpoint homolog	HUS1	AF076844	3364	1.69
Transcription elongation factor A (SII), 2	TCEA2	D50495	6919	1.69
Tumor necrosis factor type 1 receptor-associated protein	TRAP1	NM_016292	10131	1.69
Karyopherin alpha 4 (importin alpha 3)	KPNA4	AB002533	3840	1.68
Chaperonin containing TCP1, subunit 6A (zeta 1)	CCT6A	L27706	908	1.67
Immunoglobulin lambda variable 1-47	IGLV1-47	Z73663	28822	1.67
MutL homolog 3 (Escherichia coli)	MLH3	AB039667	50801	1.67
Potassium inwardly-rectifying channel, subfamily K, member 4	KCNK4	AF247042	27030	1.67
RNA-binding protein S1, serine-rich domain	RNPS1	L37368	10921	1.66
Zinc finger protein 384	ZNF384	U80738	7177	1.66
Tryptase, alpha	TPSAB1	M30038	171017	1.66
Ring finger protein 44	RNF44	AB029023	51374	1.65
Apoptosis related protein APR-3	apr-03	NM_016085	22838	1.65
Basigin	BSG	X64364	35	1.64
Aminolevulinate, delta-,synthase 2 (sideroblastic/hypochromic anemia)	ALAS2	X60364	212	1.64
Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	ACADS	Z80345	682	1.64
Sarcolipin	SLN	U96094	6588	1.63
Myogenic factor 6	MYF6	X52011	4618	1.62

DOWNREGULATED GENES

Gene's name	symbol	Accession n°	Gene ID	d
Eukaryotic translation elongation factor 1 beta 2	EEF1B2	X60489	1933	−1.97
Regulator of G-protein signaling 2, 24 kD	RGS2	L13463	5997	−1.97
Protein tyrosine phosphatase, receptor type, c polypeptide	PTPRC	Y00062	5788	−2.04
ARP2 (actin-related protein 2, yeast) homolog	ACTR2	AF006082	10097	−2.17
Similar to chloride intracellular channel protein 1 (Nuclear chloride ion channel 27) (NCC27) (p64 CLCP) (Chloride channel ABP)	LOC390363	XM_495936	390363	−2.28
High-mobility group (nonhistone chromosomal) protein 1 like 10	HMG1L5	L08048	10354	−2.37
Receptor-associated protein of the synapse, 43kD	RAPSN	Z33905	5913	−2.46
Chromosome 12 open reading frame 35	C12orf35	AK000703	55196	−2.59
Homo sapiens Bruton's tyrosine kinase	BTK	U78027	695	−2.76
Ribosomal protein L17	RPL17	X53777	6139	−2.85
Complement factor H-related 4	CFHR4	X98337	10877	−2.94
Immunoglobulin superfamily containing leucine-rich repeat	ISLR	AB024536	3671	−3.44
Low density lipoprotein receptor-related protein 5	LPR5	AF077820	4041	−3.46
Apoptosis antagonizing transcription factor	AATF	AJ249940	26574	−4.30
Anti-Mullerian hormone receptor, type II	AMHR2	U29700	269	−5.62

GENE SELECTION FOR REAL-TIME PCR ANALYSIS

The 91 genes differentially expressed in the array analysis are primarily involved into the oxygen transport, the positive regulation of the protein-kinases, the immunity and the lipid metabolism. Nevertheless, the microarray data referred only to the A.A.A patients, and because we were interested also in the carotid stenosis, we selected some genes which regulate lipid metabolism and immunity, the biological processes primarily involved in both pathologies, in order to validate with real-time PCR analyses the microarray data, and also to test the expression levels of those genes in other AAA patients and in carotid stenosis patients.

For real-time PCR analysis we have selected the following genes:

LIPID METABOLISM

- Monoglyceride lipase – MGLL – U67963 – 11343
- Free fatty acid receptor 2 – FFAR2 – AF024690 – 2867
- Adiponectin receptor 1 – ADIPOR1 – AK001484 – 51094
- Phospholipase A2, group IB (pancreas) – PLA2G1B – M21054 – 5319
- Hydroxysteroid (17-beta) dehydrogenase 14 – HSD17B14 – NM_016246 – 3738
- Acyl-Coenzime A dehydrogenase, C-2 to C-3 short chain – ACADS – Z80345 – 682

- Low density lipoprotein receptor-related protein 5 – LPR5 – AF077820 – 4041

IMMUNITY

- Toll-like receptor adaptor molecule 1 – TICAM1 – AF070530 – 7746
- Interleukin 1 receptor accessory protein-like 1 – IL1RAPL1 – AJ243874 – 11141
- Tumor necrosis factor type 1 receptor-associated protein – TRAP1 – NM_016292 – 10131

MGLL: MONOGLYCERIDE LIPASE(3p13-q13.33)

Monoglyceride lipase, also called monoacylglycerol lipase, play an important role in the hydrolysis of the monoglycerides formed during the hydrolysis of triglycerides. In adipose tissue, the stored triglycerides are hydrolyzed sequentially by triglyceride lipase and hormone sensitive lipase to diglycerides and monoglycerides, respectively. The monoglycerides formed are subsequently hydrolyzed by MGLL to fatty acids and glycerol. MGLL also catalyzes the hydrolysis of monoglycerides produced by lipoprotein lipase activity on triglycerides of circulating chylomicrons and very-low-density lipoproteins. The free fatty acids released from triglycerides by these lipases are used as a source of energy in the body. An additional role for MGLL, the participation in the metabolism of the endocannabinoid'2-arachidonoylglycerol'(2-AG; 1) has recently emerged, because seems to be responsible for the low

level of endocannabinoid activity in brain. High level of MGLL are therefore related to high levels of FFA which contribute to the activation of the immunity response and the attraction of monocytes to the adipose rich zone and to the endothelial cells of blood vessels.

FFAR2: FREE FATTY ACID RECEPTOR 2(19q13.1)

This gene encodes a member of the GP40 family of G protein-coupled receptors that are clustered together on chromosome 19. FFA2 mRNA can be detected in a variety of tissues, but the highest expression is found in immune cells such as neutrophils, monocytes, peripheral blood mononuclear cells, B-lymphocytes, and polymorphonuclear cells (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Considerable levels of FFA2 mRNA were detected in bone marrow and spleen but these are thought to reflect the expression of the receptor on immune cells (Le Poul et al., 2003),The encoded protein is a receptor for short chain free fatty acids and may be involved in the inflammatory response and in regulating lipid plasma levels.

ADIPO1: ADIPONECTIN RECEPTOR 1(1p36.13-q41)

Adiponectin receptor 1 is specific for adiponectin, an hormone secreted by adipocytes that acts as an antidiabetic and anti-atherogenic adipokine. Levels of adiponectin in the blood are decreased under conditions of obesity, insulin resistance and type 2 diabetes. Administration of adiponectin causes glucose-lowering effects and ameliorates insulin resistance. This insulin-sensitizing effect of adiponectin seems to be mediated by an increase in fatty-acid oxidation through activation of AMP kinase and PPAR-alpha. Adiponectin acts by way of two receptors, ADIPOR1 and ADIPOR2.

AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver.

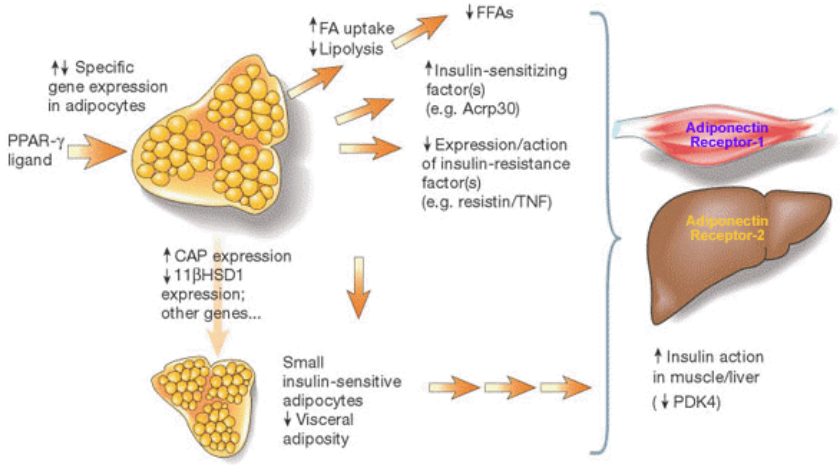


Fig.10. Action of adiponectin

Nevertheless, recent studies have demonstrated that ADIPOR1 is also expressed in peripheral mononuclear cells, approximately on 1% of T cells, 93% of monocytes, 47% of B cells, and 21% of NK cells. Moreover, is well established that adiponectin inhibits the vascular endothelial adhesion molecules production and the cytokines production during the first phase of the atherosclerosis development.

PLA2G1B: PHOSPHOLIPASE A2, GROUP 1B (Pancreas) (12q23-qter)

Phospholipases, in general, are enzymes which hydrolyze membrane phospholipids to generate products that are involved in the regulation of many cellular processes. Their activity is controlled by a wide variety of agonists, including hormones, neurotransmitters, growth factors, and cytokines. The principal phospholipases are PLA_1, PLA_2,

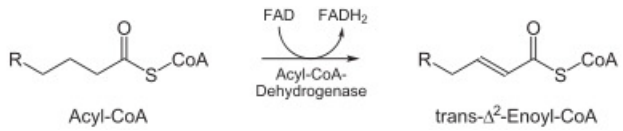
PLC, and PLD, and they cleave phospholipids at different sites. Phospholipase A2 catalyzes the release of fatty acids from glycero-3-phosphocholines. The best known varieties are the digestive enzymes secreted as zymogens by the pancreas of mammals. Other forms of PLA2 have been isolated from brain, liver, lung, spleen, intestine, macrophages, leukocytes, erythrocytes, inflammatory exudates, chondrocytes, and platelets. (Seilhamer et al., 1986). Among the fatty acids produced by PLA2 there's arachidonic acid, an omega-6 fatty acid transformed by COX1/2 into leukotrienes and prostaglandins, which are be able to modulate immune function via the lymphocyte; they are mediators of the vascular phases of inflammation and are potent vasodilators, increasing vascular permeability.

HSD17B14: HYDROXYSTEROID (17-B) DEHYDROGENASE 14 (19q13.33)

This enzyme is primarily involved in the metabolism of steroids at the C17 position and also of other substrates, such as fatty acids, prostaglandins, and xenobiotics (Lukacik et al., 2007). Due to it's capacity of producing haematic prostaglandins, it could be involved in the increase of haematic inflammatory state.

ACADS: ACYL-COENZYME A DEHYDROGENASE, C-2 to C-3 short chain (12q24.31)

This gene encodes a tetrameric mitochondrial flavoprotein, which is a member of the acyl-CoA dehydrogenase family. This enzyme catalyzes the initial step of the mitochondrial fatty acid beta-oxidation pathway.



Acyl-CoA trans-Δ^2-Enoyl-CoA

LRP5: LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 5 (11q13.4)

This gene encodes a transmembrane low-density lipoprotein receptor that binds and internalizes ligands in the process of receptor-mediated endocytosis. This protein also acts as a co-receptor with Frizzled protein family members for transducing signals by Wnt proteins (Fig.11) and was originally cloned on the basis of its association with type 1 diabetes mellitus in humans.

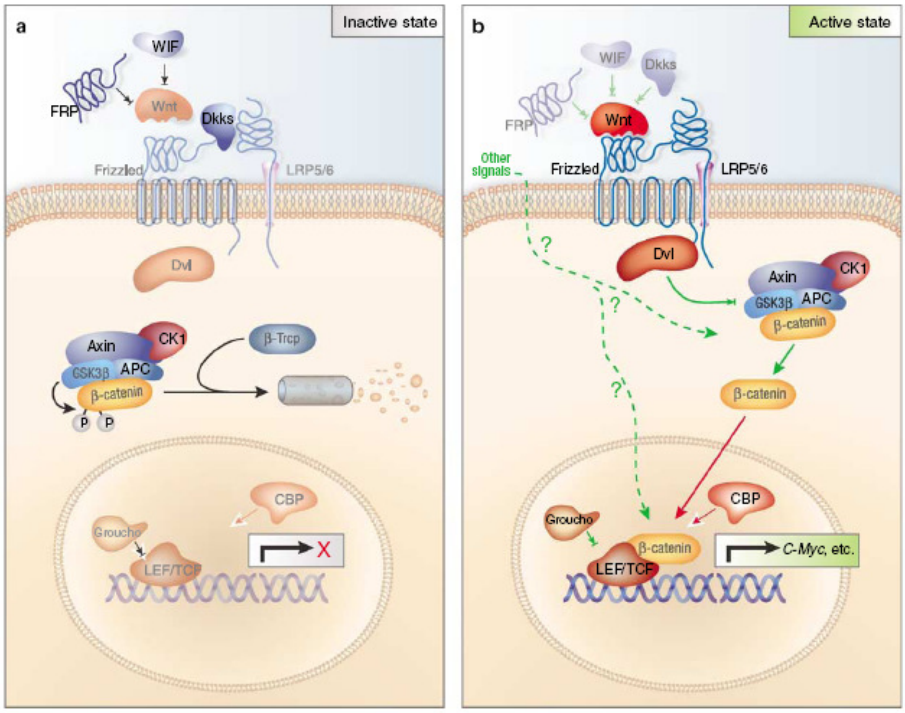


Figure 11 Schematic representation of the canonical Wnt/b-catenin signaling pathway. (a) In the absence of Wnt signal, b-catenin is recruited into the APC/ Axin/GSK3b complex, and phosphorylated by GSK3b at the N-terminal 'destruction box'. The phosphorylated b-catenin binds to b-Trcp of the proteosome machinery and is targeted for degradation. As the result, no free b-catenin enters nucleus to form transcriptional complex with LEF/Tcf and to regulate downstream gene expression. (b) Wnt binds to its Fz receptor and LRP5/6 co-receptor and activates Dvl, leading to the inhibition of APC/Axin/GSK3bmediated b-catenin degradation. Stabilized b-catenin forms a transcriptional complex with LEF/Tcf and activates downstream targets such as c-Myc. Negative regulators, such as Dkks, WIF and FRPs, inhibit the interactions between Wnt ligands and their receptors. There is crosstalk between Wnts and other signaling pathways, such as growth factors that activate receptor tyrosine kinases.

PNAS , January 7, 2003 , vol. 100 , no. 1, 229–234

The Wnt signaling pathway plays a pivotal role in embryonic development and oncogenesis through various signaling molecules including Frizzled receptors, LRP5 and LRP6, and Dickkopf proteins. In addition, the Wnt signaling is also involved in adipogenesis by negatively regulating adipogenic transcription factors (Tcfs); in fact, Wnt signalling acts as an adipogenic switch, when it is on, adipogenesis is repressed, when it is off, adipogenesis is initiated. It was demonstrated that LRP5 is highly expressed in many tissues, including hepatocytes and pancreatic beta cells; moreover, LRP5 can bind apolipoprotein E (apoE), raises the possibility that LRP5 plays a role in the hepatic clearance of apoE-containing chylomicron remnants, a major plasma lipoprotein carrying diet-derived cholesterol.

TICAM1: TOLL-LIKE RECEPTOR ADAPTOR MOLECULE 1 (19p13.3)

Innate immunity against invading pathogens relies on sensing specific molecular features expressed in microorganisms by pattern recognition receptors. This recognition is mediated by a set of germline-encoded receptors called Toll-like receptors (TLRs).

TICAM1, better known in literature as TRIF, is a Toll/IL1R(TIR) domain-containing adaptor molecule which specifically interacts with TLR3 and activates nuclear factor kappa-B (NFKB), inducing interferon-beta (IFN-B) transcription (Fig.12).

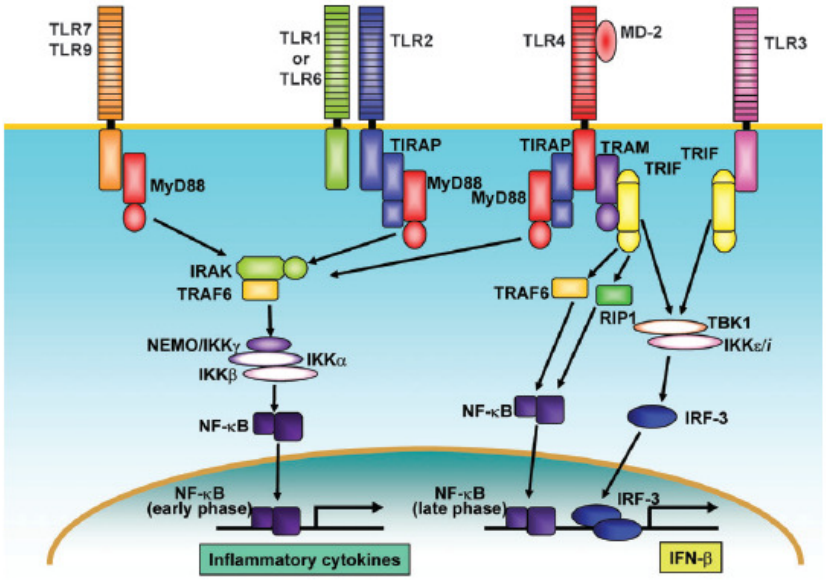


Fig. 12. TLR signaling pathway. TLR signaling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88,associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the IjB kinase (IKK) complex consisting of IKKa, IKKb and NEMO/IKKc. The IKK complex phosphorylates IjB, resulting in nuclear translocation of NF-jB which induces expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-b are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. Non-typical IKKs, IKKi/IKKe and TBK1, mediate activation of IRF-3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

International Immunology, Vol. 17, No. 1, pp. 1–14

IL1RAPL1: INTERLEUKIN 1 RECEPTOR ACCESSORY PROTEIN-LIKE 1 (Xp22.1-p21.3)

The protein encoded by this gene is a member of the interleukin 1 receptor family and is similar to the interleukin 1 accessory proteins. This gene is located at a region on chromosome X that is associated with X-linked non-syndromic mental retardation. Deletions and mutations in this gene were found in patients with mental retardation. This gene is expressed at a high level in post-natal brain structures involved in the hippocampal memory system, which suggests a specialized role in the physiological processes underlying memory and learning abilities. Actually, the involvement of IL1RAPL1in the pathogenesis of atherosclerosis isn't still demonstrated in literature, but we could associate this gene with the pathology because of it's linkage with IL-1, a pro-inflammatory cytokine involved in inflammatory reactions.

TRAP1: TNF RECEPTOR-ASSOCIATED PROTEIN 1(16p13.3)

TRAP1 encode a mitochondrial HSP90 protein; HSP90 proteins are highly conserved molecular chaperones that have key roles in signal transduction, protein folding, protein degradation, and morphologic evolution. HSP90 proteins normally associate with other cochaperones and play important roles in folding newly synthesized proteins or stabilizing and refolding denatured proteins after stress. TRAP-1 mRNA expression is increased in tumor cells resistant to chemotherapy. In fact, the stable clones expressing constitutively high TRAP-1 levels are more resistant to H2O2-induced DNA damage and to apoptosis by cisplatin, contain higher reduced glutathione (GSH)

levels than control cells; and do not release the apoptosis-inducing factor into the nucleus upon cisplatin treatment. Furthermore, high TRAP-1 levels interfere with caspase 3 activation. These results confirm the he protective role of TRAP-1 against oxidative stress and apoptosis (Montesano Gesualdi N. et al, Stress. 2007 Nov;10(4):342-50). TRAP1 also interacts with Exostosins-1 and 2(EXT1-2), the enzymes responsible of the heparan sulphate (HS) biosynthesis; briefly, HS synthesis initiates by the formation of a tetrasaccharide (glucuronicacid–galactose–galactose–xylose,GlcAβ3Galβ3Galβ4Xyl) at specific serine residues of proteoglycan core proteins (Fig. 13). This intermediate serves as a primer for the addition of N-acetylglucosamine (GlcNAca1,4), the first committed step in HS synthesis. Chain polymerization then takes place by the alternating addition of GlcAh1,4 and GlcNAca1,4 from the corresponding nucleotide sugars (UDP-GlcA and UDP-GlcNAc, respectively). HS is involved in the coagulation cascade, because blood anti-coagulation is achieved mostly by heparan sulfate proteoglycans derived from endothelial cells.

Fig. 13. Heparan sulphate biosynthesis. HS is synthesized by the formation of a linkage region on a proteoglycan protein core, followed by polymerization and sulfation of the chain. The first committed reaction for HS assembly is the attachment of the first GlcNAc unit (filled squares) catalyzed by the initiating GlcNAc transferase (aGlcNAcTI), possibly represented by the genes EXTL2 and EXTL3. The copolymerase complex elongates the chain by alternating additions of h1,4GlcA and a1,4GlcNAc residues catalyzed by EXT1 and EXT2.

REAL-TIME PCR ANALYSES

In order to provide high specificity , the selected primers were first controlled in-silico with the software primerBLAST (**http://www.ncbi.nlm.nih.gov/tools/primer-blast/**), which is be able to discover all the possible annealings between the primers and the interesting sequence.

Correct annealing

```
product length = 230
Forward primer  1    CTTAACGTGAAGGCCACCAT  20
Template        723  ....................  742

Reverse primer  1    CCCTATCCACACGTGAACCT  20
Template        952  ....................  933
```

Mismatched annealing

```
product length = 941
Forward primer  1    CTTAACGTGAAGGCCACCAT  20
Template        772  ..CCGG..C..........A  791

Forward primer  1     CTTAACGTGAAGGCCACCAT  20
Template        1712  TGGC.........T....C.  1693
```

After the selection of two couples of primers for each gene, we have performed a dissociation test, extremely useful to select optimal primer concentrations, especially to check for the presence of primer dimer product and aspecific annealing (Fig,14). In fact, ideally the experimental samples should yield a sharp peak at the melting temperature of the amplicon, whereas the NAC (no amplification controls) and NTC (non template controls) will not generate significant fluorescent signal. This result indicates that the products

are specific, and that SYBR Green I fluorescence is a direct measure of accumulation of the product of interest. If the dissociation curve reveals a series of peaks, it indicates that there is not enough discrimination between specific and non-specific reaction products, and so, to obtain meaningful data, optimization of the RT-PCR would be necessary.

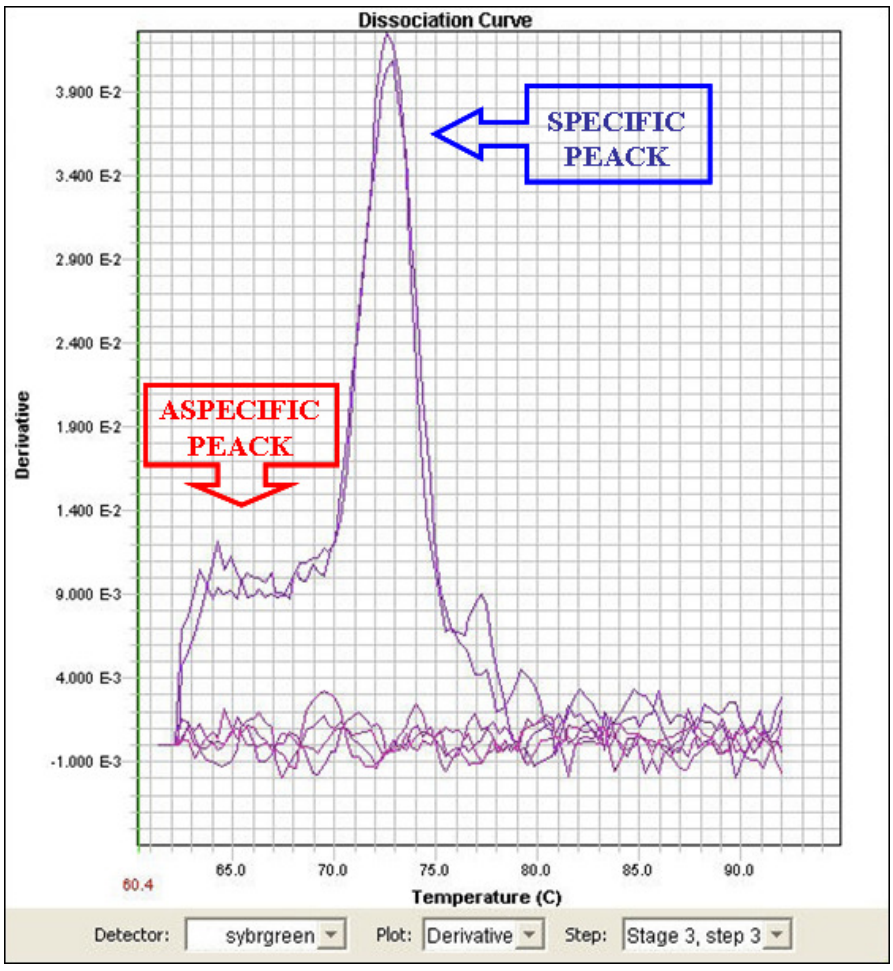


Fig.14. Diagram of the dissociation test

We used GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as a housekeeping gene because is uniformly and highly expressed in the

hematopoietic lineage (mean Ct = 21). We used the Comparative Method (or ΔΔCt method) for the gene expression quantification; this technique is ideal for primer pairs with an efficiency of 90% or greater and for genes that will show a large fold change in gene expression (5-fold or greater). For each sample, the PCR analysis was performed two times, and the sample mean Ct (Ct = copy number) was compared with the GAPDH mean Ct; the value obtained is ΔCt. The procedure was repeated for the 10 selected genes on 120 samples (40= ctrl, 40= AAA pz, 40= TEA pz), obtaining a total of 1200 ΔCt values. In order to verify the significance of this data, for each gene, the ΔCt values were analyzed with a two-***tailed*** T test (TEA pz vs ctrl and AAA pz vs ctrl, $p<0,05$). Then, the ΔΔCt values were calculated for each gene as the mean of the ΔCt values of each experimental group (ctrl, AAA pz and TEA pz) and then used in the following equation:

$$X = 2^{-\Delta\Delta Ct}$$

X is a "fold" values that represent how every single gene is differently expressed in the 3 experimental groups (ctrl, AAA pz and TEA pz).
The following tables show the obtained results.

AAA pz

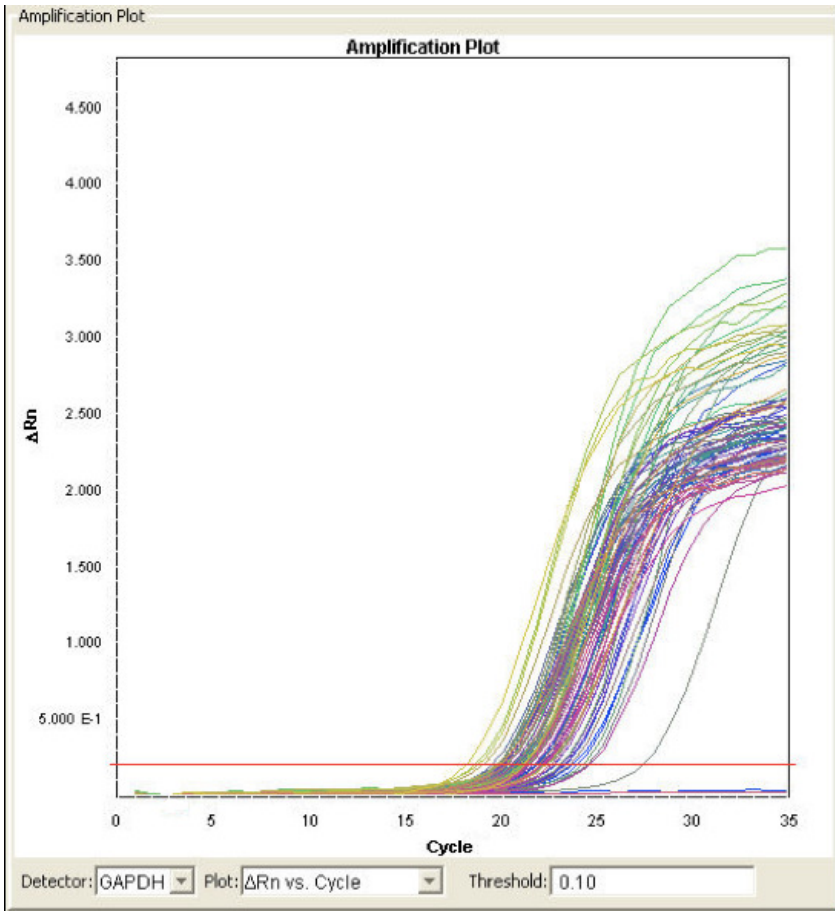
GENE	MEAN	ΔΔCt	FOLD	T TEST
MGLL	3.882	-1,434	2,70	0,000986348
FFAR2	3.387	-0,441	1,36	0,014722573
ADIPO1	6.975	-1,218	2,33	1,84182E-03
PLA2GIB	2.961	-0,665	1,59	0,00043179
HSD17B14	4.498	-2,619	6,14	2.95921E-05
ACADS	1.980	-1,782	3,44	9,62007E-05
LRP5	4.284	0,658	0,63	0,014932002
TICAM1	3.785	-0,565	1,48	0,018367989
IL1RAPL1	4.183	-2,430	5,39	3,1962E-04
TRAP1	1.618	-1.314	2,49	0,00089126

TEA pz

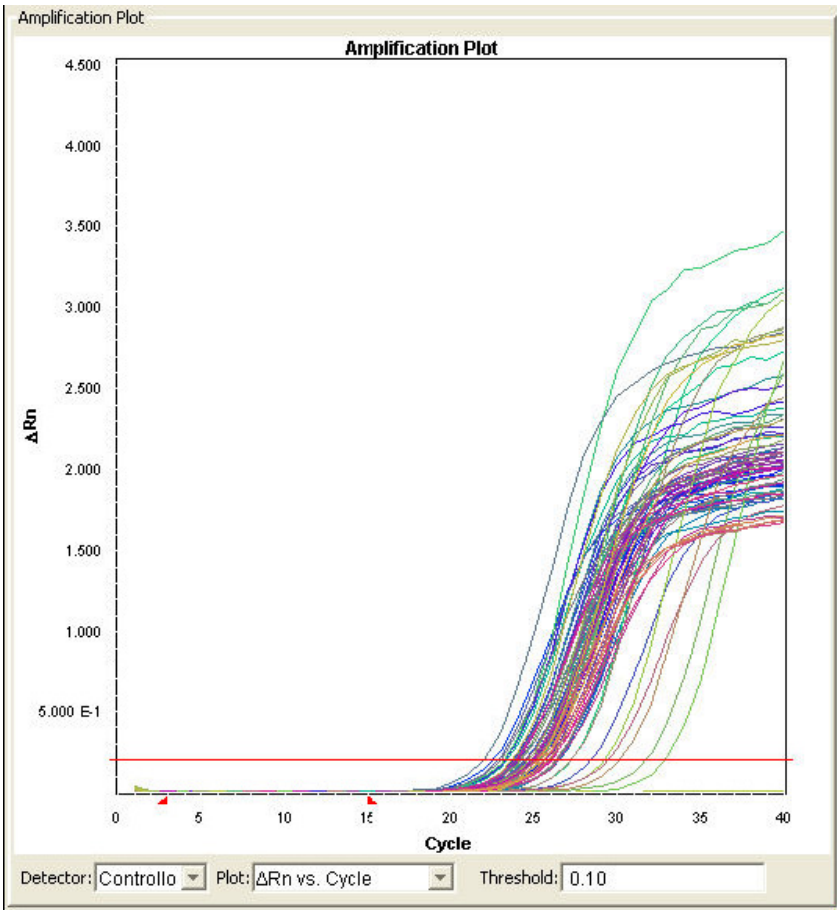
GENE	MEAN	ΔΔCt	FOLD	T TEST
MGLL	3.784	-1,532	2,89	0,0020185
FFAR2	3.279	-0,550	1,46	0,08359461
ADIPO1	6.985	-1,209	2,31	0,00070896
PLA2GIB	3.009	-0,616	1,53	0,018481044
HSD17B14	4.906	-2,211	4,63	1,0553E-04
ACADS	2.215	-1,547	2,92	3,126E-05
LRP5	4.517	-0,890	0,54	0,00475383
TICAM1	3.882	-0,467	1,38	0,02873310
IL1RAPL1	4.312	-2,300	4,93	6,45241E-04
TRAP1	1.691	-1,241	2,36	0,010343593

About AAA patients, this data confirmed the data obtained with microarray analysis; moreover, an analogue situation is observed for TEA patients, underling that the selected genes are potentially involved in the pathogenesis of both pathologies. Below the amplification plots of the real-time PCR analysis are reported.

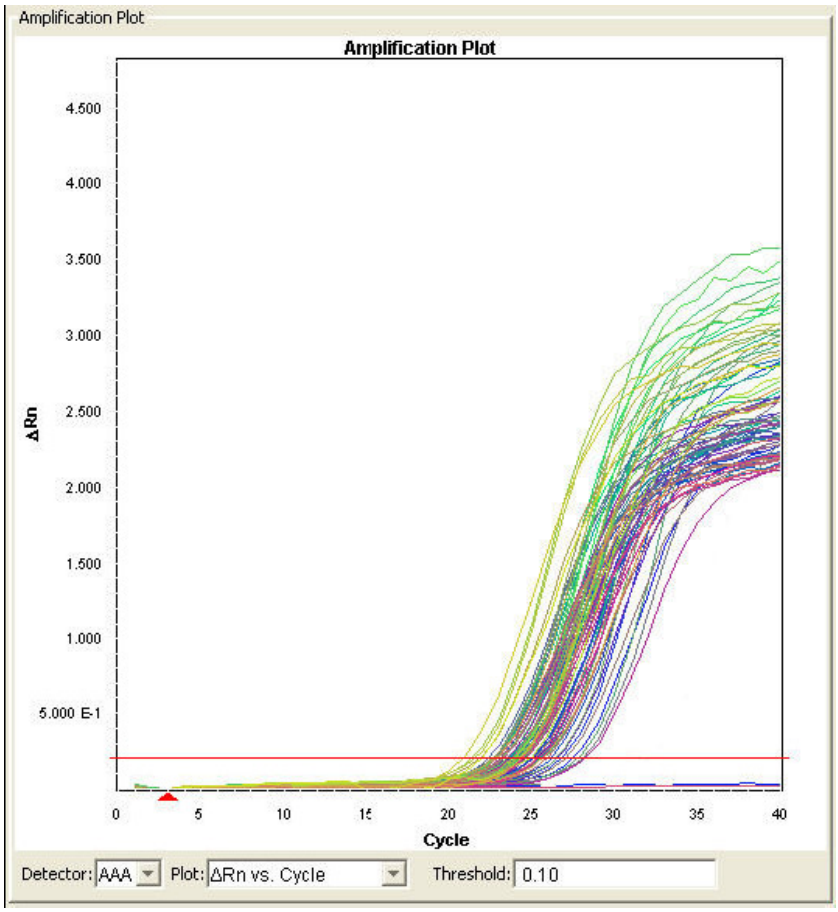
GADPDH


Amplification Plot
Amplification Plot
4.500
4.000
3.500
3.000
2.500
2.000
1.500
1.000
5.000 E-1
ΔRn
0
5
10
15
20
25
30
35
Cycle
Detector: GAPDH
Plot: ΔRn vs. Cycle
Threshold: 0.10

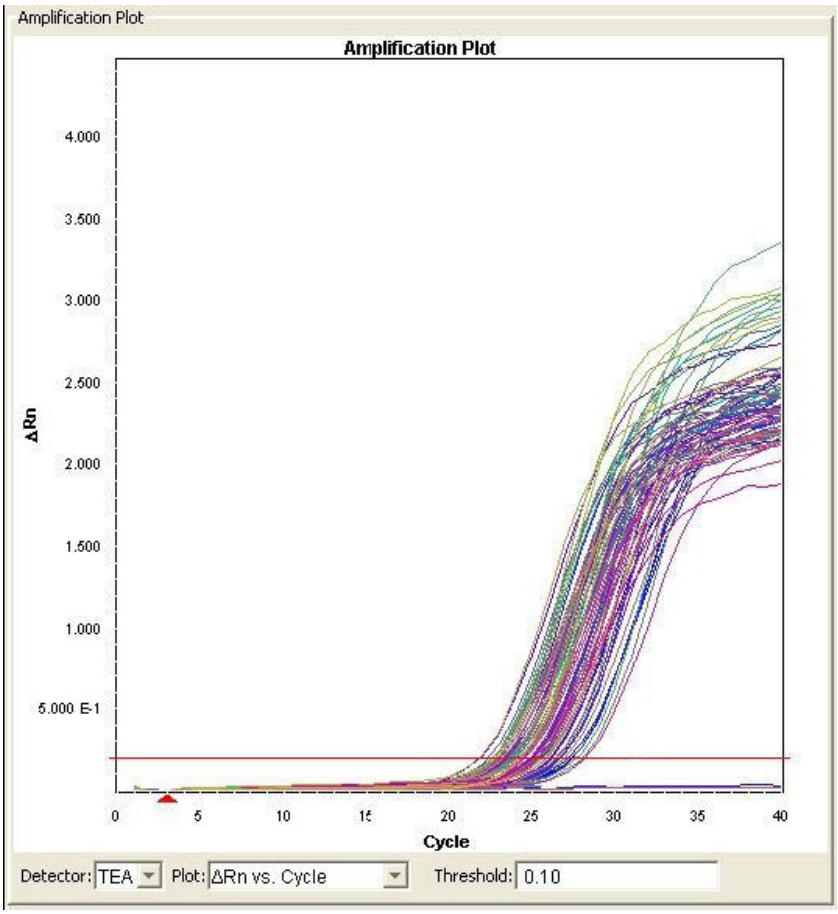
CTRL


Amplification Plot
Amplification Plot
4.500
4.000
3.500
3.000
2.500
2.000
1.500
1.000
5.000 E-1
ΔRn
0
5
10
15
20
25
30
35
40
Cycle
Detector: Controllo
Plot: ΔRn vs. Cycle
Threshold: 0.10

AAA PATIENTS



TEA PATIENTS



CONCLUSIONS

As we discussed, despite medical and surgical advances, atherosclerotic and aneurysmatic diseases are the leading cause of death in the Western world. Our work gets into the more extensive project which propose to develops a “Clinical Decision Support system”, defined as a statistical models of the pathologies, specific for each patient, based on the heterogeneous data analysis (molecular analysis, clinical analysis, clinical imaging, patient anamnesis).

We focus our attention on patients affected by aortic aneurysmatic abdominal disease and carotid stenosis. Our experimental work can be divided into 3 part: the first recruitment of patients and control subjects, the subsequently study of the gene expression among abdominal aortic aneurysm (AAA) patients with microarray technology, and the final evaluation of the differential gene expression in both AAA patients and carotid stenosis patients. About the first part, we spent a lot of time in order to select an homogeneous experimental group of patients for each pathology, in order to minimize the intrinsic differences between the patients and so to obtain relevant data. Nowadays we have collected 420 haematic samples tanks to the collaboration with professor Giorgio Maria Biasi and the Vascular Surgery Unit - S.Gerardo Teaching Hospital, Monza. The microarray analyses were performed by professor B.Giusti and professor R.Abbate group, at Careggi University Hospital, Firenze; in the Laboratory of Molecular Medicine – Department of Surgical Sciences – University of Milano-Bicocca, Monza, the last part of this work was developed. About the real-time PCR analyses some observations must be done. First, cDNA used for the analysis comes from RNA extract from PBMC's, even if we know that the

theoretically best material is the tissue sample (plaque or aortic wall) .Our choice depends on the difficulty to collect tissue samples in patients and, mostly, in control subjects. In fact, during a surgical intervention, the primary objective of the surgeon is to resolve the artery occlusive problems damaging the vessel and in particular the endothelium as little as possible, in order to avoid the development of a subsequently restenosis. Moreover, in order to have a control group, throughout a short period of time we should have collected tissue samples from advanced-age non pathologic subjects dead for traumatic events; instead, PBMC's are easily to collect both in patients and controls subjects. Secondly, even if is reported that TaqMan method is more accurate and reliable than the SYBR green method, we have chosen the second one because of the cheapness and the quick realization of a qualitative method in comparison with a quantitative one. In fact, for this first screening, we decided to use a qualitative method (SYBR green) which calculate the rate of gene expression between patients and controls samples, and not the precise amount of gene copies number for each analysis; starting from the data obtained with this method, we will use the TaqMan method for the subsequently analysis on a bigger number of subjects, referring to the standard commercial curves in order to calculate for each sample the gene copies number. Among the pull of genes resulting differentially expressed in microarray analysis, we have chosen to analyze with the real time PCR the expression of 10 genes related to lipid metabolism and inflammation; the data obtained confirm the role of those mechanisms in the development of the atherosclerosis and aneurysm, and because of the presence of the same gene expression

alterations in both pathologies, ours results underline the possibility of a common pathogenetic basis. Below, some considerations about those genes are reported. Starting with the lipid metabolism related genes, monoglyceride lipase, also called monoacylglycerol lipase (MGLL), play an important role in the hydrolysis of the monoglycerides formed during the hydrolysis of triglycerides. In adipose tissue, the stored triglycerides are hydrolyzed sequentially by triglyceride lipase and hormonesensitive lipase to diglycerides and monoglycerides, respectively. The monoglycerides formed are subsequently hydrolyzed by MGL to fatty acids and glycerol. MGL also catalyzes the hydrolysis of monoglycerides produced by lipoprotein lipase activity on triglycerides of circulating chylomicrons and very-low-density lipoproteins. The free fatty acids released from triglycerides by these lipases are used as a source of energy in the body. High level of MGLL are therefore related to high levels of FFA which contribute to the activation of the immunity response and the attraction of monocytes to the adipose rich zone and to the endothelial cells of blood vessels. Moreover, recently has been discovered that MGLL is highly expressed in CD16+ Mo, a minor subset of monocytes expanded in certain inflammatory conditions including sepsis and HIV infection and be able to migrates into tissues expressing CX3CL1 and producing pro-inflammatory cytokines. In ours patients MGLL is overexpressed in comparison with control subjects , and this is consistent with the inflammatory state of patients. Also Free fatty acid receptor 2, is overexpressed in ours patients in comparison with control subject. Has been demonstrated that FFAR2, FFA2 mRNA can be detected in a variety of tissues, but the highest expression is

found in immune cells such as neutrophils, monocytes, peripheral blood mononuclear cells, B-lymphocytes, and polymorphonuclear cells (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). This protein is a receptor for short chain free fatty acids, linked to the MGLL action, involved in the inflammatory response and in regulating lipid plasma levels. The upregulation of FFAR2, as the MGLL overexpression, confirm the immunity and lipid metabolism involment in the aneurysm and carotid stenosis pathogenesis. ADIPOR1 is an adiponectin receptor, an hormone secreted by adipocytes that acts as an antidiabetic and anti-atherogenic adipokine. Is known that a decreased plasma level of adiponectin is associated with obesity and metabolic syndrome and correlated with endothelial dysfunction. Moreover, a recent publication provide the novel evidence that upregulation of AdipoRs in ECs potentiates the antiinflammatory effect of adiponectin, suggesting that the modulation of adiponectin receptors may have potential therapeutic applications for cardiovascular desease. In ours patients ADIPOR1 is overexpressed in comparison with the control subjects, and so two hypothesis could be formulated: the first one is that this upregulation could be a cellular try to trigger a protective mechanism increasing the levels of adiponectin in the blood flow and consequently its receptor expression, in contrast the second one is that this upregulation could be a consequence of the lower levels of adiponectin in the blood flow.
PLA2G1B (phospholipase A2, group1B) is a member of the Phospholipases family, enzymes that hydrolyze membrane phospholipids to generate products that are involved in the regulation of many cellular processes. Their activity is controlled by a wide

variety of agonists, including hormones, neurotransmitters, growth factors, and cytokines. The principal phospholipases are PLA_1, PLA_2, PLC, and PLD, and they cleave phospholipids at different sites. Phospholipase A2 catalyzes the release of fatty acids from glycero-3-phosphocholines. Among the fatty acids produced by PLA2 there's arachidonic acid, an omega-6 fatty acid transformed by COX1/2 into leukotrienes and prostaglandins, which are be able to modulate immune function via the lymphocyte; they are mediators of the vascular phases of inflammation and are potent vasodilators, increasing vascular permeability. In ours patients PLA2 is overexpressed in comparison with control subjects, and this correlate with their pathological inflammatory state. Also the upregulation of HASD17B14 (hydroxysteroid (17-beta) dehydrogenase 14) is linked to the increase of haematic inflammatory state; in fact, this enzyme is primarily involved in the metabolism of steroids at the C17 position and also of other substrates, such as fatty acids, prostaglandins, and xenobiotics (Lukacik et al., 2007), and it's be able to produce haematic prostaglandins. Acyl-Coenzyme A dehydrogenase (ACADS) is a tetrameric mitochondrial flavoprotein, which is a member of the acyl-CoA dehydrogenase family. This enzyme catalyzes the initial step of the mitochondrial fatty acid beta-oxidation pathway. The overexpression of ACADS in patients could be related to a higher lipids metabolic amount. Among the 10 validated genes, the only one resulting downregulated in ours patients is LPR5 (Low-density lipoprotein receptor-related protein 5) . This gene encodes a transmembrane low-density lipoprotein receptor that binds and internalizes ligands in the process of receptor-mediated endocytosis.

This protein also acts as a co-receptor with Frizzled protein family members for transducing signals by Wnt proteins. It's known that the Wnt signaling is also involved in adipogenesis by negatively regulating adipogenic transcription factors (Tcfs); in fact, Wnt signalling acts as an adipogenic switch, when it is on, adipogenesis is repressed, when it is off, adipogenesis is initiated. A possible explanation of LPR5 downregulation in ours patients is that this could be a cellular protective mechanism, in fact the Wnt pathway inhibition is related to an increase in adipogenesis, favouring the adipocytes internalization of free fatty acids (FFAs) and so the decrease of FFAs haematic concentration which contribute to the inflammatory response. Among the "immunity" related genes, we observed an upregulation of TICAM1 (Toll-like receptor adaptor molecule 1), IL1RAPL (Interleukin 1 receptor accessory protein like-1) and TRAP1 (Tumor necrosis factor type 1 recepto-associated protein) in ours patients in comparison with control subjects. TICAM1, better known in literature as TRIF, is a Toll/IL1R(TIR) domain-containing adaptor molecule which specifically interacts with TLR3 and activates nuclear factor kappa-B (NFKB), inducing interferon-beta (IFNB) transcription, which is responsible of the increasing of proinflammatory cytokines. An upregulation of TRIF could be so related to the inflammatory state improvement. About IL1RAPL1, even if its involvement in the pathogenesis of atherosclerosis isn't still demonstrated, we could associate the upregulation of this gene with the pathology because of it's linkage with IL-1, a pro-inflammatory cytokine involved in inflammatory reactions. Lastly, TRAP1 encode a mitochondrial HSP90 protein, a molecular chaperones that have key

roles in signal transduction, protein folding, protein degradation, and morphologic evolution. TRAP-1 mRNA expression is increased in tumor cells resistant to chemotherapy. In fact, the stable clones expressing constitutively high TRAP-1 levels are more resistant to H2O2-induced DNA damage and to apoptosis by cisplatin, confirmed the he protective role of TRAP-1 against oxidative stress and apoptosis (Montesano Gesualdi N. et al, Stress. 2007 Nov;10(4):342-50). TRAP1 also interacts with Exostosins-1 and 2(EXT1-2), the enzymes responsible of the heparan sulphate (HS) biosynthesis; HS is involved in the coagulation cascade, because blood anti-coagulation is achieved mostly by heparan sulfate proteoglycans derived from endothelial cells. So, we can assume that TRAP1, through the action of EXT and HS, is maybe involved in both coagulative and antioxidant processes related to the pathologies.

So concluding, those results show that we have identify a pool of disease related genes that could be involved in the development of atherosclerotic and aneurysmatic lesions; the next step will be to better understand the role and the molecular mechanism of action of each genes, also still validating the genes expression in a bigger number of patients. At the end, we would like to set up a model of the diseases correlating the genetic data sources with all the parameters today available and used for the diagnosis and treatment of the pathologies, in order to help the decision process about the therapeutic strategy (pharmacological and/or surgical); in this way perhaps in the future will be possible to personalize the therapy of each patient according to the molecular and clinical characteristic of the pathology.

BIBLIOGRAPHY

1. Chiari H. Ueber Verhalten des Teilung-swinkels der Carotis Communis bei der Endarteritis Chronica Deformans. Verh Dtsch Ges Pathol. 1905;9:326.

2. Hunt JR. The role of the carotid arteries in the causation of vascular lesions of the brain, with remarks on certain special features of the symptomatology. Am J Med Sci. 1914;147:704-713.

3. Moniz E, Lima A, de Lacerda R. Par thrombose de la carotide interne. Presse Med. 1937;45:977.

4. Hultquist GT, Jena GF. Uber Thrombose und Embolie der Arteria Carotis und hierbei vorkommende Gehirnstorungen. Quoted in Fisher CM, Adams RD. Observations on brain embolism with special reference to the mechanism of hemorrhagic infarction. J Neuropathol Exp Neurol. 1951;10:92-93.

5. Fisher CM. Occlusion of the internal carotid artery. Arch Neurol Psychiatry.. 1951;65:346-377. 14. North American Symptomatic Carotid Endarterectomy Trial Collaborators. Beneficial effect of carotid endarterectomy in symptomatic patients with high grade carotid stenosis. N Engl J Med. 1991;325:445-453.

6. Endarterectomy for Asymptomatic Stenosis. Executive Committee for ACAS Study. JAMA. 1995;273:1421-1428.

7. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (1) N Engl J Med 1992;326:242-250.

8. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (2) N Engl J Med 1992;326:310-318.

9. Ross R, Glomset JA. The pathogenesis of atherosclerosis (first of two parts) N Engl J Med 1976;295:369-377.

10. Ross R, Glomset JA. The pathogenesis of atherosclerosis (second of two parts) N Engl J Med 1976;295:420-425.

11. Ross R. Atherosclerosis—an inflammatory disease N Engl J Med 1999;340:115-126.

12. Stary HC. Composition and classification of human atherosclerotic lesions Virchows Arch 1992;421:277-290.

13. Stary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosisA report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1994;89:2462-2478.

14. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histologic classification of atherosclerosisA report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995;92:1355-1374.

15. Schwartz SM, deBlois D, O'Brien ER. The intimaSoil for atherosclerosis and restenosis. Circ Res 1995;77:445-465.
16. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis Circulation 2002;105:1135-1143.

17. Stary HC. Natural history and histologic classification of atherosclerotic lesions: an update Arterioscler Thromb Vasc Biol 2000;20:1177-1178.

18. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk Arterioscler Thromb Vasc Biol 2003;23:168-175.

19. Ignarro LJ. Experimental evidences of nitric oxide-dependent vasodilatory activity of nebivolol, a third-generation beta-blocker Blood Press Suppl 2004;1:2-16.

20. Ignarro LJ, Napoli C. Novel features of nitric oxide, endothelial nitric oxide synthase, and atherosclerosis Curr Atheroscler Rep 2004;6:281-287.

21. Voetsch B, Jin RC, Loscalzo J. Nitric oxide insufficiency and atherothrombosis Histochem Cell Biol 2004;122:353-367.

22. Ravensbergen J, Ravensbergen JW, Krijger JK, Hillen B, Hoogstraten HW. Localizing role of hemodynamics in atherosclerosis in several human vertebrobasilar junction geometries Arterioscler Thromb Vasc Biol 1998;18:708-716.

23. Nerem RM. Vascular fluid mechanics, the arterial wall, and atherosclerosis J Biomech Eng 1992;114:274-282.

24. Gimbrone Jr. MA, Nagel T, Topper JN. Biomechanical activation: an emerging paradigm in endothelial adhesion biology J Clin Invest 1997;991809–13.

25. Traub O, Ishida T, Ishida M, Tupper JC, Berk BC. Shear stress-mediated extracellular signal-regulated kinase activation is regulated by sodium in endothelial cellsPotential role for a voltage-dependent sodium channel. J Biol Chem 1999;274:20144-20150.

25. Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature Circ Res 1999;85:753-766.

27. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress Circ Res 2000;87:840-844.

28. Steinberg DPS, Carew TE, Khoo JC, Witztum JL. Beyond cholesterolModifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915-924.

29. Khalil MF, Wagner WD, Goldberg IJ. Molecular interactions leading to lipoprotein retention and the initiation of atherosclerosis Arterioscler Thromb Vasc Biol 2004;24:2211-2218.

30. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis Circ Res 2002;91:281-291.

31. Gordon S. Pattern recognition receptors: doubling up for the innate immune response Cell 2002;111:927-930.

32. Andersen HO, Holm P, Stender S, Hansen BF, Nordestgaard BG. Dose-dependent suppression of transplant arteriosclerosis in aorta-allografted, cholesterol-clamped rabbitsSuppression not eliminated by the cholesterol-raising effect of cyclosporine. Arterioscler Thromb Vasc Biol 1997;17:2515-2523.

33. Steinberg D, Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? Circulation 2002;105:2107-2111.

34. Hansson GK. Immune mechanisms in atherosclerosis Arterioscler Thromb Vasc Biol 2001;21:1876-1890.

35. Rosenfeld ME. Leukocyte recruitment into developing atherosclerotic lesions: the complex interaction between multiple molecules keeps getting more complex Arterioscler Thromb Vasc Biol 2002;22:361-363.

36. Okamura Y, Watari M, Jerud ES, et al. The extra domain A of fibronectin activates Toll-like receptor 4 J Biol Chem 2001;276:10229-10233.

37. Tsan MF, Gao B. Cytokine function of heat shock proteins Am J Physiol Cell Physiol 2004;286:C739-C744.

38. Triantafilou M, Triantafilou K. Heat-shock protein 70 and heat-shock protein 90 associate with Toll-like receptor 4 in response to bacterial lipopolysaccharide Biochem Soc Trans 2004;32:636-639.

39. Vink A, Schoneveld AH, van der Meer JJ, et al. In vivo evidence for a role of Toll-like receptor 4 in the development of intimal lesions Circulation 2002;106:1985-1990.

40. Pasterkamp G, Van Keulen JK, De Kleijn DP. Role of Toll-like receptor 4 in the initiation and progression of atherosclerotic disease Eur J Clin Invest 2004;34:328-334.

41. Hollestelle SC, De Vries MR, Van Keulen JK, et al. Toll-like receptor 4 is involved in outward arterial remodeling Circulation 2004;109:393-398.

42. Mallat Z, Tedgui A. Current perspective on the role of apoptosis in atherothrombotic disease Circ Res 2001;88:998-1003.

43. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromesParts 1 and 2. N Engl J Med 1992;326:242-250and 310–8.

44. Theroux P, Fuster V. Acute coronary syndromes: unstable angina and non–Q-wave myocardial infarction Circulation 1998;97:1195-1206.

45. Nemerson Y. Tissue factor and hemostasis Blood 1988;71:1-8.
46. Banner DW, D'Arcy A, Chene C, et al. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor Nature 1996;380:41-46.

47. Moreno PR, Bernardi VH, Lopez-Cuellar J, et al. Macrophages, smooth muscle cells, and tissue factor in unstable anginaImplications for cell-mediated thrombogenicity in acute coronary syndromes. Circulation 1996;94:3090-3097.

48. Hutter R, Sauter BV, Fallon JT, Fuster V, Badimon JJ. Macrophages and vascular stellate cells in human carotid plaques are prone to apoptosis and tissue factor expression(abstr) J Am Coll Cardiol 2001;38(Suppl A):288A.

49. Badimon JJ, Lettino M, Toschi V, et al. Local inhibition of tissue factor reduces the thrombogenicity of disrupted human atherosclerotic plaques: effects of tissue factor pathway inhibitor on plaque thrombogenicity under flow conditions Circulation 1999;99:1780-1787.

50. Roque M, Reis ED, Fuster V, et al. Inhibition of tissue factor reduces thrombus formation and intimal hyperplasia after porcine coronary angioplasty J Am Coll Cardiol 2000;36:2303-2310.

53. Kato H. Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: basic and clinical aspects Arterioscler Thromb Vasc Biol 2002;22:539-548.

59. Kullo IJ, Gau GT, Tajik AJ. Novel risk factors for atherosclerosis Mayo Clin Proc 2000;75:369-380.

60. Salomaa V, Rasi V, Kulathinal S, et al. Hemostatic factors as predictors of coronary events and total mortality: the FINRISK '92 Hemostasis study Arterioscler Thromb Vasc Biol 2002;22:353-358.

61. Edelberg JM, Christie PD, Rosenberg RD. Regulation of vascular bed-specific prothrombotic potential Circ Res 2001;89:117-124.

62. Edgington TS. So what is critically lacking with coronary atherosclerotic plaques? Perhaps the antithrombotic control Am J Pathol 2001;159:795-796.

63. Dangas G, Badimon JJ, Smith DA, et al. Pravastatin therapy in hyperlipidemia: effects on thrombus formation and the systemic hemostatic profile J Am Coll Cardiol 1999;33:1294-1304.

64. Rauch U, Osende JI, Chesebro JH, et al. Statins and cardiovascular diseases: the multiple effects of lipid-lowering therapy by statins Atherosclerosis 2000;153:181-189.

65. Badimon L, Martinez-Gonzalez J, Royo T, Lassila R, Badimon JJ. A sudden increase in plasma epinephrine levels transiently enhances platelet deposition on severely damaged arterial wall—studies in a porcine model Thromb Haemost 1999;82:1736-1742.

66. Miller GJ. Hemostasis and cardiovascular riskThe British and European experience. Arch Pathol Lab Med 1992;116:1318-1321.

67. Muller JE, Kaufmann PG, Luepker RV, Weisfeldt ML, Deedwania PC, Willerson JT. Mechanisms precipitating acute cardiac events: review and recommendations of an NHLBI workshopNational Heart, Lung, and Blood Institute. Mechanisms Precipitating Acute Cardiac Events Participants. Circulation 1997;96:3233-3239.

68. Meigs JB, Mittleman MA, Nathan DM, et al. Hyperinsulinemia, hyperglycemia, and impaired hemostasis: the Framingham Offspring Study JAMA 2000;283:221-228.

69. Rauch U, Ziegler D, Piolot R, et al. Platelet activation in diabetic cardiovascular autonomic neuropathy Diabet Med 1999;16:848-852.

70. Sambola A, Osende J, Hathcock J, et al. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity Circulation 2003;107:973-977.

71. Osende JI, Badimon JJ, Fuster V, et al. Blood thrombogenicity in type 2 diabetes mellitus patients is associated with glycemic control J Am Coll Cardiol 2001;38:1307-1312.

72. Tschoepe D, Rauch U, Schwippert B. Platelet-leukocyte-cross-talk in diabetes mellitus Horm Metab Res 1997;29:631-635.

73. Rauch U, Crandall J, Osende JI, et al. Increased thrombus formation relates to ambient blood glucose and leukocyte count in diabetes mellitus type 2 Am J Cardiol 2000;86:246-249.

74. Kallen J, Welzenbach K, Ramage P, et al. Structural basis for LFA-1 inhibition upon lovastatin binding to the CD11a I-domain J Mol Biol 1999;292:1-9.

75. Frenette PS. Locking a leukocyte integrin with statins N Engl J Med 2001;345:1419-1421.

76. Walter DH, Rittig K, Bahlmann FH, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells Circulation 2002;105:3017-3024.

77. Soejima H, Ogawa H, Yasue H, et al. Heightened tissue factor associated with tissue factor pathway inhibitor and prognosis in patients with unstable angina Circulation 1999;99:2908-2913.

78. Gando S, Nanzaki S, Sasaki S, Kemmotsu O. Significant correlations between tissue factor and thrombin markers in trauma and

septic patients with disseminated intravascular coagulation Thromb Haemost 1998;79:1111-1115.

79. Rosenberg RD, Aird WC. Vascular bed-specific hemostasis and hypercoagulable states N Engl J Med 1999;340:1555-1564.
80. Soejima H, Ogawa H, Yasue H, et al. Angiotensin-converting enzyme inhibition reduces monocyte chemoattractant protein-1 and tissue factor levels in patients with myocardial infarction J Am Coll Cardiol 1999;34:983-988.

81. Saito Y, Wada H, Yamamuro M, et al. Changes of plasma hemostatic markers during percutaneous transluminal coronary angioplasty in patients with chronic coronary artery disease Am J Hematol 1999;61:238-242.

82. Hugel B, Socie G, Vu T, et al. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia Blood 1999;93:3451-3456.

83. Munford RS. Statins and the acute-phase response N Engl J Med 2001;344:2016-2018.

84. Blake GJ, Ridker PM. Novel clinical markers of vascular wall inflammation Circ Res 2001;89:763-771.

85. Ridker PM, Rifai N, Clearfield M, et al. Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events N Engl J Med 2001;344:1959-1965.

86. Bickel C, Rupprecht HJ, Blankenberg S, et al. Relation of markers of inflammation (C-reactive protein, fibrinogen, von Willebrand factor, and leukocyte count) and statin therapy to long-term mortality in patients with angiographically proven coronary artery disease Am J Cardiol 2002;89:901-908.

87. Burke AP, Tracy RP, Kolodgie F, et al. Elevated C-reactive protein values and atherosclerosis in sudden coronary death: association with different pathologies Circulation 2002;105:2019-2023.

88. Zwaka TP, Hombach V, Torzewski J. C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis Circulation 2001;103:1194-1197.

89. Torzewski M, Rist C, Mortensen RF, et al. C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis Arterioscler Thromb Vasc Biol 2000;20:2094-2099.

90. Pasceri V, Willerson JT, Yeh ET. Direct proinflammatory effect of C-reactive protein on human endothelial cells Circulation 2000;102:2165-2168.

91. Schonbeck U, Libby P. CD40 signaling and plaque instability Circ Res 2001;89:1092-1103.

92. Schonbeck U, Varo N, Libby P, Buring J, Ridker PM. Soluble CD40L and cardiovascular risk in women Circulation 2001;104:2266-2268.

93.Chaturvedi S, Bruno A, Feasby T, et al. Carotid endarterectomy–an evidence-based review: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology (2005) 65:794–801.

94.European Carotid Surgery Trialists' Collaborative Group. Randomised trial of endarterectomy for recently symptomatic carotid stenosis: final results of the MRC European Carotid Surgery Trial (ECST). Lancet (1998) 351:1379–87.

95.Howell SJ. Carotid endarterectomy. Br J Anaesth 2007;99:119–31

96.Anonymous. Endarterectomy for asymptomatic carotid artery stenosis. Executive Committee for the Asymptomatic Carotid Atherosclerosis Study. JAMA 1995; 273:1421–1428.

97.MRC Asymptomatic Carotid Surgery Trial (ACST) Collaborative Group. Prevention of disabling and fatal strokes by successful carotid endarterectomy in patients without recent neurological symptoms: randomised controlled trial. Lancet 2004; 363:1491–1502.

98.Chambers BR, Donnan GA. Carotid endarterectomy for asymptomatic carotid stenosis. Cochrane Database Syst Rev 2005; 4:CD001923.

99.Bond R, Rerkasem K, Rothwell PM. Systematic review of the risks of carotid endarterectomy in relation to the clinical indication for and timing of surgery. Stroke 2003; 34:2290–2301.

100.Touze E, Calveta D, Chatellierb G and Masa J.L. Carotid stenting. Current Opinion in Neurology 2008, 21:56–63.

101. F. Crusco1, A. Antoniella, D. Di Lazzaro, A. Giovagnoni Multidetector CT and coronary artery bypass grafts. Radiol med (2007) 112:1087–1099.

102.Tonkin AM. Clinical relevance of statins: their role in secondary prevention. Atheroscler Suppl 2001;2(1):21–5.

103.Ray KK, Cannon CP. Early time to benefit with intensive statin treatment: could it be the pleiotropic effects? Am J Cardiol 2005;5(96(5A)):54F–60F.

104.LaRosa JC, Grundy SM,Waters DD, et al. Treating to newtargets (TNT) investigators intensive lipid lowering with atorvastatin in patients with stable coronary disease. N Engl J Med 2005;352(14):1425–35.

105.Pignone M, Phillips C, Mulrow C. Use of lipid lowering drugs for primary prevention of coronary heart disease: meta-analysis of randomized trials. BMJ 2000;21(321(7267)):983–6.

106.Thavendiranathan P, Bagai A, Brookhart MA, Choudry NK. Primary prevention of cardiovascular diseases with statin therapy. Arch Intern Med 2006;166:2307–13.

107.Stenestrand U, Wallentin L. Swedish register of cardiac intensive care (RIKSHIA) early statin treatment following acute myocardial infarction and 1-year survival. JAMA 2001;285(4):430–6.

108. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. Nat Med 2000;6(12):1399–402.

109. Hakamada-Taguchi R, Uehara Y, Kuribayashi K, et al. Inhibition of hydroxymethylglutaryl-coenzyme a reductase reduces Th1 development and promotes Th2 development. Circ Res 2003;93(10):948–56.

110.Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001;103:2885–90

111.Laufs U, La Fata V, Plutzky J, Liao KL. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 1998;97:1129–35.

112.DichtlW, Dulak J, FrickM, et al. HMG-CoA reductase inhibitors regulate inflammatory transcription factors in human endothelial and vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2003;23(1):58–63.

113.Undas A, Brummel KE, Musial J, Mann KG, Szczeklik A. Simvastatin depresses blood clotting by inhibiting activation of prpthrombin, factor V, and factor XIII and by enhancing factor Va inactivation. Circulation 2001;103:2248–53.

114.Remick J, Weintraub H, Setton R, Offenbacher J, Fisher E, Schwartzbard A Fibrate Therapy.
Cardiology in Review 2008;16: 129–141.

115.Ruilope LM, Rosei EA, Bakris GL, Mancia G, Poulter NR, Taddei S, Unger T, Volpe M, Waeber B, Zannad F. Angiotensin receptor blockers: therapeutic targets and cardiovascular protection**.** Blood Press. 2005;14(4):196-209.

116. Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BM Renin-angiotensin system and cardiovascular risk. Lancet. 2007 Apr 7;369(9568):1208-19.

117. Godfraind T. Antioxidant effects and the therapeutic mode of action of calcium channel blockers in hypertension and atherosclerosis. Phil. Trans. R. Soc. B (2005) 360, 2259–2272

118.Murray CJ, Lopez AD. Mortality by cause for eight regions of theworld: Global Burden of Disease Study. Lancet 1997;349:1269–76.

119.DonnanGA, Fisher M, Macleod M,Davis SM. Stroke. Lancet 2008;371:1612–23.

120.Zivin JA. Factors determining the therapeutic window for stroke. Neurology 1998;50:599–603.

121.Hossmann KA. Viability thresholds and the penumbra of focal ischemia. Ann Neurol 1994;36:557–65.

122.Obrenovitch TP. The ischaemic penumbra: twenty years on. Cerebrovasc Brain Metab Rev 1995;7:297–323.

123.Astrup J, Siesjo BK, Symon L. Thresholds in cerebral ischemia—the ischemic penumbra. Stroke 1981;12:723–5.

124.Furlan M, Marchal G, Viader F, Derlon JM, Baron JC. Spontaneous neurological recovery after stroke and the fate of the ischemic penumbra. Ann Neurol 1996;40:216–26.

125.Fisher M, Garcia JH. Evolving stroke and the ischemic penumbra. Neurology 1996;47:884–8.

126.MarkusR,Reutens DC, Kazui S,Read S,Wright P, Pearce DC, et al.Hypoxic tissue in ischaemic stroke: persistence and clinical consequences of spontaneous survival. Brain 2004;127:1427–36.

127.Nagesh V,Welch KM, Windham JP, Patel S, Levine SR, Hearshen D, et al. Time course of ADCw changes in ischemic stroke: beyond the human eye! Stroke 1998;29:1778–82.

128.Baron JC. Mapping the ischaemic penumbra with PET: implications for acute stroke treatment. Cerebrovasc Dis 1999;9:193–201.

129.Read SJ, Hirano T, Abbott DF, Sachinidis JI, Tochon-Danguy HJ, Chan JG, et al. Identifying hypoxic tissue after acute ischemic stroke using PET and 18Ffluoromisonidazole. Neurology 1998;51:1617–21.

130.Martin RL, Lloyd HG, Cowan AI. The early events of oxygen and glucose deprivation: setting the scene for neuronal death? Trends Neurosci 1994;17:251–7.

131.Katsura K, Kristian T, Siesjo BK. Energy metabolism, ion homeostasis, and cell damage in the brain. Biochem Soc Trans 1994;22:991–6.

132.Nicholls D, Attwell D. The release and uptake of excitatory amino acids. Trends Pharmacol Sci 1990;11:462–8.

133.Nicoli F, Lefur Y, Denis B, Ranjeva JP, Confort-Gouny S, Cozzone PJ. Metabolic counterpart of decreased apparent diffusion coefficient during hyperacute ischemic stroke: a brain proton magnetic resonance spectroscopic imaging study. Stroke 2003;34:e82–7.

134.Schneweis S, Grond M, Staub F, Brinker G, Neveling M, Dohmen C, et al. Predictive value of neurochemical monitoring in large middle cerebral artery infarction. Stroke 2001;32:1863–7.

135.Brouns R, Sheorajpanday R, Wauters A, De Surgeloose D, Mariën P, De Deyn PP. Evaluation of lactate as a marker of metabolic stress and cause of secondary damage in acute ischemic stroke or TIA. Clin Chim Acta 2008;397:27–31.

136.Dohmen C, Bosche B, Graf R, Reithmeier T, Ernestus RI, Brinker G, et al. Identification and clinical impact of impaired cerebrovascular autoregulation in patients with malignant middle cerebral artery infarction. Stroke 2007;38:56–61.

137.Uyttenboogaart M, Koch MW, Stewart RE, Vroomen PC, Luijckx GJ, De Keyser J. Moderate hyperglycaemia is associated with favourable outcome in acute lacunar stroke. Brain 2007;130:1626–30.

138.Meldrum B, Evans M, Griffiths T, Simon R. Ischaemic brain damage: the role of excitatory activity and of calcium entry. Br J Anaesth 1985;57:44–6.

139.Choi DW. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci 1988;11:465–9.

140.Chen M, Lu TJ, Chen XJ, Zhou Y, Chen Q, Feng XY, et al. Differential roles of NMDA receptor subtypes in ischemic neuronal cell death and ischemic tolerance. Stroke 2008;39(11):3042–8.

141.Furukawa K, Fu W, Li Y, Witke W, Kwiatkowski DJ, Mattson MP. The actinsevering protein gelsolin modulates calcium channel and NMDA receptor activities and vulnerability to excitotoxicity in hippocampal neurons. J Neurosci 1997;17:8178–86.

142.Chen ZL, Strickland S. Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. Cell 1997;91:917–25.

143.Love S. Oxidative stress in brain ischemia. Brain Pathol 1999;9:119–31.

144.Kontos HA, George E. Brown memorial lecture. Oxygen radicals in cerebral vascular injury. Circ Res 1985;57:508–16.

145.Kontos HA. Oxygen radicals in cerebral ischemia: the 2001 Willis lecture. Stroke 2001;32:2712–6

146.Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996;271:C1424–37.

147.Masters BS. Nitric oxide synthases: why so complex? Annu Rev Nutr 1994;14:131–45.

148.Zhang F, Xu S, Iadecola C. Time dependence of effect of nitric oxide synthase inhibition on cerebral ischemic damage. J Cereb Blood Flow Metab 1995;15:595–601.

149.MacAllister RJ, Whitley GS, Vallance P. Effects of guanidino and uremic compounds on nitric oxide pathways. Kidney Int 1994;45:737–42.

150.Kielstein JT, Donnerstag F, Gasper S,MenneJ, KielsteinA, Martens-Lobenhoffer J, et al. ADMA increases arterial stiffness and decreases cerebral blood flow in humans. Stroke 2006;37:2024–9.
151.Calver A, Collier J, Vallance P. Nitric oxide and the control of human vascular tone in health and disease. Eur J Med 1993;2:48–53.

152.Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315–424.

153.Chan JR, Boger RH, Bode-Boger SM, Tangphao O, Tsao PS, Blaschke TF, et al. Asymmetric dimethylarginine increases mononuclear cell adhesiveness in hypercholesterolemic humans. Arterioscler Thromb Vasc Biol 2000;20:1040–6.

154.Lennon SV, Martin SJ, Cotter TG. Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. Cell Prolif 1991;24:203–14.

155.EvansMD,CookeMS. Factors contributing to the outcome of oxidative damage to nucleic acids. Bioessays 2004;26:533–42.

156.Liu PK, Hsu CY, Dizdaroglu M, Floyd RA, Kow YW, Karakaya A, et al. Damage, repair, and mutagenesis in nuclear genes after mouse forebrain ischemiareperfusion. J Neurosci 1996;16:6795–806.

157.Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood–brain barrier opening after reperfusion in rat brain. Stroke 1998;29:2189–95.

158.Montaner J, Molina CA, Monasterio J, Abilleira S, Arenillas JF, Ribo M, et al. Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. Circulation 2003;107:598–603.

159.Wei EP, Kontos HA, Christman CW, DeWitt DS, Povlishock JT. Superoxide generation and reversal of acetylcholine-induced cerebral arteriolar dilation after acute hypertension. Circ Res 1985;57:781–7.

160. Lorberboym M, Lampl Y, Sadeh M. Correlation of 99mTc-DTPA SPECT of the blood–brain barrier with neurologic outcome after acute stroke. J Nucl Med 2003;44:1898–904.

161.Bang OY, Buck BH, Saver JL, Alger JR, Yoon SR, Starkman S, et al. Prediction of hemorrhagic transformation after recanalization therapy using T2*-permeability magnetic resonance imaging. Ann Neurol 2007;62:170–6.

162.Kidwell CS, Latour L, Saver JL, Alger JR, Starkman S, Duckwiler G, et al. Thrombolytic toxicity: blood brain barrier disruption in human ischemic stroke. Cerebrovasc Dis 2008;25:338–43.

163.Hjort N, Wu O, Ashkanian M, Solling C, Mouridsen K, Christensen S, et al. MRI detection of early blood–brain barrier disruption: parenchymal enhancement predicts focal hemorrhagic transformation after thrombolysis. Stroke 2008;39:1025–8.
164.Hamann GF, Okada Y, Fitridge R, del Zoppo GJ. Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion. Stroke 1995;26:2120–6.

165.Kastrup A, Engelhorn T, Beaulieu C, de CA, Moseley ME. Dynamics of cerebral injury, perfusion, and blood–brain barrier changes after temporary and permanent middle cerebral artery
occlusion in the rat. J Neurol Sci 1999;166:91–9.

166.Kamiya T, Katayama Y, Kashiwagi F, Terashi A. The role of bradykinin in mediating ischemic brain edema in rats. Stroke 1993;24:571–5.

167.Abumiya T, Lucero J, Heo JH, Tagaya M, Koziol JA, Copeland BR, et al. Activated microvessels express vascular endothelial growth factor and integrin alpha(v)beta3 during focal cerebral ischemia. J Cereb Blood Flow Metab 1999;19:1038–50.

168.Okada Y, Copeland BR, Fitridge R, Koziol JA, del Zoppo GJ. Fibrin contributes to microvascular obstructions and parenchymal changes during early focal cerebral ischemia and reperfusion. Stroke 1994;25:1847–53.

169.Del Zoppo GJ, Hallenbeck JM. Advances in the vascular pathophysiology of ischemic stroke. Thromb Res 2000;98:73–81.

170.Rapela CE, Green HD. Autoregulation of canine cerebral blood flow during hypercarbia and during hypocarbia and during controlled (H+). Circ Res 1964;15(Suppl. 1):205–15.

171.Paulson OB, Strandgaard S, Edvinsson L. Cerebral autoregulation. Cerebrovasc Brain Metab Rev 1990;2:161–92

172.Andresen J, Shafi NI, Bryan Jr RM. Endothelial influences on cerebrovascular tone. J Appl Physiol 2006;100:318–27.

173.Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988;332:411–5.

174.Hardebo JE, Kahrstrom J, Owman C, Salford LG. Endothelin is a potent constrictor of human intracranial arteries and veins. Blood Vessels 1989;26: 249–53.

175.Ziv I, Fleminger G, Djaldetti R, Achiron A, Melamed E, Sokolovsky M. Increased plasma endothelin-1 in acute ischemic stroke. Stroke 1992;23: 1014–6.

176.Estrada V, Tellez MJ,Moya J, Fernandez-Durango R, Egido J, Fernandez Cruz AF. High plasma levels of endothelin-1 and atrial natriuretic peptide in patients with acute ischemic stroke. Am J Hypertens 1994;7:1085–9.

177.Moldes O, Sobrino T, Millan M, Castellanos M, Perez de la Ossa N, Leira R, et al. High serum levels of endothelin-1 predict severe cerebral edema in patients with acute ischemic stroke treated with t-PA. Stroke 2008;39:2006–10.

178.Oliveira-Filho J, Silva SC, Trabuco CC, Pedreira BB, Sousa EU, Bacellar A. Detrimental effect of blood pressure reduction in the first 24 h of acute stroke onset. Neurology 2003;61:1047–51.

179.Ahmed N,Nasman P,WahlgrenNG. Effect of intravenous nimodipine on blood pressure and outcome after acute stroke. Stroke 2000;31:1250–5.

180. Zazulia AR, Videen TO, Powers WJ. Symptomatic autoregulatory failure in acute ischemic stroke. Neurology 2007;68:389–90.

181.Shin HK, Nishimura M, Jones PB, Ay H, Boas DA, Moskowitz MA, et al. Mild induced hypertension improves blood flow and oxygen metabolism in transient focal cerebral ischemia. Stroke 2008;39:1548–55.

182.Leonardi-Bee J, Bath PM, Phillips SJ, Sandercock PA. Blood pressure and clinical outcomes in the International Stroke Trial. Stroke 2002;33:1315–20.

183.Del Zoppo GJ, Mabuchi T. Cerebral microvessel responses to focal ischemia. J Cereb Blood Flow Metab 2003;23:879–94.

184.Del Zoppo GJ. Microvascular changes during cerebral ischemia and reperfusion. Cerebrovasc Brain Metab Rev 1994;6:47–96.

185.Borensztajn K, Peppelenbosch MP, Spek CA. Factor Xa: at the crossroads between coagulation and signaling in physiology and disease. Trends Mol Med 2008.

186.Green D. Coagulation cascade. Hemodial Int 2006;10(Suppl 2):S2–4.

187.Willemse JL, Hendriks DF. A role for procarboxypepidase U (TAFI) in thrombosis. Front Biosci 2007;12:1973–87.

188.Willemse JL, Brouns R, Heylen E, De Deyn PP, Hendriks DF. Carboxypeptidase U (TAFIa) activity is induced in vivo in ischemic stroke patients receiving thrombolytic therapy. J Thromb Haemost 2008;6:200–02.

189.Brouns R, Heylen E, Sheorajpanday R, Willemse JL, Kunnen J, De Surgeloose D, et al. Carboxypeptidase U (TAFIa) decreases the ef-

ficacy of thrombolytic therapy in ischemic stroke patients. Clin Neurol Neurosurg 2009;111:165–70.

190.Fisher M, Francis R. Altered coagulation in cerebral ischemia. Platelet, thrombin, and plasmin activity. Arch Neurol 1990;47:1075–9.

191.Zeller JA, Lenz A, Eschenfelder CC, Zunker P, Deuschl G. Platelet–leukocyte interaction and platelet activation in acute strokewith and without preceding infection. Arterioscler Thromb Vasc Biol 2005;25:1519–23.

192.Chong ZZ, Xu QP, Sun JN. Effects and mechanisms of triacetylshikimic acid on platelet adhesion to neutrophils induced by thrombin and reperfusion after focal cerebral ischemia in rats. Acta Pharmacol Sin 2001;22:679–84.

193,Htun P, Fateh-Moghadam S, Tomandl B, Handschu R, Klinger K, Stellos K, et al. Course of platelet activation and platelet-leukocyte interaction in cerebrovascular ischemia. Stroke 2006;37:2283–7.

194.Eddleston M, de la Torre JC, Oldstone MB, Loskutoff DJ, Edgington TS, MackmanN. Astrocytes are the primary source of tissue factor in the murine central nervous system. A role for astrocytes in cerebral hemostasis. J Clin Invest 1993;92:349–58.

195.Dong Y, Benveniste EN. Immune function of astrocytes. Glia 2001;36:180–90.

196.Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol 1998;56:149–71.

197.Morioka T, Kalehua AN, Streit WJ. Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. J Comp Neurol 1993;327:123–32.

198.Buck BH, Liebeskind DS, Saver JL, BangOY, Yun SW, StarkmanS, et al. Early neutrophilia is associated with volume of ischemic tissue in acute stroke. Stroke 2008;39:355–60.

199.Grau AJ, Reis A, Buggle F, Al-Khalaf A,Werle E, Valois N, et al. Monocyte function and plasma levels of interleukin-8 in acute ischemic stroke. J Neurol Sci 2001;192:41–7.

200.DeGraba TJ. The role of inflammation after acute stroke: utility of pursuing anti-adhesion molecule therapy. Neurology 1998;51:S62–8.

201.Huang J, Choudhri TF, Winfree CJ, McTaggart RA, Kiss S, Mocco J, et al. Postischemic cerebrovascular E-selectin expression mediates tissue injury in murine stroke. Stroke 2000;31:3047–53.

202.Ehrensperger E, Minuk J, Durcan L, Mackey A, Wolfson C, Fontaine AM, et al. Predictive value of soluble intercellular adhesion molecule-1 for risk of ischemic events in individuals with cerebrovascular disease. Cerebrovasc Dis 2005;20:456–62.

203.Blann A, Kumar P, Krupinski J, McCollum C, Beevers DG, Lip GY. Soluble intercelluar adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1 and von Willebrand factor in stroke. Blood Coagul Fibrinolysis 1999;10:277–84.

204.Allan SM, Rothwell NJ. Cytokines and acute neurodegeneration. Nat Rev Neurosci 2001;2:734–44.

205.Vila N, Chamorro A. Cytokines and acute-phase response in acute stroke. Stroke 1995;26:1729.

206.Vila N, Castillo J, Davalos A, Chamorro A. Proinflammatory cytokines and early neurological worsening in ischemic stroke. Stroke 2000;31:2325–9.

207.Hallenbeck JM. The many faces of tumor necrosis factor in stroke. Nat Med 2002;8:1363–8.

208.Vila N, Castillo J, Davalos A, Esteve A, Planas AM, Chamorro A. Levels of anti-inflammatory cytokines and neurologicalworsening in acute ischemic stroke. Stroke 2003;34:671-5.

209.Stamatovic SM, Keep RF, Kunkel SL, Andjelkovic AV. Potential role of MCP-1 in endothelial cell tight junction ‘opening’: signaling via Rho and Rho kinase. J Cell Sci 2003;116:4615–28.

210.Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV. Effects of the chemokine CCL2 on blood–brain barrier permeability during ischemiareperfusion injury. J Cereb Blood Flow Metab 2006;26:797–810.

211.Adibhatla RM, Hatcher JF, Larsen EC, Chen X, Sun D, Tsao FH. CDP-choline significantly restores phosphatidylcholine levels by differentially affecting phospholipase A2 and CTP: phosphocholine cytidylyltransferase after stroke. J Biol Chem 2006;281:6718–25.

212.Schwab JM, Schluesener HJ. Cyclooxygenases and central nervous system inflammation: conceptual neglect of cyclooxygenase 1. Arch Neurol 2003;60:630–2.

213.Kawano T, Anrather J, Zhou P, Park L, Wang G, Frys KA, et al. Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. Nat Med 2006;12:225–9.

214,Manabe Y, Anrather J, Kawano T, Niwa K, Zhou P, Ross M, et al. Prostanoids, not reactive oxygen species,mediate COX-2-dependent neurotoxicity. AnnNeurol 2004;55:668–75.

215.Tomimoto H, ShibataM, IharaM, Akiguchi I, Ohtani R, Budka H. A comparative study on the expression of cyclooxygenase and 5-lipoxygenase during cerebral ischemia in humans. Acta Neuropathol 2002;104:601–7.

216.Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR, Sieber FE, Portera-Cailliau C. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. Brain Res Bull 1998;46:281–309.

217.Choi DW. Ischemia-induced neuronal apoptosis. Curr Opin Neurobiol 1996;6:667–72.

218.Leist M, Nicotera P. Calcium and neuronal death. Rev Physiol Biochem Pharmacol 1998;132:79–125.

219.Leist M, Volbracht C, Kuhnle S, Fava E, Ferrando-May E, Nicotera P. Caspasemediated apoptosis in neuronal excitotoxicity triggered by nitric oxide. Mol Med 1997;3:750–64.

220.Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281: 1312–6.

221.Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS, et al. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-d-aspartate. J Neurosci 1995;15:6377–88.

222.Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, et al. Mediation of neuronal apoptosis by enhancement of outward potassium current. Science 1997;278:114–7.

223.Koh JY. Zinc and disease of the brain. Mol Neurobiol 2001;24:99–106.

224.Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.

225.Cho BB, Toledo-Pereyra LH. Caspase-independent programmed cell death following ischemic stroke. J Invest Surg 2008;21:141–7.

226.Schaller B, Graf R. Cerebral ischemia and reperfusion: the pathophysiologic concept as a basis for clinical therapy. J Cereb Blood Flow Metab 2004;24:351–71.

227.Kidwell CS, Saver JL, Mattiello J, Starkman S, Vinuela F, Duckwiler G, et al. Diffusion-perfusion MRI characterization of post-recanalization hyperperfusion in humans. Neurology 2001;57:2015–21.

228.DietrichWD. Morphological manifestations of reperfusion injury in brain.Ann N Y Acad Sci 1994;723:15–24.

229.Pan J, Konstas AA, Bateman B, OrtolanoGA, Pile-Spellman J. Reperfusion injury following cerebral ischemia: pathophysiology, MR imaging, and potential therapies. Neuroradiology 2007;49:93–102.

230.Wong CH, Crack PJ. Modulation of neuro-inflammation and vascular response by oxidative stress following cerebral ischemia-reperfusion injury. Curr Med Chem 2008;15:1–14.

231.Arumugam TV, Shiels IA,Woodruff TM, Granger DN, Taylor SM. The role of the complement system in ischemia-reperfusion injury. Shock 2004;21:401–9.

232.Rosenberg GA, Cunningham LA,Wallace J, Alexander S, Estrada EY, Grossetete M, et al. Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: activation ofMMP-9 linked to stromelysin-1 and microglia in cell cultures. Brain Res 2001;893:104–12.

233.Bayes M, Rabasseda X. Gateways to clinical trials. Methods Find Exp Clin Pharmacol 2008;30:67–99.

234.U.S. National Institutes of Health. www.clinicaltrials.gov.

235.Whiteley W, Tseng MC, Sandercock P. Blood biomarkers in the diagnosis of ischemic stroke. A systematic review. Stroke 2009;40(5):e380–8.

236.Lederle FA, Johnson GR, Wilson SE, Chute EP, Hye RJ, Makaroun MS et al. The aneurysm detection and management study screening program: validation cohort and final results. Aneurysm Detection and Management Veterans Affairs Cooperative Study Investigators. Arch Intern Med 2000; 160:1425–1430.

237.Freestone T, Turner RJ, Coady A, Higman DJ, Greenhalgh RM, Powell JT. Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 1995;15:1145–1151.

238.Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest 2000;105:1605–1612.

239.Saraff K, Babamusta F, Cassis LA, Daugherty A. Aortic dissection precedes formation of aneurysms and atherosclerosis in an-

giotensin II-infused, apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2003; 23:1621–1626.

240.Watanabe N, Arase H, Kurasawa K, Iwamoto I, Kayagaki N, Yagita H et al. Th1 and Th2 subsets equally undergo Fas-dependent and –independent activation-induced cell death. Eur J Immunol 1997;27:1858–1864.

241.Tsuruda T, Kato J, Hatakeyama K, Kojima K, Yano M, Yano Y et al. Adventitial mast cells contribute to pathogenesis in the progression of abdominal aortic aneurysm. Circ Res 2008;102:1368–1377.

242.Tchougounova E, Lundequist A, Fajardo I, Winberg JO, Abrink M, Pejler G et al. A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2. J Bio Chem 2005; 280:9291–9296.

243.Sun J, Sukhova GK, Yang M, Wolters PJ, MacFarlane LA, Libby P et al. Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. J Clin Invest 2007;117:3359–3368.

244.Leskinen MJ, Kovanen PT, Lindstedt KA. Regulation of smooth muscle cell growth, function and death in vitro by activated mast cells —a potential mechanism for the weakening and rupture of atherosclerotic plaques. Biochem Pharmacol 2003;66:1493–1498.

245.Nakae S, Suto H, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cells enhance T cell activation: importance of mast cell-derived TNF. Proc Natl Acad Sci USA 2005;102:6467–6472.

246.Elmore JR, Keister BF, Franklin DP, Youkey JR, Carey DJ. Expression of matrix metalloproteinases and TIMPs in human abdominal aortic aneurysms. Ann Vasc Surg 1998;12:221–228.

247.Crowther M, Goodall S, Jones JL, Bell PR, Thompson MM. Localization of matrix metalloproteinase 2 within the aneurysmal and normal aortic wall. Br J Surg 2000;87:1391–1400.

248.Curci JA, Liao SX, Huffman MD, Shapiro SD, Thompson RW. Expression and localization of macrophage elastase (matrix metallo-

proteinase-12) in abdominal aortic aneurysms. J Clin Invest 1998;102:1900–1910.
249.Tamarina NA, McMillan WD, Shively VP, Pearce WH. Expression of matrix metalloproteinases and their inhibitors in aneurysms and normal aorta. Surgery 1997;122:264–271.

250.Mao D, Lee JK, VanVickle SJ, Thompson RW. Expression of collagenase-3 (MMP-13) in human abdominal aortic aneurysms and vascular smooth muscle cells in culture. Biochem Biophys Res Commun 1999;261:904–910.

251.Nollendorfs A, Greiner TC, Nagase H, Baxter BT. The expression and localization of membrane type-1 matrix metalloproteinase in human abdominal aortic aneurysms. J Vasc Surg 2001;34:316–322.

252.Goodall S, Crowther M, Hemingway DM, Bell PR, Thompson MM. Ubiquitous elevation of matrix metalloproteinase-2 expression in the vasculature of patients with abdominal aneurysms. Circulation 2001;104:304–309.

253.McMillan WD, Tamarina NA, Cipollone M, Johnson DA, Parker MA, Pearce WH. Size matters: the relationship between MMP-9 expression and aortic diameter. Circulation 1997;96:2228–2232.

254.Petersen E, Gineitis A, Wagberg F, Angquist KA. Activity of matrix metalloproteinase-2 and -9 in abdominal aortic aneurysms. Relation to size and rupture. Eur J Vasc Endovasc Surg 2000;20:457–461.

255.Defawe OD, Colige A, Lambert CA, Munaut C, Delvenne P, Lapiere CM et al. TIMP-2 and PAI-1 mRNA levels are lower in aneurysmal as compared to athero-occlusive abdominal aortas. Cardiovasc Res 2003;60:205–213.

256.Abisi S, Burnand KG, Waltham M, Humphries J, Taylor PR, Smith A. Cysteine protease activity in the wall of abdominal aortic aneurysms. J Vasc Surg 2007;46:1260–1266.

257.Lindholt JS, Erlandsen EJ, Henneberg EW. Cystatin C deficiency is associated with the progression of small abdominal aortic aneurysms. Br J Surg 2001;88:1472–1475.

258.Satta J, Juvonen T Haukipuro K, Juvonen M, Kairaluoma MI. Increased turnover of collagen in abdominal aortic aneurysms, demonstrated by measuring the concentration of the aminoterminal propeptide of type III procollagen in peripheral and aortal blood samples. J Vasc Surg 1995;22:155–160.

259.Bode MK, Soini Y, Melkko J, Satta J, Risteli L, Risteli J. Increased amount of type III pN-collagen in human abdominal aortic aneurysms: evidence for impaired type III collagen fibrillogenesis. J Vasc Surg 2000;32:1201–1207.

260.Krettek A, Sukhova GK, Libby P. Elastogenesis in human arterial disease: a role for macrophages in disordered elastin synthesis. Arterioscler Thromb Vasc Biol 2003;23:582–587.

261.Miller FJ Jr, Sharp WJ, Fang X, Oberley LW, Oberley TD, Weintraub NL. Oxidative stress in human abdominal aortic aneurysms: a potential mediator of aneurysmal remodeling. Arterioscler Thromb Vasc Biol 2002;22:560–565.

262.McCormick ML, Gavrila D, Weintraub NL. Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 2007;27:461–469.

263.Darling RC. Ruptured arteriosclerotic abdominal aortic aneurysms: a pathologic and clinical study. Am J Surg 1970;119:397–401.

264.Glima°ker H, Holmberg L, Elvin A, Nybacka O, Almgren B, Bjo¨rck CG et al. Natural history of patients with abdominal aortic aneurysms. Eur J Vasc Surg 1991;5:125–130.

265.Lederle FA, Johnson GR, Wilson SE, Chute EP, Littooy FN, Bandyk D et al. Prevalence and associations of abdominal aortic aneurysm detected through screening. Aneurysm Detection and Management (ADAM) Veterans Affairs Cooperative Study Group. Ann Intern Med 1997;126:441–449.

266.Boll AP, Verbeek AL, van de Lisdonk EH, van der Vliet JA. High prevalence of abdominal aortic aneurysm in a primary care screening programme. Br J Surg 1998;85:1090–1094.

267.The UK Small Aneurysm Trial Participants. Mortality results for randomised controlled trial of early elective surgery or ultrasonographic surveillance for small abdominal aortic aneurysms. Lancet 1998;352:1649–1655.

268.Lederle FA, Wilson SE, Johnson GR, Reinke DB, Littooy FN, Acher CWet al. Immediate repair compared with surveillance of small abdominal aortic aneurysms. N Engl J Med 2002;346:1437–1444

269.Steinmetz EF, Buckley C, Shames ML, Ennis TL, Vanvickle-Chavez SJ, Mao D et al. Treatment with simvastatin suppresses the development of experimental abdominal aortic aneurysms in normal and hypercholesterolemic mice. Ann Surg 2005;241:92–101.

270.Kalyanasundaram A, Elmore JR, Manazer JR, Golden A, Franklin DP, Galt SW et al. Simvastatin suppresses experimental aortic aneurysm expansion. J Vasc Surg 2006;43:117–124.

271.Nagashima H, Aoka Y, Sakomura Y, Sakuta A, Aomi S, Ishizuka N et al. A 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, cerivastatin, suppresses production of matrix metalloproteinase-9 in human abdominal aortic aneurysm wall. J Vasc Surg 2002;36:158–163.

272.Sukhija R, Aronow WS, Sandhu R, Kakar P, Babu S. Mortality and size of abdominal aortic aneurysm at long-term follow-up of patients not treated surgically and treated with and without statins. Am J Cardiol 2006;97:279–280.

273.Schouten O, van Laanen JH, Boersma E, Vidakovic R, Feringa HH, Dunkelgrun M et al. Statins are associated with a reduced in-

frarenal abdominal aortic aneurysm growth. Eur J Vasc Endovasc Surg 2006;32:21–26

274.Liao S, Miralles M, Kelley BJ, Curci JA, Borhani M, Thompson RW. Suppression of experimental abdominal aortic aneurysms in the rat by treatment with angiotensin-converting enzyme inhibitors. J Vasc Surg 2001;33:1057–1064.

275.Fujiwara Y, Shiraya S, Miyake T, Yamakawa S, Aoki M, Makino H et al. Inhibition of experimental abdominal aortic aneurysm in a rat model by an angiotensin receptor blocker, valsartan. Int J Mol Med 2008;22:703–708.

276.Sun J, Sukhova GK, Yang M, Wolters PJ, MacFarlane LA, Libby P et al. Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. J Clin Invest 2007;117:3359–3368.

277.Tsuruda T, Kato J, Hatakeyama K, Kojima K, Yano M, Yano Y et al. Adventitial mast cells contribute to pathogenesis in the progression of abdominal aortic aneurysm. Circ Res 2008;102:1368–1377.

278.Yoshimura K, Aoki H, Ikeda Y, Fujii K, Akiyama N, Furutani A et al. Regression of abdominal aortic aneurysm by inhibition of c-Jun Nterminal kinase. Nat Med 2005;11:1330–1338.

ACKNOWLEDGEMENTS

Ed eccoci giunti alla parte più difficile da scrivere...i ringraziamenti! già, perché trovare una frase esaustiva per ognuno di voi...e tentare di non dimenticare nessuno soprattutto, sarà una grande impresa!! Dunque...

Innanzitutto desidero ringraziare profondamente la Prof.ssa Marialuisa Lavitrano, che mi ha "cresciuto" nel suo lab dai tempi della tesi di laurea e mi ha dato la possibilità di diventare quello che sono... e assieme a lei, naturalmente tutto il lab: Roby (il capo che negli anni è diventato buonissimo... :D) , Grace, Alessia, Ste P. (oltre a tutto il resto, grazie infinitamente per il supporto durante la stesura di questo lavoro...senza di te non ce l'avrei mai fatta!!), Robert, Laura M, Manu..quelli che ora non lavorano più qui...grazie Scaglia, Bus, e ovviamente tutti i tesisti...Eros, Teo, Fabbbbbbio, Segu, l'ultimo arrivato Cinzi e tanti altri...grazie per tutte le giornate che abbiamo condiviso insieme...!!
Manz...(temevi mi fossi dimenticata di te vero?? :D)...grazie perché oltre che il compagno di lavoro più strampalato, solare e zen che conosca, sei diventato anche un grande amico... (CHETTENEREZZA... :))

Un doveroso ringraziamento va al resto dei componenti del 4°piano dell'U8...grazie ai "Micro", ai Gambacorti..(siete troppi...se provo a citarvi tutti va a finire che qualcuno me lo dimentico per forza..:D)...fiera, fierissima di far parte di questo condominio di pazzi!! :)

Grazie alla mia famiglia...sembrano ringraziamenti scontati ma non è cosi...a mio padre fino a che ha potuto esserci, a mia mamma e mio fratello che ancora mi sopportano...alle mie zie preferite Anna e Rosetta, e ai miei fratellini acquisiti Francesco, Pasquale e Rossella...perché da sempre avete deciso di camminare al mio fianco pazientemente, senza stancarvi mai, anche quando sembravo troppo distante da raggiungere o rimanevo troppo indietro...

Grazie a te amore mio...Francesco...non ci sono parole nuove per te...tutto quello che sento te lo ripeto ogni giorno..grazie perché nonostante tutte le difficoltà, la distanza, la mia ansia, nonostante a volte sia una gran cialtrona..:D..nonostante tutto tu con il tuo amore ci sei sempre stato, grazie perché rendi ogni giorno della mia vita semplice, speciale e perfetto...ti amo..

Grazie a Lara...sorellina...più passano i giorni più penso che ci abbiano separate alla nascita... :) grazie perché il tuo neurone è sempre acceso e sintonizzato con il mio...per tutto quello che abbiamo condiviso in questi anni e che condivideremo ancora...perché perdersi con te non ha prezzo, perché le cene a casa di Lara restano sempre la mia uscita preferita...per le chiacchierate in mezzo al mare (io te e le meduse...) e in generale per l'estate scorsa...fantastica!..e ancora perché con una pazienza infinita trovi la forza di ascoltarmi parlare per ore, anche quando tutto e tutti sarebbero già morti di noia probabilmente...per tutto questo e per molto altro ancora grazie..sei molto più di un'amica...(non piangere ora eh!! :D)

Grazie agli amici di sempre...quelli che da molti anni mi accompagnano nelle mie avventure...che mi sopportano...qualcuno addirittura dai tempi del liceo! grazie Chia, Cinzia. Ele S., Andrea, Elisa..per tutti i momenti splendidi che abbiamo condiviso insieme, quelli che guardando poi le foto a distanza di tempo ti fanno venire i lacrimoni...e per tutti i momenti che verranno...vi voglio troppo bene!!

Grazie al Black Marlin...e a tutte le meravigliose persone che li ho incontrato e che hanno accompagnato le mie estati (e non solo..)..ragazzi, non saprei nemmeno dire quanti sono gli anni passati insieme...ho perso il conto!cmque..iniziamo con l'appello... MILANO: Marta, Claudia (vi voglio un bene infinito!!) e rispettivi superfidanzati Giulio e Mimmo...Gerbellezza, Sentimento e Fede...il magnifico trio, l'associazione a delinquere Savino, Pisano e Ziccardi (perché nessuno è in grado di turbare la tranquillità delle giornate in spiaggia come fate voi...:D), Falco...quello che non beve nemmeno...Antonella e ancora il gruppo dei milanesi Vip: Dade, Albo, Chris, il Ferra, il Pilli...ragazzi, le bevute con voi rimarranno sempre dei momenti indelebili nella mia memoria...(chiaramente mi riferisco a quei momenti di cui mi ricordo qualcosa..:D)...FIRENZE...Theino...ti adoro lo sai!.. ROMA: Alessia (Amo, ti voglio un bene infinito..ma quante ne abbiamo combinate insieme?!?!), Simone, Luca e Angela...(Angy sei nel gruppo romani..ma è colpa del tuo fidanzato!)..perché grazie a voi oggi posso dire che i romani sono un popolo fantastico!! ...NAPOLI: Assia..(non ti vedo da un po' ma resti sempre l'amica napoletana più pazza che conosca...ti voglio troppo bene), Fabrizio, Fabio, Giulio, Ile, Filo, Rocco, i fratelli Sanniola e tutti quelli che negli anni si sono aggiunti...ragazzi, San Leonardo...il Marchese...ma che vo dic'a fa??

(spero si scriva cosi...:D) abbiamo condiviso dei momenti magici...grazie davvero di cuore...
E poi ancora tutti quelli che non ho citato (e sono una marea..), quelli che sono rimasti per poche estati, quelli che erano solo di passaggio...perché ogni passaggio al Black non resta mai inosservato e non viene mai dimenticato...perché la magia di quel posto sta proprio nel riuscire a fermare il tempo...tutti noi siamo cambiati, cresciuti...ma al Black riusciamo a dimenticarcene!!:D

E ora veniamo all'università...Ele..quante ne abbiamo passate insieme...ora purtroppo mi sei finita lontana, in Svizzera...ma sai che il bene che ti voglio è rimasto immutato nel tempo...e Cri, tu mi sei finita ancora più lontana...a Singapore..ma anche per te vale lo stesso discorso...ragazze, mi mancate da morire....però è proprio vero che l'amicizia, quella vera, resta intatta sempre e comunque!!e poi la Britt...tesoro..ultimamente ci vediamo poco, però resti sempre la mia patatina... :), Lore, Manu, Lidia Nico, Teo, Silvia...e poi ancora il gruppo evergreen con cui abbiamo condiviso cazzate, partitoni a carte e a dadi...Dario Edo Dido Corrado e tutti gli abitanti del -1 dell'U3-U4...quanta nostalgia se ripenso ai quei giorni...

Gabri...grazie...per le chiacchierate, le bevute, le risate...perché molti anni sono passati, ma io e te rimaniamo sempre io e te...l'amicizia è un dono prezioso, e tu me lo ricordi ogni giorno!

E dunque, a chiudere questa carrellata infinita di smancerie e nostalgia...Emi...ti ho lasciato per ultimo apposta perché con te le smancerie sono bandite...cosi chi legge ha il tempo di ricomporsi un attimo! :)...negli ultimi mesi sei stato una colonna portante per me...nel senso che hai portato un sacco di cazzate in più nella mia vita!! :)...grazie per le chiacchierate fino alle 3 di notte...se ho perso un sacco di ore di sonno è solo colpa tua...per tutti i Cipollini e le faccine meravigliose che mi hai passato... Zarina, le scolopendre naturalmente..(come dimenticarle...si sono intrufolate anche in questi ringraziamenti...visto?!?!e scommetto che una ti sta girando intorno proprio ora, mentre leggi..:D)..tua sorella e i gatti, i panda e la tua altissima opinione sulle donne...e ancora Amici, GF, X-factor...e LOST...grazie perché sei la mia Lostpedia preferita!! :D grazie perché entrare nel magico mondo di Emi è stata un'esperienza unica...un po' come entrare nel Sottomondo di Alice..(ora però non riesco più a uscirne...AIUTO!!) ahh e naturalmente, grazie in anticipo per le

lezioni di poker...(cosi ora è scritto...scripta manent no?!?...ora non puoi più tirarti indietro!!)..

Non so quale sia il modo canonico di concludere i ringraziamenti...io ho deciso che chiudo cosi..ringraziando me stessa...perché ho toccato il fondo tante volte ma non ho mollato mai, perché ogni giorno posso guardarmi allo specchio con fierezza...grande Lau, ce l'hai fatta anche sta volta!! ;)