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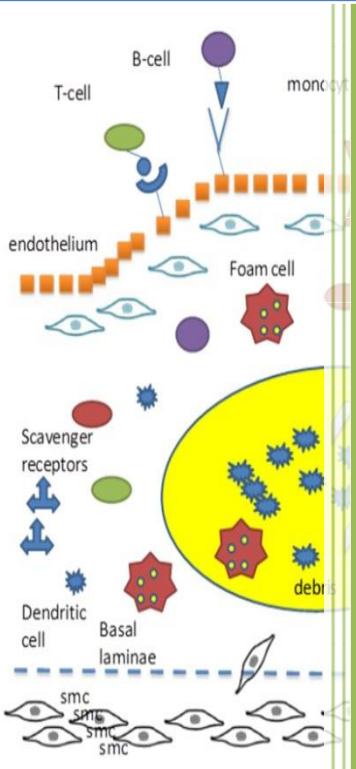
Complicate Approaches towards Complicated Atherosclerotic Plaques

Μηχανισμοί ρήξης της αθηρωματικής πλάκας



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Ευχαριστίες

Μετά τη συμπλήρωση της παρακολούθησης του διετούς μεταπτυχιακού προγράμματος σπουδών στη «Μοριακή και εφαρμοσμένη Φυσιολογία», επιθυμώ να εκφράσω το θαυμασμό μου για την έκταση και το βάθος της έρευνας που διενεργείται σε πειραματικά εργαστήρια της χώρας μας, όπως μου δόθηκε η ευκαιρία να διαπιστώσω από τις ομιλίες των εκλεκτών προσκεκλημένων ερευνητών και καθηγητών.

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Τέλος, ευχαριστώ το Θεό για την υγεία τη δική μου και των γύρω μου και γιατί μου χάρισε όλα τα ανωτέρω.

CONTENTS

Complicate Approaches towards Complicated Atherosclerotic Plaques

Abstract

Medical research is oriented to vascular events' prevention, either by the inhibition of growth or by the early detection and stabilization of the vulnerable atherosclerotic plaques. Numerous studies have been published in literature, aiming to determine vulnerability, by means of associating traditional risk factors, epidemiological data or morphological characteristics of atheromatous plaque, as these are provided by routine or highly specialized imaging techniques. All of these studies though, focus to only a few factors implicated in plaque instability.

The largest part of the current atherosclerosis research is targeted to the attempt of modelling the systemic and the local events, leading in plaque vulnerability. Of particular interest is the role of molecular signaling pathways and of gene interactions. The experimental reproducing of atheromatous plaque formation, growth and destabilization remains challenging, because of the limitations that animal models present.

An holistic approach of atherosclerotic plaque vulnerability , considering mechanical and flow parameters of the prone to disease arterial segments, the circulating molecules, the enzymatic systems and the cells contributing to plaque evolution, the dynamic transformation , function and degeneration of arterial wall components are reviewed in this study.

CHAPTER 1: ATHEROSCEROSIS

1.1. Introduction

Cardiovascular events are responsible for a large percentage of mortality and morbidity in the modern countries. AMI and stroke account for 35 % of annual deaths in USA and for a considerable percentage of disabilities.

Atherosclerosis , a chronic degenerative disease of the arterial wall, is the basic cause of the great majority of vascular events.It is a systemic disease , not uniformely nor predictably progressing in all segments of the arterial tree. Atherosclerosis starts with the formation of fatty streaks on the arterial endothelium at the age of 11 -12 years. These streaks develop to fibrous plaques at about the age of 15-30 years, depending on individual's risk for atheromatous disease. Fibrous plaques develop more rapidly in high risk individuals. The pattern of disease's progression is similar in various population groups, such as men and women[88].

1.2. Atheromatous Plaque Development

The pattern of atherosclerotic plaque formation, growth and rupture has been described in many studies. LDL molecules infiltrate vascular endothelium and anchorate to extracellular matrix. Circulating monocytes via toll like receptors attach and penetrate the endothelium to phagocyte the entrapped ox LDL molecules. Monocytes interact with specific selectins, such as P-selectin glycoprotein ligand /PSGL-1, via integrins of monocytes such as very late antigen-4/VLA-4, and lymphocyte function associated antigen 1(LFA-1) and bind to endothelial ligands VCAM-1 and ICAM-1[117].

After entering the subendothelial space, circulating monocytes transform to macrophages through a process where members of tumor necrosis factor family (TNF) and cytokines such as macrophage colony stimulating factor (MCSF) play important role [117]. The uptake of oxLDL is mediated by scavenger receptors, following which macrophages differentiate to foam cells. In the same time smooth muscle cells migrate to subintimal area and change their phenotype also to foam cells. Local inflammatory cascades become activated, apoptosis of the foam cells is increased and more cholesterol is delivered to extracellular space, to recycle inflammation. Neovascularization is triggered inside the plaque, with the new vessels presenting a monolayer wall through which red blood cells readily extravasate, segmentate and provide more cholesterol of membranic origin extracellularly. Abrupt hemorrhage from neovessels may occur leading to acute plaque augmentation and rupture. Local crowding of macrophages releasing MMP's (metalloproteinases) weaken the fibrous cap particularly at the shoulders of the plaque and predispose to rupture,subsequent intravascular exposion of extracellular matrix components and thrombus formation.The correlation of histologically

measured plaque capillary density and of intraplaque hemorrhage , with vascular events has been depicted in many studies[91].

FIGURE 1: STABLE ATHEROMATOUS PLAQUE.

The phenotype of a complicated plaque may differ, reflecting possible variability in pathogenetic mechanisms. An atheromatous plaque may be stable and present no complications for a long time. This plaque is characterized as stable and has thick fibrous cap and a small lipid core (see Figure 1). Acute coronary syndrome may be due to coronary plaque rupture or to coronary plaque erosion. Different biomarkers may be the hallmark of such variability: Patients with ACS (acute coronary syndrome) caused by a culprit plaque erosion have increased systemic levels of myeloperoxidase compared to patients with culprit plaque rupture, as this is in vivo measured with OCT. Myeloperoxidase has also increased density in thrombi that overly eroded, but not ruptured plaques in acute coronary death victims. This is a possible effect of selective accumulation of hyalouran and its receptorCD44 triggering endothelial cell erosion [71].

A great number of risk factors such as diabetes, hyperlipidemia, smoking, hypertension have been associated to atherosclerosis.

1.3. General Risk Factors

Smoking, age, hypertension, obesity, diabetes, waist circumference are some of the risk factors proposed for screening and therapy algorithms. ApoB-related lipoproteins (LDL,TG), inversely related levels of HDL, levels of homocysteine, C-reactive protein and fibrinogen are also essential risk factors for cardiovascular risk assessment. Coronary artery atherosclerosis measured by calcium score is a major predictor for heart attack[5].

The risk of vascular event determines the so called «vulnerable patient». The already mentioned traditional risk factors have been the base of Framingham study. This study provided an estimation of cardiovascular risk considering the relationship of traditional risk factors and of epidemiological parameters to the number of vascular events (heart attacks, strokes) and deaths in a steady population in a predefined period of time. The individual cardiovascular risk assessment of 8% (relatively low) to 12% (moderate risk) or 20% (high risk) annually offers only little contribution on the handling of the vascular patient[5]. Besides, these algorithms when applied in different populations prove to be weak predictors of the individual cardiovascular risk, accounting only for about 50% or even less of vascular events. This is probably due to limited risk factor consideration, while a large number of factors , such lifestyle etc that are difficult to precisely define and count and that essentially contribute to disease's progression are not included in the study.

Another study based on computed tomography- angiographic findings proved that only 55% of patients with heavy atherosclerosis were identified as high risk by Framminghам/National cholesterol educational programm risk scores. On the other hand 32% with no plaques were taking statins[37,95].

Functional vascular assessment is a recently introduced term, referring to endothelial dysfunction related to novel risk factors such as sleep apnea and mental stress [95].

In Cardiovascular Health study intima medial wall thickness, electrocardiographic changes (based on Minnesota coding system) and ankle/brachial blood pressure proved to be predictive for cardiovascular events in older individuals[5].

Smoking , active and passive, remains one of the more potent risk factors for cardiovascular disease. It accounts for almost half of the sudden cardiac deaths in males from 40 to 45 years old. Underlying mechanisms are:

- Endothelium damage
- Coagulation and fibrinolytic disorders
- Inflammation induction
- High levels of CRP, homocysteine and fibrinogen levels
- High levels of soluble adhesion molecules (VCAM-1, ICAM-1)
- HDL reduction
- Integrin expression at monocytes
- Increased platelet activity[99]

Quitting of smoking after myocardial infarction may reduce total mortality up to 36% during 3-7 years after the event[99].

The extent of subclinical atherosclerosis has proved to be a powerful determinant of cardiovascular risk. Diabetic patients have extensive subclinical atherosclerosis that is probably installed even before the diagnosis of diabetes[5].

The phenotype or the mechanisms of an acute vascular event may be modified among sexes. In women coronary artery disease presents one decade later than men. Females

have more frequent coronary attacks with nonobstructive atheromatous coronary lesions and with plaque erosion in areas of minimal plaque burden. Coronary thrombus over an eroded lesion is the predominant finding in women with ACS instead of men where a plaque rupture is the basic mechanism. Even in carotid plaques women have more stable plaques, with more vascular smooth muscle cells, less macrophage, IL-6,-8, MMP-8,-9 and less lipid. Inflammatory factors correlate with MMP-3 derived from vascular smooth muscle cells in women but not in men[40]. Vasospasm or microembolic episodes may justify the phenomenon[72].

FIGURE 2: ATHEROMATOUS PLAQUE RUPTURE.

Various combinations of risk factors have been proposed as algorithms estimating the risk of plaque rupture (see Figure 2). Yellow color grade as provided by angioscopy of atheromata has shown moderate correlation with angiographic findings and more potent with male gender , levels of LDL and hypertension[105].

It has been proposed that risk factors for cardiovascular disease should be discriminated to distal , i.e. the factors leading to atherosclerosis progression, and proximate ,i.e., the ones converting a chronic atheromatous lesion to a complicated one , mostly presenting with a vascular event[5]. Distal risk factors are the traditional risk factors that have already mentioned.

1.4. Proximal Risk Factors

The conversion mechanisms of a chronic, stable atheromatous plaque to a vulnerable one with a great risk of rupture, installing a vascular event, remains a subject of investigation.

Basic areas of research are inflammation, thrombin generation and fibrinolysis. Markers of inflammation associated with increased risk of cardiovascular events are:

- elevated CRP
- White blood cell count
- Low serum albumin (acute phase protein)
- Cytokines (TNF-a and IL-6)
- Endothelial leukocyte adhesion molecules (ICAM,VCAM,selectin)

Thrombin generation plays role in development of thrombosis. Fibrinogen and clotting factors such as factors VII, IX, X may be related to increased cardiovascular risk.

Markers of fibrinolysis have also been evaluated. PAI-1 (Plasminogen Activator Inhibitor) and d-dimers have been related to cardiovascular risk, but no strong clinical impact has been produced[5].

Apart from biological factors, the contribution of neuropsychological parameters to plaque destabilization is not negligible. Personallity type A, characterized by stress and hostility, has been related to cardiovascular events via neurohumoral factors such as cortisol and catecholamines[5].

Perioperative stress is another common triggering factor for acute coronary events. Half of these events are due to rupture of a vulnerable plaque, while the rest is due to hypotension and anemia[77].

Atheromatous plaques are formed in sites the most exposed to mechanical and flow stress. The growth of a plaque follows a more or less benign course, with the worse scenario ending in a vascular event, when a territory is deprived of blood flow. This may be due to hemodynamic or to embolic mechanisms. According a study, carotid plaques rich in MMP-9, macrophages and T-cells are prone to rupture and lead to embolic events, while the rest are involved in hemodynamic stroke mechanism[35]. A complex interaction of mechanical forces, oxidative reactions, cell to cell recognition cascades, immune system activation and molecular pathways that lead to chemical and structural transformation of atheromatous plaque's microenvironment, determine its fate.

According to AHA classification, atherosclerotic plaques may be characterized as[81]:

1. Type I : isolated macrophage-derived foam cells that contain lipid droplets
2. Type II: numerous macrophage foam cells
3. Type III: extracellular lipid deposit
4. Type IV: large lipid and necrotic core , fusion of droplets
5. Type Va : large lipid and necrotic core covered by thin fibrous cap, Vb: calcified type V plaques, Vc: V plaques with minimal lipid and necrotic core and predominant fibrosis.

No particular class for vulnerable thin cap fibroatheromata is mentioned[81].

CHAPTER 2: STRUCTURE OF ATHEROMATOUS PLAQUE

2.1 Vulnerable Plaque/ Vulnerable Patient

Most vascular events are attributed to rupture of atheromatous plaques. An atheromatous plaque that is prone to rupture is called «vulnerable». It has been estimated that 60% of AMI's are caused by the rupture of a vulnerable plaque[60]. The entity of vulnerable plaque has been defined in the frame of the PROSPECT trial[30].

The term of vulnerability has been first established and studied in cardiology, which remains pioneer in the field of vascular research. It refers to plaques that present a future risk of complication that is thrombosis, acute progression or volume expansion. Vulnerable plaques may be prone to rupture, to erosion and to intraplaque hemorrhage. They may be severely calcified or they may present a single protruding calcified nodule. Even without the presence of overt thrombus, atheromatous plaques may predispose to acute vascular events via embolic mechanisms to the distal vasculature, or by inciting a vascular spasm, or even by favouring arrythmias installation. In order to define the vulnerable plaque a number of criteria have been proposed, based on imaging and biological features of culprit plaques. Morphological criteria are

- Thin cap with underlying large lipid core (50-60%[87])
- Endothelial denudation with local platelet aggregation (20%[87])
- Major stenosis (20-30%[87])
- Presence of fissure
- Protruding calcified nodule (2%[87])
- Outward arterial remodelling
- Yellow plaque
- Acute inflammation and endothelial dysfunction

Functional criteria

- Plaque inflammation
- Oxidative stress
- Endothelial dysfunction
- Apoptosis
- MMP's activity
- Angiogenesis
- Platelet aggregation and/or activation[25].

Plaque erosion usually refers to thickened intima and a fibrous cap rich in proteoglycans, SMCs and type III collagen, but poor in inflammatory cells[78] (see Figure 4).

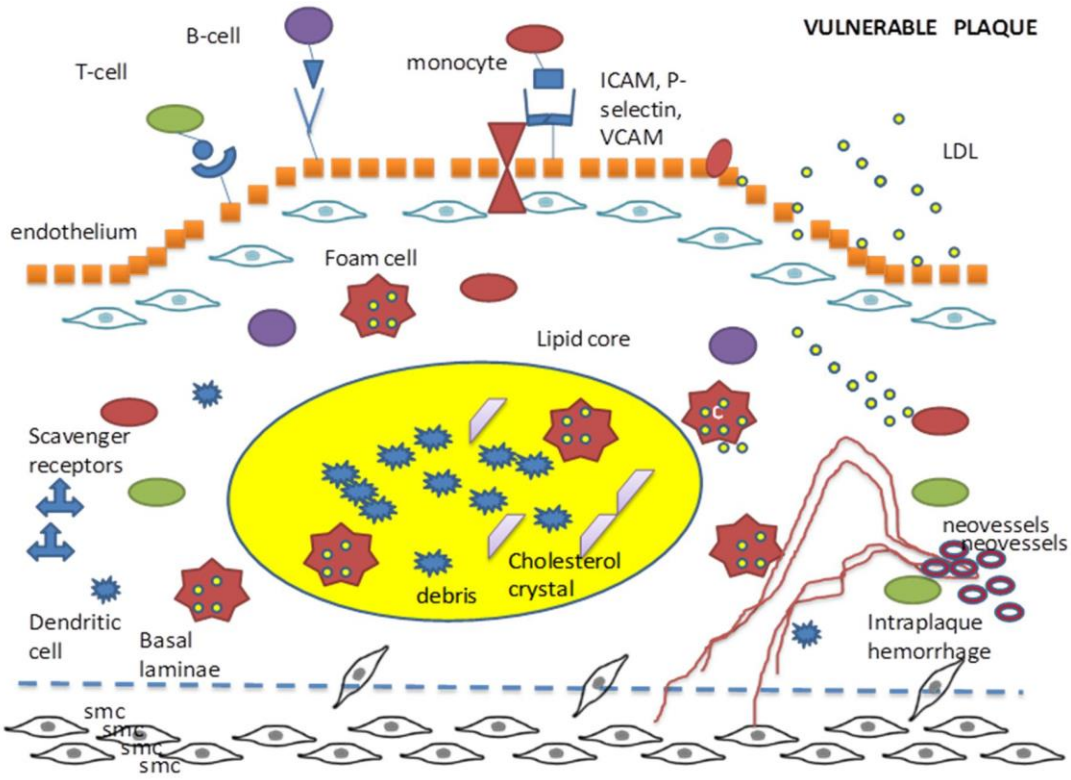

FIGURE 3: VULNERABLE PLAQUE.

It is worth to be mentioned that in 1/3 of vascular events no plaque rupture is documented. Moreover, a great number of plaque ruptures are clinically silent, with no overt vascular event occurring. The aforementioned data indicate that not a single parameter, such as complicated plaques, but a combination of variables may play important role in the presentation of a vascular event. These variables may be mechanical hemodynamic forces, the relative concentration of blood components, the thrombogenicity of the endothelium and the state of fibrinolysis[111].

Current medical research is targeted to « plaques at risk for rupture» identification, along with the design of therapies to stabilize them. The majority of studies published in literature examine plaque's vulnerability taking into consideration some of parameters- epidemiological, morphological, material or mechanical- playing role in the phenomenon. A holistic approach where different scopes of the same subject are gathered together is missing. In the present study , a wide spectrum of factors participating in plaque growth and rupture are reviewed.

2.2 Animal Models

The use of animal models for the study of atheromatous plaque progression and rupture has not yet been ideal. The most widely used animal models, mice, rabbits, pigs develop little or

not at all atheromatous lesions, while these don't lead to fatal vascular events through the same procedure like humans.

Pigs die from malignant arrythmias. This fact, along with technical considerations of providing care and feeding render this model less appropriate for atheromatous studies.

Rabbits are herbivorous and have a different cholesterol metabolism than humans [13]. Rabbits cholesterol fed do not present overt plaque rupture and fatal vascular events, unless mechanical or chemical inducers are applied. Balloon inflation and potent vasoconstrictors are used for this purpose[121].

ApoE knockout mouse is one of genetically engineered mouse models that lack genes necessary for lipid metabolism. They are hypercholesterolaemic even on a normal chow diet and develop atheromatous lesions from the early adulthood.

ApoE is an antiatherogenic lipoprotein involved in cholesterol uptake and metabolism by the liver. Through the LDL receptor in the aminoterminal region of apoE, clearance of chylomicrons . Reverse cholesterol transport to be excreted in bile, enzyme activation involved in cholesterol metabolism such as lipase, and cholesteryl ester transfer protein are facilitated. ApoE has indirect atheroprotective properties, inhibiting LDL oxidation, platelet aggregation, SMC and endothelial cell proliferation and T-lymphocyte activation. ApoE knockout mice fed with chow diet develops at 6 weeks of age fivefold cholesterol levels than the wild type[13].

Brachilcephalic (innominate) arteries of apoE mouse have a length of 2mm and a width of 0.5mm. They develop atheromatous plaque by steps similar to humans', at 42-54 weeks, with a high frequence of intraplaque hemorrhage and fibrotic conversion of necrotic zones. Plaque rupture requires fat rich diet[13].

Atherosclerotic plaques of brachiocephalic arteries of apo E mouse may rupture spontaneously after a lipid rich diet and this is why this model is used for the study of hypercholesterolemia and lesion development. Again, in ApoE knockout mice plaque rupture doesn't produce a major vascular event. Moreover, thrombus formation doesn't protrude enough to lead to lumen occlusion. Mechanical injury or chemical irritation is also used to induce vascular events[121].

The atheromatous plaques present multilayer phenotype indicating probably multiple silent ruptures with thrombus attachment and scarring. Another hypothesis supports episodic growth of the plaque with quiscent intervals. The number of buried caps may indicate the number of ruptures, with possible correlation of plaque growth and instability to the inter-rupture intervals. The shorter these intervals are, the more defective healing of the plaque occurs with subsequent growth of the lipid core and thinning of the fibrous cap. Limitations of the model are the great number of sudden animal deaths, a fact that could not be related to brachiocephalic or coronary plaque ruptures and may be attributed to malignant arrythmia development. Furthermore, the size mismatch between mouse and humans confusing the estimation of critical cap thickness , along with the strain differences in absorbing cholesterol (more effective in 129Sv mice), or in platelet content of tumor

growth factor TGF-1 (fourfold in C57BL/6 mice than in 129Sv) interfere with the interpretation of the results of the studies[9]. Another issue is that the plaque rupture of brachiocephalic artery doesn't lead to luminal thrombus formation, instead of humans. Reduced shear stress of the vascular wall in regions of flow disturbances doesn't play significant role, instead of humans. Lateral fatty streaks lead to plaque progression and not a lipid core augmentation. The formation of large necrotic core in apoE deficient mice is related more to plaque stabilization and increased cellularity, where resident smooth muscle cells transform to chondrocyte-like or osteoblast-like cells producing collagen and leading to stable fibrotic nodule formation. Intraplaque hemorrhage is noticed through lateral fissures and not due to fibrous cap disruption[70].

It is clear that atheromatous plaque development and rupture have similarities as much as differences between mice and humans. Generally Apo E knockout mice is a reliable animal model of atheromatous plaque progression , but concerning plaque vulnerability this model is not representative[13].

Similarities:

- Systolic and diastolic blood pressures
- Plaque development in regions of arterial bifurcation
- Average peak aortic root blood velocities
- Fibrinogen and tPa (tissue plasminogen activator) concentration
- Intraplaque hemorrhage in the majority of plaque ruptures
- Response to statin treatment

Differences

- Wall shear stress 20fold higher in mice than in humans
- Lack of superimposed thrombus in case of rupture
- PAI-1 (plasminogen activator inhibitor) 5-12fold lower in mice
- Burried fibrous caps as a result of silent repeated ruptures

LDL receptor deficient mouse is another strain used for the study of atherosclerosis. It presents milder hyperlipidaemic disorder than the Apo E-/- mouse on normal chow diet and develops atherosclerosis very slowly. Both models develop atherosclerosis rapidly in a high fat, high cholesterol diet[99].

2.3 Endothelial Dysfunction- The Role of NO

Endothelium is a functional system resisting adhesion and aggregation of platelets and inflammatory cells, enhancing fibrinolysis and controlling vascular tone via eNOS (endothelial nitric oxide synthase) enzyme. Nfkb regulated inflammatory molecules and adhesion molecules (ICAM-1, VCAM-1) are blocked by NO synthesis, therefore vasodilation is favoured. Traditional risk factors such as smoking, diabetes, obesity, hyperlipidemia, hypertension, lead to eNOS impairment, with subsequent endothelial activation and abolishment of the aforementioned protective effects.

Functional assessment of the arterial wall is achieved via FMD (flow mediated dilation). The method relies on NO production by endothelium after blood flow changes. FMD has proved to be independent predictor of vascular events for patients of moderate or high risk, reflecting endothelial dysfunction. Improvement in FMD indicates better prognosis, while no change of FMD after treatment indicates poor response and worse prognosis. Routinely the imaging artery lies proximally of the occluding cuff and the procedure is NO-dependent. It has been shown that even when the imaging artery is inside the ischemic field, the result is still predictive of vascular events, althogh less NO-dependent[38].

The decrease of NO bioavailability is the result of a reduction of eNOS activity or of a decrease in mRNA and protein eNOS expression. Lipoprotein-related mechanisms have been proposed: eNOS and caveolin-1 displacement intracellularly, eNOS inactivation by high interaction between eNOS and cav1,CD36-mediated depletion of cholesterol in caveolae inactivating eNOS, higher concentration of endogenous competitive synthase (ADMA), eNOS uncoupling and higher levels of superoxide anion, vascular increase in ROS [2].

Endothelial cell surface favors transmigration of monocytes via impaired junctions between endothelial cells, preferably at basal laminal areas of high concentration of LDL. Junction adhesion molecules (JAM-A and –C) are involved in altered vascular permeability [2].

2.4 Extracellular Matrix

The main pathogenic event that triggers atheromatous cascade is lipid retention and accumulation in extracellular matrix of subendothelial space. Proteoglycans such as versican, display high affinity with LDL and the greater the length and the number of proteoglycan-related glycosaminoglycan chains and their degree of sulfation, the stronger is their capacity to retain LDL particles in the intima.

Glucoprotein-LDL complexes become subject to further modification such as oxidation and enzymatic cleavage getting the subendothelial space more atherogenic[2].

2.5 Lipid Core

The size of lipid core plays role in plaque vulnerability. It is probable that the size predisposing to rupture varies among the different parts of the arterial tree. In human coronary arteries averages the 29-34% of plaque area, when in carotids reaches 40% and in aortas the 60%[39]. Lipid core is avascular. Free cholesterol and cholesterol esters, with predominance of the former are the constituents of the lipid core. The source of cholesterol is a matter of discussion. Emphasis is given to plasma lipoproteins intruding arterial wall and being phagocytosed by macrophages. Cholesterol of erythrocytes extravasating from neovessels or from intraplaque hemorhage is another source[39]. Many studies have been conducted to predict the model of plaque growth. It is likely that the size and the growth rate of necrotic core is related to oxidized LDL spatial distribution inside the plaque. Necrotic core growth is favoured in oxygen deficient areas, as cells far from the lumen die from hypoxia[74].

2.6 Fibrous Cap

The critical thickness of the cap is another point of debate. Virmani et al is considering <65μm[60] , a value approximate in other studies -<67 μm[29] , while studies using MRI/FSI measurements refer to pixel numbers[103] .The threshold of 65μm is valid for coronary vessels, while for carotids rise to 100-150μm[127]. Fibrous cap overlies the lipid core and its thinning is an independent factor increasing the risk of plaque complication. The thinning of fibrous cap is due to degradation from MMP's produced by macrophages, but in the same time, to decreased production of collagen from smooth muscle cell depletion. Unstable plaques demonstrate heavy inflammatory infiltration of the fibrous cap , with the percentage of macrophages rising to 14-17% in aorta and 26% in the coronary arteries[39]. Fibroblast activation protein has been associated with fibrous cap thinning in human coronary fibroatheromata. It is produced by aortic smooth muscle cells and contributes to collagen I breakdown[37].

An index of severity of atheromatosis is the calcium score. It refers to the proportion of calcium in the plaque area. A high calcium score in coronary vessels reflects a high risk for an ischemic event. Inversely, a high calcium score in carotid arteries represents stability. The degree and the pattern of calcification is a strong independent factor in plaque vulnerability[14]. Coronary artery calcium is better predicting coronary events, while IMT (intima media thickness) is better predictor of strokes[111].

2.7 Adventitia

The role of adventitia in atherosclerosis progression is not negligible. A great number of various cell types are resident in adventitia: mast cells, fibroblasts, macrophages, ganglionic cells, all are present in various amounts and a various functional status. Mast cells are found to be activated in infarct related coronary arteries, releasing vasoactive compounds possibly participating in neurohormonal mechanisms. Adventitial mast cell activation may lead to plaque destabilization promoting intraplaque hemorrhage, leucocyte recruitment inside the plaque and macrophage apoptosis.

Unstable atherosclerosis has also been related to heavy adventitial macrophage infiltration. Moreover, adventitia is rich in sensory C- and Aδ-fibers that play a crucial role in adventitial inflammation, by releasing peptides after local cation channel activation. TRPV1 (transient receptor potential vanilloid-1) on C-nerve endings is one of these channels that potentially may serve as therapeutic target[108].

CHAPTER 3: ATHEROMATOUS PLAQUE FUNCTIONAL STATUS

3.1 Inflammation

The role of inflammation is outstanding in atheromatous plaque progress and complications, and atheromatosis is generally considered as focal inflammatory disease of the arterial tree. It is known that enzymes produced by inflammatory cells such as MMP's, cathepsins, thryptase/chymase, degrade the matrix and the fibrous cap of an atheromatous plaque and predispose to plaque rupture and thrombus formation. Macrophages and T-cells secrete MMP's that induce tissue factor (TF) expression in endothelial cells, macrophages and smooth muscle cells, and create a prothrombotic state. Fibrinogen levels and systemic levels of inflammatory factors are also increased. Adipose tissue is another site of inflammation, where interferon γ, MCP-1(monocyte chemoattractive protein),TNFa, IL6 and PAI1 are produced and contribute to prothrombotic milieu[47]. The importance of inflammation in atherosclerotic process has been shown in Jupiter study, where assessing inflammatory state and targeting to lowering it is a therapeutic strategy[49].

A great number of inflammatory markers besides hsCRP (high sensitivity CRP), such as Il-6,-10,-18, soluble CD40 ligand, P-and E-selectin, fibrinogen, NT –proBNP have been studied with questionnable clinical impact[11]. IL-6 has been associated to poor 90 – day clinical outcome in patients with AMI (acute myocardial infarction), while , IL-10 demonstrates a protective action. NT-proBNP estimation may potentially improve a future clinical predictive model [131].

Neopterin is a byproduct of GTP (guanosine triphosphate pathway) metabolism and may serve as a marker of inflammation. In unstable plaques interferon-γ, released by activated lymphocytes, stimulates macrophages to produce neopterin. Its concentration has been found increased in unstable and complex atherosclerotic plaques from carotid and coronary specimens or angiography findings and systematically in the same patients. This is possible due to interaction of neopterin with neutrophil derived products such asMPO and HOCL that promote instability[75].

Ets2 (v-ets erythroblastosis virus E26 oncogene homolog) induces atherosclerosis vulnerability by promoting an endothelial cell phenotype of enhanced inflammatory state[50].

3.2 Neovascularization

Oxygen and nutrient supply of the arterial wall is normally provided by the adventitial vasa vasorum and by diffusion from the lumen. With increase of the plaque thickness (more of the limit of 0,35mm), these sources are inadequate to meet the needs for oxygen and nutrients. In the presence of inflammatory environment new vessels are formed. Their main origin are adventitial vasa vasorum on the base of the plaque, but a small proportion may come from the lumen through the thin fibrous cap. Hif-1 and VEGF stimulation are involved in the phenomenon.

Studies with contrast enhanced carotid ulrasound depicted association between increased adventitial vasa vasorum hyperplasia and intraplaque neovascularization to cardiovascular disease and history of vascular event respectively[116].

No matter what the origin of the neovessels is , they share the same capillary morphology with irregular pattern of expansion inside the plaque and with the same action on augmenting the risk of intraplaque hemorrhage and of plaque rupture. MMP's , growth factors and other cytokines play role in extracellular matrix degradation , capillary invasion and plaque's remodeling. Intimal-medial neovascularization is closely associated with the inflammatory reaction inside the plaque [130].

Hypoxic environment is pronounced in thickened intima more than 500μm[68].

Plaque neovascularization is associated to plaque growth and rupture, contributing to plaque vulnerability. It has been shown that plaque neovascularization has a role in plaque destabilization indepentantly of the size of the plaque[85]. Inraplaque hemorrhage (IPH) increases acutely the size of the plaque and may lead to plaque rupture. On the other hand it is considered as a precursor of plaque healing and stabilization. Stages of IPH have been proposed, where organized and amorphous type reflect a different pathogenetic mechanism and a different tendency for destabilization.

The organized type is suggested to come from neovessel rupture type, it is characterized by increased microvessel density, presence of macrophages, smooth muscle cells and inflammation and augments vulnerability. On the other hand the amorphous type consist from neovessel leakage of plasma proteins, vW factor, fibrinogen, it shows cell depletion and characterizes the most stable plaques[101].

Another correlation has been assumed between IPH and type III collagen preporderance on in diabetics of type I [48]. Accumulation of plasma contents to plaque interstitium promotes inflammation and calcium deposition. Iron and glycophorin A depositions have also a role in plaque vulnerability[110].Intimal microvessels cluster at the borders of the lesion, that is base and shoulders. Their density is two to fourfold higher in vulnerable than in stable plaques[39]. Intraplaque hemorrhage provides the lipid core a significant amount of rich in cholesterol erythrocyte membranes and increases plaque's volume and vulnerability. The RBC membrane acts as an atherogenic and inflammatory stimulus inside the plaque. This is why a plaque stable for a long time may turn inpredictably to a vulnerable one.

Metabolic syndrome and diabetes mellitus are associated with multiple metabolic toxicities producing ROS and impairing vascular endothelium and eNOS . These are potent angiogenic inducers inside the plaque[130]. Furthermore, iron accumulated from hemoglobin breakdown leads to free radical formation promoting inflammatory milieu inside the plaque. Therefore, oxidized LDL is produced and cell death is favoured[119].

It is worth to mention that altough inhibitors of angiogenesis halted atheromatous plaque progress in ApoE$^{-/-}$ mice, patients receiving antiangiogenic drugs for cancer present higher incidence of cardiovascular events. This may be indicative of protective effect of physiological VEGF concentrations, or of multiple factors implicating in plaque destabilization[99].

3.3 Ageing

Arterial wall degeneration by ageing has been depicted in many pathology studies. Arterial dilation, intimal wall thickening, increased stiffness, reduced compliance and endothelial dysfunction of various degrees are some of the age- associated findings presenting even in healthy individuals. Animal studies document that atheromatous plaques in older rabbits are more susceptible to hyperlipidemia than in younger ones. What interests is the compositon of atheromatous plaques in humans of different ages and for the same degree of luminal stenosis. In a study the plaques of the younger individuals were most fibrous and stable, while of the older ones presented greater calcification, depletion of vascular smooth muscle cells, tendency towards heavier macrophage infiltration and MMP-9 prevalence[6].

In another study, the age of atheromatous plaques has been estimated by calculating the ^{14}C content of the plaque, an element released in atmosphere at 1950's and'60's ,during atomical bomb tests, which follows predictive declination patterns. This study concluded that the age of the symptomatic plaques is 5 to 10 years, irrelative to patient's age, positively related only to plasma insulin levels, with the faster formating plaques beeing the most vulnerable ones[15].

3.4 Apoptosis/Senescence

Vascular smooth muscle cells proliferate readily at the first stages of atherosclerotic disease, while they are vanished in advanced lesions. Senescence-associated-β- galactosidase activity increases progressively , indicating that by the time the smooth muscle cells of the plaque turn to be senescent and apoptotic. Their depletion, especially at the shoulder areas, deprives fibrous cap from its underlying skeleton and predisposes to plaque's vulnerability[93]. Vascular smooth cell apoptosis contribution to atheromatous plaque formation and instability remains a matter of controversy, because of the association of the process to aneurysm formation and Marfan disease. It is possible that vsmc apoptosis in non atheromatous environment leads to aneurysmal arterial wall degeneration. On the opposite, in atherosclerotic lesions vsmc apoptosis leads to calcification, fibrous cap thinning, necrotic core augmentation and prevention of expansive arterial remodeling[98]. Macrophages can induce smc apoptosis by triggering their Fas apoptotic pathway and by secreting proapoptotic molecules such as TNFa[117].

Apoptosis of macrophages gains importance in the fate of atheromatous plaque. In the early stage of the disease , macrophage apoptotic activity proves to be beneficial , aiding at the clearance of apoptotic foam cells. This is characterized by diminished plaque cellularity and by lesion stabilization. At the progress of atheromatous plaques, the accumulation of oxidized LDL, oxysterols, TNF-a, FAS ligand, hypoxia, NO and intracellular free cholesterol render this clearance deffective. Apoptotic cells turn to be necrotic, enhancing lesion inflammation, matrix-degradeing protease secretion and increased local cellularity. Furthermore, a thrombogenic environment develops from the delivery of tissue factor and from platelet attraction by the accumulation of lysophosphatidic acid derivatives. The multifuctorial nature of macrophage death inside atheromatous lesions rises obstacles in therapeutic planning[57].

Deffective efferocytosis, e.g., clearance of apoptotic cells, is associated to plaque destabilization. Macrophages outnumber all types of cells in the intima of advanced atheromata. The phagocytosis of apoptotic foam cells includes complex interactions of macrophage receptors, apoptotic cell ligands and extracellular bridging molecules, like lactadherin (MGF-E8). Defect in each one of the aforementioned factors leads to problematic efferocytosis and necrotic core formation[8,36]. Cell- surface markers of macrophages, expressed differently in topographically discrete plaque lesions may reflect different stages of monocyte to macrophage differentiation, different lipid loading, or different adhesion to extracellular molecules . Competitive inhibition of receptors for apoptotic cells caused by oxidized cholesterol molecules has also been proposed[57]. The complexity of the procedure is obvious since selective silencing of efferocyte receptors indicates that not all of them are implicated. MERTK receptor, TG2 and Ldlr are propably more important when deffective than CD36 in plaque inflammation and necrosis. Dendritic cells have been implicated. Dendritic cells via CD8a contribute to defective clearance of apoptotic cells, although in a lesser extent. Their function as antigen presenting to T-cells and their relative role to increase the risk of plaque rupture is under investigation[8].

3.5 Hypoxia inside Atheromatous Plaque

Deficient oxygen supply inside the avascular atheroma and increased oxygen demand from the inflammatory cell metabolic activity results in low oxygen tension in this area[106]. The consequences of plaque hypoxia are:

- Plaque angiogenesis

Hypoxia induced factor in combination with vascular endothelial growth factor are potent inducers of angiogenesis inside the plaque. Fragile neovessels are prone to rupture and to augment the plaque's volume, providing the atheromatous core with lipid rich RBC membrane fragments and heme, a source of iron , which is substrate for oxidative stress. Vascular smooth cell migration is elicited and plaque destabilization is favoured.

- Altered glycose metabolism

Glycose metabolism is accelerated and shifted to anaerobic way. Oxidative environment is developed and mitochondrial and extramitochondrial apoptosis is triggered.

- Plaque's proteolysis promotion

Extracellular matrix proteolysis and remodelling is promoted and degradation of collagen allows for neovessel expansion and fibrous cap weackening. MMP and cathepsin's activation lead to further remodelling and weakening of the fibrous cap.

- Inflammation

Hypoxia triggers the formation of prooinflammatory cytokines and leukotrienes and Akt activation.

- Lipid accumulation

Hypoxia alters sterol and triglyceride metabolism, leading to lipid accumulation in macrophage foam cells. Fatty acid synthesis is increased, while lipid efflux is decreased. SCD-1 (stearoyl coenzyme A desaturase) and HMG- CoA (Hydroxylmethylglutaryl coenzyme A) expression is increased in hypoxic conditions[106].

CHAPTER 4: ATHEROMATOUS PLAQUE MECHANICAL STRESS

4.1 Mechanical Stretch /Flow Parameters

Vascular wall is susceptive to different stresses during the cardiac cycle. Shear stress is acting on endothelium, while tensile stress is acting on the whole of vascular wall. Only few studies regard the combination of both types of stress and even less the relation of stress to biological factors to incite a vascular event. Therefore, the results are confusing. It seems that shear stress renders atheromatous plaque vulnerable, but the site of rupture corresponds rather to areas of increased wall stress, even not predictably (see Figure 4).

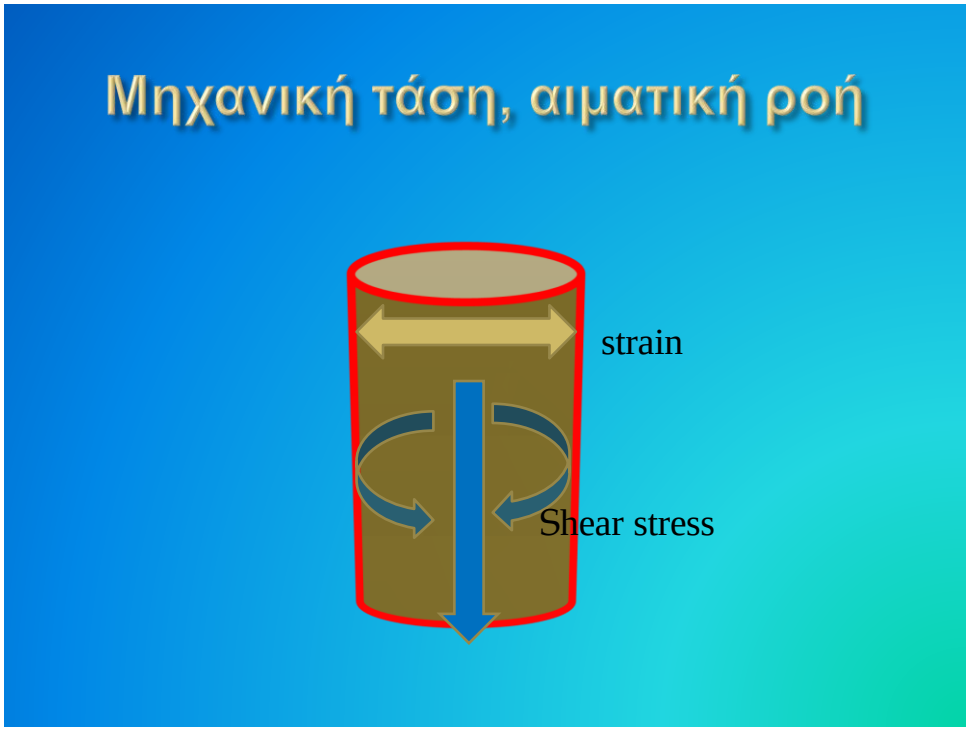

FIGURE 4: ATHEROMATOUS PLAQUE MECHANICAL STRESS.

IVUS (intravascular ultrasound) and conventional algorithms through fluid structure interaction analysis have been used to estimate the areas at risk, although experimental confirmation hasn't been possible yet[134].

The atheromatous burden is greater at sites of vessel bifurcation (see Figure 5). Even more, mechanical forces intraluminally lead to preferential development of atheromatous plaques

in sites of proximal bifurcation i.e. of the coronary tree, which are also the most vulnerable to rupture. The pattern of vessel tapering, the presence of side branches and the variance in shear stress may justify the variability in atherosclerosis distribution[54].

FIGURE 5: PLAQUE FORMATION IN SITES OF BIFURCATION.

It has been estimated that 82% of the plaque ruptures occur at the site of the maximum stress[103]. In a number of studies, stress analysis by FSI (fluid structure interaction)/MRI correlates the sites of rupture to the ones of maximal wall stress. The shoulders of the plaque have been proved as the locations the most prone for rupture. This seems to be valid independently of the thickness of the fibrous cap. Limitation of these studies is the acceptance of linearity in blood flow and of isotropy in wall properties, conditions far from reality. Moreover, as the study examines the post-rupture plaque, assumptions have to be made about the location and the thickness of the fibrous cap rupture in order to reconstruct the prerupture geometry[103]. Finally no correlations to systemic factors are included in these studies.

In another study, three dimensional analysis with MRI of symptomatic carotid plaques was used to define a computational model, representing the stress and the stretch inside the plaque. The observations revealed considerable variations in stress and stretch of vascular wall in symptomatic plaques with juxtaluminar hemorrhage[52].

Endothelial shear stress is the force directed by the blood flow to the endothelium. Low shear stress is associated to proatherogenic and proinflammatory genes upregulation, while at the same time, atheroprotective genes are downregulated. This is the way atherosclerosis initiates at the sites of low shear stress. Furthermore, low shear stress favors the thinning of the fibrous cap, promoting plaque's vulnerability. Studies with the use of simulators have

proved that LDL concentration under conditions of steady flow is higher near the endothelium than in the other areas of the vascular lumen. Particularly distal to stenosis, where, the shear stress is low, this concentration may exceed the systematic one up to 20%.This locally increased concentration of LDL molecules trigger the atherosclerotic process. Administration of statins has beneficial effects in downregulating atherosclerotic process even in the areas of low shear stress, by attenuating the local proinflammatory effects or , possibly, by ameliorating endothelial shear stress , although the latter has not been proved in the total of studies[102].

Extreme values of shear stress may induce the expression of adhesion molecules on endothelial cells and favour monocyte adhesion. High shear stresses upregulate ICAM1 production, but the very low ones increase VCAM1[74].

Another physical phenomenon capable of inducing vascular events is a spastic reaction of the vessels as a response to various mechanical or biological stimuli. Coronary artery spasm has been implicated in a considerable number of myocardial infarctions or sudden deaths. It refers to sudden localized contraction of coronary vessels, in the presence of atherosclerotic intima with enhanced response to vasoconstictors such as thromboxane, serotonin, endothelin. In case that a vulnerable plaque exists, rupture and thrombus formation ensues. This mechanical phenomenon explains Prinzmetal angina. Chronic low grade inflammation and high concentration of mast cells in vasospastic areas have been demonstrated[28].

4.2 Computational Models for Structure and Fluid Meshes[14,103]

In a number of studies, maximal plaque wall stress is higher in symptomatic than asymptomatic patients for the same degree of lumen stenosis. Maximal pressure is applied on the shoulders of the plaque, especially the proximal one[16,55]. Limitations of these studies come also from the low contrast of the fibrous cap at MR imaging, and from the technical challenge of measuring the flow rate ratio for each individual[16].

Microcalcifications may be present intracellularly or they are embedded in the fibrous cap in the form of spherical inclusions. These microcalcifications have been demonstrated at coronary vessels by high resolution micro-CT and create high wall stresses, as calculated by FSI simulations. It is estimated that increase the wall stress by 2,15- fold of its original value. They also favorate surrounding soft tissue debonding from the rigid calcium inclusion, a fact that leads to plaque destabilization [60]. This may explain that rupture may be noticed at sites of half of the maximum wall stress[84].The amount and the pattern of distribution of calcium inside the plaque may influence the risk of rupture at sites of maximal wall stress. The calcium is either diffuse inside the lipid core or in the form of small or large aggregates. The distance between the fibrous cap and the calcium aggregates (calcium gap) correlates positively with vulnerability. The thickness of the fibrous cap, the presence and the size of the lipid core and the pattern of calcification are independent factors influencing plaque rupture. Modelling the combined contribution of these factors in plaque instability using computational methods remains a challenging project [14].

Acute increase of plaque volume has been attributed in a number of studies to a change of cholesterol state from the soluble to solid form by crystallinization. The phenomenon is

triggered by the local inflammation environment, the pH shifts, temperature, saturation changes and it is accompanied by volume expansion, crystal piercing of the membrane, disruption of the fibrous cap and of the overlying intima, resulting in plaque rupture. Vasa vasorum of the plaque may be also lacerated by cholesterol crystals, provoking intraplaque hemorrhage and further plaque augmentation. The process's magnity depends on the size of the lipid core. Large lipid cores, usually in men, tend to present acute volume expansion, while small lipid cores, usually in women, tend to be eroded[30]. Statins seem to dissolve cholesterol crystals and blunt their tips in vitro, favouring plaque stabilization[20]. Besides volume expansion and possible tearing of fibrous cap, crystallized cholesterol is phagocytosed by macrophages inducing lysosomal damage, activation of NLRP3 inflammasome, activation o f caspase-1, and production of interleukine -1b, a cytokine correlated with disease severity[113].

A great number of mathematic models have been proposed to predict the growth of necrotic core and the vulnerability of a plaque. Assumptions have been builded on oxLDL distribution inside the plaque, chemoattraction of monocytes and hypoxia effects inside the lipid core. The drawback of all simulations is the reliance on stable conditions and the assumed linearity of reactions. On the other hand, in vivo, no state can be considered as given: distribution of oxLDL or cellular elements inside the plaque may be irregular or a transposition can be noticed due to vessel wall movements. Hypoxia is not linearly increased according the distance from the bloodstream, because vasa vasorum inside the plaque may be formed[74].

Outward vascular remodelling is a compensative mechanism during atherosclerotic plaque development. It is an independent predictor of major cardiac events, and it is positively correlated with large necrotic core, while negatively with fibrous tissue.

It is obvious that hypertension, hypotension and tachycardia, especially when combined to anemia, may trigger vascular events by inciting complication of plaques at risk of rupture[1].

CHAPTER 5: ATHEROMATOUS PLAQUE CELLULAR FACTORS

5.1 Progenitor Cells

Circulating endothelial cells have been associated to acute coronary syndromes[23]. There is a debate whether circulating smooth muscle cells of bone marrow origin participate in atheromatous plaque formation, instead of coming from native arterial wall layers[24]. Even in the process of plaque healing, where extracellular matrix, consisting of collagen type III and glycozaminoglycanes, fills the gap of rupture site, smooth muscle cells of local origin predominate. The process creates a constrictive remodelling of arterial wall and rapid plaque progression [87]. Imaging criteria of plaque healing refer to lumen curvature and contour roughness smoothing[96]. It is proposed that progenitor cells in the blood may interfere with atherosclerosis progression via paracrine signalling and not by differentiation into smc or endothelial cells[99].

5.2 Monocytes, Macrophages

Modified LDL particles elicite the release of chemoattractive factors from endothelial cells and the production of adhesive molecules (integrins, selectins) which favor monocyte and T-cell recruitment and their penetration into arterial wall through junctions between endothelial cells. Junction adhesion molecule (JAM-A and - C) regulates endothelial permeability. A common transcription factor, such as nfKb has been suggested as the activator of different genes, producting chemotactive and adhesion molecules.

According recent research, selective recruitment of monocyte subsets in atheromatous lesions may be related to various atherosclerosis phenotype. This is more clearly demonstrated in mice, where monocyte subsets are defined by Ly-6C receptor expression. Therefore, Ly-6C high monocytes are abundant under inflammatory conditions, promoting macrophage formation in atheromatous plaque. On the other hand, Ly-6C low monocytes have poor concentration in atheromatous lesions, have antiinflammatory properties and favor angiogenesis. In humans monocyte populations are divided according CD14 and CD16 receptors. Monocyte subsets may have predictive value in obese patients, in patients with stable coronary disease and in these with chronic renal failure. CD16+ indicates favourable prognosis, although evidence is not strong enough [2].

Monocyte biology may be modified during the various stages of atherosclerosis progress. A shift of the monocyte subset ratio may reflect an increased plaque activity and monitoring of monocyte subsets may trace individuals with sublclinical atherosclerosis. A need for a flow cytometric assay is obvious[69].

Colony stimulating factor leads to monocytes' transformation into macrophages. A variety of mechanisms are involved in modified LDL uptake by macrophages and subsequent foam cell formation:

1. Scavenger receptors class A-1 and class A-2 are involved in oxidized LDL incorporation
2. Low density lipoprotein receptor related protein-1 contributes to aggragate LDL uptake via a scavenger receptor SREBP1 and SREBP2 mechanism.
3. Fc gamma receptor favors LDL incorporation to immune complexes
4. Macropinocytosis, a liquid phase uptake mechanism non-related to scavenger receptors has been proposed for minimally oxidated LDL. Endogenous activation of toll like receptors TLR-4 by spleen tyrosine kinase is responsible.

Ox-LDL uptake from macrophages and the subsequent formation of foam cells is a procedure mediated by scavenger receptors (SRs) , such as SRAI, AII, B and CD36. The transcription of peroxisome proliferators-activated receptors γ (PPAR γ) favours ox-LDL uptake by macrophages via an increased CD36 expression. Therefore, a positive feedback mechanism is provided[135].

Lipid accumulation in macrophages results to their transformation into foam cells which release ROS, tissue factor, cytokines, growth factors,MMPs all that trigger inflammatory reactions, vascular remodelling and plaque thrombogenicity. Vascular smooth muscle cells are another source of tissue factor [2].

Monocyte subsets have been related to plaque's destabilization. $CD14^{+}CD16^{+}$ monocyte upregulation has been related to plaque rupture and acute coronary syndrome. This subtype presents increased TLR4 (toll like receptor 4) expression, a receptor of exogenous LPS (lipopolysaccharide) and endogenous heat shock protein, with important role in innate immunity and inflammatory processes. TLR4 is strongly expressed at the lipid core and the shoulders of the atheroma and, activating the accumulated monocytes, contributes to plaque's vulnerability[107].

Monocyte chemoattractant protein-1 recruits monocytes and macrophages in the vessel wall. It is detected in atheromatous lesions and regulates the local expression of interleukines, tissue factor and adhesion molecules. Inhibition of MCP-1 activity is a potential therapeutic target for complicated atherosclerosis[34].

CD36 is a transmembrane glycoprotein of macrophages that serves as scavenger receptor, mediating uptake of oxidized LDL leading to foam cell formation. CD36 overexpression shows a linear relation with the number of atherosclerotic risk factors. It seems that diabetes mellitus is the risk factor with the greater influence in CD36 expression, with the poorly controlled patients having the lower levels of glycated CD36[42].

5.3 Autoimmune reaction

Autoimmune reactions are implicated in atherosclerotic process. Chemical modification of Ldl trapped in subendothelial space, impaired or absent Treg activity and response to drugs

support the autoimmune basis of atheromatous pathology. Autoimmune diseases are characterized by intense atheromatosis[10].

5.4 T-cells

T-cells also contribute to atherogenesis. They enter the intima after binding to VCAM-1, they become activated by interferon-γ and they release inflammatory cytokines (CD40 ligand). The role of T-cell subsets is again proposed, with Th1 cells showing proinflammatory action, when on the other hand, Th2 cells and Treg attenuate inflammation [2, 31]. In a study T-cells, MMP-9 and macrophages were abundant in carotid plaques of thromboembolic vascular events compared to plaques involved in hemodynamic vascular events[35].

5.5 Dendritic Cells

The number of DC is increased in atherosclerotic arteries. Their concentration is increased in intima and adventitia and the mechanism of action has not been confirmed yet. They probably present antigens such as oxidized LDL to T naive cells, promoting T-cell recruitment and atherogenesis progression [2]. DC form clusters around vasa vasorum, at the shoulders and at rupture prone regions of the plaque. It has been shown that hyperlipidemia alters the immune function of DC but statin administration reduces their activation[117]. The number and the status of activity of DC is altered in diabetic patients. These cells are accumulated in inflammed atheromatous plaques by a mechanism where the soluble fraction of the chemokine fraktalkine plays a major role . This way their levels in plasma are declined[31].

5.6 Vascular Smooth Muscle Cells

Vascular smooth muscle cells (smc) produce the components of extracellular matrix (collagen, proteoglycans, elastin), and the enzymes responsible for these components' turnover; Lysyl oxidase for synthesis and metalloproteinases for degradation. Normally they present the non synthetic-contractile phenotype. During the process of atheromatosis, vascular smooth muscle cells acquire a synthetic- proliferative phenotype, migrate to intima where they transform to foam cells and increase synthesis of extracellular matrix , promoting intimal thickening and vascular remodelling. It has been shown that both native and modified LDL affect the migratory capacity of smc through altered expression of cytoskeleton related proteins (e.g myosin regulatory light chain dephosphorylation)

Vascular smooth cells retain the capacity to differentiate and dedifferentiate, and this balance may be used as therapeutic target. Upregulation of mir143/145 may act for plaque stabilization or for in plaque restenosis inhibition[61].

Vsmc can migrate from media to subintimal space participating in plaque formation, can regulate blood pressure by contraction, produce extracellular matrix, can phagocytose oxidized LDL and transform to foam cells. They can also produce receptors (VEGF) and cytokines that attract white cells and endothelial cells from adventitia to neovascularization. Between contractile and synthetic phenotype, a large variety of vsmc phenotypes can coexist. Phenotypical vsmc switching depends on mechanical stretching, chemical factors,

and transcriptional regulatory pathways, such as SRF (serum response factor)/myocardin axis, which is again activated by Rho/Rho-kinase pathway. [133].

5.7 Platelets

Platelets are implicated in vascular events by two ways: They become activated by shear stress (role of ER- chaperone), and by chronic exposure to traditional risk factors and adhere to intact but inflammed endothelium. Downregulation of PGI2, NO and enhanced fibronectin, ICAM-1, P-selectin,E-selectin ,CRP and von Wilebrand production contribute to the phenomenon. This procedure is mostly atherogenic and less thrombogenic. On the other hand, in sites of denuded endothelium and atherosclerotic plaque component exposion platelets aggregate and form thrombus. Circulating von Willebrand factor binds to the exposed collagen –type III and IV- of the interior vascular layers, allowing interaction with platelet glycoprotein receptors [2].

After plaque erosion, intraplaque components entering the blood stream contact with platelets and activate them. Such component is LPA, an intermediate product of phospholipid metabolism binding to LPA receptors and acting as an autocrine or paracrine mediator. AcylLPA is the most potent platelet activator and is present in mildly oxidized LDL. Platelets have three types of LPA receptors which mediate platelet cytoskeletal rearrangement via GTPase Rho and Rho kinase activation. LPA receptor antagonists , such as dioctylglycerol pyrophosphate and dioctylphosphatidic acid may have therapeutic implications, with the constraint of questionnable conclusion extraction from studies on carotid plaques only , where besides LPA-mediated platelet activation is documented in a percentage and not the total of specimens. Considering that additional plaque lipids are also thrombogenic, further studies are required to positively conclude about therapeutic strategies[122].

The local thrombus is initially of platelet origin. The deposition of fibrin stabilizes and expands the thrombotic process and larger ruptures are more thrombogenic than small ones[39].

Apart their traditional role in thrombosis and hemostasis, activated platelets release and express inflammatory mediators that interact with leukocytes and endothelial cells and promote atherogenesis. Platelet –mediated inflammation takes place through expression of TNF superfamily members , such as CD40 ligand, FasL and LIGHT, the latter being a content of fresh coronary thrombi. These ligands induce inflammatory responses to monocytes and to endothelial cells and upregulate the production of adhesion molecules and the release of cytokines, leading to plaque destabilization[12].

Platelet staining inside the plaque produced useful conclusions. Although not clinically singificant regarding symptomatic and asymptomatic patients, a study[63] of carotid plaques gave the following results:

- The presence of platelets inside the plaques is prominent in areas of intraplaque hemorrhage and close to cholesterol clefts. It is not necessarily

associated to erythrocyte leakage, probably because of thin intraplaque neovessels

- Intraplaque platelets create a proinflammatory milieu producing interleukines such as IL8 (higher concetration in more platelets and higher microvessels density)
- The short halflife of platelets is indicative of recent or ongoing bleeding

5.8 Endoplasmic Reticulum

The role of endoplasmic reticulum in sterification of cholesterol's fatty acids and in unfolded protein degradation is important in apoptotic death of foam cells and in plaque instability [2]. Endoplasmic reticulum's impaired degradation of unfolded proteins contributes to apoptotic death and plaque rupture [2]. Particularly in advanced lesions, activation of endoplasmic reticulum stress signal-transduction pathways leads to increased macrophage apoptosis increasing plaque's vulnerability[36].

5.9 Mitochondria

The regulatory role of mitochondria in oxidative stress is related to atherosclerosis progression. Increased production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical and of reactive nitrogen species (RNS) result in dysfunctional mitochondria, mitochondrial DNA damage,respiratory chain deficiency and endothelial and smooth cell apoptosis. Mitochondrial biogenesis -related genes, such as PGC-1 (peroxisome proliferator-activated receptor γ - coactivator) participate in mitochondrial dysfunction, through mechanisms not fully understood yet. Recently the role of mitochondrial fusion and fission in atherosclerosis procedure is investigated. In healthy mitochondria there is a balance between fusion and fission. Under oxidative stress and increased ROS production fission is favoured which results in mitochondrial breakdown.Furthermore, the ATP sensitive K+ ion channels regulating mitochondria ion transport by ROS and RNS production are also related to atherosclerosis progression[64].

CHAPTER 6: ATHEROMATOUS PLAQUE MOLECULAR FACTORS- SYSTEMIC/LOCAL

6.1 Complement Cascade

It is demonstrated that complement may be activated inside the plaque by all the three ways. Activation by the classic and leptin pathway has a protective effect by the removal of apoptotic cells, immune complexes and debris. On the other side, complement activation by the alternate pathway may be proatherogenic and favour plaque progression and destabilization. Complement proteins inside the vascular wall may be retained from plasma or, rather, produced locally. There is evidence that complement activation differs in the different layers of the vascular wall, with the superficial levels triggering the classic and alternate pathway. Complement polymorphisms have been associated with coronary artery disease. High levels of C3, C4 and C5a have also been related to vascular events. Studies with the C5 inhibitor pexelizumab provided conflicting results regarding its cardioprotective effects[26]. C5a elicits MMP-1 and MMP-9 expression, further promoting instability in atherosclerotic plaques[27].

6.2 Molecules, Biomarkers

Atherosclerosis is a multifactorial disease and the need of complex computational models is obvious. Although a great number of biomarkers have been directly associated to atherosclerosis development and to plaque's vulnerability, no single one, neither a combination of multiple biomarkers proved to be reliably predictive for cardiovascular risk estimation.

Cytokines, interleukines, high levels of RANTES , IL-Ira, MIP-Ialpha, MIP-Ibeta, IL-2, IL-4,5,6,7,17,PDGF-BB,VEGF and IFN-gamma are implicated in atherosclerotic process[7]. Proinflammatory cytokine Il-18 stimulates smc proliferation and migration through MMP-9 dependent mechanisms.Ap-1 and nfkb aktivation participate to the phenomenon[45].

A mumber of molecular factors have been proved to be atheroprotective.

Il-1 has an atheroprotective effect, promoting outward vessel remodelling and stabilizing atheromatous plaque. This is mediated through MMP-3 dependent mechanisms. Il-1 signaling induces MMP-3 expression. Deficiency of IL-1 in Apo-E mouse results in plaque instability, with decreased smc and collagen content and increased intraplaque hemorrhage[44].

Heat-shock protein 27 is reduced in human coronary atheromatous plaques. Experimental animal studies have shown atheroprotective effect of HSP-27 expression induction in male

and female rats. This effect is attributed to decreased cholesterol accumulation, to plaque remodelling and stabilization[115].

Humanin is a cytoprotective peptide endogenously produced inside atherosclerotic plaque as a response to inflammatory and apoptotic processes. Its concentration is greater in unstable atherosclerotic plaques[43].

Leptin is a protein mainly produced by adipose cells. Leptin expression is associated with insulin resistance, obesity and metabolic syndrome. At tissue level leptin interacts with leptin receptor. Apart from its pleiotropic hormonal actions, leptin has been implicated in atherosclerosis process, enhancing[120]:

- Endothelial oxidative stress- ROS generation
- Expression of intracellular adhesive molecules and MCP-1
- MMP-2,MMP-9 and VEGF upregulation
- SMC proliferation and migration
- Proinflammatory cytokine production[120]

Recent studies have shown that leptin may be produced inside atheromatous plaque by other cell types, such as smc and macrophages and exert autocrine and paracrine effect leading to plaque destabilization. Infection-induced TNFa triggers intraplaque leptin expression, a process possibly mediated by toll like receptors[120].

Tissue factor is expressed in circulating monocytes and in the vascular wall. Its expression is favoured by a lipid rich diet and results in increased plaque's thrombogenicity and in neovessel plaque formation. Mechanical injury and sustained inflammatory milieu promotes tissue factor expression and activity[118].

Many factors may interfere with more than one mechanisms of complicated atherosclerosis. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) , a member of tumor necrosis factor family, presents a double action:

1. Binding to his receptor , fibroblast growth factor-inducible 14, contributes to arterial wall remodelling , inflammatory response and plaque volume expansion in apoE knockout mice
2. It regulates expression and activation of tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) , which are molecules with known prothrombotic action[89].

6.3 Mmp (Metalloproteinases)

It is a family of 23 genetically determined enzymes, regulating the balance between extracellular matrix formation and breakdown. They all are characterized by a conserved catalytic segment of Zn-binding sequence. They are classified into five groups[124]:

- Collagenases (-1,-8,-13) / Gelatinases (-2,-9)
- Stromelysins (-3,-10,-11)
- Minimal MMP's (-7,-26)

- Membrane – type MMP's[123]

Their activity is of paramount importance for atherosclerotic plaque formation, expansion and rupture. MMP-2 and MMP-9 are clearly implicated in plaque destabilization[65] but also MMP-1, MMP-3, MMP-11, MMP-12[66]. The predominant MMP's source are the foam cells.MMP's share common properties, such as acting in neutral pH, production by proforms , activation by proteolytic reactions, and inhibition by a few tissue inhibitors of metalloproteinases (TIMP's) or by plasma proteins (a2 – macroglobulin)[66,124] . They degrade collagens, elastins, the core proteins of proteoglycans and glycoproteins. By this degradation they result in vascular wall weakening and atherosclerotic plaque fibrous cap thinning. Several macrophage phenotypes may produce different amounts and types of MMP's[66]. Upregulation of MMP production is achieved via a nfkb-dependent pathway[67]. MMP-9 polymorphisms show no direct correlation with plaque destabilization[100].

MMP-8 is also named neutrophil collagenase, as it is expressed and rapidly released by circulating leukocytes after exposure to inflammatory stimuli. It is also expressed and released by other cell types (endothelial cells, smooth muscle cells) , but after considerable delay. MMP-8 is activated by ROS released by activated neutrophils and by proteases (cathepsins, chymothrypsins). Its concetration in atherosclerotic plaque is 8fold than in normal vessels and it is linearly associated with the vulnerability of the plaque and the risk of vascular events. The clinical use of the MMP8 as a biomarker (plasma or serum), is under investigation , with various studies providing conflicting evidence. The role of gene polymorphisms of MMP-8 in atherosclerosis remains to be defined[124].

Excess activation of MMP-9 and MMP-2 without tissue inhibitors of metalloproteinases TIMP's compensatory elevation or activation leads to ecm (extracellular matrix) destruction, plaque's instability and restenosis, in case of previous intervention[45]. Oxysteroles may disturb MMP's/TIMP's balance, contributing to plaque destabilization and rupture[83]. ROS hyperproduction inside atheromatous plaque upregulates MMP-9 expression through activation of redox-transcription factors[83].

6.4 Collagen

Collagen is a crucial component of extracellular matrix in healthy and diseased arterial wall. It is present in all layers of arterial wall. In the intimal layer type IV collagen predominates. In the media types I, III, and V and XVIII are present. Adventitia is rich in types I and III. During atherosclerotic plaque development substantial change in extracellular matrix composition are noticed. SMC turn from contractile to synthetic phenotype and large amounts of collagen type I and III are produced. At the same time matrix degradation is enhanced with proteinase activation. Fragments of type I and type IV collagen are formed, triggering signaling in inflammatory cells and favouring calcification (collagen II and IX). Collagen receptors have been identified on smc (integrins) and on leukocytes (integrins, scavenger receptors). 3-d conformation of different collagen types may have a role in plaque destabilization[114].

The amount of collagen in the fibrous cap and in the plaque is differently measured in wet and dry tissue. Increased LH1 activity (enzyme catalyzing the hydroxylation of lysine,

responsable for helical portion of collagen) inside the plaque is a possible indicator for sites of greater plaque vulnerability [59].

Predominance of type III collagen, instead of type I, in atheromatous plaques of diabetic patients is associated to inflammation, neovascularization and plaque's instability[48].

Collagen turnover is mediated by proteases, such MMP's and cathepsins. Cathepsin K is a lysosomal enzyme secreted by macrophage foam cells and osteoclasts, playing an important role in extracellular matrix remodeling. It degrades collagen type I, which accounts for 60-70% of arterial walls'total collagen, at its C-terminal telopeptide, producing a specific fragment, CTX-1. Cathepsin K and CTX-1 are present in significant amounts in advanced coronary plaques but not in the early ones, consisting a possible index of plaque's staging. [17].

The varying predominance of different collagen types, their layering pattern in fibrous cap and their relevant tensile strength are parameters that have been studied with various methods. LIFS (laser induced fluorence spectroscopy) is one of these methods of limited penetration that investigates plaque's surface irregularities and strength, corresponding well with histopathologic findings[104].

The narrow pH profile of cathepsin K and most of other cathepsins (F,S,L), requiring acidic pericellular environment for extracellular activity may justify their preferred action in advanced atherosclerotic plaques. Most of cathepsins are expressed by macrophages, endothelial and smooth muscle cells near the fibrous cap and in regions of foam cell accumulation. They consist predictors of plaque instability. Because of the involvement of cathepsins in major histocompatibility complex (MHC) classII antigen presentation and T-cell activation by antigen – presenting cells, caution is necessary when developing cathepsin inhibitors [18].

Collagen exposed in a ruptured atherosclerotic plaque attracts platelets with the platelet-specific collagen receptor glycoprotein VI (GPVI) and initiates thrombus formation[41].

Collagen induces metalloproteinase shedding of GPVI releasing soluble sGPVI which can serve as biomarker, while GPVI inhibition in experimental stroke models proves to be protective [41].

6.5 S100 proteins

S100 proteins, especially S100A8/9 and S100A12, also called S100 calgranulins, belong to the family of calcium binding intracellular proteins. They are involved in a great number of cellular functions such as calcium homeostasis, cell growth and inflammation regulation. Furthermore, they can also be secreted and exert an extracellular cytokine effect, acting on surface receptors via an autocrine or paracrine manner. It has been shown that S100 proteins can increase atheromatous plaque vulnerability and predispose to vascular events. This is mediated via the receptor RAGE and TLR-4 in macrophages and smooth muscle cells, where atheromatous plaque size, necrotic core and medial calcification are all increased[97].

6.6 Oxidized LDL

Although many of the cardiovascular events occur in individuals with normal cholesterol levels[11], there is still a strong linear relationship between the levels of LDL cholesterol and the extent of atherosclerosis. Oxidized LDL proves to be an independant risk factor of cardiovascular disease, but the relationship between circulating LDL and oxidized LDl has not been confirmed. The size of LDL particles is another predictor of heart attacks. Smaller and denser LDL particles have greater affinity for subendothelial extracellular matrix and tendency for oxidation. The measurement of LDL particle size is NMR (nuclear magnetic resonance) spectroscopy of lipoproteins. Smaller Lp(a) are more atherogenic than larger ones, but the clinical importance of this finding is uncertain [5].

It has been suggested that apoA-1 lipoprotein, apoB-1 lipoprotein and their ratio apoB/apoA correlate better with the risk of cardiovascular disease than total cholesterol, LDL,HDL and TG in untreated patients. In patients treated with statins LDL, HDL, and total cholesterol have no predictive value[4].

OxLDL act as the dominant chemoattractant for monocytes[73].

Oxysterols are cholesterol oxidation products that induce expression and activation of MMP-9 and decrease TIMP-1, the endogenous MMP inhibitor , in human macrophages and endothelial cells. Oxysterols are provided by the diet or produced endogenously via enzymatic pathways or nonenzymatic autooxidative cholesterol modification[83].

6.7 HDL

It has been evident by epidemiologic studies that high levels of HDL are protective against vascular events mostly because of its property to remove cholesterol from the vascular tree (reverse cholesterol transport) and to deliver it to the liver to be further metabolized [2] . HDL promotes efflux of cholesterol from macrophages and has an antiinflammatory and antioxidant effect on the arterial wall [3].

Recent evidence reveals additional antiatherogenic as much as antithrombotic activities of HDL. The most important protection concerns endothelium, with favored NO production, fortified barrier integrity and antiapoptotic effect. Antithrombotic properties are mediated by platelet inactivation, impaired thrombin generation and favoured fibrinolysis [2].

6.8 Other

LpLA2 (lipoprotein associated phospholipase A2 is a hydrolytic enzyme for oxidized phospholipids, releasing lysophosphatidylcholine, a molecule with proinflammatory properties enhancing atherosclerosis. LpLA2 proved to be an independant risk factor for prediction of stroke, or recurrence of stroke in general or selected populations. Combined with other biomarkers, such as CRP its predictive value is further increased[56]. Arachidonic acid, a normal content of plasma membrane phospholipids, is another metabolic product released by phospholipase A2, having proinflammatory properties in the vascular wall and favouring lipid retention, accumulation of foam cells and plaque rupture.Arachidonic acid produces leukotrienes 5-lipoxygenase pathway. Their signaling is mediated by membrane

and cytosolic receptors CysLT and BLT. Leukotriene synthesis inhibitors and leukotriene receptor antagonists may represent a therapeutic target in the treatment of atherosclerosis[53].

Proteomic analysis of serum and plaque contents associates new proteins to complicated plaques. Osteopontin is a glycoprotein secreted by macrophages, also known as early T-lymphocyte activator1, with possible predictive role for future cardiovascular events[92]. A study than measured osteopontin levels in carotid plaques after endarterectomies concluded that it can be used as a predictor for cardiovascular events[11].

It has been attempted to predict future vascular events or restenosis after endarterectomy based on plaque histology. One of these studies was Athero-Express, but again the conclusions were of little clinical value[11].

Apo C1 is mostly expressed inside the necrotic core while ApoE around it and inside the fibrous cap. These proteins are produced by macrophages and they are associated to rupture.

Nfkb is a family of five protein products that act as nuclear transcription factors. Their action is a rapid response to various stimuli (infection, inflammation, oxidation, radiation, physical factors, cytokine and growth factors, glycosylation products). Its activity refers to transient upregulation of genes involved in adhesion molecules and cytokine production, along with cytokine crosstalk. Nfkb is crucial for immune and inflammatory modulation, proliferation and apoptosis. In its inactive form it is located in cytosol bound with IkB inhibitors.Prolonged activation of nfkb has been observed in atherosclerosis and other chronic diseases. In atherosclerosis the action of nfkb is involved in early (cytokine and adhesion factor modulation) and later stages of the disease (macrophage to foam cell transformation), playing a role in plaque's vulnerability[76]. Activation of nfkb is involved in the expression of proinflammatory cytokines (IL-6, IL-8, TNF-a). However it may also be involved in the expression of antiinflammatory interleukines (IL-10). Hepatic growth factor is a potent intrinsic nfkb inhibitor with known antiinflammatory effect. Activation of nfkb pathway is implicated in various stages of atherosclerosis[80]:

- Amplification and maintenance of inflammatory process
- Intraplaque neovascularization
- Induction of apoptosis
- Triggering of coagulation cascade[80]

RAGE (receptor for advanced glycation end products) plays important role in diabetic atherosclerosis. Rage overexpression is associated with increased expression of COX-2/mPGES-1 and concominant inflammatory reaction in diabetic plaque macrophages, inducing MMP expression. This procedure is involved in plaque destabilization[129].

6.9 Infection

Infection from HSV, CMV, Chlamydia pneumoniae and other pathogens may be associated with atherosclerosis generation and progression, by the convertion of infected endothelium

to procoagulant and proinflammatory state and mediating the excretion of adhesive molecules and inflammatory cytokines. Another possible mechanism is by triggering autoimmune responses versus epitopes of the vascular wall omologous with pathogen's epitopes. Multiple metaanalysis proved though that antibiotic administration had no favourable effect in atherosclerosis regression [46]. It is likely that infection triggers atherosclerosis in a chronic way and linearly increasing with the number of infections per year and their duration. Vaccination against influenza reduced the incidence of strokes at a percentage 50%[51].

6.10 Genes

Studies in monozygotic and dizygotic twins demonstrated strong genetic correlation of genetic factors to cardiovascular disease. Unfortunately, the multifactorial nature of atherosclerotic disease complicates the design of a reliable genetic test, since a great number of genes coding lipoprotein metabolism, macrophage function, vascular tone fibrinolytic and inflammatory pathways should be taken into account. The study of single nucleotide polymorphisms identified 160 genetic loci negatively or positively associated to risk for coronary atherosclerosis, but their correlation with plaque's instability is uncertain[99].

Genetic code impact in atherogenesis is under research. Epigenetic DNA modifications, mainly hypermethylation, predispose to atherosclerosis. These modifications are possibly caused by traditional risk factors. MicroRNA's, translation regulators, are also implicated in endothelial integrity, macrophage activation, vascular smooth cell proliferation and cholesterol metabolism. Mir's show various activities in many levels of atherogenesis, such as mononuclear cell transmigration, angiogenesis inhibition, endothelial cell adhesion and neointima progression [2]. MicroRNA's are implicated in vascular smooth muscle cell metabolism and proliferation, therefore playing role in vascular remodelling and intima formation. Mir143 and 145 have been proved to keep the balance between the proliferative and the contractile phenotype.[61].Mir126 is expressed in endothelial cells, while mir155 is expressed in monocytes. Mir155 may prevent maturation of monocytes to macrophages, therefore preventing fibrous cap degradation. Mir146 is expressed in monocytes and seems to decrease proinflammatory signalling and cytokine expression inside atheromatous plaque[62].

Gene / protein interactions and potential human atherogenic genes are involved in atherosclerosis. Caveolae, JAK/STAT pathways and S100 A9 and S100 A8 interacting proteins upregulation, indicate a proinflammatory state[7.]

Upregulation of IL6, STAT1, ISGF3GIL10RA in atherosclerotic coronary and carotid plaques leads to JAK/STAT pathway activation[7].

Acute cardiovascular events present a circadian rythm, therefore the clock genes may be involved in the process of atherosclerosis. Animal studies in ApoE-/- mice suggested that disturbed function of p53 and c-Myc genes lead to abnormal cell growth, proliferation and apoptosis.There is also evidence that overexpression of p53 favours apoptosis and leads to plaque rupture under experimental conditions[21]. Even genes involved in the expression of thrombosis related factors such as Pai-1, t-PA, TF and ET-1 follow a circadian rythm. The

circadian fluctuation of thrombolytic activity may be reversed in advanced atherosclerosis and lead to vascular events in the early morning[22].

6.11 Plaque Healing

Before a fatal vascular event a number of subclinical plaque complications such as rupture or erosion, may happen, followed by thrombosis and healing. These multiple cycles may happen 4 times before the sudden death in a percentage of 60%[88].

Vulnerable plaques may turn to stable phenotype after a vascular event. A study of carotid plaques after stroke (histology of endarterectomy specimens) proved a plaque stabilization over time, with a decrease first of IL-6 and IL-8 expression and of caspase activity , followed by a decrease of macrophages, while on the other hand, an increase of plaque smooth muscle cells was noticed[90] (see Figure 6).

FIGURE 6: HEALED ATHEROMATOUS PLAQUE.

CHAPTER 7: DIAGNOSTIC MODALITIES

7.1 Imaging Modalities Old/Modern

Imaging of atheromatous plaques was indirect at the beginning, with angiography estimating the degree of lumen stenosis, in terms of diameter reduction. Stenosis was defined as the proportion of arterial diameter at the site of maximum stenosis, related to the diameter of the normal vessel. Ambrose's classification established morphological criteria for complicated plaques in the base of eccentric or concentric stenosis, irregular or scalloped borders and multiple irregularities. Complexity of lesions has been classified also by Goldstein, referring to the presence of intrafilling defects, ulcers, irregularities and flow compromisies[1]. Angiography remains the gold standard for the most challenging diagnostic problems, or in case of planned angioplasty. Its role has been limited because of its interventional nature, the need for special equipment, the radiation exposure and the cost.

Colour duplex ultrasound is another promising method that evaluates morphological, but also, functional parameters of the vascular tree. It provides information about the shape and the extent of atheromatous plaque, about the degree of stenosis in terms of lumen diameter and internal surface and about the texture of the plaque in terms of calcification. The doppler examination provides further information concerning blood velocity in different parts of the arterial tree. Algorithms estimating the degree of stenosis combining morphological and velocity criteria are available. Colour duplex ultrasound remains a wide screening method of low cost, with no exposure to radiation and with no need for highly specialized equipment. The main drawback of the method is the technical considerations in highly calcified arterial segments and in hardly accessible areas, along with the operator-dependent nature of the examination.

Recently, traditional criteria of angiographically proven lumen stenosis greater than 50%, have been reconsidered. Expansive arterial wall remodelling caused by atherosclerotic progression may subtle the clinical impression of disease severity. On the other side, many unstable plaques are nonstenotic and asymptomatic until rupture[39].

A number of invasive technologies have been developed for the vulnerable plaque investigation. These technologies may be discriminated in three groups:

1. Microanatomy imaging
    a. High frequency intravascular ultrasound (IVUS)
    b. Virtual histology – IVUS
    c. Intravascular optical coherence tomography

    d. Intravascular magnetic resonance imaging (MRI)
2. Metabolic activity measurement
    a. Intravascular thermography
    b. Intravascular elastography
3. Chemical composition characterization
    a. Near infrared reflectance or Raman spectroscopy

The main drawbacks of these novel technologies are their invasive nature, the need for highly specified personnel and their high cost[78].

Angioscopy is a method that permits direct visualization of the interior surface of the arterial tree. The main drawbacks are the ineterventional character of the method and the size limitation of the vessels under investigation. IVUS is another modern method, interventional too, investigating the extent and the texture of atherosclerotic plaques. It is often combined with other diagnostic modalities such as virtual histology, thermography or even palpography. The resolution of IVUS (100- 250μm) is inadequate for thin fibrous cap identification (23+/-19μm) and this is a drawback of the method[73].

PROSPECT study was a large multicenter study that combined angiography, grayscale and radiofrequency virtual histology-intravascular ultrasound[54].

OCT (optical coherence tomography) measures back-reflected infrared light and provides the best resolution among the invasive imaging techniques. It also has excellent histopathological correlations. The clinical application is restricted though, due to signal attenuation from blood. Even the next generation method (OFDI-optical frequence domain imaging) still presents limited penetration[73]. Light absorption from blood limits the value of OCT and NIR (near infrared spectroscopy)[126]. NIR estimates the chemical composition of plaque components by quantative absortion at different wavelengths. This property has been well correlated to histological findings, this is, why this method is complementary to IVUS[73].

Technical improvements give a promising role in OCT, in measurement of fibrous cap thickness and even in new aspects such as macrophage infiltration and spatial (3-d) estimation. Evaluation of the therapeutic effect from statin treatment to fibrous cap thickness is also possible[82].

Inravascular electric impedance spectroscopy has been used for plaque's stability assessment. Indirect conclusions may be extracted about cellular composition of the plaque, since inflammatory environment affects the impedance of atherosclerotic lesions. Abundant neovascularization, presence of scavenger receptor class B expressing cells and increased of MMP-9 immunoreactivity give high impedance values[33].

Electron beam computed tomography (fixed detectors) detects coronary calcification. Multidetector CT is an evoluted method, providing an assessment of the coronary lumen and of the vessel wall, with valuable information about tissue texture, but with limited discrimination between lipid rich and fibrous noncalcified plaque [73]. During MDCT both radiation source and detectors rotate during patient motion[78]. Combined with ECG data,

multidetector CT angiography is a satisfactory method for associating plaque characteristics (volume, lipid rich necrotic core , fibrous cap, surface disruption) with stroke, even in moderate degree of stenosis (0-49%)[86]. It has been shown that the risk for rupture is higher in coronary plaques with low CT attenuation, positive remodelling and spotty calcification[78].

Single photon emission CT (SPECT) is another method that can identify thrombus (fibrin detection), inflammation (leukocyte and macrophage infiltration detection), and plaque angiogenesis (integrin detection)[78].

Spiral CT and MRI have also been used for the assessment of the extent of atherosclerosis of the arterial tree[5]. The main drawback of these methods is the cost along with equipment availability limitations and the fact that metallic implant, cardiac defibrillator and claustrophobic patients are incompatible with the MRI methods.

High resolution MRI for coronary plaque imaging, using T1, T2 and proton density weighing allows for discrete imaging of fibrous tissue, calcium, lipid and blood and provides information for plaque composition. The accuracy of the technique has been verified with histology ex vivo[78].

Exploitation of the increased knowledge about plaque components and their biological behavior lead to highly evolved and specified imaging modalities. Molecular MRI is one of the latest methods applied for plaque characterization. It uses paramagnetic particles conjugated with biomolecules showing elective concentration or activity inside atheromatous plaques. Such paramagnetic particles are:

- New generation gadolinium chelates (constructs with albumin, HDL and liposomes)
- Paramagnetic nanoparticles with magnetic iron oxide pretreated with polymer coatings
- Magnetofluorescent nanoparticles
- Magnetic relaxation switches (they undergo reversible structure modification in the presence of specific enzymes, they detect troponin, BNP, CRP)[78]

Molecular MRI targets to

- Imaging targets of plaque rupture such as fibrin (perfluorocarbon nanoparticles, gadolinium labeled peptides, liposome based nanoparticles)[78]
- Imaging cellular targets inside the plaque (gadofluorine, HDL-like nanoparticles)[78]
- Imaging cell surface markers (antiE-selectin antibody paramagnetic liposomes)[78]
- Imaging plaque angiogenesis (anti-integrin antibody paramagnetic liposome)[78]

The design of biocompatible 3T loopless detectors for CMR angiography has been targeted to fibrous cap thickness measurement and proved to show excellent views of vessel pathology for resolution of 80μm[58].

A great number of MRI contrast agents targeting plaque inflammation in molecular level, have been developed. Targets indicating inflammatory plaque activity and their relevant contrast agents have been the following[68]:

Targets	Contrast agents
Ox-LDL	Antibody conjugated paramagnetic micelles (caution for retention, Gd toxicity and nephrogenic systemic fibrosis)
Endothelial cells	Microparticles of iron oxide conjugated with antibodies directed against VCAM-1 / agent to P-selectin
Neovascularization	Dynamic contrast enhanced MRI (DCE) with Gd-based contrast agent
Macrophages	GRASP imaging techniques with LUSPIO (lipid coated ultra small superparamagnetic iron-oxide particles) /SPIO's[125]. They are spontaneously engulfed by macrophages, they cause a MRI-detected signal decrease that correlates with intraplaque inflammation[125].
Apoptotic cells	Annexin A5 conjugated Gd-containing micelles
Activated platelets	Single chain antibodies conjugated nanoparticles,
Thrombus[68]	Fibrin –specific Gd-perfluorocarbon nanoparticles, CEST (chemical exchange saturation transfer)

Scintigraphic imaging is a promising method for detecting cellular and metabolic changes inside the plaque which render it prone to rupture. Potential radiotracers for the molecular imaging of the vulnerable plaque are radiolabeled agents that allow the noninvasive imaging of their biodistribution, with the use of SPECT or PET cameras. Such agents are:

- LDL (slow kinetics)
- MCP-1 (binding to circulating monocytes)
- (18f) FDG (accumulation in sites of macrophage infiltration, glucose metabolism of macrophages, greater uptake in unstable plaques. Drawbacks in the study of coronary vessels because of the heart movement and because of small size and high background uptake which compromises the SNR-signal to noise ratio[125])
- Monocytes/macrophages cellular imaging (antibodies against LOX-1, against peripheral benzodiazepine receptor, against lactadherin[125])
- VCAM-1 peptide binder
- P-selectin on endothelial cells

- MMP's inhibitors
- Annexin A5 (apoptosis) binding to phosphatidylserine –normally localized at the inner membrane leaflet, externalized during apoptosis[125]

However, nuclear imaging of coronary plaques remains challenging, because of the small size of the arteries[81].

7.2 Nanotechnology

The vulnerable plaque detection remains one of the most challenging step in acute vascular events prevention. Nanotechnology provided several nanoparticle platforms that can be applied as contrast agents for molecular imaging of biomarkers possibly related to instability.These biomarkers are cell receptors, protease activity such as MMP-2,8,9 and cathepsins or oxLDL and CRP. A library of synthetic micelles and lipoprotein mimics have been constructed for such biomarker imaging. Contrast agents may be incorporated in nanoparticles either at their surface, or into their core, depending on the processing method. Gadolinium, the PET tracer Cu and other agents are used as contrast. Nanoparticles present advantages, such as circulation half life, site selectivity and modular design. Because of their large size of 10nm they cannot be excreted by the kidneys. They are cleared by reticuleondothelial system. Nanotechnology gains increasing role in vulnerable plaque treatment, with amelioration of drug eluting stents, limiting in stent restenosis and late stent thrombosis. In this frame biodegradable and bioabsorbable stents not triggering inflammatory responses are designed. A further progress has been made in mechanical and geometrical coating of stent surface to promote endothelialization by using extracellular matrix mimicking nanofibrous matrix[73].

CHAPTER 8: TREATMENT

The pharmacological treatment with lipid lowering, antihypertensive and antiplatelet drugs have achieved impressive reduce in cardiovascular and stroke morbidity and mortality. Current controversies refer only to how aggressive this therapy will be for a specific patient and how prevention with dietary interventions and encouragement to healthy lifestyle will incorporate to younger people education[5].

8.1 Dietary

Dietary lipid restriction is an established policy for treating hyperlipidemia and the notorious consequences in atheromatous plaques. Besides the halting in plaque progression, dietary lipid restriction has been proved to reduce plaque's instability by lowering tissue factor-mediated thrombogenicity and plaque's angiogenesis[118].

There is increasing evidence that fish oil and long chain n-3 polyunsatturated fatty acids (PFUA) consumption protects against cardiovascular disease and has favourable effect even when administered post infarction. It has been shown that after prolonged fish oil intake the PFUA get incorporated into advanced carotid plaques altering plaque composition and morphology towards more stable phenotype. This stability is associated with reduced number of foam cell, inflammatory markers and T-cell inside the plaque[32].

Chinese medicine provides a variety of herbs or their mixtures, potentially stabilizing atherosclerotic plaques. These herbs have multiple effects on lipid and collagen metabolism, along with antiinflammatory effects [132].

The nfkb inhibition by some natural dietary products (antioxidants, flavonoids and PUFA's) and by commonly prescribed drugs (aspirin), is a promising strategy in the clinical setting[80].

8.2 Antilipidemic

Several trials have reported a reduction of 20% in vascular events per mM of LDL reduction after statin treatment. The beneficial effect of statins is due to their pleiotropic action. According large volume of studies, statins result to[99]:

- Increase of collagen and decrease of inflammatory cells and of tissue factor inside the plaque
- Increase the fibrous cap thickness and decrease the lipid core
- Decrease hs-CRP levels
- Reduce vascular events even in the presecne of normal cholesterol levels[99].

Antilipidemic drugs have successfully targeted in 5 different points:

- Risk of clinical vascular events
- Plaque volume
- Plaque composition in cellular level (may occure after 4 months of treatment[88]).
- Plaque composition in molecular/chemical level[88].

It has been proved that the favourable effects of statin treatment may persist even 10 years after the treatment has ceased[88].

Intensive lowering cholesterol levels has been superior compared to moderate statin therapy in halting atherosclerosis progress, as this has been measured with ultrasonography or IVUS methods. This effect is stil valid after eliminating the concominant lowering in hsCRP. The clinical benefit though is less impressive, as no significant decrease in vascular events has been noticed[94].

Agressive plaque stabilization with high doses of statins may on the other hand increase in a small but significant degree the incidence of hemorrhagic stroke. This may be caused by lipid core's cholesterol liquefication and concominent leakage from unplugged holes of arterial wall. It is noteworthy that cerebral arteries have only one layer of elastic lamina, favoring fragility[30].

Lowering the plaque cholesterol and therefore preventing cholesterol crystal formation may be achieved with drugs increasing HDL levels or promoting HDL function by increasing ABC transporters.Cholesterol ester transfering protein (CETP) can significantly elevate HDL levels, and in the same time, lower LDL levels. Although the results of CETP inhibitors on the lipidemic profil are beneficial, their side effects were detrimental and led to relative studies (ILLUMINATE,dal- OUTCOMES) termination. More cardiovascular events were noted, because of a rise in blood pressure[113].

Animal studies have shown that high concentrations of ApoA-1, the main structural protein of HDL, can inhibit the progression of atherosclerosis in apoE knockout mice or after arterial transplantation. Furthermore, liver directed gene transfer of apoA-1 caused plaque regression in LDL-receptor deficient mice. In a recent study intravenous administration of human apoA-1 in apoE knockout mice with advanced brachiocephalic atheromatous plaque reduced plaque disruption and intraplaque hemorrhage.The plaque stabilization was due to composition alteration, with no change in size or lipid content. A rise in smc differentiation and a reduction of MMP-13 favoured plaque stabilization [3].

In humans it has also been confirmed that statin therapy has a stabilizing effect on atheromatous plaque as this is measured by IVUS, angiography and inflammation markers such as MMP-9 and hsCRP. This favourable effect is still valid even without lowering of blood LDL levels, as statins have a pleiotropic-antiinflammatory effect on endothelial cells, smooth muscle cells, leukocytes and platelets[4,99].This was the result of the ESTABLISH Japanese study. Statins may postpone or even cancel the need for stenting of borderline vulnerable lesions[4].The same conclusions had the Jupiter trial, where biomarkers of reduced plaque vulnerability were hsCRP and LDL levels[11].

8.3 Antivascularization

Inhibiting plaque neovascularization promotes stability of the plaque[85].

8.4 Antioxidants

Oxidative stress has been implicated in atheromatous plaque progression and conversion to instability. Unfortunately oxidative stress locally or systematically is not possible yet to be reliably measured and validated. Trials of antioxidants such as vitC,E, carotene on prevention of coronary disease have been disappointing. The favorable effects of Mediteranean diet in lowering cardiovascular risk have been attributed to antioxidant consumption from early age. In Japan, although levels of LDL are higher post-World War II, while blood pressure and smoking approach the western lifestyle, mortality from cardiovascular disease remains lower . This is possibly due to protective effects of Japanese diet which involves increased soy and fish oil consumption[5].

Antioxidants have a antinfkb effect, which is a tempting therapeutic target. For the same purpose proteasome and IkB inhibitors have been studied, but high toxicity was a side effect. ACE inhibitors, b-blockers and statins prove to have favorable effect on nfkb suppression[76].

8.5 Antiplatelet Therapy

Aspirin has been shown to be effective for secondary prevention, while positive evidence exists for the rest of antiplatelet drugs (clopidogrel, prasugrel and ticagrelor). A dual effect is achieved, first in reducing local thrombus formation and second via antiinflammatory action[99].

8.6 Antihypertensive Therapy

B-blockers reduce vascular events, especially myocardial infarction and sudden cardiac death. Their beneficial effect is attributed to ameliorating hemodynamic forces restricting turbulence and lowering arterial wall stress.

Angiotensin II blockers, apart from regulating arterial hypertension have also antiinflammatory action (angiotensin II is a proinflammatory cytokine) and improve endothelial function99.

8.7 Other Antiatherosclerotic Therapies under Investigation

- ✓ Niacin (increases HDL, antiinflammatory effect)
- ✓ Artificial HDL-like apoA1 complexes
- ✓ Omega-3 supplementation
- ✓ PPAR agonists
- ✓ Antidiabetic thiazolidindiones (rosiglitazone – reduced plaque CD4-positive lymphocyte content, increase in collagen-I content)
- ✓ Selective inhibition of Lp-PLA_2 (lipoprotein-associated phospholipase A_2)-Decrease in lysophosphatidylcholine content, necrotic area , inflammation[99]

- ✓ Selective MMP's (MMP-9) inhibition, selective cathepsin S inhibition
- ✓ Melagatran (direct thrombin inhibitor) – reduces plaque size, promotes fibrous cap stability
- ✓ Maraviroc –used in HIV-1 patients, chemokine receptor CCR5 (proatherogenic) inhibitor [99]

8.8 Vaccines

A vaccine against CETP (cholesteryl ester transfer protein) has been developed, aiming to increase HDL levels and, as a consequence, to reduce cardiovascular disease. The vaccinehas been tested in phase I and phase II studies and prove to reduce CETP activity by 15% and increase plasma HDL concentration by 12%. Whether these changes prove to be clinical significant is under investigation[109].

8.9 Gene Therapy

The use of mir143/145 for plaque stabilization or for instent restenosis inhibition has been attempted via direct dehoxyribonoucleotide injection or via viral transfer.(mirs may encapsulated in lipid vesicles to facilitate fusion with cytoplasmic, endosomal or nuclear membrane[62]). Chemical stabilization is required before injection and intraarterial delivery is more efficient than intravenous one, as the latter results in preferably renal, liver and spleen distribution. More integrins are expressed and focal adhesions become strengthened, leading to fibrous cap integrity[61].There are potential complications by mir manipulation, as mir's have been implicated in cancer. For example, upregulation of mir221 to decrease plaque angiogenesis may trigger papillary thyroid carcinoma, and the same is valid for mir155 as to nasopharyngeal carcinoma. On the other hand a number of other mirs are found to be reduced in some types of cancer, so their upregulation at least is not oncogenic[62]. Mir's are implicated in multiple steps of atheromatic lesion progression, so they can be therapeutic target in many levels: in endothelial dysfunction and in monocyte maturation (mir146), in cholesterol biosynthesis (mir33), in plaque angiogenesis (mir 200), in fibrous cap stabilization (mir 143/145),in SMC proliferation (mir 221/222)[62]. Mir145 lentivirous has been administered in apoE knockout mice where it shifted VSMC towards more stable atheromatous phenotype[123]. Considering the role of monocytes in inflammation and immunity caution should be taken with mir manipulation not to impair functions such as protection against opportunistic infections and tumor invasion [62].

Inhibition of chemokines such as MCP-1 has also been a therapeutic target[35.]

CHAPTER 9: DISCUSSION

Atherosclerosis is a systemic degenerative disease of the arterial tree, leading to chronic ischemia of the target organs, or to acute vascular events such as myocardial infarct, stroke and death.

Traditional risk factors i.e smoking, age, sex, diabetes, hypertension, hyperlipidemia, obesity, have been expeditiously studied, but their value for vascular risk prevention proved to be inadequate. Inflammatory markers, oxidative status, systemic and local biomolecules offer additional information for the understanding of the disease, but only limited benefit for the handling of the atherosclerotic patient. Similar was the fate of the results of the studies about the mechanical forces on the endothelium and the vascular wall. Genetic studies rather clouded the field than they cleared it and the state of the art in molecular imaging leads only to assumptions and not to definite conclusions about atheromatous plaque vulnerability.

The multifactorial nature of the disease's generation, evolution and tendency for complications described with the term «vulnerability», inhibits the production of reliable occlusions. No matter how large the volume of research was on the field, the individualized vascular risk remains undefinable. The need for computational models, combining epidemiological, biological and laboratory data is obvious for the individual vascular risk estimation and the follow up of the administered treatment.

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