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The human carotid atherosclerotic plaque: an observational review of histological scoring systems

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Abstract. – OBJECTIVE: The atherosclerotic plaque is a complex dynamic pathological lesion of the arterial wall, characterized by multiple elementary lesions of different diagnostic and prognostic significance. Fibrous cap thickness, lipid necrotic core dimension, inflammation, intra-plaque hemorrhage (IPH), plaque neovascularization and endothelial dysfunction (erosions) are generally considered the most relevant morphological details of plaque morphology. In this review, the most relevant features able to discriminate between stable and vulnerable plaques at histological level are discussed.

SUBJECTS AND METHODS: Retrospectively, we have evaluated the laboratory results from one hundred old histological samples from patients treated with carotid endarterectomy. These results were analyzed to assess elementary lesions that characterize stable and unstable plaques.

RESULTS: A thin fibrous cap (<65 micron), loss of smooth muscle cells, collagen depletion, a large lipid-rich necrotic core, infiltrating macrophages, IPH and intra-plaque vascularization are identified as the most important risk factors associated with plaque rupture.

CONCLUSIONS: Immunohistochemistry for smooth muscle actin (smooth muscle cell marker) and for CD68 (marker of monocytes/macrophages) and glycophorin (marker of red blood cells) are suggested as useful tools for an in deep characterization of any carotid plaque and for distinguishing plaque phenotypes at histology. Since patients with a carotid vulnerable plaque are at higher risk of developing vulnerable plaques in other arteries as well, the definition of the vulnerability index is underlined, in order to stratify patients at higher risk for undergoing cardiovascular events.

Key Words:
Atherosclerosis, Vulnerable plaque, Unstable plaque, Stable plaque, Histological samples, Retrospective observational study.

Introduction

The atherosclerotic plaque is a complex dynamic pathological lesion insurging in the arterial wall, characterized by a heterogeneous morphology, including multiple elementary lesions of different diagnostic and prognostic significance. The early roots of the modern histological characterization and classification of atheroscle-

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rotic plaques may be found in a pivotal article by Virmani et al[1] published more than 20 years ago. In that paper, the authors performed an accurate histological analysis of coronary arteries in a large cohort of 241 cases of sudden cardiac death (SCD)[1]. The study focused on the identification of the peculiar type of plaque responsible for SCD. This study firstly evidenced that only one third of SCDs were associated with plaque rupture. As a consequence, coronary thrombosis was shown to may insurge in the absence of plaque rupture. In the same study, in 19% of SCD cases, histology evidenced the presence of endothelial dysfunction and erosion of the coronary plaque, with abundant arborized smooth muscle cells (SMCs) embedded in a proteoglycan-rich matrix inside the plaque. According with these findings, Virmani et al[1] suggested a new histological classification of atherosclerotic plaques, in which the following entities were identified (Figure 1):

1. Pathological intimal thickening: the plaque is mainly formed by SMCs embedded in a proteoglycan-rich matrix, with extracellular lipids, in the absence of a necrotic core. Erosion may be superimposed, causing luminal thrombosis.
2. Fibrous Cap atheroma: it is characterized by a lipid-necrotic core, with an overlying fibrous cap. Erosion may be superimposed, with luminal thrombosis.
3. Thin fibrous cap atheroma: thin fibrous cap (< 65 micron) infiltrated by macrophages and lymphocytes, with few SMCs, and scattered hemosiderin granules. A large necrotic core is located under the fibrous cap. The thin fibrous cap may undergo cap disruption and rupture, followed by extrusion of the lipid-necrotic core, ending with luminal occlusive thrombosis.
4. Calcified nodule: eruptive nodular calcification, emerging from a fibro-calcific plaque. It may be associated with erosion and non-occlusive thrombosis[2].
5. Fibro-calcific plaque: collagen-rich plaque, with large calcifications, few inflammatory cells, leading to significant stenosis. Thrombosis is absent.

Since there was no clear evidence of a specific sequence of events characterizing the evolution of any atherosclerotic plaque, Virmani et al[1] suggested the use of descriptive terms for plaque classification. Two main elementary lesions should be analyzed with particular attention:

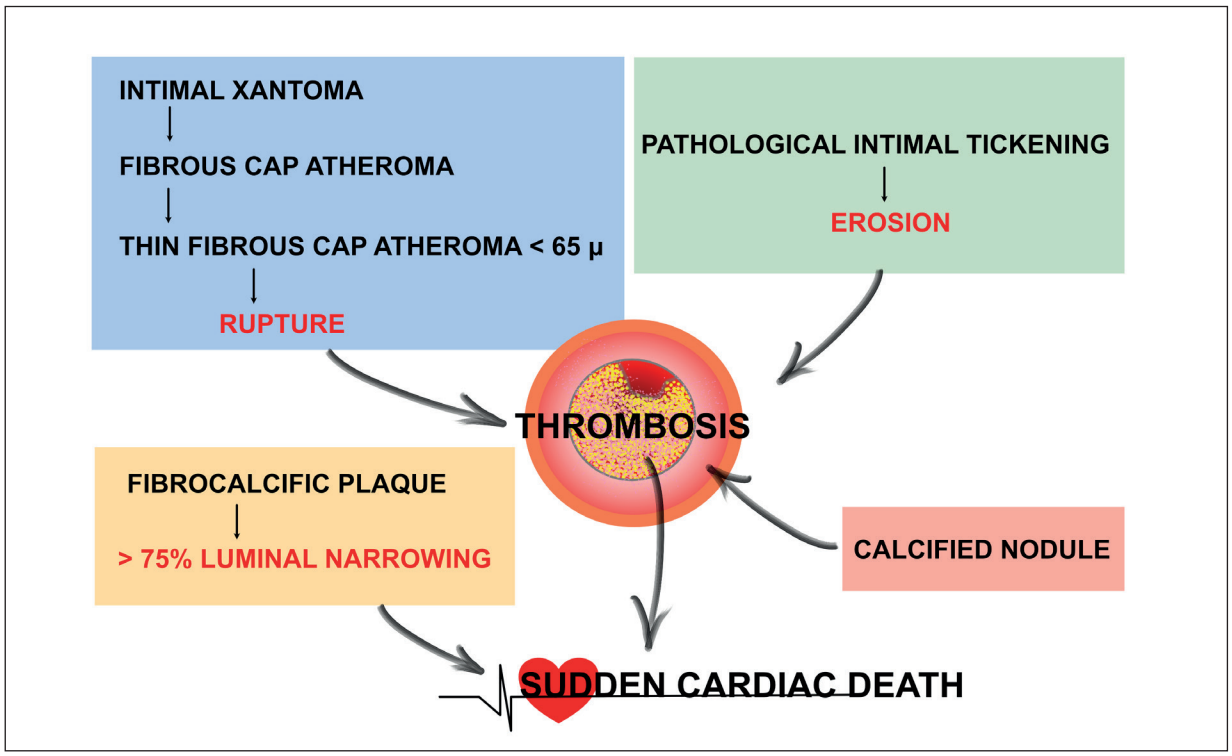

Figure 1. A simplified scheme for classification of atherosclerotic lesions.

A. The fibrous cap, a distinct layer of newly formed connective tissue, completely covering the lipid-necrotic core, consisting purely of SMCs embedded in a collagenous proteoglycan-rich matrix. The fibrous cap thickness (< or > 65 micron) and varying degrees of infiltrating inflammatory cells (monocytes, lymphocytes) allowed the distinction between a thin (< 65 micron, diffusely infiltrated by inflammatory cells) and a thick (> 65 micron, without inflammatory cells) fibrous cap. The former should be considered prone to rupture, whereas the latter should characterize a stable plaque with no tendency to rupture.
B. The lipid-rich necrotic core, a mass of large amounts of extracellular lipids, cholesterol crystals and necrotic debris, surrounded by monocytes/macrophages, including foamy cells. Other elementary lesions that may be associated are: intra-plaque hemorrhage (IPH), calcifications (large or thin), inflammation (lymphocytes, monocytes, plasma-cells, mast cells), neo-angiogenesis, also defined as intraplaque *vasa vasorum*.

In this review, the most relevant features able to distinguish between stable and vulnerable plaques at histological level are discussed.

Subjects and Methods

Data Analyzed

Retrospectively, we have evaluated the routine laboratory results of one hundred old histological samples from patients treated with carotid endarterectomy. The laboratory test results and respective clinical data were available at the Division of Pathology, of the Hospital University of Cagliari. These data were derived from laboratory routine work achieved from January 2016 to December 2018. The patient age ranged from 22 to 70 years hold.

Exclusion Criteria

We have not considered clinical data end respective laboratory results from patients with: had previous revascularization (CABG, PTCA, stenting). Patients being evaluated for other cardiac diseases (e.g., valvular disease, etiology of cardiomyopathy). Patients with unstable angina who have elevated serum cardiac biomarkers, ECG changes, etc.; those with NSTE-ACS, NSTEMI, STEMI, or definite acute coronary syndrome.

Immunohistochemistry

The immunohistochemistry results evaluated in this work were previously performed with the same laboratory procedures, in particular:

Smooth muscle actin (smooth muscle cell marker), We have used dishes with collagen-coated coverslips that have been labeled with a primary antibody, followed by a secondary antibody that has been appropriately conjugated to Alexa-488 and Alexa-350 (Invitrogen). Cells were labeled with rhodamine-phalloidin to make F-actin visible. A high-resolution digital fluorescent microscope (Leica DMI600) was used to observe the cellular localization of labeled proteins. To uniformly assess fluorescence intensity across trials, the periods of image capture, intensity gaining, and image black levels were optimized and maintained for all experiments.

CD68 (marker of monocytes/macrophages): was used the IHCeasy CD68 Ready-To-Use IHC Kit, Proteintech Group, Inc Rosemont, IL 60018, USA in according with manufacture instruction.

Glycophorin (marker of red blood cells): atherosclerotic tissue was analyzed by using Glycophorin A [JC159] kit, Biocare medical Biocare Medical 60 Berry Dr Pacheco, CA USA, in according with manufacture instructions.

Statistical Analysis

The differences between the tested formulations were compared by means of the Chi-squared test by using an online calculator, (social statistics, available at: https://www.socscistatistics.com/), with p-value set at 0.05.

Results

Each elementary lesion could be simplified in its impact, but it is reasonable to hypothesize that it is not the mere presence or absence of a specific feature to play a role but its volume and the interaction and balance with all the others. This is well explained by the calcifications that in some cases are considered as protective features whereas in other cases have been associated to a significant plaque vulnerability[3,4].

After defining the spectrum of the lesions detectable inside an atherosclerotic plaque, Virmani et al[1] described the factors responsible for the insurgence of thrombotic events in the atherosclerotic setting (Figure 2). Among these, plaque

rupture and erosion were indicated as the most relevant complications of the unstable plaque, able to trigger the activation of the hemostasis system and the thrombotic cascade, ending with occlusive thrombosis. Of some relevance in this debate is the article of Naghavi et al[5], in which the peculiarities of vulnerable plaques were well defined. In this article, starting from the definition of "vulnerable plaque", the authors well defined the vulnerable patients, underlying the necessity of a stratification of subjects with atherosclerotic lesions for the development of new risk assessment strategies. Recent studies[6] have revealed that 1 out of 20 subjects presenting with an acute coronary syndrome undergoes a recurrent ischemic event within one year.

In a meeting on the vulnerable plaque in Santorini, Greece, in 2013, it was recognized that increased understanding of pathophysiology of atherosclerotic lesions had created the need for agreement on new nomenclature of the multiple elementary lesions of the plaque[7]. In this meeting, the concept of the "vulnerable patient" was better defined, including subjects with high atherosclerotic burden, high-risk vulnerable plaques and thrombogenic blood.

In more recent years, other authors better defined the main features associated with plaque vulnerability, i.e., the histological risk factors responsible for the instability of the plaque and for its rupture[8]:

1. Thin fibrous cap < 65 micron;
2. Loss by apoptosis of smooth muscle cells;
3. Collagen loss;
4. Infiltrating macrophages;
5. Intra-plaque hemorrhage (IPH);
6. Neo angiogenesis;
7. Intra-plaque inflammation;
8. Large lipid-rich necrotic core;
9. Endothelial dysfunction with erosion;
10. Irregular luminal morphology with ulceration (> 1 mm).

In the same article, the heterogeneity of atherosclerotic plaque morphology and the high

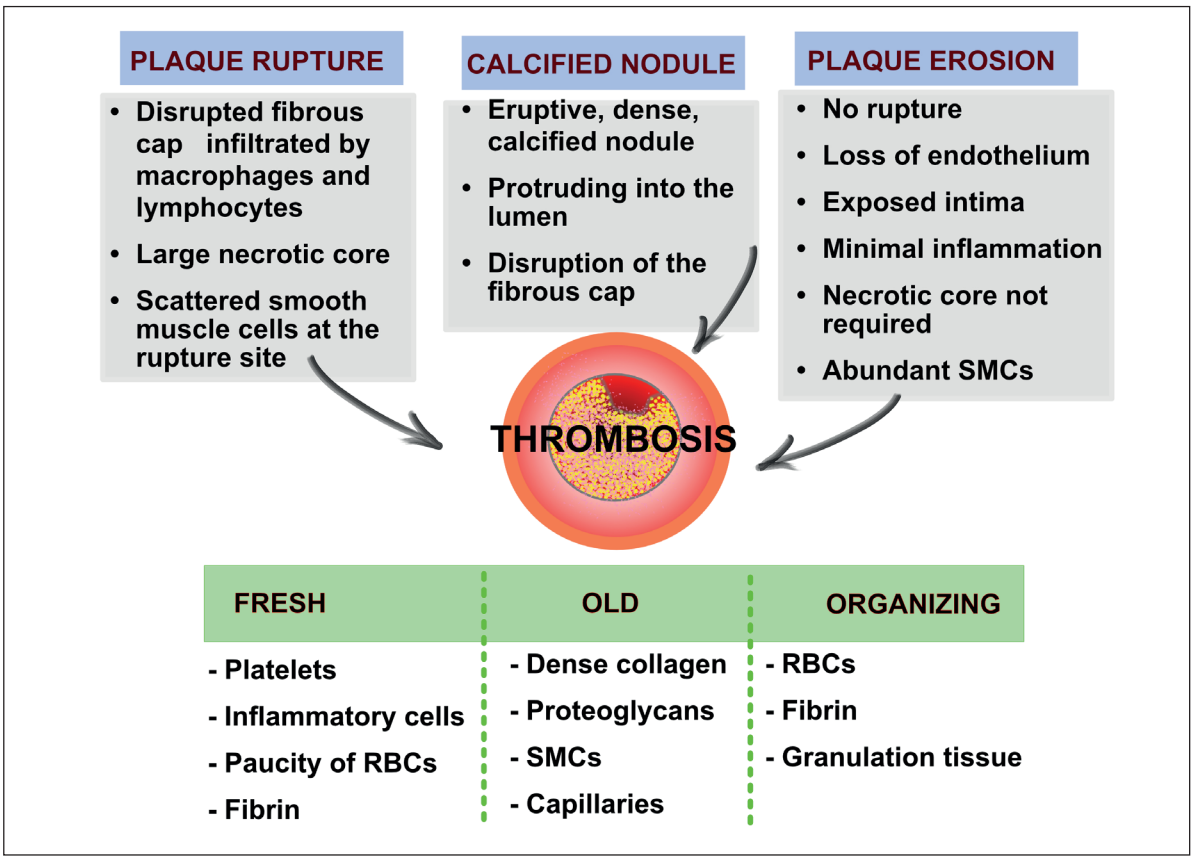

Figure 2. Pathogenesis of thrombosis in sudden cardiac death.

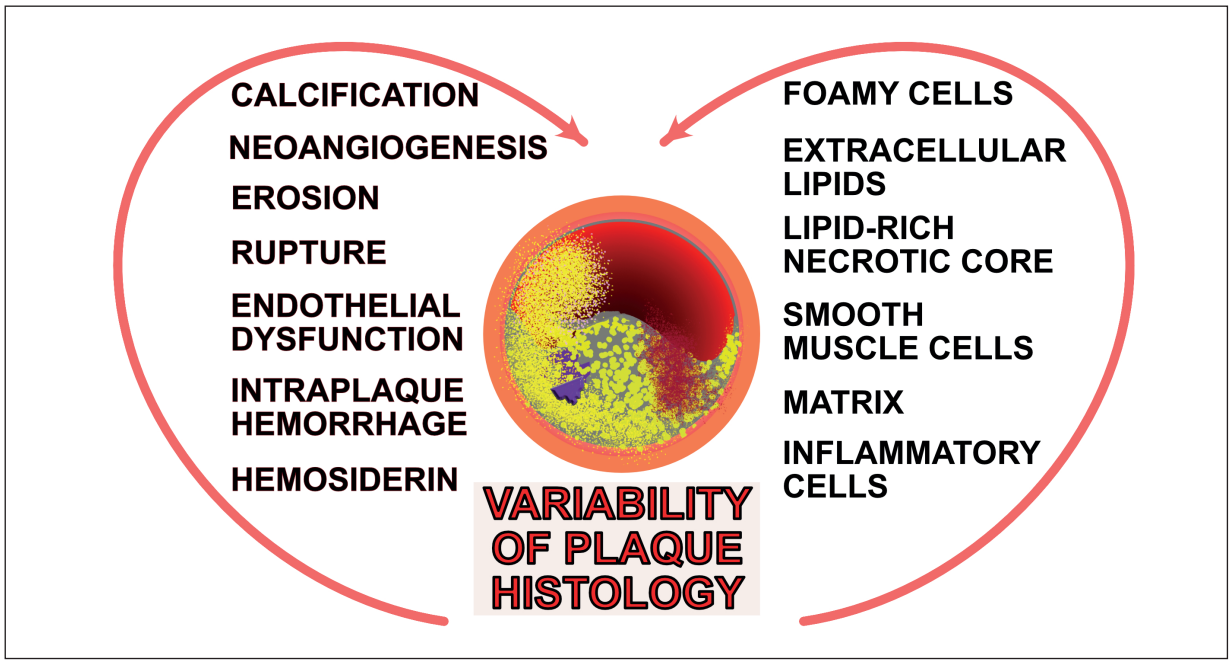

Figure 3. Heterogeneity of plaque morphology.

number of elementary lesions that may be found inside a plaque were underlined. The variable association of the multiple elementary lesions typical of the spectrum of the atherosclerotic process gives rise to a marked histological variability among plaques even in the same patient (Figure 3)[9].

On the basis of the histological changes previously reported by Virmani et al[1], the following sequential histological changes may be hypothesized to occur in the evolution of the atherosclerotic plaque (Figure 4):

1. Adaptative intimal tickening;
2. Intimal xantoma;
3. Pathological intimal tickening;
4. Fibroatheroma;
5. Fibrocalcific plaque;
6. Thin cap fibroatheroma;
7. Ruptured plaque;
8. Thrombosis-complicated plaque;
9. Healed plaque.

A new variant was introduced, in 2018, in plaque histology and evolution by Poredos et al[10]: the different vascular territories in which the atherosclerotic plaque may develop. According to the authors, atherosclerosis developing in peripheral arteries, including the superficial femoral artery, are characterized by a different clinical course mainly due to a different histological structure of the plaques. In brief, peripheral arterial disease might be characterized by fibro-proliferative plaques with more fibrotic elements, higher number of smooth muscle cells, lower density of intra-plaque vasa vasorum, lower amounts of extracellular lipids, less inflammatory cells, smaller and less frequent lipid-rich necrotic cores. These architectural specificities of peripheral plaques might explain why femoral atherosclerotic plaques are more stable and less prone to rupture, when compared to coronary

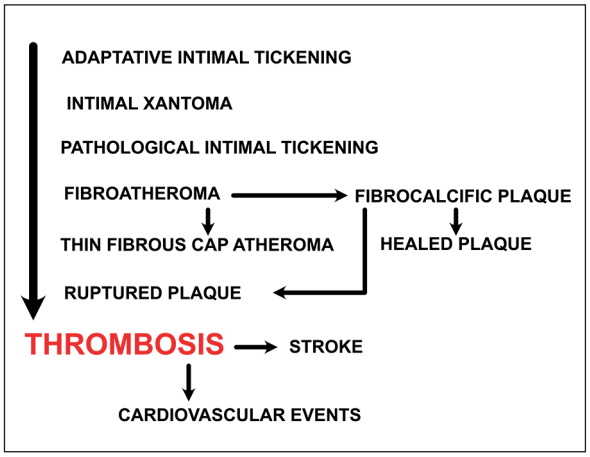

Figure 4. Putative sequential histological changes of the plaque.

and carotid plaques. Moreover, according with Poredos et al[10] hypothesis on the relevance of arterial localization on the clinical outcome of atherosclerotic lesions, superficial peripheral arteries should be characterized by a more efficient ability in remodeling of the arterial wall. This ability might allow a better compensation for the reduction of the arterial lumen caused by the plaque, providing the persistence of a sufficient blood flow even with large plaques.

A study recently published by Munger et al[11], on the application of machine learning to a better understanding of atherosclerotic lesions, introduced the Artificial Intelligence (AI)-driven molecular characterization of the plaque in the field of atherosclerosis, similarly to models of analysis already applied in the imaging field of atherosclerosis[12]. According to this study, the vulnerable plaques might be differentiated from the stable ones by the association of histological and molecular markers, allowing a molecular characterization of the atherosclerotic lesions (Figure 5). At molecular level, vulnerable plaques might be identified by high levels of cycloxigenase-2, PGE-type prostaglandins, matrix-metalloproteinases, VCAM-1, catepsins and multiple inflammatory cytokines produced by activated monocytes. An increased expression of the NF-KB signal transduction pathway, a reduced expression of genes induced by the Kruppel-like Factor (KLF) 2/4, a reduced expression of nitric oxide synthase, and the increased production of interferon-alpha by vascular smooth muscle cells might better define the molecular signature of the unstable plaque. This molecular signature, according to Munger et al[11], might allow a better characterization of the plaque and a better evaluation of the atherosclerotic process developing in other vascular districts of the same patient, allowing a stratification of affected patients in different classes of risk for future adverse events. Moreover, a better knowledge of the molecular pathways involved in plaque insurgence and development might allow a better comprehension of the factors that promote weakening of the fibrous cap, development of a large lipid-rich necrotic core and neovascularization with intra-plaque hemorrhage, the pathological events responsible for plaque rupture and thrombosis.

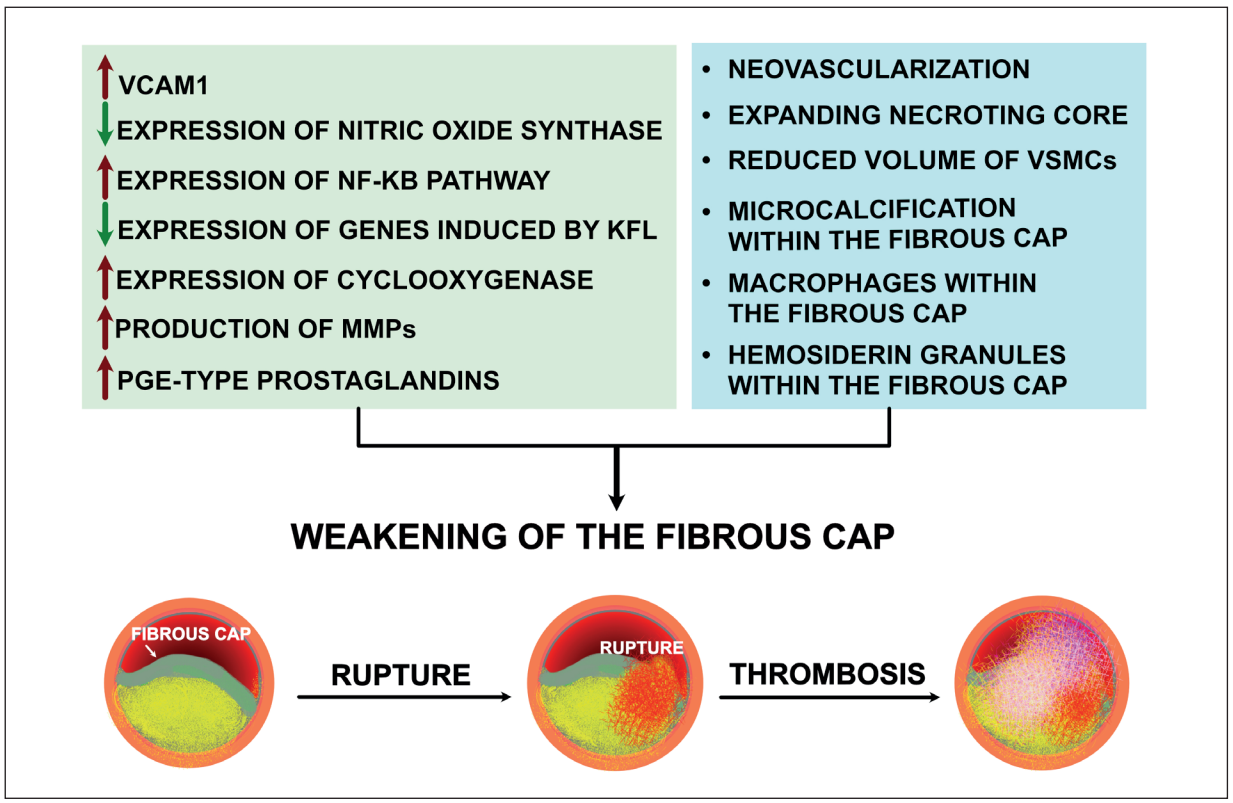

Figure 5. Morphologic/molecular characterization of vulnerable plaques: factors favoring weakening of the fibrous cap.

In a recent review entitled "the changing landscape of atherosclerosis", Libby[13] focused on the epidemiological transition of the disease, showing that the mechanisms that underpin thrombotic complications of the plaque have evolved beyond the old concept of the vulnerable plaque. According to Libby, we might add that the landscape is changing even regarding the histological classification of the atherosclerotic plaque. A more recent article by Moerman et al[14] mainly focused on the plaque composition in advanced human carotid atherosclerosis, has confirmed the following findings as typical of the vulnerable plaque: i) thin fibrous cap; ii) large lipid-rich necrotic core; iii) high inflammatory activity; iv) decreased smooth muscle cell content; v) the presence of elongated patches of macrophages in the cap, along the lumen and/or deeper in the intima. A very recent intriguing paper, by Goncalves et al[15], might add value to the histological characterization of the removed carotid plaques, and stratify patients into two main groups: at high and at low risk of developing future cardiovascular events due to plaque rupture. The authors analyzed the plaque histology of a large cohort of patients who underwent carotid endarterectomy, with the aim of identifying the histological and immunohistochemical factors that might predict an increased risk of future cardiovascular events. They proposed a vulnerability index, calculated as a ratio between the sum of %CD68 (macrophages), Oil Red O (lipids), glycophorin A (red blood cells) and the sum of % alpha-smooth muscle actin (vascular smooth muscle cells) and % trichrome stain (collagen). In this study, the risk of future cardiovascular events was two times higher among patients with a high vulnerability index (in the fourth quartile) than those with a lower vulnerability index[15].

According to these preliminary data, the proposed plaque vulnerability index appears to be a very interesting proposal, being able to predict adverse future events due to the specific type of atherosclerotic process undergoing in a specific patient. Whereas the index proposed by Goncalves et al[15] appears to be very useful for plaque analysis in clinical trials and in scientific studies, some limitations to the introduction of this index in routine clinical practice are related to its complexity and to the time necessary for the evaluation of the percentage of the stained plaque area for each used stain and immunostaining.

Discussion

One hundred slide examinations show elementary lesions in according with previous studies[14,15], such as: fibrous cap, apoptosis of smooth muscle cells, infiltrating macrophages, intra-plaque hemorrhage (IPH), neoangiogenesis, intra-plaque inflammation, lipid-rich necrotic core, endothelial dysfunction with erosion.

The landscape of the atherosclerotic plaque is changing for all physicians in understanding, diagnosing and treating this complex disease. It is the same also for pathologists, whose aim is to give clinicians and radiologists the highest number of useful information in order to get a deeper comprehension of the type of atherosclerotic process that characterizes each patient, quickly moving from a general approach towards a more tailored one.

Pathologists dedicated to the interpretation of the plaque are facing multiple challenges. First, the need of a better definition of all the histological elementary lesions occurring in a plaque. In this field, a better characterization of vascular smooth muscle cells appears mandatory. Recent data[16] evidence a previous unknown ability of vascular SMCs to dedifferentiate into multiple directions, giving rise to different cell types, including adipocytes, fibroblasts, osteoblasts and undifferentiated mesenchymal cells. Given the ability of SMCs to differentiate toward multiple morphologies, the use of antibodies against alpha-SMA should be encouraged in the evaluation of any plaque[17,18]. The second challenge regards the understanding of a more appropriate utilization of immunohistochemistry, focused on the identification of the multiple cell types involved in plaque development, progression and healing. The application of molecular characterization of the plaque represents another challenging field, with attention to the molecular pathways involved in the rupture of the thin fibrous cap and to the endothelial dysfunction responsible for the erosion. Finally, the request of a simple and fast histological plaque vulnerability or instability index, able to predict future adverse events in the atherosclerotic plaques localized in other vascular districts of the same patient, especially if they associated with other diseases[19,20], is emerging in clinical practice.

Conclusions

Finally, on the basis of our experience in this field[17,18], a better dialogue between radiologists and pathologists might allow a better charac-

terization of the plaques, improving the clinical-pathological classification of all plaques, and helping to stratify patients with slow or high risk for future adverse events by offering a new tool that could help to better understand and treat the unique atherosclerotic fingerprint that characterizes each subject by offering new target therapies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethical Statement

In this retrospective study, no individual-level personal data were collected, as only aggregate and only anonymous data are reported. All clinical reports reported a patient's informed consent authorization to use the data for a statistical or observational study. In any case, the Helsinki guidelines have complied.

Authors' Contribution

GC, CG, FD, SL designed the research work, wrote the text and first lecture of the slides; SJ, BA, SA, OG, VEP5, SA, OG, LNG, CF, CM, GY conduct a reviewer of the text; AV, CF, CP, PM, CR a second lecture of the slides and conduct the histological practical part.

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