Atherosclerosis is a complex disease, the onset of which depends on various components of the vascular system, metabolism and immune system. It generates the fibro-fatty plaque or stable plaque characterized by accumulation of lipids in the intima of the arteries, a fibrous cap covering the atheroma core which may consists in lipid-laden cells (macrophages and vascular smooth muscle cells), proteoglycans, collagen, elastin, foam cells and, sometimes, cholesterol crystals needle-like clefts, fibrin and neovessels. The progression of the plaque leads to unstable atherosclerotic lesions. The result will be a large plaque, consisting of an evident lipid core surrounded by a fibrous cap, infiltrates of immunocompetent cells and calcium deposits. Advanced plaque is characterized by macrophages invasion and by the thinning of the fibrous cap. Progression of atherosclerotic plaque can lead to its rupture and result in the occlusion of an artery or in the formation of a thrombus. Apoptosis in the fibrous cap, rich in vascular smooth muscle cells and macrophages, and its subsequent weakening seems to be an important regulator of plaque stability. In our study, we collected specimens from stable atherosclerotic plaques in the right or left internal carotid artery of patients with clinical symptoms. Histology and histochemistry were performed in specimens for cell identification and detection of structural alterations. Immunohistochemical analysis related to caspase-3 and N-cadherin was performed in order to highlight the pro-survival role of N-cadherin against apoptosis in the stable atherosclerotic plaques. Our results showed that when expression of N-cadherin is evident and strong in the fibrous cap of the atheroma, apoptosis is not detected, as reported by recent literature. The aim of our study was to acquire greater knowledge on the biological mechanisms related to plaque vulnerability in order to develop new therapies to maintain atherosclerotic plaque stability avoiding its rupture which could determine consequences such as thrombosis.

**Keywords:** Atherosclerosis, apoptosis, caspase-3, human atherosclerotic plaques, immunohistochemistry, n-cadherin, hematoxylin and eosin, masson's trichrome

### Introduction

Atherosclerosis is a complex disease, the onset of which depends on various components of the vascular system, metabolism and immune system. It is not a simple passive accumulation of lipids within the vascular wall but a complex process that is briefly described. The endothelium is a metabolically active and fundamental tissue of the vascular wall. It has a critical role in regulating the metabolism of lipoproteins and other molecules that may participate in the formation of atherosclerotic plaque. The onset and growth of atherosclerotic plaque is due to the initial adhesion, infiltration and deposition of lipoproteins in the intima of arteries, defined as “fatty streak”. Subsequently, oxidation of low density lipoprotein (LDL) takes place and this is the initiator metabolic event in the formation of the plaque, in fact it induces an inflammatory process resulting in endothelial damage [1]. The endothelial damage leads to the infiltration of leukocytes and, therefore, of macrophages that phagocyte oxidized LDL accumulating lipid in their cytoplasm, transforming themselves into foam cells, rich in cholesterol [2]. In subsequent phases, the accumulation of fibrous tissue leads to the growth of the real atheroma. If the inflammatory response is not able to neutralize or remove harmful agents, it can continue and stimulate the migration and proliferation of vascular smooth muscle cells (VSMCs) that migrate from the tunica media to the intima producing extracellular matrix that acts as a structural scaffold of the atherosclerotic plaque [1,2]. It generates what is termed fibro-fatty plaque or stable plaque which replaces the simple lipid accumulation of the initial stages. The fibro-fatty plaque is characterized by accumulation of lipids in the intima of the arteries, a fibrous cap covering the atheroma...
The specimens were collected from atherosclerotic plaques (average age of 64 years) with clinical symptoms. All patients were previously studied through the following instrumental examinations: coronary angiography, magnetic resonance angiography, computed tomography angiography, ecodoppler, echocardiography. All patients showed right or left internal carotid artery stenosis >60-70%. Patient characteristics and clinical information on smoking habit, diabetes, hypercholesterolemia, hypertension, etc., were summarized in Table 1. The specimens, excised by carotid endarterectomy (CEA), were obtained from the Complex Vascular Surgery and Transplant Centre, Policlinic of Catania and the “Fondazione Mediterranea G.B. Morgagni”, Catania. Twenty fragments of vascular tissue were obtained from the distal side of the specimens, considering them as control vascular tissue. Informed consent was obtained from each patient; the research was approved by the Local Medical Ethical Committee and conformed to the ethical guidelines of the Declaration of Helsinki. All specimens were submitted to histological, histochemistry and immunohistochemical analysis.

Table 1. Patients features and clinical information.

<table>
<thead>
<tr>
<th>Patients (n=58) features and clinical information</th>
<th>% patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>smoker</td>
<td>18</td>
</tr>
<tr>
<td>ex-smoker</td>
<td>36</td>
</tr>
<tr>
<td>diabetes</td>
<td>32</td>
</tr>
<tr>
<td>hypercholesterolemia</td>
<td>36</td>
</tr>
<tr>
<td>hypertension</td>
<td>73</td>
</tr>
<tr>
<td>antiplatelet therapy</td>
<td>27</td>
</tr>
<tr>
<td>statin therapy</td>
<td>31</td>
</tr>
</tbody>
</table>

Histology and histochemistry
The specimens were rinsed in phosphate-buffered saline (PBS), fixed in 10% buffered-formalin. After an overnight wash, specimens were dehydrated in graded ethanol, cleared in xylene and paraffin-embedded. Sections were obtained according to routine procedures, as previously described [24,25]. Transverse sections of 4-5 μm thick were cut from paraffin blocks using a rotary microtome (Leica RM2235; Leica Microsystems, Wetzlar, Germany), mounted on saline-coated slides and stored at room temperature. The sections were stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome for general cell identification and for the presence or absence of structural alterations. The sections were examined with a Zeiss Axioplan light microscope (Carl Zeiss; Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss; Oberkochen, Germany).

Immunohistochemistry (IHC)
For immunohistochemical analysis, specimens were processed as previously described [26]. Briefly, the slides were dewaxed in xylene, hydrated using graded ethanol and were incubated for 30 min in 0.3% H₂O₂/methanol to quench endogenous peroxidase activity and then rinsed for 20 min with phosphate-
buffered saline (PBS; Bio-Optica, Milan, Italy). The sections were heated (5 min x 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites. The blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA; Sigma, Milan, Italy) in PBS for 1 hour in a humid chamber. BSA was used as a blocking agent to prevent non-specific binding of the antibody. Following blocking, the sections were incubated overnight at 4 °C with rabbit monoclonal Anti-Caspase-3 antibody (ab32351; abcam, Cambridge, UK), diluted 1:100 in phosphate buffer saline (PBS; Sigma, Milan, Italy); mouse monoclonal Anti-N-cadherin antibody clone 13A9 (05-915; EMD Millipore Corporation, Billerica, MA, USA), diluted 1:400 in phosphate buffer saline (PBS; Sigma, Milan, Italy). Immune complexes were then treated with a biotinylated link antibody and then detected with peroxidase labeled streptavin, both incubated for 10 min at room temperature (LSAB+ System- HRP, K0690; Dako, Glostrup, Denmark). The immunoreaction was visualized by incubating the sections for 2 minutes in a 0.1% 3,3’-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark). The sections were lightly counterstained with Mayer’s hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in GVA (Zymed Laboratories, San Francisco, CA, USA) and observed with an Axiosplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

Xels of the analyzed fields. Statistical analysis showed that in stable atherosclerotic plaques, the mean of % stained areas by N-cadherin was much higher compared with the mean of % stained areas by caspase-3, and p-value was considered significant (p<0.05) (**Figures 4A, 4C and 4E**). No immunoreaction was observed in the negative controls treated with PBS without the primary antibodies (**Figures 4B and 4D**).

### Evaluation of immunohistochemistry

The antibodies-staining (caspase 3 and N-Cadherin) status were identified as either negative or positive. Immunohistochemical positive staining was defined as the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation by light microscopy as previously described [27]. Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at a protein level. Positive controls consisted of tissue specimens with known antigenic positivity. Sections treated with PBS without the primary antibodies served as negative controls. Fifteen fields, randomly selected from each section, were analyzed and the percentage area stained with antibodies (caspase 3 and N-Cadherin) were calculated using a software for image acquisition, morphometric and densitometric analysis (AxioVision Release 4.8.2-SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabelling in each field, expressed as % positive, dark brown pixels of the analyzed fields, as described previously [28]. Statistical significance of results was thus accomplished. Digital micrographs were taken using the Zeiss Axiosplan light microscope (Carl Zeiss, Oberkochen, Germany) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany); evaluations were made by three blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the case was reconsidered to reach a unanimous agreement.

### Statistical analysis

Statistical analysis was performed using SPSS software (SPSS® release 16.0, Chicago, IL, USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the unpaired t test with Welch correction. P-values of less than 0.05 were considered statistically significant. Data are presented as the mean±SD. Cohen’s kappa was applied to measure the agreement between the three observers and averaged to evaluate overall agreement as previously described [28].

### Results

**Histology and histochemistry**

Microscopic examination showed plaques classified as types III and IV [29]. In examined specimens, histologic analysis highlighted the typical morphology of type III (fibrous plaque) and type IV (early fibro-atheroma) lesions. Fibrous plaques were characterized by the presence of fibers and scattered smooth muscle fibroblasts and mononuclear inflammatory cells (**Figures 1A, 1B and 1C**), intra- and extra-cellular lipid deposits (**Figure 1A**), foam cells (**Figure 1C**), thick fibrous cap (**Figure 1C**), neovessels (**Figure 1B**). Fibro-atheromas were characterized by more evident atheroma with foam cells (**Figures 1D and 1E**), optical empty needle-like cholesterol crystals (**Figure 1D**) and a thick fibrous cap rich in vascular smooth muscle fibroblasts (**Figures 1D, 1E and 1F**); in some cases, scattered areas of calcification were highlighted (**Figure 1E**).

**Immunohistochemistry (IHC)**

Immunohistochemical staining was analyzed in stable atherosclerotic plaques. In particular caspase-3 immunolabelling was not observed in stable atherosclerotic plaques (**Figures 2A and 2B**). Caspase-3 immunolabelling was also absent in control vascular tissue (**Figure 2C**). The % of stained areas by caspase-3 expressed by % dark brown pixels of the analyzed fields, was considered. Obviously, from statistical analysis, caspase-3 immunostaining in atherosclerotic plaques vs. vascular tissue controls showed ap>0.05, considered not significant (**Figure 2D**). On the contrary, N-cadherin immunolabeling was detected at high levels, localized in the...
Figure 1. Sections of atherosclerotic plaques. H&E and Masson’s trichrome stainings. (A) Fibrous plaque with evident fibers and presence of scattered smooth muscle fibrocells and mononuclear inflammatory cells (red asterisk); intra- and extra-cellular lipid deposits (black arrows). (B) Fibrous plaque with evident fibers and presence of scattered smooth muscle fibrocells and mononuclear inflammatory cells (red asterisk); presence of neovessels (black arrows). (C) Fibrous plaque with evident fibers (red asterisk) and foam cells (insert); thick fibrous cap (yellow arrows) and thick muscle layers surrounding the plaque (red arrows). (D) Large atheroma (red asterisk) with optical empty needle-like cholesterol crystals (insert); thick fibrous cap (black arrows). (E) Evident atheroma with foam cells (red asterisk), thick fibrous cap (black arrows) and scattered areas of calcification (red arrows). (F) Thick fibrous cap rich in vascular smooth muscle fibrocells (black arrows). A, B, C, D, E, original magnification 2.5x; scale bars:100 µm. F and insert in C, original magnification 10x; scale bars:50 µm. Insert in D, original magnification 20x; scale bar:10 µm.

extracellular matrix of the stable plaques (Figure 3A) and in the fibrous cap, rich in VSMCs where N-cadherin immunostaining was particularly localized in the cytoplasm (Figure 3B). In the vascular tissue controls, diffuse N-cadherin immunostaining was also detected at similar levelas in the plaques, and it was particularly localized in both cytoplasm and nucleus of involved cells (Figure 3C). The % of stained areas by N-cadherin, expressed by % dark brown pixels of the analyzed fields, was considered. The mean of % stained areas by N-cadherin in atherosclerotic plaques was similar to vascular tissue controls and p-value considered not significant (p>0.05) (Figure 3D).
Figure 4. Caspase-3 and N-cadherin immunostaining in the stable atherosclerotic plaques.

(A) Fibrous plaque with none caspase-3 immunostaining, neither in the extracellular matrix nor in the cells present here. (B) Anti-caspase-3 antibody negative control. (C) N-cadherin immunostaining in the extracellular matrix and in VSMCs, particularly in the cytoplasm (insert), of the stable plaques. (D) anti-N-cadherin antibody negative control. (E) Graph. A bar chart representing a comparison of the % caspase-3 and N-cadherin positive area in stable atherosclerotic plaques (n. 58), expressed by % positive, dark brown pixels of the analyzed fields. Data are presented as mean±SD. *p<0.05. A and B, original magnification 10x; scale bars: 50 µm. C and D, original magnification 20x; scale bars: 10 µm. Insert in C, original magnification 40x; scale bar: 10 µm.

Finally, the % of stained areas by caspase-3 vs. N-cadherin in stable plaques was considered, expressed by % dark brown pi.

Discussion
From the literature, it is known that atherosclerotic plaque vulnerability is directly determined by thinning of the fibrous cap due, in turn, to apoptosis of cells present here such as VSMCs, mainly, and macrophages. Apoptosis is a complicated process involving a great number of molecules such as N-cadherin that seems to have a protective role in VSMCs, macrophages and foam-cells of stable plaque against apoptosis. As mentioned in the introduction, authors demonstrated that N-cadherin provides a pro-survival signal to VSMCs, macrophages and foam-cells, and that a soluble form of N-cadherin in, composed of the extra-cellular domain, acts as a mimetic reducing VSMCs apoptosis [14,22,23]. Other authors demonstrated that the activity of MMP-7 is up-regulated during apoptosis of VSMCs, and they observed that MMP-7 causes cleavage of N-cadherin which is associated with increased VSMCs apoptotic rates [7]. The present study was made only in stable atherosclerotic plaques that less frequently undergo rupture compared with unstable plaques. Our immunohistochemical study highlighted and confirmed data in literature related to the protective role of N-cadherin against apoptosis of VSMCs, macrophages and foam-cells in stable plaques. Our immunohistochemical results showed that high expression of N-cadherin was linked to minimal or absence of apoptosis, as expressed by none caspase-3 immunostaining in the extracellular matrix and in the fibrous cap of stable atherosclerotic plaques. In fact, we did not detect caspase-3 immunolabelling in stable atherosclerotic plaques and in vascular tissue controls, testifying that apoptosis was not ongoing. On the contrary, we detected N-cadherin immunolabelling at high levels both in the extracellular matrix and in the fibrous cap of the stable plaques, as well as in vascular tissue controls, testifying to the protective role of N-cadherin against apoptosis. Obviously, statistical analysis confirmed that. In reality, in our study we did not investigate in relation to the molecular mechanism of N-cadherin in its protective role and further investigation should be necessary to better understand. Nevertheless, we consider it important to confirm data from recent literature in order to develop new therapeutic strategies for maintaining stability of the atherosclerotic plaque, thus avoiding its rupture with the well-known inauspicious consequences.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

<table>
<thead>
<tr>
<th>Authors’ contributions</th>
<th>GM</th>
<th>RI</th>
<th>GM</th>
<th>RP</th>
<th>MAS</th>
<th>RS</th>
<th>SC</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research concept and design</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Collection and/or assembly of data</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Data analysis and interpretation</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Writing the article</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Critical revision of the article</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Final approval of article</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Acknowledgement
This study was supported by grants provided by the Department of Bio-Medical Sciences, School of Medicine, University of Catania, Catania, Italy. The authors would like to thank Prof. Iain Halliday for commenting and making corrections to the paper.

Publication history
Editor: Gjumrakch Aliev, GALLY International Biomedical Research & Consulting LLC, USA.
Received: 15-May-2014 Final Revised: 07-Jun-2014
Accepted: 13-Jun-2014 Published: 20-Jun-2014
References


Citation:
http://dx.doi.org/10.7243/2055-091X-1-4