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Immunohistochemical expression of anti-CD68 antibody in atherosclerotic plaque

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Abstract

Inflammation of the vascular wall is an essential event in the development of atherosclerosis, the main leukocytes of the inflammatory infiltrate being the monocyte/macrophages. These cells are very heterogeneous and rapidly change their function in response to signals received from the local environment. *Purpose*: The aim of this research was to study the immunohistochemical expression of the anti-CD68 antibody at the level of the atherosclerotic plaque. *Materials and Methods*: We used tissue samples obtained by endarterectomies in patients with symptomatic atherosclerotic lesions. Inflammatory reaction was highlighted in the arterial wall by immunohistochemistry using an anti-CD68 monoclonal antibody that marks normal or pathological monocytes/macrophages. *Results and Discussion*: The types of analyzed atherosclerotic lesions showed a positive reaction with the anti-CD68 antibody, varying in distribution and intensity in the vascular intima. The CD68 was positive in macrophages depending on the extent of the inflammatory reaction. *Conclusions*: The presence of many macrophages in the atherosclerotic plaques indicates a chronic inflammatory reaction, accompanied by fibroblast proliferation and connective tissue changes that influence the stability of the plaques.

Keywords: atherosclerosis, arteries, lesions, macrophages, anti-CD68 antibody.

Introduction

Cardiovascular pathology represents, due to diversity, in its current scope and especially because of its consequences, one of the most important health problems of this century, worldwide. At present, Romania occupies the top places in the world for both cardiovascular disease as well as incidence and mortality rates of vascular nature. This reality requires, as essential measures, the preventive treatment of persons with cardiovascular risk and treatment of all patients undergoing a major vascular event (myocardial infarction, stroke, etc.) [1, 2].

The medical literature brought into focus in recent years, states that endothelial dysfunction is an early marker of atherosclerosis, which results in increased vascular permeability, leukocytes and platelets adhesion, vascular smooth muscle cells proliferation, and finally causing a vasoconstrictor and pro-inflammatory status. This endothelial status could be considered as result of aggregation of all risk factors of a person, accompanying a wide range of conditions with increased cardio-vascular risk such as coronary disease, hypertension, diabetes mellitus, dyslipidemia, etc.

Vascular wall inflammation is associated with and largely due to changes in the status of leukocytes in the arterial wall. The influx of monocytes in an early stage of the process of atherosclerosis causes the most common form of vascular inflammation [3].

Macrophages are strategically located in the body tissues, where they ingest and process foreign particles, dead cells and other waste products and recruit other macrophages in response to inflammatory signals [4, 5].

Materials and Methods

We performed a retrospective study that included selected data from observation sheets and pathological results of a total number of 213 patients, aged between 33 and 78 years, hospitalized in the Cardiovascular Surgery Department of the Institute of Cardiovascular Diseases "Prof. Dr. George I. M. Georgescu", in the period 2005–2009. The severity of the symptoms at the time of hospitalization and degree of lumen stenosis, due to the presence of lesions, led to the performance of endarterectomies with the subsequent removal of plaques stenosis. The specific tissue samples were processed by the classic histopathological technique of paraffin embedding, microtome sectioned in 5 μm thick slices and stained with the classical Hematoxylin and Eosin method, which allowed us to study the distribution, the extent and the complications of the lesions. Also on the representative blocks, immunohistochemical stains were performed for the study of various factors involved in the pathogenesis of atherosclerosis. The present article refers to the immunohistochemical study of the inflammatory response of atherosclerotic plaque at different stages of development, using the anti-CD68 antibody.

CD68 antibodies are used in current practice as markers for monocytes/macrophages present in normal or pathological tissue fixed in paraffin. The CD68

antigen is a membrane glycoprotein, type 1, with a molecular weight of 110 kD. It is found in endosomes or lysosomes (long version) rather than the cell surface (short version), and it is strongly expressed by blood monocytes and tissue macrophages.

As primary antibody, we used a CD68 monoclonal antibody, clone KP1 AM416-5M. This antibody is commonly used *in vitro* diagnosis. It stains the CD68 antigen in the cytoplasm of the macrophages in different tissues fixed in formalin and embedded in paraffin.

The presence of the antigen in tissues by immunohistochemistry is a process that takes place in two steps: the first step involves the binding of the primary antibody to the antigen, followed by the detection of the bound antibody by a chromogenic system. The detection system used was Super Sensitive Non-Biotin HRP, which uses a non-biotinic polymer technology with two major components: the reagents Super Enhancer™ and Poly-HRP (Horseradish Peroxidase); the endogenous biotin problems were eliminated by the fact that this system does not use the Avidin–Biotin complex.

The sections are fixed, sectioned and attached on to special electrostatically treated slides. Subsequently they are dewaxed in toluene, pretreated with antigen retrieval and then incubated with primary antibody. The link with the primary antibody is detected by adding a secondary antibody, which is conjugated with a horse radish peroxidase polymer and DAB substrate. Once the brown color appears, the slides are submerged in water to stop the reaction and then stained with Hematoxylin. Finally, the sections are mounted in Canada balm or Enthelan. This system – unlike the conventional technique, rich in biotin – obtains signal amplification with high sensitivity by increasing the number of enzyme molecules conjugated with secondary antibodies.

The immunogen is represented by lysosomal granules of the human lung macrophages. The immunoglobulin class is IgG1 kappa mouse. This monoclonal antibody is a mouse antibody obtained from the supernatant of a tissue culture and diluted in a saline buffer solution at pH 7.6, containing 1% BSA and 0.09% sodium azide. The immunohistochemical staining technique was performed using the Super Sensitive Detection System kit (Biogenex). This process reduces or even eliminates the background noise compared to systems using Biotin–Streptavidin and also reduces the incubation time with the majority of Biogenex primary antibodies.

Positive control for the CD68 was performed on slides prepared from stomach. Negative control was used to confirm that the tested positive reaction is the result of a specific antigen-antibody link. These slides are a duplicate of the slide to be tested and working under the same conditions as this, eliminating the primary antibody. The primary antibody is replaced with an immunoglobulin (negative control serum) from the same species of the primary antibody and at the same dilution.

The primary antibody, clone KP1, required a Citra Plus antigen retrieval pretreatment by boiling in a microwave. This procedure is necessary in order to increase the intensity of staining and to reduce the background noise. At first, the slides are immersed in the solution from the drum; the oven is set to 100^0C until it begins to boil. Then it is adjusted to the 50^{th} power and the slides are kept for 10–12 minutes, removed from microwave and cooled completely in a citrate solution, followed by the normal processing.

Results

In our study, the histopathological examination of prevailed samples revealed the presence of different types of atherosclerotic plaques, with different degrees of lumen stenosis. Endarterectomy pieces, obtained from the 213 patients, were classified according to AHA classification (*American Heart Association*) in six different types of lesions. The V and VI types had the highest rate (Figure 1).

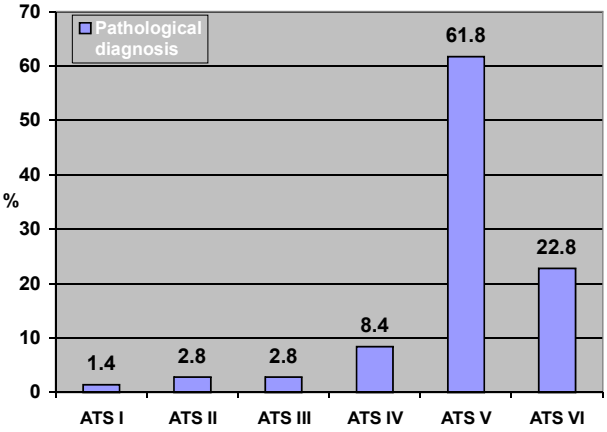

Figure 1 – ***Distribution of cases according to pathological diagnosis.***

The degree of stenosis varied a lot, sometimes going up to almost a complete reduction of the vascular lumen. Lesions were most often presented as a single plaque with a single lipid core or as stratified plaques with multiple lipid cores and fibrotic layers, with calcium deposits or predominantly fibrotic changes.

Inflammatory activity in atherosclerotic lesions, observed in optical microscopy (Figures 2 and 3), was also immunohistochemically demonstrated.

The CD68 antigen is a membrane glycoprotein, strongly expressed by blood monocytes and tissue macrophages. The analyzed lesions showed a variable degree of infiltration with foamy macrophages. Some areas showed a low density of CD68 positive immunoreactivity (Figures 4 and 5). The fibrous collagen cap revealed the presence of macrophages (CD68 positive), although other inflammatory cells were also present.

In the core of the atherosclerotic plaques, CD68 was positive in a variable number of macrophages, depending on the extent of the inflammatory reaction. The presence of many macrophages indicates a chronic inflammatory reaction, which is accompanied by changes in the cells and the extracellular matrix of connective tissue.

In stable plaques, immunohistochemical staining for the CD68 showed either a moderate zonal (Figure 6) or diffuse positive immunoreactivity (Figure 7) or an

increased zonal (Figure 8) or diffuse positive immunoreactivity (Figure 9). Near the necrotic lipid core is a large number of foamy macrophages; in contrast, the fibrous cap itself has only a few macrophages. In complicated plaques, however, we observe numerous CD68 positive macrophages in the cap of the plaques.

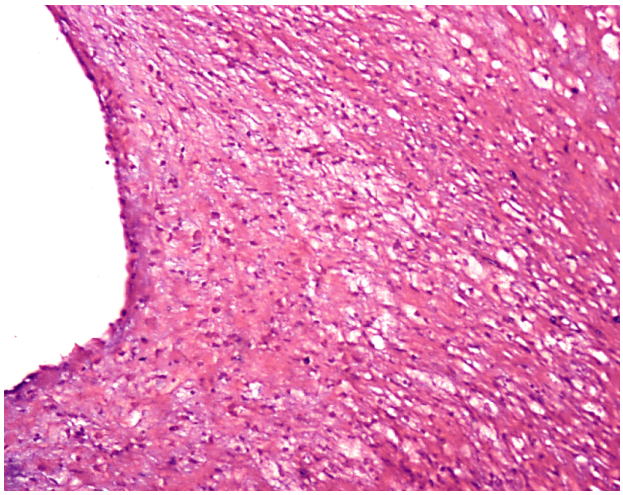

Figure 2 – ***Atherosclerotic plaque: discontinuous endothelium, foamy cells, extracellular lipid deposits (HE stain, ob. 20×).***

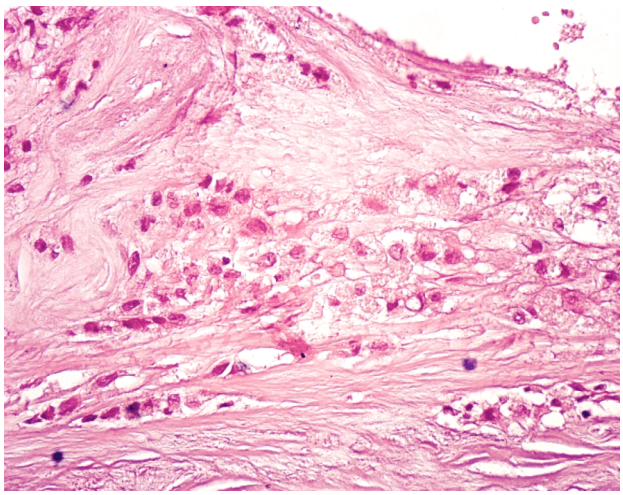

Figure 3 – ***Atherosclerotic plaque: abundant extracellular matrix, foamy cells (HE stain, ob. 40×).***

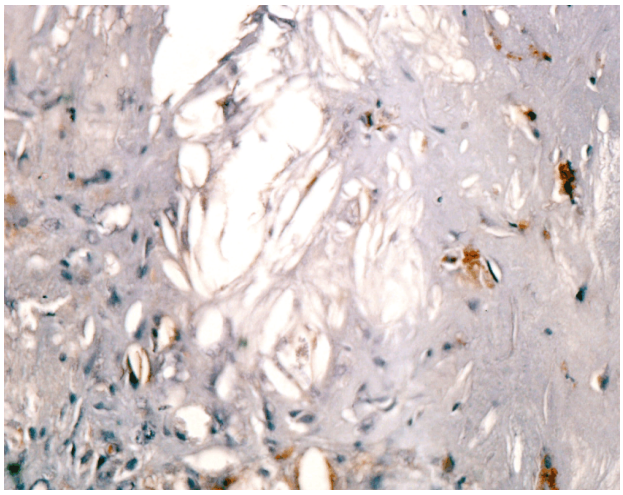

Figure 4 – ***Atherosclerotic plaque: positive immunoreactivity for CD68, low density (ob. 40×).***

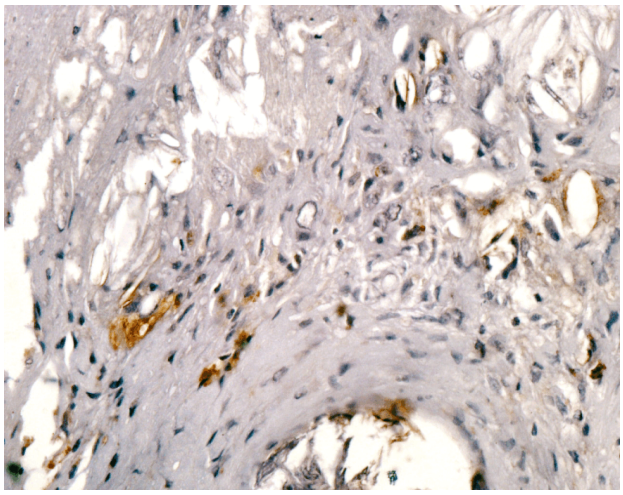

Figure 5 – ***Atherosclerotic plaque: positive immunoreactivity for CD68, low density (ob. 40×).***

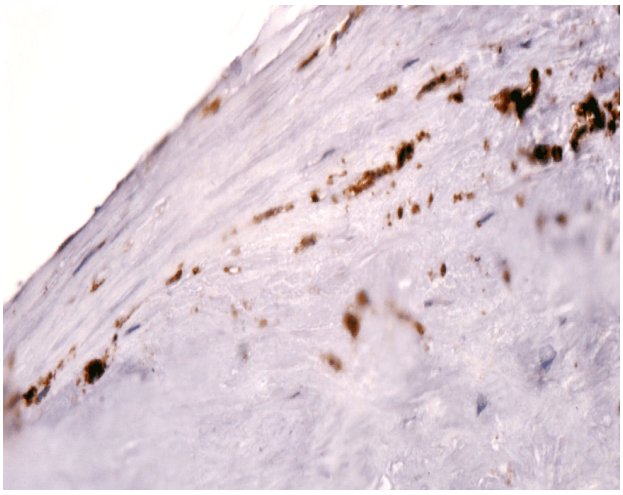

Figure 6 – ***Atherosclerotic plaque: positive immunoreactivity for CD68, moderate zonal density (ob. 40×).***

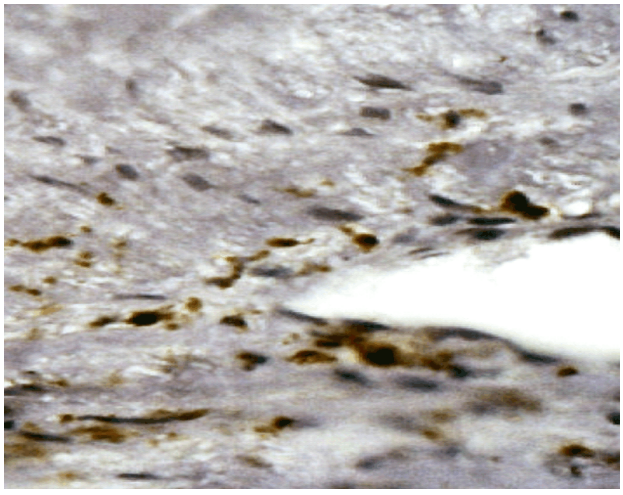

Figure 7 – ***Atherosclerotic plaque: positive immunoreactivity for CD68, moderate diffuse density (ob. 40×).***

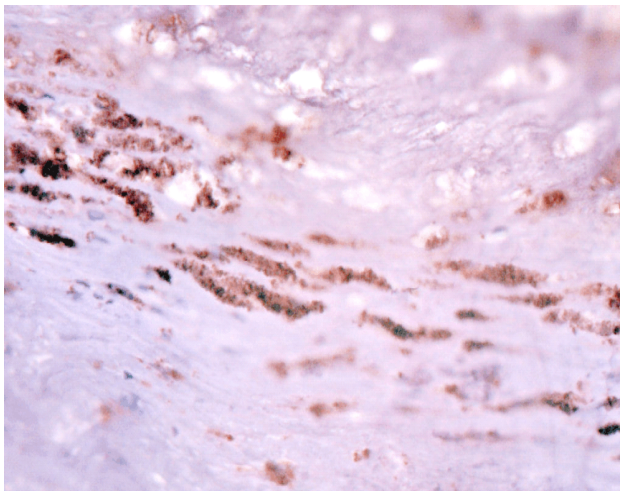

Figure 8 – ***Atherosclerotic plaque: positive immuno-reactivity for CD68, increased zonal density (ob. 40×).***

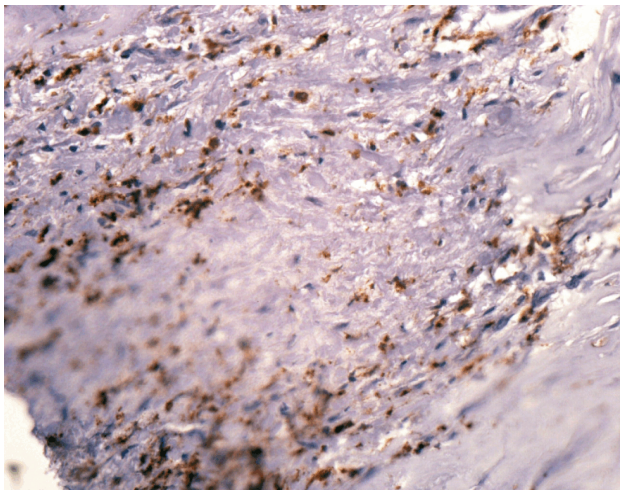

Figure 9 – ***Atherosclerotic plaque: positive immuno-reactivity for CD68, increased diffuse density (ob. 40×).***

Discussion

Atherosclerotic lesions are histologically classified into six types according to the *American Heart Association*. In the first type, foamy cells appear as isolated cells, followed by stages of fatty streaks, and fibroatheromatous plaques to more complex lesions [6]. Lesions type I and II may occur in the first decade of life, although they may be found in adults also; preatheroma or lesion type III may occur in adolescence and its structure is intermediate between the lipid streak and the atheroma, and types IV, V and VI are considered advanced lesions. In the V and VI types, vascular stenosis or thrombosis and/or bleeding may be present because of injuries and can be clinically silent or apparent. Lesion progression from I to VI happens over several decades of life, with different growth mechanisms [6].

Many researchers believe that atherosclerosis is a chronic immune-inflammatory disease in which the key events leading to impaired arterial intima are the interactions between blood monocytes and activated endothelium [7, 8]. In the early stages of atherosclerosis, monocytes migrate into the subendothelial layer where they differentiate into macrophages or dendritic cells. If the atherogenic lipoproteins are present in the subendothelial tissue, most cells become foamy macrophages. By aggregation of the foamy cells, the atheroma core can arise; as the process evolves, the center of the lesion becomes necrotic, consisting of fat, cholesterol crystals and cell debris [8].

In our study, the immunohistochemical marker CD68 was used for the assessment of the inflammatory reaction in the plaque. Physiologically, white line cells do not adhere to normal endothelium, but, early in atherosclerosis, the arterial endothelial cells begin to express on their surface selective adhesion molecules that link various types of leukocytes: ICAM-1, VCAM, ELAM [9, 10]. Of these molecules, VCAM-1 (vascular cell adhesion molecule-1) precisely binds the types of leukocytes found in human or experimental early atheromas: monocytes and T-lymphocytes. Consequent to accession by the endothelial line, monocytes, strongly stimulated by local production of chemokines and receptors of chemokines, migrate through the endothelial cells in order to locate into the intima, where they become macrophages and begin to accumulate lipoproteins (especially oxidized LDL) [11, 12]. Initially, the recruitment of monocytes and the differentiation in macrophages has a protective role by removing modified lipids, but as the fat load increases, the damage progression occurs [13–15]. It has been shown many times that macrophages express multiple metallo-proteinases and serine protease that degrade the extra-cellular matrix, which weaken and predispose to rupture the atherosclerotic plaques. They secrete many other factors such as reactive oxygen species, eicosanoids, TNF-α and IL-1, MCP-1, which further increase the leukocyte adhesion [16, 17].

Although there are various factors known to induce the chemotactic migration of monocytes, the MCP-1 (monocyte chemoattractant protein-1) is the most potent and powerful inductor of their migration into atherosclerotic lesions. The colony-stimulating factor is essential for macrophage differentiation, proliferation and survival in these lesions. A minor population of macrophages can proliferate even inside the atherosclerotic lesions, especially in the early stage [18–20]. Macrophages express a variety of receptors, particularly scavenger-receptors, and they uptake various modified lipoproteins. Subsequent accumulation of cholesterol esters in the cytoplasm leads to the formation of foamy cells in the developing lesions. Macrophages express different scavenger-receptors, but the class A type I and type II (MSR-A I, II) plays the most important role in the uptake of oxidized low density lipoprotein. In addition, macrophages and macrophage-derived foamy cells produce ceroid and advanced glycation end products (AGEs) and they accumulate these substances in their cytoplasm. The generated extracellular AGEs are up taken by other macrophages through specific receptors, including MSR-A I, II. Most cells die inside the plaques by apoptosis, while others escape from the lesions in the peripheral blood. Macrophages also play multiple roles in inducing plaque rupture, blood coagulation and fibrinolysis by producing various

enzymes, activators, inhibitors and bioactive mediators. During the development of atherosclerosis, macrophages interact with vascular endothelial cells, smooth muscle cells of the media and other inflammatory cells, particularly T-cells and dendritic cells [21, 22].

Numerous studies regarding the role of immune effectors in atherosclerosis focuses on CD4+ T-cells and macrophages, which release proinflammatory cytokines in atherosclerotic plaque [23, 24]. It was found that CD4+ cells interact with oxidized LDL and it is considered that this process makes them autoantigens. It was suggested the possible role of antibodies and autoantigens in early atherosclerosis [25, 26] and in this context the participation of immunological factors in early human hypertension it has been reported. Anti-HSP-65 and endothelial cell antibodies (AECA) increase in patients with borderline hypertension. However, anti-oxLDL and anti-lysophosphatidylcholine are low in patients with hypertension, suggesting that different autoantigens may have an anti-atherogenic function.

The CD68 was predominantly positive in the middle of atherosclerotic plaques, depending on the extent of the inflammatory reaction. In stable plaques, either a moderate zonal or diffuse positive immunoreactivity or a high zonal or diffuse immunoreactivity near the lipidonecrotic core with a large number of foamy macrophages were revealed. In contrast, in the fibrous cap itself, only a few macrophages were present. In the cap of complicated plaque, numerous CD68 positive macrophages were also observed.

Atherosclerotic lesions in experimental animals and humans contain different phenotypes of macrophages, which play different roles in mediating inflammation, clearance of dead cells, and possibly in tissue repair. The presence of lipids in the plaques changes the phenotype and biological functions of the macrophages, which activate specific sets of genes. Activation of the inflammasoms by cholesterol crystals and the interaction with oxidized lipids by specific receptors leads to an M1 inflammatory phenotype of macrophages. A new MOX phenotype develops when the oxidized phospholipids activate the response of stress genes by Nrf2. Other lipid mediators, such as fatty acid derivative nitrosilates and omega-3 fatty acids polarize macrophages of the atherosclerotic plaques with their transformation in anti-inflammatory phenotypes [27].

Conclusions

The examination of fragments obtained from our group of patients showed various degrees of infiltration with foamy macrophages. In the early stages, the CD68 positive cells were identified in arterial lumen and subsequently in the wall, suggesting recruitment of monocytes from circulation. Some areas showed a low density of CD68 positive immunoreactivity. Fibrous collagenous cap showed the presence of CD68 positive macrophages, along with other inflammatory cells.

The qualitative analysis of the inflammatory component, as evidenced by the immunohistochemical examination, allowed the identification of differences in location of inflammatory cells in the vascular wall, from intima to adventitia, which can be correlated with developmental stages of plaque formation and organization.

A deeper understanding of how lipids that accumulate in atherosclerotic plaques affect the phenotype and the functions of macrophages and thus the evolution of such lesions, will help in the future to develop new therapeutic strategies.

The complexity of atherosclerotic disease, with aspects still not completely understood, with a negative impact on the health of the population, brings constantly this topic to the attention of researchers and clinicians.

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