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Role of "Memory-like" Natural Killer cells in Human Cytomegalovirus seropositive atherosclerotic patients

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- $NKG2C^-$ $Fc\varepsilon R1\gamma^-$ Adaptive NK cell subset is significantly amplified in CAP of high-risk patients compared to their PB

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CONCLUSION

REFERENCES

ATHEROSCLEROSIS

Atherosclerosis is a chronic, progressive, inflammatory disease that affects large and medium-sized arteries, elastic and muscular, starting at the level of the tunica intima of the vessel and subsequently also involving the tunica media.

Atherosclerotic lesions, represented by atheroma in different phases of evolution, are characterized by highly specific cellular and molecular responses that can be described as an inflammatory disease. Each of them represents a different stage of the chronic inflammatory process involving arteries, which in the absence of adequate therapeutic treatment, induces advanced and complex lesions [1].

Atheroma induces in the site of development to an increase in thickness of the arterial wall, caused by the accumulation of different materials of blood and tissue origin, in particular lipids, with prevalence of cholesterol and cholesterol esters, resulting in a fibrotic reaction. An increase of the inflammatory state occurs, with an infiltration of leucocytes, particularly macrophagic cells, which take the accumulated lipids and become foam-cells, and a migration and proliferation of smooth muscle cells (SMCs) and fibroblasts, with extracellular matrix production (collagen and proteoglycans). Each lesion may show great tissue variability [2]. In fact, some atherosclerotic lesions appear predominantly dense and fibrous, others may contain large amounts of

lipids and necrotic residues, while most present combinations of each of these characteristics.

Being the atheroma characterized to of a slow and progressive expansion, it induces a double damage: the reduction of the lumen of the artery in which it has developed, possibly ending in its occlusion, and the progressive fibrosis of tunica media [3].

Although the first characterization dated to the works of ancient Egyptians, Greeks, and Romans [4], the term "atherosclerosis" was used for the first time about 160 years ago, when Lobstein, in his “Treatise on Pathological Anatomy”, described arteriopathies as characterized by a hardening of the arterial wall. Later, in 1904, Marchand coined the term atherosclerosis, which derives from the conjugation of the word *ather*o (from the Greek "atheré", meaning mush, indicating the outward appearance of atherosclerotic lesions in the advanced stage) with the word *sclerosis* (hardening). Sclerosis was the reaction of connective tissue to insults of various kinds and consisted in the reduction of the cellular component of this tissue and the simultaneous increase of the matrix, responsible for hardening and loss of elasticity [4] (Figure 1).

Therefore, the atherosclerotic process, together with other risk factors, forms the pathological substrate of cardiovascular diseases, and in particular ischemic heart disease (coronary heart disease: CHD), the leading cause of mortality and morbidity in the world.

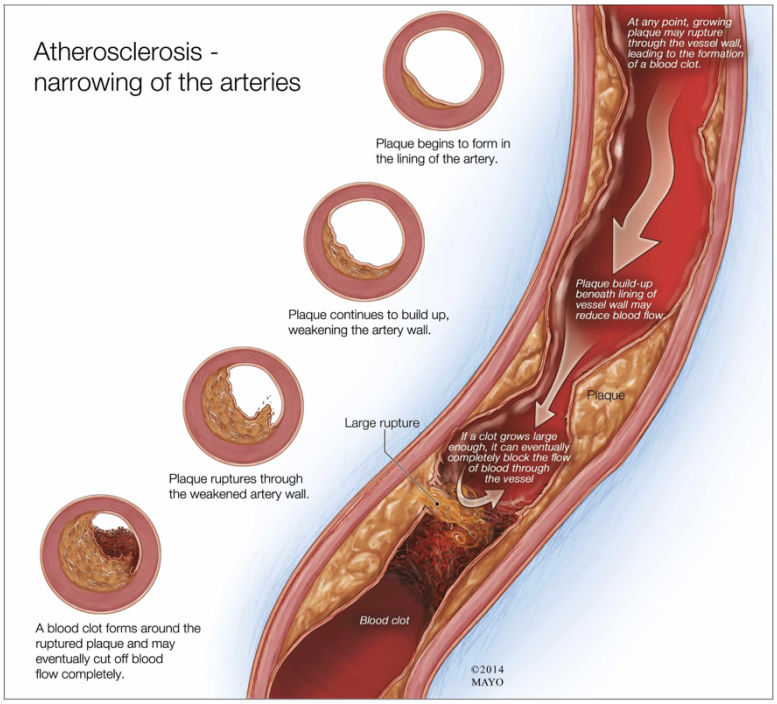


Figure 1 - Artery affected by atherosclerotic process. On the left, section of artery from initial formation of plaque to the complete occlusion by blood clot. On the right, total view of the process in the vessel (*From Simper et al, Circulation 2003).*

Epidemiology

Atherosclerosis is a systemic disease and the common cause of heart attacks, strokes and peripheral vascular disease collectively referred to as cardiovascular diseases (CVD), which are the leading cause of global mortality and a major contributor to disability.

An estimated 17.9 million people died from CVDs in 2019, representing 32% of all global deaths (Figure 2). Of these deaths, 85% were due to heart attack and stroke. Out of the 17 million premature deaths (under

the age of 70) in 2019, 38% were caused by CVDs [5].

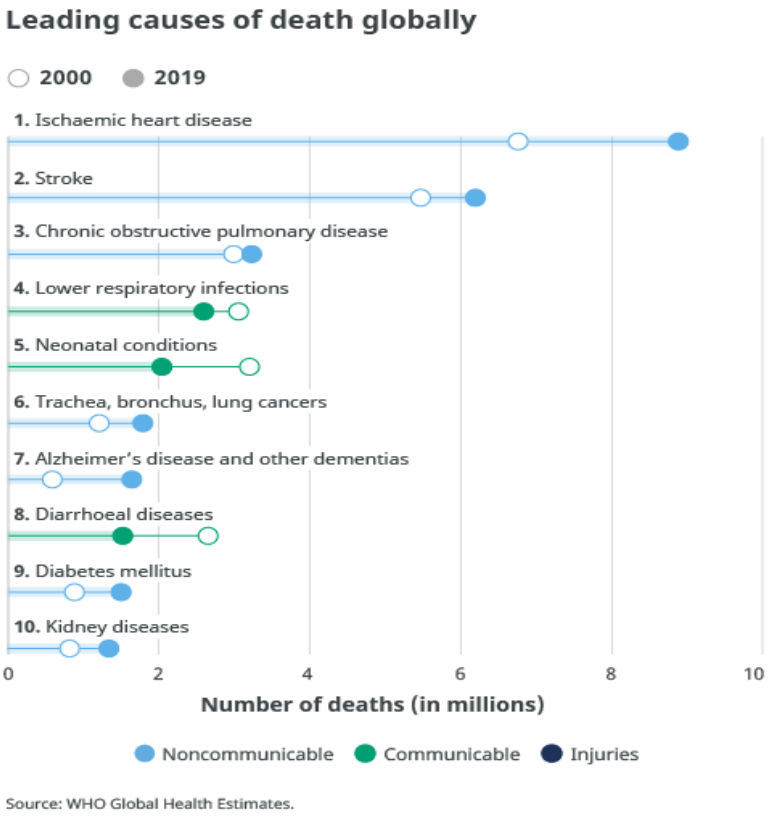


Figure 2 - World causes of death (*From WHO Global Health Estimates 2019*).

Based on National Health and Nutrition Examination Survey (NHANES) from 2017 to March 2020 data, the prevalence of CVD [including CHD, heart failure (HF), stroke, and hypertension] in adults ≥20 years of age is 48.6% overall (127.9 million in 2020) and it increases with age in both males and females. Based on 2020 mortality data, HF and stroke currently claim

more deaths each year than cancer and chronic lower respiratory disease combined. In 2020, 207.1 of 100 000 people died of HD and stroke. In 2020, 19.05 million deaths were estimated for CVD globally, which amounted to an increase of 18.71% from 2010. The age-standardized death rate per 100 000 population was 239.80, which represents a decrease of 12.19% from 2010. Overall, the prevalence of CVD was 607.64 million cases in 2020, an increase of 29.01% compared with 2010. However, the age-standardized prevalence rate was 7354.05 per 100 000, an increase of 0.73% from 2010 [6].

The Global Burden of Disease (GBD), or "global impact of pathologies", is a program of evaluation of the effect of pathologies on the world population, present in the page of the World Health Organization (WHO) (Figure 3).

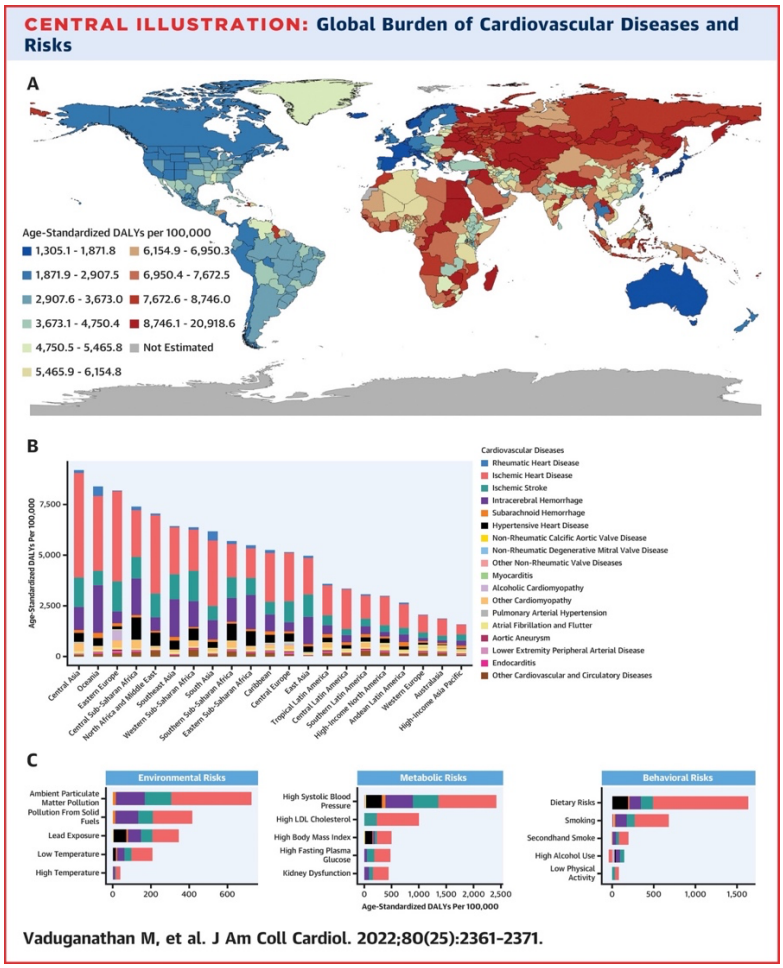


Figure 3 - Global Burden of Cardiovascular Diseases and Risks. Age-standardized disability-adjusted life years (DALYs) per 100,000 for (A) cardiovascular diseases globally, (B) specific cardiovascular diseases by region, and (C) global burden attributable to selected risk factors compared to the theoretical minimum risk level *(From Vaduganathan et al. J Am Coll Cardiol 2022, 80, 2361–2371)*

The GBD study provides data on disease mortality of 187 nations, grouped into 7 macro areas. The percentage of deaths from cardiovascular diseases was: 35.8% in High Income countries (Asia Pacific Zone, Western Europe, Australia, North America,

South Latin America); 58.2% in Eastern Europe (Central Europe, Eastern Europe, Central Asia); 35.7% in the Asian Pacific Zone (Southeast Asia, East Asia, Oceania); 20.4% in South Asia; 28.8% in Latin America/Caribbean (tropical Latin America, central Latin America, Andean Latin America, Caribbean); 42.3% in North Africa-Middle East; 8.8% in sub-Saharan Africa [7].

In Western countries, cardiovascular diseases represent the first cause of death in both sexes, followed, in order of frequency, by cancers, accidental deaths, chronic bronco-pneumopathies and diabetes.

In the United States, in 2013 [8], CVDs were cause of over 30% of all deaths, CHD of about 20% and stroke (stroke) of about 10-12% [9]. Since atherosclerosis is cause of 90% of coronary events and 60% of cerebral strokes, it represents the first cause of death. Atherosclerosis is also responsible for most cases of heart failure and of peripheral arterial disease and 30% of dementia [10].

Although these observations are limited, there is concern that an increase in mortality due to CHD can occur in the next years.

There are many prevention strategies implemented by the WHO, first the "Global Hearts Initiative", through which WHO is supporting world governments to increase prevention interventions in CVD, through three technical systems: "MPOWER" to control

tobacco use (risk factor), “SHAKE" to reduce salt use in food (risk factor) and "HEARTS" to support the management of these diseases in primary medical care.

In emergency situations, medical staff is trained to best intervene on the patient, avoiding death and providing a recovery of post-stroke [11].

Launched in September 2016, the "Global Hearts Initiative" is now an important prevention system used by many countries.

The development of efficient primary prevention strategies is essential to act on early stage of the disease, when the damage has not yet started or is limited and, therefore, potentially responsive to correction.

Although the correction and the control of risk factors have been found an excellent way to prevent the onset of the disease, or at least reduce its development, the identification and monitoring, through diagnostic-instrumental investigations, of a condition of "Preclinical atherosclerosis" (early stage of pathology in which the anatomy, but not the functionality of the vessel is altered) represent an important strategy to control the disease. Indeed, it can be considered an authentic early marker of vascular damage, revealing any structural transformation, and becoming an expression of the individual predisposition to develop atherosclerotic and consequently CVD.

Just as example, it can be useful in smokers with evident diffuse medium-intimal thickening or reduced peripheral perfusion.

Risk Factors

Risk factors are specific characteristics or conditions, in healthy patients, statistically related to the occurrence of a certain pathological status, whose correction was associated, based on epidemiological evidence, with a reduction of the incidence of the disease.

It was demonstrated that there is a direct causal relationship between the risk factor and the development and progression of atherosclerotic lesion and the related clinical events.

For this reason, the concept of global cardiovascular risk (RCVG) has been introduced, that is referred not to the individual risk factor but considers the amount of many risk factors at different levels, which affect the onset of injury and therefore of the pathology.

Atherosclerosis, similarly, to associated CVD, is a "multifactorial" pathology, as many risk factors contribute simultaneously to its development.

Risk factors affecting atherosclerotic disease were commonly distinguished into traditional and emerging [12]:

Traditional (modifiable and not): classically risk factors associated with atherosclerotic disease; distinguished in:

- modifiable or susceptible to corrections, by behavioral changes or by pharmacological interventions. They include dyslipidemia, diabetes mellitus, high blood pressure, cigarette smoke, overweight/ obesity, sedentary or absence of physical activity, alcohol use and psychosocial and behavioral factors (diet, stress).
- not modifiable or conditions that, if present, cannot be susceptible to any change, such as: age, sex, ethnic characteristics, familiarity, or genetic predisposition for CVD.

Emerging: conditions for which only a few years ago a direct connection with atherosclerotic disease was demonstrated. In this group, metabolic syndrome, inflammatory factors, hyperhomocysteinemia, microalbuminuria, prothrombotic and coagulative factors, infectious diseases can be included. Even today, there is no scientific evidence of a correlation between emerging risk factors and the onset of the disease. Furthermore, for some of these factors, there is no drug treatment yet.

Modifiable traditional risk factors

- Dyslipidemias

Conditions in which high concentrations of lipid fractions is present in the blood.

The primary event in the formation of atherosclerotic lesions is the accumulation of lipids in sub-endothelial area of the tunica intima of the artery.

Lipids are transported in the plasma through lipoproteins, complex water-soluble molecules composed of a nucleus of cholesterol and triglycerides, a superficial layer of phospholipids, free cholesterol, and specific transport proteins, the apolipoproteins.

There are different classes of lipoproteins that are distinguished by lipid content, dimensions, density in ultracentrifugation, electrophoretic mobility and proteins present on their surface. The main plasma lipoproteins are chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL or VLDL remnants), low density lipoproteins (LDL) and high-density lipoproteins (HDL). Lipoproteins are then transformed switching lipids and proteins and moving from one class to another.

An increase of plasma levels of lipoproteins results in an increase in blood lipid levels, contributing to the development and progression of the atherosclerotic process. Lipoproteins rich in triglycerides, chylomicrons and VLDLs are not considered to be atherogenic, differently from their derivatives, such as the VLDL remnants and the LDL, cholesterol-rich lipoproteins. In fact, patients with atherosclerosis and cardiovascular pathologies frequently have a lipid profile characterized by VLDL remnants and LDL elevated plasma levels [3].

The lipoproteins considered at high-risk for the atherosclerosis are the LDL, small and dense, having a high plasma half-life, and present into the bloodstream for longer time compared to the others, making the continuous release of lipids, thus supporting atherogenic process.

These lipoproteins into the bloodstream undergo changes in density and size that make them easily subjected to oxidative events, being first transformed into LDL medium oxidized (MM-LDL) and then in LDL oxidized (ox-LDL). Oxidation is considered one of the initiating factors of atherosclerotic injury [13]. Therefore, dyslipidemia contributes directly to the development and progression of CHD.

Even if both men and women are involved in the disease, there are apparent gender-specific differences. Plasma levels of total cholesterol and LDL, that transport it (LDL-C), are generally lower in pre-menopausal women than men with the same age [14]. After menopause, total cholesterol and LDL-C increase, peaking around 60. HDL-C is significantly higher even in pre-menopausal and post-menopausal women, compared to men of the same age ($P > 0.001$) and this could be considered a specific factor of protection of the gender [15]. In women, as in men, the association between LDL cholesterol and increased cardiovascular risk, as well as the benefits of its reduction in high-risk subjects, has been demonstrated.

- Diabetes Mellitus

Diabetes mellitus is defined from the American Heart Association as a “condition equivalent to coronary disease”.

Diabetes is correlated with an increased risk of myocardial infarction, stroke and arteriopathy. As a consequence, diabetic patients must be treated promptly, to maintain low levels of glycaemia (<120 mg/dl), fasting and post-prandial (<180 mg/dl), and glycosylated hemoglobin A1c (hba1c) (<6.5-7%); searching and correcting the associated cardiovascular risk factors, such as hyperlipidemia, smoking and hypertension, and applying prevention therapeutic strategies (C-LDL target <100 mg/dl) [16].

Worldwide, 2.8% or 171 million people are affected by diabetes, and in 2030 is estimated an increase of 5% [17].

Diabetes mellitus include from 90% to 95% of all cases detected in adults [18]. Cardiovascular mortality rate increases from 2 to 4 times owing to this pathology [19]. Data from the Framingham Heart Study/National Heart, Lung and Blood Institute indicated that the risk of developing CVD for diabetic women (2.5 for women, 2.4 for men) and to die for CVD (2.2 for women women, 1.7 for men) was slightly higher than the men [20].

Cardiovascular events are the leading cause of death in type 2 diabetes [21]. Diabetic women have a risk

of developing CVD three to five times greater than men [22].

The Copenhagen City Heart Study included 7,198 women, in a period of twenty years; the relative risk of heart attack in diabetics was 1.5-4.5 compared to non-diabetics [23]. In addition, diabetic women developed CVD earlier, when compared to non-diabetic women and to men of the same age.

The reason is still unknown. A recent study showed that diabetic women under 65 years have worse outcome after a myocardial infarction than men, and this probably due to an increase in the weight of risk factors [24].

- Hypertension

The maintenance of high blood pressure regimens is related to rapid progression of atherosclerotic disease and increased risk of myocardial infarction, stroke, and cardiovascular events in general.

Arterial hypertension accelerates the atherosclerotic process for mechanical stress suffered from the vascular wall (shear stress), with endothelial damage, and for the associated hormonal changes, such as the production of angiotensin II, which indirectly plays an equally important pathogenetic role. Hypertension is associated with an increased energy expenditure for the heart cells, leading to a situation of continuous work overload that cannot be long faced by the physiological mechanisms of compensation. In this

way, a slow and progressive reduction of the functional reserve of the heart muscle occurs. Therefore, the persistent increase in post-load forces in the myocardium causes the impossibility to satisfy any condition of increased metabolic demand of the tissue, especially if other risk factors are present.

It is necessary to take care of symptoms in a cardiopathic patient, monitoring and applying targeted treatment. It is necessary an integrated therapeutic approach with the correction of all risk factors, such as hypercholesterolemia, diabetes, obesity, hyperfibrinogenemia, or cigarette smoking.

Signs and symptoms of isolated systolic or systolic hypertension are not specific, but their identification and careful control reduce the incidence of cardiovascular complications.

The guidelines of the Joint National Committee VII, taken by the World Health Organization (WHO), allowed to indicate an optimal blood pressure whic levels are 120/80 mmHg. These data have also been confirmed in the Joint Guidelines of the European Society of Cardiology and the European Society of Arterial Hypertension [25].

- Cigarette smoke

It is known that smoking predisposes to atherosclerosis [26, 27].

From the Nurses' Health Study which included more than one hundred and twenty thousand healthy

nurses, emerged that four or five cigarettes a day almost doubled the risk, while twenty cigarettes a day increased the risk by six times [28].

Nicotine activates the sympathetic adrenergic system resulting in increased heart rate, cardiac work, blood pressure and a possible reduction of coronary flow for vasoconstriction. Carbon monoxide acts with a direct toxic mechanism on the endothelium that becomes more permeable to lipoproteins and causes relative hypoxia secondary to the increase of carboxyhemoglobin.

There is a close link between tobacco consumption, increase oxidation of LDL-C, platelets aggregation and endothelial changes [29]. Compared to non-smokers, women smokers die 14.5 years earlier [29-30]. Every year, in the USA, 178,000 women die for cigarette smoking. A case-control study on women younger than 44 years (n = 448) demonstrated that the chances of having a heart attack are strongly associated with the number of cigarettes, with a risk of 2.47 for women who smoked from 1 to 5 cigarettes/day, increasing to a risk of 74.6 for those who smoked more than 50 cigarettes/day compared to non-smokers (n = 1728) [31].

According to the CDC, eliminating smoking reduces the risk of CHD, stroke, peripheral vascular pathologies, and heart attacks [31]. Regular exposure to second-hand smoke increases the risk of cardiovascular disease by 25%. [32].

The 2002 World Health Report estimates that in developed countries, over 20% of cardiovascular events is due to smoking [33].

The INTERHEART case-control study estimates that 29% of cases of heart attack in Western Europe are due to smoking and that smokers and ex-smokers present almost double the risk than non-smokers [34].

- Overweight/obesity

As independent risk factors increasing the risk of atherosclerosis and CVD, literature identified overweight [with a body mass index (BMI)>25 kg/m_2] and obesity (with BMI>30 Kg/m). Obesity increases the risk to develop atherosclerotic and CVD over 2-2.5 times, respectively in men and women [35].

These conditions promote the emergence of other risk factors, such as diabetes mellitus, arterial hypertension, and alterations in atherogenic sense of lipid profile [36]. Risk is strictly connected to the measurement of abdominal circumference, which was related to the amount of perivisceral fat.

Visceral obesity is associated with a condition of insulin resistance that leads to alterations of the glucose metabolism, to hypertriglyceridemia with low levels of HDL-C, to hypertension, configuring the Metabolic Syndrome that constitutes an independent risk factor [37].

According to NCEP ATP III, in cases of Metabolic Syndrome, reference circumference values are: >88 cm in women and >102 cm in men [37,38]. Some healthy practices, as weight loss and physical activity, determine reduction of cardiovascular risk and promoted some changes in lipid structure: reduction of total cholesterol, LDL-C and triglycerides, and increased HDL-C.

- Alcohol consumption

Several population studies show a relationship between excessive alcohol consumption and mortality for CVD [39, 40]. Comparisons between different populations revealed a strong inverse connection of incidence rates of atherosclerotic disease and alcohol abuse.

It has been shown that small quantities of good red wine (about half a glass/day) could be considered protective, thanks to the antioxidant effects of substances present in it (resveratrol and other polyphenols).

Some studies have shown a reduction in mortality for CHD to 20%, in people who were used to drinking about 1 liter of wine/day. Positive consequences of this reduction were effects on the lipid pattern (increased HDL and Apo A1), on hemostatic factors (fibrinogen reduction), on metabolic changes, such as the impaired insulin secretion in diabetic patients [41, 42]. Some trials evidenced that excessive alcohol

intake is linked to increased incidence of hypertension and brain hemorrhagic events in populations of northern Europe, sadly famous for the excessive alcohol consumption, even among young people [43].

Not modifiable traditional risk factors

- Age

The development of atherogenesis begins in young people and evolves over time. Several autopsy studies have shown the existence of atherosclerotic lesions not only in juveniles but also in human fetuses conceived by hypercholesterolemic mothers [44].

After the onset of CHD, its development increases with age and literature studies define male subjects as "at risk" over 45 years of age and female subjects over 55 years of age. Often people with acute myocardial infarction under the age of 65, and in many cases even people under the age of 40, account for most deaths among men over the age of 35 in the Western world.

- Sex

In general, the incidence of coronary heart disease is higher in men than in women of childbearing age, as estrogen appears to play a protective role.

After menopause, this difference in incidence is eliminated, since estrogen deficiency in women induces unfavorable changes in the lipid profile, with increased cholesterol-LDL and reduction of HDL and

dysfunctional changes at the endothelial level. However, in recent decades, the advantage of women of childbearing age has gradually decreased due to tobacco use and lifestyle changes.

- Familiarity and genetic predisposition

A positive family history for early CHD has given an increased risk at any level of other known risk factors. Familiarity with cardiovascular disease, defined as the occurrence of a coronary event within 55 years in men or within 65 years in women in a relative of 1 grade, is considered an important independent risk factor, with a prevalence among these pts ranging from 42 to 69 % [45,46].

In epidemiological studies conducted in twins it was observed that the relative risk was 8% for the male sex if the twin died within 55 years, while for female sex relative risk up to 15% if the twin has died within 65 years [47]. It seems that familiarity weighs more in the female [48].

The relative risk varies in relation to the number of relatives involved, 2% in the presence of only one relative involved and increases to 3% in case of 2 or more first-degree relatives involved [48].

Familiarity, in addition to being an independent risk factor, has a cumulative effect, since, associated with smoking and to hypercholesterolemia, it leads to an increase in relative risk up to 14% [46,47].

A correct family history is the best way to assess the "genetic risk" of cardiovascular diseases, an important possibility to do genetic diagnosis.

There are numerous genes that can increase the susceptibility to atherosclerosis. These genetic factors are consisting in mutations and polymorphisms. These factors have been only partially identified and are generally frequent. This suggests that an individual has more "markers" that predispose him to atherosclerosis and cardiovascular risk. But only together lifestyle, environmental factors and risk factors with genetic predisposition determined global risk [49-50].

Emerging risk factors

- Metabolic syndrome

According to the definition provided by the NCEP-ATP III, this is a metabolic imbalance [40], which can be diagnosed if different conditions coexist in the same subject: increase of triglycerides >150 mg/dl; low levels of HDL cholesterol, <40 mg/dl in men and <50 mg/dl in women; hypertension with PAO >130/85 mmhg; fasting blood sugar between >100 mg/dl and <125 mg/dl; and waist circumference, >102 cm in men, and >88 cm in women.

It was a condition that includes predisposing factors, the coexistence of which increased exponentially the incidence of coronary and carotid disease.

Researchers of the University of Kuopio in Finland have studied the relationship between metabolic syndrome coronary artery disease, cardiovascular disease, and mortality from all causes. 1209 Finns between 42 and 60 years were observed for a period of 13 years. From the collected data, metabolic syndrome was associated with a statistically significant risk to overall mortality (risk ratio, HR = 1.90 to 2.10) and for cardiovascular mortality (HR from 2.60 to 3.00) regardless of cardiovascular seniority and/or diabetes.

The use of dihydroxy-methyl-glutaril-coA (HMG-coA) reductase inhibitors, which reduce hypercholesterolemia, lowered the risk of cardiovascular events in the same population over the long follow-up period [43, 51].

Ultrasound is used to detect the presence of carotid atherosclerosis in patients with metabolic syndrome. A recent study associated the presence of metabolic syndrome with the increased progression of carotid intimal mean thickening (IMT), even after correction of several risk factors, highlighting that the metabolic syndrome provided more information on the progression of preclinical atherosclerosis that goes well beyond risk factors and allows better prediction of cardiovascular events [52, 53]. Early identification, early diagnosis and prevention of metabolic syndrome are, therefore, key points in the proper

management of the patient with atherosclerotic disease.

- Inflammation

Inflammation plays a crucial role in the atherosclerotic process; not only induce this process but also participates in its progression, as well as in the genesis of plaque complications causing the development of an Acute Coronary Syndrome (ACS). After activation of the acute phase, plasma levels of certain markers of phagocytosis released into the bloodstream may indicate the severity of the ongoing inflammatory process. A recent study highlighted the importance of fibrinogen and C-reactive protein (PCR) as possible marker of cardiovascular risk [54]. PCR is an acute phase protein produced by the liver, which activates the complement system, induces the expression of tissue-factor (Factor III of coagulation or thromboplastin) and consequently activates the coagulative cascade.

PCR plasma levels are a risk indicator in asymptomatic pts with a positive history for other risk factors as well as a prognostic predictive value in pts with ACS. It is known that the serum level of PCR, usually very low in plasma concentrations, reflects the vulnerability of atherosclerotic plaque and can be considered an indicator of the cardiovascular risk marker [55].

Some observations indicate that obesity and metabolic syndrome may be accompanied by increased plasma PCR concentrations, suggesting a relationship between metabolic alterations and inflammation [56,57,58].

Several studies have found a strong correlation between PCR plasma levels and coronary heart disease death [59]. For example, in the Monica-Ausberg study, 936 middle-aged men with coronary clinical evidence found a general increase in risk of about 19% for future fatal or non-fatal cardiovascular events in subjects with high PCR levels [60].

All these epidemiological data together demonstrate the prognosis of PCR as a global inflammatory indicator to reveal cardiovascular risk factors.

As for fibrinogen (coagulation factor I), an acute phase protein produced by the liver, there is a strong association between it and cardiovascular events.

Fibrinogen increases blood viscosity, increases thrombogenesis of blood flow by improving platelet aggregation, promotes thrombosis and finally increases fibrin production resulting in an increase in thrombus size and their reduced susceptibility to lysis.

Maede and others, in a study of 1511 men, found that high levels of fibrinogen are associated with an increased risk of fatal and non-fatal cardiovascular events of 84% [61].

- Homocysteine

Homocysteine is an intermediate metabolite of myeloid hydrogen amino sulfide.

Hyperhomocysteinemia promotes atherogenesis by interfering with the complex antithrombotic systems of endothelial cells, resulting in endothelial dysfunction, platelet activation and disorders of hemostatic balance.

In addition, hyperhomocysteinemia inhibits the production of nitric oxide (NO), activates the production of reactive oxygen species (ROS) and activates pre-inflammatory factors, favoring venous and arterial thrombosis.

Hyperhomocysteinemia promotes abnormal hemostatic activation through the expression of tissue factor in endothelial cells and macrophages, resulting in activation of the extrinsic pathway of the coagulative cascade. Moreover, it has been observed that hyperhomocysteinemia is not only closely associated with hyperhomocysteinemia, but also has a positive correlation with several pro-coagulating factors, thus helping to maintain a state of hypercoagulability [62]. Consequently, an increase in serum homocysteine of 5 mmols/L appears to be equivalent to an increase of about 20 mg/dl of total cholesterolemia [63]. Thanks to diets and/or pharmacological interventions (vitamin B6 in a dose of 50 mg/day; folic acid at a dose of 0.5 mg/day and possibly vitamin B12 at a dose of 1 mg/day), plasma

homocysteine levels have been reduced and consequently the risk of cardiovascular morbidity and mortality has also decreased [64].

- Microalbuminuria

The term microalbuminuria indicates the subclinical increase in urinary excretion of albumin, with values between 30 and 299 mg/24 hours. The concomitant increase in the permeability of the capillaries would favor the transmembrane passage of albumin but also atherogenic lipoproteins within the vascular wall. Microalbuminuria has recently been included in the list of emerging cardiovascular risk factors, due to scientific disorders that have demonstrated its influence in systemic vascular damage and endothelial dysfunction.

- Infections

There is evidence that some micro-organisms such as Cytomegalovirus, Herpes Virus, Chlamydia Pneumoniae, Helicobacter Pylori, may contribute to the onset of atherosclerotic disease, as well as making atherosclerotic plaques unstable, acting like “noxae” on the vasal endothelium. It has been observed that some bacterial Heat Shock Proteins (HSPs), particularly those of Chlamydia Pneumoniae, have a strong antigenic resemblance to human HSPs, and it is therefore possible that an infection caused by Chlamydia, with subsequent localization of the

pathogen within the atherosclerotic plaque, could lead to the activation of the immune system against "auto" antigens.

In any case, if the antigen is recognized as foreign, activation of T cells occurs, which in turn produce many cytokines that modulate the various stages of the atherosclerotic process. The increased antibody titer against some of these microorganisms has been used as a predictor of cardiovascular events. The hypothesis of microbial infection in atherosclerosis is controversial, however studies so far focusing on antibiotic-based treatments have not produced significant results in reducing cardiovascular events.

Etiopathogenesis

Atherosclerosis is characterized by intimate lesions, the atheroma, protruding into the vascular lumen, capable of determining changes in normal blood flow and direct damage to the structure of the vessel. These lesions have a first focal distribution in the artery, then covering the entire circumference of the walls (Figure 4).

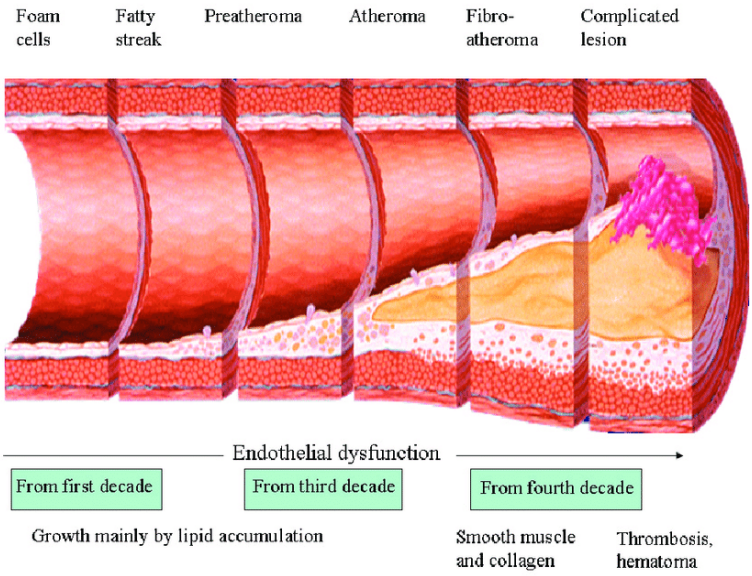


Figure 4 - Evolution and progression of human atherosclerotic lesions. From initial processes to the formation of complicated injuries (*From Stary et al. 1995*)

In the development of atherosclerotic lesions, several stages can be distinguished: the initial processes that generate clinically silent lesions, the stages of progression that lead to the formation of stenotic lesions not necessarily symptomatic; and events that generate complicated lesions, underlying chronic or acute clinical manifestations.

Structurally, the simple lesion consists of a plaque located in the intima, with a central nucleus of lipids (cholesterol and its esters) and covered by a "fibrous cap".

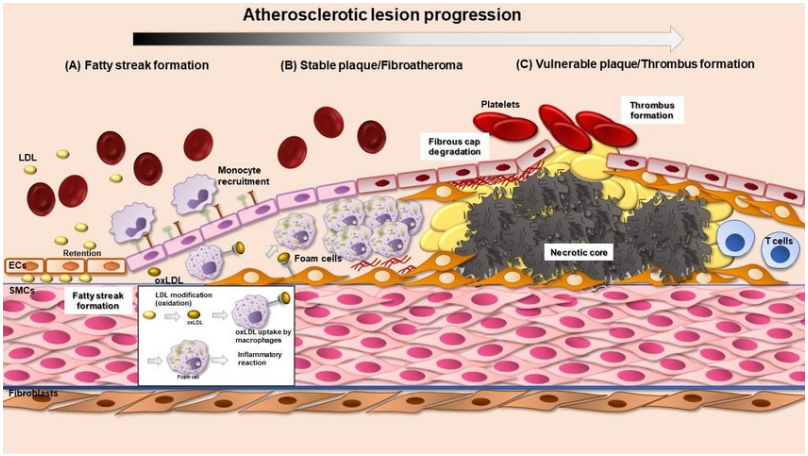


Figure 5 - Pathogenetic characteristics, and cellular elements involved in the atherosclerotic plaque formation. (*From Charla E., et.al. Cellular Signaling. 2020*).

Atherosclerotic plaques consist mainly of cellular elements: smooth muscle cells, macrophages and leukocytes, extracellular connective tissue, collagen, elastic fibers, proteoglycans, and lipid deposits inside and outside cells. In different plaques these components are present in various proportions, resulting in a wide spectrum of lesions with different degrees of instability.

The coating "fibrous cap" is formed by smooth muscle cells, a few leukocytes and relatively dense connective tissue. The area located under and next to the fibrous cap, the "shoulder" is formed by macrophages, smooth muscle cells and T cells. The nucleus, deeper and often necrotic, consists of disorganized lipid material, formed by cholesterol crystals, cellular debris, organized clots, and other plasma proteins. Lipids are basically made from

cholesterol and its esters and are present in cells that phagocytize this lipid material, called "foamy cells" (foamy cells): mainly circulating monocytes activated in the tissue in macrophages and activated smooth muscle cells. Finally, especially in the periphery of the lesions, are frequent neovascularization traits, represented by the proliferation of new blood vessels. Change in the histological characteristics of plaques depend on, in addition to the lipid content, on the number of smooth muscle cells and macrophages, the amount of collagen and other extracellular components [65].

The pathogenetic development of these lesions is still unclear; endothelium is thought to play a crucial role in the initial development of plaque. The most accredited hypothesis puts at the base of this process, a reaction to the intimal damage. Atherosclerosis would in this case represent an inflammatory response of the vascular wall [1].

A feature of primary lesions is the presence of a morphologically damaged endothelium, this allowed to focus on its importance in the development of the disease, such as dysfunction and cellular activation, with an increase in endothelial permeability. This is manifested by increased adhesion of leukocytes and monocytes, evidenced by alterations in the expression of endothelial adhesion molecules Inter Cellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecules 1 (VCAM-1) [66-67]. The main

causes of dysfunction and endothelial damage are hemodynamic changes that occur in the points of the circulatory tree, such as bifurcations, and the harmful effects of hypercholesterolemia.

Role of blood flow

In support of the role of blood flow, and its modifications from laminar to turbulent with loss of tangential force, therefore low "shear tension" is the very localization of plaques that develop more typically in the stenotic zone or at the level of bifurcations [68,69]. It is believed that the modified flow, with different levels of parietal stress, causes local endothelial dysfunction and thus predisposes to the development of lesions at these sites.

All this causes the activation of numerous pro-inflammatory and pro-atherogenic genes, from which the production of cytokines, adhesion molecules and coagulation proteins, which cause an increase in endothelial permeability, of cellular turn-over and cellular LDL endocytosis.

Role of hypercholesterolemia.

Hypercholesterolemia is closely involved in the development of plaque. In addition, the hyper lipidic state itself causes endothelial dysfunctions, for increased oxygen production of free radicals that deactivate nitric oxide, the main relaxing factor in the arteries.

Oxidative changes induced by free radicals produced by macrophages and endothelial cells contribute to the formation of LDL-oxidized, which in turn determine the formation of lesions in various ways; For example, they can be phagocytized by macrophages that turn into foamy cells, or are chemotactic for circulating monocytes, or stimulate the release of growth factors. etc.

The hypothesis that hyperlipidemia leads to the formation of lesions through oxidative stress on the endothelium is demonstrated by 55 clinical and experimental studies that highlight how antioxidant proteins and drugs, reducing oxidation, have a protective effect on atherosclerosis; in addition, cholesterol lowering, and antioxidant therapy improve endothelial function [70].

In the mechanism of plaque genesis, the adhesion of monocytes on the endothelial surface is a consequence of release from the adhesion molecules and then migration through these cells going to localize in the sub-endothelium. Here they transform into macrophages and phage the LDL-oxidized molecules becoming foam cells.

Macrophages have a multifactorial role in the progression of atherosclerosis thanks to the secretion of proteins, such as interleukin-1(IL-1) and tumor growth factor (TNF), which increase the adhesion of leukocytes. Macrophages also produce oxygen radicals that cause LDL oxidation in lesion and

process growth factors that can contribute to the proliferation of smooth muscle cells.

At the onset of injury development, smooth muscle cells migrate to the intima where they proliferate and determine the deposit of extracellular material, thus promoting plaque growth [71]. In the early stages of atherogenesis, intimal plaque consists of the aggregation of foam cells, some of which can die by releasing extracellular lipids and cellular debris surrounding muscle cells. The cellular-adipose atheroma changes progressively following the deposition of collagen and proteoglycans. The connective tissue is particularly abundant in the intimal area where it produces the "fibrous cap", which thus develops the mature fibro-atheroma [1].

Histopathological classification of carotid plaque according to AHA

American Heart Association (AHA) have made a histopathological classification of atherosclerotic lesions according to the natural progression of the lesion, a numerical staging universal system currently used.

The specific features of each stage are described below and are represented graphically (Figures 6-7).

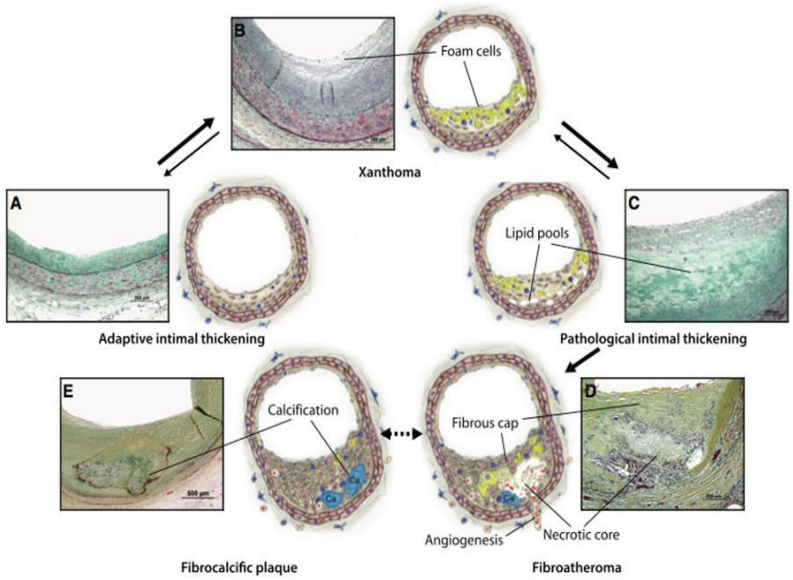


Figure 6 - Main lesion types of atherosclerosis and proposed sequence of their development. (*From Fog Bentzon et. al., Circ. Res. 2014*)

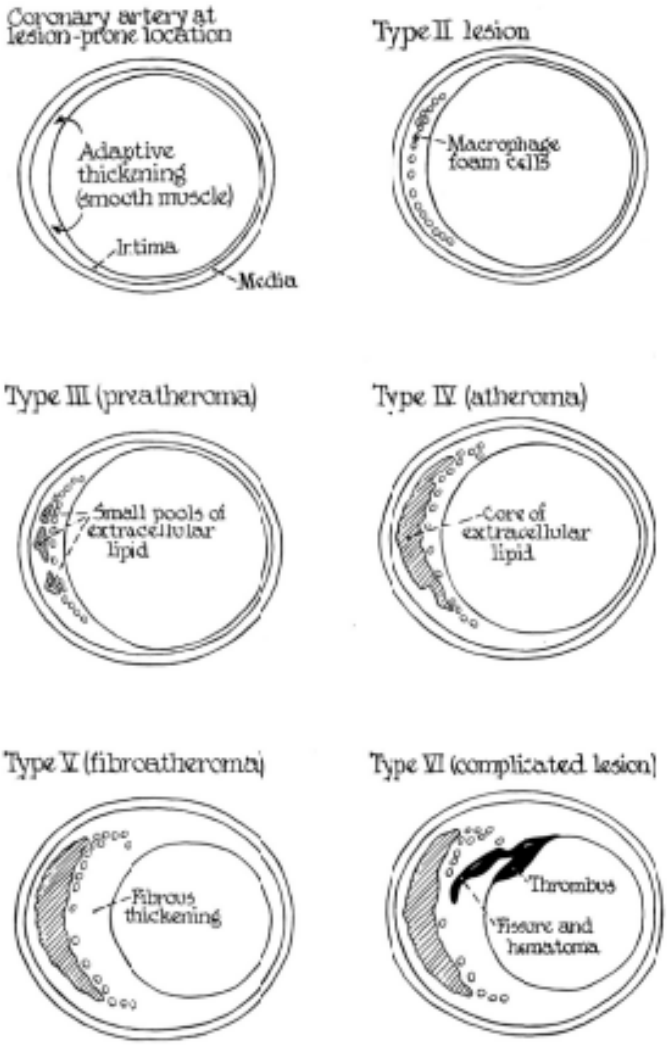


Figure 7 - Histological classification of atherosclerosis lesions, according to American Heart Association (AHA). *(From Stary C., et al. Circulation 1995).*

I-III lesions are initial lesions, silent precursors of advanced lesions (IV-VI) from which clinical events originate. In the first three decades of life the composition of lesions is predominantly lipidic and the stage is normally between I and III; after this period the evolution of plaque becomes unpredictable (stage IV-VI).

The formation of the intima lesions of the aorta and coronary arteries undergoes an acceleration at 25-30 years for men and at 40-45 years for women.

Stage I - Silent lesions characterized by a thickening defined as "adaptive", since it is considered the physiological response of the artery to the insults of blood flow. These lesions are typical of infants and children and are present in adults in parts of the arteries less susceptible to injury.

They consist of lipid deposits detectable only under electron microscope. The initial lesion has enough atherogenic lipoproteins to determine an increase in macrophages and their transformation into foamy cells, thanks to the phagocytosis of the same lipoproteins and lipids associated with them.

Stage II - Type II lesions are typical of puberty and are related to plasma cholesterol concentrations. They consist of foamy cells that accumulate in layers, T-cells, arranged in yellow streaks, approximately detectable on the surface of the intima, and smooth muscle cells; this lesion is known as "fatty steak".

Stage III- Type III lesions, called intermediate, transient or pre-atheroma lesions, are typical of young adults. In addition to foamy cells, extracellular lipid accumulations are present.

Stage IV- Type IV lesions, or atheroma, consist of a dense accumulation of extracellular lipids (lipid nucleus) occupying a well-defined region of the intima, resulting in severe disorganization of the

latter. Between the lipid nucleus and the endothelial surface, the intima contains macrophages and smooth muscle cells. The formation of the lipid core increases the fibrous tissue which subsequently modifies the structure of the intima above the lipid nucleus. In this lesion there are peripheral regions more susceptible to breakage because macrophages are abundant and, through the release of metalloproteinases (MMP), they can damage the fibrous tissue.

Stage V- Type V lesions are characterized by the formation of fibrous tissue; the lipid nucleus cover undergoes an increase in fibrous tissue, mainly collagen (fibrous cap). Type V lesions can be classified into: Va fibroateroma with prevalence of lipid core; Vb fibroateroma with calcifications; Vc, predominantly fibrous, with almost no lipid core. These lesions may narrow the arterial lumen and develop into fissure, hematoma and /or thrombus and thus be clinically relevant. Type V lesions consist of many inflammatory cells and can show intra plaque neovascularization. Type IV and V are lesions with highest morbidity rate and mortality from atherosclerotic disease.

Stage VI- Type VI lesions are defined as complicated, as they frequently induce chronic or acute clinical manifestations. They are frequent from the fourth decade of life and are distinguished in the following subtypes: VIa, with ulcerations of the endothelial surface; VIb, with presence of hematoma or

hemorrhage; VIc, with thrombosis; VIabc, with presence of the already mentioned alterations.
Complications may arise due to individual differences in risk factors and tissue reactions.

Morphological, cellular, and molecular characteristics of carotid plaque

"Vulnerable Plaque"

In the last two decades, the concept of "vulnerable plaque" has gained increasing importance, being used to determine different levels of risk in atherosclerotic cardiovascular events.

Atherosclerotic plaques can be defined as "stable" or "vulnerable", depending on their morphological and cytochemical characteristics (Figure 8).

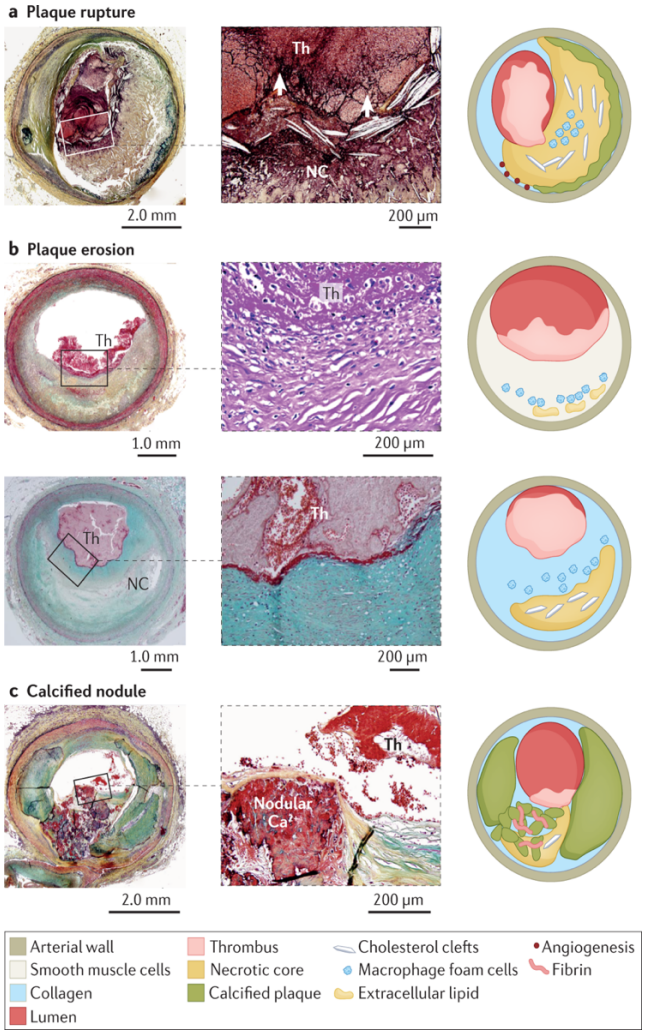


Figure 8 - Matching between stable and vulnerable plaque. Histological images comparing vulnerable plaques, specifically the moment of rupture (a) with the moment of erosion (b), and a calcific nodule or stable plaque (c). (Th: *Trombus*, NC: *Necrotic Core*, Ca^2: *Calcium*). (*From Gaba et. al, Nature Reviews Cardiology 2023*).

Stable plaques are usually characterized by a thick fibrous cap and a higher rate of calcification. The incidence of ischemic events in patients with these plaques is less frequent than in patients with vulnerable plaques. Several studies have identified

the correlation between plaque and the incidence of cerebrovascular events. In the study of Vasuri et al [72], a histological analysis of plaque was performed; and it was noted, in those plaques with higher calcification rate, the presence of a reduced inflammatory infiltrate, a smaller lipid nucleus and a lower degree of angiogenesis. Owing to their structure, these plaques were correlated with a lower incidence rate of TIA or stroke than those with low calcium level [72]. In the study of Kiran et al. [73] the calcified plaques studied are related to an 11 times lower probability of developing symptoms than those not calcified. This suggests that the presence of calcium can stabilize plaque, making it less susceptible to biomechanical stressors. The clinical application of the concept of "stable plaque" is particularly important in asymptomatic patients with high degree of stenosis.

Vulnerable plaques (also referred as "high-risk plaque" or "unstable plaque") are characterized by a large lipid nucleus, composed of lipids and cellular debris, a thin fibrous cap and a significant infiltrate of inflammatory cells.

In addition, they may show ulcerations or intra-plaque bleeding and thrombotic phenomena. The latter are associated with an increased risk of distal rupture and embolization. Molecules released by inflammatory cells, particularly MMPs, are responsible for reducing the thickness of the fibrous

cap, endothelial dysfunction, and thrombogenicity of plaque components.

Reducing the thickness of the fibrous cap has a key role in destabilizing plaque [74,75]. The fibrous cap is composed mainly of collagen. It is defined as the area between the nucleus and the lumen. The lipid nucleus is the inner region of plaque. It consists of ox-LDL, cellular debris and necrotic components [74,75,]. MMPs, a family of zinc peptidases, released and activated by pro-inflammatory cytokines [76], mediate extracellular matrix degradation and remodeling.

Plaque vulnerability is increased by intra plaque angiogenesis. The formation of new vessels was evaluated with immunohistochemical studies on a plaque sample obtained by CEA [72]. Plaque growth and progression are directly related to vessel density, while vessel morphology is related to plaque instability. The endothelial cells of the new vessels lack cellular junctions; this makes atherosclerotic plaque more incline to hemorrhage on site.

Adhesion molecules for leukocytes are expressed by the endothelial cells of the new vessels in greater quantities than endothelial cells of the normal lumen. Several studies show that symptomatic patients have newer intra plaque vessels more irregular than asymptomatic ones [72].

In Bobryshev et al study, over 90% of dendritic cells (DC) accumulated in the shoulder region at plaque

level, 70% of which expressed the CD83 receptor, a marker of dendritic activation.

In the shoulder region, DC and abundance of T cells and macrophages were identified, while many Natural Killer (NK) cells were observed in the necrotic nucleus [77]. Other predictive factors of plaque vulnerability are serum biomarkers indicating systemic inflammatory status, as PCR and MMPs [78].

Therapeutic and surgical treatment

Therapeutic treatment

Thanks to various non-invasive diagnostic techniques, most asymptomatic patients receive diagnosis of carotid stenosis and can therefore be treated. Every year patients with stroke risk that present stenosis > 80% is between 3.5% and 5%. For the treatment of asymptomatic carotid atherosclerosis (ACAS) and asymptomatic carotid stenosis (ACST), bilateral myringotomy (BMT) and carotid endoarterectomy (E-CEA) were compared in asymptomatic patients, suggesting that aggressive intervention with E-CEA produces more beneficial results than BMT alone. The limitations of both methods are that neither uses the current standard of pharmacological care for the medical treatment of carotid stenosis [79].

The best medical treatment for stroke currently includes anti-platelet agents, statins, anti-hypertensive agents, a strict diabetic control (glycemic) and changes in the lifestyle of patients.

Hypertension should be kept under control with therapies to maintain a blood pressure value average below 140/90 mmHg; patients with diabetes mellitus or kidney disease should maintain blood pressure values of <130/80 mmHg. Considering patient's comorbidity, compliant drugs can be prescribed.

The guidelines of the European Society of Vascular Surgery (ESVS) suggest that for the prevention of stroke, calcium antagonist drugs in mono therapy are more effective than angiotensin-converting enzyme inhibitors (ACE) or angiotensin receptor blockers (ARB) [80, 81].

Hypolipidemic therapy should allow plasma values of LDL cholesterol below 100 mg/dl.

In patients with many cardiovascular risk factors or co-existing CAD, a target plasma value of less than 70-72 mg/dl is preferred. Drugs, such as statins, are recommended in patients with ischemic stroke, TIA, asymptomatic carotid stenosis >50% and /or CAD with obvious atherosclerotic origin.

In addition, diabetes screening is necessary or always recommended; and pts with diabetes should be treated pharmacologically [80, 81]. Asymptomatic patients benefit from aspirin treatment; symptomatic patients should be treated with antiaggregant therapy as soon

as the diagnosis of TIA or stroke is made. Smoking should be stopped in both asymptomatic and symptomatic patients. Daily exercise should be performed, and a BMI <25 [80] should be achieved.
Symptomatic patients, however, must undergo surgery, unless the risk of the latter is excessively high (e.g., severe cardiopulmonary disease, recent cerebral infarction or with hemorrhagic conversion).

Surgical treatment

The indication for the surgical treatment of patients with carotid disease must consider neurological symptomatology; the degree of carotid stenosis; the morphology of carotid plaque; medical comorbidities; vascular and local anatomical characteristics.
Neurological symptoms and the degree of stenosis are the indications to be considered for invasive treatment. Considering medical comorbidities, vascular, local anatomical features and plaque morphology, the surgeon decides to practice carotid E-CEA (Figure 9) or carotid stent (CAS).

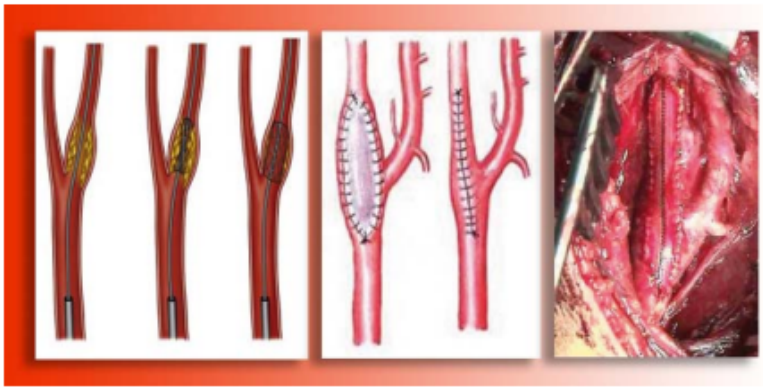


Figure 9 - Example of carotid endarterectomy (E-CEA) surgical technique. (*From Kotsis T., et.al., Int.J. of Angiology 2019*).

ESVS guidelines suggest surgery as the best option for symptomatic pts [82].

The grade of stenosis in the symptomatic patient is calculated using the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria [83]. According to the NASCET criteria, a ratio of 4 identifies a 70% of stenosis, which corresponds to 83% of the stenosis of the ECST criteria.

Data from the European Carotid Surgery Trial (ECST) and NASCET studies show that surgery increased the risk of 5 years of ischemic ipsilateral stroke in patients with stenosis below 30% (absolute risk reduction = 2.2%, p = 0.05), had no effect in patients with 30% -49% stenosis (3.2%, p = 0.6), was of marginal benefit in those with 50% -69% stenosis (4.6%, p = 0.04) and was highly beneficial in those with 70% stenosis or greater without almost occlusion (16.0%, p< 0.001) [83, 84].

Recommendation for the invasive treatment:

- Surgical treatment of carotid disease is absolutely indicated in symptomatic patients with stenosis> 70% (NASCET) and probably with stenosis> 50% (NASCET).
- Perioperative stroke mortality should be <6%.
- CEA is contraindicated in symptomatic patients with stenosis below 50%.
- CEA should be performed within 2 weeks of the last symptoms of the patient.
- CEA may be recommended for asymptomatic men under 75 with a 70-99% stenosis if the risk associated with surgery is less than 3%.
- The benefit of CEA in asymptomatic women with carotid stenosis is significantly lower than in men. The CEA should therefore be considered only in young and fit women [82].

According to the AHA guidelines, the best way to treat most asymptomatic patients with 60% to 99% stenosis is E-CEA in association with BMT [85].

NATURAL KILLER CELLS

Characteristics and role in innate immunity

Natural Killer (NK) cells are the first and best described innate lymphoid cells (ILCs). They are a subpopulation of ILC1, which play a crucial role in the innate response against viruses and intracellular

bacteria. These cells are effectors of innate immunity, which recognize and kill infected and/or damaged cells by releasing lithic molecules and secreting inflammatory cytokines [86-87-88].

NK cells form 5-20% of blood and spleen mononucleate cells, while they are rare in other lymphoid organs. The term natural killer comes from the in *vitro* ability of these cells to kill target cells without any type of activation (unlike $CD8^+$ T cells that must be activated before differentiating into cytotoxic).

In relation to cytotoxic activity, NK cells products a relevant quantity of Interferon (IFN)-γ, a pro-inflammatory cytokine that promotes the microbicidal activity of macrophages [86]; moreover, NK cells also release various cytokines and chemokines of significant importance.

Human NK cells include two subgroups of cells: in human peripheral blood (PB) most NK cells (95%) belong to the $CD56^{dim}$ $CD16^+$ subset; the secondary subgroup of NK cells in blood (5%) is characterized by $CD56^{bright}$ $CD16^-$ cells ; that representing the majority of NK cells in peripheral tissues, secondary lymphoid organs (75%) [87] and afferent lymph [88,89].

These subsets of NK cells were originally described as having different functional activities (Figure 10): $CD56^{dim}$ $CD16^+$ NK cells, that also express killer Ig-like receptors (KIRs) and high levels of perforin, that

have enhanced cytolytic activity; and $CD56^{bright}$ $CD16^{-}$ NK cells, that express low levels of perforin, no KIRs, and are able to secrete large amounts of cytokines (e.g., IFN-γ, GM-CSF, and TNF-α), but are devoid of killing activity [90].

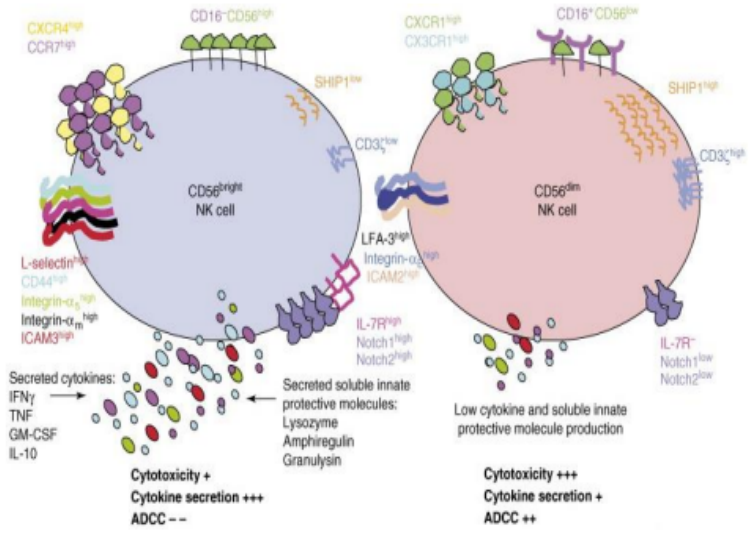


Figure 10 - Natural Killer cells feature. On the left representation of $CD56^{bright}$ NK cells; on the right representation of $CD56^{dim}$ NK cells. (*From Cooper M., et.al. Blood 2021*)

NK cells recognize and kill infected or transformed cells, as stressed cells, or tumor cells.

This ability to distinguish between altered or normal cells depends on the expression on the surface of NK cells of two types of receptors: inhibitory receptors and activating receptors.

NK cells inhibitory receptors recognize MHC (Major Histocompatibility Complex) class I molecules. These molecules are constitutively expressed by most normal cells of the body; but often, not expressed by viruses infected cells or neoplastic cells. Lack of this expression leads to loss of inhibitory signaling by NK cells.

At the same time, the infected cell expresses ligands for activating receptors, which, in turn, induce activation of the cytotoxic ability of NK cells.

NK cell activating receptors can recognize structures on both NK cell targets and normal cells, but when MHC class I molecules are recognized, the influence of inhibitory signals is dominant.

In some cases, in which the expression of activator receptor ligands has been recently induced or has increased into infected or transformed cells, activating receptors predominate over the action of inhibitory receptors and allow NK cells to also kill cells that express MHC class I molecules. Some activating receptors recognize MHC-like class I molecules expressed alone on damaged or transformed cells.

There are several families of Natural Killer cell receptors.

Inhibitory receptors

The binding of inhibitory receptors to their ligands determines a signal transduction cascade that involve phosphatase protein, which counteracts the effects of activation of protein kinases activated by activator receptors. The specificity of inhibitory receptors for MHC class I molecules (ligands for these receptors) protects normal host cells by killing from NK cells.

These belong to three main families and a common feature is the presence in their cytoplasmic tail of Immunoreceptor Tyrosine-based Inhibition Motif (ITIM). The first shown family is Ig-like killer receptors (KIR), which recognize different alleles of HLA-A/B/C (human leukocyte antigen). The binding of HLA class I molecules to KIR molecules is very important for recognition of molecules by KIR. This bond is characterized by great speed of activation and shutdown, which is thought to employ NK cells to quickly "test" the presence of MHC in many cells.

In addition, short-lived inhibitory signals generated by KIR, can stimulate NK cell to kill an MHC-negative cell target. A second family of inhibitory receptors consists of similar Ig transcripts. A member of this family, ILT-2, has a great specificity for many MHC class I alleles and contains four ITIM.

The third family of NK cells inhibitory receptors consists of heterodimers composed of C-type lectin, called NKG2A (characterized by two ITIM domains), covalently bound to the molecule CD94.

CD94/NKG2A receptors bind HLA-E, a non-classical class I MHC molecule. D94/NKG2A inhibitory receptors perform a control function that identifies the absence of HLA-E, classical MHC class I- molecules and HLA-G molecules.

Like KIR receptors some CD94/NKG2 receptors have no ITIM domains and act as NK-activating receptors. These domains are essential for the signal functions of inhibitory receptors. ITIM patterns recruit phosphatases that counteract the effects of kinases involved in signal transduction initiated by activator receptors.

As result of the binding of MHC class I molecules to NK cell inhibitor receptors, tyrosine residues from ITIM sequences of cytoplasmic tails are phosphorylated, leading to phosphatase recruitment, which remove phosphates from many proteins involved in signal translation. These events result in a reduction of signal sent by the activating receptors, which are coupled to protein kinases that add phosphates to many intracellular substrates [86].

Activating receptors

NK cell activating receptors include structurally distinct number groups of molecules for which only a few ligands are known. One of the first activating receptors identified in NK cells is CD16, a receptor to recognize the Fc portion of IgG1 and IgG3

antibodies; responsible for antibody-dependent cellular cytotoxicity (ADCC).

A newly discovered group of human NK receptors, called natural cytotoxicity receptors (NCRs), includes NKp46, NKp30 and NKp44; the first receptors that concern the cytotoxicity of NK cells have been identified and characterized [91]. Whereas NKp46 and NKp30 are constitutionally expressed, NKp44 is selectively expressed by NK cells that have undergone previous activation [92]. In recent years, transcript 3 associated with HLA-B (BAT3), protein pp65 and member B7-H6 of the B7 family have been identified as ligands recognized by NKp30 [93,94]; while leukemia-5 protein (MLL5) of the mixed lineage has been identified as one of the cellular ligands of NKp44 [95].

These proteins are members of the Ig superfamily. Although their ligands are not yet known, studies with blocking antibodies suggest their dominant role in killing neoplastic target cells. An additional activating receptor of NK cells is NKG2D, also expressed by T cells. It recognizes in humans cell surface molecules belonging to the main families of histocompatibility complexes of class I linked to the MIC chain: MIC-A, MIC-B; and also, UL16-binding protein ULBP [95,96]. All these ligands have domains homologous to the alpha and beta domains of the MHC class I.

The expression of NKG2 ligands is characteristic of normal cells but is induced by signals of cellular stress or DNA damage, or also by processes of neoplastic transformation and virus-infected cells. Therefore, NK cells can use these receptors to eliminate damaged cells and neoplastic cells. DNAM-1, an activation receptor expressed by almost all NK cells and partially shared with T cells and monocytes, specifically recognizes PVR (CD155) and Nectin-2 (CD112) [97], two members of the nectine family. Other surface activating molecules, including 2B4, NTBA and NKp80 [91], may also contribute to the activation of NK cells by acting as co-receptors capable of amplifying the activation of NK cells induced by NCR or NKG2D.

The interaction of the ligand with the NK-activating receptors determines the production of cytokines and increases migration to sites of infection and promote the killing of target cells. A shared feature of activating receptors is the presence of non-covalently bound subunits, whose cytoplasmic tails recruit kinases involved in signal transduction.

The CD16 receptor and some activating receptors are associated with subunits featured Immunoreceptor Tyrosine-based Activation Motif (ITAM).

Then the tyrosine residues of ITAM are phosphorylated by SRC kinase, and thus a phosphorylation cascade of signal transduction is generated.

The NKG2D receptor is associated with a subunit called DAP10, which has a tyrosine motif in the cytoplasmic tail. After phosphorylation, this pattern binds phosphatidylinositol-3 kinase (PI3K) and Grb2, which initiates a signal cascade other than that initiated by ITAM phosphorylation. Some members of the KIR and CD94/NKG2 receptor families, such as KIR2DS and CD94/NKG2C, do not contain ITIM domains, but associate with accessory molecules that express ITAM and send NK cell activator signals. As it is known, some of these receptors recognize MHC class I molecules and it is unclear why such potentially harmful receptors exist on NK cells.

Activating receptors bind MHC class I molecules with minor affinity than correlated inhibitory receptors; and it seems that activating receptors can bind MHC molecules related to pathological conditions.

Most NK cell activating receptors are also expressed by some subpopulations of T cells. However, the expression of these receptors is induced only in T cells activated by antigenic recognition, while the expression of receptors on NK cells is constitutive.

This highlights an important distinction between innate and adaptive immunity. In particular, the effector cells of innate immunity, such as NK cells, are fully differentiated and ready to respond to infections, whereas the effector cells of adaptive

immunity are generated only after exposure to the antigen from naive precursors.

Effector functions of NK cells

As already mentioned, NK cells kill infected cells and activate the microbicidal activity of macrophages. NK cells have granules that contain proteins responsible for killing target cells. When NK cells are activated, the exocytosis of the granules leads to the release of these proteins near the target cell. A granule protein, called perforin, facilitates the entry of other proteins, called granzymes, into the cytoplasm of the target cell. Granzymes are enzymes that induce apoptosis of the target cell. By killing infected cells with viruses and intra-cellular bacteria, NK cells eliminate "reservoirs" of infection.

IFN-γ produced by NK cells activates macrophages, increasing their ability to kill phagocytic bacteria. Finally, some tumors are targets of NK cells, perhaps because neoplastic cells do not express normal levels or the appropriate type of MHC class I molecules. This mechanism has been studied in vitro; therefore, it has been proposed that these cells also have the function of killing malignant clones in vivo [86].

Involvement of Natural Killer cells in atherosclerosis

The presence of NK cells within human atherosclerotic plaques has been documented in several studies [98,99,100]. In fact, some chemokines present in atherosclerotic lesions, released by endothelial cells and activated macrophages themselves, can directly influence the recruitment of NK cells at this site.

The Monocyte Chemoattract-1 Protein (MCP-1), that attracts monocytes, has been found in atherosclerotic lesion [101,102] and has been shown to be chemotactic also for NK cells [103].

Another chemokine observed in both human and murine atherosclerotic lesions, was defined Fractalchin (CX3CL1) [79], and it induces the migration and activation of NK cells, resulting in increased cytotoxicity and production of the pro-atherogenic cytokine IFN- γ [104].

In addition, several cytokines, such as IL-15, IL-12, IL-18 and IFN-α, capable of recruiting and activating NK cells, have been shown to have pro-atherogenic effects [105,106,107]; therefore, their atherogenic potential may be partially linked to NK cells involvement.

Through the observation of patients with severe atherosclerotic pathology, it was demonstrated that NK cells may play a role in atherosclerotic disease, as high levels of circulating NK cells have been

observed [108]. A recent study demonstrated the presence of NK cells in CAP of symptomatic patients, able to release large amount of IFN- γ [109].

IFN-γ is one of the main cytokines released by activated NK cells behaving as a pro-atherogenic cytokine, which induces plaque destabilization, apoptosis of smooth muscle cells and activation of MMPs [109,110,111,112]. IFN- γ produced by CAP-NK cells might also contribute to determine shedding by MMPs of ligand to NK cells' activating receptors. This hypothesis is further sustained by the observation that the frequency of IFN-γ+ NK cells correlated with serum levels of sMICA in symptomatic atherosclerotic patients [109].

Regardless of which subset or function of NK cells is involved, it must be stated whether cells affecting plaque can express ligands for NK cell activating receptors, since different interactions between NK cell receptor and ligand have been associated with atherosclerotic disease [113-114]. It has been reported that, while vascular smooth muscle cells are not targeted by NKs, macrophage cells were found close to NK cells within atherosclerotic lesions.

Thus, these two innate cellular populations can interact during the atherosclerotic process [112].

In line with this hypothesis, it has been previously demonstrated that both macrophages and endothelial cells of atherosclerotic vessels express NKG2D MICA/B ligands [109], further supporting the

hypothesis that macrophages can actively interact with NK cells in atherosclerotic lesions.

Surprisingly, NK cells and DC cells may also interact within atherosclerotic plaques. It has recently been shown that ox-LDL significantly promotes the interaction of NK and DC via dependent contact CD48-2B4 mechanisms [115], mediated by IL-12p70 and IFN-γ [116].

These findings suggest that the NK-DC crosstalk may also be involved in the activation of the immune system cellular network that occurs within atherosclerotic lesions, where NK cells and DC cells could interact efficiently in the presence of ox-LDL. Although, the immune response occurring within atherosclerotic lesions has not yet been fully elucidated, it is now established that the pathology begins with inflammatory processes affecting endothelial cells of the vascular wall.

During an inflammatory response, the different cells of the immune system act not only directly, but also by reciprocal action to optimize protective responses or to worsen the disease (pathological immune responses). Over the past decade, several studies have focused on the relationship between NK cells and different antigen-presenting cells, including macrophages and dendritic cells.

As previously described, within the atherosclerotic lesion activated NK cells drive the differentiation of monocytes into DC cells and stimulate the

monocytes-NK interaction; so, it could be an interesting new insight to investigate the role of NK cells in the pathogenesis of this disease.

Therefore, the reciprocal activation that occurs between NK cells and antigen presenting cells can induce an expansion of the initial inflammatory process, playing a critical role in the maintenance and progression of atherosclerotic disease.

NATURAL KILLER CELLS AND HUMAN CYTOMEGALOVIRUS (HCMV) INFECTION

HCMV and its involvement in atherosclerosis

Atherosclerosis appears to be a multifactorial disease [117]. Several epidemiological studies have established the traditional risk factors involved in this pathology [109,118]. Besides traditional factors, numerous scientific evidence has reported an association between atherosclerosis and infection from bacterial and persistent viral pathogens, including *Herpes Simplex virus* (HSV), *Human Cytomegalovirus* (HCMV), *Chlamydia pneumonia*, *Helicobacter pylori* and some other infectious agents [117]. Human cytomegalovirus (HCMV) is a common opportunistic pathogen with a worldwide prevalence of 45–100% depending on age, location,

gender, and socio-economic status [119]. Whereas in healthy individuals, generally infection does not result in morbidity, the virus can cause devastating complications in neonates and immunocompromised patients [119].

Initial infection is followed by life-long persistence characterized by periodical reactivation episodes.

The persistence of the virus is demonstrated in renal transplant recipients, who show evidence of active HCMV infection [120]. The ability of this virus to reactivate from a latent state contributes significantly to its success as a human pathogen, yet the tissue distribution of latent CMV remains poorly understood. HCMV genomes were detected in different tissue as lung, spleen, bone marrow, blood, and lymph nodes [121]. HCMV can infect a remarkably broad cell range within its host, including parenchymal cells and connective tissue cells of any organ and various hematopoietic cell types. Epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells are the predominant targets for virus replication [122]. Moreover, recent scientific knowledge shows that HCMV selects as a reservoir, in its state of latency, immune cells. HCMV viral genomic DNA was discovered in circulating $CD14^{+}$ cells (monocytes cells) from healthy seropositive subjects [123]; as well as, in general, in peripheral leukocytes [124]. Curiously, analogous studies *in vivo*, conducted into both man and mouse, suggested

that myeloid/macrophagic cells are cellular reservoir of virus in a latency status, e.g., experiments on animal model demonstrate the persistence of Murine Cytomegalovirus (MCMV) for 9 months in macrophages, after administration into the abdominal cavity [124].

Remembering that these cells, reservoir of HCMV, are among the main “actors” of the atherosclerotic process [66]; must wonder if HCMV may be involved in the pathogenesis of atherosclerosis.

Recently works suggest a certain role of CMV in the pathogenesis of atherosclerosis-associated diseases [125,126,127]. In addition, antigens as well as HCMV DNA were found in arterial smooth muscle cells, in aortic aneurism tissue, as well as coronary atherosclerotic plaques [128,129,130].

But few works and few information do not allow to understand the real involvement of the HCMV in the progression of the carotid atherosclerotic process, as the molecular mechanism induced by the virus that generate the development of this pathology.

One of these suggested mechanisms is the activation of innate immune cells present in CAP, as NK cells, that producing IFN-γ, pro-inflammatory cytokine, play a critical role in plaque instability [109,131].

"Memory like or adaptive" Natural Killer cells

NK cells provide critical host defense against viral pathogens, especially papillomaviruses and herpesviruses, as HCMV. Individuals with deficiencies in functional NK cells are highly susceptible to recurrent, systemic virus infections [132]. Despite the conventional description of NK cells indicate these cells as innate immune cells; studies over the past decade provide persuasive evidence that some NK cell responses exhibit features of adaptive immunity; including clonal-like expansion of antigen-specific effector cells and generation of long-lived memory populations capable of enhanced recall responses [133]. Antigen-specific memory responses by NK cells were first recognized in studies in mice involving MCMV. Resistance to MCMV is mediated by a subset of NK cells characterized by Ly49H, activating receptor which recognizes the virus-encoded glycoprotein, m157, expressed on the surface of MCMV-infected cells [134]. MCMV infection triggers the activation and proliferative clonal like expansion of $Ly49H^+$ NK cells, that have memory features, e.g., long-lived persistence and enhanced responsiveness to the secondary virus infection [136].

Analogous to the MCMV-specific memory responses demonstrated in mice, memory-like NK cell populations have also been described in humans in response to HCMV infection.

HCMV-seropositive individuals exhibit an expanded population of NK cells expressing the receptor complex CD94/NKG2C [137]. This is an activating receptor that, during the infection, binds UL-40 peptide-HLA-E complex, a complex formed by HLA-E specific ligand of this receptor and UL-40 peptide encoded by HCMV; and consequently, activating NK cells [136].

Expanded NKG2C^{+} NK cells population referred to as “memory-like” or “adaptive” NK cells, displays unique features, as adaptive immune properties, including long-term persistence, unique epigenetic profile and enhanced functional responsiveness to opsonizing antibodies [118,138].

Memory-like NK cells have a distinctive surface phenotype, with preferential expression of the maturation marker, CD57, and of inhibitory *immunoglobulin-like transcript 2* (ILT2) and KIRs, but reduced expression of the NCRs, NKp30 and NKp46, the intracellular signaling proteins, SYK and EAT-2, and the transcription factor, PLZF [131,138]. Remarkably, adaptive NK cells are commonly identified as FcεR1γ^{-} NK cells, many but not all of which are NKG2C^{+} cells [131,139]. In addiction of unique phenotype profile these cells also exhibit specific functional features, as enhanced ADCC functionality and IFN-γ production following exposure to antibody-coated target cells [131,138, 139].

The unique phenotype and function profile of memory-like NK cells are mirrored by epigenetic modifications at regulatory regions for the genes encoding FcεR1γ, IFN-γ, EAT-2, and PLZF [131-138]. For example, loss of silencing DNA methylation marks at a conserved non-coding sequence upstream of the IFNG promoter correlates with enhanced IFN-γ production by these cells [140, 141].

Several studies confirm that memory-like NK cells are involved in numerous infectious diseases, such as Covid-19, Hepatitis B; as well as in cancer [142, 143,144] and seem to have a protective role, thanks to their powerful functionality. Their long-term persistence and improved functional response to opsonizing antibodies, led to the belief that these cells could be used as a target vaccine [133].

On the contrary in atherosclerosis, as the immune response is known to exacerbate the pathological process; adaptive NK cells, that producing large amount of CD-16 mediated IFN-γ, proatherogenic cytokine, may be involved in plaque instability.

HYPOTHESIS AND AIM OF THE STUDY

HCMV is a DNA virus that remains latent in host cells upon primary infection [123,145]. HCMV can infect a broad range of host cells, including monocytes/macrophages, fibroblasts, epithelial, endothelial, dendritic cells, and muscle fibers [146,147]. Recent work has shown that seropositive individuals, 60-70% of the adult population in Western countries, have a varied distribution of HCMV in different tissues, predominantly in the lungs, spleen, lymph nodes, and bone marrow [121]. Remarkably, HCMV nucleic acids and/or antigens have also been detected in atherosclerotic plaques [120, 130]. In this context, HCMV has been proposed to possibly contribute to the progression of the carotid atherosclerotic plaque (CAP) [147,148,149].

NK cells are members of the innate lymphoid cell (ILC) family [150,151,152] and provide immediate immune responses against both tumor and virally infected cells, as HCMV [152].

In CAP of pts with cerebrovascular symptoms, ranging from amaurosis fugax to transitory ischemic attack (TIA) and stroke, has been demonstrated the presence of activated NK cells, stronger producers of IFN-γ, cytokine involved in plaque destabilization [109].

Notably, in individual infected by HCMV [124] have been identified “Memory-like or adaptive” NK cells, a subset of NK cells characterized by adaptive

immune features, including long-term persistence, unique epigenetic profile, and enhanced functional responsiveness (IFN- γ production) [138,140].

Having regard as reported, we hypothesize that HCMV infection/reactivation may trigger an innate immune response able to impair the stability of plaque. Specifically, we propose that HCMV-dependent NK cell activation within plaque should result in IFN-γ release and, in turn, to the induction of Metalloproteases (MMPs), which are largely produced by macrophages and critically involved in plaque destabilization [153].

This study will shed light on the role of HCMV infection in the pathogenesis of atherosclerosis. We will investigate whether and how the immune response to HCMV infection/reactivation is involved in plaque instability, with obvious spillovers in novel strategies of prevention and treatment.

MATERIALS AND METHODS

Ethics declaration

All the studies were carried out after obtaining the approval of the institutional ethics committee of the University Hospital "G. Martino" Messina and the subjects participated after signing the informed consent, according to the Declaration of Helsinki.

Patient selection and sample recruitment

A total of 64 patients, followed-up at the Unit of Vascular Surgery of A.O.U. Ospedale "G. Martino" for monitoring carotid plaque stability, was recruited for this study over a period of three years (2020 to 2023). Patients were evaluated with duplex-ultrasound [154]. As inclusion criteria for the study, all patients with CAP have a plaque stenosis higher than 50%. Patients were classified following conventional criteria as bearing high-risk plaques (High risk patients- HR patients) or low risk plaques (Low risk patients-LR patients) estimating the diameter reduction of the internal carotid artery (ICA) and evaluating plaque morphology and echogenicity [155]. The degree of stenosis was estimated according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria [156]. Pts underwent periodical follow-up for monitoring CAP stability with duplex-ultrasound; every 6 months. Pts were selected for invasive treatment according to the European Society for Vascular Surgery (ESVS) guidelines [157,158]. Carotid plaques were removed by eversion carotid endarterectomy technique. Pts with symptoms of stroke, transient ischemic attack (TIA), or amaurosis fugax within 6 months of diagnosis of carotid artery disease will be defined symptomatic. Among high-risk patients,

symptomatic complications occurred in thirteen pts (20,7%). Peripheral blood was drawn from all patients with CAP and from healthy controls of similar gender and age distribution with no evidence of carotid plaques in the ultrasonographic study. Clinical variables analyzed were age, sex, body mass index, classical vascular risk factors, and the presence of clinical atherosclerosis in other arterial territories (ischemic heart disease and peripheral vascular disease). Demographic data, symptoms, and cardiovascular risk factors (hypertension, ischemic heart disease, chronic obstructive pulmonary disease, dyslipidemia, diabetes mellitus, smoking, and chronic kidney failure); were collected into a database.

A standard clinical diagnostic test was used to analyze serum samples from patients and controls for HCMV-specific circulating IgG antibodies (DiaSorin, Saluggia, Italy). 58 patients with CAP (90,4%), were $HCMV^{+}$.

Carotid plaque and blood sample processing

After surgical removal, CAPs were widely washed in phosphate-buffered saline (PBS) to remove contaminants (cell debris and red blood cell aggregates). Then, samples were mechanically minced by scissors to obtain small fragments; and enzymatically digested with a mixture containing DNAse (250 U/mL, 100μg/ml Roche Diagnostics International Ltd., Rotkreuz, Switzerland) and

collagenase type IV (150 U/mL, 1 mg/ml Worthington, Lakewood, NJ) in RPMI 1640 for 60 min at 37° C. The suspension was filtered through a cell strainer, and subsequently, washed by centrifugation in PBS to remove residual enzyme and red blood cells. Mononuclear cells (MNCs) from plaque cell suspensions or heparinized blood samples were isolated by Ficoll- Hypaque (Sigma-Aldrich, St. Louis, Missouri) density gradient centrifugation (30 minutes, 25°C, 2100 *revolutions per minute,* rpm). Then cells were cryopreserved at − 80 °C in a freezing solution composed by 10% *dimethyl sulfoxide* (DMSO) and 90% *fetal bovine serum* (FBS), until their use.

HCMV Serostatus Analysis

Sera were processed using a serum separator tube (SST) and samples were allowed to clot for 2 h at room temperature before centrifugation for 15 min at 1,000 × g. Samples were stored at −20°C prior analysis. HCMV serological status was determined using the chemiluminescent immunoassay LIAISON® CMV IgG and IgM (DiaSorin, Saluggia, Italy).

Phenotypic Characterization of Memory-like NK Cell Subsets

Cryopreserved mononuclear cells (MCs), were thawed, washed with phosphate buffered saline (PBS)

and stained with previously defined saturating concentrations of the following fluorochrome conjugated mouse anti-human mAbs: anti-CD45 Krome Orange (clone J.33), -CD56 PE-Cy7 (clone N901 NHK1), -CD3 APC-H7 (clone UCHT1), -CD16 Pacific Blue (clone 3G8), -CD8 PC 5.5 (clone B 9.11), CD69 ECD (clone L78) from (Beckman Coulter Brea, CA); - NKG2C APC (clone REA205) from (MiltenyiBiotec Bergisch Gladbach, Germany); Grazyme FITC (clone GB11), Ki67 BB630 (clone B56), CD7 BUV805 (clone M-T701), Siglec 7 BB755 (clone F023-420), Syk BB660 (clone 4D10), PLZF PE-CF594 (clone R17-809), CD2 BB700 (clone RPA.2.10), PD1 V450 (clone EH12.1), NKG2A BV510 (clone 131411), DNAM BV605 (clone DX11), NKG2D BV711 (clone 1D11), NKp30 PE (clone 5 D12), CD45RA BUV563 (clone HI100), CD57 BB790 (clone NK-1), CCR7 BUV 563 (clone 2-L1-A), CD103 BUV 661(clone Ber-ACT8), KIR MIX BUV 737(clone DX27/DX9), CD62L BUV 805 (clone DREG-56), NKp80 PE (clone 5D12), CX3CR1PE-CF594 (clone 2A9-1), CD85j PE-Cy5 (GHI/75), TIGIT BUV 395 (clone 741182 from Becton Dickinson (BD Biosciences, San Jose, CA), for 20 min at room temperature (RT). After staining, samples were washed with PBS (Euroclone, Pero, Milano) (used for all the washing steps). Then, intracellular staining with anti-human FcεR1γ FITC (clone FCABS400F) from (Merch/Sigma Aldrich,

Darmstadt, Germany) was performed using PerFix EXPOSE from (Beckman Coulter Brea, CA) according to the manufacturer's indications. Samples were then acquired using BD FACSymphony A5 flow cytometer (BD Biosciences, San Jose, CA) and cytofluorimetric data were analyzed by FlowJo 10.8.1 software (Tree Star). Negative controls included isotype- matched irrelevant Abs.

Memory NK cell subsets were identified by antibody combinations within the lymphocyte region, determined by physical parameters (FSC and SSC).

ANTIGEN	CLONE	FUNCTION
Granzyme	GB11	NK cell Function
Ki67	B56	Proliferation
CD69	L78	Tissue residency
CD7	M-T701	Convetional NK
Siglec 7 / CD328	F023-420	Marcatore non memory
SYK / FCepsR1 / EAT-2		Marcatore non memory
PLZF	R17-809	Marcatore non memory
CD2	RPA.2.10	Adaptive NK
CD16	3G8	Adaptive NK
PD1	EH12.1	Exsaustion
NKG2A	131411	Convetional NK
CD14-CD19-FVS	MfP-9 / SJ25C1	Exclusion
DNAM	DX11	CMV Recognition NK REC
NKG2D	1D11	CMV Recognition NK REC
NKp30		Conventional NK
CD3	UCHT1	T cell-Exclusion
CD45RA	HI100	Convetianal/Adaptive NK
CD57	NK-1	Adaptive NK
CD45	HI30	Leuco gate
CCR7	2-L1-A	LN HOMING
NKG2C	134591	Adaptive NK
CD103	Ber-ACT8	Tissue residency
KIR MIX	DX27 / DX9	INHIBITORY REC
CD62L	DREG-56	PA HOMING
NKp80	5D12	NK cells
CX3CR1	2A9-1	Fractalkine receptor PA HOM.
ILT2 (CD85j)	GHI/75	Adaptive NK
CD56	NCAM16	NK cells

Table 1. Multiparametric flow-cytometry panel for Memory-like NK cells identification.

Evaluation of IFN-γ Production

Cryopreserved PBMCs were thawed and rested overnight in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin (both from Sigma-Aldrich, St. Louis, Missouri) and human Interleukin- 2 (IL-2) (MiltenyiBiotec, Bergisch Gladbach, Germany). Then, PBMCs were co-cultured with K562 cells or anti-CD16-coated P815 cells for 6 h at 37 ◦C, to

interact at an effector-to-target ratio of 1:1 in the presence of anti-CD107a. Brefeldin A and monensin both from (Merch/Sigma Aldrich, Darmstadt, Germany) were added 1 h after co-incubation to inhibit cell secretion. At the end of stimulation, cells were washed with PBS, and then stained for surface antigens. After staining, cultured cells were washed, then fixed with 1% paraformaldehyde (PFA) (Merck, Germany) for 20 min at 4°C, and permeabilized with 1% saponin (Merck, Germany). Anti-IFN-γ PE (clone 45,15) (Beckman Coulter Brea, CA) was added after fixation and permeabilization, for 30 min RT. Surface CD107a and intracellular IFN-γ expression were finally assessed by flow cytometry on effector cells.

Histological and immunohistochemical analysis of carotid plaque

To evaluate the morphological characteristic of CAP, endarterectomy specimens were fixed in 4% paraformaldehyde in 0.2 M PBS, decalcified (Shandon TBD-2 Decalcifier, Thermofisher Scientific, US), dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin (Paraplast, Supplies SPI, West Chester, PA, USA). Blocks were cut with microtome (sections of 4-5 μm) and each slice stained, with hematoxylin and eosin (HE), Sirius red (SR) and Masson's Trichrome stains.

The same specimens used for histological evaluation were employed for tissue localization, by immunohistochemistry staining, of NK cells, *Macrophagic Cells* (MCs) and HCMV. Paraffin blocks were cut (sections of 4-5 μm) and the sections placed on polysine slides (Thermo Fisher Scientific, Waltham, MA, USA), cleared with xylene and rehydrated in ethanol. Antigen retrieval was obtained in buffer citrate pH 6.0; endogenous peroxidase was blocked with 3% H2O2 in PBS.

Primary antibodies anti-Cytomegalovirus antibody (clones CCH2 + DDG9; Agilent, Santa Clara, CA, USA); monoclonal rabbit anti-CD68 antibody (clone SP251; Sigma-Aldrich, St. Louis, MO, USA); rabbit anti-NKp46 antibody (Biorbyt, Cambridge, UK) were incubated overnight at 4 ◦C in a moisturized chamber. The day after, the secondary antibodies (Vectastain, Vector, Burlingame, CA, USA) were added and 3,3′-Diaminobenzidine (DAB) (Sigma-Aldrich) was used to visualize the reaction. The sections were counterstained with Mayer's hematoxylin. Appropriate positive and negative controls were used in each test.

Cytomegalovirus/CD68 double immunostaining was performed using the EnVision™ G/2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red) (Agilent, Santa Clara, CA, USA), according to the manufacturer's protocol. Slides were photographed with a Nikon Ci-L light microscope using a digital

camera Nikon Ds-Ri2 and saved as Joint Photographic Experts Group (JPEG) with the software Adobe Photoshop 2021 (Adobe, San Jose, CA, USA).

Statistical Analysis

The frequency of NK-cell populations and the expression of IFN-γ bare presented as median (range). Bivariate analyses were performed using GraphPad Prism Version 5.01 (GraphPad Software, CA, USA). Differences between study groups were evaluated using non-parametric Mann-Whitney tests. Wilcoxon matched pair signed rank tests were used to assess changes over time. Correlations were assessed using Spearman's Rank Correlation Coefficient. P values <0.05 are considered statistically significant.

RESULTS

Clinical Characteristics of atherosclerotic patients

Out of 64 pts 24 were women (16%). Mean age was 71 ± 10 years (range 52–90). 19 patients (12,1%) were diabetic, 16 (10,2%) were smokers, 46 (29,4%) had hypertension, and 27 (17,2%) had hyperlipidemia. 49 patients were asymptomatic (31,3%) and 15 (9,6%) were symptomatic.

	Low-Risk (n=26)	High-Risk (n=38)
Age	66.5y (17.3)	73.5y (28)
Women	12 (3.1)	12 (4.6)
Stroke	0	1 (0.4)
TIA	0	3 (1.2)
Amourosis Fugax	0	0
Ischemic Heart disease	3 (0.8)	8 (3.1)
Hypertension	10 (2.6)	36 (13.7)
Smoker	2 (0.5)	14 (5.4)
Diabetes status	3 (0.8)	16 (6.1)
Hyperlipidemia	3 (0.8)	24 (9.2)
Renal Status	1 (0.3)	4 (4.2)
Plaque stenosis:		
50-70%	26 (6.8)	0
>70%	0	38 (14.5)
Symptomatic	3 (0.8)	12 (4.6)
aSymptomatic	23 (6)	26 (9.9)
HCMV seronegative pts	3	3
HCMV seropositive pts	23	35

Table 2. Clinical Characteristics of atherosclerotic patients
Categorical variables were reported as count (percentage). Continuous variable was reported as mean ± standard deviation. TIA indicates transient ischemic attack ; HCMV, human cytomegalovirus; pts, patients.

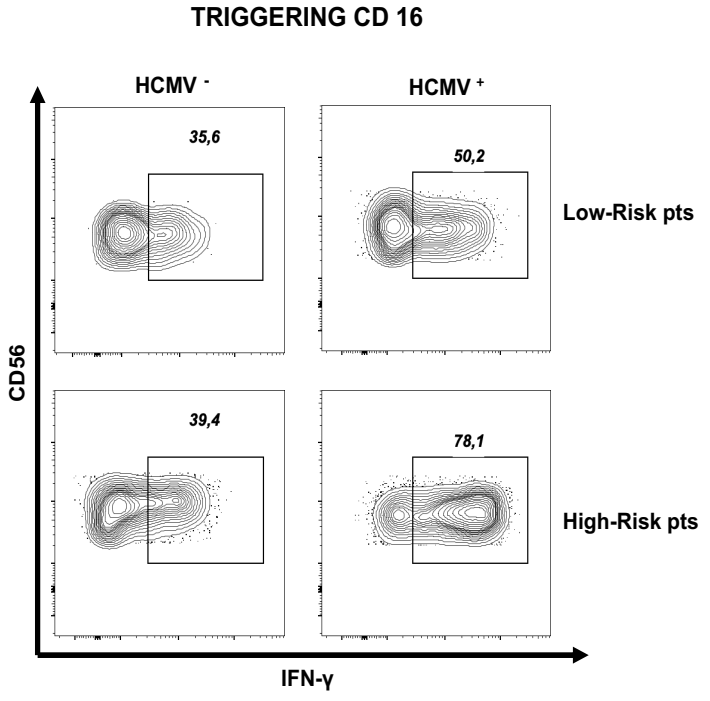
Increased CD16-mediated IFN-γ production is observed in high risk HCMV$^+$ patients

To investigate whether IFN-γ production by NK cells has a role in HCMV-induced atherosclerotic plaque progression we analyzed NK cell functionality in HCMV$^+$ and HCMV$^-$ atherosclerotic patients monitored for carotid plaque stability.

To assess NK cell functional potential, we evaluated the functional activity of peripheral $CD56^{dim}$ NK cells stimulated in vitro with P815 tumor cells, previously coated with anti-CD16, to mimic ADCC in vivo, and K562 tumor cells to reproduce natural cytotoxicity.

As displayed in Figure 11, peripheral $CD56^{dim}$ NK cells from $HCMV^{+}$ high-risk pts had higher responsiveness to CD16 in vitro engagement, and produced higher amount of IFN-γ, compared with NK cells from low-risk pts. Nevertheless, no difference was noticed by the K562-induced stimulation (natural effector functions), (data not shown).

A



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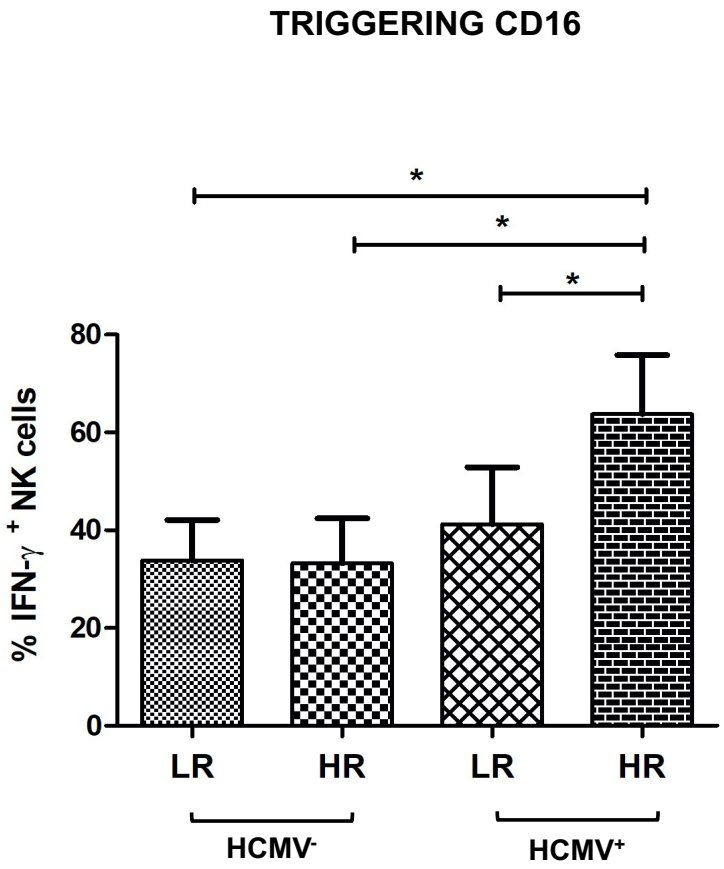


Figure 11 NK cells from HCMV$^+$ high-risk pts display enhanced CD16-mediated effector function, in terms of IFN-γ production compared to low-risk. A) Representative cytofluorimetric plots of IFN-γ release from peripheral CD56^{dim} NK cells of low-risk and high-risk pts **B)** Statistical analyses of the same data represented in figure A, from 6 HCMV$^-$ atherosclerotic pts, and 10 HCMV$^+$ atherosclerotic pts, that demonstrate the significantly higher CD16-mediated activation of NK cells from high-risk pts compared to low-risk. The Significant data are indicated as *P < 0.05. (LR: *Low-Risk pts*, HR: *High-Risk pts*).

Boosted CD16-mediated IFN-γ release in HCMV$^+$ patients is correlated to the expression of FcεR1γ$^-$

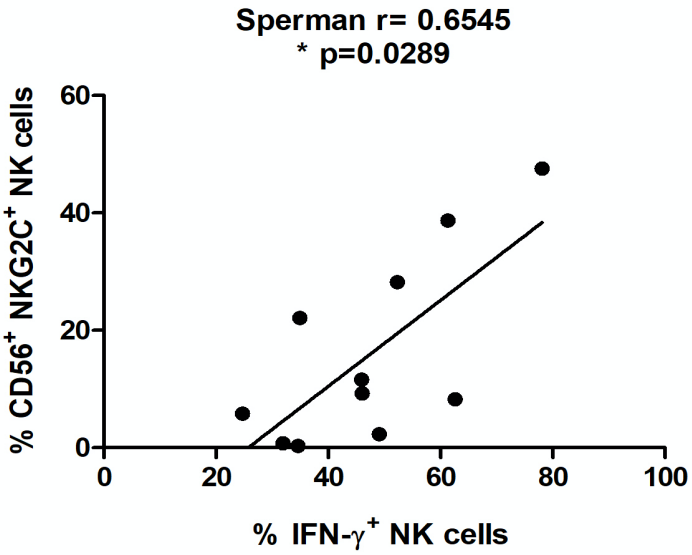
Thus, we wondered whether the potent functional behavior of NK cells in HCMV$^+$ pts might correlate with specific phenotypic features of this cell subset. HCMV infection drives phenotypic and functional reconfiguration of CD56^{dim} NK cells, in memory-like or adaptive NK cells [131]. These cells are characterized by adaptive immune properties, including long-term persistence, unique epigenetic profile and enhanced functional responsiveness, particularly regarding the capability to produce IFN-γ in response to CD16 stimulation [124,140, 131].

Human adaptive NK cells constitute a heterogeneous population commonly defined according to the expression of FcεR1γ and NKG2C [159]. [118].

As we did not observe any correlation between CD16-mediated effector function and CD16 expression (data not shown) we asked if this potent activation depended on specific features, such as phenotypic expression of NKG2C or FcεR1γ$^-$.

As shown (Figure 12) a correlation between expression of FcεR1γ$^-$ and release of IFN-γ resulted statistically significant, as well as between NKG2C$^+$ and release of IFN-γ.

A



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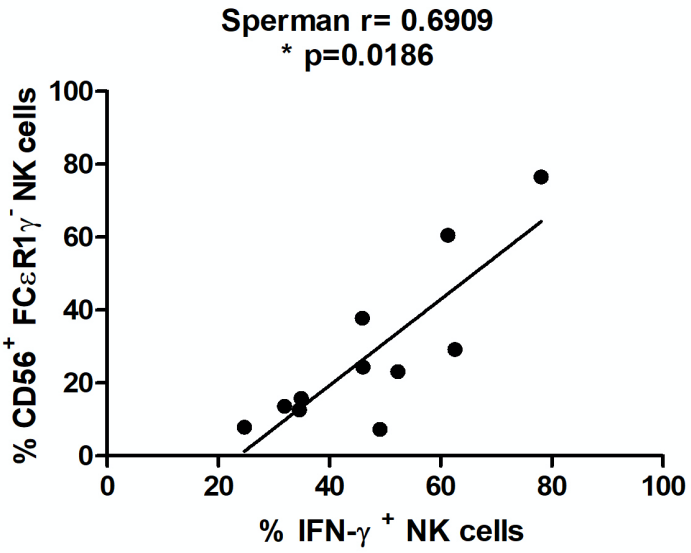


Figure 12. Correlation about IFN-γ production by CD56^{dim} NK cells and their phenotyping expression of FcεR1γ^{-}. A) Graph indicates that is present a correlation from IFN-γ release by CD56^{dim} NK cells and their expression of NKG2C marker from the same patients considered in figure 11. **B)** Significant correlation between IFN-γ release by CD56^{dim} NK cells and their expression of FcεR1γ^{-} phenotyping marker, from the

same patients considered in figure 11. The Significant data are indicated as *P < 0.05.

FcεR1γ$^-$ *Memory-like* NK cells are significantly increased in HCMV$^+$ high-risk atherosclerotic patients compared to low-risk

The lack of FcεR1γ in CD56^{dim} NK cells is an exclusive signature to identification of adaptive NK cells [159]. It has been shown that Memory-like NK cells, compared to conventional NK cells produce greater amounts of pro-inflammatory cytokines, as IFN-γ, in response to cells infected with viruses, as well as in the presence of virus-specific antibodies [140]. IFN-γ is involved in plaque instability, activating MMPs [109, 153].

In line with our precedent result, in HCMV$^+$ pts the FcεR1γ$^-$ peripheral CD56^{dim} NK cells population appeared significantly increased in high-risk atherosclerotic patients compared to low-risk as revealed in (Figure 13). Also, there was no difference between symptomatic and asymptomatic patients in the cohort of high-risk patients.

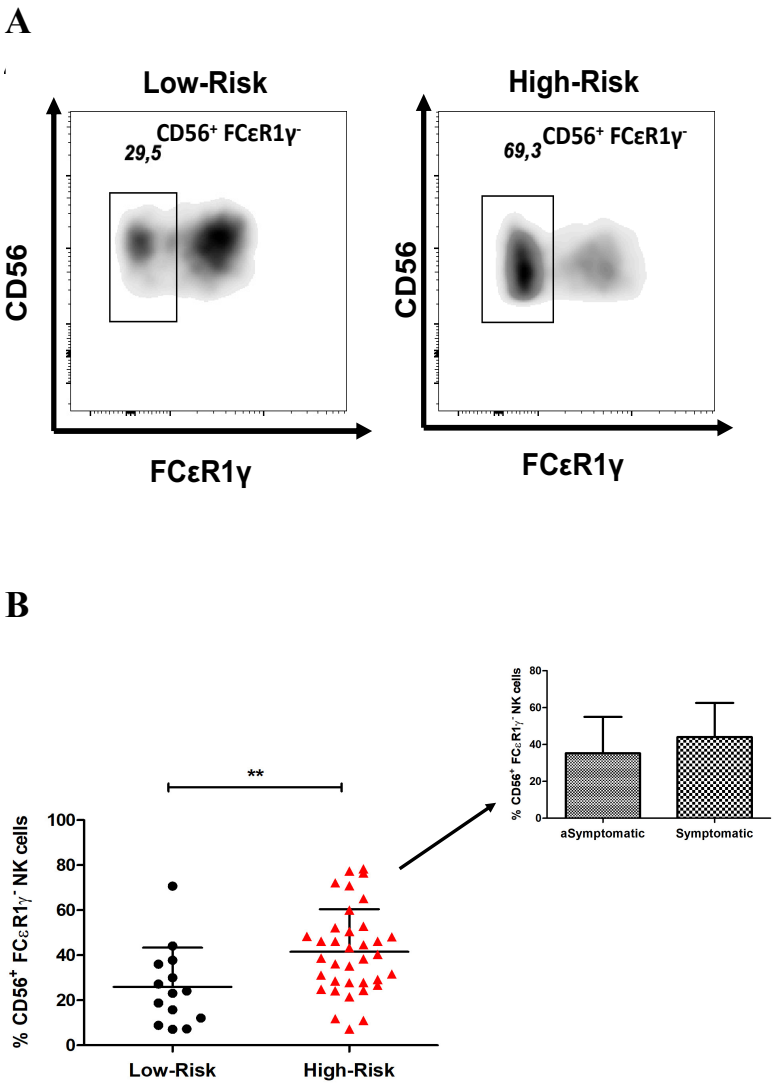


Figure 13 FcεR1γ⁻ CD56^{dim} NK cells are significantly increased in high-risk pts compared to low-risk. A) Representative flow cytometry plots of FcεR1γ expression in gated CD56^{dim} NK cells from HCMV-seropositive low-risk and high-risk pts. **B)** Statistical analysis of the same data represented in figure A, from 14 HCMV^{+} low-Risk pts and 36 HCMV^{+} high-risk pts, that indicate significant increase of FcεR1γ^{-} in CD56^{dim} NK cells from high-risk pts compared to low-risk pts. Significant data are indicated as **P < 0.005.

NKG2C$^-$ FcεR1γ$^-$ *Adaptive* NK cell subset is meaningfully amplified in PB of HCMV$^+$ high-risk patients compared to low-risk

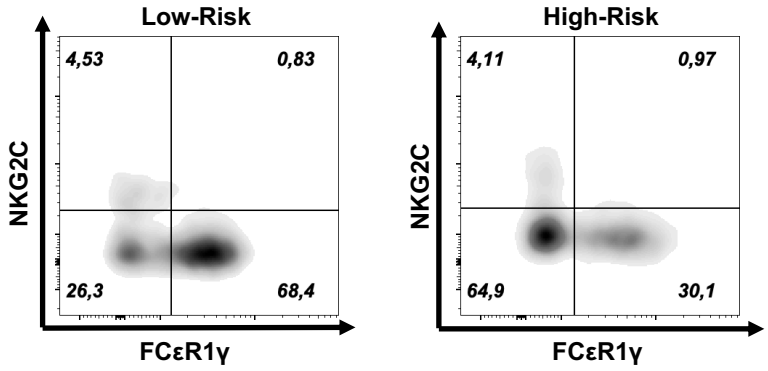
Then, we investigated the heterogeneity of human CD56^{dim} NK cell subpopulations according to the expression of FcεR1γ and NKG2C in our cohort of high-risk and low-risk HCMV$^+$ pts.

As well as shown in a recent study [160], in our cohort of pts we identified that the proportions of NKG2C$^+$ NK cells displayed a dichotomous distribution in high-risk patients that was not observed in the other group, but there was no statistical difference in the cohort of our patients (data not shown).

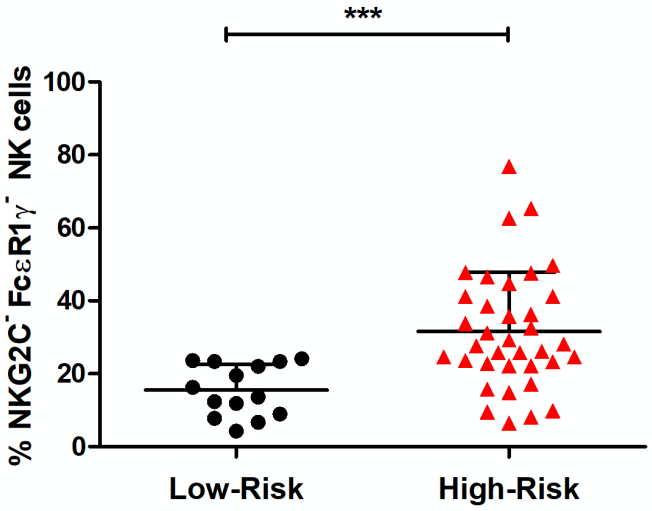
Remarkable, NKG2C$^-$ FcεR1γ$^-$ adaptive NK cells subset significantly increased in PB of HCMV$^+$ high-risk pts compared to low-risk. And as expected, the counterpart, NKG2C$^-$ FcεR1γ$^+$ NK cells, or canonical NK cells, decreased in the same pts compared to low-risk (Figure 14). Also, there is no difference between symptomatic and asymptomatic patients in the cohort of high-risk pts.

This result demonstrated the presence of memory-like NK cells in PB of HCMV$^+$ atherosclerotic pts with high-risk carotid atherosclerotic plaque. As shown in Figure 15 multiparametric analysis confirmed that the NK cells subset in high-risk pts undergoes an “adaptive” reconfiguration.

A


Low-Risk
4,53
0,83
26,3
68,4
NKG2C
FCεR1γ
High-Risk
4,11
0,97
64,9
30,1
NKG2C
FCεR1γ

B


***
% NKG2C⁻ FcεR1γ⁻ NK cells
100
80
60
40
20
0
Low-Risk
High-Risk

C

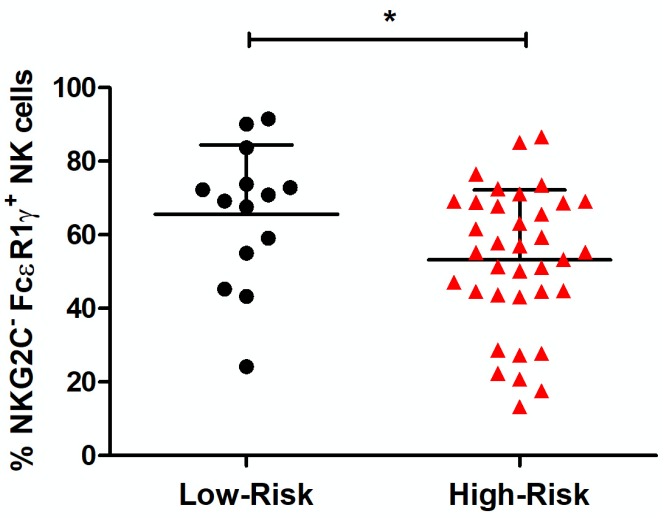


Figure 14 NKG2C$^-$ FcεR1γ$^-$ CD56^{dim} NK cells are significantly increased in PB of high-risk pts compared to low-risk. A) Representative flow cytometry plots that showed four different subpopulations stratified from peripheral CD56^{dim} NK cells for both markers NKG2C and FcεR1γ of HCMV seropositive low-risk and high-risk pts. **B) and C)** Statistical analysis of the same data represented in figure A from 14 HCMV$^+$ low-Risk pts and 36 HCMV$^+$ high-risk pts, that indicate significantly increased of NKG2C$^-$ FcεR1γ- NK cells subset in high-risk pts compared to low-risk. And in C the counterpart of NK cells that is meaningfully augmented in low-risk pts. The Significant data are indicated. *P < 0.05 and ***P < 0.0001.

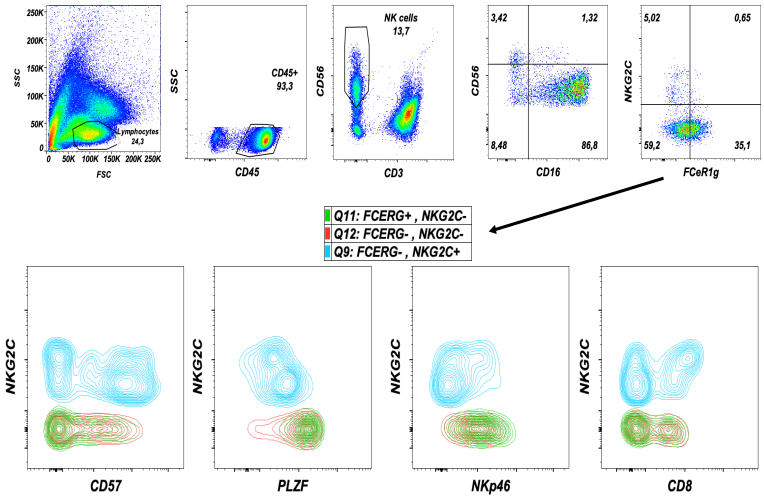


Figure 15 Phenotypic multiparametric analysis of Adaptive NK cells from PB of High-Risk pts. Multiparametric analysis in flow cytometry, from HCMV^{+} high-risk pts, showing that the subset of CD56^{dim} NK cells showed phenotypic features typical of memory-like NK cells. These cells appeared as CD56^{dim} NKG2C$^{+/-}$ NKp46^{-} PLZF^{-} CD8$^{+/-}$.

NKG2C^{-} FcεR1γ^{-} NK cell subset is significantly expanded in Follow-Up HCMV^{+} atherosclerotic patients over time

As previously described (materials and methods) low-risk pts were follow-up pts presenting a carotid stenosis >40% and monitored for carotid plaque stability by ultrasonographic analysis every 6 months. Precedent data indicated an expansion of NKG2C^{-} FcεR1γ^{-} adaptive NK cells subset in PB of HCMV^{+} high-risk pts and showed that peripheral CD56^{dim} NK cells from the same pts exhibited a significatively

heightened capability to produce IFN-γ in response to CD16 stimulation. Considering these results, we aimed at stratifying $CD56^{dim}$ NK cells in follow-up $HCMV^{+}$ pts over time for both markers NKG2C and FcεR1γ. We wanted to study the frequencies of different subpopulations in PB of follow-up pts over time, and specifically we wanted to understand if $NKG2C^{-}$ $FcεR1γ^{-}$ NK cells subpopulation might suffer a modulation in terms of frequency over time. Our goal was to identify possible low-risk pts who, having similar profile to high-risk pts, could undergo evolution in terms of plaque destabilization and consequent outcomes. As displayed (Figure 16), $NKG2C^{-}$ $FcεR1γ^{-}$ NK cells subset expanded only in pts advanced in term of plaque instability over time, defined as "progression" pts. In "basal" (first day of monitoring) phenotype profile of these pts the $NKG2C^{-}$ $FcεR1γ^{-}$ NK cells subset was present at lower frequency compared to the time of progression. Remarkably, the "stable" pts, that remain stable in terms of plaque instability over time, showed a frequency of this subpopulation very low at the first control as well as in the successive ones.

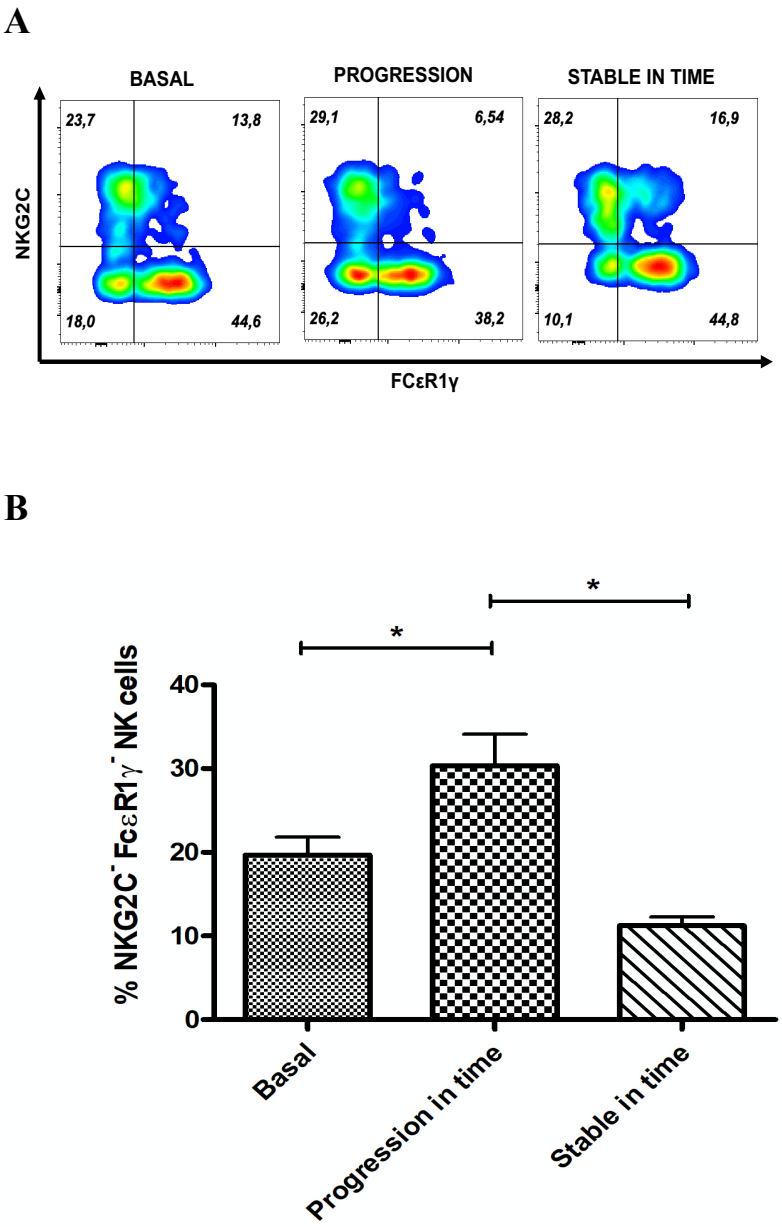


Figure 16 NKG2C$^-$ FcεR1γ$^-$ CD56^{dim} NK cells subset is suggestively amplified in PB of follow-up pts over time. A) Representative flow cytometry plots that showed four different subpopulations stratified from peripheral CD56^{dim} NK cells for both markers NKG2C and FcεR1γ of follow-up pts over time. Specifically, "Basal" was the demonstrative profile of follow-up pts at the first day of monitoring. "Progression", was the demonstrative profile of pts who undergo evolution in terms of plaque instability over the time. And "Stable in time" is the demonstrative profile of pts who remain stable in terms

of plaque stability over the time. **B)** Statistical analysis of the same data represented in figure A, from 21 $HCMV^+$ low-risk pts including 7 evolved pts, that indicate a significant increase of $NKG2C^-$ $Fc\varepsilon R1\gamma^-$ NK cells subset in "time of progression" of the evolved pts compared to their "basal time" and compared to stable pts in time. The Significant data are indicated as $*P < 0.05$.

$NKG2C^-$ $Fc\varepsilon R1\gamma^-$ *Adaptive* NK cells subset is significantly amplified in CAP of high-risk patients compared to their PB

Adaptive NK cells are characterized by the lack of FcεR1γ or gain of NKG2C expression and have strong effector functions by CD16 engagement in terms of IFN-γ release [138]. This cytokine induces activation of MMPs and consequent rupture of the plaque, causing instability [161].

Considering the presence and the amplification of memory-like NK cells subpopulations in PB of high-risk $HCMV^+$ pts (data precedent displayed); we decided to study the distribution of heterogeny $CD56^{dim}$ NK cells in CAP of high-risk $HCMV^+$ pts, that underwent E-CEA surgery, according to both markers NKG2C and FcεR1γ. This analysis might identify a possible expansion of one of these subpopulations induced by HCMV and their possible implication in plaque instability.

As revealed (Figure 18) $NKG2C^-$ $Fc\varepsilon R1\gamma^-$ NK cells subset underwent suggestive increment in terms of

frequency in CAP of high-risk pts compared to their autologous PB. This data demonstrated an amplification of memory-like NK cells specific subset in CAP of pts.

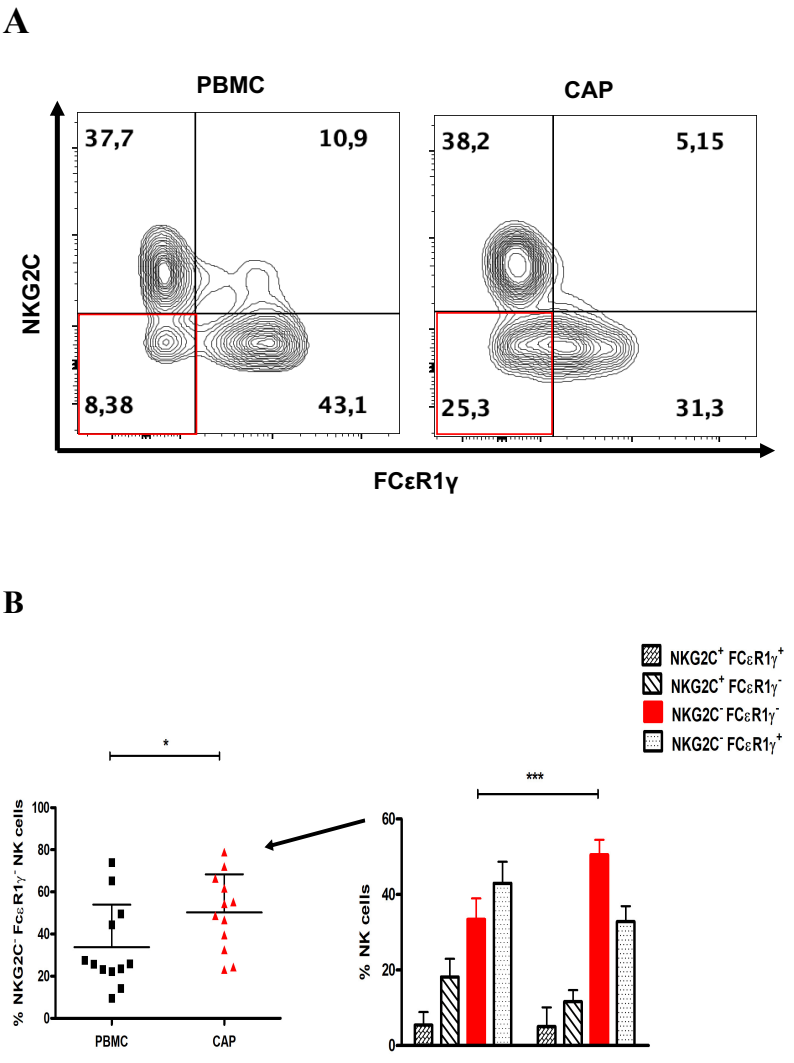


Figure 18 NKG2C$^-$ FcεR1γ$^-$ CD56^{dim} NK cells are amplified in CAP of high-risk pts compared to autologous PB. A) Representative flow cytometry plots that showed four different subpopulations stratified from peripheral CD56^{dim} NK cells for both markers NKG2C and FcεR1γ in PB and CAP of high-risk pts. **B)** Statistical analysis of the same data represented in

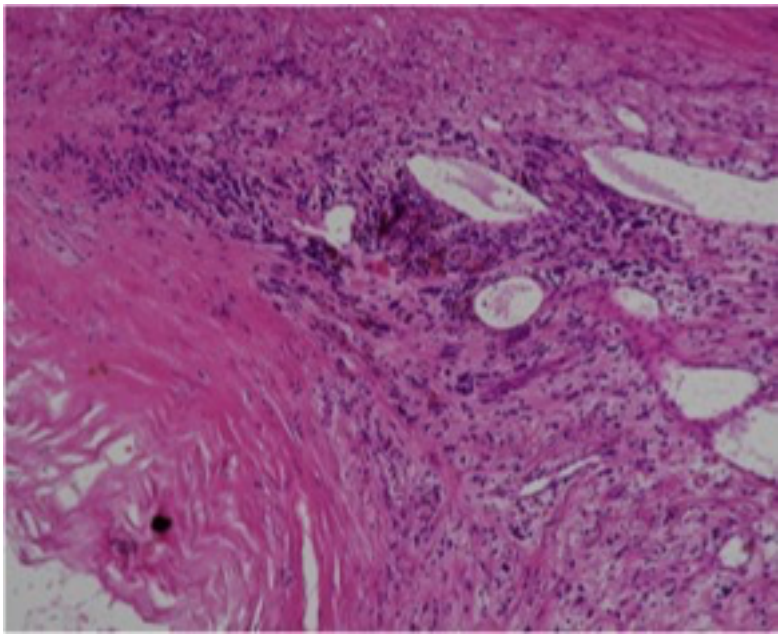
figure A, from 12 HCMV^{+} high-risk pts undergoing E-CEA, that indicate significantly increased of NKG2C^{-} FcεR1γ- NK cells subset in CAP of high-risk pts compared to their autologous PB. The Significant data is indicated as ***P < 0.0001.

NK cells and HCMV^{+} cells of macrophage origins infiltrate the atheromatous plaque

HCMV is a Herpesviridae that after primary infection may remain latent, even for a long time, and occasionally reactivate, generating numerous and different outcomes on infected subject, especially if immune-compromised [145]. The virus can infect a broad range of host cells, including monocytes/macrophages, fibroblasts, epithelial, endothelial cells, and muscle fibers, cells homing to the plaque environment [146, 162]. Recent work has shown that seropositive individuals have a distribution of HCMV in different tissues [121]. HCMV nucleic acids and/or antigens have also been detected in atherosclerotic plaques [120, 130] and in this context, HCMV has been proposed to contribute to the progression of the carotid CAP [147-149]. HCMV induce the activation of NK cells and NK cells infiltrate the CAP [109,139]. Curiously, HCMV induces a reprogramming of conventional NK cells, to display key features of adaptive cells [162]. To complete our data and demonstrate possible

implication of HCMV in instability of CAP we performed immunohistochemistry analysis, singular, or double staining. As exhibited in the images NK cells are possible infiltrated in the arterial wall containing the atheromatous plaque, complicated by hemorrhagic rupture, mainly localizes between fragmented smooth muscle cells near the plaque and near the neovascularization associated with atheroma and intermingled with macrophages (Figure 19 B). NKp46 cells (considered NK cells) and macrophages are in and around the aberrant new vessels developed in the plaque, while the onset of the bleeding events revealed by the presence of erythrocytes between the surrounding "damaged" and "dissociated" smooth muscle cells (Arrows in Figure 19 B and C). Curiously, HCMV was present intimate the CAP, and specifically, as displayed in double staining, the virus was localized inside $CD68^{+}$macrophage-like cells widely infiltrate the plaque (Figure 19 D and E).

A



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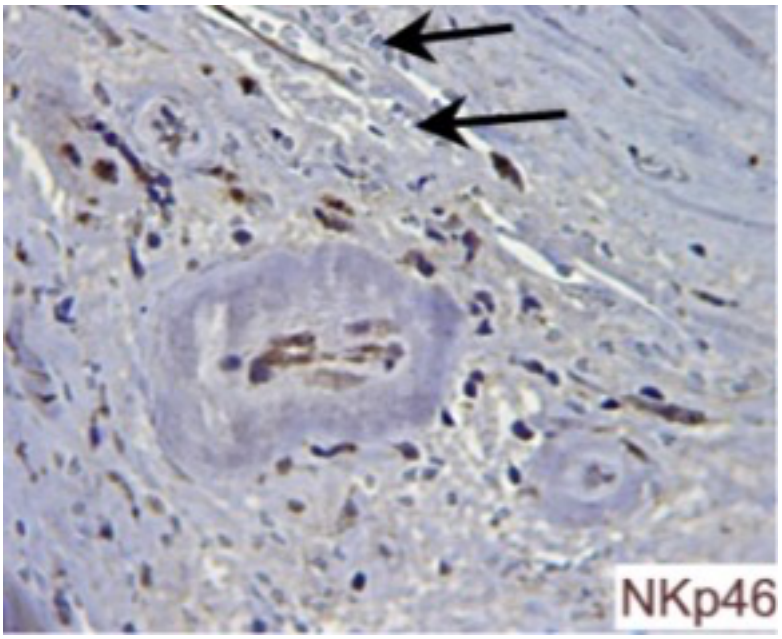

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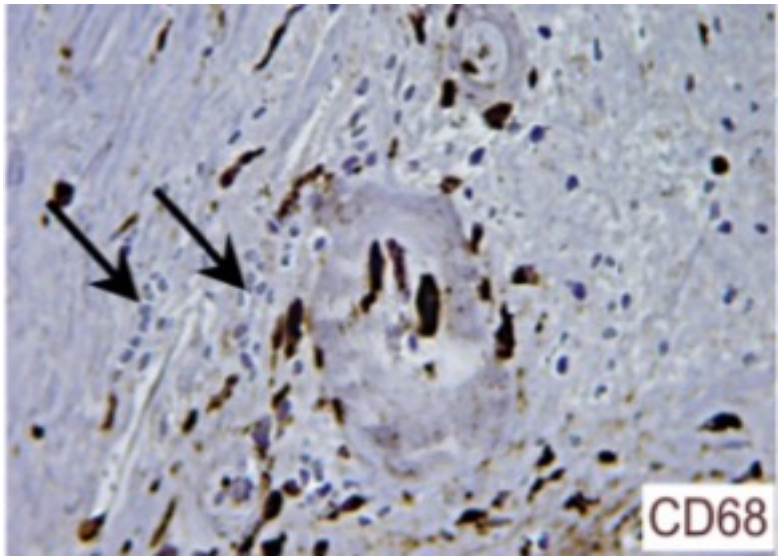

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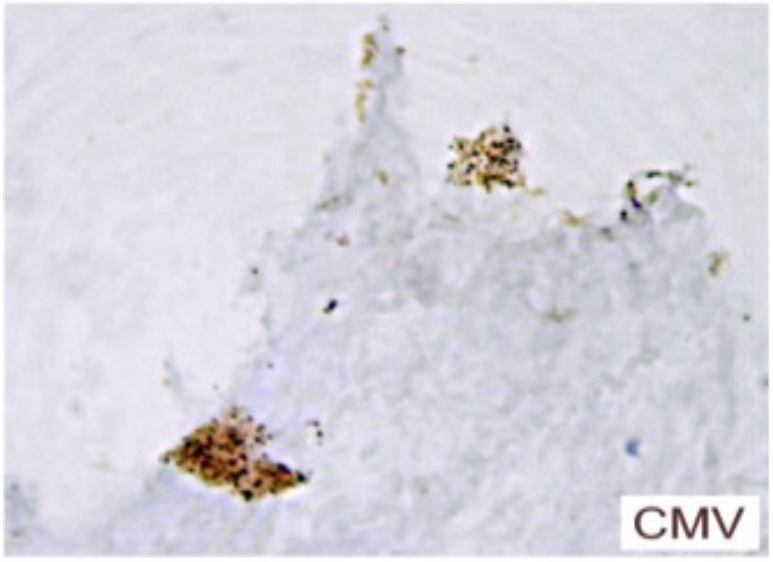

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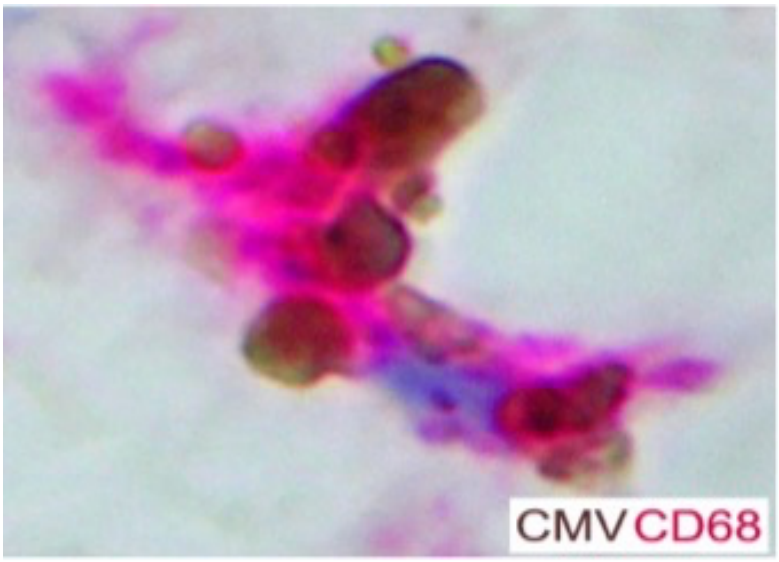

Figure 19 NK cells and HCMV$^+$ cells of macrophage origins infiltrate the atheromatous plaque A) Histological imaging obtained by HE staining, to evaluate the organization of CAP (magnification X200). Atherosclerotic tissue appears characterized by abundant fibrous tissue, smooth muscle cell accumulation within the intima, that result altered and loses normal morphology. **B)** IHC staining that represent the possible infiltration of NK cells in CAP (NKp46$^+$ cells in brown) near the neovascularization associated with atheroma (arrows indicate the erythrocytes into the blood vessels), (magnification X200). **C)** IHC staining that represent the infiltration of macrophagic cells in CAP (CD68$^+$ cells in brown) around the aberrant new vessels (arrows indicate the erythrocytes into the blood vessels), (magnification X200). **D)** IHC staining that represent the infiltration of HCMV in CAP (HCMV$^+$ cells in brown) (magnificationX200). **E)** IHC double staining that represent the colocalization of macrophagic cells (CD68$^+$ cells in red) and HCMV (HCMV$^+$ cells in brown) in CAP (magnification X400).

DISCUSSION

Most of the current knowledge on atherosclerosis considered this pathology as a chronic inflammatory process with multifactor etiology. Among these induction factors was considered viral infection, such as HCMV infection [1,13,14,14,163]. HCMV is a DNA virus with high prevalence in the adult human population with lifelong latency in different tissue and possible reactivation, thus triggering immune responses [120, 121, 130, 146, 164]. Previous papers on this topic have shown that HCMV choose, as reservoir, cells of myeloid/macrophagic origin [164] which have a crucial role in the development of atheroma [164, 165]; however, few data are currently available about the presence of HCMV in atherosclerotic plaque and its involvement in the pathogenesis of atherosclerosis.

HCMV has been proposed to activate a broad range of cells, in particular NK cells that have a key function in the response to the virus infection [150,151,152]. Natural killer cells are cytotoxic innate lymphocyte, which, as reported in recent studies, infiltrate the atheromatous plaque [109]. In this environment, they produce high amount of IFN-γ, a pro-inflammatory cytokine that was involved in plaque instability [109].

Curiously, HCMV, in PB of healthy donors, drives a phenotypic and functional reconfiguration of NK cells, which become “adaptive or memory-like” NK

cells. This subpopulation has distinct characteristics as clonal expansion, long-life property, and strong effector function via CD16 engagement, compared to their "canonical" counterpart [131, 140], and is commonly defined by either a lack of FcεR1γ and presence or not of NKG2C expression [131,159].

Although an expansion of $NKG2C^+$ NK cells has been identified in patients $HCMV^+$ with high-risk CAP; data about NK cells, in particular memory-like NK cells, their distribution in plaque, their activation and consequent functionality, are still limited.

Restricted information about the involvement of HCMV in the pathogenesis of atherosclerosis, as well as the mechanisms induced by the virus to determine plaque instability, had hindered the development of possible biomarkers useful for the detection of patients with unstable plaques at greater risk of vascular complications.

In this study, we focused on i) demonstrating the presence of HCMV in carotid atherosclerotic plaque and its involvement in plaque instability; ii) analyzing possible phenotypic and functional changes induced by the virus on NK cells compartment, present and involved in the atherosclerotic process [109]; iii) stratifying our investigations on patients with high and low risk of plaque instability.

We showed with functional assay and cytofluorimetric analyses that peripheral $CD56^{dim}$ NK cells of high-risk $HCMV^+$ atherosclerotic pts had

enhanced CD16-mediated IFN-γ release when compared to both low-risk $HCMV^+$ pts, and high-risk and low-risk $HCMV^-$ pts. Furthermore, we demonstrated that NK cells of infected high-risk pts were highly responsiveness to opsonizing antibodies and produced IFN-γ, a pro-inflammatory cytokine that caused plaque instability [109].

Considering this first result and that HCMV drive the formation of memory-like NK cells [131, 138], cells that were very stronger productors of IFN-γ [140], we wanted to investigate if this potent CD16-engagment activation of $CD56^{dim}$ NK cells from high-risk $HCMV^+$ pts depended on specific features of NK cells, example adaptive signatures. Since memory-like NK cells were commonly characterized by the lack of FcεR1γ (signaling adaptor) [140, 166] we analyzed the correlation between IFN-γ release by $CD56^{dim}$ NK cells of $HCMV^{+/-}$ atherosclerotic pts and the frequency of $CD56^{dim}$ $FcεR1γ^-$ NK cells subpopulation, and it resulted that existing a direct correlation between amount of IFN-γ production and the higher frequency of $CD56^{dim}$ $FcεR1γ^-$ NK cells subpopulation. Considering that, it has been hypothesized that NK cells of $HCMV^+$ atherosclerotic pts had adaptive features; and since these cells produced large amount of IFN-γ , it was essential to understand their presence and distribution in both low- and high-risk pts.

While memory-like NK cells were usually described as FcεR1γ^{-} and NKG2C$^{+/-}$ cells [159], we analyzed both markers in CD56^{dim} NK cells from high-risk and low-risk HCMV^{+} atherosclerotic pts. Interestingly, our data demonstrated that CD56^{dim} FcεR1γ^{-} NK cells subset was significantly increased in high-risk pts when compared to low-risk, revealing that adaptive NK cells preferentially occupied PB of high-risk pts. Stratifying peripheral CD56^{dim} NK cells also according to NKG2C marker, in high-risk pts, we showed that NKG2C^{-} FcεR1γ^{-} NK cells subpopulation resulted significantly increased respected to low-risk pts. Additionally, the same subpopulation appeared suggestively amplified in terms of frequency in carotid atherosclerotic plaque of high-risk pts compared to autologous PB, demonstrating that adaptive NK cells were present in CAP of high-risk pts and underwent a modulation in tissue when compared to PB.

Remarkably, through the follow-up of the plaque condition in low-risk atherosclerotic pts, we demonstrated that CD56^{dim} NKG2C^{-} FcεR1γ^{-} NK cells subpopulation might bring to an expansion in terms of frequency in advanced pts over time. While, at the first control of evolved pts as well as in stable pts in time (first and following controls), the frequency of this subpopulation remained very low. This interesting datum suggested a possible association between expansion of specific memory-

like NK cells subset and the progression of atherosclerotic process.

Since few papers showed the presence of HCMV in plaque [120] and the data obtained suggest a possible virus-mediated activation of innate response, specifically NK cells that infiltrate the CAP of high-risk atherosclerotic patients [109,139] we also performed immunohistochemical analyses in CAP. Remarkably, the analyses revealed the presence of HCMV in carotid atherosclerotic plaque, into cells of macrophagic origin ($CD68^+$ cells). Moreover, although NKp46 is not a mutually exclusive marker of NK cells [167, 168], but used to identify these cells in tissues [169,170], our analysis allowed us to focus on the coexistence of these cells and HCMV in the microenvironment of atherosclerotic plaque. Further investigation and the use of other markers will allow more specific investigations.

CONCLUSIONS

In conclusion, this study indicated that HCMV might play a role in the progression of atherosclerotic process by cross-priming of innate response. The evidence that $NKG2C^-$ $Fc\varepsilon R1\gamma^-$ memory-like NK cells subset was represented in both PB and CAP of high-risk $HCMV^+$ pts, but especially in patients evolved over time, suggested that adaptive NK cells

compartment was involved in the atherosclerosis phenomena. The indication that adaptive FcεR1γ$^-$ NK cells were the strongest CD16-mediated producers of IFN-γ [118], and that these cells were widely present in PB and CAP of high-risk pts and correlate with the increased release of IFN-γ, supported our hypothesis that adaptive NK cells are committed in plaque instability.

All together our data suggest the importance of investigating the presence of HCMV in atherosclerotic pts, as well as the importance of detecting in the same pts specific subsets of memory-like NK cells, virus-induced, which could become potential markers to identify low-risk pts who, having similar profile to high-risk pts, may suffer from plaque destabilization and subsequent negative outcomes.

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