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CIGARETTE SMOKE INDUCES p38 MAPK-INITIATED AND FAS-MEDIATED ERYPTOSIS

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INDEX

1. INTRODUCTION

***Dedicated to all those who will read this work**.*

Prevention, doing good and keeping healthy is the basis of everything.

We often forget that our body is a precious treasure chest that deserves our best attention.

We must learn to take care of ourselves. To have love for ourselves. For US and our loved ones.

1.1 Cigarette Smoking (CS)

The World Health Organization estimates that each year, more than 8 million people worldwide die from tobacco consumption. Most tobacco-related deaths occur in low- and middle-income countries, which are often the target of intense interference and marketing by the tobacco industry.

Tobacco can also be deadly for non-smokers. Exposure to second-hand smoke has also been implicated in negative health outcomes, causing 1.2 million deaths each year. Almost half of all children breathe air polluted by tobacco smoke and 65,000 children die each year from second-hand smoke-related diseases. Smoking during pregnancy can lead to several lifelong health conditions for children.

In the European Union, statistics shown in **Figure 1**, tobacco consumption remains the greatest preventable risk factor for health and is responsible for 700,000 deaths each year. About 50% of smokers die prematurely, resulting in an average loss of 14 years of life per smoker. Tobacco consumption is the leading cause of preventable cancer, with 27% of all cancers attributed to tobacco consumption. In addition, smokers are also more likely to suffer from a number of diseases due to their tobacco use, including cardiovascular and respiratory problems[1].

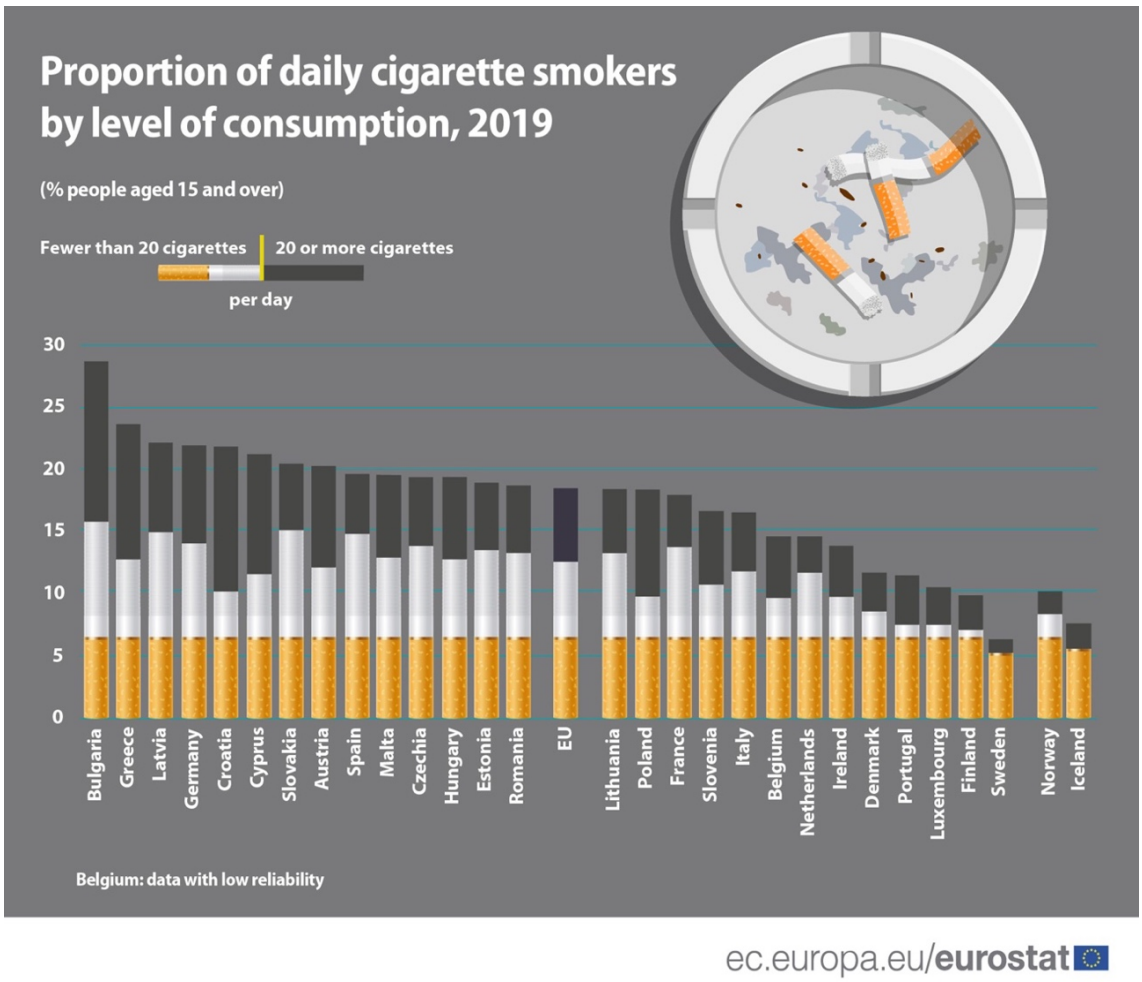

Figure 1 - 18.4% of EU population smoked daily in 2019.

12.6% of the EU population consumed fewer than 20 cigarettes per day, while 5.9% consumed 20 or more cigarettes on a daily basis. Across the EU Member States, the countries with the largest shares of daily cigarette smokers were Bulgaria (28.7%), Greece (23.6%), Latvia (22.1%), Germany (21.9%) and Croatia (21.8%). In contrast, the countries with the smallest shares of daily smokers were Sweden (6.4%), Finland (9.9%), Luxembourg (10.5%), Portugal (11.5%) and Denmark (11.7%). In Italy, it is estimated that more than 93,000 deaths (20.6% of all deaths among men and 7.9% of all deaths among women) are attributable to tobacco smoke, with direct and indirect costs amounting to more than 26 billion euro[2]. Regarding cancer, tobacco is the risk factor with the greatest impact that can be attributed to at least 43,000 deaths per year.

Data on smoking prevalence among adults are collected annually by ISTAT with the Daily Life Activities Survey and by the Higher Institute of Health with PASSI surveillance and the ISS/Doxa survey.

In 2021, according to ISTAT data, smokers, among the population of 14 years and more, are just under 10 million. The prevalence is 19%. Gender differences are strong: among men smokers are 22.9% among women 15.3%. Tobacco smoking was more common in the 25-44 age group (about 1 in 4 people)[3].

Tobacco is a plant belonging to the Solanaceae family, native to South America and imported to Europe after the discovery of the New World. The chemical composition of tobacco smoke depends on various factors such as plant variety, cultivation methods (fertilizers and pesticides) and post-harvest processing. In general, the burning of tobacco and paper, which reaches temperatures of over 800 ºC, generates two different smokes. The sidestream smoke evolves from the smoldering end of the cigarette while the smoker is not puffing, and contributes substantially to environmental tobacco smoke (ETS) while the mainstream smoke emerges from the butt end of the cigarette and is mainly inhaled by the smoker. The mainstream smoke is a mixture of more than 4000 substances, all of which are considered harmful to human health, conventionally divided into gaseous and corpusculated components (**Table 1**). In addition to nitrogen, oxygen and carbon dioxide, the gaseous component includes carbon monoxide in significant concentration (about 4% by volume) and minor but significantly harmful amounts of nitrogen oxides, ammonia, nitrosamine, hydrocyanic acid, nitriles, volatile hydrocarbons, acetaldehydes, formaldehyde and acrolein.

The corpusculated component consists of an aerosol of tar/nicotine particles (diameter 0.1 to 1.0 μm, average diameter 0.2 μm).

Tar is the sticky brown residual substance left after removal of nicotine and moisture from

the corpusculated component. It consists of a complex mixture of polynuclear aromatic hydrocarbons comprising carcinogens such as non-volatile nitrosamines, aromatic amines and benzopyrene. Some radioactive elements such as polonium-210 are also present in the tar. The use of additives to improve taste in cigarettes may be an additional source of risk[4]. In relation to the pathophysiologic effect these compounds are divided into:

irritants and oxidants; carcinogens and co-carcinogens; carbon monoxide; nicotine.

The toxic and irritating substances contained in cigarette smoke are responsible for alterations in the respiratory mucous membranes and the conjunctiva. They are responsible for the impairment of mucociliary function and the development of chronic bronchitis. This group also includes free radicals, contained in smoking. These contribute to the damage of the respiratory system, favoring the progression of chronic bronchitis towards emphysema[5] and cardiovascular damage[6], primarily by altering endothelial function.

The marked deficiency of antioxidant vitamins, in particular vitamin C, in smokers testifies to the body's attempt to resist oxidative damage. Carcinogens in smoke act either as initiators, or as carcinogens, causing the development of cancer, or as promoters, or co-carcinogens, stimulating their growth[7]. The most important carcinogens are polycyclic hydrocarbons, nitrosamines, aromatic amines, while phenols and free radicals act as promoters[8].

Table 1 - Distribution of some selected toxic substances in the main flow of cigarette smoke (mainstream - MS) and in the side stream of smoke (side stream - SS) of cigarettes without filter[9].

Substances	MS	SS		MS	SS
Gaseous component			**Particulate component**		
Carbon monoxide (mg)	10-23	2.5-4.7	Corpusculate material (mg)	10-23	2.5-4.7
Carbon dioxide (mg)	20-60	8.41	Nicotine (mg)	1-2.3	2.6-3.3
Formaldehyde (µg)	70-100	0.10-0.50	Phenol (µg)	60-120	2-3
Acrolein (µg)	60-100	8.15	Catechol (µg)	100-280	0.6-0.9
Acetone (µg)	100-250	2-5	Aniline (ng)	360	30
Pyridine (µg)	20-40	10-20	2-toluidine (ng)	160	19
3-Vinyl pyridine (µg)	15-30	20-40	2-naphthylamine (ng)	1.7	30
Hydrocyanic acid (µg)	400-500	0.10-0.25	Benzoanthracene (ng)	2-7	2-4
Nitrogen oxides (µg)	100-600	4-10	Quinoline (ng)	500-2000	8-11
Ammonia (µg)	50-130	4-130	N-nitrosodiethanlamina (ng)	20-70	1.2
N-nitrosodimethylamine (ng)	10-40	20-100	Nickel (ng)	20-80	13-30
N-nitrosopirrolidine (ng)	6-30	6-30	Polonio-210 (pCi)	0.03-0.5	-

The radioactive substances in cigarettes are also derived from fertilizers used in plantations. Deserves a focus polonium-210, which, having become volatile at temperatures of 800, adheres tenaciously to the smoke particles and with them penetrates deeply into the bronchial tree, where alpha radiation damages the DNA of the cells favoring the development of the tumor. The combined effect of exposure to polonium 210 and polycyclic hydrocarbons is very powerful[10]. Even flavorings, added to tobacco to improve its taste, otherwise unpleasant and irritating, are transformed, following combustion, into carcinogenic substances.

The carbon monoxide released and inhaled is about 20 mg and is not retained by the filter. Once absorbed, through the lungs it binds to hemoglobin with an affinity 200 times greater than that of oxygen.

The carboxyhemoglobin content in the blood of a smoker is about 10 times higher than that of a non-smoker. In addition to impaired transport and mitochondrial use of oxygen, toxic effects are also exerted on muscle fibers, especially cardiac (pro-arrhythmic effect), and endothelial cells[6].

Nicotine is an alkaloid (such as morphine, heroin, codeine, etc.) and can be absorbed through different routes: oral mucosa, respiratory mucosa, skin, and the amount depends on pH. Cigarette smoke contains nicotine in a less absorbable form but given the enormous surface area of the respiratory system, 95% of the nicotine present in smoke reaches the blood circulation of the aspiring smoker.If the smoker does not inhale instead, he absorbs an amount equal to about half (the rest remains in the environment)[11].Nicotine is responsible for the phenomenon of tobacco smoking addiction[12]. From the point of view of toxicity, it is particularly harmful for the cardiovascular system both for the stimulating effects of colinergic system (acute effects) and for interference with endothelial function (chronic effects)[13].

The tobacco industry has developed attempts to reduce the content of these toxic components while trying to preserve taste and taste. For example, for cigarettes produced in Britain between 1934 and 1979, the average tar content decreased by 49%, the nicotine content by 31% and the carbon monoxide content by 11%[14]. These results have been achieved through modifications of the genetic strains of tobacco plants and the use of fertilizers, the time and manner of harvesting, storage and maturing of leaves and processing techniques[15]. The characteristics of cigarettes are then changed in terms of packing density, length, paper porosity, ventilation, and filter filtration efficiency. More difficulties have been encountered in reducing the carbon monoxide content as this compound cannot be reduced either by a similar decrease in the levels of pre-combusted tobacco nor can it be retained by filters as the molecule is large too small. Carbon

monoxide is produced in the portion of cigarette being combusted and in the immediate vicinity of high temperature tobacco pyrolysis[16]. An attempt has been made to introduce cigarette-making methods which could alter the extent of combustion and the temperature of the tobacco, so as to encourage the lateral spread of the tobacco bar through the paper and through ventilation holes in the filter. The latter method also leads to a dilution of the smoke and a reduction of the other toxic components of the smoke.

However, as mentioned above, the way in which the cigarette is smoked critically affects the quantities of tobacco, nicotine and carbon monoxide emitted. Simplifying as much as possible, the tobacco bar acts as a fractionation column by concentrating the components towards the bottom. The corpusculate component is produced at much higher concentrations (up to 2 times) towards the end of the cigarette: from this comes the suggestion to leave butts as long as possible. The amount of nicotine absorbed depends on the depth of the aspiration: for this reason the smoker switching to lighter cigarettes does not substantially change his nicotine rate as he tends, in general, to inhale more deeply[17].

1.2 Tissue damage and smoking disordes

More than 50 years have already passed (January 11, 1964) since the publication of the first report by the Surgeon General (SG) in the United States with the first conclusions on the danger and impact of tobacco smoking on health. The "Report on smoking and health" of Surgeon General, the highest authority of the Federal Government of the United States on public health was the first government report that related tobacco consumption and health damage, including lung cancer and cardiovascular disease. The scientifically rigorous publication has laid the foundations for the fight against tobacco

addiction in the United States and worldwide[18]. In the last 50 years there have been several other update reports on the wave of numerous scientific studies that have defined better and more detailed causal relationships between, not only active smoking but also between exposure to second-hand smoke and a wide range of diseases and other harmful effects on the human body[19]. All this led, in 1998, Dr. Gro Brandtland, then Director General of the World Health Organization (WHO), to declare that "*Tobacco is one of the greatest emerging health disasters in the history of humanity*". In the 2015 SG report, the list of diseases and other adverse effects caused by smoking widens, thus reaffirming the wide consequences of tobacco smoking. In this SG report there is a summary of the conclusions of previous reports regarding the health consequences of tobacco smoking and an update, based on scientific literature, for those conditions for which the evidence was previously considered suggestive, including macular degeneration, colorectal

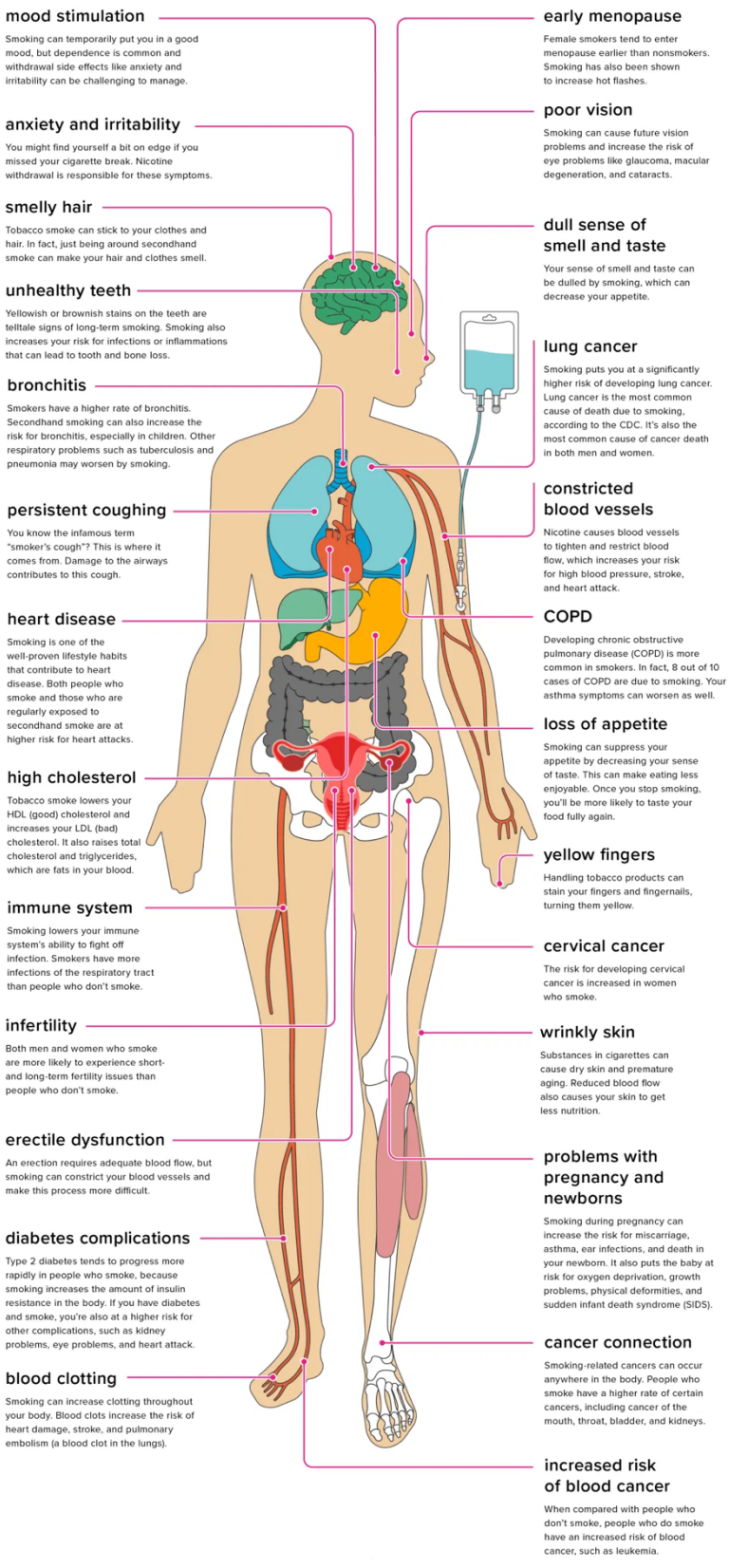

Figure 2 - Effects of smoking cigarettes on our body. healthline.com/health/smoking/effects-on-body

cancer, breast cancer, prostate cancer, and male sexual dysfunction.

This report covers the different health outcomes, due to active and passive smoking, that were not covered exhaustively in previous SG reports, including the general effects on the immune system and the development of several diseases in which the immune system plays a key role, such as tuberculosis, diabetes, rheumatoid arthritis, and systemic lupus erythematosus. All aspects reinforced by the latest SG report of 2020 in which the

benefits of smoking cessation are highlighted. Tobacco smoke, both active and passive, acts as an etiological factor transversal to various organs and systems, both for cancer and chronic inflammatory diseases, having as main target organs oropharynx, bronchopulmonary and cardiovascular (**Figure 2**). Among all the smoke-related diseases, lung cancer, chronic obstructive pulmonary disease (COPD) and cardiovascular diseases (coronary heart disease, stroke and peripheral vasculopathies), represent, worldwide, the main public health problems and are among the diseases with the highest mortality worldwide[2]. The first two diseases have common characteristics: high mortality, common risk factors, such as tobacco smoke, genetic predisposition, environmental exposures, and a common basis of inflammatory processes[20]. Tobacco consumption is the main risk factor for both diseases. In fact, for lung cancer, more than 85% of all lung cancers occur among current or former smokers, while for COPD tobacco smoking is responsible for 80-85% of cases. COPD is the fourth leading cause of death in the world with a tendency to be third by 2020 and with a current prevalence of around 10%[21]. COPD as well as being an obstructive bronchial disease, characterized by a partial or total irreversibility of the bronchial obstruction with a progressive fatal lung deterioration over time[22] is also recognised as a systemic inflammatory disease with pulmonary and extrapulmonary symptoms, including an increased risk of developing lung cancer[23]. COPD has a marked effect on the quality of life of patients and affects up to 50% of smokers[24]. Bronchopulmonary damage is caused by oxidative stress (exogenous of smoking and endogenous), release of inflammatory cytokines, increased proteasic activity due to the imbalance between protease/antiprotease and expression of autoantibodies[25] that all together in turn can lead to a picture of chronic bronchitis with alteration of mucociliary clearance and possible progressive evolution towards a picture of chronic obstructive pulmonary disease (COPD) and pulmonary emphysema. COPD

and pulmonary emphysema may be risk factors for lung cancer[20][26][27]. There is no more proven and documented cause-and-effect association in medicine than the epidemic parallel between cigarette smoke and lung cancer[28][29][30]. This evidence appears clear and inexorable if we follow the progressive increase in smoking tobacco consumption that begins with the end of the 19th century with the manufacture of the first automatic cigarette-making machine by Bonsak and the progressive increase in incidence and mortality for lung cancer in the world. In 1889 lung cancer was an extremely rare disease: only 140 cases documented in the world. Today, lung cancer is the leading cause of cancer mortality in the world, accounting for up to 13% of all cancer deaths with more than 1,400,000 deaths/year[31].

Tobacco is also the most important risk factor for the development of coronary heart disease, stroke and peripheral vasculopathies. Cardiovascular disease kills more people than any other cause of death worldwide and contributes to about 17% of all deaths from such diseases. According to the World Health Organization, tobacco use and exposure to smoking (including passive smoking) are the second leading cause of cardiovascular disease after hypertension, and smoking is also a risk factor for hypertension[6].It is estimated that the contribution of smoking to cardiovascular mortality is 430,000 deaths per year in Europe (out of a total of 1,200,000)[32]. The action of cigarette smoking on the cardiovascular system is determined by nicotine, oxidative stress, and carbon monoxide (CO)[6]. Nicotine increases LDL cholesterol values, decreases HDL and increases platelet aggregation. It also has hypertensive action and increases heart rate[33].Free radicals undermine the integrity of the endothelium and the elasticity of the vasal wall and the smooth muscle fibres of the vessels and myocardium[34]. Carbon monoxide also increases the permeability of the vasal endothelium by facilitating atherosclerotic processes and,

by binding to haemoglobin, chronically reduces tissue oxygenation. CO also causes cardiac hypertrophy[35][36], in fact decreases the bioavailability of nitric oxide, increases pro-inflammatory cytokines and adhesion molecules that, together with the activation of the sympathetic nervous system, cascade vasomotor dysfunction, increased pro-thrombotic factors, reduced fibrinolysis, platelet activation, lipid peroxidation, and chronic inflammation. Starting from the genetic predisposition all these factors play an important role together with insulin resistance on the onset and progression of atherosclerotic disease[37].

1.3 Relationship between smoking and cardiovascular disease

Cigarette smoking is a powerful risk factor for heart disease and stroke[6].Exposure to cigarette smoke has also been associated with the progression of atherosclerosis[13] (**Figure 3**). Direct exposure to cigarette smoke has been found to be associated with a 50% increase in atherosclerosis progression, while exposure to second-hand smoke is associated with a 25% increase[27]. Cigarette smoking therefore continues to represent a serious health risk and contributes significantly to cardiovascular morbidity and mortality by affecting all stages of atherosclerosis, from endothelial dysfunction to acute clinical events. Exposure to both active and passive (environmental) cigarette smoke predisposes to cardiovascular events[6].The exact toxic components of smoking and the mechanisms involved in cardiovascular dysfunction are largely unknown, but cigarette smoking has been shown to increase inflammation, thrombosis, and oxidation of low-density lipoprotein cholesterol. Experimental and clinical data support the hypothesis that exposure to cigarette smoke increases oxidative stress as a potential mechanism for initiating cardiovascular dysfunction[39]. Low tar cigarettes and smokeless tobacco have also been shown to increase the risk of cardiovascular events compared to non-smokers[40][41].

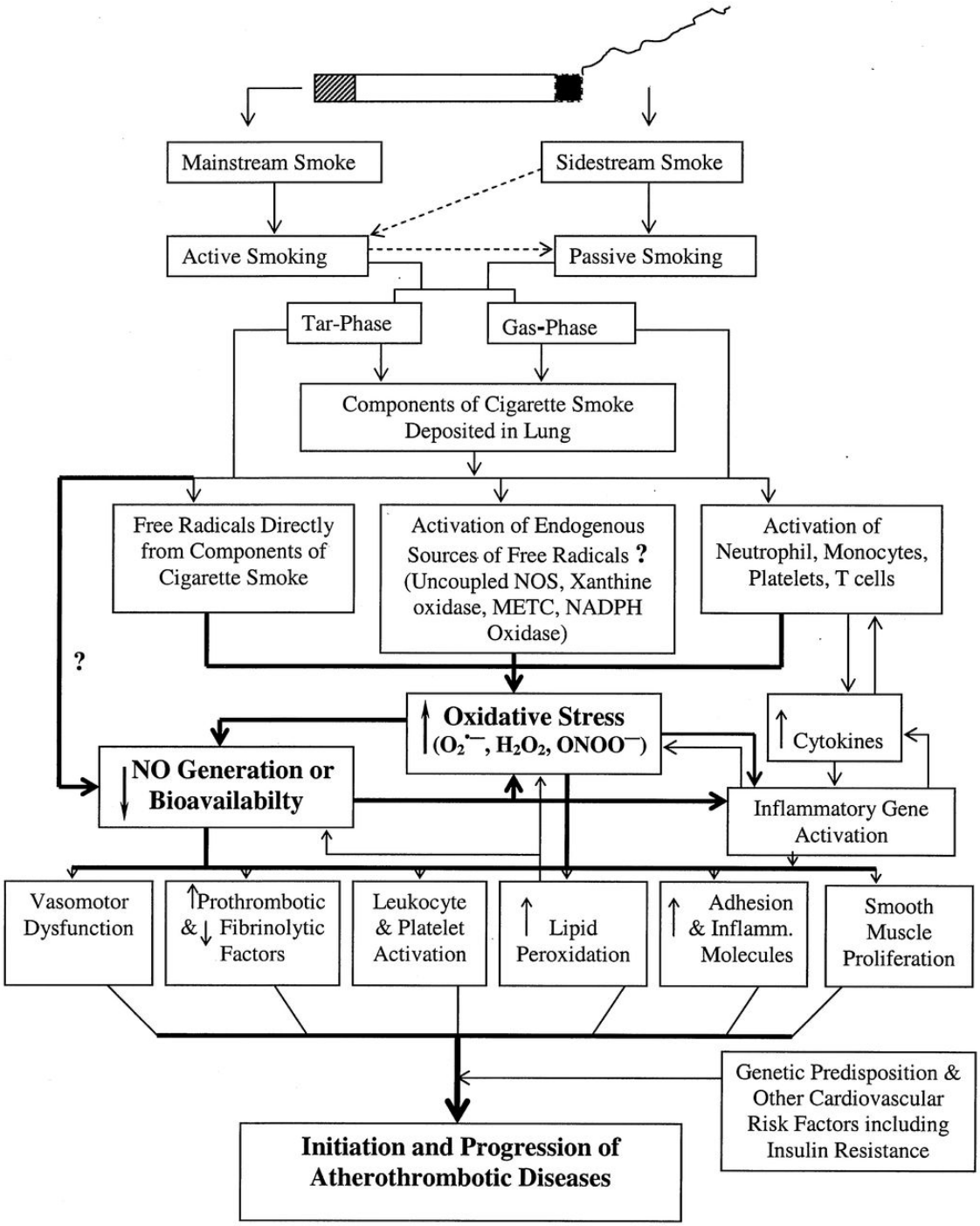

Figure 3 - Potential pathways and mechanisms for cardiovascular dysfunction mediated by cigarette smoking. The bold cells and arrows in the flow diagram represent the probable central mechanisms in the complex pathophysiology of atheromatic thrombotic disease mediated by cigarette smoke. H2O2 = hydrogen peroxide; METC = mitochondrial electron transport chain; NADPH = nicotinamide adenine dinucleotide phosphate reduced form; NOS = nitric oxide synthase; ONOO- = peroxynitrite; O 2- = superoxide[39].

Cigarette smoking predisposes the individual to several clinical atherosclerotic syndromes, including stable angina, acute coronary heart syndromes, sudden death, and stroke[39]. Aortic and peripheral atherosclerosis have also increased, leading to intermittent claudication and abdominal aortic aneurysm and also has been found to be a predictor of coronary heart injury[42]. Vasomotor dysfunction, inflammation and lipid modification are integral components for initiation and progression of atherosclerosis. These components precede the apparent structural and clinical pathological manifestations of atherosclerosis[43][44]. The inflammatory response is an essential component in the initiation and evolution of atherosclerosis. Several studies have shown that smoking causes a 20% to 25% increase in peripheral blood cell count[45]. In vivo, smoking is associated with an increase in the level of multiple inflammatory markers including C-reactive protein, interleukin-6 and cancer necrosis factor alpha in both male and female smokers[46][47]. Local recruitment of leukocytes on the surface of endothelial cells is an early event in atherosclerosis. Increases in various proinflammatory cytokines increase the interaction between leukocyte cells and endothelium leading to the recruitment of leukocytes. In fact, soluble levels of VCAM-1, ICAM-1 and E-selectin are higher in smokers[48][49].

1.4 Red Blood Cells (RBC)

Erythrocytes (from the Greek erythròs, «red» and cytos «cell»), also known as RBC, are the major cellular component of the blood (from 40% to 45%) and the main means to provide oxygen (O2) to the tissues of our body, through the flow of blood generated by the cardio-circulatory system.

Red blood cells (RBCs) absorb oxygen in the lungs and release it into the tissues through the blood capillaries. Erythrocyte cytoplasm is rich in haemoglobin, an iron-containing biomolecule that can bind oxygen and is responsible for the red colour of cells and blood. The cell membrane is composed of proteins and lipids and this structure provides essential properties for the physiological function of cells such as deformability and stability while crossing the circulatory system and in particular the capillary network.

In humans, mature red blood cells are flexible and oval biconcave discs. They lack nucleus and most organelles, in order to accommodate the maximum space for haemoglobin. We can imagine them as haemoglobin containers, where the plasma membrane forms the structure of the container. One adult produces about 2,4 million new erythrocytes per second[50]. Erythrocytes develop in the bone marrow and remain in circulation for 100-120 days before they are "recycled" by macrophage[51]. About a quarter of the cells in the human body are red blood cells. Almost all vertebrates, including all mammals and humans, have erythrocytes. Vertebrate erythrocytes consist primarily of haemoglobin, a complex metal-protein containing heme groups, whose iron atoms temporarily bind to oxygen (O2) molecules in the lungs and release them throughout the body. Oxygen easily spreads through the cell membrane of erythrocytes. Haemoglobin in erythrocytes also carries part of the carbon dioxide (CO2) which is one of the major waste products in tissues. Most carbon dioxide, however, is transported as bicarbonate ($HCO3^-$) dissolved in blood plasma[52]. Human erythrocytes have a disc diameter of about 6,2-8,2

μm a thickness at the thickest point of 2-2,5 μm and a minimum thickness at the centre of 0,8-1 μm, resulting therefore smaller than most other human cells. Adult humans have about 20-30 billion red blood cells. Women have about 4-5 million red blood cells per microliter (cubic millimetres) of blood and men about 5-6 million; people living at high altitude with low oxygen voltage have more. The red colour of the blood is due to the spectral properties of heme group in haemoglobin, and the intensity of red varies depending on the state of haemoglobin: when combined with oxygen, the resulting oxyhaemoglobin is scarlet, when oxygen has been released, the resulting deoxyhaemoglobin is a dark red, burgundy colour. Each human red blood cell contains about 270 millions of these haemoglobin molecules, each haemoglobin molecule carries four heme groups; haemoglobin makes up about one third of the total cell volume. Haemoglobin is responsible for the transport of more than 98% of the oxygen in the body (the remaining oxygen is transported dissolved in the blood plasma). The red blood cells of an average adult human male collectively store about 2.5 grams of iron, representing about 65% of the total iron in the body[53][54]. Haemoglobin also has a very high affinity for carbon monoxide, forming carboxyhaemoglobin that has a very bright red colour.

Having proteins that carry oxygen within specialized cells has been an important step in the evolution of vertebrates as it allows to obtain less viscous blood, higher oxygen concentrations and better diffusion of oxygen from blood to tissues. The size of erythrocytes is on average about 25% larger than the diameter of the capillary, and it has been hypothesized that this improves the transfer of oxygen from red blood cells to tissues[55].

Nucleus

Human erythrocytes are anucleates, meaning they lack a cell nucleus. In comparison, red blood cells of other vertebrates have nuclei.

The absence of the nucleus in red blood cells is explained by the fact that efficient oxygen transport requires erythrocytes to pass through narrow capillaries and the presence of a nucleus would limit this passage.

Membrane composition

RBCs are deformable, flexible, able to adhere to other cells and are able to interface with immune cells. Their membrane plays many roles about this. These functions strongly depend on the composition of the membrane. The red blood cell membrane is composed of 3 layers: the glycocalyces outside which is rich in carbohydrates, the lipid double layer which contains many transmembrane proteins, in addition to its main lipid constituents, and the skeleton of the membrane, a structural network of proteins located on the inner surface of the lipid double layer[56]. Half the mass of the erythrocyte membrane is made up of proteins, the other half are lipids, namely phospholipids and cholesterol[57].

Membrane lipids

The plasma membrane of these cells comprises a typical lipid double layer, similar to that found in virtually all human cells. In a nutshell, this double lipid layer is composed of cholesterol and phospholipids in equal proportions by weight. The lipid composition is important as it defines many physical properties such as the permeability and fluidity of the membrane. In addition, the activity of many membrane proteins is regulated by interactions with lipids in the double layer. Unlike cholesterol which is evenly distributed between the inner and outer leaflets, the 4 main phospholipids are arranged asymmetrically[56].

In the outer monolayer we find phosphatidylcholine (PC) and sphingomyelin (SM); in the inner monolayer we find phosphatidylethanolamine (PE), phosphatidylinositol (PI) in small quantity and phosphatidylserine (PS). This asymmetric distribution of phospholipids is the result of the function of different transport proteins. Proteins called

"flippases" move phospholipids from the outer monolayer to the inner one, while others called "flopping" do the opposite. There are also "scramblase" proteins that move phospholipids in both directions at the same time, lowering their concentration gradients independently of energy[58]. There is still considerable debate going on regarding the identity of these membrane holding proteins in the red blood cell membrane.

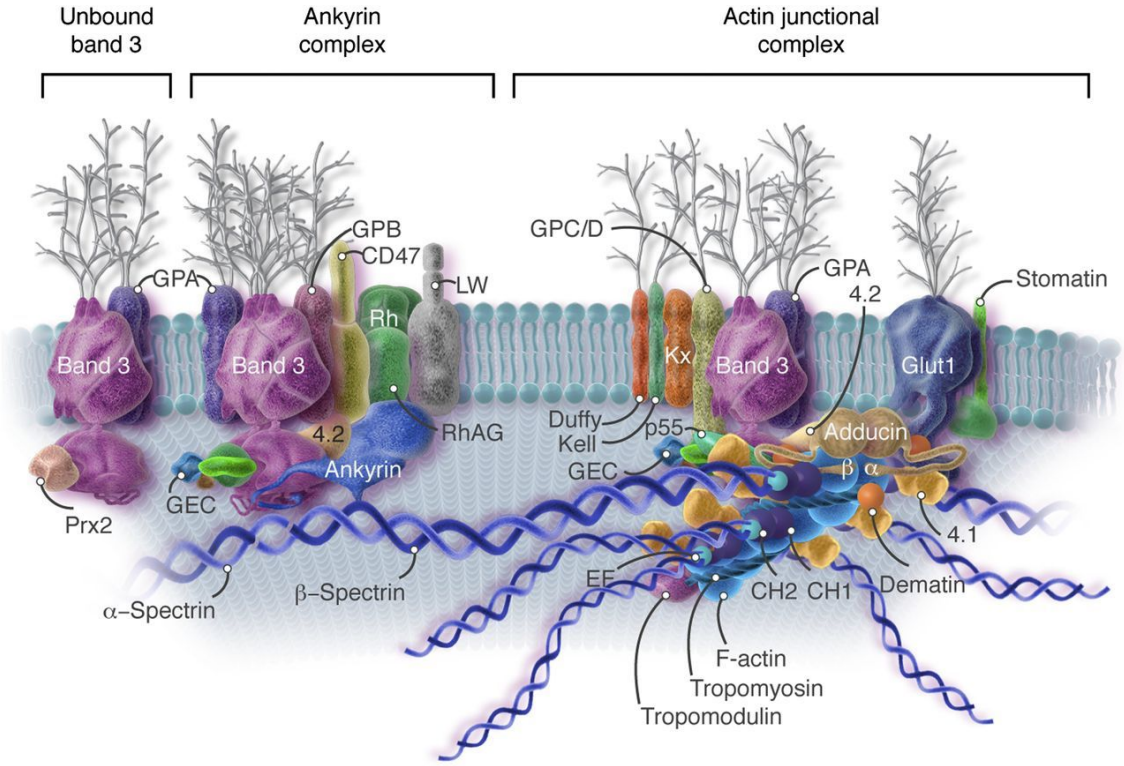

Figure 4 - The red blood cell membrane. eClinPath.com, Cornell University, hematology/physiology/erythrocytes

Maintaining the asymmetric phospholipid distribution in the double layer (such as an exclusive localization of PS and PI in the inner monolayer) is crucial for the integrity and function of the cell due to several reasons:

- Macrophages recognize and phage RBCs that expose PS to their external surface[59].

- Premature destruction of thalassaemic and falciform red blood cells has been linked to disruptions of lipid asymmetry leading to exposure of PS on the outer monolayer[60]. Exposed PS can enhance the adhesion of red blood cells to vascular endothelial cells,

effectively preventing normal transit through micro vascularization. Both PS and phosphatidylinositol-4,5-bisphosphate (PIP2) can regulate the mechanical function of the membrane, due to their interactions with skeletal proteins such as spectrin[61]. Recent studies have shown that the binding of spectrin to PS promotes the mechanical stability of the membrane[62].

Membrane proteins

Membrane skeleton proteins (**Figure 4**) are responsible for the deformability, flexibility and durability of red blood cells, allowing the compression of the red blood cell diameter to less than half the standard diameter (7-8 μm), and also allow to recover the disc shape in a similar way to an object made of rubber.

There are currently more than 50 known membrane proteins that can exist in a few hundred up to a million copies per red blood cell[56]. About 25 of these membrane proteins carry various blood group antigens, such as antigens A, B and Rh, among many others. These membrane proteins can perform a wide variety of functions, such as the transport of ions and molecules across the red cell membrane, adhesion, and interaction with other cells such as endothelial cells, such as signalling receptors, as well as other currently unknown functions[56][63]. Red blood cell membrane proteins are organized according to their function.

Transporting

Band 3 is a carrier of anions and is also an important structural component of the cell membrane of erythrocytes[64]. Each cell contains about one million copies and constitutes 25% of the cell membrane surface[40]; Aquaporin 1 - water channel[64] ; Glut1 - glucose

transporter and dehydroascorbic acid[65]; Kidd antigen protein - urea transporter[66] ; RhAG - carrier of gas[67]; Na^+/K^+ ATPase[68]; Ca^2 ATPase[69] ; $Na^+/K^+/Cl^-$ cotransporter[70] ; Na^+/Cl^- cotransporter[70] ; Na^+/H^+ antiporter[70] ; K^+/Cl^- cotransporter[70] ; channels of Gardos[70].

Cellular adhesion

ICAM4 interacts with integrins[71] ; BCAM a glycoprotein that defines the blood group. Some membrane proteins, on the other hand, establish bonds with skeletal proteins and can play an important role in regulating the cohesion between the lipid double layer and the skeleton of the membrane, which prevents the collapse of the cell membrane (vesciculating).

Surface electrostatic potential

Zeta potential is an electrochemical property of cell surfaces that is determined by the net electric charge of molecules exposed on the surface of cell membranes. The normal zeta potential of red blood cells is -15.7 millivolts (mV). Much of this potential appears to be contributed by exposed sialic acid residues in the membrane: their removal results in a zeta potential of -6.06 mV[72].

2. ERYPTOSIS

Some parts described in this chapter has also been previously published in papers in which I am the first author and of which I hold the copyright together with the co-authors. These parts are merely descriptive and appropriately quoted.

Restivo et al., Antioxidants 2021, 10, 154, doi:10.3390/antiox10020154.

Restivo et al., Int. J. Mol. science 2022, 23, 3019, doi.org/10.3390/ijms23063019.

2.1 Eryptosis in general

The average life span of circulating mature human erythrocytes is about 100–120 days. The elimination of senescent or damaged RBCs is mediated by the Band-3 protein[73], by the activation of the complement and the hemolytic process is carried out in the spleen by macrophages. Human RBCs damaged under different stressors can be removed before by programmed suicidal death, a process called eryptosis[74]. Until recently, it was thought that because they lack a nucleus and mitochondria, RBCs were unable to undergo apoptosis. This hypothesis has been denied by much experimental studies that have shown the existence of the process known as eryptosis, which regulates the elimination of damaged or senescent RBCs[75]. Eryptosis is, therefore, a physiological process and except for the depolarization of mitochondrial membrane and condensation of chromatin is mostly similar to the apoptosis of nucleated cells. Eryptosis, also, is very important from a physiological point of view, as it prevents intravasal hemolysis of RBCs, allowing their elimination without leading to break of the membranes with the consequent inflammation due to the release of intracellular contents[76]. Hematopoietic processes on the one hand, and eryptosis on the other, play a key role in maintaining an adequate number of circulating RBCs. Any de-regulation of these processes can alter the number of circulating erythrocytes, compromising the oxygenation capacity of the tissues due to a minor amount of circulating hemoglobin. The hematopoietic processes mediated by the bone marrow and the hemocateretic processes mediated by macrophages that lead to the elimination of senescent or damaged RBCs, are in constant equilibrium in healthy subjects. The responsibility for regulating this fine balance is operated by erythropoietin[77]. Therefore, the most important signs of eryptosis are cell shrinkage, membrane blebbing and exposure

of phosphatidylserine to the outer membrane sheet, characteristics that are completely stackable to the apoptotic process[78].

2.2 Mechanisms of the eryptotic machinery

There are various molecular mechanisms leading to eryptosis (**Figure 5**). One of the most frequent is increased cytosolic concentration of Ca2+ ions, but also exposure of erythrocytes to energy depletion, hyperosmotic shock and oxidative stress. Normally, Ca^{2+} homeostasis is regulated through the complementary action of Ca^{2+} pumps - which act as extrusion mechanisms dependent on ATP - and calcium channels - through which extracellular Ca2+ enters.

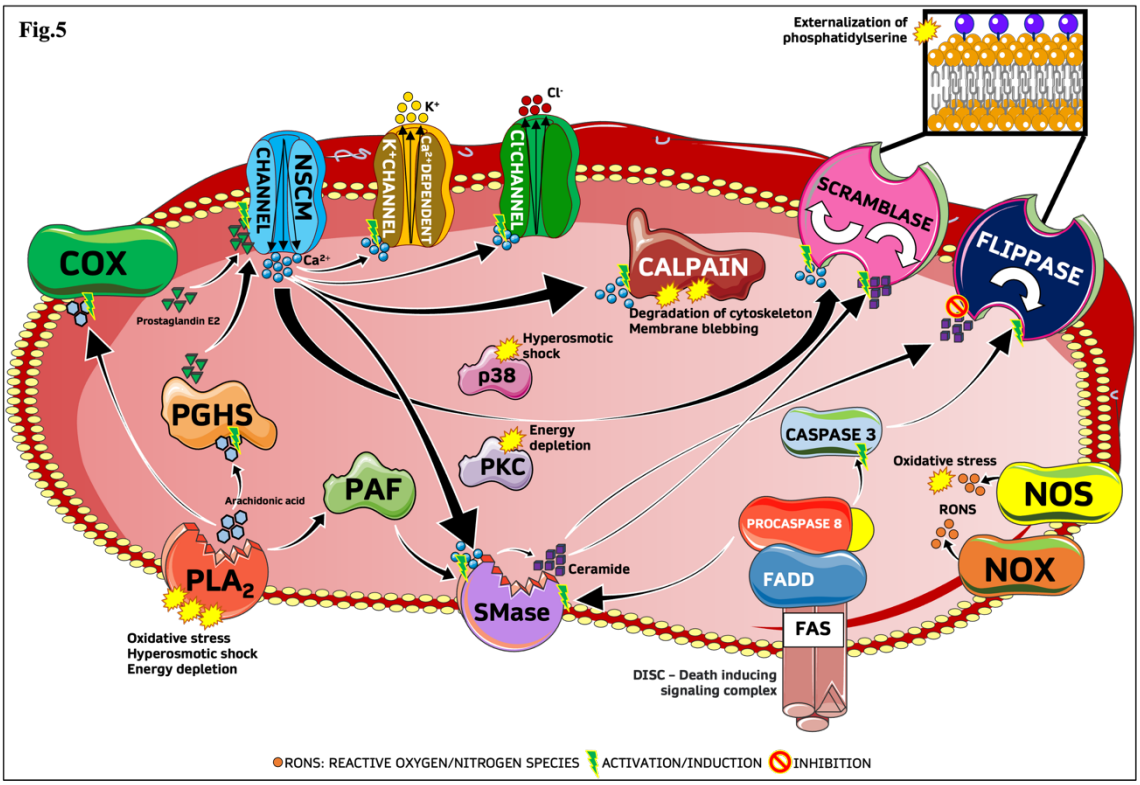

Figure 5 - Biomolecular pathways that regulate the eryptosis machinery including: Ca^{2+} influx[79], PLA2 activation, K^+ channels activation, calpain and/or caspases stimulation, PAF activation, stimulation of SM, assembly of DISC, p38 phosphorylation, PKC activation and NOX/NOS stimulation.

Some pro-eryptotic stimuli can activate phospholipase A2 (PLA2) with the production of arachidonic acid (AA), through hydrolytic catalysis of the bond in position 2' of membrane phospholipids. AA is metabolized in prostaglandin E2 (PGE2) by peroxide synthase (PGHS), or cyclooxygenase (COX). Production and stimulation by PGE2 is followed by the entrance of the Ca^{2+} ions ,in the cytosol of the erythrocyte through non-selective cationic channels[80].

The increase in the levels of Ca^{2+} ions is followed by the activation of the calcium-dependent potassium (K^+) channels (also called Gardos Channels). The activation of these channels induces the release of K^+ and Cl^- ions in extracellular space which causes hyperpolarization of the membrane. The loss of these ions causes an osmotic flow which leads to leakage of water and the consequent reduction of cell volume (shrinkage) typical of erythrocytes[81].

The increase in Ca^{2+} levels, also, stimulate calpain a cysteine endopeptidase, responsible for degradation of the RBCs cytoskeleton and membrane blebbing[82]. μ-calpain, along with the caspases, is one of the most important protease responsible for the cleavage of various membrane proteins present in erythrocytes that produce morphological alterations of the cell[83]. Although in 2001 it was shown that erythrocyte caspases were functionally active *ex vivo*[84], the role of caspase 8 and caspase 3 in the eryptotic process was elucidated a few years later, in a study in which caspases were shown to have a functional role in mature RBCs. Following a redox imbalance to which mature erythrocytes are subject, some phenomena involving caspases have been highlighted. In particular, the translocation of Fas receptor (FAS) to the membrane rafts, the formation of a complex associated with FAS, and the activation of caspase 3 and caspase 8. These events are

independent from the activation of calpain and stimulated by oxidizing species such as tert-butyl hydroperoxide or phenylhydrazine[85].

Furthermore, with the loss of Cl^-, there is still a PGE2 discharge which further increases the intracellular Ca^{2+} levels. While the activation of PLA2 leads to formation of PGE2, on the other hand it stimulates the release of the platelets activating factor (PAF). PAF is a powerful scramblase stimulator –protein used to move phospholipids from one monolayer to another of the lipid bilayer within the plasma membrane - and concurs its activation, together with the production of PGE2[86]. Together with the entrance of Ca^{2+}, PAF also stimulates the activity of membrane sphingomyelinase (SM). Sphingomyelinases are ubiquitous enzymes that catalyse the hydrolysis of cell membrane sphingomyelin, producing ceramide. The latter is a lipid that acts as a second intracellular messenger both in cell differentiation and in apoptosis. The increase of ceramide in the membrane, in fact, together with Ca^{2+} determines the activation of scramblase and inhibition of flippases. These, unlike the scramblases, are selective transmembrane proteins: they remove certain phospholipids from the outer sheet of the membrane and flip them into the leaflet exposed to the cytosol. The final effect of activating the scramblase and the simultaneous inhibition of flippases, causes phosphatidylserine (PS) to remain outsourced. This mechanism could play a key role in the characteristic adhesiveness found in eryptotic erythrocytes which can be due to impairments in the microcirculation[78][86].

Other studies have highlighted another mechanism involved in eryptosis due to energy depletion, which would seem to induce a reduced activity of the calcium-ATPase. All this leads to the reduction of Ca^{2+} efflux with consequent increase in the concentration of Ca^{2+} in the cytosol, which triggers all the mechanisms already seen previously. The lack of energy also causes the enzymatic activity of glutathione reductase to be reduced avoiding

the regeneration of reduced glutathione (GSH) with consequent depletion of antioxidant defenses of erythrocytes, which become more susceptible to oxidative stress. Under normal energy conditions, PS is maintained on the inner side of the membrane by flippase, whose activity is ATP-dependent. Consequently, a decrease in the activity of this transporter results in a greater amount of outsourced PS. In addition, other studies have also shown the involvement of the protein p38 kinase in the mechanism that triggers eryptosis following hyperosmotic shock[87]. Finally, energy depletion can lead to the activation of protein kinase C (PKC), a serine/ threonine kinase which stimulates eryptosis, by phosphorylating membrane proteins[88][89] as well as the early production of ROS by NADPH oxidase (RBC-NOX), nitric oxide synthase (RBC-NOS) and xanthine oxide-reductase (XOR) activated by oxysterols bring RBCs to eryptosis[90].

All the mechanisms mentioned, seem to lead to the maintenance of PS on the outer side of the membrane. The loss of the symmetry of the membrane and externalization of the PS are factors of stimulation of the phagocytosis activity of macrophages which ensure the removal of erythrocytes from the bloodstream[91].

2.3 Pathophysiological implication of eryptosis

"Several pathologies with relevant clinical significance are linked to excessive eryptosis, as summarized in (**Figure 5)**, mainly involving anemia, deranged microcirculation and/or increased prothrombotic risk[81][92].

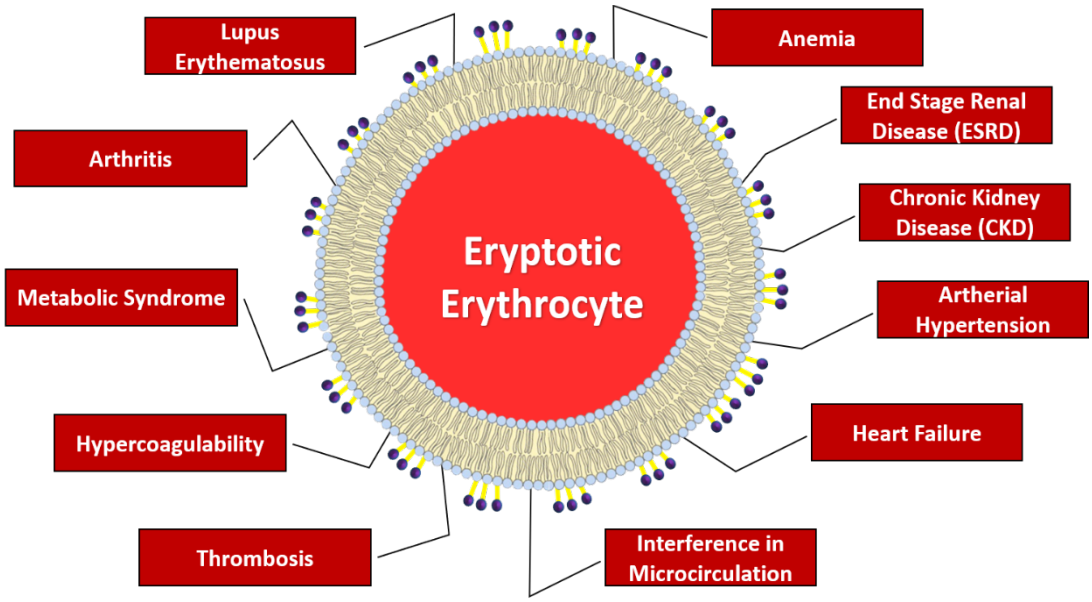

Figure 6 - Main diseases related increased eryptosis [56].

Anemia is one of the most frequent pathologies affecting RBCs, characterized, for the most part, by a decrease in levels of hemoglobin which leads to an insufficient supply of oxygen to the tissues. As mentioned above, as long as the eryptotic processes come compensated by adequate erythropoiesis, the number of erythrocytes circulating in the blood remains unaffected. In conditions where erythropoiesis is no longer able to counterbalance the accelerated loss of erythrocytes due to eryptosis, clinically evident anemia could result[79][93].

A study has highlighted the central role of eryptosis in anemia found in patients with terminal stage renal disease (end stage renal disease - ESRD). In particular, the

erythropoiesis is compromised by the renal production and release of erythropoietin (EPO)[94] and from iron deficiency[95]. Trials suggest, however, that anemia in ESRD largely results induced by an accelerated clearance of circulating erythrocytes, due to stimulation of eryptosis[96]. Thus, the condition of anemia does not normalize despite the compensation of the erythropoietin deficiency, and therefore, the persistence of anemia is due to a greater extent to an increase of eryptosis[97]. Even in chronic kidney disease (CKD), that ultimately causes kidney failure, there is a condition of anemia. The cause of anemia was initially associated with an iron deficiency[98]. More recent evidence, however, suggests that the cause of the anemia in these patients is eryptosis which reduces the average life of circulating erythrocytes.

Even liver failure and fibrosis are associated with anemia which can derive from various conditions, such as bleeding, malignant tumors, viral infections, chronic inflammation and deficiency of essential nutrients such as vitamin B12 and folate[99][7100]. It has recently been shown that also the resulting anemia from liver failure is attributable to eryptosis, which is induced by high bilirubin levels. Indeed, the high levels of conjugated bilirubin in the blood can increase the cytosolic activity of Ca^{2+} and the formation of ceramide[101].

The average lifespan of RBCs is also shortened in diabetes[102]. Anemia is prevalent in a large number of diabetic subjects and cannot be attributed to a deficiency in erythropoiesis, since the number of reticulocytes is increased[103][104]. New studies have revealed that anemia related to diabetes can derive, at least in part, from an increase in eryptosis[105][106]. The erythrocytes of diabetic patients show an increase in the activity of superoxide dismutase and ROS production. The mechanism of diabetes-induced eryptosis has been identified in glycoxidation, which consists on the glycation of biomolecules, dependent on oxidative stress. In fact, a condition of lasting hyperglycemia accelerates

the glycation of the free amino groups of the membrane proteins in RBCs. This process leads to the formation and accumulation of advanced glycation end products (AGEs), substances with a pro-oxidant nature that induce morphological alterations of the RBCs cytoskeleton culminating in the increase in PS levels on the outer membrane[107][108].

A recent study has also highlighted the involvement of eryptosis in arterial hypertension. This is characterized by high arterial pressure, both systolic and diastolic. Usually, this pathology is associated with dyslipidemia, which consists of an alteration of the concentration of triglycerides or cholesterol in the blood. Both ailments can contribute to oxidative stress which, as we have already said, can induce an increase in eryptosis. The study involved four different groups: normotensive with and without dyslipidemia, and hypertensive with and without dyslipidemia. Hypertensive patients had higher eryptosis levels, associated with an increase in Ca^{2+} levels and oxidative stress; thus suggesting that eryptosis participates in the pathophysiological mechanisms of hypertension[109].

Anemia is also a common condition in heart failure. According to a study, patients suffering from heart failure show an increased percentage of erythrocytes with externalization of PS, ROS levels and a reduced cell volume. Increased oxidative stress is one key feature of heart failure that can promote hemolysis and eryptosis[110].

In addition, eryptosis can interfere with microcirculation, as the erythrocytes that expose PS they have a greater ability to adhere to the vascular wall[111]. The eryptotic erythrocytes, due to their procoagulant phenotype, can contribute to the onset of thrombosis. Adherence to endothelial cells and platelets by erythrocytes that expose PS on their membranes is due to the ability to interact with certain surface receptors; for example, with the transmembrane ligand CXC chemokine CXCL16, also known as SR-PSOX. The latter

acts as scavenger receptor with a high affinity for PS and with oxidized low-density lipoproteins. Platelets also express CXCL16 which stimulates their activation and translates vascular inflammation into thrombo-occlusive events[112]. Moreover, platelets express CD36, a receptor that was demonstrated to be able to interact with PS. The interaction of erythrocytes with platelets can promote thrombus formation in conditions of hypercoagulability associated with various clinical disorders such as liver insufficiency and chronic kidney disease. In a model of thrombosis triggered by ferric chloride, erythrocytes have been shown to recruit platelets in the site of the injury, thus supporting the idea that they contribute actively to the pathophysiology of thrombosis[113]. The outsourcing of the PS on the cell membrane of erythrocytes serves as a platform for the assembly of prothrombinase (factor X) that stimulates the formation and the coagulation of thrombin, thus mediating the procoagulant effects of eryptotic erythrocytes. PS exposure can be further stimulated by the coagulation factors I, V and X, which cause a state of hypercoagulability[114]. Likewise, smoking may aggravate microcirculation since it has recently been reported that cigarette smokers have a higher level of circulating erythrocytes than non-smokers characterized by greater externalization of PS, a decrease in intracellular stores of GSH and an increase in C reactive protein (CRP)[115].

Obesity is also linked to an increased risk of thrombosis. Although the mechanisms involved have not yet been elucidated, it is believed that a greater aggregability of erythrocytes and a reduced deformability of the same in obese patients cause hypercoagulability disturbances. In fact, in a recent study it was shown that PS exposure in erythrocytes is significantly higher in patients with a higher body mass index than healthy subjects. Eryptosis participates in the hypercoagulability associated with obesity

and to atherosclerosis, thus underlining the importance of the pathological link between erythrocytes and endothelial dysfunction and the activation of macrophages in obesity[116].

Many of the factors aforementioned can contribute to development of the metabolic syndrome, a pathology that involves a series of metabolic factors that increase risks of cardiovascular diseases, diabetes and associated diseases such as dementia[82]. The development of the latter depends on the onset of systemic chronic inflammation. Indeed, the dysfunctional activation of the inflammatory response strongly compromises metabolic homeostasis of key tissues in energy use. A pro-inflammatory, pro-oxidant and pro-thrombotic state has been observed in patients with metabolic syndrome. Accumulation of fat and obesity are major players in the development of chronic inflammation, due to the ability of adipocytes to secrete pro-inflammatory mediators such as cytokines or certain hormones like leptin, in response to hypertrophic signals. The persistence of the inflammatory state can induce endothelial dysfunction, which represents one of the early events which then leads to atherosclerosis. Clinical evidence suggests that eryptosis plays a key role in the development of all risk factors associated with metabolic syndrome - such as hyperglycemia, dyslipidemia, hypertension and obesity - and clinical complications - such as diabetes and atherosclerosis[82]. These evidences are reinforced recently from an *in vivo* study that showed a higher level of eryptotic erythrocytes in hypercholesterolemic patients compared to normocholesterolemic[117]. In this sense, a recent *ex vivo* assay has reported that saturated fatty acids (in particular lauric acid), well-know compounds that raise circulating cholesterol levels, induce eryptosis through a Ca2+-dependent externalization of PS, cell shrinkage and granularity, oxidative stress, accumulation of lipid peroxides, and stimulation of casein kinase 1α[118].

Epidemiological studies suggest that inflammation is also one of the major causes of anemia in the elderly and chronic pathologies. Anemia in inflammatory diseases is largely due to reduced iron homeostasis and suppression of erythropoiesis by pro-inflammatory cytokines. It has recently been shown that the inflammatory cytokines induce changes in the erythrocyte membrane. A study showed that anemia in arthritis patients is due to, at least in part, the increase in eryptosis. The latter is induced by an increased oxidative stress and increased cytosolic Ca^{2+} levels. It has been hypothesized that the increased adhesion of eryptotic erythrocytes to the endothelial vascular cells can contribute to the pathophysiology of vascular occlusion and ischemia in patients with arthritis[119].

Anemia also occurs in approximately 50% of patients with systemic lupus erythematosus (SLE), an autoimmune disease. Anemia in SLE has a multifactorial etiology and it can also be triggered by autoimmune destruction of erythrocytes and from immune-mediated hematopoietic insufficiency. Also, in some patients with SLE, antibodies against erythropoietin were found, a fact by which they modify the normal production of erythrocytes. Anemia in SLE can also be caused by the reduced lifespan of erythrocytes since a significantly high percentage of circulating erythrocytes exhibit PS on their membranes. Furthermore, these patients show an increase of circulating reticulocytes, an increase in the cytosolic activity of Ca^{2+} and a high ROS production. Hence eryptosis can contribute to pathophysiology anemia in SLE[120].”[79]

3. AIM OF THE STUDY

Cigarette smoke (CS) is a well-established risk factor in the etiology of cardiovascular diseases, including diseases of the peripheral vascular system[121], ischemic heart disease[122], atherosclerosis[123], myocardial infarction[123] and stroke[124]. Knowledge of the biochemical mechanisms underlying CS-induced vascular dysfunction is still limited. Studies attribute vascular inflammation and thrombotic mechanisms to the absorption of toxic components of tobacco smoke at the alveolar level, which entering the bloodstream causes damage to endothelial cells and blood cells such as leukocytes and platelets[125][126][127][128]. In a previous study, done by my research group during my experimental thesis period, it has been reported that smokers have high levels of circulating apoptotic erythrocytes, suggesting a possible contribute of the eryptosis to the smoke-associated vascular injury[115].

During my PhD program, carried out at the biochemistry laboratory of the Department of Chemical and Pharmaceutical Biological Sciences and Technologies of the University of Palermo, I am committed to assess a causative association linking CS to eryptosis. In this *in vitro/ex vivo* study we investigated how CS extract (CSE) can directly affect the RBCs function and determined the mechanism by which this occurs. This work shows that the CSE initiates eryptosis *via* the extracellular signaling pathway with the DISC assembly leading to overproduction of ceramide by nSMase and the activation of caspase-8/caspase-3. We also report that p38 MAPK regulates the assembly of the DISC and provide preliminary results about the stimulation of the extrinsic death pathway in RBCs isolated from smokers.

4. MATERIALS AND METHODS

4.1 Preparation of CSE

CSE was prepared by a modification of a previously method published by Su et al [129]. In brief, 3 filtered commercial cigarettes, each containing 0.8 mg nicotine, 10 mg tar and 10 mg carbon monoxide according to the manufacturer's report, were smoked consecutively through an apparatus with a constant airflow (0.4 L/min) controlled by an air compressor. The smoke was bubbled through 30 ml of Ringer solution pre-heated at 37°C. The Ringer solution consists of (mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH, 5 glucose, 1 $CaCl_2$, pH 7.4. Cigarettes were combusted up to 3 mm from the filter. The smoke solution was adjusted to pH 7.4 and then filtered through a 0.22-mm filter (Millipore, Bedford, MA) to sterilize it. This solution was considered to be 100% CSE. Concentration of nicotine, one of the stable constituents, was assessed by LC–MS analysis carried out using an Ultimate 3000 instrument coupled to a TSQ Quantiva (Thermo Fisher Scientific, San José, CA) triple-stage quadrupole mass spectrometer. Nicotine mean concentration of three different CSE preparations was 32,13±1,23 μg/ml. We can therefore say that the extraction technique is reproducible.

4.2 RBCs and Treatment

Fresh blood samples were collected in heparinized tubes from non-smoking healthy male volunteers (n = 8; age range 33-66 years; normal BMI range) with informed consent, and RBCs were immediately collected by centrifugation (2000x *g*, 4 °C, 20 min) over a Ficoll (Sigma-Aldrich, Cat. No. F5415) gradient. RBCs were washed twice in Ringer solution. Aliquots of the cell pellet were used to create an hematocrit (HT) at 0.4% in the same buffer (control) or in Ringer containing CSE at appropriate concentration for the

treatment. Erythrocytes were then incubated at 37 °C, 5% CO_2 and 95% humidity for the indicated times. For treatments in nominally calcium-free Ringer, $CaCl_2$ in the solution was replaced by 50 μM cell-permeable calcium-chelating agent BAPTA-AM (Sigma-Aldrich, Cat. No. A1076).

Where indicated, pre-treatment was carried out with either neutral sphingomyelinase inhibitor GW4869 (Sigma-Aldrich, Cat. No. D1692) (15 μM), caspase 8 inhibitor Z-IETD-FMK (MedChemExpress, Monmouth Junction, NJ, USA, Cat. No. HY-101297) (25 μM) or p38 MAPK inhibitor SB203580 (MedChemExpress, Cat. No. HY-10256) (10 μM). Inhibitors were added in dimethyl sulfoxide (DMSO) at a 0.1% (v:v) final concentration at the RBCs samples (HT 0.6 %). Cells were pre-incubated for 1h at 37 °C before that CSE at appropriate amount, or Ringer (control), was added. HT was then normalized at 0.4% and the treatment was prolonged up to the set time.

Healthy volunteers (smokers and non-smokers) were recruited and the experimental study, approved by the Ethic Committee of Palermo 1-University Hospital (No. 8-09), was performed in accordance with the Declaration of Helsinki and its amendments.

4.3 Hemolysis

After treatment, RBCs were centrifuged (1800 rpm, 24 °C, 5 min) and hemoglobin concentration in the supernatant was measured by absorbance at 408 nm (Soret's band) by spectrophotometry (DU-640 Spectrophotometer Beckman, Brea, CA, USA). The absorbance value at 408 nm of the supernatant derived from similar erythrocytes lysed in distilled water was considered as 100% hemolysis.

4.4 Flow Cytometry

4.4.1 Measurement of Phosphatidylserine (PS) Externalization and Forward Scatter (FSC)

RBCs were washed once in Ringer solution, pH 7.4, and adjusted to 1.0 x 10^6 cells/mL with binding buffer following the manufacturer's instructions. In experiments designed to evaluate the percentage of PS externalization, suspension of RBCs (100µL) was incubated with 5 µL of Annexin V-FITC (eBioscience, San Diego, CA, USA, Cat No. 88-8005-74) at room temperature in the dark for 15 min. Subsequently, suspension samples of at least 1.0 x 10^4 cells were subjected to fluorescence-activated cell sorting (FACS) analysis by Epics XL™ flow cytometer, using Expo32 software (Beckman Coulter, Fullerton, CA). RBCs were analyzed by FSC, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

4.4.2 Measurement of Intracellular Reactive Oxygen Species (ROS)

Production of ROS was determined by measuring percentage changes in fluorescence resulting from oxidation of the dichloro-dihydro-fluorescein diacetate (DCFDA) (Sigma-Aldrich, Cat. No. D6883) fluorescent probe. DCFDA, at 10 µM final concentration, was added to 1.0 x 10^5 cells 30 min before the end of the treatment, in the dark. RBCs were collected by centrifugation (2000 x *g*, 4 °C, 5 min), washed, resuspended in PBS, and analyzed as reported by Attanzio et al[90].

4.4.3 Measurement of Intracellular Glutathione (GSH)

Measurement of intracellular GSH level was monitored by probe 5-chloromethylfluorescein diacetate (CMFDA) (Abcam, Cambridge, UK, Cat. No. ab145459). CMFDA, at 1 µM final concentration, was added to 1.0 x 10^5 cells 40 min before the end of the treatment, in the dark. RBCs were collected by centrifugation (2000 x *g*, 4 °C, 5 min), washed, resuspended in PBS, and analyzed by flow cytometry[130].

4.4.4 Measurement of Intracellular Calcium

Cytosolic concentration of Ca^{2+} was monitored by measuring the fluorescence change of the Fluo-3 AM (Sigma-Aldrich, Cat. No. 73881) dye used as a probe for Ca^{2+}, whose intensity is directly representative of the cytosolic levels of the ion. Briefly, Fluo-3 AM at 2µM final concentration was added to 1.0 x 10^5 erythrocytes 40 min before the end of treatment in the dark. After centrifugation, (2000 x *g*, 4 °C, 5 min) RBCs were washed, resuspended in PBS and analyzed as reported above.

4.4.5 Measurement of Ceramide

Abundance of ceramide levels was measured as follows. Briefly, after treatment, 1.0 x 10^5 erythrocytes were incubated for 1 hour at 37 °C with 1 µg/ml of a mouse monoclonal anti-ceramide antibody (Sigma-Aldrich, Cat. No. C8104) in PBS containing 0.1% bovine serum albumin (BSA). RBCs after two washing steps with PBS-BSA, were stained for 30 min with 20 µL of a goat anti-mouse, polyclonal, fluorescein isothiocyanate-conjugated, secondary antibody (Millipore, Billerica, MA, USA, Cat. No. AQ502F) diluted 1/50 in PBS-BSA in the dark. Finally, erythrocytes were collected by centrifugation (2000 x *g*, 4 °C, 5 min), washed twice, resuspended in PBS and analyzed by flow cytometer as reported

above.

4.5 Immunoprecipitation

RBCs (2.0 x 10^8 cells) were washed twice with PBS, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 1% NP-40, 2 mM PMSF, 0.5 mM DTT and 2 mg/mL lysozyme) containing phosphatase (Roche, Basel, Switzerland, Cat, No. 4906845001)- and protease inhibitors (Roche, Cat. No. 4693132001) and sonicated (2 cycles, each for 30 sec) with Labsonic LBS1-10 (Labsonic Falc, Treviglio, Italy). Lysates were centrifuged (40000x *g*, 4 °C, 1 h) and supernatants were immunoprecipitated overnight with mouse anti-FAS antibody (1:200) at 4 °C. Supernatants were then incubated with 20 µl of Protein G PLUS-Agarose (Santa Cruz, Biotechnology, Dallas, TX, USA, sc-2002) for 3 h at 4 ° C. Beads were pelleted, washed twice in lysis buffer and finally proteins were separated by SDS-PAGE for immunorecognition by western blotting.

4.6 Western Blotting

Erythrocytes (2.0 x 10^8 cells) were lysed, centrifuged as reported above and supernatants were collected. Bradford protein assay (Bio-Rad, Hercules, CA, USA, Cat. No. 5000006) was used to quantify the total protein concentration. For each sample, equal amounts of proteins were loaded (50µg/lane), separated on 10% gel by discontinuous SDS-PAGE and then electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Cat. No. IPVH00010). Blots were treated with blocking solution (5% nonfat dry milk) and then incubated overnight at 4 °C with primary antibodies in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, pH 7.4) containing Tween 20 (1%, v/v) (TBST) and 5% (w/v) BSA. Mouse monoclonal anti-FADD (Santa Cruz, sc-271748), anti-FAS (Santa Cruz, sc-74540), anti-caspase-3 (Santa Cruz, sc-56053), anti-caspase-8 (Santa Cruz, sc-81657) and

anti-p-p38 MAPK (Santa Cruz, sc-166182) primary antibodies were used at a dilution of 1:200. After washing three times with TBST, immunoblots were incubated with a 1:2000 dilution of rabbit anti-mouse IgG Antibody, horseradish peroxidase (HRP) conjugated (Sigma-Aldrich, Cat. No. AP160P) for 1 hour at room temperature. Immunoblots were then washed five times with TBST and developed by enhanced chemiluminescence (Amersham, Milan, Italy, Cat. No. RPN2232). Mouse monoclonal anti-Actin antibody (Santa Cruz, sc-8432) was used as loading control. Densitometric analysis of protein spots was measured by Quantity One Imaging Software (Bio-Rad, Cat. No. 1708265) and the results were reported as arbitrary densitometric units normalized to actin.

4.7 Cross-linking Reaction by 3,3´-dithiobis(sulfosuccinimidyl propionate (DTSSP)

DTSSP (Sigma-Aldrich, Cat. No. 803200) is a synthetic impermeable thiol-cleavable cross-linker that reacts with primary amines to form stable amide bound. For cross-linking, 1 mM DTSSP in PBS was added to RBCs samples 30 min before end of the treatment. Erythrocytes were then lysed in 1% Triton X-100, 5 mM iodoacetate, protease inhibitors, 150 mM NaCl, 15 mM EDTA and 10 mM Tris-HCl as reported by Mandal et al[59]. After immunoprecipitation with anti-Fas antibody as above described, samples were separated into two aliquots and boiled independently in SDS gel denaturing sample buffer, as reducing condition, and β- mercaptoethanol-free sample buffer, as non-reducing condition. Finally, samples were separated by 7.5% SDS-PAGE and analyzed with anti-Fas antibody.

4.8 Ex vivo Analysis

Fresh blood samples from 2 healthy non-smoking males aged 39 and 56, and 2 males of matching age who regularly smoked 15 or more cigarettes per day for 10 years were drawn, with informed consent. Subjects had normal range of BMI and they did not report any kind of disease or drug use. Plasma cotinine level, a highly sensitive and specific biomarker of tobacco smoke exposure, was analyzed using LC/MS spectrometry with a detection limit of 0.015 ng/ml. Red blood cells from the subjects were isolated as reported above and immediately used for immunoprecipitation and Western blotting analysis.

4.9 Statistical Analysis

Results are expressed as mean ± SD of n separate experiments in triplicates. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Fisher's correction for multiple comparisons using Prism version 8 (GraphPad Software Inc., San Diego, USA). In all cases, significance was accepted if the null hypothesis was rejected at the $p < 0.05$ level.

5. RESULTS

5.1 CSE induces dose-dependent eryptosis

The loss of phospholipid membrane asymmetry of erythrocytes, with the externalization of PS, is the most characteristic marker of eryptosis. To assess the eryptotic potential of extracts, human RBC were treated for 24 h with CSE from 0% to 20%. The treatment led to a dose-dependent and statistically significant PS externalization induction, measured by FACS analysis by staining with V-FITC annexin (Fig. 7).

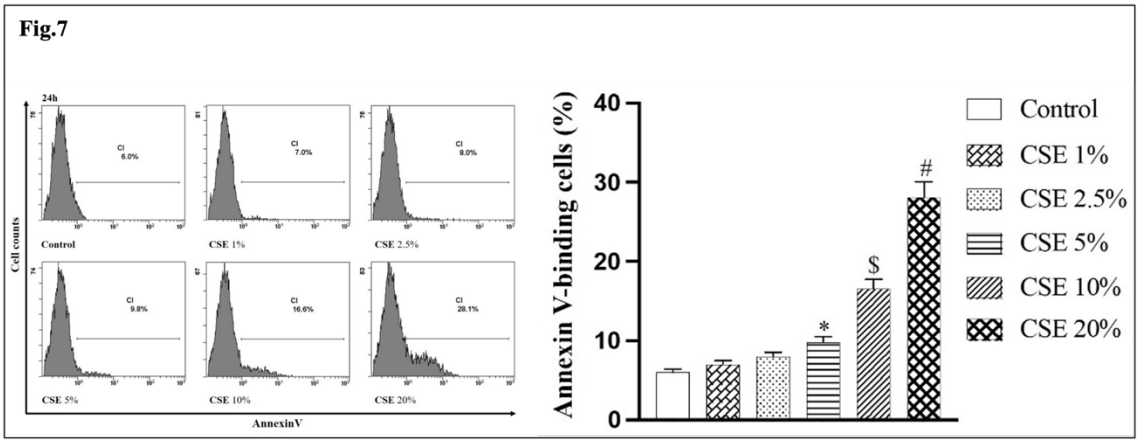

Figure 7 - CSE induces eryptosis with externalization of PS. Percentage of PS-exposing erythrocytes after 24 h incubation with CSE. RBCs incubated with vehicle were used as control. Annexin V-FITC associated fluorescence was measured by flow cytometry as reported in Methods. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p< 0.05$ (ANOVA associated with Fisher's correction).

After a 24 h exposure with either 10% or 20% CSE, Annexin V binding to RBCs accounted for 17,1±0,91% or 27,6±1,55% respectively. All other experiments were done with CSE 10% or 20%. In these conditions we also observed a sharp decrease in cell volume, determined by Forward Scatter (FSC) (Fig. 8).

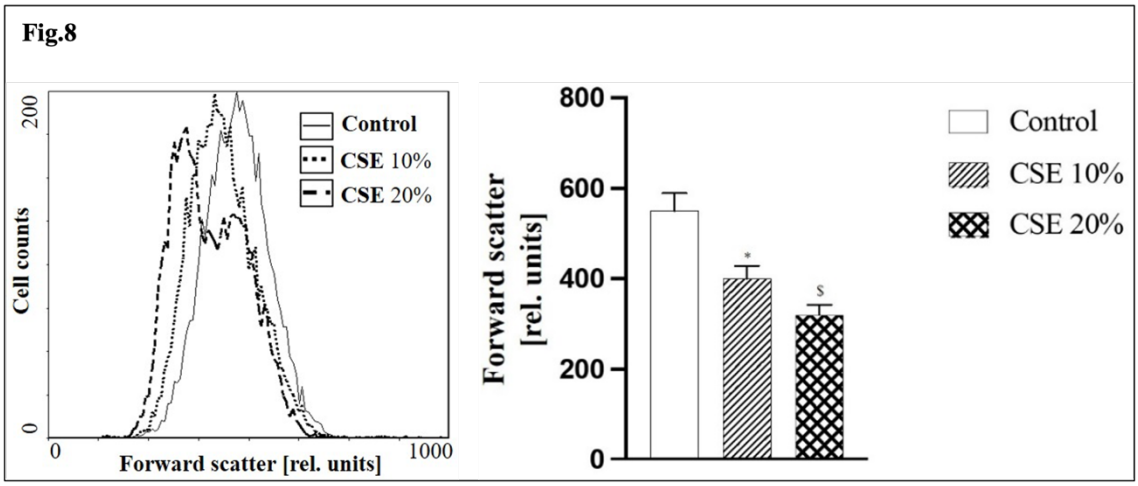

Figure 8 - CSE induces eryptosis with decrease of cell volume. Measurement of FSC after 24 h incubation with CSE. RBCs incubated with vehicle were used as control. FSC was measured by flow cytometry as reported in Methods. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

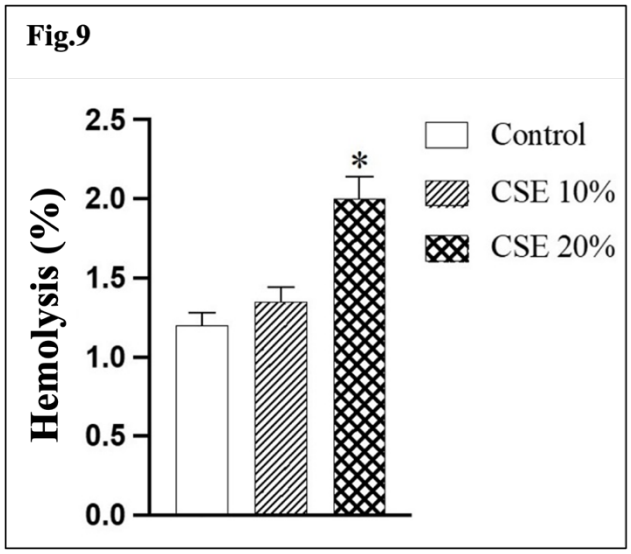

Figure 9 - CSE does not cause hemolysis. Percentage of cell hemolysis after 24 h incubation with CSE. RBCs incubated with vehicle were used as control. Hemolysis was measured spectrophotometrically as reported in Methods. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

To be sure that the predominant mechanism in CSE-induced cell death of RBCs was eryptosis, we investigated the index associated with cell necrosis by hemolysis analysis in RBSs treated with CSE 10% and 20%. Respect to the untreated cells (control), CSE treatment induced a slight but significant release of hemoglobin only at 20%, with a value increasing from 1.2%±0.1% to 2.0±0.3% (Fig. 9). Because only a negligible fraction of RBCs was lysed, it is possible to conclude that eryptosis appears to be the main CSE-induced mechanism of cell death of RBCs.

5.2 CSE induces stress-oxidative independent eryptosis

CSE contains more than 5000 oxidizing chemicals as well as a very high number of unidentified long- or short-lived free radicals[131]. Redox imbalance is a determining factor in the fate of erythrocytes in different conditions. We then measured intracellular levels of ROS and GSH in RBCs exposed to CSE. Cytofluorimetric analysis with specific probes, such as 2,7-dichlorofluorescin (DCF) and 5-chloromethylfluorescein (CMF), showed that incubation for 24 hours with 10% or 20% of CSE did not induce significant changes compared to control cells ($p>0,05$, $n=6$) intracellular levels of ROS nor caused depletion of GSH (Fig. 10a and b). With these analyses we exclude that oxidative stress is involved in eryptosis induced by CSE.

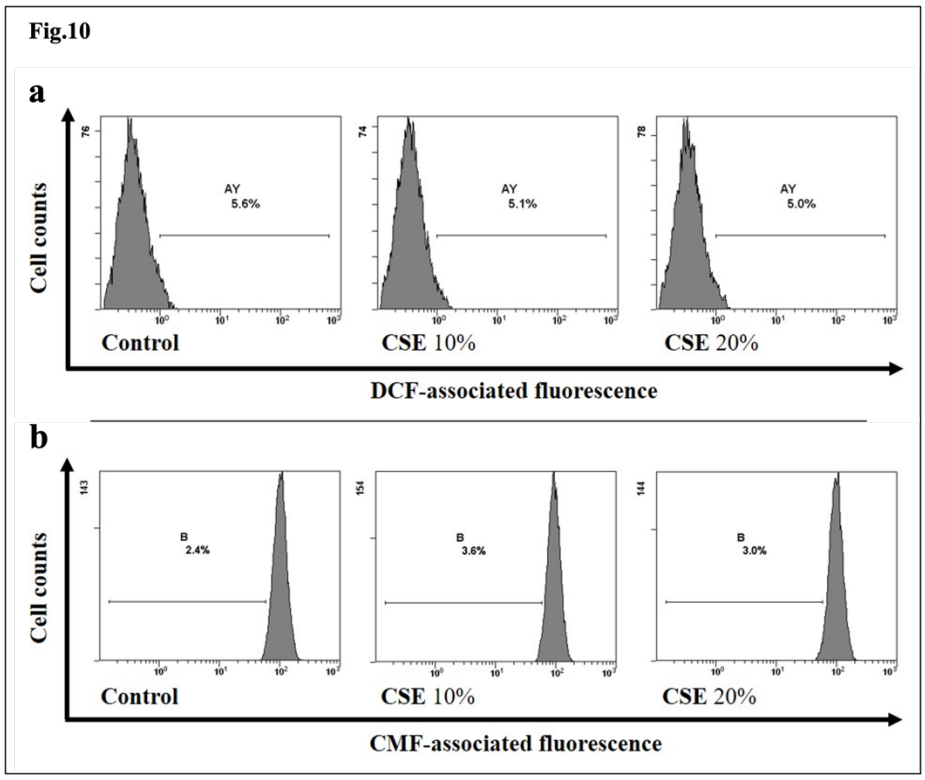

Figure 10 - CSE does not induce oxidative stress in erythrocytes. (a) ROS levels measured as DCF-associated fluorescence and (b) GSH content measured as CMF-associated fluorescence in RBCs after 24 h incubation with CSE. RBCs incubated with vehicle were used as control. Flow cytometry analysis was carried out as described in Methods. Representative images of four experiments carried out in triplicate with comparable results.

5.3 CSE induces eryptosis through extrinsic apoptotic pathway

To assess whether CSE-induced eryptosis was associated with an extrinsic death pathway that begins with an extracellular signaling, we studied the formation of death-inducing signaling complex (DISC) in membrane.

For this analysis we carried out experiments of co-immunoprecipitation in RBCs treated with CSE 10% or 20%, during an interval time of 0-6 h. RBCs were lysed with 1% of Triton X-100 and then were immunoprecipitated with anti-antibody Fas, followed by western blotting with anti-FADD antibodies or anti-caspase 8. Both proteins, caspase-8 and FADD, were found in the immunoprecipitate from RBCs treated with CSE, the longer the exposure time or the greater the CSE amount, the higher the protein amounts (Fig. 11).

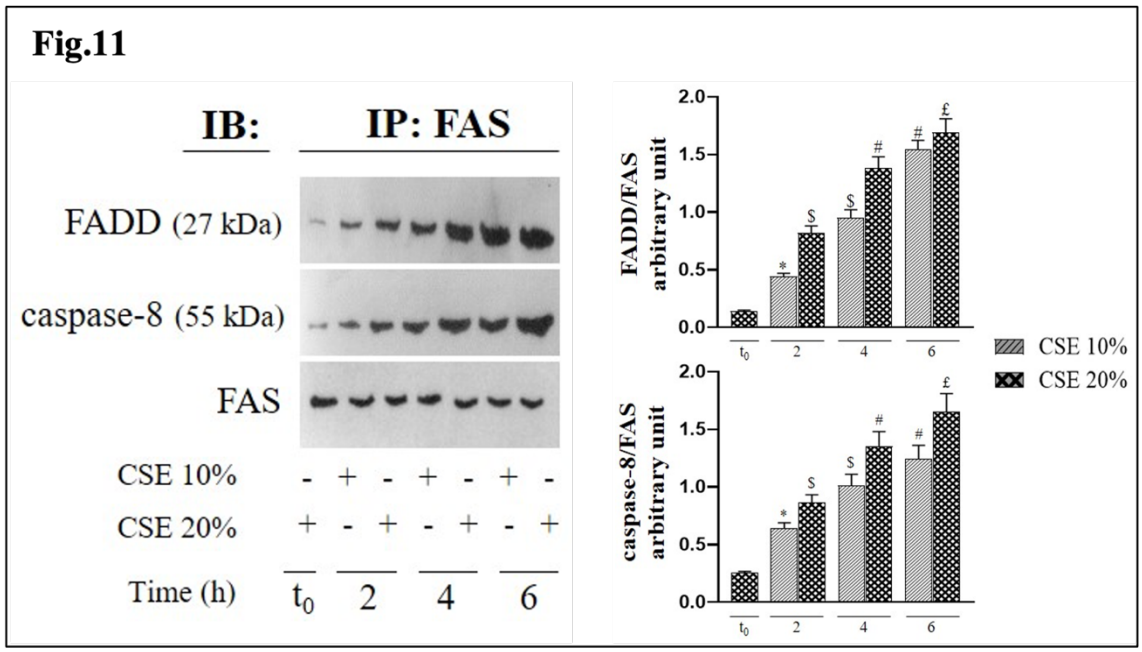

Figure 11 - **CSE induces eryptosis through extrinsic signaling pathway activation (1).** Association of Fas with FADD and caspase 8. RBCs treated at different time intervals with CSE were lysed and immunoprecipitated with anti-Fas antibody as described in Methods. The immunoprecipitates were separated by SDS-PAGE and blotted with anti-FADD or anti-caspase-8. Blots were reprobed with anti-Fas antibody to ensure equal loading of the gels. RBCs incubated with vehicle were used as control. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p< 0.05$ (ANOVA associated with Fisher's correction).

Since the aggregation of the Fas receptor is associated with the assembly of the DISC, to strengthen the obtained data we have also studied the oligomerization of this receptor. Fas receptor oligomerization was evaluated in RBCs after a 6 h treatment with CSE 20%, in the absence or in presence of cross-linker 3,3-dithiobis(sulfosuccinimidyl propionate) DTSSP. The cross-linker was used to hold back the receded Fas oligomers. Cellular lysates, therefore, were immunoprecipitated with anti-Fas antibody and separated by SDS-PAGE in reducing and nonreducing conditions. Immunoblotting with anti-Fas antibody of separate proteins revealed bands at different molecular weights: 45kDa for monomers, 85kDa for dimers and 150kDa for trimers in the not reducing gel with the RBCs treated with CSE but not in control erythrocytes (Fig. 12).

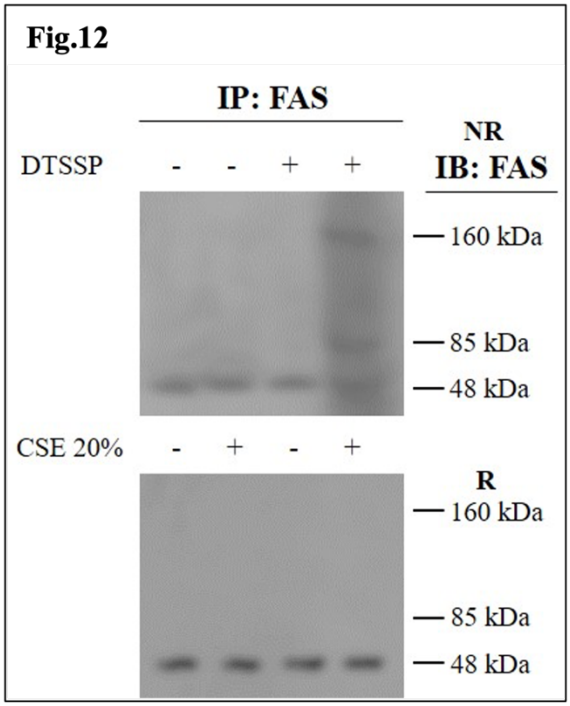

Figure 12 - CSE induces eryptosis through extrinsic signaling pathway activation (2). Oligomerization of Fas. Thirty min before the end of treatment (6 h) with CSE, RBCs were either left untreated or treated with the thiol-cleavable cross-linker DTSSP (1 mM). Then RBCs were lysed and immunoprecipitated with anti-Fas as described in Methods. Immunoprecipates were separated by SDS-PAGE under nonreducing (NR) and reducing (R) conditions and immunoblotted with anti-Fas antibody. Blots are representative of the results obtained in three separate experiments with comparable results. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

5.4 Key role of caspase-8 in CSE-induced eryptosis

Caspase-8 transduces apoptotic signals originating from DISC by undergoing autocleavage and processing the executioner caspase 3[132]. To study the involvement of caspase 8 in this eriptotic mechanism, we treated the RBCs with CSE 10% and 20% for 6 hours and after treatment the cell lysates were immunoblotted with anti-caspase-8 or caspase 3.

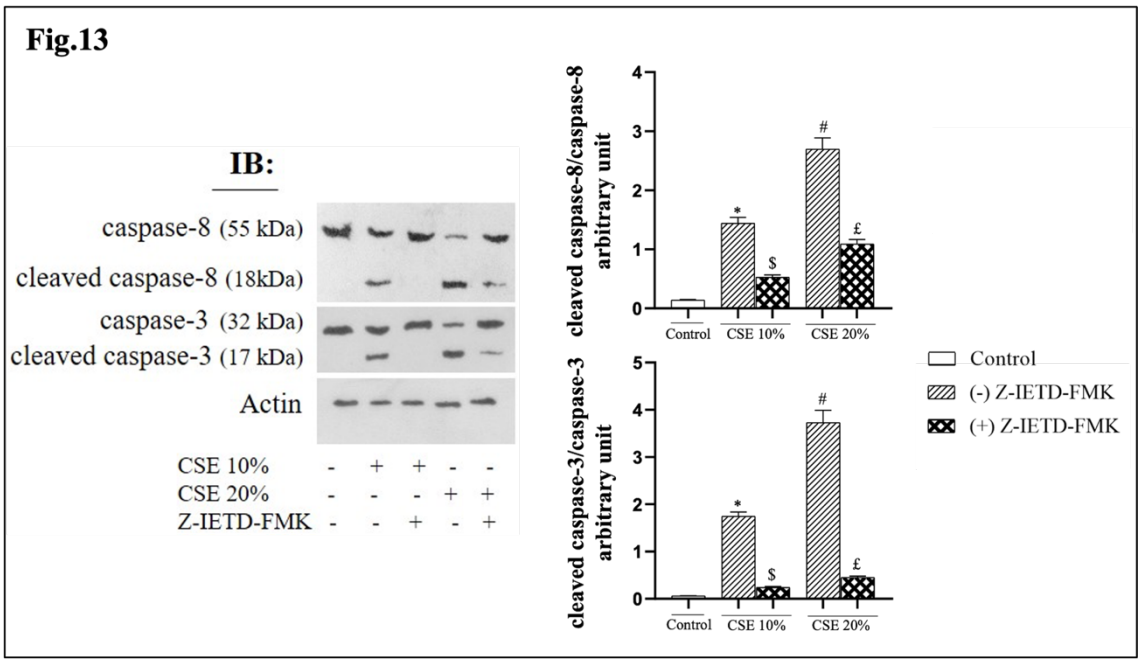

Figure 13 - Key role of caspase 8 in CSE-induced eryptosis (1). Western blot analysis of caspases-8/3 in RBCs pre-incubated for 1 h in the absence or in the presence of the caspase-8 inhibitor Z-IETD-FMK (25 μM) before the treatment with CSE for 6 h. RBCs incubated with vehicle were used as control. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p< 0.05$ (ANOVA associated with Fisher's correction).

Experiments show that CSE causes a decrease in the levels of the inactive precursors of both caspases, with the concomitant appearance of the active subunit cleaved caspase-8 p18 and the cleaved caspase-3 p17 subunit (Fig. 13). For the determination of the key role of caspase 8 in the mechanism, we pre-incubated RBCs with the specific inhibitor of caspase 8 Z-IETD-FMK, before exposure to CSE, and evaluated the cleavage of caspase 8, caspase 3 and the externalization of PS. Experiments show that the inhibitor prevented

the formation of the active subunit of caspase-3 (Fig. 13).

In addition, Z-IETD-FMK significantly reduced AnnexinV-FITC-associated fluorescence measured after treatment for 24 h with both 10% and 20% CSE (Fig. 14) compared to RBCs in the absence of the inhibitor.

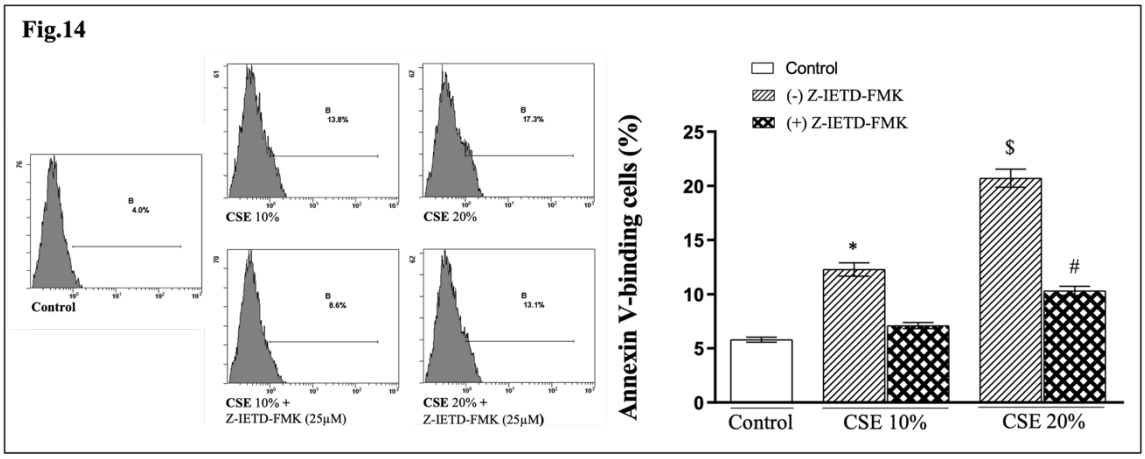

Figure 14 - Key role of caspase 8 in CSE-induced eryptosis (2). Percentage of PS-exposing cells in RBCs pre-incubated for 1 h in the absence or in the presence of the caspase-8 inhibitor Z-IETD-FMK (25 μM) before the treatment with CSE for 24h. Annexin V-FITC associated fluorescence was measured by flow cytometry as reported in Methods. RBCs incubated with vehicle were used as control. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

Interestingly, Z-IETD-FMK did not modify the release of hemoglobin caused by the CSE treatment (not shown), excluding that necroptosis, normally antagonized by caspase 8[133], was involved in the CSE-induced death of RBCs. In summary, the results described so far show that the CSE induces through Fas-dependent DISC recruitment followed by a direct involvement of the initiator caspase-8 in the activation of the executioner caspase-3.

5.5 CSE-induced eryptosis requires caspase-8-mediated ceramide formation

A sphingolipid-imbalance in membrane leading to ceramide release is a condition associated to eryptosis[134]. To evaluate the sphingolipid participation in the process, we evaluated the time-course of ceramide formation in RBCs membrane exposed to CSE 20%, in a time interval of 0-6. In the treated RBCs, compared to the control cells, we observed a rapid increase in the production of ceramide with a maximum peak after 20 minutes of treatment (Fig. 15).

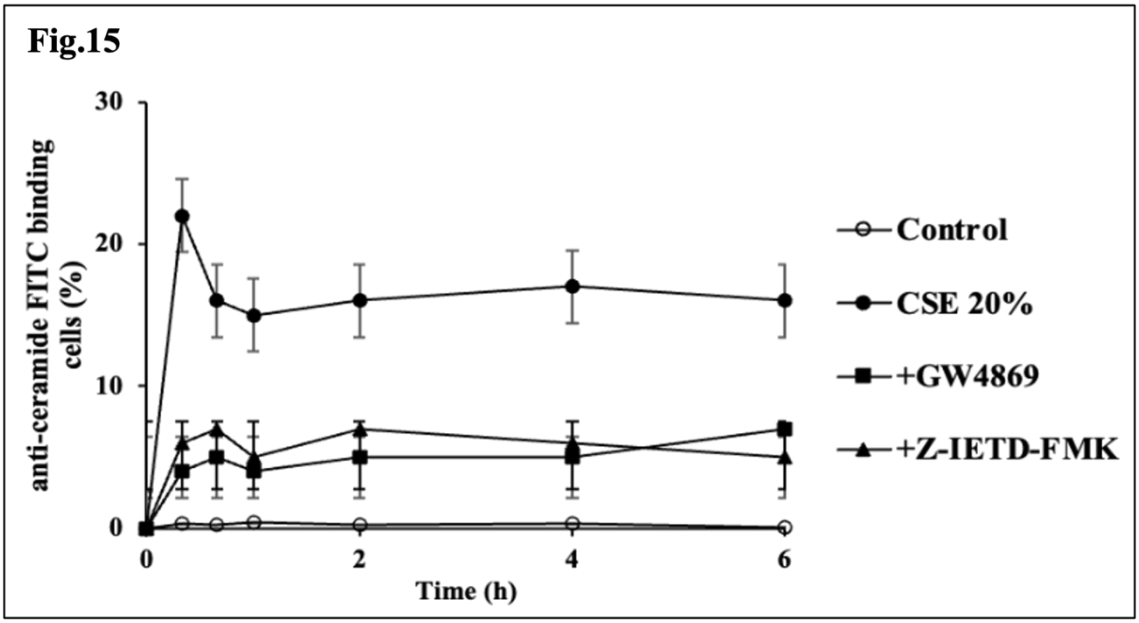

Figure 15 - CSE-induced eryptosis requires caspase-8-mediated ceramide formation. Time-course of ceramide formation in 20% CSE-treated RBCs pre-incubated for 1 h in the absence or in the presence of nSMase inhibitor GW4869 (15 μM) or caspase-8 inhibitor Z-IETD-FMK (25 μM). Flow cytometry analysis of ceramide was carried out as described in Methods. Representative of four experiments carried out in triplicate with comparable results. RBCs incubated with vehicle were used as control.

A number of factors related to morphological changes and apoptosis may elicit neutral sphingomyelinase (nSMase) activity in human RBC[135]. When the RBCs were pre-incubated for 1 hour with the nSMase inhibitor GW4869 before stimulation with CSE, the synthesis of ceramide was strongly inhibited (Fig. 15).

The decrease of ceramide, also, coincides with the concomitant net inhibition of externalized PS measured after 24 h of treatment (Fig. 16).

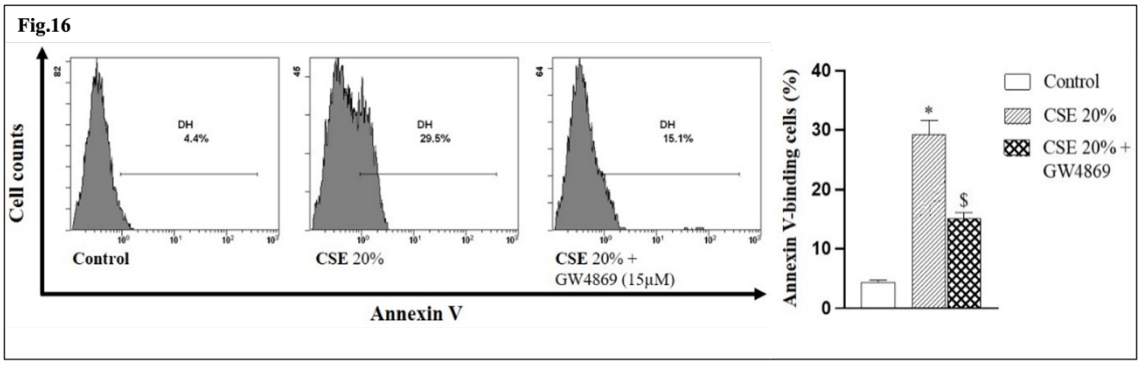

Figure 16 - CSE-induced eryptosis requires caspase-8-mediated ceramide formation. Percentage of phosphatidylserine-exposing RBCs after 1 h pretreatment in the absence or in the presence of GW4869 (15 µM) followed by 24 h treatment with CSE. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p< 0.05$ (ANOVA associated with Fisher's correction). Representative of four experiments carried out in triplicate with comparable results. RBCs incubated with vehicle were used as control. Bars with special characters are significantly different with $p< 0.05$ (ANOVA associated with Fisher's correction).

5.6 Involvement of ceramide in CSE-induced eryptosis, is independent from Ca^{2+} production

We also noted with interest that the pre-treatment of cells with caspase-8 inhibitor Z-IETD-FMK resulted in a reduction in the formation of Ceramide (Fig. 15). Our results clearly indicate nSMase's involvement in the eryptotic process, as well as ceramide generation appears to be dependent on the caspase initiator in the CSE-induced signaling pathway. It has been reported that ceramide sensitizes erythrocytes to the eryptotic activity of calcium ions[134]. Cytofluorimetric analysis in the presence of calcium-sensitive probe Fluo 3-AM provided evidence that a 24 h treatment of RBCs with CSE 10% and 20% did not cause a significant increase of the cytosolic calcium level (Fig 17).

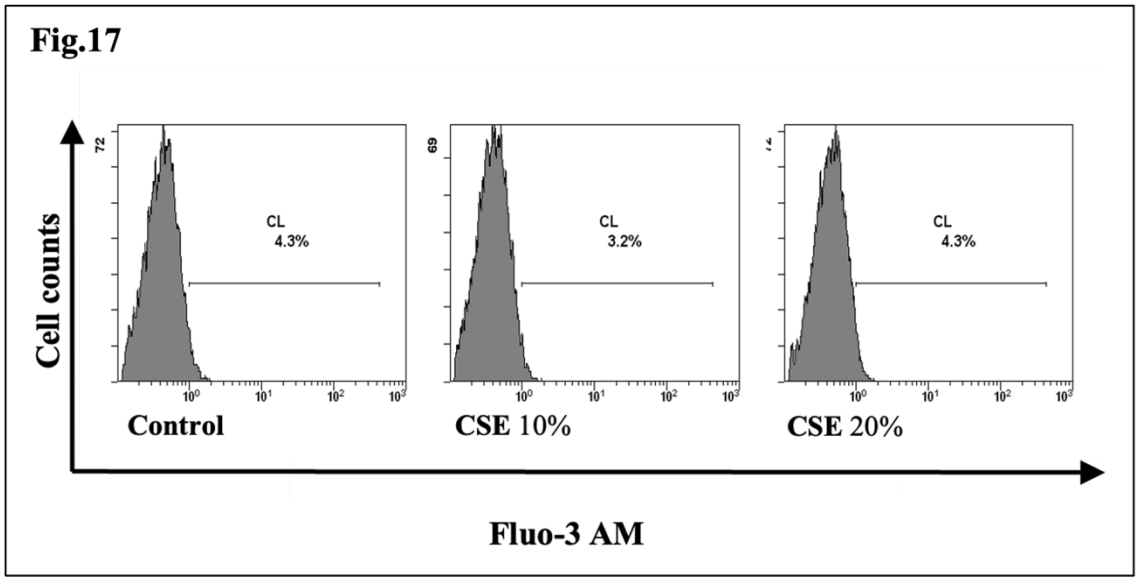

Figure 17 - ***Involvement of ceramide in CSE-induced eryptosis, is independent from Ca^{2+} production*****.** Analysis of intracellular calcium levels. Fluo-3AM-associated fluorescence in RBCs treated for 24h with CSE was measured by flow cytometry as reported in Methods. Representative of four experiments carried out in triplicate with comparable results. RBCs incubated with vehicle were used as control.

Cells exposed to CSE in the absence of extracellular Ca^{2+} and in the presence of calcium chelating agent permeable to BAPTA-AM cells (50 μM) also reported exposure of PS comparable to that of cells treated with CSE in a medium containing Ca^{2+} (not shown). These results indicated that the role of ceramide in CSE-induced eriptotic mechanism is completely independent from Ca^{2+} activity.

5.7 CSE-induced extrinsic eryptosis is initiated via p38 MAPK

Activation of p38 MAPK is associated with eryptosis induced by different cell stressors[87]. From 0 to 6 hours of exposure, treatment with CSE resulted in a marked increase in the active phosphorylated form of the signaling enzyme (p-p38) (Figure 18).

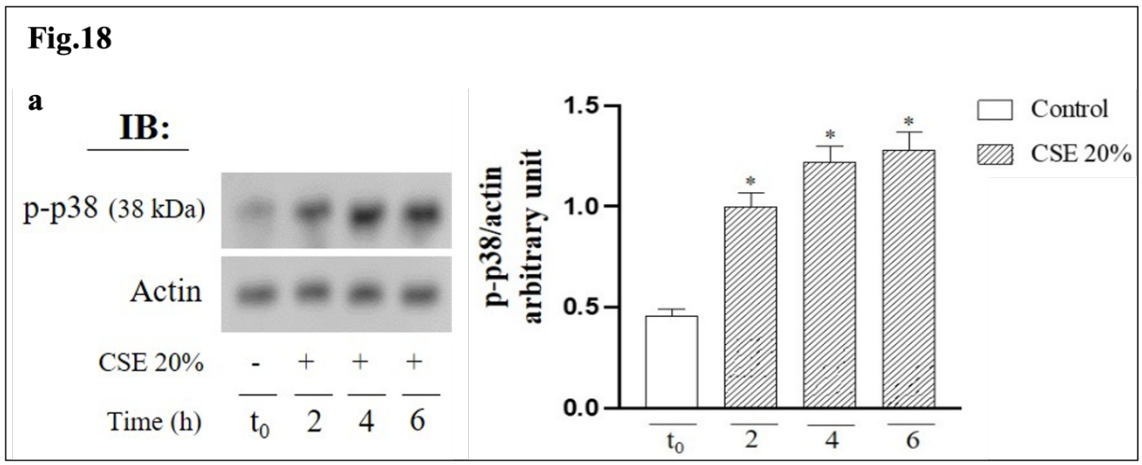

Figure 18 - CSE-induced eryptosis is dependent by p38 MAPK activation (1). Western blot analysis of p-p38 in RBC treated at different time intervals with CSE. RBCs incubated with vehicle were used as control. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

To place the involvement of p38 MAPK in the eryptotic mechanism and understand whether the enzyme is upstream or downstream of the DISC assembly, we pre-incubated RBC with SB203580, a specific enzyme inhibitor. Inhibition of p38 kinase led to a significant decrease in the amount of procaspase-8 in the immunoprecipitate with anti-Fas (Fig. 19a) and its active p18 subunit cleaved in the cell cytosol (Fig. 19b).

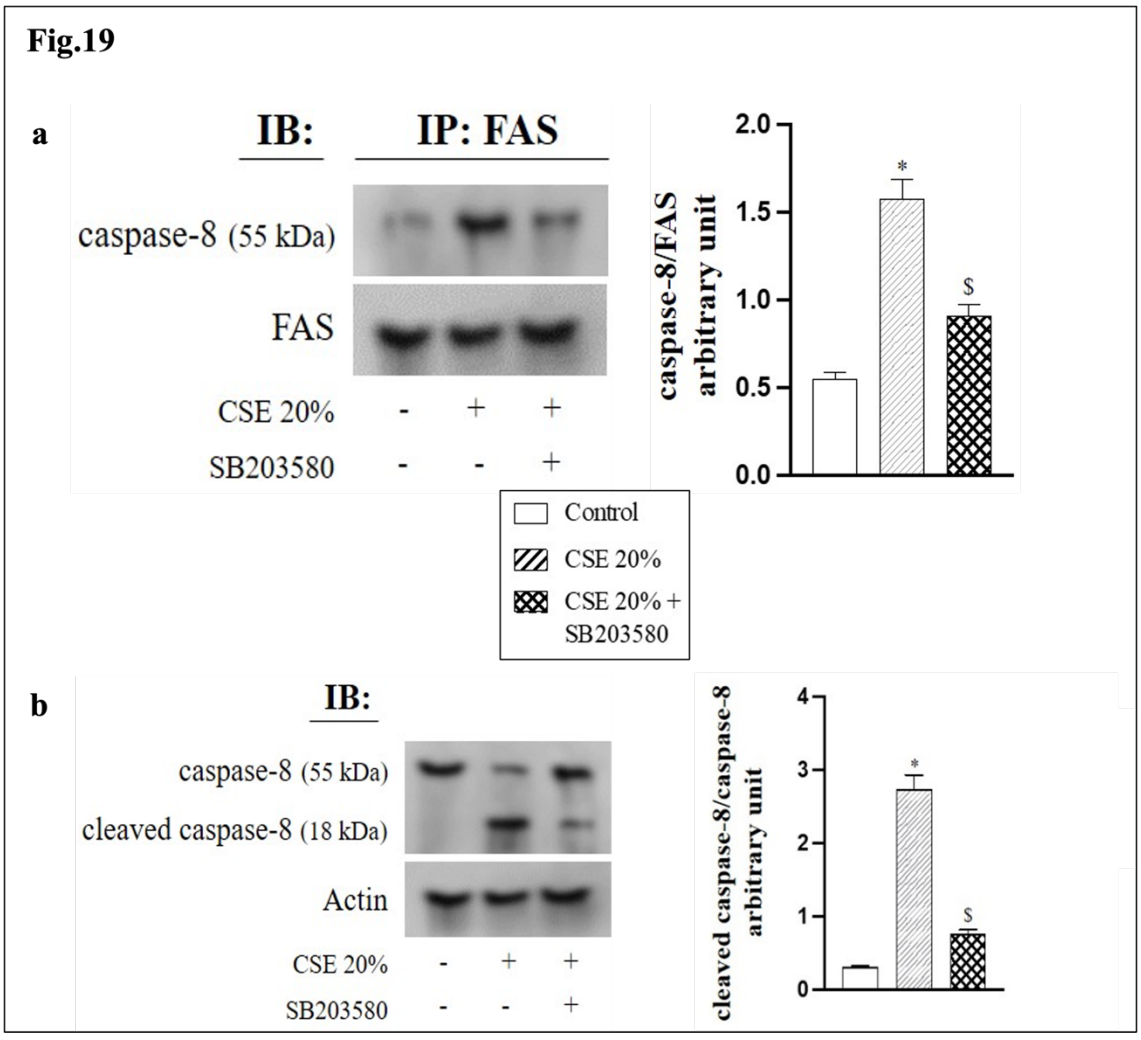

Figure 19 - CSE-induced eryptosis is dependent by p38 MAPK activation (2). (**a**) Association of Fas with caspase-8 in RBC treated with CSE in the absence or in the presence of p-38 MPK inhibitor. RBCs were pre-incubated for 1 h in the absence or in the presence of 10 µM SB203580 before 6 h treatment with 20% CSE. Immunoprecipitation with anti-Fas antibody and immunodetection of caspase-8 after electrophoretic separation of proteins were carried out as described in Methods. Blots were reprobed with anti-Fas antibody to ensure equal loading of the gels. **(b)** Western blot analysis of caspases-8 in RBCs pre-incubated for 1 h in the absence or in the presence of SB203580 (10 µM) before the treatment with CSE for 6 h, respectively. RBCs incubated with vehicle were used as control. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

Moreover, in the presence of SB203580 we also measured a strong reduction in fluorescence associated with AnnexinV-FITC in comparison with RBCs stimulated by CSE in the absence of the inhibitor (Fig. 20).

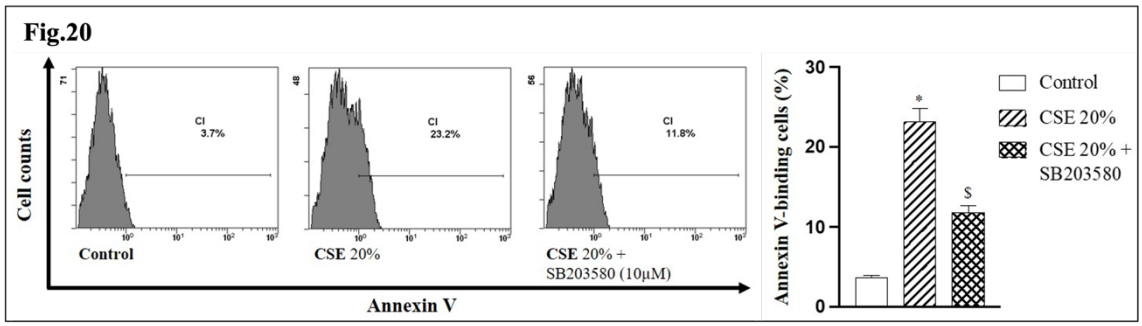

Figure 20 - CSE-induced eryptosis is dependent by p38 MAPK activation (3). Percentage of PS-exposing cells in RBCs pre-incubated for 1 h in the absence or in the presence of SB203580 (10 μM) before the treatment with CSE for 24h. Annexin V-FITC associated fluorescence was measured by flow cytometry as reported in Methods. RBCs incubated with vehicle were used as control. Values are means ± SD of six independent experiments carried out in triplicate. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

We can therefore conclude that CSE-induced extrinsic eryptosis started via p38 MAPK.

5.8 Smokers subjects have higher FADD and caspase-8 associate with Fas receptor

Smokers have high levels of eryptotic circulating erythrocytes[115]. To confirm the CSE-induced Fas-dependent eriptotic mechanism, we researched DISC characteristic proteins in the RBCs of smokers in comparison with non-smokers subjects. Fresh blood samples from 2 healthy non-smoking males aged 39 and 56, and 2 males of matching age who regularly smoked 15 or more cigarettes per day for 10 years were drawn, with informed consent. Cotinine levels measured in the plasma of subjects accounted for 440.19±18.30 ng/mL in the smoker subject number 1 and 611.83±23.11 ng/mL in the smoker subject number 2 (analysis in duplicate), while it was under the detection limit in both the non-smokers volunteers. RBCs isolated by the same healthy voluntary subjects, were analyzed by co-immunoprecipitation experiments with anti-Fas followed by immunorecognition of FADD and caspase-8.

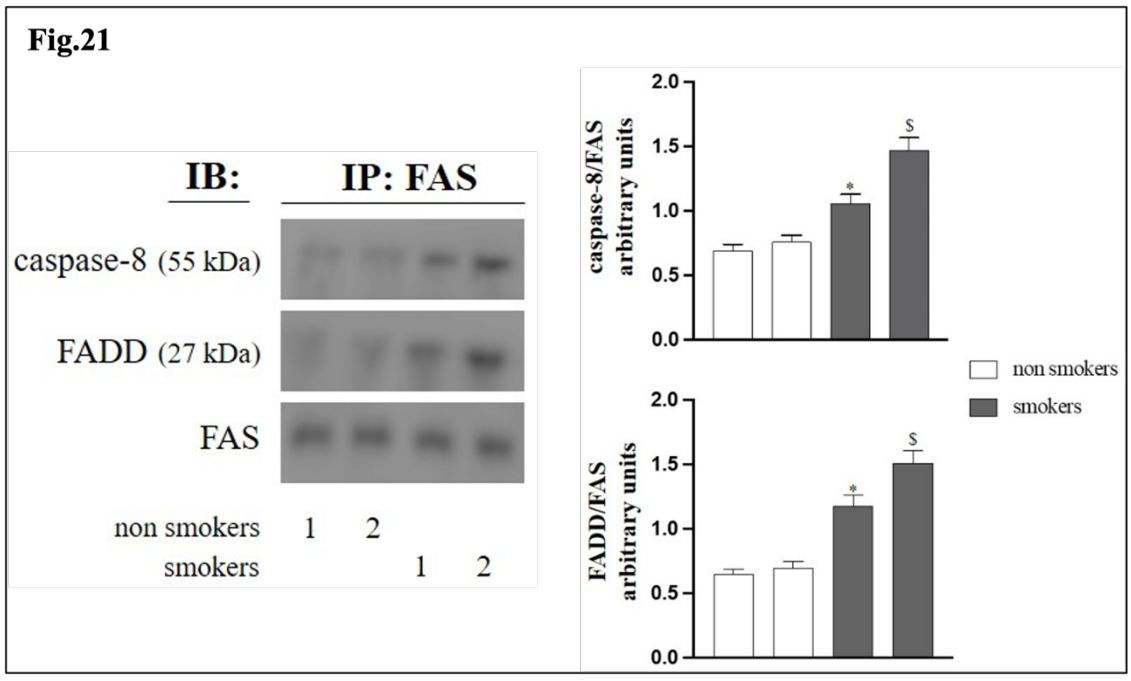

Figure 21 - Fas-associated signaling complex in erythrocytes from smoker healthy humans. RBCs isolated both from smoker and non-smoker volunteers were lysed and submitted either to immunoprecipitation with anti-Fas antibody (a). The immunoprecipitates were separated by SDS-PAGE and blotted with anti-FADD or anti-caspase 8. Blots were reprobed with anti-Fas (a) antibody to ensure equal loading of the gels. Values are means ± SD of three independent determinations. Bars with special characters are significantly different with $p< 0.05$ (ANOVA associated with Fisher's correction).

The results obtained showed a significantly greater localization of both proteins in the immunoprecipitates of RBCs isolated from smoker than non-smokers (Fig. 21). This evidence supports the hypothesis that CS may induce DISC formation in human RBCs.

In addition, the erythrocytes of smokers showed higher levels of p-p38 MAPK than those of non-smokers, suggesting the activation of the CS-dependent signaling enzyme (Fig. 22).

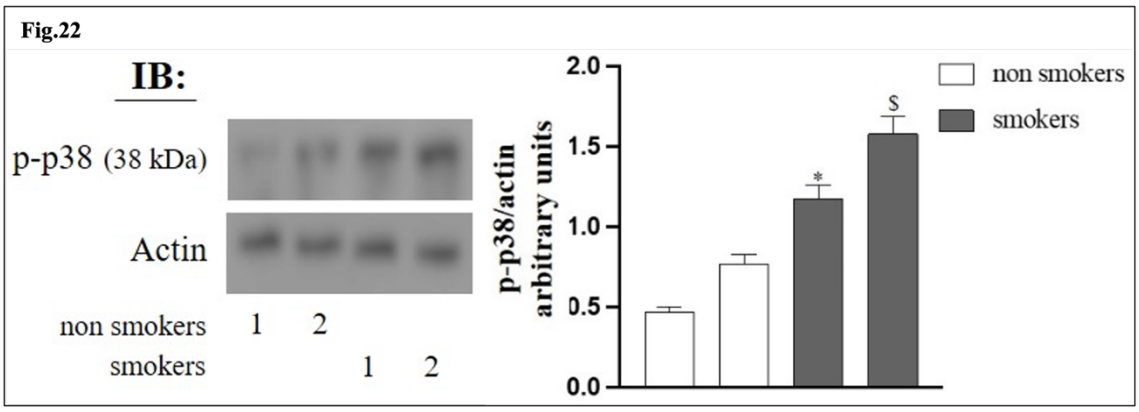

Figure 22 - p-p38 MAPK levels in erythrocytes from smoker healthy humans. RBCs isolated both from smoker and non-smoker volunteers were lysed and submitted either to western blot analysis of p-p38 MAPK (b). The proteins were separated by SDS-PAGE and blotted with anti-caspase 8. Blots were reprobed with anti-actin antibody to ensure equal loading of the gels. Values are means ± SD of three independent determinations carried out on the same blood sample of figure 21. Bars with special characters are significantly different with $p < 0.05$ (ANOVA associated with Fisher's correction).

6. DISCUSSION

Several studies link cigarette smoking to damage of the cardiovascular system leading to pathological dysfunction of endothelial cells, activation of leukocytes and platelets, as well as increase of circulating eryptotic RBCs[115][125][126][128]. Although much effort has been made to understand the pathways by which CS causes direct cytotoxicity, the exact mechanisms remain unclear and often controversial. In this study, for the first time to the best of our knowledge, we demonstrate that the exposure to the CSE induces in RBCs an extrinsic apoptotic pathway that includes the formation of DISC p38 MAPK-initiated followed by activation of caspase-8/caspase-3 *via* ceramide formation. In addition, this is the first study investigating the mechanism by which CS can affect the health and fate of erythrocytes.

CSE, simulating the various harmful components of real CS, includes high concentrations of oxidants and ROS inducers[105]. Notwithstanding oxidative stress has been recognized as key mechanism of CSE toxicity in various human cells, including alveolar macrophages[136], bronchial epithelial cells[137] and lung fibroblasts[138], we here show that CSE causes PS externalization and cell shrinkage in RBCs without affecting the ROS/GSH balance. In line with our data, other studies in endothelial cells have demonstrated that CSE treatment has no effect on GSH content[129] and that antioxidants exhibit only a minimal protective effect in CSE-triggered cell death[139]. In addition, our data exclude that activities of enzymes that generate reactive either oxygen or nitrogen species in the erythrocytes, such as NADPH oxidase, nitric oxide synthase or xanthine oxide-reductase, are up-regulated by the CSE-treatment[90].

Mitochondrial dysfunction resulting in the release of apoptogenic factors has been recognized as the main apoptotic death signaling mediated by CSE. However, molecular investigations have demonstrated that CSE can also sensitize cells toward apoptosis *via* the extrinsic pathway too[140]. Mature RBCs are devoid of mitochondria but contain all the

other characteristic components of the receptor-dependent apoptotic pathway[85]. Though it has been ascertained that the Fas/FasL pathway plays an important role in the inhibition of the expansion and differentiation of immature erythroblasts[141][142], functional role of the extrinsic apoptotic pathway in mature erythrocytes is still debated. Indeed, Fas receptor ligation with agonistic antibody was shown to have no impact on either caspase-8 activation[58] or PS exposure[143]. Conversely, Mandal and coll.[85] have clearly demonstrated that oxidative stress leads to the formation of Fas-FADD-caspase-8 complexes in RBCs membrane, followed by caspase-8 and 3 activation and PS externalization to the outer membrane leaflet. In addition, the activation of ROS-associated Fas signaling pathway has been demonstrated in erythrocytes from animals chronically exposed to arsenic or lead[144][145]. Within this controversial scenario, in the present study we demonstrate that CSE induces Fas aggregation and that the adapter protein FADD and caspase-8 co-immunoprecipitate with Fas in RBCs, consistently with formation of DISC. Moreover, the activity of initiator caspase-8 appears a prerequisite for both caspase-3 activation and PS externalization, supporting the involvement of a Fas/caspase-8/caspase-3-dependent signaling in CSE-induced eryptosis. Our data demonstrate, as a whole and for the first time, that the Fas-mediated signaling cascade in mature erythrocytes can be an integral and functional mechanism of death pathway not mediated by oxidative stress.

Several studies provide evidence that ceramide generation is involved critically in CSE-induced cytotoxicity in numerous human cells line as well as *in vivo*. nSMase activation appears required for the CS-mediated upregulation of ceramide levels and apoptosis in human airway epithelial (HAE) cells[146], rat microvascular cells[147] and in lung tissues from smokers with emphysema[122]. Furthermore, nSMase activity and ceramide formation in erythrocytes has been involved in the mechanism of Pb(II) toxicity[148]. In line with all these findings, our study shows that nSMase-dependent formation of ceramide occurs in

CSE-treated RBCs and that this lipid mediator is a key player in the eryptotic mechanism. Indeed, nSMase inhibition by GW4869 reduced both ceramide formation and PS externalization. Ceramide is considered an indispensable apoptotic signal in the Fas signaling pathway, demonstrated by the tendency of signaling receptors to cluster in lipid rafts [149]. In our laboratory we still investigate under the role of ceramide in the CSE-induced Fas aggregation in RBCs. In addition, our data indicate that ceramide generation depends on caspase-8 action, according to consolidated evidences indicating that, at early stage of Fas signaling, this initiator protease is essential to activate sphingomyelinase[150][151][152].

Heavy metals induce eryptosis *via* nSMase-dependent ceramide generation, linked to increased intracellular calcium levels and scramblase activation[149]. Interestingly, in our study eryptotic activity of CSE does not involve calcium entry and, consequently, scramblase activity can be excluded from the breakdown of PS asymmetry but the involvement of the flippase activity of aminophospholipid translocase (APLT) in the CSE-induced eryptotic mechanism deserves to be investigated.

p38 MAPK is a prominent mediator of the cellular stress response and is considered an important element of the cellular machinery triggering the apoptosis of nucleated cells[154] as well as of erythrocytes under osmotic shock[87]. It has been reported that exposure to CS induces activation of Fas-dependent signaling pathway following the phosphorylation of p38 and then apoptosis in lung tissue of rats[155]. In erythroblasts, the ASK1/JNK/p38 signaling axis is required for the Fas-mediated caspase activation that drives erythroid differentiation[156]. In the present study, we show that CSE-induced eryptosis in RBCs is initiated by activation of p38 MAP kinase leading to DISC assembly and caspase cascade. The possible role of ASK1 as upstream molecule capable of regulating p38 MAPK *via* MEKK3/MEKK6 remains to be investigated.

Although many aspects of the CSE-induced eryptotic mechanism still need to be clarified, with our data we are in the condition to draw the possible molecular pathway as represented in Fig. 23.

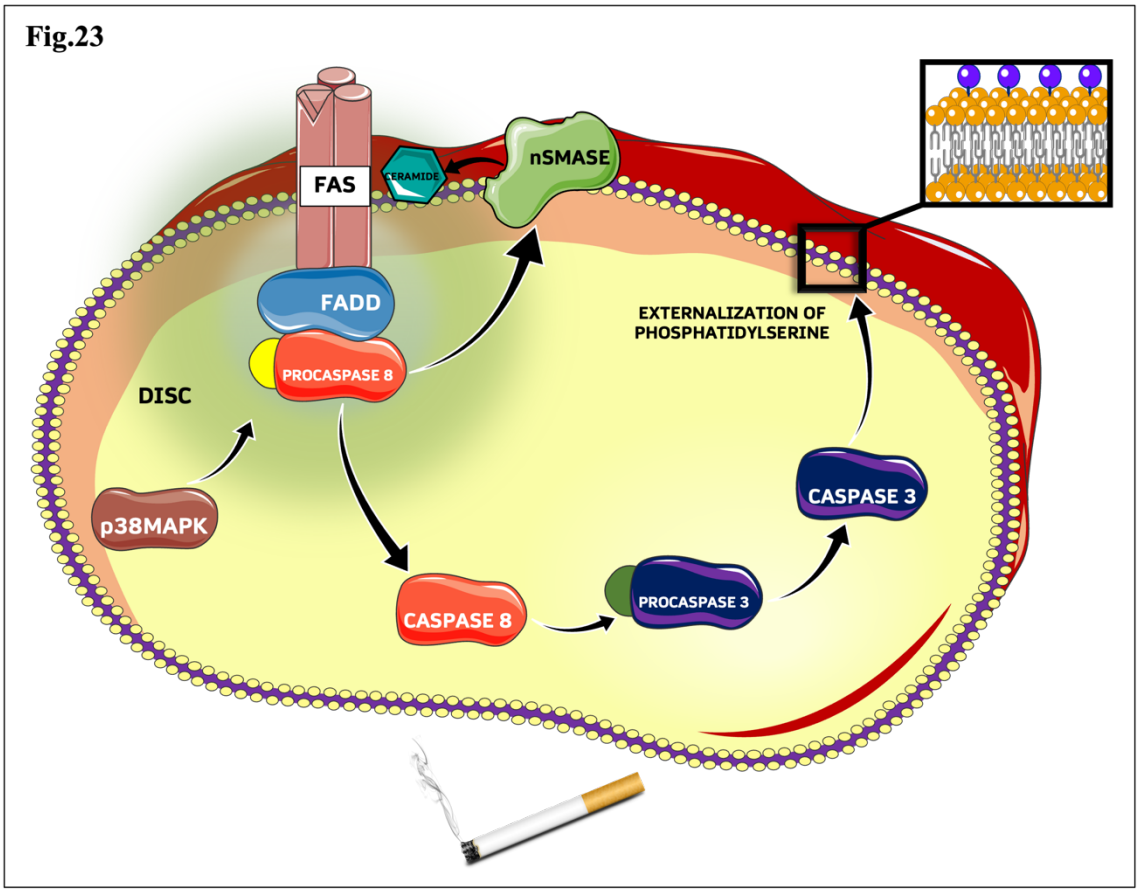

Figure 23 - Proposed mechanism for CSE-induced eryptosis.

At last, but not least, we show higher levels of Fas-associated FADD and caspase-8, as well as of p-p38MAPK levels, in erythrocytes from two smoker subjects in comparison with those from not smokers, supporting the Fas-dependent signaling pathway in CS-dependent eryptosis. Analysis on a larger sample of subjects will be necessary to unequivocally validate these findings.

7. CONCLUSIONS

In conclusion, our study provides evidence that, in mature erythrocytes, stimulation of p38 MAPK by CSE may sensitizes the cells to Fas-induced caspase activation driving the cell fate toward programmed death. In the light of the detrimental effects of eryptotic RBCs on both endothelial cells and platelets that include vascular inflammation and blood clotting, our data suggest p38 MAPK as a potential molecular target for preventing excessive eryptosis in smokers and mitigate the associated vaso-occlusive complications. Future studies aim to fill the signalling gap between the assembly of the DISC and the activation of p38 MAPK and to study the modification of membrane lipids-raft leading to DISC assembly. We also want to investigate which component of the CSE (gaseous or corpusculated phase) is most responsible for the cytotoxic effect on RBCs and whether there is a direct proportionality between high levels of cotinine and eryptosis. At last, it could be of interest investigating about new natural substances that can inhibit the eryptotic process in smokers with the aim of formulating a supplement to positively counteract diseases aggravated by eryptotic circulating red blood cells.

8. AKNOWLEDGEMENTS

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A warm thanks goes to this fantastic Spanish group that has always made me feel at home.

Valencia es mi segunda ciudad, siempre en el corazón.

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