Ekaterina Sergueevna Potapova

BCs Genetics

Longitudinal evaluation of hemorheological markers in acute inflammatory diseases

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Doutora Patrícia Napoleão
Co-orientador: Doutora Carlota Saldanha

Júri:

Presidente: Prof. Doutora Paula Maria Theriaga Mendes Bernardo Gonçalves
Arguente(s): Prof. Doutora Maria Teresa Ferreira Marques Pinheiro
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Resumo

Enfarte agudo do miocádio (AMI), sepsis e artrite reumatóide (AR) são três doenças distintas que têm como factor comum serem doenças inflamatórias. São responsáveis por milhões de mortes por ano em todo o mundo e têm, também, grande peso económico nos recursos hospitalares. Apesar de o conhecimento acerca dos mecanismos destas doenças aumentar de ano para ano, existem ainda muitas lacunas por preencher. A existência de biomarcadores que permitam diagnosticar preventivamente a doença bem como prever desfechos mais desfavoráveis são algumas destas lacunas. Este estudo foi realizado com a intenção de estudar a evolução de quatro marcadores hemorreológicos (deformabilidade eritrocitária, agregação eritrocitária, monóxido de azoto – NO e S-nitrosoglutatuão – GSNO) de modo a compreender o seu mecanismo e as diferenças existentes nos três diversos tipos de doenças inflamatórias. O estudo incidiu sobre quatro grupos: sepsis (14 doentes), AR (25 doentes), enfarte agudo do miocárdio (STEMI; 15 doentes) e controlo (CTR; 15 voluntários saudáveis). No grupo STEMI foram feitas duas medições, correspondendo a primeira à admissão no Serviço de Urgência e a segunda um mês depois. No grupo da sepsis quatro medições foram feitas a cada doente - admissão na Unidade de Cuidados Intensivos (UCI), 24 horas, 72 horas depois e alta da UCI. Observou-se uma diferença significativa dos níveis de agregação eritrocitária para 10s entre os grupos STEMI admissão e controlo. Diferenças significativas de deformabilidade, agregação e GSNO foram observadas entre os grupos sepsis (em qualquer ponto de recolha) e CTR. Para além disto, verificaram-se também variações longitudinais significativas no grupo sepsis para as concentrações eritrocitárias de GSNO. Em conclusão, verificaram-se valores anormais de alguns dos parâmetros hemorreológicos estudados nas doenças inflamatórias estudadas, ou seja, sepsis, AMI e AR. Tais resultados parecem apontar para uma relação entre o processo inflamatório a as alterações hemorreológicas no sangue.

Termos-chave: inflamação, eritrócitos, monóxido de azoto, hemorreologia.

Abstract

Acute myocardial infarction (AMI), sepsis and rheumatoid arthritis (RA) are part of the inflammatory diseases' group. Every year millions of people are affected and die because of them, all around the globe. In the past years our knowledge of these diseases has increased and a light on their pathophysiology has been shed, but there is still much more to be discovered. Biological markers that would make pre-disease diagnosis possible would change many of the outcomes for patients suffering from them. The aim of this study was to access the evolution of four hemorheological markers (erythrocyte deformability, erythrocyte aggregation, nitric oxide – NO and S-nitrosoglutathione – GSNO) in order to understand their behaviour in three types of inflammatory diseases. Four study groups were created: ST-elevation myocardial infarction (STEMI) group (15 patients), RA (25 patients), sepsis (14 patients) and the control group (CTR; 15 healthy volunteers). In STEMI group two different measurements were taken, one at time of hospital admission and the other after a month. In the sepsis group four measurements were taken throughout the internment in the Intensive Care Unit (ICU): admission, 24 hours, 72 hours after and discharge. Significant difference was observed in the 10s erythrocyte aggregation marker between the STEMI patients at hospital admission and CTR group. Significant differences of deformability, aggregation and GSNO were obtained upon comparison of sepsis patients (at all time-points) and CTR. In addition to this, the longitudinal changes of GSNO erythrocyte concentrations were significant. In conclusion, abnormal values for some of the studied hemorheological parameters were verified in inflammatory diseases, namely myocardial infarction (STEMI), sepsis and rheumatoid arthritis. The results seem to point out to a relation between inflammation and the hemorheological alterations.

Keywords: inflammation, erythrocytes, nitric oxide, hemorheology.

Contents

Figure Index

Table Index

Abbreviations

ACh – acetylcholine

ACE-inhibitor – angiotensin-converting-enzyme inhibitor

AMI – acute myocardial infarction

BMI – body mass index

CTR – control group

CVD – cardiovascular disease

EDI – erythrocyte deformability index

ESR – erythrocyte segmentation rate

GSH – glutathione

GSNO – S-nitrosoglutathione

HR – heart rate

ICU – Intensive Care Unit

IFN-ϒ – interferon-gamma

IL – interleukin

IMT – intima-medial thickness

LDL – low density lipoprotein

LME – linear mixed effects

MAP – mean arterial pressure

NF-κB – nuclear factor kappa B

NO – nitric oxide

NOS – nitric oxide synthases

RA – rheumatoid arthritis

RBC – red blood cell

RDW – red cell distribution width

SNO-Hb – S-nitrosohemoglobin

STEMI – ST-elevation myocardial infarction

TNF-α – tumour necrosis factor-alpha

1. – Introduction

1.1. – General Introduction

Inflammation is a biological mechanism that occurs as a normal response of our body to infections and injuries. When this process becomes excessive it brings a great deal of harm.

There is a large number of diseases that occur as a consequence to uncontrolled inflammation: necrotizing enterocolitis, acne, angina, pharyngitis, pyelitis, pleurisy, empyema, pelvic inflammation disease, gastroenteritis, urinary tract infection, and many others. They target different organs and have different outcomes, but they all have the same inflammatory agents: inflammatory cytokines, arachidonic acid-derived eicosanoids such as prostaglandins, thromboxanes and leukotrienes, adhesion molecules and other agents that can have a role in inflammation such as reactive oxygen species (Philip, 2006).

In this study three different inflammatory diseases are going to be approached - acute myocardial infarction, sepsis and rheumatoid arthritis - and compared in terms of hemorheological markers.

Hemorheology is the science of the physical properties of blood flow and deformation behaviour in the circulatory system.

In this study four hemorheological markers – erythrocyte aggregation, erythrocyte deformability, erythrocyte nitric oxide (NO) and S-nitrosoglutathione (GSNO) concentrations - are going to be studied in order to observe their behaviour and their differences among three inflammatory conditions, and also in healthy people.

1.2. – Inflammatory Diseases

1.2.1. – Acute Myocardial Infarction

Cardiovascular disease (CVD) is the main cause of death globally. In 2008, roughly 17.3 million people died from CVDs, which accounts for 30% of all global deaths that year (WHO, 2011). According to the World Health Organization, is it projected to remain the single leading cause of death across the globe. Therefore, an understanding of their pathophysiology is crucial and the need for

markers that could uncover the presence and imminence of the disease, as well as for efficient therapeutic agents, is urgent.

ST-segment elevation myocardial infarction (STEMI) is a specific type of acute myocardial infarction (AMI), and part of the cardiovascular diseases group. In this condition the coronary artery is completely blocked, and as a result all the heart muscle that is being supplied by the affected artery is in ischemia and can start to die depending on the duration of the ischemia. This type of myocardial infarction is detected on an electrocardiogram, as it produces an elevation in the ST segment, which means that an elevated amount of heart muscle is being damaged. It is an acute event that results from thrombosis developing on a coronary atherosclerotic plaque (Libby, 2001), as a consequence of its disruption (Libby, 2006).

The current view of atherosclerosis as a lipid storage disease has been completely substituted by the notion that it is indeed an inflammatory disease (Fig. 1.1), with extensive evidences and studies backing up this fact. Several other concepts have also changed during the last years: arteries are not viewed as inanimate conduits but as highly organized organs composed by living cells, and evidences suggest that atheromatous plaques develop within the arterial wall, instead of on it. We now realize that atherosclerosis is not a direct component of aging, and that our lifestyle and behaviour can modify the inflammatory processes that lead to the disease (Libby, 2006). Furthermore, it is believed that the occurrence of an acute coronary event is not related to the degree of stenosis nor do the lesions occur randomly, but instead is dependent of the physical properties of the plaque and its vulnerability. Vulnerable plaques are the ones who suffer plaque disruption. These are plaques whose structure and content makes them likely to undergo thrombosis in the future. Those features are: a large lipid core occupying at least 50% overall plaque volume, a high density of macrophages, a low density of smooth muscle cells in the capsule, a high tissue factor content and a thin plaque cap with disorganized collagen structure (Davies, 2000).

As referred above, disruption of an atherosclerotic plaque is the main cause of AMI. The occlusion reduces the blood flow of that fraction of the myocardium, which results in progressive tissue ischemia. Necrosis begins after approximately 30 minutes. If the perfusion of the myocardium stays for about 3 hours, profound ischemia, necrosis and acute episode of infarction occur (Collinson and Gaze, 2007). Cell death during STEMI is permanent and so the need to restore tissue perfusion is urgent. Necrosis induces generation of free radicals. They trigger a cytokine cascade initiated by the release of tumour necrosis factor-alpha (TNF-α) that results in an inflammatory response. Neutrophils are recruited to the ischemic tissue and infiltrate the endothelium, further contributing to the persistent inflammatory response (Frangogiannis *et al*., 2002). After infarction the repair and remodelling stage begins, characterized by structural rearrangement of the cardiac chamber wall. Proliferation of

interstitial fibroblasts along with deposition of extracellular matrix components leads to myocardial stiffness and diastolic dysfunction that could ultimately result in heart failure (Kapoun *et al.*, 2004).

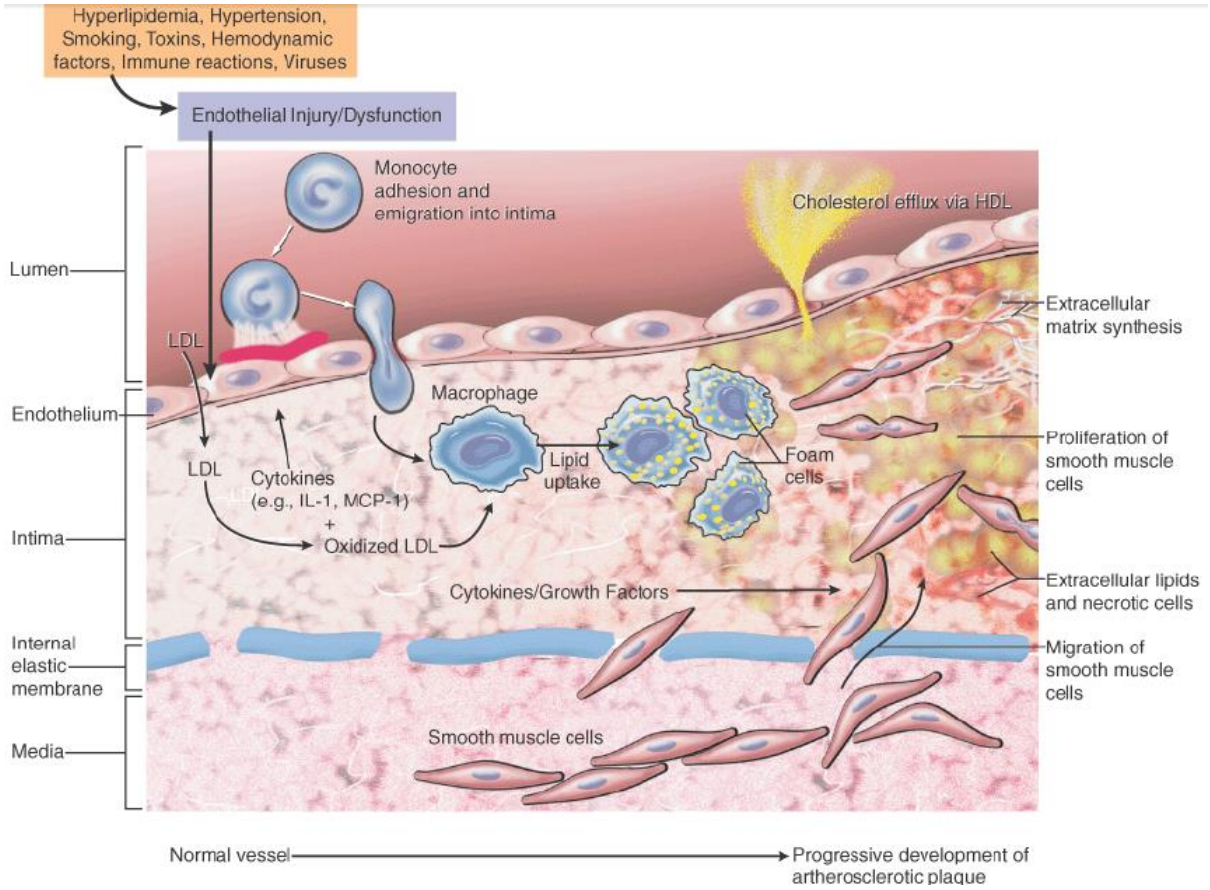

Figure 1.1 - Development of atherosclerosis. The progression of the atherosclerotic lesion from normal vessel up until the atherosclerotic plaque is formed (Adapted from Wall, 2012).

1.2.2. – Sepsis

Sepsis is another disease that is a leading cause of death (Xu *et al.*, 2010). Over the years, there has been an increase of hospitalizations with sepsis as a primary or secondary diagnosis (Hall *et al.*, 2011). It is an inflammatory disease that kills more than 6 million infants and young children, and 100,000 new mothers every year (Hall *et al*, 2011).

Sepsis is an inflammatory disease that starts with a bacterial or fungal infection. Paradoxically, it is not the infection that kills people but instead is the host immune response while attempting to fight it. The infection triggers an overwhelming immune response as the body releases chemicals into the blood to fight the infection, which triggers widespread inflammation. The most common cause of sepsis are Gram-positive bacterial pathogens but fungal organisms are increasing rapidly (Martin, 2012). Blood clots appear in several sites of the body and cause diminished blood flow, which in turn

deprives the organs from oxygen and nutrients. It also induces changes in the circulatory system, especially in the microcirculation (Hinshaw, 1996).

There are different levels of sepsis – sepsis, severe sepsis and septic shock. Cases of severe sepsis and septic shock are lower than those of sepsis. In severe cases there is acute organ dysfunction and possible failure. These cases are related to the source of infection. In septic shock there is a weakening of the heart due to a decrease in the blood pressure.

There have been longitudinal changes in the incidence of sepsis. The numbers of hospitalized patients are getting higher each year and cases of sepsis and severe sepsis are increasing in excess of the growth of population. In the developing world, sepsis is more common among the younger people and the organisms that trigger it are likely to be Gram-negative enteric pathogens and atypical pathogens (Martin, 2012). There is a differential risk for developing sepsis regarding to specific patient factors. Conditions that alter the immune system increase risk of getting sepsis. Other factors are race, ethnicity and gender: males have a higher risk than females, and though the mechanisms behind the difference among race and ethnicity are not clear, it is observed that Caucasians have lower risk of developing sepsis (Martin, 2012).

1.2.3. – Rheumatoid Arthritis

According to the World Health Organization, rheumatoid arthritis (RA) is a chronic systemic disease that affects the joints, connective tissue, muscle, tendons and fibrous tissue, resulting in an accumulation of fluid in the joints and causing pain and systemic inflammation (WHO, 2013). It is a complex systemic multifactorial inflammatory process (Scher, 2012) of unknown etiology. First coined by Sir Alfred Garrod in 1851 (Scher, 2013) it strikes between the ages of 20 to 40 and turns into a chronic disabling condition that causes pain and deformity, and possibly even severe disability. The statistic data show that the prevalence varies between 0.3% and 1% and mainly affects the female gender and people in developed countries (WHO, 2013).

Activated macrophages, T lymphocytes and plasma cells infiltrate to the synovium (thin membrane present in joints that lines the joint capsule and also secretes synovial fluid) and stimulate joint lesions. Biopsies taken from RA patients contain high concentrations of cytokines such as TNF-α, interleukins (IL)-1β, IL-6 and IL-8 (Philip, 2006). An increase of inflammatory markers is antecedent of disease progression and also joint destruction, which occurs in the first years of RA (Matsuda *et al.*, 1998).

The main goals for the treatment of RA are the control of signs and symptoms, prevention of joint damage progression and the achievement of remission (Scirè *et al*, 2009). Cardiovascular disease

(such as acute myocardial infarction) is a major source of morbidity and mortality in RA (Solomon *et al*, 2013). It is interesting to note that traditional cardiovascular risk factors do not entirely explain this connection.

1.3. – Blood Hemorheology

The blood is a two-phased liquid formed by 45% formed elements (erythrocytes, leukocytes and platelets) and 55% plasma. It carries hormones, enzymes and vitamins, oxygen to tissues, collects carbon monoxide, conveys nutritive substances (amino acids, sugars, mineral salts), gathers excreted material which is later eliminated through renal filters and participates in the defence of the organism by means of phagocytic activity of leukocytes, bactericidal power of serum and immune response of lymphocytes (Bianco, 2013). The fluidity of blood at a given shear rate and temperature is determined by rheological properties of plasma and formed elements, and by the volume fraction (hematocrit) of the formed elements (Baskurt e Meiselman, 2003).

Erythrocytes (Fig. 1.2), or red blood cells (RBC), are the most numerous blood cells: about 4-6 millions/mm^3. They have no nucleus, are rich in hemoglobin and are responsible for providing oxygen to tissues. They also play a major role in the process of inflammation. In fact, alterations of their hemorheological properties can indicate the presence of inflammation.

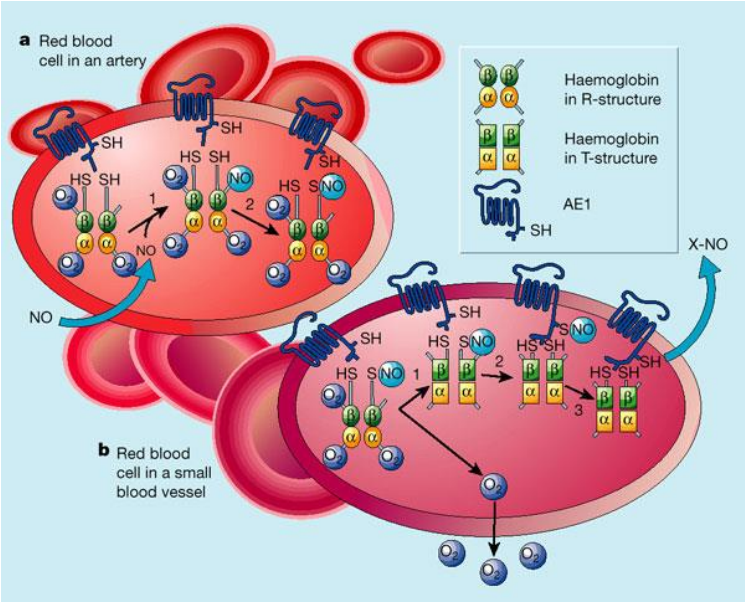

Figure 1.2 - Red blood cells in two types of vessels. This figure shows the uptake and/or release of nitric oxide by RBC in different vessels (Adapted from Gross SS, 2001).

Deformability is one of the properties of the erythrocytes, determined by 3 main factors: viscoelastic properties of the membrane (flexibility), viscosity of the content (fluidity of the cytoplasm) and geometry (surface/volume globular ratio). It is what determines the average life of the erythrocyte and the rheologic properties of the blood, being of extreme importance in the microcirculation. It is also crucial in the oxygenation of hemoglobin and tissues. Viscoelastic properties of the membrane allow it to deform greatly and recover, if the equilibrium point is not surpassed (Silva, 1982).

Erythrocyte aggregation is one of the factors that determines blood viscosity, along with the hematocrit, plasmatic viscosity and erythrocyte deformability (Silva, 1982). It affects directly the RBC distribution and the dynamic of the blood flow, especially in microcirculation. Aggregation is responsible for the shear thinning behaviour of normal human blood – fluid's viscosity decreases with increasing rate of shear stress due to aggregate's dispersion (Meiselman *et al.*, 2007). RBCs in humans tend to aggregate forming a shape that looks like a stack of coins and is called *rouleaux*. The aggregation becomes enhanced in the presence of acute phase proteins such as fibrinogen and is reversible (Baskurt *et al.*, 2009). When examining the blood of healthy donors, aggregates appear to have relatively weak attractive forces, as they break up when subjected to relatively low shear rates – 20-40 s^{-1}. The aggregating potential of cells differs with a large variation between all healthy persons, and more dense cells exhibit greater aggregation (Meiselman *et al.*, 2007).

RBC take part in severe coronary occlusion mostly in conditions of low shear rate, e.g. within the microcirculation in peri-infarct domain of myocardium (Dormandy *et al.*, 1982).

Nitric oxide (NO) is an endothelium-derived relatively stable gas with a very broad spectrum of actions: dilates blood vessels, reduces platelet and monocyte adhesion, reduces release of superoxide radicals, prevents smooth muscle cells proliferation and reduces oxidation of low density lipoprotein (LDL). All these effects are directly related to atherosclerosis and show the importance of NO in the disease. It is produced by enzymes called the nitric oxide synthases (NOS) while they convert L-arginine into L-citruline, and after formed it binds the heme group of soluble guanylate cyclase (Cooper and Brown, 2008). There are three kinds of NOS: two kinds, (nNOS and eNOS), are constitutive forms present in neuronal and endothelial cells that produce basal levels of NO, while the third form (iNOS) is an inducible form of the enzyme found in macrophages, monocytes, neutrophils and Kupffer cells (El-Sallab *et al.*, 2002). After formed, endothelial NO is released into the vessel lumen and can be scavenged by erythrocytes. It diffuses into the erythrocyte through band 3 protein and becomes fixed by the haemoglobin molecules (Fig. 1.2), generating nitrosohemoglobin. Erythrocytes scavenge NO when oxygen tension is high and liberate it when it is low (Saldanha *et al.*, 2013). It also has other roles: is a key component of the respiratory cycle, causes smooth muscle relaxation, has the ability to diffuse through the cell membrane. It directly affects RBC deformability,

so a possibility of NO having a regulatory role on erythrocyte deformability has been proposed (Bor-Kucukatay *et al.*, 2003). Under normal conditions NO has an anti-inflammatory role, but when there is a destabilization in the surrounding environment and in NO it may turn into a pro-inflammatory signalling molecule. After diffusing into erythrocytes, NO may be stored or return to the blood stream as an active S-nitrosothiol molecule. Nitrites (NO_2-) and nitrates (NO_3-) are the major stable metabolites that result from its oxidation (Kesmarky *et al.*, 1998).

S-Nitrosoglutathione (GSNO) is an endogenous nitrosothiol with a critical role in NO signalling (Fig.1.3). It is produced by the reaction of NO with glutathione (GSH) and is much more stable than NO in biological systems. It can act as a nitrosylating agent, which leads to it having anti-inflammatory effects, and it also presents antioxidant effects through redox modulation, namely down regulation of peroxynitrite and up regulation of glutathione. GSNO inhibits platelet activation, inflammatory processes in endothelial and T cells, and reduces embolization in humans. A GSNO/hemoglobin homeostasis in circulation is required for GSNO to perform its S-nitrosylation mechanisms (Khan *et al.*, 2011).

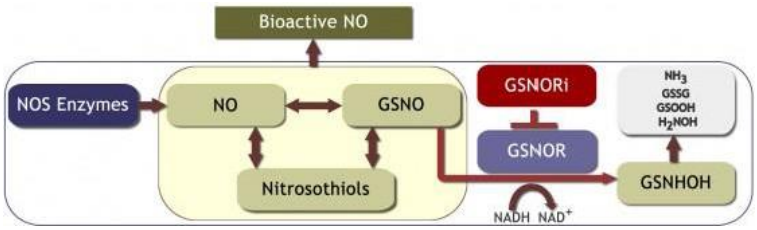

Figure 1.3 – Synthesis of GSNO. GSNO pathways along his synthesis (Adapted from Colagid, S-Nitrosoglutathione, www.wikipedia.org).

1.4. – Aims of This Study

This work is part of a broad project whose final goal is to contribute to the increase of knowledge about the pathophysiology of inflammatory diseases through the analysis and study of several markers implied in the origin and progression of these diseases. The present thesis has benefited from ongoing collaborations with medical staff from several hospitals.

The main goal of this study was to investigate the contribution of four hemorheological markers for the pathophysiology of inflammation and their evolution, and see if it can be quantified and possibly used as part of the diagnosis or even prevention methods.

The process used to reach this aim was analysis of four hemorheological markers:

- erythrocyte deformability
- erythrocyte aggregation
- NO
- GSNO

Those markers were measured in the blood of patients that suffered from two different inflammatory diseases:

- acute myocardial infarction
- sepsis

The results will then be compared with a control group constituted by healthy individuals with no sign of inflammation and also with patients suffering from a chronic inflammatory disease (patients with rheumatoid arthritis – RA).

Patients with acute myocardial infarction were monitored at the moment of their hospital admission and after 30 days, while sepsis patients were monitored at the time of their admission in the intensive care unit (ICU), 24 hour after, 72 hours after and at the time of their discharge.

This specific study of erythrocytes and their changes during various phases of inflammation may help strengthen our knowledge of these diseases and further down the road have a direct impact on the clinical procedures in order to achieve a faster, earlier and most effective diagnosis.

2. – Longitudinal evaluation of hemorheological markers in patients with acute myocardial infarction

2.1. – Role of STEMI in World's Health

AMI is a cardiovascular disease that plays a huge role in the world´s health panorama, as it is the main cause of death globally. STEMI is the most dangerous type of AMI as it blocks a great amount of hearth muscle, which ends up by being damaged.

A series of studies that involve thousands of people have been done along the years, and the knowledge of this disease and its mechanisms has improved greatly. Despite this, a lot more is still to be uncovered in order to let us achieve efficient prevention methods, diagnosis, and treatment.

STEMI starts with the formation of the atherosclerotic lesion and culminates with thrombosis – formation of a blood cloth (thrombus) – that obstructs the vessel and causes ischemia. Thrombosis of the plaque occurs by two different processes. The first one is endothelial erosion and occurs because of the extension of endothelial denudation, which makes large areas of subendotelial connective tissue of the plaque exposed. The probable explanation for this process is macrophage. When highly activated, they cause endothelial cell death by apoptosis and production of proteases. Studies linked endothelial cell loss and proximity of macrophages. The second process is plaque disruption. In this scenario the plaque cap tears and the lipid core is exposed to blood in the arterial lumen and starts to coagulate. Coagulation is fast because the lipid core is highly thrombogenic- it has tissue factor, fragments of collagen and crystalline surfaces (Davies, 2000).

Inflammation plays a key role in the mechanism of this disease by promoting several actions: the initiation of atherosclerotic lesion, its progression to complex plaque, the weakening of the fibrous cap (which renders plaque prone to rupture), and the boosting of thrombogenicity of the lipid core (Davies, 2000).

2.2. – Objectives

The aim of this chapter is to unveil the contribution of hemorheological parameters for the STEMI pathophysiology and their evolution. To achieve the proposed objective, four hemorheological markers – erythrocyte deformability, erythrocyte aggregation, NO and GSNO erythrocyte concentrations – were measured in patients with AMI upon their hospital admission and 30 days after.

Furthermore, the results were compared with patients suffering from a chronic inflammatory disease, patients with RA, and without inflammation, the control group.

2.3. – Materials & Methods

2.3.1. – Study Groups

The study groups were composed by both female and male patients and were organized according to the illness these patients presented when admitted to the hospital.

In this chapter two study groups (myocardial infarction group and rheumatoid arthritis group) are going to be analysed and compared. A control group was also created with females older than 50 years old and males. The samples were taken from October 2012 to June 2013.

ST-elevation Acute Myocardial Infarction (STEMI) Group: Composed by 15 patients (5 females and 10 males) with documented ST-elevation changes, creatine kinase 3 times above normal and with primary coronary intervention (PCI) as reperfusion therapy. STEMI patients were enrolled during the first 24 hours of hospital admission. Blood samples were taken from patients immediately after being admitted into the Serviço de Cardiologia at Hospital de Santa Marta and before the administration of IIb/IIIa inhibitors and PCI intervention. Only patients with no previous history of heart diseases and who were suffering from myocardial infarction for the first time were accepted into this group. A second sample (follow-up) was taken about a month after the episode.

Rheumatoid Arthritis Group: Composed by 25 patients (19 females and 6 males) from Consulta de Reumatologia from Hospital Egas Moniz (CHLO-EPE) without known antecedents of ischemic or pulmonary pathology and without electrocardiographic changes or other positive stress tests. Blood samples were taken from patients suffering from rheumatoid arthritis during their appointment at Hospital de Santa Marta. Patients were previously diagnosed with rheumatoid arthritis and were part of an ongoing study at the Hospital.

Control Group: Composed by 15 healthy volunteers (4 females and 11 males). Inclusion criteria for reference controls was absence of any history of cardiovascular diseases, any life threatening diseases, or any other disease or condition that would impair compliance. Blood samples were obtained by vein puncture from healthy volunteer donors at the Banco Público de Sangue do Instituto Português do Sangue (Lisbon) under an institutional agreement with Instituto de Bioquímica da Faculdade de Medicina da Universidade de Lisboa. All donors were informed and signed a written consent.

Blood samples (9 ml) from all subjects enrolled were drawn into heparin tubes and were tested within the next 3 hours.

The medical team of the patients at Hospital de Santa Marta provided all clinical data.

2.3.2. – Methods

As mentioned previously, in the present study four hemorheological parameters were measured - erythrocyte deformability, erythrocyte aggregation, NO and GSNO erythrocyte concentrations. Above are described the methodologies used for each determination (the list of materials and reagents used in this determination is presented in the Appendix B).

Erythrocyte Aggregation

Aggregation of erythrocytes in the blood samples was tested using the Myrenne Aggregometer MA-1 (Schmid-Schönbein *et al*., 1983; Rampling and Martin, 1989). The red blood cell aggregates are placed in a rotating cone plate chamber and dispersed during 10 s with a shear rate of 600 s^{-1} and after stopped. Infra red light from a light emitting diode is then applied through the cells and its intensity measured. This measurement was done twice - for 5 and 10 seconds, and then the mean for these values was calculated.

Erythrocyte Deformability

Erythrocyte deformability was measured with a Rheodyn SSD diffractometer (Bessis and Mohandas, 1975a; Bessis and Mohandas, 1975b; International Committee for Standardization in Haematology, 1986) at three different shear forces, 0.6, 6.0 and 30.0 Pa. The blood sample is added to a viscous solution of Dextran and placed in the instrument between one stationary disk and a rotating one. The rotation of the disk produces different well-defined shear forces dynamic viscosity (Pa.s), while a laser beam penetrates the solution and gives different diffraction patterns. The light intensity of these patterns is measured at two different points (A and B), equidistant from the center of the image and the erythrocyte elongation index (EEI) is obtained, in percentage, with the formula:

$$EEI(\%) = \frac{A - B}{A + B} \times 100$$

Three different shear stress forces (0.6 Pa, 6.0 Pa and 30.0 Pa) were compared when studying erythrocyte deformability. Dextran, the reagent used in the erythrocyte deformability test, is a neutral

macromolecule that allows to see the formation of rouleaux when RBC are resuspended in electrolyte solutions (Bäumler *et al.*, 1999).

Measuring the NO in erythrocytes

NO was measured with a Nitric Oxide Measuring System. The blood was centrifuged for 10 minutes and plasma was removed. A sodium chloride 0.9% at pH 7.0 solution was added to 1.5 µl of erythrocyte suspension in order to reach an Ht of 0.05%. A magnet was then put in the solution. The amino-IV sensor (Carvalho *et al*., 2004), linked to a computer and to the NO measuring system, was submerged in the solution and Ach 10^{-3} M was added. The value for NO was calculated using the peaks that the measuring system gives when adding the Ach, as this alteration is proportional to the amount of NO mobilized by Ach-stimulated erythrocyte.

Measuring the S-nitrosoglutathione (GSNO) concentration in erythrocytes

GSNO concentration was calculated using the values obtained with the Thermo Spectronic Genesys 10UV-VIS Spectrophotometer (Guevara *et al*., 1998). The blood was centrifuged at 11000 rpm and plasma was removed. Mill-Q water, ethanol and chloroform were added to the erythrocyte suspension and it was vortexed. The measurement was based on the Griess Reaction using a commercial available kit by Invitrogen. Briefly, a solution was made containing components A and B of Griess reagent kit and PBS pH 7.4 solution. Erythrocyte suspension and Mill-Q water were added to this solution. Then, $HgCl_2$ was added to this mixture in one cuvette but not in the other in order to compare. Two different controls were used: one with the Griess reagents plus PBS pH 7.4 solution and water, and the other with the same components plus $HgCl_2$. The cuvettes were left to incubate in the dark for 20 minutes and then were read in the spectophotometer at 490 nm.

2.3.3. – Statiscal Analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) program, version 2.7.0 and also R software (version 2.11.1).

Values of $p<0.05$ were considered statistically significant.

Distribution of the hemorheological markers between the various groups of patients was analyzed using the nonparametric Kruskal-Wallis test. This test does not assume that the data is normally distributed, but it is based on other assumptions: data is independent from each other and is selected randomly from the population.

Furthermore, in order to compare the longitudinal variations of STEMI patients, blood markers were repeatedly measured in the same patient at two different time-points. Consequently, the observations are inter-correlated and common statistical methods as analysis of variance or non-parametric correlations are unsuitable. For that reason, a regression algorithm that accounts the effect of repeated measures was applied. The chosen statistical method was the linear mixed effects (LME) that models the concentrations of blood markers through time considering that measures for each patient were not independent. This statistical model describes the longitudinal variations of each patient by calculating slopes and averages of the variables in each time point. Therefore, it allows to estimate the differences in average slopes between hospital admission (day 0) and day 30, giving a measure of the variation of each blood marker over time.

2.4. – Results

2.4.1. – Characterization of the Study Groups

The clinical characteristics of the patients allocated in the STEMI and in the RA study groups are listed in Table 2.1. Baseline clinical and demographical data were registered for each of the patients at the hospital: age, sex, weight, height, smoking habits, blood pressure, hyperlipidemia, hypertension, diabetes mellitus and abdominal fat.

Waist perimeter, systolic blood pressure, diastolic blood pressure and abdominal fat were measured for all the RA patients. Unfortunately, the baseline clinical data is not available for the control group due to the type of agreement established with Instituto Português do Sangue.

Blood test results that included blood cell count and CRP levels were also registered. In the STEMI group there is additional information about the culprit lesion regarding stenosis, the type, location and number of vessels approached and stents used.

All the STEMI patients were taking at least one kind of medication. Six STEMI patients (40%) had multivessel disease. In seven patients the culprit vessel was the right coronary artery, in six patients it was the left descending coronary artery, and in one it was the circumflex artery. Six patients were lost in the follow up.

Table 2.1 - Baseline clinical characteristics of the subjects enrolled. ACE – angiotensin-converting-enzyme; BMI – body mass index; BMS - bare-metal stent; DES - drug-eluting stent; LAD - left anterior descending coronary artery; LCX - left circumflex artery; RCA - right coronary artery.

	CTR (n=15)	**RA** (n=25)	**STEMI** (n=15)
Sex (f/m)	4/11	19/6	5/10
Age (y)	-	60±13	64±12
BMI (kg/m^2)	-	26±3	28±3
Waist perimeter (cm)	-	84±8	-
Systolic BP (mm Hg)	-	135±16	-
Diastolic BP (mm Hg)	-	80±8	-
Risk factors			
Smoking (n (%))	-	3 (12)	4 (27)
Hyperlipidemia (n (%))	-	16 (64)	8 (53)
Hypertension (n (%))	-	14 (56)	13 (87)
Diabetes (n (%))	-	3 (12)	4 (27)
Abdominal fat (n (%))	-	4 (16)	-
Pre-event medication			
Aspirin (n (%))	-	-	11 (73)
ACE-inhibitor (n (%))	-	-	6 (40)
β-blockers (n (%))	-	-	11 (73)
Statins (n (%))	-	-	12 (80)
Angiographic data			
Stenosis (%)	-	-	99
TIMI Class			
Normal flux (TIMI = 3) (n (%))	-	-	2 (13)
Occlusion (TMI <2) (n (%))	-	-	12 (80)
Multivesell disease (n (%))	-	-	6 (40)
Culprit vessel			
LAD (n (%))	-	-	6 (40)
RCA (n (%))	-	-	7 (47)
LCX (n (%))	-	-	1 (7)
Number of stents			
1 stent (n (%))	-	-	5 (33)
2 stents (n (%))	-	-	6 (40)
3 stents (n (%))	-	-	1 (7)
4 stents (n (%))	-	-	1 (7)
Stents type			
BMS (n (%))	-	-	7 (47)
DES (n (%))	-	-	6 (40)

Values expressed as mean±sd, except when otherwise indicated.

2.4.2. – Hemorheological Markers

Concerning the values of hemorheological and biochemical parameters obtained for the three study groups (Table A.1 in the appendix), it is possible to observe that STEMI at day 0 and RA patients did not significantly differ from the deformability values obtained in the control group (Fig 2.1).

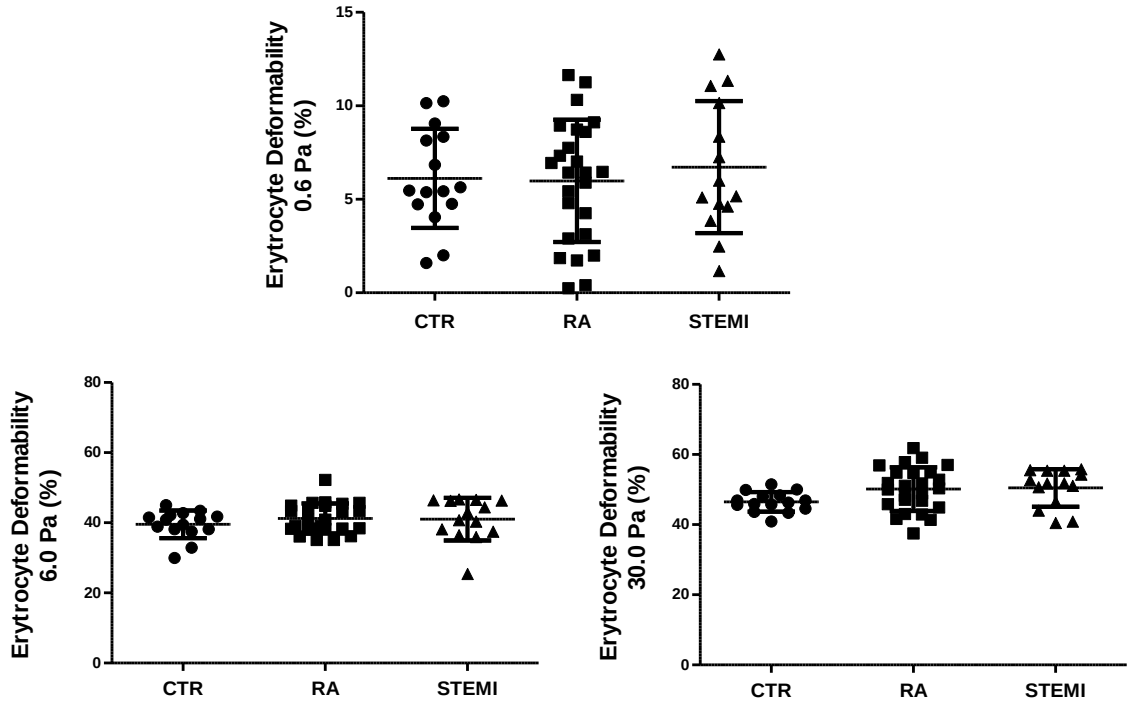

Figure 2.1 - Variations of erythrocyte deformability, at 0.6 Pa, 6.0 Pa and 30.0 Pa, between the control, RA and STEMI (day 0) groups.

After performing the statistical analysis for the erythrocyte aggregation markers, a significant difference was only observed in the values of erythrocyte aggregation at 10s between STEMI patients at hospital admission and healthy volunteers. No significant difference was obtained when comparing the RA group to the control group (Fig. 2.2).

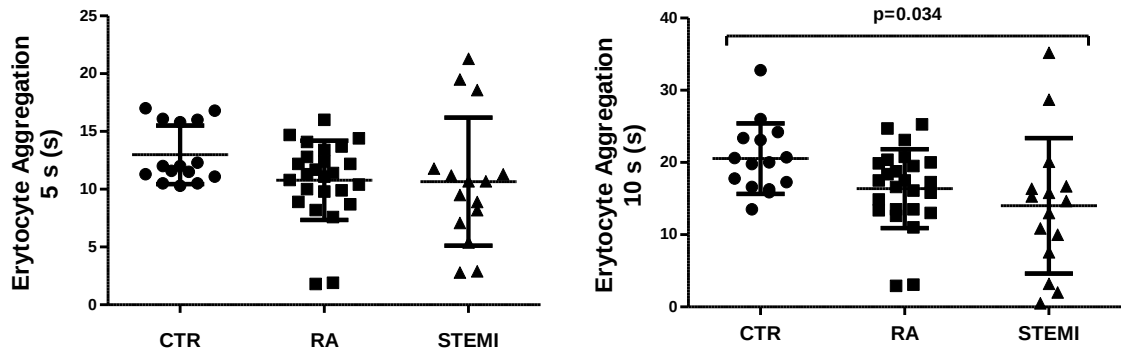

Figure 2.2 - Variations of erythrocyte aggregation at 5 s and 10 s between the control, RA and STEMI (day 0) groups.

Concerning the erythrocyte NO and GSNO concentrations, no differences were verified between STEMI patients at hospital admission, RA patients or controls (Fig. 2.3).

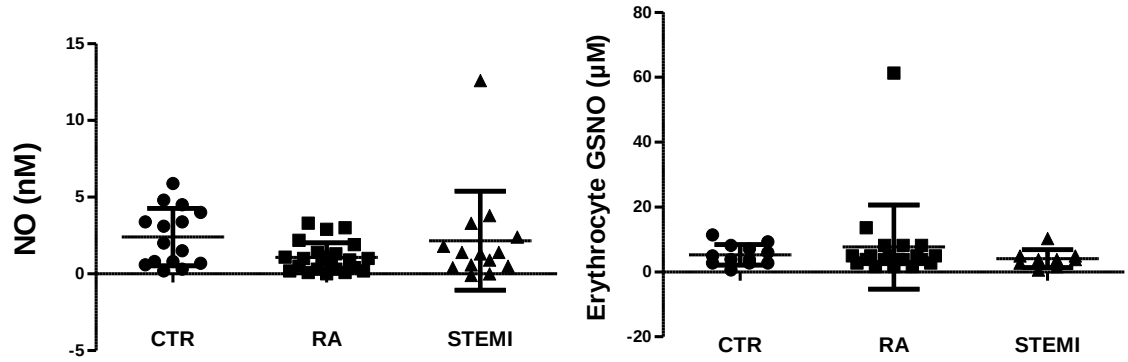

Figure 2.3 - Variations of erythrocyte NO and GSNO concentrations between the control, RA and STEMI (day 0) groups.

2.4.3. – Longitudinal Variations in STEMI Group

In STEMI patients, the longitudinal variations in the hemorheological markers were inspected using special statistical models. The linear mixed effect (LME) model was the chosen one to model each variable as a response variable over time.

The longitudinal variations in STEMI patients for erythrocyte deformability were non significant ($F>0.21$, $p>0.66$; Fig. 2.4), and the same happens for erythrocyte aggregation ($F>1.06$, $p>0.27$; Fig. 2.4).

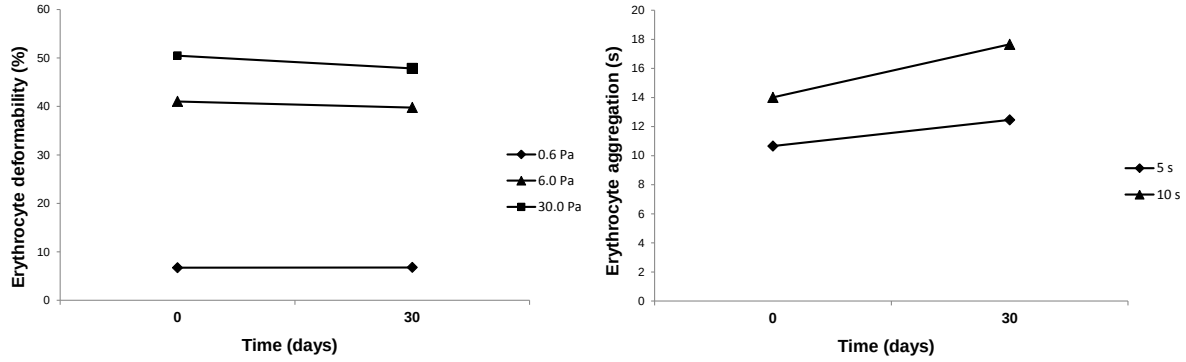

Figure 2.4 – Longitudinal variations of erythrocyte deformability (at 0.6 Pa, 6.0 Pa and 30.0 Pa) and aggregation (at 5s and 10s) of STEMI patients at hospital admission and 30 days after.

LME statistical analysis shows that there are also no changes over time in the concentrations of NO (F=0.03, p=0.87; Fig. 2.5) and GSNO (F=1.03, p=0.35; Fig. 2.5) in erythrocytes of STEMI patients.

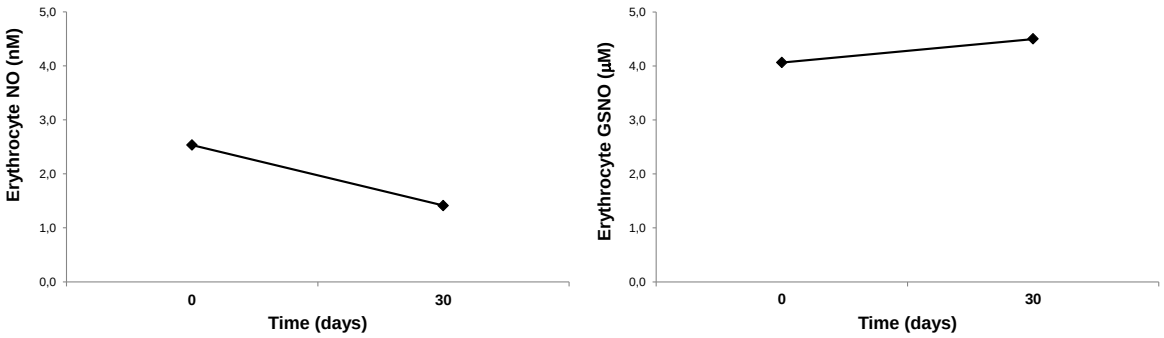

Figure 2.5 – Longitudinal variations of concentration of NO and GSNO in erythrocyte of STEMI patients at hospital admission and 30 days after.

Furthermore, the difference between the STEMI patients at day 30 and RA and control groups were also verified. Once again no variations were observed between RA and controls and STEMI group at day 30 (Table A.1 in the appendix).

2.5. – Discussion

Resulting from an atherosclerotic plaque rupture, STEMI is a condition responsible for a very high percentage of deaths around the world every year. After plaque rupture, the highly thrombogenic plaque content is exposed to the blood in the arterial lumen and the coagulation cascade is initiated (Davies, 2000), which leads to an acute inflammatory reaction. Thrombosis causes a critical reduction in the blood flow that is severely below the myocardium's need. When perfusion stays in these alarming levels for long enough, oxygen and nutrients supply to cardiac muscle is interrupted (ischemia) and partial necrosis of the muscle occurs (Collinson and Gaze, 2007).

Erythrocyte deformability is one of the most important properties of erythrocytes and plays a crucial role on the way they behave. Along with erythrocyte aggregation, it is one of the determinant factors for blood viscosity (Silva, 1982). Studies have shown a relation between cardiovascular diseases and alterations of deformability, suggesting that erythrocyte deformability index (EDI) may be potentially used as a predictor in coronary diseases (Qin *et al.*, 1998). Decreased RBC deformability is associated with high variations in red cell distribution width (RDW) – in RBC volumes – which in turn is associated with increased risk for cardiovascular diseases through impairment of blood flow in the microcirculation (Patel *et al.*, 2013). Autocrine function of vascular endothelium has a very important role regulating the RBC rheology in the blood flow (Martin, 2012).

When taking into account the pathophysiology of STEMI (e.g., decrease in the blood flow and the existence of ischemia and possible necrosis) and the data from previous studies (Qin *et al.*, 1998; Patel *et al.*, 2013) that show a correlation between erythrocyte deformability and cardiovascular diseases, a significant difference in the values of these markers was expected when comparing STEMI with the control group. However, in the present study no significant difference was observed between STEMI patients and healthy donors.

Erythrocyte aggregation is one of the determinant factors of blood viscosity. High values of plasma viscosity and an increase of erythrocyte aggregation tendency has been observed in patients with ischemic heart diseases (Kesmarky *et al.*, 1998). Erythrocyte aggregation is influenced both by extrinsic factors - levels of plasma protein, hematocrit, and shear rate - and intrinsic factors, such as RBC shape and membrane surface charge (Saldanha *et al.*, 2012).

Erythrocytes tend to aggregate more under conditions of low shear stress rates, even though the attractive forces are relatively weak. As the shear stress increases, the aggregates diminish in size and the RBCs align with the flow.

Aggregation was determined in stasis of the blood sample for 5 and 10 seconds. In this study we verified that STEMI patients at hospital admission presented lower erythrocyte aggregation than controls for the 10 second measurement.

Since thrombosis causes a diminished shear stress in the blood flow of its site, a higher erythrocyte aggregation was expected. It has been observed in atherosclerosis and other vascular diseases that oxidative processes overcome RBCs defences, which makes these oxidative-modified RBCs act at periphery as pro-oxidant bullets that can modify behaviour and fate of other vascular tissues (Minetti *et al.*, 2007). Perhaps the erythrocytes became oxidative-modified in such a degree that their aggregation lowered. Another explanation is the existence of some agent – possibly some medication taken by the patient – that has this effect on aggregation.

One could hypothesize that the inexistence of differences between groups for the erythrocyte aggregation at 5s may be due to the fact that erythrocytes need a longer time to aggregate. Kayar *et al.* (Meiselman *et al.*, 2007) performed experiments on the effects of ischemia and reperfusion in rats and observed that erythrocyte aggregation was not affected by 10 minutes of ischemia, but was significantly reduced after 15 minutes of reperfusion, equally in plasma and in dextran medium.

One of the causes for the lack of significant differences between the control and STEMI groups regarding erythrocyte deformability and aggregation may be related with the medication intake of STEMI patients.

After having an STEMI episode, the patient is treated with medication that includes aspirin, β-blockers, ACE-inhibitors, statins and platelet inhibitor agents. β-blockers are used as secondary prevention and intent to reduce cells stress response, aspirin prevents platelet aggregation and vasoconstriction, ACE-inhibitors lower blood pressure by dilatating blood vessels and statins lower the cholesterol level. All these drugs aim to revert the situation caused by atherosclerosis, so there are no more acute demonstrations of the cardiovascular disease. A month after suffering from STEMI the patient should present stabilized and normal levels of blood markers. Indeed, no significative differences between the markers of the STEMI follow up group and the control group were obtained, which corresponds to the expected results.

It has been demonstrated in several studies that aspirin has an effect in platelet function, but some results also lead to the idea that aspirin may have a direct effect on erythrocytes as well. Bouhmadi *et al.* (2000) showed that erythrocyte aggregation is increased when oral contraceptives are being taken, and that 100 mg aspirin can reverse this hyper aggregation. Before this, Yousif (1999) hypothesized the possible rheologically active role of aspirin on erythrocytes through the acetylation of intracellular proteins and saturation of the cell interior with the osmotically active drug. Other studies concluded that in patients presenting acute coronary syndromes the antiaggregant effect of aspirin is modulated

not only by platelets, but also by erythrocyte deformability and white blood cells count (Mannini *et al.*, 2006). It is also shown that aspirin exerts a clear effect on the energy of adhesion between erythrocytes (Elblbesy *et al.*, 2012). This finding needs to be studied further in order to understand the precise role that it plays in erythrocyte aggregation and deformability.

NO is a gaseous signalling molecule derived from the endothelium with a direct role in pathogenesis of inflammation. It is a very reactive molecule with a wide spectrum of effects, depending on the concentrations of NO and the surrounding environment (Korhonen *et al.*, 2005).

Under normal physiological conditions NO gives an anti-inflammatory effect. It has been observed that NO augments production of the nuclear factor kappa B (NF-kB) inhibitor – a transcription factor involved in expression of genes encoding many pro-inflammatory functions of vascular wall cells and infiltrating leukocytes (De Caterina *et al.*, 1995; Thurberg and Collins, 1998). Nitric oxide induces vasodilatation in intact endothelium that leads to an increase in blood flow, and it also is a potent neurotransmitter at neuron synapses and contributes to regulation of apoptosis (Sharma *et al.*, 2007). Winlaw *et al.* (1994) reported increased levels of plasma nitrate, the stable end-product of NO production, in patients with heart failure.

Being a signalling molecule, NO is equally responsible for some undesired effects. NO plays an important role in the regulatory functions in inflammation, such as: regulation of signalling cascades, transcription factors, vascular responses and cytokine production, proliferation and apoptosis (Korhonen *et al.*, 2005). It reacts with superoxide anion and forms peroxynitrite ($ONOO^-$), a potent oxidant that promotes vascular injury.

Endothelial dysfunction leads to a reduced production of endothelium-derived NO and therefore its normal processes like dilatation of blood vessels, reduction of platelet and monocyte stickiness undergo disturbance (Scher, 2013). Considering all the physiological and hemorheological changes that occur during STEMI – starting with endothelial dysfunction - and all the cellular mechanisms in which NO takes part and plays an important role, a difference of erythrocyte nitric oxide values was expected to be observed. None of the groups, though, showed difference in their erythrocyte NO levels.

Erythrocyte NO is produced by nitric oxide synthase (NOS) and reacts with intra-erythrocytic hemoglobin, which is directly linked to NO bioavailability and homeostatic vascular function modulation (Azarov *et al.*, 2005). It regulates RBC deformability, favouring their passage through capillaries and stimulating blood flow in microcirculation (Eligini *et al.*, 2013).

Given the relation between erythrocyte NO and their deformability (Carvalho *et al*, 2006), the fact that there was no difference between the deformability markers among the groups makes this result plausible.

GSNO, a low molecular weight endogenous S-nitrosothiol, is a source of bioavailable NO and plays a critical role in its signalling. S-nitrosylation of proteins is linked to critical aspects in cardiovascular biology: S-nitrosylation of calcium cycling and essential regulators of β-adrenergic receptor signalling helps to maintain cardiac contractility (Hare, 2003; Ozawa *et al.*, 2008), while S-nitrosylation of hemoglobin regulates blood flow and oxygen delivery (Singel and Stamler, 2004). Results obtained from GSNO studies suggest that certain concentrations of it may modulate the remodelling/inhibition of fibrin networks - this relates to STEMI as changes in the architecture of fibrin networks is becoming increasingly recognized as another risk factor for cardiovascular diseases and thrombotic complications (Bateman *et al.*, 2012).

GSNO anti-inflammatory role has been demonstrated in several diseases. It attenuates infiltration of immune cells into the central nervous system, protects against demyelination and downregulates pro-inflammatory cytokines in multiple sclerosis models (Foster *et al.*, 2009).

Given the existing relation between NO and GSNO and the physiological changes that occur during STEMI, a significant difference of GSNO in the two groups was expected. It is to point out that no differences were observed in NO as well, which makes the results for GSNO less surprising.

When comparing the results of the four studied hemorheological markers between the group of STEMI patients who just suffered from an acute myocardial infarction (the patients admitted in the hospital) and the same group but a month later (follow up), differences in the marker's values are expected to be seen. Whereas the data from the first group is collected when the patient is undergoing severe thrombosis in coronary artery and the hemorheological and physiological properties of the surrounding tissues and blood are far from being normal, the data from the second is collected when the patient is stabilized and has returned to his daily chores and activities after being treated.

After statistically analysing the data, it is concluded that no significative longitudinal variation differences exist between any of the studied hemorheological markers in STEMI patients.

Even though theoretically a significant difference is expected between STEMI patients over time, the previous lack of significant difference between the STEMI admission and the control group, aside from the 10s sd erythrocyte aggregation, creates a propensity for the fact that there are no changes in the groups values.

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease – there is a chronic activation of the innate immune system and a release of pro-inflammatory cytokines. It has been showed that patients suffering from RA are at greater risk of developing cardiovascular diseases (Van Doornum *et al.*, 2002). Occurrence of CVD in RA patients has not been associated with traditional CVD risk factors like smoking, diabetes mellitus, hypertension, dyslipidemia and cholesterol (Warrington *et al.*, 2005), which leads to the assumption that it is an independent risk for cardiovascular diseases.

RA is correlated with accelerated atherosclerosis. Association of carotid intima-medial thickness (IMT) with inflammatory markers supports the evidence that CVD outcome in RA patients is indeed linked with inflammation (Arnab *et al.*, 2013). The primary site of inflammation in RA is the synovial tissue – thin, loose vascular connective tissue that makes up the synovial membrane that surrounds joints. Cytokines such as TNF-α, IL-1β and IL-6 are released from this site of inflammation and act on distant tissues, causing insulin resistance, characteristic dyslipidemia, prothrombotic effects, pro-oxidative stress and endothelial dysfunction (Sattar *et al.*, 2003).

Being such an active signalling molecule in inflammatory processes, NO is expected to present significantly different concentrations in patients suffering from RA as it is a disease in which constant inflammation occurs. Interestingly, Saldanha *et al.*, (2011) conducted a study regarding RA patients and hemorheological parameters and concluded that erythrocyte NO production is independently associated with RA.

The fact that dyslipidaemia presented in RA is pro-oxidative, and that cytokines can directly promote oxidative modification of LDL, suggest that there is a high level of oxidized lipids in RA (Sattar *et al.*, 2003). Considering that oxidative stress reduces erythrocyte deformability, a significant difference in this marker for RA patients was expected. Although increased clotting potential with elevated levels of fibrinogen, fibrin D-dimer and von Willebrand factor is observed in RA patients (McEntegart *et al.*, 2001), data from the present study shows no significant difference in erythrocyte aggregation and deformability, or in erythrocyte NO and GSNO.

Among the molecules that can interact with the erythrocyte surface is hyaluronic acid (HA), which behaves similarly to albumin. A study has shown that HA causes significant concentration-dependent decrease in erythrocyte deformability and that it is the only plasma factor that significantly affects deformability (Luquita *et al.*, 2010). In fact those authors found a correlation between erythrocyte rigidity index and HA concentration (Luquita *et al.*, 2010). As RA patients present an elevated concentration of HA, that fact may partially explain the results obtain in the present work.

A comparison between the RA and the STEMI admission was made.

RA and STEMI are both diseases in which inflammation plays an important role. They are linked because they share the common physiology of inflammation – pro-inflammatory cytokines, activated macrophages and other molecules. Therefore we can infer that the activity and levels of hemorheological markers is very similar in both cases, which is consistent with the data obtained from the comparison of both groups. No assumptions can be made, though, as both groups initially did not show any significant differences when compared with the control group. There is also the option that the two inflammation diseases have different intensities, which combined with the different etiology of both diseases would result in different values for these markers, which is not seen in these statistical results. Once again, an influence of medication could be hypothesized, however the number of patients included in the study did not allow that kind of statistical analysis of the data.

3. – Longitudinal evaluation of hemorheological markers in patients with sepsis

3.1. – Sepsis in Today's World

Sepsis causes the death of thousands of people every year. It is a serious life-threatening disease with high rates of mortality that starts with a simple infection and can very easily escalate to death. Even though the percentage of survivors has been increasing in the past years, the number of people suffering from it has been increasing as well, which makes the mortality rates stay high. It is a true challenge for patients and physicians, and there is still a lot to be learned and many questions to be answered in clinical research. The need for awareness, precise diagnosis and effective treatment is essential.

3.2. – Objectives

The aim of this chapter is to unveil the contribution of hemorheological parameters for the sepsis pathophysiology, its evolution, and more importantly the outcomes. To achieve the proposed objective, four hemorheological markers – erythrocyte deformability, erythrocyte aggregation, NO and GSNO erythrocyte concentrations – were measured in patients in different classes of sepsis upon their hospital admission, 24h, 72h after and at UCI discharge. Furthermore, the results were compared with subjects without inflammation, the control group.

3.3. – Materials & Methods

3.3.1. – Study Groups

Sepsis Study Group: is composed by 14 patients with sepsis diagnosis at the Intensive Care Unit (ICU) of Hospital Beatriz Ângelo admission, of which 5 are females and 9 males. Blood samples were taken from patients suffering from sepsis at 4 different times during their hospitalization in the ICU of Hospital Beatriz Ângelo: the first blood collection was taken at time of the admission, the second was taken 24 hours later, the third was taken 72 hours later and the fourth was taken when the patient was discharged from the ICU. Some patients were lost to follow-up because they died or were discharged

earlier from the ICU. In the present study were included patients in three different classes of sepsis (namely, systemic inflammatory response syndrome (SIRS), severe sepsis and septic shock).

Control Group: Composed by 15 healthy volunteers (4 females and 11 males). Inclusion criteria for reference controls was absence of any history of coronary disease, dyslipidaemia or hypertension, any conditions limiting mobility, life threatening diseases, or any other disease or condition that would impair compliance and negative stress tests. Healthy volunteers were selected among the blood donors of the Banco Público de Sangue do Instituto Português do Sangue (Lisbon) under an institutional agreement with Instituto de Bioquímica da Faculdade de Medicina da Universidade de Lisboa. All donors were informed and signed a written consent.

The blood samples (9 ml) were taken from January 2013 to June 2013. The blood was collected in heparin tubes and tested within the next 3 hours.

The medical team of the Hospital Beatriz Ângelo provided all clinical data.

3.3.2. – Methods

The methods and reagents used to assess the erythrocytes aggregation and deformability, as well as the concentrations of NO and GSNO in erythrocytes were previously described in Section 2.3.2.

3.3.3. – Statistical Analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) program, version 2.7.0 and also R software (version 2.11.1).

Values of $p<0.05$ were considered statistically significant.

Tests used to compare medians and distributions of the hemorheological markers were previously described in Chapter 2.3.3. The results of the statistical analysis performed to the data of the control group also are shown in Chapter 2.3.3.

Furthermore, as described in Chapter 2, a LME analysis was made, allowing to estimate the differences in average slopes between UCI admission (0 hours) and the other time points (24 hours, 72 hours and UCI discharge), giving a measure of the variation of each blood marker over time.

3.4. – Results

3.4.1. – Characterization of the Study Groups

The clinical characteristics of the sepsis study group's patients are listed in Table 3.1. Baseline clinical and demographical data was registered for each of the patients at the hospital: age, sex, weight, height, temperature, mean arterial pressure and heart rate. Blood test results were also registered.

Table 3.1 - Baseline clinical characteristics of the subjects enrolled. BMI – body mass index; HR – heart rate; MAP – mean arterial pressure; TMP – temperature.

	CTR (n=15)	**SP** (n=14)
Sex (f/m)	4/11	5/9
Age (y)	-	66±14
BMI (kg/m^2)	-	23±4.2
TMP (^oC)	-	37±0.8
MAP (mm Hg)	-	75±18
HR (bpm)	-	101±22
Death (f/m)	-	0/4
Medication		
Norepinephrine (n (%))	-	14 (100)
Dopamine (n (%))	-	14 (100)
Dobutamine (n (%))	-	14 (100)
Epinephrine (n (%))	-	14 (100)

Values expressed as mean±sd, except otherwise indicated.

All patients were administered medication during the treatment. Seven patients were diagnosed with septic shock, three with severe sepsis and four with sepsis. Four patients died, three during their internment in the ICU and one after being discharged.

Unfortunately, the clinical baseline data is not available for the control group due to the type of agreement established with Instituto Português do Sangue.

3.4.2. – Hemorheological Markers

When looking at the values of hemorheological and biochemical parameters obtained for patients with sepsis at UCI admission (Table A.2 in the appendix) and comparing them with the control group,

we can see that values for erythrocyte deformability at all shear stress forces (0.6 Pa, 6.0 Pa and 30.0 Pa) are higher in the sepsis group (Fig. 3.1).

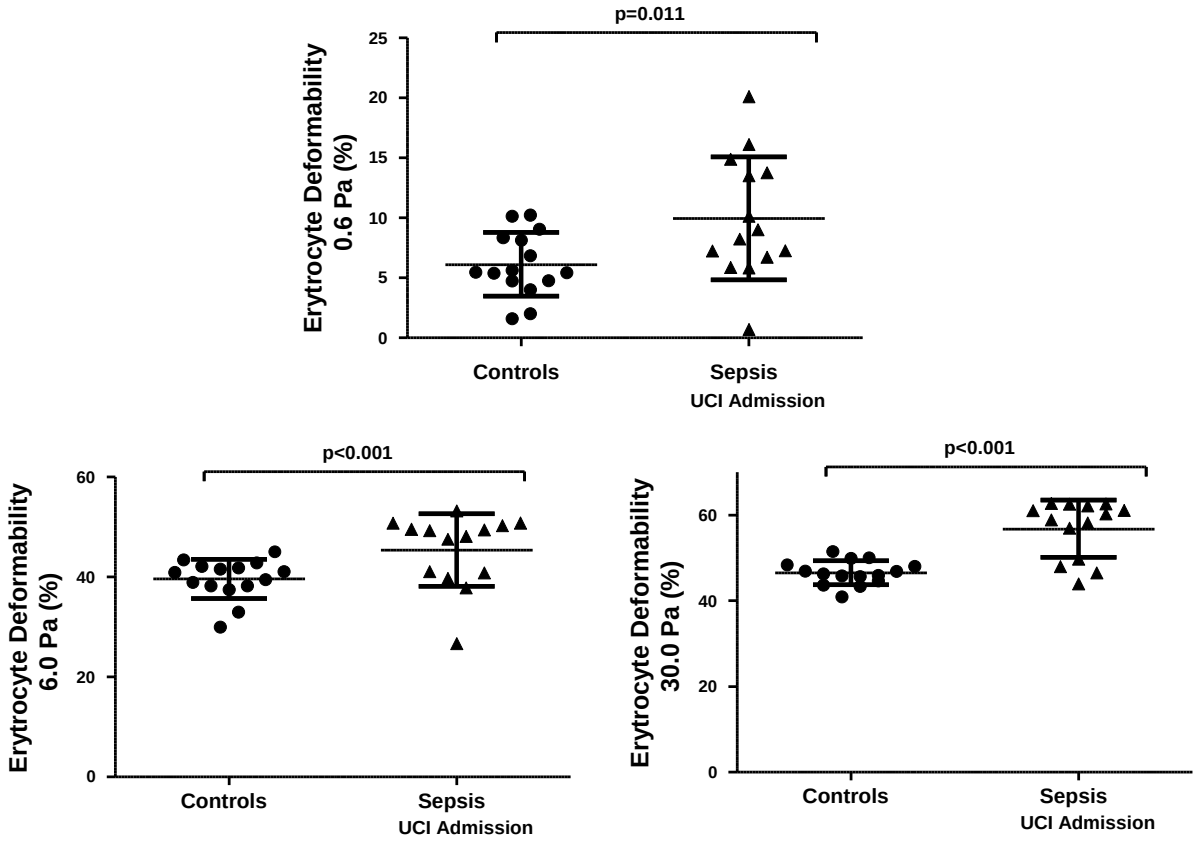

Figure 3.1 - Variations of erythrocyte deformability, at 0.6 Pa, 6.0 Pa and 30.0 Pa, between the groups of sepsis patients at UCI admission and of controls.

Concerning the values of erythrocyte aggregation, the values for 5s aggregation are slightly higher in patients with sepsis at UCI admission (Fig. 3.2). For the 10s aggregation, no significant difference was obtained between both groups.

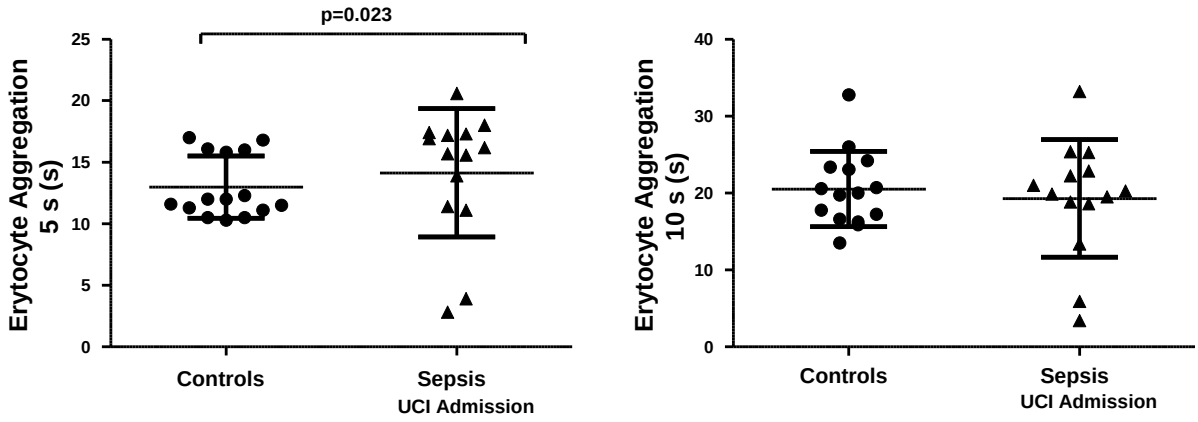

Figure 3.2 - Variations of erythrocyte aggregation, at 5 s and 10 s, between the groups of sepsis patients at UCI admission and of controls.

No significant difference was obtained for the NO and the GSNO values when comparing control group with sepsis admission (Fig. 3.3).

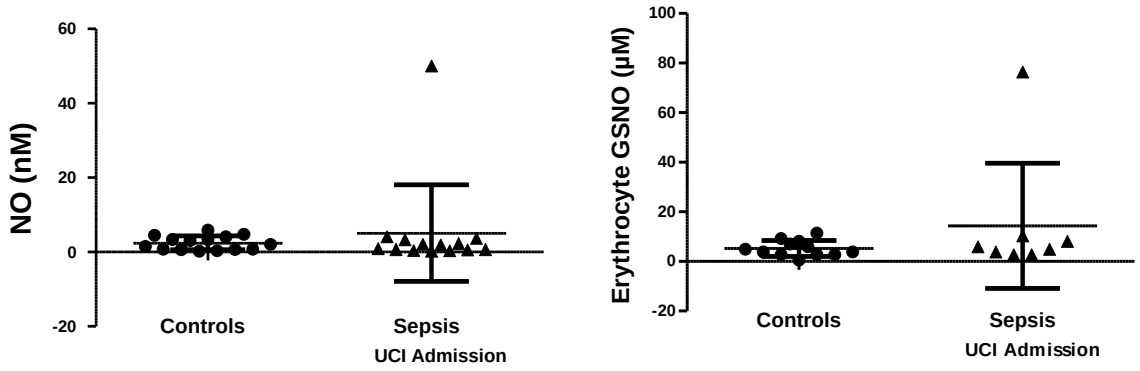

Figure 3.3 - Variations of NO and GSNO concentrations between the groups of sepsis patients at UCI admission and of controls.

3.4.3. – Longitudinal Variations in Sepsis Group

When looking at values of the hemorheological markers between sepsis patients at all the 4 time-points (Table A.2 in appendix) it is possible to verify that the erythrocyte deformability did not change over time at any shear stress rate ($F>0.48$, $p>0.71$; Fig. 3.4). However, some differences exist compared to the control values. In fact, after 24 hours the values of deformability for 6.0 Pa and 30.0 Pa are significantly higher than in healthy volunteers ($p=0.003$ and $p=0.003$, respectively). At 72 hours after UCI admission, sepsis patients present higher values of deformability, at all shear rates, than

controls (p=0.015, p=0.001 and p=0.001 for 0.6 Pa, 6.0 Pa and 30.0 Pa, respectively). At UCI discharge, differences were only found for 30.0 Pa (p=0.033).

The longitudinal variations in sepsis patients for erythrocyte aggregation were also non significant (F>0.65, p>0.34). The levels of erythrocyte aggregation for 5 s significantly differ from those of controls in patients with sepsis at 72 hours (p=0.045) and at UCI discharge (p=0.033).

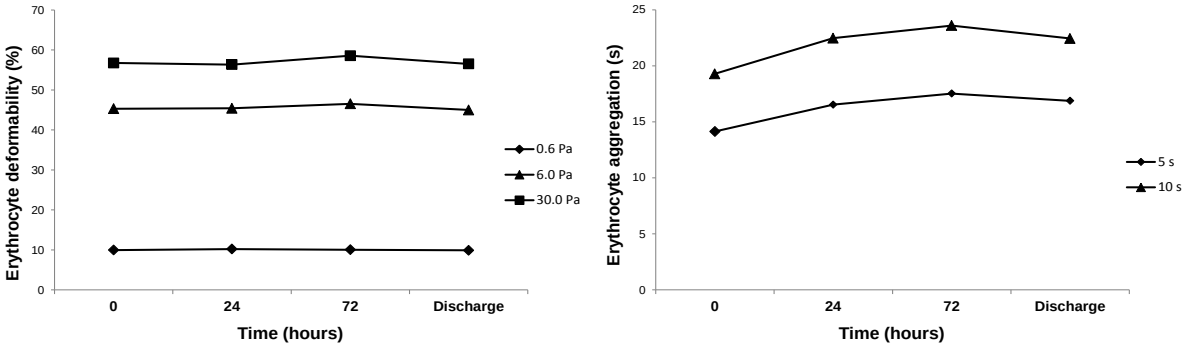

Figure 3.4 – Longitudinal variations of erythrocyte deformability (at 0.6 Pa, 6.0 Pa and 30.0 Pa) and aggregation (at 5s and 10s) in sepsis patients at four time-points.

Although the concentrations of NO in erythrocytes do not change over time (F=0.64, p=0.60; Fig. 3.5), the concentrations of erythrocyte GSNO do (F=3.27, p=0.036; Fig. 3.5). The concentrations of GSNO decrease significantly until 72 hours (p=0.049), reaching values lower than controls (p=0.015).

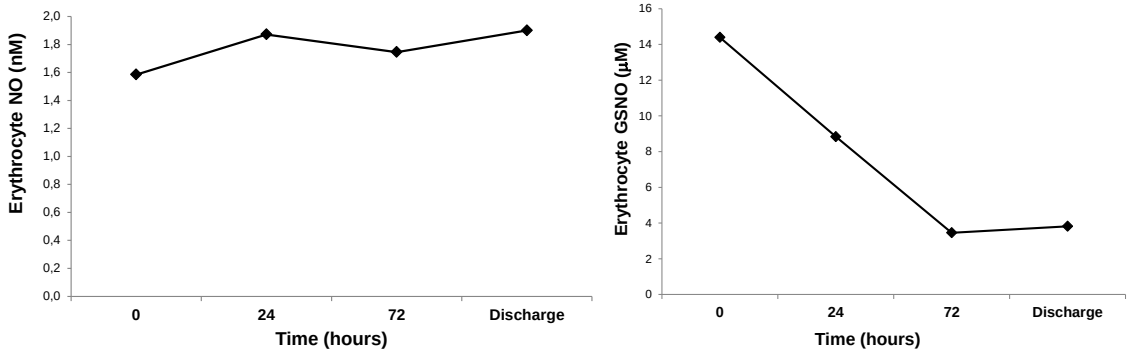

Figure 3.5 – Longitudinal variations of concentrations of NO and GSNO in erythrocyte deformability of sepsis patients at four time-points.

3.4.4. – Hemorheological Markers Association to Prognosis of Sepsis Group

Of the 14 patients that were enrolled in the ICU presenting sepsis, 4 died while being treated at the UCI.

When comparing the values between the two groups of sepsis patients it is possible to observe higher levels of 0.6 Pa erythrocyte deformability in non-survivor patients (Table A.3 in Appendix). On the other hand, 6.0 Pa and 30.0 Pa have higher values for the survivors group, except at 72 hours (Fig. 3.6). Both 5 s and 10 s aggregation have higher values for the survivors (Fig 3.6).

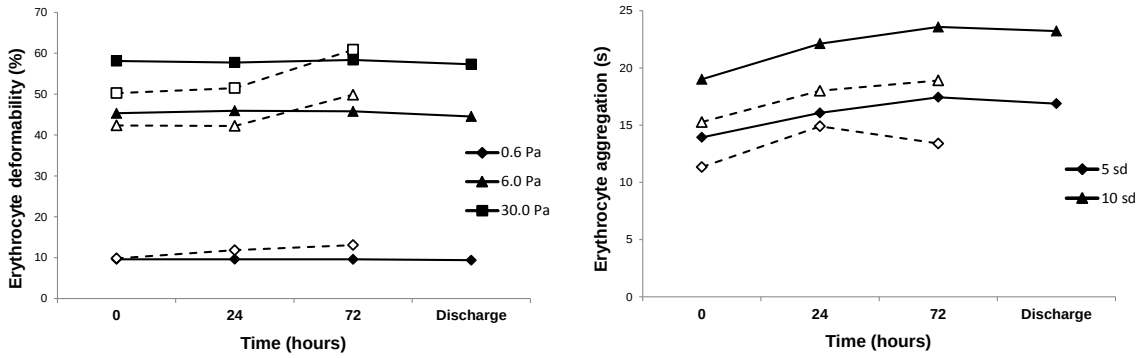

Figure 3.6 – Longitudinal variations of erythrocyte deformability (at 0.6 Pa, 6.0 Pa and 30.0 Pa) and aggregation (at 5 s and 10 s) in sepsis patients that survived (full line) and those that were dead before UCI discharge (dash line).

For the concentrations of NO and GSNO in erythrocytes, non-survivors sepsis patients at 24 hours present higher levels than survivors do (Fig. 3.7). For erythrocyte GSNO concentrations, the levels of patients that did not survive did not change much over time, contrary to the survivors (Fig. 3.7).

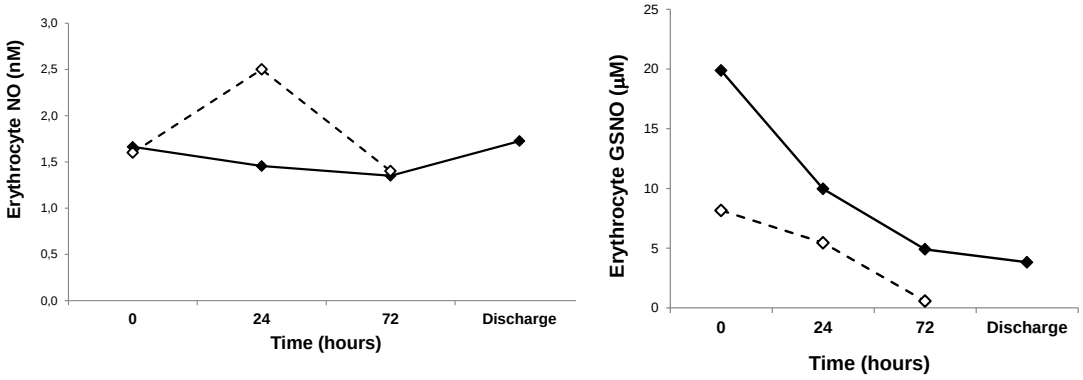

Figure 3.7 – Longitudinal variations of concentration of NO and GSNO in erythrocyte deformability of sepsis patients that survived (full line) and those that were dead before UCI discharge (dash line).

No statistical analysis was performed because of the small size of the sample, especially in what concerns the number of patients with sepsis that did not survive until discharge.

3.5. – Discussion

Sepsis is a very serious disease characterized by a systemic inflammatory response that starts with an infection. Our bodies release chemicals into the blood stream to fight the infection and this leads to an elevated inflammation. The number of people suffering from it has been increasing each year (Hall *et al.*, 2011).

Sepsis induces changes in microvascular properties related to vascular reactivity, leukocyte-endothelial cell adhesion and vascular leakage. Microcirculatory derangements like loss of capillary density also develop. These losses are translated into loss of surface area for gas exchange, maldistribution of blood flow and increased heterogeneity in the flow, which leads to disturbances in tissue oxygenation (Bateman *et al.*, 2001).

A decrease in erythrocyte deformability and an increase of erythrocyte aggregation have been observed in patients with sepsis (Bateman *et al.*, 2001; Baskurt *et al.*, 1998). Erythrocyte dysfunction, when induced by infection, has an important role in the behaviour of the immune response and can contribute to organ dysfunction in patients with sepsis (Hall *et al.*, 2011).

A previous study has shown an elevated band-3 tyrosine phosphorylation in circulating RBC in sepsis patients (Condon *et al.*, 2007). Band-3 is an erythrocyte membrane protein that affects directly cell shape, structure, function, flexibility and regulation of glucose metabolism, ion transport and cell lifespan (Zhang *et al.*, 2000). Changes in the proteins normal state originate changes in these features. Alterations of the band-3 may be linked to RBC dysfunction (Condon *et al.*, 2007). Rheological alterations that RBC suffer upon inflammation may contribute to blood congestion and impair oxygen exchange under septic conditions.

A study indicates that an erythrocyte subpopulation, consisting of 20% of circulating erythrocytes, shows a pronounced decrease in deformability when compared to the other cells (Condon *et al.*, 2003). This result indicates the possibility that only a portion of the RBC undergoes critical hemorheological changes that alter their deformability. More even, this study also demonstrates that decrease in erythrocyte deformability occurs in different degrees in a specific subpopulation.

Erythrocyte deformability was measured at three different shear forces: 0.6 Pa, 6.0 Pa and 30.0 Pa. Whole blood samples were taken from sepsis patients at four different times during their internment. Data obtained from the comparison between patients with sepsis at ICU admission and the control group showed that there really is a significant difference among the two groups for the three deformability parameters. These results match the theory that inflammation causes a disturbance in the hemorheological properties of erythrocytes - which become more rigid - and therefore causes change

in their deformability. Rigidified erythrocytes are a constant presence in blood circulation in sepsis, maybe because their amount is so high that mononuclear phagocytes don't have the capacity to phagocyte them (Powell *et al.*, 1993).

Interestingly, in sepsis patients, 24 hours after interment there are still significant differences among the deformability compared to the control group. The fact that sepsis is based on an acute inflammation spread out through the body that is very hard to fight and stop may explain why after 24 hours of treatment and monitorization there are still differences in erythrocyte deformability. Even after 72 hours of being admitted in the ICU the patient presents significant differences in deformability values. The fact that after so many hours deformability continues far from its normal values leads to the idea that even with medication and treatment the erythrocyte hemorheological parameters are still disturbed and inflammation is still happening in the body.

Even more surprising are the results of the comparison between the discharge and the control group, where significant differences are obtained once more for all the deformability measurements. Since the patients are being discharged from the ICU, deformability values were expected to be more similar to those from the control group. The fact that patients are being discharged from the ICU only means that they are no longer in a life-threatening situation, but inflammation may still be present in a lower intensity than at the acute phase.

No significant differences among the markers of erythrocyte deformability were observed when comparing patients at ICU admission and 24 hours after, which can reinforce the idea that 24 hours is a little window of time to make an inflammatory disease like sepsis regress. No differences were observed between the sepsis patients at ICU admission and 72 hours or at ICU discharge. Therefore, the difference among these time-points along with the initial assumption reinforces the results obtained from the comparisons with the control group.

Paradoxically, the three deformability measurements are the only markers whose differences are significant when evaluating the longitudinal variations of patients with sepsis. These results give the idea that sepsis patient's markers change over time, besides presenting different values from those of healthy patients. Considering that these patients were interned in the hospital and then discharged, these differences may translate the progression of the treatment – inflammation is being suppressed and therefore erythrocyte deformability undergoes changes.

An increase in erythrocyte aggregation is observed in sepsis. Aggregation is a complex mechanism, dependent of the surrounding medium composition and cellular factors such as the RBC membrane negative surface charge. Increased erythrocyte segmentation rate (ESR) is used as a criteria in diagnosis of sepsis. Elevated erythrocyte aggregation is the primary factor responsible for this increase in ESR, therefore it is directly linked to the sepsis condition. Increased erythrocyte

aggregation may be related to tissue perfusion problems. Aggregation appears to be affected mostly by plasma factors (Baskurt *et al.*, 1997).

Erythrocyte aggregation was measured in two elapsed times: during 5 seconds and 10 seconds after stopping the applied speed of $600s^{-1}$ during $10sec^{-1}$. A significant difference was obtained for the 5s measurement when comparing sepsis patients (at all time-points) with the control group, which correlates with the fact that erythrocyte aggregation is altered during sepsis.

RBC aggregates tend to form in low shear rates and may increase blood viscosity and reduce flow. With the low perfusion created RBCs damage may worsen and become more prominent (Baskurt *et al.*, 1997). The results obtained show that erythrocytes aggregate significantly in a short time – the 5s measurement. Given this, a significant difference for the 10s measurement was also expected. The fact that the 10 second measurement has no difference shows that erythrocytes somehow disaggregated after the first measurement and therefore have the same values of the healthy population. It is a curious fact that may occur due to the damage that erythrocytes suffer and that alters their physiology and consequent behaviour.

Oxidative damage to erythrocytes has been reported in sepsis patients and related to tissue ischemia and white blood cells. The damage is related to erythrocyte deformability and can indirectly affect their aggregation as impaired RBC deformability may decrease aggregation (Baskurt *et al.*, 1997).

The fact that there is a difference in aggregation between the sepsis group and the control group throughout time is consistent with the information obtained regarding erythrocyte deformability. This plus the fact that there is no significative longitudinal variations contributes to the idea that inflammation persists in patients body all through the treatment and even by the time of the discharge from the ICU.

Nitric oxide is a signalling molecule formed from L-arginine conversion by a family of 3 NOS enzymes. NO has a key role in the regulation of vascular tone and blood pressure. In normal conditions it works as a vasodilator. In sepsis, nitric oxide, along with superoxide anion and hydrogen peroxide, is released from activated phagocytes and may target erythrocytes causing a decrease in their deformability and membrane oxidation (Bateman *et al.*, 2001).

Inflammation causes an increase in levels of pro-inflammatory cytokines such as interferon-gamma (IFN-γ), which in turn leads to production of NO. Inhibition of NO synthesis can promote the resolution of shock and temporarily improve hemodynamic variables (Wang *et al.*, 2010). A high level of NO is a dangerous sign of septic shock (El-Sallab *et al.*, 2002).

In the present study, the NO in erythrocytes did not change over time in patients with sepsis, nor was it different from healthy individuals. These results go against the initially expected. The role of NO in sepsis is wide and well known and previous results have determined a significant difference in NO levels between sepsis patients and other groups. The fact that NO has been reported to modulate RBC deformability added to the significant differences obtained for the three deformability markers, reinforced the hypothesis that this marker should have shown differences between the groups (Bateman *et al.*, 2001).

The fact that erythrocytes scavenge NO when oxygen tension is high and release it when it is low (as happens in sepsis) is another reason why a significant difference was expected in this marker.

Cardiovascular abnormalities in severe sepsis are derived from the release of inflammatory mediators like NO and may contribute to shock. Studies have showed that excessive synthesis of inducible nitric oxide synthases (iNOS) leads to production of excessive NO and free radicals and results in hypotension and tissue injury in sepsis (Powell *et al.*, 1993). One possible explanation may be the existence of a compensatory mechanism so that NO produced by iNOS does not increase, resulting this way in a protective action against further injury.

Furthermore, it is known that alterations of NO derivatives inside the erythrocyte depend on both internal and external stimuli, like ACh (Saldanha *et al.*, 2013). Therefore, the inexistence of differences among groups for erythrocyte NO may be explained by the fact of NO has undergone chemical reaction and is present in the erythrocyte as other molecule.

Opposing the results from studies with a positive outcome when studying NO levels in sepsis, Terregino *et al.* (2000) did not find an increase of NO metabolites in sepsis patients. It is to note that in his study patients were presumed to have sepsis upon the arrival at the UCI and have not yet developed septic complications, which may have resulted in incorrect diagnosis.

GSNO is an endogenous low-molecular-weight nitrosothiol formed in the extracellular milieu that plays a critical role in NO signalling (Gaston *et al.*, 1993). Circulating erythrocytes contain S-nitrosohemoglobin (SNO-Hb) that results from the reaction of GSNO and hemoglobin. Researchers found out that the combination of SNO-Hb with glutathione results in relaxation of vessels (Spencer *et al.*, 2000). In vitro experiments showed that an NO exchange occurs between hemoglobin and GSNO (Romeo *et al.*, 2003). Therefore, GSNO is the main chariot in which biologically active NO is transported in peripheral blood (Liu *et al.*, 2004).

The fact that the only the comparison between the control and the 72 hours sepsis groups had any significant difference between the erythrocyte GSNO values was not expected at all. If the erythrocyte GSNO change was time related then the discharge group should have shown a significant difference as

well, as there had been even more time since the start of the treatment. The absence of difference may be due to GSNO leaving the cell, and therefore its quantification is not possible.

GSNO originates with the reaction of NO with GSH, which is an abundant molecule in the erythrocyte (Saldanha *et al.*, 2013). The lack of significative variance in GSNO levels may be because GSH was being formed in order to even the oxidative stress, or SNO-Hb, or even nitrates and nitrites.

Given the relation of NO and GSNO, and the fact that none of the groups presented a change in their NO values, the fact that only one group showed a change in the GSNO values was not a complete surprise. The results for both these markers may be related to the fact that the sample was of a small size.

4. – Conclusions & Implications

4.1. – Conclusions

The group of inflammatory diseases is vast. They have different mechanisms and cellular targets but are united under one biological process that is common to all: inflammation. This work focused on three such diseases – rheumatoid arthritis, acute myocardial infarction, and sepsis. All three of them arise big health issues, change the lives of thousands of people, are present all around the globe, and make governments spend a very large amount of money in order to treat them. Many studies have already been done in order to uncover the maximum possible that there is to know about them, but there is still a big road ahead. Prevention, efficient diagnosis and treatment are all essential not only for the patients´ wellbeing but also so that the medical teams can do the best job possible when treating these patients.

In this context, the identification of high-risk patients is of extreme importance. The present work aimed to study the relation of hemorheological parameters with acute inflammatory diseases. To achieve that four erythrocyte markers were analyzed – deformability (at 0.6 Pa, 6.0 Pa and 30.0 Pa) and aggregation (at 5s and 10s), NO and GSNO – in order to study their changes and behavior among the different groups of diseases. The fact that RBC features can be used as markers in diagnosis is a thought shared by many. The four markers used in this study can be strong candidates for this.

Patients from the STEMI group had their blood taken at two points: upon the admission in the hospital (at infarction onset), and a month later. The values from these two harvests were compared among each other in a longitudinal study, but also with a healthy control group, as well as with samples from patients suffering from rheumatoid arthritis (chronic inflammatory disease).

AMI patients at infarct onset present significant lower levels of erythrocyte aggregation (10s) compared to the controls, but no differences were observed for the other hemorheological markers. Furthermore, no longitudinal variations were verified for neither of the markers. Changes in erythrocyte aggregation, deformability, and NO have been previously observed in patients suffering from both diseases, however in the present study a possible effect of pre-event medication could influence the results.

A longitudinal evaluation was also performed in sepsis: upon hospitalization in the ICU, 24 hours, 72 hours later, and at the discharge. Changes in the erythrocyte deformability and aggregation (5s) were obtained compared to the controls. Although variations over time in sepsis patients were not observed for the majority of the studied parameters, the erythrocyte GSNO levels significantly

decreased at 72 hrs. This time-point also marks the changes between survivors and non-survivors for the studied hemorheological parameters, pointing out the importance of hemorheology in sepsis.

These results reinforce the relation between blood rheology and acute inflammation, as inflammatory processes have a direct action on erythrocytes properties, namely on the four markers that this study was based on. They also show that different conditions have different impacts on these markers, which can be related to the pathophysiology of the condition.

4.2. – Study Limitations

Obtaining the maximum number of blood samples in a short period of time was a difficulty that had to be overcome. It was a major concern as it is a sample based study, and thought the final work ended up with a decent amount of samples for a longitudinal study, it is still far from ideal in what concerns the statistical analysis. It takes a lot of coordination and commitment from the medical teams in order to retrieve blood from patients. The patients have to agree to donate the blood voluntarily, so it is also an obstacle as not everyone is willing to do so.

4.3. – Implications and Future Research

Changes in blood rheology (such as deformability and aggregation) have been observed in patients suffering from all three diseases (RA, STEMI and sepsis), so a further pursuit in the study of this topic is necessary. A bigger size sample is necessary in order to make the most accurate statistics possible. The group should ideally also contain the same sex ratio.

Parallel analysis of nitrites, nitrates, and glutathione can give more information regarding the four markers studied, and also expand the knowledge of their interrelation. Parallel studies of the molecular mechanisms of the inflammation are crucial for the understanding of the core of this process, as well as to help complement the information obtained when studying the markers.

More detailed clinical information of the patients will also improve the data obtained from every study as it can give a solid background to the researcher and help build a bigger picture of the pathophysiology of diseases.

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Appendix A

Table A.1 - Hemorheological and biochemical markers of the studied population of STEMI and AR patients and healthy volunteers.

	Control (n=15)	**RA** (n=25)	**STEMI**	
			Day 0 (n=15)	**Day 30** (n=9)
Age (y)	-	60±13	64±12	-
BMI (kg/m^2)	-	26±3	28±2.5	-
Clinical Analysis				
Total Cholesterol (mg/dl)	-	-	160±47	-
LDL-C (mg/dl)	-	-	109±40	-
HDL-C (mg/dl)	-	-	38±7.7	-
Triglycerides (mg/dl)	-	-	153±64	-
Albumin (g/l)	-	-	42±3.2	-
Glucose (mg/dl)	-	-	107±45	-
Hgb (g/dl)	-	-	13±1.9	-
Erythrocytes (x10^6/ul)	-	-	4.3±0.53	-
Hematocrit (%)	-	-	40±5.5	-
Leukocytes (x10^3/ul)	-	-	8±2.3	-
CRP (mg/l)	-	-	8.4±11	-
Troponin (ng/ml)	-	-	6.7±23	-
BNP (pg/ml)	-	-	142±230	-
Hemorheological parameters				
Deformability 0.6 Pa (%)	6±2.7	6±3	7±3.5	7±3.4
Deformability 6.0 Pa (%)	40±4	41±4	41±6	40±2.5
Deformability 30.0 Pa (%)	47±3	50±6	51±5.5	48±4.8
Aggregation 5s (s)	13±2.5	11±3	11±5.5	13±4
Aggregation 10s (s)	21±5	16±5.5	14±9.4 *	18±5.2
NO (nM)	2.4±1.9	1.1±0.95	2.5±3.4	1.4±1.8
GSNO (µM)	5.2±3.2	7.7±13	4.1±2.8	4.5±3.1

Values expressed as mean±sd. * $p<0.05$ versus CTR.

Table A.2 - Hemorheological and biochemical markers of the studied population sepsis patients and healthy volunteers.

	Control (n=15)	**SEPSIS**			
		UCI Admission (n=14)	**24h** (n=14)	**72h** (n=11)	**UCI Discharge** (n=4)
Age (y)	-	66±14	66±14	66±14	66±14
BMI (kg/m^2)	-	22.8±4.2	-	-	-
Baseline data					
TMP (^{o}C)	-	37±0.78	37±1.1	37±0.64	37±0.64
MAP (mm Hg)	-	75±18	83±14	93±22	94±12
HR (bpm)	-	101±22	94±23	98±134	73±4.2
Clinical Analysis					
Hgb (g/dl)	-	11714±6352	11771±5922	12156±6184	8250±7849
Leukocytes (/ml)	-	219214±208440	199286±174896	240667±214732	267500±195869
Platelets (/ml)	-	16±4.5	16±5.0	16±6.2	12
Time Prothromb. (seg)	-	32±19	32±28	23±20	22±30
APTT (seg)	-	563±112	646±219	483	488
Fibrinogen Clauss (mg/dl)	-	87±55	88±44	77±52	38±9.2
Urea (mg/dl)	-	2.2±2.2	1.9±1.4	1.6±1.8	3.9±5.1
Serum Creatinine (mg/dl)	-	28±33	53±83	19±10	-
Hemorheological parameters					
Deformability 0.6 Pa (%)	6.1±2.7	9.9±5.1 *	10±4.3	10±3.5 *	9.9±4.5
Deformability 6.0 Pa (%)	40±4.0	45±7.2*	45±6.8*	47±7.2 *	45±8.5
Deformability 30.0 Pa (%)	47±2.8	57±6.7 *	56±6.0 *	59±4.9*	57±5.5 *
Aggregation 5s (s)	13±2.5	14±5.2 *	16.5±6.2	17,5±3.1 *	17±2.1 *
Aggregation 10s (s)	21±4.9	19±7.7	23±11	24±7.1	22±5.3
NO (nM)	2.4±1.9	5.0±13	1.9±1.3	1.8±1.3	1.9±0.94
GSNO (µM)	5.3±32	14±25	8.8±10	3.5±3.3 *	3.8 *

Values expressed as mean±sd. * $p<0.05$ versus CTR.

Table A.3 – Longitudinal variations of hemorheological markers in sepsis patients according to the outcome at UCI discharge.

	Survivors				Dead			
	Admission	**24 h**	**72 h**	**Discharge**	**Admission**	**24 h**	**72 h**	**Discharge**
Deformability 0.6 Pa (%)	9.6±5.8	9.6±4.3	9.6±3.9	9.4±5	9.8±3.8	112±5.5	13	-
Deformability 6.0 Pa (%)	45±8.2	46±7.5	46±8.4	45±9.7	42±6.3	42±7.2	50	-
Deformability 30.0 Pa (%)	58.1±6	58±5.2	58±5.1	57±6.1	50±7.7	55±7.7	61	-
Aggregation 5s (s)	14±4.8	16±6.7	17±2.6	17±4.8	11±6.5	15±6.4	13	-
Aggregation 10s (s)	19±6.9	22±11	24±7.4	23±5.8	15±8.2	18±11	19	-
NO (nM)	1.5±1.6	1.5±1.1	1.4±1.0	1.7±1	1.6±0.6	2.5±1.2	1.4	-
GSNO (μM)	20±32	10±12	19±42	4.9±3.0	8.2	5.5±3.8	0.57	-

Values expressed as mean±sd

Appendix B – Materials and Reagents

List of the materials used in this study:

- Beakers (Normax)
- Cuvettes 1 ml, 3 ml
- Microtubes 1.5 ml, 2 ml (Eppendorf)
- Freezer at -80ºC
- Micro-pipettes (Gilson)
- Gloves
- Biofuge 15 Centrifuge (Heraeus)
- Magnet
- Myrenne Aggregometer MA-1 (Myrenne GmbH)
- Nitric Oxide Measuring System (Innovative Instruments, Inc.)
- Pipette Tips
- Refrigerator
- Rheodyn SSD (Myrenne GmbH,)
- TC Centrifuge (Sorvall)
- Syringes 3 ml
- Thermo Spectronic Genesys 10UV-VIS Spectrophotometer (Thermo Fisher Scientific)
- Vibrofix VF1 Electronic Shaker (IKA-Werk)
- Vacutainer blood collection tubes

List of the reagents used in this study:

- Acetylcholine 10^{-3}M (Sigma)
- Chloroform (Sigma)
- Dextean (Sigma)
- Ethanol 95%
- Griess Reagent (Invitrogen)
- $HgCl_2$
- Milli-Q Water (Millipore)
- NaCl 0,9% pH 7.0