Maria Inês de Jesus Freire Malho

Licenciatura em Engenharia Biomédica

Molecular characterization of the fibrinogen-erythrocyte interaction and its influence on cardiovascular pathologies by AFM-based force spectroscopy

Dissertação para obtenção do Grau de Mestre
em Engenharia Biomédica

Orientador: Prof. Dr. Nuno Correira Santos
Co-orientador: Prof. Dra. Filomena Arede Carvalho

Júri:
Presidente: Doutor Mário Forjaz Secca, Professor Associado, Universidade Nova de Lisboa
Arguente: Doutor Jorge Carvalho Silva, Professor Auxiliar, Universidade Nova de Lisboa
Vogal: Doutora Filomena Arede Carvalho, Professora Auxiliar, Universidade de Lisboa

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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To my loving family…

Acknowledgments

Reproducing the work of these last six months in words was a great challenge. However, this thesis also represents the end of a hard path to obtain this degree, for this, the next paragraphs are not easy to write either. I would like to thank everyone who guided me and accompanied me in the last five years. The write word at the write time may bring the strength in need.

First and foremost, I thank my MSc supervisor, Prof. Nuno C. Santos. Mainly for the opportunity he gave me to work in this great institute, but also for his absolute availability, not only to constructively discuss our results and methodologies, but also for giving me the chance to participate in great congresses and *IMM* courses. Furthermore, he created this fantastic team that made my working at *IMM* the most fulfilling experience I had until this date.

I would like to thank my course-coordinator, Prof. Mário Forjaz Secca, for his availability whenever I needed counseling during my time at *FCT-UNL.*

To Prof. Filomena A. Carvalho, to whom saying thank you will never be enough. Not only she taught me everything I needed to perform our essays, but also showed me great kindness and understanding whenever something did not go as expected. Her trust in my work was very important for me.

As for my college, Ana Filipa Guedes, I would like to thank all her friendship, her experience and keen eye that helped me so many times. Working with her was very gratifying.

ACKNOWLEDGMENTS

To the laboratory technician, Teresa Freitas, I thank for her assistance every time I needed. Not only she taught me great technical lab skills, but also always offered me her great kindness and care.

I would like to thank our collaborators Dr. Luís Sargento from *Hospital Santa Maria, Centro Hospitalar Norte*, who has been sending his patient's blood for our research; and Dr. Alisa Wolberg, from the School of Medicine's Department of Pathology and Laboratory Medicine, North Carolina University, for sending us her mouse's fibrinogens.

I would like to acknowledge to the *Instituto de Medicina Molecular*. Without its great conditions, this work would never be possible. This project (PTDC/QUI-BIQ/119509/2010/SFRH/BD/84414/2012) has been funded by Fundação para a Ciência e a Tecnologia – Ministério *da Ciência, Tecnologia e Ensino Superior (FCT-MCTES, Portugal)*, and *Fundação Calouste Gulbenkian.* For that, I am very grateful for their fundamental collaboration.

As for my faculty, I would like to thank *Faculdade de Ciências e Tecnologias* from *Universidade Nova de Lisboa* for providing me great knowledge, and the opportunity to work at *IMM* for my master's thesis.

To all my colleges at IMM I am grateful. They turn our working days very pleasant, with a very familiar environment.

For all the love and support, I thank my old friends Guilherme Geraldes, Inês Martins, Filipa Fouto and Ângela Paradela; and to my not so old friends Joana Nobre and Mafalda Guerra. A special thanks to Catarina Monteiro, who had to learn lots about this project for the time she spent with me. To my great far away friends Gonçalo Costa, Maria João Silva, Priscila Melo, Helena Ormieres, and Rosana Pinto, I thank all their friendship. Distance means nothing when it comes to them.

I would also like to thank my family who always believes in me. Their support has always been unconditional. Their understanding is the most important thing they could give me. For that, I dedicate this thesis to them.

Last but not least, I would like to thank Miguel Coimbra. It seems like he entered my life on the right time, and helped me through all this process. His love and friendship were essential to keep me on going.

Abstract

Cardiovascular diseases are the leading cause of death worldwide, and it is predicted that they will continue to be so, with an increasing number of deaths, mostly from heart diseases and strokes, which is expected to reach 23.3 million by 2030. Hence, it is pivotal that more studies in cardiovascular pathologies are developed.

The use of nanotechnologies in Medicine has become very promising in multiple areas, like drug delivery, gene therapy, tissue engineering and diagnoses, with the latter being the core of this project. It follows other essays developed at *Instituto de Medicina Molecular*, with the intended purpose of further studying the fibrinogen marker for erythrocytes while investigating the importance of fibrinogen blood concentration for cardiovascular diseases.

The main method used to achieve these goals was atomic force microscopy. This equipment has proved to be very promising as a highly sensitive and low operational cost diagnostic nanotool for the near future. It allows detailed understanding of the molecular mechanisms involved on the development of a disease, which is extremely relevant for the future development of new treatments.

The study of fibrinogen and erythrocyte aggregations became a matter of concern since it was found that patients who had suffered from acute cardiovascular problems had higher fibrinogen concentration in their blood. From this point on, it was

important to prove if and how the concentration of this protein is involved in the cardiovascular problem, in order to achieve a new type of functional diagnosis.

During the experimental time of this project, a thorough molecular study of the interactions between fibrinogen and its specific erythrocyte membrane receptor was performed with two different approaches:

i. A follow-up of cardiac insufficiency patients of two different etiologies: ischemic and non-ischemic. Our results show that ischemic patient erythrocytes have the highest binding force with fibrinogen, indicating that these patients represent the group with major cardiovascular risk.

ii. A fibrinogen γ chain mutant was attached to the AFM tip with the intent of studying its interaction with rodent erythrocytes. We understood that erythrocyte binding was more affected than with platelets. Therefore, we suggest that this mutant may be a marker of great importance in the characterization of the erythrocyte receptor, once it reveals that erythrocyte's binding force decreases in the absence of the $\gamma^{390-396A}$ sequence.

This new data not only contributes to the prior goal of characterizing the erythrocyte receptor for fibrinogen, but also to unveil the effect of cardiac insufficiency on erythrocyte aggregation.

Keywords: Atomic force microscopy; Force spectroscopy; Fibrinogen; Erythrocytes; Cardiac insufficiency; γ' Fibrinogen.

Resumo

As doenças cardiovasculares são a principal causa de morte a nível mundial, prevendo-se que esta condição assim permaneça, nomeadamente por doença coronária e enfarte, até atingir o número alarmante de 23,3 milhões de mortes em 2030. Posto isto, torna-se mandatório desenvolver mais a investigação em patologias cardiovasculares.

Em Medicina, o uso de nanotecnologias tem vindo a revelar-se muito prometedor em várias áreas como a administração controlada de fármacos, terapia por genes, engenharia de tecidos e diagnóstico. É neste último ponto que este projecto se enquadra, aparecendo na sequência de outros trabalhos desenvolvidos no Instituto de Medicina Molecular, com o objectivo comum de aprofundar o estudo do receptor do fibrinogénio na membrana do eritrócito, estudando paralelamente a importância da concentração de fibrinogénio no sangue como factor de risco para doenças cardiovasculares, como resposta à necessidade de criar novas terapias e métodos de diagnóstico para estas patologias.

Para tal, utilizou-se maioritariamente a técnica de espectroscopia de força por microscopia de força atómica (AFM). Este equipamento de alta sensibilidade e baixo custo de utilização permite uma melhor compreensão dos mecanismos moleculares envolvidos no desenvolvimento de uma patologia, revelando-se prometedor no desenvolvimento de novos métodos de diagnóstico funcional.

O estudo da agregação do fibrinogénio à membrana eritrocitária tornou-se de grande interesse desde que foi feita a associação entre indivíduos vítimas de

episódios cardiovasculares agudos e o elevado nível de fibrinogénio no sangue. Desta forma, é importante conseguir provar que a concentração desta proteína no sangue está relacionada com este tipo de problemas, por forma a conseguir um novo tipo de diagnóstico.

Durante o tempo experimental deste estudo, foi realizado um estudo acerca das interacções entre o fibrinogénio e o seu receptor específico na membrana dos eritrócitos através de duas diferentes abordagens:

i. Um acompanhamento de doentes insuficientes cardíacos de duas etiologias diferentes: isquémicos e não-isquémicos. Os resultados demonstraram que os eritrócitos dos pacientes isquémicos são os que apresentam maior força de rotura entre a ligação fibrinogénio-eritrócito, sugerindo que estes pacientes são aqueles que possuem o maior risco cardiovascular.

ii. Ligou-se um mutante da cadeira γ do fibrinogénio à ponta do AFM no intuito de estudar a sua interacção com os eritrócitos de ratinho. Os resultados mostraram que a ligação aos eritrócitos foi mais afectada que às plaquetas, sugerindo que este mutante pode ser um marcador muito elucidativo na caracterização do receptor dos eritrócitos, na ausência da sequência $\gamma^{390-396A}$.

Todos os resultados obtidos contribuem não só para o objectivo primordial de caracterizar o receptor do fibrinogénio na membrana dos eritrócitos, como irão contribuir para o descortínio do efeito da insuficiência cardíaca na agregação eritrocitária.

Palavras-chave: Microscopia de força atómica; espectroscopia de força; Fibrinogénio; Eritrócitos; Insuficiência cardíaca; Fibrinogénio γ' .

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Abbreviations, Symbols & Concepts

AFM	Atomic Force Microscopy
APTES	3-aminopropyl-thriethoxylan 99%
BMI	Body Mass Index
CAD	Coronary Artery Disease
CVD	Cardiovascular Disease
EF	Ejection Fraction
EGF	Epidermal Growth Factor
EPCR	Endothelial Protein-C Receptor
FDP	Fibrinogen Degradation Products
Fgn	Fibrinogen
HDL	High-Density Lipoprotein
HF	Heart Failure
HMW	High Molecular Weight
hsCRP	High sensitivity C-reactive Protein
ICAM	Intercellular Adhesion Molecule
IHD	Ischemic Heart Disease
IL	Interleukin
LAD	Leukocyte Adhesion Deficiency
LDL	Low-Density Lipoprotein

LDV	Laser Dopler Velocimetry
LMW	Low Molecular Weight
MI	Myocardial Infarction
MIDAS	Metal-Ion-Dependent Adhesive Site
NMR	Nuclear Magnetic Ressonance
NO	Nitric Oxide
PALS	Phase Analysis Light Scatter
PBS	Phosphate Buffered Saline
PRP	Platelet-rich Plasma
PSI	Plexin-Semaphorin-Integrin
PTB	Phosphotyrosine-Binding
RBC	Red Blood Cells
SNP	Single-nucleotide Polymorphisms
TD	Transmembranar Domain
TF	Tissue Factor
WLC	Worm-like-chain
WT	Wild-type
ZP	Zeta Potential
$E_{Thermal}$	Thermal Energy in Joules
F	Force in Newtons (N)
k	Spring Constant in N/m
k_B	Boltzmann Constant (1.38×10^{-23} J/K)
T	Absolute Temperature
Factor II	Prothrombin
Factor IIa	Thrombin
λ	Wavelength in meters

$\mathbf{Z}$	Zeta Potential in Volts
$\Delta\zeta$	Zeta Potential Variation

Concepts

Atheroma – Accumulation and inflammation in artery walls, mostly by macrophage cells, debris, lipids, calcium and fibrous connective tissues.

Buffy-coat – Intermediate layer between erythrocytes and plasma. It is comprised by leukocytes and platelets.

Coagulation Cascade – Process in which blood coagulates in order to stop a bleeding, i.e. to maintain hemostasis.

Dyspnea – Shortness of breath.

Glanzmann Thrombasthenia – Dysfunction in the platelet $\alpha_{IIb}\beta_3$ integrin, which results in an autosomal recessive disease causing severe bleeding disorders.

Hematocrit – Volume fraction of red blood cells in the blood.

Hemostasis – It's the action of suspending an hemorrhage, avoiding huge blood loss and vessel damage, while assuring the blood fluidity, preventing thrombi formation.

hsCRP – Blood protein synthetized in the liver as a response to inflammation (acute-phase protein).

Juctional Epidermolysis Bullosa (JEB) – JEB is one of the major forms of epidermolysis bullosa, a group of genetic conditions that cause the skin to be very fragile and to blister easily.

K_3EDTA – Anticoagulant. Blocks blood coagulation cascade by chelating calcium ions of the sample.

Kindlins – Essential regulators of integrin signaling and cell-matrix adhesion.

Oxysterols – Oxidized derivatives of cholesterol.

Prothrombotic – Describes any agent or condition that leads to thrombosis.

Rouleau – (plural: rouleaux) Erythrocytes structures (stacks) that form due to their discoid shape. They overlap each other.

Silanization – Covering of a surface through self-assembly with organofunctional alkoxysilane molecules, due to their hydroxyl group.

Talin – A cytoskeletal protein found in lymphocytes and cell-cell contact, that is capable of linking integrins to actin cytoskeleton.

1

CHAPTER 1 – Introduction

1.1. Background

The atomic force microscope is the instrument that represents the main tool for our investigation in this project. It is not only an excellent tool for recording the surface topography of a sample, but it also shows an undeniable relevance in biological studies, being a good mean for studying the strength of chemical bonds, the elastic and mechanical properties of macromolecules and the intermolecular interactions at the single molecular level.

As for the study's target, this project stands in the cardiovascular diseases research through blood cells and its interaction with the blood coagulation protein, fibrinogen. Fibrinogen plays a central role in coagulation and thrombosis. It is a high molecular weight plasma adhesion protein and a biomarker of inflammation [1, 2].

There is a growing number of reports evidencing that increased fibrinogen plasma concentration is a significant risk factor for various cardiovascular and cerebrovascular disorders. Until the recent publication of Carvalho *et al* [3],the prevailing hypothesis for the mechanism of fibrinogen-induced erythrocyte hyperaggregation was that it could be caused by a nonspecific binding mechanism, despite the published data on the changes in erythrocyte aggregation during hypertension pointing to the existence of other mechanism(s) [3].

Based on force spectroscopy measurements, the group at IMM reported the existence of a single-molecule interaction between fibrinogen and the unknown receptor on the erythrocyte membrane, with a lower but comparable affinity relative to

platelet binding [3]. The fibrinogen-platelet binding, essential for coagulation, depends on the platelet membrane receptor glycoprotein $\alpha_{IIb}\beta_3$. This publication also started the characterization process of the erythrocyte receptor. This receptor is influenced, although not as much as platelets, by calcium and eptifibatide (the $\alpha_{IIb}\beta_3$ inhibitor). Through Glazmann thrombasthenia patients studies (a rare hereditary bleeding disease caused by $\alpha_{IIb}\beta_3$ deficiency) which had the β_3 gene mutated, Carvalho *et al.* demonstrated that the erythrocyte receptor for fibrinogen should be a β_3-like.

In 2012, the same research team used an identical AFM methodology, complemented with fluorescence spectroscopy and zeta-potential measurements, to assess the changes on fibrinogen-erythrocyte interaction upon *in vivo* erythrocyte ageing [4]. Their results indicate that, when aged, erythrocytes bind less frequently, though with similar force to fibrinogen, suggesting that young erythrocyte population is the main responsible for some cardiovascular diseases associated with an increase of the fibrinogen content in the blood, which can disturb its normal flow.

Hereupon, we can say that this thesis reports a project which continues this research line.

1.2. Objectives

This project aims to acquire new insights on the identification of a number of pre-thrombotic markers for the prevention of cardiovascular diseases. To this end, studies of fibrinogen-erythrocyte interactions in cardiac insufficiency patients were performed with atomic force microscopy (AFM). All the results were compared with those obtained from healthy donors, so it can be understood if the reduction of erythrocyte-erythrocyte interactions mediated by fibrinogen is associated with the reduction and/or prevention of cardiovascular pathologies.

Moreover, the project also intends to complement previous studies of the erythrocyte membrane receptor for fibrinogen. To reach this goal, we studied the interaction between mouse erythrocytes and a natural fibrinogen γ-chain mutant via atomic force spectroscopy and Zeta potential measures, comparing those results with a wild type fibrinogen.

1.3. Thesis Outline

This thesis is comprised of six chapters.

In the first chapter a brief introduction about previous work preceding this work is made, followed by our general objectives for these experiments.

The second chapter describes the techniques and laboratorial procedures we followed for each assay. It is given a greater importance to the AFM-based force spectroscopy, as it is in this methodology that this project is based on.

Chapter three addresses several important topics that the reader should have access in order to completely understand the contents of this paper.

The fourth chapter's contents are the cardiac insufficiency assays. Firstly the cardiovascular diseases in question are described and then the methodology followed. The results and consequent conclusions are presented next.

In the fifth chapter we described the used γ-mutant fibrinogen before explaining our methodology. Then, the results and conclusions are given in the two final subchapters.

Finally, chapter six gives general conclusions about the developed work and aims for future work.

2

CHAPTER 2 – Methodologies

This chapter describes all the essential laboratory procedures we had to do in order to perform our studies. A short introduction to the AFM and Zeta potential techniques is made.

2.1. AFM – Atomic Force Microscopy

Basic Principals

The forces involved in biological processes are crucial for their understanding, which results in the need of developing a technique capable of analyzing forces at the single-molecule level. Atomic force microscopy-based force spectroscopy became a fine choice for their measurement due to its piconewton resolution. This equipment has also been progressively accepted in biological and biomedical research, as it generates high resolution images and allows the capture of transient moments. It is also used in the characterization of modified and unmodified surfaces for use in medicine and implants [5].

This microscope does not require lenses, light source to illuminate the sample or an eyepiece to look through, even though some models have an optical microscope incorporated. The main components of the AFM are its head – where the sample and optical assembly stand – and controller [5].

To understand how this equipment operates, the analogy given in Figure 1 will be considered.

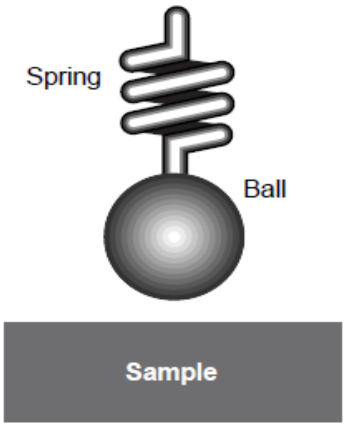

Figure 1. "Small ball on a weak spring" model [6].

The spring represents the cantilever and the ball the tip at the end of the free end of the cantilever. The cantilever movements are measured by sensitive optical methods. The tip and the sample are linked to each other by piezoelectric ceramics [6]. The cantilever is attached to a rigid substrate that can be held fixed. Then, it moves depending on the tip-sample interaction (Figure 2) at the tip: if it is attractive it deflects towards and if it is repulsive it deflects away from the sample.

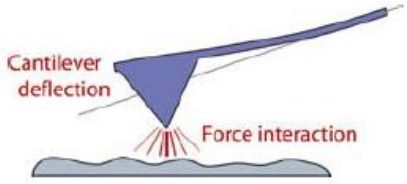

Figure 2. Interaction between tip and sample [7].

In Figure 3, a common AFM assembly is represented. The sample is mounted on a surface that can move in the x, y and z directions, typically using a piezoelectric actuator.

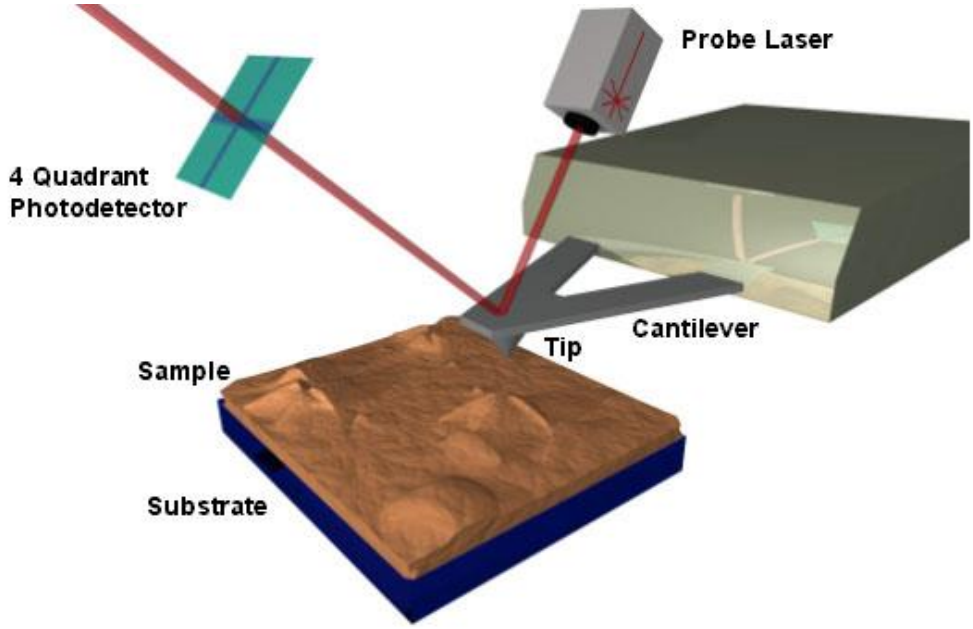

Figure 3. Schematic diagram of a common implementation of an AFM. The essential elements of an AFM are: a probe (tip) attached to a spring (cantilever); a means of measuring deflections of the spring (probe laser and photodiode); a sample; and a mean for moving the sample and probe relative to each other (piezoelectric tubes). Image taken from the Barret Group Website [1].

The cantilever deflection is detected by an optical lever, in which the movements of a laser reflected off the cantilever are detected by an optical detector, a split segment photodiode. The deflection data is passed to a computer controller that provides appropriate feedback to the stage and collects data [5].

The forces are calculated by the Hooke's Law of elasticity (Equation 1), with the deflection of the lever being the length and the cantilever stiffness the spring constant:

$$F = k \times d \qquad (1)$$

***F* represents the calculated force, *k* is the spring constant and *d* is the cantilever's deflection.**

Force spectroscopy

AFM-based force spectroscopy is the name given to the AFM quantification of the inter and intramolecular forces of interaction needed to separate the microscope tip from the sample, with piconewton resolution [5, 8].

[1] http://barrett-group.mcgill.ca/tutorials/nanotechnology/nano02.htm, accessed on September, 2013.

In this mode, the base of the cantilever is moved in the vertical direction towards the surface using the piezo and then retracted again. During the motion, the cantilever's deflection (and other parameters) is measured. The repetition of this process enables force-distance curves recordings. These curves represent the vertical cantilever deflection and include the forces measured as the probe approaches the sample and is retracted to its starting position [6].

Approaching Forces

As the cantilever approaches the sample surface, there are two phases: in the first one, the forces are too weak to give a measurable deflection, Figure 4,①; in the second phase, the attractive forces (usually Van der Walls and capillary forces) overcome the cantilever spring constant and the tip gets in contacts with the surface, Figure 4, ②.

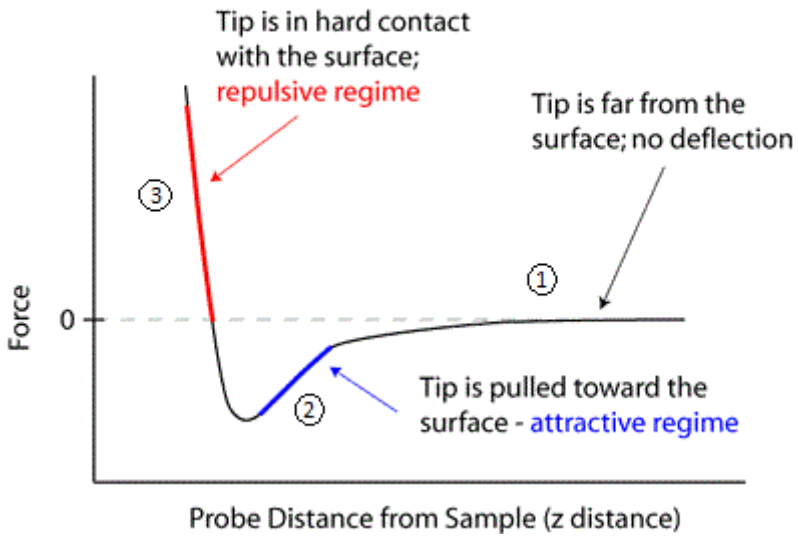

Figure 4. Approach/retraction cycle. Force/vertical cantilever deflection.

Retraction Forces

The contact between tip and sample remains while the distance between the base and the sample is such that the cantilever deflects due to repulsive forces, Figure 4, ③. As the cantilever is retracted from the surface, the tip remains in contact, and the cantilever suffers deflection in the downward sense, until its force is enough to break the tip/sample bond. Figure 5 represents the cantilever's deflection during approaching/retraction cycle.

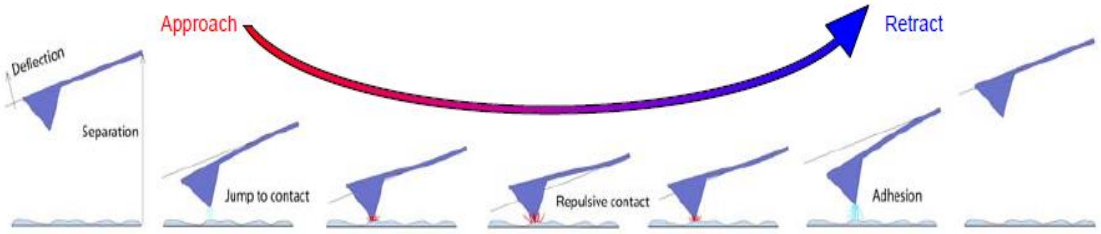

Figure 5. Approach/Retraction cycle. This schematic represents the vertical deflection of the tip during force spectroscopy [7].

Functionalized Tip

The force spectroscopy is made in liquid environment, with buffer solution, in order to mimetize the physiological medium.

To study an interaction between the sample and the tip, it is necessary to attach the case study biomolecule to it. This link must be stronger than the force that is going to be measured, avoiding detachment, but loose enough to allow the molecule to change its conformation.

2.1.1. AFM force spectroscopy tips

The tips and parameters that were used were the same as described in [3]. Force spectroscopy measurements were performed using fibrinogen functionalized OMCL TR-400-type silicon nitride tips (Olympus, Tokyo, Japan) (Figure 6).

Figure 6. OLYMPUS OMCL TR-400-type silicon nitride tips. Images taken from OLYMPUS's catalogue. The image on the left is a tip's upper-side view. The image on the right is an image taken by the AFM's video camera where the cantilever's contact with the cell is represented.

The softest triangular cantilevers, with a tip radius of 15 nm and a resonant frequency of 11 kHz, were used. The spring constants of the tips were calibrated by the thermal fluctuation method, resulting in values of 19±7 mN/m.

2.1.2. AFM tip functionalization for AFM force spectroscopy measurements

For the functionalization, AFM silicon nitride tips were cleaned with an intense ultraviolet light for 15 minutes. After cleaning, the tips were silanized in a vacuum chamber with 30 µL of 3-aminopropyl-triethoxysilane (APTES) and 10 µL of N,N-diisopropylethylamine for 1 hour in an argon atmosphere, to be coated with a self-assembled monolayer of amines. After 1 hour, the probes were rinsed with fresh chloroform and dried with nitrogen gas. The amine-terminated AFM probes were then placed in glutaraldehyde solution 2.5% (v/v) for 20 minutes and washed three times with PBS (phosphate buffered saline) pH 7.4.

Finally, the tips were placed in a fibrinogen solution to attach the fibrinogen molecules to the AFM tip. The fibrinogen sample in study is used at a concentration of 1 mg/mL for a 30 minute incubation. The functionalized fibrinogen tips can be stored in PBS buffer, but not for more than 24 hours after finishing this procedure

2.1.3. Tip calibration

Many authors have noted that the spring values indicated by the manufacturer are incorrect, since cantilever manufacturers use wide tolerances in the specified values of the force constant of cantilevers [9]. Therefore, before every AFM force spectroscopy measurements with a new tip, calibration must be performed in order to obtain the tip's spring constant.

The method JPK uses to calibrate the tip is the widely-accepted thermal method. This method returns a very reliable spring constant value in liquid environment, which causes disturbances that interfere with the thermal vibrations of the cantilever. The thermal environment of the cantilever is known and the deflection of the cantilever can be measured accurately, so the balance between them can be used to calculate the spring constant.

After loading the sample in the AFM and adjusting the AFM laser into the tip of the cantilever (the laser is well placed when the signal is at its maximum), some curves were measured in the poly-L-lysine (the glass slides' coat). The chosen curve must be a clean one, meaning no cell can be in contact with the tip (Figure 7). Thereby, we can measure the slope of the curve, which corresponds to the cantilever's deflection. Then,

the deflection sensitivity can be measured in nm/V. This voltage corresponds to the applied voltage to impede hysteresis effect [9].

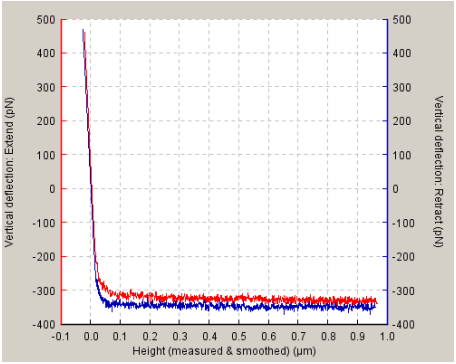

Figure 7. Calibration curve. The overlapping curves show that there is no interaction with fibrinogen.

Then, by vibrating the cantilever with no point of contact, we can measure its' resonance peak as a simple harmonic oscillator. The area under the resonance peak curve is used as a measure of the energy in the resonance. Following the equipartition theory [7] (Equation 2) we know the system's energy, which has to be equal to the thermal energy due to the absolute temperature of the system:

$$E_{Thermal} = \frac{1}{2} k_B T \qquad (2)$$

$$\frac{1}{2} k_B T = \frac{1}{2} k \langle q^2 \rangle \qquad (3)$$

Where $E_{Thermal}$ is the thermal energy in Joules, k_B is the Boltzmann constant, T is the absolute temperature in Kelvin and q is the cantilever's deflection. k represents the sought value of the spring constant in N/nm (Newtons per nanometer).

For further information about this method, please check references [7, 9].

2.1.4. Blood cells deposition for AFM studies

500µL of platelets or erythrocytes suspension were placed on a clean poly-L-lysine coated glass slide surface and allowed to deposit for 30 minutes.

Nonadherent cells were removed by two sequential washes with 1mL of buffered saline glucose citrate supplemented with calcium chloride (BSCG buffer with $CaCl_2$) 1mM. Then, the cells were loaded into the AFM and allowed to equilibrate in this buffer for 15 min before force spectroscopy measurements.

2.1.5. AFM force spectroscopy measurement

Molecular recognition was searched by intermittently pressing the cantilevers on different points of the cells adsorbed on the glass slide, 150 times per cell. The distance set between cell and cantilever was adjusted to maintain an applied force of 1 nN before retraction in every contact. Data collection for each force-distance cycle was performed at 2 µm/s, leading to a loading rate of 4 nN/s.

Each approach and retraction from a cell produces two curves as represented below.

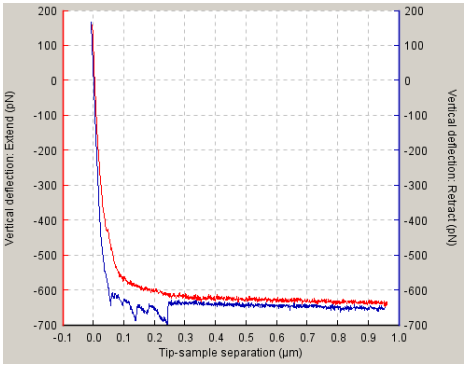

Figure 8. AFM approaching/retraction curve. The red line is the attraction curve and the blue line is the retraction curve.

2.1.6. AFM force spectroscopy curves analysis

Force curves were analyzed using the *JPK Data Processing v.4.2.27* (JPK Instruments, Berlin, Germany).

Each experiment was performed at least twice, each time on different samples and with different functionalized tips.

Histograms of the (un)binding forces of each studied fibrinogen-cell complex were constructed choosing the ideal bin size to achieve the best fitted Gaussian model peak forces, using *Origin 8* software. Selected binning sizes had 6 pN. Force rupture values ranging between 0 and 10 pN were considered to represent noise, artifacts or nonspecific interactions. According to this, the values up to 10 pN were neglected in data presentation and analysis. From each histogram, the most likely single fibrinogen molecule rupture force can be determined by fitting the distributions of the rupture forces with the Gaussian model. The maximum values of the Gaussian peaks represent a single-molecule-based statistical measure of the strength of the molecular bond.

From the collected data, not only we discover the rupture forces of the fibrinogen-cell complex and their rupture length, but also their frequency of binding. The rupture force values are defined as the force necessary to break the bond between the fibrinogen's ligand and the cell receptor. Rupture length values are defined as the distance between the cell-AFM tip contact point and the height value at the moment of the bond break. The percentage of binding represents the frequency adhesion-rupture events, calculated by the number of measured curves and the number of binding events.

2.2. Zeta Potential

In order to understand the potential stability of a colloidal sample, Zeta potential (ZP, ζ) measures were performed.

2.2.1. Basic Principals

Zeta Potential represents the potential within a particle when exposed to an electric charge. It is calculated by determining the Electrophoretic Mobility of the sample performing an electrophoresis experiment and measuring the velocity of the particles using the M3-PALS measurement technique, which is a Malvern's patented combination of Laser Doppler Velocimetry (LDV) and Phase Analysis Light Scatter (PALS). Electrophoretic mobility obtained was used to calculate Zeta-potential through the Smoluchowski equation [4]:

$$\zeta = \frac{4\pi\eta u}{\varepsilon} \qquad (4)$$

Where u represents the electrophoretic mobility, η the viscosity of the solvent, and ε its dielectric constant.

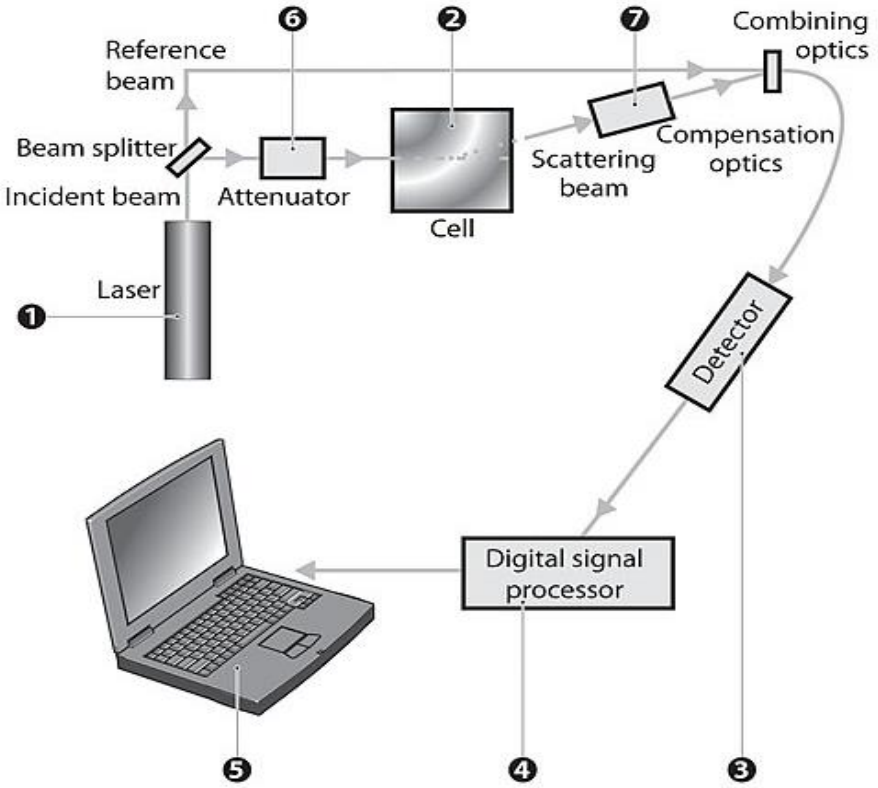

Figure 9. Optical configuration of the Zetasizer Nano series for Zeta potential measurements. Image taken from the Zetasizer Nano series's user manual.

The figure above, Figure 9, represents the procedure sequence that is necessary to measure the ZP value.

❶ is a laser that serves as a light source to illuminate the particles within the sample in ❷. The laser light is split in two beams: the reference beam and the incident beam. The incident beam interacts with the sample, suffering a 17º scattering (❼), and is subsequently combined with the reference beam. This relation is detected (❸), yielding a signal with a rate of fluctuation proportional to the speed of the particles during electrophoreses that is analyzed by a digital signal processor (❹) [10].

2.2.2. Zeta-potential sample preparation

The method adopted for Zeta-potential sample preparation was the same used in [4]. After blood cells isolation, 3.5 µL of erythrocytes were diluted in 10 mL of BSGC buffer, setting the hematocrit (kept constant during the experience) to 0.035%. The measurements were performed in the absence and presence of different soluble fibrinogen concentrations (0, 0.025 and 0.1 mg/mL) in the blood dilution. The

suspensions were filtered using a syringe filter with 0.45 mm pore size (Whatman, Florham Park, NJ) to remove any large scattering particle, which would bias the light scattering measurements. With a 1 mL syringe, the samples were injected into the cuvette.

2.2.3. Zeta-potential measurements

Measurements were conducted on a dynamic light scattering and zeta-potential equipment Malvern Zetasizer Nano ZS (Malvern, UK), equipped with a He-Ne laser (λ = 632.8 nm). The zeta-potential of the samples was determined, at 25ºC, from the mean of 15 measurements, with 60 runs each, with an applied potential of 30 V, in the absence and presence of different soluble human fibrinogen concentrations, using disposable zeta cells with platinum gold-coated electrodes (Malvern).

2.3. Human Blood Cells Isolation

Blood was collected (3mL) from adult heart failure patients into K_3EDTA anticoagulant tubes. In a Sorval TC6 centrifuge, blood was centrifuged at 220 *g* for 10 minutes at 10ºC, resulting in a two phase solution: an upper phase of plasma – platelet rich plasma (PRP) – and a lower phase of erythrocytes. The supernatant (PRP) and the buffy-coat were removed from this tube and held at 4ºC in a new falcon tube. 1 mM of BSCG buffer with $CaCl_2$ was added to the blood tube. After every buffer addition, the tube must be gently shaken. BSGC buffer is constituted by 1.6 mM KH2PO4, 8.6 mM Na2HPO4, 0.12 M NaCl, 13.6 mM sodium citrate, and 11.1 mM glucose, at pH 7.3.

The blood tube was centrifuged at the same conditions for seven minutes two more times, after repeating the cell-washing steps.

At the end of the first three centrifugations and washings, erythrocyte final suspension was prepared with the addition of BSGC buffer, to reconstitute the initial hematocrit (approximately 45%).

To isolate the platelets from PRP and erythrocytes, we washed the tubes twice as follows:

For platelets, the PRP tube was centrifuged at 1620 *g* at 10ºC for 10 minutes. At the end of each centrifugation, the supernatant was disposed and the solution ressuspended in 1mL of BSGC buffer. At the end of the two centrifugations, PRP tube was kept at 4ºC until use.

As for erythrocytes, the blood tube was centrifuged at 1620 *g* at 10ºC for ten minutes. At the end of the first centrifugation, we added 1mL of BSGC buffer. On the second centrifugation no buffer was added, and the erythrocyte mush was kept at 4ºC, until further use.

For the atomic force microscopy studies, the erythrocyte suspension was diluted 1:1000 and for platelets, PRP was diluted 1:2 with BSGC buffer + $CaCl_2$ 1 mM.

No blood was used after two days of being collected.

The study was approved by the joint Ethical Committee of the Santa Maria Hospital and Faculty of Medicine of the University of Lisbon.

Blood from heart failure patients was obtained with their previous written informed consent, following a protocol with the *Hospital Pulido Valente*. Healthy blood donors' blood from the Portuguese Blood Institute (Lisbon, Portugal) was also obtained with their previous written informed consent.

2.4. Mice Blood Cells Isolation

Blood was taken from C57BL/6 mice, with ages ranging 8 to 14 weeks, and stored in K_3EDTA anticoagulant tubes. 500 µL of this blood was transferred into an eppendorf tube. In an Eppendorf 5415R centrifuge, this tube was centrifuged at 143 *g* for 20 minutes at 10ºC, resulting in a two phase suspension: an upper phase of plasma (PRP) and a lower phase of erythrocytes. The supernatant (PRP) and buffy-coat were removed from this tube and held at 4ºC in a new eppendorf tube. 170 µM of BSCG buffer was added to the blood tube. After each buffer addition, the tube must be gently shaken.

The blood tube was centrifuged at the same conditions for 7 minutes two more times.

At the end of the first three centrifugations, erythrocyte final suspension was prepared with the addition of BSGC buffer, to reconstitute the initial hematocrit.

To isolate the platelets from PRP and erythrocytes, we washed the tubes two times as follows:

For platelets, the PRP tube was centrifuged at 574 *g* at 10ºC for ten minutes. At the end of each centrifugation, the supernatant was disposed and the solution

ressuspended in 170 µL of BSGC buffer with $CaCl_2$ 1mM. At the end of the two centrifugations, PRP tube was kept at 4ºC until use.

As for erythrocytes, the blood tube was centrifuged at 574 *g* at 10ºC for 10 minutes. At the end of the first centrifugation, we added 170 µL of BSGC buffer. On the second centrifugation no buffer was added, and the erythrocyte mush was kept at 4ºC, until further use.

For the atomic force microscopy studies, the erythrocytes were diluted 1:1000 and platelets were diluted 1:34 with the BSGC buffer + $CaCl_2$ 1 mM.

No blood was used after two days of its collecting.

2.5. Statistical Analysis

The Unpaired Student's t-test was used for statistical analysis of intergroup comparison, using *GraphPad Prism 5*. Differences were considered statistically significant for $p<0.05$.

3

CHAPTER 3 – ERYTHROCYTE-FIBRINOGEN INTERACTION AS A CARDIOVASCULAR RISK FACTOR

3.1. Introduction

The idea of plasma fibrinogen as a cardiovascular disease (CVD) marker has been studied for decades based on numerous prospective epidemiological and clinical studies, clinical observation and meta-analysis, gaining a widely spread acceptance in the scientific community [11].

As for the major incident CVD, high fibrinogen concentration was found to be associated with the unfavorable course of patients with acute coronary syndrome; myocardial infarction in an acute phase, associated with a new myocardial infarction and short-term predictor of mortality; re-stenosis after coronary stenting or balloon angioplasty; stroke; severe coronary artery disease; thromboembolism in atrial fibrillation; and atherosclerosis [1, 11-13]. More recently, studies have demonstrated that fibrinogen also plays a role as a mediator of inflammatory diseases, with a potential for selective drug targeting [14].

Erythrocytes or red blood cells (RBC) are also related to CVD incidence. Associated with high fibrinogen blood concentration, they hypperaggregate, representing the main risk factor for venous thrombosis.

In this chapter, some relevant information about platelets, erythrocytes, glycoproteins (and the role they play in blood viscosity), the fibrinogen molecule (and its function as a cardiovascular biomarker), as well as the erythrocyte-fibrinogen interaction is introduced.

3.2. Blood Cells

3.2.1. Platelets

Platelets are anucleate plasmatic cells, derived from megakaryocyte's fragments. They have a disc shape with a diameter that varies between 2 and 4 μm, with longevity from five to nine days for humans [15]. As for mice, platelet's mean diameter is 0.5 μm and its life span is approximately four days [16].

The platelet cytoplasm is constituted by actin and myosin: proteins that promote the thrombi contraction mechanism. The membrane contains proteins that are responsible for the receptor-ligand recognition. It contains receptors that mediate the communication between intra and extracellular domains. The glycoproteins enable the aggregation mediated by other molecules, like collagen or fibrin. This is highly important as it aids the platelets in their role in maintaining hemostasis, responding to vessel injuries (Figure 10), since in the event of bleeding, platelets must associate to a fibrin network, creating the blood clot [15].

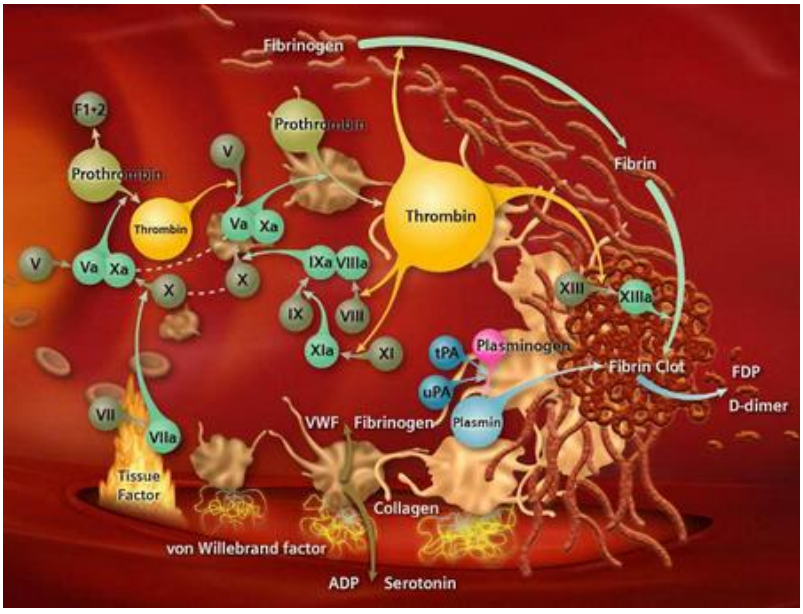

Figure 10. Coagulation cascade. A vascular injury triggers the process of coagulation by releasing collagen and tissue factor. Such intervenients activate the coagulation factors that will activate thrombin, which polymerizes fibrinogen, creating the fibrin network (extrinsic pathway).

Coagulation can also occur via intrinsic pathway in the presence of thrombin that leads to Factor XI activation. The two mechanisms converge in the formation of factor Xa [17]. Image taken from Siemens Website[2].

For further information about platelet functions, please see [18].

Platelet-rich clots (Figure 11) are formed in arteries, after rupture of atherosclerotic plaques and procoagulant agents [19].

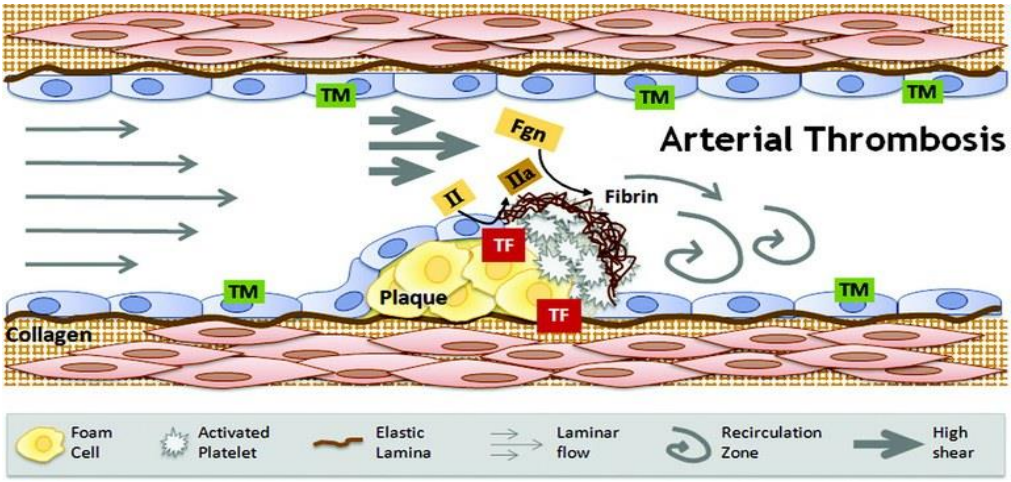

Figure 11. Arterial thrombosis – high shear environment. Image taken from Alisa Wolberg's Lab Website[3].

In Figure 11 it is possible to identify the arterial thrombosis main intervenients: fibrin(ogen) (Fgn), thrombomodulin (TM); prothrombin (II); thrombin (IIa); tissue factor (TF), collagen and platelets. These are called the "white clots" [19].

3.2.2. Erythrocytes

Erythrocytes are the most numerous cells in the blood. Their shape is bi-concave, with the periphery thicker (2.6 µm) than the center (0.8 µm) and a diameter of 7.5 µm, for humans, and they can last for 120 days in men and 110 in women. As for mice, erythrocyte's diameter is approximately 6.6 µm [20] and their life span is approximately 41 days [21]. During their maturation, these cells lose their nuclei and almost every organelle. Their main component is hemoglobin, which provides them their red color. This protein combines with oxygen, which the erythrocyte has to transport from the alveoli to all body cells [15].

Erythrocytes have a great capacity to deform in order to pass through vessels. This way they can reach all body surfaces, by means of arterioles that have a smaller

[2] http://www.healthcare.siemens.com/hemostasis/hemostasis-online-campus/interactive-hemostasis-cascade; accessed on September, 2013.

[3] http://www.med.unc.edu/wolberglab/scientific-images/arterial-thrombosis; accessed on September, 2013.

diameter than them. In some cardiovascular diseases, like hypertension, red blood cells lose this characteristic, and become more rigid, causing the blood viscosity to increase [22].

The binding between the erythrocyte membrane and fibrinogen was recently characterized [3]. In a non-ideal blood condition, where the concentration of fibrinogen is elevated, the aggregation of RBC increases and blood viscosity rises [18]. Senescence studies made by Carvalho *et al.* [4] with atomic force microscopy proved that, as aged erythrocytes suffer a decrease in the concentration of sialic acid in their membrane, this leads to a decrease in erythrocyte-fibrinogen receptor binding events (without significantly affecting the forces of each single binding) and consequently to a decrease in the aggregability capacity of RBC – less cardiovascular risk associated to these cells.

It can be observed that RBC suffer deformation in fibrinogen concentrations similar to inflammatory conditions [23]. In cases of high blood viscosity and hyperaggregation, in hyperfibrinogenemia conditions, with and without acetylcholine, nitric oxide (NO) and oxygen delivery may be compromised. However, the NO erythrocyte scavenging property is preserved in these conditions. Thereby, this can be a compensatory mechanism which represents a benefit in the intravascular resolution of inflammation [23].

Other erythrocyte aggregation factors are the hematocrit, plasma lipoproteins, osmolality and pH.

Fibrin-rich clots are formed in veins when procoagulant activity and hypercoagulant plasma are exposed on intact endothelium in reduced or static blood flow [24].

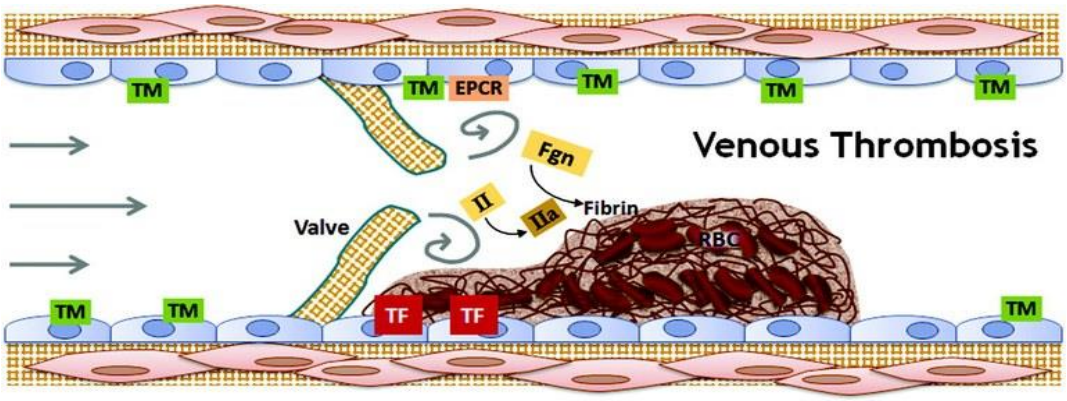

Figure 12. Venous thrombosis - low shear environment. Image taken from Alisa Wolberg's Lab Website[4].

Figure 12 shows that thrombomodulim, endothelial protein-C receptor (EPCR), prothrombin, thrombin, tissue factor, fibrin(ogen) and red blood cells are the main intervenients in venous clot formation. Venous thrombi usually form behind valve pockets, since the hypercoagulability is eased by the reduced blood flow. These are called the "red clots" [24].

For more information about RBC, see [25].

3.3. Integrins

Integrins are a large family of type I transmembrane heterodimeric glycoprotein receptors first discovered over twenty years ago. They function as the major metazoan receptors for cell adhesion and connect the intracellular and extracellular environments [26].

These glycoproteins play a very important role in many biological processes, namely in the assembly of the actin cytoskeleton and modulating signal transduction pathways that control biological and cellular functions, like cell adhesion, migration, proliferation, cell differentiation, and apoptosis [26].

Integrins have two domains, the non-covalently associated subunits, α and β, which pair to form heterodimers. Currently, there are eighteen α subunits and eight β subunits known, which pair to form at least twenty-four distinct integrin heterodimers [26].

[4] http://www.med.unc.edu/wolberglab/scientific-images/venous-thrombosis, accessed on September, 2013.

Table 1. Cell membrane integrins and their respective ligands [26].

Integrins	Ligand
$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$	Collagen
$\alpha_1\beta_1$,$\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$	Laminin
$\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta$	Fibronectin (RGD-dependent manner)

Table 2. Integrins and their corresponding binding-cell [26].

Integrins	Corresponding Cells
$\alpha_{IIb}\beta_3$	Platelets
$\alpha_6\beta_4$	Keratinocytes
$\alpha_E\beta_7$, $\alpha_4\beta_7$, $\alpha_4\beta_1$, and β_2 family	Leukocytes

3.3.1. Mutants

Mutated integrins are associated to clinical disorders. There are three well-described inherited autosomal recessive diseases in humans of this nature: mutations in the α_{IIb} and β_3 subunits are related to Glanzmann's thrombasthenia (platelet dysfunction with consequent bleeding disorders); point mutations and gene deletion in β_2 integrin subunit are associated with Leukocyte Adhesion Deficiency (LAD); and mutations in α_6 and β_4 integrin subunits result in juctional epidermolysis bullosa with skin blistering [26].

3.3.2. Structure

Each heterodimer contains an extracellular domain that binds to proteins in the extracellular environment, a single-membrane-spanning transmembrane domain, and a generally short intracellular cytoplasmic tail domain, which forms links with the cytoskeletal elements via cytoplasmic adaptor proteins [26].

Understanding and characterizing integrin structure and conformation is important to describe the mechanisms that underlie their activation. This knowledge unblocks the path for understanding integrin features like signaling and mediated adhesion.

Most of the structural data of the extracellular domains comes from high-resolution X-ray crystallography. These domains generally have large dimensions, comprehended between 80 and 150 kDa [26].

The extracellular portion of the α and β subunits contains several subdomains. The C-terminal comprises two long and extended legs and provides the connection with the transmembrane and cytoplasmic domains of each respective subunit. N-terminal stands on C-terminal [26]. This terminal is the globular bind site for ligands. Figure 13 illustrates these domains and the extended conformation state setting the platelet integrin, $\alpha_{IIb}\beta_3$, as an example.

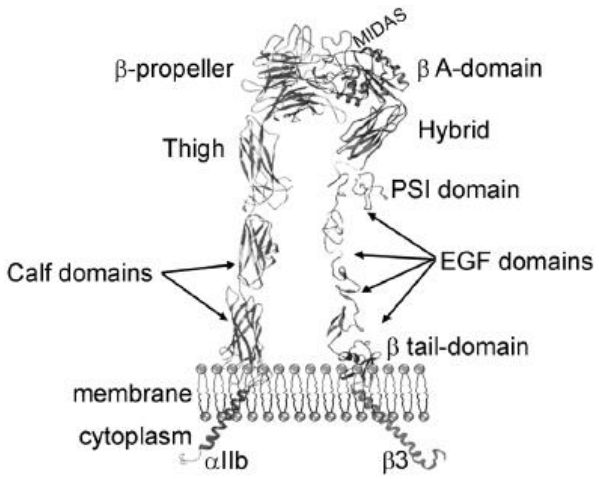

Figure 13. $\alpha_{IIb}\beta_3$ integrin representation in an extended state [27].

The α subunit head has a folded seven-bladed β propeller head domain, a thigh domain and two calf domains. Half of the α subunits contain an additional inserted I-domain, which is inserted within the β propeller domain. This domain is provided of two exclusivity features: when present, it is the only extracellular-binding site for ligands; it contains a conserved Metal-Ion-Dependent Adhesive Site (MIDAS) that binds divalent metal cations (Mg^{2+}) and plays important roles in protein ligand binding. Once a ligand binds to MIDAS, there is an alteration of the metal ion coordination and the I-domain alters from a closed, resting state to an open, active conformation. This ultimate conformation increases the ligand affinity and promotes subsequent integrin activation [26].

The β subunit head is composed by five domains: an I-like domain, structurally similar to the α subunits I-domains, a PSI (Plexin-Semaphorin-Integrin) domain, a hybrid domain; four EGF repeats, and a membrane proximal β tail (βTD) domain. The β subunit plays important roles in ligand binding in α subunits which lack the I-domain. In these integrin heterodimers, ligands bind to a crevice between the αβ subunit

interfaces, and interacts with an activated β MIDAS and the propeller domain of the α subunit [26].

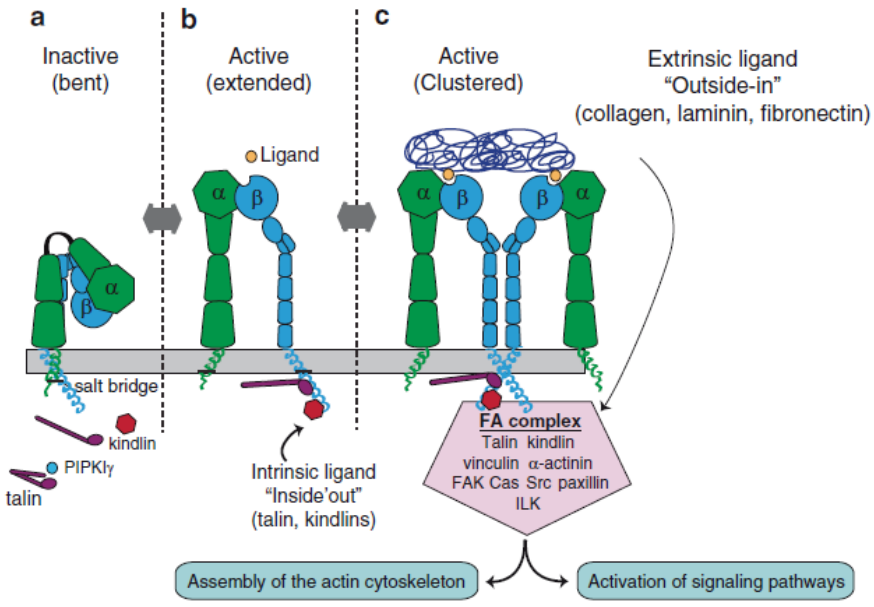

Figure 14. Integrin structure without the α I-domain. a - the integrin is in its unbound, resting state. b - Integrin is activated by proteins. c - Activated integrin binds to ligands [27].

The activation and re-conformation of the integrin is represented in Figure 14. Firstly (**a**), the integrin is in a bent conformation and the transmembrane and cytoplasmatic regions are closely associated. Then (**b**), the integrin is activated by talins and kindlins. The transmembrane and cytoplasmatic subunits separate and the extracellular domains extend. This conformation is ready to bind to ligands. Finally, when the integrin binds to ligands (**c**), they cluster at the plasma membrane. Clustering is a requisite for signaling tight focal adhesions, actin cytoskeleton assembly, and activation of other signals to control cellular functions [26].

The structural data of the transmembrane (TD) domains derives from nuclear magnetic resonance (NMR) analysis [26]. Integrin TD comprise single spanning amino acid residues structures that form α-helical coiled coils that either homo- or heterodimerize. For more information about TD, please see reference [26].

Cytoplasmic domain information has been also achieved based on NMR data [26]. This domain is the shortest of the whole integrin, with a number of largely unstructured amino acid residues comprehended between 10 and 70. The β_4 subunit represents an exception, as it contains more than 1000 amino acid residues [26]. β cytoplasmic tails are highly homologous, while α subunit tails are highly divergent. In

the membrane proximal regions of these subunits there is a salt bridge between the arginine from the α subunit and the aspartic acid from the β subunit. The salt bridge is the physical contact between the two subunits tails, supposedly to maintain integrins in their inactive, resting state. Still, these are assumptions, as the only cytoplasmic tails that have been completely characterized are the ones from the $\alpha_{IIb}\beta_3$ integrin. It is also believed that the salt bridge may not play a major role in β_1 integrin activation [26, 27].

The β integrin tails have two well-defined motifs: they represent canonical recognition sequences for phosphotyrosine-binding (PTB) domains and serve as binding sites for multiple integrin binding proteins, including talin and the kindlins [26].

3.4. Fibrinogen

Fibrinogen (Figure 15) is a protein that represents 4% of the plasma constitution [15]. It is a very long – 97.5 nm – and asymmetric molecule, with molecular weight of 340 kD, with a plasma concentration range between 2 and 4 mg/dL and plasma half-life of three to five days [1, 23]. Fibrinogen is synthesized in the liver and is secreted into the circulating bloodstream. Fibrinogen is constitutively expressed exclusively in hepatocytes and is inducible by interleukin-6 (IL-6) as part of the acute phase reactions and impaired by transforming growth factor-β [23]. Its quaternary structure is made by three pairs of nonidentical polypeptide chains – 2Aα, 2Bβ and 2γ – dimers that bind by multiple disulfide bonds [28] (Figure. 15), derived from separate genes (FGA, FGB and FGG). Subunits are grouped into three major structural regions: two D-domains and one central E-domain. As for binding sites, fibrinogen has three potential integrins binding sites: two RGD (arginine – glycine – aspartic acid) amino acid sequences within the Aα chain and a 12 amino acid sequence on the γ chain [3]. For more information about fibrinogen binding, see [29].

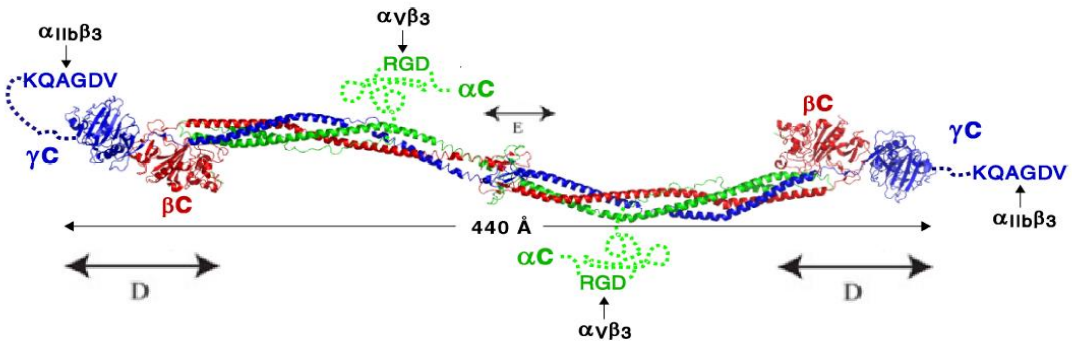

Figure 15. Fibrinogen molecule structure. Fibrinogen is formed by the central E and the distal D regions, connected by α, β and γ chains. The α chain is the green ribbon, the β chain is the

red ribbon and γ chain is the blue ribbon. The γ chain C-terminal sequence binds to the KQAGDV sequence and the α chain C-terminal binds to the RGD sequence [30].

3.4.1. Coagulation and Thrombi Formation

Fibrinogen can be considered the most important glicoprotein involved in clot formation and the only one present in plasma.

When a vessel is injured, fibrinogen polymerization into fibrin is induced by thrombin, in the presence of Ca^{2+} ions. It is this specific fibrous protein that binds with platelets in order to form the homeostatic plug. The conversion of fibrinogen into fibrin occurs via the multifaceted actions of the coagulation cascade and the proteolytic action of thrombin. Thrombin, in the presence of calcium ions, cleaves the A and B fibrinopeptides, exposing multiple polymerization sites, allowing for fibrinogen polymer formation. It is this specific fibrous protein that binds with platelets in order to form the homeostatic plug, when binding to the $\alpha_{IIb}\beta_3$ receptor in activated platelets [2]. As fibrinogen has more than one integrin-binding site, it is possible to simultaneously bind two platelets, bridging them. This fibrinogen characteristic is also very important for regulating blood hemostasis [31].

Fibrinogen and fibrin degradation products have an important role in the maintenance of homeostasis, clot formation and fibrinolysis. These mechanisms are responsible for the regulation of thrombin activation/deactivation, plasma transglutaminase activation and the activation of fibrinolysis [28]. For a further analysis of fibrin functionality, see [32].

It is important to add that fibrinogen is a determinant of plasma viscosity, among other factors, because its concentration is directly related with fibrin concentration, and consequently, the size of the formed thrombi [28]. Studies in animal models and in humans have demonstrated that the extravascular fibrinogen that is deposited in tissues upon vascular rupture is not merely a marker, but a mediator of diseases with an inflammatory component [14]. Thus, when in high concentrations, fibrinogen must be seen as a thrombogenic and atherogenic factor.

Recently, Zhmurov *et al.* [33] developed a study in the mechanisms of fibrin(ogen) forced unfolding using AFM single-molecule unfolding and simulations. They described the order of unfolding of the protein's chains and elasticity of each

chain by reversible extension-contraction of the α-helical coiled-coils. The fibrinogen nanomechanics has implications for the (patho)fisiology of fibrin clots and thrombi.

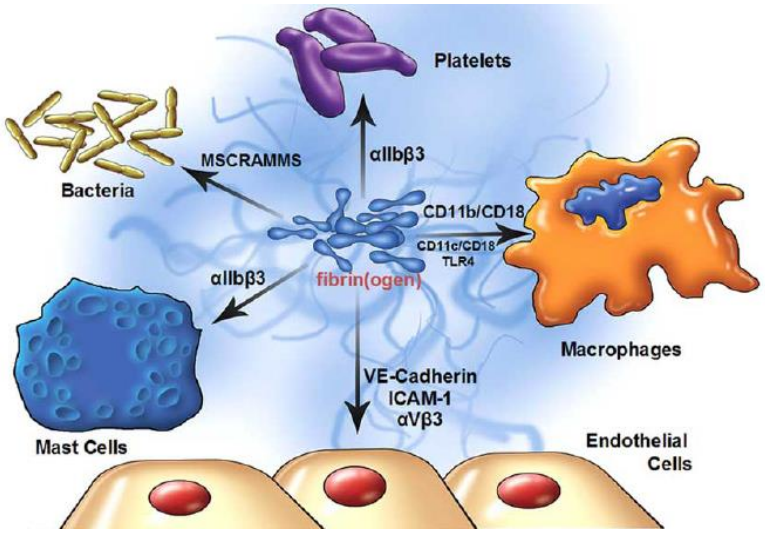

Figure 16. Fibrinogen signal transduction: cellular receptors and target cells [14].

Fibrinogen mediates several biological reactions (Figure 16). It contains multiple binding motifs for different cellular receptors and acts as the molecular link between coagulation, inflammation and immunity, regulates cell migration and capillary tube formation [14].

3.4.2. Pharmacology

Fibrinogen has the potential for selective drug targeting. This allows targeting its proinflammatory properties (inhibiting its bind to individual cellular receptors) without affecting its beneficial effects in hemostasis, since it interacts with different receptors to mediate blood coagulation and inflammation [14]. Thereby, fibrinogen provides specific targets for drug design using peptides, monoclonal antibodies or small molecules to selectively inhibit its interactions with cellular receptors

Recently, cellular targets for fibrinogen, such as monocytes and microglia were identified and fibrin was identified as a novel inhibitor of neurite outgrowth via direct activation of signaling pathways in central nervous system neurons [14].

Adams *et al.* [14] administered fibrin-derived peptides $B\beta^{15-42}$ and $\gamma^{377-395}$ on mice and concluded that the $B\beta^{15-42}$ peptide was protective against myocardial infarction, while the $B\beta^{15-42}$ peptide decreased inflammation and suppressed paralysis in animal

models of Multiple Sclerosis. Both peptides interfered with the inflammatory process without affecting the beneficial effects of fibrinogen in blood coagulation.

3.5. Biomarkers

3.5.1. Introduction

Clinical laboratory tests provide information for a variety of purposes, including diagnosis, monitoring of disease, and risk assessment. Risk assessment is based on large-scale epidemiologic studies used to identify potential markers associated with the increase or decrease of the risk of CVD. However, these studies are not always clear about the reliability of a single measurement performed in one subject. It is mandatory that more studies are performed in order to demonstrate the prognostic utility of every marker when measured in a patient, and the number of measures needed from each marker to assess individual risk. In order to have a good clinical significance, a marker of risk should have a low biological variability within a single individual, compared with inter-individual variability. A limited number of measurements should be enough to assess CVD risk [13].

Fibrinogen, as an acute phase protein, indicates inflammation existence. It is seen as a danger marker above certain blood concentrations [13].

3.5.2. Epidemiology evidence of an association with Vascular Disease

Fibrinogen plays an irrevocably important role as an independently associated biomarker in cardiovascular diseases such as atherosclerosis, as fibrinogen levels correlate with the number of coronary and extracoronary vascular beds involved in atherosclerosis; coronary artery disease (CAD), in which fibrinogen levels may be an inheritable risk factor in subjects with a strong family history of myocardial infarction (MI) and stroke, both with associated risks proportional to the fibrinogen level and, in the case of MI occurrence, fibrinogen becomes a short-term predictor of mortality [1, 2, 34].

As for medical interventions, fibrinogen levels predict stenosis after angioplasty. Preinterventional levels are more predictive than postinterventional levels [1].

Among the conditions increasing plasma fibrinogen concentration, the major risk factors for cardiovascular diseases are: diabetes mellitus, hypertension, obesity,

smoking, hyperlipidemia, sedentary lifestyle, and age. On the other hand, moderate regular alcohol consumption, polyunsaturated fatty acids and regular exercise decrease fibrinogen levels [1].

3.5.3. Fibrinogen and Inflammation

Cases of hyperglycemia can induce a chronic increase of IL-6, turning the acute phase reaction into a chronic perpetual state with increased levels of fibrinogen (leading to hyperfibrinogenemia), serum amyloid A, C- reactive protein, haptoglobin and decreased albumin [2]. Hence, fibrinogen has been extensively used as a marker to assess the presence and persistence of inflammation.

Hyperfibrinogenemia is a natural organism response to aggressive situations, like trauma and inflammation. It increases plasma viscosity, platelet and erythrocyte aggregation, decreasing blood flow and promoting ischemia [2, 28]. Thus, fibrinogen is a determinant of plasma viscosity, as its concentration is directly related with fibrin concentration, and consequently, the size of the formed thrombi [28]. Therefore, in high concentrations, fibrinogen must be seen as thrombogenic and atherogenic factor. It is also a determinant of plasma viscosity and erythrocyte aggregation and, therefore, affects blood viscosity both at high and low shear rate [1].

As previously mentioned, fibrinogen is expressed exclusively in hepatocytes and is inducible by IL-6 as part of the acute phase reaction. Fibrinogen biosynthesis is connected to that of cholesterol, because oxysterols, suppressing cholesterol biosynthesis and the uptake of LDL-cholesterol, also inhibit constitutive fibrinogen expression**.** For these reasons, fibrinogen-level lowering agents also decrease cholesterol and inflammation, as there are no therapeutic agents acting only as a fibrinogen inhibitor [1].

Infection

Fibrinogen also plays an important role in bacterial infection, as it binds to bacterial cell-wall proteins that regulate the adhesion, hosting and survival of bacteria to the host tissue [14].

3.5.4. Cardiovascular Risk

Fibrinogen may induce arterial diseases by 4 mechanisms: atherogenesis (atheroma accumulation on the vessels walls), platelet aggregation and thrombi formation, fibrin thrombi formation, and blood viscosity increase [28].

Increased plasma fibrinogen levels are associated with an increased risk of CAD, myocardial infarction and stroke. The aggravation of such conditions, are due to the inflammatory effect mediated by fibrinogen, which plays this important role in inflammation, once it is regulated by proinflammatory cytokines, like interleukin-6 [1, 2]. Besides being an inflammation aggravation factor that increases risk of CAD, MI and stroke, fibrinogen correlates with other CVD risk factors including obesity, sedentary lifestyle, diabetes mellitus, hyperlipedemia, cigarette smoking and age. However, studies performed in atherosclerotic knockout mice, in the absence of fibrinogen, showed no decreased extent of the disease, suggesting that fibrinogen seems to be just a marker rather than a mediator of vascular disease. On the other hand, fibrinogen participates in the formation of atherosclerotic plaque during the first stages of CAD, suggesting that it is a mediator factor rather than a result. Therefore, it is difficult to clarify the exact role of fibrinogen in the vascular inflammatory processes [1, 2].

As an addendum, it is important to add that the changes in circulating fibrinogen levels also represent a risk factor for Alzheimer's disease [14].

Recently, Lovely *et al.* characterized γ′ fibrinogen (an alternatively-spliced form of the clotting factor fibrinogen) as a biomarker for cardiovascular diseases and single-nucleotide polymorphisms (SNPs) exclusively in and near the fibrinogen gene locus. For further information, please see [35]. For more information about fibrinogen as a cardiovascular disease mediator, please see [1].

3.5.5. Fibrinogen and Endothelial Cells

Proinflammatory cytokines increase the synthesis of nitric oxide (NO) and favor leukocyte migration in the sub-endothelial space [2].

Fibrinogen has a non-specific binding to endothelial cells. Binding to intercellular adhesion molecule-1 (ICAM-1), fibrinogen promotes the adhesion of leukocytes, platelets, and macrophages to endothelial cells. This feature may have the drastic consequence of circulating tumor cells adhesion, creating hematogenous metastases [1].

Fibrinogen also adheres to subendothelial space, causing the accumulation of LDL and apolipoprotein A, contributing to the idea that fibrinogen is a atherosclerosis mediator [1].

Figure 17 summarizes the fibrinogen synthesis, structure and its vascular functions.

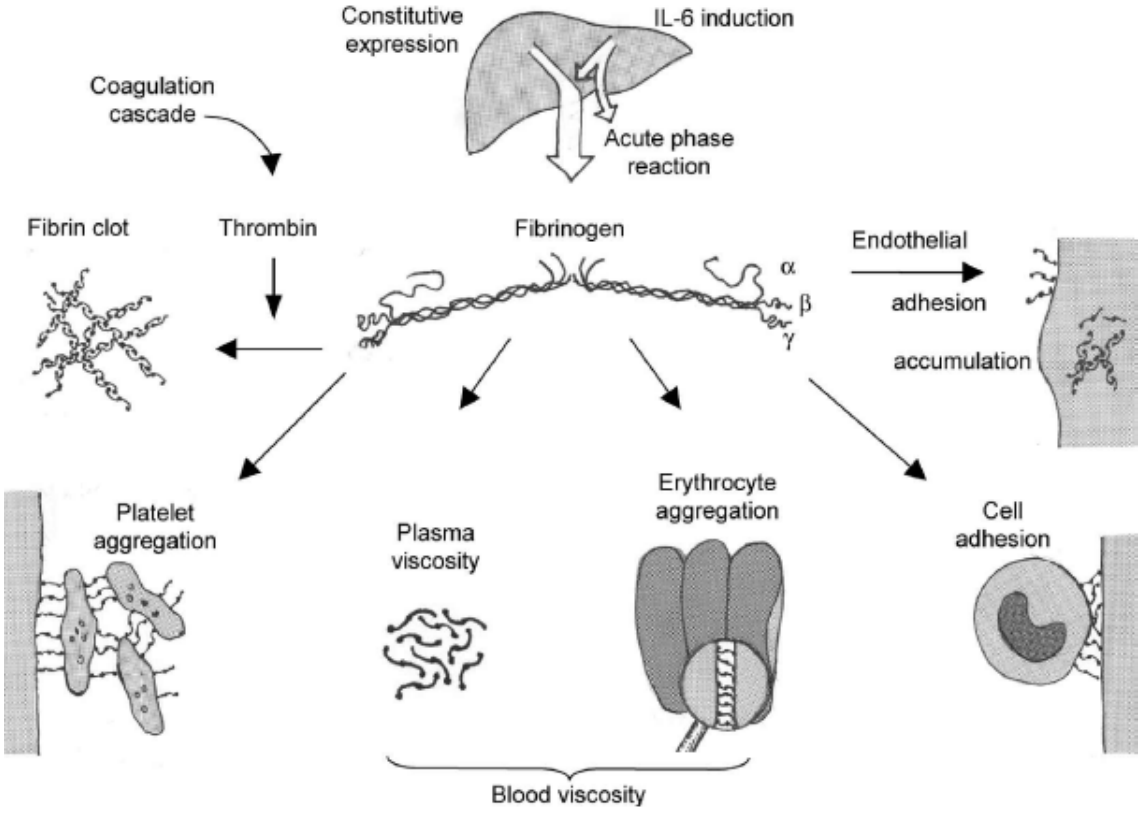

Figure 17. Fibrinogen biosynthesis, structure and vascular functions. Fibrinogen is generated in the liver under the action of IL-6, for later interaction with vascularity. Fibrinogen induces cell aggregation among endothelial tissues and vessels walls. When it interacts with thrombin, in the final stage of the coagulation cascade, forms fibrin clots that may origin thrombi. By means of its ligands, fibrinogen binds with bloods vessels and platelets creating blood clots and to erythrocytes, increasing blood viscosity [1].

3.5.6. γ' Fibrinogen

Different combinations of altered fibrinogen chains can be assembled to this molecule, especially in fibrinogens resulting from heterozygous polymorphisms or mutations. The fibrinogen γ chain, in particular, has two isoforms, the γ A (also known as γ^{50} or simply γ) isoform and the γ' (also known as $\gamma^{57.5}$ or γB) isoform that arise from alternative mRNA processing [34].

The γ' isoform chain of fibrinogen is formed by the incorporation of a splice variant of the fibrinogen γ chain. This alternative splicing event arises from an alternative processing event in the γ chain mRNA and results in the extended carboxyl terminus of the γ' chain, which contains 20 residues in place of the four carboxylterminal residues on the more common γA chain [34, 36].

The γ' fibrinogen isoform makes up approximately 7% of circulating fibrinogen, with a high affinity binding site for thrombin. Its fibrin clots, in the presence of coagulation factor XIII (also known as "fibrin-stabilizing factor"), are more resistant to fibrinolysis and have altered clot architecture compared with the common fibrinogen isoform γA/γA fibrin clots. Therefore, it is considered a risk factor for thrombosis [34, 36].

High levels of γ' fibrinogen are also associated with several forms of cardiovascular disease, not only as a general risk factor, but as a potential biomarker for assessing a patient's inflammatory state and associated cardiovascular disease risk [36]. It has been associated with several cardiovascular risk factors: age, sex, BMI (Body Mass Index), smoking, diabetes, blood glucose and triglycerides, and inversely-associated with HDL cholesterol [35]. Unlike many other CVD markers, this particular protein has biochemical properties that have the potential to actually contribute in the etiology of the disease [35]. As for genetics, which usually is an influential factor for CVD, they may not play a greater role in the association of γ' and CVD. Since γ' fibrinogen is an acute phase reactant in response to inflammation, environmental factors may play a greater role than genetics [34, 35].

Elevated levels of γ' fibrinogen have been associated with atrial thrombosis, including MI, coronary heart disease and stroke. On the other hand, γ' fibrinogen is a hemostasis inhibitor, as it does not bind to the platelet receptor $\alpha_{IIb}\beta_3$, disfavoring thrombi formation, creating a paradoxical results and conclusions between studies about this topic. Thus, the association between γ' fibrinogen and venous thromboembolism is still controversial, and may be due to genetic variation or the design of previous studies [35, 37]. To know more about relevant hypotheses in this subject, please see [34].

In 2011, Farrel *et al.* [36] conducted their studies in order to unveil more about this γ chain mutation and its relationship with CVD and inflammation process. Their results with normal mean total fibrinogen reinforced the idea of γ' fibrinogen being particularly elevated in the setting of chronic inflammation. Their results, combined with previous case-control study of CAD, also suggest that under pathological conditions there is a lack of association between γ' fibrinogen and total fibrinogen levels. Lovely *et al.* [35] suggested that γ' fibrinogen and total fibrinogen levels are under different genetic control.

In 2013, Alexander *et al.* performed a study concerning several biochemical markers. They concluded that hsCRP (an acute-phase protein) has very high biological variability, therefore, it is not a good marker of CVD. γ' fibrinogen and LDL needed two measures to assess CV risk. HDL cholesterol revealed to be the best marker in this study, as only one measure was needed to provide prognostic information [13].

To conclude, it is important to denote that it remains to be seen whether or not γ' fibrinogen is just a marker for CVD or a mediator as well.

3.6. Erythrocyte – Fibrinogen Interaction

In 1981, Rampling *et al.* [38] conducted a study aiming to understand the fibrinogen binding to erythrocyte membrane and the role of fibrinogen in rouleaux formation and erythrocyte flexibility. Their results indicated that fibrinogen does bind to erythrocyte membranes and that this bond is affected by physico-chemical factors pH and ionic strength, while it is not affected by calcium ions, plasma proteins and temperature. These results suggested that the charge interaction between the membrane and fibrinogen plays an important role in this binding. From the magnitude of the binding ratio, they calculated that approximately 2% of total fibrinogen circulates associated to erythrocytes in the blood stream and that, at normal fibrinogen concentrations (3g/l), 20.000 fibrinogen molecules bind to each red blood cell. In order to understand where the protein binds to the erythrocyte membrane, they used fibrinogen degradation products (FDP), represented in Figure 18. The X fragment lacks the C-terminal of the α chains. This loss has no significant effect in the binding of fibrinogen to the erythrocyte membranes, thus the X fragment plays no role in this linkage and is located far from the binding areas. The D fragment has one binding site and presents half the binding for fibrinogen, whether it is isolated or not. The Y has two binding sites and has a great affinity for binding, which is maintained on the E fragment although it has only one binding site.

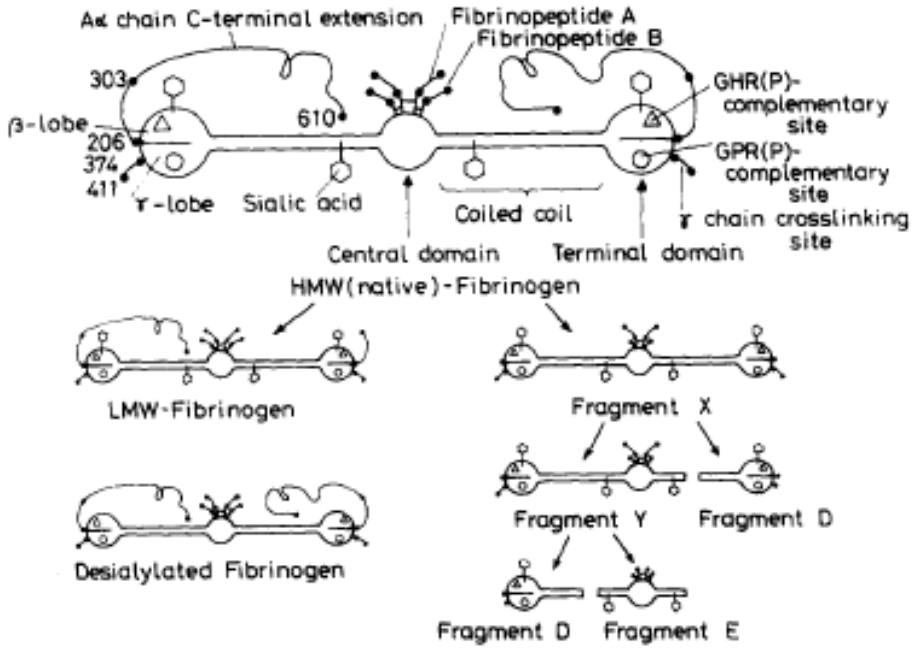

Figure 18. Schematic model of fibrinogen and fibrinogen-derived products [39].

As for the rouleaux formation and erythrocyte flexibility, this study reveals that fragments X and Y are responsible for these features, as they hold two binding sites. This characteristic allows the inter-membrane cross-linking, i.e. the same fibrinogen molecule binds to more than one erythrocyte [38].

In 1987, Meda *et al.* [39] scrutinized this topic a bit further, in order to discover the erythrocyte-binding site in the fibrinogen molecule. The 1 to 206 residues of the fibrinogen Aα-chain, by cleavage of the near terminal domain, constitutes the X fragment; 207 to 303 fragments are present in naturally occurring low molecular weight (LMW) fibrinogen, which preserves the rouleaux formation speed induced by high molecular weight (HMW) fibrinogen, meaning that this is the region responsible for the erythrocyte binding. The fibrinopeptide-containing region in the central domain (Aα and Bβ chains) revealed to be repulsive to the negative charge of the erythrocyte membrane. Sialic acid in the erythrocyte promotes aggregation. On the other hand, sialic acid from de Bβ and γ chains does not intervene in fibrinogen-induced erythrocyte aggregation. This suggests that these local structures contribute to the rouleaux formation. The fibrinogen C-terminus from the γ chain binds to platelets. As for erythrocytes, this still is a case study.

In 2002, Lominadze *et al.* [40] presented experiments on the involvement of fibrinogen specific binding in erythrocyte aggregation. They used Oregon Green 488-labeled human fibrinogen and showed that it binds with a dissociation constant, K_d, of 1.3 μM, to rat erythrocyte membranes, which was estimated to have about 1932 ± 104 binding sites. To prove whether the binding happens in or outside the erythrocyte membrane, they used red blood cells ghosts – cells with the original morphology, but void of cytoplasmic contents – and sealed red blood cells. The results showed that the specific binding occurs on the outside surface of the erythrocytes.

In 2010, Carvalho *et al.* [3] unveiled much more about this protein-membrane interaction, making use of the AFM techniques. This nanotool has been proving its benefits, since it not only shows that fibrinogen and red blood cells bind, but also can characterize this interaction.

Their results showed that a single molecule interaction between fibrinogen and an unknown receptor on the erythrocyte membrane exists. This bond is comparable to the fibrinogen-platelet bond, though fibrinogen has a weaker interaction with erythrocytes than with platelets. This study also included the characterization of the environmental conditions necessary for the binding to occur. Calcium was shown to be necessary for fibrinogen-erythrocyte binding, as it was already known for the binding to platelets. With the $\alpha_{IIb}\beta_3$ glycoprotein inhibitor eptifibatide, the same levels of inhibition were obtained for erythrocytes and platelets, but only at a higher concentration for erythrocytes. These results suggest that an integrin receptor is involved in the fibrinogen specific binding to erythrocyte membrane, which is not as calcium dependent or eptifibatide influenced as the glycoprotein $\alpha_{IIb}\beta_3$ platelet receptor, but it surely is a related integrin. The same group performed Glanzmann thrombasthenia [3] studies, where patient's cells that had a mutation in the β_3 gene (ITGB3) were used. The AFM force spectroscopy results showed that the (un)binding forces were severely impaired, stating that the integrin receptor for fibrinogen on erythrocytes membrane is a β_3-like glycoprotein [3].

Recently, Oliveira *et al.* [41] separated erythrocytes with different age fractions and incubated them with human soluble fibrinogen and/or with a blocking antibody against CD47 – a membrane protein that avoids phagocytic cells. Their results confirmed previous results from Carvalho *et al.* [4] about cell aging and their decreasing aggregation capacity, and presented new insights on CD47 as a putative mediator of the aggregation process, suggesting that the interaction erythrocyte-

fibrinogen and CD47 may contribute to RBC hyperaggregation in inflammation. In 2011, Carvalho *et al.* [4] proved through AFM methods, Zeta-potential measurements and fluorescence spectroscopy that, although the (un)binding strength is the same as for older erythrocytes, younger erythrocytes bind with higher frequency. Thus, we may assume that these red blood cell population is the main responsible for some cardiovascular diseases associated with an increase on the fibrinogen content in blood.

Currently, Peacock *et al.* [42] have been developing studies about the interaction of the RBC membrane and the fibrinogen γ chain, which have no known binding, by analyzing the effect of the erythrocyte presence in fibrin polymerization rates. On one hand, there is the γA chain C-terminus that contains an integrin binding site known to interact with platelets, while on the other there is a γ' chain that does not contain this platelet's binding site. Peacock *et al.* performed turbidity and permeation experiments and analyzed the formed clots via confocal microscopy. Results showed that γA is more affected than γ', suggesting that erythrocyte-fibrinogen interaction occurs via the lacking C-terminal receptor. This theory is in agreement with the fact that the γ chain has a ligand to the platelet receptor $\alpha_{IIb}\beta_3$, which is from the same family as the erythrocyte receptor (β_3-like integrin), and absent in the altered C-terminus of γ' [42]

4

CHAPTER 4 – Erythrocyte - Fibrinogen Interaction in Cardiac Insufficiency Patients

4.1. Introduction

Cardiovascular Diseases: Why study them?

Cardiovascular diseases are the global number one cause of death. World Health Organization[5] estimated that, in 2008, 17.3 million people died from cardiovascular diseases, representing 30% of all global deaths, of which, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke. In 2011, cardiovascular diseases killed nearly seventeen million people, of which, seven million died of ischemic heart disease and 6.2 million from stroke. In 2012, the American Heart Association [27] statistics stated that CVD claim more lives per year than cancer, chronic lung/respiratory disease, and accidents combined. The World Health Organization predicts that cardiovascular diseases will remain to be the world leading cause of death, with an increasing number of deceased, mostly from heart disease and stroke, which are expected to reach 23.3 million by 2030.

Taking this alarming information into account, it is urgent to continue studying these diseases, with the purpose of improving and creating new diagnostic and therapy methods.

In this chapter we introduce the background concepts about the diseases we studied in this project. Then, the methodologies used to perform the essays are

[5] http://www.who.int/en/, accessed on August, 2013.

described and the results are presented. Finally, conclusions are taken from those results.

4.2. Heart Failure

Heart failure (HF), or cardiac insufficiency, affects more than twenty million people in the world, with an exponential tendency to increase [43].

The annual incidence of new cases of heart failure rises from less than 0.1% among people with less than 45 years old, to 1% for those older than 65 years, to 3% for those who are more than 85 years old. Prevalence figures follow a similar exponential pattern, increasing from 0.1% before 50 to 55 years of age to almost 10% after age 80 years. The overall prevalence of HF in the adult population in developed countries is 2%. As for gender, although HF incidence is higher in men than women, the female gender represents at least one-half of the cases, probably due to longer life expectancy [43, 44].

4.2.1. Definition

Heart failure, is a clinical heterogeneous syndrome, that can be inherited or acquired, in which abnormalities of cardiac function are responsible for the inability of the heart to pump blood at an output sufficient to meet the requirements of metabolizing tissues, or the ability to do so only at abnormally elevated diastolic pressures or volumes. Many authors describe this syndrome as a developed constellation of clinical symptoms (dyspnea and fatigue) and signs (edema and rales) that condemns the patient to a poor quality of life, with frequent hospitalizations, as they experience various cardiovascular disorders and adverse events, and to a shortened life expectancy [43, 44].

HF is characterized according to the patients' left ventricular ejection fraction (EF). HF with a depressed EF, commonly referred as systolic failure, or HF with a preserved EF, commonly referred to as diastolic failure [43].

4.2.2. Etiology

Any condition that causes myocardial necrosis or produces chronic pressure or volume overload can induce myocardial dysfunction and heart failure.

Heart failure may occur as a result of several causes: impaired myocardial contractility, creating systolic dysfunction, reducing left ventricular ejection fraction;

increased ventricular stiffness or impaired myocardial relaxation, creating diastolic dysfunction which is commonly associated with a relatively normal left ventricle ejection fraction; other cardiac deficiencies, like obstructive or regurgitant valvular disease, intracardiac shunting, or disorders of heart rate or rhythm; conditions where the heart is unable to compensate increased peripheral blood flow or metabolic requirements. By all means, the left ventricle is almost always involved in the cause of this disease, even if the symptoms occur primarily as a right ventricular dysfunction.

Heart failure may result from an acute disruption of the cardiac function, like the occurrence of a large myocardial infarction or, more commonly, a chronic process. However, the major causes for HF are coronary heart disease, in which the blood vessels that supply the heart suffer a severe narrowing; hypertension; diabetes; dyslipidemia; obesity in industrialized countries; ischemia and other forms of heart disease [44].

4.2.3. Symptoms

The main symptoms of HF are fatigue and dyspnea. The first one is considered to be due to low cardiac output, as well as to skeletal muscle anomalies and other noncardiac abnormalities, like anemia. As for dyspnea, it becomes more frequent and intense as the disease progresses, occurring even at rest. The origin of this symptom is probably a result of numerous signs, being the main one the pulmonary congestion with accumulation of interstitial or intra-alveolar fluid. Other factors can include reductions in pulmonary compliance, increased airway resistance, respiratory muscle and/or diaphragm fatigue, and anemia. Dyspnea may become less frequent with the onset of right ventricular failure and tricuspid regurgitation [43].

HF may be manifested chronically, through an acute exacerbation of chronic HF, or acutely as a *de novo* manner (caused by MI, valvular disease, myocarditis or cardiogenetic shock) [44].

4.3. Ischemic Heart Disease

The most common cause of heart failure in industrialized countries is ischemic cardiomyopathy. This disease causes more deaths and disabilities and economic costs than any other illness in the developed countries [43].

4.3.1. Definition

Ischemic heart disease (IHD) is a chronic condition in which there is a lack of blood supply to the myocardium, leading to myocardium's deoxygenation. This cardiomyopathy usually occurs when oxygen supply and demand are not balanced [43].

4.3.2. Causes

The main cause for IHD is atherosclerotic disease of an epicardial coronary artery or arteries, with such severity, leading to a regional reduction in myocardial blood flow and inadequate perfusion of the myocardium supplied by the involved coronary artery [43].

The major risk factors for atherosclerosis are high levels of plasma low-density lipoprotein (LDL), low plasma high-density lipoprotein (HDL), cigarette smoking, hypertension, and diabetes mellitus [43].

4.3.3. Effects

Ischemia is revealed by episodes of inadequate tissue perfusion caused by atherosclerosis. In these episodes, the tension from the oxygen of the myocardial tissue falls, causing temporary disturbances of the mechanical, biochemical, and electrical functions of the myocardium. The ventricular contractility is affected, provoking segmental hypokinesia (body movement decrease), akinesia (loss or impairment of voluntary movements), or dyskinesia (voluntary movements diminished and presence of involuntary movements), which can reduce myocardial pump function [43].

The abrupt development of severe ischemia is related with almost instantaneous failure of normal relaxation and contraction of the muscle. The relatively poor perfusion of the subendocardium causes more intense ischemia of this portion of the wall. Gaining a bigger proportion, ischemia of the ventricle causes transient left ventricular failure and regurgitation can occur. If ischemia is transient, it may be associated with angina pectoris, if it is prolonged, it can lead to myocardial necrosis and scarring with or without the clinical picture of acute myocardial infarction [43].

4.4. Methods

The blood was received from cardiac patients from *Hospital Pulido Valente* (Lisbon, Portugal) and blood donors from the Portuguese Blood Institute (Lisbon, Portugal), after their signed consent in the participation in this research project.

This study was performed with blood samples of twenty patients (N=20), all of which suffered from heart failure, with one of two etiologies to distinguish: seven patients presented ischemia (ET2), while the other thirteen patients did not (ET1). The negative control was done with the blood of twenty healthy blood donors.

In order to prepare the blood samples for AFM force spectroscopy, we performed the blood cells isolation protocol described in Chapter 2.3. For tip functionalization, we carried out the protocol described in Chapter 2.1.2. A Human wild-type fibrinogen was used to attach to the AFM tip.

Approximately 30 minutes before loading the sample in the AFM, we deposited blood cells as described in Chapter 2.1.4. Then, we calibrated the tip as required in Chapter 2.1.3. and performed the AFM force spectroscopy measurements in agreement with the protocol in Chapter 2.1.5.

4.5. Results

After analyzing the twenty donors and twenty patients, we had 16,855 curves regarding blood donors, 6,448 from erythrocytes and 10,407 derived from platelets measure. As for cardiac insufficiency, we collected 66,354 curves. From these, 30,181 curves are due to ET1 erythrocytes and 12,842 to platelet measures; from the remaining curves, 14,867 relate to ET2's erythrocytes and 8,464 to ET2's platelets.

Binding Forces

Histograms of the (un)binding forces of each studied fibrinogen-cell complex were constructed choosing the best fitted Gaussian model peak forces to obtain the average rupture force for a single fibrinogen-cell receptor binding. Forces ranging between 0 and 10 pN were considered as unspecific interactions and were not taken into consideration.

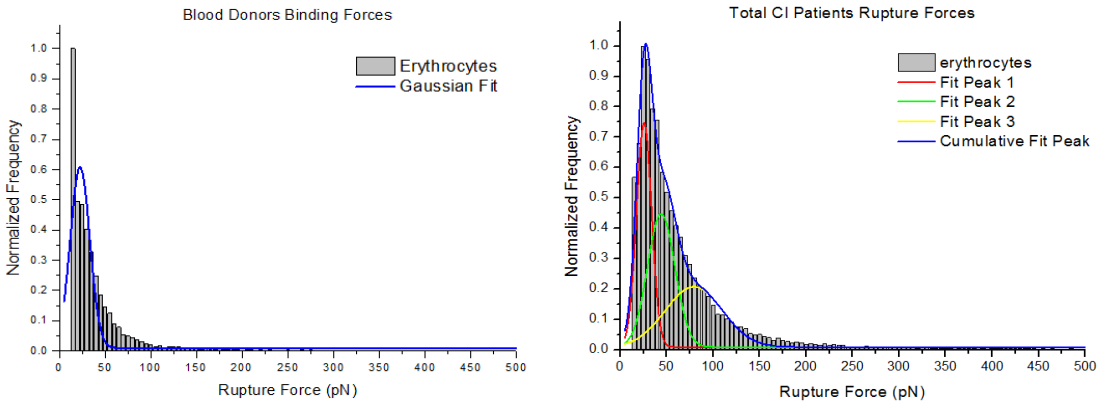

Figure 19. Blood donors and CI patients binding forces histograms. The graphic on the left represents the normalized frequency of healthy donors' erythrocytes measures. The unique Gaussian peak is at 55.2 ± 0.9 pN. The graphic on the right represents the normalized frequency counts for cardiac insufficiency erythrocytes. The three Gaussian curves used for the fitting stand at 25.6 ± 0.8 pN, the second peak at 44.1 ± 7.8 pN and the third peak at 79.2 ± 21.4 pN.

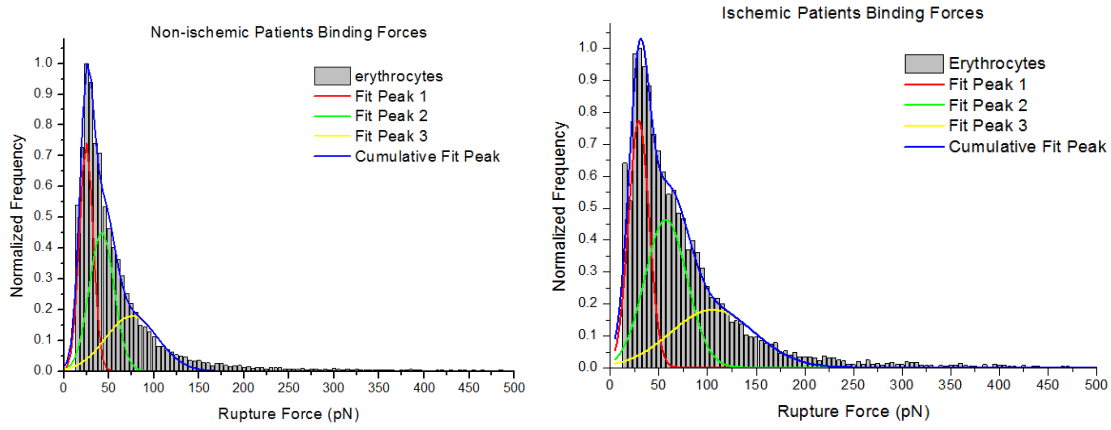

Figure 20. Ischemic and non-ischemic binding forces histograms. The graphic on the left can be fit with three rupture forces on ET1's erythrocytes. These peaks are at 27.7 ± 0.3 pN, 41.8 ± 2.0 pN and 75.1 ± 5.6 pN. On the left, the graphic refers to ET2 patients. Their erythrocytes can also be fit with three Gaussian curves. The first at 29.4 ±0 .4 pN, the second at 57.2 ± 4.0 pN and the third at 105.2 ± 24.0 pN.

The donors average erythrocyte rupture force was 42.1 ± 1.0 pN, while the patients' was 68.3 ± 0.4 pN. As for platelets, the donors' average rupture force between the cell and fibrinogen was 65.5 ± 0.8 pN and the patients' was 56.2 ± 0.5 pN. Regarding the two etiologies, ET1 patients presented average rupture forces in their erythrocytes of 63.3 ± 0.5 pN and 68.1 ± 0.8 pN in their platelets; ET2 patients had average rupture forces for their erythrocytes of 78.6 ± 0.8 pN and merely 38.1 ± 0.6 pN in their platelets.

We used the Unpaired t-test to statistically assess the differences between the fibrinogen-blood cells interaction forces for healthy blood donors and cardiac insufficiency patients.

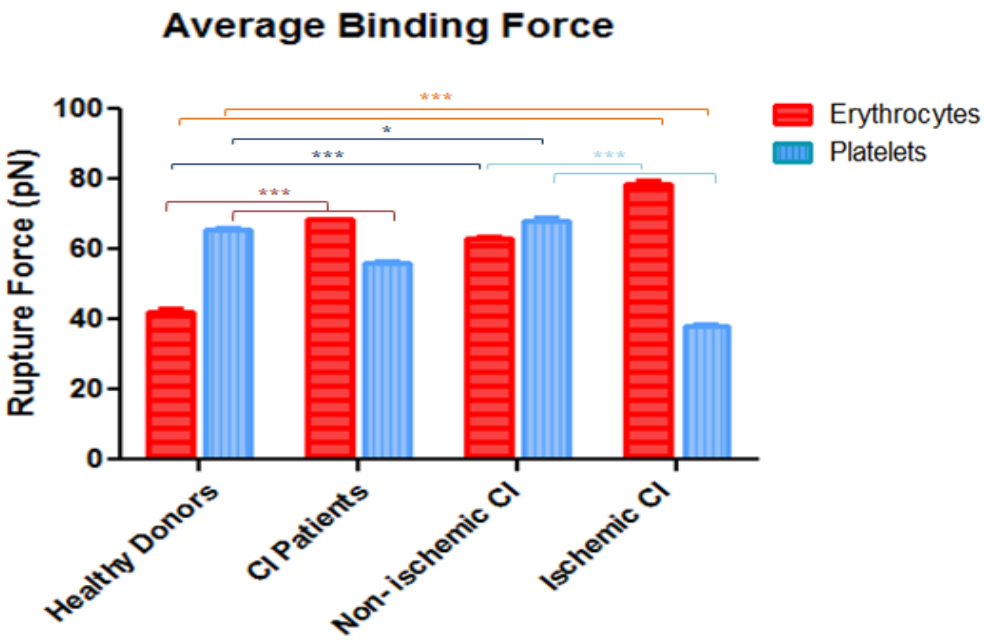

Figure 21. Binding forces comparison between all subjects. P value significance: *: 0.0018 < p < 0.05; **: 0.0001 < p < 0.0018; ***: p < 0.0001.

Regarding the etiologies, the binding forces between cell membrane and fibrinogen were all significantly different ($p<0.0001$ between all parameters, except for the comparison between the platelets from donors and ET1 patients where $p=0.0181$).

Binding Frequency

As for the binding frequency, donor erythrocytes bond 19.0 ± 1.8 % of times with fibrinogen, while patients erythrocytes bond only 15.3 ± 0.7 % of times. Donor's platelets bound 44.6 ± 1.6 % of times and patients' platelets bound to fibrinogen protein only 38.4 ± 0.8 % of times. Concerning the patients' etiologies, ET1's erythrocytes bound 15.3 ± 1.4 % and ET2's erythrocytes bound to fibrinogen with a similar frequency (15.3 ± 0.7 %). ET2's platelets bound with a frequency of 41.9 ± 1.1 %, while ET1's platelets bound with a frequency of 36.6 ± 1.5 %.

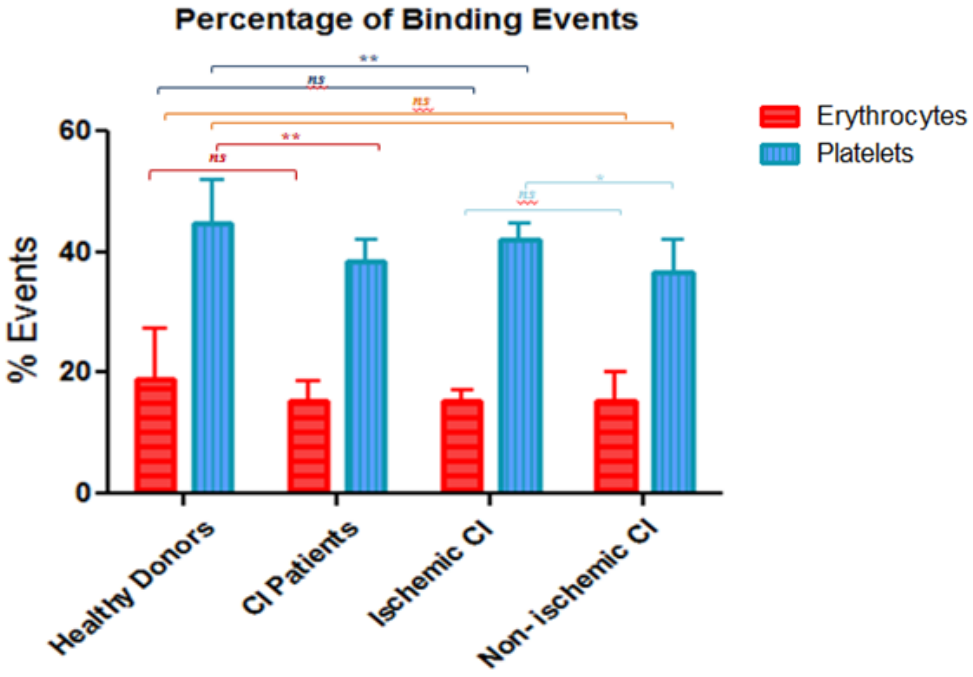

Figure 22. Percentage of events comparison between all subjects. P value significance: ns: non-significant (p > 0.05); *: 0.0018 < p < 0.05; **: 0.0001 < p < 0.0018; ***: p < 0.0001.

Applying the Unpaired t-test to these results, we verified that the binding frequency values between healthy donor and patients' erythrocytes were not significant, while the difference between donor and patients' platelet binding frequency proved otherwise, with a p value of 0.0016.

ET1's erythrocyte binding frequency was not statistically different from ET2 and donor's erythrocyte binding frequencies. ET2's erythrocytes also showed no statistical differences with the donor's erythrocytes binding frequencies.

Platelets revealed some significant divergence between the three subjects. ET1's erythrocytes presented a statistically different binding frequency from ET2 (p=0.0284) and donor's (p=0.0018) erythrocytes. As for the test between ET2 and donors' platelets, no statistically significant value was obtained.

Table 3 summarizes the AFM force spectroscopy acquired data.

Table 3. Summary of the AFM force spectroscopy measures.

	Average Binding Force (pN)		Binding Frequency (%)	
	Erythrocytes	**Platelets**	**Erythrocytes**	**Platelets**
Donors (N=20)	42.1 ± 1.0	65.5 ± 0.8	19.0 ± 1.8	44.6 ± 1.6
Total Patients (N=20)	68.3 ± 0.4	56.2 ± 0.5	15.3 ± 0.7	38.4 ± 0.8
ET1 Patients (N=13)	63.3 ± 0.1	68.1 ± 0.8	15.3 ± 1.4	36.6 ± 1.5
ET2 Patients (N=7)	78.6 ± 0.8	38.1 ± 0.6	15.3 ± 0.7	41.9 ± 1.1

4.6. Conclusions

After a thorough analysis of the obtained results, we could take some conclusions from these results.

We verified that there was a great difference between the binding forces of total patients and donors, which would be expected since these patients present higher thrombosis risks, associated with high blood viscosity, which is directly related to fibrinogen blood concentration. The main difference was on erythrocytes' binding (~26 pN, while on platelets it was ~10 pN). Binding frequency did not show great divergence between the two populations.

As for the two etiologies' binding forces, it is clear that erythrocytes and ET1 platelets maintain their high fibrinogen aggregation, while ET2 platelets suffer a great decrease in their binding forces. The binding frequency gives us insights on the effect of the pathology in the cells' membrane physiology. While erythrocytes maintain their percentage of binding, platelets increase the number of times they bind to fibrinogen, i.e. platelets' membrane seems to have more fibrinogen receptors, leading to an easier thrombi formation.

5

CHAPTER 5 – Mouse Erythrocytes-γ-Mutant Fibrinogen Interaction

5.1. Introduction

In this chapter we describe our essays with a γ' (mutant gamma chain) fibrinogen. As already explained in Chapter 3.5.6., mutated fibrinogen's γ chains are associated to cardiovascular risk [34, 36].

The fibrinogen we studied was received through a collaboration with the principal investigator of the Department of Pathology and Laboratory Medicine, School of Medicine at University of North Caroline, Alisa Wolberg. Their work lays on the study of thrombosis and bleeding disorders. To know more about this laboratory, please check their website[6]. This lab has been using this type of fibrinogen (unpublished data) on mice in order to study the fibrinogen interaction with leukocytes' integrin $\alpha_M\beta_2$, removing this receptor's ligand ($\gamma^{190-396A}$). However, the fibrinogen variant remains with its full clotting function and capacity to support platelet aggregation. This way, they evaluate if the fibrinogen sets thrombi formation through leukocyte $\alpha_M\beta_2$ receptor.

5.2. Methods

Mice blood from the *Instituto de Medicina Molecular's* rodents' facilities was used to perform these experiments.

6 http://www.med.unc.edu/wolberglab, assessed on September, 2013.

Two types of fibrinogen were used on these studies: a wild-type (WT) and a fibrinogen γ-chain mutant (γ'). Each type of fibrinogen came from two different batches. Mouse blood was isolated according to the protocol described in the Chapter 2.4. The tip functionalization and later cell deposition were performed as delineated in Chapters 2.1.2. and 2.1.4., respectively. The tip was calibrated according to 2.1.3. and AFM results were obtained as presented in Chapter 2.1.5.

For Zeta potential experiments, we prepared the sample as described in 2.2.2. and performed the measurements as presented in 2.2.3.

5.3. Results

5.3.1. AFM force spectroscopy

Erythrocytes measures consisted in 12,493 curves for WT fibrinogen and 14,258 curves for γ' fibrinogen. Regarding platelets, we obtained 9,344 curves for WT fibrinogen and 9,300 for γ' fibrinogen.

Histograms of the (un)binding forces of each studied fibrinogen-cell complex (Figure 23 and Figure 24) were constructed choosing the best fitted Gaussian model peak forces to obtain the average rupture force for a single fibrinogen-cell receptor binding. Forces ranging between 0 and 10 pN were considered noise or non-specific interactions.

Erythrocytes-WT fibrinogen interaction

Erythrocytes- γ' fibrinogen interaction

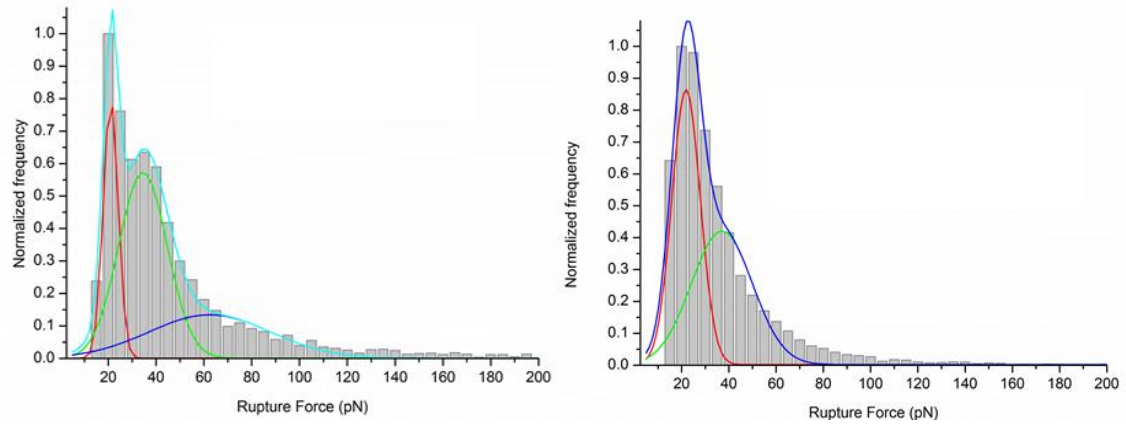

Figure 23. Rupture-force histograms for erythrocytes-wild type fibrinogen and erythrocytes-γ' fibrinogen systems. For WT fibrinogen (left), the first peak is at 21.00 $\pm$ 0.04 pN, the second peak

at 34.51 $\pm$ 0.17 pN and the third at 62.45 $\pm$ 2.33 pN. For γ-mutant fibrinogen (right), the first peak is at 21.88$\pm$0.15 pN and the second at 37.05 pN.

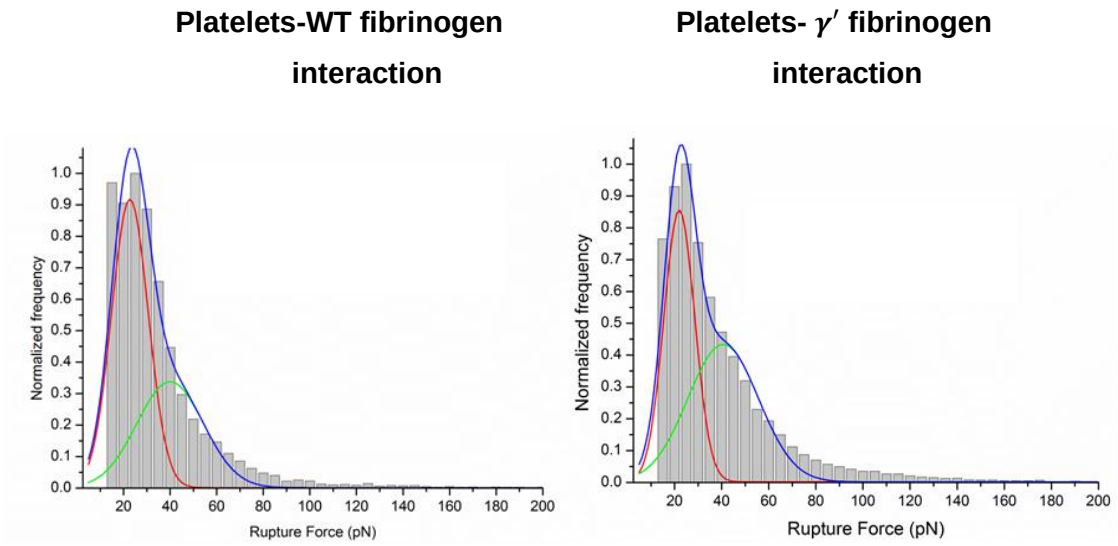

Figure 24. Rupture-force histograms for platelets-WT fibrinogen and platelet-gamma mutant fibrinogen systems. For WT fibrinogen (left), its first peak is at 22.7 $\pm$ 0.5 pN and the second at 40.0 $\pm$ 8.2 pN. The interaction between platelets and γ-mutant fibrinogen (right), yielded a first peak at 22.0 $\pm$ 0.2 pN and the second is at 40.7 $\pm$ 2.2 pN.

The AFM force spectroscopy average binding forces for erythrocytes were 52.9 $\pm$1.3 pN for WT fibrinogen and 40.2 $\pm$ 0.6 pN for γ' fibrinogen. About platelets, we obtained average binding forces of 34.0 $\pm$ 0.3 pN for WT fibrinogen and 39.2 $\pm$ 0.3 pN for γ' fibrinogen. The measured frequency of binding between fibrinogen and the erythrocyte's cell was, for WT fibrinogen, 16% and for γ' fibrinogen 20%. As for platelets, the WT fibrinogen presented 65% of binding events and γ' fibrinogen presented 62% of binding events.

After the statistical analysis, we noticed that there were no significant differences between the two fibrinogen batches. Therefore, no distinction was made for further analysis.

Table 4 and 5 summarize the acquired results.

Table 4. AFM results for the interactions of fibrinogen with erythrocytes.

Fibrinogen Type	Average Binding Force	Binding Frequency (%)
WT	53.0 ± 1.3	16
Gamma-mutant	40.2 ± 0.6	20

Table 5. AFM results for the interactions of fibrinogen with platelets

Fibrinogen Type	Average Binding Force	Binding Frequency (%)
WT	34.0 ± 0.3	65
Gamma-mutant	39.2 ± 0.3	62

5.3.2. Zeta Potential - ζ

In order to understand better how this γ-chain mutant interacts with the cell membrane we also performed Zeta potential measurements. With this methodology, we measured differences of potential that indicate electrical and physiological changes on the membrane. Zeta potential measures were performed for 0, 0.025 mg/mL and 0.1 mg/mL fibrinogen concentration with 0.035% hematocrit.

The results are presented graphically in Figure 25. It is clear that there is a large difference between the suspensions with and without fibrinogen. At lower concentrations, fibrinogen's presence effects are similar. It is on the highest concentration that the difference between WT and γ' fibrinogen becomes evident.

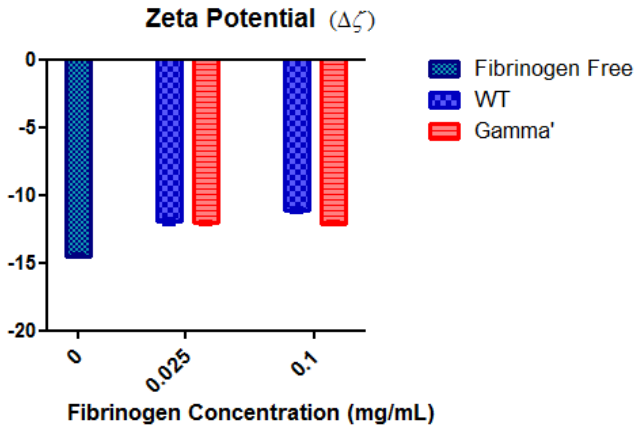

Figure 25. Zeta potential results for erythrocyte-fibrinogen interaction. Fibrinogen-free suspension presents -14.5 ± 0.14 mV. At 0.025 mg/mL of fibrinogen concentration, WT fibrinogen suspension presents -12 ± 0.29 mV and γ' suspension presents -12.1 ± 0.18 mV as Zeta-potential values. At 0.1 mg/mL of fibrinogen concentration, WT fibrinogen suspension presents -11.1 ± 0.19 mV and γ' suspension presents -12.1 ± 0.13 mV as Zeta-potential values.

The variation of the Zeta potential ($\Delta\zeta$) for each sample was calculated by subtracting from the zeta-potential value of the sample the initial value corresponding to zero fibrinogen concentration. The larger the $\Delta\zeta$, the bigger is the interaction between protein and cell membrane.

Table 6. Zeta potential variation.

	$\Delta\zeta$ (mV)	
Fibrinogen Concentration	0.025 mg/mL	0.1 mg/mL
WT Fibrinogen	2.5 ± 0.15	3.4 ± 0.05
γ' Fibrinogen	2.4 ± 0.04	2.4 ± 0.01

Table 6 shows that upon fibrinogen binding, the erythrocyte surface charge increases. For higher concentrations, the presence of γ-mutant fibrinogen reveals lower Zeta potential difference, suggesting that there is a lower binding between the cell and the protein, while WT fibrinogen, with a larger Zeta potential difference, seems to be more prone to bind to erythrocytes.

5.4. Conclusions

As mentioned before, this fibrinogen γ chain variation does not impair the platelets' clotting function. This confers with the AFM results on Table 5, where there is no significant difference between the WT and gamma-mutant binding to platelets (34.0 ± 0.3 pN and 39.2 ± 0.3 pN, respectively) and frequency of binding (62% and 65%).

On the other hand, erythrocyte data indicates that this mutation leads to lower binding forces in the interaction with γ-mutant fibrinogen (from 52.9 ± 1.3 pN for WT to 40.19 ± 0.628 pN for γ'), but higher binding frequency (from 16% to 20%).

As for Zeta potential results, we considered that the number of samples available were not enough to take many conclusions. Nevertheless, our results suggest that the γ-mutant does not bind to erythrocyte as well as the WT fibrinogen.

Fibrinogen's effect on the membrane is not very observable at low concentration, ($\Delta\zeta$ is 0.025 ± 0.15 mV for WT and 2.4 ± 0.04 mV for γ'), but, according to WT fibrinogen, when the concentration increases, it is clear that fibrinogen interacts more with the cell ($\Delta\zeta$ is 3.4 ± 0.05 mV for WT). Between both types of protein, we can distinguish that WT presents a higher affinity to the membrane, with $\Delta\zeta = 3.4 \pm 0.05$ mV, than the γ-mutant type as it does not present Zeta potential changes ($\Delta\zeta$= 2.4 ± 0.01 mV).

6

CHAPTER 6 – General Conclusions and Future Work

6.1. Conclusions

This project had duration of six months. For such a short time and for the number of essays performed, we can say that our results are quite conclusive.

Regarding the cardiac insufficiency patients, it is clear that a twenty people population is not enough to make solid conclusions, but we may start drafting some important ideas. Further investigation in this project may bring, not only notoriety for the AFM techniques, but also new clinical insights in pharmacology. Thus, it is urgent that we conclude the characterization of the erythrocyte's receptor for fibrinogen in order to develop new drugs that inhibit higher erythrocyte aggregation.

The mouse's erythrocytes measures showed that this γ-mutant does not interfere with platelets aggregability function. However, the fibrinogen's ligand to the leukocyte's receptor $\alpha_M\beta_2$ seems to interact with the erythrocyte's receptor for fibrinogen.

As for me, the author of this thesis, I found profoundly remarkable working in *IMM.* This past six months really filled me with great will to pursue this work. They were months of hard work, but when it all came together, and we started to see such sense in the acquired data, I could understand that research is even more exciting than I already thought it was. For this, I am very glad I had this opportunity of working in such an institute.

6.2. Future Work

As a closer future work, we aim at increasing the number of cardiac insufficiency patients enrolled in this study, and achieve the same number of subjects from each etiology.

Then, a one-year follow-up of myocardial infarction and hypertension patients will continue. We will perform the same AFM force spectroscopy measurements, focusing on the fibrinogen-erythrocyte interactions, and on rheological studies on erythrocyte aggregation. We shall add a new technique that can be coupled to the AFM: the cell-hesion module (already acquired) that allows cell-cell adhesion assays. This way, we can evaluate the possible association between the reduction of fibrinogen-mediated erythrocyte-erythrocyte interactions with the reduction and/or prevention of cardiovascular pathologies. Flow cytometry assays will also be performed.

This way, we expect to be able to fully identify the receptor for fibrinogen on the erythrocyte membrane and to identify which drug(s) may be successfully used to overcome the risks of fibrinogen-driven erythrocyte hyperaggregation. We expect to be able to provide new insights on the identification of some prethrombotic markers on the prevention of cardiovascular diseases. We also anticipate having a significant decrease of the erythrocyte cell elasticity and higher forces necessary to break the erythrocyte-fibrinogen-erythrocyte bridging on both types of patients, since an increase of erythrocyte aggregation on this cardiovascular disorders is expectable.

7

CHAPTER 7 – References

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