UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Immunohistochemical Identification of macrophages in a poly (L-lactic acid) film implant with standard and superhydrophobic wettability

DISSERTAÇÃO DE MESTRADO EM ENGENHARIA BIOMÉDICA

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ORIENTADORES:

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ANO DE CONCLUSÃO: 2019

DECLARO QUE ESTA DISSERTAÇÃO DE MESTRADO É RESULTADO DA MINHA PESQUISA E TRABALHO PESSOAL E DAS ORIENTAÇÕES DOS MEUS SUPERVISORES. O SEU CONTEÚDO É ORIGINAL E TODAS AS FONTES CONSULTADAS ESTÃO DEVIDAMENTE MENCIONADAS NO TEXTO E NA BIBLIOGRAFIA FINAL. DECLARO AINDA QUE ESTE TRABALHO NÃO FOI APRESENTADO EM NENHUMA OUTRA INSTITUIÇÃO.

VILA REAL, 25 DE OUTUBRO DE 2019

Samuel Sales Garcia

Dissertação apresentada à Escola de Ciências e Tecnologia da Universidade de Trás-os-Montes e Alto Douro como requisito para a obtenção do título de Mestre em Engenharia Biomédica.

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ABSTRACT

In the Tissue Engineering area, it is common the use biomaterials composed by matrices with a high percentage of collagen, which are perfect to tissue rebuilding and Regenerative Medicine. It is also known that the collagen support cell migration and vascularization process.

However, in recent decades, the use of polymers as scaffolding matrices in tissue regeneration has been widely extended. Polymers can be modified to gather the desired biodegradability and bioactivity characteristics, and they have a wide variety of mechanical characteristics, which gives them different functions in accordance with the material component. The functions that are most indorsed to these biomaterials are drug delivery and temporary prosthesis.

Thus, in this work, poly (L-lactic acid) (PLLA) films with different wettability (standard / super-hydrophobic) (S and SH, respectively) were used in an *in vivo* subcutaneous implant in rats (*Rattus norvegicus*) in order to measure the inflammatory response, using immunohistochemistry technique. The semi-crystalline polymer poly (L-lactic acid) is an aliphatic polyester which is characterized by good biodegradability, biocompatibility and versatility, and it has reasonable mechanical and processability properties in fiber formation.

After implantation, it occurs an immune response in the surgery local, and depending on the implanted materials characteristics, the inflammatory reaction will vary.

For this study 18 rats were used, 9 were implanted with PLLA S and 9 with PLLA SH. Each rat was implanted with 6 PLLA discs in 6 different sites, 4 subcutaneously and 2 intramuscularly. After collecting the samples (n=108), immunohistochemistry was performed for three antibodies: CD3 (T lymphocytes), CD163 (M2 macrophages) and CD68 (Total macrophages).

There was a decrease in the number of cells identified around the site of the implant site for both biomaterials, where it was possible to verify that the superhydrophobic PLLA promoted a minor inflammatory response.

Therefore, it was observed that for both biomaterials the inflammatory reaction decreased over time and that the adaptive immune system was not strongly activated.

Keywords: tissue engineering, poly (L-lactic acid), super-hydrophobicity, biomaterial, inflammatory response, immune-histochemical, wettability, rat model

RESUMO

Na área da Engenharia de Tecidos é comum o uso de biomateriais em matrizes com alta percentagem de colagénio indicadas em Medicina Regenerativa para a reconstrução de tecidos, pois é sabido que o colagénio favorece o processo de migração e vascularização celular.

No entanto, nas últimas décadas, o uso de matrizes poliméricas tem sido amplamente usado em Medicina Regenerativa. Os polímeros podem ser modificados correspondendo assim às características de biodegradabilidade e bioatividade desejadas e possuem uma grande variedade de características mecânicas, o que lhes confere funções diferentes de acordo com a sua composição. Estes biomateriais são muito usados como veículos na administração de medicamentos e na composição de próteses temporárias.

Assim, neste trabalho foram utilizados filmes de poli (L-ácido láctico) (PLLA) com diferentes características hidrofílicas (atandard / superhidrofóbico) (S e SH, respetivamente) em implantes *in vivo* a nível subcutâneo em ratos (*Rattus norvegicus*) para análise da intensidade de inflamação promovida com a técnica de imunohistoquímica. O polímero semi-cristalino poli (L-ácido láctico) é um poliéster alifático que se caracteriza por boa biodegradabilidade, biocompatibilidade e versatilidade, e por possuir propriedades mecânicas e de processamento na formação de fibras.

Após a implantação dos filmes de biomaterial ocorre uma resposta inflamatória da parte do organismo no local da cirurgia e, dependendo das características dos materiais implantados, a promoção de uma reação inflamatória variável.

Para este estudo foram usados 18 ratos, 9 foram implantados com PLLA S e 9 com PLLA SH. A cada rato foi-lhe implantado 6 discos de PLLA em 6 locais diferente, 4 subcutâneos e 2 a nível intramuscular. Após a recolha de amostras (n=108) realizou-se a técnica de imunohistoquímica para três anticorpos: CD3 (linfócitos T), CD163 (macrófagos M2) e CD68 (macrófagos totais).

Observou-se a diminuição do número de células identificadas em volta do local do local do implante para ambos os biomateriais, onde foi possível verificar que o PLLA super-hidrofóbico causou uma resposta inflamatória mais reduzida.

Concluímos que, para ambos os biomateriais, a reação inflamatória diminuiu ao longo do tempo e o sistema imunológico adaptativo não foi fortemente ativado.

Palavras-chave: engenharia de tecidos, poli (ácido L-láctico), super-hidrofóbico, biomaterial, resposta inflamatória, imunohistoquímica, molhabilidade, rato

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LIST OF ABBREVIATIONS AND ACRONYMS

Ab- Antibody

Ag- Antigen

CA- Contact Angle

FBMGCs- Foreign Body Multinucleated Giant cells

FDA- Food and Drug Administration

IHC- Immunohistochemistry

IM- Intramuscular

ISSO 10993- Documents defining the international harmonization of the safe use of medical devices

LHAP- Laboratory of Histology and Anatomic Pathology

MAA- Methacrylic Acid

MDPs- Macrophages and Dendritic cell Precursors

PLA- Poly (Lactic acid)

PLLA- Poly (l-Lactic acid)

PMN- Polymorphonuclear Neutrophils

RM- Regenerative Medicine

RP- Rapid Prototyping

SC- Subcutaneous

SFF- Solid Free-Form Fabrication

TE- Tissue Engineering

TERM- Tissue Engineering and Regenerative Medicine

1 INTRODUCTION

1.1 TISSUE ENGINEERING

Tissue Engineering (TE) was a term adopted in 1987, which emerged as a specified scientific area, following an independent rout from the medical evolution, but often paralleling and providing support to it [Qizhi, 2012]. It has been defined as an application of the engineering principles to the life sciences, in order to develop new methods for repair and restitution of injured or amputee [Badylak, 2004]. Tissue engineering mimic the natural tissue repair and regeneration [Correia, 2013], using not only the engineering principles, but also biological and chemical applications [Cosgriff-Hernandez, 2008]. We can divide TE in two varieties, the traditional type, which combines isolated cells with bioactive agent and a scaffold soaked with primary cells or multipotent stem cells before the implantation. On the other hand, we have the acellular type, where the scaffolds have biological structures to promote cell growth after the implantation [Cosgriff-Hernandez, 2008]. Regenerative Medicine (RM) was evolved on pair with TE, however, it is considered a broader concept, which includes the use of bioactive soluble molecules, stem cell technologies, genetic therapeutic strategies, nanotechnologies and numerous medical devices [Correia, 2013]. The objective of RM is to restore a biological function that has been modified or compromised by an injury or disease, by replacing or repairing and promoting cells, tissue or organs regeneration [Daar & Greenwood, 2007]. The importance of RM can be overseen on customized approaches, where the bioengineered products are tailored into patient needs, which can reduce recovery times and increase successful rate [Neves et al., 2016]. It is possible to see in Figure 1 the various disciplines that involve RM.

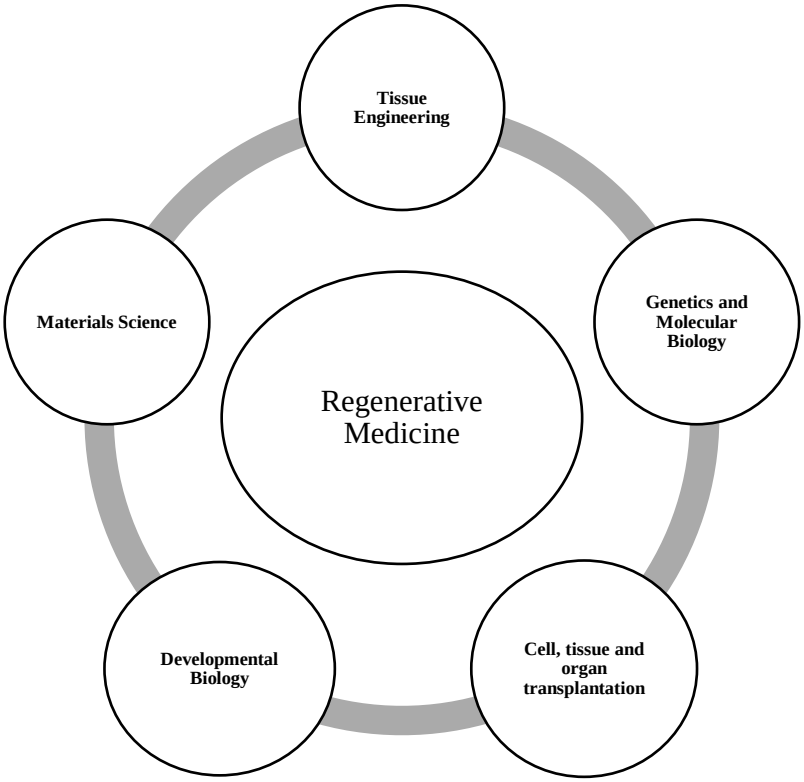

Figure 1 Disciplines in which Regenerative Medicine consist [Adapted from Daar & Greenwood, 2007].

1.2 BIOMATERIALS APPLIED TO TISSUE ENGINEERING

The implanted biomaterials are more complex than those used one decade ago as observed on Table 1. Not only because of the evolution of the materials used (metal, ceramics, synthetic polymers and biopolymers), but also related with the requirements of this, as they will interact directly with specific cells, tissues and organs [Williams, 2009]. The choice of different polymer is ordained by its application and requires a thoughtful consideration regarding its properties [Shoichet, 2010]. Some applications require biomaterial with unique physical, chemical, biological and biomechanical properties to provide efficient therapy, so both the natural polymers and the synthetic polymers could been chosen [Nair & Laurencin, 2005]. The degradable polymeric biomaterials are the favorite candidates for therapeutic

devices, like prostheses, controlled/sustained release drug delivery vehicles and three-dimensional porous structures as scaffolds for TE [Nair & Laurencin, 2007].

Table 1 A summary of the main properties and applications of synthetic polymeric biomaterials. [Adapted from Angelova & Hunkeler, 1999].

SYNTHETIC POLYMERS	
ALIPHATIC POLYESTERS	
-POLY (LACTIC ACID), POLY (GLYCOLIC ACID) AND THEIR COPOLYMERS	Used in sutures, drug-delivery systems and in tissue engineering. Biodegradable. Often copolymerized to regulate degradation time.
-POLY (HYDROXY BUTYRATE), POLY (E-CAPROLACTONE) AND COPOLYMERS, POLY (ALKYLENE SUCCINATES), ETC	Biodegradable, used as a matrix for drug-delivery systems, cell microencapsulation. Properties can be changed by chemical modification, copolymerization and blending.
POLYAMIDES (NYLONS)	Sutures, dressing, hemofiltration membranes.
POLYANHYDRIDES	Biodegradable, useful in tissue engineering and for the release of the bioactive molecules.
POLY (ORTHO ESTERS)	Surface-eroding polymers. Application in sustained drug delivery. Ophthalmology.
POLY (CYANO ACRYLATES)	Biodegradable, depending on the length of the alkyl chain. Used as surgical adhesives and glues, potentially used in drug delivery.
POLYPHOSPHAZENES	Can be tailored with versatile side-chain functionality. Made into films and hydrogels, Applications in drug delivery.
THERMOPLASTIC POLYURETHANES	Good elastomeric properties. Can be tailored by varying the starting materials. Used in permanently implanted medical devices (prostheses, vascular grafts), catheters and drug delivery systems, Initial candidates for the artificial heart.

For a scaffold to be successful after implantation, it needs a good combination of cell growth through the biomaterial, to support it, and bioreactors, to direct it [Jagur-Grodzinski, 2006]. Biocompatibility, biodegradability, mechanical properties, architecture and manufacturing technology are the requirements of scaffolds [O'Brien, 2011], as represented on Table 2.

Table 2 Main features of the scaffolds and arguments used by researchers in the field, portraying their importance.

PROPERTIES	ARGUMENTS
BIOCOMPATIBILITY	Biocompatibility of polymer-based biomaterials is of utmost importance for therapeutic uses [Hanks et al., 1996].
BIODEGRADABILITY	For the development of therapeutic devices such as temporary prostheses and three-dimensional structures, for example scaffolds, biodegradable polymeric biomaterials are the right choice [Naira & Laurencina, 2007].
MECHANICAL PROPERTIES	What makes a scaffold desirable for bone regeneration is its ability in moldability and self-harden in situ [Xua & Simon, 2005].
SCAFFOLD ARCHITECTURE	In determining bone growth rate and growth angle, scaffold architecture plays a major role, its porosity and interconnectivity being of utmost importance [Woodarda et al. 2007].
MANUFACTURING TECHNOLOGY	The scaffold's manufacturing technology and design is the core of tissue engineering [Xiong, 2002].

For a material to be successfully implanted it is intended to be bio-stable, the reaction of the host tissues to the implants have to be minimal and the graft is expected to maintain its integrity and / or to be biodegradable, meaning that it is expected that the polymer fulfills its supporting role, being degraded by the body into products that are eliminated by normal excretory pathways and replaced by host tissue [Kulkarni et al., 1971].

In order to verify the biological safety of biomaterials, regulation of ISO 10993 (documents defining the international harmonization of the safe use of medical devices) should be a model [David, 2014].

1.3 SUPERHYDROPHOBIC SURFACES

Tissue engineering has long sought the use of harmless and inert biomaterials. The objective was the biomaterial encapsulation by the host after transplantation. However, the evolution in the biomaterials knowledge has progressively moved away from bio-passive surfaces and advanced in relation to bioactive biomolecular surfaces (Figure 2), which communicate with host tissue and respond in a physiological way [Morra, 2004]. This can be observed on functionalized titanium surfaces. Which facilitate cell growth and multiplication in osteoblast cultures, as well as facilitating and contributing to cell binding and proliferation [Miyauchi et al, 2010].

Biomaterials with antibacterial surfaces are also increasingly used, with surface coatings and / or altering the surface architecture [Hasan et al., 2013].

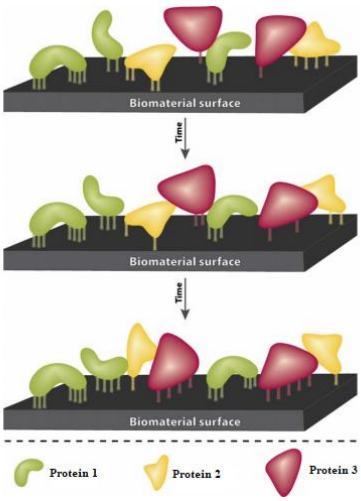

Figure 2 Evolution of the increasing protein-surface binding, when the surface is water-friendly. It is possible to see an increase in the number of connections between the proteins (1, 2 and 3) and the biomaterial surface [Adapted from Schmidt et al., 2009].

Surface wettability of a biomaterial is one of the most important parameters for the biological response to the implant. Wettability is the passivity with which a fluid spreads on a solid surface or more specifically as the fluid adheres to its surface [Menzies & Jones, 2010]. Protein adsorption is thought to be the result of solvent-protein interactions with enough energy to attract proteins from the solution. In turn, solvent-protein interactions will be related to surface water adhesion, including Van der Waal interactions, electrostatic interactions, hydrogen bonding and hydrophobic interactions. Thus, wettability affects blood coagulation, protein adsorption, platelet activation / adhesion, and cell and bacterial adhesion [Xua & Siedlecki, 2007]. In addition, modification of the surface of a biomaterial may render antibiotic use unnecessary, as it may preclude the creation of biofilm on its surface [Bazaka et al., 2012].

The higher the interfacial tension the lower the attraction between liquid and solid and the smaller the liquid scattering on the surface of the solid, as seen in Figure 3. In order to measure the wettability of biomaterials *in vitro* it is necessary to make the contact angle measurement at the liquid-solid interface [Menzies & Jones, 2010]. It is accepted by the scientific community that a surface is considered hydrophilic when a droplet of water has a

contact angle θ is <90°, hydrophobic when contact angle θ is >90° and superhydrophobic when θ is >150° with the surface [Kock-Yee, 2014]. This can be seen in Figure 4. This extreme wettability is only possible by combining surface chemistry (free surface energy) and roughness [Oliveira et al., 2018].

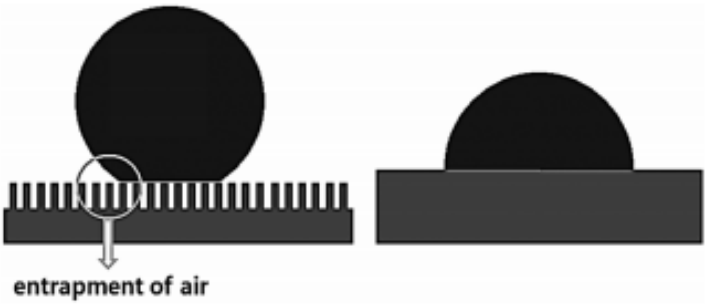

Figure 3 Biomaterial surfaces: on the left a hydrophobic surface, on the right an untreated and hydrophilic surface [Adapted from Lim et al., 2013].

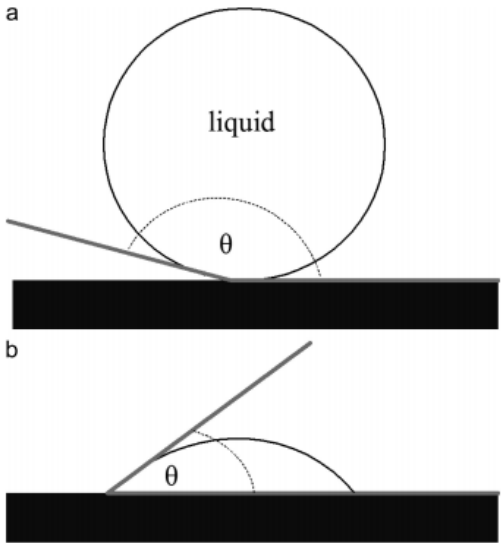

Figure 4 Contact angle θ. a) superhydrophobic surface b) hydrophilic surface [Adapted from Menzies & Jones, 2010].

1.4 PLLA- POLY (L-LACTIC ACID)

Used in the biomedical field since the years 60's, the poly (l-lactic acid) (PLLA) is chosen concerning its biocompatibility, biodegradability, excellent thermal / chemical properties and transparency [Krikorian & Pochan, 2003]. Its L-enantiomer, known as poly (L-lactide), has high tensile strength and module. Can be easily processed in thermoplastic by conventional processing techniques such as blow molding, thermoforming, injection molding and extrusion [Fundador et al., 2010]. The PLLA is a biodegradable and biocompatible synthetic polymer being used as a biomaterial in various biomedical applications [Yang et al., 2004].

The PLLA (Figure 5) is synthesized by bacterial fermentation of plant starch, is an aliphatic of polyester which is derived from lactic acid [Fundador et al., 2010]. This biodegradable thermoplastic is produced by opening the lactic acid polymerization ring [La Carrubba, 2008], or by the condensation and polymerization of all acid monomer stereoisomers pure D- or L-lactic acid or a racemic substance of both. The latter is synthesized by potato fermentation, corn, beaten sugar and sugar cane, among other vegetables sources [Krikorian & Pochan, 2003]. In the study of Fundador and colleagues [2010] nanofiber non-woven PLLA and nanofiber based PLLA were produced by electrospinning techniques, and they have concluded that as the surface of these biomaterials have an extensive area, they are suitable for biomedical applications. When PLLA poly (lactic acid) degrades, lactic acid is usually formed and enter in the tricarboxylic acid cycle and is excreted via metabolic pathways, notably water and carbon dioxide. To date no significant accumulation of residues originating from PLLA degradation has been detected in any of vital organs [La Carrubba, 2008].

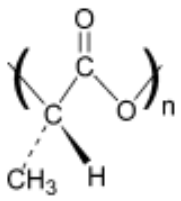

Figure 3. Chemical structure of PLLA [Adapted from Krikorian & Pochan, 2003].

Poly (lactic acid) has been used in many areas from agriculture, coating films, towels, carpets and the biomedical area, is currently used as a surgical suture material and controlled drug release devices, among other pharmaceutical and medical purposes [Mikos et al., 1994], as it has also been used as a replacement and reconstruction implant for tendon and artificial ligaments [Menzies & Jones, 2010]. The PLLA is also used as a scaffold matrix, as exemplified by the work of Ma and colleagues [2005], where the surface of the biomaterial was treated with UV-induced grafting polymerization of methacrylic acid (MAA), in order to obtain a stable layer of collagen on its surface.

1.5 PLLA- SUPERHYDROPHOBIC SURFACE

The PLLA with superhydrophobic surface is a biodegradable and biocompatible synthetic polymer with great ambitions in the biomedical area, as it is one of the few allowed by Food and Drug Administration (FDA, USA) for clinical use in human and ecological applications [Shi et al., 2008]. In Europe the European Community's Scientific Committee on Food reached the same conclusion [Conn et al., 1995], as to control cell behavior in biodegradable substrates [Alves et al., 2009]. Cell adhesion in PLLA increases with the roughness of the surface as it is shown in the study of Wan and colleagues in 2005, where the OCT 1 osteoblast-like cells show greater adhesion on smooth surfaces, however proliferation decreases. Poly (L-lactic acid) surfaces with super hydrophobic characteristics are produced based on the so-called Lotus effect, which exhibits a dual micro and nanoscale roughness

[Alves et al., 2009]. To obtain a rough and superhydrophobic surface from PLLA we can follow the method described by Lourenço and coworkers [2012]. In their technique they used flat and smooth substrates, which were produced by PLLA powder fusion. Two glass slides at 200° C were used to compress the material, followed by cooling using water. A 13% (w / v) PLLA solution in 1.4-dioxane was fused to the substrates. After cooling the substrates were immersed in absolute ethanol to induce phase separation of the molten solution, the samples were then dried under a nitrogen flow and then in a vacuum oven at 30° C for a period of 24 hours. Once the samples are completely dry, the upper part is removed, resulting in the rough superhydrophobic surface of the PLLA. Recently it has been shown that the PLLA-based superhydrophobic have potential to be used as a carrier of biomass immobilization in bioreactors, as it has been shown that rough surfaces of superhydrophobic PLLA are easily colonized by bacteria [Sousa et al., 2011]. However, in a study by Tang and colleagues [2011], it was revealed that superhydrophobic surfaces have high resistance to bacterial contamination and can thus be used in laboratory and clinical practice as antimicrobial, thus reducing the risk of device-associated infections.

Therefore, biomaterials that have superhydrophobic surfaces are used in the biomedical practice and can be used in endotracheal tubes, catheters, controlled patterns of superhydrophobic and hydrophilic regions used to construct superhydrophobic coated medical instruments to reduce bacterial adhesion when in contact with blood or body fluids, medical drug-coated instruments and disposable diagnostic devices in which the superhydrophobic surface supports droplets or facilitates fluid flow [Falde et al., 2016].

1.6 INFLAMMATORY REACTION TO BIOMATERIALS

The cellular behavior and inflammatory response depend on various characteristics and properties of the biomaterial surface, such as topography, wettability, roughness, electric load, biochemical signals, surface rigidity and functional groups [Oliveira et al., 2012]. Although the evolution made on biocompatibility, side effects such as bio-incompatibility-induced inflammation and infections may cause loss of function, necrosis and fibrosis associated with many materials and procedures [Nilsson et al., 2007]. Therefore, lesion and subsequent slight or intense inflammatory response is expected after implantation of a biomedical devices, artificial organs or biomaterials. Which could be an acute, chronic or granulomatous inflammation with foreign body reaction having granulation tissue, multinucleated giant cell and fibrosis [Anderson, 1988].

When implanted, the surfaces of biomaterials interact with surrounding tissues, which causes some degree of tissue damage (necrosis), which will initiate two major reactions, inflammation (immune response) and repair-related response (wound healing) [Kirkpatrick, 2002]. An initial contact with the blood influences the inflammatory reaction against the material, this inflammation is mediated by the complement system [Morikis & Lambris, 2001] which destroy and remove foreign substances, by direct lysis or mediating leukocyte phagocytosis by opsonization and cell lysis [Nilsson et al, 2007].

During the inflammation, the early stage of healing begins with increased endothelial permeability, infiltration of inflammatory cells and secretion of various growth factors and chemokines. Controlling wound contamination and consequent infection, as well as inducing the repair process, the inflammatory response plays an important role in the wound healing process [Chen et al., 2012].

After implantation, the biomaterial begins to absorb protein, initially fibrinogen, immunoglobulin G (IgG) and albumin, which are later replaced by high molecular weight kininogen and up to extension, by Hageman factor (factor XII) [Pankowsky et al., 1990]. These molecules are responsible for complement system and platelet activation as well granulocytes

and macrophages chemotaxis [Nilsson et al., 2007]. Immunoglobins and complement are also responsible for macrophages phagocytosis stimulation [Jenney & Anderson, 2000].

Therefore, following implantation, inflammatory (Figure 6) cells flow to the site of injury, causing a pre-matrix, which is no more than the clot involving the biomaterial and the tissue reaction. The acute inflammation (with predominance of polymorphonuclear leukocytes, namely neutrophils) and chronic inflammation (that followed and with predominance of macrophages and lymphocytes) usually lasting 2 to 3 weeks after implantation [Anderson, 2015]. Neutrophils predominate during the early days and disappear within 24 to 48 hours after injury and are then replaced by macrophages that differ from blood monocytes, which have a long life that can last up to months [Anderson, 2001].

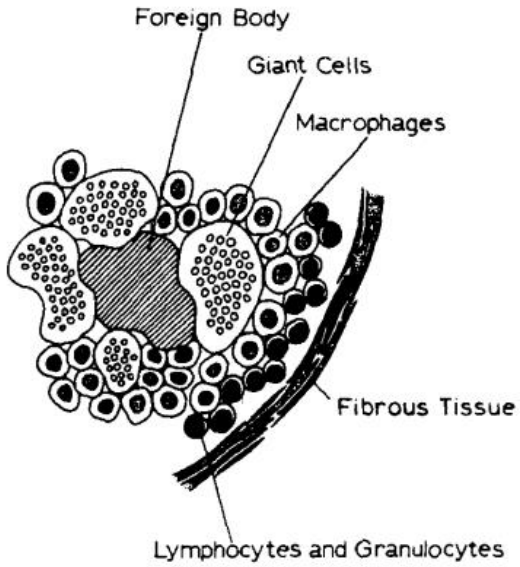

Figure 4 Foreign body granuloma, a reaction to an unknown material [Adapted from Coleman et al., 1974].
material. [Adapted from Coleman et al., 1974].

Upon implantation of a biomaterial the host initiates a sequence of events like a foreign body reaction starting with an acute inflammatory response. In many cases it leads to a rapid chronic inflammatory response and/or foreign body reaction and development of a fibrous capsule [Babensee et al., 1998]. The chronic inflammatory response is known by the phase in

which macrophages become the predominant cell type, which are responsible for wound healing and foreign body reaction [Anderson, 1993]. In chronic inflammation this is concomitant to the healing process. A specific type of chronic inflammation is the foreign body reaction [Coleman et al., 1974]. It is possible to see the inflammation phases on the Figure 7.

The macrophages have two different populations: M1 inflammatory, which inhibits cell proliferation and can cause tissue damage, and M2, which promotes cell proliferation and tissue repair [Mills, 2012].

The granulation tissue is the result of endothelial cell proliferation, maturation and capillary organization, fibroblast proliferation as well as synthesis of collagen and proteoglycans [Anderson, 1993]. The blood vessels are regulated by locally released angiogenic factors [Howdieshell et al., 2001]. The materials usually have a surface intrinsic response, which is composed of macrophages and foreign body multinucleated giant cells with varying degrees of granulation tissue [Anderson, 1993].

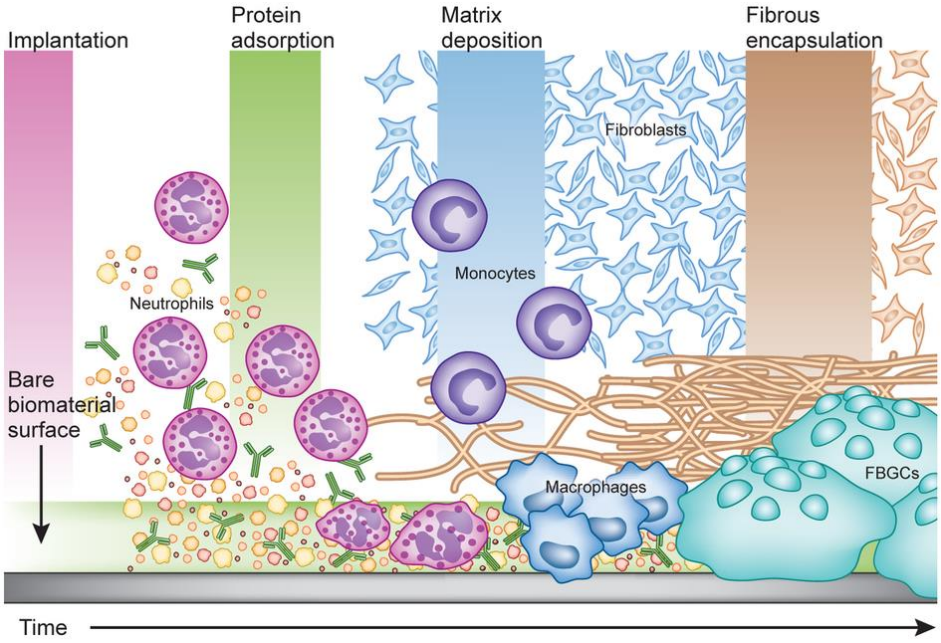

Figure 5 Temporal variation of the acute inflammation, chronic inflammatory reaction and the development of granulation tissue/repair tissue. This is the kinetics of foreign body reaction promoted by the biomaterial implantation [Babensee et al., 1998].

The process of macrophage activation and fusion to form foreign body multinucleated giant cells (FBMGC) is unique to the macrophage phenotype. Often the presence of FBMGCs is used as a histopathological marker for chronic inflammation and foreign body reaction in the host [Kao et al., 2001]. During the foreign body reaction, the monocytes recently migrated from blood differentiate into macrophages in the tissues with biomaterial and adhered to its surface. The impossibility to phagocyte the biomaterial promotes the fusion of several macrophages in the neighboring of the implanted material, forming the FBMGCs [Pires, 1998], which remain adherent at the tissue surface (isolating it) over the life of the biomaterial [Sheikh et al., 2015].

Therefore, the end-stage healing response to biomaterials is usually fibrosis in a form of scar or a capsule [Anderson, 1993]. When PLLA was implanted in subcutaneous tissue of sheep, it was found that the tissue capsule formed around the polymer is composed of fibroblasts, fibrocytes, phagocytes, some FBMGCs (Figure 8) and PMN cells.

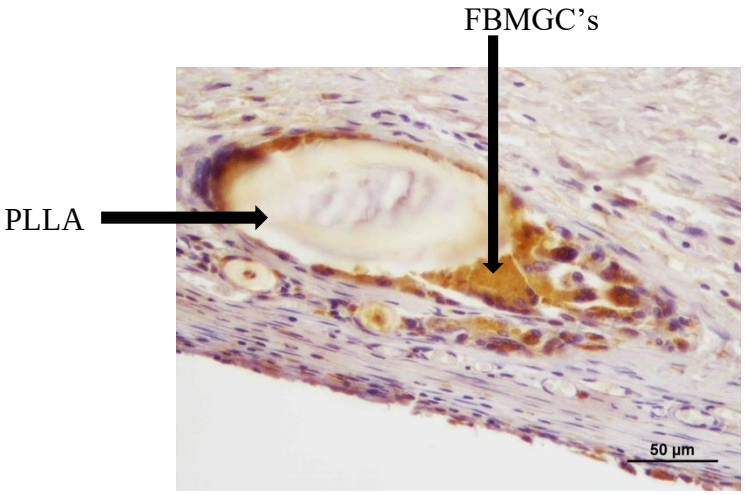

Figure 6 Microscope image of the PLLA implant zone, where foreign body multinucleated giant cells (FBMGCs) formation around the polymer, can be visualized. Immunohistochemical evaluation for macrophages (CD68), counterstained with Gill's Hematoxylin

Three months after implantation, the capsule was denser, its thickness and cell diversity increased slightly compared to the beginning and increased continuously until 6 months when it was more mature fibrous tissue [Mainil-Varlet et al., 1996].

1.7 INFLAMMATORY CELLS CHARACTERIZATION

Normal wound healing is a complex process in which damaged tissue is removed and gradually replaced by reparative tissue during a series of sequential events, which include inflammation, cell proliferation and tissue remodeling and tissue repair [Clark, 1988]. Bacteria at the site of injury release endotoxins, which are responsible for neutrophils and macrophage activation [Wolpe & Cerami, 1989].

Macrophages are known to play an important role in tissue healing and repair [Tonks, 2003], so their recruitment is essential for effective control and elimination of bacterial, viral, fungal and protozoal infections [Shi & Pamer, 2011]. The ability to mobilize monocytes (that differentiated into macrophages) and move where they are needed is essential to their role in promoting immune defense during infection and in the conduction of inflammatory diseases [Shi & Pamer, 2011]. This family cell type is a subset of circulating leukocytes that differentiate into a variety of macrophage and dendritic cells [Shi & Pamer, 2011]. Therefore, the involvement of macrophages in injuries and repairs depend heavily on the influx of circulating monocytes into the blood [Clark, 1988].

Thus, macrophage and dendritic cells precursors (MDPs) found in bone marrow differentiate into circulating monocytes, which in turn differentiate into macrophages or dendritic cells [Shi & Pamer, 2011]. Once in the tissue, monocytes differentiate into macrophages, which release growth factors that initiate inflammation and the repair (granulation) tissue formation [Clark, 1988]. Macrophages (that have highly expressed CD68 on their membrane) are known to interact with other cells and molecules by releasing numerous secretory products and expression of various surface receptors [Holness & Simmons, 1993; Sato et al., 1998].

The differentiation of monocytes into macrophages can occur in two scenarios, the first with Th1 cytokines influence, that promote the differentiation into M1 macrophages, and the second where Th2 cytokines lead to an " alternative activated" anti-inflammatory macrophage M2 phenotype [Bouhlel et al., 2007]. Type M1/M2 populations represent the two main and opposite activities of macrophages, M1 activity is inflammatory, inhibits cell proliferation and may cause tissue damage, while M2 activity is responsible for promoting cell proliferation and tissue repair [Mills, 2012]. Type M2 macrophages represent a higher percentage of macrophages in the tissues which are reported to have different gene expression profile, characterized by high expression of CD206, arginase-1, MglI and interleukin (IL)-10, involved in the repair or remodeling of tissue [Fujisaka et al., 2009]. The CD163 is a hemoglobin scavenger receptor that has high expression in M2 macrophages [Moestrup & Møller, 2004], CD163 is a receptor member of the Scavenger Receptor Cysteine-Rich (SRCR) family and it belongs to class B scavenger receptors [Fabriek et al., 2005].

In chronic inflammation the main cells found are macrophages, lymphocytes and plasma cells. The dominant cells are Th2 restricted $CD3^+$ lymphocytes, which produce IL-4, 5, 8 and 10 and tumor necrosis factor-α (TNF α) and chemokines for the regulation of local immune response [Tedla et al., 1998; Lindholt & Shi, 2006].

1.8 IMMUNOHISTOCHEMISTRY

The basis of immunohistochemistry (IHC) technique is quite simple and joint three disciplines: immunology, histology and chemistry. The fundamental concept behind IHC is the demonstration of antigens (Ag) in tissue sections by means of specific antibodies (Abs), thus forming the Antigen-Antibody complex (Ag-Ab) based on antigen-antibody interaction [Ramos-Vara, 2005]. Immunohistochemistry technique is a powerful method for locating specific antigens in formalin fixed and paraffin embedded [Schacht & Kern, 2015]. Over the years the technique has been improved increasing the ability to detect antigens in paraffin tissue sections, as the antigen retrieval methodology is able to reverse or prevent the deleterious effects of formaldehyde and increasing the sensitivity of the detection [Ramos-

Vara, 2005]. One of the most prominent advances in the technique came in the 1990s, the way recovering Ags were changed to heat fixation, exponentially increasing the number of detectable Ags in routinely fixed tissues [Ramos-Vara, 2005].

Therefore, the immunohistochemistry technique has been used for several decades, has been adapted to provide a variety of cell line and tissue type markers, with specific application to the diagnosis and classification of tumors [Taylor & Levenson, 2006] and is widely used in routine diagnostic work, as it is a very common part of scientific reports in pathology and cytology (Figure 9). Its interpretation must be based on the micro-anatomical distribution of the labeling, the proportion of positively cells, the intensity of the positive staining, as well as the color levels [Seidal et al., 2001].

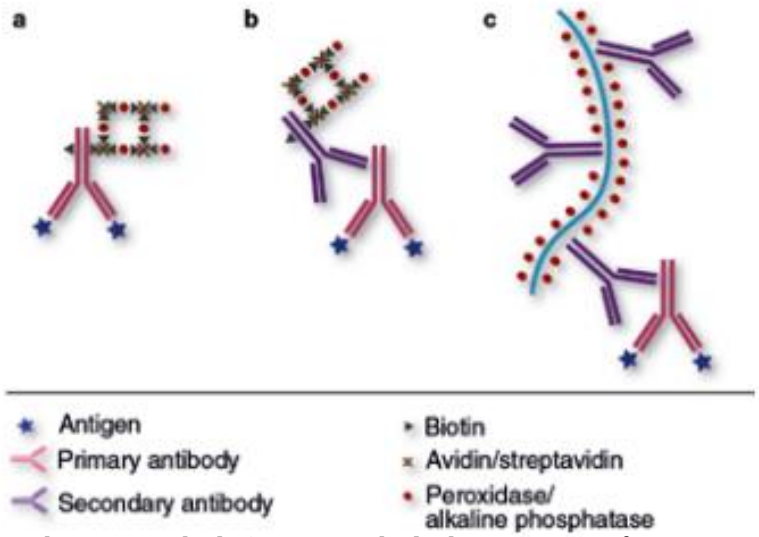

Figure 7. Immunohistochemistry methods a) Direct Method: The antigen-specific primary antibody is labeled. In this case biotin binds to streptavidin. Color visualization is achieved through horseradish peroxidase / alkaline phosphatase enzymatic reaction. b) Indirect method: The antigen specific primary antibody is not labeled but the secondary antibody that is labeled with biotin and in turn binds to the primary antibody. The visualization is the same as the direct method. Indirect method increases versatility because various unlabeled primary antibodies can be used. c) Polymer indirect method chain detection system. Biotin and streptavidin are replaced by a labeled polymeric chain, allowing for greater sensitivity and specificity [Adapted from Schacht & Kern, 2015].

Table 3 Functioning and limitations of immunohistochemistry in formalin fixed and paraffin embedded tissues. [Adapted from Schacht & Kern, 2015].

HOW IT WORKS	LIMITATIONS
• Locates specific antigens in paraffin-embedded material. Is an antigen-based antibody interaction. • Mark antigen in three steps: processing and epitope retrieval, antigen-antibody interaction and visualization through different detection systems. • Illustrates the interactions resulting from the antigen - antibody connection through optical microscopy, using a colored signal, where it is possible to observe the skin morphology and subcutaneous tissue. • It is important for skin tumors diagnosis and prognosis, as well for detection of infectious microorganisms. • It serves various purposes in physiology and pathology research.	• Not all antigens are equally detectable by immunohistochemistry. • Demanding laboratory procedure with many possible variables. Technical pitfalls can lead to false positive or false negative results. • Nonstandard methods that may have vary between laboratories. • Less sensitive and specific than PCR based.

1.9 OBJECTIVE

The aim of the present work was to evaluate the inflammatory response to PLLA standard / hydrophobic and PLLA super-hydrophobic.

Using immunohistochemistry methods evaluation of T lymphocytes (with anti-CD3 antibodies), the total macrophages (with the antibody anti-CD68) and macrophages M2 subpopulation (using the antibody anti-CD163).

2 EXPERIMENTAL SECTION

2.1 MATERIALS AND METHODS

2.1.1 POLY(L-LACTIC ACID)

A high stereoregular PLLA was from a commercially available smooth polymeric with a superhydrophobic biomimetic surfaces (Cargill Dow Polymer Mn = 69000, Mw / Mn = 1.734). The method described in Song et al. [2009] describes that a 13% (w / w) PLLA / dioxane solution was poured onto the substrate and after waiting the 4-minute evaporation period, the substrate was placed into absolute ethanol solution over a period of 1 hour. Ethanol was purchased from Panreac and Fluka Dioxane (p.a. 95%). The described process causes a specific surface structures, thereby increasing their roughness and consequently the hydrophobicity (Figure 10). The samples were dried in greenhouses at 30° C for a period of 24 hours to remove residual products. For transplantation the samples were cut in circular structures with 6 mm diameter and sterilized in ethylene oxide [Amaral, 2017].

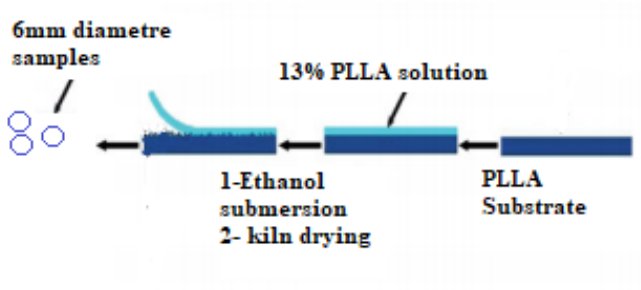

Figure 8 Schematic process of the method of production of super-hydrophobic surfaces [Adapted from Song et al., 2009].

2.1.2 ANIMALS

Eighteen male rats (*Rattus norvegicus*) with 16 weeks of age, all weighing between 350-400 g of body weight were used in this study. The animals were kept in separate cages and fed commercial rat food and water ad libitum throughout the study.

2.1.3 IMPLANTATION PROCEDURE

The rat implantation procedure was described by Amaral [2017]. The animals were divided into two groups, 9 for the implantation of the PLLA standard (S) (control group) and 9 for the implantation of PLLA super-hydrophobic (SH) (experimental group).

The anesthetic technique was made by intraperitoneal injection of medetomidine (Dexdomitor®) (0.5 mg/kg) and ketamine (Ketamidor®) (75 mg/kg). After surgery, atipamezole (Antisedan®) (1 mg/kg) was administered by intraperitoneal injection with the intention of reversing anesthesia.

The skin of the dorsal region was shaved, washed and disinfected with povidone iodine. Six para-ventral incisions were made, two subcutaneous at the scapula level, two subcutaneous at the pelvis level, and two sub-muscular at the vastus lateral muscle area. Subsequently a subcutaneous and sub-muscular pouch was created using blunt dissection with scissors. Each animal was implanted with six discs of the same type of polymeric surfaces. Then, the skin was sutured using a non-absorbable thread (Figure 11). Therefore, 108 samples of polymer, 54 PLLA S and 54 PLLA SH, were distributed among 18 rats (6 implants per rat) (Table 4).

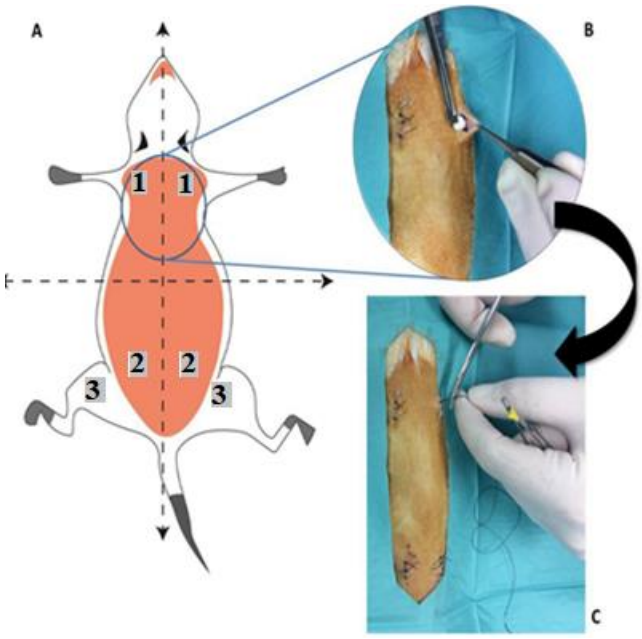

Figure 9 A) Rat (Rattus norvegicus) in ventral position; B) Subcutaneous implant insertion; C) Skin suture after implantation [Adapted from Amaral, 2017]. 1) Subcutaneous implant in the scapular region; 2) subcutaneous implant in the dorsal region; 3) intramuscular implant in the posterior limbs.

Table 4 Number of rats that were euthanized, the time after implantation, and the type of PLLA implanted.

Days after implantation	*7 Days*	*14 Days*	*60 Days*
Rats with PLLA Standard/Hydrophobic implants	3	3	3
Rats with Super-hydrophobic implants	3	3	3

2.1.4 ANIMAL EUTHANASIA AND SAMPLES HARVESTING

The animals were euthanized in 3 different time periods: one week (7 days), two weeks (14 days) and 60 days after the implantation.

For euthanasia, anesthesia was performed using the same protocol used in the implantation procedure, then a lethal dose of sodium pentobarbital was administered intraperitoneally.

After the euthanasia took place, skin and muscle samples were harvested, fixed in 10% buffered formalin and routinely processed for paraffin embedding. Each local samples were stained routinely to Hematoxylin and Eosin (H&E), and in a previous study [Amaral, 2017] submitted to evaluation with special staining for collagen and fibrous capsule thickness evaluation. Sequential 3 µm cuts, using the Leica semi-automatic microtome (Figure 12) were made to indirect immunohistochemistry to evaluate the inflammatory response to the implant.

The samples were identified with a specific number for which animal, a letter and number were assigned for the region (example: 45 R4) (Appendices). With this identification it became possible to differentiate between regions, implant type and a time period corresponding to each sample.

Figure 12 Leica Biosystems RM2245, used to cut the samples in paraffin blocks.

2.1.5 IMMUNOHISTOCHEMISTRY

Slides of each sample were subjected to indirect immunohistochemistry technique to evaluate the inflammatory response to the implant with three antibodies, anti-CD3 (T lymphocytes), anti-CD68 (total macrophages) and CD163 (M2 macrophages). The study was carried at UTAD's Histology and Anatomical Pathology Laboratory (LHAP).

Indirect Immunohistochemical Protocol

1. Put the slides in xylol for 10 to 15 minutes.
2. Hydrate the slides with decreasing alcohols (100º - 95º - 80º - 70º). 5 minutes each.
3. Pass through distilled water.
4. Heat-treated antigen retrieval (Table 5).
5. Cool slowly for 30 minutes.
6. Inactivate with 3% H_2O_2. 30 minutes.
7. Wash in PBS.
8. Incubate with universal normal serum (Ultra V Block – Ultra vision Detection System) for 5 minutes
9. Drain off excess normal serum.
10. Incubate with Primary Antibody (Table 5).
11. Wash in PBS (Phosphate Buffered Saline).
12. Incubate post-Primary (Table 5).
13. Wash in PBS.
14. Second reagent / Polymer (Table 5).
15. Wash in PBS (3 times or for 5 minutes).
16. Add DAB adding 1.6 µl of 33% H_2O_2 per milliliter immediately before use.
17. Leave for 10 min.
18. Excess DAB should be handled carefully.
19. Rinse under running water for 10 minutes.
20. Contrast with Gill's Hematoxylin for 1 to 2 minutes.

21. Rinse with warm running water for 10 minutes.
22. Dehydrate the slides with increasing alcohols (70º- 80º- 90º- 100º), 5 minutes each and finally put in xylol.
23. Mount in Entellan®.

Table 5 Clone used, target cells, dilution, incubation period, antigen retrieval and revelation system.

	Clone	*Dilution*	*Incubation*	*Antigen retrieval*	*Revelation System*
Anti- CD3 *(T Lymphocytes)*	Polyclonal (Dako)	1 : 150	2h	Citrate buffer (pH 6.0 ±0.2) 3x5 min Microwave	Ultra vision Detection System, Labvision (10 min each)
CD163 *(M2 Macrophages)*	Monoclonal ab182422 (Abcam)	1 : 1000	2h	EDTA solution (pH = 9) 3x5 min Microwave	Ultra vision Detection System Labvision (10 min each)
Anti-CD68 *(Total Macrophages)*	Polyclonal ab125212 (Abcam)	1 : 500	2h	Citrate buffer (pH 6.0 ±0.2) 2x5 min Microwave	Novolink Polymer NovoCastra™ (30 min each)

For simplification purposes the nomenclature of “A” for the intramuscular (IM) implant, “B” for the subcutaneous (SC) implant in the scapula region and “C” for the SC implant of the dorsal region were used from now on.

2.1.6 EVALUATION

After the immunohistochemical technique, for each sample, 5 photos were taken of the area around the implant using an optical microscope. After that, ImajeJ® tool was used for positive cell count in a quantitative manner (Figure 13). It was made an average of the five photos, which resulted in an average number of positive cells per field for sample.

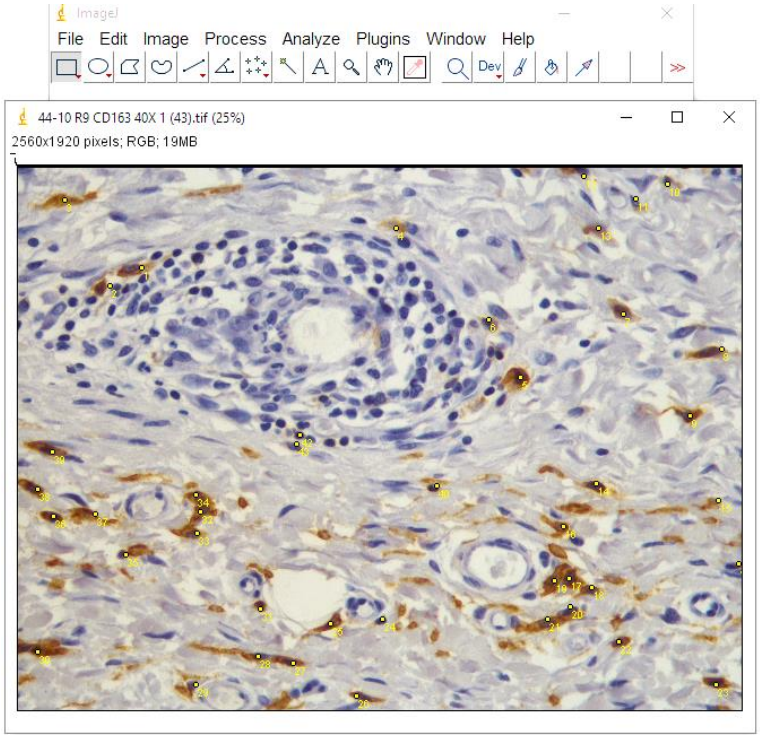

Figure 13 The positive CD163 cell counting in a subcutaneous implant on the scapular region, 7 days after the surgery, using the program ImageJ. Counterstained with Gill's Hematoxylin. Barr=50

2.1.7 STATISTICAL ANALYSIS

To prove whether the data respects a normal distribution, the values were subjected to the Shapiro-Wilk W test, where H0 would hypothesize that the values respect a normal distribution (Figure 14).

Goodness-of-Fit Test

Shapiro-Wilk W Test

W	Prob<W
0,784997	<,0001*

Note: Ho = The data is from the Normal distribution. Small p-values reject Ho.

Figure 14 Using the Shapito-Eik W Test it was proved that the data obtained did not respect a normal distribution.

Since the data did not present a normal distribution, it was decided to use a Generalized Linear Module (GLM) analysis separately for each of the antibodies. Using contrasts as a comparison between the various regions where the biomaterial was implanted, time periods and different PLLA types (Figure 15).

Effect Tests

Source	DF	L-R ChiSquare	Prob>ChiSq
PLLA types	1	0,2817722	0,5955
Days	2	26,258955	<,0001*
Regions	2	3,5348695	0,1708

Figure 15 The Generalized Linear Module test proved the lack of statistical significance for both the different regions and the PLLA types, while for the time periods (days) was found marked relevance.

2.2 RESULTS

The results presented below were organized by antibodies, first by CD3, then CD68 and finally CD163, with the three regions. At the end of each sub-chapter a table with the statistical significance of each variable is presented.

As the positions of the implants were symmetrical two by two in relation to the vertebral column, it was decided to use only 3 regions, two subcutaneous in the scapular region and in the dorsal region, as well as an intramuscular, located in the pelvic members.

T lymphocytes: CD3 labeling cell

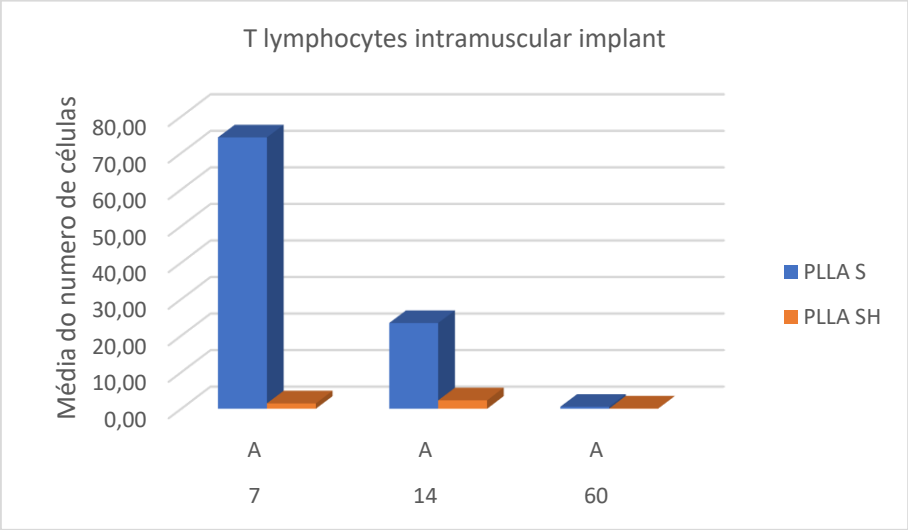

Figure 16 Mean of the number of cells (T lymphocytes, marked by anti-CD3) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on a implant.

In the analysis of the results obtained in IM implants in the 7th day after the surgery, the average of cells per field (AC/F) labeled with CD3 is much higher for PLLA S, with 74.72 AC/F, than for PLLA SH, with 1.74 AC/F. In the 14th day after the implantation, there was a decrease in the number of positive cells, with PLLA S presenting 23.47 AC/F and PLLA SH 2.30 AC/F. In the 60th day after the implantation, the averages approached zero in both PLLA. On Figure 16 a marked decrease in the AC/F can be seen over time in the application for PLLA S. Figure 17 is a photograph taken in this study of the tissues around the implant.

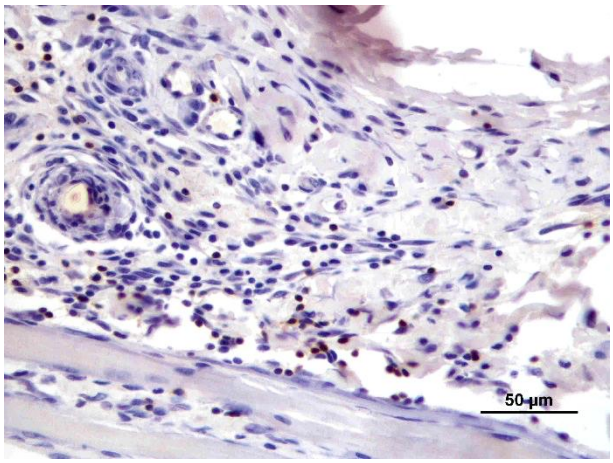

Figure 17 35 J9.1 CD3 (7 day – Standard Intramuscular)

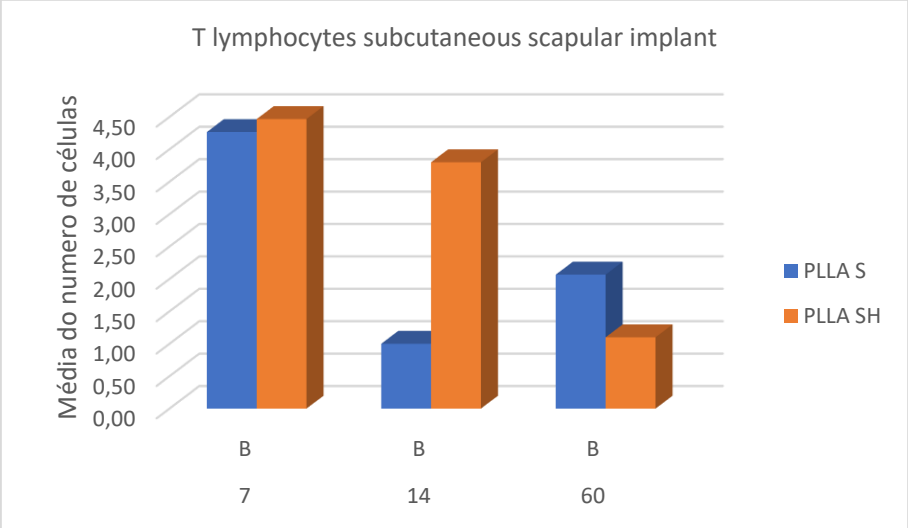

Figure 18 Mean of the number of cells (T lymphocytes, marked by anti-CD3) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the scapular region.

In B location (SC implant of the Scapular region) the CD3-positive cells there was a decrease in the number of cells for both biomaterials over time (Figure 18), except for PLLA S at 14th day, which presented higher average than PLLA SH. At day 7th, were present in the PLLA S biomaterial 4.27 AC/F and for PLLA SH 4.47 AC/F. At the 14th day, the PLLA S presented 1.00 AC/F and the PLLA SH of 3.80 AC/F. In the period corresponding to 60 days after the implantation PLLA S presented 2.07 AC/F and PLLA SH 1.10 AC/F. Figure 19 is a photograph taken in this study of the tissues around the implant.

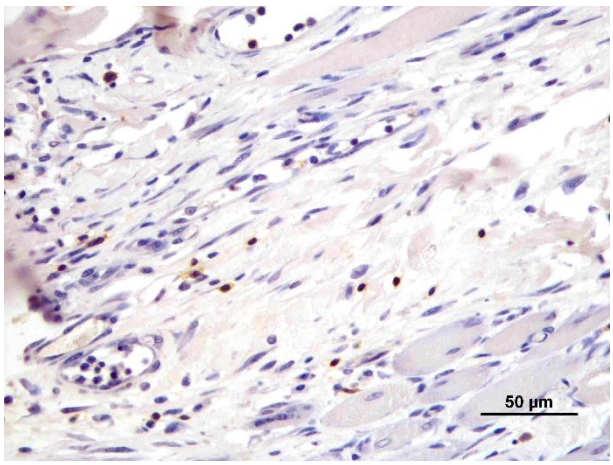

Figure 19 31 R9 CD3 (7 day – Standard subcutaneous intramuscular)

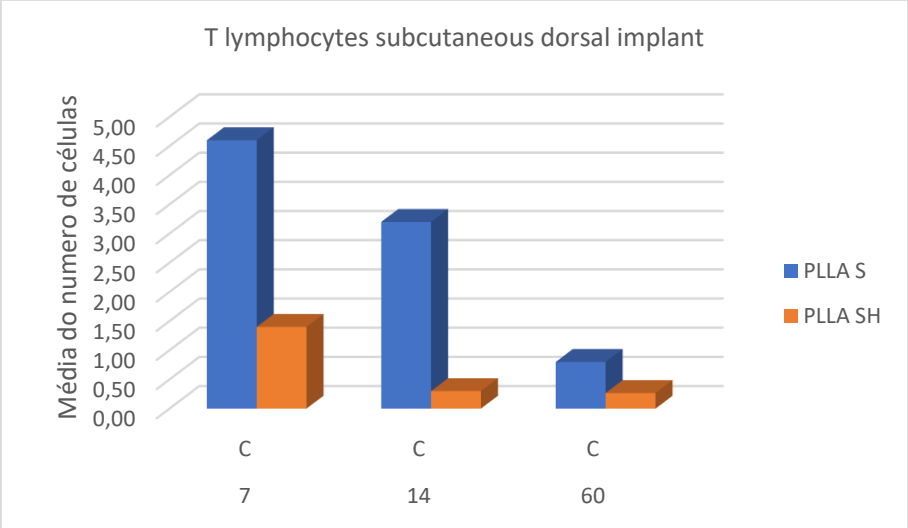

Figure 20 Mean of the number of cells (T lymphocytes, marked by anti-CD3) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the dorsal region.

For the implant located in C (for the subcutaneous (SC) implant in the dorsal region) it was observed that the number of CD3 positive cells (T lymphocytes) diminished along the time of implantation (Figure 20), at day 7, with 4.40 AC/F for PLLA S and for PLLA SH 1.40 AC/F. After 14 days was noted a slice decrease of T lymphocytes for both biomaterials, with 3.20 AC/F for PLLA S and 0.30 AC/F for PLLA SH. After 60 days of implantation, it resulted in 0.80 AC/F for PLLA S and 0.27 AC/F for PLLA SH. Therefore, there was a decrease of cells over time in both biomaterials, whereas PLLA S presented in the three moments, higher average than the corresponding PLLA SH. Figure 21 is a photograph taken in this study of the tissues around the implant.

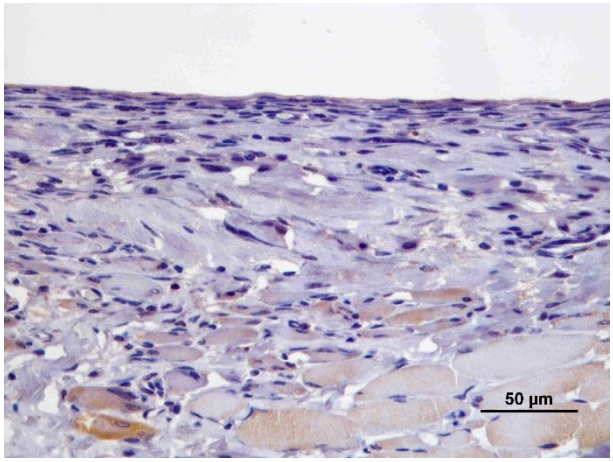

Figure 21 35 R5 CD3 (7 day – Standard Subcutaneous Dorsal)

Table 6 Effect test, P-values of the statistical significance of the various parameters for CD3.

Source	DF	L-R ChiSquare	Prob>ChiSq
PLLA Types	1	2,4453856	0,1179
Days	2	2,8080599	0,2456
Regions	2	3,8587476	0,1452

After statistical analysis of the various test parameters, it was observed that *P*-value showed that there is no significance in any of them for CD3, but it was clearly possible to see the trend of decreasing averages over time, as well as that PLLA S had, in most cases, CD3 means higher than the PLLA SH (Table 6).

CD68 macrophages positivity

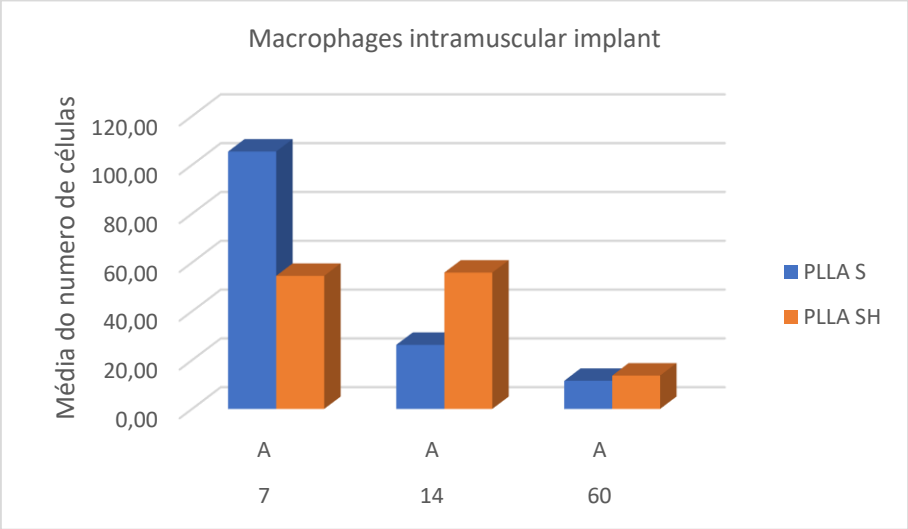

Figure 22 Mean of the number of cells (macrophages total population, marked by CD68 antibody) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the IM implant.

In the IM implant, CD68-labeled cells showed a decrease in the average of cells per field over time for both PLLA, except on the 14th for PLLA SH, where there was a slight rise of the mean compared to the 7th day. It is also verified that in the 7th the PLLA S had higher AC/F than the PLLA SH, however in the other times the PLLA SH presented slightly higher AC/F. In the 7th day the results were 105.47 AC/F for PLLA S and 54.53 AC/F for PLLA SH,

in the 14th day the results were 26.27 AC/F for PLLA S and 55.87 AC/F for PLLA SH and in the 60th day it was 11.53 AC/F for PLLA S and 13.67 AC/F for PLLA SH (Figure 22). Figure 23 is a photograph taken in this study of the tissues around the implant.

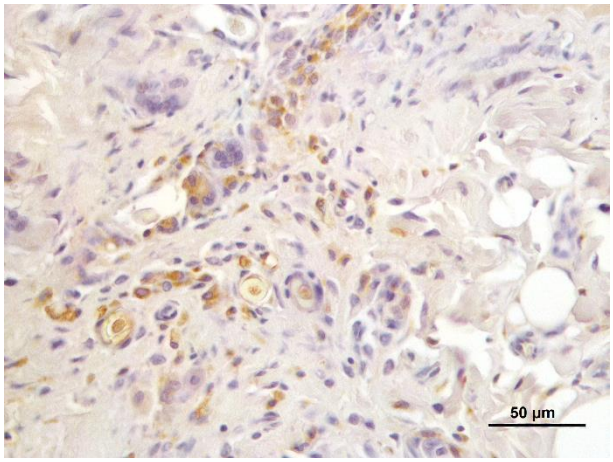

Figure 23 48 J9.1 CD68 (14 day – Superhydrophobic Intramuscular)

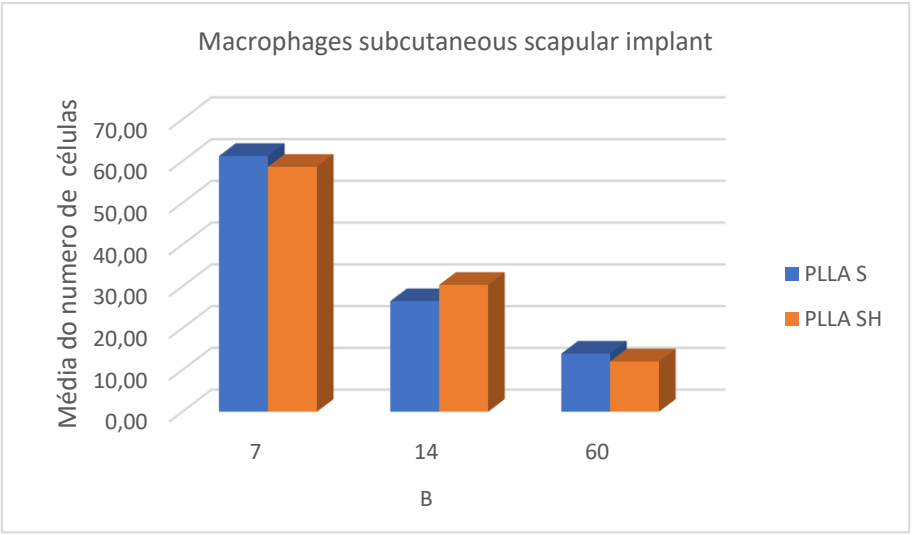

Figure 24 Mean of the number of cells (macrophages total population, marked by CD68 antibody) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the scapular region.

At the scapular implant (Figure 24), it was possible to see a decrease in the average number of cells per field over time for both PLLA types. The PLLA S averages were also higher than the PLLA SH averages at 7 days after the implantation (61.27 AC/F for PLLA S

and 58.67 AC/F for PLLA SH) and at day 60th the results were 13.93 AC/F for PLLA S and 12.07 AC/F for PLLA. However, at the 14th day after the implantation the average for PLLA SH was slightly higher than the average for PLLA S as the results were 26.50 AC/F for PLLA S and 30.40 AC/F for PLLA SH. Figure 25 is a photograph taken in this study of the tissues around the implant.

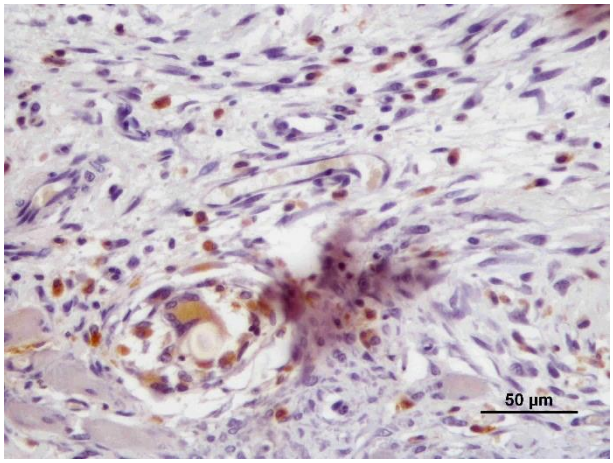

Figure 25 31 R9 CD68 (7 day – Standard Subcutaneous Scapular)

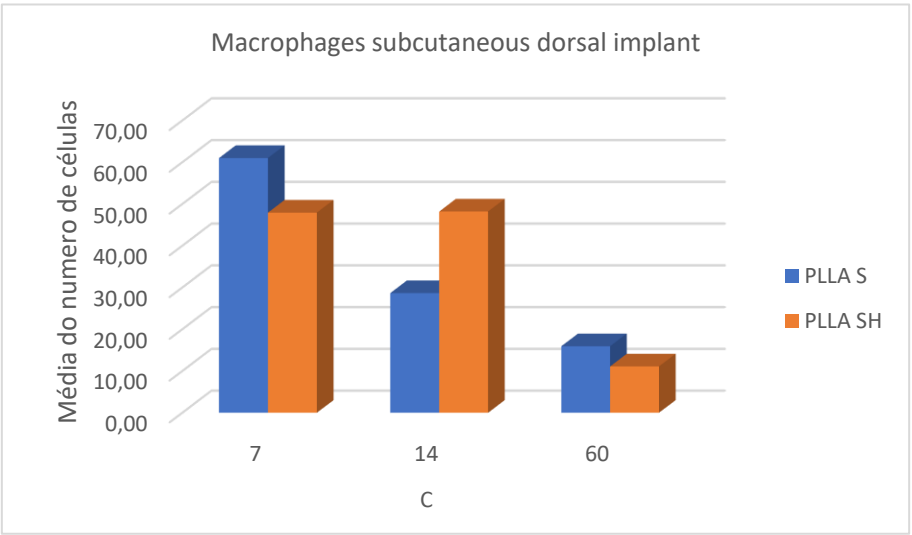

Figure 26 Mean of the number of cells (macrophages total population, marked by CD68 antibody) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the dorsal region.

The analysis of the data obtained from the B implant (scapula region SC), was observed a decrease in cell number over time for both PLLA and also that the PLLA S counting was always higher than the PLLA SH, except for the 14th days of implantation (Figure 26),

where the AC/F for PLLA SH was higher. At day 7th were counted 61.00 AC/F for PLLA S and 47.93 AC/F for PLLA SH. After 14th day, the result was 28.67 AC/F for PLLA S and 48.20

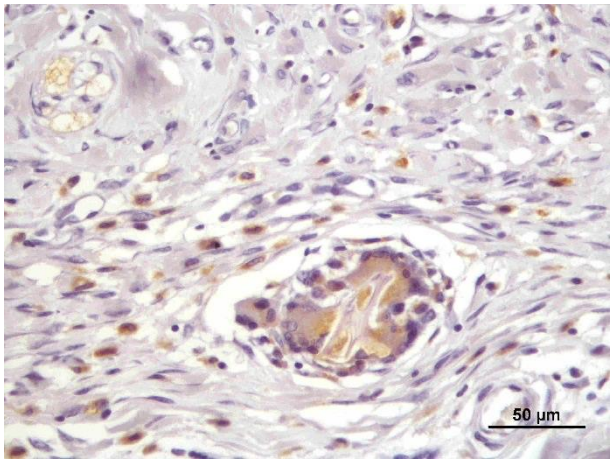

Figure 27 39 R5 CD68 (14 day – Standard Subcutaneous Dorsal)

Table 7 Effect test, P-values of the statistical significance of the various parameters for CD68.

Source	DF	L-R ChiSquare	Prob>ChiSq
PLLA Types	1	0,1055765	0,7452
Days	2	27,47913	<,0001*
Regions	2	1,6817215	0,4313

AC/F for PLLA SH, and at day 60th were observed 15.93 AC/F for PLLA S and 11.10 AC/F for PLLA SH. Figure 27 is a photograph taken in this study of the tissues around the implant.

A decrease in the AC/F of the macrophages was observed over time (Table 7). It was also possible to observe that in most cases PLLA SH had lower averages than PLLA S, however without statistical significance.

CD163 positive macrophages

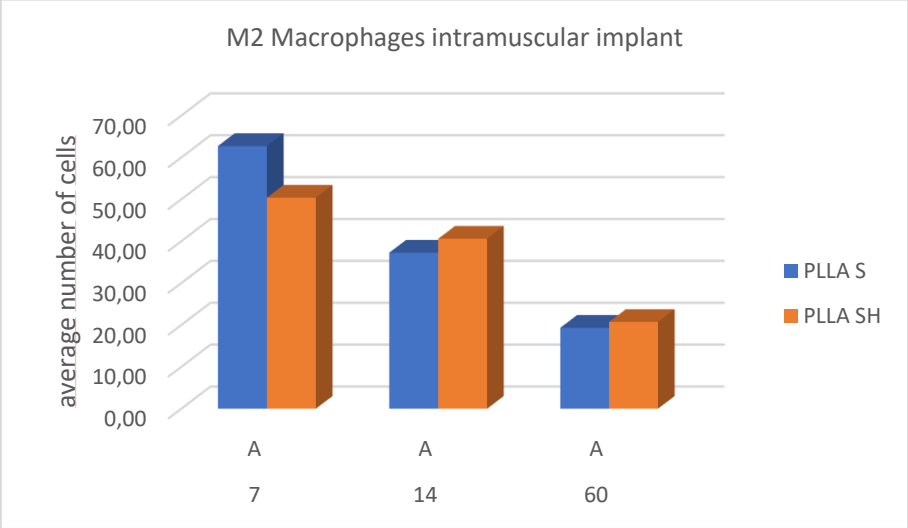

Figure 2830 Mean of the number of cells (Macrophages M2 population, marked by CD163 antibody) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the IM implant.

In the CD163-labeled at IM localization in the 7th we observed 62.73 AC/F for PLLA S and 50.40 AC/F for PLLA SH, for the 14th day were counted 37.27 AC/F for PLLA S and 40.60 AC/F for PLLA SH, in the 60th days was observed 19.33 AC/F for PLLA S and 20.73 AC/F for PLLA SH. Therefore, a decrease in cell averages per field over time was observed for both PLLA types. It was also observed that for the first period the average of PLLA S was higher than the average of PLLA SH, however at the 14th and 60th days the AC/F for PLLA SH was higher (Figure 28). Figure 29 is a photograph taken in this study of the tissues around the implant.

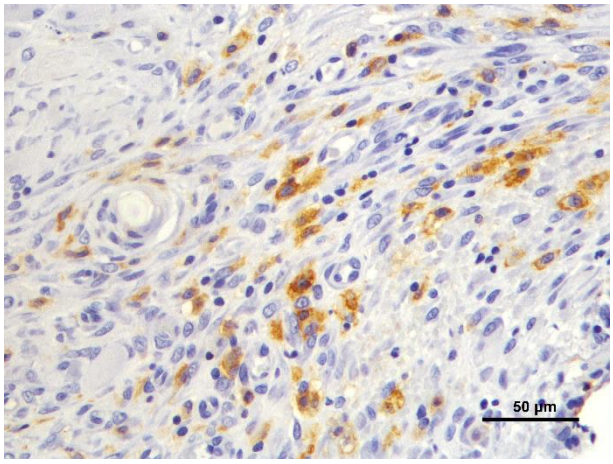

Figure 29 39 R5 CD68 (14 day – Standard Subcutaneous Dorsal)

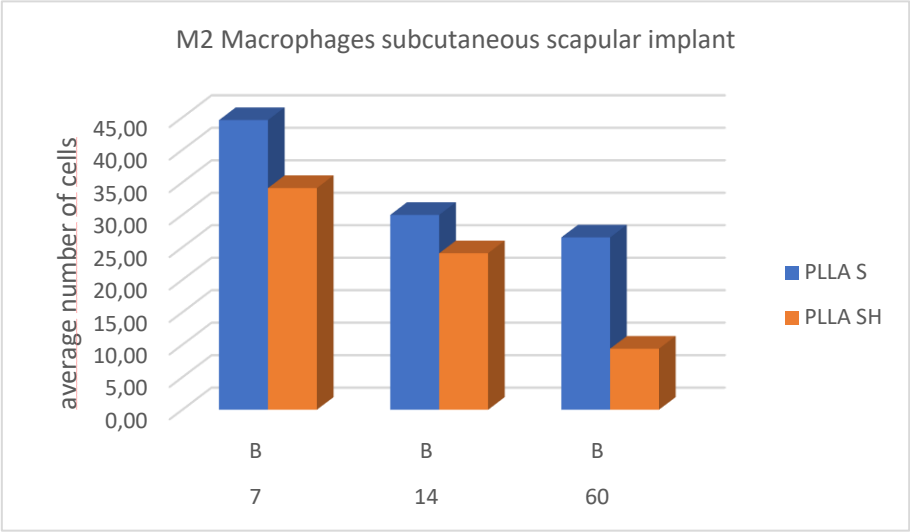

Figure 30 Mean of the number of cells (macrophages total population, marked by CD163 antibody) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the scapular region.

In the CD163-labeled at scapular region, in the 7th day the results were 44.60 AC/F for PLLA S and 34.13 AC/F for PLLA SH, at day 14th we have obtained 30.00 AC/F for PLLA S and 24.10 AC/F for PLLA SH, at day 60th we should note that PLLA S presented 26.53 AC/F and PLLA SH 9.40 AC/F. Therefore, it was observed that over time, the number of cells per field decreased for both PLLA. It was also observed that PLLA S presented higher AC/F than PLLA SH in all time periods (Figure 30). Figure 31 is a photograph taken in this study of the tissues around the implant.

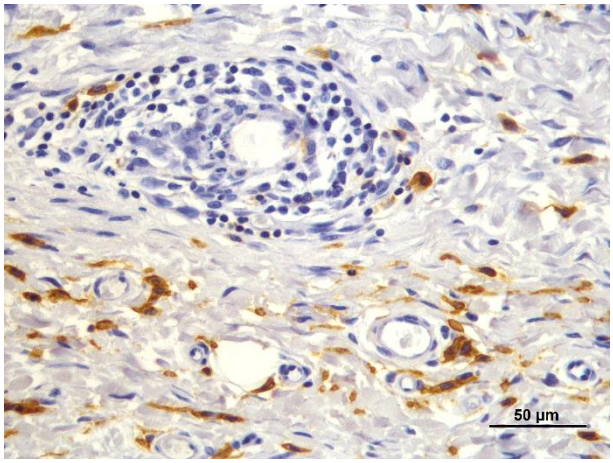

Figure 31 44 R9 CD163 (7 day – Superhydrophobic Subcutaneous Scapular)

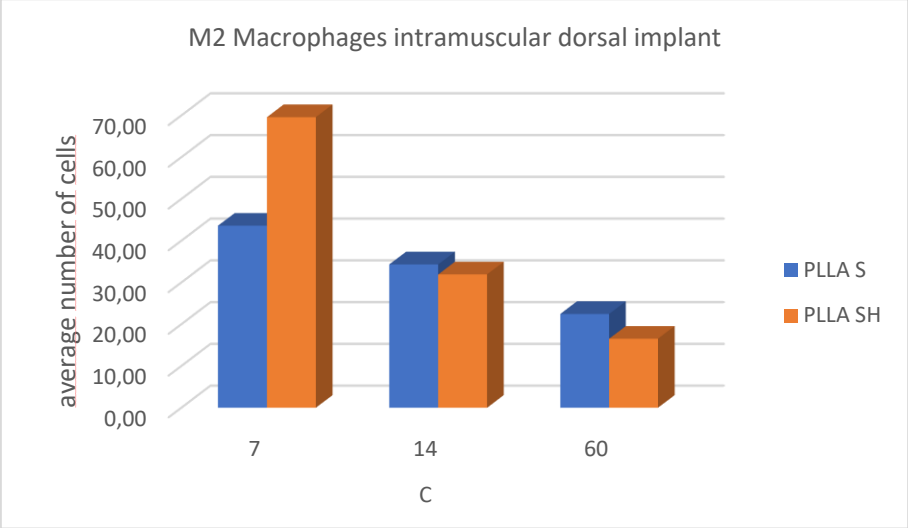

Figure 3235 Mean of the number of cells (macrophages M2 population, marked by CD163 antibody) counted for field, in different periods of time (7, 14 and 60 days) for PLLA S and PLLA SH on the dorsal region.

In the implant referring to the dorsal region for CD163, it was observed a decrease AC/F over time for both PLLA. Regarding the difference between PLLA, it should be noted that at the 7th day the PLLA SH presented higher results (43.60 AC/F) than the average of PLLA S (69.60 AC/F), in the 14th and 60th days PLLA S (34.33 AC/F at day 14th and 31.93 AC/F at day 60th) presented slightly higher AC/F than PLLA SH (22.47 AC/F at day 14th and 16.53. AC/F at day 60th) (Figure 32).

Table 8 Effect test, P-values of the statistical significance of the various parameters for CD163.

Source	DF	L-R ChiSquare	Prob>ChiSq
PLLA Types	1	0,2817722	0,5955
Days	2	26,258955	<,0001*
Regions	2	3,5348695	0,1708

A decrease in the AC/F of the M2 macrophages was observed over time (Table 8). However, although not having statistical significance, it is found that, in most cases, the PLLA SH presents means below the PLLA S.

2.3 DISCUSSION

Foreign biomaterials, such as implants, can cause immune responses which are mediated, among others, by macrophages and T lymphocytes [Gardner et al., 2013]. The immune system, with the intervention of macrophages can recognize a foreign agent and immediately initiate a protective response against this [Cooper & Alder, 2006], generally T lymphocytes activated and promote an adaptive and specific response. The presence of T lymphocytes in a lesion is the evidence of the recruitment of the adaptive immune response and, consequently, of the memory of the foreign agent, which must be effectively activated when the same agent appears over again in the body.

In the present study PLLA S implants are considered by the organism a foreign body. Nevertheless, the aim of this material is to be the most “invisible” possible to the immune system, as it could be used in several medical devices.

So, the present study has the aim to test if the wettability of this biomaterial have an improved quality to promote this “invisibility” or non-response for the immune system of the host to this material. The design of this study had the objective to evaluate the inflammation along the time for two different PLLA biomaterial with the goal to understand where they could be better suited.

The macrophages play a key role in all phases of injury response, participating in mechanisms underlying initial inflammation and subsequent repair and tissue regeneration [Oishi & Manabe, 2018]. The CD68 is a protein highly expressed by tissue and circulating macrophages. We observed that the muscle region presented more cells than SC regions at early stage, but over time the number of cells decreased in all regions. In the case of a continuous injury or failed recovery of the inflammation, macrophages may become detrimental in their activities. We have found the presence of some multinucleated giant cells, by what Oishi & Manabe said in 2018, this may indicate that there was an evolution to chronic inflammation.

The PLLA SH implants have an initial macrophage's inflammatory reaction similar in all tested sites, although the dorsal region expressed slightly higher values of cells. At the end of 60 days after implantation, the three regions demonstrate means of approximately 13 cells per field. In short, for PLLA SH the regions of the implant location do not show significant differences between them, but there is a decrease of the means over time, which indicates the prominent decrease of inflammation caused by this biomaterial over time.

In the comparison of macrophage averages per field between PLLA S and PLLA SH, it is observed that the latter has lower values than the former, both in the first period and in the last period. However, in the interim period, PLLA S has lower values than PLLA SH. We can conclude that PLLA SH has caused lower inflammatory response in the first instance, but the decrease in the number of macrophages is first started in PLLA S, 60 days after implantation, the mean of both decreases sharply, indicating no chronic inflammation formation in either of the two PLLA types.

The M2 macrophages, also called activated macrophages, in our study labeled by CD163, are essential cells in the natural wound healing process. Therefore, it is important to know its expression in the context of host response to the implanted biomaterial [Sylvestre et al., 2019]. As it can be seen from our results, for the SC implantation PLLA S located in the scapula and dorsal zone, the initial reaction was similar, with around 45 cells per field, with the values decreasing over time, and in the last period, the averages present values close to 25 cells per field. The moderate initial reaction may indicate a wound healing response from the host. In the period corresponding to 60 days after implantation, this is also verified, even though

the number of cells is halved. In the case of IM implantation, PLLA S showed a higher reaction than in previous cases, with an initial mean of 63 cells per field, but in the final period, 60 days after implantation, it showed slightly lower averages than the other implant sites.

PLLA S causes a moderate reaction in SC tissues, which is halved after 60 days, and in the IM implantation, the initial reaction is the most severe, but in the final period, lower values are observed. The PLLA S in the IM zone demonstrates a severe M2 cell reduction pattern, which may suggest that tissue in the IM zone, despite having a more prominent initial reaction, has a more effective wound healing than the remaining sites in the presence of PLLA S.

In the case of PLLA SH implants, the SC located in the dorsal zone presented the lowest means, and initially 34 cells per field were observed. However, the scapula and the IM implants showed more prominent initial reactions with means of 70 and 51 cell per field, respectively. This may indicate that PLLA SH has less wound healing effect in the dorsal area than in the other regions. The decrease in the average number of cells per field over time in the 3 regions indicates that there is small chronic wound formation.

For M2 macrophage subpopulation PLLA SH shows almost always fewer cells per field than PLLA S, in all regions and all periods except the first period of the scapula SC implant. Therefore, these results suggest that PLLA SH causes a lower wound healing effect than PLLA S.

In the present study, a T lymphocyte could be observed in the period corresponding to 7 days after implantation, in PLLA S, in the IM implant, decreasing along the time, been almost null at 60 days after implantation, suggesting that these biomaterials do not have a strong activation of its functions with this material. The same observations were made in the remaining locations. The PLLA SH implant have smaller or similar number of T lymphocytes in the tissues. This indicate the residual activation of the adaptive immune system and since biomaterials are foreign bodies, they should be biocompatible and biomimetic, having negligible adaptive immune response from the body. It has recently been recognized that the most important component for successful surgical intervention with implantation of a biomaterial is the host response to the biomaterial [Dziki & Badylak, 2018], so PLLA S and

PLLA SH are suitable for the proposed meaning, since the immune reaction to it in tissues is almost null.

2.4 CONCLUSION

This study showed that there was no statistical significance difference in the inflammation response between the PLLA SH and PLLA S. However, it has been found that PLLA SH causes lower acute inflammation than PLLA S, so it should be a bio-implant elected for purposes in which inflammation reaction is less desired, such as for medicines released devices.

We concluded that none of the biomaterials (PLLA S and PLLA SH) strongly activated the adaptive immune system. However, the immune system showed more passiveness regarding PLLA SH.

Furthermore, if we consider that the amount of M2 macrophages in the region of the injury is directly related with the wound healing effect, then PLLA SH causes less wound healing effect than PLLA S. So, for cases where wound healing effect is necessary and favorable, the use of PLLA S is recommended.

It could be concluded that both PLLA's types have excellent biocompatibility capabilities, as in all cases the inflammation decreased considerably over time.

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4 APPENDICES

Average of cell number by field and region, for different antibody used.

A - pelvic limbs; B - dorsal region; C - scapular region.

PLLA types	Days	rat identifier number	Regions	CD68 (AC/F)	CD3 (AC/F)	CD163 (AC/F)
PLLA S	7	31	A	207.2	194.8	84.2
PLLA S	7	31	B	47.8	5.6	18
PLLA S	7	31	C	39.2	11.8	34.4
PLLA S	7	35	A	37.6	14.2	46.8
PLLA S	7	35	B	46.4	6.8	51.2
PLLA S	7	35	C	49.6	1.4	43.6
PLLA S	7	36	A	71.6	13.8	57.2
PLLA S	7	36	B	89.6	0.4	64.6
PLLA S	7	36	C	94.2	0.6	52.8
PLLA S	14	37	A	57.4	65.2	60.6
PLLA S	14	37	B	no value	no value	no value
PLLA S	14	37	C	26.6	2.2	44.8
PLLA S	14	38	A	5.4	0.2	15.8
PLLA S	14	38	B	26.8	0.2	28.6
PLLA S	14	38	C	16	5	19.6
PLLA S	14	39	A	16	5	35.4
PLLA S	14	39	B	26.2	1.8	31.4
PLLA S	14	39	C	43.4	2.4	38.6
PLLA S	60	40	A	10.2	0.8	12.8

PLLA S	60	40	B	10.6	1.8	13.4
PLLA S	60	40	C	15.8	1.6	12.4
PLLA S	60	41	A	17	0.6	23.8
PLLA S	60	41	B	3	0	28.4
PLLA S	60	41	C	21.2	0	30
PLLA S	60	42	A	7.4	0.2	21.4
PLLA S	60	42	B	28.2	4.4	37.8
PLLA S	60	42	C	10.8	0.8	25
PLLA SH	7	43	A	38	1.6	43.4
PLLA SH	7	43	B	69.4	2	31
PLLA SH	7	43	C	56	2.4	44.4
PLLA SH	7	44	A	50.2	0.6	34.8
PLLA SH	7	44	B	45	9	35.2
PLLA SH	7	44	C	58.4	0.8	40.4
PLLA SH	7	45	A	75.4	2.2	73
PLLA SH	7	45	B	61.6	2.4	36.2
PLLA SH	7	45	C	29.4	1	124

PLLA SH	14	46	A	81.4	no value	49.8
PLLA SH	14	46	B	no value	no value	no value
PLLA SH	14	46	C	50.6	no value	25.8
PLLA SH	14	47	A	53	4.4	56.8
PLLA SH	14	47	B	32.2	7	17
PLLA SH	14	47	C	70	0.4	43.4
PLLA SH	14	48	A	33.2	0.2	15.2
PLLA SH	14	48	B	28.6	0.6	31.2
PLLA SH	14	48	C	24	0.2	26.6
PLLA SH	60	49	A	18.4	0	22.2
PLLA SH	60	49	B	24.6	1.8	8.4
PLLA SH	60	49	C	no value	0.2	21.2
PLLA SH	60	50	A	6.6	0.2	18
PLLA SH	60	50	B	2	0.4	10.4

PLLA SH	60	50	C	5	0	12.2
PLLA SH	60	51	A	16	0	22
PLLA SH	60	51	B	9.6	no value	no value
PLLA SH	60	51	C	17.2	0.6	16.2

Anti-CD3 labeled cells mean and standard deviation for each region and PLLA type. “A” for the IM implant, “B” for the SC implant in the scapula region and “C” for the SC implant of the dorsal region.

Regions/ PLLA types/ days after implantation	**Mean**	**SD**
A		
7th day		
PLLA S	74.26667	104.3851
PLLA SH	1.466667	0.80829
14th day		
PLLA S	23.46667	36.22172
PLLA SH	2.3	2.969848
60th day		
PLLA S	0.533333	0.305505
PLLA SH	0.066667	0.11547
B		
7th day		
PLLA S	4.266667	3.40196
PLLA SH	4.466667	3.931073
14th day		
PLLA S	1	1.131371
PLLA SH	3.8	4.525483
60th day		
PLLA S	2.066667	2.212088
PLLA SH	1.1	0.989949
C		
7th day		
PLLA S	4.6	6.2482
PLLA SH	1.4	0.87178
14th day		
PLLA S	3.2	1.56205
PLLA SH	0.3	0.141421
60th day		
PLLA S	0.8	0.8
PLLA SH	0.266667	0.305505

Anti-CD68 labeled cells mean and standard deviation for each region and PLLA type. “A” for the IM implant, “B” for the SC implant in the scapula region and “C” for the SC implant of the dorsal region.

Regions/ PLLA types/ days after implantation	**Mean**	**SD**
A		
7th day		
PLLA S	105.4667	89.72878
PLLA SH	54.53333	19.07284
14th day		
PLLA S	26.26667	27.47823
PLLA SH	55.86667	24.22753
60th day		
PLLA S	11.53333	4.936936
PLLA SH	13.66667	6.236452
B		
7th day		
PLLA S	61.26667	24.54737
PLLA SH	58.66667	12.46167
14th day		
PLLA S	26.5	0.424264
PLLA SH	30.4	2.545584
60th day		
PLLA S	13.93333	12.92646
PLLA SH	12.06667	11.50014
C	36.90588	23.90953
7th day		
PLLA S	61	29.21849
PLLA SH	47.93333	16.09513
14th day		
PLLA S	28.66667	13.81642
PLLA SH	48.2	23.09372
60th day		
PLLA S	15.93333	5.201282
PLLA SH	11.1	8.626703

Anti-CD163 labeled cells mean and standard deviation for each region and PLLA type. “A” for the IM implant, “B” for the SC implant in the scapula region and “C” for the SC implant of the dorsal region.

Regions/ PLLA types/ days after implantation	**Mean**	**SD**
A		
7th day		
PLLA S	62.73333	19.30423
PLLA SH	50.4	20.03896
14th day		
PLLA S	37.26667	22.45826
PLLA SH	40.6	22.27375
60th day		
PLLA S	19.33333	5.783886
PLLA SH	20.73333	2.369247
B		
7th day		
PLLA S	44.6	23.99083
PLLA SH	34.13333	2.759227
14th day		
PLLA S	30	1.979899
PLLA SH	24.1	10.04092
60th day	19.68	12.81764
PLLA S	26.53333	12.30664
PLLA SH	9.4	1.414214
C		
7th day		
PLLA S	43.6	9.2
PLLA SH	69.6	47.15422
14th day		
PLLA S	34.33333	13.13063
PLLA SH	31.93333	9.938477
60th day		
PLLA S	22.46667	9.069362
PLLA SH	16.53333	4.50925

In a numerology used to identify the samples, each number represents a specific mouse. J 9.1 - intramuscular sample (right side), J9.2 - intramuscular sample (left side), R4 - dorsal submuscular sample (left side), R5 - dorsal submuscular sample (right side), R8 - scapular submuscular sample (left side) , R9 - Scapular submuscular sample (right side), and the period of euthanasia.

Intramuscular samples (left side)	**Intramuscular samples (right side)**	**Dorsal samples (left side)**	**Dorsal samples (right side)**	**Scapular samples (left side)**	**Scapular samples (right side)**	**Euthanasia (days)**
31 J9.1	31 J9.2	31 R4	31 R5	31 R8	31 R9	7
35 J9.1	35 J.92	35 R4	35 R5	35 R8	35 R9	7
36 J9.1	36 J.92	36 R4	36 R5	36 R8	36 R9	7
37 J9.1	37 J.92	37 R4	37 R5	37 R8	37 R9	14
38 J9.1	38 J.92	38 R4	38 R5	38 R8	38 R9	14
39 J9.1	39 J.92	39 R4	39 R5	39 R8	39 R9	14
40 J9.1	40 J.92	40 R4	40 R5	40 R8	40 R9	60
41 J9.1	41 J.92	41 R4	41 R5	41 R8	41 R9	60
42 J9.1	42 J.92	42 R4	42 R5	42 R8	42 R9	60
43 J9.1	43 J.92	43 R4	43 R5	43 R8	43 R9	7
44 J9.1	44 J.92	44 R4	44 R5	44 R8	44 R9	7
45 J9.1	45 J.92	45 R4	45 R5	45 R8	45 R9	7
46 J9.1	46 J.92	46 R4	46 R5	46 R8	46 R9	14
47 J9.1	47 J.92	47 R4	47 R5	47 R8	47 R9	14
48 J9.1	48 J.92	48 R4	48 R5	48 R8	48 R9	14
49 J9.1	49 J.92	49 R4	49 R5	49 R8	49 R9	60
50 J9.1	50 J.92	50 R4	50 R5	50 R8	50 R9	60
51 J9.1	51 J.92	51 R4	51 R5	51 R8	51 R9	60