Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in
Scienze Biomediche Integrate e Bioetica

XXIX ciclo a.a. 2013-2014

The Pro-resolution pathway is altered in Chronic Heart Failure: implications for adaptive immunity dysregulation

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31/07/2017

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ABSTRACT

Chronic Heart Failure (CHF) is a syndrome affecting humans with a relevant immune component which contributes to the severity of the condition. Inflammation in CHF is characterized by an increased plasma level of pro-inflammatory cytokines which are the signal of the onset of acute inflammation that in absence of resolution might become chronic. Resolution of inflammation is a finely regulated process mediated by specialized pro-resolving lipid mediators (SPMs) including arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived molecules. In this work it has been shown that (DHA)-derived, also called D-series resolvins (RvDs), resolvin D1 (RvD1) and resolvin D2 (RvD2), are not able to modulate T cells immune responses in CHF patients and for this reason they could be involved in the failure of the resolution of chronic inflammation in this syndrome. Previously work has shown that RvD1 and RvD2 were able to modulate the immune activity of T lymphocytes, responsible for the adaptive immune response, in healthy humans. In this study we sought to investigate whether or not RvD1 and RvD2 are able to modulate the immune activity of T cells in CHF patients. ELISA test showed that plasma levels of RvD1 were greatly reduced in CHF patients compared with those of the healthy controls. In addition, both RvD1 and RvD2 were not able to modulate T cells immune responses in CHF patients. These results suggested that there might be a defective signaling in the pro-resolving pathway of RvDs in CHF. qRT-PCR reported a reduction of the expression in both key enzyme 15-lipoxygenase (15-LOX), involved in RvD1 and RvD2 biosynthesis, and RvD1 receptor GPR32 compared with controls, and western blotting analysis confirmed this reduction. These findings indicate that the failure of CHF patients to respond to the pro-resolving actions of RvDs is caused by defects in both biosynthetic and expression pathway of RvD1, and that this may participate in the

progression of chronic inflammation. The pro-resolution pathway might be a potential candidate to design better treatments for CHF with the aim of reducing chronic inflammation.

I. INTRODUCTION

1. INFLAMMATION

1.1 General features

Inflammation is a mechanism by which the body defends itself from the injuries and infections coming from the surrounding environment. These can be of physical, chemical, biological and environmental origin (Rankin JA., 2004). The final purpose of inflammation is to eliminate the agent that causes the disturbance and initiate repair processes of damaged tissue to restore the healthy conditions (Duronio V. et al., 2004). Inflammation starts with a vasodilation that leads to an increased permeability of blood vessels, followed by the passage of liquid from the blood vessels to the surrounding tissues and the recruitment of leukocytes in the area that has undergone the lesion. From the clinical point of view inflammation is characterized by a typical sequence of events: heat of the inflamed site (*calor*), redness (*rubor*), swelling (*tumour*), pain (*dolor*) and functional impairment (*functio laesa*) (Neville A et al, 2004), as showed in Fig. 1.

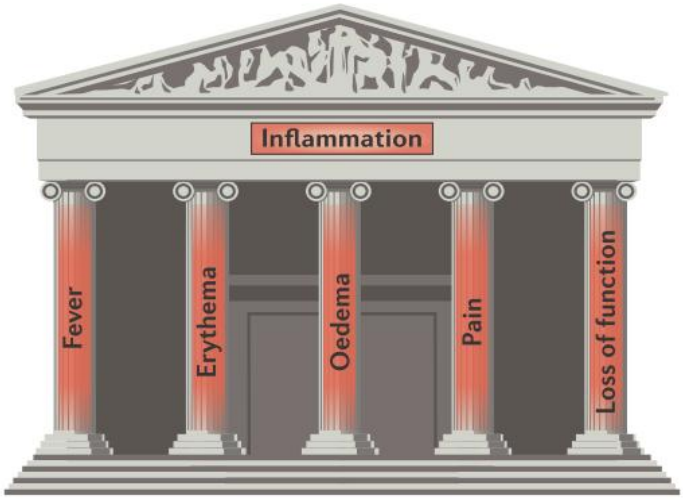

Fig. 1 Cardinal signs of inflammation. In the figure are shown the classical signs of inflammation: (*calor*), redness (*rubor*), swelling (*tumour*), pain (*dolor*) and functional impairment (*functio laesa*) (Adapted from Basil MC and Levy B.D., 2015).

This is the classical description of inflammation based on the visible manifestations induced by the inflammatory state. The redness is caused by the increased number of red blood cells that pass through the inflamed area, the heat is determined by the increased passage of blood through the dilated blood vessels, the swelling is due by the increased flow of liquid through the dilated vessels to the surrounding tissues, pain is caused by the stretching of the nerves generated by edema and the loss of function depends on the replacement of functional tissues with scar tissue. However, inflammation is effective when is able to resolve the situation in a short time and heal the tissue involved. If the inflammation persists without removing the problem it becomes chronic and could create many complications and may be responsible for the worsening of the initial situation, or even generate new chronic conditions (Aggarwal

BB. Et al., 2009). This happens because the persistence of inflammation creates a condition in which phenomena of repair and tissue destruction occur at the same time. For these reasons inflammation was initially considered as a fundamental mechanism in the healing process, but later and more recently it has also been considered as a response of the body potentially able to cause adverse effects that can damage the patient. This has led to a profound debate on the role of inflammation on diseases that affect humans and especially on how inflammation should be handled to care patients and avoid its adverse effects. These considerations highlight that inflammation is an extremely complex mechanism that potentially can be either positive or negative for the patient and therefore it should be characterized by increasingly biomedical research and managed in an optimal way by doctors. From the point of view of its duration inflammation is divided into acute and chronic.

1.2. Acute inflammation

Acute inflammation is characterized by a state of immediate response of limited duration in which the body triggers an inflammatory response aimed at eliminating the cause of the insult, which is managed for the duration necessary for the resolution of the state of physiological alteration. Acute inflammation is therefore a condition that has a beginning, a maintenance period and an end. It occurs over seconds, minutes, hours, and days (Ryan G.B. and Majno G., 1977). The acute inflammation begins with an alteration of the blood vessels in the area that undergoes the causative lesion, followed by a leakage of leukocytes from the blood vessel through the spaces between the endothelial cells, which are then released into the damaged tissue. The migration of leukocytes from blood vessels to the site of inflammation is promoted

by the presence of molecules called cell adhesion molecules (CAMs), which help the binding of neutrophils to the inner walls of blood vessels. Thanks to these ties neutrophils are arranged along to the endothelium walls and then they are subsequently squeezed through gaps between adjacent endothelial cells to reach the site of the inflammation. Leukocytes are afterwards driven by chemotactic stimuli towards the sites that have suffered the insult. These stimuli can be generated by chemokines and the complement peptide C5a. In acute inflammation leukocytes that infiltrate the injured tissue are mainly neutrophils (Kolaczkowska E. and Kubes P., 2013). In an ideal course of acute inflammation when neutrophils reach the site of the injury they kill and engulf the pathogens remaining on site and dying by apoptosis. Then come the macrophages which remove the dead neutrophils and leave the tissue heals without any lesion. Clinically acute inflammation is characterized by the classic events of inflammation such as calor, rubor, tumor, dolor and functio laesa described above. In acute inflammation the alterations of the physiological state are effectively addressed and resolved by the immune system, and they are followed by repair processes and healing of damaged tissue. Therefore at this point the inflammatory condition has been resolved by specific molecules delegated to the end of the inflammatory state. The main cells involved in acute inflammation are granulocytes polymorphonuclear leukocytes (PNM), macrophages and Natural Killer cells. Acute inflammation is a nonspecific response that starts very quickly after the injury and can be considered as the first line of defense of the organism against the injuries. The various stages of acute inflammation are shown in Fig. 2.

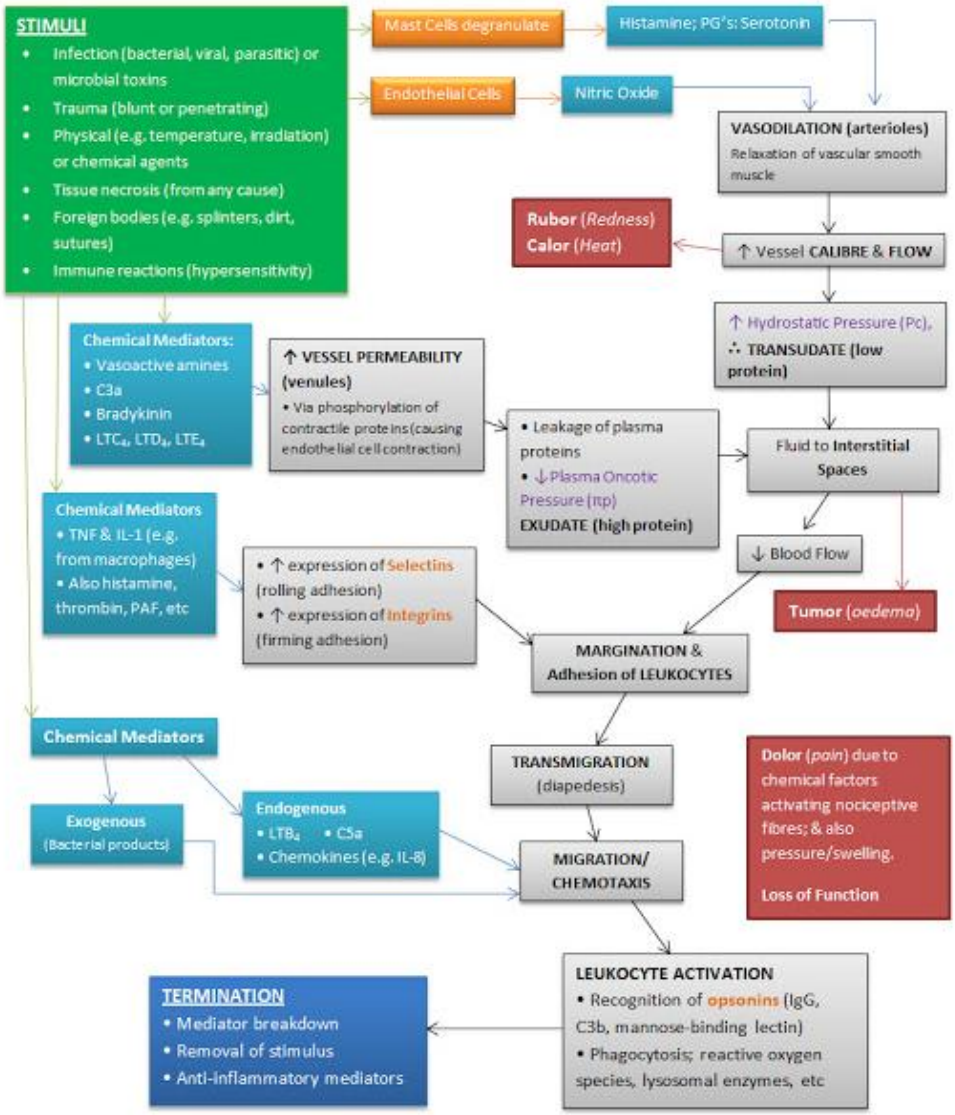

Fig. 2 Acute inflammation Flow Chart. The figure shows the various stages of acute inflammation starting from the stimuli that cause it, the general phenomena that take place as the vascular and cellular events, the biochemical mediators involved and its termination (http://www.ivline.org/2011/01/basics-of-acute-inflammation.html).

1.3. Chronic inflammation

Chronic inflammation is characterized by a state in which they are simultaneously present tissue repair attempts, active inflammation and tissue destruction. Therefore there is a persistent inflammatory state coming from acute inflammation that it was not resolved by deputies molecules (Murakami M. and Hirano T., 2012). The reasons for this continuity of the inflammatory state is the persistence of the damaging stimulus which has caused the acute inflammation, or the inability of the immune system to destroy the detrimental agent. This situation leads to a state in which remains inflammatory condition along with repair mechanisms and tissue altered remodeling so that they undergo changes that can worsen the existing pathological condition or even generate a new disease. Chronic inflammation can be induced and supported by the following events: a persistent infection that causes the passage from acute to chronic infection, a prolonged exposure to toxic agents and an autoimmune reactions and hypersensitivity, as shown in Fig. 3. The cells involved in chronic inflammation are macrophages that persist from the previous acute inflammation, B and T lymphocytes that generate an adaptive immune response and the fibroblasts responsible for the synthesis of extracellular matrix. In addition to the presence of others immune cells than in the acute inflammation, chronic inflammation is characterized by the presence of different tissue processes like angiogenesis and fibrosis (deposition of connective tissue), which are caused by the attempts to repair the damaged tissue. Hence, this prolonged state, if not stopped, can lead to harmful consequences for the body becoming a damaging, rather than helpful, mechanism. Macrophages play a critical role in chronic inflammation and can be activated by T cells that drive the transformation of monocytes into macrophages that exert their action on the inflammation sites. Also T and B lymphocytes play a pivotal role in chronic inflammation (Fujio K. et al., 2012).

Therefore compared with acute inflammation in chronic inflammation there is a major cellular component whose role becomes more important as well as other differences shown in Table 1. Acute inflammation may then undergo different destinies as shown in Fig. 3 and 4: it could be resolved and restoring the cellular and tissue conditions present before the insult that caused it, or persisting and becoming a chronic inflammation with the presence of attempts of tissue repair and fibrosis (Ueha S. et al., 2012).

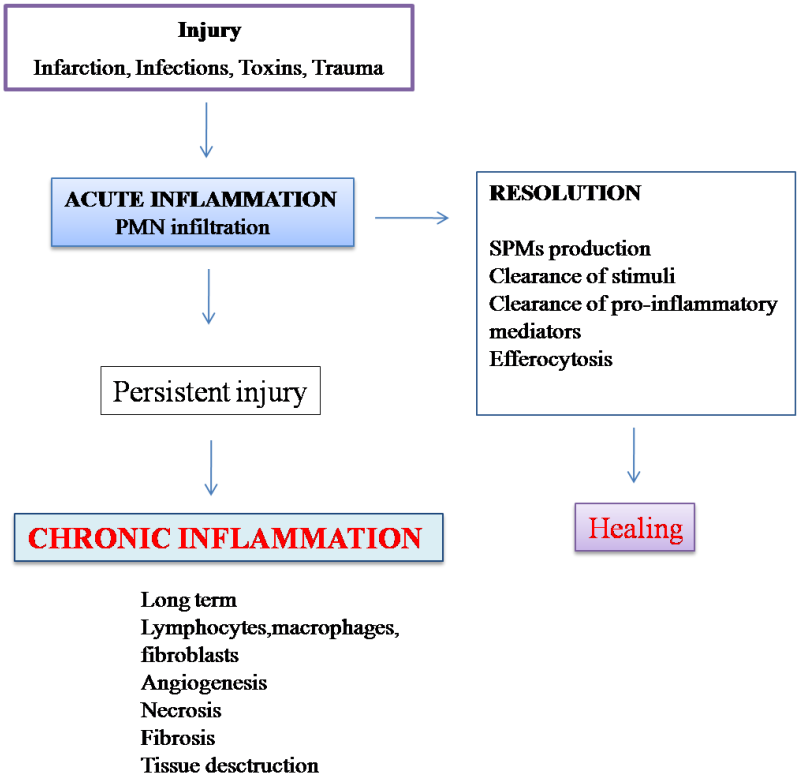

Fig. 3 Chronic inflammation. The cartoon shows the general phenomena that characterize chronic inflammation and it onset from acute inflammation.

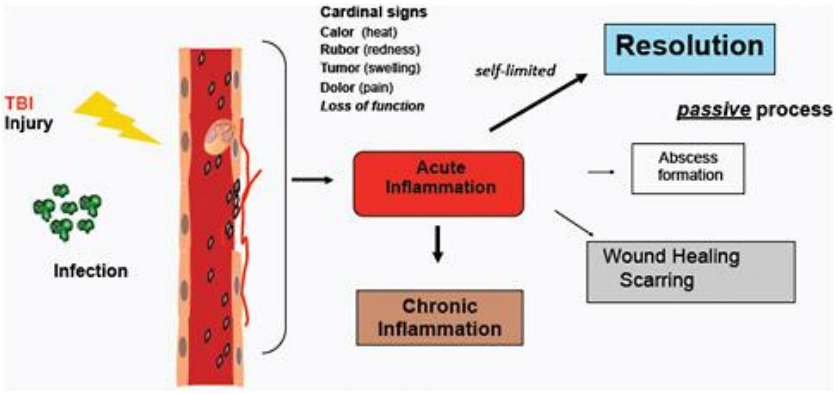

Fig. 4 Possible outcomes of acute inflammation. The figure shows the possible outcomes of acute inflammation: resolution, chronic inflammation and inflammatory diseases. (Adapted from Serhan, CN., 2011).

Table. 1 Differences between acute and chronic inflammation. Table shows the differences between acute and chronic inflammation

	Acute inflammation	Chronic inflammation
Duration	Short term	Long term
Phenomena	Vascular phenomena	Cellular phenomena
Causative agents	Bacteria, injured tissue	Persistent injury, viral, autoimmune
Immune cells	Neutrophils, macrophages	Lymphocytes, macrophages, fibroblasts
Vascular changes	Vasodilation, increased permeability	Angiogenesis
Tissue repair	No	Yes
Fibrosis	No	Yes
Outcomes	Resolution or chronic inflammation	Necrosis, fibrosis, tissue destruction

1.4. Inflammatory mediators

Inflammation is a condition in which many molecules come into play with different functions that act as mediators capable of initiating and regulating inflammation. The main molecules involved are the following and are shown in Table. 2:

- Histamine
- Serotonin
- Prostaglandins and leukotrienes
- Chemokines
- Cytokines

Histamine comes into play very early in acute inflammation and acts very quickly (Benly. P /J., 2015). It is produced in granulocytes and mast cells and has the effect of vasodilation and increased capillary permeability, two effects that favor the initiation of inflammatory response. Serotonin is essentially found in platelet granules and its main effect is similar to those of the histamine, especially vasodilatation. Prostaglandins and leukotrienes are both molecules derived from arachidonic acid, a polyunsaturated fatty acid (PUFA) Ω-6, and are important mediators in inflammation. They are produced both from leukocytes and mast cells. Prostaglandins are produced from cell membranes and their target tissues are usually the same ones where it was produced. They cause a marked effect at much lower concentrations than those of most hormones (Ricciotti E. and Fitzgerald G., 2011). Among the most important, PGE2 is a vasodilator of arterioles, decreases systemic pressure and improves cardiac and renal blood flow while PGD2 is a mesenteric, coronary and renal vasodilator. PGI2, in addition to exerting a strong platelet-like activity, is a potent vasodilator that can induce hypotension and increase heart rate reflection. The main leukotrienes are LTA4, LTB4, LTC4, LTD4,

LTE4, and LTF4. Leukotrienes are involved in asthmatic and allergic reactions, and act to sustain and amplify inflammatory processes locally (Henderson WR Jr., 1994). Chemokines are molecules that are part of the largest family of cytokines. They are responsible for chemotaxis, which is the recruitment of leukocytes in inflammation sites. Cytokines are peptidic inflammatory mediators that have the function of manage the communication signals between immune system and tissues (Zhang J. and Jianxiong An J., 2007). They are produced from a variety of cell types. Their production is transient because they act immediately and in the same environment where they are produced and generally do not find themselves in important quantities in the circles under normal conditions and act on a wide variety of cells and tissues. The most important inflammatory cytokines are TNF-α (Tumor necrosis factor)-α, IL-1 (Interleukin-1) and IL-6 (Interleukin-6), which are inflammatory cytokines, unlike IL-10 which is an anti-inflammatory cytokine.

Table. 2 Inflammatory mediators. The main mediators of inflammation.

Mediator	Source	Activity
Histamine	Mast cells, basophilis	Vasodilation, permeability
Serotonin	Platelets	Vasodilation, permeability, pain , fever
Prostaglandins	Mast cells, leokocytes	Vasodilation, pain , fever
Leucotrienes	Mast cells, leokocytes	Permeability, leukocyte adhesion, chemotaxis
Chemokines	Mast cells, leokocytes	Leukocyte chemotaxis, activation
Cytokines	Mast cells, macrophages	Endothelial activation, tissue damage, pain
Complement	Plasma	Leukocyte chemotaxis, activation

1.5. Diseases with chronic inflammation

As mentioned before if acute inflammation is not resolved it can become chronic and could be the cause of worsening of an existing illness or even be the starting cause of the onset of a new disease. Sometimes inflammation begins because of the presence of an already established disease due to disturbance suffered by tissues affected by the disease. In any case, whatever is the nature of the onset of inflammation, if it becomes chronic it can worsen the clinical conditions. There are many diseases that have a significant inflammatory component that should be addressed in a timely and decisive way. There are also diseases characterized by the presence of acute inflammation, but these are generally of short-live time, unlike diseases in which there is chronic inflammation that are often life-long debilitating illness with increased mortality and high costs for therapy and care. It is just in this case that is recognized the dual role that can have inflammation: in the case of the disease where there is acute inflammation it plays a positive role as it contributes decisively to eliminate the disease. But when there is chronic inflammation with time it becomes a negative mechanism that does not help to eliminate the disease but rather contributes to the worsening of clinical status (Straub R. and Schradin C. 2016). Therefore chronic inflammation is associated with many diseases, often fatal, that affect humans. These diseases can virtually affect all systems of the organism. Fig. 5 shows diseases that have a relevant chronic inflammation component.

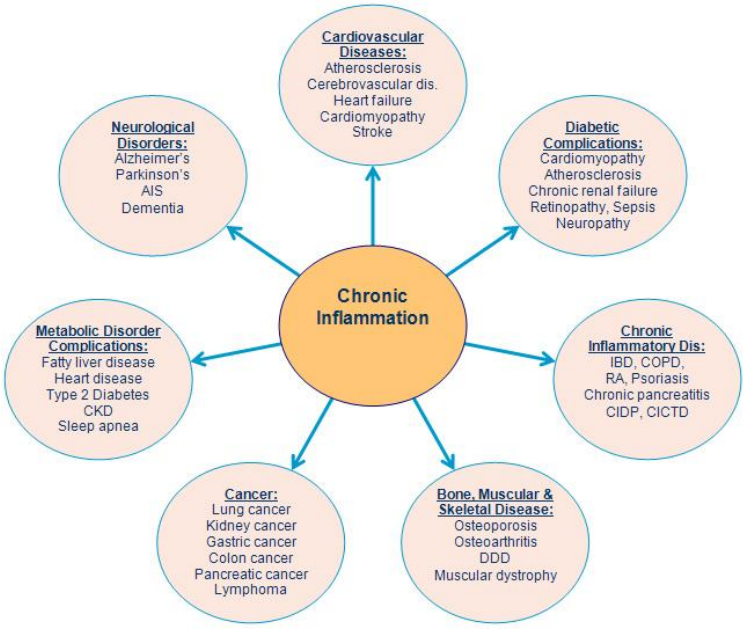

Fig. 5 Inflammatory diseases. The figure shows diseases in which chronic inflammation plays a key role (http://blog.wellnessfx.com/2013/09/16/take-deeper-look-two-biomarkers-inflammation/).

Most of the diseases listed in Fig. 5 leading to death. Since chronic inflammation plays a pivotal role in these diseases is crucial to find ways to cope as best as possible their inflammatory component with clinical interventions aimed to control, manage and reduce this immune component that should be addressed as a part of the disease. Chronic inflammation is a mechanism that disturbs the homeostasis of the tissues due to the presence of destruction and tissue repair in the inflammatory site producing fibrosis. This leads to the production of tissue damages which deteriorates cells of heart, blood vessels and the normal tissue structure. All this leads to a further exacerbation of inflammation and to a worsening of the situation. A picture just described is often considered a predisposition to the development of various forms of cancer. In fact, the presence of chronic inflammation is considered very often as a pre-cancer condition,

especially in certain tissues like the prostate. In this cancer in most patients the pro-inflammatory biomarker STAT3 it was found activated (Mora LB et al, 2002 and Tam L et al, 2007). In addition, a study shown that in 60% of colorectal cancer patients transcription factor NF-kB resulted over expressed. NF-kB plays a fundamental role in the regulation of immune response (Scartozzi M et al., 2007). This is one of the best situations in which it was demonstrated a clear association between inflammation and cancer. In fact, patients with chronic ulcerative colitis and Crohn's disease often develop colorectal cancer. Tumor cells employing some of the same molecules used by the immune system during inflammation, such as chemokines and selectins. The state of persistent inflammation creates a situation where along with the healing process take place tissue regeneration, in which leukocytes and macrophages may induce mutations in DNA of proliferating cells by the reactive oxygen species production during inflammation. Hence, the presence of reactive oxygen species at the moment of cell proliferation in which take place tissue regeneration may cause gene mutations in genes that play a key role in cell cycle as the gene encoding for the tumor suppressor protein p53, promoting the transition from a chronic inflammation state to cancer. There are some inflammatory conditions resulting from infections caused by pathogens in which it was definitely demonstrated the carcinogenic role of this condition. These situations are caused by schistosomiasis, a disease caused by parasitic flatworms called schistosomes, that increases the risk of bladder and colon cancer, the infection caused by the bacterium Helicobacter pylori which favors the development of gastric cancer, and one caused by the hepatitis C virus which predisposes to liver carcinoma. Inflammation is characterized by the presence of specific biomarkers that are responsible for the onset, maintenance and resolution of inflammation. Among the most important there are the transcription factors NF-kB and STAT3, the pro-inflammatory cytokines such as tumor

necrosis factor-alpha TNF-α, interleukin IL-1, IL-6, IL-8 and the chemokine MCP-1, enzymes such as cyclooxygenase COX-2, 5-lipoxygenase (LOX), and anti-inflammatory cytokines such as IL-4 and IL-10. These molecules, in addition to act effectively in inflammation also constitute indicators of the presence of inflammation. Chronic inflammation is an important component also of other diseases. Among these there are the Inflammatory Bowel Diseases (IBD) that affect bowel such as Crohn's disease (CD) and ulcerative colitis (UC) characterized by the presence of inflammation. In these pathologies have been found altered some of the biochemical markers typical of inflammation such as cytokines IL-2, IL-12, IL-18, IFN-γ, and TNF-α (Philip A. et al., 2008). Cardiovascular disease are also associated with chronic inflammation. In Chronic Heart Failure it has been repeatedly demonstrated the presence of high levels of TNF-α, a major pro-inflammatory cytokine, along with IL-1, IL-6 and an increased IL-8/IL-10 ratio (Thomas TH. and Advani A. 2006). Another group in which chronic inflammation is a relevant protagonist are autoimmune diseases such as rheumatoid arthritis and multiple sclerosis where were found elevated levels of cytokines such as TNF-α, IL-6, IL-1β, more precisely IL-8 in rheumatoid arthritis (Deon D et al., 2001) and increased levels of IL-1α, IL-2, IL-4, IL-6, IL-10, IFN-γ, TGF-β1, TGF-β2, and TNF-α in multiple sclerosis (Woodroofe MN. and Cuzner ML. 1993). In addition there have been found high levels of the most important biomarkers of inflammation also in other diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Young AM. Et al., 2012), in patients with diabetes (Pradhan AD et al., 2001) and in obese persons showing how these diseases are closely associated with chronic inflammation. Obesity deserves a special attention because it is a condition that leads to a series of diseases such as cardiovascular diseases in which inflammation plays a key role. It has been shown that adipose tissue is involved in the inflammation development

and that adipocytes are responsible for the production of some inflammatory biomarkers (Berg AH. and Scherer PE. 2005). On the basis of these evidences, the role played by inflammation in the pathogenesis of the major chronic human diseases has now been amply demonstrated. The inflammation is therefore a main actor in the onset of these diseases being its cause or starting after the onset of the disease worsening the clinical picture. Therefore is an imperative to consider inflammation as a mechanism of primary importance in the pathogenesis of diseases and deal with it in a very decided way trying to create new ground in research aimed to deepen its basic mechanisms to find new therapeutic strategies and on a strictly clinical level treating inflammation as a real disease.

2. RESOLUTION OF INFLAMMATION

2.1 General features

Resolution of inflammation is a finely coordinated program where are generated specific mediators termed Specialized Pro-Resolving Mediators (SPMs) that play a key role in the resolution of inflammation (Schwab JM. Et al., 2007). They act controlling and mitigating the phases of the inflammatory response after their biosynthesis, starting with the limitation of infiltration of the infection site by immune cells such as polymorphonuclear leukocytes (PMN) and continuing with the removal of these cells dead by apoptosis, a phenomenon called efferocytosis, from the site of inflammation to the lymph nodes through the action of macrophages as shown in Fig. 6. When a tissue damage or infection of a pathogenic occurs organism responds by releasing locally polyunsaturated fatty acids (PUFAs) from the phospholipidic membranes. In a few minutes prostaglandins and leukotrienes are produced from arachidonic acid to help white blood cells to achieve the infection site to develop the inflammatory response. Shortly after the beginning of the inflammatory response starts the biosynthesis of mediators of inflammation resolution phase, through an exchange in arachidonic acid metabolism that switch from the production of prostaglandins and leukotrienes to that of the production of SPMs (Levy B. D et al., 2001).

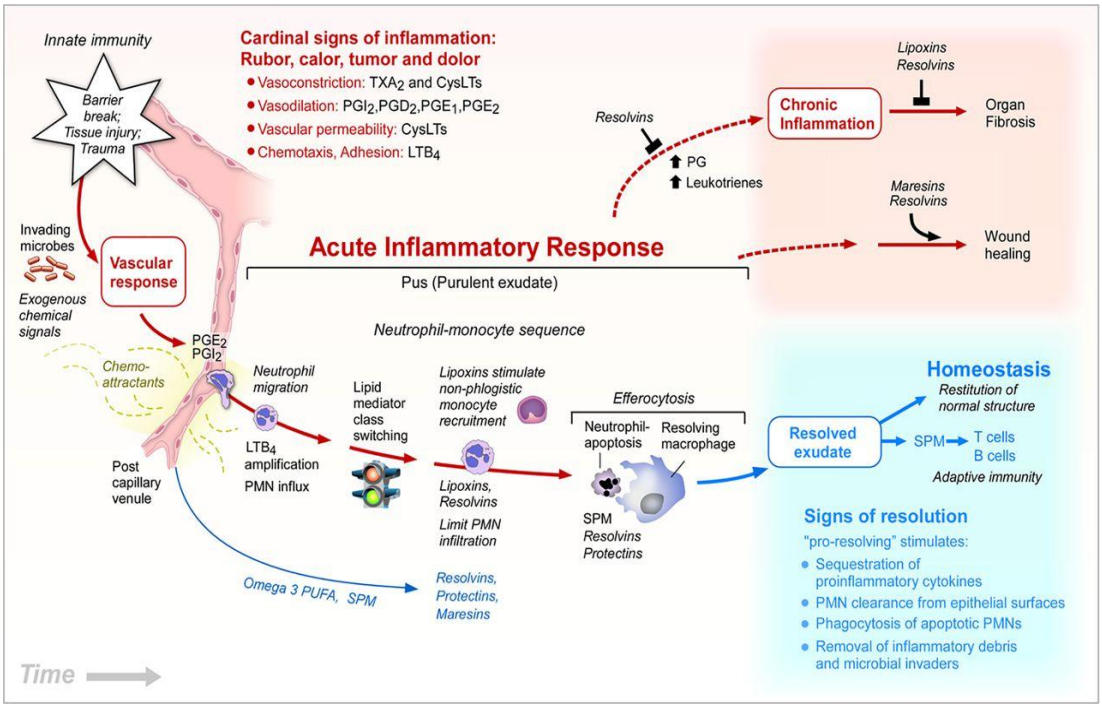

Fig. 6 Role of lipid mediators in the inflammatory response. The figure illustrates the role played by each lipid mediator in acute inflammation from the beginning to the resolution. They are shown prostaglandins E2 (PGE2) and PGI2, leucotrienes (LTX), lipoxins (LXs), resolvine (Rvs), maresins (MaRs) and protectins (PDs) each with its role (Serhan CN., 2017).

2.2 Specialized pro-resolving lipid mediators (SPMs)

SPMs are lipoxins, resolvins, maresines and protectines (Basil MC. and Levy BD. 2016) a novel families of lipid mediators that play a pivotal role in the resolution of inflammation as shown in Fig. 7. They are derived enzymatically from arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Bannenberg G. and Serhan CN., 2010). Lipoxins derived from arachidonic acid, a polyunsaturated fatty

acid Ω-6 (Serhan, C. N. et al., 1984) , while resolvins, maresines and protectines derived enzymatically from polyunsaturated fatty acids Ω-3 EPA and DHA (Serhan, C. N., 2011). SPMs were discovered in 1974 by Charles N. Serhan, Mats Hamberg and Bengt Samuelsson, who discovered that human neutrophils metabolize arachidonic acid to produce two molecules called Lipoxin (Lx)A4 and LxB4. Although at first seemed that these molecules have a pro-inflammatory action numerous studies subsequently showed that instead they had an anti-inflammatory action and then were termed specialized pro-resolving mediators (SPMs). They are produced mainly by macrophages and neutrophils and were first identified in resolving exudates. Resolvins are the most studied molecules between SPMs and are derived enzymatically from EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) that are Ω 3 PUFAs. Resolvins are also synthesized through a pathway involving aspirin called COX-2-dependent pathway. D-series resolvins are synthesized from DHA and E-series resolvins are synthesized from EPA. SPMs act by counteracting the effects of the molecules that cause inflammation beginning as leukotrienes and prostaglandins. They regulate COX-2 expression, leukotrienes and PAF formation and counteract the production of pro-inflammatory cytokines such as TNF-α and instead favor the production of anti-inflammatory ones as IL-10 (Serhan CN. et al.,2007). Moreover, they even regulate the expression of NF-kB gene. SPMs operate by interacting with five receptors of the G protein-coupled receptors (GPCRs) family (Serhan CN. and Chiang N., 2013). These receptors are expressed in different tissues, which allows SPMs to exercise their anti-inflammatory action in a targeted manner to specific cells. This information was obtained from studies in which they were used transgenic mice. Compared to drug therapy currently used the great advantage offered by SPMs is to be able to mitigate and resolve inflammation without depressing the host immune system.

This great advantage could mean address the care of inflammation without worsening the patient's condition.

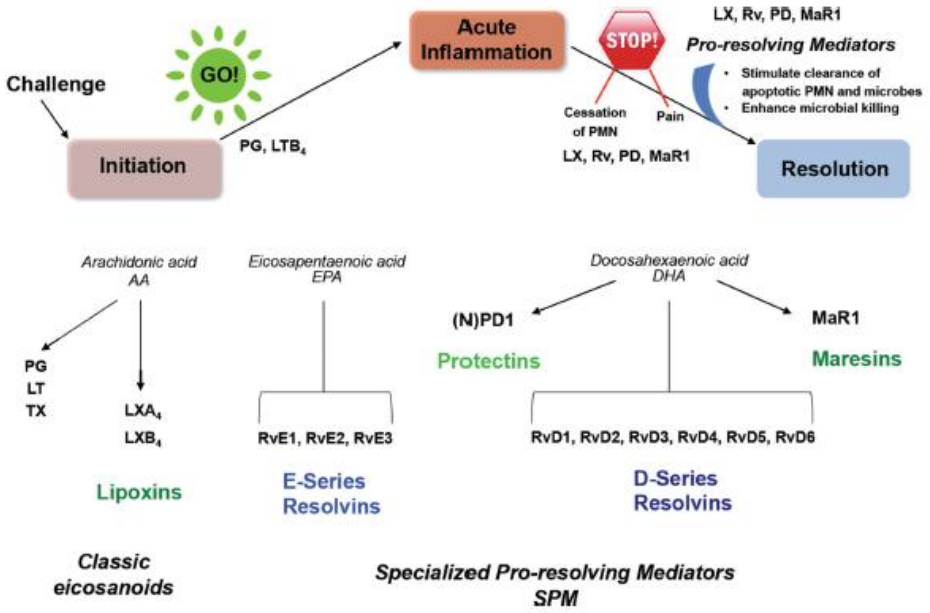

Fig. 7 Specialized pro-resolving lipid mediators (SPMs). The figure shows Specialized pro-resolving mediators (SPMs): lipoxins (LXA_4 and LXB_4) synthesized from arachidonic acid (AA), a Ω-6 polyunsaturated fatty acid (PUFA), E-series resolvins synthesized from eicosapentaenoic acid (EPA) a Ω-3 polyunsaturated fatty acid and D-series resolvins, protectins and maresines all synthesized from docosahexaenoic acid (DHA) again a Ω-3 polyunsaturated fatty acid. The figure also shows the phases of inflammation with initiation promoted by prostaglandins (PG) and leukotrienes (LTB_4) and the intervention of SPMs in inflammation resolution (Serhan CN and Chiang N., 2013).

2.2.1. AA-derived SPMs

AA-derived SPMs are Lipoxins. Lipoxin A_4 (LXA_4) and LXB_4 were the first SPMs discovered by Charles N. Serhan, Mats Hamberg and Bengt Samuelsson. They are derived enzymatically from arachidonic acid (AA), an omega-6 fatty acid and are potent anti-inflammatory mediators that act in minimal concentrations of picogram to nanogram (Serhan CN., 2005). In the last few years they have been discovered the epimers of the first two lipoxines, the epilipoxins, called 15-epi-LXA_4 and 15-epi-LXB_4. The synthesis of lipoxins occurs with two enzymatic reactions with arachidonic acid as shown in Fig. 7. The first involves attachment of an hydroperoxy (-O-OH) to form either 14,15-dihydroxy-eicosatetraenoate or 15-hydroxy-eicosatetraenoate products by the action of enzymes with 15-lipoxygenase activity such as ALOX15, ALOX12, aspirin-treated cyclooxygenase 2 or cytochrome P450s. The second enzymatic reaction, catalyzed by 5-lipoxygenase (5-LOX), forms a 5,6-epoxide from which are then produced either 5,6,15-trihydroxy- or 5,14,15-trihydroxy-eicosatetraenoates (Romano M. et al., 2015). These two enzymatic reactions may occur within a single cell which possesses both of the enzymes that catalyze the reactions, or in two different cells each of which possesses one of these two enzymes (Chandrasekharan JA. and Sharma-Walia N., 2015). This last way is called transcellular biosynthetic pathway. Lipoxins have a specific anti-inflammatory action which limits the polymorphonuclear leukocytes (PMN) recruitment on inflammation site, thus limiting their pro-inflammatory action. Also, they are potent chemo attractants via a non-inflammatory mechanism: for example, lipoxins from arachidonate activate mononuclear mobile recruitment without stimulating release of pro-inflammatory chemokines or activation of pro-inflammatory gene pathways (Serhan CN., 2007). Lipoxin LXA_4 is synthesized with two successive oxygenation reactions of arachidonic

acid as shown in Fig. 8 by the action of 5-lipoxygenase (5-LOX) e 15-lipoxygenase (15-LOX) (Gronert K. et al., 1999). This is a powerful resolving lipid mediator that acts by interacting with the G-protein coupled receptor ALX/FPR2 reducing the presence of neutrophils at the site of inflammation (Chiang N. et al., 2005), promoting apoptosis of leukocytes and the migration of monocytes/macrophages to inflamed tissue (Maddox JF. and Serhan CN., 1996). All these actions result in the reduction of inflammatory leukocytes and removal of dead cells at the site of inflammation.

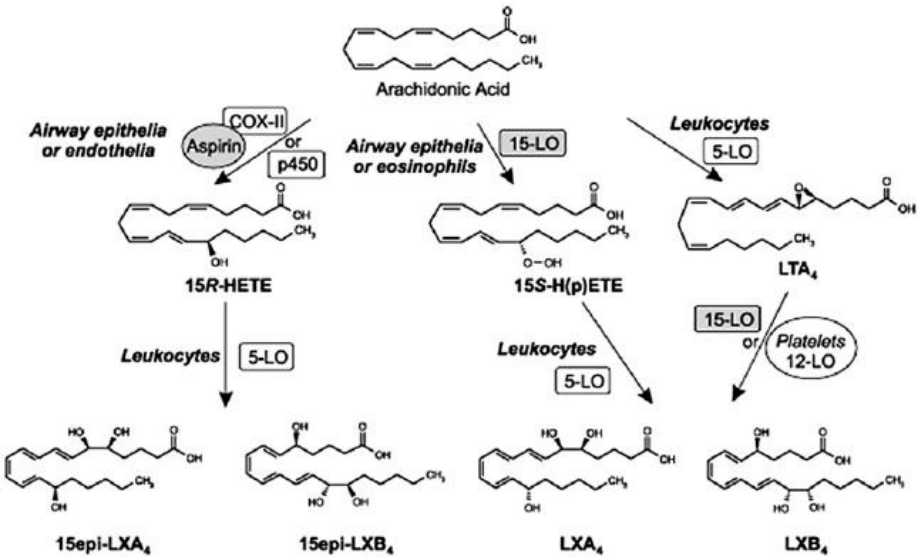

Fig. 8 AA-derived SPMs and their biosynthesis. The figure shows SPMs synthesized from arachidonic acid (AA) which are Lipoxin A_4 (LXA_4) and Lipoxin B_4 (LXB_4), the first to be discovered by Charles N. Serhan, Mats Hamberg and Bengt Samuelsson, and their epimers 15-epi-LXA_4 and 15-epi-LXB_4 (Das UN., 2012).

2.2.2. EPA-derived SPMs

EPA-derived SPMs are synthesized from omega-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) and are called E-series resolvins (Oh SF. et al., 2011). Up to now they have been identified three molecules of this series, denominated RvE1, RvE2 and RvE3. Their synthesis starts from eicosapentaenoic acid (EPA) by the action of aspirin-treated cyclooxygenase-2 (COX-2) or cytochrome P450 (CYP450) to produce the intermediate 18R-hydroperoxy-EPA, which under the action of 5-lipoxygenase (5-LOX) is transformed into RvE1 or RvE2 as shown in Fig. 9. It has been recently also discovered the production of a third molecule called RvE3 which is synthesized by the action of the 12/15-LOX. In addition, it was also identified a fourth molecule resulting from EPA called 18SRvE1, synthesized by the action of COX-2 and 5-LOX. E-series resolvins exert their action through the ChemR23/ERV receptor (Gao L et al. 2013). It has been demonstrated that the binding of RvE1 to ChemR23 receptor has the effect of trigger the signal of transduction to monocytes and dendritic cells to reduce the production of pro-inflammatory cytokine IL-12 (Arita M. et al., 2005). In addition it has been also showed that RvE1 binds to leukotriene B4 Receptor BLT1 (Arita M. et al., 2007). Therefore RvE1 bins to both ChemR23/ERV and BLT1 receptors.

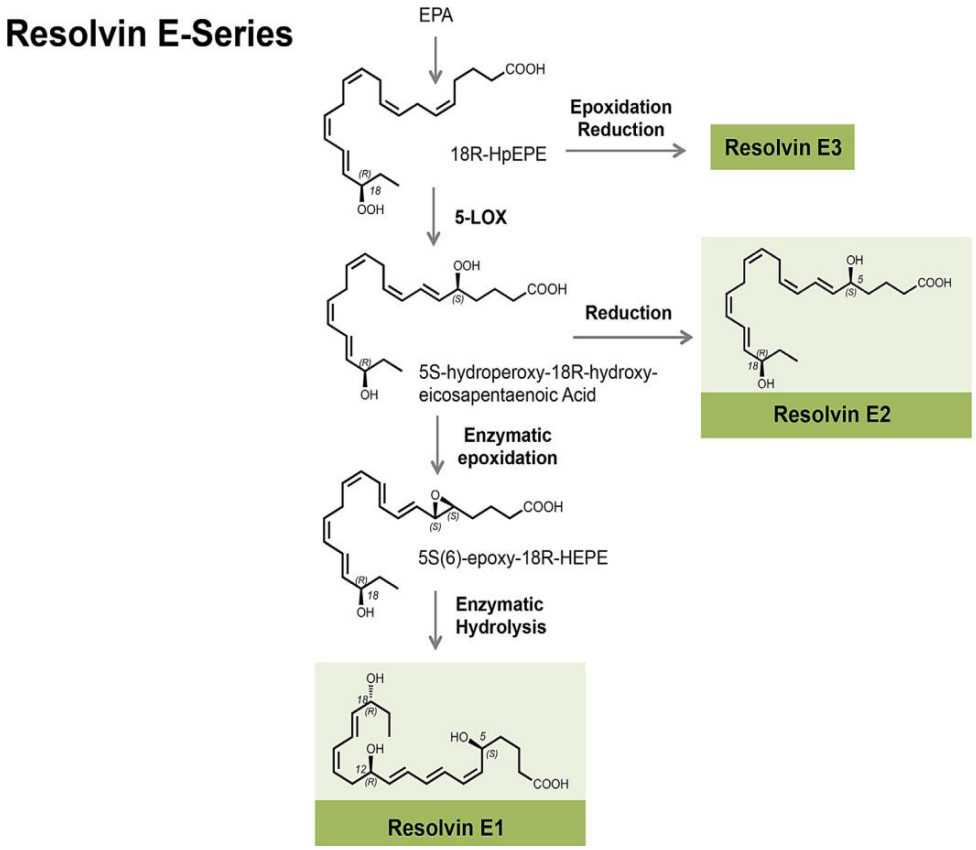

Fig. 9 EPA-derived SPMs and their biosynthesis. The figure shows the synthesis of E-series resolvins from eicosapentaenoic acid (EPA) by the action of 5-lipoxygenase (5-LOX) **(**Nan C. and Serhan CN., 2017).

2.2.3. DHA-derived SPMs

DHA is an important element of the omega-3 PUFAs found in fish oil and their beneficial effects as nutritional supplements have long been known. These beneficial effects have been further strengthened by the discovery that the D-series resolvins are synthesized from it. DHA-derived SPMs are synthesized from omega-3 polyunsaturated

fatty acid docosahexaenoic acid (DHA) and are called D-series resolvins (Serhan, C. N. and Petasis NA., 2011). D-series resolvins are synthesized from DHA through two sequential oxygenation reactions performed by the 15-LOX and 5-LOX enzymes. DHA is converted by 15-LOX to 17-HDHA and after by 5-LOX to resolvins RvD1 to RvD6 as shown in Fig. 10. D-series resolvins can also be generated in aspirin presence by aspirin-acetylated COX-2 producing the resolvins aspirin-triggered AT-RvD1 or AT-RvD2. In these cases aspirin-acetylated cyclooxygenase-2 generates 17R-HDHA, that is following transformed in AT-RvD1 or AT-RvD2 by 5-LOX (Hong S. et al., 2003). Resolvins are produced in inflammatory exudates and are the product of a transcellular biosynthesis, which occurs through the use of two cells, leukocytes and endothelial cells. It has been shown that the molecule activated by aspirin AT-RvD1 has the same effect of RvD1 in limiting the infiltration of polymorphonuclear leukocytes (PNM) in murine peritonitis (Sun YP. et al., 2007). The limitation of the infiltration of PNM is obtained through the reduction of PMN trans-endothelial migration, the earliest event in acute inflammation. D-series resolvins exert their action by means of the G protein-coupled receptors. To carry out its anti-inflammatory action RvD1, and also its aspirin-triggered (AT) epimer AT-RvD1, binds to both N-formyl peptide receptor 2 ALX/FPR2 and GPR32 receptors (Krishnamoorthy S. et al., 2010). GPR32 receptor also interacts with other D-series resolvins as RvD5, RvD3 and AT-RvD3 (Dalli J. Et al., 2013). Binding of RvD1 with receptors ALX/FPR2 and GPR32 stimulates the phagocytic activity of macrophages and helps to slow down the inflammatory action of PMNs. Until recently it was thought that RvD2 binds only to GPR18/DRV2 receptor, that is expressed in the PMNs and macrophages. By binding to macrophages RvD2 stimulates the phagocytosis and efferocytosis (Chiang N. et al., 2017). Hence, they have been identified six different D-series resolvins (Serhan CN. and Petasis N., 2011) but those

most studied and best characterized are the RvD1 and RvD2. D-series resolvins are produced and act at the site of inflammation.

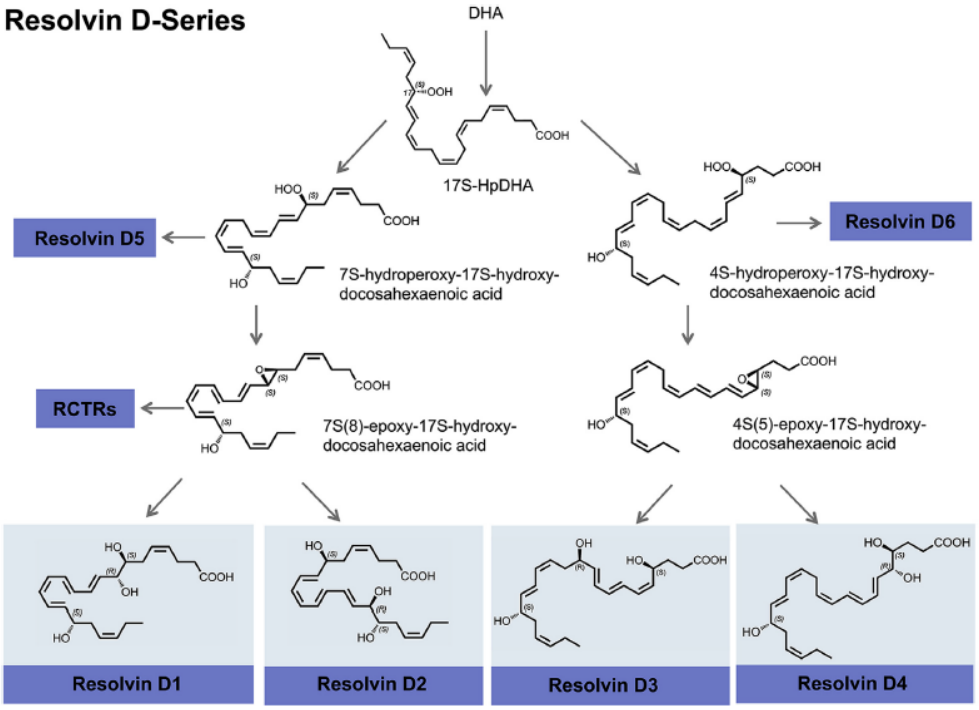

Fig. 10 DHA-derived SPMs and their biosynthesis. The figure shows the synthesis of D-series resolvins from *docosahexaenoic acid* (DHA) **(**Nan C. and Serhan CN., 2017).

3. ROLE OF RESOLVINS IN RESOLVING INFLAMMATORY STATES

Resolvins were first identified in the inflammatory exudates of mice during the study of the lipid mediators profile. As noted above, they have the ability to resolve inflammation and the generic mechanisms by which they act have been identified in several studies. Resolvins are able to dampen the production of proinflammatory mediators such as cytokines and promote the synthesis of other pro-resolving mediators. In addition, they are able to significantly reduce PMN (polymorphonucleated leukocytes) infiltration at inflammation sites, a key event in initiating and maintaining inflammation. Moreover, they are able to enhance macrophage efferocytosis which favors the removal of neutrophils dead for apoptosis from the site of inflammation as shown in Figure 11. These actions are carried out by the interaction of resolvins with specific G-protein-coupled receptors that have been described in the previous chapter.

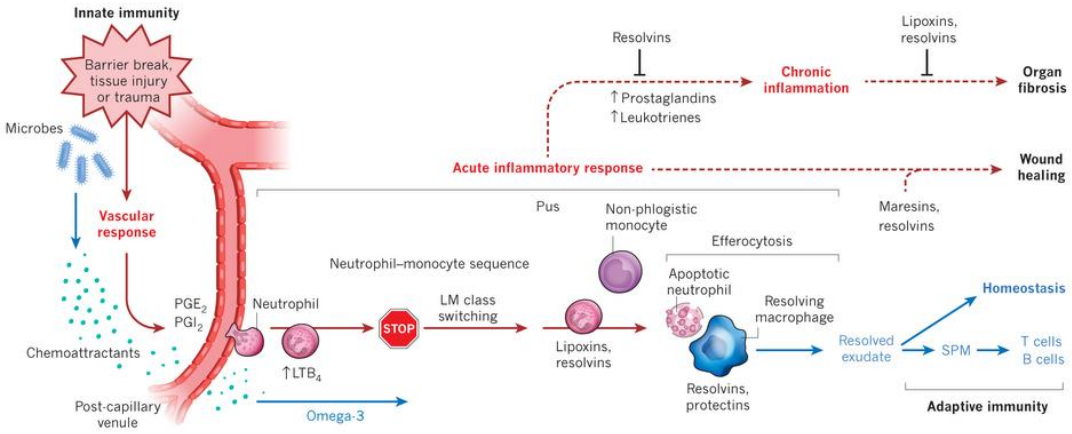

Fig. 11 Specialized pro-resolving mediators action and effects. The figure shows the role played by Pro-resolving lipid mediators in inflammation (Serhan CN., 2014).

Below are indicated some studies showing the efficacy of resolvins in controlling the inflammatory state through the mechanisms just mentioned. There are evidences on the ability of resolvins in resolving inflammatory states in both animals and humans. In mice RvD1 was able to improve pulmonary fibrosis (Yatomi M. et al., 2015) and in both mice and human cells exposed to cigarette smoke were able to markedly reduce pro-inflammatory cytokines production (Hsiao HM. Et al., 2013). Also, RvD1 reduced the eye-immuno-inflammatory reaction in rats (Rossi S. et al., 2015). Moreover, in mice RvD1 reduced leukocyte infiltration in zymosan A-induced acute peritonitis and reduced acute inflammation by regulation of the transcription control machinery in peritoneal macrophages (Recchiuti A. et al., 2014). In mice with inflammatory arthritis RvD1 showed protective effects on cartilage (Norling LV. et al., 2016) and administration of RvDs in mice with bilateral ischemia/reperfusion injury resulted in a reduction of functional and morphological kidney injury (Duffield JS. et al., 2006). In humans RvD1 increased macrophage phagocytosis of zymosan and apoptotic polymorphonucleated leukocytes cells interacting with both ALX and GPR32 receptors of phagocytes and showed a role in resolution of inflammation (Krishnamoorthy S. et al., 2010). Another study on Human Umbilical Vein Endothelial Cells (HUVECs) showed RvD1 action on inflammatory-state related proteins and genes and on the modulation of inflammatory response (Gdula-Argasińska J. et al., 2016). SPMs have been found in many human tissues as shown in Fig. 12 and even here many studies have shown their efficacy in controlling inflammation. These studies have shown that RvD2 decreased TLR4 expression to mediate resolution in human monocytes (Croasdell A. et al, 2016), SPMs attenuated cigarette smoke-induced inflammation via their pro-resolving and anti-inflammatory actions on human macrophages (Croasdell A. et al, 2015) and Aspirin-Triggered Resolving 1 (AT-RvD1) demonstrated both anti-

inflammatory and pro-resolution effects in PBMCs from patients with severe asthma (Zambalde ÉP. et al., 2016). Moreover it has been proven that D-series resolvins RvD1 and RvD2 were able to modulate T lymphocytes immune activity in humans (Chiurchiù V. et al., 2016). Indeed, many other studies have shown that in addition to RvD1 and RvD2 also other D-series and E-series resolvins are able to modulate the inflammatory response, as shown in Table. 3

Table. 3. RvDs action in murine infections and disease models.

RvDs	Disease
RvE1	Colitis
RvD5	*E.Coli* infection
RvD1, RvD5	Acute lung injury
RvD2	Cecal ligation and punture sepsis
RvD2	Burn wound sepsis
RvD3	Aging mice, peritonitis
RvD4	Skin inflammation peritonitis
RvE1	Synthesized by Candida albicans
RvD2	Reduces mortality
RvE1	Protects vs *E. Coli* pneumonia

In Table 3 is shown that RvD2 was able to be effective in cecal ligation and punture sepsis (Chiang et al., 2017, in Burn wound sepsis (Bohr et al., 2013) and in control microbial sepsis (Spite et al., 2009). Also, RvD3 was employed to demonstrate that in aging mice the process of resolution of inflammation mediated by SPMs is reduced. (Arnardottir et al., 2014) and that RvD4 had a pro-resolving actions in *S. aureus* infections and stimulated efferocytosis (Winkler et al., 2016). In addition E-series resolvins RvE1 showed its pro-resolving efficacy in colitis (Arita et al., 2005), is synthesized by the fungus Candida albicans to balance its interaction with the host (Haas-Stapleton et al., 2007) and protects vs *E. Coli* pneumonia (Seki et al., 2010).

Finally, RvD5 and Protectin D1 are effective in *E.Coli* infection (Chiang et al., 2012) and acute lung injury (Wang et al., 2011). Therefore, as demonstrated in all these studies, both D-series and E-series resolvins are very effective in controlling and modulating inflammatory response in several pathological conditions through their effects on the inflammation-resolution process. Thus, research in this field is of great interest to better understand how these SPMs exercise their action to find new therapeutic opportunities in diseases with a major inflammatory component.

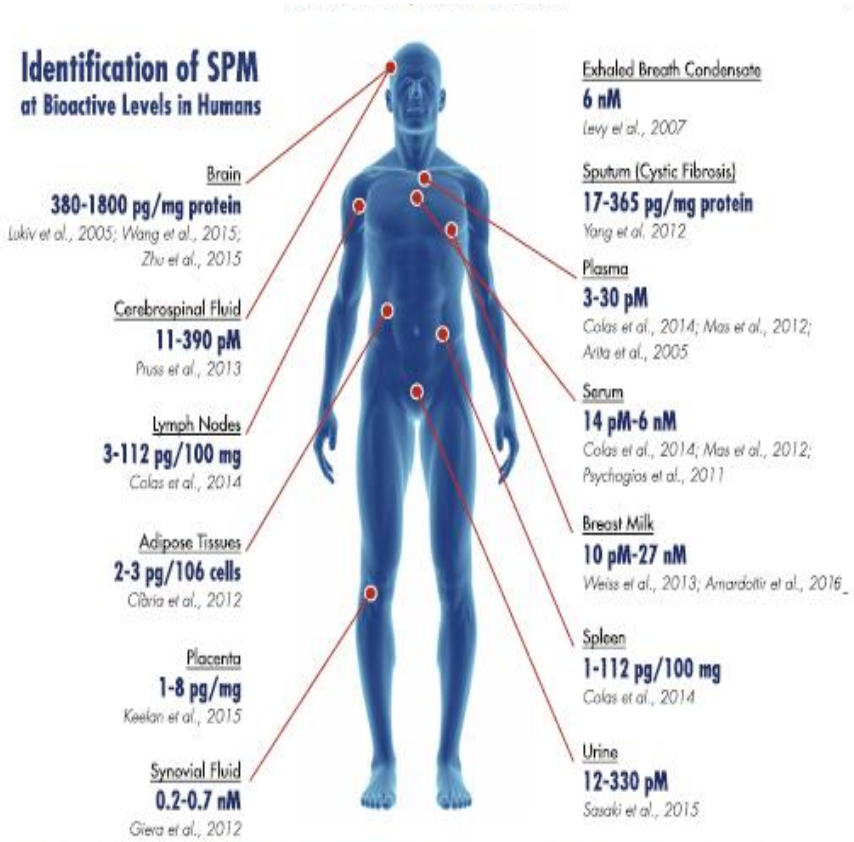

Fig. 12 Identification of SPMs in human tissues. The figure shows the points of the human body, liquids, cells and tissues, where has been detected biologically active concentrations of SPMs in *In Vivo* experimental models (Serhan CN., 2017).

4. CHRONIC HEART FAILURE (CHF)

Chronic Heart Failure is a complex syndrome that primarily affects the elderly population and is characterized by the progressive inefficiency of the heart in pumping the blood needed to satisfy the request of the organism. It develops as a result of conditions that damage the heart muscle such as myocardial infarction, hypertension, cigarette smoking, obesity, diabetes and valvular dysfunctions (Hunt SA. et al., 2005). All of these conditions cause an alteration of the propagation of the heartbeat that leads to worsen the heart capacity contraction. Although the majority of patients with heart failure is over sixty-five years it may occurs even early. It's a progressive condition which, however, is not always clinically evident, especially in the initial stages, where patients often do not show obvious symptoms. The main symptoms of heart failure are dyspnea on exertion in the early stages and even at rest in the worse stages, the edema of the lower limbs, loss of appetite and memory problems. Giving the increase of human life span it is expected that CHF will enhance its impact on world population with a progressive increase in mortality and costs for public health (Hawkins NM. et al., 2009). Data from United States shows the public health impact of CHF: about 5.7 million adults in the United States have heart failure; one in 9 deaths in 2009 included heart failure as contributing cause; about half of people who develop heart failure die within 5 years of diagnosis and heart failure costs the nation an estimated $ 30.7 billion each year (Mozzafarian D. et al., 2016).

4.1. Epidemiology

Although in the last decades it has been seen a general reduction of cardiovascular diseases, at the same time there was an increase of the prevalence of chronic heart failure. This occurred due to the increase in life expectancy and improvements in the management of the critical phases of myocardial infarction. The most recent data suggest a decrease in the incidence of CHF, caused by advances in the prevention of the syndrome, and at the same time improved therapies that lead to increased survival and prevalence (Roger V.L., 2013). Considering various studies the estimated incidence of heart failure oscillates in a range of 1-5 every 1,000 people, which increases to 30-40 for individuals with more than 75 years. The currently estimated prevalence is 1-2% of the general population in developed countries, and reaches 10% among those over 70 years of age. A third of men and more than a quarter of women over 55 is at risk of developing heart failure in the lifetime. Mortality at one year is 17% for hospitalized with unstable disease and 7% for stable outpatients. Over the last 30 years, improvements in treatments and their implementation have increased survival and reduced the hospitalization rate in patients. In patients with CHF (both hospitalized and ambulatory), most of the deaths are due to cardiovascular causes. Among people over 65 years of age presenting to primary care with breathlessness on exertion, one in six will have unrecognized CHF. After the diagnosis of CHF, survival estimates are 50% and 10% at 5 and 10 years, respectively, and left ventricular dysfunction is associated with an increase in the risk of sudden death. Available data indicated that lifetime risks are very high, regardless of gender, race, and geography (Ponikowski P. et al., 2016).

4.2. Physiopathology

CHF has several causes, but the most important is ischemic heart disease, followed by cardiomyopathy, valvular dysfunction and hypertension. Many studies on CHF have demonstrated that this syndrome has a complex physiopathology caused by dysfunctions affecting different body systems such as cardiovascular, renal and immune system (Johnson FL., 2014). The result of this complex network of dysfunctions leads to a change of the structure and functioning of the heart. The changes that occur with the onset of CHF cause reduced cardiac output and increased venous pressure along with the cellular and molecular changes that cause a progressive worsening of the condition that can lead to death. Cardiovascular disorders leading to CHF are myocardial infarction, valve dysfunction and hypertension. Myocardial infarction is the main cause of cardiovascular nature, but also the largest ever, leading to CHF (Hellermann JP. et al., 2002). The dysfunctions that occur in the heart immediately after the heart attack are those of left ventricular remodeling, with which the heart tries to maintain the same cardiac output capacity after having suffered the damage (Dargie H., 2005). These dysfunctions are early infarct expansion, subsequent infarct extension in adjacent non infarcted myocardium, and late hypertrophy in the remote LV (French B.and Kramer C., 2007). The heart has four valves: the mitral, aortic, tricuspid and pulmonary. When these valves don't work properly, as when they become stenotic or incompetent the heart muscle attempts to compensate the poor pumping action losing elasticity and efficiency (Carabello BA. and Fred A. Crawford FA., 1997). In this way valvular heart disease may lead to congestive heart failure and other complications (Maganti K. et al., 2010). Another condition that may lead to CHF is hypertension, when narrowed arteries that are less elastic make it more difficult for the blood to travel smoothly and easily causing the heart to work harder. In order to carry out this larger

work, the heart is subject to deformations and dilations that in the long run lead to the development of heart failure (Subramaniam V. and Lip GY., 2009). Often is possible to see the simultaneous presence of cardiac and renal dysfunction that may worsen each other by means of different mechanisms such as fluid overload and increased venous pressure, hypo-perfusion, neurohormonal and inflammatory activation (Metra M. et al., 2012). The simultaneous presence of cardiac and renal dysfunction may be due to common risk factors such as hypertension, diabetes, and atherosclerosis and the activation of the sympathetic nervous system and renin–angiotensin system. However many studies suggests that cardiac dysfunction may cause renal dysfunction, and vice versa. Additionally, dysfunctions of the immune system has been increasingly recognized as a pathogenic trait and as a progression marker for CHF. Briefly, the inflammatory response is characterized by activation of the immune system shortly after any events that alters the structure of the heart that leads to an increased expression of several pro-inflammatory mediators. The key role played by immune processes in the onset and progression of CHF will be widely illustrated in Paragraph 5. CHF can be classified according to different points of view. One of the most used is systolic or diastolic: the former is characterized by dilatation and systolic wall thinning of the ventricle causing his inability to contract and eject the required amount of blood (McMurray John J.V., 2010), while the latter is characterized by thickened walls with normal or reduced size of the atrium causing its inability to relax and to be filled with blood (Aziz F. et al., 2013). These two cases described fall within the definition of left-sided heart failure, because in these conditions the structures of the heart involved are left ventricle and left atrium to which comes the oxygen-rich blood from the lungs to be pushed from the left ventricle into the arteries to be distributed to all tissues (Badgett R.G. et al., 1997). When heart failure regards the structures of the right side of the heart

is called right-sided heart failure. In this case, the heart receives blood from the rest of the body through the veins that arrive at the right atrium. The blood then passes into the right ventricle that drives it to the lungs. In right-sided heart failure the blood can't get into the atrium and back into the veins, causing a series of problems including swelling and ascites (Cowger Matthews J. et al., 2008). These conditions cause a lowered cardiac output of blood that the body tries to balance with the following actions: a hemodynamic defense reaction, an inflammatory response and a hypertrophic response with ventricular remodeling. The hemodynamic defense reaction is aimed at maintaining a constant perfusion of major organs balancing the impaired cardiac output. This hemodynamic defense is implemented through an increased cardiac stimulation, water and sodium retention and arteriolar vasoconstriction and is mediated by a signaling cascade. Ventricular remodeling is a response caused by the attempt of the heart to compensate the increased pressure and volume caused by the onset of CHF, but that results in worsen the existing clinical conditions (Azevedo P.S. et al., 2016). These mechanisms initially bring benefits to the patient, but with time they are detrimental and contribute to the worsening of the clinical condition of the CHF.

4.3. Clinical manifestations and NYHA classification

The main clinical manifestations of CHF are dyspnea (shortness of breath), asthenia, fatigue, weakness, anorexia and nutritional state. Dyspnea is manifested only under strong effort in the initial stages of CHF to become evident in correspondence of light efforts with the worsening of the condition (Dubé BP et al, 2016). There are two types of dyspnea that are typical clinical manifestations of CHF, orthopnea and paroxysmal nocturnal dyspnea. The orthopnea is the dyspnea in the supine position and is caused by redistribution of liquid abdomen and lower limbs toward the chest when the patient

takes the supine position. This redistribution increases the pulmonary capillary wedge pressure causing the lifting of the diaphragm. The paroxysmal nocturnal dyspnea consists of sudden and severe attacks of breathlessness at night that wake the patient and which may not regress if the patient is put in a sitting position. It is thought to be caused by decreased arterial oxygen tension. In addition, patients report having asthenia and fatigue, which are caused by reduction of perfusion of skeletal muscles and the inability of the failing heart to increase the pumping (Kasper DL. et al., 2015). The weakness is caused by the reduced ability of the heart in pumping blood which causes insufficient supply of oxygen and nutrients in the various bodily districts. In regard to anorexia and nutritional state a very important role is played in CHF from the patient's food intake. It has been shown that some cytokines may affect the caloric intake, reducing it and causing an increase in muscle weakness resulting in cachexia (Gullestad L. Et al., 2012). Many evidences in the literature suggests that reducing dietary intake in the elderly is a multifactorial event (MacIntosh C. et al., 2000). Therefore malnutrition is a negative prognostic factor that influences the evolution of the condition (Hughes CM. Et al., 2012), in which the catabolic effect produced by cytokines plays a decisive role in producing cachexia and reducing muscle mass (Collins PF. Et al., 2013). The relationship between nutritional status and cytokine implantation in patients with heart failure (Catapano G. et al., 2008) have also been studied, always finding interesting indications of how the pathology is influenced by the patient's nutritional state. The most widely used functional classification in the world for CHF is the New York Heart Association, the NYHA classification, in which the degree of heart failure is classified according to the level of limitation of physical activity (Russell S.D. et al., 2009). The NYHA classification divides CHF into four classes, I, II, III and IV which progressively identify a condition that worsens. This classification is also used to decide which

therapeutic treatment apply to each patient. The definition of classes is based on the symptoms that occur during exercise activity:

Class I. Patient asymptomatic (no symptoms). Habitual physical activity does not cause breathlessness or fatigue.

Class II. mild heart failure. The moderate physical activity (such as climbing two flights of stairs or climbing a few stairs carrying a weight) causes breathlessness or fatigue

Class III. Congestive heart moderate to severe. The minimum physical activity (such as walking or climbing half a flight of stairs) causes breathlessness or fatigue.

Class IV. Severe heart failure. Fatigue, shortness of breath or fatigue also present at rest (sitting or lying in bed).

4.4. Diagnosis and therapy

Often the symptoms for the diagnosis of CHF are not specific of the syndrome and therefore do not help in the discrimination between CHF and other diseases. Some more specific symptoms are elevated jugular venous pressure and displacement of the apical impulse, which are still difficult to identify and interpret in obese individuals, in the elderly and in patients with chronic lung disease. Clearly diagnosis requires careful patient's medical history to see if there are some conditions that favor the onset of CHF, such as previous myocardial infarction that greatly increase the likelihood of CHF in a patient with appropriate symptoms and signs (Inamdar AA and Inamdar AC., 2016). Once detected the symptoms that may lead to suppose the presence of CHF there have to be carried out the analysis necessary to ascertain the presence or not of the syndrome. These analyses include the plasma concentration of natriuretic

peptides (NPs), such as the brain natriuretic peptide (BNP) and the N-terminal fraction of its precursor (NT-pro-BNP), which increases with the rise of the volume and ventricular pressure (Izzi V. et al., 2007), electrocardiogram and echocardiography. They are molecules produced by cardiomyocytes as a result of structural stress suffered by the cells in order to dilate the blood vessels. In particular, the main function of Atrial Natriuretic Peptide (ANP) is in the regulation of water, sodium, potassium and adipose tissue homeostasis. It's produced by the atrium of cardiomyocytes with the ultimate goal of decrease blood pressure (Widmaier EP. et al., 2008). However, the natriuretic peptide produced by the ventricular cells is of major clinical interest because the ventricles having a larger mass of the atria and they produce a greater amount of natriuretic peptides which are therefore better detectable in plasma assays (Januzzi JL., 2013). Plasma concentration of NPs is used to quantify the degree of pressure overload and volume of the ventricle and is the first diagnostic test performed in the diagnosis of heart failure. However, it must be integrated with other two fundamental tests, the electrocardiogram and the echocardiography (Shamsham F. and Mitchell J., 2000). The electrocardiogram (ECG) is an instrumental analysis which detects the electrical activity of the heart, and that outputs is a track of cardiac activity (Dosh SA., 2004). This track in a healthy individual presents a characteristic pattern which varies in the presence of heart malfunctions. The track is characterized by the presence of different specific traits denominated waves which are repeated at each cardiac cycle. It's an analysis that is always performed in both individuals at risk of developing a heart condition and in patients who have already developed an heart disease. The results of ECG should always be compared with those of previous ECG to evaluate any difference. The ECG can detect abnormalities in heart function such as atrial dysfunctions, ventricular hypertrophy, arrhythmias, previous myocardial infarction and myocardial ischemia in

progress. However, an abnormal track in electrocardiogram increases the likelihood of the diagnosis of CHF (Davie AP. et al., 1996). The most important test for the diagnosis of heart failure is, however, the echocardiography, because it is able to evaluate the function and the volume of the ventricles, the presence of abnormality of the contraction and motility, to measure ejection fraction (EF) which is the fraction of blood ejected from a ventricle of the heart with each heartbeat (Iwano H. and Little WC., 2013), to detect the presence of valvular dysfunction and to discriminate between systolic heart failure and heart failure with preserved systolic function (Senni M. and Redfield M., 2001). In fact, in the latter the value of the ejection fraction is normal EF > 50%, while in systolic heart failure ejection fraction is less than 50% with dilatation of the left ventricle. The echocardiography is therefore a test that provides a wealth of information to patients in whom there is a suspicion of heart failure, including ventricular systolic and diastolic function, valve function and pulmonary hypertension, chamber volumes and wall thickness (Hung J. et al., 2007). This is an examination based on cardiac ultrasound imaging whose information play a pivotal role on the diagnosis and treatment of heart failure. The diagnosis of heart failure is then carried out through a careful clinical evaluation in order to ascertain the presence of the typical symptoms of heart failure followed by the just described instrumental analyzes as shown in Fig. 13.

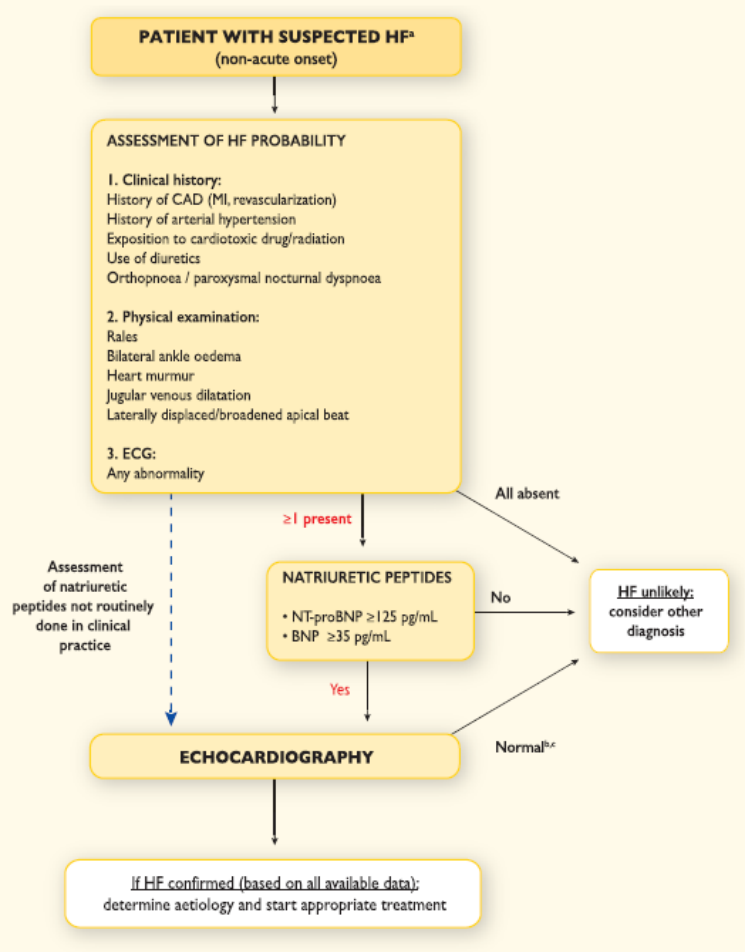

Fig. 13 Diagnostic procedure for heart failure. BNP = B-type natriuretic peptide; CAD = coronary artery disease; HF = heart failure; MI = myocardial infarction; NT-proBNP = N-terminal pro-B type natriuretic peptide **(**Ponikowski P. et al., 2016).

In the last decades new drug therapies have improved the life expectancy of CHF patients (Iacoviello M. and Antoncecchi V., 2013). The therapies used to treat patients with heart failure are intended to improve the clinical condition of the patients, their functional capacity and quality of life, as well as trying to prevent new hospitalizations and reduce mortality. For the treatment of CHF has been used certain drugs which,

however, have shown both positive and negative effects on patients, so there are regulatory bodies and clinical practice guidelines to drive doctors to prescribe the best treatment. Now the drugs more recommended by international guidelines are the following: ACE inhibitors, angiotensin II receptor blockers (ARBs) or sartans, an association between the two previous drugs, hydralazine and nitrates with long duration of action and beta-blockers (β-blockers) as shown in Fig. 14. The ACE inhibitors are also known as angiotensin converting inhibitors, have been shown to be very effective in treating CHF by improving the clinical condition of patients and increasing the quality of life and survival proving to have an effect on all NYHA classes of CHF (Demers C. et al., 2005). These drugs are angiotensin converting enzyme inhibitors (ACE, ie Angiotensin Converting Enzyme), which is part of a regulatory cascade of blood pressure (renin-angiotensin system). In particular enalapril significantly reduced mortality and hospitalizations in patients with chronic heart failure and reduced ejection fractions (SOLVD Investigators, Yusuf S. et al., 1991). The most important ACE inhibitors used in therapy are captopril, enalapril, lisinopril, perindopril and ramipril. Another major classes of drugs used in treatments of CHF are the angiotensin II receptor blockers (ARBs) or sartans. These drugs have been shown to be a viable alternative to ACE inhibitors and can also be used together with them (McMurray JJ. et al., 2003). However, even by themselves they have been shown to reduce mortality of patients and to decrease NYHA functional class (Granger CB. et al., 2003). Patients who do not respond to treatment with ACE inhibitors and sartans can be treated with hydralazine and nitrates with long duration of action that have been shown to improve the prognosis of patients and decrease their mortality. Also beta blockers have been shown to be effective in the treatment of CHF if co-administered with ACE inhibitors

and other vasodilators. In these cases they have been able to improve the prognosis, the ejection fraction and to reduce mortality (Packer M. et al., 2001).

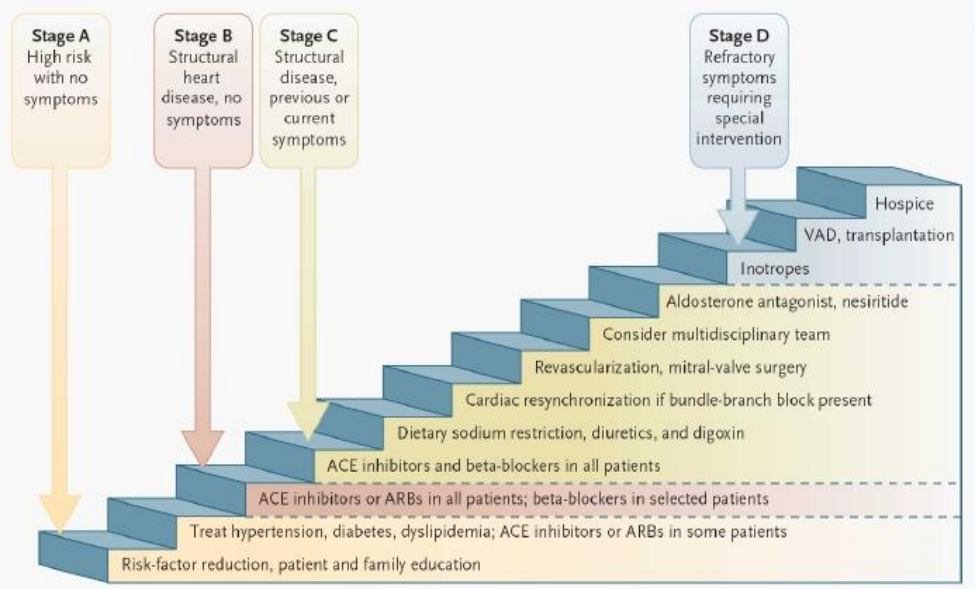

Fig. 14 Stages of Heart Failure and treatments option. (Jessup M. and Brozena S., 2003).

5. ROLE OF INFLAMMATION AND ITS RESOLUTION IN CHF

Chronic Heart Failure is a condition with an important immune component involved in the onset and maintenance of the syndrome and that may contribute in the long run to deteriorating clinical conditions of the patients. The immune system is activated by events that disturb the structure and functioning of the heart and with time may lead to CHF. The events which cause an insult to the heart can be grouped into ischemic and non-ischemic etiologies, of which ischemic origin are the major ones. In particular, it was observed that immediately after myocardial infarction, the main cause of CHF, the characteristic events of inflammation, such as activation of immune cells and production of inflammatory mediators, take place. The perturbation suffered by the heart and the subsequent reperfusion activates the innate immune responses (Liu J. Et al., 2016). This is the first and immediate immune response in which are involved neutrophils and macrophages that produce pro-inflammatory cytokines, chemokines and growth factors that are responsible for the recruitment of leukocytes at the site of tissue injury. Several studies have shown that neutrophils are involved in the innate inflammatory events that occur shortly after myocardial infarction and in other conditions such as stable angina. In this latter situation, it has been observed that neutrophils of patients exhibit a markedly increased of chemotactic activity and leukotriene B4 (LTB4) generation compared to the age-matched controls, and hence an overall increased activity (Mehta J. Et al., 1989). Further studies have highlighted the pathogenetic role of neutrophils in myocardial ischemia, where their activation causes alterations in vascular permeability and regulation of coronary circulation which in turn leads to unstable angina and myocardial infarction (De Servi S. et al ., 1991). In addition, granulocytes (PMNs) are involved in the extension of myocardial injury after infarction. They are able to release

several mediators of tissue injury that act on other cells that contribute to inflammatory response and which produce deleterious effects on cell membranes, endothelial cells and myocardium. Also, neutrophils release leukotriene C4 that have a vasoconstrictor effect on coronary arteries along with the release of thromboxane A2 by the platelets (Ricevuti G. et al., 1991). The level of neutrophils at the time of admission in patients with acute myocardial infarction was related to both the onset of CHF (Kyne L. et al., 2000) and increased risk for major adverse cardiovascular events (Haumer M. et al. , 2005), and therefore it may help in identifying those patients at greater risk of worsening the syndrome. After myocardial infarction, macrophages play an important role because they induce CC chemokines to recruit proinflammatory monocytes that differentiate into macrophages and exert phagocytotic actions, very important because it induces anti-inflammatory cascade by inhibiting leukocyte recruitment. This action has a cardioprotective effect and may induce down-modulation of proinflammatory signaling, secretion of cytoprotective mediators, activation of reparative fibroblasts and angiogenic cells (Chen B. and Frangogiannis NG., 2016). Macrophages present in myocardial infarction are derived from circulating monocytes and are implicated in inflammatory tissue remodeling, resolution of inflammation during post- myocardial infarction healing, and left ventricular remodeling. Both monocytes and macrophages are able to produce inflammatory cytokines, cathepsins and matrix metalloproteinases (Frantz S. and Nahrendorf M., 2014). Also, at the site of ischemia, macrophages remove necrotic cardiac myocytes and apoptotic neutrophils, secreting cytokines, chemokines, and growth factors, and modulate the phases of the angiogenic response. Hence, macrophages are the cells that together with neutrophils respond quickly in post-myocardial infarction wound healing regulation (Lambert JM et al., 2008). The pro-inflammatory mediators produced in the immune response phase are tumor necrosis

factor-α (TNF-α), interleukin-(IL)-6 and IL-1β. The increase in circulating cytokines levels, an important signal of immune activation, impairs cardiac performance and reduces systolic output promoting endothelial dysfunction and thromboembolic phenomena (Sharma R. Et al., 2000) and is considered a marker of this condition (Gullestad L. et al., 2012). TNF-α is produced by CD4+ T cells (Satoh S. et al., 2006), and is, as a matter of fact, the most studied pro-inflammatory cytokine in CHF (Negrusz-Kawecka M, 2002). Table 4 shows that TNF-α participates in the physiopathology of heart failure (Feldman AM. Et al, 2000), and that TNF-α LPS-responsiveness appears to relate to age in both healthy controls and CHF patients (von Haehling S. et al., 2003). The pivotal role of TNF-α in CHF was further confirmed by other works in which the subpopulation of TNF-α producing CD4 was larger in NYHA II-IV patients than in normal subjects (Satoh S. et al., 2006) and in which circulating levels of TNF-α, interleukin-6, and the soluble TNF-α receptors were enhanced and associated with increased mortality in CHF patients (Deswal A. et al., 2001). Another study showed that patients with CHF had significantly decreased levels of IL-10, an anti-inflammatory cytokine, compared with healthy control (Stumpf C. et al., 2003). However, also other cytokines are involved in heart failure, such as IL-18 that showed a serum level significantly higher in patients of NYHA class IV than in patients of NYHA classes II and III.

Table. 4 Role of pro- and anti-inflammatory cytokines in CHF

Cytokine	Effect	References
TNF- α	Participates in physiopathology	Feldman AM. Et al, 2000
TNF- α	Response related to age	von Haehling S. et al., 2003
TNF- α superfamily ligands	Enhanced gene expression	Yndestad A. et al., 2003
IL-10	Decreased levels	Stumpf C. et al., 2003
TNF- α producing CD4 cells	Larger TNF- α producing CD4 cells	Satoh S. et al., 2006
IFN-γ- positive CD4 cells	Larger IFN-γ- producing CD4 cells	Fukunaga T. at al., 2007
TNF- α and IL-6	Increased receptors expression levels	Deswal A. et al., 2001
IL-18	Increased serum levels	Yamaoka M. et al., 2002
IL-8/IL-10 ratio	Significantly plasma increased levels	Trofimov E. et al., A. 2015
IL-18	Increased plasma levels	Seta Y. et al., 2000
IL-13	Increased plasma levels	Nishimura Y. et al., 2009

In the early innate immune response there is a remarkable inflammatory cell infiltration with neutrophils and monocytes followed by lymphocytes, whose adaptive response contribute to the onset of chronic inflammatory events. Moreover, there are the molecules of the resolution of inflammation and those of angiogenesis and tissue repair seeking to reshape the ventricle. It is precisely this situation that may lead to worsen the clinical picture of the patient, because the inflammatory response is useful in trying to heal the heart after the heart attack, but its excessive presence may result in incorrect ventricular remodeling leading to CHF (Fang Lu et al., 2015). This situation favors the onset of the adaptive immune response, which is supported by B and T lymphocytes. In recent years many studies have shown the enhancement of the activity of adaptive immune response cells such as T lymphocytes in CHF, as shown in Table 5.

Table. 5 Role of T lymphocytes in CHF.

T lymphocytes	Effect	References
% T-cells expressing CD69, CD25, CD69 and CD25 markers	Significantly increased CHF patients	Yndestad A. et al., 2003
TNF-α producing CD4 and CD4/CD8 ratio	Increased TNF- α producing CD4 cells	Satoh S. et al., 2006
IFN-γ positive CD4+ cells	Increased frequency (%) of IFN-γ-positive CD4+ T cells	Fukunaga T. at al., 2007
T (Treg) cells	Treg cells decreased significantly	Li N. et al., 2010
CD4+ CD28null cells	Are associated with CHF severity	Koller L. et al., 2013
T and B lymphocytes	Decreased number of B and CD4+T-cells	Moro-García MA. et al., 2014
T (Treg) cells	Treg cells decreased significantly	Tang T.T. et al., 2010

It has been showed that the percentage of $CD4^+$ and $CD8^+$ T-cells expressing the early activation marker CD69, CD25, CD69 and CD25 was significantly increased in CHF patients compared to controls and that T-cells from CHF patients showed significantly enhanced gene expression of the TNF-α superfamily ligands, IFN-γ, IL-18 and macrophage inflammatory protein MIP-1α. (Yndestad A. et al., 2003). In addition both the absolute number of CD4+ and ratios of CD4+ to CD8+ T cells were increased in patients with heart failure (Satoh S. et al., 2006) and the frequency (%) of IFN-γ-positive CD4+ T cells was increased in patients with CHF compared with controls (Fukunaga T. at al., 2007). Also, it has been shown in two studies that CHF patients had significantly lower frequency of circulating T regulatory cells (Tregs) compared to those of healthy controls (Li N. et al., 2010 and Tang T.T. et al., 2010). Tregs cells are immunosuppressive and down-regulate induction and proliferation of T cells (Bettelli E.

et al., 2006). Moreover, it has been shown that circulating CD4+CD28null cells are associated with CHF severity (Koller L. et al., 2013) and that CHF patients showed a significantly lower levels of CD4+ T-cells compared with healthy controls (Moro-García MA. et al., 2014). This last work illustrates very well why it is of great interest to study the effect of resolvins on T lymphocytes of CHF patients. In fact, the study shown that only the lymphocytes levels were decreased, especially CD4+ T-cells. In addition, the more severe NYHA classes showed a positive correlation with the increase in the differentiation of CD4+ and CD8 + T-lymphocyte subsets. All other previous works focused on the analysis of individual cells or specific cytokines, while this work is the first one that describes simultaneously the complete profile of all immune cells on a single group of CHF patients, noting variations only on the population of T lymphocytes. These findings suggests that CHF patients have an higher degree of immunosenescence than healthy control in T-lymphocyte subpopulations that may contribute to the worsen of the disease through a compromised adaptive immune response due to accelerated aging of their immune system. On the basis of these findings, an effective therapeutic approach in CHF should seek to mainly target T lymphocytes and their altered immune responses, possibly without depressing the immune system defenses of patients. Some attempts have been made trying to hit some pro-inflammatory cytokines, and in particular TNF-α. However, these attempts to face the immune component of CHF with infliximab and etanercept targeting TNF-α failed due to adverse effects on survival and rate of hospitalization (Chung ES et al., 2003). Therefore, potentiating the anti-inflammatory mechanisms, perhaps by using resolvins, could be an alternative to contrasting inflammation. Indeed, the role of resolvins in controlling the immune response has been studied in several pathological conditions. Among these, their protective role has been reported clearly in some cardiovascular

conditions that are causes or contributory causes of CHF such as atherosclerosis and myocardial infarctions. These studies are summarized in Table 6.

Table. 6 Studies on the effect of SPMs in cardiovascular diseases (CVD). The table shows some of the most important studies on the effect of SPMs in cardiovascular diseases.

SPMs	Effect	References
RvE1	Decreased atherogenesis in rabbit	Hasturk H.et al., 2015
RvD1	Reduces atheroprogression and increases efferocytosis in mice	Fredman G. et al., 2016
RvD2 and Mar1	Decreased atheroprogression in mice	Viola JR et al., 2016
Aspirin triggered lipoxin A4 (ATL)	Decreased atheroprogression in mice	Petri MH et al., 2017
Aspirin-triggered RvD1 (AT-RvD1)	Modulates the immune response in Chagas disease patients	Ogata H. et al., 2016
RvD1	Reduces infarct size by PI3-K/Akt pathway	Gilbert K. et al., 2015
15-LOX inhibition	Attenuate 15-LOX cardioprotection	Gilbert K. et al., 2015
RvD1	Resolvin D1 improved ventricular function after myocardial infarction	Kain V. et al., 2015
RvD1	Decreases Post-Myocardial Infarct Depression	Gilbert K. et al., 2014

Over the last twenty years several studies have suggested that the onset and progression of some cardiovascular diseases such as arteriosclerosis may be sustained by defects in the immune response, where the role of SPMs has been widely demonstrated. It is now clear that the lack of control and modulation of chronic inflammatory response is one of the major causes of cardiovascular disease outbreaks (Heinz J. et al., 2017). Therefore,

it is logical to consider SPMs as a target of potential therapeutic treatments. For example, one of the mechanisms through which SPMs act in modulating inflammation is efferocytosis, which is the removal of neutrophils dead for apoptosis that have incorporated pathogenic agents by macrophages at the site of inflammation. The efferocytosis favors the resolution of inflammation. A study has shown that in atherosclerosis, this process is defective (Tabas, I., 2010). Among the most studied SPMs there are D-series resolvins, whose role in cardioprotection and in the ability to modulate innate inflammatory response, in early studies, and adaptive response, in recent studies, has been demonstrated. A recent work showed a low level of RvD1 in regions of human atherosclerotic plaques and that the imbalance caused by this defect and the pro-inflammatory leukotrienes favors the instability of the atherosclerotic plaques (Fredman G. et al., 2016). In atherosclerosis RvE1 has shown to decrease atherogenesis in rabbit (Hasturk H.et al., 2015), RvD1 showed reduction of atheroprogression in mice (Fredman G. et al., 2016), RvD2, Mar1 and Aspirin triggered lipoxin A4 (ATL) have been able to decrease atheroprogression in mice (Viola JR et al., 2016 and Petri MH et al., 2017). In myocardial infarctions Aspirin-triggered RvD1 (AT-RvD1) was able to modulate the immune response in Chagas disease patients (Ogata H. et al., 2016). This diseases is a tropical parasitic disease caused by the protozoan *Trypanosoma cruzi* that produce the enlargement of the ventricles leading to heart failure (World Health Organization. March 2013). Also, RvD1 reduced infarct size by PI3-K/Akt pathway (Gilbert K. et al., 2015) in rats, improved ventricular function after myocardial infarction (Kain V. et al., 2015) in mice and decreased post-myocardial infarct depression (Gilbert K. et al., 2014) in rats. It has been further shown that in rats the inhibition of 15-LOX, a biosynthetic enzyme of RvD1, attenuated its cardioprotective effect (Gilbert K. et al., 2015). Moreover, RvE1 protects the rat heart

against reperfusion injury (Keyes KT et al., 2010). Therefore these preliminary studies, although performed only on mice and rats, seem to confirm that also in cardiovascular diseases SPMs are able to control the modulation of inflammation and protection. Since the protective role of resolvins has already been documented in cardiovascular conditions that are causes or contributory causes of CHF, then it is plausible that they themselves may somehow be involved in CHF. Hence, it is of great interest to carry out further research to ascertain whether also in cardiovascular disease and CHF the SPMs are effective in controlling inflammation improving the clinical condition of the patients.

II. AIM OF THE STUDY

Many studies carried out as soon as after their discovery have shown that resolvins are able to resolve acute inflammation in both animals and humans. However, acute inflammation, when unresolved, can become chronic, worsening the severity of the existing conditions. It is now known that resolvins, in addition to being necessary to resolve acute inflammation, they can also prevent its chronicization by acting in both the repairing of damaged tissues and by modulating the cells typically involved in chronic inflammation such as T lymphocytes. Indeed, it has been recently demonstrated that resolvins (in particular Resolvin D1 and D2) are able to modulate the immune response of several T cell subsets. Interestingly, significant alterations in CD4+ T lymphocytes cell count and cell activities have been recently shown in CHF patients, clearly suggesting an involvement of adaptive immunity in CHF physiopathology. On the basis of this scenario, the aim of the present work was two-fold. On one hand we asked whether there is a failure in the production of Resolvins D1 and D2 during CHF and, on the other hand, we sought to investigate whether resolvins could be able to impact on T-cell dependent chronic inflammatory responses in CHF, in terms of pro-inflammatory cytokine production from both CD4+ and CD8+ T cells. The underlying mechanistic insight to resolvin-dependent effect on T cells was also investigated, in terms of leukocyte responsiveness to resolvins via their target receptors. This would open up novel approaches for the development of potential new pro-resolving treatments targeting those immune cells responsible for the chronic inflammatory events underlying CHF immunopathogenesis.

III. MATERIALS AND METHODS

1. Materials

RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) and RvD2 (7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid) were purchased from Cayman Chemical.

2. Populations of the study and exclusion criteria

Based on data from previous study (Colas RA. et al., 2014) it has been estimated the need to enroll a sample size of 23 subjects/group, total = 46, to test our hypothesis with a power of 80% at a significance level of P=0.05. The study was performed with human blood samples of CHF patients (n=27) and humans healthy controls (n=23) as shown in Table 7. The study included both sex subjects 13 males and 14 females CHF patients of average age 82,61±6,67 (mean±standard deviation) and 9 males and 14 females healthy controls of average age 78,43±4,78. The diagnosis of chronic heart failure was carried out by means of clinical criteria, BNP and NT-pro BNP serum levels and echocardiogram. The controls were selected in the same age range of the patients, namely from 70 to 90 years old, excluding subjects with any disease that could affect the immune system. Moreover, none of the controls was affected by hypertensive or ischemic heart disease.

Table 7. CHF patients enrolled in the study

	NYHA II	NYHA III	NYHA IV
n.	10	8	9
Sex	5 M/5 F	4 M/4F	4 M/5 F
Age	81,09±7.6	85,5±5,5	81,25±6,9

Data is reported as mean ± standard deviation

Patients were analyzed for their clinical parameters and after they were stratified according to NYHA (New York Heart Association) classification II (10 patients), III (8 patients) and IV (9 patients) as shown in Table 7. Both CHF patients and healthy controls gave their written informed consent to the study according to Legislative Decree n. 196/2003. Once obtained the consent of the patient, they have been performed a complete personal history, physical examination and geriatric multidimensional evaluation. Finally, a peripheral venous blood sampling of 20 ml was collected. They were enrolled CHF patients of ischemic, hypertensive or valvular origin in various stages of NYHA classification, from II to IV and healthy age matched controls were enrolled. The exclusion criteria concerned those individuals with at least one of the following characteristics: neoplastic diseases, chronic renal insufficiency stage IV and V (classification of the National Kidney Foundation - February 2002), hepatic impairment, immunological or chronic infections that can affect the immune and cytokine pattern assets, and any condition that may alter the arrangement of the immune system. It was a case-control study comparing patients with chronic heart failure and healthy subjects. The individuals of the two groups were matched for selected features,

mainly age, to be comparable, given that age dramatically impacts the immune response. Participation of the subjects was entirely voluntary and unpaid. The study was approved by the Ethical Committee of Campus Bio-Medico of Rome and was conducted according to ethical principles arising from Helsinki Declaration. Several instrumental and geriatric multidimensional evaluations were performed during the patient's recruitment.

3. Geriatric multidimensional evaluation

Geriatric Multidimensional Evaluation is a program used in order to assess the condition of the elderly by means of some parameters that describe the activities of the person as shown in Table. 9. The resources, potentialities and the needs of the elderly are also evaluated. This is essential to understand elderly conditions, creating a personalized care plan, and monitoring health status changes over time. It's a tool used by the geriatric doctors that analyzes the following functional areas of the elderly:

- Physical function, strength, motility, endurance, balance, etc.
- Cognitive function, attention, orientation, language, memory, judgment ability, ability to solve problems
- affective function, interest, quality of sleep, mood, feeling of well-being
- Social status, family support, friendship, help social, economic situation

Table. 8 Parameters examined in Geriatric Multidimensional Evaluation

Geriatric Multidimensional Evaluation	
Health status	Examination
	Pharmacological history
	Instrumental diagnostic tests
Functional state	Functional state ADL and IADL (basal and instrumental activities of daily life BARTHEL INDEX
	MNA (nutrition)
	TINETTI (motor skills)
Psychic and mental conditions	MMSE – SPMSQ (cognitive status)
	GDS (Depression)
	CAM - NEECHAM (confusion)
	BARS
Social and environmental situation	Questionnaire on housing, economic conditions and social networks

4. Spirometry

Spirometry is an analysis carried out to evaluate the respiratory function. It's a simple exam that if done with the patient's collaboration it takes no more than ten or fifteen minutes. It is performed with a instrument called spirometer and during the examination the operator helps the patient to perform the various maneuvers needed for the correct acquisition of data. Through a mouthpiece, the patient needs to drain air from the lungs into the instrument in a first step more slowly and in a second step more quickly. The examination is able to calculate the pulmonary volume and the strength of the inspiratory and expiratory muscles and thus a possible poor lung function responsible of many conditions that correlate with the respiratory difficulty of the patient. The most important of these conditions is dyspnoea (difficult breathing). It is just the discomfort

symptoms that causes the doctor to prescribe this examination, which gives indispensable indications to explain the cause of dyspnoea. There are a number of conditions and symptoms that require the execution of spirometry:

- coughing that has long been
- control of patients with respiratory problems such as asthma, chronic bronchitis, COPD (chronic obstructive pulmonary disease), emphysema and lung diseases
- respiratory function in smokers

Usually the result of spirometry falls into one of four possible outcomes: normal, obstructive, restrictive and mixed (obstructive and restrictive). Each of these in turn is classified as mild, moderate, serious or very serious. These results should be integrated with the symptoms found in the patients and with the objective examination. The main parameters that are measured by spirometry are:

- Vital capacity (VC);
- Forced vital capacity (FVC);
- Forced expiratory volume (FEV) at the interval of 0.5, 1 (FEV1), 2, and 3 seconds;
- Forced expiratory flow at 25-75% (FEF 25-75);
- Maximal voluntary ventilation (MVV), also known as Maximum Respiratory Capacity.

The first result to be evaluated in the spirometry is the ratio between the maximum expiratory volume in the first second and the vital capacity (VEMS/CV). Since VEMS is directly proportional to resistances encountered, a pathological reduction of this ratio indicates an obstruction present along the airways, which

obstructs forced exhalation. VEMS <88% in men and <89% in women indicates the presence of a obstructive ventilatory defect.

The second parameter to consider is the % of VEMS compared to the predicted value to quantify the severity of the obstruction. Obstruction is defined, considering the value of VEMS:

- Mild = 70-88%
- Moderate = 60-70%
- Moderately severe = 50-60%
- Severe = 35-50%
- Very severe = <35%
- Another parameter to be evaluated is the value of the vital capacity (CV): if the CV value is below 80% of the predicted value, there is a reduction in static pulmonary volumes. In this case, it was performed a complete spirometric examination for total lung capacity (CPT) and residual volume (VR).

5. Bioimpedentiometry

Bioimpedentiometry (BIA) is a bioelectric examination that is carried out in order to know the body composition and evaluating its nutritional status. The weight indicated by the scale does not give us information on the amount of fat and muscle mass we have, nor does it indicate whether we are well hydrated or if we are in a condition of dehydration or water retention. All this information can be known with the bioimpedentiometry. BIA is based on the principle that different types of tissue express specific electrical conductivity. All biological structures oppose a force at the flow of continuous or alternating currents that cross them, generating an impedance. The

impedance (Z), measured by the bioimpedentiometry, depends on resistance (Rz) and reactance (Xc). In particular, water is a good electrical conductor with low resistance and cells work like capacitors that accumulate and disconnect current, while fat and bones are bad conductors because of their high resistance. This is inversely proportional to the amount of body fluids, while the reactance essentially depends on the Mobile Cell Mass (ATM) or Body Cellular Mass (BCM). The measurement is carried out by positioning a pair of electrodes on the back of the hands and another pair on the back of the subject's foot; the electrodes are connected by means of wires to the measuring instrument. An alternating, imperceptible, very low intensity (800 μA) and high frequency (50 KHz) current is passed through the electrodes, which, traveling along the body, will encounter different resistances depending on the composition of the different bodily districts. The software then transforms electrical measurements into clinical data, based on algorithms that take into account the population's reference values, the anthropometric measurements of the subject, age and sex. The main parameters provided by the BIA are: Total Water (TBW, Total Body Water), Intracellular Water (ICW), Extracellular Water (ECW), Lean Mass (FFM, Free Fat Mass), Activated Mass (ATM), Cellular Mass (BMC), Extracellular Mass (ECM) and Fat mass (FM, Fat Mass). Therefore BIA provides a set of parameters that describe the state of the body and can be used both to complete a patient's state of health assessment and the state of a healthy individual who wants to maintain and improve his physical fitness.

Table. 9 Clinical data of CHF patients. Values of the clinical parameters detected in CHF patients at the time of enrollment.

	NYHA II	NYHA III	NYHA IV	UNITS OF MEASURE
PA	4,28±1.08	3,78±1,9	3,83±1,2	Degrees
FFM	48,6±10.6	50±7,7	48,17±10,1	Kg/m
BCM	21,44±7.2	15,76±2,7	19,76±7,0	Kg/m
FM	16,34±5.2	29,63±18,2	21,59±10,7	Kg/m
ECWpct	57,86±7.9	63,46±21,3	63,15±10,3	% L
ICWpct	42,38±7.8	33,48±19,9	39,95±9,6	% L
MM	26,9±8.0	23,65±17,8	28,68±8,3	Kg
MMpct	33,38±6.1	32,35±7,6	30,65±4,2	% Kg
Mbasale	1344,7±222.3	1214,62±424,6	1292,16±249,3	Kcal
BMI	23,5±2.7	30,41±7,1	27,08±6,2	Kg/m^2
BCMI	7,61±2.0	5,67±1,1	7,47±2,2	Kg
Hydration	78,18±6.4	80,45±7,1	78,11±7,1	% of FFM
SMI	8,23±1.7	9,57±0,9	8,23±1,4	% Kg
FMI	5,54±1.11	11,05±7,1	9,08±4,6	Kg/m
FFMI	17,64±2.7	19,21±1,7	18,26±2,2	Kg/m

Data is reported as mean ± standard deviation

FFM=Lean mass; BCM=Cell Body Mass; FM=Fat mass; PA=Phase angle; ECWpct= Extracellular Water Percentage; ICWpct= Intracellular water percentage; MM= Muscle mass; MMpct= Muscle mass percentage; Mbasale=Basal metabolism; BMI=Body mass index; BCMI=Cell body mass index; SMI= Skeletal mass index; FMI= Indexed fat mass; FFMI= Indexed lean mass

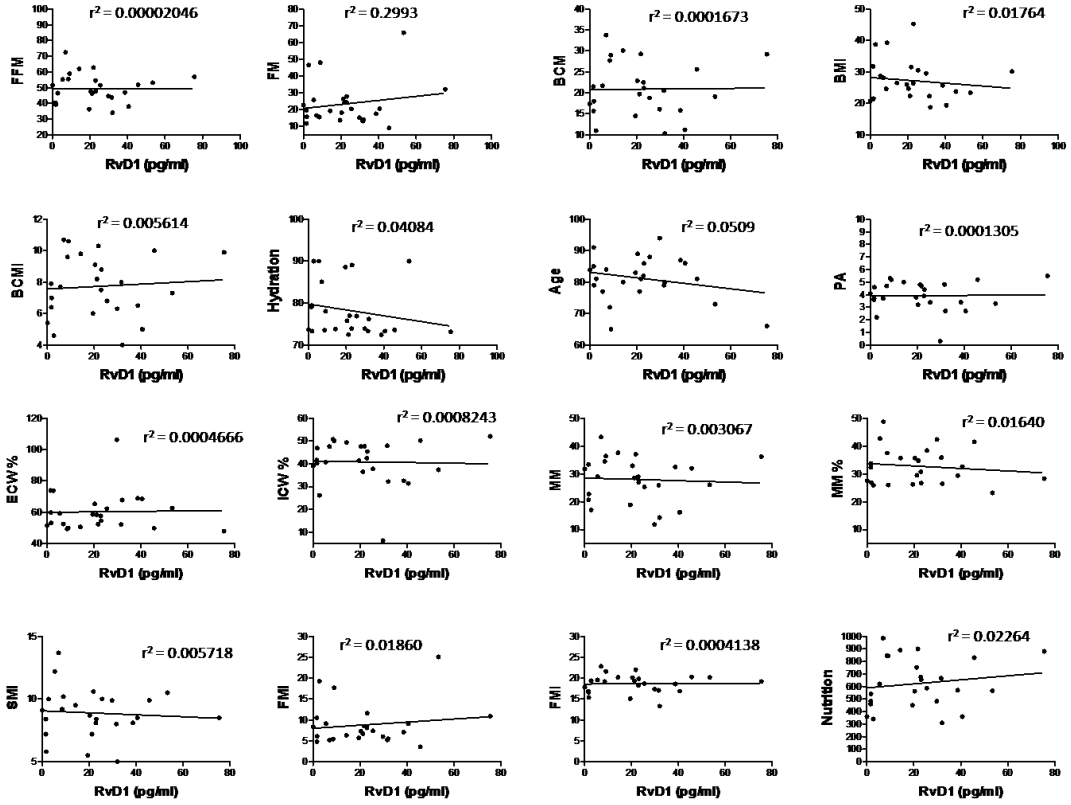

Fig. 15. Correlation analysis between the plasma values of RvD1 and the bioimpedentiometric data obtained at the time of enrollment of CHF patients.

As shown in the figure, no significant correlation was found. The analysis was carried out with the Pearson correlation test. $p > 0.05$

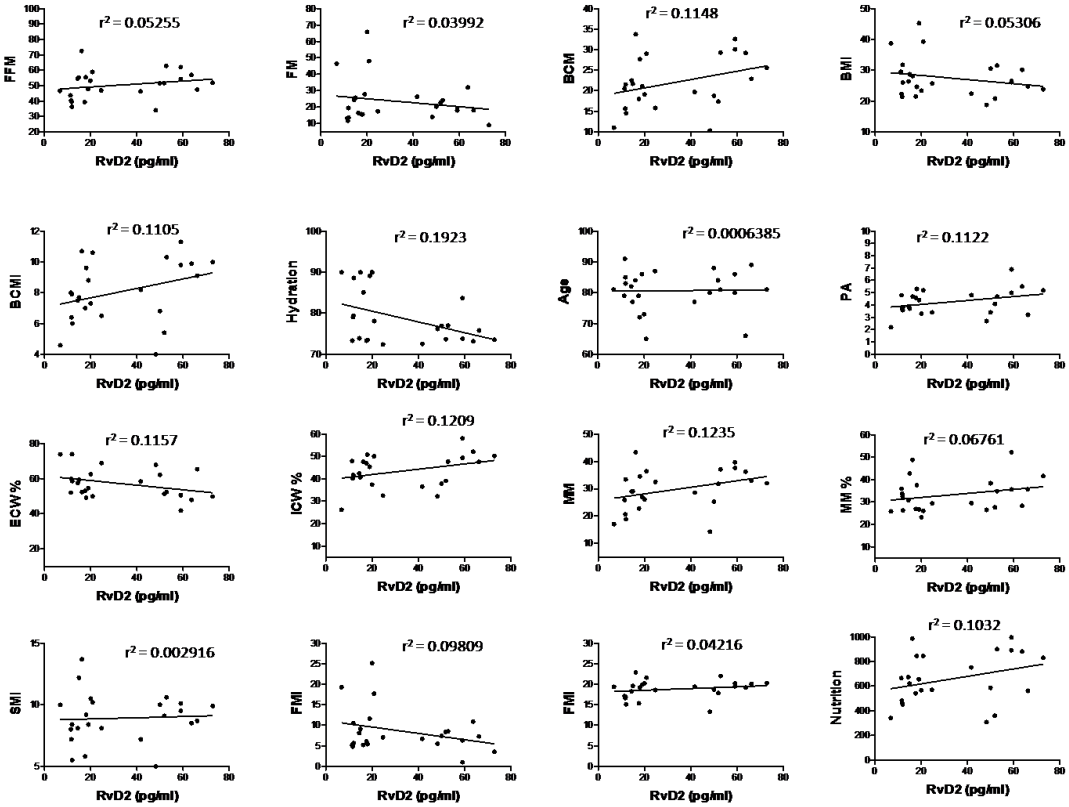

Fig. 16. Correlation analysis between the plasma values of RvD2 and the bioimpedentiometric data obtained at the time of enrollment of CHF patients.

As shown in the figure, no significant correlation was found. The analysis was carried out with the Pearson correlation test. $p > 0.05$

6. Peripheral blood mononuclear cells (PBMCs) isolation and thawing

Peripheral blood mononuclear cells (PBMCs) were separated from the whole blood by density gradient centrifugation with Ficoll-Hypaque (Amersham Biosciences) after 20 ml venous sampling as follow: the whole blood was centrifuged at 1400 RPM at 4° C to recover the plasma that was used to measure the plasma levels of RvD1 and RvD2 of CHF patients and healthy controls. The recovered plasma was then aliquoted in microvials and stored quickly at – 80° C. The remained blood containing all blood cells was diluted 1:1 with PBS (Phosphate Buffered Saline) in a 50 ml sterile Falcon. In another 50 ml sterile Falcon 15 ml of Ficoll-Hypaque (Amersham Biosciences) was placed to the bottom. The diluted blood was then deposited gently over the Ficoll making sure that the blood does not enter into the Ficoll layer. This because Ficoll is toxic and may kill the blood cells. The Falcon was then centrifuged at 1800 rpm for 20 minutes at room temperature (RT) completely without brake to allow the complete separation of the PBMCs at the end of the centrifuge. After the centrifuge the following layers appear from the bottom: red blood cells and granulocytes, Ficoll, PBMCs which form a visible ring and plasma. The visible ring containing PBMCs was recovered very carefully avoiding to take the Ficoll layer portions below and put it in another 50 ml sterile Falcon. The collected PBMCs were washed twice in PBS, respectively at 1600 rpm with half brake and at 1400 rpm without brake. Finally, the separated PBMCs were re-suspended in 10 ml of complete medium and counted. PBMCs were frozen at -80° C in freezing cell culture medium and divided into aliquots. The freezing cell culture medium was composed by fetal bovine serum (FBS) and 10% of dimethyl sulphoxide (DMSO), a cryoprotector. PBMCs were after thawed at the time of use for functional assays such as activation, modulation of T cells with resolvins, qRT-PCR and Western Blotting. At the time of the analyses, the cells have been thawed following a series of

steps to prevent the cells from becoming damaged. It begins by preparing under sterile hood 2 ml of FBS in a 15 ml Falcon for each defrosted vial and a piece of paper soaked in alcohol. The cells, which are in the microvials at -80° C, were taken to be thawed slowly immersing them halfway through the bath at 37° C until the last small ice residue. The defrosted vial (still with the last piece of icing) were quickly placed under the hood and the opening part of the vial were cleaned with the alcohol soaked paper before opening it. The contents of the vial were taken with a sterile plastic pasteur and transferred drop to drop (but quickly) into the Falcon with the FBS. Then were added 2 or 3 ml of complete cell culture medium and centrifuged at 1000 rpm for 5 minutes. The obtained pellet were resuspended mechanically by passing the Falcon on the hood grate or with fingers and was added 3 ml of complete cell culture medium. Finally PBMCs were counted.

7. Quantification of Resolvins

Plasma levels of RvD1 and RVD2 were measured in CHF patients and healthy controls by means of RvD1 and RvD2 Elisa Resolvin kit of Cayman Chemical following the manufacturer's instructions. The assay is based on the competition between free RvDs and an RvDs tracer (RvD1 linked to acetylcholinesterase (AChE)) for a limited number of RvDs specific rabbit antiserum binding sites. Plates were read by Varioskan™ LUX multimode microplate reader (Thermo Scientific). This assay has a range from 3.3-2,000 pg/ml and a sensitivity (80% B/B0) of approximately 15 pg/ml. The concentration of the RvD1 Tracer is held constant while the concentration of free RvD1 (standard or sample) varies. Thus, the amount of RvD1 Tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free RvD1 in the well. This rabbit antiserum-RvD1 (either free or tracer) complex binds to the mouse

monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is then washed to remove any unbound reagents and Ellman's Reagent, which contains the substrate to AChE, is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of RvD1 Tracer bound to the well, which is inversely proportional to the amount of free RvD1 present in the well during the incubation. The sequence of the assay steps started with the preparation of eight different solutions of standards by serial dilutions to make the standard curve. Then were made the set up of the plate where the samples should be analyzed. Into the kit there is a 96-well plate ready to use. The wells employed are the following: Blank, Total Activity, Non-Specific Binding, Maximum Binding, Standards 1-8, Samples 1 – 24. In the plate were added the following reagents: the ELISA Buffer composed by 1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA and 0.1% sodium azide, the Resolvin Ds ELISA Standard composed by resolvin Ds (RvDs) (17(S)-RvDs) in ethanol, the samples with plasma of CHF patients, the Resolvin D1 AChE Tracer composed by resolvin Ds (RvDs) acetylcholinesterase (AChE) tracer, the Resolvin D1 ELISA Antiserum composed by antiserum (anti-17(S)-RvDs rabbit IgG). After adding all reagents and plasma samples and cover it, the plate was incubated for 18 hours at 4°C. After incubation the plate were developed with 100 of Ellman's Reagent, a chemical used to quantify the number or concentration of thiol groups in a sample developed by George L. Ellman, and incubated for 90 minutes at room temperature. After the incubation the plate has been read at a wavelength between 405 and 420 nm and data were analyzed to determine the samples concentration.

8. T-cell activation and polychromatic flow cytometry

The cells have then treated with both RvD1 and RvD2 at concentration of 10.0 nmol/L. The experiment started by placing 5.0 x 10^5 PBMCs of each sample in a tube, following a grid arrangement previously established. In this case they has been employed 5.0 x 10^5 PBMCs treated with RvD1 and 5.0 x 10^5 PBMCs treated with RvD2 of each CHF patient sample. Therefore for each sample we had PBMCs used as control that have not been stimulated and not treated with resolvins, PBMCs stimulated to produce pro-inflammatory cytokines and not treated with resolvins and PBMCs stimulated and treated with RvD1 and those stimulated and treated with RvD2. After the addition of resolvins to the cells at the indicated concentration of 10.0 nmol/L, it has been waited 30 minutes before stimulating the cells with Dynabeads CD3/CD28 T Cell Expander. After the stimulation cells were incubated for 8 hours at 37° C and then analyzed by FACS (Polychromatic Flow Cytometry) to assess the levels of pro-inflammatory cytokines after treatment with RvD1 and RvD2. In order to measure the intracellular cytokine levels, secretion was inhibited by adding 1 μg/ml brefeldin A (Sigma-Aldrich), 7 hours before the end of stimulation with Dynabeads CD3/CD28 T Cell Expander (one bead per cell; Invitrogen**).** At the end of the incubation, cells were stained at cell surface with e780-conjugated anti-CD3 (eBiosciences), anti-CD4 e780 (eBiosciences), anti-CD8 v450 and Live Dead PO, made permeable with Cytofix/Cytoperm reagents (BD Biosciences), and then stained intracellularly with Phycoerythrin-Cy7-conjugated anti-TNF-α (eBiosciences), Allophycocyanin (APC)-conjugated anti-IFN-γ (eBiosciences), Phycoerythrin (PE)-conjugated anti-IL-17 (eBiosciences), anti-PercP5.5-conjugated anti-IL-2 (Biolegend), at RT for 30 minutes. Intracellular cytokines were analyzed by flow cytometry in a FACS-Cyan ADP (Beckman Coulter). For each analysis, at least

300,000 events were acquired gating on Pacific Orange-conjugated Live/Dead negative cells.

Table. 10 Surface and intracellular staining antibodies for polychromatic flow cytometry

ANTIBODY	MANUFACTURER	DILUTION
Surface staining		
e780-conjugated anti-CD3	eBiosciences	1:50
anti-CD4 e780	eBiosciences	1:50
anti-CD8 v450	eBiosciences	1:50
Live Dead PO	eBiosciences	1:50
Intracellular staining		
Phycoerythrin-Cy7-conjugated anti-TNF-α	eBiosciences	1:50
Allophycocyanin (APC)-conjugated anti-IFN-γ	eBiosciences	1:50
Phycoerythrin (PE)-conjugated anti-IL-17	eBiosciences	1:30
anti-PercP5.5-conjugated anti-IL-2	Biolegend	1:50

9. qRT-PCR

Total RNA was extracted with an RNeasy Micro kit (Qiagen). A mixture containing random hexamers, oligo(dT)15 (Promega) and SuperScript II Reverse Transcriptase (Invitrogen) was used for cDNA synthesis. Transcripts were quantified by real-time quantitative PCR on an ABI PRISM 7900 sequence detector (Applied Biosystems) with Applied Biosystems predesigned TaqMan Gene Expression Assays and Absolute QPCR ROX mix (Thermo Fisher Scientific). The following probes were used (Applied Biosystems, assay identification numbers in parentheses): ALOX-15 (Hs00167536_m1), ALOX-15 (Hs009936765_g1), ALX/FPR2 (Hs02759175_s1) and

GPR32 (Hs01102536_s1). For each sample, mRNA abundance was normalized to the amount of ribosomal protein β-actin (Hs01060665_g1).

10. Western Blotting

PBMC cells were lysed with lysis buffer and cell homogenates were subjected to 10% SDS–PAGE (50 μg/lane) under reducing conditions. Gels were then electroblotted onto 0.45-μm nitrocellulose filters (Bio-Rad, Hercules, CA, USA) and were incubated with primary anti-GPR32 polyclonal mouse antibody (1:500, clone GTX71225, GeneTex), anti-ALX/FPR2 monoclonal rabbit antibody (1:500, clone FN-1D6-A1, Genovac) or with anti-β-actin monoclonal mouse antibody (1:10.000, Bio-Rad), and then with secondary goat-antirabbit polyclonal antibody (1:2.000, Santa Cruz Biotechnologies) for GPR32 and goat anti- mouse polyclonal antibody (1:2.000 for ALX and 1:10.000 for β-actin). The target proteins amounts have been normalized to the β-actin structural protein.

11. Statistical analysis

The Power analysis was based on data from the previous study of Colas and colleagues (Colas RA. et al., 2014) and estimated the need to enroll a sample size of 23 subjects/group, total = 46, to test our hypothesis with a power of 80% at a significance level of P=0.05. We hypothesized to find a -20% RvD1 level in CHF, I e 25 pg/nl, with regard to normal values reported to be 30.9 pg/ml by Colas RA et al., 2014. On these bases the sample size has been calculated with the sample size calculator on the website

http://www.quantitativeskills.com/sisa/calculations/samsize.htm

entering the values of the following parameters:

Mean 1: RvD1 plasma levels in the literature = 30.9 pg/ml

Mean 2: Estimation of the value of the plasma level of RvD1 in CHF patients (- 20% of the values in the literature) = 25 pg/ml

SD1: standard deviation of mean 1 = 7.0

SD2: standard deviation of mean 2 = 7.0

Allocation Ratio: 1

Power: 80%

Alpha: 5%

The results of calculation were:

The sample size required for
group1=n1=23
The sample size required for
group2=n1*allocation ratio=23*1=23
The total sample size required
N=n1+n2=23+23=46

Therefore the calculated sample size was 23 CHF patients and 23 healthy controls.

All data were expressed as means ± SEM. Differences between groups were compared using Student's t-test (two groups) or one-way ANOVA (multiple groups) followed by a post hoc Bonferroni test. The criterion for statistical significance was $p < 0.05$ or less. All statistical analyses were performed with GraphPad Prism. FACS analysis was performed using the FlowJo analysis program (Treestar, Ashland, OR).

IV. RESULTS

1. Plasma levels of RvD1 and RvD2 in healthy and CHF subjects

Plasma levels of RvD1 are greatly reduced in CHF patients than in those of healthy controls.

In order to test the hypothesis that RvD1 and RvD2 could be involved in the failure of the resolution of chronic inflammation in CHF the study has been started measuring the plasma levels of RvD1 and RvD2 in both age matched healthy subjects (CTRL) and CHF patients by means of ELISA. Quantification of plasma RvD1 showed that its levels were significantly lower ($p<0.01$) in CHF patients compared to CTRL (Fig. 1.1 A). However, there were no differences between RvD2 plasma levels of CHF patients and CTRL (Fig. 1.1 B). Since CHF is clinically classified in different classes and in order to assess whether the levels of resolvins are associated to disease severity, we also showed RvD1 and RvD2 levels in stratified CHF patients (Fig. 1.2). Plasma levels of RvD1 in CHF patients NYHA class II are significantly lower ($p <0.05$) than healthy controls of more than 50% and the same reduction has been observed also in NYHA class IV, whereas in NYHA class III plasma levels of RvD1 was decreased but this reduction was not significant as in classes II and IV (Fig. 1.2 A). In any case, RvD1 plasma levels in CHF patients remained always significantly lower than those of healthy controls in all three NYHA classes. As far as RvD2, even when stratifying CHF patients, the levels of this lipid mediator did not show any significant variation in any of the NYHA classes compared to healthy controls (Fig. 1.2 B). These results suggest that in CHF patients RvD1 synthesis could be affected and therefore its action may be less effective in CHF patients than in healthy subjects.

2. Immunomodulatory activity of RvD1 and RvD2 on T cells of CHF patients

RvD1 and RvD2 are not able to modulate CD4+ and CD8+ T cell responses in CHF patients.

Since it has been shown that RvD1 and RvD2 are able to turn off the adaptive immune responses (Chiurchiù V. et al., 2016), which have recently been shown to be involved in CHF pathogenesis, and considering that in patients RvD1 decreases significantly, our hypothesis is that the major inflammatory component in CHF is due to a lack of responsiveness of the T cells to the resolvins and that thus administering them in vitro to the T cells they could be able to restore that alteration. To test this hypothesis, the potential modulation of human CD4+ and CD8+ T cells activity by RvD1 and RvD2 in CHF patients has been analyzed, in terms of the ability of RvDs to modulate the levels of pro-inflammatory cytokines. Since CD4+ and CD8+ T lymphocytes activity is responsible for the adaptive immune response, the pro-inflammatory cytokines TNF-α, IFN-γ, IL-17 production have been analyzed in activated CD4+ and CD8+ T cells in presence of RvD1 and RvD2. For each cell and cytokine the analysis was performed by considering all the patients together and stratified by class of disease. The analyzes showed that both RvD1 and RvD2 were unable to modulate the immune response of T cells for all the cytokines studied. The analysis of all CHF patients showed that in CD4+ T cells there were an increased production of TNF-α, IFN-γ, and IL-17 after treatment with both RvD1 and RvD2. Also, CD8+ T cells showed an increased production of TNF-α and IFN-γ after treatment with both RvD1 and RvD2 (Fig. 2.1 B). However, results are not statistically significant and $P > 0.05$ suggest that both RvD1 and RvD2 are not able to modulate T cells immune responses in all CHF patients taken together. When the analysis was performed stratifying patients by NYHA classes in the NYHA II

class CD4+ T cells showed that RvD1 administration after cell stimulation produced an increased level of TNF-α whereas RvD2 administration did not cause any difference. A similar trend was observed for IL-17, while the administration of both RvD1 and RvD2 did not cause any difference in the levels of IFN-γ after cells stimulation. CD8+ T cells showed an increased production of TNF-α after treatment with resolvins, especially after administration of RvD1, as happen also for IFN-γ levels (Fig. 2.2). In NYHA III class CD4+ T cells showed an increased level of TNF-α after treatment with RvD1, whereas after RvD2 administration they have not been observed differences compared to stimulated cells. The same trend has been found for IFN-γ levels where the increase after RvD1 administration was lower of that of TNF-α. Also in this case the levels of IL-17 are similar to that of TNF-α as observed for NYHA II class. CD8+ T cells showed that for TNF-α RvD1 administration after cell stimulation produced its increased level whereas RvD2 administration did not cause any difference. Also, there was an increased level of IFN-γ after administration of both RvD1 and RvD2 (Fig. 2.3). In NYHA IV class CD4+ T cells showed no differences in TNF-α levels before and after RvD1 and RvD2 administration, unlike what happens to IFN-γ where there was an increase after treatment with RvD2 and no difference after RvD1 administration. Also, IL-17 showed an increase after treatment with both resolvins, especially after RvD2 administration. CD8+ T cells showed no differences in TNF-α and IFN-γ levels before and after RvD1 and RvD2 administration (Fig. 2.4). In addition, we also investigated the expression of IL-2 cytokine, a cytokine that mainly has a mitogenic activity, namely that is responsible for T cell clonal expansion upon activation. In particular, IL-2 levels in CD4+ T cells of CHF patients did not show differences after RvD1 and RvD2 administration, while CD8+ T cells displayed an increased expression, especially after RvD2 administration (Fig. 2.1). In NYHA II class in both CD4+ T and CD8+ T cells

there was an increased level of IL-2 after treatment with RvD1 and a very small increase after treatment with RvD2 (Fig. 2.2). In NYHA III class there were no differences in IL-2 levels in both CD4+ T and CD8+ T cells after RvD1 and RvD2 administration except after RvD2 administration in CD8+ T cells (Fig. 2.3). In NYHA IV class CD4+ T cells showed an increased level of IL-2 after treatment with RvD1 but not with RvD2 where instead there was a small decrease of IL-2 level compared with cells that were not treated with resolvins. Likewise a small decrease of IL-2 level it was observed in CD8+ T cells after RvD1 administration while this was not happen after treatment with RvD2 (Fig. 2.4). Overall, results are not statistically significant ($P > 0,05$), i.e. both RvD1 and RvD2 seem generally unable to induce significant modulations of T cells immune responses even when stratifying CHF patients according to disease severity. These results demonstrate that in CHF patients, unlike to what happens in healthy subjects, both RvD1 and RvD2 not only do not reduce pro-inflammatory cytokines production but, in some specific cases, they even seem to have the tendency to increase production. This suggests that there might be some other molecular dysfunction within the T lymphocytes that does not allow them to properly respond to RvD1 and to RvD2 even when receiving it *in vitro*.

3. Analysis of the pro-resolution pathway in CHF patients

CHF patients shown defects in the pro-resolution pathway

The failure of both RvD1 and RvD2 in significantly dampening immune response of CD4+ and CD8+ T cells in CHF patients seem to be associated with the reduction in plasma levels of these molecules, particularly of RvD1. Indeed, since RvD1 was the only resolvin to be significantly reduced in CHF but whose administration to T cells

was ineffective, we next sought to investigate why RvD1 is unable to modulate the immune response of CD4+ and CD8+ T cells in CHF patients. For this purpose, the biochemical ability to synthesize this resolvin and to mechanistically respond to their action has been investigated. Initial studies were performed to analyze the ability of leukocytes of CHF and CTRL subjects to biosynthesize RvD1 by investigating the expression of its key enzymes, 15-lipoxygenase (15-LOX) and 5-lipoxygenase (5-LOX) (Fig. 3.1A), at both transcriptional and protein level. qRT-PCR analysis showed that the expression levels of 15-LOX in all CHF patients are significantly lower ($p<0.01$) compared to healthy controls (3 folds) (Fig. 3.1B). The analysis carried out stratifying CHF patients by class of disease showed a significantly ($p <0.05$) steady decline of 15-LOX expression in each class of disease compared with healthy controls (3 folds) that suggest that it is associated with the severity of the disease. In particular NYHA class II showed a significant ($p <0.05$) decrease of 15-LOX expression compared with CTRL (3 folds), whereas NYHA classes III and IV show a further decrease in the 15-LOX expression of about half compared with NYHA II class (Fig. 3.1C). On the other hand, the expression level of 5-LOX in all CHF patients was slightly higher than in healthy controls, although in a not statistically significant way (Fig. 3.1D). Accordingly, when analyzing its expression in the different NYHA classes, a similar trend was observed in all NYHA classes (Fig. 3.1E). Since 15-LOX is the first and limiting step for the biosynthesis of resolvins, our evidence of a strong reduction in CHF patients paralleled by an almost unchanged expression of its subsequent enzyme 5-LOX, explain the significant reduction in RvD1 plasma levels in CHF patients. However, since the reduced ability to biosynthesize and to actually release RvD1 was not compensated by its in vitro administration inasmuch as it was not able to reduce the inflammatory responses of T cells, we hypothesized that it could be also due to a lack of

responsiveness of leukocytes of CHF patients. Since only the levels of RvD1 were significantly altered in CHF patients and not those of RvD2, we hypothesized a major involvement of this molecule. RvD1 exerts its biological activity and its pro-resolution effects by means of the two G protein-coupled receptors, GPR32 and ALX/FPR2. qRT-PCR analysis showed that expression levels of GPR32 mRNA in all CHF patients are significantly lower ($p < 0.05$ vs CTRL) than in CTRL (3 folds) (Fig. 3.2A). The analysis carried out stratifying the patients for NYHA classes showed a significantly ($p < 0.05$) steady decrease of GPR32 mRNA expression in each class of disease compared with healthy controls (3 folds), suggesting that such decrease was associated to the severity of CHF. In particular NYHA IV class displayed the lowest expression of this receptor (Fig. 3.2B). Since gene expression levels, as expressed by mRNA content, do not usually reflect and correlate with corresponding protein levels, we also confirmed GPR32 expression by means of western blotting. As shown in Fig. 3.2C, the protein content of GPR32 in CHF patients displayed a general reduction in expression in all three NYHA classes, with the NYHA III class showing the lowest expression (3 folds) compared with healthy controls (Fig. 3.2C). On the other hand, mRNA expression of ALX/FPR2 receptor was also reduced in CHF patients compared to healthy controls (Fig. 3.2D), but not in a significant manner, as also observed in the different NYHA classes (Fig. 3.2E). Additionally, when investigating its protein, no variation in ALX/FPR2 expression was observed in any of the different NYHA classes (Fig. 3.2F). These findings suggest that GPR32 receptor is likely the responsible for the lack of responsiveness of T lymphocytes to RvD1 in CHF patients. In summary, our results highlight the impairment of the activity of RvDs in CHF patients caused by defects in both biosynthetic and receptors expression pathways that contribute to the dysregulation of adaptive immunity in CHF as shown in Fig. 3.3.

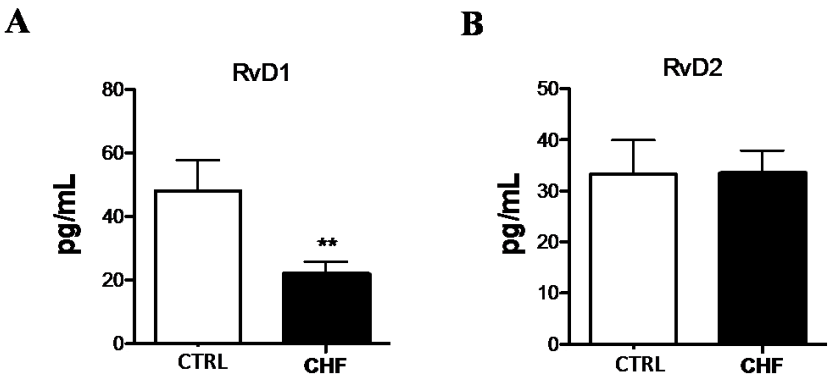

Fig. 1.1. **Plasma levels of RvD1 and RvD2 in total CHF patients and healthy controls**. Plasma levels of RvD1 (**A**) and RvD2 (**B**) in total CHF patients and healthy controls (CTRL) Data are shown as means ± SEM of 23 independent experiments. **p <0.01 vs CTRL (t-test).

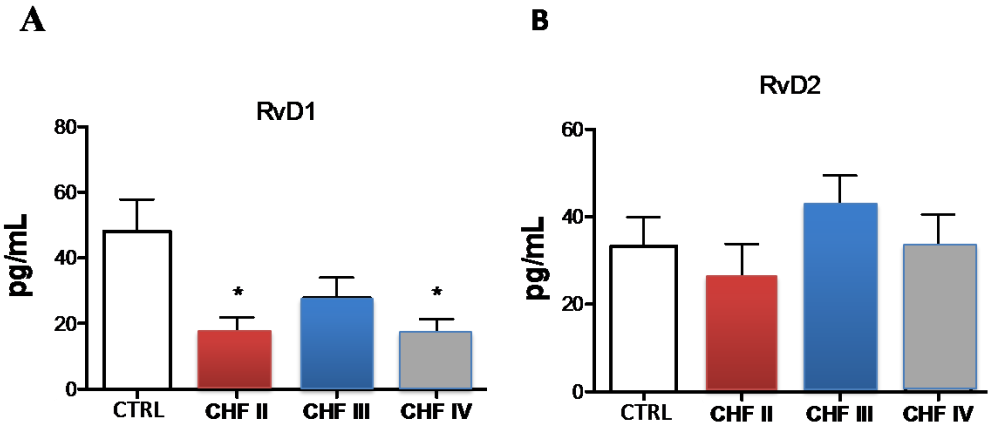

Fig. 1.2. **Plasma levels of RvD1 and RvD2 in stratified CHF patients and healthy controls**. Plasma levels of RvD1 (**A**) and RvD2 (**B**) in CHF patients stratified according to NYHA class and healthy controls (CTRL). Data are shown as means ± SEM of 23 independent experiments. *p <0.05 vs CTRL (one-way Anova).

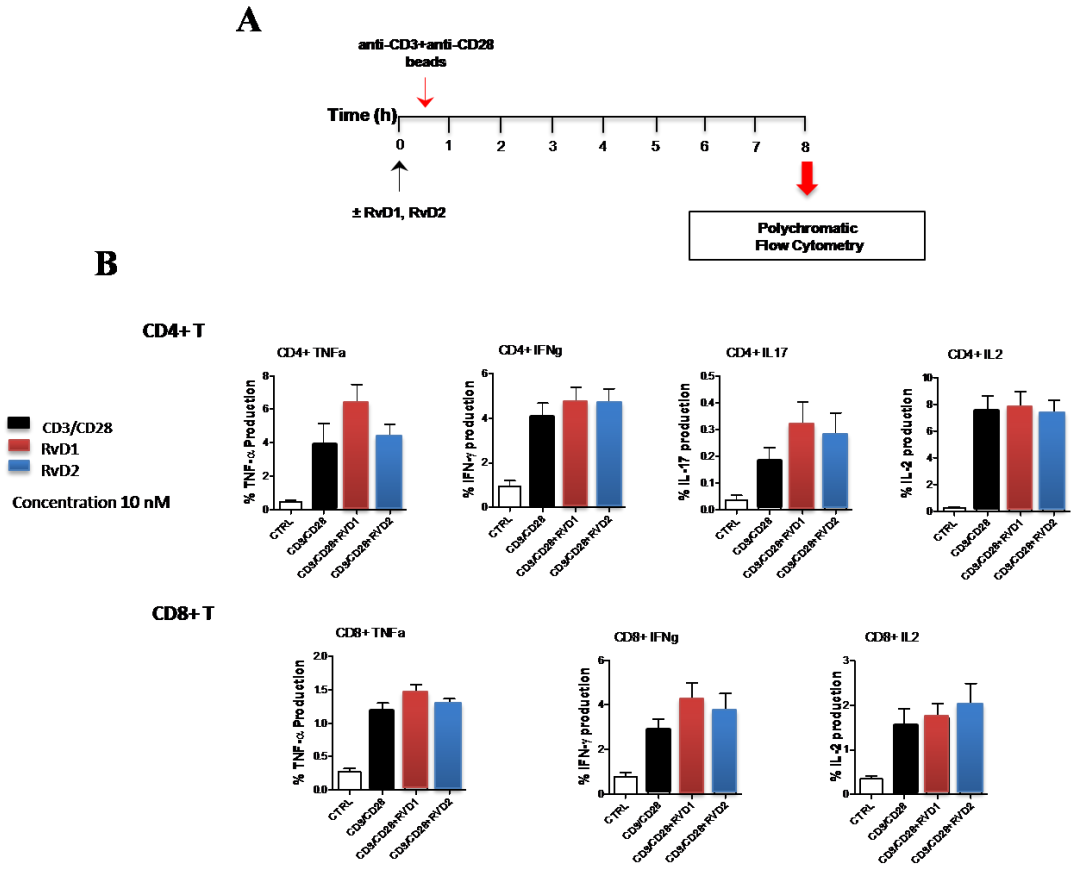

Fig. 2.1 RvD1 and RvD2 are not able to modulate CD8+ and CD4+ T cell responses in CHF patients. (**A**) PBMCs ($1x10^6$ cells/well) were treated with concentrations of 10 nmol/L of RvD1 and RvD2 for 30 min. Cells were then stimulated with Dynabeads CD3/CD28 T Cell Expander for 8 hours, stained at cell surface and intracellularly, and analyzed by flow cytometry. (**B**) Analysis of the modulation of CD4+ and CD8+ T cells immune activity by resolvins in all CHF patients. Cells were analyzed by flow cytometry, as detailed in Materials and Methods. Percentages of intracellular production of TNF-α, IFN-γ, IL-17 and IL-2 from CD4+ T cells and TNF-α, IFN-γ and IL-2 from CD8+ T cells are shown as means ± SEM of 14 independent experiments. $p > 0.05$ (one-way Anova).

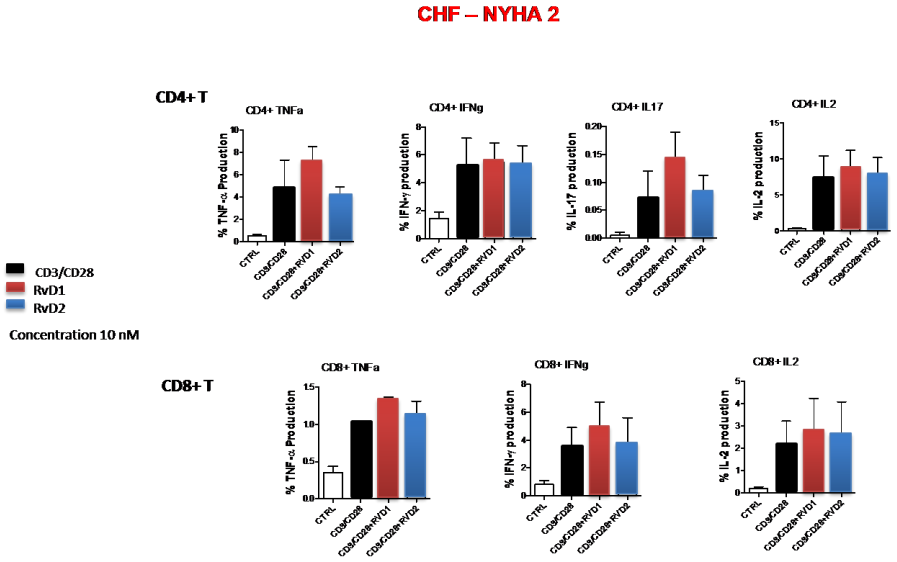

Fig. 2.2 Analysis of the modulation of CD4+ and CD8+ T cells activity by resolvins in CHF patients stratified by class of disease. CHF patients class II. Patients were stratified according to NYHA (New York Heart Association) classification II, III and IV. To test whether this result depended from having analyzed all CHF patients together and to check if it is valid also for patients of every class of disease, we performed the same analysis stratifying patients by class of disease. Cells were analyzed by flow cytometry, as detailed in Materials and Methods. Percentages of intracellular production of TNF-α, IFN-γ, IL-17 and IL-2 from CD4+ T cells and TNF-α, IFN-γ and IL-2 from CD8+ T cells are shown as means ± SEM of 14 independent experiments. $p > 0.05$ (one-way Anova).

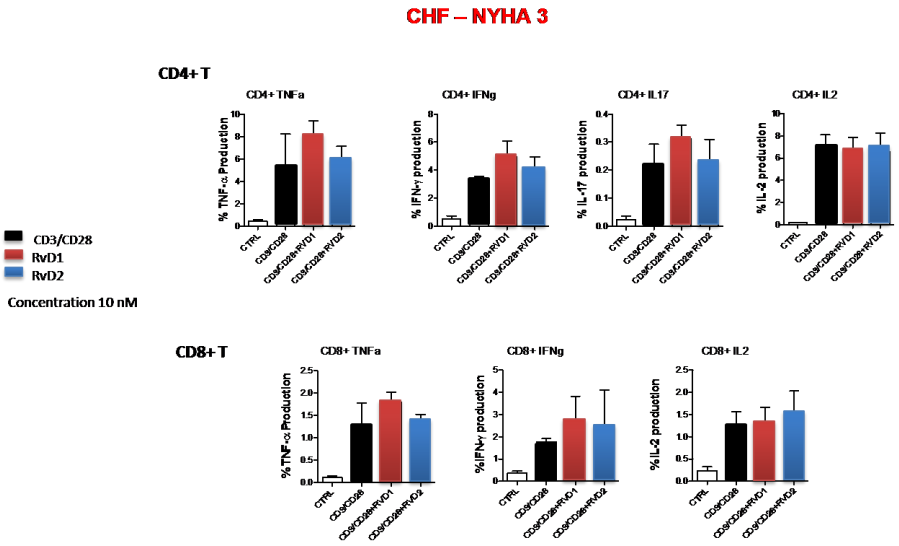

Fig. 2.3 Analysis of the modulation of CD4+ and CD8+ T cells activity by resolvins in CHF patients stratified by class of disease. CHF patients class III. Cells were analyzed by flow cytometry, as detailed in Materials and Methods. Percentages of intracellular production of TNF-α, IFN-γ, IL-17 and IL-2 from CD4+ T cells and TNF-α, IFN-γ and IL-2 from CD8+ T cells are shown as means ± SEM of 14 independent experiments. $p > 0.05$ (one-way Anova).

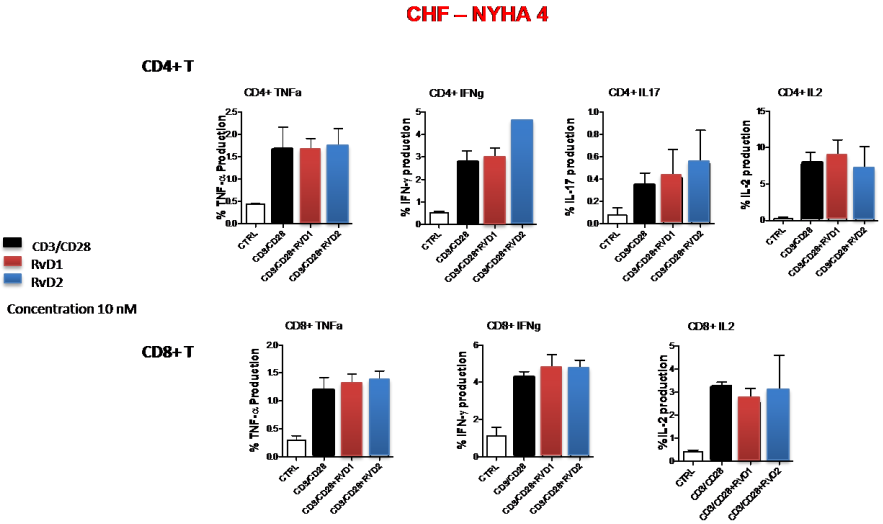

Fig. 2.4 Analysis of the modulation of CD4+ and CD8+ T cells activity by resolvins in CHF patients stratified by class of disease. CHF patients class IV. Cells were analyzed by flow cytometry, as detailed in Materials and Methods. Percentages of intracellular production of TNF-α, IFN-γ, IL-17 and IL-2 from CD4+ T cells and TNF-α, IFN-γ and IL-2 from CD8+ T cells are shown as means ± SEM of 14 independent experiments. $p > 0.05$ (one-way Anova).

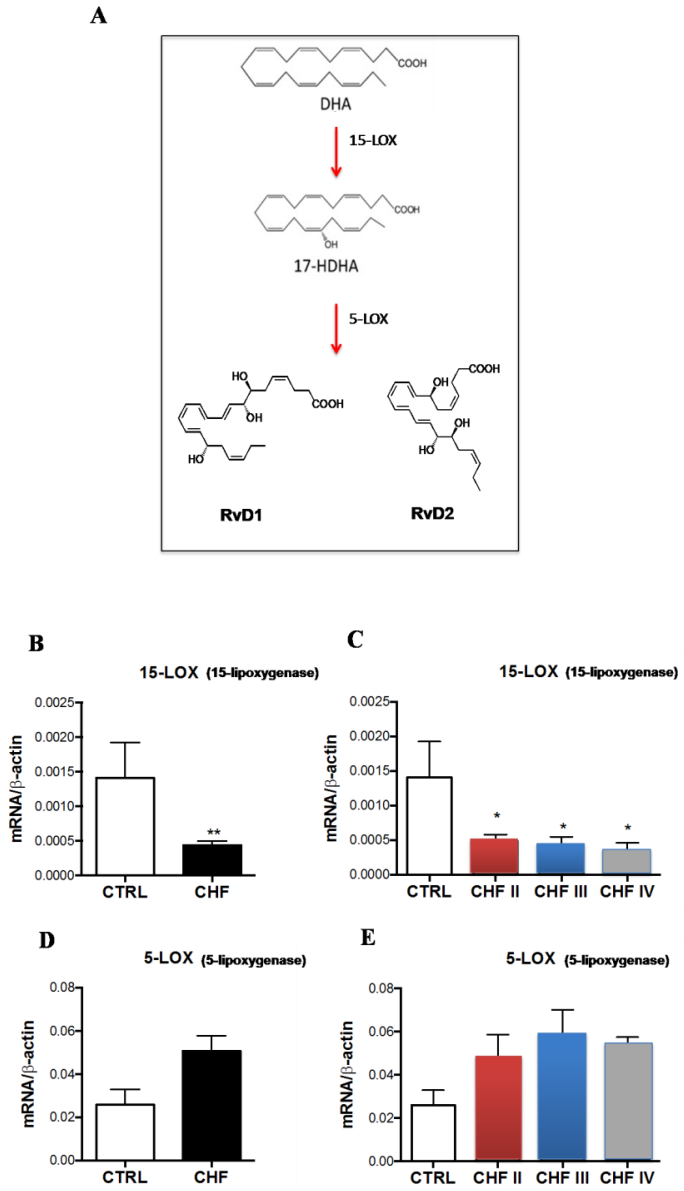

Fig. 3.1 RvD1 and RvD2 biosynthetic enzymes expression in CHF patients. (**A**) D-series resolvins biosynthetic pathway from DHA by means of 15-lipoxygenase (15-LOX) and 5-lipoxygenase (5-LOX). (**B**) 15-LOX (15-lipoxygenase) expression level in all CHF patients and (**C**) in patients stratified by class of disease. (**D**) 5-LOX (5-lipoxygenase) expression level in all CHF patients and (**E**) in patients stratified by class of disease. qRT-PCR analysis as detailed in Materials and Methods. Data are shown as means ± SEM of 23 (for 15-LOX) and 18 (for 5-LOX) independent experiments. *p <0.05 vs CTRL (one-way Anova) and **p <0.01 vs CRTL (t-test).

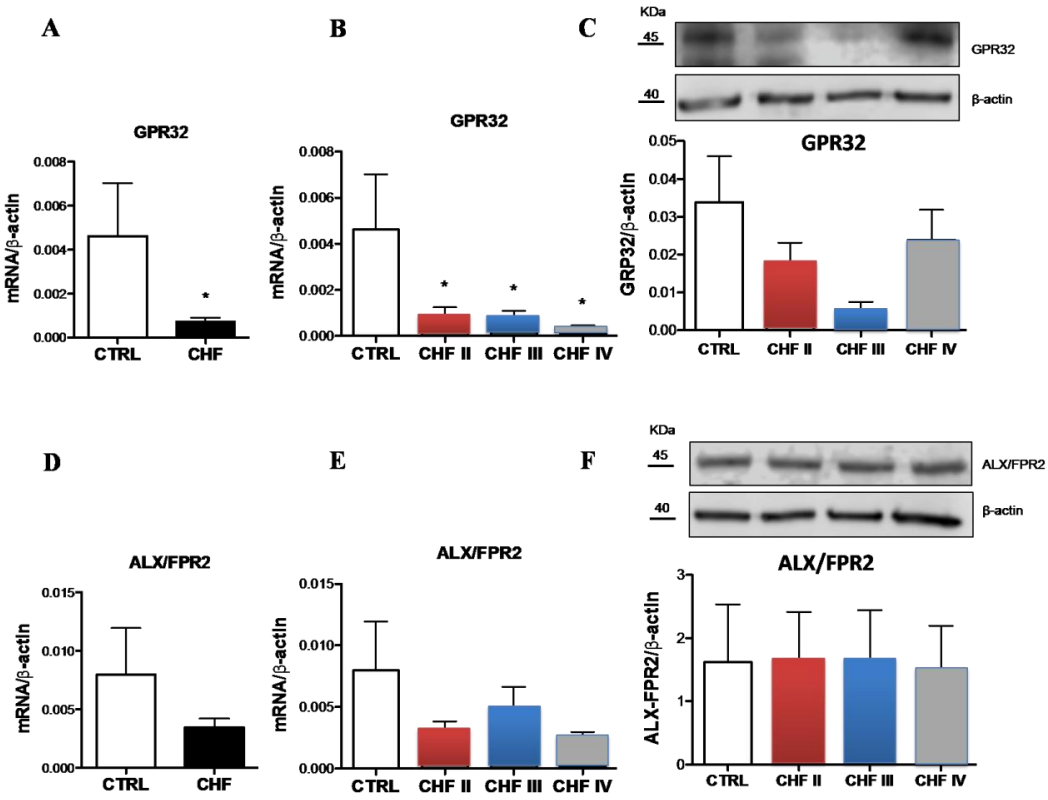

Fig. 3.2 RvD1 (GPR32 and ALX/FPR2) receptors expression in CHF patients. **(A)** qRT-PCR for all CHF patients and (**B**) for patients stratified by classes of disease and (**C**) immunoblotting analysis of GPR32 receptor. (**D**) qRT-PCR for all CHF patients and (**E**) for patients stratified by classes of disease and (**F**) immunoblotting analysis of ALX/FRP2 receptor. (qRT-PCR and immunoblotting analysis as detailed in Materials and Methods). Data are shown as means ± SEM of 13 (for qRT-PCR) or 14 (for western blotting) independent experiments. *p <0.05 vs CTRL (t-test for all CHF patients and one-way Anova for stratified patients).

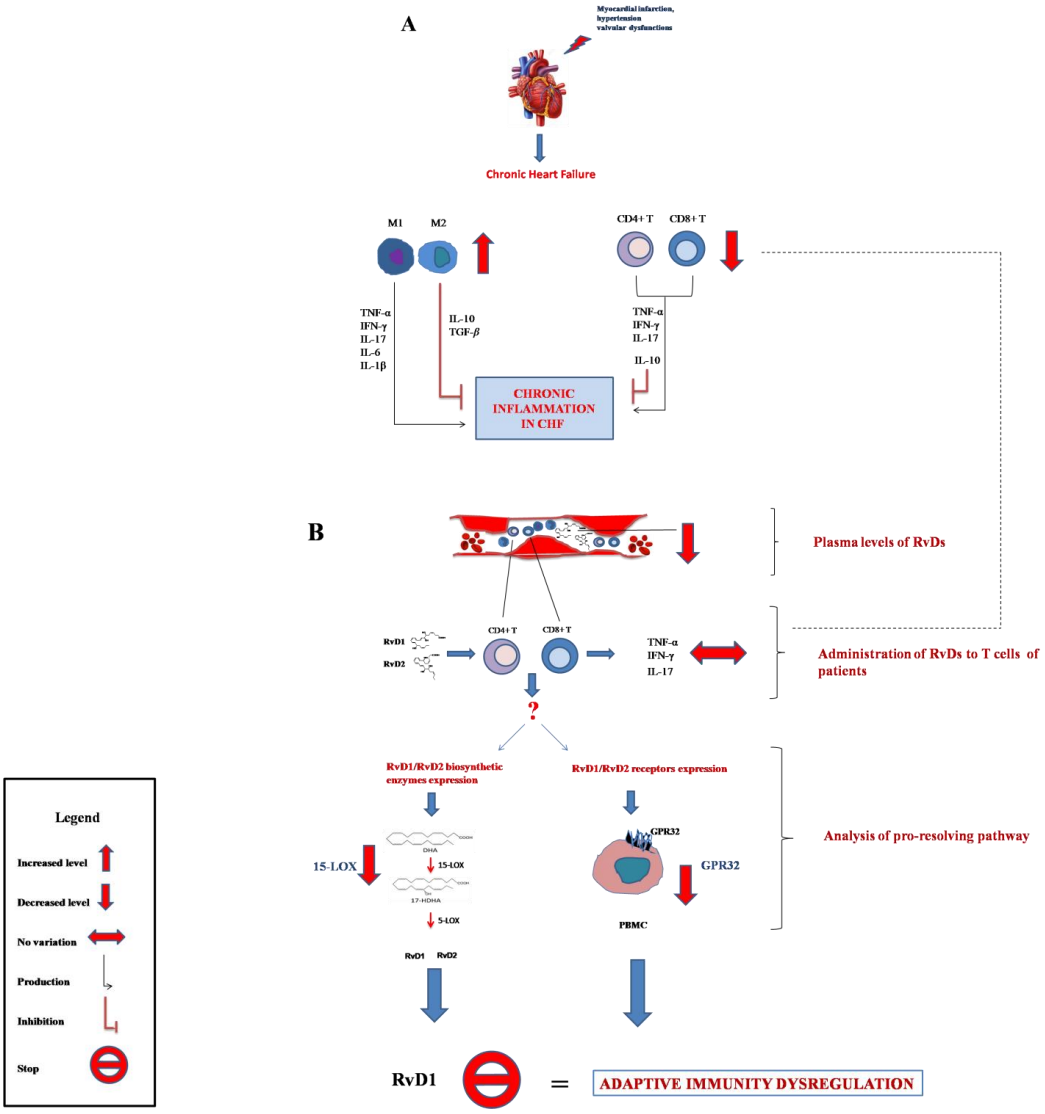

Fig. 3.3 Chronic inflammation in Chronic Heart Failure (CHF) and results of the project. The cartoon shows the immune response that occurs in CHF and the results obtained in the study. (**A**) **Events of chronic inflammation in CHF.** Myocardial infarction, hypertension and valvular dysfunctions may lead to CHF. The cells involved in chronic inflammation are neutrophils, macrophages and lymphocytes. Neutrophils and macrophages are involved in the innate immunity, but they act also in chronic inflammation. M1 macrophages produce pro-inflammatory cytokines, whereas M2 macrophages produce anti-inflammatory cytokines. Lymphocytes are responsible for

the adaptive immunity in chronic inflammation where CD4+, CD8+ and Tregs T cells play a pivotal role. CD4+ and CD8+ T cells produce pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-17 whereas Tregs cells have an anti-inflammatory action mediated by IL-10. In CHF patients, levels of neutrophils and macrophages increase, but those of CD4+ and CD8+ T cells decrease (Moro-García MA. et al., 2014), accounting for dysregulation of adaptive immune response. This is the reason why the study has been focused on CD4+ and CD8+ T cells. (**B**) **Analyses carried out and the results obtained in the study.** Plasma levels of RvD1, but not of RvD2 were found to be decreased in CHF patients compared with healthy controls. Both RvD1 and RvD2 were not able to modulate the immune response of the stimulated CD4+ and CD8+ T cells of CHF patients. The analysis of the expression of the biosynthetic pathway of RvD1 and RvD2 showed a great reduction of 15-LOX expression in CHF patients compared with healthy controls. Then, the western blotting showed a reduced expression of RvD1 receptor GPR32 in CHF patients compared with healthy controls. These results clearly indicate a dysregulation in the activity of RvDs in CHF patients, in particular of RvD1, in both biosynthetic and receptor expression pathways, that may contribute to promote chronic inflammation. Therefore, the pro-resolution pathway might be a potential candidate to design better treatments for CHF with the aim of reducing chronic inflammation.

V. DISCUSSION

In the recent years many studies have shown that resolvins are able to control the transition from acute to chronic inflammation and that unresolved inflammation plays a pivotal role in the development of several chronic inflammatory diseases, including cardiovascular diseases (Fredman G and Spite M., 2017). In the present study, we have reported the existence of an alteration in the pro-resolution pathway in CHF at the level of production of specific pro-resolving lipid mediators and of responsiveness of those adaptive immune cell populations that are actively involved in the chronic inflammatory processes underlying CHF pathogenesis. Our first finding, namely the sharp decrease of RvD1 plasma levels in CHF patients compared with healthy controls, suggests a defect in the biosynthetic pathway of this resolvin. Interestingly, RvD1 plasma levels decreased in all NYHA classes. This finding is coherent with a recent study that shown that levels of Lipoxin A4 (LXA4), another pro-resolving lipid mediator, has been found to be depressed in plasma of CHF patients in all NYHA classes (Reina-Couto M. et al., 2014). However, in the latest study, the decrease in LXA4 levels occurred consistently with the increase of CHF severity, whereas in our study the decrease of RvD1 was higher in NYHA classes II and IV than in class III. Notably, the activity of RvD1 in cardiovascular diseases has been already showed in several studies, where RvD1 has shown to improve ventricular function and to delay the onset of CHF after myocardial infarction in mice (Kain V. et al., 2015), to reduce atheroprogression and increase efferocytosis in mice (Fredman G. et al., 2016), to reduce infarct size by PI3-K/Akt pathway (Gilbert K. et al., 2015) in rats, and to decrease post-myocardial infarct depression (Gilbert K. et al., 2014) in rats. In addition, Aspirin-triggered RvD1 (AT-RvD1) was able to modulate the immune response in Chagas disease patients (Ogata H.

et al., 2016). Therefore, since RvD1 is able to modulate the immune response in cardiovascular diseases improving the clinical conditions, is reasonable to assume that the marked decrease in RvD1 detected in plasma of CHF patients in this study may significantly contribute to its inefficiency in the syndrome. RvD1 plasma levels reduction could be involved also in the balance between pro and anti-inflammatory pathways through the enzyme 5-LOX (5-lipoxygenase) activity. 5-LOX plays a key role in inflammation since it is responsible of both leukotrienes and SPMs synthesis, controlling the balance between the pro-inflammatory and anti-inflammatory pathways. When located in the nucleus, 5-LOX is phosphorylated and shifts the balance to the production of pro-inflammatory leukotrienes, mainly leukotriene B4 (LTB4); when located in the cytoplasm, the unphosphorylated 5-LOX form promotes the synthesis of pro-resolving mediators such as lipoxin A4 (LXA4). RvD1 is able to influence this balance in favor of the anti-inflammatory arm by inhibiting the phosphorylation of the 5-LOX through the calcium-activated kinase pathway (Fredman G. et al., 2014). Thus, the marked decrease of plasma levels of RvD1 found in CHF patients might contribute to perpetuate the inflammation in CHF promoting the enhancement of the production of pro-inflammatory leukotrienes. Such dramatic decrease in RvD1 plasma levels in CHF was also associated by a reduction in the expression levels of 15-LOX, the limiting enzyme responsible for RvD1 and RvD2 biosynthesis. Accordingly, the role of this key biosynthetic enzyme has been supported by a recent study reporting that the inhibition of 15-LOX in rats attenuated RvD1-dependent cardioprotective effect (Gilbert K. et al., 2015). However, we also observed increased levels of 5-LOX, the second enzyme involved in RvD1 and RvD2 biosynthesis. This result is not surprising since in previous studies it was reported that increased levels of 5-LOX occur after myocardial infarction along with recruitment of leukocytes in the area damaged by ischemia as a signal of an

increased production of eicosanoids and active resolution (Kain V. et al., 2014), confirming the role of 5-LOX in the pathogenesis of cardiovascular diseases. Since that in CHF patients a defect in RvD1 production was found both in plasma levels and in the expression of the 15-LOX biosynthetic enzyme, we wondered if this defect could be restored by administering RvD1 directly to T lymphocytes. Surprisingly, our study revealed that, contrary to healthy subjects (Chiurchiù et al., 2016), both RvD1 and RvD2 do not effectively modulate the immune response of T cells on all the pro-inflammatory cytokines analyzed, i.e. TNF-α, IFN-γ and IL-17 as well as mitogenic cytokine IL-2. Thus, not only the synthesis, but also the function of resolvins on T cells seems to be defective in CHF. We hypothesized that such lack of immunomodulatory effect of both resolvins could probably be due to a reduced T cell responsiveness of CHF patients caused by a defect in their mechanism of action. This was indeed confirmed by the observation of an altered expression in leukocytes of CHF patients of one of the two receptors of RvD1, i.e. GPR32, which progressively decreased with disease severity, at least for the mRNA expression, whereas its protein progressively decreased only in NYHA II and III. These findings, coupled with the lack of significant variations in the expression of the other RvD1 receptor ALX/FPR2, suggest that GPR32 receptor is likely the responsible for the unresponsiveness of T lymphocytes to RvD1.

In summary, both the ability to produce and release specific SPMs, particularly RvD1, and the anti-inflammatory responsiveness of CD4+ and CD8+ T cells to RvD1 are altered in CHF. The reduced expression of RvD1 receptor GPR32 likely contributes to the failure of RvD1 in curtailing the immune responses of T cells in CHF patients, accounting for the existence of numerous alterations of the pro-resolution pathway in CHF. Given that both decreased biosynthetic pathway of RvD1 and impaired expression of its GPR32 receptor were evident, it remains to be defined which of these two defects

prevails in CHF patients. If the defective biosynthesis is the main limitation, administering directly the resolvins or providing a diet rich in ω-3 fatty acid DHA, from which D-series resolvins are enzyme derived, might be a good option. Indeed, ω-3 fatty acids supplementation has been shown to increase the plasma levels of SPMs (Barden A. et al., 2014) and to produce beneficial effects on hospitalization and mortality in CHF patients (Tavazzi L. et al., 2008). However, such a strategy would not overcome the impaired receptor expression, and such a defective expression likely is the main limitation. Indeed, only RvD1 serum levels were depressed although the 15-lipoxygenase is a key biosynthetic enzyme of both RvD1 and RvD2. This result suggests that perhaps the decrease in the expression of the receptors of resolvins could be the main cause of their ineffectiveness in controlling the adaptive immune response in CHF patients. Another interesting result obtained in the analysis carried out stratifying CHF patients by class of disease is that plasma levels of RvD1 in CHF patients NYHA class II are lower than healthy controls, and they further increase from NYHA class II to class III and again decrease in patients of NYHA class IV compared with NYHA class III. Thus, although the decrease of plasma RvD1 levels occurs in all three classes of NYHA II, III and IV of CHF patients, the decrease observed in class III is significantly lower than that of the other two classes II and IV. This different behavior of class III also occurs with GPR32 receptor expression according with Western blotting analysis, although in this case the level of receptor expression decreases compared to that of the other two classes of disease, however, remaining always far below of those of the healthy controls. Therefore, the NYHA class III seems to behave differently from II and IV classes with regard to RvDs metabolism, in both biosynthetic and pro-resolving signalling pathways. However, the finding has not obvious explanation and biological plausibility and deserves to be reassessed in larger

series. A strong point in this study was that it was performed directly on human cells, unlike most other studies on the SPMs that were performed on rats or mice.

All together, our findings clearly demonstrate that there is a failure in the action of both RvD1 and RvD2 in modulating the immune response of T cells in CHF patients that can be explained with defects in the biosynthetic and pro-resolution pathway, especially with regard to RvD1. These results not only suggest that the failure of CHF patients to respond to the pro-resolving actions of D-series resolvins may participate in the progression of chronic inflammation, but also that the pro-resolution pathway might be a potential candidate to design better treatments for CHF and other cardiovascular diseases with the aim of reducing chronic inflammation. On a last note, being these endogenous mediators devoid of any cytotoxic effects on T cells (Chiurchiù V. et al., 2016), new therapies based on SPMs are expected to be well tolerated because devoid of immunosuppression.

VI. CONCLUSIONS

This study showed that the anti-inflammatory action of RvD1 and RvD2 is altered in CHF patients and that the failure in the action of RvD1 in modulating the immune response of T cells in CHF patients can be explained with defects in both the biosynthetic and pro-resolution pathway. The biosynthetic pathway showed a great decrease of 15-LOX expression in CHF patients whereas the pro-resolution pathway showed that GPR32 receptor is likely the responsible for the unresponsiveness of T lymphocytes to RvD1. In addition the study highlighted that of the two studied RvD1 and RvD2 the RvD1 is the most involved in the failure of their activity in CHF. On the basis of these results it has been concluded that the failure of CHF patients to respond to the pro-resolving actions of D-series resolvins may participate in the progression of chronic inflammation and that the pro-resolution pathway might be a potential candidate to design better treatments for CHF with the aim of reducing chronic inflammation.

List of abbreviations

CHF	Chronic Heart Failure
SPMs	Specialized pro-resolving lipid mediators
AA	Arachidonic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
RvDs	Resolvins
RvD1	Resolvin D1
RvD2	Resolvin D2
ELISA	Enzyme-linked immunosorbent assay
qRT-PCR	Quantitative real-time polymerase chain reaction
15-LOX	15-lipoxygenase
5-LOX	5-lipoxygenase
GPR32	G protein-coupled receptor 32
CAMs	Cell Adhesion Molecules
PNM	Polymorphonuclear leukocytes
PUFAs	Polyunsaturated fatty acids
PGE2	Prostaglandin E2
PGD2	Prostaglandin D2
PGI2	Prostaglandin I2
LTA4	Leukotriene A4
LTB4	Leukotriene B4

LTC4	Leukotriene C4
IL-1	Interleukin-1
TNF-α	Tumor necrosis factor-α
IL-6	Interleukin-6
STAT3	Signal transducer and activator of transcription 3
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
DNA	DeoxyriboNucleic Acid
p53	Cellular tumor antigen *p53*
MCP-1	Monocyte Chemoattractant Protein-1
COX-2	Cyclooxygenase-2
IBD	Inflammatory Bowel Diseases
CD	Crohn's disease
UC	Ulcerative colitis
IFN-γ	Interferon gamma
TGF-β1	Transforming growth factor beta 1
TGF-β2	Transforming growth factor beta 1
MaRs	Maresins
LXs	Lipoxins
PDs	Protectins
GPCRs	G protein-coupled receptors
ALX/FPR2	N-formyl peptide receptor 2
CYP450	Cytochrome P450
RvE1	Resolvin E1

BLT1	Leukotriene B4 Receptor
AT-RvD1	Aspirin-triggered resolving D1
HUVECs	Human Umbilical Vein Endothelial Cells
TLR4	Toll-like receptor 4
PBMCs	Peripheral blood mononuclear cells
NYHA	New York Heart Association
NPs	Natriuretic peptides
BNP	Brain natriuretic peptide
NT-pro-BNP	N-terminal Brain natriuretic peptide
ANP	Atrial Natriuretic Peptide
ECG	Electrocardiogram
EF	Ejection fraction
CAD	Coronary artery disease
MI	Myocardial infarction
ARBs	Angiotensin II receptor blockers
ACE inhibitors	Angiotensin-converting-enzyme inhibitor
ATP	Adenosine triphosphate
COPD	Chronic obstructive pulmonary disease
VC	Vital capacity
FVC	Forced vital capacity
FEV	Forced expiratory volume
FEF	Forced expiratory flow

MVV	Maximal voluntary ventilation
VEMS	Maximum expiratory volume per second
CPT	Total lung capacity
VR	Residual volume
BIA	Bioimpedentiometry
ATM	Activated Mass
BCM	Body Cellular Mass
TBW	Total Body Water
ICW	Intracellular Water
ECW	Extracellular Water
FFM	Free Fat Mass
ECM	Extracellular Mass
FM	Fat Mass
PBS	Phosphate Buffered Saline
FBS	Fetal bovine serum
DMSO	Dimethyl sulphoxide
AChE	Acetylcholinesterase
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetraacetic acid

VII. REFERENCES

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