ALMA MATER STUDIORUM - UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

SCIENZE MEDICHE SPECIALISTICHE

Ciclo XXVI

Settore Concorsuale di afferenza: 06/D2

Settore Scientifico disciplinare: MED/14

Circulating Fibrocytes, their role in renal fibrosis and molecular pathways involved. Possible biomarkers of fibrogenesis in chronic kidney disease

Presentata da: Dott. Pietro Fici

Coordinatore Dottorato

Prof. Sandro Mattioli

Relatore

Prof. Gaetano La Manna

Correlatore

Dott. Wainer Zoli

Esame finale anno 2014

Summary

Abstract

Circulating Fibrocytes (CFs) are bone marrow-derived mesenchymal progenitor cells that express a similar pattern of surface markers related to leukocytes, hematopoietic progenitor cells and fibroblasts. CFs precursor display an ability to differentiate into fibroblasts and Myofibroblasts, as well as adipocytes.

Fibrocytes have been shown to contribute to tissue fibrosis in the end-stage renal disease (ESRD), as well as in other fibrotic diseases, leading to fibrogenic process in other organs including lung, cardiac, gut and liver. This evidence has been confirmed by several experimental proofs in mice models of kidney injury.

In the present study, we developed a protocol for the study of CFs, by using peripheral blood monocytes cells (PBMCs) samples collected from healthy human volunteers.

Thanks to a flow cytometry method, *in vitro* culture assays and the gene expression assays, we are able to study and characterize this CFs population. Moreover, results confirmed that these approaches are reliable and reproducible for the investigation of the circulating fibrocytes population in whole blood samples.

Our final aim is to confirm the presence of a correlation between the renal fibrosis progression, and the different circulating fibrocyte levels in Chronic Kidney Disease (CKD) patients. Thanks to a protocol study presented and accepted by the Ethic Committee we are continuing the study of CFs induction in a cohort of sixty patients affected by CKD, divided in three distinct groups for different glomerular filtration rate (GFR) levels, plus a control group of thirty healthy subjects. Ongoing experiments will determine whether circulating fibrocytes represent novel biomarkers for the study of CKD progression, in the early and late phases of this disease.

Background

Human body ability to replace damaged or dead cells and repair tissue at the end of inflammatory process is essential for survival. Wound healing process has the function to restore the architecture and allow the recovery of the physiological functions of the damaged tissue. However, tissue repair could be result into two distinct processes, both involved into the repair processes. 1) the total tissue regeneration, namely the replacement of damaged cells with tissue-specific cells, restoring the physiological functions and not leaving signs of the previous damage; 2) either to fibrosis, where the chronic injury of the tissue induce hyperactivation of inflammatory cells and an excessive connective tissue deposition, leading to a permanent fibrotic scar formation and the loss of the physiological functions of the organs involved. The complete regeneration of damaged tissues occurs exclusively in the fetus but the reasons are still poorly understood, (Miller et al., 1973). In adulthood healing is more difficult, most of all in chronic disease. The body then tries to repair tissue damage by replacement of parenchymal cells and connective tissue. Over time these results in fibrosis and scarring. Fibrosis is the end-stage of chronic inflammatory reactions induced by a variety of stimuli as: persistent infection, autoimmune reaction allergic response, chemical insult, radiation and/or other tissue injury. It is characterized by the development of excessive extracellular matrix (ECM) production, cellular apoptosis and inflammatory cells infiltration, sustained by a pro-inflammatory microenvironment. (Wynn TA., 2008). The primary source of matrix synthesis are the myofibroblasts, which are fibroblast activated cells that may derive by local activated fibroblast and/or through the differentiation to activated phenotype of other cells involved into the fibrosis.. These are able to repair tissue damage by secreting extracellular matrix and develop contractile forces through their capacity to synthesize α-smooth muscle actin (α-SMA), such as to orchestrate the process by secreted cytokines and chemokins (Eddy AA., 2013). Fibrocytes, a bone-marrow derived cells, are leading actors of this process and may have both beneficial and detrimental effects on health. These can regulated the innate and adaptive immune response, by the expression and secretion of a variety of chemokines and growth factors. Also, they promote wound healing both directly and by secreting factors that activate residence fibroblast and other fibrocytes. (Bucala et al., 2007; Pilling et al., 2012)

As well in the kidney, regardless of etiology, the ultimate outcome of chronic injury is interested by a fibrogenic process. This is the final renal injury state as known as end-stage of renal disease (Risdon R.A. et al. 1968).It result into fibrotic plaques formation in the

tissue interstitium that compromise kidney function and result into tubulointerstitial injury, (Kisseleva et al., 2008).

Fibrocytes have been shown to contribute to tissue fibrosis in the end-stage of renal disease, as well as in other fibrotic disease, which appear to involved in other organ fibrogenic process including: lung, cardiac, gut and liver. This has been confirmed by different experimental results on different mice models of injury.

Moreover, the levels of circulating fibrocytes recruitment into the tissue damages correlate inversely whit the pathologic progression of the diseases (Wada T. et al., 2011), as revealed by different data by in vivo and in vitro studies of idiopatic pulmonary fibrosis and chronic kidney disease. Circulating fibrocytes are considered one of the principal source of active fibroblast/myofibroblasts, among the non-residents cells, into the kidney injury (about 35%), (LeBleu et al., 2013).

1. Circulating Fibrocytes

Circulating fibrocytes are bone marrow-derived mesenchymal cells that co-express haematopoietic stem cell antigens, markers of monocytes lineage and fibroblast products. Although their biology has come under study recently, the description of a fibrocyte-like cell population was first proposed more than 150 years ago (Cohnheim J. et al., 1867). However, it was not until 1994 that the fibrocytes were first described in a wound-healing chamber experiments, as a subpopulation of leucocytes that accumulate at site of tissue injury and show haematopoietic and progenitor cell markers, CD45 and CD34 respectively, and extracellular matrix (ECM) protein (collagen type I-IV), together. Also, these cells present in the wound exudates, were observed to adopt a morphology spindle shaped typical of fibroblast when adherent (Bucala et al., 1994). It has been proposed that fibrocytes may be a transitional stage between a monocytes and a fibroblast (Reilkoff R.A. et al., 2011).

Fibrocytes, as high plastic cell population able to adopt the phenotype of macrophages, fibroblast and activates myofibroblast, seem to contribute to both tissue injury repair and remodeling process through a series of important mechanisms (Chesney et al., 1997). They constitutively produce extracellular matrix components (collagen, vimentin and fibrin) as well as ECM-modifying enzymes, as metalloproteases (MMPs) . They can further differentiate into myofibroblast, under permissive micro-environmental condition, with expression α-SMA, favoring the contraction for the closure of the wound (Metz, 2003), also by the induction of angiogenesis (Hartlapp et al., 2001). Moreover, these produce cytokines, chemokines and growth factors that further stimulate fibroblastic hyperplasia (Chesney et al., 1998). In the setting of persisted injury that cause ongoing immune activation and impeded repair, fibrocytes increase the local inflammatory and pro-fibrotic cell populations, leading to formation of excessive fibrous connective tissue that disrupt normal tissue function also in the early phases of healing process (Bucala et al., 1994; Reilkoff R.A. et al., 2011). The functional relationship between fibrocytes and the related effector cell population of macrophages and fibroblasts has not yet been fully explicated and specific markers for distinguishing them don’t exist. The potential for overlap in the identification and function of these different cells and a lack of the subtle differences between them might impede a full understanding of the role of fibrocytes in chronic inflammation.

Normally, circulating fibrocytes comprise only a small fraction of circulating leucocytes in healthy humans, comprise between 0,1-1% of them. Increased number of these are present

in human pathologies that are characterized by both chronic macrophage-derived inflammation and persisted fibroblast activation. Such disorders include pulmonary parenchymal airway disease (Phillips et al., 2004; Keely et al. rew 2009), cardiovascular disease (Medbury et al., 2008), pulmonary hypertension (Nikam et al., 2010), autoimmune disorders (Galligan et al., 2010) and metastatic tumor (Herzog et al. 2010). Furthermore, fibrocytes are implicates in the even normal aging, and development of tissue fibrosis including liver, heart, lung and kidney fibrosis, as indicated by animal modeling and some humans studies (Reilkoff R.A. et al., 2011).

1.1. Origin and phenotypic characteristics

Expression of haematopoietic stem cell/progenitor marker CD34, the leukocyte common antigen CD45 and several markers of the monocyte lineage, concomitantly to fibroblast markers (collagen and vimentin) suggested the bone marrow-origin of fibrocytes.

Bucala and colleges indicated fibrocytes as circulating population of non-erythrocyte cells on the basis of the isolation of these from culture of peripheral blood mononuclear cells, but additional experiments from the same group and other investigator revealed that human fibrocytes may not be present in the peripheral blood and that may originate from circulating precursor (Bellini A et al. 2007). It is a monocytes "not-exclusive" precursor and it able to differentiate into different cell population as adipocytes, chondrocytes, osteoblast as well as myofibroblast (Choi Y.H. et al, 2010; Quan T.E. et al., 2004; Hong K.M. et al., 2005). It has been proposed that fibrocytes may be a transitional stage between monocytes and tissue fibroblast (Reilkoff R.A. et al., 2011).

Fibrocytes mature from a subpopulation of $CD14^+$peripheral blood mononuclear cells that express the receptor for the Fc portion of immunoglobulin G (IgG) FcγRI (CD64) and FcγRII (CD32) and do not express FcγRIII (CD16), (Pilling et al., 2003; Pilling et al., 2006; Haudeck S.B. et al, 2006). Circulating fibrocytes precursors may be present in a small fraction of the $CD14^+/CD16^-$ subset of human mononuclear cell that bear C-C motif chemokine receptor (CCR)-2 on their surface. This subset constitutes an immature subpopulation of cells that are normally present in the circulation and have been termed "inflammatory" monocytes. While in health stage, they likely serve to replenish the tissue-resident cells, during an inflammatory process they are released in high number from bone marrow into the peripheral blood and directly migrate to inflamed site (Gordon S. et al., 2005). In experimental murine models, bone marrow from enhanced green fluorescent protein (EGFP) transgenic mice was transplanted into normal mice. The analysis revealed

that the injured skin of this murine model contain numerous EGFP+ with circulating fibrocytes futures, thus validating their hematopoietic origin (Fathke C. etal., 2004).

Consisted with this, other studies showed that human fibrocytes express several haematopoietic cell markers, including CD45RO and Lymphocytes Specific Protein (LSP)-1. Also, there express several CCRs and CXC chemokine receptors (CXCRs), including CCR7 and CXCR4, such as haematopoietic progenitors (Pilling et al., 2003; Abe et al., 2001). More data confirm that fibrocytes derive from precursor of the monocyte lineage. It is supported by the evidence that these cells express the major histocompatibility complex class I and class II (MHC I and II); co-stimulatory molecules CD80 and CD86, exhibit antigen-presenting activity and active both $CD4^+$and $CD8^+$ T lymphocytes. Moreover, they lack the adhesion molecules CD11b, CD11c and CD11d (Chesney J., et al, 1997; Belmelli C., et al., 2005). Circulating and cultured fibrocytes also express CD34, often expressed by pluripotent cells, that discriminate these from tissue macrophages and fibroblasts. Otherwise, they express collagens and glycosaminoglycans as fibroblasts (Reilkoff R.A. et al., 2011).

Human fibrocytes probably represent the obligate intermediate stage of differentiation of one of these precursors of the monocytes lineage into mature fibroblast and myofibroblast at the tissue sites (Bellini A et al. 2007).

The combination of collagen production and expression of CD45 or one of the haematopoietic (CD34) or myeloid antigens (CD11b) is considered as a sufficient criterion to discriminate fibrocytes from leukocytes, dendritic cells, endothelial cells and tissue-resident fibroblasts *in vitro* and *in vivo* (Herzog E.L. et al., 2010). Moreover, an important study revealed that markers as CD45RO, 25F9 ed S100A8/A9 could help to distinguish monocyte-derived fibrocytes from monocytes macrophage and fibroblast, by culture differentiation experiments (Pilling D. et al., 2009). However, the misclassification of fibrocytes in studies of culture cells and tissue could be due for an overlap in markers expression. In this setting, morphological assessment becomes useful. The spindle-shaped morphology adopted by adherent fibrocytes distinguishes them from macrophages, which are more spherical and haven't the haematopoietic cell and inflammatory markers that are expressed by fibrocytes (Pilling D. et al., 2009)

1.2. Differentiation

Initial observation and laboratory investigations showed the development of fibrocytes from peripheral blood mononuclear cells after 10-14 days of culture in media containing

high concentrations of serum. Subsequent, other investigations revealed that, if cultured in serum-free media, cells with fibrocytes futures appeared after only 3 days (Pilling D. et al., 2003; Pilling D. et al., 2008).

Circulating fibrocytes precursor derived from a subpopulation of $CD14^+$ circulating monocytes, which comprehend a mixture of multipotent precursor able to differentiate into a number of cells other than macrophages and dendritic cells (i.e. myoblast, osteoblast, chondrocytes, adypocytes, etc.) under certain permissive condition. The development of fibrocytes and these differentiation into mature mesenchymal cells is regulated by different signals through Fcγ receptor (FcγR) and is augmented by enrichment monocytes for expression of pro-inflammatory markers such us CD11b, CD115 and GR1 (Bellini et al., 2007). Studies showed that circulating fibrocytes can be too isolated *in vitro* in serum-containing conditioning factors as tissue growth factor (TGF)-β or interleukins (ILs), although the presence of serum (i.e. such as may be encountered during in vivo injury) produces a more pro-inflammatory phenotype.

Fibrocytes development is amplified by fibrogenic cytokines signals such us platelet-derived growth factor (PDGF), interleukin (IL)-4 and IL-13. Despite, other pro-inflammatory cytokines inhibit this phenomenon, such as showed by interferon (IFN)-γ, tumor necrosis factor (TNF), IL12 culture treatment and serum amyloid P (SAP) also by innate immune stimulation with Toll-like receptor (TLR)-2 agonist (Bellini et al 2007). This was highlighted by *in vitro* studies by direct contact between T lymphocytes (inflammation regulatory cell) and $CD14^+$monocytes in co-culture assays and by their direct stimulation with fibrogenic or inflammatory factors, described above (Shao D.D. et al., 2008).

Fibrocytes are immature mesenchymal cells that do not produce large amounts of collagens or other ECM components until they differentiate into mature fibroblast and myofibroblast. Once fibrocytes have completed their maturation from monocyte precursors, further differentiation into cells ultrastructurally and phenotipically similar to mature fibroblast and myofibroblast is promoted by stimulation with TGF-β and endothelin (ET)- 1 (Bellini et al 2007). Treatment of primary culture cells of circulating fibrocytes precursor with TGF-β and ET-1 induce much more collagens and fibronectin than untreated cells, express the myofibroblast marker as α-SMA and downregulate the expression of CD34 and CD45, and of the other lymphocytes markers(Schmidt et al., 2003); same results are showed into murin model *in vivo*(Murray L.A. et al., 2011).

These data indicate that fibrocytes differentiation in both culture and disease model decreases under conditions that perpetuate inflammation and tissue injury and increase

under condition that promote wound-healing and tissue remodeling. Balance between inflammatory cytokine and profibrotic factors, TGF-β *in primis*, likely determines the ultimate fate of fibrocytes (Galligan et al., 2013).

1.3. Homing

Initial observations of fibrocytes recruitment to wound side were found to be due to specific chemokine-chemokine receptor interactions. Namely, it was shown that fibrocytes migrate to wounds in response to secondary lymphoid chemokine (SLC) also known as chemochine (C-C)-ligands 21 (CCL21), which is the ligand for (C-C) receptor CCR7. It was observed in a murin model of wound-healing assay (Abe R. et al., 2001). Afterward, other chemokine expression profile has revealed other chemokine receptors on the surface of fibrocytes, and vice versa, through the study of inflametion disease and associated disease models (Blakaja et al., 2012). Chemokines play an important role in the regulation of fibroblast precursor infiltration in response to injury (Sakai N. et al., 2006). Notably, human and murine fibrocytes look to be different. Murin fibrocytes express, CCR2 (Ekert JE et al., 2012), CCR3, CCR5(Abe R. et al., 2001), CCR7(Sakai et al., 2006) and CXCR4(Mehrad et al., 2007); Human fibrocytes express, CCR3, CCR5, and CXCR4 (Mehrad et al., 2007), as well as b1-integrine subunit and Semaphorin 7a (Gan Y. et al., 2011).

Chemokine receptors drive fibrocytes recruitment to injury site in response to different chemochine (C-C or C-X-C motifs) ligands (CCLs and CXCL), as CCL2, 3, 5, CCL21 and CXCL12, relapse by cells of the injury site and other inflammatory cells (Moore et al., 2005; Sakai et al., 2006; Ishida et al., 2007; Herzog et al., 2010).

CXCR4-CXCL12 axis is the most describe in literature. The CXCR4 is an important receptor expressed by hematopoietic stem cells, which respond to CXCL12 signal, known as stromal cell-derived factor (SDF)-1 (Tamamis P. et al., 2013). It is a strongly chemotact signal for lymphocytes and macrophages, also plays an important role in embryogenesis and angiogenesis by recruiting endothelial progenitor cells (EPCs) from the bone marrow (Saini V et al., 2010). CXCL12 is involve into $CXCR4^{+}$ fibrocytes recruitment, as demonstrated by *in vitro* and *in vivo* studies of idiopathic pulmonary fibrosis (IPF) (Murdoch, 2000; Abe et al., 2001), which showed a direct correlation between the severity of the disease states and the activity of the chemokine axis, as between the plasma levels of CCL12 and circulating fibrocytes or infiltrated percentage, in IPF patients(Mehrad et al., 2007; Andersson-Sjoland et al., 2008).

Under ureteral obstruction (UUO) mice experiments, an animal model of renal fibrosis, show a circulating fibrocytes recruitment dependent by CCR7 receptor and her ligand CCL21, as well as by CXCR4-CCL12 axis. These appear to work simultaneously but independently(Wada et al., 2011)

Also, CCR2 receptor with her ligand monocyte chemoattractant protein (MCP)-1 (CCL2), and CCR5 with her natural ligand CCL5 (also known as RANTES), or macrophage inflammatory proteins (MIP)-1α (namely as CCL3) and 1β (as CCL4) are considered potential fibrocytes homing systems. These are involve into circulating monocyte and macrophage infiltration, mainly to inflammatory response and inflammatory diseases. Self-determining studies conducted on both animal models and samples of human patients, shown important correlations between these chemotactic axis and fibrocytes recruitment in autoimmunity disease and other inflammatory process (Koch A.E. et al., 1992; Page G. et al., 2004; Yang M.H. et al., 2009). It may suggest a direct role of CCR5-CCL3/CCL4 and CCR2-CCL2 axis into circulating and tissue fibrocytes migration (Galligan et al., 2013). Furthermore, another research group has been demonstrated that CXCL16 is induced in the kidney during the pathogenesis of renal fibrosis; help the bone marrow-derived fibroblast precursors recruitment into the kidney, in a CXCL16-dependent manner. Fibroblast precursors express the CXCL16 receptor (CXCR6). Experimental investigation in vivo showed that targeted disruption of CXCL16 inhibits the recruitment of CD45-, collagen-I-, and CXCR6-positive fibroblast precursors in the kidney; and also reduces the severity of renal fibrosis and the expression of ECM protein. These to confirm that CXCL16 plays a pivotal role in the pathogenesis of renal fibrosis by recruiting bone marrow-derived fibroblast precursors into the kidney in response to obstructive injury.

Fibrocytes express several chemokine receptors, signifying their potential recruitment to the sites of inflammation where the cognate chemokines are expressed (Galligan et al., 2013). However, the signals mechanisms underlying the recruitment of circulating fibrocytes into the inflammatory site, as well in kidney injury, are incompletely understood.

1.4. Function

Fibrocytes display many functions that could influence the early phases of inflammatory process in response to tissue injury or invasion. Mostly early studies of fibrocyte biology centered on their role as a circulating source of contractile myofibroblasts. Precedent *in vivo* detection of fibrocytes transition to myofibroblast and collagen-producing phenotype in animal model of diseases (Mori L. et al., 2005; Kisseleva T et al., 2006), suggested a

circulating fibrocytes contribute to a subset of myofibroblast in wound and are integral mediators of wound-healing. Also, fibrocytes have been shown to promote angiogenesis, which is a requisite for the development and maintenance of new granulation tissue, allowing wound closure and restoration of tissue integrity. They participates in all aspects of neovascularization by secretion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL8, plateled derived growth factor (PDGF). They are also involved in the proteolysis of the basement membrane by constitutively secreting extracellular matrix degrading enzymes, as MMP-9, thus allowing for endothelial cell invasion (Blakaj A., et al., 2012).

However, beside to minimal contribution of fibrocytes to α-SMA production as shown by lineage tracing studies in several models, fibrocytes respond to important mediator of the inflammatory response and possess other properties that regulate these phenomena, promoting tissue remodeling and repair (Peng H. et al., 2012).

In experimental assays, in response to the IL-1β, a lymphocyte mitogen factor involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis, as well as to T_H1 cell cytokine release and/or viral infection, in human fibrocytes cultures downregulate collagen expression and increase production of IL-6, IL8, CCL3 and CCL4, which would promote inflammatory cell recruitment. In addition, there increase cell surface expression of leukocytes adhesion molecules, such as ICAM-1, which would increase leukocyte trafficking. However, at same condition response by increase IL10 production reducing inflammation and beginning the ECM production and would be expected to recruit inflammatory cells and promote tissue repair and remodeling (Chesney J., et al., 1998). If cultured in serum-supplemented medium, as might occur in acute injury, fibrocytes have a macrophage-like inflammatory gene program characterized by the expression of gene encoding chemokine receptor, cytokines and molecules involved in antigen presentation and lipid metabolism (Curnow S.J. et al., 2010).

Fibrocytes express of MHC class I and II molecules and CD80/CD86 co-stimulatory proteins, that allow an antigen-presenting function to $CD8^+$cells inflammatory cells (Balmelli C. et a., 2005), such as possess antigen-presenting property and under certain conditions may induce a mixed T_H1/T_H2 response in $CD4^+$ cells (Medina A. et al., 2011), suggesting an important role in early events mediating inflammation. Similarly, when these respond to the presence of apoptotic cells by increasing collagen production and repair (Peng X., et al., 2011).

This information indicate that circulating fibrocytes can respond to injury and inflammatory stimuli by take-up the functional characteristics of both classical and

alternatively activated macrophage , as well as of fibroblast and myofibroblast. Whether the inflammatory or reparative fibrocyte phenotype dominates probably depends on dynamic process governed by local factor (see fig. 1).

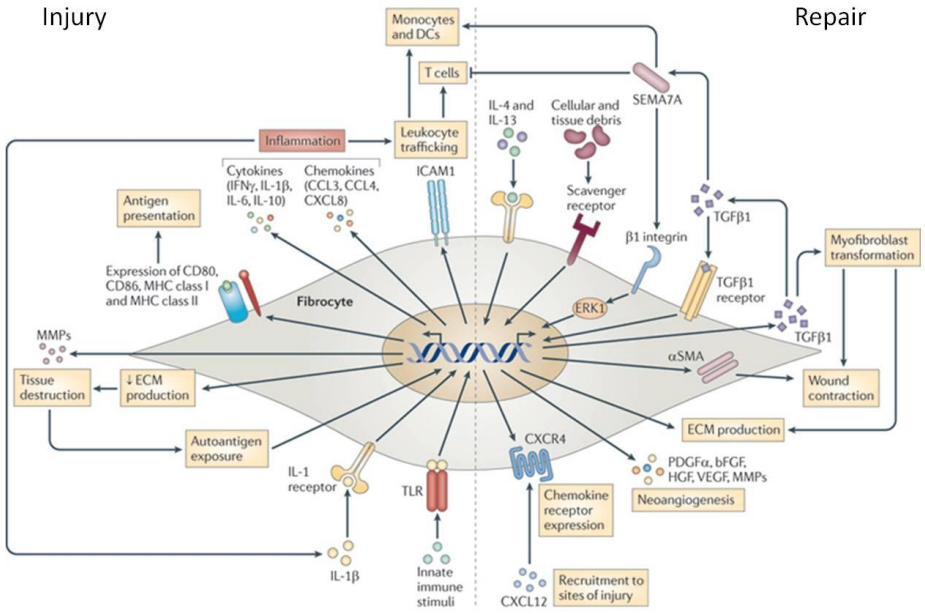

Figure n°1 : Potential roles of fibrocytes in chronic inflammatory disease. (Reilkoff LA., et al., 2011)

1.5. Fibrocytes in chronic inflammation and fibrosis

Chronic inflammatory reaction induced by a variety of stimuli, including persistent infection, autoimmune reactions, allergic responses, chemical insults, radiation and tissue injury result into a common end-stage know as fibrosis. Although initially repair process induces a generative phase, the persist of injury cause ongoing immune activation and impeded repair.

Usually, adult wound healing, often result in a dysregulated pathogenic condition, which show a chronic inflammation response characterized by replacement of normal structure whit inflammatory cells and tissue remodeling. Excess deposition of extracellular matrix components including collagen derive by both infiltrated inflammatory cells and resident fibroblast, as well as by matrix-producing cells, epithelial/endothelial-mesenchymal transition cells (EMT/EndMT cells) and bone-marrow derived cells switched to a myofibroblast phenotype. It lead to hypertrophyic tissue, hardering, and/or scarring, and fibrosis. Given the combination of ongoing inflammation and fibroblast-drive end-organ

remodeling seen in chronic inflammatory disease, circulating fibrocytes emerged as an important players in heterogeneous chronic inflammation conditions. However, most recent studies of fibrocytes have focused on describing their associations with various pathologic inflammatory conditions, but their effect and contributions to inflammatory pathologies remain unknown. The association between fibrocytes and heterogeneous chronic inflammatory conditions indicates that they have a role extending far beyond ECM production regulated by the local milieu (see fig. 1, above), (Peng H. et al., 2012).

A paradigm, based on fibrocyte futures and inflammatory process known, suggest that circulating fibrocytes recruited early during an inflammatory response have a pro-inflammatory phenotype that is induced by acute injury or autoantigen exposure, or following stimulation by local factors (i.e. IL-1β), serum factor and innate immune signals. During this state, fibrocytes amplify the immune response by both pro-inflammatory factor release and other cells recruitment (as limphocytes, macrophages and fibrocytes themselves). Later, as inflammation regresses and repair and remodeling begin, a programme of matrix production and myofibroblast transformation by fibrocytes ensues.

Increased numbers of local and/or circulating fibrocytes have been identified in diseases characterized by chronic T_H1 and T_H2 cell-mediated inflammatory responses (autoimmunity), T_H1 cell-dominant responses (cardiovascular disease) or T_H2 cell-dominant responses (asthma), (Reilkoff R.A. et al., 2011), as well as into different form of fibrosis, as pathological end-stage, including: lung, skin, liver and kidney, as well as into airway remodeling, in asthma and aging process (Peng H. et al., 2012; Mackinnon A., et al., 2013).

1.5.1. In Aging

Dysregulated inflammatory process are seen in otherwise aged individuals healthy. While the factors engage remain unclear, some studies propose a fibrocytes involvement.

Models of senescence-accelerated mice shows increased circulating and intrapulmonary fibrocytes (Xu J. et al., 2009). Col-1-luciferase mouse model proves same higher level of fibrocytes into the blood of aged mice, compared to young mice, also these imaging studies suggesting that CCR2 and CCR3 chemokine receptors could be related to fibrocytes trafficking (Scholten D. et al., 2011). Interestingly, increased fibrocyte precursors were seen in the circulation of aged healthy humans, compared to younger subjects (Mathai SK. et al., 2010). However, will be needed more studies to determine whether fibrocytes play a role in age-related tissue dysfunction.

1.5.2. In autoimmunity disorders

Emerging evidence indicates that fibrocytes have an involvement in the autoimmune effectors pathway and participate to the fibroblast-like cells in human form and mouse models of scleroderma, autoimmune thyroiditis and rheumatoid arthritis. A role for fibrocytes in autoimmune pathogenesis is suggested by studies of patients with diffuse scleroderma, a TGFβ1-driven multisystem autoimmune disorder characterized by progressive cutaneous and visceral fibrosis. Patients shown uncontrolled collagen production by activated myofibroblasts, which also secrete the chemotactic and lymphocyte-activating protein ICAM1 (Needleman B.W. et al., 1990), as well as the pro-inflammatory cytokines (as IL6, IL8, TGFb, PDGF), (Kadono T. et al., 1998). A flow-cytometric analysis of circulating or culture monocytes shows an increased number of fibrocytes, in scleroderma-related interstitial lung disease patients whit scleroderma related to lung fibrosis (Tourkina E. et al., 2011) as amniotic antisynthetase syndrome (Peng X. et al., 2011). Further support become from the recent finding of increased fibrocyte numbers in individuals with Graves' disease, an autoimmune thyroid disorder characterized by persistent inflammation, lipogenesis and fibrosis in the retro-orbital space (thyroid-associated ophthalmopathy), where the peripheral blood and retro-orbital fad pat shown a high frequency of fibrocytes and displaying high expression of several pro-inflammator mediators (Douglas RS. et al., 2011). Pro-inflammatory mediator role of fibrocytes in the pathogenesis of this disorder was suggested by a study in which CD34+, CXCR4+, CD11b+ and pro-collagen I+ cells showed high levels of the insulin-like growth factor 1 (IGF1) receptor, that lead to high-level secretion of pro-inflammatory cytokines such as TNF and IL-6 (Muller-Ladner, U. et al., 2007), and the expression of thyroid-stimulating hormone (TSH) receptor, a potential target of autoimmunity in this disease(Bahn, R.S., 2010). Fibrocytes are also associated in rheumatoid arthritis seen in the synovial inflammatory fluid of these patients (Muller-Ladner, U. et al., 2007). Although they are not found with increased frequency, these possess both the pro-inflammatory and tissue remodeling properties generally attributed respectively to synovial macrophage and synovial fibroblast. However, a role for fibrocytes in the inflammatory pathogenesis of chronic synovitis is supported by studies documenting the appearance of activated fibrocytes before the development of arthropathy in the collagen-induced arthritis murine model of rheumatoid arthritis (Galligan CL. et al., 2010)

1.5.3. In cardiovascular diseases

Cardiovascular disease follow a paradigm of macrophage-driven inflammatory changes in blood vessels that lead to late the formation of an fibrous cap, known as fibroatheroma, characterized by an accumulation of lipid and inflammatory cells inside, and a connective tissue for an increased of myofibroblasts and excessive deposition of collagen and other ECM molecules, which are stimulate by different pro-fibrotic factors produced locally by a wide variety of cells (Reilkoff R.A. et al., 2011)

The remodeling process in the arterial vessel wall results in atherosclerotic plaque (Stary HC. Et al., 1995) and stenosis of the vessel (Andreeva ER., et al., 1997). Atherosclerosis in coronary arteries could happen to myocardial infarction and fibrosis of cardiac tissue. Clinical significance of this lesion is largely dependent on its composition, which is determined by the balance between inflammation and fibrosis and affects the stability of the plaque. Fibrocytes are implicated in the development of both vascular plaques and myocardial fibrosis, although their precise contributions to disease pathology may vary (Reilkoff R.A. et al., 2011).

Fibrocytes co-expressing pro-collagen I and CD34 have been identified in the fibrous cap of human atherosclerotic lesions (Medbury, H. et al., 2008). Also, subendothelial myofibroblasts expressing the monocyte marker CD68 or CD34 have been found in lipid-rich areas of the atherosclerotic intima in human aorta (Medbury H et al., 2007), and in the fibrous cap of human carotid arteries, suggesting a fibrocyte origin for these cells (Andreeva ER et al., 1997).The accumulation of fibrocytes in the fibrous cap of the atherosclerotic lesion may have a beneficial effect in stabilizing the plaque that outweighs the detrimental increase the expantion of the fatty core, so in plaque volume (Medbury H et al., 2007). Also, several models of animal disease, as apolipoprotein E (Apoe)-null mice (model of human atherosclerosis) suggesting that fibrocytes mediate TGFβ1-induced events in this model (Buday, A. et al.2010), and that fibrocytes don't adopt the fully differentiated phenotype of either macrophages and fibroblast in atherosclerotic cup but remain relatively undifferentiated, as shown by their persistent expression of CD45 and coll Iα (Iwata, H. et al.2010). This might reflect the fact that fibrocytes are unable to form a discrete effector population given the ongoing injury and impeded repair seen in this disease.

However, in a setting of profound initial injury or persistent damages, or in presence of pro-fibrotic inflammatory stimuli, fibrocytes could contribute to develop of cardiac fibrosis and remodeling that ensue. Although fibrocytes have been found in myxomatous human

heart valves, characterized by an abnormal connective tissue composition (Barth P.J. et al., 2005), they have yet to be identified in the normal human myocardium.

Indeed, human ischaemic cardiomyopathy murine models indicates that fibrocytes might contribute to the pathogenesis through they recruited to chronically injured myocardium, where they constitute up to 3% of the total number of heart cells and involving to ECM production, although it is possible that other activities that are more typically associated with both macrophages and fibroblasts such as cytokine production, immune cell activation and angiogenesis. Thus, fibrocytes might have a protective or reparative effect in the setting of uncontrolled or persistent *in vivo* T_H1 cell cytokine exposure, whereas in response to persistent T_H2 cell cytokine exposure (as occurs in the remodeled heart) this phenotype becomes profibrotic (Reilkoff R.A. et al., 2011).

1.5.4. In Asthma and Idiopatic Pulmonary Fibrosis

Fibrocytes are associated with many form of chronic inflammatory lung disease. Elevations on circulating fibrocytes and intrapulmonary fibrocytes are seen in patient with several forms of airways remodeling including chronic persistent asthma and allergic asthma. Compelling evidence was provided by a study in which bronchoscopic lung biopsies obtained from patients with asthma after allergen inhalation showed localization of fibrocytes (coll I^+, $CD34^+$), in the airway submucosa; also, in the same study, the use of mouse ovalbumin models in murine studies of asthma have found that after allergen inhalation fibrocytes localize to the airway mucosa, were they acquire a myofibroblast phenotype in presence of inflammatory cytokines (Schmidt M. et al., 2003). The high numbers of circulating fibrocytes in patients with asthma has been registered only in those individuals with chronic airway obstruction, respect to mild asthma and health subjects, were they correlated whit basement membrane thickness, by a TGF-β1-dependent increase in proliferation and α-SMA production, and whit the disease severity (Nihlberg K., et al. 2006). However, fibrocytes were also found (in few number) in the lungs of patients with mild asthma, indicating that they might have an important role in early inflammatory progression (Nihlberg K., et al. 2006). In a murine models of obliterative bronchiolitis using heterotopic tracheal transplantation, circulating fibrocytes engrafted the tracheal allograft and differentiated into fibroblast (Harris D.A. et al., 2012)

In fibrotic lung disease, the number of accumulating fibrocytes may be as much as 25% (Strieter R.M. et al., 2009), and may significantly contribute to pulmonary fibrosis. Experimental studies on murine model of this pathology has been shown fibrocytes traffic to lung following bleomycin challenge, both human fibrocytes in SCID mice and mouse

fibrocytes in immune-competent mice (Pilling R.J. et al., 2004; Hashimoto N. et al., 2004). Elevated level of circulating fibrocytes and intrapulmonary fibrocytes have been reported in patient whit idiopathic pulmonary fibrosis (IPF), where their presence correlated whit poor prognosis (Moeller A. et al., 2009), and had increased during acute exacerbations of the disease. Similarly, high number of fibrocytes are seen in the bronchoalveolar lavage fluid of patients with Acute Respiratory Distress Syndrome (ARDS) compared to mechanically ventilated patients without ARDS, who went on to show increased mortality (Quesnel C., et al., 2012)

1.5.5. In liver fibrosis

Several investigation on mouse model of liver fibrosis had been shown an involvement of fibrocytes in the liver injury, but they role is not to be clear, and under debate. Fibrocytes were identified in the bile-duct ligation model of murine liver fibrosis, although these bone-marrow derived cells reveled in the tissue comprised a small fraction of hepatic stellate cells, which mediate collagen deposition in this model (Kisseleva T., 2006). Also, other result on experimental models indicate the fibrocyte participation to this pathology. A murine imaging study using in vivo imaging of collagen-I driven luciferase reporter gene (coll I-Luc) found that fibrocytes traffic to the disease to the engagement of the chemokine receptor CCR1 and CCR2 and differentiate into myofibroblasts (Scholten D et al., 2011); Similar populations has been identified in the spleens of mice treated whit TGF-β1, lipopolysaccharide, viral infection or carbon tetrachloride (to induce the liver injury), also these cells expressed MHC-II and stimulated naïve T cells in addiction to their modest production of ECM and α-SMA (Kisseleva T. et al., 2012), thinking of a fibrocytes participation in the early phases of fibrosis via initiation and perpetuation of host immune responses.

While similar phenomena has yet to be identified in human liver fibrosis, where there has been a suggestion that a modest portion of the myofibroblasts-like cells (no more 12%) in the liver may have bone-marrow origin. Liver biopsies for recurrent fibrosis, in male patients that had received female liver transplantation, showed a proportion of the myofibroblasts were marked whit the chromosomal markers of the recipient (Y chromosome positive), suggesting a potential circulating origin. Furthermore, histological analysis of liver biopsies showing a portion of myofibroblasts contain the Y chromosome in female patients, who have received male bone-marrow transplantation, and then to go to develop liver injury, suggested potential bone-marrow origin (Forbes S.J. et al., 2004).

However, characterization and participation in chronic liver inflammation remains to be understood.

1.5.6. In skin and systemic fibrosis

Although there are numerous reports in mice suggesting that bone-marrow derived cells may be engraft skin and produce cells of a fibroblast or myofibroblast futures (Direkze N.C. et al., 2003), the few subsequent experiments of fibrogenesis and wound healing response in mouse model showed a trifling involvement of bone marrow origin whit $CD45^+$/Coll I^+ cells (possibly fibrocytes) (Higashiyama R. et al., 2011; Barisic-Dujmovic T. et al., 2010). However there is mounting evidence that circulating fibrocytes may contribute to fibrosis in skin following irradiation (Delanian S et al., 1998) and in the skin of patients affect by nephrogenic system fibrosis disease.

Nephrogenic system fibrosis (NSF) is a disease that occurs almost exclusively in patients whit renal dysfunction, in particular in those undergoing hemodialysis for end-stage renal disease. The clinical course is characterized by symmetric and hardening of the skin and involve multiple organ as heart, lung, skeletal muscle and diaphragm, has been reported in many patients, and it can lead to death. The pathogenesis is not clear but it has been associated whit the use of gadolinium-containing contrast agents for magnetic resonance (Bellini et al. 2007), in particular in the tissue of the patients that have metabolic acidosis or other condition that favor the liberation and deposition of the toxic gadolinium ions (as renal failure) (Grobner T., 2006). Several studies, in human patients and animal models of the disease, describe the presence of vascular calcium deposit, also interstitial gadolinium and calcium phosphate storages. In the tissue involved, has been shown the presence of fibrotic lesion whit a fibro-proliferative process and a dense infiltrate composed by monocytes and abundant spindle-shaped cells, immersed in a profuse ECM. The expression of CD45 and CD34 myofibroblast-like cells of the lesion has been detect in the areas of active fibrogenesis, well in the early stage of the fibrotic process (Bellini et al. 2007). In particular, Cowper et colleagues describe double staining cells for CD45 and pro-Coll I, demonstrating that these cells are really fibrocytes (Cowper et al., 2003). Also, It has been proposed that same cells may been involved in the pathogenesis of systemic sclerosis (Postlethwaite A.E. et al., 2004). A connective tissue disease characterized by autoantibody production, microvascular stenosis and excessive collagen deposition in the skin lesions and other organ involved (Bellini et al. 2007), as demonstrated by studies described above.

1.5.7. Fibrocytes in other chronic inflammation and cancer

Reactive fibrosis are a prominent features of chronic pancreatitis (CP), chronic cystitis and gut chronic tissue inflammation, as colitis. Such diseases shown a chronic inflamed process that engage an active status of local and circulating cells engraft, leading to the release of ECM elements and tissue remodeling.

Stromal cells expressing CD34 have been found in patients with chronic cystitis (Nimphius W, et al., 2007), as in experimental models and human tissue analysis of gut inflammation and colitis in which bone-marrow derived cells has been reveled confined to the non-epithelial compartment (Lee C.Y. et al., 2011).

With reference to human tissue, unlike to health pancreatic stroma, the accumulation of fibrocytes-like cells appeared to be associated with a parallel increase in the CD34 fibrocytes and α-SMA$^+$ myofibroblast populations, in chronic pancreatitis and ductal adenocarcinoma (Barth PJ, Ebrahimsade S et al., 2002). Also, two *in vivo* studies, a model of CP by cerulean injections (Watanabe T. et al., 2009), and a mouse dietary model of pancreatitis (Akita S. et al., 2012) reveled a 20% of α-SMA$^+$ activated pancreatic stellate cells whit bone-marrow origin and pro-inflammatory factors (PDGF and TGF-β1) production by these. However, the CD34 positivity by stromal cells and their real contribution to the population of lesional myofibroblast in these pathologic conditions remains to be elucidated.

Immature monocytes with bone-marrow origin are actively recruited into solid tumor from circulation and represent an important compartment of leucocyte infiltrate in malignant lesion. The presence of fibrocytes-like stromal cells CD34$^+$ has been detected in varius human benign and invasive tumors (Bellini et al., 2007). Histological analysis of human tissue sections for CD34 and α-SMA markers, revealed that CD34$^+$/α-SMA$^-$ fibroblast are usually found in the stromal capsule of benign tumor, whereas around carcinoma CD34$^-$/α-SMA$^+$ myofibroblast are more frequent (Barth PJ, Ramaswamy A et al., 2002; Ramaswamy A et al., 2003; Chauan H. et al., 2003; Barth PJ, Schenck zu S.T. et al., 2004). Furthermore, The presences of excessive levels of TGF-β1 and ET-1 in the tumor micro-environment could trigger the CD34$^+$ fibrocytes into active myofibroblst (CD34$^-$/α-SMA$^+$). The pro-angiogenic activity of fibrocytes (Hartlapp I, et al., 2001), also suggests that these may potentially contribute to the metastatic progression of invasive carcinoma. As reveled by in vivo experimental model, CD45$^+$/ CD34$^-$/α-SMA$^+$ cells with fibrocytes phenotype, that undergoing into mature myofibroblast, have been identified in cancer-induced stroma in advance stage of an implanted large-cell neuroendocrine carcinoma in bone marrow

chimera mice (Ishii G., 2005). In conclusion, fibrocytes may be an inducer of tumor escape from host immune surveillance through they both actigen-presenting activity and the ability to regulate cytotoxic T lymphocytes (Bellini et al., 2007).

2. Chronic Kidney Disease and Renal Fibrosis

2.1. CKD epidemiology, etiology and classification

Healthy kidneys play several essential regulatory roles in the human body. They perform homeostatic functions such as the regulation of electrolytes, maintenance of acid-base balance, regulation of blood pressure (via maintaining salt and water balance). They filter the blood removing wastes (i.e. urea, creatinine, ammonium) and foreign materials (i.e. drugs). The kidneys also have an endocrine function playing a part in the production of vitamin D and erythropoietin and as part of the renin/angiotensin/aldosterone axis.

Kidney disease is defined as a heterogeneous group of disorder affecting kidney structure and function which can occur abruptly and either be resolved or become chronic and evolve into end stage renal disease (ESRD). (Levey AS et al., 2013). Is necessary to distinguish two main forms of renal failure: 1) acute kidney injury (AKI), an abrupt loss of kidney function that develops within 48 hours, which is often reversible within three months with adequate treatment; 2) chronic kidney disease (CKD), which can involve in the end-stage renal disease (ESRD). There is a complicate relationship between AKI and CKD; AKI can lead to CKD, and CKD increases the risk of AKI (Bedford M. et al, 2012).

Chronic Kidney disease (CKD) is a general term for heterogeneous disorders affecting kidney structure and function with variable clinical presentation, which is present for >3 months. The progressive nephron and GFR loss associated with progressive CKD leads to (1) abnormalities in water, electrolyte, and pH balance, (2) accumulation of waste products normally excreted by the kidney, and (3) abnormalities in the production and metabolism of certain hormones (ie, erythropoietin, active vitamin D).

Chronic kidney disease is a common and growing problem worldwide. The adjusted rates for incident and prevalent ESRD in the United States (US) in 2008 were 351 and 1,699 cases per million population (pmp), respectively (USRDS 2010). The estimated incidence and prevalence of renal replacement therapy in 25 countries of the European Union was 137 pmp, and 786 pmp, respectively (Lameire N et al., 2005). The last decade has seen a dramatic 5-6% increase per annum in the number of patients with newly diagnosed ESRD (Samuel CS and Hewintson TD. 2009).

The etiology is really heterogeneous and most risk-factor are known. The two main causes of kidney disease are systemic hypertension, that damage the small blood vessels in the kidneys, and diabetes where hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening. Hypertension and diabetes are responsible for up to two-thirds of

the cases of CKD. Glomerulonephritis, inherited diseases (i.e. polycystic kidney disease or Alport's syndrome), autoimmune diseases (i.e. systemic lupus erythematosus), amyloidosis, infections (e.g. hepatitis C, tuberculosis, HIV and parasitic infection), nephrotoxic agents (e.g. analgesics and radiological contrast media), obstructions caused by tumors, kidney stones or an enlarged prostate gland in men and repeated urinary infections are other most frequent causes of CKD. These diseases can attack and damage the tiny glomerular vessels with different mechanisms. Also accidents, injuries or complications from surgeries in which the kidneys are deprived of normal blood flow, such as trauma and a direct and forceful blow to the kidneys, can lead to kidney injury.

Kidney disease differs from most other organ systems disorders because it is often "silent". Few symptoms, often non-specific, can appear only in the late stages of the disease (Levey AS et al., 2011). As the GFR declines, a number of compensatory mechanisms are activated, of which the most important is glomerular hyperfiltration in the remaining functioning nephrons. Due to this compensatory mechanism, a patient may be totally asymptomatic despite having lost 70% of kidney function. However, glomerular hyperfiltration is associated with the development of glomerulosclerosis in the remaining functioning nephrons, which contributes to further nephron loss. Beyond a certain level of damage, the compensatory adaptation is no longer holding the hand with the loss of nephrons, with consequent reduction in glomerular filtration rate and reduced elimination of organic nitrogenous wastes (e.g. creatinine, urea). (Brenner BM, 2002).

When the GFR falls to approximately 10–15 mL/minute, nonspecific symptoms such as general malaise, weakness, insomnia, inability to concentrate, nausea and vomiting begin to appear. Other symptoms and signs that reflect generalized organ dysfunction develop as part of the uremic syndrome, such as volume overload leading to edema and systemic hypertension.

In addition to an elevated blood urea nitrogen (BUN) and serum creatinine, which are a reflection of a decreased GFR, patients with progressive CKD develop other laboratory abnormalities including hyponatremia (due to excessive water intake), hyperkalemia, hyperphosphatemia, hypocalcemia, hypermagnesemia, and hyperuricemia. Metabolic acidosis, usually with an elevated anion gap, is also common. Anemia and renal osteodystrophy can complicate later stages of the disease, while cardiovascular manifestations are the most common cause of morbidity and mortality among patients with progressive CKD.

According to KDOQI and KDIGO guidelines CKD is defined by the presence of kidney damage or decreased kidney function for three or more months**,** irrespective of the cause

(Kidney Int Suppl. 2013;3:19). Identifying patients with CKD can therefore be done without identification of the underlying cause but identifying the cause of kidney disease enables specific therapy directed at preventing further injury. The persistence of the damage or decreased function for at least three months is necessary to distinguish CKD from acute kidney disease. Kidney damage refers to pathologic abnormalities, whether established via renal biopsy or imaging studies, or inferred from markers such as urinary sediment abnormalities or increased rates of urinary albumin excretion (table n° 1). Decreased kidney function refers to a decreased glomerular filtration rate (GFR), which is usually estimated (eGFR) using serum creatinine and one of several available equations.

	Functional Criteria	**Structural Criteria**
AKI	Increase in SCr by 50% within 7 days, or increased in SCr by 0.3 mg/dL within 2 days, or oliguria	No Criteria
CKD	GFR< 60 mL/min for >3 months	Kidney damage for > 3ms
AKD	AKI or GCF<60 mL/min/1.73 m^2 for <3 months or decreased in GFR by >35% or increase in SCr by >50% for <3 months	Kidney damage for < 3 ms
NKD	GFR > 60 mL/min/1.73 m^2, stable SCr	No damage

Table n°1: Criteria for normal or not damage, AKI and CKD proposed by KDIGO guidelines. Abbreviations: AKD, acute kidney disease, AKI acute kidney injury, CKD chronic kidney disease, GFR glomerular filtration rate; NKD, no known kidney disordes; SCr, cerum creatinine. (Levey AS., et al., 2013)

In current CKD nomenclature used by KDIGO, CKD is classified based on cause, GFR category, and albuminuria category (using urine albumin-to-creatinine ratio or urine protein-to-creatinine ratio) and is outlined in 5 stages (table n°2)

Prognosis of CKD by GFR and albuminuria category

GFR categories (ml/min/ 1.73 m²) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.

Table n 2: Prognosis of CKD by GFR and albumin Categories (KDIGO Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney International Supplements (2013) 3, 19-62).

In stage 1 CKD renal function is normal but there is evidence of urine findings or structural abnormalities. Stages 2 and 3 need increasing levels of supportive care to slow and treat renal dysfunction. Patients in stages 4 and 5 usually require preparation towards active treatment in order to survive. Stage 5 CKD is considered a severe illness and requires some form of renal replacement therapy (dialysis) or kidney transplant whenever feasible.

Epidemiology and clinical evidence show link between several factors and the initiation and the progression of CKD. Hypertension, diabetes, hyperlipidaemia, obesity and smoking are risk factors or markers in the general population for the development, but at the same time contributes to the progression of injury. Genetic and familiar predisposition, age, geographical origins and sex are considered "susceptibility factors" (El Nahas AM, Bello AK, 2005).

2.2. CKD and kidney damage progression

CKD could be develop by primitive glomerular injury, as in segmental and focal glomerulosclerosis or nephropathy IgA deposits, or tubulointerstitial injury as in polycystic disease or in nephritis induced by drugs and reflux nephropathy. Moreover it could be a consequence of a pathology with different origin as hypertension, systemic dysfunction by vascular origin with involvement of the great vessels, as in bilateral renal artery stenosis, or small-vessel vasculitis.

Also metabolic diseases such as diabetes mellitus (i.e. diabetic nephropathy), lupus nephritis, renal lithiasis, infectious diseases can attack the kidneys, causing kidney failure. Patients who have recovered from an episode of acute kidney failure, whether due to acute tubular necrosis or other parenchymal diseases, may also be at risk of developing chronic kidney disease (Schrier R.W. 2010).

The progression of CKD depends by several mechanisms with common final pathway: glomerular hypertension, renal proteinuria, hyperlipidaemia, tubular cell biology, interstitial immunology fibroblast formation and fibrosis. Regardless initial renal damage, if it persist can lead to glomerulosclerosis and hyperfiltration followed by tubular interstitial injury, or conversely, with a final common pathway involving into interstitial nephritis and fibrosis (Risdon RA et al, 1968; Brenner BM, 2002).

Chronic inflammatory response with progressive loss of remaining tissue, until beyond a certain threshold of damage, it triggers a mechanism of self perpetuation and aggravation of the same, as postulated by the "*self-perpetuation hypothesis*".(Brown et al., 1997; Brenner BM, 2002).

Persisted glomerular injury produce elevation in glomerular capillary hydraulic pressure (P_{gc}) (Chen JK et al. 2005). In response, the compensatory action induced by cells factors release (i.e. aldosteron, AngII and ROS) lead to hypertension in capillary tufts as well as glomerular hypertrophy. Also, glomerular podocytes are inability to cover the increased surface, so these undergo to detachment. Segmental denudation of the capillary wall whit capillary tuft adhesions to Bowman's capsule, followed by segmental collapse, increase the single nephron glomerular filtration rate and protein leak into tubular fluid. The remaining nephrons lose their ability to autoregulate, systemic hypertension is transmitted to the other glomerulus, worsening the pathologic condition.

Hemodynamic alteration induce glomerular proteinuria, which involves into the injury of interstitum and tubular atrophy. This follow by an inflammatory response with downstream cytokine and chemokine with other concurrently pro-inflammatory and hemodynamic factors that induces an accumulation of interstitial mononuclear cells (Harris R.C. et al., 2006). The immunologic mechanisms for spreading include loss of tolerance to parenchymal self, immune deposits that share cross-reactive epitopes in both compartments and glomerular injury that reveals a new interstitial epitope (Kuncio G.S., et al, 1992).

The initial appearance of interstitial neutrophils is quickly replaced by gathering macrophages and T lymphocytes, mediated by increased cytokines production that form a nephritogenic immune response, producing interstitial nephritis, tubular damage, apoptosis

and sustained inflammatory response (Taal MV et al., 2000). Simultaneously, profibrotic growth factors and inflammatory cytokines cues induce different cellular events include mesangial cells and renal fibroblast activation, such as podocytes, endothelial and tubular epithelia cells transition to fibroblastic/mesenchymal phenotype. New interstitial fibroblasts increase the number of tissue fibroblasts kindles fibrogenesis, that lay down a collagenous matrix that disrupts adjacent vasa recta and tubular nephrons, eventually leaving (El Nahas AM, Bello AK, 2005).

For reasons yet to be fully understood, the regeneration of kidney tissue damage occurs in the fetus only, whereas the morphological and functional recovery of the parenchymal tissue in the adult is partial and tied to the attempt to compensate for the damage suffered through the healthy nephrons (Hewitson, 2009). Kidney structure is replaced with connective tissue by an excess accumulation of extracellular matrix through a chronic and auto-sustained process involving in interstitial nephritis and renal fibrosis (or scarring).

Regardless where injury begins, the kidney will go to gradually self-destruct until to stage 5 of CKD, also known as end-stage of renal disease (ESRD) (Liu Y. 2006). ESRD occurs when renal parenchyma tissue is reduced to less than 5%, as observable during an histological analysis (Bohle A. et al., 1987). That lead to a progressive and irreversible loss of kidney function until the need for replacement therapy, such us dialysis or kidney transplant (Eddy AA, 2005).

2.3. Renal Fibrosis

Conceivably, each and every one of these pathological features could contribute to the relentless progression of the destruction of renal parenchyma and loss of kidney functions.

The wide range of pathophysiologically distinct processes that lead to chronic kidney disease (CKD) converge on a common pathway that results in glomerulosclerosis, progressive interstitial fibrosis, peritubular capillary rarefaction and destruction of functioning nephrons for a tubular atrophy (Eddy A.A., 2005). Tissue injury provokes inflammation as the host defense mechanism in response. Depending on the etiology of renal failure, glomerular or interstitial infiltrated inflammatory cells become activated, and produce injurious molecules such as reactive oxygen species (ROS), as well as fibrogenic and inflammatory cytokines and growth factors.

Renal fibrosis is characterized by the development of excessive extracellular matrix (ECM) plaques in the tissue interstitium that compromise kidney function and result in eventual

tubulointerstitial injury, the ultimate outcome of chronic renal injury (Risdon R.A.et al. 1968).

Normal wound healing, inflammation is an early beneficial response to injury, and it is spontaneously resolved once the repair and recovery is completed. The connection between inflammation and fibrosis is that they are mediated in a paracrine fashion, whereby inflammatory cells secrete profibrotic cytokines that act on resident fibroblast and tubular cells. However, non resolving inflammation after chronic injury is relentless drive of fibrogenesis because it creates a vicious cycle of inflammation, tissue damages and fibrosis. Consist in a failure of the wound healing response to resolve in situation of ongoing (chronic) insult. Renal fibrogenesis is considered to be a failed wound-healing response to tissue injury, or injurious stimuli, where the balance between the pro- and anti-inflammatory factors, particularly between the self-limited repair and exuberant accumulation of extracellular matrix has been tipped in favor of the latter. What factor determines whether inflammation is a physiologic response or a driving force for tissue destruction remains elusive. Key events of kidney fibrosis, usually identified as tubularinterstitial fibrosis, is not restricted to the interstitium region. Also, take place in the glomeruli after injury, which include glomerular infiltration of inflammatory cells and implication of mesangial cells and podocytes. In many aspect, major fibrogenic mechanism are common and shared by different tissue compartment in the kidney (Liu Y. 2011).

On the basis of the sequence of destructive events, the pathogenesis of renal fibrosis is "artificially" divided into four overlapping phases: **priming**, **activation**, **execution** and **progression (**Liu Y., 2006). However, despite this arbitrary division, is a dynamic process in which many of these events occur concomitantly.

After the initial injury, the affected kidney tissue undergo a series of events in an attempt to repair and recover from the damage with a localized inflammatory response, by creating an apoptotic and pro-inflammatory niche. These processes, identified as **priming phase**, include kidney resident cell persistent stimulation by almost of renal injured described above, which leads to the production and secretion of proinflammatory factors such us cytokines and vasoactive molecules, as endoelin (ET)-1 and angiotensin (Ang)-II. Nuclear factor (Nf) kB is one of the key drive of this inflammatory process, it is triggered by connective-tissue growth factor (CTGF), hypoxia, Ang-II, aldosteron, such as for protein from tubular fluid in the both proteinuria and tubular stretch conditions. Its factor induce the production of pro-inflammatory and pro activator molecules such as plasminogen activator inhibitor (PA-1), interleukin (IL)-1, IL-6, chemokin (C-C motif) ligand 2 (CCL2), CCL5 and tumor necrosis factor (TNF)-α by the injured tubular epithelial cells (Chuang

P.Y. et al. 2013). A chemotactic gradients provide a directional signal for lymphocyte T cells, dendritic cells, monocytes/macrophages, mast cells and fibrocytes to the injury sites. The increase of expression of monocyte chemoattractant protein (MCP)-1, of the complement proteins C3 and C5, that attract monocytes and macrophages in interstitial level, and cell adhesion molecules such as intercellular adhesion molecules (ICAM)-1, the vascular cell adhesion molecules (VCAM)-1 and osteopontin-1, helping cell migration into the interstitium (Eddy A.A., 2000)

Recruitment and activation of T lymphocyte could be considered an important early event that mediates the onset of renal fibrogenesis, specially CD4+T cells (Tapmeier T.T. et al., 2010). It followed by the macrophage recruitment to the injury site, which differentiated into two broad but distinct subset categorized as either classical activated (M1) or alternatively activated (M2). In general, M1macrophage display a typical proinflammatory phenotype, produce a variety of chemokines, as well as ROS, and therefore have pathogenic function (Liu Y., 2011); M2 have been shown to mitigate renal fibrosis (Wang Y. et al. 2007), with mast cells role (Miyazawa S. et al. 2004). In addition to the soluble mediator, findings also indicate that monocytes and tubular cells might be require to induce tubular EMT, important ongoing phenomenon of fibrosis, by monocyte via an NF-kB dependent pathway (Li Q. et all., 2011). Injured tubular cells also release Danger Associated Molecular Patterns molecules, which next their effects on neighboring tubular epithelial cells and inflammatory cells through toll-like receptor to promulgate innate immune response by increasing the production of pro-inflammatory mediators and recruitment of leukocytes (Anders H.J. et al., 2007). Also, dendritic cells, which as antigen-presenting cells derive from the same bone marrow myeloid progenitor as macrophages, are abundant in normal kidney interstitium and share the disease progression.

Inflammatory status build up sustained profibrotic cytokine pressure within the local microenvironment and primes fibroblasts and tubular cells to undergo phenotypic activation or transition of other cell types and to produce a large amount of ECM components.

In the ***activation*** phase, the upsurge of profibrotic pressure in the inflamed microenvironment inevitably leads to the activation and recruitment of matrix-producing cells, known as myofibroblasts (Chuang P.Y. et al. 2013). Many cell types in the tubulointerstitium of the kidney, such as fibroblast, tubular epithelial cells, vascular smooth muscle cells and a subset of macrophages, are capable of producing ECM, during chronic renal injury response cells from five different source contribute to the pool of

myofibroblasts. These include activation of interstitial fibroblast, differentiation of pericytes, circulating fibrocytes recruitment, tubular epithelial cells and endothelial cells that undergone to transdifferentiation, acquired a mesenchymal phenotype. (Fig n°2.).

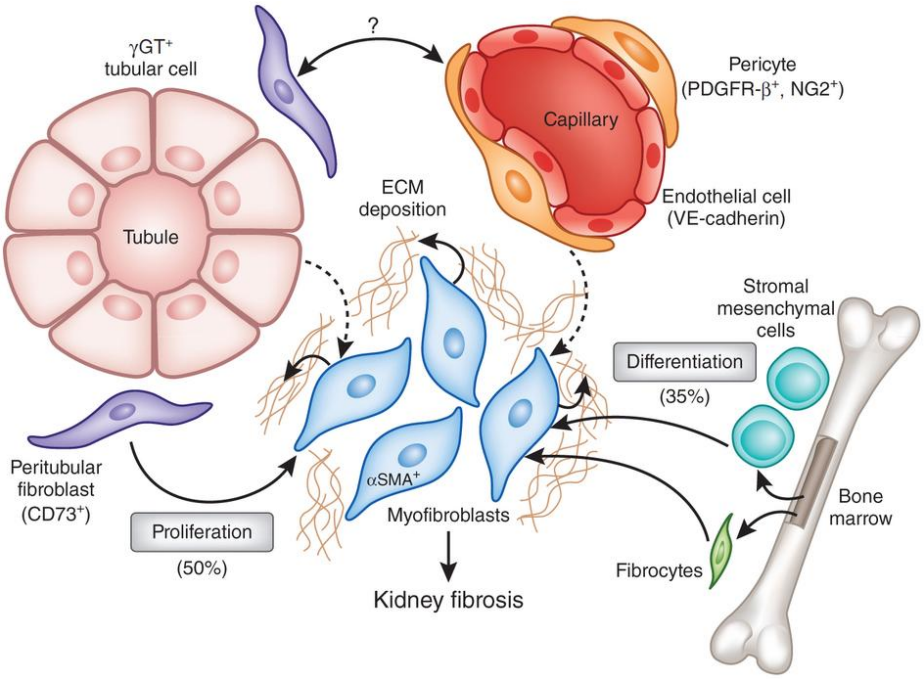

Figure n°2: Origin of scar-associated kidney myofibroblasts. Data derived from mouse kidney fibrosis models support multiple cellular origins for αSMA-positive interstitial myofibroblasts that appear de novo in response to chronic injury (Eddy AA, 2013).

Renal interstitial fibroblast are commonly regarded as the principal source of matrix production, which generate a large amount of interstitial matrix components as fibronectin, type I and Type III collagen.

In the normal Kidney, fibroblast are situated in the interstitium space form a network throughout the parenchyma, thereby stabilized the architecture (Kaissling B. et al., 2008). While, stellate shaped cells exibhit abundant rough endoplasmic reticulum, collagen-secreting granules and actin filaments, and express platelet-derive growth factor receptor (PDGFR)-β and fibroblast-specific protein (FSP)-1,a small protein that binds calcium and is associated with cytoskeleton (Liu Y., 2011).

Fibroblast activation occurs in response to cytokine cues as: PDGF, TGF-β, fibroblast growth factor (FGF)-2, l'insulin-like growth factor (IGF)-1, CTGF, and other factor as plasminogen activator (PA)-1 and retain PDGFR-β and FSP-1, in a auto-sustained systems

through both the autocrine and paracrine simuli. These promote fibroblast survival, increased myofibroblast proliferation activity and up-regulate the protein expression, in particular for ECM component, whit new filament types as vimentin, and α-smooth muscle actin (α-SMA), (Grande MT et al., 2009), wich leads to expantion of the fibroblast population and interstitial space in kidney disease. Despite α-SMA express in a few subpopulation of local fibroblast under normal condition and also during a fisiological wound-healing response, extensive studies indicate that its abundance is closely correlated with the severity of the renal fibrosis and predicts the decline of kidney function (Hewitson T.D., 2009). Vascular pericytes represent another relative contribution to myofibroblast population into the interstitial fibrosis scenary. Pericytes are a subset of the stromal cells that partially cover capillary walls, thereby stabilizing the endothelium. Following renal injury, pericytes are detached from the endhotelium, undergo migration into interstitium, proliferate, and differentiate into myofibroblasts (Lin S.L. et all., 2008; Humphreys B.D. et all., 2010). Probably, pericyte response to same stimuli of fibroblast such as cytokine, as evidenced by the presence of PDGFR-β, but more remains to be elucidate (Kaissling B. et al. 2008). Further sources of matrix-producing cells are the epithelial-mesenchymal transition and the endothelial-mesenchymal transition (EMT and EndoMT respectively), (Zeisberg M et al. 2010). EMT is a cells phenotypic conversion process that occur during embryonic development, tumor metastasis and organ fibrosis; its characterized by loss of epithelial futures and acquisition of mesenchymal markers, under the bombardment of various profibrotic cytokines, particularly TGF- β1. TGF- β1/Smad pathway such as ILK, Wnt/ β-catenin and Snail1 are all EMT-regulatory signaling that show a preferentially activation in renal tubular epithelia after injury(Hewitson T.D et al. 2009; Li Y. et al. 2009). Tubular cells with mesenchymal future can fully differentiate into interstitial myofibroblast and contribute to ECM production. EndoMT is considered to be a unique forma of EMT, as endothelial cells are a specialized type of epithelia of capillary endothelium (Li J et al., 2009; Zeisberg E.M., et al., 2008)

However, the contribution of EMT to renal fibrosis is controversial (Kriz W et al. 2011; Liu Y., 2011). Some studies described a direct involvement for both phenomena (Yang J. et al. 2001; Iwano M et al. 2002), other claimed a contrary theses (Humphreys B.D. et al., 2010; Li L. et al., 2010)

Assuming that EMT is a dynamic process in which epithelial cells and fibrocytes represent two extremes of a continual spectrum of intermediate cell phenotypes, tubular cells undergo a partial tansition, in which cells change one or two phenotypic markers, although the transitional program is activated. Such as a partial EMT, however, is associated whit

poor outcome and predicts the progression toward interstitial fibrosis in humans (Li L. et al., 2010; Herting A. et al., 2008).

Bone-marrow derived cells, known as fibrocytes are a subset of circulating monocytes with fibroblast like-future, in the periferal blood. In response to kidney injury, activated fibrocytes infiltrate renal parenchyma and participate to fibrogenesis, as shown by some studies (Sakai N. et al., 2008; Niedermeier M. et al., 2009).

Regardless of their different origins of matrix-producing cells, activated fibroblast/myofibroblast bring and to being the ***execution phase*** of renal fibrosis. Characterized by an excessive accumulation and deposition of interstitial matrix and formation of collagenous fibers, properly assembled and finally modified to resist proteolysis.

During this phase integrin-associated protein complex constitutes the molecular machinery that integrates various fibrogrogenic signal and orchestrates the matrix production and assembly. Integrins integrate the "outside-in" and "inside-out" signals between cells and their extracellular environment (Margadant C et al. 2010; Legate K.R. et al. 2009). These transmit their signal by activating the down-stream effector kinase, focal adhesion kinase and ILK. Integrines and their associated proteins constitute the core components of this machinery (Legate K.R. et al. 2006). A once, through their respectively receptors and specific downstream intracellular-signal cascade, cytokins active a host of transcription factor that act on the cognate elements in the promoter same genomic regions. Expression and synthesis of the ECM proteins is primarily controlled at gene transcription level in response to various "positive" fibrogenic cues. TGF-β1 regulates the expression of major components of this complex such as β1-integrin, ILK and PINCH, via Smad signaling (Margadant C et al. 2010; Li Y. et al, 2007; Yeh Y.C. et al, 2010; Li Y. et al., 2003). Ang II leads to upregulation of the components of this complex, through pathways either dependent or independent of TGF-β1(Yang F. et al., 2010; Carvajal G. et al., 2008; Yang F. et al., 2009). PDGF, FGF2 and CTGF participate leading to activation of ILK and production of matrix components. Such signal transduction cascade and expression of matrix gene are also regulate by a variety of microRNAs. While hepatocyte growth factor (HGF) and bone-morphogenetic protein (BMP)-7, inibith the production of matrix components, primarily antagonizing TGF-β1 (Liu Y. 2004)

A molecules machinery serves as a platform that integrates diverse fibrogenic signal and control of matrix production(Liu Y. 2011).

In the early stage of renal fibrosis, deposition of fibronectin precedes the production of fibrillar collagens of type I and type III, and regulate their assembly. This process is

followed by the production of a variety of new matrix proteins, such as secreted protein acidic and rich in cysteine (SPARC) and type IV collagen, as well as vimentin, vitronectin, thrombospondin, decorin and proteoglycan (Eddy A.A., 2005; Bradshaw A. D., et al, 2009). A collagen matrix newly synthesized is susceptible to proteolysis, and therefore the fibrosis is potentially reversible, which lead to wound-healing. While, biochemical modifications of the matrix proteins by cross-linking are induced by enzyme such as tissue transglutaminase and lysyl oxidase, rendering them stiff and resistant to proteolysis, (Eddy A.A., 2005).

Although, the accumulation of matrix protein is a prominent future of fibrosis but not the sole factor contributing to the progressive loss of renal function associated with renal failure. Many cellular and molecular events beyond the production of matrix components determine the reversibility and ultimate outcome of renal fibrogenesis, outlining the ***progression phase***. There is a shift from normal wound-healing to over-exuberant inflammatory response resulting in the undesirable consequence.

Sustained lymphocytes and macrophage infiltration, tubular epithelial cells and endothelial cells transdifferentiation, also peritubular vasculature rarefaction, tubular cells apoptosis and atrophy are present and contribute to the progressive loss of kidney function whit fibrotic scarring and renal parenchyma collapse (Zeisberg M et al. 2010; Schlondorff DO, 2008).

Damage to tubular epithelial cells induce the activation of major inflammatory and fibrogenic signaling such as NFkB, ILK, β-catenin and TGF-β1 pathways, described above, also instigates protective and regenerative responses such us autophagy and cell proliferation (Li L. et al., 2010). Normally, autophagy and apoptosis are process of "self-eating" and "cell-death" that mediates adaptation to calorie restriction, hypoxia or in inflammatory condition, as a mechanism of cell survival and protection to renal tissue omeostasis (Kume S. et al, 2010; Jiang M. et al., 2010). In up-regulate condition could be the underling mechanism that leads tubular cells decomposition. Simultaneously, cell proliferation is increased but the net mass of epithelial cells is decreased. Presumably owing to an increased rate of apoptosis and EMT as result of a defect in the cells cycle cause cells switch to a profibrotic phenotype with an increase of TGF-β expression (Koesters R et al., 2010; Yang L. et al., 2010). Furthermore, this condition diminish the effectiveness of its endogenous protective mechanism as BMP-7 and others antifibrotic factors produced by tubular cells in normal kidney (Wang S. et al., 2006), establishing a vicious cycle in patients with CKD. Moreover, chronic injury impairs the integrity of the tubular basement membrane (TBM) trough a transiently down-regulated of collagen type

IV, the major component of TBM, and the induction of matrix metalloproteinase (MMP)-2 and MMP-9 synthesis and action (Yang J. et al., 2002). MMPs are protease able to degrade the ECM component, in a normal condition of wound-healing reduce matrix accumulation. Although might protect the kidney with antifibrotic action by counterbalance to up-regulated synthesis in the advanced stage, their activation in the early stage is generally pathogenic by impairing of the TBM and faciliting EMT, as demonstrated by different study on transgenic mice (Wang X et al., 2010; Cheng S. et al., 2006).

Additionally, endothelium dysfunction due to activation of myofibroblasts from pericytes under EndoMT not only generate more matrix-producing cells but also directly leads to the pericytes deficiency and peritubular microvascular rarefaction (Schrimpf C. et al. 2011). It cause a reduction in nutrient and oxygen supply and diffusion in the scared tissue, resulting a tissue ischemia and hypoxia, already this by damages and excessive ECM production (Mimura I. et al., 2010). Chronic hypoxia, characteristic of advanced CKD, often coexists with increased oxidative stress and generation of ROS, which incites biological modification and damages on important macromolecules including proteins, lipids and carbohydrates. These modified macromolecules affect various kidney cells as pathogenic mediator and trigger renal inflammation and fibrogenic response (D'Agati et al., 2010; Negre-Salvayre et al., 2008). Hypoxia can lead to tubular EMT or apoptosis, activate resident fibroblast and impaired peritubular capillaries, thereby promoting a cycle of kidney injury and progression of renal fibrosis (Gunaratman L. et al, 2009).

Like the loss of tubules for atrophy, a decrease in the surface area of the peritubular capillaries is an histological feature of progressive renal disease that closely correlates to loss of kidney function and end-stage of renal disease. The renal fibrotic extension region is not only a marker of injury, but also predicts the loss of function and progression of damage in the kidney. (Chuang P.Y. et al. 2013)

3. Role of Circulating Fibrocytes into Renal Fibrosis

Renal fibrosis is a hallmark of progressive kidney disease, as the tubulointerstitial fibrosis is the principal cause of the renal function loss after kidney injury. However, the extent of renal fibrosis within the kidney is an independent predictor of clinical outcomes (Nath K.A., 1998).

The focal point of tissue fibrosis is a unique population of α-SMA-positive myofibroblasts that appear *de novo* in response to injury, after which they synthesize excessive quantitative of ECM.

As described above, the origin of the myofibroblasts is still the subject of intense debate (Eddy AA, 2013). However, different reports provide evidence that bone marrow-derived fibroblasts (know as circulating fibrocytes) are recruited into the kidney and contribute to kidney fibrosis as matrix-producer cells.

A mismatched kidney transplantation in humans has shown that the proportion of host-derived SMA-positive cells is approximately 30% in allografts undergoing chronic rejection compared with 10% in those without rejection, suggesting a circulating origin for these cells and drawing attention to fibrocytes involvement into the kidney injury (Grimm PC. et al., 2001). Until now, many researchers have been focused their studies to understand the mechanisms involved into the fibrocytes recruitment, but the real biological significance of these cells and their pathological role is still unclear.

One of the first studies using rodent models of renal fibrosis by under ureteral obstraction (UUO) has demonstrated that a 15% of bone marrow-derived fibroblasts were present in the kidney, 10 days after obstructive injury. Fibrocytes were identified as cells express enhanced GFP under the control of the FSP-1 promoter (Iwano M. et al., 2002).

Moreover, several studies, in same mice model using bone marrow transplantation have showed the presence of circulating-derived myofibroblasts into the kidney, in response to injury (, Lin F. et al. 2005; Roufosse C et al., 2006; Lin SL. et al. 2008). Same data were reported in a other experiment on in vivo models of kidney damage, in which even up to >30% of α-SMA-positive fibrocytes were visible into the injury site, 7 days after ischemia-reperfusion injury (Broekema M. et al., 2007).

Cells whit fibrocytes futures ($CD34^+$ spindle shaped cells) have been detected in tubulointerstitial lesions in patients with glomerulonephritis and these closely correlated with interstitial volume of the damage (Okon et al., 2003). Also, analysis of human injury kidney, as people with diabetic nephropathy, has been shown the presence of $CD45^+$/$proCol1^+$ myofibroblast into the interstitium, which correlated well with the severity of tubulointerstitial lesion. In these cases the interstitial fibrosis showed an inverse correlation

between the number of fibrocytes and kidney function, at the time of the biopsy. Into the same patients, the glucocorticoid therapy induced a significantly decreased during convalescent (Sakai N. et al., 2010).

Recent research report reveal the relative contribution to the myofibroblast pool by fibrocytes and other cells that participate to renal fibrosis. Using more than 20 distinct mouse models that were genetically engineered. The results revealed that only a 50% of active myofibroblasts occur by local tissue fibroblast, the remainder involved bone-marrow derived cells and local cells under EMT/EndMT process (LeBleu V.S. et al., 2013).

Studies on two murine model of UUO renal fibrosis, treated whit chronic infusion of Ang-II has been suggested that fibrocytes might contribute to fibrosis by an angiotensin II receptor type 1 (AT1R) and type 2 (AT2R), dependent pathway. Which both AT1R-inhibites mice and AT2R-deficient mice showed an increased circulating fibrocytes infiltration with concomitant upregulation of pro-collagen type I, than wild-type mice (Sakai et al., 2008). Also, pharmacologic inhibition of angiotensin II type 1 receptor reduced the degree of renal fibrosis and the number of fibrocytes in the kidney and in the bone marrow, confirming that the renin-angiotensin system by Ang-II play significant roles in renal fibrosis.

The same investigators examined whether the contribution of fibrocytes to renal fibrosis was dependent on their trafficking to the kidney via CCL21/CCR7 signaling. While, a renal fibrosis mice model that showed CD45+ Col I+ circulating fibrocytes, espressing CCR7 infiltrated the diseased kidneys. The inhibition of the CCL21/CCR7 signaling using a neutralizing antibody reduced the severity of the fibrocyte infiltration, the degree of kidney fibrosis (by almost 50%), and the renal expression of TGF-β1 and Coll I with a reduced number of CCR2-infiltrated cells, such as in CCR7-nulle mice (Sakai et al., 2006). Further, chemokine receptors of homing pathways resulted to be express by circulating fibrocytes, including CCR1, CCR2 and CCR5, usually envolved into migration of the principal target tissue (Galligan et al., 2013), as welle as CXCL16/CXCR16 system, which especially show in kidney injured models (Chen G. et al., 2011)

Thus, suggest that circulating fibrocytes may to be engaged to the injury site of kidney, by many chemokine-pathway, here these differentiate to myofibroblasts and express α-SMA, collagens and other ECM components, contributing to renal fibrosis. Which may be responsible for chronic persistent inflammation and immunemodulation by both the activation and trafficking of macrophages and other leukocytes, such as inducing EMT/EndoMT process in resident stromal cell, by MCP1/CCL2 and TGF-β1 expression.

Experimental Study

4. Aims

Circulating fibrocytes are the mesenchymal precursor of bone marrow origin, present with few levels in the peripheral blood of healthy subjects. As described below, different studies showed a direct correlation between increased levels of circulating fibrocytes and the progression of disease characterized by a fibrotic degeneration as well as in chronic renal disease.

To investigate circulating fibrocytes population futures, in this study, we developed and optimized a protocols of whole blood sample collection and enrichment methods of PBMCs, also we developed a flow cytometry analysis methods, a gene expression assay and a fibrocytes culture assay to detect and characterize the circulating fibrocyte cells. These laboratory methods were realized with the finally aim to investigate a correlation between the renal fibrosis progression and the different circulating fibrocytes levels, as well their activation status, in CKD patients. With these assumptions we proposed to Ethic Committee an observational (case/control) study protocol to provide the evidence that the evaluation of activated circulating fibrocytes levels measurements might be a new biomarker to monitoring the activity and progression of fibrogenesis into a CKD patients, as well in early phases.

5. Materials and Methods

5.1. Samples

Human peripheral blood was collected by venipunture from eight healthy adult volunteers, four for both sexes and with written consent, for a total volume of about 18 ml by each subject. A Ficoll-Paque system, Lymphosep (Biowest), was used to separate a poor sample of peripheral blood monocytes cells (PBMCs) from whole blood. Blood sample was diluted in phosphate-buffered saline (PBS) 1X with a ratio of 1:2 and transferred into a conical tube with ficoll medium pre-loaded.. It's system was improved with the implementation of the Leucosep tube (Greiner Bio-One, North America Inc.), a porous barrier incorporated into the tube that enables PBMCs isolations. Leucosep tube prefilled with separation medium, thanks to the barrier, prevents mixture of the sample material with the ficoll or a recontamination of the enriched cell fraction. After a centrifugation for 15 minutes at 1000 x g at real temperature (RT), lymphocytes and PBMCs are separated from whole blood, on the basis of their density, and enriched in an inter-phase above the separation medium. PBMCs fraction was harvested and washed twice with 10ml of PBS1X and centrifugated for 10 min at 250 x g at RT, to eliminate the platelets. An incubation with ACK lyses solution for 3-4 min on ice was developed to clean the samples rich for erythrocytes cells.

Commercial cell lines as HEK293T, Jurkat, and HT29 cell line (ATCC), were used to test the sensibility and specificity of the antibody, for antigen not highly expressed by PBMCs, which were selected to perform the flow cytometry analysis.

These tests samples were used to perform the preliminary experiments of flow cytometry analysis, differentiating cultures and molecular assays. It was necessary to develop a protocol analysis for the characterization and study of circulating fibrocytes, in CKD patients.

Subsequently we will develop an investigation of circulating fibrocytes in sixth patients affected by CKD, divided in four distinct groups based on GFR levels, and thirty subjects no affect (control group), will be expected between the patients afferent to the U.O. of Nefrology, Dialisis e Trasplantation of Prof. S. Stefoni, in the Policlinico S. Orsola-Malpighi Hospital of Bologna.

Group A: 20 patients affects by CKD with estimated Glomerular Filtration Rate (eGFR*) comprise between 30 and 45 mL/min/1.73 m^2 .

Group B: 20 patients affects by CKD with eGFR comprise between 15 e 30 mL/min/1.73 m^2.

Group C: 20 patients affects by CKD with eGFR < 15 mL/min/1.73 m².

Group D: 30 subjects not affect by any type of chronic renal disease with eGFR > 90 mL/min/1.73 m².(control group)

Patients and healthy controls samples will be collected during outpatients visit, by the selection futures described above. Subsequently, these will be transferred to the Bioscience Laboratory of IRST for the processing and experimental analysis. These will be analyzed into the bioscience laboratory of "Istituto Romagnolo per lo studio e la cura dei tumori" (I.R.T.S.), of Meldola (FC).

5.2. Sample collection systems

As described, circulating fibrocytes precursor are rear. Their detection is difficult and false positive results are easy. Different storage protocols were tested to preserve the sample, from the time of collection to the analysis. Blood samples were tested for all storage and analysis methods used in this study, it was necessary to develop and validate a sensitive and specific protocols. Two different collection tube systems were tested: BD Vacutainer® Blood Collection Tubes, for 9 ml, containing the anty-coaugulant EDTA (BD Bioscience, San Jose, CA) and CytoChex tubes, 9ml, containing a cell fixation solution and EDTA (Cyto Chex® BTD, Streck U.S.). The EDTA-tube system was selected because necessary to mRNA extraction and cells differentiation culture assays, while CytoChex as direct-draw blood collection tube specifically to immunophenotyping by flow cytometry.

5.3. Enrichment methods

Enrichment methods were tested to increase and improve the circulating fibrocytes detection than the total cell populations of the peripheral blood. the of the fibrocytes population. In this study we tried two cells enrichment methods, based on immunomagnetic separation systems, with monoclonal antibody directed to a specific antigen of interest, conjugated with paramagnetic beads. MACS CD34 human MicroBeads kit for magnetic labeling of cells associated to the MACS Large Cell Separation (LS) Columns (Miltenyi Biotec Gmbhy, Germany) and the EasySep™ magnet with the EasySep™ Human whole blood CD34 Positive Selection Kit (StemCell Technologies Inc.). Both the immmunomagnetic separation systems are based on a positive selection for CD34 positive cells, as marker of human hematopoietic stem and progenitor cells. These provides two work steps: an incubation phase whit anti-CD34 monoclonal antibody,

conjugated with the paramagnetic beads and a subsequently enrichment phase through the use of magnetic devices. In the first method, the samples labelled with CD34 magnetic conjugated monoclonal antibody, incubated for 30 min at 2-8°C, cells were loaded in a pre-balanced column placed in the MiniMACS separation Unit. The $CD34^+$ cells are magnetically labelled to the columns, while the negative fraction discarded as effluents. After, it's important to wash the columns for 2-3 times with 500µl of PBS 1X supplemented with 2mM EDTA and 0,5% of bovine serum albumin (BSA), namely as running buffer, to eliminate any non-specific cell. LS column are removed from separator and transferred onto a new collection tube, with 1ml of buffer to the reservoir to the column and using the plunger, supplied in the kit, positive fraction were recovered.

Otherwhise, EasySep™ system consist to a positive cells selection and/or negative cells depletionof the sample trough a three-step procedure, as described by the manufacture instruction for buffy coat sample. First, the buffy coat samples were incubated with a human CD34 monoclonal antibody (clone QBEnd10), for 15min at RT, it followed by incubation whit magnetic nanoparticles directed to the Fc portion of CD34 antibody, for 10min at RT, in EasySep RBC Lysis Buffer solution provided by the kit for both step. Which samples were transferred in tube without cup into the EasySep™ magnet and incubated for 10 minutes. So, the magnet and tube were inverted to pouring off the supernatant fraction with CD34 negatively fraction cells, while the magnetically labelled cells remained inside the tube. It step was repeated for 2-3 times whit samples resuspension in RBC lysis buffer, to improve the result. Finally, the sample tubes were removed by magnet to recover the enriched CD34 positive cell fractions in PBS 1X.

5.4. Cytometry analysis

Cytometry analysis, in a fist phase, were performed to evaluate the efficiency of the collection sample and enrichment phases. Which was important to assessment the effectiveness of the Cyto-Chex tube to retain the entire futures of the sample. Also, a cytometry method was developed to quantify the percentage of fibrocytes population of the blood, in both control and patient samples.

The PBMCs fraction, pre-fixed by Cyto-Chex tube, were washed in PBS 1X, centrifugated and resuspended in RBM buffer. Otherwise samples collected with BD Vacutainer tube were fixed using fixation buffer of the Cytofix/Cytoperm kit (BD Bioscience, San Jose, CA), following the manufactory instructions. After fixation, samples were wash in PBS 1X, centrifugated and resuspended in RBM buffer (Miltenyi). Also $1*10^6$ cells of each cell

lines describe above were used as positive control to test sensibility and specificity of each antibody to develop the fibrocytes measurement method. HEK293T were used to test the anti-Collagen Type I-FITC, clone 5D8-G9 (Milli-Mark®, Millipore), Jurkat cell line were used for anti-CXCR4-PE, rat anti-human, clone 2B11 (eBioscience), and HT29 cell line with anti-CCR7-APC, mouse anti-human, clone 3D12 (BD Pharmingen™). Positive control cell lines were treated whit same procedures describe here. While, antibody anti-CD45, CD34 and CD11b were tested directly on PBMCs samples.

$2*10^6$ cells of PBMCs and $1*10^6$ cells of CD34 positive cell fractions by both collection systems, were aliquoted to each tube for flow cytometry and processed to extracellular antibody stained. Cells were incubated with anti-CD45, PerCP-Cy 5.5, mouse anti-human, clone 104 (BD Pharmingen™), anti-CD34-PE-Cy7, mouse anti-human, clone 8G12 (BD), anti-CXCR4-PE, rat anti-human, clone 2B11 (eBioscience) and anti-CCR7-APC, mouse anti-human, clone 3D12 (BD Pharmingen™), in RBM buffer, for 15 min at 4°C. Intracellular staining was performed after cell permeabilization with Perm/Wash buffer, by Cytofix/Cytoperm kit (BD Bioscience, San Jose, CA), for 15min at RT and incubation with anti-Collagen Type I-FITC, clone 5D8-G9 (Milli-Mark®, Millipore), for 30min at 4°C. Cells incubated with irrelevant isotype-matched antibodies and unstained cells were used as controls. After, cells were washd in PBS 1X and trafered in 4 ml FACS tube. Fluorescence data were acquired on a FACSCanto flow cytometer (BD Bioscience, San Jose, CA) and there were analysed using BD FACSDiva software.

5.5. Cell cultures and *in vitro* differentiation assay

Mononuclear cells fraction obtained by whole blood collected in BD Vacutaner tube was carefully collected and rinsed twice with PBS 1X. PBMCs were suspended in DMEM supplemented with 10% of FBS, 1% penicillin/streptomycin solution and 2mM L-glutamine and seeded in cells culture flask (Corning™) at density of $1*10^6$ cells/ml and incubated for 24h at 37°C with 5%CO_2. After 24h the non adherent cells were removed, while the adherent cells, rich in monocytes cells, were collected and rinced twince with PBS 1X. 1*10 cells were collected to RNA extraction, the remainder cell fraction was re-plated at a density of $2.5*10^5$ cells/ml in DMEM serum-free supplemented with 2mM L-glutammine, 10mM HEPES (Sigma-Aldrich), 1% sodium pyruvate, 1% penicillin/streptomycin solution and ITS-3 (Sigma-Aldrich), as recommended by previously description (Pilling et al. 2003; Quan TE et al. 2007). These were seeded until 13 days in two different culture systems. classical plastic culture dishes 35mmX10mm,

and 8 cm^2 of diameter (Corning®) and µ-Dish ^{35mm} Grid-500 ibiTreat, (Ibidi®), a tissue culture dish 35mm Ø and a grid repeat with a distance of 500 µm.

Fibrocytes were measured ,at the end of differentiation treatment, by cell count, under x20 magnifications microscopy, into µ-Dish ^{35mm} Grid-500. Also, cell cultures in normal culture dish were monitored and collected to the RNA extraction.

While, HEK293T Jurkat , and HT29 commercial cell lines were cultured as described by ATCC instructions.

5.6. Gene expression assays

Total mRNA was isolated in three different phases. First $1*10^6$ peripheral blood monocyte cells from no-fixed sample tube, immediately after the enrichment phase; second from adherent cells after 24h of culture and finally after among two weeks (13 days) of culture. RNA was extracted with TRIZOL reagent (Invitrogen), following the manufactory instructions. After quantification by Nanodrop instrument, 1µg of total RNA was reverse transcribed into first strand cDNA with random primers and M-MLV reverse transcriptase, contained into DyNAmo cDNA Syntesis kit (Thermo Scientific). Real-time quantitative PCR (RT-qPCR) was performed used FastStart Universal Probe Master, normalization with ROX , and Taq-man assays Universal ProbeLibrary Probes selected by ProbeFinder assay design software (Roche). Detection and analysis were performed on an Abi Prism 7500 system (Applied Biosystems). Result of gene expression analysis was expressed as fold increase over the value of the control sample, by $2^{-\Delta\Delta Ct}$ analysis method. The expression of target genes as collagen I (Coll I), α-smooth muscle actin (α-SMA), CXCR4 and CCR7 was normalized to 18s transcript. Primer sequences are described in table n°3.

Gene	Primer Forward	Primer Reverse
Coll I	GCCTCTGCTCCCTTCTCAC	CTCTCCTGTTGCGTTGCTC
α-SMA	CCTATCCCCGGGACTAAGAC	AGGCAGTGCTGTCCTCTTCT
FN1	GCGAGAGTGCCCCTACTACA	GTTGGTGAATCGCAGGTCA
CXCR4	GGATATAATGAAGTCACTATGGGAAAA	GGGCACAAGAGAATTAATGTAGAAT
CCR7	GCACAATCTGGTTCTGATGTTC	GAGACGCTGTTGGGAACTTT
18s	GCAATTATTCCCCATGAACG	GGGACTTAATCAACGCAAGC

Tabel n°3: primers sequences for RT-qPCR to evaluate the gene expression of collagen I (coll I), alpha-smooth muscle actin (α-SMA), fibronectin (FN1), C-X-C chemochine receptor 4 (CXCR4) and C-C chemochine receptor 7

5.7. Statistical analysis

Data were expressed in mean ± SEM (standard error of the mean) unless otherwise stated. Graphpad 5.0 was used to perform the statistical analysis. Comparisons were evaluated by Student's unpaired t test. Results were considered statistically significant if p values were less than 0.05.

6. Results

Different storage systems were tested to assess the preservation of the sample futures at the moment of the blood collection, without sample alteration or degradation. Cytometry analysis of PBMCs collected with Cyto-Chex system (pre-fixation) showed no different results respect to no fixing system collection, as EDTA-tube (BD tube), for both samples obtained by same healthy volunteers. Cells processed and analysed by flow cytometry didn't showed different physical parameters or variation for expression levels of principal cells markers as CD45, CD3 and CD34 (Fig n°3).

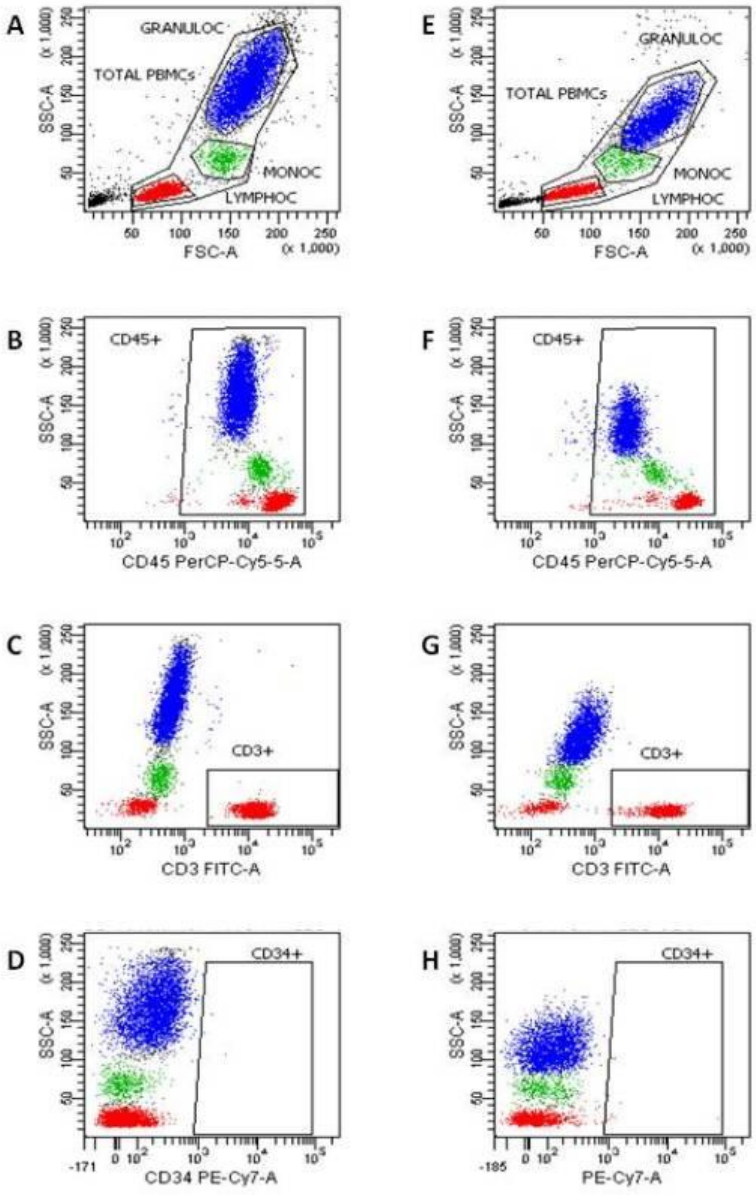

Figure n°3: Comparison between BD Vacutainer® Blood Collection Tubes, (A to D) and CytoChex tubes (E to H). Flow cytometric dot plot of physical parameters of PBMCs (A and E) and immunophenotype analysis for some principal extracellular markers CD45, CD3 and CD34, for both treatment protocols of sample. These don't show significant futures changes.

6.1. Enrichment protocols

With the final aim to obtain an high concentration of circulating fibrocytes we tried to integrate the standard protocol with an enrichment phase after the PBMCs extraction. To improve the fibrocytes detection, two different enrichment methods were tested. The two systems: MACS CD34 human MicroBeads kit and EasySep™ Human whole blood CD34 Positive Selection Kit used and tested in this study are based on immunomagnetic $CD34^+$ cells selection. Both immunomagnetic selection systems showed an effective in enriching cellular fraction $CD34^+$ but not sufficient to fibrocytes analysis. Samples processed to such enrichment showed an excessive reduction in the cell numbers, not enough to perform a good flow cytometric analysis (data not show).

6.2. Circulating fibrocytes detection by flow cytometry analysis

Antibodies used to developed the circulating fibrocytes measurement by flow cytometry were tested on positive control cell line and on PBMCs samples, for each marker. Isotype-matched antibodies controls were tested on same samples.

Results of each single antibodies showed a specific interaction with the corresponding antigen, also these didn't show a positive or detectable signal for isotype-matched antibodies tests (data not show). Thanks to this preliminary test, it was possible to define unique conditions, as time and temperature, for sample treatment and antibody staining protocols, for both extracellular and intracellular antigens.

In this develop phase of the study we tested and measurement the percentage of circulating fibrocytes expressed to the PBMCs population by flow cytometry using 4 markers to discriminate the circulating fibrocytes population (CD45, CD34, CD11b and Coll I), while CXCR4 and CCR7 were used to discriminate and evaluate the activation status of these circulating precursors(Fig n°4).

Preliminary results on the first eight healthy adult volunteers, , showed the identification of the circulating fibrocytes in the total white blood cells (WBC) sample, to confirm the specificity and sensibility of the cytometry analysis method developed. In healthy control, the percentage of fibrocytes relative to normal percentage of white blood cells (WBC) as $CD45^+$ resulted very low or absent, with a low presence of fibrocytes into the $1*10^5$total number of cells analyzed (0.04±0.01%),.

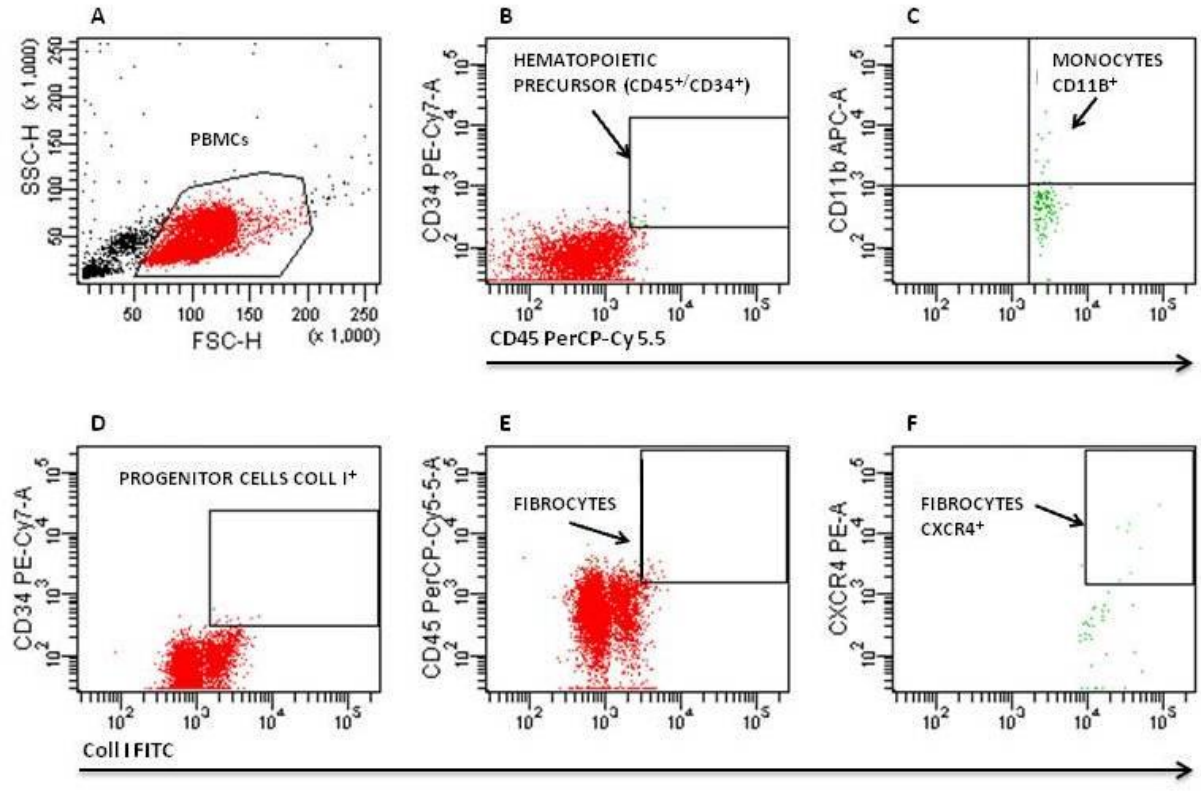

Figure n°4: Flow cytometric analysis of circulating fibrocytes in PBMCs, by healthy donor. Physical distribution of PBMCs (A), Monocytes cells positive to CD45 and CD34 (B). CD11b expression of $CD34^+/CD45^+$(C). Collagene I expression of $CD34^+$ cells, as precursors (D) and $CD45^+$ to identify Circulating Fibrocytes (E). Fibrocytes cells positive for CXCR4 (F).

6.3. Cell cultures and cell count differentiated fibrocytes

Into PBMCs cultures in conditional medium, we observed that after 13 weeks a portion of cells were differentiated into fibrocytes. These cells appear adherent and with a spindle-shaped morphology (Fig. n° 5). The number of fibrocytes were counted into the repeated grid of the ibiTreat culture dish, under x20 magnification. Their percentage reflected the flow cytometry data analysis, relatively to the total number of PBMCs seeded (among $2.5*10^5$cells).

6.4. Gene Expression analysis of principal fibrocyte markers

Total mRNA extracted by $1*10^6$ cells of total PBMCs sample and by total adherent cells in culture were collected after 24h and at the end of the 14 days of the conditioning cultures, for each healthy volunteers sample.

Gene expression of collagene I, α-SMA and fibronectin was quantified for adherent cells after 24h and 13 days of conditional culture plate. Results showed a significantly upregulation of fibrocytes markers in the samples cultured for 13 days, it compared to 24h PBMCs cultures ($p<0.05$), for the same donor sample. CXCR4 gene expression results showed a lower and unchanged levels between the different culture time samples. CCR7, however, resulted very weakly/not expressed in these samples (Fig.n°5).

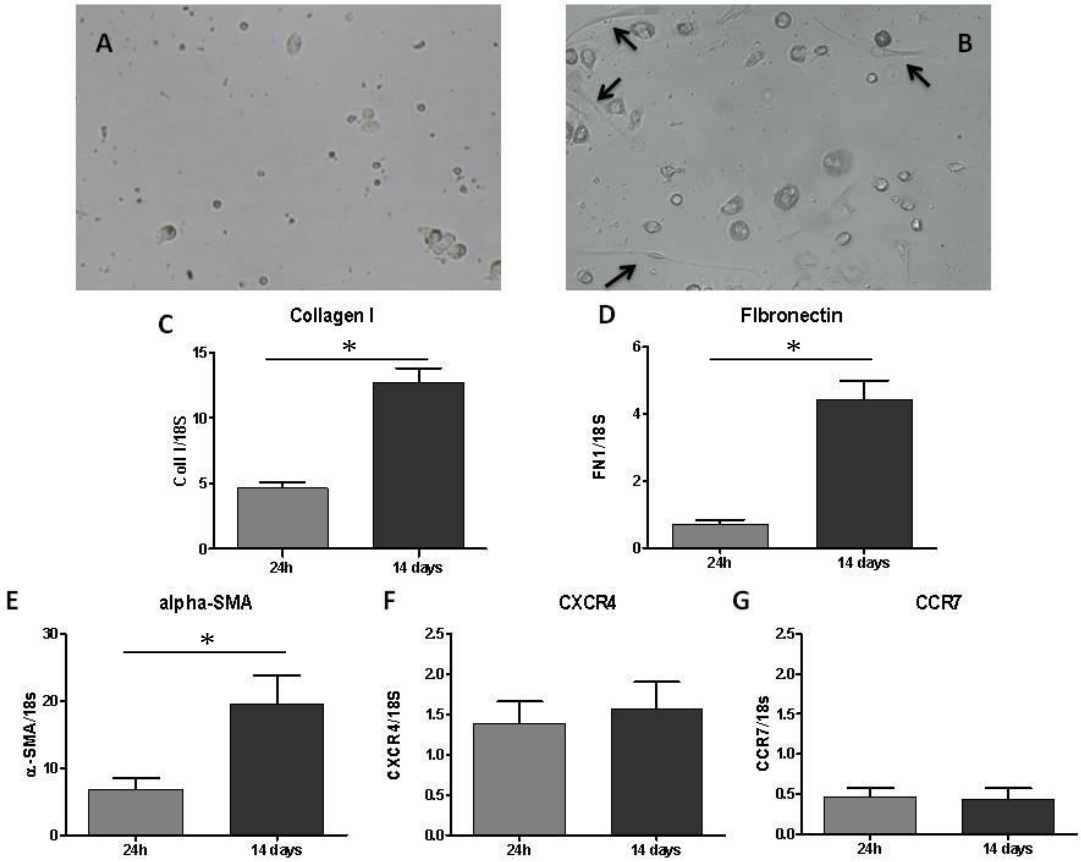

Figure n°5: Fibrocytes differentiation from PBMCs cultures (A). Cells with spindle-shaped cells, as fibroblast-like cells, are visible after 14 days of culture, by black arrows alighted (B). Gene expression levels of principal fibrocytes markers analyzed 24h and 14 days after seeding (C,D and E). Marker expression of CCR7 and CXCR4, as markers of fibrocytes activation (F and G); (p<0.05).*

7. Discussion

Circulating fibrocytes (CFs) are bone marrow-derived mesenchymal cells showing haematopoietic and progenitor cell markers (CD45, CD11b, CD34), which comprise only a small fraction of circulating leucocytes in healthy humans, comprise between 0.1-0.5% of them (Quan TE et al., 2006). However, an increase in CFs number is observed in human pathologies, characterized by chronic inflammation and persisted fibroblast activation, coupled with ECM production. A large body of evidence has shown that circulating fibrocytes might play a role in kidney fibrosis, contributing to the myofibroblast incensement (among 35%) (LeBleu VS et al., 2013). This is supported by both *in vivo* and *in vitro* evidence.

In this study, we optimized a protocol for whole blood sample collection and for the enrichment of the PBMCs fraction. Moreover, we set-up a flow cytometry assay for the detection and the characterization of circulating fibrocyte cells. Using fibrocytes culture assays and gene expression assays we improved and confirmed the CFs analysis method.

Cyto-Chex storage tube (Cyto Chex® BTD, Streck U.S.) were tested as collection system to store the sample, from the time of collection to the analysis. It is essential to preserve the cells futures of a rear and plastics cell population as circulating fibrocytes. Flow cytometry analysis on samples colleted with Cyto-Chex tube were compared to results with classical EDTA-tube. Both tube systems showed same result, without loss or alteration of the samples (Fig. n°3). While EDTA tube system was used, in this preliminary phase, because necessary to the cells differentiation culture assays and also to the mRNA extraction for the gene expression assays.

Fibrocytes are a minimal portion of circulating monocytes cells of the blood. Samples collected by healthy volunteers were processed for a PBMCs extraction by ficoll-paque system Lymphosep (Biowest), it improve with the Leucosep centrifugation tube (Greiner Bio-One, North America Inc.), specific to the mononuclear cells extraction by blood.

Due to the rarity of fibrocytes populations, we tested two method for the enrichment of $CD34^+$ cells fraction. PBMCs extracted by the whole blood samples were processed to both these immunomagnetic systems and then analyzed by flow cytometry. Cytometric analysis on $CD34^+$ cells from the blood led to good levels of enrichment, with an increase of the precursor cells into the sample. However, the CD34 antigen result expressed by a minimal fraction of cells than the total monocytes cell populations of the peripheral blood, among them are included fibrocytes. Though these samples appeared with a $CD34^+$ cells enrichment, these didn't improved the fibrocytes detection with a possible risk to reduce

the number of fibrocyte too. Probably the few start levels of the fibrocytes into the whole blood and the possible poor expretion of CD34 by CFs, represents a limit to obtain a sample rich for $CD34^+$ cells.

Circulating Fibrocytes express markers characteristic of hematopoietic cells, as CD45 and CD34, and monocytes marker as CD11b; they are unique in that they also produce extracellular matrix (ECM) protein as pro-collagen/collagen type I (Ishii G et al., 2005). Altogether, these futures represent the principal markers that allow identifying fibrocyte cells in a heterogeneous sample such as whole blood or fibrotic tissue. Furthermore, these cells express the chemokine receptors CCR2, CCR3, CCR5, CCR7 and CXCR4 (Mehrad et al., 2007; Wada et al., 2011), which are essential to the bone-marrow cell homing as well as during the recruitment into the inflammatory site. In particular, CCR7and CXCR4 chemokines axes are involved into kidney fibrosis (Sakai N. et al., 2010)

Bearing in mind this evidence, we developed and improved a common flow cytometry method for the identification of fibrocytes population. Antibodies labelled with fluorochromes directed against the principal fibrocytes markers (CD45, CD34, CD11b, pro-Coll/Coll I) were selected and tested for the detection of CFs. Also, antibody direct to the chemokine receptors CCR7 and CXCR4 were chosen and implemented in order to detect the circulating activated form of fibrocytes cells.

Positive control tests performed using cell lines, expressing a specific antigen for each antibody allowed to confirm the validity of the procedure and to define the optimal setting. Specially they allowed to define unique conditions, time and temperature, for sample treatment and antibody stained protocols, for both extracellular and intracellular antigens selected to the flow cytometry analysis of CFs. Subsequently, this method was employed for the preliminary quantification tests of fibrocytes amount in eight healthy volunteers (4 men and 4 women). The relative percentage of CFs in the total PBMCs sample obtained were in accordance with the literature (0.04±0.01%), of this analysis method.

Previous studies report that fibrocytes can be obtained after 1-2 weeks of differentiating culture of PBMCs *in vitro*. These data suggest that there is heterogeneity with respect to the ability of monocytes to differentiate in culture, specially between healthy and patients, with a higher possibility in the non healthy subjects. Moreover, as describe in literature, adding conditioned medium from PBMCs with a high yield of fibrocytes into PBMCs with a low yield of fibrocytes did not increase fibrocytes number, indicating that the number fibrocytes developed by PBMCs in some individuals may be due to the intrinsic property of their monocytes (Pilling D. et al. 2009). These monocytes may be "preprogrammed" to adopt the fibrocytes phenotype, perhaps through genetic or epigenetic changes in

progenitor cells, rather than relying purely on external influence. However, other studies confirm a direct influence of some factors as TGF-β or specific pathological condition on fibrocytes activation and differentiation (LeBLeu V.S. et al., 2013). By demonstrating the correlation between the activated circulating fibrocytes number with an increased collagen production, into a pathological condition, some study suggest a link between fibrocytes number and morbidity of the disease (Galligan C.S. et al., 2012). Probably, in patients, it does not influence the number of CFs but it affects the level of activated fibrocytes.

We have prepared PBMCs cultures by each healthy donors sample to observe if and how many monocytes differentiated into fibrocytes. After culturing adherent PBMCs for 13 days, we found the appearance of adherent spindle-shaped cells with ovoidal nucleus, as well typical fibroblast-like futures of fibrocytes. These were counted and the result confirmed the flow cytometry data (in %), compared to the total cells seeded. Conditional fibrocytes culture were employed to the gene expression assays (Fig.n°5).

Previous study demonstrated that the expression level of factors as collagen, fibronectin, vimentin and other CFs markers were enhanced in fibrocytes differentiated in culture (Pilling D. et al., 2009). In the present study, gene expression levels of principal fibrocytes markers like collagene I, α-SMA and fibronectin, were quantified for adherent cells in conditional culture plate. After 13 days of culture these were significantly upregulated compared with the same donor sample, after only 24h of culture assay (Fig n°5). However, CXCR4 gene expression results showed a lower and unchanged levels between the different culture time samples, while CCR7 resulted not expressed in these samples (data not show).

Thanks to the laboratory methods developed in this study it was possible to carry out a study of circulating fibrocytes in patients with chronic kidney disease. With these assumptions we proposed to Ethic Committee an observational (case/control) study protocol, namely “FCFR”, to investigate the role of CFs in kidney fibrosis progression into CKD patients. Sixty patients affected by CKD, divided in three distinct groups based on GFR levels, and thirty subjects not affected as control group are being recruitment. Experiments of flow cytometry analysis, in vivo culture assay and gene expression will evaluate to role of CFs in renal fibrosis of CKD pathogenesis.

8. Conclusion and futures

A flow cytometry approach coupled with *in vitro* culture assay and gene expression profiling was developed to study and characterize the CFs population, in the peripheral blood of healthy subjects. Preliminary results confirm that these method of analysis allow reliable and reproducible investigation of the fibrocytes cells fraction in whole blood sample.

Further studies using these method are ongoing, to dissect the relation between the progression of renal fibrosis and the different circulating fibrocytes levels in CKD patients, as recently suggested by previous studies (Sakai N. et al., 2010; LeBleu V.S. et al., 2013). The renal fibrotic extension region is not only a marker of injury, but it also predicts the loss of tissue function and the progression of the damage in the kidney. (Chuang P.Y. et al. 2013).

The finally aim of this study will be provide the evidence that the evaluation of activated circulating fibrocytes levels measurements might be a new biomarker to monitoring the activity and progression of renal fibrosis in CKD, as well in early phases of the disease.

Further studies aimed at understanding the biology of circulating fibrocytes, as well novel laboratory methods that allows to detect CFs with a greater sensitivity and lower costs, will be crucial for understanding the implication of CFs in the CKD pathology and to improve the diagnostic and prognostic methods used until now.

Bibliography

- Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. "Peripheral blood fibrocytes: differentiation pathway and migration to wound sites". J Immunol (2001);166:7556–7562.
- Akita S., Kubota K., Kobayashi A., Misawa R., Shimizu A., Nakata T., Yokoyama T., Takahashi M., Miyagawa S."Role of bone marrow cells in the development of pancreatic fibrosis in a rat model of pancreatitis induced by a cholinedeficient/ ethionine-supplemented diet".Biochemical and Biophysical Research Community (2012); 420: 743–749.
- Anders H.J. Heller F, Lindenmeyer M.T., Cohen C.D., Brandt U., Draganovici D, Fischereder M, Kretzler M et al. "The Contribution of B Cells to Renal Interstitial Inflammation". American Journal of Pathology (2007);170(2): 457–468.Andersson-Sjoland A, de Alba CG, Nihlberg K, et al. "Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis". International Journal of Biochemistry and Cellular Biology (2008); 40: 2129–2140.
- Andreeva ER, Pugach IM, Orekhov AN. "Collagen-synthesizing cells ininitial and advanced atherosclerosis lesions of human aorta". Atherosclerosis (1997);130:133–142.
- Andreeva ER, Pugach IM, Orekhov AN. "Subendothelial smooth muscle cells of human aorta express macrophage antigen in situ and in vitro". Atherosclerosis (1997);135:19–27.
- Bahn, R. S. "Graves' ophthalmopathy". New England Journal of Medicine (2010); 362, 726–738.
- Balmelli, C., Ruggli, N., McCullough, K. & Summerfield, A. Fibrocytes are potent stimulators of anti-virus cytotoxic T cells". Journal of Leukococytes Biology (2005); 77, 923–933.
- Barisic-Dujmovic T., Boban I., Clark S.H. "Fibroblasts/myofibroblasts that participate in cutaneous wound healing are not derived from circulating progenitor cells". Journal of Cellular Physiology (2010); 222: 703–712.
- Barth PJ, Ebrahimsade S, Hellinger A, et al. "CD34+ fibrocytes in neoplastic and inflammatory pancreatic lesions". Virchows Arch (2002); 440:128–133.
- Barth PJ, Ramaswamy A, Moll R. "CD34(+) fibrocytes in normal cervical stroma, cervical intraepithelial neoplasia III, and invasive squamous cell carcinoma of the cervix uteri". Virchows Arch (2002); 441:564–568.

- Barth PJ, Schenck zu Schweinsberg T, Ramaswamy A, et al. “CD34+ fibrocytes, alpha-smooth muscle antigen-positive myofibroblasts, and CD117 expression in the stroma of invasive squamous cell carcinomas of the oral cavity, pharynx, and larynx”. Virchows Arch (2004); 444:231–234.
- Barth, P. J., Koster, H. & Moosdorf, R. “CD34+ fibrocytes in normal mitral valves and myxomatous mitral valve degeneration”. Pathology Research Practis. (2005); 201, 301–304.
- Bellini A., Mattioli S., The role of fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest (2007); 87: 858-870.
- Blakaj A. and Bucala R. “Fibrocytes in health and disease”. Fibrogenesis and Tissue Repair (2012); 5 (Supp.1):S6.
- Blakaja A and Bucala R. “Fibrocytes in health and disease”. Fibrogenesis &Tissue Repairement (2012);5 Suppl 1: S6.
- Bohle A, Mackensen-Haen S, Gise HV. “Significance of tubulointerstitial changes in the renal cortex for the excretory function and concentration ability of the kidney: a morphometric contribution”. *Am J Nephrol* (1987)7: 421–433.
- Bradshaw A. D. ”The role of SPARC in extracellular matrix assembly”. Journal of cell comunication and signalling (2009); 3(3-4):239-46.
- Brenner BM, Meyer TW, Hostetter TH. “Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease”. New England Journal of Medicine (1982); 307(11): 652-9.
- Brenner BM. “Remission of renal disease: recounting the challenge, acquiring the goal”. Journal of Clinical Investigation (2002); 110:1753–58.
- Broekema M, Harmsen MC, van Luyn MJ, Koerts JA, Petersen AH, van Kooten TG, van Goor H, Navis G, Popa ER. “Bone marrow-derived myofibroblasts contribute to the renal interstitial myofibroblast population and produce procollagen I after ischemia/reperfusion in rats”. Journal of American Society of Nephrology (2007); 18: 165–175.
- Brown SA, Crowell WA, Brown CA, Barsanti JA, Finco DR, 1997. Pathophysiology and management of progressive renal disease”. Vet J. 154(2):93-109
- Bucala R, Spiegel L, Chesney J, Hogan M, Cerami A. “Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair”. Mol Med. (1994);1:71–81

- Bucala R. “Circulating fibrocytes: cellular basis for NSF”. J Am Coll Radiol (2008);5:36–39.
- Bucala R. “Fibrocyte: discovery of a circulating connective tissue cell progenitor”. In: Bucala R. (ed.). New insights into tissue repair and systemic fibroses. World Scientific: Singapore, 2007, pp 1-18.
- Buday, A. *et al.* Elevated systemic TGF-β impairs aortic vasomotor function through activation of NADPH oxidase-driven superoxide production and leads to hypertension, myocardial remodeling, and increased plaque formation in *apoE*- mice. American journal of Physiology, Heart and Circulatory Physiology. (2010); 299, H386–H395.
- Carvajal G. Rodríguez-Vita J., Rodrigues-Díez R., Sánchez-López E., Rupérez M., Cartier C., Esteban V., Ortiz A., Egido J., Mezzano S.A., Ruiz-Ortega M. “Angiotensin II activates the Smad pathway during epithelial mesenchymal transdifferentiation”. Kidney International (2008); 74(5):585-95
- Chauhan H, Abraham A, Phillips JRA, et al. “There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ, and invasive breast cancer”. Journal of Clinical Pathology (2003); 56:271–276.
- Chen G, Lin SC, Chen J, He L, Dong F, Xu J, Han S, Du J, Entman ML, Wang Y. ”CXCL16 recruits bone marrow-derived fibroblast precursors in renal fibrosis”. Journal of American Society of Nephrology. (2011); 22(10): 1876-86.
- Chen JK, Chen J, Neilson EG, et al. “Role of mammalian target of rapamycin signaling in compensatory renal hypertrophy”. Journal of American Society of Nephrology. (2005) 16:1384–91.
- Cheng S., Pollock A.S., Mahimarkar R., Olson J.L., and Lovett D.H. Matrix metalloproteinase 2 and basement membrane integrity: a unifying mechanism for pregressive renal injury” FASEB Journal (2006); 20: 1898-1900.
- Chesney J, Bacher M, Bender A, Bucala R. “The peripheral blood fibrocyte is a potent antigen-presenting cell capable of priming naive T cells in situ”. Proc Natl Acad Sci U S A (1997);94:6307–6312.
- Chesney J, Metz C, Stavitsky AB, Bacher M, Bucala R. “Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes”. J Immunol (1998);160:419–425.
- Chuang P.Y. Menon MC, He JC. “Molecolar target for treatement of Renal Fibrosis”. Journal of molecular medicine (2013); 91(5):549-59.

- Cowper SE and Bucala R. “Nephrogenic fibrosing dermopathy: suspect identified, motive unclear.” American Journal of Dermatopathology (2003); 25:358.
- Curnow SJ, Fairclough M, Schmutz C, Kissane S, Denniston AK, Nash K, Buckley CD, Lord JM, Salmon M. “Distinct types of fibrocyte can differentiate from mononuclear cells in the presence and absence of serum”. PLoS ONE (2010); 5: e9730.
- D’Agati and Schmidt A.M. “RAGE and the pathogenesis of chronic kidney disease”. Nature Review, Nephrology (2010); 6(6):352-60.
- Delanian S., Martin M., Bravard A., Luccioni C., Lefaix J.L. “Abnormal phenotype ofcultured fibroblasts in human skin with chronic radiotherapy damage”. Radiotherapy Oncology (1998); 47: 255–261.
- Delprat B, Ruel J, Guitton MJ, Hamard G, Lenior M, Pujol R, Puel JL, Brabet P, Hamel CP. Deafness and cochlear fibrocyte alterations in mice deficient for the inner ear protein otospiralin. Mol Cell Biol. (2005);25:847–53.
- Direkze N.C., Forbes S.J., Brittan M., Hunt T., Jeffery R., Preston S.L., Poulsom R., Hodivala-Dilke K., Alison M.R., Wright N.A.. “Multiple organ engraftment by bonemarrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice”. Stem Cells (2003); 21: 514–520.
- Douglas RS, Afifiyan NF, Hwang CJ, Chong K, Haider U, Richards P, Gianoukakis AG, Smith TJ. “Increased generation of fibrocytes in thyroid-associated ophthalmopathy.” Journal of Clinical Endocrinology and Metabolysm (2010); 95 430–438.
- Eddy A. A. “Moleclar basis of renal fibrosis”. Pediatric Nephrology, basic science review, (2000); 15: 290-301.
- Eddy AA. “Progression in chronic kidney disease”. Adv Chronic Kidney Disease (2005); 12:353-365.
- El Nahas AM, Bello AK., “Chronic Kidney disease: the global challenge”. Lancet (2005); 365: 331-40.
- Fathke C, Wilson L, Hutter J, Kapoor V, Smith A, Hocking A, Isik F. “Contribution of bone marrow-derived cells to skin: collagen deposition and wound repair”. Stem Cells (2004);22:812-822.
- Fern RJ, Yesko CM, Thornhill BA, Kim HS, Smithies O, Chevalier RL “Reduced angiotensinogen expression attenuates renal interstitial fibrosis in obstructive nephropathy in mice”. J Clin Invest (1999), 103:39–46.

- Forbes S.J. Russo F.P., Rey V., Burra P., Rugge M., Wright N.A., Alison M.R. “A significant proportion of myofibroblasts are of bone-marrow origin in human liver fibrosis”. Gastroenterology (2004); 126:955-963.
- Galligan C.L. and Fisch E.N. “The role of circulating fibrocytes in inflammation and autoimmunity”. Journal of Leukocytes Biology (2013); 93: 45-50.
- Galligan C.L., Siminovitch K.A., Keystone E.C., Bykerk V., Perez O.D. and Fish1E.N. “Fibrocyte activation in rheumatoid arthritis”. Rheumatology (2010); 49: 640–651.
- Gan Y., Reilkoff RA., Peng X., Russell TR., Chen QC., Mathai SK, Gulati M., Homer RJ., Elias JA., Bucala RJ., Herzog EL. “Role of Semaphorin 7a in TGF-b1 induced lung fibrosis, and scleroderma related interstitial lung disease”. Arthritic Rheumatoid (2011); 63: 2484-2494.
- Grande M.T., López-Novoa J.M. “Fibroblast activation and myofibroblast generation in obstructive nephropathy”. Nature Review, Nephrology (2009);5(6):319-28
- Grimm PC, Nickerson P, Jeffery J, Savani RC, Gough J, McKenna RM, Stern E, Rush DN.: “Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection”. New England Journal of Medicine (2001); 345: 93–97.
- Grobner T. “Gadolinium—a specific trigger for the development of nephrogenic fibrosing dermopathy and nephrogenic systemic fibrosis?” Nephrology Dialysis Transplantation (2006); 21: 1104–1108.
- Gunaratman L. and Bonventre J.V. “HIF in kidney disease and development”. Journal of American Society of Nephrology (2009); 20(9):1877-87.
- Harris DA, Zhao Y, LaPar DJ, Emaminia A, Steidle JF, Stoler M, Linden J, Kron IL, Lau CL. “Inhibiting CXCL12 blocks fibrocyte migration and differentiation and attenuates bronchiolitis obliterans in a murine heterotopic tracheal transplant model”. Journal of Thoracic Cardiovascular Surgery (2013); Mar 145 (3): 854-61
- Harris R.C. and Neilson E.G. “Toward a unified theory of Renal Progression”. Annual Review of Medicine (2006); 57: 356-80.
- Hartlapp I, Abe R, Saeed RW, Peng T, Voelter W, Bucala R, Metz CN. “Fibrocytes induce an angiogenic phenotype in cultured endothelial cells and promote angiogenesis in vivo”. FASEB J (2001);15:2215–2224.

- Hashimoto, N., Jin, H., Liu, T., Chensue, S. W. & Phan, S. H. “Bone marrow-derived progenitor cells in pulmonary fibrosis”. Journal Clinical Investigation (2004); 13: 243–252
- Herting A., Anglicheau D., Verine J., Pallet N., Touzot M, Ancel P.Y., Mesnard L., Brousse N, Baugey E., Glotz D., Legendre C., Rondeau E., Xu-Dubois Y.C.et al. “Early epithelial phenotypic changes predict graft fibrosis”. Journal of American Society of Nephrology (2008); 19(8):1584-91.
- Herzog EL, Bucala R. Fibrocytes in health and disease. Exp Hematol. 2010;38:548–56.
- Hewitson T.D., “ Renal tubulointerstitial fibrosis : common but never simple” Amrican Journal Physiologist of Renal Physiology (2009); 296: F1239-F1244.
- Hewitson TD, Darby IA Bisucci T, Jones CL, Becker GJ. “Evolution of tubulointerstitial fibrosis in experimental renal infection and scarring”. Journal of American Society of Nephrology (1998), 9: 632–642.
- Higashiyama R., Nakao S., Shibusawa Y., Ishikawa O., Moro T., Mikami K., Fukumitsu H., Ueda Y., Minakawa K., Tabata Y., Bou-Gharios G., Inagaki Y. “Differential contribution of dermal resident and bone marrow-derived cells to collagen production during wound healing and fibrogenesis in mice“. Journal of Investigation in Dermatology (2011); 131: 529–536.
- Hong KM, Belperio JA, Keane MP, Burdick MD, Strieter RM. Differentiation of human circulating fibrocytes as mediated by transforming growth factor-beta and peroxisome proliferatorsactivated receptor gamma. J Biol Chem. (2007);282:22910–20.
- http://kidney.niddk.nih.gov/kudiseases/pubs/yourkidneys/ (NKUDIC) National Kidney and Urologic Diseases Information Clearinghouse (2012). "The Kidneys and How They Work". National Institute of Diabetes and Digestive and Kidney Diseases. Retrieved 1 January 2013.
- http://www.kidney.org/professionals/kdoqi/guidelines_ckd/p3_pubhealth.htm. (KDOQI) Kidney Disease Outcomes Quality Initiative, “Clinical Practice Guidelines for Chronic Kidney Disease: Evaluation, Classification, and Stratification” National Kidney foundation (NKF) 2002.
- Humphreys B.D., et al. “Fate tracing reveals the pericyte and not epithelial origin of myofibroblast in kidney fibrosis. American Journal of Pathology (2010); 79: 494-501.

- Ishida Y, Kimura A, Kondo T, Hayashi T, Ueno M, Takakura N, Matsushima K, Mukaida N. “Essential roles of the CC chemokine ligand 3-CC chemokine receptor 5 axis in bleomycin-induced pulmonary fibrosis through regulation of macrophage and fibrocyte infiltration”. Am J Pathol. (2007);170:843–54
- Ishii G, Sangai T, Sugiyama K, et al. “In vivo characterization of bone marrow-derived fibroblasts recruited into fibrotic lesions”. Stem Cells (2005); 23:699–706.
- Ito Y, Aten J, Bende RJ, Oemar BS, Rabelink TJ, Weening JJ, Goldschmeding R “Expression of connective tissue growth factor in human renal fibrosis”. Kidney Int (1998); 53:853–861.
- Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. “Evidence that fibroblasts derive from epithelium during tissue fibrosis”. Journal of Clinical Investigation (2002); 110: 341–350.
- Iwata, H. Manabe I, Fujiu K, Yamamoto T, Takeda N, Eguchi K, Furuya A, Kuro-o M, Sata M, Nagai R. “Bone marrow-derived cells contribute to vascular inflammation but do not differentiate into smooth muscle cell lineages”. Circulation (2010); 122, 2048–2057.
- Jernigan SM, Eddy AA (2000). “Experimental insights into the mechanisms of tubulo-interstitial scarring”. In: El Nahas M, Harris K, Anderson S (eds) Mechanisms and clinical management of chronic renal failure. Oxford University Press, Oxford, pp 104–145.
- Jiang M. Liu K, Luo J, Dong Z. “Autophagy ia renoprotective mechanismduring in vitro hypoxia and in vivo ischemia-riperfusion injury”. American Journal of Pathology (2010); 176(3):1181-92.
- Kadono, T., Kikuchi, K., Ihn, H., Takehara, K. & Tamaki, K. “Increased production of interleukin 6 and interleukin 8 in scleroderma fibroblasts”. Journal of Rheumatology (1998); 25, 296–301.
- Kaissling B. & Le Hir M. “ the renal cortical interstitium: morphology and functional aspects”. Histochemical Cellular Biology (2008); 130: 247-262.
- KDIGO Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney International Supplements (2013) 3, 19-62.
- Keeley EC, Mehrad B, Strieter RM. “Fibrocytes: bringing new insights into mechanism of inflammation and fibrosis”. Int. J. Biochem Cell Biol.(2010); 42(4): 535-542.
- Kisseleva T, Brenner DA. “Fibrogenesis of parenchymal organs”. Pro. Am. Thoracic Society (2008); 5:338-342.

- Kisseleva T, Uchinami H, Feirt N, Quintana-Bustamante O, Segovia JC, Schwabe RF, Brenner DA. “Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis”. Journal of Hepatolology (2006); 45(3):429–438.
- Kisseleva T, von Kockritz-Blickwede M, Reichart D, McGillvray SM, Wingender G, Kronenberg M, Glass CK, Nizet V, Brenner DA. “Fibrocyte-like cells recruited to the spleen support innate and adaptive immune responses to acute injury or infection”. Journal of Molecular Medicine (Berl) (2012); 89: 997-1013.
- Koesters R., Kaissling B, Lehir M, Picard N, Theilig F, Gebhardt R, Glick AB, Hähnel B, Hosser H, Gröne HJ, Kriz W. “Tubular overexpression of transforming growth factor-beta1 induces autophagy and fibrosis but not mesenchymal transition of renal epithelial cells”. The American Journal of Pathology (2010); 177(2):632-43.
- Kriz W., Kaissling B., Le Hir M. “Epithelial mesenchymal transition (EMT) in kidney fibrosis: fact or fantasy?”. Journal of Clinical Investigation (2011); 121(2):468-74.
- Kumar, V.; Abbas, AK.; Fausto, N. “Tissue renewal and repair: regeneration, healing, and fibrosis”. In: Kumar, V.; Abbas, AK.; Fausto, N., editors. Pathologic Basis of Disease. Elsevier Saunders; Philadelphia, PA: (2005); p. 87-118.
- Kume S., Uzu T, Horiike K, Chin-Kanasaki M, Isshiki K, Araki S, Sugimoto T, Haneda M, Kashiwagi A, Koya D. “Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney”. The Journal of Clinical Investigation (2010); 120(4):1043-55.
- Kuncio Neilson EG, Haverty TP. “Mechanisms of tubulointerstitial fibrosis.” Kidney International (1992); 39:550–56
- Lameire N, Jager K, Van BW, de BD, Vanholder R. “Chronic kidney disease: a European perspective”. Kidney International Supplement (2005); 99: S30–S38.
- Leask A., Abraham D.J. “TGF-β signaling and the fibrotic response”. FASEB journal (2004); 18: 816-827.
- LeBleu, VS, Taduri G, O'Connell J, Teng Y, Cooke VG, Woda C, Sugimoto H, Kalluri R. “Origin and function of myofibroblasts in kidney fibrosis”. Nature Medicine (2013); 19, 1047–1053.
- Lee C.Y., Jeffery R., Hutchinson G., Alison M.R., Poulsom R., Wright N.A., Otto W.R., “Bone marrow cells in murine colitis: multi-signal analysis confirms pericryptal myofibroblast engraftment without epithelial involvement”. PLoS One 6 (2011); e26082.

- Legate K.R. Fässler R. “Mechanisms that regulate adaptor binding to beta-integrin cytoplasmic tails.“. Journal of cell science (2009); 122(Pt 2):187-98.
- Legate K.R. Montañez E, Kudlacek O, Fässler R. “ILK, PINCH and parvin: the tIPP of integrin signalling”. Nature Review, Molecular cell biology (2006); 7(1):20-31
- Levey AS, de Jong P.E., Coresh J, El Nahas M., Astor B.C., et al. “The definition, classification, and prognosis of chronic kidney disease: a KDIGO Controversies Conference report”. Kidney international. (2011); 80:17–28.
- Levey AS, Levin A, Kellum JA. “Definition and Classification of Kidney Diseases”. American journal of Kidney Disease (2013); 61(5):686-8.
- Li J, Qu X, Bertram JF. “Endothelial-myofibroblast transition contributes to the early development of diabetic renal interstitial fibrosis in streptozotocin-induced diabetic mice”. The American Journal of Pathology (2009); 175(4):1380-8;
- Li L. Zepeda-Orozco D, Black R, Lin F., “Autophagy is a component of epithelial cell fate in obstructive uropathy”. The American Journal of Pathology (2010); 176(4):1767-78
- Li Q. et al. “Monocytes induce proximal tubular epithelial mesenchymal transition through Nf-kB dependent up-regulation of ICM-1”. Journal Cellular Biochemistry (2011); 112: 1585-1592.
- Li Y. Dai C, Wu C, Liu Y. “PINCH-1 promotes tubular epithelial-to-mesenchymal transition by interacting with integrin-linked kinase”. Journal of the American Society of Nephrology (2007); 18(9):2534-43.
- Li Y. Zhang X, Li Y, Liu Y. “Downregulation of Smad transcriptional corepressors SnoN and Ski in the fibrotic kidney: an amplification mechanism for TGF-beta1 signaling.” Journal of the American Society of Nephrology (2003); 14(12):3167-77.
- Li Y., Tan X, Dai C, Stolz DB, Wang D, Liu Y. “Inhibition of integrin-linked kinase attenuates renal interstitial fibrosis”. Journal of the American Society of Nephrology (2009); 20(9):1907-18.
- Liao M.T., Sung C.C., Hung K.C., Wu C.C., Lo L., Lu K.C., Rahman, K. "Insulin Resistance in Patients with Chronic Kidney Disease". Journal of Biomedicine and Biotechnology (2012) : 1–5.
- Lin F, Moran A, Igarashi P. “ntrarenal cells, not bone marrow-derived cells, are the major source for regeneration in postischemic kidney”. Journal of Clinical Investigation (2005); 115: 1756–1764.

- Lin SL, Kisseleva T, Brenner DA, Duffield JS. “Pericytes and perivascular fibroblasts are the primary source of collagen-producing cells in obstructive fibrosis of the kidney”. American Journal of Pathology (2008);173: 1617–1627,.
- Liu Y. “Cellular and Molecular mechanism of renal fibrosis”. Nature Review, Nephrology (2011), 7(12): 684-696.
- Liu Y. “Hepatocyte growth factor in kidney fibrosis: therapeutic potential and mechanisms of action”. American journal of physiology, Renal physiology (2004); 287(1):F7-16.
- Liu Y. “New insights into epithelial-mesenchymal transition in kidney fibrosis”. Journal of the American Society of Nephrology (2010); 21(2):212-22.
- Liu Youhua. “Cellular and Molecular mechanisms of renal fibrosis”. Nature Reviews Nephrology (2011); 7: 684-696.
- Liu Youhua. “Renal fibrosis: new insights into the pathogenesis and therapeutics”. Kidney Int. (2006); 69: 213-217.
- Margadant C., Sonnenberg A. “Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing”. EMBO Reports (2010);11(2):97-105
- Mathai SK, Gulati M, Peng X, Russell TR, Shaw AC, Rubinowitz AN, Murray LA, Siner JM, Antin-Ozerkis DE, Montgomery RR et al. “Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype”. Laboratory Investigation (2010); 90: 812-823.
- Medbury H. Role of fibrocytes in atherogenesis. Chapter 10. In: Bucala R (ed). Fibrocytes: New Insights Into Tissue Repair and Systemic Fibroses. World Scientific Publishing Co. Pte. Ltd.: Singapore, 2007, pp 175–194.
- Medbury, H. Medbury HJ, Tarran SL, Guiffre AK, Williams MM, Lam TH, Vicaretti M, Fletcher JP. “Monocytes contribute to the atherosclerotic cap by transformation into fibrocytes. *International Angiology*. (2008); 27, 114–123.
- Medina A, Ghahary A. “Reprogrammed fibrocytes induce a mixed Th1/Th2 cytokine response of naive CD4(+) T cells”. Molecular and Cellular Biochemestry (2011); 346:89-94.
- Mehrad B, Burdick MD, Zisman DA, et al. “Circulating peripheral blood fibrocytes in human fibrotic interstitial lung disease”. Biochem Biophys Res Commun (2007); 353: 104–108.
- Metz CN. “Fibrocytes: a unique cell population implicated in wound healing”. Cell Mol Life Sci (2003);60:1342–1350.

- Miller TE, Robinson KB. “Experimental pyelonephritis: a new method for inducing pyelonephritis in the rat”.Journal of. Infection Disease (1973); 127:307-310.
- Mimura I. Nangaku M. “The suffocating kidney: tubulointerstitial hypoxia in end-stage renal disease” Nature Review, Nephrology (2010); 6(11):667-678.
- Mitch WE, Walser M, Buffington GA, et al.. A simple method of estimating progression of chronic renal failure. Lancet (1976); 2:1326–28
- Miyazawa S., Hotta O ,Doi N, Natori Y, Nishikawa K, Natori Y. “Role of mast cells in the development of renal fibrosis: use of mast cell-deficient rats”. Kidney International (2004); 65(6):2228-37.
- Moeller A, Gilpin SE, Ask K, Cox G, Cook D, Gauldie J, Margetts PJ, Farkas L, Dobranowski J, Boylan C, O’Byrne PM, Strieter RM, Kolb M. ”Circulating fibrocytes are an indicator of poor prognosis in idiopathic pulmonary fibrosis”. Am J Respir Crit Care Med. (2009);79:588–94.
- Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB. “CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury”. Am J Pathol. (2005);166:675–84.
- Muller-Ladner, U., Ospelt, C., Gay, S., Distler, O. and Pap, T. “Cells of the synovium in rheumatoid arthritis. Synovial fibroblasts”. Arthritis Research & . Therapy(2007); 9: 223
- Murdoch C. “CXCR4: chemokine receptor extraordinaire”. Immunol Rev (2000); 177: 175–184.
- Nath KA. “The tubulointerstitium in progressive renal disease”. Kidney Intenational (1998); 54:992–994.
- Needleman, B. W. Increased expression of intercellular adhesion molecule 1 on the fibroblasts of scleroderma patients. Arthritis Rheumatoid (1990); 33: 1847–1851.
- Negre-Salvayre A., Coatrieux C, Ingueneau C, Salvayre R. “Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors”. British journal of pharmacology (2008); 153(1):6-20.
- Neilson EG. “Pathogenesis and therapy of interstitial nephritis”. Kidney International (1989); 35:1257–70
- Niedermeier M., Reich B, Rodriguez Gomez M, Denzel A, Schmidbauer K, Göbel N, Talke Y, Schweda F, Mack M. “CD4+ T cells control the differentiation of Gr1+ monocytes into fibrocytes”. PNAS (2009); 106(42):17892-7

- Nihlberg K, Larsen K, Hultgardh-Nilsson A, Malmstrom A, Bjermer L, Westergren-Thorsson G: "Tissue fibrocytes in patients with mild asthma: a possible link to thickness of reticular basement membrane?" Respiratory Research (2006); 7:50.
- Nimphius W, Moll R, Olbert P, et al. "CD34+ fibrocytes in chronic cystitis and noninvasive and invasive urothelial carcinomas of the urinary bladder". Wirchows Archiv (2007);450:179–185.
- Okono K., Szumera A, Kuzniewki M. "Are CD34+ cells found in renal interstitial fibrosis?". Am J of Nephrology (2003); 23: 409-414.
- Peng H.,Herzog E.L. "Fibrocytes: emerging effector cells in chronic inflammation". Current Opinion in Pharmacology (2012): 12:1-6.
- Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM. "Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis". Journal of Clinical Investigation (2004);114:438–446.
- Pilling D, Buckley CD, Salmon M, Gomer RH: "Inhibition of fibrocyte differentiation by serum amyloid P". Journal of Immunology (2003); 171: 5537-5546.
- Pilling D, Fan T, Huang D, Kaul B, Gomer RH. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. PLoS One. (2009); 4:e7475.
- Pilling D, Tucker NM, Gomer RH. "Aggregated IgG inhibits the differentiation of human fibrocytes". Journal of Leukocytes Biology (2006); 79:1242-1251.
- Pilling D. and Gomer RH. WDifferentiation of Circulating Monocytes into Fibroblast-like Cells". Methods in Molecular Biology (2012) 904: 191-206.
- Plantinga L.C., Tuot D.S., Powe N.R. "Awareness of Chronic Kidney Disease among Patients and Providers". Adv Chronic Kidney Dis. (2010); 17(3):225-36.
- Postlethwaite AE, Shigemitsu H, Kanangat S. "Cellular origin of fibroblasts: possible implications for organ fibrosis in systemic sclerosis". Current Opinions in Rheumatology (2004); 16:733–738.
- Quan TE, Cowper S, Wu SP, Bockenstedt LK, Bucala R. "Circulating fibrocytes: collagen-secreting cells of the peripheral blood". Int J Biochem Cell Biol (2004);36:598–606.

- Quesnel C, Piednoir P, Gelly J, Nardelli L, Garnier M, Lecon V, Lasocki S, Bouadma L, Philip I, Elbim C et al. “Alveolar fibrocyte percentage is an independent predictor of poor outcome in patients with acute lung injury.” Critical Care Medicine (2012); 40:21-28.
- Ramaswamy A, Moll R, Barth PJ. “CD34(+) fibrocytes in tubular carcinomas and radial scars of the breast”. Virchows Arch (2003); 443: 536–540.
- Reilkoff RA, Bucala R, Herzog EL. “Fibrocytes: emerging effector cells in chronic inflammation”. Nature Reviews, Immunolgy (2011); 11: 427-435.
- Risdon RA, Sloper JC, De Wardener HE. “Relationship between renal function and histological changes found in renal-biopsy specimens from patients with persistent glomerular nephritis”. Lancet (1968).2:363–66
- Roberts ISD, Burrows C, Shanks JH, Venning M, McWilliam LJ. “Interstitial myofibroblasts: predictors of progression in membranous nephropathy”. J Clin Pathol (1997); 50:123–127.
- Roufosse C, Bou-Gharios G, Prodromidi E, Alexakis C, Jeffery R, Khan S, Otto WR, Alter J, Poulsom R, Cook HT. “”Bone marrow-derived cells do not contribute significantly to collagen I synthesis in a murine model of renal fibrosis. Journal of American Society of Nephrology (2006); 17: 775–782.
- Saini V, Marchese A, Majetschak M. "CXC chemokine receptor 4 is a cell surface receptor for extracellular ubiquitin". *J. Biol. Chem.* (2010) 285 (20): 15566–76.
- Sakai N, Furuichi K, Shinozaki Y, Yamauchi H, Toyama T, Kitajima S, Okumura T, Kokubo S, Kobayashi M, Takasawa K, Takeda S, Yoshimura M, Kaneko S, Wada T. “Fibrocytes are involved in the pathogenesis of human chronic kidney disease”. Human Pathology(2010); 41: 672–8.
- Sakai N, Wada T, Matsushima K, Bucala R, Iwai M, Horiuchi M, Kaneko S. “The renin-angiotensin system contributes to renal fibrosis through regulation of fibrocytes”. J Hypertens (2008); 26:780–790.
- Sakai N, Wada T, Yokoyama H, Lipps M, Ueha S, Matsushima K, Kaneko S. “Secondary lymphoid tissue chemokine (SLC/CCL21)/CCR7 signaling regulates fibrocytes in renal fibrosis”. Proc Natl Acad Sci USA. (2006);103:14098–103.
- Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. "Resistance to HIV-1 infection in caucasian

individuals bearing mutant alleles of the CCR-5 chemokine receptor gene". Nature (1996); 382 (6593): 722–5.

- Schieppati A, Remuzzi G. “Chronic renal diseases as a public health problem: Epidemiology, social, and economic implications”. Kidney Int (2005); 68(Suppl 1): S7–S10.
- Schlondorff DO. “Overview of factors contributing to the pathophysiology of progressive renal disease“. Kidney International (2008); 74(7):860-866
- Schmidt M, Sun G, Stacey MA, et al. “Identification of circulating fibrocytes as precursor of bronchial myofibroblast in ashma”. Journal of Immunology (2003); 171: 380-389.
- Scholten D, Reichart D, Paik YH, Lindert J, Bhattacharya J, Glass CK, Brenner DA, Kisseleva T. “Migration of fibrocytes in fibrogenic liver injury.” American Journal of Pathology (2011); 179:189-198.
- Schrier Robert W. Diseases of the Kidney and Urinary Tract. Clinicopathologic Foundations of Medicine (2010) Book. Editor: Lippincott Williams & Wilkins
- Schrimpf C and Duffield JS. “Mechanisms of fibrosis: the role of the pericyte”. Current Opinion in Nephrology and hypertation (2011); 20(3):297-305.
- Schrimpf C., & Duffield J.S. “Mechanism of fibrosis: the role of the pericyte”. Current opinion in Nephrology Hypertension (2011); 20: 297-305.
- Shao DD, Suresh R, Vakil V, Gomer RH, Pilling D. “Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation”. J Leukoc Biol (2008);83:1323–1333.

Sitography

- Slimani H, Charnaux N, Mbemba E, Saffar L, Vassy R, Vita C, Gattegno L. "Interaction of RANTES with syndecan-1 and syndecan-4 expressed by human primary macrophages". Biochim. Biophys. Acta (2003) 1617 (1–2).
- Stary HC, Chandler AB, Dinsmore RE, et al. “A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association”. Arterioscler Thromb Vasc Biol (1995);15:1512–1531.
- Strieter RM, Keeley EC, Burdick MD, Mehrad B. “The role of circulating mesenchymal progenitor cells, fibrocytes, in promoting pulmonary fibrosis”.

Transactions of the American Clinical and Climatological Association (2009);120:49-59.

- Taal MW, Omer SA, Nadim MK, et al. Cellular and molecular mediators in common pathway mechanisms of chronic renal disease progression. Current Opinions in Nephrology Hypertensions (2000); 9:323–31.
- Tacke F, Randolph GJ. "Migratory fate and differentiation of blood monocyte subset". Immunobiology (2006);211: 609-618.
- Tamamis P, Floudas CA. "Molecular recognition of CXCR4 by a dual tropic HIV-1 gp120 V3 loop". *Biophys. J.* (2013)105 (6): 1502–1514.
- Tapmeier T.T., et al. "Pivotal role of CD4+T cells in renal fibrosis following ureteric obstruction" Kidney International (2010), 78: 351-362.
- Tourkina E, Bonner M, Oates J, Hofbauer A, Richard M, Znoyko S, Visconti RP, Zhang J, Hatfield CM, Silver RM, Hoffman S. "Altered monocyte and fibrocyte phenotype and function in scleroderma interstitial lung disease: reversal by caveolin-1 scaffolding domain peptide." Fibrogenesis Tissue Repair (2011); 4: 15.
- United States Renal Data System, USRDS 2010 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, 2010. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD; 2010 Jan 1
- Wada T, Sakai N, Sakai Y, Matsushima K, Kaneko S, Furuichi K. "Involvement of bone-marrow-derived cells in kidney fibrosis". Clin. Exp. Nephrol. (2011); 15: 8-13.
- Wang S., de Caestecker M, Kopp J, Mitu G, Lapage J, Hirschberg R. "Renal bone morphogenetic protein-7 protect against diabetic nephropathy". Journal of the American Society of Nephrology (2006); 17(9):2504-2512.
- Wang X., Zhou Y, Tan R, Xiong M, He W, Fang L, Wen P, Jiang L, Yang J. "Mice lacking the matrix metalloproteinase-9 gene reduce renal interstitial fibrosis in obstructive nephropathy". American journal of physiology, Renal physiology (2010); 299(5):F973-F982.
- Wang Y., Wang YP, Zheng G, Lee VW, Ouyang L, Chang DH, Mahajan D, Coombs J, Wang YM, Alexander SI, Harris DC. "Ex vivo programmed macrophages ameliorate experimental chronic inflammatory renal disease". Kidney International (2007); 72(3): 290-299.
- Watanabe T., Masamune A., Kikuta K., Hirota M., Kume K., Satoh K., Shimosegawa T., "Bone marrow contributes to the population of pancreatic stellate

cells in mice". American Journal of Physiologist and Gastrointestinal Liver Physiology (2009); 297: G1138–G1146.

- Wynn TA. "Cellular and molecular mechanism of fibrosis". Journal of Pathology (2008); 214:199-210.
- Wynn TA. "Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases". J Clin Invest (2007);117(3):524–529.
- Wynn TA."Common and unique mechanisms regulate fibrosis in various fibroproliferative disease". J. Clinical Invest. (2007); 117: 524-529.
- Xu J, Gonzalez ET, Iyer SS, Mac V, Mora AL, Sutliff RL, Reed A, Brigham KL, Kelly P, Rojas M. "Use of senescence-accelerated mouse model in bleomycin-induced lung injury suggests that bone marrow-derived cells can alter the outcome of lung injury in aged mice". Journal of Gerontology. Series A. Biological Sciences Medical Sciences (2009); 64:731-739.
- Yang F, Huang XR, Chung AC, Hou CC, Lai KN, Lan HY. "Essential role for Smad3 in angiotensin II-induced tubular epithelial-mesenchymal transitino". The journal of pathology (2010); 221(4):390-401.
- Yang F. Chung AC, Huang XR, Lan HY. "Angiotensin II induces connective tissue growth factor and collagen I expression via transforming growth factor-beta-dependent and -independent Smad pathways: the role of Smad3". Hypertension (2009); 54(4):877-884
- Yang J. and Liu Y. "Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis". The american journal of pathology (2001); 159(4):1465-1475.
- Yang J., Shultz RW, Mars WM, Wegner RE, Li Y, Dai C, Nejak K, Liu Y. "Disruption of tissue-type plasminogen activator gene in mice reduces renal interstitial fibrosis in obstructive nephropathy". The journal of clinical investigation (2002); 110(10):1525–1538.
- Yang L., Besschetnova TY, Brooks CR, Shah JV, Bonventre JV. "Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury". Nature Medicine (2010); 16(5):535-543.
- Yang, L. "Peripheral blood fibrocytes from burn patients: identification and quantification of fibrocytes in adherent cells cultured from peripheral blood mononuclear cells. Lab. Invest. 82, 1183–1192 (2002).
- Yang, L., "Identification of fibrocytes in post-burn hypertrophic scar". Wound Repair Regen. 13, 398–404 (2005).

- Yeh Y, Wei WC, Wang YK, Lin SC, Sung JM, Tang MJ. “Transforming Growth Factor-β1 Induces Smad3-Dependent β1 Integrin Gene Expression in Epithelial-to-Mesenchymal Transition during Chronic Tubulointerstitial Fibrosis”. The american journal pathology (2010); 177(4):1743-1754.
- Zeisberg E.M., Potenta SE, Sugimoto H, Zeisberg M, Kalluri R., “Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition”. Journal of the American Society of Nephrology (2008); 19(12):2282-2287
- Zeisberg M., Duffield JS. ”Resolved: EMT produces fibroblasts in the kidney”. Journal of the American Society of Nephrology (2010); 21(8):1247-53