UNIVERSITA' DEGLI STUDI DI MILANO

Department of Medical Biotechnology and Molecular Medicine

PhD Course
Experimental Medicine and Medical Biotechnologies
Cycle XXXII

A multilevel approach to define the role of immune and metabolic cues on the macrophage/fibroblast interplay during fibrosis development

Thesis of
Elisa Setten
Matr. N° R11632

Supervisor: Prof. Massimo Locati

Academic year 2019/2020

INDEX

1 ABSTRACT

Alterations in tissue homeostasis occur during inflammation, oxygen deprivation, and remodeling, but they are generally reversible processes. When the cause of injury is severe, persistent or repetitive, acute inflammation could become chronic and dysregulated wound-healing could lead to fibrosis. Dynamic interplay through immune and non-immune cells, such as macrophages and tissue fibroblasts, is crucial to determinate pathological outcome.

Macrophages (Mφ) are highly plastic cells, able to assume different functional phenotypes depending on the microenvironment. Fibroblasts (Fb) are heterogeneous cells that can be activated into myofibroblasts, which are the main sources of extracellular matrix (ECM) components. In inflamed tissues and fibrotic scars macrophages accumulate in areas of hypoxia, which is known to impact on macrophages activation. In these contexts the interplay of macrophages with fibroblasts likely affects their biology. In this study, we establish an *in vitro* direct contact co-culture model with macrophages and fibroblasts in order to elucidate cell-to-cell interactions. The system set-up take into account different microenvironment alterations, including T-cells cytokine secretion and oxygen deprivation, in order to mimic adaptive immune contribution and metabolic switch during inflammation and fibrosis. To investigate the relevance of different microenvironmental cues on Mφ and Fb activation, we stimulated the co-cultures with pro-inflammatory stimuli (LPS+IFNγ)or with pro-fibrotic cytokine(IL-4) or with 1% of oxygen tension (to mimic severe hypoxia).

Model parameters will be extracted from molecular profiling approaches investigating 44 different combinations of Mφand Fb polarized into inflammatory (MI and FbI) and fibrotic (MF and FbF) settings under normoxic and hypoxic conditions, in single cell cultures and direct-contact co-cultures. A multi-level strategy let us to compare different samples, to discriminate the effect of single variable on the system and to combine up to four variables together. We found that, when cells are in direct-contact in hypoxic environment, resting and pro-inflammatory Mφ and Fb assume or maintain an enriched inflammatory signature whereas pro-fibrotic macrophages inhibit the acquisition of a pro-inflammatory phenotype of fibroblasts. Indeed, single influencing factor alone is not able to induce differences in resulting phenotype but when immune stimuli, hypoxia and co-cultivation are combined together, for a long period of time, they change Mφ and Fb transcriptional landscape. Implementation of these findings with functional assays is essential to deeper investigate this crosstalk in chronic inflammation and fibrosis to translate candidate genes into predictive biomarkers.

2 ABBREVIATIONS

Mφ = Macrophage

Fb = Fibroblast

M0 = Resting macrophage

MI = Pro-inflammatory macrophage (M1)

MF = Pro-fibrotic macrophage (M2)

Fb0 = Resting fibroblast

FbI = Pro-inflammatory fibroblast

FbF = Pro-fibrotic fibroblast

MH = Hypoxic macrophage

FbH= Hypoxic fibroblast

4H/24H = 4h/24h of hypoxia

4N/24N = 4h/24h of normoxia

SC = Single culture

CC = Co-culture

M/CC = Co-cultivated macrophage

Fb/CC = Co-cultivated fibroblast

MI/H or MF/H = Hypoxic single cultivated pro-inflammatory or pro-fibrotic macrophage

FbI/H or FbF/H = Hypoxic single cultivated pro-inflammatory or pro-fibrotic fibroblast

M0/CC or MI/CC or MF/CC = Normoxic co-cultivated resting or pro-inflammatory or pro-fibrotic macrophage

Fb0/CC or FbI/CC or FbF/CC = Normoxic co-cultivated resting or pro-inflammatory or pro-fibrotic fibroblast

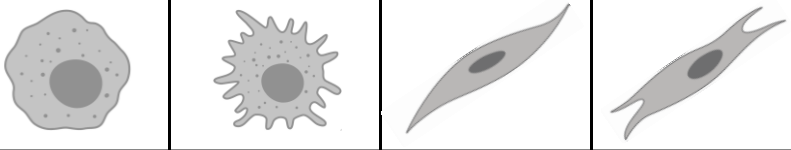
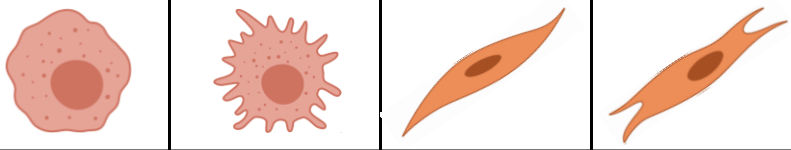
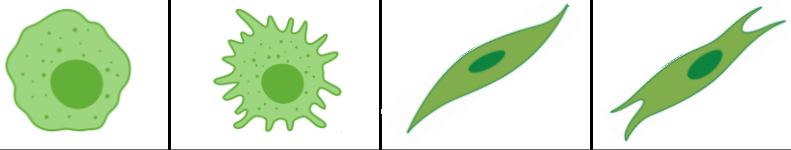
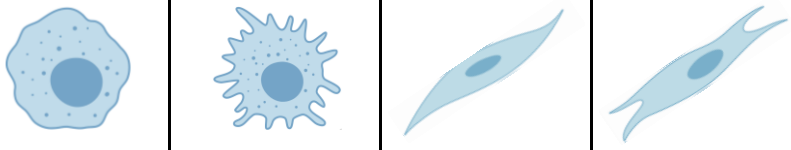
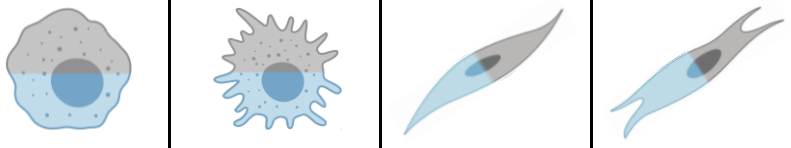
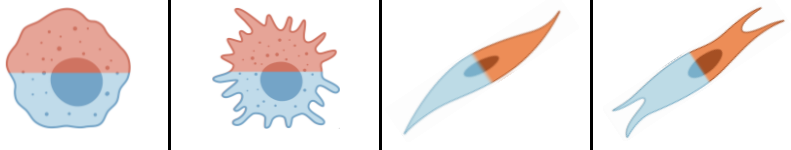
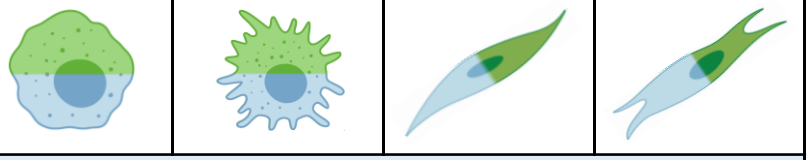
MH/CC or FbH/CC = Hypoxic co-cultivated resting macrophage or fibroblast

MI/H/CC or MF/H/CC = Hypoxic co-cultivated pro-inflammatory or pro-fibrotic macrophage

FbI/H/CC or FbF/H/CC = Hypoxic co-cultivated pro-inflammatory or pro-fibrotic fibroblast

SDEG = Significantly Differentially Expressed Genes

3 VISUAL LEGEND

Condition	Cell type			
	Macrophages		Fibroblasts	
	SC	CC	SC	CC
Resting				
Pro-inflammatory				
Pro-fibrotic				
Hypoxic				
Resting+hypoxic				
Pro-inflammatory +hypoxic				
Pro-fibrotic+hypoxic				

Time	Symbol
4h	
24h	

4 NOTE

This study originates from a more complex European project called SysMIFTA (System medicine approach to minimize macrophage-associated interstitial fibrosis and tubular atrophy in renal allograft rejection).

This project starts three years ago in 2016 and involved six different European groups expert in different fields: from anatomical pathology to digital image analysis, from immunology to mathematics. The multidisciplinary approach is the core of this work, which by integrating knowledge and information, rises to a real systemic study of IFTA.

Interstitial fibrosis and tubular atrophy are major clinical challenges in kidney transplantation. Differently from acute graft rejection, where clinical management have improved, this type of chronic reaction remain poorly understood. Implication of innate and adaptive immune response, mainly through macrophages and T cells, seems to be important in remodelling process. Moreover alternatively activated macrophages are key components of a complex network of cell-cell interaction that comprehend T cell and interstitial fibroblasts; derived tissue microenvironment changes impact on the delicate equilibrium between immunosuppressive beneficial and pro-fibrotic effects induced. Dynamic mathematical modelling of macrophage immunologic and metabolic regulation with advanced biopsy evaluation and ongoing clinical research are key components to study different stages of IFTA.

The aim of Sys-MIFTA is to translate new understanding of this network into practical benefit for patients by emerging targeted immunomodulatory therapies.

5 INTRODUCTION

5.1 Inflammation, wound-healing and fibrosis

Tissue homeostasis is a negative feedback mechanism that regulates cell population growth and dynamics in a tissue; it helps maintain healthy tissue size, responding to aberrant cell growth or death, ensuring the efficient use of resourced [1].
Cell communication within a tissue is mediated by growth factors and cytokines which control cell survival and proliferation. Cells and factors that they produced and received create a circuit that lead to maintenance of tissue homeostasis [2].
However, cells are often subjected to pressures that can compromise their fitness [3]. Alterations in tissue composition can occur during oxygen deprivation, inflammation and remodeling, but they are generally reversible [4]. All organisms have the crucial property of robustness, which is the ability of tissues and organs to maintain their functions and performances despite perturbations[5]. However, how mammalian tissues maintain population homeostasis and how pathological processes in cell composition are sustained remain poorly understood.

5.1.1 INFLAMMATION

The inflammatory response is generated by infections and mechanical or toxic damage that cause tissue injury. Damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) released by dead cells and invading organisms trigger inflammatory reaction that is characterized by the recruitment, proliferation and activation of a wide variety of different cells, including macrophages, neutrophils, natural killer cells, B and T lymphocytes, fibroblasts, epithelial cells, endothelial cells which, together, orchestrates tissue repair and homeostasis replace [6]. Indeed, generally, inflammatory response results in the protection from the spread of infection, followed by resolution: the restoration of affected tissues to their normal structural and functional state [4].
This type of inflammation is called acute inflammation: it is characterized by rapidly resolving vascular changes, oedema and neutrophilic response [7]. On the contrary, when the injury is severe, prolonged or repetitive, the acute inflammatory response can become chronic

with the enrollment of a large infiltrate of mononuclear immune cells, including macrophages, lymphocytes, eosinophils, plasma cells and non-immune cells, such as fibroblasts. Lymphocytes are mobilized to the site of injury and after activation produce cytokines that further activate macrophages and other local inflammatory cells [7].

5.1.2 WOUND-HEALING

When the injury is transient, wound-healing process takes place correctly and a controlled pro-resolving response leads to normal tissue architecture restoration and homeostasis replacement [6, 8, 9].

Specifically, when endothelial or epithelial cells are damaged, they release pro-inflammatory mediators that trigger an anti-fibrinolityic coagulation cascade with blood-clot formation and transient extracellular matrix (ECM) deposition. Platelets, exposed to ECM components, degranulate, promoting vasodilatation and increasing blood vessel permeability; myofibroblasts (or activated fibroblasts) and epithelial/endothelial cells produce matrix metalloproteases (MMPs), which disrupt the basement membrane, allowing inflammatory cells to be easily recruited to the site of injury. Growth factors, cytokines and chemokines stimulate the proliferation and recruitment of leukocytes. Neutrophils infiltrate the wound quickly and are the dominant leukocyte in the earliest stages. Concomitantly, circulating monocytes enter in the wound and differentiate into macrophages [10]. Neutrophils and macrophages together eliminate tissue debris, dead cells and any invading organisms. They also produce cytokines and chemokines, which are mitogenic and chemotactic for endothelial cells, which begin to surround the injured site. They also help new blood vessels formation. Wounds exhibit areas of marked hypoxia due to the lack of perfusion caused by vasculature damage and the great metabolic activity of infiltrating cells. Studies demonstrated that oxygen deprivation is an essential step to promote angiogenesis and other repair mechanisms in wound healing [11].

In the late phase of repair, lymphocytes appear in the wound bed and can influence wound resolution and remodeling. Activated T-cells secrete pro-fibrotic cytokines and growth factors, such as TGFβ (transforming growth factor β), IL-13 (interleukin 13) and PDGF (platelet-derived growth factor) [12, 13], which further activate the macrophages and fibroblasts, which acquire α-SMA (smooth muscle actin α) expression.

Finally, epithelial and/or endothelial cells divide and migrate over the basal layers to regenerate the damaged tissue, which complete the wound healing process.

However, chronic inflammation and repair can trigger an excessive accumulation of ECM components, which lead to the formation of a permanent fibrotic scar [7].

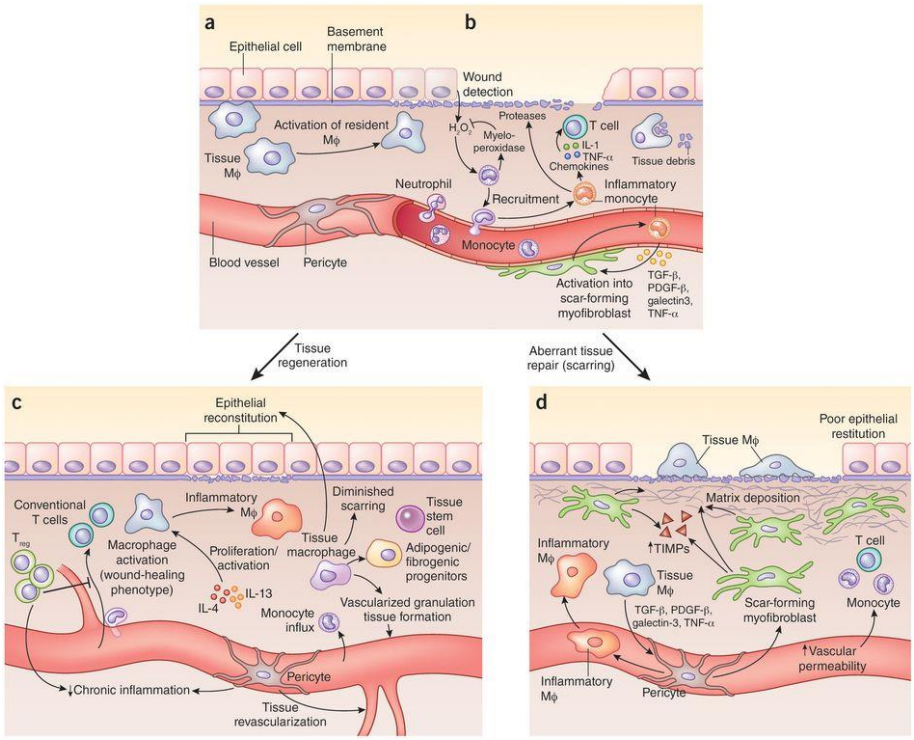


Fig. 1 General model of wound-healing. (a) Healthy tissue with normal epithelial layer and low monocyte and immune cell infiltration; (b) upon damage there is loss of epithelia, neutrophil influx, activation of resident macrophages and recruitment of inflammatory monocytes, in addition to release of inflammatory factors and activation of pericytes into myofibroblasts; (c) during tissue regeneration, epithelial layer is reconstituted, macrophages are switched in wound-healing phenotype and matix is remodeled; (d) during aberrant tissue repair, there is a continued activation of inflammatory cells, myofibroblasts proliferation and impaired epithelial regeneration [14].

5.1.3 FIBROSIS

Fibrosis is a state of continuous scaring that normally occurs during healing process, generating aggregate of ECM, myofibroblasts and macrophages that is gradually removed over weeks. Collagen turnover and ECM remodeling is regulated by various MMPs and their inhibitors, which include the tissue inhibitors of metalloproteinases (TIMPs). Fibrosis occurs when the synthesis of new collagen by myofibroblasts exceeds the rate at which it is degraded, such that the total amount of collagen increases over time [7]. Continued myofibroblasts activation and proliferation is sustained by many factors, including innate and

adaptive immune mechanisms. Chemokines, for instance, are leukocyte chemoattractants that, together with pro-fibrotic cytokines, promote the recruitment of myofibroblasts and macrophages in the site of injury. Lymphocytes, which are generally involved in chronic inflammation, are also critical players in fibrotic progression. Although chronic inflammation often precedes fibrosis, in some cases two processes are distinct. Several studies confirm the key role $CD4^+$ T cells in the progression of fibrosis and suggest the development of anti-fibrotic vaccine based on immune deviation where Th2 (T helper 2) response are switched into Th1 (T helper 1) anti-fibrotic response [15-19]. In particular, Th2 cytokines major involved in sustain of fibrosis include interleukins: IL-4, IL-5, IL-13 and IL-21. Roles of these factors will be deeper characterized later, however they are differently implied in fibrotic progression; specifically, IL-4 and IL-13 induce activation of macrophages and produce TGFβ, a crucial pro-fibrotic factor. TGFβ is a well characterized regulator of ECM and its involvement in fibrosis is been deeply studied [20]. It shows three isoforms in mammals: TGFβ1, -2, -3 with similar biological activity. However, fibrosis is mainly associated to the isoform 1 and the major sources are macrophages, which regulate both the secretion and the activation of latent TGFβ1. TGFβ1 is stored inside the cell as a disulphide-bonded homodimer, non-covalently bound to a latency-associated protein (LAP), which keeps TGFβ inactive. Binding of the cytokine to its receptors requires dissociation of the LAP, a process that is catalysed by several agents, including cathepsins, plasmin, calpain, thrombospondin, integrin-$\alpha v\beta 6$ and matrix metalloproteinases [21], many of which have become potential targets of anti-fibrotic drugs.

Macrophage secreted TGFβ promotes multiple features associated to fibrosis, such as fibroblast proliferation, activation of mesenchymal cell, including epithelial cells, into collagen-producing myofibroblast via epithelial-mesenchymal transition (EMT) and excessive production of ECM components [22].

Another important alteration is associated to the vasculature remodeling and angiogenesis [10] stimulated by pro-angiogenic factors released from macrophages (such as vasculature endothelial growth factor, VEGF) and by hypoxic areas generated after the wound lesion. Impaired angiogenesis and sprouting of hypoxia worsen fibrotic diseases.

However, there are endogenous mechanisms that slow the progression of fibrosis, mainly through regulatory T cells (Tregs) involvement [7] and IL-10 production. IL-10 is an immunosuppressive cytokine that suppresses the synthesis of type I collagens in human scar tissue-derived fibroblasts [7][23], indicating that it can directly inhibit fibrosis [7][24].

However, despite its success in some clinical studies, the mechanism by which IL-10 confers protection from fibrosis remains unclear.

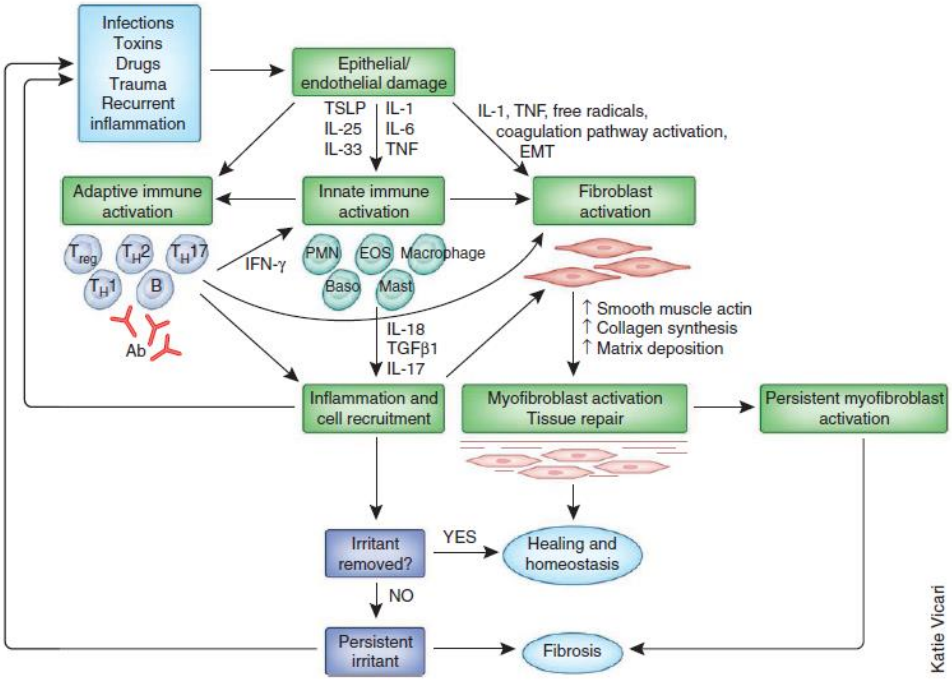


Fig. 2 Overview of wound repair and fibrosis. Epithelial and/or endothelial damage caused by various insults triggers complex interconnected wound-healing programs to quickly restore homeostasis. The coagulation pathway is triggered first, followed by acute inflammation and activation of innate immune mediators such as resident macrophages, neutrophils and dendritic cells. Epithelial and innate immune cell–derived cytokines influence the activation of the adaptive immune response. The tissue damage can also directly activate the adaptive immune response. Inflammatory and immune mediators (cytokines, chemokines and free radicals) attempt to eliminate the inciting factor while activating the resident quiescent fibroblasts into myofibroblasts that orchestrate angiogenesis and production of ECM components. Failure to adequately contain or eliminate the inciting factors can exacerbate the inflammatory response and lead to a chronic wound-healing response, with continued tissue damage, repair and regeneration, ultimately resulting in fibrosis. TSLP, thymic stromal lymphopoietin; Ab, antibody; PMN, polymorphonuclear leukocyte; EOS, eosinophil; Baso, basophil; Mast, mast cell [25].

5.1.3.1 Interstitial Fibrosis and Tubular Atrophy (IFTA)

A particular type of fibrotic disease is the interstitial fibrosis and tubular atrophy (IFTA) that often occurs after rejection of transplanted kidney. The origin of renal fibrosis can be inflammatory or immunological, obstructive, metabolic or systemic but, in any cases, the outcome is often a chronic kidney disease (CKD). In patients with CKD, the progression of disease is most closely correlated to IFTA, a fibrotic process associated with an extensive

accumulation of extracellular matrix (ECM) components in the cortical interstitium [26]. The extent of tubolointerstitial involvement is strongly correlated with the derioration of renal function; the tubolointerstitial fibrosis is a characteristic feature of chronic allograft nephropathy, which is the most common cause of kidney transplant failure [27]. In this context there are four main stages that candidates cellular participants as possible therapeutic targets: early inflammatory events with the involvement of different immune cells, like T-cell and macrophages (1), myofibroblasts activation and ECM deposition that generate interstitial scars (2), tubular epithelial cells loss their regenerative properties (3), loss of interstitial capillary integrity that compromise oxygen delivery and leads to a cascade of hypoxia-oxidant stress that worsened the fibrotic process (4) [28]. Several studies demonstrated that hypoxia in the tubulointerstitial area plays a central role in the progression of disease [29]. In particular, hypoxia increases gene expression of collagen and suppress activity of MMP2 in proximal tubular epithelial cells (PTEs) impairing ECM turnover and inducing fibrosis [30]. Moreover, decreased oxygen supply impact also in growth, activation and ECM metabolism of interstitial fibroblasts; in this cell type hypoxia promotes fibrogenic phenotype, increasing collagen production and decreasing turnover via TGFβ1-independent [31]. However, TGFβ has a crucial role in the progression of pathology: in fact TGF-b/SMAD pathway is a well known pro-fibrotic pathway and the interaction with hypoxia/HIF pathway need to be better studied[29, 32, 33] Macrophages are the major producers of TGFβ and are directly involved in myofibroblasts activation and ECM overproduction.

Indeed, during IFTA progression different cell types play a pivotal roles, also timing and environmental factors influence cell fate and disease outcome.

Next sections examine main cell players involved, starting from their biology.

5.2 Macrophages

Macrophages (Mφ) are phenotypically heterogeneous population of immune cells with a wide range of critical roles in homeostasis, surveillance, immune response, tissue injury and repair [34] [9]. They belong to the family of mononuclear phagocytes and can be distinguished into resident tissue macrophages (embryo-derived) and monocyte-derived macrophages (originated in bone marrow from a common myeloid progenitor) [9].

In homeostatic conditions, the maintenance of a resident pool of macrophages is balanced by local proliferation or recruitment and differentiation of blood monocytes [35].

The dynamic crosstalk between macrophages and their microenvironment is the key to understand the role of macrophages in healthy and diseased tissues and their behavior depends on both their origin and the stimuli they have previously encountered [36]. A commonly recognized classification, based on *in vitro* studies, divided them into two main subpopulations based on their distinct functions:

- Classically activated macrophages (M1): linked to Th1 responses and IFNγ production by antigen-activated immune cells and extended to cytotoxic and anti-tumoral properties; they produce a great amount and number of pro-inflammatory mediators.
- Alternative activated macrophages (M2): linked to Th2 response; they have anti-inflammatory properties and are involved in parasite containment, wound healing and fibrosis. Alternative activated macrophages can be subdivided into, at least, three subpopulations depending on type of stimulation and function.

M2 macrophages include also tumor-associated macrophages (TAMs) that however have a transcriptional profile that is quite distinct from those of M1 and M2 macrophages [37].

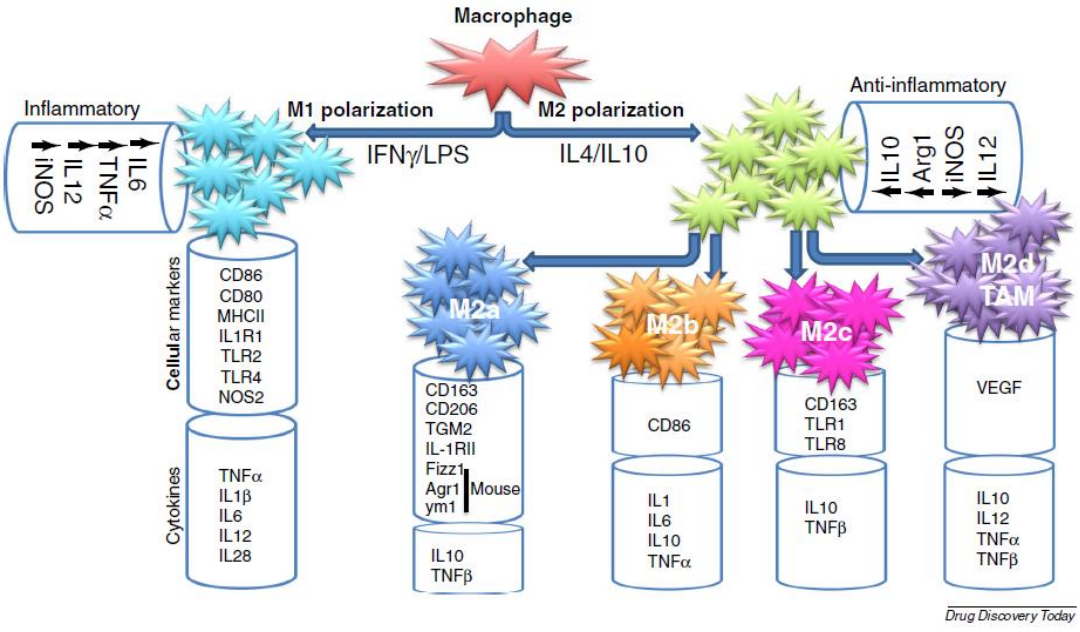


Fig. 3 Macrophage M1/M2 polarization. M1 polarized cells are induced by pro-inflammatory stimuli (interferon-gamma, IFNγ, lipopolysaccharide, LPS) and produce a pro-inflammatory, Th1 response; M2 polarized cells are induced by interleukin-4 (IL-4) and interleukin 10 (IL-10) with an anti-inflammatory, Th2 response; M2 are subdivided into four groups: M2a that show up-regulation of CD163, CD206, TGM2 molecules; M2b that upregulate CD86; M2c stimulated by interleukin-10 (IL-10) and glucocorticoids show up-regulation of CD163, TLR1 and TLR8; M2d or TAM that are associated to tumor development [38].

At molecular level, IFNγ is the main cytokine associated with M1 activation: through activation of its receptors, it can activate JAK-STAT1 (Janus kinase-signal transducers and activators of transcription) signaling and interferon regulatory factors (IRF), such as IRF-1 and IRF-8 [39]; LPS-activated macrophages are classified as M1 but the induction of M1-related genes transcription, in this case, is dependent on the autocrine production of IFNβ, which requires TRIF-dependent signaling from TLR4 (Toll-like receptor 4). TLR4 activates MyD88 and MaL/Tirap (Toll-interleukin 1 receptor domain containing adaptor protein)-dependent pathways that lead a strong pro-inflammatory cytokine and chemokine profile (IFN-β, IL-12, TNF, IL-6, CCL2, CXCL10, CXCL11). These profiles are controlled by nuclear factor of kappa light polypeptide gene enhancer (NF-κB), activator protein 1 (AP-1), IRFs and STAT1[40][37].

Through M2-promoting stimuli, IL-4 is the one that best reproduces Th2 secreted factors; it binds IL-4Rα that signals through JAK-STAT6 pathway [37]. STAT6 can act as a cofactor of PPARγ (peroxisome proliferator-activated receptor-γ) that is a master regulator of lipid metabolism in macrophages and has been known to inhibit pro-inflammatory response. Other transcription factors involved are c-Myc and IRF4. IL-4 stimulation induce transcription of transglutaminase 2 (TGM2), mannose receptor 1 (MRC1 or CD206), cholesterol hydroxylase CH25H and prostaglandin-endoperoxide synathase PTGS1, IRF4, Krüppel-like factor 4 (KLF4) and signaling modulators CISH and SOCS1[41]. IL-13 signatures are similar to IL-4 signatures but not completely overlapping [42]. Immune complexes activate M2b macrophages by inducing IL-10 secretion and activating Th2 response. Glucocorticoids (M2c) are recognized by glucocorticoid receptor (GPR) alpha, leading to nuclear translocation of the complex; into the nucleus the complex promotes or represses DNA transcription directly or by interaction with NF-κB or AP1. Targets activated by this type of stimulation include complement component 1 subunit A (C1QA), TSC22 domain family, member 3 (DSIPI), MRC1, thrombospondin 1 (THBS1), IL-10, IL1R2 and CD163 [43]. IL-10 stimulation induces M2c phenotype by the activation of STAT3; binding of ligand-receptor

complex mediates inhibition of pro-inflammatory cytokine expression, in fact it is a Th2 product and an important inhibitor of Th1 cells. Through factors activated by IL-10 are included: CXCL13, CXCL4, the recognition receptors formyl peptide receptor 1 (FPR1), TLR1, TLR8, and macrophage receptor with collagenous domain (MARCO) [40, 44].

However the dichotomous M1/M2 distinction is not representative of what takes place in an *in vivo* setting, as M1 and M2 stimuli do not exist alone in tissues. Instead, the macrophage population represents a continuum of phenotypes that stands between these two extremes[45]. Macrophages heterogeneity and plasticity are key points for their broad range of functions and explain why they are essential in different phases of initiation, repair, remodeling of wounds and in the transition between inflammatory and proliferative stages [34].
In case of acute injury, inflammatory cells, including neutrophils and monocytes, are recruited in the site of damage. This step induces a cascade of events including endothelial cell activation, cell-to-cell interactions and trans-migration into extra vascular space. Monocytes are recruited by factors released quickly after injury, such as products of coagulation cascade, factors from platelet degranulation and activated complements components. In addition pro-inflammatory cytokines, interferons, LPS or other microbial products, necrotic debris, fragments of extracellular matrix, induce monocyte activation into pro-inflammatory macrophages that contribute to the maintenance of inflammation producing themselves a large number of mediators and cytokines (IL-1, IL-6, IL-12, TNF, iNOS)[10]. When inflammation is exhausted (0-48h after injury) new tissue formation phase (2-10 days after injury) takes place with proliferation and migration of many cell types and angiogenesis [5]. In order to clear necrotic debris, macrophages acquire an anti-inflammatory, pro-resolving phenotype, switching from pro-inflammatory macrophages or differentiating from new recruited monocytes. Anti-inflammatory mediators, such as IL-1R, IL-10, and growth factors, such as TGFβ and VEGF, are released from macrophages and contribute to proliferative phase where fibroblasts and other cells promote extracellular matrix deposition, and to angiogenesis with new vessels creation. Contractile myofibroblasts contribute to wound repair in remodeling phase (starts 2-3 weeks after injury and can last several months) where many activated cells die by apoptosis or leave the site of injury and the tissue is repaired[5][10][34].

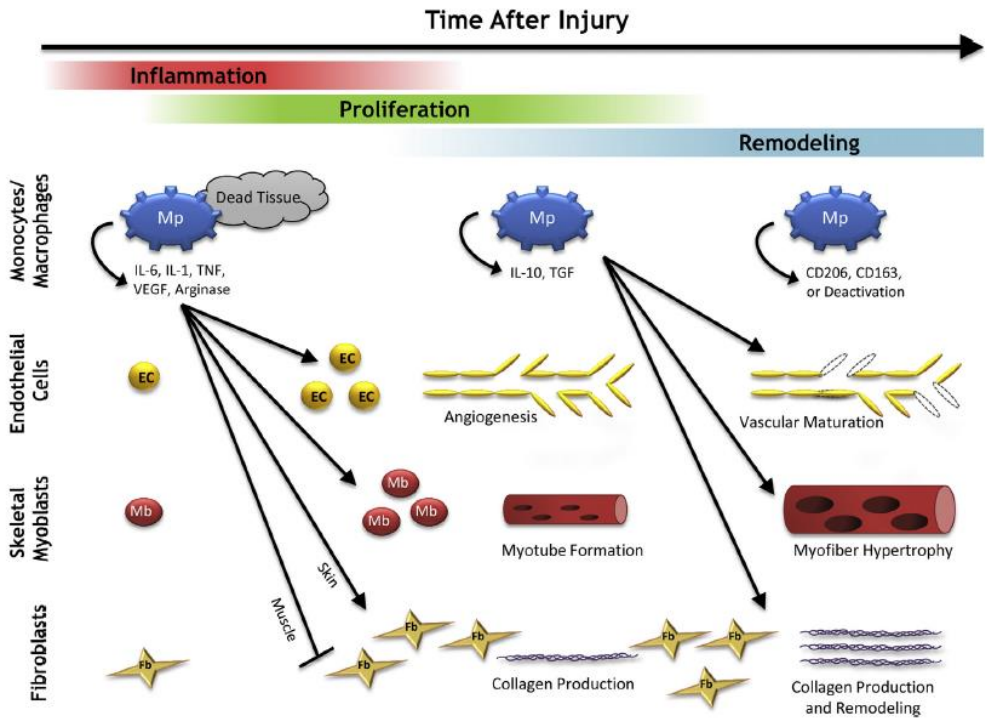


Fig. 4 Inflammation, proliferation and remodelling phases after tissue injury. Different macrophages role during tissue repair progression. Macrophages promote proliferation of endothelial cells and skeletal myoblasts by inducing vascular maturation and myofiber hypertrophy in the final stage of remodelling. Interactions with fibroblasts are tissue dependent and could promote collagen production and matrix remodelling [35].

Indeed, Mφ are active both in the initiation and resolution of inflammation. At different stages of healing, Mφ can promote debridement of the injury site, cell proliferation, angiogenesis, collagen deposition and matrix remodeling but improper regulation of these functions can impair the healing process [35].

As we previously mentioned, when wound healing process is dysregulated it could result into fibrosis. Macrophages are critical regulator of fibrotic process and play a pivotal role also in this context. They are found in close proximity to collagen-producing fibroblasts with which they interact directly or indirectly, by production of pro-fibrotic mediators, such as TGFβ1 and PDGF (platelet-derived growth factor). However, macrophages can promote fibrotic ongoing also independently from fibroblasts, by producing MMPs and TIMPs, which control ECM turnover, and secreting chemokines that recruit fibroblasts and other inflammatory cells[8].

5.2.1 MACROPHAGES IN IFTA

It is well established that macrophages play a key role in many kidney diseases and injury: they are involved in unilateral ureteral obstruction (UUO), in ischemia reperfusion injury (IRI), in Lupus nephritis, Adriamycin and Cisplatin nephrotoxicity and others [9, 46]. Macrophages are involved in acute and chronic rejection of kidney transplant; even if their contribution to T-cell mediated rejection (TMR) and antibody-mediated rejection (AMR) is commonly accepted, their role in IFTA is still under debate. Bergler et al. sustain that macrophages contribution in established IFTA is not relevant: in fact, they do not observe an increment of CD68 positive macrophage infiltration, cell proliferation and antigen presentation in comparison with acute rejection [47]. Other studies, instead, show that early macrophage infiltration in renal allograft biopsies is associate with following IFTA [9][48, 49]. *In vivo* studies of macrophages depletion by liposome clodronate attenuates kidney fibrosis; moreover, large number of double positive CD68 and CD206 macrophages are found in the active fibrotic areas of renal biopsies [49]. Macrophages switching from M1 to M2 phenotype characterizes the progression of chronic fibrosis, in fact M2 macrophages can lead to fibrotic progression in different ways; M2 macrophages are source of pro-fibrotic factors, such as TGFβ1, Fibroblast growth factor-2 (FGF2) and PDGF that promotes myofibroblasts proliferation, survival, and activation, leading to ECM overproduction. At the same time macrophages produce high level of cytokines and factors that enhance collagen production, epithelial-mesenchymal transition and local activation of pericytes [50].

However, M2 macrophages are also involved in the recovery phase of disease and depletion of macrophages at this point results in a chronic inflammation. Cao et al. report that macrophage-derived Wnt7b plays a critical role in promoting kidney regeneration via epithelial cell-cycle progression and basement membrane repair; chitinase-like protein BRP-39 promotes regeneration by limiting tubular apoptosis activating PI3K/Akt signalling pathway.

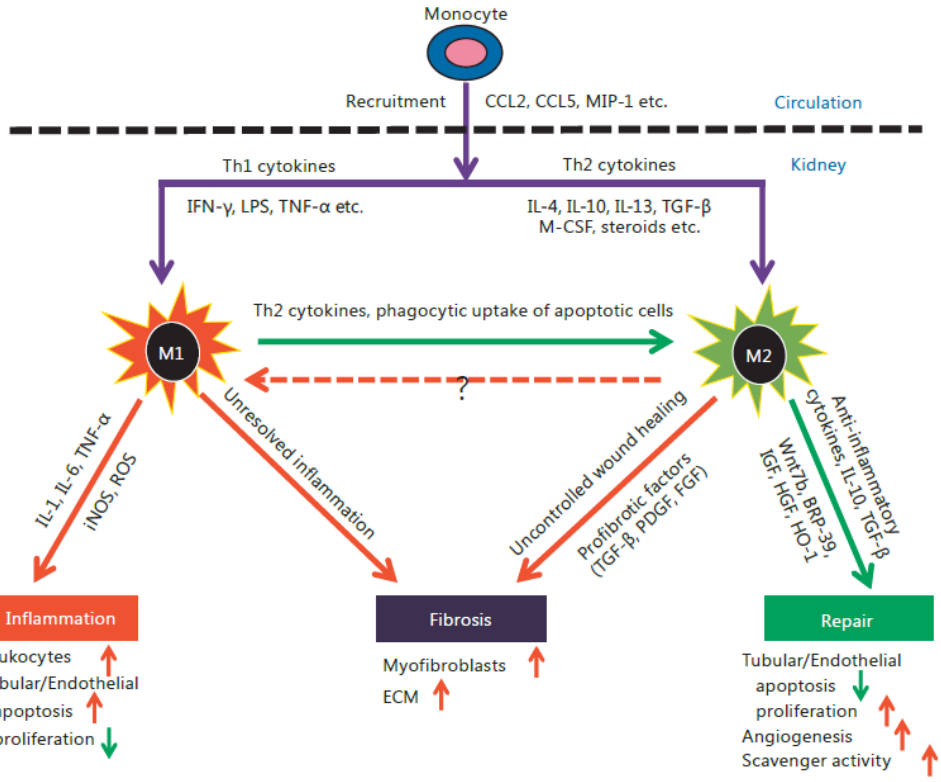


Fig. 5 Macrophage in kidney injury, fibrosis and repair. Macrophage phenotype in kidney after monocyte recruitment in site of injury. M1 macrophages are related to inflammation and, when inflammation become chronic, also to fibrosis; M1 can switch into M2 macrophage that are involved in resolution of inflammation. When macrophage switch is dysregulated it can promote fibrotic process **[50]**.

Macrophage involvement in pathological progression candidate them as good therapeutic target [50]. Many studies observe that infiltrating macrophages deletion or genetically altering of macrophage phenotype could attenuate renal diseases. Blocking M-CSF (macrophage colony stimulating factor) reduces proliferation and number of infiltrating macrophages. Alternatively, macrophages can be modulated into a protective way in order to inhibit kidney injury but it is still unknown the way to make macrophage fibrolytic in order to reduce fibrosis [9]. Furthermore, it is interesting that IL-10/TGFβ- or IL-4/IL-13-modified bone marrow-derived macrophages fail to protect against renal injury, because the anti-inflammatory phenotype of bone marrow-differentiated M2 cells is easily lost, due to the capacity of continuous proliferation, which minimizes the clinical application of autologous macrophage-based therapy by modifying the bone marrow cells of patients [50].

5.3 Fibroblasts

Fibroblasts are another cell type essential for tissue homeostasis, they are able to regulate structure and functions of healthy tissues, participate transiently in tissue repair after injury and acute inflammation [51].

Fibroblasts are elongated cells with mesodermal origin, showing fusiform or spindle-like shape, with a broad range of surface markers tissue-dependent; this complex expression pattern of surface proteins is due to the heterogeneity of this cell population that is ubiquitous in tissues and organs throughout the body. However, quiescent fibroblasts express fibroblast specific protein (FSP-1), a member of S100 family, functions as cytoplasmic calcium binding protein that can interact with cytoskeleton. Then, other markers generally attributed to fibroblast include: Thy-1 (or CD90) a glycosylphosphatidilinositol (GPI)-anchored protein involved in focal adhesion, stress fiber formation and multiple signalling pathways, it is not only expressed by fibroblasts but also by endothelial cells, neurons and hematopoietic cells; DDR2, discoid domain receptor 2, is a receptor tyrosine kinase that uses triple helix collagen I and III as a ligand, it is expressed also by other mesenchymal cells; Vimentin, is an intermediate filament protein that is expressed also in the cells with mesenchymal origin such as smooth muscle cells and bone. The various cell phenotype is dependent on anatomical site of isolation and their degree of activation. They are, specifically, involved in generation of ECM components (fibrillar collagens, fibronectins, hyaluronic acid and proteoglycans) and are essential for maintenance of normal tissue architecture [26].

Fibroblasts are also highly involved in tissue remodelling and repair: in response to increasing tension of ECM, fibroblasts proliferate and differentiate into proto-myofibroblasts that are characterized by increased expression of fibronectin and the expression of the alternately spliced ED-A isoform which is not expressed in quiescent fibroblasts. As result of mechanical tension, focal adhesion, at the end of stress fibres, evolve to larger mature focal adhesions containing alpha smooth muscle actin (α-SMA); at this point, proto-myofibroblasts differentiate into α-SMA positive myofibroblasts with contractile properties. Another marker highly expressed by myofibroblasts is FAP (fibroblast activated protein) that, unlike α-SMA, is not involved in matrix production but it is an enzyme with both dipeptidyl peptidase and endopeptidase activity, which overexpression occurs in wound healing, arthritis, atherosclerosis and tumor metastasis [52].

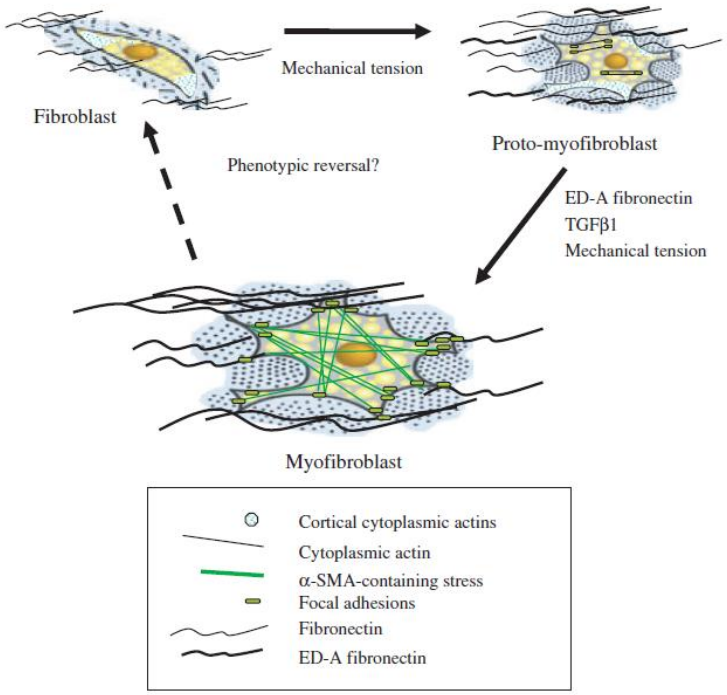


Fig. 6 Fibroblast activation in stress condition. Increased mechanical tension induces the acquisition of proto-myofibroblast phenotype with higher expression of ED-A fibronectin, TGFβ1; prolonged mechanical stress induces further differentiation into myofibroblasts characterized by the expression of α-SMA [26].

In addition to resident fibroblasts, myofibroblasts can originate from epithelial cells through epithelial-mesenchymal-transition (EMT) or by endothelial-mesenchymal transition (EndMT). Reilkoff et al. identify also a unique fibroblast-like cell derived from monocytes that have both features of macrophages and fibroblasts, called fibrocytes [53]. Finally, in some tissues, resident fibroblasts are not the only source of myofibroblasts: for example, in liver fibrosis the resident hepatic stellate cells (HSC) seems to be the major source of myofibroblasts [7].

Activated fibroblasts, upon completion of wound healing, undergo apoptosis or a particular type of programmed cell death termed nemesis (programmed necrosis). In some cases, however they persist and are associated to excessive ECM deposition, leading loss of tissue architecture, aberrant or pathological wound healing and the development of scaring [26]. In this context is important to understand the immune system influence on fibroblast activity.

Chemokines cooperate with pro-fibrotic cytokines to the development of fibrosis by recruiting myofibroblasts, macrophages and other key effector cells. Specifically, CCL3 (macrophage inflammatory protein 1α) and CC-chemokines such as CCL2 (monocyte chemoattractant

protein-1), which are chemotactic for mononuclear phagocytes, are identified as pro-fibrotic mediators. Macrophages and epithelial cells are believed to be the key sources of CCL3 [7].

As we reported before, Th1 and Th2 response are involved in the regulation of inflammation, resolution and fibrosis. Through Th2 cytokines a particular relevance is attributed to IL-4/IL-13 axis in tissue repair and fibrosis [54]. IL-4 and IL-13 elicit many similar biological responses, since they share a common receptor chain, IL-4receptor alpha (IL-4Rα), and the Janus kinase/signal transducer and activator of transcription protein 6 (JAK/STAT6) signaling pathway [37]. Different studies report that in wound healing, IL-4and IL-13 promote fibroblast chemotaxis and proliferation, myofibroblast differentiation, and production of collagen and ECM macromolecules [55]. Persistent activation of IL-4 andIL-13 signaling leads to abnormal collagen homeostasis and exert a pro-fibrotic effect mediated in great extent by TGFβ, a critical regulator of all fibrotic processes [56, 57]. IL-4 and IL-13may act on macrophages inducing an M2 phenotype that can produce TGFβ, PDGF and, through arginase upregulation, modulate polyamine and proline biosynthesis, cell growth, and collagen formation. Prasse et al. have demonstrated that M2 macrophages isolated and cultured from the bronchoalveolar lavage of idiopathic pulmonary fibrosis patients, with culture supernatants from these M2 macrophages significantly increasing collagen production by normal human fibroblastsin a CCL18-dependent manner [58]. Indeed IL-4 and IL-13 can trigger fibrosis by directly activating TGFβ production or stimulating pathways promote TGFβ signaling [57].

The canonical SMAD-signaling pathway plays a key role in controlling TGFβ-induced fibrosis, with downstream targets including connective tissue growth factor (CTGF), α-SMA, collagens, MMPs and TIMPs [54]. The pro-fibrotic effects of TGFβ include the stimulation of fibroblast chemotaxis, differentiation, proliferation, and ECM synthesis and deposition.

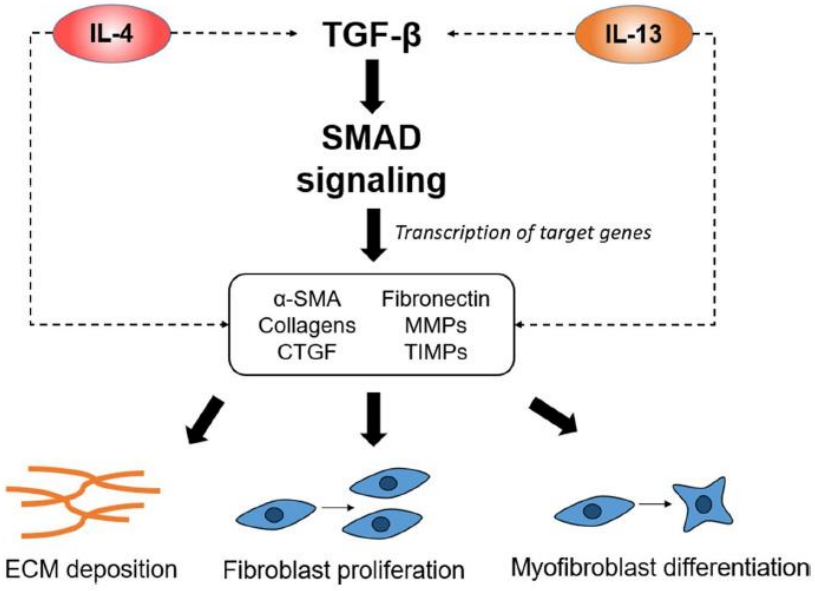


Fig. 7 Pro-fibrotic function of TGFβ. IL-4 and IL-13 cytokines activate TGFβ, which induces transcription of target genes involved in ECM deposition, fibroblast proliferation and myofibroblast differentiation, through SMAD signalling [54].

5.3.1 FIBROBLASTS IN IFTA

In kidney, fibroblasts could be divided in two groups depending on their localization:

- renal cortex fibroblasts: positively stained for vimentin, negative for the smooth muscle marker desmin and weakly positive for α-SMA [59, 60]. In normal renal cortex they are few.
- interstitial fibroblasts: they have an endocrine role since that are a source of erythropoietin (EPO) [61]. Regulation of EPO production by the kidneys is central to the control of erythropoiesis, and EPO controls erythropoiesis by regulating the survival, proliferation and differentiation of erythroid progenitor cells. Thus, the presence of normal interstitial fibroblasts is essential for homoeostasis.

In chronic progressive disease, interstitial fibroblasts assume a myofibroblast phenotype and become the major players in the formation of collagen-enriched ECM that fills the interstitium leading nephron loss and declining kidney function [26]. However, interstitial fibroblasts are not the unique source of myofibroblasts: the origin of renal myofibroblast has been at centre of debate along years. Some studies have disputed the contribution of the EMT in the emergence of myofibroblasts and fibrosis, whereas others favor the idea that vascular pericytes serve as precursors of myofibroblasts in fibrosis through EndMT. Other works suggest that myofibroblasts can originate also from bone marrow[62].

Mechanical tension contribute to fibroblasts activation by inducing them to produce collagen, laminin and fibronectin. Numerous growth factors, cytokines and hormones are involved in these differentiation processes.These include TGFβ, fibroblast growth factor (FGF), platelet-derivedgrowth factor (PDGF), interleukin-1 (IL-1), tumor necrosis factor(TNF)-a, angiotensin II and aldosterone.

The contribution of EMT to the formation of myofibroblasts in renal fibrosis is still unclear; in chronic allograft failure, were reported the loss of epithelial markers (E-cadherin, cytokeratin and zonulaoccludens 1), *de novo* expression of mesenchymal markers (vimentin, FSP-1 and α-SMA) and a collagen synthesis marker (heat shock protein 47) [27].

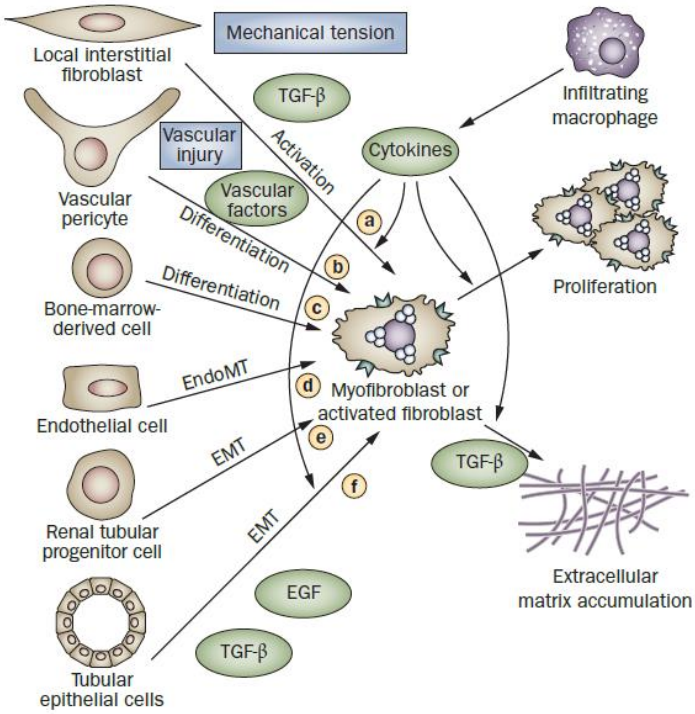


Fig. 8 Origins of myofibroblasts in kidney. a) Local interstitial fibroblasts activated by mechanical tension, cytokines and growth factors (such as TGFβ) that could be produced by infiltrating macrophages; b) vascular pericytes can tran-differentiate into myofibroblasts supported by vascular factors; myofibroblasts can originate from c) bone-marrow-derived cells, d) endothelial cells, e) renal tubular progenitor cell, f) tubular epithelial cells. Myofibroblasts induce ECM accumulation through growth factor secretion **[27].**

Therefore, given the pivotal role of myofibroblast in interstitial fibrosis, they become an interesting candidate target for therapeutic strategies. Since that EMT is a critical source of myofibroblasts some therapeutic strategies are aimed to inhibit kinases involved in this process. For instance, studies have reported that inhibition of extracellular signal-regulated

kinases 1 and2 or of the phosphatidylinositol 3 kinase–akt pathway decreased renal fibrosis and reduced expression of EMT markers in mice. Kinases recognize specific substrates through subtle differences in their catalytic structures. These differences allow the development of relatively selective inhibitors [27].

Bone morphogenetic protein 7 (BMP-7), a member of the TGFβ family, reverses renal fibrosis and myofibroblast accumulation in mice with progressive chronic renal injury and prevents TGFβ-induced EMT in mice with nephrotoxic serum nephritis [63]; in addition, it induces E-cadherin expression and decreases cell motility in cultures of adult renal fibroblasts. Hence, re-establishing a balance of pro-fibrotic and anti-fibrotic factors could be useful to design anti-fibrotic therapeutic strategies.

5.4 Hypoxia

Oxygen homeostasis represents a basic principle for human development and physiology [64]. Reduction of the normal oxygen concentrations causes metabolic alterations. In particular, molecular responses to hypoxia have been elucidated during the past several years and it is well recognized that many downstream effects of hypoxia are mediated by stabilization of the transcription factor hypoxia inducible factor 1α (HIF-1α) [65]. In normoxic conditions, hydroxylation and acetylation of the oxygen-dependent domain of HIF-1α promote a rapid degradation in proteasomes. With decreasing levels of oxygen, hydroxylation and acetylation of HIF-1α do not occur and the HIF-1α protein is stabilized. After translocation into the nucleus, HIF-1α binds with its dimerization partner HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT) to defined hypoxia-responsive elements (HREs) in regulatory regions of target genes, such as vascular endothelial growth factor (VEGF), and increases their transcription. While HIF-1α is an important mediator of hypoxia signaling, HIF-1α–independent mechanisms, such as messenger RNA (mRNA) stabilization and increased transcription by other HIF family members, also contribute to the cellular responses to hypoxia [65]. Low oxygen levels induce activation of various transcription factors such as hepatocyte nuclear factor 4 (HNF-4), nuclear factor-interleukin-6 (NF-IL-6), nuclear factor-κB (NF-κB) and members of the *fos* and *jun* (AP-1) families.

Indeed tissue hypoxia lead to cellular dysfunction and eventually cell death.

Macrophages accumulate in large number in hypoxic/ischemic tissues and respond to hypoxia by up-regulating different transcriptional factors. Macrophages must adjust their metabolic requirements to generate energy in an oxygen independent fashion. Numerous studies have demonstrated that pro-inflammatory macrophages are highly dependent on glycolysis and that anti-inflammatory and pro-homeostatic macrophages show a stronger preference for mitochondrial OXPHOS to generate ATP [66]. Hypoxia is a master regulator of glycolysis, since oxygen deficit results in limited OXPHOS and cells must rely on glycolysis to generate ATP. HIF1α is fundamental to this process, inducing the expression of glycolytic enzymes such as hexokinase II (HKII) [67], phosphofructokinase (PFKFB3) [68] and glucose transporters such as GLUT1 [69]. All these metabolic adaptations allow pro-inflammatory macrophages to develop their functions in the inflamed tissues.

The induction of HIF-1α in the context of inflammation was soon discovered to depend on the presence of NF-κB [70]. HIF-1α is also recruited to the CXCR4 promoter, which mediates the chemotactic responses to its ligand CXCL12, which is expressed in hypoxic environments [71].

On the contrary, M2 macrophages do not need this rapid switch to glycolysis and obtain much of their energy from fatty acid oxidation and oxidative metabolism, which can be sustained for longer periods. This is consistent with their functional roles, as they appear later in the inflammatory response during the resolution phase and fulfill longer-term functions such as angiogenesis and extracellular matrix remodeling. Collectively, these findings demonstrate that metabolic adaptation is central to the polarization and functional activity of macrophages during hypoxia.

Hypoxia exerts its effect also on fibroblasts mainly in a TGFβ- dependent manner: TGFβ/SMAD signaling pathway, in fact interact with HIFs, inducing fibroblasts proliferation and activation into myofibroblasts. Moreover, hypoxia can induce a fibrogenic phenotype, increasing production of collagens and decreasing turnover via TGβ-independent mechanisms that involve a heme protein oxygen sensor and activation of PKC- and TK- mediated pathways. Norman et al. have also demonstrated that fibroblasts increase the expression of TIMP-1 in response to HIF-1 activation in hypoxia [31].

5.4.1 HYPOXIA IN IFTA

Hypoxia is a well-known factor that promote kidney injury. As a result of microvascular compromise, hypoxia can promote the pathogenesis of fibrosis interacting with another key player: TGFβ. TGFβ/SMAD pathway plays a crucial role in the progression of kidney injury and its interaction with hypoxia/HIF pathway may contribute to the abnormal accumulation of ECM components, such as collagens [29]. Moreover, it is known that interstitial fibroblasts, the major ECM producing cells, have a subpopulation that produces erythropoietin (EPO) and thus possess an O_2-sensing mechanism. Norman et al. showed that hypoxia simultaneously stimulated ECM synthesis and suppressed turnover through activation of interstitial fibroblasts. Hypoxia promotes a fibrogenic phenotype, increasing production of interstitial collagens and decreasing turnover via a TGFβ-independent mechanism that involved the interstitial fibroblasts EPO-producing subpopulation. In addition, hypoxia promotes the transcription of TIMP-1 a matrix metalloproteases inhibitor strictly related to hypoxia [31].

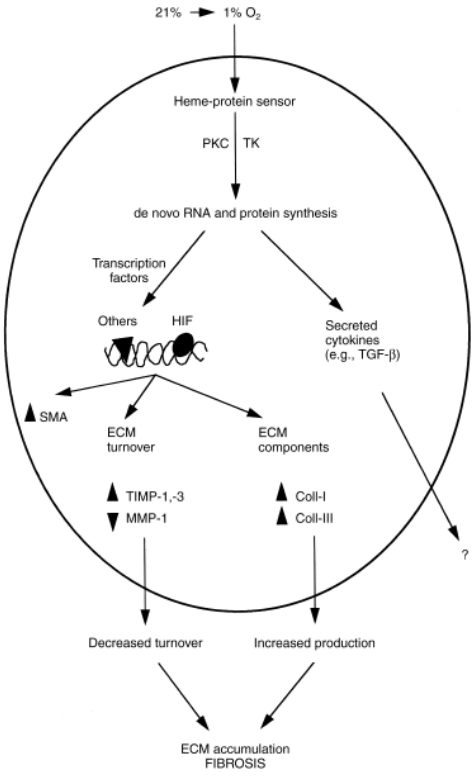


Fig. 9 Schematic representation of fibrosis induction by hypoxia. Hypoxia acts on EPO, producing interstitial fibroblast with O2-sensing mechanism. Hpoxia/HIF pathway activation induces ECM production through up-regulation of Coll-I and Coll-III and a decrease of ECM turnover through TIMP-1 and -3 up-regulation and MMP-1 downregulation [31].

5.5 Cellular circuits

Homeostasis maintenance, inflammation, wound-healing and fibrosis are orchestrated by many different cell types that interacts to each other (in direct or indirect ways) and are involved in the generation of different environments that could promote progression or resolution of inflammation. How mammalian tissues are able to maintain homeostasis and how pathological deviations in cell composition are sustained remain poorly understood.

One way in which tissues control homeostasis is by regulating growth factors availability. Growth factors are involved in cell survival and proliferation; cells can produce growth factor for themselves (autocrine signal) or for the neighbors (paracrine signal). Growth factors exchange through cells creates cell circuit. In addition to growth factor production, tissue composition can be regulated also by other extrinsic factors such as oxygen, nutrients and space availability [2].

In order to understand how single process and factor can lead to different outcome, it is important to define the circuit of cell-cell interactions.

6 AIM OF THE WORK

By this study we want to deeper understand macrophage-fibroblast interplay under different type of perturbations that take part to fibrotic disorders.
Specifically we have two main objectives:

- Immunological aim: to understand the effect of Th1-Th2 cytokines on macrophage-fibroblast crosstalk
- Metabolic aim: to understand the effect of hypoxia on macrophage-fibroblast crosstalk

7 MATERIALS AND METHODS

7.1 Human Mφ isolation and differentiation

Peripheral blood monocytes were isolated from healthy donors by different density gradient centrifugations. Lympholyte-H (Cederlane, USA) gradient was used to separate peripheral blood mononuclear cells (PBMCs) from granulocytes and red blood cells; then, a Percoll 46% gradient (Lonza, USA) was applied to distinguish monocytes from lymphocytes. Monocytes were counted and stained with anti-human CD14 (BD Horizon) and CD16 (Biolegend) to assess by flow cytometry (fluorescence activating cell sorting, FACS) the purity of separated cells (lymphocyte contamination <30%). By this staining is possible to distinguish classical monocytes (CD14+/CD16-), intermediate (CD14+/CD16+) and non classical monocytes (CD14-/CD16+).

Monocytes were then plated at determinate concentration in RPMI 1640 (Lonza, USA) 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Penicillin/Streptomicyn (P/S) and were stimulated with 100ng/ml of human M-CSF (macrophage stimulating factor; Miltenyi, 100ug/ml) for seven days. At the end of differentiation macrophages (Mφ) were stained again with anti-human CD14 and CD16 to check the purity (CD14+/CD16+ >90%).

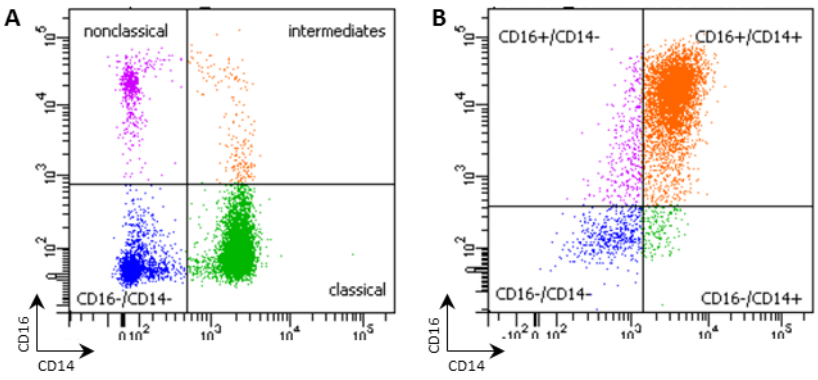


Fig. 10 FACS staining of monocytes and macrophages. The same staining (CD14/CD16) is used to identify monocytes (A) and macrophages after 7 days of differentiation (B).

7.2 Fibroblast culture

Human dermal BJ fibroblast cell line (ATCC® CRL-2522™) were cultivated in DMEM high-glucose (LONZA) 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Penicillin/Streptomicyn (P/S). Whatever this cell line is a long living line, fibroblasts used for this study are in early passages.

7.3 Polarizing stimuli and hypoxia induction

After seven days of differentiation, medium was changed and macrophages were polarized toward pro-inflammatory M1 phenotype by incubation with LPS (E.coli 055:85, 100ng/ml, Sigma) and IFNγ (20ng/ml, R&D) or into alternative M2 phenotype with IL-4 (20ng/ml, Miltenyi) for 4h or 24h. Resting macrophage, M0, were left unstimulated for the same period of time. The same type of treatment is applied to BJ fibroblasts.
Cells that were cultivated in normoxic condition were maintained at 37°C in humidified incubator at 20% O_2, 5% CO_2 in air; hypoxic treatment was performed placing cells in a different incubator (Thermo Fisher Heto) at 37°C with a mixture of 1% O_2, 5% CO_2 and 94% nitrogen.
Stimulatory treatments and hypoxia are performed simultaneously.

7.4 RNA isolation and qRT-PCR

After treatment cells were lysed with TRIzol reagent (Ambion) and RNA was extracted using DirectZOL RNA Miniprep kit (ZymoResearch) according to the manufacturer's instruction. Total mRNA amount was quantified by NanoDrop 2000c (Thermo Scientific™) and retro-transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using TaqMan Fast Advanced Master Mix 2X (Applied Biosystems) and specific Taqman probes (Thermo Fisher) reported in the following table:

Gene	probe ID	Gene	probe ID
Bnip3	Hs00969291_m1	*Tnf*	Hs00174128_m1
Cxcr4	Hs00607978_s1	*Acta2*	Hs00909449_m1
Slc2a1 (Glut1)	Hs00892681_m1	*Col1a2*	Hs01028956_m1
Vegfa	Hs00900055_m1	*Ctgf*	Hs00170014_m1
Alox15	Hs00993765_g1	*Fap*	Hs00990791_m1
Ccl5	Hs00174575_m1	*Thy1*	Hs00174816_m1
Ccl17	Hs00171074_m1	*Vim*	Hs00958111_m1
Mrc1 (Cd206)	Hs00267207_m1	*Hprt*	Hs02800695_m1

Table 1. List of TaqMan probes with gene name and ID code.

Reactions were performed on a VIIA-7 Real-Time PCR Detection System (applied Biosystems). The thermal cycling conditions were standard fast-cycling; relative expression values were calculated using ΔΔCT method normalized on *Hprt* (Hypoxanthine Phosphoribosyltransferase) as housekeeping.

7.5 Statistical analysis

Statistical analysis was performed using Prism version 7.0 (GraphPad software). Comparisons were calculated by two-way ANOVA test applying Sidak's multiple comparisons correction. The level of statistically significant difference was defined as $p \leq 0.05$.

7.6 Co-culture and FACS-sorting

For co-culture experiment, differentiated macrophages were replated directly onto adherent fibroblasts (plated 16h before), with the fibroblast:macrophage ratio at 1:2, respectively. After 24h of co-culture in basal condition (normoxia without stimuli) co-culture were stimulated (LPS+IFNγ or IL-4) and put in hypoxic incubator for 24h or 4h. Then, cells were detached and single cell suspension is prepared for FACS-sorting. Zombie Aqua Fixable Viability kit (Biolegend) was used to exclude dead cells. Next, cells were stained with anti-human CD45

(BD) in order to distinguish macrophages (CD45+) from fibroblasts (CD45+). FACS-sorting was performed on a FACSAria III cell sorter (BD Bioscence) using FACSDiva software.

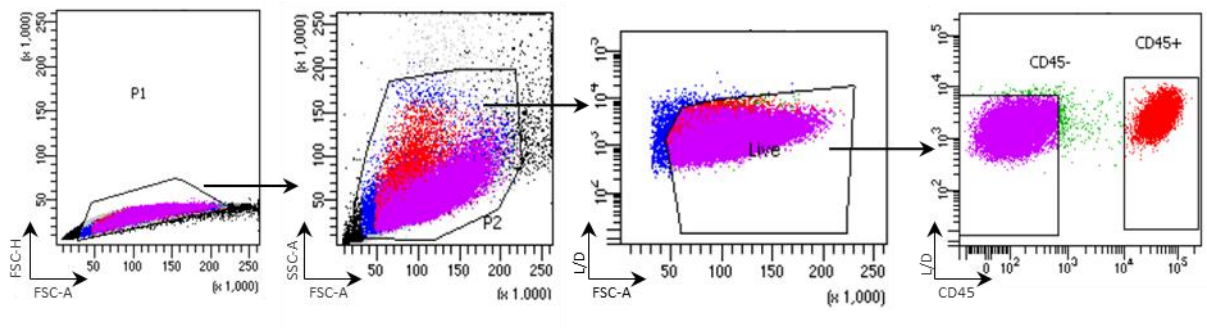


Fig. 11 Gating strategy applied to macrophages and fibroblast FACS-sorting. Co-cultivated cells were stained to identify live cells and to discriminate macrophages from fibroblast by using CD45 antibody. Gating strategy applied excludes doublets (FSC-A/FSC-H), identifies cells through physical parameters (FSC-A/SSC-A) and viability staining (FSC-A/L/D). Last dot-plot is generated on live cells and discriminate CD45- cells (fibroblasts) and CD45+ cells (macrophages).

7.7 RNA sequencing

FACS sorted samples were collected and cells were lysed by TRIzol reagent. Total RNA was isolated using DirectZOL RNA Miniprep kit (ZymoResearch) according to the manufacturer's instruction. Quantification and quality check (RNA integrity number RIN>7) were assessed by using Qubit4 (Invitrogen) instrument. Libraries preparation and processing were performed with Lexogen protocol: by using QuantSeq 3' mRNA-Seq Library Prep Kit we generated Illumina compatible libraries of sequences close to the 3' end of poly(A) RNA. Finally, NextSeq 500 System from Illumina was used to perform sequencing, producing an average of 5M reads per sample (single-end, 75 bp).

7.8 Bioinformatic analysis

Reads from RNA-sequencing were subjected to quality check and trimming using the FastqQC and BBduk tools and to alignment using the STAR method. The Phread quality score was high (more than 20) and the percentage of alignment along the reference genome

was higher than 80% along all the samples. Reads were aligned along genes using the HTseq count tool and subjected to differential expression analysis using the EdgeR Bioconductor package.

Unsupervised analysis

Firstly, data were analyzed by unsupervised analysis: dimensional reduction and correlation analysis. Three different algorithms were applied: PCA (Principal Component Analysis), t-SNE (t-distributed Stochastic Neighbor Embedding) and UMAP (Uniform Manifold Approximation and Projection). These are useful methods for dimensional reduction analysis (most used at the moment); they are not clustering algorithm but they can be used to visualize clustering. For this reason, dots were colored considering cell type and cell polarization.

The PCA is a deterministic linear statistical procedure that converts original variables, by a linear transformation, in a new set of data. Here, samples are the variables and gene expression values are the observations (the characteristics that describe the variables). The original matrix and the PCA-transformed matrix have the same dimensions. To visualize the results, only PC1 and PC2 were considered and plotted in a Cartesian coordinate system (PC1, PC2, …, PCn are ordered by variance; e.g. PC1 is characterized by the highest variance); PC1 is reported on the *x* axis and PC2 on *y* axis. Graph quality (and validity) is related to the variance associated with each principal component represented (sum of variance of PCs considered).

t-SNE and UMAP are nonlinear stochastic algorithms for dimensionality reduction, useful to visualize in a low-dimensional space (two or three dimensions) very high-dimensional dataset. t-SNE better preserves local structure, while UMAP better represents biological distances. Unlike the previous case, in which all principal components were calculated and only 2 were selected, here is fixed a priori the number of components (specifically n = 2, since the results were represented in a Cartesian plane) and only these are calculated. Seed was fixed at the beginning of the analysis (seed = 42).

After that, considering gene expression matrix, samples' correlation was calculated (Pearson correlation); only genes most expressed were taken into account for this analysis (gene expression mean > 50). Macrophages and fibroblasts were considered separately; correlation matrixes were plotted in two heatmaps.

Supervised analysis: differential gene expression analysis

Differential gene expression analysis was assessed on TMM normalized data by EdgeR (v3.24.3) and was performed in paired. For each single comparison, a design matrix was defined based on the experimental design. Significant differential expression in each gene was tested using the QL F-test; were selected only genes with a False Discovery Rate (FDR, p-value adjusted considering Benjamini-Hochberg correction) < 0.05.

For each comparison, genes were plotted in a *volcano plot* (significance, y axis, versus fold change, x axis); significance and fold change were reported in log scale (log 10 and log 2, respectively).Gene expression values of selected genes (FDR<0.05) were plotted in *heatmaps*, one for each comparison. Values are scaled by row (considering mean and standard deviation value for each gene) and each column represents a replicate.

Pathway analysis were performed on IPA software (*Ingenuity Pathway Analysis*, v01-13). Lists of differentially expressed genes for each comparison and their logFC values were used for identification of significantly enriched pathways. Only pathways with |z-score| > 2 and $\log_{10}p > 1.3$ were selected.

For higher level analysis, heatmaps and Venn diagrams were realized to visualize the results.

The 4-columns heatmaps (second level analysis) were generated on the total of significantly differentially expressed genes with |logFC|≥1 that belong to the double comparison minus genes shared between the two comparisons.

Let **A** be the list of genes differentially expressed in the first comparison (FDR<0.05 and |logFC|>1) and **B** be the list of genes differentially expressed in the second comparison (FDR<0.05 and |logFC|>1):

$$\mathbf{A \cup B \setminus A \cap B}$$

The 8-columns heatmaps (third level analysis) were generated on the total of significantly differentially expressed genes with |logFC|≥1 that belong to the two double comparison minus genes shared between the two pair of comparisons.

$$\mathbf{(A \cup B \setminus A \cap B) \cup (C \cup D \setminus C \cap D)}$$

In both cases of 4 and 8 columns heatmaps, values reported are the means of three replicates and are scaled by row.

For *Venn diagrams* only genes characterized by FDR<0.05 and |logFC|≥1 were taken into account.

7.9 Set up of hypoxia impact on macrophages and fibroblasts

The first step of the study is aimed to assess the impact of hypoxia on macrophages and fibroblasts alone. In order to obtain an indicative information about genes that respond to hypoxia in these two cell types, which we will investigate also in the co-culture system, we set up a series of experiments with the following experimental design:

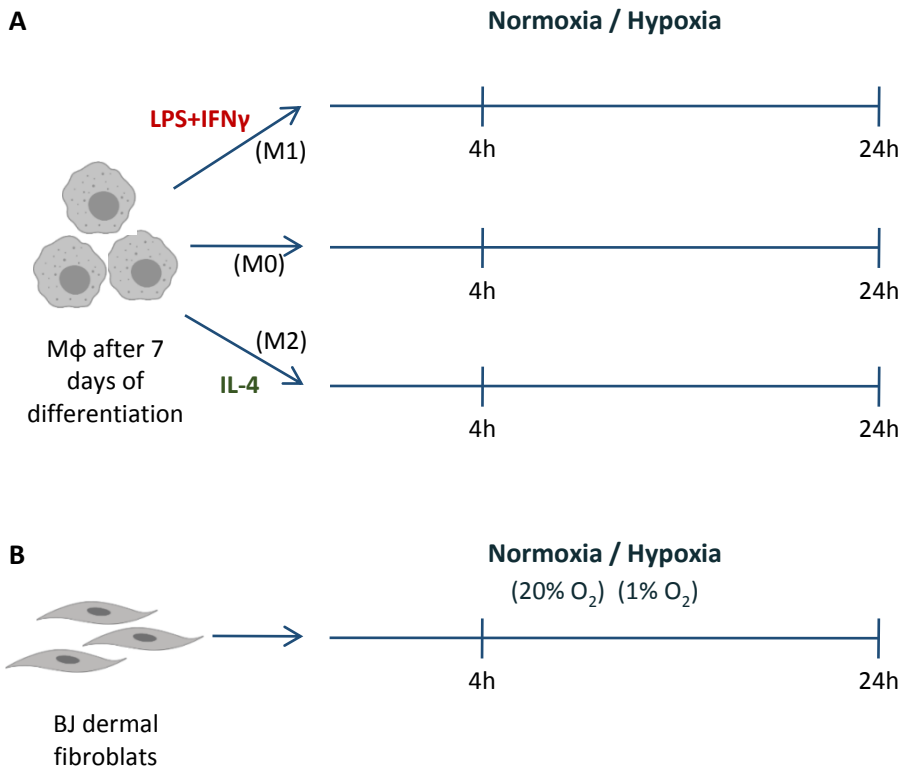


Fig. 12 Schematic representation of hypoxia induction. Macrophages are stimulated with LPS+IFNγ to obtain M1 cells, or with IL-4 to obtain M2 cells, or left un-stimulated (M0); at the same time, they were put under hypoxia (1%O_2) for 4h or 24h (A); BJ fibroblasts are stimulated by hypoxia only, for the same range of time used for macrophages (B).

7.9.1 MACROPHAGES RESPOND TO HYPOXIA INDUCING SPECIFIC TRANSCRIPTS

Since that in literature is known that different genes in different cell types are better responders to hypoxia [71-73][74][75], we decided to select some of these genes and to analyze their expression at mRNA level in our cells. In particular, we want to see how macrophage polarization could affect the response to hypoxia of these genes. Candidate genes

selected are: GLUT-1 (Glucose Transporter 1), VEGFA (Vascular Endothelial Growth Factor A), CXCR4 (Chemokine Receptor 4). All of these genes are involved in different cell functions (metabolism, angiogenesis, migration) that could be affected by oxygen deprivation. In fact, we observed a different regulation in resting macrophages with a general increase of expression of these genes under hypoxia both at 4h and at 24h. Instead, when macrophages are stimulated with LPS+IFNγ, to promote a pro-inflammatory phenotype, GLUT-1 and CXCR4 remain up-regulated under hypoxia while VEGFA responds better to the stimuli in a normoxic environment. Alternative macrophages (IL-4 stimulated) show a behavior much similar to resting macrophages.

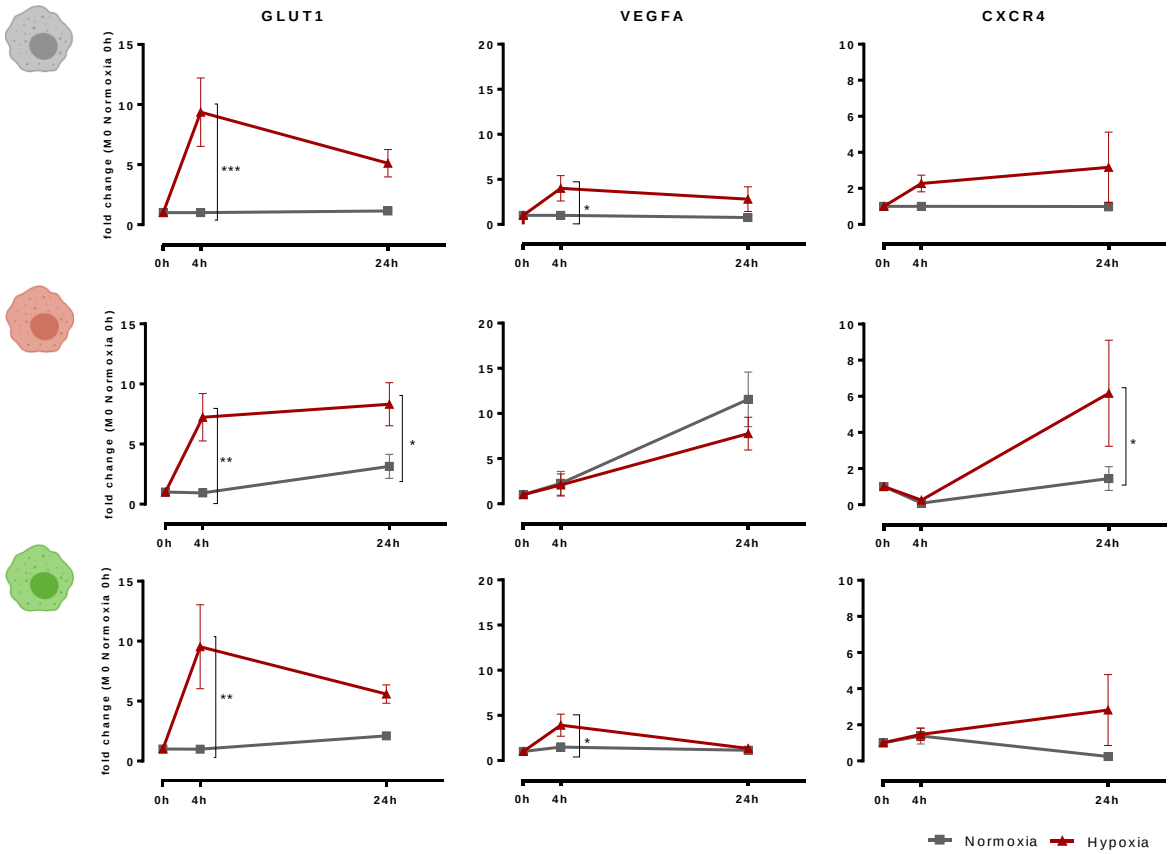


Fig. 13 Level of expression of hypoxia-responsive genes (GLUT-1, VEGFA, CXCR4) in different resting and activated macrophages. The first row is relative to M0 resting Mφ (gray), the second to M1 activated Mφ (red) and the third to M2 activated Mφ (green). For each plot is represented the fold change of mRNA expression on the M0 in normoxia at 0h; red line indicates the level of transcripts of samples under hypoxia, the gray line indicates the level of transcripts of samples in normoxia. (N=5, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

7.9.2 HYPOXIA DOWN-REGULATES OR DOES NOT ALTER THE EXPRESSION OF TYPICAL M1/M2 RELATED GENES

Macrophages respond to hypoxia by inducing specific genes, but what happen to genes related to Mφ polarization?

In order to answer to this question, we performed a qRT-PCR on some genes that are known to be involved into the promotion of pro-inflammatory (TNF, CCL5, CD80) or alternative (ALOX15, CCL17, CD206) macrophage phenotype and what we found are two possible effects: hypoxia 1) does not interfere with the expression of polarizing genes or 2) down-regulates mRNA level of expression of some polarizing genes.

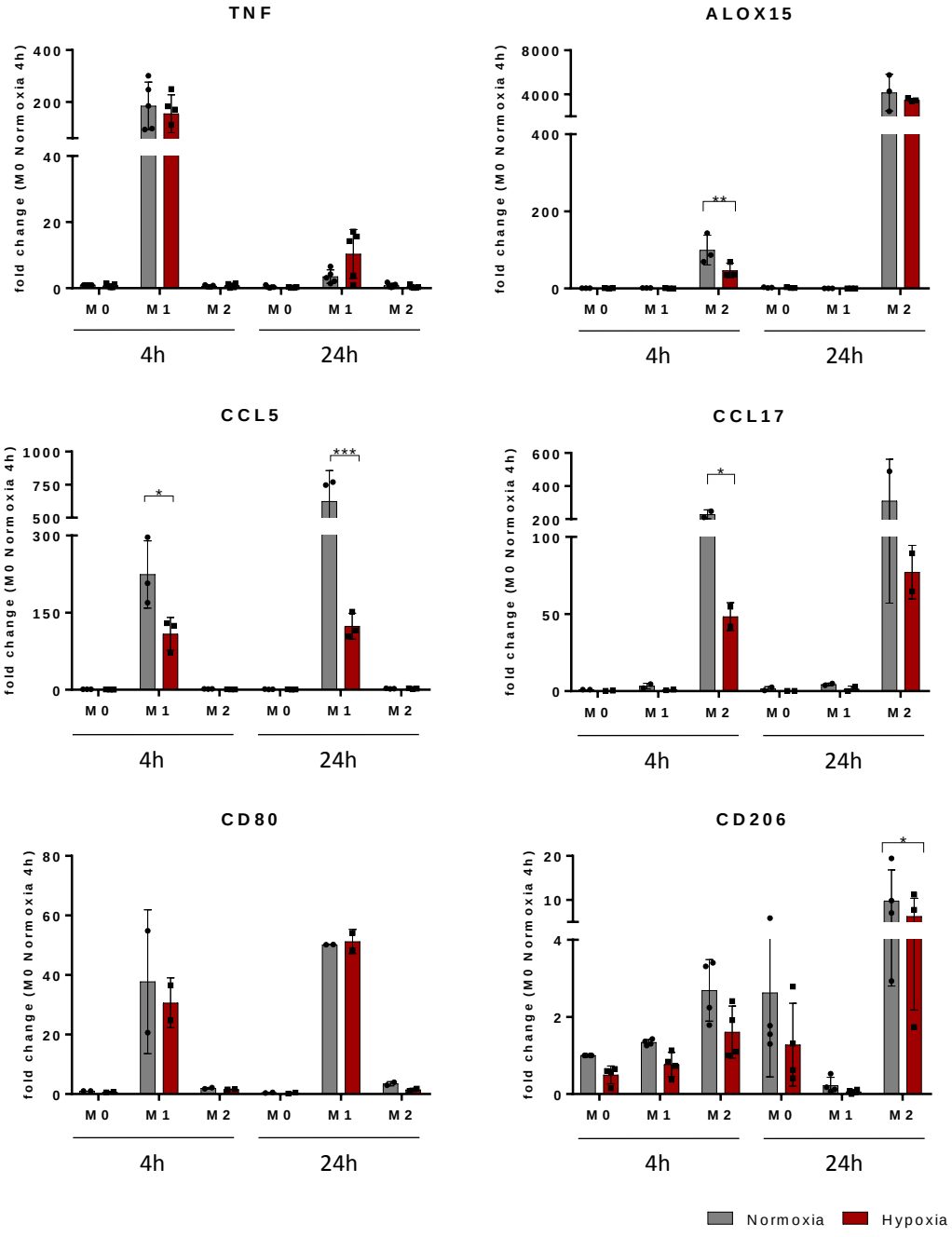


Fig. 14 Level of expression of three pro-inflammatory associated genes (TNF, CCL5, CD80) and three pro-fibrotic related genes (ALOX15, CCL17, CD206). Histograms represent level of mRNA expression as a fold change on M0 in normoxia at 4h; gray bar are related to normoxic condition, red bars to hypoxic condition. (N=4, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

7.9.3 HYPOXIA INDUCES SPECIFIC TRANSCRIPTS IN BJ FIBROBLASTS BUT DOES NOT AFFECT EXPRESSION OF FIBROBLAST ACTIVATING GENES

Similarly on what we have done for macrophages, we perform a qRT-PCR on BJ human dermal fibroblasts to assess their response to hypoxia (at 4h, 24h and 48h). In this case we did not stimulate cells with any factors.

We observe that fibroblasts (Fb) respond to hypoxia by inducing GLUT-1, VEGFA and BNIP3 (BCL2 interacting protein 3) [65]; instead fibroblast activating genes (ACTA2, CTGF, COL1A2, Vimentin, CD90, FAP) are not affected by hypoxia.

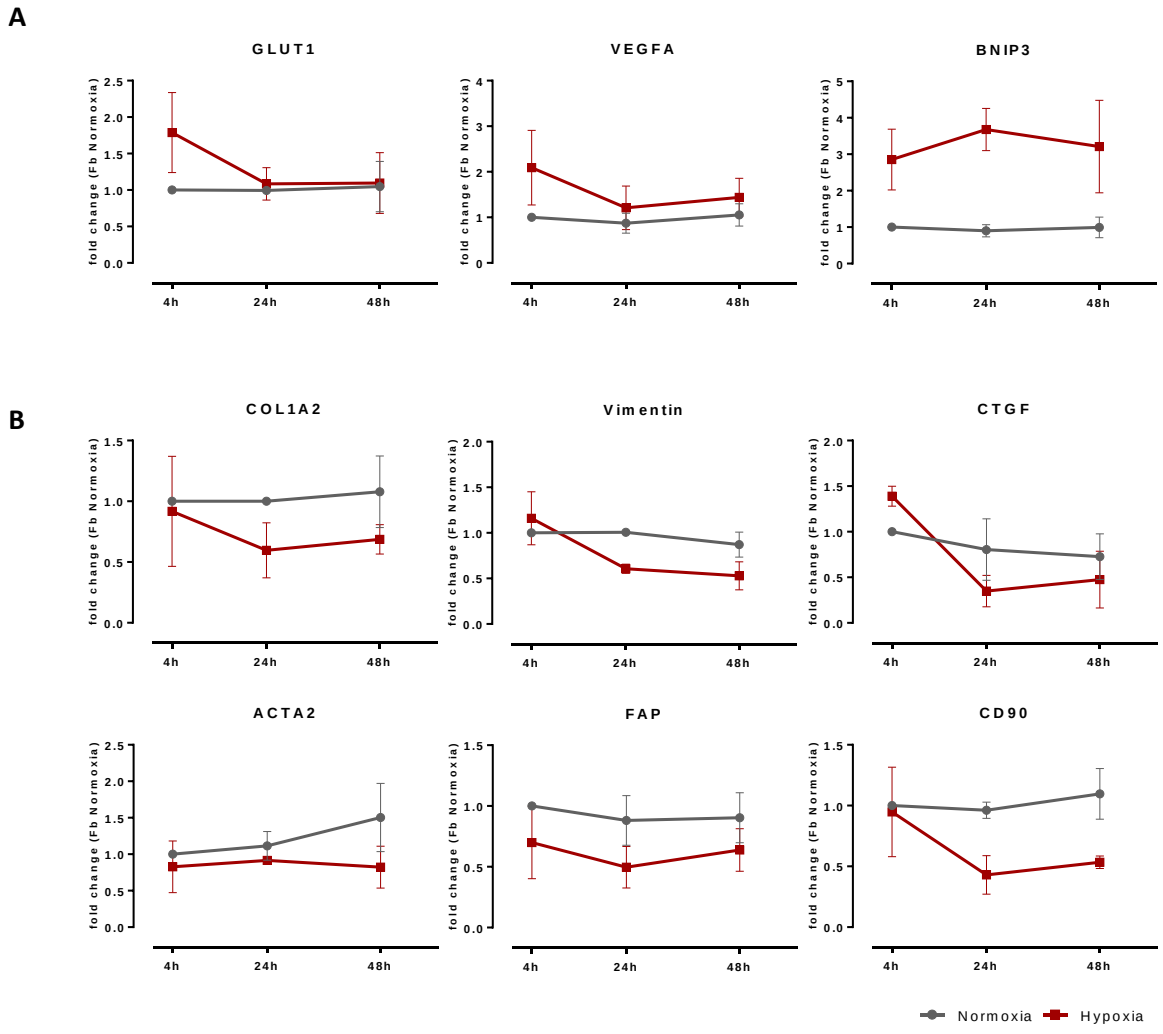


Fig. 15 Level of expression of hypoxia-responsive genes and fibroblast activating genes in BJ cells. Hypoxia-responsive genes (GLUT-1, VEGFA and BNIP3) in BJ fibroblasts at 4h, 24h and 48h of hypoxia

subministration (A); Fibroblast-activating genes (COL1A2, Vimentin, CTGF, ACTA2, FAP, CD90) (B). For each plot is represented the fold change of mRNA expression on the Fb in normoxia at 4h; red line indicates the level of transcripts of samples under hypoxia, the gray line indicates the level of transcripts of samples in normoxia. (N=5, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

7.10 Set up of co-culture experiments

In order to study the interplay between fibroblasts and macrophages we set up a direct contact co-culture system (as described in material and method section). Starting from the simplest condition, M0 Mφ and Fb0 Fb, we decide to put into the system different variables to mimic what happens in chronic inflammation disease and in the initiating phase of fibrotic process.
Moreover, since we decide to include into the analysis both early and late activated genes, we have two time points of analysis: 4h and 24h.
Indeed, we have already two variables:

1) cell type (Mφ & Fb)
2) time (4h & 24h)

and we have to add the other factors that could impact and alter the *in vivo* system:

3) LPS+IFNγ (to mimic Th1 contribution) or IL-4 (to mimic Th2 response)
4) Hypoxia (to mimic a typical metabolic perturbation that often occurs in this context)

Therefore, we need an experimental design that allow us to include all these factors.

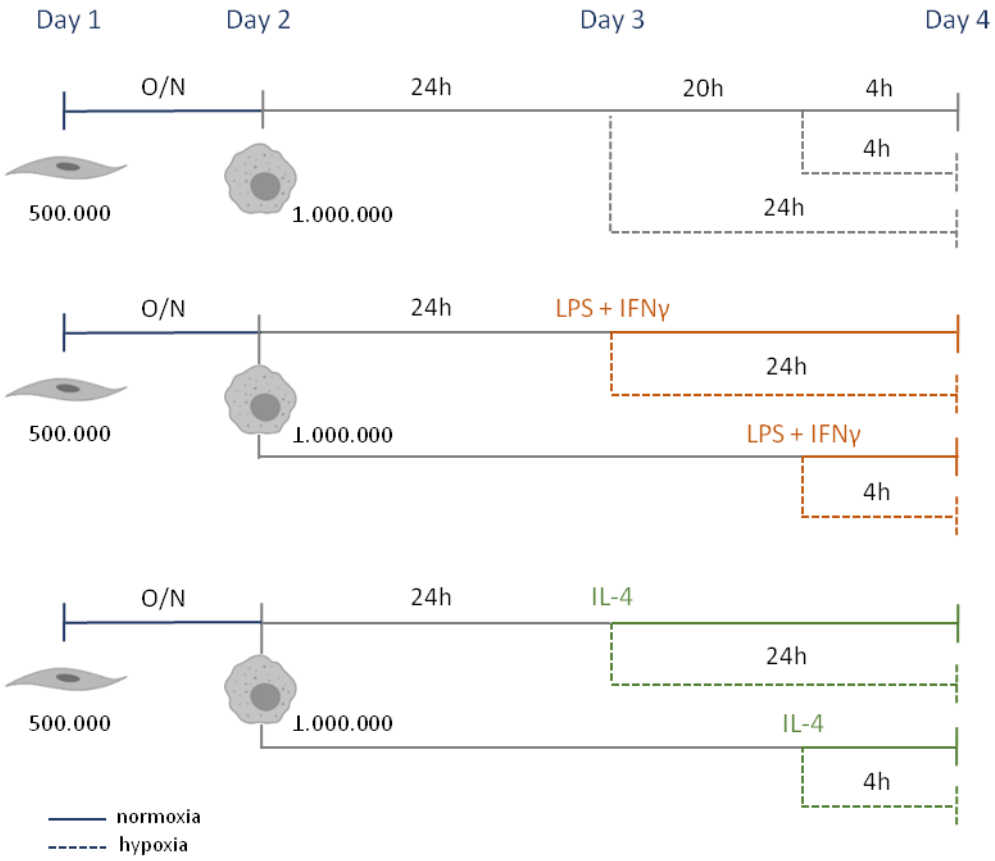


Fig. 16 Scheme of co-culture experimental design. Are reported three main lanes referred to the three different polarizing status: the first lane is referred to the co-culture without stimuli (gray); the lane in the middle is referred to the co-culture stimulated at day 3 with LPS+IFNγ (24h) or 4h before the end of experiment (in red); the last lane is referred to the IL-4 stimulated co-culture (in green). Dashed lines mean that co-culture are put under hypoxia. At the final time point (day 4) there are 11 different conditions.

Indeed we need also controls of single cultures of macrophages and fibroblasts treated in the same way of co-cultures. The following scheme represents the experimental design of controls:

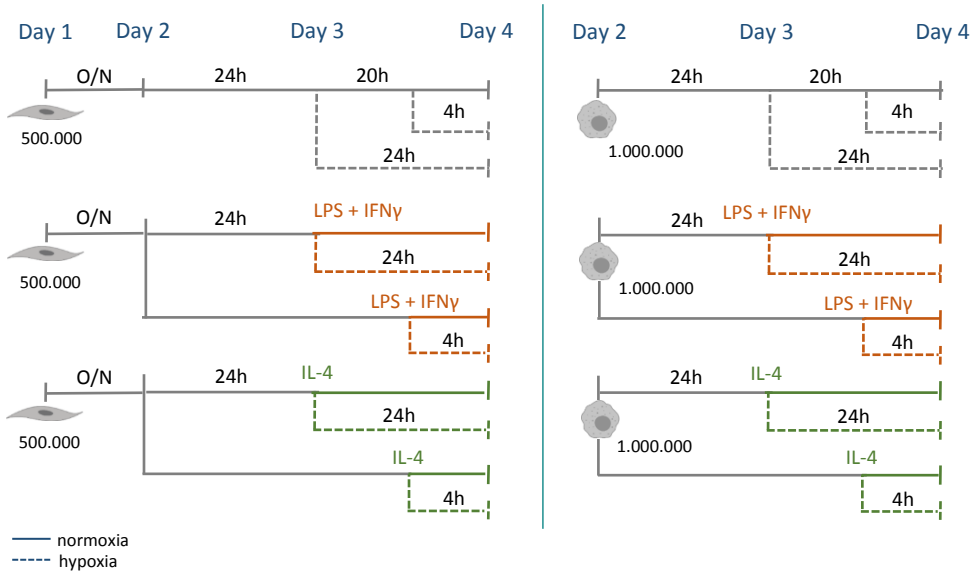


Fig. 17 Scheme of single cultures experimental design. The scheme reported is the same applied to the co-culture. On the left is reported the design used for macrophage, on the right the design used for fibroblasts.

At the end of experiment, co-cultivated cells are sorted for CD45 (as described in materials and methods) by FACS. Macrophages are collected as CD45+ fraction and fibroblasts as CD45- fraction.

After RNA extraction from co-cultivated and single cultivated samples, RNA-sequencing is performed on a total of 44 samples for each replicate (the experiment is performed in triplicate).

Since that the aim of the study is the analysis of the transcriptional profile of macrophages and fibroblasts, the second part of the work will be focused on the bioinformatics analysis of RNA-sequencing results.

7.11 Big data analysis

As previously mentioned the huge amount of samples needs to be analyzed step by step in order to avoid mistakes. We obtained, from the sequencing, a matrix of gene expression for each sample with a total of 17.650 genes.

The following table summarizes all the data that we have produced (multiplied for 3 replicates):

	single culture (SC)					
	M0	MI	MF	Fb0	FbI	FbF
normoxia 4h (4N)		**(S1)** MI 4N	**(S2)** MF 4N		**(S3)** FbI 4N	**(S4)** FbF 4N
hypoxia 4h (4H)	**(S5)** M0 4H	**(S6)** MI 4H	**(S7)** MF 4H	**(S8)** Fb0 4H	**(S9)** FbI 4H	**(S10)** FbF 4H
normoxia 24h (24N)	**(S11)** M0 24N	**(S12)** MI 24N	**(S13)** MF 24N	**(S14)** Fb0 24N	**(S15)** FbI 24N	**(S16)** FbF 24N
hypoxia 24h (24H)	**(S17)** M0 24H	**(S18)** MI 24H	**(S19)** MF 24H	**(S20)** Fb0 24H	**(S21)** FbI 24H	**(S22)** FbF 24H
	co-culture (CC)					
	M0	MI	MF	Fb0	FbI	FbF
normoxia 4h (4N)		**(S23)** MI CC 4N	**(S24)** MF CC 4N		**(S25)** FbI CC 4N	**(S26)** FbF CC 4N
hypoxia 4h (4H)	**(S27)** M0 CC 4H	**(S28)** MI CC 4H	**(S29)** MF CC 4H	**(S30)** Fb0 CC 4H	**(S31)** FbI CC 4H	**(S32)** FbF CC 4H
normoxia 24h (24N)	**(S33)** M0 CC 24N	**(S34)** MI CC 24N	**(S35)** MF CC 24N	**(S36)** Fb0 CC 24N	**(S37)** FbI CC 24N	**(S38)** FbF CC 24N
hypoxia 24h (24H)	**(S39)** M0 CC 24H	**(S40)** MI CC 24H	**(S41)** MF CC 24H	**(S42)** Fb0 CC 24H	**(S43)** FbI CC 24H	**(S44)** FbF CC 24H

Table 2. Summary table of total samples (44 x3replicates). This table represents sequenced samples (from S1 to S44) of one replicate. Samples are divided into single-cultivated (SC) from S1 to S22, on the top, and co-cultivated (CC) from S23 to S44, on the bottom. Columns represent cell type (M=macrophages, Fb=fibroblasts) and polarization status (M0=resting MΦ, MI=pro-inflammatory MΦ, MF=pro-fibrotic MΦ, Fb0=resting Fb, FbI=pro-inflammatory Fb, FbF=pro-fibrotic Fb); rows represent time and oxygen status (4N=normoxia 4h, 4H=hypoxia 4h, 24N=normoxia 24h, 24H=hypoxia 24h). Each sample is reported by a label that indicates in order: cell type-polarization status, culture status, time, oxygen status. Example: S40= MI CC 24H is a co-cultivated, pro-inflammatory macrophage that was subjected to hypoxia for 24 hours.

7.11.1 UNSUPERVISED ANALYSIS: PCA, T-SNE, UMAP AND CORRELATION HEATMAPS

To investigate the major differences in samples and to discriminate the impact of different variables, we use three unsupervised approaches mentioned before (4.8) on the total amount of samples:

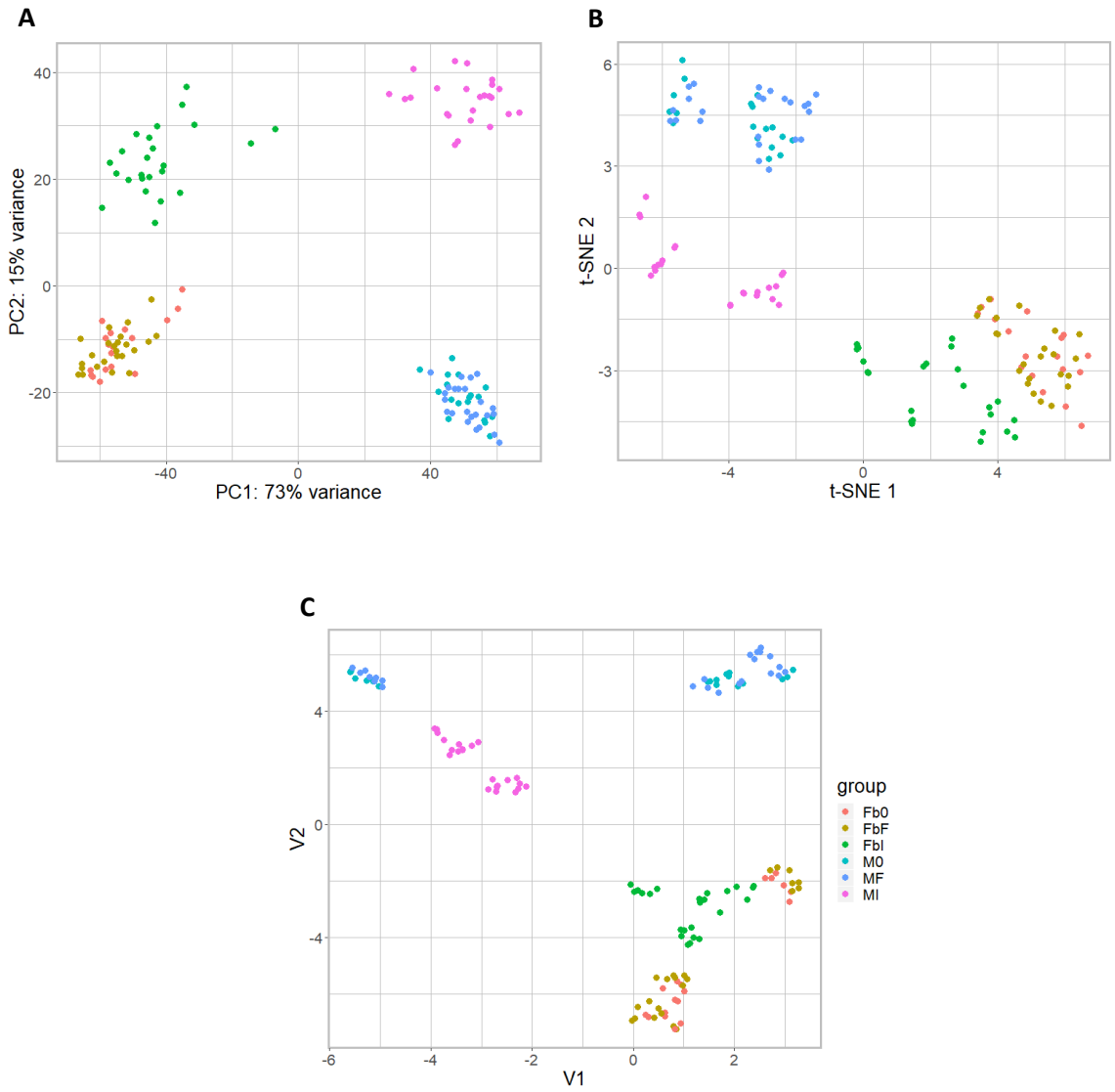
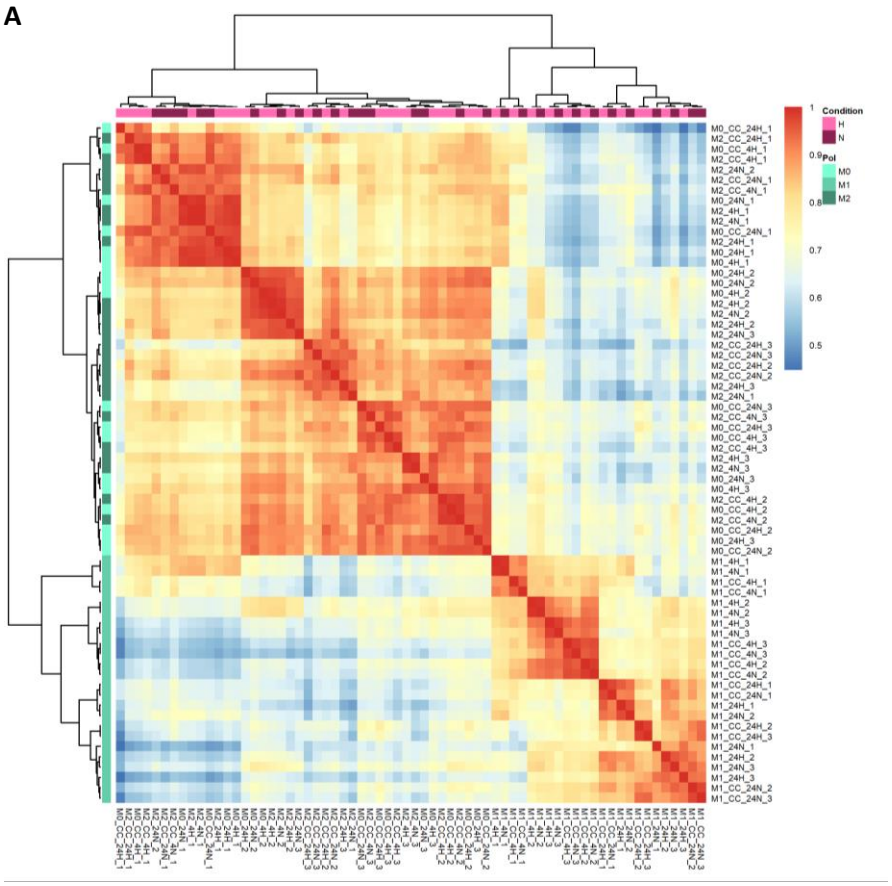


Fig. 18 Graphs of three different unsupervised analysis. Reported graphs (PCA (A), tSNE (B) and UMAP (C)) show in red and light blue resting fibroblasts (Fb0) and macrophages (M0) respectively, in gold and blue pro-fibrotic FbF and MF, in green and pink pro-inflammatory FbI and MI.

It is shown that all approaches confirm a major difference due to the cell type (PC1:73% of variance in PCA); the second level of difference that we observe is related to the pro-

inflammatory condition that both in macrophages and in fibroblasts show a degree of variance (PC2:15%).

Then, we move to the correlation analysis of samples:



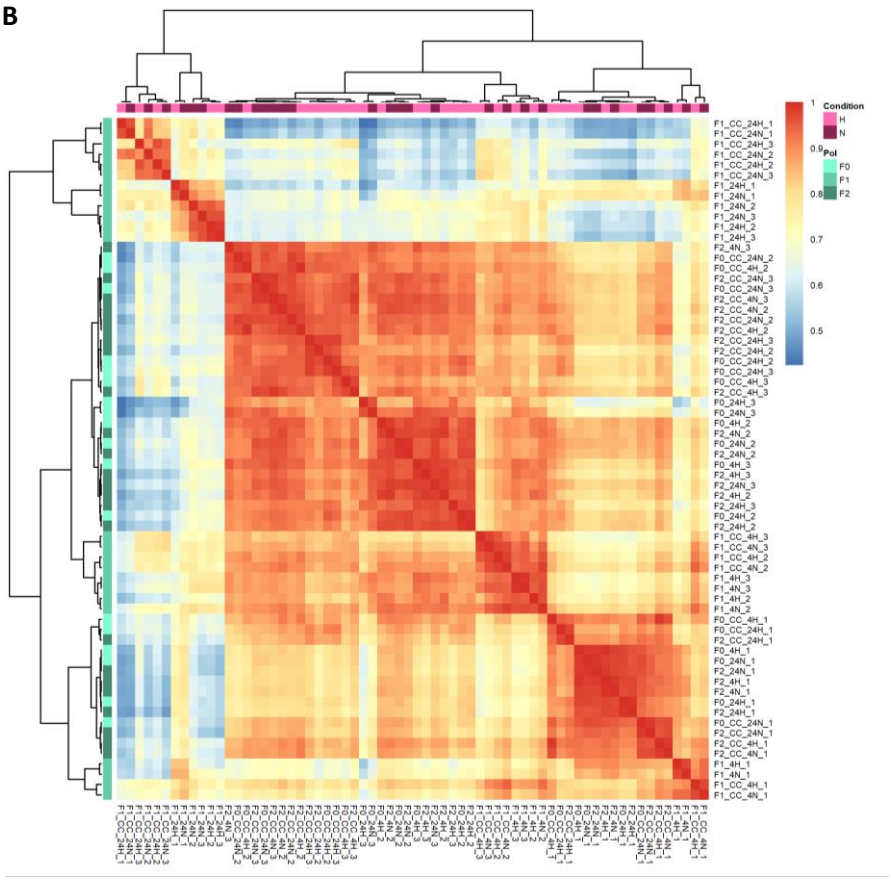


Fig. 19 Correlation Heatmaps. Correlation of transcriptional profiles through all macrophage samples (A) and fibroblast samples (B). Both the maps show in different degree of pink hypoxic or normoxic condition, and in different degree of green polarizing status (resting, pro-inflammatory, pro-fibrotic). Labels indicate each sample ID.

Since that correlation maps show a discrete degree of variability through replicates, for both macrophages and fibroblasts, we decide to proceed by using an in-paired approach for the subsequent differential analysis of comparisons.

7.11.2 SUPERVISED ANALYSIS: DIFFERENTIAL ANALYSIS

This part of the study is based on differential analysis applied to each comparison between two samples. Each simple comparison for one variable is put in comparison with another one obtaining a double comparison that take into account two variables; then, one double comparison is compared to another double comparison resulting into two double comparison with three variables. In order to simplify the analysis, we stratify the comparisons into three

levels of increasing complexity, giving the possibility to understand and analyze different comparisons by using different variables.

The following table summarize the 180 comparisons divided into three levels of complexity.

Table 3 (below) Summarizing table of differential analysis comparisons. The table reports all comparisons (numbered from C1 to C180) divided by level of complexity (row) and state of polarization (column). For each level is reported the cell type (Mφ or Fb) by row and the time point (4h or 24h) by column. The other two variables (culture and oxygen conditions) are alternatively (or combined) reported depending on the comparison; number of comparisons for pro-inflammatory and pro-fibrotic conditions is higher than in resting condition because they add the polarizing variable in the comparisons. SC=single culture, CC=co-culture, H=hypoxia, N=normoxia, 0=resting condition, I=pro-inflammatory condition, F=pro-fibrotic condition, HvsN=hypoxia vs normoxia comparison, CCvsSC= co-culture vs single culture comparison, Ivs0=pro-inflammatory vs resting comparison, Fvs0=pro-fibrotic vs resting comparison.

	CELL TYPE	POLARIZATION: resting				POLARIZATION: pro-inflammatory				POLARIZATION: pro-fibrotic			
		4h	24h	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
1st LEVEL		SC		CC		N/SC		H/SC		N/SC		H/SC	
	Mϕ	**(C1)** H vs N	**(C3)** H vs N	**(C5)** H vs N	**(C7)** H vs N	**(C15)** I vs 0	**(C17)** I vs 0	**(C19)** I vs 0	**(C21)** I vs 0	**(C47)** F vs 0	**(C49)** F vs 0	**(C51)** F vs 0	**(C53)** F vs 0
	Fb	**(C2)** H vs N	**(C4)** H vs N	**(C6)** H vs N	**(C8)** H vs N	**(C16)** I vs 0	**(C18)** I vs 0	**(C20)** I vs 0	**(C22)** I vs 0	**(C48)** F vs 0	**(C50)** F vs 0	**(C52)** F vs 0	**(C54)** F vs 0
		N		H		N/CC		H/CC		N/CC		H/CC	
	Mϕ		**(C9)** CC vs SC	**(C11)** CC vs SC	**(C13)** CC vs SC	**(C23)** I vs 0	**(C25)** I vs 0	**(C27)** I vs 0	**(C29)** I vs 0	**(C55)** F vs 0	**(C57)** F vs 0	**(C59)** F vs 0	**(C61)** F vs 0
	Fb		**(C10)** CC vs SC	**(C12)** CC vs SC	**(C14)** CC vs SC	**(C24)** I vs 0	**(C26)** I vs 0	**(C28)** I vs 0	**(C30)** I vs 0	**(C56)** F vs 0	**(C58)** F vs 0	**(C60)** F vs 0	**(C62)** F vs 0
						SC		CC		SC		CC	
	Mϕ					**(C31)** H vs N	**(C33)** H vs N	**(C35)** H vs N	**(C37)** H vs N	**(C63)** H vs N	**(C65)** H vs N	**(C67)** H vs N	**(C69)** H vs N
	Fb					**(C32)** H vs N	**(C34)** H vs N	**(C36)** H vs N	**(C38)** H vs N	**(C64)** H vs N	**(C66)** H vs N	**(C68)** H vs N	**(C70)** H vs N
						N		H		N		H	
	Mϕ					**(C39)** CC vs SC	**(C41)** CC vs SC	**(C43)** CC vs SC	**(C45)** CC vs SC	**(C71)** CC vs SC	**(C73)** CC vs SC	**(C75)** CC vs SC	**(C77)** CC vs SC
	Fb					**(C40)** CC vs SC	**(C42)** CC vs SC	**(C44)** CC vs SC	**(C46)** CC vs SC	**(C72)** CC vs SC	**(C74)** CC vs SC	**(C76)** CC vs SC	**(C78)** CC vs SC
2nd level		(HvsN)CC vs (HvsN)SC		(CCvsSC)H vs (CCvsSC)N		(Ivs0)N/CC vs (Ivs0)N/SC		(Ivs0)H/SC vs (Ivs0)N/SC		(Fvs0)N/CC vs (Fvs0)N/SC		(Fvs0)H/SC vs (Fvs0)N/SC	
	Mϕ	**(C79)** C5 vs C1	**(C81)** C7 vs C3		**(C83)** C13 vs C9	**(C85)** C23 vs C15	**(C87)** C25 vs C17	**(C89)** C19 vs C15	**(C91)** C21 vs C17	**(C109)** C55 vs C47	**(C111)** C57 vs C49	**(C113)** C51 vs C47	**(C115)** C53 vs C49
	Fb	**(C80)** C6 vs C2	**(C82)** C8 vs C4		**(C84)** C14 vs C10	**(C86)** C24 vs C16	**(C88)** C26 vs C18	**(C90)** C20 vs C16	**(C92)** C22 vs C18	**(C110)** C56 vs C48	**(C112)** C58 vs C50	**(C114)** C52 vs C48	**(C116)** C54 vs C50
						(Ivs0)H/CC vs (Ivs0)H/SC		(Ivs0)H/CC vs (Ivs0)N/CC		(Fvs0)H/CC vs (Fvs0)H/SC		(Fvs0)H/CC vs (Fvs0)N/CC	
	Mϕ					**(C93)** C27 vs C19	**(C95)** C29 vs C21	**(C97)** C27 vs C23	**(C99)** C29 vs C25	**(C117)** C59 vs C51	**(C119)** C61 vs C53	**(C121)** C59 vs C55	**(C123)** C61 vs C57
	Fb					**(C94)** C28 vs C20	**(C96)** C30 vs C22	**(C98)** C28 vs C24	**(C100)** C30 vs C26	**(C118)** C60 vs C52	**(C120)** C62 vs C54	**(C122)** C60 vs C56	**(C124)** C62 vs C58
						(HvsN)CC vs (HvsN)SC		(CCvsSC)H vs (CCvsSC)N		(HvsN)CC vs (HvsN)SC		(CCvsSC)H vs (CCvsSC)N	
	Mϕ					**(C101)** C35 vs C31	**(C103)** C37 vs C33	**(C105)** C43 vs C39	**(C107)** C45 vs C41	**(C125)** C67 vs C63	**(C127)** C69 vs C65	**(C129)** C75 vs C71	**(C131)** C77 vs C73
	Fb					**(C102)** C36 vs C32	**(C104)** C38 vs C34	**(C106)** C44 vs C40	**(C108)** C46 vs C42	**(C126)** C68 vs C64	**(C128)** C70 vs C66	**(C130)** C76 vs C72	**(C132)** C78 vs C74
3rd level						[(Ivs0)Hvs(Ivs0)N]CC vs	[(Ivs0)Hvs(Ivs0)N]SC	[(Ivs0)CCvs(Ivs0)SC]H vs	[(Ivs0)CCvs(Ivs0)SC]N	[(Fvs0)Hvs(Fvs0)N]CC vs	[(Fvs0)Hvs(Fvs0)N]SC	[(Fvs0)CCvs(Fvs0)SC]H vs	[(Fvs0)CCvs(Fvs0)SC]N
	Mϕ					**(C133)** C97vsC89	**(C135)** C99vsC91	**(C137)** C93vsC85	**(C139)** C95vsC87	**(C157)** C121vsC113	**(C159)** C123vsC115	**(C161)** C117vsC109	**(C163)** C119vsC111
	Fb					**(C134)** C98vsC90	**(C136)** C100vsC92	**(C138)** C94vsC86	**(C140)** C96vsC88	**(C158)** C122vsC114	**(C160)** C124vsC116	**(C162)** C118vsC110	**(C164)** C120vsC112
						[(HvsN)Ivs(HvsN)0]CC vs	[(HvsN)Ivs(HvsN)0]SC	(HvsN)CCvs(HvsN)SC]I vs	[(HvsN)CCvs(HvsN)SC]0	[(HvsN)Fvs(HvsN)0]CC vs	[(HvsN)Fvs(HvsN)0]SC	[(HvsN)CCvs(HvsN)SC]I vs	[(HvsN)CCvs(HvsN)SC]0
	Mϕ					**(C141)** (C35vsC5)vs(C31vs1)	**(C143)** (C37vsC7)vs(C33vs3)	**(C145)** C101vsC79	**(C147)** C103vsC81	**(C165)** (C67vsC5)vs(C63vs1)	**(C167)** (C69vsC7)vs(C65vs3)	**(C169)** C125vsC79	**(C171)** C127vsC81
	Fb					**(C142)** (C36vsC6)vs(C32vs2)	**(C144)** (C38vsC8)vs(C34vs4)	**(C146)** C102vsC80	**(C148)** C104vsC82	**(C166)** (C68vsC6)vs(C64vs2)	**(C168)** (C70vsC8)vs(C66vs4)	**(C170)** C126vsC80	**(C172)** C128vsC82
						[(CCvsSC)Ivs(CCvsSC)0]H vs [(CCvsSC)Ivs(CCvsSC)0]N		[(CCvsSC)Hvs(CCvsSC)N]I vs [(CCvsSC)Hvs(CCvsSC)N]0		[(CCvsSC)Fvs(CCvsSC)0]H vs [(CCvsSC)Fvs(CCvsSC)0]N		[(CCvsSC)Hvs(CCvsSC)N]F vs [(CCvsSC)Hvs(CCvsSC)N]0	
	Mϕ					**(C149)** (C43vsC11)vs(C39vs9)	**(C151)** (C45vsC13)vs(C41vs9)	**(C153)** C105vsC83	**(C155)** C107vsC83	**(C173)** (C75vsC11)vs(C71vs9)	**(C175)** (C77vsC13)vs(C73vs9)	**(C177)** C129vsC83	**(C179)** C131vsC83
	Fb					**(C150)** (C44vsC12)vs(C40vs10)	**(C152)** (C46vsC14)vs(C42vs10)	**(C154)** C106vsC84	**(C156)** C108vsC84	**(C174)** (C76vsC12)vs(C72vs10)	**(C176)** (C78vsC14)vs(C74vs10)	**(C178)** C130vsC84	**(C180)** C132vsC84

Moreover, since that each comparison is thought to answer to specific biological question about the impact of single factor or multiple factors together, the following table summarizes these questions and related comparisons that will be reported in the Results (8) section:

BIOLOGICAL QUESTION	COMPARISON CODE
What is the impact of hypoxia on resting cells?	C1-C2-C3-C4
What is the impact of hypoxia on co-cultivated cells?	C5-C6-C7-C8
What is the impact of co-culture on resting cells?	C9-C10
What is the impact of co-culture on hypoxic cells?	C11-C12-C13-C14
What is the effect of pro-inflammatory factors (LPS+IFNγ) on Mϕ and Fb?	C15-C16-C17-C18
What is the effect of pro-inflammatory factors (LPS+IFNγ) on hypoxic Mϕ and Fb?	C19-C20-C21-C22
What is the effect of pro-inflammatory factors (LPS+IFNγ) on co-cultivated Mϕ and Fb?	C23-C24-C25-C26
What is the effect of pro-inflammatory factors (LPS+IFNγ) on co-cultivated hypoxic Mϕ and Fb?	C27-C28-C29-C30
What is the effect of hypoxia on pro-inflammatory Mϕ and Fb?	C31-C32-C33-C34
What is the effect of hypoxia on co-cultivated pro-inflammatory Mϕ and Fb?	C35-C36-C37-C38
What is the effect of co-culture on pro-inflammatory Mϕ and Fb?	C39-C40-C41-C42
What is the effect of co-culture on hypoxic pro-inflammatory Mϕ and Fb?	C43-C44-C45-C46
What is the effect of pro-fibrotic cytokine (Il-4) on Mϕ and Fb	C47-C48-C49-C50
What is the effect of IL-4 on hypoxic Mϕ and Fb?	C51-C52-C53-C54
What is the effect of IL-4 on co-cultivated Mϕ and Fb?	C55-C56-C57-C58
What is the effect of IL-4 on co-cultivated hypoxic Mϕ and Fb?	C59-C60-C61-C62
What is the effect of hypoxia on pro-fibrotic Mϕ and Fb?	C63-C64-C65-C66
What is the effect of hypoxia on co-cultivated pro-fibrotic Mϕ and Fb?	C67-C68-C69-C70
What is the effect of co-culture on pro-fibrotic Mϕ and Fb?	C71-C72-C73-C74
What is the effect of co-culture on hypoxic pro-fibroticMϕ and Fb?	C75-C76-C77-C78
What is the effect of hypoxia when cell are co-cultivated?	C79-C80-C81-C82
What is the effect of co-culture when cells are under hypoxia?	C83-C84
What is the effect of pro-inflammatory stimuli when cells are co-cultivated?	C85-C86-C87-C88
What is the effect of pro-inflammatory stimuli when cells are under hypoxia?	C89-C90-C91-C92
What is the effect of pro-inflammatory stimuli when hypoxic cells are co-cultivated?	C93-C94-C95-C96
What is the effect of pro-inflammatory stimuli when co-cultivated cells are put under hypoxia?	C97-C98-C99-C100
What is the effect of hypoxia when pro-inflammatory cells are co-cultivated?	C101-C102-C103-C104
What is the effect of co-culture when pro-inflammatory cells are put under hypoxia?	C105-C106-C107-C108
What is the effect of pro-fibrotic stimuli when cells are co-cultivated?	C109-C110-C11-C112
What is the effect of pro-fibrotic stimuli when cells are under hypoxia?	C113-C114-C115-C116
What is the effect of pro-fibrotic stimuli when hypoxic cells are co-cultivated?	C117-C118-C119-C120
What is the effect of pro-fibrotic stimuli when co-cultivated cells are put under hypoxia?	C121-C122-C123-C124
What is the effect of hypoxia when pro-fibrotic cell are co-cultivated?	C125-C126-C127-C128
What is the effect of co-culture on pro-fibrotic cells when they are under hypoxia?	C129-C130-C131-C132
What is the impact of LPS+IFNγ when oxygen and culture status are modified?	C133-C134-C135-C136 C137-C138-C139-C140
What is the impact of hypoxia when polarizing and culture status are modified?	C141-C142-C143-C144 C145-C146-C147-C148
What is the impact of co-culture when polarizing and oxygen status are modified?	C149-C150-C151-C152

	C153-C154-C155-C156
What is the impact of IL-4 when oxygen and culture status are modified?	C157-C158-C159-C160 C161-C162-C163-C164
What is the impact of hypoxia when polarizing and culture status are modified?	C165-C166-C167-C168 C169-C170-C171-C172
What is the impact of co-culture when polarizing and oxygen status are modified?	C173-C174-C175-C176 C177-C178-C179-C180

Table 4 Biological questions and relative comparisons code answer. This table summarizes all the comparisons grouped by the biological question to which they answer. Codes used are the same that we applied in table 3.

8 RESULTS

The core of the analysis was performed at transcriptional level by comparing mRNA profile of each sample in a series of comparisons by a multi-level approach with increasing complexity, as we mentioned in the previous section (4.11.2).

The 1st level is the simplest one: it is composed by 78 single comparisons based on an analysis of genes significantly differentially expressed in the two samples under investigation; for each comparison is reported:

- The number of genes significantly differentially expressed (quantitative information)
- An heatmap with the level of expression of that genes (quantitative information)
- A volcano-plot that shows the degree of differential expression of genes reported in the heatmap (quantitative and qualitative information)
- A graph with pathways significantly enriched in the comparison (qualitative information)

The 2nd level is the intermediate: it is composed by 54 double comparisons where two variables are investigated at the same time; for each comparison is reported:

- The number of genes significantly differentially expressed (quantitative information)
- A Venn diagram that explain the double comparison based on the number of genes significantly differentially expressed (quantitative information)
- A four-columns heatmap with the union of genes significantly differentially expressed in the two comparisons subtracted by the genes that are in common (quantitative information)

The 3rd level is the more complex: it is composed by 48 comparisons, with three variables studied together in a total of three double comparisons; for each comparison is reported:

- The number of genes significantly differentially expressed (quantitative information)
- A Venn diagram that explain the two double comparisons based on the number of genes significantly differentially expressed (quantitative information)
- An eight-columns heatmap with the union of significantly genes differentially expressed in the three double comparisons subtracted by genes that are in common in each comparison (quantitative information)

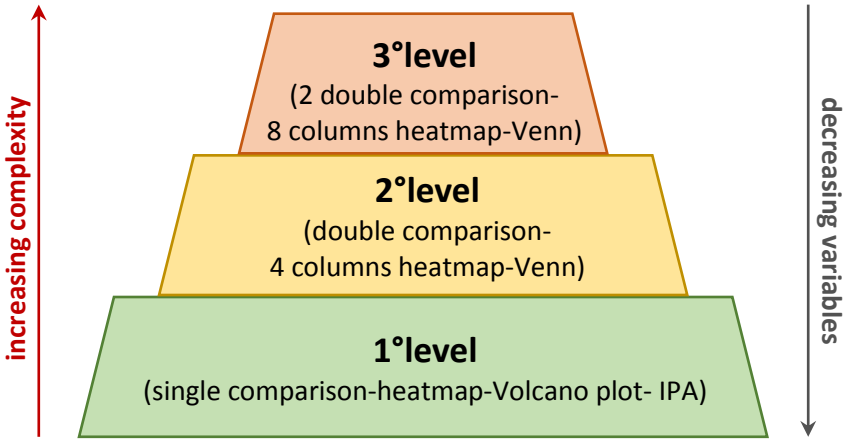


Fig. 20 Multi-level pyramid of comparisons. The scheme represents three levels of complexity of transcriptional analysis. For each level is reported the type of analysis performed, with increasing grade of complexity from the level 1 to the level 3 and decreasing number of variables considered in different comparisons, from the top to the bottom (three variables at level 3, 2 variables at level 2 and 1 variable at level 1).

8.1 FIRST LEVEL

8.1.1 RESTING CONDITION

8.1.1.1 What is the impact of hypoxia on resting cells?

[Code: C1 (S5vsS11)]

Macrophages cultivated for 4 hours (4h) under hypoxia are similar to resting macrophages cultivated in normoxia; in fact only 37 genes are significantly differentially expressed and they do not enrich a specific pathway.

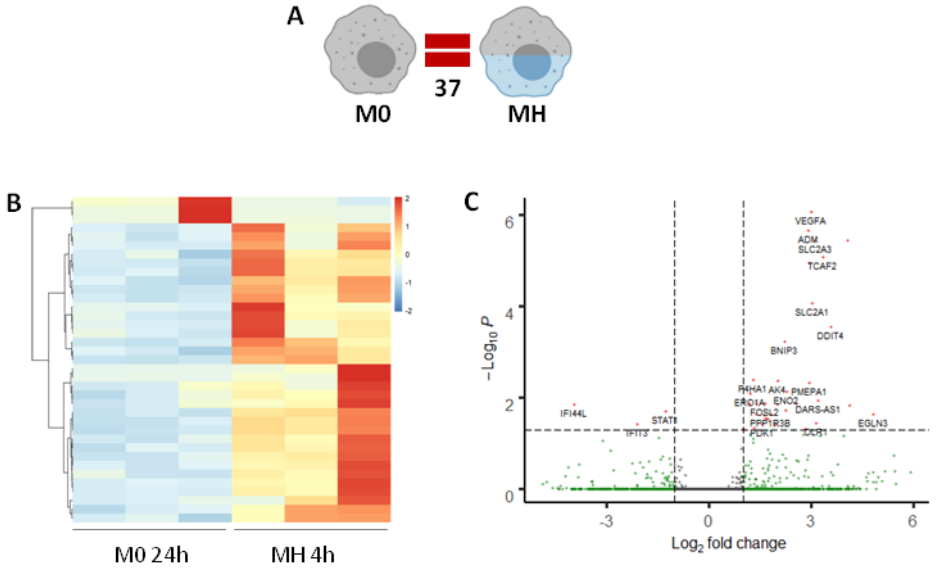


Fig. 21 Comparison M0vsMH. Schematic representation of M0vsMH comparison at 4h with the number of significantly differentially expressed genes (SDEG) (FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change < –1 or >1) (C).

[Code: C2 (S8vsS14)]

Fibroblasts under hypoxia for 4h show a different profile to fibroblasts in normoxia with 97 genes significantly differentially expressed that enrich HMGB1, PI3K/AKT, IL-8, ErbB, ILK signalling pathways.

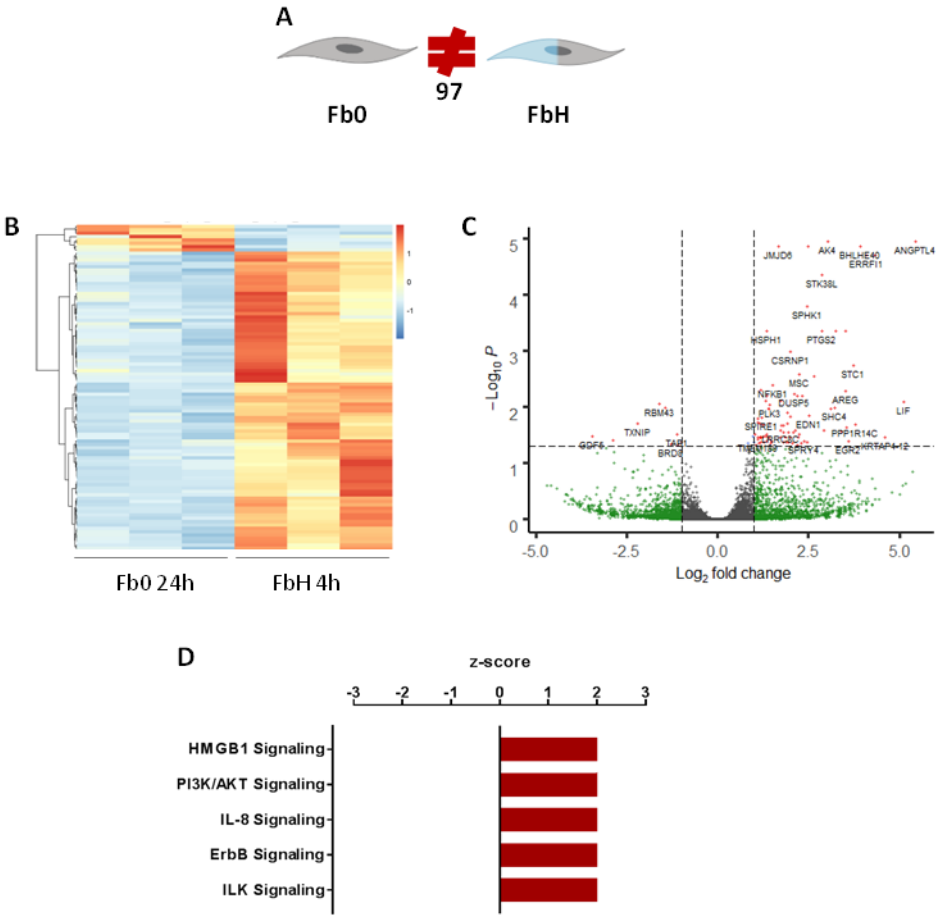


Fig. 22 Comparison Fb0vsFbH. Schematic representation of Fb0vsFbH comparison at 4h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change < –1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

[Code: C3 (S17vsS11)]

After 24h of hypoxia macrophages do not assume a different phenotype in comparison to resting normoxic macrophages.

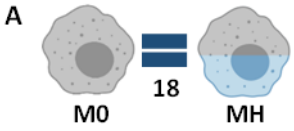


Fig. 23 Comparison M0vsMH. Schematic representation of M0vsMH comparison at 24h with the number of SDEG (FDR<0.05) (A).

[Code: C4 (S20vsS14)]

Fibroblasts under hypoxia for 24h reduce the number of SDEG in comparison to the 4h, and they are similar to the normoxic counterpart.

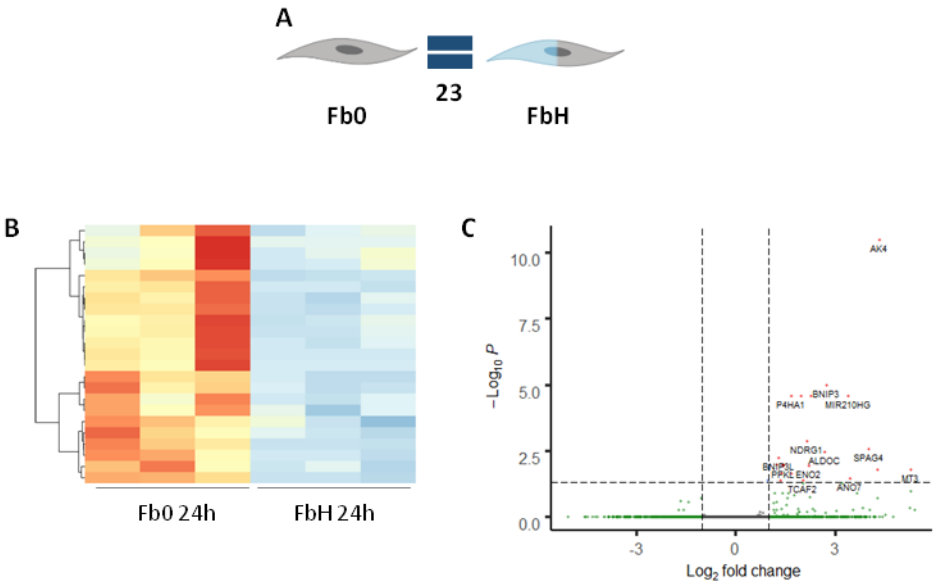


Fig. 24 Comparison Fb0vsFbH. Schematic representation of Fb0vsFbH comparison at 24h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change < –1 or >1) (C).

8.1.1.2 What is the impact of hypoxia on co-cultivated cells?

[Code: C5 (S27vsS33)]

Macrophages co-cultivated with fibroblasts in hypoxia for 4h show 44 SDEG in co-cultivated macrophages in normoxia; all these genes do not enrich a specific pathway.

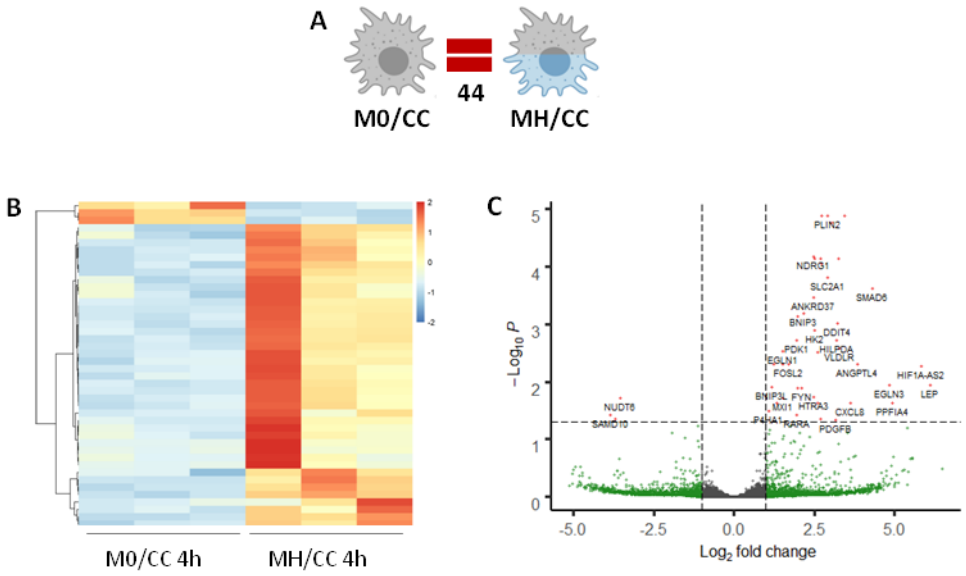


Fig. 25 Comparison M0/CCvsMH/CC. Schematic representation of M0vsMH comparison at 4h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change < -1 or > 1) (C).

[Code: C6 (S30vsS36)]

Fibroblasts co-cultivated with macrophages for 4h in hypoxia show 105 SDEG in the normoxic counterpart; in this case, we see a slightly enrichment in IL6 – HMGB- ILK signalling pathways.

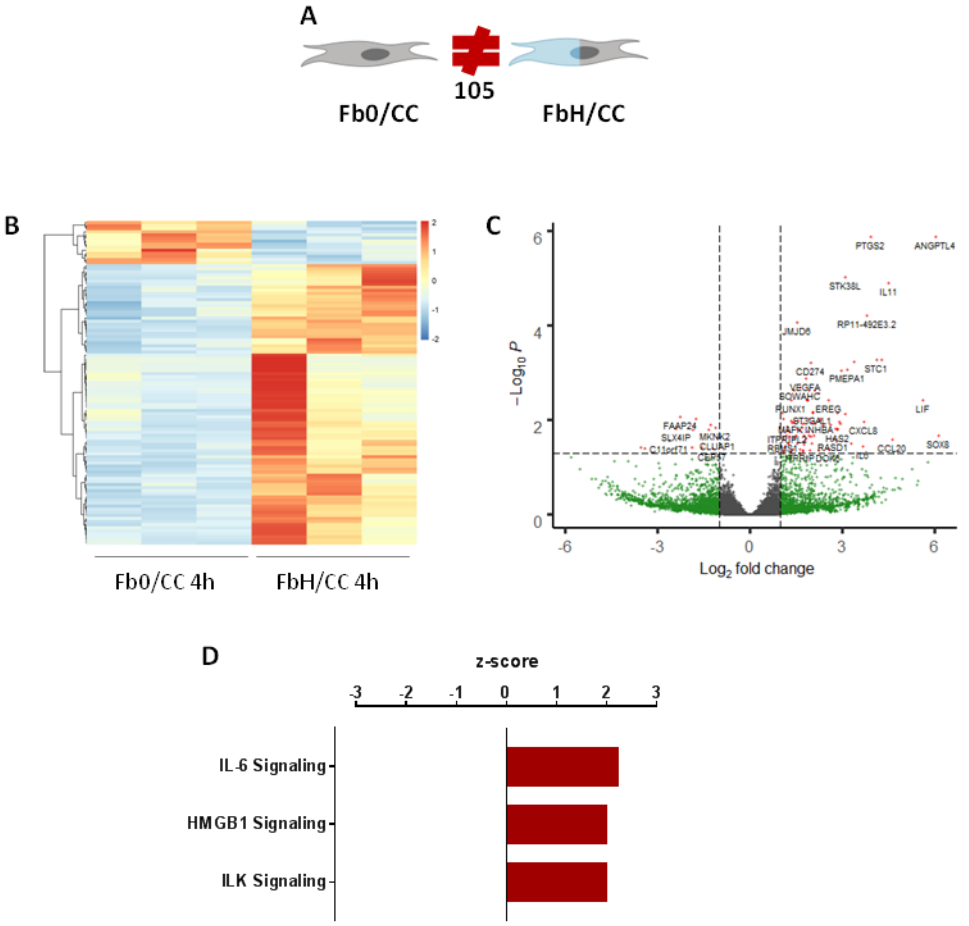


Fig. 26 Comparison Fb0/CCvsFbH/CC. Schematic representation of Fb0vsFbH comparison at 4h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change < -1 or >1) (C).IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ -2 in blue (D).

[Code: C7 (S39vsS33)]

After an extended period of time, 24h of hypoxia, macrophages co-cultivated are different in comparison with normoxic co-cultivated macrophages; there are 1139 SDEG that enrich pathways related to actin remodelling (Actin cytoskeleton signaling), extracellular matrix deposition (GP6 signaling, Integrin signalling) and proliferation (Wnt/Ca2+ pathway, PCP pathway). Activation of these pathways suggests the acquisition of a different phenotype in hypoxic co-cultivated Mϕ, which differ from the normoxic co-cultivated counterpart.

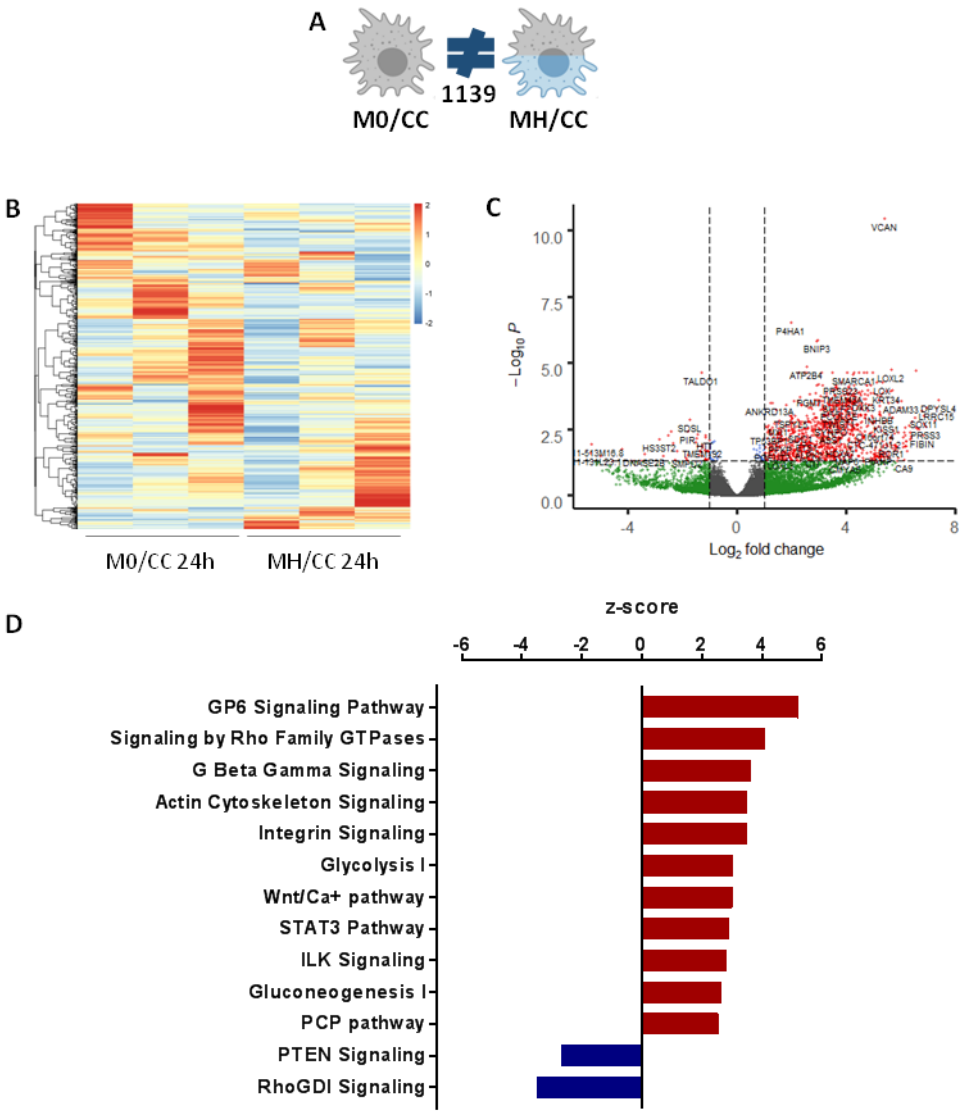


Fig. 27 Comparison M0/CCvsMH/CC. Schematic representation of M0/CCvsMH/CC comparison a 24h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change < -1 or > 1)

(C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

[Code: C8 (S42vsS36)]

After 24h of hypoxia, fibroblasts co-cultivated with macrophages show different profile from co-cultivated fibroblasts in normoxia; there are 1305 genes with a different level of expression that enrich a wide variety of pathways related to different cell function, connected to growth factors (FGF, PDGF, HGF), pro-angiogenesis (VEGF), chemokine signalling, leukocyte recruitment, pro-inflammatory signals. Similarly on what we observed for Mϕ, hypoxic co-cultivated Fb acquire a different phenotype by the activation of many pathways related to cell growth and promotion of inflammation.

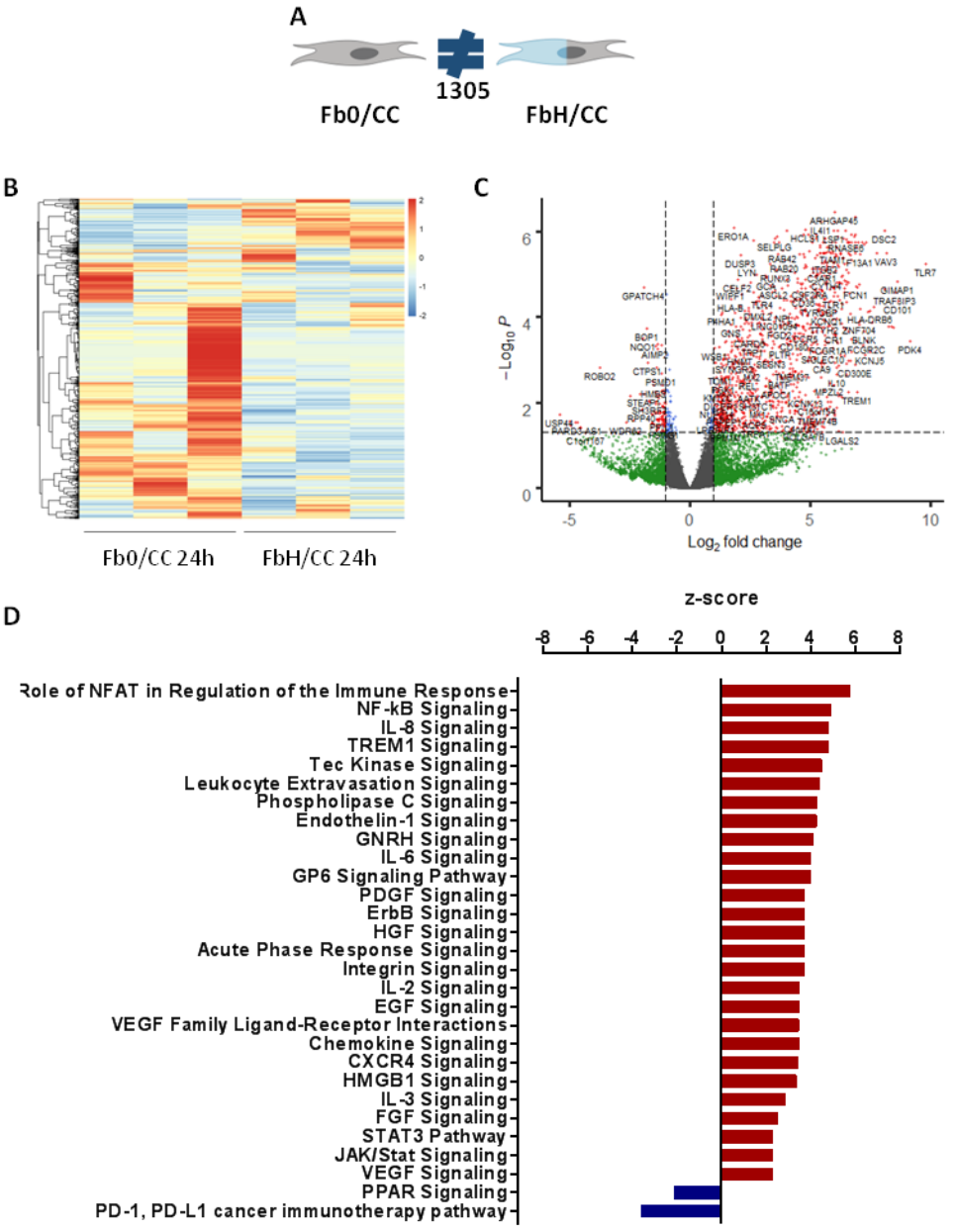


Fig. 28 Comparison Fb0/CCvsFbH/CC. Schematic representation of Fb0/CCvsFbH/CC comparison a 24h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; value are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

8.1.1.3 What is the impact of co-culture on resting cells?

[Code: C9 (S33vsS11)]

Macrophages co-cultivated with fibroblast for 24h in normoxia are the same cells to macrophages cultivated alone for the same period of time.

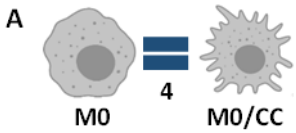


Fig. 29 Comparison M0vsM0/CC. Schematic representation of M0vsM0/CC comparison a 24h with the number of SDEG (FDR<0.05) (A).

[Code: C10 (S36vsS14)]

Fibroblasts co-cultivated with macrophages for 24h in normoxia show no differences with fibroblasts alone.

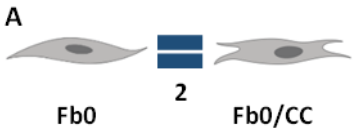


Fig. 30 Comparison Fb0vsFb0/CC. Schematic representation of Fb0vsFb0/CC comparison a 24h with the number of SDEG (FDR<0.05) (A).

8.1.1.4 What is the impact of co-culture on hypoxic cells?

[Code: C11 (S27vsS5)]

Macrophages in hypoxia for 4h and co-cultivated with fibroblast are the same of macrophages put under hypoxia for 4h alone; co-cultivation does not modify hypoxic macrophage profile (32 genes differentially expressed only).

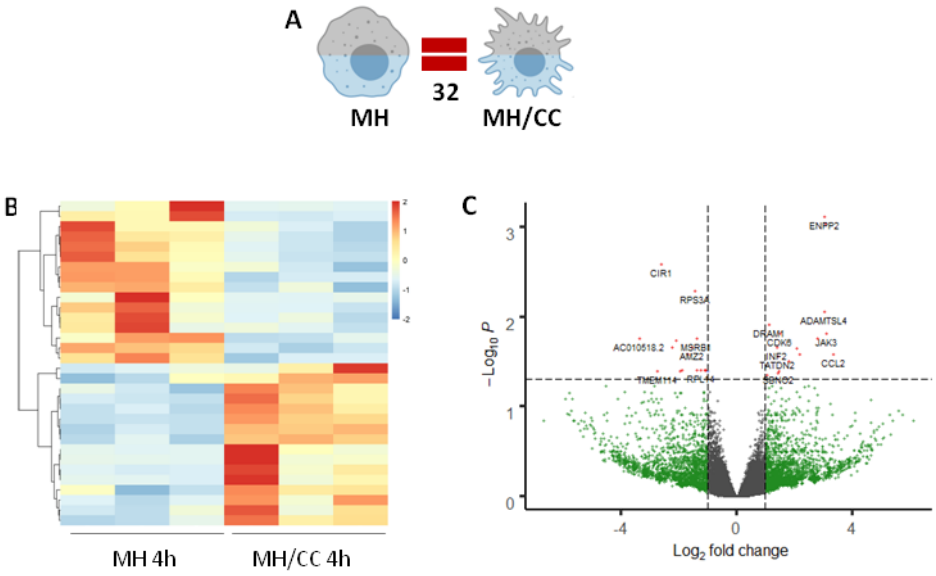


Fig. 31Comparison MHvsMH/CC. Schematic representation of MHvsMH/CC comparison a 4h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; value are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C).

[Code: C12 (S30vsS8)]

Fibroblasts under hypoxia for 4h and co-cultivated with macrophages show no difference with hypoxic fibroblast cultivated alone.

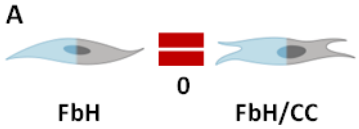


Fig. 32 Comparison FbHvsFbH/CC. Schematic representation of FbHvsFbH/CC comparison a 4h with the number of SDEG (FDR<0.05) (A).

[Code: C13 (S39vsS17)]

Macrophages in hypoxia for 24h and co-cultivated with fibroblast are different from macrophages put under hypoxia for 24h alone; co-cultivation modifies hypoxic macrophage profile by inducing different expression of 1424 genes. Pathways enriched in this comparison are related to actin remodelling, CXCR4 signalling, Wnt non canonical activation, IL-6 and IL-8 signalling.

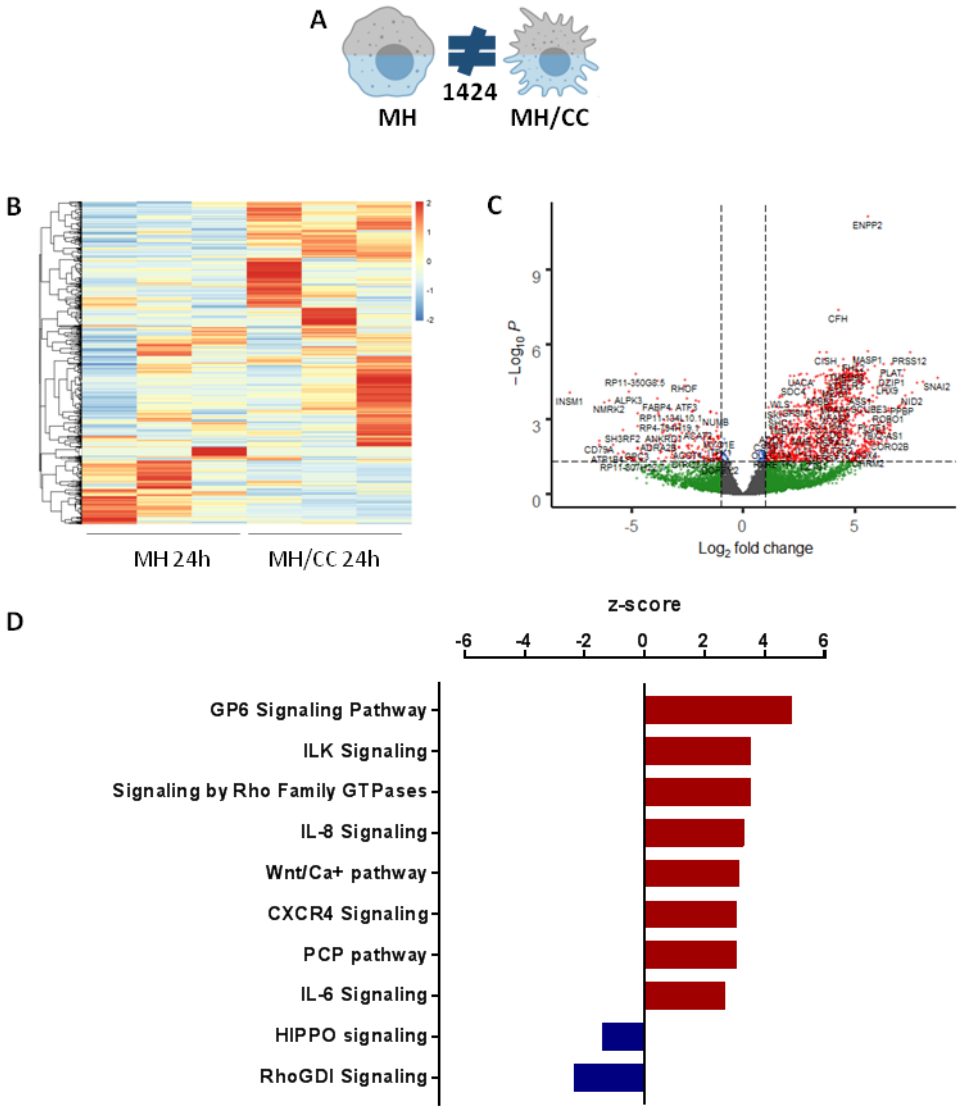


Fig. 33Comparison MHvsMH/CC. Schematic representation of MHvsMH/CC comparison a 24h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; value are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

[Code: C14 (S42vsS20)]

Fibroblasts under hypoxia for 24h and co-cultivated with macrophages show differences with hypoxic fibroblasts cultivated alone since that 1291 genes are differentially expressed. Pathways major affected are related to inflammation, angiogenesis, chemotaxis and extracellular matrix remodelling.

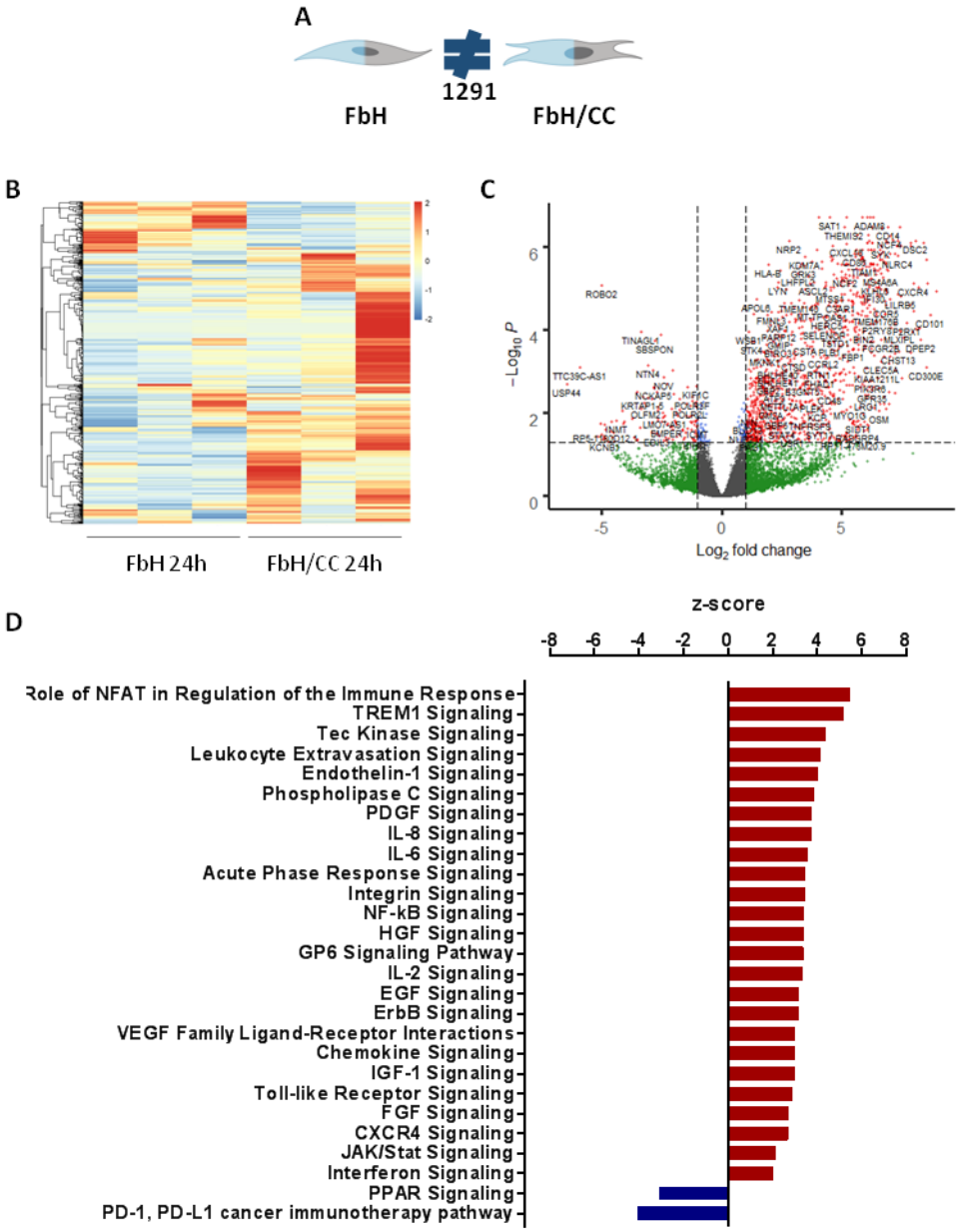


Fig. 34 Comparison FbHvsFbH/CC. Schematic representation of FbHvsFbH/CC comparison a 24h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; value are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C). IPA

analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

SUMMARY FIRST LEVEL: RESTING CONDITION

The two conditions in which emerge a significant difference are:

C7 and C8

C13 and C14

Moreover fibroblasts only differ also in other two conditions:

C2 and C6

8.1.2.1 What is the effect of pro-inflammatory factors (LPS+IFNγ) on Mφ and Fb?

[Code: C15 (S1vsS11)]

Macrophages stimulated for 4h with LPS+IFNγ show a pro-inflammatory phenotype as reported in literature; by comparing stimulated macrophages to resting cells, it is observed that 3531 genes are differentially expressed with a promotion of a pro-inflammatory phenotype.

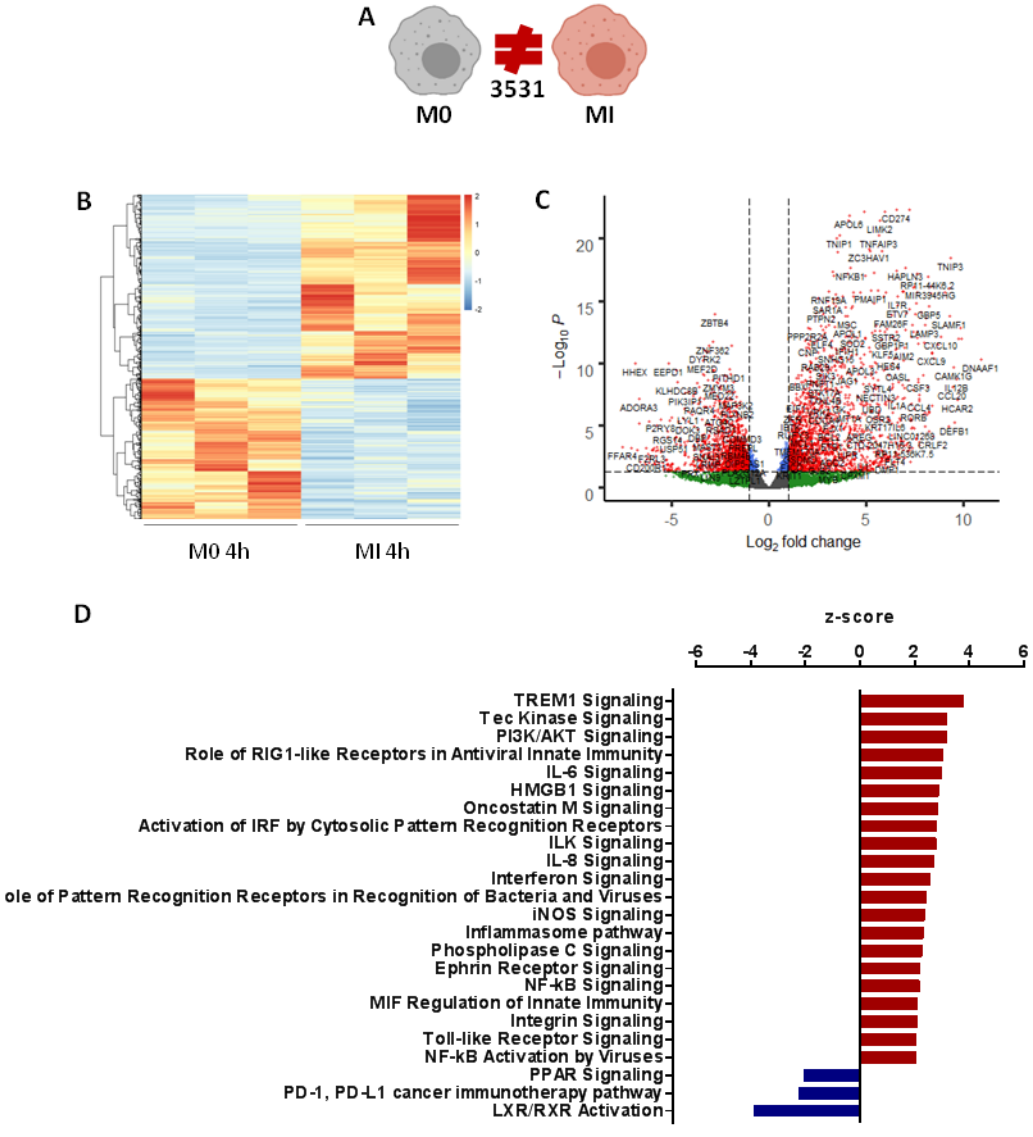


Fig. 35 Comparison M0vsMI. Schematic representation of M0vsMI comparison a 4h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; value are scaled by row and each column represent one replicate

(there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

[Code: C16 (S3vsS14)]

Fibroblasts stimulated by LPS+IFNγ for 4h show pro-inflammatory properties with a differential expression of 3983 genes in comparison with the resting condition. Pathways enriched are mostly related to inflammation.

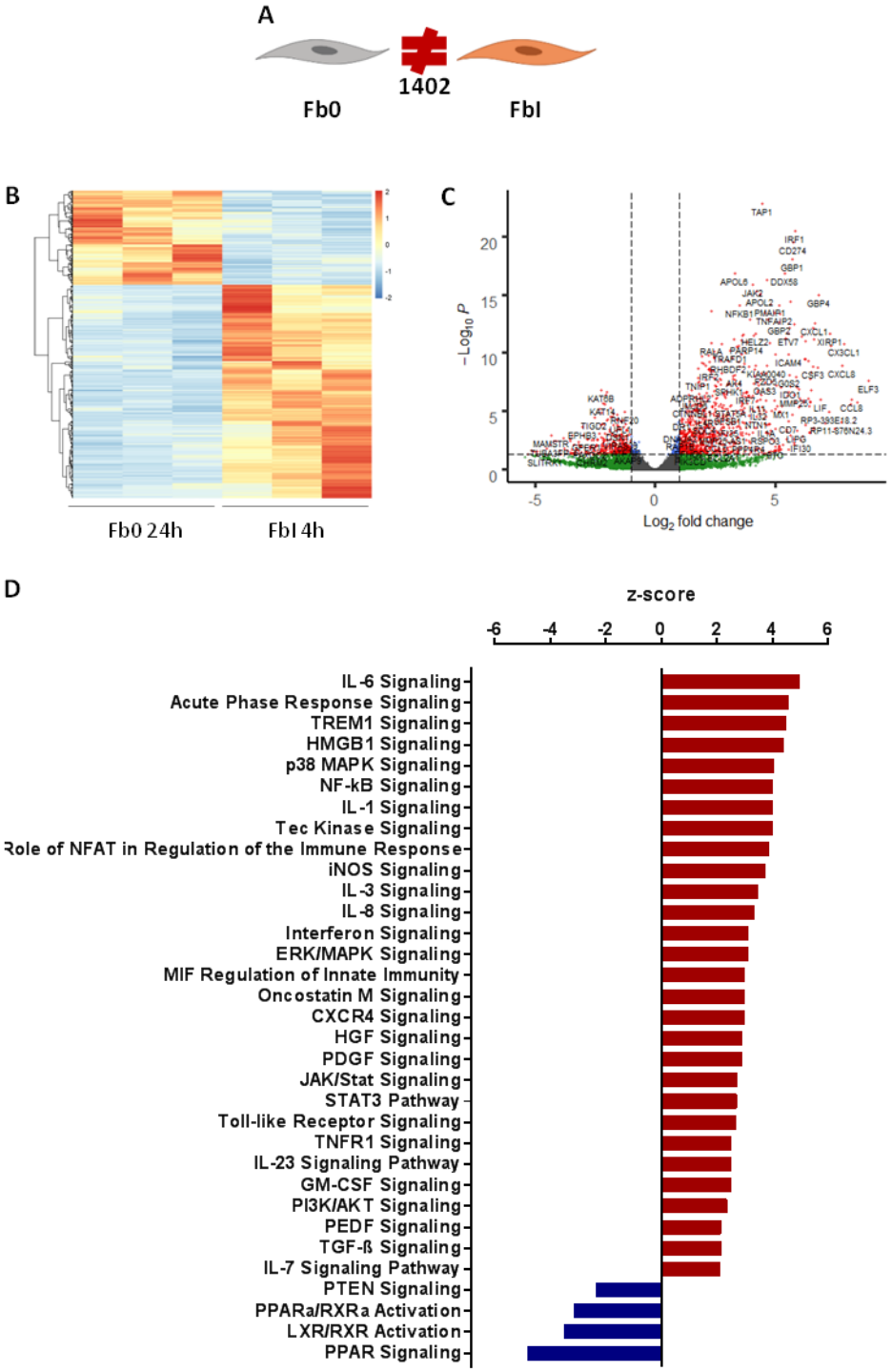


Fig. 36 Comparison Fb0vsFbI. Schematic representation of Fb0vsFbI comparison a 4h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; value are scaled by row and each column represent one replicate

(there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

[Code: C17 (S12vsS11)]

Macrophages stimulated for 24h with LPS+IFNγ maintain a pro-inflammatory phenotype as reported in literature; by comparing stimulated macrophages to resting cells it is observed that 3389 genes are differentially expressed with a promotion of a pro-inflammatory phenotype.

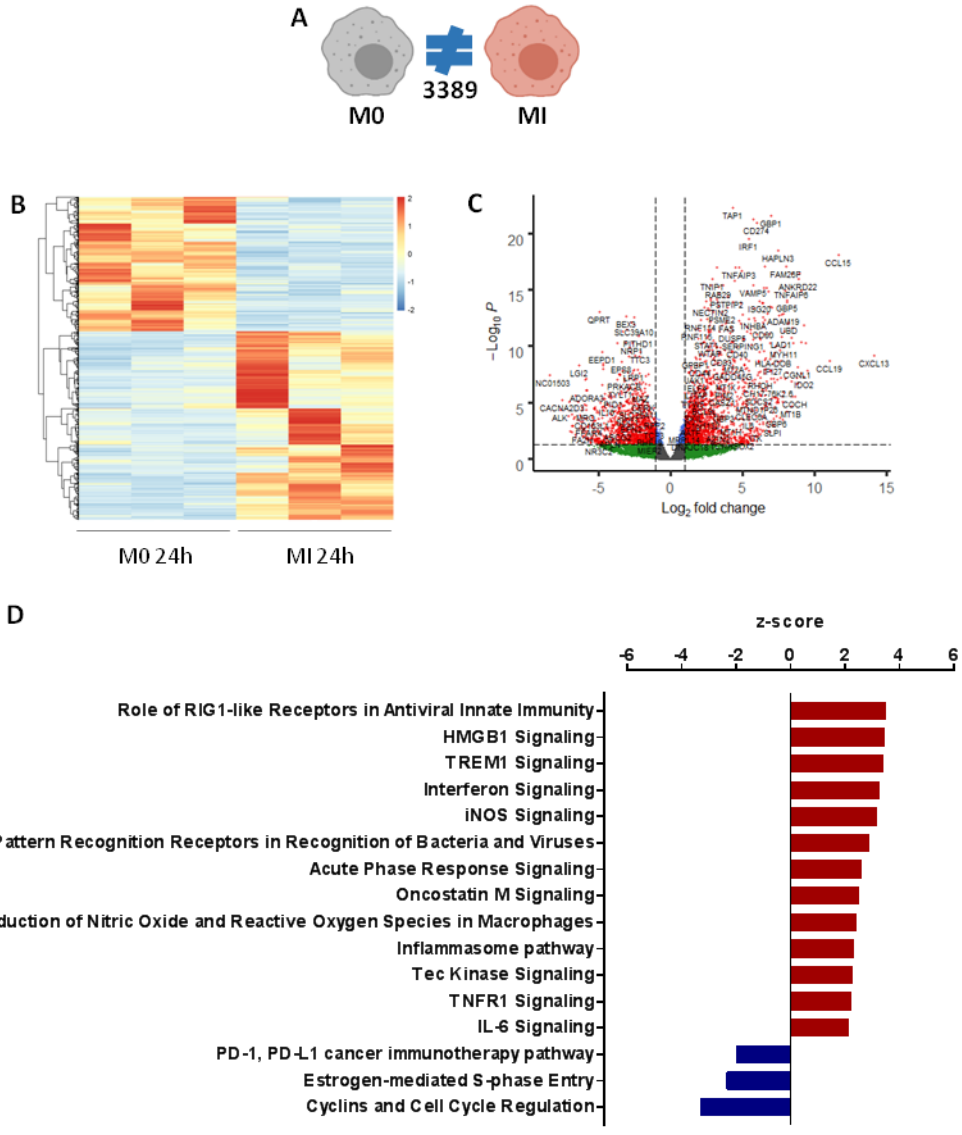


Fig. 37 Comparison M0vsMI. Schematic representation of M0vsMI comparison a 24h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; value are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

[Code: C18 (S15vsS14)]

Fibroblasts stimulated by LPS+IFNγ for 24h show pro-inflammatory properties with a differential expression of 3840 genes in comparison with the resting condition. Pathways enriched are mostly related to inflammation.

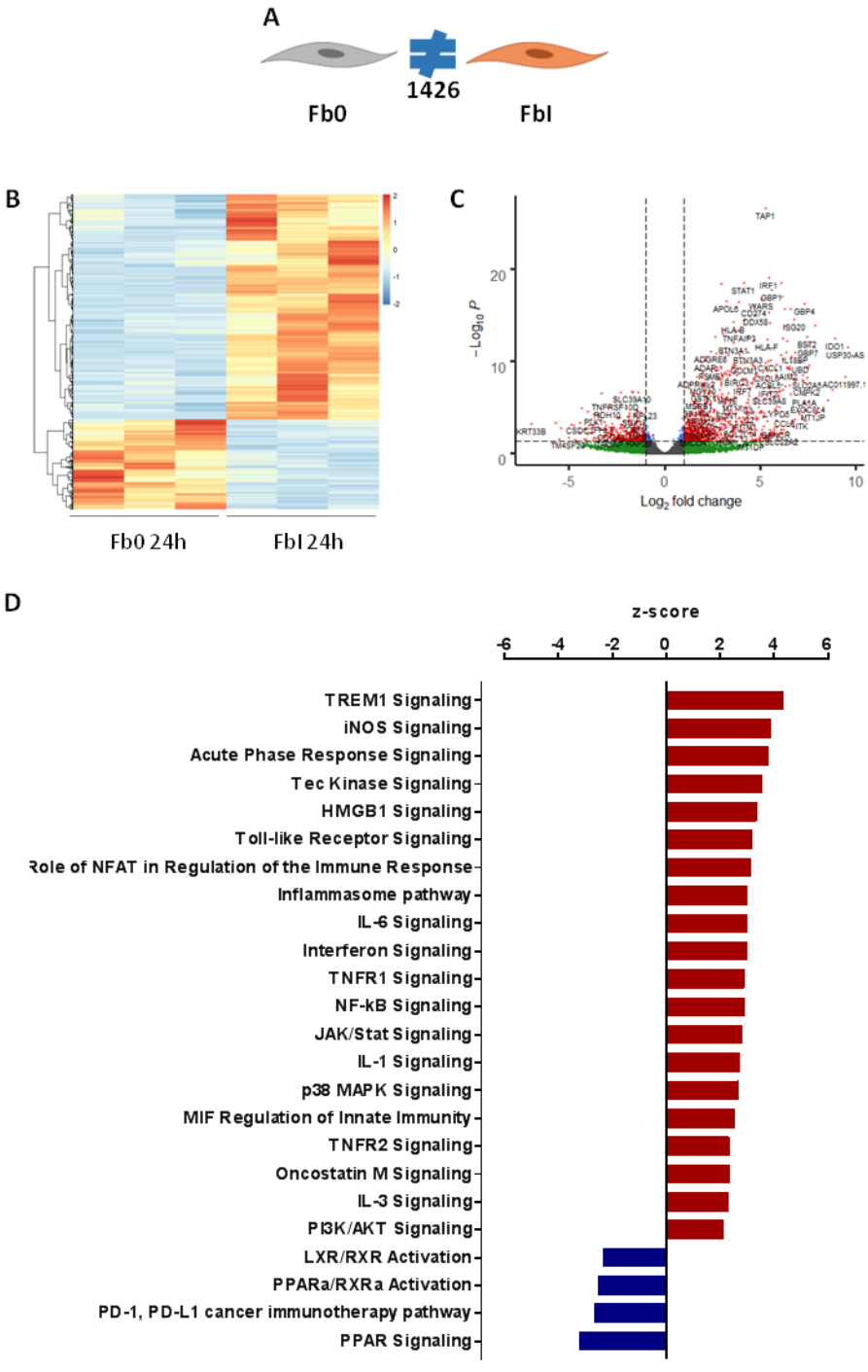


Fig. 38Comparison Fb0vsFbI. Schematic representation of Fb0vsFbI comparison a 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression

and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

8.1.2.2 What is the effect of pro-inflammatory factors (LPS+IFNγ) on hypoxic Mφ and Fb?

[Code: C19 (S6vsS5)]

Macrophages stimulated for 4h with LPS+IFNγ in hypoxic environment differ from hypoxic macrophages, which do not receive the pro-inflammatory stimulation; 2930 genes are differentially expressed with an enrichment of pathways related to inflammation.

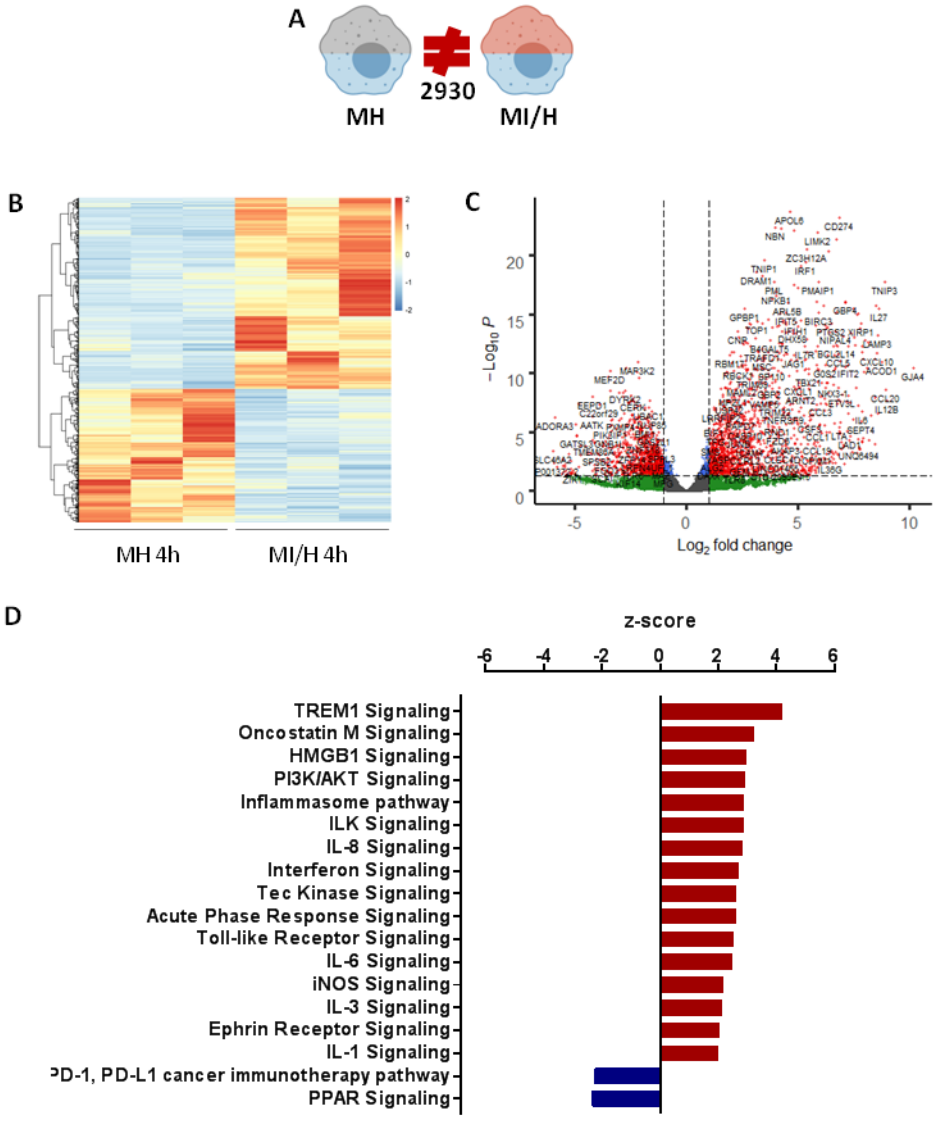


Fig. 39 Comparison MHvsMI/H. Schematic representation of MHvsMI/H comparison a 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate

(there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C20 (S9vsS8)]

Fibroblasts stimulated by LPS+IFNγ for 4h in hypoxia show pro-inflammatory properties with a differential expression of 628 genes in comparison with the hypoxic fibroblast without cytokine stimulation. Pathways enriched are mostly related to inflammation.

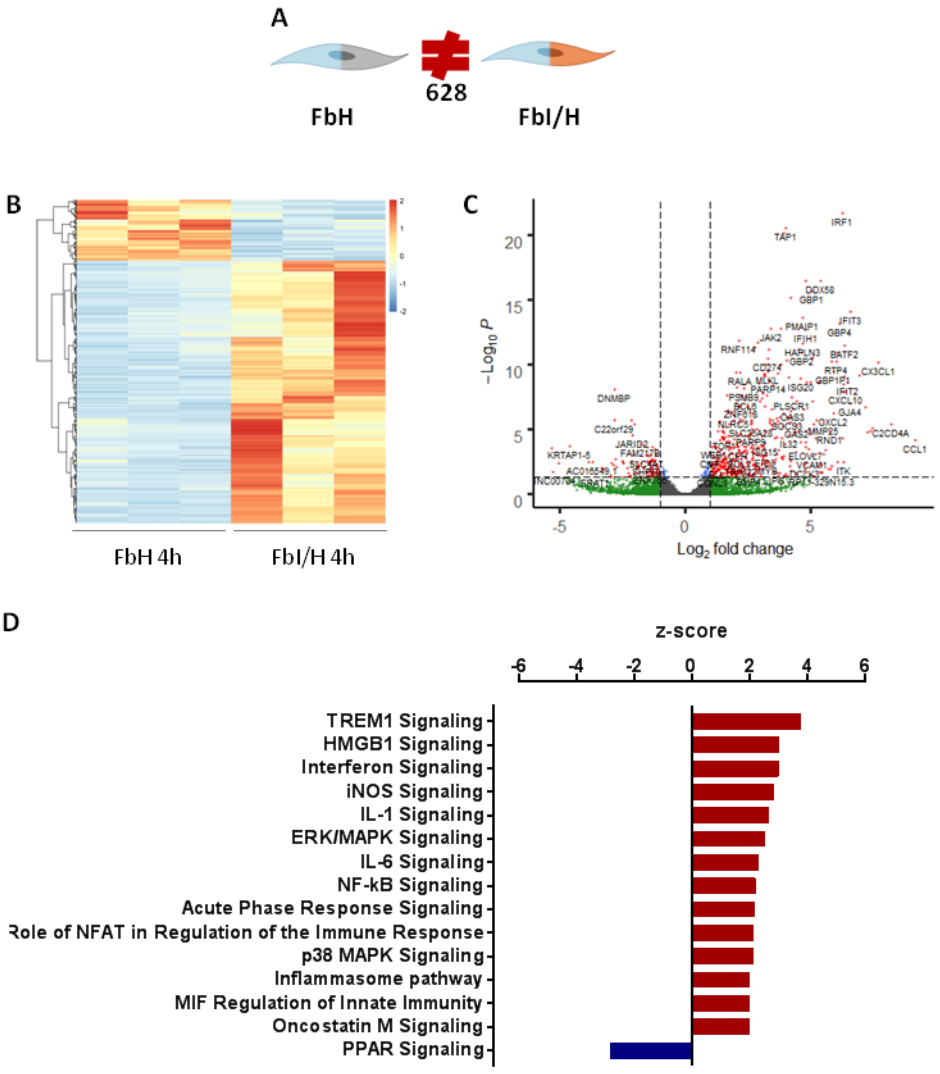


Fig. 40 Comparison FbHvsFbI/H. Schematic representation of FbHvsFbI/H comparison a 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are

showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C21 (S18vsS17)]

Macrophages stimulated for 24h with LPS+IFNγ under hypoxia maintain a pro-inflammatory phenotype; by comparing stimulated macrophages to resting cells in hypoxia it is observed that 3474 genes are differentially expressed with a promotion of a pro-inflammatory phenotype.

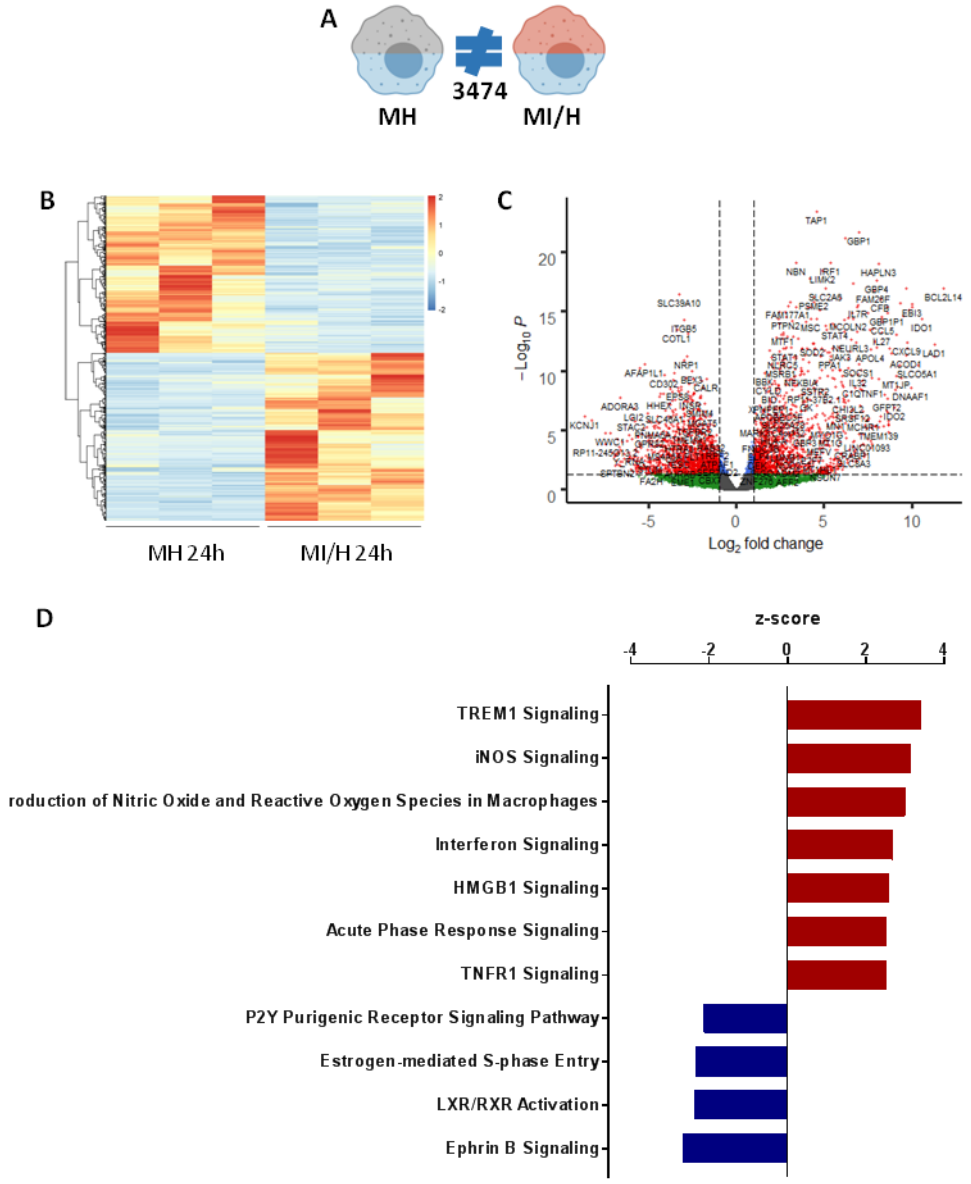


Fig. 41 Comparison MHvsMI/H. Schematic representation of MHvsMI/H comparison a 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C22 (S21vsS20)]

Fibroblasts stimulated by LPS+IFNγ for 24h in hypoxia show pro-inflammatory properties with a differential expression of 1773 genes in comparison with the hypoxic condition alone. Pathways enriched are mostly related to inflammation.

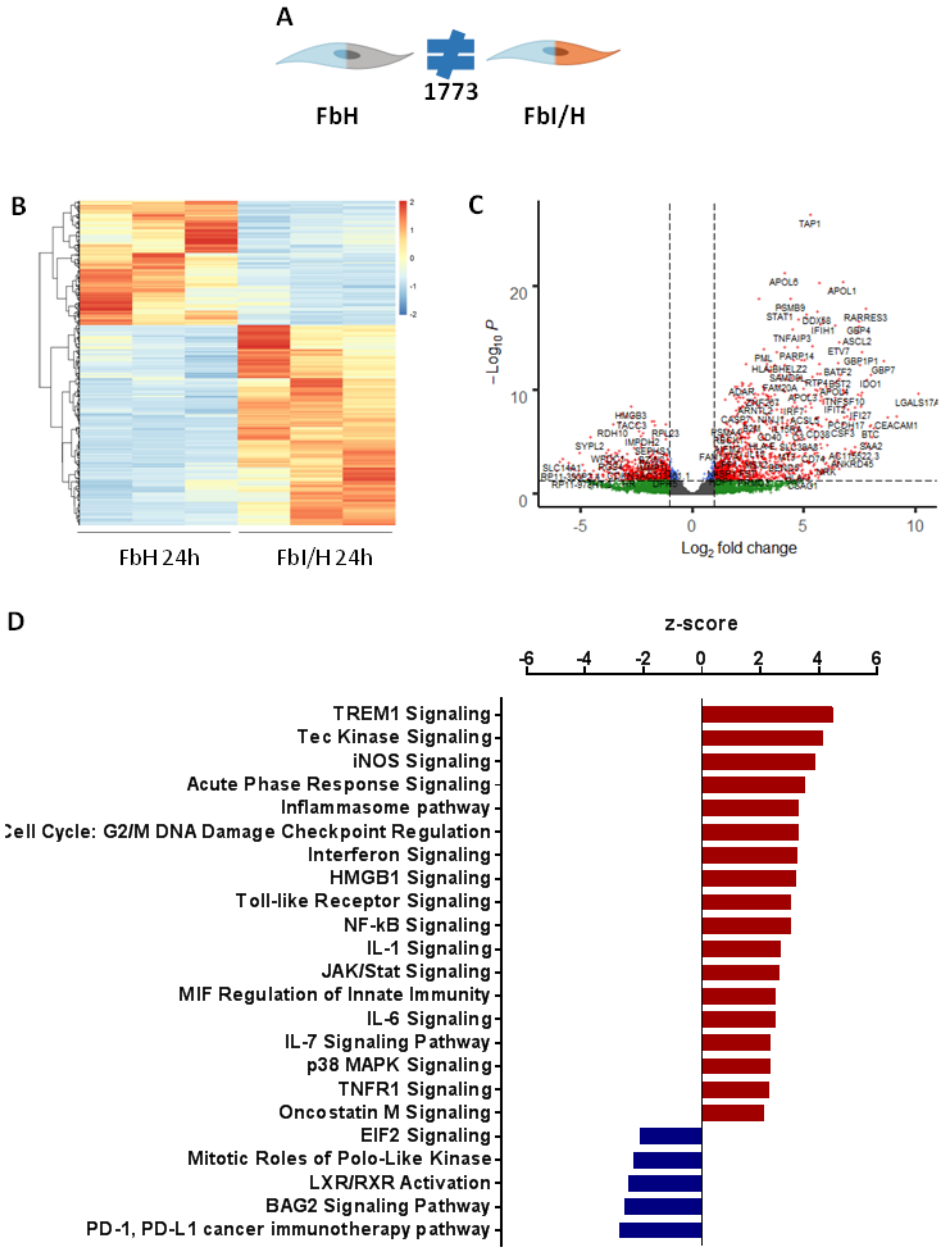


Fig. 42 Comparison FbHvsFbI/H. Schematic representation of FbHvsFbI/H comparison a 24h with the number of SDEG(FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C). IPA analysis result

is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ –2 in blue (D).

8.1.2.3 What is the effect of pro-inflammatory factors (LPS+IFNγ) on co-cultivated Mφ and Fb?

[Code: C23 (S23vsS33)]

Macrophages stimulated for 4h with LPS+IFNγ in co-culture with fibroblasts are different from co-cultivated macrophages that do not receive the pro-inflammatory stimulation; 3983 genes are differentially expressed with an enrichment of inflammation-related pathways (TREM1 signalling, IFN signalling, etc).

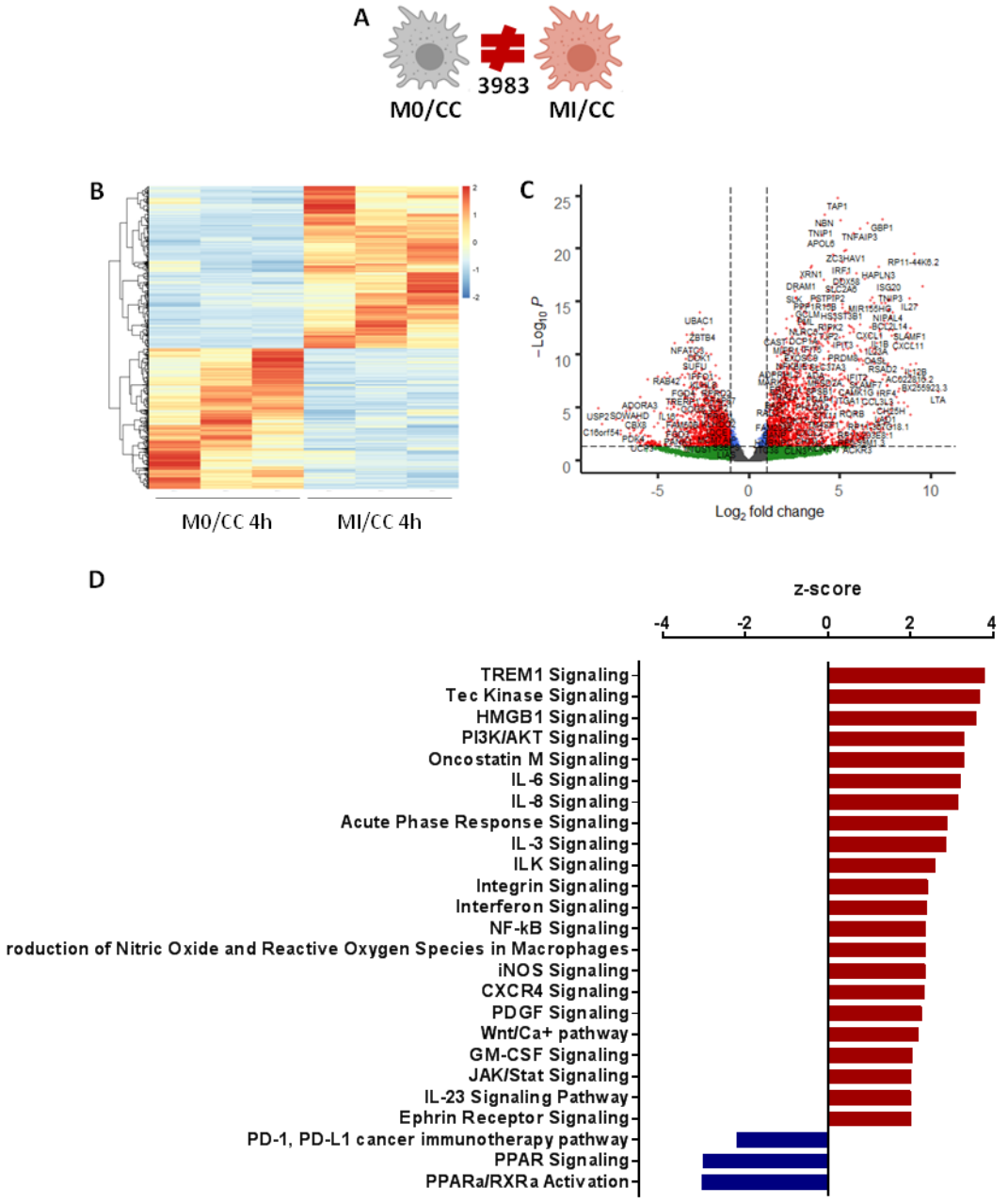


Fig. 43 Comparison M0/CCvsMI/CC. Schematic representation of M0/CCvsMI/CC comparison a 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C24 (S25vsS36)]

Fibroblasts stimulated by LPS+IFNγ for 4h in co-culture with macrophages show pro-inflammatory properties with a differential expression of 2131 genes in comparison with the co-cultivated fibroblasts without cytokine stimulation. Pathways enriched are mostly related to inflammation.

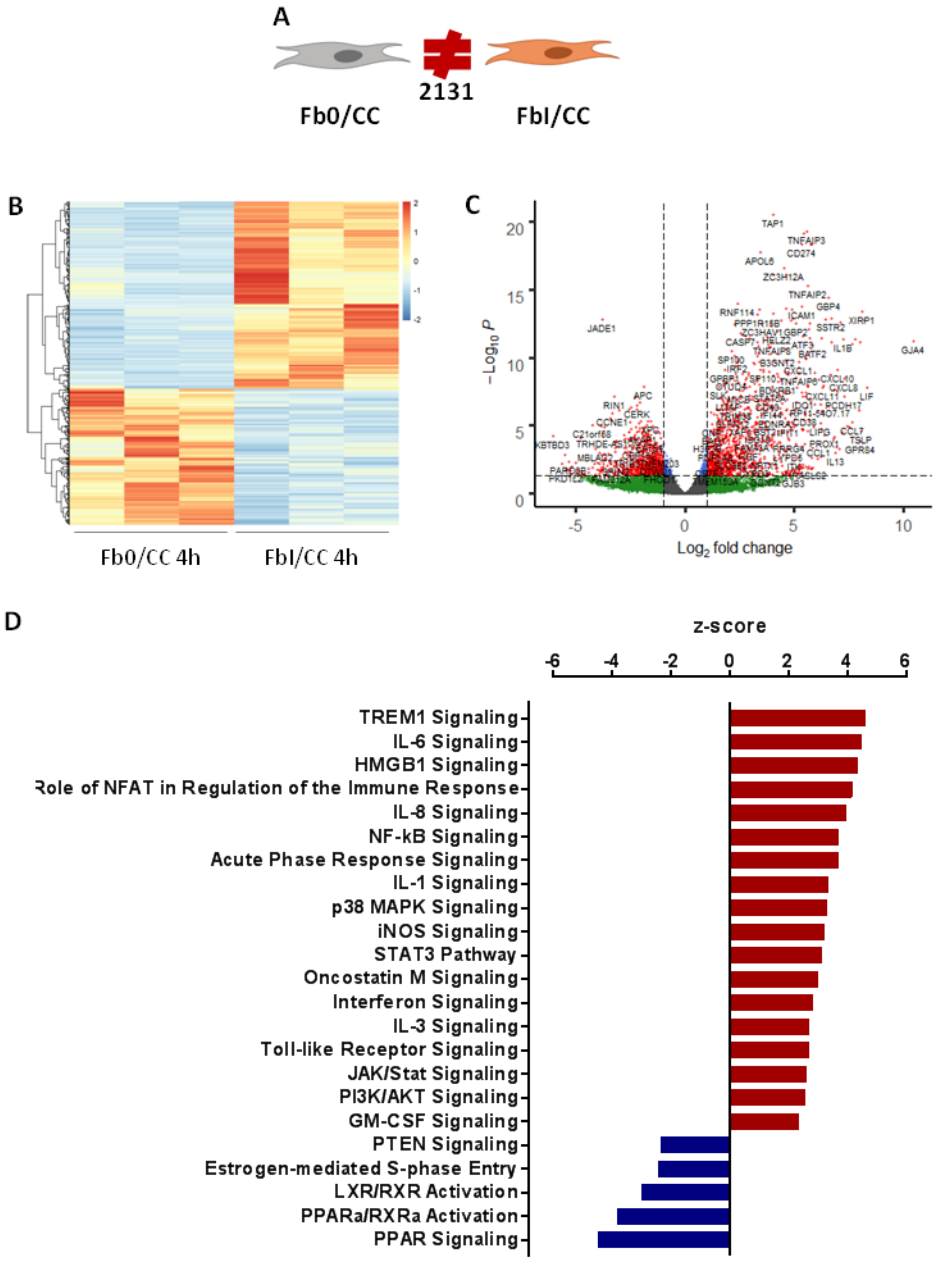


Fig. 44 Comparison Fb0/CCvsFbI/CC. Schematic representation of Fb0/CCvsFbI/CC comparison a 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; value are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C25 (S34vsS33)]

Co-cultivated macrophages stimulated for 24h with LPS+IFNγ maintain a pro-inflammatory phenotype; by comparing stimulated macrophages to resting cells in co-culture it is observed that 3840 genes are differentially expressed with the promotion of a pro-inflammatory phenotype.

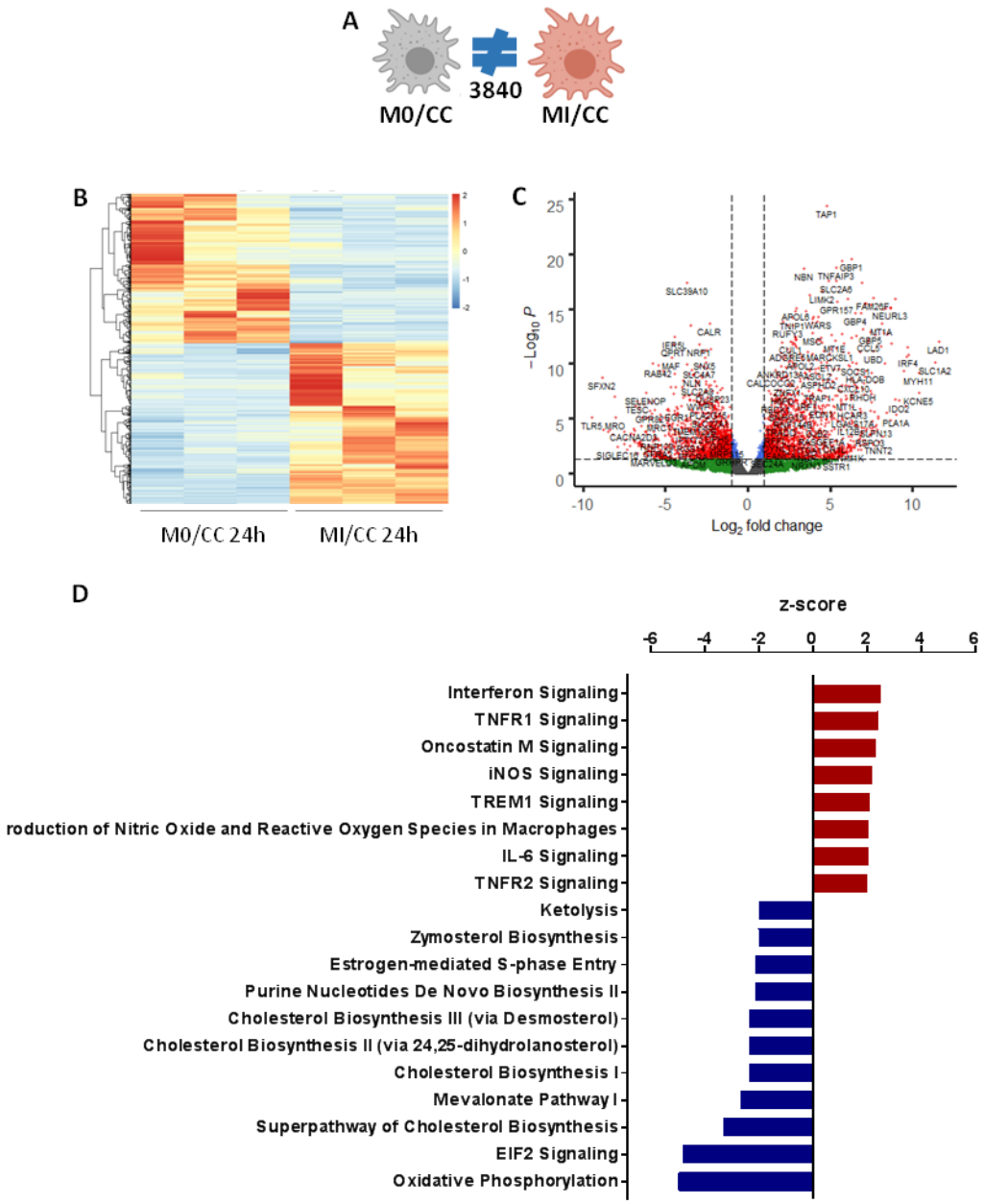


Fig. 45 Comparison M0/CCvsMI/CC. Schematic representation of M0/CCvsMI/CC comparison a 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as

$-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <-1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ -2 in blue (D).

[Code: C26 (S37vsS38)]

Co-cultivated fibroblasts stimulated by LPS+IFNγ for 24h show pro-inflammatory properties with a differential expression of 4841 genes in comparison with the co-cultivated resting fibroblasts. Pathways enriched are mostly related to inflammation.

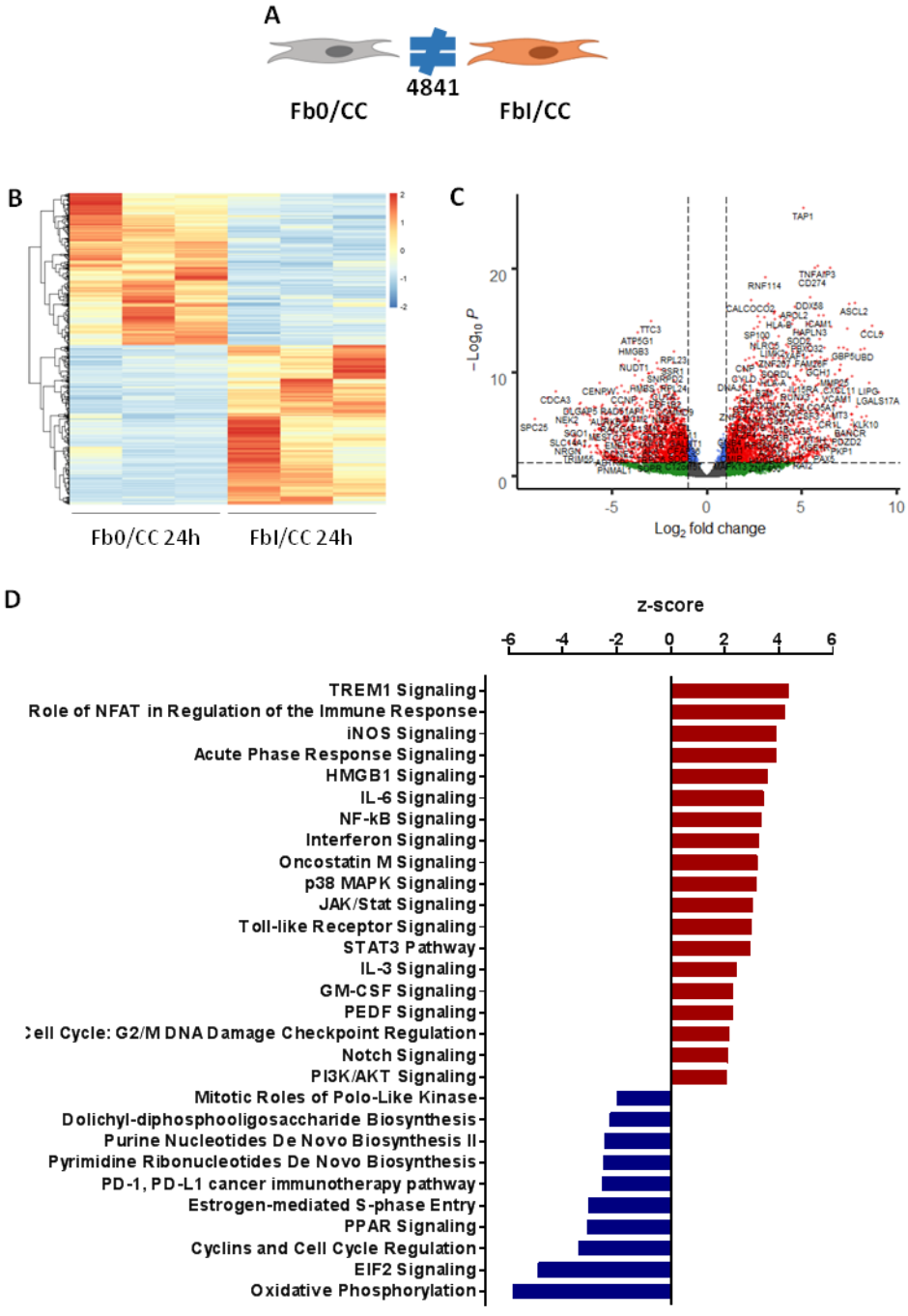


Fig. 46 Comparison Fb0/CCvsFbI/CC (below). Schematic representation of Fb0/CCvsFbI/CC comparison at 24h with the number of genes differentially expressed (FDR<0.05)(A); Heatmap of genes significantly differentially expressed; value are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|logFC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

8.1.2.4 What is the effect of pro-inflammatory factors (LPS+IFNγ) on co-cultivated hypoxic Mφ and Fb?

[Code: C27 (S28vsS27)]

Macrophages stimulated for 4h with LPS+IFNγ in co-culture with fibroblasts under hypoxia are different from co-cultivated hypoxic macrophages that do not receive the pro-inflammatory stimulation; 3574 genes are differentially expressed with an enrichment of pathways related to inflammation (TREM1 signalling, IL-6/IL-8 signalling, etc).

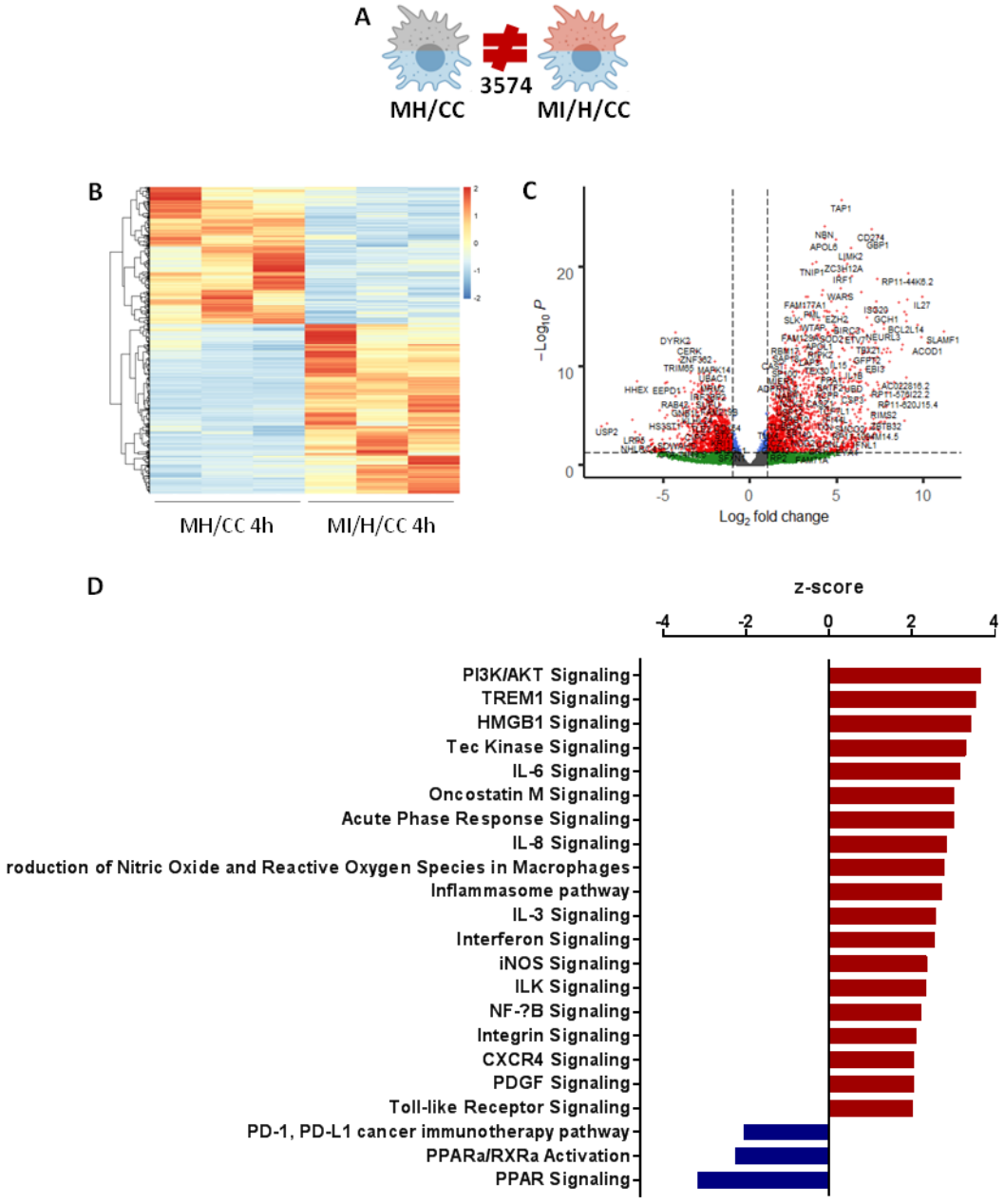


Fig. 47 Comparison MH/CCvsMI/H/CC. Schematic representation of MH/CCvsMI/H/CC comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C28 (S31vsS30)]

Fibroblasts stimulated by LPS+IFNγ for 4h in co-culture with macrophages under hypoxia show pro-inflammatory properties with a differential expression of 1141 genes in comparison with the co-cultivated hypoxic fibroblasts without cytokine stimulation. Pathway enriched are mostly related to inflammation.

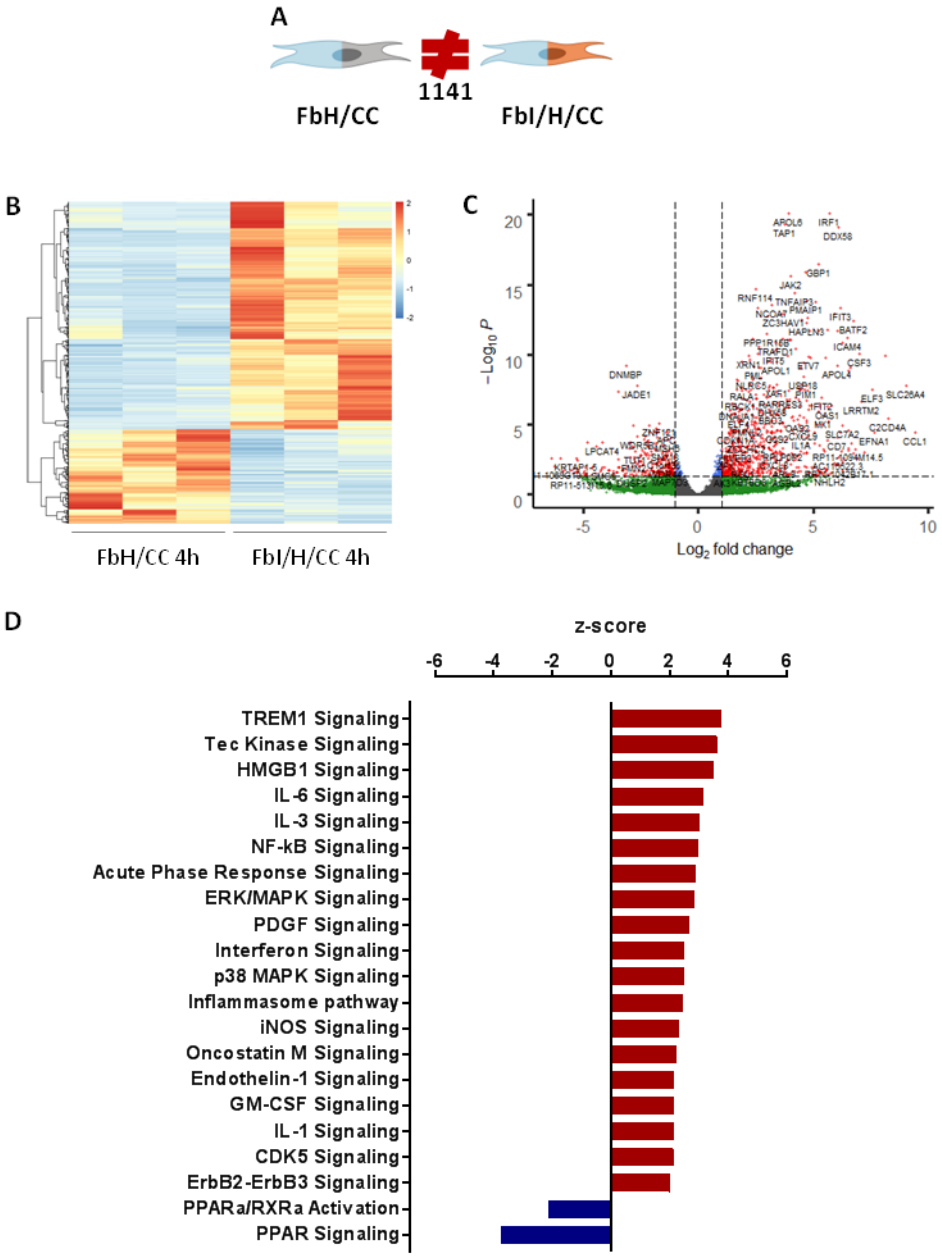


Fig. 48 Comparison FbH/CCvsFbI/H/CC. Schematic representation of FbH/CCvsFbI/H/CC comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C29 (S40vsS39)]

Co-cultivated hypoxic macrophages stimulated for 24h with LPS+IFNγ maintain a pro-inflammatory phenotype; by comparing stimulated macrophages to resting cells in co-culture under hypoxia, it is observed that 4663 genes are differentially expressed with the promotion of a pro-inflammatory phenotype.

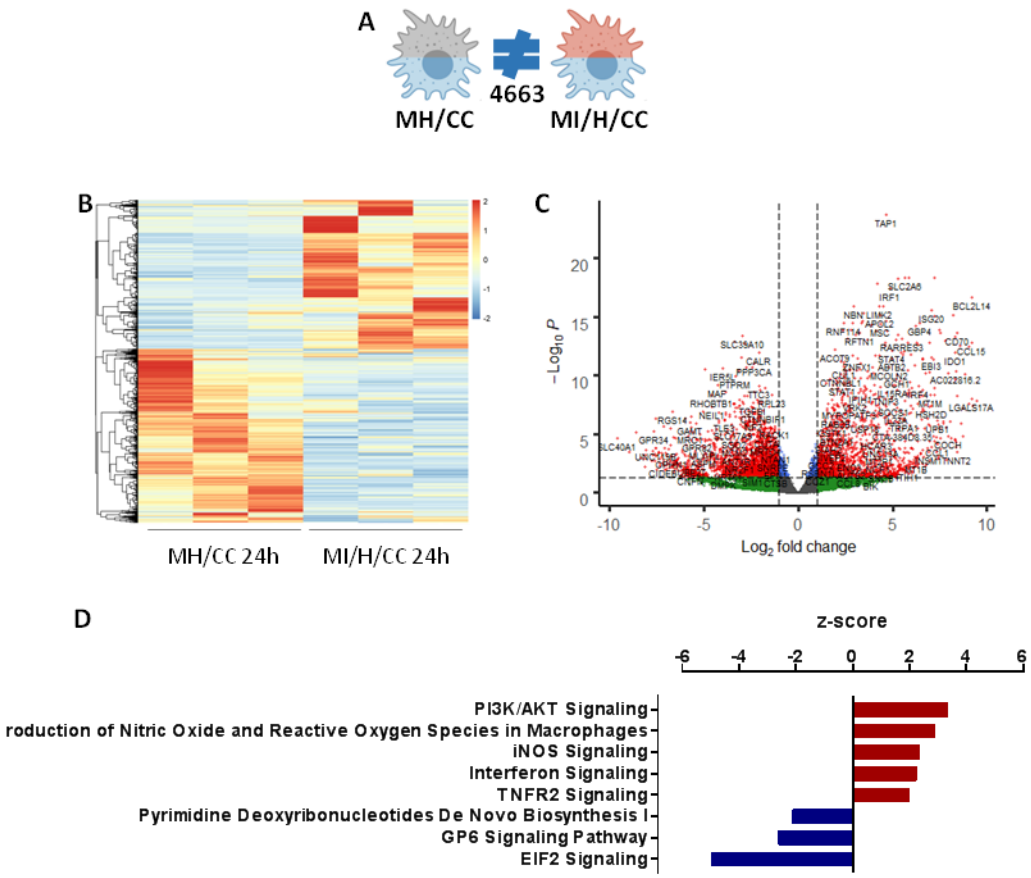


Fig. 49 Comparison MH/CCvsMI/H/CC. Schematic representation of MH/CCvsMI/H/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C30 (S43vsS42)]

Co-cultivated fibroblasts stimulated by LPS+IFNγ for 24h in hypoxia show pro-inflammatory properties with a differential expression of 4106 genes in comparison with the co-cultivated hypoxic fibroblasts. Pathways enriched are mostly related to inflammation.

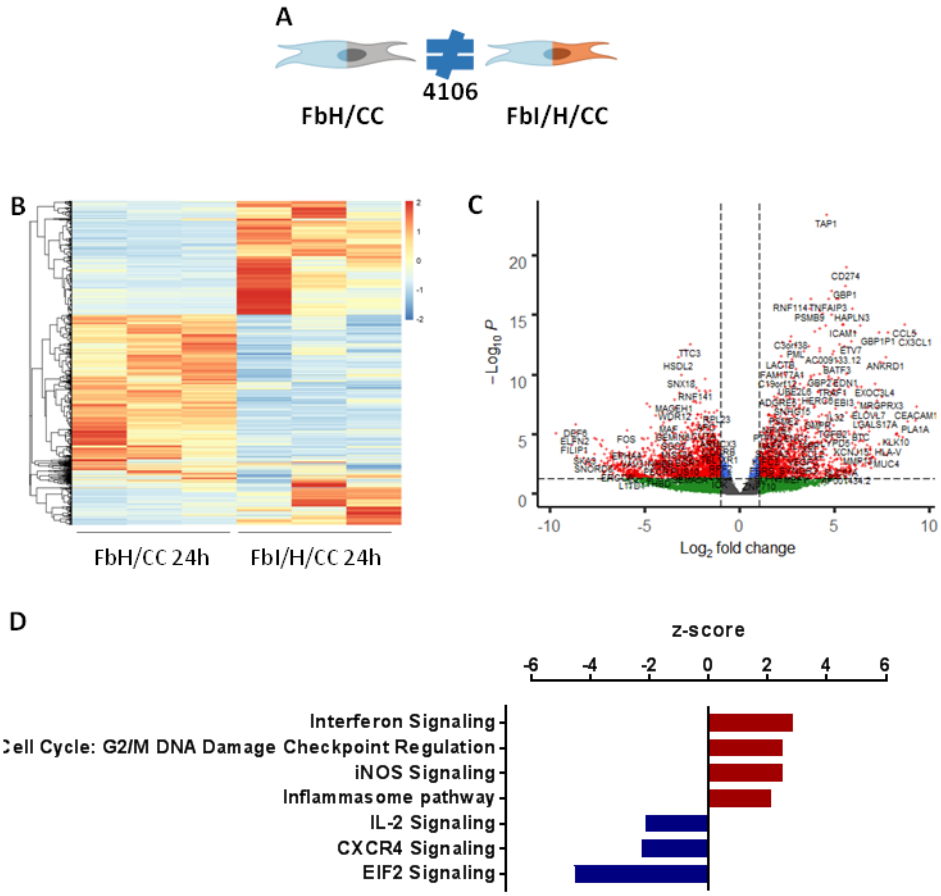


Fig. 50Comparison FbH/CCvsFbI/H/CC. Schematic representation of FbH/CCvsFbI/H/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-Log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as Log_2 fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

8.1.2.5 What is the effect of hypoxia on pro-inflammatory Mφ and Fb?

[Code: C31 (S6vsS1)]

Pro-inflammatory macrophages after 4h of hypoxia are similar to pro-inflammatory macrophages in normoxic context; 22 genes only are significantly differentially expressed.

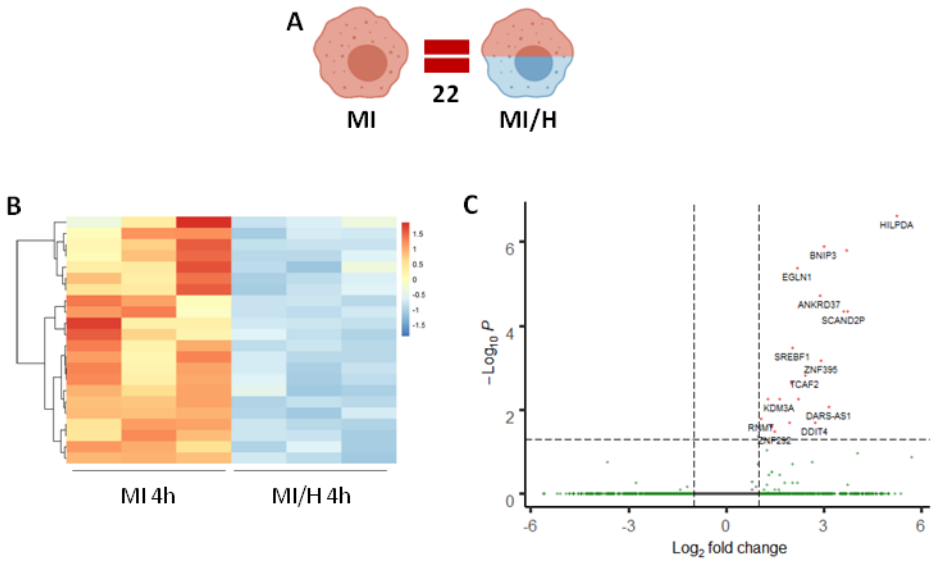


Fig. 51 Comparison MIvsMI/H. Schematic representation of MIvsMI/H comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C32 (S9vsS3)]

Fibroblasts stimulated by LPS+IFNγ for 4h under hypoxia show the same phenotype to normoxic counterpart.

Fig. 52 Comparison FbIvsFbI/H. Schematic representation of FbIvsFbI/H comparison at 4h with the number of SDEG (FDR<0.05)(A).

[Code: C33 (S18vsS12)]

Pro-inflammatory macrophages after 24h of hypoxia are similar to pro-inflammatory macrophages in normoxic context; 32 genes only are differentially expressed.

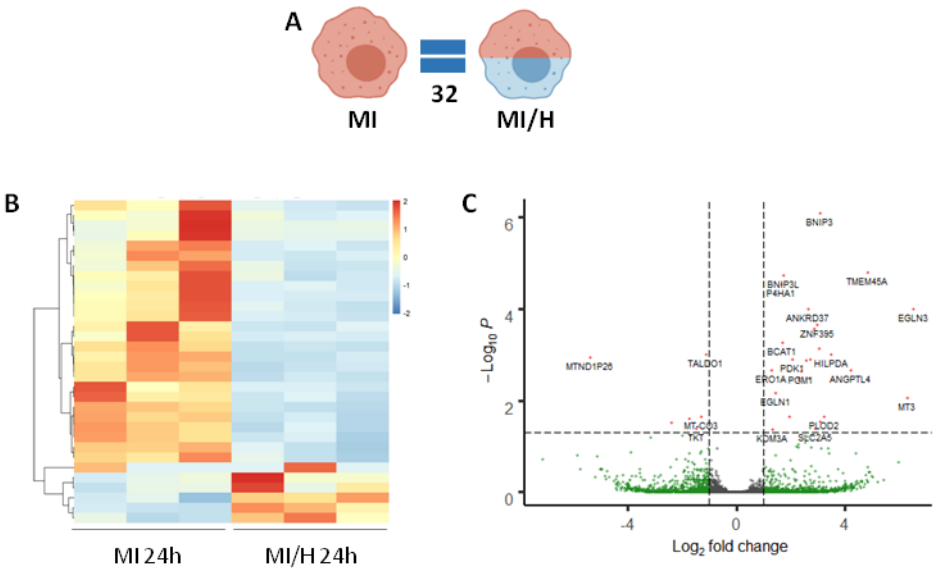


Fig. 53 Comparison MIvsMI/H. Schematic representation of MIvsMI/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of genes SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C34 (S21vsS15)]

Fibroblasts stimulated by LPS+IFNγ for 24h under hypoxia show the same phenotype to normoxic counterpart with 12 genes significantly differentially expressed.

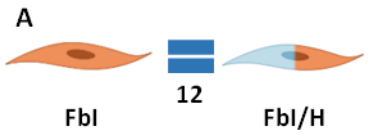


Fig. 54 Comparison FbIvsFbI/H. Schematic representation of FbIvsFbI/H comparison at 24h with the number of SDEG(FDR<0.05)(A).

8.1.2.6 What is the effect of hypoxia on co-cultivated pro-inflammatory Mφ and Fb?

[Code: C35 (S28vsS23)]

Pro-inflammatory macrophages after 4h of co-culture with fibroblasts under hypoxia are similar to co-cultivated pro-inflammatory macrophages in normoxia; 17 genes only are significantly differentially expressed.

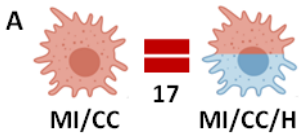


Fig. 55 Comparison MI/CCvsMI/CC/H. Schematic representation of MI/CCvsMI/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A).

[Code: C36 (S31vsS25)]

Pro-inflammatory fibroblasts after 4h of co-culture with macrophages under hypoxia are the same of co-cultivated pro-inflammatory fibroblasts in normoxia.

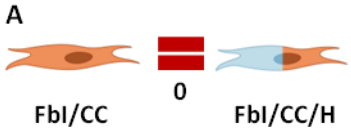


Fig. 56 Comparison FbI/CcvsFbI/CC/H. Schematic representation of FbI/CcvsFbI/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A).

[Code: C37 (S40vsS34)]

Pro-inflammatory macrophages after 24h of co-culture with fibroblasts under hypoxia are similar to co-cultivated pro-inflammatory macrophages in normoxia; 37 genes only are differentially expressed.

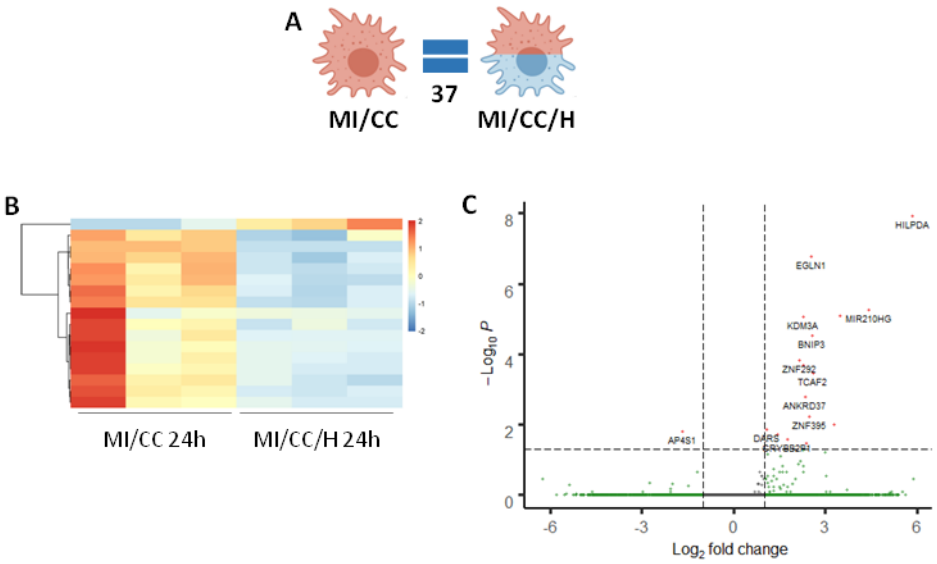


Fig. 57 Comparison MI/CcvsMI/CC/H. Schematic representation of MI/CcvsMI/CC/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C38 (S43vsS37)]

Pro-inflammatory fibroblasts after 24h of co-culture with macrophages under hypoxia are the same of co-cultivated pro-inflammatory fibroblasts in normoxia with 22 genes significantly differentially expressed.

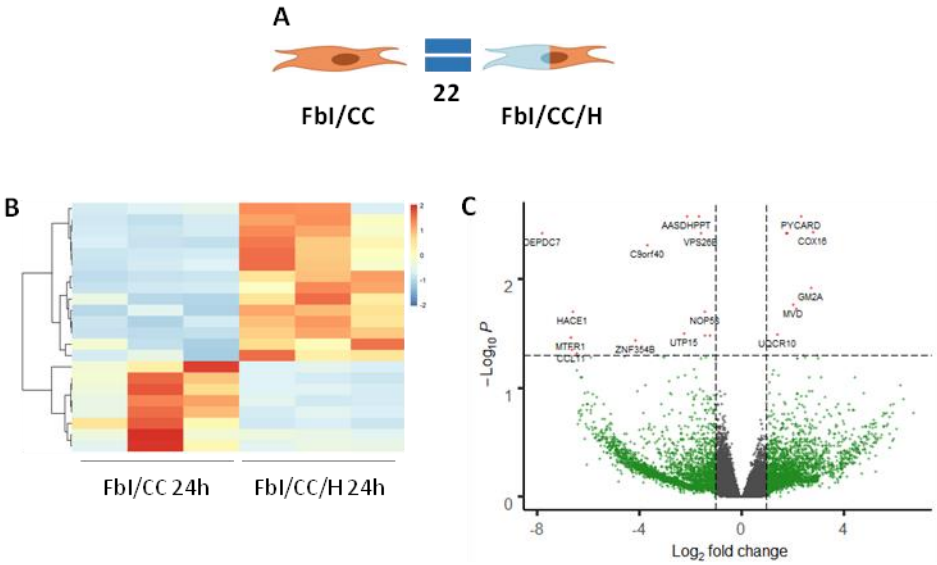


Fig. 58 Comparison FbI/CcvsFbI/CC/H. Schematic representation of FbI/CcvsFbI/CC/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

8.1.2.7 What is the effect of co-culture on pro-inflammatory Mφ and Fb?

[Code: C39 (S23vsS1)]

Pro-inflammatory macrophages after 4h of co-culture with fibroblasts are similar to pro-inflammatory macrophages alone; 14 genes only are significantly differentially expressed.

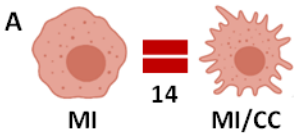


Fig. 59 Comparison MIvsMI/CC. Schematic representation of MIvsMI/CC comparison at 4h with the number of SDEG (FDR<0.05)(A).

[Code: C40 (S25vsS3)]

Pro-inflammatory fibroblasts after 4h of co-culture with macrophages are the same of pro-inflammatory fibroblasts alone.

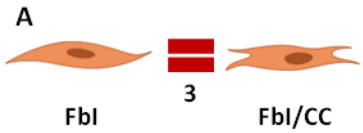


Fig. 60 Comparison FbIvsFbI/CC; schematic representation of FbIvsFbI/CC comparison at 4h with the number of SDEG (FDR<0.05)(A).

[Code: C41 (S34vsS12)]

Pro-inflammatory macrophages after 24h of co-culture with fibroblasts are different from pro-inflammatory macrophages alone; 110 genes are differentially expressed, most of them are

related to pro-inflammatory pathways but there are also a decrease in activation of pathways connected to metabolic functions.

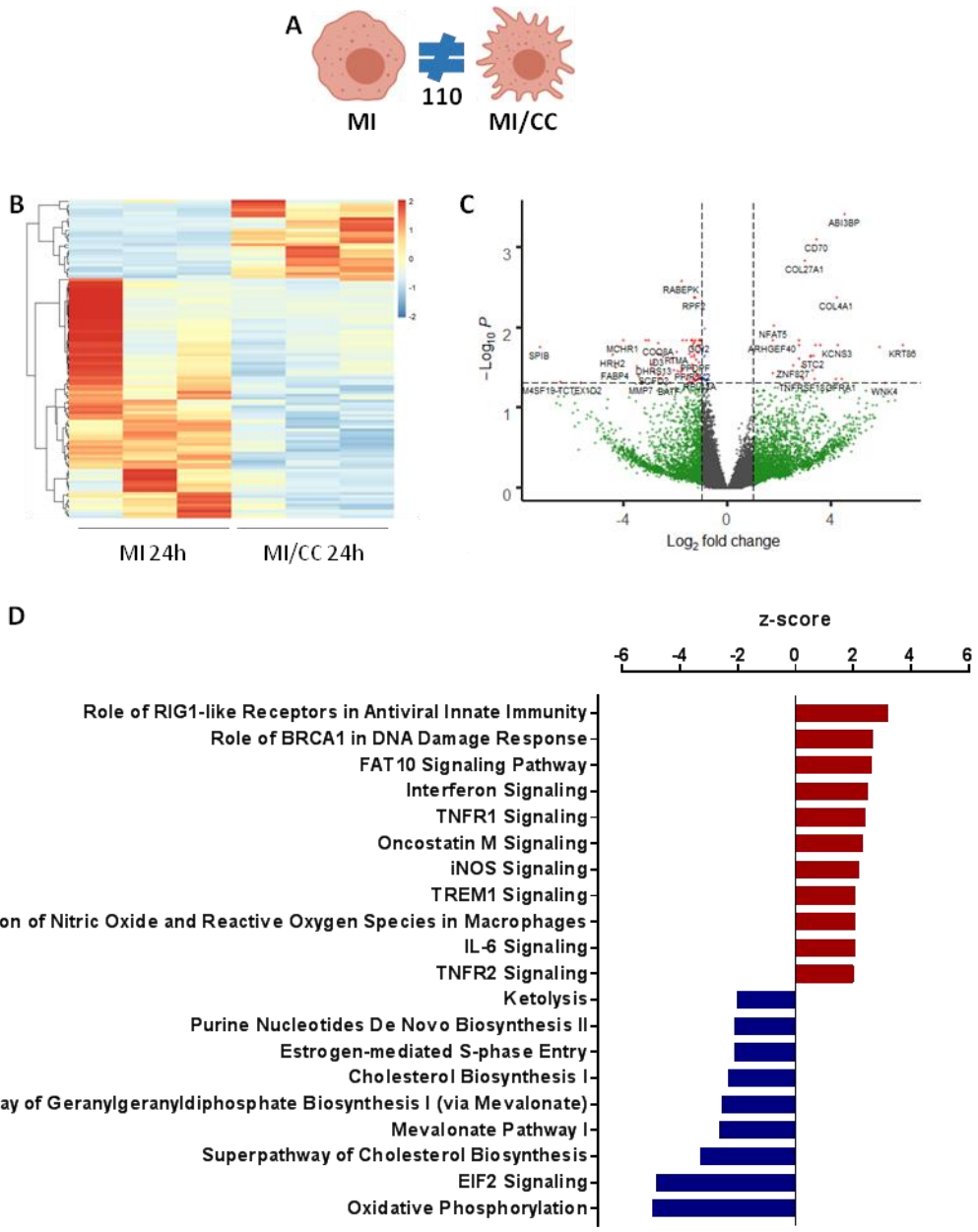


Fig. 61 Comparison MIvsMI/CC. Schematic representation of MIvsMI/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C42 (S37vsS15)]

Pro-inflammatory fibroblasts after 24h of co-culture with macrophages are different from pro-inflammatory fibroblasts alone; 1973 genes are differentially expressed, most of them are related to pro-inflammatory pathways but there are also a decrease in activation of pathways connected to metabolic functions.

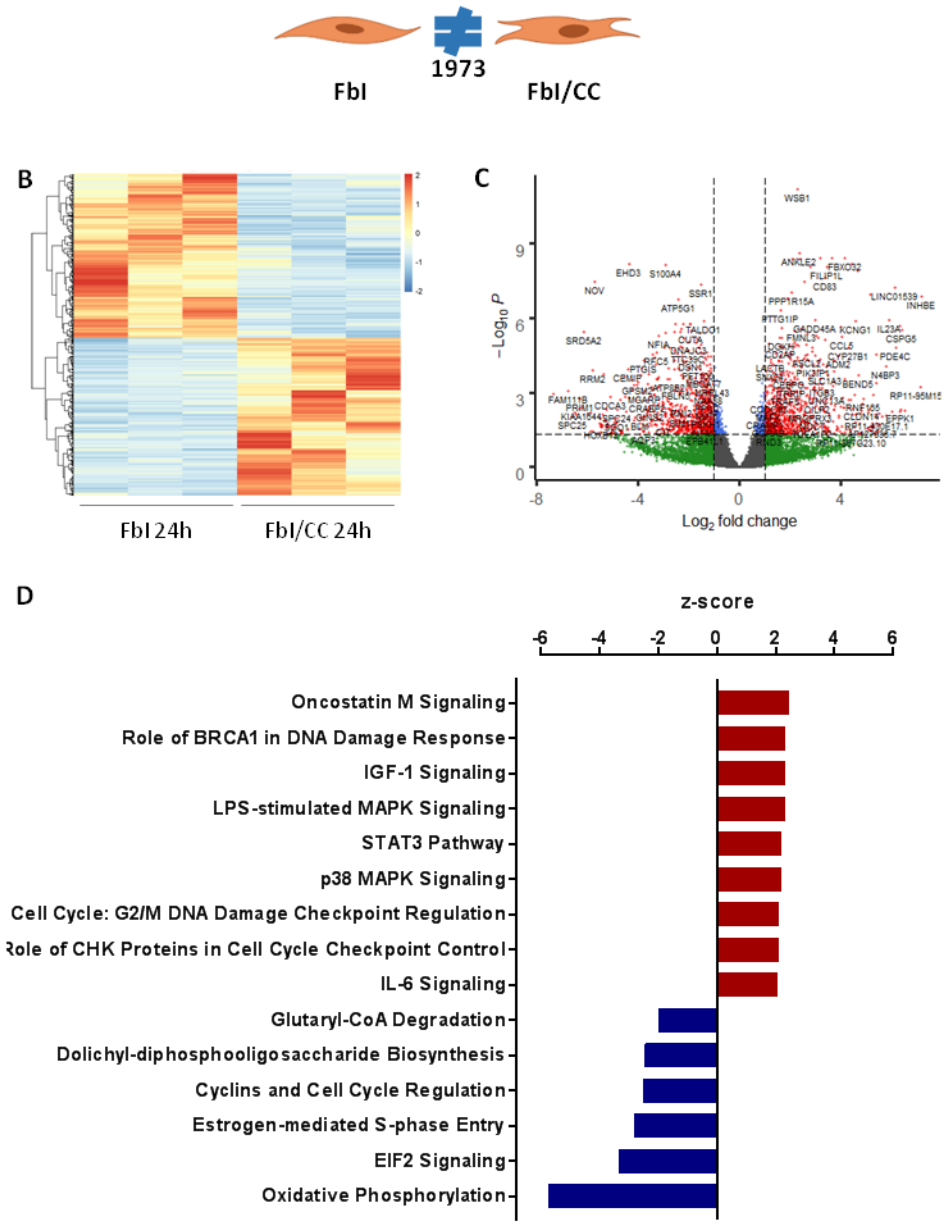


Fig. 62 Comparison FbIvsFbI/CC. Schematic representation of FbIvsFbI/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|logFC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

8.1.2.8 What is the effect of co-culture on hypoxic pro-inflammatory Mφ and Fb?

[Code: C43 (S28vsS6)]

Pro-inflammatory macrophages after 4h of co-culture with fibroblasts under hypoxia are similar to pro-inflammatory hypoxic macrophages alone; 38 genes only are differentially expressed.

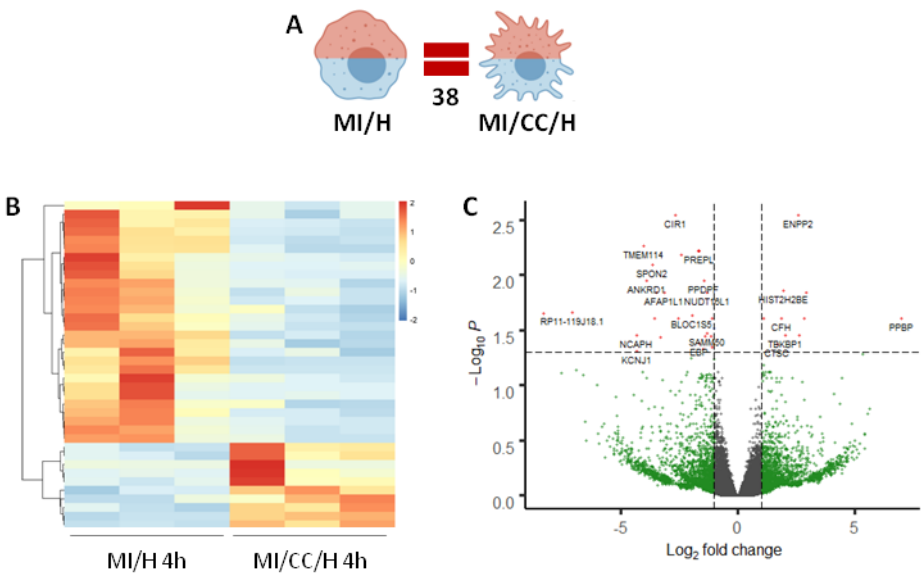


Fig. 63 Comparison MI/HvsMI/CC/H. Schematic representation of MI/HvsMI/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C44 (S31vsS9)]

Pro-inflammatory fibroblasts after 4h of co-culture with macrophages under hypoxia are similar to pro-inflammatory hypoxic fibroblasts alone (25 genes significantly differentially expressed).

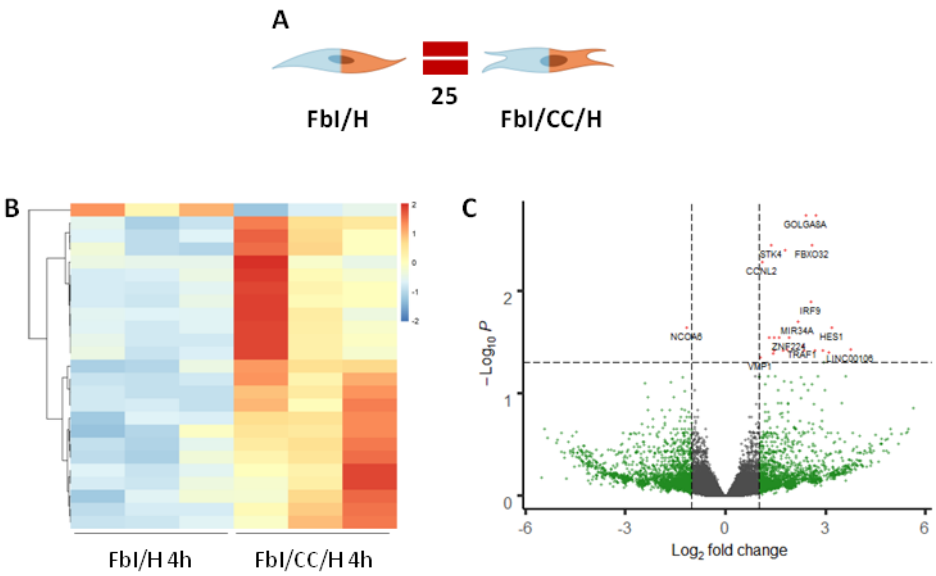


Fig. 64 Comparison FbI/HvsFbI/CC/H. Schematic representation of FbI/HvsFbI/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of genes SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C45 (S40vsS18)]

Pro-inflammatory macrophages after 24h of co-culture with fibroblasts under hypoxia are different from pro-inflammatory hypoxic macrophages alone; 943 genes are differentially expressed; in this case most of genes are related to actin remodelling, VEGF, PDGF signalling.

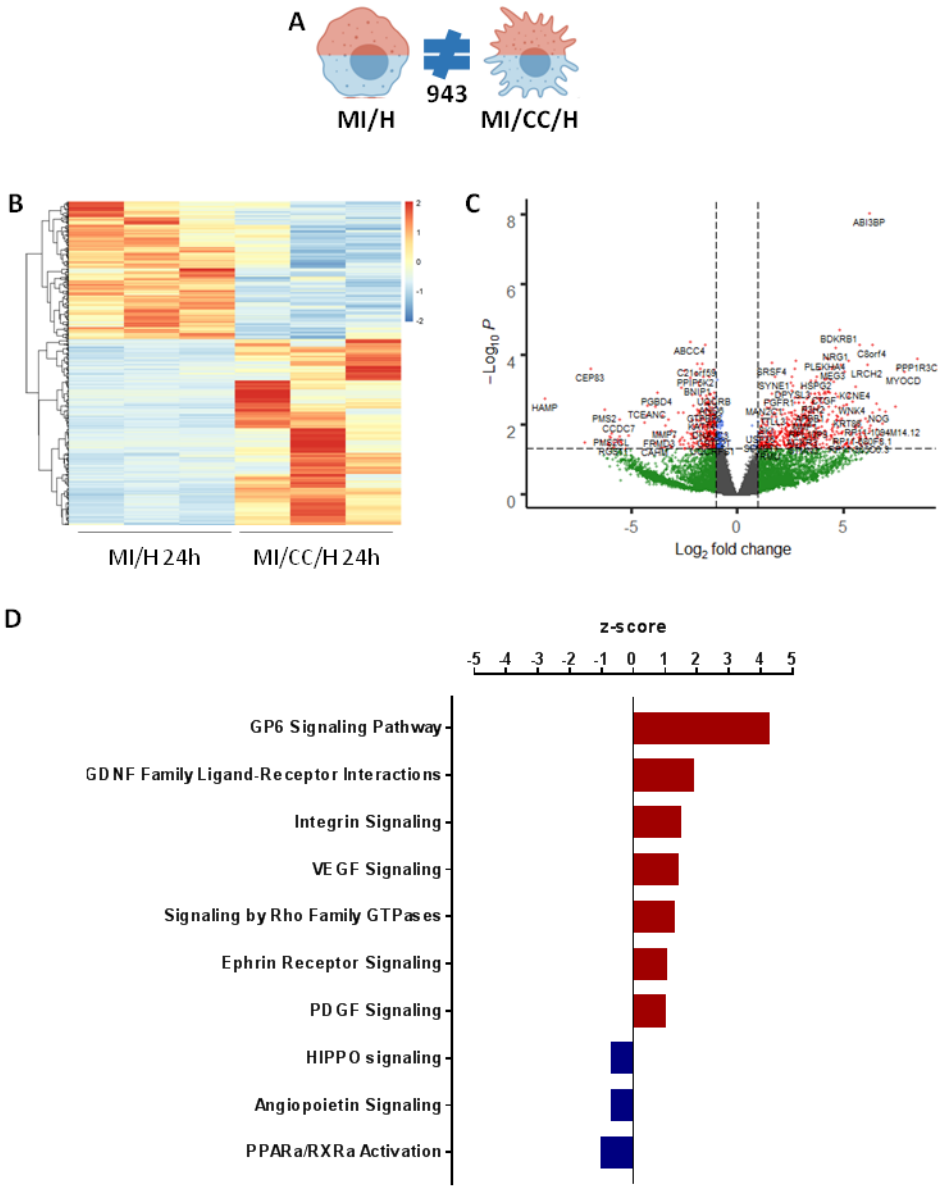


Fig. 65 Comparison MI/HvsMI/CC/H. Schematic representation of MI/HvsMI/CC/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|logFC| \geq 1$ (represented on the x axis as $\log_2$ fold change <-1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤ -2 in blue (D).

[Code: C46 (S43vsS21)]

Pro-inflammatory fibroblasts after 24h of co-culture with macrophages under hypoxia are different from pro-inflammatory hypoxic fibroblasts alone; 1900 genes are differentially

expressed, most of them are related to leukocyte extravasation signalling, IL-8 signalling, PDGF signalling.

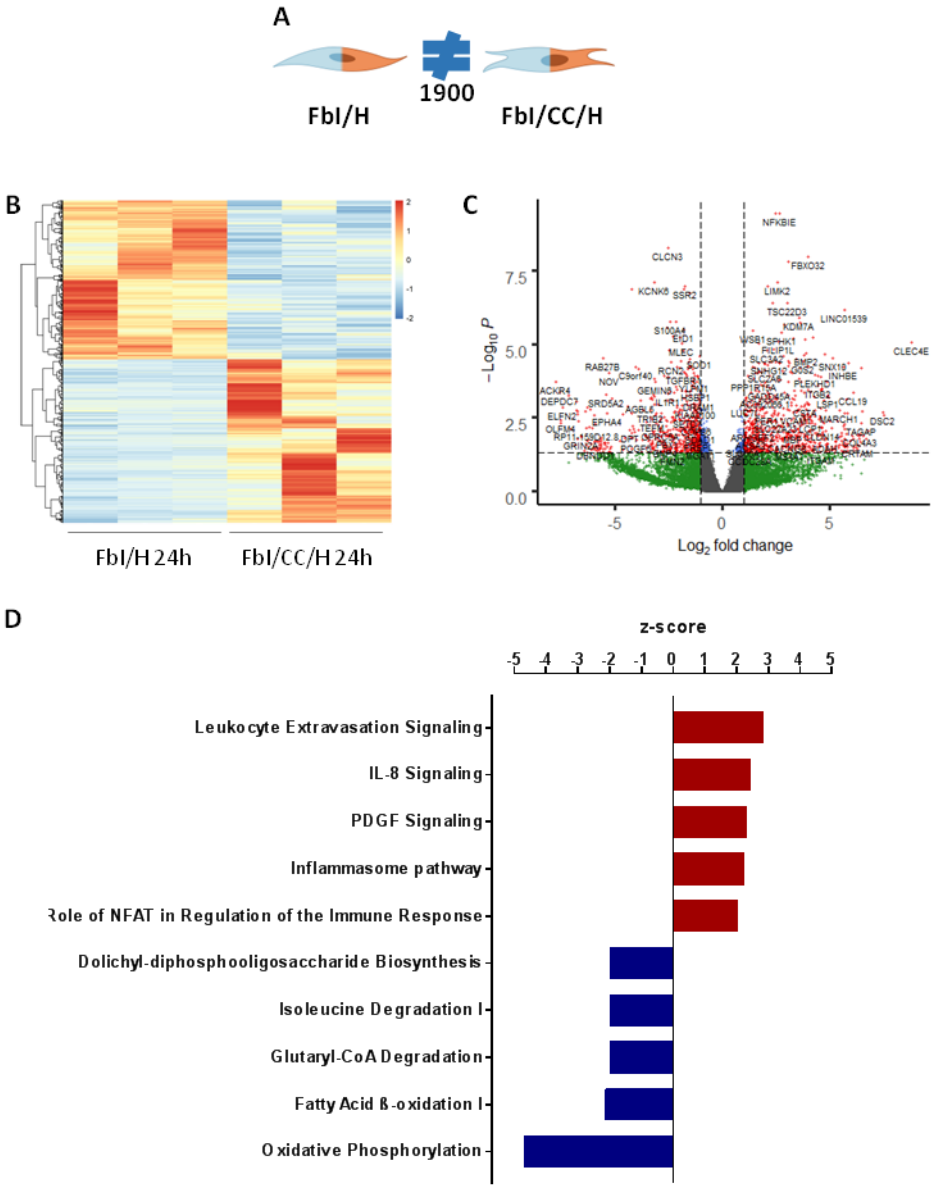


Fig. 66 Comparison FbI/HvsFbI/CC/H. Schematic representation of FbI/HvsFbI/CC/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

SUMMARY FIRST LEVEL: PRO-INFLAMMATORY CONDITION

Significant differences come up in all comparisons that are based on pro-inflammatory versus resting conditions:

C15-C16-C17-C18

C19-C20-C21-C22

C23-C24-C25-C26

C27-C28-C29-C30

Differences emerge also in other two comparisons:

C37-C38

C45-C46

8.1.3.1 What is the effect of pro-fibrotic cytokine (Il-4) on Mφ and Fb?

[Code: C47 (S2vsS11)]

Macrophages stimulated for 4h with IL-4 show an alternative phenotype as reported in literature; by comparing stimulated macrophages to resting cells it is observed that genes are significantly differentially expressed with a characteristic metabolic switch.

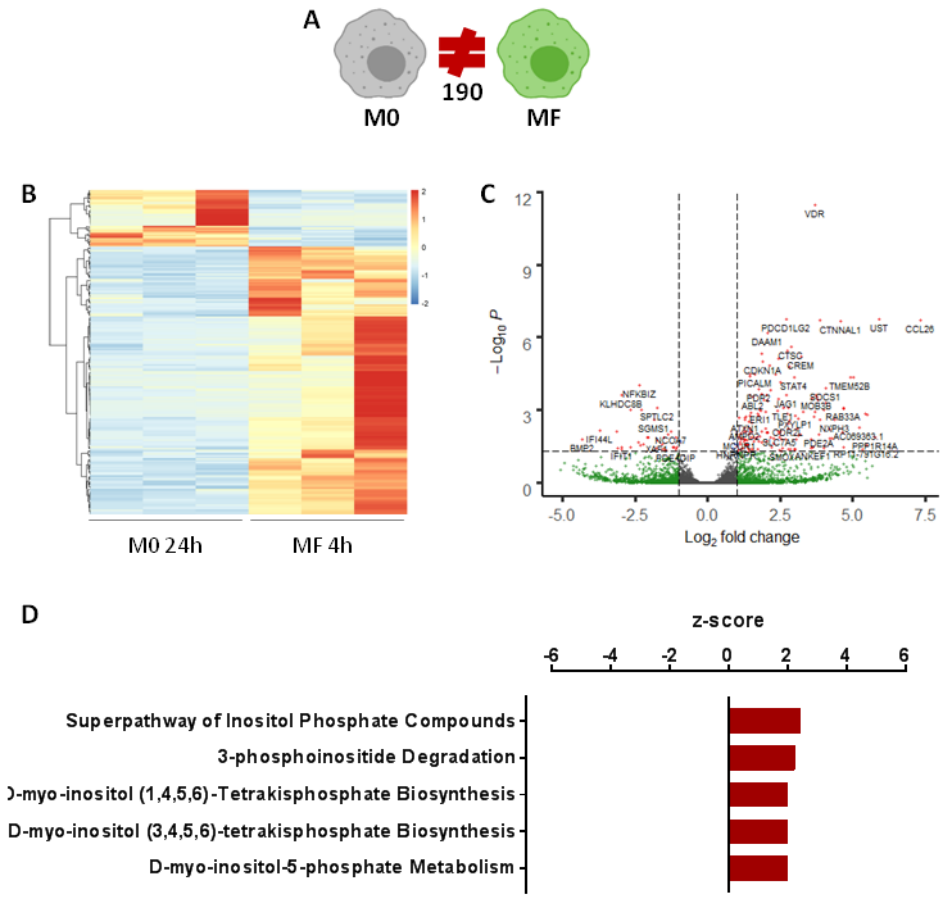


Fig. 67 Comparison M0vsMF. Schematic representation of M0vsMF comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of genes SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C48 (S4vsS14)]

Fibroblasts stimulated for 4h with IL-4 show no changing in comparison with resting fibroblasts.

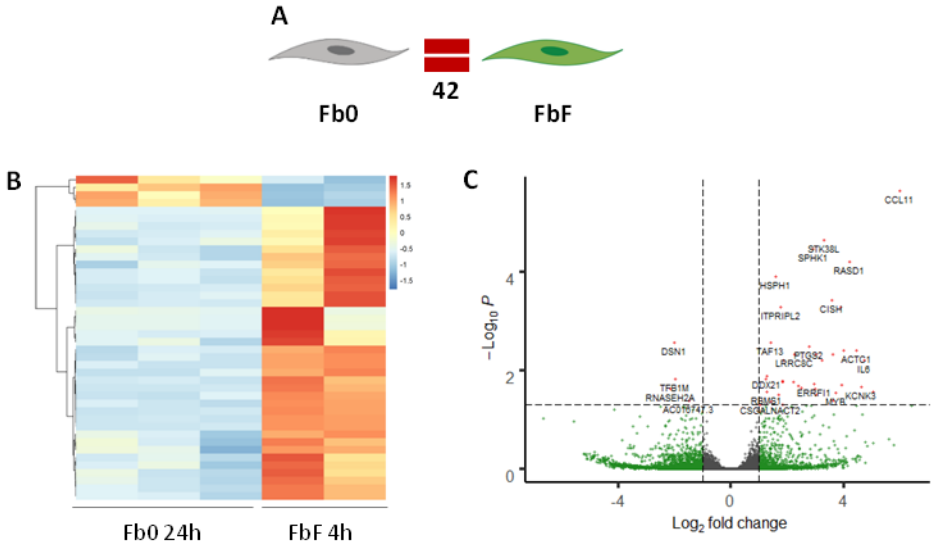


Fig. 68 Comparison Fb0vsFbF. Schematic representation of Fb0vsFbF comparison at 4h with the number of SDEG (FDR<0.05) (A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as –log10P >1.3) and a |logFC|≥1 (represented on the x axis as log2 fold change < –1 or >1) (C).

[Code: C49 (S13vsS11)]

Macrophages stimulated for 24h with IL-4 maintain the same alternative phenotype already acquire at 4h with 178 genes significantly differentially expressed.

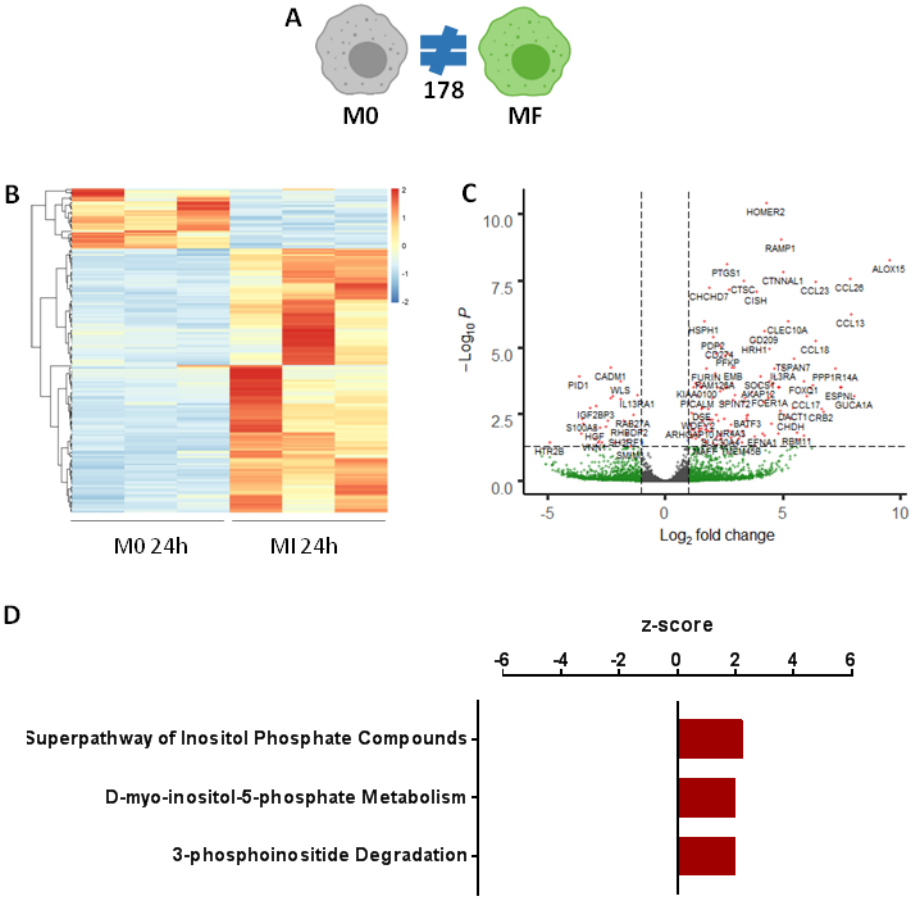


Fig. 69 Comparison M0vsMF. Schematic representation of M0vsMF comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C50 (S16vsS14)]

Fibroblasts stimulated for 24h with IL-4 show no changing in comparison with resting fibroblasts.

Fig. 70 Comparison Fb0vsFbF. Schematic representation of Fb0vsFbF comparison at 24h with the number of SDEG (FDR<0.05)(A).

8.1.3.2 What is the effect of IL-4 on hypoxic Mφ and Fb?

[Code: C51 (S7vsS5)]

Macrophages stimulated for 4h with IL-4 in hypoxic environment are different from hypoxic macrophages that do not receive the pro-fibrotic stimulation; 88 genes are significantly differentially expressed.

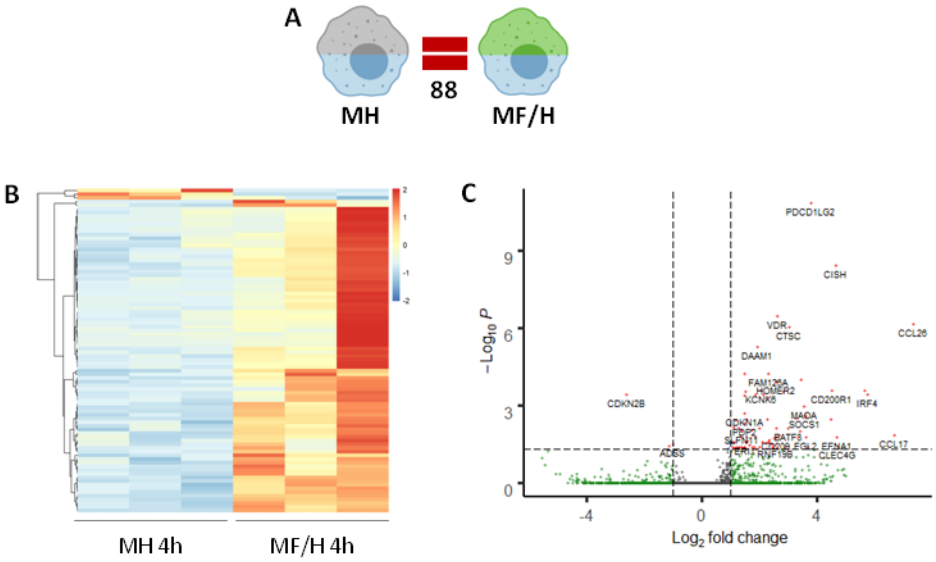


Fig. 71 Comparison MHvsMF/H. Schematic representation of MHvsMF/H comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C52 (S10vsS8)]

Hypoxic fibroblasts stimulated for 4h with IL-4 show no changing in comparison with hypoxic resting fibroblasts.

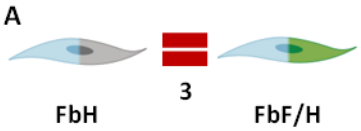


Fig. 72 Comparison FbHvsFbF/H. Schematic representation of FbHvsFbF/H comparison at 4h with the number of SDEG (FDR<0.05)(A).

[Code: C53 (S19vsS17)]

Macrophages stimulated for 24h with IL-4 in hypoxic environment are different from hypoxic macrophages that do not receive the pro-fibrotic stimulation; 144 genes are significantly differentially expressed.

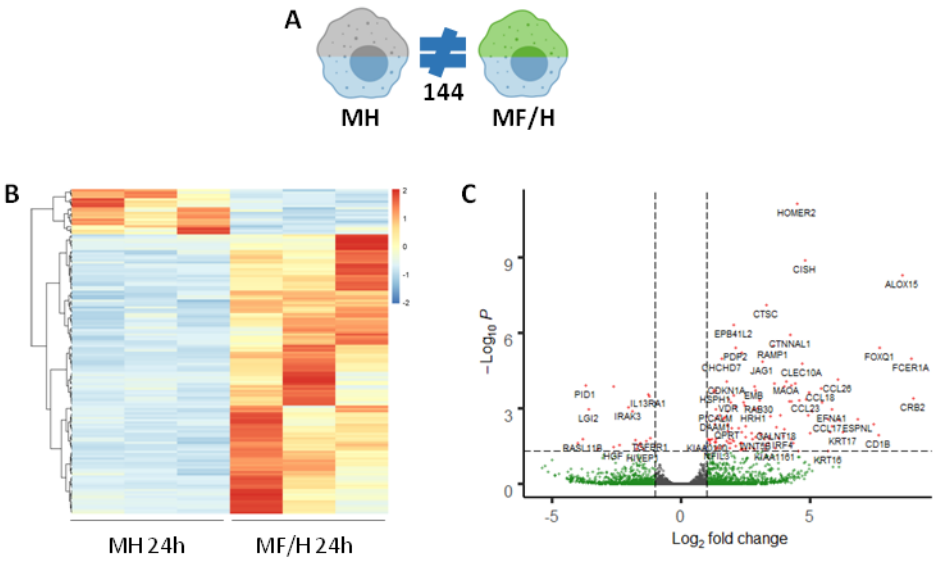


Fig. 73 Comparison MHvsMF/H. Schematic representation of MHvsMF/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|logFC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C54 (S22vsS20)]

Hypoxic fibroblasts stimulated for 24h with IL-4 show no changing in comparison with hypoxic resting fibroblasts.

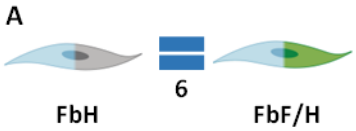


Fig. 74 Comparison FbHvsFbF/H. Schematic representation of FbHvsFbF/H comparison at 24h with the number of SDEG (FDR<0.05)(A).

8.1.3.3 What is the effect of IL-4 on co-cultivated Mφ and Fb?

[Code: C55 (S24vsS33)]

Macrophages stimulated for 4h with IL-4 in co-culture with fibroblasts are different from co-cultivated macrophages that do not receive the pro-fibrotic stimulation; 138 genes are differentially expressed with an enrichment of pathways related to metabolism and pro-fibrotic functions (Superpathway of inositol phosphate compounds, PTEN signalling, PDGF signalling).

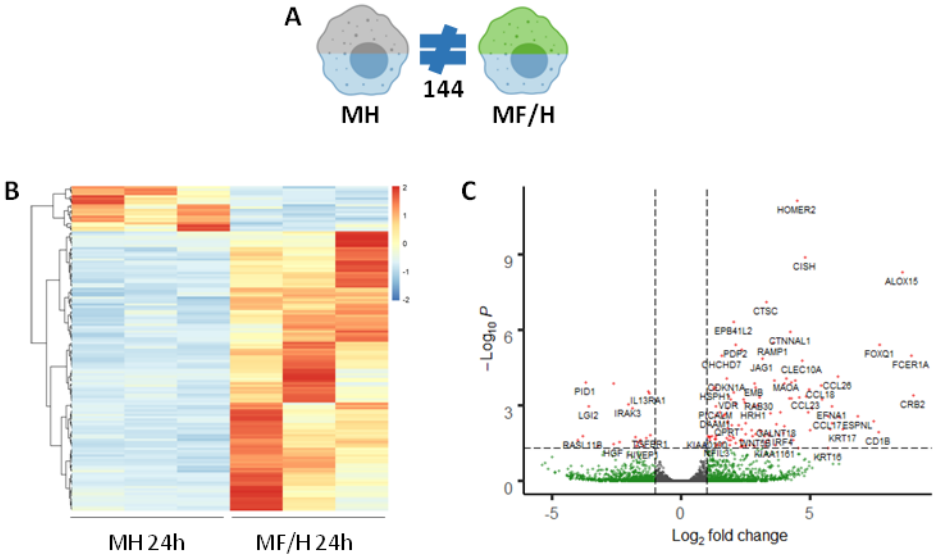


Fig. 75 Comparison M0/CCvsMF/CC. Schematic representation of M0/CCvsMF/CC comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|logFC| \geq 1$ (represented on the x axis as $\log_2$ fold change <-1 or >1)

(C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C56 (S26vsS36)]

Fibroblasts stimulated for 4h with IL-4 in co-culture with macrophages are different from co-cultivated fibroblasts that do not receive the pro-fibrotic stimulation; 133 genes are significantly differentially expressed with an enrichment of pathways related to inflammation (TREM1 signalling, IL-6 signalling etc).

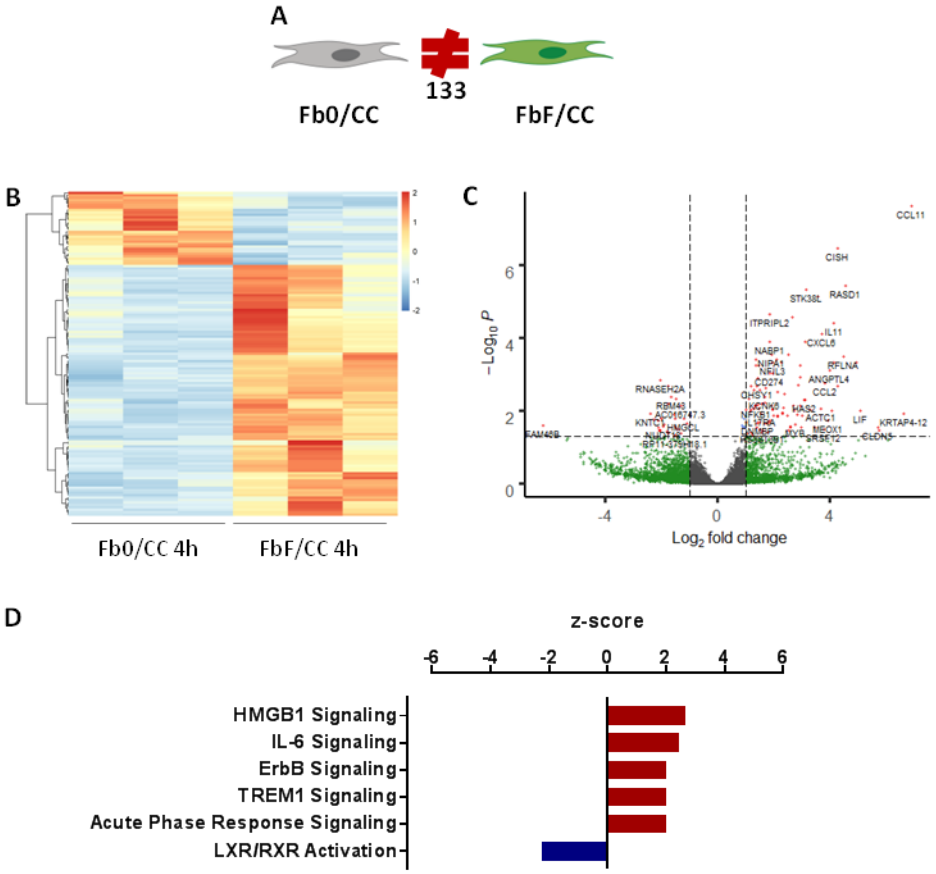


Fig. 76 Comparison Fb0/CCvsFbF/CC. Schematic representation of Fb0/CCvsFbF/CC comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C57 (S35vsS33)]

Macrophages stimulated for 24h with IL-4 in co-culture with fibroblasts are different from co-cultivated macrophages that do not receive the pro-fibrotic stimulation; 173 genes are differentially expressed with an enrichment of pathways related to PTEN signalling and T-cell exhaustion and a decreased activation of p38 MAPK and STAT3 signalling pathways.

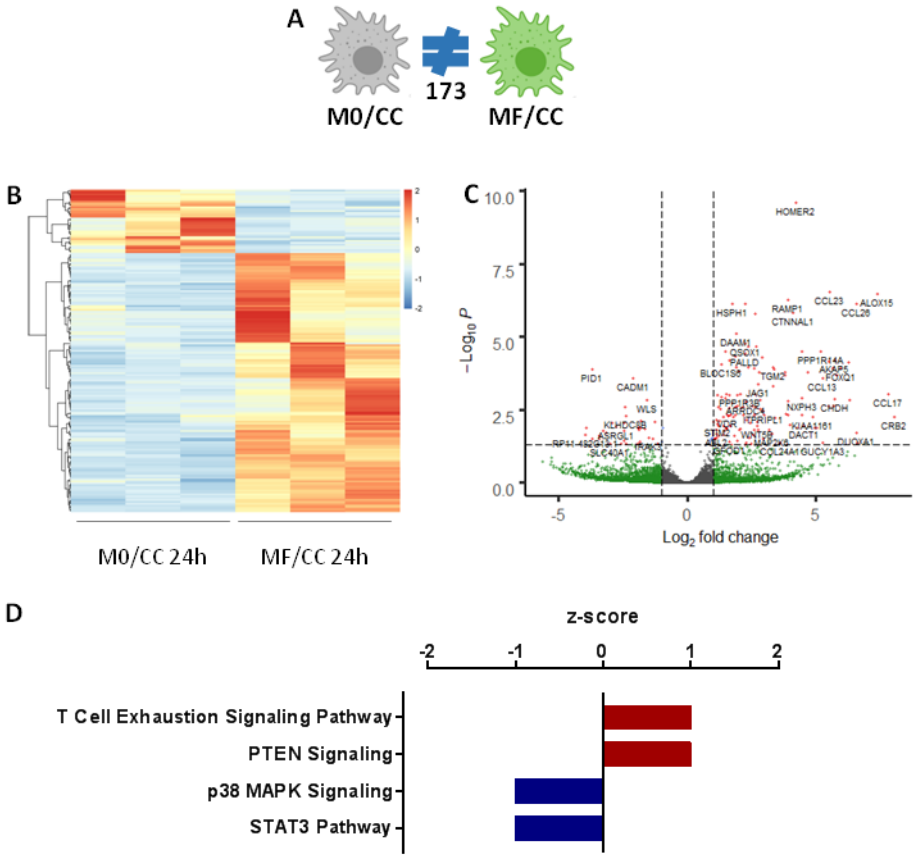


Fig. 77 Comparison M0/CCvsMF/CC. Schematic representation of M0/CCvsMF/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C58 (S38vsS36)]

Fibroblasts stimulated for 24h with IL-4 in co-culture with macrophages are equal to co-cultivated fibroblasts that do not receive the pro-fibrotic stimulation.

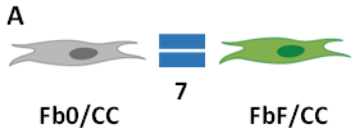


Fig. 78 Comparison Fb0/CCvsFbF/CC. Schematic representation of Fb0/CCvsFbF/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

8.1.3.4 What is the effect of IL-4 on co-cultivated hypoxic Mφ and Fb?

[Code: C59 (S29vsS27)]

Macrophages stimulated for 4h with IL-4 in co-culture with fibroblasts under hypoxia are similar to co-cultivated hypoxic macrophages that do not receive the pro-fibrotic stimulation; only 74 genes are significantly differentially expressed.

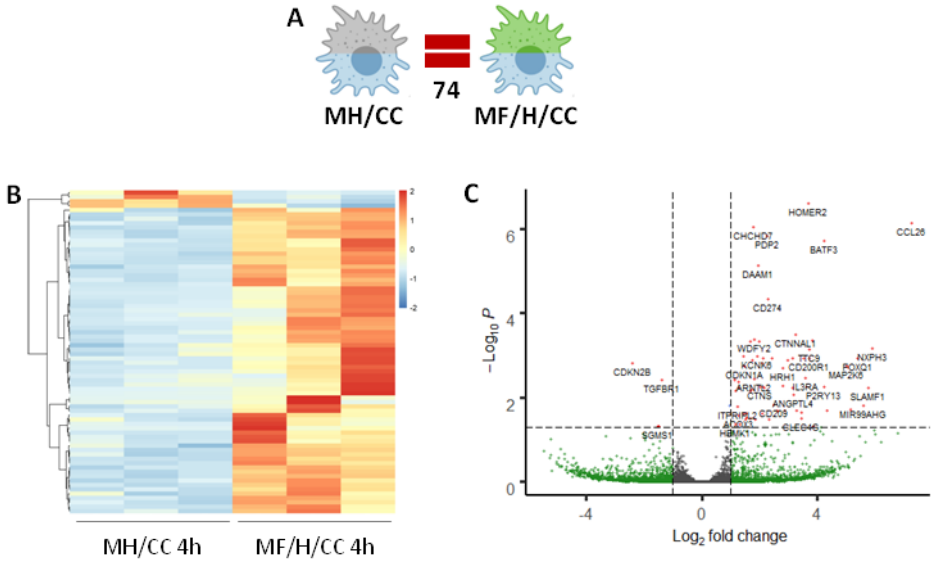


Fig. 79 Comparison MH/CCvsMF/H/CC. Schematic representation of MH/CCvsMF/H/CC comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on

the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C60 (S32vsS30)]

Fibroblasts stimulated for 4h with IL-4 in co-culture with macrophages under hypoxia are equal to co-cultivated hypoxic fibroblasts that do not receive the pro-fibrotic stimulation.

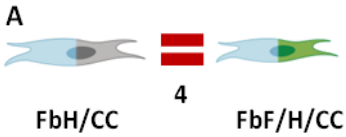


Fig. 80 Comparison FbH/CCvsFbF/H/CC. Schematic representation of FbH/CCvsFbF/H/CC comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; value are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C61 (S41vsS39)]

Macrophages stimulated for 24h with IL-4 in co-culture with fibroblasts under hypoxia are different from co-cultivated hypoxic macrophages that do not receive the pro-fibrotic stimulation; 1465 genes are significantly differentially expressed and most of them are related to pathway that are switched-off in the MF/H/CC condition. Pathways down-regulated are related to different cell function (actin polimerization, pro-inflammatory response and cell cycle regulation).

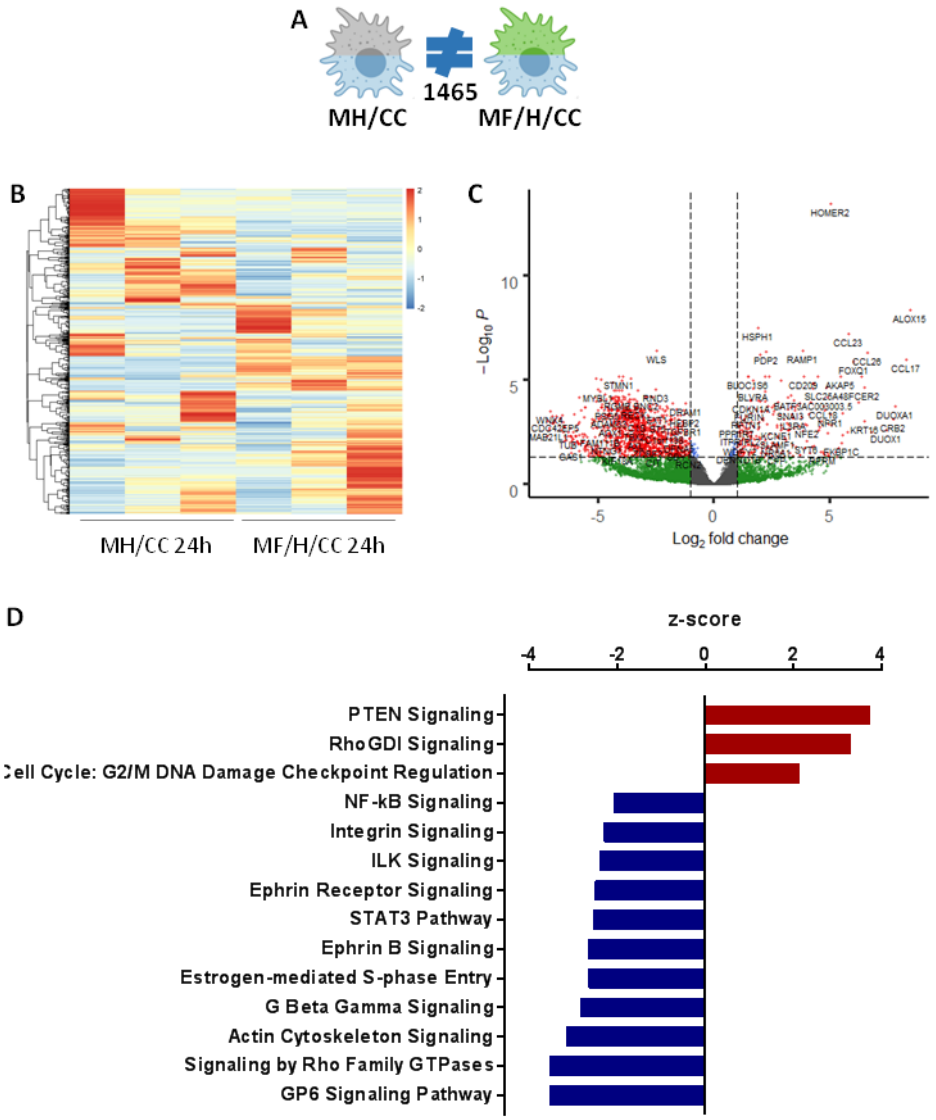


Fig. 81 Comparison MH/CCvsMF/H/CC. Schematic representation of MH/CCvsMF/H/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1)

(C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

[Code: C62 (S44vsS42)]

Fibroblasts stimulated for 24h with IL-4 in co-culture with macrophages under hypoxia are different from co-cultivated hypoxic fibroblasts that do not receive the pro-fibrotic stimulation; 868 genes are significantly differentially expressed and most of them are related to pathway that are switched-off in the FbF/H/CC condition. Pathways down-regulated are related to different cell function (cell-cell contact, actin polimerization, pro-inflammatory response and cell cycle regulation).

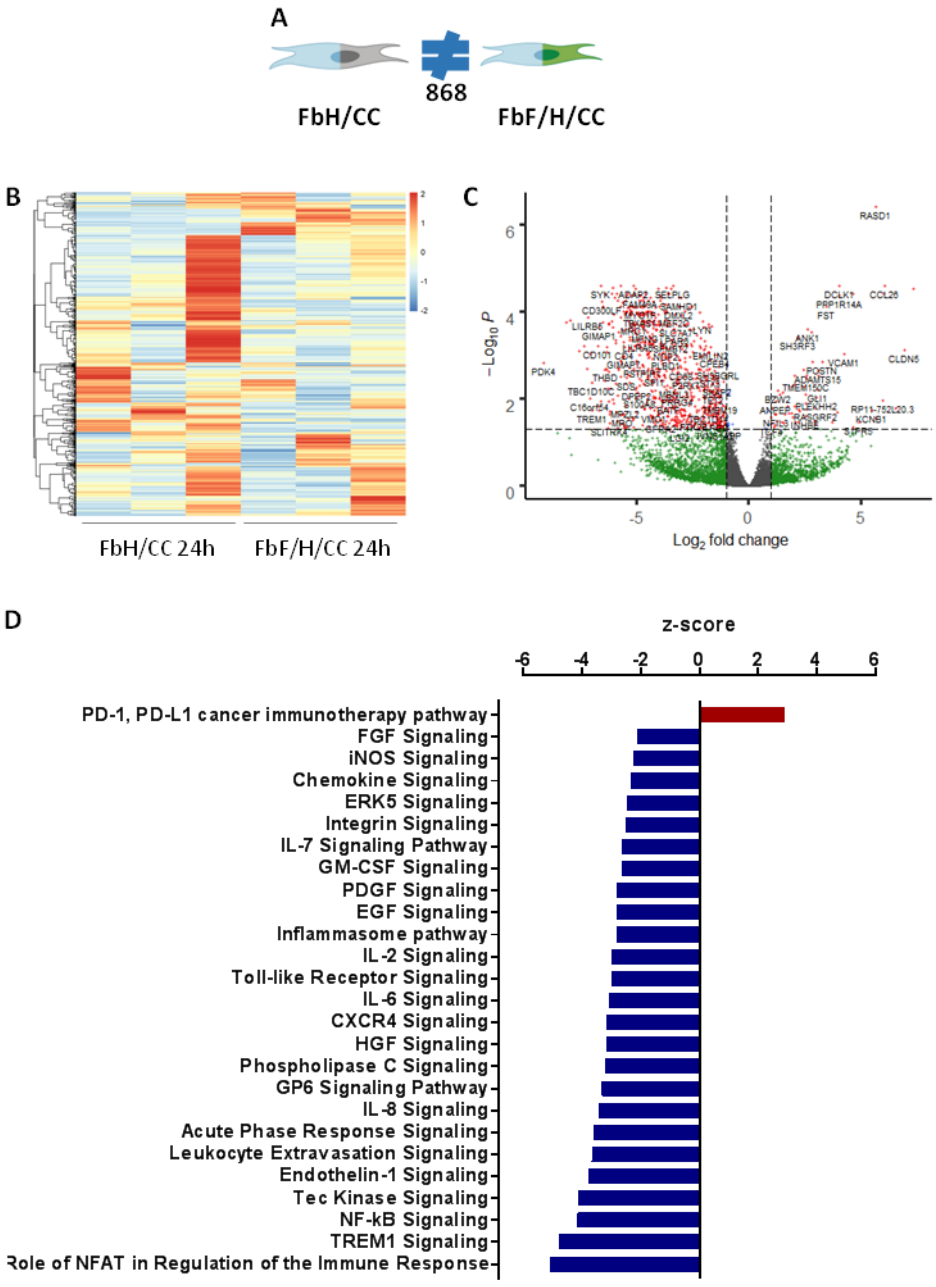


Fig. 82 Comparison FbH/CCvsFbF/H/CC. Schematic representation of FbH/CCvsFbF/H/CC comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|\log FC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C). IPA analysis result is reported as a bar graph that shows pathways positive regulated with a z-score ≥ 2 in red and pathways negative regulated with a z-score ≤–2 in blue (D).

8.1.3.5 What is the effect of hypoxia on pro-fibroticMφ and Fb?

[Code: C63 (S7vsS2)]

Pro-fibrotic macrophages after 4h of hypoxia are similar to pro-fibrotic macrophages in normoxic context; 15 genes only are significantly differentially expressed.

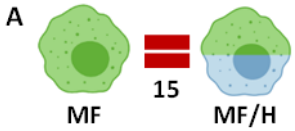


Fig. 83 Comparison MFvsMF/H. Schematic representation of MFvsMF/H comparison at 4h with the number of genes differentially expressed (FDR<0.05) (A).

[Code: C64 (S10vsS4)]

Pro-fibrotic fibroblasts after 4h of hypoxia are equal to pro-fibrotic fibroblasts in normoxia.

Fig. 84 Comparison FbFvsFbF/H. Schematic representation of FbFvsFbF/H comparison at 4h with the number of genes differentially expressed (FDR<0.05)(A).

[Code: C65 (S19vsS13)]

Pro-fibrotic macrophages after 24h of hypoxia are similar to pro-fibrotic macrophages in normoxic context; 23 genes only are significantly differentially expressed.

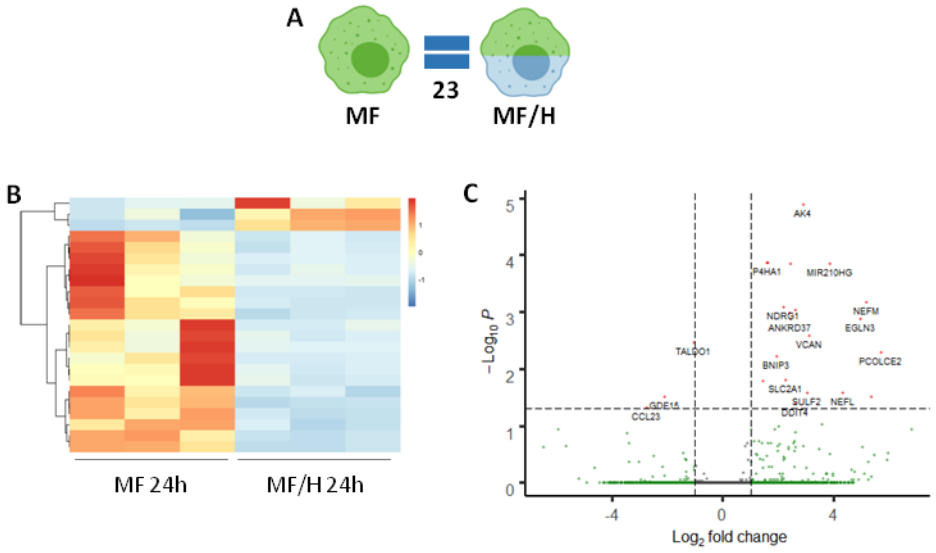


Fig. 85 Comparison MFvsMF/H. Schematic representation of MFvsMF/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C66 (S22vsS16)]

Pro-fibrotic fibroblasts after 24h of hypoxia are similar to pro-fibrotic fibroblasts in normoxia.

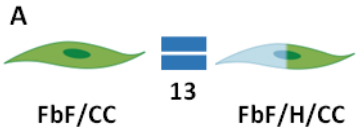


Fig. 86Comparison FbFvsFbF/H. Schematic representation of FbFvsFbF/H comparison at 4h with the number of genes differentially expressed (FDR<0.05) (A).

8.1.3.6 What is the effect of hypoxia on co-cultivated pro-fibrotic Mφ and Fb?

[Code: C67 (S29vsS24)]

Pro-fibrotic macrophages after 4h of co-culture with fibroblasts under hypoxia are similar to co-cultivated pro-fibrotic macrophages in normoxia; 30 genes only are differentially expressed.

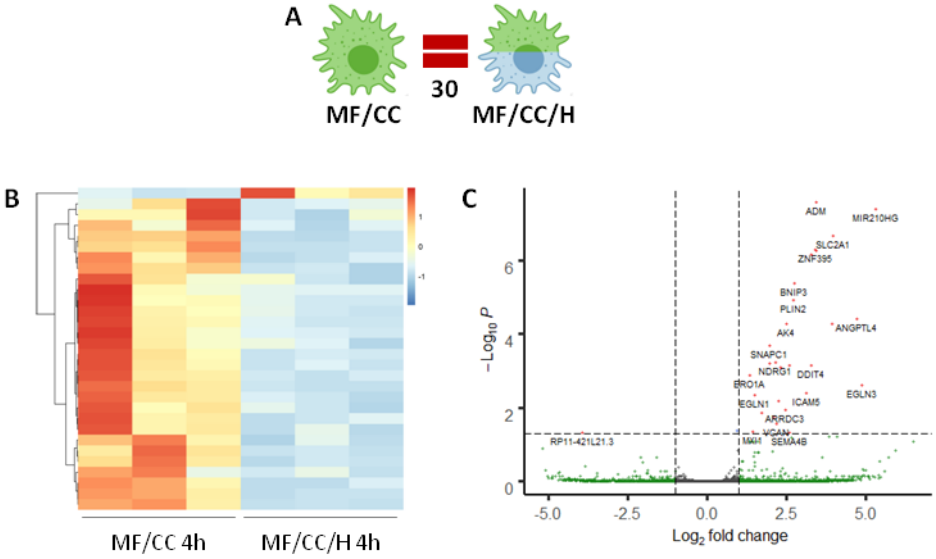


Fig. 87 Comparison MF/CCvsMF/CC/H. Schematic representation of MF/CCvsMF/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C68 (S32vsS26)]

Pro-fibrotic fibroblasts after 4h of co-culture with macrophages under hypoxia are similar to co-cultivated pro-fibrotic fibroblasts in normoxia; 6 genes only are differentially expressed.

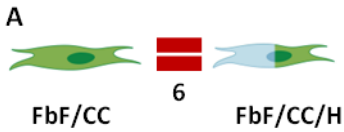


Fig. 88 Comparison FbF/CCvsFbF/CC/H. Schematic representation of FbF/CCvsFbF/CC/H comparison at 4h with the number of genes differentially expressed (FDR<0.05)(A).

[Code: C69 (S41vsS35)]

Pro-fibrotic macrophages after 24h of co-culture with fibroblasts under hypoxia are similar to co-cultivated pro-fibrotic macrophages in normoxia; 17 genes only are differentially expressed.

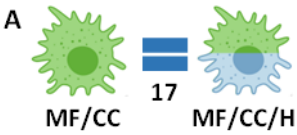


Fig. 89 Comparison MF/CCvsMF/CC/H. Schematic representation of MF/CCvsMF/CC/H comparison at 24h with the number of SDEG (FDR<0.05)(A).

[Code: C70 (S44vsS38)]

Pro-fibrotic fibroblasts after 24h of co-culture under hypoxia are similar to co-cultivated pro-fibrotic fibroblasts in normoxia; 60 genes are significantly differentially expressed.

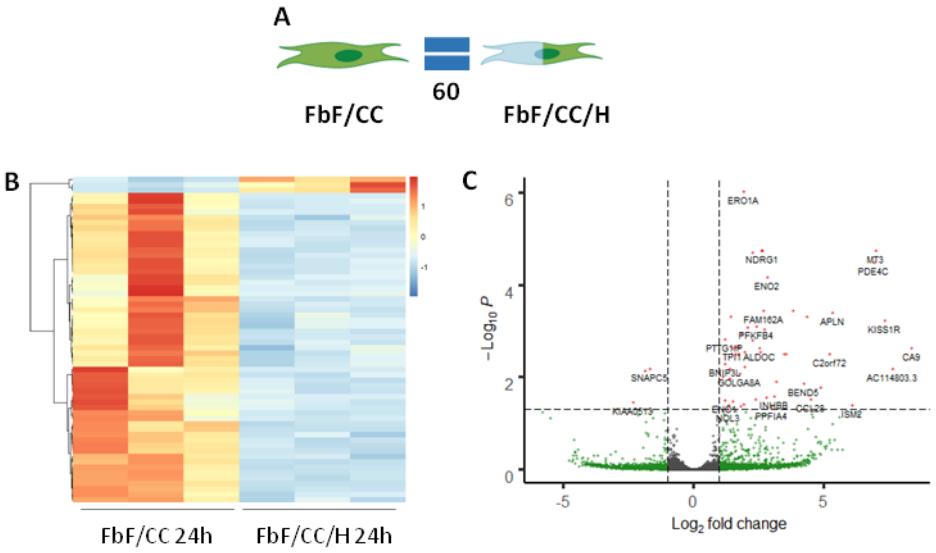


Fig. 90 Comparison FbF/CCvsFbF/CC/H. Schematic representation of FbF/CCvsFbF/CC/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of genes SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P > 1.3$) and a $|logFC| \geq 1$ (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

8.1.3.7 What is the effect of co-culture on pro-fibrotic Mφ and Fb?

[Code: C71 (S24vsS2)]

Pro-fibrotic macrophages after 4h of co-culture with fibroblasts are similar to pro-fibrotic macrophages alone; 26 genes only are significantly differentially expressed.

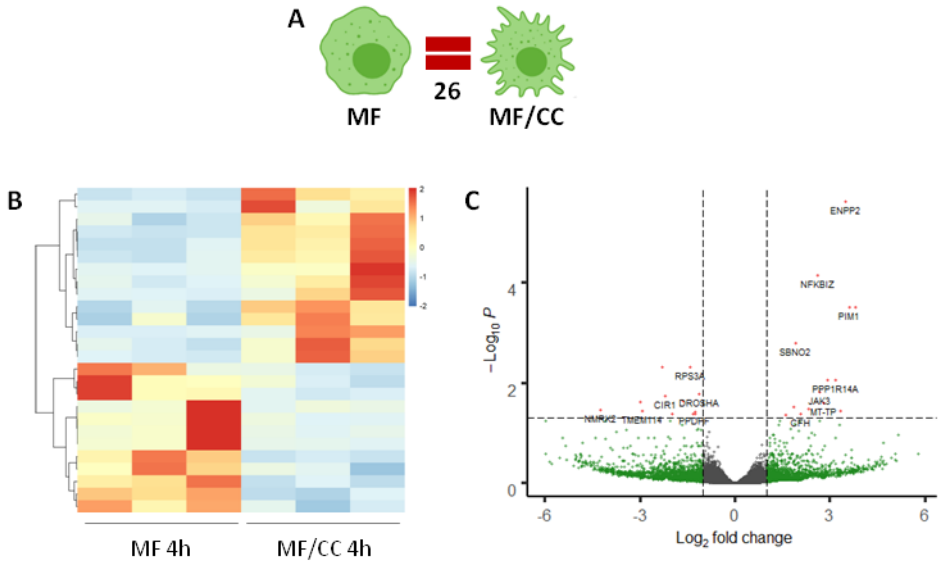


Fig. 91 Comparison MFvsMF/CC. Schematic representation of MFvsMF/CC comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C72 (S26vsS4)]

Pro-fibrotic fibroblasts after 4h of co-culture with macrophages are equal to pro-fibrotic fibroblasts alone.

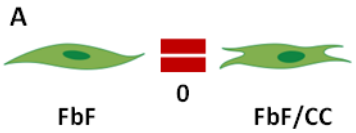


Fig. 92 Comparison FbFvsFbF/CC. Schematic representation of FbFvsFbF/CC comparison at 4h with the number of genes differentially expressed (FDR<0.05)(A).

[Code: C73 (S35vsS13)]

Pro-fibrotic macrophages after 24h of co-culture with fibroblasts are similar to pro-fibrotic macrophages alone.

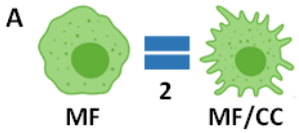


Fig. 93 Comparison MFvsMF/CC. Schematic representation of MFvsMF/CC comparison at 24h with the number of SDEG (FDR<0.05)(A).

[Code: C74 (S38vsS16)]

Pro-fibrotic fibroblasts after 4h of co-culture with macrophages are similar to pro-fibrotic fibroblasts alone.

Fig. 94 Comparison FbFvsFbF/CC. Schematic representation of FbFvsFbF/CC comparison at 4h with the number of SDEG (FDR<0.05)(A).

8.1.3.8 What is the effect of co-culture on hypoxic pro-fibroticMφ and Fb?

[Code: C75 (S29vsS7)]

Pro-fibrotic macrophages after 4h of co-culture with fibroblasts under hypoxia are similar to pro-fibrotic hypoxic macrophages alone; 93 genes are significantly differentially expressed.

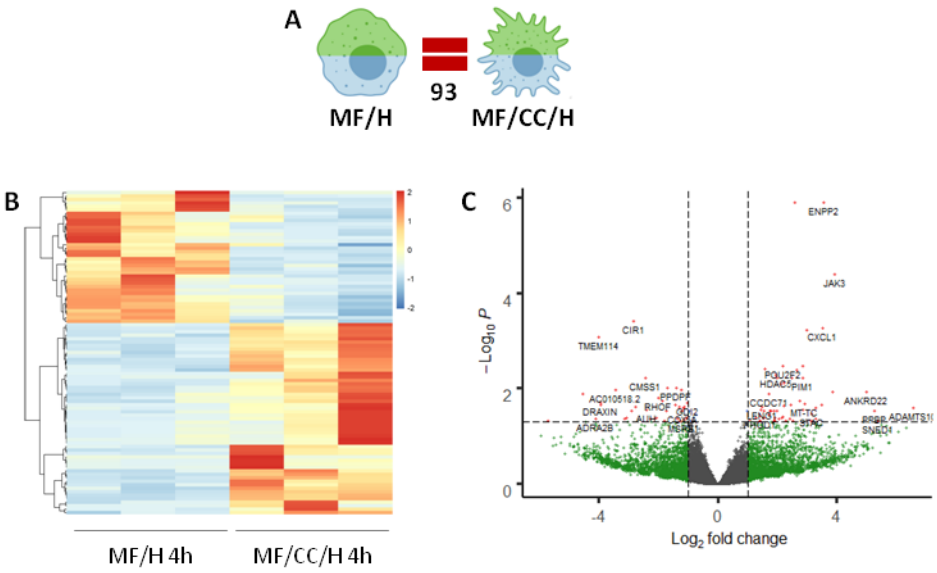


Fig. 95 Comparison MF/HvsMF/CC/H. Schematic representation of MF/HvsMF/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C76 (S32vsS10)]

Pro-fibrotic fibroblasts after 4h of co-culture with macrophages under hypoxia are equal to pro-fibrotic hypoxic macrophages alone.

Fig. 96 Comparison FbF/HvsFbF/CC/H. Schematic representation of FbF/HvsFbF/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A).

[Code: C77 (S41vsS19)]

Pro-fibrotic macrophages after 24h of co-culture with fibroblasts under hypoxia are similar to pro-fibrotic hypoxic macrophages alone; 47 genes are differentially expressed.

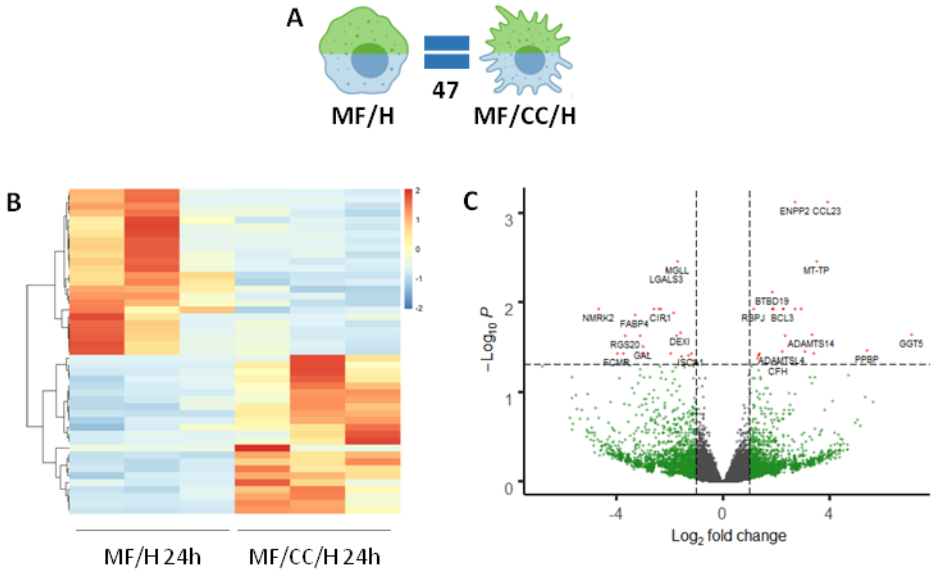


Fig. 97 Comparison MF/HvsMF/CC/H. Schematic representation of MF/HvsMF/CC/H comparison at 24h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represent one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes

significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

[Code: C78 (S44vsS22)]

Pro-fibrotic fibroblasts after 24h of co-culture with macrophages under hypoxia are similar to pro-fibrotic hypoxic macrophages alone; 23 genes are significantly differentially expressed.

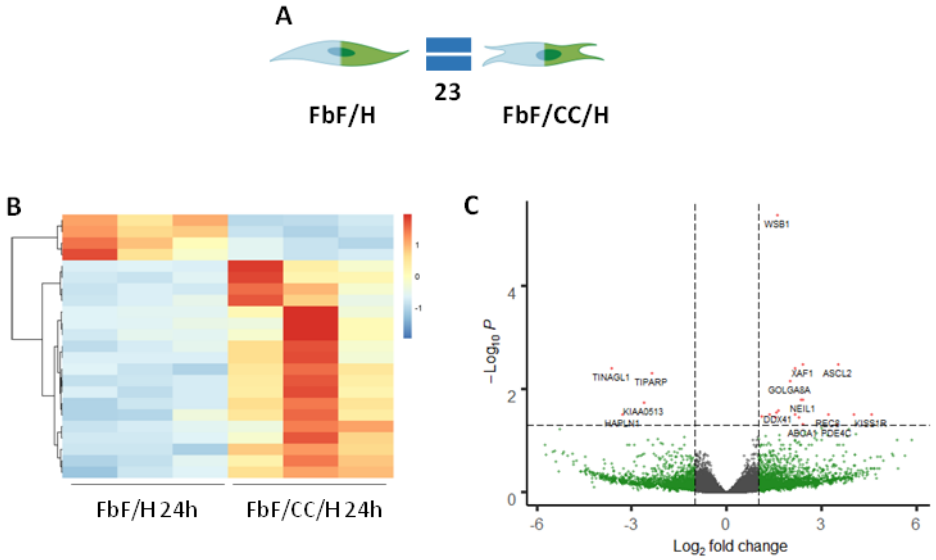


Fig. 98 Comparison FbF/HvsFbF/CC/H. Schematic representation of FbF/HvsFbF/CC/H comparison at 4h with the number of SDEG (FDR<0.05)(A); Heatmap of SDEG; values are scaled by row and each column represents one replicate (there are three replicates for sample) (B); Volcano plot represents each gene as a dot: not significative genes are showed in gray, genes significative only for the expression in green and genes significative for both expression and FDR in red. It is reported the label of that genes that have an FDR<0.05 (on the y axis represented as $-\log_{10}P$ >1.3) and a |logFC|≥1 (represented on the x axis as $\log_2$ fold change <–1 or >1) (C).

SUMMARY FIRST LEVEL: PRO-FIBROTIC CONDITION

Significant differences come up in some comparisons that are based on pro-fibrotic versus resting conditions:

C47-C49

C53

C55-C57-C56-C58

C59-C61-C60-C62

8.2 SECOND LEVEL

8.2.1 RESTING CONDITION

8.2.1.1 What is the effect of hypoxia when cell are co-cultivated?

[Code: C79 (C5vsC1)]

Macrophages under hypoxia for 4h are similar to normoxic resting macrophages (37 SDEG); when macrophages are co-cultivated with fibroblasts in hypoxic environment for 4h, they maintain a similar phenotype to co-cultivated macrophages in normoxia (44 SDEG); indeed, hypoxic macrophages are similar to their normoxic counterpart both in single and co-culture. However, if we consider genes differentially expressed in the two comparisons, we observe that 20 genes are shared, 24 are differentially expressed only in co-cultivated Mφ and 17 in the single cultivated Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 24 and 17 genes together, we obtain a 4-columns heatmap. Most of genes are up-regulated in both single and co-cultivated hypoxic macrophages, in comparison to the normoxic counterpart (C).

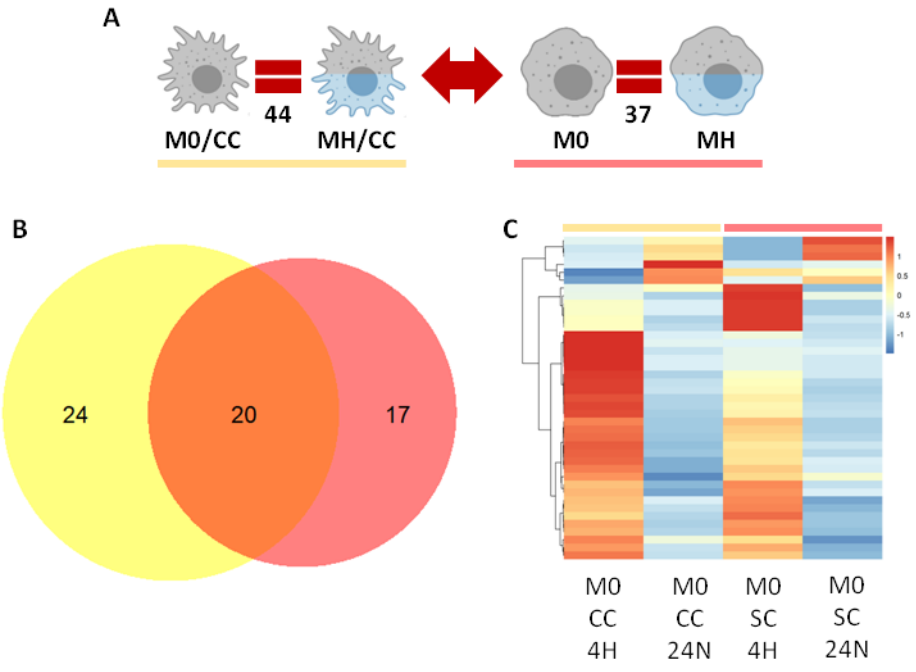


Fig. 99 Comparison (M0vsMH)CCvs(M0vsMH)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. The number of SDEG (FDR<0.05) is reported for each comparison. In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = M0/CCvsMH/CC; pink= M0vsMH) (B); the 4-columns heatmap reports Venn-diagram

total genes minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C80 (C6vsC2)]

Fibroblasts under hypoxia for 4h are different from normoxic resting fibroblasts (97 SDEG); when fibroblasts are co-cultivated with macrophages under hypoxia for 4h, they show a different phenotype from co-cultivated fibroblasts in normoxia (105 SDEG); indeed, hypoxic fibroblasts differ from their normoxic counterpart both in single and co-culture. However, if we consider genes differentially expressed in the two comparisons we observe that 52 genes are shared, 53 are differentially expressed only in co-cultivated fibroblasts and 42 in the single cultivated fibroblasts (B). By excluding genes that are differentially expressed in both comparisons and considering the 53 and 42 genes together, we obtain a 4-columns heatmap where we can observe that most of that genes are up-regulated in both single and co-cultivated hypoxic fibroblasts in comparison to the normoxic counterpart (C).

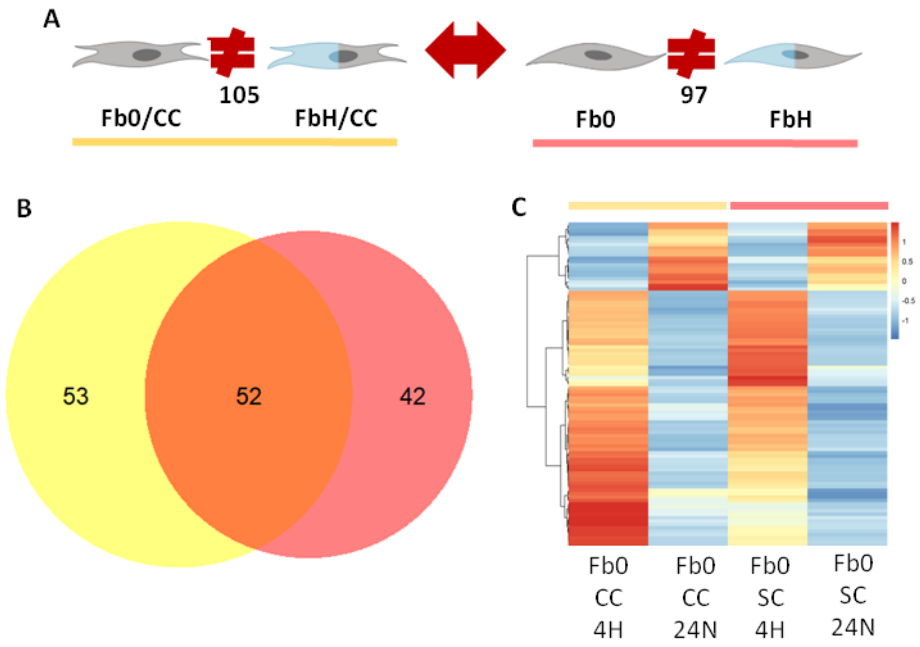


Fig. 100 Comparison (Fb0vsFbH)CCvs(Fb0vsFbH)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = Fb0/CCvsFbH/CC; pink= Fb0vsFbH) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C81 (C7vsC3)]

Macrophages under hypoxia for 24h are similar to normoxic resting macrophages (18 SDEG); when macrophages are co-cultivated with fibroblasts under hypoxia for 24h, they become completely different from co-cultivated macrophages in normoxia (1139 SDEG); indeed, co-cultivated macrophages have a different behaviour if they are under hypoxia. If we consider genes differentially expressed in the two comparisons we observe that 17 genes are shared, 1093 are differentially expressed only in co-cultivated Mφ and 1 in the single cultivated Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 1093 and 1 genes together we obtain a 4-columns heatmap (C) where we can observe that samples have an heterogeneous pattern of expression of these genes but the M0/CC 24H (first column) has a more different signature from the other three samples.

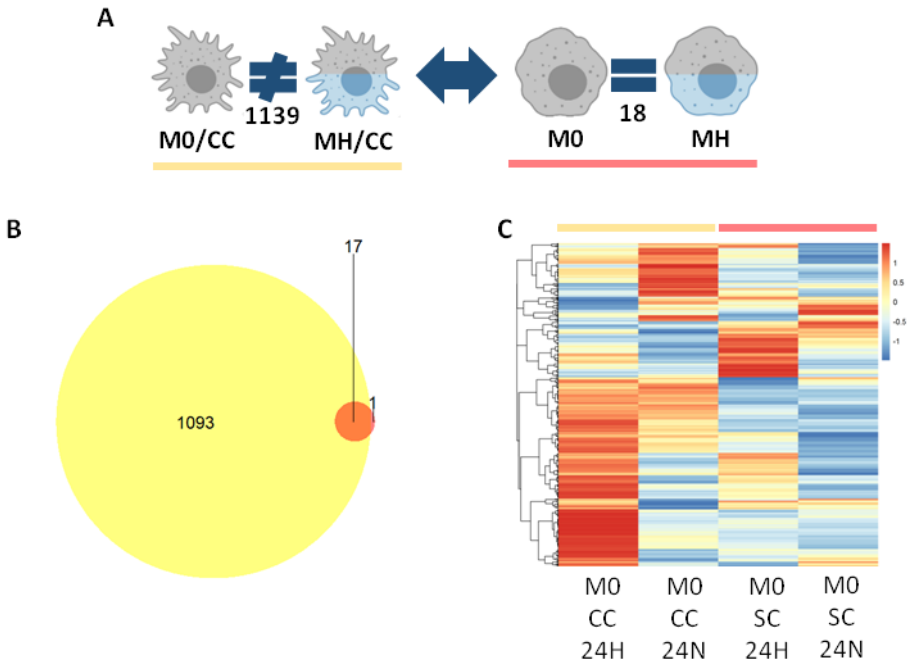


Fig. 101 Comparison (M0vsMH)CCvs(M0vsMH)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in

pink the comparison related to the single culture (A); Venn-diagram of genes SDEG with a |logFC|≥1 in the two comparisons (yellow = M0/CCvsMH/CC; pink= M0vsMH) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C82 (C8vsC4)]

Fibroblasts under hypoxia for 24h are similar to normoxic resting fibroblasts (23 SDEG); when fibroblasts are co-cultivated with macrophages and put under hypoxia for 24h they become completely different from co-cultivated fibroblasts in normoxia (1305 SDEG); indeed, hypoxic fibroblasts have a different behaviour if they are co-cultivated or alone. If we consider genes differentially expressed in the two comparisons, we observe that 14 genes are shared, 1195 are differentially expressed only in co-cultivated fibroblasts and 8 in the single cultivated fibroblasts (B). By excluding genes that are differentially expressed in both comparison and considering the 1195 and 8 genes together, we obtain a 4-columns heatmap (C) where we can observe that sample Fb0/CC 24H (first column) has a different pattern of gene expression from the other three samples.

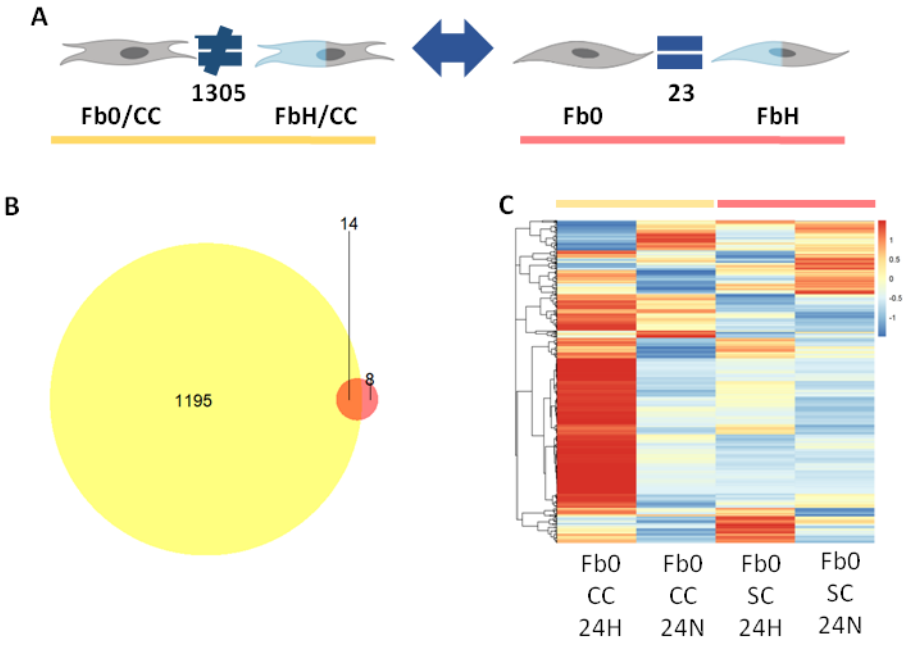


Fig. 102 Comparison (Fb0vsFbH)CCvs(Fb0vsFbH)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 24h.
For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = Fb0/CCvsFbH/CC; pink= Fb0vsFbH) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column

represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.1.2 What is the effect of co-culture when cells are under hypoxia?

[Code: C83 (C13vsC9)]

Macrophages co-cultivated with fibroblasts for 24h are similar to resting, not co-cultivated, macrophages (4 SDEG); when macrophages are co-cultivated with fibroblasts under hypoxia they become completely different from hypoxic macrophages alone (1424 SDEG); indeed, co-cultivated and single cultivated macrophages show a different response to hypoxia. If we consider genes differentially expressed in the two comparisons, we observe that 3 genes are shared, 1370 are differentially expressed only in hypoxic Mφ and 1 in the normoxic Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 1370 and 1 genes together, we obtain a 4-columns heatmap (C): samples have an heterogeneous pattern of expression of these genes but M0 SC 24H and M0 SC 24N (2nd and 4th column) have more similar pattern of expression.

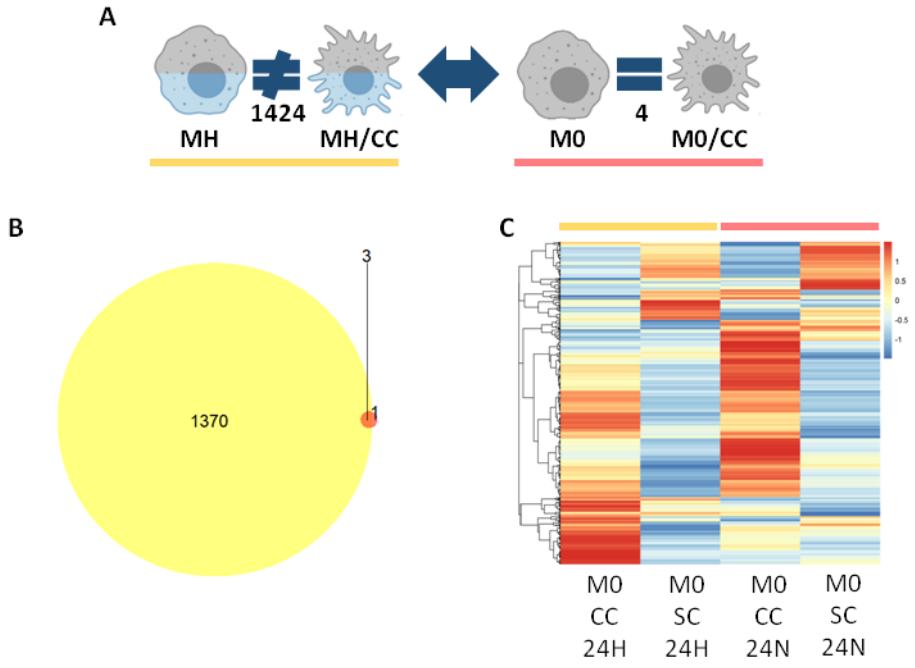


Fig. 103 Comparison (M0/SCvsM0/CC)Hvs(M0/SCvsM0/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of genes significantly differentially expressed with a |logFC|≥1 in the two comparisons (yellow = MHvsMH/CC; pink= M0vsM0/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes

of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C84 (C14vsC10)]

Fibroblasts co-cultivated with macrophages for 24h are similar to resting, not co-cultivated fibroblasts; when fibroblasts are co-cultivated with macrophages under hypoxia, they become completely different from hypoxic fibroblasts alone (1291 SDEG); indeed, co-cultivated and single cultivated fibroblasts show a different response to hypoxia. If we consider genes differentially expressed in the two comparisons, we observe 1212 are differentially expressed only in hypoxic Fb (B). By excluding genes that are differentially expressed (1) in both comparison, we obtain a 4-columns heatmap (C) where we can observe that sample Fb0 CC 24H (1st column) has a different gene expression pattern from the other samples.

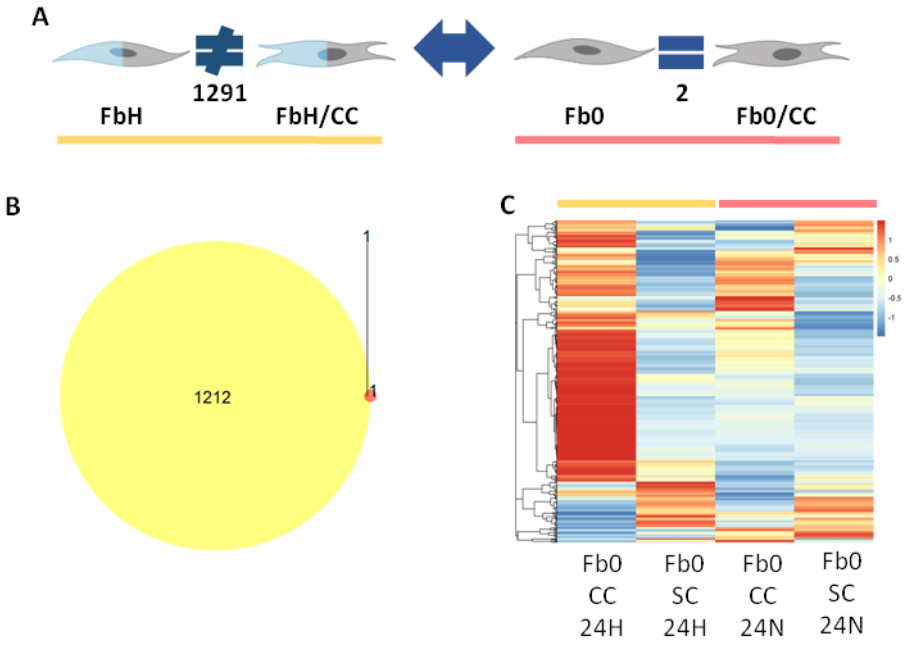


Fig. 104 Comparison (Fb0/SCvsFb0/CC)Hvs(Fb0/SCvsFb0/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbHvsFbH/CC; pink= Fb0vsFb0/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.2.1 What is the effect of pro-inflammatory stimuli when cells are co-cultivated?

[Code: C85 (C23vsC15)]

Macrophages stimulated for 4h with LPS+IFNγ acquire a pro-inflammatory phenotype (MI), different from resting Mφ (3531 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 4h, they acquire a pro-inflammatory phenotype, similarly on what happen in the single culture, in comparison to co-cultivated, not stimulated, macrophages (3983 SDEG); indeed, when macrophages are stimulated with LPS+IFNγ change their phenotype independently from cultivation condition. If we consider genes differentially expressed in the two comparisons, we observe that 2407 genes are shared, 1145 are differentially expressed only in co-cultivated Mφ and 722 in the single cultivated Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 1145 and 722 genes together, we obtain a 4-columns heatmap where we can observe that MI SC 4N and MI CC 4N (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples M0 CC 24N and M0 SC 24N (2nd and 4th column) (C).

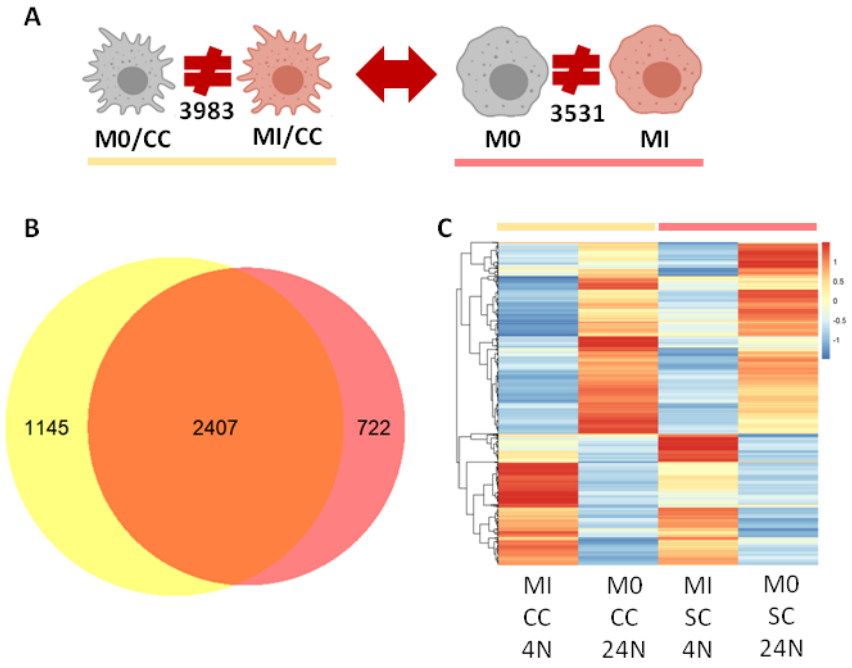


Fig. 105 Comparison (M0vsMI)CCvs(M0vsMI)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of macrophages at time 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons

(yellow = M0/CCvsMI/CC; pink= M0vsMI) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C86 (C24vsC16)]

Fibroblasts stimulated for 4h with LPS+IFNγ acquire a pro-inflammatory phenotype (FbI) different from resting Fb (1402 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h, they acquire a pro-inflammatory phenotype similarly on what happen in the single culture, in comparison to co-cultivated, not stimulated fibroblasts but increasing the number of genes significantly differentially expressed (2131); indeed, when fibroblasts are stimulated with LPS+IFNγ, they change their phenotype independently if are co-cultivated or alone; however co-culture increases the number of genes differentially expressed. Considering genes differentially expressed in the two comparisons, we observe that 1035 genes are shared, 853 are differentially expressed only in co-cultivated Fb and 229 in the single cultivated Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 853 and 229 genes together, we obtain a 4-columns heatmap where we can observe that FbI SC 4N and FbI CC 4N (1st and 3rd columns) have a similar gene expression pattern that is the opposite of the other two samples Fb0 CC 24N and Fb0 SC 24N (2nd and 4th columns) (C).

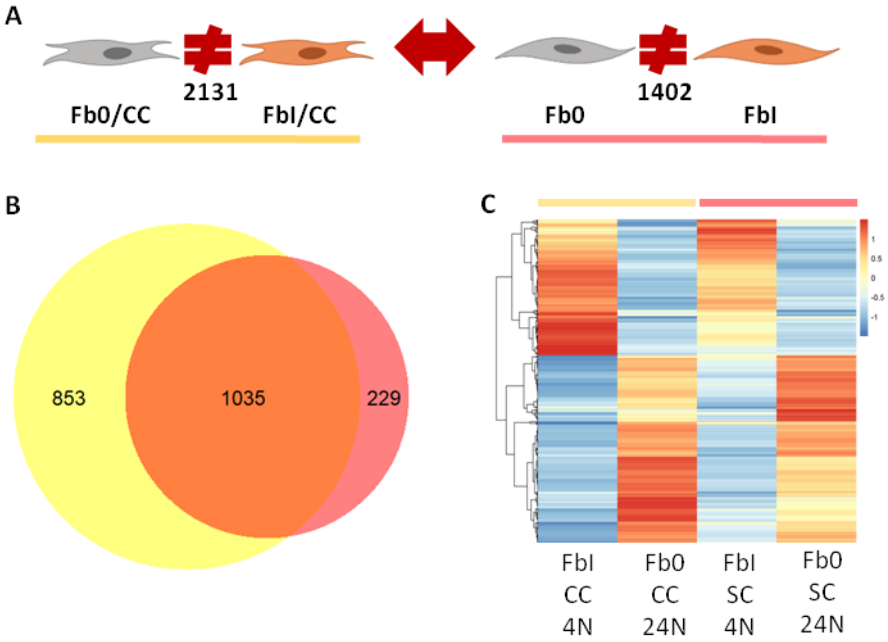


Fig. 106 Comparison (Fb0vsFbI)CCvs(Fb0vsFbI)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the

comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = Fb0/CCvsFbI/CC; pink= Fb0vsFbI) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C87 (C25vsC17)]

Macrophages stimulated for 24h with LPS+IFNγ acquire a pro-inflammatory phenotype (MI) different from resting Mφ (3389 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 24h, they acquire a pro-inflammatory phenotype similarly on what happen in the single culture, in comparison to co-cultivated, un-stimulated macrophages (3840 SDEG); indeed, when macrophages are stimulated with LPS+IFNγ, they change their phenotype independently if are co-cultivated or alone. If we consider genes differentially expressed in the two comparisons, we observe that 1996 genes are shared, 1377 are differentially expressed only in co-cultivated Mφ and 1012 in the single cultivated Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 1377 and 1012 genes together, we obtain a 4-columns heatmap where we can observe that MI SC 24N and MI CC 24N (1st and 3rd columns) have a similar gene expression pattern that is the opposite of the other two samples M0 CC 24N and M0 SC 24N (2nd and 4th columns) (C).

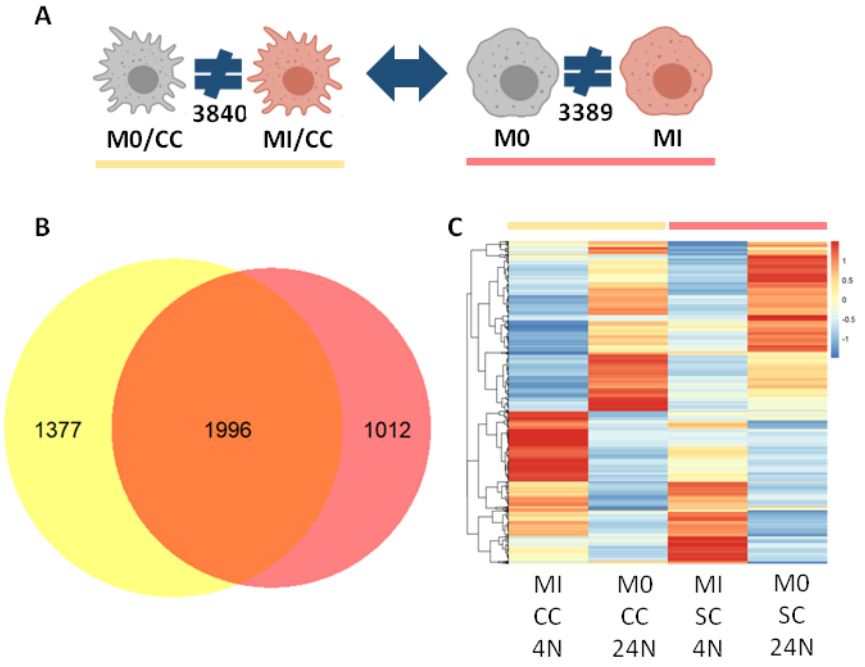


Fig. 107 Comparison (M0vsMI)CCvs(M0vsMI)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in

pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = M0/CCvsMI/CC; pink= M0vsMI) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C88 (C26vsC18)]

Fibroblasts stimulated for 24h with LPS+IFNγ acquire a pro-inflammatory phenotype (FbI) different from resting Fb (1426 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 24h, they acquire a pro-inflammatory phenotype similarly on what happen in the single culture, in comparison to co-cultivated, un-stimulated fibroblasts but with an increase in the number of SDEG (4841); indeed, when fibroblasts are stimulated with LPS+IFNγ, they change their phenotype independently from culture condition; however, in co-culture, the number of SDEG is higher. If we consider genes differentially expressed in the two comparisons, we observe that 1060 genes are shared, 3149 are differentially expressed only in co-cultivated Fb and 240 in the single cultivated Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 3149 and 240 genes together, we obtain a 4-columns heatmap where we can observe that FbI SC 24N and FbI CC 24N (1stand 3rd columns) have a similar gene expression pattern that is the opposite of the other two samples Fb0 CC 24N and Fb0 SC 24N (2nd and 4th columns) (C).

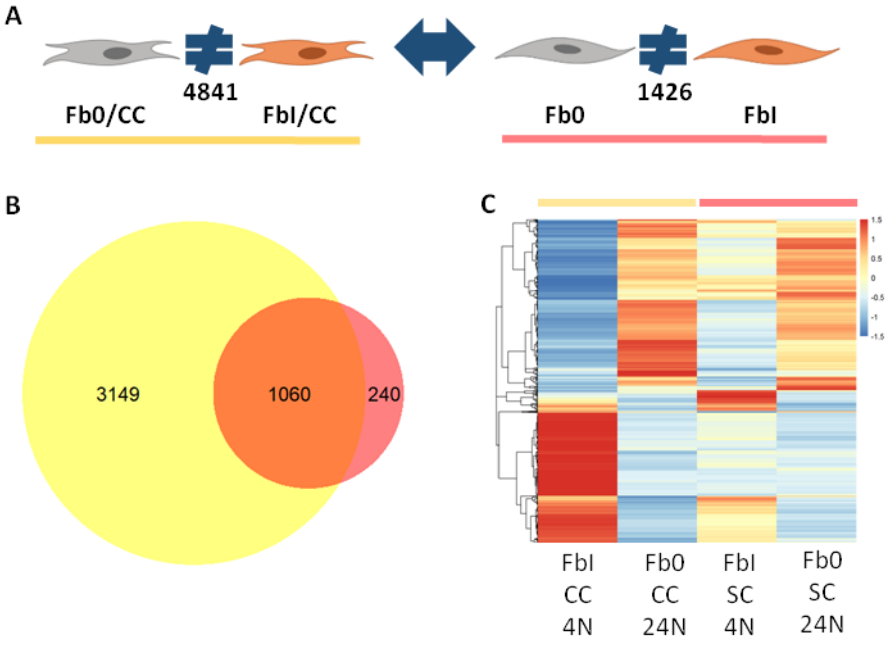


Fig. 108 Comparison (Fb0vsFbI)CCvs(Fb0vsFbI)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in

pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = Fb0/CCvsFbI/CC; pink= Fb0vsFbI) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.2.2 What is the effect of pro-inflammatory stimuli when cells are under hypoxia?

[Code: C89 (C19vsC15)]

Macrophages stimulated for 4h with LPS+IFNγ acquire a pro-inflammatory phenotype (MI) different from resting Mφ (3531 SDEG); when macrophages are put under hypoxia and stimulated with LPS+IFNγ for 4h, they acquire a pro-inflammatory phenotype similarly on what happen in normoxia, in comparison to hypoxic, not stimulated macrophages (2930 SDEG); indeed, when macrophages are stimulated with LPS+IFNγ, they change their phenotype independently from the oxygen status of environment. If we consider genes differentially expressed in the two comparisons, we observe that 2072 genes are shared, 514 are differentially expressed only in hypoxic Mφ and 1057 in the normoxic Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 514 and 1057 genes together, we obtain a 4-columns heatmap where we can observe that MI SC 4H and MI SC 4N (1st and 3rd columns) have a similar pattern of gene expression that is the opposite of the other two samples M0 SC 4H and M0 SC 24N (2nd and 4th columns) (C).

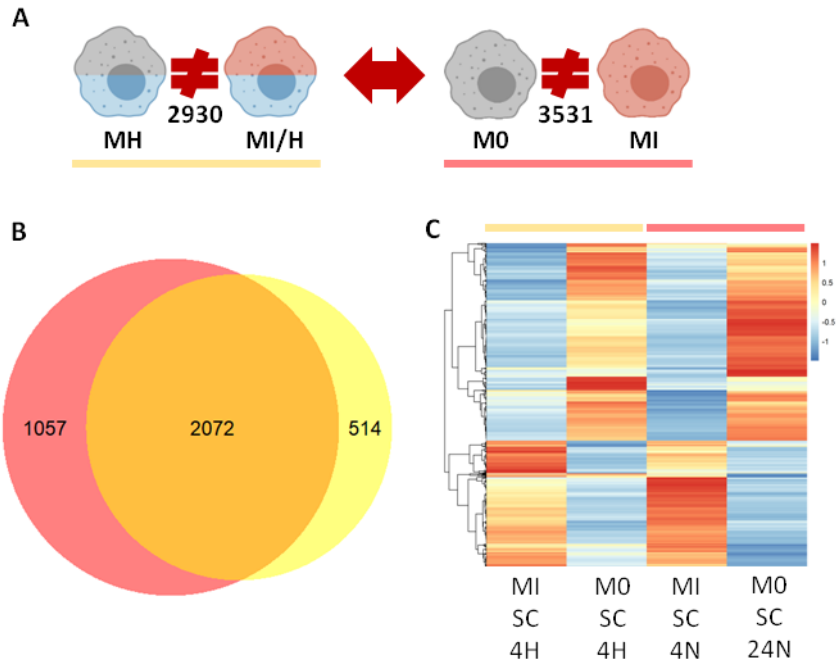


Fig. 109 Comparison (M0vsMI)Hvs(M0vsMI)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = M0vsMI; pink= MHvsMI/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C90 (C20vsC16)]

Fibroblasts stimulated for 4h with LPS+IFNγ acquire a pro-inflammatory phenotype (FbI) different from resting Fb (1402 SDEG); when fibroblasts are put under hypoxia and stimulated with LPS+IFNγ for 4h, they acquire a pro-inflammatory phenotype similarly on what happen in normoxia, in comparison to hypoxic fibroblasts not stimulated but the number of SDEG (628) is lower; indeed, when fibroblasts are stimulated with LPS+IFNγ, they change their phenotype independently from the oxygen status of environment, however under hypoxia the number of SDEG is decreased. If we consider genes differentially expressed in the two comparisons, we observe that 480 genes are shared, 108 are differentially expressed only in hypoxic Fb and 784 in the normoxic Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 108 and 784 genes together, we obtain a 4-columns heatmap where we can observe that FbI SC 4H and FbI SC 4N (1st and 3rd columns) have a similar pattern of gene expression that is the opposite of the other two samples Fb0 SC 4H and Fb0 SC 24N (2nd and 4th columns) (C).

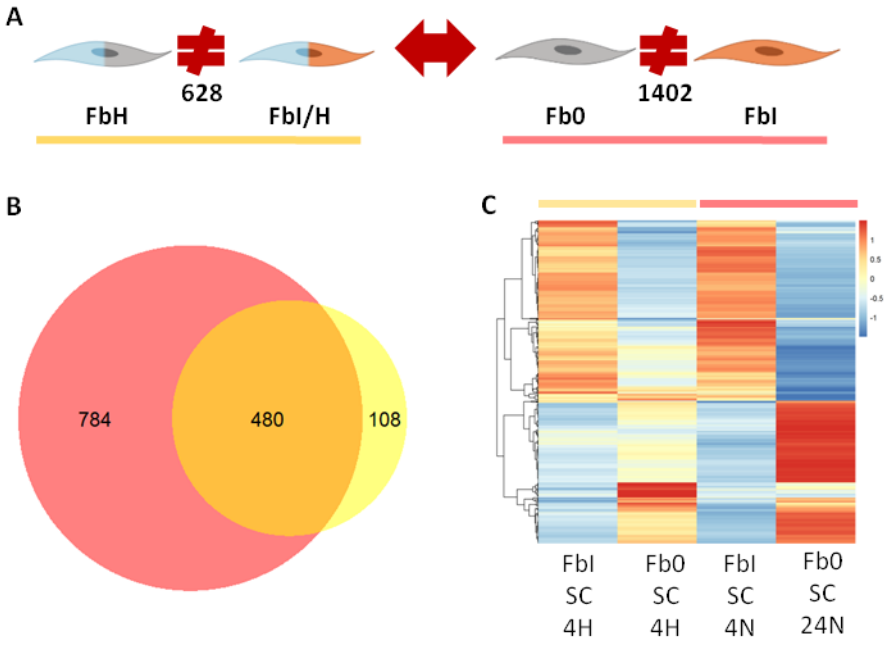


Fig. 110 Comparison (Fb0vsFbI)Hvs(Fb0vsFbI)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbHvsFbI/H; pink= Fb0vsFbI) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C91 (C21vsC17)]

Macrophages stimulated for 24h with LPS+IFNγ acquire a pro-inflammatory phenotype (MI) different from resting Mφ (3389 SDEG); when macrophages are put under hypoxia and stimulated with LPS+IFNγ for 24h, they acquire a pro-inflammatory phenotype similarly on what happen in normoxia, in comparison to hypoxic, not stimulated macrophages (3474 SDEG); indeed, when macrophages are stimulated with LPS+IFNγ, they change their phenotype independently from the oxygen status of environment. If we consider genes differentially expressed in the two comparisons, we observe that 2175 genes are shared, 897 are differentially expressed only in hypoxic Mφ and 833 in the normoxic Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 897 and 833 genes together, we obtain a 4-columns heatmap where we can observe that MI SC 24H and MI SC 24N (1stand 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples M0 SC 24H and M0 SC 24N (2nd and 4th column) (C).

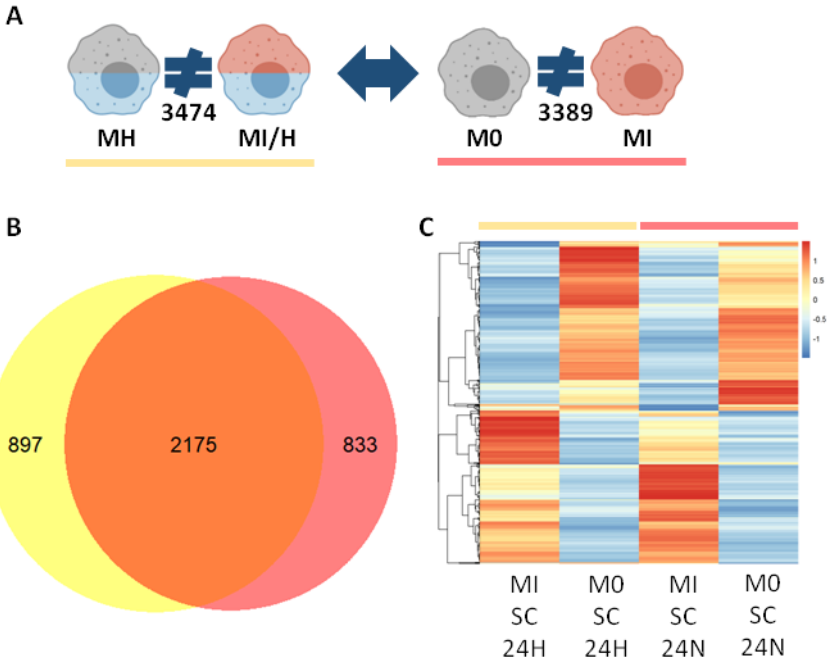


Fig. 111 Comparison (M0vsMI)Hvs(M0vsMI)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = M0vsMI; pink= MHvsMI/H) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C92 (C22vsC18)]

Fibroblasts stimulated for 24h with LPS+IFNγ acquire a pro-inflammatory phenotype (FbI) different from resting Fb (1426 SDEG); when fibroblasts are put under hypoxia and stimulated with LPS+IFNγ for 24h, they acquire a pro-inflammatory phenotype similarly on what happen in normoxia, in comparison to hypoxic fibroblasts not stimulated (1773 SDEG); indeed, when fibroblasts are stimulated with LPS+IFNγ, they change their phenotype independently from the oxygen status of environment. If we consider SDEG in the two comparisons we observe that 1036 genes are shared, 569 are differentially expressed only in hypoxic Fb and 264 in the normoxic Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 569 and 264 genes together, we obtain a 4-columns heatmap where we can observe that FbI SC 24H and FbI SC 24N (1st and 3rd column) have a similar gene expression pattern that is the opposite of the other two samples Fb0 SC 24H and Fb0 SC 24N (2nd and 4th column) (C).

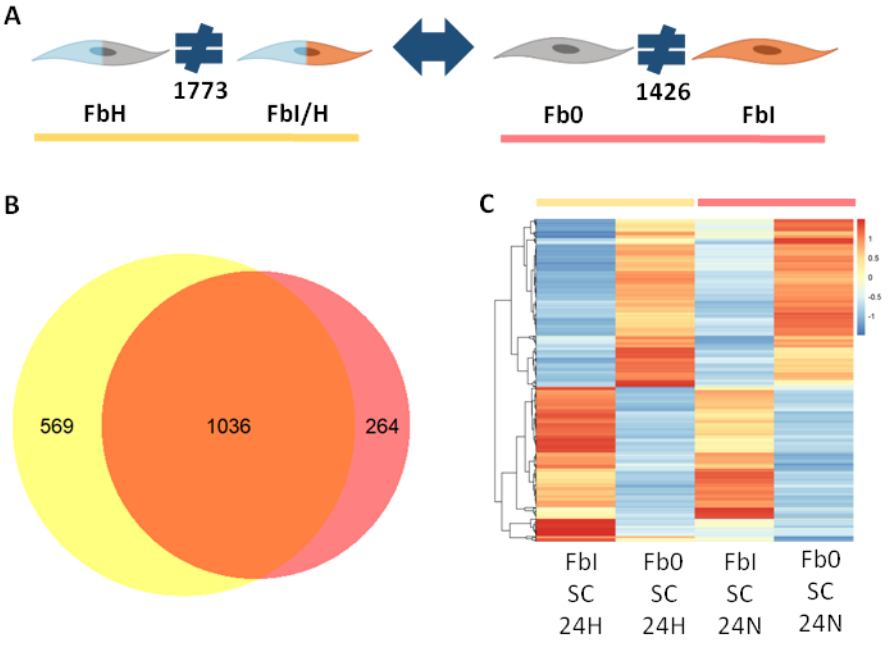


Fig. 112 Comparison (Fb0vsFbI)Hvs(Fb0vsFbI)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbHvsFbI/H; pink= Fb0vsFbI) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.2.3 What is the effect of pro-inflammatory stimuli when hypoxic cells are co-cultivated?

[Code: C93 (C27vsC19)]

Macrophages stimulated for 4h with LPS+IFNγ under hypoxia acquire a pro-inflammatory phenotype (MI/H) different from hypoxic Mφ (MH) (2930 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 4h under hypoxia (MI/H/CC), they acquire a pro-inflammatory phenotype, similarly on what happen in the single culture, in comparison to hypoxic co-cultivated, not stimulated macrophages (MH/CC) but the number of SDEG (3574) is increased; indeed, when macrophages are stimulated with LPS+IFNγ, in hypoxia, they change their phenotype independently from cultivation, with an increase of genes differentially expressed in the first case. If we consider SDEG in the two comparisons we observe that 2054 genes are shared, 1125 are differentially expressed only in hypoxic, co-cultivated Mφ and 532 in the single cultivated, hypoxic Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 1125 and 532 genes together, we obtain a 4-columns heatmap where we can observe that MI CC 4H and MI SC 4H (1st and 3rd column) have a similar gene expression pattern that is the opposite of the other two samples M0 CC 4H and M0 SC 4H (2nd and 4th column) (C).

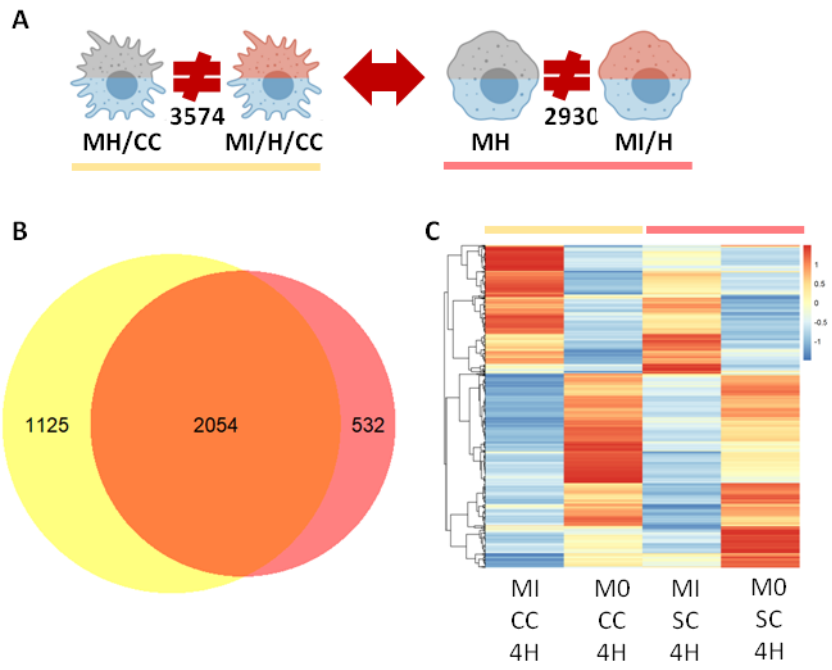


Fig. 113 Comparison (M0vsMI)H/CCvs(M0vsMI)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMI/H/CC; pink= MHvsMI/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C94 (C28vsC20)]

Fibroblasts stimulated for 4h with LPS+IFNγ under hypoxia acquire a pro-inflammatory phenotype (FbI/H) different from hypoxic Fb (FbH) (628 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/H/CC), they acquire a pro-inflammatory phenotype, similarly on what happen in the single culture, in comparison to hypoxic, co-cultivated, not stimulated fibroblasts (FbH/CC) but with an increase in the number of SDEG (1141); indeed, when fibroblasts are stimulated with LPS+IFNγ under hypoxia, they change their phenotype independently from culture condition, with an higher number of SDEG if they are co-cultivated. If we consider genes differentially expressed in the two comparisons we observe that 508 genes are shared, 535 are differentially expressed only in hypoxic co-cultivated Fb and 80 in the single cultivated hypoxic Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 535 and 80 genes together we obtain a 4-columns heatmap where we can

observe that FbI CC 4H and FbI SC 4H (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples Fb0 CC 4H and Fb0 SC 4H (2nd and 4th column) (C).

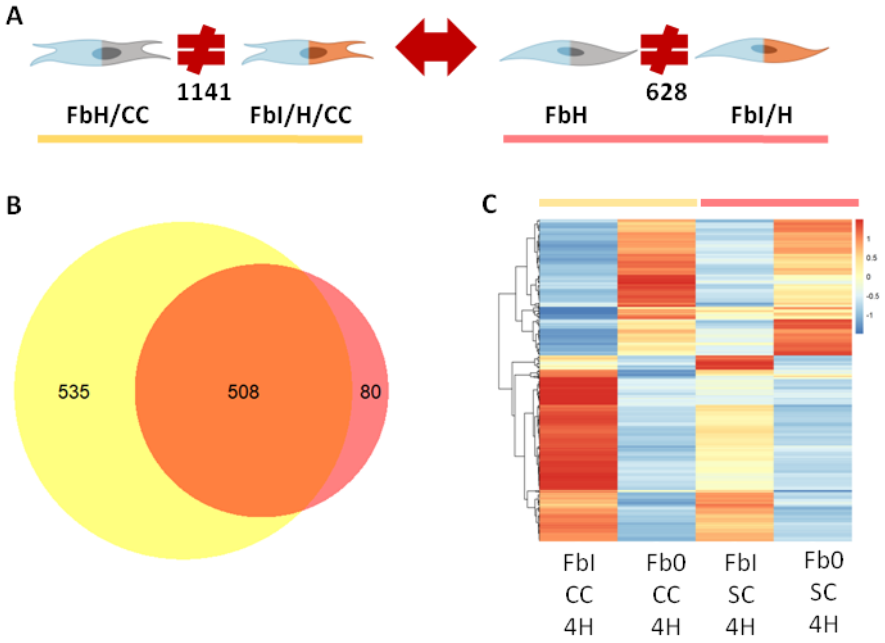


Fig. 114 Comparison (Fb0vsFbI)H/CCvs(Fb0vsFbI)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbH/CCvsFbI/H/CC; pink= FbHvsFbI/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C95 (C29vsC21)]

Macrophages stimulated for 24h with LPS+IFNγ under hypoxia acquire a pro-inflammatory phenotype (MI/H) different from hypoxic Mφ (MH) (3474 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 24h under hypoxia (MI/H/CC), they acquire a pro-inflammatory phenotype in comparison to hypoxic, co-cultivated, not stimulated macrophages (MH/CC) with 1000 genes more than the same comparison in single culture (4663 SDEG); indeed, when macrophages are stimulated with LPS+IFNγ in hypoxia, they change their phenotype independently from culture condition, with a great increase in number of SDEG if they are in co-culture. Considering genes differentially expressed in the two comparisons we observe that 1894 genes are shared, 2223

are differentially expressed only in hypoxic co-cultivated Mφ and 1178 in the single cultivated hypoxic Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 2223 and 1178 genes together we obtain a 4-columns heatmap where we can observe that all four samples have a different gene expression pattern (C).

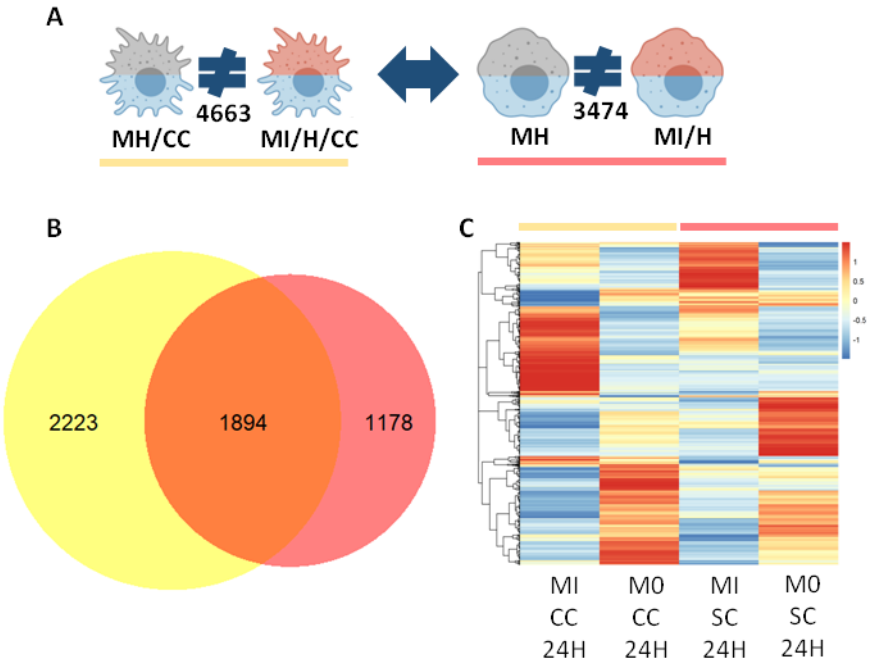


Fig. 115 Comparison (M0vsMI)H/CCvs(M0vsMI)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMI/H/CC; pink= MHvsMI/H) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C96 (C30vsC22)]

Fibroblasts stimulated for 24h with LPS+IFNγ under hypoxia acquire a pro-inflammatory phenotype (FbI/H) different from hypoxic Fb (FbH) (1773 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 24h under hypoxia (FbI/H/CC), they acquire a pro-inflammatory phenotype in comparison to hypoxic, co-cultivated, not stimulated fibroblasts (FbH/CC) with 1000 genes more than the same comparison in single culture (4106 SDEG); indeed, when fibroblasts are stimulated with LPS+IFNγ in hypoxia, they change their phenotype independently from the culture state but

with a great increase in number of SDEG if they are in co-culture. Considering genes differentially expressed in the two comparisons, we observe that 1040 genes are shared, 2579 are differentially expressed only in hypoxic, co-cultivated Fb and 565 in the single cultivated, hypoxic Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 2579 and 565 genes together, we obtain a 4-columns heatmap where we can observe that all samples have a different pattern of gene expression (C).

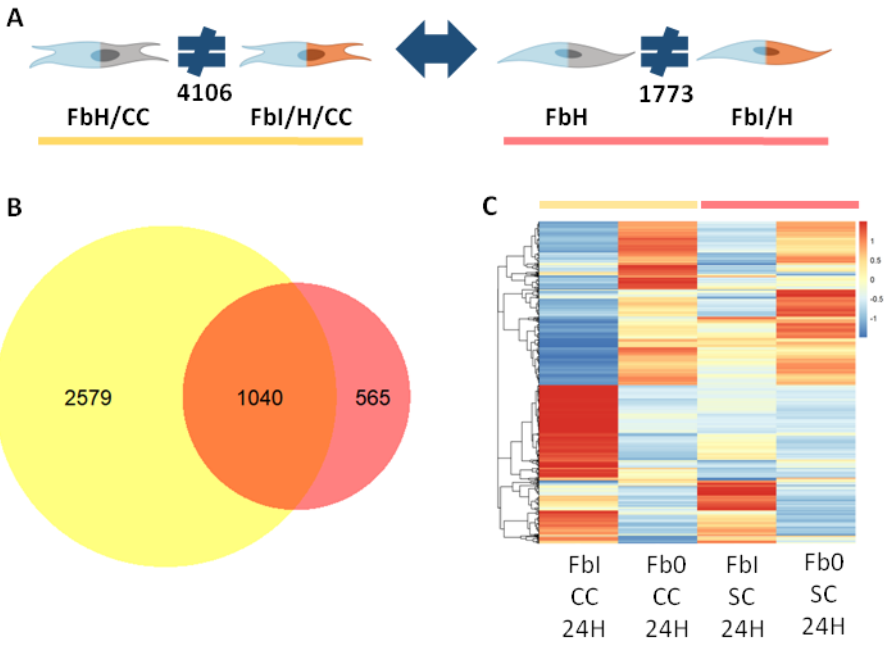


Fig. 116 Comparison (Fb0vsFbI)H/CCvs(Fb0vsFbI)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbH/CCvsFbI/H/CC; pink= FbHvsFbI/H) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.2.4 What is the effect of pro-inflammatory stimuli when co-cultivatedcells are put under hypoxia?

[Code: C97 (C27vsC23)]

Macrophages stimulated for 4h with LPS+IFNγ in co-culture with fibroblasts acquire a pro-inflammatory phenotype (MI/CC) different from co-cultivated Mφ without any stimulation (M0/CC) (3983 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 4h under hypoxia (MI/H/CC), they acquire a pro-inflammatory

phenotype, similarly on what happen in normoxia, in comparison to hypoxic, un-stimulated, co-cultivated macrophages (MH/CC) (3574 SDEG); indeed, when macrophages are stimulated with LPS+IFNγ in co-culture, they change their phenotype independently from oxygen status of microenvironment. If we consider genes differentially expressed in the two comparisons we observe that 2406 genes are shared, 773 are differentially expressed only in hypoxic, co-cultivated Mφ and 1146 in the normoxic, co-cultivated Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 1146 and 773 genes together, we obtain a 4-columns heatmap where we can observe that MI CC 4H and MI CC 4N (1st and 3rd column) have a similar gene expression pattern that is the opposite of the other two samples M0 CC 4H and M0 CC 24N (2nd and 4th column) (C).

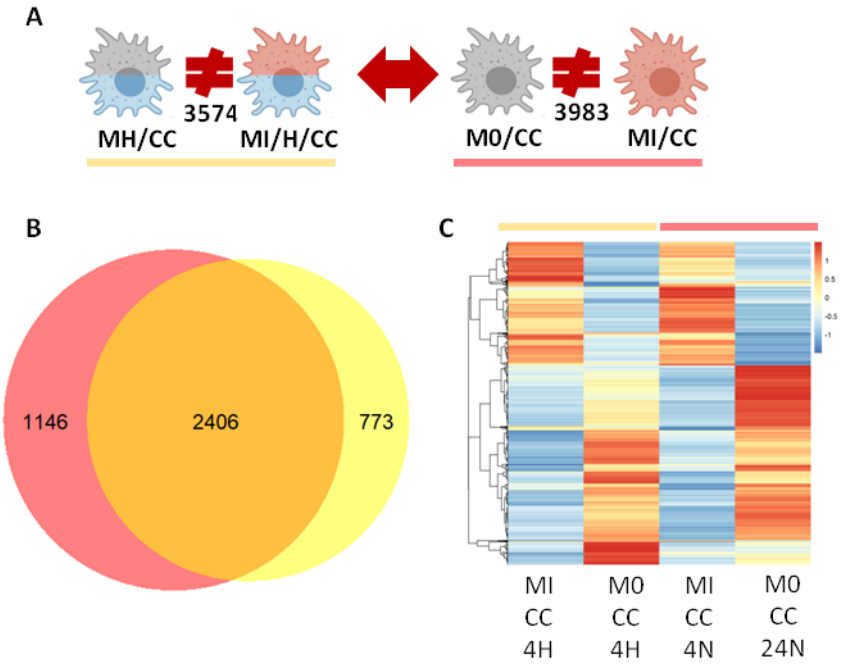


Fig. 117 Comparison (M0vsMI)H/CCvs(M0vsMI)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMI/H/CC; pink= M0/CCvsMI/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C98 (C28vsC24)]

Fibroblasts stimulated for 4h with LPS+IFNγ in co-culture with macrophages acquire a pro-inflammatory phenotype (FbI/CC) different from co-cultivated Fb without any stimulation

(Fb0/CC) (2131 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/H/CC) they acquire a pro-inflammatory phenotype, similarly on what happen in normoxia, in comparison to hypoxic, not stimulated, co-cultivated fibroblasts (FbH/CC) but with a lower number of SDEG (1141); indeed, when fibroblasts are stimulated with LPS+IFNγ in co-culture, they change their phenotype independently from the oxygen status of microenvironment but under hypoxia the number of SDEG is lower. If we consider genes differentially expressed in the two comparisons, we observe that 761 genes are shared, 282 are differentially expressed only in hypoxic, co-cultivated Fb and 1127 in the normoxic, co-cultivated Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 1127 and 282 genes together, we obtain a 4-columns heatmap where we can observe that FbI CC 4H and FbI CC 4N (1st and 3rd column) have a similar gene expression pattern, instead Fb0 CC 4H and Fb0 CC 24N have different pattern of gene expression (C).

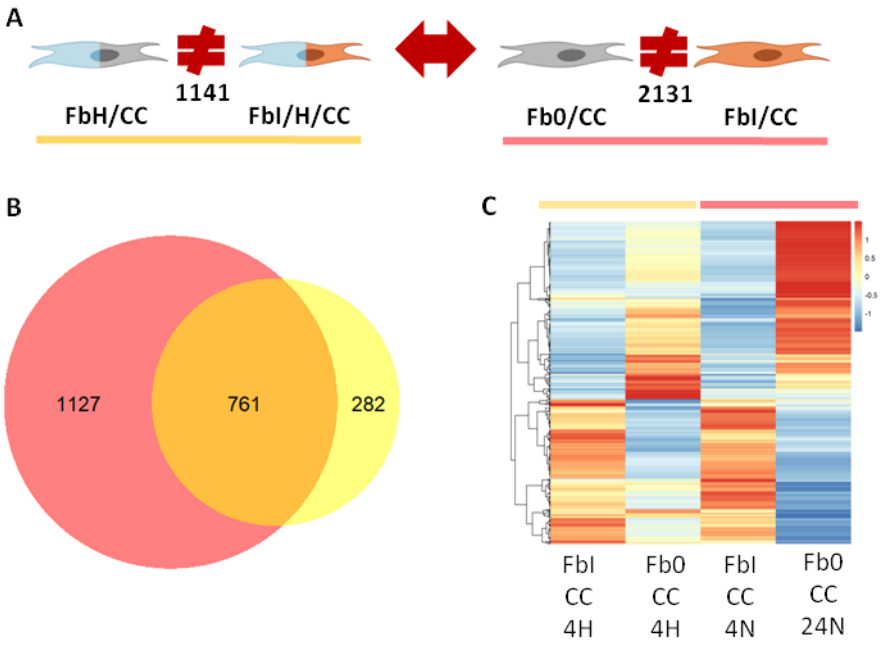


Fig. 118 Comparison (Fb0vsFbI)H/CCvs(Fb0vsFbI)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbH/CCvsFbI/H/CC; pink= Fb0/CCvsFbI/CC) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C99 (C29vsC25)]

Macrophages stimulated for 24h with LPS+IFNγ in co-culture with fibroblasts acquire a pro-inflammatory phenotype (MI/CC) different from co-cultivated Mφ without any stimulation (M0/CC) (3840 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 24h under hypoxia (MI/H/CC), they acquire a pro-inflammatory phenotype, similarly on what happen in normoxia, in comparison to hypoxic, co-cultivated, not stimulated macrophages (MH/CC) but with higher number of SDEG (4663); indeed, when macrophages are stimulated with LPS+IFNγ in co-culture, they change their phenotype independently from oxygen status of microenvironment, however with an increased number of SDEG under hypoxia. Considering genes differentially expressed in the two comparisons we observe that 2099 genes are shared, 1274 are differentially expressed only in hypoxic, co-cultivated Mφ and 2018 in the normoxic co-cultivated Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 1274 and 2018 genes together, we obtain a 4-columns heatmap where we can observe that all samples have a different pattern of gene expression (C).

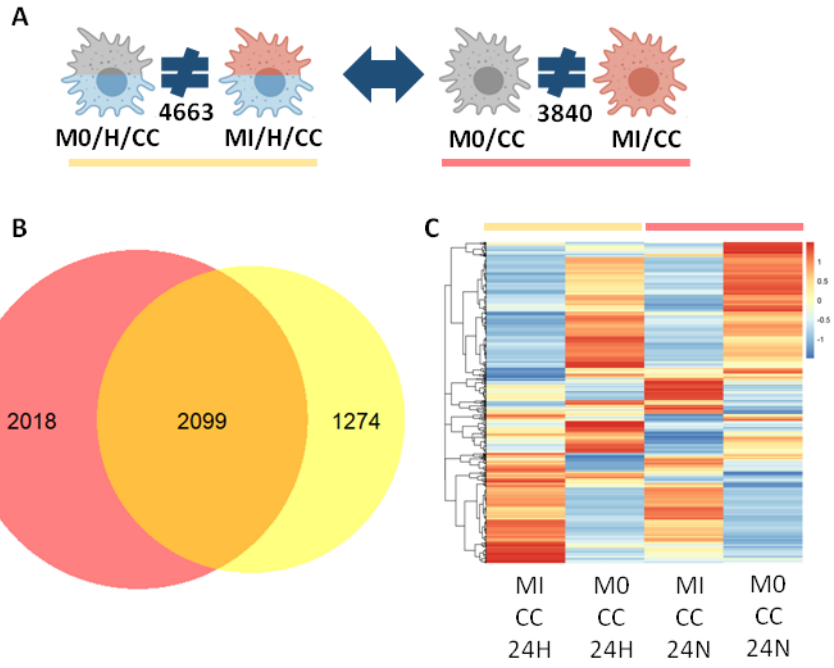


Fig. 119 Comparison (M0vsMI)H/CCvs(M0vsMI)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMI/H/CC; pink= M0/CCvsMI/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column

represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C100 (C30vsC26)]

Fibroblasts stimulated for 24h with LPS+IFNγ in co-culture with macrophages acquire a pro-inflammatory phenotype (FbI/CC) different from co-cultivated Fb without any stimulation (Fb0/CC) (4841 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 24h under hypoxia (FbI/H/CC) they acquire a pro-inflammatory phenotype, similarly on what happen in normoxia, in comparison to hypoxic, co-cultivated, not stimulated fibroblasts (FbH/CC) (4106 SDEG); indeed, when fibroblasts are stimulated with LPS+IFNγ in co-culture, they change their phenotype independently from oxygen status of microenvironment. If we consider genes differentially expressed in the two comparisons, we observe that 2119 genes are shared, 2090 are differentially expressed only in hypoxic, co-cultivated Fb and 1500 in the normoxic co-cultivated Fb (B). By excluding genes that are differentially expressed in both comparisons and considering the 2090 and 1500 genes together, we obtain a 4-columns heatmap where we can observe that FbI CC 24H and FbI CC 24N (1st and 3rd column) have a similar gene expression pattern that is the opposite of the other two samples Fb0 CC 24H and Fb0 CC 24N (2nd and 4th column) (C).

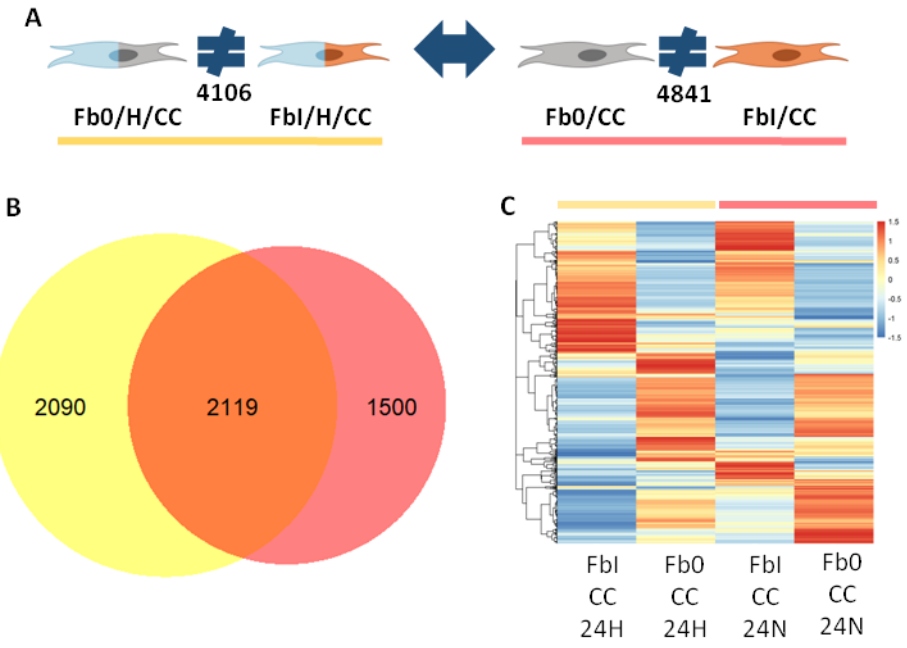


Fig. 120 Comparison (Fb0vsFbI)H/CCvs(Fb0vsFbI)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in

the two comparisons (yellow = FbH/CCvsFbI/H/CC; pink= Fb0/CCvsFbI/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.2.5 What is the effect of hypoxia when pro-inflammatory cells are co-cultivated?

[Code: C101 (C35vsC31)]

Macrophages stimulated with LPS+IFNγ and put under hypoxia for 4h (MI/H) are similar to MI Mφ in normoxia (MI) (22 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 4h under hypoxia (MI/H/CC) they remain similar to normoxic, pro-inflammatory, co-cultivated macrophages (MI/CC) (17 SDEG); indeed, when pro-inflammatory macrophages are under hypoxia they do not change their phenotype in single culture neither in co-culture. If we consider genes differentially expressed in the two comparisons we observe that 12 genes are shared, 5 are differentially expressed only in co-cultivated MI Mφ and 9 in single cultivated MI Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 5 and 9 genes together, we obtain a 4-columns heatmap where we can observe that MI CC 4H and MI SC 4H (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples MI CC 4N and MI SC 4N (2nd and 4th column) (C).

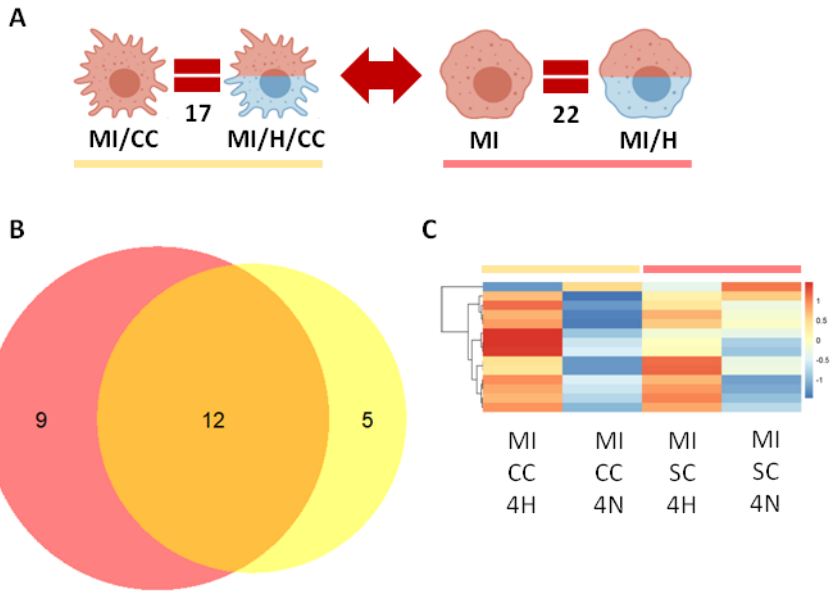


Fig. 121 Comparison (MIvsMI/H)CCvs(MIvsMI/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory macrophages at time point of 4h. For

each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MI/CCvsMI/H/CC; pink= MIvsMI/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C102 (C36vsC32)]

Fibroblasts stimulated with LPS+IFNγ and put under hypoxia for 4h (FbI/H) are the same to FbI Mφ in normoxia (FbI); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/H/CC), they remain the same to normoxic pro-inflammatory co-cultivated fibroblasts (FbI/CC); indeed, when pro-inflammatory fibroblasts are under hypoxia they do not change their phenotype in single culture neither in co-culture.

Fig. 122 Comparison (FbIvsFbI/H)CCvs(FbIvsFbI/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A).

[Code: C103 (C37vsC33)]

Macrophages stimulated with LPS+IFNγ and put under hypoxia for 24h (MI/H) are similar to MI Mφ in normoxia (MI) (32 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 24h under hypoxia (MI/H/CC), they remain similar to normoxic, pro-inflammatory, co-cultivated macrophages (MI/CC) (37 SDEG); indeed, when pro-inflammatory macrophages are under hypoxia, they do not change their phenotype in single culture neither in co-culture. If we consider genes differentially expressed in the two comparisons, we observe that 11 genes are shared, 21 are differentially expressed only in co-cultivated MI Mφ and 26 in single cultivated MI Mφ (B). By excluding genes that are differentially expressed in both comparison and considering the 21 and 26 genes together, we obtain a 4-columns heatmap where we can observe that all samples have different pattern of gene expression (C).

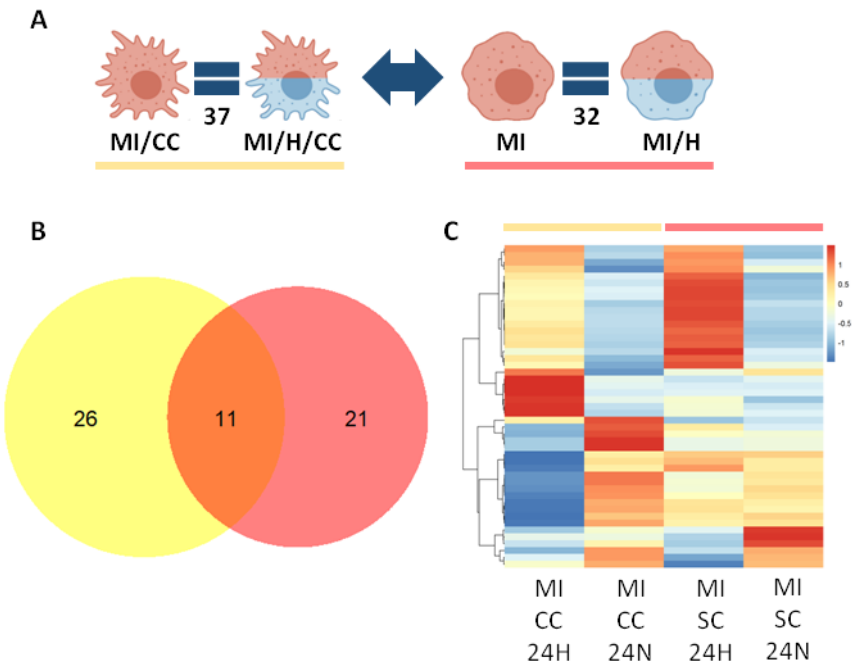


Fig. 123 Comparison (MIvsMI/H)CCvs(MIvsMI/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MI/CCvsMI/H/CC; pink= MIvsMI/H) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C104 (C38vsC34)]

Fibroblasts stimulated with LPS+IFNγ and put under hypoxia for 4h (FbI/H) are similar to FbI Mφ in normoxia (FbI) (12 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/H/CC), they remain the same to normoxic, pro-inflammatory, co-cultivated fibroblasts (FbI/CC) (22 SDEG); indeed, when pro-inflammatory fibroblasts are under hypoxia they do not change their phenotype in single culture neither in co-culture. If we consider genes differentially expressed in the two comparisons we observe that there are no genes shared, 22 are differentially expressed only in co-cultivated FbI Fb and 12 in single cultivated FbI Fb (B). Considering genes together, we obtain a 4-columns heatmap where we can observe that all samples have different pattern of gene expression (C).

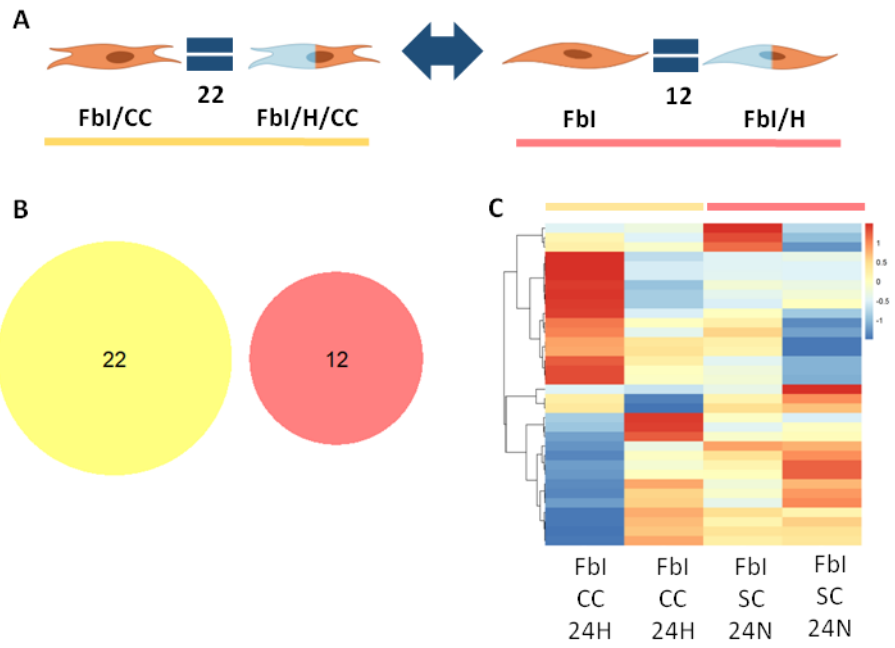


Fig. 124 Comparison (FbIvsFbI/H)CCvs(FbIvsFbI/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbI/CCvsFbI/H/CC; pink= FbIvsFbI/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.2.6 What is the effect of co-culture when pro-inflammatory cells are put under hypoxia?

[Code: C105 (C43vsC39)]

Macrophages stimulated with LPS+IFNγ and put in co-culture with fibroblasts for 4h (MI/CC) are similar to MI Mφ alone (MI) (14 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 4h under hypoxia (MI/H/CC) they remain similar to hypoxic pro-inflammatory single-cultivated macrophages (MI/H) (38 SDEG); indeed, when pro-inflammatory macrophages are co-cultivated they do not change their phenotype independently from the oxygen status. If we consider genes differentially expressed in the two comparisons, we observe that 11 genes are shared, 26 are differentially expressed only in hypoxic MI Mφ and 3 in normoxic MI Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 26 and 3 genes together, we obtain a 4-columns heatmap where we can observe that MI CC 4N and MI CC 4H (1st and 3rd

column) have a similar gene expression pattern that is the opposite of the other two samples MI SC 4N and MI SC 4H (2nd and 4th column) (C).

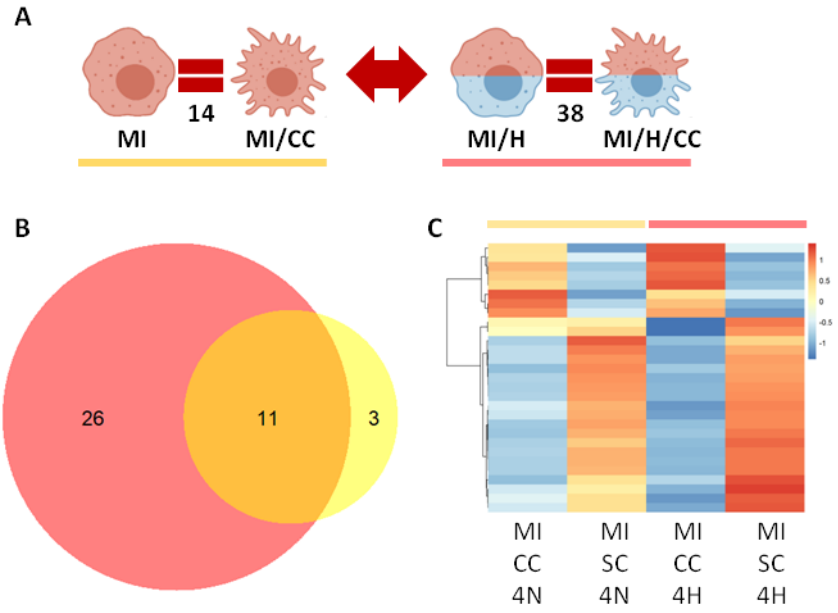


Fig. 125 Comparison (MIvsMI/CC)Hvs(MIvsMI/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MIvsMI/CC; pink= MI/HvsMI/H/CC) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C106 (C44vsC40)]

Fibroblasts stimulated with LPS+IFNγ and put in co-culture with macrophages for 4h (FbI/CC) are similar to FbI Fb alone (FbI); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/H/CC), they remain similar to hypoxic, pro-inflammatory, single-cultivated fibroblasts (FbI/H) (25 SDEG); indeed, when pro-inflammatory fibroblasts are co-cultivated they do not change their phenotype independently from oxygen status. If we consider genes differentially expressed in the two comparisons, we observe that 23 are differentially expressed only in hypoxic FbI Fb (B). By excluding genes that are differentially expressed in both comparison and considering genes together we obtain a 4-columns heatmap where we can observe that FbI CC 4N and FbI CC 4H (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples FbI SC 4N and FbI SC 4H (2nd and 4th column) (C).

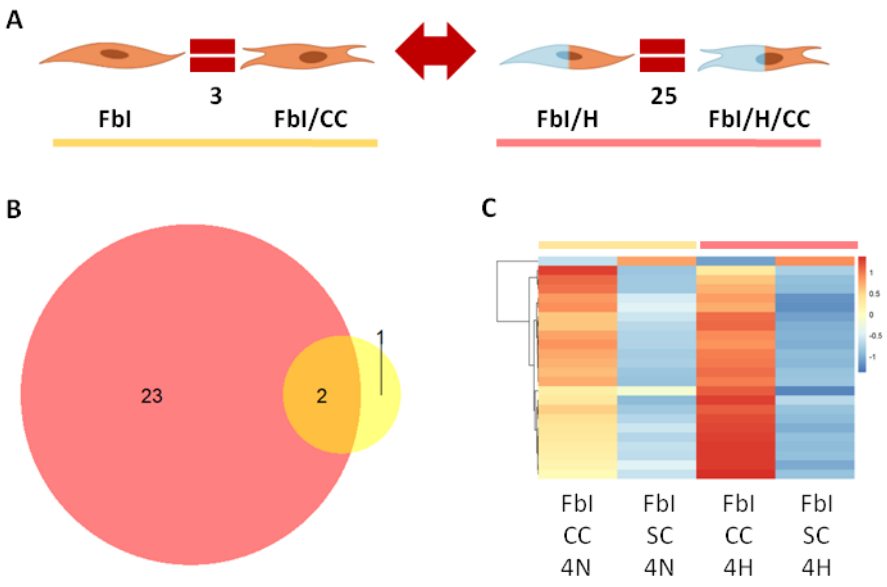


Fig. 126 Comparison (FbIvsFbI/CC)Hvs(FbIvsFbI/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbIvsFbI/CC; pink= FbI/HvsFbI/H/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C107 (C45vsC41)]

Macrophages stimulated with LPS+IFNγ and put in co-culture with fibroblasts for 24h (MI/CC) are different from MI Mφ alone (MI) (110 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with LPS+IFNγ for 24h under hypoxia (MI/H/CC), they are different from hypoxic, pro-inflammatory, single-cultivated macrophages (MI/H) with higher number of SDEG (943); indeed, when pro-inflammatory macrophages are co-cultivated, they change their phenotype both in normoxia and in hypoxia but in the last case there is an higher number of differentially expressed genes. If we consider genes differentially expressed in the two comparisons, we observe that 32 of that genes are shared, 785 are differentially expressed only in hypoxic MI Mφ and 66 in normoxic MI Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 785 and 66 genes together, we obtain a 4-columns heatmap where we can observe that MI SC 24N and MI SC 24H (2nd and 4th column) have a similar gene expression pattern, instead MI CC 24N and MI CC 24H (1st and 3rd column) have a different pattern of expression (C).

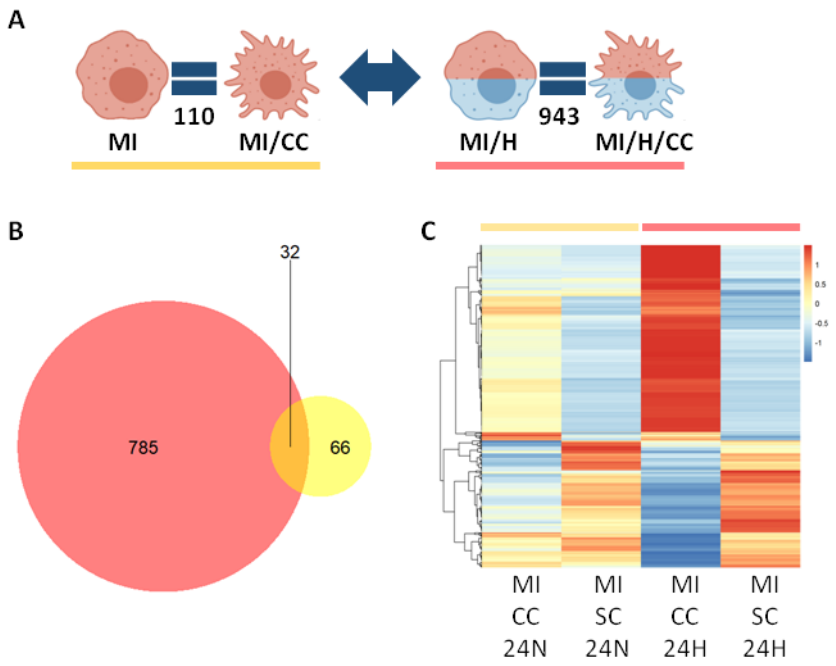


Fig. 127 Comparison (MIvsMI/CC)Hvs(MIvsMI/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MIvsMI/CC; pink= MI/HvsMI/H/CC) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C108 (C46vsC42)]

Fibroblasts stimulated with LPS+IFNγ and put in co-culture with macrophages for 24h (FbI/CC) are different from FbI Fb alone (FbI) (1973 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with LPS+IFNγ for 24h under hypoxia (FbI/H/CC), they are different from hypoxic pro-inflammatory, single-cultivated fibroblasts (FbI/H) (1900 SDEG); indeed, when pro-inflammatory fibroblasts are co-cultivated, they change their phenotype both in normoxia and in hypoxia. If we consider genes differentially expressed in the two comparisons, we observe that 630 of that genes are shared, 742 are differentially expressed only in hypoxic FbI Fb and 1012 in normoxic FbI Fb (B). By excluding genes that are differentially expressed in both comparisons and considering the 742 and 1012 genes together, we obtain a 4-columns heatmap where we can observe that FbI CC 24N and FbI CC 24H (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples FbI SC 24N and FbI SC 24H (2nd and 4th column) (C).

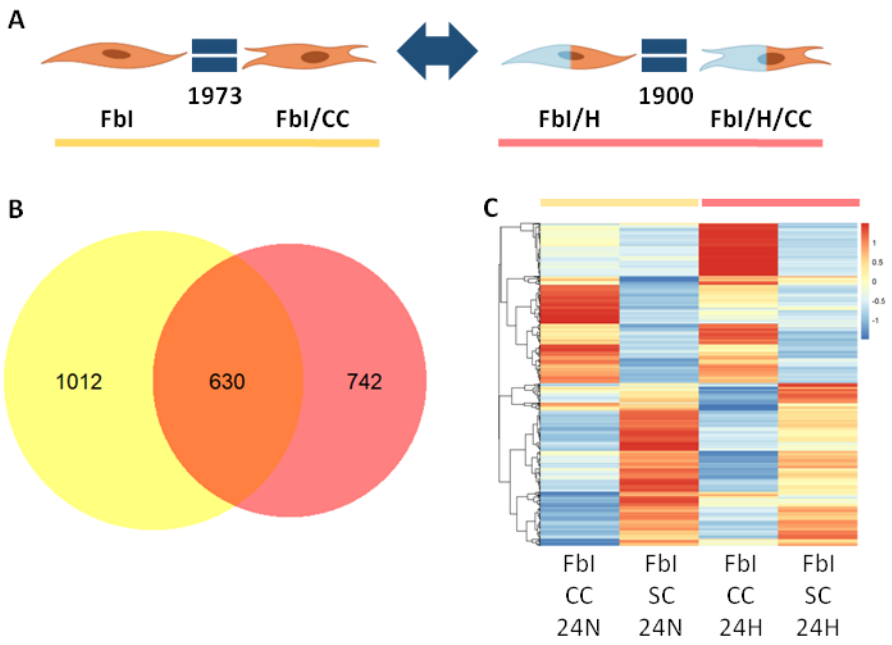


Fig. 128 Comparison (FbIvsFbI/CC)Hvs(FbIvsFbI/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of pro-inflammatory fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbIvsFbI/CC; pink= FbI/HvsFbI/H/CC) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.3.1 What is the effect of pro-fibrotic stimuli when cells are co-cultivated?

[Code: C109 (C55vsC47)]

Macrophages stimulated for 4h with IL-4 acquire an alternative phenotype (MF) different from resting Mφ (190 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 4h, they acquire an alternative phenotype similarly on what happen in the single culture, in comparison to co-cultivated, not stimulated macrophages (138 SDEG); indeed, when macrophages are stimulated with IL-4, they change their phenotype independently from the culture condition. If we consider genes differentially expressed in the two comparisons, we observe that 77 genes are shared, 61 are differentially expressed only in co-cultivated Mφ and 108 in the single cultivated Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 61 and 108 genes together, we obtain a 4-columns heatmap where we can observe that MF SC 4N and MF CC 4N (1st and 3rd column) have a similar gene expression pattern that is the opposite of the other two samples M0 CC 24N and M0 SC 24N (2nd and 4th column) (C).

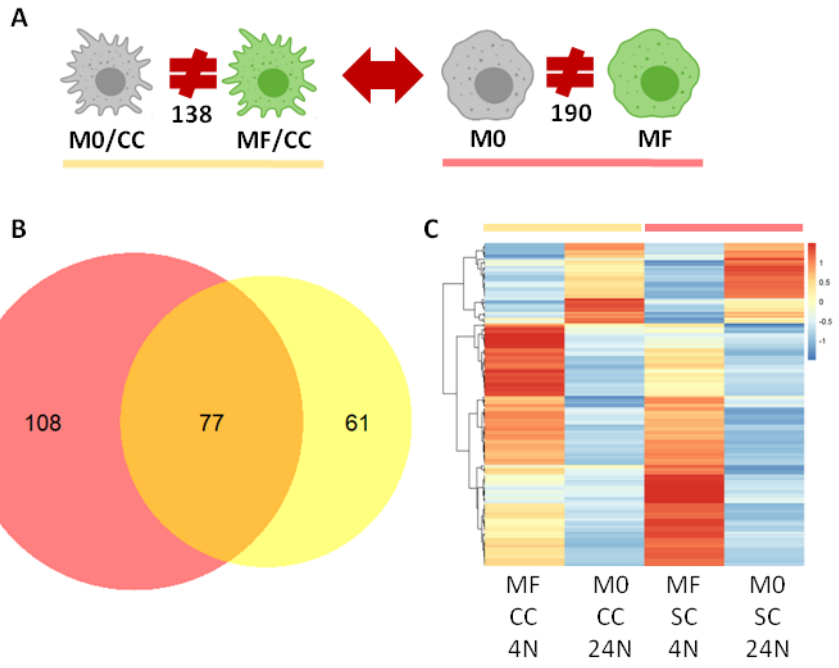


Fig. 129 Comparison (M0vsMF)CCvs(M0vsMF)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = M0/CCvsMF/CC; pink= M0vsMF) (B); the 4-columns heatmap indicates the total of

genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C110 (C56vsC48)]

Fibroblasts stimulated for 4h with IL-4 do not acquire a different phenotype (FbF) from resting Fb (42 SDEG); instead, when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 4h, they acquire a different phenotype in comparison to co-cultivated, un-stimulated fibroblasts (133 SDEG); indeed, when fibroblasts are stimulated with IL-4, they change their phenotype only in co-culture. If we consider genes differentially expressed in the two comparisons, we observe that 36 genes are shared, 92 are differentially expressed only in co-cultivated Fb and 6 in the single cultivated Fb (B). By excluding genes that are differentially expressed in both comparisons and considering the 92 and 6 genes together, we obtain a 4-columns heatmap where we can observe that Fb0 CC 24N and Fb0 SC 24N (2nd and 4th column) have a similar pattern of gene expression that is the opposite of the other two samples. Moreover, FbF CC 4N (1stcolumn) has a particular pattern that differs also from FbF SC 4N(3rd column) (C).

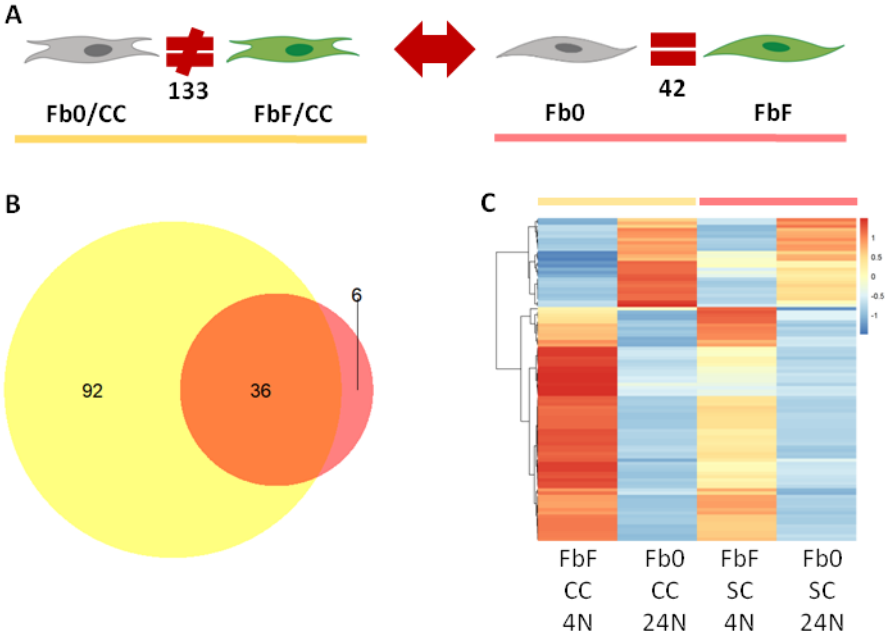


Fig. 130 Comparison (Fb0vsFbF)CCvs(Fb0vsFbF)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = Fb0/CCvsFbF/CC; pink= Fb0vsFbF) (B); the 4-columns heatmap indicates the total of

genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C111 (C57vsC49)]

Macrophages stimulated for 24h with IL-4 acquire an alternative phenotype (MF) different from resting Mφ (178 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 24h, they acquire an alternative phenotype similarly on what happen in the single culture, in comparison to co-cultivated macrophages not stimulated (173 SDEG); indeed, when macrophages are stimulated with IL-4, they change their phenotype independently from culture condition. If we consider genes differentially expressed in the two comparisons, we observe that 111 genes are shared, 55 are differentially expressed only in co-cultivated Mφ and 65 in the single cultivated Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 61 and 108 genes together, we obtain a 4-columns heatmap where we can observe that MF SC 24N and MF CC 24N (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples M0 CC 24N and M0 SC 24N (2nd and 4th column) (C).

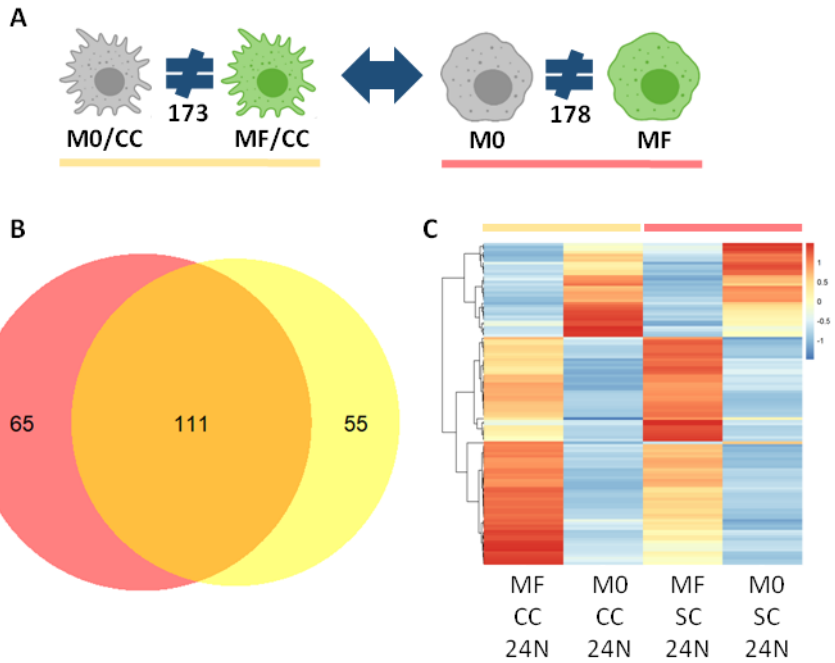


Fig. 131 Comparison (M0vsMF)CCvs(M0vsMF)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = M0/CCvsMF/CC; pink= M0vsMF) (B); the 4-columns heatmap indicates the total of

genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C112 (C58vsC50)]

Fibroblasts stimulated for 24h with IL-4 do not acquire a different phenotype (FbF) different from resting Fb; when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 24h they maintain the same phenotype of co-cultivated fibroblasts not stimulated; indeed, when fibroblasts are stimulated with IL-4 do not change their phenotype neither if they are single or co-cultivated. If we consider genes differentially expressed in the two comparisons, we observe that the 3 genes differentially expressed the single cultivated Fb are included in the 7 genes differentially expressed in co-cultivated Fb (B). Considering the 4 genes belonging to the co-cultivated fibroblast comparison we obtain a 4-columns heatmap where we can observe that FbF SC 4N and FbF CC 4N (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples Fb0 CC 24N and Fb0 SC 24N (2nd and 4th column) (C).

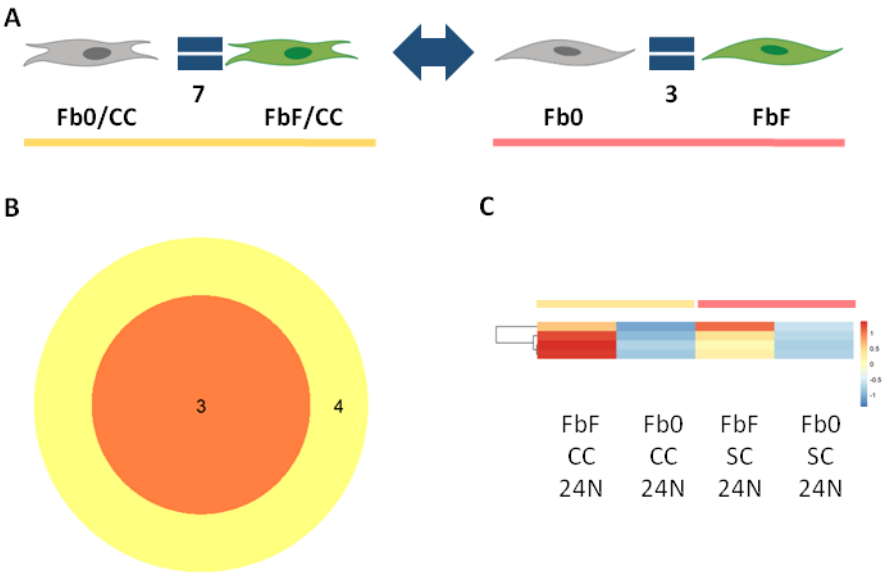


Fig. 132 Comparison (Fb0vsFbF)CCvs(Fb0vsFbF)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = Fb0/CCvsFbF/CC; pink= Fb0vsFbF) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.3.2 What is the effect of pro-fibrotic stimuli when cells are under hypoxia?

[Code: C113 (C51vsC47)]

Macrophages stimulated for 4h with IL-4 acquire an alternative phenotype (MF) different from resting Mφ (190 SDEG); instead, when macrophages are put under hypoxia and stimulated with IL-4 for 4h, they acquire an alternative phenotype in comparison to hypoxic, un-stimulated macrophages but with a lower number of SDEG (88) in comparison with the normoxic set up; indeed, when macrophages are stimulated with IL-4, they change their phenotype in normoxia with a greater extent than in hypoxia. If we consider genes differentially expressed in the two comparisons, we observe that 69 genes are shared, 16 are differentially expressed only in hypoxic Mφ and 116 in the normoxic Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 16 and 116 genes together, we obtain a 4-columns heatmap where we can observe that MF SC 4H and MF SC 4N (1st and 3rd column) have a similar gene expression pattern that is the opposite of the other two samples M0 SC 4H and M0 SC 24N (2nd and 4th column) (C).

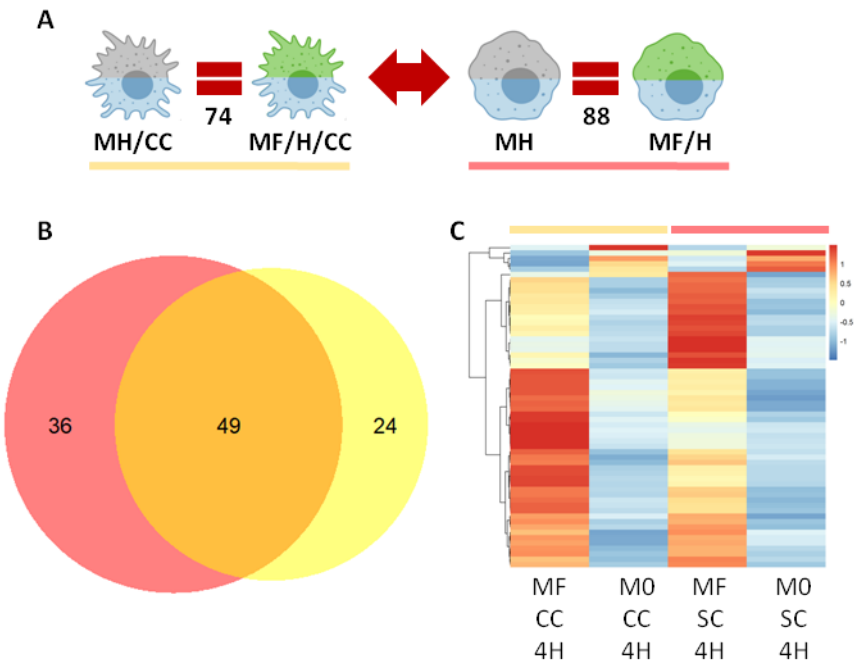


Fig. 133 Comparison (M0vsMF)Hvs(M0vsMF)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = M0vsMF; pink= MHvsMF/H) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C114 (C52vsC48)]

Fibroblasts stimulated for 4h with IL-4 (FbF) show a similar phenotype to resting Fb (42 SDEG); when fibroblasts are put under hypoxia and stimulated with IL-4 for 4h, they maintain the same phenotype, similarly on what happen in normoxia, in comparison to hypoxic, not stimulated fibroblasts; indeed, when fibroblasts are stimulated with IL-4, they do not change their phenotype independently from the oxygen status of environment. If we consider genes differentially expressed in the two comparisons, we observe that 4 genes differentially expressed in hypoxic Fb are included in the 42 genes differentially expressed in normoxic Fb (B). Considering 38 genes belonging to the normoxic Fb0vsFbF comparison we obtain a 4-columns heatmap where we observe that FbF SC 4H, Fb0 SC 4H and FbF SC 4N (1st, 2nd and 3rd column) show a similar pattern of gene expression, while the sample Fb0 SC 24N has a distinctive profile (C).

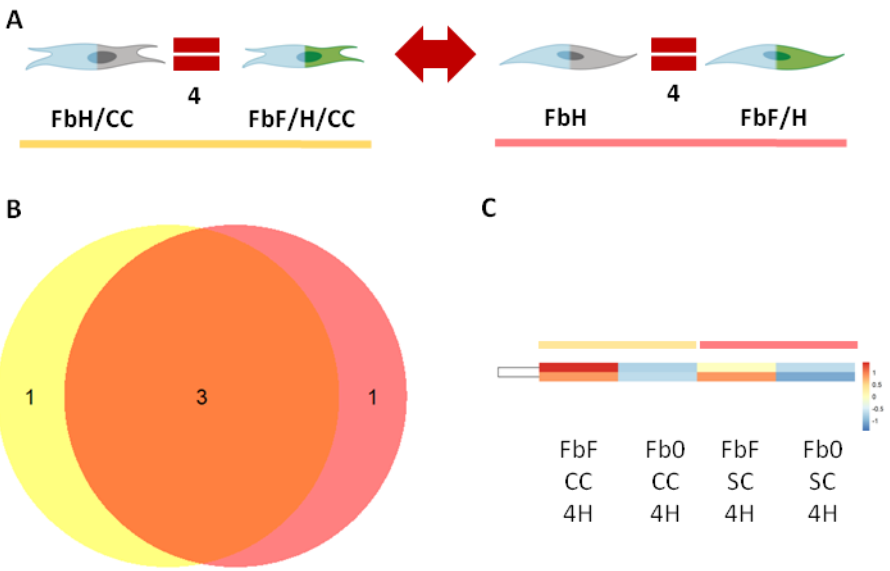


Fig. 134 Comparison (Fb0vsFbF)Hvs(Fb0vsFbF)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbHvsFbF/H; pink= Fb0vsFbF) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C115 (C53vsC49)]

Macrophages stimulated for 24h with IL-4 acquire an alternative phenotype (MF) different from resting Mφ (178 SDEG); when macrophages are put under hypoxia and stimulated with IL-4 for 24h, they acquire an alternative phenotype in comparison to hypoxic, not stimulated macrophages (144 SDEG); indeed, when macrophages are stimulated with IL-4, they change their phenotype independently from the oxygen status of environment. If we consider genes differentially expressed in the two comparisons, we observe that 107 genes are shared, 34 are differentially expressed only in hypoxic Mφ and 69 in the normoxic Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 34 and 69 genes together, we obtain a 4-columns heatmap where we can observe that MF SC 24H and MF SC 24N (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples M0 SC 24H and M0 SC 24N (2nd and 4th column) (C).

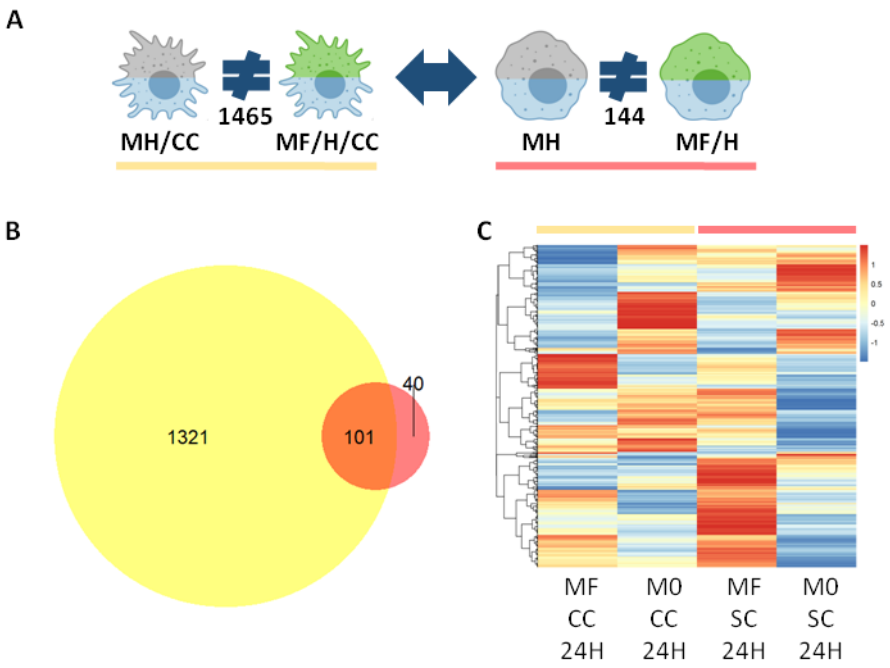


Fig. 135 Comparison (M0vsMF)Hvs(M0vsMF)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MHvsMF/H; pink= M0vsMF) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C116 (C54vsC50)]

Fibroblasts stimulated for 24h with IL-4 (FbF) show the same phenotype of resting Fb; when fibroblasts are put under hypoxia and stimulated with IL-4 for 24h, they maintain the same phenotype, similarly on what happen in normoxia, in comparison to hypoxic fibroblasts not stimulated; indeed, when fibroblasts are stimulated with IL-4, they do not change their phenotype independently from the oxygen status of environment. If we consider genes differentially expressed in the two comparisons, we observe that 3 genes differentially expressed the normoxic Fb are included in the 6 genes differentially expressed in hypoxic Fb (B). Considering the 3 genes belonging to the hypoxic FbHvsFbF/H comparison we obtain a 4-columns heatmap where we can observe that FbF SC 24H and FbF SC 24N (1stand 3rd column) have a similar pattern, different from Fb0 SC 24H and Fb0 SC 24N (2nd and 4th column) (C).

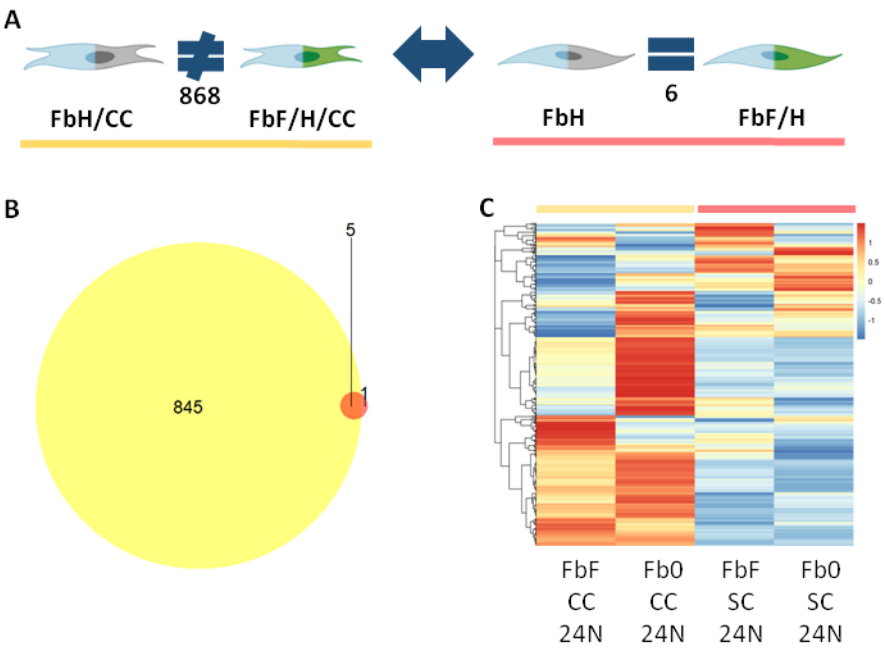


Fig. 136 Comparison (Fb0vsFbF)Hvs(Fb0vsFbF)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbHvsFbF/H; pink= Fb0vsFbF) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.3.3 What is the effect of pro-fibrotic stimuli when hypoxic cells are co-cultivated?

[Code: C117 (C59vsC51)]

Macrophages stimulated for 4h with IL-4 under hypoxia (MF/H) show a similar phenotype to hypoxic Mφ (MH) (88 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 4h under hypoxia (MF/H/CC), they acquire a similar phenotype to hypoxic, co-cultivated, not stimulated macrophages (MH/CC) (74 SDEG); indeed, when macrophages are stimulated with IL-4 in hypoxia, they do not change their phenotype independently from culture condition. If we consider genes differentially expressed in the two comparisons, we observe that 49 genes are shared, 24 are differentially expressed only in hypoxic, co-cultivated Mφ and 36 in single cultivated, hypoxic Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 24 and 36 genes together, we obtain a 4-columns heatmap where we can observe that MF CC 4H and MF SC

4H (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples M0 CC 4H and M0 SC 4H (2nd and 4th column) (C).

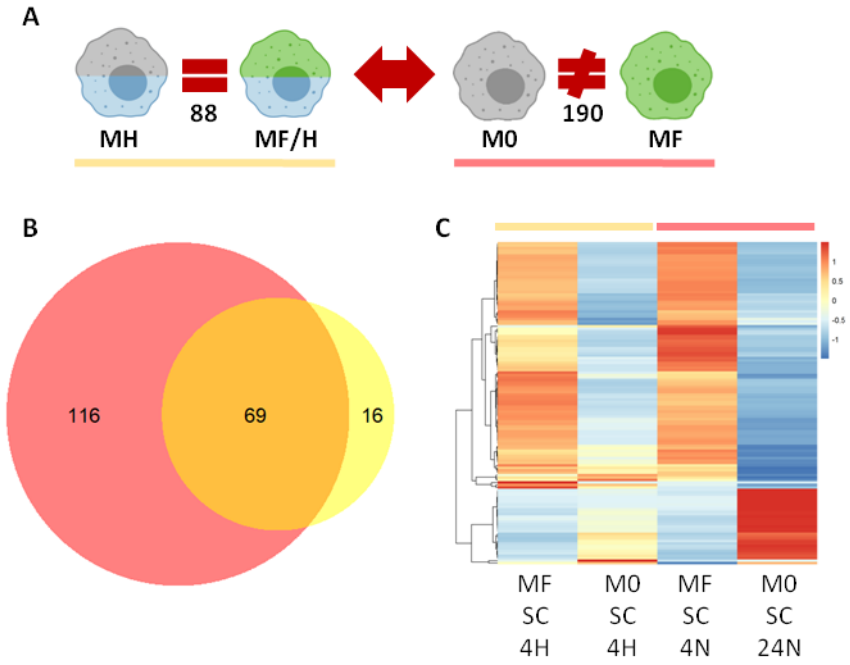


Fig. 137 Comparison (M0vsMF)H/CCvs(M0vsMF)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMF/H/CC; pink= MHvsMF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C118 (C60vsC52)]

Fibroblasts stimulated for 4h with IL-4 under hypoxia (FbF/H) are the same to hypoxic Fb (FbH); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 4h under hypoxia (FbI/H/CC), they acquire the same phenotype of hypoxic, co-cultivated, not stimulated fibroblasts (FbH/CC); indeed, when fibroblasts are stimulated with IL-4 in hypoxia, they do not change their phenotype independently from culture condition. If we consider genes differentially expressed in the two comparisons, we observe that 3 genes are shared, 1 is differentially expressed only in hypoxic co-cultivated Fb and 1 in the single cultivated hypoxic Fb (B). By excluding genes that are differentially expressed in both comparison and considering the 2 genes together, we obtain a 4-columns heatmap where we observe that FbI CC 4H and FbI SC 4H (1st and 3rd column) have a similar pattern of gene

expression that is the opposite of the other two samples Fb0 CC 4H and Fb0 SC 4H (2nd and 4th column) (C).

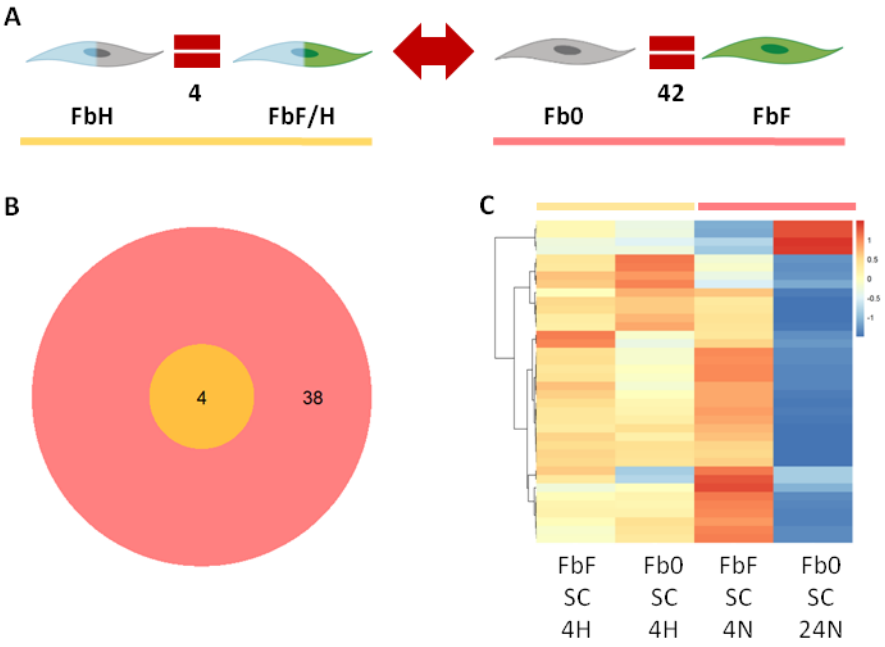


Fig. 138 Comparison (Fb0vsFbF)H/CCvs(Fb0vsFbF)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbH/CCvsFbF/H/CC; pink= FbHvsFbF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C119 (C61vsC53)]

Macrophages stimulated for 24h with IL-4 under hypoxia (MF/H) show a different phenotype from hypoxic Mφ (MH) (144 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 24h under hypoxia (MF/H/CC), the difference acquired in the single culture, in comparison to hypoxic, co-cultivated, un-stimulated macrophages (MH/CC), is increased (1416 SDEG); indeed, when macrophages are stimulated with IL-4 in hypoxia, they change their phenotype with a greater extent if they are co-cultivated. If we consider genes differentially expressed in the two comparisons, we observe that 101 genes are shared, 1321 are differentially expressed only in hypoxic, co-cultivated Mφ and 40 in single cultivated, hypoxic Mφ (B). By excluding genes that are differentially expressed in both

comparisons and considering the 1321 and 40 genes together, we obtain a 4-columns heatmap where we can observe that samples show an heterogeneous gene expression pattern (C).

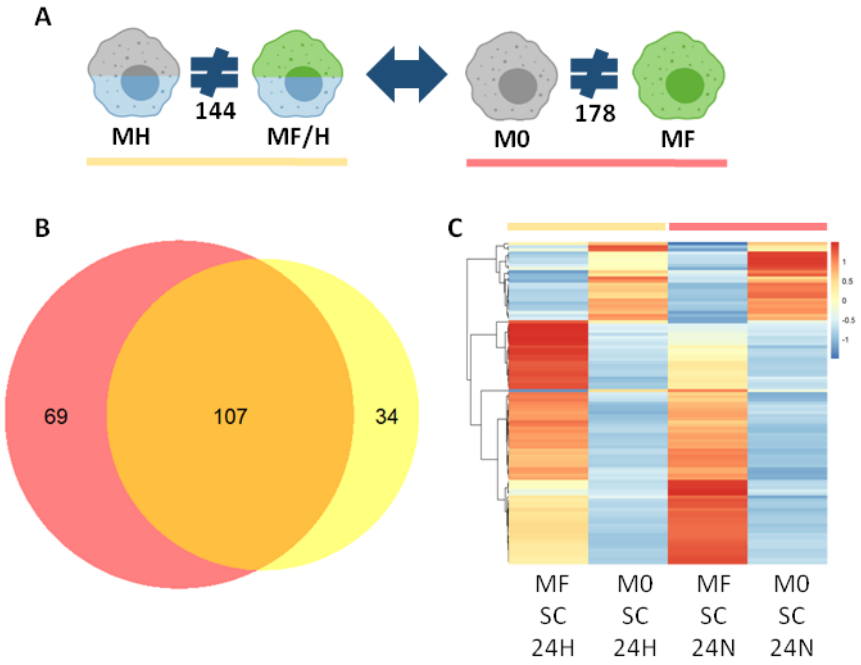


Fig. 139 Comparison (M0vsMF)H/CCvs(M0vsMF)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMF/H/CC; pink= MHvsMF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C120 (C62vsC54)]

Fibroblasts stimulated for 4h with IL-4 under hypoxia (FbF/H) are the same to hypoxic Fb (FbH); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 4h under hypoxia (FbI/H/CC), they acquire a different phenotype from hypoxic, un-stimulated, co-cultivated fibroblasts (FbH/CC) (868 SDEG); indeed, when fibroblasts are stimulated with IL-4 in hypoxia, they change their phenotype only in co-culture. If we consider genes differentially expressed in the two comparisons, we observe that only 5 genes are shared, 845 are differentially expressed only in hypoxic co-cultivated Fb and 1 in the single cultivated hypoxic Fb (B). By excluding genes that are differentially expressed in both comparisons and considering the 845 and 1 genes together we obtain a 4-columns heatmap where we can

observe that FbF SC 24H and Fb0 SC 24H (3rd and 4th column) share a similar pattern of gene expression (C).

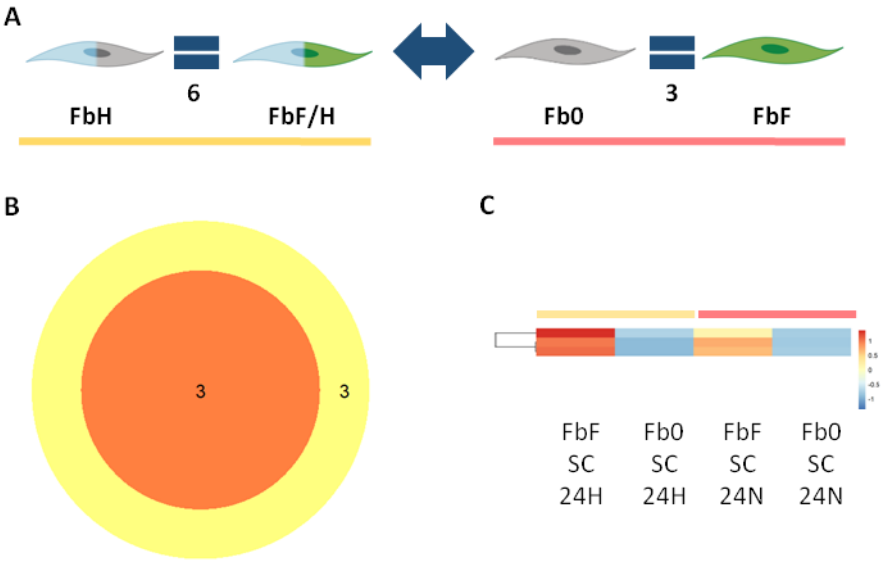


Fig. 140 Comparison (Fb0vsFbF)H/CCvs(Fb0vsFbF)H/SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbH/CCvsFbF/H/CC; pink= FbHvsFbF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.3.4 What is the effect of pro-fibrotic stimuli when co-cultivated cells are put under hypoxia?

[Code: C121 (C59vsC55)]

Macrophages stimulated for 4h with IL-4 in co-culture with fibroblasts (MI/CC) acquire a different phenotype from co-cultivated Mφ without any stimulation (M0/CC) (138 SDEG); instead, when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 4h under hypoxia (MI/H/CC), they show a similar phenotype to hypoxic, co-cultivated, not stimulated macrophages (MH/CC) (74 SDEG); indeed, when macrophages are stimulated with IL-4 in co-culture, they change their phenotype only in normoxic microenvironment. If we consider genes differentially expressed in the two comparisons, we observe that 50 genes are shared, 23 are differentially expressed only in hypoxic co-cultivated Mφ and 88 in the normoxic co-cultivated Mφ (B). By excluding genes that are differentially expressed in both

comparisons and considering the 23 and 88 genes together we obtain a 4-columns heatmap where we can observe that MI CC 4H and MI CC 4N (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples M0 CC 4H and M0 CC 24N (2nd and 4th column) (C).

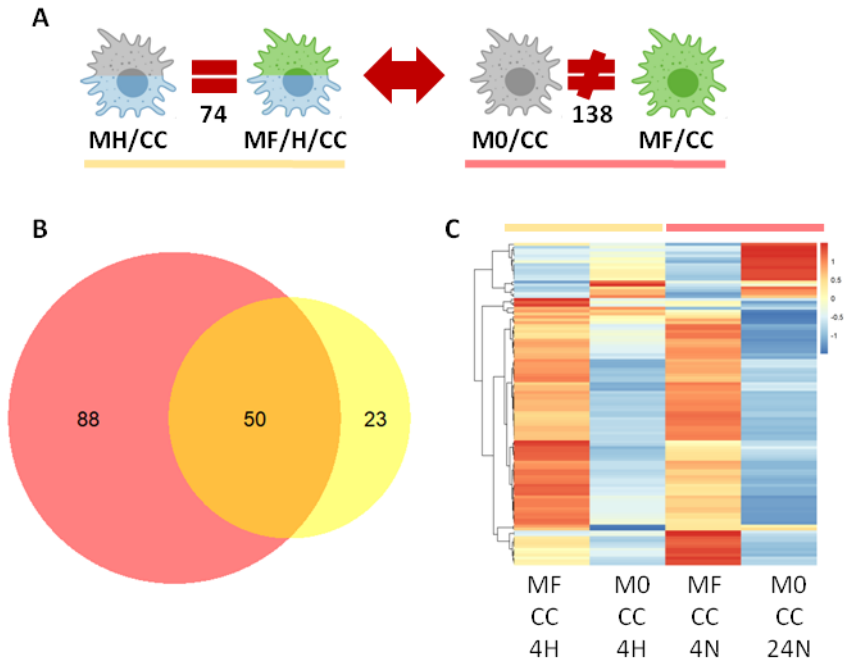


Fig. 141 Comparison (M0vsMF)H/CCvs(M0vsMF)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMF/H/CC; pink= M0/CCvsMF/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C122 (C60vsC56)]

Fibroblasts stimulated for 4h with IL-4 in co-culture with macrophages (FbI/CC) acquire a different phenotype from co-cultivated Fb without any stimulation (Fb0/CC) (133 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 4h under hypoxia (FbF/H/CC), they do not acquire a different phenotype from hypoxic, co-cultivated, un-stimulated fibroblasts (FbH/CC); indeed, when fibroblasts are stimulated with IL-4 in co-culture, they change their phenotype in normoxic microenvironment only. If we consider genes differentially expressed in the two comparisons, we observe that 4 genes differentially expressed in the comparison FbH/CCvsFbF/H/CC are included in the 133 genes differentially

expressed in normoxic, co-cultivated Fb (B). Considering the 124 genes, we obtain a 4-columns heatmap where we can observe that FbF CC 4H, Fb0 CC 4H and FbF CC 4N (1st, 2ndand 3rdcolumn) have a similar pattern of gene expression that is the opposite of sample Fb0 CC 24N (4th column) (C).

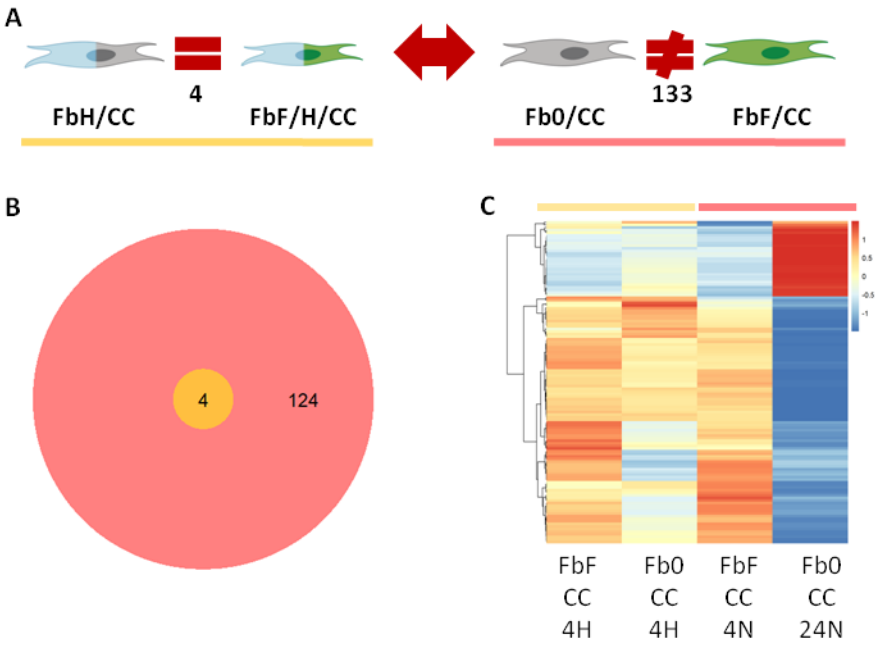


Fig. 142 Comparison (Fb0vsFbF)H/CCvs(Fb0vsFbF)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbH/CCvsFbF/H/CC; pink= Fb0/CCvsFbF/CC) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C123 (C61vsC57)]

Macrophages stimulated for 24h with IL-4 in co-culture with fibroblasts (MI/CC) acquire a different phenotype from co-cultivated Mφ without any stimulation (M0/CC) (173 SDEG); instead, when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 24h under hypoxia (MI/H/CC), they show a similar phenotype to hypoxic, not stimulated, co-cultivated macrophages (MH/CC) (1465 SDEG); indeed, when macrophages are stimulated with IL-4 in co-culture, they change their phenotype independently from the oxygen status of microenvironment. If we consider genes differentially expressed in the two comparisons, we observe that 115 genes are shared, 1307 are differentially expressed only in hypoxic co-

cultivated Mφ and 51 in the normoxic co-cultivated Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 1307 and 51 genes together, we obtain a 4-columns heatmap where we can observe an heterogeneous pattern of gene expression through samples (C).

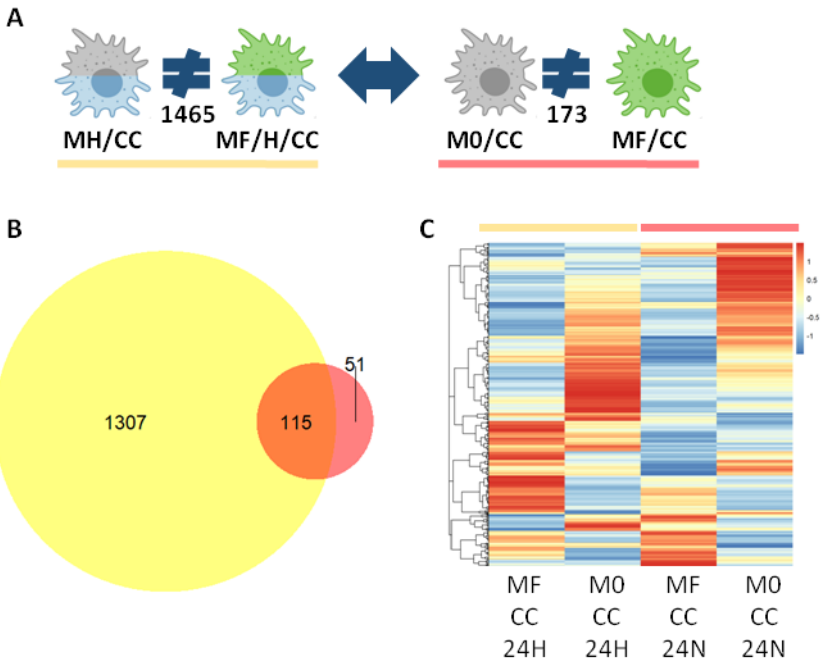


Fig. 143 Comparison (M0vsMF)H/CCvs(M0vsMF)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MH/CCvsMF/H/CC; pink= M0/CCvsMF/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C124 (C62vsC58)]

Fibroblasts stimulated for 24h with IL-4 in co-culture with macrophages (FbF/CC) show the same phenotype of co-cultivated Fb without any stimulation (Fb0/CC); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 24h under hypoxia (FbF/H/CC), they acquire a different phenotype from hypoxic, co-cultivated, not stimulated fibroblasts (FbH/CC) (868 SDEG); indeed, when fibroblasts are stimulated with IL-4 in co-culture, they change their phenotype in normoxic microenvironment only. If we consider genes differentially expressed in the two comparisons, we observe that 6 genes are shared, 844 are differentially expressed only in hypoxic, co-cultivated Fb and 1 in the normoxic, co-cultivated

Mφ (B). Considering the 844 and 1 genes we obtain a 4-columns heatmap where we can observe that FbF CC 24N, Fb0 CC 24N (3rdand 4thcolumn) have a similar pattern of gene expression (C).

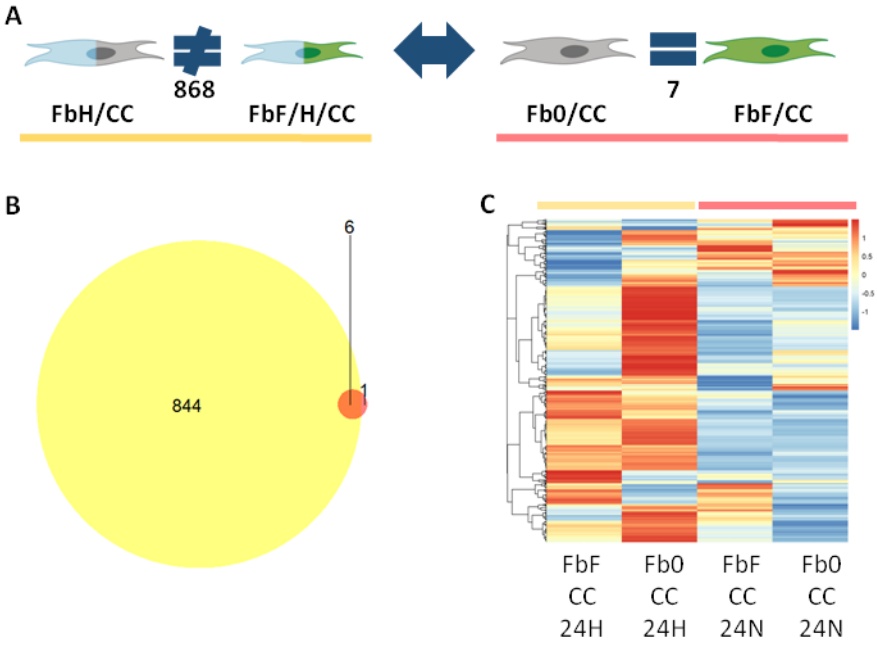


Fig. 144 Comparison (Fb0vsFbF)H/CCvs(Fb0vsFbF)N/CC. Schematic representation of double comparison of two variables: oxygen status and culture condition of resting fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbH/CCvsFbF/H/CC; pink= Fb0/CCvsFbF/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.3.5 What is the effect of hypoxia when pro-fibrotic cell are co-cultivated?

[Code: C125 (C67vsC63)]

Macrophages stimulated with IL-4 and put under hypoxia for 4h (MF/H) are similar to MF Mφ in normoxia (MF) (15 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 4h under hypoxia (MF/H/CC), they remain similar to normoxic, pro-fibrotic, co-cultivated macrophages (MF/CC) (30 SDEG); indeed, when alternative macrophages are put under hypoxia, they do not change their phenotype, independently if they are in single or co-culture. If we consider genes differentially expressed in the two comparisons, we observe that 12 genes are shared, 17 are differentially expressed only in co-cultivated MF Mφ and 3 in single cultivated MF Mφ (B). By excluding genes that are

differentially expressed in both comparisons and considering the 17 and 3 genes together, we obtain a 4-columns heatmap where we can observe that MF CC 4H (1st column) have a different pattern of gene expression in comparison to the other samples and that MF CC 4N and MF SC 4N (2nd and 4th column) have a similar pattern (C).

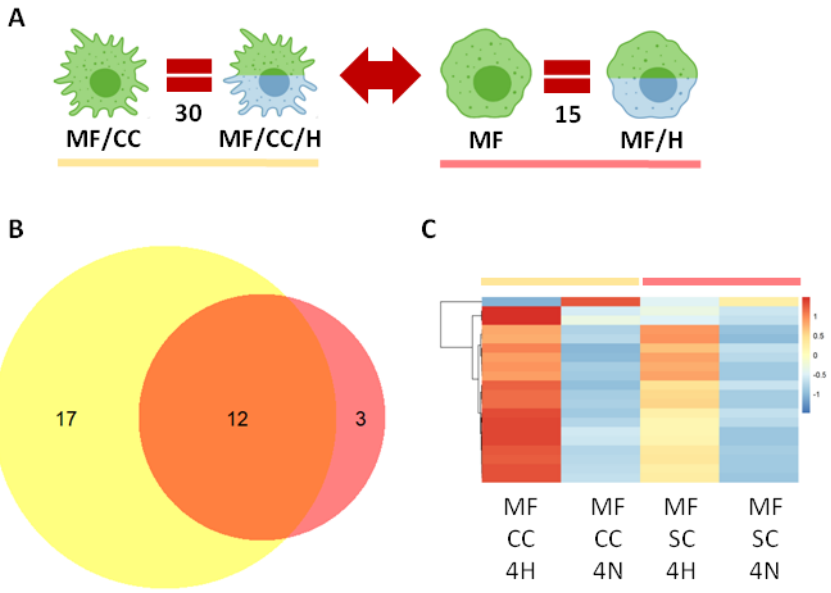


Fig. 145 Comparison (MFvsMF/H)CCvs(MFvsMF/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of alternative macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MF/CCvsMF/H/CC; pink= MFvsMF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C126 (C68vsC64)]

Fibroblasts stimulated with IL-4 and put under hypoxia for 4h (FbF/H) are the same of FbF Fb in normoxia (FbF); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 4h under hypoxia (FbF/H/CC), they remain similar to normoxic, pro-fibrotic, co-cultivated fibroblasts (FbF/CC); indeed, when alternative fibroblasts are put under hypoxia, they do not change their phenotype independently from culture condition.

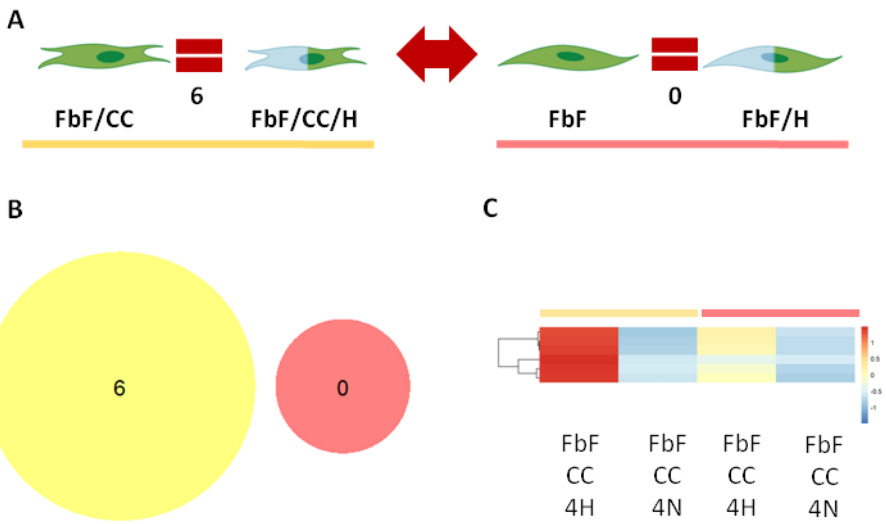


Fig. 146 Comparison (FbFvsFbF/H)CCvs(FbFvsFbF/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbF/CCvsFbF/H/CC; pink= FbFvsFbF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C127 (C69vsC65)]

Macrophages stimulated with IL-4 and put under hypoxia for 24h (MF/H) are similar to MF Mφ in normoxia (MF) (23 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 24h under hypoxia (MF/H/CC), they remain similar to normoxic, pro-fibrotic, co-cultivated macrophages (MF/CC) (17 SDEG); indeed, when alternative macrophages are put under hypoxia, they do not change their phenotype independently if they are in single or co-culture. If we consider genes differentially expressed in the two comparisons, we observe that 10 genes are shared, 6 are differentially expressed only in co-cultivated MF Mφ and 13 in single cultivated MF Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 13 and 6 genes together we obtain a 4-columns heatmap where we can observe that MF CC 24N and MF SC 24N (2nd and 4th column) have a similar pattern of gene expression (C).

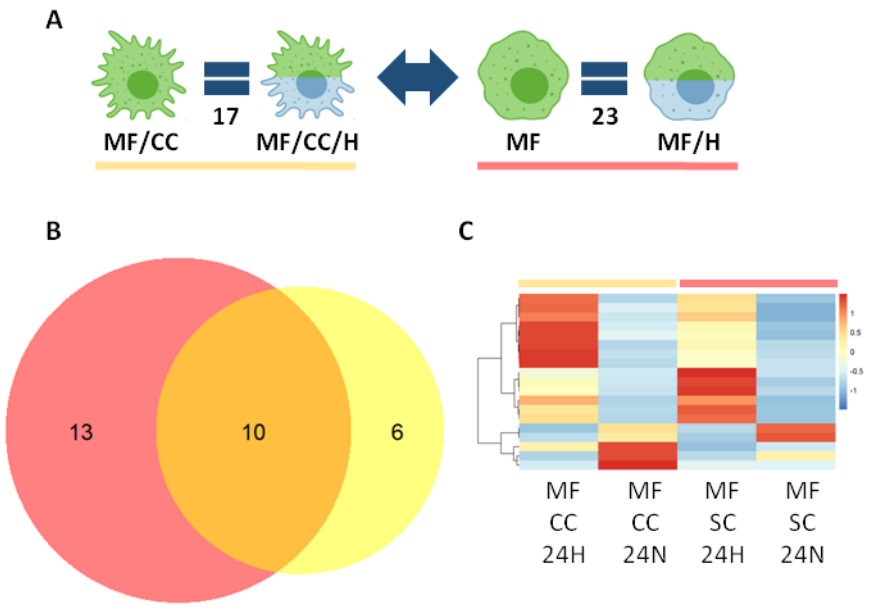


Fig. 147 Comparison (MFvsMF/H)CCvs(MFvsMF/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of alternative macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MF/CCvsMF/H/CC; pink= MFvsMF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C128 (C70vsC66)]

Fibroblasts stimulated with IL-4 and put under hypoxia for 24h (FbF/H) are the same of FbF Fb in normoxia (FbF) (13 SDEG); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 24h under hypoxia (FbF/H/CC), they remain similar to normoxic, pro-fibrotic, co-cultivated fibroblasts (FbF/CC) (60 SDEG); indeed, when alternative fibroblasts are put under hypoxia, they do not change their phenotype independently if from culture condition, but in co-culture the number of genes differentially expressed under hypoxia is increased. If we consider genes differentially expressed in the two comparisons, we observe that 12 genes are shared, 45 are differentially expressed only in co-cultivated MF Mφ and 1 in single cultivated MF Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 45 and 1 genes together, we obtain a 4-columns heatmap where we can observe that FbF CC 24H (1st column) has a different pattern

of gene expression and FbF CC 24N and FbF SC 24N (2nd and 4th column) have a similar pattern of gene expression (C).

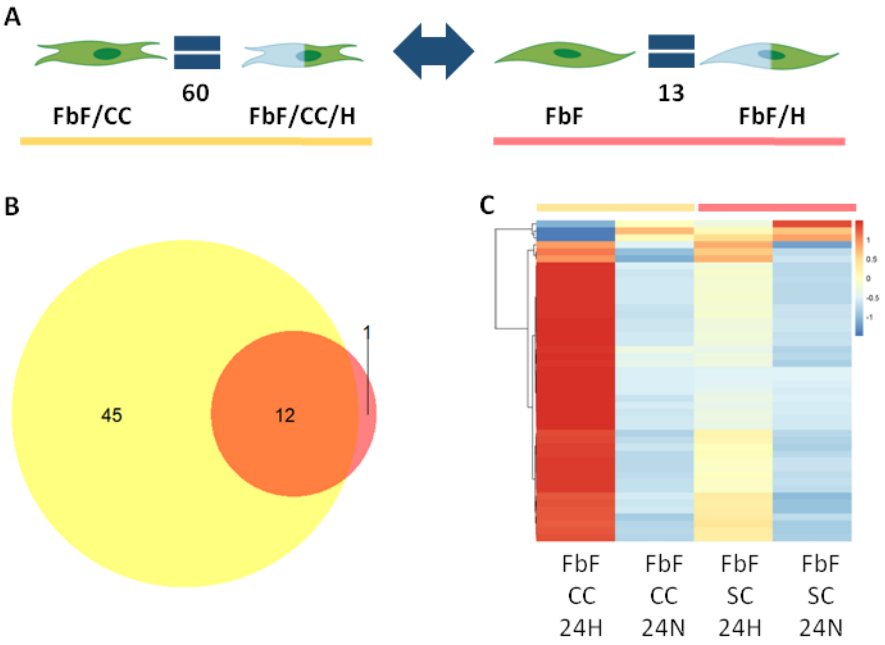


Fig. 148 Comparison (FbFvsFbF/H)CCvs(FbFvsFbF/H)SC. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to the co-culture, in pink the comparison related to the single culture (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbF/CCvsFbF/H/CC; pink= FbFvsFbF/H) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.2.3.6 What is the effect of co-culture on pro-fibrotic cells when they are under hypoxia?

[Code: C129 (C75vsC71)]

Macrophages stimulated with IL-4 and put in co-culture with fibroblasts for 4h (MF/CC) are similar to MF Mφ alone (MF) (26 SDEG); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 4h under hypoxia (MF/H/CC), they remain similar to hypoxic, alternative, single-cultivated macrophages (MF/H) (93 SDEG); indeed, when alternative macrophages are co-cultivated, they do not change their phenotype independently from the oxygen status of environment, but the number of differentially expressed genes is increased under hypoxia. If we consider genes differentially expressed in the two comparisons, we observe that 19 genes are shared, 69 are differentially expressed only in

hypoxic MF Mφ and 7 in normoxic MI Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 69 and 7 genes together, we obtain a 4-columns heatmap where we can observe that MF CC 4N and MF CC 4H (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples MF SC 4N and MF SC 4H (2nd and 4th column) (C).

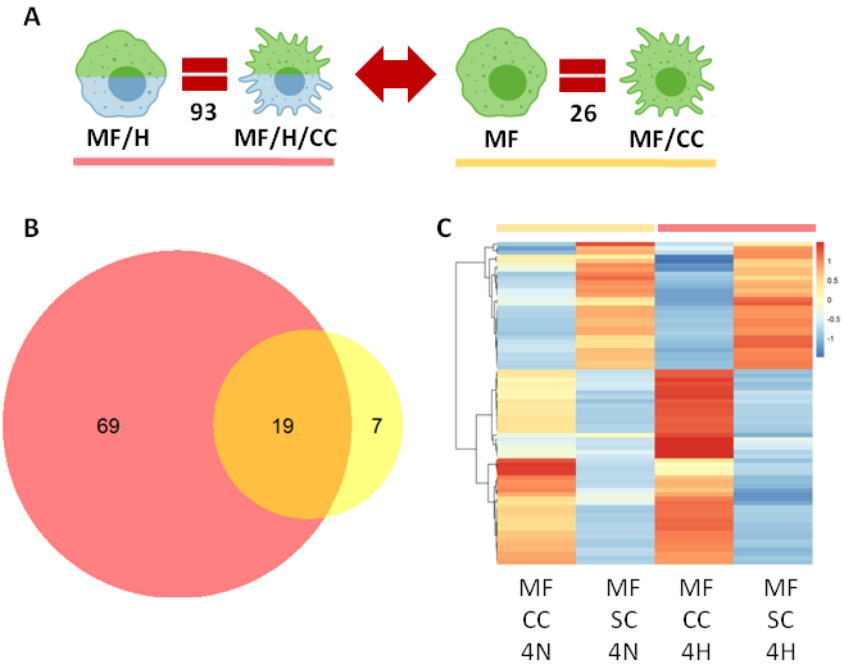


Fig. 149 Comparison (MFvsMF/CC)Hvs(MFvsMF/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of alternative macrophages at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to normoxia, in pink the comparison related to hypoxia (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MF/HvsMF/H/CC; pink= MFvsMF/CC) (B); the 4-columns heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C130 (C76vsC72)]

Fibroblasts stimulated with IL-4 and put in co-culture with fibroblasts for 4h (FbF/CC) are the same of FbF Fb alone (FbF); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 4h under hypoxia (FbF/H/CC), they remain similar to hypoxic, alternative, single-cultivated fibroblasts (FbF/H); indeed, when alternative fibroblasts are co-cultivated, they do not change their phenotype independently if they are in hypoxic or normoxic environment.

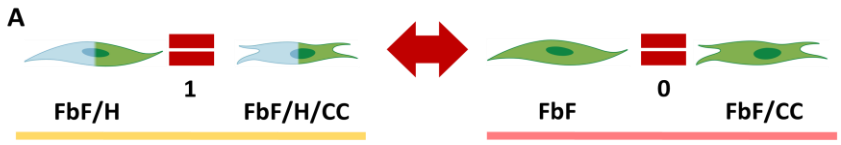


Fig. 150 Comparison (FbFvsFbF/CC)Hvs(FbFvsFbF/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05).

[Code: C131 (C77vsC73)]

Macrophages stimulated with IL-4 and put in co-culture with fibroblasts for 4h (MF/CC) are similar to MF Mφ alone (MF); when macrophages are co-cultivated with fibroblasts and stimulated with IL-4 for 4h under hypoxia (MF/H/CC), they remain similar to hypoxic, alternative, single-cultivated macrophages (MF/H) (47 SDEG); indeed, when alternative macrophages are co-cultivated, they do not change their phenotype independently if they are in hypoxic or normoxic environment, but the number of differentially expressed genes is increased under hypoxia. If we consider genes differentially expressed in the two comparisons, we observe that 46 are differentially expressed only in hypoxic MF Mφ and 1 in normoxic MF Mφ (B). By excluding genes that are differentially expressed in both comparisons and considering the 46 and 1 genes together, we obtain a 4-columns heatmap where we can observe that MF CC 24N and MF CC 24H (1st and 3rd column) have a similar pattern of gene expression that is the opposite of the other two samples MF SC 24N and MF SC 24H (2nd and 4th column) (C).

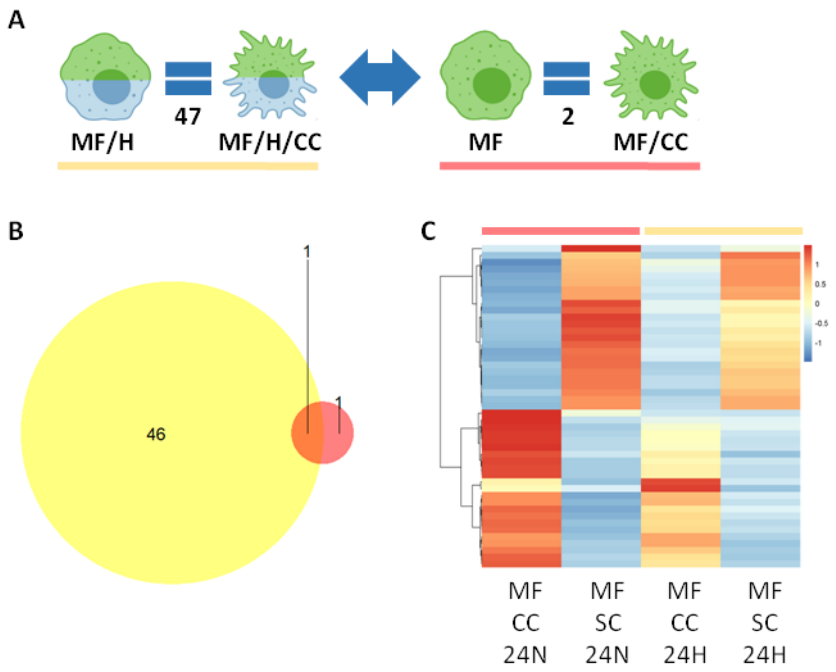


Fig. 151 Comparison (MFvsMF/CC)Hvs(MFvsMF/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of alternative macrophages at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In pink is underlined the comparison related to normoxia, in yellow the comparison related to hypoxia (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = MF/HvsMF/H/CC; pink= MFvsMF/CC) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C132 (C78vsC74)]

Fibroblasts stimulated with IL-4 and put in co-culture with fibroblasts for 24h (FbF/CC) are similar to FbF Fb alone (FbF); when fibroblasts are co-cultivated with macrophages and stimulated with IL-4 for 24h under hypoxia (FbF/H/CC), they remain similar to hypoxic, alternative, single-cultivated fibroblasts (FbF/H) (23 SDEG); indeed, when alternative fibroblasts are co-cultivated, they do not change their phenotype independently if they are in hypoxic or normoxic environment, but the number of differentially expressed genes is increased under hypoxia. If we consider genes differentially expressed in the two comparisons, we observe that 20 are differentially expressed only in hypoxic FbF Fb and 6 in normoxic FbF Fb (B). By excluding genes that are differentially expressed in both comparisons and considering the 20 and 6 genes together, we obtain a 4-columns heatmap where we can observe that FbF CC 24N (1st column) has a different pattern of gene expression that is the opposite of the other two samples FbF SC 24N and FbF SC 24H (2nd and 4th column) (C).

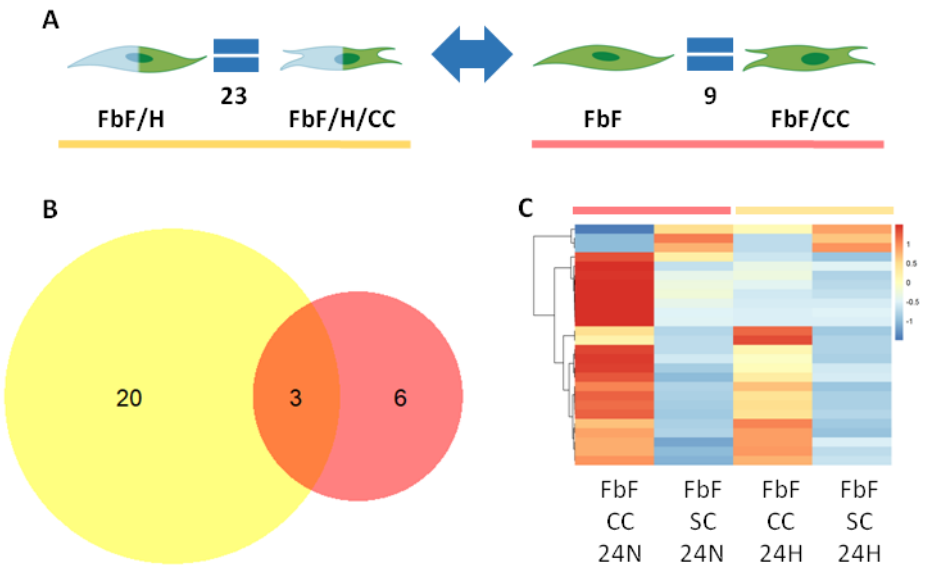


Fig. 152 Comparison (FbFvsFbF/CC)Hvs(FbFvsFbF/CC)N. Schematic representation of double comparison of two variables: oxygen status and culture condition of fibroblasts at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison related to hypoxia, in pink the comparison related to normoxia (A); Venn-diagram of SDEG with a |logFC|≥1 in the two comparisons (yellow = FbF/HvsFbF/H/CC; pink= FbFvsFbF/CC) (B); the 4-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.3 THIRD LEVEL

8.3.1 PRO-INFLAMMATORY CONDITION

8.3.1.1 What is the impact of LPS+IFNγ when oxygen and culture status are modified?

[Code: C133 (C97vsC89)]

Macrophages stimulated with LPS+IFNγ and put under hypoxia for 4h in co-culture with fibroblasts (MI/H/CC) are different from MH, co-cultivated without stimuli (MH/CC) [comparison A]; this difference is observed also in the same comparison made in normoxia (MI/CC vs M0/CC) [comparison B] (AvsB: 3574vs3983). The effect of combined hypoxia and pro-inflammatory stimuli is assessed also in single culture, where pro-inflammatory Mφ are different from resting, both under hypoxia [comparison C (MI/HvsMH)] and in normoxia [comparison D (MIvsM0)] (CvsD: 2930vs3531) with a comparable number of SDEG. If we consider these genes in the two double comparisons, we observe that pro-inflammatory Mφs remain different from their resting counterpart, independently if they are under hypoxia or in co-culture or in hypoxic co-culture. Venn diagram (B) shows that most of SDEG in the four comparisons are shared (1698). Moreover, pro-inflammatory stimuli (LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

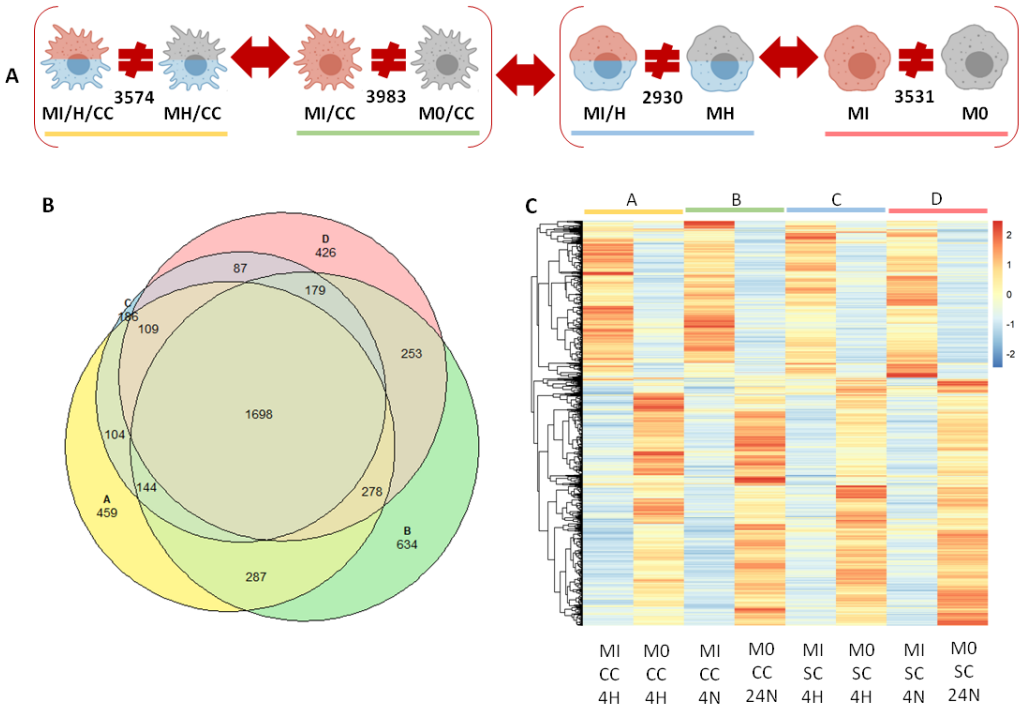


Fig. 153 Comparison [(MIvsM0)Hvs(MIvsM0)N]CC vs [(MIvsM0)Hvs(MIvsM0)N]SC. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of MIvsM0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/H/CCvsMH/CC), in green the comparison B (MI/CCvsM0/CC), in blue the comparison C (MI/HvsMH) and in pink the comparison D (MIvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C134 (C98vsC90)]

Fibroblasts stimulated with LPS+IFNγ and put under hypoxia for 4h in co-culture with macrophages (FbI/H/CC) are different from FbH, co-cultivated, without stimuli (FbH/CC) [comparison A]; this difference is observed also in the same comparison made in normoxia [comparison B] with an higher number of genes differentially expressed (AvsB: 1141vs2131). The effect of combined hypoxia and pro-inflammatory stimuli is assessed also in single culture, where pro-inflammatory fibroblasts under hypoxia (FbI/H) are different from resting (FbH) [comparison C] and are more different when they are in normoxic environment [comparison D] (CvsD: 628vs1402); however in co-culture and in single culture they have a comparable number of SDEG. If we consider these genes in the two double comparisons, we observe that pro-inflammatory Fb remain different from their resting counterpart, independently if they are under hypoxia or in co-culture or in hypoxic co-culture. The number of SDEG observed in normoxia is decreased under hypoxia, both in single and in co-culture; instead in normoxic co-culture this number is increased. Venn diagram (B) shows that 430 genes differentially expressed are shared in the four comparisons. Moreover, pro-inflammatory stimuli (LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

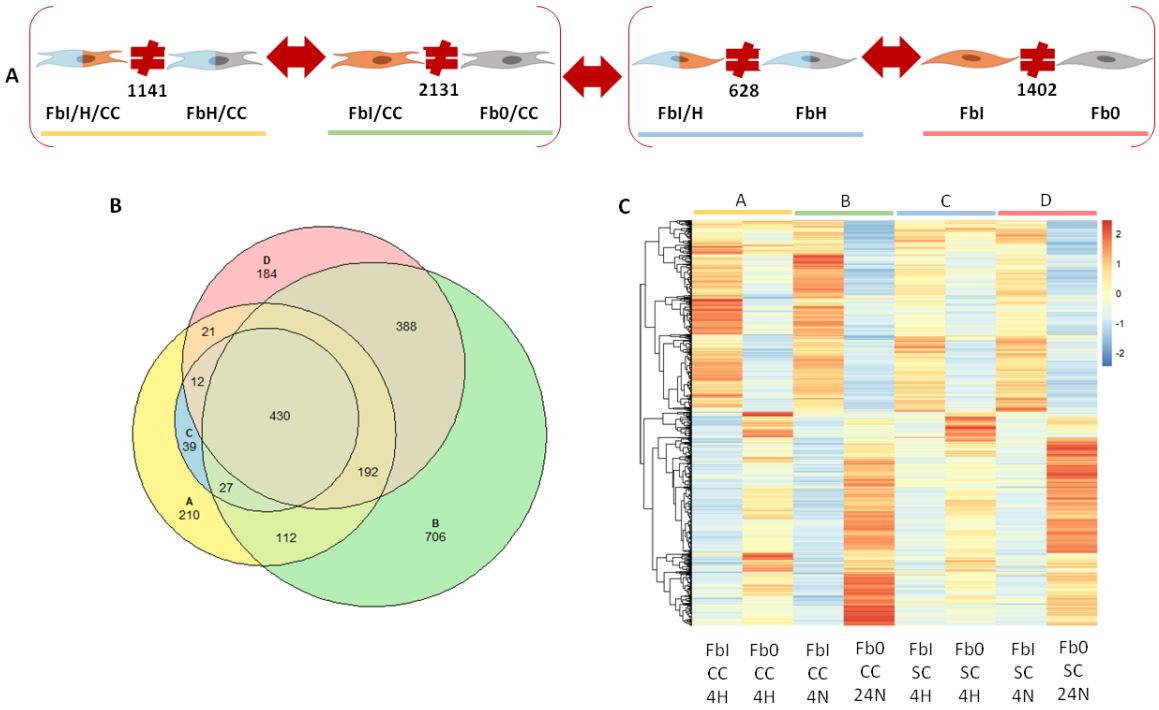


Fig. 154 Comparison [(FbIvsFb0)Hvs(FbIvsFb0)N]CC vs [(FbIvsFb0)Hvs(FbIvsFb0)N]SC. Schematic representation of two double comparison of three variables: oxygen status and culture condition of FbIvsFb0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/H/CCvsFbH/CC), in green the comparison B (FbI/CCvsFb0/CC), in blue the comparison C (FbI/HvsFbH) and in pink the comparison D (FbIvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparison and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C135 (C99vsC91)]

Macrophages stimulated with LPS+IFNγ and put under hypoxia for 24h in co-culture with fibroblasts (MI/H/CC) are different from MH, co-cultivated, without stimuli (MH/CC) [comparison A]; this difference is observed also in the same comparison made in normoxia (MI/CC vs M0/CC) [comparison B] (AvsB: 4663vs3840). The effect of combined hypoxia and pro-inflammatory stimuli is assessed also in single culture where pro-inflammatory Mφ are different from resting both under hypoxia [comparison C (MI/HvsMH)] and in normoxia [comparison D (MIvsM0)] (CvsD: 3474vs3389); with a comparable number of significantly SDEG. If we consider these genes in the two double comparisons, we observe that pro-inflammatory Mφs remain different from their resting counterpart, independently if they are under hypoxia or in co-culture or in hypoxic co-culture. Venn diagram (B) shows that most of SDEG in the four comparisons are shared (1366). Moreover, pro-inflammatory stimuli

(LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

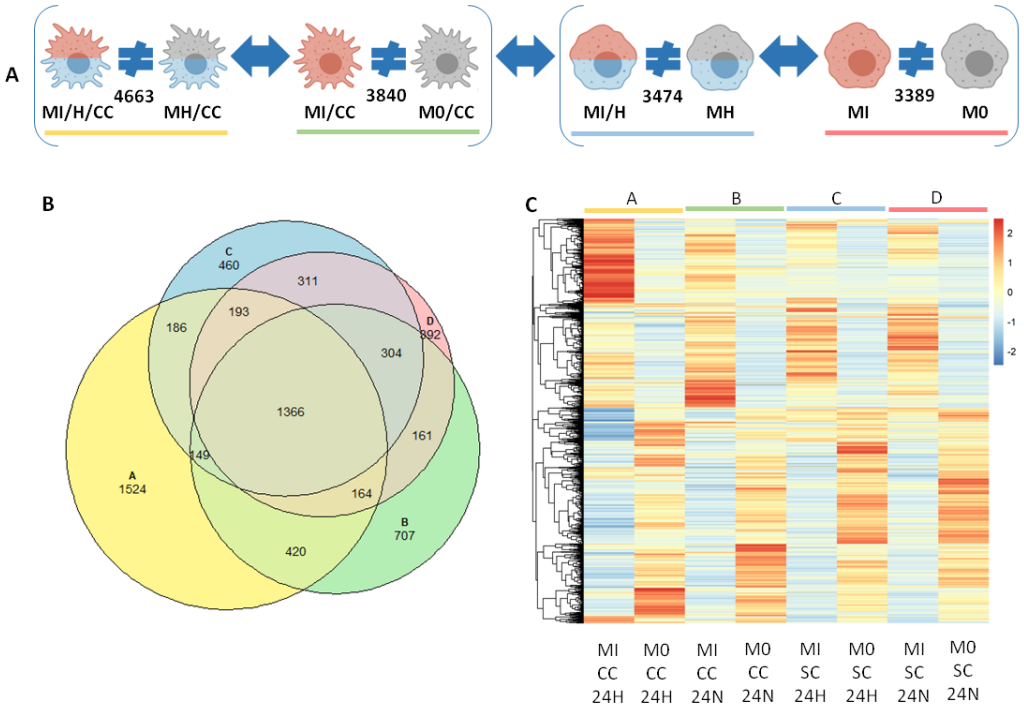


Fig. 155 Comparison [(MIvsM0)Hvs(MIvsM0)N]CC vs [(MIvsM0)Hvs(MIvsM0)N]SC. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of MIvsM0 at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/H/CCvsMH/CC), in green the comparison B (MI/CCvsM0/CC), in blue the comparison C (MI/HvsMH) and in pink the comparison D (MIvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C136 (C100vsC92)]

Fibroblasts stimulated with LPS+IFNγ and put under hypoxia for 24h in co-culture with macrophages (FbI/H/CC) are different from FbH, co-cultivated, without stimuli (FbH/CC) [comparison A]; this difference is observed also in the same comparison made in normoxia [comparison B] (AvsB: 4106vs4841). The effect of pro-inflammatory stimuli is assessed also in single culture where pro-inflammatory fibroblasts are different from resting both under hypoxia [comparison C: (FbI/HvsFbH)] and in normoxia [comparison D: (FbIvsFB0)] (CvsD: 1773vs1426); moreover, in co-culture the number of SDEG between FbI and Fb0 is higher than in single culture, independently by oxygen status. Venn diagram (B) shows that 713

genes differentially expressed are shared in the four comparisons and the difference previously explained between single and co-culture comparisons [AvsB]. Moreover, pro-inflammatory stimuli (LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

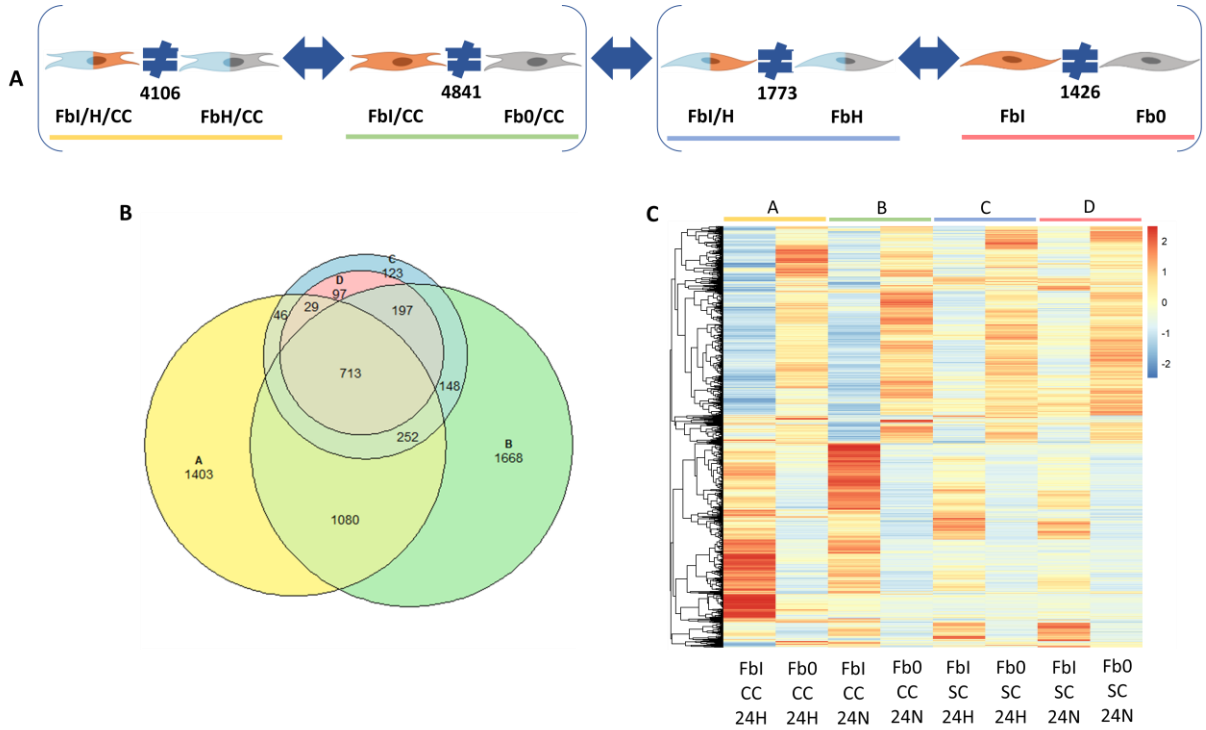


Fig. 156 Comparison [(FbIvsFb0)Hvs(FbIvsFb0)N]CC vs [(FbIvsFb0)Hvs(FbIvsFb0)N]SC. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of FbIvsFb0 at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/H/CCvsFbH/CC), in green the comparison B (FbI/CCvsFb0/CC), in blue the comparison C (FbI/HvsFbH) and in pink the comparison D (FbIvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C137 (C93vsC85)]

Macrophages stimulated with LPS+IFNγ and put under hypoxia for 4h in co-culture with fibroblasts (MI/H/CC) are different from MH, co-cultivated, without stimuli (MH/CC) [comparison A]; this difference is observed also in the same comparison made in single culture (MI/H vs MH) [comparison B] (AvsB: 3574vs2930). The effect of pro-inflammatory stimuli is assessed also in normoxic environment where pro-inflammatory Mφ are different

from resting both in co-culture [comparison C (MI/CCvsM0/CC)] and alone [comparison D (MIvsM0)] (CvsD: 3983vs3531); with a comparable number of SDEG. If we consider these genes in the two double comparisons, we observe that pro-inflammatory Mφs remain different from their resting counterpart, independently if they are under hypoxia or in co-culture or in hypoxic co-culture. Venn diagram (B) shows that most of differentially expressed genes in the four comparisons are shared (1698). Moreover, pro-inflammatory stimuli (LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

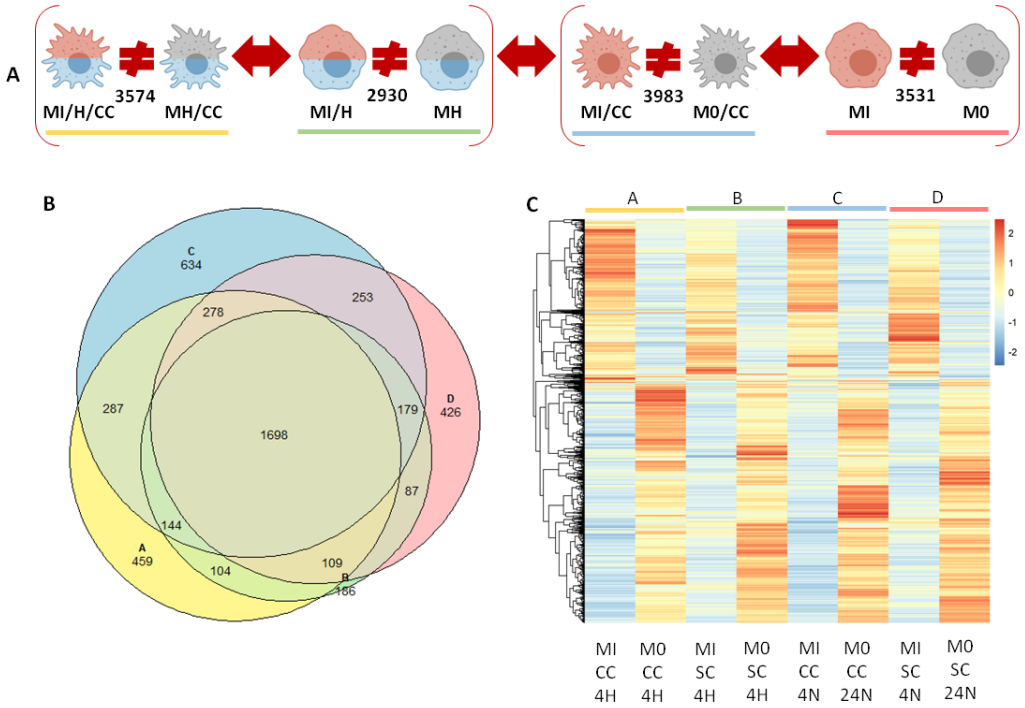


Fig. 157 Comparison [(MIvsM0)CCvs(MIvsM0)SC]H vs [(MIvsM0)CCvs(MIvsM0)SC]N. Schematic representation of two double comparisons of three variables: culture condition and oxygen status of MIvsM0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/H/CCvsMH/CC), in green the comparison B (MI/HvsMH), in blue the comparison C (MI/CCvsM0/CC) and in pink the comparison D (MIvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C138 (C94vsC86)]

Fibroblasts stimulated with LPS+IFNγ and put under hypoxia for 4h in co-culture with macrophages (FbI/H/CC) are different from FbH, co-cultivated, without stimuli (FbH/CC)

[comparison A]; this difference is observed also in the same comparison made in single culture [comparison B] but with a lower number of differentially expressed genes (AvsB: 1141vs628).

The effect of LPS+IFNγ is assessed also in normoxia, where pro-inflammatory co-cultivated fibroblasts (FbI/CC) are different from co-cultivated resting (Fb0/CC) [comparison C] but are less different when they are alone [comparison D] (CvsD: 2131vs1402). If we consider these genes in the two double comparisons, we observe that pro-inflammatory Fb remain different from their resting counterpart, independently if they are under hypoxia or in co-culture or in hypoxic co-culture. Under hypoxia the number of SDEG observed in normoxia is decreased both in single and in co-culture; instead, in normoxic co-culture this number is increased. Venn diagram (B) shows that 430 genes differentially expressed are shared in the four comparisons. Moreover, pro-inflammatory stimuli (LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

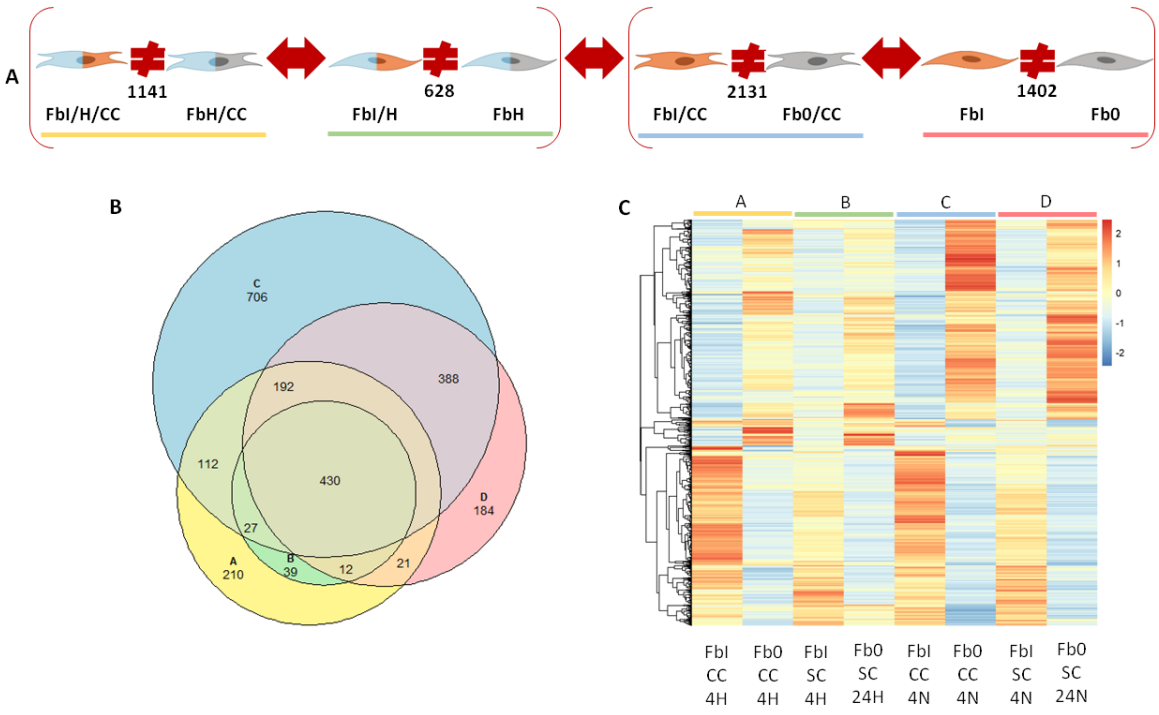


Fig. 158 Comparison [(FbIvsFb0)CCvs(FbIvsFb0)SC]H vs [(FbIvsFb0)CCvs(FbIvsFb0)SC]N. Schematic representation of two double comparisons of three variables: culture condition and oxygen status of FbIvsFb0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/H/CCvsFbH/CC), in green the comparison B (FbI/HvsFbH), in blue the comparison C (FbI/CCvsFb0/CC) and in pink the comparison D (FbIvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus

genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C139 (C95vsC87)]

Macrophages stimulated with LPS+IFNγ and put under hypoxia for 24h in co-culture with fibroblasts (MI/H/CC) are different from MH, co-cultivated, without stimuli (MH/CC) [comparison A]; this difference is observed also in the same comparison made in single culture (MI/H vs MH) [comparison B] (AvsB: 4663vs3474). The effect of pro-inflammatory stimuli is assessed also in normoxic environment, where pro-inflammatory Mφ are different from resting both in co-culture [comparison C (MI/CCvsM0/CC)] and alone [comparison D (MIvsM0)] (CvsD: 3840vs3389); with a comparable number of significantly differentially expressed genes. If we consider these genes in the two double comparisons we observe that pro-inflammatory Mφs remain different from their resting counterpart, independently if they are under hypoxia or in co-culture or in hypoxic co-culture. Venn diagram (B) shows that most of SDEG in the four comparisons are shared (1366). Moreover, pro-inflammatory stimuli (LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

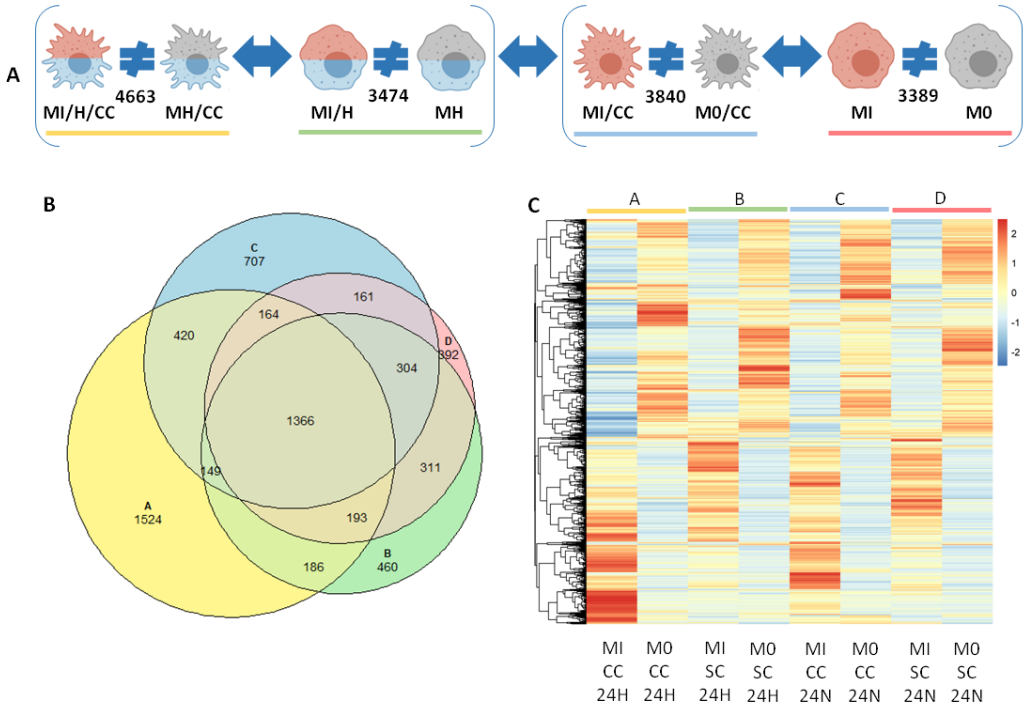


Fig. 159 Comparison [(MIvsM0)CCvs(MIvsM0)SC]H vs [(MIvsM0)CCvs(MIvsM0)SC]N. Schematic representation of two double comparison of three variables: culture condition and oxygen status of MIvsM0 at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/H/CCvsMH/CC), in green the comparison B (MI/HvsMH), in blue the comparison C

(MI/CCvsM0/CC) and in pink the comparison D (MIvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the two double comparison and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C140 (C96vsC88)]

Fibroblasts stimulated with LPS+IFNγ and put under hypoxia for 24h in co-culture with macrophages (FbI/H/CC) are different from FbH, co-cultivated, without stimuli (FbH/CC) [comparison A]; this difference is observed also in the same comparison made in single culture [comparison B: (FbI/HvsFbH)] but with a lower number of genes differentially expressed (AvsB: 4106vs1773). The effect of pro-inflammatory stimuli is assessed also in normoxia, where pro-inflammatory fibroblasts are different from resting both in co-culture [comparison C: (FbI/CCvsFb0/CC)] and in single culture [comparison D: (FbIvsFb0)] (CvsD: 4841vs1426); indeed, in co-culture the number of SDEG between FbI and Fb0 is higher than in single culture, independently by oxygen status. Venn diagram (B) shows that 713 genes differentially expressed are shared in the four comparisons and the difference previously explained between single and co-culture comparisons [AvsC]. Moreover, pro-inflammatory stimuli (LPS+IFNγ) promote a difference that is stronger than the other factors, as we can observe in the 8-columns heatmap reported (C).

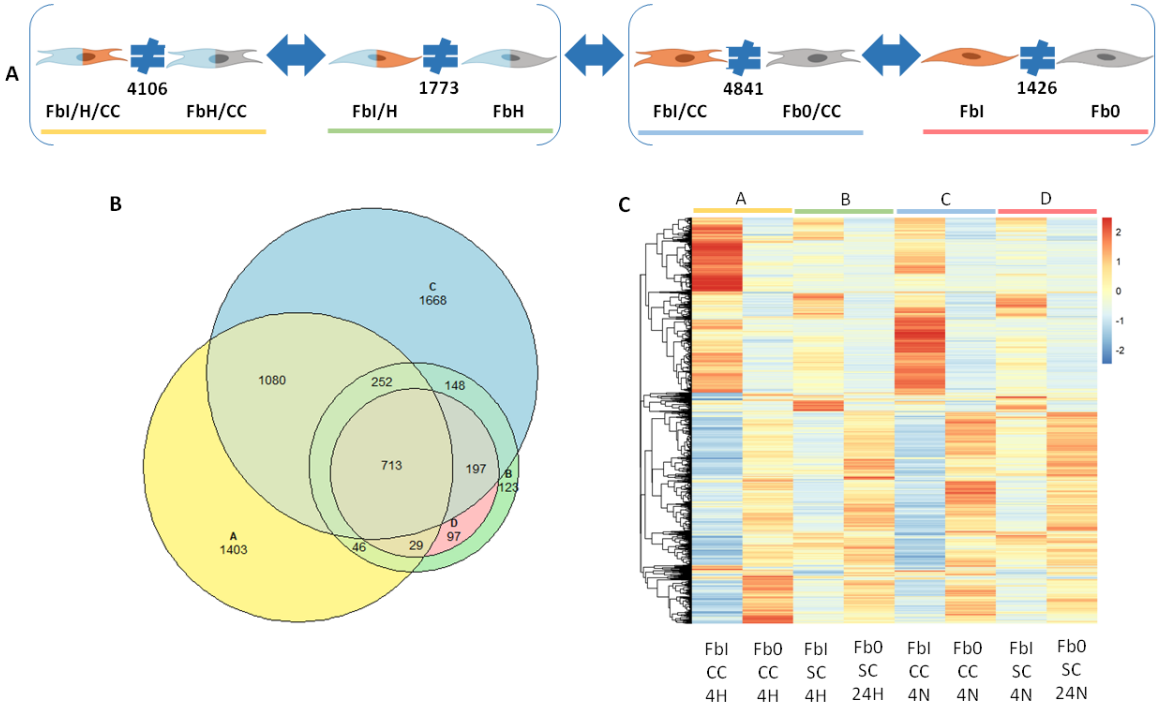


Fig. 160 Comparison [(FbIvsFb0)CCvs(FbIvsFb0)SC]H vs [(FbIvsFb0)CCvs(FbIvsFb0)SC]N. Schematic representation of two double comparisons of three variables: culture condition and oxygen status of FbIvsFb0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/H/CCvsFbH/CC), in green the comparison B (FbI/HvsFbH), in blue the comparison C (FbI/CCvsFb0/CC) and in pink the comparison D (FbIvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

8.3.1.2 What is the impact of hypoxia when polarizing and culture status are modified?

[Code: C141 (C35vsC5)vs(C31vsC1)]

Macrophages put under hypoxia and stimulated with LPS+IFNγ for 4h in co-culture with fibroblasts (MI/CC/H) are not different from pro-inflammatory Mφ co-cultivated in normoxia (MI/CC) [comparison A]; when resting Mφ are put under hypoxia in co-culture (M0/CC/H) or are co-cultivated in normoxia (M0/CC) [comparison B] they do not differentially express an higher number of genes (AvsB: 17vs44). The effect of hypoxia is assessed also in single culture where hypoxic pro-inflammatory Mφ are similar to their normoxic counterpart [comparison C (MI/HvsMI)] and hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)] (CvsD: 22vs37). Venn diagram (B) shows that 8 differentially expressed genes are shared in the four comparisons. Indeed, when macrophage with the same polarizing status are compared to observe the effect of different oxygen condition in different state of culture, we do not found any differences in the pattern of SDEG, as we can observe in the 8-columns heatmap reported (C).

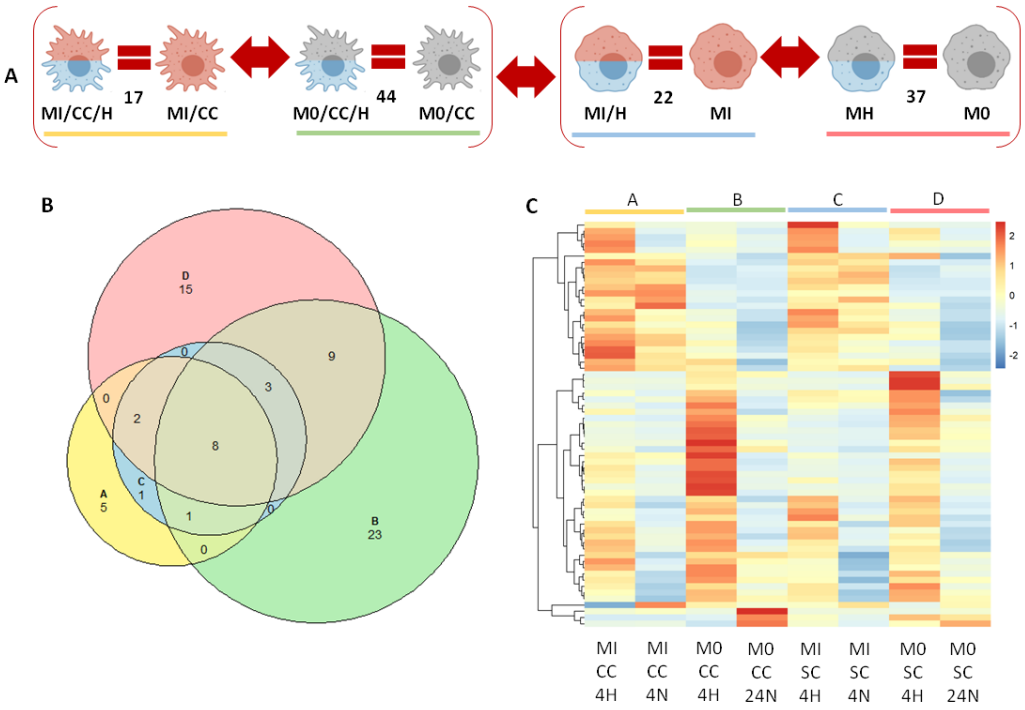


Fig. 161 Comparison [(MI/HvsMI)Ivs(MHvsM0)0]CCvs [(MI/HvsMI)Ivs(MHvsM0)0]SC. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Mφ vs normoxic Mφ at time point of 4h. For each comparison is reported the number of genes SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/CC), in green the comparison B (M0/CC/HvsM0/CC), in blue the comparison C (MI/HvsMI) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C142 (C36vsC6)vs(C32vsC2)]

Fibroblasts put under hypoxia and stimulated with LPS+IFNγ for 4h in co-culture with macrophages (FbI/CC/H) are not different from pro-inflammatory Fb co-cultivated in normoxia (FbI/CC) [comparison A]; when resting Fb are put under hypoxia in co-culture (Fb0/CC/H) or are co-cultivated in normoxia (Fb0/CC) [comparison B], they differentially express an higher number of genes (AvsB: 0vs105). The effect hypoxia is assessed also in single culture, where hypoxic pro-inflammatory Fb are similar to their normoxic counterpart [comparison C (FbI/HvsFbI)], instead hypoxic Fb0 are different from normoxic one [comparison D (FbHvsFb0)] (CvsD: 0vs97). Venn diagram (B) shows that 52 differentially expressed genes are shared in the comparisons between B and D. Indeed, when fibroblast with the same polarizing status (pro-inflammatory) are compared to observe the effect of different oxygen condition in different state of culture, we not find differences in the pattern of SDEG.

When resting fibroblast are put in different oxygen condition, they show an hundred of genes differentially expressed both in co-culture and single culture, indicating that hypoxia affect resting condition independently from culture condition. As we can observe in the 8-columns heatmap (C), columns referred to comparison A and C have the same pattern of expression of reported genes, instead comparison B and D have sample Fb0/CC similar to Fb0/SC while samples FbH/CC and FbH/SC are similar to pro-inflammatory Fbs.

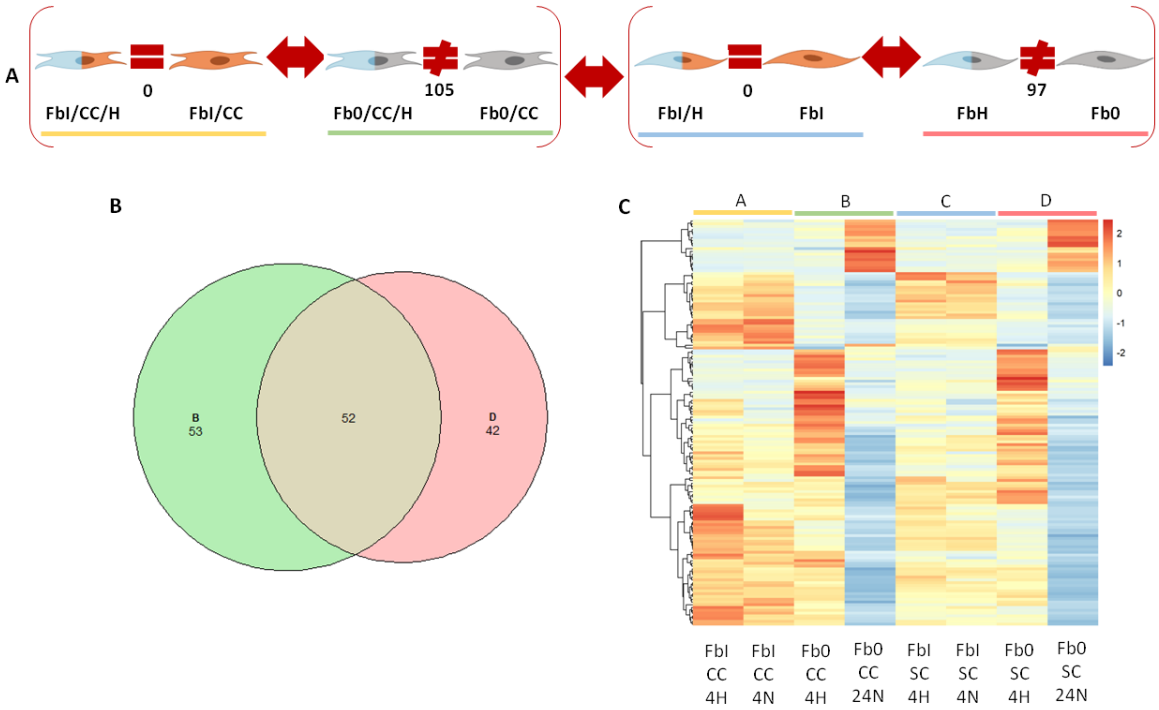


Fig. 162 Comparison [(FbI/HvsFbI)Ivs(FbHvsFb0)0]CC vs [(FbI/HvsFbI)Ivs(FbHvsFb0)0]SC. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/CC), in green the comparison B (Fb0/CC/HvsFb0/CC), in blue the comparison C (FbI/HvsFbI) and in pink the comparison D (FbHvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C143 (C37vsC7)vs(C33vsC3)]

Macrophages put under hypoxia and stimulated with LPS+IFNγ for 24h in co-culture with fibroblasts (MI/CC/H) are not different from pro-inflammatory Mφ co-cultivated in normoxia (MI/CC) [comparison A]; when resting Mφ are put under hypoxia in co-culture (M0/CC/H) or are co-cultivated in normoxia (M0/CC) [comparison B] they differentially express an higher

number of genes (AvsB: 37vs1139). The effect hypoxia is assessed also in single culture, where hypoxic pro-inflammatory Mφ are similar to their normoxic counterpart [comparison C (MI/HvsMI)] and hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)] (CvsD: 32vs18). Venn diagram (B) shows that there is only one comparison that give an high number of SDEG (comparison B with 1076 genes). Indeed, when macrophage with the same polarizing status (pro-inflammatory) are compared to observe the effect of different oxygen condition in different state of culture, we do not found any differences in the pattern of SDEG; when we consider resting Mφ, hypoxia seems to have an important effect only when they are in co-culture. In the 8-columns heatmap reported (C) we cannot appreciate this difference since that the stronger difference is given by the pro-inflammatory phenotype that is the opposite of resting phenotype (as we said before) and values are scaled by row.

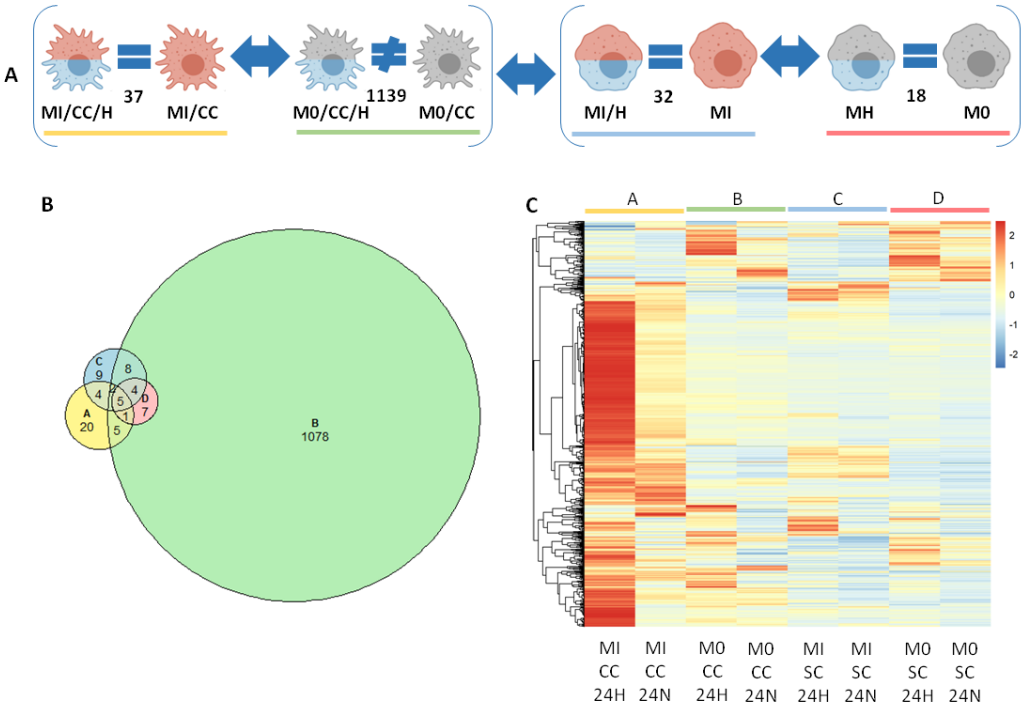


Fig. 163 Comparison [(MI/HvsMI)Ivs(MHvsM0)0]CC vs [(MI/HvsMI)Ivs(MHvsM0)0]SC. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Mφ vs normoxic Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/CC), in green the comparison B (M0/CC/HvsM0/CC), in blue the comparison C (MI/HvsMI) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C144 (C38vsC8)vs(C34vsC4)]

Fibroblasts put under hypoxia and stimulated with LPS+IFNγ for 24h in co-culture with macrophages (FbI/CC/H) are not different from pro-inflammatory Fb co-cultivated in normoxia (FbI/CC) [comparison A]; when resting Fb are put under hypoxia in co-culture (Fb0/CC/H) or are co-cultivated in normoxia (Fb0/CC) [comparison B] they differentially express an higher number of genes (AvsB: 22vs1305). The effect of hypoxia is assessed also in single culture, where hypoxic pro-inflammatory Fb are similar to their normoxic counterpart [comparison C (FbI/HvsFbI)] and hypoxic Fb0 are similar to normoxic one [comparison D (FbHvsFb0)] (CvsD: 12vs23). Venn diagram (B) shows that the comparison B gives the high number of SDEG (1186). Indeed, when fibroblast with the same polarizing status (pro-inflammatory) are compared to observe the effect of different oxygen condition, in different state of culture, we not find differences in the pattern of SDEG. When resting fibroblast are put in different oxygen condition, they show a thousand of genes differentially expressed only when co-cultivated. In the 8-columns heatmap reported (C) we cannot appreciate this difference since that the stronger difference is given by the pro-inflammatory phenotype that is the opposite of resting phenotype (as we said before) and values are scaled by row.

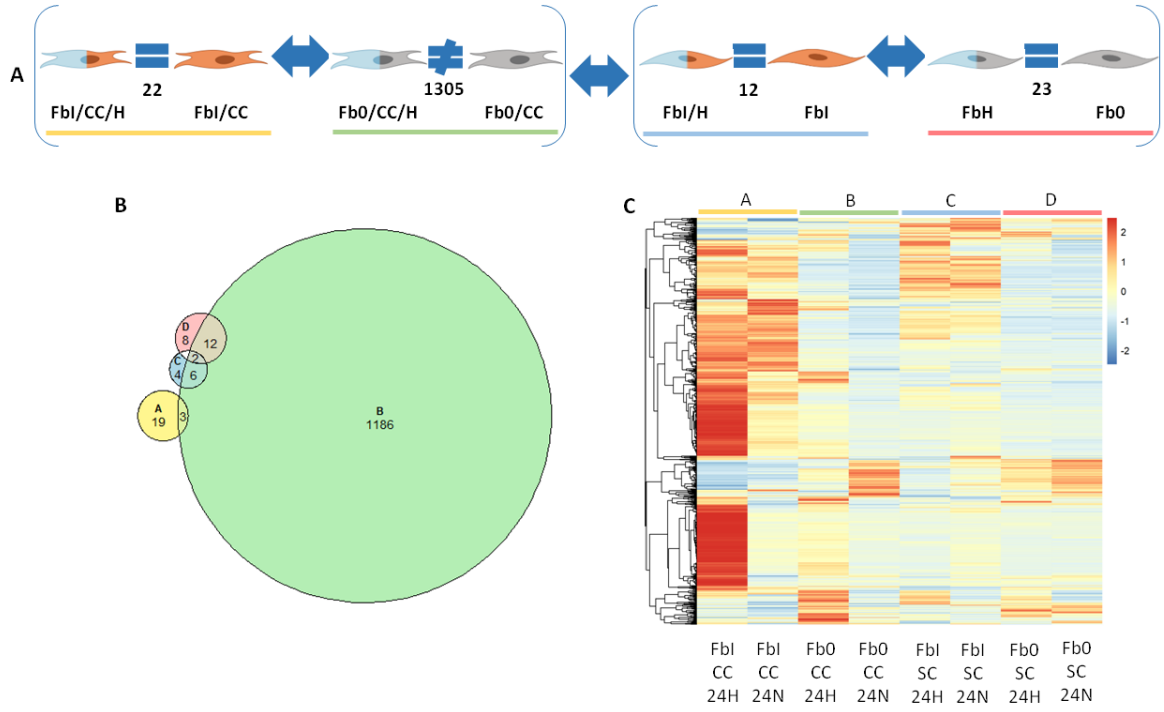


Fig. 164 Comparison [(FbI/HvsFbI)Ivs(FbHvsFb0)0]CC vs [(FbI/HvsFbI)Ivs(FbHvsFb0)0]SC. Schematic representation of two double comparison of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/CC), in green the comparison B (Fb0/CC/HvsFb0/CC),

in blue the comparison C (FbI/HvsFbI) and in pink the comparison D (FbHvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the two double comparison and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C145 (C101vsC79)]

Macrophages put under hypoxia and stimulated with LPS+IFNγ for 4h in co-culture with fibroblasts (MI/CC/H) are not different from pro-inflammatory Mφ co-cultivated in normoxia (MI/CC) [comparison A]; the same happen when MI are in single culture, in hypoxia (MI/H) or in normoxia (MI) [comparison B]: they do not differentially express an higher number of genes (AvsB: 17vs22). The effect of hypoxia is assessed also in resting macrophages where co-cultivated, hypoxic Mφ are similar to their normoxic counterpart [comparison C (MH/CCvsM0/CC)] and single cultivated hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)] (CvsD: 44vs37). Venn diagram (B) shows that 8 differentially expressed genes are shared in the four comparisons. Indeed, when macrophage with the same polarizing status are compared to observe the effect of different oxygen condition in different state of culture, we not find differences in the pattern of SDEG, as we can observe in the 8-columns heatmap reported (C).

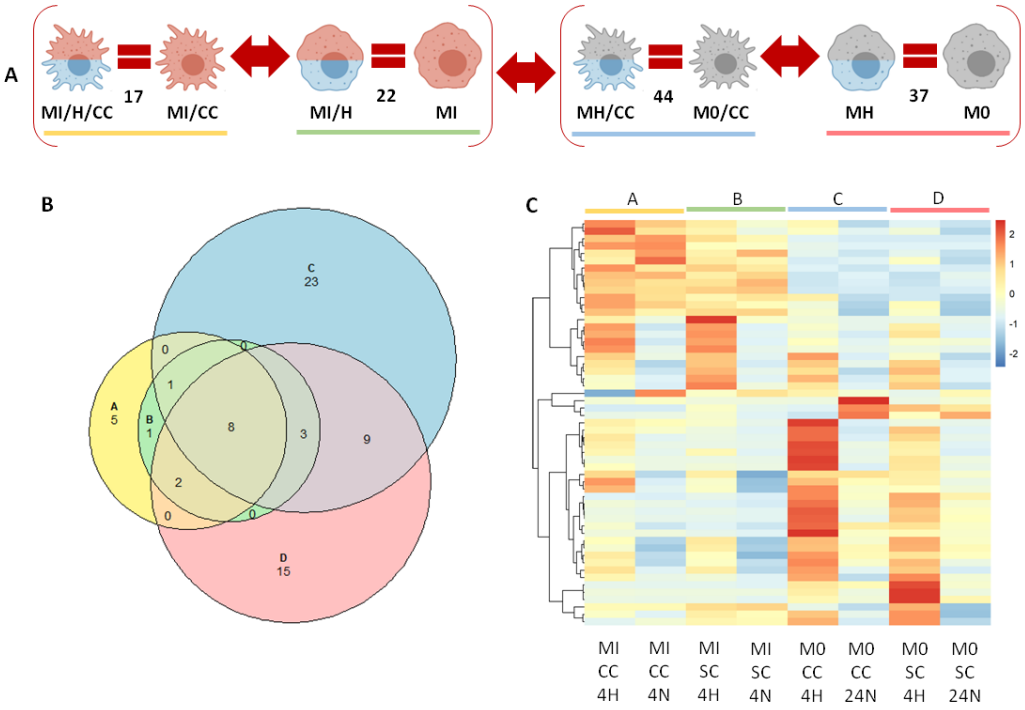


Fig. 165 Comparison [(MI/HvsMI)CCvs(MI/HvsMI)SC]I vs [(MHvsM0)CCvs(MHvsM0)SC]0. Schematic representation of two double comparison of three variables: polarizing status and culture condition of hypoxic

Mφ vs normoxic Mφ at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/CC), in green the comparison B (MI/HvsMI),in blue the comparison C (M0/CC/HvsM0/CC) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C146 (C102vsC80)]

Fibroblasts put under hypoxia and stimulated with LPS+IFNγ for 4h in co-culture with macrophages (FbI/CC/H) are not different from pro-inflammatory Fb co-cultivated in normoxia (FbI/CC) [comparison A]; when FbI are put under hypoxia in single culture (FbI/H) or are in normoxia (FbI) [comparison B] they are the same (AvsB: 0vs0). The effect of hypoxia is assessed also in hypoxic, co-cultivated, resting Fb that are different to their normoxic counterpart [comparison C (FbH/CCvsFb0/CC)]; single cultivated, hypoxic Fb0 are different from normoxic one [comparison D (FbHvsFb0)] (CvsD: 105vs97). Venn diagram (B) shows that 52 differentially expressed genes are shared in the comparison between C and D. Indeed, when fibroblast with the same polarizing status (pro-inflammatory) are compared to observe the effect of different oxygen condition in different state of culture, we not find differences in the pattern of SDEG. When resting fibroblast are put in different oxygen condition, they show an hundred of genes differentially expressed both in co-culture and single culture, indicating that hypoxia affect resting condition independently from culture condition. As we can observe in the 8-columns heatmap (C), columns referred to comparison A and B have the same pattern of expression of reported genes, instead comparison C and D have sample Fb0/CC similar to Fb0/SC and FbH/CC similar to FbH/SC.

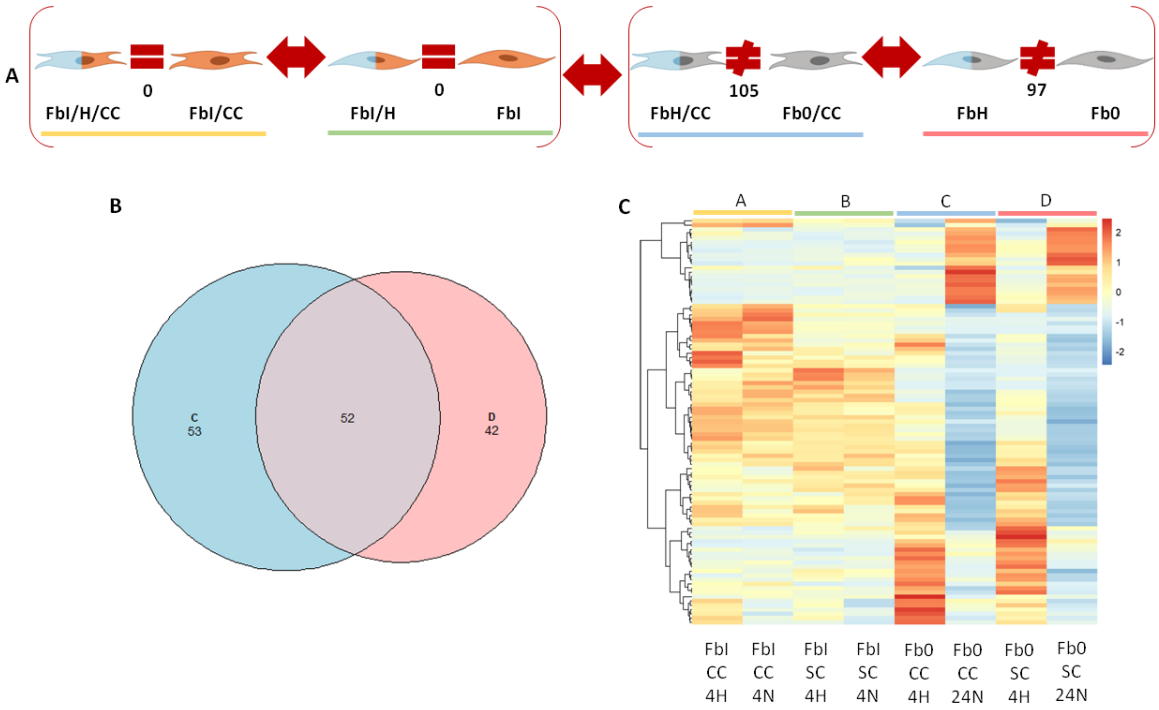


Fig. 166 Comparison [(FbI/HvsFbI)CCvs(FbI/HvsFbI)SC]I vs [(FbHvsFb0)CCvs(FbHvsFb0)SC]0. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/CC), in green the comparison B (FbI/HvsFbI),in blue the comparison C (Fb0/CC/HvsFb0/CC) and in pink the comparison D (FbHvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C147 (C103vsC81)]

Macrophages put under hypoxia and stimulated with LPS+IFNγ for 24h in co-culture with fibroblasts (MI/CC/H) are not different from pro-inflammatory Mφ co-cultivated in normoxia (MI/CC) [comparison A]; the same happen when MI are in single culture in hypoxia (MI/H) or in normoxia (MI) [comparison B]: they do not differentially express an higher number of genes (AvsB: 37vs32). The effect of hypoxia is assessed also in resting macrophages where co-cultivated hypoxic Mφ are different to their normoxic counterpart [comparison C (MH/CCvsM0/CC)], instead single cultivated hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)] (CvsD: 1139vs18). Venn diagram (B) shows that differentially expressed genes are given by comparison C. Indeed, when macrophage with the same polarizing status (pro-inflammatory) are compared to observe the effect of different oxygen condition in different state of culture, we not find differences in the pattern of SDEG. When

resting Mφ are stimulated with hypoxia, they differ from normoxic counterpart only if they are co-cultivated, meaning that hypoxia effect in resting cells is culture state dependent. In the 8-columns heatmap reported (C) we cannot appreciate this difference, since that the stronger difference is given by the pro-inflammatory phenotype, which is the opposite of resting phenotype (as we said before), and values are scaled by row.

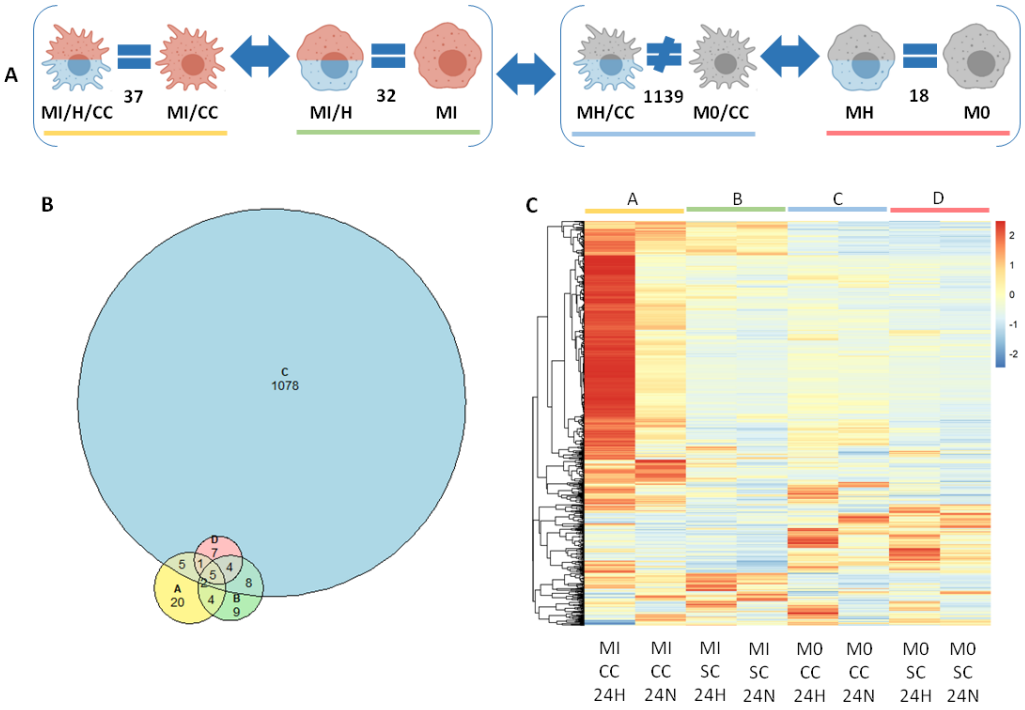


Fig. 167 Comparison [(MI/HvsMI)CCvs(MI/HvsMI)SC]I vs [(MHvsM0)CCvs(MHvsM0)SC]0. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Mφ vs normoxic Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/CC), in green the comparison B (MI/HvsMI), in blue the comparison C (M0/CC/HvsM0/CC) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C148 (C104vsC82)]

Fibroblasts put under hypoxia and stimulated with LPS+IFNγ for 24h in co-culture with macrophages (FbI/CC/H) are not different from pro-inflammatory Fb co-cultivated in normoxia (FbI/CC) [comparison A]; when FbI are put under hypoxia in single culture (FbI/H) or are in normoxia (FbI) [comparison B] they are the same (AvsB: 22vs12). The effect of hypoxia is assessed also in hypoxic co-cultivated resting Fb that are different to their

normoxic counterpart [comparison C (FbH/CCvsFb0/CC)]; single cultivated hypoxic Fb0 are not different from normoxic one [comparison D (FbHvsFb0)] (CvsD: 1305vs23). Venn diagram (B) shows that SDEG are given by the comparison C (1186). Indeed, when fibroblast with the same polarizing status (pro-inflammatory) are compared to observe the effect of different oxygen condition in different state of culture, we do not found any differences in the pattern of SDEG. When resting fibroblast are put in different oxygen condition, they show a thousand of genes differentially expressed in co-culture but not in single culture, indicating that hypoxia affect resting condition depending by culture condition. In the 8-columns heatmap reported (C) we cannot appreciate this difference, since that the stronger difference is given by the pro-inflammatory phenotype that is the opposite of resting phenotype (as we said before) and values are scaled by row.

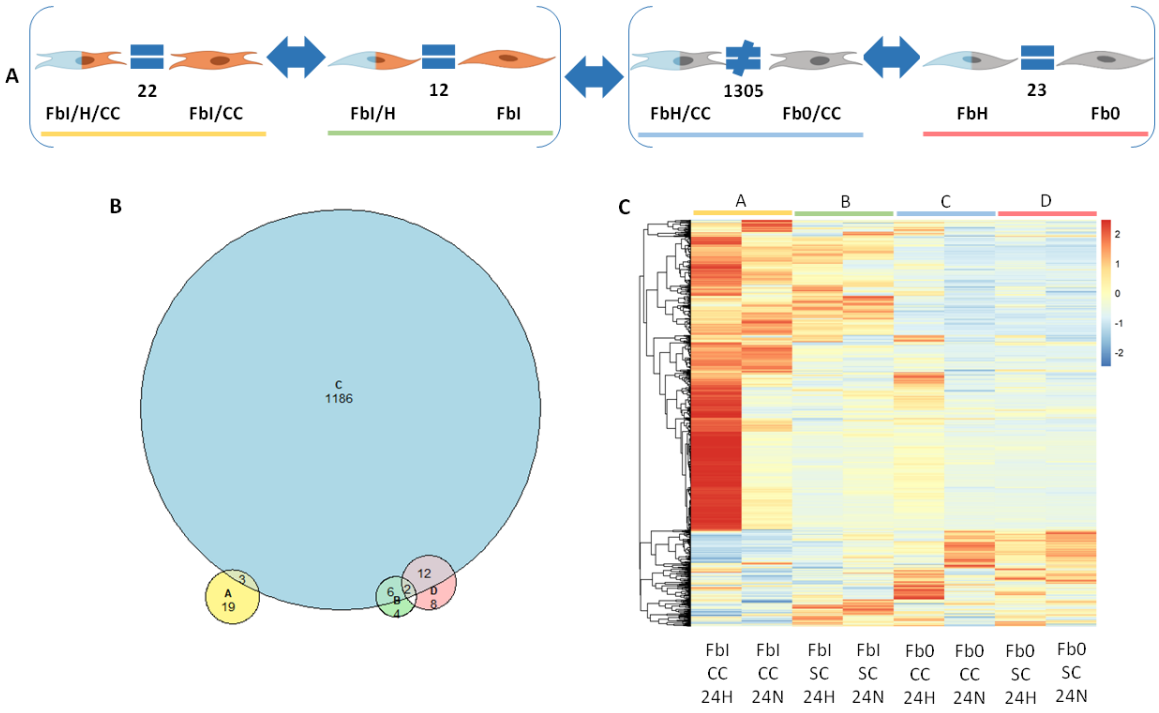


Fig. 168 Comparison [(FbI/HvsFbI)CCvs(FbI/HvsFbI)SC]I vs [(FbHvsFb0)CCvs(FbHvsFb0)SC]0. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/CC), in green the comparison B (FbI/HvsFbI), in blue the comparison C (Fb0/CC/HvsFb0/CC) and in pink the comparison D (FbHvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

8.3.1.3 What is the impact of co-culture when polarizing and oxygen status are modified?

[Code: C149 (C43vsC11)vs(39vsC9)]

Macrophages co-cultivated with fibroblast and stimulated with LPS+IFNγ under hypoxia for 4h (MI/H/CC) are not different from pro-inflammatory Mφ single cultivated in hypoxia (MI/H) [comparison A]; when resting Mφ are put under hypoxia in co-culture (MH/CC) or are single-cultivated (MH) [comparison B], they do not differentially express an higher number of genes (AvsB: 38vs32). The effect of co-culture is assessed also in normoxia where co-cultivated pro-inflammatory Mφ are similar to their single cultivated counterpart [comparison C (MI/CCvsMI)] and co-cultivated, resting M0 are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 14vs4). Venn diagram (B) shows that there are few genes in each comparison. Indeed, when macrophage with the same polarizing status are compared to observe the effect of different culture condition, in different oxygen status, we do not found any differences in the pattern of SDEG, as we can observe in the 8-columns heatmap reported (C).

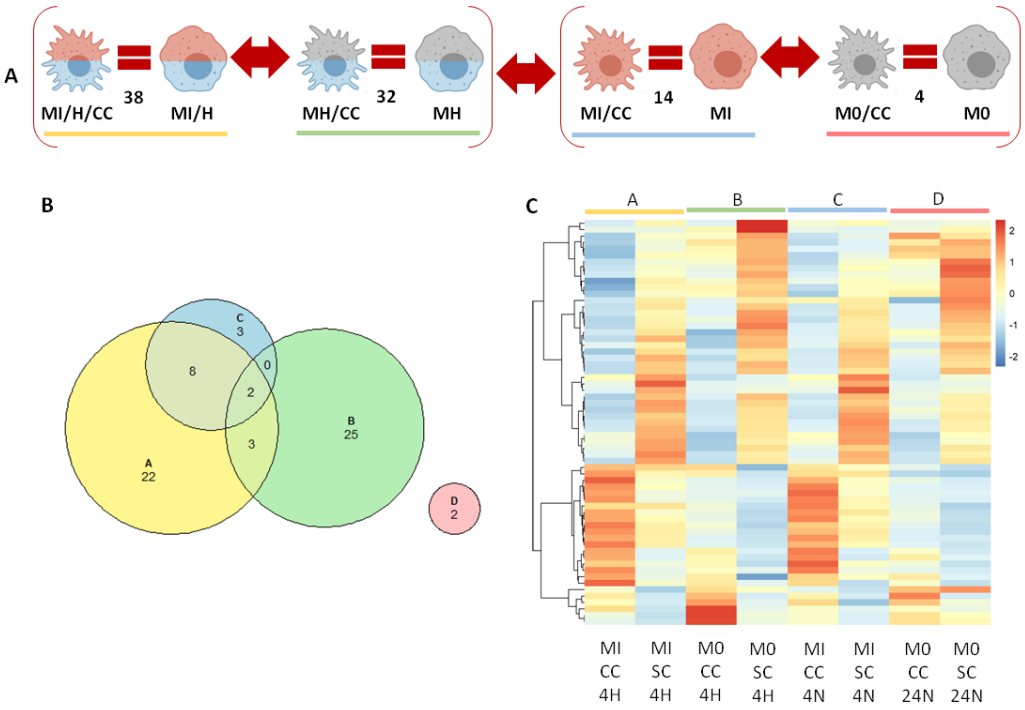


Fig. 169 Comparison [(MI/CCvsMI)Ivs(M0/CCvsM0)0]H vs [(MI/CCvsMI)Ivs(M0/CCvsM0)0]N. Schematic representation of two double comparisons of three variables: polarizing and oxygen statusof co-cultivated Mφ vs single cultivated Mφ at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/H), in green the comparison B (MH/CCvsMH),in blue the comparison C (MI/CCvsMI) and in pink the comparison D (M0/CCvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C150 (C44vsC12)vs(40vsC10)]

Fibroblasts co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/CC/H) are not different from hypoxic pro-inflammatory Fb single cultivated (FbI/H) [comparison A]; when resting Fb are put under hypoxia in co-culture (FbH/CC) or are single-cultivated (FbH) [comparison B] they are the same (AvsB: 25vs0). The effect of co-culture is assessed also in normoxic, co-cultivated FbI that are not different to their single cultivated counterpart [comparison C (FbI/CCvsFbI)]; co-cultivated resting Fb are the same of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 3vs2). Venn diagram (B) shows that there are few genes in each comparison.

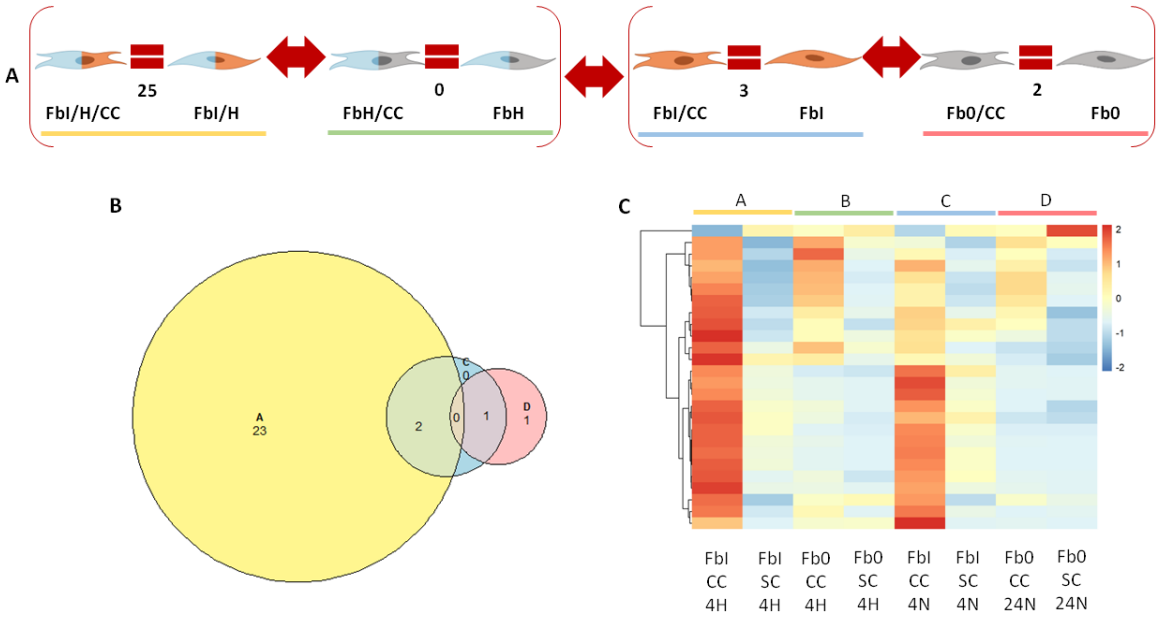


Fig. 170 Comparison [(FbI/CCvsFbI)Ivs(Fb0/CCvsFb0)0]H vs [(FbI/CCvsFbI)Ivs(Fb0/CCvsFb0)0]N. Schematic representation of two double comparisons of three variables: polarizing and oxygen statusof co-cultivated Fb vs single cultivated Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/H), in green the comparison B (FbH/CCvsFbH), in blue the comparison C (FbI/CCvsFbI) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C151 (C45vsC13)vs(41vsC9)]

Macrophages co-cultivated with fibroblast and stimulated with LPS+IFNγ under hypoxia for 24h (MI/H/CC) are different from pro-inflammatory Mφ single cultivated in hypoxia (MI/H) [comparison A]; when resting Mφ are put under hypoxia in co-culture (MH/CC) or are single-cultivated (MH) [comparison B] they do differentially express an higher number of genes (AvsB: 943vs1424). The effect of co-culture is assessed also in normoxia, where co-cultivated, pro-inflammatory Mφ are different to their single cultivated counterpart [comparison C (MI/CCvsMI)] and co-cultivated resting M0 are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 110vs4). Venn diagram (B) shows that comparisons with the higher number of SDEG are A and B. They share 256 genes, and comparison B has a thousand of genes specific for that comparison. Indeed, when macrophages with the same polarizing status are compared to observe the effect of different culture condition in different oxygen status, we find that major differences come out under hypoxia both in pro-inflammatory and, with an higher extent, in resting cells when they are

co-cultivated; in normoxia, instead, only in pro-inflammatory condition co-culture induce a difference of an hundred of genes. In the 8-columns heatmap reported (C) we cannot appreciate this difference since that the stronger difference is given by the pro-inflammatory phenotype that is the opposite of resting phenotype (as we said before) and values are scaled by row.

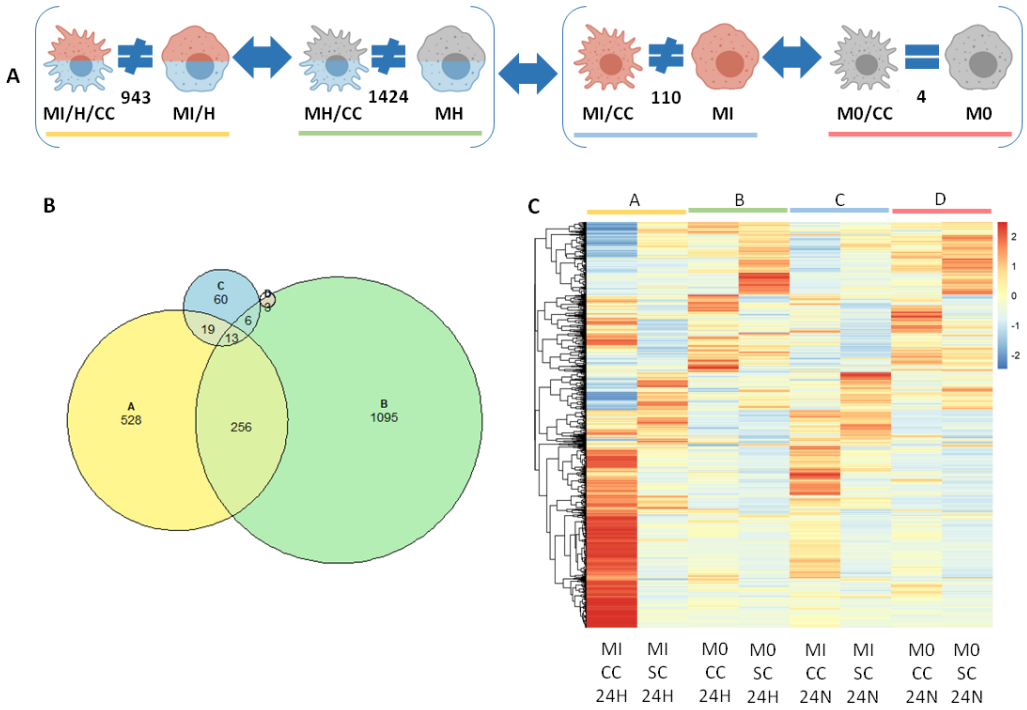


Fig. 171 Comparison [(MI/CCvsMI)Ivs(M0/CCvsM0)0]H vs [(MI/CCvsMI)Ivs(M0/CCvsM0)0]N. Schematic representation of two double comparisons of three variables: polarizing and oxygen status of co-cultivated Mφ vs single cultivated Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/H), in green the comparison B (MH/CCvsMH),in blue the comparison C (MI/CCvsMI) and in pink the comparison D (M0/CCvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C152 (C46vsC14)vs(42vsC10)]

Fibroblasts co-cultivated with macrophages and stimulated with LPS+IFNγ for 24h under hypoxia (FbI/CC/H) are different from hypoxic pro-inflammatory Fb single cultivated (FbI/H) [comparison A]; when resting Fb are put under hypoxia in co-culture (FbH/CC) or are single-cultivated (FbH) [comparison B] they are different (AvsB: 1900vs1291). The effect of co-culture is assessed also in normoxic, co-cultivated FbI that are different to their single cultivated counterpart [comparison C (FbI/CCvsFbI)]; co-cultivated, resting Fb are the same

of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 1973vs2). Venn diagram (B) shows that there is only one comparison where there are no differences (comparison D), whereas the other three comparisons share 81 SDEG. Indeed, when fibroblasts with the same polarizing status are compared to observe the effect of different culture condition, in different oxygen status, we find that major differences come out under hypoxia, both in pro-inflammatory and, with an higher extent, in resting cells when they are co-cultivated; in normoxia, instead, only in pro-inflammatory condition co-culture induce a great difference of SDEG. In the 8-columns heatmap reported (C) we can see that FbI/H/CC and FbI/CC (comparison A and C) have a similar pattern of SDEG and that it is different from the respective single cultivated counterpart; instead the other great difference between the two members of comparison B it cannot be really appreciate because the difference between pro-inflammatory phenotype and resting phenotype is stronger (as we said before) and values are scaled by row.

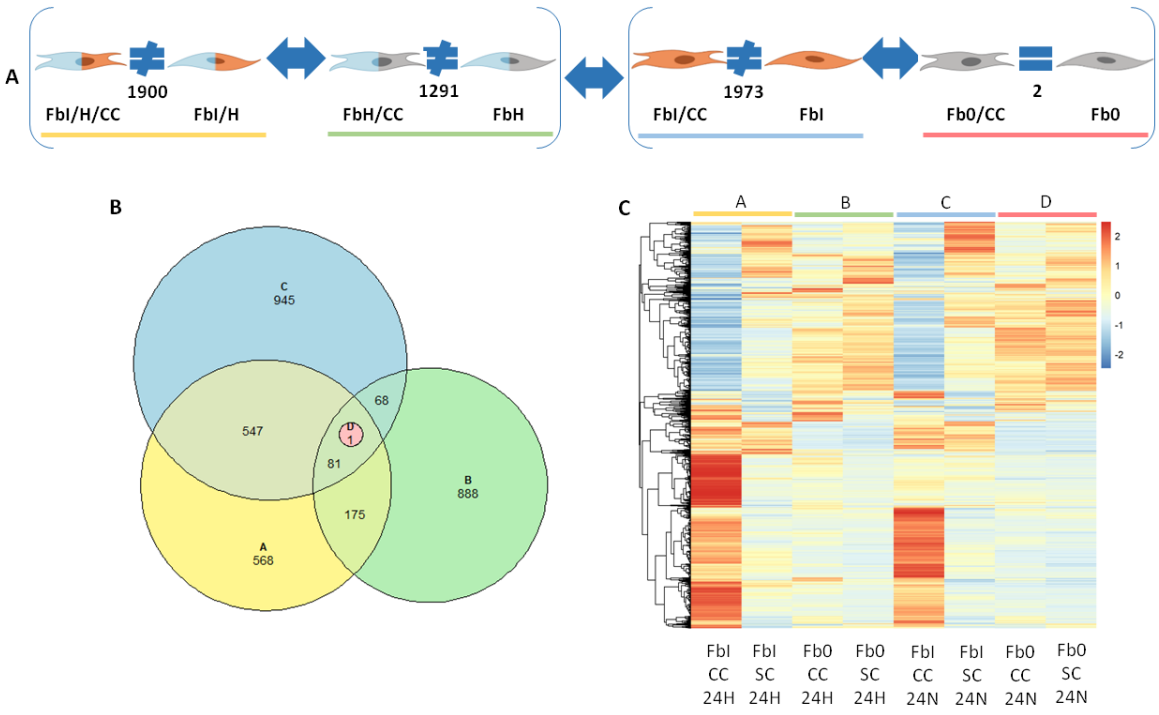


Fig. 172 Comparison [(FbI/CCvsFbI)Ivs(Fb0/CCvsFb0)0]H vs [(FbI/CCvsFbI)Ivs(Fb0/CCvsFb0)0]N. Schematic representation of two double comparison of three variables: polarizing and oxygen status of co-cultivated Fbvs single cultivated Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/H), in green the comparison B (FbH/CCvsFbH),in blue the comparison C (FbI/CCvsFbI) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total

of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C153 (C105vsC83)]

Macrophages co-cultivated with fibroblast and stimulated with LPS+IFNγ under hypoxia for 4h (MI/H/CC) are not different from pro-inflammatory Mφ single cultivated in hypoxia (MI/H) [comparison A]; when MI are put in normoxia in co-culture (MI/CC) or are single-cultivated (MI) [comparison B] they do not differentially express an higher number of genes (AvsB: 38vs14). The effect of co-culture is assessed also in resting Mφ under hypoxia that are similar to their single cultivated counterpart [comparison C (MH/CCvsMH)] and normoxic, co-cultivated resting M0 are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 32vs4). Venn diagram (B) shows that there are few genes in each comparison. Indeed, when macrophage with the same polarizing status are compared to observe the effect of different culture condition in different oxygen status, we do not found any differences in the pattern of SDEG, as we can observe in the 8-columns heatmap reported (C).

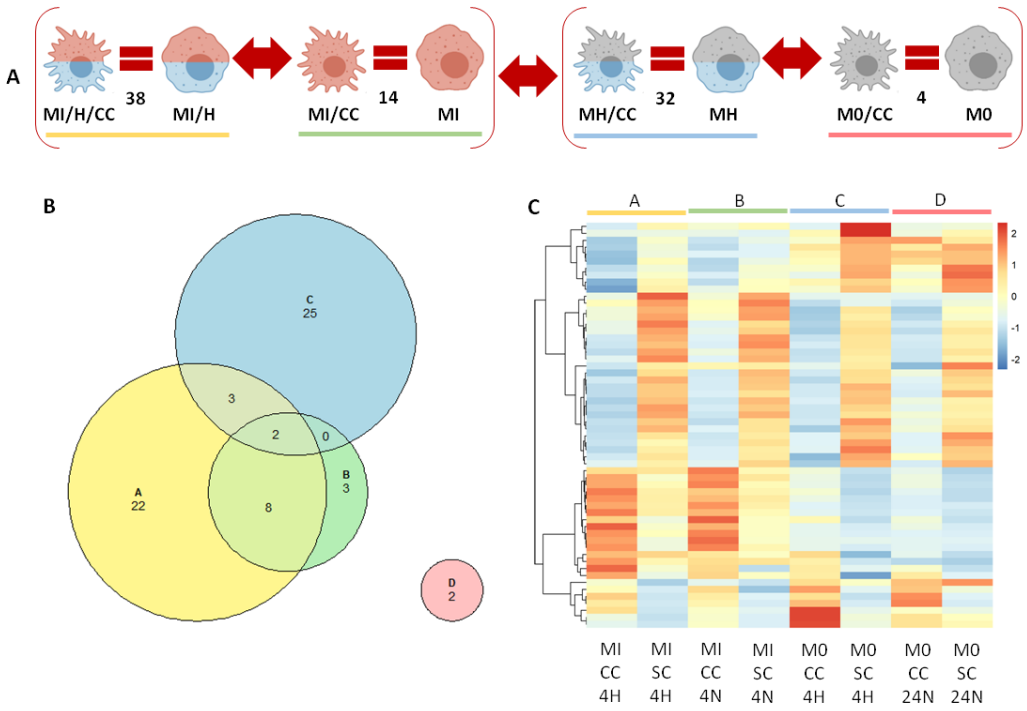


Fig. 173 Comparison [(MI/CCvsMI)Hvs(MI/CCvsMI)N]I vs [(M0/CCvsM0)Hvs(M0/CCvsM0)N]0. Schematic representation of two double comparisons of three variables: polarizing and oxygen status of co-cultivated Mφ vs single cultivated Mφ at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/H), in green the comparison B (MI/CCvsMI), in blue the comparison C (MH/CCvsMH) and in pink the comparison D (M0/CCvsM0) (A);

Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C154 (C106vsC84)]

Fibroblasts co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/CC/H) are not different from hypoxic pro-inflammatory Fb single cultivated (FbI/H) [comparison A]; when FbI are put under normoxia in co-culture (FbI/CC) or are single-cultivated (FbI) [comparison B] they are the same (AvsB: 25vs3). The effect of co-culture is assessed also in hypoxic co-cultivated resting Fb that are not different to their single cultivated counterpart [comparison C (FbH/CCvsFbH)]; normoxic, co-cultivated resting Fb are the same of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 0vs0). Venn diagram (B) shows that there are no differentially expressed genes shared through the four comparisons, and generally there are few genes in each comparison.

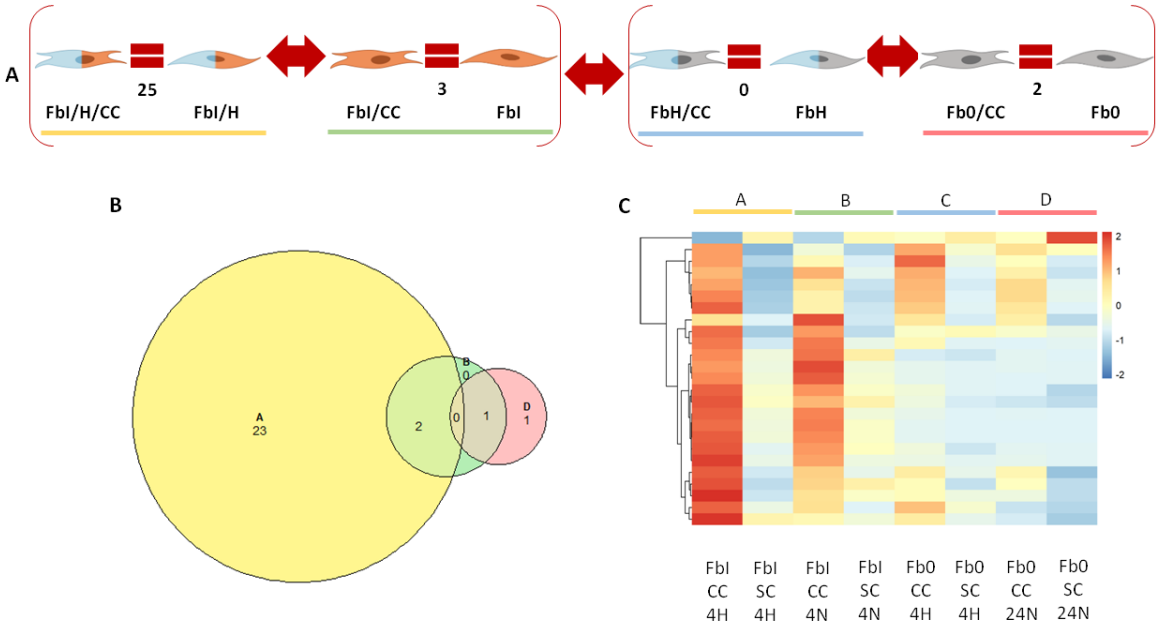


Fig. 174 Comparison [(FbI/CCvsFbI)Hvs(FbI/CCvsFbI)N]I vs [(Fb0/CCvsFb0)Hvs(Fb0/CCvsFb0)N]0. **S**chematic representation of two double comparison of three variables: polarizing and oxygen status of co-cultivated Fbvs single cultivated Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/H), in green the comparison B (FbI/CCvsFbI), in blue the comparison C (FbH/CCvsFbH) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represent a member of the two double comparison and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C155 (C107vsC85)]

Macrophages co-cultivated with fibroblast and stimulated with LPS+IFNγ under hypoxia for 24h (MI/H/CC) are different from pro-inflammatory Mφ single cultivated in hypoxia (MI/H) [comparison A]; when MI are put in normoxia in co-culture (MI/CC) or in single-culture (MI) [comparison B], they differentially express a lower number of genes (AvsB: 943vs110). The effect of co-culture is assessed also in hypoxic, co-cultivated, resting Mφ that are different from their single cultivated counterpart [comparison C (MH/CCvsMH)]. Co-cultivated, resting M0 are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 1424vs4). Venn diagram (B) shows that comparisons with the higher number of SDEG are A and C: they share 256 genes and comparison C has a thousand of genes specific for that comparison. Indeed, when macrophage with the same polarizing status are compared to observe the effect of different culture condition in different oxygen status, we find that major differences come out under hypoxia both in pro-inflammatory and, with an higher extent, in resting cells, when they are co-cultivated; in normoxia, instead, only in pro-inflammatory condition co-culture induce a difference of an hundred of genes. In the 8-columns heatmap reported (C) we cannot appreciate this difference since that the stronger difference is given by the pro-inflammatory phenotype that is the opposite of resting phenotype (as we said before) and values are scaled by row.

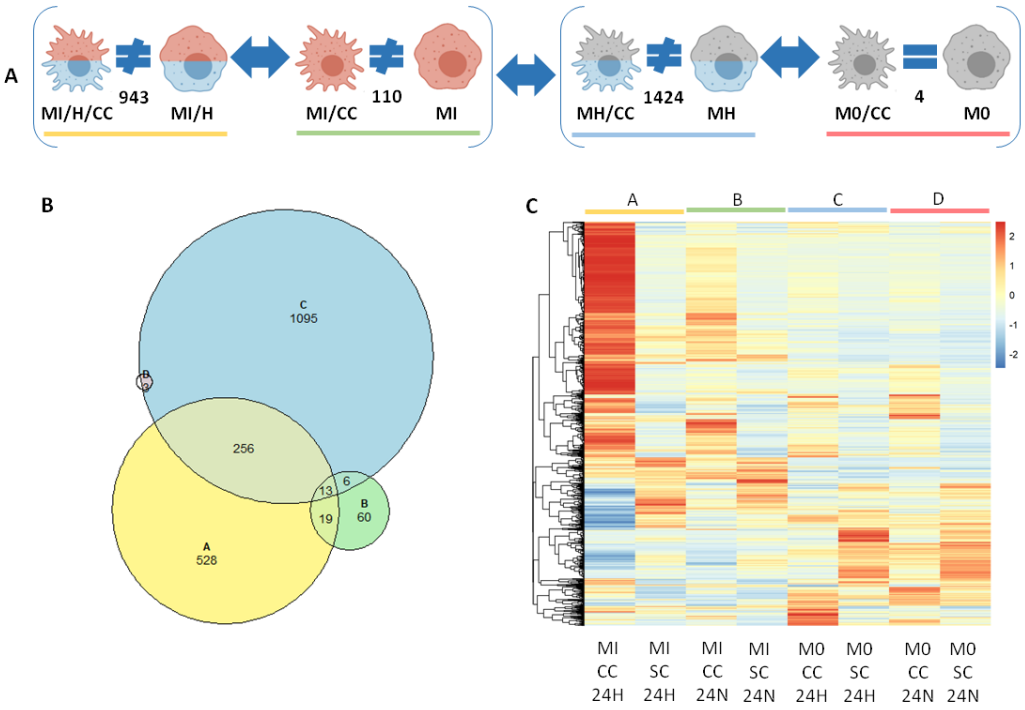


Fig. 175 Comparison [(MI/CCvsMI)Hvs(MI/CCvsMI)N]I vs [(M0/CCvsM0)Hvs(M0/CCvsM0)N]0. Schematic representation of two double comparison of three variables: polarizing and oxygen status of co-cultivated Mφ vs single cultivated Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MI/CC/HvsMI/H), in green the comparison B (MI/CCvsMI), in blue the comparison C (MH/CCvsMH) and in pink the comparison D (M0/CCvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C156 (C108vsC86)]

Fibroblasts co-cultivated with macrophages and stimulated with LPS+IFNγ for 4h under hypoxia (FbI/CC/H) are different from hypoxic, pro-inflammatory, single cultivated Fb (FbI/H) [comparison A]; when FbI are put under normoxia in co-culture (FbI/CC) or are single-cultivated (FbI) [comparison B], they show a great difference in number of SDEG (AvsB: 1900vs1973). The effect of co-culture is assessed also in hypoxic, co-cultivated, resting Fb that differs to their single cultivated counterpart [comparison C (FbH/CCvsFbH)]; normoxic, co-cultivated, resting Fb, instead, are the same of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 1291vs2). Venn diagram (B) shows that there is only one comparison where there are no differences (comparison D), whereas the other three comparisons share 81 differentially expressed genes. Indeed, when fibroblasts with the same polarizing status are compared, to observe the effect of different culture condition in different

oxygen status, we find that major differences come out under hypoxia both in pro-inflammatory and, with an higher extent, in resting cells, when they are co-cultivated; in normoxia, instead, only in pro-inflammatory condition, co-culture induce a great difference of SDEG. In the 8-columns heatmap reported (C), we see that FbI/H/CC and FbI/CC (comparison A and B) have a similar pattern of SDEG and that it is different from the respective single cultivated counterpart; instead, the other great difference between the two member of comparison C it cannot be really appreciate because the difference between pro-inflammatory phenotype and resting phenotype is stronger (as we said before) and values are scaled by row.

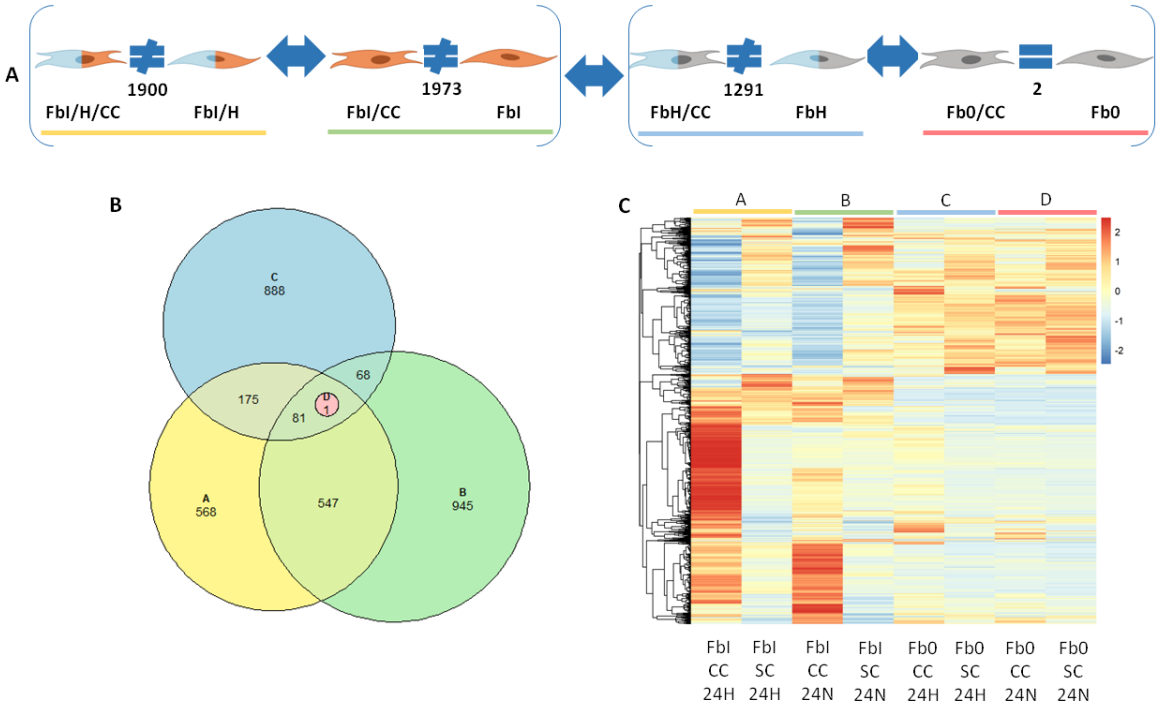


Fig. 176 Comparison [(FbI/CCvsFbI)Hvs(FbI/CCvsFbI)N]I vs [(Fb0/CCvsFb0)Hvs(Fb0/CCvsFb0)N]0. Schematic representation of two double comparisons of three variables: polarizing and oxygen statusof co-cultivated Fbvs single cultivated Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbI/CC/HvsFbI/H), in green the comparison B (FbI/CCvsFbI),in blue the comparison C (FbH/CCvsFbH) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

8.3.2.1 What is the impact of IL-4 when oxygen and culture status are modified?

[Code: C157 (C121vsC113)]

Macrophages stimulated with IL-4 and put under hypoxia for 4h in co-culture with fibroblasts (MF/H/CC) are similar MH, co-cultivated, without stimuli (MH/CC) [comparison A]; instead, the same comparison made in normoxia shows a major difference (MF/CC vs M0/CC) [comparison B](AvsB: 74vs138). The effect of combined hypoxia and pro-fibrotic stimulus is assessed also in single culture, where alternative Mφ are similar to resting M0 under hypoxia [comparison C (MF/HvsMH)] and different under normoxia [comparison D (MFvsM0)] (CvsD: 88vs190). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Mφs remain different from their resting counterpart, independently if they are in single or co-culture, but when they are put under hypoxia this difference is decreased. Venn diagram (B) shows that 36 SDEG are shared by four comparisons and that the two comparisons with higher number of SDEG are A and D. Moreover, pro-fibrotic stimulus (IL-4) promotes a difference that is stronger than the other factors but hypoxia could limit this difference, as we can observe in the 8-columns heatmap reported (C).

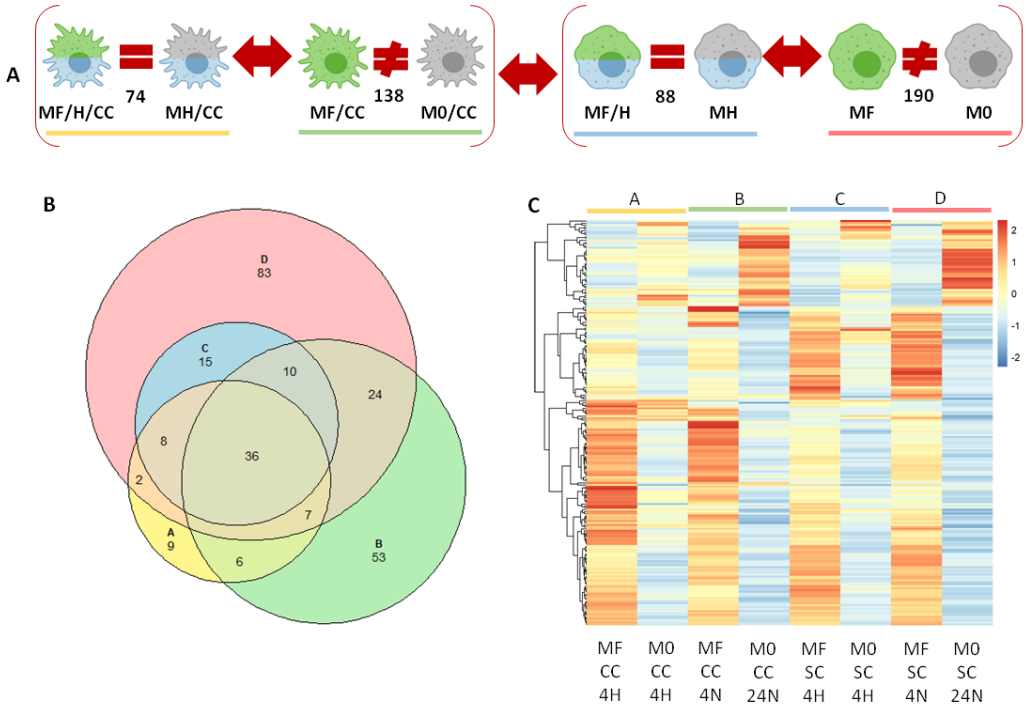


Fig. 177 Comparison [(MFvsM0)Hvs(MFvsM0)N]CC vs [(MFvsM0)Hvs(MFvsM0)N]SC. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of MFvsM0 at

time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/H/CCvsMH/CC), in green the comparison B (MF/CCvsM0/CC), in blue the comparison C (MF/HvsMH) and in pink the comparison D (MFvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C158 (C122vsC114)]

Fibroblasts stimulated with IL-4 and put under hypoxia for 4h in co-culture with macrophages (FbF/H/CC) are the same of FbH, co-cultivated, without stimuli (FbH/CC) [comparison A]; instead, the same comparison made in normoxia shows a major difference (FbF/CC vs Fb0/CC) [comparison B](AvsB: 4vs133). The effect of combined hypoxia and pro-fibrotic stimulus is assessed also in single culture, where alternative Fb are the same of resting Fb0 under hypoxia [comparison C (FbF/HvsFbH)] and remain similar under normoxia [comparison D (FbFvsFb0)] (CvsD: 3vs42). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Fbs are the same of their resting counterpart under hypoxia, independently if they are in single or co-culture, but when they are in normoxia culture condition makes the difference because only in co-culture FbF is different from Fb0. Venn diagram (B) shows that there is only comparison B with an hundred of SDEG. Indeed, pro-fibrotic stimulus (IL-4) promotes a difference only in normoxic co-culture (comparison B), moreover we can see that the other samples are similar to each other and to FbF/CC for the pattern expression of the SDEG with the exception of sample Fb0 that is much similar to Fb0/CC, as we can observe in the 8-columns heatmap reported (C).

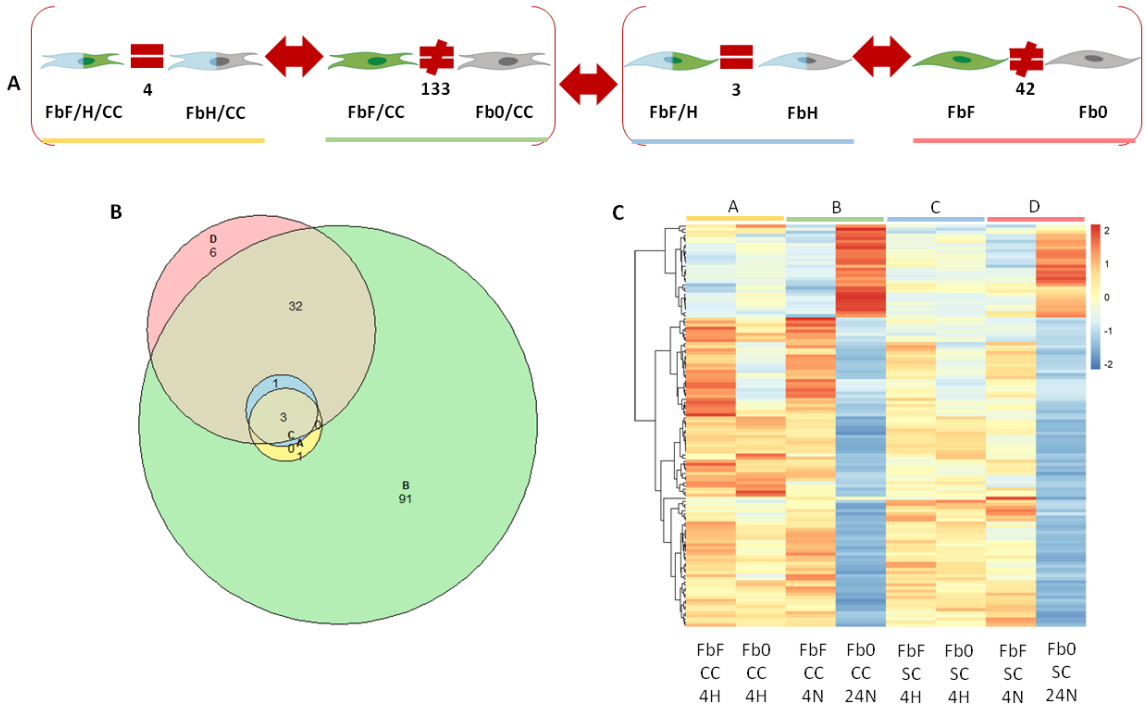


Fig. 178 Comparison [(FbFvsFb0)Hvs(FbFvsFb0)N]CC vs [(FbFvsFb0)Hvs(FbFvsFb0)N]SC. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of FbFvsFb0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/H/CCvsFbH/CC), in green the comparison B (FbF/CCvsFb0/CC), in blue the comparison C (FbF/HvsFbH) and in pink the comparison D (FbFvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C159 (C123vsC115)]

Macrophages stimulated with IL-4 and put under hypoxia for 24h in co-culture with fibroblasts (MF/H/CC) are different from MH, co-cultivated, without stimuli (MH/CC) [comparison A]; instead, the same comparison made in normoxia shows a lower difference (MF/CC vs M0/CC) [comparison B](AvsB: 1465vs173). The effect of combined hypoxia and pro-fibrotic stimulus is assessed also in single culture, where alternative Mφ are different to resting M0 under hypoxia [comparison C (MF/HvsMH)] and also under normoxia [comparison D (MFvsM0)] (CvsD: 144vs178). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Mφs remain different from their resting counterpart independently by culture and oxygen status, but when they are put under hypoxia and co-cultivated this difference is increased. Venn diagram (B) shows that 72 SDEG are shared by four comparisons and that the comparison A has 1267 SDEG in that comparison only.

Moreover, pro-fibrotic stimulus (IL-4) promotes a difference that becomes stronger when is combined with hypoxia and co-culture, as we can observe in the 8-columns heatmap reported (C).

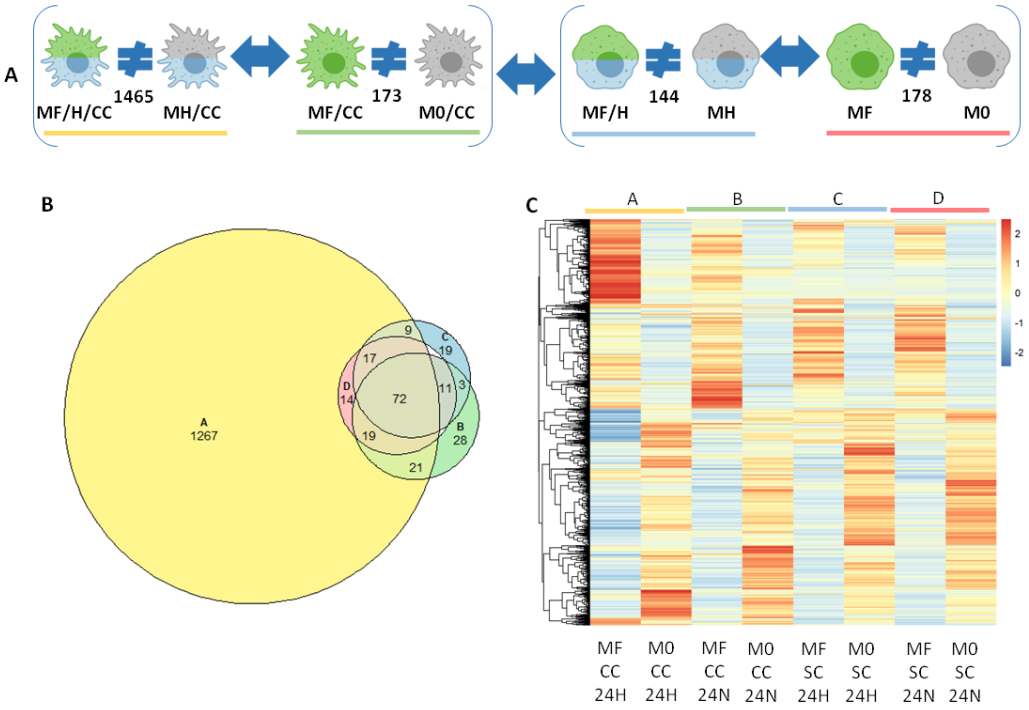


Fig. 179 Comparison [(MFvsM0)Hvs(MFvsM0)N]CC vs [(MFvsM0)Hvs(MFvsM0)N]SC. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of MFvsM0 at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/H/CCvsMH/CC), in green the comparison B (MF/CCvsM0/CC), in blue the comparison C (MF/HvsMH) and in pink the comparison D (MFvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C160 (C124vsC116)]

Fibroblasts stimulated with IL-4 and put under hypoxia for 24h in co-culture with macrophages (FbF/H/CC) are different from FbH, co-cultivated, without stimuli (FbH/CC) [comparison A]; instead, the same comparison made in normoxia shows no difference (FbF/CC vs Fb0/CC) [comparison B](AvsB: 868vs7). The effect of combined hypoxia and pro-fibrotic stimulus is assessed also in single culture, where alternative Fb are the same of resting Fb0 under hypoxia [comparison C (FbF/HvsFbH)] and remain similar under normoxia [comparison D (FbFvsFb0)] (CvsD: 6vs3). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Fbs are the same of their resting counterpart,

independently by oxygen and culture status, but when they are under hypoxia and in co-culture the difference between FbF and Fb0 comes out. Venn diagram (B) shows that there is only comparison A with eight hundred of SDEG. Indeed, pro-fibrotic stimulus (IL-4) promotes a difference that becomes stronger only in hypoxic co-culture (comparison A), even if also in the other comparisons the list of SDEG taken in account show difference in IL-4 stimulated fibroblasts in comparison to resting Fb, as we can observe in the 8-columns heatmap reported (C).

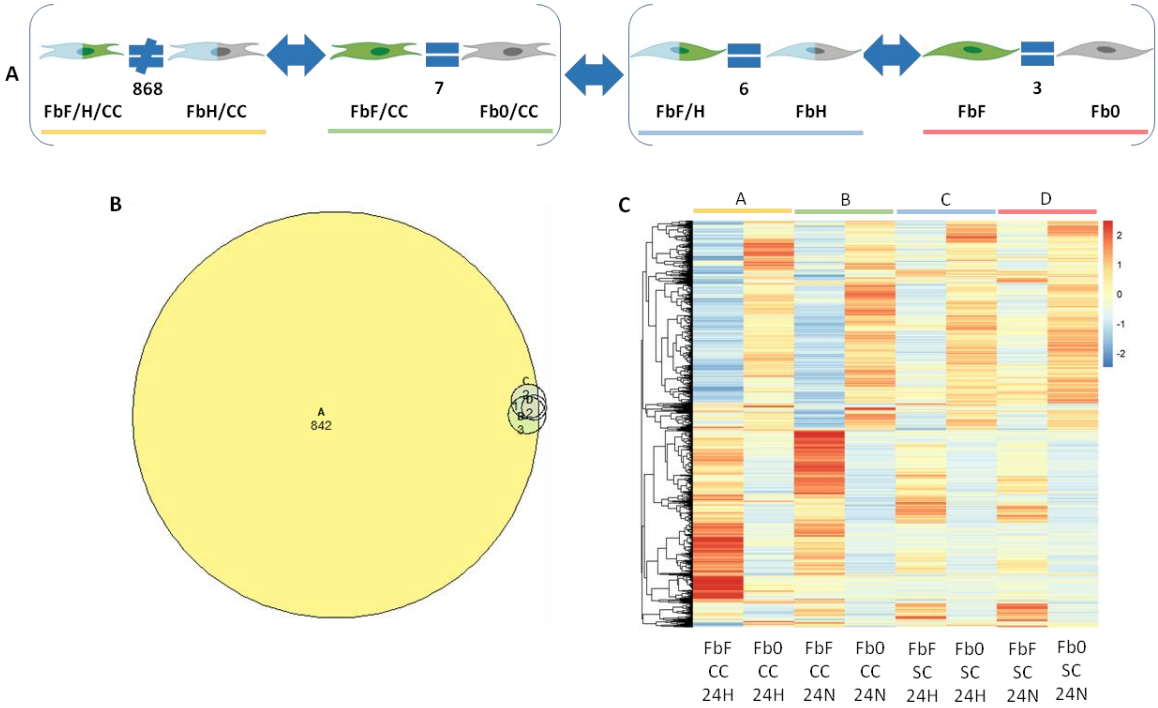


Fig. 180 Comparison [(FbFvsFb0)Hvs(FbFvsFb0)N]CC vs [(FbFvsFb0)Hvs(FbFvsFb0)N]SC. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of FbFvsFb0 at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/H/CCvsFbH/CC), in green the comparison B (FbF/CCvsFb0/CC), in blue the comparison C (FbF/HvsFbH) and in pink the comparison D (FbFvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C161 (C117vsC109)]

Macrophages stimulated with IL-4 and put under hypoxia for 4h in co-culture with fibroblasts (MF/H/CC) are similar MH, co-cultivated, without stimuli (MH/CC) [comparison A]; the same comparison made in single culture shows a similar difference (MF/H vs MH)

[comparison B](AvsB: 74vs88). The effect pro-fibrotic stimulus is assessed also in normoxia, where alternative Mφ differs from resting M0 in co-culture [comparison C (MF/CCvsM0/CC)] and also in single culture [comparison D (MFvsM0)] (CvsD: 138vs190). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Mφs remain different from their resting counterpart, independently if they are in single or co-culture, but when they are put under hypoxia this difference is decreased. Venn diagram (B) shows that 36 SDEG are shared by the four comparisons and that the two comparisons with higher number of differentially expressed genes are C and D. Moreover, pro-fibrotic stimulus (IL-4) promotes a difference that is stronger than the other factors but hypoxia could limit this difference, as we can observe in the 8-columns heatmap reported (C).

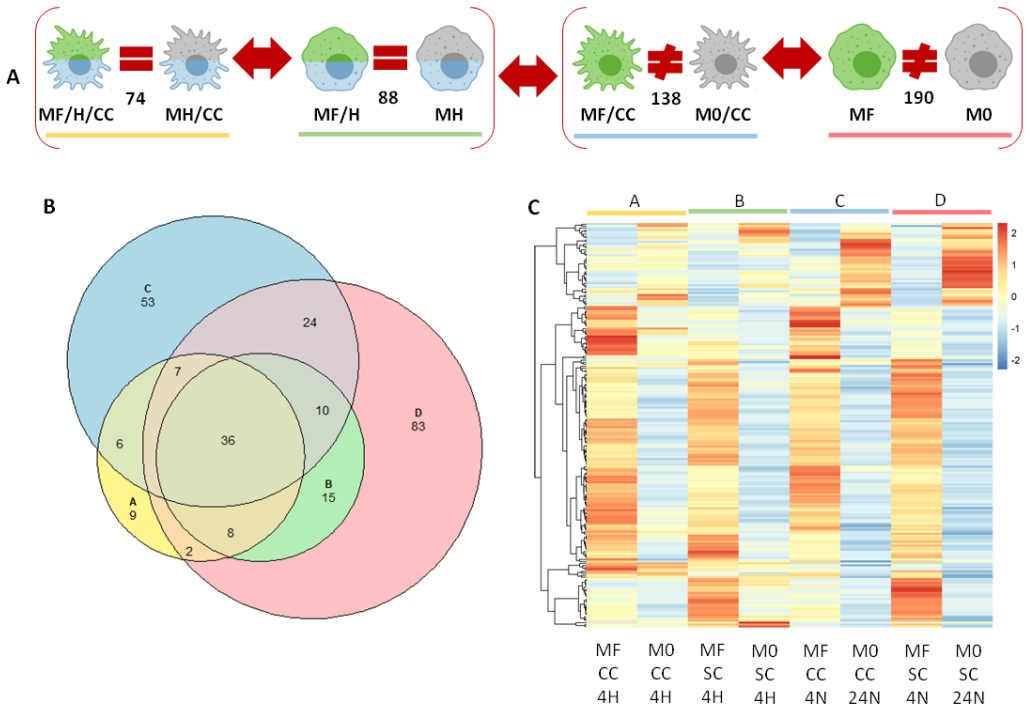


Fig. 181 Comparison [(MFvsM0)CCvs(MFvsM0)SC]H vs [(MFvsM0)CCvs(MFvsM0)SC]N. Schematic representation of two double comparison of three variables: oxygen status and culture condition of MFvsM0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/H/CCvsMH/CC), in green the comparison B (MF/HvsMH), in blue the comparison C (MF/CCvsM0/CC) and in pink the comparison D (MFvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C162 (C118vsC110)]

Fibroblasts stimulated with IL-4 and put under hypoxia for 4h in co-culture with macrophages (FbF/H/CC) are the same of FbH, co-cultivated, without stimuli (FbH/CC) [comparison A]; the same is observed for comparison made in single culture (FbF/H vs FbH) [comparison B](AvsB: 4vs3). The effect of pro-fibrotic stimulus is assessed also in co-culture under normoxia, where FbF are different from resting Fb0 [comparison C (FbF/CCvsFb0/CC)] but this difference is decreased in single culture [comparison D (FbFvsFb0)] (CvsD: 133vs42). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Fbs are the same of their resting counterpart under hypoxia, independently if they are in single or co-culture, but when they are in normoxia culture condition makes the difference because only in co-culture FbF is different from Fb0. Venn diagram (B) shows that there is only comparison C with an hundred of SDEG. Indeed, pro-fibrotic stimulus (IL-4) promotes a difference only in normoxic co-culture (comparison B), moreover we can see that the other samples are similar to each other and to FbF/CC for the pattern expression of the SDEG with the exception of sample Fb0 that is much similar to Fb0/CC, as we can observe in the 8-columns heatmap reported (C).

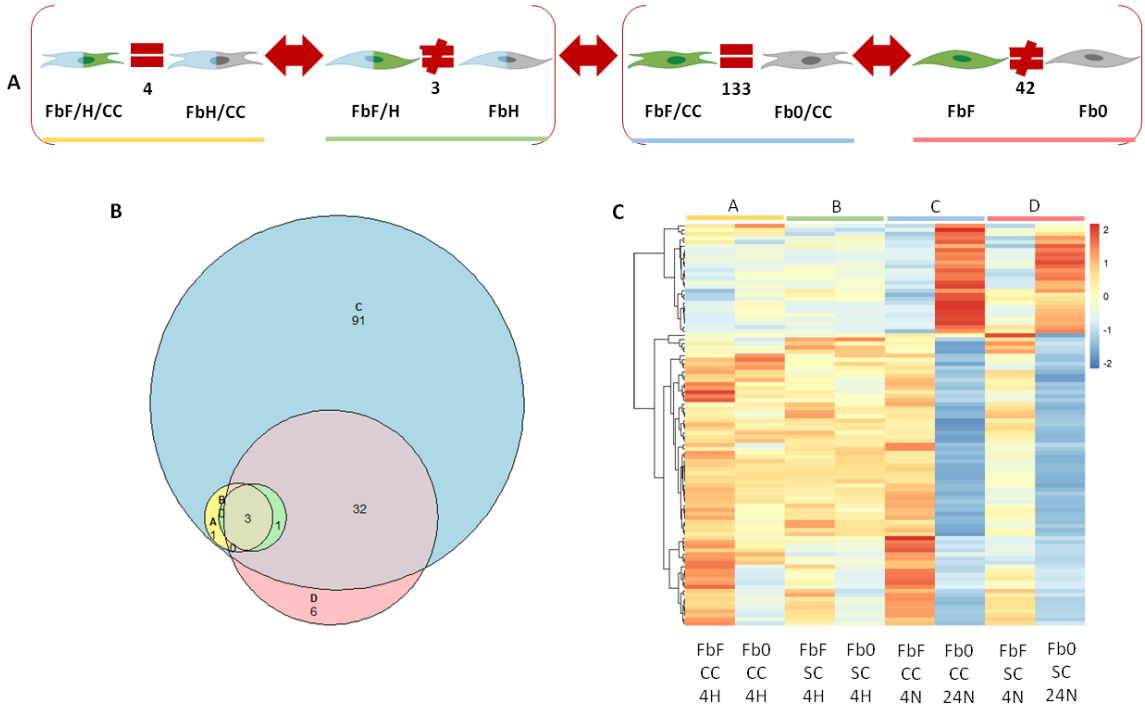


Fig. 182 Comparison [(FbFvsFb0)CCvs(FbFvsFb0)SC]H vs [(FbFvsFb0)CCvs(FbFvsFb0)SC]N. Schematic representation of two double comparisons of three variables: oxygen status and culture condition of FbFvsFb0 at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/H/CCvsFbH/CC), in green the comparison B (FbF/HvsFbH), in blue the comparison C

(FbF/CCvsFb0/CC) and in pink the comparison D (FbFvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C163 (C119vsC111)]

Macrophages stimulated with IL-4 and put under hypoxia for 24h in co-culture with fibroblasts (MF/H/CC) are different from MH, co-cultivated, without stimuli (MH/CC) [comparison A]; the same comparison made in single culture shows a lower difference (MF/H vs MH) [comparison B](AvsB: 1465vs144). The effect pro-fibrotic stimulus is assessed also in normoxia, where alternative Mφ differs from resting M0 in co-culture [comparison C (MF/CCvsM0/CC)] and also in single culture [comparison D (MFvsM0)] (CvsD: 173vs178). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Mφs remain different from their resting counterpart, independently by culture and oxygen status, but when they are put under hypoxia and co-cultivated this difference is increased. Venn diagram (B) shows that 72 SDEG are shared by the four comparisons and that the comparison A has 1267 genes differentially expressed only in that comparison. Moreover, pro-fibrotic stimulus (IL-4) promotes a difference that becomes stronger when is combined with hypoxia and co-culture, as we can observe in the 8-columns heatmap reported (C).

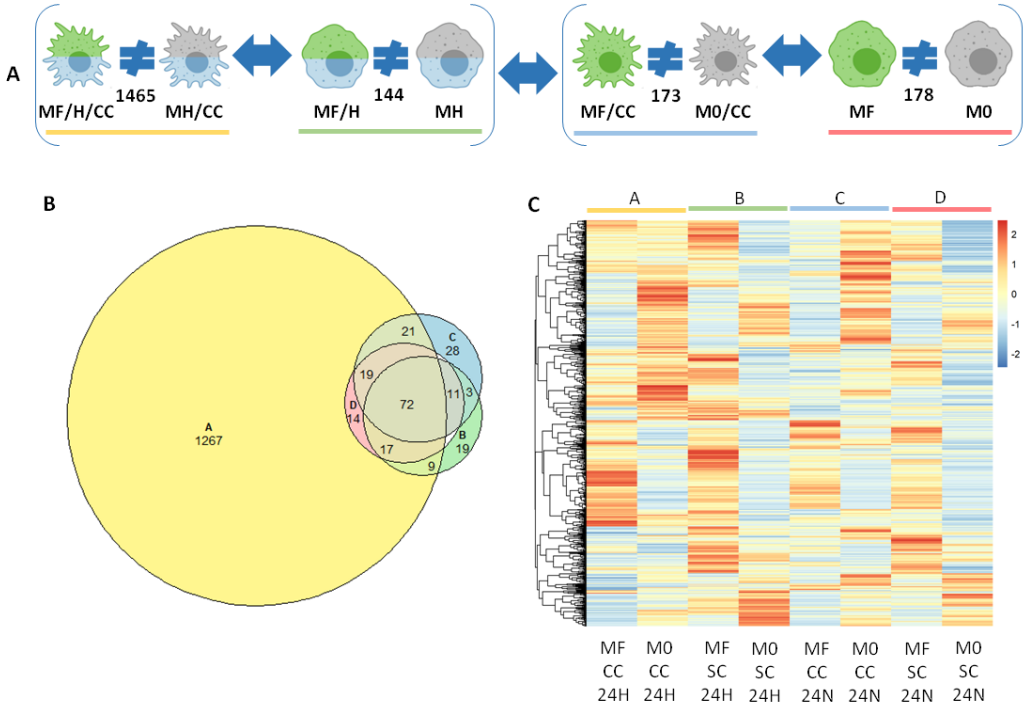


Fig. 183 Comparison [(MFvsM0)CCvs(MFvsM0)SC]H vs [(MFvsM0)CCvs(MFvsM0)SC]N. Schematic representation of two double comparison of three variables: oxygen status and culture condition of MFvsM0 at time point of 24h. For each comparison is reported the number of genes SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/H/CCvsMH/CC), in green the comparison B (MF/HvsMH), in blue the comparison C (MF/CCvsM0/CC) and in pink the comparison D (MFvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C164 (C120vsC112)]

Fibroblasts stimulated with IL-4 and put under hypoxia for 24h in co-culture with macrophages (FbF/H/CC) are different from FbH, co-cultivated, without stimuli (FbH/CC) [comparison A]; instead the same comparison made in single culture shows no difference (FbF/H vs FbH) [comparison B](AvsB: 868vs6). The effect of pro-fibrotic stimulus is assessed also in co-culture under normoxia, where FbF are not different from resting Fb0 [comparison C (FbF/CCvsFb0/CC)] and the same is in single culture [comparison D (FbFvsFb0)] (CvsD: 7vs3). If we consider these genes in the two double comparisons, we observe that pro-fibrotic Fbs are the same of their resting counterpart, independently by oxygen and culture status, but when they are under hypoxia and in co-culture the difference between FbF and Fb0 comes out. Venn diagram (B) shows that there is only comparison A with eight hundred of SDEG. Indeed, pro-fibrotic stimulus (IL-4) promotes a difference that becomes stronger only in hypoxic co-culture (comparison A), even if also in the other comparisons the list of SDEG taken in account show difference in IL-4 stimulated fibroblasts in comparison to resting Fb, as we can observe in the 8-columns heatmap reported (C).

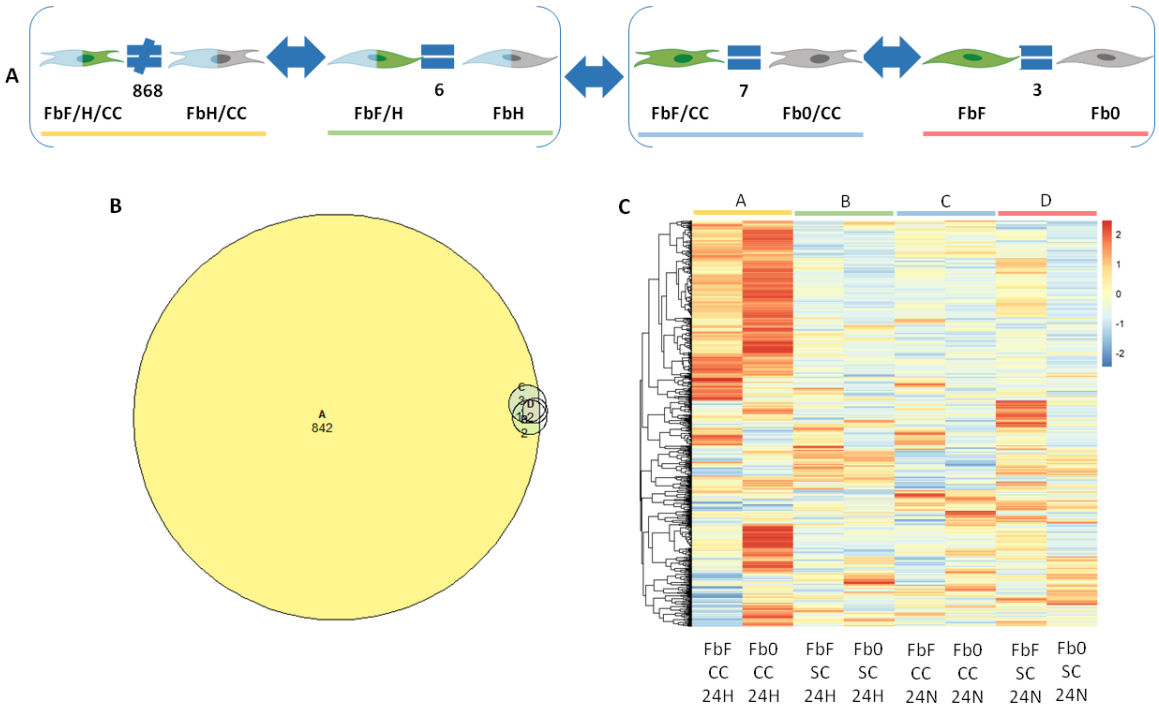


Fig. 184 Comparison [(FbFvsFb0)CCvs(FbFvsFb0)SC]H vs [(FbFvsFb0)CCvs(FbFvsFb0)SC]N. Schematic representation of two double comparison of three variables: oxygen status and culture condition of FbFvsFb0 at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/H/CCvsFbH/CC), in green the comparison B (FbF/HvsFbH), in blue the comparison C (FbF/CCvsFb0/CC) and in pink the comparison D (FbFvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

8.3.2.2 What is the impact of hypoxia when polarizing and culture status are modified?

[Code: C165 (C67vsC5)vs(C63vsC1)]

Macrophages put under hypoxia and stimulated with IL-4 for 4h in co-culture with fibroblasts (MF/CC/H) are not different from alternative Mφ co-cultivated in normoxia (MF/CC) [comparison A]; when resting Mφ are put under hypoxia in co-culture (M0/CC/H) or are co-cultivated in normoxia (M0/CC) [comparison B], they do not differentially express an higher number of genes (AvsB: 30vs44). The effect hypoxia is assessed also in single culture where hypoxic, alternative Mφ are similar to their normoxic counterpart [comparison C (MF/HvsMF)] and hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)]

(CvsD: 15vs37). Venn diagram (B) shows that 10 SDEG are shared in four comparisons. Indeed, when macrophage with the same polarizing status are compared to observe the effect of different oxygen condition in different state of culture we not find differences in the pattern of SDEG, as we can observe in the 8-columns heatmap reported (C).

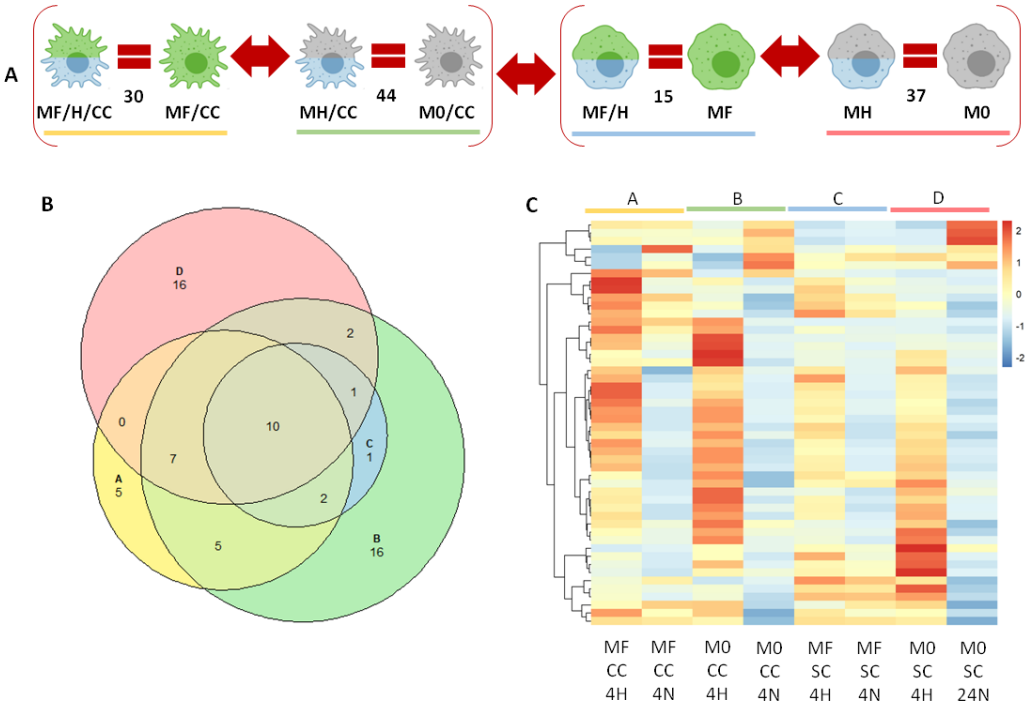


Fig. 185 Comparison [(MF/HvsMF)Fvs(MHvsM0)0]CC vs [(MF/HvsMF)Fvs(MHvsM0)0]SC. Schematic representation of two double comparison of three variables: polarizing status and culture condition of hypoxic Mφ vs normoxic Mφ at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/CC), in green the comparison B (M0/CC/HvsM0/CC), in blue the comparison C (MF/HvsMF) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C166 (C68vsC6)vs(C64vsC2)]

Fibroblasts put under hypoxia and stimulated with IL-4 for 4h in co-culture with macrophages (FbF/CC/H) are not different from pro-fibrotic Fb, co-cultivated in normoxia (FbF/CC) [comparison A]; when resting Fb are put under hypoxia in co-culture (Fb0/CC/H) or are co-cultivated in normoxia (Fb0/CC) [comparison B], they differentially express an higher number of genes (AvsB: 6vs105). The effect hypoxia is assessed also in single culture where hypoxic pro-fibrotic Fb are the same of their normoxic counterpart [comparison C (FbF/HvsFbF)], instead hypoxic Fb0 are different from normoxic one [comparison D

(FbHvsFb0)] (CvsD: 0vs97). Venn diagram (B) shows that 51 SDEG are shared in the comparisons between B and D. Indeed, when fibroblast with the same polarizing status (pro-inflammatory) are compared to observe the effect of different oxygen condition in different state of culture we not find differences in the pattern of SDEG. But, when resting fibroblast are put in different oxygen condition, they show an hundred of SDEG both in co-culture and single culture, indicating that hypoxia affect resting condition, independently from culture condition. As we can observe in the 8-columns heatmap (C), columns referred to comparisons A and C have the same expression pattern of reported genes, instead comparisons B and D have sample Fb0/CC similar to Fb0/SC and FbH/CC similar to FbH/SC.

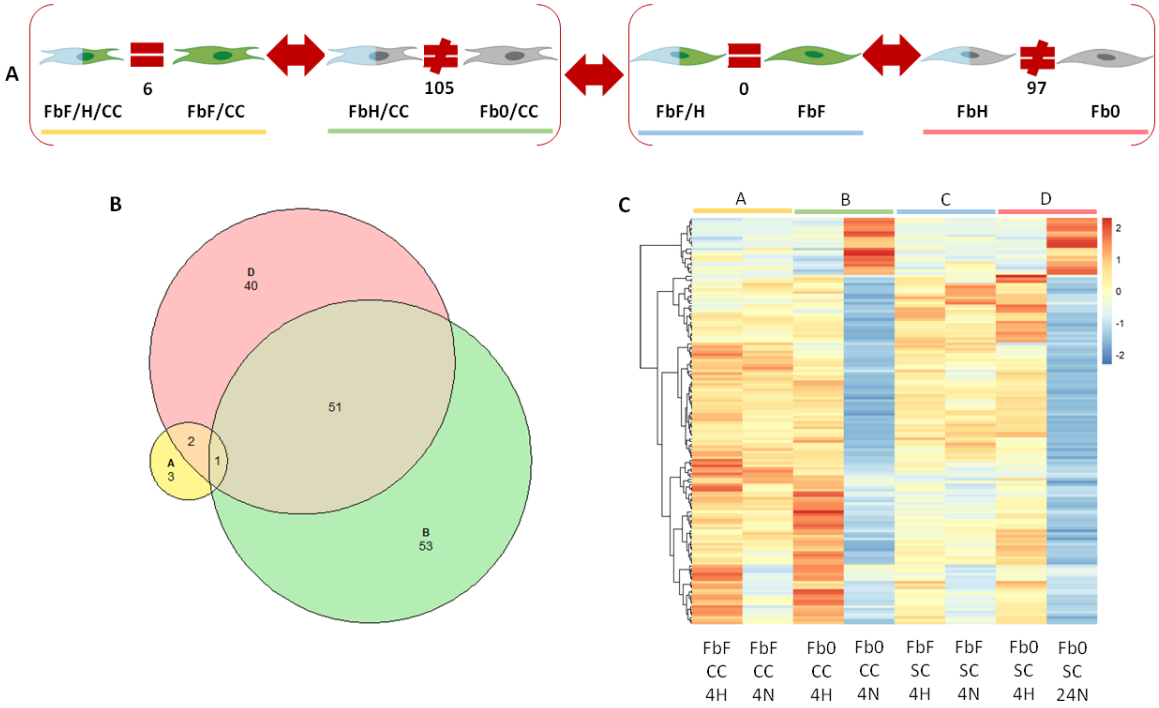


Fig. 186 Comparison [(FbF/HvsFbF)vs(FbHvsFb0)]CC vs [(FbF/HvsFbF)vs(FbHvsFb0)]SC. Schematic representation of two double comparison of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/CC/HvsFbF/CC), in green the comparison B (Fb0/CC/HvsFb0/CC), in blue the comparison C (FbF/HvsFbF) and in pink the comparison D (FbHvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C167 (C69vsC7)vs(C65vsC3)]

Macrophages put under hypoxia and stimulated with IL-4 for 24h in co-culture with fibroblasts (MF/CC/H) are not different from alternative Mφ co-cultivated in normoxia (MF/CC) [comparison A]; when resting Mφ are put under hypoxia in co-culture (M0/CC/H) or are co-cultivated in normoxia (M0/CC) [comparison B], they differentially express an higher number of genes (AvsB: 17vs1139). The effect hypoxia is assessed also in single culture, where hypoxic alternative Mφ are similar to their normoxic counterpart [comparison C (MF/HvsMF)] and hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)] (CvsD: 23vs18). Venn diagram (B) shows that there is only one comparison that give an high number of SDEG (comparison B with 1069 genes). Indeed, when macrophage with the same polarizing status (pro-fibrotic) are compared to observe the effect of different oxygen condition in different state of culture, we do not found any differences in the pattern of SDEG; when we consider resting Mφ hypoxia seems to have an important effect only when they are in co-culture. In the 8-columns heatmap reported (C) we can observe that MH/CC in comparison B has a distinctive gene expression pattern.

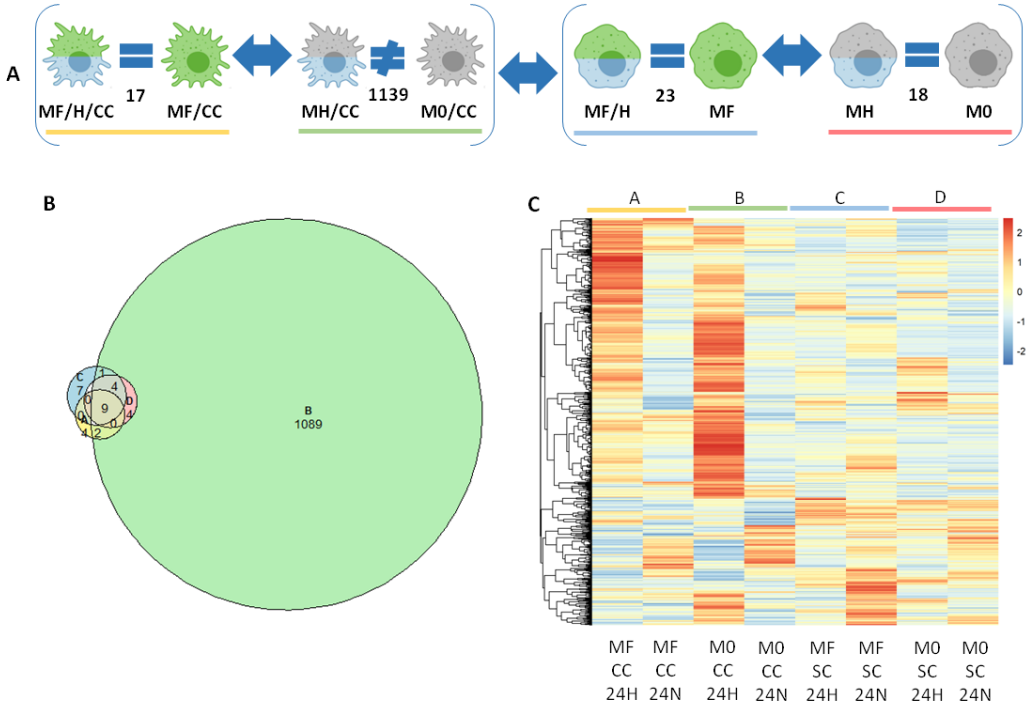


Fig. 187 Comparison [(MF/HvsMF)Fvs(MHvsM0)0]CC vs [(MF/HvsMF)Fvs(MHvsM0)0]SC. Schematic representation of two double comparison of three variables: polarizing status and culture condition of hypoxic Mφ vs normoxic Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/CC), in green the comparison B (M0/CC/HvsM0/CC), in blue the comparison C (MF/HvsMF) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG

with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C168 (C70vsC8)vs(C66vsC4)]

Fibroblasts put under hypoxia and stimulated with IL-4 for 24h in co-culture with macrophages (FbF/CC/H) are not different from pro-fibrotic Fb co-cultivated in normoxia (FbF/CC) [comparison A]; when resting Fb are put under hypoxia in co-culture (Fb0/CC/H) or are co-cultivated in normoxia (Fb0/CC) [comparison B], they differentially express an higher number of genes (AvsB: 60vs1305). The effect hypoxia is assessed also in single culture, where hypoxic pro-fibrotic Fb are similar to their normoxic counterpart [comparison C (FbF/HvsFbF)] and hypoxic Fb0 are similar to normoxic one [comparison D (FbHvsFb0)] (CvsD: 13vs23). Venn diagram (B) shows that the comparison B gives the high number of SDEG (1164). Indeed, when fibroblast with the same polarizing status (pro-fibrotic) are compared to observe the effect of different oxygen condition, in different state of culture, we do not found any differences in the pattern of SDEG. But, when resting fibroblast are put in different oxygen condition, they show a thousand of SDEG only when co-cultivated. In the 8-columns heatmap reported (C) we cannot appreciate this difference.

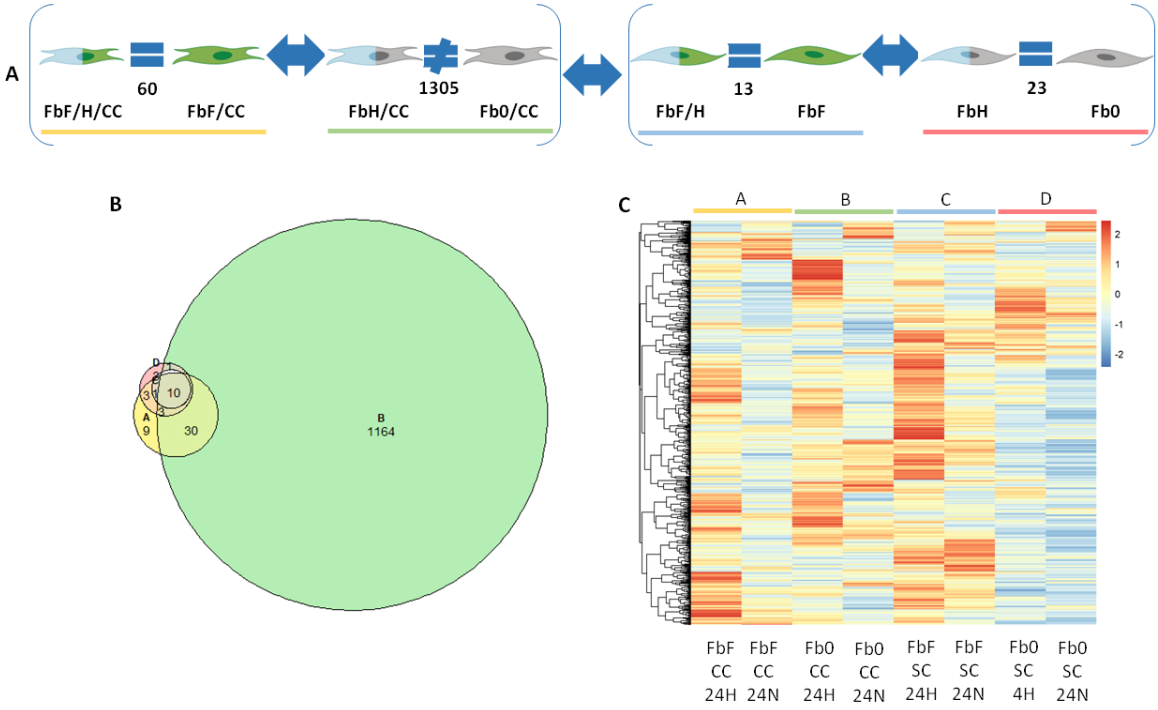


Fig. 188 Comparison [(FbF/HvsFbF)vs(FbHvsFb0)]CC vs [(FbF/HvsFbF)vs(FbHvsFb0)]SC. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In

yellow is underlined the comparison A (FbF/CC/HvsFbF/CC), in green the comparison B (Fb0/CC/HvsFb0/CC), in blue the comparison C (FbF/HvsFbF) and in pink the comparison D (FbHvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C)

[Code: C169 (C7125vsC79)]

Macrophages put under hypoxia and stimulated with IL-4 for 4h in co-culture with fibroblasts (MF/CC/H) are not different from alternative Mφ, co-cultivated in normoxia (MF/CC) [comparison A]; the same happen when MF are in single culture in hypoxia (MF/H) or in normoxia (MF) [comparison B]: they do not differentially express an higher number of genes (AvsB: 30vs15). The effect of hypoxia is assessed also in resting macrophages, where co-cultivated, hypoxic Mφ are similar to their normoxic counterpart [comparison C (MH/CCvsM0/CC)] and single cultivated, hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)] (CvsD: 44vs37). Venn diagram (B) shows that 10 SDEG are shared in the four comparisons. Indeed, when macrophage with the same polarizing status (pro-fibrotic) are compared to observe the effect of different oxygen condition, in different state of culture, we not find differences in the pattern of significantly differentially expressed genes, as we can observe in the 8-columns heatmap reported (C).

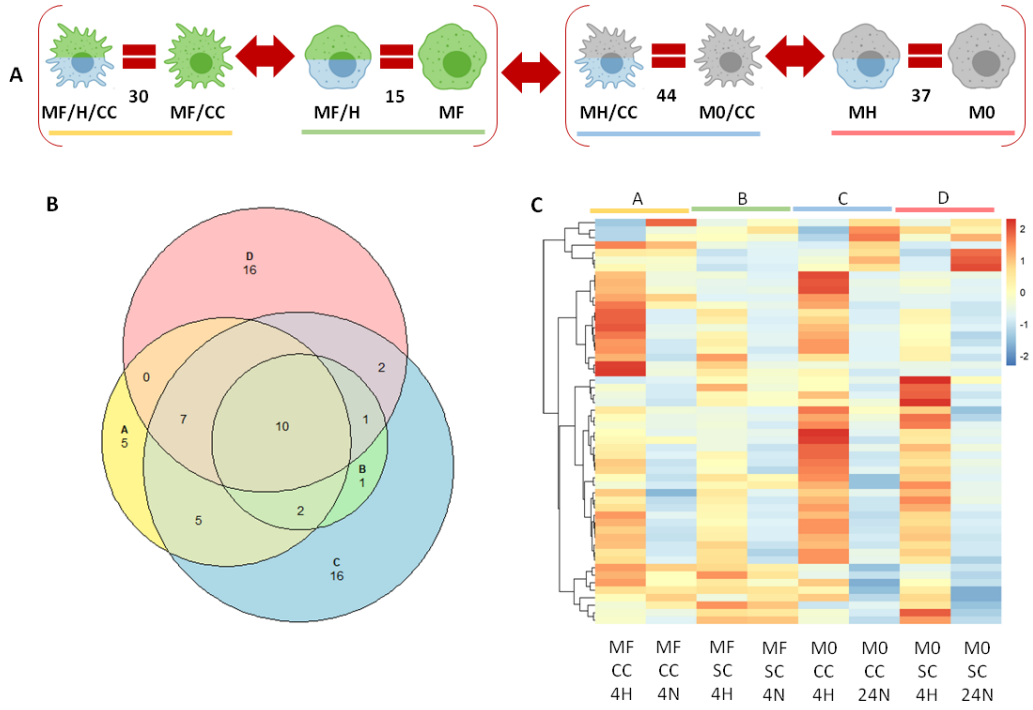


Fig. 189 Comparison [(MF/HvsMF)CCvs(MF/HvsMF)SC]F vs [(MHvsM0)CCvs(MHvsM0)SC]0. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of

hypoxic Mφ vs normoxic Mφ at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/CC), in green the comparison B (MF/HvsMF),in blue the comparison C (M0/CC/HvsM0/CC) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C170 (C126vsC80)]

Fibroblasts put under hypoxia and stimulated with IL-4 for 4h in co-culture with macrophages (FbF/CC/H) are not different from pro-fibrotic Fb co-cultivated in normoxia(FbF/CC) [comparison A]; when FbI are put under hypoxia in single culture (FbF/H) or are in normoxia (FbF) [comparison B], they are the same (AvsB: 6vs0). The effect hypoxia is assessed also in hypoxic, co-cultivated, resting Fb that are different to their normoxic counterpart [comparison C (FbH/CCvsFb0/CC)]; single cultivated, hypoxic Fb0 are different from normoxic one [comparison D (FbHvsFb0)] (CvsD: 105vs97). Venn diagram (B) shows that 52 SDEG are shared in the comparisons between C and D. Indeed, when fibroblast with the same polarizing status (pro-fibrotic) are compared to observe the effect of different oxygen condition, in different state of culture, we do not found any differences in the pattern of SDEG. But, when resting fibroblast are put in different oxygen condition, they show an hundred of SDEG both in co-culture and single culture, indicating that hypoxia affect resting condition, independently from culture condition. As we can observe in the 8-columns heatmap (C), columns referred to comparison A and B have the same pattern of expression of reported genes, instead comparison C and D have sample Fb0/CC similar to Fb0/SC and FbH/CC similar to FbH/SC.

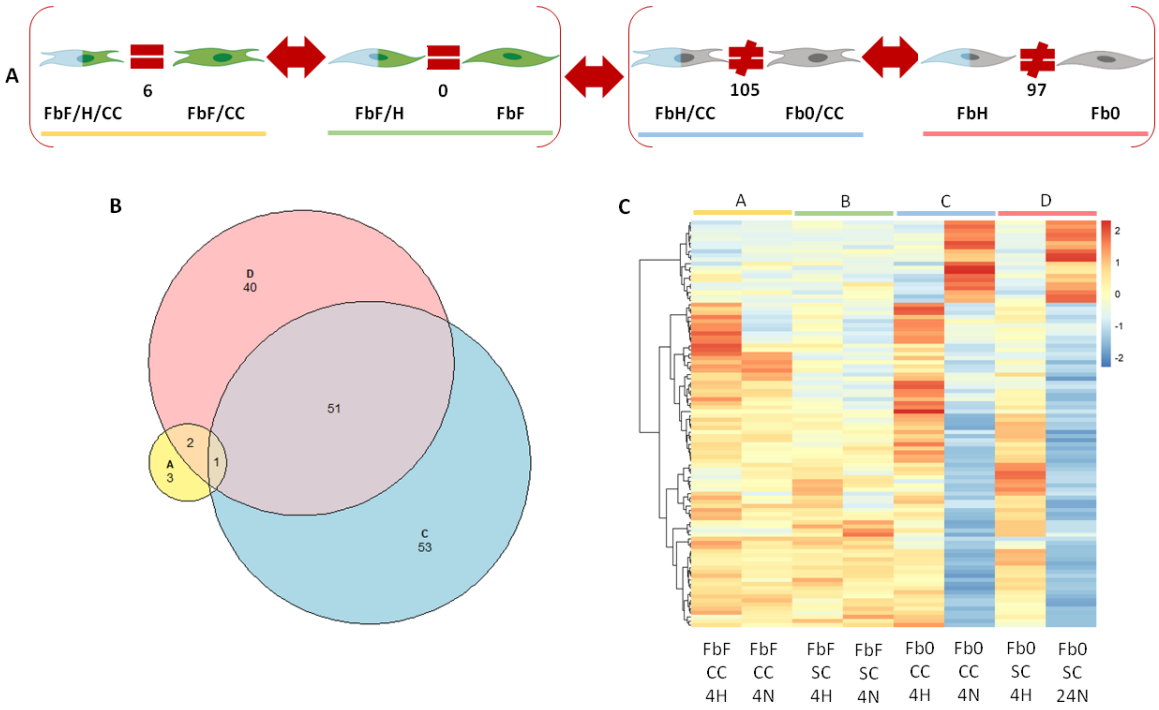


Fig. 190 Comparison [(FbF/HvsFbF)CCvs(FbF/HvsFbF)SC]F vs [(FbHvsFb0)CCvs(FbHvsFb0)SC]0. Schematic representation of two double comparison of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/CC/HvsFbF/CC), in green the comparison B (FbF/HvsFbF), in blue the comparison C (Fb0/CC/HvsFb0/CC) and in pink the comparison D (Fb HvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C171 (C127vsC81)]

Macrophages put under hypoxia and stimulated with Il-4 for 24h in co-culture with fibroblasts (MF/CC/H) are not different from alternative Mφ, co-cultivated in normoxia (MF/CC) [comparison A]; the same happen when MF are in single culture in hypoxia (MF/H) or in normoxia (MF) [comparison B]: they do not differentially express an higher number of genes (AvsB: 17vs23). The effect of hypoxia is assessed also in resting macrophages, where co-cultivated hypoxic Mφ are different to their normoxic counterpart [comparison C (MH/CCvsM0/CC)], instead single cultivated, hypoxic M0 are similar to normoxic one [comparison D (MHvsM0)] (CvsD: 1139vs18). Venn diagram (B) shows that SDEG are given by comparison C. Indeed, when macrophage with the same polarizing status (pro-fibrotic) are compared to observe the effect of different oxygen condition, in different state of culture, we do not found any differences in the pattern of SDEG. When resting Mφ are

stimulated with hypoxia, they differ from normoxic counterpart only if they are co-cultivated, meaning that hypoxia effect in resting cells is culture state dependent. In the 8-columns heatmap reported (C) we can observe that MH/CC in comparison C has a distinctive gene expression pattern.

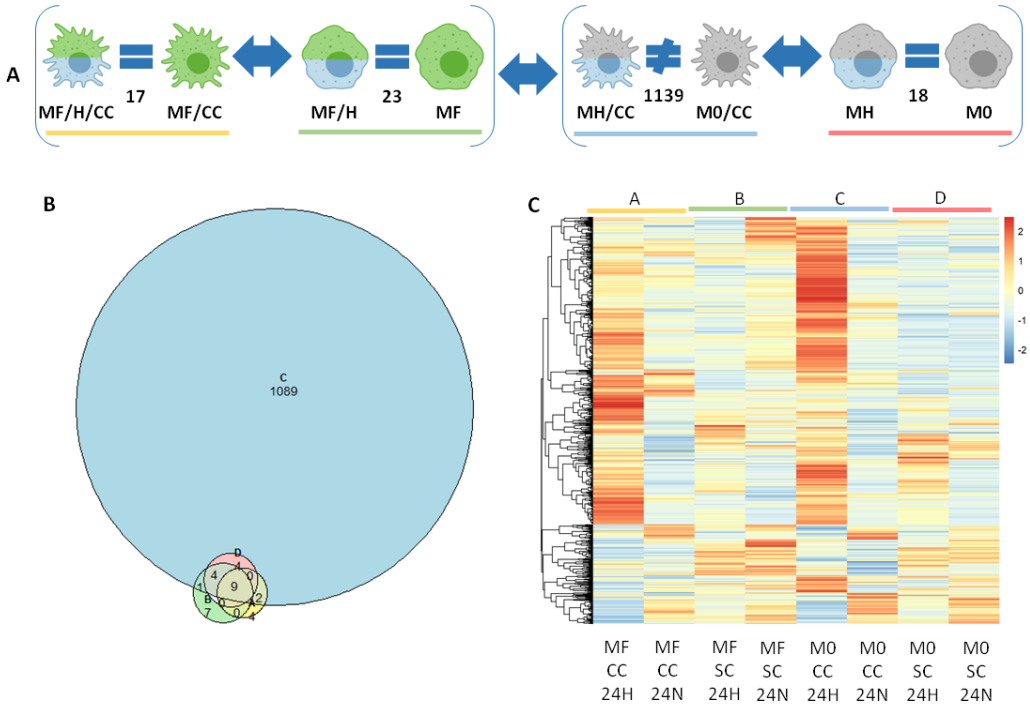


Fig. 191 Comparison [(MF/HvsMF)CCvs(MF/HvsMF)SC]F vs [(MHvsM0)CCvs(MHvsM0)SC]0. Schematic representation of two double comparison of three variables: polarizing status and culture condition of hypoxic Mφ vs normoxic Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/CC), in green the comparison B (MF/HvsMF),in blue the comparison C (M0/CC/HvsM0/CC) and in pink the comparison D (MHvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C172 (C128vsC82)]

Fibroblasts put under hypoxia and stimulated with IL-4 for 24h in co-culture with macrophages (FbF/CC/H) are not different from pro-fibrotic Fb co-cultivated in normoxia (FbF/CC) [comparison A]; when FbF are put under hypoxia in single culture (FbF/H) or are in normoxia (FbF) [comparison B], they are the same (AvsB: 60vs13). The effect hypoxia is assessed also in hypoxic, co-cultivated, resting Fb that are different to their normoxic counterpart [comparison C (FbH/CCvsFb0/CC)]; single cultivated, hypoxic Fb0 are not different from normoxic one [comparison D (FbHvsFb0)] (CvsD: 1305vs23). Venn diagram

(B) shows that SDEG are given by the comparison C (1164). Indeed, when fibroblast with the same polarizing status (pro-fibrotic) are compared to observe the effect of different oxygen condition, in different state of culture, we do not found any differences in the pattern of SDEG. But, when resting fibroblast are put in different oxygen condition, they show a thousand of SDEG in co-culture but not in single culture, indicating that hypoxia affect resting condition, depending by culture condition. In the 8-columns heatmap reported (C) we cannot appreciate this difference.

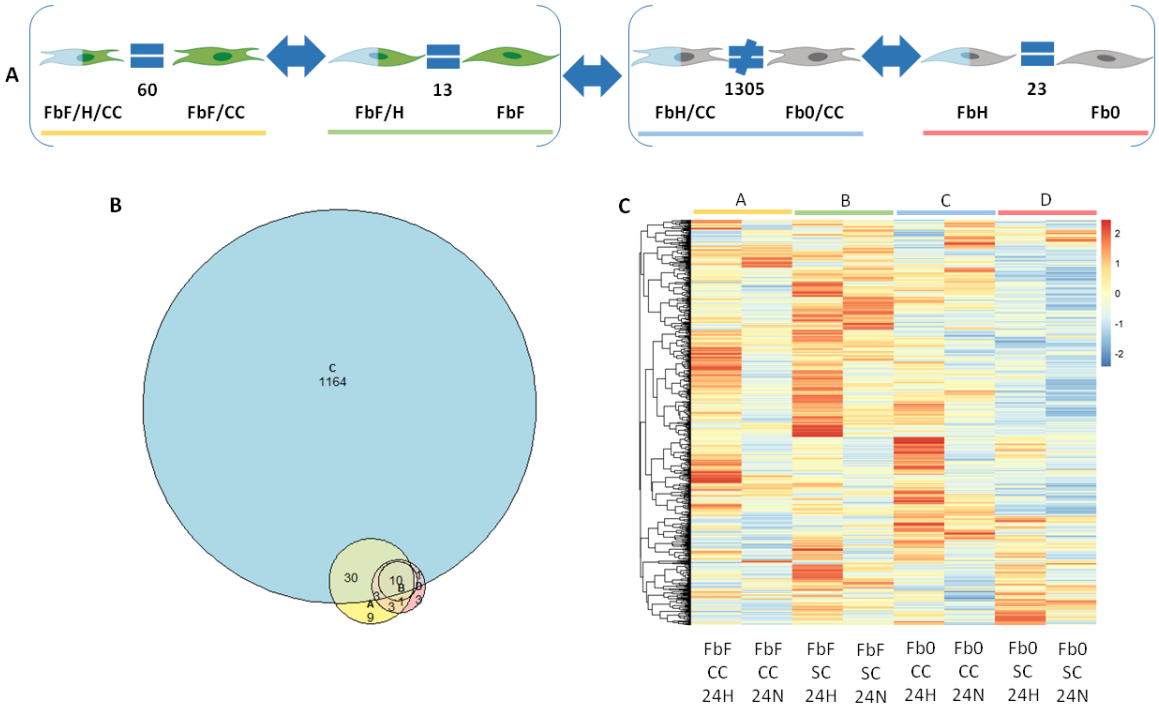


Fig. 192 Comparison [(FbF/HvsFbF)CCvs(FbF/HvsFbF)SC]F vs [(FbHvsFb0)CCvs(FbHvsFb0)SC]0. Schematic representation of two double comparisons of three variables: polarizing status and culture condition of hypoxic Fbvs normoxic Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/CC/HvsFbF/CC), in green the comparison B (FbF/HvsFbF),in blue the comparison C (Fb0/CC/HvsFb0/CC) and in pink the comparison D (Fb HvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

8.3.2.3 What is the impact of co-culture when polarizing and oxygen status are modified?

[Code: C173 (C75vsC11)vs(C71vsC9)]

Macrophages co-cultivated with fibroblast and stimulated with Il-4 under hypoxia for 4h (MF/H/CC) are not different from pro-fibrotic Mφ, single cultivated in hypoxia (MF/H) [comparison A]; when resting Mφ are put under hypoxia in co-culture (MH/CC) or are single-cultivated (MH) [comparison B], they do not differentially express an higher number of genes (AvsB: 93vs32). The effect of co-culture is assessed also in normoxia, where co-cultivated, alternative Mφ are similar to their single cultivated counterpart [comparison C (MF/CCvsMF)] and co-cultivated resting M0 are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 26vs4). Venn diagram (B) shows that there are not SDEG shared through four comparisons, and, generally, there are few genes in each comparison. Indeed, when macrophage with the same polarizing status are compared, to observe the effect of different culture condition, in different oxygen status, we find differences between single and co-culture in the pattern of SDEG in all comparisons, as we can observe in the 8-columns heatmap reported (C).

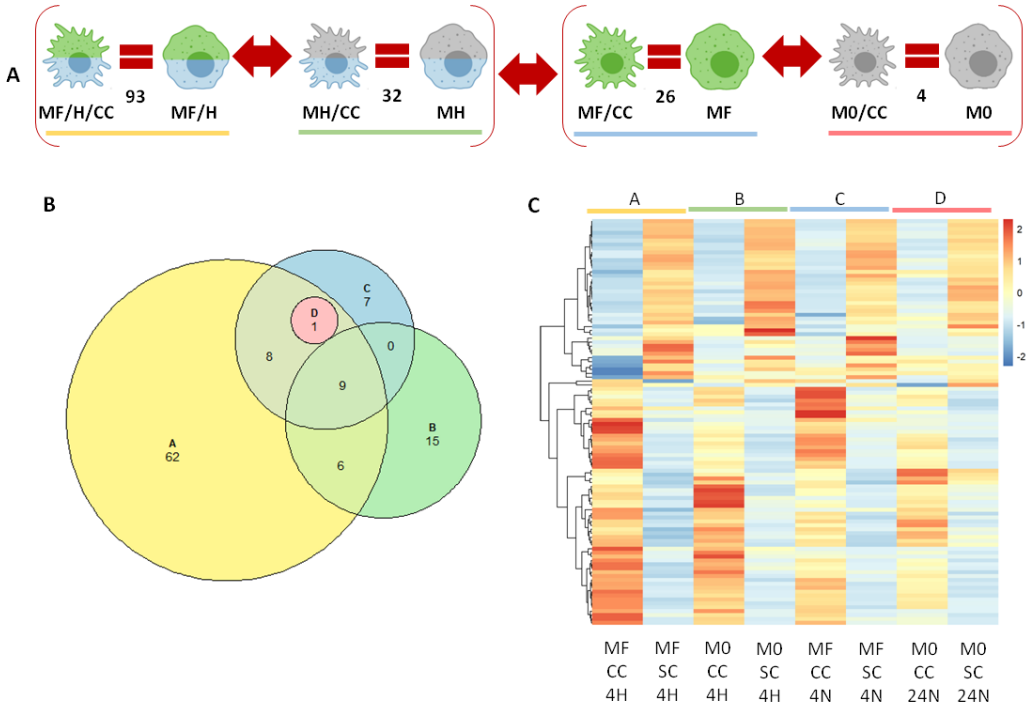


Fig. 193 Comparison [(MF/CCvsMF)Fvs(M0/CCvsM0)0]H vs [(MF/CCvsMF)Fvs(M0/CCvsM0)0]N. Schematic representation of two double comparisons of three variables: polarizing and oxygen status of co-cultivated Mφ vs single cultivated Mφ at time point of 4h. For each comparison is reported the number of SDEG

(FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/H), in green the comparison B (MH/CCvsMH),in blue the comparison C (MF/CCvsMF) and in pink the comparison D (M0/CCvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C174 (C76vsC12)vs(C72vsC10)]

Fibroblasts co-cultivated with macrophages and stimulated with IL-4 for 4h under hypoxia (FbF/CC/H) are not different from hypoxic pro-fibrotic Fb single cultivated (FbF/H) [comparison A]; when resting Fb are put under hypoxia in co-culture (FbH/CC) or are single-cultivated (FbH) [comparison B], they are the same (AvsB: 3vs0). The effect of co-culture is assessed also in normoxic co-cultivated FbF that are not different to their single cultivated counterpart [comparison C (FbF/CCvsFbF)]; co-cultivated resting Fb are the same of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 0vs2). Venn diagram (B) shows that there are not SDEG shared through four comparisons, and generally there are few genes in each comparison.

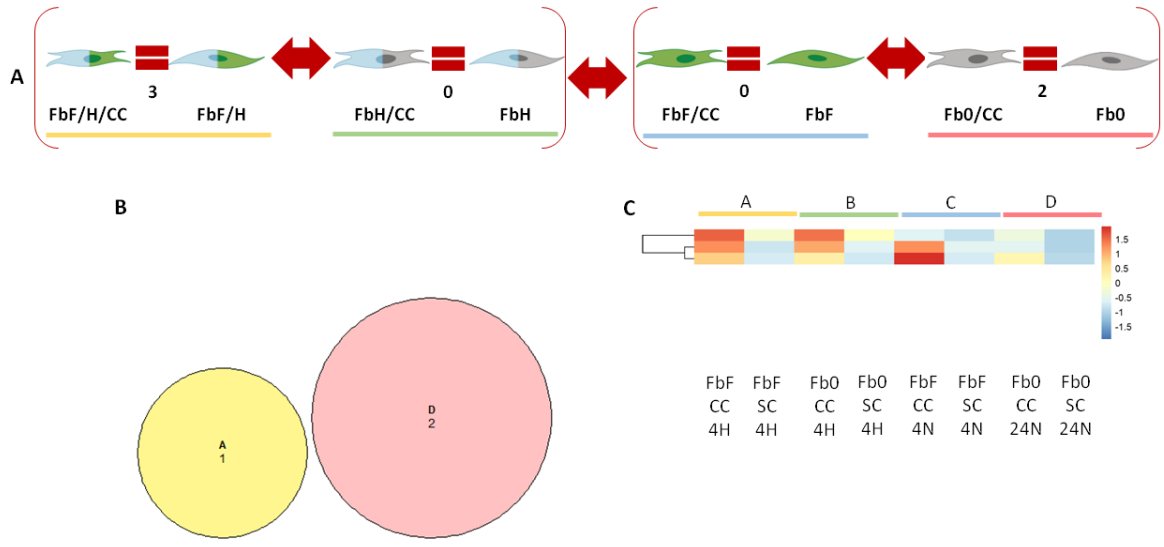


Fig. 194 Comparison [(FbF/CCvsFbF)Fvs(Fb0/CCvsFb0)0]H vs [(FbF/CCvsFbF)Fvs(Fb0/CCvsFb0)0]N. Schematic representation of two double comparisons of three variables: polarizing and oxygen status of co-cultivated Fbvs single cultivated Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/CC/HvsFbF/H), in green the comparison B (FbH/CCvsFbH),in blue the comparison C (FbF/CCvsFbF) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total

of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C175 (C77vsC13)vs(C73vsC9)]

Macrophages co-cultivated with fibroblast and stimulated with Il-4 under hypoxia for 24h (MF/H/CC) are not different from alternative Mφ, single cultivated in hypoxia (MF/H) [comparison A]; when resting Mφ are put under hypoxia in co-culture (MH/CC) or are single-cultivated (MH) [comparison B], they do differentially express an higher number of genes (AvsB: 47vs1424). The effect of co-culture is assessed also in normoxia where co-cultivated alternative Mφ are not different from their single cultivated counterpart [comparison C (MF/CCvsMF)] and co-cultivated, resting M0 are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 2vs4). Venn diagram (B) shows that the comparison with the higher number of SDEG is the A (1340 genes). Indeed, when macrophage with the same polarizing status are compared to observe the effect of different culture condition, in different oxygen status, we find that major differences come out under hypoxia only in resting cells, when they are co-cultivated. In the 8-columns heatmap reported (C) we cannot appreciate this difference since that the stronger difference is given by the pro-fibrotic phenotype and values are scaled by row.

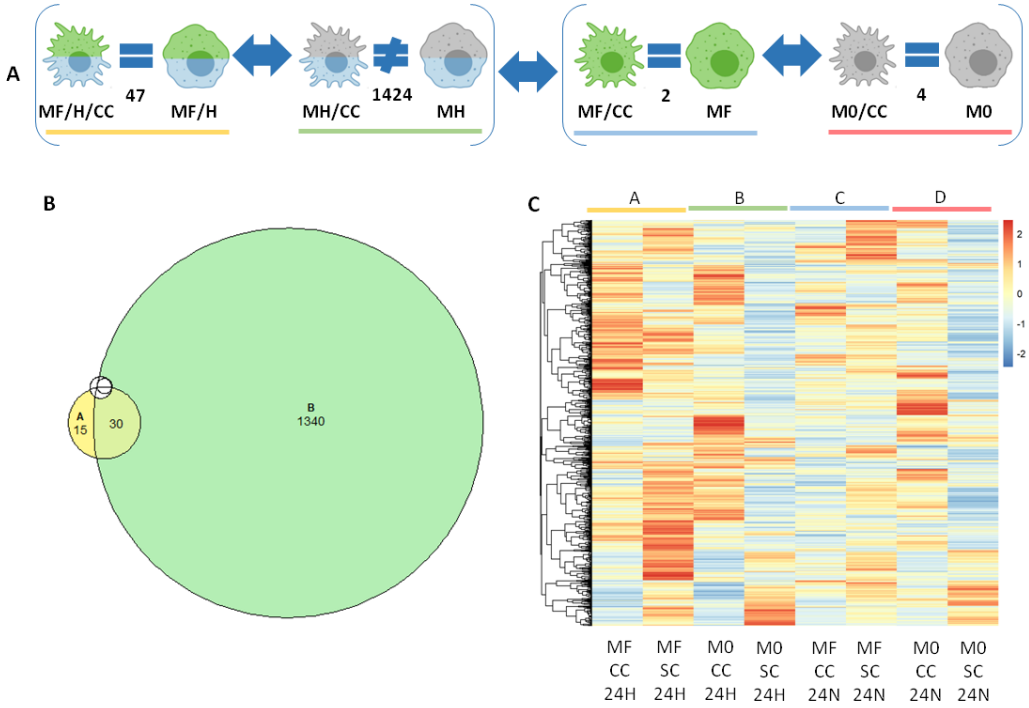


Fig. 195 Comparison [(MF/CCvsMF)Fvs(M0/CCvsM0)0]H vs [(MF/CCvsMF)Fvs(M0/CCvsM0)0]N. Schematic representation of two double comparison of three variables: polarizing and oxygen statusof co-

cultivated Mφ vs single cultivated Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/H), in green the comparison B (MH/CCvsMH),in blue the comparison C (MF/CCvsMF) and in pink the comparison D (M0/CCvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C176 (C78vsC14)vs(C74vsC10)]

Fibroblasts co-cultivated with macrophages and stimulated with Il-4 for 24h under hypoxia (FbF/CC/H) are not different from hypoxic pro-fibrotic Fb single cultivated (FbF/H) [comparison A]; when resting Fb are put under hypoxia in co-culture (FbH/CC) or are single-cultivated (FbH) [comparison B], they are different (AvsB: 23vs1291). The effect of co-culture is assessed also in normoxic, co-cultivated FbF that are similar to their single cultivated counterpart [comparison C (FbF/CCvsFbF)]; co-cultivated, resting Fb are the same of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 9vs2). Venn diagram (B) shows that there is only one comparison where there are differences (comparison B: 1197 SDEG), whereas the other three comparisons have low number of SDEG. Indeed, when fibroblasts with the same polarizing status are compared, to observe the effect of different culture condition, in different oxygen status, we find that major differences come out under hypoxia also in resting cells when they are co-cultivated. In the 8-columns heatmap reported (C) we can see that FbH/CC (comparison B) has a different pattern of expressed genes from the respective single cultivated counterpart and from the other comparisons.

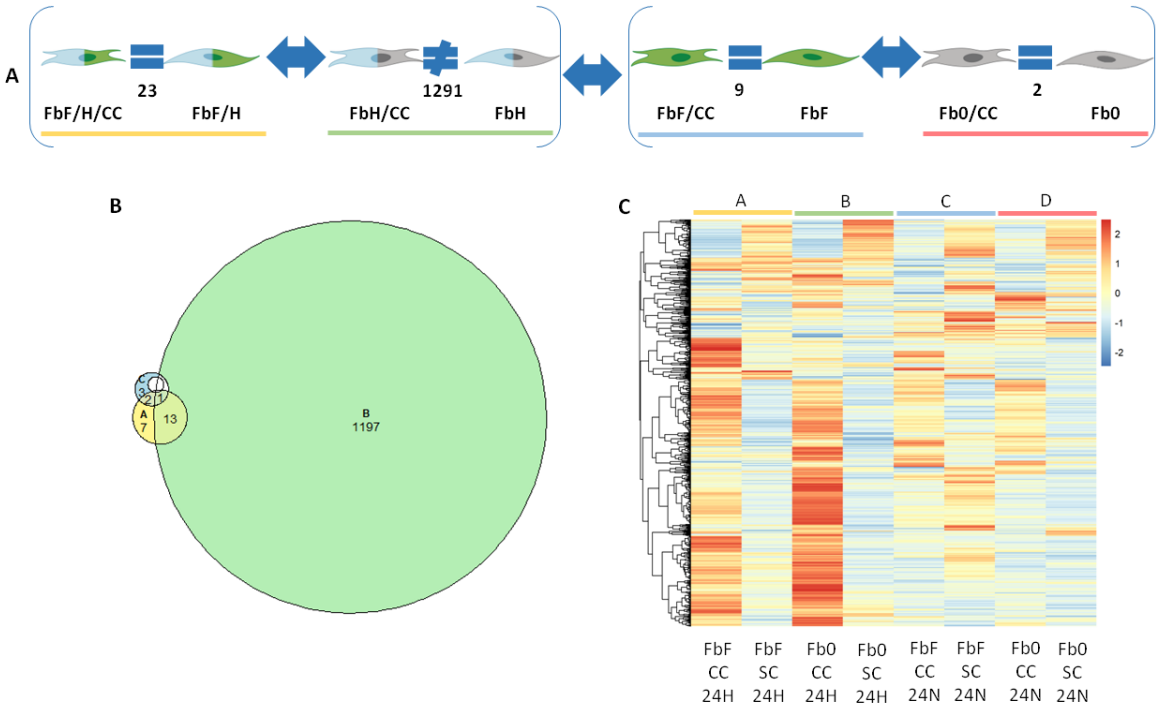


Fig. 196 Comparison [(FbF/CCvsFbF)Fvs(Fb0/CCvsFb0)0]H vs [(FbF/CCvsFbF)Fvs(Fb0/CCvsFb0)0]N. Schematic representation of two double comparison of three variables: polarizing and oxygen status of co-cultivated Fbvs single cultivated Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/CC/HvsFbF/H), in green the comparison B (FbH/CCvsFbH),in blue the comparison C (FbF/CCvsFbF) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C177 (C129vsC83)]

Macrophages co-cultivated with fibroblast and stimulated with IL-4 under hypoxia for 4h (MF/H/CC) are not different from alternative Mφ, single cultivated in hypoxia (MF/H) [comparison A]; when MF are put in normoxia in co-culture (MF/CC) or are single-cultivated (MF) [comparison B], they do not differentially express an higher number of genes (AvsB: 93vs26). The effect of co-culture is assessed also in resting Mφ under hypoxia that are similar to their single cultivated counterpart [comparison C (MH/CCvsMH)] and normoxic co-cultivated, resting M0 are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 32vs4). Venn diagram (B) shows that there are not SDEG shared through four comparisons, and generally there are few genes in each comparison. Indeed, when macrophage with the same polarizing status are compared, to observe the effect of different

culture condition, in different oxygen status, we do not found any differences in the pattern of SDEG, as we can observe in the 8-columns heatmap reported (C).

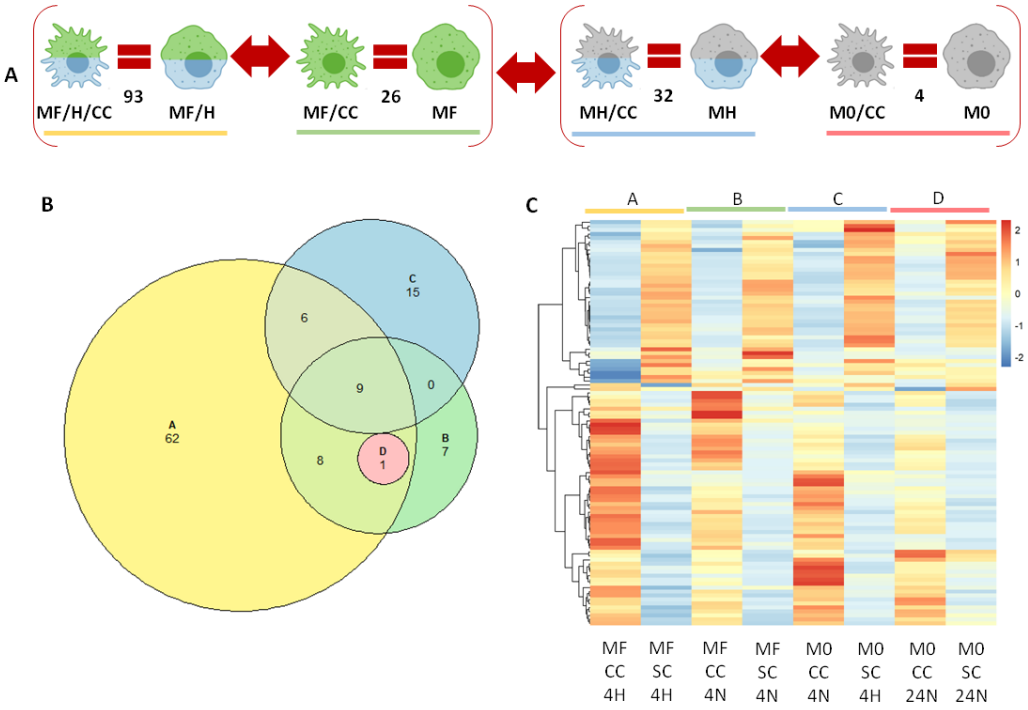


Fig. 197 Comparison [(MF/CCvsMF)Hvs(MF/CCvsMF)N]F vs [(M0/CCvsM0)Hvs(M0/CCvsM0)N]0. Schematic representation of two double comparison of three variables: polarizing and oxygen status of co-cultivated Mφ vs single cultivated Mφ at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/H), in green the comparison B (MF/CCvsMF), in blue the comparison C (MH/CCvsMH) and in pink the comparison D (M0/CCvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C178 (C130vsC84)]

Fibroblasts co-cultivated with macrophages and stimulated with IL-4 for 4h under hypoxia (FbF/CC/H) are not different from hypoxic, pro-fibrotic Fb single cultivated (FbF/H) [comparison A]; when FbF are put under normoxia in co-culture (FbF/CC) or are single-cultivated (FbF) [comparison B], they are the same (AvsB: 3vs0). The effect of co-culture is assessed also in hypoxic, co-cultivated, resting Fb that are not different to their single cultivated counterpart [comparison C (FbH/CCvsFbH)]; normoxic, co-cultivated, resting Fb are the same of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 0vs2). Venn

diagram (B) shows that there are not SDEG shared through the four comparisons, and generally there are few genes in each comparison.

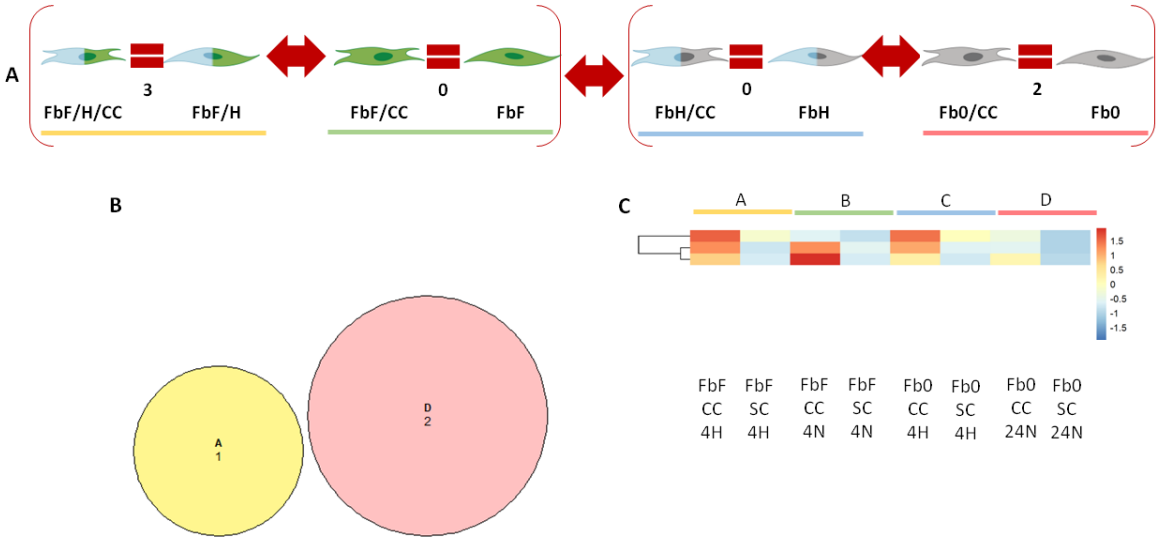


Fig. 198 Comparison [(FbF/CCvsFbF)Hvs(FbF/CCvsFbF)N]F vs [(Fb0/CCvsFb0)Hvs(Fb0/CCvsFb0)N]0. Schematic representation of two double comparison of three variables: polarizing and oxygen statusof co-cultivated Fbvs single cultivated Fb at time point of 4h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/CC/HvsFbF/H), in green the comparison B (FbF/CCvsFbF), in blue the comparison C (FbH/CCvsFbH) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C179 (C131vsC85)]

Macrophages co-cultivated with fibroblast and stimulated with IL-4 under hypoxia for 24h (MF/H/CC) are not different from alternative Mφ, single cultivated in hypoxia (MF/H) [comparison A]; when MF are put in normoxia in co-culture (MF/CC) or are single-cultivated (MF) [comparison B], they do not differentially express an higher number of genes (AvsB: 47vs2). The effect of co-culture is assessed also in resting Mφ under hypoxia that are different from their single cultivated counterpart [comparison C (MH/CCvsMH)] and normoxic, co-cultivated, resting M0, instead, are similar to single cultivated one [comparison D (M0/CCvsM0)] (CvsD: 1424vs4). Venn diagram (B) shows that there are no SDEG shared through the four comparisons, and there is only comparison C with 1340 SDEG. When resting Mφ are stimulated with hypoxia, they differ from normoxic counterpart only if they are co-

cultivated, meaning that hypoxia effect in resting cells is culture state dependent. In the 8-columns heatmap reported (C) we cannot appreciate this difference since that the stronger difference is given by the pro-fibrotic phenotype and values are scaled by row.

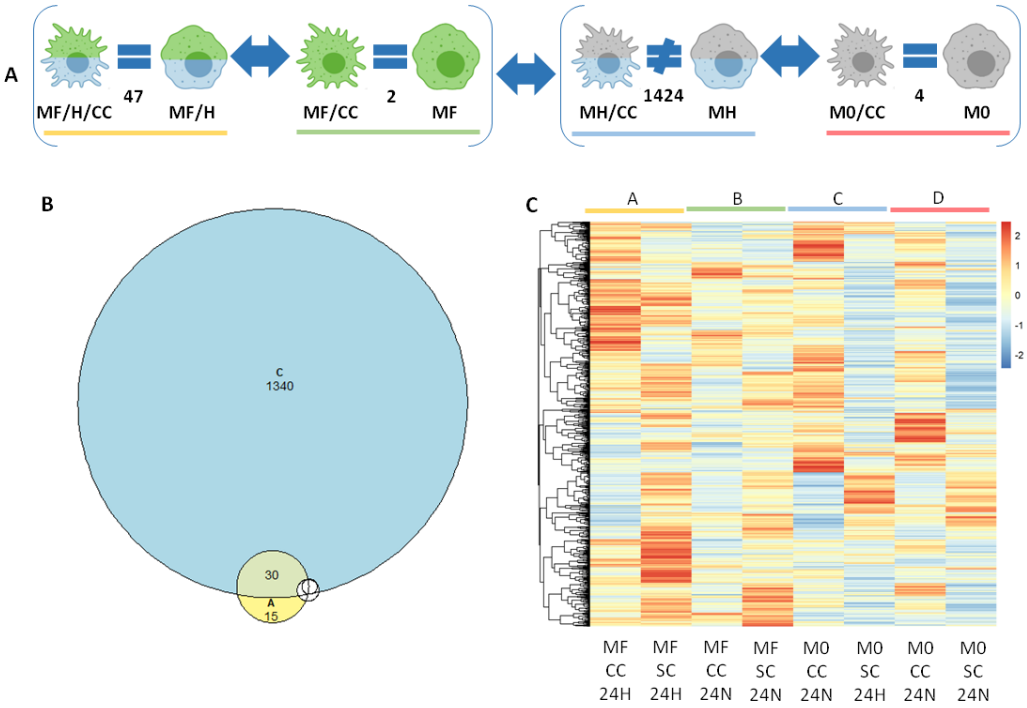


Fig. 199 Comparison [(MF/CCvsMF)Hvs(MF/CCvsMF)N]F vs [(M0/CCvsM0)Hvs(M0/CCvsM0)N]0. Schematic representation of two double comparison of three variables: polarizing and oxygen statusof co-cultivated Mφ vs single cultivated Mφ at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (MF/CC/HvsMF/H), in green the comparison B (MF/CCvsMF), in blue the comparison C (MH/CCvsMH) and in pink the comparison D (M0/CCvsM0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

[Code: C180 (C132vsC86)]

Fibroblasts co-cultivated with macrophages and stimulated with IL-4 for 24h under hypoxia (FbF/CC/H) are not different from hypoxic, pro-fibrotic Fb single cultivated (FbF/H) [comparison A]; when FbF are put under normoxia in co-culture (FbF/CC) or are single-cultivated (FbF) [comparison B], they are the same (AvsB: 23vs9). The effect of co-culture is assessed also in hypoxic, co-cultivated, resting Fb that are not different to their single cultivated counterpart [comparison C (FbH/CCvsFbH)]; normoxic, co-cultivated, resting Fb are the same of single cultivated one [comparison D (Fb0/CCvsFb0)] (CvsD: 1291vs2). Venn

diagram (B) shows that there is only one comparison where there are differences (comparison C: 1197 SDEG), whereas the other three comparisons have low number of SDEG. Indeed, when fibroblasts with the same polarizing status are compared, to observe the effect of different culture condition, in different oxygen status, we find that major differences come out under hypoxia also in resting cells, when they are co-cultivated. In the 8-columns heatmap reported (C) we can see that FbH/CC (comparison C) has a different pattern of SDEG from the respective single cultivated counterpart and from the other comparisons.

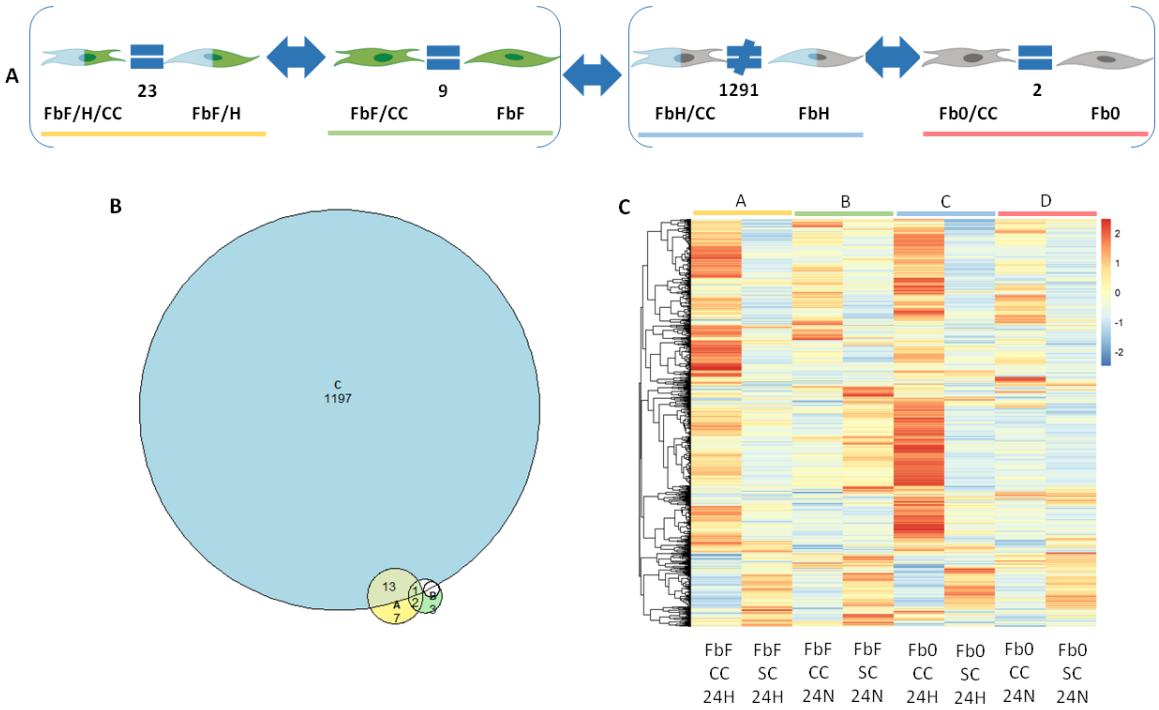


Fig. 200 Comparison [(FbF/CCvsFbF)Hvs(FbF/CCvsFbF)N]F vs [(Fb0/CCvsFb0)Hvs(Fb0/CCvsFb0)N]0. Schematic representation of two double comparisons of three variables: polarizing and oxygen statusof co-cultivated Fbvs single cultivated Fb at time point of 24h. For each comparison is reported the number of SDEG (FDR<0.05). In yellow is underlined the comparison A (FbF/CC/HvsFbF/H), in green the comparison B (FbF/CCvsFbF), in blue the comparison C (FbH/CCvsFbH) and in pink the comparison D (Fb0/CCvsFb0) (A); Venn-diagram of SDEG with a |logFC|≥1 in the four comparisons (B); the 8-colums heatmap indicates the total of genes reported in the Venn-diagram minus genes of intersection (rows); each column represents a member of the two double comparisons and is the mean value of three replicates; gene expression level is scaled by row (C).

9 DISCUSSION

The rationale of this study is connected to the Sys-MIFTA European project, which is aimed to study the interstitial fibrosis and tubular atrophy (IFTA) after kidney allograft rejection, with a multidisciplinary approach. Cellular interplay and extrinsic factors that impact on development of IFTA are object of debate of several studies [29, 47-50]. It is well recognized that progressive fibrosis is the result of myofibroblasts' ongoing activation; it is caused by cumulative damage from multiple insults, including inflammation [49]. It is also commonly accepted that T cells play a pivotal role in the initiation of acute rejection but they cannot be the only infiltrating cell population involved [47]; in fact, macrophages also are involved in acute and chronic injury. Infiltrating macrophages take part in the development of interstitial inflammation, tubular apoptosis and interstitial fibrosis through production of signalling molecules that promote tubular death and secretion of survival factors and modulators of epithelial-mesenchymal transition (EMT), which induce fibrosis progression in kidney [48]. Another key factor in pathogenesis of fibrosis [31] is hypoxia: by altering the surrounding environment, hypoxia induces important changes in fibroblasts and immune cells functions.

However, there are many aspects that need to be clarified: macrophages exert broad effects in promoting inflammation but also in myofibroblast' activation, fibrosis and tissue repair. This is due to the highly heterogeneous and plastic phenotype of macrophages. Environmental cytokines and growth factors are able to change macrophages' properties and functions, altering microenvironment and inducing different interactions with neighbouring cells, such as T cells, fibroblasts, endothelial and epithelial cells. Indeed, environmental changing, interacting cell types and timing of interactions are all factors that could impact, with a different outcome, on disease progression or resolution.

For this reason the present study is aimed to better understand the interaction between main cell types involved in the progression of fibrotic process: macrophages and fibroblasts, with the effect of different environmental factors (cytokines produced from T cells, growth factors and hypoxia) for different period of time.

In order to simplify this interplay, we used an *in vitro* system of direct contact co-culture that give us a model that loses the complexity of the tissue but, at the same time, offers a more generic description that could be applied to many contexts. Moreover, by using this model, we are able to discriminate the effect of single variable on the system and to combine up to four variables together.

Using ingenuity pathway analysis on 1st level comparisons we found that comparisons with a number of SDEG >100 are generally associated with enrichment of specific pathways. The following table underlines significant comparisons:

	CELL TYPE	POLARIZATION: resting				POLARIZATION: pro-inflammatory				POLARIZATION: pro-fibrotic			
		4h	24h	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
1st LEVEL		SC		CC		N/SC		H/SC		N/SC		H/SC	
	Mϕ	**(C1)** H vs N	**(C3)** H vs N	**(C5)** H vs N	**(C7)** H vs N	**(C15)** I vs 0	**(C17)** I vs 0	**(C19)** I vs 0	**(C21)** I vs 0	**(C47)** F vs 0	**(C49)** F vs 0	**(C51)** F vs 0	**(C53)** F vs 0
	Fb	**(C2)** H vs N	**(C4)** H vs N	**(C6)** H vs N	**(C8)** H vs N	**(C16)** I vs 0	**(C18)** I vs 0	**(C20)** I vs 0	**(C22)** I vs 0	**(C48)** F vs 0	**(C50)** F vs 0	**(C52)** F vs 0	**(C54)** F vs 0
		N		H		N/CC		H/CC		N/CC		H/CC	
	Mϕ		**(C9)** CC vs SC	**(C11)** CC vs SC	**(C13)** CC vs SC	**(C23)** I vs 0	**(C25)** I vs 0	**(C27)** I vs 0	**(C29)** I vs 0	**(C55)** F vs 0	**(C57)** F vs 0	**(C59)** F vs 0	**(C61)** F vs 0
	Fb		**(C10)** CC vs SC	**(C12)** CC vs SC	**(C14)** CC vs SC	**(C24)** I vs 0	**(C26)** I vs 0	**(C28)** I vs 0	**(C30)** I vs 0	**(C56)** F vs 0	**(C58)** F vs 0	**(C60)** F vs 0	**(C62)** F vs 0
						SC		CC		SC		CC	
	Mϕ					**(C31)** H vs N	**(C33)** H vs N	**(C35)** H vs N	**(C37)** H vs N	**(C63)** H vs N	**(C65)** H vs N	**(C67)** H vs N	**(C69)** H vs N
	Fb					**(C32)** H vs N	**(C34)** H vs N	**(C36)** H vs N	**(C38)** H vs N	**(C64)** H vs N	**(C66)** H vs N	**(C68)** H vs N	**(C70)** H vs N
						N		H		N		H	
	Mϕ					**(C39)** CC vs SC	**(C41)** CC vs SC	**(C43)** CC vs SC	**(C45)** CC vs SC	**(C71)** CC vs SC	**(C73)** CC vs SC	**(C75)** CC vs SC	**(C77)** CC vs SC
	Fb					**(C40)** CC vs SC	**(C42)** CC vs SC	**(C44)** CC vs SC	**(C46)** CC vs SC	**(C72)** CC vs SC	**(C74)** CC vs SC	**(C76)** CC vs SC	**(C78)** CC vs SC
2nd level		**(HvsN)CC vs (HvsN)SC**		**(CCvsSC)H vs (CCvsSC)N**		(Ivs0)N/CC vs (Ivs0)N/SC		(Ivs0)H/SC vs (Ivs0)N/SC		(Fvs0)N/CC vs (Fvs0)N/SC		(Fvs0)H/SC vs (Fvs0)N/SC	
	Mϕ	**(C79)** C5 vs C1	**(C81)** C7 vs C3		**(C83)** C13 vs C9	**(C85)** C23 vs C15	**(C87)** C25 vs C17	**(C89)** C19 vs C15	**(C91)** C21 vs C17	**(C109)** C55 vs C47	**(C111)** C57 vs C49	**(C113)** C51 vs C47	**(C115)** C53 vs C49
	Fb	**(C80)** C6 vs C2	**(C82)** C8 vs C4		**(C84)** C14 vs C10	**(C86)** C24 vs C16	**(C88)** C26 vs C18	**(C90)** C20 vs C16	**(C92)** C22 vs C18	**(C110)** C56 vs C48	**(C112)** C58 vs C50	**(C114)** C52 vs C48	**(C116)** C54 vs C50
						(Ivs0)H/CC vs (Ivs0)H/SC		(Ivs0)H/CC vs (Ivs0)N/CC		(Fvs0)H/CC vs (Fvs0)H/SC		(Fvs0)H/CC vs (Fvs0)N/CC	
	Mϕ					**(C93)** C27 vs C19	**(C95)** C29 vs C21	**(C97)** C27 vs C23	**(C99)** C29 vs C25	**(C117)** C59 vs C51	**(C119)** C61 vs C53	**(C121)** C59 vs C55	**(C123)** C61 vs C57
	Fb					**(C94)** C28 vs C20	**(C96)** C30 vs C22	**(C98)** C28 vs C24	**(C100)** C30 vs C26	**(C118)** C60 vs C52	**(C120)** C62 vs C54	**(C122)** C60 vs C56	**(C124)** C62 vs C58
						(HvsN)CC vs (HvsN)SC		(CCvsSC)H vs (CCvsSC)N		(HvsN)CC vs (HvsN)SC		(CCvsSC)H vs (CCvsSC)N	
	Mϕ					**(C101)** C35 vs C31	**(C103)** C37 vs C33	**(C105)** C43 vs C39	**(C107)** C45 vs C41	**(C125)** C67 vs C63	**(C127)** C69 vs C65	**(C129)** C75 vs C71	**(C131)** C77 vs C73
	Fb					**(C102)** C36 vs C32	**(C104)** C38 vs C34	**(C106)** C44 vs C40	**(C108)** C46 vs C42	**(C126)** C68 vs C64	**(C128)** C70 vs C66	**(C130)** C76 vs C72	**(C132)** C78 vs C74
3rd level						[(Ivs0)Hvs(Ivs0)N]CC vs	[(Ivs0)Hvs(Ivs0)N]SC	[(Ivs0)CCvs(Ivs0)SC]H vs	[(Ivs0)CCvs(Ivs0)SC]N	[(Fvs0)Hvs(Fvs0)N]CC vs	[(Fvs0)Hvs(Fvs0)N]SC	[(Fvs0)CCvs(Fvs0)SC]H vs	[(Fvs0)CCvs(Fvs0)SC]N
	Mϕ					**(C133)** C97vsC89	**(C135)** C99vsC91	**(C137)** C93vsC85	**(C139)** C95vsC87	**(C157)** C121vsC113	**(C159)** C123vsC115	**(C161)** C117vsC109	**(C163)** C119vsC111
	Fb					**(C134)** C98vsC90	**(C136)** C100vsC92	**(C138)** C94vsC86	**(C140)** C96vsC88	**(C158)** C122vsC114	**(C160)** C124vsC116	**(C162)** C118vsC110	**(C164)** C120vsC112
						[(HvsN)Ivs(HvsN)0]CC vs	[(HvsN)Ivs(HvsN)0]SC	(HvsN)CCvs(HvsN)SC]I vs	[(HvsN)CCvs(HvsN)SC](	[(HvsN)Fvs(HvsN)0]CC vs	[(HvsN)Fvs(HvsN)0]SC	[(HvsN)CCvs(HvsN)SC]I vs	[(HvsN)CCvs(HvsN)SC]0
	Mϕ					**(C141)** (C35vsC5)vs(C31vs1)	**(C143)** (C37vsC7)vs(C33vs3)	**(C145)** C101vsC79	**(C147)** C103vsC81	**(C165)** (C67vsC5)vs(C63vs1)	**(C167)** (C69vsC7)vs(C65vs3)	**(C169)** C125vsC79	**(C171)** C127vsC81
	Fb					**(C142)** (C36vsC6)vs(C32vs2)	**(C144)** (C38vsC8)vs(C34vs4)	**(C146)** C102vsC80	**(C148)** C104vsC82	**(C166)** (C68vsC6)vs(C64vs2)	**(C168)** (C70vsC8)vs(C66vs4)	**(C170)** C126vsC80	**(C172)** C128vsC82
						[(CCvsSC)Ivs(CCvsSC)0]H vs [(CCvsSC)Ivs(CCvsSC)0]N		[(CCvsSC)Hvs(CCvsSC)N]I vs [(CCvsSC)Hvs(CCvsSC)N]0		[(CCvsSC)Fvs(CCvsSC)0]H vs [(CCvsSC)Fvs(CCvsSC)0]N		[(CCvsSC)Hvs(CCvsSC)N]F vs [(CCvsSC)Hvs(CCvsSC)N]0	
	Mϕ					**(C149)** (C43vsC11)vs(C39vs9)	**(C151)** (C45vsC13)vs(C41vs9)	**(C153)** C105vsC83	**(C155)** C107vsC83	**(C173)** (C75vsC11)vs(C71vs9)	**(C175)** (C77vsC13)vs(C73vs9)	**(C177)** C129vsC83	**(C179)** C131vsC83
	Fb					**(C150)** (C44vsC12)vs(C40vs10)	**(C152)** (C46vsC14)vs(C42vs10)	**(C154)** C106vsC84	**(C156)** C108vsC84	**(C174)** (C76vsC12)vs(C72vs10)	**(C176)** (C78vsC14)vs(C74vs10)	**(C178)** C130vsC84	**(C180)** C132vsC84

Table 5 Summarizing table of total 180 comparisons with underlined significant comparisons. Table reports underlined in blue significant comparisons (>100 SDEG) at 4h and in yellow at 24h.

In particular, we observed that pro-inflammatory stimuli (LPS and IFNγ) are able to induce a pro-inflammatory phenotype both in macrophages and in fibroblasts [45, 76, 77] by the enrichment of inflammation-related pathways (such as NFkB signalling, IL-6 signaling, IL-8 signaling, PI3K/AKT signalling, Interferon signalling etc) [C15-C18].

Pro-fibrotic stimulus IL-4, instead, is able to induce an alternative phenotype [78, 79] in normoxic macrophages (by activation of metabolic-related pathways such as super pathway of inositol phosphate compounds) but not in fibroblasts that do not acquire a different phenotype [C47, C49].

Hypoxia alone is not able to move high number of genes both in resting and differently activated macrophages. Since that hypoxia induces transcriptional activation of HIF in a shorter period of time (1-2h), we can speculate that the majority of *hif* target genes are already up-regulated before the 4h and we cannot appreciate this increase. However, hypoxia strictly related genes that we tested previously (GLUT-1, VEGFA, CXCR4, BNIP3) are up-regulated, indicating a response to the stimulus that is not able to induce a different phenotype *per se*. In resting fibroblasts, hypoxia induces some changes at 4h only, with the activation HMGB1, IL-8, PI3K/AKT signalling pathways, but at 24h this activation is exhausted [C2,C6].

Then, if we consider the co-culture effect on normoxic, resting macrophages and fibroblasts, we do not observe a significant difference.

Moving to the analysis of two variables together, we observed that hypoxia and co-culture of resting cells for a longer period of time induce a different phenotype. Hypoxic, co-cultivated macrophages show an enrichment of activation in pathways related to ECM remodelling (GP6 signaling, Integrin signalling), cell movement (Actin cytoskeleton signalling) and proliferation (Wnt/Ca+ pathway and PCP pathway); hypoxic, co-cultivated fibroblasts, instead, show an enrichment of activation in inflammation related pathways (TREM1 signaling, IL-6 and IL-8 signaling , NFkB signalling, JAK/Stat signalling), cell growth (EGF signalling, FGF signalling, PDGF signalling), angiogenesis and leukocyte recruitment (VEGF signalling, Leukocyte extravasation signalling, CXCR4 signaling). Indeed, co-cultivation and hypoxia together are able to induce a different phenotype in resting macrophages and fibroblasts, which acquire respectively a proliferative and a pro-inflammatory phenotype [C7, C8, C13, C14].

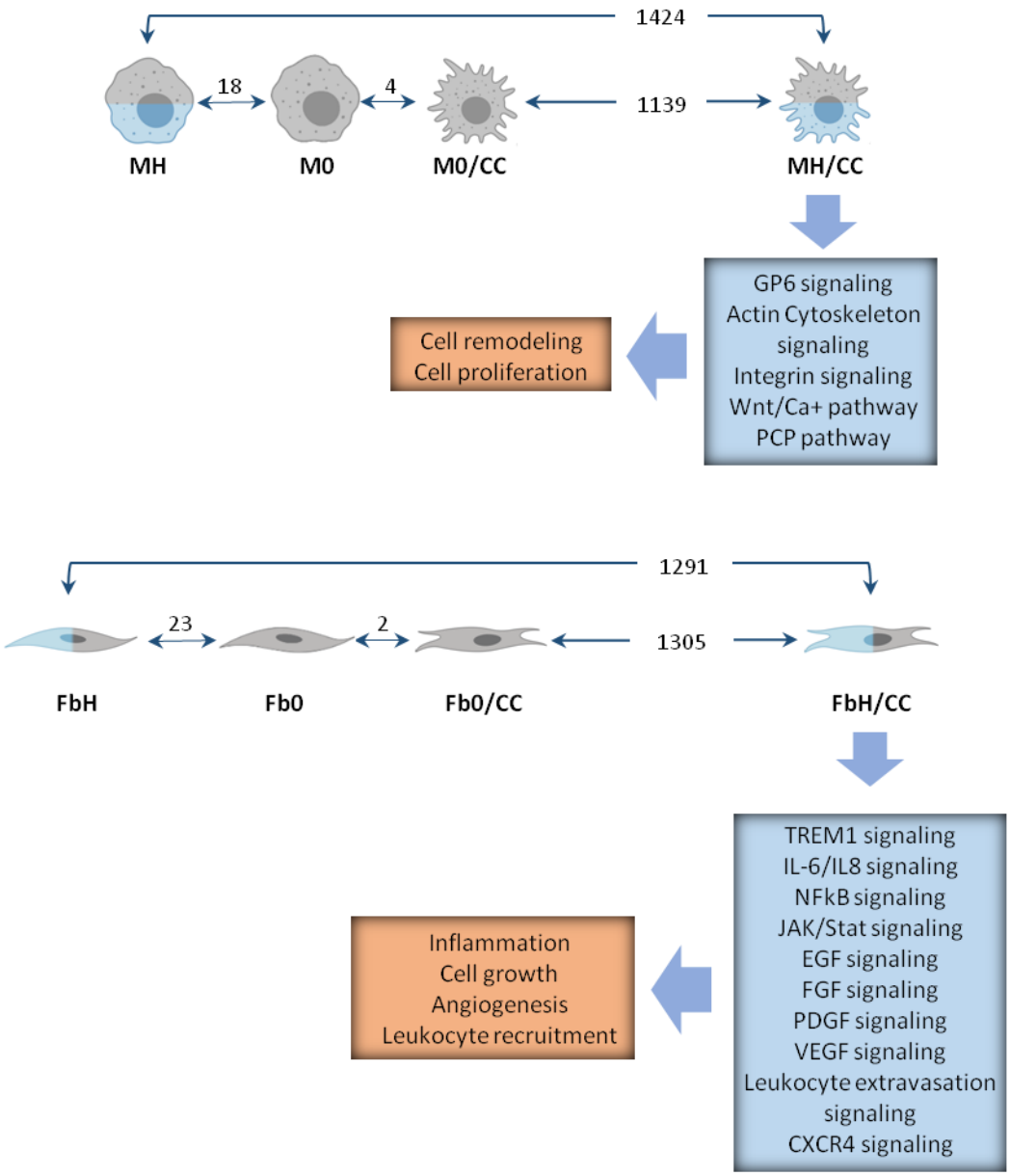


Fig. 201 Circuit of resting macrophages and fibroblasts at 24h shows activated pathways in hypoxic co-culture. Schematic representation of macrophages (on top) and fibroblasts (on bottom) at 24h of normoxia, hypoxia, single or co-culture. Numbers indicate SDEG between compared conditions. Hypoxic, co-cultivated condition shows the higher number of SDEG in comparison to hypoxic, single cultivated condition and to normoxic, co-cultivated condition. In blue box are reported activated pathways and in the orange box related enriched functions.

In pro-inflammatory context, hypoxic macrophages and fibroblasts show an enrichment of inflammation-related pathways (such as NFkB signalling, IL-6 signaling, IL-8 signaling, PI3K/AKT signalling, Interferon signalling etc) as we observed in normoxic cells [C19-C22] but they do not acquire a different signature. Hence, hypoxia and pro-inflammatory stimuli are not sufficient to induce differences in phenotype. On the other hand, if we take into

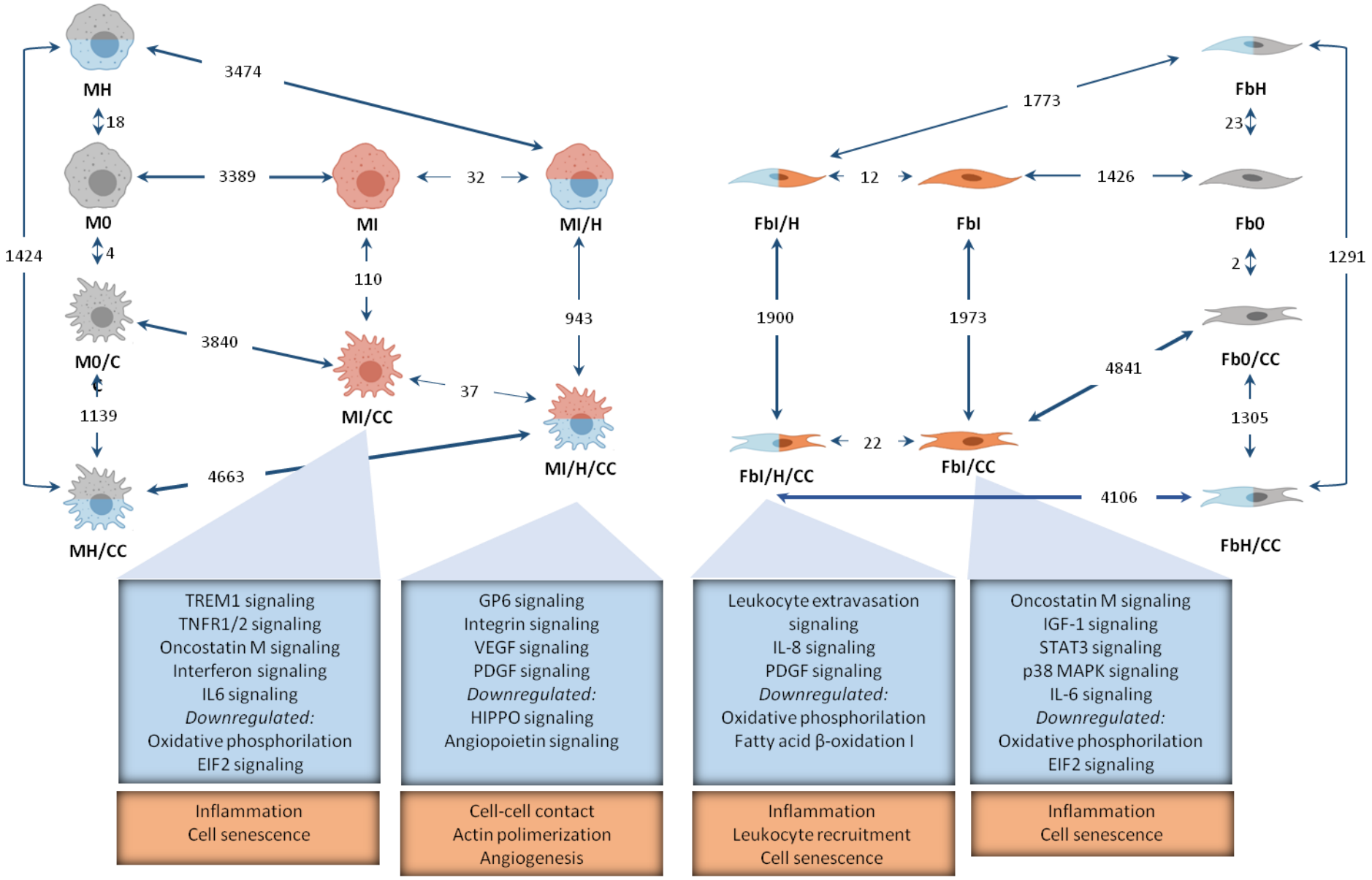
account co-cultivated cells in pro-inflammatory environment, we always find an enrichment in the pro-inflammatory genes pattern [C23-C26]; when cells are co-cultivated for a long period of time, we demonstrate that macrophages have an higher enrichment of inflammation related pathways (TREM1signaling, FAT10signaling, TNFR1/2signaling, Oncostatin Msignaling, Interferonsignaling, IL-6 signaling pathways) but also inactivation of EIF2 signaling and oxidative phosphorylation inducing cell senescence [C41]. Similarly, pro-inflammatory, co-cultivated fibroblasts show an enrichment of inflammation related pathways (IL-8 signaling, Inflammasome pathway), of leukocyte recruitment related pathways and the inactivation of oxidative phosphorylation and fatty acid β-oxidation I [C42].

These observations demonstrate that co-culture and pro-inflammatory stimuli are sufficient to promote the acquisition of different properties on pro-inflammatory macrophages and fibroblasts.

Moving to the analysis of three variables, if we take into account pro-inflammatory stimuli, hypoxia and co-culture, we observed again the acquisition of pro-inflammatory signature [C27-C30] but we find also that pro-inflammatory, hypoxic, co-cultivated macrophages enrich pathways related to cell-cell interaction (GP6 signaling, GDNF signalling, Integrin signalling), angiogenesis (VEGF, PDGF signalling) [C45]; pro-inflammatory, hypoxic, co-cultivated fibroblasts show an enrichment of activation in inflammation related pathways (Oncostatin M signaling, IGF-1 signaling, IL-6 signaling , STAT3 signalling, p38 MAPK signalling) but also the inactivation of EIF2 signaling and oxidative phosphorylation inducing cell senescence [C46].

If we look at hypoxic contribution, we do not found any differences, indicating that through co-cultivated cells, oxygen status is not a discriminating factor that induces different phenotype.

Fig.202 (below) Circuit of pro-inflammatory macrophages and fibroblasts at 24h shows activated pathways in hypoxic co-culture. Schematic representation of pro-inflammatory macrophages (on left) and fibroblasts (on right) at 24h of normoxia, hypoxia, single or co-culture. Numbers indicate SDEG between compared conditions. The weight of arrows correlates with the number of SDEG. Pro-inflammatory cells have high number of SDEG in comparison to respective resting condition. Pro-inflammatory, co-cultivated and pro-inflammatory, hypoxic, co-cultivated conditions show the higher number of SDEG in comparison to normoxic and hypoxic, single cultivated condition and to normoxic, co-cultivated condition. Are reported activated pathways (blue box) and enriched functions (orange box).


MH
M0
MI
MI/H
M0/C
MI/CC
MI/H/CC
MH/CC
3474
18
3389
32
4
110
943
3840
37
1139
4663
1424
FbH
FbI/H
FbI
Fb0
Fb0/CC
FbI/H/CC
FbI/CC
FbH/CC
1773
23
12
1426
2
1900
1973
4841
1305
22
4106
1291
TREM1 signaling
TNFR1/2 signaling
Oncostatin M signaling
Interferon signaling
IL6 signaling
Downregulated:
Oxidative phosphorilation
EIF2 signaling
GP6 signaling
Integrin signaling
VEGF signaling
PDGF signaling
Downregulated:
HIPPO signaling
Angiopoietin signaling
Leukocyte extravasation signaling
IL-8 signaling
PDGF signaling
Downregulated:
Oxidative phosphorilation
Fatty acid β-oxidation I
Oncostatin M signaling
IGF-1 signaling
STAT3 signaling
p38 MAPK signaling
IL-6 signaling
Downregulated:
Oxidative phosphorilation
EIF2 signaling
Inflammation
Cell senescence
Cell-cell contact
Actin polimerization
Angiogenesis
Inflammation
Leukocyte recruitment
Cell senescence
Inflammation
Cell senescence

Looking at IL-4 stimulation in combination with hypoxia we do not observe any differences in phenotype of macrophages and fibroblasts; however, it is important to notice that pro-fibrotic macrophages are not different from resting macrophages under hypoxia, indicating that it mediates an inhibitory effect on pro-fibrotic phenotype.

Pro-fibrotic, co-cultivated macrophages maintain their pro-fibrotic signature, while fibroblasts do not acquire a different phenotype [C55-C58], indicating that co-culture and IL-4 are not sufficient to induce changes in respective phenotype.

Considering pro-fibrotic stimulus, hypoxia and co-culture variables together, we do not find any differences, indicating that these three factors together are not able to induce a different phenotype.

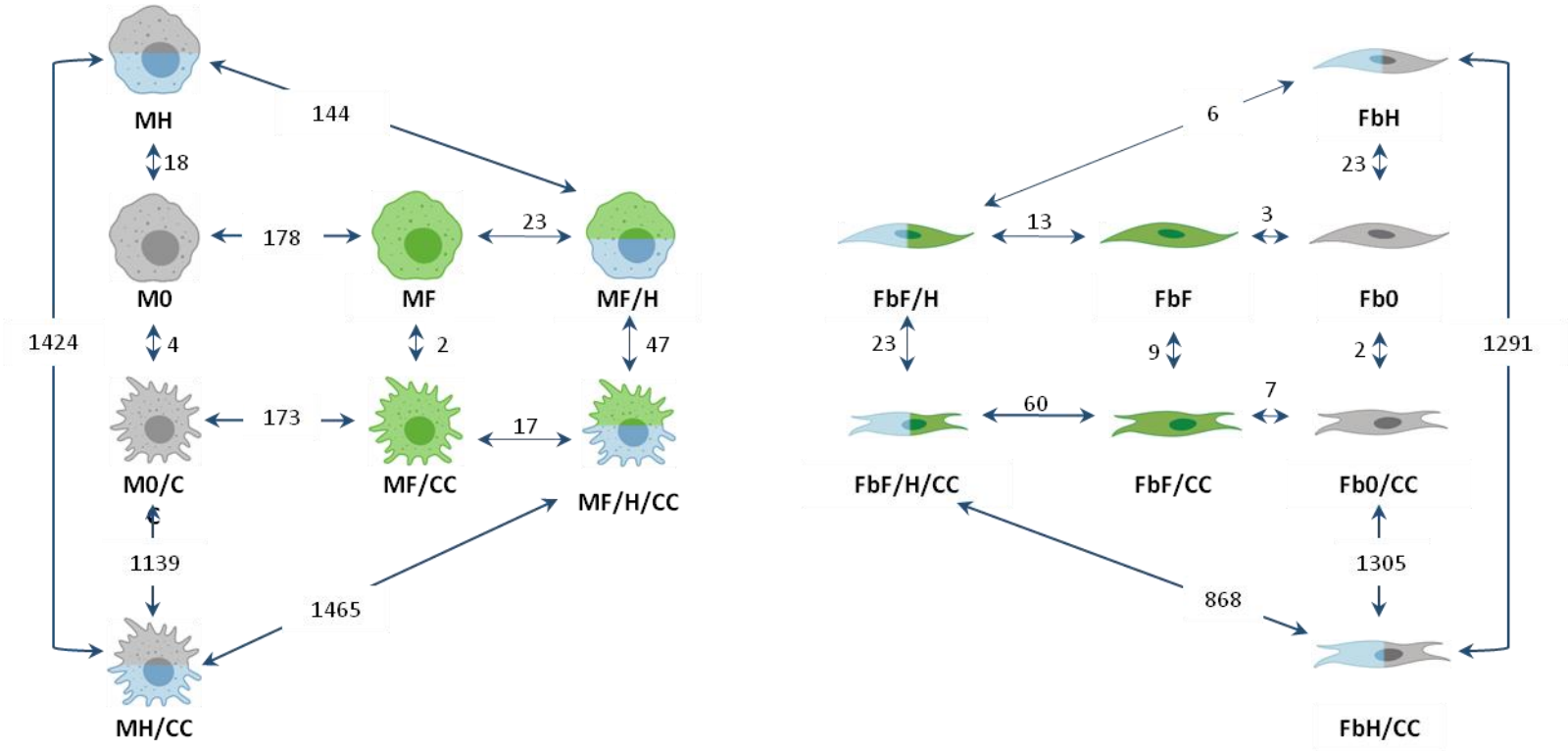


Fig. 203 Circuit of IL-4 stimulated macrophages and fibroblasts at 24h do not show activated pathways in hypoxic co-culture. Schematic representation of alternative macrophages (on left) and fibroblasts (on right) at 24h of normoxia, hypoxia, single or co-culture. Numbers indicate the SDEG between compared conditions. The weight of arrows correlates with the number of SDEG. Alternative macrophages have SDEG in comparison to respective resting condition. Fibroblasts stimulated with IL-4 do not acquire a different phenotype.

Indeed, with our *in vitro* model we have confirmed the activation of macrophages into pro-inflammatory or pro-fibrotic polarizing phenotypes by using pro-inflammatory factor and cytokine (LPS and IFNγ) that mimic Th1 response and macrophage engagement and IL-4 to reproduce Th2 activation of macrophages with pro-fibrotic properties. At the same time, we observed that fibroblasts can assume a pro-inflammatory phenotype if subjected to the same stimuli but they do not acquire a myofibroblast phenotype if treated with IL-4.

Moreover, when macrophages are close to fibroblasts in normoxic microenvironment, they seems to not be influenced one to each other; if macrophages and fibroblasts are not in contact but are in hypoxic niches, they do not acquire a different phenotype. On the contrary, when macrophages and fibroblasts are in direct contact in hypoxic environment they show an evident interplay: macrophages activate machinery to change their morphology by actin cytoskeleton remodelling and acquire a proliferative phenotype through activation of non-canonical Wnt pathway PCP (planar cell polarity); they also show transcripts for cytokines such as IL-6 and IL-8 that contribute to the acquisition of pro-inflammatory phenotype of interacting, hypoxic fibroblasts. Moreover, these fibroblasts show also the activation of pathways involved in cell growth, angiogenesis, and leukocytes recruitment and produce also factors that can activate Wnt pathway on direct contact macrophages, supporting the thesis of a direct crosstalk between the two cell types. It is important to notice that this interplay is shown only when hypoxia is present, since that in normoxic condition the two cell types seems to not talk to each other.

Instead, if macrophages and fibroblasts are in contact in inflamed tissue, they both acquire pro-inflammatory properties, suggesting that in this context pro-inflammatory factors are sufficient to promote phenotypic changes without the contribution of metabolic switch induced by hypoxia. In this condition, both cells types downregulate oxidative phosphorylation and EIF2 signaling pathway indicating the activation of senescence process that we can suppose necessary to the exhaustion of inflammation.

In hypoxic regions of inflamed tissue, macrophages that were in contact with fibroblasts assume a phenotype really similar to what we found in normoxia with, in addition, the activation of pathways related to cell-cell interaction and ECM remodelling; similarly, fibroblasts that were in contact with macrophages are similar to the normoxic counterpart with the activation of pathways related to leukocyte recruitment. Hence, we can conclude that hypoxia and direct contact of cells have a synergistic role in pro-inflammatory condition.

Finally, pro-fibrotic stimulation by IL-4 promotes an alternative phenotype in macrophages that does not change under hypoxia in direct contact with fibroblasts; fibroblasts conditioned

with IL-4 do not respond in hypoxia, neither when are in contact with macrophages. However, IL-4 conditioned fibroblasts are not resting fibroblasts, in fact resting fibroblasts change their phenotype in hypoxic environment when are in contact with macrophages. Indeed, we suggest that IL-4 *per se* has no impact on fibroblasts, but through conditioning of macrophages it is able to induce an inhibitory mechanism in fibroblasts that does not let them to acquire a pro-inflammatory phenotype. Therefore, IL-4 in our model is not able to promote fibrosis but in a co-culture system, composed by macrophages and fibroblasts, is able to block macrophages in an alternative phenotype and to inhibit fibroblasts switch in a pro-inflammatory phenotype. Indeed, we can suppose that IL-4 stimulus is able to induce differences directly on macrophages and is able to indirectly inhibit fibroblasts' change by the help of hypoxia but it is not sufficient to induce the acquisition of pro-fibrotic properties.
All these observations leading us to consider 24h of co-culture and hypoxia as factors that together induce the major changes in both cell types and the addition of Th1 or Th2 stimuli improve these changes, even if in different ways.

The 4th level of complexity of this study is represented by time; in fact, the present model can be also useful to reproduce in a simplified time-scale representation a generic inflammatory process. In case of injury, damaged tissue needs to be repaired and different cell types could be involved: macrophages and fibroblasts, together with other cells, play an important role in many steps and they could be differentially influenced by surrounding environment acquiring different phenotype. At physiological condition, macrophages and fibroblasts in connective tissue interact in a similar way of what we see *in vitro* in the direct-contact culture of resting cells, but, when tissue is injured, damaged cells recruit inflammatory cells from blood circulation, with an increase of pro-inflammatory factors released in environment. Monocytes from blood and resident macrophages differentiate into pro-inflammatory activated macrophages; at the same time, fibroblasts could acquire a pro-inflammatory phenotype. Interaction between these two cells types contribute to the maintenance of inflammatory environment that can be sustained also by Th1 cytokines, secreted by T lymphocytes (represented in our model by co-culture stimulated with LPS+IFNγ). If the injury is severe or prolonged other events could take place, such as the instauration of hypoxic niches that could aggravate tissue condition: in fact, under hypoxia, macrophages and fibroblasts interplay sustain inflammation in a double way: 1) not activated macrophages proliferate and not activated fibroblasts acquire a pro-inflammatory phenotype, 2) pro-inflammatory macrophages and fibroblasts promote leukocyte recruitment and angiogenesis. Reduction of

normal vasculature and increased leakage increase blood content and decrease blood flow, alimenting hypoxia. At this point there are two possible outcomes: healing or fibrosis, in both cases inflammatory process is stopped and occurs a switch of secreted cytokines and factors. Specifically, in our case, we mimic what happen in an IL-4 conditioned environment, where we have alternative macrophages (that could come from blood monocytes or from macrophage switch) that interact with tissue fibroblasts (*in vitro* co-culture stimulated with IL-4). The alternative macrophages-fibroblasts crosstalk shows different results in comparison to the interplay that occurs in a homeostatic condition: in fact, it seems that IL-4 exerts its effect on macrophages but not directly on fibroblasts, which are inhibited by alternative macrophages and are not still able to switch in pro-inflammatory phenotype. Indeed, in the pro-resolutive phase we have alternative macrophages and a particular type of fibroblasts that are not resting since they are blocked in the activation of pro-inflammatory pathways but neither typical myofibroblasts since that they are not able to produce growth factors and extracellular matrix proteins.

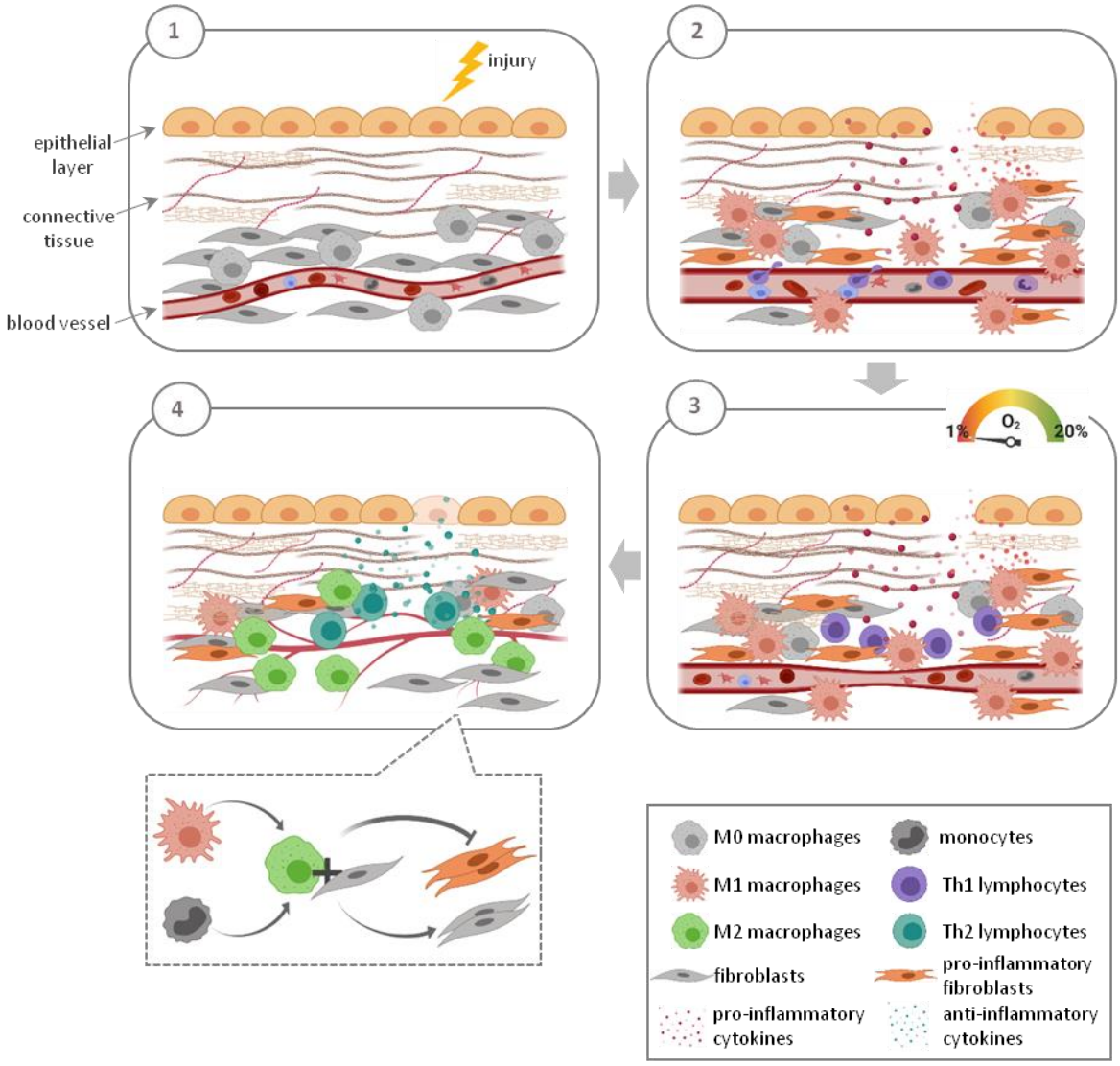


Fig. 204 Macrophage/fibroblast co-culture resembling inflammatory process in time-scale model. Schematic representation of inflammation after injury: normal tissue, before damage, shows not activated fibroblasts and resident macrophages in connective tissue; a normal vessel is reported (1). After injury pro-inflammatory factors are produced by damaged cells, leukocyte are recruited from blood vessels and monocytes initiate to differentiate into pro-inflammatory macrophages (MI), at the same time fibroblasts switch into pro-inflammatory phenotype (2). Prolonged damage induces the instauration of hypoxic niches that aliment inflammation with an increase of pro-inflammatory cells recruitment, Th1 involvement, vasculature leakage and angiogenesis (3). When damage is exhausted healing process takes place with anti-inflammatory cytokines secretion from Th2 lymphocytes that induce alternative phenotype in macrophages from blood monocytes or switch from pro-inflammatory macrophages. Interaction of alternative macrophages with fibroblasts blocks fibroblasts switch into pro-inflammatory phenotype and promote the maintenance of resting phenotype (4).

Indeed we have to say that in this study, we have mimicked the fibrotic process in a slightly way and we need an implementation by using different pro-fibrotic factors (such as FGF,

TGFβ or IL-10); moreover, we can also generate a more complex crosstalk adding another cell type, such as endothelial cell, in order to ameliorate the system that could get closer to the reality.

However, the approach of generic system with the analysis of different variables effect and a transcriptional profile of each condition could be applicable to different contexts and give the possibility of many implementations and changes.

Deeper characterization of activated pathways that we found will be done in order to identify and clarify which events and factors play a key role in the interaction between macrophages and fibroblasts in determinate conditions. Functional assays to verify proliferation activity, cellular senescence, cell-cell interaction and ECM remodelling will be the next step to validate transcriptional data that we have obtained.

10 CONCLUSION

This study presents an *in vitro* model that centred many different environmental variables that are commonly found in *in vivo* inflammatory processes. The complex experimental design let us also to compare these different variables and discerning the contribution of each of them in a basal system constituted by macrophages and fibroblasts in direct contact. The major advantage is given by the possibility to analyse single simple component interaction that differs by one variable (1st level) and simultaneously to observe the more complex picture considering two or three variables together (2nd and 3rd levels).

Starting from this complex scenario we identify a specific condition in which we obtain a different cell phenotype (both for macrophages and fibroblasts): 24h of combined hypoxia and co-culture are able together to induce significant cellular changes and the addition of pro-inflammatory or pro-fibrotic stimuli contribute, in different ways, to the acquisition of different properties.

Another important advantage is the possibility to have the complete transcriptional profile of all 44 conditions that we have, and to find target genes that could become interesting candidate marker to better understand inflammatory but also healing and fibrotic processes. Since that is a very general model could be applied in many different context implementing or changing variables.

11 BIBLIOGRAPHY

1. Teo, J.J.Y., R. Weiss, and R. Sarpeshkar, *An Artificial Tissue Homeostasis Circuit Designed via Analog Circuit Techniques.* IEEE Trans Biomed Circuits Syst, 2019. **13**(3): p. 540-553.
2. Zhou, X., et al., *Circuit Design Features of a Stable Two-Cell System.* Cell, 2018. **172**(4): p. 744-757.e17.
3. Di Gregorio, A., S. Bowling, and T.A. Rodriguez, *Cell Competition and Its Role in the Regulation of Cell Fitness from Development to Cancer.* Dev Cell, 2016. **38**(6): p. 621-34.
4. Nathan, C. and A. Ding, *Nonresolving inflammation.* Cell, 2010. **140**(6): p. 871-82.
5. Laurent, P., et al., *Immune-Mediated Repair: A Matter of Plasticity.* Front Immunol, 2017. **8**: p. 454.
6. Wynn, T.A. and K.M. Vannella, *Macrophages in Tissue Repair, Regeneration, and Fibrosis.* Immunity, 2016. **44**(3): p. 450-462.
7. Wynn, T.A., *Cellular and molecular mechanisms of fibrosis.* J Pathol, 2008. **214**(2): p. 199-210.
8. Wynn, T.A. and L. Barron, *Macrophages: master regulators of inflammation and fibrosis.* Semin Liver Dis, 2010. **30**(3): p. 245-57.
9. Cao, Q., D.C. Harris, and Y. Wang, *Macrophages in kidney injury, inflammation, and fibrosis.* Physiology (Bethesda), 2015. **30**(3): p. 183-94.
10. Koh, T.J. and L.A. DiPietro, *Inflammation and wound healing: the role of the macrophage.* Expert Rev Mol Med, 2011. **13**: p. e23.
11. Crowther, M., et al., *Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors.* J Leukoc Biol, 2001. **70**(4): p. 478-90.
12. Li, M.O., et al., *Transforming growth factor-beta regulation of immune responses.* Annu Rev Immunol, 2006. **24**: p. 99-146.
13. Parsons, C.J., M. Takashima, and R.A. Rippe, *Molecular mechanisms of hepatic fibrogenesis.* J Gastroenterol Hepatol, 2007. **22 Suppl 1**: p. S79-84.
14. Forbes, S.J. and N. Rosenthal, *Preparing the ground for tissue regeneration: from mechanism to therapy.* Nat Med, 2014. **20**(8): p. 857-69.
15. Wynn, T.A., et al., *An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection.* Nature, 1995. **376**(6541): p. 594-6.
16. Chiaramonte, M.G., et al., *An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response.* J Clin Invest, 1999. **104**(6): p. 777-85.
17. Cheever, A.W., et al., *Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis.* J Immunol, 1994. **153**(2): p. 753-9.
18. Pesce, J., et al., *The IL-21 receptor augments Th2 effector function and alternative macrophage activation.* J Clin Invest, 2006. **116**(7): p. 2044-55.
19. Reiman, R.M., et al., *Interleukin-5 (IL-5) augments the progression of liver fibrosis by regulating IL-13 activity.* Infect Immun, 2006. **74**(3): p. 1471-9.
20. Sato, M., et al., *Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction.* J Clin Invest, 2003. **112**(10): p. 1486-94.
21. Letterio, J.J. and A.B. Roberts, *Regulation of immune responses by TGF-beta.* Annu Rev Immunol, 1998. **16**: p. 137-61.
22. Ueno, M., et al., *Hypoxia-inducible factor-1α mediates TGF-β-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis.* Am J Physiol Lung Cell Mol Physiol, 2011. **300**(5): p. L740-52.

23. Wangoo, A., et al., *Interleukin-10- and corticosteroid-induced reduction in type I procollagen in a human ex vivo scar culture.* Int J Exp Pathol, 1997. **78**(1): p. 33-41.
24. Wang, S.C., et al., *Expression of interleukin-10 by in vitro and in vivo activated hepatic stellate cells.* J Biol Chem, 1998. **273**(1): p. 302-8.
25. Wynn, T.A. and T.R. Ramalingam, *Mechanisms of fibrosis: therapeutic translation for fibrotic disease.* Nat Med, 2012. **18**(7): p. 1028-40.
26. Meran, S. and R. Steadman, *Fibroblasts and myofibroblasts in renal fibrosis.* Int J Exp Pathol, 2011. **92**(3): p. 158-67.
27. Grande, M.T. and J.M. López-Novoa, *Fibroblast activation and myofibroblast generation in obstructive nephropathy.* Nat Rev Nephrol, 2009. **5**(6): p. 319-28.
28. Eddy, A.A., *Overview of the cellular and molecular basis of kidney fibrosis.* Kidney Int Suppl (2011), 2014. **4**(1): p. 2-8.
29. Kushida, N., et al., *Hypoxia-Inducible Factor-1α Activates the Transforming Growth Factor-β/SMAD3 Pathway in Kidney Tubular Epithelial Cells.* Am J Nephrol, 2016. **44**(4): p. 276-285.
30. Orphanides, C., L.G. Fine, and J.T. Norman, *Hypoxia stimulates proximal tubular cell matrix production via a TGF-beta1-independent mechanism.* Kidney Int, 1997. **52**(3): p. 637-47.
31. Norman, J.T., I.M. Clark, and P.L. Garcia, *Hypoxia promotes fibrogenesis in human renal fibroblasts.* Kidney Int, 2000. **58**(6): p. 2351-66.
32. Nangaku, M. and K.U. Eckardt, *Hypoxia and the HIF system in kidney disease.* J Mol Med (Berl), 2007. **85**(12): p. 1325-30.
33. Tanaka, T. and M. Nangaku, *Angiogenesis and hypoxia in the kidney.* Nat Rev Nephrol, 2013. **9**(4): p. 211-22.
34. Boniakowski, A.E., et al., *Macrophage-Mediated Inflammation in Normal and Diabetic Wound Healing.* J Immunol, 2017. **199**(1): p. 17-24.
35. Novak, M.L. and T.J. Koh, *Phenotypic transitions of macrophages orchestrate tissue repair.* Am J Pathol, 2013. **183**(5): p. 1352-1363.
36. Jansen, J.E., et al., *Combining Mathematical Models With Experimentation to Drive Novel Mechanistic Insights Into Macrophage Function.* Front Immunol, 2019. **10**: p. 1283.
37. Lawrence, T. and G. Natoli, *Transcriptional regulation of macrophage polarization: enabling diversity with identity.* Nat Rev Immunol, 2011. **11**(11): p. 750-61.
38. Patel, U., et al., *Macrophage polarization in response to epigenetic modifiers during infection and inflammation.* Drug Discov Today, 2017. **22**(1): p. 186-193.
39. Hu, X. and L.B. Ivashkiv, *Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases.* Immunity, 2009. **31**(4): p. 539-50.
40. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.
41. Martinez, F.O., et al., *Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences.* Blood, 2013. **121**(9): p. e57-69.
42. Scotton, C.J., et al., *Transcriptional profiling reveals complex regulation of the monocyte IL-1 beta system by IL-13.* J Immunol, 2005. **174**(2): p. 834-45.
43. Ehrchen, J., et al., *Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes.* Blood, 2007. **109**(3): p. 1265-74.
44. Park-Min, K.H., T.T. Antoniv, and L.B. Ivashkiv, *Regulation of macrophage phenotype by long-term exposure to IL-10.* Immunobiology, 2005. **210**(2-4): p. 77-86.
45. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* J Clin Invest, 2012. **122**(3): p. 787-95.
46. Guiteras, R., et al., *Macrophage Overexpressing NGAL Ameliorated Kidney Fibrosis in the UUO Mice Model.* Cell Physiol Biochem, 2017. **42**(5): p. 1945-1960.
47. Bergler, T., et al., *Infiltration of Macrophages Correlates with Severity of Allograft Rejection and Outcome in Human Kidney Transplantation.* PLoS One, 2016. **11**(6): p. e0156900.

48. Shen, B., et al., *Macrophages regulate renal fibrosis through modulating TGFβ superfamily signaling.* Inflammation, 2014. **37**(6): p. 2076-84.
49. Toki, D., et al., *The role of macrophages in the development of human renal allograft fibrosis in the first year after transplantation.* Am J Transplant, 2014. **14**(9): p. 2126-36.
50. Meng, X.M., et al., *Macrophage Phenotype in Kidney Injury and Repair.* Kidney Dis (Basel), 2015. **1**(2): p. 138-46.
51. Öhlund, D., E. Elyada, and D. Tuveson, *Fibroblast heterogeneity in the cancer wound.* J Exp Med, 2014. **211**(8): p. 1503-23.
52. Hamson, E.J., et al., *Understanding fibroblast activation protein (FAP): substrates, activities, expression and targeting for cancer therapy.* Proteomics Clin Appl, 2014. **8**(5-6): p. 454-63.
53. Reilkoff, R.A., R. Bucala, and E.L. Herzog, *Fibrocytes: emerging effector cells in chronic inflammation.* Nat Rev Immunol, 2011. **11**(6): p. 427-35.
54. Nguyen, J.K., et al., *The IL-4/IL-13 axis in skin fibrosis and scarring: mechanistic concepts and therapeutic targets.* Arch Dermatol Res, 2019.
55. Borthwick, L.A., T.A. Wynn, and A.J. Fisher, *Cytokine mediated tissue fibrosis.* Biochim Biophys Acta, 2013. **1832**(7): p. 1049-60.
56. Lee, C.G., et al., *Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1).* J Exp Med, 2001. **194**(6): p. 809-21.
57. Wilson, M.S. and T.A. Wynn, *Pulmonary fibrosis: pathogenesis, etiology and regulation.* Mucosal Immunol, 2009. **2**(2): p. 103-21.
58. Prasse, A., et al., *A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18.* Am J Respir Crit Care Med, 2006. **173**(7): p. 781-92.
59. Alpers, C.E., et al., *Human renal cortical interstitial cells with some features of smooth muscle cells participate in tubulointerstitial and crescentic glomerular injury.* J Am Soc Nephrol, 1994. **5**(2): p. 201-9.
60. Clayton, A., R. Steadman, and J.D. Williams, *Cells isolated from the human cortical interstitium resemble myofibroblasts and bind neutrophils in an ICAM-1--dependent manner.* J Am Soc Nephrol, 1997. **8**(4): p. 604-15.
61. Maxwell, P.H., et al., *The interstitial response to renal injury: fibroblast-like cells show phenotypic changes and have reduced potential for erythropoietin gene expression.* Kidney Int, 1997. **52**(3): p. 715-24.
62. LeBleu, V.S., et al., *Origin and function of myofibroblasts in kidney fibrosis.* Nat Med, 2013. **19**(8): p. 1047-53.
63. Zeisberg, M., et al., *Bone morphogenic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models.* Am J Physiol Renal Physiol, 2003. **285**(6): p. F1060-7.
64. Semenza, G.L., *Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology.* Trends Mol Med, 2001. **7**(8): p. 345-50.
65. Distler, J.H., et al., *Hypoxia-induced increase in the production of extracellular matrix proteins in systemic sclerosis.* Arthritis Rheum, 2007. **56**(12): p. 4203-15.
66. Van den Bossche, J., J. Baardman, and M.P. de Winther, *Metabolic Characterization of Polarized M1 and M2 Bone Marrow-derived Macrophages Using Real-time Extracellular Flux Analysis.* J Vis Exp, 2015(105).
67. Riddle, S.R., et al., *Hypoxia induces hexokinase II gene expression in human lung cell line A549.* Am J Physiol Lung Cell Mol Physiol, 2000. **278**(2): p. L407-16.
68. Obach, M., et al., *6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia.* J Biol Chem, 2004. **279**(51): p. 53562-70.
69. Behrooz, A. and F. Ismail-Beigi, *Dual control of glut1 glucose transporter gene expression by hypoxia and by inhibition of oxidative phosphorylation.* J Biol Chem, 1997. **272**(9): p. 5555-62.
70. van Uden, P., N.S. Kenneth, and S. Rocha, *Regulation of hypoxia-inducible factor-1alpha by NF-kappaB.* Biochem J, 2008. **412**(3): p. 477-84.

71. Schioppa, T., et al., *Regulation of the chemokine receptor CXCR4 by hypoxia.* J Exp Med, 2003. **198**(9): p. 1391-402.
72. Burke, B., et al., *Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy.* Am J Pathol, 2003. **163**(4): p. 1233-43.
73. Raggi, F., et al., *Regulation of Human Macrophage M1-M2 Polarization Balance by Hypoxia and the Triggering Receptor Expressed on Myeloid Cells-1.* Front Immunol, 2017. **8**: p. 1097.
74. Bosco, M.C., et al., *Hypoxia modifies the transcriptome of primary human monocytes: modulation of novel immune-related genes and identification of CC-chemokine ligand 20 as a new hypoxia-inducible gene.* J Immunol, 2006. **177**(3): p. 1941-55.
75. Deguchi, J.O., et al., *Chronic hypoxia activates the Akt and beta-catenin pathways in human macrophages.* Arterioscler Thromb Vasc Biol, 2009. **29**(10): p. 1664-70.
76. Martinez, F.O., et al., *Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression.* J Immunol, 2006. **177**(10): p. 7303-11.
77. Murray, P.J., et al., *Macrophage activation and polarization: nomenclature and experimental guidelines.* Immunity, 2014. **41**(1): p. 14-20.
78. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions.* Immunity, 2010. **32**(5): p. 593-604.
79. Mantovani, A., et al., *Macrophage plasticity and polarization in tissue repair and remodelling.* J Pathol, 2013. **229**(2): p. 176-85.