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Treating pain to modulate Frailty: a bench to bedside mechanism based model

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Index

Riassunto

Il dolore è ad oggi uno dei principali problemi invalidanti che grava sulla salute pubblica, con un'elevata prevalenza nella popolazione sia nella sua forma acuta che cronica. Il dolore acuto, sviluppato solitamente in seguito a un danno tissutale, ha funzione "sentinella" di difesa che, però, quando non opportunamente trattato, può evolvere in dolore cronico, perdendo la sua funzione di difesa e trasformandosi esso stesso in malattia. Si parla di dolore cronico quando diventa persistente, per più di 3 mesi, spesso accompagnato dall'insorgenza di altri sintomi quali fatica, sonno disturbato, alterazioni cognitive, con un forte impatto sull'attività lavorativa e sulla qualità di vita. Il dolore è un fenomeno cosciente caratterizzato da un'importante componente nocicettiva, ovvero il risultato di un complesso network neurologico che coinvolge pathways eccitatori ed inibitori responsabili della trasmissione ed elaborazione della percezione dolorosa, ed una componente emozionale, che rappresenta lo stato psichico da cui dipende, almeno in parte, l'elevata soggettività percettiva inter-individuale. Numerosi studi, infatti, hanno dimostrato come precedenti traumi, fisici o psicologici, la paura o l'ansia possano esacerbare la percezione dolorosa. Ciò sembra essere particolarmente vero nei casi di dolore cronico in cui pazienti che ne soffrono per lunghi periodi sviluppano ansia e depressione, le quali incidono maggiormente sulla percezione cosciente. D'altra parte, è emerso come siano proprio questi disordini cognitivi e alterazioni psicologiche a determinare un aumento del rischio di cronicizzazione del dolore, probabilmente interferendo con i trattamenti terapeutici. Ad oggi il trattamento del dolore è soprattutto incentrato sulla somministrazione di FANS ed oppioidi, con un'elevata variabilità inter-individuale in termini di efficacia, spesso con scarsi benefici sul dolore e insorgenza di effetti collaterali importanti. In questi casi risulta necessario ricorrere a strategie invasive come ad esempio l'impianto di un neuro-stimolatore midollare. Recentemente diversi

studi hanno suggerito l'esistenza di una possibile correlazione tra dolore e fragilità, una condizione multifattoriale tipica dell'anziano, definita come uno stato clinico di aumentata vulnerabilità associato a declino delle riserve funzionali e all'incapacità di ripristino dell'omeostasi a seguito di eventi stressanti. Dolore cronico e fragilità sembrano condividere diversi meccanismi, quali infiammazione e neuroinfiammazione, attivazione del sistema immunitario, età dei soggetti coinvolti; questi fattori potrebbero suggerire un'influenza reciproca.

Di fatti sembra che il dolore cronico sia un fattore stressante predisponente alla fragilità nei soggetti anziani e d'altra parte la fragilità potrebbe interferire con i meccanismi regolatori del dolore favorendone la cronicizzazione. Inoltre, la fragilità espone il soggetto anziano ad un maggiore rischio di incorrere in numerose patologie, disabilità, ospedalizzazione e nei casi più gravi anche aumentata mortalità. Pertanto, risulta fondamentale una diagnosi precoce ed un trattamento tempestivo per prevenirne il declino. Tuttavia, ad oggi restano molti interrogativi da risolvere circa i meccanismi alla base della fragilità e, data la sua multifattorialità, risulta anche di difficile diagnosi. Alla luce di ciò, lo scopo di questo progetto è valutare l'esistenza di una correlazione tra dolore e fragilità e, in tal caso, valutare se un trattamento di successo del dolore possa effettivamente prevenire o invertire una condizione di fragilità, agendo sul miglioramento dello stato psicologico del paziente e della qualità della vita nel suo complesso. Per far ciò sono stati elaborati due modelli in parallelo:

1. *Animale,* di competenza del gruppo UNIMI, che prevede l'uso di topi giovani (11 settimane d'età) e anziani (20 mesi d'età), in cui è stata indotta una condizione di Osteoartrite (OA) successivamente trattata cronicamente con 2,5 mg/kg di morfina. Dopo 14 giorni dall'induzione di OA e 7 giorni di trattamento, i topi sono stati sacrificati per poter effettuale le analisi biochimiche sul midollo spinale (L3-

L5) e su quattro aree cerebrali (ipotalamo, ippocampo, corteccia frontale e prefrontale)

2. *Umano* costituito da pazienti afferenti all'Unità di Terapia del Dolore dell'Ospedale di Parma che presentano dolore cronico e idoneità ad impiantare uno stimolatore neuromidollare, come ultima strategia terapeutica. I pazienti vengono seguiti fino ai 6 mesi successivi all'impianto.

Questi due modelli hanno permesso di esaminare da un lato l'influenza che le singole componenti (età, dolore, trattamento) possono esercitare sui comportamenti e sullo stato di fragilità, utilizzando il modello animale, dall'altro l'uomo conferisce una visione clinica globale legata al dolore e alla fragilità, intesa come alterazione psicologica, insorgenza di disabilità fisica e declino della qualità di vita, prima e dopo il trattamento interventistico. I test comportamenti sul modello animale sono stati condotti dal gruppo UNIMI; sono state valutate le soglie basali nocicettive sia nei topi adulti che anziani, oltre alla valutazione dell'impatto del dolore cronico sullo stato di fragilità. Non sono state evidenziate differenze basali tra adulti e anziani ma, a seguito dell'induzione di OA, sia adulti che anziani mostrano alterate soglie nocicettive a stimoli meccanici e dolore spontaneo, che ritornano ai livelli basali a seguito del trattamento cronico con 2,5 mg/kg di morfina. Parallelamente, lo stato di fragilità peggiora in presenza di dolore sia nei topi giovani che anziani, ritornando a valori simili a quelli basali a seguito del trattamento con morfina. Le analisi biochimiche, condotte invece da UNIPR, correlano con i risultati comportamentali e mostrano che la presenza di dolore indotto da OA induce una forte neuroinfiammazione, sia in adulti che anziani, con maggiore rilevanza negli anziani. Infatti, è stato riscontrato un aumento dell'espressione genica di TNF-α, TLR4, CD11, Iba-1 e ATF3 nel midollo spinale di topi anziani con dolore rispetto ai topi adulti con dolore. Il trattamento con morfina sembra contrastare la neuroinfiammazione

così come mostrato dalla riduzione dell'espressione di IL-1β, CD11 e ATF3, GFAP, Iba-1 nel midollo dei topi trattati, sia adulti che anziani, parallelamente al ripristino delle funzionalità motorie, delle soglie nocicettive osservate dai test comportamentali, e dell'indice di fragilità. A livello sovra-spinale, invece, è stato evidenziato un aumento di espressione di alcuni marcatori nei topi anziani rispetto agli adulti ma sembra che né il dolore né il trattamento abbiano effetto a questo livello. Questo potrebbe dipendere dalla necessità di uno stimolo doloro più inteso o una finestra temporale più ampia affinché vengano coinvolti questi centri cerebrali.

Nell'uomo, nonostante l'esiguità campionaria, è stato possibile valutare lo stato psicologico di partenza del paziente, l'intensità del dolore e la sua interferenza con la vita quotidiana, tramite la compilazione autonoma di specifici test. Lo stato di fragilità del paziente è stato valutato sulla base dell'interpretazione di alcuni sintomi tipici di questa condizione, mancando ad oggi un protocollo standard per la sua diagnosi. Nello specifico viene considerato lo stato psicologico, essendo l'ansia e la depressione tipici della fragilità, e la tendenza a catastrofizzare il dolore, oltre alla disabilità fisica che interferisce con le attività quotidiane quali dormire, camminare, piegarsi e lavorare. Infine è stato valutato l'impatto che questi fattori hanno sulla qualità della vita. Tali valutazioni sono state monitorate prima e dopo l'impianto di neurostimolazione al fine di valutare una possibile correlazione tra l'esito della trattamento e i sintomi di fragilità. In generale, è emerso un miglioramento della sintomatologia dolorosa dopo l'impianto la quale sembra correlare con il miglioramento delle disabilità fisiche, dei sintomi psicologici e della qualità di vita. Contestualmente alla valutazione clinica sono state eseguite analisi biochimiche su campioni di sangue periferico, da cui sono state allestite colture di PBMC. Dall'analisi di espressione genica effettuata prima e dopo l'impianto, fino a 6 mesi di follow-up, risulta una leggera riduzione dell' espressione

genica dei marker infiammatori IL-1, IL6 e TNF-α dopo l'impianto SCS, ma i risultati non sono significativi e necessitano di un ampliamento del numero campionario. Inoltre, con un maggior numero di campioni sarà possibile effettuare le analisi su campioni di surnatante di PBMCs stoccati, al fine di identificare quali citochine vengano rilasciate dalle cellule immunitarie differentemente stimolate.

In conclusione, una visione globale dei risultati sino ad ora ottenuti, sebbene molto preliminari, confermano il dolore cronico come fattore di rischio per l'insorgenza o peggioramento di uno stato di fragilità; gli alti livelli di neuroinfiammazione identificati nei topi anziani possono suggerire un amentato rischio di sviluppare patologie degenerative come Parkinson e Alzheimers; infine, un trattamento del dolore adeguato e personalizzato sembra essere la strategia da adottare e migliorare per agire su alcuni dei fattori tipici della fragilità, quali il ripristino delle funzionalità motorie, miglioramento dello stato psicologico e di qualità di vita.

Summary

Pain is one of most disabling conditions that affects many people every year in the word. It involves two fundamental aspects: its neurobiological nature and its subjective perception. First represents nociceptor component, excitatory and inhibitory pathways involved in transmission of painful signal and conscious perception processing, while the second is related to psychological state. It has been shown, in fact, that past trauma, previous negative experiences, fear or anxiety could influence the pain perception. Therefore, pain experience is extremely variable between people and can involve different neuronal mechanisms. Pain generally onsets as adaptive response to preserve injured tissue by other damages but, when it is not opportunely treated and persists over than 3 months, it evolves in chronic pain, losing its protection role and becoming itself disease. It strongly influences quality of life causing sleeping disturbance, reduction of mobility and sociality, work absence, and cognitive alteration including anxiety and depression. Nowadays, gold standard treatment is represented by pharmacological therapy, often resulting in low pain relief and an improved risk to incur in adverse reactions. When pharmacological treatment fails, it becomes necessary proceed with invasive approach like spinal cord neurostimulator implant. Recently, it has been suggested a relationship between pain and frailty state which appear share some mechanisms and could influence each other. Frailty is a clinical multifactorial condition characterized by high vulnerability to stressor factors and decreased capacity to maintain homeostasis. Pain is a relevant stressor that appears to be a risk factor to frailty insurgence in old patients and, on the other hand, frailty appears influence pain inhibitory mechanisms, allowing pain chronicization. Moreover, frailty exposes patients to increased risk to incur in acute pathologies, hospitalization, comorbidity and mortality and, therefore, it is important early diagnosis to avoid its exacerbation. However, until now standard protocol to frailty diagnosis and treatment

are missing due to its multifactorial nature and lack of totally knowledge about mechanisms involved. So in light of this, this project aims to investigate on possible correlation between pain and frailty and verify if a successful pain treatment could reverse or prevent frailty onset. To this purpose, in this project we have used two parallel models:

1. *Animal,* operated by UNIMI, which involved young (11 weeks old) mice and old (20 months old) mice, in which has been induced osteoarthritis (OA) and successively chronically treated with 2,5mg/kg of morphine. After 14 days from OA induction and 7 days of treatment, mice have been sacrificed in order to conduct biochemical analysis on spinal cord (L3-L5) and four cerebral areas (hippocampus, hypothalamus, frontal and pre-frontal cortex)
2. Human, which involved patients afferent to Pain Therapy of Hospital of Parma with chronic pain eligible for spinal cord stimulator implant (SCS). Patients have been followed until 6 months after stimulator implant.

Use of both models has allowed to better analysis each single component involved using mice model (age, pain and treatment), and simultaneously have a globally vision of clinical and psychological state of patients, before and after SCS implant. Perception of pain has been investigated in both animal and human models. Basal nociceptive thresholds have been evaluated before OA induction and no difference have been found between young and old mice in response to mechanical stimuli and spontaneous pain. After OA induction, a significant increment of both spontaneous pain and hypersensitivity to mechanical stimuli has been shown in all MIA mice, independently from age factor. Chronic morphine treatment has allowed resetting basal threshold in both young and old mice treated. About frailty status, it has been significant influenced

by pain, more in old than young mice; however, morphine treatment has been able to restore FI in both young and adult, alongside restore of nociceptive thresholds. Biochemical analysis, performed by UNIPR, correlate to behavioural results and, in fact, have shown that pain induced by OA dues a strong neuroinflammation in both young and old mice, with more impact in old. This is supported by increased expression levels of TNF-α, TLR4, CD11, Iba-1 e ATF3 in spinal cord of old mice with pain (MIA old) in confront to corresponding young mice (MIA young). Furthermore, morphine treatment appears contrast neuroinflammation as well as shown by gene expression reduction of IL-1β, CD11 e ATF3, GFAP, Iba-1 in spinal cord of both young and old mice treated (MIA young/old + morphine), alongside the restore of functional activities found by behavioural tests. At sovra-spinal levels, instead, in all four areas considered, it has been noticed an increase of some mediators in old mice in confront to young, but pain or treatment don't appear to have influence at this level. This could be explained by need to have a more intensive painful stimulus or a longer time in order to involve these central areas.

In human model, although few samples collected, has been possible evaluate psychological state of each patient, pain intensity and its interference with daily life, through compilation of specific tests. Frailty status has been evaluated in according to some symptoms interpretation, including psychological state, considering anxiety and depression are involved in frailty evolution, and pain catastrophizing, physical disabilities and impairment of quality of life. These considerations have been performed before and after SCS implant in order to evaluate existence of correlation between treatment outcome and frailty symptoms.

In general, it has emerged an improved of painful symptomatology that correlates whit improved of physical disabilities, psychological state and quality of life. In addition to

clinical evaluation, biochemical analysis have been conducted on peripheral blood of patient by which PBMCs' cultures have been collected. These have been performed before and after SCS implant, as well as at 3 and 6 months after implant ant it has resulted a light reduction of gene expression of inflammatory markers IL-1, IL6 e TNF-α, after implant in confront to baseline. However, these results are not significant due to low number of samples collected. With higher number of samples we aim to extend data until now obtained and perform analysis on PBMC's supernatants, in order to identify cytokines released by human immunity cells differently stimulated.

In conclusion, although results until now obtained are much preliminary, a global interpretation of them confirms chronic pain as risk factor for onset or decline of frailty status; high levels of neuroinflammation found in old mice could suggest an improved risk to develop several degenerative pathologies, including Parkinson and Alzheimer. Finally, personalized and successful pain treatment appears to be best strategy to use and improve in order to act on some frailty symptoms, including restore of mobility functionalities and psychological state and then the improvement of patients' quality of life.

Introduction

1.1 Pain

Pain is one of the most disability worldwide problem; it has been estimated that 20% of adults suffers pain globally and 10% are newly diagnosed with chronic pain every year.[1] It has commonly been accepted International Association for the Study of Pain (IASP) definition that identifies pain as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"*.[2] This assumption involves two fundamental aspects of pain: its neurobiological nature that represents nociceptor component, and its subjectively perception. It has been shown, in fact, that past trauma, previous negative experiences, fear or anxiety could influence the pain perception.[3] Therefore, pain experience is extremely variable between people and can involve different neuronal mechanisms. According to heterogeneity pain manifestation and different pathways involved, it is necessary understand which mechanisms underlying each type of pain in order to identify specific target treatments. Three main types of pain can be distinguished: nociceptive, inflammatory and pathological pain.[4]

Nociceptive pain is transient pain generated by activation of peripheral nociceptor receptors and it has a protection role against physical damage (Figure 1A); also inflammatory pain onsets in defense to tissue injury or infection, activating immune system (Figure 1B). Both of them represent an adaptive response to preserve injured tissue by other damages and generally are acute pain, resolved in a few days. However, when acute pain is not opportunely treated and persists for more time, it can develop in pathological chronic pain (Figure 1C).[5] It could happen that non-inflamed areas, near to initial injury site, become sensitive as a results of plasticity in peripheral nociceptors and central nociceptive pathways,[6] causing also activation of low-threshold fibers. Moreover, chronic pain occurs in presence of nerve damage, known

as neuropathic pain, or of alteration of nervous system functionality, named dysfunctional pain. Chronic pain is maladaptive pain that persists over than 3 months and no longer has protection role but it becomes itself disease; in fact it is itself maintained, after an initial damage and continues when it is resolved, becoming highly disabling.[7] Therefore, pain and in particular its chronicization and pathological evolution, represent a social and healthy economic problem. However, the cause of major of cases of pain is until now unknown; this underlines the necessity of more in-depth researches in order to better understand mechanisms underlying pain processes and identify more accurate and timely treatments.

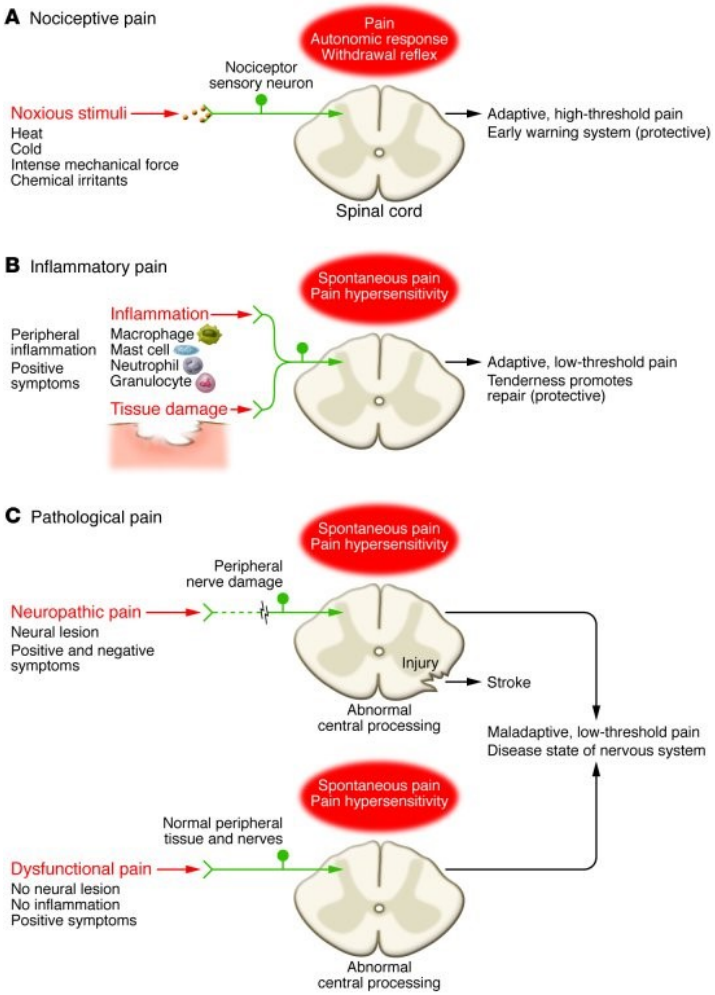

Figure 1. Pain Classification.[4]

(**A**) Nociceptive pain

(**B**) Inflammatory pain

(**C**) Pathological pain.

1.1.1 Physiopathology of Pain

Mechanism by which we can perceived a peripheral stimulus as a painful sensation is known nociception. It is activated by different stimuli, including mechanical, thermal or chemical, and includes four steps:

I. ***Transduction*** of noxious stimuli in neural electrical activity through ionic channels of nociceptors,[8] including subtype of Transient Receptor Potential Channel (TRPA, TRPM and TRPV),[9,10] Sodium isoform channel (Nav), Potassium channel (KCNK) and Acid-Sensing Ion Chanell (ASICs).[11] Nociceptors are particular peripheral nervous fiber, pseudo-unipolar, whit their cell bodies located in dorsal root ganglia (DRG) of spinal cord or trigeminal ganglia for stimuli from body or face respectively. They are primary afferent neurons and can be differentiated in two major classes according to conduction velocity, diameter, presence or not of myelination and the kind of stimulus transferred:

   Aδ: medium diameter myelinated, responsive to acute, fast (12-30 m/s) and well-localized pain. They include Type I for mechanical and heat stimuli (52-56°C) for first pain and Type II, with lower heat threshold than Type I, involved in fast responses to thermal stimulus (40-45°C).[12] There are others fasting conducting fiber (30-100 m/s), Aβ fibers, which are responsive to non painful stimuli and, therefore, they participate to pain sensation only when pathological pain occurs.

   C: small diameter unmyelinated, responsive to slow (0.5-2 m/s) non localized pain. They represent the majority of primary fibers and involve peptidergic and non-peptidergic fibers.

Both classes include many polymodal activated fibers by both high threshold mechanical and thermal stimuli.[13]

Ionic channel, opening, are responsible of stimulus transduction with influx of Na^+ and Ca^{++}, generating a potential of action.

II. ***Transmission*** of nerve impulses from periphery to the spinal cord and brain through release of neurotransmitters, glutamate as the primary in the dorsal horn, and substance P and CGRP neuropeptides, act on ionotropic receptor, as AMPA, NMDA and mGlu, of second neurons, exciting them. This synergistic action comports a post-synaptic depolarization that sustains action potentials, arriving to brain area. In particular way, glutamate binds AMPA receptors, generating a short-term depolarization and fast synaptic transmission, while neuropeptides mediate long-term and slow transmission.[8] Pain transmission is mediated by ascending pathways which take origin by secondary afferents. These cross over to the opposite side of the spinal cord giving rise to anterolateral system by which pain arrives supra-spinal areas. Collection of different cortical and supra-spinal areas contributing to conscious painful experience is known as “pain matrix”[14,3] and involves the rostral ventromedial medulla (RVM) and caudal ventrolateral medulla (CVLM), periaqueductal gray region (PAG), rostral anterior cingulate cortex (rACC), hypothalamus, amygdala, insula, and somatosensory cortex 1 and 2.[13] Anterolateral ascending pathway is shown in figure 2 and is represented by:[15]

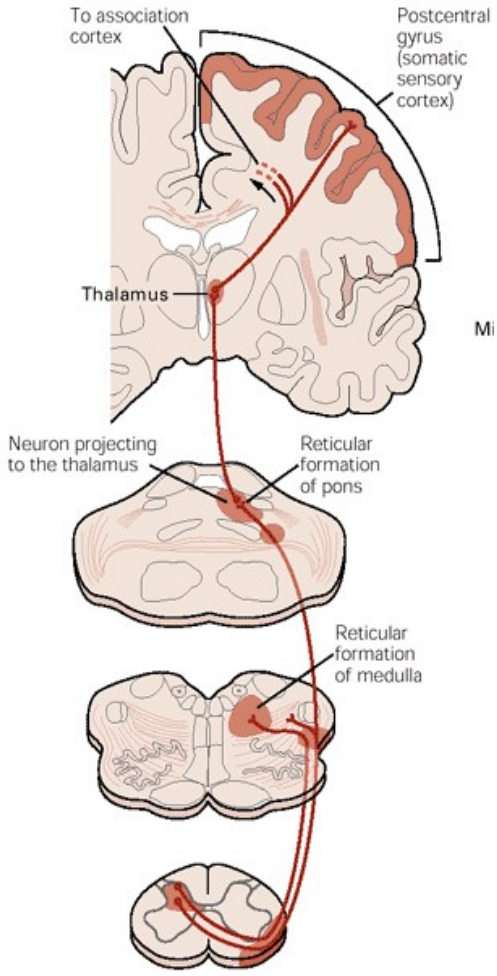

Figure 2. Anterolateral pain ascending pathway.

http://www.neurones.co.uk

Spinothalamic tract (STT): principal ascending nociceptive pathway. It has origin in dorsal horn and transmits temperature and touch signals linked to pain. Secondary neuron crosses the midline of spinal cord and ascends to VPL (Ventral Post Lateral) and VPM (Ventral Postero Medial) nuclei of the thalamus. These in turn project to primary sensory cortex.

Spinoreticular tract (STR): It has the same origin and localization of STT. It has axons that are in part directed to medulla nuclei, involved in motor control, and on the other hand to reticular formation of pons, involved in mechanism of nociception. Third order neurons of the pathways project to the thalamus.

Spinomesencephalic tract (SMT): it has origin in lamina I and V and project to mesencephalic reticular formation and PAG. Indirectly, it is also linked to amygdala and limbic system, supporting hypothesis of emotional involvement in pain perception.

III. **Modulation** of painful signals via spinal inhibitory interneurons and descending pain modulatory circuits

IV. **Perception** of pain as nerve stimuli that people recognize as conscious experience, as result of excitatory and inhibitory mechanisms control and emotional interpretation.[8]

1.1.2 Modulation Of Pain

The conscious sensorial experience of pain is the result of a complex neurological network that involves dynamic excitatory and inhibitory pathways, which can exacerbate or inhibit pain perception, respectively. Lamina V of dorsal horn of spinal cord presents GABAergic and glycinergic interneurons responsible of inhibition of pain processing between primary afferents and second neurons, according to Melzack and Wall's Gate Control Theory of Pain (1965).[16] They found that the status of this gate, open or closed, is determinate by balance between input from Aβ no-nociceptive fibers, which promote closed status activating local inhibitory interneurons, and Aδ and C fibers, which instead promote its opening. Therefore, exclusively stimuli higher than threshold, thanks to their capacity to generate a potential of action, will be transmitted to brain centers.[17]

Gate control is also regulated by descending inhibitory and facilitator pain pathways which origin from the "pain matrix" and project to dorsal horn of the spinal cord.

Particularly important is descending inhibitory pathway originated from PAG and RVM and projected, directly or indirectly, to the spinal cord in which they release serotonin, norepinephrine and endogenous opioids. Here, serotonin is able to act as both inhibitor and facilitator of pain progression, based on type of receptor activated[18,19]. There have been identified 15 subtypes of the 5-HT receptors involved in a bidirectional modulation of pain transmission, but their specific action in spinal cord is poorly clarified.[19] RVM is

the major output node in bidirectional modulation of descending nociceptive, being able to facilitate or inhibit pain, thanks to presence of two classes of neurons, “ON- cells” and “OFF-cells”.[20] PAG and RVM are closely connected with locus coeruleus, main noradrenergic site involved in inhibition of pain transmission through direct projection to pre and post-synaptic spinal cord neurons. Moreover, Gassner M and colleagues have demonstrated that an increased inhibition of pain transmission is mediated by depolarization of GABA interneurons, caused by activation of α1- adrenergic reception.[21] Endogenous opioids are released after environmental stimulus, activing descending pathway arising from PAG.[3]

1.1.3 Chronicization of Pain

Pain modulation mechanisms result impaired in many pain-related diseases, causing both abnormal pain amplification and its increased conscious perception. Tissue damage or persistent pain, associated or not to diseases (including diabetes, arthritis or tumor), cause switch from acute pain to chronic pain, resulting in nerve damage and increased neurotransmitters release. Persistent stimulation of nociceptors by noxious stimulus results in their change and occurrence of peripheral sensitization: after injury, many chemical mediators are released from primary afferent terminals and no-nervous cell, as immunity cells, fibroblasts, endothelial cells, platelets and keratinocytes. The mediators released give rise to “inflammatory soup” that includes prostaglandins E2 (PGE2), bradikinin, ATP and pro-inflammatory cytokines (TNF-α and IL-1β) that, binding G-protein coupled receptors (GPCRs), ionotropic and tyrosine kinase receptors, favor peripheral sensitization of receptors. An hypersensitivity to thermal and mechanical stimuli occurs via second messengers action, including cAMP, Proteink Kinase A and C, MAPKinasi pathway, by increasing synthesis of

neuromodulators (BDNF, SP and CGRP), ion channels and their activity (such as TRPV1, TRPA1, TTX-R, sodium and calcium channel) in primary sensory neurons. It contributes to maintenance of peripheral sensitization, evolving in a decrement of activation threshold of nociceptors, an increased response to noxious stimuli (hyperalgesia) and a nociceptive response to no painful stimuli (allodynia).[22] When allodynia is related to touch stimuli, it is named tactile allodynia and it is in turn subdivided in static and dynamic, according to type of stimulus stinging or rubbing respectively. Dynamic allodynia can be Aβ or C fibers-mediated, while static allodynia is always C fibers-mediated. This differentiation has also therapeutically strategy diversification.[22] Continuous nociceptors activation, and related neurotransmitters release, are also responsible for neurogenic inflammation onset. Vasodilation, plasma extravasation and activation of immune cells favor inflammatory soup sustainment.[23] Therefore, an extended activity of primary sensory neurons causes changes in spinal cord and brain, through alteration in central synapses and descending projections, which appear to favorite a pain state.[3] These changes induct occurrence of states known as central sensitization and wind up, in spinal dorsal horn.[24] AMPA and/or kainate receptors in a phosphorylated form allow an increased opening time with increased burst firing and consequently activation of NMDA receptors through removal of Mg^{++} block. These processes promote an increase of number of active receptors on synaptic membrane, which sustain central sensitization.[24] Consequently, it happens an increase ongoing neuron activity with decrement of excitation threshold levels.[25]

1.1.4 Neuropathic Pain

Neuropathic pain as defined as "*pain caused by lesion or disease of the*

somatosensory nervous system".[7] It is highly invalidating due to lack of an efficacy therapy; this negatively affects individual's lifestyle, his social life and psychological state. It can be caused by lesions both peripheral and central nervous system; several different etiologies have been identified:[26,27]

- Persistent pain caused by nociceptors hyperexcitability, exacerbated by sympathetic efference (SMP)
- Dismielinosis by incomplete damages of fibers with ipermyelinations or demyelination phenomenon, causing sustainment of potential of action and involvement of Aδ and C-fibers.
- Axonal neuropathy that comports increased sensibility to tactile and chemical stimuli
- Deafferentation pain, due to proximal lesions at ganglion of dorsal horn which comport denervation and consequent hypersensitivity, and riafferentation anomalies
- Central neuropathic pain that origins by abnormal activity of central neurons caused by anatomical damage or primary dysfunction by vascular, traumatic or neurodegenerative etiology
- Metabolic and viral diseases, traumatic nerve injuries, inflammation, cancer and aging

Furthermore, a relevant aspect of neuropathic pain is spontaneous pain that onsets in absence of an identifiable stimulus, because of ectopic action potential generation within the both primary sensory neurons and low-threshold large myelinated afferents. Due to peripheral and central sensitization occurrence and impairment of descending control systems, ectopic action potential is transmitted to central areas, where takes

place the conscious pain perception.[28] However, recently it has been observed an interaction between neurons, inflammatory-immune cells and glial cells that appears to be involved in neuropathic pain onset and progression.[29]

In fact, a damage at nervous afferents comports recruitment ad activation of immunity cells and glial cells in several sites:

- **in peripheral nerves** Schwann cells and macrophages sustain Wallerian degeneration distal to axonal injury (Figure 3)[30] Macrophages are first participants to inflammatory reaction after nerve damage, reinforcing by neutrophils action and macrophages recruited. Several mediators are released, included CGRP, SP, bradikinin and nitric oxide in order to increase vascular permeability and favorite immune cells permeation at site of injury. Pro-inflammatory chemokines (PGs and NGS) and cytokines (TNF-α, IL-1β and IL-6) are released. Recruitment and activation of all these cells comports the onset of neuro-inflammation cytokine-mediated state that sustains sensory abnormalities and sensitization.[31]

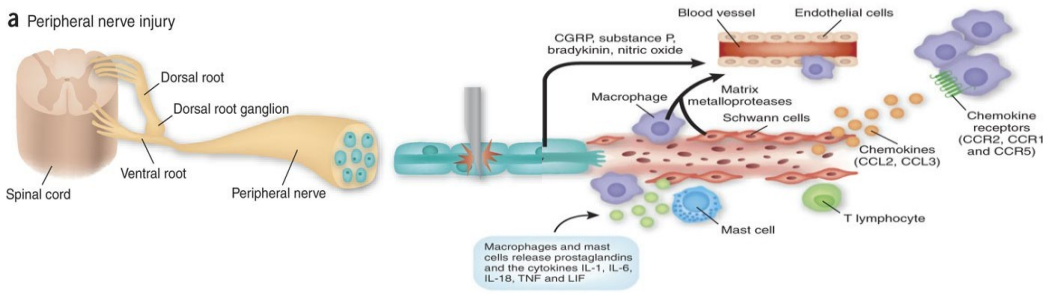

Figure 3. Inflammatory state in peripheral nerves.

Image modified from Scholz and Woolf, 2007[30]

- **In dorsal root ganglion** macrophages and lymphocytes T are triggered by chemokines released by DRG neurons and, in turn, promote inflammatory response, contributing to sensitization, releasing pro-inflammatory cytokines (TNF-α, Il-1β and IL-6) and chemokines. It occurs an increment of sodium influx and neuropeptide synthesis that results in alteration of synaptic input to spinal cord.[30]

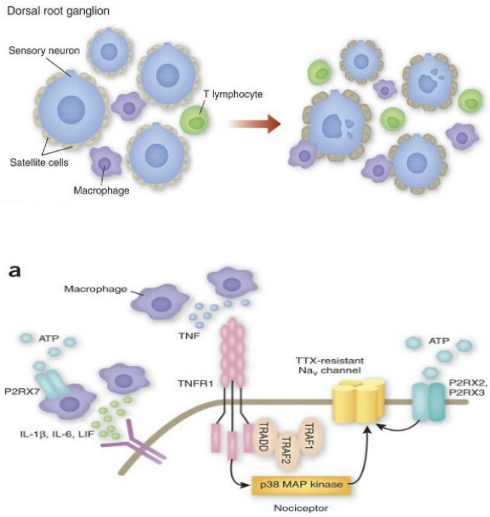

Figure 4. Immune response in DRG.

Image modified from Scholz and Woolf, 2007.[30]

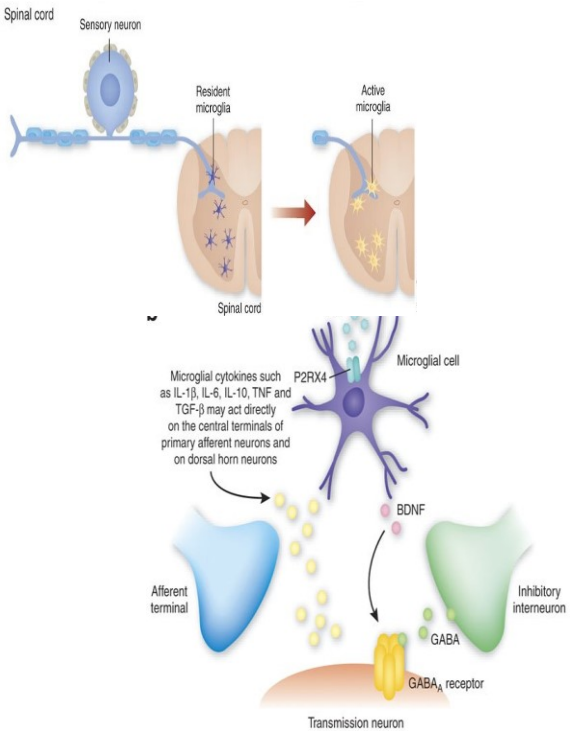

Figure 5. Microglia and astocytes activation.

Image modified from Scholz & Woolf 2007.[30]

- **In Spinal cord and supra-spinal sites** microglia activation primary occurs and successively astrocytes, with amplification of inflammatory and nociception response.[30] In fact, CGRP, SP, glutamate and ATP released by pre-synaptic terminals stimulate spinal microglia and astrocyte activation, which in turn release cytokines in the spinal cord, resulting in impairment of pain transmission, hypersensitivity occurrence and prolongation of a pain state.[32] TNF-α and IL-1β released by

astrocytes support an increment of glutamate availability that, in turn, bind AMPA and NMDA receptors, making neuronal hyperexcitation. On the other hand, brain-derived neurotrophic factor (BDNF) comports decrement of GABA and glycine release by interneurons, causing a lack of inhibition of spinal cord pain transmission and then it is favored central sensitization and chronic pain onset and maintenance.[33]

1.1.5 Multidisciplinary Pain Treatment

Pain is a multidisciplinary problem and its treatment involved a combination of biopsychosocial approach, including physiotherapy, acupuncture and psychological therapies.[34] About clinical approach, physicians usually use the World Health Organization (WHO) analgesic ladder that provides as the first step the prescription of paracetamol and the non-steroidal anti-inflammatory drugs (NSAIDs). These act inhibiting COX-1 and COX-2 inflammatory enzymes. The second and third step consist in use of weak (e.g. codeine and dihydrocodeine) and strong opioids (e.g. morphine) respectively.

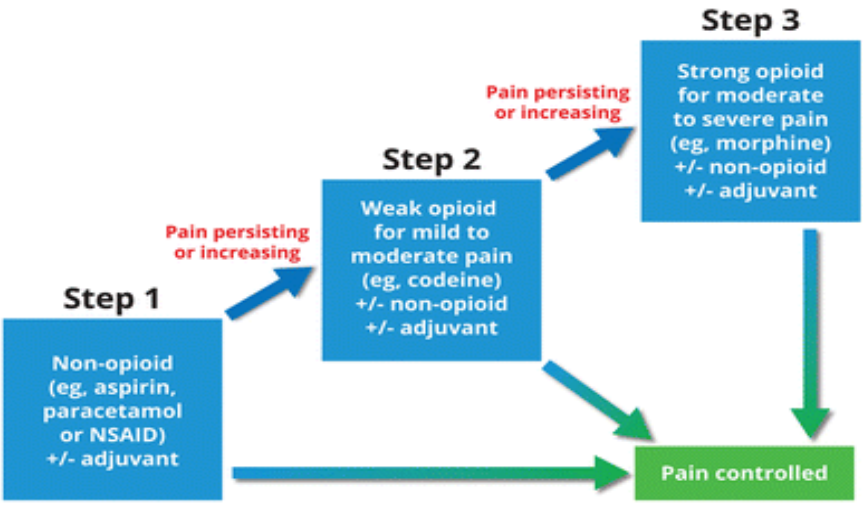

Figure 6. WHO analgesic ladder.[34]

Opioids act blocking nerve activity through G-protein-coupled opioid receptors activation. However, all of these pharmacological treatments can cause several side effects which become more serious in the third group of drugs than first, and include respiratory depression, nausea, constipation, cognitive impairment, addiction.[35] Moreover, due to cognitive impairment involvement, pain treatment could be associated with adjuvants to reduce pain-related anxiety and depression and hypnotics' administration to insomnia cure.[34] However, many patients whit pain have not relief with standard pharmacological therapies; it comports an increased risk to chronicization of pain and the necessity to appeal to invasive procedures.

1.1.5.1 Spinal Cord Stimulation

Spinal Cord stimulation (SCS) is one of the invasive procedures reserved to patients in which all other approaches have been tried without success, due to its invasively and expensively. Nowadays, 50000 neuro-stimulators are implanted every year; it appears interfere with pain nociceptive pathways but its mechanism of action is until now not completely understood. It is minimally invasive and reversible and acts on dorsal column of spinal cord, modifying pain perception. Generally, SCS system is composed from four components (Figure. 7):

- A neurostimulator implanted subcutaneously in the abdomen or in the buttock area; it is responsible of electrical pulse's generation
- One or more electrodes implanted in the epidural space
- A lead that connects the electrode(s) to the neurostimulator.
- A remote controller used to turn the neurostimulator "on" or "off" and to adjust the level of stimulation.

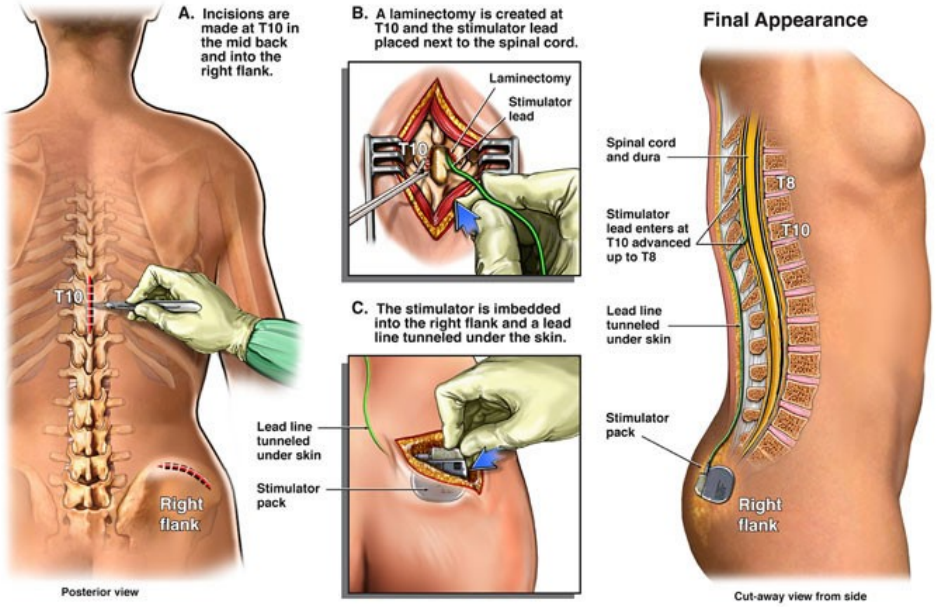

Figure. 7 Spinal cord Stimulator implant (SCS)

Main indication to SCS implant include:[36]

- Failed Back Surgery Syndrome (FBSS)
- Cervical and Lumbosacral chronic radiculopathy
- Post-Herpetic neuralgia
- Complex Regional Pain Syndrome (CRPS I and II)
- Diabetic Neuropathy
- Refractory Angina Pectoris Treatment
- Parkinson's symptomatology

People selected for SCS normally have a stimulation trial period of 2-4 weeks in order to determine suitability and degree of pain relief of this treatment, before to permanent implantation. Procedure is in local anesthesia and electrode(s) are bind with a temporary external device that patient can regulate within an impulse range established by surgeon. After period of trial if pain relief is ≥ 50%, physician will proceed

to permanent stimulator implantation.[37] Despite high initial costs, in the long run period, SCS appears more advantageous than conventional treatments.[38] However, several adverse reactions could incur, including infection of surgical wound, meningitis onset, migration of electrodes and accidental device broken. Moreover, some people have not pain relief or do not maintain it for a long time.[39] There are many factors could influence successful outcome, among which psychological state of patient appears to have a fundamental role, although it unknown which are psychopathological bases most at risk.[40] These aspects sustain the involvement of emotional component in subjective pain perception. In particular, it has been demonstrated that high pain catastrophizing values are associated with post-chirurgical chronic pain maintenance and these value appear to be useful to predict clinical status evolution and treatment outcome.[41]

1.1.6 Animal Model of Chronic Pain

About mechanisms underlying pain condition, there are several aspects which are not completely understood and better pain therapies are need. This sustains the necessity of preclinical animal models and rodents are main models used in preclinical studies.[42] They can be differentiated in following groups:[43]

- Models of Inflammatory pain and Arthritis: generally inducted with chemical irritant, including Freund's adjuvant (CFA), carrageenan and formalin. Osteoarthritis pain can be caused via monosodium iodoacetate (MIA) injection or by surgical transection of a ligament of the knee, such as anterior cruciate ligament or medial meniscus.

- Models of Neuropathic pain: nerve damage is caused by partial or full ligation, transection or compression of peripheral nerves, such as sciatic, infraorbital or trigeminal nerve. There many different models used to specific neuropathic diseases study.[44]

1.2 Frailty

Frailty is an important syndrome that involves people older than 65 age, with higher prevalence in women (9.6%) than in men (5.2%).[45] It is commonly defined as an increased vulnerability to stressors that impairs multiple, inter-related systems, leading to decrease in physiological reserves and a decline in the ability to maintain homeostasis, involving an improved risk of hospitalization, disability and mortality.[46] An unique protocol in order to objectively quantify frailty is missing due to its multidimensional nature that makes difficult standardize the procedure. Fried created valid five clinical criteria, helping in screening patients:

- muscle weakness,
- slowness,
- exhaustion,
- recent unintentional weight loss,
- sedentary lifestyle

These criteria identify the “frailty phenotype”: a subjects is identify as “frail” when at least 3 of 5 criteria are reported, while when just 1-2 of 5 criteria are identified they are “pre-frail” subjects, having an increased risk for becoming frail.[47] Fried’s criteria were completed with psychiatric symptoms introduced in the 2007 by Rockwood and

Mitnitski, identifying a “frailty index” (FI) in which are reported many aging deficit. High FI is correlated to high frailty status.[48]

1.2.1 Aetiology of Frailty

At present, it has not been found an unique cause of frailty onset; it shares common pathways with aging, including dysregulation of immune system and endocrine system, mitochondrial dysfunction, reduced physical activity and chronic inflammation state.[49] Immune system modification and inflammation are frequent in aging people, which represent main frail subjects. The aging immune system is characterized by a specific condition known as “InflammAging” that presents chronic systemic inflammatory state with elevated inflammatory molecules, such as IL6 and C-reactive protein [CRP].[50,51] An increased count of main cellular components of the innate immune system, such as white blood cell, neutrophils and monocytes have been found in frail patients supporting the hypothesis of involvement of immune system activity in frailty syndrome.[52] Modification of immune system functionality also comports a mitochondrial dysfunction with consequently increment of ROS and apoptosis impairment. It occurs a catabolic state that results in anorexia, sarcopenia and loss of adipose tissue, traits typically present of frailty patient. Finally, an alteration of circulating hormones has been identified in frailty subjects, such as insulin-like growth factor 1 (IGF-1) and dehydroepiandrosterone sulphate (DHEA-S) decrement, and cortisol increment. This contributes to physical frailty worsening.[53]

1.2.2 Mechanism Shared With Pain

Recently, it has emerged that pain and frailty may share some mechanisms[54] that

influence each other. Both are more frequent in aging and are often related to cognitive impairment such as depression. Stress can unveil a condition of frailty otherwise masked and pain could be the stressor that could precipitate or accelerate a frailty condition.[55] On the other hand, the frailty could be a risk factor for pain modulation, acting on descendent inhibitory pathway.[56] Several studies have been conducted to better understand the relationship between pain and frailty; one of the first studies on this field has been published in 2008 by Blyth and colleagues[57] and successively a casual association has been demonstrated in a cohort studies of European male old patients with chronic widespread pain (CWP). They had a worsening of frailty status after about 4 years of follow-up.[58] Association between pain and frailty has been found in other chronic pain diseases as osteoarthritis[59] and chronic low back pain.[60] Both represent a clinical and socioeconomics problem due to their multifactorial nature and the lack of efficient treatment; therefore, if this mutual influence should be confirmed, this could open a scenario that underlines the importance of targeted research on patients treatment improvement.
In fact, it has been suggested that a successful pain treatment could reverse frailty status.

1.2.2.1 Genetic Evidences

One of the factors that appear to be shared by pain and frailty is represent by genetic component.[61,62,63] Livshits and colleagues have analysed 3626 individuals selected with frailty parameters and which were acted by CWP disease. These patients have been included in a genome-wide association study. They have demonstrated a shared genetic and environmental source in a large sample of both chronic pain and frailty.[63] However, this study included a large sample of only women, then, results can't be extended to men. In a cohort with both sex samples, it has been found that genetic background influences frailty status in men more than women do, while female frailty status could be more related to environmental factors.[61] In a more large GWAS study have been found 26 independent variants potentially associated with frailty, including

genetic variants of HLA protein, acting on immune function in aging [64] and related to neuronal function such as CSMD3, ANK3, TMOD3.[63] These findings underline the involvement of immune system and cognitive impairment in frailty onset and development, suggesting a correlation with pain mechanisms too.

1.2.2.2 Immune System and Inflammation

Inflammation and neuroinflammation are mechanisms involved in both pathologies; as seen above, inflammatory molecules have been found in frail patients[50,51] and pain onset, its chronicization and maintenance, are sustained by a strong inflammation that involves several mediators and cytokines produced by different cell types in the peripheral and nervous system.[23] In fact, an increased IL-6 levels have been also found to be linked to severe painful diseases as atherosclerosis, osteoporosis, and sarcopenia.[65] The immune system and inflammation could be a common link not only between frailty and pain, but also with depression and cognitive impairment, common conditions of both pathologies in which it has been shown that the immune plays a relevant role. Several cytokines can induce depression, cognitive impairment and weakness.[61] Successful pain treatment can often reverse the immune system modifications and inflammation present in chronic pain, restoring a correct balance pro/anti-inflammatory cytokines.[27] Therefore, if this correlation should be confirmed a new therapeutically strategies could be investigate to frailty treatment.

1.2.3 Animal Models of Frailty

Aging animal models have been largely used to study aging mechanisms in order to promote longer and healthy life.[66] Recently, focus has been on developing on animal

models of frailty, using old animals.[67] Mice are major animal used to study senescence and frailty processes thanks to their similarity with humans. According to maturational rate, mice 18-24 mouths are defined "old" and correlate to human 56-59 years old.[68] To assess frail clinical status in old mice, 31 observational variables (31-items) are identified (Table 1) and FI are a value between 0-1, then a higher score underlies higher frailty status.[69]

Table 1. 31-items to determinate Frailty Clinical Index in mice models.[69]

System and Parameter	Potential Deficits
Alopecia	Hair loss due to age-related balding and/or barbering (fur trimming)
Loss of fur colour	Change in fur colour from black to grey or brown
Dermatitis	Inflammation, overgrooming, barbering or scratching causing skin erosion. Can result in open sores anywhere on the body
Loss of whiskers	Loss of vibrissae (whiskers) due to aging and/or whisker trimming
Coat condition	Ruffled fur and/or matted fur. Ungroomed appearance. Coat does not look smooth, sleek, and shiny
Tumors	Development of tumors or masses anywhere on the body
Distended abdomen	Enlarged abdomen. May be due to tumor growth, organ enlargement, or intraperitoneal fluid accumulation
Kyphosis	Exaggerated outward curvature of the lower cervical/thoracic vertebral column. Hunched back or posture
Tail stiffening	Tail appears stiff, even when animal is moving in the cage. Tail does not wrap freely when stroked
Gait disorders	Lack of coordination in movement including hopping, wobbling, or uncoordinated gait. Wide stance. Circling or weakness
Tremor	Involuntary shaking at rest or during movement

System and Parameter	Potential Deficits
Forelimb grip strength	A decline in forelimb grip strength
Body condition score	Visual signs of muscle wasting or obesity based on the amount of flesh covering bony protuberances
Vestibular disturbance	Disruption in the ability to perceive motion and gravity. Reflected in problems with balance, orientation, and acceleration
Hearing loss	Failure to respond to sudden sound (eg, clicker) indicative of hearing loss or impairment
Cataracts	Clouding of the lens of the eye. An opaque spot in the center of the eye
Corneal opacity	Development of white spots on the cornea. Cloudy cornea
Eye discharge/swelling	Eyes are swollen or bulging (exopthalmia). They may exhibit abnormal secretions and/or crusting
Microphthalmia	Eyes are small and/or sunken. May involve one or both eyes
Vision loss	Vision loss, indicated by failure to reach toward the ground when lowered by the tail
Menace reflex	Rapid eye blink and closure of the palpebral fissure in response to a nontactile visual threat to the eye. Measures the integrity of the entire visual pathway including cortical components
Nasal discharge	Signs of abnormal discharge from the nares
Malocclusions	Incisor teeth are uneven or overgrown. Top teeth grow back into the roof of the mouth or bottom teeth are long and easily seen
Rectal prolapse	Protrusion of the rectum just below the tail
Vaginal/uterine/penile prolapse	Vagina or uterus protrudes through the vagina and vulva. Penis cannot reenter the penile sheath.
Diarrhea	Feces on the walls of the home cage. Bedding adheres to feces in cage. Feces, blood, or bedding around the rectum
Breathing rate/depth	Difficulty breathing (dyspnea), pulmonary congestion (rales), and/or rapid breathing (tachypnea)

System and Parameter	Potential Deficits
Mouse Grimace Scale	Measure of pain/discomfort based on facial expression. Assessment of five facial features: orbital tightening, nose bulge, cheek bulge, ear position (drawn back), or whisker change (either backward or forward)
Piloerection	Involuntary bristling of the fur due to sympathetic nervous system activation
Temperature	Increase or decrease in body temperature
Weight	Increase or decrease in body weight

Aim of Research

The association between the occurrence of chronic pain and risk to develop frailty has been discussed and agreed upon in several recent papers.[54-63] However, major of them are observational studies and an action to modulate frailty through successful pain treatment is missing. Therefore, the main aim of my PhD project is evaluate if the successful pain treatment correlates with reverse of frail status.

For this purpose, several objectives have been proposed:

1) It has been investigated the role of pain treatment on pain relief and frailty in both older human subjects (through SCS) and animal model (through morphine administration, operated thanks to collaboration with University of Milan). The possibility to use animal models, specifically mice C57BL/6J, allows to better separate different variables involved in frailty, including aging, pain, pain treatment, in order to better understand specific casual role of each component. Moreover, understand if successful pain treatment can reverse frailty could represent good way to prevent or slow frailty in older people.

2) It has been found that pain and frailty can share inflammation, neuroinflammation and immunity system activation, therefore, it has been investigated the role of pro- and anti-inflammatory cytokines and neurological mediators, and their different gene expression, in pain/frailty condition. It has also been investigated if pain treatment module their expression. It has been performed in both mice and human samples at different time point.

3) They have been enrolled 18 patients subjected to SCS implant. Although SCS is a widely used and validated approach, there are some people which have not

pain relief and one of the prognosis factor appears to be psychological condition of patient, such as depression, anxiety, memory of trauma. Therefore, it is important identify a psychometric profile, that together molecular profile, could predict successful outcome of procedure in term of pain relief and improvement of quality if life.

Material and Methods

3.1. Animal Model

All experiments about animal models have been performed using C57BL/6J mice young (11 weeks) and old (20 months). About all of concerning animal care and experimental procedures have been operated by Department of Pharmacological and Biomolecular Science of University of Milan (UNIMI), according to guidelines of the Animal Care and Use Committee of the Italian Ministry of Health (DL 116/92 and DL111/94-B). This preclinical model has been thought in according to human factors shared (aging, pain, treatment).

Experimental groups:

- Control young mice (CTR y)
- Control young mice treated with morphine (CTR y+m)
- Osteoarthritic young mice (MIA y)
- Osteoarthritic young mice treated with morphine (MIA y+m)
- Control old mice (CTR o)
- Control old mice treated with morphine (CTR o+m)
- Osteoarthritic old mice (MIA o)
- Osteoarthritic old mice treated with morphine (MIA o+m)

Chronic pain has been chemically induced through intra-articular injection into the right knee of 1mg of monosodium iodoacetate (MIA). It is commonly used as a model of osteoarthritic pain; mild pain-like behaviour onsets after 3 days from injection and persists up to 4 weeks.[70] Differently, control mice have been treated with saline solution. Pain treatment has been provided with 2.5 mg/kg of morphine, subcutaneously injected in mice once daily, beginning with 7 day, for all the duration of the study.

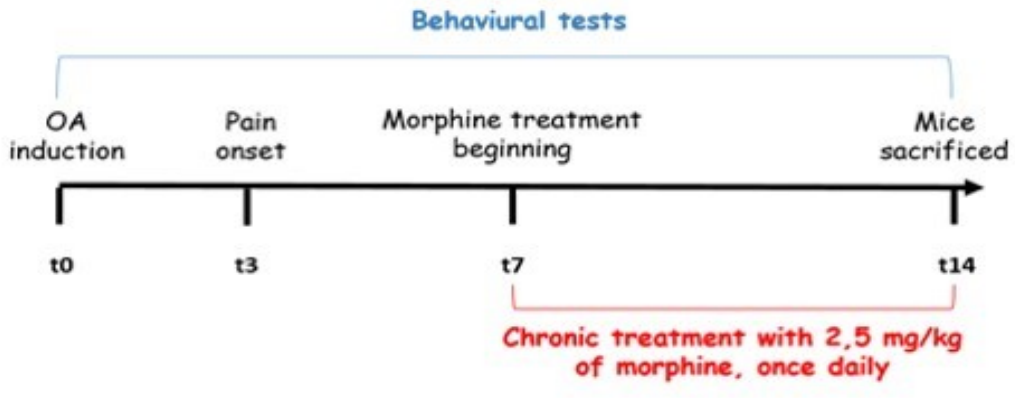

Figure 8. Experimental protocol timeline.

At t0 basal thresholds have been measured and OA has been inducted. t3=3 days after OA induction, pain onsets. t7= chronic morphine treatment beginning. t14= 7 days after morphine treatment, mice have been sacrificed.

3.1.1 Nociceptive Behaviour Test

Several behavioural tests have been conducted in order to evaluate nociceptive threshold and monitor progression of pain. Generally, tests are based on induction of a mechanical or thermal stimulus and measure the animal's reaction in term of latency to retreat from painful cause. Before to MIA induction, basal threshold have been measured (t0) through Von-Frey test and Incapacitance test; successively the same tests have been effectuated at t3, t7 and t14 in order to monitor over time the threshold responses of the animals to painful stimuli.

- **Von- Frey test**

Particularly indicated to identify alteration of pain threshold in chronic inflammatory and neuropathic states. It is used to measure mechanical allodynia. This test consists in evaluation of mechanical sensitivity on animal hind paw, via Von-Frey filament, a probe

Figure 9. Dynamic plantar Aesthesiometer.

www.ugobasile.com

with 0.5mm diameter. The probe is linked to Dynamic Plantar Aesthesiometer that has a moveable force actuator localized under a wire mesh on which animal are positioned. Von-Frey filament acts a growing force on plantar surface (10g/10sec) until animal withdraw the paw. The value of force applied (PWT) in a range of time identifies its pain threshold.[71]

- **Incapacitance test**

It is used to evaluate spontaneous pain, without external nociceptive stimuli. Rodent is positioned on a Plexiglas box, with hind paws on each of two pads, while front paws are on vertical wall. This instrument measure the weight exercised from each hind paw in order to determine the difference in accord to presence of pain or not. Values are registered in LCD display; if both values of right and left paw are between 45-55% then there is an equal distribution

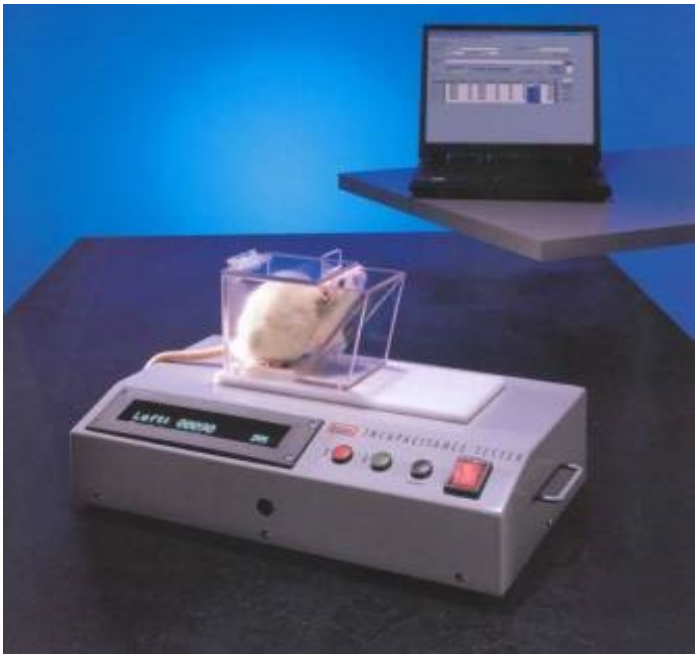

Figure 10. Incapacitance test.

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of weight, showing absence of pain. However, after of one paw damage it will register a difference values with less distribution of weight on paw injured.[71]

3.1.2 Frailty Evaluation

Frailty evaluation in animal model has been conducted by UNIMI using 31-items reported in table 1.[69] At each item has been assigned a value between 0 (absence of deficit) and 1(severe deficit). Frailty Index (FI) Score has shown as mean of all evaluations. It has been evaluated at t0, before OA induction, and in all experimental groups 14 days after OA induction. Furthermore, tests to evaluate strength, balance and motor coordination have been conducted, being some of aspects deficient in old humans.

3.1.3 Collection of Nervous Tissues.

After 14 days from OA induction (t14), mice have been sacrificed through beheading and from each mouse has been collected:

- Spinal cord (L3-L5)
- 4 Sovra-spinal Area: Hypothalamus, Frontal and Pre-frontal Cortex, Hippocampus

Tissues collected have been immediately frozen in liquid nitrogen and conserved at -80°C by UNIMI and successively sent to UNIPR to molecular analysis, such as gene expression of pro and anti-inflammatory cytokines (TFNα, IL-6, IL1B, INF-γ,IL-10, IL-4), cellular damage marker (ATF3), glial markers (CD11B, GFAP,Iba-1), macrophages

activation factor (TLR4) and neurotrophic factor (BDNF).

3.2 Human Study

18 patients with mean age of 66 years and chronic pain selected for surgery treatment (SCS) by physicians of Pain Therapy of Hospital of Parma have been enrolled, according to guideline to Good Clinical Practice (GCP) and actually laws on observational clinical studies. As explained before, SCS implant consists in a prior phase of trail and, if this will have a good outcome after 4 weeks in term of pain relief ≥ 50 then, it will pass to definitive implant. Each candidate patient has been exhaustively informed by physician about his potential involvement in this project and, if patient accepted to participate, doctor asked him to sign Informed Consent and complete the Case Report Form (CRP). It has been guaranteed them the anonymity, therefore, an ID code has been associated to each patient during first enrollment visit (t0). Successively, follow-up visits have been scheduled according to Figure 11. During each clinical visit, pain and frailty status have been evaluated through several questionnaires, autonomously completed by patient. Furthermore, 20mL of peripheral blood has been collected for successive biochemical analysis.

ELIGIBILITY CRITERIA:

- Least 18 years old
- Eligibility to SCS implant
- Sign of CRF

EXCLUSION CRITERIA

- Lack of signature of CRF
- Incapacitance to independently complete questionnaires
- Diagnosis of cognitive disease
- Addiction from drug and/or alcol

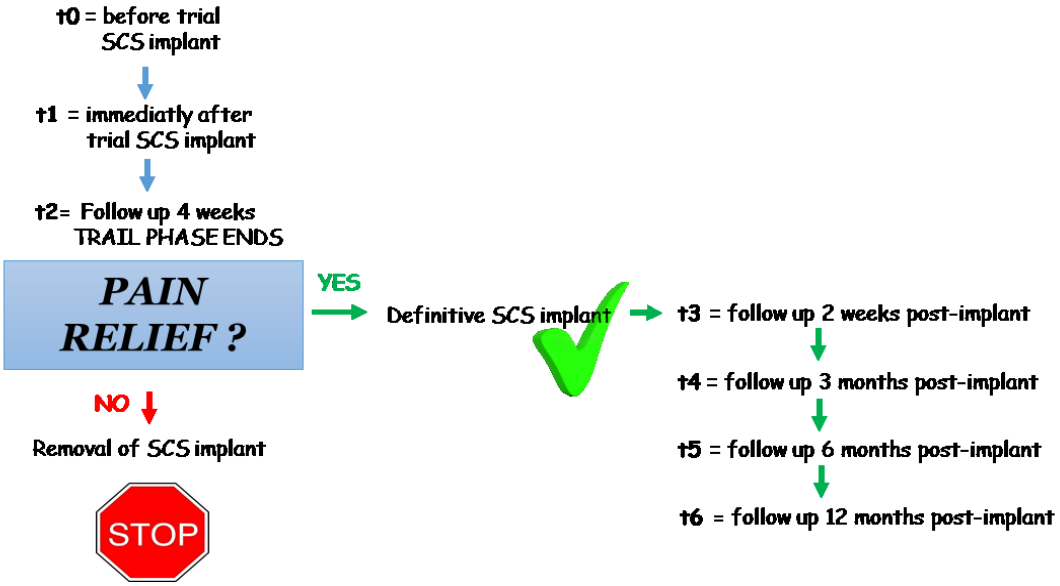


Figure 11. Follow-up visits scheduled.

3.2.1 Clinical Evaluation of Pain

At enrollment visit (t0 baseline), clinician collects several information about patients:

- Pharmacological and pathological anamnesis in order to know what is cause that allows necessary SCS implant
- Prior invasive treatments
- Through Brief Pain Inventory (BPI) it is evaluated grade and characteristics of pain: first part of questionnaire investigates sensorial component of pain

therefore patients defines location and intensity of worse, mean, minor and actual pain signing a numeric value in a range 0 (no pain) – 10 (worse imaginable pain). Last seven questions investigate pain influence on daily life, sleeping, emotions by range 0 (no influence) – 10 (totally influence).[72]

- Through Healty-related quality of life (EQ-5D-3L) it is measured quality of life. It is composed by two sections; first includes 5 items (mobility, personal care, daily activity, pain, anxiety/depression) and for each of which patients chose number value between 0 (absence of problem) and 3 (extremely limited activity). Second section evaluates healthy perceived state using *Visual Analogue Scale* (VAS) with score from 0 (worse healthy possible state) to 100 (best healthy state)[73]

These tests have been repeated during follow-up visits in order to monitor the efficiency of treatment in term of pain relief and improvement of quality of life.

3.2.2 Psychological Evaluation

Nowadays, which are psychological predictors of therapeutic outcome it is not clarified, therefore, other studies are necessary. Patients enrolled have been submitted to specific test in order to evaluate personality and temperamental tracts, including innate psychological characteristics, inherited and presents during all life course, that are not influenced by external events. Test used are:

- Adult Temperament Questionnaire (ATQ) – it includes 77 items and for each of which a value from 1 (absolutely false) to 7 (absolutely true) is chosen by patients. It examines neuroticism aspects, extroversion, effortful control and orienting sensitivity.[74]

- Pain Catastrophizing Scale (PCS)- evaluates tendency to catastrophizing pain; it includes 13 items and for each of which patients chose a value between 0 (never) and 4 (always). Total max score is 52 with a clinical threshold fixed for values ≥ 30.[75]
- Hospital Anxiety and Depression Scale (HADS) – excludes confounding variables of the anxious and depressive state (then how patients feel in that moment) from tract (innate characteristics)[76]

These tests are repeated during follow-up visits in order to monitor the psychological state evolution in parallel with painful symptomatology.

3.2.3 Frailty Evaluation

Due to lack of standardized protocol to frailty diagnosis, in this study it has been evaluated on base of psychological tests results, since anxiety and depression are some of symptoms by frailty. Moreover, physical disabilities compromise patients' quality of life, including walking, lift objects, bend, working, have been considered into interpretation of frailty status of patients before and after treatment. Changes in these fields have been evaluated at different time points and possible correlation with pain and its treatment has been investigated.

3.2.4 Biological Sample Collection

At each clinical visit 20mL of peripheral blood has been collected in EDTA vacutainer in order to evaluate cytokines production in vitro stimulation of peripheral blood

mononuclear cell (PBMCs) cultures.

3.2.4.1 Isolation of PBMCs From Whole Blood

Purification of PBMCs is conducted under laminar flow hood in order to obtain the higher grade of sterility. PBMCs have been isolated by density gradient centrifugation using Ficoll-Paque Plus. This solution has a density of 1,007m/mL and allows to separate PBMCs, which are deposited between solution and plasma forming a ring, from others blood cells including erythrocytes, granulocytes and dead cells, which have major density and then precipitate to the bottom. (Figure 12)

In detail, protocol provides:

- Dilution 1:1 of whole blood collected in EDTA with PBS 1x, gently mix by inversion
- Add 10mL of Ficoll-Paque Plus in a new 50mL tube
- Add diluted whole blood on top of Ficoll-Plaque solution, make attention to avoid to mix blood with solution
- Centrifuge at 800g for 20 minutes at room temperature
- After phase separation, collect only ring of PBMCs in a new falcon 50mL
- Wash cell adding fresh PBS 1x until a final volume of 50mL
- Centrifuge at 1500rpm for 10 minutes at 4°C
- Discard supernatant and resuspend pellet cell with 3mL of RPMI 1640 medium
- Count cell by Burker's camera: dilution 1:5 of cells in a new aliquot in which collect 20µL of cells and 80 µL of RPMI 1640 medium. Of this aliquot, 10 µL are post on Burker's camera and cells are be count at microscope. Total number of cells is obtained by mean of cells number of all four quadrants multiplied for

volume in which cells have been resuspended (5mL), for dilution factor(3mL) and for camera dilution factor(10^4)

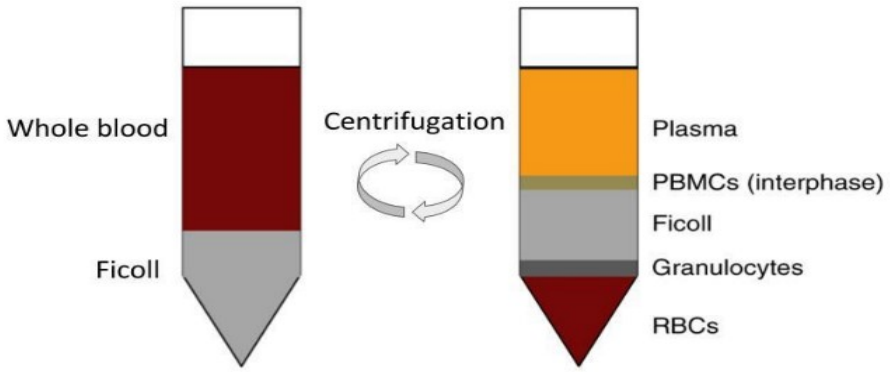


Figure 12. Stratification of whole blood after density gradient centrifugation with Ficoll-PAque Plus (Sigma). www.humancellsbio.com

3.2.4.2 Cell Cultures of PBMCs

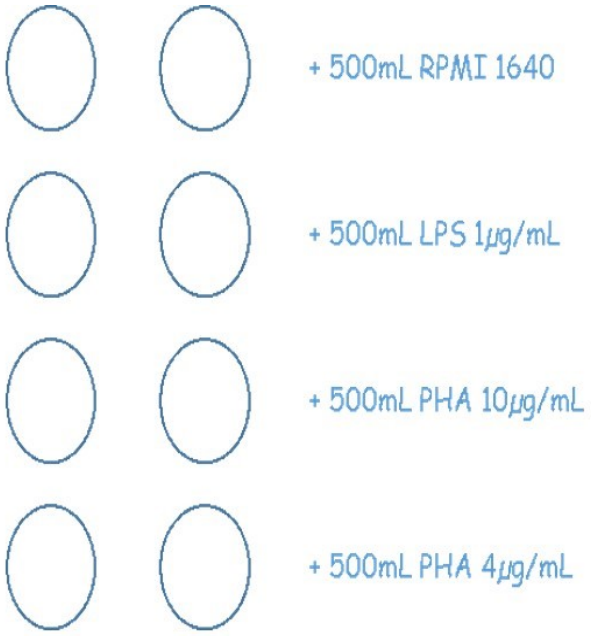


Figure 13. Design of stimulated PBMCs cultures.

Cellular cultures have been prepared in sterile multiwell plates; $2x10^6$ cells (500 µL) have been plated in each well and different stimuli have been added, as phytohemoagglutinin (PHA) for stimulation lymphocyte cytokines and lypopolisaccaride (LPS) for macrophage cytokines (Figure 13). Each condition has been plated in duplicate. Cells remaining have been stored at -80°C for

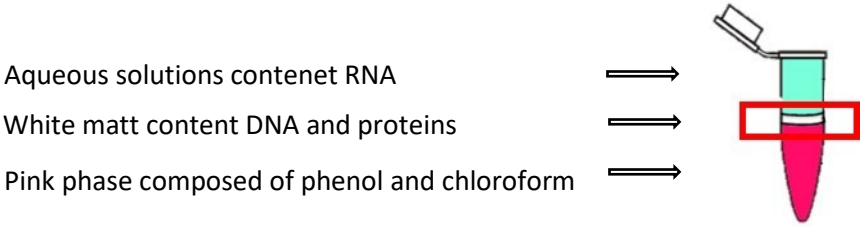
gene expression analysis. Cells plated have been incubated at 37°C in 5% CO_2 and 95% air. After 24h, supernatant has been collected and centrifuged at 13000rpm for 10minutes at room temperature. Supernatant has been transferred in a new Eppendorf and stored at -80°C for cytokines analysis.

3.3 Gene Expression

3.3.1 Homogenization of samples and total RNA extraction

Extraction of total RNA has been conducted by both mice tissues collected and humans PBMCs stored using the same protocol. First step consists in homogenization of sample with sonicator (UP50H Compact Lab Homogenizer, Hielscher ultrasonic GmbH; Teltow, Germany) in 800 µL of Trizol°, a monophasic solution of phenol and guanidine isothiocyanate that destroys cell membrane allowing leak of RNA from cells. When there are not visible tissue pieces, sample is transferred into new Eppendorf though insulin syringes. Each tube has been treated in according to following protocol:

- Add 160 µL of chloroform and manually shaken for 30 seconds, then incubate for 5 minutes at room temperature.
- Centrifuge at 12000 rpm for 15 minutes at 4°C. At this point her is a separation of the solution in three different phases:



- Transfer only aqueous solution into new Eppendorf and add 400 µL of Isopropanol to allow the precipitation of RNA.

- Manually shake for 30seconds and incubate for 10 minutes at room temperature.
- Centrifuge at 12000 rpm for 10 minutes at 4°C. At this point the bottom of the tube contains a pellet of RNA
- Eliminate supernatant and wash pellet with 800 µL of frozen Ethanol 75%
- Centrifuge at 7500rpm for 8 minutes at 4°C
- Eliminate ethanol and let dry pellet in the air
- Resuspend RNA pellet in 20ul of DEPc water
- Incubate Eppendorf at 55°C for 5minutes
- Resuspend RNA pellet and repeat incubation.
- Stored RNA at -80°C to avoid degradation of the sample

3.3.2 Digestion of Genomic DNA

Before proceeding to the quantification of total mRNAs extracted, they underwent to DNase treatment (DNA-*free*™ DNA Removal Kit, Invitrogen™), to avoid any contamination of genomic DNA, according this protocol:

- Add to each sample 2µL of DNAsi Buffer 10x and 1 µL of rDNAsi
- Vortex and spin and then incubate the samples at 37°C for 30 minutes.
- Add to each sample 2 µL of DNAsi Inactivation Reagent
- Incubate samples for 3 minutes at room temperature and then centrifuge at 12000rpm for 3minutes
- Transfer only supernatant, containing total RNA, in a new Eppendorf
- Samples could now be used for quantification of total RNA or can be stored at -80°C until use.

3.3.3 Quantification of Total mRNA

Total RNA concentration has been determined using spectrophotometer NanoDrop ND-1000.(Thermo Fhiser Scientific) with high accuracy and reproducibility. The sample retention system employs surface tension to hold the sample in place between two optical fibers. This enables the measurement of very highly concentrated samples without the need for dilutions. 1 µL of sample is dispensed directly on the base of the measure, the spectrum of absorbance (260nm) and its analysis are shown on the PC screen. Concentration of total RNA extracted and ratios of the absorbance 260nm/280nm (contamination index of protein) and 260nm/230nm (contamination index of carbohydrates or other organic substances) are measured. A sample of RNA is considered of good quality for successively analysis when its ratios is about 2.

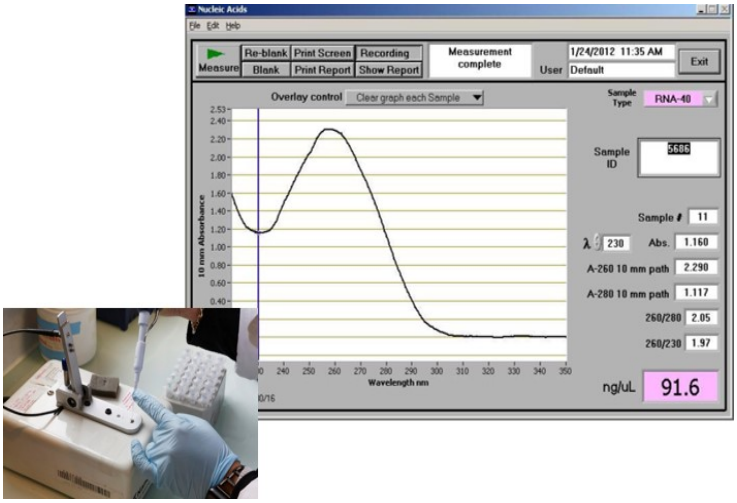


Figure 14. Nanodrop ND-100

3.3.4 Retrotranscription of mRNA

Extracted RNA is converted in cDNA using High-Capacity RNA-to-cDNA kit (Applied Biosystems). For each sample, 1000ng of RNA have been converted in cDNA in 20µL of RT reaction, in triplicate. Then, 3000ng total of RNA have been converted in cDNA for each sample. RT reaction is prepared on ice according following table 2:

COMPONENT	VOLUME PER REACTION
2 XRT Buffer Mix	10 µL
20X RT Enzime Mix	1 µL
RNA sample	up to 9 µL
Nuclease-free H_2O	Quantity Sufficient to 20 µL
Total per reaction	20 µL

Table 2. High-Capacity RNA-to-cDNA reagent kit. (Applied Biosystems)

Plate with samples are loaded on thermal cycler (Verity Applied) and reverse transcription run according program setting (Table 3). At this point cDNA can be stored for short-term (up to 24h before use) at 4°C or at -20°C for long-term.

SETTING	STEP 1	STEP 2	STEP 3
TEMPERATURE	37°C	95°C	4°C
TIME	60 minutes	5 minutes	∞

Table 3. Program to retrotranscription of mRNA

3.3.5 Real-Time PCR

Real-time PCR (Polymerase Chain Reaction) is a semi-quantitative technique that allows the study of gene expression, through specific fluorescent probes. Amplification of cDNA is monitored during all experimental phase of growth. Taq-Man chemical has been used, based on 5'-3' exonuclease activity of Taq polymerase combined with florescent marked probes. Taq-Man probes have a fluorocrome named reporter FAM

(6-carboss fluorescina) covalently linked to 5' extremity, and a QUENCHER non fluorescent (NFQ) linked to 3' extremity. When the probe is intact, QUENCHER inhibits reporter fluorescence, but during extension phase of PCR, if target is present, it is linked by probe and Taq polymerase activity removes inhibition of QUENCHER, and a fluorescent signal (FAM) is generated, directly proportional of target concentration. The amplification reaction has been performed using CFX Connect Real-Time PCR System (Biorad) and carried out in a final volume of 20µL composed by:

- 1 µL of Taq-Man probe
- 10 µL of Master Mix containing Taq-polymerase enzyme
- 7 µL of RNase-free water
- 2 µL of cDNA

Specific probe for cytokines and markers of interest, reported in Table 4, related to both animal and human samples, have been used.

PROBE MOUSE	ID NUMBER	PROBE HUMAN	ID NUMBER
BDNF	Mm01334042_m1	TNF	Hs00174128_m1
PK2	Mm01182450_g1	INF-γ	Hs00989291_m1
PK-R1	Mm00517546_m1	IL-1	Hs01555410_m1
PK-R2	Mm00769571_m1	IL-2	Hs00174114_m1
IL-1β	Mm00434228_m1	IL-4	Hs00174122_m1
IL-6	Mm00446190_m1	IL-6	Hs00174131_m1
CD11b	Mm00434455_m1	IL-10	Hs00961622_m1
GFAP	Mm01253033_m1	GAPDH	Hs02786624_g1
GAPDH	Mm99999915_g1		
TaqMan® Gene Expression Assays, Applied Biosystem			

Table 4. Taq-Man Specific probes to both species, mice and humans.

Reaction conditions:

STEP	TEMPERATURE (°C)	TIME(MM:SS)
Initial denaturation	95	3:00
Cycled Template Denaturation (40 cycles)	95	00:10
Annealing and Extensions (40 cycles)	60	1:00

Table 5. Real-Time PCR instrument program.

All Real Time PCR assays have been performed in duplicate, as endogenous control gene GAPDH has been used, while as negative control (NTC) has been used the reaction mix without cDNA. Relative quantification has been performed using the comparative threshold method. Threshold cycle numbers (C_t) of the specific gene of interest and the endogenous housekeeping GAPDH have been detected by CFX Connect Real-Time PCR System (Biorad). Gene expression values, for each gene, have been standardized on correspondent values of the endogenous control gene and relative values of calibrator sample using formula:

$$2^{-\Delta\Delta CT}$$

In which:

- ΔC_t values has been calculated subtracting GAPDH Ct to target gene of interest C_t
- $\Delta\Delta C_t$ has been calculated subtracting ΔC_t of calibrator sample to ΔC_t of each sample

3.4. Data Analysis

Results have been expressed as mean ± SEM (Main Standard Error).

Unpaired t-test has been used for frailty index analysis; while for all behavioral tests on mice, it has been conducted variance analysis *Two-way ANOVA*, followed by Bonferroni's post hoc test. About biochemical evaluations on both mice and humans, variance analysis *One-way ANOVA* has been applied, followed by Bonferroni's test post hoc test. *One- way ANOVA* has also been used to analysis results obtained by patients about pain's and psychological tests, at different time points. Successively, *Pearson's correlation* has been evaluated between pain severity and some frailty symptoms in human. $\rho = -1$ indicates negative correlation; $\rho = 0$ indicates absence of correlation; $\rho = -1$ indicates positive correlation. Values have been considered significant when $p < 0.05$.

Results

4.1. Mice Model

4.1.1 Evaluation of Nociceptive Threshold In Mice

In order to evaluation mechanical allodynia (Figure 15-A) and spontaneous pain (Figure 15-B) in different experimental conditions, basal thresholds (t0) before MIA induction have been measured. These test have been conducted in all mice groups at 3 days after MIA induction (or physiological injection for CTR groups), when painful symptomatology is onset, at 7 days after MIA induction when chronic morphine treatment starts, 3 days after treatment starts and 7 days after, when animals are sacrificed in order to biological samples collection.

CTR young and old have not basal different levels at t0 in both tests, while both MIA young and old show an important hypersensitivity in confront to CTR young and CTR old respectively.

At 3rd days, all MIA mice show a significant decrement of threshold after mechanical stimuli (A), in ipsilateral injection paw. Similarly, a little decrement of threshold pain (A) and increment of spontaneous pain have been appeared in CTR mice after physiological solution injection. This condition could be evoked by itself injection procedure and, in fact, it has spontaneously been resolved after 7 days, and threshold levels are return to basal levels.

At 7th day, pain treatment starts and mice have been treated until seven days with a single injection of 2.5mg/kg of morphine. All groups have been treated with morphine and its effect on painful symptomatology has been evaluated after 3 and 7 days. Figure 15 shows as chronic morphine treatment improves painful symptomatology. In fact, after 3 days of treatment, both MIA young and adult group have total recovery to threshold of mechanical allodynia (A) and spontaneous pain levels (B). This condition is sustained until the end of protocol, at 14th day, in which MIA and CTR appear to have

same threshold levels in both tests. Healthy mice (CTR), both young and old, don't show differences when treated with morphine respect to untreated CTR mice, in both tests.

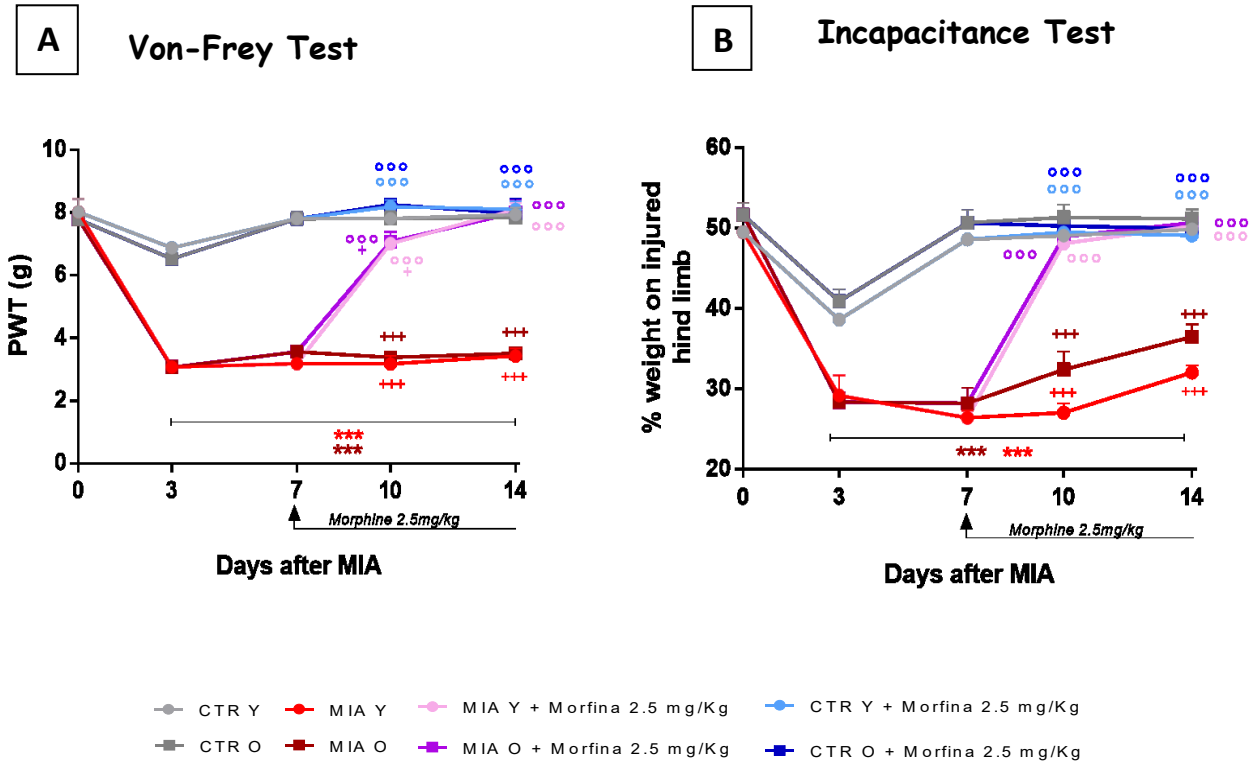


Figure 15. Effect of chronic morphine treatment on spontaneous pain. (A) Von-Frey Test. Effect of chronic morphine treatment on mechanical allodynia threshold. (B) Incapacitance Test. t0= before MIA induction (basal), t3=3 days after MIA induction, t7=morphine treatment beginning, t10= 3days after treatment beginning, t14=7days after treatment beginning and mice sacrificed. Values are expressed as mean ± SEM, n=5mice/group. Statistical analysis have been conducted by *Two-way ANOVA*, and *Bonferroni's* post-hoc test. +p<0.05, ++p<0.01, +++p<0.001 vs CTR + morphine; $p<0.05, $$p<0.01 vs young; **p<0.01, ***p<0.001 vs corresponding CTR; °°°p<0.001 vs MIA.

4.1.2 Frailty Index Evaluation

All mice used in this study have been submitted to FI evaluation; in figure 16 is shown as mice CTR, without pain, with similar age have different range of FI, in particular in old CTR mice. This indicates different healthy states independently by age. Furthermore, CTR old mice have FI higher than young; this is related to presence of some deficits age-related in old mice. In both adult and old mice OA induction comports a significant increment of FI in confront to corresponding CTR condition, more in old in which basal score was already higher than young. This confirms the role of pain on worsening of frailty status. Moreover, MIA old mice have shown a significant higher FI than MIA young mice, demonstrating a greatest impact of pain in old mice due to lack of functional resources age-related.

After chronic morphine treatment, both adult and old have shown a significant improvement of FI score, confirming our hypothesis according to which a good pain treatment could reverse or prevent frailty status.

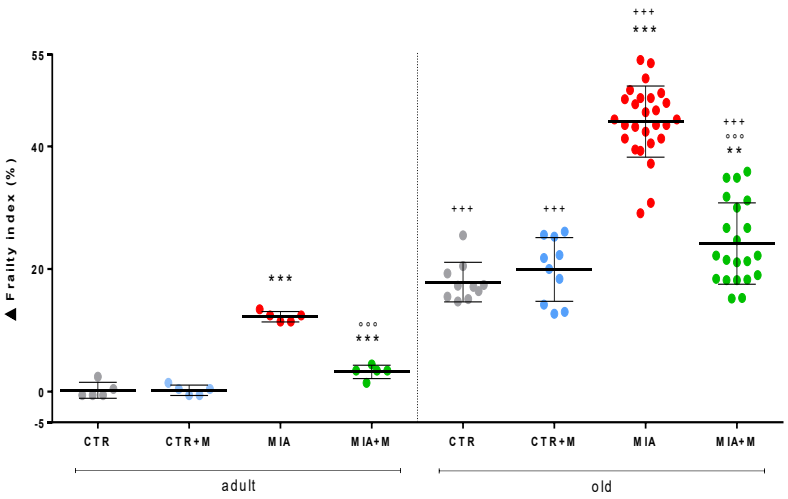


Figure 16. Frailty Index Evaluation.

Frailty Index Score in adult and old mice, 14 days after OA induction. Values are expressed as mean ± SEM. Statistical analysis have been conducted by *unpaired t-test*. **p<0.01; ***p<0.001 vs CTR; °°°p<0.001 vs MIA; +++p<0.001 vs corresponding adult.

4.1.3 Gene Expression

After 14 days from OA induction and 7days of chronic treatment, all mice have been sacrificed and biological samples have been collected in order to evaluate inflammatory component at spinal level (spinal cord L3-L5) and suvra-spinal level (hypothalamus, hippocampus, pre- and frontal cortex).

- **Spinal cord (Figure 17):** OA induces a strong neuroinflammation in MIA young group that is reflected by significant increment of IL-6, ATF3 and CD11 levels respect to its healthy control group (CTR young). MIA old, in addition to IL-6, ATF3 and CD11 increased levels, shows a significant increment of TLR4, GFAP, Iba1, respect to its healthy control group (CTR old). Pharmacological treatment allows decrement of IL-1β, CD-11 and ATF3 expression levels in MIA young, while pro-inflammatory cytokine IL-6 expression, increased by pathology, is not influenced by morphine. Also in MIA old group has been observed a decrement of GFAP, TLR4, Iba-1 in addition to IL-1B, CD-11 and ATF3 gene expression when treated with morphine. About young mice, both CTR and MIA, TLR4 gene expression does not appears to be influenced by pathology or treatment just like IL-1 β and IL-6 in both CTR and MIA old. Therefore, in general it appears that chronic morphine treatment is able to contrast neuroinflammation inducted by OA. Furthermore, an increment of gene expression levels of TNF-α, TLR4, CD11, Iba-1 e ATF3 has been shown in MIA old respect to MIA young, probably related to inflammatory state caused by aging (Inflammaging)

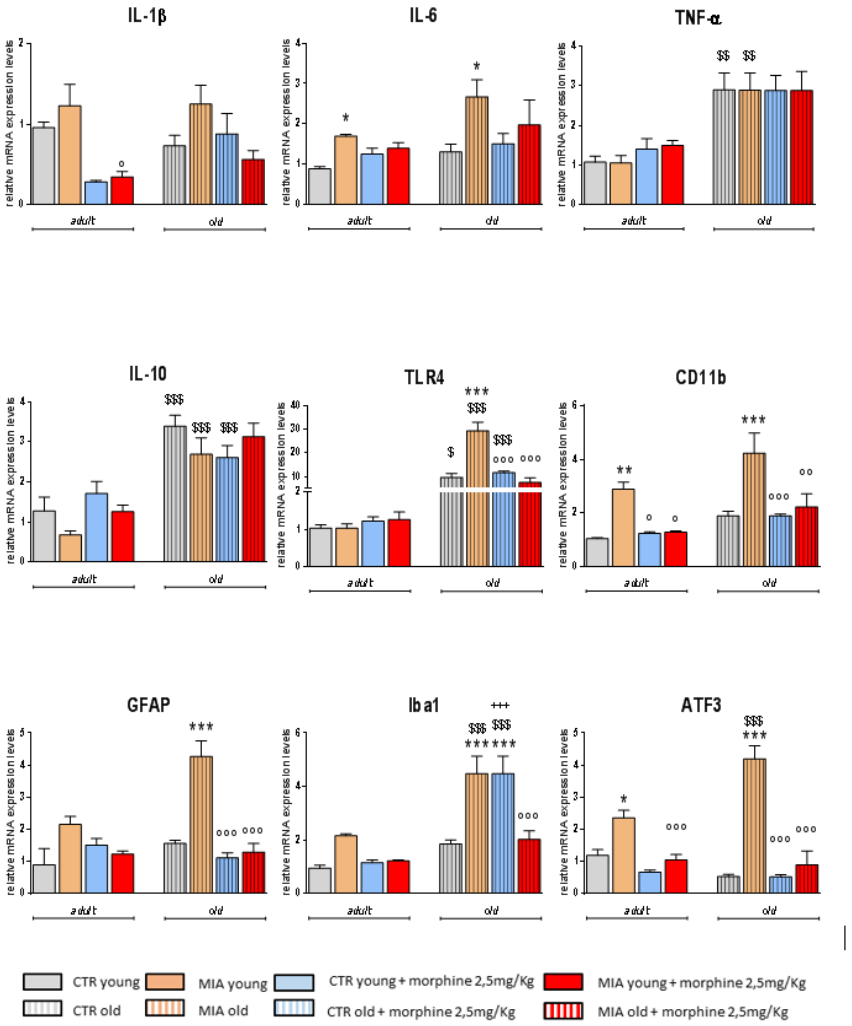


Figure 17. Gene expression in spinal cord of mice.

Results have been obtained by Real Time PCR and have been standardized on correspondent values of the endogenous control gene (GAPDH) and relatove calibrator (CTR young group). Values have been expressed as Mean of $2^{-\Delta\Delta CT}$±SEM and n=10mice/group. Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. +p<0.05, ++p<0.01, +++p<0.001 vs CTR+morphine; $p<0.05, $$p<0.01, $$$p<0.001 vs corresponding young group; *p<0.05, **p<0.01, ***p<0.001 vs corresponding CTR group; °p<0.05, °°p<0.01, °°°p<0.001 vs corresponding MIA group.

• **Hypothalamus (Figure 18):** In general, it appears that age induces increment of gene expression level of IL-6 and TNF-α; in fact, in both CTR old and MIA old it has been shown higher levels of these genes than same young condition, CTR young and MIA young respectively. BDNF expression levels shows decrement in old mice, with a significant reduction caused by morphine treatment in MIA old in confront to MIA young. However, it appears that neither pathology nor the treatment have effects on gene expression modulation at this level in old mice. About other genes analyzed, no difference have been found between groups.

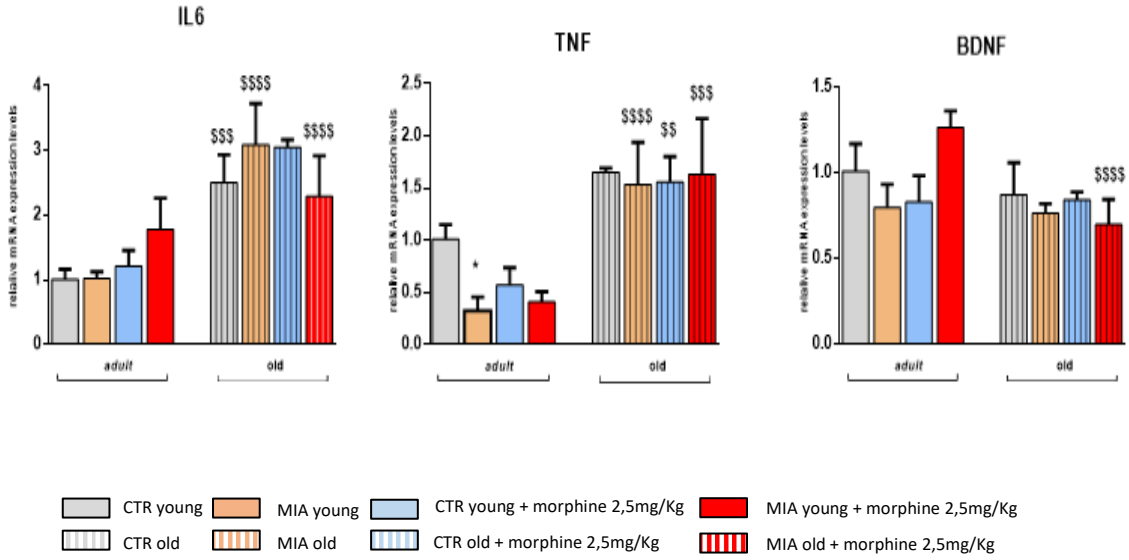


Figure 18. Gene expression in hypothalamus of mice.

Results have been obtained by Real Time PCR and have been standardized on correspondent values of the endogenous control gene (GAPDH) and relatove calibrator (CTR young group). Values have been expressed as Mean of 2 -ΔΔCT±SEM and n=4mice/group. Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. +p<0.05, ++p<0.01, +++p<0.001 vs CTR+morphine; $p<0.05, $$p<0.01, $$$p<0.001 vs corresponding young group; *$$p<0.01, $$$p<0.001 Vs corresponfing young group.

• **Pre-frontal cortex (Figure 19):** Also in this section, it appears that age contributes increasing gene expression levels of IL-6, TNF-α and BDNF as it has been shown in old mice compared to same young condition (e.g. CTR old VS CTR young, MIA old VS MIA young, ecc) . About other markers analyzed, it appears that they are not influenced neither by age, pathology than treatment, because difference in expression levels between experimental groups have not been observed.

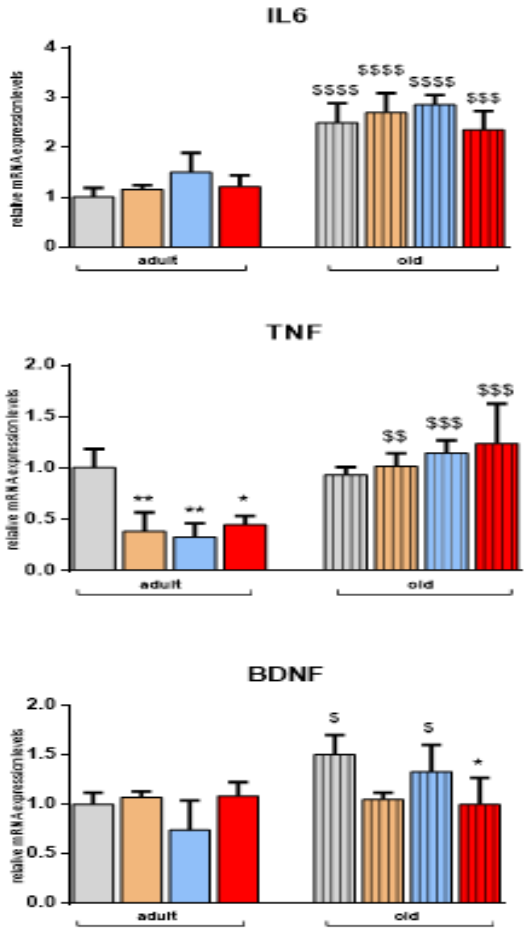
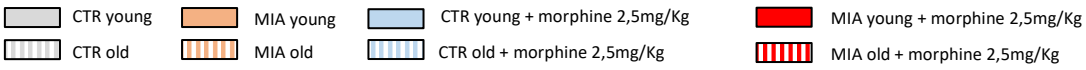


Figure 19. Gene expression in pre-frontal cortex of mice.

Results have been obtained by Real Time PCR and have been standardized on correspondent values of the endogenous control gene (GAPDH) and relatove calibrator (CTR young group). Values have been expressed as Mean of $2^{-\Delta\Delta CT}$±SEM and n=4mice/group. Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 Vs corresponfing young group.



• **Frontal cortex (Figure 20):** IL-6 and TNF-α gene expression is increased in this section too in old mice confronted to corresponding young condition, while the other markers don't appear influenced by different conditions.

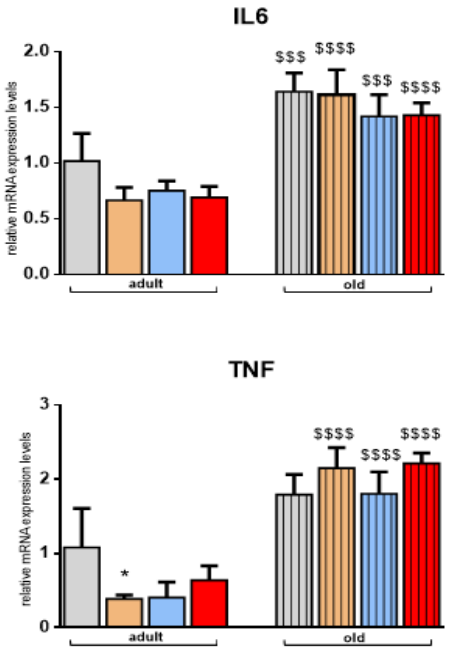


Figure 20. Gene expression in frontal cortex of mice.

Results have been obtained by Real Time PCR and have been standardized on correspondent values of the endogenous control gene (GAPDH) and relatove calibrator (CTR young group). Values have been expressed as Mean of $2^{-\Delta\Delta CT}$±SEM and n=4mice/group. Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. $$$p<0.001, $$$$p<0.0001 Vs corresponfing young group.

•**Hippocampus (Figure 21):** In this section it appears that only TNF-α gene expression is influenced by age. However, due to samples damage, it has not been possible conduct gene expression analysis on MIA old treated group. About other markers analyzed, it appears that they are not influenced neither by age, pathology than treatment, because difference in expression levels between experimental groups have not been observed.

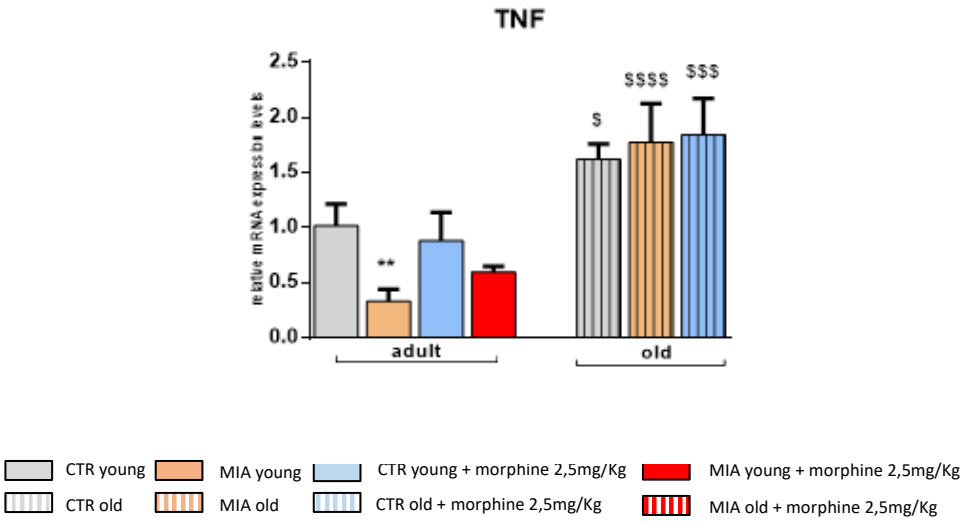


Figure 21. Gene expression in hippocampus of mice.

Results have been obtained by Real Time PCR and have been standardized on correspondent values of the endogenous control gene (GAPDH) and relatove calibrator (CTR young group). Values have been expressed as Mean of $2^{-\Delta\Delta CT}$±SEM and n=4mice/group. Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. $p< 0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001Vs corresponfing young group.

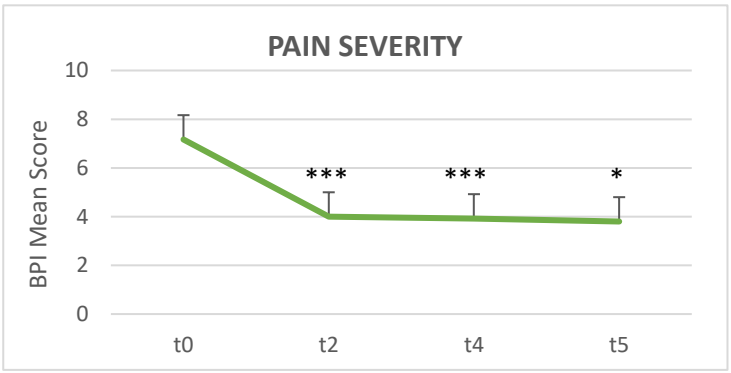
4.2 Human Study

4.2.1 Pain And Quality of Life Evaluation

At baseline (t0), 18 patients have been enrolled and have completed questionnaires. About BPI evaluation, two aspects have been investigated: pain intensity and pain interference with emotional and work life respectively. At t2, when SCS definitive implant has positioned (15 patients), it appears a significant reduction of pain severity in confront to t0 and it is also lower after 3 months (t4) and 6 months (t5) since implant than t0. (Figure 22-A). Furthermore, data related to pain interference on emotional and work life appears to have similar trend of pain severity. In fact, these parameters

resulted significant decreased at t2, t4 and t5 in confront to t0. (Figure 22 – B) These results are related to 15 patients, which decided to continue with SCS treatment; 3 of 18 initially enrolled have removed implant due to lack of benefit.

A



B

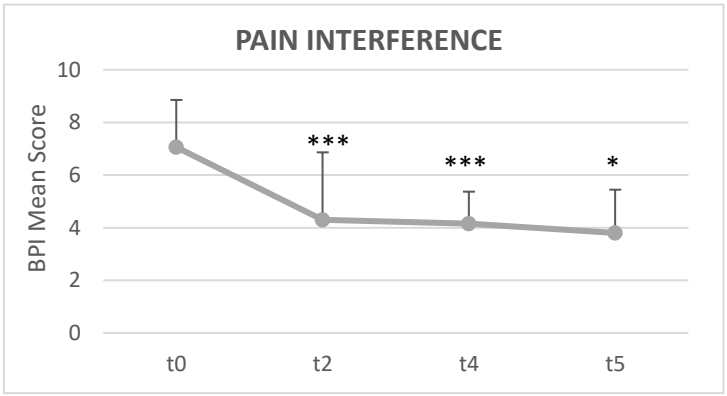


Figure 22. Evaluation of Pain severity (A) and interference with emotional and work life (B). Values have been obtained by interpretation of BPI test and shown as Mean ± SEM at baseline (t0), follow-up at 4 weeks (t2,) at 3 months post SCS implant (t4) and 6 months post SCS implant (t5). Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. *p<0,05 ; **p<0,01 ; ***p<0,001 VS t0.

European standardized Index (EQ Index) has been used to evaluate quality of life of patients at baseline and it has emerged mean value of 0,43 (range 0-1). It is shown a significant increase at t2, maintained at t4 and t5 in confront to t0. (Figure 23)

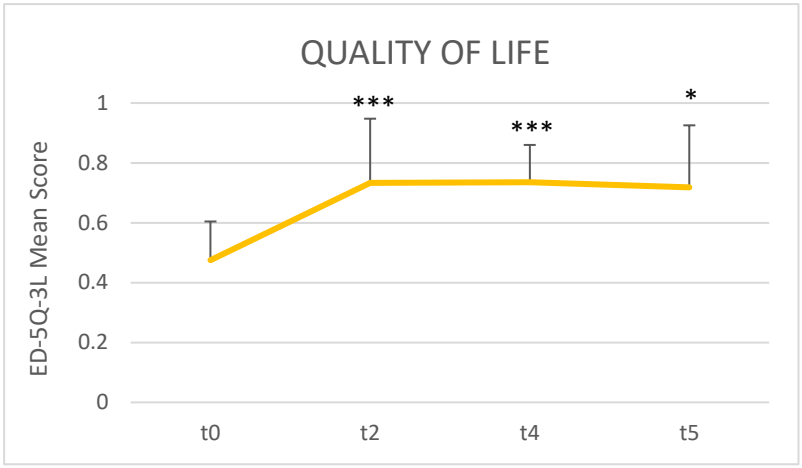


Figure 23. Evaluation of quality of life.

Values have been obtained by interpretation of EQ-5D-3L test and shown as Mean ± SEM at baseline (t0), follow-up at 4 weeks (t2,) at 3 months post SCS implant (t4) and 6 months post SCS implant (t5). Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. *p<0,05; ***p<0,001 VS t0.

4.2.2 Psychological Evaluation

Psychological evaluation has been performed during enrollment visit through specific tests, which have been repeated during follow-up visits in order to monitor possible changes, considering that cognitive diagnosed diseases represent exclusion criteria. ATQ results have not been reported due to low number of patient enrolled until now. Considering HADS Anxiety and Depression Scale (0-7= no anxious/ no depress, 8-10= borderline, >10 =anxious/depress), between patients enrolled at t0 we have found 5 non anxious, 6 borderline and 7 anxious, while about depression state there are 6 non depress, 4 borderline and 8 depresses. HADS total score is 16.07 (range 0-32). Pain Catastrophizing questionnaires has identified 50% of patients "clinically catastrophizing."

After 4 weeks SCS implant has been positioned (t2) followed by control visit after 3 (t4) and 6 (t5) months. At these time points psychological tests have been repeated by patients, which continue with procedure. As shown in figure 24 Pain Catastrophizing Total Score have significant decreased at t2 in confront to t0. It has also appeared decreased at t4 and t5 but it has not resulted significant.

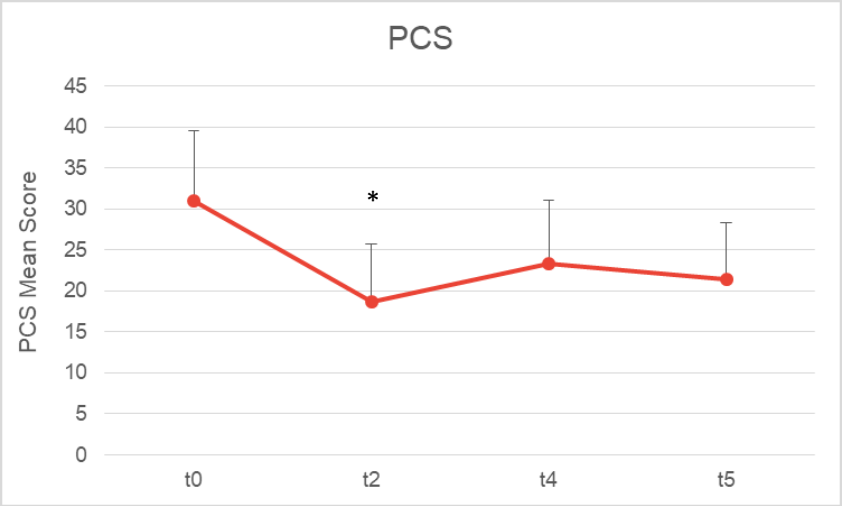


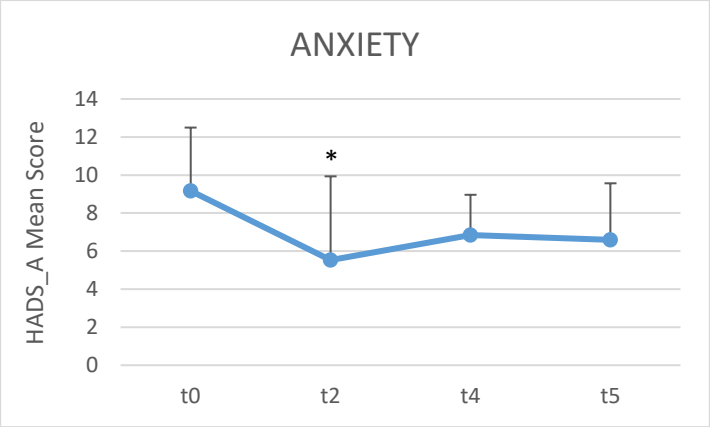
Figure 24. Evaluation of Pain Catastrophizing.

Values have been obtained by interpretation of PCS test and shown as Mean ± SEM at baseline (t0), follow-up at 4 weeks (t2,) at 3 months post SCS implant (t4) and 6 months post SCS implant (t5). Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. *p<0,05 VS t0.

Figure 25-A shows how anxiety score appears significant decreased at t2 in confront to t0. It is also decreased at t4 and t5 in confront to t0 but a lightly increment is present at t4 in confront to t2, although it does not result significant.

Depression score also appears significant decreased at t2 but not at t4 and t5, although score is lower than t0. Also in this case, a light increment has been observed at t4 in confront to t2, but it hasn't resulted significant. (Figure 25-B)

A



B

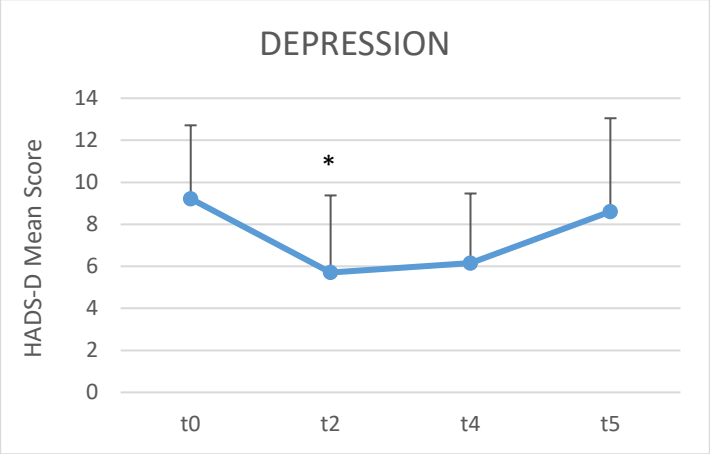


Figure 25. Evaluation of anxiety (A) and Depression (B). Values have been obtained by interpretation of HADS test and shown as Mean ± SEM at baseline (t0), follow-up at 4 weeks (t2,) at 3 months post SCS implant (t4) and 6 months post SCS implant (t5). Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. *p<0,05 VS t0.

4.2.3. Correlation between Pain and Frailty Symptoms

Successively we have investigated if pain severity and its resolution could influence some parameters shared with frailty status, including pain interference with daily life activities, quality of life impairment and psychological states. For this purpose, Pearson's correlation has been conducted between BPI severity's score and BPI-interference score (Figure 26), at different time points. A strong and significant (p<

0.01) positive correlation is emerged between variables (r = 0.99), suggesting an effect of successful pain treatment on improvement of daily activities.

A successful pain treatment appears to positively influence the quality of life; in fact a strong and significant (p<0.01) negative correlation (r = -0,99) has been found between Pain severity and Quality of life then, at t2 when pain severity significant decreases, a significant improvement of quality of life is reported. This trend is also maintained at successively time points. (Figure 27)

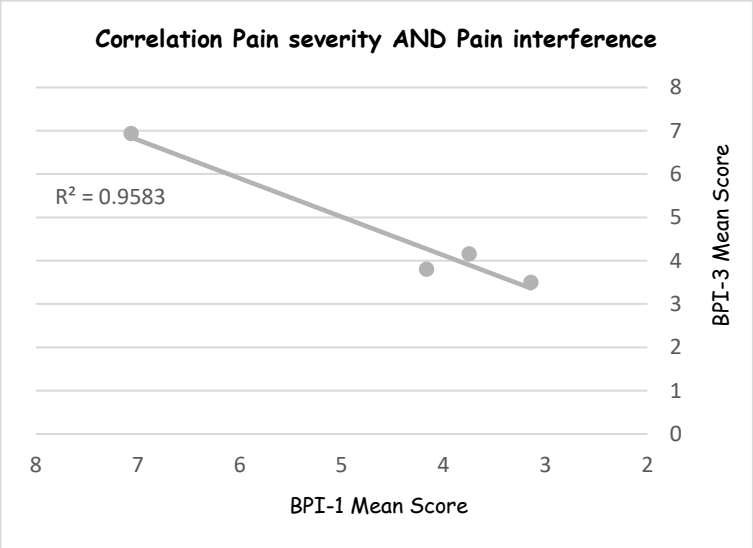


Figure 26. Evaluation of possible correlation between Pain severity and its interference in daily activities. Correlation has been calculated with Pearson's Correlation that has shown a strong positive correlation with r= 0,97 and p<0,05

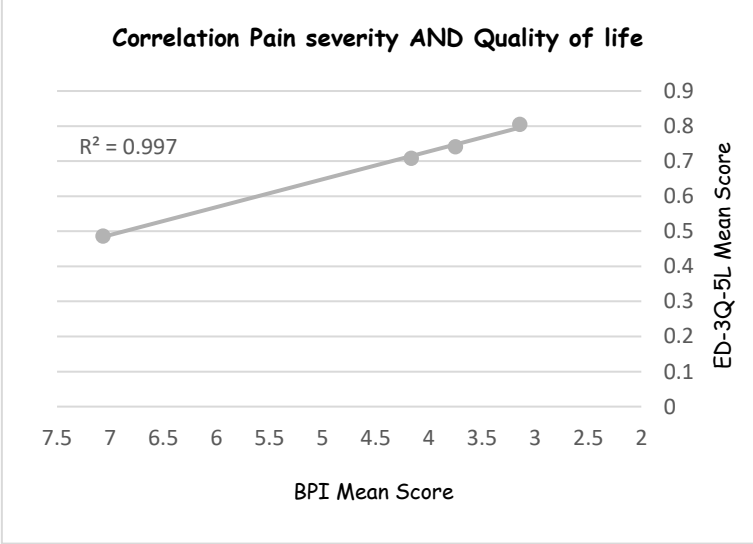


Figure 27. Evaluation of possible correlation between Pain severity and Quality of life. Correlation has been calculated with Pearson's Correlation that has shown a strong negative correlation with r= -0.998 and p<0.001

About pain influence on psychological variables examined, a positive correlation (r=0,963; p<0,05) has been found between Pain severity and Pain Catastrophizing Score. It appears that there is a parallel trends that could suggest a reduction of tendency to pain catastrophizing could be influenced by pain severity decrement, obtained thanks to good treatment outcome. (Figure 28)

About anxiety and depression states, they do not appear significant correlate with pain severity, as shown in figure 29.

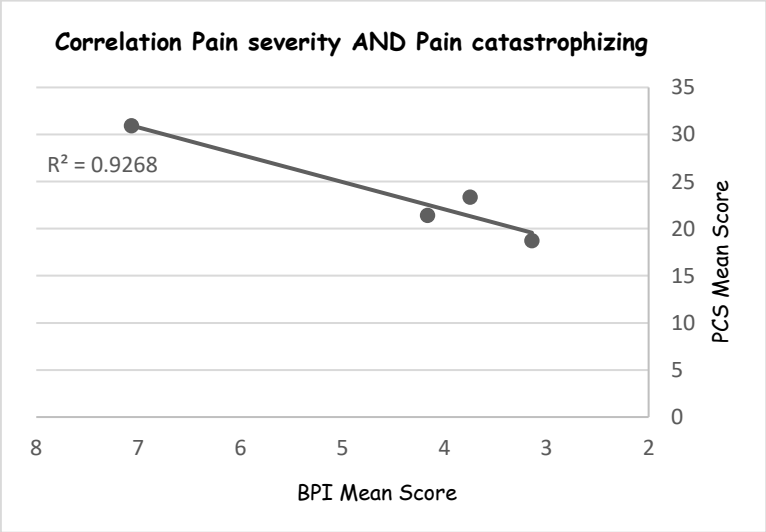


Figure 28. Evaluation of possible correlation between Pain severity and Pain Catastrophizing.

Correlation has been calcutaed with Pearson's Correlation that has shown a positive correlation with r= 0,963 and p<0,05

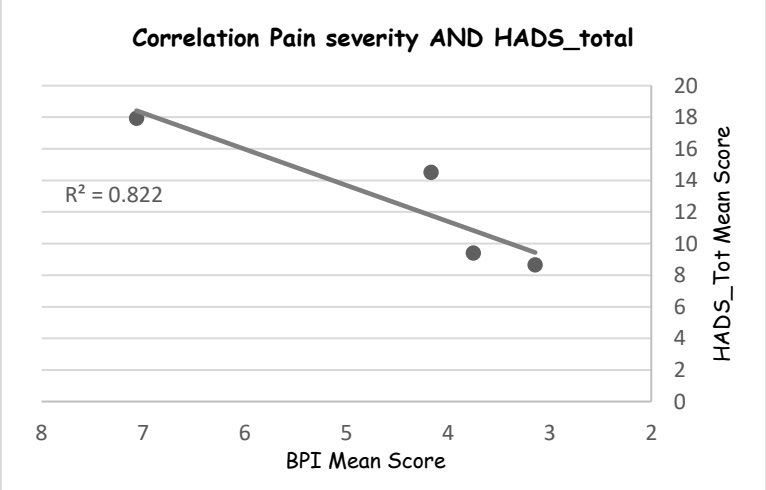


Figure 29. Evaluation of possible correlation between Pain severity and HADS_total.

Correlation has been calcutaed with Pearson's Correlation but varibles don't appear correlated.

4.2.4 Gene Expression

Nowadays, of 15 patients enrolled and with SCS definitive implant, only 8 have been analyzed for inflammatory gene expression, since enrollment visit to follow up at 6months; due to COVID-19 emergency the other patients follow-up visits and new enrollments have not been possible. Gene expression analysis of IL-1, IL-2, IL-4, IL-6, IL-10, TNF-α and INF-γ have been conducted and, as it shown in Figure 30, expression of pro-inflammatory cytokines TNF-α, IL-1 and IL-6 appears to have a decrement trend after SCS implant up to 3 months (t4). However, only IL-1 results are significant, other are not significant due to low number of samples analyzed.

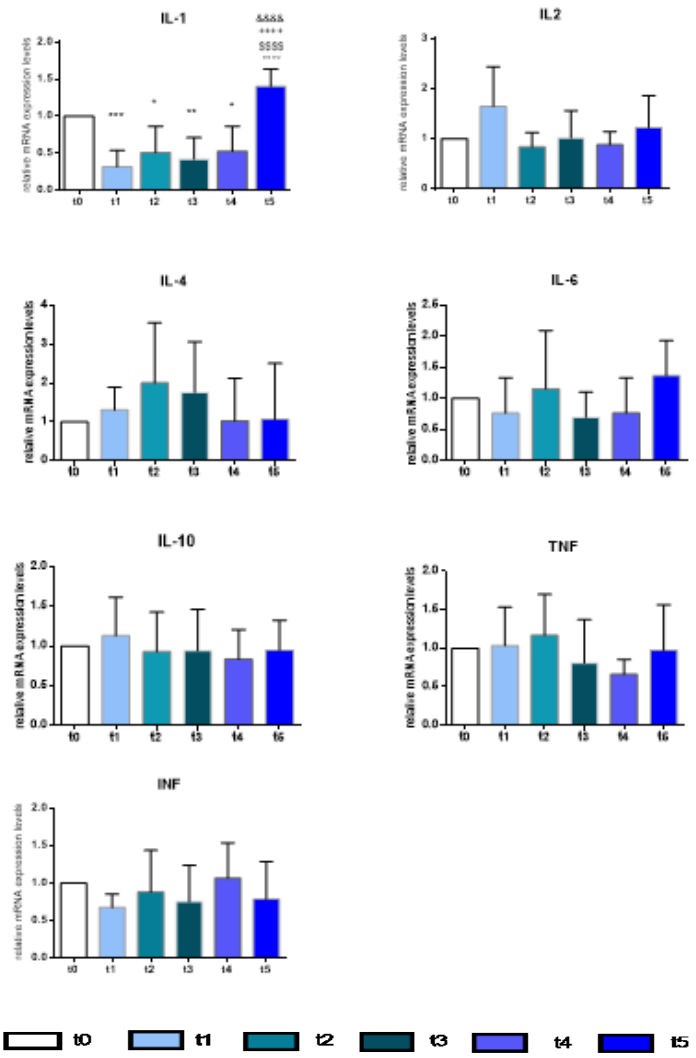


Figure 30 Effect of SCS implant on inflammatory component in human.

Results have been obtained by Real Time PCR and have been standardized on correspondent values of the endogenous control gene (GAPDH) and relative calibrator (t0 group). Values have been expressed as Mean of $2^{-\Delta\Delta Ct}$ ±SEM and n=8samples/group. Statistical analysis has been conducted with One-way ANOVA and Bonferroni's post-hoc test. *p<0.05, **p<0.01, ***p<0.001 vs t0; °°°°p<0.0001 vs t1; $$$$p<0.0001 vs t2; ++++p<0.0001 vs t3; &&&&p<0.0001 vs t4

Discussion

Chronic pain is one of most disabling conditions that affects many people every year in the word. It strongly influences quality of life causing sleeping disturbance, reduction of mobility and sociality, work absence, and cognitive alteration including onset of anxiety and depression. People main affected are more than 65 years old, in particular woman, in which are present aging physiological processes that influence or exacerbate pain sensation and management. Nowadays, gold standard treatment is represented by pharmacological therapy, often resulting in low pain relief and an improved risk to incur in adverse reactions. Causes of low efficacy of treatments can be multifactorial, including pharmacogenetics individual profile, multi treatments interference, delay to start a correct therapy, lack of completely knowledge of mechanisms underlying pain processes, its maintenance and chronicization. An example is represented by neuropathic pain, chronic pain resulting from a damage or disease within the somatosensory system. In the last years, new knowledges are emerged about neuropathic pathogenesis, adding to initially evidence that shown peripheral and central sensitization as principal mechanisms involved.[27, 28] In fact, it has been shown a pathological interaction between neurons, glia and inflammatory immune cells, with consequently released of pro- and anti- inflammatory cytokines implicated in maintenance of pain state.[29] Moreover, recently it has been suggested a relationship between pain and frailty state which appear share some mechanisms and could influence each other.[54]

Frailty is a clinical multifactorial condition that exposes patients to increased risk to incur in acute pathologies, hospitalization, comorbidity and mortality due to decreased capacity to response to stressor and to maintain homeostasis.[46] Pain is a relevant stressor that appears to be a risk factor to frailty insurgence, in old patients. Therefore, it is important found an appropriate pain treatment, personalized, timely and with low

risk of adverse effects in order to prevent its chronicization and, then, verify if a successful pain treatment could prevent or reverse frailty state too.[55]

To this purpose, in this project we have used two parallel models, animal and human, to better analysis each single component involved using mice model (age, pain and treatment), and at same time have a globally vision of clinical and psychological state of patients with chronic pain. Perception of pain has been investigated in both animal and human models. A recent research has shown a decreased capacity to activate pain inhibitory descendent pathways in old patients, suggesting an increased sensibility to pain stimuli due to alteration of central nervous system rather than peripheral, implicated in genesis and transmission of pain.[77]

About pain animal model has been used C57BL/6J mice young (11 weeks) and old (20 months) in which has been induced osteoarthritis by MIA induction at right knee by UNIMI. This approach allows having both damage structural, due to chondrocytes death and articular cartilage erosion, and central system, due to hypersensitivity onset in more distal sites. Pain and frailty states have been evaluated by UNIMI through behavioural tests. In particular, it is emerged that no basal difference are present between young and old mice in response to mechanical stimuli and with spontaneous pain. After OA induction, a significant increment of both spontaneous pain and hypersensitivity to mechanical stimuli has been shown in all MIA mice, independently from age factor, in confront to their corresponding CTR. However, through other functional test conducted by UNIMI, which results are not reported in this thesis, is emerged that pain presence is more disabling in old than young mice. To evaluate the role of treatment on pain symptomatology in different experimental conditions, it has been chosen to treat all mice whit morphine, being one of most drugs used in pain therapy. In fact, opioids use to treat non-oncologic chronic pain in old patients is

increased,[78] although they can have several side effects.[35]

All mice have been chronically treated with 2.5 mg/kg of morphine, since 7th day after OA induction to 14th day. It is emerged that morphine treatment is able to restore basal thresholds about both mechanical and spontaneous pain, in both young and old mice, after seven days. These results are parallel to restore of functional activities shown in MIA treated mice, in particular in old mice in which physical disability was more marked before treatment (data not shown). Moreover, analyzing all different mice groups about their frailty status, it has emerged that pain impact significantly frailty status in both young and old in confront to corresponding control without pain, but in MIA old mice FI has resulted higher than young and more disabling due to lack of functional available reserves aging-related. In accordance with results obtained from behavioral nociceptive tests, chronic treatment with morphine is also able to improve frailty status, sustaining the hypothesis that a successful pain treatment could influence frailty, preventing its worsening.

Recent evidences show immune system involvement in pain, therefore, we have analysed expression of some inflammatory and neuronal markers at spinal (L3-L5 area that receives afferences from knee) and supra-spinal level (hypothalamus, hippocampus, frontal and pre-frontal cortex). In fact, activation of neurons and glia cells at spinal cord has been identified with consequently increase of neuropeptides released. At spinal cord, OA induces in both MIA young and old a severe neuroinflammation in confront to corresponding CTR, showed by increased gene expression of all glial and microglial markers, sustaining an activation of non neuronal component, but also an increase of ATF3 expression level, suggesting neuronal damage presence. These results appear more evident in old mice probably due to Inflammaging.[50,51] Moreover, an increased expression of TNF-α and TLR4 has been

observed in old CTR mice in confront to young CTR, indicating the presence of an activated inflammatory pathway also when pain status is not present. Particularly evident has resulted a significant increased expression of all markers in old MIA in confront to young MIA, underlining a relevant role of aging on activation of inflammatory pathways. About opioids treatment impact on neuroinflammation, it has been identified a significant reduction in both young and old MIA treated in confront to corresponding MIA non treated and, moreover, this effect has resulted more evident in old MIA treated in confront to young MIA treated. In order to understand if this anti-inflammatory effect was related to drug or to pain relief obtained, it has been considered modulation of inflammatory mediators gene expression in old CTR mice treated with morphine without pain. The absence of gene expression variations suggests that anti-inflammatory modulation observed in MIA treated is related to pain relief rather than to drug.

At supra-spinal level, instead, it has been identified an increased level of IL-6, TNF-α and CD-11 expression in old mice in confront to corresponding young condition, but no effects has been shown related to pain or morphine treatment. This could be due to a pain stimulus not enough intense or the need to time course longer than 14 days to obtain involvement of cerebral areas considered.

About human study, until now 18 patients afferents of Pain Therapy Section of Hospital of Parma with chronic pain and eligible to SCS implant have been enrolled. 3 of them have dropped out of the study due to lack of benefit after implant. The others 15 patients have been followed since enrolment (t0) to 6 months after SCS definitive implant (t5). Patients are submitted to pain and psychological evaluation through tests during every clinical visit. Due to lack of standard protocol to frailty diagnosis, it has been assumed frailty status in accordance with cognitive impairment (including anxiety,

depression and tendency to pain catastrophizing) and physical disability (including difficulty in daily activities performance). Possible changes in pain and frailty status, and their potential correlation, have been investigate before and after implant. In general, it has been registered a global improvement of pain relief after SCS implant as well as quality of life, sustained until 6 months later. Patients have shown a significant reduction of pain interference with daily activities such as walking, lift objects, bend, and working. In order to understand if these global successes influence each other, correlation between variables considered has been investigated. It is emerged that pain severity positively correlates with pain interference in daily activities and negatively with quality of life. Therefore, when pain severity decreases, thanks to good SCS outcome, also frailty, in term of better quality of life and decrement of physically disability, improves.

About psychological state, instead, anxiety, depression and tendency to pain catastrophizing result improved after SCS implant but no correlation with pain severity has been found. These variables have been also studied in order to understand if starting cognitive state could influence good treatment outcome, but until now no differences have been identified, probably due to low number of samples as well as it has not been possible analyze ATQ results. Finally, similarly to mice model, also on human PBMCs samples, inflammatory state has been evaluated at different time point, before and after SCS implant through gene expression analysis. However, only 8 patients has been evaluated at all time points due to loss of samples collection of some patients during COVID-19 emergency. Therefore, these results are preliminary and require a collection enrichment; with higher number of samples, we will also perform protein analysis on PBMCs supernatants collected, in order to identify cytokines released by human immunity cells differently stimulated.

Conclusions

Although results obtained until now are very preliminary due to few samples collected, these could suggest that presence of chronic pain can be a risk factor to frailty onset, as well as demonstrated in old mice (UNIMI) and in old humans, in which a strong physical disability and psychological impairment have been reported, before treatment starts. Furthermore, high levels of neuroinflammation observed in old MIA mice induced by pain could suggest a favorable condition to insource of several aging diseases such as degenerative disease, including Parkinson's and Alzheimer's pathologies. Pain treatment has positively resulted to influence frailty status in both mice and humans. In the latter it has been evidenced a correlation between pain severity and some frailty symptoms, including physical disabilities and quality of life, which improve parallel to pain relief increment. However, a major number of patients is need in order to better understand the role of treatment on inflammation and psychological factors.

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