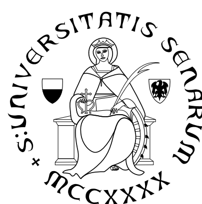

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Exploration of the relationship between diet and inflammatory mediators in chronic pain syndroms

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ABSTRACT

Introduction: Chronic pain represents a significant clinical challenge, impacting daily functioning and overall quality of life. Several studies have demonstrated the relationship between diet and chronic pain disorders, but it is still unclear how nutrition influences chronic pain. The body recognizes foods through immunoglobulin G (IgG) mapping which contributes to the creation of a personal food profile. High levels of IgG4 activate inflammatory processes. Moreover, gut inflammation amplifies the transcription of pro-inflammatory factors, increases intestinal permeability and influences various metabolic pathways, such as tryptophan metabolism (in particular the serotonin and kynurenine pathways). Exosomes, biological microvesicles, are produced by the gut and they carry signaling molecules, contributing to regulatory processes.

Methods: In the human part, in two different steps, two groups of subjects suffering non-oncological chronic pain were recruited and several questionnaires about their health, especially concerning pain, gastrointestinal conditions and psychological state, were administered. IgG4 were determined in the first step. The Dietary Inflammatory Index (DII) and tryptophan metabolites were analyzed in the second step. All subjects were asked to follow a diet for 4 weeks (IgG4 antibody-guided exclusion diet, first step, or anti-inflammatory diet, second step). In the in vitro part, a gut barrier inflammation model was set up utilizing Caco-2 cells with lipopolysaccharides (LPS) and/or serotonin. Pro-inflammatory cytokines (IL-1β and IL-6) were measured in cell supernatants. Exosomes were isolated from Caco-2 cell supernatants; they were characterized and their incorporation into nervous system cells was evaluated.

Results: 54 subjects (43 women and 11 men) were included in the first step of the study and 38 women were included the second step. In the first step, at visit 1, most subjects showed medium/high levels of IgG4 to at least one food. In the second step, all subjects had inflammatory DII values. Pain conditions and quality of life parameters were improved in the chronic pain sufferers with both nutritional approaches. In the second step, the chronic pain intensity was correlated with the subjects' gastrointestinal functions and there was a significant correlation between VAS intensity and some urinary tryptophan metabolites (quinolinic acid, QA, and 5-hydroxyindole acetic acid, 5-HIAA).

Caco-2 cells treated with LPS for 24 hours released pro-inflammatory cytokines (IL-1β and IL-6). Exosomes released by Caco-2 cells were on the order of 10^{10} particles/mL, with a size of approximately 100 nm or slightly larger, and they were positive for the markers CD-9, CD-81, and TSG-101. Exosomes released by Caco-2 cells were incorporated into central nervous system cells (neurons and astrocytes) at 6 hours.

Conclusions: Pain conditions and other quality of life parameters were improved in the chronic pain sufferers merely from a change in food consumption. Moreover, the communication between nutrition/gut and brain seems to be supported by microvesicles called exosomes.

INTRODUCTION

Pain

The official definition of pain was given by the International Association for the Study of Pain (IASP) in 1979: "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage. It is a subjective and individual experience, involving sensory (nociceptive) components related to the transmission of painful stimuli from the periphery to central structures, as well as experiential and affective components that significantly modulate the perceived pain."
Pain is present throughout the evolutionary scale as it is essential for survival. Physiological pain serves as an alarm signal perceived in the event of damage (visible or invisible) to the organism. In its absence, individuals may incur traumas or diseases that are difficult to recognize and can lead to death if left untreated. Thus, pain plays a crucial protective function by warning of potential tissue damage, which can be avoided or corrected. Physiological pain is localized, of short duration, and disappears once the damage isresolved. Additionally, it induces an emotional response and vegetative and motor reflexes,thanks to which tissue damage is prevented or limited. The affective and emotional aspects of pain perception, being individual and subjective, distinguish this sensory mode from all others. The characteristics of urgency and primordiality are responsible for the emotional nature of the painful experience.
In addition, pain can be pathological, presenting as a self-sustaining phenomenon (pain syndrome) (Mannion & Woolf, 2000).

Nociceptive fibers

Noxious stimuli activate nociceptors, specialized sensory receptors consisting of peripheral terminations of primary sensory neurons with cell bodies located in the dorsal root ganglia of the spinal cord and in the Gasser ganglion, associated with the trigeminal nerve.

The fibers responsible for pain transmission are called nociceptive fibers and come in two types: C fibers and Aδ fibers. C fibers, innervating deep tissues, are small and unmyelinated, leading to slow transmission, while Aδ fibers, innervating the skin and deep tissues, are small-caliber myelinated fibers, allowing for faster transmission of the painful stimulus. The presence of these two different types of nociceptive fibers results in the "double pain" phenomenon. The two perceptions are separated by a short temporal interval: initially, there is the "first pain," rapid and transmitted by Aδ fibers, characterized by a well-localized pricking sensation felt only at the level of the skin and mucous membranes and not exceeding the duration of the stimulus. The "second pain" is slower as it is transmitted by C fibers and is characterized by a diffuse sensation felt both at the skin level and in deep tissues, exceeding the duration of the stimulus.

Nociceptors can respond to chemical, mechanical, and thermal stimuli, leading to the identification of three main classes of nociceptors: thermal nociceptors, mechanical nociceptors, and polymodal nociceptors, as well as a fourth class, less understood, known as silent nociceptors that are distributed in the viscera. Thermal nociceptors (Aδ fibers) are activated by thermal stimuli above 45°C and below 5°C; mechanical nociceptors (Aδ fibers) are activated by high-intensity pressure stimuli applied to the skin; polymodal nociceptors (C fibers), on the other hand, can be activated by intense mechanical and thermal stimuli, as well as chemical stimuli.

Harmful stimuli depolarize the free nerve endings of afferent fibers and generate action potentials that propagate toward the central nervous system. Depolarization is enabled by receptors in the nociceptor membrane that converge the thermal, mechanical, and chemical energy of noxious stimuli into a depolarizing electrical potential. Receptors involved in pain signaling are diverse and distributed differently, both within the same nociceptive fiber and among different nociceptor subpopulations. For example, in the peripheral terminal of the ganglion neuron, there are receptors activated by thermal and chemical stimuli, including some members of the transient receptor potential (TRP) ion channel family, purinergic receptors activated by adenosine triphosphate (ATP), and prostaglandin receptors.

Pain pathways

The nociceptive sensation is transmitted to the dorsal horn of the spinal cord, where the following components are present: intrinsic reflex fibers, which induce an immediate response that affects motor neurons, leading to a peripheral change of action, and ascending

fibers (contralateral), which integrate signals at a higher level. They reach the elongated spinal cord and ascend towards the thalamus, from which they project to the sensory cortex, amygdala, insula, and cingulate gyrus. The insula processes information related to viscera and contributes to elaboration of the vegetative component of responses to painful stimuli. The cingulate gyrus is involved in the processing of emotional states accompanying pain (see Figure 1).

This process illustrates the complex network of pathways involved in the transmission and processing of nociceptive signals, encompassing both immediate reflex responses and higher-level integration, ultimately contributing to the sensory and emotional aspects of the pain experience.

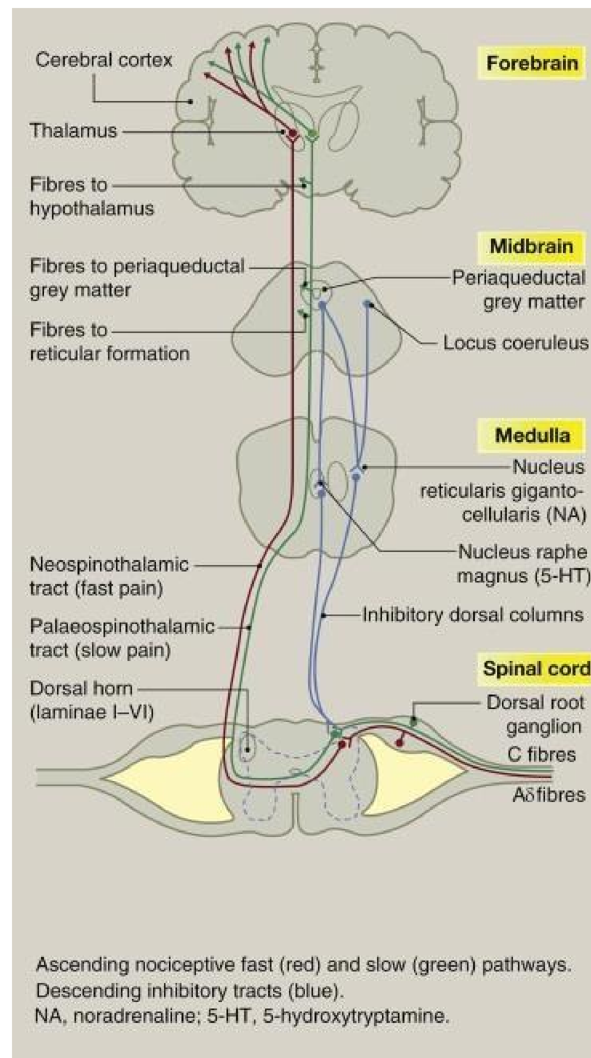


Figure 1. **Anatomy of the pain pathway.** Primary afferent nociceptors transmit noxious information to projection neurons within the dorsal horn of the spinal cord. A subset of these

projection neurons conveys information to the somatosensory cortex through the thalamus, providing details about the location and intensity of the painful stimulus (Steeds, 2016).

Nociceptive information is modulated at multiple levels by endogenous inhibitory control systems, many of which are tonically active. In specific situations, such as stressful events or conditions deemed potentially dangerous, a reduction in neural and behavioral responses to noxious stimuli may occur, leading to induced analgesia. In these circumstances, the key molecules involved are endorphins and endocannabinoids.

One of the nociception control mechanisms is the "gate control theory" proposed in 1965 by R. Melzack and P. Wall. This theory suggests the existence of neural mechanisms capable of inhibiting nociceptive transmission at the level of the dorsal horns of the spinal cord. It is based on the hypothesis of the convergence of information from different sensory modalities, with the fibers involved being nociceptive fibers and tactile and pressure fibers of type Aβ. When the latter are stimulated, they activate inhibitory interneurons that block the transmission of any painful stimuli (closed gate); conversely, if the activity of nociceptive fibers prevails, they inhibit the interneurons, allowing the painful stimulus to be transmitted (open gate).

In addition to the aforementioned theory, the presence of inhibitory modulation of nociceptive circuits by descending serotoninergic fibers from the raphe nucleus (RN) and noradrenergic fibers from the locus coeruleus (LC) has been demonstrated. These endogenous antinociceptive systems act both at the spinal level and at the level of the medial thalamus.

Various areas of the cerebral cortex can also modulate the activity of spinal nociceptive neurons, both directly and through connections with other brainstem centers. This explains the ability of cognitive-attentional factors to control pain.

Types of pain

Pain can be classified into various types depending on etiopathological and clinical characteristics. We can distinguish between acute and chronic pain. Acute or physiological pain is short-lived and essential for survival. When the nociceptive stimulus becomes prolonged (longer than 3 months), it is referred to as chronic pain. Chronic pain persists even after the resolution of the triggering cause.

Sensitization

Sensitization of the somatosensory system induced by nociceptors is adaptive as it makes the system hyper-alert in conditions where the risk of further damage is high. This sensitization is an expression of synaptic plasticity triggered in the central nervous system (CNS) by nociceptor input. Various forms of functional, chemical, and structural plasticity can sensitize the central nociceptive system to produce hypersensitivity to pain in both normal and pathological circumstances, some of which are persistent. Sensitization is represented by an increased response to noxious stimuli (hyperalgesia) or the induction of pain following innocuous stimuli (allodynia).

Sensitization can be triggered by various chemical mediators released by injured cells accumulating at the site of tissue damage. These mediators include peptides and proteins such as bradykinin, substance P (SP), nerve growth factor (NGF), as well as compounds like ATP, histamine, serotonin, prostaglandins, leukotrienes. Many of these mediators are released by different cell types, all contributing to lowering the nociceptor activation threshold. These chemicals lead to the release of two neuroactive peptides: SP and calcitonin gene-related peptide (CGRP). They play a crucial role in tissue inflammation and are also responsible for the axon reflex, a physiological process characterized by vasodilation in tissues adjacent to a lesioned skin area. Additionally, these neuropeptides lead to further histamine release by mast cells, activating polymodal nociceptors.

In addition to low-molecular-weight compounds and peptides, neurotrophins can also induce or maintain pain. For example, NGF is particularly active in painful conditions of inflammatory origin.

Central sensitization can be distinguished from peripheral sensitization, and although the two phenomena may seem comparable, they are fundamentally different. Peripheral sensitization involves a reduction in threshold and an amplification of the reactivity of nociceptors when the peripheral terminals of these high-threshold primary sensory neurons are exposed to inflammatory mediators and damaged tissue. Consequently, it is limited to the site of tissue injury. While peripheral sensitization certainly contributes to sensitizing the nociceptive system and thus to inflammatory pain hypersensitivity at inflamed sites (primary hyperalgesia), it represents a form of pain triggered by nociceptor activation, albeit with a lower threshold due to increased sensitivity to peripheral transduction. Generally, it requires an ongoing peripheral pathology for its maintenance. Peripheral sensitization seems to play a significant role in thermal alteration but not in mechanical sensitivity, which is an

important feature of central sensitization.
Unlike peripheral sensitization, central sensitization involves pathways not typically dedicated to nociception, such as large myelinated fibers of low-threshold mechanoreceptors, being recruited to produce pain. Additionally, it produces pain hypersensitivity in non-inflamed tissue by altering the sensory response elicited by normal inputs. It increases pain sensitivity long after the initial cause may have disappeared and when there may be no ongoing peripheral pathology. Since central sensitization stems from changes in the properties of neurons in the central nervous system, pain is no longer associated, as acute nociceptive pain is, with the presence, intensity, or duration of particular peripheral stimuli. Instead, central sensitization represents an abnormal state of nociceptive system reactivity. Pain is generated as a consequence of changes within the central nervous system that alter how it responds to sensory inputs, rather than reflecting the presence of peripheral noxious stimuli.
Persistent pain can be broadly distinguished into two major classes: neuropathic pain and inflammatory or nociceptive pain. Inflammatory or nociceptive pain arises from the activation of cutaneous or soft tissue nociceptors in response to tissue damage, typically inducing a massive release of inflammatory cytokines. Furthermore, increased neuroinflammatory mediators activate microglia, responding by releasing pro-inflammatory cytokines. These cytokines can induce a change in the protein profile of neurons.
Neuropathic pain, typically chronic, can be linked to various causes such as trauma, diabetes, infections, chemotherapy, etc. These conditions may lead to direct damage to peripheral or central nerve fibers. This pain often presents as a burning sensation.

Astrocytes and chronic pain

Chronic pain is an expression of peripheral plasticity regarding the peripheral nervous system (Gold and Gebhart, 2010) and central plasticity regarding the central nervous system (Ji et al., 2003). The most widely studied neuronal mechanisms are hyperexcitability and sensitization of primary sensory neurons (peripheral sensitization) and enhancement of excitatory synaptic transmission in spinal cord, brainstem, and cortical neurons (central sensitization), caused by transcriptional, translational, and post-translational regulation. Other neuronal mechanisms include disinhibition (reduced inhibitory synaptictransmission), descending pathway facilitation (e.g., from the brainstem to the spinal cord),and long-term potentiation (LTP) in the cortex and spinal cord.

In recent years there has been increased recognition of the importance of non-neuronal cells, especially glial cells, in the initiation and maintenance of chronic pain. Glial cells in the CNS consist of three major groups: astrocytes, microglia, and oligodendrocytes (Gao and Ji, 2010). Glial cells in the peripheral nervous system consist of satellite glial cells in the dorsal root ganglia and trigeminal ganglia and Schwann cells in the peripheral nerves.

Astrocytes are the most numerous cells in the CNS and constitute 20-40% of all CNS glial cells. Historically, they were thought to primarily provide structural and nutritional support to neurons; however, it is now known that they also play an active role in many critical neural processes (Ben Haim and Rowitch, 2017). Unlike other glial cells, astrocytes are the only cells in physical contact with each other through protein complexes called gap junctions, which directly connect the cytosol of adjacent cells to allow free exchange of ions and small cytosolic components (Giaume and McCarthy, 1996). Additionally, a fundamental feature of astrocytes is their extensive contact with cerebral blood vessels, allowing them to modulate blood flow during neuronal activation (Iadecola and Nedergaard, 2007); their involvement in the composition of the blood-brain barrier (BBB) is crucial for communication between the interior and exterior of the CNS.

It is estimated that a single astrocyte can ensheath 140,000 synapses and contact 4 to 6 neuronal cell bodies, as well as interact with 300 to 600 neuronal dendrites in rodents (Oberheim et al., 2009). Close contact with neurons and synapses allows astrocytes not only to support and nourish neurons but also to regulate the external chemical environment during synaptic transmission. The growing appreciation of active roles of astrocytes has led to the proposal of a "tripartite synapse" theory. It is based on the facts that glia respond to neuronal activity with an elevation of their internal calcium (Ca2+) concentration and trigger the release of chemical transmitters from glia themselves, and glial transmitters cause feedback regulation of neuronal activity and synaptic strength. According to this theory, astrocytic processes are active components of synapses, in addition to pre- and postsynaptic components (Araque et al., 1999).

The modulation of synaptic transmission in astrocytes does not seem to be solely due to Ca2+, since potassium (K+) also appears to play a fundamental role. One of the essential tasks of astrocytes is to maintain K+ homeostasis. Recently, it has been demonstrated that increases mediated by astrocytic Ca2+ receptors can modulate neural network activity through the active uptake of extracellular K+ (Wang et al., 2012). Because the extracellular concentration of K+ is an important determinant of the resting membrane potential and

thereby of neuronal activity, active uptake of K+ represents a simple yet powerful tool for rapid modulation of neural networks. In addition to K+, astrocytes are physiologically involved in the modulation of glutamate and water levels (Verkhratsky et al., 2012; Simard and Nedergaard, 2004). Astrocytes express glutamate transporters, K+ channels and water channels.

Glutamate is the primary excitatory neurotransmitter modulating nociceptive networks. The glutamate transporter 1 (GLT1) and glutamate-aspartate-transporter (GLAST), which are highly expressed in astrocytes, modulate extracellular glutamate levels to maintain homeostasis of the CNS in normal physiological conditions.

Regulation of glutamate levels in the synaptic cleft is mediated by transporters responsible for the reuptake of extracellular glutamate; GLT1 is responsible for 90% of glutamate clearance in the spinal cord (Robinson et al., 1998) and it is critical in pain signaling termination. GLT1 is downregulated in multiple models of neuropathic pain (Sung et al., 2003).

Glutamate is crucial for the chronicization of pain as it is the neurotransmitter involved in synaptic plasticity underlying the maintenance of pain. At glutamatergic synapses, astrocytically released glutamate has been suggested to play a crucial role in mediating neuronal-glial circuits. Astrocytes not only clear presynaptically released glutamates during synaptic transmission, but can also release glutamate via diverse pathways (Takano et al., 2005) in response to increased intracellular Ca2+ concentration by activation of G-protein coupled receptors expressed at the astrocytic membrane. In turn, this Ca2+-dependent glutamate release from astrocytes can be sensed by presynaptic or postsynaptic glutamate receptors such as the metabotropic glutamate receptor (mGluR) (Perea and Araque, 2007) or N-methyl-D-aspartate (NMDA) receptor (Fellin et al., 2004), both of which are known to modify presynaptic and postsynaptic activities or synaptic plasticity.

Chronic neuropathic pain can arise due to astrogliopathy, in which the normal capacity of astrocytes to maintain CNS homeostasis is disrupted. Astrogliopathy results in abnormal extracellular levels of glutamate. These astrocyte-driven pathologies lead to neuronal hyperexcitability and neurotoxicity, neuroinflammation, and chronic pain. Importantly, astrocytes promote and maintain chronic pain conditions via neuron–glial and glia–glial interactions; astrocyte-produced mediators such as cytokines and chemokines are powerful neuromodulators regulating both excitatory and inhibitory synaptic transmission in the pain circuit, leading to central sensitization (Kawasaki et al., 2008).

The Enteric Network

Interactions between the immune and gut systems

The stomach, small intestine, and large intestine are parts of the gastrointestinal (GI) tract responsible for digestion, absorption, and secretion. Histologically, the intestinal tissue consists of several compartments: mesentery and serosa, muscular layer, submucosa, lamina propria, epithelium, and lumen (Figure 2). The mesentery is the compartment through which blood vessels, lymphatic vessels, and nerve fibers pass. It also encloses the mesenteric lymph nodes, which are the lymph nodes draining the intestine. The mesentery is adjacent to the serosa, the outermost layer of the mesothelium that encapsulates and lubricates the gastrointestinal tract. The muscular layers are the outermost layers, consisting of an external longitudinal muscle layer and an internal circular muscle layer.

Peyer's patches and lymphoid follicles, immune structures, emanate from the submucosa and extend into the mucosa, which consists of the lamina propria and the innermost epithelial layer. The lamina propria contains many innate and adaptive immune cells but is also composed of connective tissue with a structural function. Peyer's patches are circular plaques, each consisting of lymph nodules (from 10 to over 200) joined by interfollicular lymphoid tissue. They are part of mucosa-associated lymphoid tissue (MALT). The epithelium lining them contains scattered M cells among enterocytes. These cells actively internalize antigens adhering to the intestinal mucosa.

The intestinal mucosa is a dynamic environment that continuously interacts with billions of bacteria and occasionally with pathogens of various kinds. This thin layer is constantly renewed (every 3-5 days). The intestinal epithelium is an active component of immunity, as below it there are immune cells necessary for the defense of the organism, anatomical structures collectively referred to as the gut-associated lymphoid tissue (GALT). This complex system is responsible for initiating and regulating the immune response at the intestinal level. The gastrointestinal tract is one of the largest surfaces of the body constantly facing the external environment. In order to maintain intestinal balance, the immune system must be able to recognize potential threats and trigger a proper inflammatory response, while also demonstrating tolerance towards foods and other harmless molecules. If this mechanism does not function as it should, there is an alteration of the immune response and the onset of chronic inflammation with the consequent production of molecules called cytokines, which can activate or inhibit the activation of defense cells.

Furthermore, there is a strong relationship between the microbiota, the intestinal mucosa, and the immune system. We know, in fact, that the type of immune response is influenced by the integrity of the intestinal barrier and the balance between normal flora and pathogenic microorganisms within the intestinal microbiota. An alteration of the intestinal bacterial flora is referred to as dysbiosis.

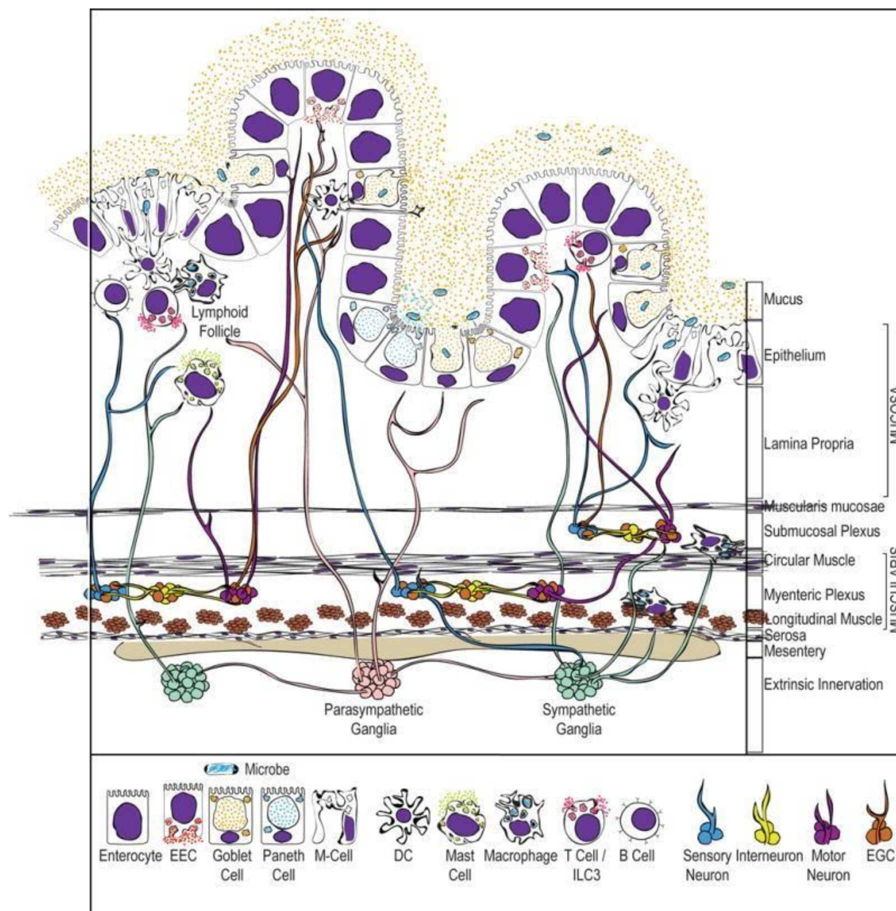


Figure 2. **Anatomy of the gastrointestinal tract** (Yoo and Mazmanian, 2017).

Gastrointestinal dysfunction is often a comorbidity with numerous non-intestinal conditions. Therefore, interactions between interdependent cellular pathways in the intestine and the periphery may underlie processes involved in health and disease. Homeostatic communication through the intestinal epithelium involves the contribution of diet and the microbiota, interacting with the mucosal immune system and the enteric nervous system (ENS).

The gut-brain connection

Our gut is intricately linked to our brain: the communication is bidirectional, meaning the brain communicates with the gut and vice versa (Figure 3). A key player in this communication is the intestinal microbiota, a community of microorganisms residing within the intestine. Organisms within the intestinal microbiota interact with each other and with the human body in complex and dynamic ways. A well-balanced microbiotic composition and its subsequent maintenance are considered crucial for human health, as disruptions negatively impact health and increase the host's susceptibility to a wide variety of diseases, including behavioral and neuropsychiatric disorders (Penders et al., 2006). The central nervous system (brain and spinal cord) communicates with the gut through a complex communication network known as the gut-brain axis, which involves:

- Cytokines;
- Hypothalamic-pituitary-adrenal (HPA) axis – primarily involved in adaptive responses to stress including the limbic system in the brain;
- Autonomic nervous system (ANS) – driving both afferent (sensory) and efferent (motor) signals;
- Vagus nerve – thought to be the main driver behind the mind-body connection;
- Short-chain fatty acids (SCFAs) – produced when the good gut bacteria ferment fiber in the colon;
- Neurotransmitters – chemical messengers which transmit signals from neuron to neuron, neuron to muscle or neuron to gland;
- Gut permeability – transportation, absorption and balance of nutrients, immunity and tolerance of foreign substances.

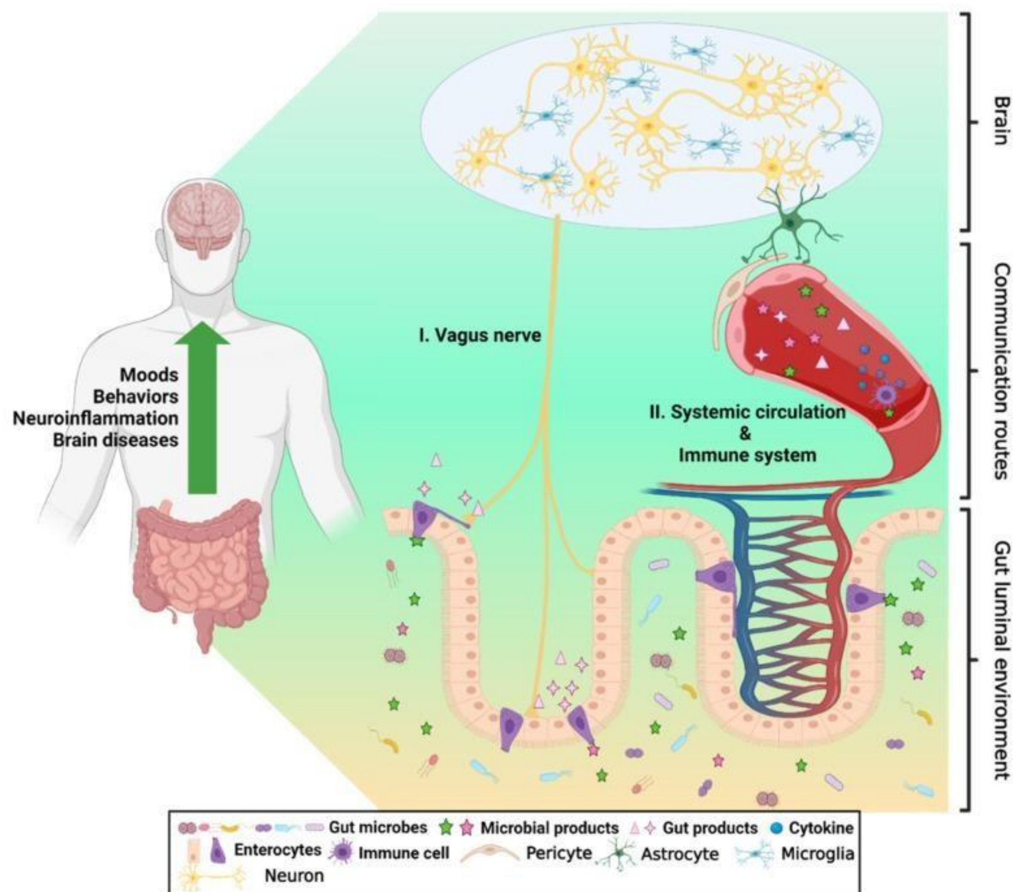


Figure 3. **Gut-brain axis.** The gut luminal environment, including the gut microbiome, affects the physiology and behavior of the brain via multiple routes (Kim et al., 2021).

Cytokines

Some cytokines regulate and modulate a wide range of inflammatory processes and can promote the induction of both autoimmune diseases and chronic inflammations. Cytokines are usually small proteins, typically released by immune cells, with a specific effect on the interaction and communication between the cells that released them and the "target" cells. Cytokines perform redundant activities, meaning that different cytokines can stimulate the same functions. This allows the body to manage many compensatory phenomena. They are often produced in a cascade mechanism activated when a specific cytokine stimulates its "target" cell to produce other cytokines with different effects. Cytokines can act both synergistically and antagonistically in relation to numerous other modulation factors. Inflammation is usually characterized by a constant increase in circulating levels of mediators such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, B-cell activating factor (BAFF), and platelet activating factor (PAF), whose combined action can affect both

innate and adaptive immune responses. Cytokines represent a signal of variation from normal homeostasis.

BAFF is a membrane protein belonging to the TNF superfamily consisting of 258 amino acids, produced and secreted by immune cells such as macrophages, monocytes, dendritic cells, and T cells, but also by adipose tissue cells, epithelial cells of salivary glands, astrocytes, and nasal and bronchial epithelial cells. The expression of BAFF increases in the presence of cytokines such as interferon INF-α, INF-γ and IL-10, and also after the activation of toll-like receptors (TLR) such as TLR4 or TLR9. BAFF is also produced by activated T cells and may play an essential role in the activation of innate immune cells, as it actively induces monocyte survival, the secretion of pro-inflammatory cytokines, and the expression of co-stimulatory molecules. At the molecular level, the binding of BAFF to its receptors activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) family for the transcription of factors that influence multiple aspects of normal and pathological cellular physiology. PAF is a phospholipid responsible for platelet degranulation and a mediator of inflammation (Reznichenko and Korstanje, 2015). PAF is secreted by a large number of immune system cells and muscle cells, except for smooth ones. Currently, there is only one known receptor for PAF, PAF-R. When it binds to its receptor, PAF activates different signals responsible for numerous biological functions such as the activation of cytoplasmic proteins that can be either stimulatory or inhibitory (Liu et al., 2016). Regarding the type of activated proteins, there will be different intracellular responses. For this reason, PAF can both promote and inhibit functions such as leukocyte activation, platelet aggregation, release of free radicals, and synthesis of cytokines involved in the inflammatory response such as IL-1, IL-6, and IL-8.

The role of the microbiome is also fundamental in cytokine production; the mucosal microbiota plays a vital role in the maturation of host immune system cells and protection against pathogens (Hansen and Sams, 2018; Belkaid and Mano, 2014). In particular, the microbiota orchestrates the local immune system in the intestine (Grigg and Sonnenberg, 2017) and shapes immune and non-immune cells located in distant sites and acting systemically (Kieper et al., 2005; McCoy et al., 2017).

HPA axis

The HPA axis is the central coordinator of the neuroendocrine stress response systems. It is composed of endocrine components in the hypothalamus, the anterior portion of the pituitary gland, and an effector organ, namely the adrenal glands. Following exposure to stress,

neurons in the paraventricular nucleus of the hypothalamus secrete corticotropin-releasing hormone (CRH) from nerve endings in the median eminence directly into the hypothalamic-pituitary portal circulation. CRH stimulates the production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH, in turn, stimulates the release of glucocorticoids from the adrenal gland cortex. Glucocorticoids modulate metabolism, as well as immune function and the brain, thereby coordinating the physiological adaptive response to stress. At the same time, various brain networks modulate the activity of the HPA axis. Specifically, CRH neurons in the paraventricular nucleus receive inhibitory control from the hippocampus and prefrontal cortex, while the amygdala and brainstem exert excitatory control. Additionally, glucocorticoids themselves exert negative feedback control of the HPA axis by regulating paraventricular nucleus and hippocampal neurons.

Behavioral stress can induce analgesia. In situations of danger (stressful situations), pain reactions can be suppressed to promote more adaptive behavioral forms. The duration of such analgesia can be on the order of minutes or hours, depending on the type of stressor. If an organism's natural response to emergency situations includes a decrease in pain sensitivity, then it becomes reasonable to assume that the pain inhibitory system, which uses opioid peptides, may be involved in this decrease. However, it is experimentally proven that stress can induce both opioid-mediated analgesia and opioid-independent forms. Every stressful physical agent increases the plasma levels of beta-endorphin, ACTH and corticosterone; however, not all stressors induce analgesia.

Furthermore, the vagus nerve is able to stimulate the HPA axis through a pathway leading to the secretion of corticotropin-releasing factor (CRF) from the hypothalamus. CRF stimulates the secretion of ACTH from the pituitary gland. This stimulation leads to the release of cortisol from the adrenal glands, aimed at reducing peripheral inflammation.

ANS

The sympathetic and parasympathetic innervation of the gastrointestinal tract and its role in modulating gastrointestinal function have been extensively examined (Jaenig, 2006). The overall effect of sympathetic activity on the intestine is inhibitory, slowing transit and gastrointestinal secretion. This inhibitory effect is largely achieved through inhibitory modulation of cholinergic transmission and a stimulatory effect on smooth muscle in sphincteric regions. Another subset of sympathetic postganglionic neurons is involved in mucosal immune modulation (Elenkov et al., 2000) and interacts with the intestinal flora and

mucosa (Lyte et al., 2010). Additionally, a noradrenaline-mediated reduction in the expression of TLR by intestinal epithelial cells has been observed (Gopal et al., 2008). Noradrenaline also plays a role in modulating the virulence of certain pathogens (Lyte et al., 2010), but the mechanisms through which intraluminal bacteria are exposed to noradrenaline released by sympathetic nerve endings are not clear.

Parasympathetic innervation consists of vagal and sacral divisions, which innervate the structures of the anterior and posterior intestine, respectively. Vagal motoneurons provide input to the stomach, small intestine, and the proximal portion of the colon. Excitatory vagal input reaches ganglia within the enteric nervous system (ENS) to mediate vago-vagal motor reflexes and the cephalic phase of gastric acid secretion; it also reaches enterochromaffin cells for the release of serotonin (5-hydroxytryptamine, 5HT) (Stephens and Tache, 1998). Finally, vagal modulation of macrophage activation through nicotinic acetylcholine receptors has been reported as part of a vago-vagal anti-inflammatory reflex (Pavlov and Tracey, 2005).

Vagus nerve

The vagus nerve is a mixed nerve composed of 80% afferent fibers and 20% efferent fibers. Thanks to its role in interoceptive awareness, it can receive information regarding the osmolarity of the intestinal lumen, the quantity of ingested carbohydrates, and the presence of certain types of metabolites produced by the intestinal microbiota. Through vagal afferent fibers, these messages are captured and sent to the CNS where, following integration processes in specific brain regions, including the amygdala, hypothalamus, and cortex, they lead to the generation of specific hormonal, motor, and behavioral responses.

An example of such signaling is the inflammatory reflex, a prototypical neural mechanism centrally integrated to modulate the immune response and maintain homeostasis. Tissue damage causes the local release of inflammatory cytokines, and their message is conveyed by vagal afferent fibers and integrated into the CNS, producing a specific response aimed at reducing peripheral inflammation. This “anti-inflammatory” response travels via vagal efferent fibers and, reaching the target, limits pro-inflammatory responses within a healthy, protective, and non-toxic range. The absence of this reflex, resulting from neural lesions or genetic ablation of essential components, leads to excessive innate immune responses and cytokine-induced toxicity.

Mechanisms mediating vagal anti-inflammatory capacities:

- HPA axis.
- Splenic anti-inflammatory pathway: The vagus nerve, by stimulating the splenic nerve, inhibits the release of TNF-α by spleen macrophages through α-7-nicotinic acetylcholine (ACh) receptors.
- Cholinergic anti-inflammatory pathway (CAIP): The vagus, interacting with enteric neurons, releases acetylcholine at the synaptic junction with macrophages. Acetylcholine binds to α-7-nicotinic ACh receptors on macrophages to inhibit the release of TNF-α. This pathway has high conductance speed, allowing immediate modulatory input to the affected inflammation site.

Several factors can reduce vagal activity and consequently impact its anti-inflammatory activity. The main ones are stress and obesity.

Stress inhibits vagal stimulation and simultaneously stimulates the sympathetic nervous system, promoting pro-inflammatory properties and inducing a prolonged increase in inflammatory cytokines even after the end of exposure to the stressful agent. Continuous exposure to multiple and repeated stressors favors an allostatic load that makes it challenging to recover parasympathetic tone.

The vagus nerve, by innervating the intestine, plays a significant role in metabolic control, conveying peripheral information on nutrient volume and type between the intestine and the brain. Depending on nutritional status, vagal neurons express two different neurochemical phenotypes that can inhibit or stimulate food intake. Chronic ingestion of calorie-rich diets reduces the sensitivity of vagal neurons to peripheral signals, contributing to hyperphagia and obesity.

Short-chain fatty acids

SCFAs are small organic monocarboxylic acids with a chain length of up to six carbon atoms. They are the main products of anaerobic fermentation by the microbiota of indigestible polysaccharides such as dietary fibers and resistant starch in the colon (Miller and Wolin, 1996; Louis and Flint, 2009; Pascale et al., 2018). Additionally, a small portion of SCFAs may derive from the metabolism of amino acids (Louis and Flint, 2017) through fermentation of proteins in the colon. They are primarily composed of acetate, propionate, and butyrate (Fernandes et al., 2014; Luu et al., 2019). After their production, SCFAs are absorbed by colonocytes (Vijay and Morris, 2014). SCFAs not metabolized in colonocytes are transported into the portal circulation and used as an energy substrate for hepatocytes

(Schönfeld and Wojtczak, 2016). Only a small fraction of acetate, propionate, and butyrate derived from the colon reaches the systemic circulation and other tissues (Cummings et al., 1987), including the CNS where they appear to have neuroactive properties (Fung et al., 2017; Tan et al., 2014; Dalile et al., 2019) (Figure 4).

In addition to crossing the BBB, SCFAs seem to play a crucial role in maintaining its integrity, central to brain development, and preserving the homeostasis of the CNS. They also play a role in microglial maturation and neuroinflammation (Erny et al., 2015). Several studies have reported that sodium butyrate is capable of reducing microglial activation and the secretion of pro-inflammatory cytokines (Patnala et al., 2017; Wang et al., 2018; Yamawaki et al., 2018) and that, in addition to modulating inflammatory cytokines, it appears to be involved in astrocyte signaling pathways.

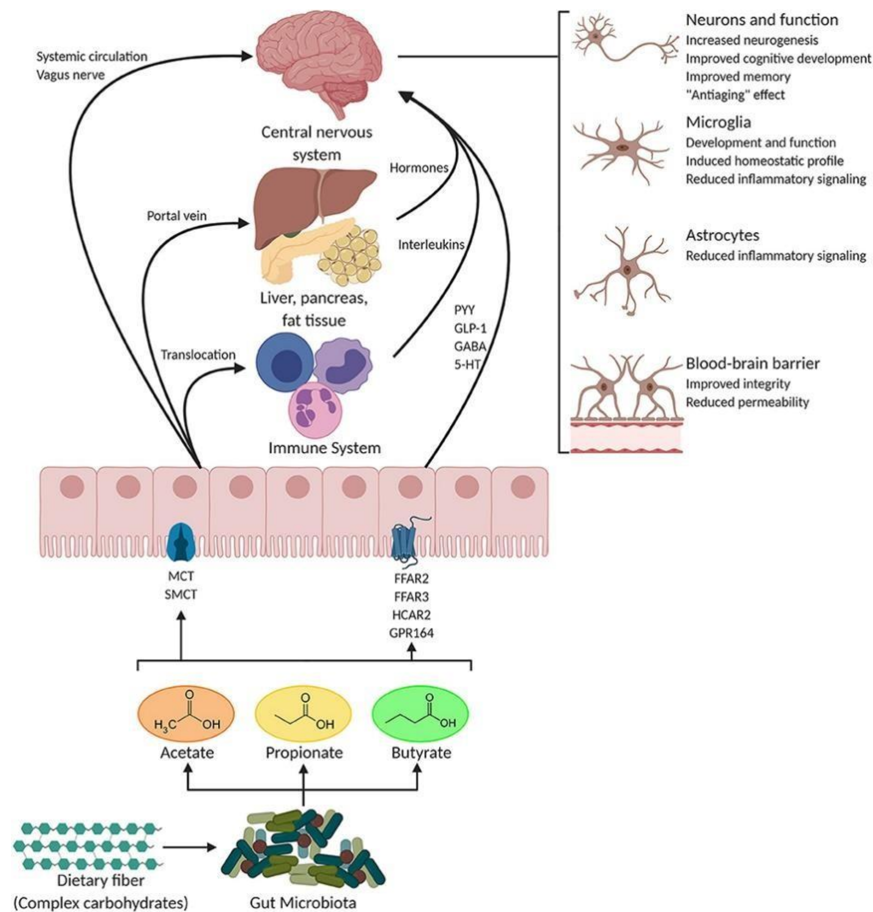


Figure 4. **Potential pathways through which SCFAs influence gut-brain communication** (Silva et al., 2020).

Neurotransmitters

The intestine can also modulate the synthesis of various neurotransmitters, particularly through the action of the microbiome. Among the neurotransmitters modulated at the intestinal level are gamma-aminobutyric acid (GABA), 5HT, dopamine (DA), and noradrenaline (NA) (Sherwin et al., 2018; Fung et al., 2017; Calvani et al., 2018). These neurotransmitters can potentially influence microglial activation and various brain functions (Abdel-Haq et al., 2019).

Serotonin:

Serotonin is a key regulator of gastrointestinal motility and secretion (Gershon and Tack, 2007; Mawe and Hoffman, 2013). The majority of serotonin (>90%) in the human body is produced by enterochromaffin (EC) cells in the intestine, where it is synthesized by the rate-limiting enzyme tryptophan hydroxylase (Tph/TPH) 1 and stored in secretory granules before release (Gershon, 2013).

Potential signal transducers involved in microbiota communication with the CNS include enterochromaffin cells, which can bind various microbial products and secrete serotonin into the lamina propria, increasing serotonin concentrations in the colon and blood (Reigstad et al., 2015; Yano et al., 2015).

Dysfunction of serotoninergic systems (Crowell, 2004) and dysbiosis of the intestinal microbiota (Ohman and Simrén, 2013) have been implicated in subgroups of irritable bowel syndrome (IBS), depression, and other systemic disorders. The effects of intestinal microbiota on serotonin homeostasis at mucosal, neuronal, and systemic levels are crucial for various pathophysiological conditions in humans.

Permeability

The gut provides an essential barrier consisting of an epithelial monolayer seated on the basal membrane. The intestinal epithelium plays a central role in the barrier function and in maintaining homeostasis. Intestinal epithelial cells (IEC) interact with both the luminal microbes as well as submucosal immune cells (Figure 5). In addition, IEC have a high turnover, and barrier integrity is dependent on continuous renewal of epithelial cells but also on the balance between protein synthesis and proteolysis. The paracellular permeability between IEC plays a major role in the regulation of intestinal permeability. The paracellular permeability of the intestinal barrier is regulated by a complex protein system called tight

junctions (TJ) (Turner et al., 2014). The TJ are composed of multiple proteins including transmembrane proteins, and in particular, occludin and the claudins family. These transmembrane proteins interact with cytosolic proteins, including the zonula occludens protein family, which also interact with the F-actin cytoskeleton. The association of these proteins is highly dynamic and contributes to regulation of the epithelial barrier.
Glutamine is the most abundant amino acid in plasma and plays a key role in maintaining the integrity of the intestinal barrier, particularly in the expression of tight junction proteins under various conditions. It has been observed that glutamine depletion leads to villi atrophy, reduced expression of tight junction proteins, and increased intestinal permeability (Bertrand et al., 2013). Furthermore, glutamine supplementation has been shown to enhance the function of the intestinal barrier in various experimental and clinical conditions. It appears that the protective effect of L-glutamine is likely mediated by transactivation of the epidermal growth factor receptor (EGFR) leading to activation of protein kinase C and mitogen-activated protein kinases, which could induce the expression of TJ proteins (Rao and Samak, 2012).

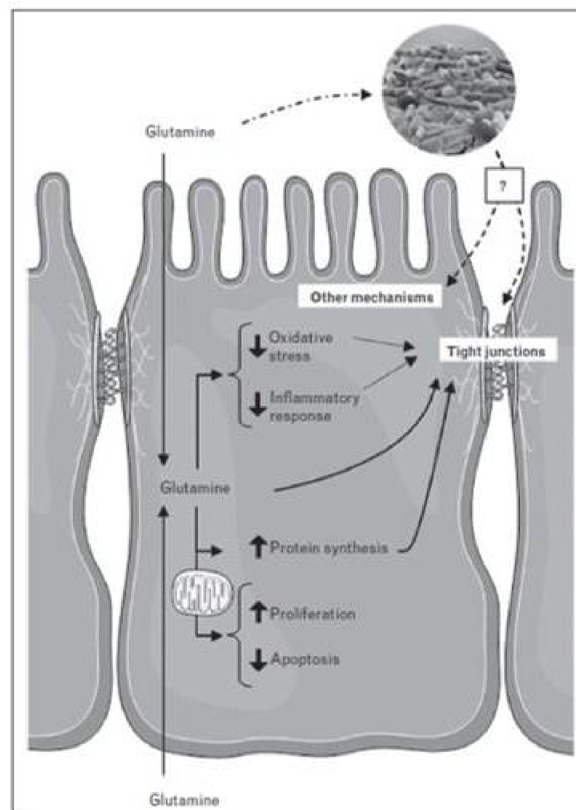


Figure 5. **Putative mechanisms of glutamine effects on gut barrier function** (Achamrah et al., 2017).

Increased permeability of the intestinal barrier can also allow the translocation of bacterial products, leading to elevated cytokine levels and subsequent induction of chronic systemic inflammation. This may impact the BBB, resulting in more intense harmful effects (Dinan and Cryan, 2017). It is now widely recognized that peripheral insults triggering a systemic inflammatory response could influence ongoing inflammation in the CNS primarily through microglial activation, the production of inflammatory molecules, and the recruitment of peripheral immune cells into the brain. This shapes an inflammatory brain environment that can significantly compromise neuronal function (Perry et al., 2003).

DIET and PAIN Syndromes

Dietary basis of inflammatory diseases

The relationship between nutrition, health, and well-being is part of the concrete experience of every individual. It involves all aspects of daily life, both physical and mental. The significant connection between nutrition and well-being now has solid scientificfoundations, and there is evidence today regarding the relationship between nutrition and inflammation. Numerous clinical symptoms can be scientifically attributed to the type of diet, and by changing dietary habits, one can often achieve improvement or resolution of many diseases by modulating inflammation through something as simple as food.

Three different causes leading to food-related inflammation, or rather three different mechanisms of action that can provoke either alone or together a pathological reaction, have been identified:

- The specific type of food used,
- The glycation process,
- The proportion of nutrients in each meal.

Each person has a personalized dietary profile defined by the level of class G antibodies (immunoglobulin type G, IgG) related to a specific food, indicating excess or constant repetition of the intake of a food or similar ones belonging to the same food group. When a particular food is eaten repetitively or excessively, the level of BAFF and other cytokines such as PAF increases in the body, thereby increasing the overall body inflammation (Kang, 2016). Finkelman (2016) explains how IgG antibodies act as protective antibodies against a possible allergic reaction; these specific antibodies for certain foods can work to activate awareness of food antigens, thus counteracting the action of immunoglobulin type E (IgE) (responsible for an allergic reaction). Food-related inflammation is not an allergic reaction. Through IgG, the immune system responds very similarly to all foods belonging to the same food group and sharing some aspects of immunological similarity. Large food groups are specific to each population, as foods vary in different parts of the world. The five major groups for the European population are: gluten and wheat, milk and dairy products, yeasts and fermented foods, nickel-containing foods, and cooked oils.

Moreover, sugars can have a strong influence on body inflammation due to the glycation process (Smith, 2017). Excessive intake of sugars (sucrose, glucose, fructose, maltose, etc.)

can induce an inflammatory condition. Another way to develop glycation, and thus induce inflammation in an organism, depends on the glycation of certain proteins or certain carbohydrates on the surface of many foods and occurs when foods are cooked at high temperatures. The inflammatory symptom from glycation can be mistaken for an allergy as it can appear suddenly. In reality, sugars and glycated substances grow gradually and progressively within the body until they reach a "threshold level" that, when surpassed, induces the symptom. The effect of glycation is to increase the overall reactivity of the immune system. Acute inflammatory reaction depends on a short-term effect, but more importantly its possible long-term effect causes damage to tissues and the accumulation of specific toxins in the body, glyco-toxins.

Thanks to the work of Lago (2007), we know that chronic inflammations can be correlated with the incorrect proportion of carbohydrates and proteins within each individual meal. There is also a clear relationship between the proportion of nutrients on the plate and the time of day they are consumed, and the imbalance of these aspects can be responsible for the production of substances derived from adipose tissue, adipokines, as well as inflammatory substances, including leptin. Mamerow (2014) explained with extreme precision that the amounts of protein needed throughout the day must be distributed in a balanced way in the three different meals. The correct proportion of different nutrients was schematically represented by Sin (2011) as the Healthy Eating Plate (Figure 6), which attributes the correct value to proteins and describes the importance of using whole grains instead of refined ones in the right quantity and of avoiding counting calories and instead emphasizing the proportion between different nutrients.

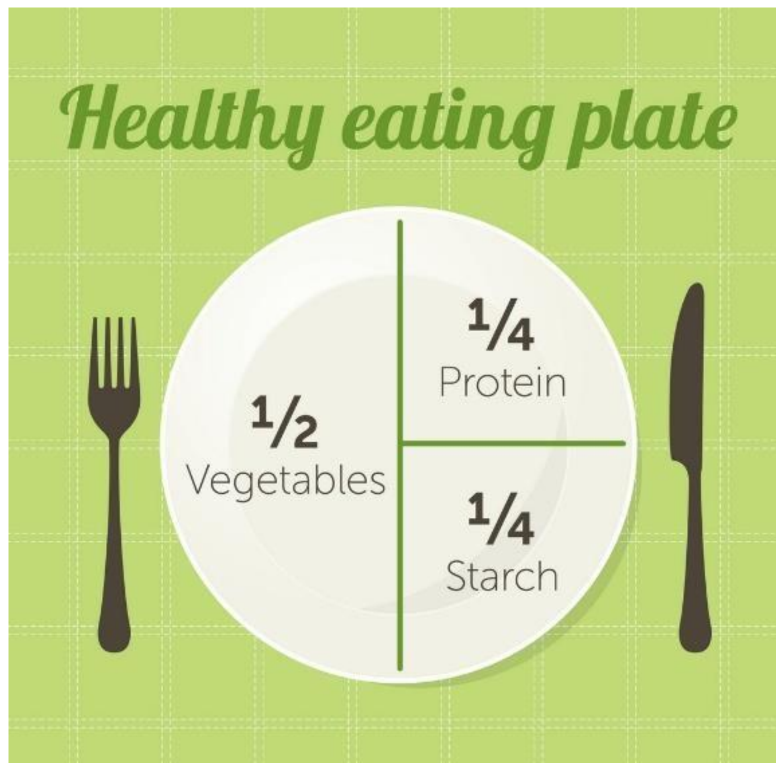


Figure 6. **Healthy eating plate.**

The intestinal microbiota plays a crucial role in intestinal inflammation. The dietary basis of inflammatory diseases is likely explained by interactions between food or bacterial metabolites and immune cells, or pathways for intestinal homeostasis. The current key metabolites playing protective roles include SCFAs, omega-3 fatty acids, and those derived from tryptophan catabolism. The diet shapes the ecology and diversity of the intestinal bacterial community (De Filippo et al., 2010; Le Chatelier et al., 2013; Turnbaugh et al., 2008). Additionally, the composition of the intestinal microbiota is correlated with many human diseases (Clemente et al., 2012; Kau et al., 2011; Rotondo and Mazmanian, 2009). In a recent study, individuals with low bacterial richness (approximately 23% of the Danish population) tended to have a more pronounced inflammatory phenotype than those with high bacterial richness (Le Chatelier et al., 2013). Low bacterial diversity has also been associated with inflammatory bowel disease (IBD) (Manichanh et al., 2006), although it is not known whether this is a cause or a consequence of the disease.

IgG4

The immunoglobulin family consists of five types, of which, under normal conditions, about 80% is made up of IgG, 15% of immunoglobulin A (IgA), 5% of immunoglobulin M (IgM),

0.2% of immunoglobulin D (IgD), and the remaining small percentage of IgE. IgG is the most abundant immunoglobulin in human serum and is composed of molecules of four subclasses designated IgG1 to IgG4, each with diverse biological properties determined by structural differences in the heavy gamma chains contained in the molecules. Low concentrations of all IgG subclasses indicate the presence of immunodeficiency. High levels of IgG4 were reported to underlie the so-called IgG4-related disease (IgG4-RD), a heterogeneous, subacute and usually silent autoimmune disease involving many organs (Al-Khalili and Erickson, 2018); nonetheless, high IgG4 in serum is not necessarily indicative of an IgG4-RD (Nambiar and Oliver, 2021). Foods have various components that may induce immune reactions, including the production of variable amounts of food-specific immunoglobulins (Ig), particularly IgG4. IgG4 are described as Ig produced by the immune system to advise about the ingestion of 'that food' (Bernardi et al., 2008; Speciani and Piuri, 2015). IgG4 is an IgG subclass induced by type 2 cytokines such as IL-4 and IL-13 (Aalberse et al., 2009). An excess of these specific food antigens activates inflammatory processes.

Ligaarden (2012) confirmed that IgG levels are indicative of excessive or repeated food intake, and Finkelman (2016) explained the relationships between the quantity of antigens, specific IgE and IgG, and the potential effects that their balance can determine. The value of IgG for foods increases depending on the diet followed. Clinical manifestations due to food arise from the interaction between the quantity of food antigen, the quantity of IgG and IgE, and the level of immune complexes. Systemic anaphylaxis can occur both through IgE-mediated and IgG-mediated pathways. The IgE-mediated pathway requires a smaller quantity of antigens and antibodies than the alternative pathway. IgG antibodies prevent IgE-mediated anaphylactic reactions by intercepting antigens before they bind to IgE associated with mast cells. Consequently, IgG antibodies can block systemic anaphylaxis induced by small amounts of antigen, even though they may instead mediate systemic anaphylactic reactions in the presence of higher quantities (Speciani and Piuri, 2015).

From the outdated concept of "food intolerance", science is transitioning to the definition of "food inflammation". Excluding allergic reactions mediated by IgE, the only recognized forms of food intolerance are gluten intolerance (celiac disease) and biochemical lactose intolerance. Everything else is now defined as food inflammation, and it is possible to control such inflammation through a varied and complete diet.

Tryptophan and its metabolites

Inflammatory subclinical or silent inflammations are among the causes of many chronic diseases. Cytokines, released due to the inflammatory process, not only influence immune cells but also various metabolic pathways. This is particularly relevant to the metabolism of tryptophan. An essential amino acid, tryptophan (Trp) is found in foods such as red meat, fish, eggs, yogurt, and many vegetables. Trp can follow multiple metabolic pathways. The most famous and long-known is the pathway leading to the synthesis of 5HT, but most Trp is used in the production of kynurenine (KYN) (Figure 7).

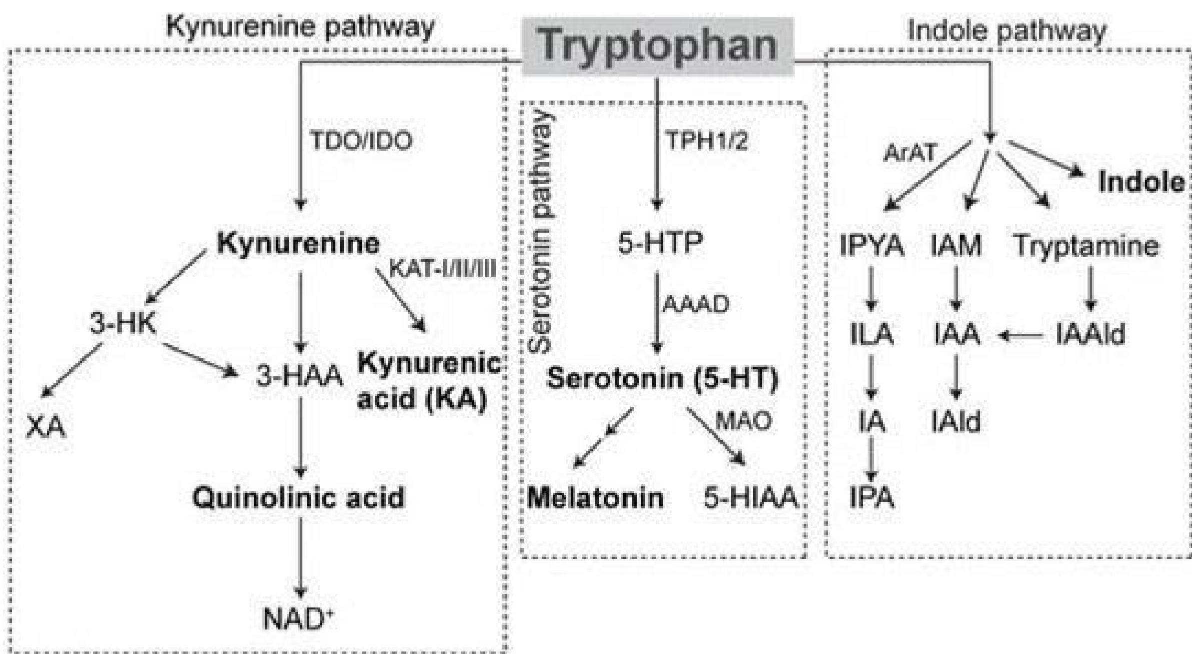


Figure 7. **The three metabolic pathways of tryptophan**: the kynurenine pathway, the serotonin/melatonin pathway, and the indole pathway (Roth et al., 2021).

The enzymes indoleamine 2,3-dioxygenase (IDO), tryptophan 2,3-dioxygenase (TDO) and kynurenine mono-oxygenase (KMO) are activated by inflammatory cytokines (IFN-α, -β, -γ, TNF-α, IL-6, PAF). Inflammations thus promote the production of kynurenine and quinolinic acid. In contrast, during the production of serotonin and melatonin, Trp is subtracted; for this reason, the synthesis of kynurenine can decrease by up to 50%.

In the digestive system, Trp is important for the regeneration of intestinal mucosa and protects against the proliferation of potentially pathogenic agents. It is also available in enterochromaffin cells for serotonin production. A deficiency of Trp in feces has been

detected in patients with inflammatory bowel diseases (Keszthelyi et al., 2013; Lamas et al., 2016). A deficiency of Trp in feces can also be caused by limited oral intake of the amino acid. When there is sufficient intake, causes of deficiency may be inflammatory reactions of the mucosa or alterations in the microbiome. In these cases, the degradation pathway of intestinal Trp is strengthened through the activation of IDO and KMO. Additionally, cofactors for the synthesis of one or the other metabolite are also crucial; for serotonin, for example, the presence of vitamin B6 and magnesium is necessary.

Serotonin is a well-known neurotransmitter and precursor of melatonin. In the CNS, it has an antidepressant, relaxing, anxiolytic effect and promotes learning and memory. Moreover, serotonin participates in blood clotting and wound healing. It is also of great importance for the intestine, where it acts on peristalsis, reabsorption, immune activity, and the sensation of enteric pain. Melatonin is the hormone of sleep that regulates the day-night rhythm. It is produced in the pineal gland (CNS), in the eye retina, and in the intestine. Melatonin also has antioxidant properties.

Tryptophan hydroxylase catalyzes the initial, rate-limiting step in the conversion of tryptophan to serotonin. Within the gut, ECs express TPH1, while serotonergic neurons express TPH2 in the CNS and ENS. TPH1 and 2 convert TPH to the intermediate L-5-hydroxytryptophan (5-HTP) and L-amino acid decarboxylase converts 5-HTP to 5HT. Within the pineal gland, TPH is converted by TPH1 to serotonin, which can be further converted into melatonin, the major endogenous regulator of sleep initiation and circadian rhythms. Serotonin is otherwise catabolized by monoamine oxidase (MAO) into 5-hydroxyindole acetaldehyde and then by aldehyde dehydrogenase to 5-hydroxyindole acetic acid (5-HIAA) which is excreted in the urine (O’Mahony et al., 2015).

5-HIAA could be a good marker of inflammation, currently used in support of clinical diagnosis in appendicitis (Bolandparvaz et al., 2004).

Serotonin has many roles, depending on the type of receptor it binds, and thus can perform different actions (Roth et al., 2021). Serotonin receptors vary in expression in target tissues and are implicated in a variety of disorders (Barnes et al., 2021). Serotonin receptors are part of the G protein-coupled and ligand-gated ion channel families. Serotonin exerts its diverse actions by binding to cell surface receptors which can be classified into seven distinct families (5-HT1 to 5-HT7) according to their structural diversity and mode of action (Kitson, 2007).

Among the many activities, serotonin also stimulates vagal nerve endings in the upper

gastrointestinal tract expressing the 5-HT3 receptor. Luminal lipid infusions promote satiety via 5-HT3- and cholecystokinin A receptor (CCK1)-dependent vagal signaling, and 5-HT3 antagonism contributes to greater food intake by humans (Zhu et al., 2001). Serotonin also appears to play a major role in abdominal pain signaling, as 5-HT3 antagonism ameliorated pain behavior and dorsal horn activation induced by colonic distension in a murine model (Giordano and Dyche, 1989; Crowell, 2004). Activation of parasympathetic neurons causes elevations in circulating serotonin (Giordano and Dyche, 1989).
Moreover, several immune cells express serotonin receptors, including B and T lymphocytes, monocytes, macrophages, and dendritic cells (DCs) suggesting an immunomodulatory role for serotonin (Herr et al., 2017). 5-HT1, 5-HT2 and 5-HT7 are receptors expressed in these cells. Serotonin stimulates production of pro-inflammatory IL-1β, while decreasing TNFα (Idzko et al., 2004). Additionally, serotonin receptor families mediate differential cytokine responses. Among these it was seen that activation of 5-HT3 receptors also stimulates IL-1β and IL-6 production (Fiebich et al., 2004). Thus, systemic effects of serotonin activation on peripheral tissues also include nociception.

Kynurenine derives from Trp via IDO, activated by inflammatory states. Very active IDO and a high level of kynurenine are found in subjects with obesity, metabolic syndrome, chronic stress, depression, chronic pain, cardiovascular diseases, neoplasms, infections, autoimmune and neurogenerative diseases (Heyes et al., 1992; Kim et al., 2012). There are also indications that low mitochondrial activity, i.e., ATP production, is accompanied by a high kynurenine/Trp ratio (Karabatsiakis et al., 2014). In rheumatoid arthritis, autoimmune diseases and viral infections, reduced IDO activity and thus a decreasing level of kynurenine are observed instead (Kang et al., 2015). Excess kynurenine inhibits the innate immune system and strengthens the adaptive system, thus reducing the effectiveness of immune defenses, as cytotoxic T cells are inactivated. Patient tolerance increases (Nguyen et al., 2014). In contrast, inhibited IDO activity and a lack of kynurenine result in a lack of immunosuppression (Kang et al., 2015).
Kynurenine is metabolized into kynurenic acid (KA) via the enzyme kynurenine-oxoglutarate-transaminase (KAT), an antagonist of the NMDA receptor with antioxidant, anti-inflammatory, and analgesic effects. In the case of inflammatory metabolism, an increase in kynurenine is important for the body for the production of anti-inflammatory agents. Kynurenine can cross the blood-brain barrier and thus reach the CNS. KAT is lacking in macrophages and microglia cells in the brain, and thus kynurenine is metabolized

exclusively into quinolinic acid (QA), an NMDA receptor antagonist. QA has neurotoxic, pro-inflammatory, and oxidative effects (Guillemin, 2012). QA can limit the integrity of the BBB. Many neuropsychiatric and neurodegenerative clinical pictures are associated with a high level of QA (Lovelace et al., 2016). In astrocytes in the brain, kynurenine can be transformed into KA, which also has a neuroprotective effect. QA from other cell types can be degraded in astrocytes (Guillemin et al., 2001). Hence, a high transformation of Trp into kynurenine also entails a high risk of neuroinflammatory and neurotoxic damage. Moreover, if the production of QA increases, the synthesis of nicotinamide adenine dinucleotide (NAD) is slowed down, and NAD is a necessary factor in many redox metabolism pathways.

The last metabolic pathway of tryptophan is the indole pathway, in which the microbiota is particularly involved. Indole-3-aldehyde, a tryptophan metabolite produced by lactobacilli, is an agonist of the aryl hydrocarbon receptor (AhR) (Zelante et al., 2013). After binding to the agonist, AhR translocates to the nucleus, where it induces the expression of some genes involved in the production of mediators important for intestinal homeostasis (Li et al., 2011; Veldhoen and Brucklacher-Waldert, 2012).

Vertebrates have developed various mechanisms to respond to food and bacterial metabolites. One mechanism occurs through metabolite-sensitive G protein-coupled receptors (GPCRs), which produce immediate biological responses to specific metabolites. Many common food and bacterial metabolites have GPCR sensors. The reason why body cells, including immune cells, use these receptors to modify their function is still uncertain, but presumably it is related to the need to perceive the availability of nutrients.

Dietary patterns and interventions to alleviate chronic pain

Chronic pain is multifaceted and can originate from several conditions mixed in thousands of combinations. The clinical result is unique. Chronic pain represents the essence of the need to create personalized medicine (Raffaeli et al., 2021; Chadwick et al., 2021). Each subject has his/her personal, familial and social history. Age and sex are likewise fundamental (Pieretti et al., 2016) and food can be added to this list of factors (Elma et al., 2022). Indeed, of particular interest among the various efforts to find causes/therapeutics to fight pain is the recent attention towards possible interactions between GI health and chronic pain conditions (Aloisi, 2023; Wilder-Smith, 2011; Erdrich et al., 2020; Casini et al., 2022). Several studies have been carried out to determine the potential benefits of foods considered to be anti-inflammatory (Dragan et al., 2020). Moreover, the modulatory role of the

microbiota has been studied (Guo et al., 2019) and several gut-related biomarkers have been determined in the blood (Clos-Garcia et al., 2019; Chen et al., 2021). All these studies have suggested a beneficial effect of specific attention to the gut and the foods commonly consumed by pain patients (Brain et al., 2021). A list of potentially pro- or anti-inflammatory foods is available and thus diets are provided that include known anti-inflammatory foods and/or eliminate known inflammatory ones (Ricker and Haas, 2017).

The presence of GI disorders in chronic pain patients is not common knowledge in medical practice, even though efforts have been made to report possible interactions among pain and GI disorders. For instance, it has repeatedly been reported that a high percentage of fibromyalgia patients complain of IBS (Sivri et al., 1996; Chang, 1998; Erdrich et al., 2020); on the other hand, among subjects with GI complaints those with fibromyalgia reported a higher number of symptoms related to several areas of the GI tract than those without fibromyalgia. The gut does not have fast nociceptors but only C fibers unable to send detailed information about inflammation and/or lesions; thus, for patients with GI disorders, there are only mild signs not always able to indicate the magnitude of the problem and its localization (Malin et al., 2009). Signs of discomfort like abdominal swelling, diarrhea, constipation, gastroesophageal reflux, etc. are common, although it is difficult to relate these signs to specific problems and they are generally treated with symptomatic drugs. Moreover, in many subjects, they can be considered 'normal' due to familial or social habits. Only recently has it become clear that these conditions could be accompanied by serious disruption of the intestinal barrier (McGuckin et al., 2009), able to increase the interactions of bacteria with the host's immune system and the release of inflammatory markers like cytokines (Chen et al., 2018). These substances can act in all body areas to impair physiological functions and induce pain.

The human body uses six categories of nutrients from food: carbohydrates, fat, protein, fiber, minerals, and vitamins. Diet therapy is a professionally prescribed diet which provides specific nutrients, antioxidants, or prebiotic supplementation for beneficial health effects. Dietary patterns in chronic pain also seem to have a positive impact on comorbidities including obesity, type 2 diabetes mellitus, cardiovascular diseases, and depression (Philpot and Johnson, 2019; Lean, 2011; Casas and Estruch, 2016).

Common diets like the Mediterranean, ketogenic, etc. have been tested in groups of pain patients with interesting results (Pagliai et al., 2020). For instance, a significant decrease in a range of symptoms and disease scores was noted after four weeks of adherence to the low

fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAP) diet (Staudacher et al., 2017).

The Western diet based on processed meat, sugary foods, refined grains, and low intake of fruits and vegetables causes an excessive production of pro-inflammatory mediators that sensitize the peripheral afferent neurons; these mediators include interleukins, histamine, TNF-α, 5HT, bradykinin, free radicals, and eicosanoids (prostaglandins, leukotrienes, and thromboxane). The imbalance of the Western diet also yields fewer anti-inflammatory mediators (Cordain et al., 2005).

The Dietary Inflammatory Index has been developed to classify a person's diet in order to help reverse the dietary imbalance by proper individualized diet therapy (Shivappa et al., 2015). The requirements of such diets are fulfilled by diets high in whole-grains, fish, fruits, green vegetables, and olive oil (Sesti et al., 2011). Recent studies have identified many dietary choices that can improve chronic pain due to components with antioxidant and anti-inflammatory properties (De Gregori et al., 2016).

Besides inflammation, low dietary intake of micronutrients, especially omega-3 fatty acids, vitamins B1, B3, B6, B12 and D, magnesium, zinc and β-carotene, is also associated with chronic neuropathic or inflammatory pain (Philpot and Johnson, 2019). Supplementation of the diet with these specific nutrients contributes to alleviation of chronic pain (Elma et al., 2020), as observed in systematic reviews on chronic pelvic pain (Sesti et al., 2011), low back pain (Mauro et al., 2000), rheumatoid arthritis or joint pain secondary to inflammatory bowel disease (Martin and Reid, 2017), migraine (Shaik and Gan, 2015), chronic non-cancer pain (Brain et al., 2019), pain in chronic liver disease (Mariqueo and Zúñiga-Hernández, 2020), and aromatase inhibitor-related arthralgia in breast cancer (Kim et al., 2018).

Of all well-known old and new lifestyle factors, dietary choices might have the greatest influence on the occurrence, maintenance, and perception of chronic pain (Casas and Estruch, 2016). However, systematic reviews and meta-analyses of the effects of diet therapy on chronic pain have provided ambiguous results (Geir et al., 2019).

Exosomes

Extracellular vesicles (EVs) are nanometer-scale membranous particles that can carry and deliver a wide range of cargo, from proteins to genetic material. Studies on these vesicles intensified after the discovery that extracellular vesicles act as conduits of intercellular messages that can influence the activity of neighboring cells, both in normal physiological processes and in pathophysiology. Furthermore, EVs have shown significant promise as biomarkers to diagnose disease states. EVs have been detected in many body fluids, highlighting their role as intercellular communication mediators thanks to their transfer ability (Kalra et al., 2013; Street et al., 2017).

Exosomes correspond to the internal vesicles of an endosomal compartment, the multivesicular body, and are released following the exocytic fusion of this organelle with the plasma membrane (Figure 8). Intracellularly, they form through budding into the interiorof the endosomal membrane in a process that sequesters specific proteins and lipids.

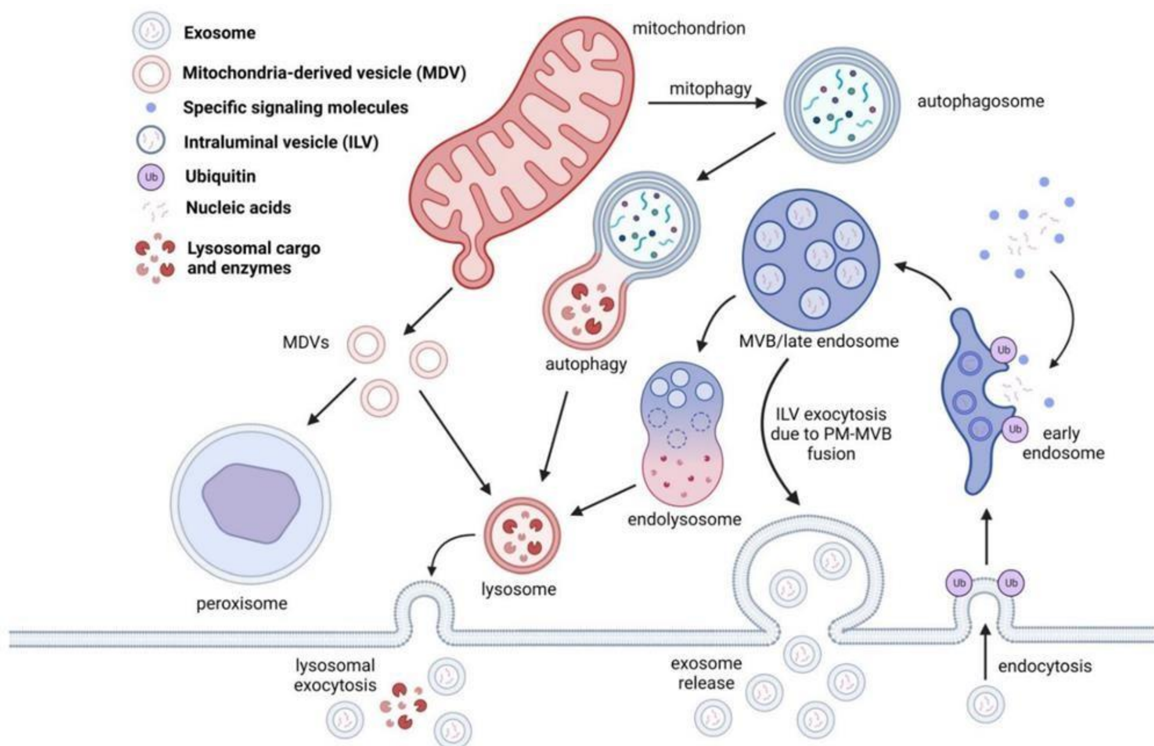


Figure 8. **Biogenesis and release of EVs.** Once the early endosome is formed, intraluminal vescicles (ILVs) are packaged with cargo tagged for degradation; an endolysosome is formed and will proceed to lysosomal degradation. In some cases, the ILVs are released during fusion of multivesicular bodies (MVB)-PM and are consequently released as

exosomes. Lysosomes are also involved in protein degradation through autophagy, mitophagy and mitochondria derived vesicles (MDVs) (Horbay et al., 2022).

Endosomes are active sorting organelles that distribute collected constituents to distinct destinations, such as apical and basolateral plasma membranes and the trans-Golgi network (Lemmon and Traub, 2000). These processes are mediated by transport vesicles that detach from the membrane tubules associated with the endosome, similar to the sorting/transport processes carried out by other organelles within endocytic and secretory pathways (Kirchhausen, 2000).

The endosomal system consists of primary endocytic vesicles, early endosomes, late endosomes, and lysosomes (Mellman, 1996). Late endosomes derive from early endosomes through a maturation process that involves a change in content and a gradual accumulation of internal vesicles. Therefore, late endosomes are often referred to as multivesicular bodies (MVBs) (Sotelo and Porter, 1959; Novikoff et al., 1964). MVBs accumulate internal vesicles that potentially have three distinct fates (Denzer et al., 2000):

1. Directing proteins and lipids associated with lysosomes for degradation;
2. Serving as temporary storage compartments;
3. Releasing internal vesicles, now called exosomes, through the fusion of the MVB membrane with the plasma membrane.

Fusion of MVBs with the cell surface has been observed in a broad range of cell types, including epithelial cells (Van Niel et al., 2001). Additionally, exosomes have been isolated from the supernatants of tissue cultures, and it has been observed that purified exosomes have a molecular composition similar if not identical to that of the internal vesicles of MVBs (Denzer et al., 2000).

Exosomes range in size from 30 to 150 nm. Compared to other EVs, such as microvesicles or ectosomes, exosomes differ in size, lipid composition, content, and cellular origin. Transmission electron microscopy studies have suggested that exosomes have a cup morphology and a bilayer lipid structure (Kowal et al., 2014). The bilayer membrane confers stability to the structure, protects cargo from degradation processes, and allows the exosomes to move across biological barriers thanks to membrane adhesive proteins (Dong et al., 2019). Exosomes have a specific gene expression profile by which it is possible to detect and differentiate them from other EVs. In particular, this specific protein pattern includes tetraspanins (CD9, CD63, CD81, and CD82), heat shock proteins (HSP60, HSP70, and

HSP90), tumor susceptibility gene 101 protein (TSG101), and ALG-2-interacting protein X (ALIX) (Bobrie et al., 2011) (Figure 9).

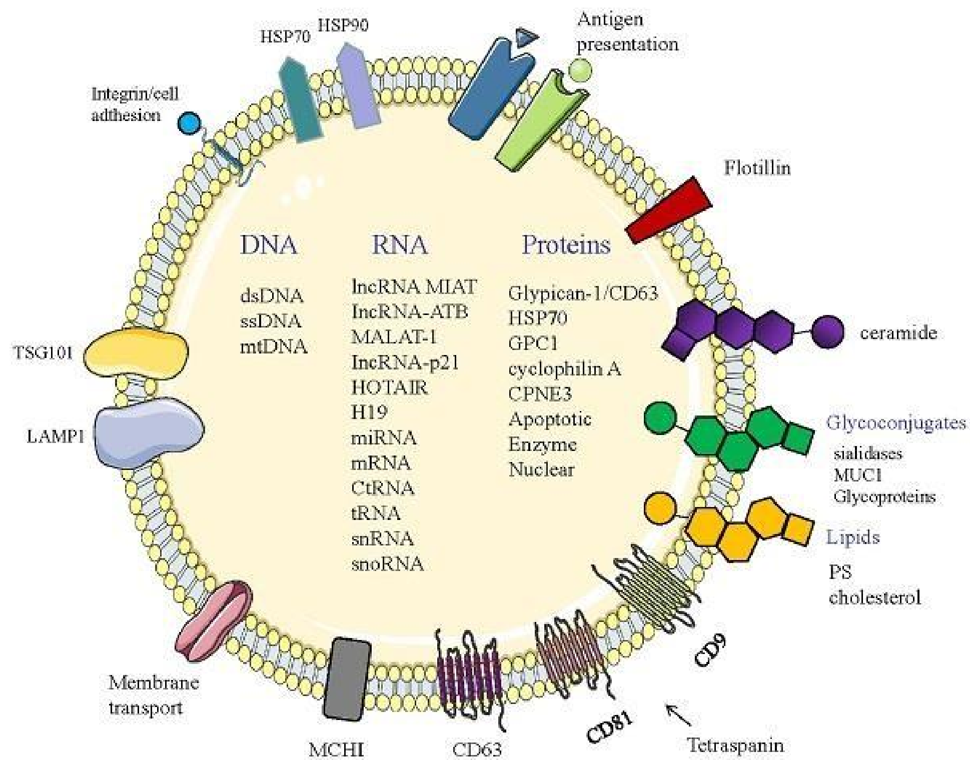


Figure 9. **Structure and composition of exosomes.** Exosomes consist of an aqueous core and a phospholipid bilayer. They carry important biomolecules such as proteins, DNA, and RNA, while the exosome membrane is rich in a variety of specific proteins: tetraspanins (CD9, CD63, CD81); major histocompatibility complexes I and II (MHC I, II); TSG101; HSP70, 90 (heat shock protein 70,90); LAMP1; surface receptor; ceramide; glycoconjugates (Luo et al., 2021).

Intestinal epithelial cells secrete exosomes

The intestinal epithelium is an absorbing surface and a physical barrier between the host and its environment. The vesicles produced by this tissue are strongly influenced by environmental conditions. Exosomes can have both autocrine and paracrine actions, thus modulating the functions of nearby cells and distant cells (Figure 10). They are crucial mediators of cell-to-cell communication and play a significant role in intercellular signaling. They encapsulate specific molecules and deliver them selectively to host cells. This mechanism can also involve cells far from the intestine, as exosomes entering the

bloodstream can contact any organ and tissue, including the CNS. In fact, exosomes are capable of crossing the BBB. Changes in intestinal physiology or the environment due to dietary patterns or microorganisms can contribute to physiological changes in the brain and vice versa.

The exosomes in the intestinal microenvironment can be classified into two groups based on their origin: those deriving from microorganisms and those from host cells.

The properties of these exosomes are influenced not only by the type of parent cells but also by the current physiological conditions of these cells. Enterocytes in the intestinal lumen are constantly exposed to various agents, including food, intestinal microorganisms, and pathogens. Changes in this dynamic environment can alter the production and properties of EVs released by the intestine, which can impact the brain's physiopathology (Van Niel et al., 2001; Zhang et al., 2021; Sugihara et al., 2019).

Inotsuka et al. (2020) treated human intestinal Caco-2 epithelial cells with γ-aminobutyric acid (GABA) and found that GABA-induced intestinal exosomes facilitated neurite growth in SH-SY5Y cells.

Intestinal EVs can also be influenced by microbial infections, toxins, and even EVs from microorganisms (Ayyar and Moss, 2021; Deng et al., 2015; Hu et al., 2013). This microbe-host interaction can have an impact on other remote organs (Chen et al., 2019).

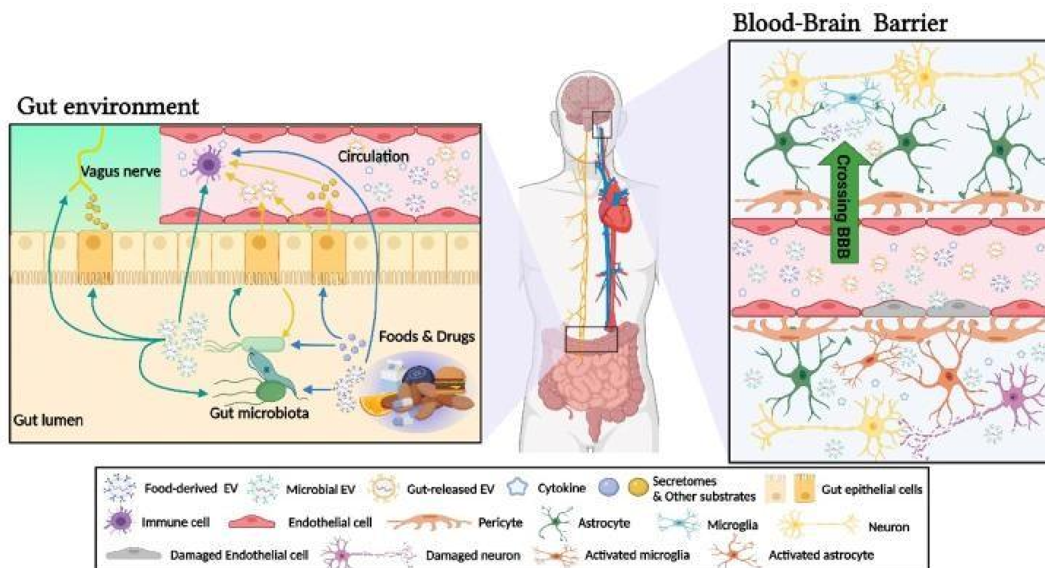


Figure 10. **Gut–brain interaction mediated by EVs in the gut microenvironment.** EVs occurring in the gut microenvironment can be categorized as either of microbial origin or of gut cell-released origin (Kim et al., 2021).

Another function of exosomes produced by intestinal epithelial cells involves their relationship with the immune system; indeed, epithelial-lymphoid interactions are crucial in the regulation of immune responses (Van Niel et al., 2001). Because the intestinal epithelium can be considered a relatively static structure in the body, despite its rapid renewal, it is tempting to hypothesize that exosomes released from this structure may be part of the link between antigens in the intestinal lumen and cells of the immune system in the lamina propria or more distally. In normal conditions, exosomes derived from intestinal epithelial cell lines express low levels of major histocompatibility complexes (MHC) class I and no class II molecules, which are strongly upregulated in inflammatory conditions (Van Niel etal., 2001).

The expression of A33 antigen, a receptor-like molecule of the immunoglobulin superfamily, on basolaterally released exosomes is intriguing, and the involvement of this molecule in antigen presentation deserves further investigation. Basolaterally released exosomes may allow antigen presentation through direct interactions with T cells or through their uptake by professional antigen-presenting cells, as hypothesized for dendritic cell-derived exosomes (Zitvogel et al.,1998).

Exosomes and pain

The involvement of exosomes in pain processes has been demonstrated by studies on different chronic pain diseases (Ju et al., 2019; Tavasolian et al., 2020). Some of these studies have suggested that exosomes could be a novel therapeutic strategy (Ren et al., 2019), transferring cargo into nearby and distant target cells. Moreover, exosomes might represent a tool to distinguish subgroups of patients with higher possibility of benefits from specific treatments (Ramanathan et al., 2019). Exosomes could represent novel non-invasive biomarkers of specific pain diseases in order to obtain an early diagnosis. The molecular pattern inside exosomes differs according to the cellular origin, epigenetic modifications, environmental state, and stress factors. Therefore, identification of a specific cargo's profile associated with pain states might help to distinguish a specific pathological state from a healthy state and to use exosomes as biomarkers of the diseases.

Thanks to their ability to transfer miRNAs, exosomes also have an important role in neuronal mechanisms such as synaptic plasticity, neurogenesis, and neuronal differentiation (Kumar and Reddy, 2016). Exosomes appear to play a role in synaptic plasticity, as observed in some models of neurodegenerative diseases (Wang et al., 2017). For example, miR-26 in

astrocyte-derived exosomes is involved in CNS diseases and synaptic plasticity (Lafourcade et al., 2016).

Exosomes are released and taken up again by neurons depending on synaptic activity. In a mouse sciatic nerve model, exosomes were quantified over time in the medial prefrontal cortex and nucleus accumbens; this revealed that their release can mimic pain sensation-like behaviors. However, the projections from the medial prefrontal cortex to the nucleus accumbens are important players in the reward circuitry and their activation inhibits pain behaviors. It was proposed that the release of exosomes from these brain areas mediates the pain threshold and allodynia (Yu et al., 2020).

A decrease of thermal hyperalgesia following a single injection of macrophage-derived exosomes has been found, suggesting a potential immunoprotective role. Three exosome-transported miRNAs (miR-21-3p, miR-146a, and miR-146b), known to be involved in the control of overactivation of the innate immune response, were overexpressed in both murine and human models (McDonald et al., 2014).

Exosomes are involved in many inflammatory diseases due to their abilities to transfer different molecules such as miRNAs and proteins acting on close or distant target tissues (Stoorvogel, 2012). Uncontrolled or unresolved inflammation can be active pathways of systemic inflammation involved in the pathogenesis of several pain diseases such as osteoarthritis, rheumatoid arthritis, inflammatory bowel diseases, and neurodegenerative diseases.

Exosomes also have an anti-inflammatory action. Indeed, exosomes derived from adipose mesenchymal stem cells were shown to play a role in chondro-protective and anti-inflammatory activities in osteoarthritis (Ju et al., 2019). Exosomes were also shown to be involved in inflammatory bowel diseases, a set of chronic disorders that occur whenintestinal homeostasis is impaired (Jiang et al., 2016). One of the many mechanisms underlying these diseases is the immune response modulated by macrophage activity.Macrophage-derived exosomes are involved in this pathophysiological mechanism throughan immunosuppressive role. In fact, exosomes of normal intestine transferred into mice withinflammatory bowel diseases were responsible for a decrease in the severity of the disease (Jiang et al., 2016). Moreover, an increased number of exosomes containing annexin A1 hasbeen found in serum from inflammatory bowel disease patients (Larabi et al., 2020).

AIM

The primary objective of this study was to assess the correlation between nutrition and chronic pain. Specifically, the effects of various nutritional approaches (exclusion diet guided by IgG4 levels and personalized approaches) on pain (pain intensity, percentage of body area experiencing pain, and pain quality), mood states, and overall quality of life.

The secondary objective of the study was to explore the connection between pain and gastrointestinal disorders (such as abdominal pain, abdominal swelling, and abdominal heaviness), as well as the relationship with body composition.

The third goal of the study was to evaluate the dietary habits of individuals experiencing chronic pain, with a particular focus on inflammatory levels in their diets.

The fourth objective was to investigate urinary tryptophan metabolites in individuals with chronic pain, specifically examining the serotonin and kynurenine pathways.

Finally, for a deeper understanding of the relationship between intestinal barrier inflammation and the nervous system, the study aimed to characterize the gut extracellular vesicles known as exosomes using an in vitro model and to evaluate their uptake in central nervous system cells.

MATERIALS AND METHODS

Human Studies

Subjects

Two groups of subjects with various types of non-oncological chronic pain were enrolled for this project. The first group consisted of male and female subjects with non-oncological chronic pain, while the second group was only non-oncological chronic pain women. The diet based on the exclusion of foods that induced high levels of IgG4 was tested in the first step of the study. The second step investigated a) the incidence of gastrointestinal symptoms in individuals with chronic pain, b) the effects of a personalized diet on the pain status, and c) the relationship between pain conditions and tryptophan metabolites. Both experimental protocols adhered to the principles of the Declaration of Helsinki of 1964 and its later amendments; the experimental procedures were approved by the University of Siena Local Ethics Committee (CAREUS).

Experimental procedure

In both steps, each subject included in the study provided informed consent for the analysis and publication of the data, and then met with the experimental team twice (visit 1 and visit 2) (Figure 11). Visit 1 was considered as basal/control, while at visit 2 all determinations were repeated to highlight possible changes.

During visit 1, subjects in both groups underwent the following general experimental procedures to evaluate:

- Pain intensity and features, with the following tests:
  - Visual analogue scale, VAS
  - Margolis test, MA
  - Italian Pain Questionnaire, QUID
- Quality of life state, with the following questionnaires:

    - Ad hoc questionnaire on smoking habits, sleep problems, menstrual cycle alterations, GI problems, birth control pill intake, allergies/intolerances, presence of other clinical problems
    - Profile of Mood States, POMS
    - Short Form-36, SF-36
- Nutritional status:
    - Anthropometric measurements
    - Bio-impedance analysis (BIA)

In the first step only, a few drops of blood were collected to analyze food-specific IgG4 levels. The analysis was conducted with one of the following tools:

- NutriSMART® rapid test, a commercial tool used to determine 57 foods.
- Analytical determination by the enzyme-linked immunosorbent assay (ELISA) method. A few drops of blood were included in a special paper system, to be sent to the laboratory.

On the basis of each food's IGg4 level, each subject received a personalized list of foods to be excluded for 4 weeks.

In the second step only:

- Feeding data were collected by means of an ad hoc questionnaire
- Gastrointestinal conditions were investigated by a gut state questionnaire:
    - Symptom Severity Scale (SSS)
- A urine sample was collected to analyze tryptophan metabolites.

In both groups, visit 2 took place after 4 weeks. All subjects repeated the same general experimental procedure as at visit 1. Questionnaires were self-administered with support from an expert clinician. Questionnaires were scored by a researcher blinded to the testing phase (visit 1 or visit 2).

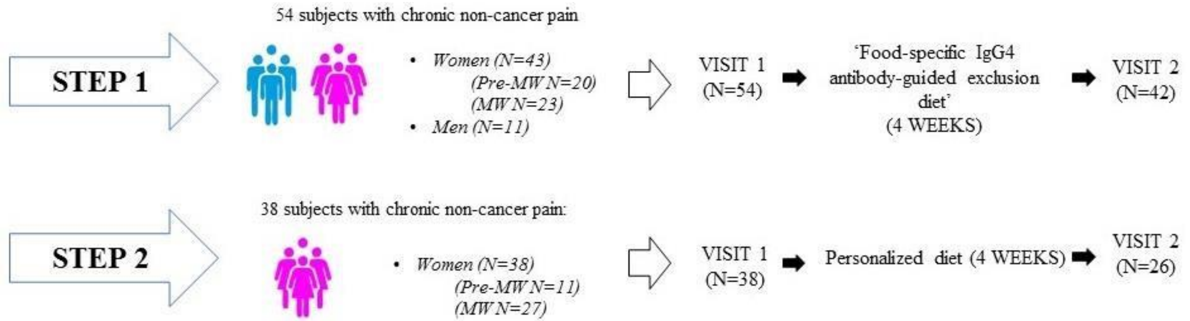


Figure 11. **Experimental procedure in the human study.**

Pain measures

Visual analogue scale

The visual analogue scale (VAS) is a unidimensional measure of pain. It is a self-reported scale consisting of a 10-cm-long horizontal line. For pain intensity, the scale is anchored at the extremes by two verbal descriptors referring to the pain status: “no pain” (score of 0) and “pain as bad as it could be” or “worst imaginable pain” or “unbearable pain” (score of 10) (Huskisson, 1974) (Figure 12). Subjects were asked to indicate the average VAS experienced in the last week at three times of the day (morning, VASm; afternoon, VASa; night, VASn) with a tick on the line at the point that best referred to his or her pain.

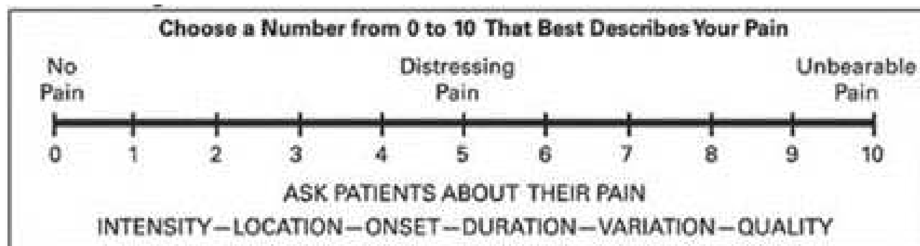


Figure 12. **Visual analogue scale.**

Margolis test

The Margolis test (MA) is a drawing rating system that evaluates percentages of pain distribution in the body. This test is composed of 45 anatomical areas each with a corresponding percentage value (0–100) of body surface in order to compute a total weighted score. Subjects were asked to indicate the areas where pain was experienced in the last week

at three times of the day (morning, MAm; afternoon, MAa; night, MAn) (Margolis et al., 1986).

Italian Pain Questionnaire

The Italian Pain Questionnaire (QUID) is the validated Italian version of the McGill Pain Questionnaire (De Benedittis et al., 1988). It provides a subjective measurement of quality and intensity of the pain experienced in the last week. It is a semantic interval scale composed of 42 pain descriptors divided into 3 major classes (sensory, S, affective, A and evaluative, E) and 16 subclasses incorporated into the fourth indicator called miscellaneous(M). The QUID represents the most parsimonious, meaningful and idiomatic set of Italian pain descriptors, providing quantitative information that can be analyzed statistically, yet preserving a close structural parallel with the MPQ. The subject has to choose only the exact words that match their current feelings from the descriptor list of the QUID. From the value acquired for each dimension (S, A, E, M) a Pain Rating Index rank value (PRIr) is obtained, and from the sum of the rank value a Pain Rating Index rank-Total (PRIr-T) is acquired. Moreover, it includes the present pain intensity (PPI), a 6-point scale where the subject has to report the pain intensity experienced on the testing day. It consists of a number–word combination chosen by the respondent, from no pain to the worst pain experienced [none, 0; mild, 1; discomforting, 2; distressing, 3; horrible, 4; excruciating, 5].

Quality of life questionnaires

Profile of Mood States

The Profile of Mood States (POMS), widely used to assess transient, distinct mood states and mood changes (McNair et al., 1971), consists of 58 words or brief phrases examining eight different dimensions of mood swings over the last week. It comprises six subscales: Tension-Anxiety (T-A), Depression-Dejection (D-D), Anger-Hostility (A-H), Vigor-Activity (V-A), Fatigue-Inertia (F-I) and Confusion-Bewilderment (C-B). Participants are asked to assess their mood state on a 5-point scale: “not at all” (0), “a little” (1), “moderately” (2), “quite a bit” (3), “extremely” (4). In each subscale, values higher (T-A, D-D, A-H, F-I, C-B) or lower (V-A) than 55 were considered significantly altered with respect to the normal population.

The POMS was chosen because it is brief, easy to administer, and useful for non-psychiatric populations, and it considers a variety of mood states, including anxiety, depression, and anger, the most prevalent symptoms among pain subjects (Kerns, 2003).

Short Form-36

Short Form-36 (SF-36) is one of the most used instruments evaluating perceived health status (Apolone and Mosconi, 1998). It is extremely sensitive to small changes in a population's perceived health status and is therefore useful in monitoring health gains at the population level.

It consists of 36 items grouped into two components and divided into eight scales: the first four scales—physical functioning (PF), role physical (RP), bodily pain (BP), general health (GH)—are included in the Physical Component Summary (PCS); the other four—vitality (V), social functioning (SF), role emotional (RE), mental health (MH)—are included in the Mental Component Summary (MCS). A scoring algorithm is used to convert the raw scores into the eight dimensions listed above. The scores are transformed to range from zero where the respondent has the worst possible health to 100 where the respondent is in the best possible health.

Gastrointestinal conditions

Symptom Severity Scale

The GI Symptom Severity Scale (SSS) was specifically designed to assess the severity of GI symptoms in a subject with the focus on abdominal pain and discomfort (Francis et al., 1997). The total scale (SSS total) is the sum of six subscales (abdominal pain intensity (API), abdominal pain frequency (APF), abdominal swelling (AS), evacuation satisfaction (ES), abdominal heaviness (AH) and interference with quality of life (IQL)). Each of the six questions generates a maximum of 100 points using prompted visual analogue scales (0, best condition - 100, worst condition), leading to a total of 600. In the SSS total, mild, moderate and severe cases were indicated by values of 90 to 210, 210 to 360 and > 360 respectively. For the analysis of this questionnaire the total scale and the individual subscales (API, APF, AS, AH, ES, IQL) were used.

The SSS is the scale best suited to measure abdominal pain because it has been correlated with physical measures (such as the pain induced by rectal distension), is reproducible and is psychometrically robust (Francis et al., 1997).

Nutritional status and body composition

Anthropometric measurements

Body mass was measured to the nearest 0.1 kg using a platform beam scale and height was measured to the nearest 0.5 cm using a stadiometer. Participants were asked to remove shoes and heavy clothes prior to weighing. Height was measured according to standard procedures. The participants were asked to stand up straight against the backboard with their body weight evenly distributed and both feet flat on the stadiometer platform, while the head was in the Frankfort horizontal plane (NHANES, 2017).

The body mass and height data were used to calculate the body mass index (BMI) using the Quetelet equation [body mass/height^2 (kg/m^2)]. The BMI is the reference indicator for epidemiological and screening studies. It is important to emphasize that the BMI, as an indicator for population studies, is unable to assess the actual body composition, nor does it allow for understanding the distribution of body fat within an individual. The human body is mainly composed of four molecular-level components: water, fat, proteins and minerals, usually in that order of decreasing amount.

Adipose tissue is by far the most varying compartment—between individuals, but also within an individual over time. BMI is the most widely used way to estimate body fat. Being a very simple and inexpensive method, it is the basis for the definition of overweight (BMI 25-30) and obesity (BMI ≥30). However, for a given BMI, the body fat percentage changes with age, and the rate of this change is different depending on sex, ethnicity and individual differences (Gallagher et al., 2000). And while BMI correlates with fat accumulation and metabolic health in large populations, it is insensitive to the actual distribution of body fat (Thomas et al., 2012). BMI values below 18.5 indicate underweight, while BMI values between 18.5 and 24.9 indicate a normal weight condition.

Bioelectrical impedance analysis

Bioelectrical impedance analysis (BIA) was used to estimate body composition with a bioimpedentiometer (Akern Srl, Florence, Italy). BIA is a simple, non-invasive technique

that measures the electrical characteristics of the human body (Figure 13), i.e., impedance (Z) and phase angle (PhA) (from those, resistance (Rz) and reactance (Xc) can also be derived). Total body water (TBW), fat-free mass (FFM), fat mass (FM), body cell mass (BCM), body cell mass index (BCMI) and extracellular water (ECW) can be estimated by means of predictive equations that include BIA variables and very often other variables, such as age, height and body mass. Impedance/resistance (Rz) and reactance (Ohm) values were examined using specific software (BodyGram 1.31). On the basis of theoretical principles, BIA is most appropriately reserved for the assessment of body hydration. This includes total body water, as well as intracellular and extracellular water. In longitudinal assessments, overhydration and dehydration, as well as the effect of respective treatments, can be followed by bioelectrical impedance vector analysis (BIVA). Assuming a fixed hydration, FFM and body cell mass can be calculated from BIA data. These two body components are measures of functional body mass, which gives rise to the second application of BIA focusing on malnutrition and rehabilitation. As BIA gives an indirect estimate of fat mass (calculated from the difference between body weight and FFM), the third application is to energy stores and thus to clinical nutrition. In addition to hydration, functional body mass and energy stores, BIA may also serve as a monitor of disease load; a low-phase angle is associated with poor prognosis. Hence, as a fourth application, BIA data reflect the severity of disease in acutely and chronically ill subjects (Kyle et al., 2004; Kyle et al., 2004). The BIA method has an error of 4–8% compared to criterion methods (Moon, 2013).

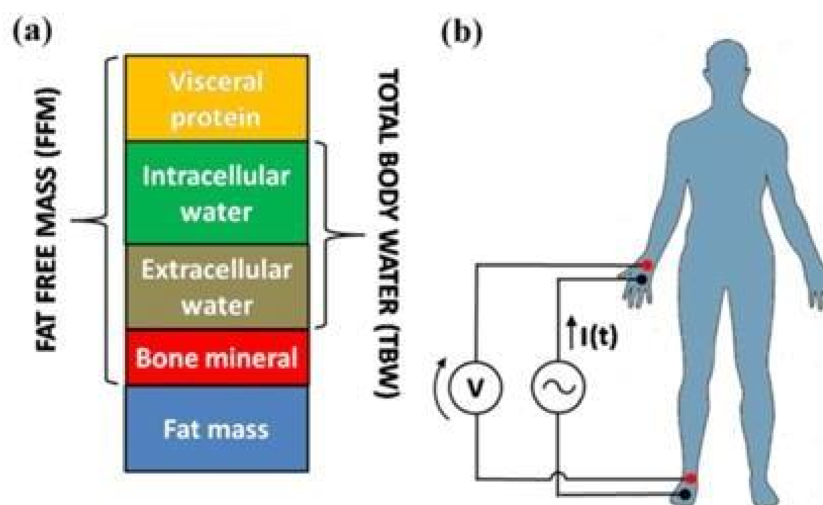


Figure 13. **Bioelectrical impedance analysis.**

Determination of specific IgG4 levels

For each subject, a few drops of blood were collected (capillary sampling) and used in the NutriSMART® rapid test (DST, Schwerin, Germany) or left to dry in an ad hoc absorbent paper for ELISA determination.

NutriSMART® test

NutriSMART® is a single-use immunoenzymatic test for food intolerances with a qualitative measurement of IgG4. It is primarily employed in cases of suspected food intolerance due to repeated or excessive consumption of one or more foods, when a specialist deems it necessary to modify a patient's diet or in other types of investigations (Casini et al., 2022). The test provides valuable insights to enable effective and timely intervention, ensuring the patient's optimal well-being.

The NutriSMART® test was immediately applied following the manufacturer's instructions. It consists of 40 wells containing food-specific antigens referring to 57 common foods. At the end of the process a three-level score was obtained: mild (reported as 1 or +), medium (reported as 2 or ++) or high (reported as 3 or +++).

ELISA determination

The analytical determinations were carried out by the ELISA method. ELISA is a solid-phase sandwich immunoenzymatic test that allows for a quantitative analysis. The serum samples were diluted. The wells of a 96-well microplate were coated with one of 80 food-specific allergens. Samples, including standards and controls, were pipetted into the wells. Less than 1 µL of serum per allergen was required. The plate was then sealed and incubated at room temperature for 1 hour. Sample-specific IgG4 antibodies bound to antigens in the wells during incubation. Unbound components of the serum were washed and patted dry after the sample incubation. Anti-human IgG4 antibodies coupled to horseradish peroxidase (HRP) were added to the wells and they bound to IgG4 antibodies from the sample, standards or controls. After another incubation at room temperature for 30 min, washing and patting dry, 100 µL of a substrate mixture consisting of equal proportions of 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxide were added. The reaction was stopped after 10 min by adding a stop solution. A yellow dye was formed and the respective intensity was related to the proportional amount of bound antibodies. The absorbance was measured

at 450 nm. The concentrations of food-specific IgG4 (U/mL; 1 U = 1.47 ng) were calculated using the standard curves of 80 types of food-specific IgG4 provided by the manufacturer. The detection limit was 0.01 U/mL.

The results were then reported to each subject who was asked to completely avoid for 4 weeks any foods with a score of 2 or 3 (NutriSMART® test) or foods showing IgG4 levels higher than 3.5 U/mL (ELISA) independently of the number of foods to be excluded.

Feeding habits and Dietary Inflammatory Index

Feeding habits of the participants were investigated regarding the composition of the meals taken during the day, daily water intake, presence of fruit and vegetables within the day, and weekly fish consumption. The 24-hour dietary recall (24HR) was used to investigate baseline dietary intake. The 24HR is a structured interview intended to acquire detailed information about the types and amounts of all the foods, beverages and dietary supplements consumed in the past 24 h (Baranowski, 2012). A key feature of the 24HR is that, when appropriate, the respondent is asked for more detailed information than first reported. For example, the preparation method or the specific type of food. In addition to other detailed descriptors, such as time of day and source of food, the portion size of each food and beverage was recorded. Food models, pictures, and other visual aids were used to help respondents judge and report portion size and to improve accuracy. Other information collected regarded eating occasions, timing and locations of meals and snacks, sources of food and beverages, and other activities such as TV and computer use during meals. The 24HR provides contextual information, such as meal and snack patterns, consumption of foods and beverage at home and away from home, and activities during meals. The 24HR took 20 minutes to complete.

MetaDieta Software (METEDA, Version Professional 4.0.1, Rome, IT) was used to analyze the 24HR data. In particular, 27 dietary parameters were extrapolated: alcohol, β-carotene, cholesterol, carbohydrates, energy, fats, fiber, folic acid, iron, magnesium, zinc, vitamin A, vitamin B-6, vitamin B-12, vitamin C, vitamin D, vitamin E, monounsaturated fatty acid, protein, niacin, riboflavin, (n-3) fatty acids, (n-6) fatty acids, polyunsaturated fatty acids, saturated fat, selenium and thiamine. The Dietary Inflammatory Index (DII) is the first instrument that correlates the foods and dietary constituents with specific inflammatory markers: IL-1β, IL-4, IL-6, IL-10 and TNF-α (Cavicchia et al., 2009). Calculation of the DII is based on the 24HR dietary intake data which are then linked to the regionally

representative world database. The calculation of DII followed previously published protocols (Shivappa et al., 2014). Briefly, the individual dietary data were first linked to a regionally representative global intake database to compute an inflammatory effect score Z value [individual reported intake-global daily mean intake)/global standard deviation]. To minimize right skewing, the Z value was then converted to a centered percentile score [(2* percentile of Z value -1)]. After that, the centered percentile score of a food parameter was multiplied by the respective inflammatory effect score to obtain a food parameter-specific DII. The individual overall DII was the sum of 27 food parameter-specific DIIs. The DII can vary from a minimum of -5.5 (anti-inflammatory range, best condition) to a maximum of +5.5 (pro-inflammatory range, worst condition). Negative values are associated with an anti-inflammatory diet, positive values with a pro-inflammatory diet (Hébert et al., 2019).

Tryptophan metabolites determination

Fourteen urine samples were collected from chronic pain subjects. Control urine samples were similarly collected from 7 age-matched control subjects (without pain). The urine was collected in sterile test tubes on the morning of the visit immediately upon the participant's arrival and stored in a -80 °C freezer until the time of analysis. Urine samples from chronic pain subjects were collected twice (visit 1 and visit 2), urine samples from control subjects were collected just once (visit 1).

Liquid chromatography coupled to high resolution tandem mass spectrometry (LC-HRMS/MS) was used to determine levels of 5HT, creatinine, Trp and its metabolites kynurenine, KA, 5-HIAA and QA as follows: 0.5 mL of urine was diluted with 0.5 mL of acetonitrile, vortexed for 60 seconds at room temperature and incubated at -20 °C for 10 min. Subsequently, samples were centrifuged for 10 min at 2 °C at 6,000 g. Afterwards 0.5 mL of supernatant was transferred to a new tube and diluted with 0.75 mL of 0.2 M acetic acid. The solution was then filtered through a 0.22 μm filter and subjected to LC-HRMS/MS analysis. The chromatographic separation was carried out on a reversed phase poroshell C18 column (100 × 2.1 mm, 2.7 μm, Agilent Technologies) kept at 35 °C using a gradient elution combining water (mobile phase A) and acetonitrile (mobile phase B) containing 0.1% formic acid. Eluting molecules were then analyzed using a quadrupole-orbitrap mass spectrometer to characterize and quantify target molecules. HRMS/MS was used to record the signal owing to the pseudo-molecular ions of the targeted molecules and to their most intense product ions. The quantification was achieved by a calibration curve based on standard

reference material at increasing concentration values. The urinary concentration of 5HT and other related metabolites was then divided by the creatinine concentration to normalize concentration values and allow the comparison between different samples.

Nutritional approach

In the first step of the study, the diet was based on the exclusion of foods with high levels of IgG4. In the second step, the food recommendations were designed by a team of nutritionists of the University of Siena who evaluated individual cases and gave personalized nutritional advice to each participant. In all subjects, the diet was evaluated according to the person's BMI, age, physical activity and food-related allergies (if reported) as suggested by the Dietary Reference Values of Nutrients and Energy for the Italian population (LARN IV Revision) (SINU, 2014). The daily intake of nutrients was based on the Mediterranean Diet pattern, whose anti-inflammatory properties are known (Maiorino et al., 2016).

Dietary caloric intake was planned as follow:

- subjects classified as normal weight, caloric intake ranging from 1700 to 1900 kcal/die;
- subjects classified as overweight, caloric intake ranging from 1400 to 1600 kcal/die;
- subjects classified as obese, caloric intake ranging from 1100 to 1300 kcal/die.

The proposed diet was composed of five meals during the day (breakfast, lunch, dinner and two snacks) with a daily setting (from Monday to Sunday) for 4 weeks.

The daily nutrient intake was divided as follow:

- 50% of the daily energy intake from complex carbohydrates with a limitation of sugars and sweeteners;
- 30% of the daily energy intake from lipids, with an adequate intake of polyunsaturated fatty acid (PUFA) derived mainly from extra virgin olive oil, fish and nuts;
- 20% of the daily energy intake from proteins ranging from 0.9 g to 1.1 g/kg/die.

Moreover, a daily intake of fiber ≥ 20 g/die derived from fruits, vegetables and starchy foods, was considered.

In vitro

The experimental procedure of the in vitro part is shown below (Figure 14).

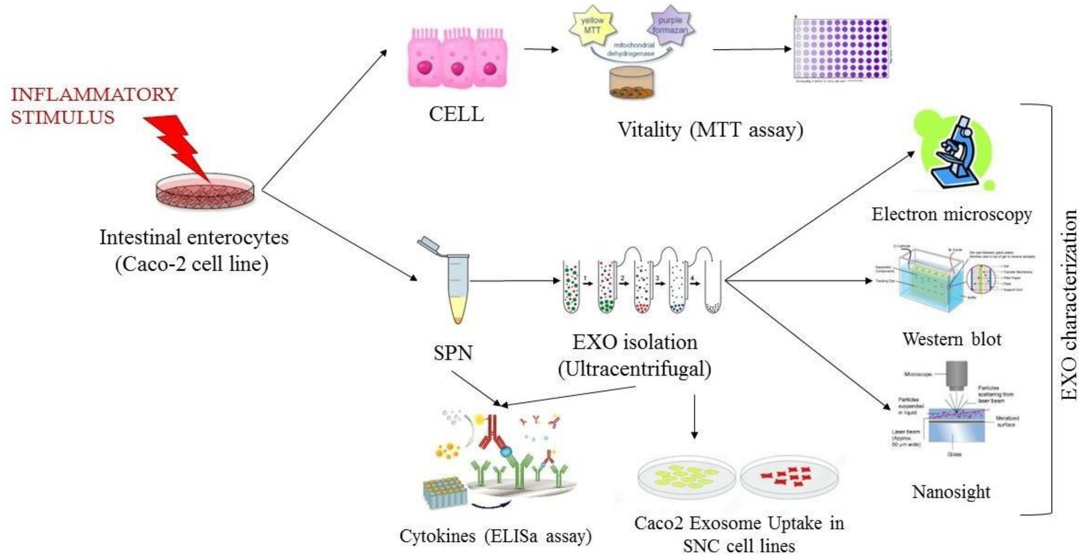


Figure 14. **Experimental procedure of the in vitro study.**

Caco-2 culture

Cancer coli-2 (Caco-2, ATCC-HTB-37) is a human epithelial cell line isolated from colon tissue derived from a 72-year-old white male with colorectal adenocarcinoma (Fogh et al., 1977). Caco-2, an adherent cell line, was used as a model of the intestinal epithelial barrier (Figure 15 (A) and (B)). Caco-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4.5 g/L) supplemented with 20% fetal bovine serum (FBS) and the following additions: 1% non-essential amino acids (NEAA) and 25 mg/mL gentamycin (complete medium). The cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2. For propagation in culture flasks, the Caco-2 cells were seeded $0.7*10^6$ in 25 cm^2 flasks. The medium was changed every 3 days. At 80% confluence, typically after 4–5 days, the cells were split 1:6 before further cultivation.

For the splitting, the Caco-2 cells were trypsinized by first rinsing twice with phosphate buffered saline (PBS) (3 mL per 25 cm^2 flask), after which 1 mL of trypsin was added and the flask was gently shaken for 1 min. The flask was incubated at 37 °C for 5 min. The incubation with trypsin should be as short as possible as this process will affect cell viability. As soon as the cells were detached, trypsinization was stopped by adding complete medium

containing FBS. The cells were transferred to a test tube, subjected to low speed centrifugation to sediment intact cells, and then resuspended in the new medium. The supernatant was transferred to a new test tube, and an aliquot was taken to count the cells. Cell viability was checked (mortality < 5%).

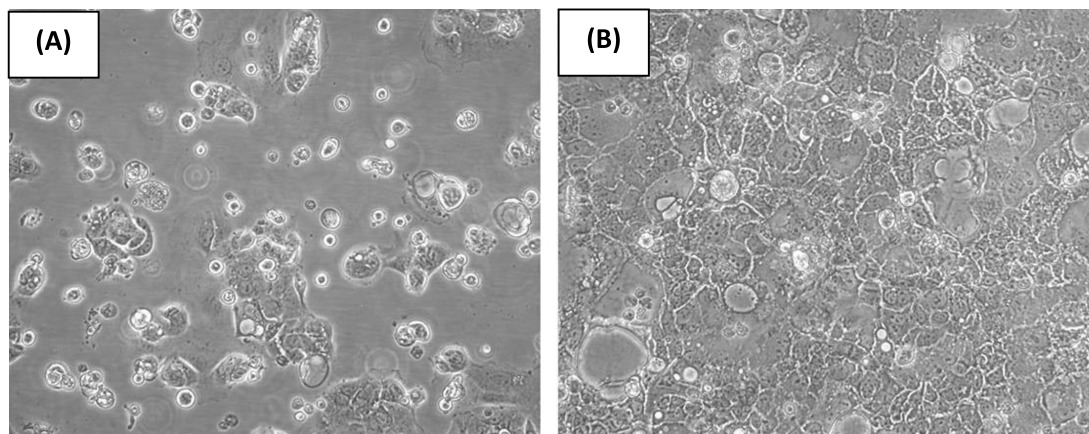


Figure 15. **Caco-2 cells. (A)** Low density; **(B)** High density.

Central nervous system cells

U-373 MG cell line

The U-373 MG cell line (Figure 16) was derived from a malignant human glioblastoma by the explant technique. The cell line was cultured in DMEM with 4.5 g/L of glucose, containing 10% heat-inactivated FBS, 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 50 mg/L gentamycin (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C in a humidified atmosphere with 5% CO_2. Cells were routinely harvested by a brief incubation in 0.05% trypsin and reseeded before reaching confluence. For the experiments, seeded cells were rested overnight and then treated in fresh medium. Cell proliferation was evaluated by counting the harvested cells in a Bürker chamber, and each sample at each treatment time was expressed as percentage of cell number at the start of the experiment. Cell viability was assessed using the trypan blue exclusion assay.

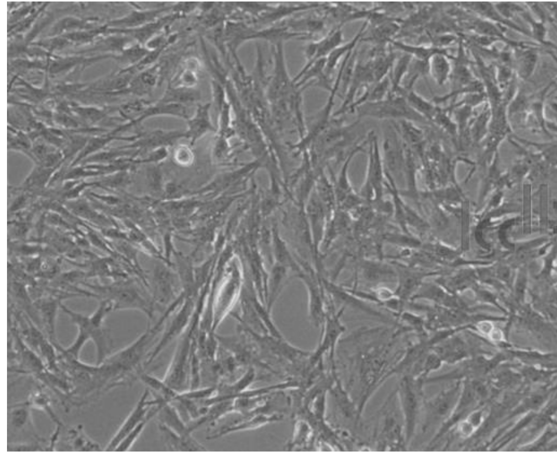


Figure 16. **U373 cell line.**

SH-SY5Y cell line

The SH-SY5Y cell line (Figure 17) is a thrice cloned subline of the neuroblastoma cell line SK-N-SH, which was established in 1970 from a metastatic bone tumor from a 4-year-old cancer patient. This cell line has been widely used as a model of neurons since the early 1980s as these cells possess many biochemical and functional properties of neurons. SH-SY5Y cells were cultured in DMEM with low glucose (1 g/L) and L-glutamine, supplemented with 10% FBS. Cells were cultured at 37 °C and 5% CO_2. Between 10 and 30 passages were used, and media were changed every 3 days.

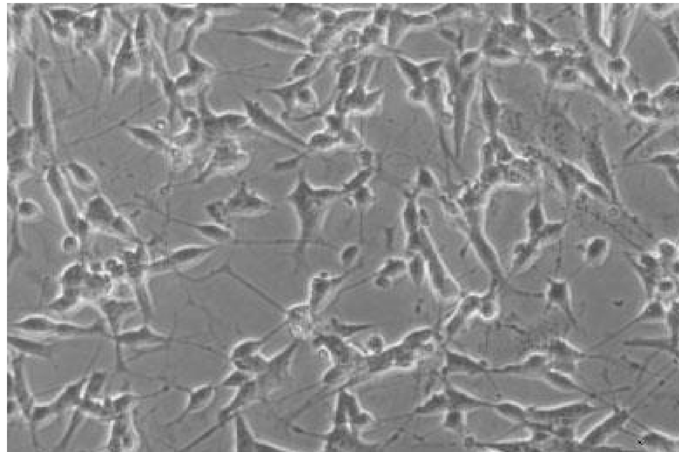


Figure 17. **SH-SY5Y cell line.**

Gut inflammation model

Lipopolysaccharides (LPS) from Escherichia coli O111:B4 and 5HT were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Euroclone (Milan, Italy) respectively. LPS was

solubilized in PBS; 5HT was solubilized in ethanol. Caco-2 (50-70 passages) monolayers were seeded into 6-well plates. After 24 hours of incubation for adherence, samples were stimulated with LPS (10 μg/mL) or 5HT (10 μM) or LPS (10 μg/mL) with 5HT (10 μM), and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 24 hours. During stimulation, exosome-free FBS was utilized. To prepare exosome-free FBS, FBS was centrifuged for 2 hours at 50,000 RPM with an ultracentrifuge. After incubation, cell lysates and supernatants were collected from the wells. Supernatants were centrifuged for 10 min at 1,300 RPM. Cell lysates and supernatants were kept at –80 °C until further use.

Caco-2 vitality

The MTT assay involves conversion of the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to insoluble purple formazan by the action of mitochondrial reductase. Formazan is then solubilized and the concentration is determined by optical density at 540 nm. Small changes in metabolic activity can generate large changes in MTT, allowing one to detect cell stress and vitality. The assay has been standardized for adherent or non-adherent cells grown in multiple wells. The protocol uses a standard 96-well plate.

Caco-2 cells were incubated with DMEM containing 5 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) for 2 hours at 37 °C, in a humidified atmosphere of 5% CO_2. Then dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added and the absorbance at 540 nm was examined using a microplate reader (Thermo Fisher Scientific - USA). DMSO is a polar aprotic solvent capable of dissolving both polar and non-polar compounds.

Pro-inflammatory cytokine levels

The quantitative determinations of IL-1β and IL-6 concentrations in cell culture supernatants and exosomes were performed using the ELISA method, employing commercial kits and following recommended protocols. The protocol followed is similar to the one explained above for the determination of IgG4. In short, wells of a 96-well microplate are coated with a specific antibody for the cytokine of interest. Samples, including standards and controls, are pipetted into the wells: if the cytokine is present, it is immobilized in the well by the antibody. After washing to remove unbound substances, a specific secondary antibody

linked to a cytokine-specific enzyme is added, and after another wash a substrate solution is added, leading to the formation of coloration proportional to the amount of cytokine bound in the initial step. Color development is then halted by a specific reagent, and the color intensity is measured at a specific wavelength with a spectrophotometer. Cytokine concentration values are obtained by interpolation on the standard curve prepared with increasing known concentrations of the cytokine. The samples were analyzed in duplicate. The results were expressed as pg/µg of total proteins.

Exosomes isolation

To isolate exosomes, 6 mL of supernatant were collected and transferred to polycarbonate tubes for use in the ultracentrifuge. Each tube was filled with cold 1X PBS and the masses of the bottles were balanced. The supernatant was centrifuged at 2,500 g at 4 °C for 30 min to remove apoptotic bodies and then further spun for 1 h at 15,000 g to eliminate the microvesicles. Exosomes were then collected by ultracentrifugation (2 hours at 100,000 g, 4°C) in an ultracentrifuge. The pellet was re-suspended with 1X PBS and centrifuged for 1 hour at 100,000 g at 4 °C. The supernatant was discarded and the pellet was re-suspended with 1 mL of PBS and filtered with a 0.22 filter. For Western blot analysis, the pellet was re-suspended with 40 µL of deionized water (H2Odd). The samples were transferred into 1.5 mL microtubes and stored at −80°C.

Exosomes characterization

NanoSight and transmission electron microscopy (TEM) were used to measure exosome particle size and concentration. Western blotting was used to identify exosomal marker proteins, CD9, CD81 (external markers) and TSG-101 (internal marker).

Analysis of exosomes by NanoSight

NanoSight nanoparticle tracking analysis (NTA) is the most common method utilized in the field for assessing the concentrations and sizes of EVs in solution. This method measures the characteristic movement of exosomes (and nanoparticles in general) in solution based on Brownian motion (Bachurski et al., 2019). It then determines information about the particles in solution by capturing scattered light post-illumination with a laser, and uses the Stokes-

Einstein equation and cell volume information to determine concentrations and sizes of vesicles.

Extracellular vesicle sizes and concentrations were determined using the NanoSight NTA instrument. A 1 mL exosome sample derived by ultracentrifugation was prepared by freshly diluting the exosome sample 1:100 in H2Odd (0.1 µm filtered) before reading. The exosome number was recorded using the following analytical settings on the NanoSight (NTA 3.4 Build 3.4.4): camera type: sCMOS; laser type: Blue488; camera level: 16; slider shutter: 1600; slider gain: 295; syringe pump speed: 30 arbitrary units; number of frames: 1498; temperature: 24.9-25 °C; viscosity: 0.888 - 0.891 cP; FPS: 25; detection threshold: 4; blur size: auto and max jump distance: auto: 13.4 - 14.1 pix.

Analysis of exosomes by the Western blot technique

Western blotting was used to identify exosomal marker proteins: CD9, CD81 and TSG101. After the isolation of exosomes, they were resuspended in 40 µL of H2Odd, and 10 µL of 1X loading buffer (LB) were added. 1X LB consisted of 12.5% Tris-HCl 0.5 M (pH 6.8), 10% glycerol, 2% SDS, 5% dithiothreitol, 0.00125% bromophenol blue. Samples were boiled for 5 min. 1/2 of the total exosomes (25 µL) was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), following the Laemmli method (Laemmli, 1970). Briefly, between two glass plates separated by spacers, two different gels, separating and stacking gel containing 10% and 4% acrylamide respectively, were sequentially poured and allowed to polymerize. Samples and molecular weight standards were loaded onto a 10% reducing gel. Electrophoresis was conducted in an appropriate running buffer (3.02 g/L Tris-HCl, 14.4 g/L glycine, 1 g/L SDS) under the following parameters: 1 hour, 300 V, 50 mA. Following the electrophoresis run, the proteins were subjected to electrotransfer onto a nitrocellulose membrane. The transfer was conducted in a suitable chilled buffer (10% methanol, 192 mM glycine, 25 mM Tris) under the following parameters: 2 hours, 100 V, 350 mA. To stain the proteins on nitrocellulose membranes post-transfer, the membranes were immerged in red ponceau solution for verification of protein transfer. The membranes were washed with H2Odd and Tris-buffered saline with 0.1% Tween® 20 (TTBS, Tween Tris-buffered saline: 0.1% Tween-20, Tris 20 mM, NaCl 150 mM). Non-specific sites on the membrane were blocked by incubation with a TTBS solution with 5% non-fat milk for 1 hour at room temperature. Then, the individual primary antibody was incubated at 4 °C overnight: rabbit anti-CD9 (1:500), rabbit anti-CD81 (1:200), rabbit

anti-TSG-101 (1:500). The primary antibodies were diluted in TTBS. After washing with TTBS, the membranes were incubated with peroxidase-conjugated anti-rabbit secondary antibody (1:6,000). The secondary antibody was diluted in TTBS. After 3 washes of 10 min each, the membranes were incubated for 1 min with the enhanced chemiluminescence system and the chemiluminescent signal was acquired using the ChemiDoc XRS instrument.Images were photographed and quantified using ImageJ. Samples were normalized to red ponceau.

Analysis of exosomes by transmission electron microscopy

TEM imaging was performed to observe the size and morphology of the isolated exosomes. 10 μg of the exosomes were diluted in 500 μL of PBS. The PBS-diluted exosomes were absorbed into 200-mesh TEM grids. After washing with distilled water, the exosome was stained with 2% uranyl acetate solution for 2 min and detected using a transmission electron microscope.

Exosome uptake in SNC cell lines

Exosomes were labeled with the green fluorescent cell linker PKH67 according to the manufacturer's instructions. Briefly, exosomes isolated from Caco-2 supernatant were centrifuged for 16 hours at 27,000 g at 4 °C. The pellet was diluted in 1 mL of diluent C, and 6 μL of PKH67 dye was diluted in 1 mL of diluent C. The dilutions were then mixed gently for 5 min, and 2 mL of 1% bovine serum albumin (BSA) was added to bind excess dye. The labeled exosomes were washed in PBS at 100,000 g for 60 min. U373 (astrocytes)and SH (neurons) were then incubated with 166 μL of labeled exosomes for 3 or 6 hours. After incubation, the cells were washed twice with PBS and fixed in 4% paraformaldehydefor 10 min without light. Cell nuclei were stained with 6-diamidino-2-phenylindole (DAPI)solution at 1 μg/mL. The exosome uptake was established using a Zeiss LSM700 confocal microscope. All the images were acquired at the same magnification (63 x) with pinholes opened to obtain 0.8-μm-thick sections. Detectors were set to detect an optimal signal below the saturation limits. Images were processed with Zen 2009 image software (Carl Zeiss, Jena, Germany). IF quantification was performed using ImageJ Software to determine Mean Fluorescent Intensity.

Statistical Analysis

All data were tested for normality using the Kolmogorov-Smirnov (K-S) normality test. The statistical differences between groups and times were determined using analysis of variance (ANOVA) followed by post-hoc Fisher's least significant difference (LSD) test when necessary. Correlations were carried out with Pearson correlation coefficient. A $p<0.05$ was considered significant. All analyses were performed with Statistica® (StatSoft Inc, Tulsa, OK, USA) software and GraphPad Prism software (Boston, MA, USA). Data are reported as the mean and standard error of the mean (SEM).

RESULTS

Human Studies

In step 1, 54 of the 60 subjects who agreed to participate in the study (46 women and 14 men) were included in it (43 women and 11 men). The age of the participants ranged between 18 and 77 years. Women were considered in menopause when menses had not been present for more than 1 year. 20 of the 43 women were in reproductive age (Pre-MW) and 23/43 in menopause (MW). Thus subjects were divided into three groups depending on sex and menopausal status (premenopausal women, Pre-MW; menopausal women, MW; Men).

In step 2, of the 40 women considered, 38 were included in the study. Due to logistic/sanitary (Covid-19) problems, some of the subjects were contacted remotely (11/38). The age of the participants ranged between 19 and 73 years. Eleven of the 38 women were in reproductive age (Pre-MW) and 27/38 in menopause (MW). Twelve of the 38 subjects withdrew before the conclusion of the project.

In both steps, all subjects signed the informed consent form and agreed to follow the diet for 4 weeks; all described high compliance regarding the diet.

Pain measures

In step 1, all subjects had suffered chronic pain longer than 1 year, and in most of them more than one pain condition could be determined. However, on the basis of the prevalence of clinical symptoms and previous diagnosis, each subject was included in one of four different pain conditions: diffuse pain (DP), headache (HA), low back pain (LBP) and neuropathic pain (NP). DP was present in 14 subjects, LBP in 17 subjects, NP in 12 subjects and HA in 11 subjects. In women, chronic pain syndromes were equally represented with a slight prevalence of HA (7/20) in Pre-MW and LBP (7/23) in MW, while in men the majority reported LBP (7/11) (Table 1).

Table 1. **Number of subjects for each pain condition in step 1.** Pre-MW (premenopausal women); MW (menopausal women); Men; NP (neuropathic pain); LBP (low back pain); HA (headache); DP (diffuse pain); TOT (Total).

GROUP	*NP*	*LBP*	*HA*	*DP*	TOT
PRE-MW	4	3	7	6	20
MW	6	7	4	6	23
MEN	2	7	0	2	11
TOT	12	17	11	14	54

In step 2, chronic pain (not related to the gut) was present in all women for years (2-25). Based on the kind of pain/pains suffered, the subjects could be divided into two main groups according to the IASP classification (ICD-11, Treede et al., 2019): 32/38 in the chronic widespread pain (CWP) group and 6/38 in the pelvic pain (PP) group (Table 2). The pain values considered were those attributed to the main painful condition.

Table 2. **Number of subjects for each pain condition in step 2.** Pre-MW (premenopausal women); MW (menopausal women); CWP (chronic widespread pain); PP (pelvic pain); TOT (Total).

GROUP	*CWP*	*PP*	TOT
PRE-MW	8	3	11
MW	24	3	27
TOT	32	6	38

Visual Analogue Scale

In step 1, VAS was carried out at three times of the day (VASm, VASa, VASn). At visit 1, several subjects reported VAS scores higher than 5 (Pre-MW: 16/20; MW: 20/23; Men: 8/11). In Pre-MW, pain levels appeared to be lower in the morning and increased in the night, whereas in MW and Men the VAS score was higher in the morning and decreased during the day (Figure 18). After 4 weeks of exclusion diet, VAS decreased in all groups

and in all determinations. The decrease was greater in Pre-MW than in the other two groups. In particular, ANOVA was applied to the VAS data with the factors group (three levels: Pre-MW, MW and Men), test (two levels: visit 1 and visit 2) and VAS (three levels: VASm, VASa, VASn repeated). A significant effect was found for test [$F_{1,89} = 8.27$, $p<0.01$], as VAS was lower at visit 2 than visit 1. Moreover, there was a significant group × VAS interaction [$F_{4,178} = 3.73$, $p<0.01$]: in the Pre-MW group, VASm was lower than VASa ($p<0.05$); in the MW group, VASm was higher than VASa ($p<0.05$) and VASn ($p<0.001$); VASm was higher in MW than in Pre-MW ($p<0.01$). No significance was found for the factor group.

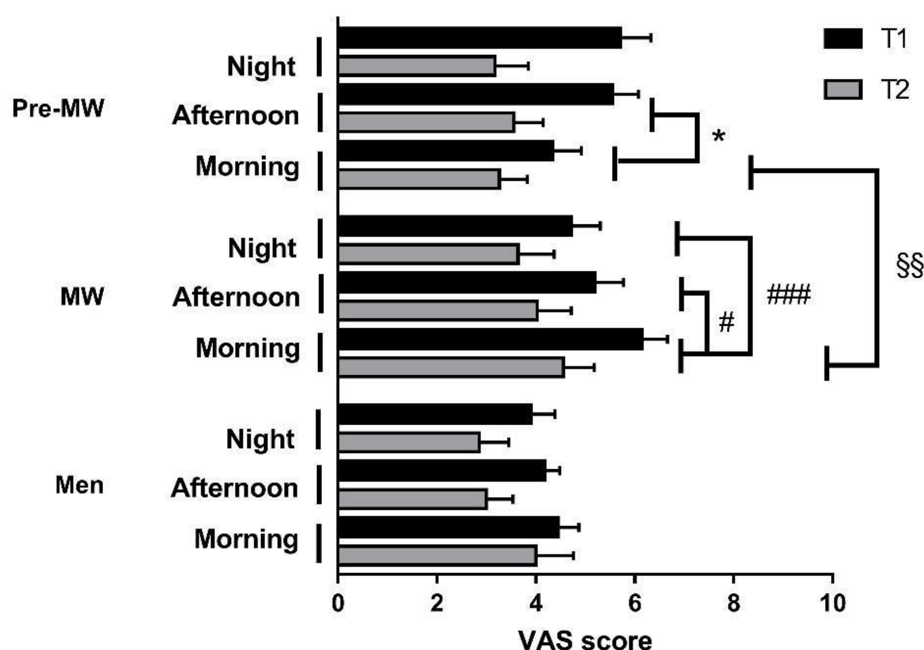


Figure 18. **Visual analogue scale (VAS) step 1** determined in Pre-MW (premenopausal women, n = 17), MW (menopausal women, n = 18) and Men (n = 7), during visit 1 (T1) and visit 2 (T2) at three times of the day (morning, afternoon, night). *p<0.05 VAS morning vs VAS afternoon in Pre-MW, #p<0.05 VAS morning vs VAS afternoon in MW, ###p<0.001 VAS morning vs VAS night in MW, §§p<0.01 VAS morning in MW vs VAS morning in Pre-MW.

In step 2, at visit 1, 24/38 subjects reported VAS scores higher than 5 (Pre-MW: 8/11; MW: 16/27). After 4 weeks of diet, the VAS mean decreased in all groups. In particular, ANOVA was applied to the VAS data with the factors group (two levels: Pre-MW and MW), test (two levels: visit 1 and visit 2) and VAS (three levels: VASm, VASa, VASn repeated). A

significant effect was found for test [F1,59 = 5.88, p<0.01], as VAS was lower at visit 2 than visit 1. Moreover, there was a significant group × VAS interaction [F2,118 = 2.89, p<0.05]: in the MW group, VASm was higher than VASa (p<0.05) and VASn (p<0.001) (Figure 19).

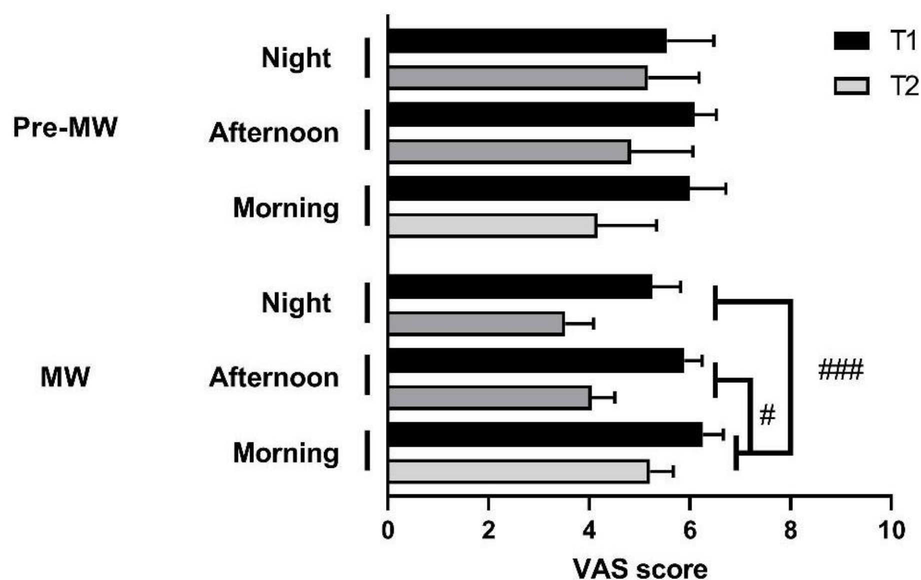


Figure 19. **Visual analogue scale (VAS) mean step 2** determined in Pre-MW (premenopausal women, n = 6), MW (menopausal women, n = 19), during visit 1 (T1) and visit 2 (T2). #p<0.05 VASm vs VASa, ###p<0.001 VASm vs VASn.

Margolis test

In both steps, the Margolis (MA) test was carried out at three times of the day (MAm, MAa, MAn).

In step 1 (Table 3), ANOVA applied to MA values with the factors group, test and Margolis (three levels: MAm, MAa, MAn) revealed a significant effect of Margolis [F2,158 = 5.24, p<0.01] since MAm was higher than MAa (p<0.01) and MAn (p<0.05), independently of group and test. The significant group × Margolis interaction [F4,158 = 2.93], p<0.05) was due to MW having higher values in the morning than in the afternoon (p<0.01). Men showed higher levels of MAm than MAa (p<0.01) and MAn (p<0.05).

Table 3. **Margolis (MA) in step 1** body area percentage in the Pre-MW (premenopausal women), MW (menopausal women) and Men groups; visit 1 (T1), visit 2 (T2); MAm (morning); MAa (afternoon); MAn (night). Values are reported as mean±SEM. #p<0.05 and ##p<0.01 vs MAm, *p<0.05 and **p<0.01 vs MAm same group.

Group (N)	**MAm**		**MAa ##**		**MAn #**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**
Pre-MW (17)	8.7±1.4	5.9±1.1	8.0±1.2	7.3±1.3	8.4±1.4	5.5±1.2
MW (18)	12.3±1.7	13.4±3.6	10.5±2.0**	9.7±3.2**	10.6±2.1	12.5±3.8
Men (6)	11.9±2.1	9.5±1.9	9.1±1.9*	6.8±2.6*	9.5±2.2*	7.0±2.2*

In step 2 (Table 4), ANOVA applied to MA values with the factors group (two levels: Pre-MW and MW), test and Margolis (three levels: MAm, MAa, MAn) revealed a significant effect of Margolis [$F_{2,120} = 4.09$, $p<0.01$] due to MAn being lower than MAa ($p<0.05$) and MAm ($p<0.001$). No significance was found for the factors group and test.

Table 4. **Margolis (MA) in step 2** body area percentage in the Pre-MW (premenopausal women) and MW (menopausal women) groups; visit 1 (T1), visit 2 (T2); MAm (morning); MAa (afternoon); MAn (night). Values are reported as mean±SEM. *p<0.05 MAn vs MAa; ***p<0.001 MAn vs MAm.

Group (N)	**MAm*** **		**MAa***		**MAn**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**
Pre-MW (6)	26.5±4.0	24.7±6.7	23.2±3.8	27.8±4.7	22.8±5.2	23.3±4.6
MW (20)	26.5±7.7	29.9±5.6	23.9±5.8	27.6±5.3	21.6±4.7	22.4±5.1

Present Pain Intensity

In step 1, at visit 1 all groups had PPI values ranging from 1.5 to 2.5, i.e., from "mild" (1) to "strong" (3) intensity (Figure 20). The exclusion diet significantly decreased (improved) this parameter, as shown by the significance of the factor test [$F_{1,86} = 5.41$, $p<0.05$] owing to the higher levels at visit 1 than at visit 2.

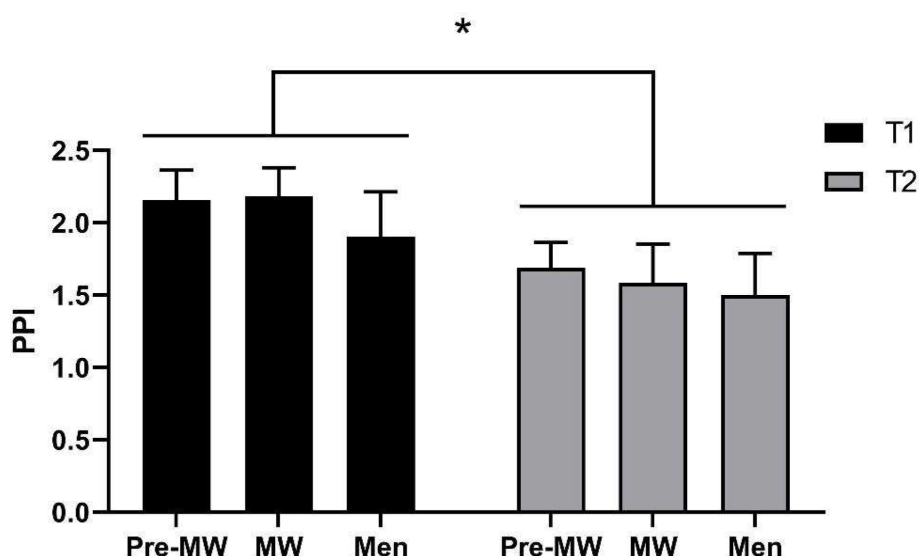


Figure 20. **Present pain intensity (PPI) in step 1** determined in Pre-MW (premenopausal women, n = 16), MW (menopausal women, n = 18) and Men (n = 7) during visit 1 (T1) and visit 2 (T2). *p<0.05 T1 vs T2.

In step 2, ANOVA was applied to the PPI values with the factors group (two levels: Pre-MW and MW) and test (visit 1 and visit 2). No significant differences were found.

Italian Pain Questionnaire

In step 1, the study of pain features by means of the QUID questionnaire revealed a high significance among groups due to the higher levels (worse) in Pre-MW than in MW and Men; indeed the values were higher in all the classes and the Pain Rating Index rank-Total (PRIr-T) values were mostly double in Pre-MW than in the other groups. In particular, as reported in Table 5, ANOVA with the factors group, test and QUID (four levels: QUIDs, sensorial; QUIDa, affective; QUIDe, emotional; QUIDm, miscellaneous, repeated) revealed a significant effect of group [$F_{2,87} = 10.71$, $p<0.001$] due to the higher values in Pre-MW than in the others (both $p<0.001$), independently of test and QUID. The factor QUID was

significant [$F_{3,261}$ = 44.12, p<0.001] since QUIDs was higher than the other dimensions (all p<0.001). No differences were found for the factor test. Similarly, ANOVA applied to PRIr-T (Figure 21) revealed a significant effect of group [$F_{2,85}$ = 12.72, p<0.001] due to Pre-MW having higher values than the others (both p<0.001).

Table 5. **Italian Pain Questionnaire (QUID) in step 1** in the Pre-MW (premenopausal women), MW (menopausal women) and Men groups; visit 1 (T1), visit 2 (T2); classes: s (sensorial), a (affective), e (emotional), m (miscellaneous). Values are reported as mean±SEM. ###p<0.001 vs other groups. ***p<0.001 vs other classes independently of group.

Group (N)	**QUID s*** **		**QUID a**		**QUID e**		**QUID m**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
Pre-MW### (16)	7.8±1.1	6.6±1.5	4.8±0.8	4.8±0.9	4.6±0.9	5.1±0.9	2.9±0.7	1.5±0.4
MW (18)	5.4±0.7	4.2±0.8	2.2±0.5	1.9±0.5	2.0±0.5	2.3±0.7	1.4±0.6	0.8±0.4
Men (7)	4.1±0.8	2.6±0.6	1.3±0.5	1.0±0.7	1.9±0.4	1.3±0.5	0.6±0.3	0.6±0.4

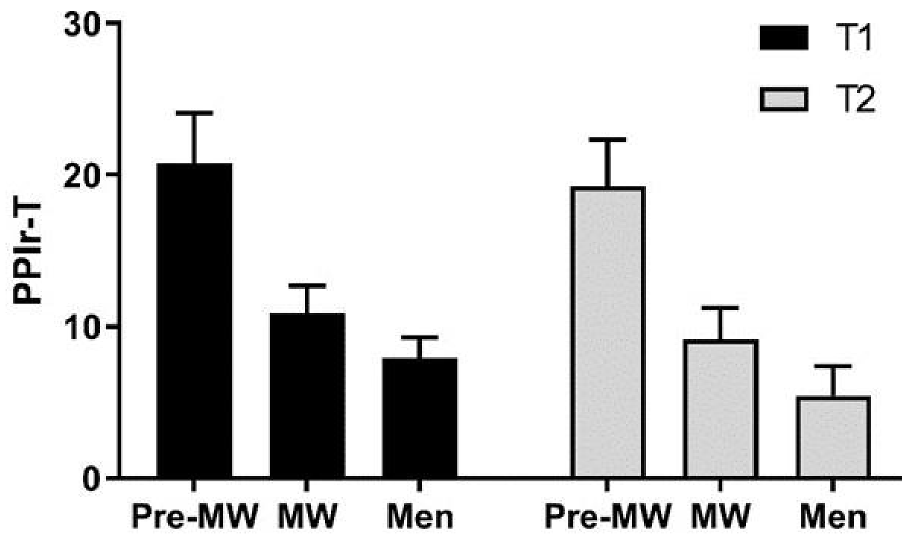


Figure 21. **PRIr-T (Pain Rating Index rank-Total) in step 1** in the Pre-MW (premenopausal women), MW (menopausal women) and Men groups; visit 1 (T1), visit 2 (T2). MW and Men groups are significantly lower than Pre-MW group (p<0.001).

In step 2, ANOVA with the factors group, test and QUID (four levels: QUIDs, sensorial; QUIDa, affective; QUIDe, emotional; QUIDm, miscellaneous repeated) revealed a significant effect of QUID [$F_{3,108} = 28.22$, $p<0.001$] since QUIDs was higher than the other dimensions (Table 6). Moreover, ANOVA revealed a significant effect of the factor test on QUIDa [$F_{1,23} = 3.96$, $p<0.05$] and QUIDm [$F_{1,23} = 12.44$, $p<0.001$] due to the higher values at visit 1 than visit 2. No differences were found for the factor group. ANOVA applied to PRIr-T (Figure 22) revealed a significant effect of test [$F_{1,23} = 5.74$, $p<0.05$] due to the higher values at visit 1 than visit 2.

Table 6. **Italian Pain Questionnaire (QUID) in step 2** in the Pre-MW (premenopausal women) and MW (menopausal women) groups; visit 1 (T1), visit 2 (T2); classes: s (sensorial), a (affective), e (emotional), m (miscellaneous). Values are reported as mean±SEM. #p<0.05, ###p<0.001 T1 vs T2; ***p<0.001 vs other classes independently of group.

Group (N)	**QUID s*** **		**QUID a**		**QUID e**		**QUID m**	
	T1	**T2**	**T1#**	**T2**	**T1**	**T2**	**T1###**	**T2**
Pre-MW (6)	9.3±2.4	8.7±1.5	4.7±1.8	3.5±2.0	4.2±0.8	4.8±1.9	4.2±1.5	1.5±0.7
MW (19)	9.5±1.4	6.1±3.1	4.7±0.9	2.7±0.7	4.8±1.1	3.1±0.9	3.4±0.7	1.2±0.9

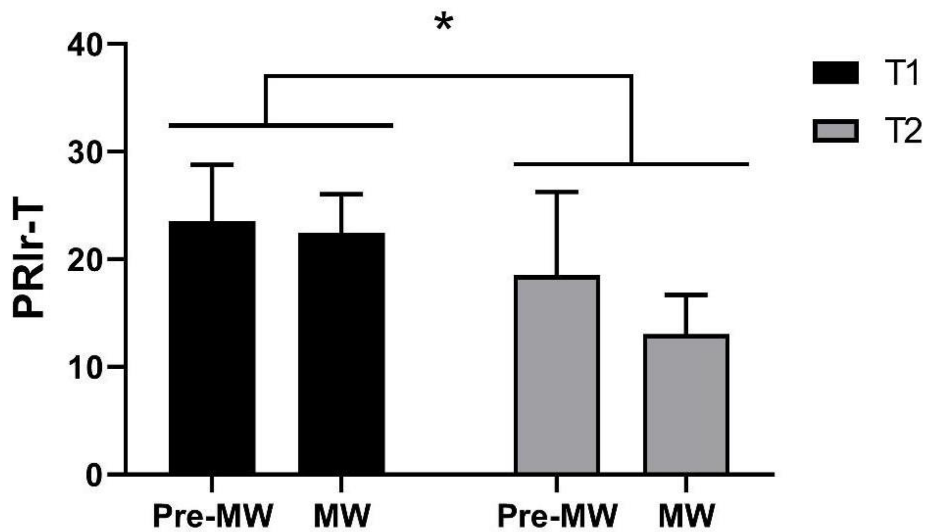


Figure 22. **PRIr-T (Pain Rating Index rank-Total) in step 2** in the Pre-MW (premenopausal women) and MW (menopausal women) groups; visit 1 (T1), visit 2 (T2). *p<0.05 T1 vs T2.

Quality of life questionnaires

Profile of Mood States

In step 1, ANOVA with the factors group, test and POMS (six levels: T-A, Tension-Anxiety; D-D, Depression-Dejection; A-H, Anger-Hostility; V-A, Vigor-Activity; F-I, Fatigue-Inertia; C-B, Confusion-Bewilderment) revealed a significant effect of group [$F_{2,67} = 5.92$, $p<0.01$] due to Pre-MW having higher (worse) values than the other groups (MW, $p<0.001$; Men $p<0.05$); moreover there was a significant effect of POMS [$F_{5,335} = 9.86$, $p<0.001$] since POMS V-A was lower than all the other scales (all $p<0.001$) and POMS F-I was higher than all the others (all $p<0.01$) except POMS A-H. The significant group × POMS interaction [$F_{10,335} = 2.91$, $p<0.001$] was due to Pre-MW having higher levels than Men and MW in all classes except POMS V-A (Table 7).

Table 7. **Profile of Mood States (POMS) in step 1** values determined in the Pre-MW (premenopausal women), MW (menopausal women) and Men groups during visit 1 (T1) and visit 2 (T2). Classes: T-A (Tension-Anxiety); D-D (Depression-Dejection); A-H (Anger-Hostility); V-A (Vigor-Activity); F-I (Fatigue-Inertia); C-B (Confusion-Bewilderment). Values are reported as mean±SEM. ###p<0.001, #p<0.05 respectively MW and Men vs Pre-MW; §§§p<0.001 POMS V-A vs all the other classes, §p<0.01 POMS F-I vs all the other classes except POMS A-H.

Group (N)	POMS T-A		POMS D-D		POMS A-H		POMS V-A §§§		POMS F-I §		POMS C-B	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Pre-MW (16)	61.9± 3.3	56.8± 2.9	63.6± 4.0	59.7± 4.9	63.2± 3.1	58.1± 4.1	44.3± 2.7	45.2± 3.0	67.1± 3.7	61.9± 3.8	60.9± 2.8	55.4± 3.2
MW (12) ###	53.6± 3.0	48.9± 1.0	51.8± 2.4	48.4± 1.8	54.9± 3.6	51.3± 1.7	47.6± 3.5	48.6± 2.8	54.7± 3.6	53.6± 2.1	47.9± 3.2	46.6± 2.6
Men (7) #	50.4± 3.0	52.3± 5.9	50.1± 3.1	54.9± 4.9	51.9± 3.8	55.6± 6.0	49.1± 2.1	47.1± 5.2	56.1± 5.3	54.4± 4.0	52.1± 3.4	53.6± 2.6

In step 2, ANOVA with the factors group, test and POMS (six levels) revealed a significant effect of POMS [$F_{5,295} = 17.07$, $p<0.001$] due to POMS V-A being lower than all the other scales and POMS F-I being higher than all the others (Table 8). No significance was found for the factors group and test.

Table 8. **Profile of Mood States (POMS) in step 2** values determined in the Pre-MW (premenopausal women) and MW (menopausal women) groups during visit 1 (T1) and visit 2 (T2). Classes: T-A (Tension-Anxiety); D-D (Depression-Dejection); A-H (Anger-Hostility); V-A (Vigor-Activity); F-I (Fatigue-Inertia); C-B (Confusion-Bewilderment). Values are reported as mean±SEM. ***$p<0.001$ POMS V-A vs all the other classes, **$p<0.01$ POMS F-I vs all the other classes.

Group (N)	POMS T-A		POMS D-D		POMS A-H		POMS V-A ***		POMS F-I **		POMS C-B	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Pre-MW (6)	66.2± 4.4	59.8± 7.2	53.7± 5.2	52.7± 6.0	60.7± 4.0	56.7± 6.1	47.7± 3.9	44.7± 4.3	70.3± 6.0	62.8± 7.7	54.7± 7.1	52.7± 7.8
MW (19)	55.3± 2.3	51.7± 2.8	57.1± 2.2	54.4± 3.3	57.5± 2.9	55.8± 3.5	43.6± 1.4	44.2± 2.3	62.1± 2.5	58.1± 3.3	57.7± 2.5	52.7± 2.6

Short Form-36

The Short Form-36 results are reported in Table 9. In step 1, ANOVA was applied with the factors group, test and SF-36/PCS (for the Physical Component Summary, four levels: PF, RP, BP, GH) or SF-36/MCS (for the Mental Component Summary, four levels: V, SF, RE,

MH). There was a significant effect of SF-36/PCS [F3,261 = 25.8, p<0.001] since PF was higher than the other PCS classes (all p<0.05), independently of group and test; moreover, the significance of SF-36/MCS [F3,271 = 6.58, p<0.001] was due to V-A being lower than the other MCS classes (all p<0.05), independently of group and test.

PCS and MCS were analyzed by ANOVA with the factors group and test. There was a significant effect of test on PCS [F1,38 = 5.03, p<0.05] due to the values being higher at visit 2 than visit 1 (Figure 23).

Table 9. **Short Form (SF-36) data in step 1** recorded in Pre-MW (premenopausal women), MW (menopausal women) and Men, during visit 1 (T1) and visit 2 (T2), for each SF-36 component (PF, physical functioning; RP, role physical; BP, bodily pain; GH, general health; V, vitality; SF, social functioning; RE, role emotional; MH, mental health). Values are reported as mean±SEM. *p<0.05 vs all the other PCS classes, §p<0.05 vs all the other MCS classes.

Group (N)	PF*		RP		BP		GH		V		SF§		RE		MH	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Pre-MW (17)	73.5 ± 5.3	74.1 ± 7.3	46.2 ± 8.6	52.9 ± 10.0	39.1 ± 4.8	49.8 ± 4.4	48.3 ± 5.0	51.5 ± 5.4	39.5 ± 4.9	45.9 ± 5.8	49.8 ± 4.5**	53.5 ± 5.9**	41.5 ± 9.3	56.7 ± 9.4	53.0 ± 4.0	51.3 ± 5.6
MW (18)	66.7 ± 4.5	73.9 ± 4.3	41.7 ± 8.2	37.5 ± 8.9	43.2 ± 3.7	48.7 ± 3.6	50.5 ± 3.3	57.6 ± 3.7	42.9 ± 4.8	53.6 ± 2.6	58.8 ± 4.6	63.1 ± 4.0	50.6 ± 7.5	62.8 ± 9.3	53.1 ± 3.9	62.9 ± 2.4
Men (7)	75.5 ± 6.3	73.6 ± 6.0	55.0 ± 12.0	64.2 ± 18.0	57.7 ± 6.1*	58.2 ± 8.6*	52.2 ± 5.7	54.0 ± 6.0	56.5 ± 3.6	47.9 ± 3.9	66.1 ± 5.6	71.2 ± 6.6	59.8 ± 13.0	57.1 ± 14.0	62.4 ± 4.2	62.9 ± 7.3

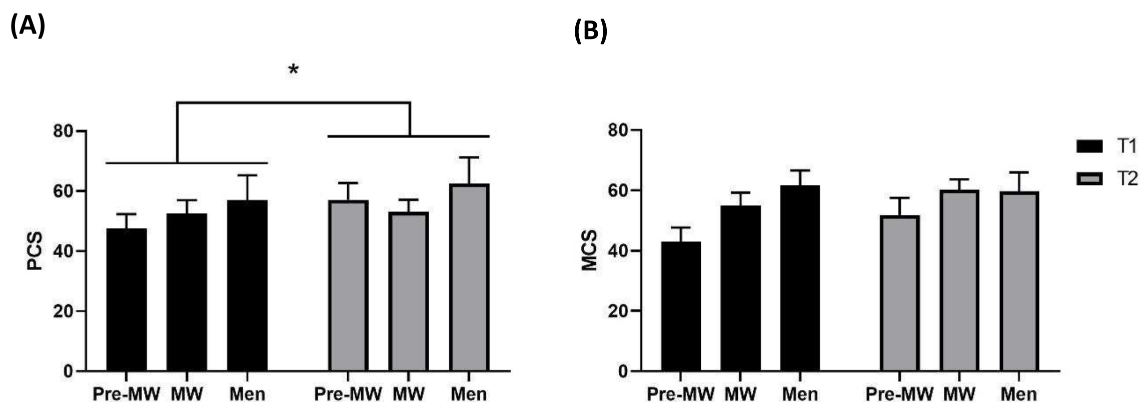


Figure 23. **(A) PCS, Physical Component Summary; (B) MCS, Mental Component Summary in step 1.** Data recorded in Pre-MW (premenopausal women), MW (menopausal

women) and Men, during visit 1 (T1) and visit 2 (T2). Values are reported as mean±SEM. *p<0.05 T1 vs T2.

In step 2, ANOVA was applied with the factors group, test and SF-36/PCS (four levels: PF, RP, BP, GH) or SF-36/MCS (four levels: V, SF, RE, MH) (Table 10). There was a significant effect of test [$F_{1,60} = 5.71$, $p<0.05$] due to higher values at visit 2 than visit 1 (improved condition). Moreover, there was a significant effect of SF-36/PCS [$F_{3,180} = 17.39$, $p<0.001$] since PF was higher than the other PCS classes (all $p<0.001$). Finally, the interaction SF-36/PCS x test was significant [$F_{3,180} = 2.87$, $p<0.05$] since only RP and BP were lower at visit 1 than visit 2. For the Mental Component, there was a significant effect of SF-36/MCS [$F_{3,180} = 8.25$, $p<0.001$] due to V-A being lower than the other MCS classes, independently of group and test.

PCS and MCS were analyzed by ANOVA with the factors group and test. The significant effect of test on both [$F_{1,24} = 7.25$, $p<0.01$; $F_{1,24} = 7.24$, $p<0.01$] was due to the higher values at visit 2 than visit 1 (Figure 24).

Table 10. **Short Form (SF-36) data in step 2** recorded in Pre-MW (premenopausal women) and MW (menopausal women), during visit 1 (T1) and visit 2 (T2), for each SF-36 component (PF, physical functioning; RP, role physical; BP, bodily pain; GH, general health; V, vitality; SF, social functioning; RE, role emotional; MH, mental health). Values are reported as mean±SEM. ***p<0.001 vs all the other PCS classes, §§§p<0.001 vs all the other MCS classes, #p<0.05 vs T2.

Group (N)	PF ***		RP		BP		GH		V§§§		SF		RE		MH	
	T1	T2	T1#	T2	T1#	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Pre-MW (6)	61.7 ± 7.7	76.7 ± 8.3	34.7 ± 5.3	62.5 ± 7.9	38.3 ± 7.3	53.3 ± 4.0	33.3 ± 11.1	42.5 ± 12.8	38.5 ± 6.5	39.2 ± 13.9	54.2 ± 13.9	60.4 ± 11.8	38.7 ± 5.9	44.5 ± 8.4	46.2 ± 10.2	56.7 ± 10.8
MW (20)	57.3 ± 5.6	65.5 ± 4.3	33.8 ± 7.5	58.7 ± 8.8	28.7 ± 2.9	50.3 ± 10.7	37.5 ± 3.2	49.8 ± 4.1	36.1 ± 3.3	45.8 ± 4.9	50.0 ± 4.3	68.1 ± 5.7	51.3 ± 8.8	62.5 ± 8.0	51.3 ± 3.9	62.2 ± 3.8

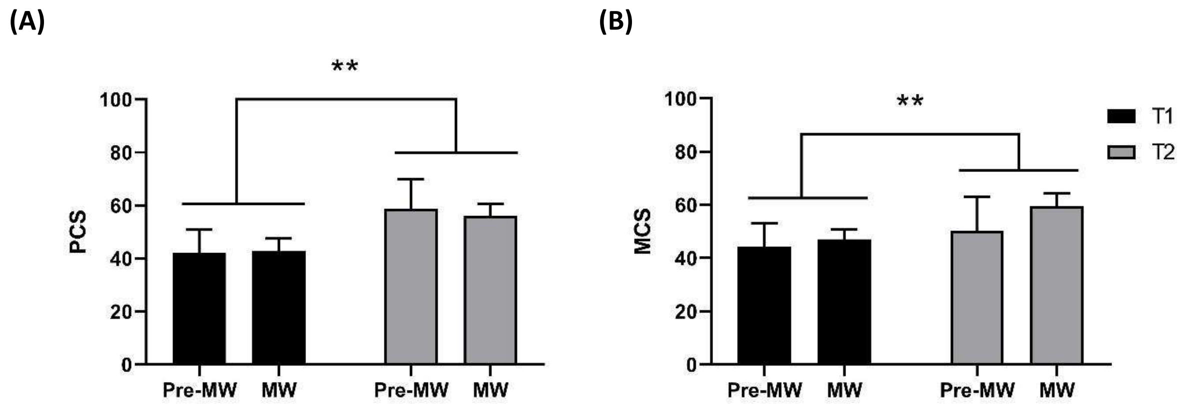


Figure 24. **(A) PCS, Physical Component Summary; (B) MCS, Mental Component Summary in step 2.** Data recorded in Pre-MW (premenopausal women) and MW (menopausal women), during visit 1 (T1) and visit 2 (T2). Values are reported as mean±SEM. **p<0.01 T1 vs T2.

Gastrointestinal conditions

GI conditions were evaluated only in step 2. The SSS questionnaire was completed by 19 women, all suffering from CWP. The SSS total score, the sum of the six subscales, was 338.95±32.41 (Figure 25 (A)). In particular, 1/19 subject was in the normal SSS total range (<90) while 3/19 had mild SSS total (90-210), 5/19 had moderate SSS total (210-360) and 10/19 had severe SSS total (>360). The six subscales were also analyzed individually: abdominal pain intensity (API) had a value of 54.21±7.99, abdominal pain frequency (APF) 49.74±8.60, abdominal swelling (AS) 58.95±6.71, abdominal heaviness (AH) 55.26±6.28, evacuation satisfaction (ES) 60.52±6.94 and interference with quality of life (IQL) 61.05±6.30 (Figure 25 (B)). In the SSS subscales, severe cases were indicated by scores higher than 60; in particular, 8/19 women reported API, APF and AS values higher than 60, 6/19 reported AH values higher than 60, 10/19 reported IQL values higher than 60, 11/19 reported ES values higher than 60.

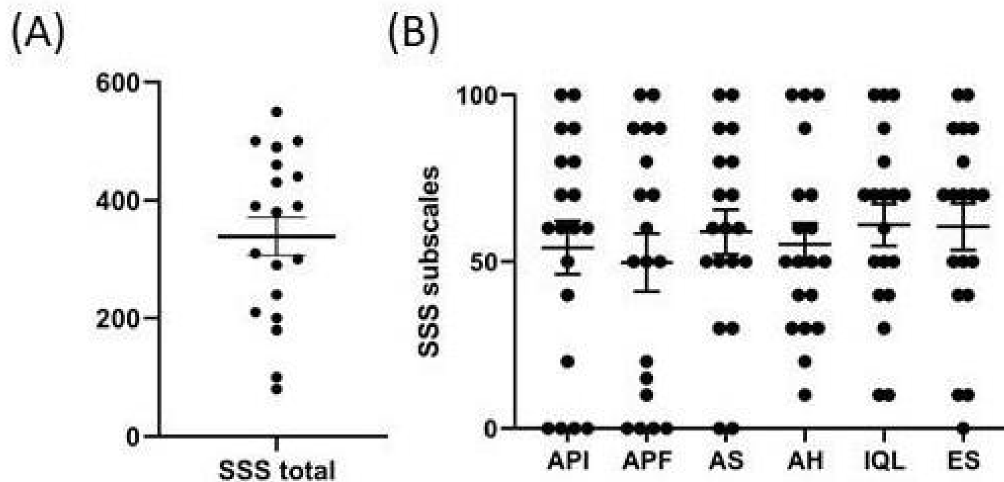


Figure 25. **Gastrointestinal (GI) Symptom Severity Scale (SSS). (A)** SSS total**; (B)** SSS subscales (abdominal pain intensity, API; abdominal pain frequency, APF; abdominal swelling, AS; abdominal heaviness, AH; interference with quality of life, IQL; evacuation satisfaction, ES). Higher values mean worse conditions. Data are reported as mean±SEM, N=19.

ANOVA applied to SSS total and single subscale values with the factors group and test revealed a significant effect of test on SSS total and on all subscales (API, APF, AS, AH, ES) except IQL [$F_{1,10} = 20.90$, $p<001$; $F_{1,10} = 10.81$, $p<0.01$; $F_{1,10} = 11.45$, $p<0.01$; $F_{1,10} = 7.52$, $p<0.05$; $F_{1,10} = 6.61$, $p<0.05$; $F_{1,11} = 18.41$, $p<0.001$ respectively] since all the values were higher at the first visit than the second visit (improved condition) (Figure 26). Moreover, SSS total of visit 2 was positively correlated with VAS mean of visit 2 [$r=0.90$, $p<0.001$, N=15] (Figure 27).

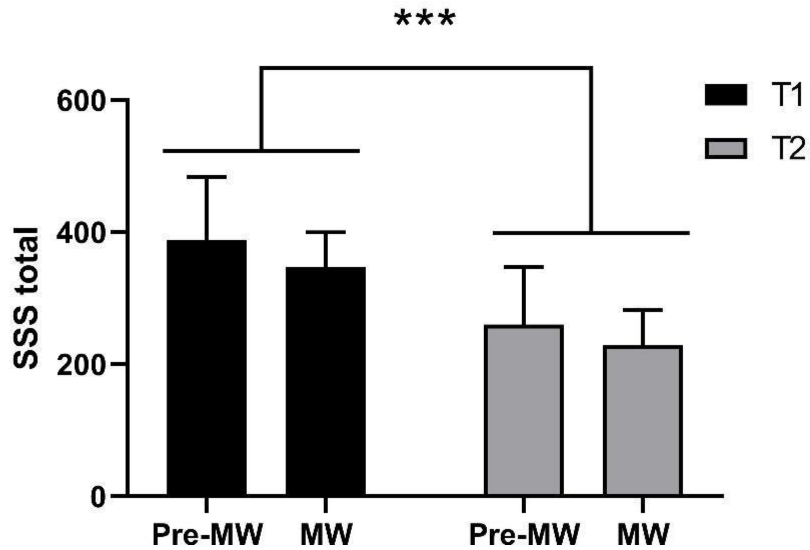


Figure 26. **Gastrointestinal (GI) Symptom Severity Scale (SSS).** Data recorded in Pre-MW (premenopausal women) and MW (menopausal women), during visit 1 (T1) and visit 2 (T2). Values are reported as mean±SEM. ***p<0.001 T1 vs T2.

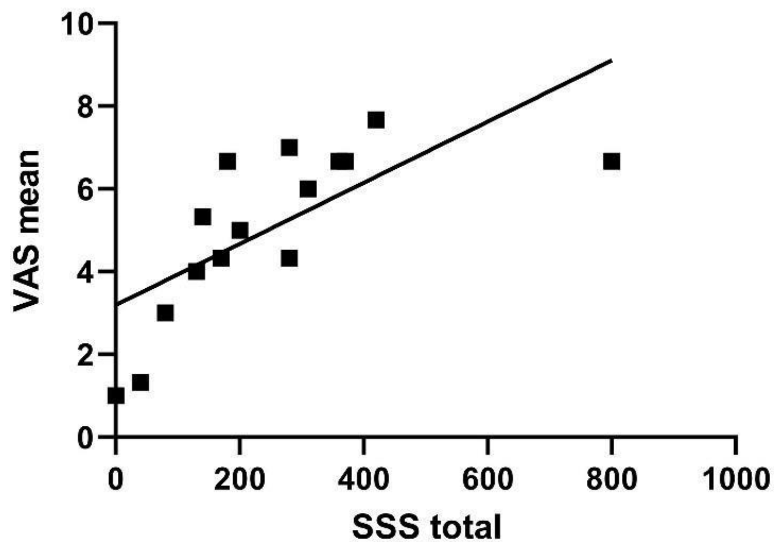


Figure 27. **Correlation between SSS total and VAS mean at visit 2.** Correlation of Symptom Severity Scale (SSS) and visual analogue scale (VAS). Pearson r analysis was used to determine the significance of the correlation (r=0.90, p<0.001, N=15).

At the end of the study, 9/15 subjects reported a reducton of GI-SSS (reduction of at least 50 points) and they were defined as "responders". No differences were found between the

responders and non-responders group (N=6) for the intensity of pain (VAS); VAS decreased in both groups.

Nutritional status and body composition

In step 1, at visit 1, BMI scores higher than 25, i.e., in the overweight/obese classes, were present in 10/19 Pre-MW, 13/22 MW and 7/10 Men (Table 11); these frequencies did not change significantly at visit 2. The BMI data were subjected to ANOVA with the factors group and test. BMI was negatively correlated with SF-36/PF (visit 1/visit 2, $r=-0.4$, $p<0.001$), indicating that higher PF (physical functioning) was related to lower BMI.
Bioelectrical impedance analysis (BIA) results are reported in Table 11; ANOVA with the factors group and test revealed a significant effect of group on total body water (TBW) and fat free mass (FFM) [$F_{2,86} = 42.08$, $p<0.001$; $F_{2,86} = 40.90$, $p<0.001$ respectively] since men had higher values than Pre-MW and MW (all $p<0.001$). There were no significant differences in body cell mass (BCM), body cell mass index (BCMI), extracellular water (ECW), phase angle (PhA) or fat mass (FM).

Table 11. **Bioelectrical Impedance Analysis (BIA) and anthropometric measures** determined in step 1, in the Pre-MW (premenopausal women); MW (menopausal women); Men, during visit 1 (T1) and visit 2 (T2); BMI (body mass index); PhA (phase angle); TBW (total body water, L); FFM (fat free mass, kg); FM (fat mass, kg).

Group (N)	**BMI**		**PhA**		**TBW**		**FFM**		**FM**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
Pre-MW (16)	27.1±1.4	26.6±1.5	5.8±0.1	5.5±0.2	35.7±1.2	34.7±1.3	48.5±1.5	46.8±1.8	23.6±2.9	23.9±3.1
MW (19)	26.7±1.0	26.4±1.1	5.4±0.1	5.5±0.1	35.5±1.0	35.4±1.2	47.9±1.3	47.5±1.3	21.5±1.9	20.5±1.8
Men (7)	26.9±1.0	27.9±1.3	6.1±0.3	5.5±0.4	47.4±1.8	48.9±1.8	63.6±2.1	63.7±2.0	18.6±2.4	22.6±2.8

In step 2, 11/38 women were in the normal BMI range (18-25) while 27/38 had a BMI over 25, i.e., 16/27 were in the overweight category (25-30) and 11/27 were classified as obese (30+) (Table 12). ANOVA was applied to BMI with the factors group and test. No significant effects were found.

BIA was carried out on 26 women. As reported in Table 12, all participants had normal fat free mass (FFM); 15/26 had high fat mass (FM) and only in 2/26 women was the total body

water (TBW) lower than the reference value. ANOVA applied to all BIA parameters with the factors group and test revealed a significant effect of test and an interaction between test and group on total body water [$F_{1,13} = 5.31$, $p<0.05$; $F_{1,13} = 6.44$, $p<0.05$ respectively] since TBW was higher at visit 2 than visit 1, in particular in Pre-MW (Figure 28).

Table 12. **Bioelectrical Impedance Analysis (BIA) and anthropometric measures** determined in step 2 in the Pre-MW (premenopausal women) and MW (menopausalwomen); during visit 1 (T1) and visit 2 (T2); BMI (body mass index); PhA (phase angle); TBW (total body water, L); FFM (fat free mass, kg); FM (fat mass, kg).

Group (N)	**BMI**		**PhA**		**TBW**		**FFM**		**FM**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
Pre-MW (21)	27.5±0.9	26.7±1.4	6.0±0.1	5.5±0.2	34.6±2.3	35.1±6.1	47.6±2.6	47.9±8.1	27.6±4.4	25.6±11.9
MW (5)	26.3±1.2	26.9±5.6	6.0±0.1	6.1±0.3	34.4±0.9	34.5±1.3	46.7±1.2	46.9±1.8	24.2±2.0	22.7±2.8

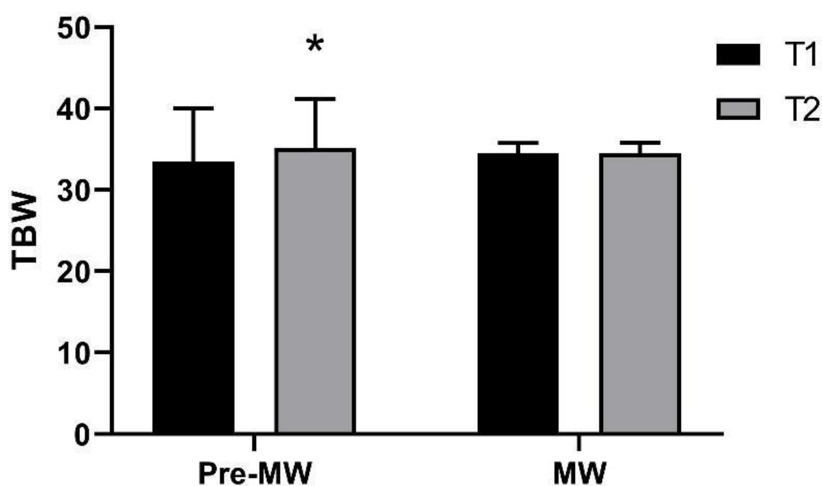


Figure 28. **TBW (total body water, in litres, L) in step 2.** Data recorded in Pre-MW (premenopausal women) and MW (menopausal women) during visit 1 (T1) and visit 2 (T2). Values are reported as mean±SEM. *p<0.05 T2 vs T1 in Pre-MW.

Determination of specific IgG4 levels

IgG4 levels were determined only in step 1. Food-specific IgG4 levels were measured in all subjects with one of two methods. The rapid test (NutriSMART®, able to test 57 foods) was used at the beginning because of its ease of use and immediate response in 23/54 subjects

(Pre-MW = 6, MW = 11, Men = 6). Later, 80 foods were tested by the ELISA method in 31/54 subjects (Pre-MW = 14, MW = 12, Men = 5). In the ELISA subjects it was possible to repeat the test after 4 weeks of the exclusion diet to assess possible changes in IgG4 levels. For the analysis, the 80 foods were grouped into 10 classes (fish, meat, cereals, vegetables, legumes, dairy products, eggs, fruit, dried fruit, various).

IgG4 levels were determined by the NutriSMART® rapid test only at visit 1, as part of a pilot study. Score values for each food ranged from 1 to 3; values equal to or higher than 2 were considered higher than normal and the subjects were asked to exclude those foods.

IgG4 levels were quantified by ELISA twice (visit 1 and visit 2). The IgG4 values ranged from 0.08 to 1259.7 U/mL; values higher than 3.50 U/mL were considered higher than normal and the subjects were asked to exclude those foods.

To determine the foods' IgG4 levels, the total sum was calculated for all subjects; Figure 29 shows the IgG4 levels determined for each of the 80 foods at visit 1 in all subjects tested with the ELISA method. Spelt and wheat showed the highest IgG4 levels in the cereals class, while casein and cow milk were highest among dairy products, and hazelnut and almond were highest in the dried fruit class. Eggs, both yolk and white (but particularly egg white), also showed very high levels.

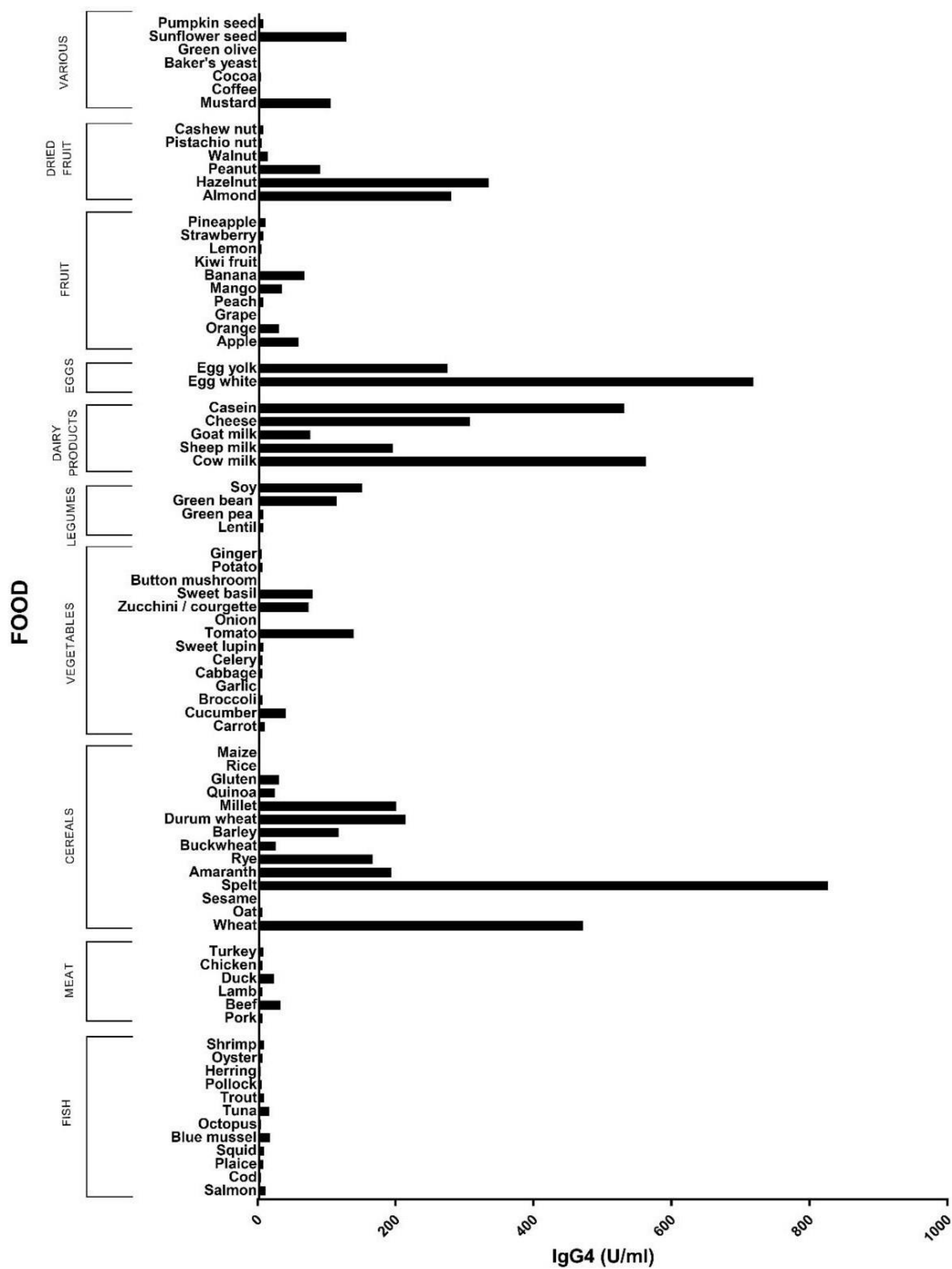


Figure 29. **Total amount of IgG4** determined (ELISA method) in the 80 foods divided into 10 classes at visit 1.

On the basis of the IgG4 levels determined at visit 1, each subject received a list of foods to be excluded as well as suggestions to replace foods and a booklet to note all changes occurring during the 4-week period.

Foods with IgG4 higher than normal (i.e., values higher than 3.50 U/mL or values of 2 or higher) were present in all subjects; in particular, 63% of subjects showed more than five foods to be excluded. Table 3 reports the list of all excluded foods and the percentages of

subjects that excluded that food. Several foods had to be excluded in a very high percentage of subjects (at least 15%) (Table 13).

Table 13. **List of foods excluded and for each food the percentage (%) of subjects excluding that food**. Exclusion was carried out with IgG4 higher than 3.5 U/mL or a score ≥ 2 in the rapid test.

FOOD	%	FOOD	%	FOOD	%
Salmon	5.6	Carrot	11.1	Apple	22.2
Blue mussel	9.3	Cucumber	11.1	Orange	16.7
Tuna	13	Broccoli	3.7	Grape	1.9
Trout	5.6	Garlic	7.4	Peach	1.9
Pork	20.4	Cabbage	1.9	Mango	3.7
Beef	27.8	Celery	11.1	Banana	35.2
Lamb	16.7	Tomato	18.5	Kiwi	11.1
Duck	7.4	Onion	7.4	Lemon	9.3
Chicken	5.6	Zucchini/courgette	14.8	Strawberry	1.9
Turkey	7.4	Sweet basil	16.7	Pineapple	18.5
Wheat	37.0	Potato	9.3	Almond	25.9
Spelt	24.1	Green pea	22.2	Hazelnut	25.9
Amaranth	13	Green bean	46.3	Peanut	14.8
Rye	16.7	Soy	20.8	Walnut	1.9
Buckwheat	9.3	Cow milk	27.8	Mustard	3.7
Barley	22.2	Sheep milk	35.2	Coffee	1.9
Durum wheat	13	Goat milk	22.2	Yeast	5.6
Millet	37.0	Casein	29.6	Cocoa	5.6
Quinoa	9.3	Egg white	42.6	Sunflower seed	5.6
Gluten	18.5	Egg Yolk	25.9	Pumpkin seed	1.9
Rice	11.1				

To know the total amount of IgG4 per subject and their changes between visits, the IgG4 values obtained by ELISA for the 80 foods were summed and ANOVA was applied to the total amount with the factors group (three levels; Pre-MW, MW and Men) and test (two levels: visit 1 and visit 2). Neither group [$F_{2,56} = 1.11$, n.s.] nor test [$F_{1,56} = 0.41$, n.s.] showed significance, indicating that all groups had the same IgG4 levels and there was no change in IgG4 from visit 1 to visit 2.

To study possible subject group differences and variations between the two visits in the different food classes, ANOVA was applied to the IgG4 values with the factors group, test and food (10 levels repeated: fish, meat, cereals, vegetables, legumes, dairy products, eggs, fruit, dried fruit, various). As shown in Figure 30, ANOVA revealed the significance of food [$F_{9,504} = 11.24$, $p<0.001$] due to IgG4 levels for cereals, dairy products, eggs and dried fruit being higher than the others. Group and test did not show significance, suggesting no influence of sex, age or time since it appears that after 1 month of food-specific exclusion the IgG4 levels were not modified.

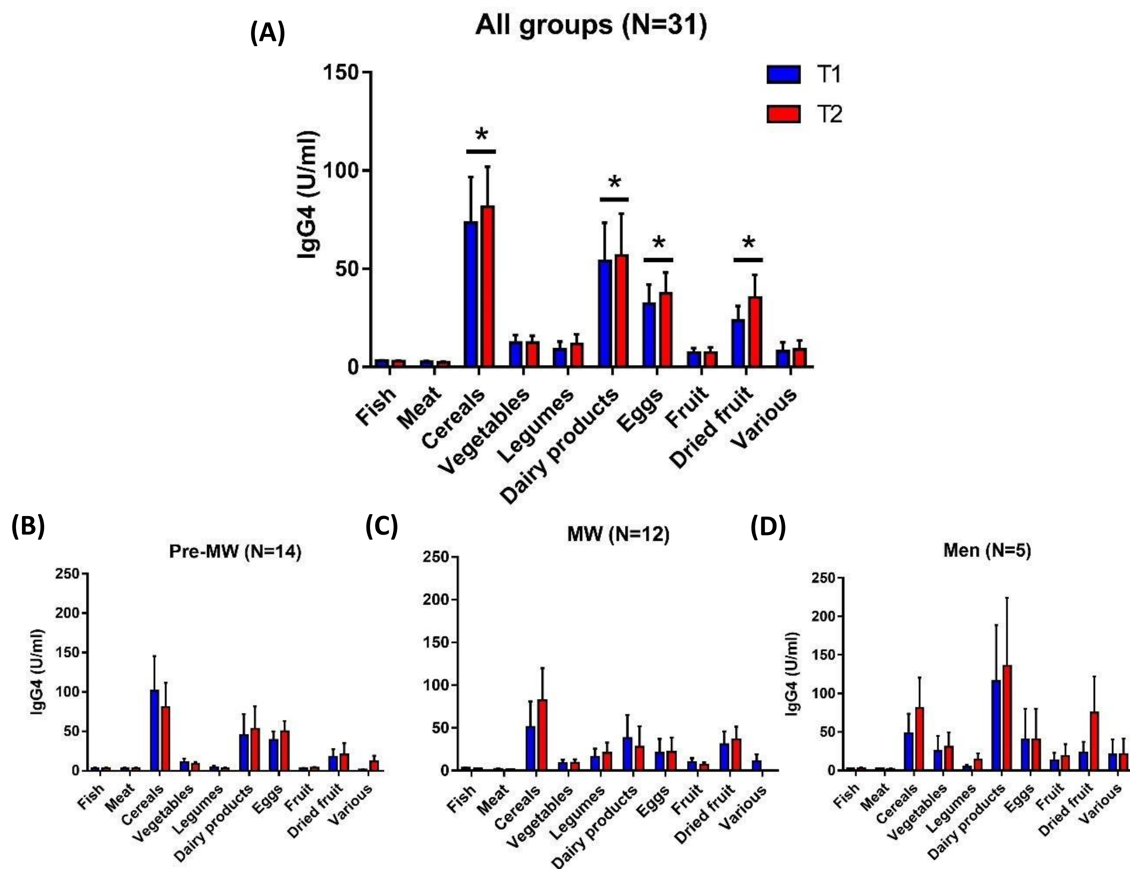


Figure 30. **IgG4 levels.** (**A**) IgG4 total for all groups; (**B**) Pre-MW (premenopausal women); (**C**) MW (menopausal women) and (**D**) Men during visit 1 (T1) and visit 2 (T2). *$p<0.05$ cereals vs all the other foods; dairy products vs all the other foods except cereals; eggs vs all the other foods except cereals, dairy products and dried fruit; dried fruit vs fish, meat, fruit and various.

Dietary Inflammatory Index

In step 2, feeding habits were investigated in more detail and the Dietary Inflammatory Index (DII) was calculated from the 24HR data. The DII was calculated only at visit 1 since by visit 2 all subjects had followed an anti-inflammatory diet.

As shown in Figure 31, in all subjects the DII showed values higher than 0, i.e., in the pro-inflammatory range. Indeed, more than 50% of subjects showed very high values (>3). ANOVA applied to the DII score with the factor menopause (Pre-MW, MW) showed no significant differences ($p>0.05$). No significant correlation was found between the DII and the pain measures ($p>0.05$).

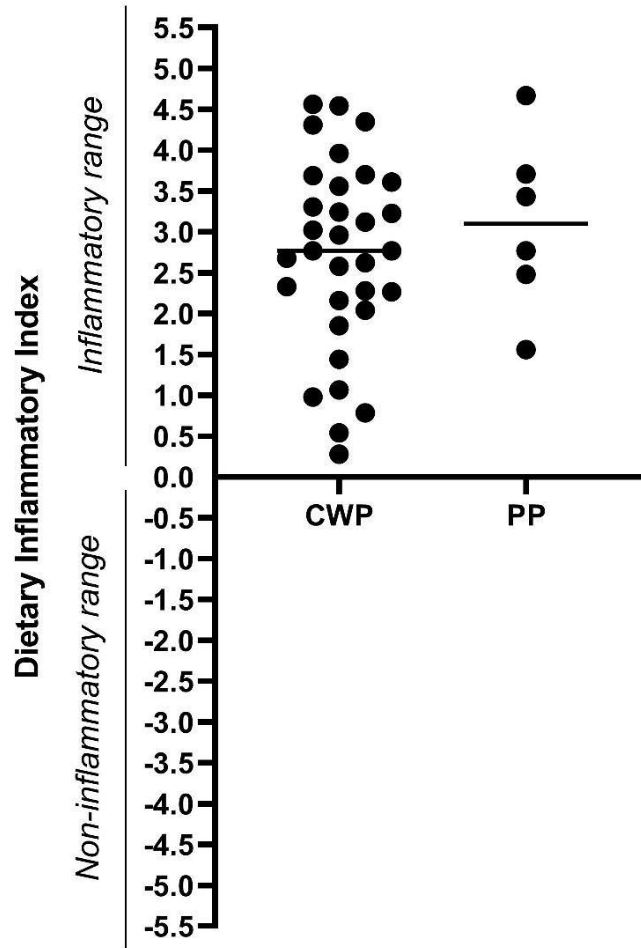


Figure 31. **Dietary Inflammatory Index (DII)** in Pre-MW (premenopausal women, N=11) and MW (menopausal women, N=27). The DII is considered non-inflammatory in the range -5.5/0 or inflammatory (0/+5.5). Data are reported as single values and as mean values.

Urinary tryptophan metabolites

Urinary tryptophan metabolites were determined only in step 2 at visit 1 (N=21, 14 women with chronic pain and 7 age-matched controls (without pain)) and visit 2 (N=8 women with chronic pain). There were higher values of 5-HIAA and QA in the women with chronic pain than in the control subjects, and in the women with pain the values were positively correlated with the VAS mean (N=14, respectively: $r=0.64$, $p<0.01$; $r=0.55$, $p<0.05$) (Figure 32 (A) and (B)). Moreover, in subjects with pain, ANOVA applied to the tryptophan metabolites with the factor VAS range (low VAS: 3-5.5; high VAS: 6-8) revealed a significant effect on 5-HIAA [$F_{1,12} = 5.23$, $p<0.05$, N=14] due to 5-HIAA being higher in women with high VAS than women with low VAS. QA showed a similar trend but the effect was not significant (Figure 32 (C) and (D)).

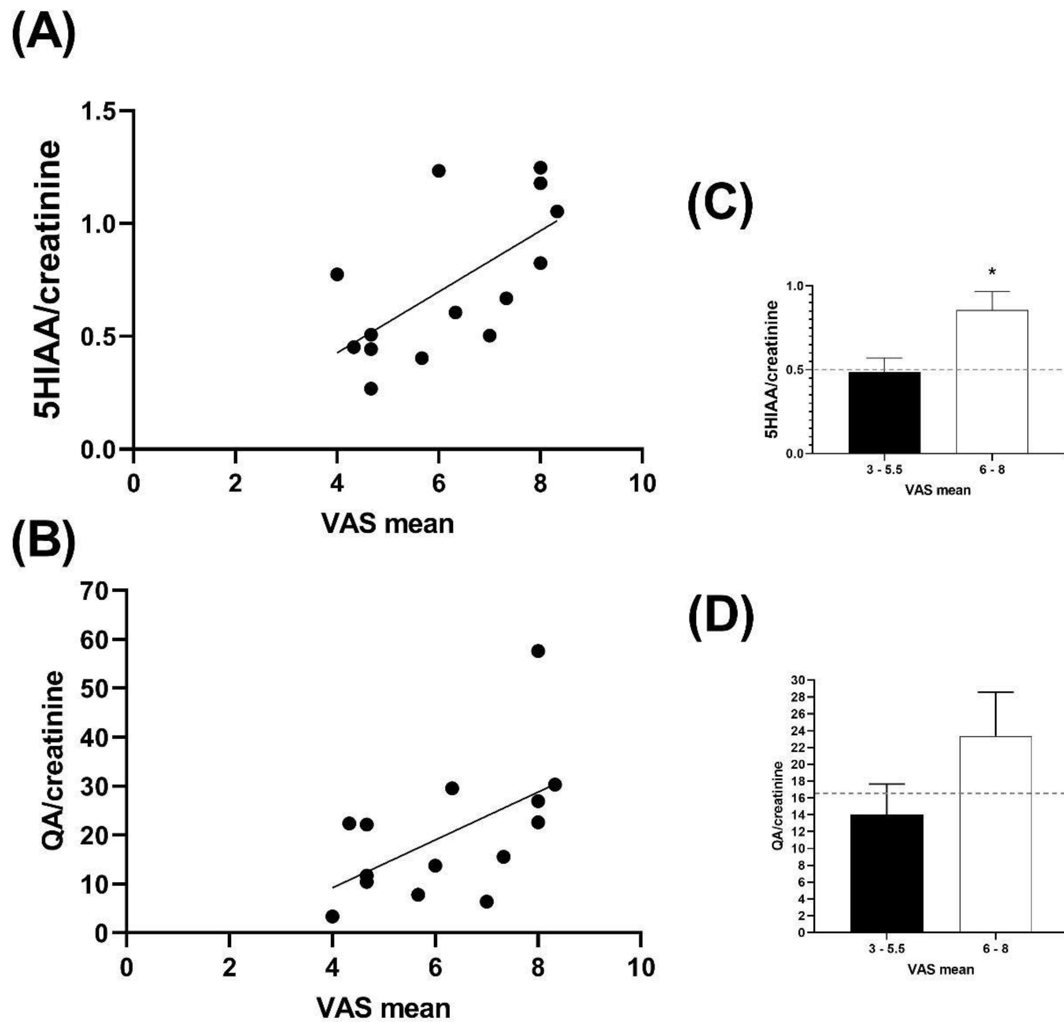


Figure 32. **Correlation of VAS mean and (A) 5-hydroxyindole acetic acid (5-HIAA)/creatinine** and **(B) quinolinic acid (QA)/creatinine** carried out during visit 1. Pearson r analysis was used to determine the significance of the correlation (N=14): **A** $p<0.01$ and **B** $p<0.05$. **(C) 5-HIAA/creatinine** and **(D) QA/creatinine** in women with low VAS range (VAS: 3-5.5) (N=5) and with high range (VAS: 6-8) (N=9). *$p<0.05$ vs VAS:3-5.5. Dashed lines indicate the mean values of the control subjects (N=7). Data are reported as mean±SEM.

5-HIAA was negatively correlated with BP/SF-36 [N=14, r=-0.74, $p<0.01$] due to the higher BP values (better condition) in women with low 5-HIAA levels (Figure 33).

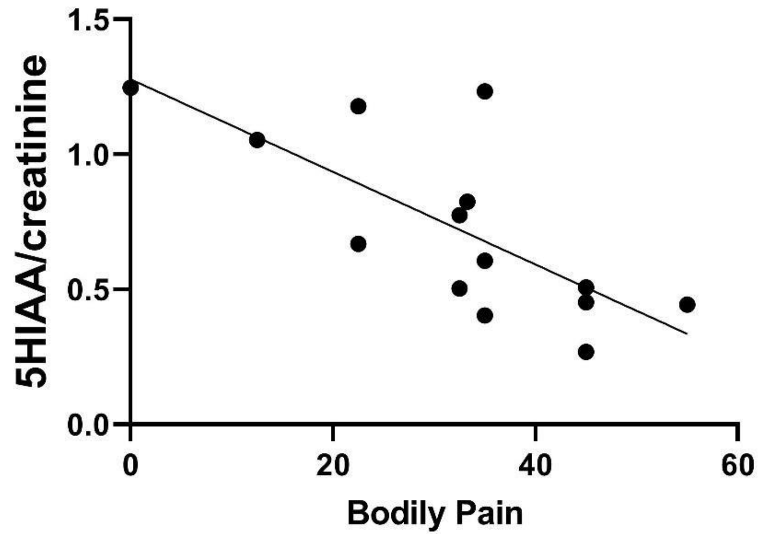


Figure 33. **Correlation of 5-hydroxyindole acetic acid (5-HIAA)/creatinine and bodily pain (BP) values (N.B. higher BP values mean better condition).** Pearson r analysis was used to determine the significance of the correlation at visit 1 ($r=-0.74$, $p<0.01$, $N=14$).

The factor group (Pre-MW and MW) was not considered due to the too low number of subjects.

Repeated measures ANOVA applied to the urinary tryptophan metabolites with the factor test showed no significant differences ($p>0.05$) (Figure 34).

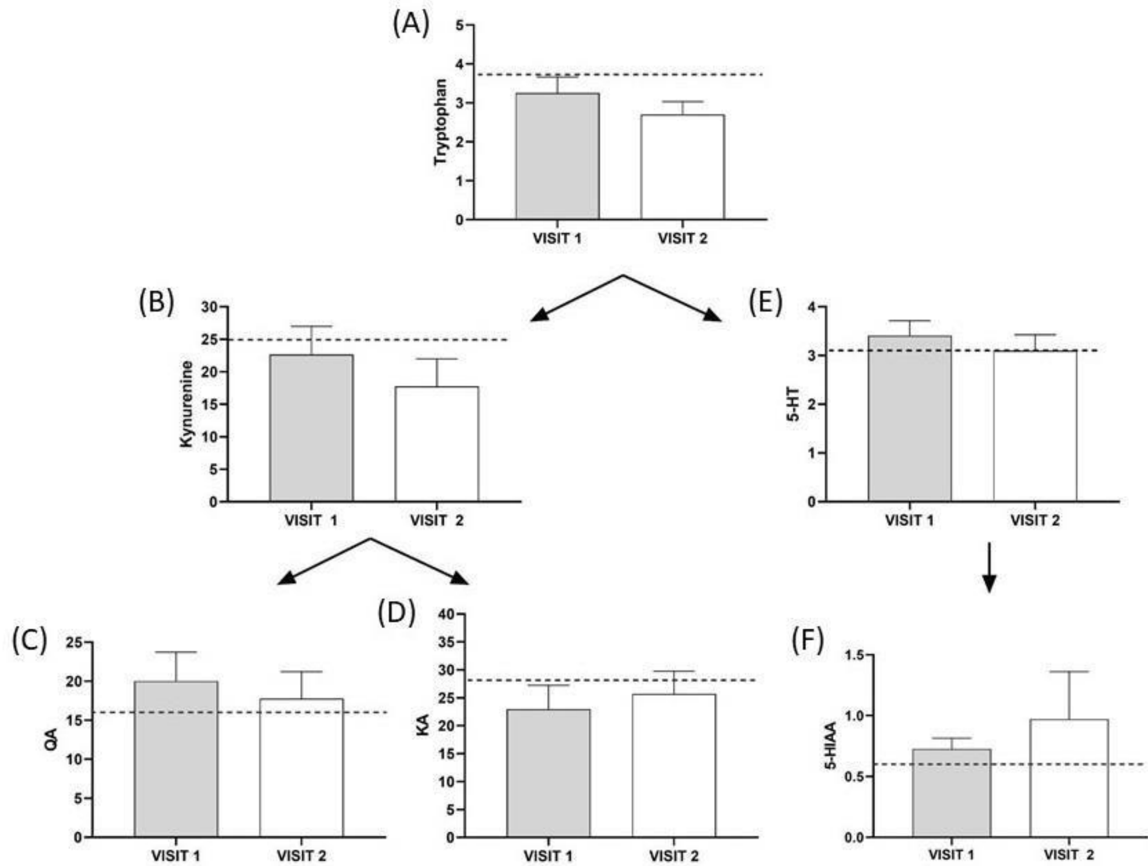


Figure 34. **Urinary tryptophan metabolites** in chronic pain subjects with data from visit 1 and visit 2: N=8**. (A)** Tryptophan, **(B)** Kynurenine, **(C)** Quinolinic acid (QA), **(D)** Kynurenic acid (KA), **(E)** Serotonin (5HT) and **(F)** 5-hydroxyindole acetic acid (5-HIAA). All measures were normalized with creatinine concentration. Dashed lines indicate the mean values of the control subjects (N=7). Data are reported as mean±SEM.

In vitro

Caco-2 vitality

The MTT assay was applied to Caco-2 cells to test the effect on cell viability of serotonin (5HT), lipopolysaccharide (LPS) and a 5HT+LPS solution. The 5HT concentration was 10 µM, LPS concentration was 10 µg/mL and the solution consisted of 5HT at 10 µM and LPS at 10 µg/mL. The cell vitality (% of control, CT) was evaluated after 24 hours of treatment and after 24 hours post-treatment. As shown in Figure 35, no cytotoxic effect was observed but the mean absorbance values revealed that the cell viability of Caco-2 cells was decreased with LPS [$F_{4,44} = 15.44$, $p<0.001$] after 24 hours of treatment. No significant effect was found with 5HT or LPS+5HT.

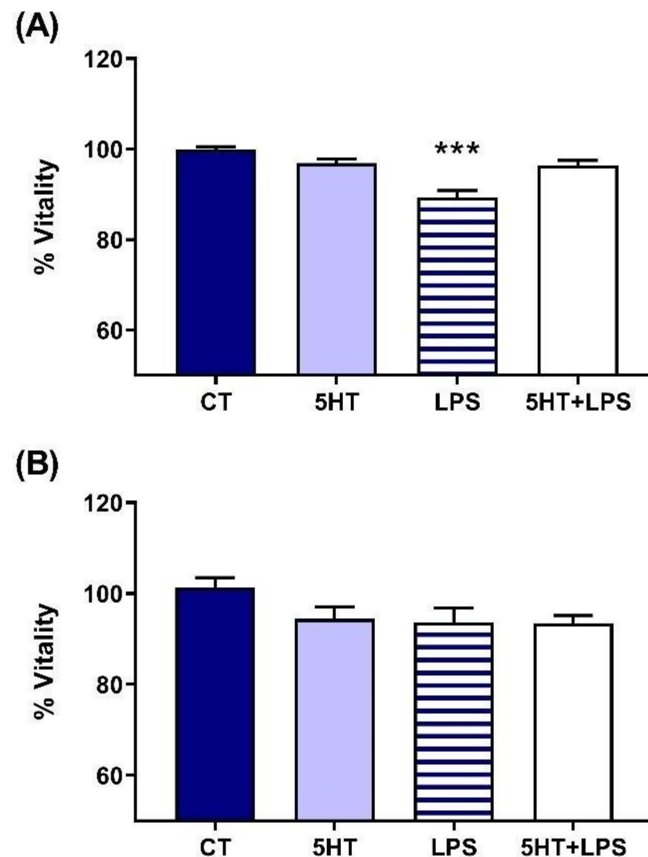


Figure 35. **Caco-2 vitality (% of control, CT).** Serotonin (5HT) 10 µM, lipopolysaccharide (LPS) 10 µg/mL. **(A)** After 24 hours of treatment (T) ***p<0.001 vs CT; **(B)** After 24 hours treatment+24 hours post-treatment (PT). Data are reported as mean±SEM.

Pro-inflammatory cytokine levels in Caco-2 supernatant

ELISA was used to test IL-1β and IL-6 levels in supernatant (SPN) of Caco-2 cells treated with 5HT, LPS and 5HT+LPS (as explained above) after 24 hours of treatment and after 24 hours post-treatment. ANOVA applied to IL-1β levels showed a significant effect of LPS after 24 hours of treatment [$F_{5,6} = 5.61$, $p<0.05$] due to IL-1β levels being higher in supernatant of cells treated for 24 hours with LPS than in CT (Figure 36).

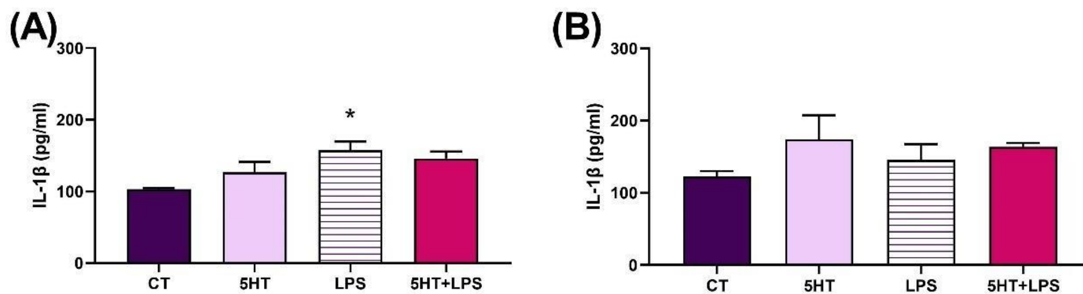


Figure 36. **IL-1β (pg/mL) in Caco-2 SPN.** Serotonin (5HT) 10 µM, lipopolysaccharide (LPS) 10 µg/mL. **(A)** SPN after 24 hours of treatment (T) *p<0.05 LPS vs CT; **(B)** SPN after 24 hours treatment+24 hours post-treatment (PT). Data are reported as mean±SEM.

ANOVA applied to IL-6 levels showed a significant effect of LPS and LPS+5HT after 24 hours of treatment [$F_{5,6} = 10.58$, $p<0.05$] due to the IL-6 levels being higher in supernatant of cells treated for 24 hours with LPS and LPS+5HT than in CT (both $p<0.05$) (Figure 37). Moreover, there was a significant effect of LPS at 24 hours post-treatment [$F_{5,6} = 5.59$, $p<0.05$] since the IL-6 levels were lower in supernatant of LPS-treated cells than in CT (Figure 37).

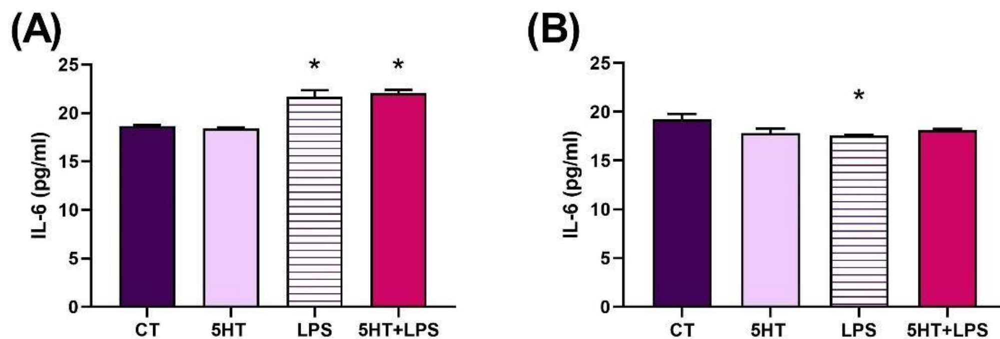


Figure 37. **IL-6 (pg/mL) in Caco-2 SPN.** Serotonin (5HT) 10 µM, lipopolysaccharide (LPS) 10 µg/mL. **(A)** supernatant after 24 hours of treatment (T), *$p<0.05$ LPS and LPS+5HT vs CT; **(B)** supernatant after 24 hours treatment+24 hours post-treatment (PT), *$p<0.05$ LPS vs CT. Data are reported as mean±SEM.

Exosomes characterization

Exosomes were isolated from Caco-2 supernatant to characterize these extracellular vesicles in different conditions (CT, LPS, 5HT and LPS+5HT groups). Firstly, the size distribution and concentration of Caco-2-derived exosomes were identified by means of NanoSight NTA. The total particles value was high, averaging between $2.43*10^9$ and $2.61*10^{10}$ per mL (Figure 38 (A) and (B)). The size distribution in the total extract was concentrated around a mean of 134.4 nm, which was in accordance with the feature of the exosomes (Figure 38 (A) and (B)).

The exosome surface markers CD9 and CD81 as well as the internal marker TSG101 were present (Figure 38 (C)).

In the TEM images, the Caco-2-derived exosomes appeared as circular particles (Figure 38 (D)) and the average diameter was around 108 nm.

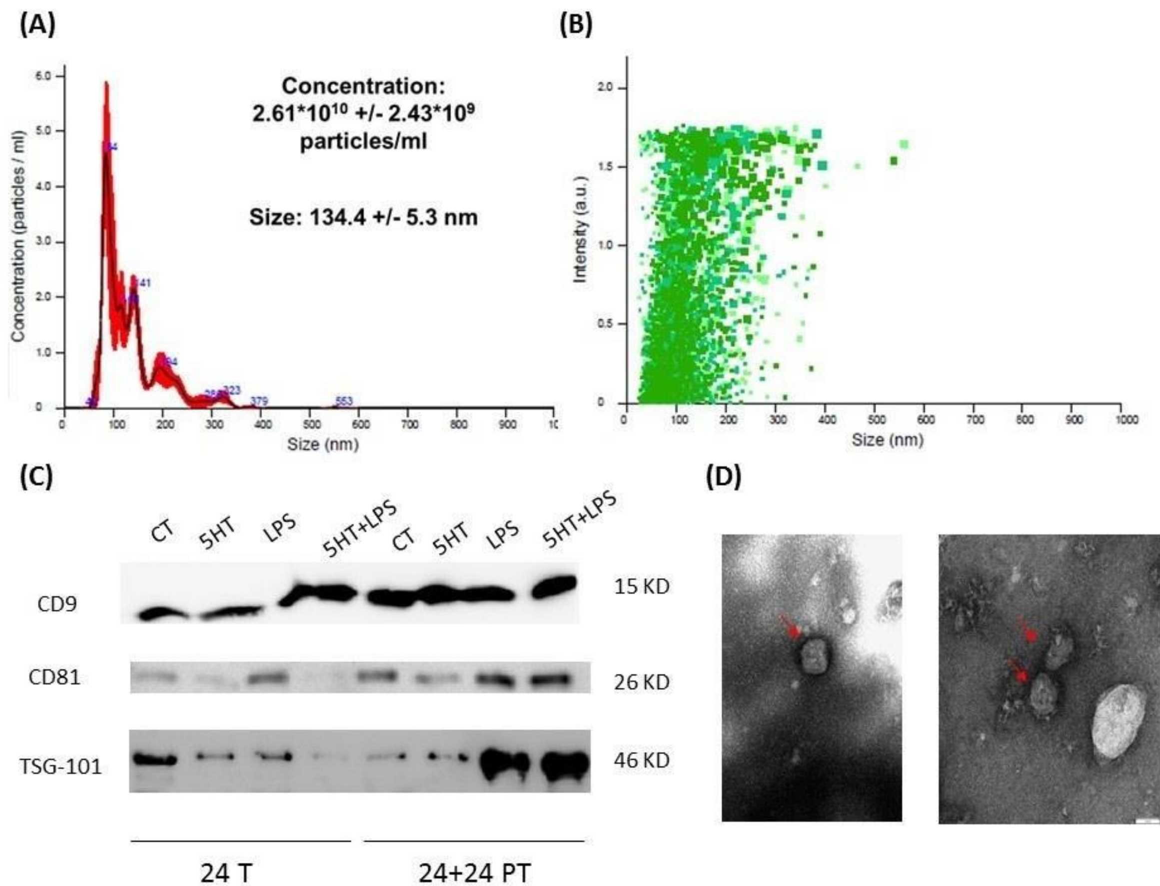
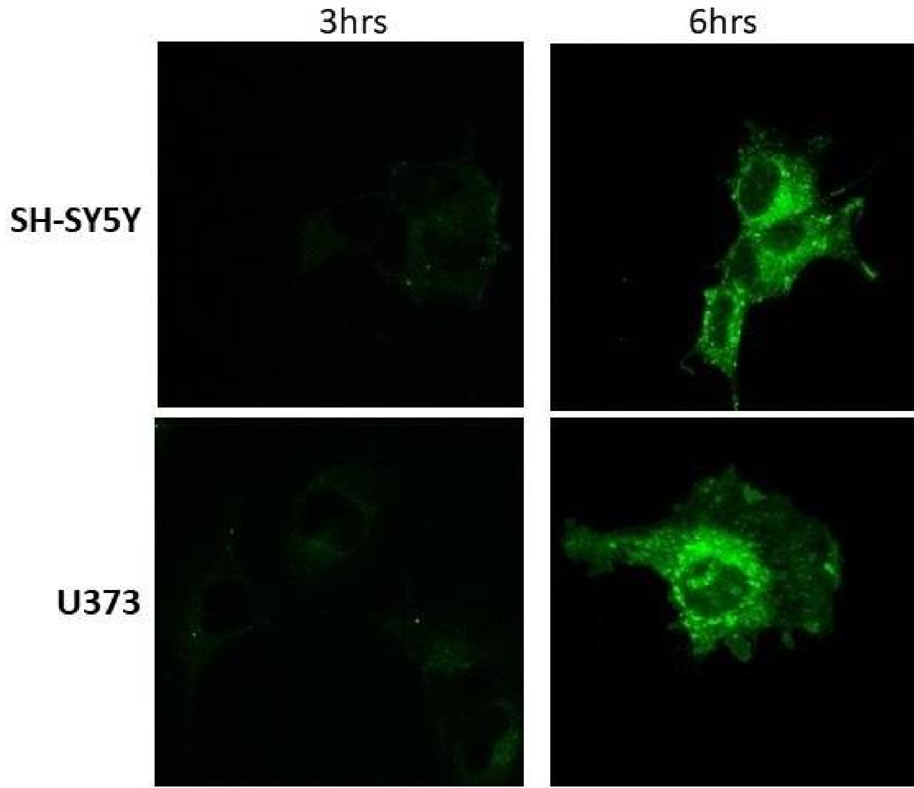


Figure 38. **Characterization of Caco-2-derived exosomes. (A, B)** Observation of the number and size of exosomes by nanoparticle tracking analysis. **(C)** Measurement of protein levels of CD9 (PM: 15 KD), CD81 (PM: 26 KD) and TSG-101 (PM: 46 KD) by Western blotting. Abbreviations: control (CT), serotonin (5HT) 10 μM, lipopolysaccharide (LPS) 10 μg/mL, after 24 hours of treatment (24 T), after 24 hours treatment+24 hours post-treatment (24+24 PT), **(D)** Images of exosome morphology from transmission electron microscopy (TEM).

Exosome uptake in SNC cell lines

Labeled exosomes were incubated with U373 and SH-SY5Y cells to assess uptake of the extracellular vesicles amd visualized by confocal microscopy. As shown in Figure 39, the fluorescence in acceptor cells had increased over time at 6 hours in both cell types.


(A)
3hrs
6hrs
SH-SY5Y
U373

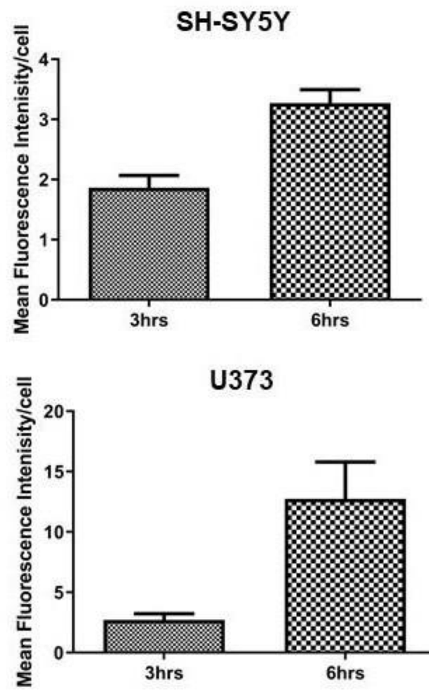

(B)
SH-SY5Y
Mean Fluorescence Intenisity/cell
4
3
2
1
0
3hrs
6hrs
U373
Mean Fluorescence Intenisity/cell
20
15
10
5
0
3hrs
6hrs

Figure39. **(A) Exosome uptake by SH-SY5Y and U373 cells** at 3 hours and 6 hours. Caco-2-derived exosomes were labeled with PKH67 (green). (**B) Mean fluorescence intensity** quantification of SH-SY5Y and U373 cells treated with exosomes labeled with PKH67 for 3 hours and 6 hours. Magnification 63 x.

Pro-inflammatory cytokine levels in exosomes

ELISA was used to test IL-1β and IL-6 levels in exosomes released by Caco-2 cells treated with 5HT, LPS and 5HT+LPS (as explained above) after 24 hours of treatment (Figure 40 (A) and (B)). ANOVA applied to IL-1β levels showed a trend towards higher levels in the LPS sample than in CT [$F_{3,4} = 5.93$, $p=0.06$] (Figure 40 (A)). No effect was found for IL-6.

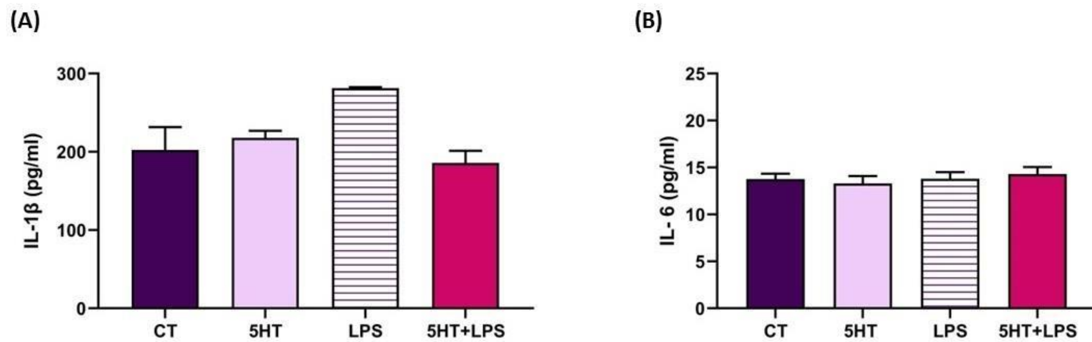


Figure 40. **(A) IL-1β (pg/mL) and (B) IL-6 (pg/mL) in Caco-2 EXO.** Serotonin (5HT) 10 µM, lipopolysaccharide (LPS) 10 µg/mL. In exosomes isolated by SPN of Caco-2 after 24 hours with the treatment. Data are reported as mean±SEM.

DISCUSSION

The main results of the present study can be summarized in three points: the first is related to the significant improvement in pain conditions in the chronic pain sufferers merely from a change in food consumption; the second involves the several pieces of evidence suggesting strong interactions between chronic pain intensity and GI health; the third highlights the communication between nutrition/gut and brain via microvesicles called exosomes.

Recently the World Health Organization (WHO) highlighted the importance of diet in relation to chronic disease management (Schulze et al., 2018). Unhealthy eating behavior and a poor diet are suggested to predict, perpetuate or underlie a variety of clinical conditions (Elma et al., 2020).

In the human part of the study, the data refer to subjects of different ages (hence of different reproductive status) and with chronic pain of different origin. Thus, the subjects were divided into groups based on sex/menopausal status. The subjects with pain were not chosen on the basis of the pathology but merely because of the presence of chronic pain. Indeed, as the study considered pain not directly related to the gut, the aim was to assess the possibility to improve the pain condition independently of the pain diagnosis. In accordance with the Classification of Chronic Pain for the International Classification of Diseases (ICD-11) by the International Association for the Study of Pain (IASP), pain was considered a pathology and not a symptom, and an attempt was made to directly modulate pain pathways regardless of the initial cause that activated them. Pain related to the intestine was never considered the "primary" pain.

Age, and in particular menopausal status, was always considered in the data analysis since estrogens play a significant role in the physiology and pathology of the GI tract, including regulation of motor and sensory functions (Mulak et al., 2014). For instance, earlier studies indicated that women have slower colonic transit than men (Degen and Phillips, 1996).

The pain condition was obtained with the most used tools able to determine pain intensity and distribution (VAS, MA), pain quality (QUID) and bodily pain (SF-36/BP). The results indicated that the pain intensity was related to GI conditions (GI-SSS). Indeed, the gut often presents disorders considered 'normal' and rarely considered by clinicians as part of the problem in pain subjects. In the present study, more than 80% of subjects reported GI

disorders. Most of them were related to feeding in both direct and indirect ways. Directly because ALL subjects showed values >0, i.e., in the inflammatory range (DII). Indirectly because the use of drugs always affects the gut functions. The inhibitory action of opioids on GI transit is well known, constipation being a common side effect of opioids; it is probably less considered that opioids inhibit GI secretions, with less mucus and thus less digestive enzymes (Khansari et al., 2013). Importantly, NSAIDs, antacids and antidepressants (able to disrupt 5HT metabolism) also have important effects on the GI tract. Therefore, due to their long-term use, it can be hypothesized that the vicious circle once started would never stop.

GI disorders can be accompanied by cellular alterations able to disrupt the regular digestion/absorption of foods/nutrients (Bielefeldt et al., 2009). This condition can have two main consequences: first, the lack of an adequate absorption of nutrients, for instance Vit. D, Vit. B12, etc.; second, poor digestion of foods will allow the arrival of undigested elements in the colon, able to significantly alter the gut microbiota with long-lasting effects (e.g., decrease of SCFAs) and to produce products (e.g., gas) that lead to other clinical consequences. These effects were summarized by the GI-SSS test in which most of the subjects showed very high values, indicating discomfort.

Several studies have hypothesized the relationship between inflammatory processes and pain (Watkins and Maier, 2005). During inflammation the immune system is activated and able to release several cytokines, which can induce a number of short- and long-lasting effects, including pain pathway sensitization (Dantzer et al., 2008). The inflammation can be acute or chronic (Pahwa et al., 2023). If the stimulus is short-lasting the resolution of inflammation will be the critical process able to protect host tissues. However, if the inflammatory stimulus persists and/or the resolution process is inadequate, prolonged or excessive inflammation will be present and will be the basis for chronic inflammation (Cotran et al., 1994). A particular type of chronic inflammation is low-grade inflammation (Rönnbäck and Hansson, 2019). This kind of low-grade increase in systemic concentrations of inflammatory markers has been observed in subjects with chronic pain (Koch et al., 2007; Parkitny et al., 2013). Low-grade inflammation can be initiated or maintained by gut damage even though a clear pathology is not necessarily manifested (Chassaing and Gewirtz, 2014). The gut barrier integrity can be affected by intrinsic and extrinsic factors, including inflammatory mediators released by the intestinal mucosa (Martel et al., 2022). Many studies have suggested that some foods, nutrients and non-nutrient food components modulate inflammation both

acutely and chronically (Calder et al., 2011). At present, there are no authorized or non-authorized health claims that specifically address the health benefit area of suppression or control of low-grade inflammation, although it is now clear that nutrition is a protagonist in this process (Minihane et al., 2015).

In the present study, the interaction between the gut and pain was also confirmed by the urinary levels of tryptophan metabolites, which were correlated with pain intensity.
Indeed, 5-HIAA is the primary urinary metabolite of serotonin and it is an index of serotonin concentration in the blood. The higher levels found in subjects with higher pain intensity support our hypothesis of serotonin involvement in chronic pain. Once released from enterochromaffin cells, 5HT stimulates local digestive functions through the enteric nervous system or is transported in the blood (Liu et al., 2021). Currently, the exact role of serotonin in GI inflammation is complex and not fully understood. Several animal and human studies have demonstrated profound changes in one or more elements of 5HT signaling in the development of a variety of symptoms in IBS, including abdominal pain and diarrhea (Linden et al., 2003; O'Hara et al., 2004; Bertrand et al., 2010). Furthermore, a study on dextran sulfate sodium (DSS)-induced colitis in mice revealed that intestine-derived 5HT has a great effect on regulation of the composition and growth of commensal bacteria in vitro (Kwon et al., 2019). Once in the blood, 5HT cannot cross the blood-brain barrier but can be captured by platelets able to release it in response to altered conditions in the body area suffering some local tissue disorders (Rust et al., 2023). These data do not support a specific inflammatory role of 5HT in in vitro studies nor a great effect on the release of cytokines from cells. Thus we have to suggest the possibility that 5HT once released in the blood can induce pain in the periphery because of its effects like vasoconstriction.

In addition to 5HT, another tryptophan metabolite of great importance is kynurenine. At least 90% of the human intake of tryptophan is converted to kynurenine for further metabolism (Badawy, 2017) by IDO. Pro-inflammatory cytokines potently induce IDO activity. Kynurenine is further catabolized into two neuroactive inflammatory mediators, KAand QA. Unlike 5HT, kynurenine catabolites can cross the blood-brain barrier, and in the brain QA acts as a neuronal NMDA channel agonist (Guillemin et al., 2005), important in the neuronal plasticity underlying the chronicity of pain (Schwarcz and Stone, 2017). The increase of the kynurenine pathway leads to a massive consumption of tryptophan and a reduction of the serotonergic pathway. However, other studies have reported that IDO knockout mice show a worsening of intestinal conditions (Ciorba et al., 2010). In the present

study, there were higher QA values in pain subjects than in controls, and QA was positively correlated with pain intensity; these results suggest a negative role of this metabolite in subjects with pain.

The BMI determined in all subjects (both steps) showed that most of them were overweight or obese (BMI>25). A weight increase could be an indirect cause of chronic pain and be related to GI disorders. Weight is known to affect pain, firstly as a result of the clear mechanical interaction at spinal and joint levels and secondly because obesity is often accompanied by an inflammatory state (Forsythe et al., 2008); moreover, being overweight often induces subjects to limit food intake with an altered consumption of nutrients.

In the present study, a large-scale determination was carried out to identify, among 80 foods, those to which the subject with pain had developed high IgG4 levels. The 80 foods were grouped into 10 main classes (fish, meat, cereals, vegetables, legumes, dairy products, eggs, fruit, dried fruit, various). Subjects showed very high IgG4 levels, particularly in four of the food groups: dairy products, eggs, cereals and dried fruit, in agreement with several studies related to allergic reactions (Bordoni et al., 2017; Reig-Otero et al., 2018; Yu et al., 2014). Indeed, these foods are well known to exert a strong stimulating action on the immune system and in most countries it is mandatory to report their presence on food labels in order to advise allergy sufferers. The subjects with pain were asked to exclude these foods and/or other ones with high IgG4 levels. This seems to be the first time that this kind of exclusion diet procedure has been applied to subjects with pain with the primary aim to improve their painful conditions. Interestingly, the IgG4 levels measured before and after 4 weeks of an exclusion diet were identical in the same subjects. Thus, it can be hypothesized that in some cases the IgG4 antibodies remain in the blood longer than 30 days after consumption of the related food has stopped.

In the present experiment, chronic pain was significantly improved by an exclusion diet with a selective absence of foods for which the subject had shown high levels of IgG4; the improvement was independent of the IgG4 levels. Chronic pain, of any origin, may have an inflammatory component (Tal, 1999) and IgG4 production is related to inflammation (Aalberse et al., 2009). Hence, the presence of IgG4 in the blood could represent a good marker to establish if a subject is consuming foods able to stimulate their increase. It is possible that food-specific IgG hypersensitivity renders subjects more reactive to a low-grade inflammatory process which would not necessarily cause symptoms per se but can

amplify other pathologies (Minihane et al., 2015). This would explain why excluding foods to which subjects have IgG antibodies might be particularly beneficial in pain despite the fact that these antibodies may also be present in the general population (Atkinson et al., 2004).

This determination allowed a personalized diet, although in practice it is difficult to be carried out due to the cost and/or the availability of a laboratory where IgG4 can be measured. Therefore, in the second part of the experiment, the diet was personalized on the basis of the nutritionists' judgment. In particular, the subject's gastrointestinal conditions were evaluated to study possible interactions. Significant correlations were found between pain scores, GI conditions and the diet, indirectly suggesting the possible factors acting to increase pain.

In the in vitro part, exosomes released by Caco-2 cells were investigated. Evidence from recent studies on exosomes indicate that these extracellular vesicles are capable of modulation of genes and cellular function and can thereby regulate inflammation and immune response. Exosomal cargo is strongly associated with the nature of the cells that produce them. Exosomal components are optimal for use as biomarkers as they can be extracted with minimal invasive techniques from most biological fluids such as blood, urine and saliva. Data on exosomes in gut inflammation are limited. In the present study, gut inflammation was reproduced in vitro using Caco-2 cells and LPS and 5HT as inflammatory agents, alone or in combination. The human gut epithelium presents a crucial interface between ingested food items and the host. The damage was evaluated after 24 hours of treatment with the inflammatory agents and then after 24 hours post-treatment. There was an approximately 30% reduction in cell viability after 24 hours of treatment with LPS. No significant reduction appeared to be caused by 5HT alone or by the combination of both agents. In the post-damage phase, cell viability did not significantly decrease. These data seem to indicate that, even though serotonin was released in large quantities in the case of intestinal inflammation, it did not induce cell death.

Two pro-inflammatory cytokines (IL-1β and IL-6) were assessed in the cellular supernatant. Both showed a significant increase after a 24-hour treatment with LPS, and IL-6 also increased with the combination of LPS and 5HT but not with 5HT alone. These data confirm that LPS had a pro-inflammatory role, whereas serotonin seems not to have had a pro-inflammatory role. However, serotonin also did not seem to have a protective role, capable

of countering the inflammatory effects of LPS. IL-1β and IL-6 could regulate intestinal permeability by regulating the expression of tight junction proteins (Al-Sadi et al., 2013; Suzuki et al., 2011). This would explain how these cytokines played a role in intestinal inflammation.

Caco-2-derived exosomes were isolated from the cellular supernatants through ultracentrifugation and then characterized. They were on the order of $2.43*10^9$ to $2.61*10^{10}$ particles per mL, with a size of approximately 100 nm or slightly larger, and they were positive for CD-9, CD-81, and TSG-101. The incorporation of exosomes released by Caco-2 cells into central nervous system cells (neurons and astrocytes) was assessed after 6 hours. After verifying, through the characterization of exosomes, that the vesicles obtained from Caco-2 cells were indeed exosomes, a new communication path between gut and brain was investigated, since these microvesicles could be powerful messages that arrive inside nerve cells. Recent data have supported the emerging role of exosomes in communication between the periphery and the central nervous system (Han et al., 2023), and their ability to pass through the blood-brain barrier (Banks et al., 2020) makes them ideal candidates for such communication. The results of this study seem to demonstrate that exosomes were indeed taken up by nerve cells, both neurons and glia (astrocytes). This could lead to the hypothesis that they release their cargo inside the nerve cells. The content of exosomes is varied (miRNA, proteins, lipids...) and depends on the cells of origin (Xie et al., 2022).

In this study, cytokines released by inflamed intestinal cells were evaluated in exosomes. The level of IL-1β but not that of IL-6 in the exosomes showed a tendency to increase in the LPS sample, suggesting that alteration of the physiology of Caco-2 cells treated with LPS can lead to a selective encapsulation of cytokines in the released extracellular vesicles. These vesicles could therefore deliver their contents to both nearby and distant cells. This result would suggest the role of extracellular vesicles in the regulation of local immune-inflammatory responses (Yin et al., 2019; Van Niel et al., 2001) and their role in the regulation of activation of inflammatory genes in other parts of the body, as observed in the liver, in spleen tissues (Wang et al., 2019) and in the central nervous system (Han et al., 2023). Thus, it can be hypothesized that IL-1β in exosomes could be released into neurons and astrocytes and it could promote neuroinflammation, a mechanism also involved in chronic pain (Ji et al., 2014). The study results are unable to explain why IL-6 does not enter exosomes like IL-1β following LPS treatment. This difference could be due to the fact that each cytokine plays both distinct and overlapping roles with other cytokines (Carlson et al.,

1999). These findings underscore the importance of understanding the effect of cytokines in various physiological contexts.

CONCLUSIONS AND FUTURE PERSPECTIVES

To summarize, the present results confirm the starting hypothesis, namely that there is a relationship between chronic pain, the GI tract and nutrition. Different nutritional approaches seem to result in a reduction of pain intensity and GI symptoms in subjects affected by various chronic pain disorders. Exosomes could be protagonists in this process, as direct carriers of messages from the periphery to the CNS.

These results focus attention on the importance of a multimodal view of subjects with chronic pain. As part of a whole-person approach to pain management, greater attention by clinicians to dietary habits and the kind of foods consumed could help to avoid pain and pain chronicization.

Further studies to explore intestinal exosomes in in vitro studies and in human samples to identify new possible biomarkers of chronic pain are warranted. Particular attention will be given to the exosome contents and their effects on CNS cells.

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