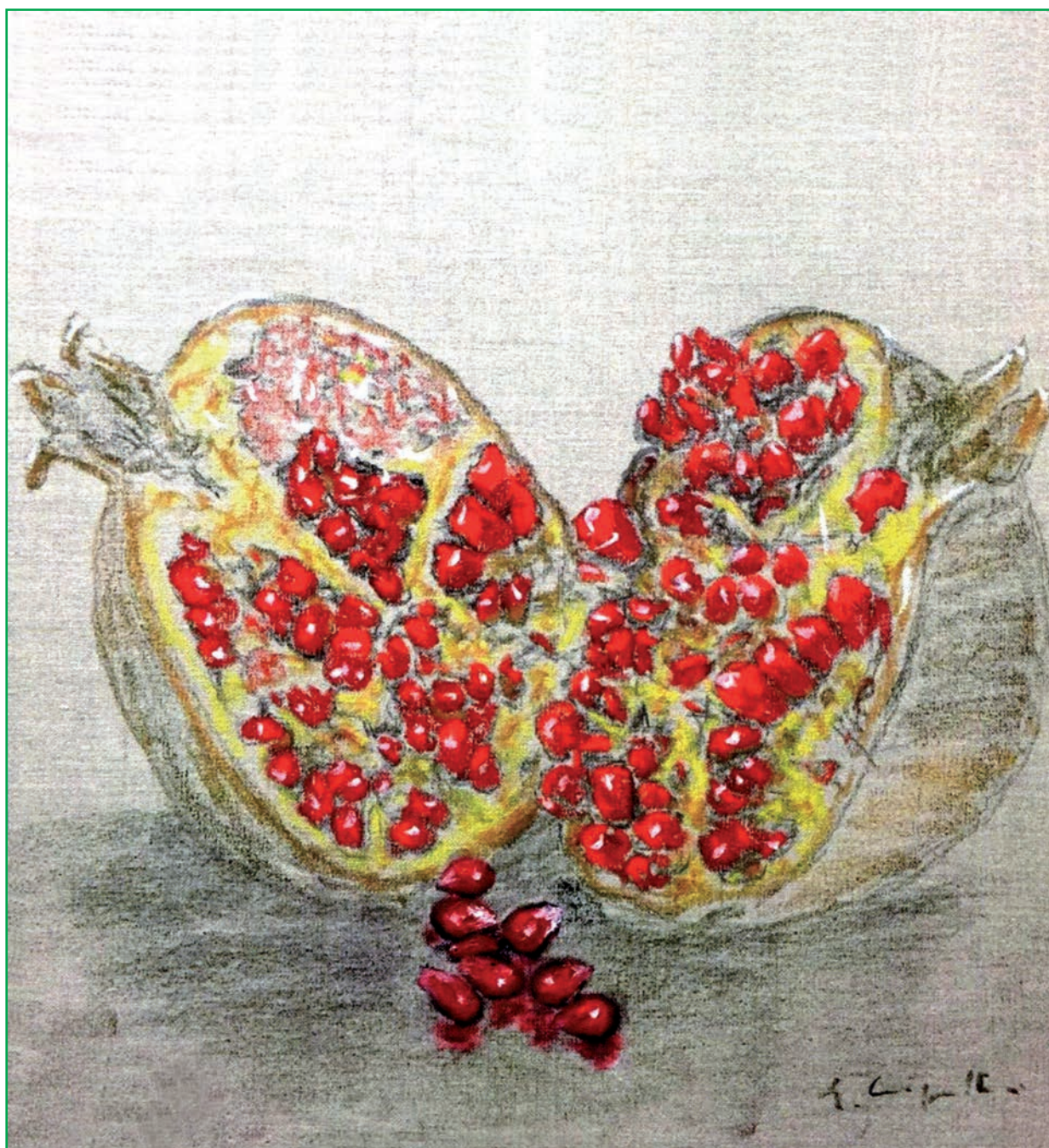




Physiological Regulating Medicine

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Scientific Director

Leonello Milani

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L. Milani

BDNF

BRAIN DERIVED NEUROTROPHIC FACTOR

– AN EXTRAORDINARY AND POWERFUL MASTER REGULATOR OF THE BRAIN

*Time it was, and what a time it was,
it was a time of innocence, a time of confidences.
Long ago, it must be.
I have a photograph.
Preserve your memories, they're all that's left you.*

– Paul Simon & Art Garfunkel. Bookends Theme, Columbia Records, 1968.

THE INCREDIBLE CASE OF HENRY MOLAISON – THE AMNESIAC WE WILL NOT FORGET

In 1953, Henry G. Molaison, 27, from Manchester, Connecticut (USA) underwent a major bilateral anteromedial temporal lobectomy, with extensive resection of the anterior 2/3 of the hippocampi, parahippocampal cortices, entorhinal cortices, piriform cortices, and amygdalae (FIG. 1), in an attempt to stop his severe, worsening, drug-resistant, tonic-clonic seizures (15-20 per day), which had afflicted him since childhood and had probably been caused by a road accident at the age of 9, which had resulted in a fractured skull and brain concussion.

– I quote Dr William B. Scoville, the expert neurosurgeon who had operated on him:
“(...) we performed a bilateral resec-

tion of the medial temporal lobes, advancing posteriorly for a distance of 8 cm from the central point of the edge of the temporal lobe, so that the temporal horn formed the lateral margin of the resection”.

The neurosurgeon had already performed several partial ablations of other areas of the brain in cases of severe psychosis (at that time they were defined as “psychosurgery procedures”) with no significant side effects (Scoville, 1951).
– The procedure carried out on Henry Molaison had had a favourable impact on his epileptic fits, which were considerably fewer and of shorter duration, but the patient had become incapable of creating new memories: he only had a blurred memory of a few events concerning his family and



– **Henry G. Molaison (1926-2008).**
Photograph taken 2 years before his bilateral anteromedial temporal lobectomy.
– **One of the most widely studied patients in the History of Medicine.**

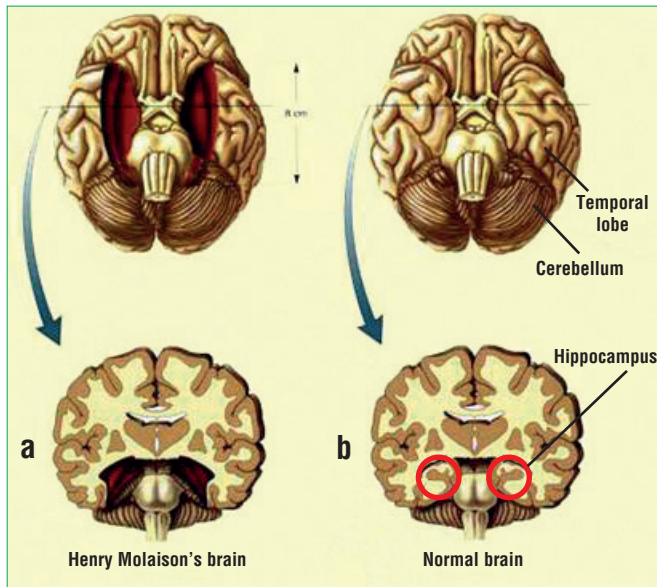


FIG. 1

a) Extensive ablation of the anterior $\frac{2}{3}$ of the hippocampi, parahippocampal cortices, entorhinal cortices, piriform cortices, and amygdalae; b) Normal brain.

Top: Inferior view of normal encephalon.

Bottom: Cross section at the level of the temporal lobe of Henry Molaison's brain (a) and normal brain (b).

childhood; nevertheless, he could play the piano, ride a bicycle and perform even complex actions that the brain transmits to the somatic musculature due to conditioned motor patterns, "motor memories", which are currently defined as "intuitive", but which the patient did not remember having learned.

The patient's motor cortex, basal ganglia, and cerebellum were intact, therefore his coordinated automatic motor skills were correctly preserved.

His ability to formulate procedural memory remained intact.

– Despite the severe memory problems, the patient performed well in intelligence and articulated language skill tests, a clear indication of how some memory functions – short-term storage (max. 1-2 minutes), vocabulary, phonemes, etc. – had not been compromised by the surgery (Corkin, 2002; Smith & Kosslyn, 2007).

Before the Molaison case, neuroscientists had considered memory to be "monolithic": the entire brain was involved in the memorisation processes. It is currently recognised that short-term memory is created by trains of electrical impulses conveyed from the cortex to the hippocampus, leaving no trace in the DNA of the hippocampal cells.

– If the experience is emotionally meaningful or repeated (*repetita iuvant*), the information stabilises, **it is imprinted in the DNA of the hippocampal cells**, which send engrams to the cortex that are now recorded and persist over time.

This is how long-term memory is formed.

In the incredible case of Henry Molaison, the hippocampus and neighbouring cortices (FIGS. 1, 2) had been partially removed, which meant that "his" time had stopped on 1 September 1953, the date of his surgery; after that date ... nothing.

– From 1955, the patient was constantly monitored for a few hours a day by neuropsychologist Brenda Milner; every day – without exception – Henry could not remember ever meeting her before. Every day throughout the many years of observation, Henry would hold out his hand to her and introduce himself: "*Hi... I am Henry, nice to meet you, Ma'am*"

Henry lived to the age of 82, in a temporary present moment, in the here and now... always and only for a few seconds, in a state of "perfect" mindfulness.

- The recent past did not exist, the future would never be remembered; there was only the fragmented remote past, which was in any event prior to the date of his surgery.

– In 2014, Molaison's brain became the focus of very detailed, sophisticated studies (Annese *et al.*, 2014); it is now preserved at the San Diego School of Medicine Institute for Brain and Society Brain Observatory, San Diego, California (USA) (2401 very thin fronto-occipital "slices"; inter: 70 μ m).

From these studies it emerged that the amount of brain tissue removed was less than what was reported at the time, but... there was no post-surgery NMRI in 1953.

– Even after his death, neuroscientists continue and will continue to learn from the "amnesiac we will not forget", which is the title of an article by Benedict Carey published in the New York Times on 4 December, 2008.

Paradoxically, the man without a memory is and will continue to be a lasting memory for all neuroscientists, and others.

BIZZOZERO'S ERROR AND THE NEUROTROPHINS

According to the classification formulated in 1894 by Giulio Bizzozero (1846-1901) (Vigliani, 2002), the cells of an organism can be divided into 3 groups:

- 1) labile, which continuously reduplicate, for example the mucous membranes, epidermis and endometrium (lining epithelial cells);
- 2) stable, which do not normally reduplicate continuously, but can undergo mitosis in response to homeostatic requirements, for example in glandular tissues or in the liver after a hepatectomy;

3) perennial, highly specialised, incapable of cell division, for example **nerve cells**.

– This classification, which was used until the final decades of the last century (Chiarugi & Bucciantie, 1968), was later radically deconstructed following the discovery of Nerve Growth Factor (NGF) (Levi-Montalcini & Hamburger, 1951).

– **NGF** is a peptide-based neurotrophic factor primarily involved in the proliferation, growth, maintenance and survival of some target neurons.

- Bizzozero's perennial cells... do not exist.

The following belong to a group of Neurotrophic Factors **not** related to NGF: **1)** CNTF (Ciliary NeuroTrophic Factor); **2)** GDNF (Glial-cell-line Derived Neurotrophic Factor); **3)** TGF α/β (Trophic Growth Factor α/β); **4)** FGFs (Fibroblast Growth Factors); **5)** EGF (Epidermal Growth Factor) and **6)** PDGF (Platelet-Derived Growth Factor).

– Since NGF, **3 further neurotrophins (NTs)** have been discovered in humans: **BDNF** (Barde *et al.*, 1982), **NT3** (Maisonpierre *et al.*, 1990) and **NT4** (NT4/5) (Hallbook *et al.*, 1991), each with a distinct profile of trophic effects on specific central and peripheral neuronal subpopulations.

- Of particular significance is BDNF (Brain Derived Neurotrophic Factor), which was first isolated by Yves-Alain Barde, Dave Edgard & Hans Thoenen in the laboratories of the Neurochemistry Section of the Max-Planck Institut für Psychiatrie in Munich - Germany.

The research group was coordinated by the Swiss Prof. Thoenen.

– There follows a quote from part of his original description of the discovery of BDNF: “(...) we are reporting on the purification of a factor, taken from the brain of pigs, which promotes the survival and growth of the sensory neurons in cultured chicken embryos (...); approximately 1 microgramme of this factor was isolated from 1.5 kg of pig brain (5 brains, *Ed.*); this factor is the first purified neurotrophic factor since NGF (Nerve Growth Factor), from which it can be clearly distinguishable due to its different antigenic and functional properties”.

Thoenen studied Neurotrophic Factors throughout his life (Thoenen, 1995, 2000; Thoenen & Sendtner, 2002), “a modest man whose discoveries have had profound impact on modern neuroscience” (Iversen, 2013).

A human brain should contain, on average **0.85 $\approx$ microgrammes** (one microgramme = a millionth of a gramme).

- Mature BDNF is a protein composed of 119 amino acids, with a molar mass of 14 kDa $\approx$ (13.2-15.19 kDa), an iso-

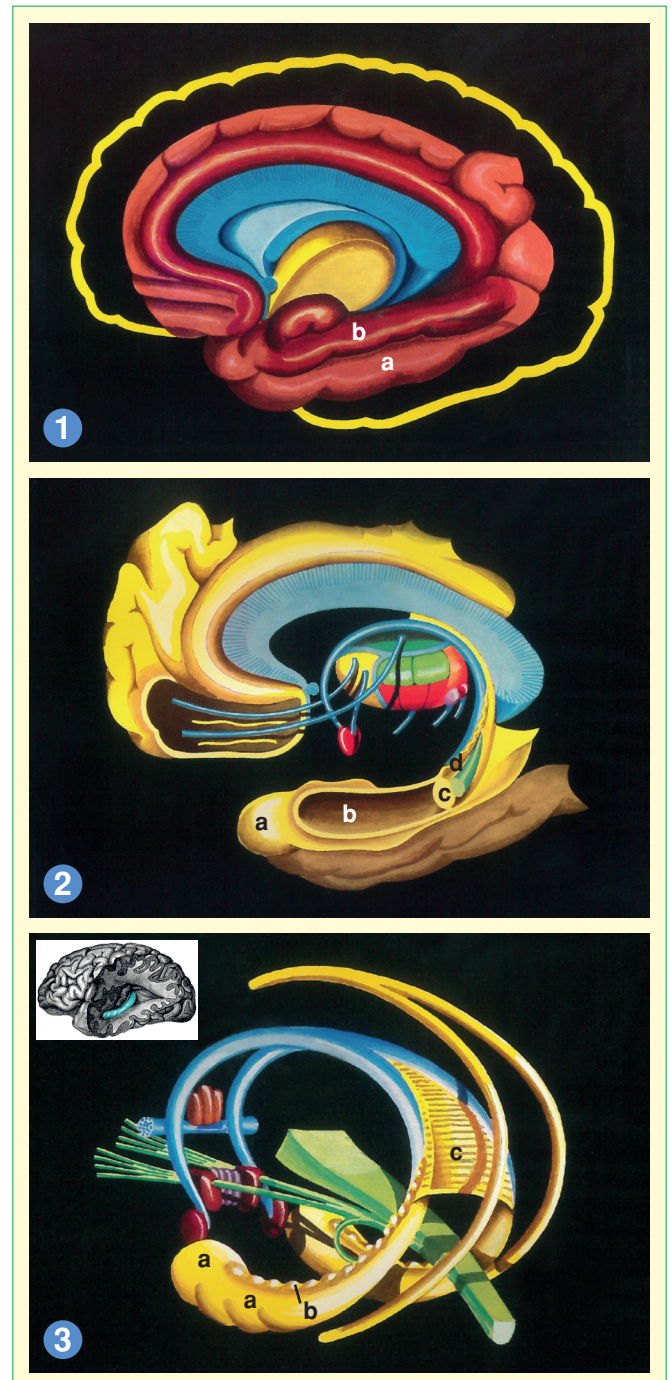


FIG. 2

The hippocampus is very difficult to successfully identify in anatomical dissection. This is because it is located deep in the temporal lobe, and because there are some differences in the terminology used by the various schools of anatomy. Moreover, its course does not follow the major axis of the temporal lobe, but is inclined from bottom to top and from the outside to the inside.

– Its correct location is shown on the 3 images.

1 left hemi-encephalon: a) temporal gyrus; b) hippocampal gyrus;

2 left semi-encephalon: a) temporal lobe, anterior portion; b) empty space that accommodates head and body of hippocampus; c) hippocampus sectioned 2/3 anterior - 1/3 posterior (tail); d) fimbria of hippocampus;

3 a) head and body of hippocampus; b) fimbria of hippocampus.

– Note that the hippocampi of the 2 hemispheres approach the mid-line and, in the region of their tails, they are paired; (c) tentorium of hippocampus.

Once attached to its main TrkB receptor, a dimer, BDNF initiates 3 distinct PATHWAYS.

From top, left: in the 1st PATHWAY, the sequential concatenation of 5 protein complexes (Shc, Grb2, Sos, GAB1, PI3K) stimulates PIP2, a phospholipid component of the cell membranes, which – in turn – stimulates another phospholipid, PIP3.

Through the intermediation of PDK1 (Phosphoinositide-dependent kinase) this produces Akt, also known as Protein-Kinase B. This facilitates neuronal survival, neuronal growth, neuroplasticity, and short-term memory (cytoplasmic activity).

Akt can enter the nucleus and modify the DNA: in this case the information materialises in the long-term memory.

– In the 2nd PATHWAY, intramembrane PIP2, stimulated by PLC γ , a protein attached to the 2nd arm of the TrkB dimer, stimulates IP3, which releases Ca⁺⁺ and produces CAMK (or MAMK), a Ca⁺⁺/modulin-dependent protein-kinase that enters the nucleus (see above for Akt).

– The 3rd PATHWAY involves the sequential linking of the first 4 protein complexes to a series of promoters, as far as ERK (signal-regulated extra cellular kinase; it also enters the nucleus to modify the DNA).

• NOTE: the table shows 2 TrkB receptors for display and graphic purposes only.

In reality, the 3 PATHWAYS occur simultaneously following the interaction of BDNF with the TrKBs found on the neurons that expose them.

electric point of 9-10 (Chao & Bothwell, 2002). It shares **50% of its amino acid sequence with 3 other NT** (Narhi *et al.*, 1993; Marco Salazar, 2014) (FIG. 3).

– All 4 human NTs share a common ancestral gene (Dos Santos *et al.*, 2011; Covaceuszach *et al.*, 2021).

BDNF is encoded by the BDNF gene which, in humans, is located on chromosome 11, band p 13.

The BDNF gene is made up of 11 exons (exon = portion of the gene that is transcribed by RNA polymerases during transcription) associated with 8 functional promoters + 1 exon that codes for BDNF (Metsis *et al.*, 1993; Timmusk *et al.*, 1993).

– The significance of this complex transcriptional organisation is not well understood; the most accredited hypothesis is that it induces **multiple stages of regulation** by means of alternative promoters, different RNA stability or different subcellular localisations of both RNA and proteins.

– BDNF is synthesised in the neuronal endoplasmic reticulum as a **PRE-PRO-BDNF** complex which subsequently migrates into the Golgi apparatus and later into the trans Golgi system, a maturation system that faces towards the internal portion of the cell membrane.

The PRE portion (18 amino acids) undergoes proteolytic cutting in the endoplasmic reticulum of the nerve cell.

Mature BDNF subsequently splits from the PRO portion (112

amino acids) due to the action of enzymes found in both the intracellular and extracellular compartments.

If this latter splitting does not occur, mature BDNF is not formed. Instead the PRO-BDNF precursor is formed, and biological activity of this differs from that of mature NT (Lee *et al.*, 2001), which induces a neurotoxic effect, as occurs in Alzheimer-Perusini disease (Chen *et al.*, 2017; Fleitas *et al.*, 2018).

Each NT binds to one or more tropomyosin-kinase receptors, member(s) of the **tyrosine-kinase family (TrKs)** (Patapoutian & Reichardt, 2001), which activate a wide range of intracellular signals, including – crucially – cyclic AMP phosphorylation (Ji *et al.*, 2005).

• In the case of BDNF, it binds and activates 2 different transmembrane receptors, both at the pre-synaptic and post-synaptic level: one with **high affinity** [TrK (pronunciation: Trak) B], mentioned above, the other belonging to the TNF superfamily (Tumour Necrosis Factor), **p 75**, with **low affinity**.

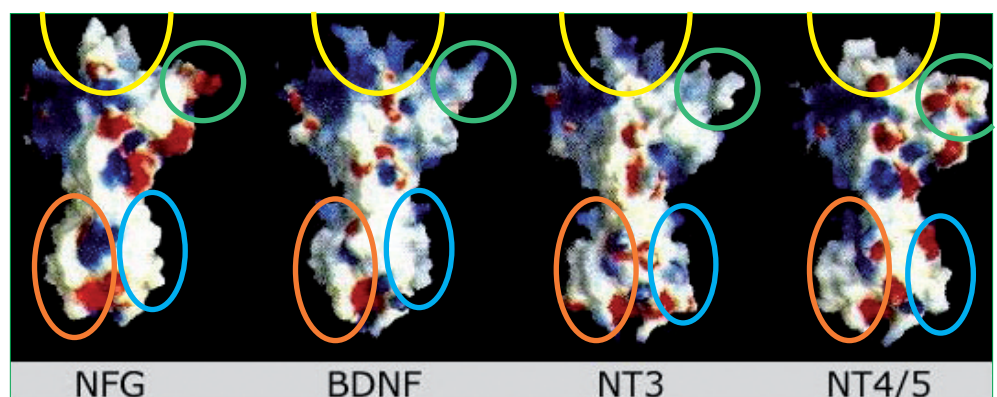
• These 2 receptors show no homology, or cytoplasmic domain, and they activate different signalling pathways. They probably appeared at different times in the course of evolution (Tettamanti *et al.*, 2010).

In the presynaptic neuron, BDNF is “packed” into dense, optically dark vesicles; it reaches the terminal plate of the neuron (nerve plate) through rapid axonal flow from the neuronal nucleus (Conner *et al.*, 1997).

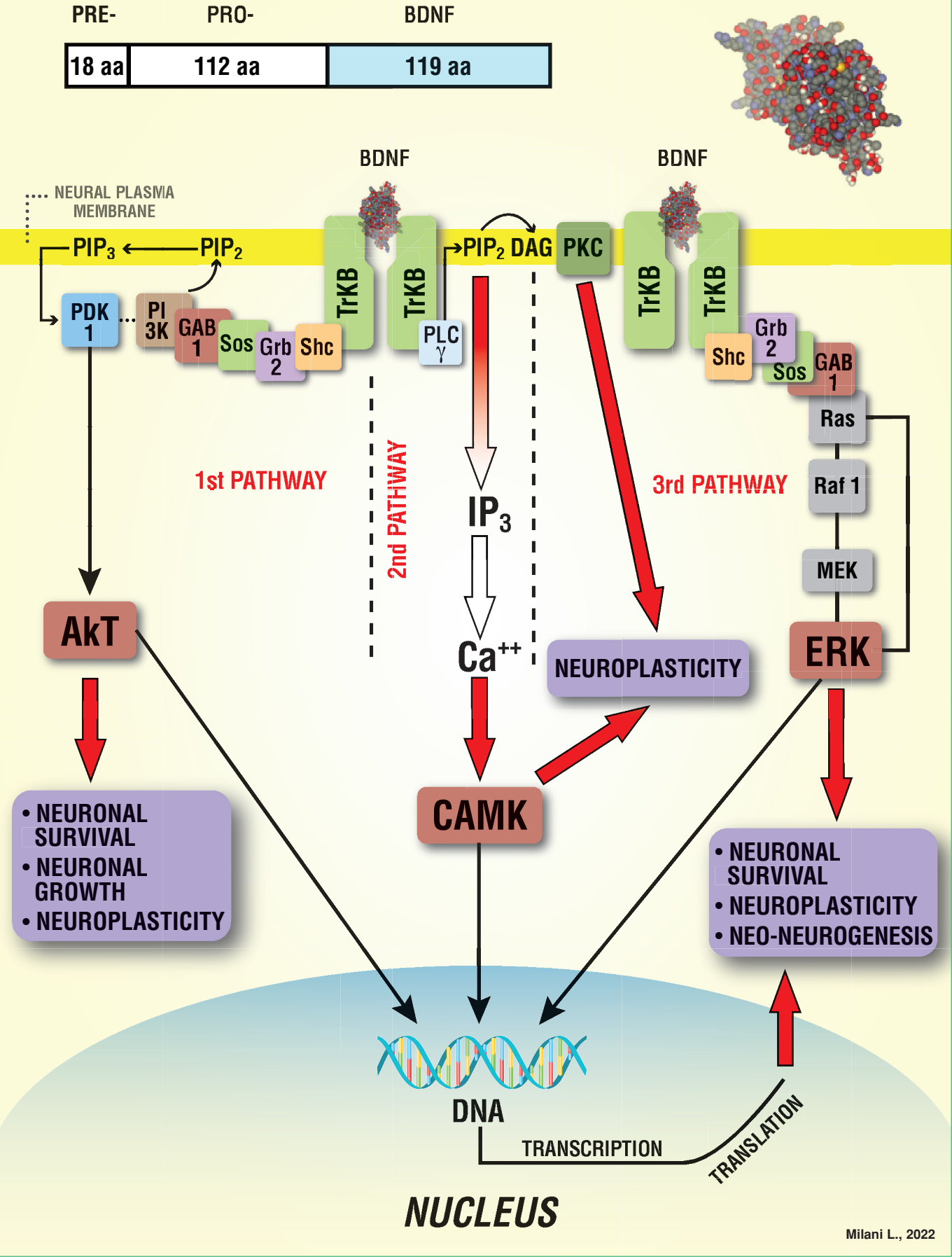
FIG. 3

Despite having a similar protein molecular structure, neurotrophins NGF, BDNF, NT3 and NT4/5 show significant morphological differences.

– Author's highlights.



TAB. 1



– Upon arrival of the electrical impulse (*spike*), BDNF binds to the 2 post-synaptic element receptors mentioned above, and there it triggers – on TrkB – **3 pathways**, with distinct cascade mechanisms.

TrkB is a transmembrane receptor with a short outer part and a long inner part, and is also a dimer (Shen & Maruyama, 2012) (**TAB. 1**).

– **1st PATHWAY:** in an 8-step process [2 of which occur inside the neuronal plasma membrane (PIP2→PIP3)] we arrive at the formation of **Akt**, also known as Protein-Kinase B: this induces survival, neuronal growth and neuroplasticity whether it remains in the cytoplasm or enters the nucleus, and stimulates transcription → translation with the help of DNA.

– **2nd PATHWAY:** **CAMK** (Ca²⁺/modulin-dependent kinase) is synthesised in a 4-step process.

This enters the nucleus directly and – from this moment on – works in the same way as Akt in the 1st Pathway, in the intra-nuclear passage.

– **3rd PATHWAY:** a 7-step process [the first 4 of which are identical to those of the 1st Pathway (Shc, Grb₂, Sos and GAB1)] results in the synthesis of **ERK** (Extracellular Signal-Regulated Kinase).

The terminal linking of 3 proteins (Ras, Raf 1 and MEK) to form a chain, identical to that which causes insulin to release glucose to the cells, thanks to DNA, also leads to the same effects as the 1st Pathway, thus highlighting the classic phenomenon of redundancy, as occurs in the cytokines (Milani, 2007).

• Synthesis:

BDNF = neuronal survival, neuronal growth, neuroplasticity, neo-neurogenesis and prevention of neuronal apoptosis.

SPECIFIC NATURE OF BDNF

- Its activity on the post-synaptic receptor depends only on the depolarisation of the pre-synaptic neuron (Mowla *et al.*, 1999; Goggi *et al.*, 2003).
- It can be transferred per se from a donor neuron to a recipient neuron (role of neurotransmitter too?) (Kohara *et al.*, 2001; Selvam, 2018).
- It excites the glutaminergic synapses and depresses the GABAergic synapses (hence the excitation) (Tanaka *et al.*, 1997).

– In Henry Molaison's hippocampi and neighbouring cortices, BDNF was overexpressed following his road accident, which explains his continuous, severe epileptic seizures and justifies the subsequent anteromedial temporal lobectomy with excision of part of both hippocampi.

The activities performed by BDNF are the most widely, best characterised of the 4 known NTs.

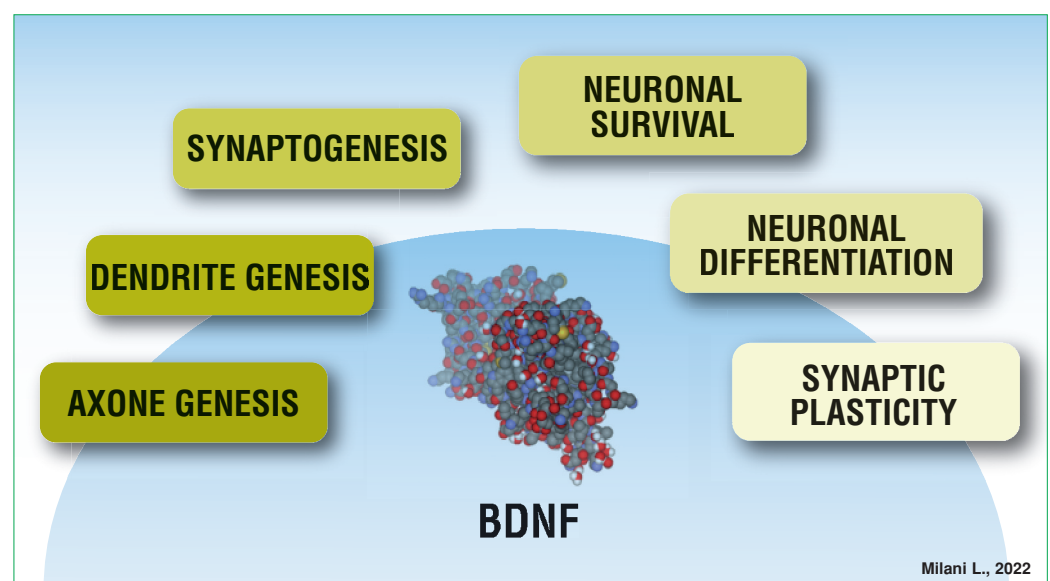
► From the extensive medical-scientific literature consulted, the biological activities **related to BDNF** are: neuronal survival, neuronal differentiation, synaptogenesis, synaptic plasticity, increase in the arborisation of dendrites and new axons: a truly... extraordinary, powerful master regulator of the brain, both in prenatal life and throughout life (**TAB. 2**).

In the brain, a high concentration of BDNF can be detected in the **cerebral cortex**, especially the **pre-frontal cortex**, which is well-developed only in humans (Milani, 2018), in

TAB. 2

BDNF

– Main biological activities.



the cerebellum, the basal ganglia and – above all – the **hippocampus**, structure belonging to the limbic brain; outside the brain, in the retina, kidneys, heart, lungs, pancreas, prostate, skeletal muscle, and saliva (Pruunsild *et al.*, 2007).

In the prefrontal cortex ... for high level thinking, in the cerebellum ... for posture, in the basal ganglia ... for the control of extrapyramidal involuntary movements and in the hippocampus ... for the consolidation of long-term memory and the control of emotions.

– Binomial Prefrontal Cortex-Hippocampus... for learning.

- Not bad for a protein made up of only 119 amino acids.

BDNF has been synthesised in all **vertebrates** since the lower Carboniferous period, 360-318 million years ago, and is almost **identical in all mammals**. It has shown extraordinary success in the course of evolution (molecular stability and highly performing activities concerned with integration in the environment) (Götz *et al.*, 1992; Tettamanti *et al.*, 2010).

– However, there are two factors that compromise BDNF function; a single substitution of methionine instead of valine (BDNF val66met) along its amino acid sequence has been linked with the susceptibility, incidence and clinical picture of certain psychiatric disorders (anxiety, depression) and neurodegenerative disorders such as glaucoma, multiple sclerosis, Alzheimer-Perusini disease, amyotrophic lateral sclerosis, Parkinson's Disease and Rett Syndrome* (Egan *et al.*, 2003; Shen *et al.*, 2018).

BDNF val66met (variant) polymorphism occurs in **30%** ≈ of the population (source: University of Milan - La Statale Insalutenews 19 October 2016) and is not associated with serum BDNF (Terraciano *et al.*, 2013).

The other genetic error is BDNF L21, associated with congenital central alveolar hypoventilation syndrome (Weese-Mayer *et al.*, 2002) ("Ondine's curse"), a rare disease characterised by a major defect in the central control of respiration.

– These genetic errors have only been demonstrated in humans.

* **Rett syndrome:** Rare developmental disease that affects the CNS.

Almost all sufferers of the disease are female (the "girl children with beautiful eyes"), with an estimated prevalence of 1/9000 adolescents.

– The syndrome is characterised by apparently normal development in the first 5-20 months, after which there is a progressive loss of acquired motor function, stereotyped hand movements, an inability to interact and socialise, and loss of speech. At the age of 20-30 years, many patients develop scoliosis, sleep and breathing disorders and epileptic seizures.

- Some changes in BDNF levels lead to psychiatric disorders, depression, bipolar disorders, psychosis, eating disorders, post-traumatic stress disorder, anxiety disorders, self-harm, and suicidal behaviour.

Of all the NTs, it has emerged that BDNF is the major regulator of many neuronal types such as sensory neurons, retinal ganglion cells, spinal motor neurons, cholinergic neurons, and some clusters of dopaminergic neurons.

BDNF is widely distributed in the CNS; its expression is decreased in many degenerative diseases, as demonstrated in post-mortem studies on Alzheimer-Perusini disease (Phillips *et al.*, 1991; Hock *et al.*, 2000), Parkinson's disease (Mogi *et al.*, 1999; Phillips *et al.*, 1999), Huntington's disease (Ferrer *et al.*, 2000), and severe depression (Lee & Kim, 2010), in which BDNF increases as a result of antidepressant therapy (Shimizu *et al.*, 2003).

- Many neurons are eliminated during the period of intrauterine development in mammals: a large percentage of neurons already formed (**20% to 70%**) undergoes degeneration; this takes place in order to regulate the optimal number of neurons in adult life. However, this number changes significantly throughout life: at the age of 50 years ≈ **most** of the neurons in the hippocampus are replaced *ex novo* and new neurons are formed, along with the associated dendritic arborisations and synapses. This is to prevent the loss of memories that the apoptotic neurons had preserved as engrams: the master regulator of these complex functions is – above all – BDNF.

– In fact, in the animal model of learning and memory, electrical stimulation of the hippocampus increases the expression of BDNF and NGF (Patterson *et al.*, 1992; Castrén *et al.*, 1993; Bramham *et al.*, 1996).

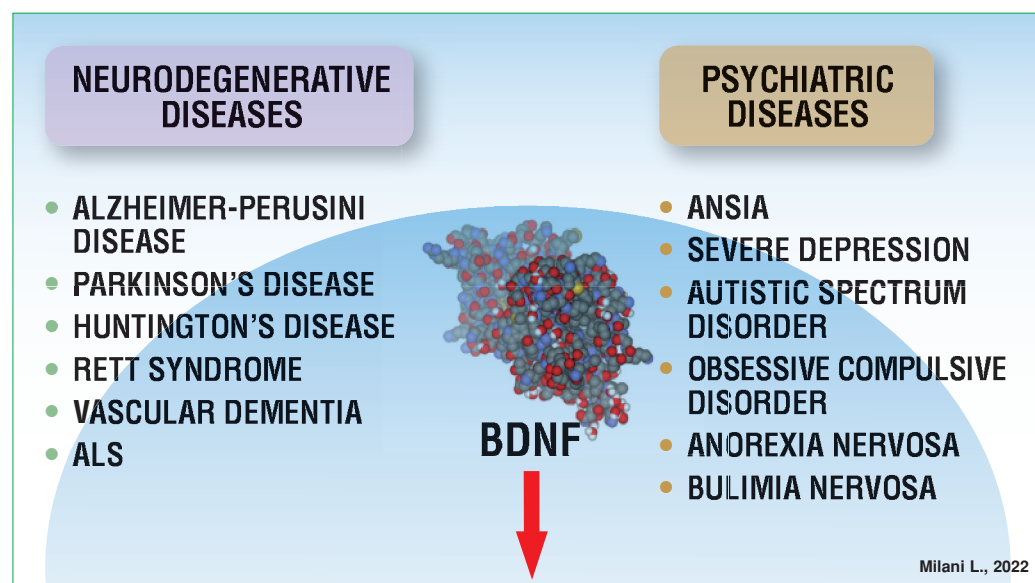
- Optimal cognitive function is dependent on dynamic, active neuronal plasticity (Herndon *et al.*, 1997; Morrison & Hof, 1997; Gonzalez *et al.*, 2016), but over the years this cracks, mainly due to the loss of dendritic spines (Morrison & Hof, 1997).

This does not take place in the entire brain, but only in some specific areas: in cerebral ageing, neuronal loss is a phenomenon localised in the **cortical, subcortical** (Smith *et al.*, 2004) and **hippocampal neurons**, where a high concentration of BDNF is expressed (Erickson *et al.*, 2010).

- ▶ This is the rationale for the progressive decline in voluntary and involuntary motor function, learning, memory and processing-critical thinking in many elderly people (Silhol *et al.*, 2008), in which a transition from **neuro-inflammation** to **neuro-degeneration** has begun (Milani, 2014, 2016; Milani &

TAB. 3

Many neurodegenerative and psychiatric diseases emerge from a decrease of BDNF in the brain.



Pelosi, 2016; Montenero & Milani 2019) and/or atherosclerosis/arteriosclerosis of the cerebral vessels (Vascular dementia).

- The *primum movens* is Low-Grade Chronic Systemic Inflammation. This is the “silent killer” to be identified and neutralised, nothing else.

BDNF

– NON-PHARMACOLOGICAL THERAPY

The conventional therapies currently available to reduce the phenomenological aspects of cerebral ageing are both pharmacological and non-pharmacological; the former, which target the neurotransmitters, with no guarantee of success, have various side effects and are expensive (Uberti & Molinari, 2018).

The gene expression of BDNF can be affected by a series of stimuli:

- **Endurance motor exercise** increases BDNF levels by 200%-300% (Salfert *et al.*, 2010).

- **Strength motor exercises** [e.g., repeated weight lifting for 5-10 min/day (Neeper *et al.*, 1995)]. The increase in BDNF in skeletal muscle is proportional to contractile intensity in aerobiosis relating to a more favourable fat/lean mass ratio, which is enhanced by the consumption of fatty acids for oxidation, and by the improvement in energy consumption of glucose (Mattheus *et al.*, 2009; Henry *et al.*, 2018).

- **Deep sleep** (Eckert *et al.*, 2017).

- **Light stimulation:** BDNF increase in the visual cortex (Castrén *et al.*, 1992).

- **Osmotic stimulation:** BDNF increase in the hypothalamus (Castrén *et al.*, 1995; Dias *et al.*, 2003).

- **Meditation, Yoga** (Cahn *et al.*, 2017), **Mindfulness** (Gomutbutra *et al.*, 2020).

- **Ketogenic diet** (Vizuet *et al.*, 2013).

- **Intermittent fasting** (Mattson *et al.*, 2020).

- **Foodstuffs:** Polyphenols (coffee, green tea, dark chocolate, blueberries) (Gravestijn & Mensink, 2021).

- **Polyunsaturated fatty acids:** Omega 3, Omega 6 (Wu, 2004).

- **Probiotics** containing Bifidobacteria (Intestine-Brain Axis) (Tian *et al.*, 2019).

- Avoiding patho-stress, social isolation, refined sugars and ethanol: these are powerful depressants that affect the production of BDNF.

BDNF

– LOW-DOSE PHARMACOLOGICAL THERAPY

A therapeutic approach using BDNF could be extremely effective in preventing and treating neurodegenerative diseases (and others) (see above), which suffer from a deficiency of this crucial NT.

Knusel *et al.* (Aut. cit., 1992) demonstrated that the administration of recombinant human BDNF in the cerebral ventricles of rats results in the neuroprotection of damaged cholinergic neurons.

Similarly, in 2001 (Chen *et al.*, 2001) it was demonstrated that intravitreal injections of BDNF in cats increased the survival of retinal ganglion cells after partial destruction of the optic nerve.

– At the time it was believed that BDNF could not cross the **Blood-Brain Barrier (BBB)**, a functional structure located between blood and brain parenchyma that selectively regulates blood flow to and from the brain, thus protecting it from intoxication and poisoning.

For this reason, experiments were necessarily designed as *in loco* procedures (e.g., as stated above, in the cerebral ventricles and the vitreous body), directly into the CNS.

– The discoverer of BDNF himself expressed scepticism about the rational therapeutic use of NTs (Thoenen & Sendtner, 2002).

- This continued until **2010**, when Klein *et al.* (2010) brilliantly demonstrated that the blood concentration of BDNF reflected what was found inside the brain: BDNF did cross the BBB.

– It did, however, present problems relating to the dose administered.

► In this respect, the **BDNF low dose** formulation (**4CH**) successfully solves the problem.

Uberti & Molinari (Aut. cit., 2018; Molinari *et al.*, 2020) demonstrate that **BDNF 4CH** (Guna Laboratories - Milan):

in vitro

- *crosses* the intestinal barrier;
- *crosses* the BEE, thereby reaching the brain tissue;
- activates the astrocytes and neurons, the 2 main types of cells involved in the ageing process;
- activates its main TrkB receptor through the recruitment of MAPK [(mitogen-activated-protein-kinase) (also defined as MAP = ERK)], a kinase that directs cellular responses to various stimuli, such as e.g. mitogens, osmotic stress, pro-inflammatory cytokines, etc. (Pearson *et al.*, 2001).

in vivo

- after the oral intake of BDNF 4CH, the drug arrives in the brain within **1 day**, reaching a peak within **2 days**;
- BDNF 4CH remains in the brain even in the absence of treatment, since it triggers the endogenous physiological systems that support the anti-ageing processes.

The authors conclude that “the use of low-dose BDNF offers a number of benefits due to the fact that this preparation has no side effects or adverse reactions, features that are necessary if it is used without the supervision of a specialist”.

In the section “BDNF – Non-pharmacological therapy”, I have also identified – among others – **deep sleep**, **Omega 3/6 fatty acids** and **Bifidobacteria** as being stimulants of BDNF gene expression.

– Therefore, for all patients who could benefit from taking **Guna-BDNF** (Brain Derived Neurotrophic Factor - human recombinant), **15-20 drops x 2/day by sublingual route for 4-6 months** (children < 6 years of age: 10 drops x 2/day by sublingual route for 4-6 months) and, in general (**TAB. 3**):

1) patients with neurodegenerative diseases (vascular and non-vascular dementia, Alzheimer-Perusini disease, Parkinson’s disease, multiple sclerosis, Huntington’s disease, Rett syndrome and others);

2) Patients suffering from psychiatric diseases [anxiety (Malzac, 2002), severe depression, depression, autistic spectrum disorders, obsessive compulsive disorder, anorexia nervosa, bulimia nervosa, and others (Supino, 2020; Melcarne, 2021)];

3) Specific learning disorders (Supino, 2019);

4) Patients suffering from sarcopenia [BDNF is actively involved in muscle regeneration and in boosting the oxidation of fatty acids; BDNF is also produced directly in striated muscle, with a peripheral endocrine function (So *et al.*, 2014; Delezie *et al.*, 2019)];

5) Cardiovascular diseases. BDNF stimulates angiogenesis and controls the survival of adult endothelial cells, vascular smooth muscle cells, and cardiomyocytes;

6) Type 2 diabetes (Krabbe *et al.*, 2007; Meek *et al.*, 2013; Davarpanah *et al.*, 2021) ...

... my best advice is therapeutic supplement, with or without overlapping with conventional drugs, with:

– **Guna-Melatonin**, 15-20 drops x 2/day;

– **Omegaformula** – Omega 3-6-9 from micronised seeds of the Baobab fruit, 3 chewable tablets/day during or after main meals;

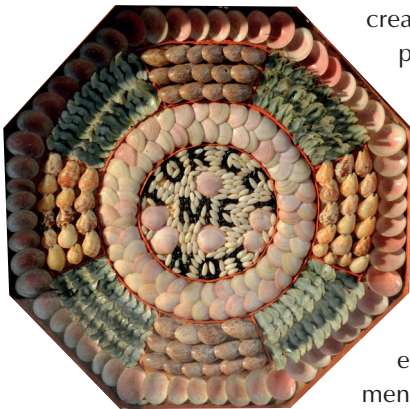
– **Proflora** (probiotic component: *Bifidobacterium* and *Lactobacillus* genera), 1 sachet/day before lunch.

Guna-BDNF is also an effective treatment for mental fatigue, chronic fatigue syndrome and neurovegetative disorders due to long-COVID (post COVID-19 brain fog, or neuro-COVID).

HI... I AM HENRY, NICE TO MEET YOU, MA'AM

All over the world, humans are living longer and seemingly healthier than just a few decades ago.

All this – however – must of course be set in the context of the basic biology of each individual, “his/her” medical history, “his/her” physiological and pathological ageing processes, “his/her” genetic programming [mitotic clock (Ehrenstein, 1998; Fossel, 2018)], “his/her” lifestyle, and the numerous, not always identifiable factors that play a part in making old age difficult – for many – and increasingly dependent on third parties.



– Adding years to life is not the same as adding life to years.

Neurodegenerative diseases, especially Alzheimer-Perusini disease and other forms of dementia that result in the progressive loss of memory, elaborate

thinking and purposeful behaviour have a negative impact on learned functioning and daily activities (emotional problems, semantic and lexical difficulties, memory loss, reduced motivation).

– This entire procession depends on the progressive atrophy of the cortical and subcortical regions that underlie the factual nature of these behaviours (including neuro-ageing).

• These brain areas, which formed in humans as an accident of evolution, these 4 brains (Milani, 2018) are “orchestrated” by BDNF which “does not marry the soma as such; it rests on that and it is from that position that it can put together the less perfect products of corporeality, turning them into Regulation” (Turco & Turco, 2019).

– It is “ageing”, impoverished BDNF that withers the hippocampus and the cortices that “communicate” with it (and ... vice versa); it is lipofuscin, neuromelanin, the Lewy and Hirano bodies, the substance β -amyloid and the Tau proteins that interrupt the neurotransmission wires.

– BDNF 4CH opens up new scenarios, new and real hopes, revitalises ageing life, breathes life into destiny, takes the edge off... “Hi, I am Henry, nice to meet you, Ma’am”.

I dedicate this work *in memoriam* to my dear friend

Dr Marlowe Elizabeth Reynolds of Berwyn, Chicago, who recently passed away.

– Marly... this goes for you.

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The editorial team would like to thank the editors of the websites that supplied the following images:

Fig. p. 3

<https://suzannecorkin.com/patient-h-m/>

Fig. 1 (author's caption)

<https://www.ilsuperuovo.it/lindimenticabile-storia-di-h-m-il-paziente-amnesico-piu-famoso-al-mondo/>

Fig. 2 - ③ (insert)

<https://en.wikipedia.org/wiki/Hippocampus>

Fig. 3 (author's highlights)

Fig. p. 12

https://www.1stdibs.co.uk/furniture/folk-art/nautical-objects/sailors-valentine-for-get-me-not-barbados-circa-1885/id-f_10642463/

– Fig. 2 is composed of 3 Tables (①, ②, ③) taken from Valzelli L. – *Profili di Psicofisiologia e Neurochimica*. Manfredi Editore, Milano; **1971**. Author's caption.

Tables 1, 2, and 3 were completed by the author.

In tables 2 and 3, the 3D molecule of BDNF is taken from:

https://en.wikipedia.org/wiki/Brain-derived_neurotrophic_factor

author

Prof. Leonello Milani

– Scientific Director of La Medicina Biologica and Physiological Regulating Medicine

– Vice President of the International Academy of Physiological Regulating Medicine

Milano, Italy



THERAPEUTICS

F. Esposito

SUMMARY

The hypothalamic-pituitary-adrenal (HPA) Axis is the primary mechanism by which the Central Nervous System interacts with the Endocrine System. In response to environmental stressors, including viral infections, the HPA Axis mobilizes the appropriate metabolic resources to meet specific demands. The final effector hormone of the HPA Axis is cortisol. Cortisol release increases markedly upon awakening and then gradually decreases throughout the day, reaching a minimum around midnight.

– Alterations in this specific pattern of cortisol secretion are associated with adverse health consequences, including sleep disorders and chronic inflammatory diseases, the origin of which may be related to a sensitized cortisol response. Diet and nutritional status play a key role in restoring order to a cortisol-related organ system that does not respond physiologically to external stimulation.

– In this article, our focus is on four components in particular: selenium (in its organic form of selenium-methionine), as a trace element constituting several selenium-proteins involved in the modulation of oxidative stress, phosphatidylserine, a membrane phospholipid promoting correct apoptosis and amplifying antioxidant enzymatic activity, vitamin D which, in synergy with selenium, mitigates the inflammatory response, and vitamin B5 whose role as an energy booster is crucial for the production of glutathione and thus for reducing oxidative stress.

KEY WORDS

STRESS, SELENIUM, PHOSPHATIDYLSERINE, VIT. D, VIT. B5, GUNAVIT B5 PLUS, CORTISOL, HPA AXIS, NUTRACEUTICS, MICRONUTRIENTS

CLINICAL MANIFESTATIONS OF STRESS-MEDIATED AND INFECTION-RELATED CORTISOL DYSREGULATION – THE ROLE OF GUNAVIT B5 PLUS IN RESTORING STRUCTURAL BALANCE AND FUNCTIONAL INTEGRITY

INTRODUCTION

Cortisol is a powerful anti-inflammatory hormone produced by the adrenal glands in the fasciculated area of the cortical portion. Its dysfunction can lead to widespread inflammation following the reactivation of an acute reaction to stress with various origins.

– Generally speaking, high levels of cortisol are associated with diets that are high in animal protein and carbohydrates with a high insulin-sensitive glycaemic index inducing the production of further cortisol.

Despite stress being an inevitable part of life, a prolonged or excessive response to pain or various factors can intensify sympathetic and neuroendocrine activity, deplete cortisol and perpetuate widespread pain and inflammation.

Following acute stress, high cortisol levels may help to reinforce fear-based emotional memories for even unexpected, previously unknown events, conditioning a physiological response to stress, making it sensitised.

– A hypervigilant response to stressful stimuli may lead to frequent or pro-



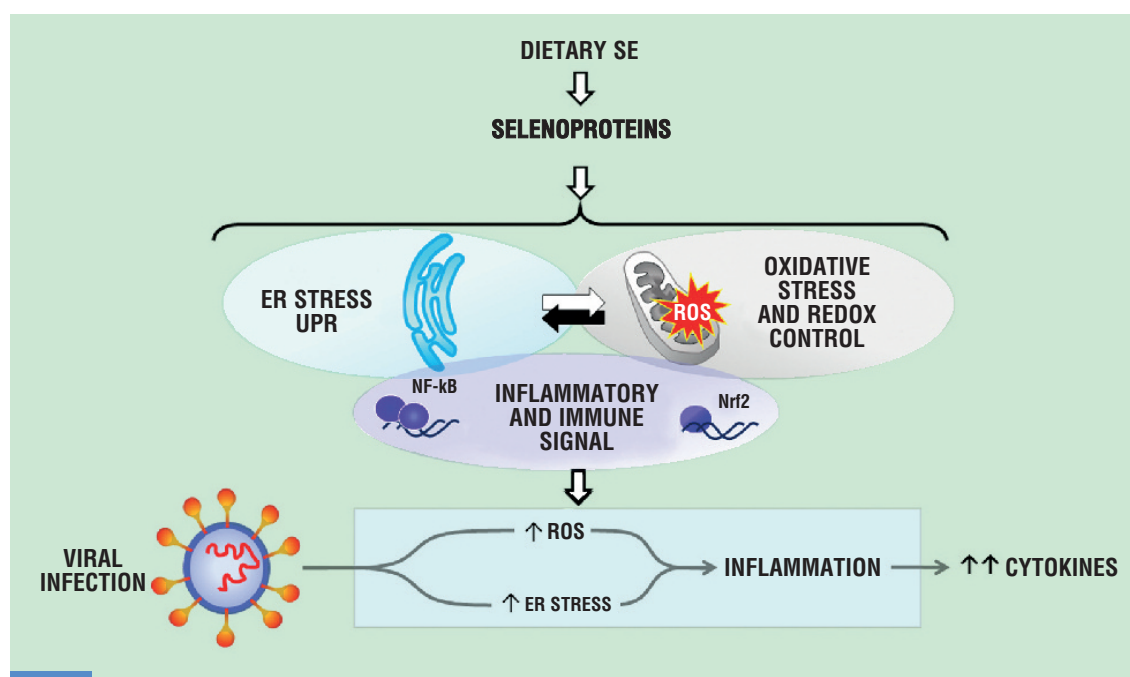


FIG. 1

Graph retrieved from: <https://www.cambridge.org/core/journals/british-journal-of-nutrition/article/selenium-and-viral-infection-are-there-lessons-for-covid19/BE3AC78D5C92725BE83C4E474ECBB548>.

longed activation of the **hypothalamic-pituitary-adrenal (HPA) Axis**.

Following a chronic reactivation of the HPA, cortisol dysfunction may encourage the transmission of pain through altered modulation or the repeated activation of nociceptors by pro-inflammatory mediators.

The reactivation of a stress response triggers the release of pro-inflammatory sympathetic catecholamines.

Cortisol's compromised anti-inflammatory functions can intensify and prolong the short-term physiological inflammatory response.

– The side effects of systemic inflammation may include **1)** autoimmune susceptibility, **2)** widespread oxidative damage, **3)** the accumulation of free radicals produced by the inflammation itself, with the related tissue degeneration.

Osteoporosis, myopathies, and idiopathic neuropathies are common manifestations of systemic inflammation, and pain is a common effect of these conditions.

– Viral infections can induce various physiological changes in the human Endocrine System, resulting in a cytokine-mediated activation of the HPA Axis to increase the production of cortisol, thus modulating the immune response.

Any virus that causes systemic viraemia and inflammation can stimulate the HPA Axis to release cortisol, through early pro-inflammatory cytokines (IL-1, IL-6, and TNF- α) and T-cell related cytokines, INF- γ , and IL-2.

These mediators act on the hypothalamic cells that produce CRH (corticotropin-releasing hormone), the anterior pituitary and the adrenal cortex to increase glucocorticoids during viral disease.

- At the moment, the Coronavirus disease (COVID-19, SARS-CoV-2) mainly involves the lungs, leading to pneumonia, which is often complicated by acute respiratory distress syndrome, and sepsis.

The pathogen enters the pneumocyte using the host's angiotensin-converting enzyme 2 (ACE2) as a receptor.

Binding results in the *down*-regulation of ACE2 and the ineffective conversion of the pro-thrombotic angiotensin II (the levels of which are directly proportional to those of cortisol and the proportion of *spike* proteins on the surface of the Coronavirus), into angiotensin I.

Furthermore, the enzyme is expressed on the arterial and venous endothelial cells of many organs, including the adrenal glands.

– It is common knowledge that the severe course of the disease is associated with an overreaction of the immune system with a mass release of cytokines and chemokines ('cytokine storm'), which can be correlated with HPA dysfunction.

Elevated cytokines also trigger the induction of hyaluronan synthase 2 (HAS2) in alveolar epithelial cells (type 2) and fibroblasts.

– Thus, the synthesis of **hyaluronan** occurs, which has a high water-binding capacity of up to 1,000 times its molecular weight, resulting in the accumulation of fluid in the lungs, which correlates with the development of respiratory distress syndrome.

– COVID-19 therefore constitutes a universal threat requiring rapid, favourable and safe measures to reduce the risk of infection, suppress the development of virulence, strengthen the immune system and support healing.

– It is known that individuals living in areas with a low selenium intake or inadequate nutrition, and patients with pre-existing comorbidities are at particularly high risk of becoming infected with COVID-19 or other viral infections.

The optimum status of **selenium** (100 µg per day) promotes increased T-cell proliferation, NK (*natural killer*) cell activity and innate cell functions.

– Selenium supports a stronger vaccine response and solid immunity to pathogens.

It also suppresses severe inflammation in tissues such as the lungs and intestines.

- Clinical studies have shown that selenium supplementation modulates the inflammatory response in patients with respiratory distress syndrome by restoring the antioxidant status of the lungs and suppressing the levels of IL-1β and IL-6.

Selenium supplementation inhibits the pathogen-induced activation of the transcription factor NF-κB and its downstream release of pro-inflammatory cytokines.

In humans, dietary selenium is incorporated into 25 selenium-proteins, including glutathione peroxidase, selenium F protein, selenium proteins K and S, and thioredoxin reductase.

These play crucial roles in molecular pathways such as **1)** the response to oxidative stress or endoplasmic reticulum stress, **2)** the response to unfolded proteins, and **3)** the immune and inflammatory response involving NF-κB and the nuclear factor erythroid-2-related factor (Nrf2 regulating the gene expression of a wide variety of antioxidant cytoprotective enzymes).

– In human studies and animal models, it has been observed that the crossed dialogue between these pathways is crucial for producing an adequate response to viral infections; low selenium concentration is linked to a less efficient response to RNA virus infection.

FIG. 1 shows how reduced selenium-protein expression, resulting from a low/sub-optimal selenium status, may alter molecular pathways involved in stress responses and contribute to an +aggressive pro-inflammatory environment leading to a worse prognosis of viral disease.

– As observed, the decline in selenium status leads to insufficient protective selenium enzymes, particularly glutathione peroxidase, an enzyme consisting of selenium and glutathione. Glutathione peroxidase is an enzyme belonging to the class of oxidoreductases.

By reducing the amount of hydrogen peroxide in water, it converts the reduced form of glutathione (GSH) into **glutathione disulfide (GSSG)**, the oxidised form of glutathione.

– In other words, the glutathione is oxidised. The oxidised GSSG is then 'recharged' or regenerated by NADPH (a reduced form of NADP⁺), turning into GSH (a reduced form of glutathione).

If there is a selenium and glutathione deficiency, an insufficient inactivation of peroxides as precursors of reactive oxygen species (ROS) occurs, resulting in a **serious disturbance to the redox balance**.

Glutathione peroxidase itself, however, interacts inhibitory with the MPRO protease of SARS-CoV-2, a protease which is necessary for the formation and production of virus components. MPRO is directly involved in the proteolysis of glutathione peroxidase and other important selenium proteins (thioredoxin reductase and glutamate cysteine ligase), which, when deactivated or structurally demolished, **do not**

guarantee the adequate removal of free radicals.

– In order for there to be a powerful regeneration and activation of the glutathione peroxidase, it is necessary to increase the amount of energy required to produce glutathione, i.e. to ensure two molecules of ATP for the production of each glutathione molecule.

To do this, it is necessary to enhance the Krebs cycle through the first component that enters it, i.e. acetyl coenzyme A.

The latter is derived from pantothenic acid, i.e. vitamin B5.

– The expected consequences of an increase in acetyl-CoA content in the mitochondria are the stimulation of pyruvate oxidation, the functioning of the Krebs cycle and the oxidation of fatty acids.

It also appears that vitamin D3 plays the role of upregulating the thioredoxin-reductase (selenium-containing protein that regenerates NADPH) and glutamate-cysteine ligase (the first enzyme of the glutathione biosynthetic pathway).

The overexpression of glutathione, glutathione peroxidase, superoxide dismutase, and the downregulation of NADPH oxidase are induced by vitamin D3 to reduce oxidative stress.

The effective over-regulation of so-called selenium proteins by vitamin D3 requires the presence of an adequate level of selenium, however.

The full potential of vitamin D3 with respect to COVID-19 or other viral infections can only be considered in combination with an optimal selenium intake.

Chronic stress also reduces **cortisol-binding globulin (CBG)**.

– CBG is a glycoprotein synthesised in the liver and secreted into the bloodstream where it binds with high affinity to glucocorticoid hormones, such as

cortisol in humans and corticosterone in laboratory rats.

In mammals, 95% of circulating glucocorticoids are bound to CBG (80%) and albumin (15%), and only 5% of the free fraction is able to enter the CNS.

During stress, the concentration of glucocorticoids increases significantly (the free fraction increases even more) once the CBG is saturated.

The observed pronounced increase in ACTH and cortisol during stress can be explained by a decrease in CBG in chronically stressed individuals.

– A membrane phospholipid known as phosphatidylserine, physiologically and temporarily externalised as a marker during apoptosis, first causes the normalisation of CBG levels under these conditions, followed by the normalisation of HPA Axis activity and reactivity.

During viral infections, another impairment of the structural and functional aspect of the cell membrane also occurs.

– Viral infections create structural membrane damage that leads to abnormal permanent exposure of phosphatidylserine on the outer cell surface (scramblases activate caspases which inactivate the flippases that bring phosphatidylserine back to the membrane after the apoptosis signal).

– The balance between the internalisation and externalisation of phosphatidylserine is equal to the balance between pro-inflammation and anti-inflammation.

If phosphatidylserine is extended out of the cell's *bilayer* phospholipid structure, apoptosis occurs and cellular activity shifts towards a suppression of immune response (a suppression that viruses promote) in favour of an extended cellular and tissue synthesis pathway, when instead there must be a correct balance between the apoptotic signal, and immediate macrophage phagocytosis, with the subsequent reentry of the phos-

phatidylserine into the membrane structure.

In such a compromised system, it would be crucial to strengthen the membrane phosphatidylserine, repairing and sustaining the cellular apoptotic defence mechanism and the apoptotic cell elimination mechanism, the first defence strategy against pathogens and the interleukin-mediated inflammatory state.

The efficient elimination of apoptotic cells from the airways by phagocytes is essential for the effective resolution of inflammation and a return to pulmonary homeostasis.

– A disruption in this process would lead to a secondary necrosis of accumulated apoptotic cells, the release of necrotic cell debris, and the subsequent uncontrolled inflammatory activation of the innate immune system by the released 'Damage-associated molecular patterns' (DAMPs).

In other words, during sepsis or inflammatory events, phosphatidylserine exposure may be over-regulated on cell surfaces throughout the body, including endothelial cells, platelets, erythrocytes, neutrophils, lymphocytes, and extracellular micro-particles.

– Within the context of a SARS-CoV-2 infection, the intracellular conditions required for the activation of scramblases can be triggered by the viral infection itself, down modulation of ACE2 and the resulting increase in angiotensin II directly related to circulating cortisol levels.

With phosphatidylserine overexpressed, such structural membrane disruption would lead to the formation of clots and the activation of the extrinsic coagulation pathway by association with factor VII.

• By administering phosphatidylserine in the appropriate dosages, the cell membrane is structurally and functionally repaired, therefore competitively preventing abnormal externalisation and thus

blocking the activation of inflammatory and pro-coagulant processes.

Furthermore, the phosphatidylserine **intensifies** the antioxidant action of glutathione peroxidase, it **regulates** the release of interleukin-1 β , it **normalises** the production levels of cortisol in response to stress, it **promotes** the differentiation of T-lymphocytes in the interleukin-10 producing form, the antagonist of pro-inflammatory Interleukins.

In appropriate dosages, phosphatidylserine safely **shuts down** or **reverses** biochemical elevations and the structural deterioration of nerve cells, it **supports** human cognitive functions such as short-term memory formation, long-term memory consolidation, the ability to learn and recall information, attention focus, and concentration.

It also supports locomotory functions, especially quick reactions and reflexes.

► At appropriate dosages and in synergy, the administration of selenium (in the organic selenium-methionine form), phosphatidylserine, vitamins B5 and D3 (**GunaVit B5 PLUS**) triggers a virtuous mechanism aimed at combating the symptoms related to **chronic stress** and **infections** and those consisting of chronic fatigue, muscular and joint pain, insomnia, hair and memory loss, difficulties concentrating, increased histamine reactivity, atypical skin rashes, dyspnoea, synovitis and thyroiditis.

A vitamin B5 deficiency also lowers the available acetylcholine, the neurotransmitter also used by the Parasympathetic Nervous System.

– The uncontrolled and increased sympathetic tone subsequently causes hypertension, tachycardia, atrial arrhythmias, and a 'hyperadrenergic' state known to influence heart disease, and strokes.

– A vitamin B5 deficiency leads to a reduced production of cortisol where it acts as an anti-inflammatory mediator,

thus leading to increased arthritic pain, myalgia, fatigue, headaches, depression, insomnia, and widespread 'pro-inflammatory' effects on the Immune System.

The combination of vitamins D and B5 creates an intestinal environment favourable to the repopulation of *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* that make up the normal human microbiome.

A deficiency in the action of glutathione peroxidase promotes angiotensin II-induced left ventricular hypertrophy, dilatation, and ventricular dysfunction, highlighting a crucial physio-pathological role for ROS in the development of cardiovascular diseases.

– When the cortisol-mediated response is repeated and exacerbated, cortisol can *invade* the receptors for mineralocorticoids, thus triggering an aldosterone-like reaction.

In the case of COVID-19, activation (associated with cortisol) of the mineralocorticoid receptor in the virus-infected epithelial and endothelial cells stimulates the release of ATP, which then acts on the purinergic receptors.

In the lungs, this can cause a non-productive cough through the purinergic receptors on the affected vagal nerves.

On the other hand, in endothelial cells it can stimulate: **1)** the exocytosis of the Weibel-Palade Bodies* containing angiotensin-2, which is involved in the pathogenesis of acute respiratory distress syndrome (ARDS), and **2)** the Von Willebrand factor (VWF), which mediates platelet adhesion to the endothelium and thus coagulation.

* **Editor's note:** Weibel-Palade Bodies are cytoplasmic organelles found in the endothelium cells of blood vessels, and of the heart.

CONCLUSIONS

Nutritional therapy is an important part of patient care, for surviving viral infections or other stress-promoting conditions, and for a better and shorter recovery as a result.

In addition, controlling malnutrition and optimal nutritional supplementation are essential steps to ensure the Immune System works in the best way possible.

– Patients with malnutrition are more likely to belong to poor socio-economic groups.

Therefore, nutritional supplementation is important for at-risk groups and fragile individuals such as the elderly with relatively weak immune systems.

People with a weak immune system are more prone to bacterial and/or viral infections.

– Early and adequate nutritional supplementation, together with pharmacological treatment and clinical collaboration, are essential when it comes to restoring both the structural and functional balance in health, and promoting a general state of well-being. ■

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author

Dr. Felice Esposito

- Clinical Nutritionist
- Certified Sonographer (Ultrasound Internist and Doppler)

Borgosatollo (BS), Italy



D. Lepori

SUMMARY

The aim of this study is to evaluate the effects of the administration of Guna Collagen Medical Devices in acute and chronic pain symptoms associated with Osteoarthritis.

The goal of this study is to evaluate how the administration of district-specific MDs can modulate pain and improve functional and motor responses.

– After ascertaining the patient's clinical state and requesting, if possible, the suspension of anti-inflammatory, analgesic or similar drugs, we proceeded with the administration of subcutaneous, locoregional infiltration, according to the Mesotherapy technique.

The infiltrations were carried out on a weekly basis (1 session/week x 12 weeks) in 20 patients suffering from pain related to their osteoarthritic condition of varying clinical entity (from moderate to severe).

The 20 patients presented the following complaints: 5 knee pain, 7 low back pain, 5 neck pain, 3 shoulder-related pain.

The assessment parameters used were the WOMAC questionnaire for knee pain, the VAS scale for pain and the VAS scale for subjective sensation of functional improvement in movement (0-10). The questionnaires were given to the patients before the start of the therapy (T0) and after the 1st, 2nd, 3rd, 6th and 12th week of treatment. Therapy involved the use of specific Collagen MDs, administered alone without other drugs, using 5 ml syringes, 30G x 12mm needles. At each treatment data were collected according to the WOMAC questionnaire and the two VAS Scales adopted.

At the end of the treatment period, following the processing of the collected data and the observation of the patients for 6 months after the therapy, it was observed a significant reduction in perceived and evoked pain as well as a general improvement in the functional condition of the joints.

KEY WORDS

GUNA COLLAGEN MEDICAL DEVICES, MD-NECK, MD-SHOULDER, MD-LUMBAR, MD-KNEE, NECK PAIN, SHOULDER PAIN, LOW BACK PAIN, KNEE PAIN, JOINT PAIN, OSTEOARTHRITIS

GUNA COLLAGEN MEDICAL DEVICES IN THE TREATMENT OF PAIN IN OUTPATIENT CASES OF OSTEOARTHRITIS

OSTEOARTHRITIS PAIN

Pain accounts for a large part of the professional life of an outpatient doctor; it is a common complaint that goes hand in hand with the most diverse clinical situations, becoming the reason for the medical consultation.

In chronic diseases, pain has a significant impact on the patient's quality of life and can limit, for example, mobility, the capacity to work, psycho-physical well-being, and social relationships.

– In osteoarticular diseases, such as **Osteoarthritis (OA)**, the pain is often difficult to bear.

– It is therefore very important to protect patients from acute pain, and above all from chronic pain, as much as possible.

Various methods are used to classify pain and its extent; that of the IASP (International Association for the Study of Pain) and the WHO, among the most accepted, both stigmatise the major impact that pain has on the quality of life of those who suffer from it.

Certain types of pain can be identified from a classification point of view: acute, chronic, procedural, movement-evoked, referred, and spontaneous pain, etc.

They have in common the nervous pathway and its physio-anatomical hierarchy: there are a number of medical, psychological and psychiatric facets to the pain.

In osteoarticular diseases it is linked to the overactivation of painful type **A Delta** and **c nerve fibres**.

These somatic nociceptive fibres are located in the periosteum, endosteum, joint capsule and peri-articular structures.

It goes without saying that, especially in chronic diseases, there is also a significant psychophysiological-somatomorphic component, with major affective-emotional implications that influence the perceived pain.

– Most of the painful conditions associated with osteoarticular pictures present multifactorial signs and symptoms, often correlated with chronic degenerative diseases, most frequently OA.

In OA, pain symbolises a vast array of distressing clinical conditions.

What these situations have in common is persistent pain that may or may not be related to disease patterns of inflammation and degeneration.

– The joints are subject to acute or chronic inflammatory processes related to an assortment of problems that develop and accumulate over time.

The most common causes are:

- work-related (tiring, repetitive, or sedentary jobs, performed with unnatural postures, etc.)
- morpho-functional disorders (dysplasia, scoliosis, changes in the joint status, etc.)
- traumatic
- iatrogenic/post-operative

– metabolic/food-related/endocrine.

In recent years, the medical community is increasingly coming round to the idea that degenerative processes have a multifactorial basis, like in the case of OA, thus abandoning the previous notion which inevitably associated them with ageing.

What all these conditions have in common is that they activate inflammatory processes that are manifested in the form of damaged ligaments, tendons, aponeurosis, planking of the joints and intra-articular cartilage, and affect the collagen component.

In fact, collagen is the main component of all structures that make up the joints (extra and intra).

OSTEOARTHRITIS

OA is defined as degenerative joint disease; it involves the destruction and potential loss of joint cartilage, damage to the subchondral bone, which goes into eburnation, damage to the joint margins with narrowing of the joint space, and the development of osteophytes and geodes on the joint heads.

It is the most common joint disease in the adult population. It is generally diagnosed around the 5th/6th decade of life and is almost omnipresent in multiple joints by the 8th decade, even if not

always symptomatic, and can be easily confirmed by radiological diagnostics.

Below the age of 40, OA has repeated/chronic traumatic causes and mainly affects the male gender.

Normally only half of OA patients have symptoms, pain being the most prominent.

OA is damage to the joint cartilage tissue, which is primarily made up of collagen.

OA can be primary, often idiopathic, or secondary (multiple causes that change the microenvironment, metabolism and the characteristics of the joint cartilage).

The bone areas affected by OA are:

– **Large joints, 95%:** spine 54%; knee 27%; hip 7%; shoulder, elbow, hand and fingers 7%.

– **Small joints, 5%:** talus calcaneal joint 4%; metatarsophalangeal joint of 1st finger 1%.

The majority of the joints affected by OA are subjected to a static load (large joints), while the minority are subjected to a dynamic load.

It should be pointed out that, under normal conditions, the joints do not undergo enough friction to damage the bone heads and joint cartilage, whether by habitual use, overuse or trauma.

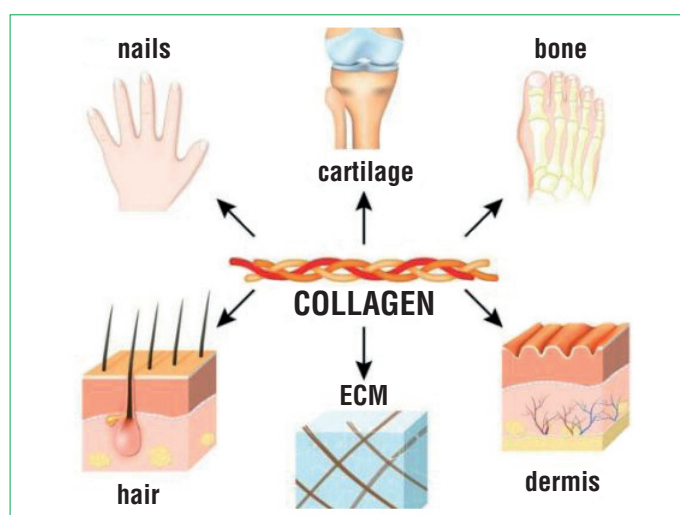
– The intra-articular hyaline cartilage is not vascularised; it is made up of water and collagen proteins (95%) and chondrocytes (5%).

Since cartilage does not have blood or lymph vessels, the metabolism and health of joints are related to the “squeezing” of joints, i.e. the physiological compression and decompression of hyaline cartilage during movement, which ensures metabolic regulation by diffusion.

The inflammation at the root of OA generates sclerosis and rigidity, thus creating a vicious circle that progressively aggravates the joint damage.

Whatever the cause, one can always

FIG. 1



observe a discrepancy between the physical load and resulting mechanical stress with the capacity of the joints to absorb and cushion this work; there is therefore a progressive thinning of the joint cartilage, exposure of the bone, inflammation of the joint capsules and oedema in the periarticular soft tissue.

In OA, there is an increasing reduction in the physiological ability of the joint components to slide freely over one another.

– The correct functioning of joints depends on the quality of their cartilage components; if their mechanical and metabolic properties are preserved, the joint is less likely to undergo OA and deterioration, and cause pain.

Over time, many factors contribute to a reduction in the metabolic capacity of collagen to maintain the physiological dynamics of synthesis and degradation.

Being bedridden for a long time, immobility or mobility that is not sufficient to provide mechanical stimuli to the joints give rise to a deficit in the ability to synthesise collagen and therefore in the physiological regeneration of cartilage and successful management of joint lubrication (primarily hyaluronic acid). Movement, in fact, is one of the factors that regulates the metabolism of cartilage and collagen based on mechanical “squeezing” in relation to the static-dynamic load on the joint.

OA has long been considered a degenerative disease with an age-related basis; in recent years, however, the disease tends to be considered within the context of a multifactorial framework that generates a complex aetiology and pathogenesis of the joint damage, with a biochemical-cellular basis involving the chondrocytes, collagen and cartilage.

– It has been observed that one of the main factors that cause OA is the suffering of chondrocytes leading to the release of enzymes and signal molecules, which gradually induce an abnormal turnover of the elements that make up the joint structures.

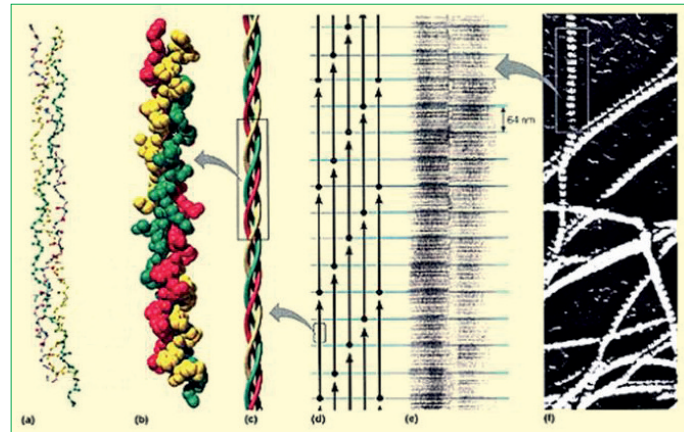


FIG. 2

THE IMPORTANCE OF COLLAGEN

Collagen represents approximately 7% of the weight of an adult organism.

It is so abundant in the human body that it constitutes approximately 30% of the total proteins; it is found almost everywhere (skin, teeth, bones, tendons, joints, serous membrane and vessel walls, etc.) (FIG. 1); it has significant metabolic and connective support functions and also performs biochemical functions associated with tissue homeostasis, such as, for example, a **buffer function**.

– As stated above, the importance of collagen lies in the fact that it is the most widely-represented, versatile and ubiquitous protein-based structure in the body.

Collagen plays an active role, rather than a passive role as a mere structural element; this is because it can adapt its metabolism according to the body weight and pressure to which it is subjected, e.g. FACIT (Fibril Associated Collagens with Interrupted Triple Helices).

Collagen also has **bioconductor** and **biosensor** characteristics, with **piezoelectric** and **vector information transfer** properties.

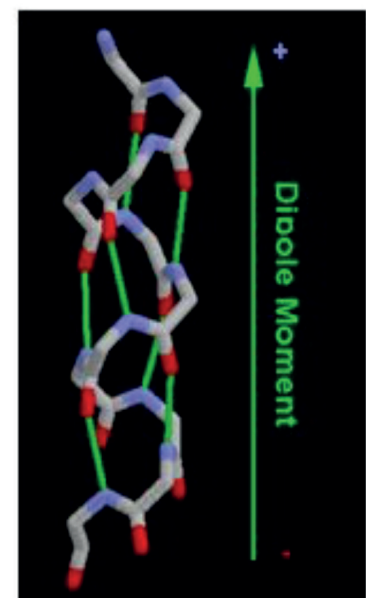
It also interacts with water to generate ion exchanges: these biophysical properties give collagen a powerful metabolic role in the structures formed from it (FIGS. 2, 3, 4).

The basic unit of collagen is tropocollagen, consisting of three laevorotatory peptide helices; this unit is bonded to glucose and galactose, which are connected to specific amino acids.

This basic structure gives collagen its vast range of properties, especially the repetition of specific amino acid triplets; each type of collagen has a specific variation of the amino acid triplets.

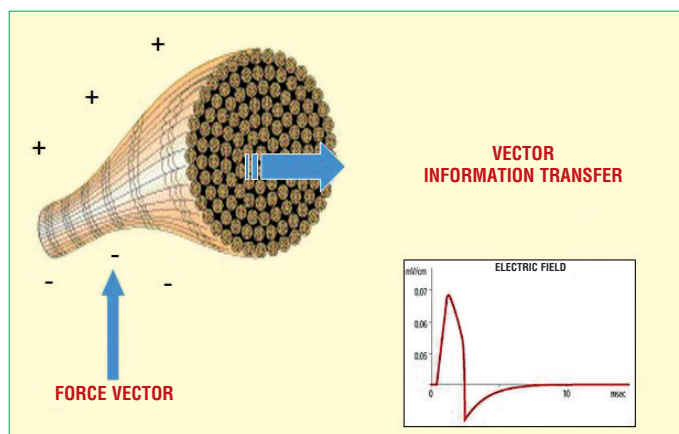
Tropocollagen gives rise to procollagen, which polymerises to form protocollagen outside the cell by means of a self-catalytic self-assembly mechanism involving certain oxidases.

FIG. 3



RCSB PDB
PROTEIN DATA BANK

FIG. 4



The individual units of procollagen are ready to self-assemble in turn, giving rise to mature collagen that has periodicity (true micro-metamerism) (FIG. 2).

GUNA COLLAGEN MEDICAL DEVICES: THE NEW THERAPEUTIC APPROACH TO PAIN IN OSTEOARTICULAR DISEASES

The Guna Collagen Medical Devices (MDs) are an effective therapeutic aid, considering the high concentration of collagen in the structures involved in joint diseases such as OA.

The administration of porcine-derived

collagen has beneficial effects on the management/reduction of certain lytic enzymes, the most important being the MMP-9, MMP-13 metalloproteases and Cathepsin K.

– Many studies highlight the importance of MMPs and other lytic enzymes in the genesis and chronic maintenance of joint damage on an enzymatic-inflammatory basis, where a collagen metabolism imbalance favours its degradation rather than neosynthesis. There are other collagenolytic enzymes in addition to MMPs, for example other collagenases and ROS, often in a broader context of low grade inflammation based on many factors.

– There is considerable evidence in the medical-scientific literature (over 40 articles) concerning the positive results shown by the intraarticular and/or periarticular infiltration of Collagen MDs, which improve the response of tissue to MMPs and lead to reparative processes in the joint and periarticular tissue (www.collagenmd.guna.com).

– It was therefore decided to use Collagen MDs in selected patients with confirmed OA.

Guna Collagen Medical Devices are injectable medical devices based on highly biocompatible collagen of porcine origin, with the utmost histocompatibility with human collagen.

The molecular selection is of high quality due to the use of tangential-flow filtration, sterilisation of the collagen fibres, and control of the molecular weight.

The collagen obtained in this way is highly pure and bioavailable (TAB. 1).

The Collagen MDs also contain ancillary biological molecules that increase specific joint tropism and the effectiveness of collagen on the intended target area. Their possible uses fall within the context of secondary prevention and therapy.

TAB. 1

Guna Collagen Medical Devices.

GUNA Medical Device		COMPOSITION
BODY DISTRICT SPECIFIC	MD-NECK	Collagen + Silica
	MD-THORACIC	Collagen + <i>Cimicifuga</i>
	MD-LUMBAR	Collagen + <i>Hamamelis</i>
	MD-SHOULDER	Collagen + <i>Iris</i>
	MD-HIP	Collagen + Calcium Phosphate
	MD-KNEE	Collagen + <i>Arnica</i>
	MD-SMALL JOINTS	Collagen + <i>Viola</i>
	MD-POLY (Multi-articularity)	Collagen + <i>Drosera</i>
	MD-ISCHIAL (Sciatica)	Collagen + <i>Rhododendron</i>
TISSUE-SPECIFIC	MD-MUSCLE	Collagen + <i>Hypericum</i>
	MD-NEURAL	Collagen + <i>Citrullus</i>
	MD-MATRIX (Extra-Cellular Matrix)	Collagen + Citric acid, Nicotinamide
	MD-TISSUE (Soft tissues)	Collagen + Ascorbic acid, Magnesium gluconate, Pyridoxine hydrochloride, Riboflavin, Thiamine hydrochloride

Another reason for deciding to use the Collagen MDs was the lack of side effects. They are very safe, easy to handle, quick and straightforward to use, and therefore also suitable for an outpatient setting.

– Moreover, they are well tolerated by patients according to the literature.

MATERIALS AND METHODS

Twenty patients (13 M and 7 F) suffering from OA were enrolled in this trial.

All patients were diagnosed in a specialist consultation prior to being recruited for the trial.

– The patients (60-85 years) were divided into groups according to the area affected by the arthritic damage and pain.

Patients M: **5** with **Gonarthrosis**, **5** with **Lumbar Spondyloarthritis**, and **3** with **Cervical Spondyloarthritis**.

Patients F: **3** with **Shoulder OA**, **2** with **Lumbar Spondyloarthritis**, and **2** with **Cervical Spondyloarthritis**.

– After signing the informed consent form (purpose, methods, treatment procedure and products for the study concerned), all patients underwent **infiltrative Mesotherapy treatment** on a weekly basis for 12 consecutive weeks, at which times the specific MD for the affected area was administered.

Questionnaires were issued to the patients on the **1st, 2nd, 3rd, 6th, and 12th weeks**.

All the patients filled in 2 VAS (Visual Analogue Scale) questionnaires, one for the pain and the other for the assessment of perceived subjective improvement.

The 5 M patients suffering from Gonarthrosis also filled in the WOMAC (Western Ontario and McMaster Universities Arthritis Index).

To take part in the study, the patients were asked to discontinue any ongoing therapies where possible, to avoid affecting the outcome of the results.

PATIENTS M

• **Gonarthrosis Group:**

5 patients, average age 70.8 years (min. 60 - max. 82), with previous diagnosis of confirmed Gonarthrosis.

– **Treatment: MD-Knee.**

• **Lumbar Spondyloarthritis Group:**

5 patients, average age 72.4 years (min. 61 - max. 85), diagnosed with Lumbar Spondyloarthritis with disc problems, osteophytic processes and straightening of the lumbar spine.

– **Treatment: MD-Lumbar.**

• **Cervical Spondyloarthritis Group:**

3 patients, average age 73.3 years (min. 68 - max. 79), with a diagnosis of confirmed Cervical Spondyloarthritis.

– **Treatment: MD-Neck.**

PATIENTS F

• **Scapulohumeral OA Group:**

3 patients, average age 68.3 years (min. 68 - max. 72), with a clinical picture that was variable but attributable to a process of Scapulohumeral Osteoarthritis.

– **Treatment: MD-Shoulder.**

• **Lumbar Spondyloarthritis Group:**

2 patients, average age 67.5 years (min. 66 - max. 69), with a confirmed diagnosis of Lumbar Spondyloarthritis.

– **Treatment: MD-Lumbar.**

• **Cervical Spondyloarthritis Group:**

2 patients, average age 69 years (min. 67- max. 71), with a confirmed diagnosis of Cervical Spondyloarthritis.

– **Treatment: MD-Neck.**

RESULTS, DISCUSSION AND CONCLUSION

At the end of the 12 weeks of treatment, all 20 patients had completed the therapeutic cycle.

The data collected were processed using the statistical method of mean dis-

tribution (FIG. 5), a statistical study of simple modality, effective and highly suited to an outpatient experiment.

– This statistical study is based on the average of the values collected in order to eliminate or reduce errors relating to min. and max. distribution values; this enables an objective, fairly reliable assessment of the results obtained in the population in question.

As shown by the results in TABS. 2 and 3, there was a gradual, contained improvement in the first 3 weeks of treatment (reduction in perceived pain, and a modest improvement in overall subjective perceived pain).

– The improvement and elimination of pain are fundamentally different **after the 6th week of treatment** by infiltrative mesotherapy when compared with the initial conditions; the results obtained are maintained up to the 12th week, with further improvements.

Based on the data obtained at the end of the 12th week, we can confirm that the treatment had a very positive effect on the pain and the general well-being of the patients.

The very low invasiveness and speed of administration of the MDs also resulted in almost total adherence to the therapy, generating good feedback, and satisfaction from the patients.

The infiltrative treatment using Guna Collagen MDs is certainly of great im-

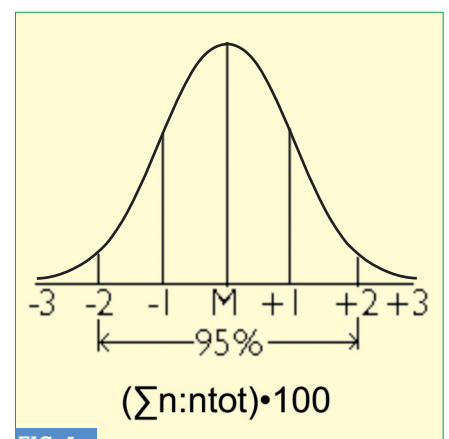


FIG. 5

Average distribution of statistical values; distribution bell curve with summation.

portance in the treatment of OA and pain.

This area is increasingly appealing to practitioners, since there is a gradual increase in joint diseases in the population.

– There are many strategies for managing OA (viscosupplementation, PRP, oral collagen, etc.), but, among the infiltrative treatments, Guna Collagen

Medical Devices stand out in terms of quality and safety.

Based on our experience and the literature, we can confirm that they have unique characteristics that are ideal for the treatment and management of OA, including in an outpatient setting (very important aspect).

Guna Collagen Medical Devices have an excellent formulation profile.

They are highly purified products combined with ancillaries that enhance their therapeutic action; no adverse effects have been reported in 10 years, and they have a broad range of applications (13 MDs can be combined in various ways, if necessary).

– Moreover, their therapeutic effect can be detected after fewer sessions, and last for longer periods, than other therapeutic strategies. They can also be administered to patients with comorbidi-

A																				
	WOMAC baseline	WOMAC 1st week	WOMAC 2nd week	WOMAC 3rd week	WOMAC 6th week	WOMAC 12th week		VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week		VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week
1	59	59	55	53	45	38	1	9	8	8	7	5	4	1	0	0	2	2	5	7
2	56	55	52	53	46	32	2	9	9	9	8	6	4	2	0	1	2	3	5	6
3	53	53	50	49	38	20	3	8	7	7	8	7	5	3	0	1	1	2	3	7
4	51	50	50	48	41	28	4	8	7	6	6	5	3	4	0	2	2	3	5	8
5	52	52	51	49	41	26	5	7	7	6	6	5	3	5	0	1	1	2	4	8
	54.2	53.8	51.6	50.4	42.2	30.8		8.2	7.6	7.2	7	5.6	3.8		0	1.0	1.6	2.4	4.4	7.2

B															VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week
1	9	9	8	8	5	4	1	0	1	1	2	4	7	1	0	1	1	2	4	7
2	8	7	7	6	4	2	2	0	2	2	3	5	8	2	0	2	2	3	5	8
3	9	9	8	8	6	4	3	0	1	2	2	4	7	3	0	1	2	2	4	7
4	7	7	5	6	3	1	4	0	1	1	2	6	8	4	0	1	1	2	6	8
5	6	5	5	5	3	2	5	0	2	2	3	5	8	5	0	2	2	3	5	8
	7.8	7.4	6.8	6.6	4.2	2.6		0	1.4	1.6	2.4	4.8	7.6		0	1.4	1.6	2.4	4.8	7.6

C															VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week
1	9	9	9	8	6	3	1	0	1	1	2	4	7	1	0	1	1	2	4	7
2	9	8	7	7	5	2	2	0	0	1	1	5	8	2	0	0	1	1	5	8
3	8	8	8	7	5	2	3	0	1	2	2	6	8	3	0	1	2	2	6	8
	8.6	8.3	8	7.3	5.3	2.3		0	0.7	1.3	1.7	5	7.6		0	0.7	1.3	1.7	5	7.6

TAB. 2

Patients M (13) divided according to disease.

A – Gonarthrosis (5) – from left to right: WOMAC score for Gonarthrosis, VAS for perceived pain and VAS for perceived subjective improvement.

B – Lumbar Spondyloarthritis (5) – left: Perceived pain score on VAS; right: Perceived subjective improvement on VAS.

C – Cervical Spondyloarthritis (3) – left: Perceived pain score on VAS; right: Perceived subjective improvement on VAS.

TAB. 3

Patients F (7) divided according to disease.

A – Scapulohumeral Arthritis (3) – left: Perceived pain score on VAS; **right:** Perceived subjective improvement on VAS.

B – Lumbar Spondyloarthritis (2) – left: Perceived pain score on VAS; **right:** Perceived subjective improvement on VAS.

C – Cervical Spondyloarthritis (2) – left: Perceived pain score on VAS; **right:** Perceived subjective improvement on VAS.

A													
	VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week		VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week
1	8	8	7	7	4	2	1	0	0	1	1	3	7
2	9	8	8	7	3	1	2	0	1	2	2	4	8
3	8	7	7	7	4	2	3	0	2	2	2	4	8
	8.3	7.6	7.3	7	3.6	1.6		0	1	1.6	1.6	3.6	7.6

B													
	VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week		VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week
1	7	7	6	6	4	2	1	0	2	2	3	5	8
2	8	7	7	6	4	2	2	0	2	3	4	6	9
	7.5	7	6.5	6	4	2		0	2	2.5	3.5	5.5	8.5

C													
	VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week		VAS baseline	VAS 1st week	VAS 2nd week	VAS 3rd week	VAS 6th week	VAS 12th week
1	9	9	8	7	4	2	1	0	2	3	2	4	8
2	9	9	9	7	5	2	2	0	2	2	3	5	8
	9	9	8.5	7	4.5	2		0	2	2.5	2.5	4.5	8

ties who are undergoing specific individual therapies, depending on the case.

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The author would like to thank the editors of the websites from which the images are taken:

Fig. 1

<https://www.healthintegratori.com/collagene-integratore-dalla-cartilagine-alla-bellezza-della-pelle/> ©

Fig. 2

https://besport.org/sportmedicina/equilibrio_corporeo_tessuto_connettivo.htm ©

Fig. 4

https://besport.org/sportmedicina/equilibrio_corporeo_tessuto_connettivo.htm ©

author

Dr. Dimitri Lepori

– Medical expert in Integrated Medicine and Neural Therapy

Florence, Italy



EXOSOMES, THE SHUTTLES OF INTERCELLULAR COMMUNICATION

– THEIR EMERGING ROLE AND DIAGNOSTIC AND THERAPEUTIC POSSIBILITIES

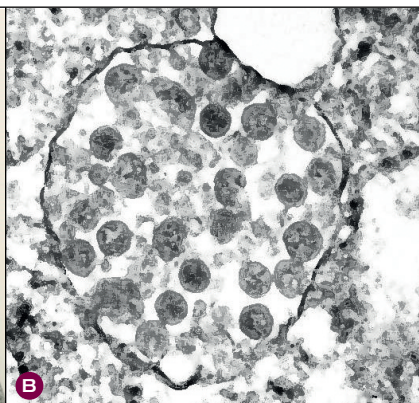
*When a wise man points at the moon,
the fool looks at the finger.*

THE SHEEP, THE RAT AND “ALICE IN BLUNDERLAND”

In her historical review article published in *Cell*, May-June 2005 (Johnstone, 2005), a few years before her death and about 20 years after her discovery of **exosomes** and their expulsion from the plasma membrane of mature sheep reticulocytes (Pan & Johnstone, 1983), **Rose Johnstone**, born Mamelak, of Polish origin (Lodz-Poland, 1928 – Montréal-Canada, 2009), ironically defines the entire research journey

that led to her crucial discovery as Alice in a “Land of Errors” (*Alice in Blunderland, an Iridescent Dream*), referring, in the first part of the title of a paragraph, to the title of the famous short story by John Kendrick Bangs (1907), a parody of the better known “Alice’s Adventures in Wonderland” by Lewis Carroll (1865).

– The word “*blunder*” means error, misunderstanding, misstep, mistake, to underline the many difficulties and unforeseen events she encountered while carrying out of her experiments and those of her young Chinese collaborator **BinTao Pan** in the Department of Biochemistry of the Faculty of



A – Rose Mary Johnstone pictured at the time of her discovery of exosomes in 1983 (McGill University Historical Archives, Montreal – Canada).

B – Multivesicular body (MVB) containing approximately thirty intraluminal vesicles (ILVs). The membrane of the MVB fuses with the plasma membrane of the mother cell, thus releasing ILVs. – These, having reached the extracellular space, are termed exosomes (EXO).

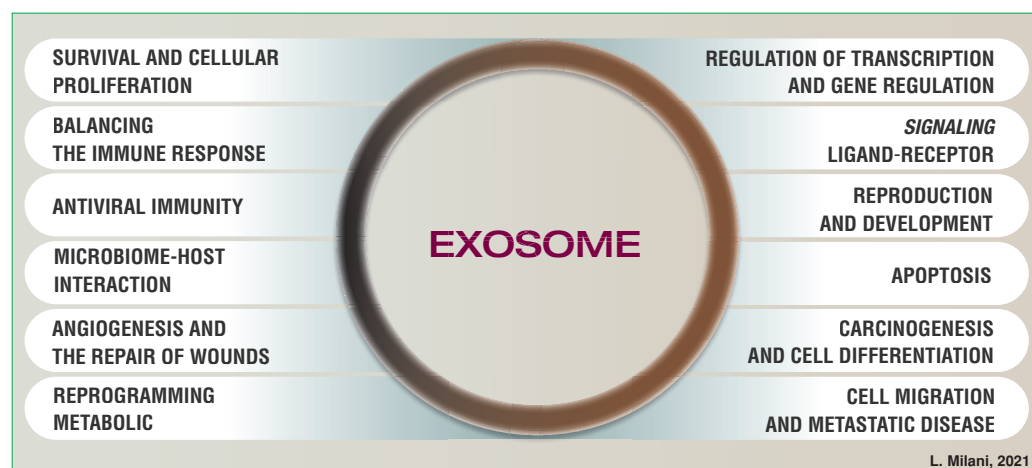


FIG. 1

Main physiological and pathological activities performed by exosomes.

Medicine at McGill University in Montreal Canada, and to highlight the fact that, at the time of the discovery and for many years to follow, exosomes were considered nothing more than “waste”, “refuse”, cellular “garbage” to be eliminated from the cytoplasm. Surely an unusual, original title for a paragraph, full of the many inevitable unknowns and errors involved in lengthy research and a key biological discovery under the electron microscope (SEM).

– Less than one week [at the very same time (Harding *et al.*, 2013)] after Pan & Johnstone’s publication, the *Journal of Cell Biology* published a paper by **Harding, Heuser & Stahl** (1983) on their research at the University of Washington in St. Louis USA, in which some tiny vesicles ejected (*jettisoned* in the original English = thrown out) into the extracellular space by the mature reticulocytes of rats are photographed under a SEM.

- The term “*exosome*” is mentioned in neither Pan & Johnstone’s article nor that of Harding *et al.*; it was coined 4 years later by Professor Johnstone (Johnstone *et al.*, 1987), although the same name had been used 6 years earlier in reference, however, to some isolated cell membrane fragments in body fluids (Trams *et al.*, 1981) which, strictly speaking, had nothing to do with exosomes.

It should be noted that the Johnstone and Stahl research groups were independent, and that both were conducting experiments on the formation and internalisation of transferrin receptors in mature reticulocytes.

The discovery of the production of tiny nanoparticles (thereafter termed exosomes) expelled into the extracellular space by reticulocytes (young erythrocytes) is to be considered completely unexpected and purely fortuitous.

– Although the paternity (or rather: maternity) of the discovery of **exosomes (EXO)** is still debated today (Harding *et al.*, 2013), it is generally attributed to Johnstone, for two reasons; her publication pre-dates that of Harding *et al.*, even if only by a few days, and the term “*exosome*” was revived by the brilliant Canadian researcher in reference to what she had

discovered 4 years earlier.

- Rose M. Johnstone was the first and only woman to hold the Chair of Biochemistry in the Faculty of Medicine at the McGill University of Montreal-Canada.

She is also remembered as a highly active promoter of women’s rights in the academic and scientific world, a commitment she maintained with continuity and determination even after retirement, until her death at the age of 81 years.

– As mentioned above, EXOs were initially considered as by-products of cellular metabolism and their role went unrecognised: that they had a significant impact even on the cells close to the mother cell.

In fact, from 1983 (the year of the discovery) to 2000, the number of publications on EXOs was extremely small; only in the early years of the new millennium, about 20 years **after** their discovery, did researchers focus their attention on EXOs and interest literally exploded.

- There are now approximately 8500 publications, 2 dedicated scientific societies (American Society for Exosomes and Microvesicles and International Society for Extracellular Vesicles), a specialised journal [Journal of Extracellular Vesicles (impact factor 2019: 14.976)], 2 databases (Exocarta and Vesiclepedia), and one research portal (exRNA).

EXOSOMES

– THE MYSTERIOUS MESSENGERS

EXOs are tiny microvesicles (nanoparticles) that tend to be rounded or oval shaped, with a **diameter of 50-100 nm*** generated by the membrane of the intracellular multivesicular

* **1 nanometre (nm)** = 1×10^{-9} m = 1 billionth of a metre = 10 ångström.
In the past the nanometer was called a millimicron (1/1000 micron).

On average, an exosome is 1000 times smaller than the cell that generates it.

– One million exosomes could fit onto the tip of a pencil.

bodies (see page 33, [FIG. B](#) and [FIG. 3](#)) through the process of expulsion into the extracellular space, genuine shuttles carrying a load of proteins (Simpson *et al.*, 2009), lipids (Vidal *et al.*, 1989), and nucleic acids (Valadi *et al.*, 2007; Waldenstrom *et al.*, 2012) able to “deliver” their contents to the recipient cells they encounter, and reprogramme them.

EXOs are, and represent, a new method of **intercellular communication** which plays a key role in many cellular processes, for example the immune response, the first to be identified (Raposo *et al.*, 1996; Greening *et al.*, 2015), signal transduction (Gangoda *et al.*, 2015), antigen presentation (Mittelbrunn *et al.*, 2011) and many others (Fernandes *et al.*, 2020) ([FIG. 1](#)).

– EXOs can be released by virtually **all** eukaryotic cells (Ruivo *et al.*, 2017), cells with a defined nucleus, isolated from the remaining the cytoplasm by a membrane.

Recently, EXOs have also been photographed after their expulsion from prokaryotic cells (Kalluri & LeBleu, 2020).

- EXOs are generated by all **animal** and **plant cells**; by varying their load according to the type of cell from which they are derived, EXOs can provide – among other things – valuable prognostic information in a very wide range of diseases, such as: **1**) chronic inflammation (Lesser *et al.*, 2016; Hessvik & Llorente, 2018); **2**) diseases of the lipid metabolism (Record *et al.*, 2014); **3**) cardiovascular diseases (Kishore *et al.*, 2016); **4**) liver diseases (Sung *et al.*, 2018); **5**) kidney diseases (Gonzalez-Calero *et al.*, 2014); **6**) neurodegenerative diseases (Howitt & Hill, 2016); **7**) cancer (Salem *et al.*, 2016), and others.

EXOs have been isolated from various human body “fluids”, for example blood (Caby *et al.*, 2005), urine (Pisitkun *et al.*,

2004), saliva (Ogawa *et al.*, 2011), sweat (Wu *et al.*, 2018), sperm (Ronquist & Brody, 1985; Park *et al.*, 2011; Aalberts *et al.*, 2012), milk (Admyre *et al.*, 2007), amniotic fluid (Asea *et al.*, 2008), cerebrospinal fluid (Vella *et al.*, 2008), ascitic fluid (Andre *et al.*, 2002), and bile (Masyuk *et al.*, 2010).

– These EXOs, with specific genomic, protein and lipid profiles, reflect the cellular origin as “fingerprints” or “signatures” of the cells that generated them and, as a result, have great potential use as biomarkers of many diseases (see below).

Although biological fluids are relatively simple to obtain (“liquid” biopsies) and rich in EXOs, these have not yet been used as biomarkers in current clinical practice (Zhang *et al.*, 2019).

It is only a matter of a few years, since dedicated studies are developing exponentially all over the world. Having one or more biomarkers of a disease before it becomes clinically evident means directing its course and prognosis.

EXOs derived from neoplastic cells contain RNA that can be incorporated into healthy cells, thus promoting the spread of the primary tumour (Skog *et al.*, 2008).

Although most research has been carried out on cells of mammalian species, some have shown that strains of *Leishmania* and *Mycobacterium* are also able to generate EXOs and thus regulate host defence (Silvermann *et al.*, 2010).

– In a similar way, this “pathway” enables the dissemination of molecules used by pathogens in an attempt to subvert the host's immune response and enable infections/infestations to persist or make them difficult to resolve.

In humans, as in parasites, the proteins contained in the exosomal cargo and transported some distance away remain intact and maintain their catalytic activity like the native

	EXOSOMES	MICROVESICOLES	ECTOSOMES	MEMBRANE PARTICLES	EXOSOME-LIKE VESICLES	APOPTOTIC VESICLES
ORIGIN	ENDOSOMES	PLASMA MEMBRANE	PLASMA MEMBRANE	PLASMA MEMBRANE	INTERNAL COMPARTMENTS	ND
DIMENSIONS	50-100 nm	100-1000 nm	50-200 nm	50-80 nm	20-50 nm	50-500 nm
SEDIMENTATION	100,000 g	10,000 g	160,000-200,000 g	100,000-200,000 g	175,000 g	1,200 g, 10,000 g or 16,000 g
LIPIDIC COMPOSITION (partial)	CHOLESTEROL SPHINGOMYELIN CERAMIDES EXPOSED PHOSPHATIDYLSERINE	EXPOSED PHOSPHATIDYLSERINE	CHOLESTEROL DIACYL-GLYCEROL EXPOSED PHOSPHATIDYLSERINE	ND	DO NOT CONTAIN LIPIDS	ND
MAIN PROTEIN MARKERS	TETRASPANINS (CD63, CD9) ALIX TSG 101	INTEGRINS, SELECTINS	PROTEOLYTIC ENZYMES NO CD63	NO CD63	TNF RECEPTOR (TNF R1)	HISTONES

TAB. 1

Physical and chemical characteristics of different types of vesicles discovered in the extracellular milieu.

– Apoptotic vesicles are only found in the extracellular milieu following apoptosis of a cell; ND = not defined.

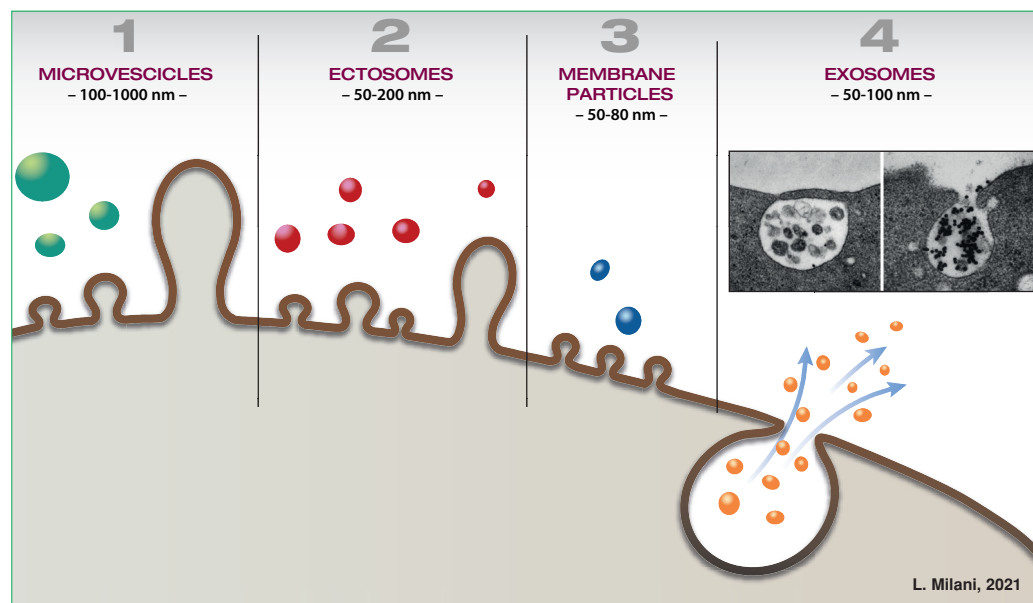
– Adapted from Javalet, 2016, adapted from Théry, 2009 (see References).

FIG. 2

Microvesicles (1), ectosomes (2), and membrane particles (3) result from a budding (exvagination) of the cell membrane and mainly contain the cytosol of the cell that generated them.

Exosomes (4) are – instead – released directly into the extracellular space thanks to an invagination of the cell membrane, passing – as a result – through a gap in the cell membrane which, subsequently, closes again.

– Each exosome, like 1, 2, and 3, has a double membrane.



ones (they are the same!), modifying the physiology and fate of the recipient cells (Colombo *et al.*, 2013; Kalra *et al.*, 2016).

• This intercellular communication physiologically enables the harmonious coordination of an organism's cellular functions as a whole, and thus promotes broader functional homeostasis as per Cannon (Cannon, 1929) and the allostatic state as per Sterling & Eyer (Sterling & Eyer, 1988).

BIOGENESIS OF EXOSOMES

To clarify the terminology and correct a few mistranslations or dubious interpretations, it is important to specify that cells secrete different types of extracellular vesicles: the first 3 are formed from a **budding** of the plasma membrane (**microvesicles**, **ectosomes**, and **membrane particles**) (TAB. 1; FIG. 2).

• On the other hand, EXOs have an **intracellular origin** and are released into the extracellular space by fusion of a mature multivesicular body with the cell membrane which, after expelling the EXOs, closes.

It never requires a continuous solution between the cytoplasm and extracellular "fluid".

There are also some recent descriptions of so-called EXO-like structures (see TAB. 1) of intracellular origin, whose function has still not been clearly defined (Fernades *et al.*, 2020; Lu & Huang, 2020).

– Briefly and chronologically, the formation of EXOs basically takes place in **4 stages** (FIG. 3):

• **Stage 1:** formation per **internal budding** of the cellular membrane of a small endocytic vesicle.

• **Stage 2:** enlargement of the endocytic vesicle, forming an **endosome** that generates a few small intraluminal vesicles (ILV) inside it.

• **Stage 3:** formation of a multivesicular body (MVB).

MVBs are therefore formed during the maturation of the endosome (see Stage 2), a process through which the ILVs (see Stage 2) are generated by invagination of the bordering membrane. MVBs had already been observed precisely 20 years before the discovery of EXOs (Fuchs, 1963; Policard *et al.*, 1963) but, again, no biological significance had been attached to them.

– At this point, the MBVs can meet 2 fates: either they **3a)** merge with a lysosome, a cell organelle containing lytic enzymes that can break down organic components (digestive system of the cell) or they **3b)** migrate towards the cell membrane. The proteins and/or lipoprotein complexes necessary to the transport of MVBs inside the cell have recently been identified (Hurley & Hanson, 2010; Hanson *et al.*, 2012; Henne *et al.*, 2012).

• **Stage 4:** fusion of the MVBs with the cell membrane and release of ILVs into the extracellular space; from this point on, the ILVs are defined as EXOs.

From Endosome to EXOs, progressive invaginations of the plasma membrane, forming more and more, and smaller and smaller elements, are performed by the so-called ESCRT (Endosomal Sorting Complex Requiring for Transport) machinery, which is made up of 4 multiprotein complexes (0-I-II-III) (FIG. 4).

Intracellular Calcium is the true regulator of the fusion of MVB to the cell membrane, leading to the expulsion of EXOs (Savina *et al.*, 2003; 2005). This role is aided by the ALIX protein (Baietti *et al.*, 2012), whose three-dimensional structure has been determined (Fisher *et al.*, 2007; Larios *et al.*, 2020).

– This dynamic model, whose main points were formulated and published by Harding, Heuser & Stahl in 1983, has withstood the test of time, as claimed by one of the 3 authors, Philip Stahl “a distinguished plenary speaker” who had coordinated the experiment at the time, in a 2014 Congress organised by ISEV (International Society for Extracellular Vesicles) in Rotterdam The Netherlands and, more recently, confirmed in one of his publications (Stahl & Raposo, 2019).

EXOSOMES – THE CARGO

At each stage, from endocytic vesicle to EXO, selected quantities of various types of RNA, proteins and lipids are loaded into it.

Approximately 4400 proteins, 200 lipids, 1700 types of messenger RNA (**mRNA**) and 800 microRNA (**miRNA**) have so far been identified in the various EXOs examined (Mathivanan *et al.*, 2012; Kim *et al.*, 2013).

– Of particular interest is the role played by RNAs, powerful gene regulators. Indeed, RNA contained in EXOs **can modify the gene expression of the recipient cells** (Valadi *et al.*, 2007; Nolte-’t Hoen *et al.*, 2012).

The various types of RNA are not coding inside EXOs, but they certainly interfere with the RNA of the recipient cells. This is how tumours metastasise remotely (Minciacchi *et al.*,

2015). This is how EXOs produced by the healthy cells of a specific organ regenerate diseased cells of that organ in a patient (**exosomal regenerative therapy**).

EXOs basically contain 5 types of miRNA:

1) MicroRNAs (miRNA) - which consists of 20-22 nucleotides. These miRNAs have been proposed as non-invasive biomarkers, for example of cholangiocarcinoma (Puik *et al.*, 2017), small cell lung cancer (Hu *et al.*, 2020) and non-small cell lung cancer (Grimolizzi *et al.*, 2017).

miRNAs represent 76% of all map readings; they are transferred to the recipient cells, resulting in a transient or persistent phenotypic modification (Mittelbrunn *et al.*, 2011), and genotypic modification through RNA-dependent DNA polymerase = reverse transcriptase = retrotranscriptase.

2) lncRNA - consisting of 200 nucleotides involved in the regulation of cell differentiation, the cell cycle and epigenetics (Hu *et al.*, 2015), angiogenesis, metastasis, and neoplastic growth (Hewson & Morris, 2016).

3) CirRNA (circular RNA), a biomarker with diagnostic, prognostic, predictive properties.

4) Messenger RNA (mRNA).

5) other types of RNA (long non-coding RNA; piwi interacting RNA, transfer RNA, small nuclear RNA, small nucleolar RNA).

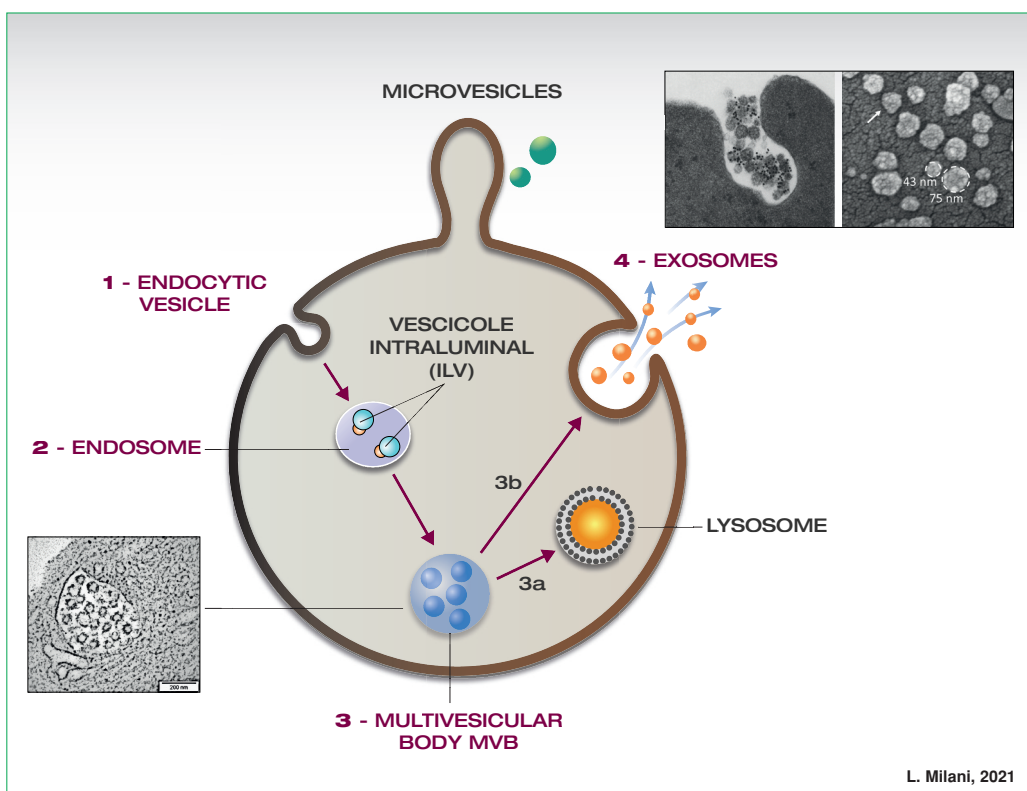


FIG. 3

Generation of exosomes and microvesicles.

– Multivesicular bodies (MVB) (3) are formed during the maturation of endosomes (2), a process through which the intraluminal vesicles (ILV) are generated by invagination of the bordering membrane of the endosome. MVBs can fuse with lysosomes (3a) in order to break down their contents, or (3b) fuse with the cell membrane, releasing ILVs which, once they have crossed the limit imposed by the cell membrane, take the name of exosomes.

– Exosomes are miniaturised “fingerprints” of the cells that generated them.

L. Milani, 2021

– In addition to RNA, EXOs can also contain fractionated DNA (Sharma & Johnson, 2019; Spada *et al.*, 2020).

In addition to RNA, EXOs contain proteins such as the **4 tetraspanins** involved in the penetration of EXOs into recipient cells and fusion events, the **Heat Shock Proteins (HSPs)** involved in presentation, antigen binding and the formation of MVB (release of EXO), and **Cell Adhesion Proteins**.

The EXO cargo is also rich in lipids such as cholesterol, sphingomyeline, ceramides, phosphatidyl-serine, and arachidonic and phosphatidic acids.

– The typical composition of an EXO is shown in **FIG. 5**.

INTERCELLULAR COMMUNICATION

Cells normally communicate with neighbouring cells through direct contact (gap junctions, surface proteins), or remotely (hormones, chemokines, cytokines), or via electrical and chemical signals (nucleotides, short-chain peptides and lipids).

– EXOs have completely different, peculiar ways of communicating: from parent cell to recipient cells they penetrate directly into them, near or far. Once they have penetrated the recipient cells, they stimulate them using ligands located on

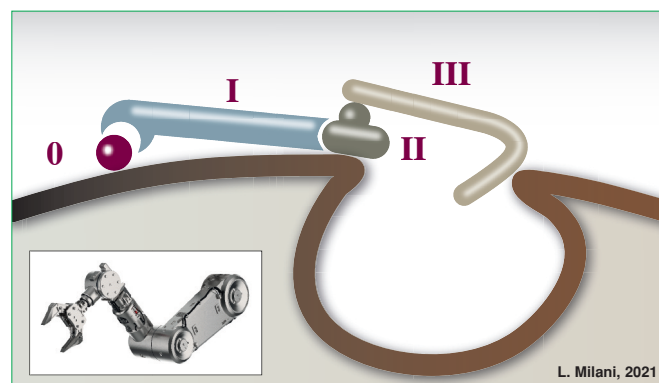


FIG. 4

Schematic graphics of the ESCRT (Endosomal Sorting Complex Requiring for Transport) machinery, made up of IV protein subunits in line (0, I, II, III), which “manufactures” the invaginations and “welds” the membranes from endosome to EXO.

Like a robotic arm, ESCRT-I pulls ESCRT-0 towards itself by “curling” the plasma membrane, while ESCRT-II pushes it down. When an endovesicle has formed, ESCRT-III approaches the 2 lipoprotein neck flaps of the parent membrane.

• **ESCRT is an ancient phylogenetic mechanism. It also found in the Archaea that appeared on Earth 3.5 billion years ago, the prokaryotes that can survive in extreme environmental conditions.**

Nature loves patterns and repeats them.

– Adapted from Raiborg & Stenmark, 2009 (see References).

their surface, transfer the activated receptors, and **epigenetically reprogramme** them by delivering their cargo.

A true... home delivery.

– This unique, emerging behaviour in Biology and Medicine accounts for, for example, their enormous importance in immunology, such as activation, suppression, immune tolerance, and antigen presentation (Raposo *et al.*, 1996; Zitvogel *et al.*, 1998; Clayton *et al.*, 2011; Zhang *et al.*, 2011; Smyth *et al.*, 2013; Okoye *et al.*, 2014); EXOs derived from mesenchymal stem cells have shown enormous potential in reducing the extent of myocardial infarction (Lai *et al.*, 2010; Liu *et al.*, 2017), in renal infarction (van Koppen *et al.*, 2012), in the healing of wounds (Zhang *et al.*, 2015), in liver disease (Tan *et al.*, 2014; Jiang *et al.*, 2018), in the protection of neurons (Xin *et al.*, 2012), in the reduction of retinal damage caused by laser surgery (Yu *et al.*, 2016), in addition to the control of viral pathogenicity, and the treatment of premature ageing, to name the just most striking examples.

EXOSOMES AND LIVER DISEASES

The therapeutic efficacy of EXOs from mesenchymal stromal stem cells (MSCs) and hepatocytes has been studied in animal models *in vitro* and *in vivo*.

Below is an updated and exhaustive list of the most significant bibliographic references, in ascending chronological order:

1. **HEPATIC FIBROSIS** - Masyuk *et al.*, 2013; Charrier *et al.*, 2014; Hyun *et al.*, 2015; Chen *et al.*, 2016; Nojima *et al.*, 2016; Lou *et al.*, 2017; Shen *et al.*, 2017; Qu *et al.*, 2017; Chen *et al.*, 2018 a; Mardpour *et al.*, 2019; Povero *et al.*, 2019; Rong *et al.*, 2019; Fiore *et al.*, 2020; Bruno *et al.*, 2020 a;b.
2. **HEPATIC CARCINOMA** - Kogure *et al.*, 2011; Fonsato *et al.*, 2012; Sun *et al.*, 2018; Li *et al.*, 2020.
3. **HEPATITIS, DRUG-INDUCED HEPATOTOXICITY** - Li *et al.*, 2013; Lemoine *et al.*, 2014; Tan *et al.*, 2014; Abbas & Hamideh, 2014; Brigstock, 2015; Cai *et al.*, 2017; Chen *et al.*, 2018 b.
4. **NON-ALCOHOLIC STEATOHEPATITIS (NASH)** - Bruno *et al.*, 2020 b.
5. **LIVER REGENERATION AFTER PARTIAL HEPATECTOMY** - Herrera *et al.*, 2010.
6. **BIOMARKER OF LIVER DISEASES** - Wang *et al.*, 2017; Barile & Vassalli, 2017.

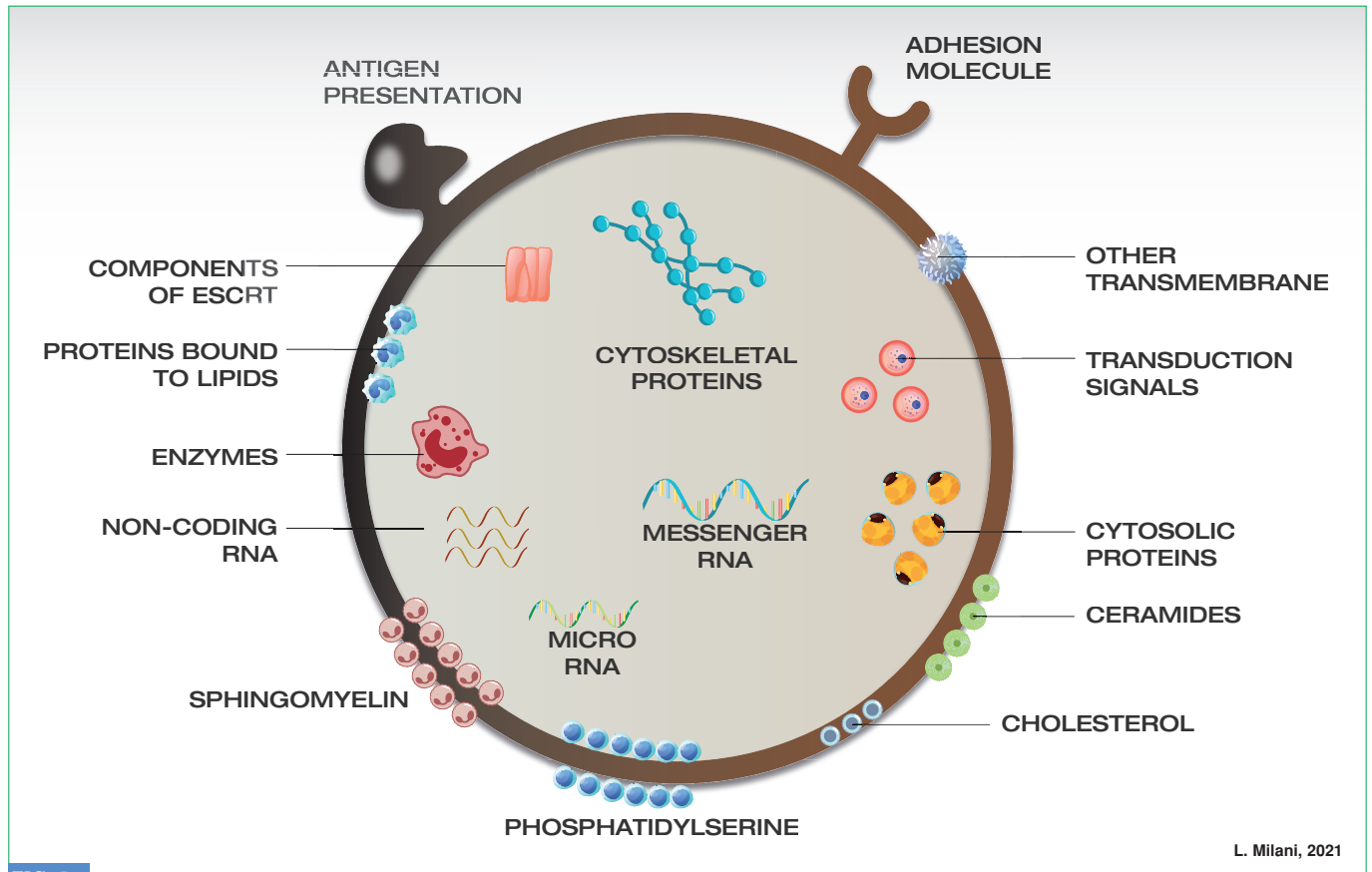


FIG. 5

L. Milani, 2021

Typical composition of an exosome. The cargo.

These data from the most recent scientific literature emphasise the major therapeutic impact offered by EXOs in the treatment of liver diseases, their antidegenerative, antispasmodic effects, and their potential use as biomarkers of liver diseases.

► In this context, we should mention **Epatoguna** (Guna Laboratories - Milan, Italy), a food supplement based on hepatic lyophilisate processed intact from healthy young swine according to the Neorland® method, choline and green tea.

– The hepatic lyophilisate in Epatoguna is a source of well-preserved exosomes that are biologically active on human stem cells, as can be clearly seen in the results of the experiment conducted by the research group coordinated by Prof. C. Ventura – CNR, Bologna, Italy (Ferroni *et al.*, 2019).

– I quote the authors (from the discussion in the article):
1) “*Neorland® swine’s liver contains exosomes; 2) these are internalised by human cells and are biologically active*”.
Neorland® hepatic lyophilisate is found exclusively in Epatoguna.

– **Choline** is a lipotropic substance that prevents the deposition of lipid in the liver parenchyma.
Diets with a low choline content favour NAFLD (Non-Alcoholic Fatty Liver Disease), the current, more correct definition

of the “old” hepatic steatosis.

The nutraceutical intake of choline boosts the synthesis of very low density lipoproteins (VLDL), thus facilitating the transport of triglycerides outside the cell and avoiding their accumulation in the cytoplasm of the hepatocytes (Lombardi *et al.*, 1968; Yasuda *et al.*, 2020).

– **Green tea** [(*Camellia sinensis* (L.) Kuntze] (titrated at 95% in epigallocatechin-3-gallate) protects the liver parenchyma and reduces inflammatory markers; it lowers hepatic triglycerides (Xu *et al.*, 2020), ALT (Yu *et al.*, 2017), and regulates insulin resistance (Pham *et al.*, 2014).

THE BOTTLE ON THE BEACH

The discovery that lymphocytes also have receptors for hypothalamic hormones (CRF, GHRH, LHRH), hormones (cortisol, prolactin,



GH, melatonin), neuroendocrine peptides (enkephalins, β -endorphin, somatostatin, NGF, ACTH, substance P, CGRP), neurotransmitters (adrenaline, noradrenaline, acetylcholine, dopamine) which, in turn, release neuroendocrine peptides, and that immune function is also affected by some hormones, neurotransmitters and other messengers (in Milani, 2018) strongly suggests that there is a powerful **information exchange** between P, N, E and I.

Each one of the 4 “performers” in the PNEI superSystem is **only** effective if it is interconnected at the same time as the other 3, thus ensuring “true” psycho-organic homeostasis. None other.

– Each of these 4 “performers” acts remotely through electrochemical mechanisms, synapses, ligands, etc. or modifications of its own cells or those of the recipient, not necessarily target cells.

– Everything in Nature is information.
The Whole itself is information, in formation.

Networks and networks of networks are the guarantors of alignment of the expressions of Life.
They oversee the domains of coherence.

– A single hypo/hyper imbalance between them alerts the entire individual in a compensatory attempt to restart the jammed, highly complicated clock.

• A 5th has recently been added to these 4 Systems: the exosomal/microvesicular system which, through short and long range intercellular communication, transfers microRNA and messenger RNA that affect the recipient cells along their own “journey”, carried in bodily fluids, reaching their destination as... beached messenger bottles - Neptune’s treasure.

– Studies on EXOs have literally exploded exponentially in the last 5 years, opening up new, unexpected, fascinating opportunities in early diagnostics (biomarkers) (Simpson *et al.*, 2009; Heubner *et al.*, 2015; Fais *et al.*, 2016) and in therapy as drug delivery systems (El Andaloussi *et al.*, 2013; Johnsen *et al.*, 2014; Vader *et al.*, 2016; Batrakova *et al.*, 2016; Ingato *et al.*, 2016) or as EXOs processed from healthy **animal** or **plant tissue** that are biocompatible with human tissue.

– The therapeutic effect of stem cells is most likely mediated by the release of the EXOs they produce: these have the ability to recapitulate the effect of stem cells and – consequently – bring further life to their practical applications... regenerative medicine, the dream – now more and more tangible – of every doctor and every patient. ■

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The editorial team would like to thank the editors of the websites that supplied the following images:

Fig. p. 33 (left)

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Fig. 2 (photo insert)

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Fig. 3 (photo insert bottom left)

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Fig. p. 39

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author

Dr. Orlando Ferroni

- Veterinary doctor
 - Researcher in Tissue biology
- Cremona, Italy



*R. Tassinari,
C. Cavallini, V. Taglioli,
E. Olivi, C. Ventura*

SUMMARY

Chronic Liver Diseases are a global health problem, accounting for 2 million deaths per year. In this context, many efforts have been made to identify new treatments able to restore, but in particular to preserve, liver function in the lifetime. Epatoguna is a food supplement, rich in choline and green tea extract, mainly composed of swine-lyophilized liver Neorland®. It's well known that liver contains a great amount of micro- and nano-vesicles, exosomes, arising from all the cell types contained in the organ.

– In this paper, we present our preliminary results regarding exosome isolation from lyophilized liver and their biological action on human stem cells. Exosomes from Neorland® lyophilized liver were immune-separated and quantified with ELISA. After that, human mesenchymal stem cells were treated with isolated exosomes. Human stem cells were able to internalize swine exosomes, as highlighted by BODIPY staining, and moreover exosome-treated cells displayed a cell cycle arrest, not later than 24 hours after exosome administration. These preliminary data pave the way for new experiments, aimed at a comprehensive understanding of the role played by swine-lyophilized liver-derived exosomes in preserving a healthy liver in humans.

KEY WORDS

EPATOGUNA, LYOPHILIZED SWINE LIVER-DERIVED EXOSOMES, HUMAN MESENCHYMAL STEM CELLS, UPTAKE

EPATOGUNA – SWINE-LYOPHILIZED LIVER NEORLAND® IS THE SOURCE OF WELL-PRESERVED EXOSOMES, BIOLOGICALLY ACTIVE ON HUMAN STEM CELLS

INTRODUCTION

Chronic liver diseases (CLDs) constitute a global health problem.

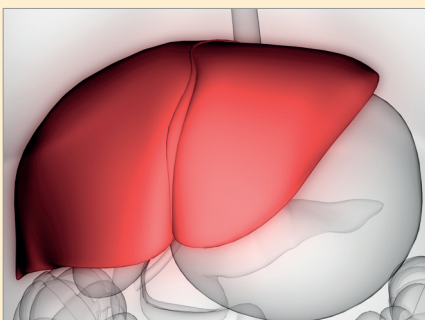
– Despite considerable advances in their understanding and management, millions of people still suffer from chronic diseases associated with this vital organ.

It is currently estimated (and probably underestimated) that **844 million** people are carriers of CLDs, with a mortality of 2 million patients per year (1).

– These diseases present a worldwide clinical problem, comparable with that of other chronic diseases such as dia-

betes (422 million patients; 1.6 million deaths per year), lung diseases (650 million patients; 6.17 million deaths per year) and cardiovascular diseases (540 million patients; 17.7 million deaths per year) (2).

– The liver reflects an individual's state of health; the progression of a pathological state towards a chronic condition is determined by a combination of different factors [infections (HBV and HCV), alcohol abuse, genetic factors, etc.]. CLDs are not often recognised at an early stage, because the symptoms are initially scarce and generic; even abnormalities that can be detected by biological examinations are limited (2).



It is therefore essential to implement prevention programmes, and identify therapeutic strategies that prevent the progression of liver diseases.

– **Epatoguna** is a food supplement based on **lyophilized swine liver, choline** and **green tea**.

Neorland® lyophilized swine liver is obtained through a controlled lyophilization process specially designed to increase bioavailability and prevent the possible denaturation of the active ingredients, obtained from the fresh intact liver of young swine.

– This special procedure ensures the bioavailability of specific natural nutrients, for example the complete **vitamin pool** (especially group B vitamins), **minerals** (e.g. Iron) and essential **amino acids**, which help support and regulate hepatic metabolic processes.

It has also been hypothesised that the liver has a significant micro/nanovesicle content, notably **exosomes**, which are derived from all components of the animal's liver, including particularly numerous stem cells in young swine.

• Out of all the components of Epatoguna, we decided to study the microvesicle content, particularly exosomes, and their action in *in vitro* models.

– The cells that make up an organism are not entities in their own right; they must be able to both *perceive* and *relate* to the surrounding environment and pass “information” to the outside. There are many different mechanisms by which cells exchange information, and they vary according to the type of cell, but the secretion of signalling molecules, which make up the so-called cellular “secretome”, is common to all cells.

The cellular “secretome” is made up of “naked” molecules (cytokines, chemokines, growth factors, etc.), and various types of extracellular vesicles (EVs), such as microvesicles, microparticles, nanovesicles, and exosomes.

Eukaryotic cells contain compartments surrounded by membranes, called endosomes, which have various functions.

One particular subclass of these, called the Multivesicular Bodies (MVBs), in turn contains vesicles with membranes. These can undergo various fates, including that of being released into the extracellular space, hence the name of **exosomes**.

Exosomes are a distinct population of extracellular vesicles with a diameter ranging between 30 nm and 150 nm. They have specific features (3) and specific endosomal proteins on the membrane, including CD9, CD63, CD81 and others, not found on other types of secreted vesicles which, by contrast, are formed by evagination from the plasma membrane (4).

Although the exosomes have been extensively studied, the exact mechanism by which they are secreted and the regulation of this process are still not well-defined (5).

Exosomes have been isolated *in vivo* from various biological fluids, e.g., blood, and *in vitro* from various types of cultured cells.

Exosomes mediate interaction between cells through nucleic acids (coding and non-coding RNA) and the proteins they contain, which can be transferred to neighbouring cells by endocytosis processes mediated by membrane antigens (6-9).

The mechanism by which exosomes are internalised by the recipient cells has as yet to be clarified.

This is because extracellular vesicles can be incorporated by various mechanisms, including phagocytosis, endocytosis, pinocytosis and fusion of the plasma membrane.

As mentioned, exosomes are secreted by cells specifically to mediate intercellular communication, since they are able to transfer various types of molecules to recipient cells, including proteins and different types of coding and non-coding RNA.

– This enables them to play a fundamental role in the **regulation** of both **physiological** and **pathological processes**, modifying the regulatory networks of the cell (10).

Exosomes are secreted by various types of cells and tissues. One part of the molecules they contain is always the same and is essential to the structure and sorting of the vesicles; however, they are partially differentiated in relation to cell-specific proteins and RNA, which probably reflect both their own biological function and that of the cell from which they originated (11).

The function of most of the proteins contained in exosomes is not well understood; however, many studies show that they clearly differ from those found in all other microvesicles secreted by cells (12).

Nucleic acids (mRNA and miRNA) are not active within the exosome, but become active when internalised by the recipient cell.

The study of exosomal miRNA has revealed a remarkable **specificity** according to the **cell from which it originates**, and has highlighted major differences, for example between different types of cancer cells (13,14).

– This pronounced variability of content suggests a selective process for the internalisation of miRNA, mediated by the cell that generates the exosome.

Many studies have investigated the mechanisms by which exosomes, within the same species, enter the recipient cells, release their contents (cargo) and mediate physiological and pathological processes.

On the other hand, the way in which they facilitate communication between cells of different species has not been well-researched (15), although it is known, for example, that approximately **80%** of the proteins found in the exosomes of mice and humans are conserved (homologous) between the two species (16).

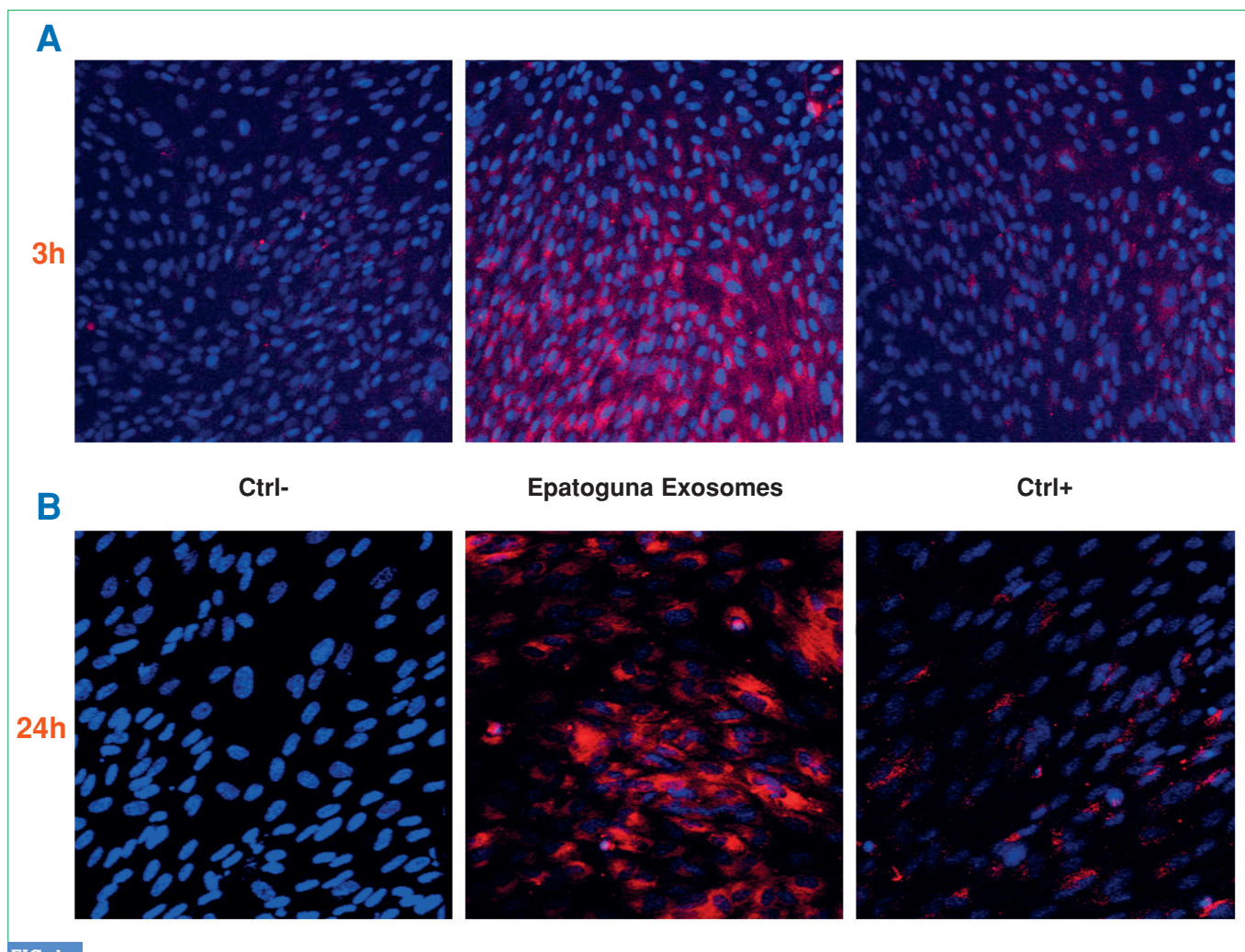


FIG. 1

In vivo study of exosome uptake.

– Photomicrographs of human mesenchymal cells isolated from foetal membranes obtained from full term deliveries (hFM-MSCo), taken 3 hours (A) and 24 hours (B) after the following treatments: Ctrl- = untreated cells; Epatoguna Exosomes = cells placed in contact with exosomes stained with BODIPY; Ctrl+ = positive control consisting of cells placed in contact with BODIPY dye only.

The images were acquired using a Nikon Ti-E microscope and analysed with Nis (Nikon) software.

– Mouse exosomes can be transferred into human mast cells, and the RNA within them is translated into murine proteins in the recipient cell (17). Some studies have also been conducted on exosomes found in milk (18).

There is some evidence that the exosomal cargo of bovine milk, particularly the nucleic acids (RNA), is transported to circulating immune cells.

– Some types of miRNA and mRNA appear to be able to regulate the expression of human genes or be translated into biologically active proteins, respectively (19).

► The aim of the work we have carried out is to investigate the presence of ex-

osomes in Neorland® lyophilized swine liver and, if they are found, to determine whether these nanovesicles could be biologically active on human cells, particularly stem cells.

MATERIALS AND METHODS

One milligramme of lyophilized swine liver is resuspended in Ringer-lactate buffer (Krebs-Ringer Solution, Alfa Aesar) for 1 hour, and kept under constant agitation.

Collagenase 1A (Sigma-Aldrich) at a concentration of 0.5 mg/ml is then added, and the mixture is kept at 37°C for a further hour, with constant stirring.

At the end of this time, scalar filtrations are carried out, using a cell-grinder (Sigma-Aldrich) and cell strainer (Falcon). A further 3 filtrations are carried out using syringe filters, until a pore size of 0.2 µm is reached.

– The eluate is added to Exo-Quick-TC (System Biosciences, LLC) and stored overnight at 4°C.

The following morning, the preparation containing the exosomes is centrifuged at 3000 x g for 15 minutes, according to the protocol, and the pellet is resuspended in PBS buffer.

The sample is treated with Rab5b Exo-Flow Capture Kit (System Biosciences, LLC), according to the protocol.

Magnetic beads carrying the Rab5b ex-

osomal antibody are then added to the sample.

Using a magnetic support, the beads with the bound exosomes are held still while the sample is subjected to successive washings.

On completion of the process, the exosomes are released from the beads and eluted in 200 µl of the elution buffer provided in the kit.

Quantification is performed using an ELISA kit (Porcine CD81 antigen ELISA Kit, MyBioSource).

The uptake of porcine exosomes in human cells is investigated using **Mesenchymal Stem Cells** isolated from foetal membranes (FM-MSCs), by viewing them under a fluorescence microscope (Nikon Ti-E).

The exosomes are stained with BODIPY

1 TR ceramide (Molecular Probes™, Life Technologies, Carlsbad, CA, USA), according to the protocol, and purified from the excess dye by means of columns (Exosome Spin Columns, MW 3,000; Invitrogen, Life Technologies). The exosomes stained with BODIPY are added to the culture medium.

The cells are identified by vital staining of the nuclei using NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher). The proliferation is assessed using the MTT method (Thiazolyl Blue Tetrazolium Bromide, Sigma Aldrich).

The cells are added with MTT, left to incubate at 37°C for 2 hours, the dye solubilised in a lysis buffer, and the absorbance determined using a spectrophotometer (MultiskanGO, Thermo Fisher) at 570nm.

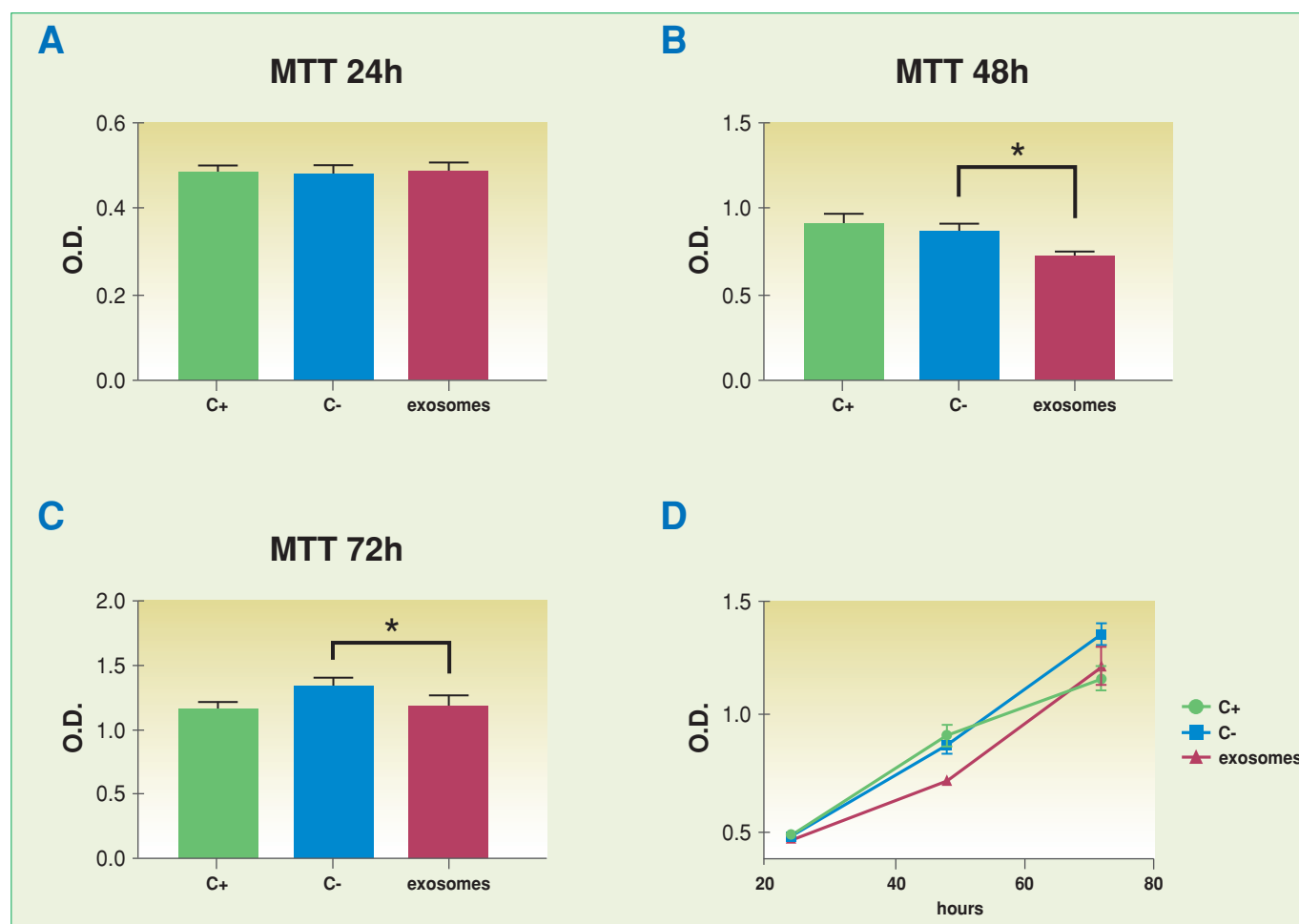
A statistical analysis is carried out using the one-way ANOVA method, with Tukey correction.

$p < 0.05$ data are considered significant.

RESULTS

The lyophilized swine liver preparation is resuspended in a neutral buffer, Krebs-Ringer Solution, and subsequently digested with collagenase IA in order to release the nanovesicles trapped in the collagen, a protein that occurs extensively in the liver.

Since exosomes are nanovesicles with a diameter of a few nanometres (30 to 150 nm), the digestate undergoes several filtration steps, initially using large-pore filters to remove all coarse compo-



TAB. 1

Cell growth analysis.

A-B-C – On the abscissa axis: C+ (positive control) = cells cultured in standard FBS; C- (negative control) = cells cultured in exosome-free FBS; exosomes = cells cultured in exosome-free FBS with the addition of exosomes derived from swine liver.

On the ordinate axis: absorbance (O.D. – Optical Density) measured at 24hrs (A), 48hrs (B) and 72hrs (C). * = significant differences where $p < 0.05$.

D: XY representation of values shown in A, B, C.

nents, then increasingly selective filters, thus enriching the solution in nanovesicles as much as possible.

– Exosomes can be isolated by various methods, for example ultracentrifugation, microfluidics, and many others. For the purpose of this work, we made use of the ability of some molecules to polymerise, such as polyethylene glycol (PEG), embedding the microparticles present in the solution to make them heavier.

The polymer is added to the solution from which the microvesicles are to be isolated, left to incubate for at least one night at 4°C, and centrifuged the following day.

The sediment (pellet) that formed after centrifugation, containing the exosomes, is washed and resuspended in a sterile isotonic buffer (PBS).

– Exosomes are nano-vesicles that present specific proteins on the membrane that surrounds them (including, for example, CD9, CD81, HSP70, etc.).

The purification method we use is based on magnetic beads, which bind antibodies directed towards one of these proteins, specifically Rab5b.

The beads are placed in contact with the solution that virtually contains exosomes, so that the antibody present on the bead can bind to the exosome to be isolated.

A magnet is used to remove the beads from the solution containing all the other particles co-precipitated by the polymer. Once the beads have undergone several washings, the exosomes are released using the Elution Buffer and remain resuspended in it.

– Once isolated by magnetic separation, the exosomes are quantified in an ELISA assay. The ELISA test is based on specific antigen-antibody recognition. The kit uses an antibody found on exosomes, CD81, an alternative to Rab5. This stratagem, i.e. using two different antibodies to isolate and quantify the exosomes, enables us to **eliminate** the possibility of also isolating free proteins

with the beads, those not bound to the microvesicles.

In fact, if no exosomes were present, the ELISA would be completely negative for CD81, since there be no single CD81 proteins in solution as they would have been lost during the purification process.

Once the presence of nanovesicles was detected, we investigated whether or not they had a potentially biologically active role.

– To determine whether or not the exosomes derived from lyophilized swine liver were recognised and internalised by human cells, we used mesenchymal stem cells (**MSCs**) isolated from a human placenta during a full term birth (kindly donated by Prof. Laura Bonsi and Prof. Francesco Alviano, University of Bologna).

Foetal membrane-derived MSCs (FM-MSCs) are particularly plastic cells and have all the stem cell markers required to define a population as “mesenchymal” (20).

MSCs can also differentiate into many cell types, including osteoblasts, chondroblasts, adipocytes and **hepatocytes** (21).

For the purpose of these experiments, human mesenchymal cells were cultured in exosome-depleted serum (Exosome-depleted FBS one shot, Gibco), in order to avoid confusing them with the swine exosomes administered in culture.

The first matter to be investigated was whether or exosomes isolated from lyophilized swine liver could be **recognised** by human cells and **internalised** by them.

– To test this hypothesis, we used a dye with a high affinity for lipophilic membranes, belonging to a class of fluorescent dyes whose basic structure consists of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene, i.e. the BODIPY 1 TR ceramide, using a published method already practised in our laboratory (22).

More specifically, the exosomes are

stained with BODIPY 1 TR ceramide and, after removing the excess dye, i.e. the dye not bound to the exosomes, thanks to the purifying columns (Exosome Spin Columns, MW 3,000; Invitrogen, Life Technologies), they are added to the culture medium.

The cells are viewed under a microscope after 3 and 24 hours.

– As can be seen in [FIG. 1A](#), after 3 hours the cells (highlighted by the blue colour of the nuclei) treated with the exosomes begin to turn red, and already a significant difference between these and both the negative control (without dye) and positive control (dye only) can be seen.

The positive control is treated with a dye solution, with no exosomes, and undergoes the same purification as the sample with exosomes, through the Exosome Spin Columns. This proves that the staining of the analysed sample is **only** caused by the BODIPY retained by the exosomes, and not by the possible contamination of free dye.

The cells are once again observed 24 hours after the process ([FIG. 1B](#)).

The images show a striking result: the staining of the cells treated with exosomes (stained with BODIPY) is evident, whereas the two controls, positive and negative, appear the same as the previous day.

• The result of this experiment provide evidence that human cells, in this case stem cells, are able to **recognise** and **internalise** exosomes of porcine origin, **completely eluding the problem of species diversity**.

– Having clarified this very important point, we asked ourselves if, in addition to entering the cells, exosomes had any effect on them.

The first test carried out (many others are currently in progress) assessed the impact on cell growth.

The growth of the cells was assessed us-

ing MTT; MTT is a colorimetric assay that relies on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) to formazan, a blue coloured substance.

This test is commonly used to assess the toxicity of substances on the mitochondria.

If the substance to be tested has no direct effect on the mitochondria, then, with some approximation, the number of mitochondria can be correlated to the number of cells.

Based on this assumption, the MTT assay is commonly used in the assessment of cell growth.

As in [TAB. 1A](#), the cells treated with a single dose of exosomes show no significant difference when compared with the positive control (cells kept in non-exosome-depleted FBS) and negative (cells kept in FBS purified of exosomes) after 24 hours.

– By contrast, after 48 hours ([TAB. 1B](#)), the growth of mesenchymal cells treated with exosomes begins to stop, with an effect that continues for up to 72 hours ([TAB. 1C](#)).

As deduced from the graph in [TAB. 1D](#), exosomes have no toxic effect on cells. In fact, the absorbance values (OD) increase steadily, revealing an overall increase in the number of cells in all three samples.

DISCUSSION

The preliminary data presented here provide two very important pieces of information:

1. Neorland® lyophilized swine liver **contains** exosomes; **2.** these are **internalised** by human cells and are **biologically active**.

Recent studies have investigated the effect of lyophilized on the preservation of exosomes, so that they can be biolog-

ically active even after a period of storage.

Exosomes are normally stored at -80°C (23), but there is some evidence that this is not an optimal condition for long-term storage.

This is because after 4 days of storage at this temperature, the exosomes are found to be morphologically different to those freshly isolated (24).

– Even storage at 4°C to 37°C changes the morphology of these vesicles, making them smaller, which possibly has a significant effect on their biological action (25).

Lyophilization is a process commonly used to preserve many types of organic material, from proteins to plasma, as it increases long-term stability (26).

In relation to the storage of exosomes, lyophilization has been found to be a technique of choice. Studies have confirmed that this procedure preserves integrity and functionality (27).

Our preliminary data confirm that exosomes are not destroyed by the Neorland® lyophilization procedure, and remain **intact** and **biologically active**.

The cells used in this study are human mesenchymal cells isolated from full term placenta (20).

Mesenchymal stem cells are adult cells that can be isolated from various tissues, since they are found in almost all organs.

They are defined as such by the presence of some surface markers (CD29, CD44, CD73, CD90, CD105) and the absence of others (CD14, CD34, CD45 and HLA-DR), and also their ability to differentiate into other cell types (osteocytes, chondrocytes, and adipocytes). In addition to their differentiation potential, they also have an intrinsic immunomodulating capacity.

Their function within the organs from which they are obtained is not always

known, but the potential demonstrated *in vitro* makes them particularly interesting, not only because of their use in autologous transplant, but above all the possibility of identifying treatments that could improve the regenerative capacity of the organ to which they belong.

The liver also has mesenchymal stem cells (liver-derived human MSCs LHM-SCs) which possess specific features that differ from those isolated from other organs; for example greater secretion of protective factors (28).

These cells can differentiate into mature hepatocytes; however, the mechanism by which this occurs *in vivo* is still unknown, although it is clear that they are involved in various physiological and pathological processes (29).

– In this context, it is particularly interesting that exosomes derived from swine liver are able to terminate the growth of mesenchymal stem cells.

Several studies are in progress in our laboratory to investigate this fact.

– Firstly, an assessment of the dose-dependence of this action is taking place.

Moreover, we are analysing the changes in gene and protein expression of the recipient cells, with a view to understanding whether, in addition to the termination of a cycle, there is a differentiation stimulus. It has already been shown that the addition of exosomes extracted from differentiated cells can change the fate of mesenchymal stem cells, and switch the phenotype from adipocytic to osteocytic and vice versa (30).

Regulation could also occur through the miRNA found in the exosome, as already demonstrated in other models, where it was observed that exosomes derived from hematopoietic cells can facilitate the differentiation of embryonic cells *in vitro* by inhibiting a specific pathway (31). ■

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corresponding author

Dr. ssa Claudia Cavallini

– GUNA ATTRE (Advanced Therapies and Tissue Regeneration), Acceleratori di Innovazione, CNR (National Research Council)

Bologna, Italy



L. Cristaldi

SUMMARY

Chronic Venous Insufficiency (CVI) is due to a state of prolonged venous hypertension in the skin and subcutaneous tissue, predominantly in the lower limbs; the bicuspid venous valves are responsible for directing blood from the superficial veins of the lower limbs to the heart via the deep venous system, from the bottom up, against the force of gravity.

In CVI, these valves are unable to perform this task, so there is a reversal of the flow (reflux) of blood from the Deep Venous System to the Superficial Venous System, which creates a continuous pressure on the walls of the veins that can lead, in an advanced phase of the disease, to the wearing out and dilation of the wall itself (varices) and to the leakage of fluid into the surrounding tissues (oedema).

– The aim of this clinical study is to verify whether the combined treatment with PRM medicines and Guna Collagen Medical Devices can provide more important and lasting clinical results than the treatment with phlebotonic drugs.

KEY WORDS

CVI, MD-MUSCLE, MD-TISSUE, ANTI AGE VEIN, GUNA MATRIX, GUNA LIVER

TREATMENT OF CHRONIC VENOUS INSUFFICIENCY WITH GUNA COLLAGEN MEDICAL DEVICES AND PRM THERAPY

VENOUS DISEASE IN MEDICINE

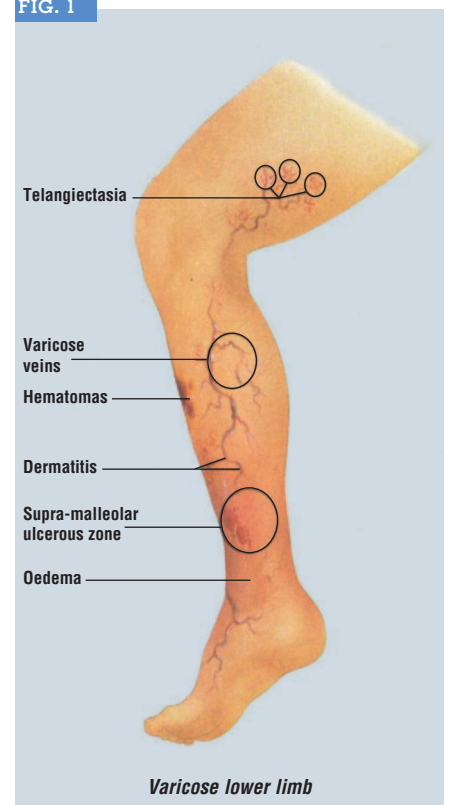
Venous disease is one of the oldest, most widespread medical conditions that affect humans. The first description of the disease appears in the Ebers Papyrus, considered to be the first Treatise on surgery and dating back to approximately 1550 BC (18th dynasty of Egypt).

– The first description of varicose vein surgery is attributed to Aulus Cornelio Celsus (1st century AD).

The only one of his four-part work *Artes* that has survived intact is *De Medicina*, in which the author gives a detailed discussion of Diet, Pharmacological Therapy and Surgery.

– In 1628, William Harvey (1578-1657), following the teachings of his master Gerolamo Fabrici d'Acquapendente (1533-1619), published the volume *Exercitatio Anatomica de Motu*

FIG. 1



Truncal varicosis

– Failure of the great saphenous vein and consequent varicosis of the branch in the anterior region of the leg.

**Reticular varicosis****Telangiectasia**

– Intracutaneous ectasia.



FIG. 2

Cordis et Sanguinis in Animalibus, in which he identified the structure and function of the venous valves and defined the physiological mechanisms of blood circulation.

WHAT IS CVI

Chronic Venous Insufficiency (CVI) is the result of prolonged venous hypertension in the skin and subcutaneous tissue, mainly affecting the lower limbs.

Under normal conditions, the venous bicuspid valves are responsible for directing blood upwards: from the superficial veins of the lower limbs towards the heart via the Deep Venous System, against the force of gravity.

– In CVI, these valves are unable to perform this task, so there is a reversal of the flow (reflux) of blood from the Deep Venous System to the Superficial Venous System, leading to **superficial venous hypertension**.

As a consequence, blood stagnates in the veins of the lower limbs and creates continuous pressure on their vein walls. In the advanced stages of the disease, CVI can lead to vein wall wear out and dilation (varicose veins) and to fluid leakage into the surrounding tissues (oedema), causing endothelial damage. CVI can be subdivided into:

- **organic**, caused by an actual disease of the veins (varicose veins, the re-

sults of venous thrombosis, etc.)

- **functional**, an expression of functional overload in the veins which, although normal, are stimulated to overwork (disorders of the sural pump due to problems with posture, lymphoedema, etc.) (FIG. 1).

CVI – EPIDEMIOLOGY AND RISK FACTORS

The prevalence of CVI is **10-15%** in adult men and **50-55%** in women.

- In the age range that goes up to 50-60 years old, the disease primarily affects females, while beyond this age-group there is no significant difference between men and women.

CVI is more common in individuals of Caucasian ethnicity due to a genetic predisposition associated with various environmental and social factors that lead to, or exacerbate, venous hypertension [(e.g. pregnancy, constipation, oral contraceptives, incorrect footwear, unsuitable clothing, a sedentary lifestyle, obesity, alcohol, smoking) and/or orthopaedic abnormalities (high arches or flat feet, postural disorders of the lumbar spine and/or knee)].

The phenomenon is increasing in highly urbanised and heavily industrialised populations.

CVI is a chronic progressive disease; in addition to the unquestionable aesthetic

implications, it must be considered a true social phenomenon, which causes ever-increasing costs for healthcare facilities.

CVI – CLASSIFICATION AND STAGING

In classifying and staging CVI, reference is made to the acronym **C.E.A.P.**, which was introduced in 1996 and later became established in literature.

– C.E.A.P. stands for components of venous disease: **C** = Clinic: clinical signs (grade 0-6); **E** = Etiology: etiological distinction into congenital, primary, secondary; **A** = Anatomy: vein segments affected, perforating veins, superficial veins, deep veins; **P** = Pathophysiology: pathophysiological dysfunction, reflux, obstruction, reflux, and obstruction.

- The clinical classification is as follows:

C0s: no visible or palpable clinical signs of venous disease

C1: telangiectasia (small intracutaneous reticular varicose veins <1 mm) and reticular (varicose veins hypodermic <3 mm)

C2: varicose veins; subcutaneous >3 mm

C3: oedema; fluid retention

C4a: dystrophic lesions of venous origin (dyschromia, eczema, hypodermatitis)

C4b: dystrophic lesions of venous origin

(hypodermatitis, lipodermatosclerosis, *atrophie blanche*)

C5: C4 + healed ulcer

C6: C4 + open ulcer.

CVI – CLINICAL SIGNS

Varicose veins are the most common manifestation of superficial venous disease.

From a morphological point of view, the following types of varicose can be distinguished (FIG. 2):

- **Truncal varicosis**

Great saphenous vein, small saphenous vein

- Complete truncal varicosis: venous reflux starts from the first valve, at the junction of the venous trunk (crosse).

- Incomplete truncal varicosis: insufficiency of the main venous trunk with sufficient crosse, and venous reflux affecting another vessel.

- Collateral varices: inflow through insufficient lateral branching.

- Varices from perforating vein: insufficient inflow through a perforating vein.

- **Reticular varicosis**

Subcutaneous tortuous vein which belongs to neither the main trunk nor the collateral branches; diameter: <3 mm.

- **Telangiectasia**, Intracutaneous ectasia.

PHLEBOEDEMA

A slight swelling of the limbs in the evening is not uncommon.

After a long day of work, the volume of the leg can increase by up to 100 ml; after a long flight, with no movement, this can be as much as 200 ml.

Oedema that does not disappear spontaneously within a few hours, or after a walk, is considered pathological.

Bilateral and poorly symptomatic types of oedema are mostly systemic (cardiac, renal, hepatic).

Phleboedema is always associated with disorders and/or clinical signs such as dilated superficial veins, varicose veins, and cutaneous dystrophy.

Chronic phleboedema is only partially

reversible. It results in an induration that can be detected on palpation and affects all structures in the integument, a condition defined as **lipodermatosclerosis (LDS)**.

LDS can develop into atrophy.

The leg has the appearance of an upside-down bottle of *champagne*.

In some cases, a secondary lymphoedema develops (FIG. 3), presenting acral thickening of the epidermal fold, hyperkeratosis and papillomatosis.

CUTANEOUS DYSTROPHIES

Cutaneous dystrophy mainly refers to damage caused by chronic venous hypertension, or failure to lower the pressure in the veins when walking.

It is localised in the area of the primary varices, particularly in regions with insufficient perforating veins, in the leg and the distal foot.

- The clinical picture becomes rapidly dominated by secondary disorders: allergic inflammation and irritation in the case of chronic oedema and loss of epidermal barrier function, microvascular thrombosis, local ischaemia and reperfusion, intoxication due to accumulation of Iron, etc.

The pathophysiology can be recognised from the clinical picture: dermo-epidermitis, hypodermatitis, hyperpigmentation, and ulceration. Reorganisation of



FIG. 3

the epidermal vessels can partly be thought of as a consequence of the reparative response: telangiectasia of the thighs, retromalleolar and paraplantar varices, and loss of capillaries in atrophic areas with capillary expansion. *Atrophie blanche* is a result of *capillaritis alba* and eventually of an acute, intensely painful circumscribed ischaemia, associated with inflammation or microvascular thrombosis.

The *ulcus cruris* occurs spontaneously due to minor traumas which often go unnoticed. Some ulcers can be healed by compressive therapy, while others persist for years (FIG. 4).

JOINT DYSFUNCTIONS

Joint dysfunction is an inevitable consequence of CVI.

Of particular importance is the functional limitation in the mobility of the ankle joint, initially associated with



FIG. 4

FIG. 5



pain, then subsequently aggravated by a narrowing of the joint capsule. This leads to the so-called phlebological equinism, with a resulting difficulty in walking (FIG. 5).

Patients normally compensate for the problem by wearing high-heeled shoes.

The lesion causes a vicious circle, in which the pump function of the calf muscles is severely compromised.

ACUTE VENOUS DISEASES – CLINICAL SIGNS

VARICOSE HAEMORRHAGE

The rupture of varicose veins is a dramatic, albeit harmless, complication of a particular form of varicosis: thin-walled intracutaneous venous aneurysms can develop painless fissures due to minor trauma.

The raised venous pressure, lack of musculature in the vascular walls, and small size of the opening often result in extensive bleeding.

The acute phase can be resolved by elevating the lower limb and applying a compression bandage.

– The most effective treatment for these varicose veins is sclerotherapy with effective eccentric compression.

PHLEBITIS

The term phlebitis (FIG. 6) refers to a series of disease patterns that differ from a nosological and clinical point of view.

The occurrence of a reddish thrombus in a varicose vein is inevitable in cases of varicose thrombosis, with little inflammation in the venous wall but intense inflammation in the surrounding area.

The inflammation of a superficial non-varicose vein affects the entire vascular wall, and the surrounding area to a much lesser extent; the thrombus is reduced or absent.

In varicothrombosis, a thrombus tends to grow and become stratified, with possible development in the Deep Venous System; phlebitis in a non-varicose vein tends to migrate, occasionally passing into a different venous segment (migrating phlebitis).

The autonomous development of deep vein thrombosis and oligosymptomatic pulmonary embolism are not uncommon in both forms of venous inflammation.

– Frequent ultrasound examinations are prescribed in both cases.

In the case of varicothrombosis, removal of the thrombus by incision or heat procedure on the varicose veins generally leads to rapid improvement.

FIG. 6



DEEP VENOUS THROMBOSIS

The signs of acute venous thrombosis depend on the patient's situation and the extent of the thrombosis itself.

– In bedridden patients, the signs are so subtle that diagnosis is often delayed.

Typical warning signs are cyanosis, dilated superficial veins and raised skin temperature.

Ambulatory patients rapidly show signs of compartment syndrome: hardening of the affected parts (sole of the foot, calf), tenderness in the muscles, reduced mobility, pain when placing the foot on the ground, walking, or during active or passive flexion and extension of the foot.

Obvious oedema, i.e. a difference between the circumference of the calf and that of the contralateral of >3 cm, is only to be expected in cases of ascending thrombosis (FIG. 7) if the thrombosis has reached the common femoral vein.

On the other hand, descending thrombosis typically causes rapid, major swelling, and is not generally characterised by compartment syndrome in the calf.

MATERIALS AND METHODS

The patients in our case study were examined from a clinical and instrumental point of view both at the start and end of treatment.

– The following criteria were considered in the assessment:

gender, age, weight, height, family history of CVI, family history of venous thromboembolic disease, oestrogen-progestin therapy, hormone replacement therapy, pregnancy, work activity, weight gain, history of deep vein thrombosis (DVT) or superficial vein thrombosis (SVT).

The objective assessment was carried out through careful analysis of the lower limbs (condition of the skin, examination, palpation).

The subjective assessment was carried out using a VAS representing the pain level, on which the patient places a point corresponding to the degree of pain.

The Trendelenburg manoeuvre enables the formulation of a clinical diagnosis of insufficiency of the large and small

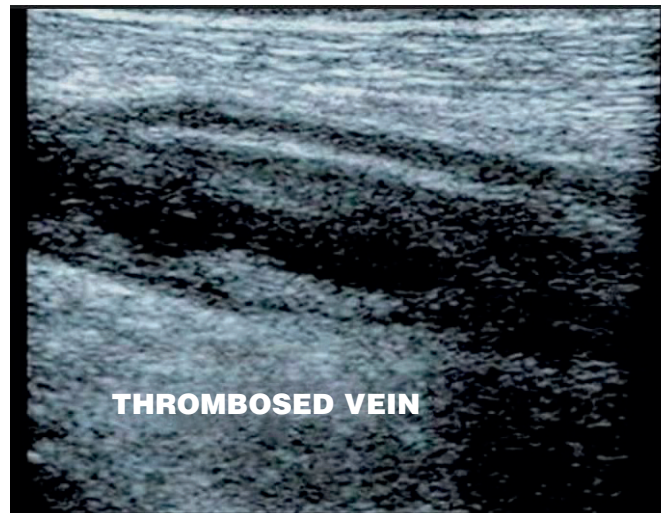


FIG. 7

saphenous veins in the region of the valves at the crosse of the perforating veins.

A haemodynamic study using colour doppler echocardiography is performed to assess the level of venous incontinence and the reduced rate of flow in the iliac, femoral, and popliteal veins.

- Nineteen patients with a C.E.A.P. classification, 13 female and 6 male, were enrolled in the study. 2-3.

The patients, aged between 35 and 55 years old, were monitored from March to June 2021.

– Inclusion criteria: F and M patients with a clinical and instrumental diagnosis of CVI, C.E.A.P. 2-3.

– Exclusion criteria: pregnancy; breastfeeding; current or previous DVT within one year of diagnosis; destructive or ablative treatment of the saphenous axis; other ongoing therapies for the same disease.

All the patients were given the following therapy:

- Outpatient treatment: **MD-Muscle** 1-2 vial(s) + **MD-Tissue** 1-2 vial(s). Treatment by injection in acupoints M5, M6, M9, M10 (see page 60) and parallel to the varicose vessel using 30G x 4 mm needles, 1/week.
- Home therapy: **Anti Age Vein**, 5 pellets x 2/day; **Guna Liver**, 5 pellets/day; **Guna Matrix**, 30 drops/day diluted

in 1.5 litres of drinking water throughout the day for 90 days.

- 18 - 21 mm/Hg elastic-compression stocking.

Patients were also monitored from the nutritional point of view.

They were encouraged to consume foods rich in substances recommended for this disease: cherries, berries, blueberries, blackberries, and currants (rich in proanthocyanidins, anthocyanidins and bioflavonoids that boost the resistance of small vessels, counteracting the permeability of the capillaries and inflammatory reactions in the connective tissue).

Buckwheat, a source of flavonoids, was also included in the diet.

This food is rich in rutin, a glycoside of quercetin, which has properties that strengthen the capillary wall.

Foods containing rutin can prevent the onset of bleeding and improve microcirculation, with pronounced anti-inflammatory, antioxidant properties. In addition to rutin, buckwheat contains other flavonoids (vitexin and isovitexin).

– Chilli, ginger, garlic, and onion were also recommended.

Due to their ajoene and adenosine content, garlic and onion are particularly effective as thromboxane inhibitors.

Apples, oranges, grapefruit, and lemon were also recommended for their rutin content, and fresh pineapple, rich in

bromelain, as an ingredient that supports fibrin metabolism.

The diet also included natto, a soy-based food rich in probiotics, vitamin K2 and nattokinase, an enzyme that reduces the formation of blood clots and dissolves those already formed, and omega-3 fatty acids, which lower cholesterol and stimulate the release of blood thinning enzymes.

Finally, grape seed oil, rich in polyunsaturated fatty acids (which have a laxative and cholesterol-lowering effect); associated with the anthocyanosides in leaves, it helps protect the blood vessels and improves elasticity in the tissues due to its ability to stabilise elastin and collagen.

– Foods whose consumption was reduced or ruled out included pepper, aubergines, tomatoes, potatoes, dairy products, and red meat (saturated fats), to avoid overloading the liver and strengthen the integrity of the connective tissue and the muscle components of the venous walls.

THERAPEUTIC PROPERTIES OF THE PRM MEDICINAL PRODUCTS USED

– **Anti Age Vein** is the PRM medicinal product indicated in venous stasis, venous varices, thrombophlebitis, phlebothrombosis, and haemorrhoids.

– **Guna Matrix** is a medicinal product specifically formulated for its pronounced detoxification effect on the ECM (extra-cellular matrix).

Its composition facilitates a coordinated action targeted at the deactivation, cleavage, and centrifugation of ECM toxins.

The primary effect of the medicinal product is exerted on the ECM, with a secondary effect on the kidneys (where it channels the toxin load removed by the ECM).

– **Guna Liver** is an effective medicinal product in diseases affecting the organs of the digestive system; it is a co-adjuvant in the treatment of liver disorders. Guna Liver contains active ingredients that help relieve gastroenteritis, meteorism, liver disease, and chronic vomiting.

– **Guna MD-Muscle** and **Guna MD-Tissue** are injectable medical devices made with Type I collagen of porcine origin.

- MD-Muscle contains Collagen, Heparin, NaCl, and Water for injection.
- MD-Tissue contains Collagen, Ascorbic Acid, Magnesium Gluconate, Pyridoxine hydrochloride, Riboflavin, Thiamine hydrochloride, NaCl, and Water for injection.

WHY USE GUNA COLLAGEN MEDICAL DEVICES

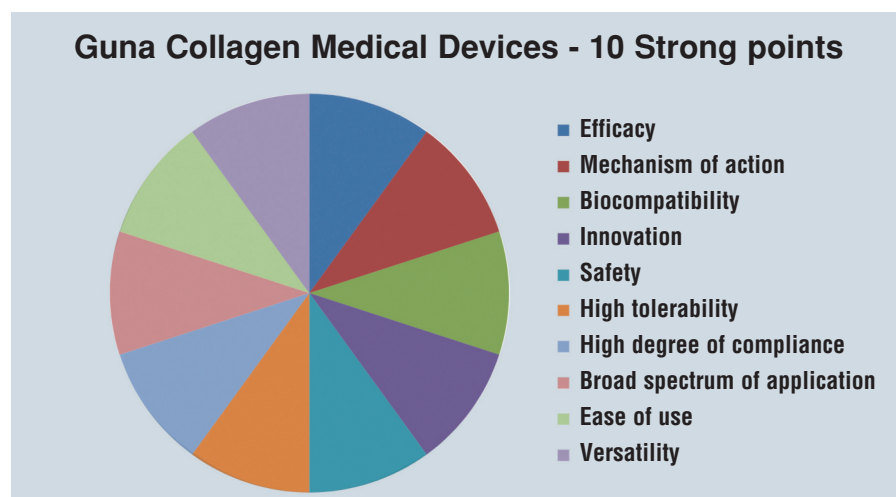
The advantages of the Guna Collagen Medical Devices are (TAB. 1):

- **Efficacy:** they restore and strengthen damaged anatomical structures; improving mobility and function, and acting directly on pain.
- **Mechanism of action:** they perform supportive actions in their specific areas of use.
- **Biocompatibility:** total compatibility between type I collagen of porcine origin and human collagen.
- **Innovation:** the collagen used is the outcome of innovative production processes developed in the Guna Laboratories.
- **Safety:** chemical and physical properties that ensure safety in clinical use.
- **High tolerability:** no side effects, allergic reactions, or problems due to drug interactions have been recorded so far.
- **High degree of compliance:** due to the mechanism of action and the absence of side effects, the treatment is well tolerated and satisfies patient expectations.
- **Broad spectrum of application:** they can be used in the treatment of various diseases in various fields of medicine.
- **Ease of use:** they can be used for subcutaneous, intra-dermal, local intramuscular, peri- and intra-articular injections.
- **Versatility:** they can be administered individually or in combination with one another; they are not contraindicated in patients receiving drug therapy, before or after surgery.

MD-Muscle and **MD-Tissue** were used in this study to manage the repair and remodelling of the venous vessels, essential to the functional recovery phase. The main difference between the venous and the arterial wall are that the former has more elastin and less collagen.

– Collagen Medical Devices act as bio-scaffolds.

TAB. 1



The deposition of newly synthesised collagen fibrils in the damaged region, secondary to the loco-regional infiltration of Collagen Medical Devices, produces a significant improvement in the mechanical qualities of the injured tissue; the anisotropic properties of the tissue are particularly well-restored.

Anisotropy is a mechanical property of collagen that describes the ability of its fibres to propagate the tensile forces to which they are subjected in a single preferred direction.

– This property, which is fundamental to many biological functions, is gradually lost when the collagen fibres are damaged due to ageing, wear, or trauma, particularly when the H+ bonds between the collagen fibres, essential to their orientation, are distorted. The optimal formation and distribution of collagen fibre is not only essential to structural integrity and functionality; it also plays a central role in transmitting tensile forces to the fibroblasts.

RESULTS

All patients reported heaviness in the lower limbs, which disappeared within 3 weeks after starting treatment.

At the start of the treatment, one male patient reported a sense of tension and weight in the calves, particularly after long periods sitting or standing, with ankle oedema in the evenings, itching and noticeable varices.

– After one month of treatment the pain and swelling disappeared; after 3 months the varices were much less evident.

All the 13 female patients, had pain, swelling, reticular varices and noticeable varices.

– After only 1 month of treatment the pain and swelling disappeared; 3 months after the start of treatment the varicose veins were much less evident.



FIG. 8

Start of the treatment.



FIG. 9

End of the treatment.

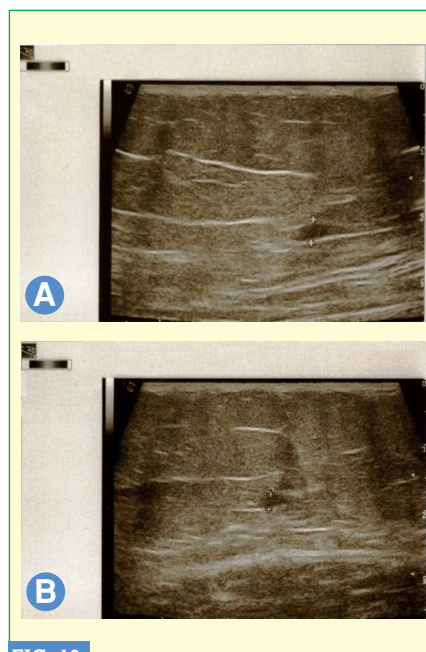


FIG. 10

Male patient before the treatment (A) and after the treatment (B).

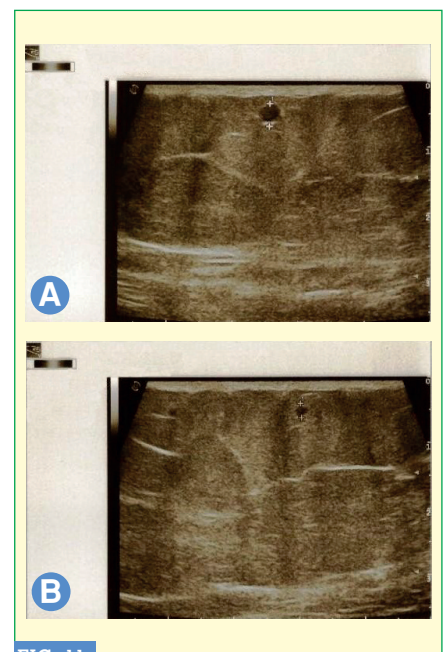


FIG. 11

Female patient before the treatment (A) and after the treatment (B).

CONCLUSIONS

This clinical study showed a clear improvement in symptoms (**pain, swelling, and heaviness**); the **reduced size** of the

varices was also appreciated.

– Moreover, owing to the observance of dietary instructions, the patients lost 10-20% of their body weight. Due to the synergistic combination of

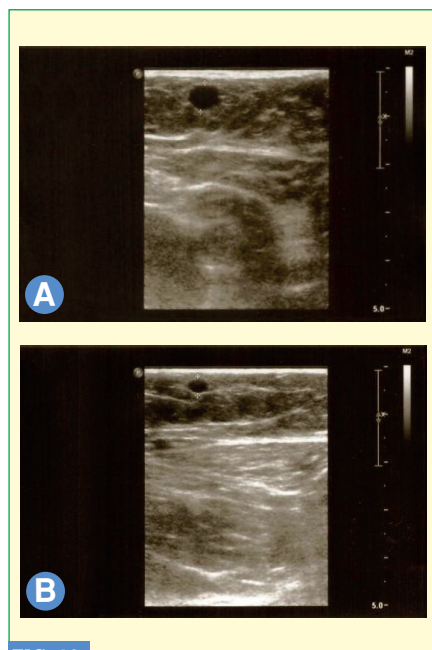
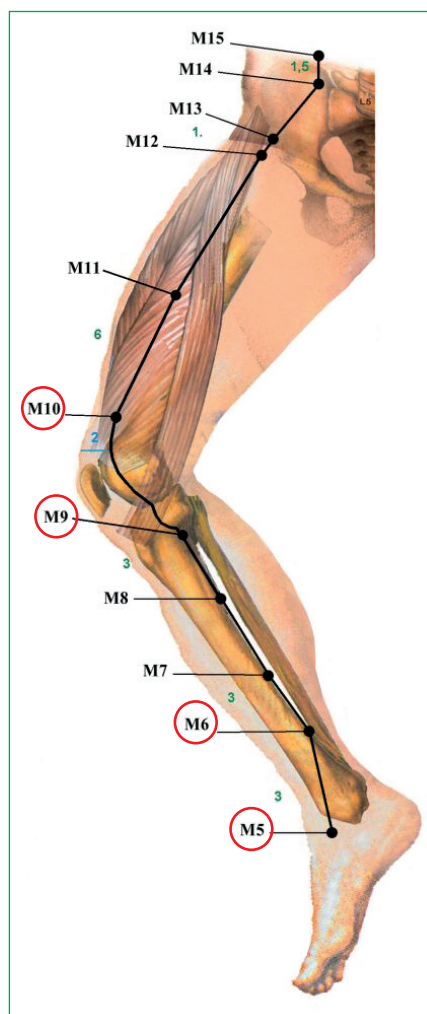


FIG. 12

Female patient. VGS before the treatment (A) and after the treatment (B).



<http://freeyourenergy.it/lelemento-terra-meridiano-dello-stomaco-della-milza/>

PRM medicinal products - Guna Collagen Medical Devices, the treatment proposed here proved effective in treating the symptoms of CVI in patients with a C.E.A.P. classification 2-3 (FIGS. 8-12). ■

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author

Dr. Lidia Cristaldi

– Specialist in Angiology
– Azienda USL Bologna Hospital for Rare Diseases Polyclinic Mengoli; Reference Centre for Diseases of the Circulatory System; Reference Operative Unit; Territorial Angiology
Cesena, Italy