PROPOSALS for an INTEGRATED IMMUNOTHERAPY PROTOCOL in ONCOLOGY and CHRONIC CONDITIONS such as PERSISTENT LYME DISEASE

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1. Introduction

The proposals contained in this document derive from more than 30 years of scientific research and observation in the fields of molecular oncology and neurosciences. These proposals aim at establishing a protocol that has the objective to provide strong support to the immune system in the context of a type of immunotherapy directed at the innate arm of the immune system.

1.1. The innate immune system

Innate immune responses are not specific to a particular pathogen or to a particular type of cancer cell in the way that the adaptive immune responses are. They depend on a group of proteins and cells of the immune system (macrophages and dendritic cells on one side, natural killer cells on the other side) that recognize conserved features of pathogens and abnormal cells, and become quickly activated to help destroy pathogens or cells perceived as abnormal. Whereas the adaptive immune system arose in evolution less than 500 million years ago and is confined to vertebrates, innate immune responses have been found among both vertebrates and invertebrates, as well as in plants, and the basic mechanisms that regulate them are conserved throughout evolution.

1.2. The innate immune system in cancer

In recent years, roles of the immune system in immune surveillance of cancer have been explored using a variety of approaches. The roles of the adaptive immune system have been a major emphasis, but increasing evidence supports a role for innate immune effector cells such as natural killer cells in tumor surveillance. Defined innate immune interactions in the cancer context include recognition by innate cell populations and also by dendritic cells and macrophages in response to damage-associated molecular patterns. The goal of this protocol is to potentiate these immune responses.

1.3. Information about the complementarity of this protocol

It is important to notice that this protocol has not to be considered an alternative to conventional methods of addressing diseases or standard of care, but rather as a complementary approach that aims at maximizing the therapeutic effects of conventional approaches, at the same time reducing the side effects of those approaches by supporting the immune system that is involved in all the

mechanisms of defense. Because of this, the proposals here described can be implemented in conjunction with any other type of therapeutic approach, whether conventional or complementary.

2. Immune system reconstitution by imuno®

2.1. Scientific background on imuno®

imuno® is a novel type of immune supporting compound that targets the innate immune system. At variance with other immune stimulating molecules that target only one type of cells (*e.g.* macrophages), imuno® targets all cells of the innate immune system and, because of this, helps to direct the adaptive immune system against cancer cells, pathogens, cells infected by viruses, cells harboring abnormal proteins or pathogens.

imuno® is based on the non-covalent, multi-molecular assembly of three components with known healthy properties that are assembled through a novel proprietary procedure. One of the major novelties of imuno® that sets it apart from all other immune stimulating approaches is the use of a novel type of low-molecular-weight chondroitin sulfate (LMW-CS), a glycosaminoglycan that is endowed with a number of healthy properties ranging from anti-inflammatory to anti-viral, neuro-protecting, and immune stimulating.

The healthy properties of CS have been described in a number of peer-reviewed papers and it is known that CS contains the active site of GcMAF (the moiety N-acetylgalactosamine). It has been hypothesized that CS may be responsible for the health effects attributed to GcMAF (*https://www.ncbi.nlm.nih.gov/pubmed/27515218*).

2.2. Low-molecular-weight chondroitin sulfate (LMW-CS)

LMW-CS represents an evolution of CS that is extracted from bovine cartilage and, if compared with CS from animal origin, LMW-CS has much higher purity and, most important for its biological function, is highly controlled and homogeneous at variance with animal-derived CS that is unfractionated. Unfractionated means that it is a mixture of CS molecules with different molecular weights, on average, high molecular weight. LMW-CS, however, has constant charge density and molecular mass parameters; it has a much more homogeneous profile and lower polydispersity; it has a constant lower molecular weight that allows much greater bio-availability.

LMW-CS has a protein content of 0,1% and an almost no risk compared to animal-derived CS where current pharmacopoeia limits allow a protein content up to 6.0%. In other words, LMW-CS is 99.9% pure as compared to animal-derived CS where a mere 94% purity is allowed.

LMW-CS has demonstrated clinical activity much higher than the best animal-derived unfractionated CS. LMW-CS is able to significantly decrease Gamma-GT activity by approximately 31% and plasmatic C-Reactive Protein levels by about 9%. These results demonstrate significant anti-inflammatory properties *in vivo*. Consistent with these results, pro-inflammatory cytokines are also decreased. The greater efficacy of LMW-CS in reducing parameters of inflammation is related to its lower molecular mass with respect to CS of extractive origin. As far as safety is concerned, in toxicity studies, Sprague Dawley rats were gavaged with LMW-CS at dose levels of 0, 250, 500 and 1000 mg/kg body weight/day for 90-days. No mortality or significant changes in clinical signs, body weights, body weight gain or feed consumption were noted. Similarly, no toxicologically relevant treatment-related changes in hematological, clinical chemistry, urinalysis and organ weights were noted. Macroscopic and microscopic examinations did not reveal treatment-related abnormalities. In vitro, mutagenic and clastogenic potentials as evaluated by Ames assay, chromosomal aberration test, and micronucleus assay, did not reveal genotoxicity of LMW-CS. In a pharmacokinetic study in humans, LMW-CS showed higher absorption as

compared to unfractionated CS of animal origin. This feature can be attributed to low molecular weight. The results of sub-chronic toxicity study supports the no-observed-adverse-effect level (NOAEL) for LMW-CS as 1000 mg/kg bw/day, the highest dose.

The main feature of LMW-CS that sets it apart from unfractionated CS of animal origin and from all current products containing CS, is the homogeneous low molecular weight.

Presence of non-animal LMW-CS makes imuno® "vegan", a feature that is nowadays greatly appreciated and is not shared by other compounds featuring unfractionated CS of animal origin.

2.3. Other components

The other two components of imuno®, Vitamin D3, and phosphatidylcholine (PC) are endowed with a number of healthy properties that are amplified by the multi-molecular assembly occurring in imuno®.

2.4. Peer-reviewed literature

The rationale for the design of imuno® and details on the scientific background can be found in a peer-reviewed paper published in a major scientific journal dedicated to integrative cancer therapeutics, see Ruggiero and Pacini 2018

3. Mechanism of action

There are two mechanisms of action at work in imuno®, with particular reference to its effects when injected subcutaneously or around a lesion

(*Please notice; injections of supplements is considered an off-label use in some Countries and is subjected to certain restrictions. Make certain that you are following the rules and regulations of the Country where you operate*).

3.1. The effects of each ingredient amplified by combination in a single multimolecular structure.

3.2. The effect of the oil/water emulsion on the immune system.

3.1. The effects of each ingredient amplified by combination in a single, original, multi-molecular structure

The original proprietary procedure enables non-covalent binding of PC to LMW-CS thanks to the sulfation pattern of LMW-CS that is distributed on a higher number of molecules as compared with that of unfractionated CS, thus allowing a higher number of electrostatic interactions. Such a feature is not present in any other compound based on unfractionated CS.

Vitamin D3, being a lipophilic molecule of small size, is intercalated in the structures constituted by PC and LMW-CS is a manner similar to the assembly in cell membranes.

The multi-molecular assembly described above, favors bio-availability of all the ingredients of the imuno® formula thus amplifying the known healthy properties of each one with particular reference to immune system modulation as well as anti-inflammatory, anti-cancer and neuroprotective properties. The proprietary procedure of manufacture also brings the advantage of the formation of nano-emulsions. It is well assessed that nano-particulate delivery systems offer

plenty of advantages over conventional dosage forms which include improved efficacy, reduced toxicity, enhanced biodistribution, and improved patient compliance.

3.1.1. Role of nanosizing

Nano-emulsions solve one of the major problems of supplement or drug administration and ensures the efficacy of relatively low doses. Thus it is known that the challenge of drug delivery is the liberation of drug agents at the right time in a safe and reproducible manner, usually to a specific target site. Conventional dosage forms, such as orally administered pills and subcutaneous or intravenous injection, are the predominant routes for drug administration. But pills and injections offer limited control over the rate of drug release into the body; usually, they are associated with an immediate release of the drug.

Consequently, to achieve therapeutic levels that extend over time, the initial concentration of the drug/supplement in the body must be high, causing peaks (often adjusted to the stay just below known levels of toxicity for the drug or supplement) that gradually diminish over time to an ineffective level. In this mode of delivery, the duration of the therapeutic effect depends on the frequency of dose administration and the half-life of the drug. This peak and valley delivery is known to cause toxicity in certain cases, most famously with chemotherapy drugs for cancer. This is the reason why certain supplements, such as Vitamin D3 for example, are administered in very high doses, just below the toxicity threshold, in order to achieve some biological effect. The reason for such a greater efficiency at much smaller doses is that nanoscale molecules, and hence nano-emulsions, have far larger surface areas than similar masses of larger-scale materials. As surface area per mass of a material increases, a greater amount of the material can come into contact with surrounding materials, thus affecting reactivity.

In addition, nano-emulsions control and sustains release of the ingredients of imuno® during the transportation and at the site of localization, optimizing organ distribution of the molecules and subsequent clearance so as to achieve an increase in supplement therapeutic efficacy and reduction in side effects.

The system can be used for various routes of administration including oral, nasal, parental, according to the needs and in compliance with laws, rules and regulations governing the use of supplements and therapeutic approaches.

A significant advantage is that the dose and side effects can be conveniently reduced to attain maximum therapeutic benefits.

In addition, as far as the anti-cancer properties of the ingredients of imuno® are concerned, the rationale of using nanoparticles/nano-emulsions for tumor targeting is based on the following characteristics:

3.1.2. Nanoparticles will be able to deliver a concentrated dose of anti-cancer molecules in the vicinity of the tumor targets via the enhanced permeability and retention effect or active targeting by ligands on the surface of nanoparticles.

3.1.3. Nanoparticles will reduce exposure of healthy tissues by limiting drug distribution to the target organ.

3.1.4. Bio-distribution of nanoparticles is rapid, within 3 hours, and involves mononuclear phagocytic system (MPS/macrophages) and endocytosis/ phagocytosis process. Such propensity of MPS for endocytosis/phagocytosis of nanoparticles provides an opportunity to effectively deliver therapeutic agents to these cells thus achieving the original goal of macrophage activating factors such as GcMAF.

3.2. The effect of the oil/water emulsion on the immune system

In this regard, the mechanism of action of imuno® can be compared to that of Freund's Incomplete Adjuvant. Freund's adjuvant is a solution of antigen emulsified in mineral oil and used as an immune-potentiator (booster). The complete form, Freund's Complete Adjuvant is composed of inactivated and dried mycobacteria, whereas the incomplete form lacks the mycobacterial components (hence just the water in oil emulsion). In the field of immunotherapy of cancer, mechanisms by which adjuvants promote anti-tumor immunity generally rely on the stimulation of innate immunity via the pattern-recognition receptors, such as Toll-like receptors, upon which innate immune cells prime robust and sustained adaptive immune responses against the tumors (*https://www.ncbi.nlm.nih.gov/pubmed/27006304*).

3.2.1. imuno®, in addition to the biological effects due to its individual components, stimulates the innate immune system through a mechanism analogous to that of oil/water-based adjuvants. It is generally assumed that incomplete and complete Freund's adjuvant act by providing a complex set of signals to the innate compartment of the immune system, resulting in leukocyte proliferation and differentiation. Early events include rapid uptake of adjuvant components by dendritic cells, enhanced phagocytosis, secretion of cytokines by mononuclear phagocytes (macrophages), and transient activation and proliferation of CD4+ lymphocytes (*https://www.ncbi.nlm.nih.gov/pubmed/11739546*).

3.3. Peer-reviewed literature

The mechanism of action of imuno® is described in detail in Ruggiero and Pacini 2019

4. Directions for use

4.1. Before starting: The Ruggiero Treatment Evaluation Scale (RTEC)

4.1.1. Rationale for the use of The Ruggiero Treatment Evaluation Scale (RTEC)

imuno® works on the immune-neuro-endocrine axis as an adaptogen and, therefore, it may rebalance a number of functions that pertain to the spheres of neurology, psychiatry, psychology, and cognition. These effects are of importance not only for those conditions where symptoms pertaining to these spheres are prominent such as, for example, neuroborreliosis, chronic fatigue syndrome or neurodegenerative disease, but also for cancer.

In fact, diagnosis and treatment of cancer is known to influence psychological well-being to a significant degree. Rates of psychological distress are elevated for most individuals who have been diagnosed with cancer when compared to population norms. Common psychological reactions to cancer are mood and anxiety-related concerns. Elevated rates of depression and anxiety in response to a cancer diagnosis is often attributable to uncertainty regarding mortality and well as going through arduous treatments and concerns related to functional interference and body-image or other self-concept related distress. Understanding how individuals react psychologically to cancer is important to support their overall well-being and maximize the quality of life during treatment and beyond. While the prevalence of psychological disturbance in reaction to cancer is relatively high when compared to population norms, many individuals report fairly stable psychological well-being through the cancer trajectory and some even report improved psychological wellbeing.

In order to evaluate the effects of imuno® on these states, a dedicated questionnaire termed "The Ruggiero Treatment Evaluation Scale (RTEC)" has been developed with the goal of evidencing

those effects of imuno® that may escape the attention of the Therapist focused primarily on the specific symptoms of the disease for which imuno® is used.

4.1.2. How to evaluate the effects imuno® using RTEC

It is worth noticing that the effects of imuno® on the immune-neuro-endocrine axis may be slow and progressive; since imuno® works by rebalancing physiological mechanisms these effects may go unnoticed unless specifically addressed. The RTEC has been developed precisely to address these aspects and provides a useful tool to assess the efficacy of the treatment in addition to the specific analyses or lab test that evaluate the primary disease.

The RTEC has to be compiled by the patient, not the Therapist. First assessment has to be performed before starting the treatment with imuno® and at regular intervals thereafter. As a rule of thumb, the second assessment should occur not earlier than eight weeks after starting the treatment.

The Ruggiero Treatment Evaluation Scale (RTEC)

The Ruggiero Treatment Evaluation Scale (RTEC) is 65-item diagnostic assessment tool developed by Dr. Ruggiero. The RTEC is designed to evaluate the effectiveness of treatments for sleep disturbances, anxiety, and other psychological disorders as well as conditions involving perceived energy levels and general wellbeing. The questionnaire, which is completed by the subject, takes about 15 minutes to complete. The RTEC is successful in measuring interventional effects as well as tracking changes over periods of time. The RTEC should be completed before the treatment that is to be evaluated and at regular intervals such as, for example, every month. A Decrease in RTEC score indicates improvement.

Today's date: ..... Name of the subject:..... Age:.....Male ☐ Female ☐

Section 1. Please circle the letters to indicate how true each phrase is: [N] Not true; [S] Somewhat true; [V] Very true Score: V=2; S=1; N=0	
N S V 1. I have trouble falling asleep	N S V 9. I often think something is wrong with my body
N S V 2. I have trouble staying asleep	N S V 10. I am a shift worker and/or my sleep schedule is irregular
N S V 3. I take (drugs/supplements) to help sleep	N S V 11. My legs are restless and/or feel uncomfortable before bed
N S V 4. I use alcohol to help sleep	N S V 12. I have been told that I am restless or that I kick my legs in my sleep
N S V 5. I have medical conditions that disrupt my sleep	N S V 13. I have unusual behaviors or movements during sleep
N S V 6. I am losing interest in hobbies or activities	N S V 14. I snore
N S V 7. I often feel sad, irritable, or hopeless	N S V 15. I have been told that I stop breathing, gasp, snort or choke in my sleep
N S V 8. I often feel nervous or worried	N S V 16. I have difficulty staying awake during the day

Section 2. Please circle the letters to indicate how true each phrase is: [N] Non descriptive; [S] Somewhat descriptive; [V] Very descriptive Score: V=2; S=1; N=0	
N S V 1. I am not able to relax	N S V 11. Most of the times my muscles are tense, aching, or sore
N S V 2. I tend to focus on upsetting situations or events happening in my life	N S V 12. I often have sweaty or cold, clammy hands
N S V 3. I feel fearful for no reason	N S V 13. I spend a lot of time wondering why I feel the way I do
N S V 4. Usually I am not as happy as the people around me	N S V 14. I am afraid of crowds, being left alone, the dark, strangers, or traffic
N S V 5. Often I have diarrhea, constipation, or other digestive problems	N S V 15. I often faint or feel like fainting
N S V 6. I often have a dry mouth	N S V 16. I have difficulty swallowing or have a "lump in throat" feeling
N S V 7. When someone snaps at me, I spend the rest of the day thinking about it	N S V 17. I experience twitching, trembling or shaky feelings
N S V 8. No matter what I do, I can't get my mind off my problems	N S V 18. I am easily irritated
N S V 9. I am easily alarmed, frightened, or surprised	N S V 19. I feel futile/useless
N S V 10. I often experience shortness of breath or choking feelings	N S V 20. I often think about all the things I have not yet accomplished

Section 3. Please circle the letters to indicate how true each phrase is: [N] Not true; [S] Somewhat true; [V] Very true Score: V=0; S=1; N=2	
N S V 1. I never experience unusually long periods of fatigue	N S V 4. I have never substantially reduced my previous levels of occupational, educational, social, or personal activities because of persistent fatigue
N S V 2. When I feel fatigued it is because of some obvious ongoing physical exertion that I am aware of	N S V 5. I have never experienced impairment in short-term memory or concentration, severe enough to cause substantial reduction in previous levels of personal activity
N S V 3. My fatigue goes away after I have rested normally	

Section 4. Please circle the letters to indicate how true each phrase is: [N] Non descriptive; [S] Somewhat descriptive; [V] Very descriptive Score: V=0; S=1; N=2	
N S V 1. I like most parts of my personality	N S V 10. I feel as if I've done all there is to do in life in a very satisfying way
N S V 2. When I look at the story of my life, I am pleased with how things have turned out so far	N S V 11. For me, life has been a continuous process of learning, changing, and growth
N S V 3. Some people wander aimlessly through life, but I am not one of them	N S V 12. I think it is important to have new experiences that challenge how I think about myself and the world
N S V 4. The demands of everyday life never get me down	N S V 13. People would describe me as a giving person, willing to share my time with others
N S V 5. I never feel disappointed about my achievements in life	N S V 14. I never give up trying to make big improvements or changes in my life
N S V 6. Maintaining close relationships has never been difficult or frustrating for me	N S V 15. I am not influenced by people with strong opinions
N S V 7. I live life one day at a time with satisfaction and don't really worry about the future	N S V 16. I have experienced many warm and trusting relationships with others
N S V 8. In general, I feel I am in charge of the situation in which I live	N S V 17. I have confidence in my own opinions, even if they are different from the way most other people think
N S V 9. I am good at managing the responsibilities of daily life	N S V 18. I judge myself by what I think is important, not by the values of what others think is important

Section 5. Please circle the letters to indicate how true each phrase is: [N] Non descriptive; [S] Somewhat descriptive; [V] Very descriptive Score: V=0; S=1; N=2	
N S V 1. I am able to concentrate	N S V 4. I enjoy normal activities
N S V 2. I am capable of making decisions	N S V 5. I face up to problems
N S V 3. I capable of overcoming difficulties	N S V 6. I feel reasonably happy

5. Ways of Administration

imuno®can be administered through a variety of routes according to the clinical judgment of the Therapist who takes care of the administration.

Please notice: the following considerations are NOT medical advice and they have to be considered only as a starting point for a reflection.

5.1. Dose

In general terms, the initial dose could be 0.1-0.2 mL and it can be increased or decreased according to the clinical judgment. In the case of subcutaneous injections, the initial frequency should be once every 3 to 5 days according to clinical judgment.

5.2. Injections

(*Please notice; injections of supplements is considered an off-label use in some Countries and is subjected to certain restrictions. Make certain that you are following the rules and regulations of the Country where you operate*).

The way of administration depends on the anatomical area to be targeted.

For example, in the case of visible/palpable tumors, the dose could be split in 2 or 3 aliquots and injected subcutaneously in the close proximity of the tumor. If the procedure is performed under ultrasound guidance, as it is recommendable, a rapid increase in blood flow should be observed at the echo-color-Doppler. This is due to the release of nitric oxide by cells of the immune system (macrophages and natural killer cells) and should correspond to a cancer cell-killing effect. Please notice that imuno® shows anti-aggregant/anticoagulant properties and these properties need to be taken into account in order to avoid bleeding.

In the case of tumors that cannot be reached with subcutaneous injections, the lymphatic route could be exploited, and imuno® could be injected in the proximity of the nodes pertaining to the anatomical area where the tumor is located. imuno® should be injected subcutaneously in an area of the body whose lymph is drained to the nodes in the proximity of the tumor. A similar approach, targeting the lymphatic system, could be used in persistent Lyme disease.

5.3. Suppositories

In the case of liver lesions, whether primary or metastatic, the rectal route is preferable. In this case, the best strategy is to inject the imuno® into wax or glycerin suppositories or to dilute it to prepare an enema. imuno® can be injected into Bravo suppositories.

The same strategy can be applied to prostatic or pelvic lesions.

5.4. Nebulization

In the case of lung or brain lesions, whether primary or metastatic, nebulization can be used. In this case, imuno® can be dissolved in 5-7 ml of saline and administered with a common nebulizer. Given the release of nitric oxide, a drop in blood pressure can be expected and caution should be exercised to avoid fainting. If an ultrasound system is available, you can observe an increase in the blood flow in the spleen right after the nebulization. This is due to the release of nitric oxide by cells of the immune system that are resident in the spleen.

5.4.1. Ultrasound

In the case of brain lesions, the concomitant use of transcranial ultrasound may synergize with the effects of imuno® administered by nebulization. For the use of ultrasound as a therapeutic approach, please see Ruggiero and Klinghardt 2017

These different routes of administration are not mutually exclusive and can be used in concomitance, according to clinical judgment.

5.5. Direct delivery of imuno® to the brain through the olfactory nerve

5.5.1. Introduction and rationale for nasal delivery of molecules to the brain bypassing the blood-brain barrier; this approach may be used for neuroborreliosis and/or neurologic conditions.

It is well known that delivery of molecules (drugs or supplements) to the brain is rather difficult because of the blood-brain barrier (BBB) that proves rather impermeable to a great number of molecules endowed with biological activity. For example, poor brain penetration of tarenflurbil (TFB) was one of the major reasons for its failure in phase III clinical trials conducted on Alzheimer's patients. TFB, or Flurizan or R-flurbiprofen, is the single enantiomer of the racemate NSAID flurbiprofen that is a rather common drug used to counteract inflammation and pain. The rationale was that TFB could have ameliorated brain inflammation that is common in Alzheimer's disease; however, lack of brain penetration caused the failure of the experience.

A similar problem affects Quetiapine (QTP), marketed as Seroquel, which is an antipsychotic approved for the treatment of schizophrenia, bipolar disorder, and along with an antidepressant to treat major depressive disorder. Also, in this case, the major problem was the poor penetration of the drug inside the brain because of the obstacle posed by the BBB.

Therefore, there has been a considerable effort to develop effective delivery systems that enable the delivery of molecules to the brain.

Recent studies demonstrate that nano-emulsions of otherwise non-deliverable molecules enable the direct passage of such molecules to the brain via the nasal mucosa and, more precisely, via the olfactory cranial nerve whose terminations pass through the ethmoid bone.

Please notice that imuno® was designed to be a nano-emulsion.

A recent study was designed with the aim of improving drug delivery to the brain through intranasally delivered nanocarriers. TFB was loaded into two different nanocarriers i.e., poly (lactide-co-glycolide) nanoparticles (TFB-NPs) and solid lipid nanoparticles (TFB-SLNs). The particle size of both the nanocarriers (<200nm) as determined by dynamic light scattering technique and transmission electron microscopy, assured transcellular transport across olfactory axons whose diameter was ≈200nm and then paving a direct path to the brain. TFB-NPs and TFB-SLNs resulted in 64.11±2.21% and 57.81±5.32% entrapment efficiencies respectively which again asserted the protection of drug from chemical and biological degradation in the nasal cavity. In vitro release studies proved the sustained release of TFB from TFB-NPs and TFB-SLNs in comparison with pure drug, indicating prolonged residence times of drug at targeting site. Pharmacokinetics suggested improved circulation behavior of nanoparticles and the absolute bioavailabilities followed this order: TFB-NPs (i.n.)>TFB-SLNs (i.n.)>TFB solution (i.n.)>TFB suspension (oral). Brain targeting efficiency was determined in terms of % drug targeting efficiency (%DTE) and drug transport percentage (DTP). The higher %DTE (287.24) and DTP (65.18) were observed for TFB-NPs followed by TFB-SLNs (%DTE: 183.15 and DTP: 45.41) among all other tested groups. These encouraging results proved that therapeutic concentrations of TFB

could be transported directly to the brain via olfactory pathway after intranasal administration of polymeric and lipidic nanoparticles (*Eur J Pharm Sci. 2016 May 13. pii: S0928-0987(16)30170-1*).

Two important conclusions can be drawn from this study: lactide-co-glycolide nanoparticles proved more efficient than all other vehicles and 200 nm was the critical threshold of size for the emulsion nanoparticles to be efficiently delivered to the brain

It is worth noticing that the biochemical features of lactide-co-glycolide nanoparticles are superimposable to those of low molecular weight glycosaminoglycans such as LMW-CS because of the high concentration of negative charges on the molecule surface. The efficiency of LMW-CS in enhancing absorption has been recently demonstrated (*Int J Pharm. 2014 Apr 25;465(1-2):143-58*).

Therefore, since imuno® is composed of LMW-CS it may prove an efficient molecular complex to deliver bioactive compounds to the brain exploiting both the trans- and the intracellular pathways pertaining to the olfactory nerve.

Another recent study evaluated the possibility of improved drug delivery of QTP using a nano-emulsion system that was developed for intranasal delivery. Effects of different vehicles of Emalex LWIS 10, PEG 400 and Transcutol P, as co-surfactants, were studied on isotropic region of pseudoternary-phase diagrams of nanoemulsion system composed of capmul MCM (CPM) as oil phase, Tween 80 as surfactant and water. Phase behaviour, globule size, transmission electron microscope (TEM) photographs and brain-targeting efficiency of quetiapine nano-emulsion were investigated. In vitro dissolution study of optimised nanoemulsion formulation, with mean diameter 144 ± 0.5 nm, showed more than twofold increase in drug release as compared with the pure drug. According to results of in vivo tissue distribution study in Wistar rats, intranasal administration of QTP-loaded nanoemulsion had shorter T max compared with that of intravenous administration (AAPS PharmSciTech. 2016 May 20).

In other words, a nano-emulsion delivered through the nasal route was more efficient in targeting the brain than intravenous administration.

It is well acknowledged that delivery of nano-emulsions to the brain through the nasal route involves the olfactory nerve at the level of the mucosa in the region corresponding to the cribriform plate of the ethmoid bone.

The olfactory nerve (*nervus olfactorius*) is the first cranial nerve and contains the afferent nerve fibers of the olfactory receptor neurons, transmitting nerve impulses about odors to the central nervous system, where they are perceived by the sense of smell. Derived from the embryonic nasal placode, the olfactory nerve is somewhat unique among cranial nerves because it is capable of some regeneration if damaged. The olfactory nerve is sensory in nature and originates on the olfactory mucosa in the upper part of the nasal cavity. From the olfactory mucosa, the nerve (actually many small nerve fascicles) travels up through the cribriform plate of the ethmoid bone to reach the surface of the brain. Here the fascicles enter the olfactory bulb and synapse there; from the bulbs (one on each side) the olfactory information is transmitted into the brain via the olfactory tract.

Such a route to administer molecules directly to the brain without passing through the circulation and, hence, without being stopped by the BBB, is currently investigated for a number of other molecules in addition to those mentioned above. For example, haloperidol is a commonly prescribed antipsychotic drug currently administered as oral and injectable preparations *i.e.* using administration routes identical to those that can be used for imuno®.

A recent study aimed to prepare haloperidol intranasal mini-emulsion helpful for psychiatric emergencies and exhibiting lower systemic exposure and side effects associated with non-target site delivery. Haloperidol mini-emulsions were successfully prepared by spontaneous emulsification adopting 23 factorial design. The effect of three independent variables at two levels each namely; oil type (Capmul®-Capryol™90), lipophilic emulsifier type (Span 20-Span 80) and HLB value (12-14) on globule size, PDI and percent locomotor activity inhibition in mice was evaluated. The optimized formula (F4, Capmul®, Tween 80/Span 20, HLB 14) showed globule size of 209.5±0.98nm, PDI of 0.402±0.03 and locomotor inhibition of 83.89±9.15% with desirability of 0.907.

Also, in this case, the critical features were the size of the globules around 200 nm and the physico-chemical features of the emulsifiers.

Biodistribution study following intranasal and intravenous administration of the radiolabeled 99mTc mucoadhesive F4 revealed that intranasal administration achieved 1.72-fold higher and 6 times faster peak brain levels compared with intravenous administration, thus confirming the general principle that delivery of molecules to the brain is achieved more efficiently with intranasal administration than with intravenous injections.

Drug targeting efficiency percent and brain/blood exposure ratios remained above 100% and 1 respectively after intranasal instillation compared to a maximum brain/blood exposure ratio of 0.8 post intravenous route. Results suggested the CNS delivery of major fraction of haloperidol via direct transnasal to brain pathway that can be a promising alternative to oral and parenteral routes in chronic and acute situations. Haloperidol concentration of 275.6ng/g brain 8h post intranasal instillation, higher than therapeutic concentration range of haloperidol (0.8 to 5.15ng/ml), suggests possible sustained delivery of the drug through nasal route (*Eur J Pharm Sci. 2016 May 3. pii: S0928-0987(16)30156-7*).

In short, very recent evidence point to the nasal route of administration of nano-emulsions as the most effective way to target molecule to the brain avoiding the obstacle posed by the BBB and effectively by-passing the barrier.

Several devices can be used to deliver drugs in general, and imuno® in particular, using the nasal route to directly access the brain.

It is important to establish which nostril is pervious and which side of the olfactory mucosa contains the highest amount of olfactory nerve fibers. In order to do so, it may be useful to assess the degree of olfactory sensitivity (sense of smell) in each nostril by having the subject smell coffee beans in a small cup, first from one nostril (keeping the other one closed with the fingers) and then from the other and having the subject evaluate which nostril is more sensitive. The evaluation of sensitivity is achieved by assessing the number of coffee beans that are necessary for the subject to smell the coffee smell. In other words, if from the right nostril the subject smells coffee when in the cup there are only three beans, and, *vice-versa*, from the left nostril she/he needs 6 beans to smell, this means that the right nostril is more sensitive or more pervious and this is the nostril that has to be used.

Administration of the imuno® is achieved with the head slightly tilted and it is advisable that the subject remains in such a position for 10 minutes.

5.6. Guidelines for nasal administrations

5.6.1. Instruct patient to clear or blow nose gently unless contraindicated (*e.g.*, risk of increased intracranial pressure or nosebleeds).

5.6.2. Position Patient:

5.6.3. Help patient to supine position and position head properly.

5.6.4. Tilt head back over edge of bed or place small pillow under patient's shoulder and tilt head back.

5.6.5. Support patient's head with non-dominant hand.

5.6.6. Instruct patient to breathe through mouth.

5.6.7. Hold dropper or spray 1 cm above the chosen nostril and instill prescribed number of drops toward midline of ethmoid bone.

5.6.8. Have patient remain in supine position 10 minutes.

5.6.9. Offer facial tissue to blot runny nose but caution patient against blowing nose for several minutes.

5.6.10. Assist patient to a comfortable position after medication is absorbed.

5.6.11. Dispose of soiled supplies in proper container and perform hand hygiene before and after the procedure.

5.6.12. Observe patient for onset of side effects 15 to 30 minutes after administration.

6. Tools to evaluate the efficacy of the proposals listed above and monitor efficacy of treatments

6.1. Ultrasonography of the spleen with echo-color-Doppler

The effectiveness of the therapeutic approaches described in these proposals can be assessed by evaluating vasodilation that is consequent to activation of cells of the immune system in the spleen.

This criterion is useful to evaluate the immediate action of the therapeutic approaches described in these proposals as well as the function of the immune system declines with age and in the presence of chronic diseases.

This procedure is safe, rapid and inexpensive and has been validated in a number of papers including the original studies describing the effectiveness of GcMAF. Since imuno® can be considered a type of immunotherapeutic approach in the same line of GcMAF, such a procedure can be used for imuno® as well.

(https://www.ncbi.nlm.nih.gov/pubmed/24982371
http://thescipub.com/abstract/10.3844/ajisp.2017.114.126).

6.2. PINI (Prognostic Inflammatory Nutritional Index) score

This score is an excellent measure of the nutritional and inflammatory status of the subject. Assessment of the PINI score may be helpful in assessing the individual condition as far as nutrition and inflammation are concerned. More and more links are being found almost daily between environmental factors, obesity, oxidative stress, inflammation, systemic maladies, and aging as well as with central nervous system disorders like Alzheimer's disease, Parkinson's disease, Huntington's and cancer. Therefore, the PINI score should be as low as possible.

This score was developed in 1985 to help insurance companies estimating the life expectancy of elderly people.

https://www.ncbi.nlm.nih.gov/pubmed/3922909

In 2012, Fabris *et al*. demonstrated that PINI score predicted the life expectancy of last stage cancer patients who did not undergo any therapy. Those with a high PINI score had a mean life expectancy of about 6 months *vs* close to 5 years for those with a low PINI score.

http://thescipub.com/abstract/10.3844/ajisp.2012.65.70

PINI score is based on inexpensive blood analyses that, in many cases, do not require a prescription.

6.2.1. The PINI score is calculated as follows:

$$\frac{\text{alpha-1 acid-glycoprotein (mg/dl) x CRP (mg/dl)}}{\text{albumin (g/dl) x prealbumin (mg/dl)}}$$

The score should decrease progressively during the implementation of this protocol.

A simplified version of the PINI score can be obtained by analyzing serum albumin and C-reactive protein (CRP). With the implementation of the protocol, CRP should decrease and albumin increase.

Evaluation of changes of the PINI score may prove useful in cancer as well as in persistent Lyme disease and/or chronic neurodegenerative conditions.

6.3. Autonomic Response Testing (ART)

This test has to goal to evaluate the results of this protocol using an improved version of applied kinesiology. ART represents an evolution and an expansion of the technique that was originally proposed by Omura in 1985 (*https://www.ncbi.nlm.nih.gov/pubmed/6124084*), and subsequently validated in a number of studies including two randomized-order blinded studies registered as a clinical trial (*https://www.ncbi.nlm.nih.gov/pubmed/6747487 https://www.ncbi.nlm.nih.gov/pubmed/27903263*).

In a paper published in 2016, ART, a safe, rapid and inexpensive test, was utilized by independent researchers in the diagnosis and treatment of painful scars. In this study, the Authors state that "In our experience, ART produces useful and consistent information most of the time" (*https://www.ncbi.nlm.nih.gov/pubmed/27458497*). The validity of ART was further confirmed in another independent investigation focused on breast cancer (*https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5134821/)*. At variance with the test proposed by Omura, ART takes into account the entirety of the autonomic response and not only the strength or the resistance of muscles. This is particularly important in the study of parasympathetic activity and in the evaluation of the balance between sympathetic and parasympathetic activities. A core principle of the test relies on the “Resonance Phenomenon Between Identical Substances” (*http://thescipub.com/abstract/10.3844/ajisp.2017.114.126*).

ART may prove particularly useful in assessing the efficacy of this protocol in persistent Lyme disease.

6.4. Nagalase

Nagalase is a good indicator of low-grade systemic inflammation and may be associated with a number of condition where the proposals quoted above may prove useful. One of the most reliable laboratories for this test is the RED Labs in Belgium. With the implementation of the proposals quoted above, nagalase should decrease.

Nagalase testing may prove useful in cancer as well as in persistent Lyme disease and/or chronic neurodegenerative conditions.

6.5. TKTL1 and Apo10 scores

TKTL1 plays a crucial role in ovarian cancer metabolism and its expression predicts poor prognosis (Krockenberger *et al.*, 2010); therefore, a decrease in the expression of TKTL1 may be interpreted as a sign of decreased aggressiveness of cancer itself. It should be noticed, however, that TKTL1 expression is not unique for ovarian cancers and it appears that TKTL1 belongs to a group of metabolic genes involved in the glycolytic pathway that is significantly up-regulated in a variety of tumor cells in cancer patients and plays active roles in tumor progression (Furuta *et al.*, 2010). The cumulative TKTL1 score after treatment with imuno® is expected to decrease.

Apo10 is a marker of abnormal apoptosis and proliferation and it represents an independent marker for poor survival for certain carcinomas (Grimm *et al.*, 2013). Consistent with these observations, it has been recently proposed that overcoming drug resistance of Apo10-positive cells in precursor lesions and tumors by natural compounds may act as sensitizers for apoptosis or could be useful for chemoprevention (Grimm *et al.*, 2015). The cumulative Apo10 score after treatment with imuno® is expected to decrease.

Evaluation of TKTL1 and Apo10 scores in response to treatment may prove useful in cancer.

7. The Ruggiero-Klinghardt Protocol and imuno®

imuno®, by rebalancing the immune-neuro-endocrine axis may prove useful in persistent Lyme disease with particular reference to those situations where the agents responsible for the infection are latent or hidden in sanctuaries that make them inaccessible to therapies. To this end, the Ruggiero-Klinghardt Protocol should be implemented before starting treatment with imuno® with the goal of mobilizing the infectious agents so that the immune system may recognize and fight them. Following is a step-by-step description of the Ruggiero-Klinghardt Protocol adapted for the use with imuno®.

7.1. Autonomic Response Testing (ART).

7.1.1. Perform traditional medical examination involving the collection of anamnesis, the study of previous laboratory and imaging results, and objective examination.

7.1.2. Collect biological samples for laboratory tests before performing Autonomic Response Testing (ART). (Details for the laboratory tests are given in step 7.8).

7.1.2.1. Follow the instructions of the specialized laboratory as far as modalities of the collection of biological samples (urine, stools, blood, serum, and breath) are concerned. The type of matrix has to be selected according to the indication provided for by ART. The anatomical localization indicated by ART will dictate the most suitable matrix.

7.1.3 Perform ART to examine all the aspects pertaining to the autonomous response and not only muscle strength or resistance.

7.1.4. Narrow the spectrum of diagnostic hypotheses and identify organs or the areas of the body that need to be studied by diagnostic ultrasonography in the next step 7.2.

7.1.5. Record the findings of ART that will be used for comparison in step 7.4.

7.1.6. Repeat steps 7.1.3 - 7.1.5 with a different couple of Therapist and Assistant and evaluate and record consistency and reproducibility.

7.2. Diagnostic Ultrasonography.

7.2.1. Use an ultrasound system with color-Doppler application and with a linear and a convex transducer.

7.2.2. Study the organs or the areas of the body indicated by ART.

7.2.3. Look for abnormalities in morphology, echostructure, vascularization, and blood-flow.

7.2.4. Record as many images as possible at different levels of magnification.

7.3. Application of therapeutic ultrasounds.

7.3.1. Select the appropriate pulsed sequence, frequency, and duration of treatment.

7.3.1.1. For the spleen select pulsed sequence indicated as 50%, frequency of 1 MHz, for 3 minutes.

7.3.1.2. For the deep cervical nodes and the vagus nerve, pulsed sequence indicated as 20%, frequency of 3.3 MHz, for 90 seconds on each side of the neck.

7.3.1.3. For other nodes identified by ART and ultrasonography, pulsed sequence indicated as 20%, frequency of 3.3 MHz, for 90 seconds in correspondence of each node.

7.3.1.4. For the brain, pulsed sequence indicated as 10%, a frequency of 3.3 MHz, for 90 seconds on each side of the head using the temporal acoustic window.

7.3.2. Apply therapeutic ultrasounds with slow circular movements in order to send the ultrasound waves to the targeted organ or structure. Use abundant gel.

7.3.3. Invite the patient to exercise or to breathe slowly and deeply for about 5 minutes after the last application of therapeutic ultrasounds.

7.4. Second ART.

7.4.1. Perform ART; compare the results with those obtained in step 1 and record the results and the comparisons.

7.4.2. Evaluate and record consistency and reproducibility with a different couple of Therapist and Assistant and compare the results with those obtained in step 7.1.6.

7.5. Biological sample collection.

7.5.1. Six hours after step 7.3, collect biological samples as in step 7.1.2.1.

7.6. Patient treatment with imuno®.

7.6.1. Treat the patient according to the indications given above as adapted to the specific condition identified with the previous steps.

7.6.2. Perform ART to fine-tune the dose and way of administration of imuno®.

7.6.2.1. Confirm the potential efficacy of the treatment fine-tuned by ART in the preceding step, using diagnostic ultrasonography (color-Doppler) as described above in "Tools to evaluate the efficacy of the proposals listed above and monitor the efficacy of treatments".

7.6.3. Apply targeted therapeutic ultrasounds as described in step 3 with the goal of exploiting the known therapeutic effects of pulsed ultrasounds that comprise anti-inflammatory effects, enhanced lymphatic drainage, and optimization of imuno® uptake and utilization.

7.7. Evaluation of efficacy and assessment of end-point.

7.7.1. Repeat steps 7.1 to 7.6 to evaluate the efficacy of the treatment and its end-point.

7.8. Laboratory tests; please see "Tools to evaluate the efficacy of the proposals listed above and monitor the efficacy of treatments".

7.8.1. The type of test to be performed according to the Ruggiero-Klinghardt Protocol has to be chosen according to the individual needs; for example, the test defined EliSpot may be preferred in selected patients

7.8.2.1. Examples of laboratory tests for persistent Lyme disease.

7.8.2.2. Examples from different laboratories

Analysis		Result	Units	Reference Range	Chart
Borrelia EliSpot					
1 Borrelia b. Full Antigen	!	**12**	**SI**		
0-1 = negative 2-3 = weak positive > 3 = positive					
1 Borrelia b. OSP-Mix	!	**16**	**SI**		
0-1 = negative 2-3 = weak positive > 3 = positive					
1 Borrelia burgdorferi LFA-1	!	**3**	**SI**		
0-1 = negative 2-3 = weak positive > 3 = positive					

	Remark	Borrelia sequence confirmed at GIGA Sequencing University of Liege	
Bartonella	Bartonella	Negative	Negative
Borrelia	Borrellia	**Positive**	Negative
CD57	CD57 Absolute count	**35**	60,00 - 360,00 cells/µl
CYMV	Cytomegalovirus	0	0,00 - 50,00 Cpies/million cells
EPBV	Epstein-Barr virus	0	0,00 - 50,00
HHV 6	Human Herpesvirus 6	0	0,00 - 50,00 copies/million cells

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Analysis	Result	Units	Reference Range	Chart
Borrelia burgdoferi Elispot				
Borrelia burgd. fully antigen	**+ 20**	SI	< 2	
Borrelia peptide mix	**+ 10**	SI	< 2	
Borrelia LFA-1	**+ 3**	SI	< 2	
Diagnosis Borrelia				
The Elispot indicate a cellular activity against Borrelia burgdorferi.				
Anaplasma phagocytophilum Elispot				
Ehrlichia Elispot LTT	**+ 14**	SI	< 2	
Diagnosis Ehrlichia				
The Elispot indicates cellular activity against Ehrlichia.				
Chlamydia pneumoniae Elispot				
Chlamydia pneumoniae Elispot LTT	**+ 70**	SI	< 2	
Diagnosis Chlamydia pneumoniae				
The Elispot indicates cellular activity against Chlamydia pneumoniae. A cross-reaction in the Elispot-LTT between Chlamydia trachomatis and Chlamydia pneumonia can not be ruled out.				
Chlamydia trachomatis Elispot				
Chlamydia trachomatis Elispot LTT	**+ 101**	SI	< 2	
Diagnosis Chlamydia trachmatis				
The Elispot indicates cellular activity against Chlamydia trachomatis. A cross-reaction in the Elispot-LTT between Chlamydia trachomatis and Chlamydia pneumonia can not be ruled out.				
Cytomegalo-Virus Elispot				
Cytomegalo Virus Elispot LTT	**+ 7**	SI	< 2	
diagnosis cytomegalovirus				
The Elispot indicate cellular activity against Cytomegalo-Virus.				

7.9. Considerations on the use of the Ruggiero-Klinghardt Protocol

The critical steps in the Ruggiero-Klinghardt Protocol are represented by ART and therapeutic ultrasound. ART is used to achieve different purposes. Thus, the initial ART (step 7.1) has the goal to identify the organs or the areas of the body that require further investigation; to narrow the diagnostic hypotheses and provide information on the underlying pathology *i.e.* the presence of pathogens, neoplastic cells, abnormal cells or toxicants. ART is then repeated as step 7.4; this second ART has the scope of evaluating whether therapeutic ultrasounds used in step 7.3 were successful in mobilizing pathogenic noxae from sanctuaries or reservoirs and making them "visible" to the Therapist performing ART. Thirdly, ART of step 7.6.2 (specific patient treatment) serves the scope of fine-tuning the therapeutic choice. The other critical step (step 7.3) is represented by the use of therapeutic ultrasounds that, in the context of the Ruggiero-Klinghardt Protocol, have the role to "squeeze" at the cellular and molecular level the organs or the tissues that may offer a hide to pathogens or other noxae. Such an effect is then confirmed by the following step 7.4 as described above. The Ruggiero-Klinghardt Protocol is designed as a recursion of diagnostic procedures that serve to confirm each other with the goal of achieving accurate and early diagnosis in elusive conditions.

The following clinical observation shows an example of diagnosis obtained in an elusive case of persistent Lyme co-infection. A patient with a history of angina with negative results at conventional cardiology tests (such as stress echo etc.) had his urine samples collected before and after application of therapeutic ultrasounds, that is in step 7.1.2, and, subsequently, in step 7.5.1. In the urine sample collected at step 7.1.2, PCR-based DNA analysis did not evidence any pathogen among those studied in the Lyme panel even though ART had indicated the presence of Bartonella species. It is worth noticing that the patient had also tested negative for the presence of antibodies against Bartonella Henselae (IgG/IgM), and negative also in the Western Blot test (CDC criteria) in the blood sample obtained the same day, but before implementation of the protocol. This patient was one of the several cases where ART suggested a diagnosis that was not confirmed by laboratory tests. However, DNA test performed on the urine sample collected as per step 7.5.1, clearly evidenced one well-identified pathogen, Bartonella bacilliformis, a pathogen that may cause of endocarditis, albeit this infection is commonly diagnosed only post-mortem. In other words, the application of the protocol enabled laboratory confirmation of ART findings; confirmation that had not been possible without implementing the protocol.

The Ruggiero-Klinghardt Protocol borrows the "shock and kill" approach that is used to eliminate the reservoirs of HIV that are responsible for the latency and persistence of the virus. This "shock and kill" strategy pursues the goal of stimulating HIV replication in a latent viral reservoir; at first sight, such a strategy may appear counterintuitive as the objective of pharmacological antiretroviral therapies is to block, not to stimulate, HIV replication. However, the rationale behind this approach, as in the Ruggiero-Klinghardt Protocol, is to render the hidden virus "visible" to the immune system and to the specific approaches such as imuno®. Thus, the scope of the Ruggiero-Klinghardt Protocol is to render pathogens, toxicant, neoplastic cells or cells infected by viruses that would otherwise be inaccessible to diagnostic and therapeutic tools, "visible" so that they can be identified and fought by the Therapist and by the body's immune system.

The Ruggiero-Klinghardt Protocol represents a novelty in the field of diagnostics and therapeutics because it aims at achieving a higher degree of precision combining in an integrated and logically sequential manner, techniques and procedures that have been used for decades. In respect to the existing methods the Ruggiero-Klinghardt Protocol offers the advantage of being safe and relatively inexpensive since it does not require sophisticated instruments; because of this, it can be implemented in different parts of the world.

Other applications of this protocol are in the field of neurodegenerative and neurodevelopmental conditions with particular reference to autism. Thus, there is preliminary evidence indicating that a number of pathogenic noxae that were undetected in autistic subjects can be evidenced with the application of this protocol.

7.10 Peer-reviewed literature

The Ruggiero Klinghardt Protocol