LOW DOSE MEDICINE

The new Paradigm

Innovative therapies in Dermatology

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terapie d'avanguardia

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GUNA S.p.a.
Advanced Therapies
Company Introduction

Dear Doctors,

Through the points of our "Manifesto", I'm pleased to guide you for a brief journey in our history, our corporate philosophy, our commitment to research, our successes, and our goals and meta-goals we have reached in these 30 years since the inception of Guna S.p.a. In addition, I would like to share with you those goals and meta-goals we are targeting to achieve in the future.

Encounters with eminent personalities of global Dermatology have led us, in recent years, to invest significant resources in finding new pharmacological solutions to complex diseases such as *Psoriasis*, *Vitiligo* and *Atopic Dermatitis*, for which, again, Medicine has not offered definitive therapeutic responses and for which millions of people in the world carry upon their shoulders the burden of physical, psychological and social suffering that are caused by these diseases.

Today, we present to you not only our Company, but the results of our pre-clinical and clinical research. We are aware of being at the commencement of a long journey; however, more importantly, we are also aware of being on the right track and look forward with great enthusiasm to what lies ahead and to the ability to create positive, lasting change in the lives of millions of people across the globe.

Included in this brochure, along with the presentation of our Company, you will find a chapter dedicated to the fundamentals of *Low Dose Medicine* and Research in this field. One chapter, by *Prof. Dr. Torello Lotti*, illustrates the possibilities of the Medicine of low doses in the treatment of dermatological chronic autoimmune inflammatory diseases, the steps taken thus far, and those steps to be taken from today.

Thank you for your time, attention, and dedication,

Alessandro Pizzoccaro
President and Founder of Guna S.p.a.

The Manifesto

Vision-Mission-Values

A Pharmaceutical
Company

Guided by
ethical principles

Devoted to
scientific research

Focused on
education and scientific information
addressed to doctors and pharmacists

Aimed at promoting
biological medicinal products:
-unique -effective -innovative -without side effects

Targeted at affirming the values and the model of
Human Centered Medicine
in harmony with Nature

Contributing to make the World a
better place to live

A Pharmaceutical Company

GUNA: advanced therapies

From the dream of a person-centered Medicine in harmony with nature, from a modern and visionary idea of effective drugs with no side effects, from the project of a company founded on solid ethical principles and devoted to research, Guna S.p.a. was founded in Milan in 1983.
Today, with over 260 employees (production, logistics, administration, marketing, export, research and development, medical-scientific information) and a presence in over 30 countries on 4 continents, Guna is one of the world's most intriguing companies in the field of Biological Pharmaceuticals, Nutraceuticals and Cosmeceuticals.

Guna S.p.a. is the leader in Italy in the segment of biopharmaceuticals.

1983 — GUNA was founded in Milan.

1983 — Creation of the magazine "The Biological Medicine".

1989 — Construction of the first production facility.

1993 — Begins production of cytokines, hormones, neuropeptides and growth factors in activated low doses.

1996 — Founding of the Italian Academy of Biological Medicine.

2001 — Begins international development through Eastern Europe.

2003 — International expansion continues through Central America and South America.

The Production
30 years of Quality, Technology and Innovation

Technological innovation is the principle which has inspired Guna since its beginning.
The new production facility, opened in 2008, covers an area of 65,000 square feet on four levels and represents futuristic technological and pharmaceutical solutions.
Guna Laboratories are authorized in accordance with the international requirements of **Good Manufacturing Practice** (GMP) and all the production procedures are approved in accordance with **ISO 9001: 2008** and **ISO 13485: 2012** ***Medical Devices*** rules.

Over 800 products are part of Guna's wide range:

- **Drugs**
- **Food Supplements**
- **Medical Devices**
- **Cosmeceuticals.**

Since 2006 more than
70 products are FDA *listed.*

2005 — GUNA Inc. was founded in the United States.

2006 — Over 70 products are FDA *listed.*

2006 — Guna obtains the Quality Certification UNI EN ISO 9001: 2000.

2008 — Inauguration of the new production facility in Milan.

2009 — Publication of the work "Gariboldi et al. *Low dose oral administration of cytokines for treatment of allergic asthma*". Pulmonary Pharmacology & Therapeutics 22 (2009) 497-510) which highlights the scientific community's ability to treat disease with low doses of activated cytokines.

2012 — The University of Milan founded the CREBION - Interdepartmental Research Centre for the Study of Biological Effects of Nano-concentrations, of which Guna is an Official Partner.

2013 — Guna begins the research into the effects of low doses of cytokines and growth factors in skin diseases.

Guna Medicines
30 years of Efficacy, Safety, and Scientific Progress

Guna Laboratories are known throughout the world for their medicines which are **effective and without side effects**. Studies of Molecular Biology and Biochemistry, applied to medicinal plants and to biological molecules (neuropeptides, cytokines, hormones and growth factors), have led Guna to the discovery of the extraordinary therapeutic possibilities of active ingredients used in its products.
The use of **low doses** is the fulcrum on which revolves Guna Pharmacology. Efficacy without side effects is the result of the encounter between low dosages of active principles and the innovative pharmaceutical technique called ***SKA (Sequential Kinetic Activation)***, developed and standardized by Guna Laboratories.
The SKA technology allows the use of lower dosages of biological molecules (under the minimum dose considered pharmacologically active), giving them an effectiveness comparable to the doses normally used in Pharmacology.

Guided by ethical principles

It is not only important what we do,
it is equally important the way we do it.

We are aware that gain is the target of a company, and we recognize this aspect of our work without any hypocritical attempt to argue otherwise, but we are equally convinced that the search for gain cannot and should not be separated from ethics and meta-social objectives and that business can have a human face.

We believe that there can be a model of business where interests of the shareholders and the interests of the community can coexist because, as the community evolves and improves the quality of life for its people, a dynamic company improves its position and expands its activities.

As a company, we know we have many responsibilities: we must invest in scientific research, in employment, in technological innovation, in the well-being of our employees and in the improvement of the land in which we live; we know we have to work every day with intensity, skill and passion to improve the health of people living in the countries where we do business and, in general, promote a more conscious approach to health and disease.

We want our medicines to be accessible to everyone, and that the access to health becomes a basic human right recognized everywhere in the World: therefore, we decided to make available our know-how to all. Guna is a **patent-free** and **copyleft** company: we think, in fact, that the circulation of ideas and information are the engines of progress.

Global Customer Satisfaction: we want to be sure that our customer is always satisfied with the services we offer, from the quality of the production process to the therapeutic results of each individual patient.

Devoted to Scientific Research

We want to be sure of the effectiveness of our products

Scientific Research and **Clinical Trials** are one of the most important pillars underpinning the success of Guna in the global pharmaceutical field.

Every year Guna invests an abundant amount of capital in Basic and Clinical Research.

The Research and *Development Center* of Guna S.p.a. collaborates with some of the most prestigious Italian and foreign Universities, supporting, in particular, the research regarding the biological activity of low doses and contributing to the creation of a new pharmacological paradigm.

Through the **Basic Research** on isolated cell lines and animal models, Guna has led the way to the understanding of action mechanisms of activated nanoconcentrations, demonstrating their effectiveness and safety. In addition, over the past eight years, a great push was given to **Clinical Research** for the definitive validation of the therapeutic effects on patients.

Through close collaboration with some of the most prestigious Italian Research Institutes and some of Italy's most renowned Universities (*Istituto Superiore di Studi Sanitari, Rome; Hospital "Fatebenefratelli", Rome; "Polyclinic Institute", Milan; "Sapienza" University, Rome; Hospital "City of Health and Science" Turin, etc.*), Guna launched in 2010 the Clinical Research Project, initiating numerous trials on a number of high prevalence diseases and developing advanced therapeutic approaches. Today, numerous basic and clinical research studies are published in high impact factor international journals.

Thanks to the efforts in the field, in 2015, the Guna pipeline includes numerous ongoing clinical research projects, while others are already under construction and others almost completed, including studies on Crohn's Disease, Rheumatoid Arthritis, Vitiligo, Fibromyalgia, Rotator Cuff Syndrome, Knee Osteoarthritis, and Recurrent Respiratory Infections (RRI) in pediatric population.

Guna is an official partner of C.R.E.B.I.O.N. (*Interdepartmental Research Centre for the Study of Biological Effects of Nano-Concentrations*) of the University of Milan.

Focused on education and scientific information addressed to Doctors and Pharmacists

We work to form expert physicians and conscious patients

Since its founding, Guna has been characterized for the strategic role assigned to the training of Doctors and Pharmacists and to patient information.

Independently or through the support of Medical associations, Guna organizes annually, just in Italy alone, more than 600 teaching days, structured as workshops or annual or multi-year courses. Such commitment to education has been and still is indispensable to provide, in replacement of the assigned institutes of higher learning, an adequate knowledge of Low Dose Medicine, not yet included in university programs, but requested by doctors, pharmacists and patients more and more.

In recent years and on the driving force of the rising research achievements in the field of low dosages, there has been a growing interest by academic institutions regarding Low Dose Medicine. Since 2011 several Italian universities, including Parma, "Sapienza" Rome, Siena, Novara, "G. Marconi" Rome, have established Postgraduate Courses and Master Degree Programs.

In parallel, outside Italy, the therapeutic success of Guna medicines, their innovative spirit and research results, have brought Low Dose Medicine into the classrooms of some of the most important universities of the World: Loyola University of Chicago, the University of Wisconsin, Madison, the Miller Medical School in Miami, Arizona State University in Phoenix and the Royal Society of Medicine in London. Every year thousands of doctors all over the world attend seminars which are sponsored by Guna and its partners.

Passion, expertise, teacher experience, along with an agility that is oriented to a practical, immediate teaching style make Guna courses a benchmark of excellence for medical updating.

Correspondingly, a network of Medical-Scientific Representatives and Pharmacy Consultants has been established which is exemplified by professionalism and expertise that strengthen the knowledge of the scientific basis of Low Dose Medicine and its clinical application possibilities.

Aimed at promoting biological medicinal products:

Unique - effective - innovative - without side effects

Original medicines, innovative technologies

Innovations, research, experimentation, effectiveness, and absence of side effects are the characteristics that have driven Guna's products to affirm itself in the world.

Guna has developed and continues to research innovative medicines for a wide spectrum of diseases in numerous therapeutic areas.

We produce only natural and effective drugs with no side effects, which are able to physiologically stimulate the biological processes underlying the maintenance or recovery of homeostatic conditions and respect the health of every single person.

For this reason the road for a product to be a "Guna product" is very long:

Only one idea in 1000 becomes a Guna product.

Therapeutic areas

- Allergology
- Anti Ageing And Regenerative Medicine
- Dermatology
- Gastroenterology
- Clinical Immunology
- Aesthetic Medicine
- Preventive Medicine
- Ophthalmology
- Oncology
- Pediatrics
- Rheumatology
- Pain Therapy

Targeted at affirming the values and the model of Human Centered Medicine in harmony with Nature

Improving patients' life – generating wellbeing

- We believe that every patient is first of all a man who suffers.
- We think that health is not just absence of disease.
- We believe that man is mind, body and spirit.
- We think it is possible to act on the causes and symptoms of the disease together.
- We think that there can exist drugs without side effects.
- We think that Nature is the most extraordinary "pharmaceutical industry".
- We think that a Medicine that understands the intimate mechanisms of both Biology and human Physiology is able to develop a new generation of drugs: biological, effective, respectful of the human being and in harmony with Nature.

. . . We believe that this Medicine already exists.

A NEW PARADIGM IN MEDICINE THE LOW DOSE MEDICINE

Scientific basis, Fundamentals of Methodology, Pre-clinical and clinical research

Alessandro Perra
Scientific Director - Guna S.p.a.

Per minima ad maxima

Since 1994 Guna S.p.a. has invested significant financial and intellectual resources, firstly into the study of a new therapeutic concept and secondly, into the research and development of pharmacological principles which are the players of this new vision of Medicine. The results of pre-clinical and clinical research and newly emerged therapeutic scenarios have brought Guna S.p.a. to the limelight of the interest of the Italian[1] and international[2] scientific community, accelerating the worldwide expansion of the company. From Guna's idea of a human centered Medicine and in harmony with Nature, *Low Dose Medicine* (LDM) was born.

[1] *in 2012 the University of Milan established the C.R.E.B.I.O.N. - Interdepartmental Research Center for the Study of Biological Effects of Nano-Concentrations and, since 2011, several Italian universities, including Parma, "Sapienza" Rome, Siena, Novara, "G.Marconi" Rome, have established Postgraduate Courses and Master Degree Programs in Low dose Medicine.*

[2] *lectures and seminars were held at Loyola University of Chicago, Miller Medical School in Miami, Arizona State University in Phoenix, the University of Wisconsin Madison, the Royal Society of Medicine in London, to name only some of the most prestigious foreign institutions.*

The Low Dose Medicine

Low Dose Medicine (LDM) was born from the merging of Molecular Biology with Psycho-Neuro-Endocrine-Immunology (PNEI), and was developed in recognition of research results in the field of the pharmacology of low doses.

Low Dose Medicine is a person-centered Medicine, based on three guiding principles:

- to treat the man and not just the disease;
- to act on the causes and not just the symptoms;
- to consider man as a whole mind-body and in his individuality.

Low Dose Medicine starts from an original idea in the medical field: to bring back a sick organism to the original physiological condition through the use of the same biological molecules normally present in the body and that, in healthy conditions, monitor and guide body functions.

In fact, many of these molecules are known and studied by Molecular Biology, which, not surprisingly, defines them as signaling (messenger) molecules, namely substances that are able to transport to different cells in the body the "right instructions" for their proper operation.
These molecules are *neuropeptides*, *hormones*, and *cytokines*, along with *growth factors*, fundamental regulatory molecules, and tissue stimulating molecules.

Signaling Molecules and the P.N.E.I. network

Since the 1970s, research in the fields of Physiology and Molecular Biology has given increasing evidence to the critical role of signaling molecules in all physiological and pathological processes.
It is recognized that these substances play a decisive role in determining the state of health or of illness and it is now established that each disease is the expression of mutated concentrations, in excess or deficiency, of these substances; all the medical research is moving toward the study of signaling molecules, which determine the fate in a positive (healing) or negative (progression) way of numerous pathological conditions, and the possibility of their application for therapeutic purposes.

In hand with the acquisitions on the signaling molecules, in the last years we have witnessed in the medical field, the gradual abandonment of the separatist and scotomized conception of the biological functions of the body, giving way to a more unified one in accordance with the guiding principles of Psycho-Neuro-Endocrine-Immunology (P.N.E.I.) [1-4].

P.N.E.I. approach represents a genuine paradigm shift in Medicine: from a strictly biomedical and specialized view of health and disease to a deeply interdisciplinary one. The main unifying P.N.E.I. element is identified in the cross-talk between the psychoneuroendocrine systems and the immune system *(Figure 1)*.

This sophisticated cross-talk is mediated by a complex network of signaling molecules (cytokines, hormones, neuropeptides and growth factors) which are the vehicle of the biological information necessary for the complex and efficient regulation of cellular responses to stimuli. An altered cross-talk due to an imbalance between specific signal molecules is fundamental, for example, in inflammatory, allergic and autoimmune diseases onset [5-7]; restoring the physiological concentration of signaling molecules is the key point to recover the homeostatic equilibrium.
In homeostatic conditions (corresponding to a healthy state), in fact, the concentrations of these molecules in the extra-cellular matrix (ECM) [8; 9] are comprised in a specific physiological range (from nanograms/ml to picograms/ml) and diseases can be considered as expressions and consequences of changed concentrations of these fundamental substances [10-13] *(Figure 2)*.

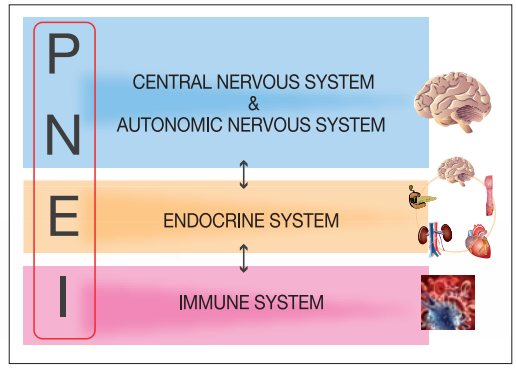

Figure 1 - Network P.N.E.I. and bi-directional *cross-talk* between the systems.

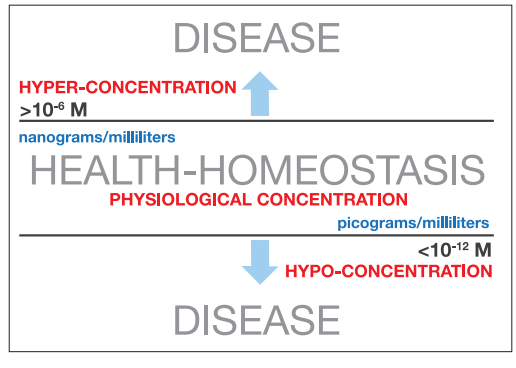

Figure 2 - Range of physiological concentration of signaling molecules at the level of the extracellular matrix.

The use of biological molecules, which control and drive cellular functions in order to restore the original homeostatic physiological condition, is the core of Low Dose Medicine.

For a deep understanding of Low Dose Medicine, it is necessary to consider some key points related to cross-talk between the systems of P.N.E.I. network (and between the cells) mediated by signaling molecules:

1. The cross-talk between cells, organs and systems is always bi-directional, such as the effects of the alteration of the cross-talk itself [14-16];
2. The intercellular signaling occurs through the diffusion of signaling molecules in the extracellular matrix (ECM): states of pathological alteration of the ECM lead to a deterioration in the quality of the communication between cells and, in general, between organs and systems [17,18];
3. The ligands-receptors interaction is crucial for the efficacy of the signal transduction in terms of quality and potency: substrate concentration and binding properties such as affinity and saturation phenomena are key parameters [19, 20].

The signaling molecules used in LDM are orally administered and their activity is systemic (working on complex cell signaling pathways). Scientific literature reports that cytokine oral intake is effective in modulating immune response [21-23] and a possible action mechanism involves M cells at intestinal epithelium level. Signaling molecules are taken by M cells from intestinal lumen and presented to immune T cells within Peyer's patches lymph nodes [24] inducing an appropriate immune response *(Figure 3)*.

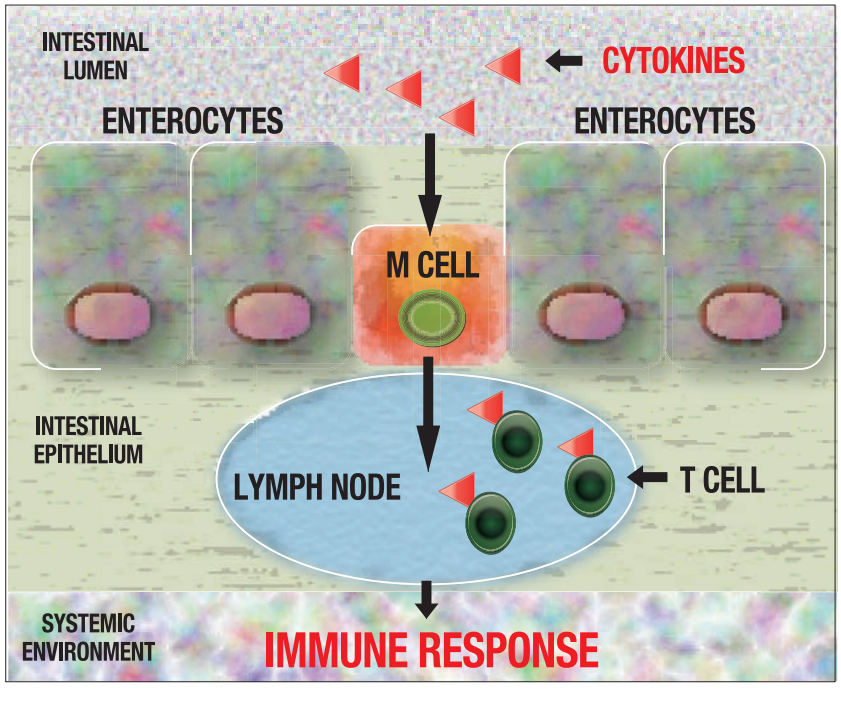

Figure 3 - The role of M cells in the processes of absorption of signaling molecules at the level of the intestinal lumen.

How *"low"* must a *low dose* be (to be effective and safe)?

A critical point of signaling molecules (and peptides in general) oral administration is represented by their low bioavailability (typically less than 1-2%); an effective drug delivery system is requested in order to improve this key parameter. The use of *physiological low doses* (nanograms-picograms) *(Figure 2) per os* in LDM is made possible by the application of *SKA technology* (Sequential Kinetic Activation), a particularly sophisticated *drug delivery system*, based upon the principles of Quantum Physics ("release activity": ability of the basic substance to release its activity in the aqueous milieu) [25], which allows the nano-concentrations to be active, even below the actually considered minimum effective dose, with therapeutic results comparable to those induced by high concentrations. The action mechanism of SKA low dose cytokines, hormones, neuropeptides and growth factors consists in sensitization or activation of some units of cellular (or plasmatic) receptors in virtue of their high dilution, [practically in their physiological working range between 10^{-6} molar (microgram) for hormones [8] and 10^{-12} molar (picogram) for the other signaling molecules [9] *(Figure 4)*]. This receptors sensitization allows the trigger of chain reactions (complex systems) and a restart of the biological function of the whole P.N.E.I. network. SKA low dose molecules work by bringing to the system information able to activate auto-regulation mechanisms.

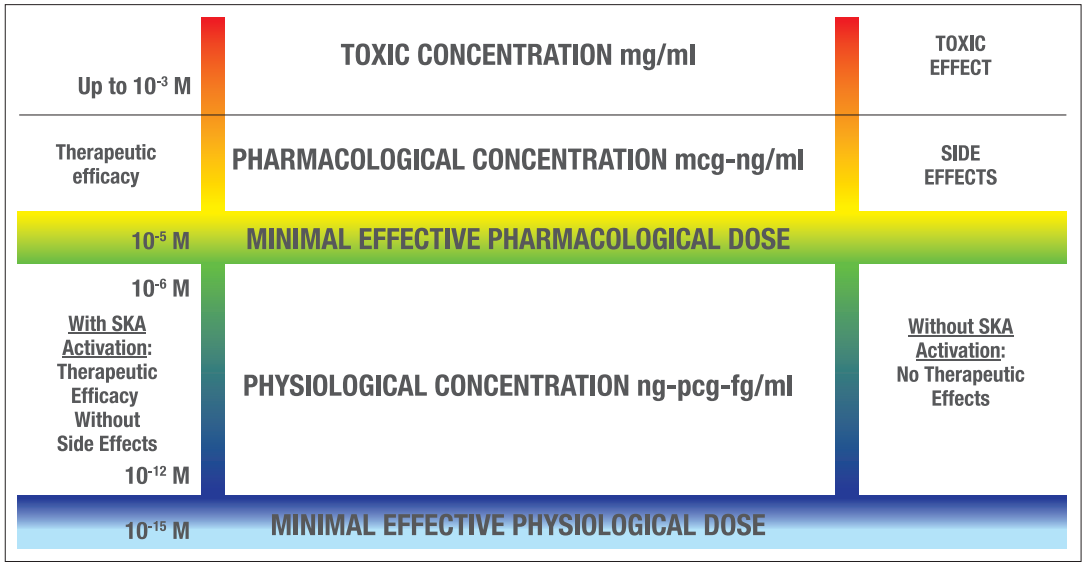

Figure 4 - Relationship between signaling molecules concentration and their effects.

Given these premises, from a clinical point of view, the therapeutic approaches are:

1. To restore the homeostasis of a pathologically *down-regulated* cellular pathway administering the **same** cytokine, hormone, neuropeptides or growth factor (**low dose SKA**) that is physiologically involved in the cellular signaling;
2. To use **antagonistic** molecules (**low dose SKA**) in order to down-regulate the molecules pathologically *up-regulated*, restoring their homeostatic concentration *via* negative feedback mechanisms, according to the principle of "opposing" molecules.

The ability to correct, for example, alterations of the immune system with the use of cytokines or endocrine disorders with the use of hormones, represents one of the most fascinating and innovative research fields in Molecular Biology applied to Medicine; unfortunately the clinical application of this knowledge has always been brought to a standstill by the side effects that these substances show when used at high doses, those normally used until now.

Bio-Tech and low dose

The latest knowledge in the field of biotechnology production of human recombinant proteins allowed GUNA S.p.a. to realize medicines of highest quality and efficacy. Thanks to the aforementioned pharmaceutical technique called **SKA** (**Sequential Kinetic Activation**), discovered, codified and standardized by Guna Laboratories, it is now possible to use low dose of hormones, neuropeptides, cytokines and growth factors with therapeutic results comparable to those induced by high concentrations but without side effects.
The Guna SKA method inaugurates a new era within the possibility of clinical use of signaling molecules; the "scientific dream" to use biological molecules such as cytokines, hormones, neuropeptides or growth factors at low dosages (the only possible not to induce side effects) becomes today possible thanks to the particular pharmaceutical procedure used by Guna in the production of these molecules.

A new frontier in the fields of pharmaceutical industry and Molecular Biology is born and it is developing all over the world; Italian researchers and Italian industry are cutting-edge in this field.

The Research

Basic and clinical research has underpinned the thesis of Low Dose Medicine: in November 2009, in fact, the journal *Pulmonary Pharmacology & Therapeutics* published the first paper on the effects of low dose SKA cytokines in an animal model of allergic asthma (Gariboldi *et* al. *Low dose oral administration of cytokines for treatment of allergic asthma.* Pulmonary Pharmacology & Therapeutics 22 (2009) 497-510) [26].
Since 2009, new publications [26-30] have followed the paper published by Gariboldi and colleagues *(Table 1)*.

Year	Authors	Journal	Research type	Title	Tested molecules
2009	Gariboldi S. *et* al.	Pulmonary Pharmacology & Therapeutics	***In vivo*** **basic research**	Low dose oral administration of cytokines for treatment of allergic asthma.	**IL-12** **IFN-γ**
2012	D'amico L. *et* al.	Journal of Cancer Therapy	***Ex vivo*** **basic research**	Low Dose of IL-12 stimulates T Cell response in cultures of PBMCs derived from Non Small Cell Lung Cancer Patients.	**IL-12**
2013	Cardani D. *et* al.	Gastroenterology Research	***In vivo*** **basic research**	Oral administration of Interleukin-10 and Anti-IL-1 Antibody ameliorates experimental intestinal inflammation.	**IL-10** **Anti IL-1**
2014	Radice E. *et* al.	International Immunopharmacology	***Ex vivo*** **basic research**	Low-doses of sequential-kinetic-activated interferon-gamma enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study.	**IFN-γ**
2014	Roberti ML. *et* al.	Journal of Biological Regulatory & Homeostatic Agents	**Clinical Trial**	Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in *Psoriasis Vulgaris*.	**IL-4** **IL-10** **IL-11**
2015	Luchetti P.	Minerva Medica Oftalmologica (in press)	**Clinical Trial**	Increasing of visual function in patients with retinal atrophy treated with Low Dose Medicine drugs. Monocentric retrospective observational study.	**NT3** **NT4** **NGF**
2015	Galli E. *et* al.	Submitted	**Clinical Trial**	Clinical and immunological evaluation of long-term treatment with Low Dose Medicine in pediatric population affected by *chronic Atopic Dermatitis*. Experimental randomized double-blind at two stages clinical trial.	**IL-12** **IFN-γ**
2015	Fiorito F. *et* al.	Submitted	**Clinical Trial (veterinary)**	Clinical improvement in feline herpesvirus-1 infected cats by oral low doses of Interleukin-12 and Interferon-gamma.	**IL-12** **IFN-γ**
2015	Barygina V. *et* al.	Submitted	***In vitro*** **Basic Research**	The role of IL-4, IL-10, b-FGF and β-endorphin in modulating intracellular redox status and proliferation rate in human skin keratinocytes subjected to oxidative stress.	**IL-4,** **IL-10,** **b-FGF** **β-endorphin**

Table 1: Main papers published and submitted in the field of Low Dose Medicine since 2009.

Basic research studies on *low dose* SKA cytokines with stimulatory activity of immune cell response.

In the papers published by D'Amico *et* al. and Radice *et* al. the LDM approach is proposed and verified in two *in vitro/ex vivo* models based upon the stimulation of different immune cell subpopulations collected from oncologic patients.
Both works are designed to evaluate the effect of specific low dose SKA cytokines (involved in both differentiation and stimulation of immune cells) capable of stimulating the immune response in presence of neoplastic disease.

- D'Amico and colleagues [D'Amico L, Ruffini E, Ferracini R, Roato I (**2012**) *Low Dose of IL-12 stimulates T Cell response in cultures of PBMCs derived from Non Small Cell Lung Cancer Patients.* Journal of Cancer Therapy 3: 337-342] conducted a study on *ex vivo* PBMCs obtained from the peripheral blood of patients with **Non Small Cell Lung Cancer** (NSCLC). The aim of this study was to assess immunostimulatory and immunomodulatory activity of low dose **SKA IL-12 (0.01 pg/ml)** on the subpopulation of T-lymphocytes. Low dose SKA IL-12 was proved to be capable of stimulating both $CD4^+$ and $CD8^+$ T lymphocytes and in particular the increase of $CD4^+$ T cells expressing IFN-γ and simultaneously the increase of the cytotoxicity of the $CD8^+$ T lymphocytes was observed. It was also found that the action of IL-12 is also directed to the Treg cells with the function of down-regulation, particularly important because of the increase of this subpopulation in the oncologic patient. The work also showed a significant increase of lytic activity against H1373 cells exerted by $CD8^+$ lymphocytes.
D'Amico and colleagues also indicated the concentration of 0.01 pg/ml as the more active.

- Radice and colleagues [Radice E, Miranda V, Bellone G (2014) *Low-doses of sequential-kinetic activated interferon-gamma enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study Intern.* Immunopharm 19(1): 66-73] conducted a study on *ex vivo* natural killer cells obtained from the peripheral blood of patients with **early stage colorectal carcinoma (CRC)** - in the presence or absence of metastasis - and from healthy donors. The aim of this study is to assess immunostimulatory and immunomodulatory activity of low doses of IFN-γ on PB-NK cells.

In particular, the lytic ability of PB-NK cells suitably stimulated with **IFN-γ** in conventional dosage (1 ng/ml) or **low-dose SKA IFN-γ (0.25 fg/ml)** is evaluated.
The PB-NK cell activity is depressed in increasing manner in relation to the development stage of the tumor; both administration of IFN-γ at the conventional dosage of 1 ng/ml and SKA IFN-γ low dose (0.25 fg/ml), enhances the cytotoxicity of PB-NK cells from healthy volunteers and in patients affected by early stage CRC, demonstrating the non-inferiority of the LDM treatment.

Study	Type	Cytokines	Positive Control	Placebo Control	Results
D'Amico L. *et* al. Low Dose of IL-12 stimulates T Cell response in cultures of PBMCs derived from Non Small Cell Lung Cancer patients.	***Ex vivo*** **Basic Research**	SKA low dose rIL-12 (1/0.01 pg/ml)	rIL-12 (10 ng/ml)	(vehicle)	• Stimulation of $CD4^+$ and $CD8^+$ T-cells. • Increased number of $CD4^+$ T-cells secreting IFN-γ. • Increased lytic activity of $CD8^+$ T-cells. • Treg suppression.
Radice E. *et* al. Low-doses of sequential-kinetic-activated interferon-gamma enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer.	***Ex vivo*** **Basic Research**	SKA low dose IFN-γ (0.25 fg/ml)	(rIFN-γ 1 ng/ml)	—	• Reduction of PB-NK cells cytotoxicity related with tumor progression stage. • Low dose SKA IFN-γ activates PB-NK cells in early stage CRC. • Low and high doses of IFN-γ show the same activity on PB-NK cells.

Table 2: Synopsis of the key points of the studies of basic research on low dose SKA cytokines with stimulatory activity on the response of immune cells.

The most relevant topics of the two works are resumed in *table 2*.

From the studies of D'Amico *et* al and Radice *et* al it is clear that the use of low dose cytokines SKA is highly effective in the proposed models. The presented studies provide in both cases the comparison with an internal positive control given by the presence of a suitable group treated with the same cytokines but in conventional doses. In both cases non-inferiority of the low dose SKA treatment is demonstrated when compared to conventional one; additionally, in the work of D'Amico and colleagues the high-dose IL-12 treatment (10 ng/ml) leads to a concomitant down-regulation of CD4+ cells and, in particular of Th1 lymphocytes, an event that is not recorded in the low-dose treatment.
Therefore, both studies suggest a **profile of efficacy and safety** of LDM highlighting the absence of the adverse effects normally attributed to the tested cytokines (when administered at high doses) [31,32].

Basic research studies and clinical trials on *low dose* SKA cytokines with rebalancing activity on the Th1/Th2 response.

Numerous pathologies with an important inflammatory component are characterized by the presence of a shift in the immunological balance which is mainly reflected in an imbalance between the cytokines expressed by the two major lymphocyte subpopulations: Th1 and Th2.
Depending on the prevalence of an immune response attributable to one of the two lymphocyte types, cytokine profiles will be accordingly altered. The predominance of a Th2 response is classically associated with allergic diseases with a strong inflammatory component (e.g., bronchial allergic asthma) while the prevalence of Th1 response is linked to autoimmune inflammatory diseases such as *Psoriasis Vulgaris* or chronic inflammatory syndromes like *Crohn's disease*.
In this context, **6 studies** (**3 of basic research and 3 clinical trials**) were produced in order to test the potential of the therapeutic approach based on Low Dose Medicine on the balance of the immune response.

- Gariboldi *et* al . [Gariboldi S, Palazzo M, Zanobbio L, Dusio GF, Mauro V, *et* al (**2009**) *Low dose oral administration of cytokines for treatment of allergic asthma.* Pulm Pharmacol Ther 22(6): 497-510] studied the immunological mechanisms of **allergic bronchial asthma** in a suitable animal model in order to verify the effectiveness of the use of *low dose SKA* cytokines (**IL-12 and IFN-γ 0.1 fg/ml**) to rebalance the Th1/Th2 response.
In this paper some basic immunological parameters altered in the presence of bronchial allergic asthma were evaluated *in vivo*:

1. the quantitative/qualitative composition of both immune cells panel (eosinophils, neutrophils and mononuclear cells) was evaluated in BALF (Broncho-Alveolar-Lavage-Fluid) of animals;
2. the expression of a typical panel of cytokines (IL-4, IL-5, IL-13, IL-17) and a specific antibody (IgE-OVA) were evaluated in the BALF and in plasma.

Collected data showed the efficacy on Th1/Th2 switch modulation of low dose SKA cytokines treatment. Great importance was recognized of the fundamental role played by the Sequential Kinetic Activation of the studied cytokines: in fact no biological effect can be attributed to these cytokines in the absence of SKA procedure.

Also the synergistic effect of the combined use of IL-12 and IFN-γ, compared to the use of the individual cytokines, emerged clearly from the study.

The study also includes a detailed dose-response screening aimed at identifying the minimum effective concentration for the tested cytokines. Another intriguing effect of low dose cytokines was described; low doses SKA of IL-12 and IFN-γ (0.1 fg/ml) are able to induce the secretion of the same cytokines by splenocytes and CD11+ DC cells (only IL-12) in vitro with a detected concentration in the order of nanograms.
These data clearly describe the immunomodulatory attitudes of low dose messenger molecules exerted through the direct stimulation of immune cells with a final rebalancing effect on Th1/Th2 cytokines expression.

- Cardani and colleagues [Cardani D, Dusio GF, Luchini P, Sciarabba M, Solimene U, *et* al (**2013**) *Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation.* Gastroenterology Research 6(4): 124-133] investigated in a validated *in vivo* murine model the immunological mechanisms underlying the **inflammatory bowel**

diseases (**IBDs, e.g. *Crohn's disease***). The analysis of the panel of Th1/Th17 cytokines selected for the study (TNF-α, IFN-γ, IL-12, KC, and IL-17) clearly shows that in the model of the disease there is a marked upregulation of these proinflammatory cytokines. The use of **low dose of SKA IL-10 and anti IL-1 monoclonal antibody** (**both at a concentration of 0.01 pg/ml**) proves to be able to significantly reduce the expression of all the inflammatory markers and to increase the endogenous production of IL-10, typical Th2 anti-inflammatory interleukin, inducing a rebalance of the Th1/Th2 switch. Other physiological and histological parameters evaluated in the study are improved by low-dose SKA treatment.

- Barygina V. *et* al [*The role of IL-4, IL-10, b-FGF and β-endorphin in modulating intracellular redox status and proliferation rate in human skin keratinocytes subjected to oxidative stress.* (submitted)] evaluated the effects of low dose SKA IL-4, IL-10, b-FGF, β-endorphin and anti-IL-1 antibody in the modulation of intra- and extra-cellular oxidative stress and on the proliferation of human keratinocytes.
Oxidative stress was induced on human keratinocytes immortalized cell line (HaCaT) by incubation with 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH).
The HaCaT cells were then treated with **10 fg/ml of IL-4 SKA**, **SKA IL-10**, **b-FGF SKA**, **β-endorphin SKA** or **anti-IL-1 SKA** for 24 hours and the proliferation index and the intra- and extra-cellular oxidative status was measured by fluorimetric test and flow cytofluorimetry in combination with the fluorescent dye H2DCFDA (DCF), respectively.

Preliminary results (presented to *the Master Class in **Vitiligo and pigmentary disorders** - Amritsar, India 28 to 30 November 2014*) have shown a significant reduction of intra-cellular oxidative stress, in particular with low dose SKA IL-4, IL-10, b-FGF of 18±4%, 31±3% and 26±2%, respectively, and the extracellular oxidative stress, in particular with low dose SKA IL-4 and b-FGF of 28±4% and 37±5%, respectively, and an increase of proliferation index in particular with low dose SKA of b-FGF and Anti IL-1 antibody, respectively of 23±4% and 22±3% compared to control.

- Roberti *et* al [Roberti ML, Ricottini L, Capponi A, Sclauzero E, Vicenti P, et al. (2014) *Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris.* J Biol Regul Homeost Agents 28(1): 133-9] investigated the possibility of using specific **low dose SKA cytokines** (**IL-4; IL-10; IL-11, at a concentration of 10 fg/ml**) for the therapy of a typical autoimmune disease with a clear inflammatory component such as ***Psoriasis Vulgaris***. The efficacy of treatment with low-dose cytokines was evaluated both in terms of improvement of the condition of psoriatic lesions and in the quality of life through a multicenter double-blind placebo-controlled clinical study on a significant number of patients and conducted through the use of internationally validated rating scales PASI (Psoriasis Area Severity Index) and DLQI (Dermatology Life Quality Index) for the evaluation of the extent of the lesions and to determine the quality of life respectively. The clinical trial has shown that the three SKA cytokines (10 fg/ml) are able to improve significantly both the PASI score (Friedman test: $p = 0.00960$) and the DLQI score (Friedman test, $p = 0.00007$) between the baseline and after 3 months and between baseline and after 6 months.
The obtained results allowed the authors to identify some key points on the activity of the tested cytokines on *Psoriasis Vulgaris*: they are effective and safe from a therapeutic point of view and also have a long-term action, which extends into the first months after the end of treatment. This feature may be crucial in view of the treatment of chronic diseases.

- Galli E. *et* al [*Clinical and immunological evaluation of long-term treatment with Low Dose Medicine in pediatric population affected by chronic atopic dermatitis. Experimental randomized double-blind at two stages clinical trial.* (submitted)] studied the effectiveness of a treatment with **low dose SKA cytokines** (**IL-12 10 fg/ml and IFN-γ 10 fg/ml**) and a low dose drug with connective drainage action (*Galium-Heel*® - Baden Baden, Germany) in a pediatric population affected by chronic ***Atopic Dermatitis***.

The experimental, randomized controlled double-blind two-stage study included children with atopic light to mild dermatitis (assessed by index SCORAD - SCOring Atopic Dermatitis, which was not to exceed the value of 40, with a minimum score of 6), with a number of relapses ≥ 4/year with the appearance of skin lesions for at least six months when inserted in the study.
All the children had to present an acute phase of disease at the enrollment time. Both children with atopic dermatitis IgE mediated (specific *in vivo* and/or *in vitro* tests with positive results) and non-IgE mediated (specific *in vivo* and/or *in vitro* tests with negative results) were included.
As primary outcome was assessed reduction in the severity of *Atopic Dermatitis* through the SCORAD index with a percentage waiting for improvement of 30%, whereas, as a secondary outcome, the following parameters have been taken into consideration: lengthening of the "disease-free interval", tolerability and compliance of treatment and management of adverse events, Skin Prick Test to major inhalant allergens and food, Skin Prick by Prick Test to the main food allergens, Patch Test to the main food allergens, mites and Nickel, total and specific IgE against the main inhalant allergens and food, characterization of lymphocyte subpopulations by cytofluorimetry with monoclonal antibodies, cellular and serological study of pro-and anti-inflammatory cytokines (IL-10, IL-13, IL-12 and IFN-γ).

Preliminary results (presented at the *XXVI National Congress S.I.P.P.S. - Italian Society of Preventive and Social Pediatrics - Verona, 27-29 November 2014*) show that the group treated with low dose cytokines has a decreased SCORAD score between T0 and T8 by 54%, a decrease that continues in the follow up reaching 64%. At the same observation period, the treated group showed significantly reduced use of symptomatic drugs (antihistamines and topical corticosteroids).
The study also showed a progressive improving in the quality of life (itching and nocturnal disturbances) of subjects treated with low dose SKA cytokines during the investigation period.

- Fiorito F. *et* al [*Clinical improvement in feline herpesvirus-1 infected cats by oral low doses SKA of Interleukin-12 and Interferon-gamma (10 fg/ml).* (submitted)] evaluated the effectiveness of low-dose cytokine SKA against ***infection by Feline herpesvirus-1*** (***FHV-1***), a common pathogen of cats that causes a picture of rhinitis, conjunctivitis and corneal ulcer. Until now, it had been observed that conventional antiviral drugs, and high doses of cytokines are poorly effective against FHV-1 infection.

31 unvaccinated cats, positive for FHV-1 (shown by test PCR - Polymerase Chain Reaction) were enrolled. 16 cats were treated for 6 months with oral administration of **low dose SKA IL-12 and IFN-γ (10 fg/ml)** and 15 cats with antibiotic therapy. At 2, 6 and 12 months after the end of treatment nasal, oropharyngeal and conjunctival swabs were analyzed.
A panel of blood tests and the overall clinical condition were also evaluated.
At follow-up, in the group treated with low dose SKA cytokines the PCR test was negative in 13/16 cats (81.25%, $p < 0.001$) with a significant improvement of both general and specific clinical symptoms, except in 3/16 cats (18.75%), which showed a FHV-1 PCR positive. In the control group, however, 15/15 cats were PCR-positive, with little or no improvement in clinical symptoms.
These results show that the low dose therapy with SKA cytokines represents an innovative approach for the treatment of FHV-1 infection in cats and suggest the possibility of extension of these treatments also in other animal species.

Clinical trials on *low dose SKA* growth factors with stimulation activity of the tissue trophism

In the case of growth factors, the use of low-dose physiological SKA goes in the direction of enhancing the biological action of the same growth factor, through the up-regulation of trans-membrane receptors present on target cell surface. The rationale of their clinical use is configured in the treatment of hypotrophy and atrophy conditions.

- Luchetti P. [*Increasing of visual function in patients with retinal atrophy treated with Low Dose Medicine drugs. Monocentric retrospective observational study*. (In press on Minerva Medica Oftalmologica)] investigated the efficacy of oral administration of ***low dose SKA growth factors, NT3, NT4*** and ***NGF*** (***0.01 pg/ml***), in combination with low dose of injectable drugs vascular stimulation, in increasing the visual function in people with ***retinal diseases.***

29 eyes of patients aged between 18 and 70 years were observed and evaluated, all presenting a severe form of retinal central or peripheral atrophy documented by OCT (Optical Computed Tomography).

Some subsets of hereditary-degenerative diseases, with atrophic evolution, were defined within the observed population. For the diagnosis and evaluation of the results they have made use of microperimetry (Nidek MP1) and standard ISCEV electroretinograms (International Society for Clinical Electrophysiology of Vision).

The neurotrophins, in addition to their well-known and described role of trophic factors for neuronal survival (in brain development and in adult brain) and in addition to their involvement in neurodegenerative processes (in the adult brain and in brain aging) exert multiple actions related to the synaptic activity and plasticity phenomena.

Neurotrophins are traditionally considered proteins with trophic action for survival and neuronal differentiation. The whole design of the therapeutic protocol has pursued a variety of objectives, namely: activation of the capillary, activation of the removal processes of amorphous scar material, reactivation of enzymatic processes, regeneration of specific tissue.

The analysis of results show the statistical significance of the increase of the obtained response after the period of therapy.

The highlights of the works described on the activity of rebalancing of Th1/Th2 subsets by low dose SKA cytokines and stimulation of tissue trophism by low dose SKA growth factors are summarized in *Table 3*.

Study	Type	Cytokines/ antibodies	Positive control	Placebo control	Results
Gariboldi S. *et* al. Low dose oral administration of cytokines for treatment of allergic asthma.	***In vivo* basic research**	IL-12; IFN-γ (100 ng; 1 ng; 10 pg; 100 fg; 1 fg; 0.01 fg; 0.0001 fg /dose)	X IL-12; IFN-γ (500 ng/ dose)	Control group	• SKA low doses are effective and safe on Th1/Th2 balancing. • Non-activated cytokines are ineffective. • The association of cytokines shows synergistic effects.
Cardani D. *et* al. Oral administration of Interleukin-10 and Anti-IL-1 antibody ameliorates experimental intestinal inflammation	***In vivo* basic research**	IL-10; Anti IL-1 (50 fg/Kg)	—	Control group	• The association of SKA low dose IL-10 and Anti IL-1 is effective in countering inflammation related to IBD.
Barygina V. *et* al. The role of IL-4, IL-10, b-FGF and β-endorphin in modulating intracellular redox status and proliferation rate in human skin keratinocytes subjected to oxidative stress.	***In vitro* basic research**	IL-4; IL-10; b-FGF; β-Endorphin; Anti-IL-1 (10 fg/ml)	—	Untreated group	• IL-4, IL-10 and b-FGF show a significant antioxidant effect. • Anti-IL-1 and b-FGF show a significant positive effect on cell proliferation.
Roberti ML *et* al. Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in *Psoriasis vulgaris*.	***Multicentre double-blind placebo-controlled RCT***	IL-4; IL-10; IL-11 (10 fg/ml) 20 drops twice a day for each cytokine for 3 months consecutively	—	(vehicle)	• The association of SKA low dose IL-10, IL-11 and IL-4 is effective in the treatment of *Psoriasis Vulgaris*. • SKA low dose Interleukins show a long-term action.
Fiorito F. *et* al. Clinical improvement in feline herpesvirus 1 infected cats by oral low doses of Interleukin-12 and Interferon-gamma	***Clinical trial (veterinary)***	IL-12; IFN-γ (10 fg/ml) 10 drops a day each cytokine for 6 months consecutively	pradoflo-xacina	—	• Neutralization of viral load in 13/16 animals. • Improvement of clinical symptoms in all considered parameters.
Luchetti P. Increasing of visual function in patients with retinal atrophy treated with Low Dose Medicine drugs. Monocentric retrospective observational study.	***Observational retrospective clinical trial***	NT3; NT4; FGF (0.01 pg/ml) 15 drops twice a day each growth factor for 6 months consecutively	—	—	• The statistical results clearly indicate that the proposed treatment was able, within the limits of the sample, to get an early functional recovery. • The data lead to consider the need for prolonged and continued treatment for the maintenance of the achieved therapeutic results.
Galli E *et* al. Clinical and immunological evaluation of long-term treatment with Low Dose Medicine in pediatric population affected by *Chronic Atopic Dermatitis*. Experimental randomized double-blind at two stages clinical trial.	***Double-blind double-stage RCT***	IL-12; IFN-γ (10 fg/ml) 15 drops twice a day each interleukin for 8 months consecutively; children under 5 years: 8 drops twice a day each interleukin for 8 months consecutively	—	(vehicle)	• The group treated with low dose cytokines SKA has a decrease of SCORAD score between T0 and T8 of 54%, a decrease that continues in the follow-up until it reaches 64%. • The study also showed a progressive improvement of the quality of life of patients receiving low-dose SKA cytokine treatment.

Table 3: Synopsis of the key points of the studies of basic research and clinical trials on low-dose SKA cytokines with rebalancing activities on Th1/Th2 ratio and clinical studies testing low dose SKA growth factors with stimulating activity on tissue trophism.

Ongoing research

- Migliore A. *et* al
Ospedale San Pietro Fatebenefratelli – Roma.

Randomized controlled trial on the maintenance of low disease activity with **SKA low dose administration of cytokines Interleukin-4 (10 fg/ml) interleukin 10 (10 fg / ml) Anti-Interleukin-1 (10 fg / ml)**, compared with standard therapy (DMARDS), in treating ***Rheumatoid Arthritis***.

Research in pipeline

- Torta R. *et* al
Azienda Ospedaliero-Universitaria.
Città della Salute e della Scienza - Torino.

Fibromyalgia and chronic low grade inflammation: efficacy and safety of **low dose Interleukin 10 SKA (10 fg/ml)**. Double-blind placebo-controlled randomized clinical trial.

Synopsis of research on Low Dose Medicine

The analyzed articles report the experimental evidence of the effectiveness of LDM approach on diseases involving the immune system. All the papers show the ability of the signaling molecules to modulate the responses of immune cells in a highly selective fashion; especially, the immunostimulatory and immunomodulatory skills of the tested cytokines are clearly described.
The ability to act in a refined manner on the Th1/Th2 balance is crucial for the management of diseases with diametrically opposed cytokine imbalances such as *Bronchial Allergic Asthma* (which shows a Th2 predominance) [33,34], *Crohn's disease* [35,36] and *Psoriasis Vulgaris* [37,38] (Th1-driven diseases).

One of the key issues emerging from the analyzed scientific works is the effectiveness of treatment with low dose molecules in spite of the fact that they operate at lower concentrations than those generally considered pharmacologically effective.
The use of cytokines and other signaling molecules has often collided with the need of high dosages, but these high concentrations show a wide range of side effects in addition to the proper pharmacological effects.

The classical minimum active dose is generally found between the lowest pharmacological one (10^{-5}) and the highest physiological one (10^{-6}) *(Figure 4)*; low dose pharmacology moves within the range of physiological concentrations of signaling molecules acting below the concentrations at which adverse effects appear but equally reaching appreciable therapeutic results. The ligand-receptor binding properties are crucial to explain how low dose SKA signaling molecules can be effective. Receptor affinity for its specific ligand is fundamental for the activation of

postreceptorial downstream [39, 40], in fact ligand saturation generally induces the receptor freezing and/or its down-regulation. Low dose molecules are able to induce a direct physiological receptorial stimulation of immune cells (as described by Gariboldi S. and colleagues) modulating the responses within the homeostatic range; LDM realizes one of the cardinal points of P.N.E.I. approach to the disease: to restore a physiological panel of signaling molecules.

From a pharmacological point of view the revised works highlight the importance of the activation of low dose molecules through the process of drug delivery known as SKA (Sequential Kinetic Activation): low dose molecules not processed with this activation procedure are totally ineffective as described by Gariboldi S *et* al. SKA activation is fundamental in order to overcome the conceptual wall represented by the minimum pharmacologically effective dose inducing an activity release effect exerted by low dose molecules by interaction with the aqueous vehicle.

Conclusions on medical research in *Low Dose Medicine*

Five years of scientific research on Low Dose Medicine have allowed researchers to provide data of a different nature, scientifically relevant, able to demonstrate:

1) The validity of the theoretical concepts underlying the LDM;
2) The centrality of the pharmaceutical technological process called SKA (Sequential Kinetic Activation);
3) The experimental and clinical effectiveness of low dose SKA signaling molecules.
4) The immunomodulatory and immunostimulant ability of cytokines and the trophic activity of growth factors;
5) The safety of the preparations tested.

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LOW DOSE MEDICINE FROM ITALIAN RESEARCH AN INNOVATIVE IMMUNO THERAPEUTIC APPROACH FOR SKIN DISORDERS

P.N.E.I. and Low Dose Cytokines and Growth Factors Therapy

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Psycho-Neuro-Endocrine-Immunology (P.N.E.I) and Low Dose Medicine (LDM): Affirming the model of "Human Centered Medicine"

From the second half of the 1970s the development of Psycho-Neuro-Endocrine-Immunology (P.N.E.I.), the discipline that studies the functional relationships between the nervous, immune and endocrine systems by R. Ader, offered the opportunity and the conceptual tools to study both physiological and pathological biological processes in accordance with an unified vision of the body functions and, at the same time, identifying the P.N.E.I. mechanisms that manage the control of these functions. Taken all together, the Central Nervous System, the Autonomic Nervous System, the Endocrine and Immune Systems constitute the P.N.E.I. network [1-4] (*Figure 1*).

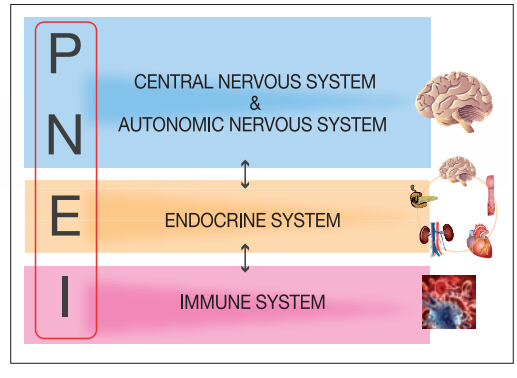

Figure 1 – P.N.E.I. Network and bi-directional *cross-talk* among Systems.

The bi-directional cross-talk between the systems within the P.N.E.I. network is managed by a relevant number of signaling (messenger) molecules which are the carriers of the "biochemical information" necessary for the homeostatic regulation of all the biological responses within the network. A break in the cross-talk due to an imbalance between specific signaling molecules is fundamental for the onset of inflammatory, allergic and autoimmune diseases [5; 6].
In the last 30 years the research in the fields of Molecular Biology and physiopathology has identified in hormones, neuropeptides, cytokines and growth factors the signaling molecules involved in both physiological and pathological processes in clear accordance with the principles of P.N.E.I.

Low Dose Therapy: from nano-concentrations a new hope for skin diseases and pigmentary disorders

Low Dose Medicine (LDM) highlights the importance of the role played by inflammation and, in particular, by low grade chronic inflammation, in the etio-pathogenesis of a wide spectrum of diseases. Some of the more serious and complex dermatological chronic inflammatory autoimmune diseases can today be addressed with LDM. In fact, considering these skin diseases as systemic, the LDM acts on them through the bio-regulation of the Immune System via oral systemic administration of signaling molecules, that is the only possible access to the restoration of cross-talk between the immune cells themselves and between the immune cells and skin cells.
The loss of the immunological balance is characterized by a shift between Th1/Th17- and Th2- mediated immune response due to an altered amplification of Th1/Th17 and Th2 lymphocyte subsets.
The Th1/Th2 switch, observed in autoimmune skin diseases, results in the hyper-production of Th1-related cytokines: *Alopecia Areata*, *Vitiligo* and *Psoriasis Vulgaris* present a characteristic over-expression of proinflammatory cytokines (mainly derived from Th1/Th17 lymphocytes), typical of organ-specific autoimmune diseases [7, 8].
Since the 1970s anti-cytokine therapy was proposed and tested for the treatment of skin autoimmune diseases mainly counteracting the expression of Th1 proinflammatory cytokines such as IFNs, IL-1 and TNF-a. The therapeutic use of Th2 cytokines and specific monoclonal antibodies was applied for *Alopecia Areata*, *Psoriasis Vulgaris* and *Atopic Dermatitis* treatment.

However, side effects due to high dosages normally used for these molecules have hindered the development of possible new drugs [9, 10]. The most important and limiting pitfalls connected with the use of high dosage cytokines and other signaling molecules are, still today:

- the need of high doses of active molecules in order to reach the therapeutic goal
- the low compliance of systemic administration performed by *iv* and subcutaneous injections.

Currently, the availability of low dose SKA signaling molecules (cytokines, growth factors, hormones, and neuropeptides) and the development of LDM theoretical and clinical approach make it possible to use lower doses of activated molecules (active range between picomoles and femtomoles) [11, 12] with therapeutic outcomes comparable to those induced by high dosages but without side effects [13-17].
In the field of Dermatology, the first studies on low dose cytokines are beginning to be published.
A multicenter double-blind placebo-controlled clinical study performed by **Roberti ML. *et* al.** - *Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in Psoriasis vulgaris* - [17] described the efficacy of specific low dose SKA cytokines (IL-4; IL-10; IL-11 at the concentration of 10 fg/ml - GUNA S.p.a.; Milan, Italy) for the therapy of ***Psoriasis Vulgaris***.

The two outcomes chosen for the evaluation of the treatment with low dose SKA cytokines were:

- the presence and extension of psoriatic lesions;
- the improvement of the quality of life.

These two parameters were evaluated using the rating scales PASI (Psoriasis Area Severity Index) and DLQI (Dermatology Life Quality Index) respectively.

Roberti and colleagues clarified some aspects of low dose SKA cytokine action against *Psoriasis Vulgaris* and the results of the study allowed the researchers to confirm that low dose SKA cytokine administration is effective, safe and long-lasting, which are crucial aspects for a hypothetic treatment of other chronic diseases such as ***Vitiligo*** [18-22].

Therapeutic protocol for *Psoriasis Vulgaris*

- **GUNA Interleukin-4**: 20 drops twice a day for 3 months consecutively
- **GUNA Interleukin-10**: 20 drops twice a day for 3 months consecutively
- **GUNA Interleukin-11**: 20 drops twice a day for 3 months consecutively

The therapy cycles can be repeated according to the clinical history of every single patient, the severity of the disease and the individual response of each patient. All the medicines can also be administered all together, dissolved in a little water, keeping them at least for 30 seconds in the mouth, preferably far from meals. In children below 6 years the dosage is 10 drops (instead of 20 drops such as in adults) twice a day for at least 3 months consecutively.

Another important study regarding ***Atopic Dermatitis***, in the pediatric population is stimulating great interest in the scientific community. Preliminary data presented at *XXVI SIPPS Congress (Società Italiana di Medicina Preventiva e Sociale – Verona, November 27-29th, 2014)* have shown the efficacy of low dose SKA Interleukin-12 and IFN-γ (both at 10 fg/ml) as reported in the study *"Clinical and immunological evaluation of long-term treatment with Low Dose Medicine in pediatric population affected by chronic atopic dermatitis. Experimental randomized double-blind at two stages clinical trial"* performed by **Galli E. *et* al** (Ospedale San Pietro Fatebenefratelli - Roma).

Therapeutic protocol for *Atopic Dermatitis*

- **GUNA Inteleukin-12**: 15 drops twice a day for 8 months consecutively (children under 5 years, 8 drops twice a day for 8 months consecutively)
- **GUNA Interferon-γ**: 15 drops twice a day for 8 months consecutively (children under 5 years, 8 drops twice a day for 8 months consecutively)
- **Galium -Heel**: 15 drops twice a day for 8 months consecutively (children under 5 years, 8 drops twice a day for 8 months consecutively)

The therapy cycles can be repeated according to the clinical history of every single patient, the severity of the disease and the individual response of each patient. All the medicines can also be administered all together, dissolved in a little water, keeping them at least for 30 seconds in the mouth, preferably far from meals.

In recent years, researchers and clinicians operating in the field of LDM have investigated the possibility of treatment with *SKA low dose* cytokines, growth factors and neuropeptides in ***Vitiligo***.
The results are extremely encouraging and drawing new scenarios for the care of this dramatic disease.

Oxidative stress and *Vitiligo* onset

Vitiligo is a skin disorder characterized by a progressive depigmentation which is caused by the loss of melanocytes (or a reduction in its activity) at the cutaneous level. The causes of melanocyte loss (or a reduction in its activity) are still unclear, but a relevant number of observations lead researchers to ascribe cellular immunity as having an important role in *Vitiligo* etio-pathogenesis. In *Vitiligo*, the observed imbalance in cytokine expression at cutaneous lesions level, mainly on the border of affected area with healthy skin, is probably due to a shift of the immune system with a prevalence of Th1/Th17 (high IL-1, mainly at perilesional level, [23] and IL-17) response instead of a Tregs/Th2 one (low IL-4 level) and may be part of etiology of this autoimmune disease [24]. TNF-α also plays a pivotal role in oxidative stress-mediated cytotoxicity directed against melanocytes and keratinocytes [25] (*Figure 2*).

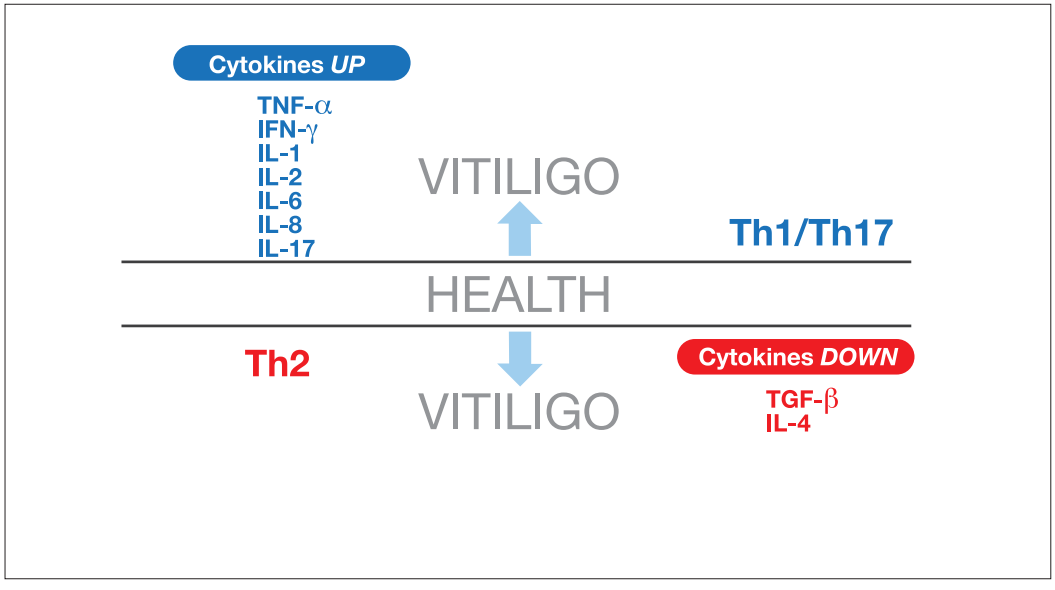

Figure 2 – Up- and down-regulated cytokines in *Vitiligo*.

In *Vitiligo*, also high levels of free oxygen radicals (reactive oxygen species: ROS) are detected and contribute to skin structure damage [26]. Hyper-sensibility to ROS is recognized as pivotal for the disease onset: the uncontrolled accumulation of free radicals is one of the most important triggers for keratinocytes and melanocytes death, which leads to the disruption of epidermal unit of melanization homeostasis and consequent depigmentation. Melanocytes, in healthy conditions, react against ROS over-expression producing some typical phase II enzymes such as hemeoxygenase-1 (HO-1), superoxide dismutase (SOD) and catalase. Antioxidant enzymes synthesis is induced by the nuclear translocation of NF-E2-related factor (Nrf2) which binds AREs sequences within the enzyme genes; in *Vitiligo*, this synthetic pathway is compromised and in particular Nrf2/ARE/HO-1 axis is impaired [27].
An alteration in anti-oxidative response is detected also in keratinocytes located in *Vitiligo* lesional skin. The protease-activated receptor-2 (PAR-2) enhances both inflammation and Nrf2-mediated response against oxidative stress; at skin level, PAR-2 activation promotes melanin uptake from keratinocytes and anti-oxidant enzymes expression (e.g.: quinone oxidase, NQO-1, a phase II enzyme acting as ROS scavenger), both mechanisms are fundamental for cellular protection against oxidative triggers [28]. The breakdown of PAR-2/Nrf2 crosstalk is linked with the onset of skin diseases such *Atopic Dermatitis* and *Vitiligo*: in lesional keratinocytes, PAR-2 expression is impaired, resulting in reduced Nrf2 nuclear translocation and subsequent defective anti-oxidant response.

In summary, decreased antioxidant enzymes activity and increased ROS levels, due to chronic inflammatory conditions, driven by interleukin-2 (IL-2), may be linked with Nrf2 pathway alteration in *Vitiligo* both in melanocytes and keratinocytes subsets.

Keratinocytes-melanocytes crosstalk: the role of *basic-Fibroblast Growth Factor*

Keratinocytes are the major source of cytokines at the epidermal level with proliferative, immunological and inflammatory properties. The sophisticated mechanism of cross-talk between keratinocytes and melanocytes is essential for proper skin pigmentation; the numerical ratio between keratinocytes and melanocytes is 10 to 1 and the first ones produce a set of signaling molecules essential for the maintenance and functionality of the second ones.
The cytokine network between keratinocytes and melanocytes includes:

- SCF (stem cells factor)
- ETs (endothelins)
- b-FGF (basic-fibroblast growth factor)

These keratinocytes-derived cytokines are fundamental for melanocytes growth, differentiation and migration; in particular, b-FGF exerts a paracrine signaling function between keratinocytes and melanocytes [29]. b-FGF is not only a mitotic and migrating factor but it is also involved in radical oxygen substances detoxifying processes by inhibiting ROS (and related damages) via activation of PI3K/Akt and consequent inhibition of NF-kB nuclear translocation [30] (*Figure 3*).

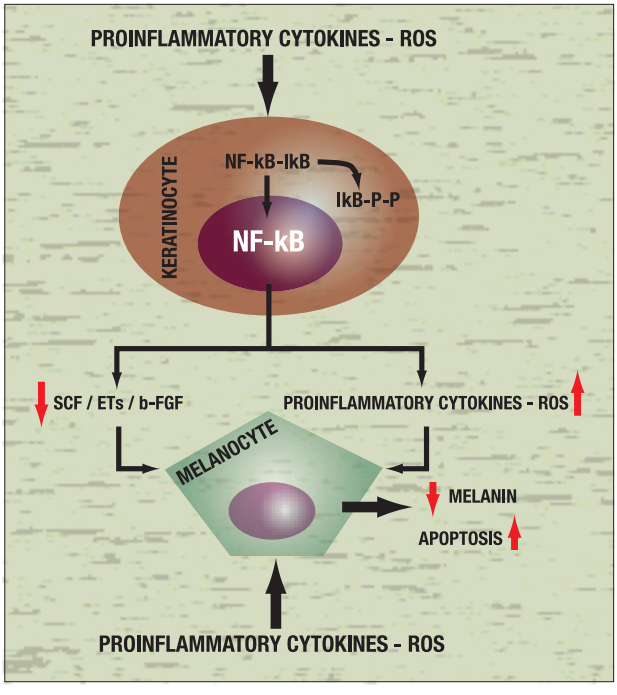

Figure 3. Schematic representation of proinflammatory cytokines and ROS effects on keratinocytes/melanocytes crosstalk

In a great number of skin diseases (such as *Atopic Dermatitis*, *Psoriasis* and *Vitiligo*) a decisive inflammatory component is still present. Focusing our attention on *Vitiligo*, the alteration of immune response (Th1/Th17 up-regulation), the increased ROS production and the onset of an autoimmune response (primarily due to altered proteic synthesis and DNA damage with consequent generation of auto-antigens) are key events in the onset of this skin disease.

As previously described, both keratinocytes and melanocytes are directly damaged by the altered skin microenvironment which characterizes *Vitiligo* in terms of cytokine network alteration.

The keratinocytes-melanocytes cross-talk break-down is central for skin depigmentation in vitiliginous areas. Incorrect autoimmune response and oxidative stress affect keratinocytes viability and, consequently, reduce the production of trophic factors, essential for melanocytes viability. Impaired melanocytes stimulation results in reduction of melanin synthesis and in an increased cell death ratio.

Taken together, these alterations in dermis cellular composition and function result in the depigmentation phenomena which represent the expression and the picture of the etio-pathogenetic process sustaining *Vitiligo*.

Biologic activity of *b-FGF* in high and low doses

b-FGF, as noted, has targeted a number of cell types exerting a trophic action or regulating cellular proliferative and pro-active mechanisms.
These biological characteristics make it a potential molecule with pharmacological properties resulting from its ability to modulate both the growth phase and that of differentiation and migration of a set of target cells. In particular the use of human (or bovine) recombinant b-FGF has been tested in a number of clinical trials that provided for both the systemic use via continuous infusion and topical use [31-33].
These studies are mainly directed to the evaluation of the applicative use of b-FGF as an enhancer of bone repair and soft tissue lesions healing by direct stimulation of fibroblasts and the dosages used are in the order of micrograms. Some side effects, although not supported by statistical significance, are associated to the treatment with high doses of this growth factor.

Shifting the focus from the direct effects of b-FGF on the fibroblasts to the hypothetical use of b-FGF as a signaling molecule between different cell subsets within the same tissue is greatly interesting. Acting as a messenger mediator, b-FGF plays its biological functions in a concentration-dependent manner. In particular, the dose-response ratio is not linear but follows a typical bell-shaped curve with a peak of activity in a concentration range identified between nanograms and picograms. This physiological biphasic effect, which characterizes the b-FGF activity, aimed at:

- promoting chemotaxis/migration of mast cells in the mouse model [34]:
- facilitating thymocytes adhesion to marrow stromal cells as a signal of the myelopoietic increase in a murine model [35]:
- stimulating the production of adhesion factor VCAM-I in vitro by human endothelial cells (umbilical cord) in a dose-dependent manner [36]:
- producing a significant increase (compared to controls) of the radiopaque shadow of dental implants three weeks after the execution of the operation, with the administration of b-FGF in nanograms; the same shadow, however, decreases with the use of b-FGF in micrograms [37].

Low Dose Medicine for *Vitiligo* management: the role of *low dose SKA* b-FGF and other cytokines and neuropeptides.

For a correct epidermal pigmentation, it is essential that the keratinocyte produces the signaling molecules that induce proliferation and survival of melanocytes, in order to maintain a proper melanin production [38].
The interruption of this signaling results in the loss of melanocytes viability, resulting in their numerical reduction and in the epithelium depigmentation [39, 40], this latter characterized by the appearance of white patches, the classic macroscopic expression of *Vitiligo*.
All these evidences suggest the crucial therapeutic role of *low dose SKA GUNA-FGF* (Guna S.p.a. Italy) orally administered (very interesting can be the overlapping between systemic

administration of *low dose SKA b-FGF* and topic administration of b-FGF in high dosage; thanks to the action mechanism of *low dose SKA* b-FGF we can have the upregulation of transmembrane receptors on melanocytes and the consequent boosting effect of topical administration).

But the therapeutic use of only b-FGF allows acting only on the last step of the etiopathogenetic cascade of *Vitiligo*. Since *Vitiligo* is a systemic chronic autoimmune inflammatory disease, where the onset of the disease is due to an etiologic loop between oxidative stress and chronic inflammatory phenomena, secondary to upregulation of Th1/Th17 cytokines (IL-1, IL-17, IFN-γ and TNF-α), only acting on these "*origin sin*" we can assert to propose an original and innovative treatment (*Figure 4*).

Acting at the origin of the inflammatory phenomena, counteracting pro-inflammatory cytokines with specific *low dose SKA* cytokines (IL-10, Anti-IL-1 and IL-4) and, at the same time, stimulating melanocytes to produce melanin *via* up-regulation of transmembrane receptors through *SKA low dose* b-FGF, represents the hypothesized new LDM approach for ***Vitiligo*** treatment.

Preliminary data provided by **Barygina V. and colleagues** (presented at *Master Class in Vitiligo and pigmentary disorders – Amritsar, India, November 28-30th ,2014*) have shown that the research in the field of low dose applied to Dermatology is headed in the right direction. In this basic research study, oxidative stress was induced in immortal line of human keratinocytes HaCaT by incubation with 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH). Further, HaCaT cells were treated with 10 fg/ml SKA concentrations respectively of IL-4, IL-10, b-FGF, β-Endorphin and anti-IL-1 for 24 hours; at the end of the treatment the proliferation rate and the intracellular/extracellular oxidative status were measured by fluorometric assay and by flow cytometry in combination with H2DCFDA (DCF) fluorometric dye, respectively.

Conversely, the incubation with b-FGF and Anti-IL-1 led to a notable increase in the cell proliferation rate (23±4% and 22±3% vs control, respectively).

Concomitantly with basic research, numerous clinical experiences on *Vitiligo* are ongoing in Italy and all over the world and some clinical studies are in the pipeline.

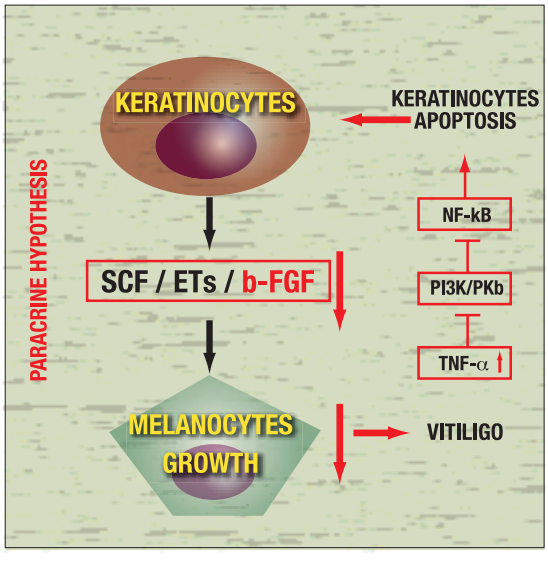

Figure 4 – Keratinocytes/Melanocytes interplay and inflammation in *Vitiligo* onset

Therapeutic protocol for *Vitiligo*

- **GUNA-FGF**: 20 drops twice a day for 6 months consecutively
- **GUNA Interleukin-4**: 20 drops twice a day for 6 months consecutively
- **GUNA Interleukin-10**: 20 drops twice a day for 6 months consecutively
- **GUNA Anti-IL-1**: 20 drops twice a day for 6 months consecutively

The therapy cycles can be repeated according to the clinical history of every single patient, the severity of the disease and the individual response of each patient. All the medicines can also be administered all together dissolved in a little water, keeping them at least for 30 seconds in the mouth, preferably far from meals. In children below six years the dosage is 10 drops (instead of 20 drops such as in adults) twice a day for 6 months consecutively.

Conclusions

A large number of dermatological diseases have, among its etiological components, an altered immune response caused by the imbalance between Th1/Th17- and Th2-driven responses.
An innovative strategy for the treatment of these diseases can be based on the rebalance of this altered cytokine profile. High doses of recombinant signaling molecules such as cytokines, antibodies, neuropeptides and growth factors are effective against some immune aspects of skin autoimmune diseases but mild to severe dose-dependent and time-dependent side effects are still present.
The possibility of a fine adjustment of the immune response through the use of suitably identified signaling molecules is an extraordinary therapeutic opportunity for chronic inflammatory autoimmune diseases of the skin.

The availability of *low dose SKA-activated* cytokines and the LDM approach (validated by a growing number of scientific evidences in terms of efficacy and safety) induces us to postulate a new therapeutic approach based on systemic oral administration of low doses of activated cytokines and growth factors, which represents an innovative strategy for the treatment of dermatological diseases characterized by an immune Th1/Th2 imbalance such as *Atopic Dermatitis, Psoriasis Vulgaris* and *Vitiligo*.

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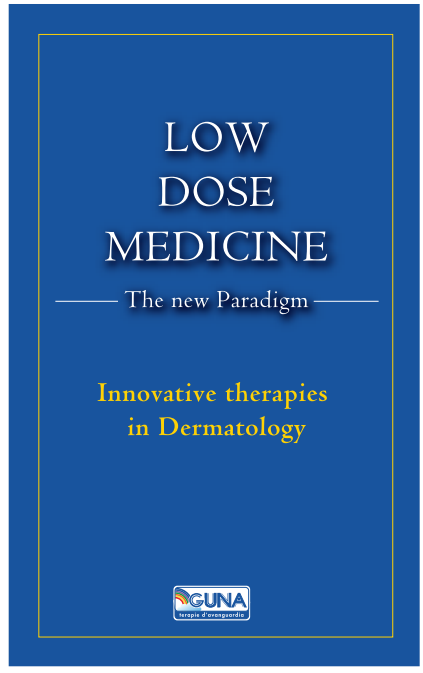

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