UP TO DATE

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THE HISTORY OF LOW DOSE MEDICINE RESEARCH REVIEW OF PRECLINICAL AND CLINICAL STUDIES WITH LOW DOSE SKA CYTOKINES SINCE 2009

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SUMMARY

Since the second half of the 80s the development of the Psycho-Neuro-Endocrine-Immunology concepts resulted in a change of perspective, from a separatist point of view to a unifying one, relating to the interpretation of the biological functions of the body. A key point was the recognition of the importance of continuous cross-talk between cells, organs and Systems in both physiological and pathological conditions based on the fine regulation of the levels of a large number of messenger molecules.
Interpreting the pathological phenomenon as an imbalance in intercellular signaling, the administration of low physiological doses of messenger molecules (which act as homeostatic modulating agents) can be considered an intriguing and innovative approach in order to restore the correct intracellular signaling and consequently to restore health. These concepts are the milestones of Low Dose Medicine.

- Five years of scientific research in the field of Low Dose Medicine demonstrated the validity of the conceptual approach and efficacy and safety of the therapeutic intervention based on the oral administration of low doses of activated messenger molecules.
This review summarizes for the first time the Low Dose Medicine scientific studies published since 2009 and gives a comprehensive overview of the basic and clinical research methodological approaches and results, highlighting the effectiveness of the experimental and clinical use of low dose activated messenger molecules.

KEY WORDS **PSYCHO-NEURO-ENDOCRINE-IMMUNOLOGY, LOW DOSE MEDICINE, SEQUENTIAL KINETIC ACTIVATION, IMMUNE SYSTEM, INFLAMMATION, TH1/TH2 BALANCE, AUTOIMMUNE DISEASES, HISTORY OF MEDICINE.**

INTRODUCTION

Since the 70s the research in the fields of Physiology and Molecular Biology has given increasing evidence of the critical role of signaling molecules such as **hormones**, **neuropeptides**, **cytokines** and **growth factors** in all physiological and pathological processes.

– In homeostatic conditions (corresponding to a healthy state) the concentrations of these molecules in the extra-cellular matrix are comprised in a specific physiological range and diseases can be considered as expressions and consequences of changed concentrations of messenger molecules [1-4].

In recent years we have witnessed, in the medical field, the gradual abandonment of the separatist conception of the biological functions of the body.

It has given way to a more unified vision in accordance with the guiding principles of Psycho-Neuro-Endocrine-Immunology (PNEI) [5-8].

– The PNEI approach represents a paradigm shift from a strictly biomedical view of health and disease to an interdisciplinary one.

The main unifying PNEI element is identified in the **cross-talk** between the Psycho-Neuro-Endocrine Systems and the Immune System. It is mediated by a complex network of signaling molecules which are the vehicle of the biological information necessary for the complex and efficient regulation of cellular responses to stimuli.

An altered crosstalk due to an imbalance between specific signal molecules is fundamental, for example, in inflammatory, allergic and autoimmune diseases onset [9-11]; restoring the physiological concentration of messenger molecules is the target to recover the homeostatic equilibrium.

– Some key points of PNEI cross-talk based on messenger molecules should be considered:

1. The cross-talk between cells, organs and Systems is always bidirectional,

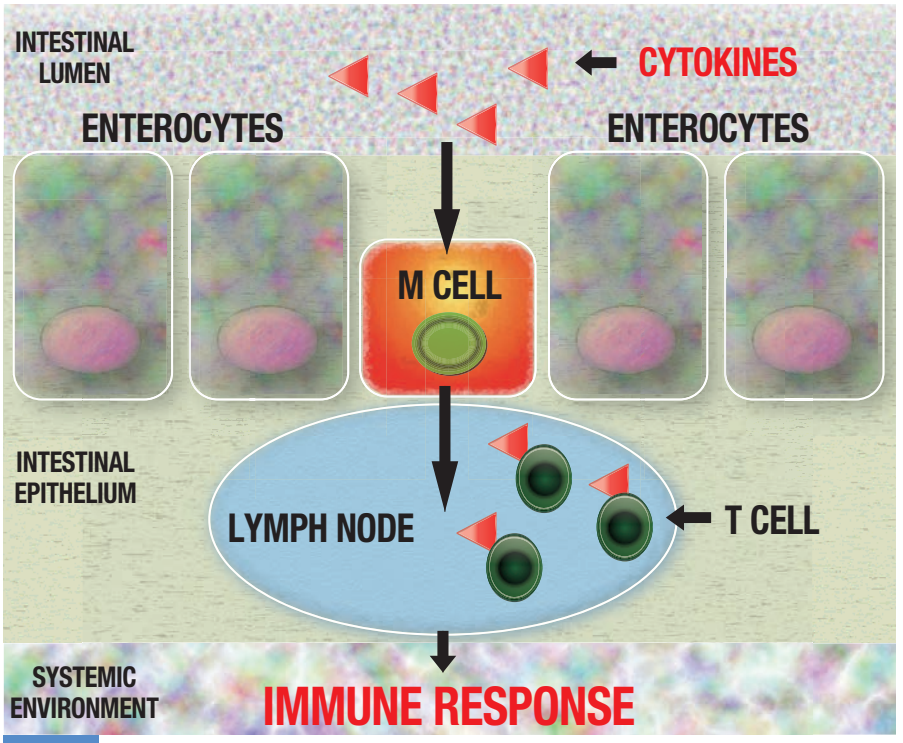

Figure 1

Hypothetical pathway of cytokines-induced M cells-mediated immune response after *per* os administration.

as the effects of the alteration of the cross-talk itself [12-14].

2. The intercellular signaling occurs through the diffusion of signal molecules in the extracellular matrix (ECM): states of pathological alteration of the ECM leads to a deterioration in the quality of the communication between cells and, in general, between organs and Systems [15,16].
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 [17,18].

The use of biological molecules which control and drive homeostatic functions in order to restore the starting physiological conditions (homeostasis) is the **core of Low Dose Medicine** (LDM).

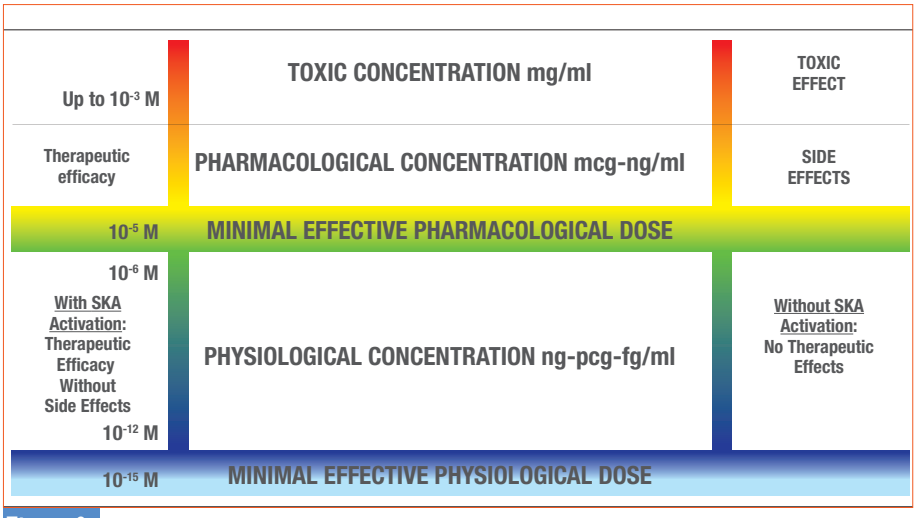

Figure 2

Biological effects of different doses of messenger molecules.

LDM represents an innovative medical paradigm born from the fusion of the most recent knowledge in the fields of Molecular Biology, PNEI and nano-concentrations research.
Instead of active compounds with potential pharmacologic side effects, the messenger molecules used in LDM are orally administered substances of the human body and, specifically, members of cell signaling pathways.

Scientific literature reports that cytokines oral intake is effective in modulating immune response [19-21] and a possible action mechanism involves M cells at intestinal epithelium level.

Messenger molecules are taken by M cells from intestinal lumen and presented to immune T cells within Peyer's patches lymph nodes [22] inducing an appropriate immune response (Figure 1).

A critical point of messenger molecules (and peptides in general) oral administration is represented by their low bioavailability (typically less than 1-2%) [23]; an effective drug delivery system is requested in order to improve this key parameter.

The use of low doses of active molecules per os in LDM is made possible by the application of **SKA technology** (**Sequential Kinetic Activation**), a drug delivery system 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

Year	Authors	Journal	Title	Research Type	Tested Molecules
2009	Gariboldi S. *et* Al.	***Pulmonary Pharmacology & Therapeutics***	Low dose oral administration of cytokines for treatment of allergic asthma	**Basic research *in vivo***	**IL-12 IFN-γ**
2012	D'Amico L. *et* Al.	***Journal of Cancer Therapy***	Low Dose of IL-12 stimulates T Cell response in cultures of PBMCs derived from Non Small Cell Lung Cancer Patients	**Basic research *ex vivo***	**IL-12**
2013	Cardani D. *et* Al.	***Gastroenterology Research***	Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation	**Basic research *in vivo***	**IL-10 anti IL-1**
2014	Radice E. *et* Al.	***International Immunopharmacology***	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	**Basic research *ex vivo***	**IFN-γ**
2014	Roberti M.L. *et* Al.	***Journal of Biological Regulatory & Homeostatic Agents***	Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris	**Clinical *trial***	**IL-4 IL-10 IL-11**

Table 1
List of the major published works in the field of Low Dose Medicine since 2009.

10^{-6} (microgram) for hormones [24] and 10^{-12} (picogram) for the other messenger molecules [25] (Figure 2).

This receptors sensitization allows the trigger of chain reactions (complex systems) and a restart of the biological function of the whole PNEI network.

-SKA low dose molecules work by bringing to the system an amount of information able to activate auto-regulation mechanisms.

From a clinical point of view, the hypothetical therapeutic approaches are:

1. To enhance a pathologically down-regulated cellular pathway using the same cytokine, hormone, neuropeptides or growth factor which are physiologically involved in the impaired signalling.
2. To use antagonistic low dose molecules in order to re-equilibrate a biological effect according to the principle of "opposing" molecules.

SCIENTIFIC LITERATURE SUPPORTS LDM APPROACH

Low Dose Medicine is not only a novel therapeutic theory based on hypothesis.

It is a new scientific paradigm based on a growing body of experimental evidences which clarify the physiologic and biochemical concepts underpinning the use of low doses of messenger molecules.
Scientific research has validated the theoretical principles of LDM: in November 2009, in fact, Pulmonary Pharmacology & Therapeutics published the first paper on the effects of SKA activated low-dose cytokines in the treatment 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 [27-30] followed the paper published by Gariboldi *et* Al. (Table 1) extending available data regarding LDM therapeutic design, efficacy and safety.

– The research works described in this review can be classified on the basis of the previously described therapeutic approach (Table 2).

BASIC RESEARCH STUDIES ON CYTOKINES WITH STIMULATORY ACTIVITY OF THE IMMUNE CELLS RESPONSE

In the papers published by D'Amico *et* Al. and Radice *et* Al. the LDM approach is proposed and verified in two *ex vivo* models based upon the stimulation of different immune cells subpopulations collected from oncologic patients.

– Both works focus on the pathologic responsiveness reduction of particular classes of immune cells in the presence of tumor disease and on the ability of specific low dose SKA cytokines (involved in the differentiation and stimulation of immune cells) to stimulate the immune response.

– D'Amico *et* Al. 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 immune-stimulatory and immune-modulatory activity of low dose SKA IL-12 on the subpopulation of T lymphocytes.
Low dose SKA IL-12 was proved to be capable of stimulating both CD4+ T lymphocytes and CD8+ cells and in particular the increase of CD4+ T cells expressing IFN-γ and simultaneously the increase of the cytotoxicity of the CD8+ T lymphocytes has been observed

D'Amico *et* Al. analyzing the dose-response data, also indicated the concentration of **0.01 pg/ml** as the most active; higher concentrations are quite ineffective (1 pg/ml) or show opposite action (10 ng/ml).

– Radice *et* Al. conducted a study on *ex vivo* Natural Killer cells obtained from the peripheral blood of patients with **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.
The lytic ability of PB-NK cells suitably stimulated with IFN-γ in conventional dosage (1ng/ml) or low-dose SKA IFN-γ (0.25 fg/ml) is evaluated.

The PB-NK cell activity is depressed increasingly 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.

The most relevant topics of the two works are resumed in Table 3.

▶ 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 treatment is demonstrated when compared to conventional one; additionally, in the work of D'Amico *et* Al. the high-dose IL-12 treatment (10 ng/ml) leads to a concomitant down-regulation of CD4+ cells and, in particular of Th1lymphocytes, 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 TRIAL ON LOW DOSE 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 or chronic inflammatory syndromes like Crohn's disease.

In this context, three studies 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. have studied the immunological mechanisms of **allergic bronchial asthma** in a suitable animal model in order to verify the effective-

"Enhance" approach	"Re-balance" approach
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. *Journal of Cancer Therapy.* ***2012*** *Sep; 3:337-342.*	Gariboldi S. *et* Al. – Low dose oral administration of cytokines for treatment of allergic asthma. *Pulm Pharmacol Ther.* ***2009*** *Dec;22(6):497-510.*
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. A preliminary study. *Intern. Immunopharm.* ***2014*** *19(1):66-73.*	Cardani D. *et* Al. – Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation. *Gastroenterology Research.* ***2013*** *Aug; 6(4): 124-133.*
	Roberti M.L. *et* Al. – Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris. *Journal of Biological Regulators & Homeostatic Agents.* ***2014****; 28(1): 133-139.*

Table 2
Classification of research papers according to the therapeutic approach.

Study	Research Type	Cytokine	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	**Basic research *ex vivo***	**rIL-12** (1/0.01 pg/ml)	X rIL-12 (10 ng/ml)	X (vehicle)	• Stimulation of $CD4^+$ and $CD8^+$ T cells. • Increased CD4+/IFN-γ Tcells. • Increased CD8+ T cells lytic activity. • T-reg cells 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. A preliminary study.	**Basic research *ex vivo***	**IFN-γ** SKA *low dose* (0.25 fg/ml)	X (rIFN-γ 1 ng/ml)	---	• PB-NK cytotoxicity decreases with tumor progression. • SKA low dose IFN-γ activates PB-NK cells in early stages of CRC • Low dose and high dose IFN-γ show the same activity on PBNK cells

Table 3
Synopsis of D'Amico *et* Al. and Radice *et* Al. basic research papers topics.

ness of the use of low dose cytokines to rebalance the Th1/Th2 response.
Gariboldi *et* Al. evaluated *in vivo* some basic immunological parameters altered in the presence of bronchial allergic asthma:
(1) the quantitative/qualitative composition of both immune cells panel (eosinophils, neutrophils and mononuclear cells) was evaluated in bronchial alveolar fluid (BALF) 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 to the fundamental role played by the Kinetic Sequential 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 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 immune-modulatory 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 *et* Al. instead have 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 up-regulation of these pro-inflammatory cytokines.

The use of low dose of SKA IL-10 and anti IL-1 antibody 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 treatment.

– Roberti *et* Al. investigated the possibility of using specific low dose 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**.

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 of a significant number of patients and conducted through the use of internationally validated rating scales PASI (Psoriasis Area Severity Index) and DLQI (the lesions and the quality of life respectively).

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 dis-

eases. Relevant topics of the cited works are resumed in Table 4.

HIGHLIGHTS OF LDM RESEARCH

The analyzed articles report the experimental evidence of the effectiveness of LDM approach on diseases involving the Immune System.

All the articles show the ability of the messenger molecules to modulate the responses of immune cells in a highly selective fashion; especially, the immune-stimulatory and immune-modulatory 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 the 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 signal molecules has often collided with the need of high dosages, realizing concentrations which 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. 2); the LDM is studied in order to descend within the range of messenger molecules physiological concentrations, aiming to obtain appreciable therapeutic results **operating below the concentrations at which the side effects appear**.

The ligand-receptor binding properties are crucial to explain how low doses of signaling molecules can be effective.

Receptor affinity for its specific ligand is fundamental for the activation of postreceptorial downstream [39-41].
In fact ligand saturation generally induces the receptor freezing and/or its down-regulation.

Low dose molecules are able to induce a direct physiologic receptorial stimulation of immune cells (as described by Gariboldi *et* Al.) modulating the responses within the homeostatic range; LDM realizes one of the cardinal point of PNEI approach to the disease: to restore the physiological panel of messenger 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: low dose molecules not processed with this activation procedure are totally ineffective as described by Gariboldi *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.

Study	Research Type	Cytokine/ antibody	Positive control	Placebo control	Results
Gariboldi S. *et* Al. – Low dose oral administration of cytokines for treatment of allergic asthma	**Basic research *in vivo***	**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)	X Control group	• SKA low doses are effective and safe in Thl/Th2 rebalance. • Non-activated cytokines are ineffective. • Cytokines association shows synergic effect.
Cardani D. *et* Al. – Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation	**Basic research *in vivo***	**IL-10 anti IL-1** 50 fg/Kg	---	X Control group	• SKA low dose IL-10 and anti IL-1 association is effective against IBD-related inflammation
Roberti M.L. *et* Al. – Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris	**Multicenter doubleblind placebo-controlled RCT**	**IL-4 IL-10 IL-11** 10 fg/ml; 40 drops/day each	---	X (vehicle)	• SKA low dose IL-10, IL-11 and IL-4 association is effective against psoriasis vulgaris • SKA low dose Interleukins show a long time action

Table 4
Synopsis of the topics of Gariboldi *et* Al., Cardani *et* Al. and Roberti *et* Al. research.

CONCLUDING REMARKS ON LOW DOSE MEDICINE

Five years of scientific research on LDM have allowed the researchers to provide assets of diverse and scientifically relevant data which can prove:
I - the validity of the theoretical concepts underpinning the LDM approach;
II - the centrality of the pharmaceutical process of molecules setting up called SKA;
III - the effectiveness of the experimental and clinical use of activated messenger molecules at low dosages;
IV - the immune-modulatory and immune-stimulatory ability of the tested cytokines;
V - the safety of the tested preparations. ■

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