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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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Abstract

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 an 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 healthy conditions; 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 summarize 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.

Keywords: Psycho-neuro-endocrine-immunology; Low dose medicine; Sequential kinetic activation; Immune system; Inflammation; Thl/Th2 balance; Autoimmune diseases; History of medicine.

Abbreviations:

PNEI: Psycho-Neuro-Endocrine-Immunology; LDM: Low Dose Medicine; SKA: Sequential Kinetic Activation; ECM: Extra Cellular Matrix; PBMC: Peripheral Blood Mononuclear Celi; PU¬NK: Peripheral Blood-Natural Killer; NSCLC: Non-Small Cell Lung Cancer; CRC: Colorectal Carcinoma; IL: Interleukin; IFN- γ: Interferon-γ; IBDs: Inflammatory Bowel Diseases; TNF-α: Tumor Necrosis Factor-α; PASI: Psoriasis Area Severity Index; DLQI: Dermatology Life Quality Index.

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]. 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 psychoneuroendocrine 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 cross-talk 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 bi-directional, 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). 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).

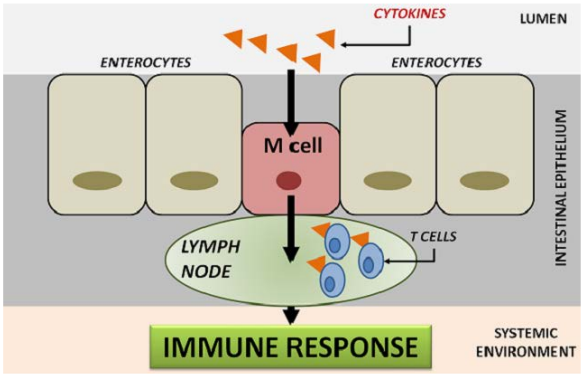

Figure 1: Hypothetical pathway of cytokines-induced M cells-mediated immune response after per os administration.

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 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 information able to activate auto-regulation mechanisms.

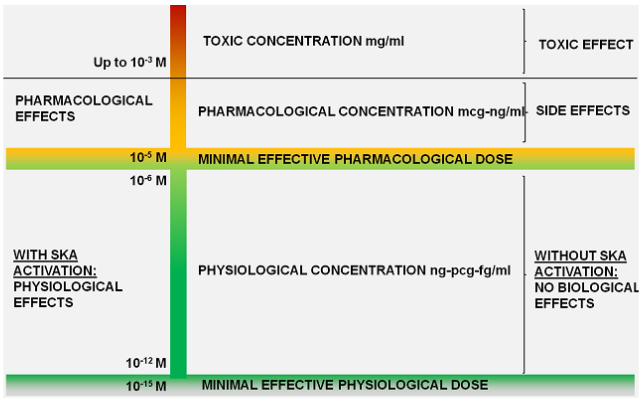

Figure 2: Biological effects of different doses of messenger molecules.

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 allergie 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).

Year	Authors	Journal	Research Type	Title	Tested Molecules
2009	Gariboldi, et al. [26]	Pulmonary Pharmacology & Therapeutics	Basic research *in vivo*	Low dose oral administration of cytokines for treatment of allergie asthma	IL-12 IFN-γ

2012	D'amico, et al. [27]	Journal of Cancer Therapy	Basic research *ex vivo*	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, et al. [28]	Gastroenterology Research	Basic research *in vivo*	Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation.	IL-10 α-IL-1
2014	Radice, et al. [29]	International Immunopharmacology	Basic research *ex vivo*	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

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

"Enhance" approach	**"Re-equilibrate" approach**
D'Amico 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 et al. (2014) Low dose oral administration of cytokines for treatment of allergie asthma. Pulm Pharmacol Ther 22: 497-510.
Radice 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(I):66-73.	Cardani et al.(2013) Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation. Gastroenterology Research 6: 124-133.
	Roberti et al. (2014) Immunomodulating treatment with low dose Interieukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris. J Biol Regul Homeost Agents 28: 133-139.

Table 2: Therapeutic approach-based research works classification.

Basic research studies on cytokines with stimulatory activity of the immune cells response

In the papers published by D'Amico and Radice the LDM approach is proposed and verified in two *in vitro/ex vivo* models based upon the stimulation of different immunitary cells subpopulations collected from oncologie 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 immunostimulatory and immunomodulatory 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. It was also found that the action of IL-12 is also directed to the T-reg cells with the function of down-regulation, particularly important because of the increase of this subpopulation in the examined pathology. D'Amico, et al. analyzing the dose-response data, also indicated the concentration of 0.01 pg/ml as the more 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 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. 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 *in vitro/ex vivo* 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 Thl 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].

Study	Type	Cytokine	Positive control	Placebo control	Results
D'Amico et al. Low Dose of 1L-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)	XrIL-12 (10ng/ml)	X (vehicle)	• Stimulation ofCD4+ and CD8+ T cells. • Increased CD4+/IFN-γ Tcells. • Increased CD8+ T cells lytic activity. • T-reg cells suppression.
Radice et al. Low-doses of sequential-kinetic-activated interferon-gamma enhance the *ex vivo* cytotoxicity Of peripheral blood naturai killer cells from patients with early-stage colorectal cancer	Basic research *ex vivo*	SKA low dose IFN-γ (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 shows the same activity on PB-NK cells

Table 3: Synopsis of D'Amico et al. and Radice et al. basic research papers topics.

Basic research studies and clinical trial on low dose cytokines with rebalancing activity on the Thl/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 allergie asthma) while the prevalence of Thl 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 effectiveness of the use of low dose cytokines to rebalance the Thl/Th2 response.

Gariboldi et al. evaluated *in vivo* some basic immunological parameters altered in the presence of bronchial allergic asthma: (i) the quantitative/qualitative composition of both immune cells panel (eosinophils, neutrophils and mononuclear cells) was evaluated in bronchial alveolar fluid (BALF) of animals; (ii) 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 Thl/Th2 switch modulation of low dose SKA cytokines treatment. Great importance was recognized of 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 immunomodulatory attitudes of low dose messenger molecules exerted through the direct stimulation of immune cells with a final rebalancing effect on Thl/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 diseasc). The analysis of the panel of Thl/Thl7 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 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 Thl/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 (Dermatology Life Quality Index) for the evaluation of the extent of the lesions and to determine 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 diseases. Relevant topics of the cited works are resumed in Table 4.

Study	Type	Cytokine/antibody	Positive control	Placebo control	Results
Gariboldi et al. Low dose oral administration of cytokines for treatment of allergie 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 et al. Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Injlammation.	Basic research in vivo	IL-10 anti IL-l 50 fg/Kg		X control group	• SKA low dose IL-10 and anti IL-1 association is effective against IBD-related inflammation
Roberti et al. Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in Psoriasis vulgaris.	Multicenter double-blind placebo-controlled RCT	IL-4; IL-10; IL-1110 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 Gariboldi et al., Cardani et al. and Roberti et al. research.

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 immunitary 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 Thl/Th2 balance is crucial for the management of diseases with diametrically opposed cytokine imbalances such as Bronchial Allergie Asthma (which shows a Th2 predominance) [33,34], Crohn's Disease [35,36] and Psoriasis Vulgaris [37,38] (Thl-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 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 S, et al.) modulating the responses within the homeostatic range; LDM realizes one of the cardinal point of PNEI approach to the disease: to restore 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.

Concluding Remarks on Low Dose Medicine

Five years of scientific research on LDM has 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 immunomodulatory and immunostimulatory ability of the tested cytokines; (v) the safety of the tested preparations.

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