GUNA Scientific Department

LOW DOSE MEDICINE

Highlights on basic and clinical research studies

1st EDITION
SEPTEMBER 2017

GUNA
terapie d'avanguardia

1st Edition: September 2017

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"Falsehood flies,
and truth comes limping after it"

Jonathan Swift (1667 - 1745)

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ALLERGOLOGY

DERMATOLOGY

FOREWORD

Scientific research and **clinical trials** are important pillars underpinning the success of Guna S.p.a. in the pharmaceutical industry.

Every year Guna invests significant resources in **basic and clinical research.**

Guna cooperates with prestigious universities in Italy and abroad by supporting research on the biological activity of low dosages of active principles, thus contributing to build a new paradigm in Pharmacology.

Research studies on isolated cell lines and animal models led Guna to the understanding of the action mechanisms of activated nanoconcentrations, demonstrating their effectiveness and safety. Moreover, over the past eight years, great emphasis has been given to Clinical Research for the definitive validation of the therapeutic effects on patients and of safety.

Guna activated collaborations with some of the most prestigious Italian Research Institutes and some of Italy's most renowned Universities (among others: *Istituto Superiore di Studi Sanitari, Rome; Hospital "Fatebenefratelli", Rome; "Polyclinic Institute" Milan; "Sapienza" University, Rome; Hospital "City of Health and Science, Turin; University of Florence; University of Pavia; University of Naples; University of Novara*). In 2009 Guna launched the Clinical Research Project, and started numerous trials on several high prevalence diseases treated with advanced therapeutic approaches.

The engagement of Guna in research studies continues on an ongoing basis. At the beginning of 2017, many studies are in progress or in the pipeline, and this commitment will be increasingly intense in the future.


International Immunopharmacology
Journal of Cancer Therapy
Gastroenterology Research
MINERVA OFTALMOLOGICA
Journal of BIOLOGICAL REGULATORS & Homeostatic Agents
PULMONARY PHARMACOLOGY & THERAPEUTICS
Drug design, Development and Therapy
Dovepress
CIMID
Der Hautarzt
Journal of Ovarian Research
Dermatological Science
JDS
MINERVA CARDIOANGIOLOGICA
Translational Oncology
BMC Musculoskeletal Disorders


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INTRODUCTION

Since the mid-80s, the development of concepts expressed by Psycho-Neuro-Endocrine-Immunology determined a shift in the interpretation of the human body's biological functions, from a strictly organicistic/specialized view to the modern concept of cellular network and the importance of the continuous cross-talk between cells, organs and systems, in both physiological and pathological conditions. Particular attention is drawn to messenger molecules and their important role, thus paving the way to a possible new solution in the clinical field: **the opportunity to make use of the same messenger molecules as medicines, in order to bring the sick organism back to its original physiological conditions.**

During the following years, research in physiology and in molecular biology provided more and more evidence of the crucial role of these molecules, e.g. hormones, neuropeptides, cytokines and growth factors, in all physiological and pathological processes, and opened new scenarios in the field of pharmacology.

However, a setback in the development of new medications has come from the negative side effects that these molecules may cause, when administered in concentrations as high as those normally used in pharmacology.

In the early 90s, in Italy, a new medical and pharmacological approach was developed: "Low Dose Medicine". This was made possible by innovative pharmaceutical technologies that allow a reduction in the concentration of these molecules in the pharmaceutical preparations. It was then observed, through basic research studies on cells and on animal models, and later on through clinical trials, that very low doses of hormones, neuropeptides, cytokines and growth factors are able to produce the same biological effects (therefore, therapeutic effects) as higher doses normally used in pharmacology, but without the negative side effects linked to such higher concentrations.

BASIC PRINCIPLES OF LOW DOSE MEDICINE

Low Dose Medicine (LDM) stems from the merging of Molecular Biology, and Psycho-Neuro-Endocrine-Immunology (PNEI), and it has been developed thanks to advancements in **low-dose pharmacology.**

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

- To address the person and not just the disease
- To act on the cause and not just on the symptom
- To consider a person as a whole, and in his/ her individuality.

The focus on each human being as a mind-body whole is a key concept in Low Dose Medicine. Each individual patient is always and constantly central for defining the appropriate low-dose therapy. On the other hand, the etiology, i.e. the origin of the disease, instead of its symptoms, is the ultimate target of any low-dose therapy.

- *To recover or to maintain the original physiological conditions (homeostasis) is the first and basic principle of Low Dose Medicine.*

THE P.N.E.I. APPROACH

Low Dose Medicine stems from an innovative idea in the medical field: to bring back a sick organism to its original physiological conditions by using those biological molecules (signaling molecules) that are normally present in the body and that, in healthy conditions, monitor and guide the body's physiological functions. These molecules are **cytokines, hormones, neuropeptides** and growth factors.

Moreover, Low Dose Medicine uses very low doses of natural substances derived from plant, animal or mineral sources and tries to investigate their properties through the magnifying lens of Molecular Biology.

Along with the discoveries on signaling molecules, medical science has undergone a shift from a mechanical view of the body's physiological functions to a more unified and complex concept, in accordance with the principles of **Psycho-Neuro-Endocrine-Immunology**. This vision has strongly influenced the development of Low Dose Medicine.

- *A unified and global vision of the human body is the second principle of Low Dose Medicine.*

P.N.E.I. SYSTEMS AND BIDIRECTIONAL CROSS-TALK

The key to understanding the P.N.E.I. approach is the cross-talk between the Psycho-Neuro-Endocrine systems and the immune system.

This sophisticated cross-talk is mediated by a complex network of signaling molecules, which are the vehicle of the biological information necessary for such a complex and efficient regulation of cellular response. An altered cross-talk due to an unbalanced concentration (an excess or a deficiency) of certain signaling molecules is crucial, for instance, in inflammatory, allergic and autoimmune diseases. Restoring the physiological concentration of signaling molecules is key to recovering the homeostatic balance.

- *The use of physiological concentrations of biological molecules able to direct and control cellular functioning, and therefore to restore the original physiological conditions, is the third principle of Low Dose Medicine and it is at the heart of any low dose therapy.*

BIOAVAILABILITY OF SIGNALING MOLECULES: S.K.A. TECHNOLOGY

An issue in the administration of signaling molecules is their low bioavailability. An effective drug delivery system is required for improving this key parameter. **The use of physiological low doses of these molecules is made possible by the application of a special technology called S.K.A. (Sequential Kinetic Activation).** S.K.A. technology is based on the principles of quantum physics, and in particular on release activity, i.e. the ability of a substance to release its activity in an aqueous medium. This means that S.K.A. technology allows these ultra low concentrations of messenger molecules, which are contained in a water-alcohol solution for oral use, to be effective even if concentration is below what is usually considered as the minimum effective dose, with therapeutic results comparable to those induced by higher concentrations.

S.K.A. technology is the key for obtaining innovative pharmaceutical products, while low doses of signaling molecules are the active principles of Low Dose Medicine.

This modern and refined technological process allows to obtain the effective, safe, and synergistic pharmaceutical products on which Low Dose Medicine is based.

SIGNALING MOLECULES ACTIVATED BY S.K.A. TECHNOLOGY: ACTION MECHANISMS

The action mechanism of low-dose, SKA-activated cytokines, hormones, neuropeptides, growth factors, is the sensitization and activation of a few units of cellular or plasmatic receptors. This takes place because the molecules are highly diluted. **Physiological concentrations in our body are of the order of nanograms/ milliliter** (for instance, hormones), **up to fentograms/milliliter** (as is the case for other signaling molecules).

Low-dose, SKA-activated molecules work on the whole P.N.E.I. system, by providing information able to activate self-regulation mechanisms.

The ability to correct an alteration in the immune system by administering certain cytokines, or to restore an endocrine imbalance by using specific hormones, represents one of the most fascinating and innovative fields of research in Molecular Biology applied to Medicine.

LOW DOSE MEDICINE AND SCIENTIFIC RESEARCH: A WINNING PARTNERSHIP

Ten years of scientific research in the Low Dose Medicine field have proven the validity of this approach and the efficacy and safety of therapeutic intervention based on the oral administration of low doses of activated messenger molecules. This long journey started in 2009, when the journal Pulmonary Pharmacology & Therapeutics published the first article on the effects of low-dose cytokines in an animal model of allergic asthma (Gariboldi S. *et al. Low dose oral administration of cytokines for treatment of allergic asthma.* Pulmonary Pharmacology & Therapeutics 22 (2009) 497-510).

Since 2009, new publications have followed the paper published by Gariboldi et al. (TABLE 1).

Having travelled this long and often rough, but always exciting, path, we can now say that scientific literature supports the Low Dose Medicine approach and that what used to be mere scientific theory has now lain the foundations for a new medical paradigm.

LIST OF THE MOST IMPORTANT PUBLICATIONS IN THE LOW DOSE MEDICINE FIELD (2009 - 2017)

YEAR	AUTHOR	JOURNAL	RESEARCH TYPE	TITLE	SUBSTANCE / MEDICATION
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 antibodies
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	Clinical Trial	Increasing of visual function in patients with retinal atrophy treated with drugs of Low Dose Medicine. Monocentric retrospective observational study.	· NT3 · NT4 · NGF
2015	Barygina V. et al.	Journal of Dermatological Science	*In vitro* basic research	Treatment with low-dose cytokines reduces oxidative-mediated injury in perilesional keratinocytes from vitiligo skin.	· IL-4 · IL-10 · b-FGF · β-endorphin
2015	Lotti T. et al.	Journal of Biological Regulatory & Homeostatic Agents	Clinical Trial	Vitiligo: successful combination treatment based on oral low dose cytokines and different topical treatments.	· IL-4 · IL-10 · Anti-IL-1 antibodies · b-FGF
2015	Radice E. et al.	Translational Oncology	*Ex vivo* basic research	Enhancement of the immunomodulatory functions of ex vivo-generated dendritic cells from early-stage colon cancer patients by consecutive exposure to low doses of sequential kinetic activated IL-4 and IL-12. A preliminary study.	· IL-4 · IL-12

YEAR	AUTHOR	JOURNAL	RESEARCH TYPE	TITLE	SUBSTANCE / MEDICATION
2015	Lotti T.	Der Hautarzt	Clinical Trial	Successful combination treatment for Psoriasis with phototherapy and Low Dose Cytokines. A spontaneous observational retrospective clinical study.	· IL-4 · IL-10 · Anti-IL-1 antibodies
2016	Barygina V. et al.	Journal of Dermatological Science	*In vivo* basic research	Low dose cytokines reduce oxidative stress in primary lesional fibroblasts obtained from psoriatic patients	· IL-4 · IL-10 · b-FGF · β-endorphin
2016	Fiorito F. et al.	Comparative Immunology, Microbiology and Infectious Diseases	Clinical Trial (veterinary)	Clinical improvement in feline herpesvirus 1 infected cats by oral low dose of interleukin-12 plus interferon-gamma.	· IL-12 · IFN-γ
2016	Genazzani A. et al.	Frontiers in Gynecological Endocrinology	Observational pilot study	Pharmacological and Integrative Treatment of Stress-Induced Hypothalamic Amenorrhea	· β-Estradiol
2016	Uberti F. et al.	Cell Tissues Organs	*In vitro* basic research	Stimulation of the Non-neuronal Cholinergic System by Highly Diluted Acetylcholine in Keratinocytes	· Acetylcholine
2017	Martin Martin S. et al.	Drug Design, Development and Therapy	Clinical Trial	An open randomized active-controlled clinical trial with low-dose SKAcytokines versus DMARDs evaluating low disease activity maintenance in patients with rheumatoid arthritis.	· Anti IL-1 antibodies · IL-10 · IL-4
2017	Carello R. et al.	Italian Journal of Pediatrics	Clinical Trial	Long-term treatment with Low-Dose Medicine in chronic childhood eczema. A double-blind two-stage randomized control trial	· IL-12 · IFN-γ

TABLE 1
List of the most important publications in the low dose medicine field since 2009. Updated August 2017.

MAIN FINDINGS FROM THE RESEARCH CONDUCTED IN THE LOW DOSE MEDICINE FIELD

All of the experiences conducted to date (**TAB. 1**) show, in particular, the ability of signalling molecules to modulate immune cell response in a highly selective manner and, more specifically, they clearly describe the immunostimulatory and immunomodulatory capacities of the cytokines tested.

The ability to act on the Th1/Th2 balance is essential for the management of diseases characterised by imbalances in the concentration of cytokines with diametrically opposite biological activity, such as allergic bronchial asthma (which shows Th2 predominance) and Crohn's disease and Psoriasis Vulgaris (conditions in which Th1 response is predominant).

One of the key points emerging from the scientific papers analysed is the efficacy of treatment with low-dose molecules, despite the fact that they work at concentrations lower than those usually considered pharmacologically active. The use of cytokines and other signalling molecules is often hampered by the need to use high doses, which takes these substances to final concentrations such as to induce a vast range of collateral effects, in addition to the desired pharmacological effects.

The conventional minimum active dose for these molecules is usually between the lowest pharmacologically active dose (10^{-5}) and the maximum physiological concentration (10^{-6}).

Low-dose pharmacology, on the other hand, works within the range of physiological concentrations of signalling molecules (10^{-9} - 10^{-15}) and, therefore, beneath the concentrations at which adverse events occur, whilst nevertheless achieving appreciable therapeutic results.

The properties of the ligand-receptor bond are fundamental for explaining how low-dose SKA signalling molecules can be effective. The receptor's affinity for its specific ligand is fundamental for activating the signalling pathways downstream of the receptor. In conditions of ligand saturation, receptor blockade and/or down-regulation are usually induced.

Low-dose molecules, on the other hand, are able to induce direct physiological receptor stimulation on the immune cells (as described by Gariboldi S. *et al.*, 2009) by modulating response within the homeostatic range. LDM achieves one of the key points of the PNEI approach to disease: restoring the physiological signalling molecule network.

From a pharmacological point of view, the papers analysed highlight the importance of the activation of low-dose molecules through the drug delivery process known as SKA (Sequential Kinetic Activation): low-dose molecules not prepared using this activation procedure are completely ineffective, as described, once again, by Gariboldi S. et al., 2009. SKA activation is fundamental in order to overcome the conceptual barrier constituted by the lowest pharmacologically effective dose. SKA technology is able to induce a release effect of the activity exerted by the low-dose molecules through interaction with the aqueous vehicle.

Ten years of scientific research on Low Dose Medicine have allowed researchers to provide different types of scientifically-relevant data that is able to demonstrate:

I. the validity of the theoretical concepts forming the basis of the LDM approach;

II. the key role played by the pharmaceutical technological process known as SKA (Sequential Kinetic Activation);

III. the efficacy of the experimental and clinical use of low doses of SKA-activated signalling molecules;

IV. the immunomodulatory and immunostimulatory capacities of the cytokines tested and the trophic activity of growth factors;

V. the safety of the preparations tested.

LOW-DOSE MEDICINE: A NEW ERA FOR PHARMACOLOGY FOR A NEW APPROACH TO TREATMENT

By applying SKA technology, biological molecules that are pharmacologically active at low doses are able to remodulate the neuro-immuno-endocrine network, whose imbalances cause a number of medical conditions. The option of being able to use biological molecules with immunomodulatory properties paves the way for the effective treatment of illnesses with a high social impact, for which current treatments are largely symptomatic and non-decisive.

LDM provides the instruments for supporting, in an integrated way, the treatments that are currently considered the gold standard for many diseases. The possibility of using low-dose SKA signalling molecules without side effects makes it possible to intervene on many networks in a physiological and pleiotropic manner, whilst simultaneously acting on the crucial aspects of the diseases' onset (or trigger) mechanism.

The most modern techniques for producing human recombinant proteins have made it possible to produce effective medicinal products able to meet high-quality production standards.

SKA pharmaceutical technology makes it possible to produce low-dose medicinal products able to obtain the same therapeutic effects as those containing high doses of signalling molecules, without the adverse effects usually linked to high doses.

The SKA production method opens a new era in the clinical use of signalling molecules of biotechnological origin.

LDM is attracting ever-greater interest from the scientific world and preclinical and clinical studies are paving the way for new treatment scenarios for many diseases, thereby giving new hope to millions of patients.

In the light of the results of research and the consistency of the critical mass of studies conducted, we can now confidently state that from Evidence-Based Medicine, ***Low Dose Medicine*** has now entered the era of Efficacy-Based Medicine.

STATISTICS

BASIC STATISTICS

Applying statistics to research in the biomedical field (considered as including both basic and preclinical research) provides a kind of guarantee regarding the reliability of the results and their analysis. Correct statistical analysis is the presupposition for a realistic interpretation of the results of any study.

The statistical instruments used in scientific publications evolve constantly: the purpose of the following paragraph is to provide some general direction for the interpretation of the statistical information provided in the next pages.

DEFINITION OF STATISTICS

Statistics is a science that aims to make unthinkable events comprehensible and measurable using numbers.

THE STATISTICAL PROCESS

A statistical investigation includes the following phases:

1. **Sampling**

Selecting the population of subjects to study.
The population may be a:

- *Sample population* (usually chosen by randomisation);
- *Objective population* (selected using set criteria, for example, subjects suffering from a certain disease).

2. **Data collection**

3. **Data presentation**

- Graph: the data collected are presented as graphs such as histograms, bar charts, line or curve graphs, pie charts, etc. (**FIG. 1**).

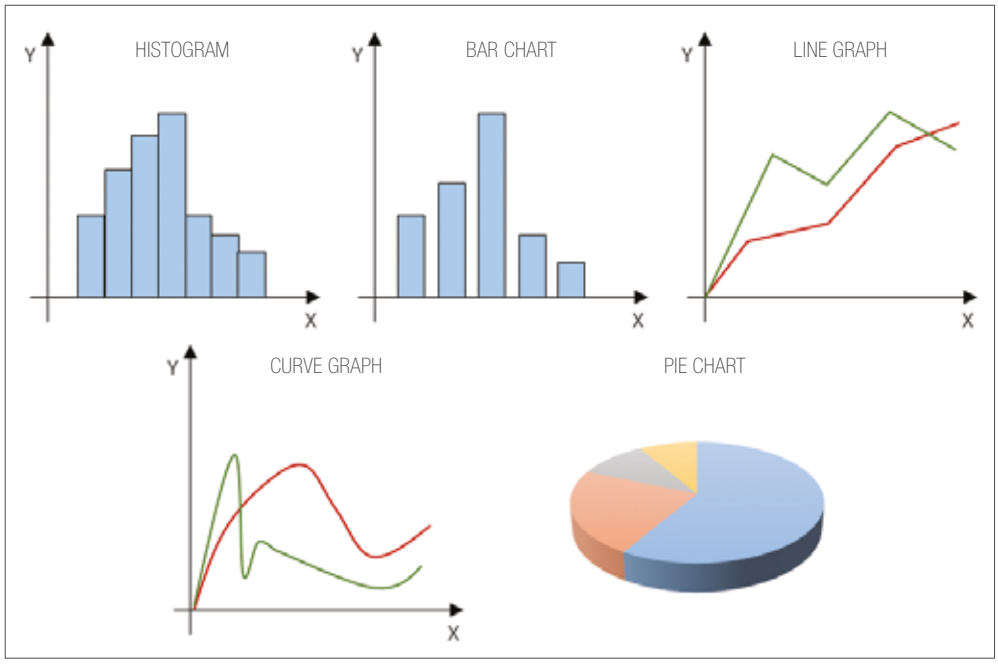

FIG. 1
EXAMPLES OF DATA PRESENTATION USING GRAPHS

- Numerical: the data are represented by characteristic numerical values:
  - » *Mean* (the sum of the individual cases divided by the number of cases added together);
  - » *Range* (highest and lowest value of the dataset collected);
  - » *Median* (the mid-value of the values collected).
  - » *Mode* (the value that appears most frequently);
  - » *Standard deviation* (SD) (indicates the dispersion of the values around the mean): it is represented graphically with a "T-shaped" or a "double T-shaped" bar, for example, on the histogram, column or point of the dotted line (**FIG. 2**).

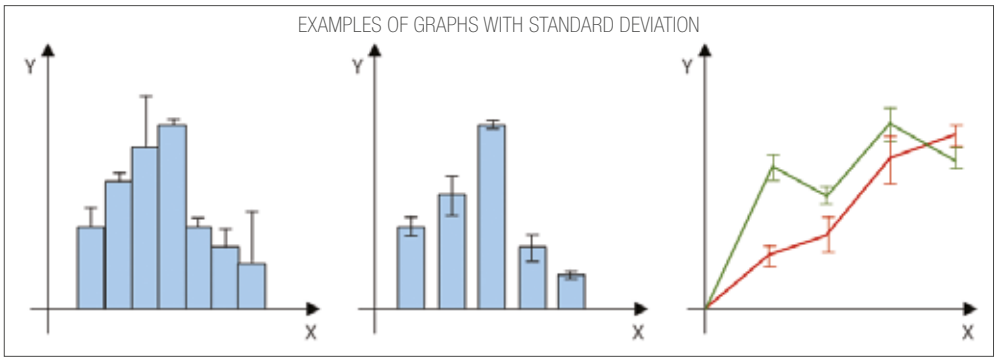

FIG. 2
EXAMPLES OF DATA PRESENTATION USING GRAPHS WITH STANDARD DEVIATION

- Distribution of a population: the values collected and indicated graphically can be presented as two forms of distribution (**FIG. 3**):
  - » *normal* (or Gaussian or bell) distribution, in which the mean, median and mode coincide; the mean and standard deviation describe the population correctly;
  - » *non-normal distribution* in which the mean, median and mode do not coincide; the mean and standard deviation do not describe the population correctly. In these cases (non-normal distribution), the distribution of the population is expressed with percentiles. With percentiles, the median coincides with the 50th percentile (mid-point). The 25th percentile (median – 25%) and the 75th percentile (median+25%) can be used, as can the 5th percentile (median – 45%) and the 95th percentile (median+45%).

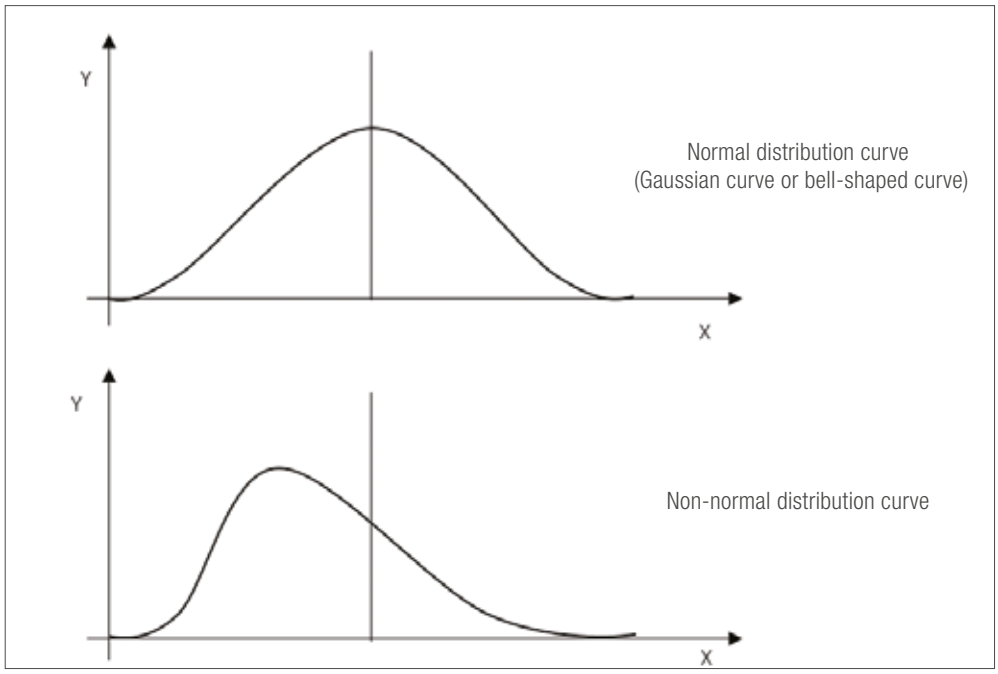

FIG. 3
EXAMPLES OF
DISTRIBUTION CURVES

Data processing

There are a great number of data processing tests, including parametric and non-parametric tests.

1. *Parametric tests* are tests that can be applied when data distribution is normal. Parametric tests include:

   » Student's test: Student's test (or t-test) is a type of analysis used to verify whether there are any differences between two means.

   » Analysis of variance test (ANOVA): the analysis of variance is a method developed by Fisher. The test can be used to establish whether two or more sample means can be obtained from populations with the same parametric mean.

2. *Non-parametric tests* are tests that do not need the values compared to have a certain distribution (e.g. Gaussian). The purpose of nonparametric methods is to compare samples whose distribution trend is unknown or cannot be hypothesised. They are suited to even very small samples.

   Nonparametric tests include:

   » The Mann-Whitney U-test: the Mann-Whitney U test may be used when the 2 fundamental assumptions (normal distribution and large sample size) for the use of parametric tests are not satisfied.

   » Friedman test: is the nonparametric equivalent of the ANOVA test.

CONFIDENCE LIMITS

The calculated mean of a sample population is not always representative of the general population of collected data. There are usually two factors that influence the difference between two means (sample population and general population):

- the number of members of the sample population (the larger the sample, the less likely the calculated mean is to differ from that of the general population);
- the intrinsic variability of the parameter studied.

The discordance between the mean of the sample population and the mean of the general population is usually referred to as the **Standard Error (SE).**

Standard Error provides a probabilistic estimate of the difference between the mean of the tested sample and that of the general population considered.

The **confidence limits** are the values between which it can be presumed that the actual population mean falls. Confidence limits can be:

- 68% (m ± 1 SE)
- **95% (m ± 2 SE)**
- 99% (m ± 3 SE)

The most commonly used confidence limit is 95%.

SIGNIFICANCE TESTS

This is a statistical method that makes it possible to establish whether two results are significantly different. Significance is based on a hypothesis, known as the **null hypothesis**, according to which there is no significant difference between the two results. It is then established whether the null hypothesis is true or false.

According to convention, a probability (P) greater than 5% ($P>0.05$) considers the null hypothesis to be true, whereas a probability lower than 5% **($P<0.05$)** considers the null hypothesis to be false i.e. it **confirms the statistical significance of the result**. Moreover, the more the value of P is lower than 5% (e.g. $P>0.01$, $P<0.005$, $P<0.001$), the more the difference observed between the two results is significant (real).

The value of **P** represents the probability of erring by claiming that there is a real difference between 2 results. Significance is indicated (in both the charts and the text) by using asterisks in a number that is proportionate to significance [$P<0.05$ (*); $P<0.005$ (**), etc.] next to the data series it refers to.

INDEX OF ABBREVIATIONS

ACTH	Adreno Cortico Tropic Hormone
AMP	Antimicrobial peptide
Anti-IL-1	Anti-IL-1 antibodies
APCs	Antigen-presenting cells
AUC	Area Under the Curve
BALF	Bronchoalveolar Lavage Fluid
b-FGF	Basic-Fibroblast Growth Factor
Caco-2	Human Epithelial Colorectal Adenocarcinoma Cells
CD	Crohn's Disease
$CD4^+$ (T cells)	T cells positive for cluster of differentiation 4
$CD8^+$ (T cells)	T cells positive for cluster of differentiation 8
CDAI	Clinical Disease Activity Index
CRC	Colorectal Carcinoma
CRF	Corticotropin Releasing Factor
CRP	C-Reactive Protein
DA	Dopamina
DAS28	Disease Activity Score 28
DCs	Dendritic cells
DHEA	Deidroepiandrosterone
DLQI	Dermatology Quality of Life Index
DMARDs	Disease Modifying Antirheumatic Drugs
DSS	Dextran Sodium Sufphate
E2	Estradiol
ELISA	Enzyme-Linked Immunosorbent Assay

ESR	Erythrosedimentation Rate
FACS	Fluorescence-activated Cell Sorting
FHV-1	Feline herpesvirus-1
FSH	Follicle Stimulating Hormone
FU	Follow-up period
GABA	Gamma-Aminobutyric Acid
GHA	Global Health Assessment
GnRH	Gonadotropin-releasing Hormone
HA	Hyaluronic Acid
H2DCFDA (DCF)	2',7'-dichlorodihydrofluorescein diacetate
HPA	Hypothalamic-Pituitary-Adrenal Axis
HPG	Hypothalamic-Pituitary-Gonadal Axis
IBDs	Inflammatory Bowel Diseases
HT-29	Human Colorectal Adenocarcinoma Cell Line
IFN-γ	Interferon-γ
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-12	Interleukin-12

IL-17	Interleukin-17
ISCEV	International Society for Clinical Electrophysiology of Vision
K562	Human Myelogenous Leukemia Cells
KC	Murine IL-8 homologue
LDA	Low Disease Activity
LDM	Low Dose Medicine
LH	Luteinizing Hormone
LU_{30}	Lytic Units 30%
MHC-I	Major Histocompatibility Complex-I
MHC-II	Major Histocompatibility Complex-II
MLR	Mixed Lymphocytes Reaction
MoDCs	Monocyte-derived Dendritic Cells
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NGF	Nerve Growth Factor
Nidek's MP1	Nidek's MP1 Retinal Microperimeter
NK	Natural Killer cells
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
NSCLC	Non-Small-Cell Lung Carcinoma
NT3	Neurotrophin-3
NT4	Neurotrophin-4
OA	Osteoarthritis
OCT	Optical Coherent Tomography
PASI	Psoriasis Area Severity Index
PBMCs	Peripheral Blood Mononuclear Cell

PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PNEI	Psycho-Neuro-Endocrine-Immunology
PRL	Prolactin
RLU	Relative Luminescence Unit
ROS	Reacting Oxygen Species
SAA	Serum Amyloid A
SCORAD	Scoring Atopic Dermatitis
SDAI	Simplified Disease Activity Index
SF36	36-Item Short Form Survey
SKA	Sequential Kinetic Activation
SOD	Superoxide dismutase
T3	Triiodothyronine
Th1	T-helper 1
Th2	T-helper 2
Th17	T-helper 17
TJ	Tight Junctions
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor-α
Treg	Regulatory T cells
UC	Ulcerative Colitis
VAS	Pain Visual Analog Scale
ZO-1	Zonula Occludens-1

TABLE OF CONCENTRATIONS

Molar concentrations	Exponent (in referring to the gram unit)	Number of molecules/liter	Number of molecules/µl
Millimoles (mmol - mg/L)	- 3	$1x\ 10^{21}$	$1x\ 10^{15}$
Micromoles (µmol - µg/L)*	- 6	$1x\ 10^{18}$	$1x\ 10^{12}$
Nanomoles (nmol - ng/L)	- 9	$1x\ 10^{15}$	$1x\ 10^{9}$
Picomoles (pmol - pg/L) **	- 12	$1x\ 10^{12}$	$1x\ 10^{6}$
Femtomoles (fmol - fg/L)	- 15	$1x\ 10^{9}$	$1x\ 10^{3}$
Attomoles (amol - ag/L)	- 18	$1x\ 10^{6}$	1
Zeptomoles (zmol - zg/L)	- 21	1000	1/1000
Yoctomoles (ymol - yg/L)	- 24	1	1/1000000

(*) THE CONCENTRATION OF GUNA LOW DOSE SKA D6 HORMONES FALLS WITHIN THE MICROMOLE RANGE.

(**) THE CONCENTRATION OF THE GUNA LOW DOSE SKA 4CH NEUROPEPTIDES, CYTOKINES AMD ANTIBODIES FALLS WITHIN THE PICOMOLE RANGE.

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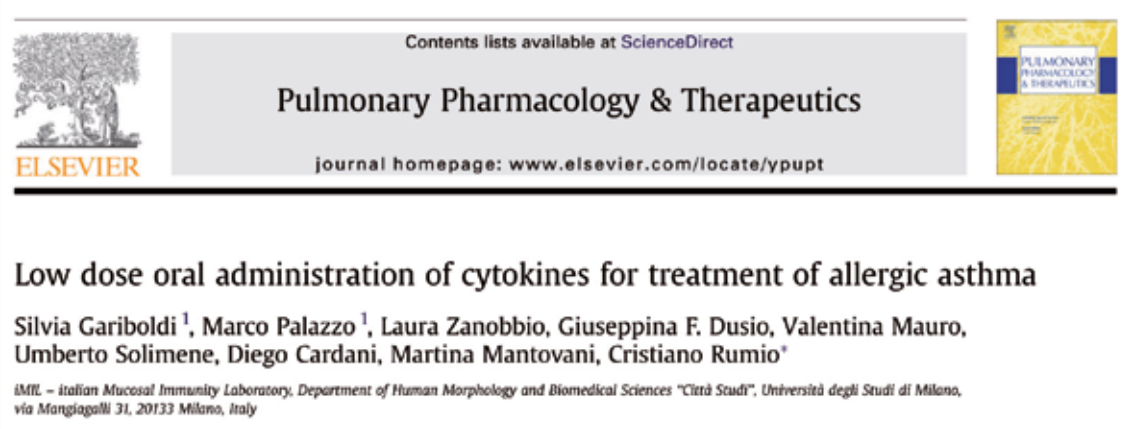
GUNA HIGHLIGHTS

Allergology

LOW DOSE ORAL ADMINISTRATION OF CYTOKINES FOR TREATMENT OF ALLERGIC ASTHMA



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Low dose oral administration of cytokines for treatment of allergic asthma

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FOREWORD

The article ***"Low dose oral administration of cytokines for treatment of allergic asthma"*** is the result of a two-year research study carried out by the Department of Human Morphology, University of Milan, Italy.

The work opens new prospects in the treatment of allergic diseases and has shown the therapeutic effects of a combination **of low-dose SKA Interleukin-12 and Interferon-γ (GUNA®-IL 12, GUNA®-IFNGAMMA. Guna S.p.a. - Milan, Italy)** in the treatment of allergic asthma in an animal model.

INTRODUCTION

Allergies, as many other pathologies affecting the Immune System, can be considered as an alteration of what is known as the ***immune system balance***.
In this specific case it is a switch from Th1 to Th2, i.e., Th2 lymphocytes are hyperexpressed on Th1 lymphocytes.
A major role in Th lymphocytes (T helper lymphocytes) function is played by cytokines, molecules capable of controlling the function of Th lymphocytes, our immune system's "orchestra conductors", on which depends the evolution of many pathologies, including allergies.
The use of cytokines to modify various alterations affecting the immune system is one of the most fascinating and groundbreaking research fields of Molecular Biology applied to Medicine.

The research study published on ***Pulmonary Pharmacology & Therapeutics*** is based on the fact that allergic patients typically show a **hyper-expression of Th2 lymphocytes** and therefore an excess synthesis of two cytokines produced by the latter: **Interleukin-4 (IL-4) and Interleukin-5 (IL-5)**.

These are allergy-predisposing interleukins: IL-4 induces the up-regulation of Th2 and B lymphocytes (increasing IgE production); IL-5 induces the differentiation of eosinophil granulocytes: this immunological situation is the real, deep seated prerequisite for the onset of allergies. Conversely, IL-12 sustains the up-regulation of Th1 lymphocytes, while **IFN-γ** inhibits the down-regulation of Th2 lymphocytes (**TABLE 1**).

The administration of these cytokines has positive therapeutic effects in terms of **Th1/Th2 switch** rebalancing, resulting in resolution of allergic asthma symptoms.

Therefore, a therapy based on an IL-12 and IFN-γ combination is successful as it produces an increase in IL-12 and IFN-γ blood levels and at the same time a decrease in IL-4 and IL-5 blood levels.

The study has shown the therapeutic effects of **oral administration of low dose IL-12 and IFN-γ on mice (GUNA®-IL12, GUNA®-IFN-Y, Guna S.p.a. - Milan, Italy). These low doses have been indentified with a dilution of 4CH (which corresponds to 0.01 pcg/ml equivalent to a 1 fg/dose in a mouse)** and have been activated by a special pharmaceutical technology called **SKA (Sequential Kinetic Activation).**

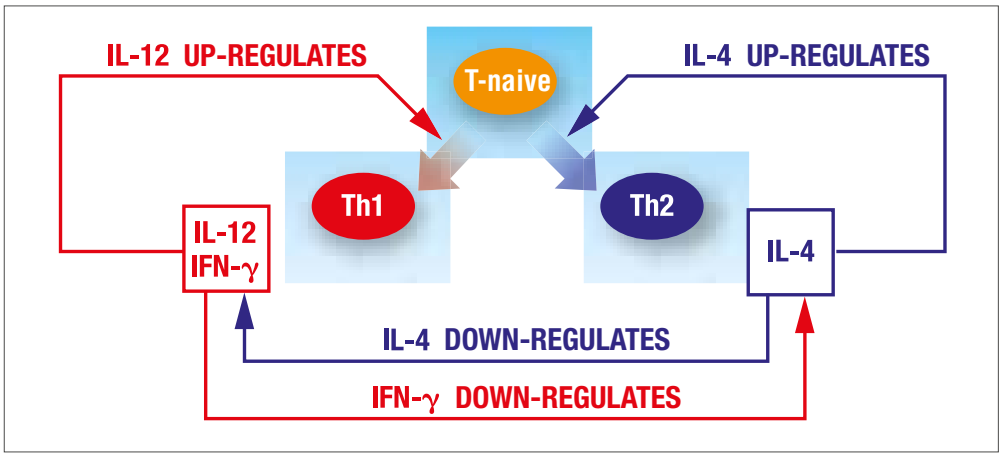

TABLE 1
CROSS REGULATION
BETWEEN TH1 AND TH2

RESULTS

The low doses activated by SKA used in this study have shown the same effects as high doses **(1 µg/ml equivalent to 100 ng/dose in a mouse) but no negative side effects in**:

- RESTORING Th2 LYMPHOCYTES STANDARD ACTIVITY LEVELS (**FIG. 1**)
- RESTORING Th1 LYMPHOCYTES STANDARD ACTIVITY LEVELS (**FIG. 2**)
- DRASTICALLY REDUCING EOSINOPHIL GRANULOCYTES (**TABLE 2**)
- REDUCING BRONCHIAL HYPER-REACTIVITY
- SIGNIFICANTLY INHIBITING SPECIFIC IGE
- DRASTICALLY REDUCING CLINICAL SYMPTOMS

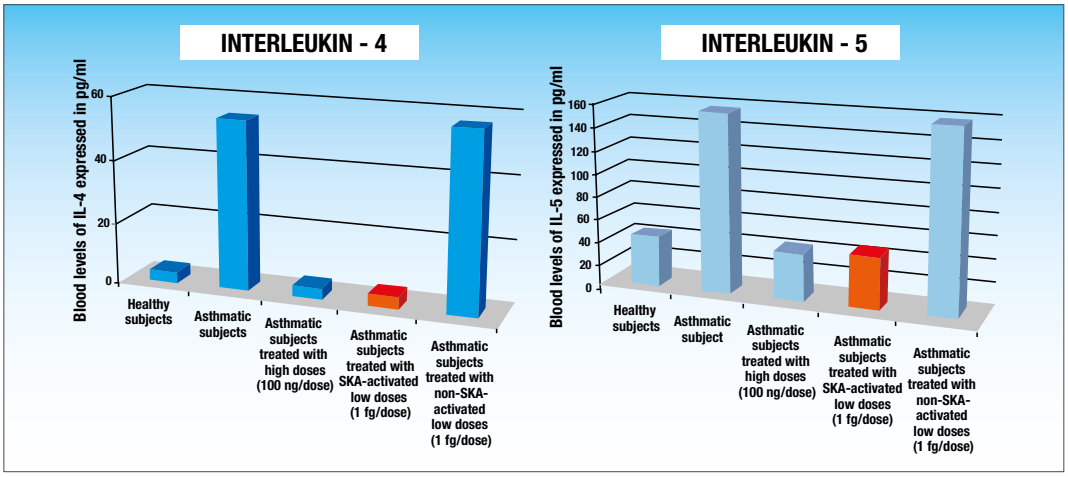

FIG. 1
IL-4 AND IL-5 BLOOD LEVELS AFTER ADMINISTERING IL-12 AND IFN-γ AT DIFFERENT CONCENTRATIONS AND ACTIVATED OR NON-ACTIVATED BY *SEQUENTIAL KINETIC ACTIVATION*

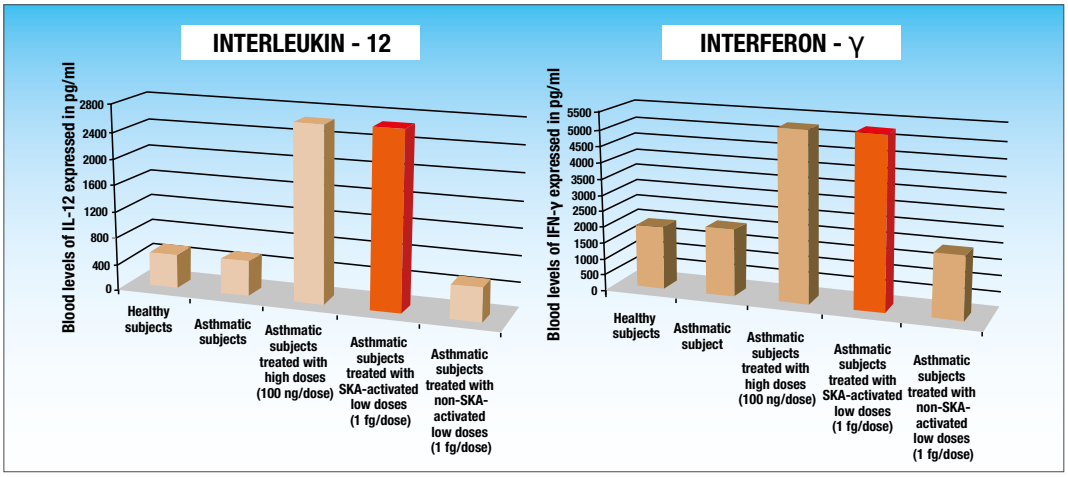

FIG. 2
IL-12 AND IFN-γ BLOOD LEVELS AFTER ADMINISTERING IL-12 AND IFN-γ AT DIFFERENT CONCENTRATIONS AND ACTIVATED OR NON-ACTIVATED BY *SEQUENTIAL KINETIC ACTIVATION*

GROUP	NUMBER OF EOSINOPHIL GRANULOCYTES IN THE BRONCHOALVEOLAR LAVAGE FLUID (BALF) ON DAY 20TH
Healthy mice	0
Asthmatic mice	20,188 ± 0,613
Asthmatic mice treated with high dose (100ng/dose)	0
Asthmatic mice treated with SKA Low Dose (1fg/dose)	0
Asthmatic mice treated with non SKA Low Dose (1fg/dose)	19,567 ± 0,685

TABLE 2
NUMBER OF EOSINOPHIL GRANULOCYTES IN THE BALF (BRONCHOALVEOLAR LAVAGE FLUID) AFTER ADMINISTERING IL-12 AND IFN-γ AT DIFFERENT CONCENTRATIONS AND ACTIVATED OR NON-ACTIVATED BY *SEQUENTIAL KINETIC ACTIVATION*

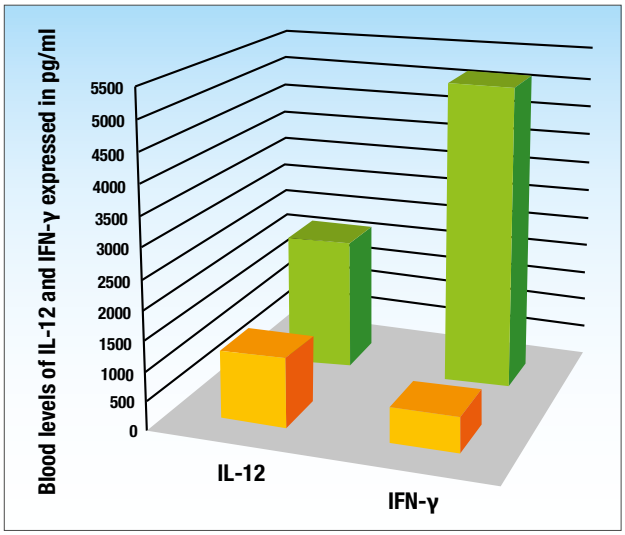

FIG. 3
IL-12 AND IFN-γ BLOOD LEVELS AFTER ADMINISTERING ONLY LOW DOSE OF SKA IL-12 OR BOTH IL-12 AND IFN-γ SKA LOW DOSE

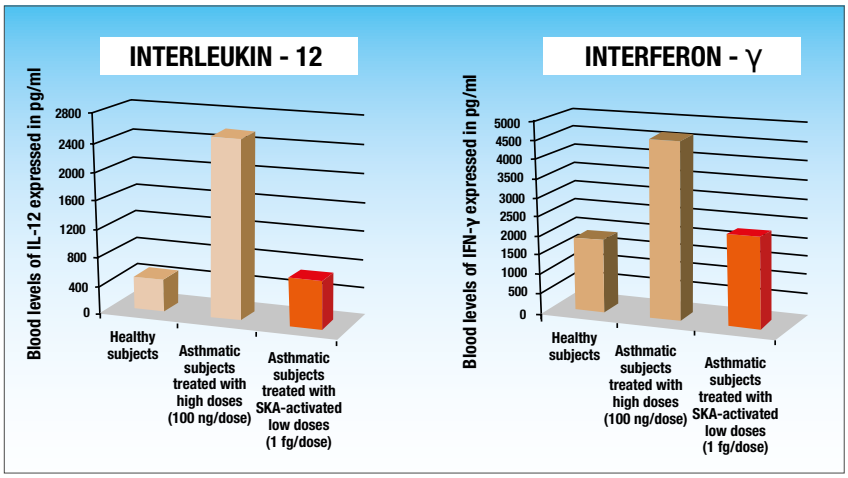

FIG. 4
IL-12 AND IFN-γ BLOOD LEVELS 13 DAYS AFTER THE END OF THE TREATMENT BASED ON HIGH DOSE ADMINISTRATION OF IL-12 AND IFN-γ OR SKA LOW DOSE

CONCLUSIONS

The innovating element of the Italian researchers' study, which has been described as an **"INTRIGUING WORK"**, adding that **"… THE STUDY SUGGESTS A NOVEL APPROACH FOR THE CURE OF ASTHMA"** by the referees of the above mentioned Journal, is the discovery of a **special combination of the two cytokines, i.e., interleukin-12 and interferon-γ**, whose combination has showed greater efficacy compared to the sum of the effect of each individual cytokine (**FIG. 3**).
On the other hand, the "scientific dream" of using biological molecules such as low-dose cytokines (the only ones that have no side effects, such as, for example, a Th1 hyperpolarization - **FIG. 4**) has come true. This has been possible by the special pharmaceutical technique used to produce the molecules employed in the study.

It has been demonstrated that only the low doses prepared according to the SKA method have the same therapeutic effects as high-dose pharmacological concentrations, but without the latter's side effects.

Low doses not activated according to the SKA procedures have shown no biological activity or therapeutic effect.

The various activated and non-activated dilutions were provided to the Department of Human Morphology of the University of Milan by **Guna S.p.a. Laboratories**, where the **SKA (Sequential Kinetic Activation)** method of preparation has been standardized.

BIBLIOGRAPHICAL REFERENCE

Gariboldi S, Palazzo M, Zanobbio L, Dusio GF, Mauro V, Solimene U et al. Low dose oral administration of cytokines for treatment of allergic asthma. Pulm Pharmacol Ther 2009;22:497-510.

GUNA HIGHLIGHTS

Allergology

LONG-TERM TREATMENT WITH LOW-DOSE MEDICINE IN CHRONIC CHILDHOOD ECZEMA. A DOUBLE-BLIND TWO-STAGE RANDOMIZED CONTROL TRIAL

Carello et al. Italian Journal of Pediatrics (2017) 43:78
DOI 10.1186/s13052-017-0393-5

Italian Journal of Pediatrics

RESEARCH Open Access

Long-term treatment with low-dose medicine in chronic childhood eczema: a double-blind two-stage randomized control trial

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FOREWORD

The results of the clinical trial titled ***"Long term treatment with Low-Dose Medicine in chronic childhood eczema. A double-blind two-stage randomized control trial"*** are the outcome of a project lasting approximately four years that involved dozens of investigators working in 3 different units: UO di Selezione Clinica [Clinical Selection Unit], composed of paediatricians from the Lazio region, Dipartimento di Allergologia Pediatrica [Department of Paediatric Immunology] of Ospedale Fatebenefratelli in Rome and UO di Biostatistica [Biostatistics Unit] of Istituto Superiore di Sanità [National Institute for Health].

This important and challenging study demonstrated the immunomodulatory activity of the combination Galium-Heel® (Manufactured by Biologische Heilmittel Heel GmbH - Baden-Baden, Germany) plus Interleukin (IL)-12 (GUNA®-IL 12) plus interferon (IFN)-gamma (GUNA®-IFN-γ) at sub-nanomolar concentrations (low doses), manufactured by Guna S.p.a. - Milan, Italy, as treatment for childhood eczema.

The results of this clinical study provide new therapeutic options for the treatment of Atopic Dermatitis (eczema) and **suggest possible uses for Low-Dose Medicine in the treatment of various allergic conditions.**

INTRODUCTION

Atopic dermatitis is the most common chronic inflammatory condition in childhood. This illness, which usually appears within the first five years of life, affects more than 20% of children. Genetic, epigenetic and environmental factors, as well as skin barrier function disorders, innate or adaptive immune system alterations and other pathogenetic mechanisms that are still not yet fully understood underlie the onset of the disease.

The disorders caused by Atopic Dermatitis, first and foremost intense pruritus and sleep disorders, constitute a frustrating condition for sufferers and their parents that has a negative impact on academic performance and on family and social life, as well as non-negligible psychological and socioeconomic repercussions.

The conventional treatments for moderate or severe eczema are often unable to control skin lesion activity and, in some cases, more aggressive treatments associated with side effects are needed to provide patients with relief. By exerting a modulatory activity, Low-Dose Medicine provides excellent therapeutic support able to re-establish immune homeostasis between the different lymphocyte populations and to improve the patients' conditions.

The purpose of this two-stage, double-blind, randomised, placebo-controlled clinical study was to verify the clinical efficacy and the safety of long-term therapy with Low-Dose Medicine in subjects with mild/moderate chronic eczema.

Subjects in the acute phase of the disease were screened by the UO di Selezione Clinica [Clinical Selection Unit] and enrolled by Dipartimento di Allergologia Pediatrica [Department of Paediatric Immunology] of Ospedale Fatebenefratelli in Rome (UOIA).

The enrolled subjects were split into 2 study groups:

➔ Group A, which took a placebo solution

➔ Group B, which took
- Guna®-Interleukin 12 (Guna®- IL 12)
- Guna®-Interferon-γ (GUNA®-IFN-γ)
- Galium-Heel®

SCHEME OF TREATMENT

The treatment preparations (Galium-Heel®, Guna®-IL 12, and Guna®-IFN-γ) and the placebo preparations were administered orally (8 drops for children aged 5 years or under; 15 drops for children over 5 years of age) twice a day for 6 non-consecutive months in each stage (FIG. 1).

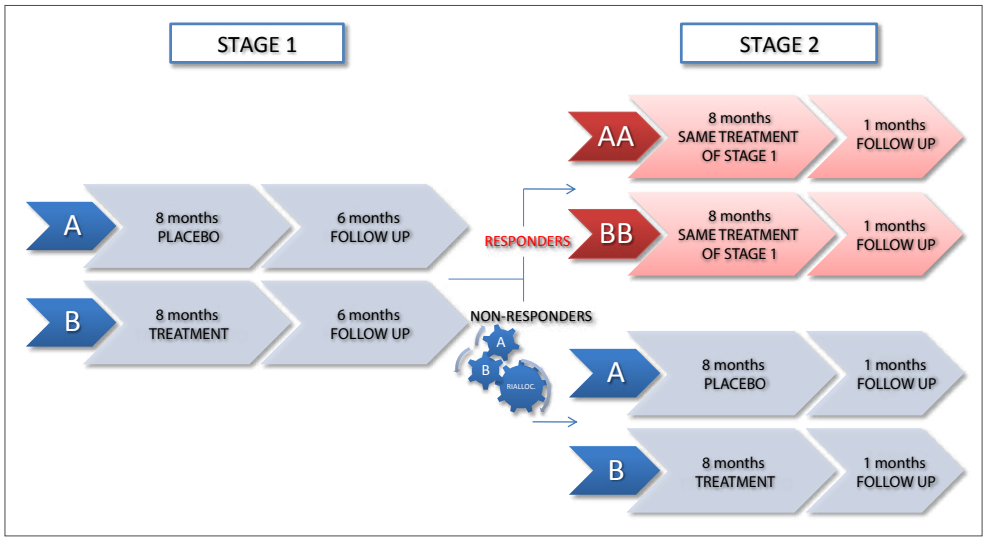

FIG. 1
STUDY DESIGN

After the 1st stage, the responders (who were identified clinically), also received the same treatment in the 2nd stage (AA; BB).

The NON-responders were randomised and re-allocated to use placebo or the active treatment (Groups A and B), which became AA; BB; AB or BA.

- AA, children who received placebo in both the 1st and 2nd stage of the study.
- BB, children who received the active treatment in both the 1st and 2nd stage of the study.
- AB, children who received placebo in the 1st and the active treatment in the 2nd stage of the study.
- BA, children who received the active treatment in the 1st and placebo in the 2nd stage of the study.

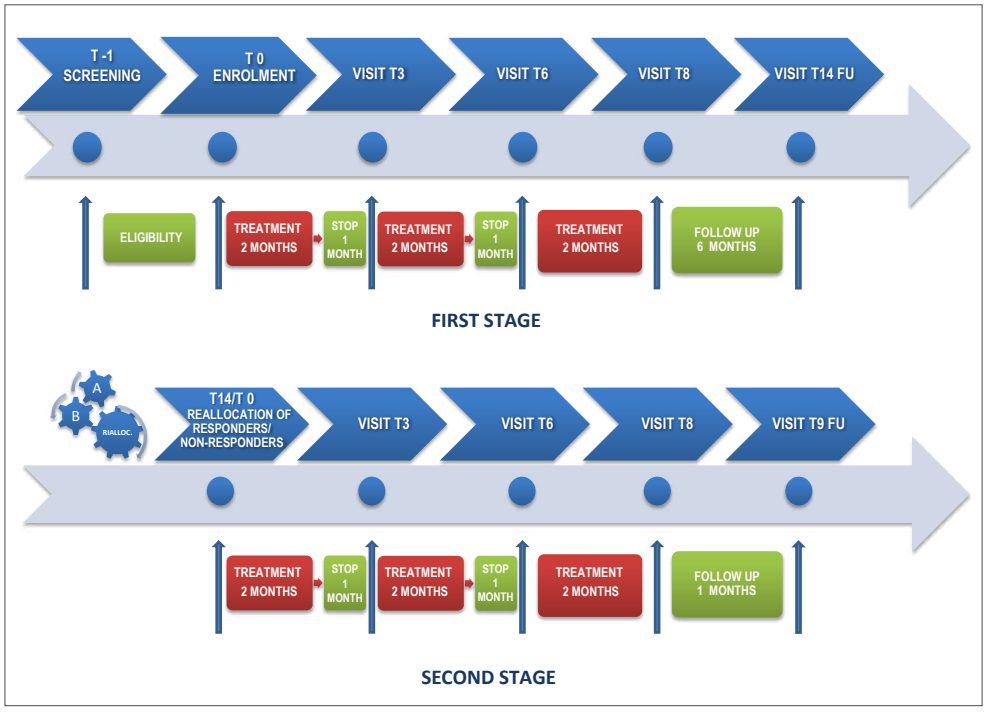

FIG. 2
VISITS AND TREATMENTS DURING STAGE 1 AND STAGE 2

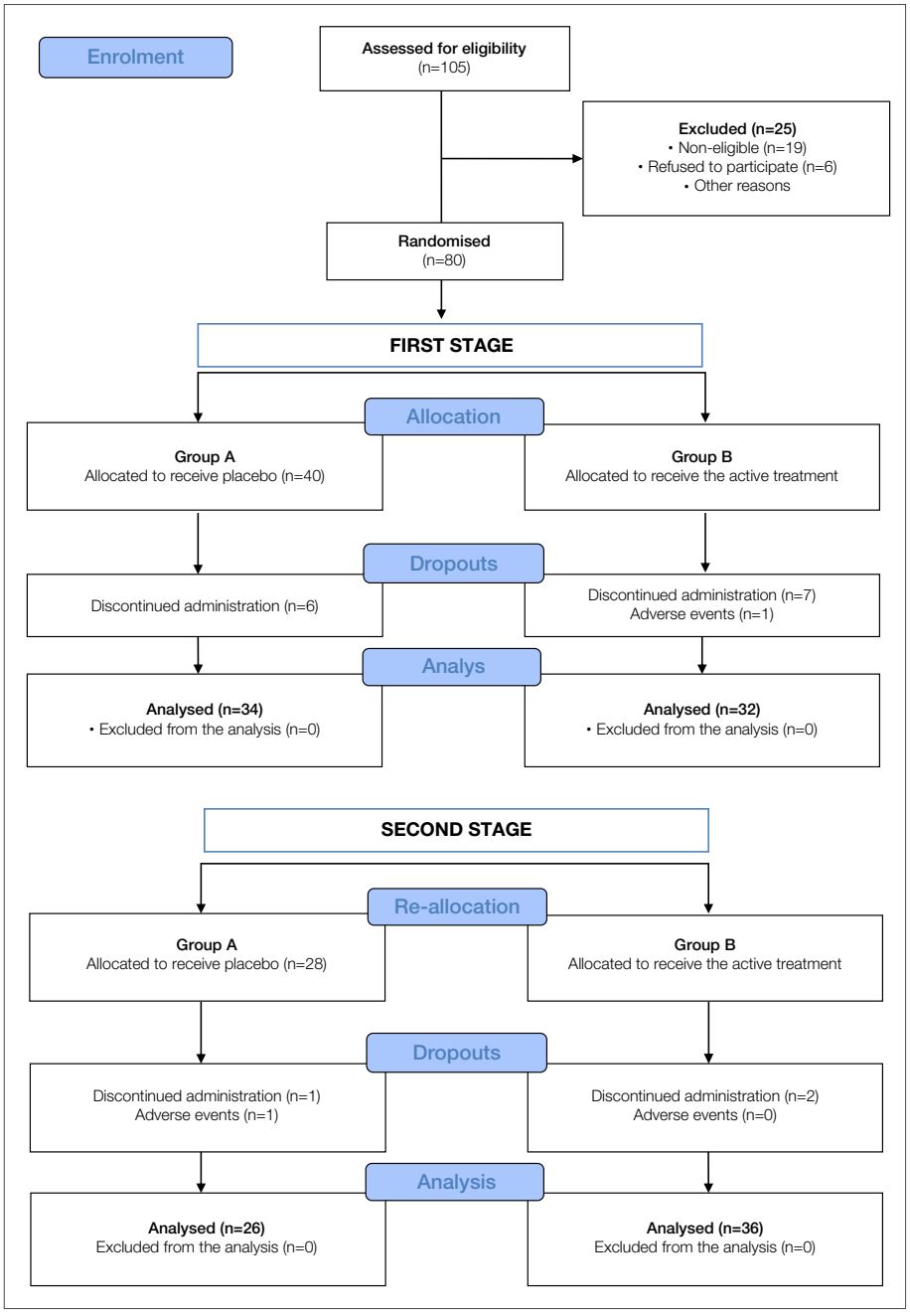

FIG. 3
FLOW CHART FOR THE FIRST AND SECOND STAGES OF THE CLINICAL STUDY (CONSORT 2010)

During the study, subjects were allowed to be administered conventional treatments (topical steroids, antibiotics or systemic or topical antihistamines) when needed in order to provide relief from symptoms. A clinical diary filled out by the parents recorded the use of conventional treatments throughout the study.

The study's primary objective was to verify the subjects' clinical improvement by means of the improvement in the SCORAD [Scoring Atopic Dermatitis] score. The secondary objectives were to:

- estimate the disease-free interval;
- monitor changes in pruritus and sleep disorder symptoms;
- verify the frequency of use of conventional symptomatic therapy in both study Groups;
- monitor the safety and tolerability of long-term treatment.

The data obtained were statistically analysed by the UO di Biostatistica [Biostatistics Unit] of Istituto Superiore di Sanità [National Institute for Health], which took an active part in developing the study's design and monitored all steps of the trial closely.
At the end of the study, an *"intention-to treat analysis"* and a "*per-protocol analysis"* were conducted.
An *"interim analysis"* was conducted at the end of the 1st stage of the trial, in order to assess the study's design.

As shown in the flow chart, 66 of the 80 subjects were included in the statistical analysis of the 1st stage of the study: 40 in Group A and 40 in Group B (**FIG. 3**).

RESULTS

TABLE 1 shows the values for the clinical and demographic variables for the two Study Groups at enrolment.

VARIABLES	GROUP A	GROUP B
SCORAD (mean) ±SD	13.93±8.28	14.71±7.64
% Boys	55.0	52.5
average age (months) ±SD	68.97±43.64	78.57±50.86
median age (months)	64.5	60.5
age at diagnosis (months)	51.2	68.2
IgE kU/l (mean) ±SD	373.5±929.4	409.6±561.4
% positivity to prick test	42.5	47.5

TABLE 1

TABLE 2 shows the SCORAD score values at the different time points of the 1st Stage for Groups A (placebo) and B (active treatment) and 2nd stage for Groups A (placebo), B (active treatment), AA (placebo/placebo) and BB (active treatment/active treatment).

In the 1st stage of the study, the SCORAD score dropped between T0 and T14 in both Groups.

- In Group A (placebo), it dropped by 41% between T0 and T8 and by 53.2% between T0 and T14.
- In Group B (active treatment), the SCORAD scores dropped by 54% between T0 and T8 and by 64% between T0 and T14.

The intragroup difference between T0 and T14 is significant for both Groups.

At the end of the 1st stage, the responders continued with the same treatment they took during the 1st stage. The subjects classified as NON-responders were randomised and re-allocated to the placebo or active treatment groups (AA; BB; AB; BA) (**TABLE 2**).

FIRST STAGE					
PLACEBO GROUP A	**N**	**MEAN**	**SD ±**	**% CHANGE VS. T0**	**P-VALUE**
T0	40	13.93	8.284		
T3	37	11.00	11.081		
T6	34	10.15	11.712		
T8	33	8.15	4.147	41.5	
T14FU	32	6.53	3.213	53.2	0.0001
total	177	10.18	8.830		
GROUP B TREATMENT					
T0	40	14.71	7.646		
T3	35	9.74	10.248		
T6	35	8.00	10.454		
T8	33	6.79	4.083	54.0	
T14FU	33	5.30	3.820	64.0	0.0001
total	177	9.17	8.469		

SECOND STAGE					
GROUP AA PLACEBO/PLACEBO	**N**	**MEAN**	**SD ±**	**% CHANGE VS. T0**	**P-VALUE**
T0	22	6.23	3.131		
T8	22	5.86	6.319		
T9FU	22	4.64	8.301		
GROUP BB TREATMENT/TREATMENT					
T0	27	5.22	3.745		
T8	27	5.07	3.050		(T8/T9) 0.049
T9FU	27	3.52	2.592		(T0/T9) 0.058

TABLE 2

A total of 1482 clinical diaries were collected and analysed.

TABLE 3 shows the data obtained from the clinical diary analysis regarding pruritus and sleep disorders.

- In Group A, there was an albeit non-significant reduction in pruritus and sleep disorders between T0 and T8, whereas the presence of both symptoms remained high, but not significant, between T9 and T14, compared to the baseline.
- In Group B, the pruritus score dropped between T0 and T8 and remained stable between T9 and T14. In the same Group, sleep disorders dropped by 50 % between T0 and T8 ($p<0.001$) and were completely absent at T14.

GROUP A - PLACEBO	T0	T8	T14 follow up
Pruritus			
N	23	23	18
Mean	24.91	19.74	32.61
SD±	22.52	24.11	39.99
Sleep disorders			
N	23	23	18
Mean	9.13	5.87	13.33
SD ±	18.36	16.74	30.24

GROUP B - TREATMENT			
Pruritus			
N	21	21	13
Mean	24.33	15.76	15.67
SD±	22.45	17.61	20.33
Sleep disorders			
N	21	21	13
Mean	5.62	2.95	0.000
SD±	14.56	11.27	0.000

TABLE 3

TABLE 4 shows the differences in the use of conventional symptomatic medicinal products between Group A and Group B for the 1st stage at time-points T0 -T8 and T9 -T14.

Subjects who used steroids or antihistamines at least once were considered as having been treated with this type of medication (FU=follow-up). The use of symptomatic medicinal products (expressed as person-months) was analysed in both Study Groups using the clinical diaries.

- ➔ During the 1st stage, Group A took medicines for 30 person-months between T0 and T8 and between T9 and T14; Group B took symptomatic medication for a period of 16 person-months between T0 and T8 and for a period of 9 person-months between T9 and T14.
- ➔ Therefore, Group B took medication for a far shorter time than Group A between T0 and T8.
- ➔ Group B took medication for a significantly shorter time than Group A during the period T9-T14 (Fisher's exact test = 0.001).

The safety and tolerability of the treatment was confirmed. Just two adverse events were observed, one in Group A and one in Group B. The analysis of the event in Group B, using the method described by Naranjo et al., did not reveal any relationship between the use of the medicinal product and the event.

VARIABLES	GROUP A T0-T8	GROUP B T0-T8	DIFFERENCE BETWEEN GROUP A AND GROUP B	GROUP A T9-T14FU	GROUP B T9-T14FU	DIFFERENCE BETWEEN GROUP B AND GROUP A
% decrease of SCORAD	42.5	54	-11.5	19.9	21.9	-2.0
conventional therapy (months/person)	30	16	-14	30	9	-21
% patients treated with steroids	32.1	23.1	-9.0	17.9	15.3	-2.6
% patients treated with antisthamines	35.7	19.2	-16.5	32.1	11.5	-20.6

TABLE 4

CONCLUSIONS

SCORAD score

- In the 1st stage of the study, disease severity dropped in both Group A and in Group B (53.2% vs. 64%). The improvement in Group A can be partly explained by the greater use of conventional symptomatic treatments and the use of topical detergents with softening and moisturising properties.
- The improvement in the SCORAD score was far more obvious in Group BB, which received the active treatment in both the 1st and 2nd stages of the study.

These results are in line with the hypothesis that Low-Dose Medicine acts progressively, by modulating the Immune System until homeostasis is achieved.

DISEASE-FREE INTERVAL

- The follow-up data show an improvement in SCORAD scores in the two Groups in both study stages. However, the effects are more obvious in Group B.
- In Group BB, homeostasis is maintained during follow-up.

Group B maintained a low SCORAD score, with far lower use of medicinal products than Group A, during the follow-up period.

The results for the disease-free interval support the hypothesis that Low-Dose Medicine acts progressively by modulating the Immune System.

PRURITUS AND SLEEP DISORDERS

- Pruritus and sleep disorders improved in Group A between T0 and T8, and worsened greatly between T8 and T14 FU.

These data confirm that eczema is a complex disease in which psychological aspects play an important role.

- Pruritus and sleep disorders improved in children in Group B between T0 and T8 and between T0 and T14 FU.

These data confirm the immunomodulatory activity of Low-Dose Medicine, even in periods in which the treatment is not taken (carryover pharmacological effect).

The subjective symptoms improved in Group B between T0 and T8 and this improvement is maintained during the T8 - T14 period. This information is very important if we consider that the consumption of symptomatic medicinal products was significantly lower in Group B than in Group A (9 person-months vs. 30 person-months).

- Sleep disorders improved significantly in Group B (T0/T8 - 1st stage) and disappeared in the 2nd stage.

USE OF CONVENTIONAL SYMPTOMATIC MEDICATION

- Group A took symptomatic medication for 30 person/month between T0 and T8 and between T9 and T14. Group B took symptomatic medication for 16 person/month between T0 and T8 and for a period of 9 person-months between T9 and T14.

This information explains the improvement in the SCORAD score for Group A in the 1st stage of the study.

SAFETY AND TOLERABILITY

- The data regarding safety and tolerability confirmed the data obtained in previous studies.

To conclude, this study suggests than Low-Dose Medicine represents an efficacious therapeutic option for the clinical treatment of Atopic Dermatitis.

THE PRELIMINARY RESULTS OF THIS STUDY WERE PRESENTED AT THE CONGRESS

"4th JOURNEY THROUGH ECZEMA"

Naples (IT) - June 2014

BIBLIOGRAPHICAL REFERENCE

Carello R, Ricottini L, Miranda V, Panei P, Rocchi L, Arcieri R, Galli E. Long term treatment with Low-Dose Medicine in chronic childhood eczema. A double-blind two-stage randomized control trial. Ital J Pediatr. 2017.

GUNA HIGHLIGHTS

Dermatology

LOW DOSE CYTOKINES REDUCE OXIDATIVE STRESS IN PRIMARY LESIONAL FIBROBLASTS OBTAINED FROM PSORIATIC PATIENTS

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Letter to the Editor

Low dose cytokines reduce oxidative stress in primary lesional fibroblasts obtained from psoriatic patients

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FOREWORD

Psoriasis vulgaris is a systemic inflammatory disease with a pathogenesis that is still unclear.
The disease is characterised by the appearance of erythematous squamous plaques on the skin. These plaques are characterised by keratinocyte proliferation (incorrect differentiation) and a marked presence of inflammatory infiltrate.
On an immune level, this translates into **an unbalanced T-cell response to the Th1/Th17 subclones, with a consequent reduction in Th2/Treg subclones. Due to this imbalance between the T-cell populations, Psoriasis is classified and an inflammatory condition with an autoimmune component.**

INTRODUCTION

Clinical evidence (Roberti ML et al. Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris. J Biol Regul Homeost Agents 2014; 28: 133-9.) shows that low-dose cytokines reduce the inflammatory component, which plays an important role in the onset and progression of Psoriasis, more effectively than conventional recombinant cytokines at high doses normally used in pharmacology.
This basic research study assessed the **effect of low-dose SKA cytokines on oxidative stress in primary lesional fibroblasts from psoriatic patients.** Fibroblasts are fundamental for the creation of a micro-environment in which the keratinocytes are able to proliferate and differentiate correctly. Excessive oxidative stress phenomena in the fibroblasts compromise this cell-cell cross-talk.

RESULTS

The results of the study show that oxidative stress is high in the fibroblasts present in psoriasis lesions (LES) and, at the same time, they confirm that treating LES fibroblasts with low-dose SKA IL-4, IL-10, b-FGF and β-Endorphins (GUNA®-IL 4, GUNA®-b-FGF, GUNA®-BETA ENDORFIN. GUNA S.p.a., Milan, Italy) reduces oxidative stress in the ex vivo/in vitro model proposed.

➔ **FIG. 1 -** Assessment of the total ROS (Reactive oxygen species) production in primary fibroblasts obtained from samples of skin lesions of psoriatic patients (LES) and skin samples from healthy donors (CTR).
Total ROS production was assessed by Flow Cytometry (FACS) using a dedicated fluorescent probe (H2DCFDA).

- **FIGS. A and B:** fluorescence emission after 48 hours' incubation with low-dose SKA IL-4, IL-10, b-FGF and β-Endorphin at a concentration of 10 fg/ml. No effects were observed in the control fibroblasts (CTR), whereas fluorescence emission was reduced by the low-dose treatment in the lesional fibroblasts (LES).
- **FIG. C:** quantitative analysis of ROS production detected by flow cytometry, in which ROS production is expressed as a percentage of the CTR fibroblast value. The values shown (mean ± SD) represent five independent experiments, each of which was conducted in triplicate.

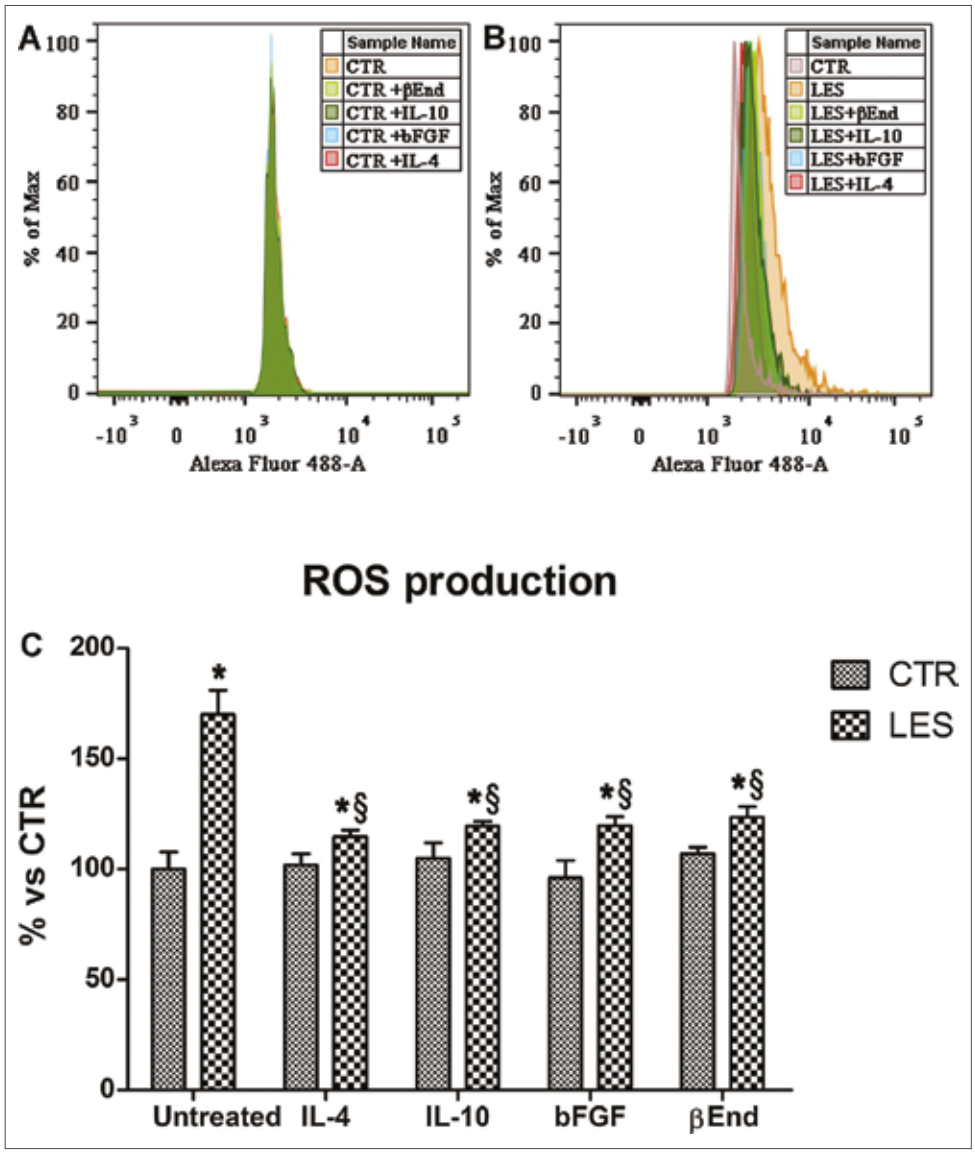

FIG. 1
ROS VALUES IN THE SKIN OF PSORIATIC PATIENTS WHO WERE AND WERE NOT TREATED WITH LOW-DOSE CYTOKINES

* SIGNIFICANT DIFFERENCE ($P \leq 0.05$) COMPARED TO THE VALUE MEASURED IN THE CONTROL FIBROBLASTS (CTR); § SIGNIFICANT DIFFERENCE ($P \leq 0.05$) COMPARED TO THE UNTREATED LES FIBROBLASTS.

➔ FIG. 2 - NADPH oxidase values in the lesional fibroblasts (LES) of psoriatic patients and healthy donors (CTR). NADPH oxidase was measured by luminometric assay performed after 48 hours' incubation with low-dose SKA IL-4, IL-10, b-FGF and β-Endorphin at a concentration of 10 fg/ml.

Of all the cytokines, b-FGF alone led to a significant reduction in NADPH oxidase activity on the LES fibroblasts.

FIG. 2C shows the percentage values normalised in relation to the control values (CTR) of the area under the curve (AUC) of NADPH oxidase activity.

- Extracellular ROS production was significantly higher in the LES fibroblasts than in the CTR fibroblasts.
- The values shown (mean ± SD) represent five independent experiments, each of which was conducted in triplicate.

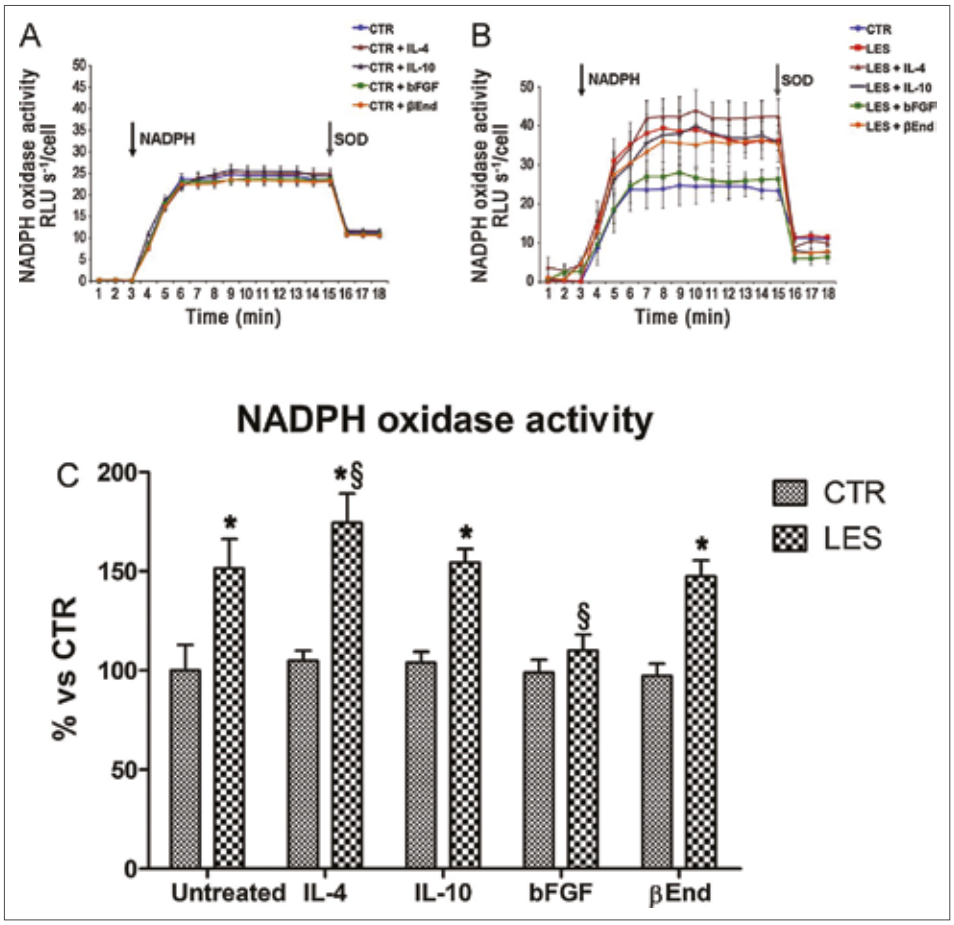

FIG. 2
NADPH OXIDASE VALUES IN THE SKIN OF PATIENTS WHO WERE AND WERE NOT TREATED WITH LOW-DOSE CYTOKINES

* SIGNIFICANT DIFFERENCE ($P \leq 0.05$) COMPARED TO THE VALUE MEASURED IN THE CONTROL FIBROBLASTS (CTR); § SIGNIFICANT DIFFERENCE ($P \leq 0.05$) COMPARED TO THE UNTREATED LES FIBROBLASTS.

CONCLUSIONS

This paper, which was published in the Letters to the Editor section of the Journal of Dermatological Science, shows that treatment with low-dose SKA IL-4, IL-10, b-FGF, and β-Endorphin significantly reduces oxidative stress in human fibroblasts obtained from psoriasis skin lesions. This translates into a considerable reduction in one of the main triggers of Psoriasis: chronic inflammatory state.

In the light of these observations, it can be concluded that the hypothesis of treating Psoriasis with low-dose intervention now has important additional experimental grounds. Moreover, this article confirms, from a action mechanism point of view, the clinical results obtained by the research described in the next pages (Roberti ML et al. Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris. J Biol Regul Homeost Agents 2014; 28:133-9).

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Barygina V, Becatti M, Lotti T, Taddei N, Fiorillo C. Low dose cytokines reduce oxidative stress in primary lesional fibroblasts obtained from psoriatic patients. J Dermatol Sci. 2016;83(3):242-4.

GUNA HIGHLIGHTS

Dermatology

IMMUNOMODULATING TREATMENT WITH LOW DOSE INTERLEUKIN-4, INTERLEUKIN-10 AND INTERLEUKIN-11 IN PSORIASIS VULGARIS.

JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS Vol. 28, no. 1, 133-139 (2014)

IMMUNOMODULATING TREATMENT WITH LOW DOSE INTERLEUKIN-4, INTERLEUKIN-10 AND INTERLEUKIN-11 IN PSORIASIS VULGARIS

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FOREWORD

The results of the clinical study titled ***"Immunomodulating treatment with low dose interleukin-4, interleukin-10 and interleukin-11 in Psoriasis Vulgaris"*** represent the outcome of a two-year project involving a number of experimenters who selected, enrolled, and observed patients affected by Psoriasis Vulgaris in Italy.

This explorative study has demonstrated the immunomodulating activity of low-dose SKA interleukin-4, interleukin-10 and interleukin-11 (GUNA®-IL 4, GUNA®-IL 10, GUNA®-IL 11. Guna S.p.a. - Milan, Italy) in the treatment of this disease.

The opportunity for clinical applications of a combination of low doses of biological molecules paves the way for the treatment of other immune-related pathologies.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease affecting 2-3% of the world population. It is characterized by hyperproliferation and hyperplasia of the superficial layers of the epidermis.

Inappropriate signals released by the immune system determine an altered keratinocyte differentiation, resulting in the formation of desquamating, thickened, inflamed and erythematous plaques.

Psoriasis is a multifactorial disease in which environmental triggering factors overlap with genetic factors.
The pathogenesis of the disease is still not completely clear, and it is generally classified as a **Th1-polarized autoimmune disease** with high levels of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1, IL-2, IL-6, IL-8, IL-17, IL-12, IL-18, IL-22 and IL-23.

The aim of this multicenter, double-blind, randomized, placebo-controlled clinical trial, alternated according to a cross-over model, was to verify the safety and the effects of the administration of immunomodulating cytokines such as IL-4, IL-10, and IL-11, at a concentration of 10fg/ml and activated by SKA technology, on the inflammatory process and on the quality of life of patients.

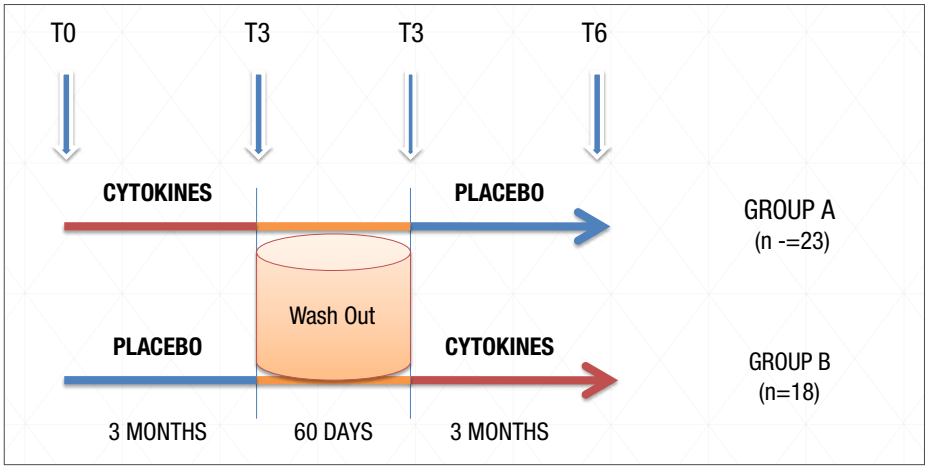


FIG. 1
STUDY DESIGN

The trial involved **48 patients**, divided into two groups and followed up for 8 months. Each patient received, according to a cross-over model, either the experimental treatment or placebo, alternatively (**FIG.1**).

The clinical endpoint (Psoriasis Area Severity Index - PASI) and quality of life (Dermatology Quality of Life Index - DLQI) were evaluated in 41 subjects. No adverse event was reported during the whole trial.

TREATMENT SCHEME

- **Guna®-Interleukin 4 (GUNA®-IL 4):** 20 drops, twice daily for 3 consecutive months.
- **Guna®-Interleukin 10 (GUNA®-IL 10):** 20 drops, twice daily for 3 consecutive months.
- **Guna®-Interleukin 11 (GUNA®-IL 11):** 20 drops, twice daily for 3 consecutive months.

SELECTION CRITERIA

INCLUSION CRITERIA

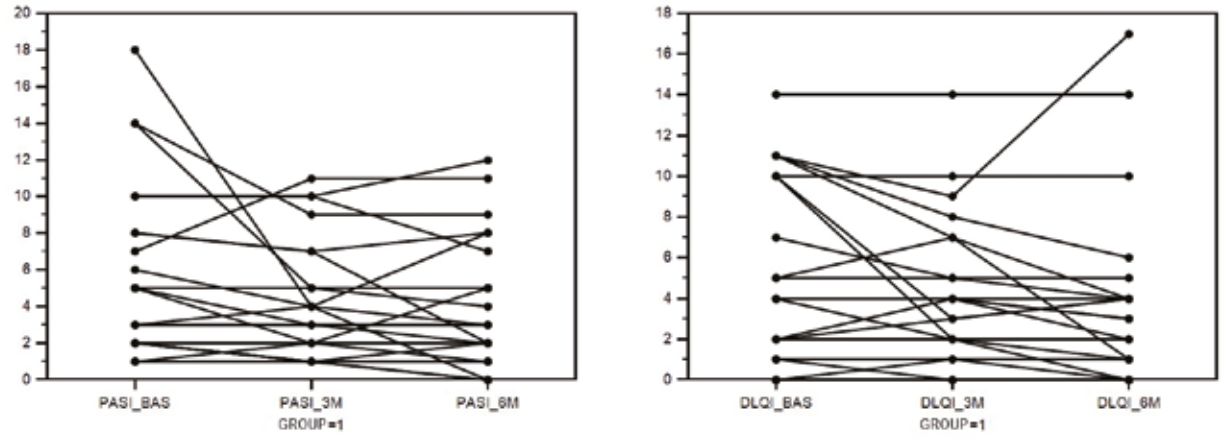
- Age > 18 years
- slight and moderate Psoriasis (PASI score < 30) at screening

EXCLUSION CRITERIA

- Subjects affected by con guttate psoriasis, palmoplantar, erythrodermic or pustular psoriasis at screening.
- Subjects who underwent topical or systemic therapies for psoriasis before screening, during the fifteen days, or three months, respectively.
- Subjects under treatment for other diseases with topical and/or systemic steroid therapies, anti-TNF and other immunomodulating or immunosuppressive therapies.

RESULTS

TABLE 1 shows PASI and DLQI scores of patients (n=23) in Group A (treated with low-dose SKA cytokines in the first stage).

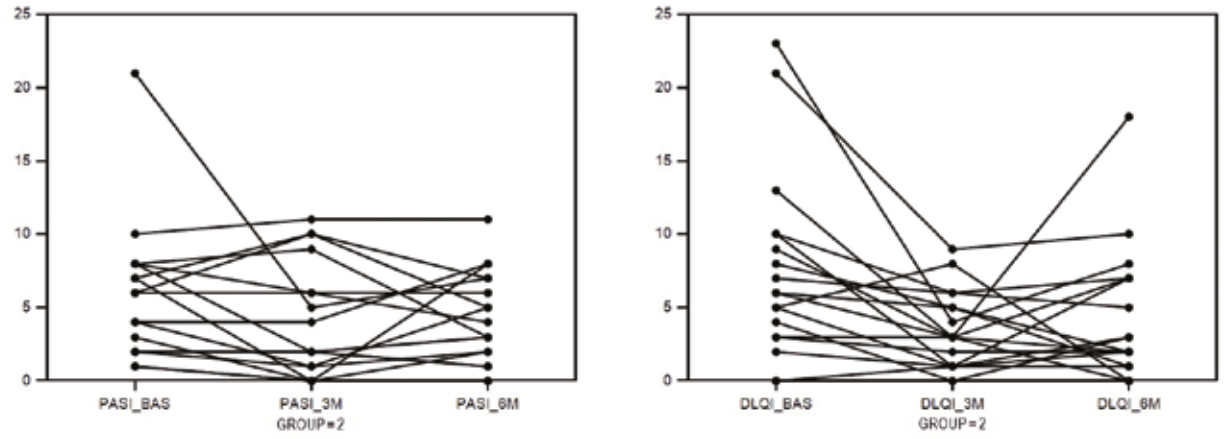


PASI AND DLQI SCORES IN GROUP A SUBJECTS. DATA ARE EXPRESSED IN MEDIAN WITH RANGE (MINIMUM AND MAXIMUM)

N=23	Basal	3 months	6 months	p
PASI	5 (1-18)	4(1-11)	3 (0-12)	p<0.05 (bas. vs 6 m.)
DLQI	4 (0-14)	4 (0-14)	3 (0-17)	p<0.05 (bas. vs 6 m.)

TABLE 1

TABLE 2 shows PASI and DLQI scores of patients (n=18) in Group B (treated with placebo in the first stage).



PASI AND DLQI SCORES IN GROUP B SUBJECTS. DATA ARE EXPRESSED IN MEDIAN WITH RANGE (MINIMUM AND MAXIMUM)

N=18	Basal	3 months	6 months	p
PASI	5 (1-21)	2 (0-11)	3.5 (0-7)	p>0.05 (bas. vs 3/ 6 m.)
DLQI	6 (0-10)	3 (0-9)	2.5 (0-18)	p<0.05 (bas. vs 3/ 6 m.)

TABLE 2

Psoriasis is a multifactorial disease with a very important psychological component.

- **In Group A, the administration of cytokines in the first stage stabilised the Immune System, making it less sensitive to psychological "intrusions", which led to a progressive and stable improvement at the end of the study (P<0.05).**
- **Group B, which was treated with placebo in the first stage, was most likely subject to the Hawthorne effect, which is consistent with the disease studied. In this group, as there was no immune "control" resulting from the early administration of cytokines and a greater placebo-induced halo effect was observed.**

N.B.: The results of the trial suggest that the drug has a carryover effect (continuation of the pharmaceutical effect even after the end of medicinal product administration) and, consequently, the statistical methods usually used for crossover studies were considered to be inappropriate and it was decided to compare the two Groups between the baseline and 6 months.

CONCLUSIONS

- The simultaneous administration of low-dose cytokines (IL-4, IL-10 and IL-11) at a concentration of 10 fg/ml **significantly reduces the severity** of Psoriasis Vulgaris and significantly **improves the quality of life** of affected patients.
- The study showed that low-dose cytokines administered at a concentration of 10 fg/ml have a dependent **carryover effect in chronic diseases.** The administration of these molecules leads to a progressive improvement in the disease and this improvement continues for a period that is yet to be determined, even after the discontinuation of administration (carryover pharmacological effect).
- The administration of low-dose SKA IL-4, IL-10 and IL-11 cytokines at a concentration of 10 fg/ml with a posology of 20 drops twice daily is **safe**.

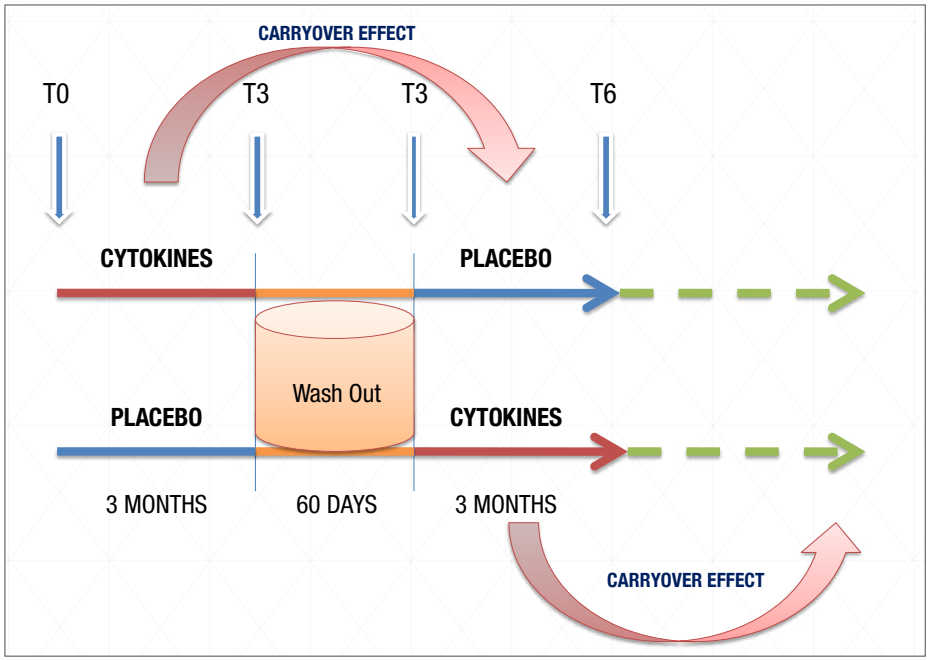


FIG. 4
CARRYOVER EFFECT

SAPIENZA
UNIVERSITÀ DI ROMA

THE RESULTS OF THIS STUDY WERE PRESENTED DURING THE

XXI GIORNATE DI DERMATOLOGIA CLINICA

CLINICAL DERMATOLOGY DAYS, 21st edition
Rome Congress & Expo Center (Rome, IT) - January 29th-31st, 2015

BIBLIOGRAPHICAL REFERENCE

Roberti ML, Ricottini L, Capponi A, Sclauzero E, Vicenti P, Fiorentini E et al. Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris. J Biol Regul Homeost Agents 2014; 28: 133-9.

GUNA HIGHLIGHTS

Dermatology

SUCCESSFUL COMBINATION TREATMENT FOR PSORIASIS WITH PHOTOTHERAPY AND LOW DOSE CYTOKINES. A SPONTANEOUS OBSERVATIONAL RETROSPECTIVE CLINICAL STUDY.

Leitthema

Hautarzt
DOI 10.1007/s00105-015-3698-6

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Erfolgreiche Kombinationsbehandlung der Psoriasis mit Phototherapie und niedrig dosierten Zytokinen

Spontane retrospektive klinische Beobachtungsstudie

FOREWORD

Psoriasis is a chronic skin disease with an important inflammatory and autoimmune component, which affects approximately 2 - 3% of the world's population. Most patients have Psoriasis Vulgaris (characterised by erythematous squamous plaques). Psoriasis is associated with a poor quality of life and severe comorbidities.

The purpose of this **spontaneous observational retrospective clinical study** was to **assess the pharmacological activity** of an innovative treatment based on the use of **laser therapy** (UVA-1 radiation) with or without the administration of **low-dose SKA cytokines Interleukin 4, Interleukin 10 and low-dose SKA anti-IL-1 antibodies (GUNA®-IL 4, GUNA®-IL 10, GUNA®-ANTI IL 1. Manufactured by Guna S.p.a, Milan, Italy)** in patients with Psoriasis Vulgaris. The results obtained showed that the two treatments have a considerable additive effect.

INTRODUCTION

In the early stages of disease, **the effect of the trigger stressors causes the death of healthy keratinocytes.** This leads to the release of the antimicrobial peptide (AMP) LL37, which binds with the DNA/RNA fragments released after keratinocyte apoptosis. These nucleic acids are recognised by the toll-like receptors (TLR) 7, 8 and 9 present on the surface of the dendritic cells (DC), causing their activation. **The activation of dendritic cells causes Th1, Th17 and Th22 lymphocyte clonal expansion, with the consequent production of pro-inflammatory cytokines such as IL-17, TNF-α, IFN-γ and IL-21, which induce and drive the typical immunological imbalance and progression of Psoriasis.** The Th22 sub-population produces IL-22, which acts by stimulating keratinocyte proliferation (**FIG. 1**).

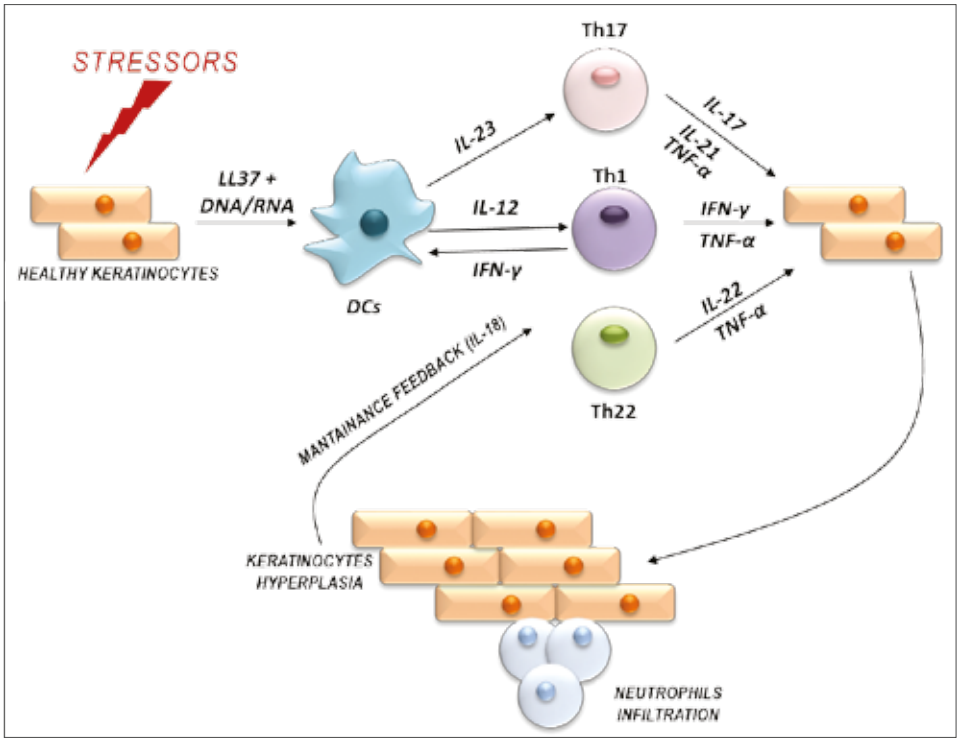

FIG. 1
IMMUNOLOGICAL PATHOGENESIS OF PSORIASIS

Local skin inflammation and increased keratinocyte proliferation are fundamental factors for disease onset and for the formation of the typical skin lesions.
The hyperplastic keratinocytes in turn produce large quantities of **pro-inflammatory cytokines** (IL-1β, IL-6, IL-8, TNF-α) that, by further stimulating the T-cells, **induce a disease self-maintenance mechanism.**
In this mechanism, a key role is played by IL-17, which, in addition to mediating the autoimmune response typical of psoriasis, induces the production of SAA (Serum Amyloid A), an acute phase inflammatory protein.
SAA binds with TLR-2 and TLR-4 (expressed by the keratinocytes); their activation causes NF-kB nuclear translocation and the **consequent activation of the pro-inflammatory cascade** that leads to the production of IL-1β, IL-6; IL-8 and TNF-α (**FIG. 2**).

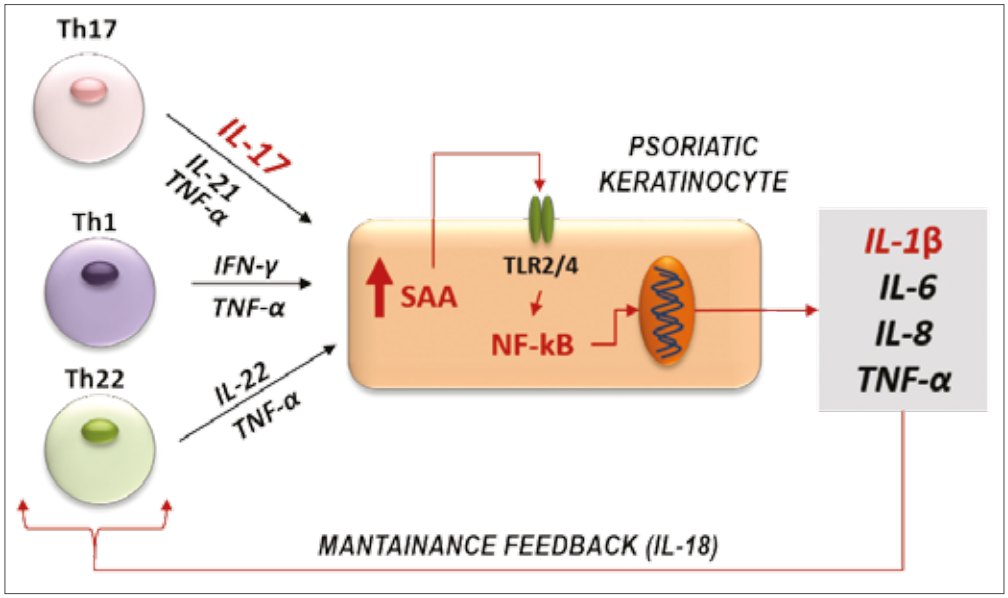

FIG. 2
PRO-INFLAMMATORY MECHANISMS IN THE PSORIATIC KERATINOCYTE

FOCUS: THE ROLE OF INTERLEUKIN 1

IL-1β plays a key role in maintaining the inflammatory micro-environment in psoriasis lesions. Psoriatic keratinocytes present an altered response to IL-1β.

In psoriatic keratinocytes, the expression of IL-1β and IL-1Ra (the endogenous IL-1 receptor antagonist) is reduced, whereas IL-1β levels are about 7 times higher; the reduced numerical ratio between IL-1Ra and IL-1 fully reflects the insufficiency of the IL-1Ra anti-inflammatory activity in Psoriasis.

STUDY DESIGN

- **Type of study:** spontaneous, observational, retrospective clinical study.
- **Subjects assessed:** 32 patients (male and female, 27 to 70 years of age) with Psoriasis Vulgaris.
- **Treatment period:** November 2014 to April 2015.

DATA COLLECTED

- **PASI (Psoriasis Area Severity Index) values** of 15 patients treated with UVA-1 (18 irradiations - 2 irradiations/week -) **(UVA-1 data group).**
- **PASI values** of 17 patients treated with UVA-1 (18 irradiations - 2 irradiations/week) plus oral co-administration of GUNA®-IL 4; GUNA®-IL 10 and GUNA®-ANTI IL 1 (20 drops twice daily) for the duration of treatment **(UVA-1 + CYTO data group).**

The PASI scores evaluated were collected at the first treatment session (T0) and after 10 weeks, i.e. one week after the last treatment session (T10).

All patients used white petroleum jelly alone as topical treatment to minimise any erythema caused by the UVA-1 treatment.

TREATMENT SCHEMES

- Group 1

  UVA-1 18 irradiations (2 irradiations/week for 9 weeks; 120 J/cm2 administered by an Alba 355 UVA- 1 355 nm laser, manufactured by Elettronica Valseriana, Casnigo, Italy).

- Group 2

  UVA-1 18 irradiations (2 irradiations/week for 9 weeks; 120 J/cm2 administered by an Alba 355 UVA- 1 355 nm laser, manufactured by Elettronica Valseriana, Casnigo, Italy) plus **GUNA®-IL 4, GUNA®-IL 10 and GUNA®-ANTI IL 1 (20 drops of each medicinal product twice daily) for 9 consecutive weeks.**

RESULTS

The data collected describe the two main results obtained:

- Confirmation of the efficacy of laser therapy with UVA-1.
- The fundamental activity of low-dose SKA cytokines and antibodies in boosting the efficacy of UVA-1 laser therapy.

More specifically, the mean PASI scores recorded for each patient at T0 were 8.5 (SD ± 0.9) in the UVA-1 Group and 8.3 (SD ± 0.7) in the UVA-1 + CYTO group.

At T10, the mean PASI scores recorded were 3.3 (SD ± 0.4) in the UVA-1 Group and 1.2 (SD ± 0.3) in the UVA-1 + CYTO Group. The PASI score improved considerably for all subjects assessed.

A statistically significant difference was observed between the Groups treated with UVA-1 alone at T10 and with UVA-1+CYTO at T10 (*** p <0.0001), showing that low-dose cytokines considerably increase the efficacy of UVA-1 laser therapy (FIG. 3).

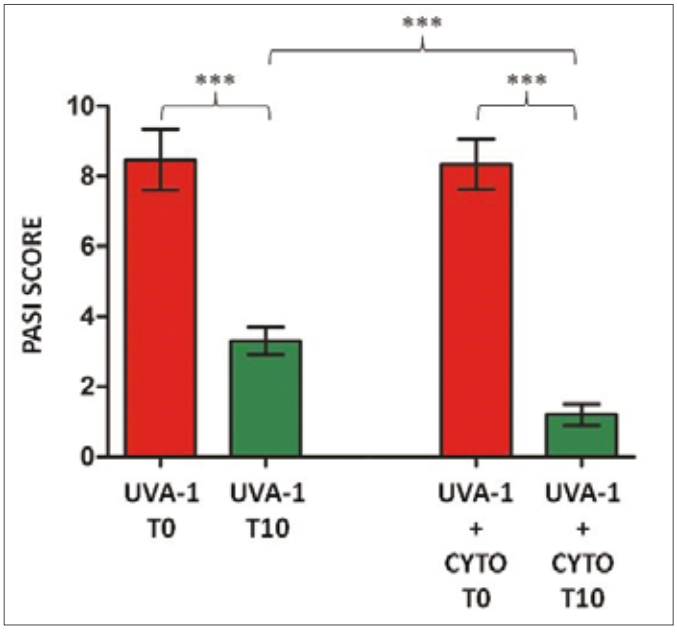

FIG. 3
REDUCTION IN THE PASI SCORE OBSERVED IN EACH PATIENT GROUP

CONCLUSIONS

Despite the limited number of patients and quantity of data collected and assessed, this retrospective study bore **a number of interesting results:**

- The high efficacy and tolerability of UVA-1 laser therapy were confirmed. The reduction in the PASI score shows the efficacy of monochromatic radiation at 355 nm for the treatment of skin diseases with an inflammatory component, such as Psoriasis.
- **On the basis of the strong theoretical grounds described, the co-administration of low-dose cytokines significantly improves the results obtained with laser therapy.**
- **It can be postulated that the Immune System rebalancing action of low-dose medicinal products complements and considerably improves cell response to UVA-1 radiation.**

The combination of laser therapy and oral administration of low-dose SKA medicinal products is efficacious and no adverse event was observed. The therapeutic results evaluated contribute to increasing the corpus of clinical data available as regards both the efficacy of laser therapy and the confirmation of the validity of the Low Dose Medicine approach for Psoriasis (see: Roberti ML, Ricottini L, et al. Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris. J Biol Regul Homeost Agents 2014; 28: 133-9).

These two treatment options could form the foundations for an innovative strategy for the treatment of inflammatory skin diseases such as Psoriasis Vulgaris. The data described encourage additional studies to confirm the results of this preliminary clinical observation.

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Lotti T. Successful combination treatment for psoriasis with phototherapy and low-dose cytokines: A spontaneous, retrospective observational clinical study. Hautarzt. 2015;66(11):849-54.

GUNA HIGHLIGHTS

Dermatology

TREATMENT WITH LOW-DOSE CYTOKINES REDUCES OXIDATIVE-MEDIATED INJURY IN PERILESIONAL KERATINOCYTES FROM VITILIGO SKIN

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Treatment with low-dose cytokines reduces oxidative-mediated injury in perilesional keratinocytes from vitiligo skin

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FOREWORD

Vitiligo is a disease with a very complex aetiology that is usually considered a chronic autoimmune inflammatory disease affecting approximately 0.5-0.8% of the population of Western countries. The incidence in Asian countries is higher, more specifically 10 times higher in India. **Vitiligo presents as areas of depigmentation with a varying extent that, as time goes by, tend to get larger.**

The pathogenesis of Vitiligo is also complex as it involves inflammatory and immune mechanisms, as a key role is played by oxidative stress phenomena. The presence of a micro-environment characterised by a persistent inflammatory condition mediated by the classical Th1-derived cytokines, in particular IL-1, IL-6 and TNF-α, and by intense oxidative stress caused by reactive oxygen species (ROS), is in turn the main causal factor of the progressive loss of keratinocyte function and therefore fuels the self-sustaining process, which leads to disease maintenance and progression (**FIG. 1**).

Increased levels of IL-1 are found in patients with active lesions, especially in the perilesional area, where the inflammatory phenomena are most intense, which makes a considerable contribution to disease progression.

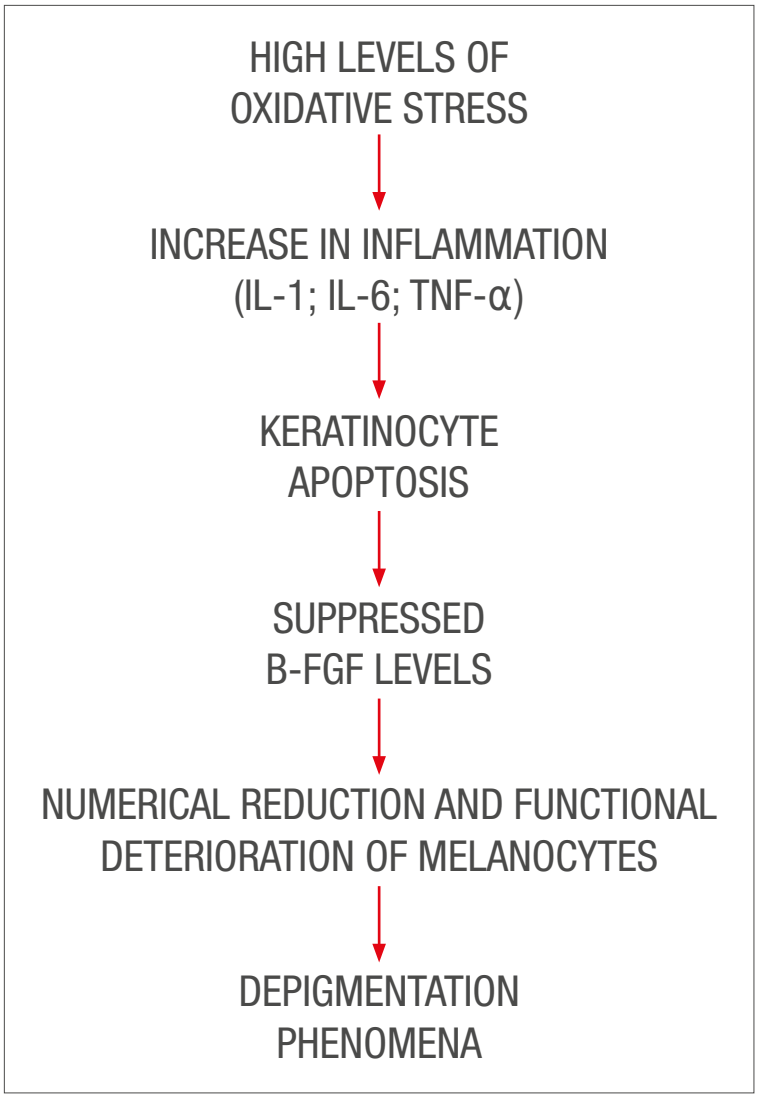

FIG. 1
SUMMARY OF THE MAIN BIOLOGICAL EVENTS RESPONSIBLE FOR THE ONSET OF VITILIGO

INTRODUCTION

The central importance of inflammatory phenomena, and oxidative stress in particular, urged the investigators to assess the effects of low-dose SKA IL-4, IL-10, b-FGF and β-Endorphin (GUNA®-IL 4, GUNA®-IL 10, GUNA®-BETA ENDORFIN. Guna S.p.a, Milan, Italy) in the modulation of intra- and extra-cellular oxidative stress and proliferation of human keratinocytes.

The in vitro study was conducted on keratinocytes obtained from healthy donors (CTR) and on primary perilesional keratinocytes (PL) obtained from biopsies of the depigmented skin of vitiligo patients.

The cells were then treated with 10 fg/ml of low-dose SKA IL-4, IL-10, b-FGF and β-Endorphin for 48 hours and the proliferation index and the intra- and extra-cellular oxidative status were measured by fluorometric assay and flow cytometry using H2DCFDA (DCF) fluorescent dye, respectively.

Primary keratinocytes harvested from healthy subjects were cultivated and used as control cells.

To aid a clear understanding of the experiments and relative charts, TABLE 1 provides a key for the cell treatment study group breakdown and the assays conducted. For all groups, the cells were incubated with the low-dose SKA cytokines at a concentration of 10 fg/ml for 48 hours.

TREATMENT	KERATINOCYTES FROM HEALTHY DONORS (CTR)	KERATINOCYTES FROM PATIENTS (PL)
-------	CTR	PL
+ GUNA®-IL 4 (10 fg/ml)	CTR+IL-4	PL+IL-4
+ GUNA®-IL 10 (10 fg/ml)	CTR+IL-10	PL+IL-10
+ GUNA®-FGF (10 fg/ml)	CTR+bFGF	PL+bFGF
+ GUNA®-Beta Endorfin (10 fg/ml)	CTR+β-END	PL+β-END

TABLE 1
BREAKDOWN OF TREATMENT GROUPS

ASSAYS CONDUCTED

- Luminometric assay of NADPH oxidase activity for the quantification of the levels of extracellular oxidative stress.
- Flow cytometry assay of ROS (reactive oxygen species) generation for the quantification of the levels of intracellular oxidative stress.
- Fluorimetric cell viability assays.

RESULTS

The results showed:

- **A significant reduction in extra-cellular oxidative stress** (reduction in NADPH oxidase activity), in particular **with low-dose SKA IL-4 and b-FGF** cytokines (**FIG. 2**).
- **A significant reduction in intra-cellular oxidative stress** (reduction in ROS production), in particular with **low-dose SKA IL-4, IL-10 and b-FGF** cytokines (**FIG. 3).**
- **An increase in the proliferation index expressed as a percentage of the untreated PL keratinocytes,** especially with **low-dose SKA b-FGF and beta-Endorphin** cytokines, compared to the control group (**FIG. 4**).

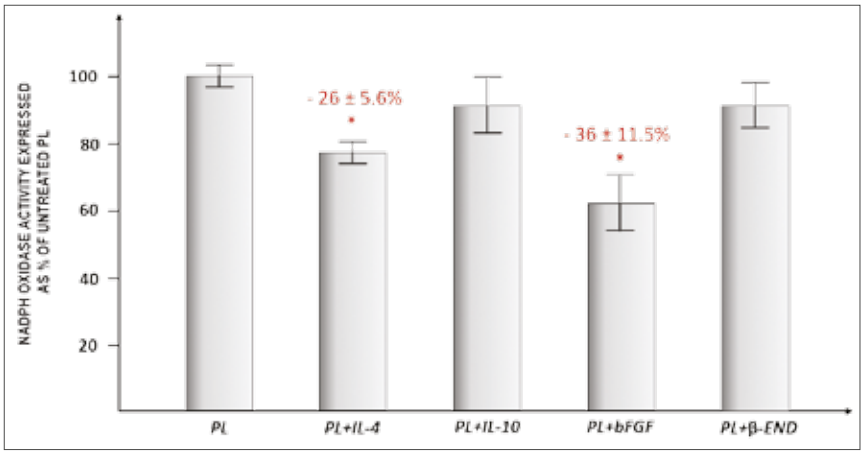

FIG. 2
QUANTITATIVE ANALYSIS OF NADPH OXIDASE ACTIVITY. THE VARIATION IN NADPH OXIDASE ACTIVITY LEVELS IS EXPRESSED AS A PERCENTAGE OF THE VALUE OBSERVED IN UNTREATED PL KERATINOCYTES (100%). * SIGNIFICANT DIFFERENCE (P≤ 0.05) COMPARED TO UNTREATED PL KERATINOCYTES. THE VALUES SHOWN (MEAN ± SD) REPRESENT FIVE INDEPENDENT EXPERIMENTS.

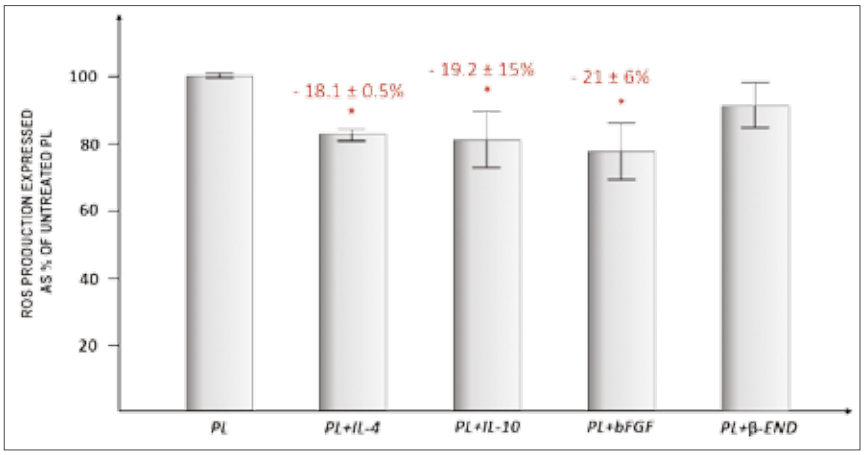

FIG. 3
QUANTITATIVE ANALYSIS OF ROS PRODUCTION. ROS PRODUCTION IS EXPRESSED AS A PERCENTAGE OF THE VALUE OBSERVED IN THE UNTREATED PL KERATINOCYTES (100%). * SIGNIFICANT DIFFERENCE (P ≤ 0.05) COMPARED TO THE UNTREATED PL KERATINOCYTES. THE VALUES SHOWN (MEAN ± SD) REPRESENT FIVE INDEPENDENT EXPERIMENTS.

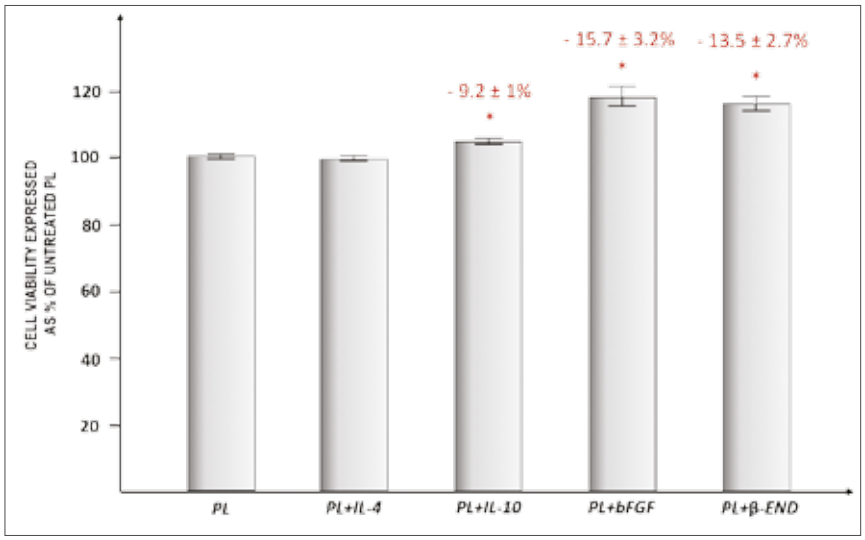

FIG. 4
CELL VIABILITY ANALYSIS THE VIABILITY OF THE PL KERATINOCYTES FOLLOWING TREATMENT WITH IL-4, IL-10, B-FGF OR B-ENDORPHIN WAS ASSESSED BY ASSAYING THE REDUCTION IN RESAZURIN (DYE INDICATING CHANGES IN PH AND OXIDATION-REDUCTION). VIABILITY IS EXPRESSED AS A PERCENTAGE OF THE VALUE OBSERVED IN THE UNTREATED PL KERATINOCYTES (100%). THE VALUES SHOWN (MEAN ± SD) REPRESENT FIVE INDEPENDENT EXPERIMENTS.

CONCLUSIONS

The paper published in the Journal of Dermatological Science shows that treatment with **low-dose SKA IL-4, IL-10, b-FGF, and β-Endorphin** significantly reduces oxidative stress phenomena in human keratinocytes in the perilesional area of vitiligo skin lesions. This translates into a considerable reduction in one of the main triggers of Vitiligo: chronic inflammatory state.

The main points brought to light in the paper are:

- **Efficacy on one of the fundamental aetiological factors of Vitiligo: inflammation**
- **Synergy of action**
- **Selective action**

In the light of these observations, it can be concluded that the hypothesis of treating Vitiligo with Low Dose Medicine now has sturdy experimental grounds.

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GUNA HIGHLIGHTS
Dermatology

VITILIGO: SUCCESSFUL COMBINATION TREATMENT BASED ON ORAL LOW DOSE CYTOKINES AND DIFFERENT TOPICAL TREATMENTS

JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS Vol. 29, no. 1 (S), 53-58 (2015)

VITILIGO: SUCCESSFUL COMBINATION TREATMENT BASED ON ORAL LOW DOSE CYTOKINES AND DIFFERENT TOPICAL TREATMENTS

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FOREWORD

The study titled ***"Vitiligo: successful combination treatment based on oral low dose cytokines and different topical treatments"*** originated from the curiosity of a group of researchers from University centres in five countries (Italy, Germany, Czech Republic, Bulgaria and Romania) to verify the efficacy of Low-Dose Medicine (LDM) in the dermatology field, more specifically, in the treatment of Vitiligo. This retrospective observational study confirmed the efficacy of the combination of **low-dose SKA basic-fibroblast growth factor, interleukin 10, interleukin 4, and anti-interleukin 1 antibodies (GUNA®-FGF, GUNA®-IL 10, GUNA®-IL 4, GUNA®-ANTI IL 1. Manufactured by Guna S.p.a. - Milan, Italy)** administered orally as monotherapy or co-administered with other local or systemic treatments.

The opportunity to co-administrate low doses of these molecules, which are easy to manage and without dose-dependent toxic effects by means of SKA technology, aroused the curiosity of the authors and made this study possible.

INTRODUCTION

Vitiligo is characterised by depigmented areas of **skin** caused by an absence of melanocytes. The origin of this disease is currently unknown and understanding of its pathogenesis is poor (**FIG. 1**).

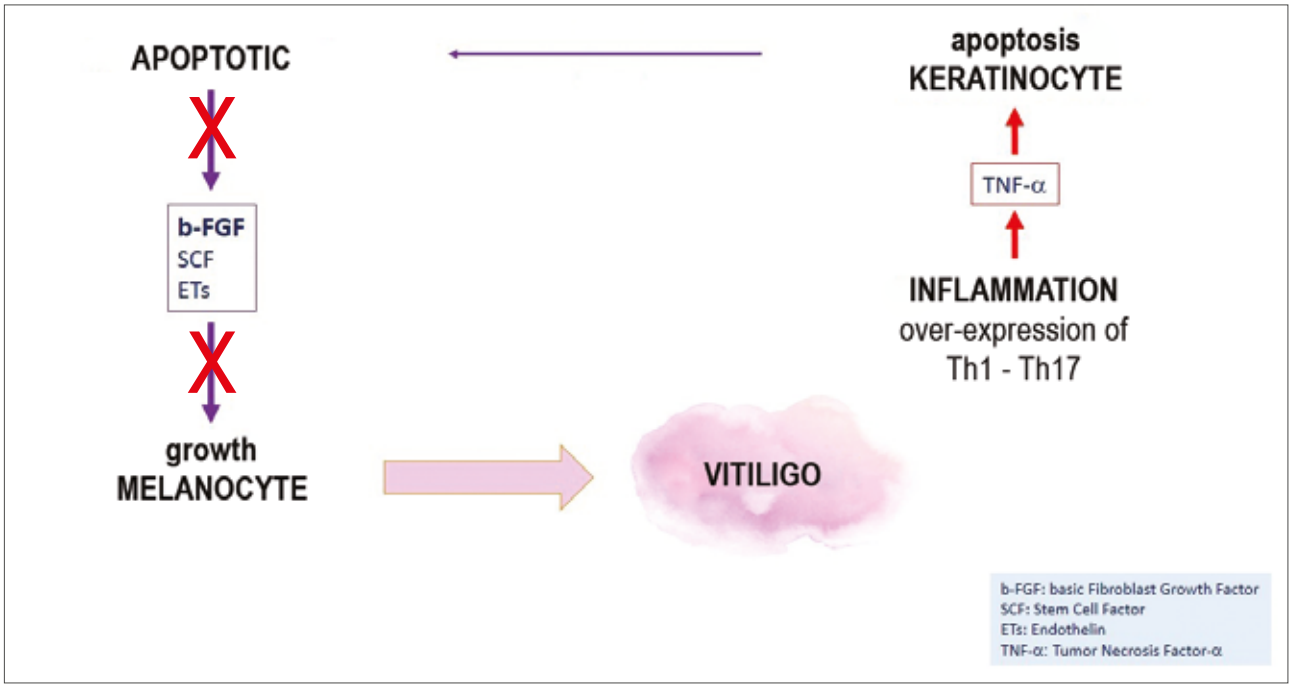

FIG. 1
PARACRINE HYPOTHESIS FOR THE PATHOGENESIS OF VITILIGO

Vitiligo has a profound impact on both the physical and mental health of those affected and includes loss of skin photoprotection, impairment of skin immunity and considerable deterioration in quality of life.

The unsatisfactory efficacy of the pharmacological or surgical treatments available for this condition constantly urges researchers to test new treatment strategies. The non-surgical repigmentation therapies include **narrowband UVB radiation, broadband UVB, topical or oral treatment with corticosteroids** and other treatments deriving from the herbal remedy tradition. Combined therapies are also used in clinical practice, including **psoralene plus UVA radiation** and UVA radiation combined with potent corticosteroids. The most recent and efficacious approaches include **targeted UVB microphototherapy and targeted laser therapy.**

For some time now, researchers and clinicians working in the LDM field have been treating Vitiligo patients with **low-dose cytokines, growth factors and neuropeptides,** with very encouraging results.

The purpose of this retrospective observational study was to assess the efficacy and safety of the oral administration of low-dose SKA drugs (GUNA®-FGF, GUNA®-IL 10, GUNA®-IL 4 and GUNA®-ANTI IL 1) as monotherapy or in combination with other systemic or local treatments (oral administration of *Gingko biloba* extract, targeted 311nm narrowband microphototherapy, topical administration of betamethasone propionate and exposure to sunlight) (TABLE 1).

TREATMENT GROUPS

- 20 subjects - GUNA®-FGF
- 20 subjects - GUNA®-FGF + targeted 311nm narrowband microphototherapy
- 20 subjects - GUNA®-FGF + topical Betamethasone propionate 0.05%
- 20 subjects - GUNA®-IL 10 + GUNA®-IL 4 + GUNA-ANTI IL 1
- 20 subjects - GUNA®-IL 10 + GUNA®-IL 4 + GUNA®-ANTI IL 1 + targeted 311nm narrowband microphototherapy
- 20 subjects - GUNA®-IL 10 + GUNA®-IL 4 + GUNA®-ANTI IL 1 + topical Betamethasone propionate 0.05%
- 20 subjects - Targeted 311nm narrowband microphototherapy
- 20 subjects - Topical Betamethasone propionate 0.05%
- 20 subjects - Gingko biloba 240 mg/day
- 20 subjects - Exposure to sunlight 30min/day with SPF 15 sunscreen

TREATMENT SCHEMES

Betamethasone propionate 0.05% for topical use, applied twice daily.

LDM drugs for oral use (GUNA®-FGF, GUNA®-ANTI IL 1, GUNA®-IL 4, GUNA®-IL 10), 20 drops of each drug administered twice daily, for 9 consecutive months.

TABLE 1

The retrospective analysis was conducted **on 200 subjects** affected by the disease. The subjects considered belonged to 10 different Groups, all with an impairment of no more than 15% of the body surface area, who had followed different treatment protocols and completed the prescribed treatments correctly (**TABLE 2**).

AGE RANGE	MIN: 21	MAX: 55		
GENDER	MALE 44%	FAMALE 56 %		
DURATION OF DISEASE, IN YEARS	MIN: 2	MAX: 9		
PREVALENCE OF PHOTOTYPE, IN %	TYPE II: 46%	TYPE III: 32%	TYPE IV: 20%	TYPE V: 2%

TAB. 2
TABLE SHOWING THE RELEVANT CHARACTERISTICS OF THE SUBJECTES OBSERVED

RESULTS

This retrospective observational study considered data collected between June 2014 and March 2015 regarding 200 subjects who had completed 10 different therapies for the treatment of Vitiligo.

TABLE 3 shows the clinical results, i.e. the actual percentage of repigmentation of the skin surface obtained after the different treatments.

Treatment groups (20 subjects each group)	Excellent (>75%)	Considerable (50-75%)	Moderate (25-50%)	Limited (<25%)
GUNA®-FGF	21%	23%	30%	26%
GUNA®-FGF + targeted 311nm narrowband microphototherapy	66%	11%	15%	8%
GUNA®-FGF + topical Betamethasone propionate 0.05%	64%	20%	10%	6%
GUNA®-IL 10 + GUNA®-IL 4 + GUNA®-ANTI IL 1	37%	8%	32%	23%
GUNA®-IL 10 + GUNA®-IL 4 + GUNA®-ANTI IL 1 + targeted 311nm narrowband microphototherapy	63%	23%	7%	7%
GUNA®-IL 10 + GUNA®-IL 4 + GUNA®-ANTI IL 1 + topical Betamethasone propionate 0.05%	55%	25%	14%	6%
Targeted 311nm narrowband microphototherapy	51%	20%	19%	10%
Topical Betamethasone propionate 0.05%	37%	19%	30%	14%
Gingko biloba 240 mg/die	6%	19%	25%	50%
Exposure to sunlight 30min/day with SPF 15 sunscreen	26%	31%	20%	23%

TABLE 3

CONCLUSIONS

The results obtained by this retrospective observational study show that a percentage of subjects of between 74% (GUNA®-FGF) and 77% (GUNA®-IL 10 + GUNA®-IL 4 + GUNA®-ANTI IL 1) respond to LDM treatment as regards the chosen objective, i.e. the percentage of skin surface repigmentation higher than 25%.

The data show that both approaches, i.e. the b-FGF-mediated restoration of signalling between keratinocytes and melanocytes and the control of the inflammatory response mediated by the signalling molecules IL-10, IL-4 and anti-IL-1 antibodies, constitute a valid approach to the treatment of Vitiligo.

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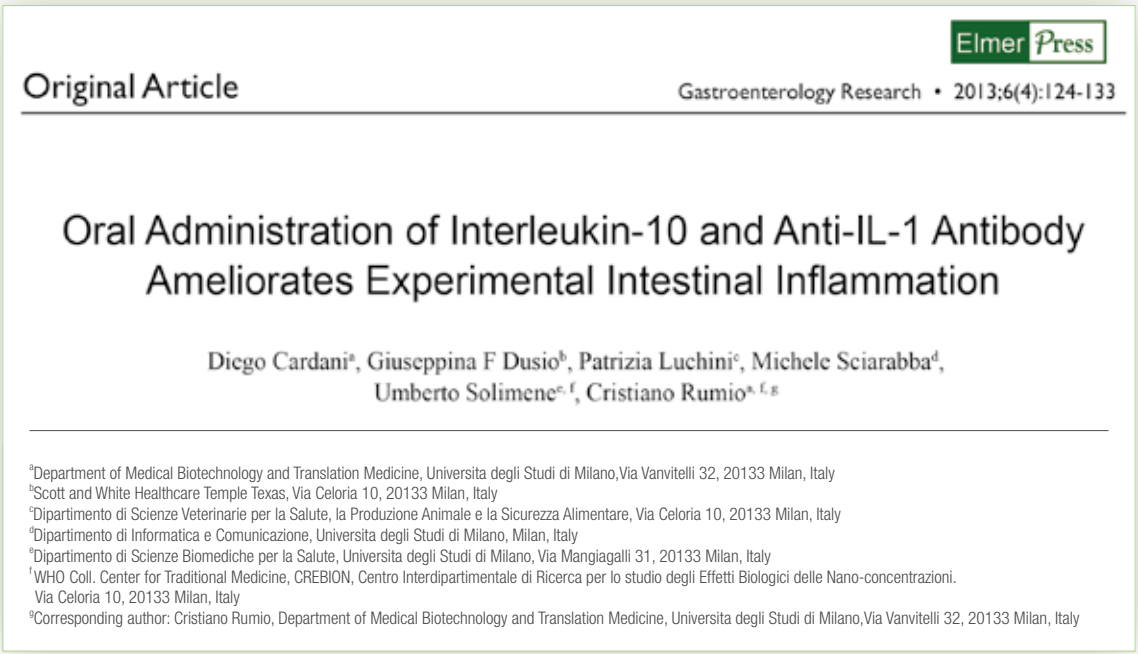
GUNA HIGHLIGHTS

Gastroenterology

ORAL ADMINISTRATION OF INTERLEUKIN-10 AND ANTI-IL-1 ANTIBODY AMELIORATES EXPERIMENTAL INTESTINAL INFLAMMATION



Elmer Press

Original Article

Gastroenterology Research • 2013;6(4):124-133

Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation

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FOREWORD

Inflammatory bowel diseases (IBDs) are a group of chronic diseases affecting the gastrointestinal tract.

Two of the most common forms of inflammatory bowel disease are:
- **Ulcerative colitis (UC),** which affects the large intestine
- **Crohn's disease (CD),** which can affect the whole GI tract

The pathogenesis of inflammatory bowel disease is not completely clear, although current theories postulate that the inflammation of the mucosae could be caused by an exaggerated reaction by the mucosal immune system, i.e. the loss of inflammatory homeostasis that governs the delicate immune balance between maintaining the structure and function of the intestinal epithelium, the gut flora and pathogenic microorganisms.

INTRODUCTION

The research group headed by Prof. C. Rumio studied the effect of a solution containing **low-dose SKA IL-10 and anti-IL-1 antibodies (GUNA®-IL 10, GUNA®-ANTI IL 1. Guna S.p.a. - Milan, Italy)** on the modulation of intestinal inflammation in an animal model of IBD.
The study was conducted using BALB/c mice split into three groups to receive the following treatments:

- **UNTR Group:** control animals
- **DSS Group:** animals treated by oral administration of 2% Dextran sodium sulphate (DSS - a potent surfactant) in order to induce experimental inflammatory disease.
- **DSS+IL-10/anti-IL-1 Group:** animals treated with 2% DSS and, simultaneously, with low-dose SKA IL-10 and anti-IL-1 antibodies.

RESULTS

The parameters assessed were:
- **colon morphology** (histology)
- **the transepithelial electrical resistance of the colon** (measured in Ω/cm^2)
- **the levels of pro- and anti-inflammatory cytokines** (ELISA assay of IFN-gamma; TNF-alpha; IL-10; IL-12; IL-17; KC)
- **tight junction architecture** [production and distribution of Zonula Occludens-1 (ZO-1) tight junction protein, evaluated by immunofluorescence]

The researchers observed that the oral administration of low-dose SKA IL-10 and anti-IL-1 antibodies restores intestinal barrier function during intestinal inflammation whilst reducing the levels of pro-inflammatory cytokines, increasing IL-10 concentrations and improving tight junction architecture.

FIG. 1A - Protective effects of oral co-administration of low-dose SKA IL 10 and anti-IL 1 antibodies in an animal model of induced chronic colitis. Evaluation of the resistance of the colon (measured in Ω/cm^2) assessed with Ussing Chamber in different treatment groups.
The graph shows the average values with relevant standard deviation (SD). The statistical analysis indicates the signinficat results obtained with the administration of low-dose medicaments. Trans-epithelial resistance, which is an indicator of intestine permeability, were restored to normal levels, while the treatment with DSS only reduces resistance significantly, thus highlighting the damage to the epithelium.

FIG. 1B - Histological evaluation in the different treatment groups. The scale from 0 to 3 indicates the intensity of the mechanical damage with structure impairment (0 = absence of damage; 3 = serious damage).
The administration of low-dose medicaments protects the intestinal structure effectively, thus reducing damage to "absent" or "mild" in the scale.

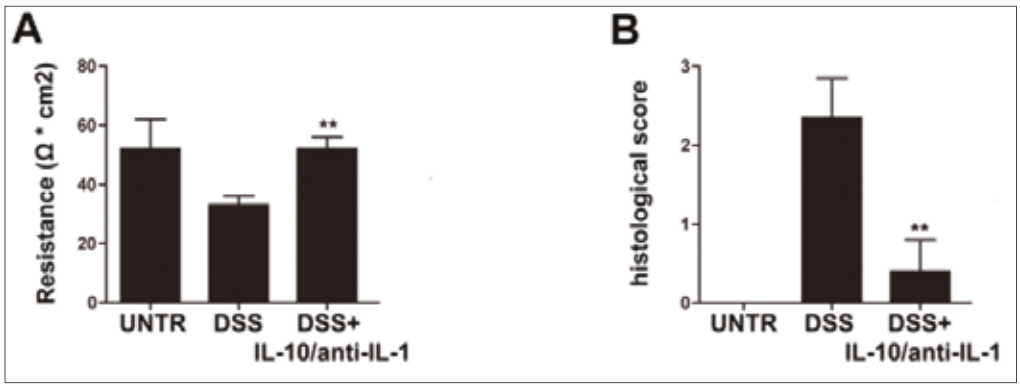

FIG. 1
EVALUATION OF
COLON RESISTANCE

FIGS. 2 and 3 show the validity of the treatment from a histological and histochemical point of view, confirming the instrumental data concerning permeability provided in Figure 1-A, which are closely linked to intestinal structure. The panels correspond with the following treatments: A = UNTR; B = DSS; C = DSS + IL-10/anti-IL-1.

FIG. 2 - When observing the sections of colon stained with H&E, it can be seen that the structure of the colon in Panel C is practically identical to the same control section of Panel A. Panel B shows the damage caused by the surfactant that, like a soap, causes epithelial inflammation by damaging the folds of the colon.

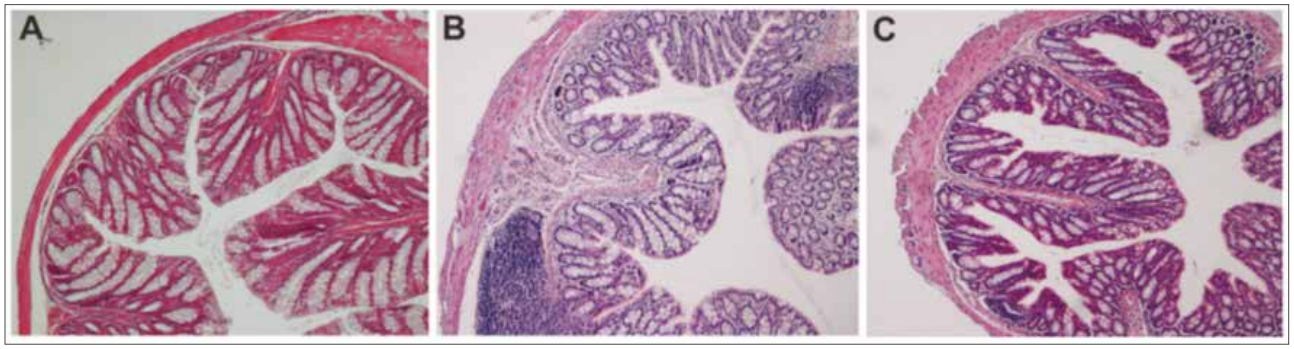

FIG. 2
HISTOLOGICAL EVALUATION OF INTESTINAL MUCOSAL INTEGRITY

FIG. 3 - When observing the sections of colon in which the tight junction protein ZO-1, a fundamental component of the tight junctions (TJ), has been labelled by immunofluorescence, it can be observed that in Panel C the distribution and quantity of protein expressed are similar to those in control Panel A. In Panel B, the tight junction damage is shown by the presence of reduced ZO-1 and its disorganisation (loss of the honeycomb structure).

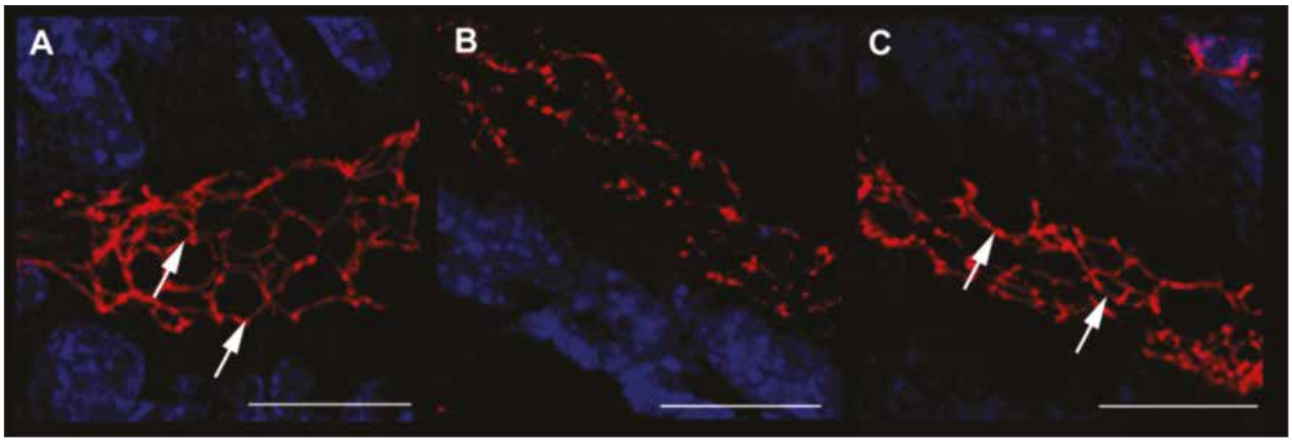

FIG. 3
HISTOCHEMICAL EVALUATION OF THE INTEGRITY OF THE TIGHT JUNCTION SYSTEMS

FIG. 4 shows the data regarding the expression of the main pro-inflammatory cytokines TNF-alpha; IL-12; IL-17; KC (murine homologue of human IL-8) and the most important anti-inflammatory cytokine, IL10.

The panel provides a graph showing the results obtained and, in short, it can be seen that all pro-inflammatory markers are significantly reduced by the low-dose treatment. At the same time, IL-10 is increased by the low-dose treatment, as expected.

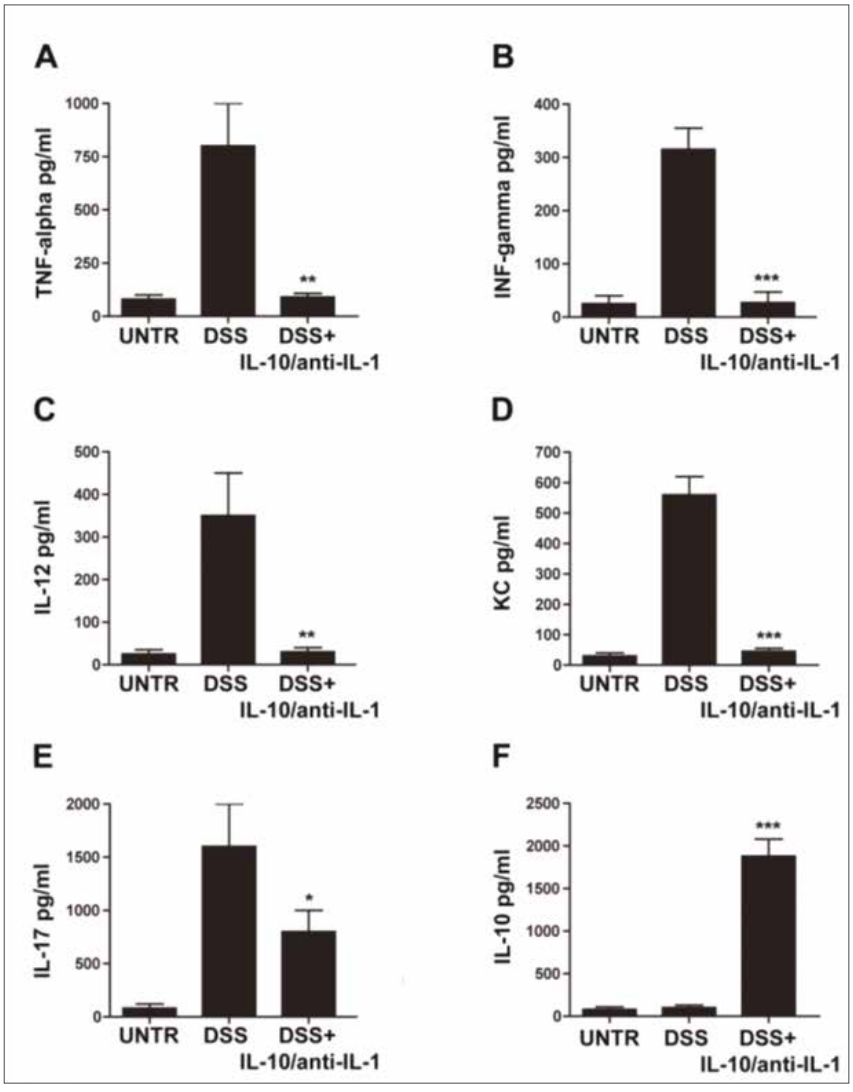

FIG. 4
EVALUATION OF PRO- AND ANTI-INFLAMMATORY CYTOKINE EXPRESSION

CONCLUSIONS

In the experimental model used, **low-dose SKA IL-10 and anti-IL-1 antibodies proved highly effective in protecting the intestine from the chemical damage induced by Dextran Sodium Sulphate** in both structural terms (histology, immunofluorescence of the tight junction systems and transepithelial resistance) and biochemical terms (cytokine levels).

The morphological and functional data collected clearly indicate that the low-dose drugs experimented are able to protect the gastrointestinal tract from DSS-induced damage.

The results obtained could favour the definition of a therapeutic strategy in patients with inflammatory bowel disease.

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GUNA HIGHLIGHTS

Gynaecology

PHARMACOLOGICAL AND INTEGRATIVE TREATMENT OF STRESS-INDUCED HYPOTHALAMIC AMENORRHEA

Bollettino di Ginecologia Endocrinologica
Vol. 10.24.32, 2016

Terapia integrativa e farmacologica dell'amenorrea ipotalamica da stress

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FOREWORD

The research group headed by AD. Genazzani (Centro di Ginecologia Endocrinologica [Centre of Endocrinological Gynaecology], Clinica Ostetrica Ginecologica [Obstetrics & Gynaecology Clinic], Università di Modena e Reggio Emilia) published in the journal **Frontiers in Gynecological Endocrinoloy** (Volume 3: Ovarian Function and Reproduction - From Needs to Possibilities) a review titled: ***"Pharmacological and Integrative Treatment of Stress-induced Hypothalamic Amenorrhea"***, previously published in Bollettino di Ginecologia Endocrinologica. The topic is that of **stress-induced hypothalamic amenorrhoea**, dealt with from both an aetiological-clinical point of view and a therapeutic point of view, placing special emphasis on the **Psycho-Neuro-Endocrino-Immune (PNEI)** aspects associated with the disorder's pathophysiology. The article is of interest from a **Low-Dose Medicine (LDM)** standpoint as, amongst the various treatment options, it describes an approach based on priming with low doses of oestrogens **(GUNA®-Beta Estradiol - Guna S.p.a. - Milan, Italy)**.

INTRODUCTION

In adolescents, amenorrhoea lasting 3-4 or more months is a relatively common occurrence and is usually due to alterations in hormone levels (oestrogen deficiency and high levels of luteinising hormone, LH).
In the absence of these conditions and other systemic or endocrine factors, amenorrhoea is considered to be caused by hypothalamic blockade. This research clearly shows the role played by stress in the onset of hypothalamic amenorrhoea and metabolic, physical or psychological stressors are often also present (**FIG. 1**).

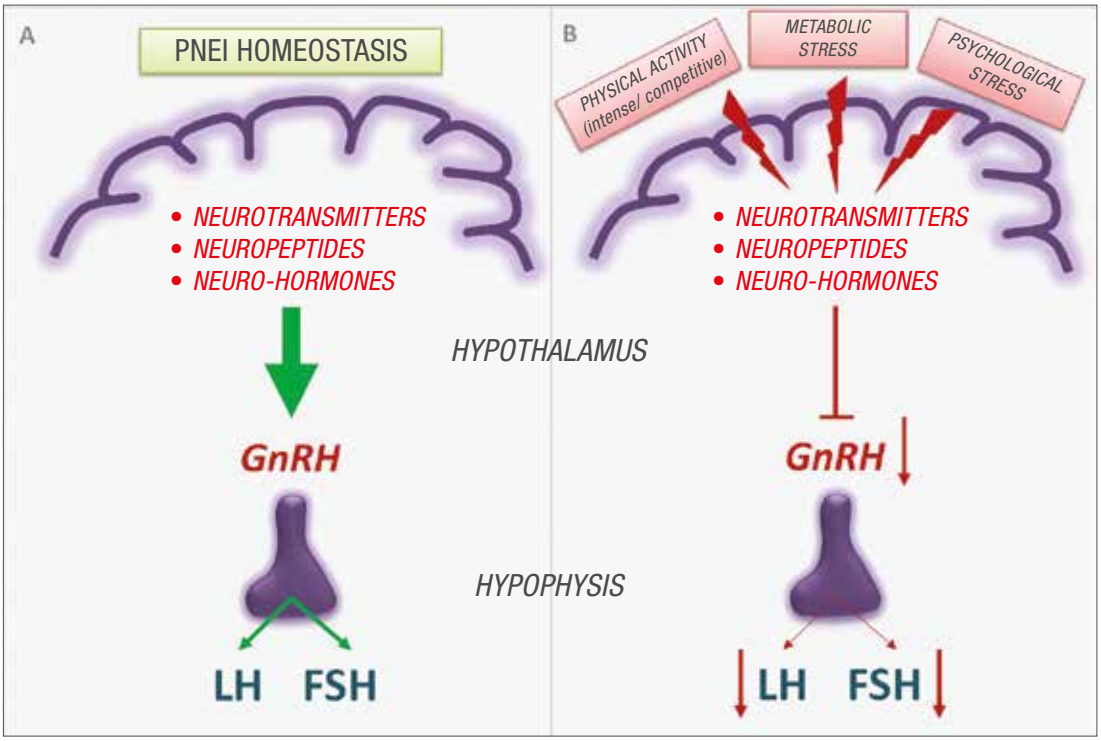

FIG. 1
SIMPLIFIED DESCRIPTION OF THE HYPOTHALAMIC-PITUITARY AXIS AND ITS ALTERATION INDUCED BY STRESSORS

For at least a million years, the survival and evolution of the human race has been based on self-protection and preservation mechanisms. Of these mechanisms, the blockage of reproductive functions in the presence of unfavourable physiological and environmental conditions was (and still is) one of the most important, in order to guarantee the survival of the species. Stress-induced reproductive function inhibition represents the reminiscence and, in some ways, the evolution of this ancestral defence mechanism.

In short, **the presence of metabolic and/or physiological stressors translates into the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis, with a consequent elevation in ACTH (adrenocorticotropic hormone) and cortisol levels.**
An increase in ACTH is induced under the stimulation of CRF (Corticotropin-Releasing Factor), which also **suppresses the secretion of GnRH (Gonadotropin-Releasing Hormone) and LH,** thereby suppressing Hypothalamic-Pituitary-Gonadal (HPG) axis function. CRF also stimulates the central secretion of **β-endorphin**, which exerts an additional inhibitory function on GnRH (**FIG. 2**).

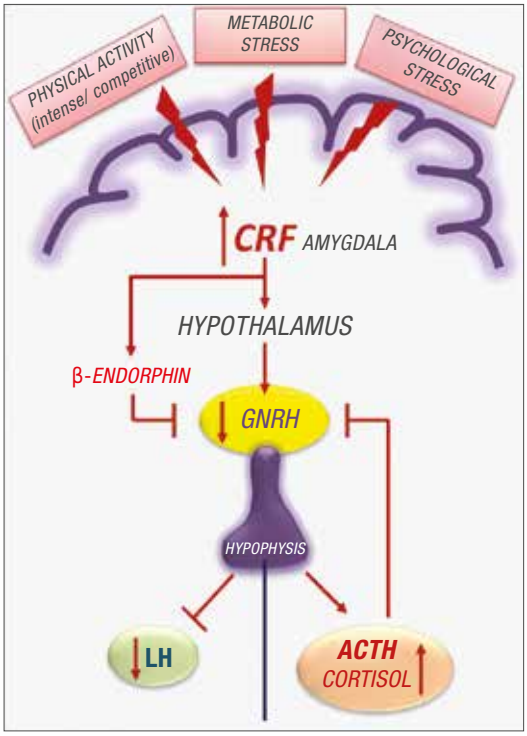

FIG. 2
HPG AXIS INHIBITION MECHANISM

Repeated and/or chronic stressors cause the onset of hypothalamic amenorrhoea: they alter hypothalamic neuroendocrine activity and the secretion of pituitary hormones. Opioid peptides have been identified as the main mediators between stressor event and amenorrhoea. Opioids are produced to "sedate" stress conditions, however, when over-expressed, they have an inhibitory effect on the pulsatile secretion of GnRH and, consequently, LH.

This inhibition can be reversed by using opioid receptor antagonists (naltrexone). Hyperactivation of the dopaminergic system [which can be restored using dopaminergic receptor antagonists (metroclopramide)] and of the serotonergic system [which can be partially restored using serotonin reuptake inhibitor (fenfluramine)] also occur in the presence of hypothalamic amenorrhoea. In addition to the interaction between the opioid, serotonin and dopamine systems, metabolic signals represent the other large group of stressors in hypothalamic amenorrhoea. A deficient diet and excessive energy consumption lead to severe weight loss. The relative percentage of this loss is a decisive factor in the inhibition of the Reproductive axis and is associated with a suppression of the levels of insulin and circulating T3 ("low T3 syndrome"). The effect of an extreme dietary regime takes the form of FSH and LH secretion inhibition.

In the light of these considerations, the therapeutic actions for the treatment of stress-induced hypothalamic amenorrhoea must act on one or more of the parameters mentioned and listed below:

- Neurotransmitters, neuromodulators (DA, GABA, 5HT, etc.)
- Various hormones (PRL, thyroid hormones)
- Diet and/or energy balance (protein, glucose, physical exercise)

To these we can add the option of low-dose oestrogen priming, as described below.

RESULTS

Ovarian steroids play an important role in the modulation of both hypothalamic and pituitary functions. Over the past 30 years, it has been proven that the use of low-dose weak oestrogens (epimestrol first, followed by oestriol) is effective in activating GnRH-induced LH secretion by the gonatropic cells. Epimestrol restores LH secretion in women with oligomenorrhoea or amenorrhoea and, similarly, oestriol (2 mg a day for 8 weeks) effectively induces an increase in plasma LH levels and in the amplitude of LH peaks, thereby also causing a significant increase in LH response to the administration of GnRH. Oestriol is thought to induce better hypothalamic-pituitary axis function by acting on various factors such as an increase in the sensitivity or number of GnRH receptors in anterior pituitary cells, an increase in the amount of GnRH secreted for each hypothalamic secretory peak and greater synthesis of LH by the gonadotropic cells.

The preliminary data obtained using GUNA®-Beta Estradiol administered orally (1µg twice daily, equal to 20 drops twice daily), would appear to confirm this result. The administration of low doses of oestradiol made possible by the use of GUNA®-Beta Estradiol are effective in determining a significant increase in the plasma levels of LH and FSH after 45/90 days of the last expression of reactivation of the HPG axis (FIG. 3).

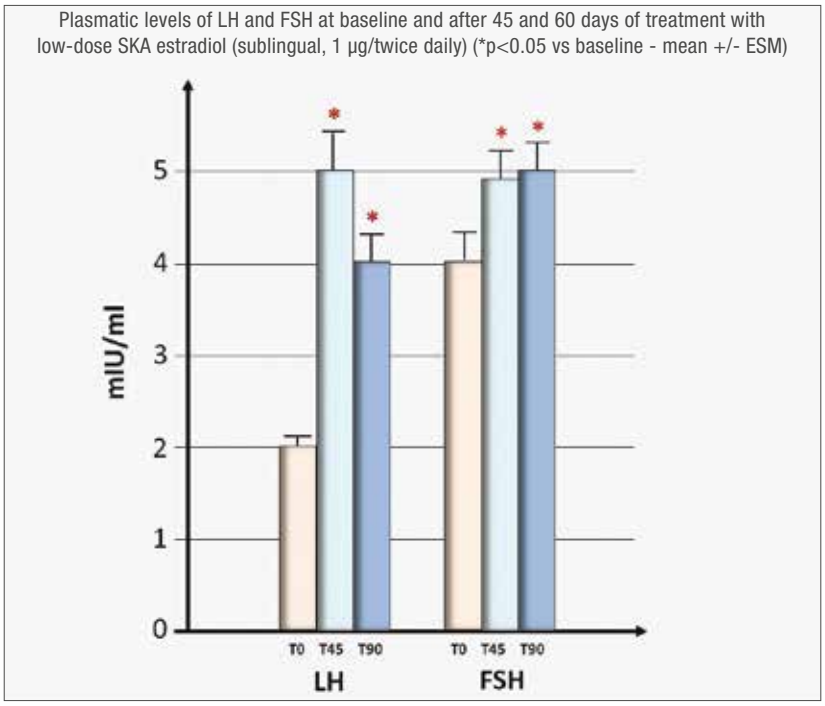

FIG. 3
EFFECT OF THE ADMINISTRATION OF GUNA®-BETA ESTRADIOL ON PLASMA LEVELS OF LH AND FSH

CONCLUSIONS

The use of low-dose SKA oestradiol induces an effect on the hypothalamus and pituitary that is similar to that hypothesised for the administration of oestriol, i.e. increased sensitivity to GnRH and greater expression of GnRH receptors, which therefore permits an increase in the synthesis and secretion of LH and FSH.

Although these data are preliminary, they show the potential capacity for HPG axis reactivation of the low-dose SKA drug GUNA®-Beta Estradiol and therefore contribute to the recovery of reproductive functions in the case of hypothalamic amenorrhoea.

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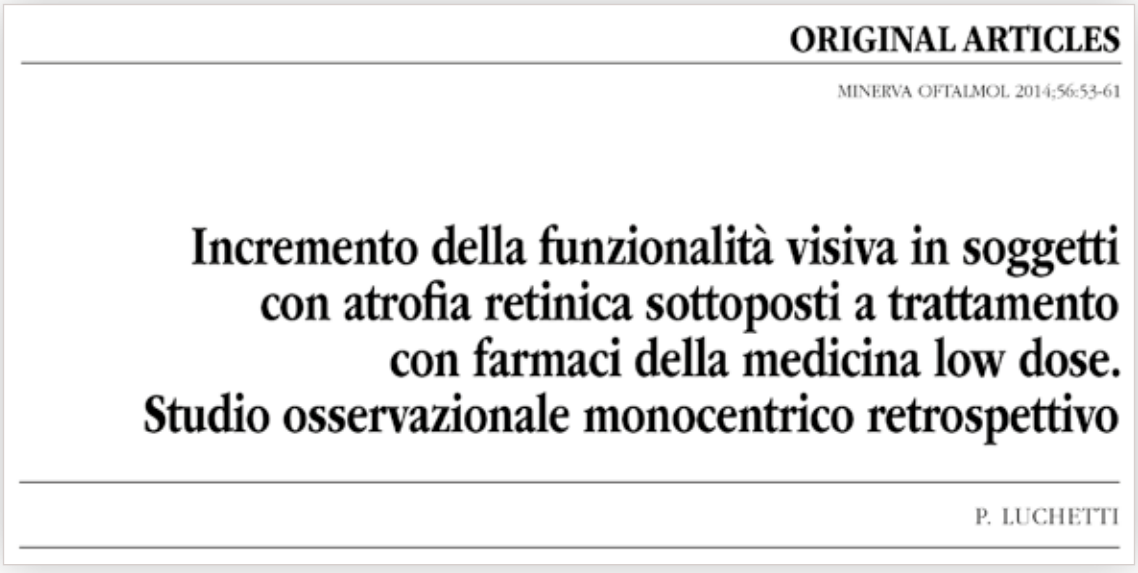
Genazzani AD, Prati A, Bonacini R, Despini G. Terapia integrativa e farmacologica dell'amenorrea ipotalamica da stress. Bollettino di Ginecologia Endocrinologica 2016;10:24-32.

Published in English as: Genazzani A.D., Despini G., Chierchia E., Benedetti C., Prati A. (2016) Pharmacological and Integrative Treatment of Stress-Induced Hypothalamic Amenorrhea. In: Genazzani A., Tarlatzis B. (eds) Frontiers in Gynecological Endocrinology. ISGE Series. Springer, Cham.

GUNA HIGHLIGHTS

Ophthalmology

INCREASING OF VISUAL FUNCTION IN PATIENTS WITH RETINAL ATROPHY TREATED WITH DRUGS OF LOW DOSE MEDICINE. MONOCENTRIC RETROSPECTIVE OBSERVATIONAL STUDY.



ORIGINAL ARTICLES

MINERVA OFTALMOL 2014;56:53-61

Incremento della funzionalità visiva in soggetti con atrofia retinica sottoposti a trattamento con farmaci della medicina low dose. Studio osservazionale monocentrico retrospettivo

P. LUCHETTI

FOREWORD

There is currently no established therapy for the treatment of retinal atrophy, which often represents the end-stage of many retinal diseases. However, Low-Dose Medicine provides a number of medicaments that could potentially be useful for the regenerative therapy of these forms.

The purpose of the study was to evaluate the efficacy of medicinal products (administered as combinations via both retrobulbar injection and via the sublingual route) in promoting a regenerative phase in the retinal cells.

The study investigated the efficacy of retrobulbar injection of Retina suis Injeel, Solanum compositum and Ubichinon compositum - Biologische Heilmittel Heel GmbH Baden-Baden, Germany - (one peribulbar injection a week for 10 weeks) combined with oral administration of low-dose SKA growth factors (0.01 pg/mL) **GUNA®-NT3, GUNA®-NT4 and GUNA®-NGF - Guna S.p.a. - Milan, Italy** - (15 drops twice daily for a continuous six-month period).

MEDICINAL PRODUCTS USED IN THE STUDY PROTOCOL AND THEIR SCOPE

- **Retina suis Injeel:** used in the homotoxicological field to treat choroiditis in sight disorders, myopia and degenerative retinal disease.
- **Solanum compositum:** used in homotoxicological medicine to stimulate blood supply.
- **Ubichinon compositum:** used in homotoxicological medicine to restore mitochondrial respiration (in particular, oxidative phosphorylation) to reduce the production of ROS (quenching effect due to the presence of low-dose quinones).
- **GUNA®-NT3; GUNA®-NT4; GUNA®-NGF:** used in this protocol to improve visual function in subjects with neuroretinal diseases.

INTRODUCTION

Retinal atrophy constitutes a dramatic unmet therapeutic need and no established therapeutic approach able to initiate the restoration of sight is known.

The role of neurotrophins deserves a special mention: in addition to their known and documented role as trophic factors required for neuronal survival (in developing and adult brains) and their involvement in neurodegenerative processes (in the adult brain and cerebral ageing), neurotrophins exert a multitude of actions associated with synaptic activity and plasticity phenomena.
The study involved the evaluation of patients aged between 18 and 70 years of age, all of whom had a severe form of central or peripheral retinal atrophy, as documented by OCT (Optical Coherence Tomography) (**FIG. 1**).

The study involved the use of microperimetry (Nidek's MP1) and electroretinography according to International Society for Clinical Electrophysiology of Vision (ISCEV) standards, in order to avoid subjectivity phenomena in patients at the first visit (T0) and after 6 months' therapy (T6).

At T0 and at T6, patients underwent MP1 microperimetry MP1 and ISCEV-standard foveal electroretinography. At the start of the study, subjects had a full ophthalmological examination with medical history and an OCT (**FIG. 2**), which was subsequently repeated as an end-of-study assessment.

Fluorangiography was used in those cases in which it could play a documentary or diagnostic role. The same techniques were used to examine patients with heredodegenerative forms, but focussing on the areas involved.

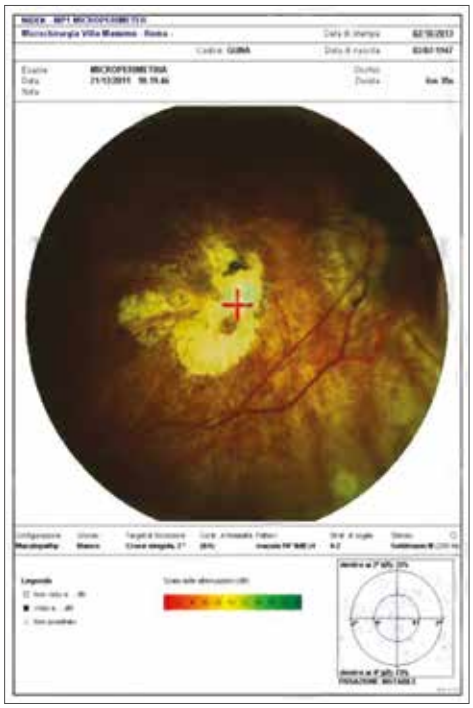


FIG. 1
EXAMPLE OF A RETINOGRAM
SHOWING THE AREA
OF MACULAR ATROPHY

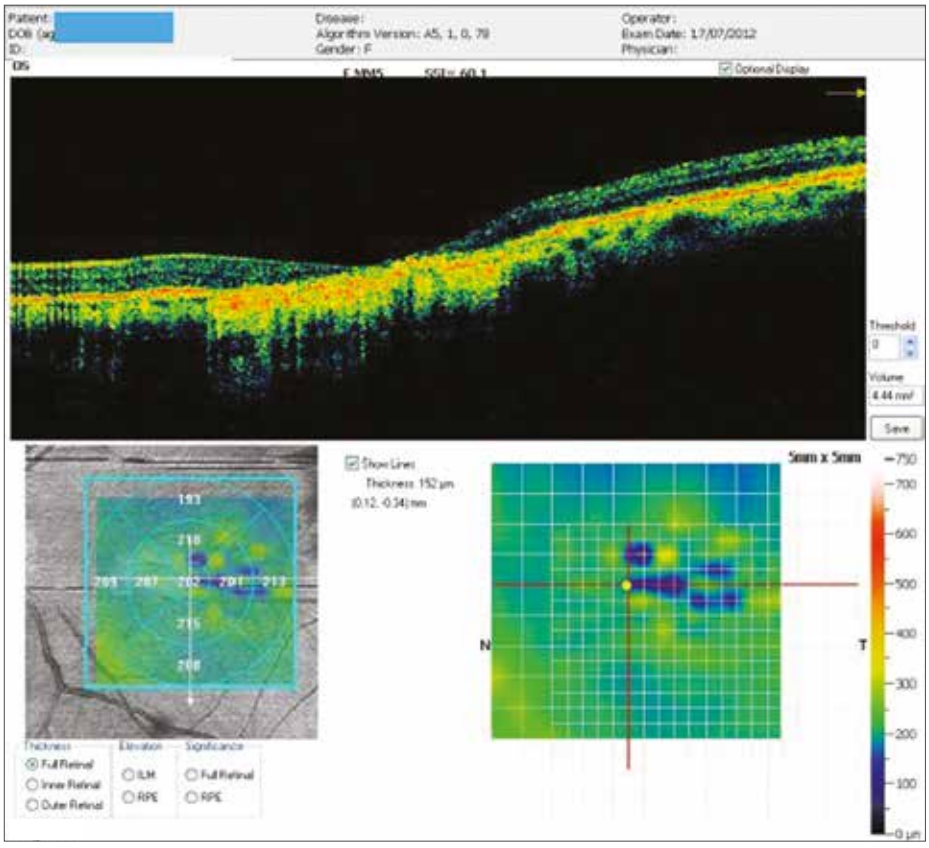


FIG. 2
EXAMPLE OF OPTICAL COHERENCE TOMOGRAPHY SHOWING CONSIDERABLE CELL LOSS
IN THE MACULAR AREA. THE MACULAR THINNING IS SHOWN IN BLUE ON THE THICKNESS MAP

RESULTS

The protocol's entire therapeutic arrangement pursued the following objectives:

- **reactivation of the microcirculation** with activation of amorphous scar tissue removal processes
- **reactivation of the enzymatic processes**
- **regeneration of the specific tissue**

At the end of treatment, 29 eyes (15 in male subjects and 14 in female subjects) were evaluated. The statistical analysis of the results made it possible to observe the considerable statistical significance in the increases in response obtained after the treatment period.

The microperimetry data showed that (FIGS. 3-4):

- **68.18% of eyes had improved**
- 22.7% were unchanged
- 9.09% had worsened

The ERG showed an increase in wave negativity (increase in cone response) in 82.1% of the eyes examined.

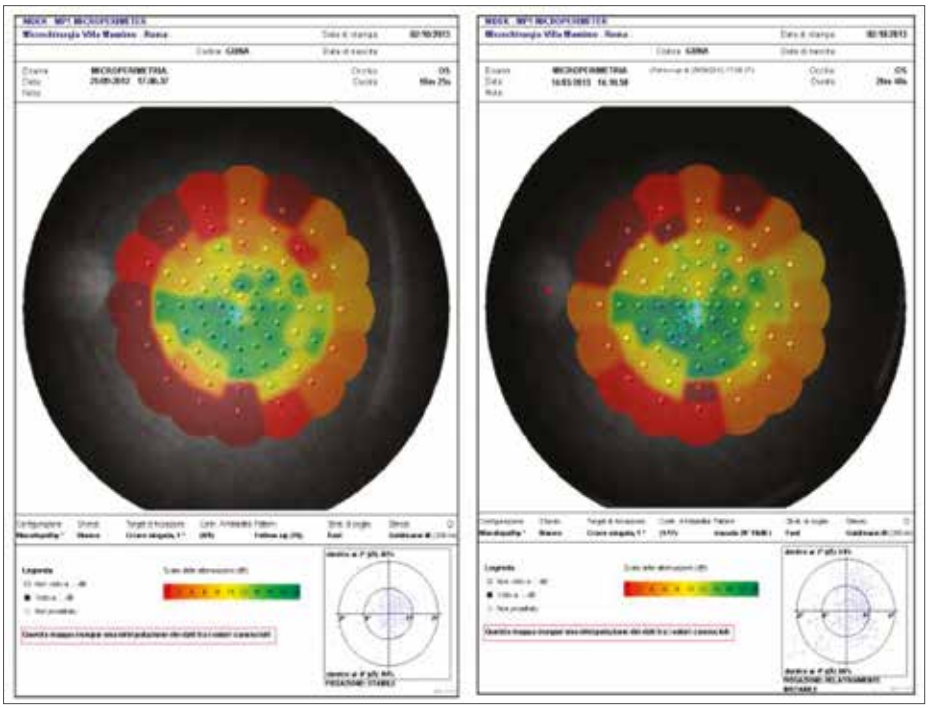


FIG. 3
EXAMPLE OF MICROPERIMETRY WITH DIFFERENTIAL MAP IN A PATIENT WITH ATROPHIC MACULOPATHY

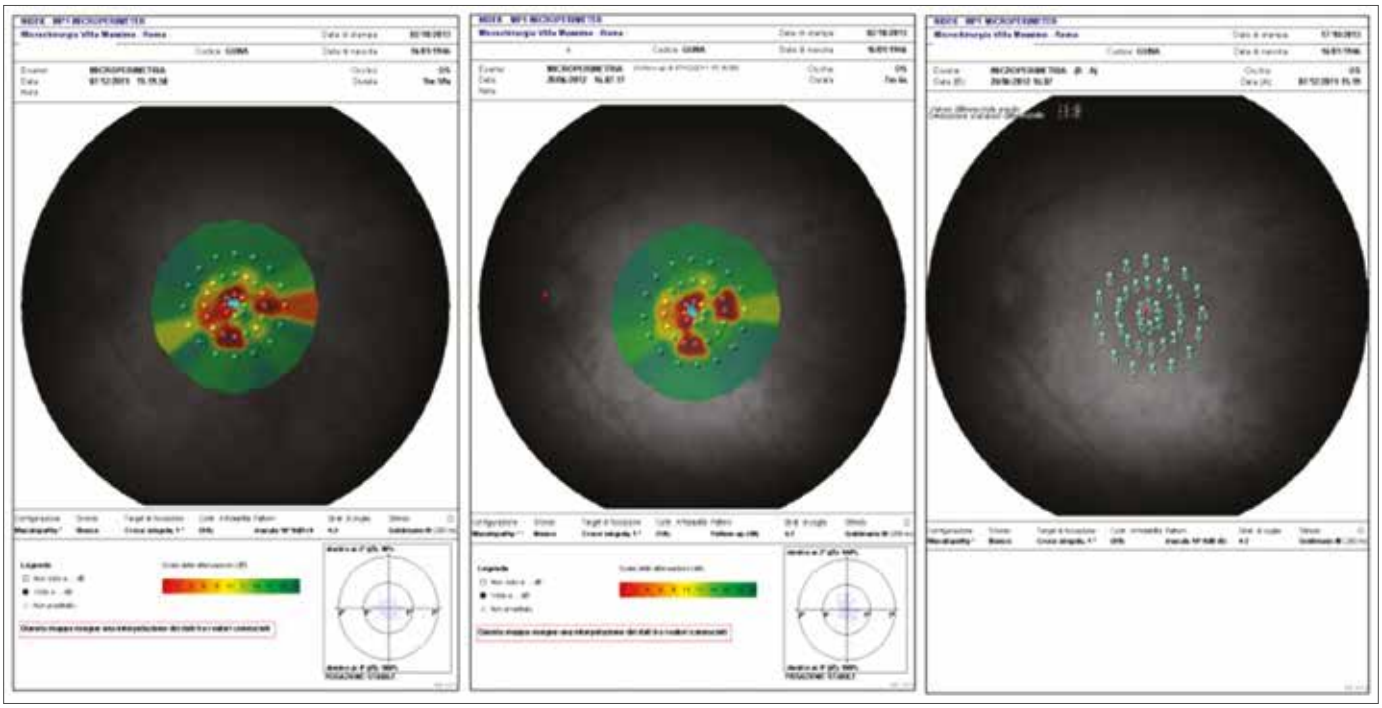


FIG. 4
EXAMPLE OF MICROPERIMETRY IN PATIENT WITH PIGMENTARY RETINOPATHY (TIME 0 AND END-OF TREATMENT ASSESSMENT).

CONCLUSIONS

Despite the limited size of the sample and a certain difficulty of administration, the very promising results make **Low-Dose Medicine treatments the most interesting option for neuroretinal cell regeneration.** The complete absence of alternative treatment options also allows us to consider these treatments the therapy of election in these difficult cases.

Outside the study, it was observed that six months after discontinuation of the therapy described, 10 out of 12 of the re-assessed eyes showed a partial clinical regression, whereas, those subjects who chose to continue treatment after one year maintained valid instrumental response after discontinuation, even in the long-term, thereby demonstrating that the retinal tissue is able to self-maintain a regenerative trend.

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Luchetti P. Incremento della funzionalità visiva in soggetti con atrofia retinica sottoposti a trattamento con farmaci della medicina low dose. Studio osservazionale monocentrico retrospettivo. Minerva Oftalmologica 2014;56(3-4):53-61.

GUNA HIGHLIGHTS

Oncology

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, 3, 337-342
doi:10.4236/jct.2012.324044 Published Online September 2012 (http://www.SciRP.org/journal/jct)

Scientific Research

Low Dose of IL-12 Stimulates T Cell Response in Cultures of PBMCs Derived from Non Small Cell Lung Cancer Patients*

Lucia D'Amico[1], Enrico Ruffini[2], Riccardo Ferracini[3], Ilaria Roato[1#]

[1]CeRMS (Center for Research and Medical Studies), A.O. Città della Salute e della Scienza di Torino, Torino, Italy; [2]Department of Toracic Surgery, A.O. Città della Salute e della Scienza di Torino, Torino, Italy; [3]Department of Orthopaedics, A.O. Città della Salute e della Scienza di Torino, Torino, Italy.
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FOREWORD

The research project that concluded with the publication titled ***"Low dose of IL-12 stimulates T cell response in cultures of PBMCs derived from Non-Small Cell Lung Cancer Patients"*** originated from the decision by a group of researchers belonging to CeRMS (Center for Research and Medical Studies), Dipartimento di Chirurgia Toracica [Department of Thoracic Surgery] and Dipartimento di Ortopedia [Department of Orthopaedics], A.O. Città della Salute e della Scienza di Torino, to investigate the action of low-dose molecules in the oncology field.

In the past, the handling difficulties and severe dose-dependent side effects of molecules such as cytokines has prevented their clinical use. The possibility of a therapeutic potential through the use of very low concentrations of IL-12 aroused the researchers' enthusiasm and made this project possible.

This research demonstrated, in vitro, the pharmacomodulatory activity of low-dose SKA IL-12 **(GUNA®-IL 12. Manufactured by Guna S.p.a. - Milan, Italy)** on the Immune System, and on T cell populations in particular, in peripheral blood mononuclear cells (PBMCs) derived from **Non-Small Cell Lung Cancer patients.**

The results obtained make it possible to design further studies, in order to develop new therapeutic strategies for the treatment of cancer and many other immunodeficiency disorders.

INTRODUCTION

Lung cancer is the first cause of cancer death worldwide. The most common type is Non-Small Cell Lung Cancer, whose prognosis is often unfavourable.

Tumour-induced immune tolerance suppresses certain specific defence mechanisms, it down-regulates the activity of $CD4^+$ T helper (Th) cells and causes an imbalance in the cytokines they produce, leading to disease evolution.

IL-12 is an important immunoregulatory cytokine that exerts potent antitumour activity, by inducing the differentiation of naive T cells into Th1 cells. Th1 polarization promotes antitumour immunity through the expression of pro-inflammatory cytokines, including IFN-γ, cytotoxic $CD8^+$ T cell expansion and NK cell activation (FIG. 1).

The aim of this study was to evaluate the ability of low-doses of IL-12 (10 ng/ml; 1 pg/ml; 0.01 pg/ml) to modulate T cell subpopulations in cultures of peripheral blood mononuclear cells (PBMCs) derived from subjects with NSCLC.

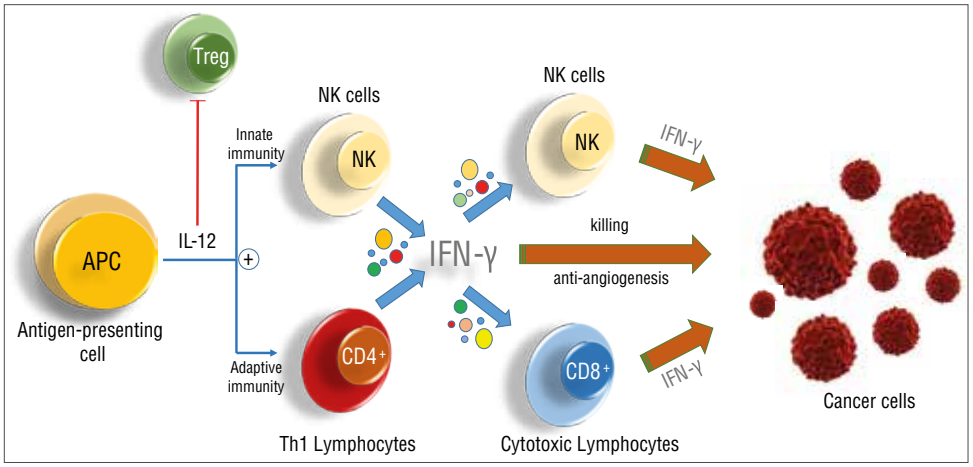


FIG. 1

RESULTS

LOW-DOSE IL-12 SUPPRESSES THE ACTIVITY OF REGULATORY T CELLS

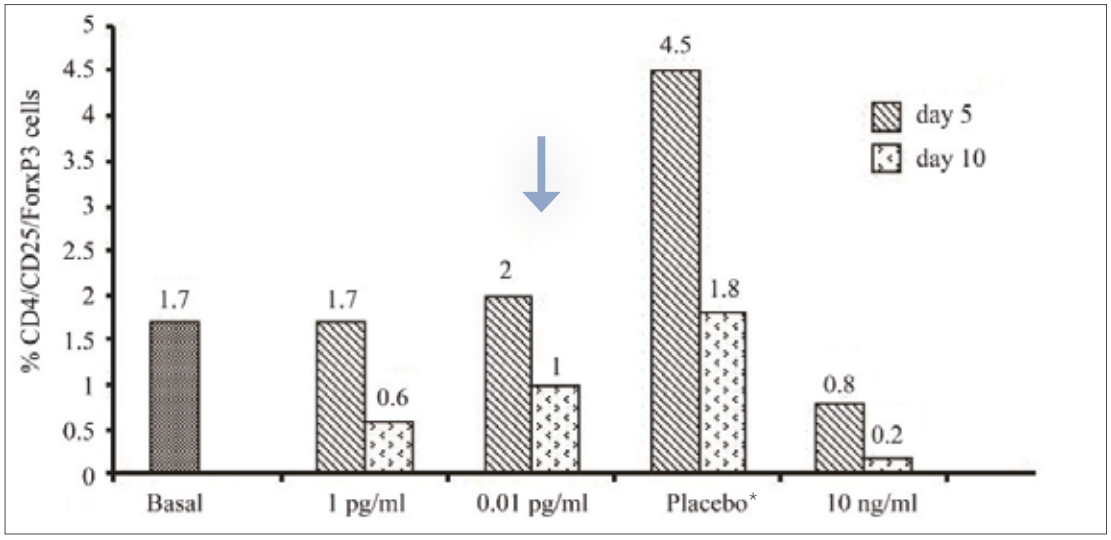


FIG. 2

* *placebo = non-treated*

The histogram shows regulatory T cell modulation during culture with and without IL-12. The number above the bar represents the mean plus standard deviation. **Regulatory T cells do not increase after stimulation with IL-12 at any dose and they decrease at the tenth day of culture (FIG. 2).**

LOW-DOSE IL-12 STIMULATES CD4⁺ T CELLS IN PBMC CULTURES

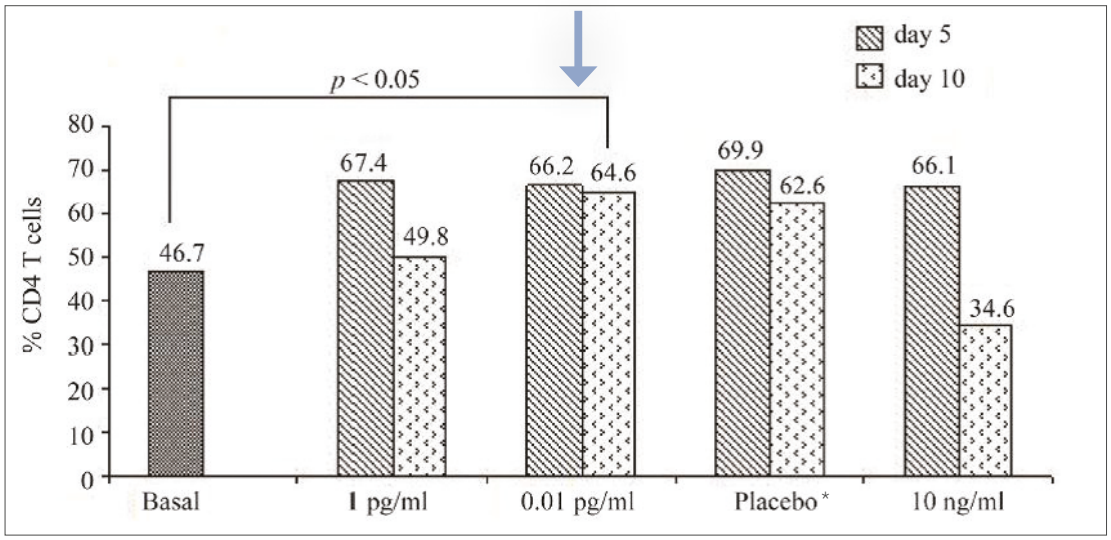


FIG. 3

* *placebo = non-treated*

The histogram shows the percentage of CD4⁺ T cells at baseline and after 5 and 10 days in the various culture conditions. The number above the bar represents the mean plus standard deviation. At day 5, there is no significant difference between the different culture conditions. **On day 10, the number of CD4⁺ T cells remains significantly high with low-dose SKA IL-12 at a concentration of 0.01 pg/ml (Guna S.p.a. - Milan, Italy) compared to the baseline (FIG. 3).**

LOW-DOSE IL-12 INCREASES THE IFN-γ PRODUCED BY THE CD4+ T CELLS

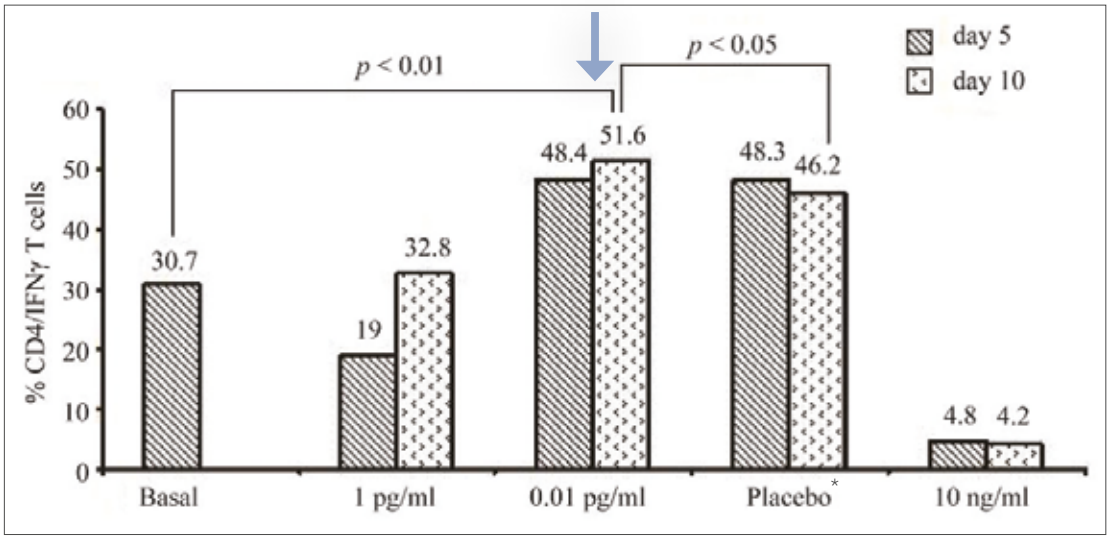


FIG. 4

* *placebo = non-treated*

The CD4+/IFN-γ produced by T cells increases after stimulation Low-dose SKA **IL-12 at the concentration of 0.01 pg/ml** (**Guna S.p.a. - Milan, Italy**) compared to the baseline. Furthermore, **at day 10, CD4+/IFN-γ remains significantly high with 0.01 pg/ml of IL-12**, as it does with placebo, whereas the other concentrations depress CD4+/ IFN-γ levels. The number on the bar represents the mean plus standard deviation (**FIG. 4**).

LOW-DOSE IL-12 INDUCES THE LYSIS OF H1373 CELLS BY CYTOTOXIC CD8+ T CELLS

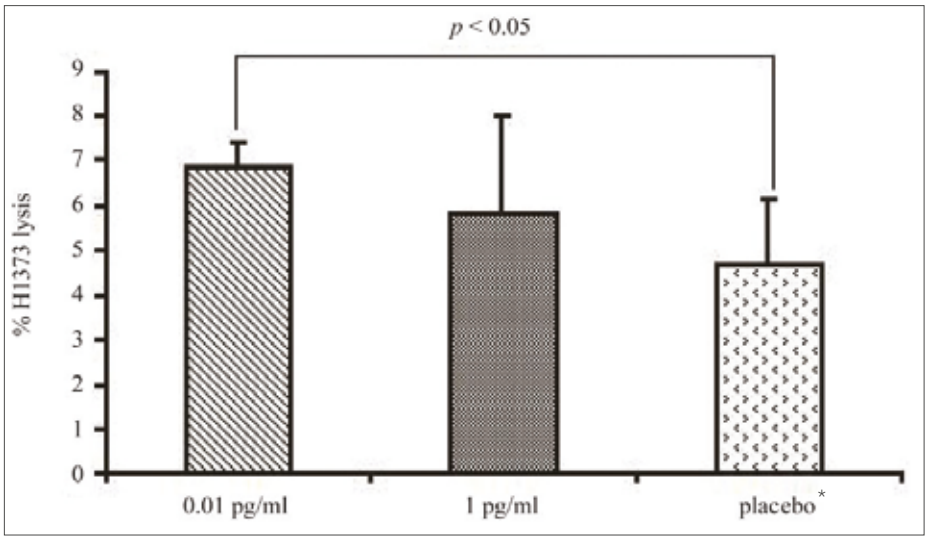


FIG. 5

* *placebo = non-treated*

The histogram shows the mean value of H1373 cell lysis by PBMCs isolated from 5 subjects with NSCLC.
IL-12 at 0.01 pg/ml increases the lysis of H1373 cells induced by PBMCs (FIG. 5).

CONCLUSIONS

This study shows that treating cultures of PBMCs derived from subjects with NSCLC with low-dose SKA IL-12 allows to modulate T cell subpopulations.

More specifically, the following were observed:

- an increase in $CD4^+$ Th1 cells and cytotoxic $CD8^+$ T cells
- an increase in IFN-γ as a consequence of the increase in $CD4^+$ Th1 cells
- inhibition of Treg cell response

This action of low-dose SKA IL-12 translates into an inhibition of the proliferation of pulmonary adenocarcinoma cells in vitro.

FUTURE HEALTH SUMMIT

THE RESULTS OF THIS STUDY WERE PRESENTED DURING

"FUTURE HEALTH SUMMIT"

Dublin (IE), May 26th, 2016

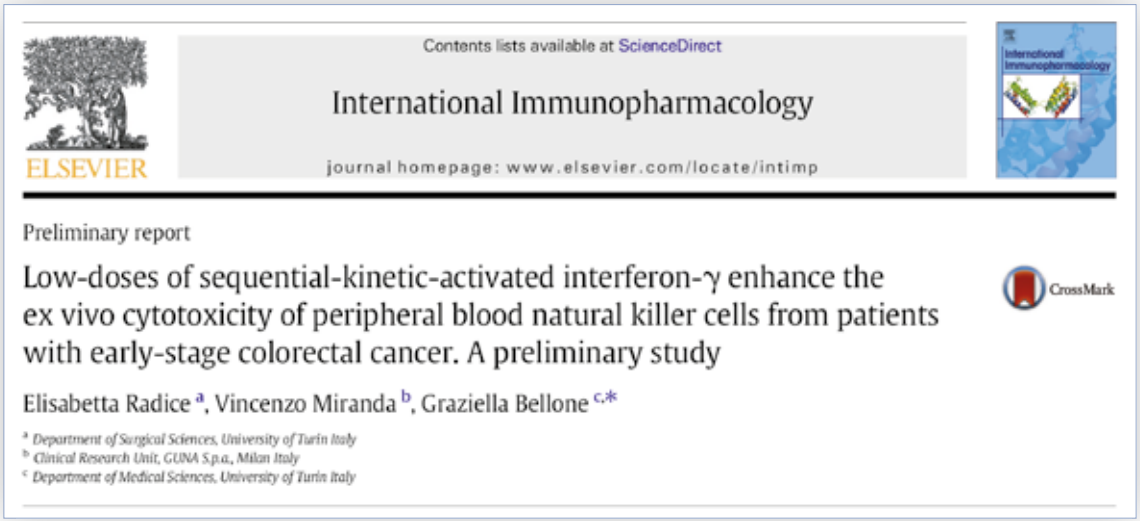
BIBLIOGRAPHICAL REFERENCE

D'Amico L, Ruffini E, Ferracini R, Roato I. 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;3:337-342.

GUNA HIGHLIGHTS

Oncology

LOW-DOSES OF SEQUENTIAL-KINETIC-ACTIVATED INTERFERON-γ ENHANCE THE EX VIVO CYTOTOXICITY OF PERIPHERAL BLOOD NATURAL KILLER CELLS FROM PATIENTS WITH EARLY-STAGE COLORECTAL CANCER. A PRELIMINARY STUDY



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Preliminary report

Low-doses of sequential-kinetic-activated interferon-γ enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study

Elisabetta Radice [a], Vincenzo Miranda [b], Graziella Bellone [c,*]

[a] Department of Surgical Sciences, University of Turin Italy
[b] Clinical Research Unit, GUNA S.p.a., Milan Italy
[c] Department of Medical Sciences, University of Turin Italy

FOREWORD

The scientific publication titled ***"Low-doses of sequential-kinetic-activated interferon-γ enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study"*** was based on scientific grounds and the decision by researchers at Dipartimento di Scienze Chirurgiche [Department of Surgical Sciences] of Università di Torino, Dipartimento di Scienze Mediche [Department of Medical Sciences] of Università di Torino and the Clinical Research Unit of Guna S.p.a in Milan. The aim was to investigate the activity of biological molecules with sub-nanomolar concentrations in the oncology field.

This research, which lasted approximately two years, demonstrated the pharmacological activity of low-dose SKA Interferon-γ on the Immune System, in particular on the increase in the cytotoxic activity of the natural killer (NK) cells of subjects with colorectal cancer.

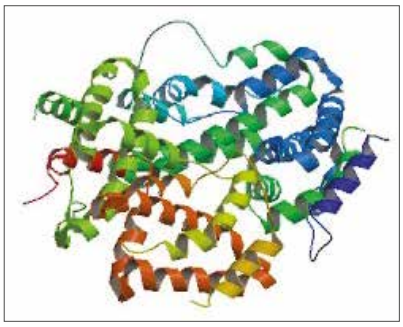

FIG. 1
HUMAN IFN-Y

The use of IFN-γ has always met with obstacles in clinical practice due to the difficulties handling it and its severe dose-dependent toxic effects. However, in the cancer field, this molecule represents a potent tool for fighting the disease. The current possibility of being able to use low-dose SKA Interferon-γ (**GUNA®-IFN GAMMA - Guna S.p.a. - Milan, Italy**) made this research project possible (**FIG. 1**).

The results of this research encourage researchers to devise new treatment strategies for colorectal cancer and for the treatment of many other immunodeficiency disorders.

INTRODUCTION

In vivo and *in vitro* studies have shown that NK cells are able to eliminate malignant cells without the classic restriction of the Major Histocompatibility Complex (MHC) and therefore play a crucial role in tumour immunosurveillance. NK cells are able to release cytokines, including Interferon-γ (IFN-γ), which promotes early inflammatory response and supports adaptive immune response.

Like IL-12, IFN-γ is able to:
- **inhibit the proliferation of Th2 cells;**
- **promote Th1 antitumour response;**
- **initiate specific cytotoxic response ($CD8^+$ T cells) to the tumour.**

In addition, IFN-γ is able to induce:
- **MHC-I in tumour cells;**
- **enhance their immunogenicity;**
- **indirectly increase their angiostasis;**
- **directly cause an antiproliferative and pro-apoptotic tumour cell response (FIG. 2).**

This potent immunoregulatory action suggests it could be used in cancer therapy. However, its side effects restrict its clinical use.

This exploratory study uses NK cells obtained from the peripheral blood of healthy donors and patients with nonmetastatic and metastatic colorectal cancer (CRC) in order to discover, in an ex-vivo model, whether exposure to **low-dose SKA IFN-γ (10 fg/ml)** is able to boost the antitumour activity of these cells, by comparing this response to that obtained by exposing NK cells to a standard dose of recombinant IFN-γ (rIFN-γ) (1ng/ml).

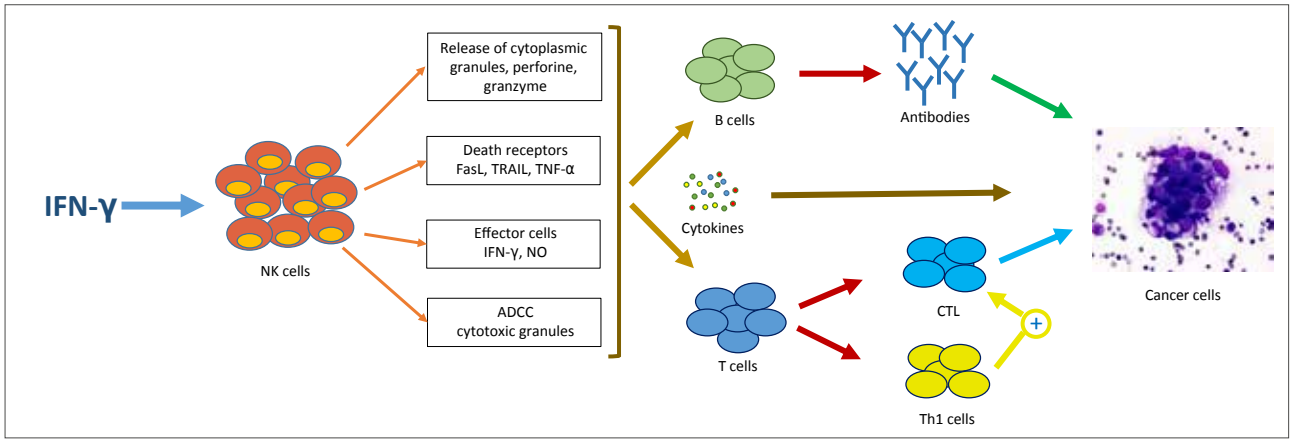

FIG. 2
IFN-γ AND NK CELLS IN TUMOUR IMMUNOSURVEILLANCE

The activity of the NK cells obtained from the peripheral blood (PB-NK) of healthy donors, patients with nonmetastatic CRC and patients with metastatic CRC was evaluated against target Myeloid Leukaemia (K562), Epithelial Colorectal Adenocarcinoma (Caco-2) and Colon Adenocarcinoma (HT-29) cell lines.

NK cell activity was expressed numerically in lytic units 30% (LU_{30}). LU_{30} represents the number of effectors (NK) required to produce 30% specific toxicity against $5x10^3$ target cells.

RESULTS

NK CELL ACTIVITY IN HEALTHY DONORS AND IN PATIENTS WITH CRC

- The PB-NK cells obtained from healthy donors and from both groups of patients with CRC are able to destroy CRC Caco-2 and HT-29 cells, albeit to a lesser extent than for K562 cells (**FIG. 3**).

These results show that the cytolytic mechanisms of PB-NK cells are almost completely preserved in the early stages of CRC (nonmetastatic CRC patients), but tend to lose efficacy as the disease progresses (metastatic CRC patients).

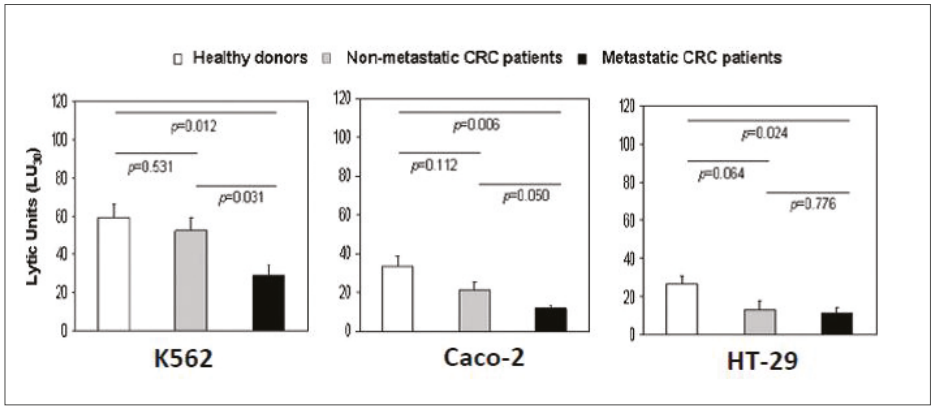

FIG. 3
NK CELL ACTIVITY IN HEALTHY DONORS AND PATIENTS WITH CRC

NK CELL ACTIVITY AFTER STIMULATION WITH SKA IFN-γ SKA AND RIFN-γ IN HEALTHY DONORS AND PATIENTS WITH NONMETASTATIC AND METASTATIC CRC IN THE THREE TARGET CELL LINES K562, CACO-2 AND HT-29

- The pre-treatment of PBL from healthy donors with low-dose SKA IFN-γ or rIFN-γ causes a significant increase in NK cell cytotoxicity compared to the control (FIG. 4-A).
- The pre-treatment of PBL from patients with nonmetastatic CRC with low-dose SKA IFN-γ causes a significant increase in NK cell cytotoxicity compared to the control, albeit to a lesser extent than that obtained with pre-treatment with rIFN-γ on the three cell lines (FIG. 4-B).
- Conversely, the PBL from patients with metastatic CRC, show a significant reduction in NK cell activity, with poor sensitivity to both treatment with rIFN-γ, and treatment with low-dose SKA IFN-γ (FIG. 4-C).

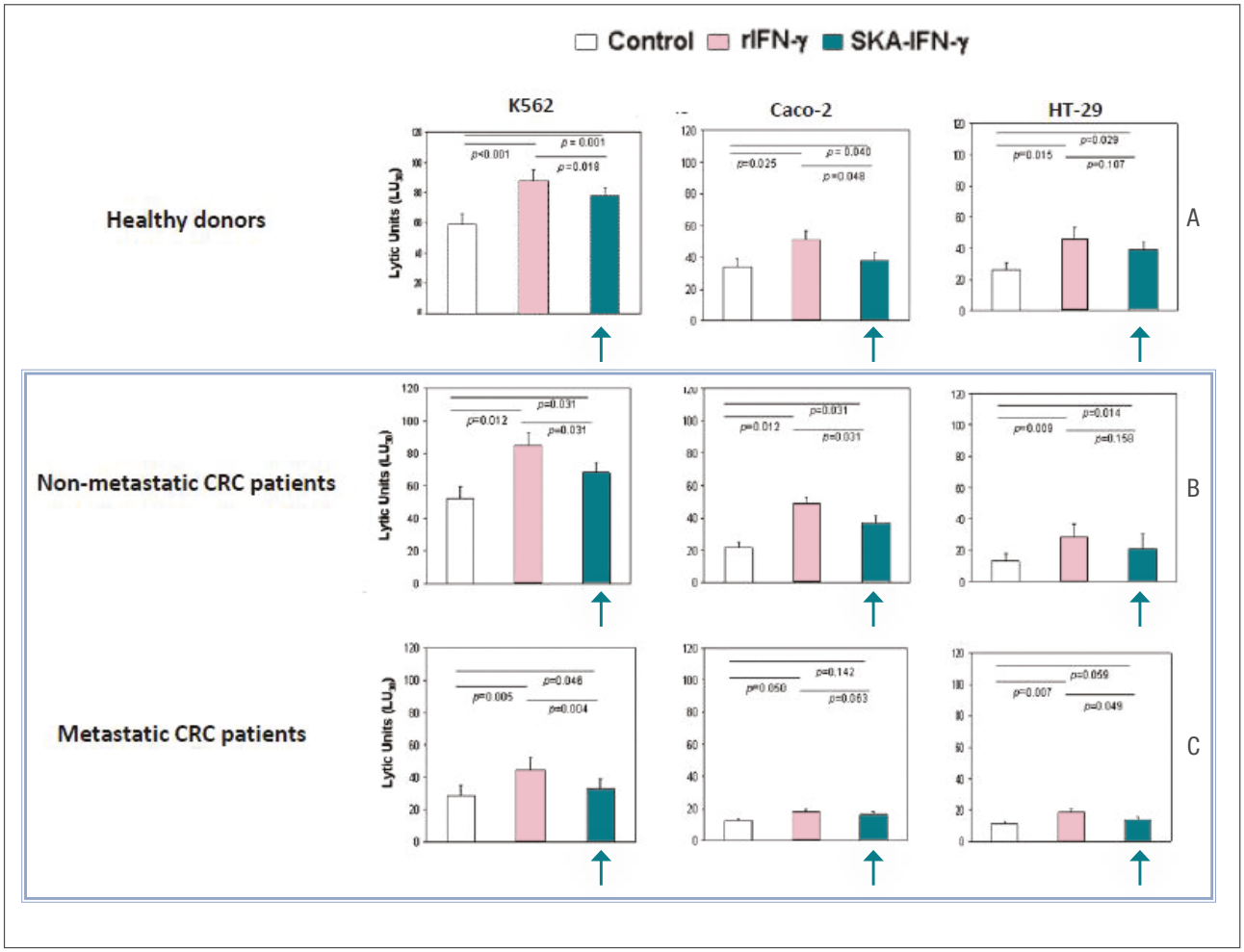

FIG. 4
NK CELL ACTIVITY AFTER STIMULATION WITH SKA IFN-γ AND RIFN-γ FOR HEALTHY DONORS, PATIENTS WITH NONMETASTATIC CRC AND PATIENTS WITH METASTATIC CRC IN THE THREE TARGET CELL LINES K562, CACO-2 AND HT-29

CONCLUSIONS

- The cytolytic capacity of NK cells from subjects with CRC is almost completely maintained in the early stages of the disease, but tends to lose efficacy at it progresses.
- Low-dose SKA IFN-γ makes it possible to take NK cells to a good degree of activation in the early stages of CRC, on a systemic and/or local level. However, this activation was not observed in the NK cells of subjects with advanced-stage disease, either after treatment with low-dose SKA IFN-γ or after treatment with rIFN-γ.

These results primarily show the immunomodulatory capacity of low-dose SKA IFN-γ and pave the way for the possibility of formulating a novel, safe and feasible therapeutic approach for increasing the killing activity of NK cells in patients with early-stage CRC.

THE RESULTS OF THIS RESEARCH STUDY WERE PRESENTED AT

3rd CONFERENCE OF TRANSLATIONAL MEDICINE ON PATHOGENESIS AND THERAPY OF IMMUNE-MEDIATED DISEASES

Milan (IT), September 29th - October 1st, 2014

BIBLIOGRAPHICAL REFERENCE

Radice E, Miranda V, Bellone G. Low-doses of sequential-kinetic-activated interferon-γ enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study Intern. Immunopharm 2014;19:66-73.

GUNA HIGHLIGHTS

Oncology

ENHANCEMENT OF THE IMMUNOSTIMULATORY FUNCTIONS OF EX VIVO-GENERATED DENDRITIC CELLS FROM EARLY-STAGE COLON CANCER PATIENTS BY CONSECUTIVE EXPOSURE TO LOW DOSES OF SEQUENTIAL-KINETIC-ACTIVATED IL-4 AND IL-12. A PRELIMINARY STUDY

Translational Oncology
www.transonc.com
Volume 8 Number 4 August 2015 pp. 327–338 327

Enhancement of the Immunostimulatory Functions of *Ex Vivo*–Generated Dendritic Cells from Early-Stage Colon Cancer Patients by Consecutive Exposure to Low Doses of Sequential-Kinetic-Activated IL-4 and IL-12. A Preliminary Study

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FOREWORD

The scientific publication titled ***"Enhancement of the Immunostimulatory Functions of Ex Vivo-Generated Dendritic Cells From Early-Stage Colon Cancer Patients by Consecutive Exposure to Low Doses of Sequential-Kinetic-Activated IL-4 and IL-12. A Preliminary Study"*** was the result of a study lasting over two years conducted by Dipartimento di Scienze Chirurgiche [Department of Surgical Sciences] of Università di Torino, by Dipartimento di Scienze Mediche [Department of Medical Sciences] of Università di Torino and by the Clinical Research Unit of Guna S.p.a in Milan.

The team of researchers showed, for the first time, the positive effects of the combined sequential use of sub-nanomolar concentration (low-dose), SKA-activated, IL-4 and IL-12 (GUNA S.p.a. - Milan, Italy) on the Immune System of patients with Colon Cancer.

The combined sequential use of these biological molecules had never been studied before by any international research group because of the impossibility of clinical application due to the severe negative side effects observed at pharmacological doses. The availability of these low-dose molecules produced by Sequential Kinetic Activation (SKA), made this project possible.

These first important results have opened a new frontier in the treatment of colon cancer and could, in the future, lead to a new, completely novel and side effect-free approach to the disease.

INTRODUCTION

Dendritic cells (DCs) have always been considered the most potent antigen-presenting cells (APCs). They are able to uptake, process and present different types of antigens to naive T cells.

Recent studies have shown that DCs have high functional plasticity which could translate into an immunostimulatory or immunosuppressive potential, or both, depending on the sequence and on the combination of microenvironmental stimuli, which affect their differentiation, maturation, activation and polarisation.

Because of their ability to coordinate all the elements of the Immune System and to express a specific immune response in tumour cells, DCs have for some time been considered a fundamental target for cancer therapy.

Cancer cells are able to escape the Immune System by means of various mechanisms, including: the inhibition of dendropoiesis, the inhibition of DC maturation, the induction of their apoptosis, the induction of DC "protumour growth" and the expression of immune checkpoints.
The functional properties of DCs depend on their state of maturation.
Only mature DCs express major histocompatibility complex type 2 (MHC-II) and costimulatory molecules and activate Th1 type immune response (FIG. 1).

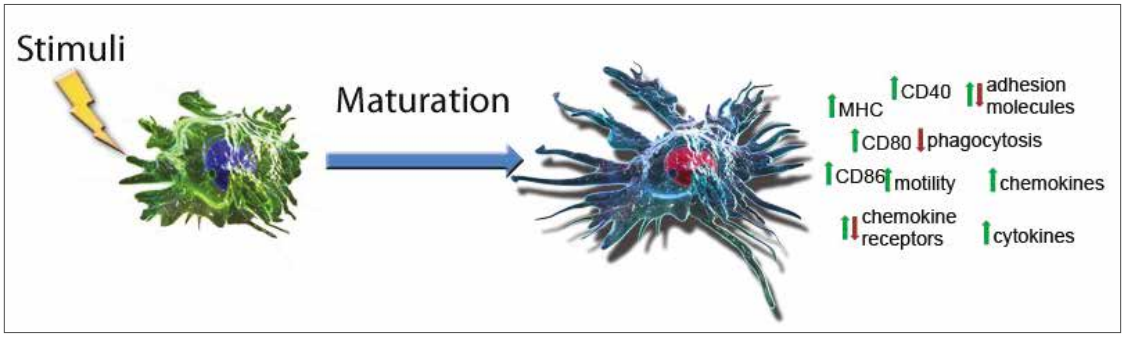
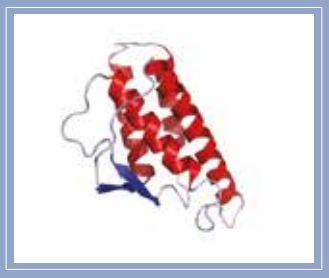
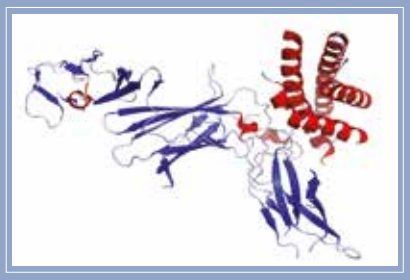


FIG. 1
DC MATURATION AND EFFECTOR MECHANISMS

This study compares low-dose IL-4 and IL-12 prepared according to the SKA method (GUNA®-IL 4, GUNA®-IL 12), with standard doses of the same recombinant human interleukins (rh) on the functional activity of DCs obtained from monocytes (MoDCs) generated *ex-vivo* from patients with primary colon cancer, patients in the metastatic phase and healthy donors (FIG. 2).



- **IL-4** is a DC maturation and growth factor with opposite effects depending on the time of application and concentration.



- **IL-12** is produced primarily by professional APCs, including monocytes/macrophages and DCs.
It stimulates Th1 differentiation and the production of IL-2, IFN-γ and TNF-α.
It induces and boosts cell-mediated immune response and cytotoxic activity (NK and $CD8^+$).

FIG. 2

Recent evidence suggests that immune type signals are able to "guide" DC maturation and activation (FIG. 3).

The MoDCs were exposed to SKA IL-4 (0.5 fg/ml) and to SKA IL-12 (2 fg/ml) individually in a combined sequential manner in parallel with rhIL-4 (50 ng/ml) and rhIL-12 (1 ng/ml).

The mixed lymphocyte reaction (MLR) was used to assess Immune System activation and T cell proliferation in response to the MoDCs. IL-12p70 and IFN-γ expression was also evaluated as an indicator of Th1 immune system activation.

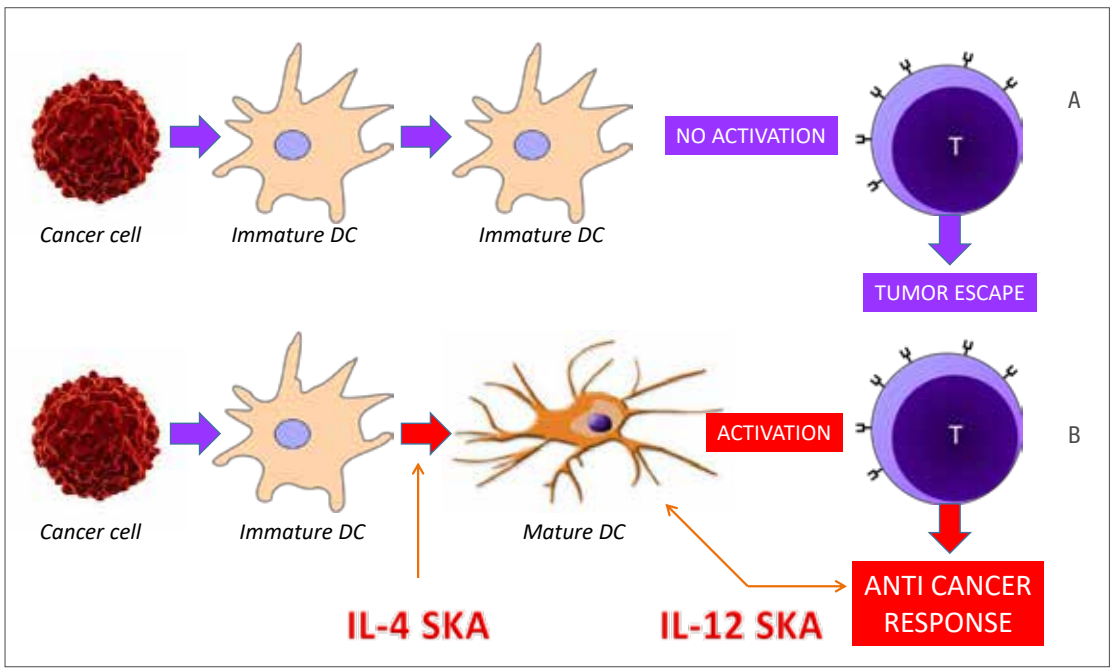


FIG. 3
DC ACTIVATION MECHANISMS

RESULTS

BASELINE STATUS OF MoDC ALLOSTIMULATORY ACTIVITY IN SUBJECTS WITH COLON CANCER AND HEALTHY DONORS

- After incubation with MoDCs deriving from subjects with Colon Cancer, naive T cells show a very low proliferative response (FIG. 4-A).
- After incubation with MoDCs deriving from healthy donors, naive T cells show a proliferative response about 10 times greater, showing the **marked functional defect of the MoDCs of the subjects with cancer** (FIG. 4-A).

BASELINE STATUS OF MoDC ALLOSTIMULATORY ACTIVITY IN SUBJECTS WITH METASTATIC AND NONMETASTATIC COLON CANCER

- After incubation with MoDCs deriving from subjects with Colon Cancer, naive T cells show a very low proliferative response that is significantly lower in metastatic subjects (FIG. 4-B).
- There is a **significant correlation between the MoDCs' antigen-presenting capacity and disease staging** (FIG. 4-B).

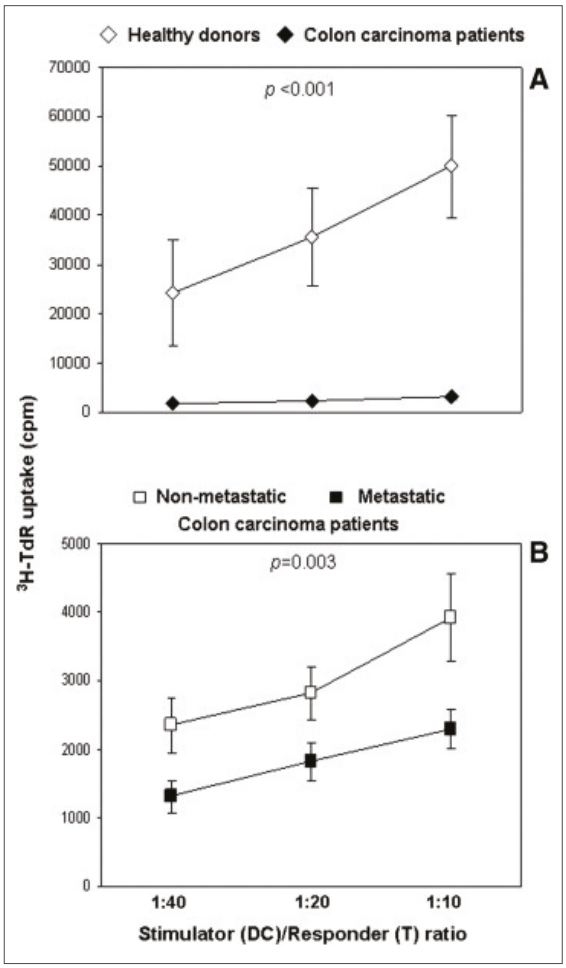


FIG. 4
MODC ALLOSTIMULATORY ACTIVITY IN SUBJECTS WITH COLON CANCER (METASTATIC AND NONMETASTATIC) AND IN HEALTHY DONORS

EFFECT OF PRE-TREATMENT WITH LOW-DOSE SKA IL-4 (48 HOURS) FOLLOWED BY EXPOSURE TO LOW-DOSE SKA IL-12 (24 HOURS) VS. STANDARD-DOSES OF RHIL-4 AND RHIL-12 ON MODC ALLOSTIMULATORY ACTIVITY IN SUBJECTS WITH COLON CANCER AND HEALTHY DONORS

- Exposing the MoDC of subjects with colon cancer to rhIL-4 or rhIL-12 significantly increases their functional activity compared to the untreated cells.
  Combined sequential treatment leads to more potent stimulation in these subjects (FIG. 5).
- When the MoDCs of the same subjects are treated with low-dose SKA IL-4 or low-dose SKA IL-12, there is no significant increase in their antigen-presenting capacity compared to the untreated cells, when the low-dose SKA cytokines are used individually.
  Sequential treatment with the same interleukins, on the other hand, leads to potent, near-significant stimulation (FIG. 5).
- **In healthy donors, MoDC exposure to low-dose SKA IL-4 or low-dose SKA IL-12 significantly increases their allostimulatory capacity compared to the control,** although rh interleukin has a greater effect (**FIG. 5**).
- **Combined sequential exposure of healthy donor MoDCs results in a significant increase in their functional capacity compared to the control, to SKA IL-4 and to SKA IL-12 (FIG. 5).**
- Combined sequential treatment with rhIL-4 and rhIL-12 enhances MoDC allostimulatory capacity in a marked manner compared to both the control and the cells treated with SKA IL-4/SKA IL-12 (**FIG. 5**).

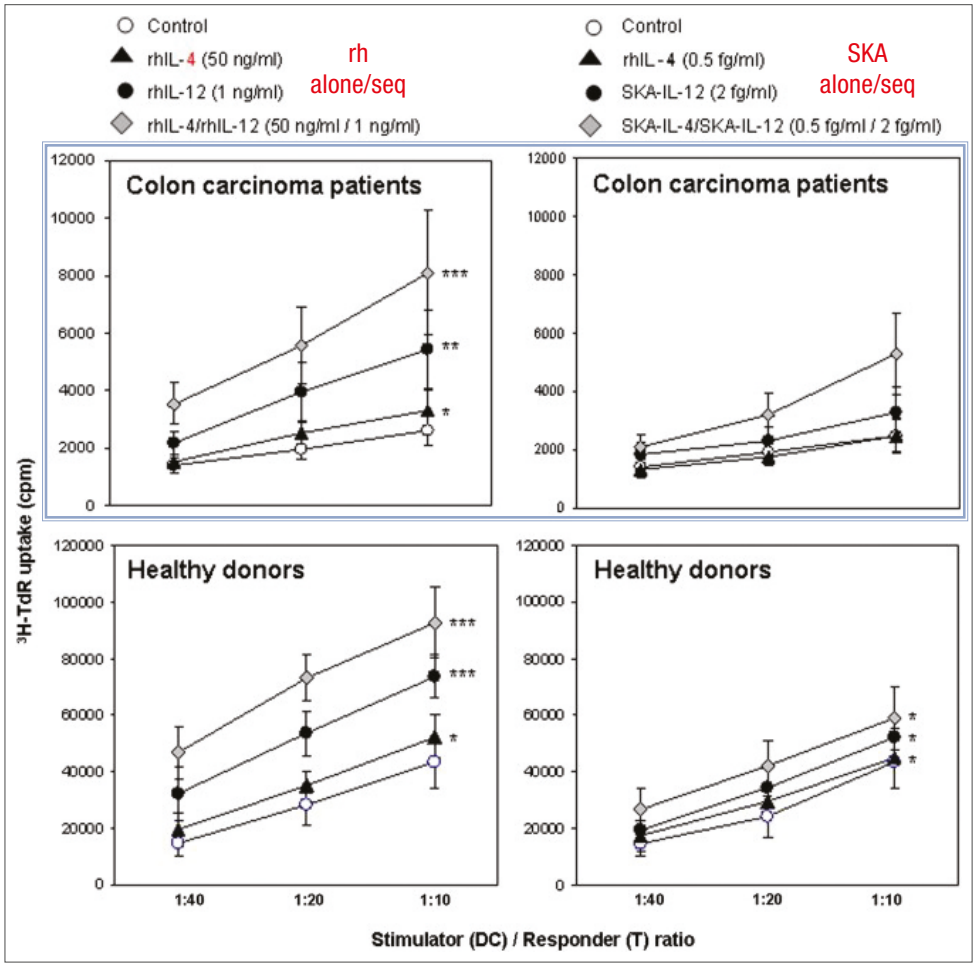


FIG. 5
EFFECT OF PRE-TREATMENT WITH LOW-DOSE SKA IL-4 (48 HOURS) FOLLOWED BY EXPOSURE TO LOWDOSE SKA IL-12 (24 HOURS) VS. STANDARD-DOSES OF RHIL-4 AND RHIL-12 ON MoDC ALLOSTIMULATORY ACTIVITY IN SUBJECTS WITH COLON CANCER AND HEALTHY DONORS

EFFECT OF THE DIFFERENT CYTOKINE PREPARATIONS, ADMINISTERED AS SINGLE AGENTS AND AS A COMBINED SEQUENTIAL TREATMENT, ON THE ALLOSTIMULATORY ACTIVITY OF THE MoDCs OF SUBJECTS WITH NONMETASTATIC AND METASTATIC COLON CANCER

Exposing the MoDCs of subjects with non-metastatic colon cancer to rhIL-4, rhIL-12 or the rhIL-4/rhIL-12 combined sequential treatment significantly enhances their functional activity compared to the untreated cells.

Conversely, the functional activity of the MoDCs of subjects with metastatic Colon Cancer only increases after exposure to rhIL-4 followed by rhIL-12, and nevertheless to a far lesser extent than in nonmetastatic subjects.

Combined sequential exposure to low-dose SKA IL-4 and low-dose SKA IL-12 of the MoDCs of subjects with nonmetastatic colon carcinoma, but not those of metastatic subjects, induces a significant activatory response compared to controls, despite not achieving the same response induced in healthy subjects.

The same SKA interleukins used as single agents did not have any significant effect on the functional activity of MoDCs in subjects with nonmetastatic colon cancer.

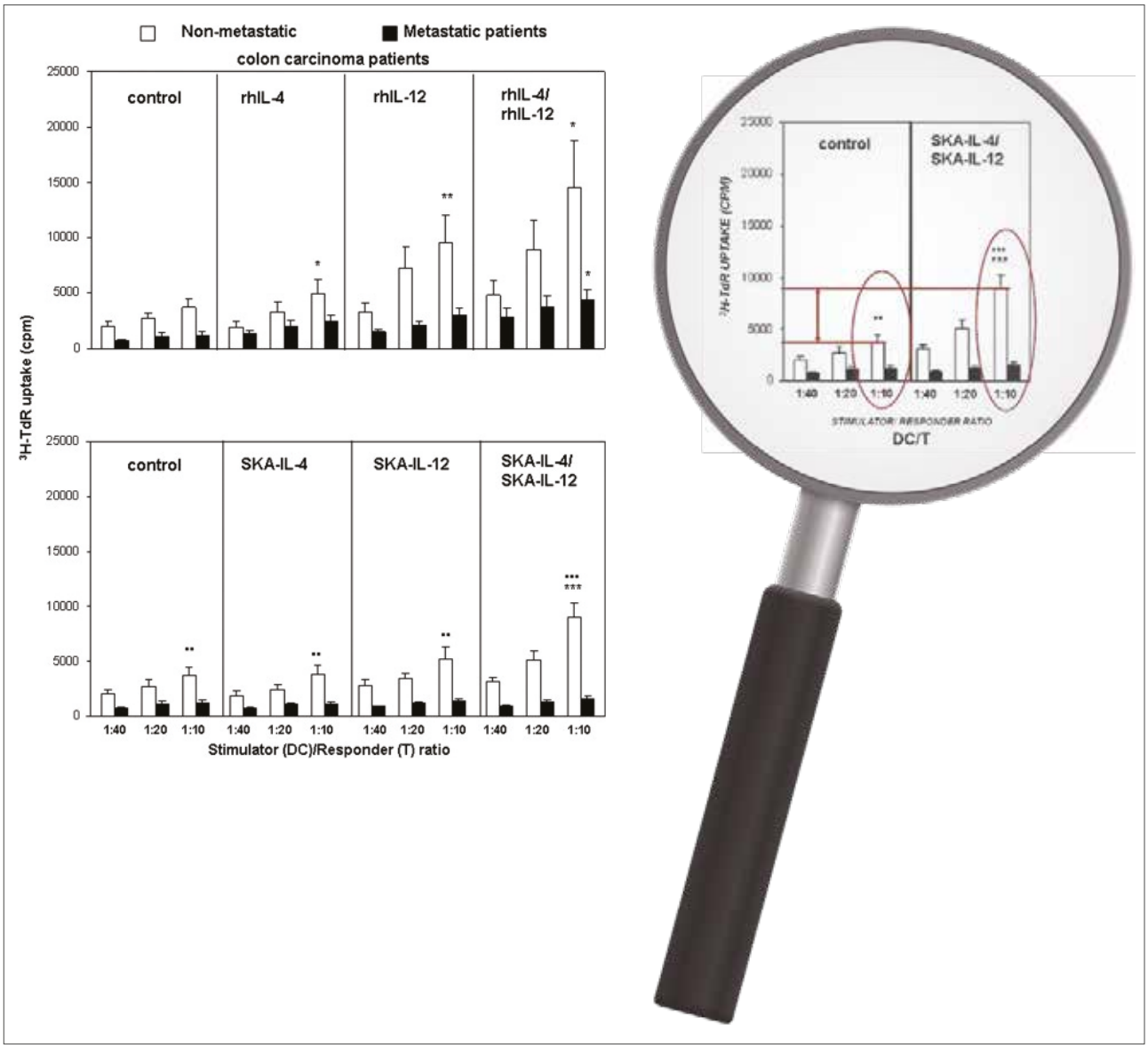


FIG. 6
EFFECT OF THE DIFFERENT CYTOKINE PREPARATIONS, ADMINISTERED AS SINGLE AGENTS AND AS COMBINED SEQUENTIAL TREATMENTS, ON THE ALLOSTIMULATORY ACTIVITY OF MoDCs IN SUBJECTS WITH NONMETASTATIC AND METASTATIC COLON CANCER

INDUCTION OF RECEPTOR COMPLEX IL-12P70 AND OF THE EXPRESSION OF IFN-γ IN MoDCs EXPOSED TO:
» rhIL-4 AND rhIL-12 AS SINGLE AGENTS AND COMBINED SEQUENTIAL TREATMENT
» LOW DOSE SKA IL-4 AND SKA IL-12 AS SINGLE AGENTS AND COMBINED SEQUENTIAL TREATMENT
IN SUBJECTS WITH NONMETASTATIC COLON CANCER AND HEALTHY DONORS

- RECEPTOR COMPLEX IL-12P70

HEALTHY DONORS

MoDCs of healthy donors cultivated with naive T cells produce negligible levels of biologically active IL-12p70, whereas **MoDC treatment with rhIL-4, rhIL-12 and, above all, with the combined sequential rhIL-4/rhIL-12 treatment induces a significant secretion of IL-12p70 compared to the control.**

A slight, non-significant increase in the production of IL-12p70 is observed in the MoDCs exposed to SKA IL-4 compared to the control. On the contrary, **MoDC exposure to SKA IL-12 as a single agent or in combined sequential SKA IL-4/SKA IL-12 treatment significantly increases IL-12p70 secretion compared to the control (FIG. 7-C).**

PATIENTS WITH NONMETASTATIC COLON CANCER

The MoDCs of patients with non-metastatic Colon Cancer respond in a less marked but significant manner compared to those of healthy donors when exposed to the rh interleukins.

Combined sequential low-dose SKA IL-4/IL-12 treatment alone induces a significant increase in IL-12p70 secretion compared to the control (FIG. 7-A).

- INTERFERON-γ

HEALTHY DONORS

The untreated MoDCs of healthy donors (control) brought into contact with naive T cells are unable to express relevant quantities of IFN-γ, whereas the treatment with rhIL-4, rhIL-12 or combined sequential rhIL-4/rhIL-12 induces a significant production of IFN-γ by T helper cells compared to the controls.

The same cells exposed to low-dose SKA IL-12 or to combined sequential low-dose SKA IL-4/IL-12 treatment (but not low-dose SKA IL-4 as a single agent) are able to induce the T helper cells to express a significant quantity of IFN-γ compared to controls (**FIG. 7-D**).

PATIENTS WITH NONMETASTATIC COLON CANCER

The MoDCs of patients with non-metastatic colon cancer only partially recover, compared to healthy donors, their capacity to induce the production of IFN-γ by T helper cells when exposed to rhIL-4, rhIL-12 or combined sequential rhIL-4/rhIL-12 compared to the controls.

Exposing MoDCs to SKA interleukins has a slight but statistically significant positive impact on the secretion of IFN-γ by the T helper cells, which supports the evidence that MoDCs, when activated by the combined sequential treatment with IL-4 and IL-12, are able to promote the differentiation of naive T cells into T helper cells that produce and express IFN-γ (**FIG. 7-B**).

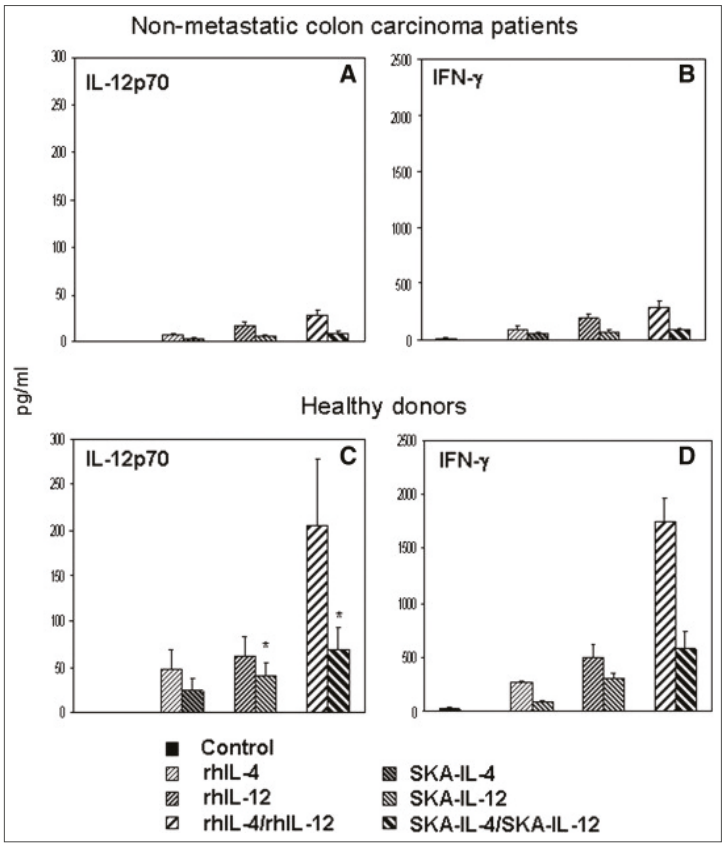


FIG. 7
INDUCTION OF RECEPTOR COMPLEX IL-12P70 AND EXPRESSION OF IFN-γ IN MODCS EXPOSED TO:
- rhIL-4, rhIL-12 AS SINGLE AGENTS AND IN COMBINED SEQUENTIAL TREATMENT
- SKA IL-4 AND SKA IL-12 AS SINGLE AGENTS OR IN COMBINED SEQUENTIAL TREATMENT

IN SUBJECTS WITH NONMETASTATIC COLON CANCER AND HEALTHY DONORS

The DC differentiation and maturation process is complex and one in which IL-4 and IL-12 play a key role; however, the effects of these two cytokines appear to be dramatically different depending on whether they are used at pharmacological doses or low-doses (FIG. 8).

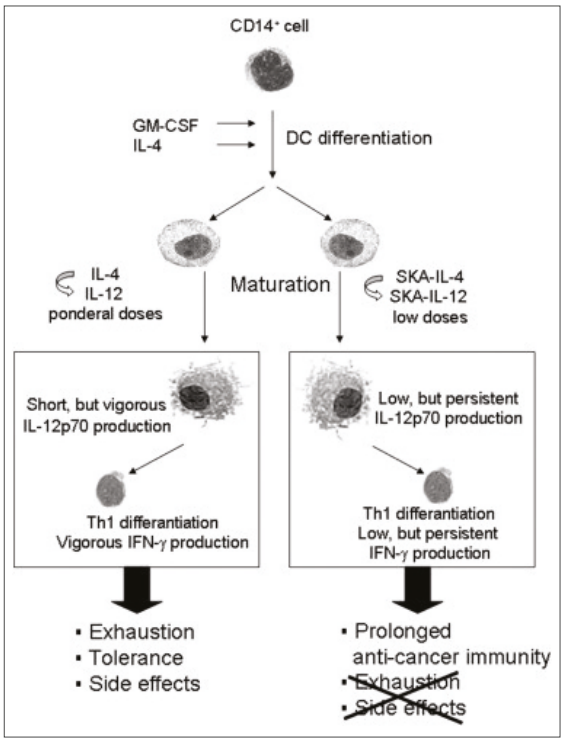


FIG. 8
EFFECTS OF DIFFERENT CONCENTRATIONS OF IL-4 AND IL-12 ON DC DIFFERENTIATION AND MATURATION

CONCLUSIONS

Compared to combined sequential treatment with pharmacological concentrations of rhIL-4 and rhIL-12, which are inapplicable from a therapeutic standpoint, **combined sequential MoDC exposure to low-dose SKA IL-4 and SKA IL-12 leads to a finer activation and regulation of the Immune System, and in particular to:**

- **PROLONGED ANTI-TUMOUR ACTIVITY**
- **ABSENCE OF CELL EXHAUSTION**
- **ABSENCE OF TOLERANCE**
- **ABSENCE OF ADVERSE EFFECTS**

THE RESULTS OF THIS RESEARCH WERE PRESENTED AT

3rd WORLD CONGRESS ON PHARMACOLOGY

Birmingham (UK), August 8-10, 2016

BIBLIOGRAPHICAL REFERENCE

Radice E, Bellone G, Miranda V. Enhancement of the Immunostimulatory Functions of Ex Vivo-Generated Dendritic Cells from Early-Stage Colon Cancer Patients by Consecutive Exposure to Low Doses of Sequential-Kinetic-Activated IL-4 and IL-12. A Preliminary Study. Transl Oncol. 2015 Aug;8(4):327-38.

GUNA HIGHLIGHTS
Rheumatology

AN OPEN RANDOMIZED ACTIVE-CONTROLLED CLINICAL TRIAL WITH LOW-DOSE SKA CYTOKINES VERSUS DMARDs EVALUATING LOW DISEASE ACTIVITY MAINTENANCE IN PATIENTS WITH RHEUMATOID ARTHRITIS

Drug Design, Development and Therapy

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An open randomized active-controlled clinical trial with low-dose SKA cytokines *versus* DMARDs evaluating low disease activity maintenance in patients with rheumatoid arthritis.

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[3]Department of Internal Medicine, San Pietro Fatebenefratelli Hospital, Rome, Italy

FOREWORD

The publication titled ***"An open randomized active-controlled clinical trial with low-dose SKA cytokines versus DMARDs evaluating low disease activity maintenance in patients with rheumatoid arthritis"*** is the final result of a project lasting several years. It originated from the need and curiosity of researchers belonging to the Unità Operativa di Reumatologia [Rheumatology Unit] of Ospedale San Pietro Fatebenefratelli di Roma to investigate the immunomodulatory action of very low-dose biological molecules in the management of patients with Rheumatoid Arthritis.

The results of the study provide valuable data regarding the immunomodulatory activity of combination therapy with low- dose SKA IL-4 plus IL-10 plus IL-1 antibodies (GUNA®-IL 4, GUNA®-IL 10, GUNA®-ANTI IL 1 - Guna S.p.a. - Milan, Italy) and indicate novel therapeutic approaches for this disease, whilst also suggesting possible uses in the treatment of other autoimmune diseases.

INTRODUCTION

Rheumatoid arthritis is a chronic autoimmune inflammatory disease that affects the joints and leads to disability in approximately 30% of affected subjects after just 3 years. In certain subjects, this condition also damages other organs, including the skin, eyes, lungs, heart, kidneys, salivary glands, nerve tissue, bone marrow and blood vessels.

The chronic inflammation that underlies the disease leads, within a short period of time, to the erosion of the joint cartilage and bone, with consequent joint deformity causing symptoms that include pain, heat, swelling, sensitivity and joint stiffness, which present especially upon waking and after periods of inactivity.

The first line of treatment for this condition are disease-modifying anti-rheumatic drugs (**DMARDs**) and biologics. At the current time, these medicinal products are the most efficacious therapy able to induce remission or low disease activity (LDA), as they are able to inhibit the action of the inflammatory cytokines expressed during the acute phase.

However, their high cost, the progressive loss of efficacy over time and the biological risk associated with prolonged treatment have led the medical and scientific community to evaluate new therapeutic options for maintaining the remission or low disease activity obtained with these drugs.

It was in this context that this open randomised, active-control prospective study was conducted, in order to evaluate whether treating subjects with Rheumatoid Arthritis with low-dose SKA IL-4, IL-10 and anti-IL-1 antibodies (Guna S.p.a. - Milan, Italy) is able to maintain the LDA or disease remission obtained with biological drugs.

Parameters such as Disease Activity Score (DAS28), Clinical Disease Activity Index (CDAI), Simplified Disease Activity Index (SDAI), Pain Visual Analog Scale (VAS), Global Health Assessment (GH), Erythrosedimentation Rate (ESR), C-Reactive Protein (CRP) and use of nonsteroidal anti-inflammatory drugs (NSAIDs) were evaluated at baseline and every three months until the end of the study (**FIG. 1**). The safety of the treatments administered was monitored for the entire duration of the trial.

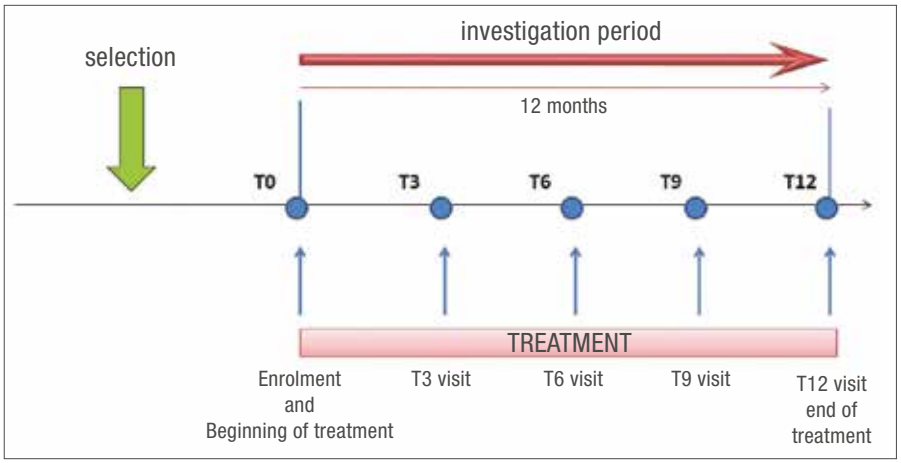


FIG. 1
STUDY DESIGN

ELIGIBILITY CRITERIA

INCLUSION CRITERIA

- Rheumatoid arthritis diagnosed according to ACR criteria
- Duration of disease < 3 years
- 28-joint disease activity score (DAS28) < 3.2 after treatment with biologics or DMARDs
- Subjects able to comply with study procedures
- Subjects able to sign the informed consent form

EXCLUSION CRITERIA

- Age < 18 years
- Duration of disease > 3 years
- Presence of comorbidities that could contraindicate therapy with biologics or immunosuppressive drugs (active infections or history of tuberculosis or tumour).

After the screening procedures, 39 subjects who had achieved LDA or disease remission after treatment with DMARDs or biologics were enrolled (FIG. 2).

After randomisation, subjects were split into two study groups:

- **Group A started taking GUNA®-IL 4, GUNA®-IL 10 and GUNA®-Anti IL 1 in 10 fg/mL SKA formulations, administered at a dose of 20 drops per day for 12 consecutive months.**
- **Group B started or continued taking DMARD therapy (FIG. 2).**

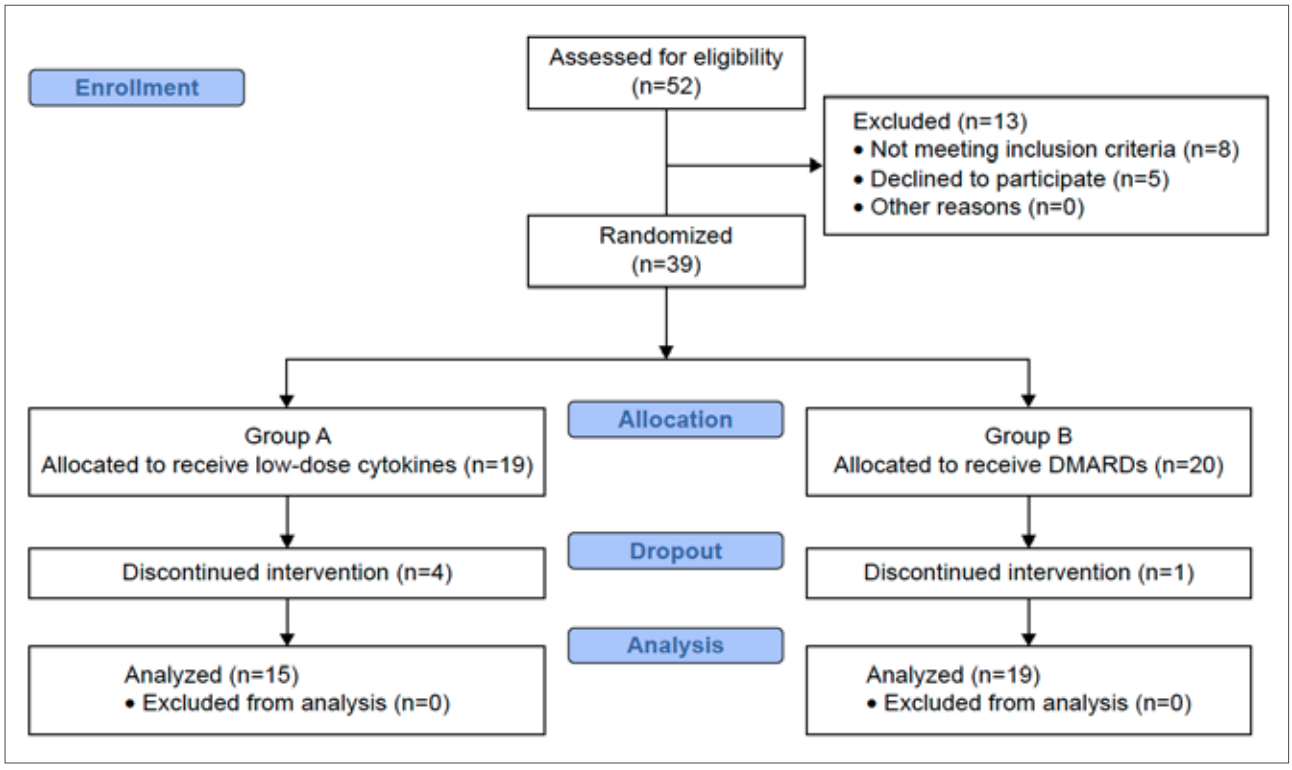


FIG. 2
52 SUBJECTS UNDERWENT SCREENING. 39 OF THEM WERE ENROLLED. 5 SUBJECTS DID NOT COMPLETE THE STUDY

RESULTS

PRIMARY ENDPOINTS

Maintenance of LDA

LDA, evaluated by DAS28-CDAI and SDAI, **was maintained at 12 months in 66.7% of subjects treated with low-dose cytokines** (Group A) (n=10) and in 42.1% of patients treated with DMARDs (Group B) (n=8); the difference between groups was not statistically significant (Fisher's exact test: p = 0.185).

Disease Activity Score (DAS28)

The DAS28 values were similar in the two groups at baseline (Mann-Whitney U test: p = 0.3991), and at 12 months (Mann-Whitney U test: p = 0.1030). Group A maintained constant DAS28 values (Friedman test: p = 0.41604), whereas the DAS28 values of Group B rose (Friedman test: p = 0.00198), with a significant difference (Conover test: p <0.05) between T0 and T9, T0 and T12, T3 and T9, T3 and T12 (**FIG. 3**).

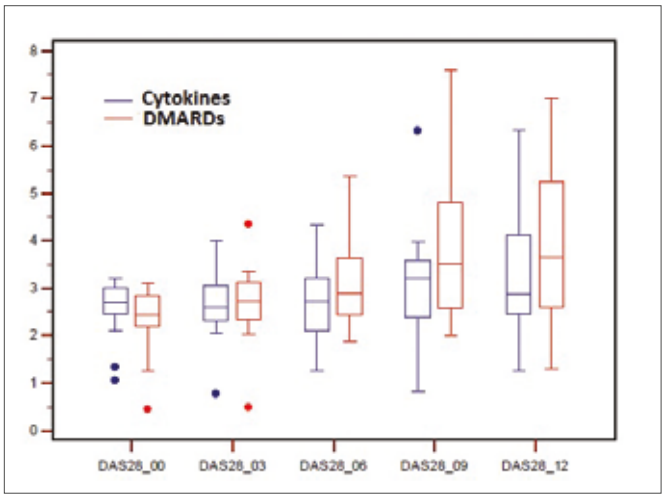


FIG. 3
DISEASE ACTIVITY SCORE DAS28
BLUE: TREATMENT WITH CYTOKINES
RED: TREATMENT WITH DMARDs

Clinical Disease Activity Index (CDAI)

The CDAI values were similar in the two groups at baseline (Mann-Whitney U test: p = 0.7317) and at 12 months (Mann-Whitney U test: p = 0.0510). The values for Group A remained stable over time (Friedman test: p = 0.84645), whereas in Group B the values rose (Friedman test: p = 0.00004), with a significant difference (Conover test: p <0.05) between T0 and T6, T0 and T9, T0 and T12, T3 and T9, T3 and T12, T6 and T9, T6 and T12 (**FIG. 4**).

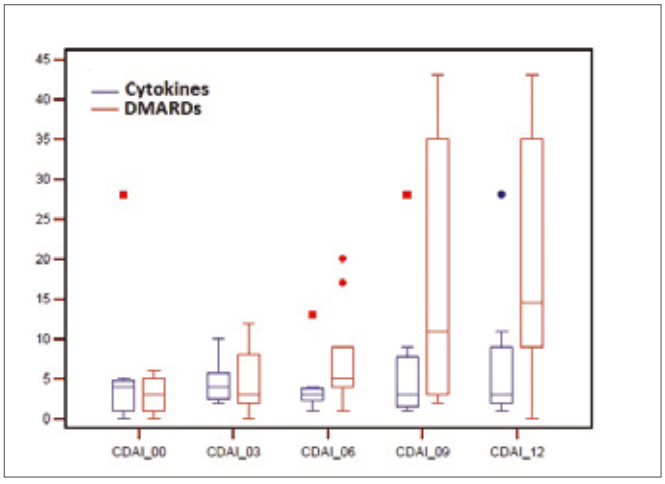


FIG. 4
CLINICAL DISEASE ACTIVITY INDEX CDAI
BLUE: TREATMENT WITH CYTOKINES
RED: TREATMENT WITH DMARDs

Simplified Disease Activity Index (SDAI)

There was no significant difference in the SDAI values between the two groups at baseline (Mann-Whitney U test: p = 0.9223) or at 12 months (Mann-Whitney U test: p = 0.0790). Group A showed a steady intra-Group trend (Friedman test: p = 0.56774), whereas a significant intra-Group difference was observed in Group B (Friedman test: p <0.00001 and Conover test: p <0.05) between the following time-points: T0 and T6, T9 and T0, T0 and T12, T3 and T9, T12 and T3, T6 and T9, T6 and T12 (**FIG. 5**).

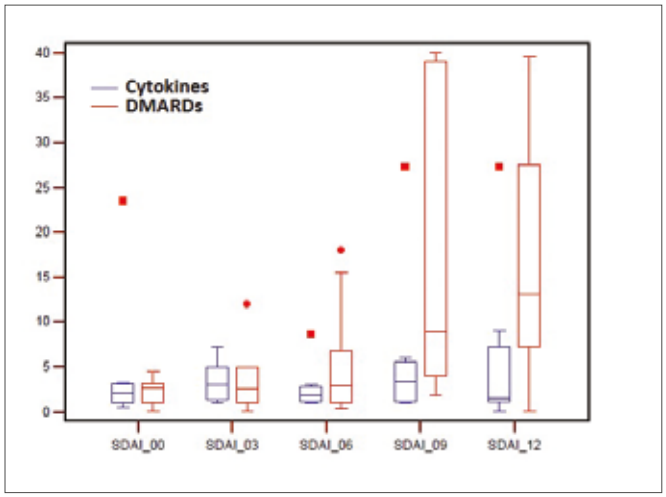


FIG. 5
SIMPLIFIED DISEASE ACTIVITY INDEX SDAI
BLUE: TREATMENT WITH CYTOKINES
RED: TREATMENT WITH DMARDs

SECONDARY ENDPOINTS

Pain Visual Analogue Scale (VAS)

The Pain VAS values were similar for both groups at baseline (Mann-Whitney U test: p = 0.7336) and at 12 months (Mann-Whitney U test: p = 0.1772).
Subjects maintained constant levels, without any intra-Group differences, as shown by the Friedman test, p values were 0.79490 in Group A and 0.12474 in Group B (**FIG. 6**).

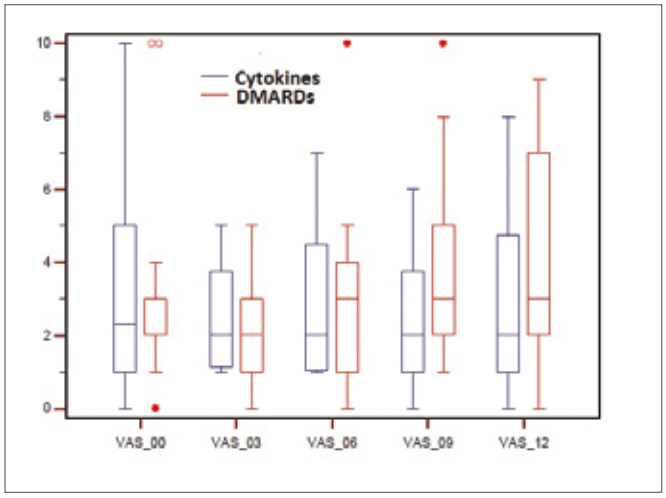


FIG. 6
PAIN VISUAL ANALOGUE SCALE
BLUE: TREATMENT WITH CYTOKINES
RED: TREATMENT WITH DMARDs

Global Health Assessment (GH)

There was no significant difference in the GH values between the two groups at baseline (Mann-Whitney U test: p = 0.4998) or at 12 months (Mann-Whitney U test: p = 0.3269).
Subjects maintained steady values in both groups (Friedman test: p = 0.19770 in Group A and Friedman test: p = 0.05608 in Group B) (**FIG. 7**).

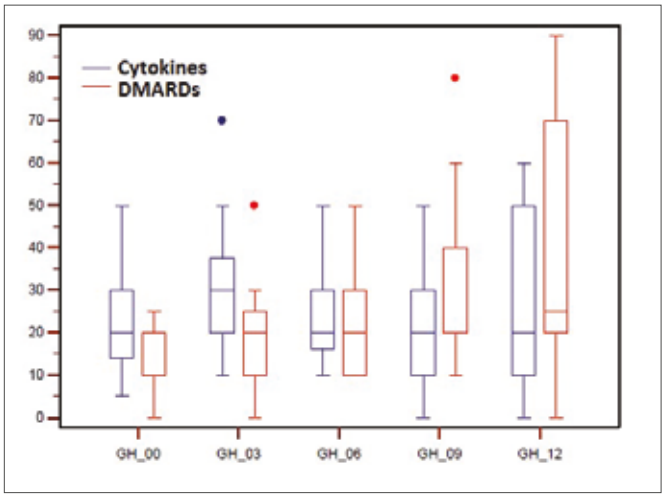


FIG. 7
GLOBAL HEALTH ASSESSMENT GH
BLUE: TREATMENT WITH CYTOKINES
RED: TREATMENT WITH DMARDs

Erythrocyte Sedimentation Rate ESR

There was no significant intra-Group difference in the ESR values at baseline (Mann-Whitney U test: p = 0.7153) or at 12 months (Mann-Whitney U test: p = 0.0699).
Similarly, no significant intra-Group difference was observed; the p values of the Friedman test were 0.53603 in Group A and 0.08022 in Group B (**FIG. 8**).

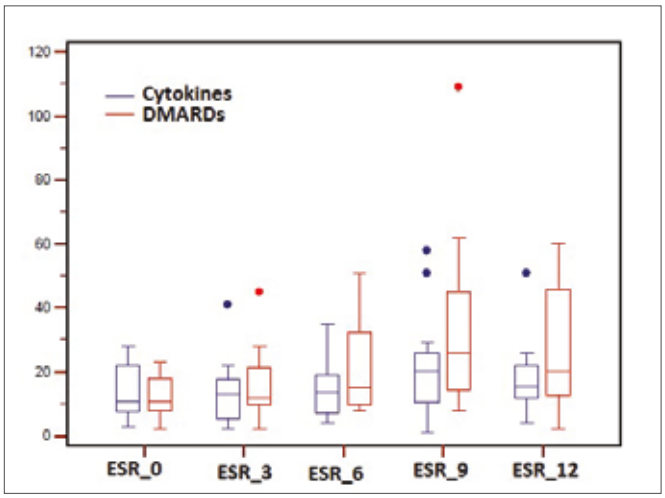


FIG. 8
ERYTHROCYTE SEDIMENTATION RATE ESR
BLUE: TREATMENT WITH CYTOKINES
RED: TREATMENT WITH DMARDs

C-Reactive Protein (CRP)

The mean CRP values were lower in Group A at baseline (Mann-Whitney U test: p = 0.0078), but similar at 12 months, without any statistically significant difference (Mann-Whitney U test: p = 0.0966).
Subjects showed constant intra-group levels, the Friedman test was p = 0.69002 in Group A and p = 0.22356 in Group B (FIG. 9).

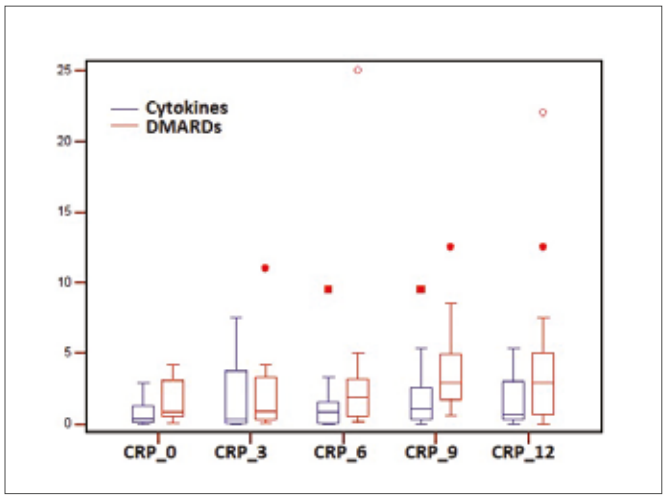


FIG. 9
C-REACTIVE PROTEIN (CRP)
BLUE: TREATMENT WITH CYTOKINES
RED: TREATMENT WITH DMARDs

Use of NSAIDs (Celecoxib 200mg/day)

No difference was observed in the use of Celecoxib between the two study Groups at the various time-points, although the small sample, due to the limited percentage of subjects taking the drug, did not make it possible to obtain a certain result.

Predictors of the efficacy of combined cytokine therapy in the maintenance of disease remission or LDA

The statistical analysis (analysis of covariance) regarding the impact of treatment on the condition of remission or LDA provided by the DAS28 values did not show any significant differences (Friedman test: p=0.530).

Assessment of safety

A total of 34 subjects underwent treatment for a consecutive 12-month period. No adverse event was recorded by Investigators in either study Group.

CONCLUSIONS

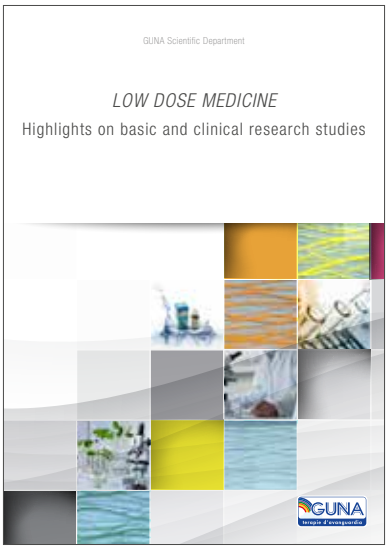
The results obtained from this clinical study show that:

- The use of low-dose SKA cytokines/antibodies administered simultaneously via the oral route showed good efficacy in maintaining LDA in subjects with Rheumatoid Arthritis, following remission obtained with biologics or DMARDs.
- The difference between the two Groups can be observed from the sixth month of treatment from the start of remission, when the Group treated with low-dose SKA cytokines/antibodies maintains the clinical signs consistent with remission in a homogeneous manner, whereas the Group treated with DMARDs gradually deteriorates, with a vast range of intra-Group variability.
- The study suggests that therapy with low-dose SKA cytokines/antibodies may act by inducing a progressive regulation and stabilisation of the Immune System, justified by the more limited dispersion in the Group treated with low-dose SKA cytokines/antibodies.
- Safety was confirmed as being excellent and no adverse event was reported amongst treated subjects.

THERAPY WITH LOW-DOSE SKA CYTOKINES AND ANTIBODIES REPRESENTS A NEW OPPORTUNITY TO BE EXPLORED IN THE MANAGEMENT OF SUBJECTS WITH RHEUMATOID ARTHRITIS, EVEN IN THE ERA OF BIOLOGICS.

BIBLIOGRAPHICAL REFERENCE

Martin-Martin LS, Giovannangeli F, Bizzi E, Massafra U, Ballanti E, Cassol M, Migliore A. An open randomized active-controlled clinical trial with low-dose SKA cytokines versus DMARDs evaluating low disease activity maintenance in patients with rheumatoid arthritis. Drug Design, Development and Therapy 2017;11:985-994



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