**RAPPORTO FINALE SUI RISULTATI DEL PROGETTO COMUNE DI RICERCA**
**FINAL REPORT ON RESULTS OF JOINT RESEARCH PROJECT**

**1. Accordo /Agreement**

CNR / SAV
anni/years 2010/2012

**2. Titolo del progetto**
**Modelli *in vitro* ed *in vivo* per lo studio dei meccanismi infiammatori e di stress ossidativo collegati all'artrite: nuove prospettive terapeutiche**

**2. Title of the project**
***In vitro* and *in vivo* models of arthritic processes for studying the mechanisms of inflammation and oxidative stress link-up. New perspectives for arthritis therapy**

Parole chiave (massimo 3)
Key words (max. 3)
**Rheumatoid arthritis – inflammation – oxidative stress**

(solo per parte italiana)
Area scientifica / Scientific area (tabella 1/ table1)
**Dipartimento Agroalimentare**

**3. Responsabili del progetto**
**Project leaders**


| **Responsabile italiano** | **Project leader** |
|---|---|
| GIAN LUIGI RUSSO<br>PRIMO RICRCATORE – CNR | BAUEROVÁ KATARÍNA<br>PhD - RESEARCH CO-ORDINATOR |
| istituto di appartenenza<br><br>ISTITUTO SCIENZE DELL'ALIMENTAZIONE<br><br>indirizzo<br>via Roma 64, 83100, Avellino<br>Tel.0825 299331 Fax 0825 781585 Cell 3299064414 - e-mail: glrusso@isa.cnr.it | affiliation<br><br>INSTITUTE OF EXPERIMENTAL PHARMACOLOGY AND TOXICOLOGY<br><br>address<br>Dúbravská cesta 9, 841 04 Bratislava, Slovakia<br>Phone/Fax: (+4212) 59410666, e-mail: katarina.bauerova@savba.sk |


**4. Obiettivi del progetto**
**4. Aims of the project**

1) To provide a model of adjuvant arthritis (AA) in male Lewis rats to test new combinatory therapy against RA using synthetic and natural substances with antioxidant activity. AA allows to monitor the disease process in the acute and subchronic phases;

2) To produce glycosaminoglycans mimetics for *in vivo* experiments in AA Lewis rats;

3) To i*n vivo* detect inflammatory and oxidative markers during adjuvant arthritis and correlate them with the physiopathology of RA.

## 5. Risultati ottenuti per obiettivo (1 pagina)
## 5. Achieved results (one page)[1]

The Slovak unit has common research with all Italian Institutions included in the project. The role of oxidative stress (OS) in rheumatoid arthritis (RA) by means of the model of adjuvant arthritis (AA) in Lewis rats was investigated. AA is a model of polyarthritis widely used for preclinical testing of antiarthritic substances. This model is characterized by arthritic, inflammatory, OS and immune parameters in time profile during 28 days to study the antirheumatic effect of selected substances.

On the basis of previous cooperation between the Bratislava and Torino groups plasmatic HNE- and MDA-protein adducts were first monitored in AA and the common results were published **(1,2,4)**. A pilot study focused on immunohistochemical detection of HNE-modified proteins in the joints of rats with AA was also performed. Slices of decalcified paraffin embedded rat joints from arthritic control group and healthy control group were assessed. Our results indicate an increased presence of HNE-modified proteins in joints of arthritic rats compared to controls, which could be related with noticeable inflammatory infiltration of "arthritic" joints. The most visible cytoplasmatic positivity was found for granulocytes and histiocytes. These preliminary results will help us to optimise this immunohistochemical method for detection of HNE-modified proteins in joint slices and use it for experiments with rat AA and combination therapy of methotrexate (MTX) and antioxidants. We suppose that MTX and/or antioxidants should be able to reduce the amount of HNE-modified proteins in joints of arthritic rats.

Further levels of plasmatic isoprostanes in AA were assessed by using a GC/MS method (Bratislava and Siena groups). The preliminary results obtained from comparison of the arthritis animals to healthy control were presented at the *3rd* International conference on osteoimmunology in Fira **(5)**. We examined also the eventual antioxidant and anti-inflammatory effect of pinosylvin (PIN) - 3,5-dihydroxy-trans-stilbene (an analogue of resveratrol) on the progression of AA in rats in monotherapy and in combination with methotrexate (MTX). Data obtained in Bratislava demonstrated that PIN potentiated both the anti-arthritic (decrease of hind paw volume) and the antioxidant effect of MTX (TBARS in plasma). Arthritic animals showed increased OS, also evaluated as plasma levels of $F_2$-isoprostanes. $F_2$-Isoprostanes can nowadays be considered the most reliable markers of OS *in vitro* and *in vivo* and can be used to evaluate the oxidative status in a number of human pathologies, including RA. PIN alone or in combination with MTX strongly reduced $F_2$-Isoprostanes levels (about 50%). Further in Siena PIN was also tested for its ability to stimulate antioxidative stress response through activation of Nrf2-target proteins. In particular, heme oxygenase (HO-1) plays an important role in AA showing anti-inflammatory and antioxidant functions. HO-1 was measured in supernatants from tissue homogenates by Western blot analysis. The data show a significant decline in HO-1 (about 40%) in the lung from AA rats. In these animals, PIN alone increased the levels of HO-1 by about 30% more than MTX. Moreover, the combination therapy was the most effective in increasing the levels of HO-1 (3-fold in respect to AA values). No changes were observed in the liver. Finally, since NF-κB plays an important role in inflammation (cytokine release, MMP expression, etc.) occurring in RA, we assessed whether PIN was able to suppress its activation. In our model, we observed a marked increase in NF- κB in the lung and liver from AA animals. This increase was strongly reduced by PIN alone as well as in combination with MTX. These results suggest that the anti-inflammatory activity of PIN can be mediated by suppression of NF-κB activation. These preliminary results demonstrate that PIN is able to reduce OS in AA rat model. In fact, the combined administration of PIN and MTX suppressed arthritic progression more effectively than did MTX alone. This natural compound may then be useful in the treatment of RA. The above reported results were presented at two international conferences **(6,7)** and will be prepared for publication in a journal.

In cooperation with Avellino unit a pilot experiment with quercetin (QUE) in rat adjuvant arthritis (AA) was performed. At the end of the experiment after evaluation of clinical markers of the disease (arthrogram and changes in body weight) no significant differences between arthritis control group and QUE treated arthritis group were found. Nevertheless, a significant reduction of γ-glutamyltransferase (GGT) activity by QUE treatment in joint homogenates was monitored. All inflammatory markers (CRP, IL-1beta and MCP measured in plasma) were improved by administration of QUE. Despite that QUE not improve clinical parameters, its administration is suppressing the systemic inflammation and in this way it is beneficial for the immunological status of the arthritic animals. QUE could be a good candidate for combination therapy with DMARDs. The data will be prepared for publication.

The activity in cooperation with Modena unit included the analysis of healthy animals, untreated arthritic animals, arthritic animals treated with chondroitin sulfate (CS) daily, 14 days before AA induction

until the end of the experiment (day 28). CS was capable of significantly reducing the severity of arthritis along with OS, a consequence of chronic inflammatory processes occurring in AA. The CS pre-treatment regimen was effective throughout the whole subacute phase, while treatment from day 1 proved effective only in the chronic period. The effects were confirmed by improved total antioxidant status and GGT activity. CS administered under a pre-treatment regimen was also able to reduce the production of pro-inflammatory cytokines, C-reactive protein (CRP) in plasma, phagocytic activity and the intracellular oxidative burst of neutrophils. CS proved to be effective in slowing down AA development and in reducing disease markers, thus supporting its beneficial activity as a drug for RA in humans **(3,8).**

[1]Numbers in brackets are referred to publications listed below

## 6. Prodotti del progetto / Results obtained

| | n./no. |
|---|---|
| **Pubblicaz. scient. su riviste internaz./ scientific publications on international reviews con IF 3, senza IF 0** | **3** |
| Pubblicaz. in atti congressi internaz./ publications in international congress proceedings | |
| Pubblicazioni in atti congressi nazionali / publications in national congress proceedings | |
| Pubblicazione libri nazionali / Publication of national books | |
| Pubblicazione libri internazionali / Publication of international books | |
| **Altre pubblicazioni / other publications (abstracts presented at international conferences)** | **5** |
| Brevetti / Patents | |
| Prototipi / Prototypes | |
| Strumentazione / Equipment and /or Devices | |
| Programmi software / Software | |
| Banche dati / Data bases | |
| Protocolli / Protocols | |
| Nuovi Materiali / New Materials | |
| Nuovi processi / New processes | |
| Cataloghi/inventari/repertori / Catalogues/Inventories | |
| Atlanti/Carte/Mappe / Atlases/Charts/Maps | |
| Progetti di ricerca / Reserch project | |
| Trasferimento innovazioni / Knowledge transfer | |
| Laboratori congiunti / Joint laboratories | |
| Alta formazione / Training | |
| **Altro / Other (seminari / lectures)** | **5** |

## 7. Informazioni dettagliate sui risultati indicati sub 6
## 7. Detailed information on results indicated under point 6

**Publications on journals with impact factors** (the names of scientists involved in the project are indicated in **bold**)

**1. BAUEROVÁ, Katarína - PAULOVIČOVÁ, Ema** - MIHALOVÁ, Danica - **DRÁFI, František** - ŠTROSOVÁ, Miriam - **MASCIA, Cinzia - BIASI, Fiorella** - ROVENSKÝ, Jozef - KUCHARSKÁ, Jarmila - GVOZDJÁKOVÁ, Anna - **PONIŠT, Silvester**. Combined methotrexate and coenzyme Q10 therapy in adjuvant-induced arthritis evaluated using parameters of inflammation and oxidative stress. In *Acta Biochimica Polonica*, 2010, vol. 57, no. 3, p. 347-354. (1.262 - IF2009).

**2. PONIŠT, Silvester** - MIHALOVÁ, Danica - JANČINOVÁ, Viera - ŠNIRC, Vladimír -

ONDREJIČKOVÁ, Oľga - **MASCIA, Cinzia - POLI, Giuseppe** - STANČÍKOVÁ, Mária - NOSÁĽ, Radomír - **BAUEROVÁ, Katarína**. Reduction of oxidative stress in adjuvant arthritis. Comparison of efficacy of two pyridoindoles: stobadine dipalmitate and SMe1.2HCl. In *Acta Biochimica Polonica*, 2010, vol. 57, no. 2, p. 223-228. (1.262 - IF2009).

**3. BAUEROVÁ, Katarína - PONIŠT, Silvester - KUNCÍROVÁ, Viera** - MIHALOVÁ, Danica - **PAULOVIČOVÁ, Ema - VOLPI, Nicola**. Chondroitin sulfate effect on induced arthritis in rats. In *Osteoarthritis and Cartilage*, 2011, vol. 19, no. 11, p. 1373-1379. (3.953 - IF2010).

**Abstracts at international conferences:**

**4. PONIŠT, Silvester - BAUEROVÁ, Katarína** - MIHALOVÁ, Danica - JANČINOVÁ, Viera - ONDREJIČKOVÁ, Oľga - PEREČKO, Tomáš - ŠTROSOVÁ, Miriam - KUCHARSKÁ, Jarmila - **MASCIA, Cinzia - PAULOVIČOVÁ, Ema** - STANČÍKOVÁ, Mária - TRUNOVÁ, Oľga. Substances affecting redox balance of the organism tested on adjuvant arthritis - an animal model of rheumatoid arthritis. New combinations with immune-suppressive drugs in prospective treatment of rheumatoid arthritis. In *Programme & Abstracts. : annual meetings. Society for Free Radical Research (SFRR Europe), "Free Radicals and the Environment", September 12-15th. The European Environmental Mutagen Society (EEMS), "Environmental Mutagenesis in the North", September 15-18th*. abstract p. 70-71. ISBN 978-82-8082-428-8.

**5. BAUEROVÁ, Katarína - PONIŠT, Silvester - PAULOVIČOVÁ, Ema** - MIHALOVÁ, Danica - NOSÁĽ, Radomír - DRÁBIKOVÁ, Katarína - JANČINOVÁ, Viera - DRÁFI, František - GVOZDJÁKOVÁ, Anna - VANKO, Marián - BEZÁKOVÁ, Lýdia - SIGNORINI, Cinzia - **GARDI, Concetta - BIASI, Fiorella** - STANČÍKOVÁ, Mária - ROVENSKÝ, Jozef. The role of redox imbalance in relation to immunological processes in adjuvant arthritis. In *Interactions of the immune and skeletal systems : 3rd International conference on osteoimmunology. June 20-25, 2010, Fira, Santorini, Greece*. - Fira : Nomikos Conference Center, 2010, abstract p. 68-69.

**6. VECCHIO, Daniela - ACQUAVIVA, Alessandra - AREZZINI, Beatrice - GARDI, Concetta - PONIŠT, Silvester - DRÁFI, František** - MIHALOVÁ, Danica - NOSÁĽ, Radomír - **BAUEROVÁ, Katarína**. Activity of pinosylvin administered in monotherapy and in combination with methotrexate on the development of rat adjuvant arthritis. In *4eme Symposium International Nutrition, Biologie de l'Oxygene et Médecine: radicaux libres et vieillissement: des aspects fondamentaux aux applications cliniques. 15 - 17 Juin 2011, Paris, France*. - Paris : Oxygen Club of California, 2011, abstract p. P14.

**7. ACQUAVIVA, Alessandra - DRÁFI, František - VECCHIO, Daniela - PONIŠT, Silvester - AREZZINI, Beatrice** - MIHALOVÁ, Danica - **GARDI, Concetta** - NOSÁĽ, Radomír - **BAUEROVÁ, Katarína**. Pinosylvin administered in monotherapy and in combination with methotrexate reduces oxidative stress in adjuvant arthritis rat model. In *Second International Conference on Environmental Stressors in Biology and Medicine : October 5-7, 2011, Centro Didattico, Policlinico Le Scotte, Universita di Siena, Siena (Italy)*, abstract p.58.

**8. BAUEROVÁ, Katarína - PONIŠT, Silvester -** MIHALOVÁ, Danica - **PAULOVIČOVÁ, Ema** – **VOLPI, Nicola**. The protective effect of chondroitin sulfate on induced arthritis in rats. In *OARSI 2010, world congress on osteoarthritis. Brussels 23-26 september 2010, Belgium.*

**Lectures:**

A lecture for Siena PhD student's school was given by **Dr. Bauerova** during her in Italy in year 2010. The topic of the lecture was:

- "OXIDATIVE STRESS AND IMMUNOLOGICAL PROCESSES IN ADJUVANT ARTHRITIS (AA). APPLICATION OF AA RAT MODEL FOR EVALUATION OF NEW ANTIRHEUMATIC DRUGS"

Furthermore, in occasion of exchange visits, the Symposium "Oxidative stress and natural compounds in autoimmune disorders and cancer" (June 2, 2011, Assembly Hall - SLOVAK ACADEMY OF SCIENCES,

Bratislava) was organized by the Slovak Unit in the frame of the SAV-CNR bilateral project. In this occasion, the following lectures have been given by members of CNR-SAV consortium:

- "OXIDATIVE STRESS, INFLAMMATION AND TISSUE REMODELING" by **Prof. C. Gardi**;
- "APOPTOGENIC ACTIVITY OF FLAVONOID QUERCETIN IN HUMAN LEUKEMIAS" **by Dr. GL Russo**;
- "THE ROLE OF OXIDATIVE STRESS IN THE DEVELOPMENT OF ARTHRITIS. BENEFICIAL EFFECT OF NATURAL COMPOUNDS" by **Dr. S. Ponist**.

During his stay in Bratislava in 2012, **prof. N. Volpi** has given a lecture on June 21 - Thursday in the meeting room of Institute of Experimental Pharmacology and Toxicology in Bratislava with the topic:

- "PLASMATIC AND URINARY GLYCOSAMINOGLYCANS CHARACTERIZATION IN MUCOPOLYSACCHARIDOSIS II PATIENT TREATED WITH ENZYME-REPLACEMENT THERAPY WITH IDURSULFASE".

## 8. Formazione di giovani ricercatori
## 8. Training of young researchers

The CNR-SAV program 2010-12 has also been the occasion for young scientists from both Countries to spend short time abroad. Since one of the goal of the bilateral programs is to implement formation of the young generation of scientists, we strongly hope to have the opportunity to pursue this goal, already initiated in this program, will continue with the request for renewal (2013-15) that we are going to present.

Regarding the SAV unit, in 2011 two scientists were staying in Italy: Mgr. Kuncirova in Siena and Dr. Ponist in Torino. During these stays, results from arthritic and healthy animals obtained in Italian and Slovak laboratories were compared, future experiments were planned and common publication (posters, lectures and manuscripts) were prepared. In details, the of Dr. Ponist at University of Torino (Prof. Biasi and Poli) performed a pilot study focused on immunohistochemical detection of HNE-modified proteins in the joints of rats with AA. Slices of decalcified paraffin embedded rat joints from arthritic control group and healthy control group were assessed. In October 2012 Mgr. Viera Kuncirova visited the laboratory of prof. Gardi at university of Siena with the aim to prepare the samples for measurements of NF-κB and HO-1/β-actin in different tissues obtained from arthritic animals treated with herbal antioxidants.

Regarding the Italian units, unfortunately the limited budget available (only three visits, one per year have been financed by CNR) does not allow a large participation of young scientist to the scientific activity coordinated by SAV in Bratislava. Despite these difficulties, dr. A. Acquaviva from the laboratory of prof. Gardi at university of Siena, travelled from Italy to Slovak from 09/09/2012 to 10/10/2012, to learn the set up procedure of *in vivo* AA model in Lewis rat and to discuss and prepare part of her PhD Thesis.

We strongly hope to have more opportunity for young Italian scientists if the next application will succeed.

## 9. Motivazione degli sviluppi della collaborazione negli anni successivi
(eventuali estensione ad altri paesi, collaborazioni multilaterali, contratti nazionali o internazionali)
## 9. Reasons for cooperative project developments in the following years, if any
(extension to other countries, multilateral collaboration, national or international contracts

The results obtained in the 2010-12 program have been published and will continue to be published on international, peer-reviewed journals. The main application of the novel knowledge will be beneficial in better understanding of joint inflammation and to improved its therapy. These studies will allow new insights to clarify the action mechanisms of the selected compounds for potential therapeutic applications in the future. For these reason, the CNR-SAV team decided to apply for the renewal of the project.

The next bilateral project (2013-15) will represent an unique opportunity to improve knowledge of a worldwide largely diffuse disease, such as rheumatoid arthritis. The project fulfills all the basic

criteria to reach successful outputs: 1. social importance: RA is chronic disease affecting approximately 1% of the population; resistance to canonical therapy and adverse effects are examples of urgent needs to try new clinical approach which may also reduce the social cost for the national health care agencies; 2. novelty: the future proposal is novel since it surfs the wave of "combination therapies" which represent a new approach in the field of the therapeutic use of naturally occurring molecules. One of the advantages of the combination strategy is to ameliorate the symptoms of the disease lowering the doses of first line drugs. This may diminish the side effects of the traditional therapy and reduce its social cost for the governments; 3. interdisciplinarity: the Italian and Slovak groups involved in this proposal are characterized by a high level of interdisciplinarity. Shortly, SAV will provide the experimental models (rats and primary cells) and the competence in pharmacology which are essential in a project aimed to improve the cure of a human pathology. On the other side, all the Italian groups involved possess a long lasting experience in the study of the molecular bases of human diseases related to oxidative stress. Their expertise in biochemistry, cell and molecular biology will allow the definition of the molecular mechanisms triggered by the combined treatments in both cellular and animal models of RA. An added value of the future proposal is that it originated from a previous and successful experience in the twin bilateral project CNR-SAV 2010-12. Here, the team, taking advantage of the exchange visits, had the opportunity to focus on new and most important aspects of RA which will be explored in the next proposal. The Italian-Slovak groups involved know each other's and established mutual interactions based not only on common scientific interests, but also on reciprocal esteem and friendship.

(firma del responsabile italiano del progetto)

(signature of the Slovak project leader)
(anche fax)

IL DIRETTORE

(firma del direttore)

Prof. Raffaele Coppola

date: OCTOBER 1st, 2012

TABELLA 1

1 – Dipartimento Terra e Ambiente
2 – Dipartimento Energia e Trasporti
3 – Dipartimento Agroalimentare
4 – Dipartimento Medicina
5 – Dipartimento Scienze della Vita
6 – Dipartimento Progettazione Molecolare
7 – Dipartimento Materiali e Dispositivi
8 – Dipartimento Sistemi di Produzione
9 – Dipartimento Tecnologie dell'Informazione e delle Comunicazioni
10 – Dipartimento Identità Culturale
11 – Dipartimento Patrimonio Culturale

**NOTA BENE:**

NELLE PAGINE CHE SEGUONO E' RIPORTATO IL PROGRAMMA DI RICERCA COSI' COME SARA' PRESENTATO ALL'AUTORITA' COMPETENTE DEL **SAV** DAL RESPONSABILE SCIENTIFICO SLOVACCO **Dr. BAUEROVA.**

POICHE' LE DUE SCHEDE PROGETTO, QUELLA **CARTACEA DEL SAV**, RISPETTO A QUELLA **ON-LINE DEL CNR**, DIFFERISCONO SOSTANZIALMENTE IN QUANTO A NUMERI DI CARATTERI DISPONIBILI, LA PARTE CARICATA SUL SITO CNR RISULTA MENO ESTESA E DETTAGLIATA RISPETTO A QUELLA PRESENTATA AL SAV.

PERTANTO, A BENEFICIO DELLA CHIAREZZA E PER MAGGIORE UNIFORMITA', SI E' RITENUTO UTILE ALLEGARE ALLA DOMANDA PRESENTATA AL CNR ANCHE LA VERSIONE ESTESA PRESENTATA AL SAV

Il responsabile Italiano,
Gian Luigi Russo

National Research Council of Italy

SLOVAK ACADEMY OF SCIENCES

# CNR – SAS project proposal
# for the years 2013 - 2015

| **1. Title of the project** |
|---|
| **Phytochemicals in ameliorating rheumatoid arthritis therapy: from preclinical studies to clinical applications**<br>***Acronym: PhytoArt*** |
| **2. Name, address of co-partner organization in Slovakia** |
| Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences,<br>Dubravska cesta 9, 84104 Bratislava, Slovak Republik |
| **3. Name, position, address, tel. and fax number of the project leader in Slovakia** |
| **PharmDr. Katarína Bauerová, PhD**<br>Research co-ordinator, Institute of Experimental Pharmacology and Toxicology<br>Slovak Academy of Sciences, Dubravska cesta 9, SK-84104 Bratislava, Slovak Republic<br>Phone/Fax: (+4212) 59410666<br>E-mail: katarina.bauerova@savba.sk |
| **4. Names of Slovak co-workers of the project (members of the Slovak team)** |
| MVDr. Štefan Bezek, DrSc<br>Ing. Lucia Račková, PhD<br>PharmDr. Silvester Poništ, PhD<br>PharmDr. František Dráfi, PhD<br>MSc. Viera Kuncirova (PhD student) |
| **5. Name and address of co-partner organization in Italy** |
| Institute of Food Sciences, National Research Council<br>via Roma 64, 83100 Avellino, Italy |
| **6. Name, position, address, tel. and fax number of the project leader in Italy** |
| **Dr. Gian Luigi Russo**<br>Institute of Food Sciences, National Research Council<br>via Roma 64, 83100 Avellino, Italy<br>Phone: +39 0825 299331<br>Fax: +39 0825 781585<br>Mobile: +39 3299064414<br>E-mail: glrusso@isa.cnr.it |
| **7. Names of Italian co-workers of the project (members of the Italian team)** |
| Prof. Fiorella Biasi<br>Prof. Concetta Gardi<br>Dr. Paola Ungaro<br>Dr. Maria Russo<br>Dr. Carmela Spagnuolo<br>Dr. Stefania Bilotto<br>Dr. Alessandra Acquaviva<br>Dr. Tina Guina |

Dr. Marco Maina
Dr. Viviana Vastolo
Sig. Umile Gardi

**8. Short description of the project**

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting approximately 1% of the population worldwide. Patients with RA have a significantly reduced life quality (degeneration of muscles and joints, muscle weakness, persistent pain) and they need a lifelong therapy. Resistance and adverse effects frequently occur during antiarthritic therapy. There is thus an urgent need for introduction of new substances into medical practice.

Reactive oxygen and nitrogen species (ROS, RNS) can contribute to the pathogenesis of RA in a variety of ways, including induction of membrane oxidation and instability, irreversible damage to proteins and DNA, cartilage damage and induction of bone resorption. In addition, it has recently been appreciated that ROS/RNS can also modulate a variety of signaling events that control gene expression and affect cellular processes that participate in chronic inflammation.

In the past, our team in the frame of the CNR-SAV bilateral project using an animal model of arthritis induced by adjuvant (AA), monitored oxidative stress (OS) and inflammation, using different clinical and biochemical/immunological markers. We have simultaneously assessed the efficacy of the administered experimental substances with regard to their ability to reduce OS and inflammatory processes. In the experiments on AA rats, we observed a beneficial effect of administration of two endogenous compounds - coenzyme $Q_{10}$ and chondroitin sulphate - and one compound related to the herbal polyphenols - pinosylvin. A further aim was to find a potential enhancement of the antirheumatic effect of methotrexate (MTX), a basic antirheumatic drug very often used worldwide in rheumatology practice. Coenzyme $Q_{10}$ and pinosylvin were selected for assessment of a combinatory therapy with MTX. The already performed experiments on AA confirmed the hypothesis of the beneficial effect of adding a suitable immunomodulator/antioxidant compound to the MTX therapy. Safety and efficacy of these approaches calls for further more detailed research not only in preclinical but also in clinical conditions. The socio-economic aim is to provide new knowledge on "traditionally old" natural compounds in medical practice.

The substances of plant origin (arbutin, curcumin, quercetin, pterostilbene and pinosylvin), mostly synthesized, were studied in pilot studies in our AA model. We particularly monitored their ability to reduce the swelling of hind paws, and their effect on some markers of OS, not published in the scientific literature so far. Among the studied compounds, the most effective substance of plant origin was pinosylvin (PIN). Therefore it was selected to test its possible effectiveness in the combination therapy with MTX. In the new project, we would like to study not only PIN, but also other polyphenols as quercetin, N-feruloylserotonin and standardized green tea extract and its main secondary metabolites. Tea polyphenols, known as catechins, usually account for 30-40% of the dry weight of the solids in brewed green tea. The most well described in the literature is the (-)-epigallocatechin gallate (EGCG). It has been already reported that green tea consumed within a balanced controlled diet improves the overall antioxidative status and protects against oxidative damage in humans. Much interest has been centered on the role of green tea antioxidant activity in regards to the aging process and degenerative diseases like cancer, cardiovascular diseases and diabetes. We would like to place the emphasis to study its therapeutic potency in RA at clinical level (as adjuvant therapy of patients treated with MTX) and preclinical level (adjuvant arthritis model and cell cultures of chondrocytes and granulocyte/macrophage lineage cells). The main focus will be given to combinatory therapy with MTX.

*PhytoArt* is articulated in three years and includes three workpackages: 1. study of combination therapy (MTX plus selected phytochemicals) in Lewis rat, a model of AA; 2. study of combination therapy (MTX plus selected phytochemicals) on primary cell cultures; 3. pilot phase-1 clinical trial on subjects affected by RA treated with a combined therapeutic protocol.

## 9. Reciprocal benefit of the bilateral cooperation

The bilateral project between SAV and CNR represents an unique opportunity to improve knowledge of a worldwide largely diffuse disease, such as rheumatoid arthritis. The project fulfils all the basic criteria to reach successful outputs: 1. social importance: as reported above, RA is chronic disease affecting approximately 1% of the population; resistance to canonical therapy and adverse effects are examples of urgent needs to try new clinical approaches which may also reduce the social cost for the national health care agencies; 2. novelty: the present proposal is novel since it surfs the wave of "combination therapies" which represent a new approach in the field of the therapeutic use of naturally occurring molecules. One of the advantages of the combination strategy is to ameliorate the symptoms of the disease lowering the doses of first line drugs. This may diminish the side effects of the traditional therapy and reduce its social cost for the governments; 3. interdisciplinarity: the Italian and Slovak groups involved in this proposal are characterized by a high level of interdisciplinarity. Shortly, SAV will provide the experimental models (rats and primary cells) and the competence in pharmacology which are essential in a project aimed to improve the cure of a human pathology. On the other side, all the Italian groups involved possess a long lasting experience in the study of the molecular bases of human diseases related to oxidative stress. Their expertise in biochemistry, cell and molecular biology will allow the definition of the molecular mechanisms triggered by the combined treatments in both cellular and animal models of RA. An added value of the present proposal is that it originated from a previous and successful experience in the twin bilateral project CNR-SAV 2010-12. Here, the team, taking advantage of the exchange visits, had the opportunity to focus on new and most important aspects of RA which generated the present proposal. The Italian-Slovak groups involved know each others and established mutual interactions based not only on common scientific interests, but also on reciprocal esteem and friendship. The previous program has also been the occasion for young scientists from both Countries to spend short time abroad. Since one of the goals of the bilateral programs is to implement formation of the young generation of scientists, we strongly hope to have the opportunity to pursue this goal, already initiated in the previous program, with the approval of the present proposal.

## 10. Distribution of the research activities among the participating institutions ("Who will do what ?")

The work will be distributed within the three years foreseen by the project and will essentially include three workpackages (WPs):
WP-1: Study involving an animal model of AA;
WP-2: Study on primary cell cultures;
WP-3: Clinical studies on subjects affected by RA

**First year**

Groups involved:
SAV: K. Bauerová, S. Poništ, F. Dráfi, V. Kuncirová
CNR (University of Siena, UNISI): C. Gardi, A. Acquaviva, U. Gardi,
CNR (University of Torino, UNITO): F. Biasi, T. Guina , M. Maina
CNR (Institute Food Sciences, Avellino, ISA): GL Russo, M. Russo, C. Spagnuolo, S. Bilotto

**WP-1: Animal model of AA: to test combination of different natural compounds associated with first line drugs.**
Taking advantage of the experience maturated during the previous bilateral program, we will pursue the evaluation of the effectiveness of natural compounds in the well-established model of

adjuvant arthritis (AA) represented by Lewis rats, in which the disease is induced by intradermal injection of heat-killed *Mycobacterium butyricum* suspended in incomplete Freund adjuvant into the base of the tail. To test the effect of the combination therapy, methotrexate (MTX) will be administered *per os* simultaneously with the adjuvant therapy twice a week in the dose of 0.3 mg/kg b.w. The selection of compounds will include those actually under investigation as part of the previous bilateral program (e.g., quercetin and pinosylvin) and others whose possible efficacy in AA is supported by published experimental evidence, such as N-feruloylserotonin and standardized green tea extracts. The results obtained in WP-1 will be essential to decide the protocols for clinical studies described in WP-3.

The selected compounds will be administered for 4 weeks after the induction of experimental arthritis. To monitor the development of AA and to evaluate the pharmacological effects of the studied substances, we will assess clinical parameters (changes in body mass of animals, hind paw volume and arthritic score), biochemical indicators (activity of GGT in spleen and joint homogenates, plasmatic level of TBARS, total antioxidant status in plasma and plasmatic level of CRP - measured by spectrophotometry), and immunological markers (plasmatic levels of relevant cytokines: mainly IL-6, IL-1α, Il-1β and chemokine MCP-1 assessed by ELISA method). Plasmatic/blood markers will be measured on selected days in time profile. Tissue markers were assessed at the end of the experiment at day 28 **(SAV).**

Plasma and tissues isolated from AA rats treated as reported above, will be transferred to the Italian groups involved in WP-1 to assess different markers of inflammation and redox activity. Detection of $F_2$-isoprostanes ($F_2$-Iso) will be performed by the group lead by Prof. C. Gardi (**UNISI).** In this task, levels of 8-epi-$PGF_2\alpha$ (the most represented isomer of the series of isoprostanes) will be evaluated in plasma from AA rat model (provided by Dr. Bauerová from SAV). The levels of $F_2$-Iso will be analysed by gas chromatography mass spectrometry tandem (GC-MS/MS) that represents the most reliable technique for this assay. In addition, the ability of selected compounds to stimulate antioxidant response through activation of HO-1 (heme oxygenase-1) will be evaluated by ELISA assay in plasma of treated rats, as well as in tissue samples**.** Since the existence of a functional link between activation of the transcription factor NF-κB and HO-1 expression, the activation of the former in relevant tissue samples, following the treatments occurred in AA rats, will be also evaluated (**UNISI**).

The group lead by Prof. F. Biasi will measure the level of matrix metalloproteases (MMPs) in samples of treated Lewis rats (provided by Dr. Bauerová from SAV). MMP1, MMP2 and MMP9 will be evaluated in plasma by both ELISA and zymography. The levels of related MMP inhibitors TIMP1 and TIMP2 (tissue inhibitors of metalloproteases) will be analyzed by both Western blot and ELISA. In addition, NADPH oxidase isoforms (NOX2 and NOX4) and MMP levels will be assessed in relevant tissue samples provided by SAV (e.g., brain, spleen and hind paw joint) (**UNITO**).

Since the selected phytochemicals possess well-known and characterized antioxidant properties, the effect of combination therapies on AA induction in Lewis rat will be verified on canonical markers of serum/plasma antioxidant status in treated rats, such as total antioxidant capacity (ABTS method), total hydroperoxide levels (FOX assay), while, in erythrocytes (if available) catalase/superoxide dismutase activities and reduced glutathione will be measured. In addition, depending on the phytochemicals selected, an attempt will be made to measure the concentration of free aglycones in the serum/plasma of rats to obtain information on the bioavailability of the bioactive compounds (**CNR-ISA**).

**Second year**

Groups involved:
SAV: L. Račková, Š. Bezek, V. Kuncírová, K. Bauerová
CNR (University of Siena, UNISI): C. Gardi, A. Acquaviva, U. Gardi,
CNR (University of Torino, UNITO): F. Biasi, T. Guina, M. Maina
CNR (Institute of Experimental Endocrinology and Oncology "G. Salvatore", Napoli, IEOS): P. Ungaro, V. Vastolo
CNR (Institute Food Sciences, Avellino, ISA): GL Russo, M. Russo, C. Spagnuolo, S. Bilotto

**WP-2: *In vitro* study on the protective role of selected phytochemicals on isolated rat primary cells injured with oxidative insults: study of the molecular mechanisms of protection**

In order to study at molecular level how the phytochemicals selected in WP-1 may exert potential protective effect(s) against AA in terms of antioxidant and anti-inflammatory activities, in the present WP-2, we will establish cellular models represented by primary rat cells, such as chondrocytes and cells of granulocyte/macrophage lineage, which are involved in the physiopathology of AA. To this aim, chondrocytes and cells of granulocyte/macrophage lineage will be isolated from rats (**SAV**) and exposed to physiological concentrations of cellular oxidants. The viability and proliferation rate will be evaluated by colorimetric methods (MTT, CyQUANT®, Griess reagent) or trypan blue dye test. Apoptosis, necrosis, autophagy, cell cycle arrest will be assessed by fluorescence microscopy and/or flow cytometry, followed by measurement of specific biochemical markers (e.g., caspase activation and DNA fragmentation for apoptosis; LDH release for necrosis; Cyto-ID® for autophagy; Cdks activity and cyclins expression for cell cycle) (**SAV and CNR-ISA**).

The ability of phytochemicals to interfere with the molecular mechanisms of inflammation in PRC will be evaluated by the Prof. Gardi (**UNISI**). The levels of 8-epi-$PGF_2\alpha$ will be measured *in vitro* in primary rat chondrocytes or cells of granulocyte/macrophage lineage (provided by SAV). The effects of selected phytochemicals on $F_2$-Isoprostane production will also be tested. At molecular level, the compounds showing antioxidant and antiinflammatory properties will be tested for their ability to stimulate antioxidative stress response through activation of Nrf2. Nrf2 is the key transcription factor regulating the antioxidant response. It mediates transcriptional regulation of various antioxidant genes including HO-1, whose products have been demonstrated to contribute to the scavenging of ROS and to exhibit anti-inflammatory effects. Nrf2 and HO-1 will be measured in chondrocyte cultures or cells of granulocyte/macrophage lineage by qPCR and Western blot analyses. By Chromatin Immunoprecipitation (ChIP) analysis **CNR-IEOS** will evaluate if the compounds showing antioxidant and antiinflammatory properties are able to induce histone modifications on Nrf2 gene promoter region. To this end, cellular lysates obtained from control and treated cells will be immuno-precipitated with antibodies recognizing histone H3 lysine 4 di-methylation (H3K4me2) and histone H3 lysines 9/14 acetylation (two histone marks of active transcription), as well as histone H3 lysine 9 di-methylation (H3K9me2) an histone mark of repression. In addition, since NF-κB plays an important role in inflammation (cytokine release, MMP expression) occurring in RA, the availability of new compounds able to suppress activation of NF-κB is of particular interest. **UNISI** will also assess it if the tested compounds modulate inflammatory cytokines (such as TNF-α, TGF-beta, IL-8, IL-1β) or MMP activity, through the suppression of inflammatory transcription factor NF-κB.

ROS generation through NADPH oxidase activation represents a key issue in understanding the molecular association between oxidative stress and inflammation in the pathogenesis of RA. NADPH oxidase is a tissue-specific multiprotein complex closely associated to cell plasma membrane. The two NADPH isoforms known to be present in inflammatory sites are NOX2 and NOX4. The evaluation of ROS generation and the analysis of different NOX isoforms activated in primary rat chondrocytes and/or cells of granulocyte/macrophage lineage (provided by SAV) will

be performed by Prof. Biasi (**UNITO**). ROS formation and NOX activities will be evaluated in cells exposed to cellular oxidants and treated with increasing concentrations (1-10 μM) of selected phytochemicals (e.g., EGCG, quercetin or N-feruloylserotonin). Intracellular ROS formation will be visualized by laser confocal microscopy using specific fluorescent probes (dihydroethidium and 2',7'-dichlorofluorescin-diacetate). Based on the molecular mechanism by which cytoplasm and membrane protein subunits assemble, NADPH oxidase activity of different NOX isoforms will be analyzed by Western blot. As mentioned above, MMP expression is under the control of NF-κB and MMPs play a role in structural changes of cartilage. These enzymes are produced by activated chondrocytes and fibroblasts of arthritic joints, and by invading leucocytes in inflamed synovium. MMPs cleave proteoglycans and collagen fibers, thus contributing to cartilage destruction. Based on this evidence, the protective effects of phytochemicals investigated will be evaluated by measuring changes in MMPs activity, as well as that of their tissue inhibitors TIMPs, on cultured granulocytes, and/or chondrocytes and/or cells of granulocyte/macrophage lineage exposed to cellular oxidants. These analyses will be performed by zymography, ELISA and Western blot methods.

**Third year**

Groups involved:
SAV: K. Bauerová and selected clinicians
CNR (University of Siena, UNISI): C. Gardi, A. Acquaviva, U. Gardi,
CNR (University of Torino, UNITO): F. Biasi, T. Guina, M. Maina
CNR (Institute Food Sciences, Avellino, ISA): GL Russo, M. Russo, C. Spagnuolo, S. Bilotto

**WP-3: Combination therapy between methotrexate and phytochemicals against rheumatoid arthritis: from preclinical studies to potential clinical application**

A phase I clinical trials will be designed on RA patients treated with methotrexate and one of the phytochemicals investigated in WP-1 and WP-2. The ideal molecule should satisfy these main criteria: 1. efficacy demonstrated after preclinical studies (WP-1 and WP-2); 2. availability of data on its safety; 3. approval for human use. The selection of patients, individual treatment and collection of samples will be performed in close cooperation with practical rheumatologists and under the care of the responsible ethical committee (**SAV**).

Blood samples will be collected from patients (serum and plasma) in order to determine endpoints of the clinical protocol. The clinical proinflammatory markers (e.g., CRP and the key cytokines) will be analyzed in plasmatic samples of RA patients (**SAV**). The analysis of the antiinflammatory and antioxidant effect of combined therapy will also include determiantion of $F_2$-Isoprostanes which are nowadays considered the most reliable markers of oxidative stress *in vitro* and *in vivo* and can be used to evaluate the oxidative status in a number of human pathologies, including RA. Levels of 8-epi-$PGF_2\alpha$ will be evaluated in plasma of patients (provided by SAV) (**UNISI**). Since key features of the pathophysiology of RA are mainly cartilage destruction mediated by proinflammatory cytokines produced by inflamed synovium and invading leucocytes, which cause release of MMPs, the analysis of MMPs concentration in combination with the detection of an array of pro-inflammatory cytokines in serum/plasma of patients included in the study (provided by SAV), will be considered diagnostic biomarkers of the protective effect of the combination therapy tested (**UNITO**). Finally, as for WP-1, **ISA-CNR** will measure on plasma/sera of patients enrolled in the clinical trial biomarkers of the antioxidant status, such as total antioxidant capacity (ABTS method), total hydroperoxide levels (FOX assay), while, in erythrocytes, catalase/superoxide dismutase activities and reduced glutathione will be measured. In addition, depending from the phytochemicals selected, an attempt will be made to measure the concentration of free aglycones in the serum/plasma to obtain information on the bioavailability of the bioactive compounds.

**11. Duration of the project**
**January 2013- December 2015**

**12. Estimation of the annual travel/stays (proposal)**

**A) Slovak institution (mobility from SAS to Italy**

| | | |
|---|---|---|
| *Number of planned trips: (Total):* | | **9** |
| Thereof: | For the year one: | 3 |
| | For the year two: | 4 |
| | For the year three: | 2 |
| *Duration of planned stay(Total):* | | **90 days** |
| Thereof: | For the year one: | 30 |
| | For the year two: | 40 |
| | For the year three: | 20 |

**B) Italian institution (mobility from Italy to SAS)**

| | | |
|---|---|---|
| *Number of planned trips (Total):* | | **7** |
| Thereof: | For the year one: | 2 |
| | For the year two: | 3 |
| | For the year three: | 2 |
| *Duration of planned stay(Total:)* | | **70 days** |
| Thereof: | For the year one: | 20 |
| | For the year two: | 30 |
| | For the year three: | 20 |

**13. Short CV of the Slovak project leader**
**Bauerová Katarína, PharmDr, PhD**

**Current position:** Senior Researcher in Pharmacology;
Head of the Department of Pharmacology of Inflammation

**Place of work:** Institute of Experimental Pharmacology and Toxicology,
Slovak Academy of Sciences
**Institute address:** Slovakia, 841 04 Bratislava, Dúbravská cesta 9

**Place and date of birth:** Slovakia, Bratislava, 08/06/1961
**Parents:** JUDr. Ján Bauer, Erika Bauerová, neé Markovičová

**Education background:**
1979-84 Faculty of Pharmacy, Comenius University, Bratislava
1984-88 PhD study in Pharmaceutical Sciences
1989 PhD degree
1996 post-gradual study (specialization of 1st degree) in Pharmaceutical Technology

**Professional practice:**

| | |
|---|---|
| Fac. of Pharm. Comenius Univ. | 01/09/1984 - PhD study |
| Inst.Exp.Pharmacol.&Toxicol. _ | 01/08/1988 - research assistant |
| | 01/01/1994 - researcher |
| | 01/07/1997 - senior researcher |
| | 01/04/2007 - Head of Dept. of Pharmacokinetics |
| | 01/03/2008 - Head of Dept. of Pharmacology of Inflammation |

**Field of study:**

- Study of global burden diseases. First, research into pharmacokinetics of relevant drugs, later specialization in pharmacological intervention in rheumatoid arthritis and inflammation
- Externally - in the years 2005-2008 - as expert in the State Institute for Drug Control concerned with safety control of the market for medical devices and health care utilities and member of the EC workgroup involved in problems of pharmacovigilance
- Continuing external lecturing activities in the Faculty of Pharmacy, Comenius University and in the
  Slovak University of Health

**Publication activity:** *in extenso* 73 publications, including 47 in CC database, 4book chapters, 277 citations

**Membership in scientific societies:**

- 1999-2011 Vice-president of the Slovak Pharmaceutical Society, member of the organizing committees of more than 15 national and international symposia and conferences,
- 1989-99 Association of Pharmacists in Bratislava – various functions in the committee; "Award of V.J. Zuffa" for the development of the Pharmacy in Slovakia

**Email:** katarina.bauerova@savba.sk

## 14. Short CV of the Italian project leader

**Dr. Gian Luigi Russo**

Born in Massa Lubrense (NA), Italy, on January, 13th, 1961

**Actual position:** Senior Research Scientist – Head of Food & Human Health division (*responsabile Commessa Alimenti e Salute dell'Uomo Dipartimento Agroalimentare del CNR*) at the Institute of Food Sciences

**Education and Professional Experience**

**1986.** Academic degree (Laurea) in Biological Science at the University of Naples "Federico II".
**1993.** Doctorate in Biochemistry at University of Naples and Bari, Italy
**1991-93.** Post-doctoral Fellow, Cold Spring Harbor Laboratory, New York (NY, USA).
**1995-96.** Research Scientist, Stazione Zoologica "Anton Dohrn", Naples.
**1997-present.** Associated Investigator, Stazione Zoologica "Anton Dohrn", Naples.
**1997-Dec 01.** Research Scientist (*ricercatore III liv*), Institute of Food Sciences, National Research Council, Avellino, Italy
**Dec 01-present.** Senior Research Scientist (*primo ricercatore*), Institute of Food Sciences, National Research Council, Avellino, Italy.

**Dr. Russo** has a broad background in biochemistry, cellular and molecular biology. As a postdoctoral fellow at Cold Spring Harbor Laboratory (NY, USA), he carried out work on cell cycle regulation by protein kinases in malignant cell lines. At Biological Station Anton Dohrn of Naples, he expanded his research to include regulation of cell division cycle during meiosis. As Senior Scientist at National Research Council in Italy (Institute of Food Sciences), he laid the groundwork on how several non-nutritional components present in the regular diet, or molecules derived from food transformation, exert, at molecular level, a protective effect towards degenerative pathologies. The results of his activity are documented by more than 70 original articles on peer-reviewed journals (H-index 2012=22 [according to Scopus]) with a total impact factor of about 270 (ISI). In addition, dr. Russo successfully administered national and European projects (e.g. staffing, research protections, budget), collaborated with other researchers, and produced several peer-reviewed publications from each project I participated. In summary, dr. Russo possesses a demonstrated record of accomplished and productive research projects in areas of high relevance in the field of Food and Health.

**Editorial / Peer reviewing activity**:

- Editorial Advisory Board of *Biochemical Pharmacology Journal.*
- Editorial Advisory Board of *Nutrition, Metabolism & Cardiovascular Diseases.*

**Teaching and educational activity**

- 1993-1996: Professor of Genetics and Biochemistry of Nucleic Acids at Second University of Naples, Italy.
- 1999-2006: Professor of Biochemistry and Applied Biotechnology at University of Naples, "Federico II", Italy.
- Teaching activity in CME and master courses in the field of cancer biology, chemoprevention, nutrition.