

# Progetto ordinario

Ministero della Salute – Direzione Generale della Ricerca Scientifica e Tecnologica - Allegato B2

Fondazione G.B. Bietti Roma

## Form 1 - General information about the project

**INSTITUTION PRESENTING THE PROJECT:** Fondazione G.B. Bietti Roma

**TITLE OF THE PROJECT (max 300 caratteri):** Biomarkers of inflammation and innate immunity in diabetic retinopathy

### SCIENTIFIC COORDINATOR:

**Name and Surname:** Varano Monica  
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### ADMINISTRATIVE COORDINATOR

(TO BE FILLED ONLY BY REGIONI, PROVINCE AUTONOME E AGENZIA DEI SERVIZI SANITARI REGIONALI)

**Name and Surname:** Lorito Nicola  
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**LIST OF PARTICIPATING UNITS (UNITÀ OPERATIVE COINVOLTE): Name of the Institutions and of their Legal Representative (if needed an attachment should be added)**

- Fondazione G.B. Bietti IRCCS , Stirpe Presidente
- Istituto Superiore di Sanità - Dipartimento di Biologia Cellulare e Neuroscienze , Garaci Enrico
- Istituto Superiore di Sanità - Dipartimento di Ematologia Oncologia e Medicina Molecolare , Garaci Enrico

**FORM 2 – DESCRIPTION OF THE PROJECT (SUMMARY OF THE ACTIVITIES OF ALL THE PARTICIPATING UNITS**

**WHAT IS ALREADY KNOWN ON THE SUBJECT (INCLUDE THREE RELEVANT REFERENCES IN PEER REVIEWED JOURNAL) (MAX 20 LINES)**

Diabetic retinopathy (DR) is a microvascular complication of diabetes mellitus (DM) and the leading cause of blindness in working-age adults. It is estimated that more than 300 million people worldwide are affected by diabetes and more than 10% of these patients will develop irreversible visual impairment within 15 years of diagnosis (1). Therapies for DR are still very limited and involve the use of invasive surgical techniques. Clinical and experimental evidence has clarified that hyperglycemia is a primary pathogenic factor for DR, however the molecular and cellular processes involved in mediating hyperglycemia-induced retinal injury remain to be established. The identification of these processes is, therefore, very critical to the development of new therapeutic approaches (2). DR is characterized by an initial period of neuro-vascular injury and increased permeability followed by active proliferation of new vessels. Both of these processes involve increases in the expression levels of the angiogenic and permeability factor Vascular Endothelial Growth Factor (VEGF) (2). It has been shown that VEGF requires the production of the leukocyte adhesion molecule ICAM-1 to induce the early subclinical vascular changes in experimental diabetes, thus suggesting that chronic, low-grade, subclinical inflammation may be responsible for many of the vascular lesions characteristic of DR. This experimental evidence is also supported by studies showing that signs of inflammation are found in human tissues from patients affected by DR. In particular, recently published data show that severity of retinopathy is positively correlated with levels of triglycerides, cholesterol, hemoglobin A1c as well as inflammatory cytokines and chemokines (i.e. Interleukin 6 and 8, RANTES and SDF-1 $\alpha$ ) (3) further supporting the hypothesis that inflammatory processes contribute to the development of DR.

1) World Health Organization Fact Sheet N 138. Geneva, Switzerland: WHO; 2002

2) RB Caldwell, M Bartoli, et al. Vascular endothelial growth factor and diabetic retinopathy: role of oxidative stress. *Curr Drugs Targets* 6:511-524, 2005

3) Meleth DM, Agron E, Chan C-C, Reed G, et al. Serum inflammatory markers in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2005; 46:4295-4301

**WHAT THE PROJECT ADDS TO THE INFORMATION ALREADY AVAILABLE (MAX 10 LINES)**

Experimental data suggest that the increased production of inflammatory mediators in the diabetic retina may be mediated by hyperglycemia-induced interaction of immune cells with the retinal vascular endothelium. It is interesting to note that there is a retinal immune privilege controlled by the "fine tuned" interplay of cellular and humoral interactions. The retinal immune privilege has not yet been characterized, but it appears clear that hyperglycemia may alter the maintenance of this system and its restoration may have therapeutic value for DR. The results of the proposed studies will contribute both to the elucidation of the molecular and cellular mechanisms involved in the maintenance of the retinal immune privilege and in the understanding of diabetes effects on these mechanisms; therefore furthering our knowledge of DR pathogenesis.

**DETAILED DESCRIPTION OF THE PROJECT'S MAIN AND SECONDARY OBJECTIVE(S) (max 40 lines )**

The main objective of this research proposal is to elucidate the cellular and molecular mechanisms involved in the pathogenesis of DR. Clinical and experimental data have shown that sub-clinical chronic inflammation is associated with and possibly causative of the neurovascular injury characterizing the early stages of DR. Of particular interest is the observation that also the production and activity of the angiogenic and permeability factor, VEGF, which is a hallmark of DR, may be part of an inflammatory response and also result in the induction of inflammatory mediators such as the leukocytes adhesion molecule ICAM-1. Therefore, inflammation is a key player in DR pathogenesis. Indeed, this is a devastating event in the eye and anti-inflammatory activities are maintained to control ocular inflammation. This "innate immunity", also known as "immune privilege", is present in the retina but has not been characterized yet. We hypothesized that hyperglycemia stimulates chronic inflammation and progression of DR by overcoming this anti-inflammatory barrier. Information available on the immune privilege of the central nervous system (CNS) may help our understanding of the retinal immune privilege. The brain responds to inflammatory stimuli (i.e. infection, aging) by activating a local innate immune response system involving infiltrating cells, resident glial cells and neurons. The induction of this protective system is tightly regulated by pattern recognition receptors

(PRRs) expressed by professional and amateur phagocytes. Among the PRRs relevant to our studies is CD14 and the family of the Toll Like Receptors, particularly TLR4 which responds to LPS-like molecules. In some diseases, these protective mechanisms lead to neurodegeneration on the ground that several innate immune molecules have neurotoxic activities. Several key regulatory mechanisms have now been evidenced in the control of CNS innate immunity that may apply also to the retinal tissue. Among these neuroimmune regulatory proteins (NIRegs) are CD95L, CD47, CD200, CD55, CD46 and HMGB1, this latter is also a ligand for the receptor for advanced glycation end-products (RAGE) which is up-regulated in DR and has been involved in its pathogenesis. All these molecules could be harnessed to explore novel therapeutic avenues. Therefore, we designed studies aimed at elucidating the mechanisms of retinal innate immunity and also at characterizing its role in DR pathogenesis. These studies involve clinical and experimental approaches and will be developed in three phases.

First phase. We will perform clinical studies to measure circulating levels of PRRs and NIRegs in diabetic patients selected for type of diabetes and degree of retinopathy. These values will be correlated with circulating levels of inflammatory mediators (chemokines, cytokines, VEGF and sICAM-1)

Second Phase We will analyze the molecular interactions and the intracellular signaling events associated with specific PRRs and NIRegs of which expression pattern changes in relation to disease stages. Particular attention will be addressed to the role of reactive oxygen species (ROS) in the modulation of the expression and/or the activity of these players.

Third Phase. We will test the activity of known negative modulators of inflammatory and oxidative responses in order to determine whether normalization of retinal vascular reactivity and retinal tissue responses are associated with a "normal" expression pattern of the analyzed PRRs and NIRegs.

The results of our studies will potentially set the stage for the development of new diagnostic tools and lead to the identification of new pharmacological targets.

## METHODS

SPECIFY: (whenever applicable) a) Patients/population; b) Intervention(s)/Analytical procedures; c) Indicator(s); d) Study design; e) Statistical analysis (MAX 2 PAGES)

First Phase (Contribution of U.O.01 and U.O.02)

We will perform clinical studies to measure circulating levels of PRRs and NIRegs in diabetic patients selected for type of diabetes and degree of retinopathy. These values will be correlated with circulating levels of inflammatory mediators (chemokines, cytokines, VEGF and sICAM-1). During the first phase of our study we will measure the circulating levels of PRRs (TLRs, CD14) in diabetic patients selected on the basis of type of diabetes (DT1 or DT2) and degree of retinopathy (no DR, DR and proliferative diabetic retinopathy PDR). These values will be correlated with circulating levels of inflammatory mediators (chemokines, cytokines, VEGF and sICAM-1) and with differential expression patterns of immune cells surface antigens selected within the NIRegs family of regulators. These experiments are designed based on the rationale that all the antigens that will be analyzed are also present on the surface of circulating immune cells and platelets (CD47). Because diabetes is a systemic disease alteration of circulating levels of inflammatory mediators as well as antigens associated with immune regulation may represent local expression pattern profiles.

Patients (contribution U.O.01)

All the procedures in patients will be performed in conformity with Ethical Committee recommendations and upon informed consent The patients will be selected as follows:

- 1) Patients with type I diabetes (DT1) (total=90 subjects) presenting evidence of
  - 1a) Diabetic retinopathy (DT1-DR, 30 subjects)
  - 1b) Proliferative diabetic retinopathy (DT1-PDR, 30 subjects)
- or
- 1c) with >10 years of diabetes but with no signs of DR (DT1-N, 30 subjects)
- 2) Patients with type II diabetes (DT2) (total=90 subjects) presenting evidence of
  - 2a) Diabetic retinopathy (DT2-DR, 30 subjects)
  - 2b) Proliferative diabetic retinopathy (DT2-PDR, 30 subjects)
- or
- 2c) with >10 years of diabetes but with no signs of DR (DT2-N, 30 subjects)
- 3) Volunteer healthy controls population (NP, 30 subjects)

DT1 and DT2 will be diagnosed following the indications of the WHO/ADA 2000. Diagnosis of DR will follow a complete eye exam comprised of visual acuity (EDTRS table), analysis of the anterior segment by biomicroscopy, ophthalmoscopic exam and fluoroangiography. Blood samples will be collected and processed to separate cellular components and the plasma and then will be delivered to U.O.02 for further processing and analysis.

Cytokine and chemokines assay (contribution U.O.02)

Circulating levels of chemokines and cytokines will be determined in plasma samples by using a Multiplex array, which allows the contemporaneous determination of 30 chemokines, 9 angiogenic factors and 30 different cytokines. These arrays are

analyzed by the use of a Bioplex Platform which is already available at the facility of U.O.02.

Assay for PRRs and NIRegs (contribution U.O.02)

The expression pattern profile of PRRs and NIRegs will be measured by fluorescence cell sorting (FACS analysis).

Second Phase (Contribution of U.O.01 and U.O.03)

We will analyze the molecular interactions and the intracellular signaling events associated with specific PRRs and NIRegs of which expression pattern changes in relation to disease stages. Particular attention will be addressed to the role of reactive oxygen species (ROS) in the modulation of the expression and/or the activity of these players. During this phase we will analyze the molecular interactions and the intracellular signaling events associated with specific PRRs and NIRegs of which expression pattern changes in relation to disease stages. Particular attention will be addressed to the role of reactive oxygen species (ROS) in the modulation of the expression and/or the activity of the analyzed PRRs and NIRegs. Indeed oxidative stress has been critically involved as key mediator of the deleterious effects of hyperglycemia on the retinal microvessels. Cellular (retinal cells exposed to elevated glucose concentration) and animal models (rat model of type 1 diabetes) will be used to disclose those molecular signaling events and bioassay (cell death, inflammation and selective gene expression by RNA silencing) will be employed to determine the involvement of these classes of proteins also in the regulation of blood retinal barrier (BRB), leukocytes entrapment in retinal microvessels.

Third phase (Contribution of U.O.02 and U.O.03)

We will test the activity of known negative modulators of inflammatory and oxidative responses in order to determine whether normalization of retinal vascular reactivity and retinal tissue responses are associated with a "normal" expression pattern of the analyzed PRRs and NIRegs. In vitro and in vivo models will be employed during this last phase. Treatments of cells and animals will be performed by using the drugs statins and the flavonoid trans-chalcone, both of these pharmacologically active classes of compounds have shown potential therapeutic values because of their documented ability to halt inflammation and oxidative stress in a number of tissues including the microvascular endothelium.

## **GENERAL TRANSFERIBILITY AND POTENTIAL IMPACT OF RESULTS (max 1/2 page)**

Diabetic retinopathy is the leading cause of blindness in working age adults worldwide, therefore, its impact on public health is tremendous. It is estimated that more than 300 million people worldwide are affected by diabetes and more than 10% of these patients will develop irreversible visual impairment within 15 years of diagnosis. The costs for supportive structures for the visually impaired patient are relevant and the progressive increase in the number of diabetic patients is also increasing the social and financial impact of this visual threatening disease. In addition, because DR affects a population of individuals during their most productive age, greatly increases the social significance of DR. Most of the known therapies are limited by invasive surgical procedures with considerable side effects. A better understanding of DR pathogenesis will provide new tools for therapeutic intervention as well as will provide opportunity for early diagnosis and treatment. In this context, the potential impact of our studies is significant.

## **OUTPUT(S) OF THE PROJECT (max 1/2 PAGE)**

(DESCRIBE THE OUTPUTS THAT THE PROJECT WILL PRODUCE SPECIFYING WHEN - DURING THE PROJECT - THEY WILL BECOME AVAILABLE Example(s) of output: ANIMAL MODELS, METHODOLOGIC WORK-PACKAGES, OTHER DELIVERABLES

The goal of this research proposal is two fold: 1) we want to elucidate the cellular and molecular mechanism involved in the maintenance of the retinal immune privilege, and 2) we want to characterize the effects of hyperglycemia on these mechanisms, therefore, identify new tools for therapeutic intervention and for early diagnosis. The studies conducted on patients will provide critical information that will be available, at least in part, at the end of the first year. Most of the studies, however, will provide complete information at the end of the two years of analysis, due to the complexity of the approaches as well as to the multiplicity of the biological targets investigated.

## **MILESTONES ALONGSIDE THE PROJECT**

(LIST UP TO TEN MILESTONES WITH RELEVANT RESULTS EXPECTED DURING THE PROJECT) (MAX 1 PAGE)

The overall goal of this research proposal is to characterize the molecular and cellular mechanisms involved in DR pathogenesis. Our immediate focus is the elucidation of the retinal innate immunity the effects of diabetes on this mechanism. A number of other objectives for our studies is listed below:

- 1) To establish a clinical relevance between PRRs expression pattern and initiation and progression of DR
- 2) To establish a clinical relevance between NIRegs expression pattern and initiation and progression of DR
- 3) To establish a direct cause-effect relationship between PRRs and NIRegs and hyperglycemia-induced retinal inflammation
- 4) To test drugs which could potentially control inflammation in DR by normalizing PRRs and NIRegs expression pattern

## **TIMETABLE OF THE PROJECT**

See attached file

## **OVERALL COSTS OF THE PROJECT**

2) Analysis of PRRs and NIREgs on retinal cells in culture

3) Animal studies using rat models of type I diabetes.

The following is a description of the different populations (patients, cell types and animals) in study for which U.O.01 will be responsible.

Patients selection (First Phase)

The patients will be selected as follows by U.O.01

1) Patients with type I diabetes (DT1) (total=90 subjects) presenting evidence of

1a) Diabetic retinopathy (DT1-DR, 30 subjects)

1b) Proliferative diabetic retinopathy (DT1-PDR, 30 subjects)

or

1c) with >10 years of diabetes but with no signs of DR (DT1-N, 30 subjects)

2) Patients with type II diabetes (DT2) (total=90 subjects) presenting evidence of

2a) Diabetic retinopathy (DT2-DR, 30 subjects)

2b) Proliferative diabetic retinopathy (DT2-PDR, 30 subjects)

or

2c) with >10 years of diabetes but with no signs of DR (DT2-N, 30 subjects)

3) Volunteer healthy controls population (NP, 30 subjects)

Cells

Cellular studies will be conducted by U.O.01 and U.O.03 which also will be in charge of maintaining the different cell types in culture and perform the appropriate treatments. In our experiments we will use human retinal endothelial cells (obtained from Dr. Bartoli Dept of Ophthalmology, University of South Carolina, USA), human retinal pigmented epithelial cells (hRPE, transformed cell lines from different sources, or low passage primary cultures from Dr. Richard Hunt, Department of Pathology, University of South Carolina, USA) and retinal organ cultures (from rat and bovine eye cups to be used as source of photoreceptors) and neuroglia preparation (the last two from U.O.03). Retinal cells in culture will be grown with media containing different doses of D-glucose (25mM = high glucose, HG; 5mM = normal glucose, NG) or L-Glucose as osmotic control (25mM, LG) will be transfected with different silencing RNAs using standard procedures or using viral carriers (adenovirus or retroviral cassettes). Dr Bartoli and members of U.O.03 have an extensive experience in gene delivery and retinal cell cultures preparation and expansion.

Animal model of diabetes. All animal experiments will be carried-out following protocols previously approved by our Institutional Committees and in conformity with the recommendations of the National Eye Institute of USA and the Association for Research in Vision and Ophthalmology for the humane use of animals in research. Experimental diabetes will be induced in Sprague Dawley rats by intraperitoneal injections (max 2, 60mg/Kg body weight) of streptozotocin in citrate buffer (0.1 mM, pH 4.5). Age-matched normoglycemic rats controls will be injected with saline. Blood glucose values equal to or higher than 350mg/dl will be considered diabetic.

## METHODS (max 1 pagina)

SPECIFY (whenever applicable): a) Patients/population; b) Intervention(s)/Analytical procedures; c) Indicator(s); d) Study design; e) Statistical analysis

) Patients selection All the procedures in patients will be executed in strict compliance with Ethical Committees recommendations and upon signed informed consent. The population of patients enrolled in the study by U.O.01 will be selected as described above. DT1 and DT2 will be diagnosed following the indications of the WHO/ADA-2000. Diagnosis of DR will follow a complete eye exam comprised of visual acuity (EDTRS table), analysis of the anterior segment by biomicroscopy, ophthalmoscopic exam and fluorescein angiography. Blood samples will be collected and processed to separate cellular components and the plasma and then will be delivered to U.O.02 for further processing and analysis.

2) Cellular studies. After treatments the cells will be analyzed for the following molecular parameters:

Oxidative stress parameters. (El-Remessy AB, JCS 2005). We will determine the levels of reactive oxygen species (ROS) using dichlorofluorescein imaging. Fluorescence emission will be analyzed in a fluorescence microplate reader. Samples pretreated with PEG-SOD or PEG-SOD+catalase are negative controls. Lipid hydroperoxides will be measured using a commercially available kit (Cayman). Nitrotyrosine levels will be assayed by dot-blot analysis.

Production of VEGF and ICAM-1.(Bartoli M, FASEB 2003). VEGF and ICAM-1 expression will be measured at mRNA and protein levels by Western blotting and quantitative Real Time PCR, respectively.

Apoptosis of retinal cells. (El-Remessy, JCS 2005) We will measure apoptosis of retinal cells in response to the different treatments with A-beta by assessing caspase 3 activity, and by observing alterations in cell nucleus morphology after staining of unfixed cells with bis-benzimide.

4. Animal studies

All the animal procedures will be performed in conformity with regulations of the U.S. National Institute of Health (NEI and Association for Research in Vision and Ophthalmology) for the humane use of laboratory animals in research. STZ-diabetic rats and age-matched normoglycemic control rats will be sacrificed after 4, 6, 8 and 16 weeks of diabetes. The retinas will be isolated and processed for molecular or histochemical analysis. Some animals will be treated with statins (fluvastatin 5mg/Kg/day) and with the flavonoid trans-chalcone (250m/Kg/day) administered orally.

Protein and RNA analysis (Bartoli, FASEB 2003, Platt DH FRBM 2006) Proteins and RNA will be extracted from frozen rat retinas and processed according specific protocols. We will measure retinal expression of the selected PRRs and NIREgs at both protein and mRNA levels by using Western blotting analysis and quantitative Real Time PCR (respectively). VEGF and ICAM-1 expression will be also measured as described above.

Immuno-histochemistry technique will be employed following for retinal immunolocalization of PRRs and NIREgs.

Oxidative stress parameters: We will determine the level of reactive oxygen species using DCF imaging of retinal frozen sections followed by Metamorph Imaging System analysis. Lipid hydroperoxide will be measured using a commercially available kit (Cayman) and nitrotyrosine formation by slot blot analysis.

Apoptosis. Detection of apoptotic cells in retinal frozen sections will be visualized by TUNEL assay and by measuring retinal layers thickening.

BRB Permeability Analysis in vivo will be measured as extravasation of bovine serum albumin (BSA)-Alexa-Fluor 488 conjugate (Molecular Probes). Densitometric analysis of the images will be obtained using Metamorph Imaging System and fluorescein density will be normalized versus plasma levels.

Ex vivo Retinal Leukocyte Entrapment. Leukocytes entrapment in retinal capillaries and arterioles will be visualized by perfusion through the right atrium with concanavalinA (Vector Labs).

Statistical analysis Comparisons among groups will be analyzed by one and two way ANOVA. Power analysis will be also performed to establish n values to achieve statistical significance with  $p < 0.05$ .

**PERSONNEL Dedicated to the Project activities (in person-months):**

Permanent staff	
Qualification* [ a ]	person-months dedicated
Medico Chirurgo Ricercatore	4

a: (giurista; statistico; economista; medico; ..)

Project staff	
Qualification* [ aaa ]	person-months dedicated
Medico Chirurgo Ricercatore	10
Biologo Ricercatore	10
Assistente Ricercatore	10

Equipment of participating units dedicated to the project:	
Type	Days/project-length
Freezer, refrigerators	250
Bioplex	150
Refrigerated centrifuges	90
Fluoroangiograph, ophthalmoscope,	200

**COSTS OF THE PARTICIPATING UNIT**

SPECIFY (whenever applicable): a) Patients/population; b) Intervention(s)/Analytical procedures; c) Indicator(s); d) Study design; e) Statistical analysis

Retinal preparations. The eyes will be enucleated from rats under deep anaesthesia and incised just dorsal of the ora serrata. The retina will be carefully peeled off the retinal pigment epithelium: the retinas immediately dissected will be used for protein studies, and for organ or cell culture experiments.

Retinal tissue cultures. Retinas will be isolated and cultered h a according to a modification of method described by Mertsch K. Retinas dissected from rats will be placed flat onto the insert of a 6-well Transwell plate (Corning, Corning, NY). The tissue will be covered with DMEM containing 10% FCS and antibiotics/antimycotics and placed in a tissue culture incubator at 37°C. The medium will contain glucose (either 5 or 30 mmol/l) or the correspondent osmotic control mannitol, or AGE preparations, for 48-72 hours. Specific inhibitors for different signal transduction pathways will be also used.

Cell cultures. Primary retinal cultures will be obtained from embryonic or neonatal rats. Cultures will be grown to obtain a mixed cell population, composed of neuronal, microglial and Müller cells, as previously described . In addition, experiments will be conducted on purified cell cultures, enriched in either neuronal, or microglial or Müller cells. Cells will be exposed to diabetic milieu and the different modulators, as for retinal tissue cultures,.

Immunolabelling. Eye bulbs will be fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), 0.12M in sucrose for 24h. After washes and cryoprotection in 30% sucrose, they will be embedded in OCT (optimal cutting temperature), frozen in liquid nitrogen and sectioned at a cryostat. Serial, 10-15-µm-thick sections will be collected on poly-L-lysine-coated glass slides, Cell cultures will be fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), 0.12M in sucrose, and permeabilized with 0.2% Triton X-100. Immunostaining will be performed using the following primary antibodies: Thy.1 for ganglion cells, vimentin for Müller cells, GFAP for Müller cells and/or astrocytes, GABA for amacrine and horizontal cells, rhodopsin for phoreceptors, occluding and ZO-1 for endothelial tight junctions, OX42 for microglial cells. Primary antibodies will be revealed by peroxidase- or fluorochrome-conjugated secondary antibodies. Microglia will also be evidenced with Griffonia simplicifolia lectin-peroxidase conjugate.

Morphometric analysis. Morphometric analysis will be performed in order to quantify modifications on digital images of immunolabelled cell cultures or sections using a specific software (Optilab, Graftek, France). Samples will be examined at a Nikon Optiphot microscope equipped with a color camera. At least four fields for each sample will be randomly chosen and recorded at 200x magnification, obtaining frames of 0.12 mm<sup>2</sup> by two separate observers. The immunostained area will be measured and the cells will be counted in each frame. The mean immunostained area per cell will be calculated. Measures obtained for each coverslip by the two observers will be averaged to produce a single mean value for each experiment

Apoptosis. After fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), 0.12 M in sucrose, apoptosis will be evaluated by the terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay and Hoechst stain. The TUNEL assay will be performed using the DeadEnd kit (Promega, WI, USA). DNA fragmentation will be visualized by diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. Cells undergoing apoptotic cell death will be quantified by counting TUNEL-positive nuclei in bright field at a Nikon Optiphot microscope. The presence of apoptotic profiles will also be assessed by staining with Hoechst 33258.

**PERSONNEL Dedicated to the Project activities (in person-months):**

Permanent staff	
Qualification* [ a ]	person-months dedicated
Medico Chirurgo Ricercatore	3
Biologo Ricercatore	3

a: (giurista; statistico; economista; medico; ..)

Project staff	
Qualification* [ aaa ]	person-months dedicated
Biologo Ricercatore	10

Equipment of participating units dedicated to the project:	
Type	Days/project-length
Laminar flow hoods, incubators	180
Microscopes, confocal microscopy	60
Refrigerators, freezer	190

culating immune cells obtained from the selected patients. Flow Cytometry analysis will be performed by U.O.02 for these studies.

## METHODS (max 1 pagina)

SPECIFY (whenever applicable): a) Patients/population; b) Intervention(s)/Analytical procedures; c) Indicator(s); d) Study design; e) Statistical analysis

### Multiplex ELISA-based assays

The expression levels of a number of different regulatory molecules (including chemokines, cytokines and growth factors) will be measured by means of a multiplex analysis based on the Luminex technology. Such analysis measures the expression of about 30 different factors simultaneously, with a sensitivity close to the one of an ELISA assay. Beads of different colors are "activated" with different antibodies and their binding to the corresponding antigens is measured by a fluorescent reaction.

The following cytokines and factors can be simultaneously measured, in a serum/plasma sample of human or mouse origin:

IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN- $\gamma$ , TNF- $\alpha$ , IL-1b, IL-5, IL-7, IL-12, IL-13, IL-17, G-CSF, MCP-1, MIP-1b, IL-1ra, IL-9, IL-15, Eotaxin, FGF-2, IP-10, MIP-1a, PDGF-bb, Rantes, VEGF.

A panel of 9 angiogenic growth factors can also be measured by a complementary technique, based on the simultaneous detection of growth factors present in serum/plasma/cell lysates and other samples, using specific antibodies immobilized on a plastic surface (Searchlight Technology).

Both techniques are currently used in the laboratories of U.O.02 and specific protocols have been generated to allow the accurate measurement of samples derived from blood and ocular samples.

### Flow Cytometry

The surface expression of PRRs and NIREgs on circulating immune cells and platelets will be determined using flow cytometry. For flow cytometry U.O.02 as available a Becton Dickinson LSRII instrument. Cell sorting is also possible by using a BD FACS Aria instrument. Whole blood anticoagulated with EDTA will be obtained from the selected patients. The cellular component will be isolated by dextran sedimentation and hypotonic lysis to eliminate red blood cells. Specific primary antibodies directed against PRRs and NIREgs will be employed to detect and quantify these surface antigens.

## PERSONNEL Dedicated to the Project activities (in person-months):

Permanent staff	
Qualification* [ a ]	person-months dedicated
Medico Chirurgo Ricercatore	4

a: (giurista; statistico; economista; medico; ..)

Project staff	
Qualification* [ aaa ]	person-months dedicated
Biologo Ricercatore	10

Equipment of participating units dedicated to the project:	
Type	Days/project-length
Bioplex platform	180
Becton Dickinson FACS Aria	120
Becton Dickinson LSR II	120
Beckman PF2D	60

## COSTS OF THE PARTICIPATING UNIT

Costs items and brief description	Total	Part covered by MoH funds [ a ]
1. Permanent staff	€ 30.000,00	None

**First Phase (24 months)**

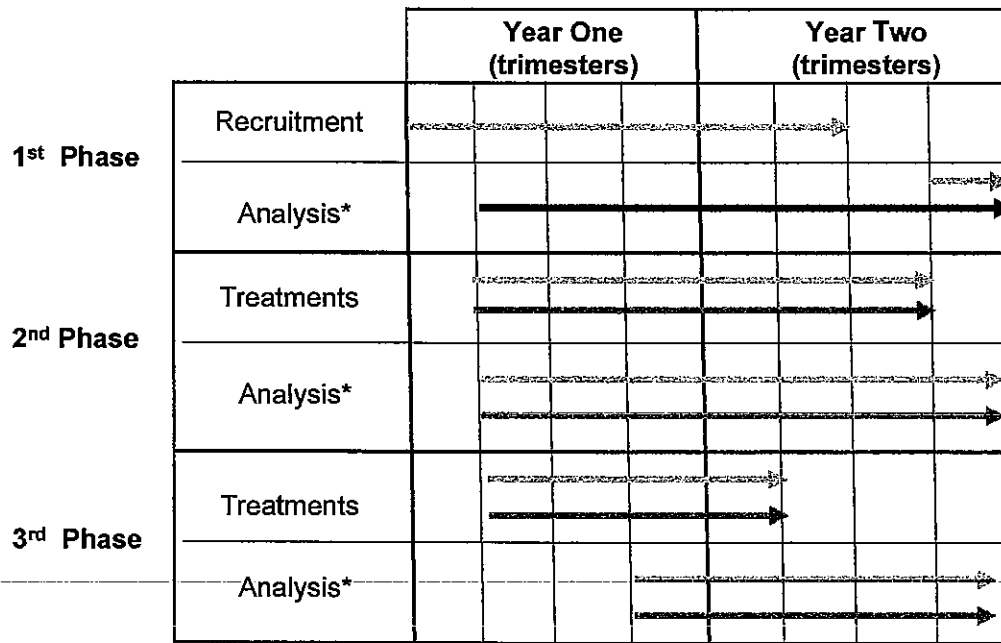
Patients Recruitment and Sample Collection (U.O.01) **18 months (1-18)**  
 Analytical Procedures and Data Analysis (U.O.02) **21 months (3-24)**

**Second Phase (24 months)**

Set Up Tissue cultures and treatments (U.O.01- U.O. 03) **18 months (3-21)**  
 Set Up animal models and treatments (U.O.01 - U.O. 03) **18 months (3-21)**  
 Analytical procedures and Data Analysis (U.O.01) **21 months (3-24)**  
 Analytical procedures and Data Analysis (U.O.03) **21 months (3-24)**

**Third Phase (24 months)**

Set Up animal models and treatments (U.O.01) **21 months (1-21)**  
 Analytical Procedures and Data Analysis (U.O.01) **12 months (12-24)**



- Contribution U.O.01
- Contribution U.O.02
- Contribution U.O.03

\*include data analysis

## Monica Varano

Fondazione G.B. Bietti per lo studio e la ricerca in Oftalmologia-IRCCS

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Monica Varano was born in Rome on August 12, 1958. She got the Medical degree on July 19, 1984 with the maximum score with laude. She got the diploma in Ophthalmology on July 15, 1988 with the maximum score with laude. Between 1982 and 1989 she attended the II Oculistic Clinic of La Sapienza University of Rome.

National Medical License Date and Number: 26/04/1985 Num 35928.

Starting in 1989 she works at the "Fondazione G.B.Bietti per lo Studio e la Ricerca in Oftalmologia", where she is the responsible for the department "Medical Retina". She is specialized in the diagnosis of vitro-retinal diseases (fluorescein angiography, indocyanine green angiography, micro-perimetry, photodynamic therapy with Visudyne). She attended courses abroad and in particular at the Ophthalmology Department of Laser and Angiography of Cretell University, Paris.

At present she is the scientific secretary of the S.I.R. (Italian Society of Retina). Starting 1997 she organized and conducted courses on fluorescein angiography, ICG, laser and photodynamic therapy at the Fondazione G.B.Bietti. She has been the international leader for the study of the age-related macular degeneration and she also was the team co-ordinator of the phase III multicentric study "VIT Verteporfin in Italy: An open label, multicentre, efficacy and safety study of the treatment of predominantly classic subfoveal choroidal neovascularization, secondary to age related macular degeneration using photodynamic therapy with Verteporfin" which started at the end of 1999 and began the use of photodynamic therapy in Italy. Actually she is principal investigator for **Mont Blanc** Trial "A 12 month randomized, double-masked, controlled, multicenter, phase II study assessing safety and efficacy of verteporfin(Visudyne photodynamic therapy administered in conjunction with Lucentis versus Lucentis monotherapy in patients with subfoveal choroidal neovascularization secondary to age-related macular degeneration"; **Lucentis+PDT in AMD** "12 months case series open study to assess the safety and efficacy of intravitreal injection of Lucentis used in combination with Visudyne in naïve subjects with subfoveal CNV to AMD"; **Quality of vision index (QOVI )study** "Observational multicenter study to evaluate quality of vision with the correlation of functional and psychometric VFQ-25 tests".

At present Monica Varano is the leader of a Reading Centre for the photodynamic therapy.

On the grounds of these researches the "Fondazione G.B.Bietti per lo studio e la ricerca in Oftalmologia" obtained the nomination of referring centre for the age related and myopic macular degeneration being able to use Visudyne vials furnished by the Region for research trials as per Ministerial Decree.

Between 1999-2001 she performed 80 operations of photodynamic therapy with Visudyne for macular degenerations at the Ophthalmic Hospital for VIT study, 870 operations of photodynamic therapy at the S.Domenico Hospital and 53 operations at the Via Livenza Centre using Visudyne vials furnished by the Region for research trials as per Ministerial Decree.

Since 1992 she is in charge of retinal angiography and laser therapy of S.Domenico Hospital where she performed 10.000 fluorescein angiographies and 3000 indocyanine green angiographies and 3000 argon and krypton treatments for peripheral retinal pathologies, vascular pathologies and macular degenerations. In order to carry out the project for the establishment of a study centre of maculopathies Monica Varano organizes angiography courses, laser and photodynamic therapy courses at Via Livenza, basic theoretical-practical courses, advanced interactive courses based on clinical cases. Dr.Varano was also the teacher for about 100 courses mostly on functional diagnosis, angiographic diagnosis and laser therapy of the vascular and degenerative diseases of chorioretinal and macular region. She is the author of about 80 scientific publications both on national and international magazines, regarding the diagnosis and treatment of eye vasculopathies, with a particular interest to the degenerative diseases of the retina. Dr.Monica Varano has cooperated to the drawing up of some chapters of various books and she is the author of some monographies.

She is member of the following Scientific Societies:

"Società Oftalmologica Italiana" (Italian Society of Ophthalmology)

Italian Society of Retina (SIR)

Italian Society of Laser in Ophthalmology (SILO)

The Association for Research in Vision and Ophthalmology (ARVO)

Jules Gonin Club

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See the original document for original contributions.

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## *Curriculum vitae et studiorum*

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### EDUCATION

1991: Ph.D. in Pharmacology (with full remarks).  
1991: Specialization in Ophthalmology, University of Napoli (with full remarks, 50/50 cum lauda).  
1987-Graduation in Medicina e Chirurgia, University of Napoli (with full remarks, 110/110 cum lauda and plauso).

### PROFESSIONAL EXPERIENCE

2003 to present: Senior Researcher at Dipartimento di Ematologia, Oncologia e Medicina Molecolare, Istituto Superiore di Sanità, Roma  
1996- 2003 Staff Scientist in the Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata, Rome, Italy  
March 1991 - April 1996: Senior Scientist at Istituto Mario Negri Sud  
1994-1996: Visiting Scientist at the Thomas Jefferson University, Jefferson Cancer Institute, Philadelphia  
- 1988-1991: Ph.D. training in Pharmacology, Istituto Mario Negri Sud

### LIST OF RECENT PUBLICATION RELEVANT FOR THE PROJECT

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