PATHOLOGY-SPECIFIC PLATFORMS

CELLULAR ASSAY PACKAGES FOR DRUG DISCOVERY PROJECTS ON SELECTED PATHOLOGICAL INDICATIONS
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Autoimmune Diseases

RHEUMATOID ARTHRITIS

Background
Rheumatoid arthritis is a chronic systemic inflammatory disease of unknown aetiology, affecting approximately 1% of adult general population.

The triggering event in the pathogenesis of RA is thought to be the activation of T cells by unknown antigens, which results in proliferation of synoviocytes, endothelial cells and other proinflammatory cells, as well as induction of autoantibody formation and secretion of proinflammatory cytokines and proteases.

Pathology Model
Human fibroblasts treated with synovial fluid derived from patients (patient stratification according to therapeutical indication is possible) will be used as a model of rheumatoid arthritis. In our lab we have recently demonstrated that exposure to synovial fluid from patients at different time points (i.e. from 24hours to 7 days) better mimics in vitro the RA pathophysiological condition, with respect to classical treatment with TNFα (Casnici et al. Mediators of Inflammation, 2014).

Readouts
The following parameters will be taken into consideration:

- **Immunofluorescence**: qualitative evaluation of the expression of cytoskeletal markers in order to evaluate cytoskeletal rearrangement.
- **Inflammatory cytokines**: quantitative evaluation of the production of selected panels of pro inflammatory cytokines by multiplex analysis (i.e. IL6, IL10, IL15, TNFα ecc...)
- **Gene expression**: quantitative evaluation of the expression of selected panels of genes of interest (i.e. IL6, IL10, IL15, TNFα etc....) in challenged cells.
- **Morphological analysis**: qualitative analysis of alterations in cell morphology in challenged cells;
- **Intracellular calcium dynamics**: time-lapse acquisition of intracellular calcium variations following exposure to synovial fluid in the presence/absence of selected Client’s compounds.
- **Proliferation**: quantitative evaluation of fibroblasts proliferation following exposure to synovial fluid, and modulation by selected Client’s compounds.
MULTIPLE SCLEROSIS

Background
One of the many open questions in multiple sclerosis research is whether inflammation in the CNS is initiated by an autoimmune attack, triggered by unidentified environmental factors, or represents a response to axonal degeneration and myelin degradation secondary to processes that are intrinsic to the CNS. Lesions characterized by microglial activation and hypoxia-like characteristics, as well as cortical lesions and the slowly progressive chronic phase of the disease, are likely driven by activated myeloid cells. However, at present it is not clear what keeps the microglial cells activated. It is possible that the T cells found throughout the CNS of patients with multiple sclerosis provide constant stimuli, i.e. by pro-inflammatory cytokines, which activate microglia.

Pathology Model

In order to recreate an in vitro pathological scenario mimicking MS condition, microglia cells will be exposed to detrimental challenges known to activate microglia in the neuroinflammatory scenarios leading to MS (i.e. hypoxia and LPS exposure) and functional parameters will be quantitatively evaluated. Moreover, the effect of the CLIENT’S compound in the modulation of the detrimental scenario will be quantitatively monitored in a dose response fashion.

Readouts
The following parameters will be quantitatively evaluated:

Biochemical Characterization
- Cell viability and toxicity: (i.e. MTT assay)
- Cell migration: (i.e. chemotactic chamber)
- Total ROS production: (i.e. DCF-DA fluorescent assay)
- Phagocytic activity: (i.e. Vybrant Assay)
- Mitochondrial damage: (i.e. HCS Mitochondrial Health assay)
- Cytokine production by inflammatory panel on multiplex ELISA: (i.e. IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, TNF-α, IFN-γ, EGF, MCP-1)
- NO production: (i.e. Griess assay)

Functional Characterization
- Membrane permeability (i.e. Yo-Pro1 uptake)
- Microvesicle shedding
Background
The Blood-Brain Barrier (BBB) is a highly selective permeability barrier that separates the circulating blood from the brain extracellular fluid in the central nervous system (CNS). The Blood-Brain Barrier allows the passage of water, some gases, and lipid soluble molecules by passive diffusion, as well as the selective transport of molecules such as glucose and amino acids that are crucial to neural function.

Chronic inflammatory states, as those typically occurring in aging diseases, lead to tissue degeneration and membrane permeability thus favouring immune cell crosstalk within the central nervous system. This crosstalk is crucial in the onset of neuroinflammatory events which characterize the early steps of neuronal degeneration.

Pathology Model
A model of in vitro Blood-Brain Barrier (BBB) using primary rat brain endothelial cells will be used (Maria Deli ref.). BBB will be subjected to oxygen glucose deprivation (OGD) to mimic stroke conditions and beside ROS, cell viability and eNOS functionality, BBB integrity will be evaluated.

Readouts
The following morphological, biochemical and functional parameters will be quantitatively assayed:

1. **Biochemical Characterization**
   - Cell viability and toxicity: (i.e. MTT assay)
   - Mitochondrial damage (i.e. HCS Mitochondrial Health assay)
   - Total ROS production (i.e. DCF-DA fluorescent assay)
   - NADPH-dependent superoxide formation (i.e. Dihydroethidium (DHE) staining).
   - Inflammatory profile: a detailed analysis of pro inflammatory and angiogenic factor production will be characterized (i.e. IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL10, VEGF, TNF-α, IFN-γ, EGF, MCP-1 etc...).

2. **Morphological Characterization**
   - The morphological analysis of membrane integrity will be monitored by means of confocal microscopy on immunofluorescent-labeled cells; zona occludens and tight junction will be quantitatively evaluated.

3. **Functional Characterization**
   - BBB permeability: i.e. FITC-dextran tracer.
   - Trans-endothelial electric resistance.
ENDOTHELIAL BARRIER

Background
The key mechanism of endothelial dysfunction is the imbalance of endothelium-derived nitric oxide (NO) production and reactive oxygen species (ROS) generation, resulting in a decline in the bioavailability of NO and excessive accumulation of ROS. This finally leads to oxidative stress and cellular injuries.

Pathology Model
Endothelial cells (i.e. Human Umbilical Vein Endothelial Cells HUVEC-2 or aortic endothelial cells HAEC) will be exposed to metabolic stress (i.e. oxygen glucose deprivation) in the presence/absence of the CLIENT’s compound.

Both static and dynamic in vitro microfluidic models will be taken into consideration in order to evaluate endothelial barrier integrity.

Readouts
The following quantitative parameters will be taken into consideration:

1. Molecular Biology
Gene expression profile: endothelial cells or aortic endothelial cells will be cultured in vitro and tested for their angiogenic properties in the presence/absence of CLIENT’s compound. Ranibizumab will be used as control. The expression of main angiogenic molecules will be quantitatively evaluated.

2. Biochemical Characterization
   - Total ROS production (i.e. DCF-DA fluorescent assay)
   - NADPH-dependent superoxide formation (i.e. Dihydroethidium (DHE) staining)
   - Cell viability and toxicity: (i.e. MTT assay)
   - Mitochondrial damage (i.e HCS Mitochondrial Health assay)
   - Inflammatory profile. A detailed analysis of pro inflammatory and angiogenic factor production will be characterized (i.e. IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, TNFα, IFN-γ, EGF, MCP-1 etc...).
Background

Alzheimer’s disease (AD) is the most common cause of dementia among ageing population. However, the mechanisms regulating synaptic dysfunction in AD are not fully understood, and require an in depth analysis of the crosstalk mechanisms ongoing during an inflammatory event, between the neuronal and non-neuronal cells present in the microenvironment, playing a crucial role in disease onset.

Pathology Model

In order to evaluate the CLIENT’s compound modulatory activity of the neuroinflammatory events leading to neurodegeneration in AD, hippocampal neurons will be exposed to either Abeta oligomers, or to glial Abeta-primed glial medium. Both the direct effects on hippocampal neurons as well as the microglial-mediated effects will be taken into consideration.

Primary hippocampal neurons in culture
Readouts

The following parameters will be analyzed:

**Step 1 - Direct Neuronal Damage:**

Primary neuronal cultures isolated from Sprague Dowley rats will be exposed to Abeta oligomers in the presence / absence of CLIENT’s. The following parameters will be analyzed:

1. **Morphological Characterization**
   - Qualitative evaluation of neuronal cytoskeletal disruption
   - Quantitative evaluation of dendritic branching
   - Quantitative evaluation of neurite elongation modulation

2. **Biochemical Characterization**
   - Quantitative evaluation of neuronal cell death (i.e. PI/DAPI/Calcein AM)
   - Quantitative evaluation of caspase activation (i.e. 3 or 8 or 9)
   - Quantitative evaluation of DNA degradation (i.e. TUNEL staining)

3. **Analysis of Oxidative Stress**
   - Quantitative evaluation of total ROS production
   - Quantitative evaluation of NO production

**Step 2 - Glial Pro-Inflammatory Phenotype:**

Microglia cells (i.e. BV2) will be challenged with Abeta oligomers in the presence/absence of CLIENT’s compound. The following parameters will be quantitatively monitored on glial cells:

1. **Biochemical characterization**
   - Quantitative evaluation of metabolic activity
   - Inflammatory cytokine production: (i.e. IL1 beta, TNFalpha, IL6)
   - Total ROS production
   - Quantification of NO production

2. **Functional characterization**
   - Quantitative evaluation of phagocytic potential
   - Quantitative evaluation of intracellular calcium dynamics
   - Quantitative evaluation of membrane permeability
   - Quantitative evaluation of microvesicle shedding
AMYOTROPHIC LATERAL SCLEROSIS

Background
ALS is a fatal neurodegenerative disease that is characterized by a progressive, selective loss of motor neurons (MN) in brain and spinal cord. The mechanisms of selective and age-dependent MN degeneration in ALS have not been defined. Recent studies suggest that both the sustained glutamate excitotoxicity as well as chronic hypoxic conditions results in increased intracellular oxidative toxicity which contributes to death of MN. The molecular pathways remain largely unknown.

Pathology Model
Compounds will be administered to a well-established motor neuron cell line (NSC-34) differentiated into motor neurons by exposure to Retinoic acid and low serum levels for 4 days as reported in literature. NSC-34 is produced by fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma. These cells express many properties of motor neurons, including choline acetyltransferase, acetylcholine synthesis, storage and release and neurofilament triplet proteins. Moreover, NSC34 spinal cord motor neurons expresses glutamate receptor proteins and generate action potentials. NSC34 neurons have been widely used to study mechanisms of neuron signalling and neuron degeneration.

Cells will be challenged with high glutamate concentrations (2-10mM) in the presence of compounds (concentrations will be jointly decided with the Client).

Readouts
Following stimulation, cells will be quantitatively assayed for the following parameters

- Quantitative evaluation of cell viability
- Quantitative evaluation of mitochondrial damage
- Quantitative evaluation of caspase 3 activation
- Quantitative evaluation of neurofilament phosphorylation by in-cell western analysis
Batten’s Disease

Background
Batten’s disease is a genetic, rare, and fatal lysosomal storage disorders. One of the main characteristic of Batten’s disease is the neurodegeneration of a brain area due to neuroinflammation. In fact, abnormal interaction between neurons and microglia, the resident immune cells within the central nervous system, has been identified as a crucial step in the onset of the pathology. Currently, microglia’s role in the onset, development, and therapy of Batten’s disease remains unfortunately unknown. This is a critical issue since activated microglia not only may either positively or negatively influence neuronal survival, via the production of growth factors or pro-inflammatory mediators, but it may also act as an ideal target for early diagnosis.

Pathology model
By culturing microglia, astrocytes and neurons on separated microchambers interconnected by microfluids, it is possible to recreate in vitro the different combinations of intercellular crosstalk in different pathophysiological scenarios, and thus understand in detail the specific contribution of each cell type in the complex biological crosstalk of a specific disease scenario such as Batten’s disease.

Microglia, astrocytes and neurons from different brain areas (i.e. cortex and hippocampus) of either Cln3−/− or wt animal models will be cultured in separate chambers in the microfluidic platform.

Readouts
All cell types in separate chambers of the microfluidic platform will be monitored for viability and oxidative stress, in order to compare standard culturing conditions on glass coverslips with culturing of cells on microdevices, as previously reported for wt neurons.

In particular, all cell types in separate chambers of the microfluidic platform will be monitored for proliferation, activation state (i.e. membrane permeability), motility, phagocytic activity, microvesicle shedding, mitochondrial damage (i.e. HCS assay) and inflammatory cytokine production (multiplex ELISA). The most physiologically relevant conditions as well as more relevant readouts for the evaluation of Batten disease model in vitro will be identified by comparing wt and KO data.
ISCHEMIA

Pathology model
Primary cortical neurons isolated from Sprague Dawley rats will be exposed to oxygen glucose deprivation (OGD) for 1h in presence absence of CLIENT’s compound and to reoxygenation for 24 hours as a model of cerebral ischemia.
Complicating the biological scenario, as cerebral microenvironment plays a crucial role in pathogenesis of cerebral ischemia, neurons will be put in microfluidic communication with glia from different brain areas (such as cortex or hippocampus) and then subjected to OGD and reoxygenation following the protocol described above, in presence absence of CLIENT’s compound.

Readouts
After reoxygenation the following parameters will be evaluated:

Step 1 - Direct Neuronal Damage:
- Morphological characterization
  - Qualitative evaluation of neuronal cytoskeletal disruption
  - Quantitative evaluation of dendritic branching
  - Quantitative evaluation of neurite elongation modulation
- Biochemical characterization
  - Quantitative evaluation of neuronal cell death (i.e. PI/DAPI/Calcein AM)
  - Quantitative evaluation of caspase activation (i.e. 3 or 8 or 9)
  - Quantitative evaluation of DNA degradation (i.e. TUNEL staining)
- Analysis of oxidative stress
  - Quantitative evaluation of total ROS production
  - Quantitative evaluation of NO production
  - Mitochondrial membrane potential (MMP) (JC-10 dye by flow cytometry)

Step 2 - Glial Pro-Inflammatory Phenotype:
- Biochemical characterization
  - Quantitative evaluation of metabolic activity
  - Inflammatory cytokine production: (i.e. IL1 beta, TNFalpha, IL6)
  - Total ROS production
  - Quantification of NO production
- Functional characterization
  - Quantitative evaluation of phagocytic potential
  - Quantitative evaluation of membrane permeability
  - Quantitative evaluation of microvesicle shedding
PARKINSON’S DISEASE

Background
The precise cause of Parkinson’s Disease (PD) is unknown, but there is a consensus that an inflammatory event is involved in the initiation of neurodegeneration, and that chronic neuroinflammation is a sustaining and exacerbating reason for the loss of the dopaminergic neurons.

Recent findings have revealed that the functional interaction between astrocytes, microglia and neurons govern both the sequence of inflammatory events (i.e. cascades of inflammatory mediators) and the pathological outcome (damage or absence of damage) to neurons. Among the proinflammatory molecules, cytokines play a central role in the self-propagation of neuroinflammation in PD.

In spite of the evidence indicating that inflammation might influence the pathogenesis of PD, there is considerable debate concerning which molecules are synthesized and released, how astrocytes and microglia interact reciprocally and with neuronal cells within the neurovascular unit, and how the kinetic responses and the precise connectivity of the inflammatory cascades are regulated.

Pathology Model

In order to evaluate the CLIENT’s compound modulatory activity of the neuroinflammatory events leading to dopaminergic neuron degeneration, physiologically relevant cell cultures of dopaminergic (DA) neurons will be either directly intoxicated with 6-OHDA or exposed to 6-OHDA primed glial medium. Both the direct effects on dopaminergic neurons as well as the microglial-mediated effects will be taken into consideration.
Readouts

The following parameters will be analyzed:

**Step 1 - Direct Neuronal Damage:**

- **Morphological characterization**
  - Qualitative evaluation of neuronal cytoskeletal disruption
  - Quantitative evaluation of dendritic branching
  - Quantitative evaluation of neurite elongation modulation

- **Biochemical characterization**
  - Quantitative evaluation of neuronal cell death (i.e. PI/DAPI/Calcein AM)
  - Quantitative evaluation of caspase activation (i.e. 3 or 8 or 9)
  - Quantitative evaluation of DNA degradation (i.e. TUNEL staining)

- **Analysis of oxidative stress**
  - Quantitative evaluation of total ROS production
  - Quantitative evaluation of NO production

**Step 2 - Glial Pro-Inflammatory Phenotype:**

- **Biochemical Characterization**
  - Quantitative evaluation of metabolic activity
  - Inflammatory cytokine production: (i.e. IL1 beta, TNFalpha, IL6)
  - Total ROS production
  - Quantification of NO production

- **Functional Characterization**
  - Quantitative evaluation of phagocytic potential
  - Quantitative evaluation of membrane permeability
  - Quantitative evaluation of microvesicle shedding
Background
Breast cancer is the second most common cancer in women. It can occur in both men and women, but it is very rare in men. Each year there are about 2,300 new cases of breast cancer in men and about 230,000 new cases in women. Many solid tumor types, including breast cancer, exhibit a functional hierarchy of cancer cells of which only a small subpopulation of replenishing stem-like cells (CSCs) can give rise to the differentiated cells that comprise the bulk tumor. The treatments today available for breast cancer are not able to deplete CSCs population in breast cancer. This could be one of the main reasons for the failure of pharmacological treatments in most cases.

Pathology Model

Human breast cancer cells will be cultured in presence or absence of CLIENT’s compounds. After treatment with the compounds, cells will be cultured in a specialized Spheroid Formation ECM to drive spheroid formation of cells. Upon completion of spheroid formation, the spheroid is embedded in an invasion matrix composed of basement membrane proteins. This matrix forms a hydrogel network on which invasive cells can travel. This model of 3D spheroid culture will allow to evaluate the CLIENT’s compounds effect on spheroid formation (index of tumour growth) and invasion (index of metastatic potential).

Readouts
The following parameters will be evaluate:

**Step 1 – Sphere formation and invasion:**
- Quantification of size of neurospheres as index of tumour formation
- Quantification of cell invasion by image analysis software

**Step 1 – GSCs characterization after sphere disgregation:**
FACS analysis for GSCs markers (CD44+/CD24low/ESA+)

Mammosphere formation

Mammosphere invasion
GLIOBLASTOMA

Background
Glioblastoma multiforme (GBM) is a IV grade astrocytoma, a highly malignant and aggressive primary brain tumor. Despite progress in clinical therapies and the understanding of molecular mechanisms underlying GBM pathogenesis, the prognosis of this tumor remains unknown. Currently, anti-neoplastic treatment combines chemotherapy, temozolomide (TMZ), radiotherapy and resectional surgery. Despite its frontline status, GBM patients commonly exhibit resistance to TMZ treatment. The identification of tumor cells with stem-like traits from human glioblastoma tissues and cell lines reveals a new and promising therapeutic target against GBM. This cell type, called glioblastoma stem cells (GSCs), is considered playing a critical role in GBM initiation, progression, and recurrence.

Pathology Model
Human glioblastoma cells will be cultured in presence or absence of CLIENT’s compounds. After treatment with the compounds, cells will be cultured in a specialized Spheroid Formation ECM to drive spheroid formation of cells. Upon completion of spheroid formation, the spheroid is embedded in an invasion matrix composed of basement membrane proteins. This matrix forms a hydrogel network on which invasive cells can travel. This model of 3D spheroid culture will allow to evaluate the CLIENT’s compounds effect on spheroid formation (index of tumour growth) and invasion (index of metastatic potential).

Readouts
The following parameters will be evaluate:

Step 1 – Sphere formation and invasion:
- Quantification of size of neurospheres as index of tumour formation
- Quantification of cell invasion by image analysis software

Step 1 – GSCs characterization after sphere disgregation:
FACS analysis for GSCs markers (CD133 and nestin)
CARDIOGENIC SHOCK

Background
Cardiogenic shock is a life-threatening medical condition characterized by insufficient perfusion of tissue to meet the demands for oxygen and nutrients. The condition involves increasingly more pervasive cell death from oxygen starvation (hypoxia) and nutrient starvation (e.g. low blood sugar). Because of this, it may lead to cardiac arrest (or circulatory arrest), which is an abrupt stopping of cardiac pump function.

Pathology model
Primary cardiomyocytes will be isolated from the heart of P2 Sprague Dawley rats. Cells will be cultured in vitro, either in the presence/absence of primary activated macrophages, and challenged with 12 hours oxygen glucose deprivation followed by 2 hours of reperfusion. Cells will be either cocultured in static conditions, or tested in dynamic micro environmental conditions, flushing activated macrophages on primary cardiomyocytes.

Readout
The following parameters will be analyzed:

- Quantitative evaluation of cell metabolism
- Quantitative evaluation of cell survival
- Quantitative evaluation of mitochondrial damage
- Expression of troponin C
- Qualitative evaluation of cell morphology
- Quantitative evaluation of selected genes of interest
- Quantitative evaluation of beating rate
- Quantitative evaluation of intracellular calcium dynamics
SEPTIC SHOCK

Background
Septic shock is a medical condition as a result of severe infection and sepsis, though the microbe may be systemic or localized to a particular site results in inadequate blood supply to the body’s tissues, resulting in ischemia and organ dysfunction. Cardiac dysfunction in sepsis is characterized by decreased contractility, impaired ventricular response to fluid therapy, and in some patients ventricular dilatation. Current data support a complex underlying physiopathology with a host of potential pathways leading to myocardial depression. Circulating factors such as cytokines (TNF-alpha, IL-1beta), lysozyme c, endothelin-1 have direct inhibitory actions on myocyte contractility. Nitric oxide has a complex role in sepsis-induced cardiac dysfunction

Pathology model
Primary cardiomyocytes will be isolated from the heart of P2 Sprague Dawley rats. Cells will be cultured in vitro, either in the presence/absence of primary activated macrophages, and challenged with either MMP9, Elastase or LPS. Cells will be either cocultured in static conditions, or tested in dynamic microenvironmental conditions, flushing activated macrophages on primary cardiomiocytes.

Readout
The following parameters will be analyzed:

- Quantitative evaluation of cell metabolism
- Quantitative evaluation of cell survival
- Quantitative evaluation of mitochondrial damage
- Expression of troponin C
- Qualitative evaluation of cell morphology
- Quantitative evaluation of selected genes of interest
- Quantitative evaluation of beating rate
- Quantitative evaluation of intracellular calcium dynamics
VASOGENIC EDEMA

Background
Vasogenic edema is characterized by an increase in extracellular fluid volume due to increased permeability of brain capillary endothelial cells to macromolecular serum proteins (e.g., albumin). The key mechanism of endothelial dysfunction is the imbalance of endothelium-derived nitric oxide (NO) production and reactive oxygen species (ROS) generation, resulting in a decline in the bioavailability of NO and excessive accumulation of ROS. This finally leads to oxidative stress and cellular injuries.

Normally, the entry of plasma protein-containing fluid into the extracellular space is limited by tight endothelial cell junctions, but in the presence of massive injury, there is increased permeability of brain capillary endothelial cells to large molecules. Vasogenic edema can displace the brain hemisphere and, when severe, lead to cerebral herniation.

Pathology Model
Endothelial cells will be exposed to metabolic stress (i.e. oxygen glucose deprivation) in the presence/absence of the CLIENT’s compounds.

Readouts
The following parameters will be taken into consideration:

- Total ROS production (i.e. DCF-DA fluorescent assay)
- Cell viability and toxicity: (i.e. MTT assay)
- Mitochondrial damage (i.e HCS Mitochondrial Health assay)
- Inflammatory profile. A detailed analysis of pro inflammatory and angiogenic factor production will be characterized (i.e. IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, TNF-α, IFN-γ, EGF, MCP-1 etc...).
AGE-RELATED MACULAR DEGENERATION

Background
Recent evidence implicates the immune system in the development of Age-Related Macular Degeneration (AMD), as immune-related proteins are found in drusen from AMD eyes. Excessive activation of inflammatory and immunological cascade with subsequent induction of damage, persistent activation of resident immune cells, accumulation of byproducts that exceeds the normal capacity of clearance giving origin to a chronic local inflammation, alterations in the activation of the complement system, infiltration of macrophages, T-lymphocytes and mast-cells from the bloodstream, participate in the mechanisms which originate the drusen.

Pathology Model
In order to evaluate the CLIENT's compound ability to modulate retinal degeneration in an in vitro model of AMD, a human line of retinal pigment epithelial cells, microglia, macrophages and T-lymphocytes (all of human origin) will be cultured in separate chambers in a microfluidic platform. Optimal cell density will be evaluated for each cell population. Cell purity and viability will be tested by specific markers. Immunoreactive cells will be activated by either LPS or IFN gamma and put in microfluidic communication soluble factor, cell-cell mediated crosstalk. Particular attention will be given to the quantification of oxidative stress. Measuring oxidative stress is crucial for the pathogenesis of AMD; in fact, recent evidence showed that the autoimmunity component might be related to oxidative stress. Furthermore, complement components will be quantified in supernatant and cellular lysates. It has been postulated a role for an alteration of the complement pathways in the pathogenesis of AMD. In fact, drusen include elements of the complement system belonging to all pathways: classical, alternative and lectin pathways. In addition, a strong association between advanced AMD and high levels of the complement components C3, CFB, CFI, CFH, and factor D (CFD) and activation fragments Bb, C3a, C5a, iC3b, and SC5b-9 has been found in the peripheral blood of patients.

Readouts
The following parameters will be taken into consideration:

- Cell purity (immunocytochemistry)
- Cell viability (MTT assay)
- Deposition of druse (i.e. quantification by electron microscopy)
- Quantitative evaluation of proliferation rate
- Quantitative evaluation of cell's activation state (i.e. membrane permeability)
- Mitochondrial damage (i.e. HCS Mitochondrial Health assay)
- NO production (i.e. Griess assay)
- Total ROS production (i.e. DCF-DA fluorescent assay)
- Cytokine production (multiplex ELISA)
Inflammation

CHRONIC ABACTERIAL PROSTATITIS

Background
Chronic abacterial prostatitis, or chronic pelvic pain syndrome (CP/CPPS), is a prevalent, yet poorly understood entity characterized by pelvic or perineal pain, irritative voiding symptoms, and sexual dysfunction. It is the most common urologic diagnosis in men less than 50 years of age. Chronic abacterial prostatitis accounts for more than 90–95% of clinical prostatitis. The impact of this condition on health status and quality of life is significant, as CP/CPPS patients demonstrate impaired quality of life and daily functioning impairment. Despite its prevalence and sickness impact, little remains known about its etiology and treatment.

The pathogenesis of CP/CPPS is still poorly understood but neurologic, immunologic, and endocrine dysfunctions have been proposed to be involved in disease development. Recently, evidence indicating an autoimmune component in the pathogenesis of CP/CPPS has begun to emerge. Patients with CPPS often have white blood cells in expressed prostatic secretions (EPS), implicating inflammation as a potential etiologic mechanism. In these patients, inflammatory infiltrates are exclusively mononuclear, consisting predominantly of lymphocytes, with a virtual absence of neutrophils, basophils, eosinophils, and mast cells.

These evidence are further supported by the observation that patients with CP/CPPS show higher levels, compared to controls, of seminal plasma proinflammatory cytokines, such as interleukin 1b, IL-6, and tumour necrosis factor a, and chemokines such as IL-8.

Pathology Model
In order to induce a chronic inflammation in prostatic cells mimicking CP/CPPS, activated macrophages (derived from human monocytes)-conditioned medium will be applied to normal human prostatic cells. The U937 monocyte cell line will be cultured in appropriate medium. To achieve macrophage differentiation and cytokine production, cells will be treated with phorbol acetate at a final concentration of 16 nM for 16 h. After allowing the cells to rest for 2–3 h, lipopolysaccharide (LPS) will be added (10 ng/ml), and the cells will be incubated for 24 h. The human prostatic normal cells (RWPE-1) will be put in communication with activated macrophages for 4 wks, in order to induce chronic inflammation, either in the presence or absence of CLIENT’s compounds.

Readouts
The following parameters will be taken into consideration:
- Quantitative evaluation of cell vitality
- Quantitative evaluation of cell proliferation
- Quantitative evaluation of caspase activity
- Quantitative evaluation of inflammatory mediators by multiplex ELISA
- Quantitative evaluation of oxidative stress by Total ROS analysis
IMMUNE CELL ACTIVATION

Macrophage Activation
Human PBMCs will be collected from blood samples of healthy donors and M2 cells will be isolated by beads separation protocol. Isolated cells will be then exposed either to the CLIENT’s proprietary molecules for defined timings or to control Antibody, along with a positive (i.e. LPS) or negative (to be defined by CLIENT) control.

Stimulated cells will be then processed for the following activation parameters:

1. **Molecular Biology**
   - Quantitative gene expression of target inflammatory genes (max 10 genes)

2. **Biochemical Characterization**
   - Inflammatory cytokine production (i.e. IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, VEGF)
   - Total ROS production (i.e. DCF-DA fluorescent assay)
   - Phagocytic activity (i.e. Vybrant Assay)
   - Mitochondrial damage (i.e HCS Mitochondrial Health assay)
   - Cell viability and toxicity: (i.e. MTT assay)

Mast Cells Activation
Mast cell activation will be monitored by quantitative analysis of allergen-induced histamine release. In particular, the following parameters will be measured:

1. **Antigen induced release of β−hexosaminidase**
Mast cells play essential roles in provoking the pathogenesis of allergic reactions via the regranulation process, measuring the degree of degranulation reflects the level of mast cell activation.

β−Hexosaminidase released by these cells during this process has been reported to be a suitable marker for determining the degree of degranulation (Guo et al 2009).

2. **Antigen-induced release of Tryptase**
Degranulation, the secretion of cytoplasmic granules, is a key step in the inflammatory response of leukocytes (e.g. basophils, neutrophils, eosinophils, and mast cells). In addition to histamine, these secretory granules contain many proinflammatory mediators including heparin, cytokines, chemokines, and many proteases.

Tryptase, a tetrameric serine proteinase, has emerged as the major component of mast cell granules, comprising up to 20% of the total protein of mast cells derived from lung, colon and skin tissue (he et al 1998, 2004). Because it is stored almost exclusively in mast cells, tryptase is a popular indicator of mast cell activation and a target for therapeutic intervention in allergic diseases.
INTERSTITIAL CYSTITIS

Background
Bladder pain syndrome (BPS)/interstitial cystitis (IC) is a chronic pain syndrome characterized by pain, pressure, or discomfort perceived to be bladder-related and with at least one urinary symptom. Although the etiology and pathogenesis of BPS/IC are unknown, numerous theories including infection, inflammation, autoimmune disorder, toxic urinary agents, urothelial dysfunction, and neurogenic causes have been proposed. In addition to the uroepithelial disruption, visceral inflammation also remains a central pathological process in BPS/IC. Inflammation within the urinary bladder viscera is characterized by increased vasculature, mucosal irritation that may result in barrier dysfunction, and infiltration of inflammatory mediators. The proliferation and activation of mast cells, in particular, have received considerable attention in the urinary bladder immune response. Mast cells secrete vasoactive chemicals to promote innate and autoimmunity and their increased activity has been widely demonstrated in BPS/IC. The subsequent exposure in the bladder interstitium to vasoactive chemicals, inflammatory mediators, and neuropeptides from visceral inflammation may lead to afferent nerve hyperexcitability and neurogenic inflammation (Yu et al., 2008).

Pathology Model
In order to test the Client’s compound modulatory effect on a BPS/IC model, T24 human bladder carcinoma cell line will be challenged in vitro with protamine sulphate (100ng/ml), either in the presence/absence of the Client’s compound, and the modulatory action exerted on the challenged cells will be quantitatively monitored by means of biochemical, molecular, metabolic and functional assays.

Readouts
The following parameters will be taken into consideration:

- Quantitative evaluation of cell vitality
- Quantitative evaluation of cell proliferation
- Quantitative evaluation of caspase activity
- Quantitative evaluation of inflammatory mediators by multiplex ELISA
- Quantitative evaluation of oxidative stress by Total ROS analysis
Kidney

KIDNEY FAILURE and EDEMA

Background

Edema forms in patients with kidney disease for two reasons:

1. A heavy loss of protein in the urine
2. Impaired kidney (renal) function

In this situation, the patients have normal or fairly normal kidney function. The heavy loss of protein in the urine with its accompanying edema is termed the nephrotic syndrome. Nephrotic syndrome results in a reduction in the concentration of albumin in the blood (hypoalbuminemia). Since albumin helps to maintain blood volume in the blood vessels, a reduction of fluid in the blood vessels occurs. The kidneys then register that there is depletion of blood volume and, therefore, attempt to retain salt. Consequently, fluid moves into the interstitial spaces, thereby causing pitting edema.

Podocytes are essential for normal functioning of glomerular filtration barrier (GFB) of the kidney. Damage or loss of podocytes causes glomerulosclerosis nephrotic syndrome in humans. In fact, disruption of the GFB is characterized by proteinuria, hypoalbuminemia, edema, and hyperlipidemia.

Pathology Model

In order to evaluate the anti-inflammatory activity of CLIENT’s compounds, human podocytes will be challenged with selective in vitro noxious stimuli mimicking renal failure and quantitative evaluation of podocyte viability will be monitored.

In particular, a conditionally immortalized human podocyte cell line will be cultured at 33C in their undifferentiated status, then differentiated at 37C. Cells will be challenged with either H2O2 (in order to mimic oxidative stress) or endothelin (in order to mimic endothelial conditioned podocyte damage).

Readouts

The following parameters will be taken into consideration:

- Viability of podocytes
- Quantitative evaluation of metabolic activity
- Evaluation of oxidative stress (i.e. ROS production)
- Quantitative evaluation of mitochondrial damage
Background
Chemotherapy-induced neuropathic pain is one of the most serious complications associated with anti-cancer drugs. It leads to a lower quality of life and dysfunction of the sensory, motor, and autonomic systems, and often causes patients to discontinue chemotherapy. Chemotherapy-induced neuropathic pain is usually misdiagnosed and undertreated, due to a lack of consensus and unclear pathophysiology, for which many mechanisms have been suggested, including mitochondrial dysfunction and various pain mediators.

To date, no agents have been shown to effectively prevent chemotherapy-induced neuropathic pain, and long-term management of pain is therefore becoming one of the most challenging aspects of treatment for neurologists and oncologists.

Pathology Model
In order to recreate in vitro the chemotherapy induced neuropathic pain model, we will challenge the primary sensory neurons (Primary cultures of DRG neurons from Sprague Dawley rats) with a well-known chemotherapeutic agent, namely vincristine.

Readouts
The following parameters will be taken into consideration:
- Cell viability
- Axonal degeneration
- LDH release
- IL1beta/TNF-alpha production
- Morphological modulation
- Electrophysiological properties
DIABETIC NEUROPATHY

Background
Diabetic painful neuropathy affects over 40% of adult diabetic patients. The pathology has been associated with a number of modifiable and non-modifiable risk factors, including the degree of hyperglycemia, lipid and blood pressure indexes, diabetes duration, and height. Diabetic neuropathy affects all peripheral nerves including pain fibers, motor neurons and the autonomic nervous system. With the exception of tight glucose control, there are no specific treatments for diabetic neuropathy, and current therapeutical strategies are rather aimed at reducing pain and other symptoms (Options for pain control include tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors (SNRIs), antiepileptic drugs (AEDs), etc.).

Pathology model
Primary cultures of DRG neurons, harvested from normal Sprague Dawley rats, will be cultured in presence of high concentration of glucose (e.g. 60mM) in order to recreate diabetic condition in vitro. Increasing evidence indicates that one of the major causes of diabetic peripheral neuropathy is an over-production of reactive oxygen species which leads to oxidative stress, mitochondrial dysfunction, neuronal damage and finally apoptosis.

Neurons isolated from dorsal root ganglia (DRG) of mammals such as rodents and hamsters rendered diabetic by treatment with drugs such as streptozotocin and alloxan have been used for study of diabetic neuropathy. Primary culture of DRG neurons from normal untreated rodents are now the preferred in vitro model given they mimic events occurring in vivo and permit detailed molecular analysis. Furthermore the use of primary cultures of DRG neurons has been adopted to obtain conspicuous data relative to changes in morphology such as reduction in neurite extension, changes in the activity of enzymes involved in the tricarboxylic acid cycle, electron transport chain, antioxidant systems and molecular events involved in mitochondrial dysfunction leading to apoptosis. DRG primary cultures are also convenient systems to trace the time kinetics of the molecular events occurring during cell death, due to oxidative stress. Most of these events have been shown to peak between 1-3 h after treatment with glucose due to sudden increase in ROS and associated stress. These observations concur with in vivo studies.

Readouts
The following assays will be performed:
- Cytoskeletal disruption
- Morphological Analysis- structural test in high content automated microscopy
- Functional analysis - functional test on neuronal network electrophysiological activity (MEA system)
Respiratory Diseases

CHRONIC OBSTRUCTIVE PULMONARY DISEASE
2D MODEL

Background

Chronic obstructive pulmonary disease (COPD) is an umbrella term that is used to describe chronic lung disease and includes the familiar terms of chronic bronchitis, small airways disease and emphysema. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases’ (Global Initiative for Chronic Obstructive Lung Disease, 2009). Pathologically the small bronchi (structures 2-4mm in diameter), small airways (<2mm in diameter) and the lower airway lung parenchyma are the main sites in which chronic bronchitis, small airways disease and emphysema develop.

Inhalation of cigarette smoke, occupational and environmental pollutants are the main causes of COPD affect all major compartments of the lung, including the central and peripheral airways, the parenchyma and the pulmonary vasculature.

Smoke exposure can directly injure the lung through the action of toxicants found within smoke but also through the attraction, activation and the release of pro-inflammatory mediators from cells of the immune system. These mediators, which can act locally to damage tissue, can also perpetuate the inflammatory response through the attraction of further inflammatory cells to the site of injury. If the exogenous (cigarette smoke) and endogenous (inflammatory cells) oxidants outweigh the lung’s antioxidant capacity, this can lead to injury and further inflammation. Thus, oxidative stress and direct toxicant induced tissue injury drives inflammation and in susceptible individuals drives the disease process and the subsequent development of COPD.

Recent studies have demonstrated that cigarette smoke extract (CSE) is able to inhibit fibroblasts recruitment and proliferation and to alter fibroblast-mediated collagen gel contraction in vitro. Furthermore the cigarette smoke is known to induce epithelial-mesenchymal transition and this transition is likely to be mediated by fibroblasts.

Pathology Model

Initially, 2D coculture of human airway epithelial cells (A549) along with human lung fibroblasts (HLF-1) will be seeded on polycarbonate transwell. The coculture will be exposed to cigarette smoke extract (CSE) in order to evaluate the effect of CLIENT’s compound on cigarette smoke lung damage.
A549 cells cocultured with HLF-1

In order to confirm the effect of the compounds in a more complicated and pathophysiological scenario, a 3D coculture will be used. A mix of collagen gel and HLF-1 will be cast into the culture wells and upon collagen polymerization, A549 cells will be added for coculture. The cells will be then challenged by CSE in presence or absence of CLIENT’s compounds.

**Readouts**

The following parameters will be taken into consideration for 2D model:

- Cell morphology and epithelial integrity (E-cadherin)
- Inflammatory mediator dosage (e.g.: IL-6, IL-8, MMP-1)
- mRNA expression of gene implicated in mucin production (e.g.: MUC1, MUC5, MUC5B)
- Oxidative stress measurement
- Epithelial-mesenchymal transition (α-SMA immunocytochemistry)

Or 3D model:

1. Gel contraction assay
Skin Diseases

PSORIASIS

Background
Psoriasis is a common, chronic relapsing/remitting immune-mediated skin disease characterized by red, scaly patches, papules, and plaques. There are five main types of psoriasis: plaque, guttate, inverse, pustular, and erythrodermic. Plaque psoriasis is the most common form and typically manifests as red and white scaly patches on the top layer of the skin. The causes of psoriasis are not fully understood. Psoriasis develops when the immune system mistakes a normal skin cell for a pathogen, and sends out faulty signals that cause overproduction of new skin cells. Though many treatments are available, psoriasis can be difficult to treat due to its chronic recurrent nature. A new generation of targeted immune therapies is being subjected to rigorous investigation in order to advance treatment options for psoriasis.

Pathology Model
Primary Normal Human Epidermal Keratinocytes (NHEK) will be cultivated in a hydrogel matrix containing a mixed 3D culture of fibroblasts and macrophages (i.e. collagen based matrices or more complex commercial systems, such as Qgel). After 3 days of immersed culture conditions, the epidermis will be airlifted for 10 days, allowing differentiation and formation of a horny layer. In order to induce psoriasis, macrophages will be activated by adding IFNγ to the medium (i.e. 200U/ml overnight).

Readouts
The following parameters will be taken into consideration:

- **Immunofluorescence**: qualitative and/or quantitative evaluation of the expression of selected markers (i.e. filaggrin, involucrin, keratin, loricrin etc...) by acquisition of confocal images or by Odyssey scanner (i.e. Li-Cor).
- **Inflammatory cytokines**: quantitative evaluation of the production of selected panels of pro-inflammatory cytokines by multiplex analysis (i.e. TNFα, IL-12, IL-17, IL-19, IL-20, IL-22, IL-23, IL-24, VEGF etc...)
- **Gene expression**: quantitative evaluation of the expression of selected panels of genes of interest (i.e. NF-κB and inflammatory and structural genes) in challenged cells. A transcriptomics analysis of challenged cells can also be taken into consideration.
- **Morphological analysis**: qualitative analysis of alterations in cell morphology in challenged cells (e.g. Masson’s trichrome staining)
- **Vitality assay**: quantitative evaluation of cell viability with MTT assay
- **Oxidative stress**: quantitative evaluation of total ROS production in challenged cells
MELANOMA

Background
Melanoma is a malignant tumour of melanocytes. Melanoma is less common than other skin cancers, however it is much more dangerous if it is not found in the early stages. It causes the majority (75%) of deaths related to skin cancer. The treatment includes surgical removal of the tumour.

Pathology Model
2D culture of melanoma cells will be cultured in vitro and analysed by FACS in order to assess cancer stem cells content (CSC) (CD133 positive cells). Moreover, cells will be monitored for inflammatory cytokine production as well as selected expression of target genes. A transcriptomics analysis of challenged cells can also be taken into consideration.

Moreover, both primary and secondary spheroid formation (ie. Cultrex® 3-D Spheroid Assay) and invasion assay (ie. Cultrex® 3-D Spheroid Cell Invasion Assay) to mimic in vivo 3D conditions will be taken into consideration. In particular, the interaction between cancer cells and niche cells from the microenvironment (fibroblasts, endothelium etc....) will be deeply investigated, in both 2D and 3D cultures.

For what concerns the 2D, cells will be microfluidically connected on MicroTISSUE, an innovative multiparametric platform which enables to dissect the contribution of single cells in the microenvironment. For what concerns 3D, cells will be included in a matrix mimicking the in vivo extracellular matrix (ECM) in order to evaluate the capability of the cells to degrade ECM and migrate in response to microenvironment stimuli.

Readouts
Cocultures of cancer cells with mesenchymal stem cells (MSC) will be taken into consideration in order to evaluate the effect of MSC on tumor progression, metastatic potential and CSC proliferation.
DERMATOSIS

Background
Dermatosis is defined as a disorder involving lesions or eruptions of the skin that are acute (lasting days to weeks) or chronic (lasting months to years). Acute lesions are relatively common and exhibit a wide range of clinical conditions. Usually, these conditions are triggered by local or systemic immunologic factors (e.g., allergic reaction); however, the exact etiology remains unclear. Acute inflammatory dermatosis conditions include erythema multiforme (EM), pruritus (urticaria), and eczema.

Pathology Model
Commercial reconstructed human epidermis (RhE) models will be used: EpiDerm (MatTek corporation). Oxazolone will be applied to keratinocytes in order to induce skin sensitization (i.e. 0.4% for 6h).

Readouts
The following parameters will be taken into consideration:

- **Immunofluorescence**: qualitative and/or quantitative evaluation of the expression of selected markers (i.e. E-Cadherin, ICAM-1, Neurotrophin 4 etc.) by acquisition of confocal images or Odissey scanner.
- **Inflammatory cytokines**: quantitative evaluation of the production of selected panels of pro inflammatory cytokines by multiplex analysis (i.e. IL-6, IL-1α, IL-8, IP-10, TARC, MCP-1, RANTES ecc...)
- **Gene expression**: quantitative evaluation of the expression of selected panels of genes of interest (i.e. E-Cadherin, ICAM-1, Neurotrophin 4 etc....) in challenged cells. A transcriptomics analysis of challenged cells can also be taken into consideration.
- **Morphological analysis**: qualitative analysis of alterations in cell morphology in challenged cells.
- **Vitality assay**: quantitative evaluation of cell viability with MTT assay
- **Oxidative stress**: quantitative evaluation of total ROS production in challenged cells.