

## AISAL symposium

### *“Imaging and translational Medicine”*

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## MAIN LECTURES:

### 1. Advanced neuroimaging techniques to investigate neurodegenerative dementias: clinical and preclinical data

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Thank to its ability to image soft tissue *in vivo* non invasively with detailed anatomical resolution, conventional magnetic resonance imaging (MRI) has shown high sensitivity in detecting macroscopic abnormalities occurring in several neurological conditions (i.e., cerebral-vascular disease, multiple sclerosis, brain tumours), thus providing a substantial contribution in both diagnosis and therapeutic addressing. However, when patients present with cognitive decline, conventional MRI is typically used mainly to exclude the presence of a potentially reversible cause (i.e., brain tumours, subdural haematoma, normal pressure hydrocephalus), and does not provide any substantial support in the diagnosis of degenerative dementias. Due to the advent of tailored therapies, an early diagnosis of dementia (in its different forms) is becoming more and more critical for a correct clinical management of this kind of patients. As a consequence, instrumental techniques are warranted with the ability to address a timely diagnosis as well as to monitor the clinical evolution of patients with cognitive decline. Over the last few years, several advanced MRI techniques have been introduced to assess brain tissue modifications both, at a macro- and microscopic level. First attempt to investigate brain changes in patients with dementia was based on the volumetric assessment of specific brain structures, known to be involved by pathological processes. Manual segmentation of medial temporal lobe structures has allowed to identify and monitor over time the local volumetric loss in patients with Alzheimer's disease (AD). Medial temporal lobes are indeed primarily implicated in memory processes, whose impairment represents the earliest and prominent disability in patients with AD. However, AD, as well as other forms of neurodegenerative dementia, present with more complex clinical features, with a correspondent pathological damage which is widely distributed across the whole brain. In addition, volumetric techniques based on manual segmentation are strongly operator dependent, and therefore poorly reproducible. To overcome these limitations, quantitative volumetric techniques based on automatic algorithms have been introduced. These techniques represent an useful tool to investigate patients with dementia in an unbiased fashion. A semiautomated rigid body coregistration technique was used to longitudinally compare age-related changes over 1 year time in a group of AD patients compared to healthy elderly controls. Significantly greater rates of volume loss were found in AD patients, with a wide separation between patients and controls. More complex algorithms, including linear and/or non linear forms of spatial normalization have also been developed, with the ability to perform region by region comparisons within a common stereotaxic space. Voxel-based morphometry (VBM) is probably one of the most promising techniques. It is a spatially-specific and unbiased method of analysis of MR images reflecting regional grey (GM) and white matter (WM) volumes at a voxel scale. VBM has been successfully applied to AD in cross sectional studies. Areas of decreased GM density were demonstrated in brain regions of AD patients not previously investigated using semi-quantitative approaches based on regions of interest (i.e., hippocampus) measures. Moreover, a similar pattern of decreased GM density, albeit of a lesser degree, was found in patients with mild cognitive impairment (MCI), a condition which is associated with a high conversion rate to dementia.

Longitudinal VBM analyses applied to follow-up studies, have shown the ability to identify, at baseline, different patterns of GM density that might predict in patients with MCI a more rapid evolution to AD. VBM has also been successfully used to investigate associations between regional GM loss and clinical, neuropsychological, and psychopathological features of patients with AD. In a recent work, it has been shown that not only cognitive disabilities, but also psychiatric and behavioural aspects are likely to be part of the neurodegenerative processes occurring to AD brains. Finally, VBM has been used to investigate patients suffering from other forms of cognitive decline, such as dementia with Lewy bodies (DLB) and frontotemporal dementia. Patterns of atrophy with a different distribution from those observed in AD patients were reported, suggesting the potential of VBM to be employed for monitoring the evolution of dementia in its different forms.

Diffusion tensor MRI (DT-MRI), by providing rotationally invariant measurements of both, magnitude and directionality of water diffusion (mean diffusivity and anisotropic indexes), represents a potentially powerful quantitative technique able to assess microscopic structural tissue characteristics and abnormalities. DT-MRI might therefore be able to characterise *in vivo* the earliest stages of the neurodegenerative pathological damage, giving insights of their etiopathology. Several DT-MRI studies on AD patients have been published so far, the most consistent ones showing specific patterns of WM changes related with different areas of the association cortex. These findings fit well with the cognitive impairment observed in AD patients and showed strong correlations with neuropsychological measures. Recent investigations that combined DT-MRI and VBM, have also highlighted the anatomical relationship between GM and WM damage, thus supporting the remarkable role that brain disconnection (together with atrophy) plays in AD pathology. DLB has also been investigated using DT-MRI. Specific patterns of cortico-subcortical microstructural changes were detected with a completely different distribution from that previously observed in AD patients. Such abnormalities were mainly located in the occipital lobes and basal ganglia, thus providing some insights of specific DLB symptoms (visual hallucination and parkinsonism), and suggesting neuroradiological markers of potential diagnostic value. DT-MRI, thanks to the structural complexity of the brain tissue, can also be used to reconstruct *in vivo* the principal WM tracts, and to perform measures of microscopic tissue integrity inside them. A recent investigation of the uncinate fasciculus (a WM tract connecting the frontal and temporal lobes) has shown that a selective involvement of this tract is likely to be responsible for impairment in higher level functions at the more advanced stages of both, AD and DLB.

Functional MRI allows the brain activation to be investigated *in vivo* during neuropsychological task performing. It may be employed to investigate abnormal patterns of brain activation under specific tasks involving different cognitive domains. Such an approach has the potential to provide quantitative information of prognostic value. Moreover, it might be useful to assess the evolution of cognitive deficits in the different forms of dementia, and to monitor clinical trials. A recent investigation in patients with amnesic MCI (which is currently considered as a prodromal clinical stage of AD) has demonstrated the presence of abnormal activations in networks related to cognitive domains and emotion processing systems in the absence of any evident clinical deficit. This suggests that functional MRI has the potential ability to detect changes of brain functioning at a preclinical stage. Another interesting functional MRI approach is the so-called 'resting-state' functional MRI. This technique is reliable and simple to be used. This makes 'resting state' functional MRI particularly valuable, especially when used to investigate subjects with cognitive decline, and poor compliance in performing tasks. 'Resting state' functional MRI allows changes of functional brain connectivity to be assessed as a function of the underlying pathological processes. Functional disconnection of brain regions related to cognition is likely to play a relevant role in determining clinical manifestations and neuropsychological deficits in patients with cognitive decline at different stages. Recently, the combination of VBM and 'Resting state' functional MRI applied to a group of patients with AD at different clinical stages, has shown how functional disconnection in the posterior cingulate gyrus

precedes the occurrence of GM loss in the same anatomical area. This evidence further supports the idea that the primary involvement of GM tissue is only one aspect of AD pathology, and that brain disconnection is likely to be responsible for some critical steps of AD evolution. Indeed, it is conceivable that GM involvement of the medial temporal lobes causes the memory deficits observed in AD at early stages (i.e., amnesic MCI). With the progressive worsening of the disease, this regional GM loss in temporal areas may cause (in its turn) a deafferentation of the posterior cingulate gyrus, which will eventually become secondarily atrophic. The cingulate gyrus is one of the major pathways connecting the limbic system with the rest of the brain. In this perspective, therefore, the implication of the posterior cingulate cortex seems a critical step for the clinical progression of cognitive disabilities, whose accumulation eventually results in fully developed dementia. However, not all patients with AD present with the same ratio of brain tissue damage and clinical symptoms. The concept of cognitive/brain reserve has been introduced as an additional factor interfering with the clinical evolution of dementias. The basic idea is that some individuals develop a cognitive reserve over life, according to their intellectual and physical activities. Education has been identified as a parameter which is highly associated with cognitive reserve. In a recent research, using VBM in a large cohort of patients with AD and MCI, it has been found a significant interaction between the distribution of regional GM loss and the level of formal education. By dividing the cohort of patients (homogeneously distributed for their level of cognitive impairment) in those with high and those with low education, the former group revealed a more remarkable pattern of GM loss in the medial temporal lobe structures. Conversely, patients with low education were more atrophic than those with high education in highly associative regions, such as the supramarginal gyrus. A possible interpretation of these data is that brain plasticity (as a function of cognitive reserve) tends to delay the clinical onset of AD and to compensate for cognitive disabilities. Indeed, patients with high education levels require a more remarkable atrophy in the medial temporal lobes (the core of AD pathology) to show the same cognitive disability of patients with low education. Conversely, patients with an high education level are likely to compensate for cognitive deficits by recruiting other areas of the association cortex. These data are relevant not only to better understand the pathophysiology of AD, but also to prompt new therapeutic approaches based on cognitive rehabilitation. Indeed, brain plasticity is known play a role not only during development, but also in aging. Acetylcholine, whose depletion is one of the most relevant neurochemical features of AD, is not only implicated in synaptic neuronal transmission, but plays also a role in neuronal plasticity. This means that the Anti-cholinesterase inhibitors, which represent the major pharmacological treatment for AD, do not have only a symptomatic, but also a pathogenetic effect. A recent DT-MRI study provides some evidence *in vivo* on the potential effect played by Anti-cholinesterase inhibitors on brain plasticity of AD brains. This study is based on the investigation of anatomical brain connectivity. This quantity is achieved by running tractography in each voxel of the brain, and by counting the number of streamlines passing through each voxel. In other word, anatomical connectivity expresses the strength of structural connectivity of each voxel with the rest of brain. This quantity can be used to compare different groups of subjects. The most surprising effect of this recent study was to highlight not only brain regions of reduced connectivity in AD patients, but also regions of increased connectivity. This increase of connectivity was present not only in AD patients compared to controls, but also in AD compared to MCI patients. The only difference between the two patient groups, AD and MCI, was the administration of Anti-cholinesterase inhibitors in the former group only. An explanation for the unexpected finding (increased connectivity in AD patients) was therefore the pharmacological modulation of brain plasticity in AD.

In conclusion, MRI with its various quantitative techniques is contributing in increasing our understanding of the pathophysiological mechanisms underlying the neurodegenerative forms of cognitive decline. MRI has the unique ability to detect brain changes *in vivo*, thus allowing to clarify the relationship between *post mortem* findings and clinical aspects of the neurodegenerative diseases.

Further, MRI provides a unique tool to investigate the effect of new treatments with the potential ability of modifying the clinical course of dementias. Finally, non-conventional MRI techniques have the potential to eventually provide neurobiological markers of diagnostic and prognostic value to be applied to single subjects in clinical routine.

## 2. Biomarkers in neuroimaging of brain disorders: from mice to men

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A biomarker, is a measurable indicator of a biological state that can be used to assess normal, physiologic processes as well as abnormal changes in the course of diseases and responses to treatment.

While biochemical and molecular markers measured on biologic specimens have been introduced since a long time in the clinical routine and medical research for different organs, systems and clinical conditions, they have had a more limited value in the study of Central Nervous System (CNS). In the past four decades, the development of advanced tomographic diagnostic imaging techniques, has opened the way to the introduction of a new class of biomarkers, the so-called imaging biomarkers that may be able to provide earlier detection of many brain diseases and to monitor disease changes over time by exploring the brain non invasively.

In fact many in vivo imaging techniques can reliably and noninvasively assess different aspects of neuroanatomy, neurochemistry, physiology, and pathology, and can provide a series of indicators, that could be correlated to activity and amount of inflammatory and demyelinating processes, as well as to global and regional brain atrophy and cognitive decline, and to neurotransmission processes (neuroreceptor density and binding ability), to tumor size, vascularization and biological aggressiveness.

Diagnostic Imaging modalities can be classified in two main groups, those providing predominantly structural information such as MRI (Magnetic Resonance Imaging), CT (Computed Tomography), US (Ultrasound) and those providing mainly functional information, such as PET (Positron Emission Tomography), SPECT (Single Photon Emission Computed Tomography) and optical imaging. However, in addition to structural information MRI can also provide in vivo functional data regarding tissue perfusion, water diffusion and tissue chemistry, using MRS (Magnetic Resonance Spectroscopy) applications.

As a result different imaging biomarkers are being tested in the assessment of many CNS disorders including neurodegenerative processes (such as Alzheimer's and Parkinson's disease), inflammatory diseases (including demyelinating diseases, such as Multiple Sclerosis), brain tumors, and neurodevelopmental abnormalities. In addition to their contribution to the assessment of the extent of disease, imaging biomarkers can also be used to improve the selection of new drugs and innovative therapeutic approaches by demonstrating early structural and functional changes associated with drug administration.

Among the morphologic imaging modalities, MRI is particularly tailored for the assessment of normal and abnormal brain structure. MRI can differentiate the normal tissue components of the brain: grey matter and white matter as well as Cerebrospinal fluid (CSF), using computer assisted procedures for tissue segmentation and classification. Therefore, different volumetric indicators of global and local brain degeneration (atrophy) can be obtained through MRI studies, in addition to conventional linear and area measurements. Of the many post-processing procedures currently available, voxel-based morphometry (VBM) can analyze whole-brain data from multiple subjects without a priori hypothesis, to obtain unbiased information concerning regional abnormalities and can be used to compare different groups of subjects, and could possibly contribute to individual patient characterization.

As an example, detailed computer assisted measurements of medial temporal lobe structural changes and atrophy may represent valuable biomarkers for early detection of degeneration in Alzheimer's disease. Rates of atrophy can also be measured with serial MRI studies. Tissue classification and volume measurements can also be applied to the assessment of tumor size in the course of treatment. Likewise, computer assisted procedures can be used to measure the amount of damaged white matter, the volume of active lesions and associated brain atrophy in demyelinating diseases such as multiple sclerosis.

On the functional and molecular imaging side, PET, SPECT and optical imaging can provide measurements of biologic functions, such as glucose and oxygen utilization, protein synthesis, nucleic acid synthesis, detection of cell or tissue specific molecules and receptors, and allow the study of neurotransmission processes.

Therefore many different imaging biomarkers can provide a combination of measurements of structural and functional data concerning different CNS diseases.

In brain tumors, size measured with MRI can serve as a morphologic imaging biomarker, while tumor metabolism and proliferation can be assessed with PET.

Tumor angiogenesis and blood flow can be evaluated with contrast enhanced MRI or PET, while MRS can provide information concerning specific metabolite patterns and their modifications following treatment therefore obtaining potential prognostic indices of tumor grading.

In neurodevelopmental diseases, MRI, in addition to quantitative measures of tissue components (grey and white matter and CSF) can obtain detailed information concerning fiber tracts development using Diffusion Tensor Imaging protocols (DTI) capable of identifying fiber bundles within the CNS.

In general, imaging biomarkers could be potentially helpful in addressing the inherent limitations associated with conventional end points in both preclinical and clinical trials. Outcome measures based on imaging biomarkers measurement could be more reliable or complement standard clinical measures, increasing the possibility to detect small effects and reducing sample size.

Translational research is receiving strong contribution from imaging and animal models of CNS diseases are important in understanding the causes, physiopathologic mechanisms and progression of brain disorders as well as treatment effects. The utilization of imaging biomarkers in this setting can be particularly helpful to conduct longitudinal studies in the same animal reducing research costs, saving time and providing valuable follow up data, in a relatively short time frame.

However imaging of small animals, in particular of mice, is a special technical challenge given the smaller size and the need of high spatial resolution to obtain results comparable to those achievable in humans. Therefore dedicated imaging equipment is required for small animal imaging, in particular for brain studies, including high field, small bore MRI scanners and dedicated high resolution PET/SPECT systems.

The availability of equivalent imaging equipment for both human studies and small animal studies is helpful for translational applications using advanced tomographic imaging techniques such as MRI/MRS, CT, PET and SPECT. (Unlike the previously mentioned techniques, optical imaging can only be applied to small animals, since light photons emitted from the brain cannot be detected in humans).

In conclusion, as a variety of imaging biomarkers of neuroanatomy, neurochemistry, physiology and pathology become available, their role in early detection and monitoring disease changes over time and response to treatment in brain disorders will likely increase with a potentially strong boost from studies on small animal models of brain diseases; a multiparametric structural and functional biomarker approach seems to be the most promising methodology to improve diagnosis and prognosis, and to develop innovative and patient-tailored therapies.

### **3. Molecular imaging for drug development in translational cancer research**

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The identification of molecular pathways playing a key role in tumor growth and progression and the unraveling of human genome provided a plethora of new targets for drug development in oncology. In addition to conventional anticancer drugs, new compounds directed against molecular targets such as growth factors, receptors, enzymes and other mediators of cell growth and apoptosis are now available and many others are going to be developed. Proving the clinical efficacy of these new drugs interacting with molecular targets has two important implications. The first is that not all patients are eligible for targeted therapy but only those in whom the target is expressed. The second is that drug-target interaction results in the inhibition of target function that in turn causes a cytostatic effect rather than a cytotoxic effect. Therefore the classical criteria of tumor response based on reduction of tumor burden may not be appropriate for the evaluation of efficacy of targeted therapy. Recently, the extraordinary development of imaging technologies, including hybrid systems such as PET/CT, allowed the visualization of biochemical, molecular and physiological pathways in tumors and organs of patients and animal models. In vivo evaluation of complex biological processes such as proliferation, apoptosis, angiogenesis, metastasis, gene expression, receptor/ligand interactions, transport of substrates and metabolism of nutrients in human cancers is feasible by using PET/CT and radiolabeled molecular probes. These imaging technologies can be employed in pre-clinical and clinical experimental settings for establishing drug efficacy thus accelerating the drug development process. Here we provide prominent examples of how molecular imaging can be used for drug screening, selection of patient for a given treatment, real-time monitoring of therapy, early detection of drug resistance and finally for tailoring therapy.

#### 4. Pre-clinical molecular Imaging

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Positron emission tomography (PET) is a nuclear medicine procedure that allows the quantification of different metabolic/biological functions. In particular, PET has acquired a relevant role in the diagnostic flow chart of many forms of human tumours providing functional data on disease extent, response to therapy, early identification of recurrence and contributing in many cases to changes in the patients' clinical management.

Animal models of human tumours are useful tools to study the mechanisms underlying human disorders. Considering the many similarities with human genome, the relatively low costs and the rapid rate of reproduction, mice are the most commonly used animals for pre-clinical studies in oncology.

Since the introduction of the first dedicated PET scanners (SA-PET) for small animal imaging (1995), the number of pre-clinical studies using this imaging modality have increased exponentially. In fact, SA-PET presents several advantages, namely that is whole-body and non-invasively provides functional data of biological processes in all body tissues and organs.

Being non-invasive, SA-PET can be repeated in the same animal over time. This allows the reduction of the total number of animals employed for each experiment (and subsequently lowers total costs) limiting the inter-animal variability (since each animal can be used as its own control).

Moreover, SA-PET provides an accurate characterization of different biological processes such as metabolism (glucose, amino acids and fatty acids), cell surface receptor expression status, apoptosis, angiogenesis and gene expression. SA-PET can detect even picomolar concentrations of the tracer, leading to excellent sensitivity in the measurement of variations in uptake, and allowing the detection of viable tumour cells at a very early stage. Many positron-emitting tracers are currently available to study these biological processes. Virtually, all molecules can be labelled with a positron-emitting isotope; especially in the case of <sup>11</sup>-carbon that does not change the molecular structure of the label.

SA-PET images can be interpreted both visually (sites of increased tracer uptake other than physiological distribution are considered pathologic) and semi-quantitatively. Semi-quantitative analyses using standard uptake values (SUV) allow a direct comparison of the metabolic changes in a given lesion over time or in response to a potentially curative new drug. The possibility to quantify tracer uptake represents a major advantage of SA-PET over other imaging modalities such as optical imaging.

Another advantage of SA-PET in pre-clinical studies is the possibility to employ this procedure for testing the efficacy of novel drugs. In fact, SA-PET early tumour engraftment in a laboratory animal is crucial for both the selection of tumour-bearing candidates to be treated with the new drugs, since small and well vascularized tumour lesions represent the best setting in which to test a potentially curative new drug. SA-PET can also assess the metabolic changes in response to treatment. Overall, the employment of SA-PET to pre-clinically assess the efficacy of new drugs may reduce the time between the development of a new compound and its commercialisation. Furthermore SA-PET may provide biodistribution and kinetics data of novel imaging probes, that are specific for the biological process under study, before human use.

From a technical point of view, SA-PET is performed in the anesthetized animal (sevoflurane gas anesthesia) after the injection of the radiotracer (0.2-0.5mCi) in the tail vein, although retro-orbital and

intraperitoneal routes have also been described) and the uptake time (depending on the tracer employed). However, protocols for animal imaging vary widely. Factors influencing PET imaging in small animals other than the administration route include animal preparation, mode of anaesthesia and room temperature that may interfere with tracer biodistribution. For example, keeping the animals warm before and after  $^{18}\text{F}$ -FDG injection leads to decrease brown fat and muscle uptake that may interfere with image reading.

All available SA-PET systems are 3D PET scanners that can acquire both static and dynamic acquisitions of single bed or multi-bed scans in the anesthetized animal. Based on clinical algorithms, several fully 3D reconstruction algorithms have been developed in the system workstations.

SA-PET imaging using different radiotracers may allow visualization of tumour cells pathways metabolism, angiogenesis, apoptosis and surface cells receptors expression.

Imaging of tumour cells metabolism relies on the use of small analogues of target molecules of a relevant biological pathway that are labelled with a positron-emitting isotope. The most commonly used tracer for clinical and pre-clinical metabolic studies is  $^{18}\text{F}$ -FDG (fluorine- $^{18}$ -fluorodeoxyglucose), an analogue of glucose labelled with fluorine.  $^{18}\text{F}$ -FDG enters the cells via the glucose transporters, and is trapped inside the cells through phosphorylation by hexokinase (since it cannot undergo further metabolism through the glycolytic pathway).  $^{18}\text{F}$ -FDG can be considered as a marker of increased cells metabolism since highly metabolic cells demonstrate increased glucose metabolism. Another factor influencing glucose consumption is oxygen availability: although the use of glucose is higher in hypoxic tissues studies, tumour cells present an increased FDG uptake even in the presence of adequate oxygen availability (aerobic glycolysis) a feature considered a hallmark of cancer cell behaviour.  $^{18}\text{F}$ -FDG SA-PET has been reported to early detect tumour cells engraftment in experimental animals in many different types of tumours, helping to select adequate candidates for testing new and expensive drugs, and can be useful to assess disease extension and variations of tumour metabolism over time. By comparing the metabolic activity of a lesion before and after treatment, SA-PET may provide valuable information regarding the efficacy of new therapeutic drugs, overcoming the well-known limits of using size as a parameter for evaluation of therapy response.

Specific PET tracers have been designed to study cells proliferation.  $^{18}\text{F}$ -FLT (3'-Deoxy-3'-[( $^{18}\text{F}$ )]fluorothymidine ([( $^{18}\text{F}$ )]FLT) is a pyrimidine analogue that reflects the activity of a thymidine kinase-1 (TK1) during the S-phase of DNA synthesis while is a very poor substrate for thymidine kinase-2 (TK2).  $^{18}\text{F}$ -FLT uptake significantly correlates with proliferation Ki67 index levels and has been proposed for in-vivo monitoring tumour response to therapy. Other PET tracers for imaging cell proliferation are FMAU ( $^{18}\text{F}$ -1-2'-deoxy-2'-fluoro- $\beta$ -D-arabinofuranosylthymine) and Choline. Due to the low marrow uptake and urinary excretion, FMAU has been proposed for studying bone metastasis and the pelvic region, however the increased levels of circulating thymidine in mice plasma have limited its use for the significant competitive inhibition of radiolabelled thymidine and thymidine analogues that leads to generally low tissues uptake in mice and rats.

Being an essential element of phospholipids of the cell membrane, choline labelled with  $^{11}\text{C}$  or  $^{18}\text{F}$  has been proposed as a marker of tumour cells lipids metabolism. In fact, actively proliferating tumour cells present an elevated level of phosphatidylcholine and an up-regulation of choline kinase that catalyses the phosphorylation of choline.

The formation of new vessels is a hallmark of cancer cells: angiogenesis is essential to tumour growth and is at the basis of tumour progression. Tumour cells stimulate angiogenesis directly through the release of cytokines and growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), leading to the formation of abnormal vessels inside the tumour, with increased tortuosity and branching.

PET probes specifically targeting angiogenesis have been therefore proposed to study tumour angiogenesis. In particular, antibodies directed against VEGF have been used in animal models of

human tumours using different labelling isotopes ( $^{89}\text{Zr}$ ,  $^{124}\text{I}$ ) and more recently VEGF itself has been directly labelled with  $^{64}\text{Cu}$ -DOTA. Although  $^{64}\text{Cu}$ -DOTA-VEGF has been reported to present a high binding-affinity to the receptor, VEGFR expression varied in different tumour sizes and stages. This observation clearly limits the possibility of translation of such findings to the clinic since the time window of high VEGFR expression is quite narrow.

Another potential target for PET probes to image angiogenesis are  $\alpha\text{v}\beta 3$  integrins, that are necessary for the formation, survival, and maturation of new blood vessels. Small peptides with the RGD (arginine-glycine-aspartic) sequence are ligands of integrin  $\alpha\text{v}\beta 3$  and have been labelled with  $^{68}\text{Ga}$  to target integrin  $\alpha\text{v}\beta 3$  expression.

The identification of hypoxic cells fraction within a tumour mass is relevant since hypoxic cells are more resistant to radiation therapy than well-oxygenated cells and receive lower concentrations of chemotherapeutic drugs, as a consequence of lower vascularization. Nitromidazoles are compounds that enter cells by passive diffusion, are reduced by nitroreductase enzymes and are therefore trapped inside the cells with reduced tissue oxygen partial pressure. When oxygen is sufficient in normally oxygenated cells, the parent compound is quickly regenerated by reoxidation and metabolites do not accumulate. Several PET nitromidazoles tracers are currently available to image hypoxia in animal tumour models such as  $^{18}\text{F}$ -FMISO,  $^{18}\text{F}$ -EF3,  $^{18}\text{F}$ -EF5,  $^{18}\text{F}$ -FAZA, and  $^{18}\text{F}$ -FETNIM.

Alternative PET agents for the assessment of intra-tumoural hypoxia are based on a metal complex of ATSM (diacetyl-bis(4N-methylthiosemicarbazone) and radioactive copper. Pre-clinical comparison between  $^{18}\text{F}$ -FMISO, the most commonly used hypoxia tracer, and  $^{64}\text{Cu}$ -ATSM showed that  $^{64}\text{Cu}$ -ATSM uptake is more rapid than FMISO. However  $^{64}\text{Cu}$ -ATSM lipophilicity is a potential limitation since its uptake by tumour cells may be influenced by regional blood flow.

Another distinctive feature of cancer cells is apoptosis. Annexin V (AnxV), an endogenous human protein, specifically binding to membrane-bound phospholipids, has been proposed as an imaging agent of apoptotic cells. However, since AnxV in-vivo manifests a low signal/noise ratio, a novel family of low-molecular weight probes (ApoSense) have been designed. These compounds selectively pass through the membrane and accumulate within the cytoplasm of apoptotic cells from the early stages of the death process; on the contrary these compounds do not enter intact cells. A new PET probe belonging to the ApoSense family and labelled with  $^{18}\text{F}$  has been designed (NST-732; [(5-dimethylamino)-1-naphthalenesulfonyl- $\alpha$ -ethyl-fluoroalanine) for imaging apoptosis: its main advantage relies on the ability to accumulate inside the apoptotic cells from the early stages of the disease process. Moreover, the uptake of NST-732 is parallel (or perhaps even preceding) caspase (a marker of mitochondrial death) activation, and can be completely blocked when the pro-apoptotic trigger is co-administered with a caspase inhibitor.

Surface cells receptors have also been employed as potential targets for PET tumour imaging: PET probes binding to specific receptors on tumour cells allows the detection of disease sites and the selection of receptor-bearing cases that may take advantage of receptor-targeted therapies. Although many PET probes have been designed for use for this purpose in humans, a smaller number has been used in pre-clinical studies of human cancer. One of the receptors that has been more extensively studied for its direct implication in tumour growth is EGFR. In fact EGFR signalling contributes to a number of processes important to cancer development and progression (activation of cells proliferation, angiogenesis, metastatic spread, enhanced cells survival) and EGFR has been reported to be over-expressed in many forms of human cancer. However, the results obtained from preliminary studies using PET probes binding to EGFR ( $^{18}\text{F}$ -Gefitinib,  $^{124}\text{I}$ -IPQA,  $^{68}\text{Ga}$ -DOTA-hEGF) are yet not definitive. Estrogen receptor expression has also been used as target for breast cancer imaging and to predict the likelihood of response to hormonal therapy:  $^{18}\text{F}$ -fluoroestradiol ( $^{18}\text{F}$ -FES) has been successfully used to evaluate estrogen receptor expression in-vivo and has been reported to correlate with results obtained in vitro by radio-ligand binding.

In conclusion, current evidence supports the role of SA-PET to study the metabolic pathways of cancer in animals models of human tumours. SA-PET allows the performance of longitudinal studies in the same experimental animal with a reduction of the total costs and provides in-vivo functional data on tissues and organs biological processes. In particular specific PET probes have been designed to target the biological hallmarks of tumour cells (such as proliferation, angiogenesis, apoptosis and receptors expression). In this regard, it is apparent that advances in the radiopharmaceutical design of new tracers are strictly linked with the possibility to assess specific biological pathways in different tumour types. Certainly the possibility to use SA-PET for new drugs efficacy studies is very appealing, since the metabolic changes often precede the reduction in tumour size and allow to differentiate viable tumour cells from scar tissue. Finally the employment of specific PET probes targeting gene expression may represent a new field of SA-PET applications in pre-clinical research.

## **5. Preclinical validation of therapeutic and diagnostic nanoparticles using in-vivo small animal imaging**

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Developments of nanotechnology allows to create new probes combining multimodality detection properties and/or therapeutic properties. For example, lipidic structures going from liposomes to nanoemulsions, dendrimers or inorganic particles.

In vivo imaging becomes a very interesting tool in the validation process of these objects especially for evaluation of the biodistribution analysis, targeting, and efficacy. Since properties of these probes are multiple, and since each imaging modality has their own advantages and limitation, it becomes very interesting to cross validate these probes using a multimodality strategy.

## **Role of tissue niche on muscle regeneration**

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One of the most exciting aspirations of current medical science is the regeneration of damaged body parts. The capacity of adult tissues to regenerate in response to injury stimuli represents an important homeostatic process. Regeneration of adult skeletal muscle is a highly coordinated program that partially recapitulates the embryonic developmental program. However, muscle regeneration is affected in several pathological conditions. Although stem cell therapy has not yet solved the major problem related to cell transplantation, namely the capacity to survive and to improve muscle regeneration, recent studies are beginning to elucidate the signals and mechanisms whereby regenerating muscle recruits circulating cells to sites of injury or degeneration. These cells need not be stem cells as long as they maintain sufficient plasticity to participate in muscle repair, either by rebuilding the damaged tissue or by instructing resident precursors.

Thus, one of the crucial parameters of tissue regeneration is the microenvironment in which the stem cell populations should operate. Stem cell microenvironment, or niche, provides essential cues that regulates stem cell proliferation and that directs cell fate decisions and survival.

It is therefore plausible that loss of control over these cell fate decisions might lead to a pathological transdifferentiation or cellular transformation. Current advances in stem cell biology justify a cautious optimism, yet the presence of stem cells seems to be not sufficient to guarantee an efficient tissue regeneration and repair. Specific factors are required to trigger stem cells toward a specific lineage, to improve their survival, and to render them effective in contributing to tissue repair. Studies on stem cell niche led to the identification of critical players and physiological conditions that improve tissue regeneration and repair.

These evidences suggest that while stem cells represent an important determinant for tissue regeneration, a “qualified” environment is necessary to guarantee and achieve functional results.

Cellular and molecular bases of muscle regeneration.

Muscle regeneration is a coordinate process in which several factors are sequentially activated to maintain and preserve muscle structure and function upon injured stimuli.

It is generally accepted that the primary cause of functional impairment in muscle is a cumulative failure to repair damage related to an overall decrease in anabolic processes.

Aging, cancer, AIDS, heart failure and genetic myopathies are all characterized by alterations in metabolic and physiological parameters and the inability to regenerate and repair the injured muscle represents a serious complication in such pathologies.

Although adult skeletal muscle is composed of fully differentiated fibers, it retains the capacity to regenerate in response to injury and to modify its contractile and metabolic properties in response to changing demand.

Regeneration is therefore an important homeostatic process, which guarantees the maintenance of muscle integrity and plasticity.

The major role in the growth, remodeling and regeneration is played by satellite cells, a quiescent population of myogenic cells that reside between the basal lamina and plasmalemma and that are rapidly activated in response to appropriate stimuli.

RT-PCR analysis, gene targeting strategies and molecular imaging revealed that satellite cells present a heterogeneous profile of gene expression depending on the functional stage of the myogenic program. Quiescent satellite cells express the tyrosine kinase receptor c-Met, a calcium-dependent cell adhesion molecule M-cadherin and the beta isoform of myocyte nuclear factor (MNF), but do not express the Myogenic Regulatory Factors or other terminal differentiation markers. Once activated satellite cells up-regulates c-Met and M-cadherin and activates the expression factors involved in the specification of the myogenic program such as Pax-7, desmin, MNF $\alpha$ , myf-5, and MyoD.

The activated satellite cells proliferate as indicated by the expression of factors involved in cell cycle progression such as and by incorporation of BrDU and [3H] thymidine. Ultimately the committed satellite cells fuse each other or to the existing fibers to form new muscle fibers during regeneration and muscle repair.

More recently other factors such as NCAM, VCAM, Sca1, CD34 and Bcl2 have been reported to be potential molecular markers of quiescent and activated satellite cells.

Transition from cell proliferation to differentiation is characterized by expression of several genes induced during skeletal muscle differentiation, such as cardiac and slow-twitch skeletal muscle Ca<sup>2+</sup>-ATPase (Atp2a2), slow-twitch skeletal muscle troponin T (Tnnt1), Igf2, nicotinic cholinergic receptor alpha polypeptide 1 (Chrna1), fibroblast growth factor receptor 4 (Fgfr4), Peg1/Mest, p57Kip2, cardiac troponin T2 (Tnnt2), H19, transforming growth factor beta1 induced transcript 4 (Tgfb1i4), nicotinic cholinergic receptor gamma polypeptide (Chrng), Igf1, myogenin and cardiac/slow-twitch skeletal muscle troponin C (Tncc). In contrast, the final stage of muscle differentiation involves the activation of other factors, such as Bex1, H19, p57Kip2, Igf2, Peg1/Mest, Peg3/Pw1, and Zac1, most of them have previously been shown to be regulated during skeletal muscle development.

More recently, it has been suggested that other “non-muscle” stem cell populations can participate to muscle regeneration and in some way contributing to maintain the pool of satellite cells. These stem cell populations could either reside within muscle, or recruited via the circulation in response to homing signals emanating from injured skeletal muscle. These populations include endothelial-associated cells, interstitial cells and bone marrow-derived side population cells. At first sight the origin of non muscle-derived stem cells, able to make muscle, appears to be mainly restricted to the hemovascular system (hematopoietic, endothelial, pericytes).

Nevertheless, if skeletal muscle possesses a stem cell compartment it is not clear why the aged muscle fails to regenerate. Either the resident muscle stem cells drastically decrease during aging or perhaps the senescent muscle is a prohibitive environment for stem cell activation and function.

Several evidences suggested that with age or under pathological conditions, the systemic environment is less effective in maintaining the myogenic fate of muscle stem cells and, instead, facilitates conversion to a fibrogenic fate. *In vivo*, this is associated with impaired muscle regeneration and an enhanced fibrotic response. This hostile microenvironment might prevent the activation of resident stem cells and thus might also reduce the success of exogenous cell therapies.

Among growth factors, insulin-like growth factor-1 (IGF-1), Hepatocyte growth factor (HGF) transforming-growth factor- $\beta$  (TGF- $\beta$ ), and fibroblast growth factor (FGF), play important role in the induction and modulation of the myogenic program during muscle regeneration and potentially modulates the microenvironment in which the stem cells reside.

IGF-1 has been implicated in many anabolic pathways in skeletal muscle and it plays a central role during muscle regeneration. Unlike other growth factors IGF-1 also stimulates myogenic differentiation and generates a pronounced hypertrophy of the muscle cells *in vivo* and *in vitro*, suggesting that this

growth factor can regulate both proliferative and differentiative responses in muscle cells, the two stages which guarantee the achievement of the regenerative program.

In addition, the local form of IGF-1 (mIGF-1) promotes an increased recruitment of proliferating bone marrow cells to injured transgenic muscles and modulates the inflammatory response, accelerating the functional rescue of injured skeletal muscle.

These data implicate mIGF-1 as a powerful enhancer of the regeneration response, mediating the recruitment of bone marrow cells to sites of tissue damage and augmenting local repair mechanisms. In addition, our results suggest that while stem cells represent an important determinant for tissue regeneration, a “qualified” environment is necessary to guarantee and achieve functional results.

In this context, therapeutic applications of adult stem cells to aged or pathological tissue repair in the context of regenerative medicine will require an increased understanding of stem-cell biology, the environment of the aged/pathological tissue and the interaction between the two.

## ORAL PRESENTATIONS:

### 1. Regenerative Stem Cell Therapy in an Animal Model of ALS Disease: MRI Longitudinal Study

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The ability to track stem cell transplants in the brain by *in vivo* neuroimaging will undoubtedly aid our understanding of how these cells mediate functional recovery after transplantation. One major challenge for the development and refinement of stem cell transplantation is to map the spatial distribution and rate of migration *in situ*. Here we report the MRI monitoring of human Skeletal Muscle Stem Cells (hSkMSCs) transplanted into the brain of Wobbler (Wb) mice, an Amyotrophic Lateral Sclerosis (ALS) mouse model (Boillée S. *et al.*, 2003). The homozygous mice develop progressive motor dysfunction with loss of motor neurons and astrogliosis preferentially in the cervical tract of spinal cord. In order to monitor hSkMSCs homing and engraftment, through longitudinal studies, we labelled cells before injection with MRI Contrast Agent Endorem™, a aqueous colloid of superparamagnetic iron oxide associated with dextran (SPIO). SPIO-labelled SkMSCs survive long-term *in vivo* and differentiate in a manner identical to that seen for unlabeled cells. Since motor neuron degeneration affects several areas of central nervous system (CNS), in order to make the transplanted cells migrate and home throughout the CNS, we injected SPIO-labelled hSkMSCs into the lateral ventricles. Our study demonstrates that hSkMSCs labelled with SPIO survive in large numbers, are able to differentiate into neuronal and glial lineages, express neural markers, and appropriately respond to microenvironmental cues after transplantation to the Wb brain. Using MRI, we are able to detect the SPIO-related signal into the Wb brain until sacrifice. Moreover, using histology, we show, interestingly, that upon transplantation, SPIO-labelled hSkMSCs migrate and integrate in a manner appropriate for their location. In order to identify the colocalization between SPIO signal and hSkMSCs engraftment, we performed histology with Anti-dextran and human specific neural marker antibodies. Sixteen weeks after transplantation, we detected hSkMSCs migrated into the parenchyma of the brain and along the ependyma of the spinal cord. This migration remained visible for up to 18 weeks. Data are confirmed by histological analyses. Knowledge of SkMSCs migration patterns and information obtained from diffusion-tensor imaging and MR spectroscopy might help to characterize the Wb model and improve the design of future clinical transplantation efforts.

## **2. High frequency ultrasound evaluation of subcutaneous tumours in CD-1 nude mice**

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In the last decade high frequency ultrasound (US) turned out as a very important preclinical tool for the study of complex pathological models such as tumours. The possibility to repeat tests in the same animal, the large amount of information supplied and the high procedural safety are the main advantages of this technique. These features make US deeply compliant with 3R rules (Replacement, Reduction and Refinement in animal studies). A431 (human epithelial carcinoma) and PC3 (human prostate carcinoma) tumours subcutaneously implanted in CD-1 nude mice were analysed. Anaesthetised animals (isoflurane 1.0 %) were examined by a high frequency US (Visualsonics-CAN) equipped by a 40 MHZ scanhead. Both 2D and 3D images of the tumours were taken. Moreover, a contrast agent specific for ultrasound procedures (MicroMarker, Visualsonics) was intravenously administered (50  $\mu\text{L}/\text{animal}$ ) to assess the percentage of tumour microcirculation (PA%). 3D reconstruction images showed a volume range between 110- and 165  $\text{mm}^3$  for A431 and between 30-80  $\text{mm}^3$  (low growth) for PC3 tumours. The values of PA% were 5 and 8%, respectively. Single exams required about 30 min and no problems occurred in the welfare of animals during the experimental phase and further recovery period. The results demonstrated that this procedure can be used in the development of new antitumour compounds. More specifically, the quantification of the microcirculation of the tumour is mandatory to assess the processes of angiogenic modulation as the main marker of the efficacy of new drugs. Among other techniques (MRI, PET, Micro-TC, etc.) high frequency US turned out an helpful tool of the modern translational medicine able to transfer biomedical knowledge from preclinical to clinical field, speeding up the approval of new therapeutic compounds.

### 3. Target therapy and cardiosafety in breast cancer: our experience

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The development of cardiac dysfunction during the treatment of breast cancer is not a recent observation. Ventricular dysfunction induced by Anthracyclines has a clinical evolution worse than the cardiac dysfunction induced by other causes. Anthracyclines effective in breast cancer in all clinical settings, have a toxicity profile that may limit their use. Ventricular remodeling is a progressive phenomenon, whose course is facilitated by sequential stress, until the heart fails to compensate and the heart failure, clinically evident, occurs. There is recent evidence that the damage of cardiomyocytes occurs at doses much lower than those commonly considered to be "fairly safe". Unfortunately when the reduction of ejection fraction (LVEF) occurs, myocardial damage has already occurred. Regarding the study of ventricular remodeling, the most widely used diagnostic method is Color Doppler Echocardiography which studies left ventricular systolic and diastolic function. We are implementing in the daily practice the technique of Tissue Doppler, Strain, Strain Rate and Speckle tracking also in the study of the cardiotoxicity of Trastuzumab. In a mouse model of doxorubicin toxicity, it was possible to identify a reduction of 25% in the systolic endocardial velocity and a reduction of 33% in the strain rate to predict left ventricular systolic dysfunction and mortality. Further studies showed a significant reduction of the strain rate, even after very low doses of anthracyclines, in patients with normal ejection fraction. The use of Tissue Doppler in target therapy is an area of active research. The challenge in the field of prevention of cardiotoxicity of anthracyclines and target therapy is to identify also minimal and initial myocardial damage before the reduction of ejection fraction. We think that the expanding use of tissue Doppler will provide a successful tool for diagnosis of initial cardiotoxicity, in order to modify the dosage and the schedule of the medication.

#### **4. Evaluation of tumoral progression with bioluminescence and micro-PET analysis in MYCN amplified neuroblastoma murine models**

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Neuroblastoma is the most common paediatric extracranial tumor and originates from neural crest. The 25% of cases presents amplification of MYCN oncogene, prognostic factor associated with a bad prognosis and responsible of tumor progression and drug resistance control. It is therefore extremely important to create preclinical murine models that quite represent the pathology in a way to evaluate the disease progression and its response to pharmacological treatment with non invasive methods. In this study two MYCN amplified Neuroblastoma (MA-NB) murine models were setting up: the Xenograft Orthotopic and the TH-MYCN, subsequently analyzed with Micro-PET and BioLuminescence Imaging.

Orthotopic model was obtained by injection of 4 Luciferase competent MA-NB cell lines, in the adrenal medulla of NOD/SCID mice. The tumor progression was analyzed with Bioluminescent Imaging (BLI) starting from the day of injection, every week. Micro-PET analysis were conducted on TH-MYCN mice with <sup>18</sup>F-FDG and <sup>18</sup>F-DOPA radiotracers. The <sup>18</sup>F-FDG uptake in the tumor of homozygous mice was analyzed by Standardized Uptake Value (SUV) index, starting from the fourth week of age, once every four days. All animals were sacrificed and each sample was used for histology, immunohistochemical and molecular analysis of MYCN and N-Myc levels.

Orthotopic model shows a 100% incidence. IMR-5 and BE2(c) show a shorter progression and latency period, respectively. The comparison of two radiotracers have highlighted the higher informativity of the <sup>18</sup>F-FDG. Homozygous TH-MYCN mice shows a 100% incidence, four weeks latency and five weeks progression periods. Histological analysis confirmed the accordance between imaging results and the presence of disease. All the collected tumor samples shows amplification and overexpression of MYCN oncogene.

The real time monitoring by non invasive imaging technique allows the early detection of MA-NB onset and progression in two complementary tumoral models. Moreover, the definition of a specific trend offer the possibility to define an optimal temporal window useful to evaluate the effectiveness of new therapies against MA-NB.

## 5. DEXA AND CT APPLICATIONS FOR BONE ANALYSIS IN MICE MODELS

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Quantitative bone analysis is the reference technique to study age or disease-related bone loss, to evaluate mineralization in fracture repair and to test new therapies for bone mineralization. Several methods for evaluating bone mineral density and concentration are available, such as dual energy X-ray absorptiometry (DEXA) and quantitative computed tomography (QCT). The mouse has become the primary laboratory model in bone research field, and bone metabolism and diseases can be accurately evaluated, in vivo and in a longitudinal and non invasive way, using dedicated small animal DEXA or high resolution microCT ( $\mu$ CT) scanners. The purpose of this paper is to describe our experience of DEXA and  $\mu$ CT applications in the laboratory mouse to perform bone analysis.

We used the Lunar Piximus densitometer (GE Medical Systems) and the Explore Locus  $\mu$ CT scanner at 45  $\mu$ m spatial resolution. Mice were anesthetized with isoflurane 1,2% plus oxygen 0,8 L/min or by intraperitoneal injection of ketamine 100 mg/kg + xylazine 10 mg/kg and positioned prone with fully extended limbs. DEXA and  $\mu$ CT dataset were analyzed by proprietary softwares, to obtain 2D or 3D reconstructions of bone and serial measurements of bone mineral density (BMD) and content (BMC) in whole body or selected regions of interest (ROI). We phenotypized with DEXA twenty eight C57BL/6J mice, wild type and knock-out for MoKA gene. BMD ( $\text{gr}/\text{cm}^2$ ) and BMC (gr) were measured in the femur. The role of BMP4 protein in bone regeneration was characterized by DEXA and  $\mu$ CT (BMD,  $\text{mg}/\text{cm}^3$ ; BMC, mg) in twenty five athymic nude mice, injected intramuscularly with FG-Ad BMP-4 or FgAdBMP-4TK and treated with ganciclovir. Whole body vibration (WBV) effects on bone in growing mice were evaluated with DEXA in thirty five C57BL/6J mice.

Student t-test revealed BMD < 10% in knock-out compared to wild type female mice ( $p = 0,0013$ ) for MoKA gene. BMD ( $\text{mg}/\text{cm}^3$ ) and BMC (mg) changes over time were measured to demonstrate ectopic bone formation into the quadriceps femoris induced by BMP4 expression. Statistical analysis of BMD and BMC with a Mann & Whitney test showed a significative increase of these parameters for total body ( $p=0.002$  and  $p=0.02$  respectively) and femur ( $p=0.001$ ) in mice treated with WBV.

DEXA and  $\mu$ CT are feasible and non invasive techniques to accurately perform in vivo quantitative bone analysis and to monitor over time changes of bone parameters in laboratory mice.

## 6. Ultrasound Contrast Agents for imaging and gene therapy

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Ultrasound contrast agents, also known as microbubbles (MB), are a powerful research tool for diagnosis and therapy of animal model of human diseases. MB enhances ultrasound (US) applications, allowing for quantitative analysis of molecular biomarkers, perfusion studies, microvasculature targeting, gene and drug delivery. Aim of our study is to illustrate the importance of ultrasound contrast agent for gene therapy in a mice model of prostate adenocarcinomas in small animal research. Targeson's ultrasound contrast agents consisting of small, stabilized lipid microbubbles (< 10 µm), which were prepared to contain adenoviruses expressing the melanoma differentiation-associated gene-7/interleukin-24 (Ad.mda-7), were injected intravenously in the tail vein of nude mice to enhance contrast of the blood pool signal and to target the adenoviruses to xenografted prostate adenocarcinomas. Viral targetization was achieved through the intrinsic characteristics of sonoporation and cavitation of microbubbles exposed to the appropriate US physics parameters such as acoustic power (Mi >0,3-0,6). Prostate tumor xenografts were established on both flanks of 420 nude mice. Tail vein injections of the MB/adenoviral complexes and sonoporation of prostate tumors started 10 weeks after tumor xenograft establishment when tumors reached an approximate volume of 150–200 mm<sup>3</sup> and were performed for 4 weeks. US MBs are viable candidates for gene delivery/therapy. MB/Ad.mda-7 complexes targeted to tumor prostate cells using US dramatically reduced tumor burden in xenografted nude mice. In conclusion, US with MBs is a non-invasive and relatively inexpensive modality and it can be performed rapidly. Moreover, this technique could be easily applied on humans, for example molecular imaging with US targeted MB could be potentially useful in the early diagnosis and treatment of tumor formations enhancing conventional treatment protocols.

## 7. Generation of bioluminescent mll-positive acute leukemias mice reveals different mll-related tumor progressions

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The MLL (Mixed-Lineage Leukemia) gene is a common target for chromosomal aberration in human acute leukemias (ALs), is frequent in infant and is associated with poor prognosis. Currently more than 60 fusion gene partners are known, and the most frequent translocations are t(9;11) MLL-AF9, t(4;11) MLL-AF4 e t(11;19) MLL-ENL. To evaluate the leukemic progression of MLL-related ALs, we developed bioluminescent (BL) xenograft mouse models of MLL-AF9, MLL-AF4 and MLL-ENL ALs. Bioluminescent imaging (BLI) is based on the reaction of the enzyme luciferase with his substrate, D-luciferin, in the presence of ATP, molecular oxygen and Mg<sup>2+</sup>, and the result is the emission of light proportional to the expression of the enzyme luciferase. *In vivo* BLI is simple, sensitive, with virtually no background noise, and is well tolerated by the laboratory animals. Acute myeloid leukemia (AML) MLL-AF9 cell lines (THP-1 from infant patient and MOLM-13 from adult patient), and acute lymphoblastic leukemia (ALL) MLL-AF4 (SEM) and MLL-ENL (KOPN-8) cell lines were stably transduced with *Photinus pyralis* firefly luciferase expression plasmid pMMP-LucNeo. 5-10x10<sup>10</sup> luciferase-expressing cells were intravenously injected in 6 weeks old NOD/SCID immunodeficient mice. Mice were monitored once a week after intraperitoneal injection of 150mg/kg D-luciferin with a CCD camera system (Berthold Night Owl LB 980) under isoflurane anaesthesia, both in prone and supine position. The BL curves were computed by the average of at least 10 mice signals, as the sum of both prone and supine acquisitions for each mouse (photon flux (ph/s) with the Windlight software (Berthold). BLI revealed to be a non-invasive, sensitive, rapid and affordable method that enables the early detection of tumors, either superficially or in deep tissues, preceding the appearance of evident symptoms and blood dissemination. Our MLL-related ALs mouse models recapitulate the different course of the infant and adult human MLL-AF9 AML, and the rapid aggressiveness of the human MLL-ENL and MLL-AF4 ALL. The parallelism between the results of BLI and the clinical course of MLL-related ALs supports our proposed bioluminescent MLL-related acute leukemias mouse models as suitable tools for the study of MLL-related ALs, and moreover for the bioluminescent evaluation of therapeutics in drug development and preclinical studies.

## 8. Innovative Burkitt's Lymphoma therapy monitored by in vivo bioluminescence imaging.

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In human Burkitt's Lymphoma (BL) BRG cells, a t(8;14) translocation, placing c-myc near the E $\mu$  enhancer of the H chain locus, causes tumor expansion. Earlier, we showed that a peptide nucleic acid complementary to the E $\mu$  sequence (PNAE $\mu$ ), specifically inhibited the expression of translocated c-myc and impaired the growth of BRG cells-induced subcutaneous tumors in CB17 SCID mice.

In this study, the therapeutic potential of PNAE $\mu$  was evaluated in a systemic mouse model. BRG-BL cells transfected with the firefly luciferase gene (BRG-BL-Luc cells) were inoculated intravenously into SCID mice resulting in a preferential expansion, similar to the one of human adult patients, in the abdominal cavity, central nervous system and bone marrow.

The mice were chronically injected intraperitoneally either with PNAE $\mu$ wt or with control PNAE $\mu$ mut (both 0.2mM in 100 ml of phosphate buffered saline) starting 1 week after the inoculation. Once a week both group of mice were injected intraperitoneally with D Luciferin (15mg/kg), and anesthetized with continuous exposure to 1–3% isoflurane inducing a linear response in photons/second for exposure times ranging from 1 to 5 min. A colorimetric coded reference bar, from purple: minimum to red: maximum, allows a 'first glance' intensity quantization of the luminescence. Bioluminescent signals from BRG-BL-Luc cells, as detected by the camera system were recorded, integrated, digitalized, displayed and quantified (in photons/second) using the Living Image (Xenogen) software. The treatment was stopped when the control animals developed severe neurological symptoms. As detected both by inspection at necropsy and imaging, overall tumor growth in PNAE $\mu$ -treated mice decreased by >80%. Tumor cells growth in the PNAE $\mu$  or control PNAE $\mu$  treated groups was calculated (photons/seconds $\pm$ s.d.) by measuring the luminescence emitted in each mouse by the BRG-Luc cells, during tumorigenesis at increasing times and number of treatments, with 2.5 min of exposure and with a homogeneous sensitivity setting.

These data were confirmed by histological and immunohistochemical studies that showed, only in PNAE $\mu$ -treated mice, a substantially reduced BL cell growth at the major sites of invasion and vast areas of necrosis in the lymphomatous tissues, with concomitant c-myc expression downregulation. Altogether, the data support the therapeutic potential of PNAE $\mu$  in human adult BL.

## 9. Development of a non-invasive method gastric emptying rate measurement in mice using bioluminescence molecular imaging

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Bioluminescence *in vivo* imaging (BLI) is a powerful tool in preclinical research allowing the real time monitoring of different physiological and pathological conditions in living intact animals using bioluminescent (BL) reporter gene technology. The development of new drug acting on gastrointestinal motility requires the used of predictive animal models suitable for preclinical structure-activity studies. Gastric emptying in mice is usually measured with invasive techniques requiring animals sacrifice; alternatively expensive and complex technologies such as scintigraphy, magnetic resonance imaging (MRI) and <sup>13</sup>C-acetic acid breath test can be used. A non-invasive, highly sensitive new test for gastric emptying time measurement has been developed using luciferase-expressing bacterial cells as a biomarker of the liquid content of the stomach.

A new thermostable red-emitting luciferase was chosen as reporter gene to transform *E. coli* cells. Bioluminescent bacteria were administered to fasting mice, after a solid meal, and in response to different doses of metoclopramide and hyoscine butylbromide. BL imaging allowed to evaluate the real time 2D spatial and temporal distribution of bacteria along the gastrointestinal tract in whole animal and to calculate GE rate in basal conditions and following pharmacological stimulation. The gastric emptying has been monitored with a Berthold LB981 low-light imaging system using an ultrasensitive CCD camera by collecting an image every minute for up to 30 minutes.

The administered BL bacteria were easily imaged and localized in the stomach and subsequently followed in the duodenum and upper intestine allowing to accurately calculate GE. GE after the test meal was significantly slower (T1/2 16±3 min) than that obtained in fasting conditions (T1/2 2±1 min); administration of hyoscine butylbromide (1 mg/Kg b.w.) significantly (p<0.05) increased T1/2 that was delayed up to 25±4 min; metoclopramide (1 mg/Kg b.w.) significantly (p<0.05) accelerated T1/2, that was achieved within 8±2 min.

A new method involving the use of a suspension of bacterial luminescent cells acting as a floating nanobeads markers of gastric liquid content to monitor gastric emptying has been developed. Bioluminescence *in vivo* imaging technique gave results comparable with the other imaging techniques employed until now and can be easily applied for pharmacological studies and drug discovery.

## **10. Microtomographic evaluation in preclinical orthopaedic studies**

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Computerized microtomography (micro-CT) is a non destructive technique that allows the tridimensional study of bone and biomaterials. Actually in micro-CT images it is possible to see the internal structure of a small object with an high resolution and without any preparation or chemical fixation. Besides specialized software and hardware implement the evolution of image analysis techniques and enable a large applicability, an high calculation intensity and a rigorous statistical approach. The sections obtained from a micro-CT acquisition are used in the analysis and the measurement of microstructural parameters. In the field of preclinical orthopaedic studies, micro-CT technique can be used in several different kinds of evaluations. There are carried out analysis on biomaterials before the implantation to evaluate porosity or mechanical characteristics; analysis on metallic implants, often utilized in orthopedics and dentistry, where the study of bone growth through bone regeneration and the integration with the surrounding bone; analysis on polymeric or ceramic implants to study principally the bone regeneration in the defects or the loss of bone substance; analysis of bone morphology that is extremely important because a lot of pathologies alter the bone microarchitecture (for example osteoporosis or pathologies and therapy that affect bone tissue. Besides, after a biomaterial implantation it is important not only the quantitative evaluation of bone growth, but also the quality of regenerate bone. The possibility to create virtual 3D models of samples based on micro-CT sections allows realistic visualizations of them and the complete understanding of structures that normally are observed only in a bidimensional mode typical of classical histological sections. These models can be used to assemble filmed sequences of the analyzed sample in movement. Therefore there is the believe that computerized microtomography can result, especially for preclinical evaluation of implant materials destined to musculoskeletal system, a very valid and non destructive method. This technique results very useful both characterized devices in pre-implant phase and characterized the same devices in the explant phase to evaluated possible deformations and/or degradations.

## 11. Usefulness of small animal positron emission tomography/computed tomography to non invasively analyse insulin sensitivity in diet induced obese mice

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We analysed the usefulness of small animal Positron Emission Tomography/Computed Tomography (PET/CT) combined technique, as a new tool to *in vivo* study insulin sensitivity in peripheral organs of mice. Standard techniques available to study insulin sensitivity of individual organs require animals sacrifice and complicated biochemical analysis of glucose uptake on explanted tissues. Using a glucose radioactive analogue (<sup>18</sup>F-FDG) we propose PET/CT as a new method to get metabolic information of peripheral organs by a simple image analysis approach. A software-based-method has been used to overlap PET and CT images in the three spatial dimensions. CT images provide the anatomical reference of the metabolic active structures detected by PET, thus allowing to correctly quantify PET signal. Three groups of mice were analysed after administration of different diet regimens leading to progressive obesity levels: standard chow diet (SD), high fat diet (HFD, 40 % energy from fat), super high fat diet (SHFD, 60 % energy from fat). Each group of animal underwent two repeated PET/CT scans, the first in fasting state (basal state), the second after insulin administration (0,7U/k). Glucose levels were measured during PET/CT procedures with a glucometer. Standardized uptake values (SUV) of brown adipose tissue (BAT), white adipose tissue (WAT), skeletal muscle, myocardium were calculated on PET/CT images. Analysis of glucose levels respectively revealed a slight and a severe compromising of insulin sensitivity in HFD and SHFD groups. SUV analysis was in agreement with glucometric test; insulin treatment in normal SD mice induced a statistically significant increase of <sup>18</sup>F-FDG uptake in myocardium, WAT, BAT when compared to the basal fasting condition, whereas insulin effect on <sup>18</sup>F-FDG uptake was lower in HFD group, and completely absent in SHFD group. Interestingly, <sup>18</sup>F-FDG uptake in skeletal muscle was not affected by insulin treatment in the three groups of animals, probably as a consequence of a counter-regulatory hormonal mechanism induced by hypoglycaemia. Even if future studies are needed to further validate imaging data with gold standard methods, these data highlight the usefulness of PET/CT technique to non invasively monitor insulin sensitivity in mice model of diet induced obesity. The opportunity to longitudinally evaluate the same animals makes PET-CT an advantageous approach for the screening of new drugs against obesity related metabolic dysregulations.

## 12. “*In vivo*” homing of labelled neural stem cells in a mouse model of spinal cord injury

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The use of adult stem cells in cell-mediated therapies is an area of considerable interest within tissue regeneration research. Treatment efficacy evaluation is generally based on functional recovery end points, skipping the evaluation of important parameters, such as the distribution of injected cells, localization at the target organ, cell survival and differentiation. Hence the need of new strategies for the visualization and localization of stem cells *in vivo*. Here we tested a multiple labelling approach for *in vivo* visualization by MRI, SPECT and Optical Imaging of murine neural stem cells (NSC) in a mouse model of traumatic spinal cord injury.

NSC, isolated from the subventricular zone of adult mouse brain, were labelled for 24h with 200µg Fe/ml of SPIOs in presence of Protamine Sulphate and analyzed for iron content, viability, morphology and differentiation capability. Labelled cells were injected either locally, at the site of the injury, or systemically into the tail vein and followed by MRI for more than a month to visualize NSC localization at the lesion site. Initial cell distribution was also followed by scintigraphy after cell labelling with <sup>111</sup>In-oxine (60 µCi/10<sup>6</sup> cells). Cells localization, distribution and viability over time were also analyzed *in vivo* by Optical Imaging after injection of NSC infected with a viral vector expressing luciferase under a PGK promoter (PLW). After imaging, mice were perfused with PFA and spinal cords extracted to perform *ex vivo* MRI and histopathological analysis.

Iron oxide labelling procedure did not significantly perturb viability and proliferation rate of NSC. SPECT imaging showed initial distribution to lung, spleen and liver. NSC, infected with the viral vector PLW, were detected by OI at the site of the injury after intramedullary injection, and at the same site one week after *i.v.* injection. MRI showed on both RARE T2-W and FLASH images a hypointense signal due to Fe+ labelled cells at the injury site three weeks after *i.v.* injection. Iron presence was confirmed on *ex vivo* MR images and on histological sections of perfused spinal cords.

Adult NSC can be efficiently labelled without significantly perturbing their physiological features and self-renewal capability. The labelled NSC were visualized *in vivo* by Scintigraphy, MRI and OI providing information on their initial distribution, localization at the spinal cord injury site and survival.

### **13. A rat model of intestinal infarction due to venous occlusion: usefulness of 7T micro-RM imaging.**

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Acute mesenteric ischemia (AMI) is a potentially fatal vascular emergency with overall mortality of 60% to 80% and an increasing incidence. The poor prognosis of gut ischemia is partially due to the lack of specific findings, either clinical or radiological, that leads to delayed diagnosis and ineffective treatment. Early diagnosis seems to be the shortest way to reduce mortality rate. Therefore, the aim of this study is to validate a rat model of acute intestinal ischemia due to venous occlusion, in which MR imaging patterns are related to the evolution of intestinal morphodynamism and histological analysis.

The study was conducted on 32 Sprague Dawley rats. After anaesthesia, a laparotomy was performed and the superior mesenteric vein (SMV) isolated. Then the rats were randomly divided in two groups: in the first (n=15), “control” animals underwent the SMV occlusion by a tight ligation and, after macroscopical monitoring, rats were sacrificed at different timing and the bowel removed for histological analysis; in the latter (n=17), a loop (3-0 gut) was tied loosely around the vessel and the tips tunnelled from the abdominal cavity through a tube to the posterior cervical area without occluding the vessel. 3 days after surgery, basal MR abdominal scans were collected for each rat using a 7T micro-MR (Bruker Biospec 70/16 US); then the loop was squeezed pulling the external tips in order to occlude the vessel and MR sessions were repeated after 5 min, 4 and 8 hrs.

The macroscopical monitoring of rats belonging to the first group showed a clear mesenteric vascular congestion at the first time-point (5 min after ligation of SMV), erosion and frank ulcers with segmental changing in colour and diameter of intestinal loops (*Spastic and Hypotonic Reflex Ileus*) at the second time-point (4 hrs) and worsening of these findings at the third (8 hrs). Instead, rats in the second group were scanned using a micro-MR with RARE T2 sequences: no evidence of pathological patterns was detected at the first time-point, while significant bowel wall thickening (> 1,5 mm) and mesenteric hyperintensity were found at following time-points (4 and 8 hrs). After experimentation, rats were sacrificed and the entire bowel removed for histological analysis on hematoxylin-eosin stained sections: vascular congestion in the submucose lamina with no sign of lysis or inflammation was present 5 minutes after ligation (first time-point), while destruction of the free portion of the ‘villi’, presence of dilated capillaries and inflammatory cells were found at 4 hrs, eventually structural destruction of the ‘villi’ with sparing of glandular ‘criptae’, haemorrhage, presence of inflammatory cell and necrotic material was detected at the third time point.

Compared to histological analysis and macroscopical evidences, MR imaging can correctly detect morpho-functional alterations of ischemic gut. MR succeeded to early identify the signs of venous mesenteric ischemia already 4 hrs after SMV occlusion. Its future application in early diagnosis of mesenteric venous ischemia is highly reasonable.

## **14. MRI quantitative microvessels characterization based on protein binding contrast agent**

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The potential of the protein-binding contrast medium B22956/1 (Bracco Imaging S.p.a) in the assessment of tumour microvasculature development during antiangiogenic therapy has been investigated by Dynamic Contrast-Enhanced MRI (DCE-MRI) in an experimental cancer model.

PC-3 cells, a human prostate cancer line known to be hypovascularized, was implanted in 16 NCR athymic nude mice. Animals were assigned randomly to a control (Vehicle) or drug-treated (Axitinib™) group. Axitinib™ is a potent receptor tyrosine kinase inhibitor that targets VEGFRs at subnanomolar concentrations with known activity on PC-3 xenografts. Tumour growth was monitored by means of calliper measurements. MRI was performed at baseline (T0) and after seven days of treatment (T1). The transendothelial permeability (kTrans) to B22956/1 (injected at the dose of 0.1 mmol/Kg) and the fractional plasma volume (fPV) were estimated from the kinetic analysis of dynamic MR data using a two-compartment model; the Initial Area Under the Curve (IAUC) was calculated in several time windows after contrast agent injection ranging from 1 to 30 minutes.

Tumours grew more slowly (p-value < 0.05 Mann-Whitney U Test) in the Axitinib™-treated group. The kTrans determined with B22956/1 decreased significantly in the treated group compared to baseline (p-value < 0.05 Mann-Whitney U test), while no significant differences were observed in the control group. Significant differences were also observed for kTrans between treated and control group at T1 while no differences were observed at T0. The value of IAUC decreased significantly in the treated group compared to baseline (p-value < 0.05 Mann-Whitney U test) provided that the MRI dynamic acquisition is extended for at least 5 minutes post contrast agent injection. Significant differences were also observed in the distribution of the IAUC parameters between treated and control groups at T1.

In summary with the use of B22956/1 the therapeutic effects of a VEGFRs inhibitor in an hypovascularized tumour can be monitored by DCE-MRI. B22956/1's sensitivity to microvascular changes, granted by its high relaxivity and by its distinctive pharmacokinetic, suggests several potential clinical applications particularly in the therapy follow-up.

## POSTER SESSION:

### 1. Imaging application for preclinical cancer studies

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Eterothopic and orthotopic injection of human tumor cells into immunodeficient animals like nude mice has been used to create experimental models of human cancers.

In this work we show an eterothopic model of kidney cancer and lung cancer by fibrosarcoma and orthotopic model of pancreatic cancer using three different imaging techniques.

To perform eterothopic model we injected subcutaneously SN12C (human kidney cancer cell line) in nude mice transfected with pEGFP to monitoring tumor growth and lymph node metastasis. In the other model we injected HT1080 transfected with pEGFP in tail vein of nude mice to establish a model of lung cancer of metastasis. The evaluation of tumor growth and lymph node metastasis was done with Macroscopy Fluorescence (*Leica*) and confirmed with histological analysis.

However, spontaneous metastasis is rarely observed when tumors are placed in subcutaneous sites.

Orthotopic models are now regarded as more likely to duplicate the process of local tumor growth and metastatic spread in patients. So in order to overcome this problem we perform an orthotopic model of pancreas injecting MiaPaca-2 in the pancreas.

The tumor growth and progression was monitored by MRI and Vevo2100 Ultrasound (*Visual Sonics*).

We show in our work that through imaging that it possible monitor tumor progression and therapeutic efficacy of drugs through preclinical studies.

## 2. Molecular Imaging by micro-PET to evaluate the antitumoral activity of natural molecules for the treatment of Alveolar Rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma. Of the two main subtypes of RMS, alveolar (ARMS) and embryonal (ERMS), the alveolar one is associated with a worse prognosis and is characterized by translocations involving fusion proteins PAX3-FKHR and PAX7-FKHR. Sulforaphane (SFN), initially discovered as an activator of phase 2 enzymes, is an isothiocyanate that plays an important role as an anticancer agent that interacts and modulates several critical cellular targets in the tumorigenic process. In this study, a mouse model of ARMS was set up for the preclinical evaluation of SFN, TRAIL and their combination effect. The microPET was used to monitor in real time the tumor growth and the response to treatment by the use of TBR.

The *in vivo* analysis was conducted on xenograft mice inoculated s.c. with 10 million rhabdomyosarcoma alveolar cells and treated i.p. for 21 consecutive days with saline (control group), with SFN (treated group) and SFN + TRAIL at two scalar concentrations (group treated in combination). The TBR was calculated at regular intervals of seven days for the duration of treatment.

The positivity of the inoculated cells was seen by the micro-PET after only two days after injection. The SFN alone resulted in a reduction of tumor growth, as well as TRAIL at the highest concentration but the combination of the same concentration of TRAIL with SFN showed instead the disappearance of the signal in micro-PET, leading the value of TBR under the threshold of positivity.

The micro-PET is a useful tool through which it's possible to study the ongoing growth of tumor in mouse models, to decide the time of treatment and to monitor, in real time, the effect of potential drugs or the antitumor activity of molecules corresponding to the decrease of TBR. The promising results obtained by the combination of SFN with TRAIL represent the bases for future association studies in which the SFN could be used not only as an anticancer agent but also as a potential chemo-preventive agent.

### **3. Use of micro-MR 7T in the suspect of acute arterial mesenteric ischemia: evaluation of an animal model**

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Mesenteric ischemia is an uncommon but often underestimated cause of nontraumatic acute abdomen and its global prevalence is around 0,1% of all hospital recovers. Despite of our better knowledge and improved diagnostic techniques bowel infarction is still frequently a fatal disease, with reported mortality rates between 50% and 90%. This poor outcome has remained static for many decades and is associated with a variety of contributing factors. Of particular relevance is the late presentation, late or incorrect diagnosis, wrong therapies.

Thanks to a simple and versatile and inexpensive animal model, we reproduced this pathology, inducing intestinal ischemia without laparotomy. The aim of the study was to identify MR imaging patterns of lesions due to acute occlusion of Superior Mesenteric Artery (SMA), relating these radiological findings to macroscopical monitoring and histology.

30 Sprague Dawley rats were anesthetized, a laparotomy was performed, and cranial mesenteric artery isolated. In a first group of “control” animals (n=15), the artery was directly occluded by a tight ligation and during the following 8 hours macroscopical lesions were monitored by photocamera. Eventually the bowel was excised for histological analysis. In a second group (n=15), a loop (3-0 gut) was tied loosely around the vessel and the tips exposed on the rat back without occluding the artery. After 3 days from surgery, basal MR (Bruker Biospin) abdominal scans were collected for each rat of this group. Afterwards the loop was tied by external tips to occlude the vessel and MR session was repeated 4 and 8 hours later. The animals were then sacrificed and the entire intestinal package processed for histological analysis on hematoxylineosin–stained sections.

No animals died before the end of the study. One rat of the second group was excluded from analysis because showed SMA showed stenosis but not complete occlusion at 8 h after ligation in the angio-MR sequences. Abdominal MR scans showed no gas in the abdominal cavity and no signs of bowel or mesentery irritation. T2 MR sequences identified several injury signs of vascular occlusion like loops dilatation, decrease of intestinal wall thickness and peritoneal fluid. These changes were paralleled by histological alterations as highlighted by ex-vivo examination, with no significant differences in the histological analysis of same intestinal tracts between the two groups.

This animal model could represents an useful and highly reproducible tool to evaluate by imaging the evolution of intestinal ischemic lesion and assess the effectiveness of new therapeutic modalities. Compared to histological analysis, MR imaging can correctly identify morpho-functional alterations of intestinal ischemia due to acute occlusion of SMA.

#### 4. Management of anaesthesia in 60 *Macaca fascicularis* undergoing medical imaging procedures

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Non-human primates (NHP) are important models for a wide variety of biomedical research. When medical imaging is needed in these animals, anaesthesia is often a prerequisite to avoid gross movements. A large number of drugs combinations is reported in the literature for NHP. However, but there is little information on major and minor complications that can occur in the peri-anaesthetic period. The aim of this study is to critically review the management of anaesthesia for imaging in NHP and to analyze undesirable events encountered, in our experience, with standard anaesthetic protocols. Twenty *Macaca fascicularis* have been anaesthetized on 60 occasions to perform positron emission tomography (PET) or magnetic resonance imaging (MRI). Twenty-eight procedures were performed on healthy animals, 23 on parkinsonian subjects and 9 in parkinsonian subjects after porcine embryonic neurons transplantation. A dissociative anaesthetic combination (ketamine-xylazine or ketamine-medetomidine-midazolam) was used; all animals were intubated, connected to a breathing system and allow to spontaneously breath oxygen 100%. Cardiovascular and respiratory functions were monitored during the procedure and in the post-anaesthetic period, and physiological data and undesirable events were recorded. Most common events observed were: mild hypothermia (22 cases), prolonged recovery (over 30 minutes after extubation in 7 animals), stiffness (4 parkinsonian animals), post-extubation hyper salivation (2 animals) and sporadic II degree AV blocks (2 animals): all the events were considered minor complications with no impact on patient's health. One animal showed post-extubation retching and hiccup on the first anaesthetic; on the second anaesthetic, the same animal had a cardio-respiratory arrest after extubation that was promptly and successfully reversed by standard resuscitation technique with no long term consequences. In 3 animals with nasal obstruction, anticipation and prevention of possible complications and modification of the peri-operative management resulted in uneventful procedures. Observation and cautious monitoring before, during and after general anaesthesia enable the prompt recognition of potential problems and rapid intervention before these may progress to serious life-threatening situations preventing disastrous consequences. A continuous reassessment of the events occurred in the peri-anaesthetic period provides important information enabling appropriate changes to improve of animal welfare and scientific outcome.

## **5. Generation of Medulloblastoma Bioluminescent Mouse Models and identification of the different tumor progression in desmoplastic, classic and anaplastic variants**

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Medulloblastoma (MB) is the most common pediatric primary tumor of central nervous system. Currently only 50-60% of the patients are successfully cured and many of the patients who survive exhibit serious side effects due to the very aggressive therapies they underwent. The purpose of this work is the development of a preclinical mouse model of MB to further investigate the mechanisms involved in the neoplasia and to evaluate new therapeutic agents.

The mouse models were created using three MB cell lines derived from three different histological variants: DAOY (desmoplastic variant), D341 (classic variant), D556 (anaplastic variant). The tumor onset and progression were evaluated with a bioluminescence molecular imaging technique based on luciferase reporter gene extracted from the North American firefly (*Photinus pyralis*). In this regard, before being grafted in mice's cerebellum, the tumor cells were transfected to permanently express luciferase gene. Afterwards, mice were monitored every seven days by a CCD camera able to detect photons emission after intraperitoneal injection of D-Luciferin.

The mouse models have shown an incidence of a 100% and different latency and tumor progression. Desmoplastic variant characterized by the presence of nodules and low cellular density, correlates with a more favourable outcome. Mice were monitored for three months with an endpoint at 108 days. Classic variant with small cells and low neuroblastic differentiation reveals an intermediate outcome with an endpoint at 66 days. Anaplastic variant with pleiomorphic nuclei, prominent nucleoli, abundant cytoplasm and intense mitotic activity, reveals a worse outcome with an endpoint at 17 days. At the end of the study, an histological characterization of the tumor has been performed.

*In vivo* molecular bioluminescence imaging allows to follow tumor evolution since the first phases of tumor development, showing good sensibility and specificity. Thus, the created xenograft orthotopic bioluminescence mouse models, reflecting the same clinical course of the histological variants of medulloblastoma, represent a valid preclinical model to study new drugs for the treatment of this neoplasia.

## 6. Feasibility of High Resolution Ultrasound guided Microinjection in mice uterus

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The mouse is an important model for exploring the developmental consequences of altering gene expression using viral vectors, to study cell lineage or migration and to validate gene therapies. Accurate injection in specific sites currently requires surgical visualization of organs with many possible complications. This approach is impractical for repeated injections. High Resolution Ultrasound (HRU) allows non invasive visualization in real time of mouse embryo. Our first goal was to determine whether the progression of the needle as it traversed the uterus wall and if the position of the needle tip could be visualized. We hereby describe our approach to test the feasibility of HRU guided needle microinjection in mice uterus.

Microinjection was performed in 5 C57Bl/6J pregnant mice at embryonic day 14.5. Mice were anesthetized with isoflurane 2% and Oxygen 0,8 L/min. Body temperature was monitored at 35–38°C. To perform in uterus microinjection a laparotomy was required. Abdominal skin was tricotomized and cleaned with 70% ethanol. A 2 cm midline incision was made along the linea alba and the uterine horns were exteriorized to record implantation sites and then repositioned in the abdominal cavity, except a short segment containing 1–3 implantation sites. We positioned uterine horn on a sterile gauze soaked with sterile PBS. Then, HRU was performed with a 55 MHz probe to focus embryos amniotic cavity. Microinjection was performed in amniotic cavity with an automatic microinjector equipped with a capillary glass. The needle was advanced with the use a micromanipulator under echo guidance until the needle tip was in the desired location. Then, 69 nL of contrast solution was injected and the accumulation of contrast was documented as a video clip. After microinjecting all sites, the maternal abdomen is closed using 8-0 silk, using a continuous suture. HRU was performed in the following two days to test fetuses health status.

In uterus microinjection was well tolerated from both mother and fetuses. In the process of externalization, an average of 8 fetuses were counted. After 21 days of pregnancy, an average of 8 healthy mice were born, confirming the absence of embryos mortality after ultrasound guided in uterus microinjection. We were able to visualize and guide the needle into the amniotic cavity. The availability of real time in utero imaging of mouse embryos made possible to perform Ultrasound guided injection of cells into precise location in different stage of embryo development.

## **7. Role of MRI in detecting gut injury induced by ischemia–reperfusion in a rat model**

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Purpose of the study was to investigate the right evolution of damages in ischemia-reperfusion injury of rat ileum, relating it to MR imaging findings. Experiments were performed on 22 Sprague Dawley male rats (250–300 g; Harlan, Italy). After anaesthesia, a median laparotomy was performed and, using an operating microscope, the superior mesenterial artery was carried out; then the vessel was clipped in non-traumatic way for 1 hour. After removing the clip, reperfusion was permitted for 6 hours. MRI images were acquired at different timing with a 7T micro MRI scanner. MRI scans were performed at T0 (basal), at the end of ischemic period (T1, 1 hour), after 3 (T2) and 6 (T3) hours after reperfusion. Histological analysis was performed on reperfused intestinal tissues removed at the different timing by different rats. MRI showed the evolution of ischemia-reperfusion damages: during all the ischemic period, imaging findings were similar to pure arterial mesenteric ischemia. During the reperfusion period, MRI showed injury signs strictly associated with venous mesenteric ischemia. Histological analysis on segments of ileum taken at different timing confirmed the injury showed by MRI.

MRI has proven to be a valuable tool both in diagnosis of mesenteric ischemia and in the early detection of damage due to reperfusion. MRI shows clearly the broad range of lesions induced after ischemia-reperfusion and confirmed by pathologists.

## 8. Small animal pet imaging of brown adipose tissue functional activity with <sup>11</sup>C-Meta-Hydroxyephedrine

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Brown adipose tissue (BAT) differentiates from white adipose tissue due to its unique ability to burn energy as heat; peripheral sympathetic nervous system (SNS) is the main regulator of BAT functions through a norepinephrine (NE) mediated signaling.

In the last year, raising experimental evidences on laboratory animals have demonstrated that pharmacological stimulation of BAT functional activity decrease energy accumulation from the diet, thus counteracting diet-induced obesity. Very recently, it has been demonstrated that not only animals, but also adult humans possess a functionally active BAT, highlighting BAT as a new target for anti-obesity research. The experimental PET tracer <sup>11</sup>C-meta-hydroxyephedrine (HED) is a NE radioactive analogue widely used to study noradrenergic cardiac function with PET.

*In vitro* analysis of HED uptake on explanted tissues pointed out this tracer as actively taken up also by other peripheral tissues as BAT. Based on this evidence we sought if *in vivo* PET imaging of BAT with HED tracer was possible, and if it could be a reliable measure of peripheral sympathetic activity.

To this aim, we studied by PET/CT, HED biodistribution in the BAT of mice analyzed in different conditions: basal conditions, cold exposure, chemical and surgical sympathectomy.

Analysis of mice PET/CT fusion images in basal conditions, demonstrated the presence of an active area of physiological uptake of HED at the level of BAT. To analyze if this signal reliably reflects sympathetic activity, we exposed animals to a cold stimulus, known to induce an over-activation of NE signaling in the BAT. As expected, cold exposed animals exhibit a drastic increase of HED uptake in BAT; moreover, when sympathetic function of the same animals was downregulated by chemical sympathectomy (6 OH-DOPAMINE), tracer uptake was reduced. Finally, when sympathetic activity was almost completely abolished by a procedure of surgical resection of sympathetic nerves surrounding BAT, PET signal in BAT area of cold exposed animals was not different from those detected in animals analyzed in basal state.

To our knowledge, these are the first data demonstrating the possibility to non invasively get direct information on SNS functional activity of BAT.

PET/CT imaging approach will allow repeated longitudinal evaluations of BAT SNS activity in the same animals after pharmacological or nutritional intervention.

## 9. Identification of $\beta_3$ -adrenoceptors in the myenteric plexus: healthy vs inflamed gut

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Experimental models are useful to study inflammation-evoked neuroplasticity<sup>1</sup>.  $\beta_3$ -adrenoceptor ( $\beta_3$ -AR) agonists showed protective effects in experimental models of intestinal inflammation, possibly by increasing blood flow and modulating gut motility<sup>2,3,4</sup>. The aim of this work was to analyze: 1)  $\beta_3$ -AR distribution within the myenteric plexus of healthy rats; 2) the chemical coding (namely, ChAT for the cholinergic excitatory pathway, NOS for the nitroergic inhibitory pathway and somatostatin for secretomotor neurons) of  $\beta_3$ -AR positive neurons; 3) the effect of DNBS-induced intestinal inflammation on  $\beta_3$ -AR distribution and chemical coding. Colitis was induced in SD rats by a single intrarectal administration of DNBS (30 mg/rat). The intestine (the distal ileum and the whole proximal and distal colon) was removed after 6 days. Whole mount preparations of myenteric plexus were obtained by removing the mucosa, submucosa and circular muscle layer. Myenteric ganglia were visualized by immunofluorescence: PGP-9.5 (1:50) was used to quantify the number of neurons/ganglia, PGP-9.5 and  $\beta_3$ -AR (1:50) to quantify the percentage of  $\beta_3$ -AR positive neurons/ganglia. ChAT (1:50), NOS (1:300) and somatostatin (1:100) were used to analyze the chemical coding of  $\beta_3$ -AR neurons. In healthy rats,  $\beta_3$ -AR positive neurons per ganglion were 28% in the ileum, 18% and 23% in the proximal and distal colon, respectively; of these, ~90% were ChAT positive, 37% and 17% were NOS-positive in distal and proximal colon respectively, and ~2-3% were somatostatin positive. In the inflamed colon, a decreased number of neurons/ganglion and an up-regulation of  $\beta_3$ -AR positive neurons only in the distal colon was observed, while the chemical coding of  $\beta_3$ -AR neurons was unchanged. DNBS-induced colitis represents a useful model to study neuroplastic changes occurring in IBD. Visualization of myenteric ganglia by immunofluorescence allowed characterization of  $\beta_3$ -AR positive neurons and analysis of their chemical coding in healthy and inflamed gut. Our results are consistent with previous studies indicating that activation of  $\beta_3$ -ARs can regulate gut motility through inhibition of cholinergic excitatory pathways and activation of nitroergic inhibitory pathway and induce analgesia through somatostatin release. Finally, the presence of  $\beta_3$ -AR on myenteric neurons bears potential for therapeutic application in IBD.

## 10. *In vivo* imaging of NF- $\kappa$ B pathway in acute lung inflammation mouse model

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NF- $\kappa$ B plays a central role in immunity, inflammation, development and cell survival. Under normal conditions, NF- $\kappa$ B activation is subjected to several layers of regulation, check-points and feed-back controls and is rapid and transient in nature. Molecular events leading to the activation of NF- $\kappa$ B have been the subject of intense research for more than 20 years, largely because of the well-documented involvement of deregulated NF- $\kappa$ B activation in a variety of human disorders. Elevated NF- $\kappa$ B activity is a hallmark of various autoimmune and inflammatory diseases. It is easy to understand the importance of monitoring the NF- $\kappa$ B in non invasive way in lung inflammation mouse model. We used a simple system for *in vivo* gene delivery, in order to create a mouse expressing the gene in the lung (a plasmid containing human NF- $\kappa$ B reporter elements and luciferin gene as a reporter). The DNA has been IV delivered in Balb-C female mice at the concentration of 40 ug per mouse using *in vivo*-jetPEI™ from Polyplus as a transfectant agent. The transient transgenic mice had been imaged using IVIS Spectrum from Caliper Life Sciences for monitoring NF- $\kappa$ B activation at different time points. All the mice had been imaged using bioluminescence in order to check the basal activity of the NF- $\kappa$ B. The mice had been divided in two groups: the control one, where they received the saline solution IT and the treated one, where they got LPS at 30ug/mouse IT. Each mouse has been monitored at 2, 5 and 24 hours after treatments. The mice of LPS group at 2 hours showed a 5-fold increase of photons compared to the saline group.

These data showed that it is feasible to monitor NF- $\kappa$ B *in vivo* in a non-invasive way by BLI and to create a new *in vivo* tool for drug discovery process.