Scientific Report for Exchange Visit Grant

Purpose of the visit

The host group's main focus is translational research directed towards developing new strategies to regenerate tissues by addressing specific clinical problems. The group has multiple European and international collaborations and grants, and contributes to EU directives for clinical use of stem cells. Group members, as well as academic collaborators' backgrounds span the entire spectrum, from clinicians to biologists, chemists and materials engineers, reflecting the multidisciplinary approach to regenerative medicine and its facilitation from 'concept to patient'.

The regenerative medicine approach for successful long-term restoration of tissues such as bone depends on the use of self-renewing stem cells and their ability to differentiate into a desired target tissue that will integrate with existing host tissue. Hence, the group's research targets innovative viable systems for bone, cartilage and nerve repair and regeneration. The focus is on directed differentiation of stem cells and cell-seeded scaffolds [hydroxiapatites, brushite-tricalcium phosphate (B-TCP)] systems for soft and hard tissue regeneration. Examples of specific projects include: engineering of the intervertebral disc; osteoinductive bone grafts for maxillo-facial and orthopaedic applications; and the vascularization of these systems.

Our group of the Bone and Mineral Metabolism Laboratory at the Instituto de Investigación Sanitaria-Fundación Jiménez Díaz (ISS-FJD) in Madrid (Spain) focuses on studies related to parathyroid hormone (PTH) related protein (PTHrP) properties as a cytokine in the kidney and bone. In the latter tissue, we have demonstrated that several PTHrP domains can exert osteogenic actions in different models of osteoporosis *in vivo* and in osteoblastic cells *in vitro*. Our group has recently demonstrated that PTHrP exerts osteogenic effects in experimental models of diabetes- and glucocorticoid-related osteopenia (1, 2). Recently, we have opened a new perspective in relation with this peptide actions in bone regeneration using experimental models and implanted biomaterials coated with PTHrP peptides. Also of interest, systemic administration of a synthetic PTHrP (1-34) analog (RS-66271; Roche Bioscience, Palo Alto, CA) was shown to counteract the prednisone-induced impairment of an ulna defect healing in rabbits (3). A more direct approach to stimulate bone repair using this rationale, however, might be the local delivery of osteogenic factors into the injured bone site. We recently characterized the uptake and release kinetics of PTHrP (107-111) loaded into these matrices with different degree of hydrophobicity on their silica surface. Our findings demonstrate that this short peptide in the C-terminal sequence of PTHrP confers osteogenic activity, *in vitro* and *in vivo*, to both unmodified and organically-modified SBA-15 (4, 5). Our recent findings support the notion that PTHrP might be envisioned as a new therapeutic strategy to improve bone healing.

The purpose of Dr. Daniel Lozano Borregón's visit has been to learn different tissue engineering methodologies presently unavailable in our group at the ISS-FJD, with a practical perspective towards bone regeneration. Specifically, we were interested in the following aims:

- To develop more relevant *in vitro* cell models from a translational perspective (human primary cells including stem cells in three-dimensional as well as in co-culture systems). This includes cell selection, expansion and modulation of cells in combination with factors to induce osteogenic cell differentiation.

- To study strategies to use growth factors in tissue engineering (to replace and repair damaged tissue that would otherwise not heal).

- To evaluate the biocompatibility and biological interactions of putative materials as implants.

- To study the deformation and compression of these biomaterials.

- To analyze the osteogenic action of PTHrP (1-36) loaded onto B-TCP as scaffolds containing human osteoblastic cells *in vitro.*

References

1- Lozano D, de Castro LF, Dapía S, Andrade-Zapata I, Manzarbeitia F, Alvarez-Arroyo MV, Gómez-Barrena E, Esbrit P. Role of Parathyroid Hormone-Related Protein in the Decreased Osteoblast Function in Diabetes-Related Osteopenia. Endocrinology 2009 150: 2027-35.

2- Luis F. de Castro, Daniel Lozano, Sonia Dapía, José R. Caeiro, Enrique Gómez-Barrena, and Pedro Esbrit. Role of the N- and C-terminal fragments of parathyroid hormone-related protein as putative therapies to improve bone regeneration under high glucocorticoid treatment. Tissue Eng part A 2010 16: 1157-1168.

3- Bostrom MP, Gamradt SC, Asnis P, Vickery BH, Hill E, Avnur Z, Waters RV. Parathyroid hormone-related protein analog RS-66271 is an effective therapy for impaired bone healing in rabbits on corticosteroid therapy. Bone 2000 26:437-42.

4- Daniel Lozano, Miguel Manzano, Juan Carlos Doadrio, Antonio J. Salinas, María Vallet-Regí, Enrique Gómez-Barrena, Pedro Esbrit. Osteostatin-loaded bioceramics stimulate osteoblastic growth and differentiation. Acta Biomater 2010 6: 797-803.

5- Trejo CG*, Lozano D*, Manzano M, Doadrio JC, Salinas AJ, Dapía S, Gómez-Barrena E, Vallet-Regí M, García-Honduvilla N, Buján J, Esbrit P. The osteoinductive properties of mesoporous silicate coated with osteostatin in a rabbit femur cavity defect model. Biomaterials 2010 31: 8564-8573.

Description of the work carried out during the visit

The first part of the short term visit was devoted to acquire the necessary knowledge to manage the new techniques to be used in the proposed strategies as outlined above. Subsequently, we performed a series of tests with different biomaterials in the presence or absence of human osteoblastic cells. Finally, we performed some experiments in B-TCP coated with PTHrP (1-36) together with human osteoblastic cells.

The different tissue engineering methodologies learned during the visit were the following:

1. Bioreactor [Electroforce 5100 Biodynamic (BOSE)]

The ElectroForce® BioDynamic® test instrument (Fig. 1) provides precise characterization of biomaterials and biological specimens in a cell culture media environment. The instrument can be used for the evaluation of a variety of specimens, including biomaterials, cell-free scaffolds and cell-seeded scaffolds, native tissue samples and tissue-engineered constructs. Its compact design makes it suitable for the incubator and for long term experiments in a cell culture laboratory.

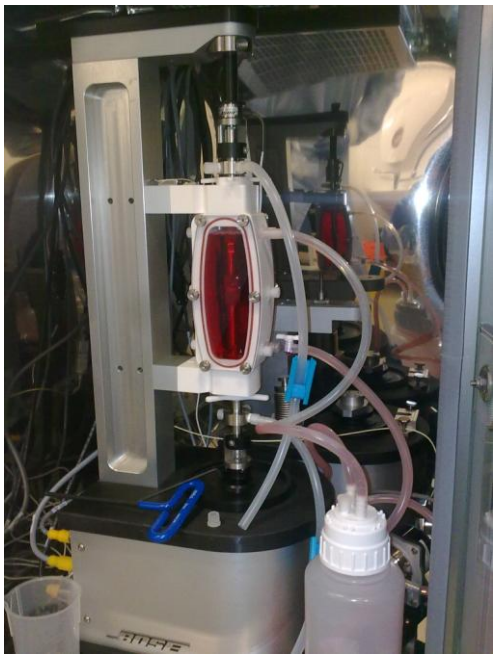

Figure 1. The ElectroForce® BioDynamic® test instrument.

BioDynamic test instrument combines mechanical testing and specimen stimulation in one system. Single specimens can be mechanically loaded under tension/compression, torsion, cyclic hydrostatic pressure, and pulsatile or steady flow. Measurements include displacement, load, torque, rotation, pressure, strain, diameter, pH, dissolved oxygen, carbon dioxide, lactate/glucose, and temperature.

I acquired the know-how of the bioreactor use, and as a test incubation, hydroxyapatites and B-TCP coated with human osteoblastic cells were placed into the bioreactor for up to 48 hours (Fig 2.).

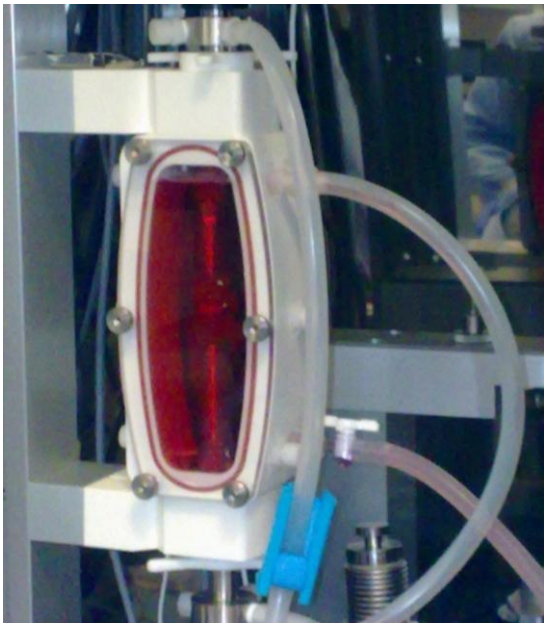

Figure 2. Presence of biomaterial coated with human osteoblastic cells into the ElectroForce® BioDynamic®.

2. Cell Counter [Particle count and size Analizer. Beckman Coulter]

In addition to reporting both cell count and concentration results, this Cell Counter (CC) (Fig. 3) adds the ability to provide size distribution of the cell population. This CC device uses the Coulter Principle (Electrical Sensing Zone Method) of counting and sizing of cells. The CC displays the entire size distribution graph, and the statistical analysis in selectable areas of the resulting graph. In addition, this instrument displays the cumulative count and the cumulative number % above and below a size determined by the cursor positioned on the graph.

Figure 3. The Cell Counter, Particle count and size Analizer. Beckman Coulter.

3. Alamar blue assay

Proliferation of human osteoblastic cells was determined using the alamar-Blue™ assay (Life Technologies), which is based on the use of a redox indicator, that quantifies cell proliferation. As cells grow, there is an increase in metabolic activity giving rise to a reducing environment in the surrounding culture medium, whilst growth inhibition produces an oxidizing environment. Reduction causes colour change of alamar-Blue™ indicator from non-fluorescent (blue) to fluorescent (red). Proliferation of cultured human osteoblastic cells was performed using several 3D porous scaffolds. Tissue culture plastic and medium supplemented with 10% IMS were used as 2D negative (non toxic) and positive (toxic) controls, respectively.

The tested scaffolds were placed in 24-well plates, cells were micro-seeded at a total density of 1.9 10^5 cells per scaffold. For these cell culture studies, a total of 1 ml primary human osteoblastic cells medium was added to each well. Cells were cultured under standard conditions with the medium being replaced every 2–3 days.

Proliferation was measured on 4, 7, 14, 21 and 28 days post-seeding. In brief, the medium was removed and replaced with 1 ml phenol red-free medium supplemented with 1/10-dilution of filtered Alamar Blue™ stock solution (Serotec, Oxford, UK). After 4 h incubation in standard conditions, 100 µl aliquots from each well (in triplicate) per scaffold was sequentially removed and added to 96-well plates (eight replicates for each test scaffold). Absorbance was read on a fluorescent plate reader on emission wavelength of 590 nm (excitation wavelength 560 nm).

4. Collagen carrier for migration assay

A collagen gel was prepared under aseptic conditions by mixing 125 ml of 10_DMEM per 1 ml of type I rat tail collagen (First Link UK Ltd., Birmingham, UK). NaOH solution was added dropwise until there was a colour change from orange to pink/purple. This solution was poured out into 6 well plates, in order to give a gel depth of 5 mm, and incubated at 37 C, 5% CO2 for 20 min to allow the collagen gel to set. 2 10^5 cells in 1 ml of medium was added to the collagen gel surface and the plates incubated overnight. The surfaces of the gels were checked for cell adherence and proliferation 24 h later. Excess culture medium was removed from the collagen gel wells, and a sterile cork borer was used to make holes in the gel of a slightly smaller diameter than the disks. The hydroxyapatite discs of pore size 150 and 500 mm (82% porosity) were press fitted into the holes with the surface of the gel and the top surface of the hydroxyapatite discs at the same height and in apposition to each other. Three ml of culture medium was added and the samples were incubated for 7 days and then processed for light microscopy.

5. Instron 5569 Load Frame

The Instron 5569 mechanical tester (Fig. 4) is capable of a wide variety of mechanical testing methods. Instrumental control and data collection and analysis are performed through Instron's Merlin software.

Figure 4. The Instron 5569 mechanical tester.

This machine is used for testing tension and compression of a wide range of materials by moving the crosshead in an upward or downward direction via drive system. The test specimen is secured between the rigid frame base and the moving crosshead. The applied load is measured by a load cell mounted between the test specimen and the crosshead (Fig. 5). There are two different load devices (2kN and 50kN capacity) to provide a range of load measurement capabilities.

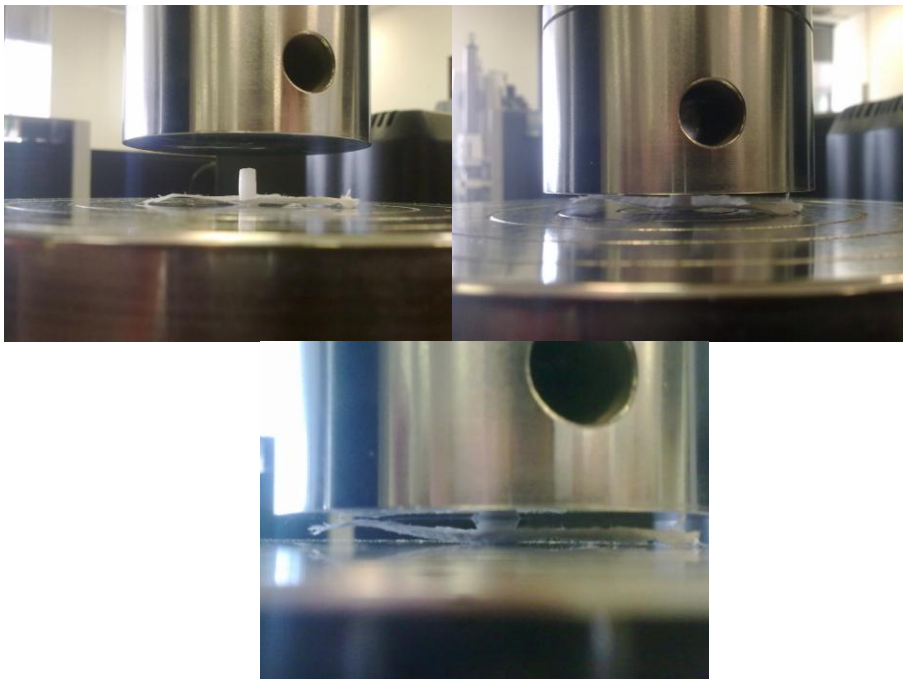

Figure 5. Different pictures of the Instron 5569 mechanical tester performed a mechanical test with a hidroxiapatite.

6. Dynamic Mechanical Analyzer (DMA 8000, Perkin Elmer)

DMA (Fig. 6) is a device that is widely used to characterize a material's properties as a function of temperature, time, frequency, stress, atmosphere or a combination of these parameters.

Figure 6. Dynamic Mechanical Analyzer.

The DMA 8000 permits operation in a "Constant force (TMA) mode" vs. time or temperature. Applications such as expansion coefficient, softening and penetration, or extension or contraction in tension geometry provide valuable data equivalent to many commercial stand-alone TMA instruments. The DMA 8000 is using an ultra-efficient cooling system. The instrument can cool to - 190 ºC in 15 minutes using less than 1 liter of liquid nitrogen, providing industry-leading performance. The Humidity Generator and Controller is a powerful and flexible option, which delivers the capability to apply and accurately control relative humidity to the sample environment in the DMA 8000. These innovative pockets allow powdered or non-self supported materials, such as powdered drugs, gels, natural products like tea, coffee, herbs, etc. and low viscosity materials to be investigated by DMA.

In the second part of the visit, I carried out different experiments using human osteoblastic cells that were seeded into B-TCP, loaded or not with PTHrP (1-36). The materials were loaded with PTHrP (107-111) (Bachem, Bubendorf, Switzerland) by soaking them in a solution of this peptide (100 nM) in phosphate-buffered saline (PBS) at 4 ºC under stirring for 24 h. We determined cell proliferation (Alamar Blue) at 5 and 8 days and the analysis of gene expression at day 5.

Description of the main results obtained

I gained experience in the development of several human primary cell models with a translational perspective, including cell selection and expansion of these cells with osteogenic factors, in the context of tissue engineering. In addition, I learned to perform different biological and mechanical tests (for example, deformation and compression) of several materials (HA, B-TCP, etc..) and I learned to evaluate the biocompatibility and biological interactions of these putative materials as implants. Finally, I analyzed several osteogenic actions of PTHrP (1-36) loaded onto B-TCP as scaffolds containing human osteoblastic cells *in vitro.* Preliminary results using Alamar blue shown that the human osteoblastic cells seeded into B-TCP, in the presence or absence of PTHrP (1-36), maintained cell viability throughout the period of study. The presence of the peptide though increased cell proliferation at different times (5-8 days) (Figs. 7 and 8). On the other hand, the proliferation of these cells when seeded in non-porous B-TCP [B-TCP(-)] was significantly lower than in the aforementioned materials

.

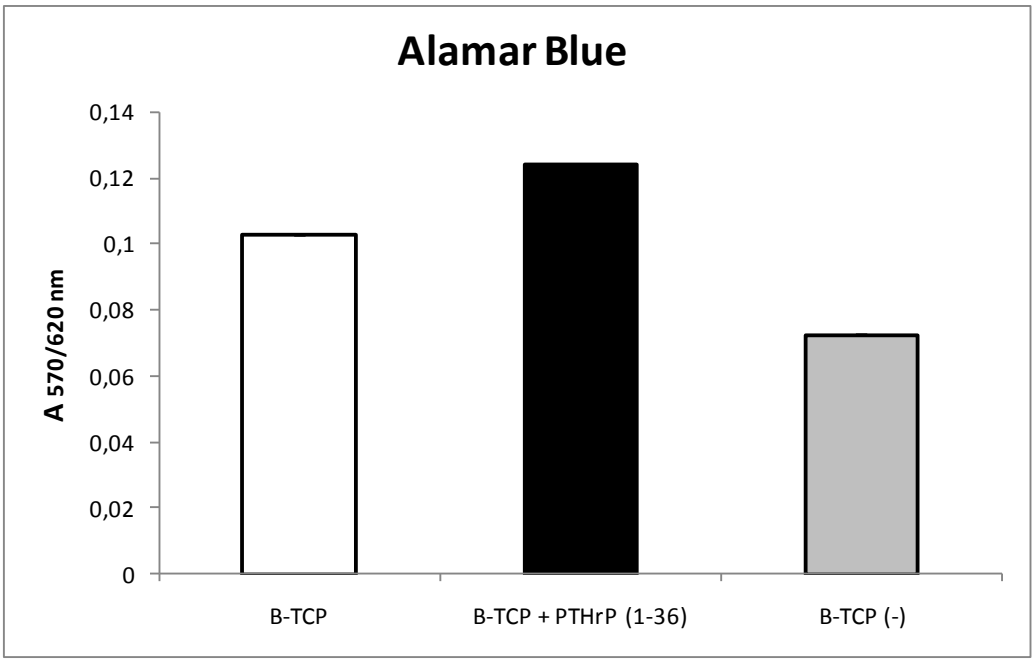

Figure 7. Proliferation rate (measured by Alamar Blue) of human osteoblastic cells seeded into the B-TCP material in the presence or absence of PTHrP (1-36) at day 5 of culture. B-TCP (-) is the same material than B-TCP but without porous.

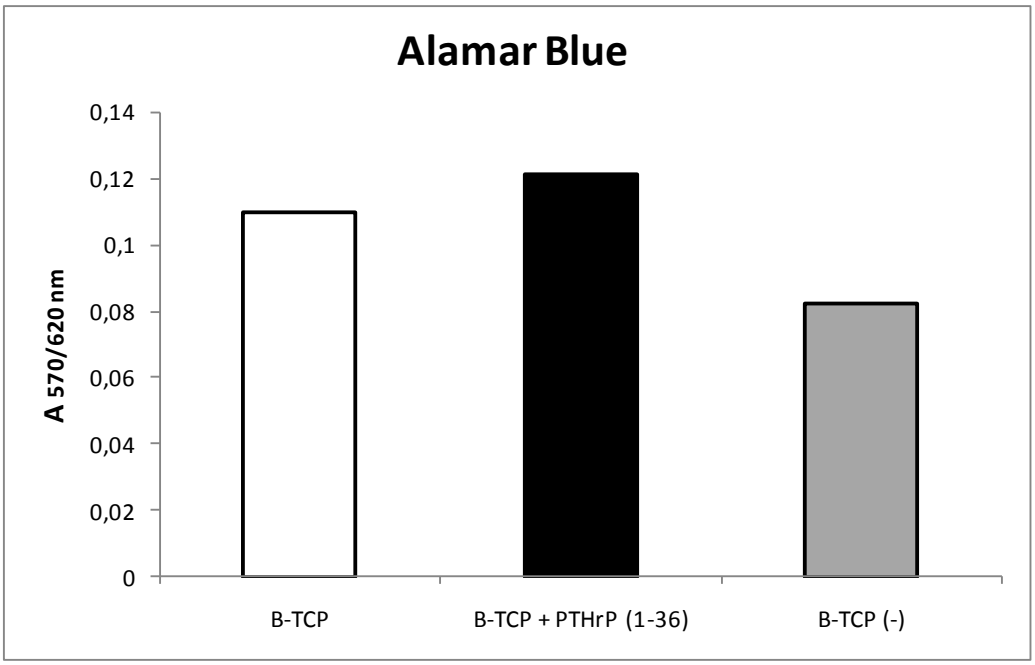

Figure 8. Proliferation rate (measured by Alamar Blue) of human osteoblastic cells seeded into the B-TCP material in the presence or absence of PTHrP (1-36) at day 8 of culture. B-TCP(-) is the same material than B-TCP but without porous.

In addition, the presence of PTHrP (1-36) increased the gene expression of Osterix, Runx2 and vascular endothelial growth factor (VEGF) in human osteoblastic cells seeded into the B-TCP materials at day 5 of culture.

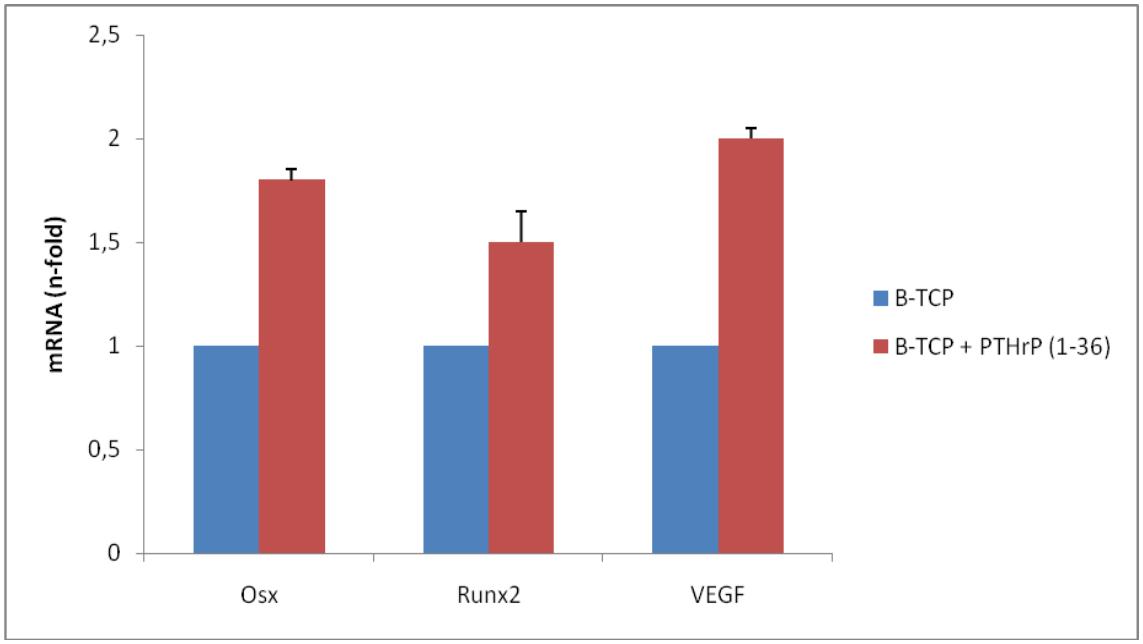

Figure 9. Changes in the gene expression levels (by real time PCR) of Osterix (Osx), Runx2 and vascular endothelial growth factor (VEGF), induced by B-TCP, loaded or not with PTHrP (1-36) in human osteoblastic cells for 5 days

Future collaboration with host institution *(if applicable)*

This short visit has allowed the granted visitor (Dr. Lozano) and his group in Madrid to establish a collaboration with the host group in London, which opens new avenues for testing new putative therapeutic agents using tissue engineering strategies to improve bone regeneration.

Projected publications/articles resulting or to result from the grant (ESF must be acknowledged in publications resulting from the grantee's work in relation with the grant).

This oponed collaboration is due to produce scientific rewards (e.g., meetings contributions and putative manuscripts in learned Research journals) in the near future.