Università degli Studi del Piemonte Orientale

Scuola di Medicina

Dottorato di Ricerca in Scienze e Biotecnologie Mediche (ciclo XXX)

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PhD student annual report

Demonstration of small DNA virus reactivation in kidney transplant recipients with pre-malignant and malignant lesions.

Introduction and objectives

Kidney transplant (KTx) is the best therapy for patients with an end-stage renal disease (ESRD), yielding to a better patient survival and rehabilitation if compared with chronic dialysis. The continuous improvement of the efficacy of anti-rejection drugs has greatly contributed to the prolonging of long-term survival of transplant recipients. Nowadays, 5-year patient survival rates are around 90% after kidney transplant and 70% after liver transplant. However, the prolonged chronic use of immunosuppressive drugs is well known to increase the risk of opportunistic diseases, particularly infection-related malignancies. Transplant recipients have a doubled risk of developing cancer compared to the general population and the natural history of cancer in these patients tends to be more aggressive than usual (1, 2). Such immunosuppression-associated solid tumours are a major cause of death in transplant recipients, raising health-economic issues. Most of these cancers are caused by reactivated viruses whose oncogenic potential is suppressed by immunological reactions in healthy individuals (3). Some types of cancer have a well-accepted association with infectious organisms, including anogenital cancer with human papillomavirus (HPV), Merkel cell carcinoma with Merkel cell polyomavirus (MCPyV), immune suppression-related Non-Hodgkin's lymphoma (NHL) with Epstein Barr Virus (EBV), Kaposi's sarcoma (KS) with KS Herpesvirus/HHV8.

Other types of cancer do not have well-accepted associations with infectious organisms, including non-melanoma skin cancer (NMSC) with HPV (4, 5), and BK polyomavirus with urinary tract carcinoma (6, 7).

NMSCs are the most common cancer type in KTRs. In contrast to the general population, squamous cell carcinomas (SCC) are more common than basal cell carcinomas (BCC). Indeed, in KTRs the squamous cell/basal cell carcinoma ratio is approximately 3-5 to 1 compared to 1 to 3-4 in the general population (8, 9, 10).

Human papillomavirus (HPV) and human polyomavirus (HPyV) share a common morphology and structural organization. The virions are small non-enveloped icosahedral particles of ≈50 nm diameter, with a circular double-stranded DNA genome of 8 and 5 kb respectively, wrapped around host cell-derived histones. Over 180 different types of HPV have been identified to date and classified into several phylogenetic groups. Of these, mucosal HPVs belonging to the α-genus and associated with infections of mucosal epithelia are the best characterized and can be grouped into 'low-risk' and 'high-risk' types, depending on the relative propensity of the resulting neoplasms to undergo malignant progression. Cutaneous β-HPVs are evolutionarily distinct from the α-genus and appear to cause widespread unapparent or asymptomatic infections in the general population. However, in immunosuppressed patients and in individuals suffering from the rare inherited disease Epidermodysplasia Verruciformis (EV), these viruses can spread unchecked and have been implicated in the development of skin cancer.

A similar scenario can be envisaged for HPyVs whose causal association with human tumors is also difficult to be established because they may also be constituents of the human skin microbiome and present as DNA in many normal human tissues during the lifelong latent infection which follows HPyVs primary infection.

The aim of my research project is to determine the association between the reactivation of small DNA viruses (HPV and HPyV) and the development of solid cancers (NMSC and urinary tract carcinoma respectively) in a cohort of kidney transplant recipients. We would like to determine the extent of viral reactivation in these tumours in order to strengthen the causal link between ubiquitous virus reactivation, occurring in the context of long lasting iatrogenic immunosuppression, and cancer development (11). While this link is well established for high-risk alpha HPV and genital cancer, association between β-HPV and skin cancer on one hand and BK with renal and urinary tract cancer on the other hand is still a matter of debate.

Materials and Methods

- **Patients**

972 patients, who underwent kidney transplantation at our University-Hospital of Novara from 1976, were included in this retrospective study. 60 patients have developed skin cancers, 10 anogenital cancers, 10 renal cell carcinomas and 3 urinary bladder carcinomas. Out of the 60

patients who developed skin cancers, we analysed 159 lesions, corresponding to 222 FFPE blocks, from 38 KTRs. Out of the 13 patients who developed urinary tract carcinoma, 3 experienced a BKV associated nephropathy (BKVAN) before the diagnosis of cancer.

- **β-HPV DNA detection in the skin lesions and genotyping**

DNA from formalin-fixed paraffin-embedded tissue from 17 KTRs was extracted by using the *QIAamp Tissue Kit (Qiagen)*. The DNA quantity and quality was then determined by *NanoDrop Spectophotometer UV-Vis 2000c (Thermo Scientific)*. The genotyping results were performed by PM-PCR RHA method at DDL *(Delft Diagnostic Laboratory, Voorburg, Netherlands)*.

- **Visualization of HPV and HPyV viral protein expression by immunostaining and DNA by Fluorescent In Situ Hybridization**

Immunofluorescence staining for the viral life-cycle markers E4 (early protein) and L1 (late protein) of HPV was performed on the FFPE sections of skin by using in-house produced rabbit polyclonal antibodies raised against the HPV E4 (kindly provided by J. Doorbar) and L1 (our laboratory) proteins. Co-staining for the proliferation marker MCM7 (minichromosome maintenance protein) was performed with a commercial monoclonal antibody (Neomarkers). HPV and BK genomes were visualized by FISH analysis on the FFPE samples. Both these techniques have been previously developed in our laboratory (12).
Immunohistochemical staining for the SV40 large T antigen was performed with a commercial monoclonal antibody (Roche Diagnostics) on the FFPE sections of carcinoma and non-pathological area of kidney and urinary tract.

Results

- **β-HPV and skin cancer**

DNA extracted from lesions was tested to determine the HPV genotypes present. The results show that out of 111 FFPE blocks analysed from 17 patients, 94 were HPV positive (85%) and 86 samples (77%) could be genotyped as containing at least one of the 25 β-HPV genotypes available on the reverse hybridization assay. The majority of them harbored multiple β-HPV genotypes. The most frequently observed genotypes are HPV5 and 8 (both belonging to species 1). Then, 222 FFPE blocks, obtained from skin lesions of 38 patients from our cohort of KTRs, were analysed for the presence of active β-HPV infection, visualized in immunofluorescence by using antibodies raised against E4 and L1 proteins. Out of these, E4-positive cells were found in 9 lesions from 5 patients and corresponded to four actinic keratosis lesions, two keratoacanthomas and the adjacent pathological area of two squamous cell carcinomas and one basal cell carcinoma.

Most of the immunofluorescence positive tumours displayed a DNA virus-induced cytopathic effect characterized by the presence of ballooning epithelial cells with enlarged granular cytoplasms and large nuclei. These findings suggest that β-HPV replication might be activated in these lesions, very likely as a consequence of immunosuppression, and might play a role in the early stages of skin carcinogenesis in KTRs patients (Figure 1).

In all the specimens, the viral E4 protein displayed the expected cytoplasmic localization in the middle-superficial layers of the epithelium together with an increase of the cellular proliferation marker MCM7 in the basal and suprabasal layers. The disappearance of E4 positivity in the adjacent normal epithelium constantly overlapped with a reduction in MCM7 expression, which was restricted to the basal layer. These findings support the hypothesis that β-HPV replication drives the cells above the basal layer to enter the cell cycle to facilitate the amplification of its genome.

As further proof that the viral life cycle was being completed, all the E4-positive areas showed expression of the major coat protein (L1), which also occurred in a subset of E4-positive cells in the upper layers.

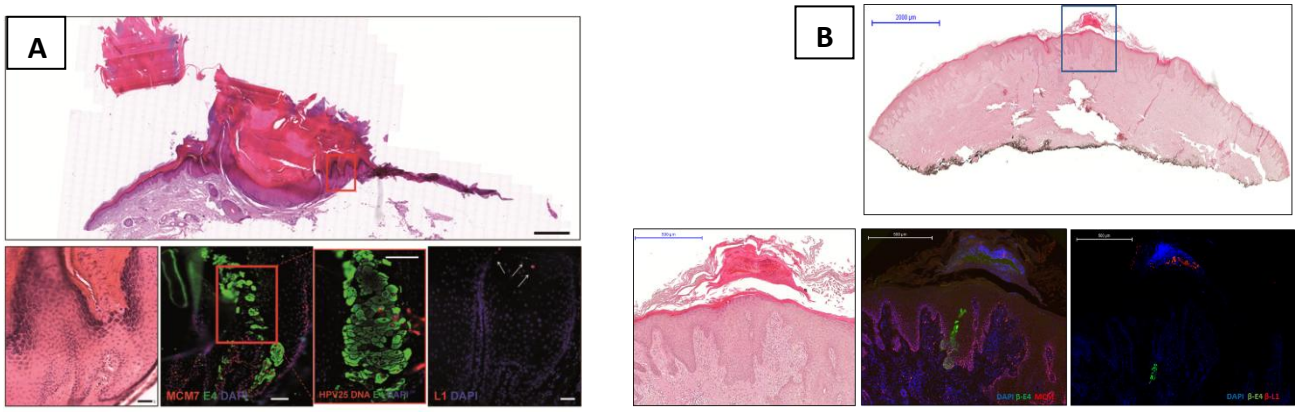

Figure 1. *Distribution of the viral and cellular markers E4, L1 and minichromosome maintenance protein 7 (MCM7) in an actinic keratosis (A) and a keratoacanthoma (B) from two kidney transplant recipients.*

- **BK Virus and urinary tract carcinoma**

We were able to analyze 10 renal malignancies and 3 urinary bladder tumors from 13 patients. Among them, 3/13 (23.1%) experienced a BKV associated nephropathy (BKVAN) before the diagnosis of cancer. Two patients developed a renal cell carcinoma and one a bladder cell carcinoma. None of them reported a BKVAN after the diagnosis of cancer.

All the tumors (n=13) have been analyzed for the expression of the LT (Large T) antigen by immunohistochemistry. Only one tumor corresponding to a bladder cell carcinoma,from a patient who was previously diagnosed with BKVAN was positive for the SV40 Large T antigen and the BKV infection was also confirmed by FISH analysis (Figure 2).

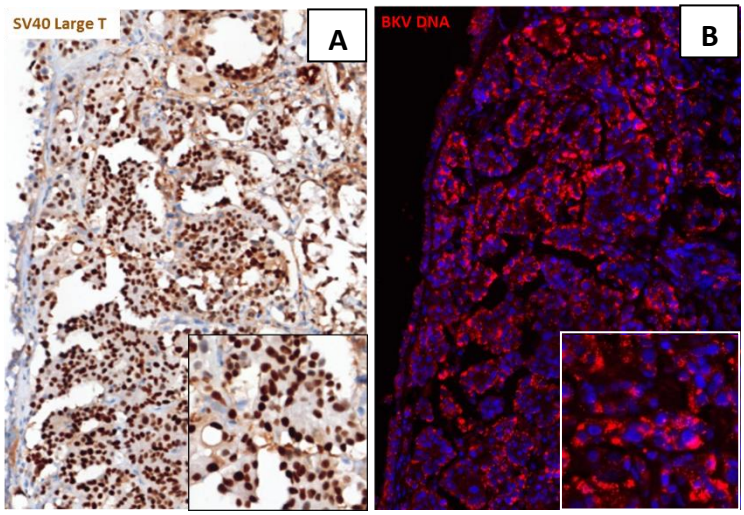

Figure 2*. IHC staining for SV40 Large T antigen on the bladder cell carcinoma (A); FISH analysis (B).*

Moreover, in another patient (diagnosed with BKVAN) we have observed BKV infection by FISH and Large T antigen staining only in the ureter of the native kidney and in the kidney biopsy taken to diagnose nephropathy but not in the tumour (Figure 3). BK infection in this patient has been further characterized by Alberto Peretti, a post doc from our laboratory currently working at the National Cancer Institute NIH (Bethesda-USA) in the laboratory of Chris Buck. Urine and serum from this patient have been processed by sequential sedimentation and density gradient ultracentrifugation for virions purification. DNA has been extracted and subjected to random-primed rolling circle amplification (RCA) to further enrich for viral sequences. Subsequently, the deep sequencing analysis was performed using Illumina Nextera XT sample preparation kit and the Illumina MiSeq platform. The results obtained by Dr. Peretti revealed the presence, both in serum and urine, of the BKV IVc2 genotype carrying a mutation in the VP1 gene.

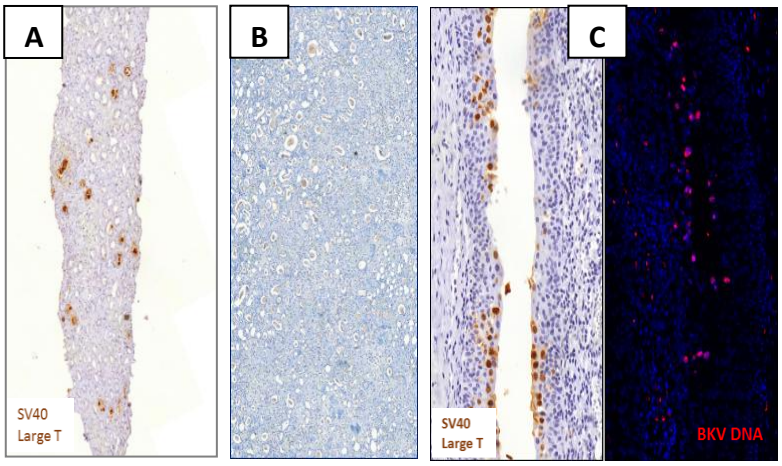

Figure 3. *IHC staining for SV40 Large T Antigen on the renal biopsy of transplanted kidney (A) and on the renal cell carcinoma (B); IHC staining for SV40 Large T antigen and FISH analysis on the ureter of native kidney (C).*

Conclusions

To our knowledge, this is the first study in which the association between skin lesions and β-HPV infections has been assessed at both DNA level by PCR and at the protein level by IF analysis. To date, a direct evidence of active β-HPV infection in tumors from non-epidermodysplasia verruciformis patients is still missing and the major criticism against a direct involvement of these viruses in skin cancer in the general population is that they do not seem to be maintained in high-grade tumors, such as squamous cell carcinoma and they are also present in the general population. Although the present study do not directly demonstrate a causal role of these viruses, the detection of E4 and L1 positivity in (i) actinic keratosis and keratoacanthoma, which are widely regarded to be squamous cell carcinoma precursors or in situ carcinoma, and (ii) the adjacent pathological epithelium of two squamous cell carcinoma and one basal cell carcinoma, clearly shows that β-HPV are actively replicating in the lesional skin of organ transplant recipients.

Increased MCM7 expression extended into the upper epithelial layers is a common feature of all the E4-positive areas, indicating that cells were driven into the cell cycle in areas of productive viral infections. The stimulation of basal cell proliferation may contribute, in association with other transforming agents, such as UVB irradiation, to the transformation process without necessarily being maintained in the more advanced disease. β-HPV may act by a "hit and run" mechanism of carcinogenesis.

The second aim of this study is to determine whether there might be any association between the reactivation of HPyV and the development of urinary tract carcinomas. The finding of 13 urinary tract cancers in our study cohort led us to investigate all the FFPE blocks from the urinary tract that were available including those corresponding to the non-neoplastic areas, the renal pelvis and ureter. Only in two patients, diagnosed with BKVAN, it was possible to visualize the presence of SV40-T antigen in the tumour or in other areas of the urinary tract.

Several lines of evidence have already suggested that the BKV may play a significant role in the pathogenesis of renal and bladder carcinoma in association with other risk factors. In fact, few studies have reported the expression of the SV40 LT antigen in some high-grade urothelial and renal tumours arising in kidney transplant recipients (13). Despite these observations, the potential causative role of HPyV in this cancer is far from clear and more investigations are needed to figure out an oncongenic activity of these viruses.

BIBLIOGRAPHY

1) Pfister, H., and Ter Schegget, J. (1997). Role of HPV in cutaneous premalignant and malignant tumors. Clin Dermatol *15*, 335-347
2) Berkhout, R.J., Bouwes Bavinck, J.N., and ter Schegget, J. (2000). Persistence of human papillomavirus DNA in benign and (pre)malignant skin lesions from renal transplant recipients. J Clin Microbiol *38*, 2087-2096.
3) Schulz, T.F. (2009). Cancer and viral infections in immunocompromised individuals. Int J Cancer *125*, 1755-1763.
4) Bouwes Bavinck, J. N., Neale, R. E., Abeni, D., Euvrard, S., Green, A. C., Harwood, C. A.,de Koning, M. N., Naldi, L., Nindl, I., Pawlita, M., Pfister, H., Proby, C. M., Quint, W. G., ter Schegget, J., Waterboer, T., Weissenborn, S., and Feltkamp, M. C. Multicenter study of the association between betapapillomavirus infection and cutaneous squamous cell carcinoma. Cancer Res, *70:* 9777-9786, 2010.
5) Asgari, M.M., Kiviat, N.B., Critchlow, C.W., Stern, J.E., Argenyi, Z.B., Raugi, G.J., Berg, D., Odland, P.B., Hawes, S.E., and de Villiers, E.M. (2008). Detection of human papillomavirus DNA in cutaneous squamous cell carcinoma among immunocompetent individuals. J Invest Dermatol 128, 1409-1417.
6) Alexiev B.A., Randhawa P., Vazquez Martul E., Zeng G., Luo C., Ramos E., Drachenberg C.B., Papadimitriou J.C. (2013). BK virus-associated urinary bladder carcinoma in transplant recipients: report of 2 cases, review of the literature, and proposed pathogenetic model. Human Pathology 44, 908-917.
7) Bulut Y., Ozdemir E., Ozercan H.I., Etem E.O., Aker F., Toraman Z.A., Seyrek A., Firdolas F. (2013). Potential relationship between BK virus and renal cell carcinoma. J Med Virol 85, 1085-1089.
8) Nindl, I., Gottschling, M., and Stockfleth, E. (2007). Human papillomaviruses and non-melanoma skin cancer: basic virology and clinical manifestations. Dis Markers *23*, 247-259.
9) Pfister, H., Fuchs, P.G., Majewski, S., Jablonska, S., Pniewska, I., and Malejczyk, M. (2003). High prevalence of epidermodysplasia verruciformis-associated human papillomavirus DNA in actinic keratoses of the immunocompetent population. Arch Dermatol Res *295*, 273-279.
10) Hampton, T. (2005). Skin cancer's ranks rise: immunosuppression to blame. JAMA *294*, 1476-1480.
11) Borgogna C, Lanfredini S, Peretti A, De Andrea M, Zavattaro E, Colombo E, Quaglia M, Boldorini R, Miglio U, Doorbar J, Bavinck JN, Quint KD, de Koning MN, Landolfo S,

Gariglio M. (2014). Improved detection reveals active β-papillomavirus infection in skin lesions from kidney transplant recipients. Mod Pathol 27, 1101-1115.

12) Borgogna C., Zavattaro E., De Andrea M., Griffin H. M., Dell'Oste V., Azzimonti B.,Landini M. M., Peh W. L., Pfister H., Doorbar J., Landolfo S., and Gariglio M. (2012). Characterization of beta papillomavirus E4 expression in tumours from Epidermodysplasia Verruciformis patients and in experimental models. Virology, 423, 195-204.

13) Alexiev B.A., Randhawa P., Vazquez Martul E., Zeng G., Luo C., Ramos E., Drachenberg C.B., Papadimitriou J.C. (2013). BK virus-associated urinary bladder carcinomain transplant recipients: report of two cases , review of the literature, and proposed pathogenetic model. Hum Pathol, 44, 908-17

LESSONS

- "Tissue engineering: the state of the art" – 14th November 2014 – Dott.ssa Francesca Boccafoschi - Department of Health Sciences, University of Eastern Piedmont.
- "Regenerative Medicine" – 21st November 2014 – Prof. Maria Prat - Department of Health Sciences, University of Eastern Piedmont.
- "Ribosomopathies" – 25th May 2015 – Prof. Steve Ellis – Medical School, University of Louisville (Kentucky)
- "Basis of scientific research" – 10th June 2015 – Prof. Nicoletta Filigheddu – Università del Piemonte Orientale (Italy)

SEMINARS

1. "Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders" – 06th November 2014 - Prof. Andrew L. Snow - Department of Pharmacology Uniformed Services University of the Health Sciences Bethesda (Maryland, USA).

2. "Optical coherence tomography from bench to bedside shening the light during percutaneous vascular intervention" – 17th November 2014 - Dott. Secco Gioel Gabrio – Department of Health Sciences, University of Eastern Piedmont.

3. "La scoperta del bosone di Higgs" – 25th November 2014 - Dott. Roberta Arcidiacono - DiSCAFF, University of Eastern Piedmont - Dott. Marta Ruspa - Department of Health Sciences, University of Eastern Piedmont.

4. "Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori" – 27th November 2014 - Prof. Laura Baglietto - Inserm - Centre for Research in Epidemiology and Population Health, Unit: Nutrition, Hormones and Women's Health, Paris.

5. "Humoral responses to HCV infection and clinical outcomes" – 28th November 2014 - Dott. Arvind Patel - Programme Leader, MRC Centre for Virus Research, University of Glasgow (UK).

6. "Uncovering the role β-HPV in field cancerization: a collaboration in progress" – 4th December 2014 – Prof. Girish Patel, Cardiff.
7. "Focus on deliver: from basics of NAFLD to hot topics in HBV & HCV infections" – 5th December 2015 – Prof. Rifaat Safadi M.D.
8. "From the legend of Prometheus to regenerative medicine" – 11th December 2015 – Prof. Antonio Musarò
9. "Pregi e difetti dei nuovi anticoagulanti orali nella pratica clinica" – 16th December 2015 – Prof. Giancarlo Agnelli
10. "Microglia microvescicles: messengers from the diseased brain" – 17th December 2014 - Dott. Roberto Furlan, San Raffaele University, Milan.
11. "Anticancer strategy Targeting cancer cell metabolism in ovarian cancer" – 19th January 2015 - Prof. Dr Yong-Sang Song, MD, PhD Director Cancer Research Institute, Gynecologic Oncology Chariman, Cancer Biology Interdisciplinary Program Professor, Obstetrics and Gynecology, College of Medicine Seoul National University.
12. "Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states" – 20th January 2015 - Dott. Tonino Alonzi, PhD, Lab. Of Gene Expression and Experimental Hepatology, Istituto Nazionale per le Malattie Infettive "L. Spallanzani" IRCCS, Rome.
13. "Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis" – 21st January 2015 - Prof. Valeria Poli - Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University of Turin.
14. "Myeloid cells as therapeutic target in cancer" – 27th January 2015 - Prof. Antonio Sica - DiSCAFF, UPO, Novara.
15. "Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells" – 11th March 2015 – Prof. Darko Bosnakovski - Associate Professor, University "Goce Delcev" Stip, Faculty of Medical Sciences, Krste Misirkov bb, 2000 Stip R. Macedonia.
16. "Signal control in iNKT cell development and function" – 09th April 2015 - Prof. Xiaoping Zhong, MD, PhD - Associate Professor, Department of Pediatrics-Allergy and Immunology Duke University, Medical Center, Durham (North Carolina, USA).
17. "Actin-based mechanisms in the control of gene expression and cell fate" – 21st April 2015 – Prof. Piergiorgio Percipalle – Associate Professor, Department of Cell and Molecular Biology, Karolinska Institutet (Solns, Sweden).
18. "An integrated approach to the diagnosis and treatment of ovarian cancer" – 7th May 2015 – Prof. John McDonald, MD, PhD – Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Biosciience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, USA).
19. "Conflicting interests and scientific communication" – 14th May 2015 – Prof. Kathleen Ruff – RightOnCanada Founder, Senior Advisor to the Rideau Institute (Ottawa, Canada).
20. "Recent developments in (cutaneous) Human Polyomavirus research" – 5th June 2015 – Mariet C.W. Feltkamp – Associate Professor of Medical Virology, Department of Medical Microbiology, Leiden University Medical Center (Leiden, The Netherlands).
21. Miniworkshop on "Biotechnology for Dermatology" – 9th July 2015 - Dr Gwenaël ROLIN, PhD - Clinical Research Engineer - Thomas LIHOREAU - Ingénieur hospitalier, Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical

Investigation Center (CIC INSERM 1431), Besançon University Hospital; INSERM UMR1098, FED4234 IBCT, University of FrancheComté, Besançon, France.

22. "High-tech product preservation and operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach" – 15th July 2015 - Dr. Ing. Marco Fatta, Phd – COMECER Group (Italy).

23. "Le cellule staminali nel danno renale acuto e nel trapianto di rene" – 28th July 2015 - Dr. Vincenzo Cantaluppi, MD – Facoltà di Medicina e Chirurgia, Università di Torino (Italy).

24. "Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)" , "Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD)" – 3rd September 2015 - Prof. Darko Boshnakovski, PhD – University Goce Delcev Stip, Faculty of Medical Sciences (Stip, R. Macedonia).

MEETING'S PARTICIPATION

- ICGEB "DNA Tumour Virus Meeting 2015" - 21-26 July 2015 - Trieste, Italy

ORAL PRESENTATION

- ICGEB "DNA Tumour Virus Meeting 2015" 21-26 July 2015- Trieste, Italy

  "Detection of HPV and HPyV infection in tumours from a cohort of kidney transplant recipients"

  Calati F., Musetti C., Cena T., Magnani C., Olivero C., Peretti A., Boldorini R., Doorbar J., Stratta P., Gariglio M. and Borgogna C.

POSTER

- SIM (Società Italiana di Microbiologia) 27-30 September 2015 – Napoli, Italy

  "Restriction of HPV18 replication in the nucleus by IFI16: what about innate sensing?"

  DeAndrea M., Albertini S., Calati F., LoCigno I., Olivero C., Borgogna C., Johnson K.E., Chandran B., Landolfo S., Gariglio M.