Università degli Studi del Piemonte Orientale

Scuola di Alta Formazione

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

Dott.ssa Silvia Albertini Tutor: *Prof.ssa Marisa Gariglio*

Anno Accademico 2014-2015

PhD annual report

The intracellular DNA sensor IFI16 gene as a candidate antiviral factor for Human Papillomavirus replication.

Introduction and objectives

The innate immune system provides the first line of defense against invading pathogens. Most cells in the body have the ability to recognize molecular hallmarks of infection, or pathogen-associated molecular patterns (PAMPs), by means of pattern recognition receptors (PRRs). Upon activation with PAMPs, the PRRs then initiate cell-signaling cascades resulting in the secretion of pro-inflammatory cytokines and interferons (IFNs), which in turn act to control the infection locally, alert surrounding cells to the danger, and promote the recruitment and activation of immune cells *(1-4).* The major PAMPs recognized during viral infections are virus-derived nucleic acids.

HPV are small, non-enveloped, double-stranded DNA (dsDNA) viruses that selectively infect keratinocytes in stratified epithelia of both skin and mucosa *(5-6).* They infect cells in the basal layer of stratified epithelia and virion production is dependent upon epithelial differentiation. High-risk HPV infections can persist despite viral activity in keratinocytes. This indicates that HPV has developed mechanisms to effectively evade or suppress the host's innate and/or adaptive immune response. Indeed, several studies on the spontaneous immune response to HPV have shown that HPV-specific cellular immunity develops quite late during persistent HPV infections and often are of dubious quality in people with progressive infections *(7-9).*

During infection of its target cells, HPV must be coming in contact with the host innate immune system's pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and absent in melanoma 2 (AIM2)-like receptors

(ALRs). HPV entry into target cells is mediated by endocytosis, followed by rapid transit of the viral genome still associated with the capsid protein L2 through the Golgi apparatus and Endoplasmic Reticulum to nuclear pores and subsequent delivery of the viral genome into the nucleus. As HPV DNA is protected by viral capsids in the cytoplasm prior to nuclear exposure, nuclear sensing is a critical aspect of defence against these nuclear replicating viruses. In this regard, the nuclear PRR IFI16 might be biologically relevant in sensing the viral genome once released in the nucleus. As demonstrated by our groups, the host nuclear factor IFI16 acts as a sensor of foreign DNA and antiviral restriction factor for human cytomegalovirus (HCMV) *(11)* and herpes simplex virus (HSV-1) *(12-14)*. In our study, we aim to determine whether (i) IFI16 has also a profound effect on HPV18 replication, (ii) the nuclear sensor IFI16 detects the HPV genome, and (iii) this sensing leads to antiviral cytokines activation/production.

Materials and methods

ChIP Chromatin Immunoprecipitation assay. ChIP assays were performed using the Shearing Optimization kit and the OneDay ChIP kit (Diagenode Europe, Seraing, Belgium). Extracts were sonicated using the BioruptorH Twin (Diagenode). Immunoprecipitation was performed with 2 µg of unmodified histone H3, acetyl-histone H3, dimethyl-histone H3, and dimethyl-histone H3. DNA solution was used to perform qPCR with primer amplifying the HPV18 long control region (LCR) and the p811 promoter.

Cell cultures. NIKS cells were cultured in presence of J2 3T3 fibroblast feeder in Ham's F-12 medium/Dulbecco's modified Eagle's medium (DMEM) (3:1), supplemented with 2.5% fetal bovine serum (FBS), 0,4 ug hydrocortisone per ml, 8.4 ng cholera toxin per ml, 5ug insulin per ml, 24 ug adenine per ml, 10 ng epidermal growth factor per ml, 100 units penicillin, and 100 ug streptomycin per ml, in presence of (γ-irradiated) feeder cells. U2OS and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% PSG.

U2OS- and NIKS-mcHPV18 cells line. U2OS- and NIKS-mcHPV18 cells were obtained by electroporation of U2OS or NIKS cells with a Nucleofector II Amaxa (Biosystems) with 2 µg of HPV18 minicircles according to the manufacturer's instructions. Minicircles were generated as previously described by Ustav (21).

Inhibition of IFI16 expression. NIKS-HPV18 were transiently transfected with the Nucleofector II Amaxa (Biosystems) according to the manufacturer's instructions with a pool of IFI16 small interfering RNAs (siRNA IFI16) or control siRNA (siRNA ctrl) as negative control (final concentration: 300 nM; Qiagen).

IFI16 overexpression. NIKS-HPV18 and U2OS mcHPV18 were infected with an adenoviral vector expressing IFI16 (AdV IFI16) or with the control plasmid AdV LacZ (MOI of 30 PFU/ml) and after 48h viral DNA levels were measured.

Growth of primary keratinocytes in organotypic raft cultures. Organotypic cultures were grown in specialized culture chambers on a collagen base, formed by mixing normal human neonatal fibroblast with type I collagen in Ham's F-12 medium containing 10% FBS and penicillin/streptomycin. NIKS cells were plated on the collagen base and after 15 days, raft cultures were harvested and fixed in 10% buffered formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H and E) for histological examination.

Detection of viral DNA and IFI16 expression in monolayer and organotypic raft cultures. FISH was performed on monolayer and raft sections together with the IFI16 staining in order to detect in situ viral DNA amplification and the localization of the IFN-inducible protein. This technique has been established in our laboratory (16).

Real-time quantitative reverse transcription-PCR. Total RNA was extracted with QIAamp Mini Kit (Qiagen) and 1mg was retrotranscribed using the iScript cDNA Synthesis Kit (BioRad). Reverse-transcribed cDNAs were amplified in duplicate using Sso Fast Eva Green Supermix (BioRad). Antiviral and pro-inflammatory cellular cytokines were analized (IFN α, IFN β, IFN λ_1, IFN $\lambda_{2,3}$, ISG54/IFIT2, IL-6, IL1β).

Proximity Ligation Assay for IFI16. Cells were grown on microscope slide, fixed with 4% paraformaldehyde and permeabilized with 0,5% Triton X-100 in PBS. Samples were incubated with primary antibodies that bind to two different epitopes of IFI16. Secondary antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS) were added to the reaction and incubated. When the two oligonucleotides are in close proximity they are ligated in a closed circle and act as primer for the Rolling Circle Amplification. This reaction incorporates a fluorescent molecule that can be visualized by fluorescence microscopy.

Results

IFI16 downregulation enhances HPV18 replication. NIKS cells silenced by specific siRNA for IFI16 have a higher viral yield, as demonstrated by viral DNA copy number that is significantly increased in NIKS-siIFI16 compared to control cells (NIKS-siCTRL) (Figure 1a). Analogs results were obtain in U2OS cells where IFI16 was stably kockout using CRISPR technology as described by Johnson et al. (12) (Figure 1b).

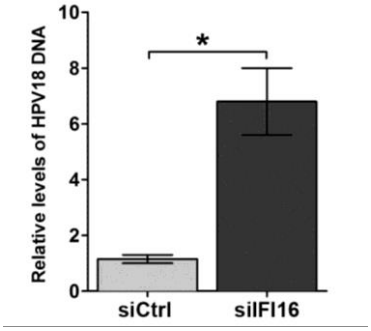


Figure 1a. NIKS-HPV18 cells were electroporated with a mixture of four different small interfering RNAs (siIFI16) or scrambled control siRNA (siCtrl). After 24 h, the cells were transferred into methylcellulose medium for 72 h to allow differentiation.To measure viral replication, total cellular DNA was analyzed by qPCR after DpnI digestion. The levels of glyceraldehyde 3-phosphate dehydrogenase were used to normalize the HPV18 levels.

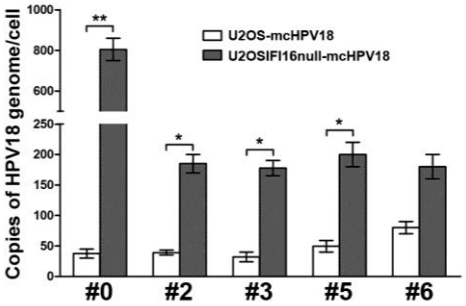


Figure 1b. U2OS IFI16-knockout cells and the parental cell line were electroporated with HPV18 minicircle DNA and, at the indicated passage (#0, #2, #3, #5, or #6), total cellular DNA was extracted. Viral replication was measured by qPCR of the DpnI-digested DNA. The levels of glyceraldehyde 3-phosphate dehydrogenase were used to normalize the
HPV18 levels.

IFI16 overexpression inhibits HPV18 replication. IFI16 overexpressing cells through an adenoviral vector (AdV IFI16) have a lower viral yield compared to cells infected with an adenoviral vector carrying the LacZ gene (AdV LacZ) used as a control.

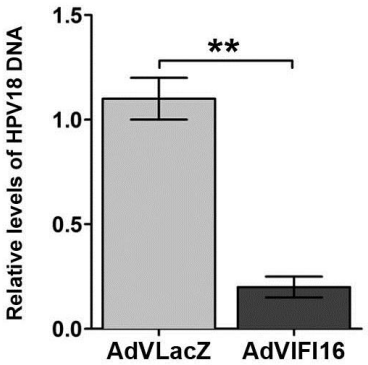


Figure 2a. NIKS-HPV18 cells were infected for 24 h with AdVIFI16 or AdVLacZ and then
transferred into methylcellulose medium for 72 h to allow differentiation. To measure viral replication, total cellular DNA was analyzed by qPCR after DpnI digestion. The levels of glyceraldehyde 3-phosphate dehydrogenase were used to normalize the HPV18 levels.

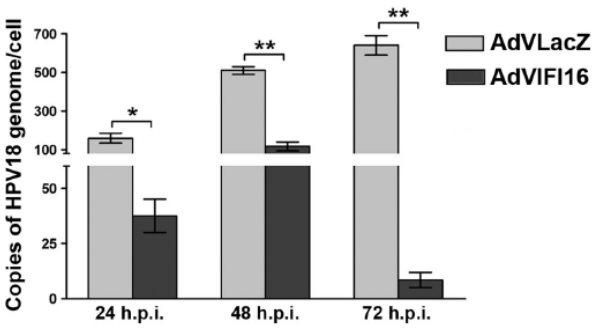
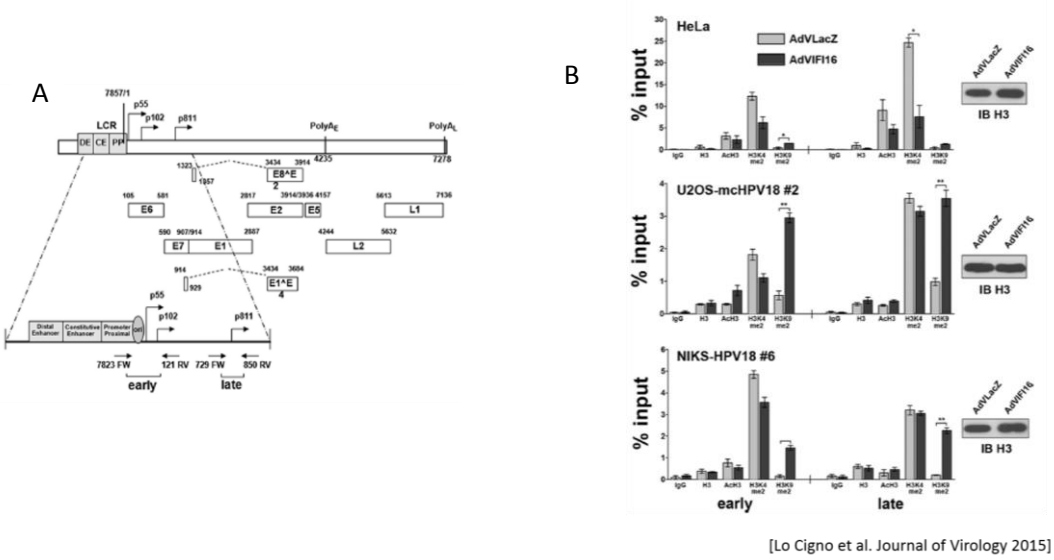


Figure 2b. U2OS cells were infected for 24, 48 and 72h with AdVIFI16 or AdVLacZ. To measure viral replication, total cellular DNA was analyzed by qPCR after DpnI digestion. The levels of glyceraldehyde 3-phosphate dehydrogenase were used to normalize the HPV18 levels.

IFI16 promotes heterochromatin association with viral DNA. Chromatin modification, such as the demethylation of histone H2 lysine 4 (H3K4), are associated with actively transcribing genes, whereas the demethylation of histone H3 lysine 9 (H3K9) is a mark of heterochromatin (18-20). To examine whether IFI16 expression could modulate chromatin structure, cells were infected with an adenoviral vector expressing IFI16 for 24h. Chromatin Immunoprecipitation analysis showed a reduction in the association of euchromatin marks and an increase of heterochromatin marks with the viral promoter region.



[Lo Cigno et al. Journal of Virology 2015]

Figure 3: (A) Linear depiction of the HPV18 genome with the early (p55 and p102) and late (p811) regulatory regions expanded. (B) HeLa, U2OS-mcHPV18, and NIKS-HPV18 cells were infected with AdVIFI16 or AdVLacZ. Extracts were prepared 24h after infection and ChIP was carried out using antibodies to unmodified histone H3 (H3), AcH3, dimethylated lysine 4 of H3 (H3K4me2), dimethylated lysine 9 of H3 (H3K9me2), or IgG as a control.
Lysate samples taken prior to immunoprecipitation (10µl) were used for Western blot analysis (IB) with the antibodies to H3 to monitor equal loading.

The IFI16 protein is down-regulated in HPV18 positive cells and doesn't colocalize with the viral genome. To visualize if there could be any recognition of the HPV18 genome by IFI16 and colocalization, U2OS-mcHPV18 cells (Figure 4a) and raft sections (Figure 4b) were analyzed by immunofluorescence (anti-IFI16 antibodies) in association with FISH (HPV18 genome). HPV18 positive nuclei usually displayed lower expression level of IFI16 than in cells in which the HPV genome was undetectable by FISH. In addition, we failed to detect any colocalization between the IFI16 and FISH signal.

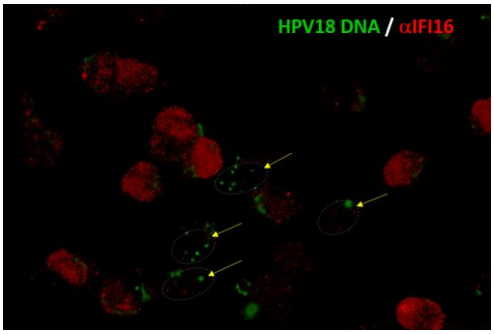


Figure 4a: Fixed cells were double stained for the presence of viral genome by DNA-FISH (green) and for IFI16 expression (red) by immunofluorescence.
Yellow arrows indicate the FISH signal.

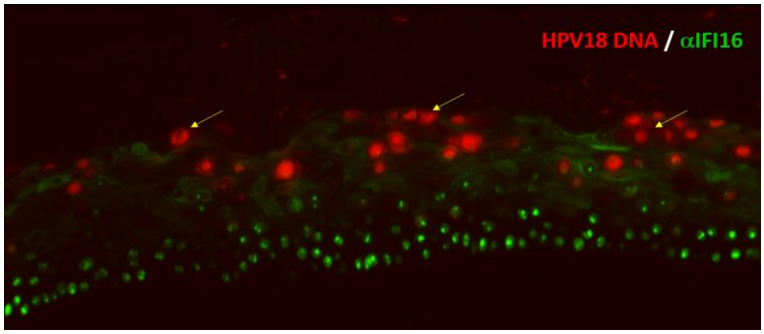


Figure 4b: NIKS-mcHPV18 grown in organotypic raft cultures (day 14). Sections were double stained for the presence of viral genome amplification by DNA-FISH (red) and for IFI16 expression (green) by immunofluorescence.

To analyze an earlier stage of infection, cells nucleofected with EdU- or FITC-labelled minicircles at 6h post nucleofection were analyzed by immunofluorescence to localize the IFI16 protein. As shown in Figure 5, the cells positive for the viral genome signal showed reduced levels of the IFI16 protein and again there wasn't any colocalization with the IFI16 fluorescent signal.

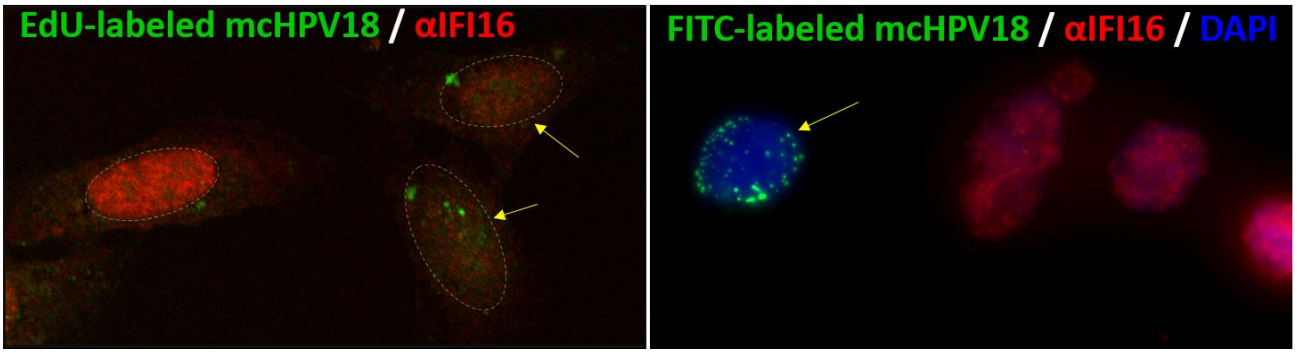
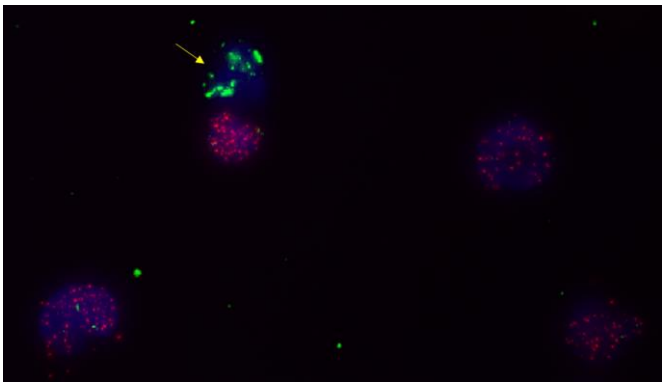


Figure 5: HPV18 minicircles (green) were labeled either with EdU Click-iT® assay (left) or with Label-IT Tracker ® (right). IFI16 expression was detected by immunofluorescence with an antibody to IFI16 (red). Yellow arrows indicate the labeled HPV18 minicircles.

Moreover, to increase the sensitivity of the technique, IFI16 was detected by Proximity Ligation Assay (PLA) (Figure 6). Even in this case, there wasn't any colocalization.



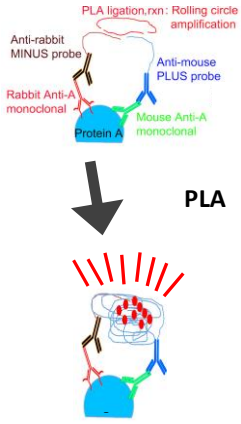


Figure 6: Label-IT Tracker ® mcHPV18. *The red dots depict IFI16.* Yellow arrows indicate the labeled HPV18 minicircles

The antiviral and pro-inflammatory cytokines are poorly induced in HPV18-positive cells. NIKS and NIKS mcHPV18 cells were grown for 6 and 12h in methylcellulose to induce differentiation. Extracted RNA was used to evaluate the production of pro-inflammatory and antiviral cytokines by RT-PCR. The results showed that the presence of HPV18 failed to stimulate the activation of cellular immune response.

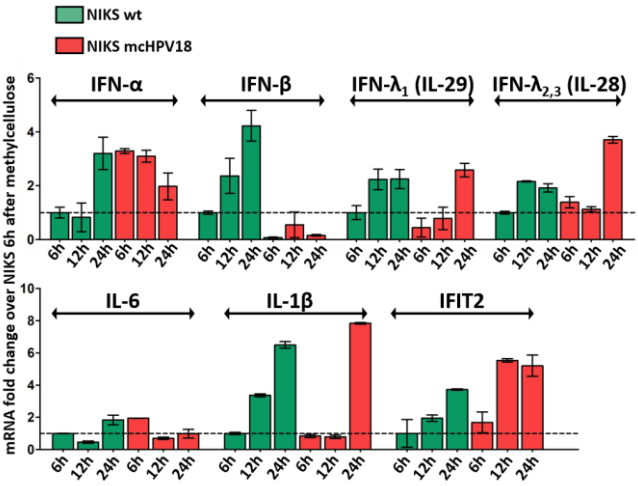


Figure 7: Total RNA was extracted at 6-12-24h after differentiation in methylcellulose and analyzed by Real Time RT-PCR to measure cellular production of antiviral and pro-inflammatory cytokines. The values were normalized to Glucuronidase Beta (GUSB) and are shown as folds change relative to WT NIKS cells differentiated for 6h.

Conclusion

In this study, we developed an in vitro model to study the role of IFI16 in controlling HPV replication. The results obtained clearly demonstrated that IFI16 acts as a restriction factor for HPV18 replication and the overexpression of IFI16 restricts chromatinized HPV DNA through epigenetic modifications. These results have been published in Journal of Virology, August 2015 *(15).*

In contrast to Herpesvirus positive cells, the presence of HPV failed to activate the innate immunity of the host cells. Moreover, consistent with the lack of IFN expression, the costaining for IFI16 by immunofluorescence and HPV18-DNA by FISH, performed in both monolayer and raft cultures, showed that in HPV18 FISH-positive nuclei IFI16 disappeared from the nucleus. In addition, we also failed to detect any relocalization of the IFI16 protein into the cytoplasm. Consistent with the lack of relocalization of the IFI16 in protein that is necessary for triggering the innate immune response, we failed to detect any induction of antiviral cytokines in HPV18-infected cells. We are currently performing experiments to clarify the molecular mechanisms through which HPV18 escape IFI16 sensing activity.

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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 PARTECIPATION

- CRISPR 2015 Oxford, 23-24 March 2015 - Oxford, UK.
- ICGEB "DNA Tumous Virus Meeting 2015" 21-26 July 2015 - Trieste, Italy.

ORAL PRESENTATION

- ICGEB "DNA Tumous Virus Meeting 2015" 21-26 July 2015 - Trieste, Italy.
  "Restriction of HPV18 replication in the nucleus by IFI16: what about innate sensing?"
  S. Albertini 1, I. Lo Cigno 1, M. De Andrea 1,2, C. Borgogna 1, A. Peretti 3, G. Gherardi 2, K. E. Johnson 4, B. Chandran 4, S. Landolfo 2, M. Gariglio 1

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?"
  M. De Andrea, S. Albertini, F. Calati, I. LoCigno, C. Olivero, C. Borgogna, K. E. Johnson, B. Chandran, S. Landolfo, M. Gariglio

PUBLICATION

- Lo Cigno I., De Andrea M., Borgogna C., Albertini S., Landini M. M., Peretti A., Johnson K., Chandran B., Landolfo S., Gariglio M. (2015) The Nuclear DNA Sensor IFI16 Acts as a Restriction Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral Promoters. Journal of virology 89, 7506-7520.