UNIVERSITY OF PIEMONTE ORIENTALE "AMEDEO AVOGADRO"

PhD in Sciences & Medical Biotechnology:

Targeted therapies in lung adenocarcinomas: new methodologies and new markers

PhD annual report 2014/2015

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1.INTRODUCTION

1.1 Lung cancer: epidemiology, etiology and classification

Lung cancer is the leading cause of cancer related deaths worldwide. Nowadays it is the neoplastic disease with the highest mortality and concerning incidence it is the second one only after sex related tumors (i.e. prostate and breast cancers) (Travis W D et al, 2004; Siegel R et al, 2015). Its mortality rate is mainly due to the high metastatic potential that makes this disease difficult to be diagnosed before the advanced stage. Lung cancer incidence is higher in men than in women but in the last years male incidence has decreased mainly thanks to the diffusion of no-smoking campaigns. In 2013, men highest incidence rates were observed in North America, East Asia, Central-Eastern and Southern Europe (from 48.5 to 56.5 new cases per 100,000 inhabitants). While in less developed countries, the highest rates were seen in West Asia, South Africa, and the Caribbean (from 25.7 to 32.2 new cases per 100,000 inhabitants). In women, the worldwide incidence rates of lung cancer are lower than those for men, and in 2013 the highest rates were seen in North America and in Northern Europe (from 35.8 to 37 new cases per 100,000 inhabitants) (Ridge C A et al, 2013).

The overall survival for lung cancer is poor, but data from 1999 to 2006 showed that women have better survival compared with men across all ages, irrespective of the histologic subtype. Indeed, in 2006, the 5-year survival rate for women with lung cancer was 19% compared to 14% for men (Ridge C A et al, 2013). Furthermore, Eurocare 4 data show a 5-year survival from lung cancer diagnosis in about 20% of cases diagnosed from 1978 to 2002 (Herbst R S et al, 2007).

Lung cancer carcinogenesis is influenced by interactions of genetic and epigenetic factors that lead to an uncontrolled growth of abnormal cells in lung tissue. The main driver is tobacco smoking, but there are also other factors (e.g. hormonal imbalance, viruses, genetic factors, genetic individual susceptibility and other environmental factors) which have been proposed to predispose to lung cancer. All these factors can cause genetic and global transcriptome changes. As a result, cells are characterized by aberrant pathways activation that can persist long term leading to dysplasia and clonal patches. Afterward, other additional changes result in angiogenesis, early stage cancer, metastasis and, at the end, invasion brings to advanced lung cancer (Herbst R S et al, 2008).

Lung cancers classification has been developed following Word Health Organization (WHO) standards and International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) recommendation. Lung tumors can be subdivided into Non-Small-Cell Lung Cancers (NSCLCs) and Small-Cell-Lung Cancers (SCLCs). The first ones are classified in Adenocarcinoma (AC), Squamous Cell Carcinoma (SCC) and Large Cell Carcinoma (LCC). Eighty-five percent of lung cancers are NSCLCs and the remaining (15%) are SCLCs. Among NSCLCs, AC represents the histotype with the highest rate (60%). SCC and LCC constitute the 25% and 15% of

NSCLCs, respectively (Herbst R S et al, 2008). Concerning classification, on the basis of morphological features, pathologists are used to classify lung cancer with a grade that describes the differentiation of the tumor: G1 indicates an high differentiated cancer, G2 a medium differentiated cancer and G3 a low differentiated cancer. Another way to characterize lung cancer is the TNM classification, where T indicates the differentiation of the primary tumor, N the lymph node invasion and M the presence of metastasis (Travis W D et al, 2004).

1.2 AC: general characteristics and molecular data

As mentioned, AC histotype is the most common among lung cancers. Patients affected by such histotype are frequently women and Asiatic. Indeed, AC histotype represents 42% of lung cancers in women and 28% in men. ACs are anatomically characterized by neoplastic cells producing mucus and organized in different structures, such as beads, ducts or solid clumps. Lung cancer cells can cover alveolar wall bringing to the development of Bronchiolar Alveolar Carcinoma (BAC). Usually, AC is identified as a grey nodule with irregular borders that blend in the lung parenchyma located near visceral pleura (Corrin B, 2000). AC frequently leads to the development of distant metastases in liver, bone, central nervous system, adrenal glands through blood and in loco regional lymph nodes through the lymphatic system (Corrin B, 2000). AC histotype is identified by the histochemical marker TTF1 (Thyroid Transcription Factor-1) and in lung cancers the most important molecular markers are the following genes: EGFR (Epidermal Growth Factor Receptor), HER2 o ErbB2 (Human Epidermal growth factor Receptor 2), KRAS (Kirsten Rat sarcoma viral oncogene homolog), BRAF, ALK (Anaplastic Lymphoma receptor tyrosine kinase) and ROS1 (Herbst R S et al, 2008; Bos M et al, 2013; De la Bellacasa P R et al, 2013) (Figure 1).

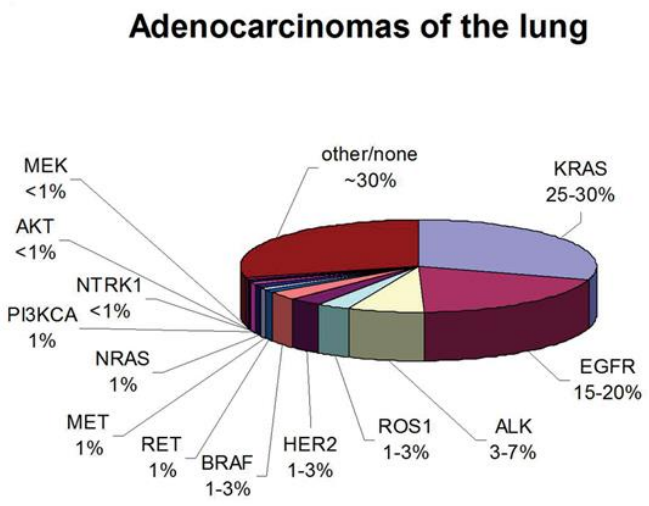

Figure 1: Pie chart representing the frequency of oncogenic alterations in lung AC (Gerber D E et al, 2014).

1.2a) EGFR

EGFR gene is located on the short arm of chromosome 7 (region 7p11) and encodes for a tyrosine kinase (TK) receptor of 170kDa. This transmembrane glycoprotein binds to the epidermal growth factor (EGF) and the binding induces receptor dimerization and tyrosine auto phosphorylation that leads to the creation of specific binding sites for the protein members of RAS-RAF-MEK or PI3K-Akt-mTOR pathways. Therefore, ligand binding to the receptor leads to the activation of these two pathways that are related to transcriptional activation of genes involved in cellular proliferation, differentiation, metastasis, angiogenesis and programmed cell-death (Koudelakova V et al, 2013). EGFR genetic alterations are observed in 15-20% of AC patients and are represented by mutations occurring in exons 18, 19, 20 and 21 (Gerber D E et al, 2014). In particular; 80% of EGFR genetic alterations are located in exons 19 and 21. The most widespread alterations of EGFR in lung cancers are point mutations (e.g: G719C, G719S, G719A in exon 18; T790M in exon 20; L858R, L861Q, L861R in exon 21) deletions (e.g: ΔE746-A750, ΔL747-T751 and ΔL747-P753 in exon 19) and insertions (e.g: D770-N771insNPG, D770-N771insSVQ and D770-N771insG in exon 20). EGFR mutations mainly occur in women of Asiatic origin.

1.2b) HER2

HER2 gene is situated on the long arm of chromosome 7 (region 17q21-17q22) and encodes for a member of EGFR family. This protein has no a ligand binding domain and therefore cannot bind growth factors, indeed an HER2 growth factor has not been found yet. However, it tightly binds other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signaling pathways, such as those involving RAS-RAF-MEK, PI3K-Akt-mTOR and JAK-STAT. The activation of these pathways leads to the promotion of cellular proliferation and angiogenesis (Cappuzzo F et al, 2005; Garrido Castro A C and Felip E, 2013; Martin V et al, 2013). In primary lung cancer, HER2 alterations are mainly represented by mutations occurring in exon 20, identified in 1-3% of all the ACs (Gerber D E et al, 2014). HER2 mutations, as EGFR, mainly occur in women. HER2 gene amplification, the main mechanism of alteration of this gene, in other cancer types (i.e: breast and gastric cancer) may occur as a mechanism of resistance to specific targeted therapies (see below).

1.2c) KRAS

KRAS gene is located on the short arm of chromosome 12 (region 12p12) and encodes for a GTPase protein of 21kDa, involved in the intracellular transductional pathways regulated by TK receptors which are related to apoptosis, cell growth and proliferation. KRAS protein activity is influenced by the exchange between GDP and GTP. The binding of GTP to KRAS leads to the activation of this protein and the recruitment of RAF proteins. KRAS binding to RAF leads to the phosphorylation of MAP2K and MAP2K-2 and, consequently, to the activation of MAPK (Mitogen-Activated Protein Kinase). In lung cancer, KRAS

alterations are represented by point mutations, mainly occurring in exon 2 (codons 12-13) and rarely in exon 3 (codons 59-61) or 4 (codon 146) (Koudelakova V et al, 2013). They are identified in 10-40% of AC patients and are mutually exclusive with EGFR and HER2 genetic alterations (Gerber D E et al, 2014). KRAS mutation occurs predominantly in smoker patients.

1.2d) BRAF

BRAF gene is situated on the long arm of chromosome 7 (region 7q34) and encodes for a protein of 94 kDa belonging to the RAF family of serine/threonine protein kinases. This protein plays an important role in regulating the MAPK signaling pathway, which affects cell division, differentiation, and secretion. In lung cancer BRAF alterations are mutations located in exons 11 and 15 and they have been found in 2-4% of ACs (Herbst R S et al, 2008; Michaloglou et al, 2008; Gerber D E et al, 2014). Half of BRAF mutations are represented by the V600E change (exon 15). The remaining 50% of mutations are mainly represented by G469A and G466V (40%) in exon 11 and D594G (10%) in exon 15. BRAF mutations mainly occur in heavy smokers.

1.2e) ALK

ALK gene is located on the short arm of chromosome 2 (region 2p23) and encodes for a TK receptor of 140kDa which belongs to the insulin receptor superfamily. It plays an important role in the development of the brain and exerts its effects on specific neurons in the nervous system. This gene has been found to be rearranged in NSCLC and in a series of cancers, including anaplastic large cell lymphomas and neuroblastoma. Chromosomal rearrangements are the most common genetic alterations in ALK gene, resulting in creation of multiple fusion genes essential for cancer development. The most common rearrangement is the EML4/ALK (both genes are situated on chromosome 2) thus representing a paracentric inversion. This inversion characterizes 3-7% of AC cases. Rearrangements involving EML4 and ALK genes are characterized by a huge number of variants; literature reports more than 13 variants of this kind of inversion. Other rare rearrangements are ALK/KIF5B, ALK/TGF and ALK/KLC1, representing only 1% of the rearranged cases (Rikova K et al, 2007; Takeuchi K et al, 2009). ALK rearrangements are usually detected in young women.

1.2f) ROS1

ROS1 gene is situated on the long arm of chromosome 6 (region 6q21-22) and encodes for a TK receptor belonging to the insulin receptor superfamily whose molecular weight is 82kDa. ROS1 plays an important role in the regulation of PI3K-Akt-mTOR and RAS-RAF-MAPK pathways, which are related to cell proliferation and differentiation. ROS1 alterations in cancers are generally characterized by rearrangements and in lung cancer the most common ones are ROS1/FIG, ROS1/SLC34A2, ROS1/CD74, ROS1/EZR, ROS1/LRIG3, ROS1/SDC4 and ROS1/TPM3, cumulatively identified in about 1% of all NSCLC cases and in 1-3% of AC subtype (Gerber D E et al, 2014). These alterations are

mutually exclusive with ALK rearrangements and are associated with young female patients (Yoshida et al, 2013).

1.3 Therapies

Surgical resection is the first approach in patients affected by early staged AC; in patients with advanced NSCLC, tumor resection is combined with chemotherapy.
Currently, the most diffused treatment for lung cancer is platinum (cis-platinum or carboplatinum)-based doublet chemotherapy from four to six cycles. This combination is characterized by the administration of platinum (Pt) and a couple of cytotoxic molecules (Gemcitabine, Paclitaxel, Docetaxel, Vinorelbine or Pemetrexed) (Cufer T et al, 2013; Shepherd F A et al, 2013). Several studies have compared platinum-based doublets containing these cytotoxic molecules and survival rates have been similar in all the trials. From 2008, Pt doublet chemotherapy brought to the reduction of the mortality rate of nearly 23% (Cufer T et al, 2013). A clinical trial of 2009 showed that, in patients treated with four cycles of chemotherapy, without progression, a maintenance cure with a single cytotoxic molecule leads to the increase of Overall Survival (OS) and Progression-Free Survival (PFS) (Ciuleanu T et al 2009). Recent data demonstrate that the administration of monoclonal antibodies (Bevacizumab) against the Vascular Endothelial Growth Factor (VEGF) in second and third line and in combination with Pt doublet chemotherapy leads to an advantage in lung cancer treatment. Indeed the trial ECOG 4599 showed that the antibody Bevacizumab administered with carboplatinum and Paclitaxel may increase PFS and OS of nearly two months (Sandler A et al, 2006; Zarogoulidis K et al, 2013).
Radiotherapy can also be recommended before surgery to reduce tumor dimensions and after surgery to reduce the risk of local relapse (Shepherd F A et al, 2013).

1.4 Targeted therapies

Chemotherapies are limited by their lack of specificity and by frequent and potentially severe dose-limiting toxicities. Therefore, there is an urgent need for more effective, better-tolerated treatments that specifically target the process pivotal to tumorigenesis and metastasis. In order to solve these problems, recent years have seen rapid progress in the development of new treatment strategies for advanced NSCLC, in particular the introduction of molecular targeted therapies (Kaneda H et al, 2013).
In the treatment of lung ACs, Gefitinib and Erlotinib, small-molecule tyrosine kinase inhibitors (TKIs) targeting EGFR, have been recently introduced. TKIs are small molecules acting as reversible inhibitors of EGFR through their competitive binding to the TK intracellular domain in proximity of the ATP/Mg^{2+} binding site. This binding inhibits TK activity that is enhanced by activating mutations of EGFR. The most diffused genetic alterations in EGFR correlated with sensibility to TKIs are ΔE746-A750 in exon 19; L858R,

L861R and L861Q in exon 21; G719A, G719C and G719S in exon 18 and V765A, T783A and S768I in exon 20. On the other hand, rearrangements (e.g. Δ770-N771insNPG, Δ770-N771insSVQ, Δ770-N771insG) and other mutations (T790M, V796L, N771T) in exon 20 are associated with resistance to TKIs. Last alterations are classified as primary resistance, happening before the administration of chemotherapy. Furthermore there are also mechanisms of secondary resistance that happen after the administration of the treatment. The most common molecular mechanism inducing secondary resistance is the mutation T790M in exon 20. Indeed, T790M is found in 50-70% of ACs resistant to TKIs (Murray S et al, 2012). To overcome TKIs resistance some trials have studied the efficacy of the treatment with irreversible inhibitors, such as Afatinib and Dacomitinib (Kaneda H et al, 2013). More mechanisms of acquired resistance to EGFR TKIs are represented by HER2 or MET gene amplification.

Other molecular therapies that are used in NSCLC care are Crizotinib and Ceritinib, two TKIs approved by the U.S. Food and Drug Administration (FDA) when in NSCLC there is evidence of ALK rearrangement by FISH (Fluorescent In Situ Hibridization) test. Crizotinib and Ceritinib inhibit ALK by binding this membrane receptor in the ATP binding site, blocking downstream molecular pathways (Bergethon et al, 2012). The treatment with these drugs generates secondary resistance too. Consequently some trials have studied other ALK inhibitors that are not yet approved (e.g. CH5424802 and LDK378) (Kaneda H et al, 2013). Some clinical trials show that Crizotinib and Ceritinib give a better OS also in patients rearranged in ROS1, but the study of the effects of them on ROS1 rearranged patients are still in progress (Bergethon K et al, 2012).

Besides EGFR TKIs and ALK inhibitors, researchers are going to investigate if there could be other molecular markers associated with specific targeted therapies that may help patients care. For example, HER2 is an important molecular marker in gastric and breast carcinomas. In these cancers, HER2 molecular alterations are associated with sensibility to Trastuzumab, a monoclonal antibody that inhibits HER2 through the binding of the extracellular receptor domain. Some clinical trials concerning lung cancers do not show a better OS in patients mutated in HER2 and treated with Trastuzumab. Nevertheless a recent study reports in one patient mutated in HER2 a good response to both TKIs and Trastuzumab (Cappuzzo F et al, 2005; Garrido-Castro A C et al, 2013). Beside HER2, also BRAF could be another molecular marker in NSCLC, indeed Dabrafenib and Vemurafenib, BRAF inhibitors that are currently administered to BRAF-mutant melanomas with great success, seem to be associated with an increment of OS in NSCLCs with BRAF mutations, especially those with V600E mutation (Paik P K et al, 2011; Gautschi O et al, 2013).

Finally also KRAS could be a potential new molecular marker in lung AC. The prognostic and predictive role of KRAS is controversial, even though the majority of USA studies indicate KRAS as a negative predictive marker of response to EGFR TKIs (Garrido-Castro et al, 2013). Nowadays no targeted therapies against KRAS have been approved but in a phase II clinical trial of 2013, Selumetinib, a MEK inhibitor, showed promising results on patients with advanced NSCLC.

1.5 ROR1 and cancer

The receptor tyrosine kinase-like orphan receptors (ROR1 and ROR2) are transmembrane proteins that are part of the receptor TK family. ROR proteins are made of an extracellular domain consisting of an immunoglobulin-like motif, a Cysteine-Rich frizzled Domain (CRD), a kringle domain and an intracellular domain characterized by a TK domain, a Proline-Rich frizzled Domain (PRD) and a Serine/Threonine-Rich Domain (S/TRD1 o 2). ROR proteins are named "orphan receptors" because their endogenous ligand has not been discovered yet. These receptors are expressed at high levels during embryo development playing an important role in skeletal and neural organogenesis but are not expressed in normal adult tissues (Rebagay et al, 2012). ROR1 is upregulated in cancer, in particular in B-cell Chronic Lymphocytic Leukemia (B-CLL), B-cell Acute Lymphocytic Leukemia (B-ALL) and Mantle Cell Leukemia (MCL) (Baskar S et al, 2008; Shabani M et al, 2008). In 2012, ROR1 overexpression was observed also in breast cancers by immunohistochemistry (IHC) (Zhang S et al, 2012). Furthermore, again at protein level, it has been shown that ROR1 is expressed in lung AC and in this histotype ROR1 is associated with TTF-1 expression. On this basis, some studies have hypothesized that TTF1 may induce ROR1 expression and, consequently, the activation of PI3K-AkT-mTOR pathway that regulates cells survival (Yamaguchi T et al, 2012). Recent studies indicate ROR1 as a new potential molecular marker for targeted therapies. ROR1 is a good candidate for the formulation of new targeted therapies because it is not expressed in adult normal tissues but only in cancer cells. In order to block ROR1 TK activity there are two possible approaches. The first one is represented by the administration of monoclonal antibodies able to block ROR1 binding domain; the second one is the treatment with TKIs that recognize ROR1 TK domain (RebagayG et al, 2012; Gentile Aet al, 2011). The development of TKI against ROR1 could be promising because literature reports aminoacid substitutions in TK domain that modify ROR1 activity, indeed these aminoacids could be a good target for the formulation of ROR1 TKIs. Concerning antibodies, in vitro experiments have reported that they do not induce the expected apoptosis in lymphoma cell lines. Nevertheless, 5 different antibodies tested on leukemia cell lines gave better results because they caused cytotoxicity in cancer cell but not in normal tissues. Furthermore, cells treated with these monoclonal antibodies showed a better response to Rituximab, a targeted therapy against the protein CD20 (Yang J et al, 2012). Both treatments are being studied but tests on B-ALL cell lines showed that ROR1 inhibition makes cells more sensitive to the treatment with Dasatinib, a Src inhibitor (Bicocca V T et al, 2011).

2.AIM

The aim of this doctorate is to find new methodologies for the analysis of molecular markers typical of lung AC and that are essential for the administration of molecular targeted therapies. The need of new methodologies is essentially due to the particular features of lung AC samples available for molecular diagnosis. Indeed, lung AC specimens are generally constituted by poor quantity and poor quality of tumour material. Very often, only small biopsies are available and, with the current methodologies (i.e. Sanger sequencing and FISH), it is really difficult to estimate the molecular profile accurately. Furthermore, cancer cells are often dispersed in a high quantity of normal cells, leading these cases as difficult to be considered representative of the tumour. In addition, there is also the problem that the interpretation of ALK and ROS1 gene status by FISH requires highly trained personnel and therefore there is a need to find a more objective way to evaluate these markers. In order to solve all the problems caused by these limits we decided to test and optimize, on patients affected by lung AC, new methodologies that will permit to obtain a better identification of mutations in patients affected by lung AC. The majority of the new methodologies that we are intended to use are developed by a Danish company, PentaBase and are characterized by Real-time PCR assays based on oligonucleotides with higher specificity, higher sensibility and higher replicability than Sanger sequencing for EGFR, KRAS, BRAF, HER2 and as efficient as FISH but observer-independent for ALK and ROS1. We think that with these new approaches we will be able to identify a larger number of mutations or genetic alterations in all the molecular markers earlier mentioned in comparison with the previous methodology based on direct sequencing and FISH analyses. Therefore, a higher proportion of patients will benefit from targeted therapies. Furthermore we proposed to test another Real-time-based PentaBase kit, focused on the analysis of T790M mutation in blood samples. This test could be important because the T790M mutation in EGFR gene is the main reason of secondary resistance to EGFR TKIs and because this assay may permit to analyse blood samples. Consequently it is less invasive than the current analysis based on the need of tumour tissue availability.

Finally, since about 50% of AC cases display a normal gene status sequences for the aforementioned markers, we decided to study ROR1 expression in patients affected by lung AC in order to define if it could be a target for the formulation of new molecular targeted therapies. We propose to evaluate its expression and to see a potential correlation between this marker and the most relevant lung cancers molecular alterastions (i.e. EGFR, KRAS, BRAF, HER2, ALK and ROS1) and the clinical-pathological features. This possible new marker could be important for all the patients that cannot be treated with the current therapies against alterations in the standard known molecular markers.

The project was scheduled as follows:

AIM1: Study of new methodologies for a better identification of mutations in all the known target genes for current tailored therapies in lung AC (EGFR, KRAS, BRAF, HER2, ALK and ROS1).

1. Characterization of molecular markers in a cohort of lung AC patients by standard methods (i.e. Sanger sequencing and FISH).
2. Optimization of the new methodologies developed by PentaBase (Denmark).
3. Characterization of the same cohort with the new optimized methodologies.
4. Comparison between the old and the new assays.

AIM2: Study of a new methodology for a better identification of EGFR T790M mutation in AC.

1. Characterization of T790M mutation in blood samples and in their associated tissue samples in a different cohort with Sanger sequencing.
2. Characterization of the same cohort with the new assay.
3. Comparison between the old method of characterization of T790M mutation (Sanger sequencing) and the new one (PentaBase kit).

AIM3: Definition of ROR1 as a new target for new therapies.

1. Evaluation of ROR1 expression through Real-time PCR in a cohort of patients affected by lung AC.
2. Characterization of the typical molecular markers of lung AC in our cohort and association of them with ROR1 expression.

3.PATIENTS, MATERIALS AND METHODS

3.1 Patients

Our cohort includes 220 patients affected by lung AC. The samples are constituted by small biopsies and resections. All the patients will be characterized for EGFR, KRAS, BRAF and HER2 genetic alterations, for ALK and ROS1 gene rearrangements, for TTF-1 protein expression and for ROR1 gene expression.

3.2 Mutational status of EGFR, KRAS, BRAF and HER2 by Sanger sequencing

Genomic DNA was extracted from 7-μm formalin-fixed, paraffin-embedded tissue sections using QIAamp Mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions and the DNA was amplified by Polymerase Chain Reaction (PCR) experiments. We searched for point mutations, deletions or insertions in EGFR exon 18 (including codons 709 and 719), exon 19 (including codons from 746 to 753), exon 20 (including codons 768, 770, 771, 776 and 790) and exon 21 (including codons 858 and 861).
We investigated KRAS point mutations in exon 2 (including codons 12 and 13), BRAF point mutations in exon 11 (including codons 466 and 469) and in exon 15 (including codon 600). Furthermore we analyzed HER2 genetic alterations paying attention to insertions in exon 20. The direct sequencing (Sanger method) of PCR products was done using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the results were analysed with an appropriate software (SeqScape Software Version 2.5, Applied Biosystems). Each sequence reaction was performed at least twice, starting from independent PCR reactions.

3.3 ALK and ROS1 gene status by FISH

FISH was performed on 4-μm formalin-fixed, paraffin-embedded tissue sections treated using the Paraffin Pretreatment kit II (Pretreatment Reagent VP 2000, Abbott Molecular AG, Baar, Switzerland) according to the manufacturer's instructions. The ALK FISH assay was done using LSI ALK Dual Colour Break Apart Rearrangement Probe (Abbott Vysis©, Illinois, North Chicago, USA) and the ROS1 FISH assay was performed using SPEC ROS1 Dual Colour Break Apart Probe (Zytovision©, Bremerhaven, Germania). The signals were evaluated with a fluorescent automated microscope (Zeiss© Axioplan 2 Imaging, Oberkochen, Germany) equipped with a 100W UV lamp; an AxioCam camera (Zeiss© AxioCam MRm) and single, double, triple band pass filters.
ALK probe hybridizes in the 2p23 region and it is characterized by a dual colour (Spectrum Green and Spectrum Orange) break apart methodology that permits to recognize ALK downstream and upstream sequences of the ALK usual breakpoints

(Figure 2). The analyses are done counting cell by cell and observing the number of coloured fluorescent signals viewed in the tissue.

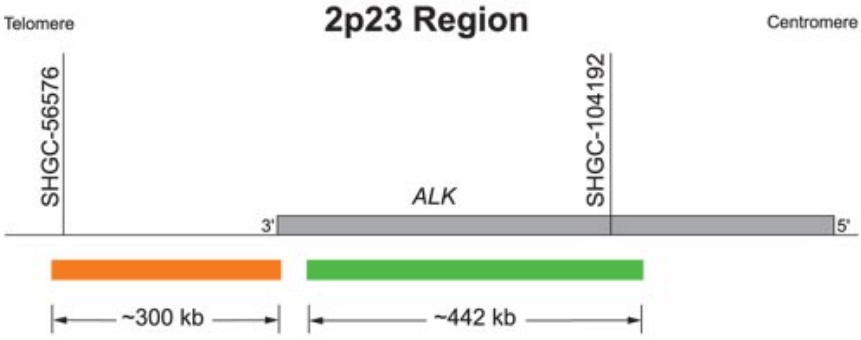

Figure 2: Schematic representation of LSI ALK Dual Colour Break Apart Rearrangement Probe (Abbott Vysis©).

If the cell is characterized by two fusion signals (i.e. two yellow signals) there is no ALK rearrangement. On the contrary if the red signal is separated by the green one, the cell has ALK rearrangement. In this case, if there is an intrachromosomal translocation (e.g. EML4/ALK inversion) the signals are less than 2/3 diameters distant. On the contrary, if the distance is more than 3 diameters, there is an interchromosomal translocation.
ROS1 probe hybridizes in the 6q22 region and it is a dual color break apart probe too. The green signal hybridizes next to the breakpoint cluster region (BCR) and the red one is distal (Figure 3). The interpretation of the signals is the same as that of ALK.

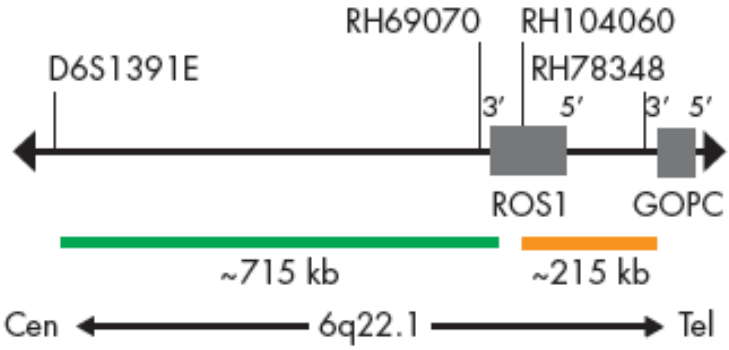

Figure 3: Schematic representation of SPEC ROS1 Dual Colour Break Apart Probe (Zytovision©) Cen: Centromere; Tel: Telomere.

In the analysis of both genes, a tissue is considered positive for ALK or ROS1 rearrangements if the rearranged cells are more than 15%.

3.4 TTF-1 immunohistochemistry

TTF-1 expression, the principal lung AC marker, was tested by immunohistochemistry (IHC) assays. We used a mouse monoclonal antibody against TTF-1 (M3575, Dako©, Glostrup, Denmark). TTF-1 signal is localized in the nucleus because it is a transcriptional factor. TTF-1 expression is reported on medical reports with values that indicate the intensity, the distribution and the percentage of positive cells.

3.5 ROR1 expression analysis

ROR1 expression was evaluated by Real-time PCR assays. To quantify the amplified cDNA we used a TaqMan fluorescent probe (Applied Biosystems) that recognizes the target gene (i.e. ROR1) and a TaqMan probe marked with a different fluorochrome that recognizes a reference gene (i.e. the RN18S1 housekeeping gene, which encodes for the 18S rRNA). The reference gene is an internal control that must always be expressed and at the same level in both normal and cancer tissues. TaqMan probes are characterized by a quencher (Q), a fluorochrome located on 3'-end, and a reporter, a fluorochrome located on 5'-end. During the Real-time PCR amplification process, the probe recognizes the denaturated cDNA strand in ROR1 and DNA polymerase cleaves the probe bringing to the split of Q and R. This division results in a fluorescent signal that is proportional to the number of DNA molecules obtained because the Q cannot mask R fluorescence when they are far away.
For the amplification, we used 100 ng and the test was repeated three times for each sample.
The data were analyzed considering the threshold cycle for each sample in both cancer and normal tissues. We used the Livak method (Livak et al, 2001), that consists in the calculation of the $2^{-\Delta\Delta Ct}$ value. $\Delta\Delta Ct$ is the difference between the sample ΔCt and the control ΔCt. Sample ΔCt is the difference between the Ct of the target gene (ROR1) and the Ct of the reference gene in tumor tissue. Control ΔCt is the difference between the Ct of the target gene (ROR1) and the Ct of the reference gene in normal tissue.
We fixed the value of 1 as cut-off so in all the samples with $2^{-\Delta\Delta Ct}$ value major than 1, ROR1 has been considered overexpressed.

3.6 Statistical analysis

The comparisons between genetic alterations and the association of them with clinic-pathological characteristics were evaluated through the two-tailed Fisher's exact test. We set a value of statistical significance equal to $p=0.05$.

4.RESULTS

In the first year we started AIM 2 and 3. Indeed, we characterized the whole cohort in which we analysed ROR1 expression and in which we are going to test the new methodologies for a better identification of the standard molecular markers: EGFR, KRAS, BRAF, HER2, ALK and ROS1.

4.1 Cohort

Our cohort is constituted by 220 patients (biopsies or tumor resections) that have been analyzed for the molecular markers with clinical relevance (EGFR, KRAS, BRAF, HER2, ALK, ROS1 and TTF-1). Forty-eight percent (106/220) of the cohort is characterized by women and 52% (114/220) by men. All the patients are affected by lung AC. Tumoural grade was available in 181 cases and the cohort was subdivided in 16% (29/181) G1, 36% (65/181) G2, 42% (76/181) G3, 2% (4/181) G1/G2, 3% (6/181) G2/G3; one patient is particular because it is characterized by two different regions, one with G1 grade and the other one with G3 grade. As regard to TNM classification, the cohort was subdivided as follows: 45% (69/154) pT1, 37% (57/154) pT2, 15% (24/154) pT3 and 3% (4/154) pT4. Lymph node status was described by these percentages: 56% (55/99) pN0, 17% (17/99) pN1, 24% (24/99) pN2 and 3% (3/99) pNx. In conclusion, only three patients have been evaluated for metastasis grade and they have all been classified as M1.

4.2 EGFR sequencing analysis

We analyzed EGFR molecular status by direct sequencing (Sanger method). We focused our attention on the characterization of exons 18, 19, 20 and 21 which encode for the TK domain and whose mutations are linked to the sensitivity to EGFR inhibitors. Ten patients were not evaluable because of bad quality of DNA (high degradation status). EGFR alterations were detected in 16% (33/210) of patients. More in details 1 patient displayed the G719A change in exon 18. Seventeen patients were characterized by genetic alterations in exon 19, in particular 1 patient displayed the L747P mutation, 1 case the S752F change, 1 sample two distinct mutations (E746G and L747S), 13 patients presented deletions (1 ΔL747-K751, 2 ΔE746-S752, 2 ΔL747-S752, 8 ΔE746-A750) and 1 case has an insertion (ins745-746). Three samples were characterized by mutations in exon 20, in particular 1 sample was D880N and two cases had insertions (i.e. H773-V774insNPH and D770_N771insG). Finally, 11 patients displayed mutations in exon21: 9 cases were mutated for L858R, 1 for Y827F and 1 for T847I. In addition to these alterations, we found one double mutated patient characterized by a deletion in EGFR exon 19 (ΔE746-A750) and one point mutation in EGFR exon 20 (T790M).

4.3 KRAS sequencing analysis

We analyzed KRAS exon 2 by direct sequencing and we could obtain molecular characterization for 212 patients, 8 samples being no evaluable because of DNA degradation. We found point mutations in 36% (70/212) of evaluable cases. In particular, 93% (65/70) of mutated samples were characterized by changes in codon 12 and 7% (5/70) in codon 13. Concerning codon 12, 21% (15/70) of mutated patients displayed the G12V mutation, 51% (36/70) the G12C mutation, 9% (6/70) the G12A change, 9% (6/70) the G12D mutation and 3% (2/70) the G12R mutation. Only 5 patients were mutated in codon 13, specifically 3% (2/70) showing the G13C mutation, 3% (2/70) the G13D change and 1% (1/70) the G13R mutation (1%).

4.4 BRAF sequencing analysis

The analyses of BRAF were focused on exons 11 and 15. Molecular information was obtained by Sanger sequencing in 185 patients, 35 cases being not evaluable because the DNA was highly degraded. Six patients (3%) displayed BRAF mutations, 3 in exon 15 and 3 in exon 11. Concerning BRAF exon 15, all the mutated cases had the V600E change whereas those mutated in exon 11 presented three different mutation types: G466V, G469A and G469V.

4.5 HER2 sequencing analysis

HER2 gene was characterized by direct sequencing in the whole cohort but 28 patients turned out to be not evaluable because their DNA was too degraded. Only a patient (1/192; 1%) showed a genetic alteration in HER2 gene. The detected mutation was the duplication of codons 775-776 after codon 775.

4.6 ALK/ROS1 FISH analysis

FISH analyses are still in progress for both ALK and ROS1 rearrangements. Nevertheless, we obtained some preliminary data that will be extended in the next year of. So far we have analyzed ALK rearrangements in 48 samples but 20 resulted not evaluable because of the high fixation and the bad conditions of the tissue. Among the evaluable samples, 1 patient (1/28; 4%) showed an ALK rearrangement. The positive sample is characterized by an intrachromosomal translocation because the signals observed by the fluorescent microscope are less than 2/3 diameters distant. On the contrary, we did not find samples with interchromosomal translocations or amplification in ALK gene.
Up to now, we have analyzed 96 samples for ROS1 chromosomal status, but 29 of them turned out to be not evaluable for the same reasons described for ALK. We observed that

only one patient is rearranged in ROS1 (1/67; 1%) showing an interchromosomal translocation because the signals observed by the fluorescent microscope are more than 2/3 diameters distant. We did not observe intrachromosomal translocations or amplification in ROS1 gene.

4.7 TTF-1 expression

The expression of TTF-1 protein was investigated through an IHC assay that allows the analysis of TTF-1 nuclear expression. IHC analyses are still in progress. To date, we have analyzed 143 patients, with 13 not evaluable probably for bad storage or bad fixation of tissue blocks. Overall, we observed that 82% (106/130) of patients are positive for TTF-1 expression and the remaining 18% (24/130) of patients are negative for TTF-1 expression.

4.8 ROR1 expression

ROR1 expression was tested by Real-time assays using a TaqMan fluorescent probe. As mentioned, enough tumor and normal tissue samples were available in 96 cases. In 34 patients the extracted RNA had a low concentration so they resulted not evaluable. By comparing the values of ROR1 in cancer and in normal tissue of the same patient, we observed that 15% (9/62) out of the evaluable patients were classified as positive for ROR1, being characterized by a $2^{-\Delta\Delta Ct}$ value > 1. Conversely, 85% (53/62) out of the evaluable patients is negative for ROR1 expression because the $2^{-\Delta\Delta Ct}$ value was < 1

4.9 Statistical analysis

In order to study the correlation between pathological characterization and molecular alterations we applied the two-tailed Fisher's exact test. On the basis of this test we did not find any statistical significant correlation between clinical-pathological data and molecular characteristics (i.e. EGFR, KRAS, BRAF, HER2, ALK and ROS1 alterations; TTF-1 and ROR1 expression).
Association studies between the different molecular markers that we have analyzed revealed a statistical significant correlation between KRAS and EGFR (Table 1) and between KRAS and TTF1 (Table 2). EGFR and KRAS mutations are mutually exclusive, because only three patients showed changes in both genes (Table 1). In particular these cases were characterized as follow: Δ746-750 in EGFR exon19/G12V in KRAS exon 2; Y827F in EGFR exon 21/G12C in KRAS exon 2; D800N in EGFR exon20/G12C in KRAS exon 2. As for KRAS and TTF-1, KRAS mutations are more associated with TTF-1 positive cases than with the negative ones (Table 2).
No correlation was found between ROR1 expression and clinic-pathological characteristics or the other molecular markers.

		EGFR		
		wt	mut	
KRas	wt	114	30	p=0.0002
	mut	82	3	

Table 1: Association analyses between the molecular data of EGFR and KRAS mutations by direct sequencing. The correlation between EGFR and KRAS is statistical significant because the p value, calculated by the two-tailed Fisher's exact test, is less than 0.05 ($p<0.05$). In particular, these two molecular markers are mutually exclusive, because only three patients showed changes in both genes. mut: mutated; wt: wild-type.

		KRas		
		wt	mut	
TTF-1	pos	69	29	p=0.009
	neg	10	15	

Table 2: Association analyses between the molecular data of KRAS and TTF-1 expression obtained by direct sequencing and IHC respectively. The correlation between KRAS and TTF-1 is statistical significant because the p value, calculated by the two-tailed Fisher's exact test, is less than 0.05 ($p<0.05$). In particular, KRAS mutations were predominantly detected in TTF-1 positive than in TTF-1 negative cases. mut: mutated; neg: IHC negative; pos: IHC positive; wt: wild-type.

4.10 New methodologies

The development of new methodologies (such as PentaBase assays) to better characterize lung AC patients are in progress. KRAS kits are nearly completed while EGFR, HER2, ALK and ROS1 ones are still under testing. Concerning BRAF, we will focus only on the V600E change that is the most important targetable mutation in this gene. In the next years, as soon as all the assays will be ready and validated, we will investigate our cohort.

4.DISCUSSION

Lung cancer is the neoplastic disease with the highest mortality and concerning incidence it is second only to sex related tumors (i.e. prostate and breast cancers) (Travis W D et al, 2004). These data highlight the importance to find new treatments that can improve the care and the survival of patients affected by such neoplastic disease, in particular by the AC histotype, the most common lung cancer subtype. Nowadays, the standard care for advanced lung cancer is tumour resection combined with four to six cycles of platinum doublet-based chemotherapy, constituted by platinum and a couple of cytotoxic molecules (Gemcitabine, Paclitaxel, Docetaxel, Vinorelbine or Pemetrexed) (Cufer T et al, 2013; Shepherd F A et al, 2013). However, chemotherapies are related to lack of specificity and to frequent and potentially severe dose-limiting toxicities.

On these bases, recent years have seen the development of targeted therapies against specific molecular markers in order to solve the limitations of chemotherapies and to improve patients' follow-up. In lung cancer, the most important targetable markers are EGFR, KRAS, BRAF, HER2, ALK and ROS1. At the moment, only inhibitors of EGFR, ALK and ROS1 have entered into clinical practice (Kaneda H et al, 2013). In particular, the drugs that have been approved are all TKIs, two against EGFR (Gefitinib and Erlotinib) and two against ALK and ROS1 (Crizotinib and Ceritinib). EGFR inhibitors are recommended for patients affected by EGFR mutant lung AC. Concerning ALK and ROS1, the administration of Crizotinib or Ceritinib is approved when there is evidence of ALK or ROS1 rearrangement by FISH (Bergethon et al, 2012). Due to the availability of targeted therapies resulted efficient in other cancers types (i.e: melanoma, breast cancer), clinical trials are evaluating the efficacy of BRAF and HER2 inhibitors in BRAF or HER2 mutant cases. Case reports showing benefits of these therapies in these patients have been published. In addition, a newly targeted therapy against KRAS mutations (Selumetinib) is under evaluation in lung cancer. However, only about half of all lung cancers are characterized by alterations in the aforementioned markers, so it is highly advisable to identify new putative targetable markers. In this light, ROR1 seems to be promising. Therefore, the present project has one philosophy: to increase the care of patients affected by lung AC. To do this, we propose two strategies: to develop new methodologies to better characterize cancer samples; and to study ROR1 expression in order to define if this TK receptor could be a target for development of new molecular targeted therapies. Indeed, the test and validations of new methodologies based on a Real-time approach and represented by a higher specificity, higher sensibility and higher replicability with respect to the current ones (Sanger sequencing and FISH), could permit to extend the number of patients that may benefit from targeted therapies. On the other hand, the study of ROR1 expression could define a molecular marker for new therapies and so it could permit to obtain a specific care also for the patients that display no alterations in all the previously described standard molecular markers.

The need of new methodologies characterized by higher specificity, higher sensibility and higher replicability is essentially due to the characteristics of lung AC samples. Frequently

AC specimens are constituted by poor quantity and poor quality of tumour material: i.e. only small biopsies and only few sections are left for the molecular characterization after morphological and IHC evaluation made by the pathologist. In this context, standard analyses (direct sequencing and FISH) results may be not representative of the tumour. Furthermore it has been demonstrated that AC and, more generally, lung cancer is a heterogeneous disease. In addition to these problems, some assays may be difficult to be interpreted. Indeed, nowadays the gold standard for the analysis of ALK and ROS1 rearrangements is FISH, but the interpretations of FISH results are objective and require highly trained scientists. So the introduction of new assays based on different technologies could permit to have a more objective interpretation of results concerning ALK and ROS1 gene alterations. For all these reasons, we decided to test and validate new assays for all the common molecular markers that are target for tailored therapies in lung AC. To date KRAS kit validations are nearly completed, whereas EGFR, HER2, ALK and ROS1 ones are still under evaluation. BRAF kit, focused on V600E, the only targetable mutation of this gene, has been already validated too. At the moment we have proceeded with the characterization of a cohort of 220 cases using current methodologies (Sanger sequencing and FISH). In the next years we are going to test these new methodologies on the cohort.
By direct sequencing we observed that mutations rates are superimposable with the percentages reported in literature for the AC lung cancers (Gerber D E et al, 2014). Association studies between molecular markers genetic status and clinic-pathological characteristics did not show significant correlations, with the exclusion of EGFR and KRAS: these mutations seem to be mutually exclusive in agreement with literature data (Koudelakova V et al, 2013). When we added the analyses of TTF-1 expression we found TTF-1 positive cases in 80% of our cohort in agreement with the literature. Interestingly, we found that TTF-1 positive cases are significantly associated with a KRAS wild-type genotype.

For the second aim, we have analysed a subgroup of the cohort (96 patients), because only in these cases sufficient material from paired normal and tumour tissues were available for RNA extraction. We choose to study ROR1 as candidate for new drugs because this TK receptor has two important characteristics that permit to define it as a potential good marker for new targeted therapies. Firstly it is expressed during development, playing an important role in skeletal and neural organogenesis, and it is upregulated in cancer (B-CLL, B-ALL, MCL and breast cancer) but it is not expressed in normal adult tissues (Baskar S et al, 2008; Rebagay et al, 2012; Zhang S et al, 2012). This expression profile is perfect because if ROR1 is not expressed in normal tissues the new targeted therapies, against ROR1, will be active only against cancer cells, while the normal tissue will not be involved in drugs negative effects. Secondly literature reports that ROR1 expression is associated with TTF-1 expression. As TTF-1 is the principal marker of AC histotype and ROR1 is associated with this transcriptional factor we believe that ROR1 could be important for this histotype. This particular association defines ROR1 as a putative new marker for new targeted therapies in lung AC.

We evaluated ROR1 expression by Real-time PCR in order to have a clear and objective picture of ROR1 status in AC lung cancers. Our data indicates that ROR1 is overexpressed in 15% of the cases. Our data do not mirror those reported in the only study investigating ROR1 expression in lung cancer. Indeed a study of 2012 reported ROR1 IHC positivity in 77% of the analyzed lung AC cohort (Zhang S et al, 2012). This difference could be justifiable because to asses ROR1 expression we used a different methodology (Real-time) from the one applied in that study (IHC). Furthermore, the antibody used in literature was developed in their lab and not commercially available. Alternatively, the discrepancy may be explained by ethnic differences: our cohort includes only Caucasian patients whereas the cohort of Zhang and colleagues only Asiatic patients. On the other hand, it is well known that AC lung cancers from Caucasian and Asian origin are different, being the percentage of EGFR mutations extremely high in Asia with respect to Western countries. Therefore, a similar difference also for ROR1 expression cannot be excluded. In the future, in order to clarify this difference, we are going to analyze ROR1 in our cohort with IHC (using commercially antibodies) and to compare our results with those of the literature. After ROR1 expression studies, we focalized our attention on the association between ROR1 expression results obtained by Real-time and the genetic alterations in the molecular markers that have been analyzed by direct sequencing or FISH. By applying the two-tailed Fisher's exact test no correlations were found between ROR1 expression and all the other molecular markers status. These data do not correspond to literature that reports a positive correlation between TTF-1 and ROR1 expression. We can justify this difference because the methodology that has been used in literature to compare ROR1 and TTF-1 expression is IHC and we do not have ROR1 expression data obtained by IHC to evaluate association between these proteins.

Overall, next year will be focused on the test of validated assays based on Real-time approach and on the analysis of ROR1 by IHC.

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ELENCO FORMAZIONE I ANNO PHD

Partecipazioni a congressi e seminari

Congresso: 80th Annual Meeting of the Swiss Society of Pathology, Losanna (Svizzera), Novembre 6-8/11/2014. "MYC heterogeneity in de novo DLBCL cases with rearrangement" (A.Valera**,** D.Soldini, L.Colomo, A.Riva, **S.Epistolio**, I.Dlouhy, E.Haralambieva, E.Campo, L.Mazzucchelli, V.Martin). **Presentazione orale del poster da parte del primo autore**.

Congresso: Quinta giornata della ricerca clinica della Svizzera Italiana. IOSI, Bellinzona (Svizzera), 27.02.2015. "Significance and clinical relevance of non-small cell lung cancers carrying two concomitant molecular hits" (**Epistolio S,** Shanane N, Martin V, Molinari F, Zappa F, Schiavone G, Wannesson L, Sessa F, Tibiletti MG, Mazzucchelli L, Frattini M). **Poster.**

Congresso: 13th International Conference on Malignant Lymphoma. Palazzo dei congressi, Lugano (Svizzera), 17-20/06/2015. "Impact on survival of MYC genetic alterations but not MYD88^{L265P} mutation in primary testicular DLBCL" (A.Valera, U.Vitolo, **S.Epistolio**, A.Riva, M.Ponzoni, P.Rafaniello Raviele, D.Novero, A.Chiapella, F.Molinari, V.Martin, A.Tucci, S.Storti, V.Pavone, H.Gomez, M.Balzarotti, G.Martinelli, M.Martelli, F.Bertoni, L.Mazzucchelli, E.Zucca). **Poster senza partecipazione al congresso**.

Simposio: "Il trattamento del melanoma". Ospedale Italiano, Lugano, Svizzera, 03.02.2015.

- "Aspetti biomolecolari ed immunologici del melanoma". Dr. Milo Frattini, ICP, Locarno (Svizzera).
- "Terapia del melanoma avanzato: dati clinici" Dott. Paolo Ascierto, Istituto nazionale tumori IRCCS-Fondazione Pascale (Italia).
- "Casi clinici" Dott. Antonello Calderoni, Dott.ssa Cristina Mangas, Dott. Vito Spataro, Dott. Alexandre Christinat.

Simposio: "PAP-test, HPV e vaccino: cosa sta cambiando". Hotel Parco Paradiso, Lugano, Svizzera, 10.09.2015.

- "PAP-test: nessuna evoluzione senza qualità" Prof. Luca Mazzucchelli, ICP, Locarno (Svizzera).
- "Lesioni squamose intraepiteliali: possiamo predirne l'evoluzione?" Dott. Franco Filciniti, ICP, Locarno (Svizzera).
- "HPV-test: quale test e quando" Dott.ssa Francesca Molinari, ICP, Locarno (Svizzera).
- "Lesioni ghiandolari della cervice uterina: problematiche" Dott.ssa Jessica Barizzi, ICP, Locarno (Svizzera).

- "Fine del PAP test dopo il vaccino?" Prof. Luca Mazzucchelli, ICP, Locarno (Svizzera).
- "Current aspects and future trends in the prevention of cervical cancer" Dott.ssa Amanda Hebert, Guy's & St Thomas' NHS Foundation Trust, London (United Kingdom).

Seminario: "Non Hodgkin limphoma genes, target, therapy and mechanism of tumor resistance". IRB, Bellinzona (Svizzera) 22.01.2015.

Seminario: "Il trattamento delle metastasi extraepatiche mediante radiologia interventistica non convenzionale". Dr. Med. Pierre Bize, CHUV Losanna. IOSI, Bellinzona (Svizzera) 04.02.2015.

Seminario: "Personalized treatment in breast cancer: still far away?". Prof. Fabrice André, Institut Gustave Roussy, Villejuif. IOSI, Bellinzona (Svizzera) 11.03.2015.

Seminario: "Pharmacokinetic and Pharmacodynamic modelling: towards individualized dosing of anticancer drugs" Dr. Markus Jörger, Kantonsspital, St Gallen. IOSI, Bellinzona (Svizzera) 13.05.2015.

Seminario: "Nodulo polmonare-cancro polmonare-versamento pleurico: il ruolo della chirurgia toracica" Dr. R Inderbitzi, Clinica St.Anna, Lucerna. IOSI, Bellinzona (Svizzera) 01.07.2015.

Seminario: "Simulatori melanocitari di melanoma" Dr.ssa Sandra Leoni Parvex, ICP, Locarno (Svizzera) 08.09.2015.

Seminario: "Seminar in ICP", ICP, Locarno (Svizzera) 24.09.2015.

- "EGFR mutation status in cell-free DNA supernatant of bronchiolar washing and brushing" Dott. Akihiko Kawahara, Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Fukuoka (Japan).
- "Heterogeneity of anaplastic lymphoma kinase gene rearrangement in non-small-cell lung carcinomas: a comparative study between small biopsy and excision samples" Dott. Hideyuki ABE, Department of Diagnostic Pathology, Kurume University Hospital, Kurume, Fukuoka (Japan).
- "Utility of LBC in endometrial cytopathology" Dott. Yoshiaki Norimatsu, Faculty of Health Science, Department of Medical Technology, Ehme (Japan).
- "Assessment of endometrial pathology: diagnostic reproducibility in LBC samples" Dott. Joshinobu Maeda, Department of Pathology, Toyama Red Cross Hospital, Toyama (Japan).

Pubblicazioni

"Impact of MYC genetic heterogeneity on protein expression in diffuse large B-cell lymphoma with 8q24 rearrangement." Alexandra Valera, Davide Soldini, Lluis Colombo, Alice Riva, **Samantha Epistolio**, Olga Balagué, Ivan Dlouhy, Eugenia Haralambieva, Elias Campo, Luca Mazzucchelli, Vittoria Martin. **Article submitted to Modern Pathology.**