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Sputum Cytometry as diagnostic tool for early lung cancer detection

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1. ABSTRACT

Lung cancer is the leading cause of cancer related deaths worldwide. Several studies have evaluated the relationship between chronic bronchitis and lung cancer. Chronic obstructive pulmonary disease refers to chronic bronchitis and emphysema, a pair of two commonly co-existing diseases of the lungs. The leading cause of both lung cancer and COPD is well recognized in tobacco use. The aim of our study is to assess the capability of Image Cytometry to identify preneoplastic lesions that occur in smokers using 5cER as diagnostic parameter that could help clinicians in lung cancer's early detection. In our study, the sputum of 80 smokers, aged 50-80 years, was collected. Out of the total, 79% had a diagnosis with typical diagnostics procedure (both COPD of various grade and lung cancer), 21% had no definitive diagnosis. In the first case, 5cER value confirmed both cancer and no-cancer diagnosis with sensitivity and specificity of 73% and 86%. In the second case, the identification of lung cancer or no lung cancer patients had a sensitivity and specificity of 72% and 67%. Moreover, our aim is to identify predictive markers and to understand if they have a correlation with ploidy status. Preliminary data show that same genes have positive correlation ($r > 0,5$) and same negative correlation ($r < - 0,5$). The future perspective of our study is to increase the number of samples to improve our data, to enlarge molecular studies using new technologies as microarray or RNA-Sequencing.

2. SMOCKED-RELATED LUNG DISEASE

Lung disease is the result of a series of mutational events that have been studied from many investigators. These kinds of events have a common start point: an inflammatory process that begin when agents (e.g. tobacco, asbestos fiber, radon gas, air pollution and second-hand smoke) interact with cells of an organ and modify its physiological network.

It has long been known that tobacco smoking plays a central role in pathogenesis of chronic obstructive pulmonary disease (COPD) and lung cancer.

Tobacco smoking is one of the most severe and widespread health problems worldwide. The tobacco-induced pulmonary cellular network presents a unique environment in which carcinogenesis proceeds along with the surrounding lung inflammatory, structural and stromal cells **(1)**.

Smoking-induced epithelial abnormalities can serve both as targets for abnormal inflammatory responses as well as initiators of deregulated inflammation. Cytokines, chemokines and growth factor released from alveolar macrophages, lymphocytes, endothelial cells and fibroblast may act to promote epithelial dysfunction and malignant progression **(1)**.

The principal factor that acts as negative signal for bronchial epithelium is oxidant's product generated from the smoke produced by cigarette.

Reactive oxygen species (ROS) and reactive nitrogen species are mediators of cell signaling in pulmonary cells and their concentrations are increased by a variety of agents, in particular by cigarette smoke. When ROS interact with the cell, they stimulate several pathways, but in particular MAPK cascade **(2)**.

A mechanicistic model shows that oxidants are the primary incentives of all MAPK pathway's and nuclear transcription factor's (such as AP-1) activation **(2)**. This activation modifies the physiological gene expression in lung cells, and could trigger several processes like cell death, tumor development and differentiation. **(2)**.

However, chronic exposure to tobacco causes an increased production of proteolitic enzymes by the neutrophils and the production of a class of enzymes, named metalloproteinases (MMP), by the macrophages **(3)**. Bronchial epithelium plays an

important role in the respiratory tract as a structural barrier and a defense against foreign agents. Cigarette smoking modifies the physiological aspect of bronchial epithelium. Smokers' bronchial epithelium presents an elevated concentration of pro-inflammatory cytokines, peptides, amines and adhesion molecules **(3)**. Moreover, the modified integrity of the epithelium facilitates the aggression by microbes. Alveolar macrophages, an important class of macrophages present in lung environment, are recruited from cigarette smoke and an influx into airways lumen has been observed. Macrophages are responsible of MMP production, a proteolitic enzyme that may be responsible of lung tissue's destruction. In smokers, an altered balance MMP/TIMP has been observed: macrophages of smokers release less TIMP1, an inhibitor of MMP, which is important for saving lung tissue structure**.** Cigarette smoke is able to induce apoptosis in bronchial cells and in alveolar wall and the augmentation of apoptotic material cause impaired phagocytosis and could change the apoptosis process in necrosis, causing persistent inflammation in smokers **(3).** Oxidative stress and cigarette smoke stimulate neutrophils to release high quantity of enzymes and mucin**.** The presence of high concentration of eosinophils was documented, and their efflux into airways appears to be a rapid reaction that could be normalized after smoking cessation **(3).**

A large network of pulmonary and systemic cytokines is involved in chronic inflammation of smokers. IL-6, IL-8, IL-1β and TNFα are active causes in smokers of the inflammation of the respiratory tract and the circulatory system (**3**)**.**

Chronic Obstructive Pulmonary Disease

Cigarette smoking is the predominant cause of chronic obstructive pulmonary disease (COPD), with an estimated attributable lung cancer risk factor exceeding 80% in smoking affected individuals **(4).** COPD represents a biomarker of cumulative exposure dose level and tissue susceptibility. Chronic cigarette smoking retards mucociliary clearance of foreign particulates and respiratory tract secretions, evokes an inflammatory response accompanied by fibrosis in the membranous and respiratory bronchioles, and cause mucus gland hypertrophy, hyperplasia and dysplasia in the proximal airways **(4).** Several recent studies have shown that comorbidities in patients with COPD have a larger impact on morbidity and mortality than COPD itself. Based

on this data it has been proposed that COPD and all its comorbidities should be grouped and addressed as "chronic systemic inflammatory syndrome" **(5)**.

The global prevalence of COPD in adults aged over 40 is estimated to be 9-10% **(6)**.

COPD refers to chronic bronchitis and emphysema, a pair of two commonly co-existing diseases of the lungs characterized by the narrowing of the airways, which leads to a limitation of the airflow to and from the lungs causing shortness of breath. In contrast to asthma, the limitation of airflow is poorly reversible and usually gets progressively worse in the long run. COPD is diagnosed on the base of fixed airflow obstruction that is not due to bronchiectiasis, cystic fibrosis or tubercolosis.

Spirometry measures the forced expiratory volume in one second (FEV_1), which is the greatest volume of air that can be breathed out in the first second of a large breath. It also measures the forced vital capacity (FVC), which is the greatest volume of air that can be breathed out in a whole large breath. Normally, at least 70% of the FVC comes out in the first second (i.e. the FEV_1/FVC ratio is >70%). In COPD, this ratio is less than normal, (i.e. FEV_1/FVC ratio is <70%) even after a bronchodilator medication has been administered. Spirometry can help to determine the severity of COPD.

Airflow limitation is caused by the combination of parenchymal destruction and airway obstruction. Pathologic studies have disclosed the pathogenesis of COPD by showing that small airways, of 2 mm internal diameter or less, are the major site of airway obstruction due to the effects caused by the remodelling and thickening of the airway walls and the luminal occlusion induced by mucus and inflammatory exudates.

Genetic studies demonstrate that, in addition to tobacco, also genetic factors contribute to COPD **(7)**. In particular, one of the most important genetic risk factor is severe deficiency of α1-antitrypsin. Moreover, recent study demonstrated the presence of new genetic alterations and in particular a very significant study demonstrated the relationship between COPD and SNPs at the CHRNA3/5 locus (a-nicotinic receptor locus), independently of smoking behaviors. **(8)**.

The histopathology of COPD includes various alterations. Central and peripheral airways and lung parenchyma show the majority of the abnormalities such us mucous gland hypertrophy, inflammation, increased wall thickness, loss of alveolar attachments, emphysema, infiltration of CD8+ lymphocytes, which are the same clinical alterations present in patients with COPD **(7).** They are, however, different in each patient, in fact

in same patients predominates parenchymal emphysema, while in others the increased wall thickness.

Inflammatory changes in the peripheral airways have been identified as the initial steps in COPD. Inflammatory cells may contribute to the airway remodelling via the secretion of proteases, fibrotic or mitogenic growth factor and cytokines **(9).**

Cytokine network plays a key role in orchestrating the inflammation of COPD. Differences in cytokine patterns are involved in the cell recruitment and in the regulation of T cells account for the different patterns of inflammation. Cytokines, such as TNFα and IL6, may play a role in amplifying inflammation and thus determining disease severity. Moreover, growth factors are responsible for the persistence of inflammatory cells and for the changes that occur in patients with COPD **(10)**.

Inhaled agents activated epithelial cells and macrophages to release multiple cytokines, which stimulate fibroblast proliferation, resulting in fibrosis in the small airways. These cells also produce proinflammatory cytokines, which amplify the inflammation, and chemokines, which attract circulating cells into lung environment. Growth factors, such as EGF, TGF-β and FGF, stimulate fibroblast growth, resulting in fibrosis of small airways, and mucus hypersecretion (**10**).

Activated macrophages, T-cells and mast cells produce and secrete MMPs that can damage the epithelial barrier. Recent studies have shown that levels of MMPs, especially MMP-9, are elevated in bronchial alveolar lavage fluid from patients with COPD, compared to normal controls **(9)**. However, in contrast to normal lungs, the repair process is disturbed in COPD due to a protease-antiprotease imbalance in MMPs-TIMPs, which are involved in the turnover of extracellular matrix. Inflammatory cells release various proteinases that exceed the proteinase inhibitor defense of the lung. Uncontrolled proteinases degrade the extracellular matrix protein components of the alveolar walls leading to its destruction and loss and to airspace enlargement **(11)**.

Other changes during COPD occur in the chromatin remodelling process and transcription factor's activation **(6)**. In particular, Ito et al. demonstrated for the first time a decrease in HDAC2 expression and activity in lung macrophages, biopsy and blood cells in patients with COPD. HDACs also play an important role in the regulation of transcription activity of the components of NF-kB. The activation of this factor occurs after the oxidants presence in environment as well as after the inhibition of

HDACs activity. The results of its activity are the production of several inflammatory factors.

Lung Cancer

As previously mentioned, lung cancer is the leading cause of cancer-related deaths over the world, among men and women characterized by a very high mortality rate **(12)**.

Lung tumors can be divided into two histological groups: non-small cell lung cancer (NSCLC) (80.4%) and small cell lung cancer (SCLC) (16.8%) **(13)**. NSCLC, consisting mainly of adenocarcinoma, squamous cell and large cell carcinoma, accounts for almost 80% of lung cancer cases, whereas SCLC is slightly more common and all known cases are due to cigarette smoking.

Abnormal chest imaging and/or non-specific symptoms are the initial signal of lung problems. Bronchoscopy (cytopathologic examination from suspect area) is generally used as an initial diagnostic tool, even if its sensitivity is low **(14)**.

Different factors contribute to lung cancer formation: tobacco smoke, ionizing radiation and viral infections are the most common causes. However, the mechanisms involved in lung carcinogenesis remain to date mainly unknown **(15)**.

DNA content is the most significant cellular parameter able to monitor the cell proliferation and the neoplastic transformation. The prognostic value of DNA ploidy in lung cancer is controversial. For example, in RIDTELC Lung Study, the presence of cells with abnormal DNA content was used as a predictive parameter for lung cancer. In the study, it was demonstrated that alterations of normal DNA content of lung cells is an expressive mark that occurs in early stage of cancer development **(16).**

As in most other cancers, several genes are involved in lung cancer disease, which is initiated by the activation of oncogenes or inactivation of tumor suppressor genes **(17)**.

The epidermal growth factor receptor (EGFR) expression, which regulates cell proliferation, apoptosis, angiogenesis and tumor invasion **(18)** is altered in NSCLC.

The proto-oncogene KRAS is often mutated and is responsible for 10–30% of lung adenocarcinomas **(18, 19)**. Cyclin D1 are amplified and over-expressed in 2.5–10% and 5% of NSCLC, respectively **(20)**. ERBB2 (also known as HER-2/neu) or BCL2 over-expression are involved in 25% of cases **(21)**.

In addition, the inactivation of tumor suppressor genes plays a key role in lung carcinogenesis, as for example the tumor suppressor gene *TP53* that is mutated in 60–75% of lung cancers, including both NSCLC and SCLC **(22)**.

Most studies have shown the overexpression of SRC, a tyrosine kinase protein, in NSCLS **(23, 24)**. SRC is also overexpressed and activated in epithelial tumors, and the levels of expression or activation generally correlate with disease progression, although activating mutations are rare **(25, 26)**.

The role of RB family genes in lung cancer malignancy has been long examined but remains unclear to date. Rb/p105 is mutated in about 90% of human SCLC. When RB1 is not mutated, alterations in members of the RB pathway have been found in human tumors, for example $p16^{INK4a}$, an upstream activator of the RB proteins (Rb/p105, p107 and p130), is frequently inactivated in lung adenocarcinomas, lung carcinoma type where Rb is not mutated (**27**).

Genetic polymorphisms are also indicated to be involved in lung carcinogenesis, e.g. interleukin-1 **(28)**, cytochrome P450 **(29)**, apoptosis promoters such as caspase-8 **(30)** and DNA repair protein **(31)**.

Nowadays, epigenetic modifications are well recognized in lung cancer tumorigenesis. A great number of aberrantly methylated genes have been identified in lung cancer. A well-studied example is the aberrant promoter methylation of the tumor suppressor gene p16 which leads to gene silencing, an early event in tumorigenesis **(32, 33)**. In many types of cancer, the fragile histidine triade gene is targeted by genetic alteration. This gene has been described as tumor suppressor. In lung tissue, promoter methylation or loss of heterozygosity at FHIT locus produce inactivation of gene functions **(34)**.

COPD and Lung Cancer

COPD and lung cancer represent two important challenges for basic scientists, applied scientists and clinicians **(35)**.

There is a lot of evidence in literature that assess the relationship between COPD and lung cancer.

High ROS concentration of oxidants and ROS contained in cigarette smoke activate inflammation pathway in lung epithelial cells. A physiological response from antioxidant and metabolic enzymes usually inactivate many potential toxic species, but

during those processes an incremented of ROS concentration has been detected. Various genes related with this inflammation state, such as NF-kB, contribute to transactivate inflammation-related genes that play a central role in COPD and lung cancer. This will result in genetic activation or silencing of gene that contribute to activate the process of apoptosis, matrix degradation, incomplete tissue repair (in COPD), excessive DNA damage and incomplete DNA repair (in lung cancer) **(36).**

Association between COPD and lung cancer were studied both in never smokers and smokers.

A prospective study of lifelong nonsmokers provides further evidence on the association between COPD and risk of lung cancer. This study assess also that there is a significant association between the sex of the individual and the occurrence of the disease. In fact, men with disease tend to experience an elevated but not significantly higher risk of lung cancer occurrence than women **(37)**.

The relationship between sex, COPD and lung cancer has been extensively studied, and the fact that women with COPD have higher risk to develop lung cancer is clear **(38).** It is related to hormonal and enzymatic outcomes. There are evidences that assess a hypothetical model on the reasons that make female, and in particular female smokers, more vulnerable to COPD and lung cancer. Cigarette smoke up-regulates a number of CYP enzymes in lungs, which metabolize component of cigarette smoke into chemical intermediate. They generate ROS that could activate the inflammation state and cell damage in lung, binding to DNA local cells with the result of DNA adducts and impair cellular replication and DNA repair. Women are particularly vulnerable to these processes for three reasons: estrogen can up-regulate expression and activity of CYP enzymes in lungs, they have smaller airways and increased bronchial responsiveness may cause an increase of the particle deposition in lung, DNA repair capacity is decreased, and p53 mutation in women has higher frequency. **(39)**.

In particular, the NSCLC lung cancer is related with COPD. Several studies confirmed the relationship between this subtype of lung cancer and COPD **(40)**. Researchers assess that the presence of COPD indicates an increased risk of more the fourfold to have squamous cell carcinoma **(40)**.

The progression from COPD to lung cancer is facilitated by impaired clearance of carcinogenic substances from chronic airflow limitation.

The association between lung cancer and COPD is not only caused by tobacco **(41)**. In literature, lot of evidence shows a high risk of lung cancer related to COPD in never-smokers. Family history of chronic bronchitis and emphysema are associates with lung cancer. One potential mechanism is lung infection leading to inflammation, COPD exacerbation and accelerated lung function decline **(41)**.

3. TOOLS FOR SCREENING IN LUNG CANCER DIAGNOSIS

Abnormal chest imaging and/or non-specific symptoms are the initial signal of lung problems. This kind of signals, unfortunately, appears when the disease is at an advanced stage. This fact represents the cause of low 5-year survival rate: over 90% of patients dying within five years of diagnosis **(42).**

For this reason, researchers focused their works in the development of new diagnostic assays that could help clinicians in early detection of this kind of cancer.

Of more than 215,000 patients with newly diagnosed lung cancer predicted in 2008, only approximately 15% expected to survive for 5 years **(43).** Screening has proven to have a crucial role in reducing the mortality rate for malignancies. In breast and colon cancer, screening was able to help the clinicians to improve the 5-year survival rate. In lung cancer, instead, screening has not the same results because the disease is characterized by difficult identification in early stage.

For lung cancer, in fact, large screening trials in the 1970s and 1980s, using chest x-rays and sputum cytology failed to establish a significant decrease in patient mortality **(44-46)**.

A renewed optimism about a 10-years survival rate arose with x-ray computed tomography (CT) in radiologic routine. Thanks to this, a 10-years survival rate of 92% was reported in a trial of 31,000 high-risk patients **(47)**. CT has led to major advancements in cross-sectional imaging due to advanced scan speed, improved spatial resolution, and the possibility to reconstruct multiple series from single data acquisition **(48)**. In the 2000s, several studies focused on the CT technique **(Table 1).**

Sputum Analysis

Chest X-Ray, low-dose computed tomography, and fluorescence bronchoscopy represent good tools in lung cancer diagnosis, but are also considered expensive and invasive technique. Also, bronchial brushing specimens are collected via invasive procedure (bronchoscopy).

Since cigarette smokers usually have higher quantities of sputum containing exfoliated cells from the bronchial tree, and since sputum represents the most easily accessible biological fluid characterized by a non-invasive collection, its analysis represents a promising area of research in early lung cancer diagnosis.

With sputum sample is possible to prepare slides for imaging test, to isolate cells form mucus and to analyze their molecular characteristics. In a sputum sample, it is reasonably possible to find cells with the same characteristic of the lung environment. For this reason, in sputum sample, it can be easily found, for example, dysplastic cell in substantially greater numbers than cancer cells **(49)**. This is positive especially for small tumors, or for tumors in early stage.

The main use of the sputum sample is the "slide use". Several studies use conventional cytology and image cytometry in addiction to other techniques (e.g. bronchoscopy or CT), to confirm the diagnosis morphologically. However, several researchers have been focused their studies on molecular characterization of sputum **(Table 2)**.

Exfoliative cytology may be used to identify early stage of cancer and to prevent cancer mortality. The first use of this kind of analysis is the screening for cervical cancer by Papanicolau. The Papanicolau idea was adapted to lung cancer by Saccomanno for microscopic examination of sputum **(50)**.

There are many clinical trials that use "slide sputum analysis" in early lung cancer diagnosis.

Two randomized studies initiated in the 1970s compared sputum cytology and X-ray: Johns Hopkins Lung Project and Memorial Sloan-Kettering Lung Study. Even if they did not pursue strong results with the dual screening, they found however a modest positive results. Both studies showed a modest benefit among the heaviest smokers and a moderate reduction in deaths due to squamous cell and large cell lung cancer. They illustrated that in dual-screen over half of the squamous cancers were detected **(51)**.

Another trial, the Mayo Lung Project, provides strong evidence on the positive combination of sputum cytology and chest x-ray **(52)**. In this study, at the end of a 6-years screening, researchers discovered 40 additional lung cancer cases in comparison to the control study. Data were also confirmed in an extended follow-up of the study **(52, 53)**.

Microscopy type of cytometry, Image Cytometry, could improve cytology results studying the ploidy status of patients.

Alterations of DNA content represent an early modification causing normal cells to become cancerous **(54)**. Feulgen reaction onto slide permits to analyze DNA content of every cell from the sputum sample. Normal epithelial cells in sputum consist mostly of diploid cells with a DNA Index of 1.0. Detection of DNA aneuploidy has diagnostic and prognostic significance in various cancers, including lung **(55, 56)**.

A multicenter validation trial was conducted collecting sputum samples from patients with high risks of lung cancer. Smears from sputum were place onto slide; DNA was stained with Feulgen reaction, and analyzed with automated cytometry systems. Pre-clinical results show that DNA cytometry using LungSign test detects stage I lung cancer: the test could achieve nearly 50% sensitivity at 90% specificity **(57, 58)**.

Using comparable technique, Li et al. found sensitivity and specificity of 75% and 50% **(59).**

In RIDTELC Lung Study, malignancy grade and 5cER was used as a predictive parameter for lung cancer. In total, 2,480 heavy smokers (0.30 pack-years), aged 50–74, with no previous cancer in the last 5 years, received chest radiology, conventional sputum cytology and sputum cytometry screening **(16)**. The same group conducted a screening for lung cancer in random exposed uranium miners, the correlation between data from automated sputum cytometry and conventional cytology was examined. As a result, 120 specimens were classified by cytometry as suspicious and 18 were classified as highly suspicious. In the highly suspicious group, only 9 samples were classified as tumor cells positive, in the suspicious group only 73. Of the 1,358 classified as benign by cytometry, only 45 samples were classified by cytology as dysplasia. In final diagnosis, 23 tumors were detected. The sensitivity of cytometry was 87% at a specificity of 92%, while cytology's sensitivity was 83% and specificity 97% **(60)**.

Another study, conducted in Czech Republic, analyzed about 6,000 patients with high risk of lung cancer development using chest x-ray and sputum cytology. In this study, the authors showed a significant increase in the number of cancer detection, including more instances of early stage of disease **(61, 62).**

The "slide sputum analysis" based on an optimizing and standardizing protocol for specimen collection and preparation would likely improve the performance analysis.

For example, Boecking et al. reported an increased sensitivity from 68% to 85% based on the numbers of specimens analyzed per patient **(63)**.

Molecular studies in lung cancer, as well as in all kinds of cancer, using gene analysis approaches, showed that this method is ready to be used in clinical studies. The information that researchers obtain by molecular studies could be important in new methods of diagnosis, prevention and treatment of the disease. This analysis should focus on the clarification of malignant characteristics of the tumor **(64)**.

Lung cancer is genetically heterogeneous and develops from multistep process. This process is characterized by various molecular alterations. So, the opportunity to create a panel of possible molecular biomarkers represents the goal of various studies. In this study, researcher use different technique to understand and describe what kind of alterations usually develop in lung cancer.

There is a lot of evidence that SCLC and NSCLC have acquired different genetic and epigenetic lesions. Alteration in tumor suppressor genes, for example, appears especially in early stage of disease **(65)**.

Molecular studies, based on genomic and proteomic assay have approximately 80% sensitivity and specificity. There are several examples of successful identification of predictive assays for benefit from chemotherapy (ERCC1, RRM1, p27Kip1, and p53 expression) or targeted therapies (epidermal growth factor receptor [EGFR], gene copy number, EGFR activating mutations, EGFR protein expression, and serum proteomic profile). These markers should be prospectively tested in clinical studies before they can be routinely used in the clinic **(66).**

To improve the diagnostic value of sputum cytology/cytometry, researchers focused on the analysis of genetic abnormalities using microscopic technique, such as FISH.

By doing that, scientists could analyze at the same time both the morphological abnormalities and the genetic alteration. It is unlikely that early-stage lung cancer will exfoliate detectable cancer cells in sputum, it is hypothesized that genetic and epigenetic changes in exfoliated cells may reflect increased cancer risk **(67)**.

Combining molecular analysis with conventional cytology, the sensitivity and the specificity in the detection of lung cancer increase respectively to 60 % and 90 %.

By adding FISH to conventional cytology, Li et al. improved the sensitivity to 76% and the specificity to 92% in early identification of lung cancer. Using FISH, they assess the

diagnostic role of same gene deletion. They showed that deletion of both HYAL2 and FHIT might represent a good marker to improve the diagnosis of lung cancer at an early stage **(68)**. They tested this probe in combination with sputum cytology, and they showed that the sensitivity of the tests was higher in comparison to cytology alone.

To confirm this data, Jiang et al. developed a mini-chip assay in which they tested sputa for four genetic probes (HYAL2, FHIT, p16 and SP-A) and three centromeric probes, and tested this assay with CT. The association of both techniques led to improvements in diagnosis of stage I Non Small Cell Lung Cancer, especially central kinds of tumors **(69)**.

Similarly, Varella-Garcia et al. **(70)** identified a panel of 4 DNA targets that improves sensitivity in lung cancer detection 18 month before diagnosis, using EGF, MYC, CEP6 and 5p15, they had a sensitivity of 76%.

Katz et al., used FISH for genetic alteration in order to evaluate whether the accuracy of diagnosing lung cancer by evaluating sputa for cytological atypia and genetic abnormalities was higher than conventional cytology alone. When the two techniques were combined, the model to predict lung cancer had 74% sensitivity and 82% specificity; using only cytology, the model had 37% sensitivity and 87% specificity. So, the specificity was the same, while the sensitivity increased. Consequently, for diagnostic model, both techniques are used to improve the results of the diagnosis **(71)**.

Gene and proteins expressions are not only studied with slide methods. In particular, aberrant gene promoter methylation was considered to understand the degree of influence that this alteration could have in cancer progression. There is a lot of evidence that supports aberrant methylation as marker for early lung cancer detection in sputum **(72)**.

In a study by Shivapurkar et al., using real time PCR, 11 genes (3-OST-2, RASSF1A, DcR1, DcR2, P16, DAPK, APC, ECAD, HCAD, SOCS1, and SOCS3) were tested and promoter methylation level in sputa from cancer patients and control patients were analyzed. It was found that only 3-OST-2 and RASSF1A methylation promoter gene increased in advanced tumor stage. After this quantitative analysis, it appears to be a promising biomarker to identify lung cancer in early stage, and the use in clinical studies is recommended **(73)**.

Liu et al. studied the effects caused by smoke exposure in a region of China. They studied the effects of tobacco in the methylation of p16, RASSF1A, MGMT and DAPK. It was found that promoter methylation of p16 gene occurred frequently both in patients exposed to smoke than in patients with lung cancer already diagnosed. Moreover, they also found promoter methylation in three other genes of the study **(74).**

miRNAs are stably present in the sputum, as demonstrated by Xie et al. **(75)**, and for this reason they are a main candidate biomarker.

miRNAs present in sputum of cancer patients are under or over-expressed in comparison to those present in sputum of cancer free patients **(76)**. In fact, analysis of a panel of candidates miRNAs (using Real Time PCR) indicating the presence of lung cancer, showed that their expression was highly variable because miR-21, miR-182 **(76),** miR-205, miR-210 and miR-708 **(77)** were over-expressed in cancer samples. Specifically miR-205 is considered useful for the diagnosis of lung squamous cancer and miR-210 is associated with cancer aggressiveness and over-expression of miR-708, frequently used in stage 1 non-small-cell lung cancer. On the contrary, sputum analysis detected under-expression of miR-126, miR-139, miR-429 in the lung cancer patient group compared with the cancer free patients.

DNA Image Cytometry

DNA image cytometry has gained wide recognition in pathology and cytopathology as a mean to obtain information about diagnosis and prognosis of human cancer (**78**).

It is established that chromosomal aneuploidy is an early event in tumorigenesis, caused by genetic instability. The cytometric equivalent of that is DNA aneuploidy. Clearly, DNA quantification can therefore serve as prognostic marker; moreover, changes in DNA ploidy may indicate therapeutic effects.

After quantitative staining of DNA, the nuclear IOD is the cytometric equivalent of its DNA content. The quantity of nuclear DNA may be influenced by the following mechanism: replication, polyploidization, gain or deletion. Furthermore, the DNA content of a cell is regularly changed throughout the cell cycle. All these effects have to be taken into consideration when a diagnostic interpretation of DNA histograms is performed (**79**).

In normal cells, the changes of chromatin appearance in the nucleus may reflect changes in the activation patterns of the genes. These changes may either be related to cell cycle or to metabolic state or differentiation status of the cell. In cancer cells changes of chromatin are common alongside with the progression of the disease (**78**).

Image cytometric (IC) DNA analysis is a comparable technique to Flow Cytometry for the detection of DNA ploidy abnormalities; IC is advantageous as the set-up cost is low, only a small number of nuclei are required, and it is more sensitive for the analysis of tetraploid cell populations (**80**).

The basic aim of diagnostic DNA cytometry is to identify DNA stem-lines outside the normal (euploid) regions as abnormal (or aneuploid) at defined statistic level of significance. So, DNA image cytometry should give information about the presence of rare cells with abnormally high DNA content, that most likely result from genomic alteration.

Since DNA IC results in nuclear IOD values in arbitrary units equivalent but not identical with nuclear DNA content, the quantification of nuclear DNA requires a rescaling of IOD values by comparing them with those obtained from cells with known DNA content, called reference cells. In general, there are two kinds of reference cell systems: external and internal. Whereas the external reference cells are very easy to be identified by the investigator, often are not easy to be prepared in parallel with clinical samples, on the other hand the internal reference cells have the advantage of sharing all preparatory steps with the analysis cells collected in the clinical specimens (**79**). With Image Cytometry cells are stained specifically for DNA and digital images of microscope fields are acquired, typically with a CCD camera. For stoichiometric DNA stains, where stain uptake is proportional to the DNA amount, at any point of image the DNA amount is proportional to the optical density (OD). The total amount of the DNA in the nucleus is the sum of optical density values of all pixels over the nucleus (Integrated Optical Density – IOD) (**78**).

In image analysis densitometry, the microscope field is captured by a microscope-mounted CCD or digital camera connected to a computer via a "frame-grabber" board. As with all digital images, these photos are displayed as a series of pixels, each with specific color and intensity. A color image of stained nuclei can be made into a single linear scale of pixel intensity by converting the image to gray scale or by analyzing only

one of three constituent "channels" that make up the color pixel. In each case, pixel intensity is distributed on scale from 0 to 255. A measurement of a slides section lacking nuclei provides measure of incidence light. Integrated Optical Density is calculated from pixel values along the 256-value scale.

Feulgen reaction represents the standard method in DNA content analysis used in Image Cytometry. The most commonly stain used in the Feulgen Reaction is the Schiff's reagent. This reaction uses strong acid to generate free aldehyde groups engendered in the deoxyribose residues of the DNA molecules (splitting off the purine bases A and G resulting in a derivative called apurinic acid), to which a fucsin molecule decolorized with SO_2 can bind and regain its pink color (**81**).

In the acquisition step, a field containing nuclei must be located and brought into focus. Once the focus is optimized, an area of the slide that is free of nuclei should be selected. This area can be used to adjust the brightness and color balance of the microscope and camera. After selecting the exposure, the camera should be "white balanced" on the white area of the slide. Once completed, a field containing nuclei for measurement can be relocated and acquired. The green channel should be used for IOD measurements because it includes the absorption peak for the Feulgen-DNA dye complex, and therefore gives the highest IODs and the most accurate estimate of the genome size. Once the image is obtained, it is necessary to set a threshold of pixel's value which is to be included in the measurements. The threshold is used only for outlining the objects to be measured. All objects within the density of the threshold should be highlighted. Any non-nuclear objects should be omitted, as should misshapen, broken, overlapping, or otherwise anomalous nuclei.

As with all densitometric and fluorometric methods, image analysis-based techniques involve the conversion of units less IOD values to absolute genome size, by the comparison of ratios with standard of previously estimated DNA content (**81**).

Histograms were analyzed according to the European Society for Analytical Cellular Pathology guidelines as follows:

- a specimen was defined as diploid when there was only one peak (which was 2c, or DI>0.9–1.1) during the G0 or G1 phase, when during the peak of the G2 phase the number of 4c nuclei did not exceed 6% of the total, or when the number of nuclei with a DNA content of 4.5c did not exceed 1% of the total.

- a specimen was defined as DNA tetraploid when there was a population of 4c nuclei (DI>1.9–2.1) representing 46% of the total, in stage G2 of the cell cycle. The term 'DNA tetraploid' generally means a DNA content indistinguishable from the one belonging to tetraploid cells, with a percentage of these cells disproportionately higher than the ones in the S phase fraction.
- a specimen was defined as aneuploid when there was a population of nuclei with abnormal DNA content, separated from the diploid peak (DI 1.1), and representing 2.5% of the total or when the number of nuclei with a DNA content of 5c or 9c exceeded 1% of the total. Aneuploid cases were further divided into near-diploid aneuploid (1.1–1.29) and aneuploid (1.30–1.89) (**82**).

Moreover, two parameters were calculated from DNA-values of the nuclei: the rate of 5c-exceeding nuclei (5cER) and 2c-deviation index (2cDI). The 5cER is the rate (in %) of aneuploid nuclei with a DNA-amount > 5c. These are different from normal separating mitotic nuclei. Nuclei are called euploid if their DNA-amount is in the range of 2c ± 0.25c. 2cDI is defined as the sum of all squared deviations of DNA-amount of all epithelial-cells (ci) from the mean value (2c) divided by the number of cells (**83**).

Table 1. Clinical trial on early lung cancer detection

STUDY	Assay	YEAR
NLST, USA	CT and CXR	2003
LSS, USA	CT and CXR	2004
DEPISCAN, France	CT and CXR	2007
LUISI, Germany	CT	2008
DANTE, Italy	CT	2008
ITALUNG, Italy	CT	2009
DLCST, Denmark	CT	2009
NELSON, Netherland and Belgiun	CT	2009

CT, computed tomography; CXR, chest-X ray

Table 2. Example of "cyt"-sputum trials in which microscopy assays is within diagnostic assay

STUDY	State	Test
Johns Hopkins Lung Project	USA	Cyt and CXR
Memorial Sloan-Kettering Lung Study	USA	Cyt and CXR
Mayo Clinic	USA	Cyt and CXR/CT
RIDTELC STUDY	Germany	CXR and Cyt and IC
Czech Study	Czech Republic	Cyt and CXR
LungSign (multicenter trial)	USA	CXR and IC

Cyt, sputum cytology; CXR, chest-X ray; CT, computed tomography; IC, sputum cytometry

4. MATERIALS AND METHODS

Patients and sputum collection

A mixed group of 83 patients were selected at the Institute of Respiratory Disease of University of Sassari: patients with lung cancer, COPD of various status (I – IV), and a control group of healthy patients. From every patient, personal and anamnestic data (generic pathology, lung pathology, FEV1 value, professional exposure to lung-disease agents, hereditary) were collected (**Table 3**).

The subjects were instructed to swallow saliva and to expectorate sputum only from the deeper airways, into a 50 ml vial filled with 25 ml Saccomanno-preservative and 0,1% of the reducing agent dithiothreitol (DTT). DTT cleavages the disulfide bounds and effectively dissolves sputum specimens. Spontaneous sputum was collected for three day in the same vial. The group of patients enrolled in this study was composed as follows: healthy patients (6), patients with asthma or other lung disease (9), patients with COPD I (15), COPD II (11), COPD III (8) and COPD IV (9), and patients with lung cancer at various stages (25). Moreover, within these patients, a group of 14 did not have an initial diagnosis, but were classified as Lung Opacity patients. Afterward, after a subsequent diagnostic study, the majority of these patients were diagnosed with cancer. The samples were labeled and brought to the laboratory.

Sputum processing

Sputum sample arriving at the laboratory in Saccomanno-DTT solution were completely liquefied and could be treated like a blood smear for a monolayer preparation. After centrifugation for 15 min at 500 g, supernatant was decanted. Cell pellet was resuspended in 1 ml Saccomanno Solution (Diapath, Italy) and two drops of the cell suspension were applied to 2 slides, smeared like a blood sample, avoiding distortion by using capillary forces only. The slides were air-dried over night. After a second centrifugation for 15 min at 500 g, supernatant was decanted and cell pellet was stored at -80°C for molecular use. As in table 1, not all sample were processed because there was too much saliva in the sputa, or because there was a small amount of cells in the sputa.

Staining

Slides for cytometry were stained according to a modified Feulgen reaction (**84**), using Feulgen Kit (Diapath, Italy). With this reaction, the purine bases were removed by hydrolysis in 5N HCl at room temperature. Slides were than stained in thionin solution and rinsed in sodium-bisulfite solutions. After dehydration in increasing alcohol concentration and xylene, the specimens were cover-slipped using Pertex (Bio Optica, Italy) for permanent mounting.

DNA Image Cytometry

The nuclei of Feulgen-stained cells were evaluated for DNA ploidy using a Olympus BX51 Microscope (Olympus Corporation, Japan) connected with a CCD camera (Optronics, USA) and an IBM Pentium 4/PC.

A minimum of 250 nuclei was measured for each slide, and at least 30 internal (or external) control cells were analyzed. The lymphocytes were used as control cells. The use of internal control cells can sometimes be more laborious than the use of external standard cells, but the ploidy value obtained is more accurate. DNA content was assessed by measuring the amount of a specific and a stoichiometric dye bound to nuclear chromatin. The amount of stain was determined by measuring the Integrated Optical Density (IOD) of each informative nucleus, which is the logarithm of the incident light intensity to transmitted light intensity ratio summed over the nucleus area. Measurements were made using a magnification of ×20. This analysis configuration allows operator-dependent selection and measurement of DNA content. Images were analyzed with ImageJ Software (NIH, USA) and 5cER value was elaborated for each patients. CV value of diploid control cells was < 5%. After the analysis of healthy and tumor samples, a 5cER value (percent of nuclei with DNA-Index of more than 5 fold of normal haploid DNA-Amount) was set borderline to 5%. The samples were classified as follows: not relevant (not significant nuclei), benign (5cER < 2.5), suspicious (2.5< 5cER < 5) and malignant (5cER > 5).

Total RNA extraction, cDNA synthesis and Real Time PCR

Total RNA was extracted from sputum sample using TRIZOL® Reagent (Invitrogen) according to manufacturer's protocol. 1µg of RNA was used for cDNA production with random primers, using M-MLV Reverse Transcriptase (Invitrogen, USA) following the manufacture's protocol. 20µl of reaction was diluited in 200µl of sterile water.

Real Time was performed using 4µl of cDNA and 250nM primers diluted in *Power* SYBR® Green PCR Master Mix (Applied Biosystem, USA), to a final volume of 10µl. Accumulation of fluorescent products was monitored using a BioRad IQ 5 System (Bio-Rad Laboratories, USA).

Each data point was obtained from at least three independent experiments. Transcripts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a reference. To ensure specific PCR amplification, every real time PCR run was followed by a dissociation phase analysis (denaturation curve) and by gel electrophoresis. ΔΔCT method was used to calculate relative changes in gene expression; primer efficiency was calculated for every target using five x 10- fold serial dilutions of PCR products. Specific primers sequences are reported in **table 4**.

Statistical analysis

The statistical correlation between Image Cytometry results (5cER value) and Real Time PCR results (gene expression value) was calculated as Pearson's correlation coefficient (a measure of the strength of the linear relationship between two variables). The pair(s) of variables with positive correlation coefficients and P values below 0,05 tent to increase simultaneously. For the pairs with negative correlation coefficients and P values below 0,05, one variable tent to decrease while the other increased. For pairs with P values greater than 0,05, there was no significant relationship between the two variables. Statistical analysis was done with Sigma Stat 3.1 Software (Systat Software, Inc.)

Table 3. Patient's classification

	Total sample collected	*Processed*
Healthy	6	4
Other	9	7
COPD I	15	12
COPD II	11	8
COPD III	8	6
COPD IV	9	7
Lung Cancer	25	19

Table 4. Real Time PCR primers sequences

	FORWARD-SEQUENCE	*REVERSE-SEQUENCE*
GAPDH	GAAGGTGAAGGTCGGAGT	CATGGGTGGAATCATATTGGA
p16	GGAAGGTCCCTCAGACATC	GCAGTTGTGGCCCTGTAG
EZH2	GCGGGACGAAGAATAATCATGG	CCAAAATTTTCTGACGATTGGAAC
Cyclin D1	CAGAGGCGGAGGAGAACAA	AGGCAGTCCGGGTCACAC
CXCR4	GGTGGTCTATGTTGGCGTCT	TGGAGTGTGACAGCTTGGAG
FHIT	TTGCCAACCAGTTTGAAGACCG	CTGCCTGTCTGAGCCGTTTAG
p53	TCAACAAGATGTTTTGCCAACTG	ATGTGCTGTGACTGCTTGTAGATG
EGFR	GCTCTGCCCGGCGAGTCGGG	AAAAGTGCCCAACTGCGTGA
Her2	CCCTCTGAGACTGATGGCTACG	GGGCCGAACATCTGGCT
MGMT	CCTGGCTGAATGCCTATTTC	GATGAGGATGGGGACAGGATT
hnRNP A1	GTGCTGTGTAAAGTTAGTCTACTC	CCAACAATCACTTTTATAACCATTCC
p21	CTGTCTTGTACCCTTGTGCCTC	AATCTGTCATGCTGGTCTGCC
p27	TCCATTTATCCACAGGAAAGTG	CCATACACAGGCAATGAAATAC
Cyclin E	AGTGTGGGAGCCAGCCTTGG	GACTTCCTCTCTATTTGCCCAG
hTERT	CTGTACTTTGTCAAGGTGGATGTGA	GTACGGCTGGAGGTCTGTCAAG
Shh	GTGGCCGAGAAGACCCTA	CAAAGCGTTCAACTTGTCCTTA
NANOG	TTGGAGCCTAATCAGCGAGGT	GCCTCCCAATCCCAAACAATA
SOX2	GGAGCTTTGCAGGAAGTTTG	GGAAAGTTGGGATCGAACAA
CD133	TCTGTGAACCTTACACGAGCAA	CATTCCCTGTGCGTTGAAGTA
WNT 1	CCCTAACCGGTGCGCCCTGGTGCC	AGCGCCCAGAGCCCCATGGCCTGC
WNT2	ACTCTCAGGACATGCTGGCT	ACGAGGTCATTTTTCGTTGG
WNT 5a	GGGAGGTTGGCTTGAACATA	GAATGGCACGCAATTACCTT
WNT 7a	GGAGGGTCCTTTTCCTGGGT	ATATTGCTGTGATGAGGCCC
βCatenin	TGCAGTTCGCCTTCACTATG	ACTAGTCGTGGAATGGCACC
Notch1	GGACATCACGGATCATATGGACC	GAGGGTTGTATTGGTTCGGCACC

5. RESULTS

Image Cytometry Analysis

The result of Image Cytometry experiments is a curve where increasing ploidy corresponds to aggravation of the lung disease. As expected, patients originally classified as healthy have a 5cER value equal to 0, so they have no cells with abnormal DNA content. Patients with lung cancer diagnosis, instead, showed a 5cER value of 5 or more. This confirms the borderline diagnostic value of 5 about 5cER. In patients with COPD or with non-bronchitics disease, the presence of cells with abnormal DNA content was reported, but usually with 5cER < 5. Abnormal results were obtained in a patient who had no significant disease of the lungs (asthma), but it had a 5cER value equal to of 8 (high value considering the patient's healthy conditions). After reviewing the patient's medical records it was discovered a history of breast cancer and at the time of the expectoration she had lymph node metastases, which have clearly influenced the cytometric evaluation (**Fig. 1**). The results of Image Cytometry were then compared with diagnoses of patients, and sensitivity and specificity of our experiments were evaluated. Cytometry experiments showed a sensitivity of 73% and a specificity of 86% (**Fig. 2A**). A group of patients (14) had no definitive lung cancer diagnosis, but a diagnosis of Lung Opacity. These patients, usually, have a definitive diagnosis of Lung Cancer. The sensitivity and specificity of cytometry experiments, in this case, were performed by comparing 5cER with subsequent final diagnosis of these patients, the values were respectively at 72% and 67% (**Fig. 2B**).

RealTime PCR experiments

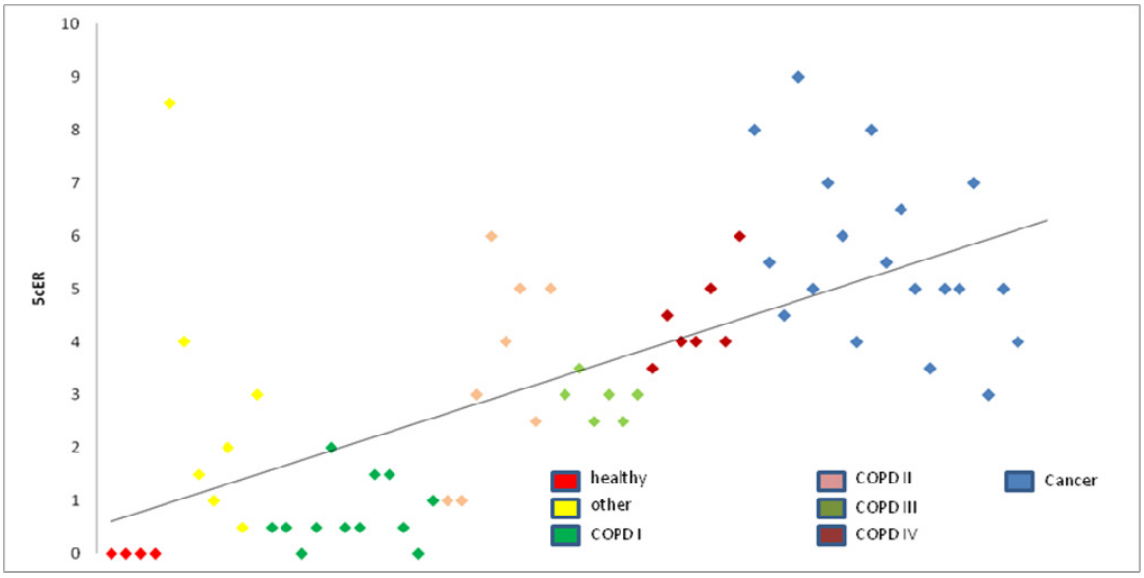
Molecular analysis was performed on different classes of genes. In particular, genes widely used in lung cancer (p53 and EGFR), genes extensively studied in recent years (p16, CXCR4, Her2, hnRNP A1, CycD1, CycE, MGMT, p21, p27), genes that have been reflected in other tumors but not exclusively in the lung (EZH2, FHIT) and, finally, genes useful for the identification of Cancer Stem Cell (Shh, Nanog, Sox2, CD133, WNT1, WNT2, WNT5A, WNT7A, Notch, β-Catenin, hTERT) were analyzed. Molecular analysis was performed using Real Time PCR experiments, in which the

expression of genes in 'pathological' patients was compared with healthy patients' gene expressions. The first step consisted in highlighting the most significant changes in patients with a diagnosis of lung cancer (**Fig. 3A**). As shown in **Figure 3B**, not all changes highlighted, and not all genes, were significant in patients with cancer. In particular, significant alterations were the reduction of the expression (and/or absence of expression) of p16, FHIT, Shh, Wnt7A, and the increased expression of EZH2, CXCR4, p27, CycE, WNT1, WNT2, WNT5A and β Catenin. The lack of expression is linked to the performance of Real Time PCR: with this technique, the changes in expression using the primers to wild-type form of genes were highlighted. If the gene resulted mutated, or epigenetic alterations were present on the promoter, it was not possible to highlight it and the analysis of Real Time PCR resulted as "not detectable" (for example, methylation of the p16 promoter was seen in the sputum of COPD patients and is positively correlated with cigarette smoking, which implied DNA methylation in the evolution of COPD (**85**)). The alterations previous described were then studied in patients with COPD and other lung diseases (**Fig. 4A**), with the aim to underline if they could be significant in pathological stage. As shown in **Figure 4B**, some changes may be "generic" or non-related at COPD stage (reduction/loss of p16 and Shh, increased WNT5A, p27 and β Catenin). The changes related to the expression of other genes appeared to be specific, especially in the late stages of COPD.

Statistical correlation

Pearsons' Coefficient was used to analyze correlation between 5cER value and gene expression level of each patient. In **table 5** Pearsons' Coefficient (and its relative p-value) of each gene is shown. It is important to note that for some genes (p16, EZH2, CXCR4, CycE, WNT1A, WNT2A, WNT5A and β Catenin) positive or negative correlation (that is greater than 0.5 in positive correlation, and less of - 0.5 in negative correlation), was stronger in comparison to the others. This result, in regards to gene expression analysis, gives to these genes a possible role of markers in combination with 5cER value.

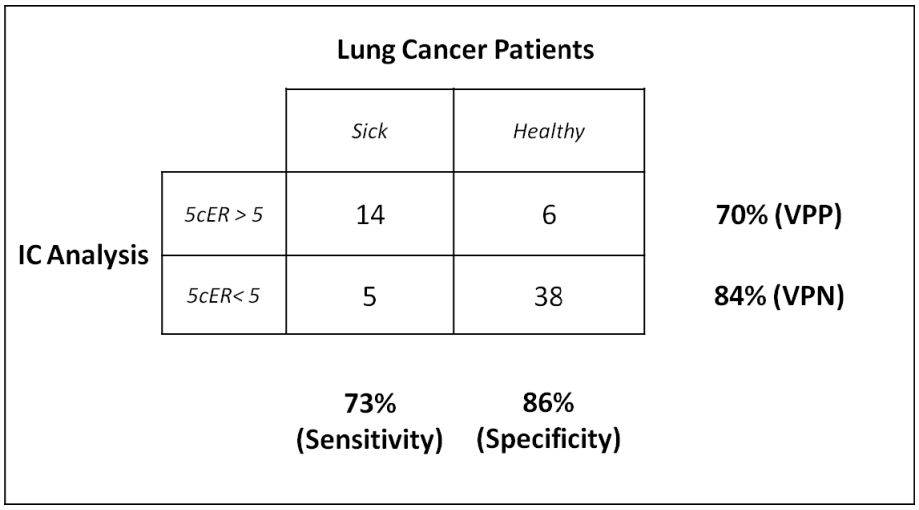
Figure 1



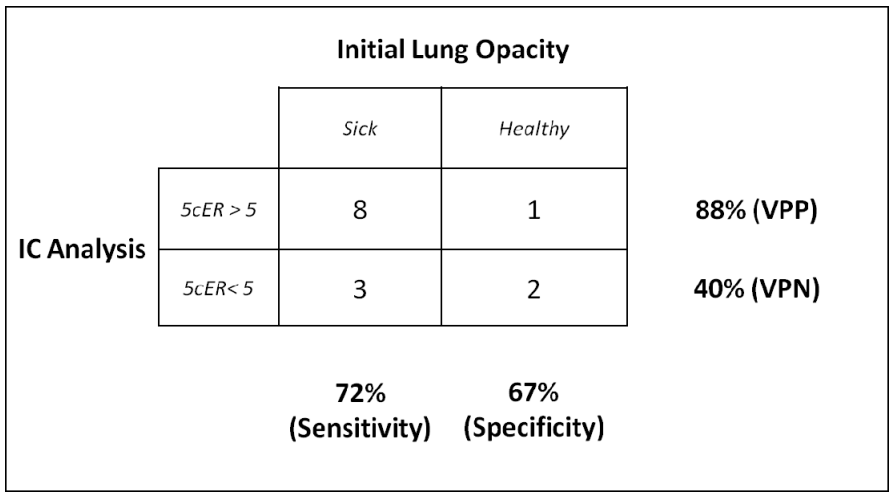
5cER value. It's evident the increasing of 5cER value with aggravation of lung disease. Especially in COPD IV patients, the presence of 5cER value > 5 confirms high risk of these patients to develop lung cancer.

Figure 2

A



B

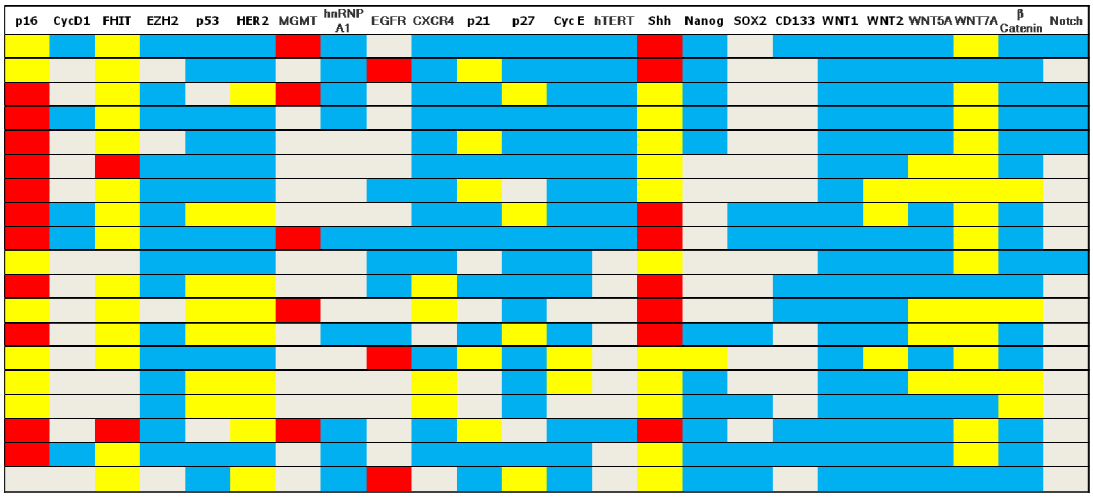


Sensitivity and specificity of Image Cytometry analysis.

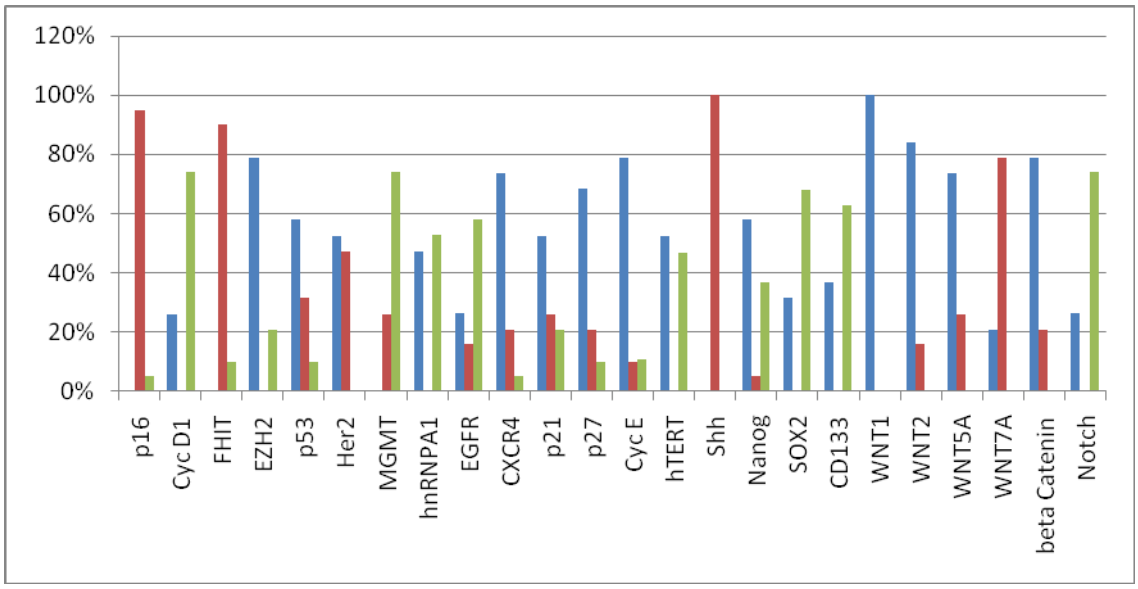
A) Sensitivity and specificity in all patients analyzed. B) Sensitivity and specificity in patients with Lung Opacity. Sick or healthy classification is due to the final clinical diagnosis

Figure 3

A



B

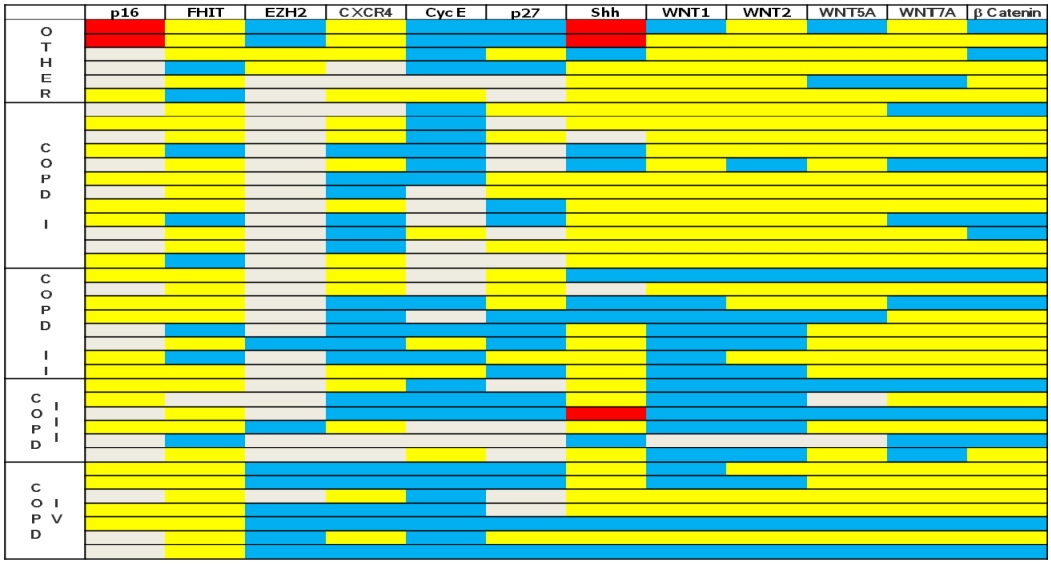


Gene expression analysis in patients with lung cancer diagnosis.

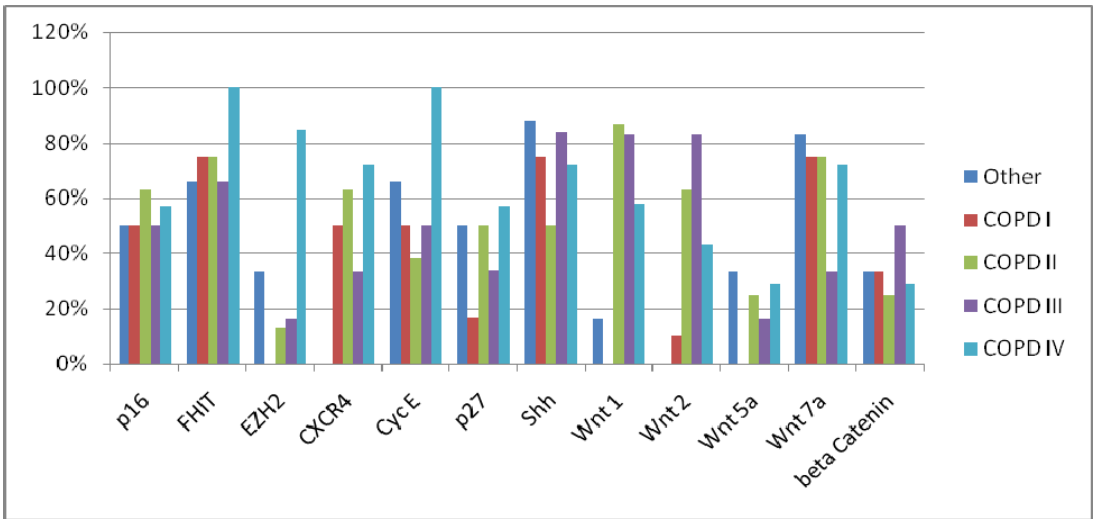
Gene expression is studied compare with healthy patients. A) Each lane is a patient. In yellow is shown reduced expression, in red loss of expression, in gray no variation of expression, in blue increased expression. B) Rate of kind of alteration about each gene in lung cancer patients. In blue increased expression, in red decreased or loss expression, in green no variation of expression.

Figure 4

A



B



Gene expression analysis in patients with COPD or other lung disease.

Gene expression is studied compare with healthy patients. Alterations evidenced in lung cancer are studied in these patients. A) Each lane is a patient. In yellow is shown reduced expression, in red loss of expression, in gray no variation of expression, in blue increased expression. B) Rate of alteration about each gene in each disease stage.

Table 5

	Pearsons' Coeff.	p-Value
p16	-0,54301113	< 0,001
EZH2	0,51715577	< 0,001
CycD1	0,36657177	< 0,005
CXCR4	0,517097476	< 0,001
FHIT	-0,30706043	< 0,05
p53	0,283047985	< 0,05
EGFR	0,112040124	> 0,1
Her2	0,357461182	< 0,005
MGMT	-0,336583899	< 0,05
hnRNP A1	0,423220508	< 0,001
p21	0,170729308	> 0,1
p27	0,367856411	< 0,01
CycE	0,642431932	< 0,001

	Pearsons' Coeff.	p-Value
hTERT	0,380958804	< 0,01
Shh	0,127774225	> 0,1
Nanog	0,1753004	> 0,1
SOX2	-0,028914648	> 0,5
CD133	0,307775489	< 0,05
WNT1	0,550877552	< 0,001
WNT2	0,601615778	< 0,001
WNT5A	0,559159913	< 0,001
WNT7A	-0,27776895	< 0,05
β Catenin	0,601721972	< 0,001
Notch	0,064342735	> 0,5

Correlation between gene expression and 5cER.

The pair(s) of variables with positive correlation coefficients and P values below 0,050 tents to increase together. For the pairs with negative correlation coefficients and P values below 0,050, one variable tents to decrease while the other increases. For pairs with P values greater than 0,050, there were no significant relationships between the two variables

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Lung cancer has been described as an epidemic disease with high mortality, effecting males and females, among old and young generations. The high-risk population is vast and growing (**16**). It has been well demonstrated that patients with early stage lesions have a much better prognosis than those in advanced stage. However, until now, there have been no accepted screening strategies to improve the early detection rate of lung cancer (**16**). Recently, population-based trials of CT screening were launched in several countries to assess the utility of CT in the early detection of lung cancer. Patients with COPD are three to four times more likely to develop lung cancer than smokers with normal lung function. Lung cancer is found in 40%–70% of patients with COPD, particularly in severe disease, and is a common cause of death in COPD patients (**86**). In a large prospective trial of almost half a million individuals, lung cancer was found more common in patients with COPD who have never smoked (**86**). The most likely explanation to the increased risk of lung cancer in COPD is the presence of chronic inflammation, with increased production of growth factors and angiogenic factors. Stopping smoking in COPD patients reduces but does not eliminate the risk of lung cancer, probably because the inflammation persists even after smoking cessation (**86**). The reduction of cancer-related morbidity and mortality in developing countries should now become an urgent global priority. Developing countries already have enormous limitations in resources and are unable to cope with an escalating cancer burden. Additionally, this major problem in developing countries is not in the best interests of the developed world and should be viewed as a global crisis. Urgent efforts are now needed to curb the widespread use of tobacco and thereby effectively reduce the incidence of cancer, particularly in underprivileged regions of the world (**87**). The non-invasiveness represents the goal that all clinical trial focusing in cancer detection should pursue. The detection of cancer in early stage of its progression requires a large-scale screening. In lung cancer detection, targets are smoking people, over 60, with previous history of cancer, and with COPD (**42**).

A good screening program have to be inexpensive, to require the use of sensitive technology, and to exploit a non-invasive sample acquisition. For the lung, sputum represents the best possible sample to be used. Good separation of cells in sputum

sample allows the collection of sufficient material to realize “slide” and molecular assay.

DNA content is the most significant cellular parameter able to monitor the cell proliferation and the neoplastic transformation. In cancer cells of solid tumor is often visible an alteration of DNA content, first cause of genetic alteration (chromosomic and/or sub-chromosomic), fundamental for the development of the disease. There are many clinical evidences on the correlation between DNA content alteration and poor prognosis. Ploidy is an expression of DNA content of tumor cells, which are classified as diploid if their DNA content is similar to non-transformed cells, or aneuploid if it is grossly abnormal (**54**). 5cER represents a cytometric value indicating the presence of cells with abnormal DNA content.

As shown in figure 1, 5cER was significantly different among groups with healthy diagnosis and groups with various stage of lung disease, in particular with lung cancer diagnosis. It was higher, with the progression from normal to malignant lesions, which suggested that the high level of 5cER was related to the respective stage of development of pulmonary malignancy. Morphological abnormalities of cancer cells may be reflected by DNA aneuploidy more than by their proliferative activity.

Nevertheless, using 5cER alone to predict the presence of lung cancer has its limitations, because not all lung cancers contain aneuploidy. Many new compounds that target the molecular pathology of advanced lung carcinoma are now undergoing clinical trials. However, it is likely that a greater understanding of the molecular and cellular pathobiology that distinguishes smokers with premalignant bronchial lesions and lung cancer from smokers with and without COPD is needed to unravel the complex molecular interactions between COPD and lung cancer. Overall these studies may allow the discovery of new molecular targets of the early carcinogenesis process that in the foreseeable future may render the early diagnosis and treatment, and maybe even the prevention, of invasive lung carcinoma a reality (**88**). So, in this study a group o genes that have a role in lung cancer was analyzed, in particular, p53, EGFR, CXCR4, MGMT, hnRNP, p21, p27 (**88**), p16, Her2 (**89**), CycE (**90**), CycD1 (**91**), FHIT (**34**), EZH2 (**92**). Moreover, stem cells and their relationship to cancer progression is a new and expanding area of research. Recent studies suggested that endogenous stem cell

signaling and differentiation paths are maintained within cancer types, and that disruption of this signaling mechanism may initiate specific lung cancers (**85**).

The "molecular" results of this study show that some of these genes could be predictive markers of cancer progression. In particular, loss (or reduction) of p16 and FHIT and increase of EZH2, CXCR4, CycE, WNT1, WNT2, WNT5a and β Catenin (alteration described in lung cancer patients) are alteration that could be expressive of lung cancer progression, because their presence is relevant in lung disease as COPD, and because there is correlation between ploidy variation and its expression (fig. 3, 4, 5).

The study is in its early stages, and the number of patients is relatively low (<100), but the results are promising.

In conclusion, DNA image cytometry might be a suitable tool for identifying a population with a particularly high risk of developing lung cancer, in particular in heavy smokers. 5c-exceeding rate could serve as a parameter to monitor the progression of a pre-invasive lesion. This parameter, combined with clinical aspect of patients, could be a useful parameter for early detection of lung cancer by automated sputum Image Cytometry. For the early diagnosis of lung cancer, other parameters should be investigated in order to increase the diagnostic sensitivity of image cytometry. Given the heterogeneous nature of lung cancer and COPD, monitoring of only one or a few genes is probably of limited value. So, our future aim will be to perform gene analysis using "transcriptomics" assays, such as array and/or RNA Sequencing, in order to analyze the whole transcriptomes present in the cells and identify molecular biomarkers for early detection of lung cancer. By understanding the common signaling pathways involved in COPD and lung cancer the hope is that treatments will be developed that not only treat the underlying disease process in COPD, but also reduce the currently high risk of developing lung cancer in these patients (**88**).

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