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Epidermal growth factor receptor and anaplastic lymphoma kinase mutations detected by immunohistochemistry in lung adenocarcinoma in patients from Johannesburg

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CANDIDATE'S DECLARATION

I, Naseema Ismail Vorajee (Student number: 9102330w) am a student registered for the degree of MMed (Anatomical Pathology) in the academic years 2014/2017.

I hereby declare the following:

I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.

I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.

I have followed the required conventions in referencing the thoughts and ideas of others. I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.



Signature ***Date: 19/07/2017***

DEDICATION

I dedicate this research report to
my beloved husband Junaid,
my darling sons Aadil and Isa,
and my dear parents.

ABSTRACT

Lung cancer is the leading cause of cancer related death worldwide. Despite the availability of conventional cytotoxic chemotherapy, prognosis even with treatment is poor. Treatment targeted to specific molecular alterations called Tyrosine kinase inhibitors (TKI) has been found to be effective in certain subtypes of non small cell carcinoma (NSCC) that have the epidermal growth factor receptor (EGFR) gene mutation and the anaplastic lymphoma kinase (ALK) translocation. EGFR mutations are seen in 18-25% of lung adenocarcinomas (AC)s and are represented in more than 90% of cases by the E746_A750 deletion on exon 19 and the L858R point mutation on exon 21. The ALK translocation is seen in 2-7% of lung cancers, and involves the EML4-ALK fusion gene product. Immunohistochemistry (IHC) has the potential of being used as an initial screening tool that can facilitate a shorter diagnostic time and fast track treatment options. Little is known about the mutational status of patients with these mutations in South Africa. This study examines the use of IHC as a means of detecting the most common EGFR mutations and the ALK translocation in lung cancer.

Method: Biopsies of patients from the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and Helen Joseph Hospital (HJH) sent to the National Institute for Occupational Health (NIOH) from 1st January 2008 to 30th June 2014 were reviewed. A total of 3901 histology reports were accessed. There were 297 lung carcinomas comprising 117 (39%) ACs and 128 (46%) squamous carcinomas (SCC)s. A total of 119 biopsies comprising 107 ACs (90%), 4 (3%) adenosquamous carcinomas (ADSCC)s, 1 (1%) large cell carcinoma (LC) and 7 (6%) NSCC (nos.) comprised the cohort. One hundred and eleven biopsies were available for mutational analysis, as 8 biopsies were excluded due to insufficient tissue availability. The mutation specific antibodies, EGFR SP111 and EGFR SP125 were used to detect the E746_A750 deletion and the L858R point mutations respectively. The high affinity ALK D5F3 antibody was used for the detection of the EML4-ALK translocation.

Results: The majority of patients in the study were Black males (61%). There were no Asians. The mean age was 58 years with a SD of 11.5. Most patients (76%) were younger than 65 years. The EGFR IHC stain was positive in 10/111 (9%) biopsies, of

which 8 were from Blacks, 6 were from males, 4 were from smokers and 2 were from non-smokers. There was however no significant difference between the proportions of Black or White and male or female among those who tested positive to EGFR and those who tested negative ($p>0.05$) and no significant association was found between the variables age, sex and smoking history ($p>0.05$). The ALK IHC was positive in 8 (7%) patients. All 8 patients were Black, six were male, four had a smoking history and two patients were non-smokers. A significant association was found between race and positive ALK IHC ($p=0.03$). There was no significant association with age ($p=0.081$). The acinar growth pattern was found in 80% of the EGFR IHC positive biopsies, with 10% of biopsies showing either lepidic, solid or micropapillary patterns. Although several growth patterns were seen in ALK IHC positive biopsies, there was a slight predominance of the acinar and solid growth patterns.

Discussion: The EGFR IHC was positive in 9% of patients, which is half the number of cases described in literature from Western and Eastern countries, where the prevalence is usually more than 18%. There may be several reasons for the lower rate of EGFR IHC positivity. Antigen degradation, intratumoral heterogeneity and a low sensitivity of the EGFR IHC antibody may have contributed. The demographic profile of patients with positive result following EGFR IHC differs from the literature although the results were not found to be statistically significant. The sensitivity of the EGFR IHC test as described in the literature ranges from as low as 61% to 100%. A good correlation between EGFR IHC and EGFR polymerase chain reaction, in confirming the presence of the EGFR mutation, is described with biopsies that show strong positive cytoplasmic staining with IHC. Strong positive cytoplasmic staining with EGFR IHC was found in these 10 biopsies. The ALK IHC result was positive in 7% of patients, which is on the upper limit of the 2-7% rate recorded in most literature. A significant association was found with Blacks. Although an association with younger patients and the ALK mutation was found, this was not statistically significant. As ALK IHC is associated with an almost 100% sensitivity and specificity, there is a possibility that the ALK mutational rate in South African Black patients may be higher than the rate in other international populations. SCC is the more common subtype of lung cancer (46%) compared to AC

(39%) in this group of patients from Johannesburg. This differs from studies in Cape Town and international studies, where a shift in trend from SCC to AC is observed. **Conclusion:** This study confirms the presence of the EGFR mutation and ALK translocation in patients with adenocarcinoma from Johannesburg using immunohistochemistry. We have proposed a diagnostic algorithm for patients with lung cancer in South Africa where EGFR IHC and ALK IHC can be used as rapid initial screening tests to identify patients with the EGFR mutation and ALK translocation respectively, provided established guidelines for IHC interpretation are followed. This approach allows patients with lung cancer who have the EGFR and ALK mutations to be fast tracked towards receiving targeted therapy.

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NOMENCLATURE and ABBREVIATIONS

AC: Adenocarcinoma
ADSCC: Adenosquamous carcinoma
ALK: Anaplastic lymphoma kinase
ATS: American Thoracic Society
CMJAH: Charlotte Maxeke Johannesburg Academic Hospital
CISH: Chromogenic in situ hybridization
EGFR: Epidermal growth factor receptor
ERS: European Respiratory Society
FISH: Flourescence in situ hybridisation
HJH: Helen Joseph Hospital
IASLC: International Association for the study of Lung Cancer
IHC: Immunohistochemistry
LC: Large cell carcinoma
NHLS: National Health Laboratory Service
NIOH: National Institute for Occupational Health
NSCC: Non small cell cancer/carcinoma
NSCLC: Non small cell lung cancer/carcinoma
PCR: Polymerase chain reaction
SCC: Squamous cell carcinoma
SCLC: Small cell lung carcinoma
TKI: Tyrosine kinase inhibitor
WHO: World Health Organisation

Chapter 1: INTRODUCTION

1.1 Background

Lung cancer is the most common visceral cancer in males worldwide and has been for several decades, with an estimated 1.8 million new cases recorded for 2012 by the International Agency for Research on Cancer, 58% of which occurred in the less developed regions (Ferlay *et al.,* 2012). Lung cancer was found to be responsible for approximately one in five deaths related to cancer, with an estimated mortality of 1.59 million.

The mortality of lung cancer recorded for South Africa in 2012 was 13.7%; the most common cause of cancer related death in males (surpassing prostate cancer) and the third most common cause of cancer related death in females (following cervical and breast cancer) (Ferlay *et al.,* 2012).

The overall prognosis of patients diagnosed with lung cancer is poor. The five year survival for lung cancer of all stages in the United States in 2004 was 16.8% (Ridge, McErlean and Ginsberg, 2013). The poor prognosis is as a result of late presentation when surgery is precluded and poor results obtained from standard platinum-based chemotherapy regimens. There is therefore a need to identify lung cancer at an earlier stage and improve current treatment regimens. Lung cancer screening programmes for early detection of lung cancer are not well established and may not be a feasible option in a resource poor country such as South Africa. The search for a more effective treatment regimen prompted the undertaking of several clinical trials, which in the beginning of the 21st century saw the success of

targeted therapy using a certain group of drugs referred to as “Tyrosine kinase inhibitor (TKI) therapy” in some patients diagnosed with lung cancer (Cheng *et al.,* 2012). Patients who showed a favourable response were found to have non small cell carcinoma (NSCC) of the lung. NSCC and small cell lung carcinoma (SCLC) are histologic subtypes of lung cancer. NSCC is more common than small cell lung cancer (SCLC) and is further sub-classified into squamous carcinoma (SCC), adenocarcinoma (AC), adenosquamous carcinoma (ADSCC) and large cell carcinoma (LC). Patients with NSCC who showed a positive response to TKIs were found to have either AC, ADSCC or LC subtypes, and on further investigation, were found to have a mutation in the epidermal growth factor receptor (EGFR) gene. Studies also revealed that most patients with lung cancer who had the EGFR mutation had a similar demographic profile: they were female, non-smokers and predominantly of Asian descent (Cheng *et al.,* 2012).

Similarly, in 2007 treatment success with another TKI, crizotinib, was found in some patients with NSCC. On further investigation, these patients were found to harbour a particular mutation involving the anaplastic lymphoma kinase (ALK) gene (Soda *et al*., 2007). The EGFR mutation was absent. Patients with lung cancer who responded to crizotinib were also found to have a similar demographic profile with respect to younger age (less than 65 years) and non-smoking history however there was no association with Asian ethnicity (Kwak *et al*., 2010).

Targeted therapy has thus changed the approach to patients with lung cancer. Current international recommendations by Lindeman *et al.* (2013) include testing for the presence of the EGFR mutation and ALK

translocation in lung resection specimens for all lung ACs including any mixed lung cancer that may have an AC component such as adenosquamous carcinoma (ADSCC) and combined small cell carcinoma with an AC component (a combined small cell carcinoma has both SCLC and NSCC components. The NSCC component may be an AC, SCC or LC (Travis *et al.,* 2015)). Testing is also recommended on tumours that do not show any clear morphological or immunohistochemical differentiation. These tumours are referred to as NSCC (not otherwise specified, nos.) if found on a small biopsy specimen and large cell carcinoma (LC) if found in a resection specimen. This approach allows patients with lung cancer, who may have an AC component that is difficult to diagnose morphologically or with immunohistochemistry, a chance of benefitting from targeted therapy. These molecular tests are not recommended for lung cancers in resection specimens that do not have an adenocarcinoma component, such as pure squamous cell carcinoma (SCC) and small cell carcinoma (SCLC), as most of these tumours were found not to harbour the EGFR mutation or ALK translocation (Lindeman *et al.,* 2013).

The EGFR mutation and ALK translocation are found in a subset of patients with lung cancer who usually do not have a smoking history. Smoking however remains a significant contributor to the incidence of lung cancer. A shift in trend in lung cancers from being predominantly of the squamous subtype to the adenocarcinoma subtype has been observed in several developed countries as a result of a change in smoking practices. Other possible causes of lung cancer include occupational exposure to chemicals, dust and fibres. A recent study by Kradin, Lafrate and Christiani

(2017) describes the presence of the EGFR mutation in three patients who had no smoking history but were exposed to asbestos fibres.

1.2 Problem statement

1). Patients in South Africa who have lung cancer present at an advanced stage (Nanguzgambo *et al*., 2011)

2). Response of lung cancer to standard chemotherapy is poor

3). Targeted therapy is promising for patients who have the specific mutations

4). Little is known about the EGFR mutation and ALK translocation rate in NSCC in South Africa and if these mutations are present, do they occur in lung cancers from patients who have a similar demographic profile as described in literature from other countries. Knowledge on the prevalence of the EGFR mutation in the South African population is limited to a single study (Chan, 2015) whilst there is no data on the ALK translocation.

5). It is uncertain whether the shift in trend in lung cancer from SCC to AC observed in other countries is true for South Africa.

6). Little is known about the relationship between the presence of the EGFR mutation and ALK translocation and occupational history.

1.3 Rationale

1). By identifying the EGFR mutation and ALK translocation in patients with lung cancer, we can identify patients who may benefit from targeted therapy.

2). Testing for these mutations is not routinely performed by the National Health Laboratory Service (NHLS) and there is no current protocol or recommendations for the testing of these mutations in South Africa. Current

international recommendations for the detection of the EGFR mutation include polymerase chain reaction (PCR) and fluorescence in situ hybridisation (FISH) for the detection of the ALK translocation (Lindeman *et al*., 2013). Immunohistochemistry (IHC) is rapid, convenient, accessible and affordable and may be used as an initial tool to identify these mutations.

1.4 Aim

The aim is to evaluate the use immunohistochemistry to detect specific mutations in lung cancer in South African patients for which targeted therapy may be a treatment option.

1.5 Objectives

1. To determine the prevalence of the EGFR mutation in lung AC, ADSCC, LC, NSCC (nos.) and combined tumours with an AC component using EGFR IHC.
2. To determine the prevalence of the ALK translocation in lung AC, ADSCC, LC, NSCC (nos.) and combined tumours with an AC component using ALK IHC.
3. To test the association between patient demographic characteristics (race, sex, age, smoking history and occupational history) and the presence of the EGFR mutation.
4. To test the association between patient demographic characteristics (race, sex, age, smoking history and occupational history) and the presence of the ALK translocation.

5. To test the association between the morphological patterns of the different subtypes of NSCC (AC, ADSCC, LC, NSCC (nos.) and combined tumours with an AC component) and the presence of the EGFR mutation.
6. To test the association between the morphological patterns of the different types of lung cancer (AC, ADSCC, LC, NSCC (nos.) and combined tumours with an AC component) and the presence of the ALK translocation.
7. To determine the prevalence of SCC and AC.

1.6 Significance

There is only one published report by Chan (2015) on the EGFR mutational status of lung cancer in the South African population.

Currently, targeted therapy for lung cancer is only available for selected patients who are either in the private sector or enrolled in clinical trials. If continued success with targeted therapy is shown, we expect targeted therapy drugs to become part of the standard treatment programme for all patients with lung cancer.

The National Institute for Occupational Health (NIOH) is a division of the NHLS, which serves the public sector hospitals that treat more than 80% of the population of South Africa. The development of an affordable, accessible and effective method of detection of the EGFR mutation and ALK translocation is needed. This is particularly relevant to South Africa, where resources are limited and patient follow up is poor.

Chapter 2: LITERATURE REVIEW

1). Lung cancer classification

Historically, lung cancer has been classified by the World Health Organisation (WHO) into two main types: small cell lung carcinoma (SCLC) and non small cell lung carcinoma (NSCLC), which was based on differences in behaviour and treatment (Travis *et al.,* 2004). Although NSCLC was further sub-classified into squamous carcinoma (SCC), adenocarcinoma (AC), large cell carcinoma (LC) and adenosquamous carcinoma (ADSCC), the lack of effective treatment against these different subtypes made sub-classification by pathologists less important.

Over the past decade and a half however, there have been several advances with regards to targeted therapy against specific mutations in lung adenocarcinoma. These advances made it necessary for pathologists to accurately sub-classify lung cancer and submit tissue for mutational analysis. In order to accurately sub-classify lung cancer, well defined and uniform criteria and terminology was required. This led to the formation of an international panel of experts from the clinical, radiological, surgical and pathological disciplines. This panel provided a multidisciplinary approach to the histological diagnosis of lung AC and provided recommendations and guidelines for further mutational analysis in patients diagnosed with lung AC (Travis *et al.,* 2011). Subsequently, the revised and updated version of the *WHO classification of tumours of the Lung, Pleura and Heart* by Travis *et al.* (2015) was published, which reflects these guidelines.

This version saw the terms “Non small cell carcinoma” (NSCC) and “Non small cell lung carcinoma (NSCLC)” being used interchangeably whilst the sub-classification of these carcinomas into AC, SCC, ADSCC and LC remained unchanged. There was also the introduction of the variant “NSCC not otherwise specified (nos.)”, which is a diagnosis made on small biopsy specimens when there are no clear morphological or immunohistochemical features of either an AC or SCC. The diagnosis of “NSCC, nos.” in a small biopsy is equivalent to the diagnosis of LC in a resection specimen. The last notable change was the replacement of tumours previously referred to as “Bronchioloalveolar carcinoma” with either “Adenocarcinoma with a lepidic pattern” if the tumour cells are non-mucinous or “Invasive mucinous adenocarcinoma” if the tumour cells are mucinous (Travis *et al.,* 2015).

2). Prognosis and treatment of lung cancer

Worldwide, NSCC is the more common type of lung cancer and accounts for the high mortality rate associated with lung cancer (Herbst, Heymach and Lippman, 2008). The poor survival is attributed to late presentation. Nanguzgambo *et al*. (2011) found that 78% of patients from Cape Town presented with at least stage 3A (locally advanced) NSCC. This late presentation precludes surgery based therapy and until the late 1990s, the treatment of advanced lung cancer involved platinum-based chemotherapy regimens, irrespective of histological subtype (Pfister *et al.,* 2004). Even with the use of third-generation cytotoxic drugs, the outlook was dismal as the increased response rate was found to be associated with an increased toxicity but no improvement in survival (Azim *et al.,* 2009). In light of this, a search for a more effective method of treatment was undertaken.

3).Targeted therapy

Advances in therapy have led to the development of molecularly targeted agents that inhibit specific pathways in tumour growth and progression, resulting in improved survival rates. It was shown in early studies from 2004 and 2005 that patients with advanced NSCC, mainly AC, who showed an improved response following treatment with EGFR TKIs gefitinib and erlotinib, had lung cancer with the EGFR mutation (Lynch *et al*., Paez *et al*. and Pao *et al*., 2004 and Shepherd *et al*., 2005). The presence of the EGFR mutation was also found to be a prognostic factor independent of EGFR TKI treatment and patients with lung AC that had the EGFR mutation had improved response rates and prolonged survival, even when they received chemotherapy (Eberhard *et al.,* 2005). In contrast, it was shown that lung cancers without the EGFR mutation responded better to conventional chemotherapy compared to EGFR TKI therapy (Mok *et al.,* 2009).

The importance of these studies is twofold: they highlight the importance of accurately classifying lung cancer into its different types and subtypes and correctly identifying NSCCs that harbour the EGFR mutation and those that do not, in order to facilitate selection of the most appropriate therapy.

4). Lung cancer trend: Squamous carcinoma versus adenocarcinoma

The incidence of lung cancer in the world has increased from the 20th century onwards as a result of the increasing use of tobacco cigarettes. During the peak of this tobacco-related epidemic, SCC was found to be the most prevalent subtype of lung cancer (Travis *et al*., 2004). AC has been described in both smokers and non-smokers. Between 1965 and 2004,

cigarette smoking in adults in the United States decreased from 42% to 21% because of legislated restrictions on tobacco products, resulting in a corresponding decrease in the number of SCC compared to AC. There was a suggestion that the increase in the number of AC was related to changes in smoking practices, such as deeper inhalation of cigarette smoke whilst cigarette design contributed as well (Devesa, Shaw and Blot, 1991; Wynder and Hoffmann, 1998 and Travis *et al*., 2004). AC is currently the more common subtype of lung cancer in developed countries (Ridge, McErlean and Ginsberg, 2013).

There is limited published data on the prevalence of lung cancer from South Africa. Early literature published in 1990 by Willcox, O'Brien and Abratt found a higher prevalence of SCC in patients with lung cancer from Cape Town. This was similar to a study published in 2013 by Mukansi, Smith and Feldman *et al.,* who looked at lung cancer patients admitted to hospitals in Johannesburg between 1992 and 1998. The shift in trend from SCC to AC as described internationally was however reflected in a study published in 2010 by Koegelenberg *et al.,* where almost twice as many lung AC cases compared to SCC were found in the Western Cape.

From the above, it appears that although there may be a shift in trend from SCC to AC in patients from the Cape, there may still be a higher prevalence of SCC in patients from Johannersburg. This research report may shed further light on which subtype of lung cancer is most prevalent in patients from Johannesburg.

5). Lung cancer mutations

Lung cancer, like most cancers, is characterized by a number of genetic and epigenetic alterations that involve the activation of oncogenes and the inactivation of tumour suppressor genes (Figure 2.1, Modified from Cheng *et al.,* 2012). The activation of oncogenes may occur through mutations (involving EGFR, KRAS, BRAF and ERBB2), translocations (involving ALK, ROS1 and RET) and amplifications (involving MET and FGFR1) (Travis *et al.,* 2015). The activation of oncogenes occurs as a result of specific mutations called “driver mutations” whilst the inactivation of these mutations result in cancer cell death. The EGFR and Kirsten rat sarcoma (KRAS) mutations and the ALK translocation are driver mutations in lung cancer.

According to Cheng *et al*. (2012) from data obtained from the Lung Cancer Mutation Consortium in the United States, approximately 64% of all AC cases harbour somatic driver mutations. 25% of ACs have mutations that involve the KRAS gene, 23% have mutations that involve the EGFR gene and 6% of cases harbour the ALK translocation, a transforming fusion gene product EML4–ALK. The mutation frequency of BRAF is 3%, PIK3CA 3%, MET amplifications 2%, ERBB2 (Her2/neu) 1%, MAP2K1 0.4%, and NRAS 0.2%. Approximately 36.4% of lung adenocarcinomas do not harbour currently detectable mutations (**Figure 2.1**, Modified from Cheng *et al.,* 2012).

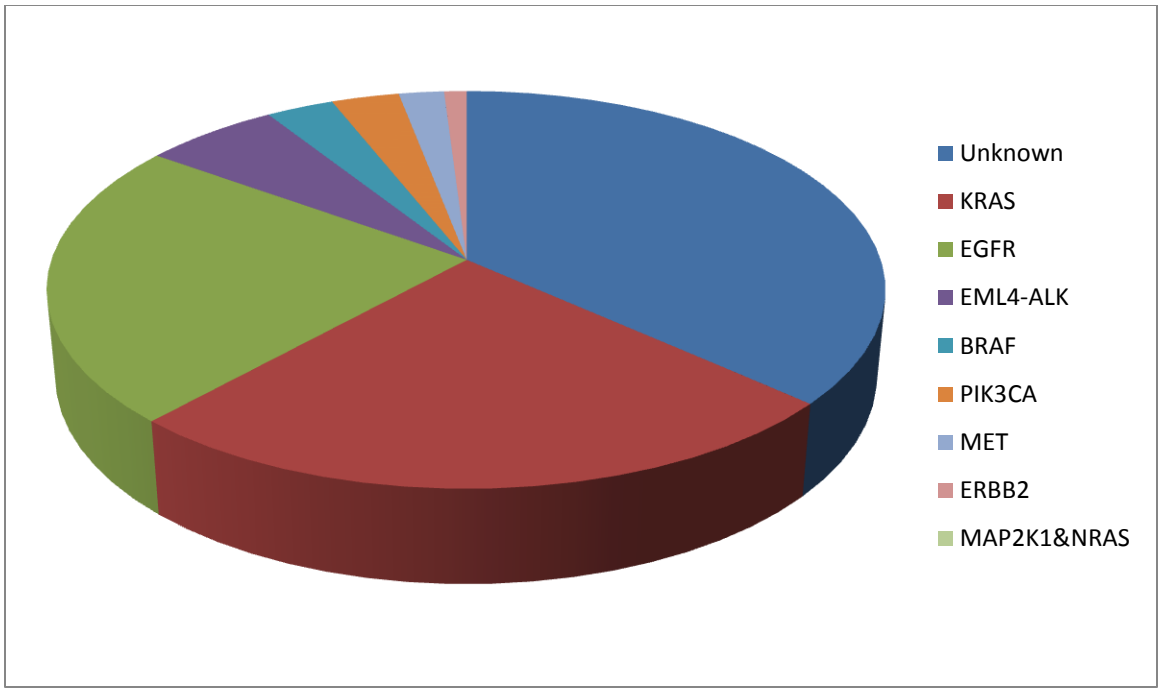

Figure 2.1 (Modified from Cheng *et al.,* 2012): Frequency of major driver mutations in lung AC

Most studies confirm that the oncogenic drivers for lung AC, i.e. mutations involving EGFR, K-RAS and ALK genes are mutually exclusive (Suzuki *et al.,* 2006, Soda *et al.,* 2007, Gandhi *et al.,*, Rodig *et al.,* and Wong *et al.,* 2009 and Inamura *et al.,* and Kwak *et al.,* 2010) with the exception of isolated case studies by Boland *et al.* (2013) and Kadota *et al.* (2014).

Pure SCC (without an AC component) do not harbour the EGFR, ALK and KRAS mutations (Rekhtman *et al.,* and Heist *et al.,* 2012). SCLCs with the EGFR mutation were discovered to be combined SCLC with an AC component (Tatematsu *et al.,* 2008).

i). KRAS

The most prevalent mutation found in lung AC involves the Kirsten rat sarcoma viral oncogene homolog (KRAS). KRAS mutations are seen in

approximately 25-30% of lung AC. It is an oncogene that encodes a GTPase downstream of EGFR. Although more than 90% of KRAS mutated lung AC have a history of tobacco use, this mutation has been described in approximately 5% of non-smokers (Rodenhuis *et al.,* 1987 and Lindeman *et al.,* 2013). KRAS transversion mutations (G→T or G→C) are more common in former/current smokers whilst KRAS transition mutations (G→A) are found in patients who have never smoked (Riely, Marks and Pao, 2009). Kadota *et al.* (2014) found that lung ACs with mucin (invasive mucinous AC and extracellular mucin) were more commonly associated with the KRAS mutation compared with non-mucinous ACs.

Several recent studies, such as the study by Kim *et al.* (2012) including earlier studies by Massarelli *et al*. (2007) and Ladanyi and Pao (2008) have shown that patients with AC that have the KRAS mutation have a more than 96% chance of disease progression and its presence was an important predictor of poor response to EGFR TKI therapy. Furthermore, mutations in KRAS have been proposed to be one of the mechanisms of primary resistance to EGFR TKI therapy (Pao *et al.,* 2005). An effective therapeutic agent against this mutation is not available although clinical trials using candidate drugs are currently underway.

Testing for the KRAS mutation is recommended by Lindeman *et al*. (2013) only if adequate tissue is available for subsequent molecular (EGFR and ALK) analysis, if required.

ii). EGFR

a) Background of EGFR

The second most prevalent mutation, seen in approximately 18-30% of lung AC, involves the EGFR gene. EGFR was first reported on the cell membrane of fibroblasts by Carpenter *et al*. (1975) and has since been shown to be expressed in normal epithelium, mesenchyme and neurogenic tissue. EGFR belongs to the erbB family of receptor tyrosine kinases. These include erbB1 (also known as EGFR), erbB2 (Her2), erbB3 and erbB4. EGFR and its family of receptor tyrosine kinases are important in carcinogenesis as they modulate cell proliferation, apoptosis, cell motility and neovascularisation (Lynch *et al.,* 2004, Paez *et al.,* 2004, Gupta *et al.,* 2009, Bethune *et al.,* 2010 and Inamura *et al.,* 2010). EGFR undergoes a conformational change once it binds to a specific ligand, leading to downward signal transduction that, depending on the pathway, will result in cell proliferation, cell maintenance by inhibition of apoptosis, cell differentiation and motility. This occurs through auto activation of EGFR itself or through two downstream intermediate pathways that involve RAS and PIK3CA (Cheng *et al.,* 2012).

The Ras/Raf/MAPK pathway promotes cell proliferation and survival, while the PI3K/AKT pathway is associated with cell growth, inhibition of apoptosis, invasion and migration (Inamura *et al.,* 2010). Early studies have shown that EGFR expression in NSCC is associated with reduced survival, frequent lymph node metastasis and poor chemosensitivity (Veale *et al*., 1993, Fontanini *et al*., 1998). Inhibition of EGFR by TKIs is associated with an increase survival in patients with advanced NSCC (Shepherd *et al.,* 2005).

b) Genetic structure of EGFR

The EGFR gene is 200kb and is found on chromosome 7p12. EGFR has an N-terminal extracellular ligand binding domain, a transmembrane lipophillic portion and a C-terminal intracellular region containing tyrosine kinase and regulatory domains. It comprises 28 exons and 27 introns. Exons 1 to 16 encode the extracellular domain, exon 17 encodes the transmembrane domain and exons 18 to 28 encode the intracellular domain.

c) EGFR mutation, amplification and overexpression

There are three types of EGFR alterations as described by Cheng *et al*. (2012) and Shepherd *et al*. (2005): namely mutation, copy number gain/amplification and protein overexpression.

EGFR amplification, defined as more than five EGFR signals per nucleus, was found in 52% of EGFR mutant lung cancers and in only 6% of tumours without the EGFR mutation. Tumours with amplified EGFR had a solid growth pattern and were more aggressive (Bethune *et al.,* 2010). Bethune *et al*. (2010) suggested that EGFR mutations occur as early events in carcinogenesis whilst gene amplification occurs later. Even though patients with lung AC that have EGFR gene amplification may show improved survival following treatment with TKIs, EGFR gene amplification was less sensitive and specific and thus not suitable for determining which patients to select for EGFR TKI therapy. Approximately 50% of EGFR mutated cases have an increase in EGFR copy number whilst 75% of cases with an increased gene copy number have EGFR mutations (Li *et al.,* 2008). This study supported findings by Ladanyi and Pao (2008). Li *et*

al.'s study also showed that although overexpression of total EGFR (as detected using IHC) was found in 40-80% of lung tumours, it was not a successful prognostic marker and its presence was independent of the EGFR mutation.

In summary, Ladanyi and Pao (2008) and Li *et al.* (2008) concluded that it was important to determine EGFR mutational status rather than EGFR amplification for the following reasons:

1). Mutant EGFR is linked to ligand-independent increased downstream signalling, unlike simple overexpression of EGFR

2). If both EGFR mutation and EGFR gene copy amplification are present, it is the mutant EGFR allele that is preferentially amplified, which suggests that it is the mutation that drives the selection for copy number gains

3). EGFR mutation is more closely linked to risk factors (negative smoking history) and demographic features (Asian, female) compared to EGFR amplification

4). Response rates of patients with EGFR mutation to EGFR TKI were high, irrespective of gene copy number whilst the response rates were low in the absence of the EGFR mutation

5). EGFR mutation status was a better predictor of patient outcome in patients' treatment with EGFR TKIs compared to EGFR copy number.

Mutations in EGFR can occur in either the extracellular or intracellular domains of the protein but the majority show mutations in the intracellular tyrosine kinase domain (Paez *et al.,* 2004).The tyrosine kinase domain is encoded by exons 18 to 24, whilst the C-terminal domain is encoded by exons 25 to 28. More than 90% of the EGFR tyrosine kinase domain

mutations occur as short in-frame deletions in exon 19 (as the E746-A750 15bp deletion) or as point (or missense) mutations in exon 21 (the latter results in arginine replacing leucine at codon 858 (L858R)) (Lynch *et al.,* 2004, Paez *et al.,* 2004 and Pao *et al.,* 2004). Less frequent mutations include point mutations (G719) in exon 18 and point mutations and in-frame insertions in exon 20. Although the most common deletion in exon 19 includes the 15bp deletion (delE746-A750 and delL77-T751insS) and the 18bp deletion (del747-P753insS), there are more than 20 other variants of the exon 19 deletion. These include deletion sizes of 9 base pairs (bp), 12bp and 24bp. (Brevet, Arcila and Ladanyi, 2010 and Cheng *et al.,* 2012).

d) <u>EGFR and lung cancer subtypes</u>

Numerous authors have reported on the prevalence of EGFR mutations in NSCC. The literature shows a much higher prevalence of EGFR mutations in AC compared to SCC. It has been suggested that SCCs that have the EGFR mutation may be ADSCC or a solid/ poorly differentiated AC with squamoid features (Rekhtman *et al.,* 2012, Travis *et al.,* 2011).
Similarly, EGFR mutations have not been described in SCLC except in combined tumours where SCLC is combined with an AC component (Tatematsu *et al.,* 2008). Other subtypes such as LC and NSCC (nos.) may also harbour this mutation as shown by Kim *et al.* (2012) in one of the largest studies that assessed the frequency of the three oncogenes EGFR, ALK and KRAS in patients with AC, SCC, LC and NSCC (nos.) who were never smokers. They concluded that the EGFR mutation was most frequent in patients with AC, followed by wild-type (no mutations), then the ALK translocation and lastly, the KRAS mutation. This study also confirmed that

most patients with SCC did not harbour any mutation (wild-type) and that the frequencies of the EGFR mutation and ALK translocation were equal in patients with LC whilst the frequencies of the EGFR and KRAS mutations were equal in patients with NSCC (nos.).

e) EGFR and demographic profile

A particular demographic profile for patients with EGFR mutated lung AC has been described. This includes patients who are young (mostly less than 65 years), female, never/non-smokers and of Asian ethnicity (Paez *et al.,* 2004, Pao *et al.,* 2004 and Shigematsu *et al.,* 2005).

Lung AC with the EGFR mutation was found to be more common in Asians compared to non-Asians. The prevalence of lung AC with EGFR mutations from patients who were from Asia, namely Korea, Taiwan, China and India was 24%, 51% and 38% and 26% respectively (Jang *et al.,* 2009, Huang *et al.,* 2011 and Doval *et al.,* 2013) whilst studies from Europe and America, showed a lower prevalence of 11-19% (Rosell *et al.,* 2009, Smits *et al.,* 2012, Cortes-Funes *et al.,* 2005 and Reinersman *et al.,* 2011).

The degree of exposure to tobacco smoke was found to be inversely related to the presence of the EGFR mutation in patients with lung AC (Tokumo *et al.,* 2005). Huang *et al.* (2011) found that significantly fewer EGFR mutations were found in patients who smoked more than 15 pack years. But although lung AC with the EGFR mutation was found to be more common in females and patients without a smoking history (Tokumo *et al.,* 2005, Rosell *et al.,* 2009 and Kim *et al.,* 2011,), these mutations were also found in some smokers and Korean males with AC (Sun *et al.,* 2012).

Similar results were found in a South African study by Chan (2015) who showed smoking to be inversely proportional to the EGFR mutational status in a study population of 94 females and 75 males from Johannesburg.

Several studies from different countries, namely Europe, North America and Asia, were reviewed by Lindeman *et al.* (2013) in an attempt to formulate molecular testing guidelines for lung cancer patients. The authors confirmed that lung cancer with the EGFR mutation was more common in females who were non-smokers. Similar demographic characteristics were found in patients who had lung cancer with the ALK translocation. The recommendations made by Lindeman *et al.* (2013) state that when selecting patients for EGFR mutation and ALK translocation testing, patients should not be excluded based on their clinical characteristics such as age, sex, ethnicity and smoking history, despite the associations as noted above, as not all patients with the EGFR mutation and ALK translocation will fit into this demographic profile.

Although there was no significant difference in the rate of the EGFR mutation in the African–American population (17%) compared to the American Caucasian population (13%) according to Reinersman *et al.* (2011), the KRAS mutation was found to be more prevalent in American Caucasians. This finding was not confirmed by Araujo *et al.* (2015), who found the rate of the EGFR and KRAS mutation similar in African-American and American Caucasians. Chan (2015) found the EGFR mutational rate to be 61% in South African Caucasians and 19% in South African Blacks.

f) EGFR and morphological profile

A specific morphological profile for lung ACs that have the EGFR mutation has been described and this includes ACs with a predominantly lepidic growth (previously called non-mucinous bronchioloalveolar carcinoma (BAC)) (Erman *et al.,* 2005) and those with micropapillary and papillary growth patterns (Ninomiya *et al.,* 2009 and Inamura *et al.,* 2010). The hobnail cell type (characterized by cells that have cytoplasmic protrusions and a tadpole/hobnail appearance) was also found. These characteristics, according to Ninomiya *et al.* (2009), may be used as good predictors of EGFR mutation in lung AC.

Sun *et al*. (2012) on the other hand, revealed that EGFR mutations were more common in lung AC that had mixed patterns: mixed acinar and lepidic pattern, followed by a mixed papillary and acinar pattern, mixed solid and acinar, micropapillary and acinar patterns when compared to pure mucinous and solid tumours.

In summary, the literature shows that although the lepidic, micropapillary and papillary growth patterns are seen in most lung cancers with the EGFR mutation, mixed growth patterns such as the acinar and solid patterns may be found.

g) EGFR and EGFR TKI therapy

An article entitled “update in lung cancer” published by Spira, Halmos and Powell (2015), states that it is well established that up front EGFR TKI therapy (gefitinib and erlotinib) supersedes the efficacy of platinum based chemotherapy and has become the current standard of care. Patients who

have the two most common EGFR mutations (E746-A750 deletion and the L858R point mutation) show a positive response rate between 48 and 90% following treatment with EGFR TKIs (Ilie *et al.,* 2010).

Treatment involves two options: the first option is preventing ligand binding to the extracellular domain using monoclonal antibodies and the second option is inhibition of intracellular tyrosine kinase activity by TKIs. The monoclonal antibody cetuximab targets the extracellular domain of EGFR, preventing ligand binding, blocking ligand-activated signal transduction and receptor dimerization. The limitation of this pathway is that since only ligand binding is inhibited, autophosphorylation of the tyrosine kinase domain through constitutive activation may still activate downstream pathways and promote carcinogenesis (Cheng *et al.,* 2012).

EGFR TKIs gefitinib (IRESSA®, Astra Zeneca UK) and erlotinib (Tarceva®, OSIL Genentech USA), bind the ATP-binding pocket of the intracellular domain, preventing autophosphorylation and intracellular downstream signalling (Ruschoff *et al.,* 2013) and thus are more effective than the monoclonal antibody cetuximab.

The European Medicines Agency (EMEA) supports the approval of gefitinib for patients with advanced and metastatic AC with the activating EGFR mutations (Ilie *et al.,* 2010) whilst erlotinib is approved for the treatment of AC in Western countries (Eberhard, Giaccone and Johnson, 2008)

Careful selection of patients for gefitinib treatment is important as patients may be at risk of developing major clinical side effects such as acute lung damage and interstitial pneumonia (Inamura *et al.,* 2010).

EGFR TKIs are available in South Africa to a limited number of patients through private medical schemes. The majority of patients in South Africa who are attended to in Government hospitals do not receive EGFR TKI therapy as it is not on the Essential Drug List.

h) Testing for the EGFR mutation

There are several available methods for the detection of EGFR mutation. These methods include molecular and non-molecular (immunohistochemical) techniques. The molecular analyses include DNA sequencing and several indirect methods (see later).

PCR is the most common method used to detect the presence of the EGFR mutation in lung AC (Angulo *et al.,* 2012). Advantages of PCR include the ability to identify both common, uncommon and new activating EGFR mutations and the ability to identify mutations that may carry resistance to EGFR TKI therapy, such as the exon 20 EGFR insertion, the KRAS mutation which confers primary resistance and the T90M mutation for acquired resistance (Otto *et al.,* 2012).

The disadvantages of PCR are:

1. It is an expensive procedure due to expensive tests and reagents (NHLS cost as at 27/02/2017 = R634.41, value added tax inclusive)
2. It is time consuming with a prolonged turnaround time
3. It is not widely available in pathology laboratories

4. It is a complex procedure required specially trained personnel
5. Its sensitivity depends on the
    a. quality of the extracted DNA
    b. tumour cellularity: mutation must be present in approximately 20% of all the DNA in the sample to allow for adequate macro-microdissection. The mutation may be missed if the sample contains <25% of tumour cells (Ladanyi and Pao, 2008)
    c. contamination of sample by non-neoplastic material such as mucin, lymphocytes, and non-neoplastic cells
    d. contamination with non-mutated allele
    e. decalcification, which usually results in DNA degradation affecting mutation detection

Most of the methods used in the literature are PCR based and perform best using fresh tissue instead of formalin fixed wax embedded tissue. Formalin fixation can lead to nucleic acid degradation and decreased amplicon length resulting in artefacts. PCR from formalin fixed wax embedded tissue requires a larger amount of tumour sample with a high ratio of tumour tissue to normal tissue content to reliably detect tumour-specific mutations. Macro or microdissection may be used to increase the ratio of tumour to normal tissue. This may not be possible on small biopsy samples such as endobronchial biopsies.

Indirect methods include:

a. High resolution melting analysis
b. Fragment analysis
c. Restriction fragment length polymorphism
d. The amplification refractory mutation system

e. Mass spectrometry based MassArray platform
f. Flourescence in situ hybridization (FISH)
g. Chromogenic in situ hybridization (CISH)

Indirect sequencing methods may have a higher sensitivity compared to direct sequencing and may therefore be used on specimens with low cellularity. These methods are very sophisticated, not readily available and require technical expertise (da Cunha Santos *et al.,* 2011). FISH requires specialized equipment for dark field fluorescent microscopy and is limited to the detection of EGFR copy number and not the detection of specific mutations.

In summary, there is a need for a method of detecting the EGFR mutation that is easy to perform and interpret, a method that requires minimal expertise and equipment, a method that provides results within a short time frame and a method that is available in most routine diagnostic pathology laboratories in South Africa that serve approximately 80% of the South African population. These criteria are fulfilled with immunohistochemistry (IHC).

IHC is a method that localises antigens in tissue sections by using labelled antibodies. The antigen-antibody reaction is visualised by using markers such as florescent dyes, enzymes or colloidal gold. When antigen-antibody binding occurs, a coloured reaction is formed. This indicates a positive result with IHC (Eberhard, Giaconne and Johnson, 2008).

There are three types of immunohistochemical tests for EGFR: total EGFR, phosphorylated EGFR and mutation-specific EGFR. Only testing for the EGFR mutation using mutation specific antibodies is recommended.

In 2009, Yu *et al.* were the first to develop two monoclonal antibodies against the two most common mutations involving the EGFR gene in lung AC, i.e. the SP111 antibody that detects the exon 19 15bp deletion E746-A750 and the SP125 antibody that detects the exon 21 L858R point mutation, in New Zealand rabbits.

IHC has the following advantages:

1. It is used routinely in pathology laboratories
2. Pathologists are familiar with interpretation of the IHC result
3. It is less expensive than PCR (NHLS cost as at 27/02/2017 = R456.29, value added tax inclusive)
4. It is a rapid procedure; results are available within 24 hours
5. It can provide reliable results on limited material. Small biopsy and cytology specimens and metastatic tumours with too few malignant cells may prove technically challenging for micro or macro dissection which is required for molecular tests (Allo *et al.,* 2014). Most patients with NSCC present late when surgery is precluded and thus most diagnoses of NSCC are made on small transbronchial and endobronchial biopsies. This limited material may be the only tissue available for diagnostic, prognostic and predictive testing. IHC, which requires the least amount of tumour tissue, may be very useful in this situation (Angulo *et al.,* 2012).

6. It is reliable even if there is excessive non-neoplastic elements which may obscure the tumour
7. Decalcification does not have any detrimental effect on IHC (Hasanovic *et al*., 2012).

The main disadvantages of EGFR IHC are:

1. Sensitivity and specificity of EGFR IHC compared to PCR. Mutation specific antibodies are specific for the two most common EGFR mutations only and do not detect other mutations in the EGFR gene (such as non 15bp deletions in exon 19, exon 18 G719 point mutation, exon 20 mutations and T790M resistance mutation).
2. The absence of a universally accepted system of scoring positive IHC results.

i) <u>EGFR IHC sensitivity and specificity</u>

Numerous studies were undertaken to determine the reliability of the mutation specific antibodies SP111 and SP125 in detecting the most common EGFR mutations, the E746_A750 deletion in exon 19 and the L858R point mutation in exon 21 respectively, compared to PCR.

Table 2.1 Sensitivity and Specificity of mutation specific antibodies SP111 and SP125

	SP111	SP125
Sensitivity (%)	61 (Ambrosini-Spaltro *et al.,* 2012) 63 (Simonetti *et al*., 2010) 71 (Seo *et al*., 2014) 82 (Kato *et al*., 2010) 85 (Brevet, Arcila and Ladanyi, 2010)	75 (Kato) 80 (Seo *et al*., 2014) 95 (Brevet, Arcila and Ladanyi, 2010) 100 (Simonetti *et al*., 2010) 100 (Ambrosini-Spaltro *et al.,* 2012)
Specificity (%)	99 (Seo *et al*., 2014) 99 (Brevet, Arcila and Ladanyi, 2010) 100 (Kato *et al*., 2010) 100 (Simonetti *et al*., 2010) 100 (Ambrosini-Spaltro *et al.,* 2012)	90 (Seo *et al*., 2014) 97 (Kato *et al*., 2010) 99 (Brevet, Arcila and Ladanyi, 2010) 100 (Simonetti *et al*., 2010) 100 (Ambrosini-Spaltro *et al.,* 2012)

As seen in **Table 2.1,** almost all studies confirmed a high specificity of 90-100% and a sensitivity which ranged from 61-100% for the SP111 and SP125 antibodies. The sensitivity of the SP111antibody was lower than the SP125 antibody.

The findings of Seo *et al*. (2014) were supported by several similar studies undertaken by Kato *et al*. (2010), Kitamura *et al* (2010), Kozu *et al*. (2011), Hofman *et al*. (2012) and Hasanovic *et al*. (2012).

The lower sensitivity found with the E746-A750 antibody in several of the above studies may be as a result of the presence of non-15bp deletions. The E746-A750 antibody detects mainly the 15bp deletion (which accounts for approximately 65-75% of deletions in exon 19 whilst it has a lower sensitivity in detecting non-15bp deletions (Brevet, Arcila and Ladanyi, 2010).

j) Scoring for IHC stains with EGFR mutation specific antibodies

A scoring system is used by pathologists when evaluating IHC stained slides, in order to assess staining intensity and distribution of positive stained cells.

Correlation studies using IHC and molecular assays, as shown by Ambrosini-Spaltro *et al.* (2012) and Brevet, Arcila and Ladanyi (2010) showed a poor correlation with the presence of the EGFR mutation and 0/1+ staining patterns. Only IHC scores of 2+ and 3+ correlated well with the presence of the EGFR mutation and were regarded as significant. By disregarding staining patterns of 1+ and only taking into account 2+ and 3+ staining patterns, the positive predictive values were raised to 100% with a minimal reduction in sensitivity. These results were confirmed by several additional studies which further confirmed the usefulness of mutation specific IHC as a screening method to detect patients for EGFR TKI therapy (Hasanovic *et al.,* 2012 and Allo *et al.,* 2014).

While there is no universally accepted scoring system for assessing EGFR IHC, the following scoring system has been recommended by Ambrosini-Spaltro *et al*. (2012), Hofman *et al*. (2012) and Seo *et al.* (2014):
0: no staining/focal staining in <10% tumour cells
1+: faint diffuse cytoplasmic staining in >10% tumour cells
2+: moderate cytoplasmic staining with focal membranous reinforcement
3+: strong cytoplasmic staining with focal or diffuse membranous reinforcement

k) Intratumoural heterogeneity

Opinion is divided on whether intratumoural heterogeneity is significant and may affect testing for the EGFR mutation. Whilst Eberhard, Giaccone and Johnson (2008), Taillade *et al.* (2007) and Kitamura *et al.* (2010) found that intratumoral heterogeneity may account for false negative results with IHC and therefore suggest multiple biopsies from different areas of the tumour be taken, this was not supported by Sun *et al*. (2012) and not recommended by Lindeman *et al*. (2013).

l) International recommendation

Following a consensus meeting in 2013 with the College of American Pathologists, the International Association for the Study of Lung Cancer and the Association for Molecular Pathology, Lindeman *et al*. (2013) published guidelines for molecular testing of patients with lung cancer that have the EGFR mutation and ALK translocation as a step towards targeted therapy using Tyrosine Kinase Inhibitors.

Although PCR is recommended for the detection of the EGFR mutation, Lindeman *et al*. (2013) state that if “scoring cut offs are set stringently to ensure a high positive predictive value, IHC with EGFR mutation-specific antibodies could be used as an initial screen to identify most patients who are candidates for EGFR inhibitors.” It is thus recommended that EGFR IHC be limited to a screening process. All negative biopsies should be referred for additional molecular analyses whilst strongly positive biopsies may be referred for EGFR TKI therapy. These recommendations were supported by several other studies (Ambrosini *et al.,* 2012, Brevet, Arcila and Ladanyi, 2010 and Seo *et al.,* 2014).

iii) Anaplastic lymphoma kinase gene (ALK)

a) Background

The third important genetic alteration seen in 2-7% of lung AC cases involves the Anaplastic Lymphoma Kinase (ALK) gene (Wong *et al.,* 2009 and Paik *et al.,* 2012). ALK is expressed in the central and peripheral nervous systems, testes, skeletal muscle, basal keratinocytes and small intestine. ALK plays a role in neuronal development and differentiation during embryogenesis and its expression remains low throughout adult life (Shackelford *et al.,* 2014).

b) Genetic structure of ALK

The ALK protein is a member of the insulin receptor superfamily of tyrosine kinase receptors and resides on chromosome 2p23. The ALK protein consists of an extracellular ligand-binding domain, a transmembrane domain and a single intracellular tyrosine kinase domain.

c) ALK activation

ALK activation occurs through three mechanisms: 1) formation of fusion proteins, 2) ALK over expression and 3) ALK point mutations. Morris *et al*. (1994) were the first to identify the NPM-ALK translocation in anaplastic non-Hodgkin lymphoma and since then, ALK translocations and mutations have been described in several tumours. Some solid tumours (inflammatory myofibroblastic tumours, squamous cell carcinomas and NSCCs) activate ALK signaling by creating unique oncogenic fusions of the ALK gene with a variety of partners through chromosomal translocation (Hallberg and Palmer, 2013). Up to 20 variants of the ALK translocation

have been identified (Shaw and Engelman, 2013). The most common fusion in NSCC results from the joining of exons 1-13 of echinoderm microtubule associated protein-like 4 (EML4) to exons 20-29 of ALK forming the EML4-ALK fusion gene.

Soda *et al.* (2007) were one of the first to describe the ALK translocation resulting in the EML4-ALK fusion gene. This fusion gene product leads to dimerisation of the ALK tyrosine kinase domain and subsequent proliferation, changes in cytoskeleton, migration and survival of tumour cells (Shaw *et al.,* 2011 and Soda *et al.,* 2007 and 2013).

The presence of the ALK translocation is mutually exclusive of EGFR and KRAS mutations, with the exception of individual case reports. In a study by Boland *et al.* (2013) which evaluated 25 cases of lung AC with the ALK translocation, a single case was also found to have the EGFR mutation and four cases had MET mutations.

d) ALK and lung cancer subtypes

The ALK translocation has been found predominantly in AC; however it has also been described in ADSCC and in a very small percentage of SCC (Wong *et al*., 2009, Rodig *et al*., 2009, Ali *et al*., 2013 and Paik *et al*., 2012).

e) ALK and demographic characteristics

Numerous studies have shown a strong association of the ALK translocation with young patients and patients who were never smokers or

were light smokers (<10 pack years) (Inamura *et al.,* 2010, Wong *et al.,* 2009, Paik *et al.,* 2012 and Ali *et al.,* 2013).

There are conflicting results in the literature regarding sex; the majority of patients of Asian ethnicity appear to be female whilst Western countries including the United States of America (USA) show a predominance of males (Shaw *et al.,* 2009). Despite the presence of the ALK translocation in patients with certain demographic profiles as described above, Rodig *et al*. (2009) also found ALK rearranged tumours in older patients and patients with a smoking history. Wang *et al.* (2014) found that the ALK translocation in their study was not associated with non-smokers.

In summary, demographic characteristics may therefore not necessarily accurately predict the presence of the ALK translocation and testing should not be restricted to patients based on their demographic characteristics. This is similar to the recommendations for testing for the EGFR mutation.

When compared with the demographic characteristics of patients with lung cancer who have the EGFR mutation, the absence of a smoking history is the single consistent common characteristic, whilst ethnicity, sex and age are not.

f) <u>ALK and morphological profile</u>

Besides the above association with certain clinical characteristics, the ALK translocation has also been associated with certain AC morphological patterns; the most common includes the solid/sheet-like growth pattern (Rodig *et al.,* 2009, McLeer-Florin *et al.,* 2012 and Popat *et al.,* 2012) whilst

the cribriform (McLeer-Florin *et al.,* 2012), papillary (Hutarew *et al.,* 2014), acinar (Wong *et al.,* 2009, Hutarew *et al.,* 2014) and lepidic (Yamamoto *et al.,* 2012) patterns have also been described. Signet ring morphology also predominated in most of these studies, including the study by Zhang *et al.* (2014).

Both EGFR mutated and ALK translocated ACs may have papillary growth patterns. The hobnail cell type is more commonly seen in lung ACs with the EGFR mutation whilst the signet ring cell type is more common in lung ACs that have the ALK translocation.

g) <u>ALK translocation prognosis and treatment</u>

The ALK translocation was not found to be a favourable prognostic factor and ALK positive patients have a generally poor outcome, similar to that of the general population of NSCC patients with advanced cancer (Shaw *et al.,* 2011 and Shaw and Engelman, 2013). The presence of the ALK translocation is associated with increased lymph node metastasis (Paik *et al.,* 2012).

NSCCs with the ALK translocation are resistant to EGFR TKI (erlotinib and gefitinib) therapy (Shaw *et al.,* 2009) but show an increased sensitivity to ALK TKI (crizotinib) therapy. Patients with lung AC who have the ALK translocation and are treated with crizotinib have an overall response rate of 57%. 72% of the patients treated were found to have a progression-free survival of more than 6 months and 92% of patients treated showed tumour shrinkage (Kwak *et al.,* 2010).

Crizotinib is currently approved by the USA Food and Drug Administration (FDA) for use in patients with advanced NSCC with the ALK translocation and the use of this drug has been further supported by the 2013 guidelines of the American Society of Clinical Oncology (ASCO), the European Society for Medical Oncology and the National Comprehensive Cancer Network (NCCN) (Lindeman *et al.,* 2013).

Second generation ALK inhibitors (ceritinib and alectinib) showed similar results to the first generation ALK inhibitor (crizotinib) particularly regarding central nervous system (CNS) penetration. Most relapses on crizotinib occurred in the CNS (Spira, Halmos and Powell, 2015).

h) Testing for the ALK translocation

There are three methods available to detect the ALK translocation: fluorescence in situ hybridisation (FISH) assay using the dual labelled break apart probes, reverse transcriptase polymerase chain reaction (RT-PCR) and IHC (Zhang *et al.,* 2014 and Wang *et al*., 2013).

FISH is used to detect chromosomal location and copy number of specific genes in tissue sections. This method uses fluorescence-tagged DNA probes that correspond to the gene to detect all cellular copies of the gene on tumour serial sections by fluorescence microscopy. FISH is the method that is being used in initial clinical trials to detect the ALK translocation and has been FDA approved for this purpose. The main advantage is the use of archival material for analysis.

There are however many disadvantages with FISH analysis:

1. Interpretation of the result requires special skill as the signal may be subtle and easily missed leading to false negative results
2. A minimum number of neoplastic cells is required to be present in the sample in order to yield a positive result that can be detected
3. FISH does not allow one to differentiate neoplastic tissue from non-neoplastic tissue so there may be uncertainty regarding the origin of the signal seen
4. FISH requires dark-field fluorescence microscopy which is not routine equipment in a laboratory
5. FISH has a longer turnaround time compared to IHC

Although RT-PCR has been described by Wang *et al*. (2013) as the most sensitive method for the detection of the ALK translocation as it involves the use of unique primers that only hybridize with the specific fusion chimeric transcript, this method is not ideal as a screening tool as the specific fusion partners are unknown. The second main disadvantage is that RT-PCR requires high quality RNA, which may be difficult to obtain from wax embedded tissue, where the RNA is usually substantially degraded. It may also be difficult to confirm the presence of tumour cells in the PCR test sample (Murakami, Mitsudomi and Yatabe, 2012 and Wang *et al*., 2013). RT-PCR is not available in many routine pathology laboratories.

Zhang *et al.* (2014) detected the ALK translocation by all three methods above and found the detection rate of IHC (35.7%) comparable to that of FISH (35.5%) and better than RT-PCR (27.9%).

IHC is widely available in NHLS Anatomical Pathology laboratories and is used as the preferred method for screening and diagnosis in routine pathology practices as discussed before. False negative results with ALK IHC were found to be highly dependent on the type of antibody clone and detection method used (Murakami, Mitsudomi and Yatabe, 2012). ALK IHC using high affinity antibodies and the use of an OtiView Amplification Kit greatly improved the sensitivity of the ALK IHC (Wallander *et al., 2012,* Hutarew *et al*., 2014 and Zwanepoel *et al*., 2014). ALK IHC using highly specific ALK antibodies has the added advantage of being able to detect complex ALK translocations which may be missed with RT-PCR and FISH, as shown by Mino-Kenudson *et al*. (2010) and Peled *et al*. (2012), respectively.

Chromogenic in situ hybridization (CISH), which uses bright-field light microscopic techniques to assess gene copy number is an alternative to FISH and IHC. CISH is accurate and reproducible and has several advantages over FISH and IHC:

1. CISH is easier to use because it uses bright-field microscopes instead of fluorescence microscopes which are more expensive
2. CISH reagents are more stable than FISH reagents which fade over time preventing a sample from being examined more than once
3. CISH does not require a high-resolution digital camera to capture micrographs of the sample before the fluorescence fades as with FISH
4. CISH allows the tissue sample to be visualised, whilst FISH does not

5. IHC may have false-negative and false-positive results. The CISH reference probe will only be positive if the assay has worked. If there is no signal for the reference probe, the assay has failed

A recent study by Nitta *et al.* (2013) showed promising results using Bright field dual ALK IHC-in situ hybridization assay.

i) <u>ALK IHC sensitivity and specificity compared with FISH</u>

There are several different ALK antibody clones. These include ALK1, ALK01, SP8 and high affinity clones, D5F3 (from Ventana OptiView) and 5A4. Numerous authors have confirmed a much higher sensitivity of the high affinity clones compared to other clones. The high affinity clones stained ALK translocated cases with strong intensity without false positive or false negative cases (Wallander *et al., 2012,* Hutarew *et al*., 2014 and Zwanepoel *et al*., 2014). Similar studies by Boland *et al.* (2009), Paik *et al.* (2012), Minca *et al.* (2013), Shan *et al*. (2014) and Wang *et al*. (2014) confirmed 100% sensitivity with a specificity range of 95% to 100% using the high affinity ALK antibody clones with good correlation between ALK IHC and ALK FISH.

Detection of the ALK translocation by ALK IHC using the high affinity antibody clones leads to a significant decrease in the number of cases referred for FISH analysis, resulting in a significant decrease in time, cost and work, without compromising diagnostic quality and accuracy.
Based on these findings, we chose the high affinity D5F3 ALK antibody clone for this study.

j) Scoring of ALK immunohistochemical staining pattern

The scoring system used for ALK IHC as determined by interpretation guidelines from Roche Ventana is based on the following criteria:

0 = no cytoplasmic staining

1+ = weak intensity cytoplasmic staining

2+ = medium intensity cytoplasmic staining

3+ = strong granular cytoplasmic staining

In order to maintain a high degree of specificity and sensitivity, Roche Ventana recommend that biopsies showing 0, 1+ and 2+ staining be regarded as negative and only biopsies showing strong granular cytoplasmic staining be regarded as positive as they correlate well with ALK FISH.

k) International recommendation

The European Union has approved the use of IHC as a standard diagnostic test for ALK positive lung cancers and the Ventana ALK (D5F3) IHC assay has been approved to detect the ALK translocation in the European Union and some Asian countries (Reck *et al.,* 2013).

The general recommendation by numerous authors is to use ALK IHC as an initial screening method for the detection of the ALK translocation. There is good correlation with a 3+ IHC result and the presence of the ALK translocation whilst biopsies that show less intense staining may or may not have the translocation. Paik *et al.* (2012) showed that all 3+ biopsies were FISH positive, 65% of 2+ biopsies were FISH positive and all of the 1+ biopsies were FISH negative for the ALK translocation.

Based on this and similar studies, the recommendation by numerous authors (Lindeman *et al*., 2013, Cabillic *et al*., 2014, Selinger *et al*., 2013 and Shan *et al*., 2014) is that cases that show a positive ALK IHC result be subjected to FISH analysis for confirmation of the ALK translocation although ALK immunohistochemistry may be considered as a screening methodology to select specimens for ALK FISH testing if carefully validated. Negative ALK IHC biopsies from patients with demographic characteristic or morphological features suggestive of the ALK translocation (such as never smokers with advanced lung AC or lung AC with signet ring morphology) and are found to be EGFR and KRAS wild type should be referred for ALK FISH analysis for the ALK translocation (Selinger *et al.,* 2013). Cabillic *et al*. (2014) concluded that this approach will have significant economic impact, with a shorter turnaround time compared to screening all patients with lung AC using ALK FISH.

iv) Other mutations

Additional mutually exclusive oncogenes include Mesenchymal-epithelial transition factor (MET) tyrosine kinase and avian erythroblastosis oncogene B (ERBB2). MET overexpression, amplification and point mutation has been identified in NSCC. MET amplification is known to be associated with EGFR TKI therapy resistance (Spira, Halmos and Powell, 2015). Other mutations include BRAF, HER2, LKB1, P53, NRAS, PIK3CA and TTF1 amplification (Herbst, Heymach and Lippman, 2008 and Greulich, 2010).

v) Stepwise approach for mutational analysis

An algorithmic approach towards mutational analysis of patients with lung cancer is suggested in **Figure 2.2**. The approach begins with exclusion of

the KRAS mutation, since this mutation is the most common mutation found in patients with lung AC and a positive KRAS result will preclude further mutational analysis (KRAS mutation is mutually exclusive of the EGFR mutation and ALK translocation). The use of a rapid and inexpensive KRAS assay is supported by Lindeman *et al*. (2013) provided adequate tissue is available to allow for subsequent EGFR and ALK testing if needed.

Biopsies that are negative for the KRAS mutation will then be submitted for EGFR and ALK IHC. All strongly positive EGFR IHC biopsies may be forwarded for EGFR TKI (gefitinib or erlotinib) therapy whilst negative and weak to moderately positive EGFR IHC biopsies require EGFR PCR. Lindeman *et al*. (2013) recommend that all biopsies, even those showing positive staining with EGFR IHC be referred for EGFR PCR confirmation. They do however also state that IHC with EGFR mutation-specific antibodies may be used as an initial screen to identify patients who may be candidates for EGFR TKI therapy provided, “scoring cut offs are set stringently to ensure a high positive predictive value.” Seo *et al*. (2014) recommend direct referral for EGFR TKI therapy of these positive biopsies without PCR confirmation.

All ALK IHC positive biopsies that show strong granular cytoplasmic staining may be forwarded for ALK TKI (crizotinib) therapy whilst weak to moderate positively stained biopsies and specific negative biopsies (biopsies from patients who have demographic features and ACs that have morphological patterns suggestive of the ALK mutation) need to be referred for ALK FISH analysis.

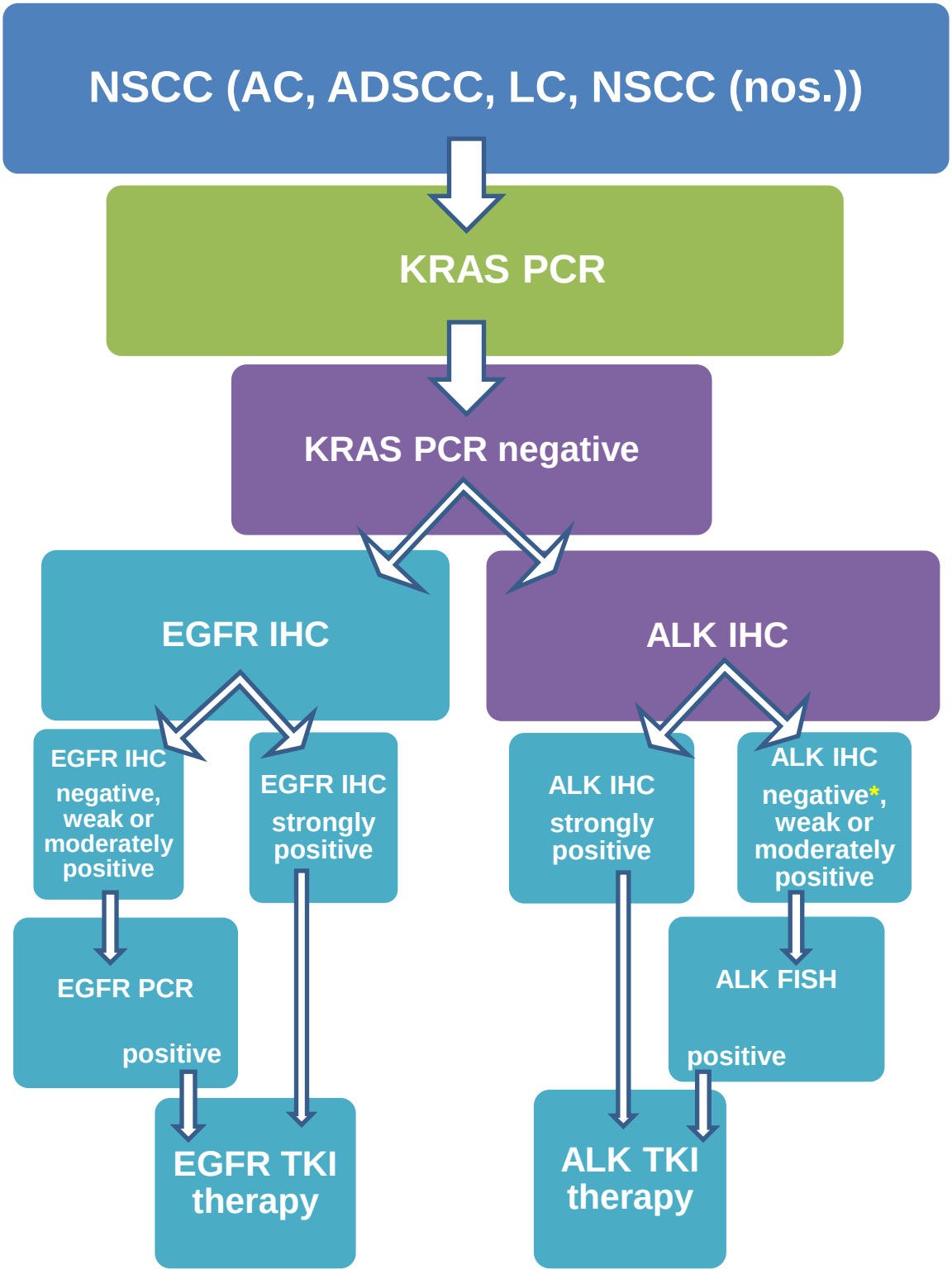

Figure 2.2 Diagnostic algorithm for the mutational analysis of patients with lung cancer

*Only ALK negative biopsies from patients with suggestive demographic characteristics & ACs with certain morphological patterns

Chapter 3: METHOD

3.1 Study type

3.2 Target population and sampling technique

3.2.1 Target population

3.2.2 Sampling technique

3.3. Data collection and Materials

3.3.1 Reliability of data

3.3.2 Selection of representative cases

3.3.3 Data collection

3.4 IHC

3.4.1 Slide preparation for IHC

3.4.2 EGFR and ALK IHC methodology

3.4.3 IHC analysis

3.5 Ethics

3.6 Statistical analysis

Method

3.1 Study type: Consecutive descriptive case series.

3.2.1 Target population: All patients from CMJAH and HJH who had biopsies that were submitted to the pathology division of the NIOH, between the period of 1st January 2008 and 30th June 2014.

3.2.2 Sampling technique:

- Study setting: prior to August 2016 when the computerised TrakCare system was implemented, all histology case reports at the NIOH were filed together with the requisition and bronchoscopy reports. These cases did not receive SNOMED codes. Immunohistochemistry was

requested from the CMJAH NHLS laboratory when required, however a systematic approach for ordering IHC was not followed. The majority of specimens received at the laboratory were for diagnostic purposes and were therefore small specimens rather than resection specimens following curative surgery.

- All biopsy reports received at the NIOH from 1st January 2008 until 30th June 2014 were reviewed manually from the NIOH case report files.
- Neoplastic reports were separated from non-neoplastic reports.
- Neoplastic reports were then separated into AC, SCC, ADSCC, LC, NSCC (nos.), combined tumours (with an adenocarcinoma component), SCLC and "Other". (Other= mesothelioma, sarcoma, lymphoma, salivary gland tumours, metastatic carcinomas, thymoma, hamartoma and cytology specimens).

3.3.1 Reliability of data

- The original diagnosis of lung AC, SCC, ADSCC, LC, NSCC (nos.), combined tumours (with an adenocarcinoma component) and SCLC were confirmed by reviewing the haematoxylin and eosin (H&E) sections, special stains and immunohistochemistry and by ordering additional immunohistochemistry where necessary, as determined using diagnostic criteria described in the 2015 *WHO Tumours of the Lung, Pleura and Heart Classification* (Travis *et al*., 2015) and classified into histological subtypes according to the International Association for the study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) (Travis *et al*., 2011) classification.

Lung cancer cases were sub-classified using the following criteria:

AC = positive TTF1 and/or Napsin A staining of malignant cells, irrespective of P63 and CK5/6 staining

SCC = malignant cells that show definite squamous differentiation, represented by intercellular bridges and/or cytoplasmic keratinization OR negative TTF1 and/or Napsin A staining with diffuse positive P63 and/or CK5/6 staining

ADSCC = a carcinoma comprising separate adenocarcinoma (confirmed with TTF1 positive and/or Napsin A positive malignant cells) and squamous carcinoma (P63 and/or CK5/6) components

LC = a resection specimen with an undifferentiated carcinoma that shows negative TTF1 and/or Napsin A staining and negative P63 and/or CK5/6 staining of malignant cells

NSCC (nos.) = a small biopsy (endobronchial/transbronchial/ transthoracic/ pleural) with an undifferentiated carcinoma that shows negative TTF1and/or Napsin A staining and negative P63 and/or CK5/6 staining of malignant cells

Combined tumour (with an AC component) = a biphasic lung cancer showing morphological and immunohistochemical characteristics of both Small cell lung cancer and lung AC

SCLC = carcinoma with morphological characteristics of a small cell neuroendocrine carcinoma and positive staining with neuroendocrine markers

AC with a lepidic growth = AC with a predominant lepidic growth AND positive TTF1 or Napsin A stain

3.3.2 Selection of cases

- All biopsies with the diagnosis of AC, ADSCC, LC, NSCC (nos.) and combined tumours (with an adenocarcinoma component) were selected. SCC and SCLC biopsies were excluded.
- The H&E stained slide of each of the selected cases were reviewed to ensure representation of malignant cells. Biopsies with very sparse representation of malignant cells (less than 5 cells per group or less than 5 isolated cells) were excluded.
- Tissue blocks from each biopsy were retrieved. Biopsies with missing tissue blocks were excluded.

3.3.3 Data collection

- A data base sheet was prepared as follows:
    - Each case was allocated a study number beginning with biopsies selected from the 1st of January 2008 and ending with biopsies from the 30th June 2014
    - The original pathology number was retained
    - The demographic data i.e. race, age and sex obtained from the requisition form and /bronchoscopy report submitted with the specimen were recorded
    - The hospital that submitted the biopsy (CMJAH or HJH) was recorded
    - The biopsy type (transbronchial, endobronchial, transthoracic, open lung (wedge resection or lobectomy), pleural, lymph node or mediastinal) as indicated on the requisition form and /bronchoscopy report was recorded

- The smoking history was obtained from the requisition form and /bronchoscopy report. Medical records were accessed, where available, for cases without a smoking history
- The pathological diagnosis (AC, ADSCC, LC, NSCC (nos.) or combined tumours (with an AC component) was recorded

3.4.1 IHC slide preparation

- 5 recut sections at 4 microns were made from the tissue block of each case for the three IHC stains (EGFR SP111, EGFR SP125 and ALK D5F3) with 2 spare slides

3.4.2 EGFR and ALK immunohistochemistry

- Roche Ventana primary antibodies with the Ventana detection kits and a Ventana BenchMark XT and BenchMark ULTRA automated slide stainer were used, according to the manufacture's protocol
- IHC for the EGFR mutations included mutation specific antibodies that were the ready to use rabbit monoclonal anti EGFR E746-A750 (clone SP111, Ventana Medical Systems, Inc.) and the anti-EGFR L858R antibodies (clone SP125, Ventana Medical Systems, Inc.).
- 4µm thick sections were cut and transferred to poly-l-lysine coated adhesive slides. The slides were baked on the Benchmark GX for 8 minutes at 60°C. The slides were then deparaffinised using EZ Prep (Ventana Medical Systems, Inc.) at 75°C for 4 minutes. Heat antigen retrieval was performed using Ventana CC1 containing Tris/Borate/EDTA at pH 8-8.5. SP111 was incubated for 72 minutes and SP125 for 64 minutes. The antibodies were supplied as pre-dilutes from Ventana and incubated at 37°C for 16 minutes (for

SP111) and 16 minutes (for SP125). Ultraview DAB detection kit was used for visualisation of the antibodies.

- ALK D5F3 IHC for the ALK translocation (the EML4-ALK fusion protein) was purchased from Roche diagnostic laboratory.
- For ALK (D5F3) immunohistochemistry, the sections were baked on the Benchmark GX for 12 minutes at 65 °C, then deparaffinised with EZ Prep from Ventana at 75 °C for 4 minutes. Heat antigen retrieval was applied on the sections using Ventana CC1 incubated for 92 minutes at 100 °C. ALK (D5F3) was supplied pre-diluted from Ventana and incubated at 37 °C for 32 minutes. OptiView detection kit and amplification kit (Ventana /Roche) were used for visualisation of the antibody, to enhance the intensity of the staining and eliminate artefacts. The OptiView amplification kit builds a molecular tree with DAB chromogen bound to an amplification multimer that is linked to multiple haptens on top of the primary antibody. The signal intensity is thus stronger than conventional stained slides, without background staining. This allowed for strong clean signals, allowing confident identification of positive and negative samples, obviating the need for a subjective IHC scoring based on staining intensity and percentage of stained cells.
- These tests were performed in the presence of appropriate positive and negative controls. Positive controls for the EGFR mutation were provided courtesy of Dr C Maske of Lancet laboratory on biopsies that were proven to harbor the respective mutation using PCR. A section of the appendix, courtesy of Mr A Lobanji of CMJAH NHLS laboratory, was used as a positive control for the ALK fusion protein

(Roche diagnostics recommend the use of positively stained neural tissue within the wall of the appendix as a positive ALK control).

- Negative controls were provided by omission of the primary antibody and incubation with immunoglobulins of the same species.

3.4.3 IHC analysis

- EGFR SP111 and SP125:

Scoring system as recommended by Ambrosini-Spaltro *et al*. (2012), Hofman *et al*. (2012) and Seo *et al.* (2014):
0: no staining/focal staining in <10% tumour cells
1+: faint diffuse cytoplasmic staining in >10% tumour cells
2+: moderate cytoplasmic staining with focal membranous reinforcement
3+: strong cytoplasmic staining with focal or diffuse membranous reinforcement
Negative EGFR IHC= 0 and 1+; Positive EGFR IHC= 2+ and 3+

- ALK D5F3 IHC:

Scoring as per guidelines by Roche Ventana:
0 = no cytoplasmic staining
1+ = weak intensity cytoplasmic staining
2+ = medium intensity cytoplasmic staining
3+ = strong granular cytoplasmic staining

- The staining pattern (membranous and/or cytoplasmic) was recorded
- The extent of the staining (patchy or diffuse) was recorded
- The nature of the stain (granular or homogenous cytoplasmic) was recorded.

- The original H&E stained slide for all positive biopsies was reviewed and the morphological growth pattern (acinar/ lepidic/ papillary/ solid/ signet/ cribriform/ micropapillary) was recorded. Carcinomas with mixed patterns were also recorded.

3.5 Ethics

Ethics clearance was received from the University of the Witwatersrand Human research ethics committee, clearance certificate no M140943 (Annexure 9).

3.6 Statistical analysis

Data management and analysis were done using Stata version 14.1 software. Means and standard deviations were calculated to summarize the age variable by sex and outcome variable. A vertical graph box was drawn to describe the dispersion of age of the participants over the categories of the outcome variable (EGFR and ALK). The Student t-test with equal variances was used to test equality of age means between the negative and positive EGFR and ALK groups at 0.05 level of significance. The immediate two-sample proportion test, using EGFR and ALK as the group variable, was used to compare proportions of different levels of categorical variables between the negative and positive EGFR and ALK groups at 0.05 level of significance, and as such testing the hypothesis of equality of proportions. The two-sample proportion test was chosen because smoking status and race are categorical variables. A bivariate exact logistic regression was used to estimate odds ratios for factors associated with the EGFR and ALK variable. The exact logistic regression was chosen instead of the regular logistic regression because of the small

sample size of this study and because some of the cells formed by the outcome (EGFR and ALK) and the independent variables sex, race and smoking history had no observations. P-values were generated to assess the significance of the association between the outcome and the covariates at 95% confidence level.

Chapter 4: RESULTS

During the study period 3901 biopsies were received and after exclusion (see **Figure 4.1**), 111 biopsies were available for assessment of mutational status.

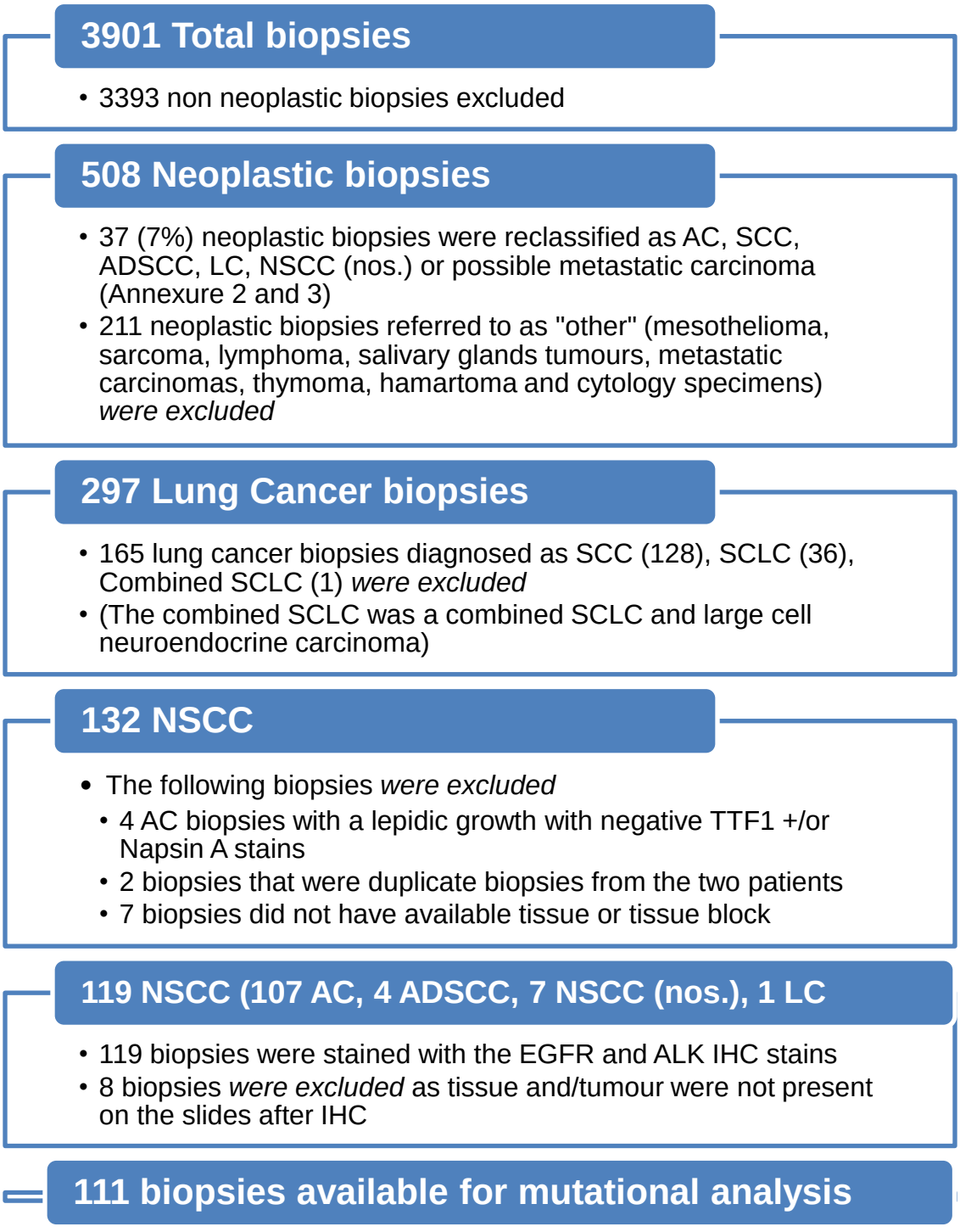

Figure 4.1 Vertical flow diagram on selection of cases for EGFR and ALK IHC

Of all 119 patients with NSCC, most patients were Black (61%) and males (61%). There were no Asians. Adenocarcinoma comprised 90% of the histological subtype (**Table 4.1**).

Table 4.1 Demographic data, biopsy type, smoking and occupational history on 119 NSCC biopsies

	(n) (Total 119)	(%)
Hospital		
CMJAH	86	72
HJH	33	28
Race		
Black	72	61
White	35	29
Other (coloured)	12	10
Sex		
Male	72	61
Female	47	39
Age	mean 57 (SD 11.5)	
< 65 years	91	77
> 65 years	27	23
Biopsy type		
Small biopsies*	75	63
Large biopsies**	9	8
Pleural biopsies	33	28
Lymph node biopsies	2	2
Occupational history		
Not available	107	90
Miner^	8	7
Other^^ and Non-miner	4	3
Smoking history		
Not available	18	15
ExSmoker	6	5
Smoker	71	60
Non-smoker	24	20
Biopsy diagnosis		
AC	107	90
ADSCC	4	3
LC	1	1
NSCC (nos.)	7	6

*Small biopsies = endobronchial biopsies (52) and transbronchial biopsies (12), transthoracic core biopsies (9) and mediastinal biopsies (2). **Large biopsies = wedge resections (6) and lobectomy (3). ^Miner = asbestos (2), platinum (2), coal (1), gold (1), commodity unknown (2). ^^Other = Flour mill worker

Of the 111 biopsies available for testing, there were 10 biopsies (9%) that showed positive staining with the EGFR IHC and 8 biopsies (7%) that showed positive staining with the ALK IHC (**Table 4.2**). All biopsies that showed positive staining with the EGFR and ALK IHC were adenocarcinomas. None of the other NSCC subtypes showed positive staining, although the number of biopsies in each of the other subtypes was small.

Table 4.2 Mutational status by lung cancer type

Lung cancer type	EGFR SP111 + n(%)	EGFR SP111 - n(%)	EGFR SP125 + n(%)	EGFR SP125 - n(%)	**EGFR Total + n(%)**	**EGFR Total - n(%)**	**ALK +n(%)**	**ALK -n(%)**
AC	8(8)	92(92)	2(2)	100(98)	**10(10)**	**90(90)**	**8(8)**	**92(92)**
ADSCC	0	4	0	4		**4**	**0**	**4**
LC	0	2	0	2		**2**	**0**	**2**
NSCC (nos.)	0	5	0	5		**5**	**0**	**5**
Total n(%)	8(7)	103(93)	2(2)	111(98)	**9(10)**	**101(91)**	**8(7)**	**103(93)**

The majority of biopsies were from male patients (63.06%) who were Black (60.06%) (**Figure 4.2**). The mean age was 56.84 ± 9.75 years among males and 58.71 ± 10.88 years among females. 67.57% of the participants had a positive history of smoking while 13.5% did not have any smoking information available.

Refer to Annexure 4a and 4b for details on the demographic data, smoking history and occupational history of biopsies showing positive staining with EGFR and ALK IHC and Annexure 5, histogram showing the parametric distribution of age for EGFR and ALK analysis.

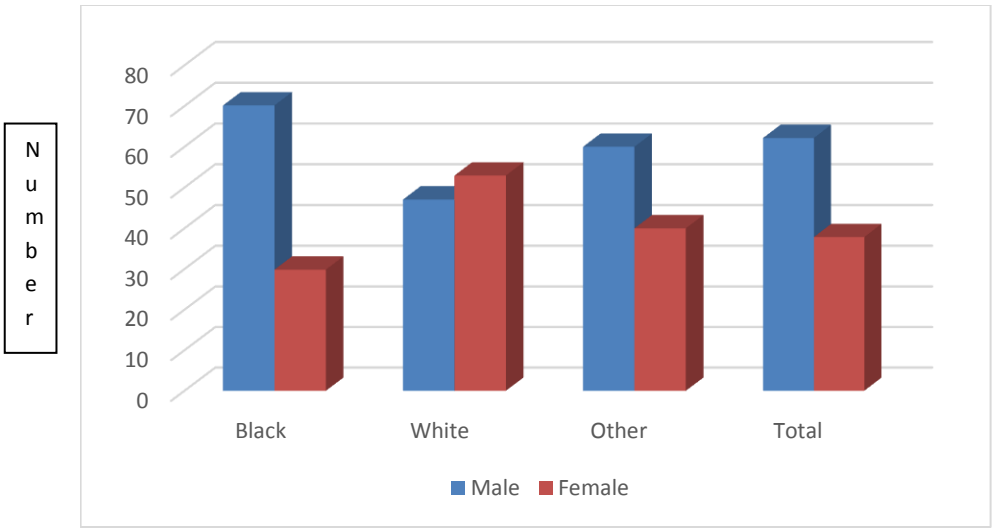

Figure 4.2 Frequency distribution of biopsies for EGFR and ALK IHC by sex and race

EGFR IHC analysis

As shown in **Table 4.3**, no significant difference between mean age among biopsies that had positive and negative EGFR IHC results was found (student t-test, p= 0.83). **Table 4.4** shows the association between the EGFR IHC and the demographic characteristics of patients, smoking history and occupational history. Ten biopsies had a positive EGFR IHC result (9.05%), eight of them were from Black patients. Of the biopsies from patients with a smoking history, 5.33% had a positive EGFR IHC result (two-sample proportion test, p= 0.051). However this association with smoking was not found to be statistically significant when an adjustment for the other features (age, sex and race) was made (odds ratio 1.553, p= 0.378) (**Table 4.5**). Occupational history was not included in the regression model because of the high number of missing values (100 of 111). A single patient with a positive gold mining history was found to be EGFR IHC positive whilst the two patients with asbestos exposure were not EGFR IHC positive.

Table 4.3 Relationship between age, sex and EGFR IHC result

	Male					**Female**					**Total**				
EGFR test	n	mean	SD	min	max	n	mean	SD	min	max	**N**	**mean**	**SD**	**min**	**max**
Negative	64	56.73	9.68	38	76	37	58.73	11.23	28	78	**101**	**57.47**	**10.27**	**28**	**78**
Positive	6	58	11.37	36	67	4	58.5	7.94	48	67	**10**	**58.20**	**9.64**	**36**	**67**
Total	**70**	**56.84**	**9.75**	**36**	**76**	**41**	**58.71**	**10.88**	**28**	**78**	**111**	**57.54**	**10.18**	**28**	**78**

Table 4.4 Association between EGFR IHC, demographic characteristics, smoking history and occupational history

Characteristics		**EGFR Negative n(%)**	**EGFR Positive n(%)**	**Total n(%)**
	Biopsies	**101(90.99)**	**10(9.01)**	**111(100)**
Age	mean (SD)	57.47 (9.78)	58.2 (9.64)	57.54(10.18)
Sex	Male	64 (91.43)	6(8.57)	70(100)
	Female	37 (90.24)	4(9.76)	41(100)
Race	Black	59(88.06)	8(11.94)	67(100)
	White	33(97.06)	1(2.94)	34(100)
	Other	9(90)	1(10)	10(100)
Smoking History	Smoker	71(94.67)	4(5.33)	75(100)
	Non-smoker	16(76.19)	5(23.81)	21(100)
	Unknown	14(93.33)	1(6.67)	15(100)
Occupational History	Not available	91(91.00)	9(9.00)	100(100)
	Miner (nos.)	2(100)	0	2(100)
	Non-miner	2(100)	0	2(100)
	Asbestos exposure	2(100)	0	2(100)
	Platinum miner	2(100)	0	2(100)
	Flour mill worker	1(100)	0	1(100)
	Gold miner	0	1(100)	1(100)
	Coal miner	1(100)	0	1(100)

Table 4.5 Bivariate exact logistic regression of features associated with EGFR IHC

Independent variable	odds ratio	p-value	95% confidence interval	
Age	1.007	0.856	0.945	1.077
Sex	1.152	1.000	0.224	5.223
Smoking History	1.553	0.378	0.61	3.652
Race	0.57	0.513	0.111	1.883
Occupational History	1.162	0.5303	0.664	1.729

ALK IHC analysis

The mean age of the patients tested for ALK IHC was 56.91 ± 9.81 years among males and 58.81 ± 10.76 years among females (**Table 4.6**). The difference between mean age among biopsies that had positive and negative ALK IHC results was not significant (student t-test, p= 0.071) (**Table 4.7).** Among these, 50% of patients had a history of smoking, although there were no significant difference in the proportions of smokers among those who tested positive to ALK and those who tested negative. Nevertheless, there was significant difference between the proportions of Black patients among those who tested positive to ALK and those who tested negative (two-sample proportion test, p= 0.017) even when race was corrected against the other features (sex, age and smoking history) (odds ratio 0.155, p= 0.03) (**Table 4.8**). The regression model confirms the association between age and ALK IHC (odds ratio 0.941, p= 0.081), which is not significant. Occupational history was not included in the regression model because of the high number of missing values (100 out 111). The two biopsies from patients with asbestos exposure were not ALK IHC positive.

Table 4.6 Summary measures of age by ALK IHC and sex

	Male					Female					Total				
ALK test	n	mean	SD	min	max	n	mean	SD	min	max	**N**	**mean**	**SD**	**min**	**max**
Negative	63	57.08	9.72	36	76	40	59.75	9.77	30	78	**103**	**58.13**	**9.78**	**30**	**78**
Positive	6	55.17	11.48	41	71	2	40	16.97	28	52	**8**	**51.38**	**13.59**	**28**	**71**
Total	**69**	**56.91**	**9.81**	**36**	**76**	**42**	**58.81**	**10.76**	**28**	**78**	**111**	**57.64**	**10.18**	**28**	**78**

Table 4.7 Association between ALK IHC, demographic characteristics, smoking history and occupational history

Characteristics		**ALK Negative n(%)**	**ALK Positive n(%)**	**Total n(%)**
	Biopsies	**103(92.79)**	**8(7.21)**	**111(100)**
Age	mean (SD)	58.13 (9.78)	51.38 (13.59)	57.64(10.18)
Sex	Male	63 (91.3)	6(8.7)	69(100)
	Female	40 (95.24)	2(4.76)	42(100)
Race	Black	59(88.06)	8(11.94)	67(100)
	White	34(100)	0	34(100)
	Other	10(100)	0	10(100)
Smoking History	Smoker	70(94.59)	4(5.41)	74(100)
	Non-smoker	20(90.91)	2(9.09)	22(100)
	Unknown	13(86.67)	2(13.33)	15(100)
Occupational History	Not available	92(92.93)	7(7.07)	99(100)
	Miner (nos.)	2(100)	0	2(100)
	Non-miner	3(100)	0	3(100)
	Asbestos exposure	2(100)	0	2(100)
	Platinum miner	2(100)	0	2(100)
	Flour mill worker	0	1(100)	1(100)
	Gold miner	1(100)	0	1(100)
	Coal miner	1(100)	0	1(100)

Table 4.8 Bivariate exact logistic regression of features associated with ALK IHC

Independent variable	Odds Ratio	P-value	95% Conf. Interval	
Age	0.941	0.081	0.876	1.008
Sex	0.528	0.711	0.05	3.141
Smoking History	1.642	0.371	0.583	4.244
Race	0.155	0.03	0	0.866
OH	1.159	0.546	0.327	1.763

The acinar morphological growth pattern was the most common pattern found in the EGFR IHC positive biopsies (**Table 4.9 and Figure 4.3 (A)**). Hobnail cells were not a feature in biopsies that showed positive staining with the EGFR IHC. The acinar and lepidic patterns were equally found in the ALK IHC positive biopsies. Only a single ALK positive biopsy contained cells with a signet ring morphology (**Table 4.9**).

Table 4.9: Morphological patterns of lung ACs with positive EGFR and ALK IHC

Morphological pattern	EGFR IHC		ALK IHC	
	(n)	%	(n)	%
Acinar	8	80	3	38
Lepidic	1*	10	1**	13
Solid	1	10	3**	38
Papillary	0		1**	13
Micropapillary	1*	10	1	13
Signet ring cells	0	0	1	13
Total	10		8	

* the same tumour showed a mixed lepidic and micropapillary pattern

** the same tumour showed a mixed solid, lepidic and papillary pattern

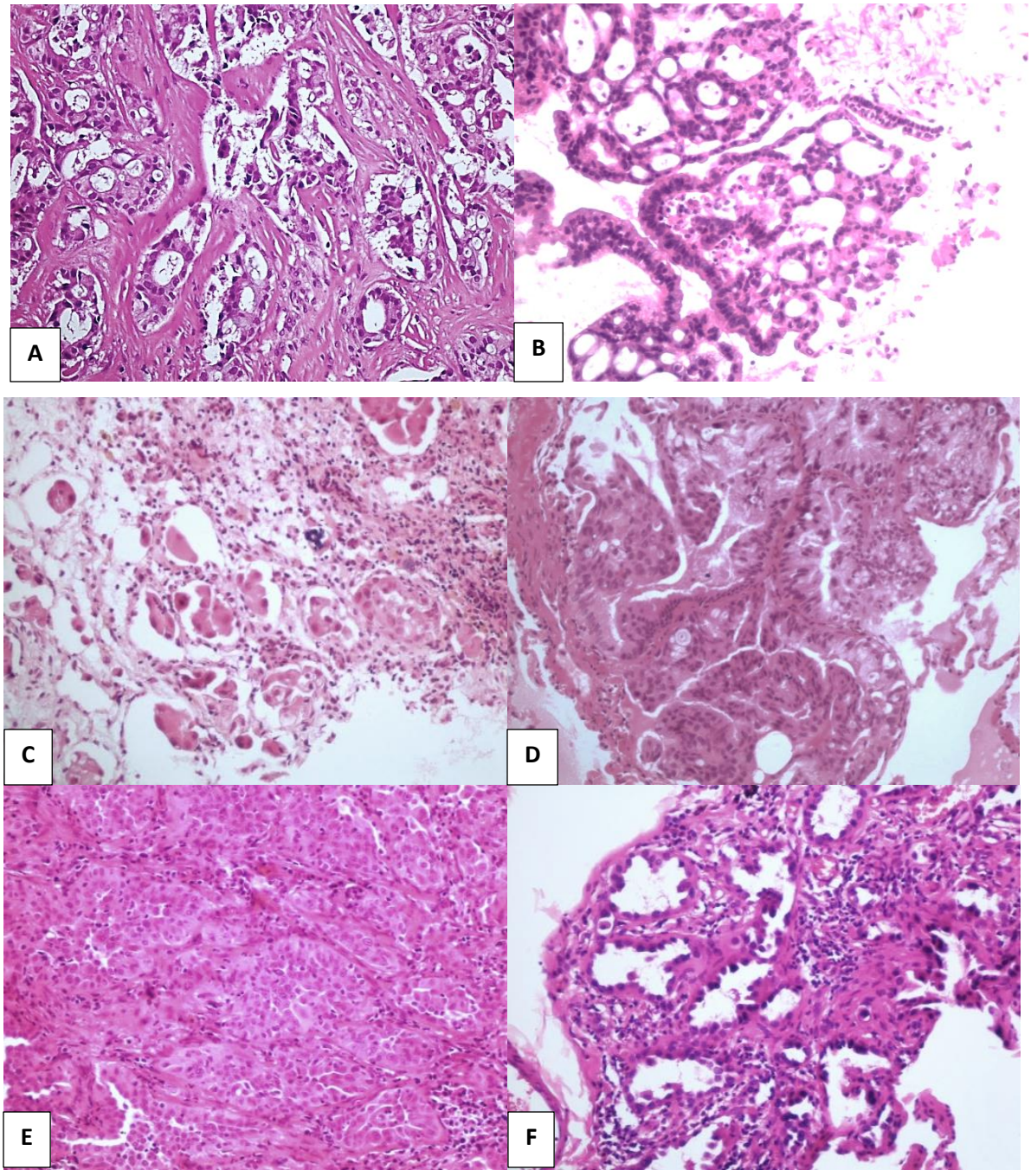

Figure 4.3 Morphological growth patterns in EGFR and ALK IHC positive lung adenocarcinomas (Haematoxylin and Eosin stained sections at 10X magnification). (A), Acinar. (B), Cribriform. (C), Micropapillary. (D), Papillary. (E), Solid. (F), Lepidic

All EGFR IHC positive biopsies showed strong cytoplasmic staining with at least focal membranous reinforcement (Figure 4.4 (A), whilst biopsies that

showed positive ALK IHC showed strong granular cytoplasmic staining (Figure 4.4 (B)). Refer to Annexures 6 and 7.

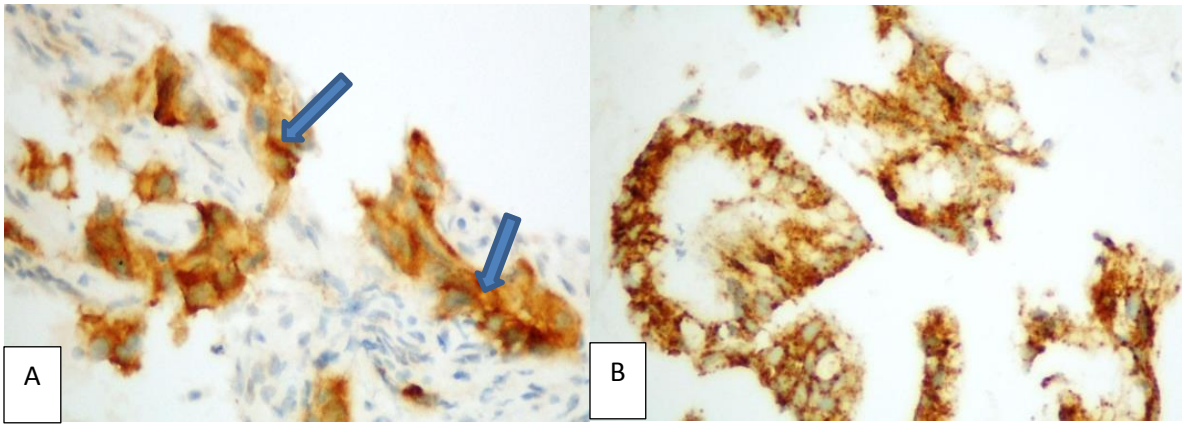

Figure 4.4 Immunohistochemical staining patterns using mutation specific EGFR antibody and High affinity ALK antibody (D5F3) at 20X magnification. (A), EGFR IHC positive case with diffuse homogenous cytoplasmic staining and focal membranous staining (arrow). (B), ALK IHC positive case with diffuse granular cytoplasmic staining

SCC is the more common overall subtype of lung cancer (46%) compared to AC (39%) in this group of patients from Johannesburg.

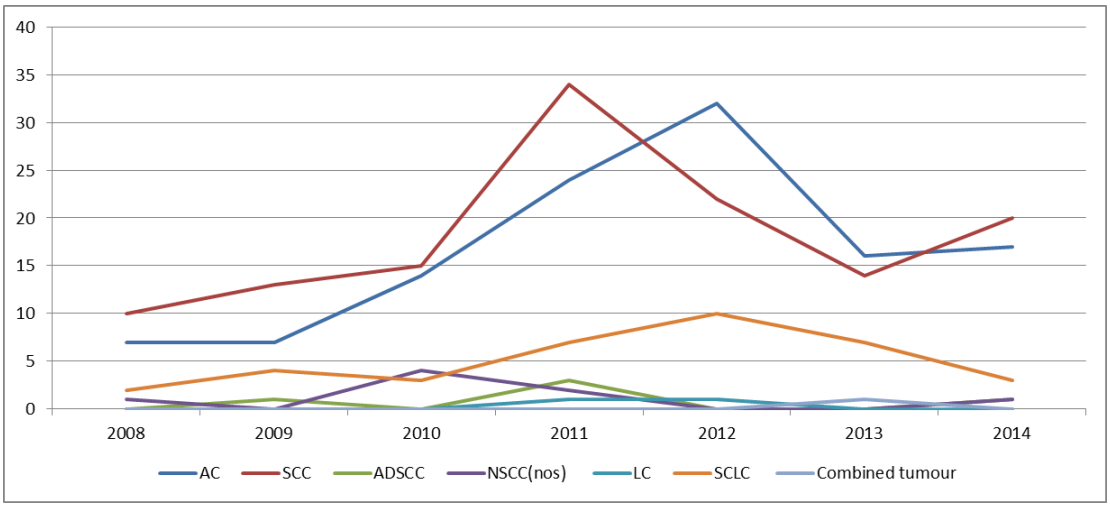

Figure 4.5 Lung cancer trend over six and a half years in patients from Johannesburg Hospital (Charlotte Maxeke Johannesburg Academic Hospital/ CMJAH and Helen Joseph Hospital/HJH)

Chapter 5: DISCUSSION

According to Lindeman *et al*. (2013), guidelines from the College of American Pathologists, the International Association for the Study of Lung Cancer and the Association for Molecular Pathology, recommend that EGFR and ALK testing be undertaken on all ACs and mixed lung cancers with an AC component, regardless of histologic grade. This includes LCs in resection specimens, NSCCs in small biopsy specimens and combined carcinomas with an AC component.

The EGFR mutation

More than 90% of EGFR mutations occur as short in frame deletions in exon 19 (as detected using the EGFR 111 antibody) or as point mutations in exon 21 (as detected using the EGFR 125 antibody) and testing for the presence of both mutations is necessary.

The EGFR mutation rate in this study is 9%, which correlates with a study of French patients by McLeer-Florin *et al* (2012) but is significantly less than the 24-51% incidence described in literature from Eastern countries (Korea, Taiwan, China and India) (Jang *et al.,* 2009, Huang *et al.,* 2011, Doval *et al.,* 2013) and less than the 11-19% incidence described in literature from Europe and America (Cortes-Funes *et al.,* 2005, Rosell *et al.,* 2009 and Smits *et al.,* 2012).

This EGFR mutation rate of 9% is also less than the 21.8% found by Chan (2015) in a study of 76 lung cancer patients from Johannesburg. There are

several reasons that may account for the discrepancies in the EGFR mutational rate between the two South African studies. Although Chan's study (2015) was also based on patients from Johannesburg, they were demographically different. Selection bias in Chan's study may have played a role as the patients selected for EGFR mutational analysis were predominantly White (71%) females who were never smokers or former light smokers, who were referred from private medical facilities. Patients in the current study are patients who presented to Government hospitals in Johannesburg and were diagnosed with lung cancer on biopsy specimens sent to the NIOH pathology division. These biopsies were from predominantly Black male patients who had a strong smoking history. The demographic profile of patients with lung cancer in this report is representative of the demographic profile of patients with lung cancer in South Africa (Ferlay *et al.,* 2012). The results in this study may therefore be more reflective of the mutational status of the South African population compared to the previous study by Chan (2015).

The EGFR IHC mutation rate of 9% is however also lower than the EGFR mutation rate of 22.6% found in the subgroup of Black patients in Chan's study (2015). There may thus be additional reasons responsible for the lower mutational rate observed.

The most significant difference with most of the published literature including Chan's (2015) is the use of EGFR PCR, which is a more sensitive method for the detection of the EGFR mutation, instead of EGFR IHC.

Added to this, certain factors may have affected the reliability of the IHC result, as described by Atkins *et al*. (2004) and Eberhard and Ladanyi (2008). These include appropriate fixative medium and duration of fixation of specimens, time from when the slide is prepared from the tissue block to when the stains are applied, antigen retrieval techniques, antibody detection protocols and the size and quality of the tissue sections used. Although the recommended fixation time for optimal EGFR expression is between 8 and 24 hours in 10% neutral-buffered formalin, we do not have control over the time it takes for specimens in formalin to reach the laboratory. There may be delays of between 24 and 48 hours before a specimen is received. Antigen retrieval may be affected by prolonged storage of unstained recut slides due to a time-dependent loss of immunoreactivity from antigen degradation. This may be seen in slides that were stored even for just 3 months. We experienced unforeseen delays in performing the IHC tests once slides were cut which may have affected antigen integrity. Tissue blocks were not recut as the majority of biopsies received were small specimens that had minimal tissue available and for cost implications. Intratumoral heterogeneity may have also contributed to false negative results, as shown by Kitamura *et al*. (2010) and as most biopsies received at the NIOH pathology division are small specimens rather than resection specimens, we cannot exclude that there may have been areas within the tumour that were not represented on the biopsy that may have had the EGFR mutation.

There were no Asians in our study population. The majority of patients whose biopsies showed positive EGFR IHC, were Black (80%), male (60%) patients who are younger than 65 years of age (80%). The association

between age, sex, race and the EGFR mutation as detected by EGFR IHC was however not statistically significant. A statistically significant association (p= 0.051) was found in biopsies from patients who had a smoking history and a positive EGFR IHC result. Although this is an unusual finding as the majority of studies have shown an association with non-smokers (Cheng *et al*. 2012), it does reflect the findings of a study of Korean patients by Sun *et al*. in 2012. The high percentage of smokers in the study population (67.57%) may have contributed. These results suggest that smoking may have a stronger association with the EGFR mutation in South African patients in contrast with patients from Asia, Europe and America. It should however be noted that when smoking was corrected with other features (age, race and sex), the association was no longer found significant. The small number of biopsies may have contributed to this.

In order to ensure reliability of the interpretation of the EGFR immunohistochemical stains used, we adhered to guidelines outlined by Brevet, Arcila and Ladanyi (2010), Ambrosini-Spaltro *et al.* (2012), Hasanivic *et al*. (2012) and Allo *et al*. (2014). These authors confirm good correlation between EGFR IHC positive results and the presence of the EGFR mutation if these guidelines are adhered to.

Although a range of morphological growth patterns of EGFR positive lung ACs were represented in this study, a dominant morphological growth pattern was not observed.

The findings in this report support the international recommendation that the demographic profile of patients and the morphological features of the tumour should not dictate whether a patient's biopsy is submitted for EGFR mutational analysis (Lindeman *et al*., 2013).

The ALK translocation

The ALK translocation rate is 7%. This is the upper limit of most recorded literature, which ranges between 2 and 7% (Wong *et al.,* 2009 and Paik *et al.,* 2012). The high affinity ALK IHC antibody, D5F3, has a high sensitivity and specificity when compared with ALK FISH for the detection of the ALK translocation (Hutarew *et al.,* 2012) and the results of this study supports the sensitivity of the ALK IHC in detecting the ALK translocation. A positive result of 7% suggests that the ALK translocation may be more prevalent in patients with lung AC from South Africa than other countries.

The demographic characteristics of patients whose biopsies showed positive staining with the ALK IHC is similar to those showing positive staining with EGFR IHC, i.e Black (100%) males (75%) younger than 65 years of age (88%). The majority of patients with lung cancer in this study were Black and a statistically significant association between race and the ALK mutation (p= 0.017) was found. There is an association with younger patients and positive ALK IHC, but this was not found to be statistically significant (p= 0.081).

The predominantly acinar and solid growth patterns observed in the ALK IHC positive biopsies is in line with the patterns described by Travis *et al.,* (2015). The signet ring morphology however cannot be used as a

determinant of the ALK mutation as it is present in only a single ALK IHC positive biopsy. Most of the biopsies received were small biopsy specimens and accurate identification of the morphological growth pattern was not always possible.

In view of the findings of this study, we re-evaluated the suggested algorithm on page 41 and recommend that in the absence of adequate tissue; molecular analysis be restricted to EGFR and ALK analysis. We recommend that patients with biopsies that show strong (3+) positive EGFR IHC staining be referred for EGFR TKI therapy whilst less positive staining and negative biopsies be subjected to PCR to assess for the presence of the EGFR mutation. Similarly, patients with biopsies that show strong (3+) positive ALK IHC staining be referred for ALK TKI therapy whilst equivocal biopsies that show less strongly positive staining be subjected to FISH. ALK IHC negative biopsies do not need additional mutational analyses unless the patient is young (less than 65 years old), with a non-smoking history or the biopsy has a predominantly acinar or solid growth pattern with signet ring cells.

<u>Updated guidelines for sub-classifying lung cancer</u>

7% of lung cancer biopsies that were diagnosed prior to the publishing of the latest WHO classification for lung cancer (Travis *et al*. 2015) were reclassified. These guidelines therefore assure that a correct final diagnosis can be made by following a standard protocol for lung cancer diagnosis, using morphological features and immunohistochemistry.

Lung cancer trend in South Africa

Smoking remains a significant contributor to the incidence of lung cancer in South Africa despite anti-tobacco measures taken by the Department of Health and The National Council against Smoking. The significance of smoking is reflected in the results of this research report, which shows that 60% of patients diagnosed with lung AC, ADSCC, LC or NSCC (nos.) were smokers and SCC remains the more common subtype of lung cancer. The shift in trend towards AC in lung cancer as seen internationally and in patients from Cape Town is not observed in this study of patients from Johannesburg. Greater effort therefore needs to be made to reduce tobacco smoking and the burden of lung cancer.

Limitations:

The limitations of the study are the following:

1). The number of lung cancer biopsy samples that were available in this study for immunohistochemical analysis was small compared with most international literature. Chan's study comprised 170 biopsies from patients who had clinical features suggestive of the EGFR mutation, with a yield of 37 positive cases (21.8%). Even though exact logistic regression was chosen instead of the regular logistic regression, no significant correlation was found with most of the features.

2). The delay between cutting of the slides and the actual application of the IHC antibodies may have compromised epitope preservation and integrity, contributing to false negative IHC results.

3). Smoking history was not available for all cases. The association between smoking and the ALK mutation was affected by the absence of a complete smoking history for all ALK positive biopsies.
4). Occupational history was not available for the majority of cases. The association between asbestos fibre exposure and the EGFR mutation and ALK translocation could not be assessed.
5). EGFR and ALK IHC were only performed on lung cancers that were diagnosed as AC, ADSCC, LC and NSCC (nos.). Most biopsies received were small biopsy specimens (63%) instead of resection specimens. The yield of positively stained cases may have been higher if EGFR and ALK IHC had been applied to all lung cancers, even those diagnosed as pure SCC or SCLC, provided certain suggestive clinical features such as young age and a non-smoking history were present. This is the recommendation by Lindeman *et al*. (2013) which is based on the presence of tumour heterogeneity that may not be reflected on a small biopsy specimen. However, resource constraints prevented us from assessing all lung cancers and the tests were confined to the specific subtypes as noted above.

Chapter 6: CONCLUSION

Although targeted therapy for patients with lung cancer that have the EGFR mutation and ALK translocation is only available in South Africa at present in the clinical trial setting and for some patients in the private sector, it is envisaged that this method of treatment may become more readily available and be part of the standard treatment regimen for patients with advanced NSCC in the future.

This study confirms the presence of the EGFR mutation and ALK translocation in patients with AC from Johannesburg using IHC.

We have modified the proposed algorithmic approach for the mutational analysis of patients with lung cancer in a resource constrained country such as South Africa, where EGFR and ALK IHC performed on limited biopsy material is used as rapid initial screening tests to identify patients with the EGFR mutation and ALK translocation, provided appropriate guidelines for IHC interpretation are adhered to (**Figure 2.2**). IHC tests are more cost effective than molecular analysis with a saving of 28% per patient at current tariff rates. Patients with ACs that are IHC positive can be fast tracked towards receiving targeted therapy.

1). EGFR mutations and ALK translocations are seen in 9% and 7% of patients with lung adenocarcinoma in this cohort of patients from Johannesburg

2). This study of 111 patients shows no significant correlation between EGFR mutation and the following variables: age, sex, race, histological tumour subtype and smoking history

3). The ALK mutation shows a significant correlation with Black patients

4). The ALK mutation in lung adenocarcinoma appears higher in the South African population (7%) than in some other international population groups (2-7%)

5). Squamous carcinoma is more common (46%) than adenocarcinoma (39%) in this study of primary lung carcinoma and the global shift in trend towards adenocarcinoma is not demonstrated in this study group

Recommendations for future research

1. The EGFR mutational rate was lower than expected. Future studies should compare EGFR IHC with PCR. It is our intention to do this should EGFR PCR become available at the NHLS.
2. To assess the ALK mutational rate with ALK FISH and compare the sensitivity of the ALK IHC used in this study with ALK FISH.
3. Accurate sub-classification of NSCC is the first step towards identifying the subset of lung cancers that may require further mutational analysis. We recommend consistent adherence to the guidelines as determined by Travis *et al*. (2011 and 2015).

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Annexure 1: Data collection sheet

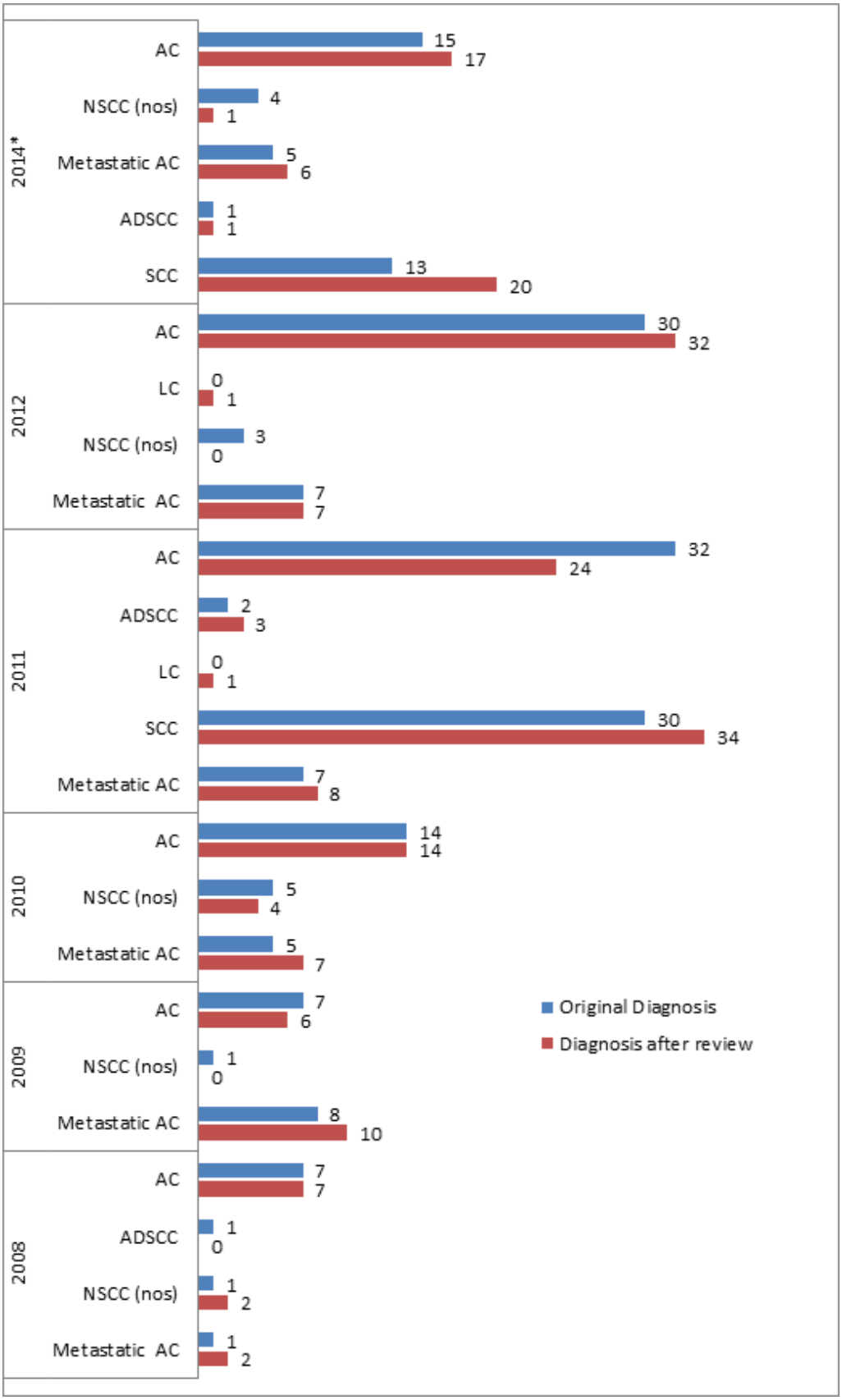
Variable Names and Variable values	
Study no:	
Path no:	
Year of biopsy:	
Hospital:	CMJAH = 1 HJH = 2
Patient demographics	
Race:	Black = 1 White = 2 Coloured = 3 Indian = 4 Uncertain= 5
Age:	
Sex:	Male = 1 Female = 2 Uncertain= 3
Smoking hx	Not available= 1 ex-smoker = 2 current smoker/recently stopped smoking = 3 non-smoker= 4
Occupation history OH	Not available=1 miner (nos.)=2 not a miner=3 asbestos exposure=4 platinum miner=5 Flour mill=6 gold miner=7 coal miner= 8
Biopsy (BX) site:	Transbronchial=1 endobronchial=2 transthoracic=3 pleural=4 lymph node=5 lobectomy=6 open lung biopsy/wedge=7 mediastinal mass = 8
Biopsy (BX) diagnosis:	(AC)Adenocarcinoma=1 (ADSCC)Adenosquamous carcinoma=2 (LC)large cell carcinoma=3 (NSCLC)Non small cell lung cancer (nos.)=4 combined tumour=5 (SCC)Squamous carcinoma=6
	(SCLC)Small cell carcinoma=7 Carcinoma (favour metastatic)=8
ALK/EGFR SP125/EGFR111: negative=1 positive=2 no tumour on slide=3 no tissue/block for IHC=4 Not done=5	
TTF1/Napsin A/CK7/CK20/P63/CK5/6: negative=1 positive=2 no tumour on slide/block=3no tissue/block for IHC=4 IHC Not done/No slide=5	

Case No	Path no	Yr	Hosp	Race	Age	Sex	OH	Smoking Hx	Bx site	Bx dx	ALK	EGFR SP125	EGFR SP111	TTF1	Napsin A	CK 7	CK 20	P 63	CK 5/6

Annexure 2a: Reclassification of cancers based on recommended guidelines by *Travis et al.* (2011 and 2015) from 01 January 2008 to 30 June 2014

Year	Original diagnosis	(n)	Diagnosis after review (n)
2014	AC	15	17
	NSCC (nos.)	4	1
	Metastatic AC	5	6
	ADSCC	1	1
	SCC	13	20
2012	AC	30	32
	LC	0	1
	NSCC (nos.)	3	0
	Metastatic AC	7	7
2011	AC	32	24
	ADSCC	2	3
	LC	0	1
	SCC	30	34
	Metastatic AC	7	8
2010	AC	14	14
	NSCC (nos.)	5	4
	Metastatic AC	5	7
2009	AC	7	6
	NSCC (nos.)	1	0
	Metastatic AC	8	10
2008	AC	7	7
	ADSCC	1	0
	NSCC (nos.)	1	2
	Metastatic AC	1	2

Annexure 2b: Reclassification of some cancers based on recommended guidelines by *Travis et al.* (2011 and 2015) from 01 January 2008 to 30 June 2014



Annexure 3a: Total biopsies received from CMJAH and HJH for the period 1st January 2008 to 30th June 2014

Year	Total biopsies(n)	Neoplastic biopsies (n)	Non-neoplastic biopsies (n)
2014	355	76	279
2013	680	67	613
2012	847	121	726
2011	792	123	669
2010	537	51	486
2009	507	47	460
2008	183	23	160
TOTAL	3901	508	3393

Annexure 3b: Biopsies received from CMJAH and HJH from 1st January 2008 to 30th June 2014

Year	NSCC					SCLC	Combined tumour *	Other***
	AC	SCC	ADSCC	NSCC(nos.)	LC			
2014	17	20	1	1	0	3	0	34
2013	16	14	0	0	0	7	1**	29
2012	32	22	0	0	1	10	0	56
2011	24	34	3	2	1	7	0	52
2010	14	15	0	4	0	3	0	15
2009	6	13	1	0	0	4	0	22
2008	7	10	0	1	0	2	0	3
Total	117	128	5	8	2	36	1	211

* Combined tumour = combination of a SCLC and NSCC

**This tumour comprised a SCLC and a large cell neuroendocrine carcinoma (it did not contain an adenocarcinoma component)

*** Other= mesothelioma, sarcoma, lymphoma, salivary glands tumours, metastatic carcinomas, thymoma, hamartoma and cytology specimens.

Annexure 4a: Demographic data, smoking history and occupational history on biopsies positive for the EGFR mutation

Year	Race	Age	Sex	Smoking history	Occupational history	Positive IHC
2009	B	67	M	No	NA	EGFR SP111
2011	B	61	F	NA	NA	EGFR SP111
2011	B	48	F	No	NA	EGFR SP111
2012	W	64	M	No	NA	EGFR SP111
2012	B	62	M	Yes	NA	EGFR SP111
2013	B	63	M	Yes	Yes**	EGFR SP111
2014	B	36	M	No	NA	EGFR SP111
2014	B	67	F	No	NA	EGFR SP111
2013	C	58	F	Yes	NA	EGFR SP125
2013	B	56	M	Yes	NA	EGFR SP125

** patient worked in a gold mine

B = Black, W = White, C = Coloured, M = Male, F = Female

NA= Not available

Annexure 4b: Demographic data, smoking history and occupational history on biopsies positive for the ALK mutation

Year	Race	Age	Sex	Smoking history	Occupational history	Positive IHC
2009	B	44	M	Yes	Yes *	ALK
2010	B	71	M	yes	NA	ALK
2011	B	54	M	No	NA	ALK
2011	B	57	M	Ex-smoker	NA	ALK
2012	B	28	F	NA	NA	ALK
2014	B	64	M	Yes	NA	ALK
2014	B	52	F	No	NA	ALK
2014	B	41	M	NA	NA	ALK

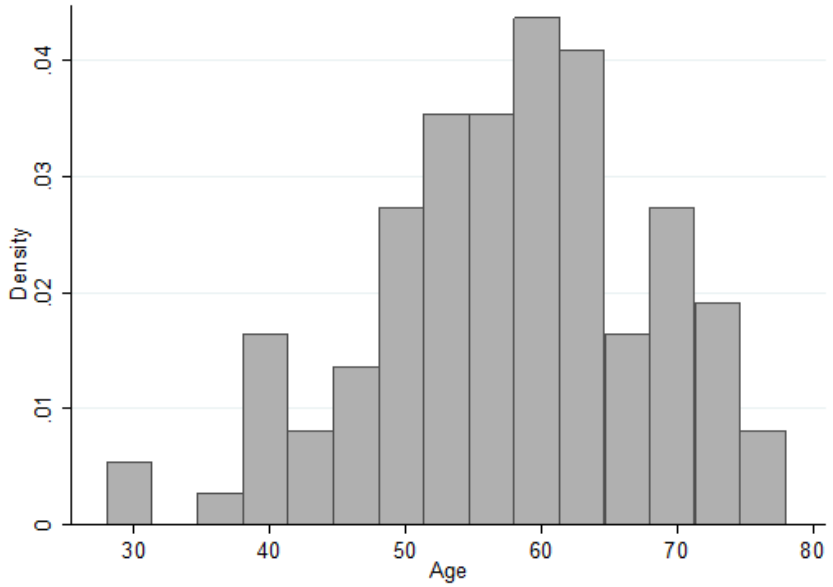
* patient worked in a flour mill

B = Black, W = White, C = Coloured, M = Male, F = Female

NA= Not available

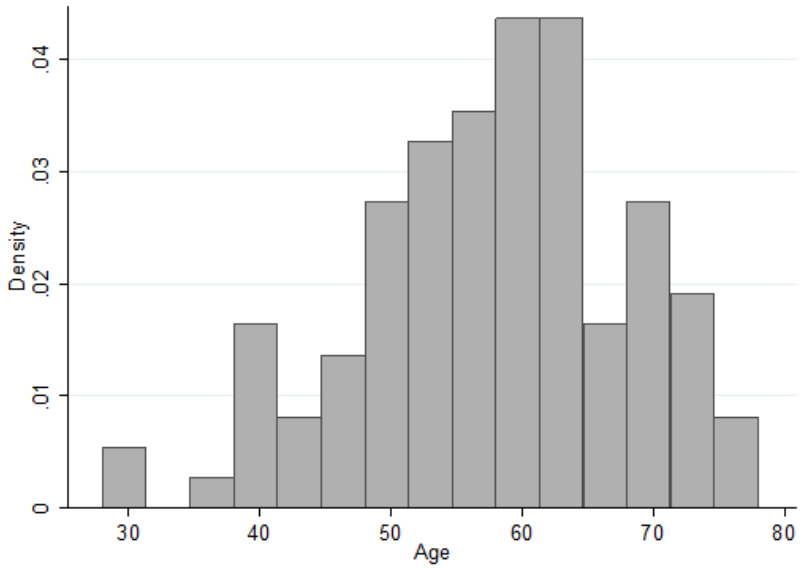
Annexure 5: Histogram

Histogram of age for EGFR



Age is consistent with a normal distribution, swilk test p= 0.3577

Histogram of age for ALK



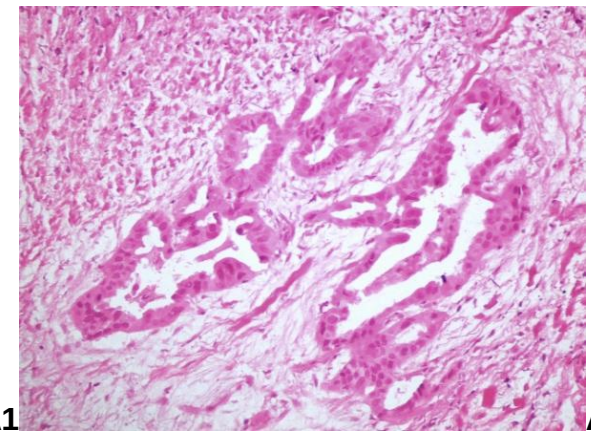
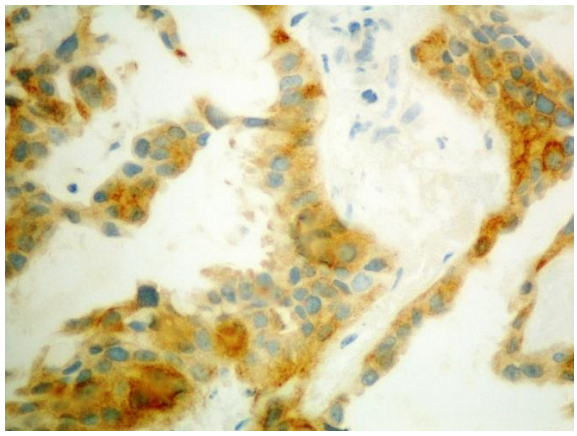
Age is consistent with a normal distribution, swilk test p= 0.3008

Annexure 6: **Morphological pattern and scoring of IHC on biopsies positive for the EGFR and ALK mutation**

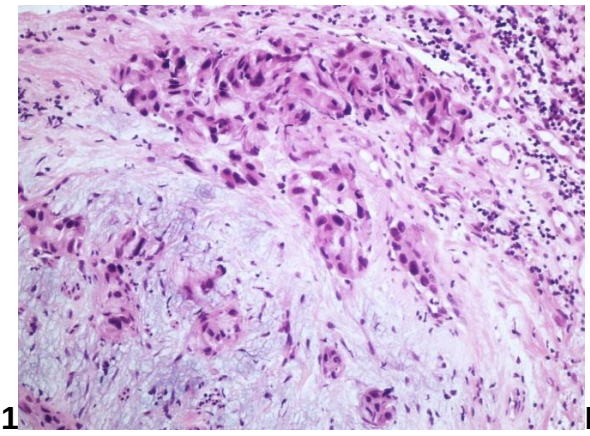
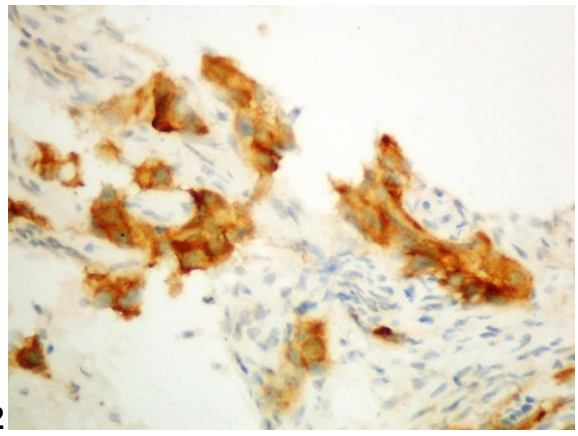
Positive IHC	Morphology	Staining pattern	Staining intensity
EGFR SP111	Acinar	Patchy homogenous cytoplasmic & membranous	2+
EGFR SP111	Acinar	Diffuse homogenous cytoplasmic & membranous	3+
EGFR SP111	Lepidic, micropapillary	Patchy homogenous cytoplasmic & membranous	3+
EGFR SP111	Solid	Patchy homogenous cytoplasmic & membranous	3+
EGFR SP111	Acinar	Diffuse homogenous cytoplasmic & membranous	3+
EGFR SP111	Acinar	Patchy homogenous cytoplasmic & membranous	3+
EGFR SP111	Acinar, nests	Patchy homogenous cytoplasmic & membranous	3+
EGFR SP111	Acinar	Patchy homogenous cytoplasmic & membranous	2+
EGFR SP125	Acinar	Patchy homogenous cytoplasmic & membranous	2+
EGFR SP125	Acinar	Patchy homogenous cytoplasmic & membranous	2+
ALK D5F3	Acinar	Diffuse granular cytoplasmic	3+
ALK D5F3	Solid	Patchy granular cytoplasmic	3+
ALK D5F3	Acinar, cribriform	Diffuse granular cytoplasmic	3+
ALK D5F3	Solid	Patchy granular cytoplasmic	3+
ALK D5F3	Papillary	Diffuse granular cytoplasmic	3+
ALK D5F3	Acinar	Diffuse granular cytoplasmic	3+
ALK D5F3	Solid, lepidic, papillary	Patchy granular cytoplasmic	3+
ALK D5F3	Micropapillary	Patchy granular cytoplasmic	3+

Annexure 7: **Photomicrographs showing morphological patterns and IHC staining on biopsies positive for the EGFR and ALK IHC**

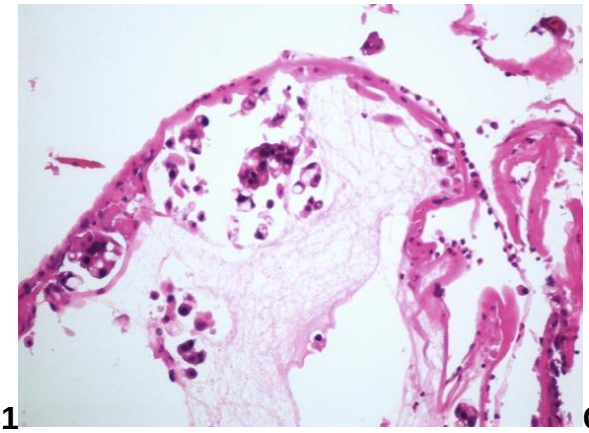
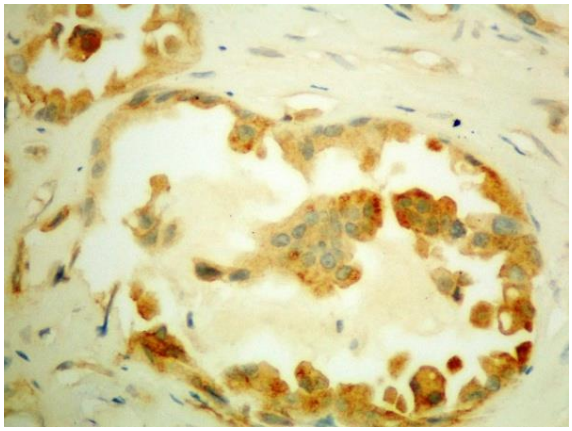
EGFR IHC: SP111 positive biopsies (A-H)

A1  **A2** 

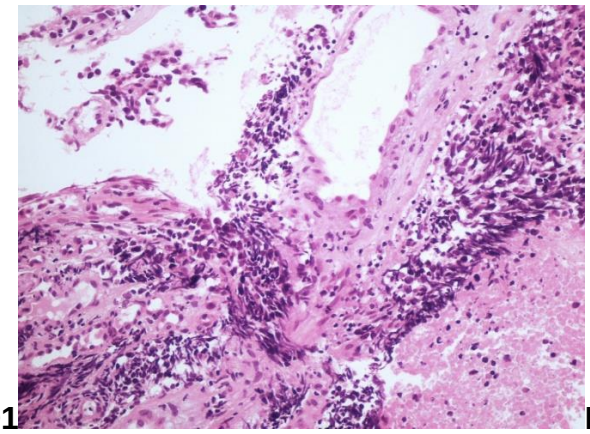
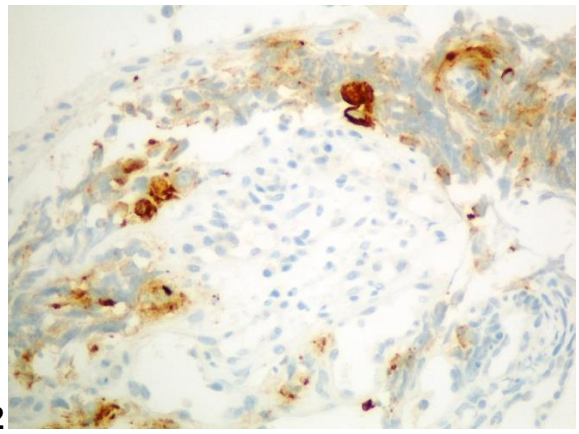
Adenocarcinoma with acinar growth pattern at 10X magnification (A1) Haematoxylin and eosin, (A2) EGFR IHC with 2+ moderate, patchy cytoplasmic & focal membranous staining

B1  **B2** 

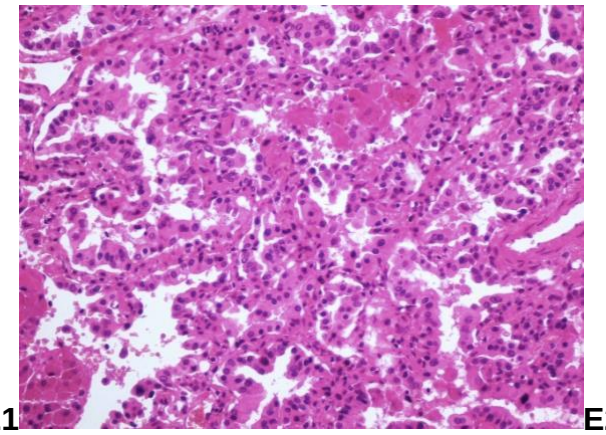
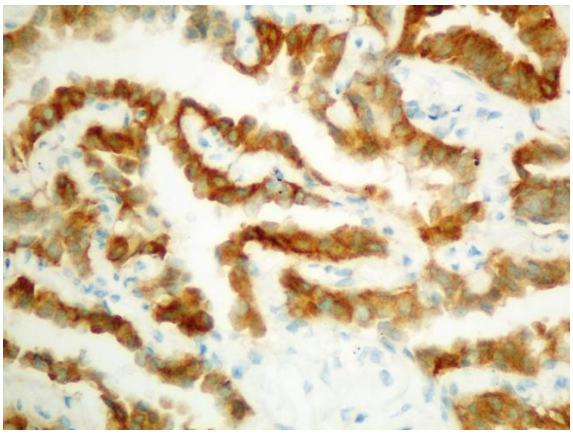
Adenocarcinoma with acinar growth pattern at 10X magnification (B1) Haematoxylin and eosin, (B2) EGFR IHC with 3+ strong, diffuse cytoplasmic & membranous staining

C1  **C2** 

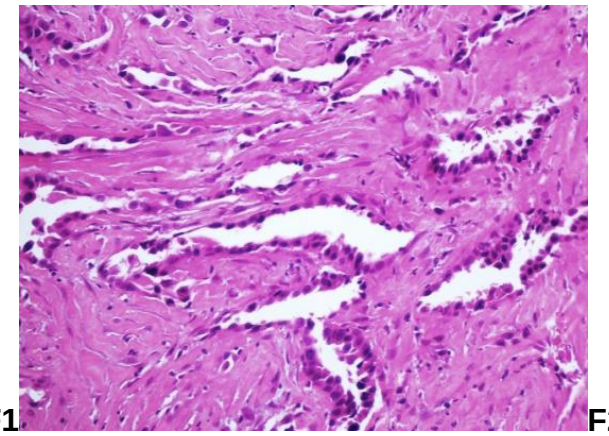
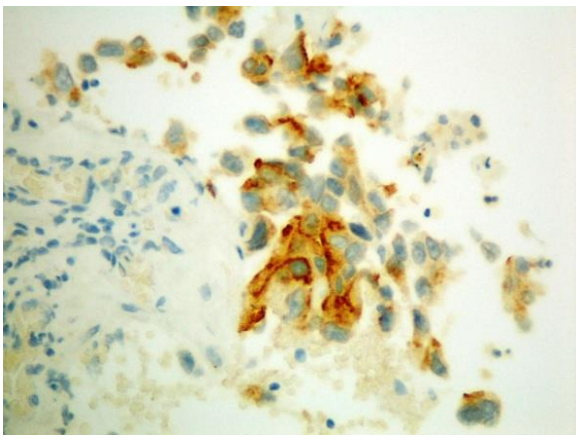
Adenocarcinoma with micropapillary growth pattern at 10X magnification (C1) Haematoxylin and eosin, (C2) EGFR IHC with 3+ strong, patchy cytoplasmic & focal membranous staining

D1  D2 

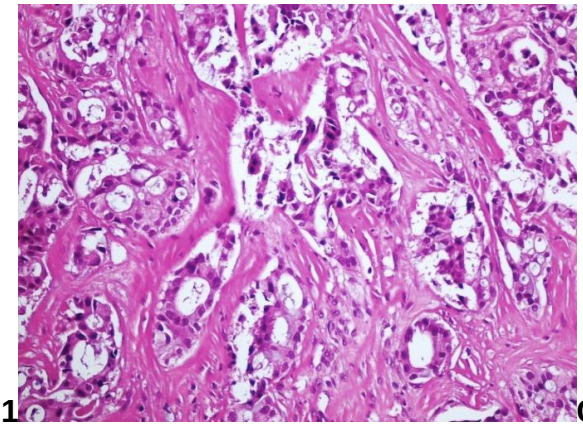
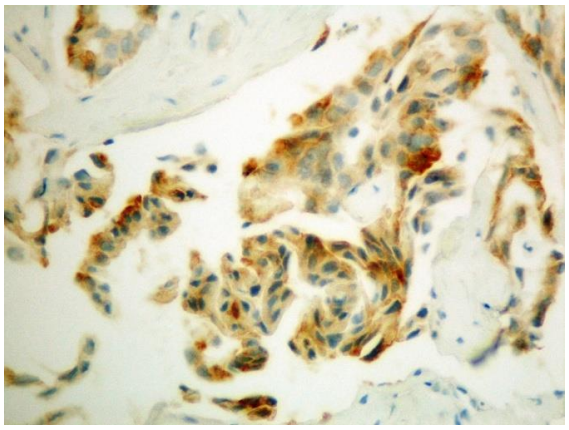
Adenocarcinoma with solid growth pattern at 10X magnification (D1) Haematoxylin and eosin, (D2) EGFR IHC with 3+ strong, patchy cytoplasmic & focal membranous staining

E1  E2 

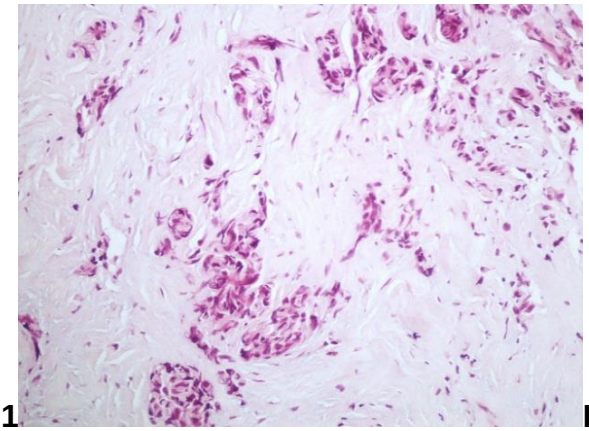
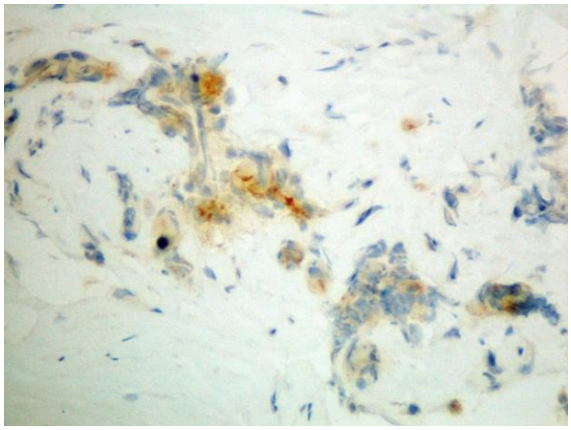
Adenocarcinoma with acinar growth pattern at 10X magnification (E1) Haematoxylin and eosin, (E2) EGFR IHC with 3+ strong, diffuse cytoplasmic & focal membranous staining

F1  F2 

Adenocarcinoma with acinar growth pattern at 10X magnification (F1) Haematoxylin and eosin, (F2) EGFR IHC with 3+ strong, patchy cytoplasmic & focal membranous staining

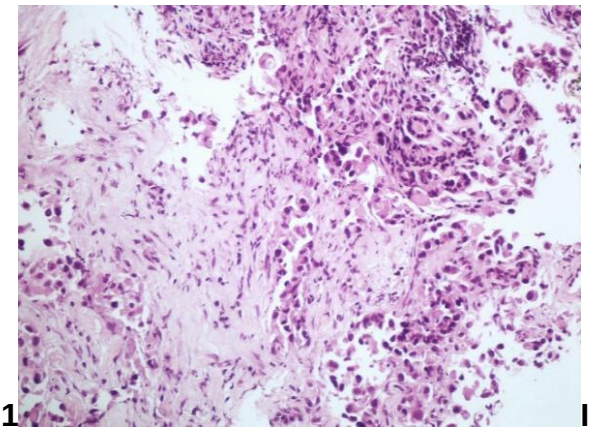
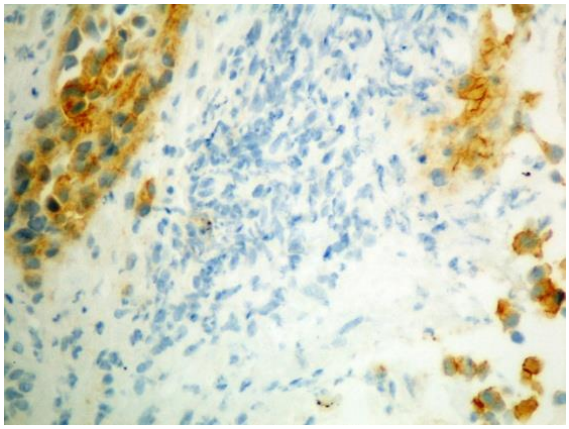
G1  G2 

Adenocarcinoma with acinar growth pattern at 10X magnification (G1) Haematoxylin and eosin, (G2) EGFR IHC with 3+ strong, patchy cytoplasmic & focal membranous staining

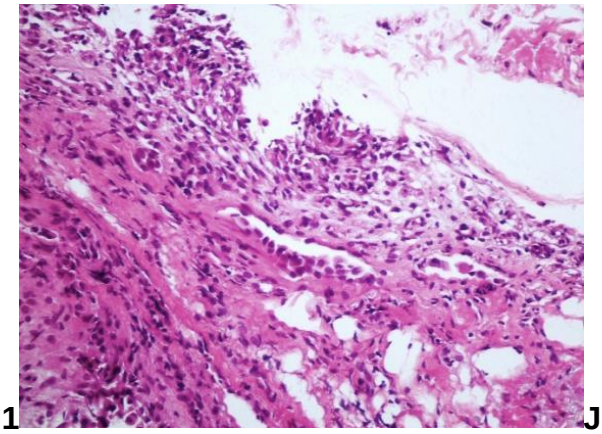
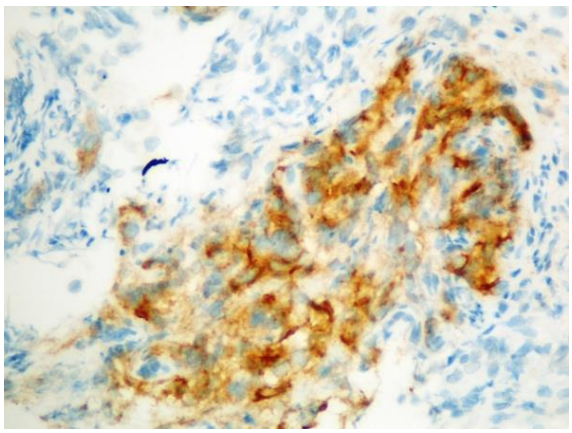
H1  H2 

Adenocarcinoma with acinar growth pattern at 10X magnification (H1) Haematoxylin and eosin, (H2) EGFR IHC with 2+ moderate, patchy cytoplasmic staining)

EGFR IHC: SP125 positive biopsies (I-J):

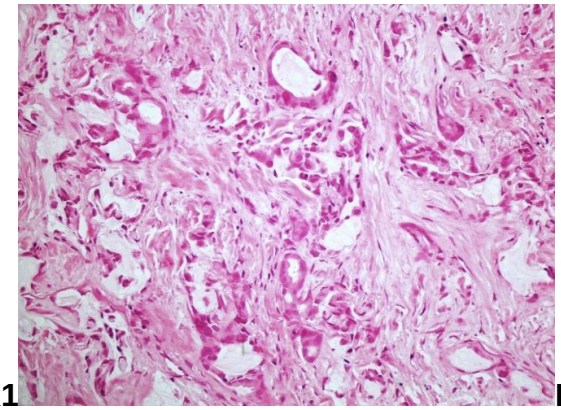
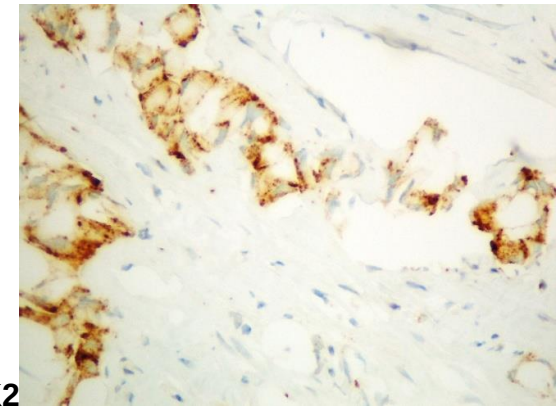
I1  I2 

Adenocarcinoma with acinar growth pattern at 10X magnification (I1) Haematoxylin and eosin, (I2) EGFR IHC with 2+ moderate, patchy cytoplasmic &focal membranous staining

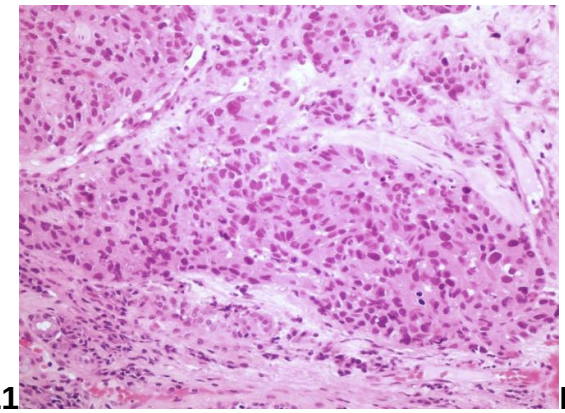
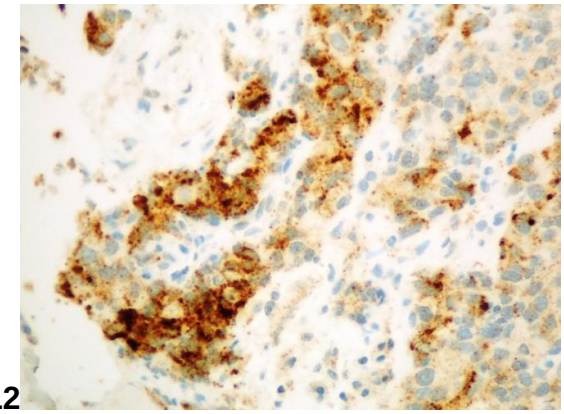
J1   J2

Adenocarcinoma with acinar growth pattern at 10X magnification (J1) Haematoxylin and eosin, (J2) EGFR IHC with 2+ moderate, patchy cytoplasmic & focal membranous staining

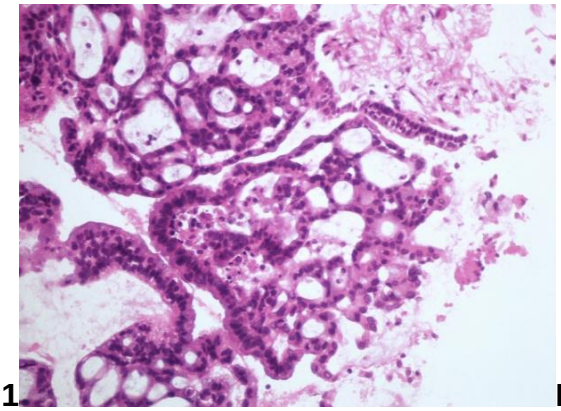
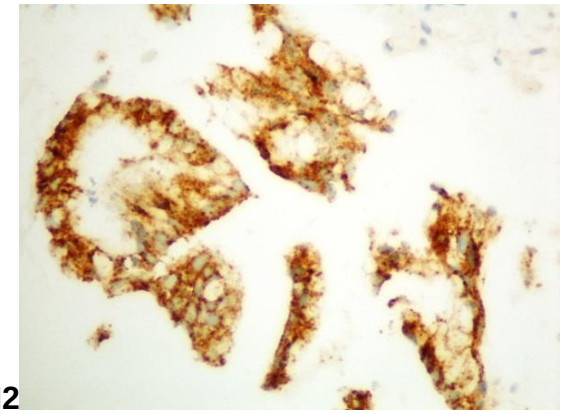
ALK IHC positive biopsies (K-R):

K1   K2

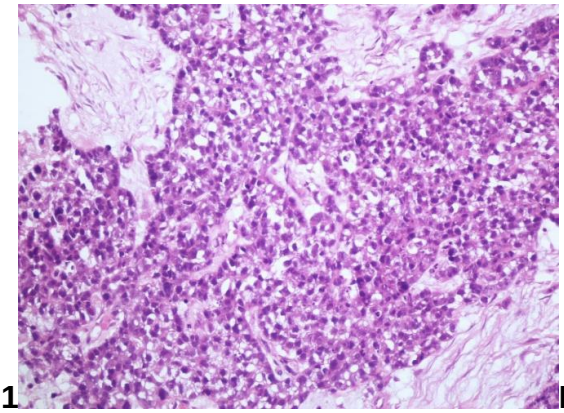
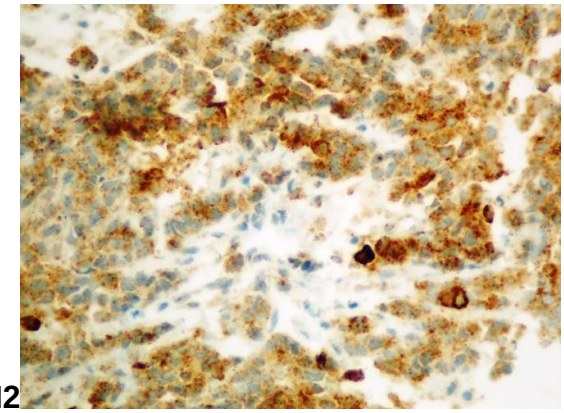
Adenocarcinoma with acinar growth pattern at 10X magnification (K1) Haematoxylin and eosin, (K2) ALK IHC with 3+ strong diffuse granular cytoplasmic staining

L1   L2

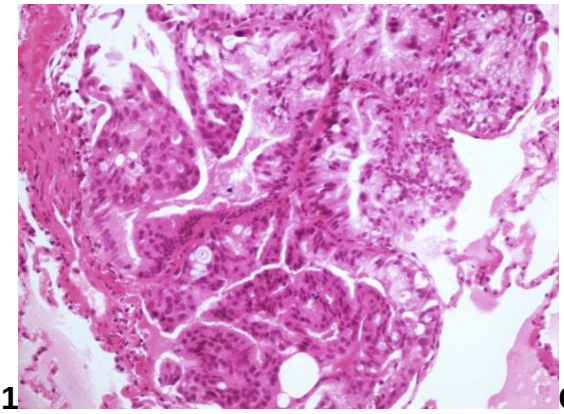
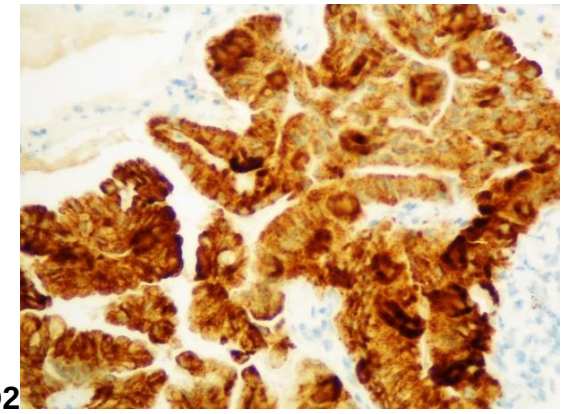
Adenocarcinoma with solid growth pattern at 10X magnification (L1) Haematoxylin and eosin, (L2) ALK IHC with 3+ strong patchy granular cytoplasmic staining

M1  M2 

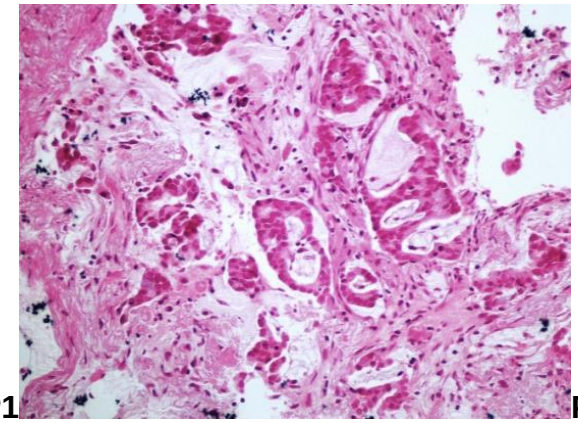
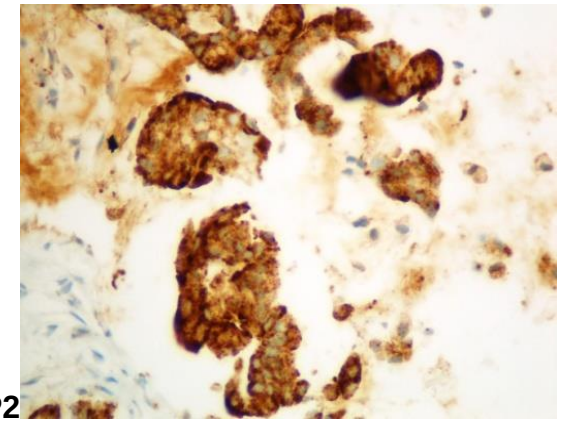
Adenocarcinoma with cribriform growth pattern at 10X magnification (M1) Haematoxylin and eosin, (M2) ALK IHC with 3+ strong diffuse granular cytoplasmic staining

N1  N2 

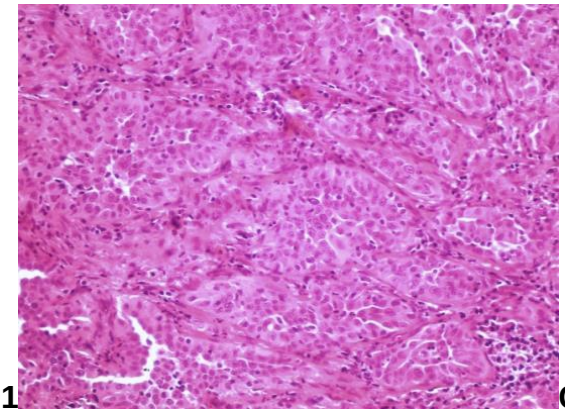
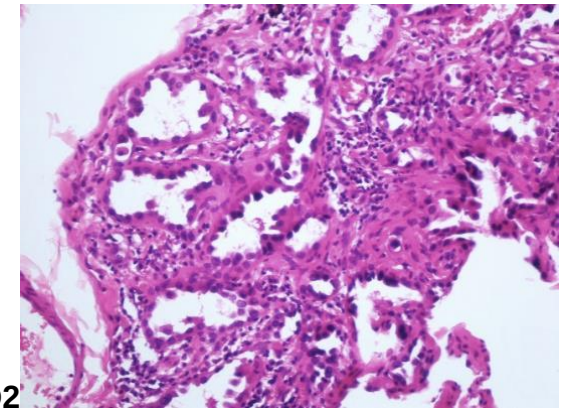
Adenocarcinoma with solid growth pattern at 10X magnification (N1) Haematoxylin and eosin, (N2) ALK IHC with 3+ strong patchy granular cytoplasmic staining

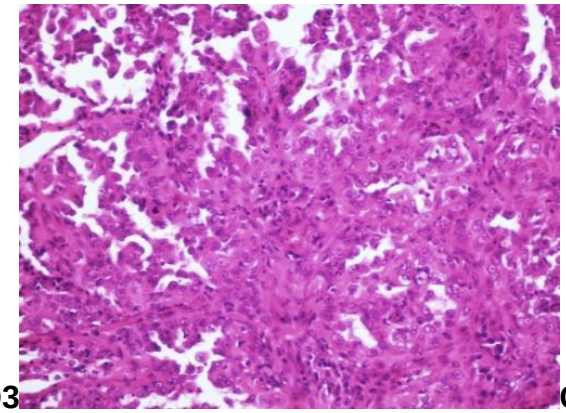
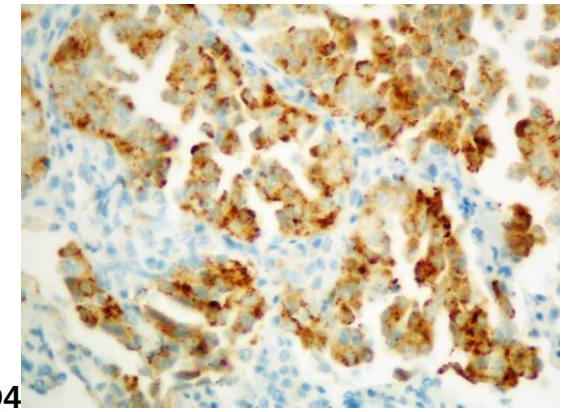
O1  O2 

Adenocarcinoma with papillary growth pattern at 10X magnification (O1) Haematoxylin and eosin, (O2) ALK IHC with 3+ strong diffuse granular cytoplasmic staining

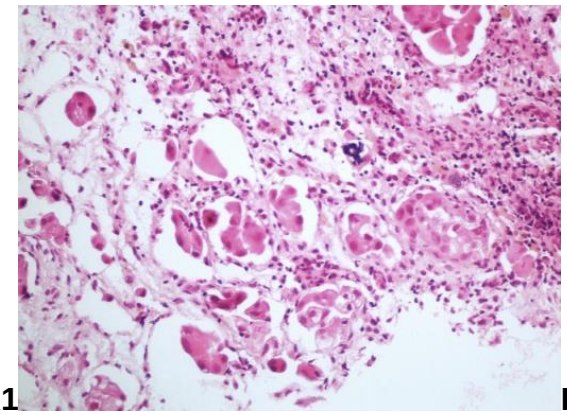
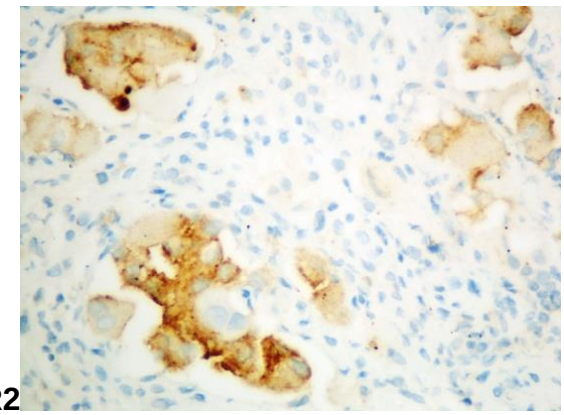
P1  P2 

Adenocarcinoma with acinar growth pattern at 10X magnification (P1) Haematoxylin and eosin, (P2) ALK IHC with 3+ strong diffuse granular cytoplasmic staining

Q1  Q2 

Q3  Q4 

Adenocarcinoma at 10X magnification (Q1) Solid, (Q2) lepidic, (Q3) papillary growth patterns, Haematoxylin and eosin; (Q4) ALK IHC with 3+ strong patchy granular cytoplasmic staining

R1  **R2** 

Adenocarcinoma with micropapillary growth pattern at 10X magnification (R1) Haematoxylin and eosin, (R2) ALK IHC with 3+ strong patchy granular cytoplasmic staining

Annexure 8: Approval letter from Dr Sophia Kisting, NIOH executive director

NATIONAL INSTITUTE FOR OCCUPATIONAL HEALTH
Division of the National Health Laboratory Service

Office of the Executive Director
25 Hospital Street, Constitution Hill, Johannesburg, 2000
Tel: +27 (0)11 712 6522 Fax: +27 (0)11 712 6523
Reference:

4 November 2014

RE: MMed Research Project Dr N Vorajee

I hereby grant permission for Dr Naseema Vorajee to utilise the National Institute for Occupational Health (NIOH) database at the Pathology division of National Institute for Occupational Health for the purpose of her MMed research project in Lung Cancer.

Yours Sincerely,

Dr Sophia Kisting
Executive Director
National Institute for Occupational Health (NIOH)
National Health Laboratory Service
Tel: +27(0)11 712 6522/6413 | Cell: +27 (0)82 609 5406 | Fax: +27 (0)11 712 6523
sophia.kisting@nioh.nhls.ac.za | www.nioh.ac.za | www.nhls.ac.za

NATIONAL INSTITUTE FOR OCCUPATIONAL HEALTH
Division of the National Health Laboratory Service

NIOH 60th Anniversary

Annexure 9: Ethics Clearance certificate: M140943

R14/48 Dr Naseema Vorajee

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140943

NAME: (Principal Investigator)	Dr Naseema Vorajee
DEPARTMENT:	Pathology National Health Laboratory Service NIOH Pathology Division
PROJECT TITLE:	Immunohistochemical Screening for Epidermal Growth Factor Receptor and Anaplastic Lymphoma Kinase Mutation in Lung Adenocarcinoma in South Africa
DATE CONSIDERED:	03/10/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof J Murray and Prof J Phillips
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	06/10/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I agree to submit a yearly progress report.

Principal Investigator Signature

Date 07/10/14

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Annexure 10: Protocol approval

UNIVERSITY OF THE
WITWATERSRAND
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Ms Thokozile Nhlapo
E-mail: thokozile.nhlapo@wits.ac.za

09 January 2015
Person No: 9102330W
PAG

Dr NI Vorajee
54 Headford Avenue
Crosby
2092
South Africa

Dear Dr Vorajee

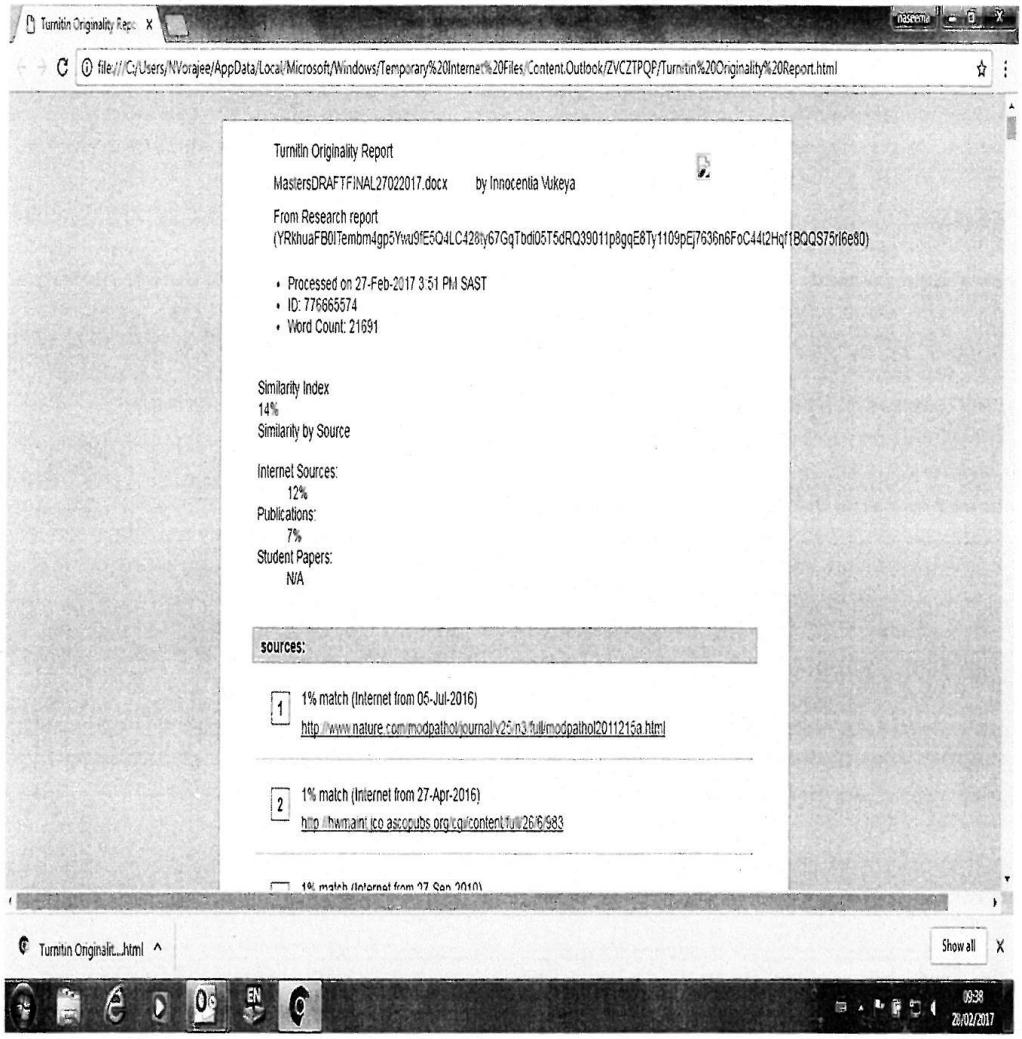
Master of Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *Immunohistochemical screening for Epidermal growth factor receptor and Anaplastic lymphoma kinase mutations in lung adenocarcinoma in South Africa* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

Annexure 11: Turnitin



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Prof J. I. Phillips

J Phillips 28/02/2017

Supervisor 2