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The significance of cytology, PCR, and culture from EBUS-TBNA for diagnosis of tuberculosis of the mediastinal lymph nodes

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1. Deutsche Zusammenfassung

Die Bedeutung der Histologie, PCR und Kultur Ergebnisse durch EBUS-TBNA für die Diagnose der Tuberkulose der mediastinalen Lymphknoten

1. Einleitung

Die Tuberkulose stellt weltweit eine der häufigsten Todesursachen der Welt dar und nahm durch die verstärkte Migrationsbewegung, die vor allem zwischen 2015 und 2016 stattfand, auch in Deutschland etwas zu. Die Asklepios Fachklinik in München Gauting verfügt über eine der größten Tuberkulosestation Deutschlands. Hier werden neben den häufigeren pulmonalen Formen auch alle anderen Manifestationen behandelt. Die extra-pulmonale Tuberkulose stellt aufgrund der erschwerten mikrobiologischen Diagnostik, die meist nur durch invasive Verfahren beinhaltet, weltweit immer noch ein großes Gesundheitsproblem dar. Lymphknoten sind die häufigste Lokalisation der extra-pulmonalen Tuberkulose. Wir untersuchten welchen Beitrag die EBUS-TBNA Technik (endobronchiale Ultraschallgesteuerte transbronchiale Feinnadelaspiration) in der mikrobiologischen Diagnostik mediastinaler und hilärer Lymphknotentuberkulosen leisten kann. Wir analysierten retrospektiv im Zeitraum von 01/2015 bis 04/2018 die histologischen und mikrobiologischen Ergebnisse der durch EBUS-TBNA gewonnenen Materialien von 127 Patienten mit gesicherter Tuberkulose. Darüber hinaus untersuchten wir, inwieweit die Analyse der Lymphknoten mit farbkodierter Elastographie bei der Unterscheidung zwischen normalem Lymphgewebe und befallenen Lymphknoten hilfreich ist.

2. Ergebnisse

Tuberkulose in den Atemwegen

Bei 48 Patienten (37.8%) konnte die Tuberkulose aus dem Atemwegsmaterial bestätigt werden, bei 79 waren diese Materialien negativ. Dies beruht vor allem darauf, dass bei Nachweis von Tuberkuloseerregern im Sputum in der Regel keine weiterführende Bronchoskopie durchgeführt wird. Bei 39 der 48 Patienten mit bakteriologisch gesicherter Lungen-TB (81.3%) konnte der Befall der mediastinalen Lymphknoten

ebenfalls erhärtet werden, wobei die Kultur mit 68.7% die höchste Sensitivität aufwies, gefolgt von der PCR mit 64.5% und der Histologie mit 50%.
In dieser Gruppe ergab die Histologie bei 24 Patienten eine granulomatöse Lymphadenitis mit oder ohne Nekrosen. Unter diesen hochselektierten 24 Fällen (bakteriologisch bestätigte Lungen-Tuberkulose kombiniert mit passender Histologie in den TBNAs) ergab sich eine Sensitivität von 67% für die PCR und von 75% für die Kultur. Im Vergleich konnte in der Gruppe der 79 Patienten ohne bakteriologische Diagnosesicherung aus Atemwegsmaterialien eine mediastinale Lymphknotentuberkulose bei 45 mittels Kultur (57.0%), 39 mittels PCR (49.4%), sowie 51 (64.5%) über die histologischen Veränderungen gestellt werden.

EBUS TBNA- Vergleich der Kultur, Histologie und PCR

In 78 Fällen (61.4%) mit gesicherter bakteriologischer Tuberkulose aus den EBUS TBNA Materialien fiel die Kultur positiv aus. Unter allen kulturell gesicherten EBUSTBNAs fiel die PCR in 65.4% positiv aus, die Histologie wies in 55. 1% typische Veränderungen auf. In 3% der Fälle fand sich eine unspezifische Nekrose. Typische Zytologie umfasste epithelioide Granulomatose mit Nekrose (43 Patienten) sowie epithelioide Reaktionen ohne Nekrose (32 Patienten). In diesen histologisch typischen Fällen konnten wir eine Sensitivität für die Kultur von 57.3% und für die PCR von 45.3% vermerken. In 29 EBUS-TBNAs fand sich histologisch unauffälliges Lymphknotengewebe, hier fiel die PCR aber in 15 Fällen positiv aus (51.7%), die Kultur sogar in 19 Fällen (65.5%). In unserer Studie erzielten wir eine Sensitivität von 59.3 % für PCR Ergebnisse.

Elastographie

Wir analysierten die Ergebnisse der EBUS-Elastographie bei Patienten mit gesicherter Lymphknotentuberkulose (n= 23) um den Stellenwert bei benignen Differentialdiagnosen zu beleuchten. Wir verwenden ein 4-stufiges Elastographie-Scoring (1: >80% grün, gelb, rot 2: 50- 80% grün, gelb, rot 3: 50- 80% blau 4: >80% blau). Es ergab sich eine Verteilung mit Fehlen von Typ 1, 26% Typ 2, 52% Typ 3 und 22% Typ 4. Die 6 Lymphknoten mit Typ 2 waren in der histologischen Aufarbeitung als unauffälliges LK-Gewebe eingestuft worden, wiesen aber in 83% eine positive Kultur bzw. in 50% eine positive PCR auf. Bei den LK mit Typ 3 fand sich in 75% eine

passende Histologie, die Kultur war in 92% positiv, die PCR in 58%. Obwohl der Elastographie–Score 4 malignen Prozessen zugeschrieben wird, konnten wir diesen Typ bei 5 Patienten (22%) mit Nachweis einer Tuberkulose finden. Hier ergab sich bei vier Patienten (80%) eine passende Histologie, die Kultur war in 80% positiv, die PCR in 100% (eine negative Kultur trotz positiver PCR).

Gamma Interferon Test (IGRA)

Gamma- Interferon Tests (IGRA) können nicht nur als diagnostische Testmethode durchgeführt werden. Wir führten IGRA bei 87 Patienten durch, wobei 78 Probanden (89%) ein positives IFN- γ Testergebnis zeigten. Es ist wichtig zu erwähnen, dass der Interferon- γ Test in 11% der Fälle falsch negative Ergebnisse erbrachte, was impliziert, dass eine Infektion nicht durch ein negatives IGRA Testergebnis ausgeschlossen werden kann.

3. Schlussfolgerung

Die aktuelle retrospektive Erhebung zeigt den hohen diagnostischen Wert der EBUS TBNA bei der Diagnostik der intrathorakalen tuberkulösen Lymphadenopathie. Die Ergebnisse lassen den Schluss zu, dass die EBUS-TBNA eine sinnvolle Erweiterung der Diagnostik zum Nachweis einer Tuberkulose darstellt. Unabhängig von der Histologie liefert sie sehr gutes Material für kulturelle und molekulargenetische Diagnostik. Die Sensitivität der PCR ist gut, erlaubt aber nicht den Ausschluss einer Tuberkulose. Eine unauffällige Histologie schließt in diesem Patientengut den Befall eines mediastinalen Lymphknotens nicht aus. In Übereinstimmung mit unseren Erfahrungen fielen die QFT-TB-Testergebnisse vor allem bei Patienten mit kürzlich erfolgter Einwanderung aus Tuberkulose Endemie Ländern, sowie nach längerer Exposition gegenüber infizierten Patienten positiv aus. Daher können IGRAS im Rahmen eines Tuberkulose Screenings genützt werden. In Betrachtung der elastographischen Ergebnisse, kamen wir zu der Schlussfolgerung, dass der Nachweis harter Lymphknoten (ELST-Score 3 oder 4) nicht nur malignen Prozessen vorbehalten ist, sondern sich auch bei tuberkulöser Lymphadenitis in hoher Zahl findet. Die Notwendigkeit weiterer histologischer und mikrobiologischer Abklärung einer tuberkulösen Infektion kann somit durch eine elastographische Untersuchung nicht ausgeschlossen werden. Der elastographische Typ 2 war histologisch mit

unauffälligem Lymphknotengewebe assoziiert, lieferte aber eine hohe Anzahl kulturell positiver Befunde. Daher konnte kein spezifischer elastographischer Typ mit Tuberkulose in Verbindung gebracht werden, weshalb kein diagnostischer Algorithmus für die Elastographie festgelegt werden konnte.

2. English Summary

Summary- The significance of cytology, PCR and culture from EBUS- TBNA for diagnosis of tuberculosis of the mediastinal lymph nodes

1. Introduction

Extra pulmonary tuberculosis still poses a major health problem globally due to difficulties in diagnosis and treatment monitoring as well as the lack of systematic microbiological assessment. This study was conducted to improve early diagnosis by evaluating the significance of EBUS TBNA, when assessing extra- pulmonary tuberculosis. Furthermore, we evaluated the possibility of using elastography to differentiate between ordinary lymphatic tissue and lymph nodes being affected by tuberculosis.

Information was retrieved from 127 patients in the period between January 2015 and April 2018, with a majority of young males (mean age 32 years), which can be seen as an effect of the increased migration trend that took place between the years of 2015 and 2018 in Germany. The strength of this retrospective study is that it only included patients with a proven tuberculosis. This condition could be ensured by either the proof of tuberculosis in the airway of the patient or in the lymph nodes. EBUS TBNA was conducted to serve the purpose of obtaining material for detailed cultural and cytological examination to elaborate cytopathological findings.

2. Results

Bacterial proof from the airways

Overall, 30.6% of the patients with a subsequently proven tuberculosis infection of the lymph nodes received positive results regarding pulmonary tuberculosis signs (bacteria in the sputum or bronchial lavage), which can be explained by the fact that when pulmonary assessment already showed cultural proof of a tuberculosis infection, in general no bronchoscopy assessment or EBUS TBNA was conducted. In total 48 patients showed a positive cultural result of the airways (37.8%). Out of the 48 patients who showed signs of proven pulmonary tuberculosis, 39 patients (81.3%) could be categorized as mediastinal lymph node tuberculosis positive (positive PCR, cultural growth, typical histopathological pattern). Cultural proof showed a sensitivity of 68.7%, followed by PCR analysis with a sensitivity of 64.5% and histology with a sensitivity of

50%. In 24 cases we found bacteriologically proven pulmonary tuberculosis in combination with a matching histology. Taking into consideration these 24 highly selective cases a sensitivity of 67% could be obtained for analysis by PCR and a sensitivity of 75% was observed for cultural assessment. These findings could be compared to patients who showed no signs of pulmonary tuberculosis. This group included 79 patients out of whom 57.0% showed positive cultural findings, 49.4% showed a positive PCR and 64.5% obtained typical histopathological findings. All these 79 patients showed proof of mediastinal lymph node tuberculosis.

EBUS TBNA

Regarding the results obtained through EBUS- TBNA from bacteriologically proven tuberculosis, 78 patients showed a positive cultural result (61.4%). Out of these 65.4% obtained a positive PCR, while only 55.1% showed a typical histology, with a total of 3% with unspecific necrosis with a lack of granulomatous reaction. Typical cytology included epithelioid granulomatosis with necrosis, which was found in 43 patients, while 32 patients showed epithelioid reactions without necrosis. Considering these patients with typical histopathological results, cultural assessment led to a sensitivity of 57.3%, while PCR analysis showed a sensitivity of 45.5%. In addition, it is important to note that histology including necrosis (33 out of 43 cases, sensitivity= 76.7%) showed a significantly higher rate of positive cultural results, than cases without necrosis (10 out of 32 cases, sensitivity= 30.3 %). Regarding the 29 cases with normal lymphatic tissue 15 still showed positive PCR results (51.7%), whereas 19 patients even obtained a positive cultural result (65.5%). Overall, PCR analysis led to a sensitivity of 59.3% for microbiological assessment.

Assessing IFN- γ test results

Interferon- γ Release assays (IGRA) cannot only be performed as a diagnostic test method but can also be used during follow-up investigation while on anti-tuberculosis therapy. We performed IGRA on 87 patients, with 78 subjects (89%) showing a positive IFN-γ test result. It is important to mention that the Interferon- γ test showed false-negative results in 11%.

Evaluation of elastography findings

We obtained elastography findings from 23 patients that showed a bacteriologically proven tuberculosis from lymph nodes. In this study we distinguished between four different types. Type one and two indicating benign or inflammatory tissue (red, yellow, green), while type three and four indicating mostly blue stiffer areas. In our case the distribution showed no Type 1 cases, 26% of Type 2 cases, 52% of Type 3 cases and 22% of Type 4 cases. Type two elastography findings showed mostly histopathological unspecific lymphatic tissue. It included 83% of the cases with a positive cultural result and 50% with a positive PCR result. Type three indicated 75% positive histopathological findings, 92% positive cultural results and 58% positive PCR results. Type four elastography patterns, showed positive cultural results in 80% and a positive PCR in 100% of the cases.

3. Conclusion

It is important to clearly identify the tuberculosis infection and to improve diagnostic methods for a quick elaboration of laboratory confirmation of the disease. EBUS TBNA is highly valuable in providing material for further cytopathological and cultural analyses and provides a safe and cost effective first-line investigation method for intrathoracic lymphatic tuberculosis. PCR based tests can provide a rapid confirmation of diagnosis, suggesting an improvement for early diagnosis.

Nevertheless, negative test results do not exclude a possible tuberculosis infection and culture analysis remains the preferential reference method. Also, it is important to regard histopathological results with caution and to bear in mind that a negative histopathological result can never rule out a tuberculosis infection. Considering QFT-TB test results, they mostly turned positive in cases of patients with recent immigration from tuberculosis endemic countries, as well as after a long duration of exposure to infected patients. Therefore, Interferon- γ release assays me be implemented for tuberculosis screenings purposes.

Elastography results cannot be used to differentiate between normal lymphatic tissue and lymphatic tissue modified by a tuberculosis infection. Elastography results do not regularly correlate with microbiological, histopathological, and cultural results. Most importantly it is indispensable to mention that the presentation of hard tissue (colored in blue), should not lead to the exclusion of tuberculosis further along the line.

3. Introduction

Tuberculosis is an infectious disease that mostly affects the lungs but can also affect other sites. It is one of the ten most deadly diseases worldwide, caused by the bacillus *Mycobacterium tuberculosis* (World Health Organization, 2018).

Mycobacterium tuberculosis was discovered on March 24, 1882, by Dr. Robert Koch. During this time tuberculosis killed one out of seven people in Europe and the United States, leading Koch´s discovery to be one of the most important steps towards controlling this disease (Barberis, 2017).

Diagnosis of extra-pulmonary disease remains especially hard due to the difficulty of reaching the disease site. Therefore, we tried to evaluate the significance of EBUS-TBNA in the diagnosis of intrathoracic tuberculosis of the mediastinal lymph nodes. We conducted a retrospective study with a cohort of 116 patients, comparing the amount of positive lymphatic tuberculosis results obtained from EBUS TBNA in patients with or without bacteriologically proven pulmonary tuberculosis. Our primary endpoint was to evaluate the quantity of matching histopathological, cultural and PCR results. In addition, we evaluated the amount of corresponding Interferon-γ test results in bacteriologically proven tuberculosis cases. Furthermore, we looked at the possibility of using elastography to differentiate between ordinary lymphatic tissue and lymph nodes being affected by tuberculosis.

4. WHO definition of tuberculosis

Tuberculosis is a highly infectious disease, which mostly affects the lungs. The disease is caused by bacteria from the *Mycobacterium Tuberculosis Complex*. Most notably tuberculosis causative agent is M. tuberculosis, but this infection can equally be caused by *M.bovis, M.microti, M.canetti, M.mungi* or *M.africanuum*. MTC contains known pathogens of humans and mammals. The bacterium is transmitted through coughing, sneezing, or spitting of infected patients with open lung tuberculosis.

Today tuberculosis is a disease which mostly occurs in low- and middle-income countries due to low health standards and an underdeveloped and underfinanced health care system that struggles to overcome one of the world's most deadly diseases. Considering the world´s population about one quarter of the world´s

population carries a tuberculosis infection. This latent infection results in the lack of any symptoms. The people are (not yet) ill with the disease and cannot transmit the infection. Nevertheless, about 10% of this latent infected population will later fall ill and develop an active disease and therefore be capable of further transmitting the disease during their lifetime. Those with a compromised immune system have a higher chance of getting sick. Risk factors include an HIV infection, malnutrition, diabetes, and smoking. When left untreated about 50% of these infected symptomatic patients do not survive the disease (World Health Organization, 2018).

Tuberculosis primarily infects the lungs but can affect different other body parts- then called extra-pulmonary tuberculosis, such as amongst others, the lymph nodes, the pleura, the central nervous system, the genitourinary system, the bones, and joints. A more serious, widespread form of tuberculosis, which is spread via hematogenous pathways, is called “disseminated tuberculosis or military tuberculosis”. Only about 15-20% of the tuberculosis cases worldwide are extra-pulmonary. Classic military tuberculosis is defined to be millet like seeding of *Mycobacterium tuberculosis* in the lung. This typical pattern can be seen on a Chest X- ray but is only present in 1- 3% of the cases. Extra-pulmonary tuberculosis generally more often affects patients with a weakened immune system (World Health Organisation, 2019).

Typical symptoms include chronic cough, night sweats, fever, weight loss, general weakness or consumption and blood-containing coughs. In the case of extra-pulmonary tuberculosis, a wide range of different other symptoms can occur. In most cases symptoms develop slowly and may be mild for some months, leading to a delay in seeking care and can result in the transmission of the bacteria to other people. People with an active tuberculosis may infect up to 5- 15 others throughout the course of a year (World Health Organisation, 2019).

Diagnosis of active tuberculosis is based on radiological findings, e.g., chest X-rays, symptoms, and detection of TB-bacteria by microscopic examination, culture, or molecular techniques. Additionally, latent tuberculosis can be examined by conducting a tuberculin skin test (TST) and/ or Interferon gamma release assay (World Health Organisation, 2019).

Tuberculosis is curable and preventable. Despite cost-effective and well-implemented options for vaccination (Bacille Calmette Guerin BCG)), as well as carefully monitored

drugs (including different combinations of antibiotics) the tuberculosis epidemic continues to spread in third world countries. This might be because the BCG vaccine is only effective against some severe tuberculosis forms, like tuberculous meningitis, but fails to withstand the pulmonary tuberculosis forms. In addition, more and more drug-resistant strains of the *Mycobacterium tuberculosis* are appearing. Moreover, the coinfection with HIV increases the likelihood of an infection.

In 2014, the World Health Assembly adopted WHO's End TB Strategy to eliminate the global TB epidemic by 2035, by reducing 90% of TB cases. This target should be achieved by the introduction of new diagnosis, treatment, and prevention methods, as well as further improvement of the vaccination options (World Health Organization, 2018). Ending the tuberculosis epidemic by 2030 is furthermore among the health targets of the United Nations Sustainable Development Goals (SDGs) (World Health Organization, 2018). Globally, tuberculosis incidences are falling about 2% per year. A cumulative reduction of 9% could be achieved between 2015 and 2019, which only accounts for half of the wanted reduction of 20% between 2015 and 2020. Between 2000 and 2019 an estimated 60 million lives were saved through tuberculosis diagnosis and treatment (World Health Organisation, 2019).

5. Epidemiology

5.1. Epidemiologic data worldwide

Although there has been remarkable progress in tuberculosis treatment as well as diagnosis methods, it remains one of the most common infectious diseases worldwide. Worldwide around one quarter of the population suffers from tuberculosis bacteria, with around 5-10% of these latently infected patients actually suffering from a symptomatic active tuberculosis infection, therefore being considered being ill with the disease. More than one third of tuberculosis cases remain undiagnosed (World Health Organisation, 2019).

With approximately 10.0 million patients in 2019, who fell ill with tuberculosis, this disease is a major health problem, with a high mortality rate in developing countries. Considering these tuberculosis cases, 88% were adults, 56% were male (6.5 million males, 3.2 million females and 1.2 million children were reported to be infected). Moreover, in 2019, 87% of the tuberculosis cases originated in eight high burden

countries including India, Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa. Tuberculosis is still the ninth leading cause of death and the number one deadliest infectious disease worldwide.

An HIV coinfection correlates with a higher chance of death due to a tuberculosis infection. Approximately 10% of the patients, who fell ill with tuberculosis, were HIV positive. Considering these patients, a third originated in Africa. In 2019 there were an estimated 1.4 million tuberculosis deaths amongst HIV negative people including 208 000 deaths among HIV positive patients (World Health Organisation, 2019).

There were 476.774 reported cases of HIV positive patients with tuberculosis infections, of whom 85 % were reported to be on antiretroviral therapy (ART). (World Health Organization, 2017). HIV positive patients do not only show a higher incidence of tuberculosis cases, but also account for higher rates of active disease, extra-pulmonary manifestations, and drug-resistant tuberculosis (Narang et al., 2015). HIV infected patients are more prone to reactivation of primary tuberculosis as well as obtaining tuberculous infections from exogenous bacilli. In several Indian studies, HIV positive patients constitute around 50% of the cases of extra-pulmonary tuberculosis. The most common site of tuberculous infection in HIV positive and therefore immunosuppressed patients are lymph nodes, followed by neurological, pleural, pericardial and abdominal sites (Sharma & Mohan, 2004).

Despite tuberculosis incidence falling during the past years, by about 2% per year, it remains one of the deadliest diseases worldwide and treatment and diagnosis have to be improved in order to decrease these numbers. Most deaths by tuberculosis can be prevented by early diagnosis and treatment. In 2016 global treatment success rates were about 83%. Tuberculosis preventative treatment has been expanding in recent years, especially in risk groups like HIV positive patients and children under five years of age. Nevertheless, most people suitable for tuberculosis preventative treatment are not accessing it (World Health Organization, 2017).

In a world of globalization and increased migration with asylum seekers, immigrants from all different countries and refugees as well as a lot of movement due to different work options, it has become more important than ever to analyze a patient's travel anamnesis to be able to estimate the risk of a tuberculosis infection. To evaluate the possibility of a tuberculosis infection, it is important to differentiate between high- as

well as low-prevalence countries. All North American countries, Central and Western Europe are considered low prevalence countries with incidences around 10 per 100,000 inhabitants. Over 95% of tuberculosis cases can be found in non-industrialized parts of the world, such as Africa, Southeast Asia as well as countries of the former Soviet Union, where patients mostly get infected in their childhood. This distribution of infectious cases is due to bad health and accommodation, as well as the HIV epidemiology in developing countries (World Health Organization, 2018).

5.2. Epidemiological data of tuberculosis infection in Germany

In defiance of the worldwide decrease in tuberculosis incidences, German tuberculosis epidemiologic data shows an increase in newly diagnosed tuberculosis infections in Germany. Tuberculosis re-emerging as a major health problem during recent years, can mostly be seen because of an increase in HIV infections as well as higher immigration rates in developed countries (Deveci et al., 2016). In 2016, 5915 tuberculosis cases were registered in Germany, resulting in an incidence of 7.2 newly diagnosed infections per 100 000 inhabitants. Considering demographic data men are more frequently affected than women, with a male tuberculosis incidence of 9.9 cases per 100 000 inhabitants, which is more than twice as high as the female incidence of 4.6 incidences per 100 000 inhabitants. The highest incidence rates can be observed within a young age group of between 20 and 24 years. The mortality rates of tuberculosis infections in Germany rose to 0.12 per 100 000 inhabitants. The lethality in 2016 of 1.7% dropped a little compared to the lethality of 1.9% the year before in 2015.

When looking at the origin of tuberculosis patients, it is striking that there is a high discrepancy between incidences of patients of German or foreign origin, with incidences of foreign patients being 19 times higher. Patients from Germany showed an incidence rate of 2.2, while foreign incidence stood at 42.6. This gap can especially be seen when regarding tuberculosis cases of young adults, where 69.1% of all reported tuberculosis cases in Germany were foreign born. The most common native countries in 2016 were Somalia, Eritrea, Afghanistan, Syria, and Romania.

An increasing number of tuberculosis infections were detected throughout a legally prescribed tuberculosis screening that is obligatory for refugees and asylum seekers

coming to Germany. The screening according to §32 (4) IfSG is an important instrument in early tuberculosis detection especially for people from high-risk countries, as tuberculosis numbers especially incline in these high-risk groups, making tuberculosis surveillance an important matter for the government. In the year of 2016, 1310 cases were detected through active case-finding. Active case finding requires systematic screening of persons at high risk of developing a tuberculosis infection. For example, people that live in close contact with infected patients. 973 of the 1310 cases that were detected by screening, were detected by screening at the borders. This screening includes an obligatory chest X-ray. In some cases, screening can also include an Interferon- γ release assay, for example when patients are pregnant and/ or under 15 years old.

Nevertheless, most of the cases could still be detected through passive case-finding. Passive case-finding implies that individuals that are aware of their symptoms are examined by health workers, who recognize the symptoms as tuberculosis typical. Overall, three quarters of all tuberculosis incidences could be revealed by passive case-finding.

Cases of foreign tuberculosis patients in Germany are increasing every year, while local cases are decreasing. This process correlates to the migration that has taken place in Germany during recent years. After tuberculosis incidences increased in 2015 due to a peak in migration and demographic movement, new incidences in 2016 declined as reported by the *Robert Koch Institute*.

Moreover, pediatric tuberculosis incidences in Germany have increased over the last few years, leading to the conclusion that better transmission conditions can be found in Germany, which can mostly be associated with expanding migration. In 2016, 233 children under 15 years were infected by tuberculosis, which marks an increase in incidence of 19% compared to 2015. The highest incidence of 2.9 per 100 000 inhabitants can be reported for children under the age of five. Uniform with adult epidemiology, children diagnosed with tuberculosis in Germany mostly originate from foreign countries. Also, the rate of children diagnosed with resistant tuberculosis bacteria peaked in 2016 at a rate of 30.5% of all infant tuberculosis diagnoses (Robert Koch Institut, 2016).

Concerning patients originating from the Western world tuberculosis incidences decrease due to an effect called tuberculosis of the elderly, describing the fact that most tuberculosis patients in Germany and other Western countries get ill due to a reactivation of a primary infection dating back decades. In contrary most cases of newly infected (and not necessarily symptomatic) patients, that can be found in the Western World are related to a travel case history, mostly concerning refugees, immigrants and people from poorer Eastern countries searching for work in the West (World Health Organization, 2018).

5.3. Multi-drug resistant Tuberculosis

Drug resistance can be defined by the growth of more than one percent of the *Mycobacterium tuberculosis* in the presence of the lowest concentration of the drug tested. According to a study by Davidson et al. drug resistance rates for lymph node tuberculosis include up to 17.6% of new cases and 55.5% of previously treated cases. In the group of previously treated patients 22% showed signs of multi-drug resistance. HIV positivity as well as previous treatment can count as risk factors for drug resistance and can be targeted through drug susceptibility testing (Davidson & Muthulakshm, 2018). The rate of patients suffering from all forms of drug-resistant tuberculosis infections (monoresistance, polyresistance and multi-drug-resistance), including resistance to second-line tuberculosis medication was 12.8% of all recorded tuberculosis cases in Germany in 2016.

Monoresistance means that one of the first-line anti- tuberculosis drugs fails to work against tuberculosis, for example in Rifampicin resistant TB. In contrast, polydrug resistance means that more than one first-line anti tuberculosis drugs (except both Isoniazid and Rifampicin). Multi-resistant strains (MDR TB) of bacteria show resistance to at least two first-line drugs against tuberculosis such as Rifampicin and Isoniazid. In Germany in 2016, the rate of multi-drug resistant tuberculosis was about 2.7%. Most of the multi-resistant tuberculosis infections recorded in Germany concerned patients with origins in the former Soviet Union. Another form of drug resistance in TB is called extensively drug-resistant TB (XDR TB). XDR TB is a rare type of MDR TB that is resistant to at least four of the core anti-tuberculosis drugs. These include Isoniazid and Rifampin, plus Fluoroquinolone (such as Levofloxacin and Moxifloxacin) and at

least one of three injectable second-line drugs (i.e., Amikacin, Kanamycin, or Capreomycin). This leaves patients with much reduced treatment options that are less effective. These types of TB mostly affect patients with HIV infection or other conditions that reduce their immune system (Robert Koch Institut, 2016).

Worldwide resistance to anti-tuberculosis medication has fluctuated between 11.5% and 13.6% in the past few years for any drug-resistance, as well as between 1.6% and 2.7% for multi-drug resistant tuberculosis infections (World Health Organization, 2017), (Herold, 2017). In 2016, 129 689 people started treatment against multi-drug resistant tuberculosis, which showed an increase compared to 125 629 patients in 2015. Treatment success remains extremely low and only accounts for about 54% globally. Demonstration of a resistant organism greatly alters anti tuberculosis treatment, and the duration of therapy as wrong treatment may induce selection of further drug-resistant strains (Navani et al., 2011). Resistance rates increase significantly every year, with a problematic augmentation of multi-drug resistant tuberculosis infections. In 2016, 600 000 new cases were reported with resistance to Rifampicin, a first line drug against tuberculosis. Out of these 600 000 cases, 490 000 patients showed multi-drug resistance. Almost half of the multidrug resistant cases (47%) were reported in India, China, and the Russian Federation (World Health Organization, 2017).

5.4. Risk factors for a tuberculosis infection

Social factors that contribute to the rising number of infections are close contact with infected individuals, for example during incarceration or living in small, crowded places, such as refugee accommodations, as well as homelessness and unemployment. For adults the risk of getting infected after contact with an infected person is about 10-15% (World Health Organization, 2018). Known medical factors that contribute to a higher risk of infection are alcohol and intravenous drug abuse, renal insufficiency, HIV coinfections and other autoimmune diseases, such as diabetes mellitus (American Thoracic Society & Center of Disease Control and Prevention, 2000). The higher risk of TB infection in HIV positive individuals is presumably due to the lack of CD 4 positive helper cells. In immunocompetent patients TH1 cells produce Interferon γ (IFN- γ) as well as Interleukin 2, which contribute to anti-mycobacterial immunity. In comparison TH2 cells mostly produce Interleukin 3, Interleukin 4, Interleukin 5, Interleukin 6 and Interleukin 10, which do not grant resistance to infection with mycobacteria

tuberculosis. In patients with an HIV infection less IFN-y is produced, while similar amounts of Interleukin 4 and Interleukin 10 are provided, leading to a higher susceptibility to fall victim to a tuberculosis infection (Sharma & Mohan, 2004).

Moreover, malignancy, especially hematological malignancies like leukemia and lymphoma apparently have an influence on the risk of tuberculosis infection. Additionally, a weak immune system can account for a big risk for tuberculosis infections. This risk is often due to patients receiving immunosuppressive medication or patients that are complicated by other diseases that disturb the immune system, as well as old age as it weakens the immune system (Xie et al., 2014). Furthermore, poor access to health care and chest X-ray suggestive of previous tuberculosis, can count as risk factors for tuberculosis (American Thoracic Society & Center of Disease Control and Prevention, 2000). Another big risk factor that can especially be found in Germany and other Western countries are TNF- alpha antagonists, used for treatment of rheumatic diseases. These drugs can lead to aberrant granuloma formation and exacerbation of chronic infections (Xie et al., 2014). Since 2006, national guidelines recommend a tuberculosis screening including tuberculin skin tests, chest X-rays and Interferon-gamma release assay for patients before starting TNF-alpha antagonists (Fonseca et al., 2008).

6. Clinical representation

Around 80% of tuberculosis patients primarily contract a lung infection, while 20% of patients show a primary extra-pulmonary tuberculosis infection. The most frequent manifestation of extra-pulmonary tuberculosis is a hilar lymphadenopathy with big swollen lymph nodes (Herold, 2017). 80 % of lung tuberculosis infections are open, meaning that tuberculosis bacteria can be found in the sputum, while one fifth of patients show closed tuberculosis infections. Khandkar reports that infections of the lymph nodes occur at a younger age of 25- 34 years, whereas pulmonary tuberculosis shows two peaks: one around 25-34 years old and another at over 65 years (Farer et al, 1979), (Khandkar et al., 2015).

6.1. Symptoms associated with a tuberculosis infection

Tuberculosis can affect every organ, which leads to difficulties in the diagnosis of tuberculosis as symptoms can differ in patients. Therefore, it is important to consider radiologic diagnosis whenever a patient shows symptoms of a prolonged cough. Infiltrations in the upper pulmonary lobe, cavernous formations, dilatation of the hilum as well as a picture of miliary tuberculosis can be radiologic signs associated with tuberculosis (Herold, 2017).

In general, tuberculosis can be associated with more general symptoms like fever, abnormal fatigue, weakness, and night sweat, as well as bronchopulmonary symptoms like coughing with or without sputum production, dyspnea, and chest pain. As symptoms of tuberculosis infection can be very unspecific, it is important to confirm bacterial growth before making the final diagnosis (Herold, 2017). A third of the patients show no symptoms at all (World Health Organization, 2018).

Moreover, localized signs, depending on the infected organ system, such as back pain for vertebral tuberculosis, abdominal pain for abdominal tuberculosis and other specific signs such as pleuritic pain and extensive pleural effusion can be found mostly in extra pulmonary tuberculosis. In some cases, fever of unknown origin can be the only diagnostic clue that may lead the examiner to suspect a tuberculous infection (Baussano et al., 2004). In cases of unspecific clinical representation, clinical suspicion and laboratory confirmation are essential prerequisites of precise diagnosis and non-delayed treatment (Narang et al., 2015).

6.2. Treatment

Every active tuberculosis infection needs to be treated. The aims of treatment of tuberculosis are first to cure the patient, as well as to prevent death from active tuberculosis or the relapse of tuberculosis. Another goal is to reduce transmission of tuberculosis to others and to prevent development of drug resistance.

Treatment consists of an intensive treatment phase with a combination of four different substances for two months, including Isoniazid, Rifampicin, Pyrazinamide and Ethambutol (HRZE), followed by a four-month continuation therapy phase with Isoniazid and Rifampicin (HR) (World Health Organization, 2010).

7. Diagnostic methods of tuberculosis infections

When suspecting a tuberculosis infection, a detailed history and physical examination of the patient is indispensable. This examination also includes a blood sample, a tuberculin test or Interferon gamma test, imaging techniques such as radiology, fine needle aspirations and molecular tests. These explorations help to detect tuberculosis infections at a relatively early stage and offer the possibility of starting empirical treatment prior to the final diagnosis. This final diagnosis can be achieved by the verification of tuberculosis bacteria through cultural detection and incisional biopsy (Deveci et al., 2016).

Tuberculosis bacteria can be diagnosed by a series of different methods including cultural growth of *Mycobacterium tuberculosis* from samples of sputum and bronchial secretion, urine and feces when suspecting urogenital or gastrointestinal tuberculosis or puncture of the lymph nodes. It is also possible to conduct histopathological analysis or the amplification of nucleic acids through the method of polymerase chain reaction (PCR) to detect a potential infection with *Mycobacterium tuberculosis*. (Herold, 2017) Bacterial evidence of tuberculosis is essential to exclude alternative diagnoses such as sarcoidosis, lung cancer or lymphoma (Navani et al., 2011).

7.1. Immunological tests

7.1.1. Interferon- γ release assays

A method of rapid diagnosis is the Interferon- γ release assay, which is an immunological in vitro test method. During a tuberculosis infection antigen presenting cells lead to the activation of T-lymphocytes which causes secretion of Interferon- γ. Memory cells which were previously immunized, can be reactivated rapidly and release Interferon- γ. This fact can be used in the Interferon- γ release assay by stimulating T-lymphocytes in vitro with antigens from *Mycobacterium tuberculosis* bacteria. If the patient has an infection with tuberculosis bacteria a secretion of Interferon- γ can be shown, which especially helps detect asymptomatic infections and latent tuberculosis cases. After a tuberculosis infection, it takes about eight weeks for a test conversion, which describes the outcome of the results turning positive. There are different sets of Interferon gamma tests all working with the same principle including T-SPOT.TB, QuantiFERON- TB® and QuantiFERON- TB® Gold, which was used in our

investigation. The method of QuantiFERON- TB® Gold In-Tube test kit is based on the whole-blood Elisa test of Interferon gamma released by T-cells. In order to perform the test 4 ml of heparinized whole blood is incubated with antigens for 20 hours. From a concentration of 0.35 IU/ml, the test is considered positive (Xie et al., 2014).

7.1.2. Tuberculin skin tests

Another T-cell related reaction is the tuberculin skin test (TST) after Mendel Mantoux, in which an enlargement of the skin induration of the forearm, shows a reaction of the T- lymphocytes to an antigen of *Mycobacterium tuberculosis*.

The test is performed by injecting antigenic material from *Mycobacterium tuberculosis*, the tuberculin extract, intracutaneous. The injection spot has to be marked in order to later on decipher the diameter of skin induration. In a next step the skin reaction is regarded 72h later. In the case of an infection with Mycobacterium tuberculosis, a delayed immune reaction (Type IV) occurs, in which T- cells react to the presented antigen and cause a local reaction- a skin induration.

An induration diameter of over 10 mm indicates a positive outcome- and therefore a potential infection with *Mycobacterium tuberculosis*. Concerning reliability, the tuberculin skin test has a high sensitivity with limited specificity. Suspicious outcomes (5-9 mm induration) can occur after BCG vaccination. Therefore, mandatory Bacille Calmette Guerin vaccination in some countries may increase false positivity of the TST. False negativity may also be due to other chronic infections, malnutrition, metabolic diseases, virus vaccinations, immunosuppressive drugs, and malignancy, sarcoidosis, or stress reactions. Moreover, false positive test results may occur in newborns or elderly people as well as in consequence of inadequate test application. Likewise, HIV co-infections, poor nutritional status, recent viral and bacterial infection as well as vaccination with a live virus may reduce the response to tuberculin skin tests. An induration of under 4 mm accounts for a negative result. A sole skin redness without induration can also be seen as negative. False negative reactions occur in at least 20% of all patients with active tuberculosis. Also, TST and IGRA cannot distinguish between active or latent tuberculosis infections (Deveci et al., 2016).

TST is widely used to screen high-risk subjects, to show a delayed hypersensitivity against the mycobacterial antigen. (Herold, 2017) The test turns positive two to ten weeks after the mycobacterial infection (Deveci et al., 2016).

Nevertheless, the specificity of the Interferon-gamma assay is significantly higher than the tuberculin skin test. Likewise, the Interferon- γ assay is less influenced by immunosuppression and HIV infection (Fonseca et al, 2008), leading to the conclusion that the Tuberculin skin test is a valuable but non-specific assessment criteria of tuberculosis (Deveci et al., 2016).

Although a positive tuberculin skin test or Interferon- γ release adds weight to the diagnosis of tuberculosis, these tests also turn positive in some cases of sarcoidosis, therefore indicating that these diagnosis methods cannot be seen as specific for tuberculosis. In addition, some of these cases can spontaneously clinically improve which could be mistaken for improvement under anti-tuberculosis treatment (Navani et al., 2011).

7.2. Imaging

Next to cultural verification, the most important diagnosis method for lung tuberculosis is radiology. The typical radiological findings in pulmonary tuberculosis include infiltrations mostly in the upper pulmonary lobe, signs of cavitation, calcification as well as dilatation of the pulmonary hilum. Tuberculous cavitation is the hallmark of the established disease and is characterized by central elucidation and low attenuation, a peripheral rim enhancement and a draining bronchus. These can arise by the melt in of caseous necrotic tuberculous tissue (Herold, 2017). Tuberculous cavitation can mostly be found in the upper lobe of the lung or the apices. The connection of tuberculous cavitation with the airways results in the release of many infectious bacilli, which can cause transmission of the infection to a new susceptible host. Furthermore, infectious droplets can deposit in the lower part of the lung, leading to the formation of Ghon foci, representing calcified healed tuberculous granuloma (Ong et al., 2014).

Regarding imaging in the case of equivocal lymphadenitis, the change of size, shape, echogenicity, and morphology of the lymph nodes strongly indicates a tuberculous infection. These sonographic criteria are mostly applied in search of cervical

lymphadenitis and therefore concentrate on nodal hilum and cortex areas in the attempt to differentiate between malign and benign cervical lymph nodes. There are three different patterns of nodal involvement depending on the stage of infection. In an early stage the lymph nodes are homogenous and after the use of intravenous contrast fluid they enhance evenly. A sign of the pejoration of the disease leads to the second and most common tuberculosis associated pattern, a lymph node with a central area of necrosis. The nodes with a center of low attenuation and a strong rim enhancement can clearly be characterized in CT. A third tuberculosis associated pattern that can mostly be seen after treatment are fibro calcified lymph nodes. In CT imaging these nodes show homogenous surfaces and calcification can therefore not be detected.

Apart from chest X-rays, ultrasound, computerized tomography (CT) and magnetic resonance imaging (MRI) can be performed both on suspected pulmonary tuberculosis as well as lymphadenitis. Normal imaging results should however not exclude a tuberculosis infection as cause of the disease pattern. Ultrasound is mostly used as an imaging technique in combination with fine needle aspiration or in EBUS TBNA, resulting in high specificity and sensitivity. CT and MRI account for valuable complementary techniques to demonstrate the sites, patterns, and extent of the infection. However, they are not sufficient to make a certain diagnosis (Deveci et al., 2016).

7.3. Hematological tests

Although there is no specific blood test for tuberculosis, general infectious blood sample shifts in combination with other tuberculosis symptoms strongly indicate a tuberculosis infection. Anemia, hyponatremia, increased ALP, elevated ESR, leukocytosis and thrombocytosis are associated with chronic disease condition and cause doubt about a non-specific inflammatory response (Deveci et al., 2016).

7.4. Histopathology

Although implementing an invasive procedure to gain the material through biopsy or needle aspiration, histopathologic methods are still one of the most important diagnostic instruments of pulmonary tuberculosis as well as lymphadenitis (Deveci et

al., 2016). The typical histologic observation in tuberculosis patients includes epithelioid granulomatosis with caseating necrosis. The granuloma is built up of an epithelioid seam with Langerhans giant cells on the inside, while we can find a lymphocyte seam on the outside. Moreover, we can find granulomatous inflammation and calcification. These histopathological findings may lead to the diagnosis of tuberculosis; however, a typical histopathology is not present in all tuberculosis cases (Xie et al., 2014). In comparison, normal lymphatic tissue can be described by benign oval-shaped lymph nodes being surrounded by a fibrous capsule (Warwick & Williams, 1973).

In tuberculous lesions, different mechanisms of pathogenesis of necrosis are known to explain cell death induced by *Mycobacterium tuberculosis*. Different mycobacterium strains are associated with macrophage apoptosis, while other virulent strains inhibit apoptosis and facilitate necrosis. Necrotic lesions sometimes show an abundance of acid-fast bacilli. Granulomatous reactions activate bacterial growth by further recruiting macrophages.

Another specialty indicated in tuberculosis infections in HIV positive patients shows that the coinfection with the HIV Virus alters the host response. Granulomas show a poorer cellular organization, with less lymphocytes and macrophages. In addition, a larger area of necrosis can be observed in these cases with higher amounts of acid-fast bacilli present in the tuberculosis granulomas (Narang et al., 2015). Nevertheless, typical granulomatous reactions and caseation are less likely to be found in HIV positive patients, due to an impaired T-cell function (Deveci et al., 2016).

7.4.1. Fine needle aspiration

Fine needle aspiration is mostly used in lymphadenitis. A fine needle is inserted in a swollen, malignant looking lymph node to obtain material for cultural, molecular, or cytological assessment (Deveci et al., 2016). The aspiration procedure is performed using a 22/23- G needle attached to a 10ml syringe mounted on a handle. Subsequently, both wet fixed and air-dried smears are prepared. In cases with a cytological diagnosis with granulomatous or necrotizing lesions, Ziehl Neelsen staining is performed to detect acid-fast bacilli (Narang et al., 2015).

Fine needle cytology can show the typical histopathological pattern of granulomatous nodes with central caseating necrosis strongly suggesting a tuberculosis infection. The sensitivity and specificity of fine needle aspiration cytology is therefore respectively high. Fine needle aspiration also accredits material for Ziehl Neelsen straining in acid fast bacilli, mycobacterial culture, and molecular PCR testing. Ziehl Neelsen straining has a specificity of 100% with a sensitivity of 46- 78% (Deveci et al., 2016). Fine needle aspiration is a fast, cost effective and minimal invasive technique to find histopathological and cytomorphological patterns and pathologic manifestations of chronic infections (Narang et al., 2015).

7.5. Bacteriological assessment

7.5.1. Culture

In order to have a definitive diagnosis it is important to prove the presence of a bacteria from the *Mycobacterium Tuberculosis Complex*. Mycobacteria possess a waxy coat which permits the bacteria to be acid proof and guarantees a slow reproduction and resistance against noxae (Herold, 2017).

A definite proof of *Mycobacteria tuberculosis* can be made by using culture to isolate the bacteria. Nevertheless, a negative culture result should not exclude a tuberculosis infection. According to Deveci et al. isolation of mycobacteria by culture is only possible in 10- 69% of the cases, when punctuating lymph nodes. Another negative factor of using culture for tuberculosis detection is the long duration until the assessment of cultural results, which can take up to six to eight weeks, leading to a delay of treatment initiation.

Cultural results can be obtained from both broth culture and culture on solid medium. Suspension including tissue samples or liquid samples have to be inoculated on slants and incubated at 37°C in order to create a direct sputum-based culture. These slants can then be inspected weekly in order to examine cultural growth. If no cultural growth can be found after 12 weeks these samples can be seen as culturally negative. In general, cultural results could be obtained more quickly by evaluating broth culture, with an average of 25 days, than culture on a solid medium with an average of 34 days (Noussair et al., 2009).

7.5.2. Molecular testing

Polymerase chain reaction (PCR) is a nucleic acid amplification test (NAAT), which provides rapid specific and sensitive diagnosis of *Mycobacterium tuberculosis*, throughout molecular testing. NAAT can detect *Mycobacterium tuberculosis* (MTB) in less than two hours. In comparison standard cultures can take between two to six weeks in order for *Mycobacterium tuberculosis* to grow, with another three weeks in addition in order to detect a resistance against Rifampicin. Therefore, NAAT cannot only help to speed up tuberculosis diagnosis, but also helps to adapt the treatment due to drug resistance. (Deveci et al., 2016).

To conduct a nucleic acid amplification (NAA) test, a sputum sample is collected from the patient with suspected tuberculosis. The sputum is mixed with the reagent that is provided with the assay. As a next step a cartridge containing this mixture is put in the GeneXpert machine. All processing from this point on is fully automated. During the process the DNA is automatically multiplied by PCR in order to detect different genetic loci and mutations. PCR allows the targeted amplification of specific DNA segments from a mixture of DNA molecules. A primer, a nucleotide sequence, on the “sense” strand of the DNA and another on the “antisense” strand, flank the DNA segment that is to be amplified. The DNA polymerase replicates after the addition of the primer and fills the strands with free nucleotides (elongation).

For example, one NAAT test is the Xpert® MTB/ RIF assay which contributes to rapid diagnosis of a tuberculosis infection and can simultaneously detect a resistance to Rifampicin (RIF). Genetic loci associated with Rifampicin resistance should furthermore be assessed for Multidrug Resistance. If a Rifampicin resistance is confirmed, rapid molecular resting for other mutations associated with drug resistance is needed, as Rifampicin resistance often co-exists with INH resistance.

It can show a sensitivity up to 98% for smear-positive, culture-positive tuberculosis. As an initial test replacing smear microscopy Xpert® MTB/RIF pooled sensitivity was 89%, with a pooled specificity of 99%. As an add-on test following a negative smear microscopy result, the Xpert® MTB/RIF pooled sensitivity was 67%, with a pooled specificity of 99%. Studies comparing smear microscopy and PCR testing showed more sensitive results for NAATs. For smear microscopy, the pooled sensitivity was 65%, while it was 88% for Xpert® MTB/RIF. Nevertheless, it is important to always

interpret the NAAT tests along clinical and laboratory findings, as well as imaging. It does not replace the need for smear with microscopy for acid-fast bacilli and culture for *Mycobacterium tuberculosis* (Steingart et al., 2014).

8. Extra-pulmonary Tuberculosis

8.1. Definition

Extra-pulmonary tuberculosis is defined as a tuberculosis manifestation other than of the lungs, with no detectable primary pulmonary tuberculosis. If any signs of lung manifestation of the *Mycobacterium tuberculosis* occur, the tuberculosis is categorized as pulmonary tuberculosis according to the World Health Organization (WHO). For example, miliary tuberculosis can be classified as pulmonary tuberculosis as lesions in the lungs occur among other places of manifestation elsewhere on the body. In comparison, pleural effusion can be characterized as strictly extra-pulmonary tuberculosis with no radiographic abnormalities in the lungs (Sharma & Mohan, 2004). Around half of the extra-pulmonary tuberculosis affects the lymph nodes (Robert Koch Institut, 2016). Tuberculosis bacilli enter the lymphatic system, as well as the blood stream to reach other extra-pulmonary organs. This can lead to the infection of virtually any other organic system (Deveci et al., 2016).

8.2. Epidemiology

Regarding the manifestation of tuberculosis, tuberculosis of the lungs is still the most common form of tuberculosis seen in Germany with 75% of all manifestations. With an incidence of 4.1 per 100 000 inhabitants open lung tuberculosis is more common than the closed form of lung tuberculosis with an incidence of 1.3 per 100 000 inhabitants. Extra-pulmonary tuberculosis was registered in 1468 cases in 2016, which accounts for about 25% of all tuberculosis incidents in Germany (Robert Koch Institut, 2016).

Even though there has been a steady decline in respiratory forms of tuberculosis, extra-pulmonary tuberculosis has been steadily increasing in developed countries. It has been suggested that this uneven decline of pulmonary tuberculosis and incline of extra-pulmonary tuberculosis could be due to the modification of the demographic structure of the population in industrialized countries. In these countries we see a co-

existence of younger immigrants and elder native residents. Furthermore, people who originated from high-prevalence tuberculosis countries are more likely to get tuberculosis infections on extra thoracic sites. Moreover, younger people and women are more likely to get non respiratory infections (Baussano et al., 2004). However, cases were also detected in the extremes of age, which might be due to reduced ability of the immune system to contain the bacilli locally in the lung parenchyma which becomes a problem in perimenopausal women. Furthermore, it has been suggested that endocrine factors, smoking habits as well as previous exposure to tuberculosis count as related factors in the incidence of extra pulmonary tuberculosis (Narang et al., 2015).

According to Gonzalez and Teeter et al. this phenomenon equally occurs more often in patients with HIV coinfections, with 70% of seropositive patients being affected by extra-pulmonary tuberculosis, compared to 15- 30% of seronegative patients. This condition is mostly associated with an immunocompromised status due to the human immunodeficiency virus infection (Gonzalez et al., 2003). Overall, a poor immune system often due to socioeconomic factors plays an important role in the formation of extra-pulmonary tuberculosis (Narang et al., 2015).

Tuberculous lymphadenitis is most common in black and Asian populations and foreign-born patients regarding Germany as the country of investigation. In addition, it is supposed to be more common in females than in men, according to various studies. Extra-thoracic tuberculosis sights may coexist with pulmonary tuberculosis. Tuberculosis cannot only affect every organ in the body, but often we can also see coexistence of chest radiographic changes, as well as hilar lymphadenopathy and mediastinal widening, accounting for simultaneous presence of different tuberculosis infections (Gonzalez et al., 2003).

8.3. Sites of extra-pulmonary tuberculosis

There are a notable number of different extra-tuberculous sites, as tuberculosis can nearly affect any organic system in the body. Extra-pulmonary tuberculosis forms include tuberculosis of the central nervous system, intestinal tuberculosis, osteoarticular tuberculosis, genitourinary tuberculosis, disseminated tuberculosis, tuberculosis of the skin and subcutaneous tissue as well as lymph node tuberculosis.

Furthermore, extra-pulmonary and extra-nodal sites affected are breast, chest and abdominal wall, salivary glands, and soft tissue. The most common form is peripheral tuberculous lymphadenopathy ranging from hilar to mediastinal lymphadenopathy, frequently with coexisting radiographic changes. In a study conducted by Narang et al., lymph nodes as an extra-pulmonary site of tuberculosis show a predilection of cervical lymph nodes (81.4%) followed by axillary lymph nodes (3.7%) and inguinal lymph nodes (1.6%). Multiple lymph node groups were involved in 2.5% of the cases (Narang et al., 2015).

8.3.1. Cervical lymphadenitis

The most common form of extra-pulmonary tuberculosis is cervical lymphadenitis. A patient is defined to have lymph node tuberculosis if either a positive culture for *Mycobacterium tuberculosis* or typical histopathological results is obtained. Mediastinal and hilar lymph nodes are primarily involved (Yew & Lee, 1995).

Diagnosis of cervical lymphadenitis remains difficult, which highlights the importance of employing differential diagnosis for this disease pattern. Concerning differential diagnosis other granulomatous lymphadenitis have to be considered, such as non-tuberculous mycobacteria (*M.avium, M.scrofulaceum, M.hameophilum*), as well as taking sarcoidosis, tularemia, toxoplasmosis, fungal disease, cat-scratch disease and neoplasms into account. This differential diagnosis can show similar histopathological conditions and cytology. Next to tumor, non-tuberculous mycobacteria is seen to be one of the most common causes of lymphadenopathy in adults in the developed world according to Sharma et al. Nevertheless, *Mycobacterium tuberculosis* is still an important differential diagnosis regarding pathogens for lymphadenitis among children in the industrialized world. In comparison Asians and Hispanic patients as well as African Americans show high predilection for tuberculosis infections. Considering non tuberculosis mycobacterial lymphadenitis, both sexes are equally affected (Deveci et al., 2016), (Sharma & Mohan, 2004). Cervical lymphadenitis is generally more common in females as well as the younger population, whereas pulmonary tuberculosis can mostly be found in elderly male patients.

Clinical presentation of cervical lymphadenitis may differ depending on individual factors, age, gender, and previous illnesses. Most frequently unilateral posterior

cervical or supraclavicular painless humps may be discovered one or two months after an infection. This chronic painless mass in the neck can be persistent and usually grows over time. The duration of an infection prior to the clinical manifestation may vary from three weeks to eight months, which hinders the early detection of the disease. Additionally, to hump formation, about 10% of patients show fistula formations. Initially the nodes are discrete, but the inflammation around the lymphatic glands results in matting and the fixation of the lymph nodes. In the course of the disease aggravation caseous collections can form, which can lead to the perforation of the deep fascia. This results in subcutaneous caseous collections called collar-stud abscess. As the disease progresses the overlying skin may be involved, resulting in sinus or ulcer formation, scrofuloderma and tuberculous dermatitis. These conditions yield a low therapeutic potential, often remaining unhealed for years. Healing may conclude in the formation of scars or calcification. The enlarged lymph nodes may vary in size. If necrosis has occurred, they may have a cystic consistency due to abscess formation. Lymph nodes are generally not tender until after secondary bacterial infection (Deveci et al., 2016), (Sharma & Mohan, 2004).

Peripheral tuberculosis lymphadenopathy can be classified into five different stages. These five stages include stage one: enlarged, mobile, firm lymph nodes showing non-specific reactive hyperplasia and stage two: large, rubbery nodes fixed to the surrounding tissue including periadenitis, an inflammation of the surrounding tissue of the gland. Stage three appears as central softening due to abscess formation, which is provoked by caseous necrosis. Stage four presents as the previously described collar-stud abscess, leading to stage five which includes sinus tract formations. Tuberculous sinus may appear with thin, blue edges including a watery discharge (Sharma & Mohan, 2004).

Symptoms that are particular for tuberculosis manifestation in mediastinal and cervical lymph nodes include cough and dysphagia. Furthermore, classical general infectious symptoms such as night sweats, weakness and fever can be seen to different extents. These symptoms are most common in HIV positive patients, who also tend to show multiple symmetrical lymphadenopathies in contrast to the representation of focal asymmetric magnifications of lymph nodes in HIV negative patients. Furthermore, intrathoracic, intraabdominal, and associated pulmonary disease is more common in HIV positive patients (Deveci et al., 2016), (Sharma & Mohan, 2004).

Lymph nodes that were mostly affected by extra pulmonary tuberculosis include mostly anterior and posterior cervical lymph nodes, followed by axillary and inguinal lymph nodes. Another lymph node station that can be affected by tuberculosis are supraclavicular, submental, and pre-auricular lymph nodes. Overall, peripheral lymph nodes are more likely to be affected (Noussair et al., 2009)

8.3.2. Tuberculous pleural effusion

The pleural effusion is the second most common site of tuberculosis. Nevertheless, despite its high incidence in poorer countries, like India, it is not common in the Western world. Pleural effusion can mostly be detected by the proof of typical tuberculous pleural granulomas through pleural biopsies and by cytological assessment of the lymphatic effusion. Differential diagnosis includes sarcoidosis and malignant pleural effusion. Malignant and tuberculous lesions may coexist due to the reactivation of a latent tubercular infection in the case of suppression of the immune system (Chakravorty et al., 2005). Patients with tuberculous pleural effusion show signs of an acute illness with symptoms of pleuritic chest pain, cough, breathlessness, and dyspnea. Diagnosis methods include chest radiography, mostly revealing unilateral pleural effusions. In addition, ultrasonography and contrast-enhanced CT scans of the chest may be useful in distinguishing between the different fibrin bands, septations and regular pleural thickening (Sharma & Mohan, 2004). In addition, bronchoscopic evaluation or sputum smear culture can be used, yielding a result of approximately 52% positive sputum cultures (Lee, 2015).

8.4. Diagnosis methods for extra-pulmonary tuberculosis

Non respiratory tuberculosis is defined by identifying acid fast bacilli and / or isolating *Mycobacterium tuberculosis* in culture samples in extra-pulmonary sites, as well as clinical, laboratory und radiological findings (indurations over 15 mm). Also, histopathological findings are considered. Microbiological identification is obtained by acid fast stain or through culture identification. Nevertheless, some patients show both negative culture and smear, although still suffering from a tuberculosis infection. In these cases, conventional methods and cytological evaluations must be combined with PCR techniques and clinical investigation, as monitoring by smear microscopy and

culture is not adequate. Monitoring through clinical assessment must be ensured. To obtain tissue for cultural and histopathological evaluation different methods including needle biopsy, excision, endoscopy, laparoscopy, biopsies under guidance of ultrasound, computed tomography (CT) or endoscopic ultrasound can be used to ascertain the diagnosis. The relative sensitivities and specificities, as well as the different therapeutic benefits must be considered, when evaluating different choices of diagnostic approaches.

In addition, it is important to evaluate the existence of conjoint pulmonary manifestation of tuberculosis infections, as about 10 to 15% of extra-pulmonary tuberculosis cases may show pulmonary involvement. Nevertheless, the yield of sputum culture in extra-pulmonary tuberculosis yields low diagnostic value. The sensitivity of sputum culture varies from 28- 50% for abdominal tuberculosis, 10- 11% for tuberculosis pericarditis, 24- 29% for tuberculous meningitis as well as 5- 14% for tuberculous lymphadenitis (Lee, 2015).

While chest X-rays have no diagnostic value, CT and MRI are strongly recommended as imaging techniques. Furthermore, fine needle aspiration in combination with cytology, molecular PCR tests and cultural examination are valuable for the definite diagnosis of cervical lymphadenitis (Deveci et al., 2016), (Sharma & Mohan, 2004). Mediastinal lymph node sampling is usually conducted by transbronchial needle aspiration and endoscopy but can now also be performed by EBUS TBNA yielding a high sensitivity compared to conventional mediastinoscopy (Navani et al., 2011). Other imaging techniques, such as gastrointestinal endoscopy, laparoscopy, cystoscopy, colonoscopy, and different biopsies under visual guidance can achieve a precise anatomical localization of different extra-pulmonary tuberculosis sites. Clarifying the exact diagnosis is important to exclude alternative diagnoses such as lymphoma, carcinoma and sarcoidosis (Noussair et al., 2009).

8.4.1. Immunological tests

Tuberculin skin tests do not show sufficient evidence in the diagnosis of extra-pulmonary tuberculosis, as not all tuberculosis infections show a tuberculin positive result and the test only yields a low sensitivity, with a wide range between correctly diagnosed positive patients and patients who did not get a positive tuberculin test

result, but still suffer from tuberculosis. Interferon gamma release assays on blood samples conducted on patients with extra-pulmonary tuberculosis showed a sensitivity of 72% and a specificity of 82% for QuantiFERON® TB- Gold, in a meta-analysis conducted by Ji Yeon Lee. Respectively a sensitivity of 90% and a specificity of 68% has been found for T-SPOT.TB. T. SPOT.TB has been shown to be more sensitive for patients with chronic forms of extra-pulmonary tuberculosis, such as lymph node or osteoarticular tuberculosis. It is a less appropriate method for acute tuberculosis forms, such as tuberculosis meningitis. QuantiFERON® TB has the highest sensitivity for tuberculous lymphadenitis. Nevertheless, this test method is less useful for tuberculosis meningitis or pleural tuberculosis, especially when testing on blood samples. In this case, it is therefore more convenient to perform an Interferon gamma assay on pleural effusion, with a sensitivity of 75% and a specificity of 82%. Overall Interferon gamma release assays show limited use for tuberculous pericarditis and peritonitis (Sharma & Mohan, 2004), (Lee, 2015).

8.4.2. Bacteriological assessment

When diagnosing extra-pulmonary tuberculosis, the emphasis is on microbiological assessment. This includes microscopy with light microscopy (Ziehl- Neelsen staining) or fluorescence microscopy (Auramin staining), cultural growth of *Mycobacterium tuberculosis* and NAAT. The use of nucleic acid amplification tests in the detection of extra pulmonary lymph nodes has been greatly discussed, with the aim of getting a more rapid and safe diagnosis. Unlike pulmonary tuberculosis, extra pulmonary tuberculosis does not yet show a clear recommendation for using PCR samples. Nevertheless, the rapid diagnosis, especially of life-threatening manifestations of tuberculosis, such as tuberculous meningitis may be a major advantage of nucleic acid amplification. Also, nucleic acid amplification (NAAT) can be conducted on less than 10 mycobacteria detected in the specimen (Noussair et al., 2009).

Analytical sensitivity of NAAT is expected to be extremely high, as many copies of gene targets may be present per genome. Sensitivities of PCR analysis range between 42 and 100%, while specificities can range between 85 and 100% depending on the use of different PCR targets, such as IS6110, 65kDa, TRC4, GCRS and others. Therefore, a positive PCR result can be considered a clear assumption of the diagnosis

of tuberculosis, while a negative nucleic acid amplification testing cannot provide enough evidence to exclude a diagnosis. For example, the Xpert® MTB/ RIF assay which is a new automated cartridge-based nucleic acid amplification testing method useful for the diagnosis of extra-pulmonary tuberculosis (Daley et al., 2007). According to Ji Yeon Lee, a meta-analysis showed overall sensitivity of 83.1% and a pooled specificity of 98.7 % for the diagnosis of extra-pulmonary tuberculosis with Xpert® MTB/RIF. Nevertheless, Xpert® mostly showed higher sensitivity for the detection of lymph node samples, while the detection of tuberculous meningitis showed moderate sensitivity and tuberculosis in pleural fluid could rarely be detected (Lee, 2015).

With the intention of evaluating the accuracy of PCR in the detection of extra pulmonary tuberculosis sites, Noussair L. et al. concluded a study evaluating the use of PCR in combination with preliminary broth culture. The PCR results showed a high specificity and a positive predictive value, but also a low variable sensitivity and negative predictive value. Therefore, a positive result correlates with a positive tuberculosis result with high certainty, while a negative result cannot exclude tuberculosis with certitude. Nevertheless, PCR results do not account for a high clinical value and tuberculosis treatment is hardly ever commenced or ceased according to nucleic acid amplification tests. It is therefore noteworthy to say that sole PCR application to extra pulmonary tissue without any prior cultural examination has a relatively low sensitivity and negative predictive value.

Nevertheless, according to Daley et al. the sensitivity and specificity of PCR used in extra pulmonary tuberculosis were highly heterogeneous throughout a meta-analysis of various studies, depending on different methods and population. Therefore, the data cannot be relied on to make any clinical recommendation, proposing that treatment should neither be started nor stopped solely based on PCR results. Mostly this is due to nuclear acid amplification methods quickly evolving and technology constantly changing, increasing speed as well as decreasing cost, which results in the variability of test results. Only the fact that sampling a larger amount of specimen leads to a higher probability of amplification of rare templates and therefore proposing a minimum of 20μl, was consecutive in all compared analyses (Daley et al., 2007).

Nucleic acid amplification can yield low positive results in targeting tuberculosis infections, due to the presence of different PCR inhibitors. These inhibitors can include

blood, host proteins and eukaryotic DNA, which can reduce amplification during nucleic acid amplification.

Furthermore, it is important to note that the most target-oriented combination of nucleic acid amplification is a combination of two PCR sampling methods using different assays, for example IS6110 and devR assays. In addition, larger volume of body fluid can enhance the possibility of detecting a tuberculosis infection in patients. Chakravorty et al. concluded that PCR and microbiological results can increase the accuracy of extra-pulmonary tuberculosis in the context of clinic-pathological characteristics. This study also concluded that PCR efficiency was superior in pleural tissue and pleural fluid compared to lymph node tissue (Chakravorty et al., 2005).

8.4.3. Histopathology

In addition to PCR and culture, histopathological analysis can be conducted. A typical histology includes typical granuloma with caseation and necrosis. Nevertheless, there can be discrepancies between histopathological and microbiological results, which can be due to a non-uniform distribution of bacilli in the samples taken (Noussair et al., 2009). Concerning histopathological patterns, we can differentiate between caseous necrosis only, caseous necrosis with degenerated inflammatory cell, caseous necrosis with epithelioid cell granulomas or epithelioid cell granulomas without any necrosis. The most evident combination of results is a positive cultural result as well as a histology indicating epithelioid granulomatous reactions including Langerhans type giant cells and caseation necrosis. In addition, hypo- cellularity, and epithelioid cells with or without the presence of acid-fast bacilli must be taken into account (Dasgupta et al., 2017). Histopathological examination requires the specimen to be placed in a solution of formalin. This solution destroys the mycobacteria, which makes it necessary to submit fresh biopsy material for culture assessment (Lee, 2015).

The gold standard is the combination of culture, histopathological analysis and response to tuberculosis treatment. PCR results lack the sufficient sensitivity to be used as the sole criteria to identify a positive tuberculosis infection. Therefore, PCR results and histopathological results should always be compared when available to decide whether a treatment should be initiated or not. Furthermore, it is important to mention that it is always important to interpret the results in the context of clinical

suspicion. Nevertheless, in cases with patients that indicate a high clinical suspicion and positive PCR results with no histopathological or cultural proof having been obtained, the positive PCR result can indicate a need for tuberculosis treatment. In addition, in cases with no other positive diagnostic criteria, an Interferon-gamma release assay may be useful to detect an extra- pulmonary tuberculosis (Noussair et al., 2009). Other studies, such as the Korean study of extra-pulmonary diagnosis methods by Ji Yeon Lee suggest that PCR yields a high diagnostic value for the diagnosis of extra-pulmonary tuberculosis. The combination of histopathological results, culture and PCR results can achieve a sensitivity of 91% in the evaluation of pleural effusion. Likewise, regarding the diagnosis of tuberculous lymphadenitis yields a specificity of 94- 100% and a sensitivity of 82.4- 100%, when combining fine needle aspiration cytology and PCR (Lee, 2015).

8.4.4. Tissue attainment

To obtain specimens to practice cultural and histopathological analysis on tissue in lymphadenopathy, fine needle aspiration cytology is an accurate first line method. After aspirating material with a 21- or 22-gauge needle, smears can be prepared that can later be stained in Ziehl- Neelsen to prove the presence of acid-fast bacilli. Fine needle aspiration cytopathology (FNAC) confirms the diagnosis in most cases. However, this technique still includes some limitations as fine needle aspiration is a blind procedure and the needle may not always be able to reach the concerned region. Therefore, if all diagnostic references show negative results in discrepancy to a highly suspicious clinical representation, it is strongly advised to repeat the procedure by trying to punctuate a different part of the lymph node. During the excision biopsy of a lymph node by fine needle aspiration it is useful to utilize CT scans to localize intrathoracic and intraabdominal lymphadenopathy. Another helpful aid can be video-assisted thoracoscopic surgery (VATS), especially in intrathoracic lymphadenopathy and pleural disease (Sharma & Mohan, 2004), (Dasgupta et al., 2004).

In addition, it is extremely important that all examinations are done under aseptic precautions, as optimal clinical preparation shows massive contribution to the accuracy of the results (Chakravorty et al., 2005). It is important to mention that a positive test result can strengthen a tuberculosis diagnosis, while a negative test result can never

rule out this diagnosis, which makes diagnosis especially of extra-pulmonary tuberculosis extremely hard. Another two criteria that are important to evaluate whether a patient is suffering from tuberculosis or not are a clinical presentation compatible with tuberculosis, as well as a positive response to anti-tuberculosis treatment (Sharma & Mohan, 2004).

8.4.5. Diagnosis of extra-pulmonary tuberculosis in different body fluids

As extra pulmonary tuberculosis includes various sites different biopsy samples have to be investigated, including mostly lymphatic tissue, but also bone marrow, digestive tissue, skin samples and organic fluids. As tissue biopsies can involve very invasive and inaccessible procedures, the investigation of body fluids provides a valuable diagnostic alternative. Organic fluids can include pleural fluid, ascetic fluid, cerebrospinal fluid, and urine. As body fluid analysis might show atypical features, negative results cannot rule out a tuberculosis infection. For example, polymorph nuclear cells may predominate at first, when symptoms have only been present for less than two weeks but may later shift towards a clear lymphocytosis (Noussair et al., 2009).

In the pleural fluid, the pericardial fluid, and the cerebrospinal fluid an elevated number of cells can be recognized, with a total count of about 100 to 500 lymphatic cells in the cerebral spinal fluid and 1000 to 5000 cells in the pleural fluid. Cerebrospinal fluid shows moderately raised cells and elevated protein (0.5- 3.0 g/L), with low plasma glucose levels <50%. Leukocytosis can also be found with about 10- 1000x 10^3 cells with mostly lymphocytes. Cerebrospinal fluid is mostly clear but can be xanthochrome in the coexistence of spinal meningitis and spinal block (Lee, 2015). Protein is usually high over 2.5 g/dl in pleural, pericardial, and cerebral fluids and glucose can be less than serum concentration. Mycobacterial culture unfortunately often shows negative results, with positivity of pleural fluid in 12- 70%, positivity of pericardial fluid in 25- 60% and positivity of cerebrospinal fluid in 40- 80%.

Urine examination is mandatory in patients with genitourinary tuberculosis but yields low probability of receiving a correct result. Also, smears and cultural examination of cold abscess must be examined if possible.

8.4.6. Difficulties in Diagnosis of extra-pulmonary tuberculosis

Diagnosis of cervical lymphadenitis is difficult as symptoms are mostly uncharacteristic and there is a lack of specific clinical and radiological features. Clinical representation is predominantly non-specific especially among coinfection with human immunodeficiency virus (HIV). Another important aspect to mention concerning diagnosis difficulties in extra pulmonary tuberculosis is their paucibacillary nature, concluding in the lack of positive smear microscopy as well as an increase in the time required to obtain a positive cultural result in solid and/ or liquid medium. This time can be up to 60 days for cultural growth on solid medium and up to 45 days in broth culture. In addition, obtaining material for histopathological and cultural assessment nearly always requires an invasive procedure, which makes it important to get useable material with a limited number of procedures, predominantly through only one procedure if possible. Therefore, in some cases the excision biopsy of an entire lymph node to sample a larger amount of tissue, would improve the diagnosis of the tuberculosis infection. In some cases, representative tissue is not accessible.

The problem of attaining adequate amount or volume of samples, as well as the difficulty of equal apportioning of the samples, for example to conceive histologic, cytological, and microbiological tests as well as PCR examination and biochemical analysis, results in another limitation of quick diagnosis of extra-pulmonary tuberculosis. Also, in extra pulmonary an irregular distribution of specimen can be found, with a high tendency of bacilli clumping together.

Cultural results of extra-pulmonary tuberculosis more often show negative results, when compared to cultural results in pulmonary tuberculosis. This is since extra-pulmonary tuberculosis includes only a small number of bacilli, subsequently leading to a low sensitivity of acid-fast bacillus smear and culture. In addition, cellular background makes it difficult to detect bacilli. This effect is further enhanced when assessing formalin-fixed paraffin-embedded tissue for cultural testing. Also, the cultural assessment only comprises a small fraction of the biopsy. According to Chakravorty et al. acid-fast bacillus staining was only positive in about 10% of patients, when using standardized methods. Furthermore, histopathological results are not always determinative (Noussair et al., 2009). This effect might be due to the loss of immune function, which can result in the demonstration of higher purulent responses and therefore less well-formed granulomas (Lee, 2015). Also, nucleic acid amplification

results are often negative due to the presence of different PCR inhibitors (Noussair et al., 2009), (Chakravorty et al., 2005).

These aspects lead to rapid diagnosis of extra-pulmonary tuberculosis being extremely challenging and result in the lack of an efficient universal sample processing method for different types of extra-pulmonary tuberculosis. These limitations of conventional techniques often result in the delay of therapy in patients with extra pulmonary tuberculosis, as well as the unnecessary continuation of presumptive treatment according to clinical presentation in patients, who consequently show no tuberculosis infection in later results (Noussair et al., 2009). Clarifying the exact diagnosis is important to exclude alternative diagnoses such as lymphoma, non-tuberculous mycobacterial disease, fungal infections, brucellosis, syphilis, carcinoma, and sarcoidosis, also causing lymphadenitis with partly granulomatous infection. Therefore, cautious investigation of the granulomatous reactions must be ensured in order to obtain the correct diagnosis (Lee, 2015), (Daley et al., 2007).

An aspect that further complicates the early diagnosis of tuberculous lymphadenitis is the behavior lymph nodes show during treatment. According to Daley et al. 21% of patients showed signs of residual adenopathy after treatment was complete, while 7% showed signs of persisting enlarged lymph nodes, 7% had transient enlargement of the lymph nodes and 14% developed new lymph nodes during therapy. Another 25% of the patients showed signs of paradoxical deterioration during treatment (Daley et al., 2007).

9. EBUS-TBNA

Endobronchial ultrasound-guided transbronchial needle aspiration is a minimally invasive technique for diagnosis of intrathoracic tuberculous lymphadenopathy by mediastinal lymph node sampling. EBUS TBNA has been an important technique in the diagnosis of sarcoidosis and malignancy for years as for example, in lung cancer staging, but can now also be efficiently used in the diagnosis of tuberculosis. It can be utilized for real time sampling of mediastinal, paratracheal and peri bronchial lesions and does not only provide information about the bronchial tissue, but also the tissue surrounding the bronchia. The aim of this study was to describe the importance of EBUS TBNA as a diagnostic utility for intrathoracic lymph node tuberculosis. Another

form of EBUS TBNA is radial EBUS, which is used to detect peripheral lesions not accessible for classic endoscopy. To undertake this examination a high-frequency ultra-sound probe (20 MHz) is inserted in the flexible bronchoscope which provides a view of the peri bronchial structures. This results in the possibility of differentiation between benign, malign, and inflammatory changes. The diagnostic reliability of radial EBUS is clearly higher than of bronchoscopy. The detection success rates increase in case of malignancy of the lesion, a lesion size greater than 2 cm diameter, a positive bronchus sign seen in CT, a location of the mass near the hilum as well as a central positioning of the mini-probe in the lesion (Kaid et al., 2018).

9.1. Importance of EBUS TBNA

EBUS TBNA is safe and well tolerated for elderly patients. It generally provides access to all lymph nodes commonly affected by intrathoracic tuberculosis and lymphadenitis, most commonly the subcarinal lymph node (station 7), followed by the paratracheal lymph node station (4R) as well as hilar lymph nodes 10 and 11. EBUS TBNA generally yield a higher number of nodal stations than traditional bronchoscopy (Navani et al., 2011). The sensitivity of EBUS-TBNA for diagnosis of intrathoracic tuberculosis ranged from 0.5 to 0.95 and the specificity ranged from 0.917 to 1.0 (Wu et al., 2015). EBUS TBNA can therefore be an accurate alternative to mediastinoscopy, which has until now been considered the gold standard for obtaining tissue samples from mediastinal lymph nodes.

Although mediastinoscopy can establish accurate diagnosis, it includes limitations such as not being able to access all mediastinal stations as well as an associated morbidity of 1-2% with the procedure. The posterior subcarinal and hilar nodes are not accessible with traditional techniques of bronchoscopy (Navani et al., 2011). Moreover, to pursue a mediastinoscopy general anesthesia must be executed, whereas EBUS TBNA can sometimes be performed solely under local anesthesia. Likewise, EBUS TBNA has also emerged as an important tool for aspiration of hilar nodes and nodes with a diameter <10 mm (Detterbeck et al., 2007), (Navani et al., 2011).

Today EBUS TBNA is the primary method for inspecting mediastinal and hilar lymph nodes and is available in all large endoscopy centers, as it can offer direct visualization of the target lymph nodes. The EBUS puncture bronchoscope also enables an

ultrasound guided fine needle aspiration of extra bronchial structures, such as lymph nodes and allows bronchial washing to be conducted during the same procedure. In many cases two or more samples from different nodal stations must be obtained (Kaid et al., 2018). Therefore, EBUS TBNA can be seen as an effective and safe first-line diagnosis method for differentiating intrathoracic lymphatic lesions, which still present a great challenge due to the lack of specific symptoms and radiologic characteristics (Navani et al., 2011).

The vast majority of cases with tuberculous lymphadenopathy have negative sputum smear and cultures. Therefore, histopathological, and cultural evidence are crucial for clinical diagnosis. It mainly manifests as the painless swelling of lymph nodes. Currently it is mostly diagnosed by lymph node biopsies that could be replaced by needle aspiration through EBUS-TBNA, which concludes in histopathological analysis as well as corresponding proof of bacteria culture (Kiral et al., 2015).

9.2. Differential diagnosis

9.2.1. Tumor diagnosis

The values of EBUS TBNA in lung cancer diagnosis are indisputable, as patients with the suspicion of central lung tumor as well as hilar or mediastinal lymph node metastases can be diagnosed with high reliability. The reliability is significantly higher than in conventional bronchoscopy. The diagnostic rate for lung cancer with endobronchial ultrasound guided transbronchial aspiration is approximately 95%. Diagnostic mediastinoscopy is only used in the case of negative histopathological EBUS TBNA results with remaining suspicion of lymph node metastases. In addition, lung cancer of the central respiratory tract can also be detected. Also, hilar, and mediastinal lymph node metastases can be more accurately diagnosed by EBUS TBNA, as imaging techniques, ones like PET CT are not accurate enough. EBUS TBNA shows a higher accuracy, nevertheless it must be considered that paraaortic lymph node stations 5 and 6 are currently not accessible through EBUS TBNA. EBUS TBNA is used to define the N stage in the TMN stage. The most common site of metastases for lung cancer is the left adrenal gland. Therefore, EBUS TBNA via the esophagus can be used as a primary method for diagnosis confirmation, as well as metastasis staging in lung cancer. Surgical clarification must be considered if negative

EBUS TBNA results are combined with continuous suspicion of lymph node involvement. Tumor exploration must be considered, whenever suspicious clinical, laboratory or diagnostic criteria occur. Characteristic malignant sonographic parameters are a round shape, heterogenous texture, and absence of a central hilum as well as necrosis of the lung parenchyma (Kaid et al., 2018).

9.2.2. Benign granulomatosis lung diseases

The two most common benign diseases that include mediastinal and hilar lymph node enlargement are sarcoidosis and tuberculosis. Sarcoidosis is the most common benign cause of an enlargement of the mediastinal and hilar lymph nodes in the Western world. Diagnosis always takes into account specific symptoms, radiological findings and histopathological verification. Bronchoscopic diagnosis includes BAL; endobronchial biopsy of the mucosa and the transbronchial biopsy. The sonomorphological presentation of sarcoidosis typically shows multiple slightly enlarged lymph nodes pressed against another, with preserved lymph node hila. Another differentiation criterion for lung cancer includes the fact that less material can be obtained through sampling and therefore more samples are required to obtain sufficient biopsy material for histopathological analysis.

Globally seen tuberculosis is the most common cause of thoracic lymphadenopathy. Tuberculosis can occur as a primary complex or a part of a reactivation. EBUS TBNA is a sensitive and safe method for diagnosis confirmation of tuberculosis, by looking at biopsy specimens for PCR, culture, and molecular diagnostics. To improve diagnostic accuracy immune-histochemical staining can be used (Kaid et al., 2018).

9.3. Different lymph node stations

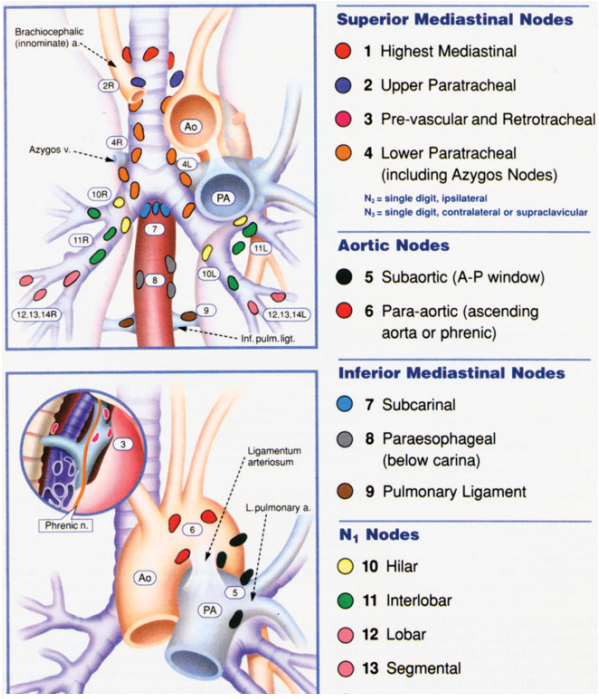


Figure 1 Journal of Thoracic Oncology 2009 4, 568-577DOI: (10.1097/JTO.0b013e3181a0d82e) Copyright © 2009 International Association for the Study of Lung Cancer Terms and Conditions

The different accessible lymph node stations include 2R, 2L, 4R, 4L, 7, 10R, 10L, 11R and 11L. To identify the correct nodal stations precise knowledge of the lymphatic stations is required. The exact lymph node positions are described by the IASLC, the International Association for the Study of Lung Cancer. 2R and 2L describe the location of the paratracheal lymph nodes. Both lymph nodes have the apex of the lung as an upper border with a lower border of the intersection of the caudal margin of the innominate vein with the trachea on the right side and respectively the superior border of the aortic arch on the left side. 4R and 4L describe the tracheobronchial lymph nodes, located in the area superior to the carina. On the right side these lymph nodes are located medial to the azygos vein. On the left side, the lymph node stations are located in the area surrounded by the medial wall of the aortic arch. The subcarinal lymph node station 7 is located in the area below the carina, where the trachea bifurcates to the two main bronchi. The hilar lymph nodes 10 are located among the right and left main bronchi. The upper border is the azygos vein on the right side and the pulmonary artery on the left side. The lower border can bilaterally be seen as the interlobar region. The last lymph nodes that are often assessed are the interlobar nodes 11, located between the lobar bronchi (Rusch et al., 2009). In HIV positive patients the most common lymph node stations affected are the stations 7, 4R and 11L (Prasag et al., 2018).

In EBUS TBNA it is important to switch to a sagittal or coronal visualization. Lymph nodes at each station are assessed according to different categories including the short-axis diameter (in mm), the shape (round or oval), the margin (distinct or indistinct) and the echotexture (homogenous or heterogenous). Most lymph nodes with a tuberculosis infection, show an oval shape, distinct margin and heterogenous

appearance on endosonographic examination. Furthermore, the presence of central hilar structures, coagulation necrosis sign and central intranodal vessels can account for signs of a tuberculosis infection.

On the left side the tracheal guide structures on the ultrasound image are the aortic arch with its three arterial branches (the common arterial trunk, the subclavian artery, and the brachiocephalic trunk). The brachiocephalic trunk crosses in front of the trachea and the brachiocephalic vein can account for the paratracheal border between lymph node stations 4R and 2R. The azygos veins mark the border between the stations 4R and 10R and can be seen in the right low paratracheal positions. On the right side the lymph nodes of the 4R station are in a more ventral position, mostly directly at the junction of the azygos vein and the superior Vena Cava. In addition, the esophagus has to be delimited from the subcarinal lymph nodes. The hilar lymph nodes are located below the upper lobe carina. On the right side it is important to differentiate between the superior location and the inferior location located below the carina of the middle lobe. Moreover, lymph node station 8 and more caudal station 9 can be accessed via a paraoesophageal approach. The paraaortic lymph node stations 5 and 6 can neither be reached by a conventional bronchoscope nor by EBUS- TBNA (Kaid et al., 2018). Neighboring vascular and intranodal vessels are avoided to avoid harming the surroundings, using color Doppler images (Madan et al., 2014). If this is not possible new reports suggest that EBUS-TBNA can be safely performed through the vessel (Kaid et al., 2018).

9.4. Complications

The complication rate of EBUS TBNA is very low, with about 0.05% of all examinations as reported by Kaid et al. Problems include bleeding as the most common difficulty, as lymph nodes are highly perfused. According to studies bleeding mostly occurs in the sub carinal lymph nodes, as these are the most perfused. Anticoagulation therapy with Marcoumar must be paused prior to taking biopsy of the lymph nodes. Likewise, dual antiplatelet therapy should be avoided if possible. In very rare cases (0.03%. 0.52%) a pneumothorax induced by EBUS TBNA has been reported. Therefore, a chest X-ray or a thoracic ultrasound must be taken after every EBUS TBNA procedure. Additionally, special attention has to be paid to mediastinitis, mediastinal-esophageal

fistula, pneumonia, bacteremia, sepsis and respiratory failure, which are very rare complications, but have to be taken into account when performing EBUS TBNA (Kaid et al., 2018). The risk of infection seems to be higher in larger necrotic lymph nodes (Navani et al., 2011).

9.5. Elastography

Elastography techniques are a diagnostic tool for tissue sampling used for the characterization of mediastinal and hilar nodal stations to evaluate lung cancer and other thoracic malignancies. It is a real-time technique describing elasticity of intrathoracic lesions that gained importance for the discrimination of benign and malign lymph nodes. Stain elastography can equally be used for diagnosis of mediastinal granulomatous disease and malignant lymphoma (Dietrich et al., 2015).

Endobronchial ultrasound guided trans-needle aspiration can be used to select lymph nodes. To evaluate the elastic characteristics of a certain tissue high frequency endoscopic ultrasound is being used to grade tissue deformation under linear compression. Elasticity describes the ratio between formability and pressure. Elastography can be described as electronic palpation. Therefore, elastography measurement takes the compression of the probe, as well as the compression of the surrounding structures, for example blood vessels, into account (Dietrich et al., 2015).

Biopsies taken for elastography assessment must be prepared directly after specimen collection in order to allow extensive molecular and histopathological diagnosis. The biopsy block must be immediately put in formalin or methanol-water solution, which causes lyses of the erythrocytes and therefore facilitates evaluation of histopathological evaluation (Kaid et al., 2018).

Stain elastography is a non-invasive technique which describes the relative stiffness of tissues through imaging, as it is displayed as a color map. We differentiate between four different colors: red, yellow, green, and blue. In general, differentiation of benign or malign lymph nodes depends on size measurements and topographic distribution. These methods can be complemented by elastography findings (Cui et al., 2013). When using elastography diagnosis it is important to say that this method can only be used as a supplement to prior examination, as there is a high chance of false-positivity,

for instance in fibrotic lymph nodes, as well as false- negativity possible in soft tissue necrosis. Elastography cannot replace biopsy but can give additional information when suspecting malignancy or inflammation (Kaid et al., 2018).

Limitations of elastography assessment are the lack of exact replicability, as the exact evaluation of the amount of color in each field is highly subjective. Therefore, sole reliance on visual judgement to assess elastography patterns and the resulting lack of objective evaluation, results in significant discrepancies between individuals concerning visually based judgements. Another bias could be the preformed expectations of the viewer as to whether a tissue is benign or malign. In addition, elastography can show false-positive results due to scarring and calcification of the lymph node (Mavi, 2017).

10. Methods

10.1. Study design

Diagnosis of extra pulmonary tuberculosis remains a worldwide problem, due to the lack of reliable data among other things. Therefore, we tried to evaluate the significance of EBUS-TBNA in the diagnosis of intrathoracic tuberculosis. We conducted a retrospective study with a real live cohort of 116 patients. Our study did not include a control group.

Only patients with a proven tuberculosis diagnosis were included in the study. Inclusion criteria consisted of either bacteriological proof of tuberculosis from the airways (as from the sputum or the bronchial lavage) or the lymph nodes and/ or cytological proof of typical tuberculosis radiological patterns. All patients with suspected tuberculosis infections that could not be bacteriological or cytological proven were excluded from our study. In addition, all patients with EBUS TBNA results showing any other diagnosis apart from tuberculosis (for example: sarcoidosis) were also excluded from the study. Diagnosis was established based on diagnostic algorithms and clinical representation provided by the Asklepios hospital.

The primary endpoint of our study was to ascertain whether a tuberculosis infection could be detected by using EBUS TBNA. In addition, elastography patterns of tuberculosis infected patients were analyzed to identify tuberculosis typical patterns.

Comparisons were made between patients with a positive or negative sputum and bronchial lavage result. In detail, we compared the amount of positive lymphatic tuberculosis results obtained from EBUS TBNA in patients with bacteriologically proven pulmonary tuberculosis to patients with no pulmonary involvement. Besides, we evaluated the quantity of matching histopathological results for cultural positive and negative EBUS TBNA results. Furthermore, patients with positive or negative cultural and PCR results from EBUS TBNA results were evaluated based on the corresponding Interferon- γ test results.

We conducted a case study among 116 patients. All patients were diagnosed with tuberculosis and tuberculous lymphadenopathy between January 2015 and March 2020 in the Asklepios hospital in Gauting, Munich. Demographic data, pathological findings and microbiological results were recorded. Ethical approval was given by the Ludwig- Maximilian-University of Munich, Germany (after § 15 of the code of medical ethics, no ethical guidance was needed in this study).

10.2. Immunological assessment

Immunological assessment of the samples at the Synlab in Gauting was performed using the QuantiFERON- TB® Gold IT Interferon- gamma release assay, as well as the QuantiFERON- TB® Gold *plus*. The QuantiFERON- TB® Gold IT measures the concentration of Interferon-gamma released by CD4+ T-cells after stimulation with tuberculosis antigens. For stimulation the mixture of synthetic peptides including ESAT-6, CFP-10, and TB7.7 (Rv2654c) to stimulate T-cells was used. The ESAT-6 and CFP-10 antigens contained in QFT-GIT are epitopes for both CD4+ and CD8+ T-cells, but mainly stimulate CD4+ T-cells to release IFN- γ. The QuantiFERON- TB® Gold *plus* assay furthermore analyzes the amount of Interferon-gamma released by CD8+ T-cells throughout the test, by additionally evaluating their response to ESAT6/CFP10 peptides. These peptides were contained in two tubes, tube one mostly containing long peptides to stimulate CD4+ T-cells, while tube 2 mostly represented short peptides stimulating both CD4+ as well as CD8+ T-cells.

To perform the Interferon- γ release assays a whole blood sample was needed. Therefore, a 10-mL whole blood sample was drawn in two blood-collecting tubes containing lithium-heparin, to prevent blood coagulation. The blood samples were

transported to the Synlab laboratory at controlled temperature (22 ± 5 °C). As a next step a 1-mL sample was poured into each assay tube and incubated at 37 ± 1 °C for 16–24 h within 16 h after collection. Testing procedures for QFT-Plus and the conventional QFT-GIT require 5 tubes in all. The first two tubes TB1 and TB2 are needed for the QuantiFERON- TB® Gold *plus* assay. An additional tube was used for the conventional antigen tube of the assessment by QuantFERON- TB® Gold IT. In addition, two control tubes one positive (mitogen) and one negative (no antigen, nil) are needed.

After incubation, the tubes were centrifuged at 2.330 × g for 15 min. A total of 300 µL of plasma was derived after centrifugation and stored at −80 °C until the enzyme-linked immunosorbent assay (ELISA) was performed. The standard QuantiFERON-TB® ELISA kit was used for ELISA. The frozen plasma sample was defrosted at room temperature and re-centrifuged at 1.750 × g for 15 min. The optical density of each well was measured on a plate reader. The concentration of released IFN- γ in each tube was calculated by subtracting the value of the nil negative control tube. If the coefficient of variation for the result was less than 15% and the correlation coefficient for the standard curve was greater than 0.98, the assay was technically valid. All the results were interpreted by referring to a 4-point standard curve. Conventional cut-off was at 0.35 IU/mL or >25% of the nil for QFT-GIT. Any result greater than this can be considered as a positive test result. This can be derived by subtracting the nil from the antigen.

10.3. Bacteriological assessment

10.3.1. Culture

Tuberculosis diagnostics from clinical specimens (sputum, bronchial aspirates, biopsies etc.) was performed at the mycobacteriology laboratory SYNLAB Gauting, which is a WHO Supranational Reference Laboratory of tuberculosis. Diagnostics included smear microscopy, PCR for *Mycobacterium tuberculosis (M.tb)* DNA as well as cultural examination.

10.3.1.1. Pre-treatment of clinical specimens

All specimens were pre-treated using the N-acetyl-L-cysteine (NALC)-NaOH method according to German national guidelines (DIN 58943-3). This pre-treatment serves for liquefaction of the specimens and for elimination of non-mycobacterial microorganisms such as bacteria and fungi (decontamination). In case of tissues or biopsies, the specimens were additionally homogenized by grinding in a sterile mortar. After decontamination, samples were centrifuged at 3.000 g to concentrate the mycobacteria. The sediment was then re-suspended in 1.5 ml of 0.5 M phosphate buffer (pH 6.8).

10.3.1.2. Smear microscopy of clinical specimens

Smears were prepared from decontaminated samples, heat-inactivated and stained with Auramin O for detection of acid-fast bacilli (AFB) using the Fluo RAL kit (RAL diagnostics, BioRepair, Sinsheim, Germany). AFB were visualized with a fluorescence microscope (magnification ×400). To detect even small numbers of acid-fast rods, at least 300 fields of vision were screened. With samples containing more than 104 AFB per ml, the bacilli can be expected to be detectable by direct microscopy. Microscopic results were evaluated qualitatively (negative/positive) and semi-quantitatively (scanty, 1-12 AFB in the smear; 1+, 4-50 AFB per 100 fields; 2+, 5-50 AFB per 10 fields; 3+, 5-50 AFB per field; 4+, >50 AFB per field) (DIN 58943-32).

10.3.1.3. Inoculation and reading of mycobacterial cultures

For cultural examination, one liquid and two solid culture media were inoculated according to German national guidelines (DIN 58943-3): (1) 0.5 ml of the pre-treated specimen was inoculated into MGIT tubes (MGITTM, Becton-Dickinson, Heidelberg, Germany) containing liquid 7H9 according to Middlebrook and (2) approximately 100 µl each were inoculated onto Loewenstein-Jensen and Stonebrink solid culture media. The inoculated culture media were incubated in incubators at 36°C +/- 1°C. MGIT tubes were analyzed using the semi-automated growth indicator system MGIT 960 (Becton-Dickinson). The examination of lymph nodes and other tissue specimens from the body periphery included the incubation of an additional MGIT culture at 30°C +/- 1°C. Cultures were incubated for up to 8 weeks (30°C cultures for up to 12 weeks). When a

culture turned positive (as indicated by the MGIT 960 automat or by visual detection of bacterial colonies on solid media), staining for presence of AFB was performed by modified Kinyoun staining using the Quick-TB kit (RAL diagnostics, Bio-Repair, Sinsheim, Germany).

10.3.1.4. DNA line-probe assays for differentiation of mycobacteria and detection of genetic resistance markers

When a primary culture turned positive with AFB, the isolate was differentiated by DNA line probe assays (Genotype CM, Genotype MTBC, Hain Lifesciences) which are based on the DNA-strip technology (multiplex PCR followed by reverse hybridization of amplicons to DNA samples immobilized on filter strips). In brief, 0.5 ml of bacterial suspension was heat-inactivated, and DNA was extracted using GenoLyse (HAIN Lifesciences). Genotype PCR mixes were prepared with 5 µl of the DNA lysate and subjected to PCR cycling. Biotin-labeled amplicons were then hybridized to respective Genotype filter strips and visualized by a streptavidin-alkaline phosphatase detection system. Interpretation of hybridization patterns followed instructions by the manufacturer. Genotype CM allowed differentiation of the most frequent mycobacterial species such as *M. intracellulare* group, *M. avium, M. kansasii* or *M. abscessus* complex; Genotype MTBC differentiated the members of the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. africanum, M. bovis ssp. bovis, M. bovis ssp. caprae, M. bovis BCG* and *M. microti*).

10.3.1.5. Moleculargenetic detection of genetic resistance markers

If *Mycobacterium tuberculosis* complex was detected from culture, testing of resistance markers for RMP and INH was performed using the line probe assay GenoType MTBDRplus (if testing has not yet been performed on the direct material). GenoType MTBDRplus can identify RMP resistance markers in the mutational hot spot region of the rpoB gene (RRDR, RMP resistance determining region) and INH resistance markers in the katG gene and the inhA promotor. In case of detection of at least resistance to RMP, screening of resistance markers for second-line drug (fluorchinolones, injectable drugs) as well as ethambutol (EMB) was additionally performed using the GenoType MTBDRsl (HAIN lifesciences). Targeted resistance

genes included gyrA, gyrB (fluorchinolones), rrs, eis (injectable drugs) and embB (EMB). If necessary, specific mutations underlying RMP or pyrazinamide (PZA) resistance were detected by Sanger sequencing. Primers used were rpoB-f / rpoB-r (5'-ggg agc gga tga cca ccc a-3' / 5'-gcg gta cgg cgt ttc gat gaa c-3') for rpoB sequencing (RMP) and pncA-f / pncA-r (5'- aag gcc gcg atg aca cct c-3' / 5'- gtg tcg tag aag cgg ccg at-3') for pncA sequencing (PZA).

10.3.1.6. Phenotypic drug susceptibility testing of *Mycobacterium tuberculosis*

For each patient with culture confirmed tuberculosis, phenotypic drug susceptibility testing (DST) was performed at least once, usually from the first *Mycobacterium tuberculosis* isolate of a patient. Standard testing included the first-line drugs RMP, INH, EMB and PZA as well as the second-line drug Levofloxacin. In cases of resistance to at least one first-line drug, the spectrum was extended to Moxifloxacin, amikacin, Capreomycin, Kanamycin and Prothionamide. If indicated (pre-XDR-/XDR-TB), PAS, Linezolid, Clofazimine, Bedaquiline and Delamanid were additionally tested. As routine method, semi-automated DST was done in MGIT tubes using the BACTEC MGIT 960 SIRE/PZA system (Becton-Dickinson). In brief, bacterial suspensions with concentrations corresponding to McFarland standard 0.5 were prepared and inoculated into drug-containing MGIT tubes or diluted 1:100 (PZA: 1:10) and inoculated into drug-free MGIT tubes to serve as growth controls. Concentrations of drugs in the drug containing MGIT tubes each corresponded to the "critical concentration" according to recommendations of WHO (World Health Organization, 2018) The critical concentration is defined as the lowest concentration of a test substance in the culture medium at which the growth of *Mycobacterium tuberculosis* bacterial indicates resistance to the respective anti-tuberculous drug. DST in MGIT at the critical concentration of the drugs provided qualitative results (susceptible or resistant). If required, minimum inhibitory concentrations (MIC) were analyzed using different drug concentrations prepared in 1:2 dilution steps from an initial drug solution.

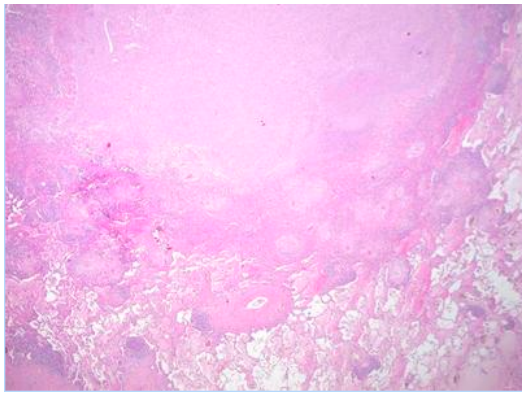
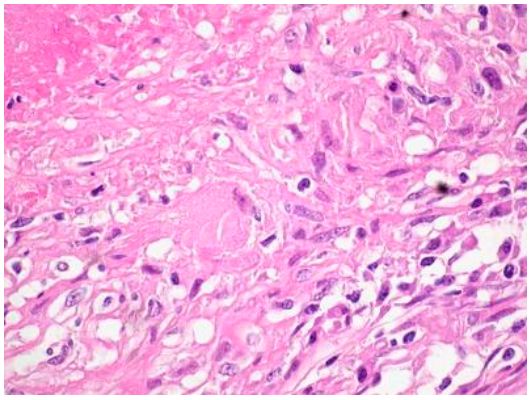
10.3.2. PCR

Mycobacterium tuberculosis complex PCR can be retrieved directly from clinical specimens. Direct detection of DNA of *Mycobacterium tuberculosis* complex was routinely performed using the semi-automated FluoroType MTB assay (Hain Lifesciences, Nehren, Germany) (Hofmann-Thiel & Hofmann, 2014). In brief, 0.5 ml of decontaminated clinical specimen was heat-inactivated, and DNA was extracted by lysis using FluoroLyse (Hain Lifesciences). PCR mixes were freshly prepared from FluoroType AM-A and AM-B reagents and 6 µl DNA each (patient samples, positive or negative control) was added. Mixes were loaded into the FluoroCycler instrument for PCR and automated analysis of melting curves. Results from valid runs were reported as “MTB complex DNA detected” or “No MTB complex DNA detected”. In case of newly diagnosed tuberculosis, screening for genetic resistance markers for rifampicin (RMP) and isoniazid (INH) was performed using the line-probe assay MTBDRplus (HAIN Lifesciences).

Alternatively, PCR detection of *Mycobacterium tuberculosis* complex DNA as well as RMP resistance markers was performed using the fully integrated cartridge based Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) system. Therefore, 0.5 ml of decontaminated sample was mixed with 1.5 ml sample reagent (Cepheid), transferred to test cartridges, and loaded into the GeneXpert instrument for fully automated real-time PCR. Results were given as “MTB detected” (high, medium, low, or very low) or “MTB not detected”. With positive cases, RIF resistance status was given as “RIF resistance detected” or “No RIF resistance detected”.

10.4. Histopathology

For histopathologic assessment the probe was preserved in formalin or Cytolyt, which is a methanol-water solution. It was later colored in HE (Hämatoxylin-Eosin). We especially tried to find signs of granulomatous inflammation. Another sign of a tuberculosis infection is a necrosis, classically a necrosis with central caseation and/ or calcification. Surrounding the center of caseation, we can find a wall of epithelioid cells as well as Langhans giant cells, with horseshoe formed nuclei.

a.  b. 

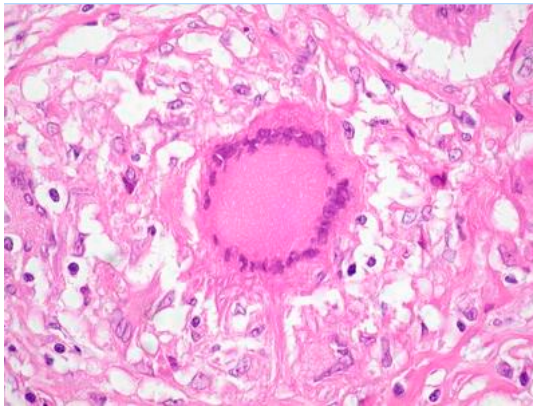
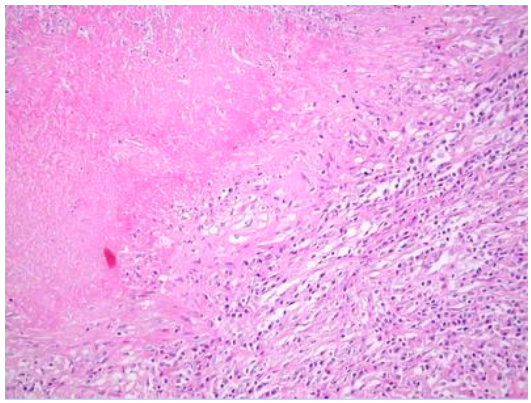
c.  d. 

Figure 2. ***a****. granulomatous inflammation, with several granulomas that form a bigger isolated circular focus, which is called a tuberculoma; the eosinophilic area in the middle of the granuloma shows necrosis;* ***b****. raised edge of the epithelioid granuloma with plasma cells, lymphocytes and epithelioid cells;* ***c****. Langhans giant cells, which show a formation of confluent macrophages;* ***d****. the raised edge of the granuloma shows the transition passage between the central area of caseous necrosis and a region of epithelioid cells, with chronic phlogistic infiltration.*

10.5. EBUS TBNA

At the clinic in Gauting we used a BF- UC 180F EBUS- bronchoscope from Olympus. EBUS-TBNA was performed according to the presence of the following signs: enlarged unilateral mediastinal/ hilar lymph nodes (>1 enlarged mediastinal or hilar lymphadenopathy with <1cm short-axis); tracheobronchial intrapulmonary parenchymal lesions based on computer tomography (CT) and clinical or radiological features. Moreover, elevated density values on native CT, increased FDG uptake in PET CT as well as pathological ultrasound findings are conditions which count as indications for EBUS TBNA. All lymph nodes with a diameter over

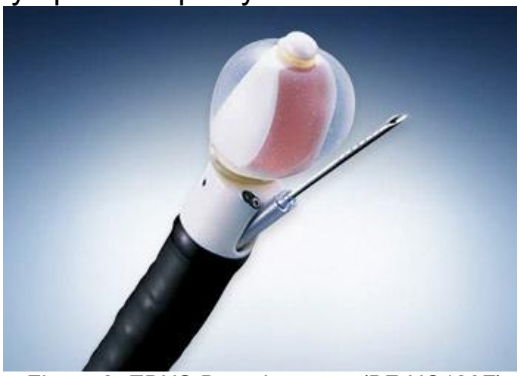


Figure 3: EBUS Bronchoscope (BF-UC180F)

one centimeter were punctuated. In further detail, conspicuous lymph nodes included exemplars with a distorted spherical shape, a heterogeneous echo-structure, a missing central hilum as well as parenchymal necrosis. In addition, it is important to only consider EBUS-TBNA, when there are no contraindications to the procedure (Detterbeck et al., 2007).

10.5.1. Procedure

Patients were prepared by fasting the day before the procedure. History of cardiac and respiratory diseases, diabetes mellitus, arterial hypertension, bleeding disorders, previous anesthetic conditions and medication history was recorded for every patient. Other relevant investigations were performed for the underlying clinical diagnosis of tuberculosis. During the procedure heart rate, blood pressure and pulse oximetric saturation were monitored. For examination it is important to look at the sagittal and coronal CT layers.

Endobronchial ultrasound guided transbronchial needle aspiration can be performed in a flexible or a rigid way. A combination of these techniques is also possible. At the Asklepios hospital in Gauting the most common procedure is a rigid bronchoscopy in combination with flexible EBUS TBNA. All EBUS-TBNA procedures were performed under general anesthesia. EBUS TBNA procedures under conscious sedation with local anesthesia like lidocaine can also be performed but are not state of the art in the Asklepios hospital in Gauting.

Before EBUS-TBNA could be conducted a general bronchoscopy was performed to inspect airways. Following the standard bronchoscopy an EBUS TBNA was performed with a special bronchoscope including a linear ultrasound transducer for real-time ultrasound guidance. The location, the shape and the structure of the lesion were examined with ultrasound. A balloon filled with water was used to connect to the bronchial interface. Based on computer tomographic findings target lymph nodes are punctured with a 22-gauge medical steel needle with a syringe connected proximally. A dedicated bronchoscope with a convex shaped ultrasound transducer at its tip can be used to sample mediastinal lymph nodes under direct visualization, scanning at a frequency of 7.5 MHz. The needle is pushed out from the distal top of the bronchoscope and suction is applied to the needle. Then the needle is moved to and

from the lymph node to aspire tissue. In addition, location, number, and size of the lymph nodes must be recorded and systematic assessment of mediastinal and hilar nodes is conducted.

Each lymph node was punctuated at least three times to obtain enough biopsy cylinders for the performance of molecular and cultural testing. Fan-shaped biopsies are taken. The three punctuates include one biopsy taken without aspiration to obtain a specimen for histopathological examination. The specimen is put on a slate to create a smear and preserved with formalin. Cytological specimens were prepared as slides without alcohol fixation to be used for microscopy and staining of acid-fast bacilli. The serial sections were cut and placed on slides before being stained with hematoxylin and eosin (HE). In addition, cell blocks were placed in formalin and taken for centrifugation. Another biopsy was taken with aspiration. The aspirate is put into a suspension with a 0.9% NaCl solution to form a heterogeneous mixture. These two samples were immediately sent to the pathological institute. Histological as well as microbiological assessment was conducted. The third punctuate was also taken with aspiration in order to achieve a specimen for tuberculosis analysis. Another suspension is created to perform microbiological and histopathological assessment by coloring the solution with the Ziehl- Neelsen staining for bacteriological proof of tuberculosis bacteria. The specimen is placed in sterile saline and colored with auramine-rhodamine staining. Later, the presence and number of granulomas, the average number of epithelioid cells in granulomas, the presence of necrosis, stain for acid-fast bacilli (AFB) and the final impression (tuberculosis vs. sarcoidosis) is evaluated. In addition, a PCR was conducted.

Rapid on-site evaluation (ROSE) of the biopsies can ensure that the examiner acquires enough material for diagnosis but is not available at the Asklepios clinic. According to CHEST guidelines and recommendations in the case of absence of ROSE, a minimum of three separate punctuations of the sampling site are strongly recommended (Wahidi et al., 2016).

Diagnosis is easier for the lower lobe than the upper lung lobe due to reduced flexibility and bending of the EBUS device in the upper lobe. The lymph nodes of the lower mediastinum (8 and 9) are only accessible throughout transesophageal approach, while the lymph nodes of stations 2L, 4L, 7, 10L and 11L can be visualized and

punctuated via transbronchial approach. Furthermore, the right paratracheal stations 2R, 4R, 10R and 11R can equally only be approached by transbronchial ultrasound guided techniques. As the lymph node 5 is hard to reach due to its low location, EBUS TBNA can be performed and additionally bronchoscopy is used.

10.5.2. Elastography

Elastography measurements in Gauting hospital were conducted during the EBUS-TBNA process. Endosonographic sampling of all clinically relevant mediastinal nodal stations includes identifying different features such as hypoechoicity, roundness, diameter >10 mm and distinct margins. Throughout the high frequency endo-sonography the texture deformation from compression with the ultrasound head showed a varying amount of tissue stiffness. Deformation images were generated by using a palpation transducer to repetitively apply minimal pressure to the tissues. Subsequently, tissue displacement was tracked between different frames of the site and the surrounding tissue, which was later used to calculate an axial gradient of the displacement. When regarding tissue deformation according to stiffness, tissue which was stiffer experienced less deformation under stress compared to softer tissue. Elasticity measurements were expressed in kPA.

We evaluated color differences between different elastography patterns. The elastography patterns can be overlaid by a B-mode scan to divide the results into four groups. Type 1 represents mostly green, red, and yellow areas (>80% green/ red/ yellow). Type two represents by majority green, red and yellow patterns (by definition 50%- 80% green/ red/ yellow). Type three is represented by majority by a blue pattern, which indicates a score of 50%- 80% of blue pattern. Type four indicates nearly only blue patterns, over 80% blue. Red and yellow tissue indicates mostly benign tissue with a high elasticity. Benign “normal” lymph nodes contain a hard cortex and a softer hilum and medulla. Blue and green predominantly indicate fibrosis or malignancy, as the tissue shows more stiffness. In the case of complete lack of green and blue coloring, a benign cause can be assumed, while a high amount of green and blue coloring indicates a high chance of malignancy or inflammatory disease. Whereas, blue indicates malignancy, green can mostly be seen in inflammatory diseases, for example sarcoidosis (Dietrich et al., 2015).

The elastography patterns were compared with the final pathological results from EBUS-TBNA. Generally, we must differentiate between a qualitative and a quantitative analysis. Quantitative analyses compare the elastography analyses of the site to the surroundings of the tissue, creating a strain ratio.

Score	Definition	Clinical definition
1	>80% green/ red/ yellow	"mainly green/ red/ yellow"
2	80-50% green/ red/ yellow	"by majority green/ red/ yellow"
3	50-80% blue	"by majority blue"
4	>80% blue	"nearly only blue"

Figure 4: Elastography patterns described by He et al (Chin Med J 2015;128:2720-5)

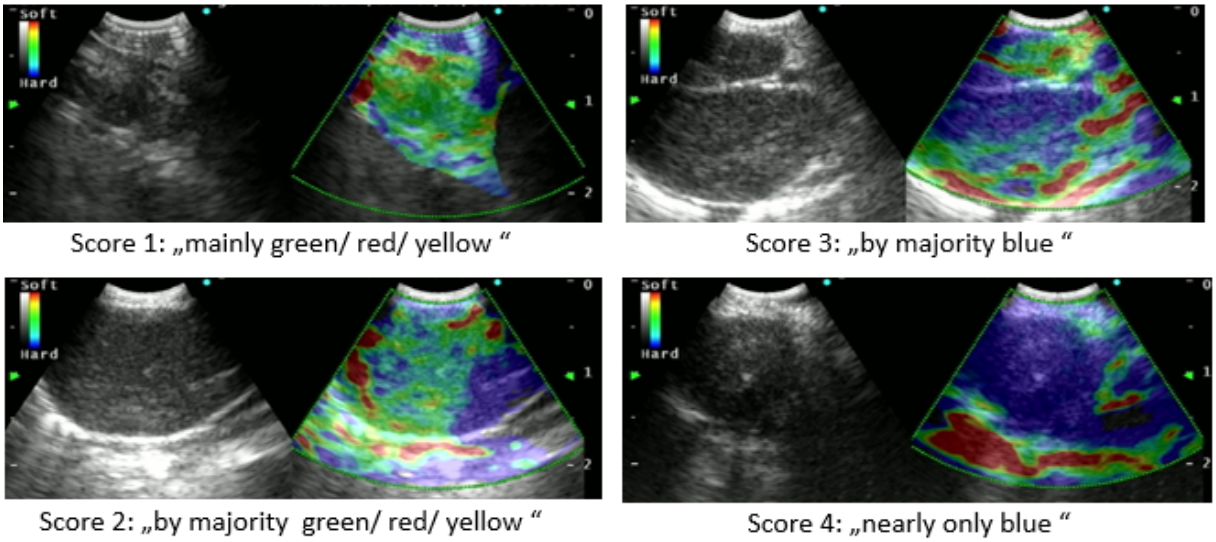


Figure 5: Elastography findings (Asklepios Klinik Gauting)

11. Results

11.1. Social demographic findings

Out of 127 recorded cases, 93 were males and 34 were females. Age ranged between 15 and 95 years, with a mean of 32, 43 years.

Demographic data including country of origin and time spent in Germany were recorded. Countries of birth were categorized according to the UN M49 classification scheme. Most of the patients were refugees from Somalia (33 people). Furthermore, a great deal of patients originated in Afghanistan (11), Eritrea (16) and Germany (8), followed by Pakistan (7), India (7) and Nigeria (4). Additionally, we recorded cases

from Eastern European countries such as Bosnia and Herzegovina (1), Bulgaria (1), Romania (4), Serbia (3), Iraq (2), Bangladesh (1) and Turkey (4) as well as from the Russian Federation (2), along with patients from Vietnam (3). Furthermore, patients originated from other African countries like Senegal (3), Guinea (1), Ethiopia (2), Congo (1), Cameron (1), Syria (3), Sierra Leone (2), Mali (1) and Central Africa (1). Apart from Germany, cases from other European countries, like Poland (1), Greece (1) and France (1) were recorded. Alongside these countries cases from Venezuela (1) and Thailand (1) were recorded.

Considering mean age, a remarkable difference between Germany and African or Eastern European countries can be seen. While in Germany the mean age of patients was around 66. 0 years; the mean age in all the other countries mentioned in the paper was around 31. 5 years. Considering that most of our patients originated in Somalia, Afghanistan, Eritrea, and Pakistan as well as Germany, we calculated the mean age for the countries mentioned above. Patients from Somalia and Eritrea showed the youngest mean age of around 20- 21 years. (Somalia= 20. 9 years; Eritrea= 20. 6 years). Patients from Pakistan and Afghanistan were generally slightly older, with a mean age of 27. 1 years for Pakistani and an average age of 32. 0 years for Afghans.

Concerning the time spent in Germany the average was around 7. 9 years. It is important to distinguish between patients originating from Germany or any other country, as patients originating from Germany spent most of their life in Germany. Nevertheless, in view of the origin countries and the augmented migration movement in Germany in recent years, the majority of patients had only been in Germany for a few weeks. For example, patients who originated in Somalia generally spent 7. 8 months in Germany, while cases reported with Eritrean origins spent an average of 6. 0 months in Germany at the time being inpatients at the Asklepios hospital in Gauting. In contrast, most of the patients originating from Germany had spent most of their life in Germany, which means that the time spent in Germany could be considered many years. Germans spent an average of 66. 0 years in Germany at the time of record. In comparison, regarding all other patients apart from Germans, the average time spent in Germany was 4.0 years.

11.2. Microbiological results

11.2.1. Overall microbiological assessment

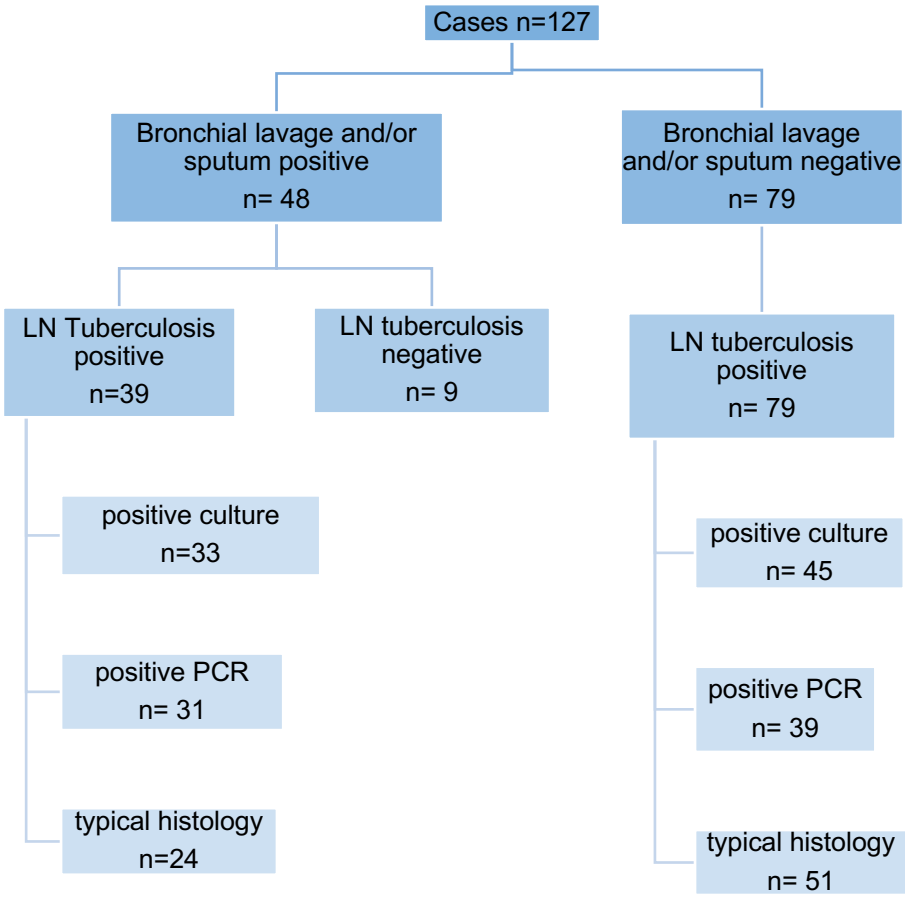


Figure 6: Microbiological results comparing patients with bacteriologically confirmed pulmonary tuberculosis to patients with no pulmonary evidence

We included 127 patients with confirmed TB who had an EBUS procedure. Of these, 48 also had a positive finding in the respiratory material, the others were only obtained from the lymph nodes. It is important to look at whether patients show signs of pulmonary involvement, as pulmonary tuberculosis is considered to be an open tuberculosis- meaning that patients can easily spread the disease through the air and infect others. Patients with an open lung tuberculosis have therefore to be isolated. Proven pulmonary involvement was classified as positive cultural results from the sputum and/ or the bronchoalveolar lavage (BAL).

48 out of 127 patients (**37.8%)** showed microbiological evidence of *Mycobacterium tuberculosis* in sputum and/or bronchoalveolar lavage. The remaining 79 patients (**62.2%**) were bacteriologically negative for tuberculosis at sputum and BAL. The 48 bacteriologically positive cases comprise of 20 cases with positive sputum samples

only, 16 cases with positive bronchoalveolar lavage positive only and 12 cases with positive sputum and bronchial lavage samples. This leads to the conclusion that only about one third of the patients showed cultural proof of a pulmonary tuberculosis- therefore considered an open tuberculosis.

In the group of patients with positive pulmonary tuberculosis results (48 patients), 39 patients (**81.3%**), showed signs of lymphatic tuberculosis, while 9 patients (18. +7%) of all pulmonary positive cases, did not show signs of lymph node involvement. Positive signs of lymphatic tuberculosis involvement included a positive PCR for tuberculosis bacteria and/ or a positive *Mycobacterium tuberculosis* cultural growth from lymph node material. Alongside these criteria a typical histopathological pattern obtained from the lymph node has also been defined as a positive sign of lymphatic tuberculosis.

Out of 48 patients who showed pulmonary signs, 31 obtained positive PCR outcomes, which accounts for about 64.5%. Additionally, in 33 cases a positive culture for tuberculosis bacteria could be attained from the EBUS aspirates, which is equivalent to a percentage of 68.7% of all patients showing signs of pulmonary tuberculosis infection. This leads to the conclusion, that about two thirds of the patients showed cultural proof from the lymph nodes, as well as the airways. Therefore, these patients can be considered to not only have a pulmonary tuberculosis, but also an extra-pulmonary tuberculosis infection. Concerning the histopathological findings 24 patients (50%) who showed tuberculosis infection of the lungs also showed typical histopathological findings in the punctuated lymph nodes, including only cases with bacteriologically confirmed tuberculosis infections.

These findings can be compared to patients who showed no signs of pulmonary infection, and who therefore suffered from isolated tuberculosis of the lymph node, compared to patients with documented evidence of tuberculosis bacteria in sputum or bronchial lavage, being adversely affected by an open tuberculosis infection. This group of patients included 79 cases (out of a total number of 127 patients) who showed lymph node infection, determined by proof of the prior mentioned criteria. It is important to mention that this group of patients only suffered from extra- pulmonary tuberculosis, therefore having a reduced risk of contagion through the airways.

A positive PCR for tuberculosis bacteria extracted from the lymph nodes was obtained from 39 patients, accounting for 49.4% of all patients with no signs of pulmonary

tuberculosis. In addition, 45 patients (57%) showed positive cultural findings. Alongside these findings, a typical histopathological pattern could be reported in 51 cases, leading to the conclusion that about 64.5% of the patients showed typical histopathological patterns in the punctuated lymph nodes.

Comparing the different sensitivities for PCR, culture, and histology it is important that not all patients received all three of those diagnostic measures. This leads to an overall sensitivity of **61.4%** (78 positive out of 127 patients) for cultural growth from material extracted from the lymph nodes by EBUS TBNA: Concerning PCR results, a sensitivity of **57%** (70 positive out of 123 patients) was measured. Also, we measured a sensitivity of **70%** (75 specific histologic patterns out of 107 patients) for histologic assessment. These results already show us that although all these patients were diagnosed with an extra- pulmonary tuberculosis, not all the diagnostic criteria were positive in every case. Overall histopathological assessment led to the highest sensitivity for detecting a tuberculosis infection in the lymph nodes by EBUS- TBNA, directly followed by cultural assessment. In Table 1, you can see a brief overview of our results, showing the amount of typical cultural, PCR and histological results from our study.

TB PROVEN FROM SPUTUM/ BRONCHIAL SPECIMEN N= 48	**LYMPH NODE TB DIAGNOSED N= 39**	**EBUS-TBNA WITH POSITIVE CULTURE N= 33**	**EBUS-TBNA WITH POSITIVE TB-PCR N= 31**	**EBUS-TBNA WITH TYPICAL HISTOLOGICAL FINDINGS N= 24**
BRONCHIAL SPECIMEN AND SPUTUM NEGATIVE N= 79	Lymph node TB diagnosed n= 79	EBUS-TBNA with positive culture n= 45	EBUS-TBNA with positive TB-PCR n= 39	EBUS-TBNA with typical histological findings n= 51
SUM N= 127	**n= 118**	**n= 78**	**n= 70 (out of 123)**	**n= 75**

Table 2: Brief overview of the results of this study: In 127 patients with proven TB an EBUS-TBNA was performed. Bronchial lavage and/ or sputum positive= positive cultural results from the airways (positive culture for Mycobacterium tuberculosis either from the sputum, the bronchial lavage or both; LN tuberculosis diagnosed= includes at least one of the following three factors: positive culture obtained by EBUS-TBNA punctuation, positive results of a TB- PCR of the mediastinal lymph nodes, typical histological findings including granulomatous reaction with/ or without caseating necrosis (Type 1 or 2 histologic results)

11.2.2. *Positive cultural results having been obtained by EBUS TBNA*

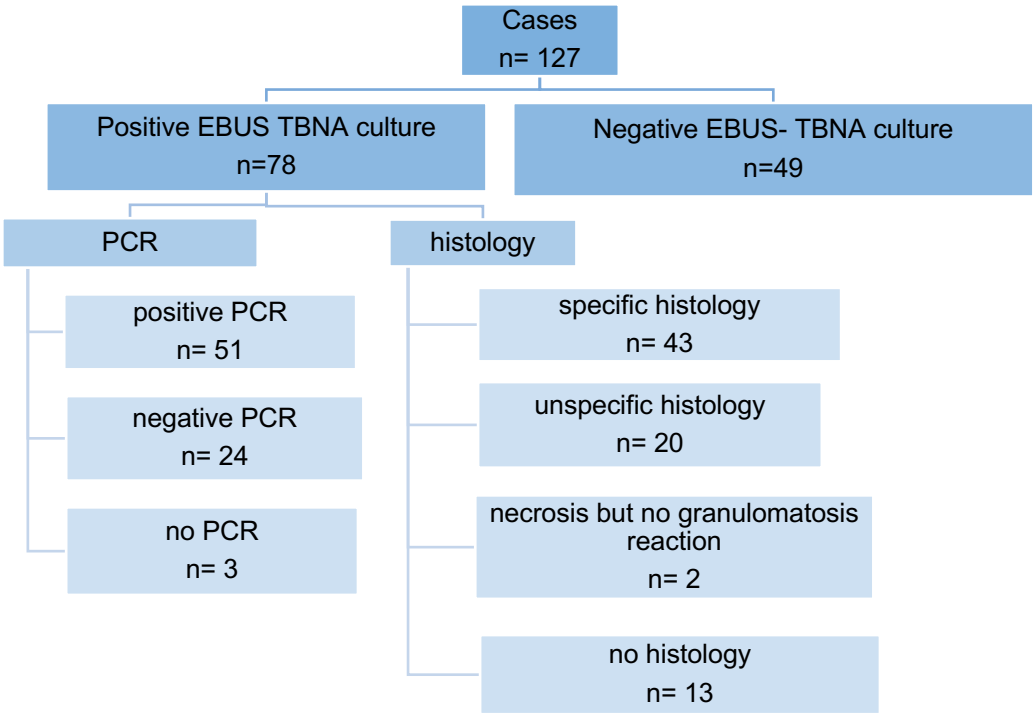


Figure 7: Comparing cultural results obtained by EBUS- TBNA

Furthermore, we explored the patient group including all patients with positive cultural results obtained by EBUS TBNA. The primary goal of the study was to find out as to whether EBUS- TBNA could be a diagnostic measurement that helps to obtain cultural and histopathological findings from the mediastinal lymph nodes to assess the presence of cervical lymphadenitis. Therefore, it is important to take a close look at the patients who received a positive culture from EBUS- TBNA. These patients showed clear signs of extra- pulmonary tuberculosis. Overall, out of 127 cases, 78 patients (**61.4%**) obtained positive cultural results, while 49 patients (**38.6%**) received negative cultural results. This shows that a positive culture for tuberculosis could be found in about two thirds of the patients with a proven tuberculosis, having undergone an EBUS- TBNA evaluation.

Out of the cultural positive patient collective, 51 patients showed positive PCR results, while 24 showed negative PCR results, therefore leading to **68%** of the patients yielding positive PCR results. In three cases no PCR had been conducted.

Histological assessment has been conducted in 65 out 78 patients with a positive cultural result for TB obtained by EBUS- TBNA from the lymph nodes. 13 patients did not undergo any histopathological analyses. Concerning histopathological findings, we

had to distinguish between specific and non-specific histopathological results. Specific tuberculosis histology could be proven in 43 cases, accounting for **66%** of the cultural positive EBUS TBNA cases, who underwent histopathological assessment. Furthermore, a total of 20 non-specific histopathological findings, therefore regarded as normal lymphatic tissue samples, were obtained, while 2 samples showed further unspecific results, with necrosis and lack of granulomatosis tissue. Therefore, overall results that showed no specific tuberculosis pattern accounted for **31%** of the patients with positive cultural results obtained by EBUS TBNA. In 3% of the cases the samples showed necrosis, but no signs of granulomatosis. Therefore, that a negative histopathological result can never out rule a tuberculosis infection.

11.2.3. Polymerase Chain reactions

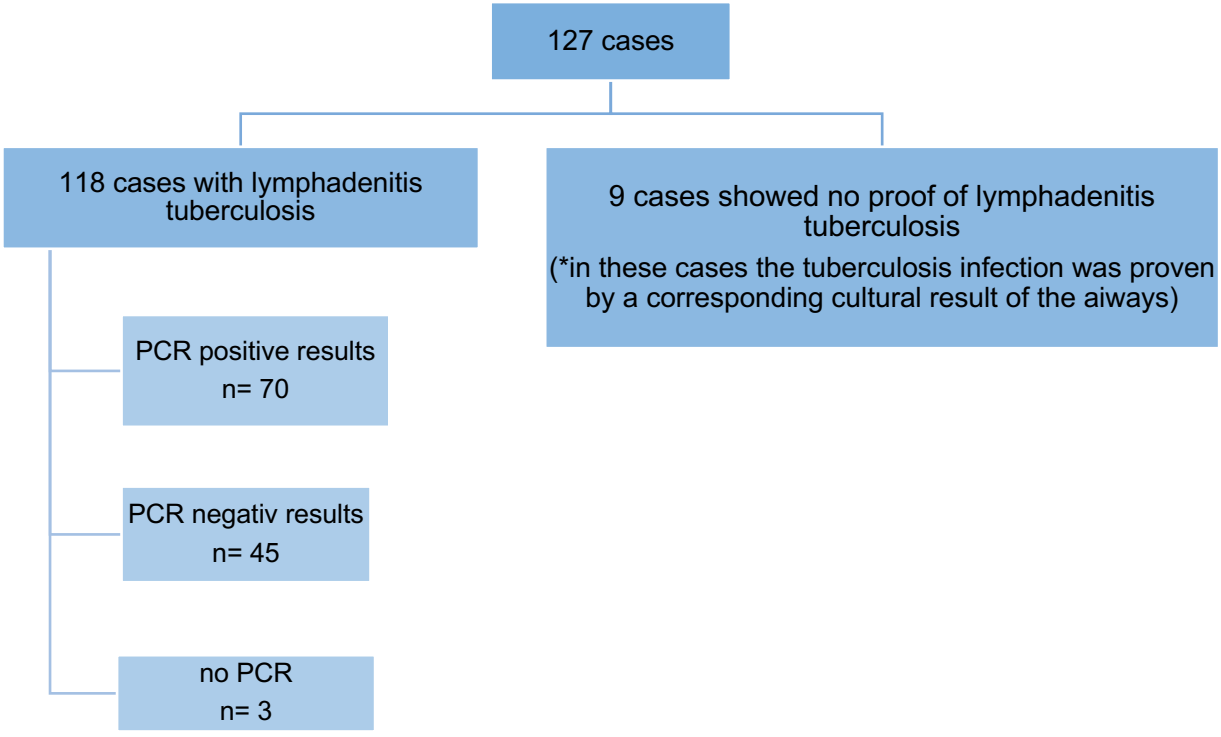


Figure 8: *Comparing cases with proven tuberculous infection to cases with no infection of the lymph node*

Evaluation by NAAT can be a fast and effective method to determine whether a patient is infected with *Mycobacterium tuberculosis* or not. Therefore, we took a special look at the 123 patients out of 127 patients that underwent PCR evaluation. The other four patients did not undergo any PCR assessment. In 118 cases proof of tuberculous infection of the lymph nodes was found. In nine cases only proof of a pulmonary

tuberculosis could be obtained. Out of the 118 cases with positive results for lymphadenitis tuberculosis, 70 patients showed positive PCR results, while 45 did not. In 3 cases patients did not undergo any nucleic acid amplification assessment. Therefore, this indicates that PCR shows a sensitivity of **61%** for the assessment of extra- pulmonary tuberculosis considering only patients with a proven extra-pulmonary tuberculosis infection.

11.3. Histological findings

11.3.1. Overall histopathological findings

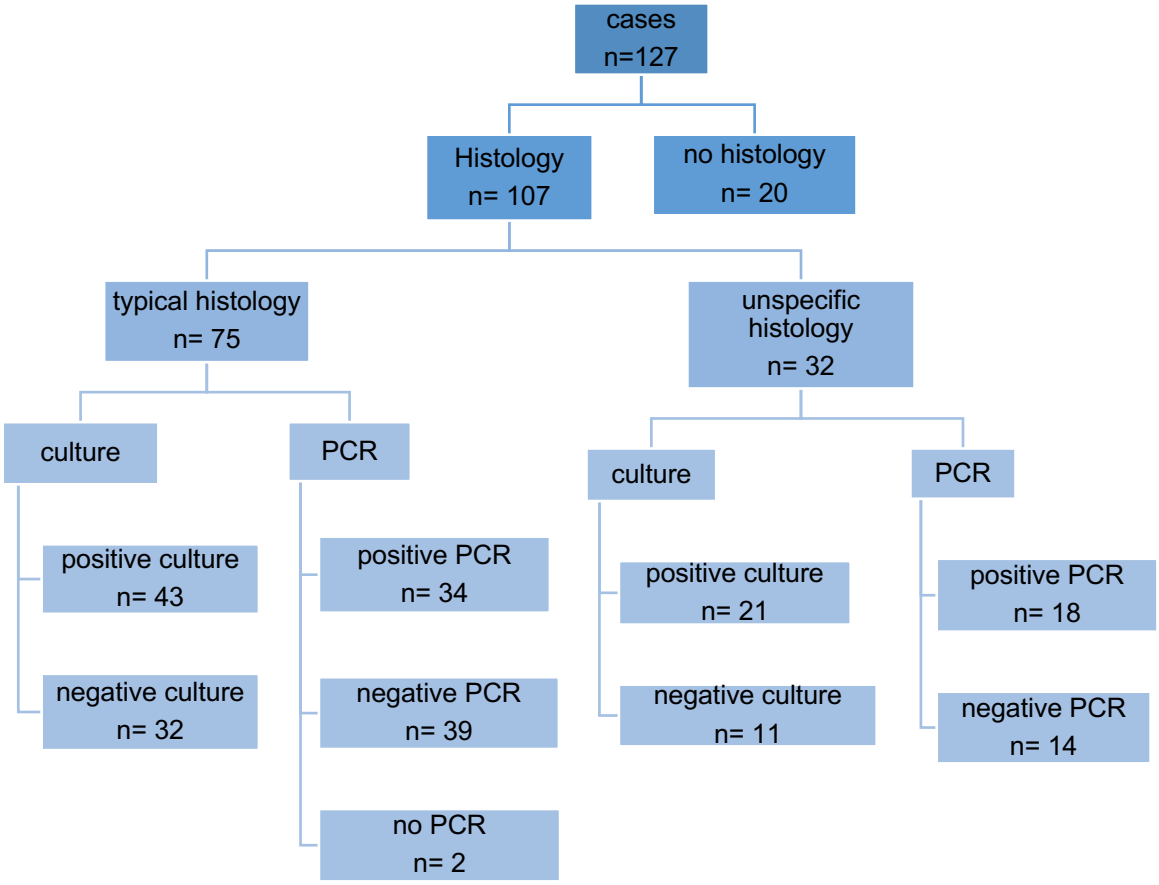


Figure 9: Comparison of overall histopathological results

Cytomorphological features of the representative lesions might be valuable in providing clues regarding possible tuberculosis infections. Therefore, a histopathologic assessment is an indisputably valuable method of evaluating chronic infections. In our study comprising 127 patients in total 107 patients underwent histopathological examination. In 20 patients no documentation of histopathological assessment could be attested.

Specific histopathological findings included two different classes. Class I included the majority of patients with specific histopathological findings with a total of **43** patients (40. 2%). It considered the typical histopathological patterns for tuberculosis including epithelioid granulomatosis with necrosis. Class II with **32** reported cases 29. 9%), included all cases with epithelioid granulomatosis without necrosis. Therefore, specific patterns for tuberculosis infections (Class I and Class II) numbered 75 patients in total (sensitivity of **70%**). Amongst all cases that showed specific histologic patterns, 43 showed positive cultural results, accounting for **57.3%** of the patients, while 32 patients did not show a positive cultural reaction. A positive molecular finding could be detected in 34 cases (**46.6%**), while 39 cases showed a negative PCR reaction. In 2 cases no PCR could be conducted from the lymph node material.

When breaking down the cases with a typical histology, we end up with 43 patients with a histology of Class I, while Class II comprised 32 cases. Regarding only patients in the first most typical class of histopathologic pattern, 33 patients (**76.7%**) showed positive cultural results while 10 showed negative cultural results. The PCR turned out positive in 28 cases (**65%**), while 15 PCR tests concluded in negative results. In comparison, the cultural results from the Class II histologic pattern (32 patients in total) turned out positive in 10 cases (**31%**), while 22 cases accounted for negative cultural results. In 6 cases (**20%**) the PCR assessment turned out to be positive for *Mycobacterium tuberculosis*, while 24 cases resulted in negative PCR results. In addition, two patients did not undergo PCR assessment. Regarding these results it is important to consider the fact that cultural proof was found more than twice as often in patients who additionally to a granulomatous reaction showed signs of necrosis. This fact will later be closely regarded in the discussion.

“No specific histopathological findings” included no granulomatous reaction with necrosis as well as any other unspecific stadium, which does not provide any relevant clues for tuberculosis detection. A total of 32 patients showed unspecific histopathological results. Of these 32 cases, 21 (**65.6%**) did indicate a positive culture, while 11 patients showed negative cultural results. In comparison to the cultural results, 18 patients (**56.2%**) showed positive molecular results, while the execution of PCR led to negative results in 14 cases. Therefore, a negative histopathological result, cannot dismiss a tuberculosis infection and further research is necessary.

The unspecific histopathological results can again be divided into unspecific histology, mostly regarded as normal lymphatic tissue; and tissue including no granulomatosis reactions with necrosis. The group of patients, who showed unspecific, normal lymphatic tissue included 29 cases, out of which 15 (**51.7%**) showed positive PCR results while, 14 showed negative results. Moreover, 19 patients (**65.5%**) were found to have positive cultural results, whereas 10 patients yielded negative cultural results.

In comparison, three patients displayed unspecific findings, as in no granulomatosis reaction, but still showing signs of necrosis. While all three patients in this group showed positive molecular PCR results, none of the patients obtained negative PCR results. Moreover, two of the patients in this field obtained positive cultural results, while one case provided cultural results with a negative outcome. This again leads to the question of whether patients with necrosis but apart from this an inconspicuous histopathological finding are more likely to actually have a tuberculosis infection.

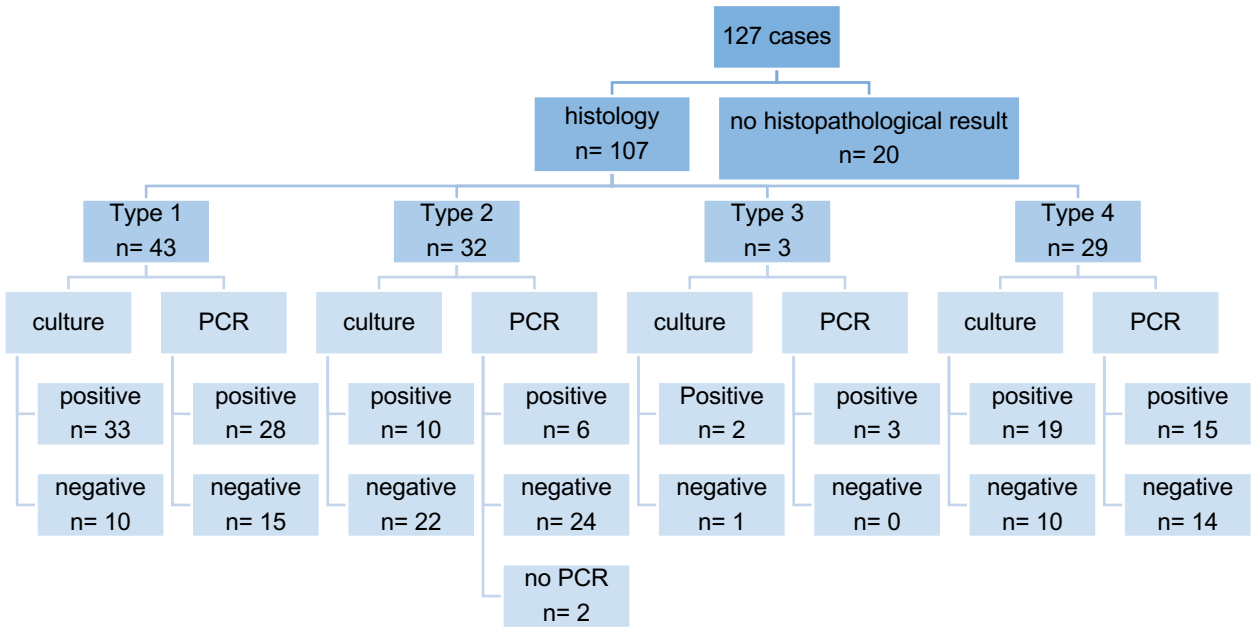


Figure 10: Subdivision of the histopathological types

11.3.2. *Histopathology in bacteriologically confirmed tuberculosis*

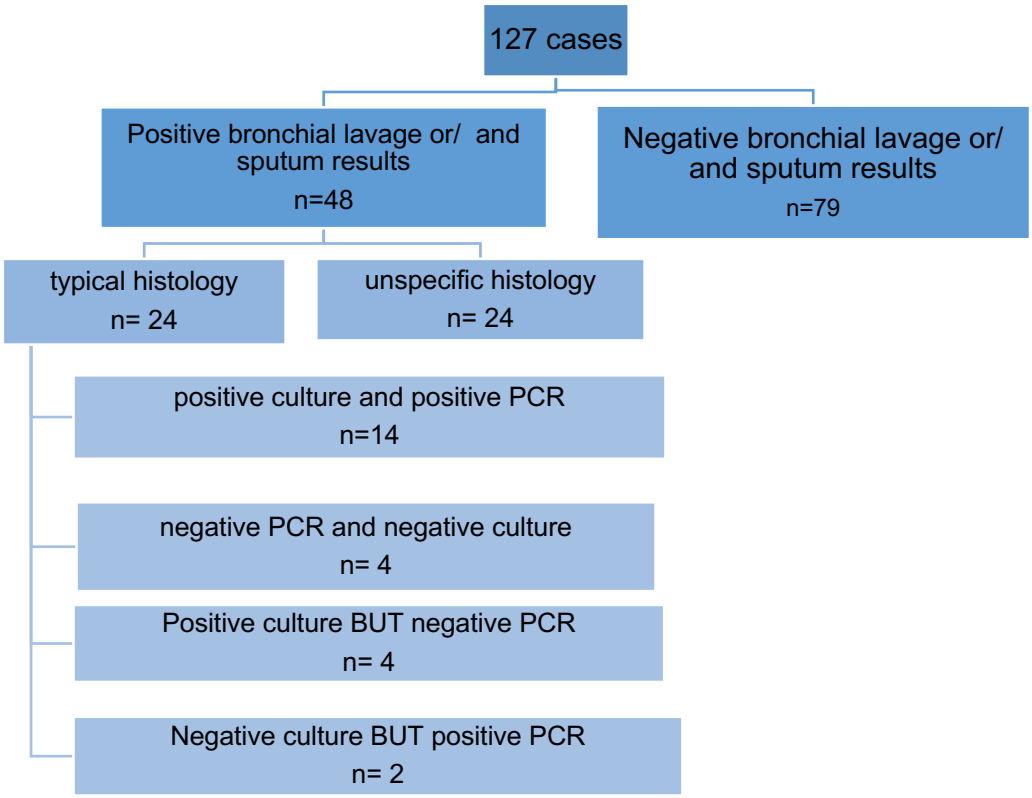


Figure 11: Histological results obtained from bronchial lavage and/ or sputum

Another important aspect we considered was patients who showed positive bronchial lavage or sputum results, therefore considered as having a bacteriologically confirmed pulmonary tuberculosis. Among this group of patients, including **48** results, equal amounts of findings showed typical histopathological results (24) or non-specific histopathological results (24) from the lymph nodes.

We took a closer look at the patients with a proven pulmonary tuberculosis, yielding typical histopathological results. These patients can be considered as highly specific as they not only show cultural proof in the airways, but also show histopathological signs of a tuberculous lymphadenitis. In these cases, we can assume, that the lymph nodes are affected by tuberculosis in 100%. Therefore, are this lymph nodes highly appropriate to calculate the sensitivities of the PCR and culture.

Among these **24 highly specific** patients we were able to distinguish between four different groups. The first group being patients providing both positive cultural as well as PCR results. This was by far the biggest group with 14 patients overall (**58.3%**). This group was followed by a group of four patients (16.7%) showing positive cultural

but negative PCR results. An equivalent number of results was also obtained for four patients (16.7%) showing neither positive cultural nor PCR results. Furthermore, two results for patients with positive molecular PCR results, but negative cultural results were recorded (8.3%). This leads to an overall sensitivity for PCR of **66.7%** and a sensitivity of **75%** for culture.

11.4. Immunological results

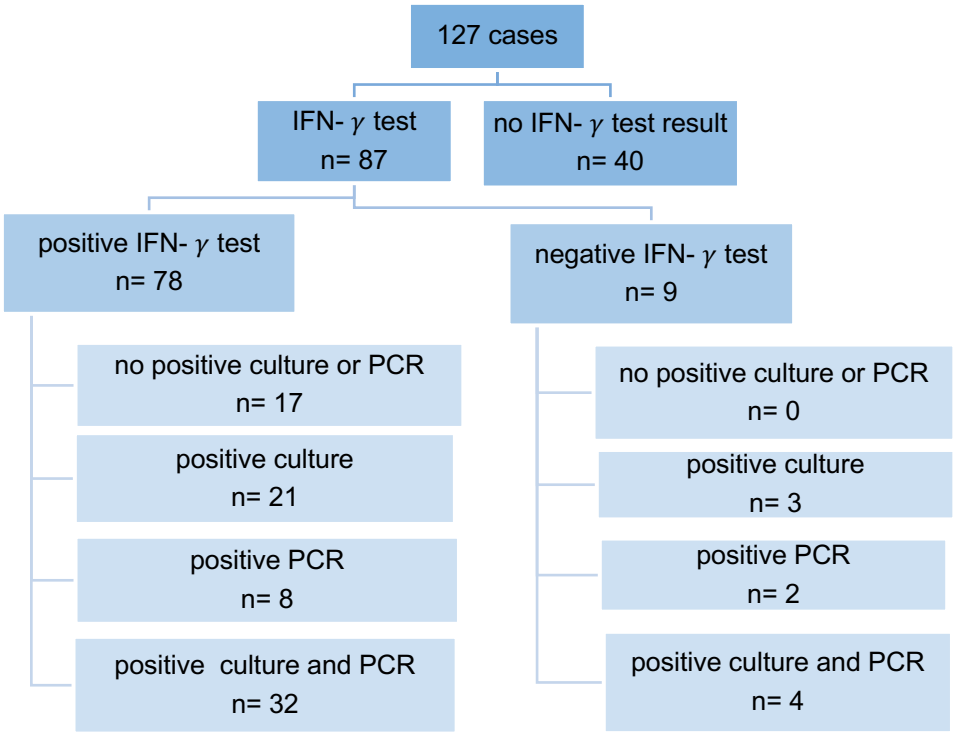


Figure 12: Comparing cases with positive and negative Interferon- y release assay results

The sensitivity of IGRAs is not very high in active diseases due to a difference in the cellular immune response during the progress from an infection to a disease. We wanted to find out the sensitivity of the IGRAs in isolated lymph node tuberculosis. Therefore, we tried to evaluate whether a positive Interferon- γ test generally correlates with a positive culture and PCR result from material obtained by EBUS TBNA in patients with a bacteriologically proven tuberculosis infection. From a total of 127 subjects, an Interferon- γ test was carried out in 87 cases (68.5%). Out of the 87 Interferon- γ tests conducted overall 78 tests (**89.7%**) showed positive results. Regarding this group with positive Interferon- γ test results, 61 cases (78.2%) showed

positive cultural or PCR results for lymph node tuberculosis. These 61 cases comprised 32 patients showing positive cultural and PCR results, 21 patients showing only positive cultural results and 8 patients indicating only positive PCR results. In contrast, 17 patients did not show any positive PCR or cultural results.

In comparison a total of 9 Interferon- γ tests showed negative results. Within this group, four results correlated with positive cultural and PCR results, while 3 cases only showed positive cultural results and 2 only showed positive PCR results. Thus, a negative Interferon- γ test cannot rule out a tuberculosis infection. In 40 cases no Interferon- γ release assay was conducted throughout the clinical investigation.

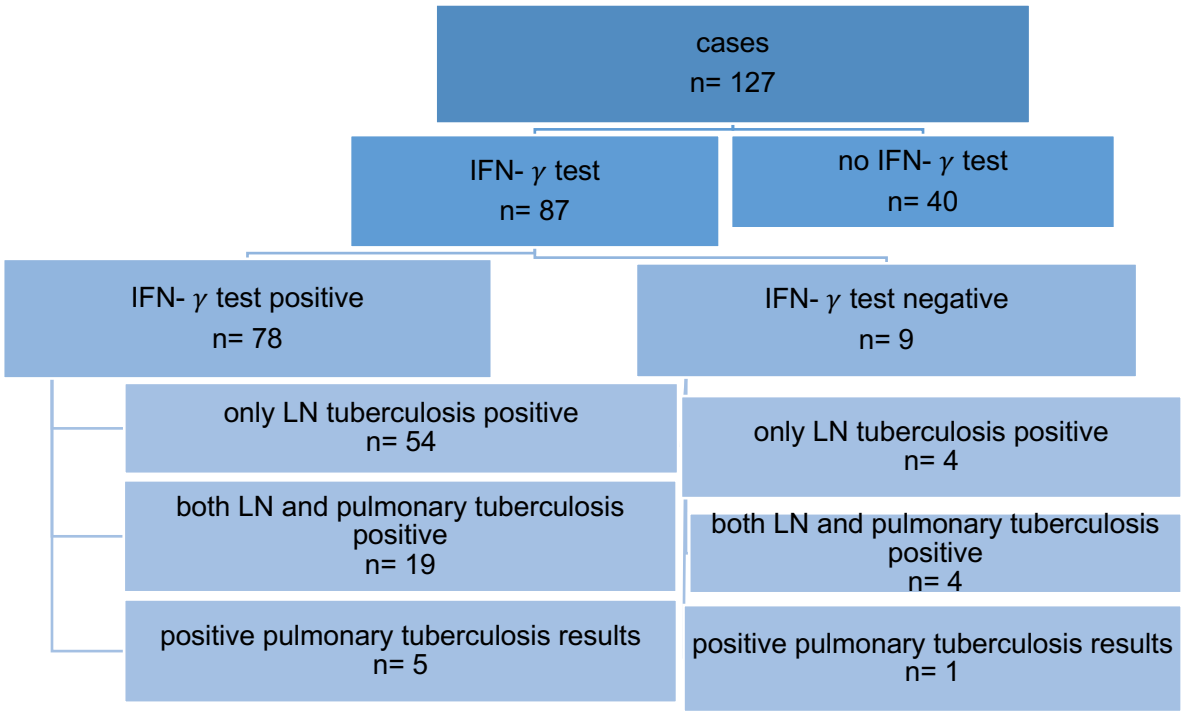


Figure 13: Breakdown of positive Interferon- y release assay results

When analyzing the Interferon- γ release assay results, we can distinguish between results that correlated with a positive result for a pulmonary tuberculosis, for a lymphadenitis tuberculosis or for both above. Concerning the 78 positive Interferon γ-test results, in 54 cases only an extra-pulmonary tuberculosis of the lymph nodes could be found (with no signs of pulmonary involvement, no positive sputum/ BAL). In 5 cases we could only obtain typical material for tuberculosis from the airways, suggesting a pulmonary tuberculosis. In 19 cases with a positive Interferon- γ result it was possible to obtain tuberculosis typical results from the airways, as well as the lymph nodes.

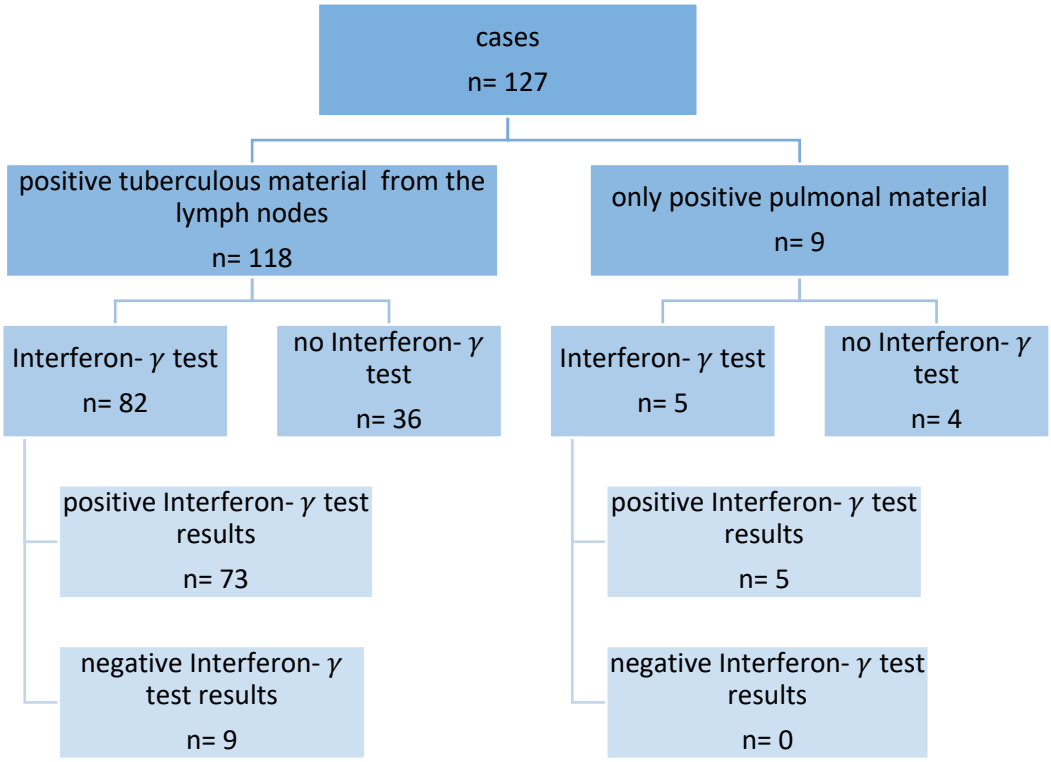


Figure 14: Comparing patients with a proven lymphadenous tuberculosis to patients with a proven pulmonary tuberculosis in consideration of Interferon- y release assay tests

Recapitulating overall results, this leaves us with a collective of 127 patients, of which 118 got a proven result with a tuberculous lymphadenitis. Out of these 118 patients, 82 patients obtained an Interferon- γ release assay assessment, while 36 did not. In 73 cases the Interferon- γ test turned positive, while it showed negative results in 9 cases. Therefore, the Interferon- γ release assay identified 73 infected patients with an extra-pulmonary tuberculosis of the lymph nodes correctly, accounting for a sensitivity of **89%.** This can again be broken down, by regarding only patients who showed signs of tuberculous lymphadenitis, but no signs of pulmonary involvement. In this group of patients 50 patients underwent IGRA. Out of these 50 cases, 46 cases turned out positive, accounting for an even higher sensitivity of **91%**.

Nine patients only showed signs of a pulmonary tuberculosis as no proof of a tuberculous involvement could be obtained. Out of these nine patients we obtained five Interferon- γ test results, which turned out positive in all five cases.

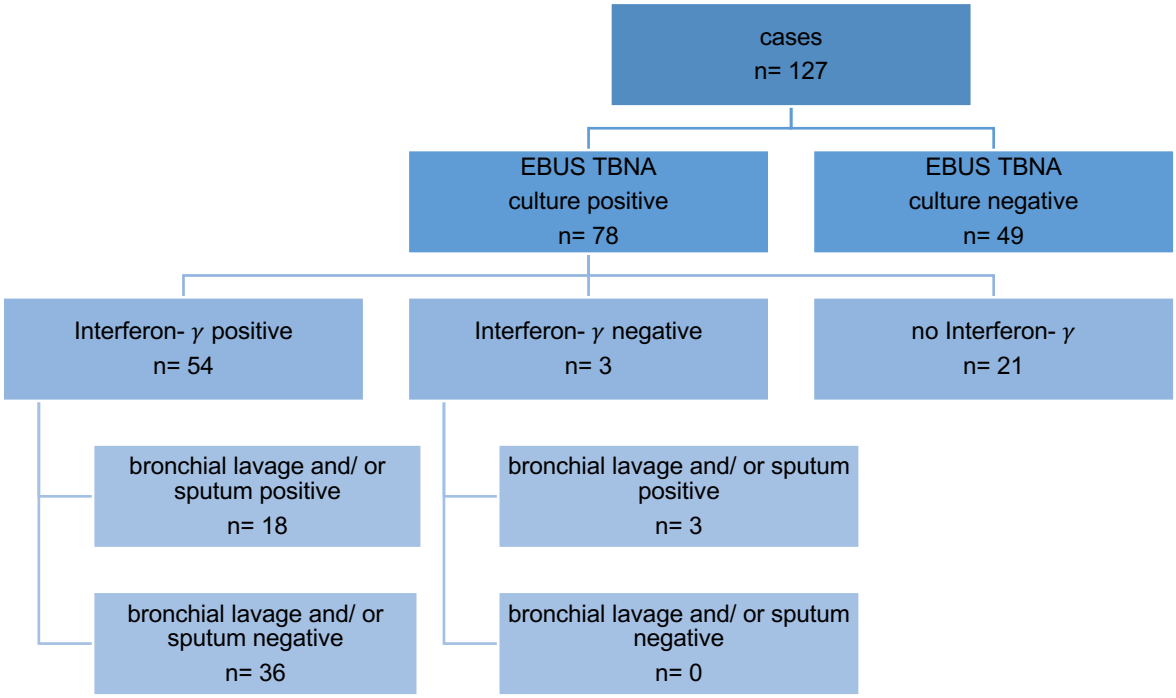


Figure 15: Subdivision of Interferon- y release assay results of bacteriologically confirmed patients by EBUS TBNA

Another aspect that interested us concerning Interferon- y assays, was the question as to whether an Interferon-gamma release assay can especially detect an extra-pulmonary tuberculosis. Therefore, we took a closer look at the 78 patients (out of 127 patients in total) who received a positive EBUS TBNA result. Concerning this patient collective, 57 overall underwent Interferon- gamma release testing. The remaining 21 patients did not receive this immunological testing. Out of the 57 patients tested, the Interferon-y release assay turned positive in 54 cases (**94.7%**), while three patients received negative results. Out of 54 cases with a positive Interferon y release assay, 18 patients also showed a positive bronchial involvement. This was not the case in the remaining 36 cases. Contrary to these findings, the bronchial lavage and/ or the sputum showed only positive results in the three cases with negative Interferon-gamma test results. Therefore, in the case of patients with a positive EBUS TBNA cultural result and no bronchial involvement (36 cases) the Interferon y release assay turned positive in 100% of the cases. This can be a sign for, that the IGRAs have a high sensitivity in incipient and subclinical forms of tuberculosis.

11.5. Elastography

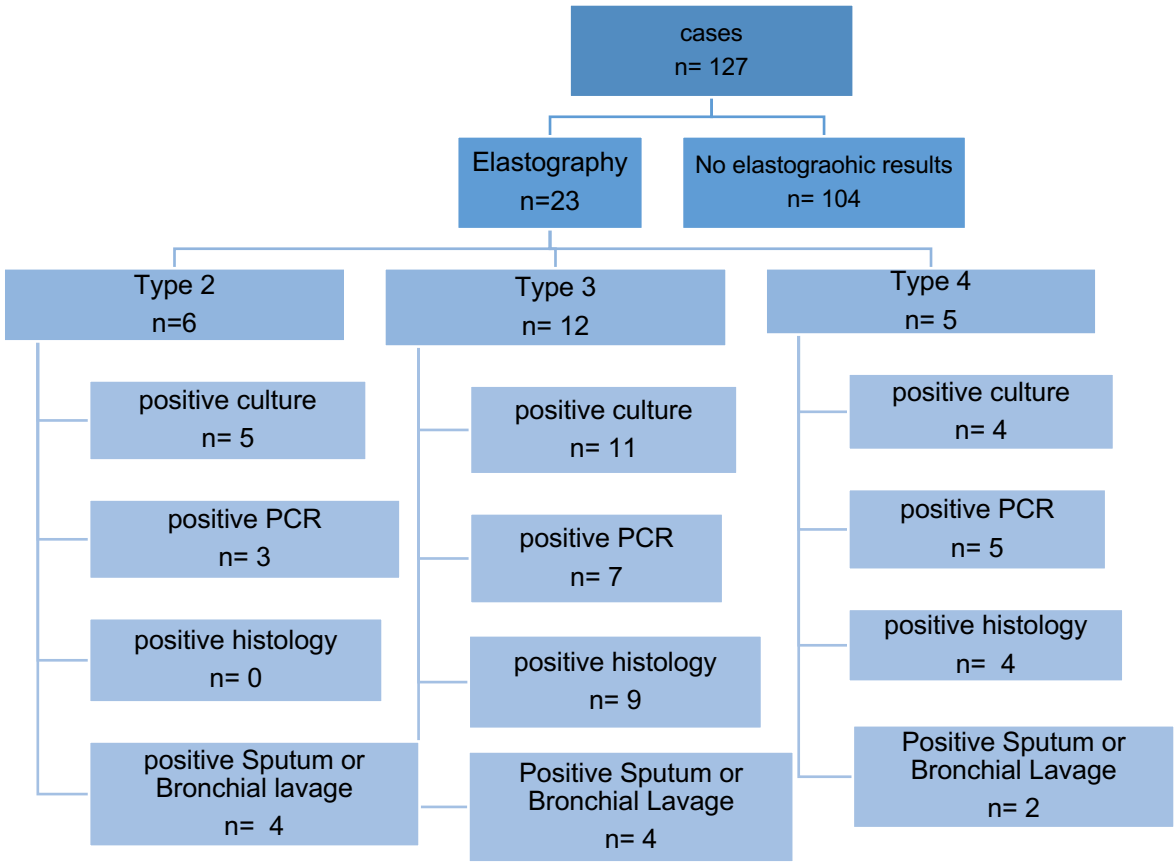


Figure 16: Breakdown of elastography results

In this study we obtained elastography findings from 23 out of 127 patients. Endobronchial elastography was taken from hilar and mediastinal lymph nodes and used to differentiate between normal lymph node tissue and malignancy. Elastography is commonly used to differentiate between malign or benign lesions, for example in tumor diagnosis. In this section, we tried to identify correlations between elastography findings and findings obtained through EBUS- TBNA, in other words the correlation between elastography results and histologic, microscopic, and cultural findings.

We differentiated between predominantly red and yellow elastography findings, indicating soft tissue; green elastography pictures indicating mostly inflammatory diseases, such as sarcoidosis and blue elastography findings indicating malign tissue.

Predominantly green and blue tissue findings can presumably be associated with tuberculosis, as these colors can mostly be found in harder tissue, indicating disease.

In order to score elastography pictures which mostly include more than one color, we divided elastography findings into four different types. Type one indicated that over 80 % of the sample showed green, red, and yellow fragments. Type two was used for samples with 50 - 80% of green, red and yellow coloring. Type three mostly indicated a picture which was predominantly colored blue (50 - 80% blue), while type four showed a nearly all blue sample, with over 80% blue overall.

Elastography findings in this study included six mostly yellow, red, and green pictures (Type 2), 12 mostly blue and green pictures (Type 3) and five predominantly blue elastography findings (Type 4). No picture with > 80% red, yellow, and green color has been included in this clinical study, which would have indicated a Type 1 sample.

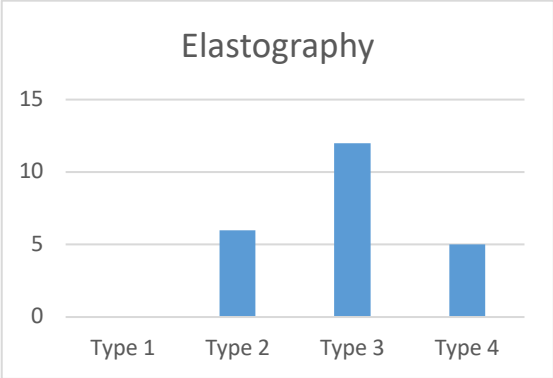


Figure 17: Elastography results

The six Type 2 elastography findings accounted for five positive cultural findings and one negative cultural finding. Furthermore, this group of elastography results indicated three positive PCR findings and three negative PCR results. The histopathological findings included five typical results and two negative results. Another aspect we looked at, was sputum and bronchial lavage, which were positive in four patients, while indicating negative results in two patients.

Concerning the Type 3 elastography findings, a clear trend can be seen with 11 positive cultural results and one negative cultural result, therefore indicating 91.7% positive cultural results in this group. Likewise, out of the 12 mostly green and blue elastography findings mentioned above, nine patients showed typical histopathological findings and three non-specific histopathological findings. In contrast, the PCR results did not show an image as clear as the other methods with seven positive PCR results and five negative results. Concerning the bronchial lavage and the sputum analyses, a positive result was obtained in four patients, while eight patients showed a negative microscopic result.

Predominantly blue elastography findings, indicating a Type 4 reaction, were obtained from five patients overall, yielding a clear tendency towards positive cultural, histopathological, and molecular results. Out of five cases four showed a positive

cultural result. Furthermore, four cases showed a specific histopathological result. All cases indicated a positive PCR. Concerning the bronchial lavage and the sputum analysis, more patients (three) showed a negative result than a positive one (two patients).

12. Discussion

Extra pulmonary tuberculosis still poses a major health problem globally due to difficulties in diagnosis and treatment monitoring. Despite considerable efforts to control the burden of the disease, it is hard to significantly reduce numbers of infections due to hurdles in precise diagnosing (Narang et al., 2015). Lymph nodes as the most common site of extra pulmonary tuberculosis are often affected, but repeatedly lack systematic microbiological assessment. In addition, improving early diagnosis methods for tuberculosis is of increasing importance due to an enhanced rate of multi-resistant tuberculosis forms. This study was therefore conducted to evaluate the significance of EBUS TBNA, when assessing extra pulmonary tuberculosis. The utilization of EBUS TBNA for the diagnosis of mycobacterial lymph node infection evolved in 2009, although only a few studies have reported the accuracy of this technique for evaluating tuberculous lymphadenitis (Geake et al., 2015). Furthermore, we evaluated the possibility of using elastography to differentiate between ordinary lymphatic tissue and lymph nodes being affected by tuberculosis.

Clinical suspicion and precise diagnosis as well as timely treatment are essential to control the disease. Considering molecular diagnostics, it is possible to use PCR, cultural assessment as well as cytology for diagnosis of extra pulmonary tuberculosis.

This study is notable as it not only reflects the population of Germany, but many other Western health facilities. These include predominantly low tuberculosis prevalence countries with an incidence of less than 10 per 100.000 where over 90% of the patients are mostly migrants from tuberculosis endemic countries. Therefore, this study with specially selected patients can be used to demonstrate the efficacy of tuberculous lymphadenitis diagnosis via EBUS TBNA in the Western world.

After obtaining approval from the ethical commission, information was retrieved from 127 patients in the period between January 2015 and April 2018. The strength of this

retrospective study is that it only included patients with a proven tuberculosis. This condition could be ensured by either the proof of tuberculosis in the airway of the patient, therefore either in the sputum or the bronchial lavage or both, or the proof of tuberculosis bacteria in the lymph nodes (PCR or culture). Another aspect that we considered, where patients with typical histologic results, as in epithelioid granulation with central caseating necrosis. Nevertheless, we excluded patients with histopathological typical results only as our goal was to deliberately focus on patients with a bacteriologically proven tuberculosis infection. EBUS TBNA was conducted to serve the purpose of obtaining material for detailed cultural and cytological examination to elaborate cytopathological findings. In Figure 18 we can see a brief breakdown of the most important results we have obtained throughout the course of our study.

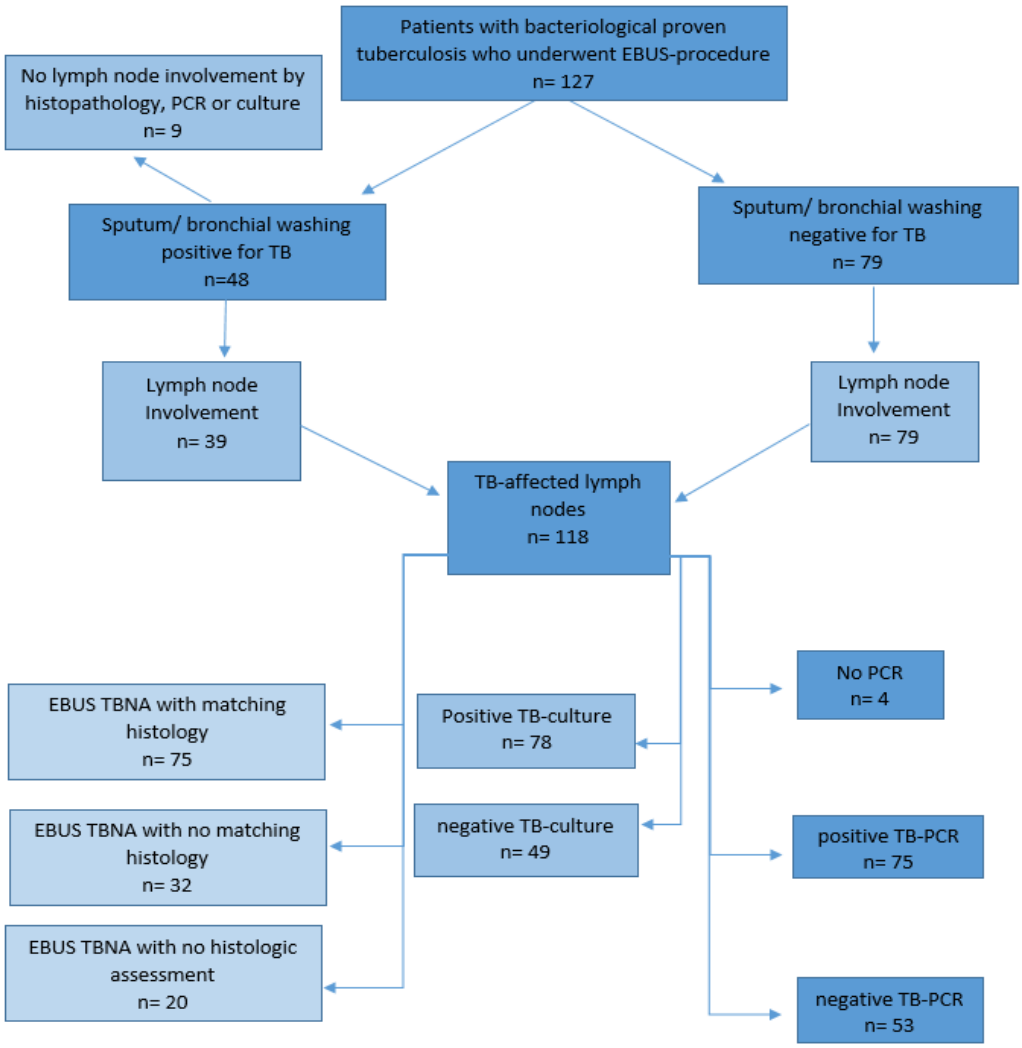


Figure 18: Brief overview of the results of our study

Sociodemographic findings

During the period of record EBUS TBNA was conducted on 127 patients. Most patients were male. This higher prevalence of extra pulmonary tuberculosis in the male population is in contrast with the much-described higher prevalence of females developing extra pulmonary tuberculosis (Narang et al., 2015). Several factors are supposed to be determining for this sex preference, including endocrine factors, smoking and previous exposure to tuberculosis. These factors were not all explicitly considered in our study, nevertheless a clear tendency of male patients could be observed. Furthermore, from the information available in the patient´s health records, cases were further classified among age, sex, and site of lesion. Most of our patients included young males (mean age 32 to 43 years), however even cases in the extremes of age could be detected (15 years to 95 years). These findings correlate with previous studies, for example a study lead by Davidson et al. supposing that extra-pulmonary tuberculosis has mostly been found in a younger age group, with a peak between 11 to 20 years, closely followed by the age group of 21 to 30 years (Davidson & Muthulakshm, 2018).

The fact that our study mostly included young male patients can be seen as an effect of the increased migration trend that took place between the years of 2015 and 2018 in Germany and especially in Bavaria, which is located close to the German border. Due to the refugee movement that peaked in 2015 mostly young male refugees tried to flee to Germany in the hope of starting a new life and finding work to provide for their family at home. According to data according to the BAMF- “Bundesamt für Migration und Flüchtlinge”, which is the German federal office of migration and refugees, the migration movement exploded in 2015 with 1.125.419 people from non- European states migrating to Germany. Considering the people that left Germany that year, this leaves us with a net migration of +859.816 persons in 2015. This is more than double the net migration that Germany experienced in 2014 with +337.421 citizens. Nevertheless, this extreme peak of migration continuously decreased in the following years with +34.884 persons in 2016 and +278.036 people migrating to Germany in 2017. Mostly people from Syria, Iran and Iraq tried to seek asylum in Germany. (Bundesamt für Migration und Flüchtlinge, 2019).

Taking a closer look at the individuals that moved to Germany there is a clear predominance of young men seeking asylum. In the period from January to April 2019, 73. 5% of asylum requests were sought by people under the age of 30 years. Out of this group of asylum seekers, 47.6% were minors. The largest group of asylum seekers included people under the age of four with 14.041 out of 51.370 during the period between January and April (27.3%). The second largest and most representative group for our study were people from 18 to 25 years of age accounting for 15% of the applicants (7724 persons). In addition, 5545 (10.6%) immigrants between 25 and 30 years applied for asylum. In both groups a clear predominance of males can be observed. In the first group 5202 of 7724 persons were male (67.3%), while the slightly older group included 3456 males (62.3%). Overall, 57. 7% of the initial applicants were males. In comparison, all asylum seekers over the age of 40 accounted for only 11.2% of all asylum seekers. In these older age groups less gender-specificity can be observed (Bundesamt für Migration und Flüchtlinge, 2019).

During the immigration process at the border a radiologic screening of the lung in one plane (mostly lateral X-ray) is to be conducted. In children under the age of fifteen as well as pregnant women immunological tests such as the Interferon- γ tests or Tuberculin tests are used. If there are any radiological, immunological, or clinical signs of tuberculosis, a bacteriological analysis of at least three different sputum samples is performed. In addition, a physical examination and a detailed investigation of the medical history must be carried out. In order to undergo these tuberculosis examinations, the refugees concerned are admitted to hospitals, for example the clinic in Gauting, which specializes in tuberculosis treatment. This is very important to reduce the possibility of spreading infections (Bundesverband der Ärztinnen und Ärzte des Öffentlichen Gesundheitsdienstes e.V., 2019).

This previously described migration movement is likely the reason why mainly young men are part of our study, which leads to a biased gender distribution in this study. This stands in contrast to most studies which were conducted in non-European countries like India and suggested that mostly female patients are affected by extra-pulmonary tuberculosis. It is important to mention that in these countries tuberculosis infection rates clearly exceed Germany´s infection rates, and the tuberculosis patient collective includes different distribution of age groups and sex. Therefore, it is difficult

to compare our sociodemographic findings, with the sociodemographic findings from other studies from countries with a much higher tuberculosis incidence rate.

Pulmonary involvement in tuberculous lymphadenitis

Out of 127 patients, 48 cases with positive tuberculosis material in the airway were obtained, while 79 showed negative results regarding pulmonary tuberculosis. Therefore, only 37.8% of the patients with a subsequently proven tuberculosis infection received positive results regarding pulmonary tuberculosis signs. Pulmonary tuberculosis has reportedly been proven when mycobacteria tuberculosis was either found in the sputum, the bronchial lavage or both. These 48 cases included 39 cases with subsequently positive proof of an infection of the lymph nodes. Therefore, 39 out of 127 patients showed both positive proof of and TB infection in the lymph nodes, as (30.7%) well as the airways.

These circumstances can be explained by the fact that when pulmonary assessment already showed cultural proof of a tuberculosis infection, in general no bronchoscopy assessment or EBUS TBNA was conducted. This is since whenever pulmonary signs of a tuberculosis infection, such as a positive bronchial lavage and/ or sputum result are present, the infection is considered a primary pulmonary tuberculosis and even finding other manifestations of tuberculosis elsewhere in the body, would not change the treatment plan. Therefore, only in cases where no cultural proof could be obtained by airway examination, but a strong suspicion for a tuberculosis infection remained, an EBUS TBNA was conducted. This was also true when the culture from the sputum or the bronchial lavage took a longer time to grow. In such cases an EBUS TBNA was conducted in the meantime to assess lymph nodes close to the central airway system for cultural examination. In these specific cases, it was therefore possible that sputum culture later turned out to be positive, leading to both positive cultural results from the sputum or the bronchial lavage as well as from the lymph nodes. Patients with positive cultural results in the sputum or bronchial lavage suffer from a primary pulmonary tuberculosis. In these cases, the lung is always infected, while extra-pulmonary tuberculous sites can also be found. In cases with only positive cultural results from the lymph nodes, a primarily extra-pulmonary tuberculosis occurs, in this study cultural

assessment was taken from the mediastinal lymph nodes, therefore positive cultural results in these examinations indicated the presence of a cervical lymphadenitis.

As bronchoalveolar lavage and sputum diagnosis have a low yield in patients with isolated tuberculosis lymphadenopathy (Navani et al., 2011), it is not surprising that only about a third of the patients in our study obtained a positive cultural result when analyzing the previously mentioned specimen.

Most specific cases with pulmonary cultural proof and typical histopathological pattern

Out of the 48 patients who showed signs of proven pulmonary tuberculosis 39 patients could be categorized as mediastinal lymph node tuberculosis positive, this accounted for 81. 3% of the cases in this group. Cultural proof showed a sensitivity of 68. 8%, followed by PCR analysis with a sensitivity of 64.6% and histology with a sensitivity of 50%.

Taking into consideration the 24 highly selective cases (bacteriologically proven pulmonary tuberculosis in combination with a matching histology found by conducting EBUS TBNA), a sensitivity of 67% could be obtained for analysis by PCR and a sensitivity of 75% was observed for cultural assessment.

These findings could be compared to patients who showed no signs of pulmonary tuberculosis. This group included 79 patients out of whom 57% showed positive cultural findings, 49.4% showed a positive PCR and 64.5% obtained typical histopathological findings. All these 79 patients showed proof of mediastinal lymph node tuberculosis.

General EBUS TBNA results

Regarding the results obtained through EBUS- TBNA from bacteriologically proven tuberculosis 78 patients overall showed a positive cultural result, which can be associated with a sensitivity of 61.4% for culture. In order to give an overview of the results of our study, we can compare the sensitivity of EBUS TBNA for cultural assessment we obtained, to the results from other studies. Multiple previous reports have shown the efficacy of EBUS TBNA for diagnosis of mediastinal mycobacterial

infection. Nevertheless, only a few have examined its diagnostic accuracy and most importantly the option of micro bacterial confirmation of tuberculosis infection.

We compared various other studies mostly from endemic countries describing the use of EBUS TBNA for diagnosis of mediastinal tuberculous lymphadenitis. For example, Ye et al. conducted a meta-analysis comparing the sensitivities of EBUS TBNA from 8 different studies with a total of 809 patients. The sensitivities ranged from 50% to 95%, with a pooled sensitivity of 0.8. Special attention must be paid to the pooled sensitivity of EBUS TBNA for the assessment of intrathoracic tuberculous lymphadenopathy of 87% (Ye et al., 2015). In comparison, we obtained a sensitivity of 61% for cultural assessment throughout EBUS- TBNA. Nevertheless, scrutinizing the significance of the comparison between these results is inevitable, as most analyses including the report conducted by Ye et al. show the sensitivity of a composite microbiological and clinicopathological assessment. In comparison, our study focused more on analyzing the sensitivity of only the cultural evaluation of the ability of EBUS TBNA to detect tuberculosis. Therefore, the results of our study show lower sensitivity rates. When considering the sensitivities for cultural positive diagnosis, most studies report sensitivities between 38% and 46%. Our study was clearly able to exceed these diagnostic values (Geake et al., 2015).

One multicenter study by Navani et al. showed more similarities with our study composition leading to increased comparability. The study included 156 patients with confirmed diagnosis of tuberculosis or unequivocal clinical and radiological response to anti-tuberculosis treatment during the follow-up of six months. EBUS TBNA was able to diagnose tuberculosis 94% of the time, including a positive culture or positive clinicopathological diagnosis. Nevertheless, a positive culture could only be obtained in 47% of the cases, with a medium time of culture turning positive ranging from eight to 16 days. This study only included patients with previously diagnosed tuberculosis, therefore EBUS TBNA was not used to confirm a clinical suspicion, thus lacking the possibility to obtain a negative predictive value or report diagnostic accuracy (Navani et al., 2011). The difference between the results of Navani et al. and our study might be due to a more specific patient collective in our case, as we have a selection bias due to inclusion of only confirmed TB-cases, so false-negative patients were potentially excluded. In addition to a shorter transport time of the tissue samples as the Gauting hospital laboratory is located at the same facility.

During our investigation, another study by Geake et al. that managed to confirm the diagnostic sensitivity for microbiologic diagnosis of tuberculosis by EBUS TBNA, came to our attention. The study was conducted in Australia and was more representative of Australian and other Western health care facilities. In this study the calculated sensitivity for cultural assessment through EBUS TBNA was 62% (24/ 39 tuberculosis cases). Therefore, this study showed very similar results to our study with a sensitivity of 61%. If cases with in- synch histopathological findings from EBUS TBNA allowed the diagnosis of a highly probable tuberculosis, the sensitivity of EBUS TBNA even increased to 92% (36/ 39 cases) now including twelve more cases that showed a negative cultural, but positive histopathological result (Geake et al., 2015).

Special notice must be taken of the most specific group of patients, which included 24 patients with positive cultural results when regarding the sputum or bronchial lavage as well as a typical histology present in the examination of the lymph nodes, after obtaining tissue from the lymph nodes through EBUS TBNA. In this group of patients, 14 patients obtained a positive cultural result when assessing the lymph nodes through EBUS TBNA. This leads to an even higher sensitivity for cultural examination in this group of patients, with a sensitivity of 75%. There is no gold standard that can be used to calculate the sensitivity of PCR and culture. However, 100% of our patients with confirmed tuberculosis from the airways and suitable histology of the lymph nodes have tuberculosis of the lymph nodes. That is why they are so well suited for the sensitivity calculation of PCR and culture. Conversely, the patients in whom we have cultural pathogens in the EBUS- TBNA sample are suitable for calculating the sensitivity of the histopathology.

For better evaluation, it is also possible to compare the results obtained by EBUS TBNA to a conventional TBNA. Reported composite diagnostic sensitivity (Cultural and histopathological assessment in combination with a high clinical suspicion) varies from 65% to 83%. The sensitivity for positive cultural results is even more significant. Results obtained by conventional TBNA only show a sensitivity of 9. 5% to 27%, respectively marking a lot lower diagnostic value than EBUS TBNA: In addition, conventional TBNA is not often used due to the lack of training in the technique as well as the risk of inadvertent vascular puncture as it is a “blind” procedure (Geake et al., 2015).

Comparing culture and histopathological results

Considering all patients with a positive cultural result obtained through EBUS TBNA, which was the case for 78 patients, 68% obtained a positive PCR, while 66.2% showed a typical histology, with a total of 3% with unspecific necrosis with a lack of granulomatous reaction. This shows that a positive histopathological result cannot always be obtained from infected lymph nodes. It is therefore very important to always take at least three samples when puncturing the lymph nodes to increase the diagnostic yield of puncture.

Our results show corresponding statements with other studies, such as a study by Eom et al. In this study five out of seven lymph nodes showed histopathological non-specific EBUS TBNA results, four subsequent cases indicated positive TB results through PCR analysis. This also suggests that a negative histopathological result cannot rule out a tuberculosis infection and that histology and cultural or NAAT results do not always correlate (Eom, et al., 2015). Another study indicating that a tuberculosis infection does not always show coherent cytological results is a study by Samaila et al. During their study, they evaluated specimen obtained by fine needle aspiration from tuberculosis positive patients to evaluate the histopathological correlation between the results. Out of 48 patients with extra pulmonary tuberculosis, mainly with affected cervical lymph nodes, 23 (47.9%) of the aspirates were purulent, 18 (37.5%) were blood stained and 7 (14.6%) had caseous material from a macroscopic perspective. Microscopically all specimen showed epithelioid cells, while pathognomonic caseation could only be found in 56.3 % and multinucleated giant cells only in 25% of the cases. AFB only showed positive results in 47.9 % of the cases. This study therefore showed that patients with proven tuberculosis infections do not always show coherent histopathological findings. Nevertheless, in this study the histopathological results show an increased tendency towards a typical tuberculous histopathological report, while our study reported a low yield in the diagnostic accuracy of cytology (Samaila & Oluwole, 2011).

A Tunisian study was published in 2018 with the aim of comparing cytological findings with Ziehl- Neelsen staining and culture. Cytological diagnosis was confirmed as being conclusive if both epithelioid granulomas and necrosis were present. Among 171 patients with tuberculous lymphadenitis, 142 patients showed correlating

histopathological findings (83%). In contrary, *Mycobacterium tuberculosis* was detected in only 38 cases (22.2%), 22 positive on ZN stain and 16 with a coherent culture. Considering the 16 culture positive cases, 14 showed granulomas with necrosis (87.5%), indicating a high diagnostic yield for cytological assessment (Rammeh et al., 2018).

In comparison, a study by Geake et al. showed that typical histopathological results may also occur in cases with a negative cultural result, also indicating that histology and culture do not always correlate. In this study in 12 out of 39 cases a typical histology (9 cases with necrotizing granulomatous inflammation, 3 cases with non-necrotizing granulomatous inflammation but adequate clinical history) could be obtained (Geake et al., 2015).

In conclusion, it is important to regard histopathological results with caution and to bear in mind that a negative histopathological result can never rule out a tuberculosis infection. Likewise, patients with seemingly correlating histopathological results, such as epithelioid granulomas with necrosis still have to be culturally assessed to obtain a confirmed bacteriological diagnosis. Therefore, it is important to always search for *Mycobacterium tuberculosis* bacteria for diagnosis confirmation.

Evaluating PCR analysis

Another important point of investigation was the significance of PCR analysis throughout EBUS TBNA in the diagnosis of extra-pulmonary tuberculosis. In our study, a total of 70 patients obtained positive PCR results throughout EBUS TBNA examination. This included 31 patients with positive cultural results for bronchial lavage or sputum analysis as well as 39 patients who did not show positive cultural results regarding bronchial lavage or sputum. This results in a sensitivity of 53% for microbiological assessment in our study when using EBUS TBNA to access lymph nodes. These results are mostly consistent with the results of the study from Navani et al., which showed that microbiological investigation yielded a positive result in 53% of the cases (Navani et al., 2011). In comparison, results by Tadesse et al. showed an increased sensitivity of 63.4% with a specificity of 100%. This higher sensitivity suggests the use of Xpert MTB/RIF assays for the use of initial diagnosis when testing lymph node tissue suspicious of lymphadenitis tuberculosis. However, due to the small

number of samples, the calculated performances require confirmation with larger studies (Tadesse, 2018). Also, a study conducted by Eom et al. in 2015 suggested a sensitivity of 56% for the detection of a tuberculosis infection via PCR analysis of an EBUS TBNA punctuate (Eom et al., 2015). In contrast, sensitivity of NAAT (nucleic acid amplification testing) was only 38% in culture confirmed cases according to a study by Geake et al (Geake et al., 2015). Recapitulating the previously presented studies, PCR analysis shows a wide range of sensitivity ranging from 38% to 63%, some studies even proposed sensitivities of up to 90% for detecting tuberculosis with NAAT from lymph node material (Tadesse, 2018). Nevertheless, these high results should be treated with caution as the representative studies only included a very low number of patients.

PCR based tests can detect DNA from dead bacilli in patients with a tuberculosis infection in a shorter time-period than by cultural assessment, suggesting an improvement for early diagnosis. Nevertheless, negative test results cannot rule out a tuberculosis infection, and culture remains the preferential reference method. However, the rapid confirmation of diagnosis as well as the possibility of obtaining fast genotypic susceptibility testing does demonstrate the soaring importance of PCR evaluation. NAAT especially can play an important role in the diagnosis of smear negative extra-pulmonary tuberculosis. Studies including elevated numbers of patients undergoing PCR evaluation of tuberculosis bacilli should be conducted.

Histopathological results

In this study of 127 patients a total of 107 patients underwent histopathological examination. Typical cytology included epithelioid granulomatosis with necrosis that was found in 43 patients, while 32 patients showed epithelioid reactions without necrosis. Out of all 75 patients with a positive histopathological pattern for tuberculosis only 43 showed positive cultural results and only 34 showed a positive PCR.

Unspecific histopathological findings were obtained in 32 cases. These can be divided into unspecific histology mostly being regarded as normal lymphatic tissue (29 cases) one the one hand and tissue including no granulomatosis reactions with necrosis (3 cases) on the other. In 29 cases punctuation with EBUS TBNA led to histopathological

unspecific lymphatic tissue. Out of these 29 patients, 15 still showed positive PCR results (51.7%), whereas 19 patients even obtained a positive cultural result (65.5%). This indicates that cytology is not as sensitive as expected and patients with a negative histopathological result in EBUS TBNA cannot automatically be regarded as free of any tuberculosis infection.

Although in various previous studies, cytomorphological analysis of different cases with tuberculosis infections mostly showed the typical epithelioid granulomas with correlating central caseous necrosis to be the most common pattern (Narang et al., 2015). this could not be confirmed in our study. Only 43 patients out of 107 that underwent histopathological analysis showed typical granulomatous reactions with necrosis. In our study we did not further investigate whether the present necrosis, showed signs of central caseation or other distribution. Another 32 showed granulomas without necrosis, whereas the remaining 32 patients showed an unspecific cytology. Therefore only 40, 2% of the patients showed typical histopathological patterns. This result remains in contrast to previous studies, yielding a much higher rate of histopathological typical tuberculosis results, than for example proposed by Davidson et al. who observed that in 79% of clinical suspects of tuberculous lymphadenitis typical histopathological evidence of granulomas with necrosis could be found (Davidson & Muthulakshm, 2018).

In our study 75 patients showed either Type 1 or Type 2 histopathologic patterns (granulomatous reaction with or without necrosis), accounting for 70% of the patients who underwent histopathological assessment. Out of these 75 patients, 43 patients showed positive cultural results. Therefore only 57.3% showed a correlating positive cultural result with typical histopathological patterns.

In addition, lesions that included necrosis did show a significantly higher prevalence of positive cultural results, than having been supposed in other studies. Navani et al. proposed that lesions compromising necrosis showed a higher probability of acid- fast bacilli in contrast to lesions lacking necrosis (Navani et al., 2011). When regarding the impact of necrosis on the prevalence of positive cultural results one needs to consider three different groups of patients. First there is the group with a histology that is most specific for tuberculosis, granulomatous reactions with necrosis. In this group, which included 43 patients, 33 showed a positive cultural result, resulting in a sensitivity of

76.7%. This is significantly higher than the results obtained by other studies for example by Navani et al. who obtained positive cultural results in 44% of the cases in this group of patients with a typical histopathological pattern. Furthermore, regarding the 32 patients who showed granulomas but no necrosis, 10 patients showed positive cultural results, resulting in a sensitivity of 31.1%. This clearly shows that the proof of necrosis has an impact on whether positive cultural results can be obtained or not. This result is compatible with results by Navani et al. who obtained positive cultural results for patients who only showed granulomas but no necrosis in 37% of the patients with bacteriologically proven tuberculosis (Navani et al., 2011). This finding indicates that the presence of necrosis on EBUS TBNA samples may be associated with a higher yield of positive culture results for tuberculosis.

To conclude, EBUS TBNA can be seen as an effective method for the diagnosis of intrathoracic lymphatic tuberculosis. This result is coherent with the findings of Navani et al. showing that EBUS TBNA yields diagnosis of tuberculosis with a sensitivity of 94%. These findings are especially important since for patients with isolated lymphadenopathy due to tuberculosis, traditional techniques for diagnosis, such as bronchoscopy, sputum culture and radiology yield low precision for diagnosing an intrathoracic tuberculosis. In this case a definite diagnosis of tuberculosis is important to diminish the risk of mistakenly using a wrong therapy (Navani et al., 2011).

Assessing IFN- γ test results

During our study, we also evaluated the role of immunological tests, when assessing tuberculosis infections. In total, we performed Interferon- gamma tests on 87 patients, while 40 patients did not undergo immunological assessments. Regarding the 87 tested subjects, 78 subjects showed a positive IFN-γ test result. All these patients suffered from a confirmed tuberculosis infection. Confirmation criteria have been mentioned above. All the patients included in the study were treated with anti-tuberculosis drugs after the diagnosis was confirmed.

Therefore, regarding the diagnostic value of immunological testing in this case, IFN-γ test showed a sensitivity of 89.7%, which largely exceeds the expected sensitivity proposed by other studies. For example, we can compare this result to a study by

Dyrhol-Riise et al. In this study, researchers evaluated the diagnostic value of QuantiFERON-TB® Gold In-tube (QFT-TB) assays for assessing suspected tuberculosis infections, to detect active or latent tuberculosis infections. In this study of 481 patients who were suspected of having a tuberculosis infection (which was later confirmed), only 148 patients showed a positive result in the Interferon- γ test assay. This results in a sensitivity of only 30.8% of the cases (Dyrhol-Riise, 2010).

Nevertheless, regarding the possibility of discovering extra-pulmonary tuberculosis the sensitivity of the Interferon- γ release assay was only 70. 1%. This correlates with 61 out of 87 cases where the Interferon- γ release assay has been conducted, who showed either a positive culture and/ or a positive PCR result obtained from lymph nodes by EBUS TBNA. It is important to mention that the Interferon- γ test showed negative results in five cases, with cultural and/ or PCR proof of cervical lymphadenitis. Therefore, in 5. 7% of the cases false-negative results were retrieved in our setting. This can be compared to a study by Kim et al. who retrospectively analyzed the medical records of 163 patients with extra-pulmonary tuberculosis. In this study, false negative results accounted for 8. 6% of the patients with a tuberculous lymphadenitis. Therefore Interferon- gamma results should be regarded with caution to avoid ruling out an infection due to a negative Interferon-gamma result (Kim et al., 2018).

Another potential problem is that Interferon- γ tests tend to stay positive after therapy. Therefore, false- positive results can appear when having been treated with anti-tuberculosis drugs after an infection and while there is no current infection. This can be seen in the study by Dyrhol-Riise et al. still showing positive Interferon- γ results in 87. 5% of the cases after three months and 84. 6% of the cases after fifteen months after the end of anti-tuberculosis treatment. In addition, false positive results can appear after the BCG vaccination (Dyrhol-Riise, 2010). This indicates that the diagnostic value of the Interferon- γ test is drastically limited in these cases and should only be regarded with caution. The test should not be used to determine the effectiveness of anti-tuberculosis therapy.

Nevertheless, consistent with our experiences, also according to Dyrhol-Riise et al. the QFT-TB test results mostly turned positive in cases of patients with recent immigration from tuberculosis endemic countries, as well as after a long duration of exposure to infected patients, (Dyrhol-Riise, 2010). These findings, as well as the high sensitivity

we obtained from our recent study, can justify the implementation of Interferon- γ release assays at tuberculosis screenings, which take place at the German border prior to immigration. These screenings aim to reduce the rapid spread of tuberculosis infections when individuals with open lung tuberculosis infections are not immediately treated.

However, other studies, for example one conducted by Takwoingi et al., propose a higher sensitivity compared to previous studies, with a sensitivity of 67.3% for the Interferon- y release test (QFT-GIT). Nevertheless, this implies that 33 people out of a 100 with active tuberculosis were missed out, when conducting the Interferon- y release assay, which is still not precise enough to rely on the Interferon- y test results in the case of a suspected tuberculosis infection. In addition, the chance of correctly detecting a tuberculosis infection with this test is lower in HIV co-infected patients. Therefore, this test is not good enough to out- rule any tuberculosis infection and should only be used with caution in any clinical setting. Hence a positive Interferon- γ release assay test can help to weigh the odds in favor of a tuberculosis infection over other differential diagnosis if the result is interpreted with awareness of its limited validity (Takwoingi et al., 2016).

Interferon- gamma release assays may have a reduced sensitivity concerning diagnosis of active tuberculosis. This is because the progression of infection to disease might affect immunological test results. IGRAS are based on cellular immune response. For the assays a sample of peripheral blood is taken to measure the IFN- γ production by peripheral blood cells, predominantly a certain subgroup of T-lymphocytes. Nevertheless, only 2- 5% of the lymphocytes of the human body circulate in the blood. Especially the number of effector memory T- cells, which are the source of the early production of IFN- γ are very low in the blood. In the case of an active tuberculosis infection, these memory T- cells are recruited to the infection site. Thus, even less memory cells are present in the peripheral blood in the case of an active tuberculosis (Lange et al., 2009).

These findings show that the Interferon- y release assay can be used to screen individuals to increase the chance of detecting a tuberculosis infection. Nevertheless, it is not sensitive enough to rule out any tuberculosis infection and results can only be used to supplement more accurate diagnosis methods.

Evaluation of elastography findings

Another aspect we considered in evaluating different methods for the diagnosis of tuberculosis is elastography. Elastography is a minimally invasive technique for the distinction between benign and malign tissue, by evaluating its stiffness. Malign tissue is stiffer than benign tissue. We tried to evaluate the informative value of elastography in the diagnosis of benign differential diagnosis, including tuberculosis.

Overall, we obtained elastography findings from 23 patients that showed a bacteriologically proven tuberculosis from lymph nodes during the recorded period from December 2017 until April 2018. In this study we distinguished between four different types. Type 1 and 2 mostly indicating benign or inflammatory tissue, while Type 3 and 4 indicated stiffer and therefore more malign tissue. In our case the distribution showed no Type 1 cases, 26% of Type 2 cases, 52% of Type 3 cases and 22% of Type 4 cases. The six lymph nodes included in Type 2 of the elastography showed histopathological unspecific lymphatic tissue, whereas 83% of the cases with Type 2 elastography findings indicated a positive cultural result and 50% obtained a positive PCR result. In comparison, regarding the biggest group of 12 lymph nodes with a Type 3 elastography pattern, a high prevalence of positive histopathological findings (75% typical cases) could be found. In addition to cytology, cultural results were positive 92% of the cases and the PCR turned positive 58% of the cases. Type 4 elastography patterns, which could be found in five cases, showed positive cultural results in 80% of the cases and a positive PCR 100% of the cases. This negates the above suspicion that Type 4 of the elastography analysis only correlates with malign tissue, therefore no lymph nodes with tuberculosis infection should show only blue colors. Controversially, we found five cases that indicated Type 4 elastography findings and still showed positive cultural and microbiological results. One case indicated a positive PCR result correlating with a negative cultural result.

In conclusion, this elastography distribution shows that the detection of stiff lymphatic tissue (ELST-Score 3 or 4) does not as previously assumed only correlate with malign tissue, but can also be found in large numbers in tuberculosis cases. Type 3 shows the highest prevalence. Together with Type 4, these elastography patterns regularly show a cytological result including granulomatous lymphadenitis. Type 2 correlates with an unspecific histopathological lymphatic tissue, but controversially shows high

cultural results. These results demonstrate that elastography results cannot be used to differentiate between normal lymphatic tissue and lymphatic tissue modified by a tuberculosis infection. Elastography results do not regularly correlate with microbiological, histopathological, and cultural results.

13. Limitations

This study had some limitations, since it was a retro perspective study, we could not obtain all information regarding HIV status, socioeconomic factors, smoking and comorbidities from the patients included in this study. A more detailed study with inclusion of these factors would give further insight into the problematics of extra-pulmonary tuberculosis and would improve the understanding of sociodemographic factors, as well as age and sex prevalence. To better understand the role of immunodeficiency and HIV in the prevalence of tuberculosis, we would propose case control studies with different groups comparing immunosuppressed and non-immunosuppressed patients with tuberculosis.

Moreover, our study only included 127 patients. However, to achieve more significant results concerning tuberculosis diagnosis, a higher number of patients would be desirable. Nevertheless, it should be mentioned that the low number of patients in our study was since we only included a very specific collective of patients, which was not the case in other studies about tuberculosis regarding the significance of culture and PCR analysis throughout EBUS TBNA for tuberculosis diagnosis.

Furthermore, it is important to mention that a selection bias is present in this study, as only patients with a suspected extra-pulmonary tuberculosis were chosen on whom we conducted an EBUS- TBNA. This leads to the conclusion that only the sensitivities for the different diagnostic measures in combination with an EBUS- TBNA could be assessed and not the sole value of the EBUS- TBNA investigation itself.

When referring to NAAT, it is important to mention that test characteristics vary a lot between studies and results are likely to be highly dependent on the particular assay used. Therefore, comparison between different test results might be difficult. In our study the Gene Xpert PCR was used, which is supposed to have good outcomes considering the diagnosis of tuberculosis (Geake et al., 2015).

When regarding elastography, even less patients were included in our study, leaving us with a patient collective of 23 patients. To better understand the role of elastography patterns in the diagnosis of tuberculosis, it is therefore highly recommended to include more patients in subsequent study designs.

Also concerning elastography findings, one always must bear in mind that elastography evaluation does not have a standardized assessment system and has to be visually evaluated. Therefore, results may depend greatly on the viewer, preventing the possibility of objective evaluation. Therefore, results vary significantly between different viewers. It would be recommended to have elastography patterns evaluated by different viewers, when conducting another study on the use of elastography for differentiation of different benign lesions, such as tuberculosis to increase objectivity.

14. Conclusion

Despite great efforts to control the burden of the disease, extra-pulmonary tuberculosis remains a major health problem globally. To achieve the eradication of tuberculosis, it is important to clearly identify the tuberculosis infection and to improve diagnosis methods for quick elaboration of laboratory confirmation of the disease. Finding incipient or subclinical forms of tuberculosis before the patients are infectious could be one step forward in achieving the goal of eradication.

Our study tries to augment the understanding of the use of EBUS TBNA in the diagnosis of tuberculosis infection of the hilar and mediastinal lymph nodes with the help of analyzing cultural and histopathological patterns. EBUS TBNA is highly valuable in providing material for further histopathological and cultural analyses and provides a safe and cost effective first-line investigation method for intrathoracic lymphatic tuberculosis. Especially when cultural and histopathological results are combined with clinical suspicion, EBUS TBNA shows high diagnostic value. Therefore, in the case of clinical suspicion of a tuberculosis infection it is important to establish the performance of EBUS TBNA assessment for improved diagnosis algorithms. Nevertheless, additional research is needed to enhance our knowledge regarding the optimal performance of this effective procedure.

As we could show that the histopathological findings are not able to rule out tuberculous infection and the finding of granulomatous epithelioid lymphadenitis is not specific, the additional recovery of material for a microbiological assessment is crucial.

Additionally, we tried to explain the correlation of different elastography results correlating with tuberculosis infection and the use of elastography for the differentiation of benign differential diagnosis including tuberculosis. Considering all the cases diagnosed with extra- pulmonary tuberculosis that underwent elastography, no specific elastography type could be associated with tuberculosis and therefore no diagnostic algorithm involving elastography could be established. Nevertheless, it is important to mention that the presentation of hard tissue (colored in blue), should not lead to the exclusion of tuberculosis diagnosis further along the line.

It is important to mention again at this point, the restriction that we have only included confirmed tuberculosis cases and that the sensitivities of the different presented methods (Culture, PCR, and histopathology) may therefore be overestimated in some cases.

15. Key information

The current study demonstrates a high diagnostic value for EBUS-TBNA in intrathoracic tuberculous lymphadenopathy. Adequate histopathological samples could be obtained in 75 cases and positive cultural results could be obtained for 78 patients, concluding that EBUS TBNA is a safe and effective procedure for obtaining tissue samples from mediastinal lymph nodes.

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17. List of figures

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19. Affidavit

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Eidesstattliche Versicherung

Victoria Kauer

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

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