Host genetics in tuberculosis susceptibility

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HOST GENETICS IN TUBERCULOSIS SUSCEPTIBILITY

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1. INTRODUCTION

1.1. Definition of tuberculosis

Tuberculosis (TB) is one of the oldest diseases that affects humans, caused by the bacteria of *Mycobacterium tuberculosis* complex. Most commonly it affects the lungs (pulmonary tuberculosis), but other organs can be also affected (extrapulmonary tuberculosis). Tuberculosis that is caused by strains susceptible to drugs is mostly curable if treatment is started on time and if drugs are correctly chosen. If it is not treated, it may be fatal within 5 years in 50-65% of cases (1).

1.2. Brief history of tuberculosis

It is not exactly clear when tuberculosis first appeared. One study from 2008 reported that tuberculosis infection was present already about 9000 years ago (2).

Around 460 BCE, the father of modern medicine, Hippocrates, recognized and described the disease called phthisis, the Greek term for tuberculosis, as the most widespread disease of the time. He believed that tuberculosis is hereditary in nature.

The greatest epidemics across Europe were occurring during the 18th and 19th centuries. In 1882 Robert Koch isolated the agent causing tuberculosis for the first time and one year later he named it *Mycobacterium tuberculosis*. The disease outcomes were detrimental - about a third of patients died within 1 year of diagnosis, and over half died within 5 years. The 5-year mortality rate among sputum smear-positive cases was 65%. Of the survivors at 5 years, 60% had undergone spontaneous remission, while the remainders were still excreting tubercle bacilli (1). Without treatment and cure, the isolation of the sick people inside the sanatoriums and away from the other population was the main method of preventing the spread of the disease.

Another problem that doctors were facing was a lack of diagnostic tests that would show a presence of *Mycobacterium tuberculosis*. Discovery of x-rays in 1895 by Wilheld Konrady von Rontgen allowed doctors to assess the disease severity, extent and progression of this disease.

Albert Calmette and Camille Guerin discovered the vaccine called BCG (Bacille Calmette- Guerin) in the beginning of the 20th century. This vaccine soon came to be widely

used. Schatz, Bugie and Waksman introduced the Streptomycin in 1944, the first drug and antibiotic that was truly effective against Mycobacterium Tuberculosis. Seven years later, the first mycobacterial drug Isoniazid was introduced and later on also some other (rifampicin). Those drugs were a huge success in the treatment of tuberculosis and the number of sick people drastically declined.

1.3. Morphology and charactersitics of *Mycobacteria*

Mycobacteria are aerobic, rod-shaped non spore-forming bacteria. They do not stain easily, but after being stained, they resist decolourization by acid or alcohol. Because of that they are called "acid-fast" bacilli. For identification of acid- fast bacteria Ziehl- Neelsen staining technique is used (3).

Three main groups of *Mycobacteria* are known: first one is *Mycobacterium tuberculosis* complex that includes *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, and *Mycobacterium canetti*. Majority of tuberculosis in humans is caused by *Mycobacterium tuberculosis*. Other organisms may also cause tuberculosis, but it is less frequent. The second group is *Mycobacterium leprae*, causing leprosy. The final, third group are non-tuberculous mycobacteria which includes all the other mycobacteria that can cause clinical manifestations similar to those of tuberculosis. Most non-tuberculous mycobacteria are not spread by airborne means from person to person and do not cause clinically significant diseases in immunocompetent people (4).

1.4. Epidemiology

As much as 1.4 million tuberculosis deaths were recorded in 2015, and an additional 0.4 million deaths resulting from TB disease among people living with HIV (By the International Classification of Diseases system it is specified that when a HIV-positive person dies from TB disease, the underlying cause is classified as HIV and not TB) (5).

Alongside HIV, tuberculosis is the second main cause of death from an infectious disease and one of the major public health threats. Most of the deaths (95%) occur in the developing, low-income countries. Every year over 100,000 children die due to tuberculosis

in these countries (4). According to the World Health Organization, most cases are estimated to be in Asia and Africa, with the highest incidence in India (6).

1.5. Transmission

As already mentioned above, the spread of *Mycobacterium tuberculosis* occurs predominantly through the air. Humans are the only reservoir for these bacteria and when an infected person coughs, sneezes, or speaks near another person, they inhale the droplet nuclei and person-to-person spread occurs. There may be as many as 3000 infectious nuclei per cough (1). Those infected nuclei can remain in the air for some time. It is believed that people that were in the close contact with the infected individual have higher chances of being infected. On the other hand, it is still not completely understood why some people infected with *Mycobacterium tuberculosis* never develop the disease, but some do.

1.6. Pathogenesis

Primary tuberculosis is the first infection with *Mycobacteria tuberculosis*. Once the person inhales the organism, it gains entry to the lungs, more specifically to alveoli. Host's innate responses react immediately and activate the alveolar macrophages to ingest and destroy the bacilli. The bacteria then are trapped in macrophages, which do not manage to effect successful counteraction, and in that case, the bacteria continue to survive in the macrophages and starts to multiply and grow. If the growth and replication of bacilli are successful, macrophage will eventually rupture and bacilli will be released. Other phagocytes are then activated to control the infection by ingesting ruptured phagocytes and their spread content. They become infected themselves, and in this way, the infection is expanding through the lungs. This process induces chronic inflammatory response and formation of granulomas that contain clusters of macrophages, epithelioid cells, giant cells and lymphocytes. Over some time, the centres of granulomas become necrotic, resembling soft cheese- a phenomenon called caseous necrosis. Caseating granulomas are the hallmark lesions of tuberculosis and are rarely seen in other diseases. Other features of tuberculosis infection are also fever, night sweats and weight loss caused by production and activation of different cytokines, including interleukin 1 and tumour necrosis factor (7).

After the initial contact with the infection, only a fraction of people develops an active disease. In the majority of people, the immune system manages to control the infection and the patient develops cell-mediated immune memory to the bacteria. This is termed the latent tuberculosis (8).

1.7. Clinical manifestations

Tuberculosis can be classified as pulmonary, extrapulmonary or combined. Pulmonary tuberculosis can be further divided into primary disease or post primary disease.

1.7.1 Pulmonary tuberculosis

1.7.1.1 Primary disease

After the inhalation of *M. tuberculosis* for the first time, primary disease develops. It commonly occurs in infants, children, and immunosuppressed adults. Some people remain asymptomatic after the primary exposure to tubercle bacilli and in some people flu-like symptoms develop- usually cough, fever and chest pain. Characteristic lesions called *Ghon focus* also develop at this time of first exposure and usually heal spontaneously. A *Ghon lesion* together with hilar adenopathy is called a *Ranke complex* (9). With correct treatment, the disease is under the control and there is no symptoms or signs of the disease. The problem is that it is not possible to eliminate all the bacilli from the body and they can remain silently in the body for years, or even decades. For as long as the person is immunocompetent, there will be no clinical signs of the disease. In the case of the disease that suppresses the cell-mediated immunity, *M. tuberculosis* may reactivate and grow, divide and cause post primary, or so-called secondary symptomatic disease.

In immunocompromised people, young children or old patients with other comorbidities those lesions may persist and the disease will rapidly progress to clinical illness. The initial lesion increases in size, pleural effusion develops due to penetration of bacilli into the pleural space and in the most severe cases, the central portion undergoes necrosis, and cavitation develops. Sometimes the bacilli enter the lymphatic system and cause hilar or paratracheal lymphadenopathy. Those enlarged lymph nodes may compress bronchi and cause partial obstruction of airways (1).

Hematogenous dissemination can follow primary infection. In some individuals,

immune response fails or is not robust enough to fight against the infection and miliary tuberculosis develops.

1.7.1.2. Posts primary disease

Post primary disease can be also called secondary, adult-type or reactivation disease. It is actually the reactivation of the tuberculosis after the primary disease and does not occur in all patients. It is caused by bacilli that survived in the primary lesions. They favour well-oxygenated places to grow, so usually the most affected are upper lobes of the lungs (mostly apex), where the oxygen content is the highest. At the beginning, secondary infection is mainly asymptomatic and if it is not treated on the time, the disease will silently progress and worsen. Post primary disease is characterized by chronic tissue lesions, cavity formation and satellite lesions. Cavities contain high number of organisms- between 10^9 and 10^{10} organisms and people with cavitary disease individuals are highly infectious (9).

Symptoms that develop during this stage of the disease are weight loss, fever, night sweats and cough. At the beginning, they might be non-specific. Cough may be non-productive or productive, containing the mucous, pus or blood. It may present also with the massive haemoptysis. In this case, patient has most probably far-advanced lesions. In some cases finger clubbing may develop (10).

1.7.2. Extrapulmonary tuberculosis

There are many different presentations of extrapulmonary tuberculosis, but the most common is lymph node tuberculosis. Other types are pleural tuberculosis, skeletal tuberculosis, genitourinary tuberculosis, gastrointestinal tuberculosis, pericardial tuberculosis, central nervous system tuberculosis, HIV associated tuberculosis and miliary tuberculosis.

1.7.2.1. Lymph node tuberculosis

Lymph node tuberculosis or so-called tuberculous lymphadenitis is most common presentation of extrapulmonary tuberculosis in both HIV- negative and HIV- positive patients. It accounts for 35% of all cases worldwide and is especially common in HIV-infected patients (1). In the past, the main pathogen causing tuberculous lymphadenitis was *M. bovis*, but recently it is mainly caused by *M. tuberculosis*. Patients usually present with enlarged lymph nodes, usually in cervical regions (in the past cervical lymphadenitis was called *scrofula)*, even though other regions can be also affected. Diagnosis is usually confirmed by lymph node biopsy and microbiological examination (10).

1.7.2.2. Miliary tuberculosis

Miliary tuberculosis or so-called disseminated tuberculosis occurs because of hematogenous spread of tubercle bacilli. Genevan physician and writer John Jacobus Manget was the first person using the term miliary tuberculosis. In 1700s he found many small white nodules in the lungs of the patients with disseminated tuberculosis that resemble millet seeds (11).

Miliary tuberculosis can develop from a progressive form of primary disease or can be a product of reactivating disease. In some individuals, the immune system is not strong enough and fails to protect the body from infection (10). People with the great risk of getting miliary tuberculosis are individuals with the weakened immune system or very young or old people. The risk of dissemination is determined mainly by mycobacterial virulence factors and host immune defences. Furthermore, studies showed that 30% to 80% of patients with miliary tuberculosis had some underlying medical conditions as concurrent childhood infections (measles, tonsillitis), malnutrition, HIV/AIDS, gastrectomy, alcohol abuse, malignancy, corticosteroids or other iatrogenic immunosuppression, connective tissue disorders (with or without iatrogenic immunosuppression), end-stage renal disease, diabetes mellitus, solid organ or bone marrow transplantation, silicosis and pregnancy (11).

Clinical presentation is non-specific. Patients usually present with night sweats, weight loss, nausea, fatigue, low-grade fever, abdominal pain and cough. During the physical examination of the abdomen, usually nothing is obtained, but sometimes hepatomegaly, splenomegaly as well as lymphadenopathy can be found. During the eye examination, the choroidal tubercles can be found. They are characteristic for miliary tuberculosis in up to 30% of cases (1). In two thirds of patients, multiple small white nodules (0.5 to 1 mm in diameter) are seen on the chest radiograph. If it is negative and there is no nodules seen, diagnosis of tuberculosis should not be excluded- especially in old and immunocompromised (HIV-positive) people (9). The biggest problem with military tuberculosis is to diagnose it on time. There are no specific markers for disseminated tuberculosis and findings are as already mentioned- nonspecific. If it is not diagnosed and treated on the time, it can be rapidly fatal.

1.7.2.3. HIV associated tuberculosis

Most powerful known risk factor predisposing people to tuberculosis infection is human immunodeficiency virus (HIV) (12). Tuberculosis is responsible for 24% of all HIV-

related mortality, making it the most common cause of death among people with HIV infection (1).

HIV positive patients are at an increased risk for developing tuberculosis disease from the recent infection. HIV also speeds up the reactivation of latent infection and accelerates the progression. On the other hand, tuberculosis also expedites the progression of the HIV infection. It is recommended for all HIV positive patients to be screened for both- active and latent tuberculosis. HIV infected patients with tuberculosis usually present with minimal symptoms or even without them. It is also common for those patients to present with extrapulmonary tuberculosis, which makes establishing the diagnosis even more difficult (13).

1.7.3 Latent tuberculosis

People being infected with *Mycobacteria tuberculosis* may develop cell mediated immune memory to this bacterium. People with latent tuberculosis are neither contagious, neither symptomatic (8). It is believed that one third of the whole population has latent tuberculosis (10). Unfortunately, it is not possible to predict who will develop an active (contagious and symptomatic) tuberculosis and who will remain with latency. Latent diagnosis can be diagnosed with the tuberculin skin test and chemoprophylaxis can be given to these individuals in the form of isoniazid, administered for six to nine months (14).

1.8. Diagnosis of tuberculosis

The diagnosis of tuberculosis begins with the clinical suspicion. Medical history should also be obtained. On the x-ray, upper lobe cavitation may be seen. Doctor should also check for general symptoms of tuberculosis, as enlarged lymph nodes, fever, nausea, fatigue, anorexia with weight loss, hemoptysis and cough that may be productive or nonproductive. None of those symptoms is specific, so other conditions such as malignancy should be excluded. In patients with weakened immune system, finding on the chest radiograph can be non-specific, for example lower lobe cavitations can be found. Computerized tomography is also an important tool in the detection of tuberculosis. It shows the extent and the progression of the disease and involvement of the lymph nodes and organs.

Specimens that can be taken for establishing the diagnosis of tuberculosis can be from different sites. Most common they consist of fresh sputum, gastric washings, urine, pleural fluid, cerebrospinal fluid, joint fluid, biopsy material or blood. All the specimens that are not sterile need to be liquefied with *N*-acetyl-L-cysteine–NaOH digestion-decontamination method with which other bacteria are being killed. This procedure is not needed for sterile fluid specimens that can be directly examined and cultured (3).

1.8.1. Sputum smear microscopy

Examination of the sputum is one of the oldest and one of the most important methods in diagnosis of active tuberculosis. The sputum is examined under the microscope for acid-fast bacilli by staining. Fluorescence microscopy with auramine-rhodamine stain is more sensitive than traditional acid-fast stains, such as Ziehl-Neelsen, and is the preferred method for clinical material (3).

Ziehl-Neelsen is the common stain for light microscopy and even though it is not the best test available on the market, it is still commonly used in practice. It is inexpensive and easily available. The main problem is its sensitivity that requires 5.000 - 10.000 bacilli per milliliter of sputum (15).

Three sputum smears are recommended. Negative smears do not definitely exclude the infection, but if acid-fast organisms are found in the specimen, this is the evidence of mycobacterial infection.

1.8.2. Culture of *Mycobacterium tuberculosis* complex

Culture remains the most accurate and sensitive test for diagnosing tuberculosis. Positive culture is needed for diagnosis, but negative results do not rule it out. The main problem with culturing the *M. tuberculosis* is its slow growth. It growths very slowly, so 4-8 weeks are usually needed to detect the growth (1). Culturing multiple samples has higher sensitivity than culturing only one specimen does. In adults, the first culture detects 85,8% of cases confirmed to have *M. tuberculosis*, the second culture adds a further 11,9% and the third specimen 2.3% with subsequent cultures adding smaller numbers (16).

There are several methods of culturing that can be used: solid, liquid, commercial, or non-commercial media. Most commonly used is egg- based non-commercial Lowenstein–Jensen medium. The main disadvantage of the culturing is that it is time consuming. As mentioned before, tubercle bacilli are not fast growers, it takes a lot of time to see the results,

and during this time, the disease is worsening and progressing.

1.8.3. Drug- susceptibility testing

For all patients, the isolate should be tested for susceptibility to isoniazid and rifampicin to see if there is any drug resistance. Patients that are resistant to any of the standard treatment will not react to those drugs and will need some other medications. Further susceptibility testing for second-line anti-tuberculosis drugs-especially the fluoroquinolones is mandatory (1).

1.8.4. Detection of the DNA

Nucleic acid amplification techniques such as the polymerase chain reaction (PCR) offer the rapid detection of *M. tuberculosis* in clinical specimens. Those tests have high sensitivity (55% to 90%) and specificity (about 99%) and are used mainly for rapid confirmation of tuberculosis (3). The main disadvantage is that those assays are expensive, not easily available and therefore not widely used.

1.8.5. Serological tests

There are many serological tests available on the market, but tests showed, that they have low sensitivity and specificity and are therefore of no clinical value (1).

1.8.6. Tuberculin skin testing for diagnosis of latent M. tuberculosis infection

This test is most commonly used for screening for latent tuberculosis infection. It is also called Mantoux test or PPD test (purified protein derivative test). It is recommended and given only for the patients in the high-risk group, such as recent (<5 years) immigrants from developing countries, healthcare workers, prisoners, alcoholics, chronic prednisone users (>15 mg/day), close contacts of those with tuberculosis and HIV-positive persons (17).

Five tuberculin units (antigens) is injected intradermal –usually in the forearm. Reaction should be read 48 to 72 hours after injection. Only the degree of induration (raised area) should be measured in millimetres for a positive Mantoux test result. Erythema does not count and should not be obtained and measured.

Tuberculin skin test interpretation depends on two factors: person's risk of being affected and measurements of induration in millimetres (18).

If there is no induration, the result is 0 mm and the test is marked as negative. 5 mm

induration size indicates positive latent tuberculosis result for HIV positive patients, chronic prednisone (steroid) users, patients with abnormal x-rays that are consistent with old granulomas and patients that are in close contact with people with tuberculosis (health care workers excluded). 10 mm induration size indicates positive test for HIV positive workers, chronic steroid users and people with abnormal x-ray consistent with old granulomas. 15 mm or more of measured induration size is positive in people with no known risk factor for tuberculosis (17).

False- negative reactions commonly occur in immunosuppressed patients and false-positive reactions can be due to previous BCG vaccination, infection with non - tuberculous mycobacteria or wrong interpretation of reaction (1).

Sometimes if the result is false negative and you suspect so, two-stage PPD can be done. Usually the second test is done one week after the first one. If it is negative, you exclude the diagnosis of latent tuberculosis and if it is reactive (>10 mm) it is true positive. It is important to know that with the repeated testing person cannot become positive, if she was PPD-negative before (19).

Tuberculin test should not be used during the active tuberculosis due to its low sensitivity, specificity and inability to distinguish between the active and latent tuberculosis. The only contraindication is previous severe reaction to tuberculin skin test.

1.8.7. Interferon - gamma release assay (IGRA)

IGRA measures T cell release of IFN-y in response to stimulation with specific tuberculosis antigen. Two highly specific antigens are available on the market: ESAT-6 and CFP-IO (1). IGRA is not able to distinguish between the active and latent tuberculosis and should be used to screen asymptomatic patients with the risk of tuberculosis infection. Specificity and price are higher compare with the tuberculin skin test, but the sensitivity is similar. There is no cross-reaction with BCG vaccination and the previous vaccination with BCG vaccine will not make IGRA positive (17).

1.9. Prevention

Prevention is the key in stopping the transmission of tuberculosis. The best way to stop the transmission is an early detection and treatment of active tuberculosis. During the active state of the disease, the person is highly contagious and with the proper and effective treatment, the transmission can be significantly reduced. Another goal is prevention of active disease in the people with known latent tuberculosis and prevention of infection in high-risk group people. The vaccination with the only vaccine currently available – BCG vaccine is another important strategy.

1.9.1. BCG vaccine

BCG (Bacille Calmette-Guérin) is the only licensed vaccine for tuberculosis. It is a live attenuated vaccine derived from *M. bovis.* In babies it reduces the risk of disseminated and central nervous system tuberculosis, but its efficiency in adults is very variable (8).

In most high-income countries, where the incidence of the tuberculosis is low, only newborns being in the high - risk group for getting tuberculosis are vaccinated. In the countries where the incidence of the tuberculosis is still high (mostly low-income countries) the vaccination with the BCG vaccine at birth is still routinely recommended. It is contraindicated to vaccinate HIV positive children (20).

BCG vaccine is relatively safe and serious complications are very rare. Three weeks after the vaccination erythemous macula appears and slowly transforms into the scar during following three months. Most common side effects are ulceration at the vaccination site and regional lymphadenitis. Those are localized and usually self - limited complications. Most serious, but luckily very rare complication is disseminated infection that occurs in ten cases per ten million doses administered. Usually it occurs in immunocompromised people and is commonly fatal (1, 21).

1.10. Treatment

1.10.1. Treatment of drug susceptible tuberculosis

As mentioned above, mycobacteria grow very slowly and they are resistant to most antibiotics. It is also well known, that those species have an ability to develop new forms of

resistance. They can be even completely resistant to many drugs. The wall of *Mycobacteria* is rich in lipids and functions as a permeability barrier and is therefore hardly permeable or even impermeable to many agents. (22). Mycobacterial species are also intracellular pathogens and therefore inaccessible for drugs that penetrate these cells poorly. Because of all those reasons long treatment with different kind of drugs is required to overcome the infection (22). Goals of the treatment are elimination of the symptoms, prevention of relapses and transmission and better quality of life. Treatment for all forms of drug susceptible tuberculosis is divided into two crucial phases: initial or bactericidal and continuation or sterilizing phase. With this regimen, more than 90% of people infected with tuberculosis can be cured (1).

During the initial phase the patients receives a standard combination of four major drugs that are considered first- line agents for the treatment of tuberculosis: rifampicin, isoniazid, pyrazinamide and ethambutol. Treatment with only one drug can lead to bacterial resistance, requiring four drugs use. Those drugs together should be taken for eight weeks. Daily dosages for person with 50 kilograms or more are rifampicin 600mg, isoniazid 300mg, pyrazinamide 2g and ethambutol 15mg/kg (23).

Isoniazid has the bactericidal activity and it inhibits synthesis of mycolic acids, one of the essential component of mycobacterial cell wall. It is the most active drug from the treatment scheme. It is toxic to liver and it is its most common side effect. During the treatment with isoniazid, liver enzymes should be checked and followed-up. If only minor increase of liver enzyme occurs, treatment can be continued, but in the case of potentially lethal isoniazid-induced hepatitis, treatment should be immediately stopped. The main symptoms of isoniazid- induced hepatitis are jaundice, loss of appetite, vomiting and pain in the right upper quadrant. Another side effect of the isoniazid can be peripheral neuropathy. To prevent it, pyridoxine should be given with isoniazid (22).

Rifampicin also has bactericidal activity and it inhibits the RNA synthesis. Probably the most shocking side effect of rifampicin for patients is that it stains body fluids into the orange colour. Other side effects can be rash, nephritis, thrombocytopenia, cholestasis and flu-like syndrome. (22).

Ethambutol obstructs the formation of the cell wall and therefore has bacteriostatic activity against susceptible mycobacteria. Its most common adverse effect is optic retrobulbar neuritis (8).

Pyrazinamide is another drug with bacteriostatic activity used for treatment of drug-susceptible tuberculosis. Unfortunately, its mechanism of action is not completely understood. It is believed that under acidic conditions pyrazinamide is converted into the active pyrazinoic acid that disturbs transport functions of the cell membrane. Its most dangerous side effect is hepatotoxicity, but it can also cause hyperuricemia, acute gout and nausea. (22).

Since all of those drugs in some extent cause liver toxicity, liver enzymes should be carefully monitored. If hepatotoxicity occurs and those drugs cannot be tolerated well, changing the treatment regimen should be considered. Fluoroquinolones are excreted via the kidneys therefore causing less damage to the liver. During initial phase the goal is to kill the majority of bacilli, prevent further transmission of the disease and prevent the emergence of drug resistance (1,10).

During the second, continuation phase patient should stop taking pyrazinamide and ethambutol, but continue with isoniazid and rifampicin at the same doses for another four months. These two drugs will eliminate the persisting *Mycobacteria*. It is important to tell the patient that disappearing of symptoms does not mean that the disease is gone and that he or she should not stop taking medications when symptoms are gone and person feels better. If the treatment is not completed it means that there are still alive bacteria in the body and relapse will occur- commonly with drug-resistant disease (22, 23).

1.10.2. Treatment of drug resistant tuberculosis

Drug-resistant tuberculosis can be primary or acquired. When patient is infected from the start with the stain that is drug resistant, we are talking about primary drug resistance. Acquired resistance on the other hand usually develops because of inappropriate treatment regimen - monotherapy (1). The incidence of drug resistant tuberculosis is increasing and it is estimated that around 8% cases of Mycobacteria tuberculosis nowadays are resistant to isoniazid (10). By WHO definition, multi –drug resistant tuberculosis is caused by bacteria that do not respond to, at least, isoniazid and rifampicin, the two most powerful anti- TB medicines. Those types of tuberculosis that are resistant to either isoniazid or rifampicin or both of them, require different kind of treatment than susceptible strains of tuberculosis. They are treated with second line treatment regimens. When tuberculosis is additionally resistant even to fluoroquinolone and second line injectable drugs, we are talking about extensively resistant multi – drug tuberculosis (24).

Drug resistant tuberculosis should be treated with at least four drugs to which organism is likely to be susceptible. Drugs are chosen from the five groups based on efficacy, safety and cost. First group of drugs are first- line drugs in the treatment of tuberculosis: isoniazid, pyrazinamide, and ethambutol. If the stain is susceptible to any of those drugs, that drug should not be stopped. High dose should be given and drugs should be taken orally. Second groups are fluoroquinolones and if possible, levofloxacin or moxifloxacin should be used. In the third group are the injectable drugs, which should be used in the following order: capreomycin, kanamycin, and then amikacin. If susceptibility is suspected, at least one of the drug from this group should be always used. In the group four there are second-line drugs and should be used in the following order: thioamides, cycloserine and then aminosalicylic acid. In the last, fifth group there are agents which role is not well understood in the treatment of tuberculosis: clofazimine, amoxicillin with clavulanate, linezolid, carbapenems, thioacetazone, and then clarithromycin (25).

WHO does not recommend using the drugs from the fifth group routinely. Those drugs should be used only when adequate regimen with the medications from the group one to four cannot be made. Usually they are used in extensively resistant multi – drug tuberculosis.

1.11 Genetics of respiratory infections

It is known that individual responses to different respiratory diseases vary and depend on the host genetics profile. A lot of research studies about the genetics of respiratory infections have been done and most of them show unclear results.

In 2015, a systematic review and meta- analysis of 386 studies, out of 24,823 studies from for bibliographic databases was made (26). This extensive study was looking for host genetic factors in different infections- tuberculosis, influenza, respiratory syncytial virus, SARS-*Coronavirus* and pneumonia. Majority of the points extracted from this research were connected to tuberculosis. In total, they found one single-nucleotide polymorphism from *IL4* gene for all respiratory infections, and role of *TLR2* gene and *CCL2* gene in tuberculosis (26).

The study of Patarčić et al. also identified other previous studies that suggested various genetic backgrounds in the definition of the tuberculosis risk. However these studies mainly failed in numerous aspects. Most commonly these studies were underpowered to

detect a true effect, they had problems in diagnosis and case definition, but even data analysis and reporting, meaning that the entire field of research had suffered from substantial methodological problems (26).

Based on the inconsistent findings of previous studies and the fact that the exact correlation between the genetic background and tuberculosis is still not proven, future investigation is needed in order to show a possible connection.

2. OBJECTIVES

The main objective of this study was to investigate the genetic factors and their ability to be used as the predictor for development of tuberculosis.

3. MATERIALS AND METHODS

This study was based on the data gathered on the sample of the 10,001 Dalmatians biobank (Figure 1). The main objective of this program is to create a comprehensive resource for the study of genetic, environmental and social determinants of health and diseases with emphasis on chronic diseases, which are the leading cause of death in Croatia and other developed countries (27). The main approach of this method is the analysis of the association of a large number of genetic markers with the observed trait (28).

The postal invitations were sent to all adult island inhabitants (aged 18 or more years), inviting them to participate in health examinations free of charge, during which time they would also participate in the research study that aimed to collect relevant information on the island inhabitants and develop a biobank. The efforts to include additional subjects were supplemented by the appearance in the local newspapers, radio stations and through contact with the local physicians, who were also encouraged to promote the idea of participation in their registered subjects. The target sample size was about 1,000 subjects, and this target sample size was reached after only five weeks of the field work.

All of the subjects were firstly informed about the study, its goals, methodology, content and risks, and were provided an opportunity to decide if they would like to participate. After decision, they were then read the informed consent, to which they could have responded positively or they could have also rejected it (a total of two people have rejected participation after all the relevant information was relayed on to them). The approval for this study was issued by the Ethical board of the Medical School of Zagreb and the Multi Ethics Research Committee from Scotland.

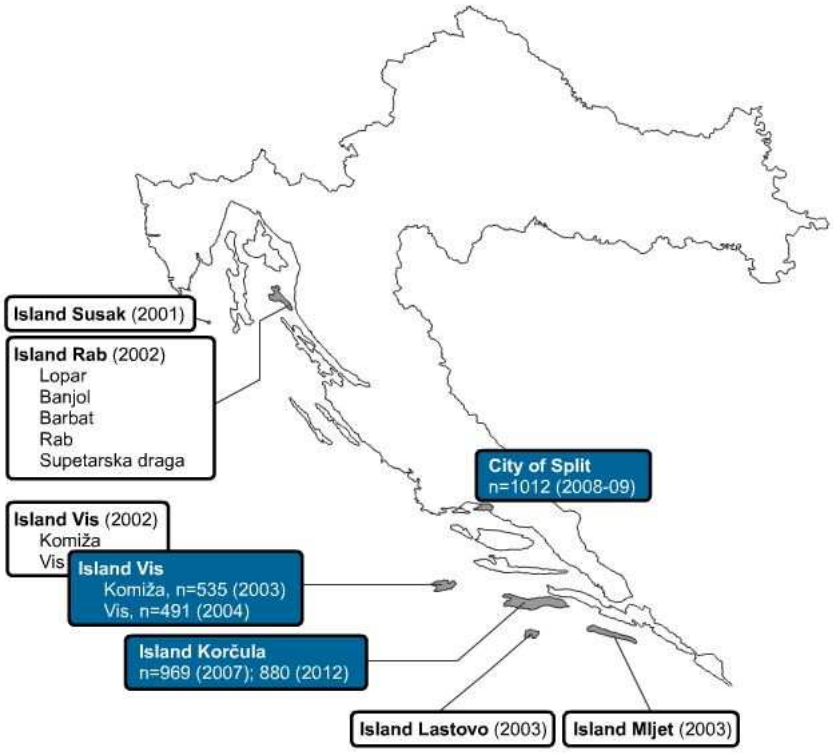

Figure 1. The map of the 10,001 Dalmatians project sites

For purpose of this study, we took the data from the Vis island sub-sample, where we had encountered the most cases of tuberculosis, therefore aiming to maximize the chances of the detection and reducing the extent of possible between-island diversity of genetic background. We used the medical records as the source of information on tuberculosis (TB). Any hospital admission was therefore considered as a positive TB diagnosis, while subjects who did not have TB-related hospital admission were considered as controls.

For purposes of the DNA extraction, we used a peripheral blood sample, with DNA extraction process completed by the Tepnel Nucleon kit (UK). After extraction, Helmholtz Centre for genotyping was used to perform genotyping, with Illumina HumanHap 300 platform, with 317,058 genetic markers in the analysis. We then used genome-wide association analysis to discover candidate genes implied in the tuberculosis diagnosis. The target variable was used in the binary form (positive TB in medical history or control), with additional adjustment for age and sex effects, as well as the familial relatedness (due to extensive pedigree structure in the investigated population). All analysis were performed using R package (http://www.r-project.org), with significance set at $P<4*10-8$ due to multiple testing.

4. RESULTS

We analysed the data for 924 subjects, with 536 men (58.0%) and overall median age of 57 years (range 18-93 years). Median age for men was 56 (18-87), while for women median age was 52 (18-93), without significant difference (P=0.429).We have recorded median years of schooling of 11.0 (with a range of 1-20), and assessed the median socioeconomic status as 9.0 (range 0-16). We have recorded 28 tuberculosis cases (3.0%), most commonly being diagnosed during childhood of the elderly subjects. We had more detailed information for 22 cases, with the average age of acquiring TB being 25.0±9.9 years. In addition, seven cases had another infectious disease record in their hospital admission medical history (25.0%), most commonly pneumonia or severe sinusitis cases. This suggest a significant overrepresentation of the coinfection under the assumption that such coinfection rates are very rare ($P<0.001$ for 2% expected coinfection rates), rare ($P=0.008$ for 10% coinfection rates) and holds up significance all the way up to coinfection rate of 12.6% ($P=0.048$). Co-occurrence of TB and asthma or severe allergy also showed an interesting pattern, with four recorded cases (14.3%).

The genome-wide association analysis had suggested three significant results (Table 1), out of which there was one gene involved in the TB prediction. This was a CECR6 gene, located at chromosome 22 (Table 1). Other than this results that had reached formal significance level, there were three more genes that were having suggestive P values – DSCAM, FOXK2 and KLF8 (Table 1, Figure 2).

Table 1. Genome-wide association analysis results

rs	Chromosome	Effect allele	Minor allele frequency	N	BETA	P	Gene
rs10515081	5	A	3%	917	0.09	4.96E-10	*NA*
rs3924359	16	A	6%	914	0.07	3.44E-09	*LOC105371275*
rs971768	22	A	7%	917	0.06	1.38E-08	CECR6
rs5953425	X	G	19%	902	0.03	2.94E-07	*NA*
rs17687319	6	G	9%	917	0.05	8.48E-07	*NA*
rs2911730	8	G	9%	917	0.04	2.19E-06	*NA*
rs6504989	17	G	13%	917	0.04	2.37E-06	*NA*
rs10057220	5	A	10%	917	0.04	2.46E-06	*NA*
rs11909247	21	G	5%	917	0.06	2.84E-06	DSCAM
rs3794716	17	A	6%	917	0.05	5.45E-06	FOXK2
rs16999738	21	A	3%	916	0.07	8.97E-06	DSCAM
rs4982925	14	A	5%	917	0.05	9.16E-06	*LOC101927045*
rs1869288	2	G	12%	917	0.03	1.04E-05	*NA*
rs13011060	2	A	22%	917	0.03	1.14E-05	*LOC101928278*
rs9320845	6	A	9%	911	0.04	1.21E-05	*LOC105377979*
rs3922927	X	A	6%	917	0.04	1.25E-05	KLF8
rs16940235	15	C	4%	917	0.06	1.38E-05	*LOC105370956*
rs1034442	6	A	9%	917	0.04	1.47E-05	*LOC105377978*
rs1919870	6	A	9%	915	0.04	1.50E-05	*LOC105377979*

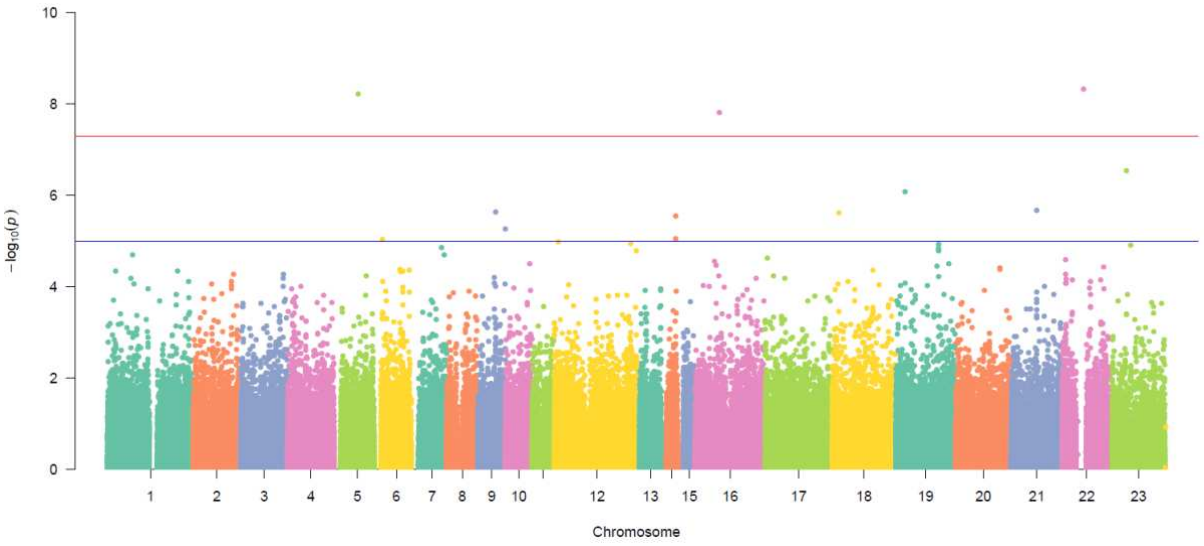

Figure 2. Manhattan plot of the TB GWAS in the island of Vis sample from the 10,001 Dalmatians project

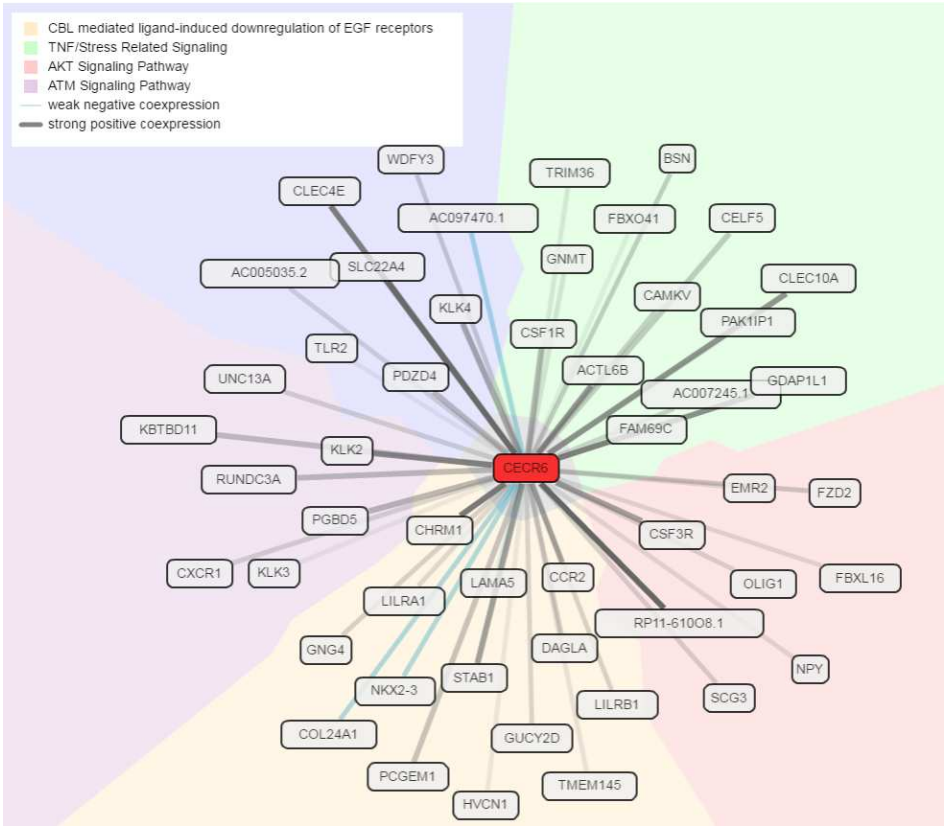

Figure 3. BioCarta pathway involving CECR6 gene

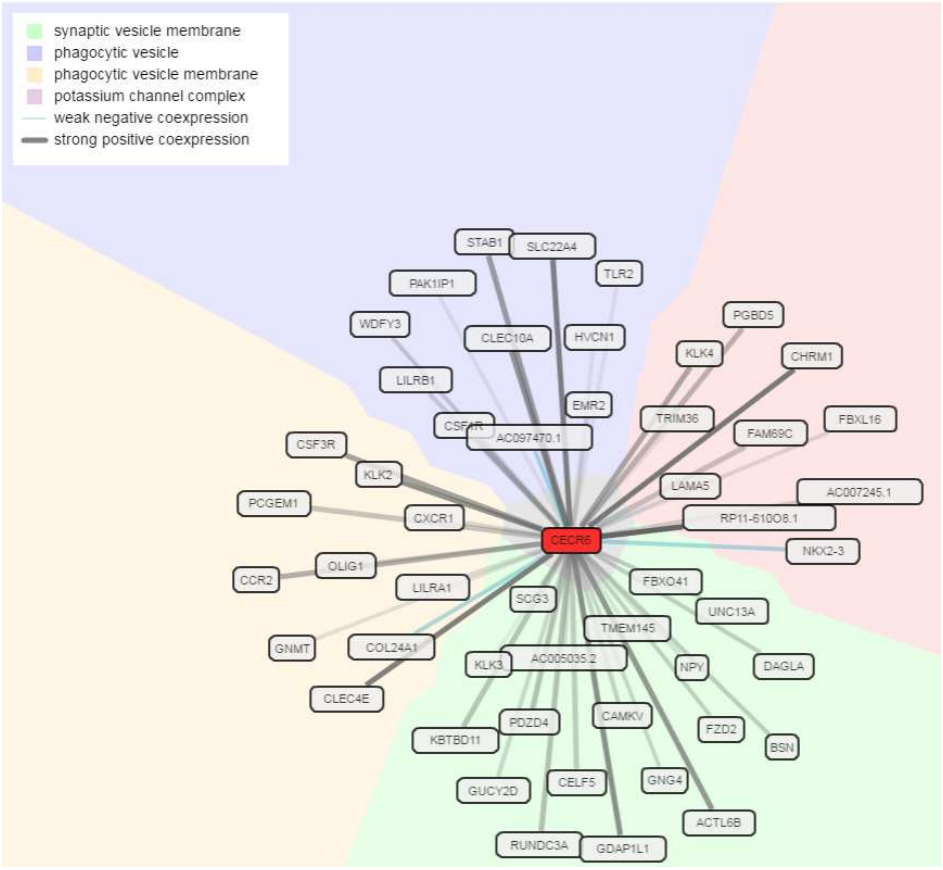

Figure 4. Gene ontology network of CECR6 gene

5. DISCUSSION

The results of this study are showing a significant relationship of the CECR6 gene with an individual tuberculosis risk. This is the first mention of the relationship of this gene with TB in research papers, although some previous studies have implied a role of this gene in an individual infection response (29).

In addition, GWASdb disease associations option suggests that this gene is likely implied in human immunodeficiency virus infection disease (risk score of 0.746), viral infections (0.228), other infectious agent disease (0.145) or immune system disease (0.055) (30), providing further support to the hypothesis that this is a causal gene for tuberculosis pathogenesis.

The development of the gene network suggests that this gene is linked to TNF/stress related signalling, AKT signalling pathway implied in survival and response to extracellular signals and ATM signalling pathway involved in major events in the cell (Figure 3). All these pathways point towards an important role of this gene in the cellular stress response, including that in CBL mediated downregulation, which has previously been implied in cancer, thus providing a possible explanation for a frequent co-occurrence of TB with cancer (31).

Analysis of the Gene Ontology database indicated a role of this gene in synaptic processes, but also in phagocytosis, further supporting possible role of this gene in TB pathogenesis (Figure 4).

Other functions of the gene include Cat eye syndrome, involved in various extent of defects of the iris of the eye, which usually comes in combination with various other morphologic disturbances and malformations, including anal atresia, preauricular deformations, cardiac defects, kidney problems, short stature and scoliosis (32).

Most notably, there is a single claim that links the CECR6 gene to tuberculosis, as a part of the patent US 20140080732 A1, where CECR6 is listed as one of the genes that were implied in the tuberculosis development (33).

However, this gene is very close to the IL17RA gene, which has previously been implied in tuberculosis pathogenesis in numerous studies (34, 35). Therefore, it might simply be a reflection of the proximity of these two genes, therefore requiring a dedicated sequencing and follow-up study to clearly separate between the functions of these two genes and creating an opportunity to establish a causal association of the gene with tuberculosis risk. All these

sources indicate that the mutation in this gene are associated with the protective effect, possibly by interfering with the mechanisms of the *Mycobacterium* entry into the cell (36).

There were three more suggestive genes found in this study - DSCAM, FOXK2 and KLF8. DSCAM was previously implied in HIV risk determination (37), but also stress response (38). FOXK2 has been described to have an IL2 enhancing/promoting function, further supporting the plausibility of the involvement in the tuberculosis pathogenesis (39).

Lastly, KLF8 was implied as a transcription factor, involved in numerous activities in relation to cell cycle (40). When taken together, these results suggest a complex genetic background underlying tuberculosis pathogenesis. This is further implicated in frequent co-infections with other infectious disease, further pointing towards a common pathway in affected individuals that makes them more susceptible to various infections.

Interestingly, mean age of tuberculosis development and hospital admission in this study was early on in adulthood, as opposed to the expected later life occurrence. This could either be a consequence of elderly people not participating in the study (selection bias), or simply lack of reliable medical records (information bias). There appears no support for the population-specific sub-structuring, which is another mechanism that can affect the results. It arises as the consequence of hidden genetic clusters in a population, but previous studies have shown a high level of population genetics homogeneity on the islands, therefore negating this hypothesis (41).

The limitations of this study include the use of a single population and lack of any kind of replication. In addition, as the study was performed in an island population, these results could be population-specific, especially having in mind a long-lasting co-evolution of TB with humans, and possibly pointing towards a population-specific ways of handling a strong selective pressure, such as TB.

All the above claims clearly point towards a role of the CECR6 gene in tuberculosis pathogenesis, and most likely involving phagocytosis mechanisms. Based on these results and after an un-linked replication, this gene could indeed become one of the biomarkers and elements used to assess and predict an individual tuberculosis prediction.

6. CONCLUSIONS

The results of this study suggest that CECR6 gene may have a role in tuberculosis pathogenesis. The biological function of the gene is in line with this claim, as it has been implied in several processes that might related to tuberculosis. Three additional genes, which did not reach formal statistical significance (DSCAM, FOXK2 and KLF8) also, seem to have roles that might easily be related to tuberculosis pathogenesis. Finally, all four of reported genes are in close proximities to genes of established roles in the immune system function, possibly reflecting their physical linkage.

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8. SUMMARY IN ENGLISH

Host genetics in tuberculosis susceptibility

Objectives: The aim of this study was to investigate the genetic background underlying increased risk of developing tuberculosis.

Methods: A subset of the 10,001 Dalmatians study data were used for purpose of this study. We performed a genome-wide association analysis of the subjects who had a medical record of hospital admission due to tuberculosis. All other subjects who were not admitted to hospital for tuberculosis treatment were considered as controls.

Results: A single significant result (at the formal significance level cut-off value of 3*E-08), belonging to the CECR6 gene was recorded. Additionally, DSCAM, FOXK2 and KLF8 yielded nearly significant result, supporting the previous idea of complex genetic background risk definition.

Discussion: This is the first report of the CECR6 gene in tuberculosis pathogenesis, which is in line with the biological functions previously described for this gene. The additional three genes also seem to have roles that can easily be associated with tuberculosis. The genetic architecture underlying tuberculosis seems to be very complex, and requires larger-scale analyses in order to be able to develop a diagnostic tools and provide individualized prevention measures in the future, by focusing on individuals that have the highest underlying risk for contracting and developing clinically relevant disease.

9. SUMMARY IN CROATIAN

Genetika domaćina u osjetljivosti na tuberkulozu

Cilj: Istražiti genetsku podlogu domaćina kao čimbenika rizika za nastanak tuberkuloze.

Metode: Koristili smo podatke iz projekta 10.001 Dalmatinac, I to populacije otoka Visa, u kojoj smo zabilježili najveći broj slučajeva tuberkuloze. Ispitanici bez podatka o bolničkom liječenju smatrani su kontrolama.

Rezultati: Rezultati su ukazali na ulogu CECR6 gena u nastanku tuberkuloze, što je novi genetski mehanizam koji predviđa individualni rizik nastanka tuberkuloze. Još tri gena su bili sugestivni, potvrđujući složenu situaciju I otežano predviđanje ishoda bolesti.

Zaključak: Iako smo opisali novi genetski mehanizam, rezultati ukazuju na veliki opseg varijabilnosti u određivanju osobnog rizika, koji je potencijalno dodatno kompliciran mogućim lokalnim rezultatima ko-evolucije patogena i domaćina. Okrupnjavanje istraživačkih pokušaja I stvaranje konzorcija će omogućiti detaljniji uvid I osigurati uvjete za razvoj individualiziranog pristupa predviđanju pojave bolesti.

10. CV

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