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THE ROLE OF LABORATORY METHODS IN DIAGNOSIS AND FOLLOW-UP OF PATIENTS WITH TUBERCULOSIS

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Summary
Objective. To conduct a literature review regarding the role of laboratory methods in diagnosis and follow-up of patients with tuberculosis for establishing the need for developing new diagnostic techniques and methods.
Material and methods. A systematic literature review was conducted through which the electronic sources published in PubMed and HINARI collection of health literature, and printed material searched according to the Universal Decimal Classification (UDC) were evaluated. The results were grouped and systematized according to the level of scientific evidence.
Results. Microbiological methods are recommended in all patients with tuberculosis, for diagnosis and follow-up. The most used immune test is the tuberculin skin test. The disorders of the immune are currently evaluated according to the white cell blood count. Immune research could not provide relevant and reproducible biomarkers.
Conclusions. Dynamic approach for the research on immune biomarkers in longitudinal studies, on a large number of patients and multianalyte assays, could provide predictive immune biomarkers to be used for the correction of the anti-TB treatment and intensification of immune modulating treatment.
Keywords: microbiology, immunology, tuberculosis, diagnosis, follow-up

Introduction

Tuberculosis (TB) is one of the priorities of the health system of any state, and prevention and control are national strategic objectives, based on the principles and pillars of the End TB Strategy [1]. The active disease is one of the leading causes of death from a single infectious agent, ranking HIV/AIDS at the top of the list with the 10 most frequent causes. Globally an estimated 10 million (8,9-11 million) people fell ill with TB in 2019, with an estimated 1,2 million deaths among HIV negative and in addition 208.000 death among people living with HIV. Men accounted 56%, women 32%, and children aged less than 15 years – 12% [2]. According to the World Health Organization (WHO) Global report, the Republic of Moldova (RM) is one of the countries in the WHO European Region where TB control is a priority and one of the 30 countries in the world with the highest burden of multidrug-resistant tuberculosis (MDR-TB) [2]. MDR-TB is a form of TB caused by *Mycobacterium tuberculosis* (MBT) resistant to the treatment with at least two first-line anti-TB drugs: isoniazid (HIN) and rifampicin (RIF) established through the conventional cultures on solid Lowenstein-Jensen either liquid MGIT BACTEC [3]. The reports provided by the Moldovan National Agency for Public Health recorded a global incidence of TB (number of new cases and relapses) 43.9/100.000 population (1.762 cases) in 2020; 71.6/100.000 population (2.877 cases) in 2019; 75.1/100.000 population (3.016 cases) in 2018; 83.3/100.000 population (3.352 cases) in 2017 and 88.5/100.000 population (3.569 cases) in 2016 [4, 5]. It was established an important decrease in patient notification between 2019 and 2020, which was mostly due to the SARS-CoV-2 pandemics and subsequently reduced accessibility to specialized healthcare services [6]. One of the major public health problems in the RM is the increasing rate of MDR-TB among newly diagnosed patients, which requires advanced microbiological methods for timely detection [7]. In the WHO Global TB report was estimated that in 2019 in the RM the incidence of MDR-TB was 14/100.000 population, the rate of primary MDR-TB was 30%, and acquired MDR-TB 60% among culture-confirmed patients [2]. In the report, the incidence of MDR-TB among the former Soviet countries was 3-6 times higher than in Western European countries in 2019 [2]. The EDCC report on TB surveillance and monitoring established that one-third (34,5%) of all cases registered in the European Member States in 2019 had a foreign origin, and most of them were born in Eastern Europe [8]. The report indicated that in 2019 – 10 countries were mostly MDR-TB burdened: Bangladesh, China, India, Indonesia, Myanmar, Nigeria, Pakistan, the Philipines, the Russian Federaion and South Africa, but India, China and the Russian Federation accounted for more than 50% of the global number [8]. The actual Moldovan epidemics is defined by a high rate of late diagnosed patients, with long evolution, which constitutes a reservoir of infection and continuously endangers the healthy population [9]. Delayed diagnosis is mostly due to barriers, which encounter the patients for accessing the specialized healthcare institutions

and laboratory facilities for performing microbiological investigations, and low sensibility of conventional methods to detect MBT, as well [10]. The above-mentioned arguments supported the opportunity to carry out a review study with the aim to update and systematize the latest information on the laboratory methods for diagnosis and follow-up of TB patients for identifying the need for developing the new diagnostic methods.

The objectives were:

1. To provide updated information about the microbiological, including molecular genetic methods used for diagnosis of tuberculosis and follow-up of patients during the anti-TB treatment.

2. To present the advances in immunological methods for diagnosis of infection with M. tuberculosis.

3. To update on the latest advances published in the studies about the immune reactivity and immune disorders during active tuberculosis.

4. To establish the need for developing the new diagnostic and follow-up methods.

Material and methods

This paper constituted a systematic literature review. As the material for the research were used the electronic sources published through the PubMed web interface and HINARI collection of health literature. For searching the printed sources the Universal Decimal Classification (UDC) method was used. Bibliographic resources were systematized by subject and content. The results were grouped and systematized according to the relevant criteria and the level of scientific evidence (high, low and very low), and the grading of recommendations (recommended, consider to apply, optional recommendation) [11].

Results

In the RM, the diagnosis of TB is based on several methods: 1) microbiological examination of sputum or other biological products depending on the clinical context, which includes the microscopic examination of the smear stained by the Ziehl-Neelson or fluorochrome method to detect acid-fast bacilli (AFB), examination of the sputum by the XpertMTB/RIF genetic molecular method and cultural methods which detects *Mycobacterium tuberculosis* (MBT), which is a strong recommendation with a high level of evidence; 2) radiological examination of the thoracic cage is recommended by the National Clinical Protocol (NCP) as a compulsory examination and reveals a polymorphic aspect, composed of a complex of proliferative, exudative, necrotic lesions, and fibrous changes; 3) blood count is recommended by the NCP as a compulsory examination of any suspected patient and can show iron deficiency anemia, leukocytosis, lymphocytosis in limited forms of tuberculosis, and lymphopenia in severe forms; systemic inflammatory syndrome assessed by: increased in erythrocytes sedimentation rate, acute phase proteins (C-reactive protein, fibrinogen, ceruloplasmin); 4) biochemical assays are recommended by the NCP as optional examinations, and can demonstrate hepatocytolytic syndrome, nitrogen retention, disturbances of hydro-electrolytic balance and increased oxidative stress indicators; 5) Elisa test for HIV infection screening is mandatory to be done in all patients investigated for TB and monitored according to the recommendations of the NCP [12].

Microbiological methods for diagnosis and follow-up of patients with tuberculosis

According to the national protocol, the detection of suspects for TB will be done mainly through the direct addressing of the symptomatic patient to the primary healthcare service. The family doctor will perform the primary evaluation, then will refer the patient to the microbiological laboratory for investigation with the scope to establish the diagnosis of tuberculosis [13].

The microbiological network in the RM consists of 1 National TB Reference Laboratory of the Institute of Phthisiopneumology (the 3rd level), 2 Regional TB References Laboratories localized in Balti, Vorniceni and Bender (the 2nd level) and 59 microscopic centers (the 1st level) [14]. The sputum collection is the first step to be performed in any suspected with pulmonary TB (PTB) patient. It is realized in any healthcare facility where the symptomatic patient comes. At least two sputum samples will be collected. Microscopic investigation through the Ziehl-Neelsen staining and GeneXpert MTB/Rif assay will be performed only from the first harvested sputum sample [14]. High number of studies showed that the low sensibility (20-40%) of the microscopy at the Ziehl-Neelsen staining was increased by the implementation of the molecular genetic GeneXpert MTB/Rif assay (GeneXpert) [14-17]. The mentioned method increased the sensibility of the microbiological diagnosis by 50-80%, contributing to the detection of MBT DNA and *rpo*B gene responsible for the resistance against rifampicin (RIF) [18]. As a consequence, a new term in actual clinical nomenclature was created, which is rifampicin-resistant TB (RR-TB). The RR-TB is the resistance to RIF detected using the genotypic or phenotypic methods with or without resistance to other first-line anti-TB drugs [19].

In the RM, despite the national implementation of rapid molecular genetic methods, the "gold standard" in the diagnosis of TB remained the culture, which uses both solid (Lowenstein-Jensen, Ogawa) and liquid (Middlebrook 7H) media [14]. The culture is much more sensitive than smear microscopy and allows to test the drug susceptibility of MBT to anti-TB drugs. The sensibility of cultures methods vary from 30% to 50% in different studies [14-20]. While a positive microscopic result requires 5000-10000 AFB/ml of sputum, the culture methods, on solid or liquid media, can detect from 10 to 100 viable MBT/ml of sputum [14]. The main advantage of the liquid media is a shorter delay (3-8 weeks) compared with solid media (12 weeks), by this way contributing to an earlier onset of the treatment according to the drug-susceptibility [20].

There are different types of automatic devices for the culture of MBT on selective liquid media: Bactec TB 460, Bactec MGIT 960, BacT/ Alert3D. Bactec 460 TB is a semi-automatic radiometric detection system that uses a liquid medium with ^{14}C-marked palmitic acid. Mycobacteria

catabolizes palmitic acid and releases $^{14}CO_2$, which is automatically quantified by the system. Radioactive carbon dioxide ($^{14}CO_2$) is measured quantitatively. The rate of $^{14}CO_2$ production is directly proportional to the rate of MBT replication and the positive result can appear in 4-25 days. The Bactec MGIT 960 system is based on the colorimetric principle to automatically detect the fluorescence in tubes with liquid culture medium Middlebrook 7H12. The tubes in the system are read every 60 minutes. Initially, fluorescence is inhibited by O_2 from the culture medium. The growth of MBT decreases the level of O_2 in the tube and stops the fluorescence. The tube is marked positive by a sensor when fluorescence is detected. A positive result for MBT appears in 3-21 days (average 14 days), and for non-tuberculosis mycobacteria in 7 days. The MB/BacT-Alert method uses the liquid medium Middlebrook 7H9, based on the colorimetric principle that monitors the replication of MBT through the CO2 accumulation. The reflectometric device detects the changes of the color from dark green to yellow and reacts when MBT density is 10^6-10^7 colony forming units/ml medium. Besides the detection of MBT growth, the cultures on solid and liquid media, allow performing the drug-susceptibility testing (DST) [21]. In the RM, the drugs to which compulsory is tested the susceptibility are the 1st line anti-TB drugs: isoniazid (HIN), rifampicin (RIF), ethambutol (EMB), and streptomycin (STR) [14]. The standard culture-based test for pyrazinamide (PZA) susceptibility is difficult to perform and an alternative DST method is the detection of the *pnc*A mutations by PCR methods [22]. The obtained results are very important to determine, which anti-TB drugs are effective and should be prescribed. There are currently various methods of DST. The direct method in patients with a positive result to the microscopic examination involves the cultivation on solid or liquid media a control sample and a study sample with anti-TB drugs of a direct sputum inoculum (or other clinical samples) after decontamination. In indirect methods, the DST is performed after isolation of the MBT culture from a clinical specimen and is widely used in RM [14].

The standard methods for determining the resistance against the anti-TB drugs using the Löwenstein-Jensen solid medium are the absolute concentrations method (Meissner) and the proportions method (Canetti). The absolute concentrations method (Meissner) of DST used in Moldovan reference laboratories is the indirect method and is applied to previously isolated strains of MBT [14]. The first step is the inoculation of MBT suspension on a drug-free media and then in media containing graded drug concentrations (INH, RIF, STR). The minimum inhibitory concentration is the lowest concentration of the drug that inhibits growth. The disadvantage of the method is the long duration required to obtain the results (8 weeks). The proportions method (Canetti) determines the increase in percentage (number of colonies) of inoculum on reference media without anti-TB drug compared to an increase on culture media containing the critical concentration (or range of concentrations) of the tested anti-TB drug. The resistance coefficient method (Mitchison) is the assessment of the growth of the patient's strain and the sensitive reference strain, performed in 5 tubes with different concentrations of each anti-TB drug (INH, RIF, STR and ETB). Resistance is defined as the ratio between the minimum inhibitory concentration of the test strain and the reference strain. Using the liquid medium such as Bactec 460 radiometric method allows the testing of sensitivity to the 1st and 2nd line anti-tuberculosis drugs; Bactec MGIT 960 colorimetric method is recommended for testing the susceptibility to the 1st and 2nd line anti-TB drugs and MB BacT/Alert or VersaTrek [14]. According to the results of the DST the patients are diagnosed with mono-resistant TB, poly-resistant tuberculosis, MDR-TB (RR/MDR-TB) or extensively drug-resistant TB (XDR-TB) [3, 12, 23]. Mono-resistant TB is defined as the infection caused by MBT resistant against one 1st line anti-TB drug, excluding the rifampicin. Poly-resistant TB means resistance to two and more 1st line anti-TB drugs with the exception of the combination of INH+RIF. MDR-TB is the infection with strains resistant to at least INH+RIF [20]. International surveys on drug resistance demonstrated that mono-resistant TB and poly-resistant TB are more frequent than MDR-TB, however not in the high burden countries, such as the RM [13]. A national study showed the predominance of mono-resistance against STR in 81% and against INH in 17% of patients. A very low rate of patients (around 2%) was established with resistance against EMB [24].

In Moldovan clinical practice, the standard treatment for drug-susceptible TB is initiated on the basis of the clinical signs and radiological abnormalities compatible with TB [12]. The confirmation of TB by positive culture and results of DST usually obtained in four to eight weeks. The molecular-genetic methods based on polymerase chain reaction (PCR) help to speed up the decision-making process in the diagnosis of TB and the initiation of an adequate treatment according to the results on the resistance to rifampicin [16-20]. Currently, in the international clinical practice, the following PCR methods are used to identify MBT nucleic acids: Amplicor PCR (Roche Molecular Systems), transcription-mediated amplification (MTD amplification method), GenoType Mycobacteria Direct Assay method (Hain Lifescience), displacement chain amplification method (BD Probe Tec), the loop-mediated isothermal amplification method (LAMP; Eiken Chemical Co) [14, 16-22]. The Xpert MTB/RIF method is a molecular-genetic method that detects MBT DNA and mutations in the *rpoB gene* responsible for RIF resistance and was implemented in the RM in 2014 [20, 21]. GeneXpert Cepheid equipment is provided by Sunnyvale LTD and included a machine for inserting several cartridges (4-16), a computer, a soft for reading the results, and a scanner for reading the barcode. Reagent cartridges are used to perform PCR inside the cartridges between the reagents. The results are assessed by the system through the measure of the fluorescence signals. The types of results obtained are: 1) MTB detected - DNA of the MTB detected; DNA of the MTB detected and RIF resistance detected - mutation of the *rpoB* gene responsible for RIF resistance identified; MTB detected and the *rpoB* gene mutation undetermined; 2) MTB undetected - no DNA of the MBT identified; 3)

Invalid - the presence or absence of DNA of the MBT cannot be determined; 4) Error - lack of result. The result depends on the number of MBT, sputum collection methods, handling, storage, and transportation. The positive result does not necessarily indicate the presence of viable MBT. It can be obtained by evaluating patients who were treated and further is eliminating MBT after completion of the treatment. For the listed reasons, evaluation of the patient by the GeneXpert MTB/RIF during the anti-TB treatment and after its completion is not recommended. The test is simple, non-laborious, requires a short time to perform, the result is obtained in 2 hours, and does not require biosecurity conditions. It is used in regional laboratories and primary healthcare facilities, however, the main disadvantage is the cost of maintenance, reagents, and cartridges [14, 18-20].

The PCR techniques are used to establish the presence of gene mutations responsible for resistance to anti-TB drugs. So, the resistance to INH is determined by the mutations in *katG* and *inhA* genes, to RIF in *rpoB* gene, to EMB in *embB* gene, to PZA in *pncA* gene, to STR in *rrs*, *rpsL*, *gidB* genes, to quinolones in *gyrA* gene [22-35]. A high level of evidence proved that the main mechanism for acquiring the drug resistance to the anti-TB drugs consists in chromosomal mutations in genes, under the influence of inadequate therapy with anti-TB drugs, such as patient's incompliance, treatment short duration, monotherapy, or individualized treatment [36, 37].

In the RM the new microbiological methods for diagnosis of TB were implemented gradually. The earliest was implemented MGIT 960 Bactec method in 2005 followed by ProbeTec in 2008. The molecular genetic method MTBDR plus was used in 2009, the MTBDRsl in 2011, MTBDR *plus version 2* in 2012 and the last implemented was GeneXpert MTB/Rif in 2014 [14].

The microbiological methods are essential in clinical and therapeutic follow-up. According to the NCP, which is based on the WHO recommendations, in patients with drug-susceptible TB, sputum smear microscopy and conventional cultures will be used at the end of the 2nd, 3rd, and 5th /end of the treatment in all new cases and at the end each month of the previously treated cases (relapse, recovered after lost to follow-up and failure). The follow-up of patients with MDR-TB will require microbiological investigations at the end of each month in the intensive phase (first 6 months) and at the end of every trimester in the continuation phase (12 to 18 months) [14]. The investigations have to be performed compulsory in specific periods as recommended by the NCP [14]

Immunological methods for diagnosis of infection with Mycobacterium tuberculosis

For the diagnosis of infection with MTB in the RM is widely used the tuberculin skin test (TST) with 2 tuberculin units (TU) of tuberculin PPD-L [12]. The TST is the oldest, cheapest, and simplest test for assessing the body's delayed-type hypersensitivity reaction to mycobacterial antigens. The technique and results of the intradermal test were described in 1908 by Charles Mantoux (1877-1947). The principle of the method consists of the intradermal introduction on the anterior surface of the middle third of the left forearm of 2 TU and the measure after 72 hours the size of the papule appearing at the injection site. The indications for the TST are to investigate: 1) children who were in contact with TB patients; 2) children with clinical signs suspected with TB; 3) children at high risk to get the MBT infection; 4) children before admission into the foster care, ancillary schools, other institutions with risk for MBT infection. The contraindications for TST are not established, but certain conditions (acute infections, chronic diseases during the overheating, convalescence, allergies, and cutaneous rashes) allow rescheduling [12]. Injection of tuberculin in an allergised organism can cause several reactions, classified – local: edema, infiltration, blisters, tissue necrosis; focal – edema, congestion, local bleeding and general – fever, hypotension, and vascular collapse. According to the NCP, the local reaction to TST are differentiated into several results: negative, meaning a red point at the injection site, or hyperemia or presence of papule with the diameter less than 4 mm in BCG unvaccinated and less than 9 mm in BCG vaccinated individuals. The negative reaction is established in people who have not been infected either naturally or artificially through the BCG vaccination. The negative TST (fals negative) does not exclude the infection in the following situations: pre-allergic period – up to 12 weeks after infection, severe forms of TB with suppression of the immune response or neoplasms, lymphogranulomatosis, and other diseases associated with suppression of the immune response [12]. The false negative result occurs in situations of temporary immunosuppression by acute infections (measles, influenza, whooping cough, etc), cachexia, trauma, surgery, menstruation, pregnancy, the period after birth, irradiation, treatment with immunosuppressive drugs or immunomodulators, etc. The positive TST is established by the papule from 5 mm in unvaccinated and from 10 mm in BCG vaccinated till 16 mm in children and 20 mm in adults. The positive result of the TST cannot differentiate latent infection from active TB. Hyperergic result is established by the papule with a diameter of more than 17 mm in children and 21 mm in adults, or bladder-necrotic reaction or lymphangitis and regional lymphadenopathy. According to the moment when the positive result was obtained, there are differentiated the tuberculin conversion and booster effect. Tuberculin conversion, is defined by a series of two TST performed in a calendar year, the first being negative and the second positive. It means that during the last year occurred the primary infection. The booster effect reflects the increase of the size of the TST when performed at a short period of time. Some conditions can determine the false-negative results of the TST. Most of them are related to immunosuppression: fever, cachexia, viral, and bacterial infections, recent vaccination, neoplastic diseases, vulnerable age (newborns and elders), and treatment with immunosuppressive drugs. Rarely technical factors could be involved: expired tuberculin, incorrect dilutions, biochemical denaturation, subcutaneous or intramuscular injection, tamponade, massage of the injection site or reading errors. The false-positive result of the TST is noted and is caused

by infection with non-tuberculous mycobacteria or reading errors [12, 38]. TST cannot differentiate the latent TB infection (LTBI) from active TB, as well the natural infection from the post-vaccination (BCG) infection [39]. LTBI is a condition of the body in which MBT are kept in latent form (dormant) and the person is conventionally healthy. Indicators of LTBI establishing a high level of evidence are the positive/hyperergic result of the TST, either conversion, and the positive result of the *in vitro* interferon-γ release test (IGRAs assay) [39]. To differentiate the natural occurred from iatrogenic infection (BCG vaccination) are evaluated the size of the papule, color, evolution, previous BCG vaccination and the post-BCG vaccination scar, presence of paraspecific reactions, and contact with a TB patient, as well. Children who were in contact with a TB case, and in which the LTBI was confirmed and the active TB was excluded will undergo the chemopreventive treatment with INH 10 mg/kg body weight for 6 months (strong recommendation, low level of evidence), according to the NCP [12].

Low specificity (from 57% to 71%) of TST determined the development of serological *in vitro* methods for diagnosis of the MBT infection, based on the production of interferon-gamma (IFN-γ) released by T-lymphocytes stimulated with mycobacterial antigens, knew as IGRAs assays [38, 39]. The amount of IFN-γ is detected through ELISA method (enzyme-linked immunosorbent assay) or ELISPOT method (enzyme-linked immunospot assay). The Quantiferon-TB Gold Assay uses ESAT-6 and CFP-10 antigens. IGRAs tests are attractive, fast, and simple methods. The sensitivity varies from 1% to 60% and the specificity from 53% to 98.7% and has high-cost implications. WHO recommended before introducing the IGRAs tests to ensure that the products fulfill local or international requirements. As well was recommended to perform further research in different geographical areas before implementing the IGRAs [39].

Immune reactivity and disorders of the immune system in tuberculosis

Mycobacterial infection and active TB in humans are cyclical [40]. The penetration of MTB by air in the lungs of an uninfected person causes the development of a lesion during 3 to 8 weeks, which is called the primary affect (primary focus). The lymphatic vessels are affected and lymphangitis and intrathoracic lymphadenopathy occur. Simultaneously, the organism acquires delayed hypersensitivity (DHS), also known as hypersensitivity type IVth, which causes a rapid and brutal response against a new infection with MBT. The morphological substrate of DHS represents the cell-mediated response in which the effector immune cells are circulating monocytes that migrated into the inoculated tissue and transformed into macrophages, epithelioid cells, giant Langhans cells, and T-lymphocytes (CD4+ and CD8+ phenotype) [41]. The localization in the cockade of the enumerated cells is characteristic of tuberculous granuloma (TBG). The center of TBG consists of caseous necrosis, surrounded by a crown of macrophages, epithelioid cells, giant Langhans cells, and lymphocytes. At the cross-section of TBG, the caseous necrosis is surrounded by a crown of giant Langhans cells, surrounded at the periphery by macrophages, epithelioid cells, lymphocytes, and a crown of the B lymphocytes and neutrophil leukocytes. Caseous necrosis is a necrotic lesion of the tissue with a low concentration of O_2 and develops an unfavorable environment for the growth and multiplication of MBT. After phagocytosis of the MBT, foamy macrophages lose their capacity to phagocyte and release the TNF-ά and other pro-inflammatory cytokines, stimulating tissue necrosis. Due to the hypoxic intracellular environment, MBT internalized in the foamy macrophages, is induced in the dormant state, also called latent form (L-form). Within 5 to 10 years the TBG is completely replaced by connective tissue impregnated with calcium salts and the calcination (post-TB changes) is constituted. Under certain conditions, usually associated with the immunosuppression of the host, the foamy macrophages lose their ability to keep MBT dormant and allow their reactivation, spread into the body, and development of active TB [41]. The clinical and radiological manifestations of the disease closely correlates with the degree of immune disorders [40]. Innate immune response to MTB infection starts with the activation of macrophages through Toll-like receptor 2 (TLR-2) and the release of pro-inflammatory and antimicrobial mediators (TNF-α, IL-1β, IL-6, IL-12, IFN-γ, IL-10, TGF-β, IL-4) [42]. Presentation of MBT antigens by activated macrophages on their surface is realized through the molecules of the histocompatibility classes I and II [40-42]. Infected macrophages and CD8+ lymphocytes are recognized by CD4+ cells and destroyed. The deficiencies of innate response contribute to the development of severe TB, straight linked with clinical symptomatology [40].

TBG allows the organism to preserve the intracellular MTB in the latent form [41]. The deficiencies of the innate immunity and the failure to constitute the TBG determine severe evolution accompanied by high clinical expressiveness and unfavorable treatment outcomes [40]. It was established important differences of the immune reactivity in the infection with different strains of MBT [40]. Some studies established that in MDR-TB the absolute count of CD3+CD4+ and CD3+CD8+ cells is increased and the content of CD19+ is diminished compared with the normal values and drug-susceptible TB [40-42]. Also, some studies on infected animals confirmed the diminished count of CD3+ which predicted the rapid death [43-45]. Published reports showed that CD25-expressing cells among CD4+ lymphocytes are the memory cells and are responsible for polyclonal activation during MBT infection. They are able to inhibit the production of cytokines, especially IFN-γ, and inhibit the activity of other CD4+ cells using the cell-mediated mechanism, which finally leads to deficiency of DHS [45-46]. Regulatory T cells (Tregs) are a specialized subpopulation of T-lymphocytes that suppress the immune response at any stage by activating perforin/granzyme and Fas/Fas ligand-induced apoptosis. They act by inhibiting the T cells proliferation, and cytokine production and play a critical role in maintaining homeostasis and self-tolerance. Some reports showed that in MDR-TB the lymphocyte apoptosis is more activated through the induction of Treg cells [45, 47]. In addition to this, during the infection with drug-resistant strains of MTB,

immune cells react slowly against the pathogen, since no fast activation of T-lymphocytes by phytohemagglutinin (PHA) at the dose of 0.01 mg/ml and tuberculin (PPD) 50, 500 and 5000 TU occurs. In the meanwhile, in drug-susceptible TB, the activation of T-lymphocytes is more evident and is manifested by a prominent proliferative response of cells and high pro-inflammatory cytokine secretion (IL-2 and IFN-γ) [47, 48].

Recent pathogenetic studies noted important differences between the immune disorders according to the clinical forms of TB and anti-TB drug susceptibility. So, pulmonary MDR-TB is characterized by a lower functional activity of lymphocytes assessed at the blast transformation responses (BTR) to mitogens, such as PHA and PPD, lower rate of CD4+, CD8+, CD20+, CD25+ cells, as well as a higher serum concentration of IL-8, compared with drug-susceptible TB, mono-, either polyresistant TB. Also, was accompanied by a higher concentration of anti-TB antibodies, immunoglobulins IgG, IgM, and IgA, and lower functional activity of the neutrophils assessed through the nitro-blue tetrazolium test, increased adenosine deaminase activity, and neopterin serum concentration [48, 49]. It was demonstrated that in MDR-TB TB there is an increased number of apoptotic lymphocytes and decreased T-cells proliferation [49]. Is was presumed that was caused by the reduced antigen-specific Th1 response. As the consequence was observed an increased functional activity of B-lymphocytes and high production of Th2-profile cytokines, especially of IL-1 beta, IL-4 and IL-10, and TNF-α which can induce or potentiate apoptosis of various immune cells (monocytes, B and Th1 cells, dendritic cells), which finally will contribute to immunosuppression and unfavorable clinical evolution of TB [40, 44, 45, 49].

In this context, NCP recommends to perform compulsory complete blood count and to tests the biomarkers for HIV infection [12]. According to the severity of the disorders, immunological investigations can be recommended [51-54]. Low-level evidence provided by immunological research, frequently performed *in vitro*, or involving animal research and a low number of case-series, lack of cohort studies, could not provide immune biomarkers and reproducible immune tests to evaluate the immune state of the patients with TB [52]. Moreover, the immune disturbances showed that are more dependent by the antigens load and tissue damage than on the spectrum of anti-TB resistance [63]. As well, lack of longitudinal studies targeting the evolution of the disease and antigen-specific peripheral immune response could not provide relevant biomarkers to be used in the patient follow-up during the anti-TB treatment [52, 53].

Discussions

Our research was focused on the assessment of the role of laboratory methods for diagnosis and follow-up of patients with tuberculosis in order to establish recommendations for primary healthcare physicians and specialists. The research involved the study of all available open sources - systematic reviews and cohort studies, which provided a high level of evidence and strong recommendations, as well as case-control studies, animal research, and in vitro research, with low level of evidence not providing recommendations.

The Republic of Moldova is a middle-income country, high MDR-TB burden country, and the guidelines for the clinical management of TB, in adult and pediatric patients, are provided by the national clinical protocols, which are based on the standardized internationally recommended approach, regardless of clinical, laboratory, and epidemiological features [2, 12, 14, 55]. The National TB programs are approved every 5 years according to the updated global TB control strategy [56]. The second pillar of the actual Strategy - End TB Strategy is to promote, enhance and intensify research and innovation implementation, especially on low- and middle-income countries, sharing the innovation, development of networks for research and capacity building [1].

The compulsory recommendation, provided by the national clinical guideline in the management of TB patients is to perform microbiological investigations of all suspected patients using conventional methods – smear microscopy and culture on solid and liquid media. Smear microscopy to detect acid-fast bacilli and molecular genetic tests GeneXpert MTB/Rif should be done in every symptomatic patient referred to the primary health care service, either specialized service [12]. This approach is highlighted in the TB manuals and national clinical standards [12, 20, 55]. The evaluation of the resistance against the anti-TB drugs with the standard methods of the Löwenstein-Jensen solid medium or BACTEC medium is crucial for identifying the adequate drugs, with which patients should be treated [14, 20]. MDR-TB is the infection with MBT strains resistant to at least INH+RIF, the most potent 1st line anti-TB drugs and develops as the consequence of chromosomal mutations in genes, under the influence of inadequate therapy, therapeutic incompliance, short duration of treatment, monotherapy, or individualized anti-TB treatment [26, 36, 57]. There were no identified studies showing the role of immune disorders in acquiring the anti-TB drug resistance.

The most used immunological test to establish MBT infection in RM, as well in all high TB burden countries is the Mantoux test (TST test) [12, 53, 54]. The recommendation according to which TST is applied is the evaluation of children who were in contact with TB patients and symptomatic children with suspected signs [12, 55]. However, the results of the TST are estimative and do not allow properly to confirm or exclude the infection [58]. Moreover, a range of factors contributes to the erroneous interpretation, false negative and false positive results [12]. Promising due to a high specificity are IGRAs test, which is based on the synthesis of interferon-gamma (IFN-γ) by T-lymphocytes stimulated with mycobacterial antigens, but is not recommended in high TB burden geographical areas [58].

Immune studies, mostly performed in vitro and a limited number of case-control studies on human subjects could not provide relevant immune biomarkers to diagnose and assess the dynamics of the symptomatic stages of MBT infection, either the follow-up during the anti-TB treatment [59]. For this purpose, there is a need to be performed longitudinal studies, on a large number of patients and multianalyte assays [60].

Conclusions

Microbiological methods are the leading investigations to be done compulsory in all patients suspected with tuberculosis, for diagnosis and follow-up.

Smear microscopy and molecular genetic tests GeneXpert MTB/Rif are strongly recommended to be performed in every symptomatic patient referred to the primary health care and specialized services.

The tuberculin skin test (TST) with 2 tuberculin units (TU) of tuberculin PPD-L is strongly recommended to be performed as an immune test in specific categories of pediatric subpopulation and national implementation of IGRAs need further research.

Currently, the immune state is evaluated according to the white blood cells from the complete blood count, pro-inflammatory biomarkers and the test for HIV.

As recommendation, we propose a dynamic approach for the research on immune biomarkers performed before and during the anti-TB treatment, in longitudinal studies, on a large number of patients and multianalyte assays.

The selected immune biomarker research should provide additional information for the correction of the anti-TB treatment, intensification of immune modulating treatment, and improvement of the treatment outcomes.

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