Residência Pediátrica
Publicação Oficial da Sociedade Brasileira de Pediatria

Submitted on: 03/30/2017
Approved on: 04/25/2017

ORIGINAL ARTICLE

Use of biomarkers in pediatric tuberculosis

Christiane Mello Schmidt[1], Adriano Queiroz[2], Claudete Aparecida Araújo Cardoso[3]

Keywords:
biomarkers, pharmacological, tuberculosis, child.

Abstract

In this article, we review the concept of biomarkers and biosignature and their potential use in pediatric tuberculosis, with application in the development of new vaccines and new laboratory methods that allow a more accurate diagnosis and evaluation of the response to treatment. Emphasis is also given to methods which include the dosage of antibodies, cytokines, transcriptomics, proteomics and metabolomics.

[1] Master's Degree in Child and Adolescent Health - Professor of Pediatrics in the Department of Maternal and Child Health of the School of Medicine of the Fluminense Federal University, Niterói, RJ, Brazil.
[2] Post-Doctorate in Public Health - Researcher at Gonçalo Moniz Research Center/FIOCRUZ - CPQGM/FIOCRUZ, Rio de Janeiro, RJ, Brazil.
[3] Post-doctorate in Public Health - Assistant Professor of Pediatrics in the Department of Maternal and Child Health of the School of Medicine of the Fluminense Federal University, Niterói, RJ, Brazil.

Correspondence to:
Claudete Aparecida Araújo Cardoso.
Universidade Federal Fluminense. Av. Marquês do Paraná, nº 303, Centro. Niterói - RJ. Brazil. CEP: 24033-900. E-mail: claudetecardoso@id.uff.br

INTRODUCTION

Tuberculosis (TB), the etiological agent of which was identified in 1882 as *Mycobacterium tuberculosis*, still represents a public health challenge worldwide[1,2]. The difficulties in controlling this disease are associated with poverty, delayed diagnosis of active TB cases, lack of compliance with treatment, infection by resistant strains, HIV coinfection, inadequate contact control, and the lack of treatment of latent TB infection (LTBI) cases[1].

Within the scope of pediatrics, the diagnosis of pulmonary TB is a challenge in clinical practice, because children may present with nonspecific signs, clinical symptoms, and radiological changes. In this age group, lesions secondary to TB are commonly paucibacillary, bacteriological confirmation is difficult. Children's inability to expectorate only adds to this difficulty. In addition, the risk of illness is greater in the first 2 years of infection, and children aged < 5 years are more likely to develop severe forms of the disease[3].

TB in children can be classified as confirmed TB, unconfirmed TB, and unlikely TB. Confirmed TB refers to cases in which the culture for *M. tuberculosis* and/or the rapid molecular test for bacterial DNA screening are positive. In cases of unconfirmed TB, there is no bacteriological confirmation and patients have at least two of the following findings: symptoms or chest X-ray with findings suggestive of TB, history of contact with TB, evidence of a *M. tuberculosis* infection or lack thereof, and appropriate response to specific treatment. A positive result on a tuberculin test and/or on an interferon-gamma release assay (IGRA) is considered as evidence of TB infection. In cases of unlikely TB, patients do not have bacteriological confirmation, nor do they meet the criteria for unconfirmed TB[4].

This classification, recently proposed by Graham et al.[4], (2015) for the standardization of case classification in pediatric TB studies, demonstrates the difficulty that still exists in defining these cases and how new diagnostic methods may help to confirm cases of TB with negative bacteriology, thus avoiding unnecessary treatment of these patients. However, laboratory control of treatment response in patients with unconfirmed or unlikely TB also has its limitations. Cases of extrapulmonary TB also tend to be paucibacillary[5]. Situations in which there is no bacteriological confirmation are still a challenge in pediatric clinical practice.

Distinguishing between LTBI and active TB may be difficult, and the available laboratory methods, such as the tuberculin test and IGRA, do not allow this distinction[6]. However, distinguishing between these two types of cases is crucial. First, the diagnosis of LTBI, in which the patient is infected and shows no signs or symptoms of pulmonary or extrapulmonary TB, enables the use of only one drug (isoniazid) for 6 months, according to current indications by the Brazilian Ministry of Health[7].

This measure decreases the patient's risk of becoming ill. In addition, the difficulty in distinguishing between the two situations may lead to the wrong treatment of a case of LTBI with three or four drugs, depending on the age of the patient and on the use of monotherapy, with isoniazid for cases of active TB. Both treatments, when administered incorrectly, contribute to the increase in morbidity of the affected population.

The BCG vaccine, which is available in single dose use for children at birth, protects only against disseminated forms of the disease (miliary TB and tuberculous meningoencephalitis), and rates of protection do not exceed 80%[8].

In light of these factors, the use of biomarkers to better understand the biological characteristics of the etiological agent and its interaction with the host may aid in the development of new laboratory methods that contribute to addressing these challenges and to consequently improve the control of the disease.

BIOMARKERS AND BIOSIGNATURES: A DEFINITION

Biomarkers are defined as molecules that measure and express a normal biological process, a pathogenic process, and therapeutic responses to pharmacological intervention[9,10]. Biomarkers may originate from the pathogen or the host and may be detected *in vivo* or *in vitro* by a single measurement in a sample or by a stimulus. When it is characteristic of a disease, the biomarker or biosignature may be used as a diagnostic test[9].

A biosignature is defined as a set of biomarkers.

In a disease as complex as TB, which still presents so many challenges, the measurement of several rather than a single biomarker appears to be more effective in its control[11].

BIOMARKERS IN TUBERCULOSIS: POTENTIAL APPLICATIONS IN PEDIATRIC CLINICAL PRACTICE

The discovery of new biomarkers opens up the perspective of their use in different challenging clinical situations involved in the improved control of TB.

Some characteristics are important for a good biomarker: it must be able to distinguish between healthy individuals and individuals with active TB, to reach normal levels with treatment, and exhibit reproducible clinical outcomes in different populations[10]. Another important aspect of biomarkers is the possibility of their being measured in other clinical specimens, such as blood, urine, and exhaled air[12].

The development of small portable devices for point-of-care tests to measure biomarkers in samples other than sputum is a necessity for the initial care of patients with suspected TB. Because in children and patients with TB/HIV

coinfection the quality of the collected sputum is impaired and smear microscopy sensitivity is low, a rapid test that can be performed using other samples, is moderately to highly sensitive, and exhibits high specificity can be of great use in public health[2].

Also in terms of diagnosis, it would be interesting to have a marker that indicates, which individuals are at greater risk of disease or progression of LTBI to active TB[13].

With respect to treatment, biomarkers may be useful in detecting cases with susceptible of receiving shorter therapeutic regimens, in the prediction of the risk of relapse, and in the assessment of response to treatment[9].

With regard to the development of new vaccines, biomarkers have the potential to identify molecules that are more immunogenic, to quantify T-cell reactivity to vaccine antigens, and to determine the durability of this response in relation to vaccine dosage and possible adjuvant use[13].

INTERACTION OF *MYCOBACTERIUM TUBERCULOSIS* WITH THE HOST

Infection by *M. tuberculosis* triggers a humoral immune response, with the synthesis of antibodies of various isotypes (IgA, IgM, and IgG) against the antigens of mycobacteria, and a cellular immune response, with the activation of monocytes, macrophages, and lymphocytes[11]. Thus, specific antibodies and products released by activated cells (enzymes, cytokines, transcriptomes, proteomes, and metabolomes) are potentially useful biomarkers for improving our understanding of this etiological agent and its interaction with the host.

There is a complex interaction between epidemiology, the infectious agent, and the host in relation to the development of the disease. Such complexity of interaction is explained by the fact that the body's response to an infectious disease depends on multiple factors. The greatest challenge is defining a biomarker that reflects this interaction, can serve as a tool for distinguishing between healthy children and those with active TB, will return to normal levels after treatment, and is replicable in other populations.

MEASURING ANTIBODIES AGAINST *MYCOBACTERIUM TUBERCULOSIS* ANTIGENS

The measurement of serum levels of specific antibodies in cases of pediatric TB are interesting, because this analysis does not require the collection of material from the infection site and can be measured quickly and relatively inexpensively using simple devices[14,15].

When the diagnostic tests involving immunoglobulin level measurement are applied to pediatric patients, it is necessary to consider some aspects of the immune system exhibited in this age group. In infants and young children, the immune system is still in development. Newborns initially respond to antigens with a higher IgM synthesis. Protection by IgG is mainly provided by the transfer of this isotype across the placenta, and IgA is mainly supplied by breast milk[16,17]. IgM, IgG, and IgA immunoglobulin levels reach values close to those of adults at 2, 6, and 10 years of age, respectively[18]. Several studies on the use of serology as a method for TB diagnosis have been conducted with adult individuals[15]. Achkar and Ziegenbalg (2012) published a review, including 23 articles, on pediatrics. In their review, large variations were found between antibody levels; sensitivity ranged from 14% to 85%, and specificity ranged from 86% to 100%.

The authors emphasized the difficulty in comparing the results of these studies resulting from differences in their methodologies, such as patient age, use of different antigens (Ag5, old tuberculin, A60 [ANDA-TB] 16KDA, 30 KDA, 38 KDA, PPD, ESAT-6, TBLG, LOS, DAT, PGBTb1, Ag 85 complex, and CFP 10), different isotype evaluated (IgM, IgG, IgA, and IgE), isolated or combined, use of commercial kits or in house methods, and type of TB diagnosed (confirmed, unconfirmed, or unlikely)[4,15].

With regard to the type of TB, serology studies show that the level of antibodies in patients with cavitary lesions and consequently higher bacillary loads is probably higher because of the greater antigenic stimulus and greater inflammatory response[19,20]. It is important to note that cavitary lesions are less frequent findings in cases of pulmonary TB in children than among adolescents and adults[21].

Another aspect to be considered regarding the use of this method in pediatric TB is the relationship between the levels of antibodies against mycobacterial antigens and previous vaccination with BCG. These antibody levels are influenced by the child's age, time since BCG vaccination, and the antigen and isotype tested[15]. Some studies found higher levels of a given antigen in vaccinated children than in unvaccinated children[22]. However, several authors have shown that prior history of BCG vaccination does not influence the levels of antibody against different antigens[15].

Currently, the World Health Organization does not recommend the use of serological tests for the diagnosis of TB in different age groups[23].

CYTOKINES

Cytokines, which are secreted by cells activated by *M. tuberculosis* antigens, have the potential to be used as biomarkers in TB, especially to distinguish LTBI from active TB and other pulmonary diseases. To be potential biomarkers, the molecules being evaluated must show certain characteristics, such as being upregulated in cases of active TB relative to healthy individuals and those with LTBI, and being secreted by cells specifically stimulated by specific *M. tuberculosis* antigens. Some cytokines are released by nonspecific T-cells and macrophages, and are elevated in various diseases. High levels of the following cytokines have been detected in patients

with TB: I-309, CXCL9, IL-10, IL-6, IL-7, IL-8, G-CSF, TGF-β1, CCL2, IL2, IL13, and TNF-Fα[24].

The measurement of interferon-gamma-inducible protein 10 (IP-10) has been evaluated in research on pediatric TB. Jenum et al. (2016) obtained promising results in a study with children in India, in which they associated the analysis of this cytokine with gamma interferon[25].

Comparing the results of studies on cytokines in TB can be difficult, because there is a difference between the cytokines and the methods used to identify them[24].

IGRAs are commercially available for use in clinical practice. They have the advantage of not being influenced by prior BCG vaccination or by previous contact with other mycobacteria. However, this laboratory method does not distinguish between LTBI and active TB. This method also has limitations when used in children aged < 2 years[6]. In Brazil, it is not available for use in public health care centers.

TRANSCRIPTOMES

Partial DNA reading results in RNA molecules known as transcripts. A collection of transcripts is referred to as a transcriptome. An evaluation of transcriptomes enables an analysis of the genes in a given cell are or are not activated under different circumstances. Most transcripts are formed by messenger RNA, which are directly involved in protein synthesis[26].

A prospective study conducted in South Africa, Malawi, and Kenya with children with suspected TB, some of whom were HIV-infected, identified a 51-transcript signature that was able to distinguish between TB and other diseases. Sensitivity was found to be 82.9% and specificity was 83.6% for the diagnosis in patients with TB confirmed by culture. Sensitivity was 62.5%-82.3% in children highly likely to have TB, 42.1%-80.8% in children likely to have TB, and 35.3%-79.6% in children suspected of having TB, but with negative cultures[27].

PROTEOMES

The complement of proteins expressed by a given cell in various situations is called a proteome. Initially, *M. tuberculosis* proteins were identified using immunological and biochemical methods. In the 1990s, new technologies such as 2-D electrophoresis and mass spectrometry have enabled the identification of protein composition of this microorganism[28].

The analysis of different cellular components allows identifying immunogenic, virulence, latency or activity markers that may be useful in the development of new vaccines, drugs, and tools for a more accurate diagnosis of TB[28].

Some examples of proteins that have been isolated include Rv0899 (which enables bacterial survival at a low pH), Rv2246 (which is involved in pathogenesis and in the synthesis of the mycolic acid present on the cell wall of *M. tuberculosis* cells), and Rv2873 (a lipoprotein present in higher quantities in *M. bovis* than in *M. tuberculosis*)[28-31].

METABOLOMES

Metabolomics is the study of the complement of metabolites in a cell, organ, or organism. Like other "-omics," metabolomics is a large-scale tool that has the advantage of providing a picture of an organism at a given instant. Its detection capacity is limited to small molecules-those with an atomic mass of < 3,000. Steve Oliver of the University of Manchester described metabolome as "a group of low-molecular-weight metabolites that are context-dependent and that vary according to the physiological, pathological, or developmental stage of the cell, tissue, organ, or organism[32]."

In metabolomic analysis, a wide variety of chemical compounds are studied, including sugars, amino acids, steroids, fatty acids, phospholipids, and organic acids. These metabolites may be divided on the basis of solubility in aqueous solutions. Conventional metabolomic methods emphasize water-soluble molecules found in cytoplasmic extracts. Hydrophobic compounds such as fatty acids and cholesterol are typically found on the cell membrane and are the object of study within a subspecialty of metabolomics known as lipidomics[33].

One component of *M. tuberculosis* that can be wielded for the development of new diagnostic tools is the cell wall. Approximately 40% of *M. tuberculosis* cell-wall dry weight consists of lipids, and one third of its genome is devoted to lipid biosynthesis and metabolism[33-35]. A recent lipid analysis showed that *M. tuberculosis* contains approximately 5,000 lipid species[33].

M. tuberculosis continually remodels the lipid content of its cell walls in response to stressful conditions imposed by the host's immune response[35]. Changes in the lipid composition of the cell wall result in a higher concentration of less immunogenic lipids on the cell wall, an alteration that appears to contribute to the ability of *M. tuberculosis* to establish long-term infection[35-36].

Another mechanism observed during infection and one which is also dependent on *M. tuberculosis* lipids is the change in lipid homeostasis in the host's cells. Some *M. tuberculosis* lipids are able to stimulate the accumulation of lipid droplets containing cholesterol, triacylglycerol, and phospholipids, resulting in the formation of foamy macrophages. This condition appears to create an environment conducive to *M. tuberculosis* survival in the intracellular medium[37].

Metabolomics and lipidomics allow the detection of the byproducts of bacterial metabolism and metabolic changes occurring in the host. This information can be used to identify TB biomarkers. In a pilot study, Frediani et al.[38] performed a plasma metabolomic analysis of individuals diagnosed with active TB and healthy contacts.

In the study, the authors identified several metabolites that were quantitatively distinct between these two groups. The putative identification of these metabolites revealed, among other findings, that infected individuals exhibited

increased levels of the amino glutamine, the cell-wall glycolipid of *M. tuberculosis* (trehalose-6-mycolate), and a D-series resolvin[38].

In another study, a metabolomic analysis identified 20 metabolites that were consistently able to distinguish individuals with active TB from those with latent TB. In addition to providing biomarkers for TB metabolites, this tool showed that *M. tuberculosis* infection changes the metabolites associated with anti-inflammation, immunosuppression, and stress[39].

It is possible that the lipid changes that occur in the host as a result of *M. tuberculosis* infection can be detected on the basis of a lipid analysis using the serum or plasma of TB patients. The detection of the *M. tuberculosis* glycolipid trehalose-6-mycolate (Frediani et al.[38]) establishes the perspective for (1) the development of new diagnostic tools based on the detection of lipid compounds from *M. tuberculosis* and (2) the identification of other cell-wall lipids of *M. tuberculosis* in the serum of patients with TB that may enable a better understanding of lipid changes in *M. tuberculosis* during infection.

When considered together, changes in the lipid composition of the cell wall of *M. tuberculosis* and changes in lipid homeostasis in the host during TB infection can be detected by lipid analysis. This information may be useful for the identification of potential biomarkers capable of predicting the progression of latent TB to the active form of the disease[38].

FINAL CONSIDERATIONS

In a disease as complex as TB, the discovery of new biomarkers contributes to a better understanding and the potential resolution of the challenges involved in prevention, diagnosis (which is fundamental in children), treatment, and clinical outcomes. The proper management of this health issue in pediatric patients may result in lower morbidity and mortality among affected population affected by the disease.

ACKNOWLEDGMENTS

We would like to thank Professor Clemax Couto Sant'Anna from the Federal University of Rio de Janeiro (UFRJ), our researcher of reference in pediatric TB, for his contribution to our research on tuberculosis biomarkers in children and adolescents.

We would also like to thank Dr. Lee Riley from the University of California, Berkeley, for setting an example of academic rigor and encouraging and supporting the research on tuberculosis biomarkers conducted by the pediatric tuberculosis studies group at Fluminense Federal University.

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