Biomarkers, 2012; 17(1): 1–8

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2011.628048

informa
healthcare

REVIEW ARTICLE

Host biomarkers of clinical relevance in tuberculosis: review of gene and protein expression studies

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Abstract

Context: Identification of clinically relevant biomarkers is required for better diagnosis, prevention and treatment of tuberculosis.

Objective: In this review, potential host biomarkers in blood or blood cells in tuberculosis were identified by a systematic approach.

Methods: A total of 55 articles were selected from PubMed and Google Scholar that analyzed gene and or protein expression in humans in active and or latent TB. Articles were scored according to certain criteria and categorized as strong or weak studies. Biomarkers reported by more than one article or by a single strong article were identified as potential biomarkers.

Results: Six most promising markers (IP-10, IL-6, IL-10, IL-4, FOXP3 and IL-12) were identified based on their presence in both mycobacterial antigen-stimulated and -unstimulated samples.

Conclusions: With this review we hope to provide a reliable guideline for biomarker studies in tuberculosis.

Keywords: Differential expression, active TB, latent TB, blood, peripheral blood mononuclear cells (PBMCs)

Introduction

Tuberculosis (TB) is a serious public health problem causing morbidity and mortality worldwide. The global morbidity and mortality is estimated at 9.4 million incident cases and 1.3 million deaths annually (WHO 2009). Coexistence of HIV and TB, emergence of drug resistant strains of *Mycobacterium tuberculosis* and lack of a protective vaccine are the main limitations in controlling TB epidemic. Human infection with *M. tuberculosis* can progress to active disease, be contained as latent infection, or be eradicated by the host response. Although these are not fixed distinct states, but rather are spectra along which patients can progress clinically (Wallis et al. 2010) and be diagnosed as such. Of the billions of people infected with *M. tuberculosis* generally only 10% would develop active disease. In the remaining 90% of the individuals the pathogen is not eliminated but contained in distinct foci by the immune system leading to an asymptomatic latent infection (Kaufmann & McMichael 2005). Existing tests however neither differentiate between latent and active TB nor identify latent TB individuals at risk of developing active disease.

Efforts to control TB are hindered by gaps existing in diagnosis, prevention and treatment. There are no rapid, cheap and accurate tests available for TB diagnosis (Agranoff et al. 2006). Current diagnostic gold standard culture of *M. tuberculosis* is lengthy and takes weeks to yield results. Sputum smear microscopy is affordable but achieves a sensitivity of only 50–70% and requires two or more patient visits. Tuberculin skin test (TST) can be uncertain by prior BCG vaccination, latent TB and cross reactivity to non-pathogenic environmental mycobacteria. IFN-γ release assays (IGRAs) and DNA amplification methods are costly and need particular skill and hence difficult to implement in low socioeconomic background where TB is particularly present. Moreover IGRAs cannot discriminate between latent and active TB (Lange et al. 2009) and have a limitation in TB diagnosis in

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(Received 17 June 2011; revised 23 September 2011; accepted 26 September 2011)

HIV-positive individuals (Aabye et al. 2009). Prevention would play a major role in TB control. Currently, there is a lack of an effective TB vaccine and if correlates of protective host immune response are known then it would help in developing better vaccines aiding disease prevention (Kaufmann 2010). Emerging resistance to current TB drugs necessitates development of newer and better drugs. Hence "biomarkers" indicating disease status would boost development of better drugs, vaccines and diagnostics (Parida & Kaufmann 2010).

Literature on large scale transcriptome or proteome profiling studies has gained momentum in recent years with each study reporting a set of differentially expressed genes in diverse clinical forms of TB. These studies have been carried out across various geographical regions and have employed various tools to arrive at a common aim—to distinguish latent and active TB patients based on their blood/blood cell transcriptome or proteome profile. However, all these studies are "standalone" with a need to analyze these studies as a whole to discern a common pattern specific to latency or active disease. Therefore, in this article we present an overview of protein and gene expression studies that identify differentially expressed genes or proteins in human host in latent and active TB. Based on our review, potential biomarkers were identified based on the number of articles reporting them and also based on the strength (articles were scored using specific criteria as strong and weak studies) of the study that reported them. We are in agreement with the suggestion (that has been made earlier) that a set of biomarkers comprising a TB biosignature would be better to detect TB than a single marker when used in an algorithmic format. Further validation of these biomarkers on a large sample set in multiple geographical locations holds key to effective control of TB.

Methods

Search strategy

We conducted a search of PubMed and Google Scholar for articles published between January 1995 and September 2010. The query terms included (Cytokines or Genes or Proteins or Biomarker or Biomarkers) and (Latent Tuberculosis or Active Tuberculosis). The search was limited to studies published in English. The search yielded a total of 2158 articles of which 46 were found to be relevant. Additional studies were identified by scanning the reference lists of articles identified by the above search strategy and were included if found relevant. Finally, around 55 research papers were selected for this review.

Study selection

Studies involving subjects with pulmonary TB and latent TB were included. However studies on subjects with extrapulmonary TB, multidrug-resistant tuberculosis (MDR-TB) or TB coupled with other complications like HIV or diabetes were not included in this review. All studies on whole blood, peripheral blood mononuclear cells (PBMCs), plasma, serum or other subset cells derived from PBMCs, identified by the search were considered as relevant. Studies performed in animals, cell lines, lung cells, pleural fluid mononuclear cells (PFMCs) or bronchoalveolar cells were excluded. For *in vitro* stimulation experiments all studies using mycobacteria or mycobacteria-derived antigens (whole mycobacterial lysate, purified protein derivative (PPD), ESAT-6, CFP-10, TB 7.7, 30 kDa or 32 kDa antigen) were included. However studies using putative mycobacterial antigens and non-specific antigens like lipopolysaccharide (LPS), phytohemagglutinin (PHA), cytokines or ionomycin were not considered. Only gene or protein expression studies were considered as relevant for this review and therefore studies on linkage, polymorphism or on other genetic aspects were not considered. QuantiFERON-TB test (QFT)/T-SPOT.TB studies (which also report on IFN-γ expression) have not been included. Also only microarray based studies which were not validated by additional techniques like real time PCR have been excluded. Immune response studies on BCG vaccinated or unvaccinated normal subjects were also not included.

Data extraction

Papers were sub-divided on the basis of the methods used for studying the expression profile. Thus of the 55 papers, 15 papers were based on detection at gene transcript level and 33 at the protein level. The remaining 7 papers had detected both RNA and proteins. Data on all the genes expressed in a given condition at an accepted level of significance was collected and tabulated. All relevant data related to samples, antigens, methodology etc. was also abstracted and compared.

Quality assessment strategy (scoring) for the research articles

The papers were graded as strong and weak studies based on nine pre-defined criteria as done previously by Kaido et al. to grade studies on randomized controlled trials in hepatocellular carcinoma and liver surgery (Kaido 2007, Kaido & Uemoto 2008). Any disagreement in defining criteria was resolved by consensus or by consulting an expert. The 55 articles were divided between two reviewers and were graded independently. The criteria for assessment included year of publication, sample size, type of controls used, study type, study design, ethnic diversity of the individuals recruited for study, geographical location/locations and screening test used to identify the patients and controls. The articles were assigned a score of 1 or 0 depending on the fulfillment or non-fulfillment of the criteria except in case of year of publication, sample size, screening test and study type. For assessment based on year of publication the articles were divided in four groups and were assigned a score of 2 if published between 2008 and 2010, 1.5 for 2005–2007, 1 for 2002–2004 and 0.5 for the rest. The rationale for taking "year" as one of the criteria was that—generally advancement in technology takes place with time and sophisticated and better techniques are applied in more current articles than the older ones. For assessment based on sample size the average sample

size of each paper was calculated. The papers were then grouped into 4 categories based on their sample sizes and scored accordingly. Studies with sample sizes ≥60 were assigned the highest score of 2, 40–60 were assigned a score of 1.5, 20–40 were given a score of 1 and studies with sample sizes below 20 were given 0.5. Like wise studies were graded as qualitative, semi-quantitative, relatively or absolutely quantitative and were assigned—0.5, 1, 1.5 or 2, respectively. Finally the scores for each paper were summed up and the mean score was calculated. All the scores showed normal distribution pattern. The mean score for the papers was 6.8. All studies with scores >6.8 were classified as strong studies and studies with scores <6.8 were classified as weak studies. See Supplementary appendix (Table A6 and A7) for detailed grading criteria and scores.

Results

Differential gene and protein profiling in latent and active TB: Overview of various approaches utilized in different articles

Definition of active TB, latent TB and healthy controls

Each paper utilized different test methods and various criteria to interpret the test results for defining the three groups—active TB, latent TB and healthy controls according to BCG vaccination status and disease prevalence in a population. Classifications (latent, active and healthy controls) used by the author were accepted and used for our analysis in this review.

Differential gene expression profiling

Largely blood derived cells were used as samples for analysis: whole blood or PBMCs being the most widely used specimens for differential gene expression profiling in TB patients. In one of the papers by Jacobsen et al. (2010) the authors have utilized T-cells for gene analysis. In some studies whole blood was directly utilized to isolate RNA for analyzing gene expression or PBMCs were isolated from whole blood and then gene expression was analyzed.

Lot of variation was found with respect to stimulation of samples between various articles. In some papers, researchers have stimulated PBMCs *in vitro* with live mycobacteria, whole cell lysate, purified protein derivative or other specific mycobacterial antigens prior to analysis of gene expression. Others have used unstimulated PBMCs or whole blood for the study. Outcomes could be different depending upon whether the samples were unstimulated, stimulated and also the method of stimulation (antigen type and time of stimulation). Moreover the precise stage of illness the patient was undergoing when the cells were drawn would also differ leading to variation in results. Microarray analysis and real time PCR were the most common methods utilized for differential genomic profiling in TB patients. The data generated in microarray was validated by quantitative real time PCR. Table 1 enlists the various methods and tissues employed for gene expression profiling in TB.

Differential protein expression profiling

Cytokine multiplex bead assays, ELISA, protein array (Su et al. 2010) and flow cytometry were employed to quantitatively determine cytokines/chemokines in TB patients and controls (Table 1). Some of the studies analyzed the cytokine expression without any stimulation while others stimulated blood or PBMCs with mycobacterial antigens. Serum/plasma or culture supernatants of PBMCs isolated from individuals were utilized for protein expression profiling (Table 1).

Lists of differentially expressed genes and proteins

In vitro stimulation of blood/blood cell samples would possibly yield diverse gene expression pattern when compared to that of unstimulated samples. Therefore, all the articles were divided into two groups based on cells being tested after stimulation with mycobacterial antigens or unstimulated. Data from both stimulated and unstimulated groups was separately analyzed and differentially expressed genes or proteins were classified as below:

i. Genes or proteins upregulated or downregulated in active TB compared to healthy control.
ii. Genes or proteins upregulated or downregulated in latent TB compared to healthy control.
iii. Genes or proteins upregulated or downregulated in active TB compared to latent TB.

The entire set of differentially expressed markers in these three groups is given in Supplementary appendix (Tables A1–A5). From these huge repertoires of biomarkers, we identified some strong candidates for future validation. The criteria to select the strong candidate biomarkers were: (1) Reported to be differentially expressed by more than one article or (2) Reported by a single strong study (as assessed by us using certain criteria explained in Supplementary appendix). The complete list of potential candidate TB biomarkers identified by our analysis is shown in Table 2.

Potential biomarkers for active TB (differentially expressed between active TB patients and healthy controls)

Amongst the several potential active TB markers (Table 2); IL-10, IL-6 and IP-10 appear to be the most promising candidates for diagnosis of active TB as they were upregulated in active TB in both unstimulated and stimulated samples. IL-10 and IL-6 are both Th2 cytokines while the

Table 1. List of various methods and tissues used for host gene and protein expression studies in TB.

Type of study	Methods used	Tissues used
Gene expression	• Microarray • qRT-PCR • Semi-quantitative RT-PCR	• Whole blood • PBMCs • T-cells
Protein expression	• ELISA • Flow cytometry • Cytokine bead assay • Protein array	• Serum • Plasma • Supernatant of cultured PBMCs

Table 2. Potential biomarker candidates for TB.

	Unstimulated		Stimulated	
Condition	Up	Down	Up	Down
ATB vs. HC	**IP-10** (Azzurri et al. 2005, Pokkali & Das 2009, Su et al. 2010)	IFN-γ/IL-4 (Wassie et al. 2008)	CCL-2/MCP-1 (Hasan et al. 2009a,b, Ruhwald et al. 2009, Su et al. 2010)	
	IL-6 (Pokkali & Das 2009, Verbon et al. 1999)	IL4δ2/IL-4 (Wassie et al. 2008)	**IL-6** (Hussain et al. 2002, Mattos et al. 2010, Su et al. 2010)	
	IL-10 (Deveci et al. 2005, Guyot-Revol et al. 2006, Verbon et al. 1999)		**IP-10** (Ruhwald et al. 2009, Ruhwald et al. 2007, Ruhwald et al. 2008)	
	IL-8 (Pokkali & Das 2009, Su et al. 2010)		**IL-10** (Hasan et al. 2009a,b, Hussain et al. 2002, Sai Priya et al. 2009)	
	Neopterin (Immanuel et al. 1997, Turgut et al. 2006)		IL-2 (Mattos et al. 2010, Ruhwald et al. 2007)	
	IL-4 (Dheda et al. 2005)		TNF-α (Hasan et al. 2009a, Mattos et al. 2010)	
	IL4δ2 (Dheda et al. 2005)		MCP-2 (Ruhwald et al. 2009, Ruhwald et al. 2008)	
	FOXP3 (Guyot-Revol et al. 2006)		IL-1RA (Ruhwald et al. 2009)	
	PTX3 (Azzurri et al. 2005)		MCP-3 (Ruhwald et al. 2009)	
	TGF-β1 (Guyot-Revol et al. 2006)		IFN-γ (Ruhwald et al. 2007)	
ATB vs. LTB	IL-10 (Demissie et al. 2004, Handzel et al. 2007, He et al. 2010)	IL4δ2 (Demissie et al. 2004, Fletcher et al. 2004)	**FOXP3** (Chen et al. 2010, Roberts et al. 2007, Wu et al. 2007)	**IL-12α** (Wu et al. 2007, Wu et al. 2008)
	FOXP3 (Burl et al. 2007, He et al. 2010)	GZMA (Maertzdorf et al. 2010)	VEGF (Chegou et al. 2009, Matsuyama et al. 2002)	**IL-12β** (Wu et al. 2007, Wu et al. 2008)
	IL-4 (He et al. 2010, Seah et al. 2000)	Eotaxin (Djoba Siawaya et al. 2009a)	**IL-4** (Roberts et al. 2007, Talreja et al. 2003)	IL-2 (Biselli et al. 2009, Sargentini et al. 2009)
	IL-6 (Chen et al. 2010, Djoba Siawaya et al. 2009a)	TEX264 (Mistry et al. 2007)	MCP-1 (Frahm et al. 2010, Hasan et al. 2009a)	IL-17 (Chen et al. 2010, Sutherland et al. 2009)
	KL-6 (Djoba Siawaya et al. 2009a, Djoba Siawaya et al. 2009b)	**IL-12** (Demissie et al. 2004)	TNF-α (Stern et al. 2009, Sutherland et al. 2010)	IL-13 (Sutherland et al. 2009)
	SOCS3 (Jacobsen et al. 2010, Mistry et al. 2007)	IFN-γ (Demissie et al. 2004)	IL-12(p40) (Sutherland et al. 2009, Sutherland et al. 2010)	IL-10 (Sutherland et al. 2009)
	DEFA4, LTF, CD64, BPI, FPR1, Rab33A, DEFA1 (Jacobsen et al. 2007)	IP-10 (Whittaker et al. 2008)	EGF, sCD40L TGF-α (Chegou et al. 2009)	IL-18 (Sutherland et al. 2009)
	IL-2RA, JAK3, MYC, PIM1, CIS		IFN-γ (Handzel et al. 2007)	IFN-γ (Chen et al. 2010)
	FCGR1B, GBP1, GBP5 CASP1 (Maertzdorf et al. 2010)		CCL-1 (Thuong et al. 2008)	MIP-1β (Chegou et al. 2009)
	LY6G6D, NOLA3, KIAA2013 (Mistry et al. 2007)		IL-15 (Frahm et al. 2010)	
	TGF-β (Chen et al. 2010)			
	IP-10 (Djoba Siawaya et al. 2009a)			
	sIL-2R (Handzel et al. 2007)			

(Continued)

Table 2. Continued.

	Unstimulated		Stimulated	
Condition	Up	Down	Up	Down
LTB vs. HC			IFN-γ (Nemeth et al. 2010, Sutherland et al. 2010) IP-10 (Petrucci et al. 2008) TNF-α (Nemeth et al. 2010) IL-2 (Nemeth et al. 2010) IFN-γ/TNF-α (Nemeth et al. 2010) IFN-γ/IL-2 (Nemeth et al. 2010) IL-13 (Sutherland et al. 2010)	

Markers reported by two or more separate research articles or by studies rated as strong after the scoring are shown below.
Markers identified in both stimulated and unstimulated blood samples in each category are indicated in bold.
ATB-active tuberculosis, LTB-latent tuberculosis, HC-healthy control.

protective immune response in TB is the Th1 type. IL-10 is implicated in hindering cellular immune response associated with active TB (Othieno et al. 1999, Delgado et al. 2002). *M. tuberculosis* itself has been shown to induce IL-6 (Zhang et al. 1994), which, in turn, has been described to impair macrophage responses to IFN-γ (Nagabhushanam et al. 2003). IP-10 (IFN-γ inducible protein 10 KD) which is also known as CXCL10 (IFN-γ inducible cytokine) has been suggested as a better alternative to IFN-γ as it is expressed in higher quantities (Ruhwald et al. 2009). These three biomarkers should be evaluated in future for their potential in active TB diagnosis. Furthermore, our analysis have identified additional seven promising biomarker candidates in unstimulated group category—IL-8, neopterin, FOXP3, IL-4, IL-4δ2, TGF-β1 and PTX3 (Table 2) (Immanuel et al. 1997, Verbon et al. 1999, Azzurri et al. 2005, Deveci et al. 2005, Dheda et al. 2005, Guyot-Revol et al. 2006, Turgut et al. 2006, Pokkali & Das 2009, Su et al. 2010). These biomarkers would be ideal for developing a point of care test (POCT) as additional steps in the procedure like *in vitro* stimulation of blood samples would not be required. Although this test may have less specificity, it can be clinically useful as a triaging tool to narrow down the number of samples to be tested by a more sophisticated test like Xpert MTB/RIF test. Such a two step approach would definitely bring down the costs of diagnosis and can be implemented in low economic settings. In stimulated group the further potential candidates such as CCL-2, TNF-α, IL-2, MCP-2, IL-1RA and MCP-3 were identified (Table 2) (Hussain et al. 2002, Ruhwald et al. 2007, Ruhwald et al. 2008, Hasan et al. 2009a,b, Ruhwald et al. 2009, Sai Priya et al. 2009, Mattos et al. 2010, Su et al. 2010). Thus, these markers should be validated for their potential as biomarkers for active disease in a whole blood based assay utilizing *in vitro* stimulation with mycobacterial antigens. IGRAs alone cannot discriminate between active TB disease and latent TB infection or successfully cured disease. Currently they can only be used as aids in diagnosing active TB disease along with sputum smear microscopy and radiological examination (Mazurek et al. 2010, Lui et al. 2011).

A rapid immunological test that can detect active TB cases accurately would be very useful for early detection and treatment. However, whether the above mentioned markers can differentiate between active TB and latent infection needs to be evaluated in future. Also our analysis identified IFN-γ as an ambiguous marker for active TB. In majority of reports IFN-γ was upregulated in active TB as compared to control in both unstimulated and stimulated samples (Verbon et al. 1999, Deveci et al. 2005, Hasan et al. 2009b). However a few reports also indicated downregulation of IFN-γ (Hasan et al. 2009a, Pokkali & Das 2009). However this disparity can be explained by the fact that their study population was in a region of high TB prevalence (India and Pakistan) and around 70% of the healthy controls were TST positive indicating latent infection.

Potential biomarkers for correlates of protection (differentially expressed between active and latent TB)

Majority of the people infected with *M. tuberculosis* do not develop the disease and remain asymptomatic. However the protective host immune responses to TB that help to contain the pathogen are far from being understood. One school of thought suggests that identifying the protective immune responses in such healthy infected individuals would probably define correlates of protective immunity in TB (Demissie et al. 2004). A list of potential candidate biomarkers in this category is given in Table 2. Of these, FOXP3 and IL-4 (both upregulated) whereas IL-12 (downregulated) are most promising biomarkers differentially expressed in active TB in comparison to latently infected individuals as they were identified in both unstimulated and stimulated groups. Our analysis showed that two papers classified as strong studies (Demissie et al. 2004, Chen et al. 2010) showed

downregulation of IFN-γ in active TB as compared to latent TB in both stimulated and unstimulated conditions. However one strong study (Handzel et al. 2007) reported upregulation of IFN-γ in active TB compared to latent TB. Hence its status as a protective marker appears a little ambiguous at this stage. FOXP3 is a marker for Treg cells that are involved in beneficial attenuation of immunopathology but are also involved in downregulation of protective responses to infection (Burl et al. 2007). IL-4 is the characteristic cytokine of Th2 response which is considered non-protective in TB. Active TB patients showed less expression of Th1 cytokine—IL-12 compared to healthy infected individuals. However, active TB patients overexpressed IL-4 (Th2 cytokine) in comparison with latent TB individuals. Significantly, the IL-4 antagonist—IL4δ2 was downregulated in active TB compared to latent TB highlighting its role as biomarker of protection. These biomarkers are important due to two reasons: (1) Discriminating TB disease and infection and (2) Identifying correlates of protective immunity. These protective immunological correlates would in turn be useful for vaccine efficacy studies.

Genes or proteins upregulated or downregulated in latent TB compared to healthy control

The data for unstimulated samples was not available for this category. IFN-γ, TNF-α, IL-2, IP-10, IFN-γ/TNF-α, IFN-γ/IL-2 and IL-13 were identified as strong candidate biomarkers (Table 2) by our study that were upregulated in latent TB compared to healthy uninfected controls in stimulated blood samples (Petrucci et al. 2008, Babu et al. 2010, Nemeth et al. 2010, Sutherland et al. 2010). These biomarkers would again find an application in diagnosis of a latent TB infection.

Conclusions

Gene expression of PBMCs and detecting the products directly from blood may reflect the systemic, physiological changes that generate TB disease (i.e. symptoms) and therefore in theory can differentiate infection and illness. Research on transcriptomic and proteomic analysis in TB has picked up in recent years leading to identification of several potential biomarkers for TB diagnosis, vaccine development and drug development. Biomarker research in TB has been very effectively highlighted by quite a few research articles recently (Doherty et al. 2009, Parida & Kaufmann 2010, Wallis et al. 2010, Walzl et al. 2011). However in this review we used a very different approach—to actually narrow down on the potential biomarkers from a plethora of differentially expressed genes and or proteins reported. Our analysis identified IL-6, IL-10 and IP-10 as the most promising markers for active TB while FOXP3, IL-4 and IL-12 were identified as the most promising biomarkers for immunological correlates of protection. However, the concentrations of biomarkers detected in the blood/PBMCs varied significantly between different articles; for e.g. average concentration of IP-10 in unstimulated blood samples from active TB patients was 182 pg/ml in one study while it was as high as 1960 pg/ml in another study. Thus, a consensus was not found with respect to the detection level of various biomarkers which needs to be established through future studies which may employ a standard laboratory protocol. Once the TB biomarkers are validated, the next step necessitates development of a novel detection technology for detection of biomarkers. We suggest that the biomarkers which can be detected at unstimulated levels should be preferred for a POCT due to lesser no of steps involved and therefore an easier implementation of the test—which is an obvious necessity in POCTs. Developing a POCT for TB requires integration of biomarker discovery and validation studies with novel detection technology. Two technologies (among others) have promise to be developed into a POCT-1. Lateral flow immunochromatography and 2. Loop mediated isothermal amplification (LAMP) test. However, technologies that allow multiplex detection of biomarkers are needed considering the fact that TB biosignature would comprise of a set of biomarkers rather than a single one. Alternatively the Cepheid Gene Xpert system could be considered and can be explored for establishing quantitative detection of biomarkers.

Evaluating and validating correlates of protection appears premature at this stage, however as more and more data from various clinical studies of newer and effective TB vaccines comes in a clear picture would emerge.

Future directions

The review highlights certain promising candidate biomarkers for TB research. Validation of these biomarkers in a large set of individuals across populations could identify a TB biosignature as well as "immunological correlates of protective immunity". It appears a little early to postulate possible algorithms for actual clinical use; nevertheless this review highlights the need for a coordinated approach and better utilization of clinical resources.

Acknowledgments

S.H.J. and A.S.G. searched the articles. A.S.G. planned and developed the article outline. S.H.J. tabulated and compiled differentially expressed genes from various articles. A.S.G. wrote introduction, results and discussion while S.H.J. wrote methods section. J.K. conceptualized the study, provided critical comments and suggestions for preparing the manuscript. The authors thank Dr. Anura V. Kurpad (Dean, St. John's Research Institute, Bangalore, India) and Dr. Prem Mony for their suggestions in making the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest.

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