

# Algorithm Design for a Cytokine Release Assay of Antigen-Specific In Vitro Stimuli of Circulating Leukocytes to Classify Leprosy Patients and Household Contacts

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**Background.** Immunological biomarkers have often been used as a complementary approach to support clinical diagnosis in several infectious diseases. The lack of commercially available laboratory tests for conclusive early diagnosis of leprosy has motivated the search for novel methods for accurate diagnosis. In the present study, we describe an integrated analysis of a cytokine release assay using a machine learning approach to create a decision tree algorithm. This algorithm was used to classify leprosy clinical forms and monitor household contacts.

**Methods.** A model of *Mycobacterium leprae* antigen-specific in vitro assay with subsequent cytokine measurements by enzyme-linked immunosorbent assay was employed to measure the levels of tumor necrosis factor (TNF), interferon- $\gamma$ , interleukin 4, and interleukin 10 (IL-10) in culture supernatants of peripheral blood mononuclear cells from patients with leprosy, healthy controls, and household contacts. Receiver operating characteristic curve analysis was carried out to define each cytokine's global accuracy and performance indices to identify clinical subgroups.

**Results.** Data demonstrated that TNF (control culture [CC]: AUC = 0.72; antigen-stimulated culture [MI]: AUC = 0.80) and IL-10 (CC: AUC = 0.77; MI: AUC = 0.71) were the most accurate biomarkers to classify subgroups of household contacts and patients with leprosy, respectively. Decision tree classifier algorithms for TNF analysis categorized subgroups of household contacts according to the operational classification with moderate accuracy (CC: 79% [48/61]; MI: 84% [51/61]). Additionally, IL-10 analysis categorized leprosy patients' subgroups with moderate accuracy (CC: 73% [22/30] and MI: 70% [21/30]).

**Conclusions.** Together, our findings demonstrated that a cytokine release assay is a promising method to complement clinical diagnosis, ultimately contributing to effective control of the disease.

**Keywords.** algorithm design; cytokine assay; differential diagnosis; household contacts; leprosy.

Leprosy is an infectious disease caused by *Mycobacterium leprae*, and approximately 200 000 new cases are registered each year worldwide [1]. It initially affects peripheral nerves and skin, with patients showing clinical, immunological, and pathological

manifestations [2]. The diagnosis of leprosy remains predominantly clinical. However, the scarcity of early signs and symptoms and the similarity to other dermatological diseases often lead to significant delays, inconclusive diagnosis, and lack of appropriate early treatment [3]. Specificity and sensitivity of serological and molecular laboratory assays are variable, and efforts to search for novel methods or host biomarkers expressed in subclinical and early leprosy cases are still needed [4]. Marçal et al previously showed that immunoglobulin G and immunoglobulin G1 tests against leprosy IDRI diagnostic-1 (LID-1) and natural octyl disaccharide bound leprosy IDRI diagnostic-1 (NDO-LID) antigens were accurate in discriminating patients with multibacillary (MB) leprosy from their household contacts (HHC), showing both higher sensitivity and specificity through receiver operating characteristic (ROC) curve [5]. Gama et al also identified a higher

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frequency of positive slit skin smear by quantitative polymerase chain reaction for MB than paucibacillary (PB) HHC [6]. The ideal biomarker for early laboratory diagnosis should reflect subclinical infection before the appearance of dermatologic/neurological signs and symptoms. Advances in the leprosy field have made the screening of putative biomarkers possible, including specific antigens, metabolic targets, and immunological parameters [7]. Evaluation of the cell-mediated immune response to *M leprae* infection based on cytokine profiles—interferon gamma (IFN- $\gamma$ ), tumor necrosis factor (TNF), interleukin 4 (IL-4), and interleukin 10 (IL-10)—has been classically used to discriminate leprosy clinical presentations (PB vs MB) [8]. However, the complexity of the clinical spectrum of leprosy has shown that this classical dichotomic profile is not universally observed in real life. Cytokines play a vital role in activating host–pathogen interaction and in the immunopathogenesis of leprosy [9, 10]. The PB form of the disease correlates with T-helper 1 (Th1; ie, interleukin 2, interleukin 12, TNF, IFN- $\gamma$ ) cytokines as well as T-helper 17 immunity [9]. The proinflammatory response in PB patients leads to bacterial control. MB form is typically associated with an anti-inflammatory cytokine (IL-10, transforming growth factor beta [TGF- $\beta$ ], IL-4) response, with increased numbers of *M leprae* bacilli as well as antibodies against bacteria antigens [11]. The close parallel between the ability of the host to establish effective immunity to *M leprae* and the interindividual variability in clinical manifestations allow leprosy a good model of human immunoregulatory disease using host immune markers as correlates of disease [12]. Recent studies have emphasized novel strategies applied to leprosy early diagnosis, providing evidence that cytokines can be useful complementary diagnostic and prognostic tools as well as showing that household contacts should be monitored to assess putative subclinical infection [13, 14]. It is well known that HHC of patients with leprosy present a higher risk of illness, especially MB contacts, due to the time and degree of exposure to *M leprae*, which justifies the importance of monitoring this population [1].

In the present study, we have applied a model of an *M leprae* antigen (MI)–specific cytokine assay in vitro and describe a decision tree algorithm of integrated analysis of immunological parameters as a putative approach for classification of the leprosy clinical form as well as to monitor HHC for the potential development of early signs of leprosy. It is important to clearly define whether a patient has MB vs PB leprosy, as this impacts the treatment. In cases when the disease classification is unclear, cytokine profiling may help define treatment. Our findings demonstrated that a cytokine release assay contribute to better control of the leprosy through earlier and more accurate diagnosis.

## MATERIALS AND METHODS

### Study Population

This cross-sectional investigation was carried out in Governador Valadares, eastern Minas Gerais State, Brazil, a hyperendemic area for leprosy (1.9 cases/10 000 inhabitants) [1]. The study

enrolled 30 patients with leprosy, before multidrug therapy, of both sexes (15 females and 15 males), ranging from 8 to 92 years old. The patients were further categorized into 2 subgroups, according to the Brazilian guidelines for leprosy management [15], referred to as follows: (1) L(PB): patients with tuberculoid-tuberculoid/borderline-tuberculoid disease (n = 15); and (2) L(MB): patients presenting borderline-borderline/borderline-lepromatous/lepromatous-lepromatous disease (n = 15). A group of healthy controls (HC) was also enrolled, comprising 69 subjects of both sexes (37 females and 32 males), ranging from 7 to 92 years old. The household contacts (HHC, n = 61) of PB or MB patients were referred to as HHC(PB) (n = 28; 18 females and 10 males, age ranging from 7 to 65 years) and HHC(MB) (n = 33; 17 females and 16 males, age ranging from 8 to 92 years), respectively. The study was submitted and approved by the Ethics Committee at Univale (research protocol number 022/09–009). All participants provided written informed consent before inclusion in the study.

### Whole Blood Sampling and Peripheral Blood Mononuclear Cell Culture In Vitro

Heparinized whole blood samples (10 mL) were collected from each participant and used to isolate peripheral blood mononuclear cells (PBMCs) for cytokine quantification upon in vitro cultures. PBMCs were isolated by 1.077 Ficoll Hypaque density gradient (GE Healthcare), washed twice with phosphate-buffered saline, and resuspended in RPMI 1640 (Gibco Invitrogen Corporation) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL), all purchased from Gibco Invitrogen. Cell count viability was assessed in Neubauer chamber microscopy upon 0.4% Trypan blue staining (BioWhittaker). Aliquots of  $2 \times 10^5$  viable cells per well were cultured in duplicate, at 37°C in 5% carbon dioxide in the humid atmosphere in the absence (unstimulated culture [CC]) or the presence (antigen-stimulated culture [MI]) of sonicated *M leprae* (original suspension: 348  $\mu$ g/mL). To measure IL-4 and IL-10, the supernatant was harvested at 24 hours, and for IFN- $\gamma$  and TNF, at 5 days after culture.

### Enzyme-Linked Immunosorbent Assay for Cytokine Measurements

The quantification of TNF, IFN- $\gamma$ , IL-4, and IL-10 in PBMC culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA), in accordance with the manufacturer's instructions (BD Bioscience). In brief, supernatants and standards were incubated in 96-well plates coated with cytokine-specific monoclonal antibodies. Wells were washed, and streptavidin–horseradish peroxidase conjugate mixed with a biotinylated antihuman cytokine antibody was added to each well. The TMB substrate solution was added and the reaction stopped with 2N sulfuric acid solution before optical density measurement at 450 nm in ELISA reader. The minimum

detectable levels for TNF, IFN- $\gamma$ , IL-4, and IL-10 provided by the manufacturer were 2 pg/mL, 1 pg/mL, 2 pg/mL, and 2 pg/mL, respectively. Cytokine levels were expressed as picograms per milliliter according to the standard curves.

### Statistical Analysis

Statistical analyses were carried out using Prism GraphPad software (version 8.01) for pairwise comparisons between groups and multiple comparisons among subgroups. Mann-Whitney test was employed for pairwise comparative analysis between patients with leprosy and HC. The Kruskal-Wallis test with Dunn posttest was used for multiple comparisons and sequential pairwise analysis among subgroups. In all cases, significant differences were considered at  $P < .05$ .

Biomarker signature analysis was carried out as previously reported by Mota et al [16] and Silveira-Nunes et al [17], modified as follows: Continuous variables expressed in picograms per milliliter were converted into categorical data using the global median values intrinsic for nonstimulated CC or MI as the cutoff edge to classify low (lower than the global median) and high (above the global median) values. The proportion of subjects (%) with high biomarker levels was then calculated for each study group. Those biomarkers with proportions of subjects above the cutoff superior to 50% were underscored and considered for comparative analysis among groups.

MedCalc software (version 18.6) was used to define the performance analysis and ROC curve assemblage. The ROC curve parameters were used to define each cytokine's global accuracy, defined by the area under the curve (AUC). Performance indices were also assessed for each cytokine, including sensitivity, specificity, negative likelihood ratio ( $LR^-$ ), and positive likelihood ratio ( $LR^+$ ). WEKA software (version 3.6.11) was used to construct the decision tree classifiers based on the set of attributes from nonstimulated and MI cultures. The leave-one-out-cross-validation (LOOCV) values were also employed as performance indices, referred to as "rotation estimation" mathematical validation model to generalize results of a given statistical analysis to an independent data set. GraphPad Prism, Microsoft Excel, and PowerPoint software packages were used for figures.

## RESULTS

### Cytokine Secretion by PBMCs From Patients With Leprosy and Healthy Controls Upon In Vitro Culture

The cytokine secretion pattern of PBMCs was determined upon in vitro culture carried out in the absence (CC) or in the presence of antigen-specific stimuli (*M leprae*-stimulated cultures); the results are presented in Figure 1. Data analysis demonstrated that lower levels of TNF but higher levels of IFN- $\gamma$  were detected in the supernatant of unstimulated PBMCs from patients with leprosy compared to HC. Upon *M leprae* stimuli in vitro, PBMCs from patients with leprosy sustained the higher levels

of IFN- $\gamma$  production. No differences were observed for the IL-4 and IL-10 secretion profile between patients with leprosy and HC, neither for the unstimulated nor the *M leprae*-stimulated cultures (Figure 1).

### Cytokine Secretion by PBMCs From Leprosy and Household Contact Subgroups Upon In Vitro Culture

Aiming at further characterizing the cytokine profile of PBMCs from distinct leprosy patients and the HHC, subjects were clustered into subgroups according to operational classification, referred to as L(PB), L(MB), HHC(PB), and HHC(MB). The results are provided in Figure 2.

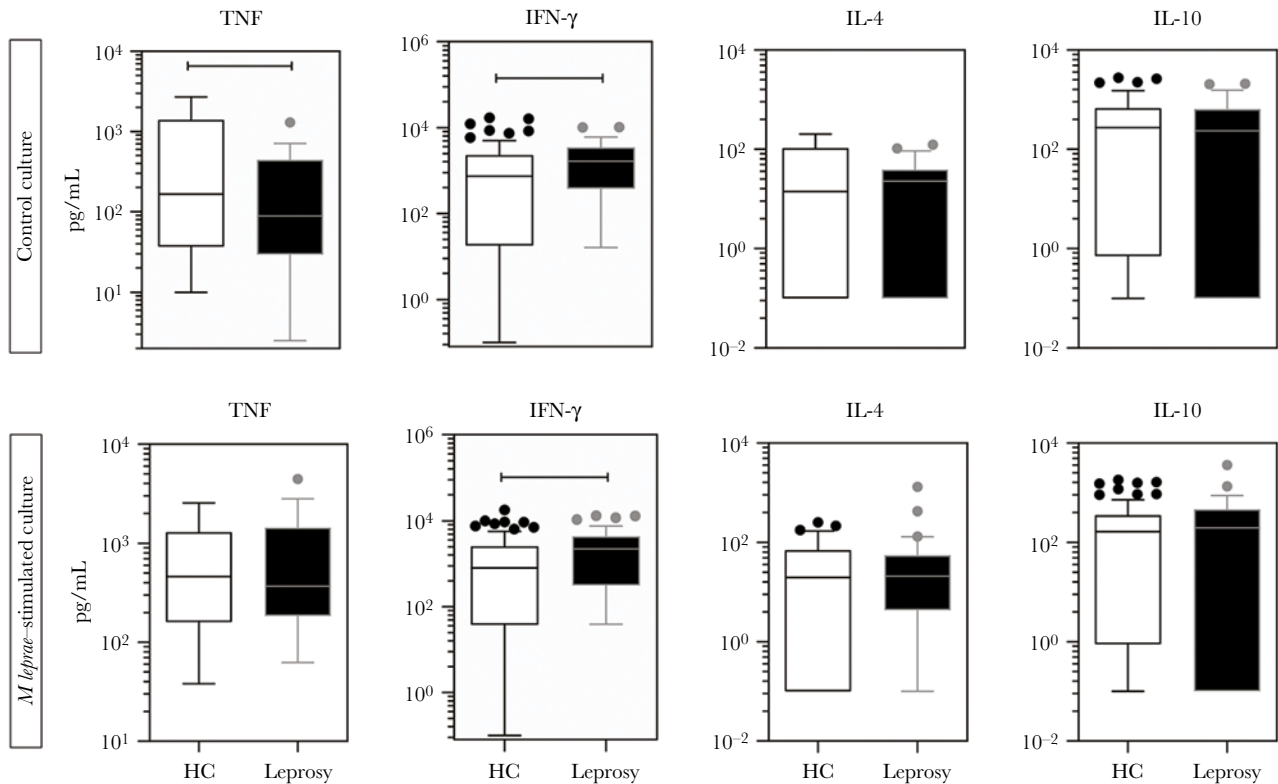
Comparative analysis on HHC and leprosy subgroups with the reference values observed for HC demonstrated that IFN- $\gamma$  was higher in L(PB) in CC and in L(PB) and L(MB) in MI cultures. Moreover, while IL-10 was higher in the L(PB) group, L(MB) displayed lower levels as compared to HC (Figure 2).

Data analysis further showed that HHC(PB) presented higher levels of TNF in both CC and MI cultures as compared to HHC(MB) and as compared to L(PB) in CC culture. Moreover, in the MI culture, HHC(PB) displayed higher IFN- $\gamma$  and IL-10 but lower IL-4 than HHC(MB). In general, HHC(MB) exhibited lower cytokine production patterns (TNF, IFN- $\gamma$ , and IL-10), except for the higher levels of IL-4 as compared to L(MB) and HHC(PB). The cytokine profile of L(MB) was characterized by lower IL-10 level as compared to L(PB) (Figure 2).

### Functional Biomarker Signatures in Patients With Leprosy and Healthy Controls

To further characterize the cytokine profile of leprosy patient clinical forms [L(PB) and L(MB)] and household contact subgroups [HHC(PB) and HHC(MB)], the panoramic cytokine signatures of PBMCs upon in vitro culture were constructed to assess the contribution of each cytokine for the overall proinflammatory or regulatory profile. For this purpose, radar charts were assembled for comparative analysis of cytokine signatures of patients with leprosy and HHC observed in CC and MI cultures (Figure 3). This approach allowed identifying cytokines with the proportion of subjects above the 50th percentile in each group, tagged as relevant biomarkers for further analysis. Using this strategy, data analysis pointed out that the HHC(PB) group displayed a polyfunctional profile composed of proinflammatory (TNF, IFN- $\gamma$ ) and regulatory (IL-10) cytokines, whereas HHC(MB) exhibited a prominent contribution of IL-4, a typical T-helper 2 (Th2)-specific cytokine (Figure 3).

The cytokine profile of L(PB) also illustrated a polyfunctional pattern comprised of most cytokines evaluated, except for TNF in the MI culture. On the other hand, noteworthy was the impaired capacity of L(MB) to produce cytokines, as seen by a reduced frequency of high cytokine producers except for a slighter production of IFN- $\gamma$  (Figure 3).



**Figure 1.** Cytokine secretion by peripheral blood mononuclear cells (PBMCs) from healthy controls (HC) and patients with leprosy upon in vitro culture. Analysis of tumor necrosis factor (TNF), interferon gamma (IFN- $\gamma$ ), interleukin 4 (IL-4), and interleukin 10 (IL-10) was carried out upon in vitro culture of PBMCs from HC (n = 69) and patients with leprosy (n = 30), in the absence of exogenous stimuli (nonstimulated culture) and in the presence of *M leprae* antigen (MI-stimulated culture). The results are presented in boxplot format, indicating the median values (min-max) of cytokine concentrations (pg/mL) and the outliers underscored by dots according to Tukey analysis. Mann-Whitney test was employed for pairwise comparative analysis. Significant differences are indicated by connecting lines for differences at  $P < .05$ .

#### Performance of Selected Biomarkers to Identify Subgroups of Patients With Leprosy and Household Contacts

ROC curve analysis was carried out to estimate the performance of each cytokine to categorize patients with leprosy and HHC, and the performance indices are provided in Figure 4. Data mining was first applied to identify those attributes with higher performance based on the combined analysis of the area under the ROC curve ( $AUC \geq 0.70$ ), with outstanding sensitivity and specificity as well as a valuable likelihood ratio. Based on these criteria, TNF from MI and CC cultures was selected as the top 2 biomarkers to segregate HHC(PB) from HHC(MB) (Figure 4). The results indicated that, using a cutoff of 100 pg/mL, TNF levels from CC culture presented an  $AUC = 0.72$ , sensitivity = 82, specificity = 79,  $LR^- = 0.2$ , and  $LR^+ = 3.8$  to cluster HHC(PB) from HHC(MB). Moreover, using a cutoff of 355 pg/mL, TNF levels from MI culture showed an elevated global accuracy with  $AUC = 0.80$ , sensitivity = 89, specificity = 79,  $LR^- = 0.1$ , and  $LR^+ = 4.2$  to cluster HHC(PB) from HHC(MB) (Figure 4).

Additionally, IL-10 from CC and MI cultures was selected as the top 2 biomarkers to classify L(PB) from L(MB) (Figure 4). The results indicated that, using a cutoff of 298 pg/mL, IL-10 levels from CC exhibited an  $AUC = 0.77$ , sensitivity = 67, specificity = 80,  $LR^- = 0.4$ , and  $LR^+ = 3.3$  to cluster L(PB) from

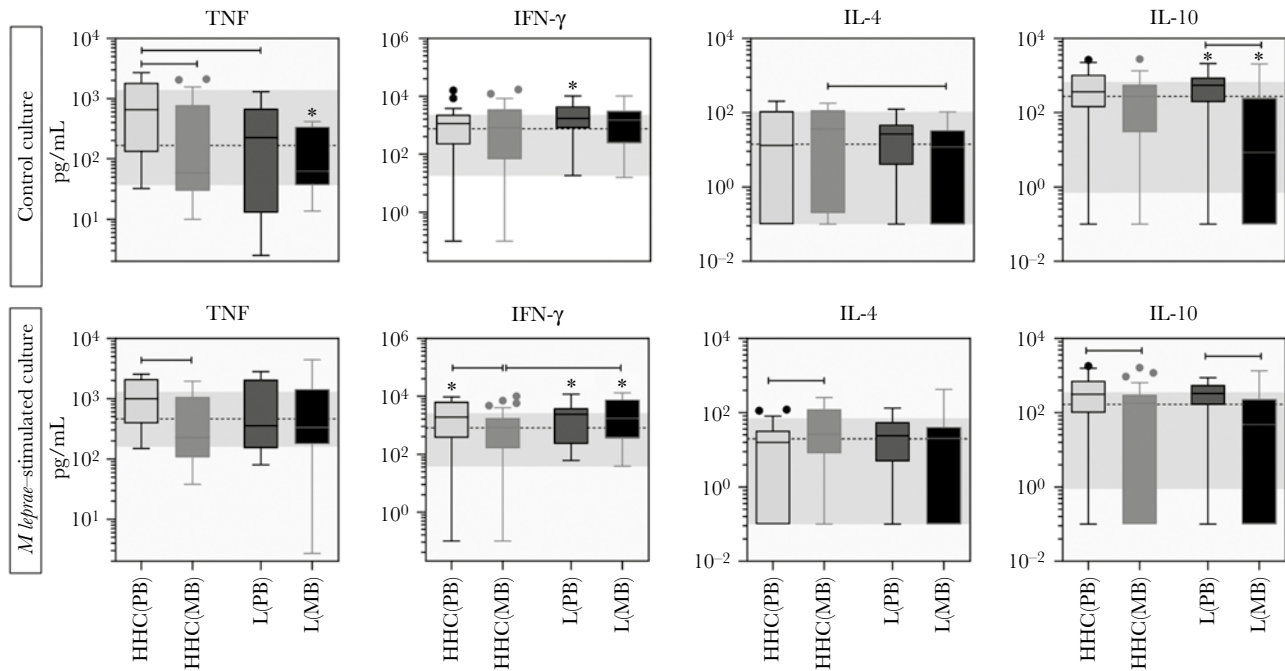
L(MB). Moreover, using a cutoff of 233 pg/mL, IL-10 levels from *M leprae*-stimulated culture presented a global accuracy with  $AUC = 0.71$ , sensitivity = 67, specificity = 73,  $LR^- = 0.5$ , and  $LR^+ = 2.5$  to classify L(PB) from L(MB) (Figure 4).

#### Proposal of Decision Tree Algorithm to Classify Patients With Leprosy and Household Contacts

Based on the overall global accuracy observed for the cytokines evaluated in CC and MI cultures, decision tree algorithms were developed to categorize subgroups of patients with leprosy and HHC (Figure 5). In this case, a decision tree classifier based on TNF production in CC culture revealed a moderate accuracy (79% [48/61]) to identify subjects with  $TNF \leq 100$  pg/mL as HHC(MB) and those with  $TNF > 100$  pg/mL as HHC(PB). Noteworthy was the decision tree classifier based on TNF production in the MI culture, which displayed an elevated full accuracy (84% [51/61]) to identify subjects with  $TNF \leq 355$  pg/mL as HHC(MB) and those with  $TNF > 355$  pg/mL as HHC(PB), with a cross-validation value by a leave-one-out strategy of 80% (Figure 5).

Complementary decision tree algorithms, using the IL-10 production in CC and MI cultures, also displayed a moderated accuracy to cluster L(PB) from L(MB). A decision tree classifier based on IL-10 production in CC culture revealed a full accuracy





**Figure 2.** Cytokine secretion by peripheral blood mononuclear cells (PBMCs) from household contacts (HHC) and leprosy patient subgroups upon in vitro culture. Analysis of tumor necrosis factor (TNF), interferon gamma (IFN- $\gamma$ ), interleukin 4 (IL-4), and interleukin 10 (IL-10) was performed upon in vitro culture in the absence of exogenous stimuli (nonstimulated culture) and in the presence of *M leprae* antigen (MI-stimulated culture), using PBMCs isolated from clinical subgroups, including HHC of people with paucibacillary [HHC(PB), n = 28] or multibacillary [HHC(MB), n = 33] leprosy as well as patients with paucibacillary [L(PB), n = 15] or multibacillary [L(MB), n = 15] leprosy in comparison to healthy controls (HC). The results are presented in boxplot format, indicating the median values (min-max) of cytokine concentration (pg/mL) and the outliers underscored by dots according to Tukey analysis. The reference values of HC are provided as median (dashed line) and 25th and 75th interquartile ranges (gray background). Multiple comparisons among groups were carried out by Kruskal-Wallis test followed by Dunn posttest for sequential pairwise comparisons. Additionally, Mann-Whitney test was also employed for pairwise comparative analysis. Significant differences are underscored by connecting lines for differences between HHC and leprosy subgroups and by \* as compared to HC reference values. In all cases, significance was set at  $P < .05$ .

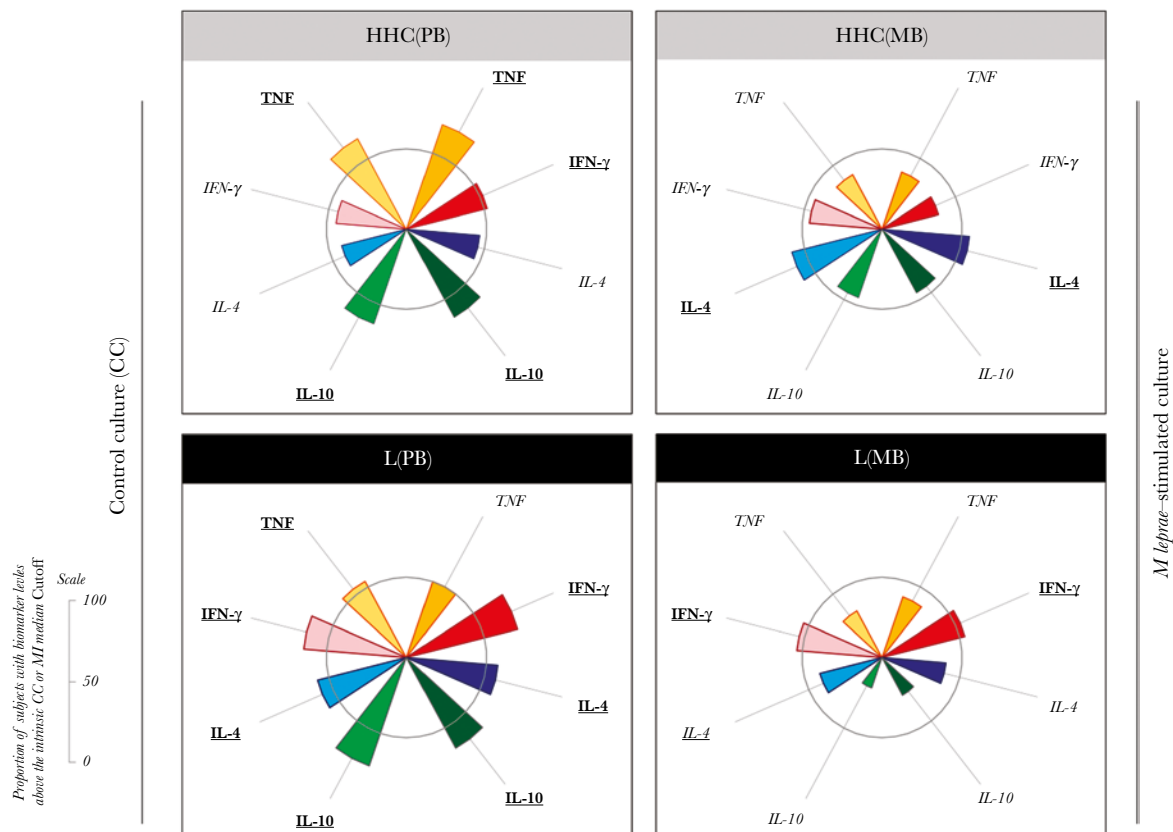
of 73% (22/30) to identify subjects with IL-10  $\leq 298$  pg/mL as L(MB) and those with IL-10  $> 298$  pg/mL as L(PB). Moreover, the IL-10 production in the MI culture displayed an accuracy of 70% (21/30) to classify subjects with IL-10  $\leq 233$  pg/mL as L(MB) and those with IL-10  $> 233$  pg/mL as L(PB) (Figure 5).

## DISCUSSION

Recent advances in *M leprae*-specific laboratory tests for early differential diagnosis have helped detect subclinical infections in the endemic populations [4, 18–20]. However, to date, the lack of commercially available laboratory tests for conclusive early differential diagnosis of leprosy represents a serious gap in public health attention in endemic countries [1]. Serological and molecular tests have been used isolated or in a stepwise algorithm for the follow-up of HHC, who are at high risk of developing the disease. However, these methods' accuracy is variable, especially in the identification of leprosy cases among contacts [15, 21, 22]. The use of immunological biomarkers as a complementary tool to detect leprosy in early stages and subclinical infection among contacts may help reduce deformities caused by delayed diagnosis [8]. In this sense, cytokine release assays have gained power as additional diagnostic tools with elevated accuracy [23, 24].

Our finding demonstrated an overall lower TNF but higher IFN- $\gamma$  production in patients with leprosy than HC (Figure 1). Moreover, we demonstrated an association between cytokine profiles and leprosy clinical forms as well as HHC of PB and MB patients (Figure 2), which we confirmed with categorical analysis of cytokine signatures (Figure 3). In general, according to the cytokine signature analysis, the L(MB) group displayed an overall profile of low cytokine production as compared to L(PB). Previous studies have demonstrated that patients with lepromatous leprosy present T-cell unresponsiveness/nergy to *M leprae* antigens [25]. Our group has also demonstrated that L(PB) displayed a polyfunctional profile characterized by an enhanced percentage of IFN- $\gamma^+$ , IL-4 $^+$ , and IL-10 $^+$  T cells, whereas L(MB) exhibited a more restricted cytokine profile [13]. Obviously, leprosy is not merely a bacteriological disease but an immunological one as well, with molecular crosstalk between Th1, Th2, monocyte lineage, and regulatory T cells (Tregs) throughout the leprosy spectrum [9].

The global accuracy (AUC), sensitivity, specificity, and likelihood ratio were employed as performance indices (Figure 4). The overall performance further allowed the identification of putative biomarkers for potential categorization of HHC and patients with leprosy (Figure 4), with TNF and IL-10 being the



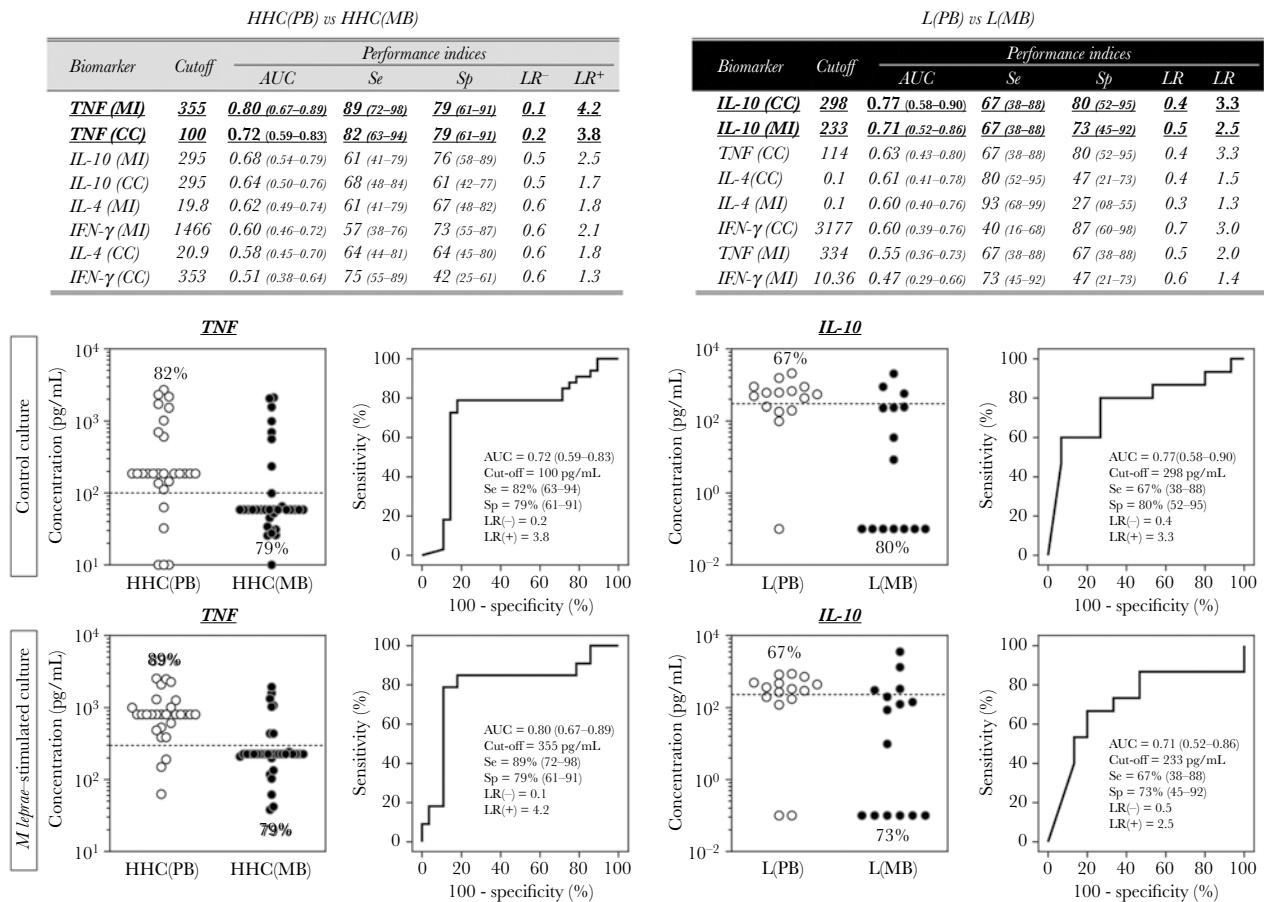
**Figure 3.** Cytokine signature in household contacts (HHC) and patients with leprosy (L) after in vitro peripheral blood mononuclear cell (PBMC) culture. Cytokine signature analysis was carried out as described in the Materials and Methods. Continuous variables (pg/mL) were converted into categorical data using the global median values intrinsic for nonstimulated control culture (CC: tumor necrosis factor [TNF] = 114 pg/mL; interferon gamma [IFN- $\gamma$ ] = 1.268 pg/mL; interleukin 4 [IL-4] = 24 pg/mL; interleukin 10 [IL-10] = 291 pg/mL) or *M leprae*-stimulated culture (MI: TNF = 438 pg/mL; IFN- $\gamma$  = 1.177 pg/mL; IL-4 = 22 pg/mL; IL-10 = 215 pg/mL) as the cutoff edge. The proportion of subjects with high biomarker levels was calculated for each study group and the data are presented in radar chart. Each axis represents 1 cytokine measured in the supernatant from CC (left bars) or MI (right bars) of PBMCs from HHC(PB [paucibacillary]), HHC(MB [multibacillary]), L(PB), and L(MB). Biomarkers with proportions of subjects above the 50th percentile are highlighted in bold underlined text.

most accurate biomarkers to classify leprosy and HHC contact subgroups. TNF has been reported as a relevant cytokine in the PB form of the disease in which is observed a host resistance to *M leprae* with the decrease in bacillary load associated with a Th1 response pattern. On the other hand, IL-10 appears together with IL-4 and TGF- $\beta$  as the hallmarks of a Th2 lymphocyte response, classically associated with the lepromatous clinical form, modulating the microbicidal response of macrophages and facilitating the bacillus survival [1, 26].

A decision tree classifier model applied to define the attributes' accuracy as biomarkers has been also constructed in this study. Our findings were further confirmed using the model of LOOCV. In this sense, higher TNF production in HHC(PB) was described, whereas HHC(MB) exhibited lower TNF production. The Th1-type immune response (TNF) predominance observed in HHC(PB) has already been reported [27, 28]. As the HHC(MB) are exposed to a larger antigenic stimulus, it may drive their adaptive immunity toward lower TNF profile. HHC(MB) constitutes the group of individuals at the highest risk of developing leprosy; therefore, this profile may be

indicative of early subclinical disease [29]. Indeed, de Carvalho et al demonstrated that the continuous exposure of HHC(MB) to live *M leprae* downregulates the specific cellular immune response against the pathogen [30]. These data corroborate the literature that reinforces the greater predisposition of HHC(MB) to develop the disease [7, 31, 32].

Our results also demonstrated that L(PB) and L(MB) could be classified apart within their respective groups, according to the IL-10 profile, with moderate accuracy in both CC or MI cultures (Figure 5). These data were further confirmed by LOOCV analysis. The decision tree algorithm showed that L(PB) was identified by higher IL-10 production, whereas L(MB) exhibited lower IL-10 production. Classically, it has been postulated by Amorim et al that a predominant type 2 immune response is observed in lepromatous clinical form, characterized by high IL-4 and IL-10 that downregulate the macrophage microbicidal activity, leading to higher bacillary load [33]. A prominent humoral response, induced by type 2 immunity, is observed in these individuals, which has been investigated through the production of antibodies against specific *M leprae* antigens [33].

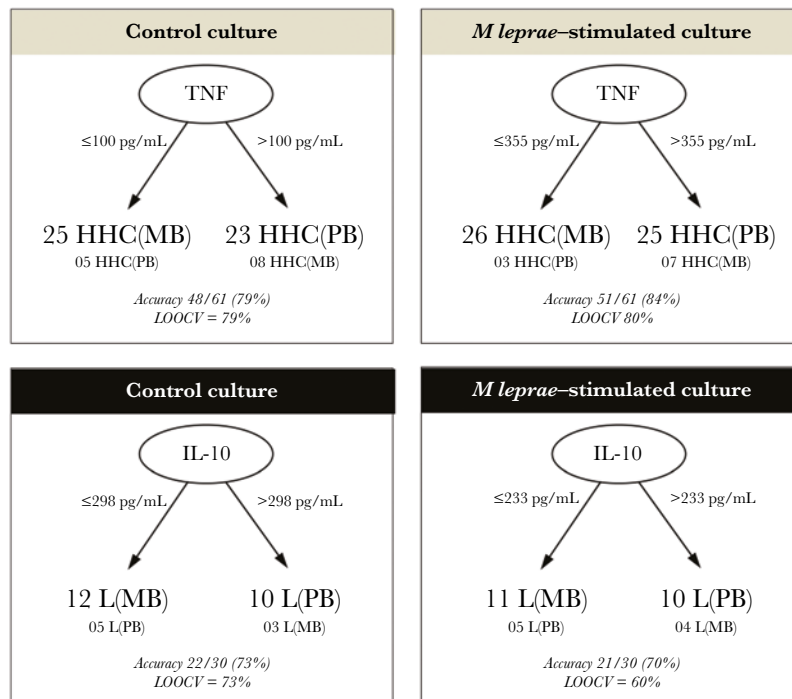


**Figure 4.** Performance of selected biomarkers to identify subgroups of patients with leprosy (L) and household contacts (HHC). The performance of cytokines (tumor necrosis factor [TNF], interferon gamma [IFN-γ], interleukin 4 [IL-4], interleukin 10 [IL-10]) secreted by peripheral blood mononuclear cells upon in vitro culture to classify HHC(PB [paucibacillary]) vs HHC(MB [multibacillary]) (left panels) and L(PB) vs L(MB) (right panels) was calculated by receiver operating characteristic (ROC) curve analysis. The area under the curve (AUC) indicates the global accuracy of each biomarker using specific cutoffs. The performance indices including sensitivity (Se), specificity (Sp), negative likelihood ratio (LR<sup>-</sup>), and positive likelihood ratio (LR<sup>+</sup>) are provided in the inserted tables. Scatterplots illustrate in detail the performance of top 2 biomarkers to classify subgroups, including TNF for HHC(PB) vs HHC(MB) and IL-10 for L(PB) vs L(MB) with high accuracy (AUC > 0.7). The frequencies of subjects classified in each subgroup are provided in the figure. The dotted line represents the cutoffs selected by the ROC curve analysis. CC indicates control culture; MI indicates *Mycobacterium leprae*-stimulated culture.

However, our results demonstrated that L(PB) could be differentiated by presenting higher IL-10 production than L(MB). In PB patients, IL-10 production can be attributed to the presence of multifunctional CD4<sup>+</sup> T lymphocytes and/or Treg cells [26, 34]. In fact, L(MB) exhibited an overall downregulation of all cytokines evaluated (Figures 2 and 3). Moreover, it is important to mention that the classical binary view of the immune response may not reflect the immunological state of all patients classified as L(MB), especially given the wide spectrum of disease manifestations. It is possible that a cytokine dysregulation model, with overall low cytokine production and T-cell unresponsiveness, may be a more appropriate model to understand the immune responses observed in L(MB) [25].

The present study has some limitations. The sample size was small and should be validated by additional analyses with a larger study sample. Another limitation is the use of isolation

of PBMCs. The use of whole blood samples would improve this assay, reducing the length of time needed for the test. In addition, it is necessary to validate the proposed model against another laboratory test to verify its ability to diagnose and classify patients [7, 11, 35, 36]. This approach combined with an algorithm design for antigen-specific in vitro assay of PBMC stimuli with subsequent cytokine measurements by ELISA could increase diagnostic sensitivity for leprosy. There is a very wide distribution of age in the study population enrolled for the present investigation. It is well known that there are likely age-related differences in cytokine production. Age-dependent changes affect the organism's ability to maintain homeostasis and to tackle stress effectively across a variety of cells, tissues, and organs, including the immune system [37]. Most experimental data on immune changes with aging show a decline in many immune parameters when compared to young healthy



**Figure 5.** Decision tree algorithms were constructed to classify subgroups of patients with leprosy (L) and household contacts (HHC) according to the operational classification (paucibacillary [PB] or multibacillary [MB]). Tumor necrosis factor (TNF) production in nonstimulated control culture and *M leprae*-stimulated culture were used as root attribute to cluster subgroups of HHC. Interleukin 10 (IL-10) production in nonstimulated control culture and *M leprae*-stimulated culture were used as root attributes to cluster subgroups of patients with leprosy. Global accuracy and leave-one-out cross-validation (LOOCV) are provided in the figure.

subjects. Together, immunosenescence and inflammaging are suggested to stand at the origin of most of the diseases of the elderly, such as infections, cancer, autoimmune disorders, and chronic inflammatory diseases. Whereas it is conceivable that global immune changes may lead to various diseases, it is also obvious that these changes may be needed for extended survival/longevity [38]. Unfortunately, in the present investigation we did not have the possibility to cover this relevant age-related aspect of the immune system. Further studies are still required to explore this matter.

In conclusion, our data demonstrated that cytokine-based assays might represent a useful approach with elevated accuracy to complement clinical diagnosis in reference laboratories. The proposed decision tree algorithms could be useful in identifying subgroups of household contacts, which may suggest early subclinical infection in contacts as well as provide an evidence of leprosy clinical form (PB vs MB), thereby supporting rational therapeutic regimens and ultimately contributing to effective control of the disease. Currently, the conventional diagnosis of leprosy is typically based on clinical evaluation alone, especially when histopathological analysis is not available. Laboratory diagnosis can help differentiate leprosy from other dermatological/neurological diseases, especially in cases of suspected recurrence, and determine an appropriate treatment duration. According to Grossi et al [39], in Brazil there is a strong tendency for health professionals to

classify patients with leprosy as having MB leprosy. Moreover, despite the practical application of an existent classification approach based on number of lesions as MB and PB that do not require sophisticated methods, several misclassifications based on this method can still be observed. The advantage of the proposed algorithm based on immunological markers as complementary information to classify leprosy patient subgroups is to provide additional data that could be associated with other clinical and laboratory findings to reach a more accurate diagnosis. In this sense, the application of the proposed algorithm could be helpful to support health professionals to make an accurate leprosy operational classification and decide which treatment to use for patients with PB or MB leprosy considering reducing the likelihood of mistreatment. It is important to mention that translation of this data into clinical care of patients and their contacts still requires further studies and validation. As future perspective, we are currently working on a device that could include these laboratorial findings together with clinical features to improve the performance of the Brazilian health services to better classify patients with leprosy and to choose the most appropriate treatment.

## Notes

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