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# Identification of bovine tuberculosis biomarkers to detect tuberculin skin test and IFNγ release assay false negative cattle

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## Abstract

Bovine tuberculosis (bTB) is an important animal and zoonotic disease, which causes severe economic losses. The main focus of this study was to assess the predictive power of previously identified biomarkers of bTB in infected animals that were negative to the tuberculin skin test (TST). We studied 16 animals with bTB, in which the disease was confirmed by necropsy, and 16 healthy animals. The level of expression of ten biomarkers (*CXCL9*, *THBS1*, *MMP9*, *IL-22*, *CXCL10*, *IFNγ*, *IL-17*, *FYVE*, *CD14*, *IL-1R*) was evaluated by RT-qPCR upon stimulation or not of peripheral blood mononuclear cells with PPDb (purified protein derivative of bovine tuberculin). In this

assay, *CXCL9*, *THBS1*, *MMP9*, *IL-22* and *IFNγ* changed their expression level depending on the bTB status. In addition, we evaluated different biomarker candidates simultaneously to infer the animal condition. By performing an analysis with classification trees, we found that the sturdiest combination was *IL-22*, *IFNγ* and *IL-1R*. On the other hand, *CXCL10*, *IFNγ* and *IL-22's* expression distinguished between bTB positive animals that were negative to TST (TST false negative animals) and the bTB negative groups. Thus, these biomarkers are promising candidates to be tested as an ancillary diagnostic assay. In addition, the expression of *CXCL10* and *IL-22* exhibited also significant differences between the bTB positive animals that were undetectable by IFNγ release assay (IGRA) and TST tests (TST and IGRA false negative animals) and the bTB negative groups. Therefore, *CXCL10* and *IL-22* constitute candidate biomarkers that could complement the two most widely used diagnostic tests.

Keywords: biomarkers, bovine tuberculosis, diagnosis

## 1. Introduction

Bovine tuberculosis (bTB) is a zoonotic disease caused by members of the *Mycobacterium tuberculosis* complex, mainly by *Mycobacterium bovis*, which represents a risk to public health and to livestock economy. The bacteria mainly infects cattle, but can be transmitted to many mammals, including humans.

In Argentina, bTB prevalence rate declined from 6.7% to 0.3% from 1969 to 2016 (http://www.senasa.gov.ar). Despite this trend, control programs did not succeed in the eradication of the disease. Nowadays bTB is still a common disease in developing countries, causing important economic losses due to livestock deaths and trade restrictions. The control of the disease is based on a test-and-slaughter policy. This eradication program is extremely costly and thus, accurate diagnostic tests are essential. The most widely used tests to diagnose bTB are the single tuberculin skin test (TST) and an ancillary test, the interferon-gamma (IFNγ) release assay (IGRA).

TST consists on the measurement of increase in skin fold thickness three days after injection of purified protein derivatives (PPD) from *M. bovis*. Different sensitivity and specificity values have been reported among *M. bovis* infected cattle ranging from 55% to 99% for the TST, influenced by the biological activity of the PPD and the inoculation site (Monaghan et al., 1994, de la Rua-Domenech, 2006). TST is the most frequently used diagnostic test due to its high availability, low costs, and because for a long time it was the only method to detect bTB. However, TST sometimes fails to differentiate cattle infected with virulent *M. bovis* from those animals sensitized with bacteria from the *M. avium* complex or environmental mycobacteria. The single intradermal comparative cervical tuberculin test (SICCT) is an alternative method that increases the

specificity of TST. SICCT consists of the simultaneous injection of both bovine and avian PPDs (PPDb and PPDa) on a same vertical plane of the shoulder with a defined distance separating the sites of injection. Despite this improvement in specificity, various factors (potency, purity, PPD dosage and interpretation of the result) strongly influence the sturdiness of the test. In addition, Amos et al. (Amos et al., 2013) have recently shown that the genetic background of the animal can also influence TST reaction.

The IGRA test involves the incubation of whole blood with PPD antigens and then the IFNγ production is measured in the collected plasma, using a capture ELISA. In previous works this test presented a sensitive between 73.0% and 100%, and its specificity varied between 85.0–99.6% (Wood and Jones, 2001), but its cost is higher than that of TST.

Both TST and IGRA tests detect the early cell-mediated immune response in bTB infection. However, at advanced stages of the disease, the cell-mediated immune response tends to decrease as opposed to an increasing humoral immune response and these tests can therefore give false negative results (de la Rua-Domenech et al., 2006). For this reason, late stage diseased animals may give false negative results. In addition, there are many other factors that can lead to variable sensitivity values of the IGRA and TST tests (errors introduced by misplacing and/or misreading the TST, biological activity of the PPD, early level infection, etc.). Therefore, novel biomarkers of infection to distinguish infected from healthy animals when TST and IGRA tests fail are urgently needed.

In order to find new biomarkers, in a previous work we assayed by microarrays the gene expression profile of bovine peripheral blood mononuclear cells (PBMCs) from animals infected with *M. bovis* upon specific stimulation (Blanco et al., 2012a). In that study,

more than 5,930 genes changed their transcription level in infected cattle compared to naïve animals. Further experiments performed with PBMCs from experimentally infected cattle validated the microarray results on a subset of selected genes (*CD14*, *IL-1R*, *THBS1*, *MMP9* and *FYVE*) (Blanco et al., 2012a). In another study our group also found that the level of expression of *IL-17* was positively associated with pathology in infected animals (Blanco et al., 2011). This study supported the use of *IL-17* as a potential biomarker of bTB infection. In addition to the biomarkers found by our group, Aranday-Cortes et al. (Aranday-Cortes et al., 2012) also observed that the transcription of *CXCL9*, *CXCL10* and *IL-22* was significantly increased in PBMCs from infected cattle compared to healthy animals following PPD stimulation.

In the present study, we evaluated the expression of *IL-17*, *CD14*, *IL-1R*, *THBS1*, *MMP9*, *FYVE*, *CXCL9*, *CXCL10* and *IL-22* as biomarker candidates of bTB infection in naturally infected and healthy cattle.

## 2. Material and Methods

### Ethics statement

The Institutional Animal Care and Use Committee (CICUAE) of CICVyA-INTA, whose regulations are in agreement with the European Union Laws for protection of experimental animals, authorized this study. Necropsies were performed in slaughterhouses authorized by the National Service of Agricultural and Food Health and Quality (SENASA).

### Selection of sampled animals

Blood samples of TST negative cattle were taken from six herds persistently infected with bTB from Argentina. Twelve of these animals presented lesions indicative of bTB

in different organs during post mortem inspection at slaughterhouses and, thus, were considered positive animals (bTB+). Four TST+/bTB+ were also included in the analysis. In every animal of the bTB+ selected group infection was also confirmed by bacteriological culture and/ or tissue PCR using primers IS*6110* (Hermans et al., 1990; Zumárraga et al., 2005) (data not shown).

The bTB negative (bTB-) group (n=16) included bovines from a herd that has been historically free of bTB. In this herd, TST is performed every six months to monitor the continuity of its bTB free status.

**Mononuclear cell preparation**

15 ml of heparinized blood extracted from each animal were used for PBMC preparation by gradient centrifugation over histopaque 1077 (Sigma Aldrich) following the manufacturer's instructions. PBMCs were incubated in RPMI (Roswell Park Memorial Institute) supplemented with bovine fetal serum (10%, Internegocios) and Antibiotic- Antimycotic 1X (Thermo Fisher) in the presence or not of PPDb (20 µg/mL, Biocor) at 37ºC for 16 hs.

**RNA preparation and RT-quantitative PCR (RT-qPCR)**

After incubation as described above, PBMCs were resuspended in 1 ml of Trizol (Sigma). Each sample was treated twice with chloroform (200 µl), centrifuged for 5 min at 9,000 g and the nucleic acids present in the aqueous phase (upper phase) were precipitated by the addition of isopropanol and subsequent incubation at -80ºC. The pellets were washed up with ice-cold ethanol 70% and resuspended in RNAse-free water. Finally, RNA solutions were cleaned up by the addition of one volume of LiCl (10M) and the samples were chilled at -80°C for 16 hours. The RNA pellets were

washed up again with ice-cold ethanol 70%, resuspended in RNAse-free water and treated with DNAseI (Ambion) following the manufacture's protocol.

The RT reactions were performed as described previously (Blanco et al., 2012a). All primers were designed using Primer 3 Software (bioinfo.ut.ee/primer3-0.4.0/). The primer sequences are shown in Table S1. The qPCR reactions were carried out with Taq Platinum DNA polymerase (Invitrogen, Life Technologies) and SYBR reagent (Thermo Fisher, Life Technologies) following the manufacturer's specifications. The reactions were performed using standard cycling conditions on Applied Biosystems StepOne plus SDS. Each reaction was performed in duplicate, and qPCR data was analysed as described previously (Pfaffl et al., 2002). The non- stimulated condition was used as the calibrator and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene. *GAPDH*'s expression has demonstrated to be stable between the different conditions and analysis of Ct data performed using Bestkeeper software (Pfaffl et al., 2004) shown acceptable expression stability (SD=0.98 and CV=5.02). In addition, *GADPH* has been used as a reference gene in previous studies of our group (Blanco et al., 2017, 2012b, 2011; Vázquez et al., 2017) and others (Buddle et al., 2003; Rhodes et al., 2007; Widdison et al., 2006).

**Interferon gamma release assay**

Heparinized blood samples were dispensed in 200 µl aliquots into individual wells of a 96-well plate. The wells contained whole blood plus 20 µg/ml of PPDb, PPDa, 1 µg/ml of pokeweed mitogen (Sigma-Aldrich) or sterile PBS 1X. The blood cultures were incubated for 16 h and plasma was harvested and stored at −80 °C. IFNγ concentration in stimulated plasma was determined using a commercial ELISA kit (Bovigam; Prionics, Zurich, Switzerland). The absorbance was read at 450 nm. A result was

considered positive if the PPDb O.D.450 minus the PPDa O.D.450 was ≥ 0.1 and the PPDb O.D.450 minus the unstimulated control O.D.450 was ≥ 0.1.

**Tuberculin skin test**

All animals were tested for TST reactivity. The animals were intradermally injected with 0.1 ml of PPDb and the reactions were read as described in Eirin et al. (Eirin et al., 2015). PPDb (32,500 IU/ml) was obtained from the National Service of Agricultural and Food Health and Quality (SENASA, Buenos Aires, Argentina). Trained veterinarians performed all TSTs using reagents with proven efficacy, thus ensuring the accuracy of the results.

**Statistical analysis**

Graphics were constructed by using GraphPad prism 5.03 software (GraphPad Software, San Diego California USA). The non-parametric Mann Whitney test was used to study significant differences between groups. For multiple comparisons a non-parametric ANOVA (Kruskal-Wallis) with Dunn´s post-test was performed.

**Classification trees**

The algorithm C5.0 was used to build classification trees. The process of fitting these models has two phases. In the first phase, called training, the algorithm is presented with a subset of animal data organized as a matrix with cases as rows (animals) and each biomarker as a column, plus a vector of observed results (bTB positive (bTB+) and negative (bTB-)). The algorithm develops a tree, or rules, to predict the results based on the data matrix. In the second phase, called testing, the tree is used to predict the cases that were left out during training. The tree performance is estimated by comparing the

predictions for these cases versus the real values. The training-testing process is repeated several times alternating the data used for training and testing to avoid the chance of obtaining an unusually good or bad classification tree. Since our dataset was relatively small, we used the leave-one variant for validation. That is, we used n-1 animals for training and one for testing and repeated the process n times until we iterated through all combination of training-testing datasets. We also repeated the whole process permuting the order of columns since this could affect the choice of best predictor by the classification algorithm. The leave-one-out method is a well-established procedure for biomarker and diagnostic test development, especially when the datasets are of a small or medium size. This method has been used and validated in various previous works for biomarkers identification (Arthur et al., 2014; Borrebaeck, 2017; Pakpour et al., 2017; Phillips et al., 2007).

The tree performance was analysed with a confusion table that summarizes the number of cases that were correctly predicted as healthy (true negatives, TN) or with bTB (true positives, TP) and the counts of wrong predictions. There are two types of wrong predictions, animals that were predicted to be bTB+ but were healthy (false positive, FP) and animals that were bTB+ but were predicted as healthy (false negative, FN). We calculated three parameters to estimate the quality of the prediction model: accuracy defined as (TP + TN) / total cases; sensitivity, which is the proportion of sick animals detected [TP / (TP + FN)]; and specificity, the proportion of healthy animals detected [TN / (TN + FP)].

## 3. Results

### Cattle sampling

In order to assess the ability to predict the bTB infection status of previously identified candidate biomarkers in TST false negative cattle, blood samples of twelve TST-/bTB+ animals were taken from high prevalence herds of Argentina. Four TST+/bTB+ were also included in the analysis. Some animals presented lesions in only one organ (focal lesions), whereas others were in various organs (multi- organic lesions) (table 1). IGRA test was performed to all bTB+ animals (n=16) and we observed that all IGRA test negative animals (n=8) were also TST negative.

**Validation of individual host markers in naturally infected cattle**

Firstly we assessed the performance of previously identified bTB biomarker candidates (*IL-17*, *CD14*, *IL-1R*, *THBS1*, *MMP9*, *FYVE*, *CXCL9*, *CXCL10* and *IL-22*) in blood samples from the selected population of healthy and infected animals. The gene expression was determined in PBMCs by measuring mRNAs by RT-qPCR after stimulating the cells with PPDb. We also measured *IFNγ* mRNA expression as a positive control of the study and, as expected, the expression of this gene was significantly up regulated in the infected animals (figure 1). *CXCL9*, *THBS1*, *MMP9* and *IL-22* presented significant differences in fold change values between the infected and healthy animals (figure 1 and table 1). On the other hand, although *IL-17* and *CXCL10* did not exhibit significant differences, their fold change values in most of the bTB+ animals were higher than the ones from the bTB- animals.

**Level of expression of individual genes in bTB+/TST- and bTB+/IGRA- animals**

We then separately analysed the expression of the candidate biomarkers in animals that presented lesions in organs (bTB+), but were negative to TST and IGRA test. The transcription of *IFNγ*, *CXCL10* and *IL-22* exhibited significant differences between the

bTB+/TST- and bTB- groups (figure 2, table S2). Thus, these biomarkers distinguished infected animals in TST false negative bovines and can therefore complement the TST test.

In addition to their ability to differentiate between bTB positive and negative animals in the group bTB+/TST- (group of TST false negatives), *CXCL10* and *IL-22* exhibited significant differences between bTB+/IGRA- and bTB- animals (figure 2, table S2). Since all IGRA negative animals are also negative to the TST test, *CXCL10* and *IL-22* constitute two candidate biomarkers that can distinguish between healthy and infected animals when the two bTB standard diagnostic tests give false negative results.

**Prediction of animal condition with classification trees**

The statistical analyses of the gene expression data with the non-parametric Mann-Whitney test revealed significant differences between bTB positive and negative animals for five genes; however, the scatter plots showed a considerable overlap between the distributions for both conditions. This means that animals with a given condition could present biomarker values that corresponded to the other condition. As an alternative to increase the accuracy of predictions, we considered inferring the condition of an animal through the simultaneous analysis of several candidate biomarkers. To this purpose, we built classification trees with the C5.0 method (Pandya and Pandya, 2015; see Materials and Methods). Thus, gene combinations that rendered the best prediction trees were *IFNγ, IL-22, CXCL10* and *IFNγ, IL-22, IL-1R*. We selected the *IFNγ, IL-22, IL-1R* combination for the next analysis, because this combination was five times more frequent than the other. Figure 3 shows the decision tree obtained.

We used the results of leave-one-out validations to build a confusion table to analyse model performance (see Materials and Methods, Table 2). The overall accuracy of the classification tree was 65% and the sensitivity and specificity were 75% and 87.5%, respectively.

## 4. Discussion

In this study we detected differential transcription of genes encoding CXCL9, IL22, THBS1, MMP9 and IFNγ between healthy and naturally infected bTB cattle. These results are consistent with previous studies showing up regulation of *CXCL9* and *IL-22* (Aranday-Cortes et al., 2012, Blanco et al., 2012a) and down regulation of *THBS1* and *MMP9* (Blanco et al., 2012a) in bTB+ animals. In addition, the expression of *IFNγ*, *CXCL10* and *IL-22* statistically differentiated bTB+/TST- from bTB- animals. These results encourage further assessment of their potential as new bTB biomarkers. CXCL9 (MIG) is a chemokine that has proven to be useful as biomarker to detect tuberculosis in humans (Chavez et al., 2016; Jacobs et al., 2016; Park et al., 2017). This is consistent with the fact that *CXCL10* (IP-10) and *CXCL9* are under the regulation of IFNγ (Kasprowicz et al., 2011). Although *THBS1*, *MMP9* and *IL-22* have been previously identified as correlates of bTB in experimentally infected cattle (Aranday-Cortes et al., 2012, Blanco et al., 2012a), this is the first work in which they were evaluated as biomarkers of bTB in naturally infected cattle.

Another relevant outcome of this study is the finding that the expression of *CXCL10* and *IL-22* not only identified bTB+ in TST- animals but also in IGRA- cattle. Thus, *CXCL10* and *IL-22* could probably be valuable targets in bTB diagnosis when TST and IGRA tests failed. In fact, *CXCL10* has been previously employed as an additional read-out for IGRA test in human tuberculosis (Whittaker et al., 2008; Ruhwald et al., 2008)

and its use increased overall test sensitivity compared to IFNγ alone (Ruhwald et al., 2011). Intriguingly, although the fold change values of *CXCL10* in most of the bTB+ animals were higher than the ones from bTB- animals, no statistically significant differences were observed between healthy and infected animals. On the contrary,, two recently published works by Parsons et al. (2016) and Sheridan et al. (2017) support the use of *CXCL10* as marker of bTB since its expression was found to be significantly different between naturally infected cattle and control animals. One possibility of the differences observed between our group and Parsons`s study is the fact that while we tested mRNA, Parsons et al. measured CXCL-10 at the protein level. However, Sheridan et al. observed significant differences in CXCL-10 levels between infected and control animals measuring mRNA. Thus, rather than differential expression at the transcriptional and post transcriptional levels, it is probable that in our hands no significant differences were observed because of the variability between animals and the small sample size.

As previously mentioned, *CXCL9* expression differentiated bTB+ from healthy cattle. However, the expression of this chemokine was not statistically different between the bTb+/TST- and bTB- groups or between the bTB+/IGRA- and bTB- groups. Therefore, *CXCL9* did not show a good performance as a complementary diagnostic tool of TST or IGRA tests.

Because of the overlap between the distributions of bTB+ and bTB- animals in most of the candidate biomarkers, we considered the use of several biomarkers simultaneously to infer the condition of an animal. One frequent solution to this problem is the use of logistic regression, in which the values of the biomarkers are used to predict the probability of having the disease. However, in this case, we could not fit logistic regression models, because some cases were predicted with probabilities of 0 or 1,

rendering unstable models. Instead, we used classification trees. With the samples herein selected, the IGRA test presented a sensitivity of 0.5 (it detected 50% of the bTB+ animals) and the built classification tree based in *IFNγ*, *IL-1R* and *IL-22* had a sensitivity of 0.75. Hence, depending on the type of samples under consideration, the test described here may increase the accuracy of blood-based diagnostic tests for bTB when applied alongside IGRA assay. Although it is difficult to use an RT-qPCR based assay at the herd level, this test could be a useful ancillary tool to be applied in those animals suspicious of having the disease that give negative results to TST and IGRA tests.

Although the herein evaluated biomarkers could potentially be used to detect false negatives of the current diagnostic methods, to validate these biomarkers as diagnostic tools a larger number of animals need to be tested. Also, it is essential to confirm the differential biomarker production between healthy and infected animals at the protein level. Thus, ELISAs to detect these protein biomarkers may be developed and used as ancillary blood tests of TST and IGRA for ante-mortem detection of bTB.

## 5. Conclusions

This study demonstrated a differential expression of *CXCL9*, *THBS1* and *MMP9* as correlates of bTB, and indicates the potential use of *IL-22* and *CXCL10* to identify bTB animals in herds suspicious of having the disease when TST and IGRA test fail. In addition, the use of classification trees allowed us to define a set of cytokines whose level of transcription can be used to increase the accuracy of IGRA test.

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**Conflicts of interest**

The authors declare no conflict of interest.

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Supplementary data

Supplementary material 1

Supplementary material 2

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Figure legends

Figure 1: Fold change of gene expression in PBMCs from infected and healthy animals upon PPDb stimulation. Fold change values from individual animals are represented by circles (infected animals) and squares (healthy animals). Median and interquartile ranges are shown. Relative gene expression was calculated using the 2-ΔΔCt method with E correction, using *GAPDH* mRNA expression as reference gene and the non-stimulated condition as the calibrator. The biomarkers from the left panel are expected to be upregulated in bTB positive animals,

whereas the ones on the right should be downregulated. Data were analysed using a two tailed unpaired Mann Whitney test (*p<0.05, **p<0.01, ***p<0.001).

Figure 2: Fold change values of individual biomarkers upon PPDb stimulation in bTB+/TST- (n=12), bTB+/IGRA- (n=8) and bTB- groups of animals. Median values and interquartile ranges are indicated for each biomarker. Significance was determined by the Kruskal-Wallis test with Dunn's multiple comparison test (*p<0.05, **p<0.01).

Figure 3: Decision tree using the C5.0 algorithm. The tree can be interpreted as a set of rules. If the biomarker *IFNγ* shows a value greater than 3.64, the animal is infected (n=10). If its value is 3.64 or lower, but *IL-22* is higher than 12.54, the animal is also infected (n=3). Then, if *IL-22* is 12,54 or lower and *IL-1R* higher than -1.65, the animal is healthy (n=10). Finally, if *IL-1R* shows a value of -1.65 or lower and *IL-22* is -1.04 or lower, the animal is healthy (n=4). The last combination contained four infected animals and one healthy.

Table1: Dataset used for subsequent analysis. All the infected animals (bTB +, 16 animals) were confirmed by necropsy. ND: not determined. FL: focal lesion. MOL: multi- organic lesions.

| Animal no. | bTB | Type of | IGRA | TST | FOLD CHANGE | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *IFNΓ* | *IL-22* | *IL-17* | *MMP9* | *THBS1* | *FYVE* | *CD14* | *IL-1R* | *CXCL9* | *CXCL10* |
| 1399 | + | FL | + | + | 44.1 | -1.82 | 2.3 | -2.94 | -11.11 | -1.61 | -1.12 | 33. | 109.07 | -1.18 |
| 1131 | + | FL | + | + | 22.9 | 102. | 5.2 | 1.38 | -6.25 | 2.34 | 1.68 | - | 74.58 | 4.26 |
| 7995 | + | MOL | - | - | 3.95 | 5.88 | 1.1 | -1.61 | -1.92 | -1.25 | -1.59 | - | 19.06 | 10.04 |
| 13235 | + | MOL | - | - | 3.23 | 194. | 10. | -1.59 | -1.94 | -1.45 | -2.13 | - | 1.36 | 54.59 |
| 3838 | + | MOL | - | - | 4.44 | 353. | 3.7 | 1.96 | 1.94 | 2.01 | 2.1 | 2.3 | -1.59 | 33.88 |
| 1050 | + | MOL | + | - | 5.94 | 17.6 | 9.2 | -3.36 | -4.46 | -1.19 | -1.09 | - | 198.51 | 25.98 |
| 1094 | + | FL | - | - | 1.88 | 16.3 | 4.1 | 1.04 | 1.21 | 1.79 | 2.28 | - | 30.57 | 11.64 |
| 1286 | + | FL | - | - | 4.59 | 127. | 7.4 | -1.92 | -5.61 | 1.3 | -1.01 | - | 52.53 | 30.67 |
| 4707 | + | MOL | - | - | 13.1 | 27.6 | 1.2 | -1.71 | -3.47 | -2.33 | 1.12 | 1.1 | 11.14 | 6.07 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5219 | + | MOL | + | - | 2.83 | 5.97 | 1.3 | -2.04 | 1.22 | -1.8 | 1.1 | - | 1.21 | 1.24 |
| 6420 | + | FL | - | - | 1.15 | 5.54 | 2.3 | -1.3 | -3.35 | 1.01 | -1.23 | -1.7 | 1 | -1.9 |
| 6752 | + | MOL | - | - | - | 13.0 | - | -1.77 | -1.87 | -1.81 | -1.48 | 1.4 | 2.1 | 3.03 |
| 2690 | + | MOL | + | - | 2.49 | 3.3 | 1.8 | -1.59 | -1.95 | -1.37 | -1.89 | - | -1.01 | 1 |
| 4308 | + | MOL | + | - | 19.7 | 2.5 | - | -2.68 | -10.82 | 9.42 | -2.25 | - | 74.74 | 103.7 |
| 75630 | + | FL | + | + | 23.9 | 7.04 | 2.5 | -3.11 | -46.29 | -1.37 | -1.86 | - | 55.89 | 18.17 |
| 75370 | + | MOL | + | + | 9.03 | 4.78 | 1.0 | -1.74 | -3 | -1.91 | -1.04 | - | 2.71 | 1.17 |
| 258 | - | ND | ND | - | 1.21 | 5.97 | 1.7 | 1.14 | -1.35 | -1.1 | 1.57 | - | 1 | -1.29 |
| 188 | - | ND | ND | - | 1.14 | -1.88 | 1.2 | -1.09 | -1.27 | -1.18 | -1.13 | - | 1.36 | 1.56 |
| 187 | - | ND | ND | - | - | -1.34 | - | -1.24 | 1.6 | 1.03 | -1.19 | - | -1.93 | -1.99 |
| 230 | - | ND | ND | - | 1.37 | 2.34 | 1.5 | -1.07 | 1.38 | 1.25 | 1.33 | 1.3 | 4.69 | 1.35 |
| 290 | - | ND | ND | - | 3.64 | 12.5 | 2.3 | 1 | 1.22 | 2.5 | 1.29 | - | -1.01 | 1.75 |
| 294 | - | ND | ND | - | - | 4.56 | 1.4 | -1.54 | -1.85 | -1.32 | -1.5 | 1.1 | 1.91 | 1.45 |
| 238 | - | ND | ND | - | - | 7.57 | - | -1.86 | -1.65 | -3.73 | -3.13 | - | 5.94 | 19.38 |
| 193 | - | ND | ND | - | 2.46 | 1.67 | 1.9 | -1.37 | 1.48 | 1.37 | -1.01 | 1.3 | 1.16 | 4.75 |
| 177 | - | ND | ND | - | 1.52 | -1.16 | - | -1.84 | -1.37 | -1.62 | -1.92 | - | 1.21 | 1.27 |
| 180 | - | ND | ND | - | 2 | 9.5 | 1.4 | -1.31 | 1.46 | 1.01 | 1.5 | 1.7 | -1.33 | 1.89 |
| 184 | - | ND | ND | - | 2.21 | 4.6 | 1.8 | 1.06 | 2.16 | 1.61 | -1.12 | 1.4 | 1.4 | 1.53 |
| 231 | - | ND | ND | - | 1.5 | -1.42 | 1.4 | -1.3 | -2.34 | 1.18 | -1.08 | -1.1 | -1.01 | 1.21 |
| 205 | - | ND | ND | - | - | -1.04 | - | -1.03 | 1.2 | -1.18 | 1.15 | -1.7 | 1.47 | -1.3 |
| 220 | - | ND | ND | - | 1.04 | 7.62 | 3 | -1.81 | -3.47 | -1.02 | -1.62 | - | 3.8 | 3.25 |
| 222 | - | ND | ND | - | 3.34 | 5.11 | 2.2 | -1.01 | -2.46 | -1.29 | -1.26 | -1.4 | 25.79 | 3.93 |
| 244 | - | ND | ND | - | 1.43 | 1.04 | 1.2 | 1.18 | -1.87 | -1.52 | -1.32 | 1.0 | 5.38 | 1.81 |

**Table 2.** Confusion matrix of the aggregated results from the leave-one-out validations.

| | **Observed condition** | |
|---|---|---|
| **Predicted** | bTB - | bTB + |
| bTB - | 14 | 4 |
| bTB + | 2 | 12 |



**Highlights**

- There is a differential expression of CXCL9, THBS1 and MMP9 as correlates of bovine tuberculosis.
- IL-22 and CXCL10 can potentially be used to identify bovine tuberculosis animals in herds suspicious of having the disease when the tuberculin skin test and the IFN-γ release assay fail.
- The level of transcription of a set of cytokines (IFN-γ, IL22, IL1R) can be used to increase the accuracy of the IFN-γ release assay.

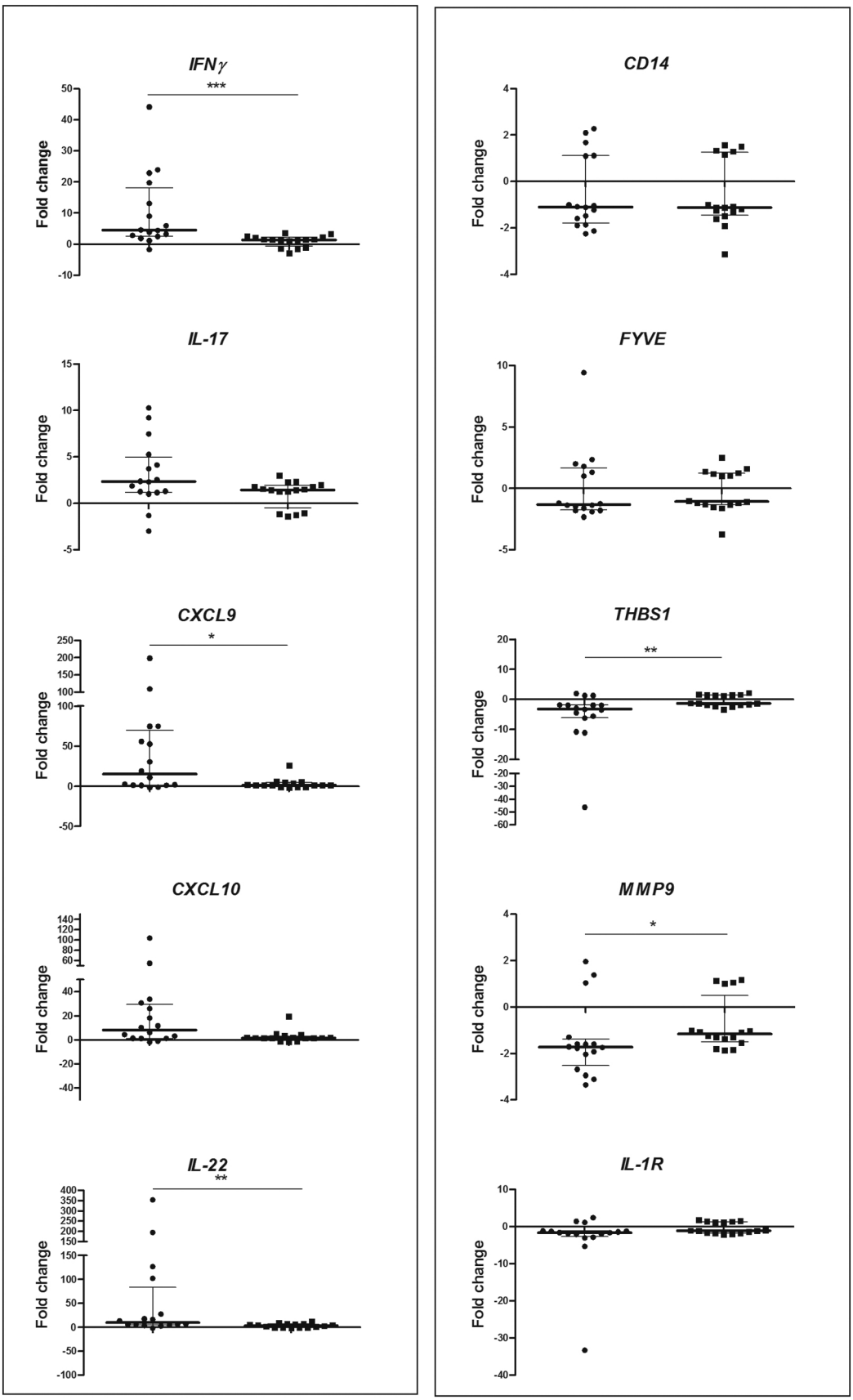
Upregulated genes in bTB+ animals
Down regulated genes in bTB+ animals
IFNγ
***
CD14
IL-17
FYVE
CXCL9
*
THBS1
**
CXCL10
MMP9
*
IL-22
**
IL-1R
Fold change
bTB+ animals
bTB- animals

Figure 1

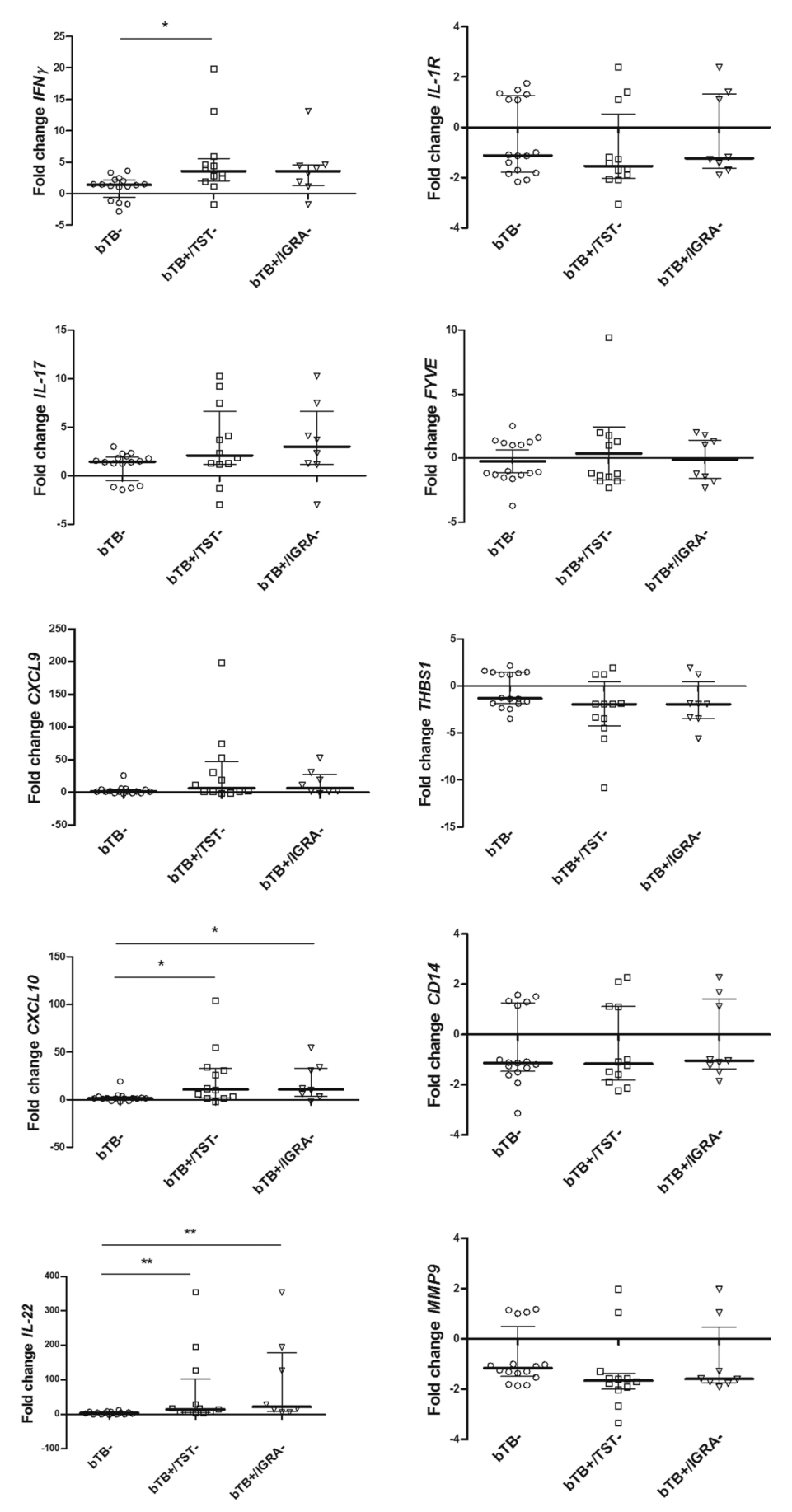
Fold change IFNγ
Fold change IL-1R
Fold change IL-17
Fold change FYVE
Fold change CXCL9
Fold change THBS1
Fold change CXCL10
Fold change CD14
Fold change IL-22
Fold change MMP9
bTB-
bTB+/TST-
bTB+/IGRA-
*
**

Figure 2

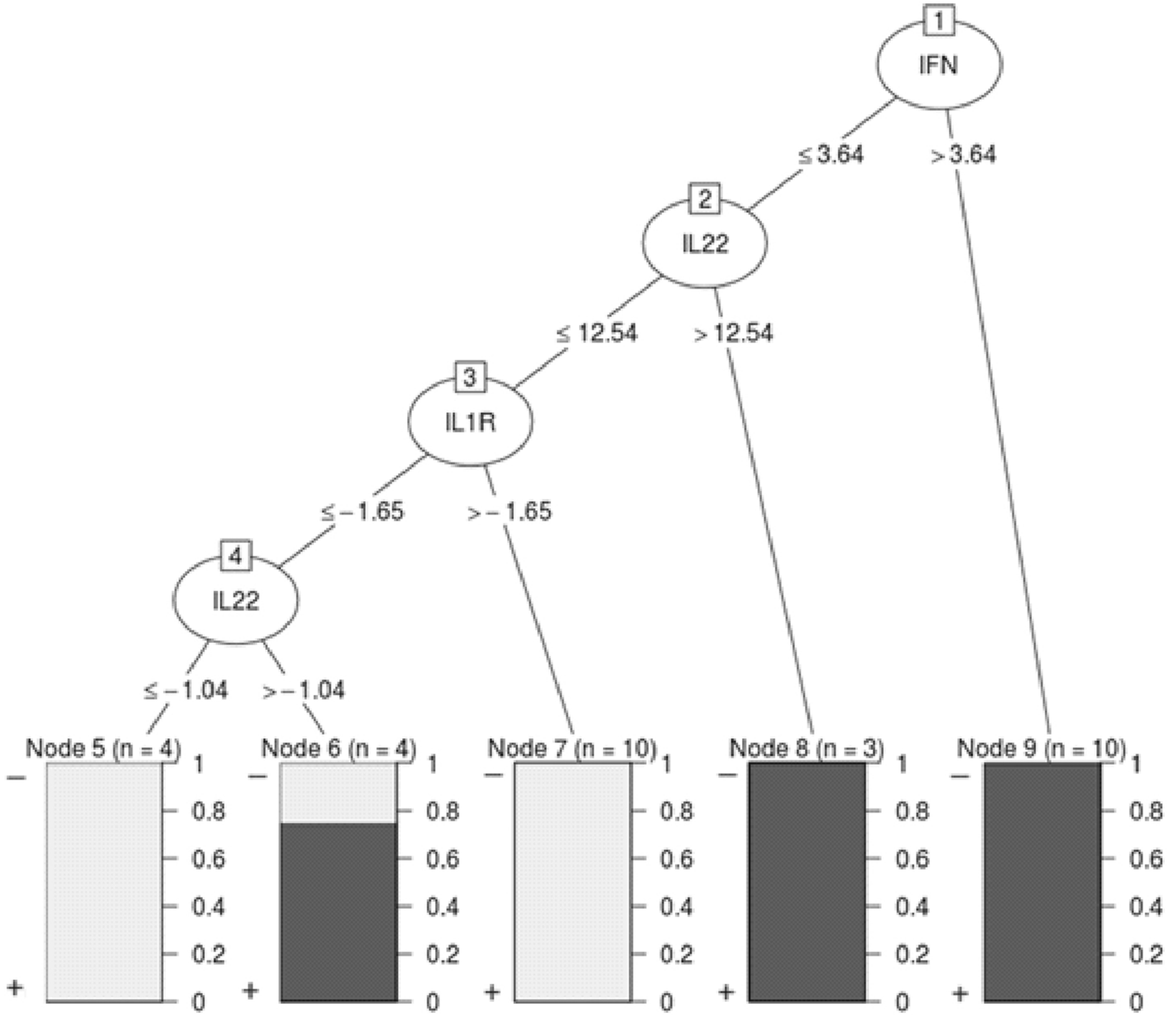
1
IFN
≤ 3.64
> 3.64
2
IL22
≤ 12.54
> 12.54
3
IL1R
≤ −1.65
> −1.65
4
IL22
≤ −1.04
> −1.04
Node 5 (n = 4)
Node 6 (n = 4)
Node 7 (n = 10)
Node 8 (n = 3)
Node 9 (n = 10)
1
0.8
0.6
0.4
0.2
0
−
+

Figure 3