Title page

THE CLINICAL RECOVERY OF TUBERCULOSIS PATIENTS UNDERGOING SPECIFIC TREATMENT IS ASSOCIATED WITH CHANGES IN THE IMMUNE AND NEUROENDOCRINE RESPONSES

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Running title

Immune-endocrine changes in TB treatment

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ABSTRACT

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a health problem worldwide. Patients with pulmonary TB show a neuro-immune-endocrine imbalance characterized by an impaired cellular immunity together with increased plasma levels of cortisol, pro- and anti-inflammatory cytokines and markedly decreased dehydroepiandrosterone (DHEA) levels. Extending these findings, we now investigated the immune-endocrine profile of TB patients undergoing specific treatment. Patients (n=24) were bled at diagnosis (T0), 2, 4, 6 months after treatment initiation and 3 months following its completion. At T0 TB patients showed increased plasma levels of IL-6, C reactive protein, IFN-γ and TGF-β. These mediators decreased during treatment, reaching levels similar to those from healthy controls (n=26). Specific treatment led to an increased lymphoproliferative response along with clinical improvement. Newly diagnosed patients had low levels of DHEA, with increased cortisol amounts and Cortisol/DHEA ratio, which normalized upon specific treatment. As regards glucocorticoid receptors (GR), TB patients at diagnosis presented a reduced mRNA GRα/GRβ ratio in their peripheral blood mononuclear cells. Further, multivariate analysis showed that Cortisol/DHEA ratio was positively associated with inflammatory mediators for which this ratio may constitute a disease biomarker. Anti-mycobacterial treatment results in a better immune-endocrine scenario for the control of physiopathological processes accompanying disease development and hence implied in clinical recovery.

INTRODUCTION

Tuberculosis (TB) is a major public health problem, constituting a leading cause of death due to an infectious agent, *Mycobacterium tuberculosis* (Mtb) [1]. Pulmonary TB is the commonest clinical manifestation ranging from mild to severe lung compromise. The cellular immune response (IR), with the production of cytokines like IFN-γ and TNF-α is involved in both disease control and pathology [2].

The chronic nature of this disease encompasses a protracted immune response that while being inefficient to eliminate mycobacteria favors chronic inflammation, along with metabolic and neuroendocrine changes likely to affect the host defense. Newly diagnosed TB patients have a neuro-immune-endocrine (NIE) imbalance characterized by increased plasma pro- and anti-inflammatory cytokines and impaired cellular IR, together with increased cortisol levels and markedly declined DHEA concentrations, resulting in a high cortisol/DHEA ratio [3, 4]. This ratio was inversely correlated with the mycobacterial-driven *in vitro* production of IFN-γ, showing that the balance between cortisol and DHEA is partly responsible for the immune disturbances seen in TB patients [5]. At the same time, some mediators involved in TB immunopathology, like TGF-β, were found to inhibit the *in vitro* production of DHEA [6].

As regards adrenal steroids these hormones are well-known for their immunoregulatory effects, wherein cortisol inhibits cellular immune reactions and proinflammatory cytokine production [7, 8], whereas DHEA displays anti-inflammatory activity along with Th1-stimulating effects [9–11].

Alterations during TB comprise changes in the level of glucocorticoid -GC- receptor (GR) expression isoforms [12]. The GR is composed of two main isoforms GRα and GRβ, the former one being biologically active whereas GRβ lacks the ability to bind cortisol and seems to function as an inhibitor of GRα-mediated transcriptional activation through the formation of GRα/GRβ heterodimers. TB patients also showed alterations in the 11β-hydroxysteroid dehydrogenase type 1 transcript (11βHSD1) [12]. Glucorticoid activity at the cellular level is influenced by the presence of 11βHSDs which catalyze the interconversion of active GCs. 11βHSD1 shifts cortisone to cortisol to facilitate GR-mediated action, whereas 11βHSD2 plays an important role in aldosterone target tissues, like the kidney, as it rapidly inactivate GCs by converting cortisol to cortisone. By inactivating endogenous glucocorticoids, 11βHSD2 allows aldosterone to access to the otherwise nonselective mineralocorticoid receptor (MR) occupancy by GC. In the kidney, 11βHSD2 is mainly expressed in collecting ducts, colocalizing with MR [13].

The bulk of evidence gathered so far can be taken to imply that both immune and neuroendocrine responses are involved in TB physiopathogenesis. As such, a thorough knowledge about the NIE changes seen in patients throughout the course of specific treatment and the ensuing clinical recovery will help to get a better understanding on the mechanisms implied in disease development and resolution. This information is also helpful for delineating the development of novel therapeutic approaches or prognostic biomarkers. According to this, we have now investigated the immune-endocrine profile of TB patients from the time of diagnosis to treatment completion and clinical recovery. We analyzed the relation between clinical and laboratory surrogates, i.e., hormones, cytokines, acute phase proteins, and lymphocyte populations. We also wanted to find out whether these immune-endocrine parameters showed some relation with disease evolution.

MATERIALS AND METHODS

Subjects

Between January 2010 and January 2016, 96 patients with newly diagnosed TB who agreed to participate in the study were recruited, but only 24 of them complied with the clinical and laboratorial follow-up protocol. All cases were HIV negative, with lung TB being diagnosed by clinical, radiological and bacteriological findings. Patients with multidrug resistant tuberculosis were excluded. Severity was classified into mild (n=2), moderate (n=13) or advanced (n=9) disease, as previously described [14]. Anti-tuberculosis therapy consisted of six months of rifampicin and isoniazid, initially supplemented by two months of pyrazinamide and ethambutol. Age-matched healthy controls (n=26) living in the same area and in no contact with TB patients were included (HCo). Exclusion criteria included diseases affecting the hypothalamus-pituitary-thyroid- or gonadal-axis, direct compromise of the adrenal gland, pregnancy, age under 18, and systemic or localized diseases requiring treatment with corticosteroids or immunosuppressants. The study protocol was approved by the Ethical Committee of the Faculty of Medical Science, National University of Rosario (Resolution nº 4397/2009) and the Centenario Hospital of Rosario (Resolution nº 124). The study was conducted in accordance with the 1964 Helsinki declaration and its later amendments. All volunteers gave their written consent prior to participate in the study.

Sample collection

Blood samples (50 ml) were obtained from TB patients at the time of diagnosis (T0), 2, 4, and 6 months (T2, T4, T6) after starting anti-tuberculous treatment, and three months following treatment completion (T9). All samples were obtained between 8:00-9:00 a.m. using EDTA as anticoagulant. Aprotinin (100 U/ml; Sigma-Aldrich, USA) was added to the plasma and samples were preserved at -70ºC. One blood sample (50 ml) was obtained from HCo and processed in the same way.

Mononuclear cell isolation and in vitro stimulation

After blood centrifugation the buffy coat was separated and diluted 1:1 in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing standard concentrations of L-glutamin, penicillin, and streptomycin (culture medium, CM). Cell suspension was layered over a Ficoll-Paque (density 1.077, Amersham Biosciences, NJ, USA) and centrifuged at 400g for 30 min. Peripheral blood mononuclear cells (PBMC) were obtained and resuspended in CM containing 5% of heat-inactivated AB human sera (PAA Laboratories GmbH, Austria). Cells were cultured in quadruplicate in flat-bottomed microtiter plates ($2x10^5$ cells/well in 200 µl) with or without addition of Mtb strain H37Rv gamma irradiated (Mtbi; 8µg/ml, Colorado University, USA). Concanavalin A (ConA; 2.5 µg/ml, Sigma-Aldrich, USA) was used as a proliferation positive control. PBMC cultures were incubated for 5 days at 37ºC, in a 5% CO_2 humidified atmosphere and pulsed with 3H-thymidine for 18 hr. Proliferative responses are expressed as the stimulation index (SI).

Flow Cytometry

A sample of EDTA anticoagulated whole blood was incubated with BD Tritest CD4FITC/CD8PE/CD3PerCP Reagent (BD Biosciences, San Jose, CA, USA) and isotype controls, according to manufacturer instructions [15]. Another sample of PBMC was incubated with anti-CD4-FITC, anti-CD25-PEcy5.5 and anti-human FoxP3-PE (BD Biosciences, San Jose, CA, USA) [15]. Stained cells were analyzed with a FACSAria II flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of positive cells and the mean fluorescence intensity (arbitrary units) for a specific marker were calculated using FACSDiva software (BD Biosciences, San Jose, CA, USA). For purpose analysis 30000 events were recorded.

Cytometric bead array (CBA)

Cytokine analysis was done using the Human Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, CA, USA) which allowed for the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A (Detection limits -Dl-: 2.6; 4.9; 2.4; 4.5; 3.8; 3.7 and 18.9 pg/ml respectively). CBA analysis was performed according to manufacturer instructions. Cytokine plasma levels were calculated using BD CBA software (version 4.0, BD Biosciences).

Evaluation of immunological mediators and Hormones

Cytokines and hormones levels were measured in plasma using commercially ELISA kits: IFN-γ, IL-4, TGF-β (BD optEIA, BD Biosciences, San Jose, CA, USA, Dl: 4.7; 2 and 125 pg/ml respectively), IL-17 (Cytoscreen, BioSource Europe, Belgium, Dl: 2 pg/ml), IL-6 (ELISA-Invitrogen, Carlsbad, CA, USA, Dl: 0.07 pg/ml), cortisol and DHEA (EIA, DRG Systems, Germany, Dl: 2.5 and 0.108 ng/ml, respectively). C reactive protein (CRP) levels were measured by high sensitivity Turbitest (Wiener Lab., Rosario, Argentina, Dl: 2.5 mg/l).

RNA isolation, cDNA synthesis and qPCR

Total RNA was isolated from PBMC using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA pellets were dissolved in DEPC sterile water and stored at -80°C. RNA quantity and integrity was assessed as performed earlier [12]. cDNA was synthesized from 2 µg of total RNA by extension of oligodT primers (Invitrogen, Carlsbad, CA, USA) with M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania) in a final volume of 40 µl DEPC sterile water. cDNA was stored at -80°C until use it. qPCR was performed with the Stratagene Mx3000P QPCR System (Amersham, USA) using 5 µl of cDNA dilution, 0.4 µM of each primer and 4 µl of 5xHOT FIREPol EvaGreen qPCR Mix Plus (Rox) (Solis BioDyne, Tartu, Estonia), final volume of 20 µl. Thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C followed by 45 PCR cycles of denaturing at 95°C for 15 s, 30 s for annealing at 60°C and 20 s for elongation at 72°C. Fluorescence readings were performed during 10 s at 80°C before each elongation steps. To normalize the expression of every gene, the transcript of PPIA [peptidylprolyl isomerase A (Cyclophilin A)] was used as an endogenous control in each mononuclear cell sample [16].

Serially diluted cDNA samples synthesized from Jurkat and NCI-H295R cell line expressing GRα and GRβ, and 11βHSD1 and 11βHSD2 mRNA, respectively [17, 18], were used as relative external standards in each run, as performed previously [12]. Similarity and homogeneity of PCR products from samples were confirmed by automated melting curve analysis (MxPro QPCR Software for Mx3000P, Agilent Technologies, USA), which revealed melting temperature values

of the PCR products. Selected primers are detailed in Table 1. Data were expressed as arbitrary units -AU-: relative quantity of the transcript respect to PPIA [peptidylprolyl isomerase A (Cyclophilin A)].

Statistical analysis

Between-group comparisons (TB vs. HCo) were performed by non-parametric Kruskall–Wallis analysis of variance followed by a post-hoc test when applicable, since some variables under analysis deviated from normal distribution. Paired comparisons during treatment were done by Friedman analysis of variance. Correlations were analyzed by the Spearman's rank test. A multivariate Principal Component Analysis using Spearman´s rank correlation (SAS software, 9.2 version) was applied for conjoint variations at the different time points. Data were considered statistically significant when $p<0.05$.

RESULTS

There were no sex- or age-related differences and degree of BCG vaccination between study groups, although the body mass index (weight/height^2) -BMI- was significantly decreased in TB patients (Table 2). Clinical laboratory data did not differ between groups, except for triglyceride and cholesterol levels, which were decreased in TB patients at T0 (Table 2). Levels of liver enzymes among treated patients remained within normal values.

Lymphoproliferation assays

In line with previous findings, newly diagnosed patients had a decreased Mtbi-driven proliferation compared to HCo ($p<0.01$, Figure 1). Such difference was no longer detected during treatment (Figure 1).

Lymphocyte subpopulations in TB patients during specific treatment

TB patients had increased white blood cells at T0 ($p<0.05$, vs. HCo), without changes during follow-up. They also showed increased and decreased relative numbers of neutrophils and lymphocytes, respectively ($p<0.05$ vs. HCo), that recovered throughout treatment. Flow cytometry studies showed decreased relative numbers of CD4+ and CD8+ T lymphocytes in newly diagnosed patients (Table 3) that reached normal levels upon starting treatment.

As reported [15], untreated patients had increased percent values of CD4+CD25+FoxP3+ T cells compared to HCo (Table 3). Such increase was even more noticeable at T2 and T4 evaluations, particularly at the latter time point. After treatment termination, Treg values decreased to levels recorded in HCo (Table 3).

Plasma immune mediators during TB treatment

At diagnosis, TB patients had increased plasma levels of IFN-γ, IL-6, CRP and TGF-β (Figure 2). Concentrations of IFN-γ declined during treatment to levels similar to HCo by T6 (Figure 2A). Levels of IFN-γ at T0 were found negatively associated with BMI ($r=-0.55$; $p<0.05$; $n=14$) and CD4+ T cell counts ($r=-0.54$; $p<0.05$; $n=14$). IL-6 levels started to decrease reaching similar concentrations to the HCo group by T6 (Figure 2B). Increased IL-6 levels at diagnosis correlated negatively with BMI ($r=-0.65$; $p<0.03$; $n=19$), and positively with IFN-γ levels ($r=0.80$; $p<0.02$; $n=14$). TB patients had increased CRP levels at diagnosis, which dropped to normal levels at T2 (Figure 2C). As expected, CRP and

IL-6 correlated positively (r=0.57; p<0.02; n=17). Levels of TGF-β lowered to values recorded in HCo (Figure 2D), following treatment initiation.

Changes in Cortisol and DHEA levels during TB treatment

Increased cortisol concentrations either at diagnosis or throughout treatment (p<0.05 vs. HCo), reached normal values at T9 (Figure 3A). At diagnosis, levels of IL-6 and cortisol were positively correlated (r=0.54; p<0.05; n=18). There was a significant increase of cortisol levels in moderate and severe patients at T0 (Fig. 4A and 4B). Cortisol levels remained augmented in moderate patients, until T9 when they lowered to HCo values (Figure 4A). In severe cases augmented cortisol values persisted until T2 (Figure 4B).

DHEA levels appeared decreased in untreated TB patients (p<0.001, Figure 3B) mostly in severe cases (Figure 4D). Moderate TB patients showed higher DHEA levels than severe ones at T0 [moderate, 4.5 ng/ml (3.7-6.7) n=13; severe, 2.7 ng/ml (2.1-4.0) n=9, p<0.03, median and 25-75 percentiles]. Specific treatment led to a recovery of DHEA levels (Figure 3B) although T9 levels situated below those from HCo (p<0.05). Further analysis by severity revealed a similar pattern in moderate patients (Figure 4C). Severe cases displayed a significant increase of DHEA levels from T2 afterwards (T0 different from T2, T4 and T6 p<0.05), but their values remained lower than controls (Figure 4D). The relative increase of DHEA was similar in both groups [i.e., increase in T2 respect to T0: moderate, 82.8% (49.9-129.4) n=13; severe, 103.3% (45.3-189.0) n=9, median and 25-75 percentiles]. There was a negative correlation between IL-6 and DHEA, at diagnosis (r=-0.62; p<0.03; n=17), and T2 (r=-0.40; p<0.05; n=19).

Untreated patients showed increased Cortisol/DHEA ratio (Figure 3C), particularly in severe cases (Figures 5A and 5B). Such unbalanced Cortisol/DHEA ratio from severe cases normalized by T4 (Figure 5B).

Relative levels of mRNA expression for GRα, GRβ along with 11βHSD1 and 11βHSD2 enzymes in PBMC from TB patients

Expression of GRα in PBMC from TB patients (n=16) was similar to those recorded in HCo (n=16), except for T4 evaluation where its expression was significantly higher than baseline values (T0, p<0.05). GRβ levels, which were increased at diagnosis, lowered to normal values during treatment (p<0.05 vs. T0).

Among patients, GRα transcript levels correlated inversely with IL-6 at diagnosis (r=-0.62; p<0.04; n=16). Expression levels of GRα at T2 were negatively correlated with IFN-γ concentrations (r=-0.63; p<0.03; n=14). The GRα/GRβ ratio was only decreased at T0, suggesting that cortisol resistance may disappear as patients undergo treatment (Table 4).

While search for 11βHSD2 transcripts (enzyme that catalyzes the conversion of cortisol to cortisone) was unsuccessful in most PBMC samples, 11βHSD1 transcript levels in PBMC from TB patients at T0 were significantly higher than values recorded at T2, T4, T6 and T9, and those from HCo (Table 4).

Multivariate analysis for principal components

To ascertain variables more likely to differentiate individuals, the multivariate analysis for principal components was applied. Variables included age, BMI, erythrocyte sedimentation rate (ERS), CD4+ T cells, IFN-γ, IL-6, CRP, TGF-β, Cortisol, DHEA, Cortisol/DHEA ratio, and GRα. Results are depicted as a biplot graphics, summarizing data from the

first two components which account for approximately 65-70% of the observed variance for selected variables, at each time-point evaluation. Variables conforming an angle below 90°, and showing a greater distance from the origin, represented those more likely to show a positive association. By contrast, variables located to form angles above 90° and distant from the origin were negatively associated.

As shown in Figure 6A, at T0 inflammatory-related variables were positively associated each other and with the Cortisol/DHEA ratio, but correlated negatively with BMI (Figure 6A). This association disappeared upon treatment initiation (Figure 6B). There was a negative correlation between DHEA, ERS and CRP levels. Strikingly the Cortisol/DHEA ratio remained associated with some proinflammatory parameters at T2 and T4 (Figure 6B and C). At T6 ERS, CRP and IL-6 were positively related (Figure 6D). The correlation with the latter cytokine also remained significant at T9, in addition to a positive association between CRP and Cortisol/DHEA ratio (Figure 6E), and both adrenal steroids (Figure 6E).

DISCUSSION

Control of an infectious process depends on the type and magnitude of the defensive response that appear beneficial during the initial phase, but may become harmful if prolonged due to pathogen persistence, as is the case of TB [19]. Some immune mediators released during infections (TNF-α, IL-1, IL-6) [20] induce important neuroendocrine modifications leading to changes in defense mechanisms and energy regulation [21], likely to impact on clinical manifestations, like the weight loss seen in TB patients [4,19]. In fact, the decline in BMI from newly diagnosed TB patients was associated with inflammation [22], given the inverse correlation between BMI and IL-6, while treatment-associated clinical improvement was accompanied by a significant increase in the BMI (data not shown).

Some groups reported low circulating levels of CD4+ T cells in patients with pulmonary TB [23,24], but in our case TB patients had decreased amounts of both lymphocyte populations which further recovered during treatment, as reported by Al-aska and cols. [25]. Factors accounting for this may have to do with increased apoptosis, homing changes [24], increased amounts of Treg cells and proinflammatory cytokines displaying pro-apoptotic capacity (i.e., IFN-γ) [26], together with augmented cortisol concentrations known to affect the functional capacity and survival of T lymphocytes [27]. In this regard, decreased CD4+ T cells at T0 were negatively associated with cortisol levels and IFN-γ. In line with the study by *Wang et al* [28] in TB patients with radiological cavities, our patient series with moderate or severe cavitary disease also had a diminished lymphoproliferation at diagnosis. Noticeably, after two months of treatment they restored their mycobacterial-driven proliferation.

As reported earlier, newly diagnosed TB cases had augmented levels of IL-6, CRP, IFN-γ and TGF-β. During Mtb infection, presence of IL-6 is essential for IFN-γ production by cells of innate immunity [29]. Macrophage activation by IFN-γ is essential to destroy Mtb, however an excess of IL-6 may counteract the activating effects of IFN-γ [30]. The relationship between these cytokines is underlined by the positive correlation shown at T0 and T2. As expected, patients with TB showed a positive association between IL-6 and CRP the time when the concentration of both proteins was highest. During treatment CRP levels declined sharply to controls values.

The cellular pattern observed in TB patients at diagnosis was compatible with a Th1 profile, essential for containing mycobacteria [31,32], since plasma levels of IL-2, IL-4, IL-17 and IL-10 were undetectable in a high number

of samples. Treatment-mediated clinical improvement was accompanied by a fall of these mediators, in line with other studies [33, 34].

As reported earlier, TB was characterized by low levels of DHEA along with increased cortisol concentrations [3,35], which may reflect adrenal alterations addressed to preserve cortisol production in an attempt to counteract the inflammatory response during active disease. TGF-β levels, which may also exert suppressive functions [36] were increased at diagnosis and dropped to controls values at T2. We have shown a cross-talk between TGF-β and DHEA [37,38], since this cytokine inhibited DHEA synthesis by human adrenal cells [3,6], whereas DHEA inhibited the otherwise increased production of TGF-β by PBMC from severe TB cases [14]. Findings at the end of the follow-up period (T9) showed increased DHEA, decreased Cortisol/DHEA ratio and normalized cortisol values, compatible with a better adrenal function linked to the disappearance of potentially harmful endogenous compounds [39]. Notably, treated patients with severe TB never reached normal values of DHEA, whereas moderate cases did so by T2. DHEA may be beneficial in TB because of its anti-inflammatory and Th1-promoting effects [40]. It cannot be ruled out that changes in plasma concentrations of DHEA during anti-tuberculosis treatment may be associated with anti-TB treatment. For instance, Rifampicin increases the metabolism of cortisol and may also affect steroidogenesis [41,42].

In line with other studies indicating that the relation between cortisol and DHEA, rather than the individual concentrations is more representative of disease activity [43], in our case the increased ratio seen in TB patients at diagnosis [3, 19] fell significantly by T2, when clinical improvement was evident.

The coexistence of high concentrations of proinflammatory mediators and modestly increased cortisol may not only imply a relative deficiency in cortisol production but also the presence of a GC resistant state [3]. Newly diagnosed patients with progressive disease, seem to also have some GC resistance [12], considering the low GRα/GRβ ratio, along with the increased expression of 11βHSD1. Such resistant state seems to disappear during treatment, allowing cortisol to exert its biological action partly reflected in the inverse correlation between this hormone and IL-6, at T4. The GRα/GRβ ratio at T0 was decreased at the expense of an increased GRβ isoform, that when forming a heterodimer receptor with GRα inhibits ligand binding, hindering the hormone to exert its biological action. Evidence points out that increased GRβ transcript is favored by inflammatory cytokines [44].

For the multivariate analysis, we selected variables of relevance to get some insight about parameters that may define patient groups at different stages of the study. Despite we could not establish variables differentiating groups of patients; the Cortisol/DHEA ratio was positively associated with inflammatory markers such as IL-6, IFN-γ, CRP and ERS. Patients started to show a clinical improvement and less inflammation by T2, but the Cortisol/DHEA ratio continued to be correlated with CRP and ERS. In this way, the Cortisol/DHEA ratio emerges as a marker of inflammation-related disease activity.

The assessment of systemic mediators is a strategy trying to assess whether their variations are related to the patient's clinical status. This assessment is less consistent when a single measurement is performed but becomes much more valuable when performed sequentially. This is the case of the present study, which not only extends some former observations but also provides novel information about modifications encompassing specific treatment. Collectively, the results suggest that anti-bacillary treatment involves a series of changes not only in the immune profile, but also in the neuroendocrine one, showing a correlation with the favorable course of TB linked to the processes underlying disease resolution.

Our study was performed in two centers with a relatively reduced sample size. Further studies in a larger sample in patients with a good clinical response to antituberculosis treatment or not will help to better ascertain the potential prognostic value of some clinical or laboratorial measures.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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FIGURE LEGENDS

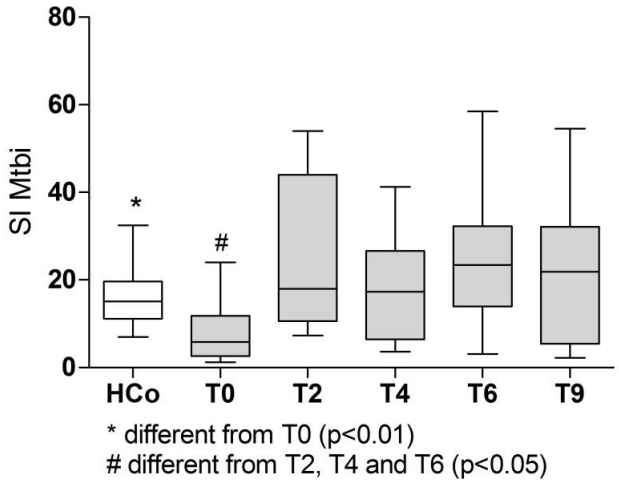


Figure 1. Lymphoproliferative response in patients with TB undergoing specific treatment. Boxes represent median (line) and interquartile range, with minimal and maximum values. **HCo (n=26):** healthy controls; **T0:** time at diagnosis; **T2, T4 and T6:** 2, 4 and 6 months following the initiation of anti-bacillary treatment; **T9:** three months following treatment completion. **SI**: stimulation index (average of counts per minute –cpm- in stimulated cultures/ average of cpm in unstimulated cultures); **Mtbi:** *M. tuberculosis* killed by gamma irradiation. A total of 24 TB patients were studied at all time points.

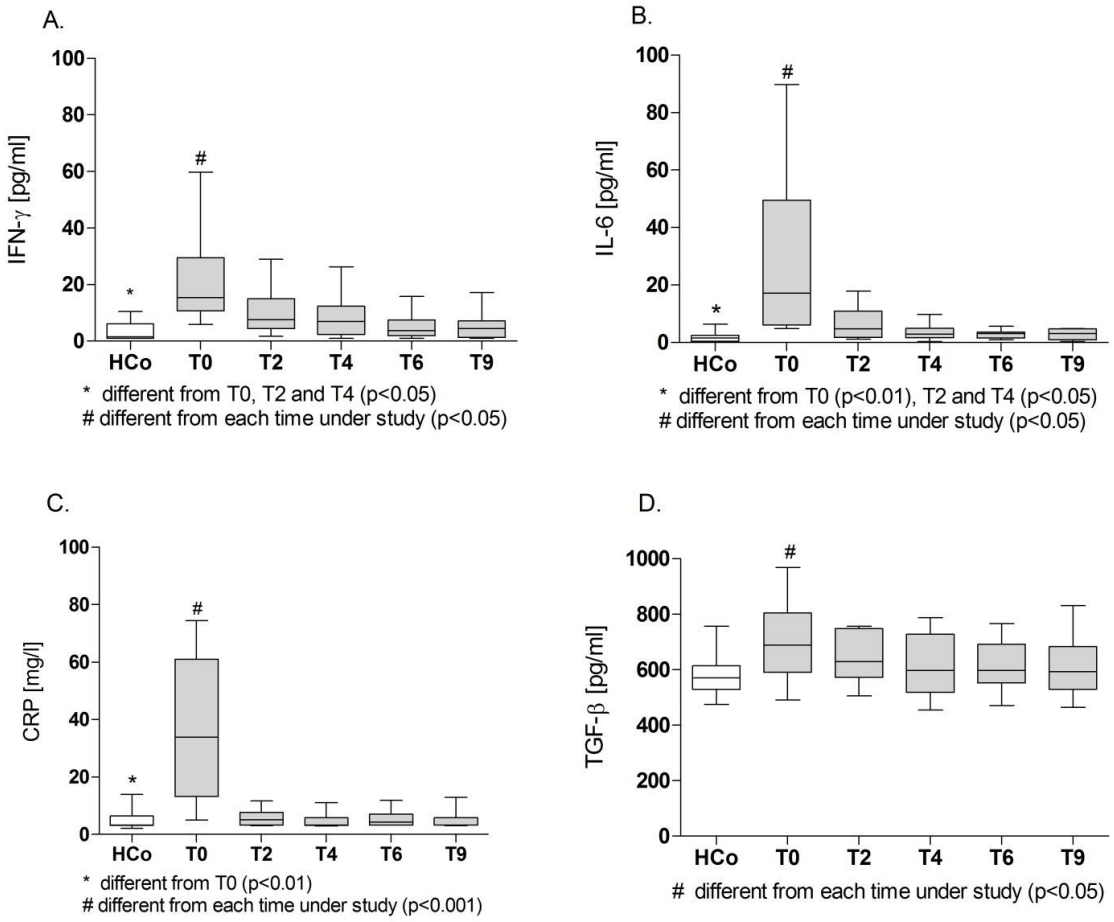


Figure 2. Plasma levels of pro- and anti-inflammatory mediators in patients with TB undergoing specific treatment. IFN-γ (**A**), IL-6 (**B**), C reactive protein (**C**) and TGF-β (**D**). Boxes represent median (line) and interquartile range, with minimal and maximum values. **HCo (n=26):** healthy controls; **T0:** time at diagnosis; **T2, T4 and T6:** 2, 4 and 6 months following the initiation of anti-bacillary treatment; **T9:** three months following treatment completion. A total of 24 TB patients were studied at all time points.

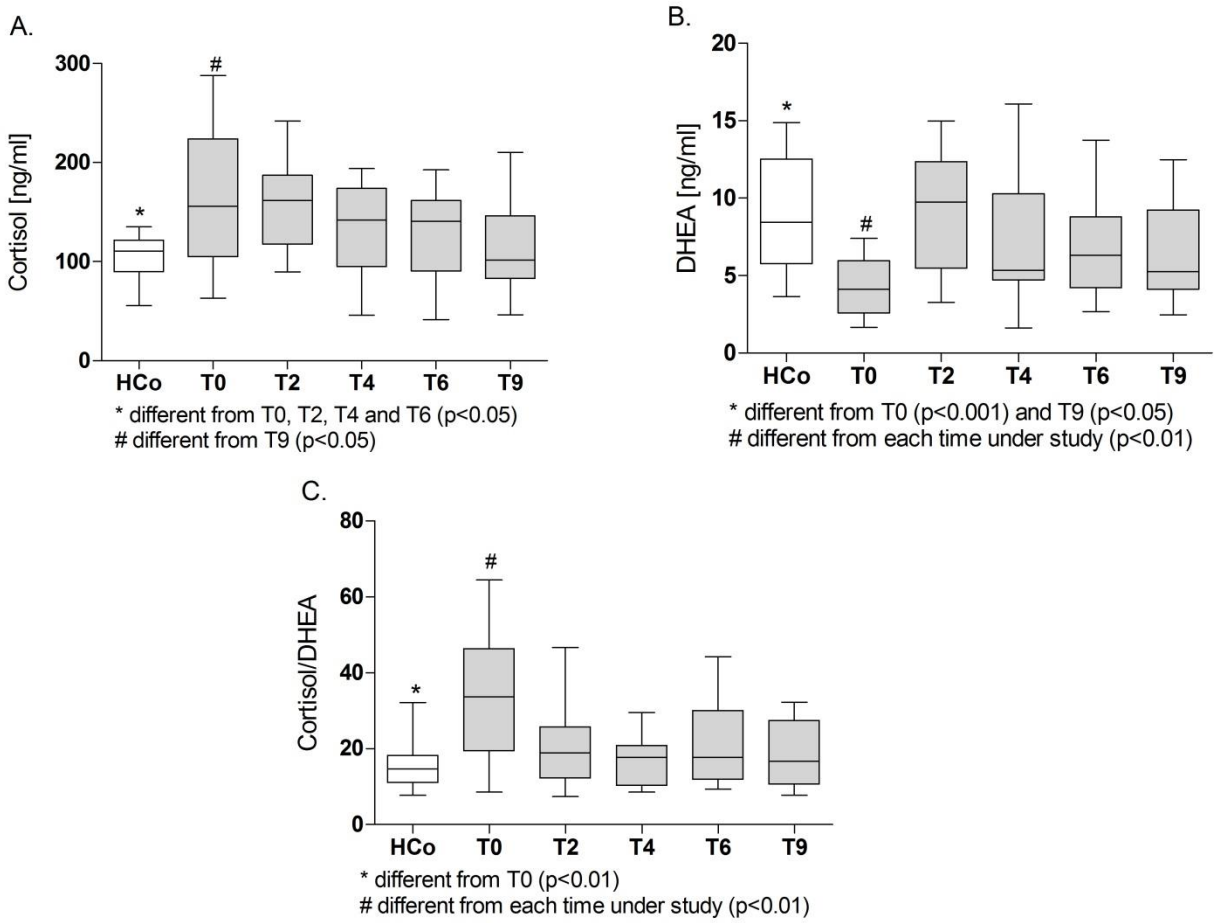


Figure 3. Plasma levels of Cortisol (A), DHEA (B) and Cortisol/DHEA ratio (C) in patients with TB undergoing specific treatment. Boxes represent median (line) and interquartile range, with minimal and maximum values. **HCo (n=26):** healthy controls; **T0:** time at diagnosis; **T2, T4 and T6:** 2, 4 and 6 months following the initiation of anti-bacillary treatment; **T9:** three months following treatment completion. A total of 24 TB patients were studied at all time points.

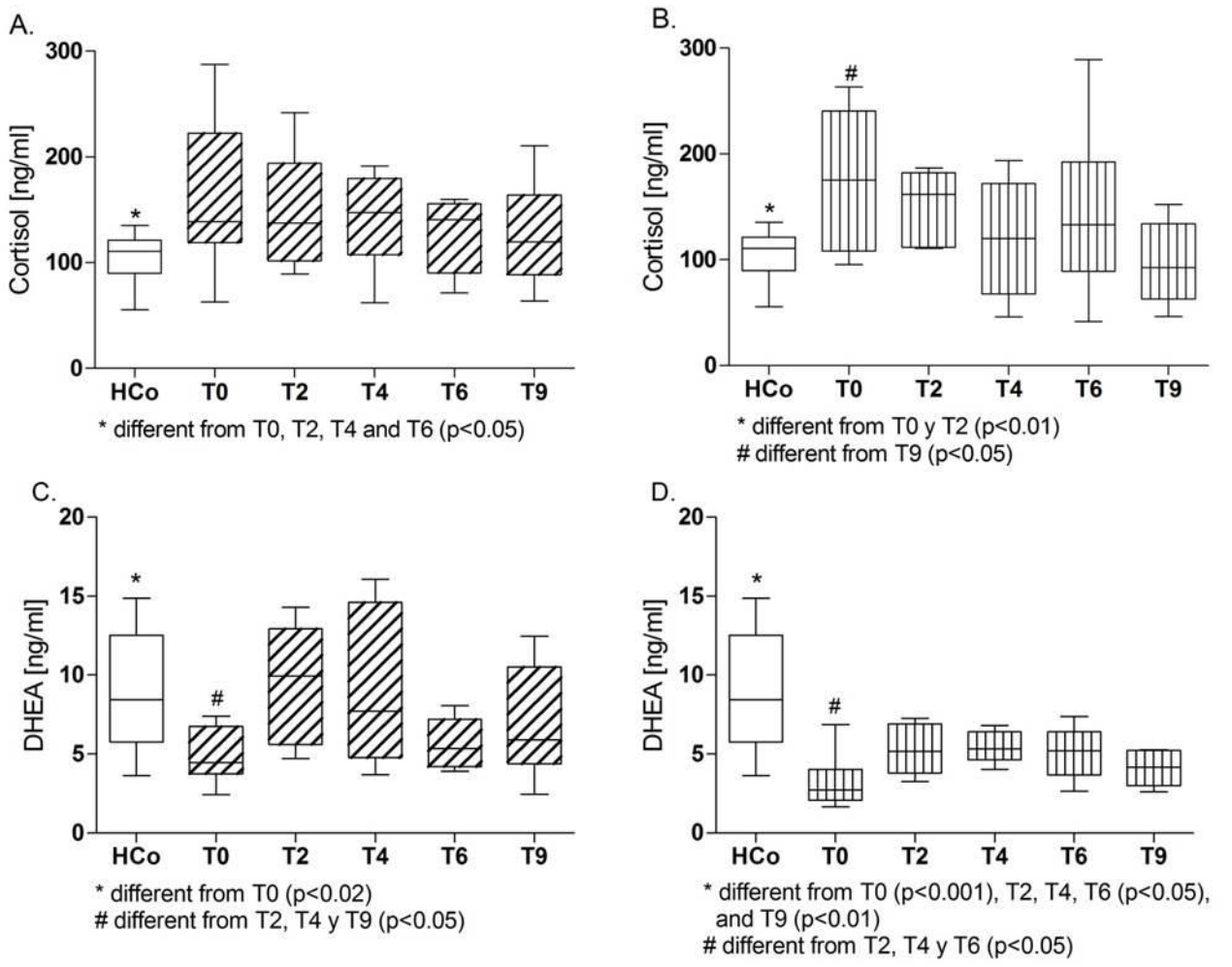


Figure 4. Plasma levels of Cortisol and DHEA in patients with moderate (A and C) and severe (B and D) tuberculosis during specific treatment. Boxes represent median (line) and interquartile range, with minimal and maximum values. **HCo (n=26):** healthy controls; **T0:** time at diagnosis; **T2, T4 and T6:** 2, 4 and 6 months following the initiation of anti-bacillary treatment; **T9:** three months following treatment completion. A total of 24 TB patients were studied at all time points.

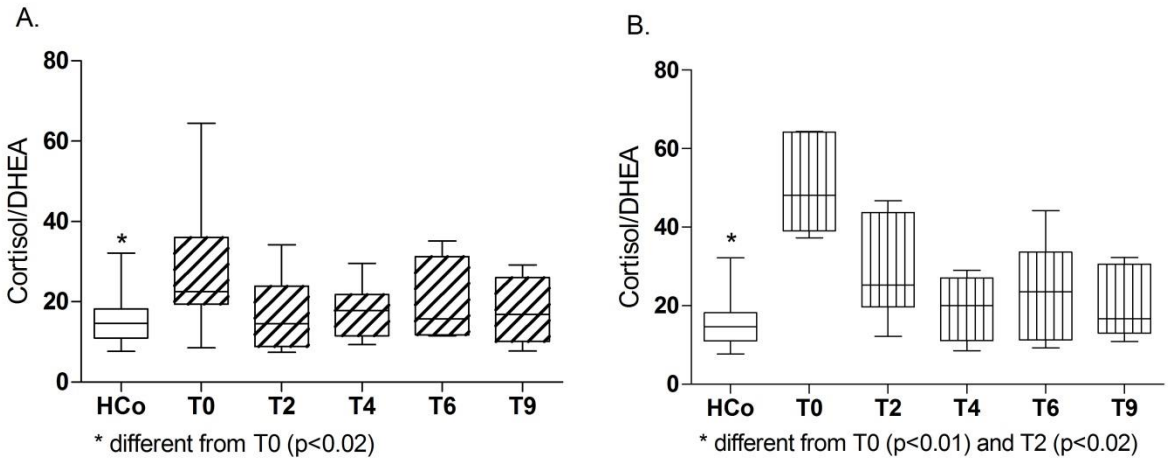
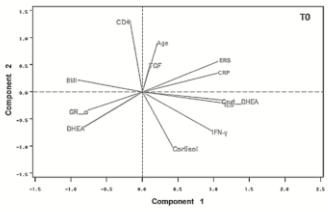


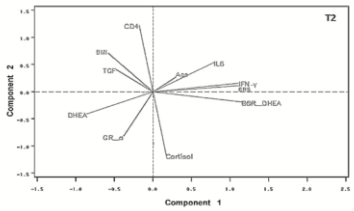
Figure 5. Cortisol/DHEA ratio in patients with moderate (A) and severe (B) tuberculosis. Boxes represent median (line) and interquartile range, with minimal and maximum values. **HCo (n=26):** healthy controls; **T0:** time at diagnosis; **T2, T4 and T6:** 2, 4 and 6 months following the initiation of anti-bacillary treatment; **T9:** three months following treatment completion. A total of 24 TB patients were studied at all time points.

A)



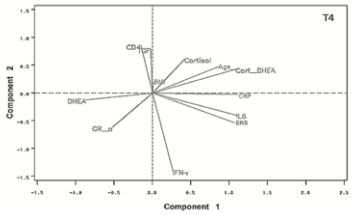
Correlations	T0	p
IL-6 vs. CRP	r= 0.80	<0.01
IL-6 vs. ERS	r= 0.82	<0.01
IL-6 vs. IFN-γ	r= 0.82	<0.01
IL-6 vs. Cortisol/DHEA	r= 0.87	<0.01
CRP vs. ERS	r= 0.81	<0.01
CRP vs. IFN-γ	r= 0.59	<0.05
Cortisol/DHEA vs. CRP	r= 0.71	<0.01
Cortisol/DHEA vs. IFN-γ	r= 0.78	<0.01
Cortisol/DHEA vs. ERS	r= 0.73	<0.01
DHEA vs. CRP	r= -0.63	<0.05
DHEA vs. ERS	r= -0.68	<0.05
IL-6 vs. BMI	r= -0.59	<0.05
CRP vs. BMI	r= -0.56	<0.05
IFN-γ vs. BMI	r= -0.58	<0.05
Cortisol/DHEA vs. BMI	r= -0.59	<0.05

B)



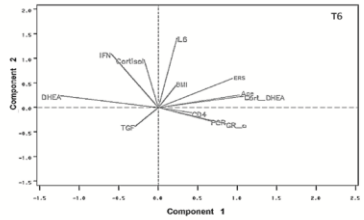
Correlations	T2	p
IL-6 vs. IFN-γ	r= 0.68	<0.05
CRP vs. ERS	r= 0.91	<0.01
CRP vs. IFN-γ	r= 0.82	<0.01
IFN-γ vs. ERS	r= 0.74	<0.01
Cortisol/DHEA vs. CRP	r= 0.89	<0.01
Cortisol/DHEA vs. IFN-γ	r= 0.67	<0.05
Cortisol/DHEA vs. ERS	r= 0.82	<0.01

C)



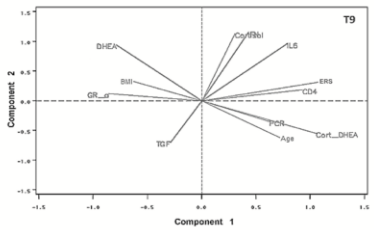
Correlations	T4	p
IL-6 vs. CRP	r= 0.76	<0.01
IL-6 vs. ERS	r= 0.66	<0.05
PCR vs. ERS	r= 0.76	<0.01
Cortisol/DHEA vs. CRP	r= 0.64	<0.05
Cortisol/DHEA vs. ERS	r= 0.58	<0.05

D)



Correlations	T6	p
IL-6 vs. ERS	r= 0.57	<0.05
CRP vs. ERS	r= 0.65	<0.05

E)



Correlations	T9	p
IL-6 vs. ERS	r= 0.68	<0.01
Cortisol/DHEA vs. CRP	r= 0.69	<0.01
DHEA vs. Cortisol	r= 0.56	<0.05

Figure 6. Multivariate analysis from principal components and correlations between clinical parameters, cytokines, hormones and transcripts in TB patients during specific treatment. T0: time at diagnosis (A); **T2, T4 and**

T6: 2 (B), 4 (C) and 6 (D) months after the initiation of anti-bacillary treatment; **T9**: three months following treatment completion (E). **CRP**: C reactive protein; **ERS**: erythro sedimentation rate; **BMI**: body mass index; **GR:** glucocorticoid receptor, **r**: Spearman correlation coefficient.

Table 1. qPCR nucleotide primer sequence

Transcript	Forward primer	Reverse primer	Size
CycA	CycA-F	CycA-R	101 bp
PPIA GeneID: 5478	5'-ggt cct ggc atc ttg tcc at-3'	5'-ttg ctg gtc ttg cca ttc ct-3'	
GRα	GR-F	GRα-R	159 bp
NR3C1, GeneID: 2908 **Transcript variant 1**	5'-gaa gga aac tcc agc cag aac-3'	5'-gat gat ttc agc taa cat ctcg-3'	
GRβ	GR-F	GRβ-R	144 bp
NR3C1, GeneID: 2908 **Transcript variant 6**	5'-gaa gga aac tcc agc cag aac-3'	5'-cta ttt ttt gag cgc caa gat tgt-3'	
11βHSD1	11βHSD1 F	11βHSD1 R	158 bp
HSD11 1, GeneID: 3290	5'cac cat gtg cgc aaa agc at-3'	5'-tgc aga ata ggc agc aac ca-3'	
11βHSD2	11βHSD2 F	11βHSD2 R	132 bp
HSD11B1, GeneID: 3291	5'-tcg cgc ggt gct cat cac-3'	5'-gta cgc agc tcg atg gca cc-3'	

Table 2. Main features of study groups

Parameters	**Study groups**		**P**
	HCo (n=26)	**TB (n=24)**	
Age	50.5 (25.8 - 57.3)	45 (21.5 - 54.8)	Ns
Sex (Male/Female)	24/02	22/02	Ns
BMI	27.4 (25.1 - 30.8)	19.9 (18.3 - 23.8)	p<0.001
BCG (%)	95%	80%	Ns
Glycemia (mg/dl) [rv:70-100]	89,0 (83,0 - 97,5)	95,5 (82,0 - 104,0)	Ns
Triglycerides (mg/dl) [rv: < 200]	120,0 (87,0 - 138,0)	94,0 (62,0 - 128,0)	p<0.05
Total cholesterol (mg/dl) [rv: 130-200]	172,0 (147,0 - 185,5)	147,0 (126,0 - 172,0)	p<0.05
GOT (IU/L) [rv: 10-38]	20,5 (18,0 - 24,3)	18,5 (15,8 - 28,8)	Ns

Data are represented as median and interquartile range. **BMI:** body mass index (weight/height^2); **BCG:** Bacillus Calmette–Guérin vaccination; **HCo:** healthy controls;

TB: patients with pulmonary tuberculosis; **GOT:** glutamic oxaloacetic transaminase; **rv:** reference value; **Ns:** not significant.

Table 3. Lymphocyte subpopulations in TB patients during specific treatment

Parameters	**HCo (n=26)**	**TB T0 (n=24)**	**T2 (n=24)**	**T4 (n=24)**	**T6 (n=24)**	**T9 (n=24)**
LTCD4+ (%)	53.25 43.98-55.53	38.35 * 32.60-52.60	51.30 # 43.00-59.00	51.70 # 38.75-60.00	55.40 # 49.50-62.20	58.80 *# 51.00-66.40
LTCD8+ (%)	35.40 28.08-39.75	25.15 * 21.80-33.43	31.10 24.03-36.18	30.95 24.80-34.75	28.60 21.00-37.25	31.30 20.05-35.80
LT CD4+ CD25+ FoxP3+ (%)	1.60 1.00-2.30	2.20 * 1.80-2.80	3.20 Ŧ# 2.20-4.68	5.80 Ŧ ٱω 2.58-6.53	3.30 * 2.10-4.40	2.20 1.00-2.65

Data are represented as median and interquartile range. **HCo**: healthy controls; **TB:** patients with pulmonary tuberculosis; **T0:** diagnosis; **T2, T4 y T6:** 2, 4 and 6 months of initiation of anti-bacillary treatment; **T9:** three months following treatment completion. * different from HCo $p<0.05$; Ŧ different from HCo $p<0.01$; # different from T0 $p<0.05$; ٱ different from T0 $p<0.01$; ω different from T9 $p<0.05$.

Table 4. Relative expression of mRNA for glucocorticoid receptors (GRs: GRα, GRβ) and the 11βHSD1 enzyme in TB patients undergoing specific treatment

Parameters	**HCo (n=16)**	**TB T0 (n=16)**	**T2 (n=16)**	**T4 (n=16)**	**T6 (n=16)**	**T9 (n=16)**
GRα (AU)	29.5 22.6-31.1	25.6 24.4-36.1	32.3 25.6-35.4	32.7 # 29.5-37.6	27.9 23.8-35.6	25.7 23.3-31.8
GRβ (AU)	0.4 0.4-1.0	1.7 * 1.0-2.2	0.6 # 0.4-1.3	1.3 0.4-2.0	0.6 # 0.4-1.6	0.4 # 0.4-1.1
GRα/GRβ	51.05 27.1-63.1	17.24 * 12.0-35.5	61.34 # 19.3-76.0	27.01 # 13.9-70.1	44.81 # 22.8-67.2	56.36 # 25.9-65.3
11βHSD1 (AU)	0.5 0.4-0.8	0.7 * 0.6-1.1	0.5 # 0.3-0.8	0.6 # 0.2-0.8	0.5 # 0.2-0.7	0.5 # 0.1-0.7

Data are represented as median and interquartile range. **HCo:** healthy controls; **TB:** patients with pulmonary tuberculosis; **T0:** diagnosis; **T2, T4 y T6:** 2, 4 and 6 months of initiation of anti-bacillary treatment; **T9:** three months following treatment completion. **AU:** arbitrary units, relative quantity of the transcript respect to PPIA [peptidylprolyl isomerase A (Cyclophilin A)]. **GR:** glucocorticoid receptor. **11βHSD1**: 11-beta Hydroxisteroid Dehydrogenase type 1. *$p<0.05$ different from HCo; $^{\#}p<0.05$ different from TB patients at T0.