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IMMUNOLOGICAL ASPECTS

An adverse immune-endocrine profile in patients with tuberculosis and type 2 diabetes

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SUMMARY

Diabetes is a risk factor for the development of pulmonary tuberculosis (TB) and both diseases present endocrine alterations likely to play a role in certain immuno-endocrine-metabolic associated disorders. Patients with TB, or with TB and type 2 diabetes (TB + T2DM) and healthy controls (HCo) were assessed for plasma levels of cortisol, dehydroepiandrosterone (DHEA), estradiol, testosterone, growth hormone (GH), prolactin, insulin-like growth factor-1 (IGF-1), cytokines (IL-6, IL-10, IFN-γ) and the specific lymphoproliferative capacity of peripheral blood mononuclear cells. All patients had higher levels of cortisol with a reduction in DHEA, thus resulting in an increased cortisol/DHEA ratio (Cort/DHEA). Increased prolactin and particularly GH levels were found in both groups of TB patients. This was not paralleled by increased concentrations of IGF, which remained within the levels of HCo. Estradiol levels were significantly augmented in patients TB, and significantly more in TB + T2DM, whereas testosterone levels were decreased in both groups of patients. IFN- γ and IL-6 concentrations were significantly increased in all TB, even further in TB + T2DM; while IL-10 was equally increased in both groups of TB patients. The *in vitro* specific proliferative capacity was decreased in both groups of patients as compared to that of HCo. The adverse immune-endocrine profile of TB seems to be slightly more pronounced in patients who also have T2DM.

1. Introduction

Pulmonary tuberculosis (TB) is a major cause of mortality around the world. In 2014, 1.5 million people died of TB (0.4 million HIV-positive) and 9.6 million people worldwide are estimated to have contracted the disease during this period [1]. The clinical manifestations are greatly influenced by the immune response to *Mycobacterium tuberculosis*, its etiologic agent, but the mechanisms underlying the outcome of the disease are not fully understood [2]. Endocrine responses during chronic infections such as lung tuberculosis are worth studying since some of the cytokines produced during this disease are likely to affect endocrine mechanisms that, in turn, influence the course of the infectious process [3,4]. In fact, proinflammatory cytokines released from affected tissues that reach the central nervous system are known to influence the secretory activity of the hypothalamic–pituitary–adrenal (HPA) axis. The adrenal gland is responsible for the release of glucocorticoids (GCs), which generally inhibit or modulate inflammation, as well as dehydroepiandrosterone (DHEA), a steroid that counteracts GCs effects on cytokine production, but also exerts itself potent anti-inflammatory effects [3,5]. Interactions between the endocrine and the immune system also involve the hypothalamic–pituitary–gonadal (HPG) axis, since macrophages and lymphocytes have receptors for gonadal steroids and these hormones can affect macrophage and lymphocyte development and function [6,7].

By evaluating hormonal and cytokine levels in patients with TB, we have previously shown imbalanced immune-endocrine responses in which levels of pro-inflammatory cytokines, cortisol and estradiol concentrations were increased whereas testosterone and

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DHEA amounts were diminished in patients, together with an increased Cort/DHEA ratio even more pronounced in those with a severe disease [8].

Endocrine disturbances can also contribute to the pathology of patients with TB, as illustrated by the detrimental influence of type 2 diabetes mellitus (T2DM) on TB development. Several studies have shown that T2DM may be associated with an increased risk of developing active TB, whereas TB patients with concomitant diabetes are at higher rates of treatment failure and death [9–11].

Type 2 DM is characterized by the failure of beta cells to compensate for insulin resistance, with inflammatory or immunological factors being implied in such alterations [12]. Thus, the simultaneous occurrence of both diseases may impose a particular set of alterations in immune and endocrine parameters that is worth exploring.

On these bases, we analyzed the blood levels of several cytokines, hormones and the specific immune response to mycobacteria in the context of the TB and diabetes association. Studies included the assessment of the plasma levels of IL-6, IFN-γ and IL-10, adrenal (cortisol, DHEA) and gonadal (estradiol, testosterone) steroids, hormones involved in immune-metabolic effects like growth hormone (GH), prolactin and insulin-like growth factor-1 (IGF-1), in parallel to the specific lymphoproliferative capacity of peripheral blood mononuclear cells.

2. Materials and methods

2.1. Sample population

Patients (6 females and 29 males) with no HIV co-infection and newly diagnosed pulmonary TB of moderate to severe degree were included. Pulmonary TB diagnosis was based on positive clinical symptoms and radiological chest results as well as sputum smear positivity for acid fast bacillus (AFB) by Ziehl Neelsen staining and a confirmatory positive culture for Mtb on Lowenstein–Jensen medium. Fourteen of these patients were diagnosed as also having T2DM (TB + T2DM). Criteria for diabetes diagnosis were hyperglycemia (based on two fasting glucose levels greater than 125 mg/dL or a random glucose level equal to or higher than 200 mg/dL) evaluated on EDTA-anticoagulated blood specimens. According to current guidelines and considering the patient age, we estimated that all TB + T2DM patients had T2DM [13–15]. Most of them had a previous diabetes diagnosis and were under conventional treatment.

The control group was composed of 20 healthy controls (HCo), sharing the same socioeconomic conditions of TB patients, without any known prior contact with TB patients, as well as no clinical or radiological evidence of pulmonary TB. Patients and HCo had no other respiratory disease, nor immunocompromising diseases.

All patients started anti-TB treatment shortly after blood sample collection (1–3 days later), for which they were untreated at the time that studies were carried out.

Samples were obtained at 8 a.m. to avoid differences due to circadian variations. Exclusion criteria included disease states that affect the adrenal glands, the HPA or HPG axes, or requiring corticosteroid treatment, pregnancy, and age below 18 years. The body mass index (BMI) was also calculated (weight/square of height). The protocol was approved by the Bioethic Committee of the School of Medical Sciences, National University of Rosario. All participants gave their consent to participate in the study.

2.2. Lymphoproliferation

Peripheral blood mononuclear cells (PBMC) were obtained from fresh EDTA-treated blood. After centrifugation, the buffy coat was separated and diluted 1:1 in RPMI 1640 (PAA Laboratories GmbH, Austria), containing standard concentrations of L-glutamin, penicillin, and streptomycin (culture medium, CM). The cell suspension was layered over a Ficoll-Paque Plus gradient (density 1.077, Amersham Biosciences, NJ, USA), and centrifuged at 400 *g* for 30 min at room temperature (19–22 °C). PBMC recovered from the interface were washed three times with CM, and resuspended in CM containing 10% heat-inactivated pooled normal AB human sera (PAALaboratories GmbH, Germany). Cells were cultured in quadruplicate in flat-bottomed microtiter plates (2×10^5 cells/well in 200 μl) with or without addition of γ-irradiated H37Rv *M. tuberculosis* strain, (Mtb; 8 μg/ml) kindly provided by Dr J. Belisle (Colorado State University, Fort Collins, CO, U.S.A.) PBMC cultures were incubated for 5 days at 37 °C, in a 5%, CO_2 humidified atmosphere and pulsed with ^{3}H-thymidine for 18 h before cell harvesting. The average counts per minute (cpm) of stimulated and non-stimulated cultures were calculated.

2.3. Quantification of cytokines and hormones in plasma

Plasma was obtained from EDTA-treated blood. Samples were centrifuged at 2000 rpm during 30 min and the plasma stored at −20 °C. Cortisol (DRG Diagnostics, detection limit 2.5 ng/ml), DHEA (DRG Diagnostics, detection limit 0.108 ng/ml), IFN-γ (BD Pharmingen, detection limit 4.7 pg/ml), IL-10 (BD Pharmingen, detection limit 3.9 pg/ml) and IL-6 (DRG Diagnostics, detection limit 2 pg/ml), prolactin (DRG Diagnostics, detection limit 0.35 ng/ml), hGH (DRG Diagnostics, detection limit 0.17 μIU/ml), insulin-like growth factor-1 (Quantikine, R&D Systems, detection limit 0.026 ng/ml), testosterone (DRG Diagnostics, detection limit 0.083 ng/ml) and estradiol (DRG Diagnostics, detection limit 9.714 pg/ml) plasma concentrations were determined using commercially available ELISA kits according to the manufacturer instructions. All samples were processed individually and assayed in duplicate.

2.4. Statistical analysis

Comparisons between groups were made by nonparametric methods: Kruskall-Wallis followed by Dunn's test for multiple comparisons, if applicable. Qualitative variables were compared by the chi square test. Associations between variables were analyzed using the Spearman correlation test. A value of $p < 0.05$ was considered as indication of significant differences.

3. Results

The subject profile is shown in Table 1. There were no between-group differences in age and sex distribution, while the presence of the BCG scar was less prevalent among TB patients. Both groups of TB patients (with or without T2DM) had a BMI lower than HCo ($p < 0.001$, Table 1). Data from *in vitro* proliferation of PBMC from patients and HCo are also presented in Table 1. PBMC from HCo had higher median proliferative responses to Mtb than both groups of TB patients ($p < 0.02$), which was more decreased in TB patients without T2DM. As commented, TB and TB + T2DM patients had a similar degree of disease severity. Measurements on HbA1c levels indicated that TB + T2DM had poorly controlled diabetes (means ± standard error of the mean of % HbA1c): HCo = 5.54 ± 0.09; TB = 5.78 ± 0.15; TB + T2DM = 9.58 ± 1.06 ($p < 0.001$).

Results from the analysis of cytokine levels in plasma are depicted in Figure 1. TB patients had increased amounts of IFN-γ respect to HCo, which was more pronounced in those with concomitant T2DM and statistically significant from HCo and TB

Table 1
Main features of subjects participating in the study.

Parameters	HCo (n = 20)	TB (n = 21)	TB + T2DM (n = 14)	Overall p value
Age (years)	47 (39.5–51.7)	52 (30.0–62.0)	53 (46.0–62.7)	ns
Sex distribution (F/M)	3/17	3/18	3/11	ns
BCG scar (%)	100	67	57	<0.01
BMI	27.1 (25.4–30.7)	20 (18.3–22.1)	21.8 (20.3–29.0)	<0.001
Lymphoproliferation (cpm)	12744 (7517–23647)	4085 (1304–5788)	7682 (1278–17074)	<0.02

Results are shown as median (25–75 percentiles).
F: females; M: males; BCG scar: Bacillus Calmette-Guerin scar. n.s.: not significant.
The body mass index (BMI) was calculated as follows: weight/square of height (kg/m^2).
cpm: the average counts per minute of stimulated cultures.
Baseline cpm: HCo = 855.3 (549–1064); TB = 486 (272–656); TB + T2DM = 399 (304–1052).

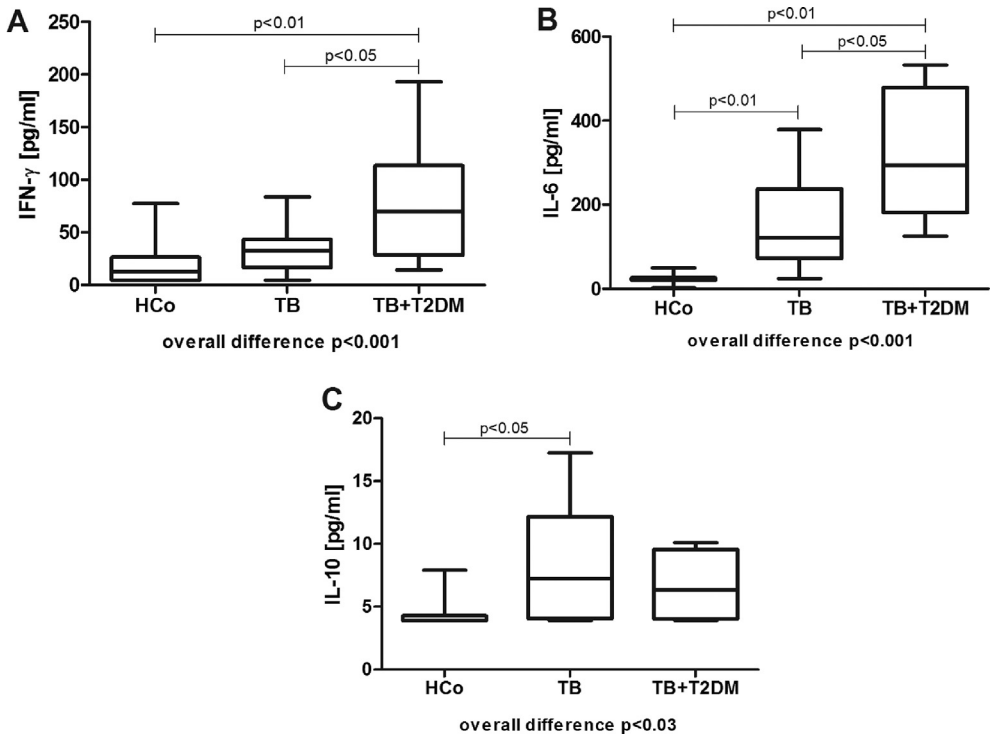

Figure 1. Plasma levels of IFN-γ (A), IL-6 (B) and IL-10 (C) in healthy controls (HCo), patients with pulmonary tuberculosis with type 2 diabetes (TB + T2DM) or not (TB). Box plots show median values, 25–75 percentiles of data in each group with maximum and minimum values.

patients (panel a). TB patients also showed increased systemic IL-6 levels, which were even higher in the TB + T2DM group (panel b). While IL-10 concentrations were higher in both groups of patients than in HCo, only the group of TB patients attained statistical difference (panel c).

According to the purpose of this study, circulating levels of adrenal steroids were also compared. As seen in Figure 2 panel a, TB patients presented significantly higher concentrations of cortisol. The highest levels were found in TB patients with T2DM, and these values were statistically significantly different from those of the TB group without T2DM. DHEA concentrations were significantly lower in both groups of TB patients as compared to those of the HCo, with no differences in relation to the presence or absence of T2DM (Figure 2 panel b). Imbalances in cortisol and DHEA levels resulted in an increased Cort/DHEA ratio in both groups of TB patients compared to HCo (Figure 2 panel c).

Both patient groups had significantly increased circulating levels of GH when compared to HCo, with a nearly significant tendency to be even higher in the TB + T2DM patients respect the TB group (Figure 3 panel a). No between group differences were detected in the levels of IGF-1 in plasma (Figure 3, panel b). When analyzing prolactin concentrations, only the group of TB patients without T2DM attained a statistical difference respect to HCo (Figure 3 panel c).

Estradiol levels were significantly augmented in TB patients, much more in those with accompanying diabetes (Figure 4, panel a), whereas testosterone levels in plasma of both groups of TB patients situated below the ones seen in HCo (Figure 4, panel b).

Since cytokines and hormones are likely to influence the production of each other, the relation between them was investigated. There were no associations in HCo, for which eventual correlations were determined in both groups of TB patients. The levels of IL-10 were positively associated with GH, as did the Cort/DHEA ratio with IL-6 in the group of patients with TB only (Table 2). The Cort/DHEA ratio also correlated positively with estradiol, in this case also in TB + T2DM patients (Table 2). As seen in the same Table, positive correlations were also found between concentrations of estradiol with two cytokines, IFN-γ (only in TB + T2DM patients) and IL-6 (both patient groups).

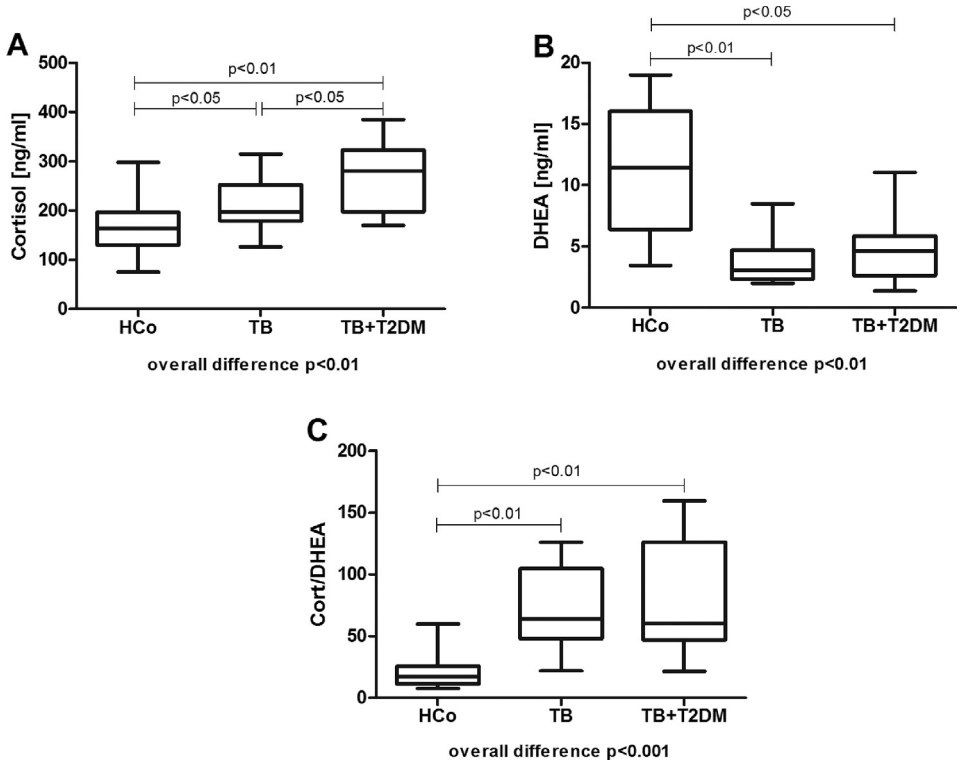

Figure 2. Plasma levels of cortisol (A), dehydroepiandrosterone (DHEA; B) and the Cortisol/DHEA ratio (C) in healthy controls (HCo), patients with pulmonary tuberculosis with type 2 diabetes (TB + T2DM) or not (TB). Box plots show median values, 25–75 percentiles of data in each group with maximum and minimum values.

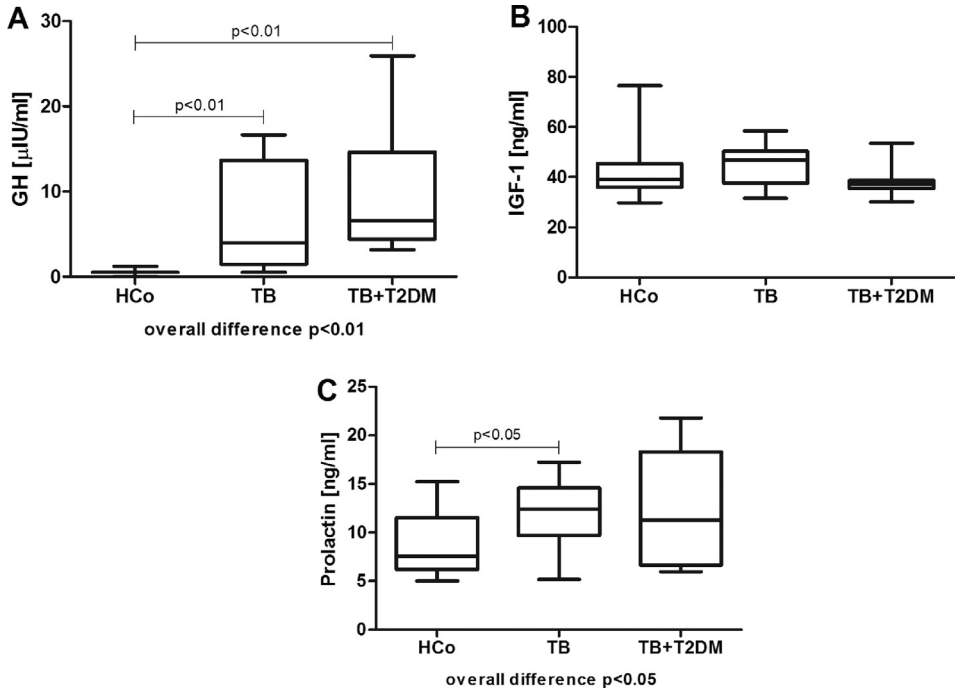

Figure 3. Plasma levels of growth hormone (GH; A), insulin like growth factor 1 (IGF-1; B), and prolactin (C) in healthy controls (HCo), patients with pulmonary tuberculosis with type 2 diabetes (TB + T2DM) or not (TB). Box plots show median values, 25–75 percentiles of data in each group with maximum and minimum values.

4. Discussion

The endocrine and immune systems interact in a bidirectional way. Hormones can affect immune functions, and in turn the immune response influences neuroendocrine functions [3,5]. The HPA axis is a key element in the communication between the endocrine and immune systems, for which it has to be finely tuned. Chronic activation of the HPA axis can affect the susceptibility or

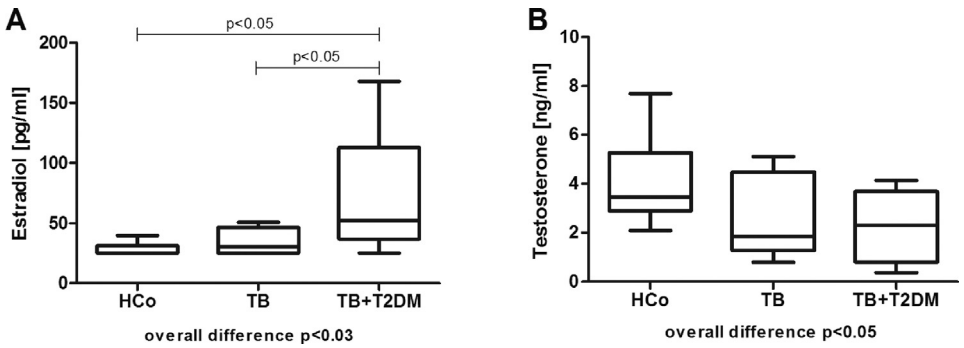

Figure 4. Plasma levels of estradiol (A) and testosterone (B), in healthy controls (HCo) and patients with pulmonary tuberculosis with type 2 diabetes (TB + T2DM) or not (TB). Box plots show median values, 25–75 percentiles of data in each group with maximum and minimum values.

Table 2
Correlation analysis of hormone and cytokine plasma levels in tuberculosis patients with (TB + T2DM) or without (TB) type 2 diabetes.

Pairwise correlations	TB		TB + T2DM	
	r coefficient	p value	r coefficient	p value
IL-10 vs. GH	0.77	<0.005	−0.20	n.s.
Cort/DHEA vs. Estradiol	0.70	<0.005	0.77	<0.04
Cort/DHEA vs. IL-6	0.63	<0.02	0.75	n.s.
Estradiol vs. IFN-γ	0.19	n.s.	0.79	<0.03
Estradiol vs. IL-6	0.67	<0.006	0.86	<0.02

n.s.: not significant.
In addition, a highly significant correlation between BMI and the mycobacterial-driven lymphoproliferative response was observed in the group of TB + T2DM patients ($r = 0.83$, $p < 0.0008$).

severity of infectious diseases, mainly through immunosuppressive effects of glucocorticoids. In general terms, cortisol downregulates a wide range of biological processes, such as cell proliferation, inflammation, and immunity, in addition to inhibiting the trafficking of T and B lymphocytes, eosinophils, basophils, macrophages, and monocytes.

The larger increase in the concentration of cortisol detected in the TB + T2DM patients included in our study as compared to those of patients with TB alone, may be the consequence of their simultaneously augmented concentrations of IFN-γ and IL-6, since both cytokines can stimulate the activity of the HPA axis [3,5]. Our results on cytokines levels corroborate former reports indicating that TB patients with concomitant diabetes have a more substantial increase in compounds with pro-inflammatory activities as compared with TB patients lacking this co-morbidity [16,17].

Despite extensive research, many aspects of T2DM pathogenesis remain unclear, although a basic link to an inflammatory component is now evident. Patients with T2DM present higher amounts of mediators involved in the inflammatory response [18–20], and the pro-inflammatory cytokine TNF-α can induce a state of insulin-resistance, reinforcing the evidence of an inflammatory component in T2DM pathogenesis [21]. In line with this immune-endocrine concept, patients with diabetes show hyperactivation of the HPA axis, resulting in elevated blood glucocorticoids [22], as observed in rodents with experimental diabetes [23–25]. Extending these findings, we now report that a similar scenario takes place in TB patients with accompanying T2DM.

Confirming earlier results showing decreased, respectively augmented, concentrations of testosterone and estradiol in TB patients [8], a further increase in estradiol levels was found in TB + T2DM patients. Sex steroids have important effects on the immune system. For example, estrogens are able to drive pro-inflammatory Th-1-associated responses [6,7], whereas testosterone exerts inhibitory effects [26,27]. These findings fit well with the view of an intensified inflammatory response in TB patients with associated T2DM.

This profile of immune-endocrine alterations, reported here for the first time, is likely to bear a close relation with the patient condition and the physiopathological mechanisms underlying the development of the disease. For example, the increased BMI which was seen in diabetic patients [28] was not present when they also have TB.

The response to an infectious agent encompasses the development of immune and inflammatory responses involving a broad range of clinical and biochemical changes. In this way, the increased risk that diabetes imposes on the development of TB may not simply rely on an altered specific immune response but on a dysregulated defensive reaction that goes beyond the immunological mechanisms. In support of this view, a recent study of transcriptional profiles in PBMC from patients with TB, some of them also with diabetes, found that transcriptional levels of the FPR gene, which encodes the receptor of powerful chemotactic factors, N-formylated peptides, prevailed among cases with both diseases [29].

Besides the central importance of IFN-γ in the defense against mycobacterial infection [30,31], this cytokine also plays an important role in the inflammatory reaction accompanying this disease, and the increased amount of circulating IFN-γ detected in our patients might be more harmful than protective. Regarding IL-6, although this cytokine coordinates some anti-inflammatory activities, it is also well-known for its pro-inflammatory effects, and it may thus be two-fold detrimental, not only because of its inflammation-promoting effect [32], but also for its capacity to inhibit macrophage responses to IFN-γ [33].

Studies of *in vitro* IFN-γ production in TB + T2DM patients are controversial. Some studies revealed no differences [34], whereas other reports showed a lower [35] or greater production [16] when compared to TB patients without T2DM. Variations in patient situations, quality of diabetes control and experimental conditions may account for such differences. In our case, the coexistence of T2DM and TB is compatible with a non-defective ability to produce IFN-γ. As stated, the increased concentrations of this cytokine in TB + T2DM as compared to those of patients with TB alone may reflect a more robust inflammatory response, coincident with the increased amounts of estradiol and IL-6, and the positive correlations between these mediators. This profile was not compensated by an equivalent increased level of IL-10, which is well known for its anti-inflammatory effects [36].

Patients with TB and T2DM did not show a poorer lymphoproliferative response than TB patients lacking this co-morbidity, implying a non-extensive defect in the specific cell-mediated

immune response. The significant association of the mycobacterial-driven proliferation with the BMI of TB + T2DM patients also points out to a particular and close relation between metabolic status and the cellular immune response, deserving further exploration. Additional data in favor of a distinct pattern of immune-endocrine relations are provided by a set of associations only detected in TB patients without T2DM, like IL-10 with GH, and the Cort/DHEA ratio with IL-6. Reasons for these relations remain speculative but it is clear that reciprocal influences between hormones and cytokines are different depending on the coexistence of T2DM.

DHEA, the other major steroid produced by the adrenal cortex, has been shown to inhibit the effect/production of proinflammatory cytokines [37]. On the other hand, DHEA seems to favor Th1 reactions while inhibiting Th2 immune responses. At the same time, a growing body of evidence indicates that DHEA has potent anti-glucocorticoid properties [37]. It has been proposed that DHEA favorably affects the course of experimental tuberculosis in mice [38]. Also administration of DHEA sulfate improved IFN-γ production in mice immunized with heat shock proteins from *M. tuberculosis* [39]. Given the role of DHEA in counterbalancing some actions of cortisol, the relation between both steroids provides a better indicator of overall effects. The increased cortisol levels in parallel to decreased DHEA concentrations observed in patients with TB, particularly in those with T2DM, may result in a situation of functional hypercortisolemia. In terms of inflammation, this adrenal steroid pattern may be still inefficient for a suitable control of inflammation, considering that the increase in cortisol levels may not be sufficient to compensate for the limited anti-inflammatory effect of reduced amounts of DHEA.

Unlike findings during chronic stress, in which stress hormones like cortisol, PRL and GH are usually reduced [40], the levels of these hormones appear frankly increased in TB patients. Thus, in view of the known effect of pro-inflammatory cytokines on endocrine functions (3,5,40), it may be inferred that immune-derived products released during the anti-tuberculous immune response may contribute to such a biased profile.

Regarding blood levels of the pituitary hormones, both PRL but particularly GH appeared increased in TB patients, a little higher in the TB + T2DM group. The stimulatory effects of these hormones on cell-mediated immune reactions are well established [41,42] and hence they may be beneficial in infections with intracellular pathogens like TB. Nevertheless, it does not seem to the present case since levels of IGF-1, whose production is induced by GH and mediates many of its effects [40], remained within normal range suggesting that there is a certain degree of GH resistance in these patients. The increased levels of GH may represent an attempt to improve cell-mediated immune responses, rendered unsuccessful by the counteractive and complex mechanisms underlying the pathology of this disease.

Infections and immune and endocrine responses are intricately interconnected, representing important integrated physiologic circuits for the regulation of defensive reactions and the coexistent inflammation. These interactions attempt to warrant immune competence to cope with the infection and metabolic collapse, but they might be sometimes disadvantageous. The results of the present investigation indicate that the adverse immune-endocrine profile of TB is more pronounced when this disease is associated with diabetes type 2, in favor of a more intense immune-inflammatory reaction and the resulting endocrine response. These findings add novel information about the detrimental role of diabetes during TB infection, and provide a stimulating background for a better understanding of such abnormalities that might contribute to both morbidity and mortality.

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Competing interests: None declared.

Ethical approval: The protocol was approved by the Bioethic Committee of the School of Medical Sciences, National University of Rosario.

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