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Assessment of Selected Endocrine Hormone Profiles in different stages of Tuberculosis infection

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This is to certify that the thesis prepared by Yosef Tsegaye, entitled: **Assessment of Selected Endocrine Hormone Profiles in different stages of tuberculosis infection** and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Clinical Chemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations

CMI	Cell-mediated immunity
DHEA	Dehydroepiandrosterone
EPTB	Extra pulmonary tuberculosis
GC	Glucocorticoids
GCR	Glucocorticoid Receptor
GH	Growth hormone
HPA	Hypothalamus–Pituitary–Adrenal Axis
Hsp	Heat shock protein
IFNγ	Interferon Gamma
IGRA	Interferon Gamma Release Assay
IL-1	Interleukin-1
INKT	Invariant natural killer cells T cells
LTBI	Latent Tuberculosis Infection
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
MTB	Mycobacterium Tuberculosis
NK	Natural killer cells
PTB	Pulmonary Tuberculosis
QFT-GIT	QuantiFERON-TB Gold In-Tube test
TB	Tuberculosis
TLR	Toll like Receptor
TNF-α	Tumor Necrosis Factor-α
TST	Tuberculin Skin Test
WHO	World Health Organization

Abstract

Background: Hormones often act as immunomodulators. Immune and endocrine interaction during infectious diseases may determine the failure or success of the immune response. This is particularly true for an infection like tuberculosis, in which pathogen and immune system coexist in a continuous interaction. Hormonal changes are likely to occur since some of the cytokines produced during this disease could affect endocrine mechanisms that, in turn, influence the course of infectious/inflammatory processes. This communication pattern exists due to the fact that cytokine producing cells as well as hormone-producing cells share common receptors and ligands. Profiling hormone levels in the different stages of TB infection or disease could therefore provide important insight in the understanding of the disease that could ultimately contribute in the development of prognostic biomarker pools.

Objective: to assess selected endocrine hormone profiles in different stages of tuberculosis infection from repository plasma samples collected over a period of time at the Armauer Hansen Research Institute (AHRI) laboratory.

Methods: A Comparative cross sectional study was conducted to analyze the levels of plasma endocrine hormones in randomly selected stored plasma samples from September to December, 2018 GC, at AHRI, Addis Ababa Ethiopia. A total of 226 plasma samples, collected from pulmonary and extra pulmonary TB patients, particularly TB lymphadenitis cases, close household contacts of PTB patients and leprosy patients, were retrieved from AHRI biorepository using convenient sampling and evaluated for selected endocrine hormones (DHEA, cortisol, testosterone, estradiol, growth hormone and leptin) using ELISA. Data has been entered, cleaned and analyzed using GraphPad Prism 7 and SPSS version 20.0.

Results: Plasma cortisol level was significantly higher in PTB, TBLN and leprosy patients compared to both LTBI uninfected groups ($P < 0.0001$, $P = 0.0011$, $P = 0.0044$, respectively) and infected groups ($P < 0.0001$, $P = 0.0135$, respectively). The levels of DHEA and leptin were significantly low in PTB patients compared to LTBI uninfected groups ($P < 0.0446$, $P < 0.0001$, respectively). Similarly, levels of leptin was significantly lower in TBLN and leprosy patients compared to healthy controls (LTBI uninfected groups) ($P = 0.0023$, $P < 0.0001$, respectively). On the other hand, plasma levels of DHEA, estradiol, testosterone and leptin significantly increased in PTB patients following treatment, whereas the concentration of cortisol and human growth hormone declined significantly after treatment ($P= 0.0001$, $P=0.03$, $P=0.0003$, $P=0.002$, $P=0.045$, $P=0.045$ respectively).

Conclusion: TB Patients are characterized by increased plasma levels of cortisol and decreased DHEA and leptin levels. Our study also indicated that treatment results in increased DHEA, leptin, estradiol and testosterone and decreased cortisol and human growth hormone among pulmonary TB patients. This alteration of hormones during TB disease suggests that hormones might influence the immune response to *M. tuberculosis* and therefore the course of the disease.

Key words: Hormone, Tuberculosis, immunity latency, disease, infection

1. Introduction

1.1. Background

1.1.1. Introduction to Hormones

Hormones are chemical substances having a specific regulatory effect on the activity of a certain organ or organs. Hormones travel through the blood to distant tissues and organs, where they can bind to specific cell sites called receptors. By binding to receptors, hormones trigger various responses in the tissues/cells containing cognate receptors. On the basis of their chemical nature, hormones are classified as peptides, steroids, and amino acid derivatives (1).

Mechanism of action of hormones comprises two components. Protein hormones interact with a receptor on the outer surface of cell membrane and they signal via second messengers generated by interacting with receptors at the cell surface. Steroid hormones pass through cell membrane and interact with intracellular receptors and the hormone receptor complex eventually binds to a segment of chromatin, which induces formation of messenger RNA that in turn enters the cytoplasm and initiates the synthesis of protein or peptides that carry out the action attributed to the hormone (2).

Their functions can be broadly grouped into several categories: reproduction and sexual differentiation; development and growth; maintenance of the internal environment; and regulation of metabolism and nutrient supply. A single hormone may affect more than one of these functions and each function may be controlled by several hormones. For example, glucocorticoids (GCs), such as cortisol, are important both in growth and nutrient supply and are also modulators of immune function (3) .

1.1.2. Immune-Endocrine Interaction

Immuno-endocrine interaction is characterized by presence of a two way communication between the immune and endocrine systems and facilitates optimum responses in the host during infections. This interaction is happened between different hormones and immune cells and is in part achieved through changes in secretion patterns of hypothalamic hormones induced by inflammatory cytokines (4).

1.1.2.1.The Hypothalamus Pituitary Adrenal Axis

The hypothalamic-pituitary-adrenal (HPA) axis plays a fundamental role in the maintenance of basal and stress-related homeostasis. This neuroendocrine axis consists of three distinct components located in the hypothalamus, the pituitary gland and the adrenal cortex. GCs, the end- products of the HPA

axis, exert their diverse actions in virtually all tissues through their ubiquitously expressed glucocorticoid receptors (GCR) (5).

Glucocorticoid receptors have been identified throughout the immune system and in circulating T-cells, B-cells, and macrophages. High circulating concentrations of corticosterone suppress innate (i.e. NK activity), cell-mediated (i.e. cytokine production), and humoral (i.e. antibody production) immune responses in laboratory rats and mice (6, 7).

Evidences from different studies indicated that hormones have role both in innate and adaptive components of immune system. In response to an infection, cytokines like interleukin (IL)-1, IL-6, and TNFα, produced by immune cells, can activate the hypothalamus–pituitary–adrenal (HPA) axis, which results in the secretion of cortisol and DHEA (8). This activation of the HPA axis by different cytokines indicates that the immune system is functionally integrated with endocrine systems (9).

1.1.2.2.The hypothalamic Pituitary Gonadal axis

In addition to their role in reproduction and sexual differentiation sex hormones such as estradiol and testosterone has role in immune system. The elevated susceptibility of males to bacterial infections can be related to the usually lower immune responses presented in males as compared to females. This dimorphic sex difference is mainly due to the differential modulation of the immune system by sex steroid hormones through the control of proinflammatory and anti-inflammatory cytokines expression, as well as toll-like receptors (TLRs) expression and antibody production (10).

Different animal and human studies showed modulation of the immune response by steroid hormones both physiologically and pathologically. Experimental study done in mice showed that testosterone reduce the activity of natural killer (NK) cells (11). Testosterone also decreases the expression of macrophage and monocyte Toll-like receptor 4 (TLR4) which is important in innate immune responses (12). Additionally, its effect in increasing the synthesis of anti-inflammatory cytokines such as IL-10 and inhibiting synthesis of proinflammatory cytokines shows its role in decreased immune response seen in males as compared to females (13, 14).

On the other hand estrogens also interact with immune cells through estrogen receptors in different immune cells including lymphocytes, macrophages, granulocytes, and mast cells, illustrates that there are direct connections between the endocrine and immune systems and that endocrine factors can directly modulate the expression of target genes in immune cells (15). In opposite to testosterone function estradiol contributes to resistance against infections by enhancing NK cell cytotoxicity and

stimulating the synthesis of proinflammatory cytokines such as IL-1, IL- 6, and TNF-α (16, 17). Estradiol also inhibits the production of IL-4, IL-10 (18).

1.1.2.3.Growth hormone

Pituitary hormone such as Growth hormones (GH) also known for its role in immune function. Many GH effects are mediated through induction of IGF-1 (19). Different in vivo and in vitro studies are done to determine the effects of GH in immune function but data are generally inconsistent and controversial (20, 21). GH is a human macrophage-activating factor which primes monocytes for enhanced production of H_2O_2 in vitro (22). Human Study showed that adults with severe GH deficiency due to panhypopituitarism revealed significantly decreased natural killer cell activity as compared with healthy controls, however, 18 months of GH replacement did not lead to any changes in natural killer cell activity, despite restoration of normal healthy IGF-1 levels (23).

1.1.2.4.Leptin

Leptin is an adipocyte-derived hormone/cytokine that links nutritional status with endocrine and immune functions(24). Several studies in animal models with genetic abnormalities in leptin or leptin receptor (LepR) revealed that leptin-deficiency associates with consistent impairment in macrophage functions in terms of phagocytosis and the expression of pro-inflammatory cytokines both in vivo and in vitro, and these conditions are reverted by exogenous leptin administration (25).

Leptins deficiency is also associated with impaired dendritic cells function which is important cell in antigen presentation to T cells (26). The immunomodulatory role of leptin has been described also in neutrophils. Indeed, it has been shown that human neutrophils only express the short form of the leptin receptor, which is enough to signal inside the cell, enhancing the expression of CD11b and preventing neutrophils apoptosis (27, 28).

In addition to its role in innate immune system leptin has also been demonstrated to modulate adaptive/ immune response. Evidences from animal study indicates that leptin participates in the maintenance of thymic maturation of double positive CD4+/CD8+ cells, reducing thymic apoptosis as well as preventing glucocorticoids-induced apoptosis in thymocytes (29). Leptin is also able to modulate regulatory T (Treg) function. It has been reported that freshly isolated Tregs produce and secrete leptin and express high amounts of the LepR (30).

1.1.3. Tuberculosis

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB) that is responsible for more deaths worldwide than any single pathogen with an estimated 10.0 million new cases (range, 9.0–11.1 million) and 1.3 million deaths(range, 1.2–1.4 million) among HIV-negative people, and there were an additional 300 000 deaths from TB (range, 266 000–335 000) among HIV-positive people, annually in 2017 (31). A relatively small proportion (5–10%) of the estimated 1.7 billion people infected with M. tuberculosis will develop TB disease during their lifetime. However, the probability of developing TB disease is much higher among people infected with HIV; it is also higher among people affected by risk factors such as undernutrition, diabetes, smoking and alcohol consumption. Overall, about 90% of cases occur among adults, with more cases among men than women. The male:female ratio among adults is approximately 2:1.(31). The majority of infected individuals develop latent infection defined as having evidence of MTB infection by immunologic tests (TST) or IFN-γ release assay (IGRA) without clinical signs or symptoms of disease and a normal chest radiograph. Although most patients with latent infection will not die of TB, the greatest danger is in reactivation (active TB after remote infection) cases and the subsequent silent spread to close contacts (32).

1.1.4. TB and Hormones

The immune response against bacterial, viral, and parasitic infections is paralleled by a significantly altered hormonal response both in experimental models and human patients (33). Interactions between immune and endocrine system during infectious diseases may determine the failure or success of the immune response. This is particularly true for an infection like TB, in which pathogen and immune system coexist in a continuous interaction (34).

TB is associated with different type of altered endocrine hormones. Hormonal changes are likely to occur since some of the cytokines produced during this disease could affect endocrine mechanisms that, in turn, influence the course of infectious/inflammatory processes (35). This communication pattern exists due to the fact that cytokine-producing cells as well as hormone-producing cells share common receptors and ligands (36).

In-depth understanding of the immune-endocrine interaction has been established in other diseases such as rheumatoid arthritis (37). Endocrine hormone profiles are not fully characterized in TB disease, and very limited studies are done to assess endocrine hormone profiles across spectrum of TB disease;

this study therefore aims to assess selected endocrine hormone profiles in TB patients and apparently healthy individuals from repository plasma samples collected over a period of time.

1.2. Statement of the problem

Hormones often act as immunomodulators, altering the sensitivity of the immune system, either as immunostimulators, immunosuppressors or immunoregulators. Several cytokines produced during TB not only exert a direct effect on immunocompetent cells, but may also influence immune cells indirectly, due to their ability to affect several neuro-endocrine mechanisms, among them the stimulation of the hypothalamus-pituitary-adrenal axis (38). Recent studies have shown that specific hormone profiles particularly, steroid hormones, correlate with TB treatment outcomes (4) and increased levels of cortisol and growth hormones and reduced levels of dehydroepiandrosterone (DHEA) was observed in TB patients compared to healthy controls (39).

Tuberculosis is about twice as prevalent in men as in women with a global male:female sex ratio for TB of 1.7:1 in 2017 (31). Sex bias is historically attributed to epidemiological factors, such as cultural and socioeconomic impacts leading to barriers in accessing healthcare (40), however, the definitive cause of this bias is unknown but biological differences between the sexes are shown to affect susceptibility to mycobacterial infection(41). Sex hormones could be a significant factor for sex bias in TB. Testosterone impairs pro-inflammatory cytokine production(42), whereas oestrogens are a pro-inflammatory inducer (43). In TB-infected mouse models, non-castrated males had higher mortality, higher bacilli burdens, less inflammation in lung compartments and lower cytokine production compared to female and castrated male mice (44).

A common feature of TB is that the immune response fails to definitely eradicate the pathogen, likely due to complex mechanisms of immune evasion mechanisms that limit the protective host response. Such a particular host-mycobacteria relationship results in the establishment of a chronic infection, during which a broad range of regulatory mechanisms are likely to operate (38). In majority of immunocompetent persons, infection with *Mycobacterium tuberculosis* (MTB) is initially contained by host defenses, resulting in latent TB infection (LTBI). However, persons with LTBI can progress to active TB at any time, often many years or even decades after initial infection, thereby serving as a source of new infections (45).

Despite a number of efforts, currently there are no sufficiently validated biomarkers to aid the evaluation of new tuberculosis vaccine candidates, the improvement of tuberculosis diagnostics or prognostic marker. Hormones could play a role in the spectrum of this disease, in which their interactions with immune system during infectious diseases may determine the failure or success of the immune response (34), therefore understanding hormone levels in the TB disease spectrum could

provide important insight in the understanding of the disease that ultimately contribute to the development of biomarker pools. And endocrine hormone profiles are not fully characterized in TB disease. This study possibly provides data on selected endocrine hormone profiles in the TB disease spectrum, spanning over healthy controls, latently infected individuals and active TB patients.

1.3.Significance of the study

Global control of TB can only be achieved through the concerted effort in the development of effective vaccines, improved diagnostics, and novel and shortened therapeutic regimens. The findings of this study will contribute to the identification of additional biomarkers that add to the existing research efforts in developing potential host markers for diagnostics, prognostics or vaccine development initiatives. In addition, as part of a postgraduate program, this work will contribute to the development of capacity building program nationally.

2. Literature Review

2.1. Hormone Profiles in TB Disease

2.1.1. Hypothalamic–Pituitary–Adrenal Axis and TB Disease

Evidences showed that in response to an infection, cytokines like IL-1, IL-6, and TNFα, produced by immune cells, can activate HPA axis, which in turn results in the secretion of cortisol and DHEA (5). Cortisol facilitate Th2 activity, partly by inhibiting Th1 cells, its natural antagonist DHEA is able to both favor Th1 cytokine production and interfere with Th2 cytokine synthesis (46, 47). Infection with intracellular pathogens like TB requires expression of Th 1 cytokine like IFN-y which is a central macrophage-activating cytokine involved in the immune protection against MTB (48). Its inhibition by cortisol might results in susceptibility to MTB infection and disease progression.

In addition, alteration in GC/DHEA balance impacts anti infectious immunity. An increased GC/DHEA ratio favors a Th2 response, which impairs the immune response against intracellular pathogens favoring an enhanced susceptibility. In contrast, a diminishing GC/DHEA balance seems to support Th1 immune response and favors host resistance against intracellular infection as shown in figure 2 (33).

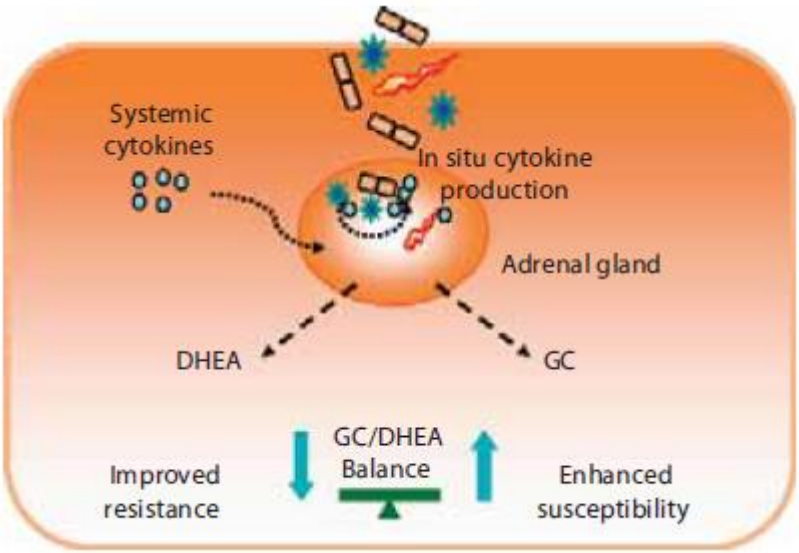


Figure 1. Role of Glucocorticoid (GC)/Dehydroepiandrosterone (DHEA) balance upon intracellular infections [Picture adapted from (33)].

Different studies shows alteration of HPA hormones in TB patients. A cross sectional study done by Del Rey et al in 2007 on 49 subjects (consisted of 30 HIV-negative newly diagnosed, untreated male TB patients and 19 male, age-matched healthy volunteers without contact with TB patients (Controls)) to determine endocrine and cytokine responses in humans with pulmonary tuberculosis revealed that

TB patients show a twofold increase in IFN-γ and IL-10 and a 10-fold increase in IL-6 plasma levels, accompanied by a 50% decrease in DHEA and modest increase in the concentration of Cortisol in plasma when compared to healthy controls (P value< 0.05) (35).

Another study done in Argentina in 2007 on 39 subjects (25 newly diagnosed TB patients and 14 healthy controls) to investigate the relationship between cortisol and DHEA levels and the immune response to mycobacterial antigens showed that TB patients had a higher IL-10 production, a decreased lymphoproliferation and a trend to reduced IFN-y synthesis, in relation to healthy controls. In this study Active disease was also characterized by increases in the plasma levels of GC and reduced concentrations of DHEA which resulted in a higher cortisol/DHEA ratio with respect to the healthy control group. In this study the authors conclude that the drop in DHEA and the concomitantly elevated cortisol, emerges as a relevant link between adrenal hormones and the immune alterations seen in TB patients (49).

2.1.2. Hypothalamic–Pituitary–Gonadal Axis and TB disease

Mycobacterial infections occur more frequently in males than in females. This TB gender difference is seen in adults of all the ages, but not in children or young adolescents which indicates involvement of sex steroids in immunity (40). This dimorphic sex difference is mainly due to the differential modulation of the immune system by sex steroid hormones through the control of proinflammatory and anti-inflammatory cytokines expression, as well as TLRs expression and antibody Production (10).

Immune cells, including T cells, B cells, dendritic cells (DCs), macrophages, neutrophils and natural killer (NK) cells, express cognate intracellular receptors which suggests that sex steroid hormones might have a role in the regulation of immunity (50). Sex steroid hormones exert their function by binding to either specific intracellular receptors that act as ligand-dependent transcription factors (classical mechanism) or membrane receptors that stimulate several signal transduction pathways (non-classical mechanism) (51).

Experimental studies suggested that androgen deprivation due to the castration of male mice leads to an increase in the absolute number of T lymphocytes in the peripheral lymph nodes and an increase in the proliferation of these cells following antigen recognition (52). On the other hand iNKT from female mice produce more IFN-y than to cells from male mice in response to in vivo challenge with iNKT cell ligand α-Galcer (53).

In humans proinflammatory cytokines produced during infections affects the hypothalamic pituitary gonadal axis. Even though there are limited studies done on the role of sex hormones in TB disease

there are some studies that tries to assess profile of sex hormones in TB patients. In expermental study done in Mexico in 2015 to investigate the implication of pro-inflammatory cytokines in the impaired production of gonadal androgens by patients with pulmonary tuberculosis (n=36) revealed that Patients showed decreased levels of testosterone in presence of high amounts of LH, together with augmented IFN-y, IL-6 and TGF-b levels. In addition in this study in vitro treatment of Leydig cells with these cytokines led to a remarkable reduction of testosterone production (54).

On the other hand in a comparative study done in Turkey in 2016 on 68 subjects (38 TB patients and 30 healthy controls) to determine relationship between tuberculosis and female hormone levels in post-menopausal women showed increased level of estrogen in TB patients (n=38) as compared to Healthy controls (n=30) (55). Another study done in 2017 in South Africa by Kleynhans and his colleagues on 37 TB patients also shows increased estradiol concentration and decreased testosterone level in TB patients (4). Del Rey et al also showed reduced amounts of testosterone and modest increases in the concentration of estradiol accompanied by increased levels of IFN-Ɣ, IL-10, IL-6 in TB patients as compared to healthy controls (35).

2.1.3. Other hormones involved in TB disease

In addition to adrenal hormones and sex steroids, studies also show alterations of different hormones in TB patients. Studies showed decreased plasma level of leptin (56), and markedly increased plasma level of human growth hormone among TB patients, though the increase in GH levels in TB patients was not followed by increased IGF-1 concentrations (35). This is unusual since this factor is normally induced by GH and mediates many of its effects. This finding indicates that TB patients may develop a degree of GH resistance. In any case, the effect of increased levels of GH can be viewed as an attempt to improve cell-mediated immune responses, which are more efficient than humoral responses to cope with the infection with MTB (35).

3. Objectives

3.1. General Objective

- To assess selected endocrine hormone profiles in different stages of tuberculosis infection from repository plasma samples, collected over a period of time at the Armauer Hansen Research Institute (AHRI) laboratory.

3.2 Specific objective

- To assess selected endocrine hormone profiles (DHEA, Cortisol, Testosterone, Estradiol, Growth hormone and Leptin) in plasma samples of PTB and EPTB patients, LTBI, Leprosy and healthy controls using ELISA.
- To compare plasma level of selected endocrine hormone profiles (DHEA, Cortisol, Testosterone, Estradiol, Growth hormone and Leptin) in PTB patients before and after anti-TB treatment.

4. Hypothesis

- There is a significant difference in plasma concentration of selected endocrine hormone levels across different spectrum of TB infection/disease.

5. Materials and Methods

5.1. Study area

The study was conducted at AHRI using stored plasma samples that have been collected over a period of time from a spectrum of TB disease.The plasma samples used for this study were biorepository samples that were collected from a cohort of voluntary adult, HIV negative TB patients (PTB and EPTB), their close household contacts Quantiferon test or Tuberculin skin test positive (QFT/TST positive), leprosy patients and healthy community controls (QFT/TST negative), who resided in Addis Ababa, which is the capital city of Ethiopia and from Hossana and Butajira Hospitals areas, 230 and 120 km, respectively, southwest of Addis Ababa, Ethiopia.

5.2. Study design and period

A Comparative cross sectional study was conducted to analyze the levels of plasma endocrine hormones in randomly selected stored samples from September to December, 2018 GC, at AHRI, Addis Ababa Ethiopia.

5.3. Population

5.3.1. Source population

The source population were all plasma samples of TB patients, their close household contacts, leprosy patients and healthy controls collected from Addis Ababa, Hossana and Butajira Hospitals.

5.3.2. Study Population

The study population were all plasma samples of TB patients, their close household contacts, leprosy patients and healthy controls collected from Addis Ababa, Hossana and Butajira Hospitals and which meets the inclusion criteria.

5.4. Inclusion and Exclusion Criteria

5.4.1. Inclusion criteria

- HIV negative individuals.
- Samples with sufficient amount (>1ml) for analysis and with required linked data (Socio demographic and Clinical characteristics) were included in the study.

5.4.2. Exclusion criteria

- Severely malnourished, anemic and individuals with chronic debilitating illness were excluded from the initial enrollment phase of the previous studies.
- Icteric and turbid and unlabeled samples were excluded from the study.

5.5. Study variables

5.5.1. Dependent variables

- Plasma level of Cortisol, DHEA, Growth hormone, Estradiol, Testosterone and Leptin.

5.5.2. Independent variables

- Age
- Sex
- BMI
- Type of Tb disease
- BCG status

5.6. Measurement and Data collection

5.6.1. Sample Size and Sampling Technique

Convenient sampling method was used to select a total of 226 plasma samples from AHRI biorepository facility. Accordingly, 32 PTB patients (32samples each before and after treatment), 35 EPTB, 40 Leprosy patients, 40 LTBI, 47 healthy controls) were included in this study to assess the levels of selected endocrine hormones in the study population.

5.6.2. Data collection procedure

Plasma samples were retrieved from AHRI biorepository using convenient sampling method. These plasma samples have been collected from a cohort of voluntary adult, HIV negative TB patients, their close household contacts, leprosy patients and healthy community controls over a period of time from Hossana, Butajira and Addis Ababa. All samples had been collected before 10:00 am every day and plasma aliquoted and stored at −80°C. Linked archived data was extracted from AHRI data management center using structured data extraction sheets.

5.6.3. Laboratory analyses

5.6.3.1. Plasma sample preparation and quantification of hormones concentration using ELISA

Plasma samples were stored at -80^{o}C of AHRI laboratory until further assayed by enzyme-linked immunosorbent assay (ELISA). Plasma concentrations of DHEA, Cortisol, Testosterone, Estradiol, Growth hormone and Leptin were measured by ELISA according to the manufacturer's instructions (Eagle Bioscience, Italy).

Briefly, plasma samples were retrieved from -80ºC freezers, thawed on ice, centrifuged at 1500 rpm for 5 min, supernatants transferred into newly labeled 1.5 ml eppendorf tubes and kept at 4ºC until assayed by ELISA. 96-well pre-coated ELISA plates (Eagle Bioscience, Italy) were incubated with sample for 1 hour at room temperature (DHEA and HGH) at 37^0c (cortisol and testosterone),for 2 hours at 37^0c for estradiol and 1.5 hour for leptin and washed with diluted phosphate buffer wash solution followed by addition of enzyme-substrate conjugates for antibody binding. Finally a resultant color change developed that will later be read as absorbance density (OD) using ELISA reader (read at 450nm against a reference wavelength at 620nm). All laboratory assa0ys were done using commercially available kits following the manufacturer's instructions and standard operating procedures. Results were interpreted according to manufacturer's instruction. Concentrations of hormones in the plasma samples were finally extrapolated from the standard curve and all statistical analyses were done using extrapolated values (annex II).

5.7. Data Quality Assurance

Pre-analytical

Protocol for sample and reagent preparation was strictly followed. The serum or plasma concentrations of several hormones after 33 freeze-thaw cycles and more than 5 years storage at -70°C, has no meaningful effects on the plasma and serum (57).The Plasma samples used in this study were collected with in the last 10 years and stored at -80°C . According to the above evidence, no effect on the stability of the stored plasma sample for measurement of concentration of hormones.

Analytical

All laboratory assays were done using commercially available kits following the manufacturer's instructions and standard operating procedures. Besides, known standards and controls were run together with all samples in duplicates to minimize intra-variability. These controls were treated as unknowns and values determined in every test procedure performed.

Post-Analytical

Results were interpreted according to manufacturer's instruction. The results were registered in log books and data collection sheets. In relation to data entry and analysis, the data was entered and cleaned accordingly. The laboratory results were checked by senior personnel working in AHRI laboratory.

5.8. Data analysis and interpretation

Plasma level of each hormone (cortisol, DHEA, growth hormone, estradiol, testosterone and leptin) were considered as dependent variables and others such as age, sex, BMI, BCG status and clinical presentations were considered as independent variables. Linked archived data were retrieved from

AHRI data management center, cleaned and imported to GraphPad Prism 7.01 and SPSS version 20 for statistical analyses. Comparisons between groups were done using non-parametric statistics (One-way ANOVA (Kruskal-Wallis test) followed by Dunnett's multiple test comparison). Correlation between hormone levels with clinical status was done using multiple regressions. Nonparametric Wilcoxon signed test was performed to compare median difference in selected endocrine hormone profiles of PTB patients before and after treatments. Data was summarized by using descriptive statistics, frequency tables and figures. P-value less than 0.05 was considered statistically significant.

5.9. Operational definitions

In the prior studies conducted at AHRI, the study groups were operationally defined as follows:

PTB patients: Newly diagnosed, bacteriologically confirmed pulmonary tuberculosis patients with typical signs and symptom complex of TB (such as cough for more than 2 weeks, night sweats, weight loss, reduced appetite) (58-62).

LTBI: Apparently healthy individuals who were screened for latent TB infection (LTBI) and became positive by quantiferon (QFT), an interferon-gamma release assay and who were (not) contacts of PTB patients (58-62).

TBLN patients: Refers to any bacteriologically confirmed or clinically diagnosed case of TB involving the lymph nodes (63, 64).

Leprosy patients: A person with one or more of the following clinical features, and who is yet to complete a full course of treatment: hypo-pigmented or reddish skin lesion(s) with definite loss of sensation, involvement of the peripheral nerves (definite thickening with loss of sensation), and skin smears positive for acid fast bacilli (65, 66) .

Healthy controls: Apparently healthy individuals who had no history of contact nor lived with a known TB patient and tested to be negative by QuantiFERON TB test (60, 62)

5.10. Ethical considerations

This study was ethically approved by the ethics committee of the Addis Ababa University, Department of Medical Laboratory Sciences (DRERC) and the AHRI/ALERT Ethics Review Committee (AAERC). Only anonymized samples were used, while retrieving samples from AHRI biorepositories, to ensure privacy and confidentiality of study participants. In addition, institution permit was obtained from AHRI to access biorepository samples and linked archived data.

5.11. Dissemination of results

The findings of this study will be summarized and presented in a form of thesis to the Armauer Hansen Research Institute and Department of Medical Laboratory Science, College of Health Science, AAU as a partial fulfillment of MSc degree in Clinical Laboratory Sciences. In addition, the findings will be presented on annual conferences of professional societies and other appropriate national and international scientific conferences. Manuscript will be submitted to peer-reviewed journals for publication.

6. Results

6.1. Socio-demographic and clinical characteristics of study population

The proportion of different study groups was relatively similar, where males (54.6%) were slightly larger in proportions (Table 1). The median age of the study population was 23 years, range in years (12, 60). Among 32 PTB patients, 96.9% (31/32) had cough, 87.5% (28/32) had night fever, 71.9% (23/32) had poor appetite and 84.4% (27/32) had weight loss. Of the TBLN patients, 24 (68.6%) had weight loss and 15 (43%) had neck swelling as a major chief complaint. Other complaints included swelling at cervical (25.7%, 9/35), inguinal (14.3%, 5/35), axillary (8.6%, 3/35), clavicular (5.7%, 2/35) and submental (2.9%, 1/35) glands. All of the leprosy patients included in this study were lepromatus leprosy patients.

Table 1 Socio-demographic and clinical characteristics of study participants

Variables	**Study groups**					
	Healthy controls (n=47)	LTBI (n=40)	PTB (n=32)	TBLN (n=35)	Leprosy (n=40)	Total
Sex						
Male (%)	27	22	18	12	27	106
Female (%)	20	18	14	23	13	88
Total	47	40	32	35	40	194
Age (years)						
<=19	27	13	3	8	3	54
>20	20	24	27	25	32	128*
Total	47	37*	30*	33*	34*	182
BMI (Kg/m2)						
<18.49	26	22	25	*	*	
>18.5	21	18	7			
Total	47	40	32			
BCG Scar						
Present	20	13	7	5	3	48
Absent	27	25	24	30	24	130
Unknown	0	2	1	0	13	16
Total	47	40	32	35	40	194

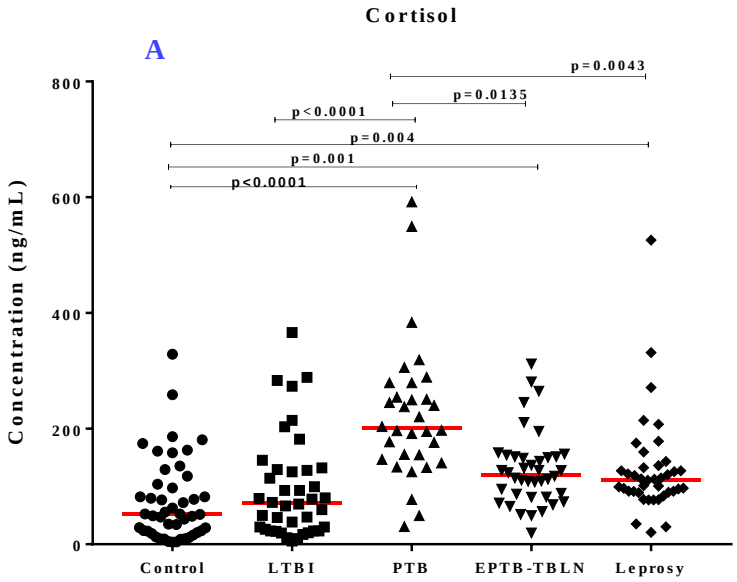
BMI: Body mass index, BCG: Bacilus Calmette-Guerin, LTBI: Latent tuberculosi infection, TBLN: Tuberculosis lymphadnitis, PTB: Pulmonary tuberculosis, * indicates that there are missed data.

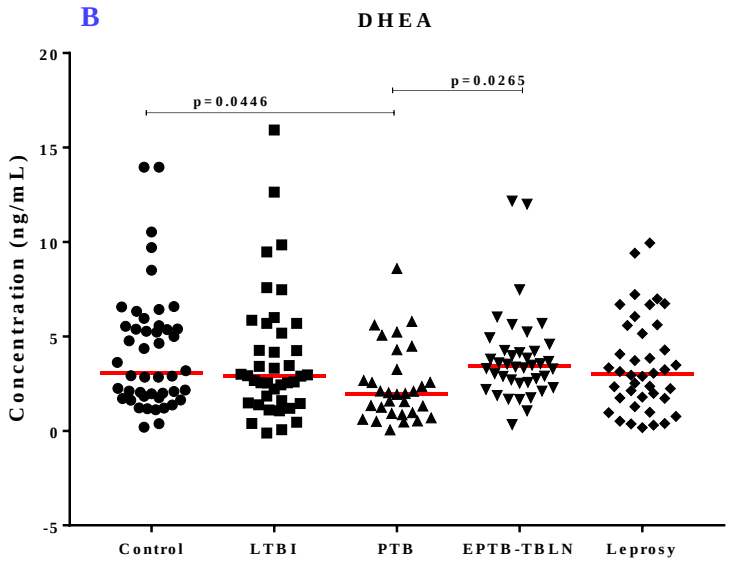
6.2. Plasma levels of hormone profiles (Cortisol, DHEA, Human Growth hormone, Estradiol, Testosterone & Leptin) between study groups

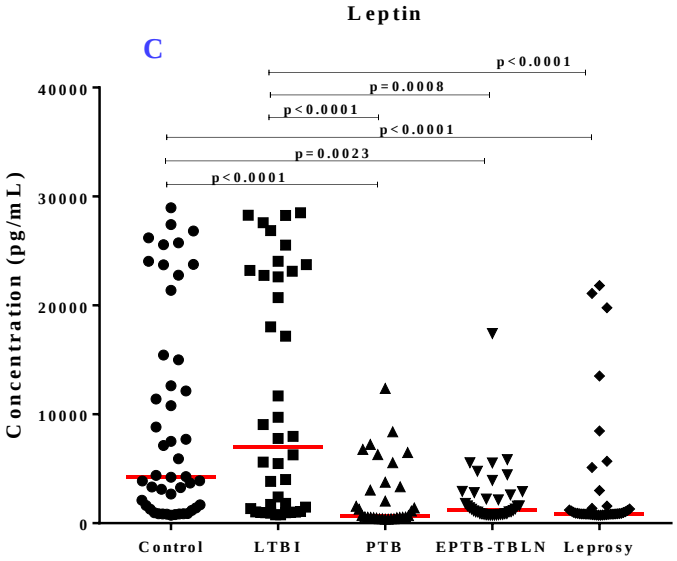
Comparison between groups was done using non-parametric statistics, namely Kruskal-Wallis test followed by Dunnett's multiple test comparison. Plasma cortisol level was significantly higher in pulmonary, extrapulmonary TB patients and leprosy patients compared to healthy controls ($p < 0.0001$, $p = 0.0011$ and $p = 0.0044$, respectively) (Figure 2, Panel A). Similarly, cortisol level was significantly higher in PTB patients compared to LTBI groups and TBLN patients ($p < 0.0001$ and $p=0.0135$, respectively) (Figure 2, Panel A). Plasma samples from leprosy patients were considered as experimental controls for the whole analyses to see if the measured hormone level was TB-specific or common to other mycobacterial chronic illnesses like leprosy. Plasma cortisol level was significantly higher in pulmonary TB patients compared to leprosy patients ($p = 0.0043$) (Figure 2, Panel A).

On the other hand, plasma DHEA level was significantly lower in pulmonary TB patients compared to healthy controls and TBLN cases ($p < 0.0446$ and $p = 0.0265$, respectively); but this was not significantly different when compared to leprosy patients (Figure 2, Panel B). Similarly, plasma leptin level was significantly lower in PTB, TBLN and leprosy patients ($p < 0.0001$, $p = 0.0023$, $p < 0.0001$, respectively) compared to healthy controls (Figure 2, Panel C). On the other hand, this level was lower in PTB, TBLN and leprosy patients compared to LTBI group ($p < 0.0001$, $p = 0.0008$, $p < 0.0001$, respectively).

Plasma human growth hormone was significantly higher in TBLN patients ($p = 0.0002$) compared to leprosy patients; however, no apparent difference was observed between the other groups. Overall, no apparent difference was observed in the plasma levels of testosterone and estradiol between the different groups.


Cortisol
A
Concentration (ng/mL)
800
600
400
200
0
p = 0.0043
p = 0.0135
p < 0.0001
p = 0.004
p = 0.001
p≤0.0001
Control
LTBI
PTB
EPTB-TBLN
Leprosy


B
DHEA
Concentration (ng/mL)
20
15
10
5
0
-5
p = 0.0446
p = 0.0265
Control
LTBI
PTB
EPTB-TBLN
Leprosy


Leptin
C
Concentration (pg/mL)
40000
30000
20000
10000
0
p < 0.0001
p = 0.0008
p < 0.0001
p < 0.0001
p = 0.0023
p < 0.0001
Control
LTBI
PTB
EPTB-TBLN
Leprosy

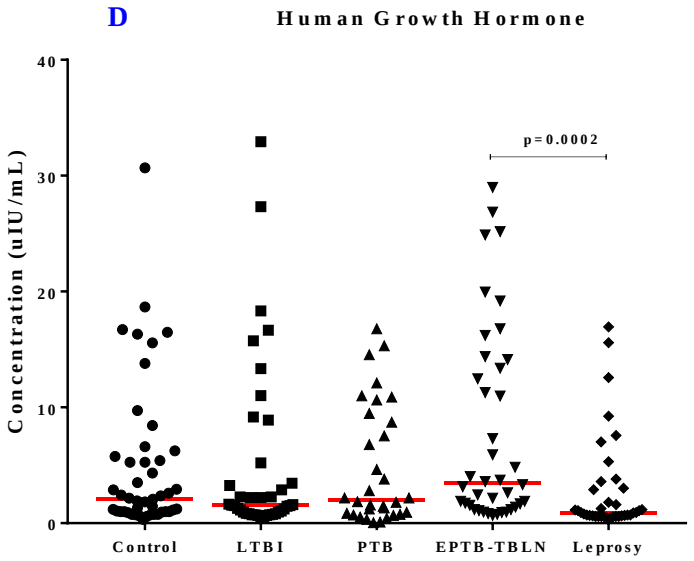

D
Human Growth Hormone
Concentration (uIU/mL)
40
30
20
10
0
p = 0.0002
Control
LTBI
PTB
EPTB-TBLN
Leprosy

Figure 2 . Plasma levels of hormone profiles in different groups of participants. Horizontal red lines represent median values. Plasma levels of hormones that are significantly associated are indicated with p-values, comparisons between groups were performed by non-parametric methods (Kruskal-Walis test). LTBI: Latent tuberculosis infection, PTB: Pulmonary tuberculosis, EPTB-TBLN: Extra pulmonary tuberculosis-TB lymphadenitis.

6.3. Plasma levels of hormone profiles (Cortisol, DHEA, Human Growth hormone, Estradiol, Testosterone & Leptin) in PTB patients before and after anti-TB treatment

The median plasma level of hormones in TB patients was assessed before and after anti-TB treatment, showing a significantly elevated level of all at follow-up, i.e. after anti-TB treatment, except for cortisol and growth hormone (Figure 3).

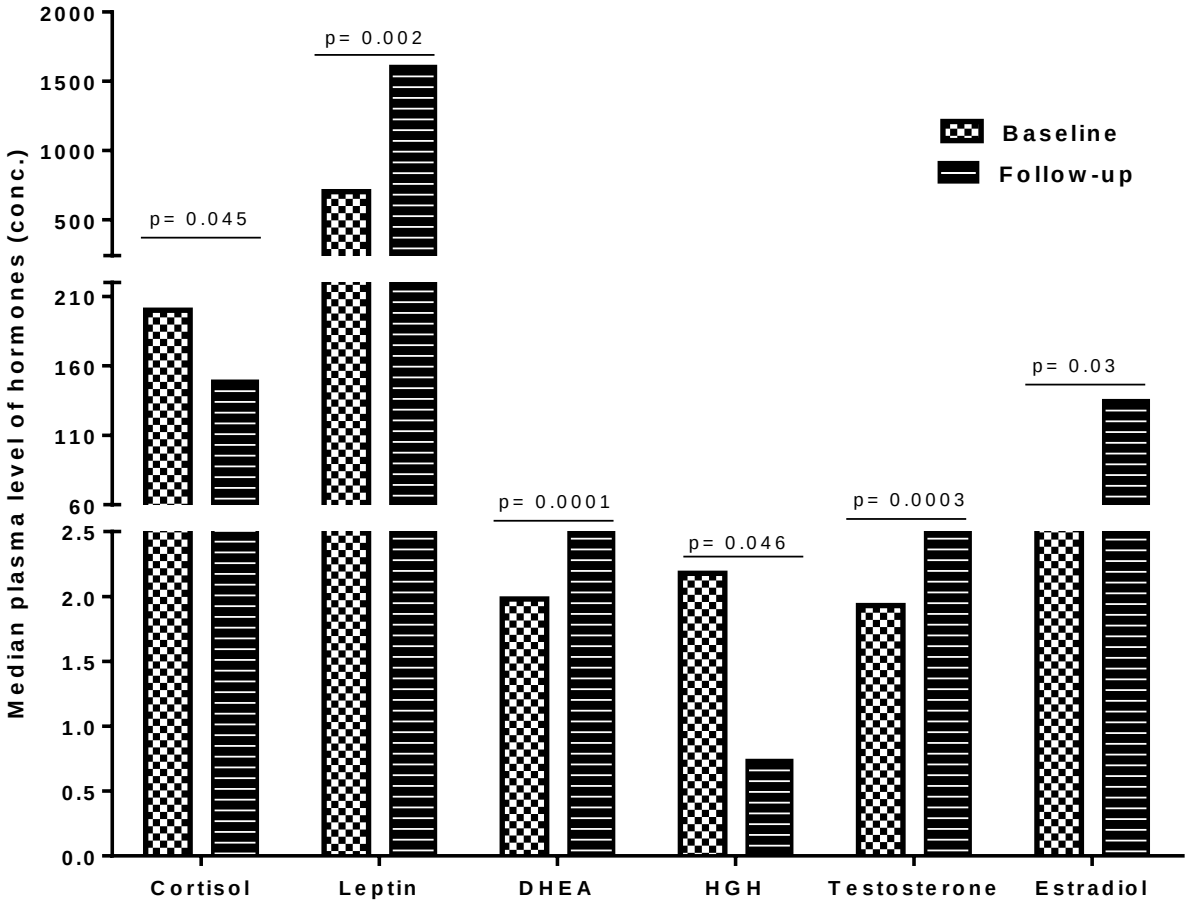


Figure 3. Plasma levels of hormones in PTB patients before and after anti-TB treatment.

6.4. Multiple linear Regression analysis results of independent variables with hormones

To assess whether some of the independent variables such as age, sex, BMI or clinical presentations are predictors of the hormone levels, we performed multiple regression on the dependent and independent variables. As summarized in table 2 in this study only cortisol and testosterone was found to be significantly predicted by independent variables.

Table 2: Multiple linear regression analysis of hormones with independent variables

Independent Variables	**Outcome variables**	**Statistics** **R^2** **P value**
Age	Cortisol	0.237 0.004
Gender	Cortisol	0.237 0.036
BMI	Cortisol	0.237 0.0001
	Testosterone	0.670 0.0001

7. Discussion

Hormones can affect immune functions, and in turn the immune response influences neuroendocrine functions (7). In this study, we investigated plasma levels of selected endocrine hormones (cortisol, dehydroepiandrosterone, leptin, human growth hormone, testosterone and estradiol) in different spectrum of tuberculosis disease (PTB patients, TBLN patients, latently infected and non-infected individuals and leprosy cases). To our knowledge, this is the first study to examine plasma level of hormones in different spectrum of tuberculosis disease in Ethiopia.

Overall, this study indicates that active TB disease is characterized by increased plasma levels of cortisol and reduced concentrations of DHEA that resulted in a higher cortisol/DHEA ratio with respect to both the healthy control group and latently infected groups. Supporting our findings, previous studies have also shown a higher concentration of cortisol in TB patients compared to healthy controls and this level increases as disease severity increases (35, 56). DHEA concentration, on the other hand, is reported to be lower in TB patients compared to controls and this level also decreases as disease severity increases (35). However, the later studies didn't examine the hormone levels among extrapulmonary TB and leprosy patients. A higher ratio of cortisol to DHEA in TB patents indicates that the immunity in this group of patients might also be modulated. Previous studies have shown that cortisol facilitates Th2 activity, partly by inhibiting Th1 cells, and its natural antagonist DHEA is able to favor Th1 cytokine production and interfere with Th2 cytokine synthesis (46, 47). Since infection with intracellular pathogens like TB requires expression of Th 1 cytokine like IFN- γ which is a central macrophage-activating cytokine involved in the immune protection against MTB (48), the increment in plasma cortisol and decrement in DHEA level could impair the immune response against TB favoring an enhanced susceptibility and disease progression (33).

In addition to this, the plasma level of both cortisol and DHEA were significantly changed after treatment, where cortisol levels significantly decreased and DHEA level increased after treatment. Similarly, others have shown that cortisol concentrations decreased during TB treatment in cured patients, but remain unchanged in patients with a failed treatment outcome, whereas DHEA concentrations gradually increased in the cured group during treatment (4). The finding of our study is also supported by former study done by Bongiovanni et al in which plasma cortisol concentration was significantly decreased after anti TB treatment (67). This alteration indicates the role these hormones play in the immunopathology of TB. Supporting this, studies have also shown that DHEA favorably affects the course of experimental tuberculosis in mice (68) and also administration of DHEA sulfate improved IFN-g production in mice immunized with heat shock proteins from MTB (69).

In line with other reports (56, 70, 71) the TB patients included in our study displayed significantly decreased plasma level of leptin compared to both healthy controls and latently infected groups. Since leptin concentration is related to the adipose tissue mass (72), the reduced leptin levels found in our study and previous reports might be linked to the weight loss that accompanies the disease. In spite of sharing similar socio-economic and environmental conditions with active TB patients, the latently infected individuals also didn't show a significant difference in plasma level of leptin as compared to healthy controls. Similarly, chronic stimulation with pro-inflammatory cytokines are also shown to suppresses leptin production in TB patients (73). In the same line our study showed a significantly improved plasma levels of leptin in PTB patients who were on anti-TB treatments and this was in agreement with a recent study (4). On the other hand, few studies have also shown an increased or no change in the levels of leptin in TB patients (74, 75). Reasons for this difference might be genetic and environmental or predisposing factors. In addition to this, the previous study sample has been collected after the initiation of treatment. In general, the decrement in leptin hormone among TB patients and its change upon treatment in the present and former studies indicated that leptin might be linked with wasting seen in TB patients.

In this study, we also assessed plasma levels of testosterone and estradiol among male and female participants, but were not statistically significant. However, other studies have shown a significantly decreased testosterone level among TB patients (35, 39). that was also associated with other pro-inflammatory cytokines such as IFN-y, IL-6 and TGF-β on the impaired testosterone production in TB patients compared to healthy controls (54). However, no difference was noted for estradiol, which might be related to effects of menstrual cycle phases and co-factors that limits the interpretation of estradiol hormone in female participants in our study. However, in line to earlier studies (4),our study showed that both testosterone and estradiol concentration were significantly increased after treatment.

In this study, we have also assessed plasma level of growth hormones (GH) across different study groups. Studies have shown that GH could prime human monocytes to kill MTB, acting as a human macrophage-activating factor (22). In our study, plasma levels of human GH was significantly higher in EPTB-TBLN patients compared to leprosy patients but no statistically significant difference was observed between other groups. In this study we saw a slightly increased levels of GH among TB patients, though it was not statistically significant, however other studies showed the concentration of growth hormone significantly increased among TB patients as compared to healthy controls (56). The effect of increased levels of GH can be viwed as an attempt to improve cell-mediated immune responses, which are more efficient than humoral responses to cope with the infection with M.

tuberculosis. Supporting this view our finding shows that plasma level of GH significantly decreased after treatment.

In general, decreased levels of powerful anabolic hormones, such as testosterone, DHEA and a decrease in leptin concentration and increased cortisol hormone could mediate the progressive debilitation and other disabling symptoms that characterize TB. In addition the alteration of these hormones upon treatment gives insight that assessment of hormones in conjunction with other biomarkers among TB patients might have prognostic value.

8. Limitations of the study

As this study involved analysis of hormones from repository plasma specimens and that levels of hormones could significantly be affected by some intrinsic factors such as dietary intake and menstrual cycles/ovulation times in female participants, etc., the observed difference in the study groups may not be accounted for these possible factors.

9. Conclusion

In conclusion, our study indicated that TB Patients are characterized by significantly increased plasma Cortisol and decreased DHEA and Leptin level, whereas plasma level of human growth hormone, testosterone and estradiol didn't showed significant difference between groups. Our study also indicated that TB treatment results in increased DHEA, leptin, estradiol &Testosterone and decreased cortisol and human Growth hormone among plumonary TB patients. This alteration of hormones during TB disease and upon treatment suggests that hormones might influence the immune response to MTB and therefore the course of the disease.

10. Recommendation

A large scale longitudinal study that combines immuno-endocrinological and metabolic profiling is required to have full picture on the role of hormones in TB immunology and their application as a biomarker of treatment response.

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Annexes

Annex I: Data Extraction sheet

I. Socio-demographic characteristics

1. Age (Years)
2. Sex Female ☐

   Male ☐
3. Study/geographic area

   Addis Ababa ☐

   Hossana ☐

   Butajira ☐

II. Study Group

1. Healthy Controls ☐
2. Latently Infected(LTBI) ☐
3. Pulmonary TB ☐
4. Extrapulmonary TB ☐
5. Leprosy ☐

III. Clinical characteristics

1. BCG status

   Vaccinated ☐

   Not Vaccinated ☐
2. BMI (Kg/m^2) _____
3. Disease sign and symptoms

   a. Pulmonary TB

   1. Cough☐ 2. Fever ☐ 3. Loss of weight☐ 4. Shortness of breath☐

   5. Loss of appetite☐ 6. Night sweats ☐ 7. Other,_____

   b. Extrapulmonary TB

   1. Chief complaints/ site of swelling

      A. Neck swelling ☐ B. Cervical☐ C. Inguinal ☐

      D. Supraclavicular ☐ E. Axillary Swelling☐ F. Others _____

2. Cough ☐ 3. Fever☐ 4. Loss of weight ☐ 5. Shortness of breath☐

6. Loss of appetite ☐ 7. Night sweats ☐ 8. Other,_____

Annex II: Standard operating procedure (SOPs)

Stadard operating procedure for Cortisol ElISA

1. **Principle of The Method:** The Cortisol (antigen) in the sample competes with the antigenic cortisol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Cortisol coated on the microplate (solid phase). After incubation, the bound/free separation is performed by a simple solid phase washing. Then, the enzyme HRP in the bound fraction reacts with the substrate (H_2O_2) and the TMB substrate develops a blue color that changes in to yellow when the stop solution (H_2SO_4) is added. The color intensity is inversily proportional to the Cortisol concentration in the sample. Cortisol in the sample is calculated through a caliberation curve.
2. **Procedure**
   - 2.1. Allow all reagents to reach room temperature (22-28 ^{0}C) for at least 30 minutes.
   - 2.2. Add 20ul Calibrator C_0-C_4 , sample and control on the respective wells.
   - 2.3. Add 200ul of conjugate
   - 2.4. Incubate for 1 hr at 37^{0}C
   - 2.5. Wash 6X with 300ul of diluted wash solution
   - 2.6. Add 100ul of TMB substrate
   - 2.7. Incubate 15 minutes in the dark at room temperature (22-28 ^{0}C)
   - 2.8. Add 100ul of stop solution
   - 2.9. Shake the microplate gently. Read the absorbance at 450nm againist a reference wavelength at 620-630nm or against blanck with in 5 minutes
   - 2.10. Concentrations of hormones in the plasma samples will be finally extrapolated from the standard curve.

Stadard operating procedure for DHEA ElISA

1. **Principle of The Method**: The essential reagents required for this immunoenzymatic assay are a biotinylated anti DHEA antibody, the DHEA antigen conjugated with the enzyme HRP (horseradish peroxidase), the DHEA antigen present in the sample, and a micro-plate coated

with Streptavidin (highly specific for Biotin). The quantity of native antigen is unknown, while the antibody and the antigen linked to HRP are in excess.

In the first part of the DHEA ELISA Assay Kit these three components are mixed together; a competitive reaction for the anti DHEA antibody between the native antigen and the antigen linked to HRP develops. Simultaneously to their formation, the complexes are fixed to the microplate wells through the interaction between the Streptavidin and the biotinylated antibody. The DHEA ELISA Assay Kit reagents in excess that have not reacted are eliminated in the washing steps. In the last part of the assay, the enzyme HRP linked in the wells reacts with the Substrate (H_2O_2) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H_2SO_4) is added. The color intensity is inversely proportional to the native antigen DHEA in the sample. DHEA concentration in the sample is finally calculated through a calibration curve.

2. Procedure

2.1. Allow all reagents to reach room temperature (22-28 ^{0}C) for at least 30 minutes.

2.2. Add 25ul Calibrator C_0-C_5 , sample and control on the respective wells (In duplicates).

2.3. Add 50ul of DHEA enzyme reagent. Shake gently the microplate for 20-30 seconds to mix.

2.4. Add 50ul of DHEA Biotin reagent. Shake gently the microplate for 20-30 seconds to mix.

2.5. Incubate 1 hr at room temperature.

2.6. Wash 5X with 350ul of diluted wash solution

2.7. Add 100ul of TMB substrate

2.8. Incubate 20 minutes in the dark at room temperature (22-28 ^{0}C)

2.9. Add 50ul of stop solution

2.10. Shake the microplate gently. Read the absorbance at 450nm againist a reference wavelength at 620-630nm or against blanck with in 5 minutes.

2.11. Concentrations of hormones in the plasma samples will be finally extrapolated from the standard curve.

Stadard operating procedure for Testosterone ElISA

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1. **Principle of The Method:** The Testosterone (antigen) in the sample competes with the antigenic Testosterone conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Testosterone coated on the microplate (solid phase). After

incubation, the bound/free separation is performed by a simple solid phase washing. Then, the enzyme HRP in the bound fraction reacts with the substrate (H_2O_2) and the TMB substrate develops a blue color that changes in to yellow when the stop solution (H_2SO_4) is added. The color intensity is inversily proportional to the Testosterone concentration in the sample. Testosterone in the sample is calculated through a caliberation curve.

2. **Procedure**

2.1. Allow all reagents to reach room temperature (22-28 0C) for at least 30 minutes.

2.2. Add 25ul Calibrator C0-C4 , sample and control on the respective wells.

2.3. Add 100ul of conjugate

2.4. Incubate for 1 hr at 37^0C

2.5. Wash 6X with 300ul of diluted wash solution

2.6. Add 100ul of TMB substrate

2.7. Incubate 15 minutes in the dark at room temperature (22-28 0C)

2.8. Add 100ul of stop solution

2.9. Shake the microplate gently. Read the absorbance at 450nm againist a reference wavelength at 620-630nm or against blanck with in 5 minutes

2.10. Concentrations of hormones in the plasma samples will be finally extrapolated from the standard curve.

Stadard operating procedure for Estradiol ElISA

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1. **Principle of The Method**: 17b-Estradiol (antigen) in the sample competes with the antigenic 17b-Estradiol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti 17b-Estradiol coated on the microplate (solid phase). After incubation, the bound/free separation is performed by a simple solid phase washing. Then, the enzyme HRP in the bound fraction reacts with the substrate (H_2O_2) and the TMB substrate develops a blue color that changes in to yellow when the stop solution (H_2SO_4) is added. The color intensity is inversily proportional to the 17b-Estradiol concentration in the sample. 17b-Estradiol in the sample is calculated through a caliberation curve.

2. **Procedure**

2.1. Allow all reagents to reach room temperature (22-28 ^{0}C) for at least 30 minutes.

2.2. Add 25ul Calibrator C_0-C_5 , sample and control on the respective wells.

2.3. Add 200ul of conjugate

2.4. Incubate for 2 hr at 37^0C

2.5. Wash 5X with 300ul of diluted wash solution

2.6. Add 100ul of TMB substrate

2.7. Incubate 30 minutes in the dark at room temperature (22-28 ^{0}C)

2.8. Add 100ul of stop solution

2.9. Shake the microplate gently. Read the absorbance at 450nm againist a reference wavelength at 620-630nm or against blanck with in 5 minutes.

2.10. Concentrations of hormones in the plasma samples will be finally extrapolated from the standard curve.

Stadard operating procedure for HGH ElISA

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1. **Principle of The Method**: In this method, the calibrators and the patient specimens and/or controls (containing the native HGH antigen) are first added to the streptavidin coated wells. Biotinylated monoclonal and enzyme labeled antibodies are then added: this antibodies have high affinity and specificity and are directed against distinct and different epitopes of HGH. Reaction between the various HGH antibodies and native HGH occurs in the microwells with out competion or steric hiderance, forming a soluble sandwich complex.

   After equilibrium is attained the antibody bound fraction is separated from unbound antigen by decantation or aspiration. Then the activity of the enzyme HRP present on the surface of the well is quantified by reaction with the TMB substrate to produce color. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. HGH in the sample is calculated through a caliberation curve.

2. **Procedure**

   2.1. Allow all reagents to reach room temperature (22-28 ^{0}C) for at least 30 minutes.

   2.2. Add 50ul Calibrator C_0-C_5 ,sample and control on the respective wells.

   2.3. Add 100ul of conjugate

2.4. Incubate 1 hr at room temperature

2.5. Wash 5X with 300ul of diluted wash solution

2.6. Add 100ul of TMB substrate

2.7. Incubate 15 minutes in the dark at room temperature (22-28 ^{0}C)

2.8. Add 100ul of stop solution

2.9. Shake the microplate gently. Read the absorbance at 450nm againist a reference wavelength at 620-630nm or against blanck with in 5 minutes.*

- *Read the microplate at 450nm and at 620nm. Read again the plate at 405nm and 620nm. Find out the wells whose Ods at 450nm are higher than 2.0. Select the corresponding Ods read at 405nm and multiply this values at 405nm by the conversion factor 3.0 (where OD 450/ OD 405= 3.0), that is: OD 450nm = OD 405nm*3.0

2.10. Concentrations of hormones in the plasma samples will be finally extrapolated from the standard curve.

Stadard operating procedure for Leptin ElISA

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1. **Principle of The Method**: A monoclonal antibody specific for Leptin A has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Leptin present is bound by the immobilized antibody. Following incubation unbound samples are removed during a wash step, and then a detection antibody specific for Leptin is added to the wells and binds to the combination of capture antibody- Leptin in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Following incubation and wash steps a substrate is added. A colored product is formed in proportion to the amount of Leptin present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven Leptin standard dilutions and Leptin sample concentration determined.
2. **Procedure:.**
   2.1. Prepare all reagents and working standards as directed in the previous sections
   2.2. Add 100 µL of Standard, control, or sample, per well. Cover with the adhesive strip provided. Incubate for 1.5 hours at 37°C.
   2.3. 1st Wash 4X with 350ul of diluted wash solution

2.4. Add 100 µL of the working solution of Biotin-Conjugate to each well. Cover with a new adhesive strip and incubate 1 hour at 37°C.

2.5. 2nd Wash 4X with 350ul of diluted wash solution

2.6. Add 100 µL of the working solution of Streptavidin-HRP to each well. Cover with a new adhesive strip and incubate for 30 minutes at 37°C Avoid placing the plate in direct light.

2.7. 3rd Wash 4X with 350ul of diluted wash solution

2.8. Add 100 µL of Substrate Solution to each well. Incubate for 10-20 minutes at 37°C. Avoid placing the plate in direct light.

2.9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

2.10. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.(optionally 630nm as the reference wave length;610-650nm is acceptable).

2.11. Concentrations of hormones in the plasma samples will be finally extrapolated from the standard curve.

Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. candidate: Yosef Tsegaye (B.Sc.)

Signature:_____

Date of submission:_____

This thesis has been submitted with our approval as advisors.

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Date: _____

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Advisor: Samuel Kinde (MSc, PhD candidate)

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Date: _____

Place: Addis Ababa, Ethiopia.

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Signature: _____

Date: _____

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