CLINICAL
INVESTIGATION

Possible impact of variations in some Cytokine levels during menstrual cycle in women of reproductive age infected with Pulmonary Tuberculosis at Nnewi, Nigeria

Abstract

Objective: The second leading cause of human mortality from infectious diseases worldwide is Mycobacterium tuberculosis (Mtb). This chronic infection is accompanied by prolonged cytokine production, which might affect the immuno-reproductive communication and favour the establishment of an adverse state. This was a prospective study designed to evaluate possible impact of some cytokine variations on menstrual cycle in TB infected females.

Methods: A total of 90 premenopausal females aged (18-45) years were randomly recruited and grouped into 30 Symptomatic TB, 30 Symptomatic TB females on ATT and 30 Control females. Blood samples were collected at follicular (Fp) and luteal phases (Lp) of menstrual cycle for determination of IL-8, IL-6, TNFα, IL-4, CD4+ T-cells, and Absolute Lymphocytes counts using enzyme-linked immunosorbent assay (ELISA), Cyflow SL Green Cytometer and Sysmex K21N Hematology Analyzer respectively.

Results: There was significantly higher IL-6, IL-8, IL-4 and TNFα with lower CD4 T-cells and Abs Lym counts in TB and TB on ATT compared to Control females at both phases of menstrual cycle ($P < 0.05$). All the cytokines were significantly lowered with higher CD4 T-cells in TB on ATT compared to TB females at both phases ($P < 0.05$). Hypogonadism correlated positively with pro and anti-inflammatory cytokines

Conclusion: The study revealed significant cytokine alterations which suggest active inflammatory process while CD4 T-cells and Abs Lym dropped showing some degree of derangement in cellular immunity at both phases of menstrual cycle; which tends to normalize on treatment. This may affect the reproductive potentials in these women.

Keywords: Pulmonary tuberculosis ▪ Premenopausal women ▪ Cytokines; Menstrual cycle ▪ Immune response

Nkiruka Rose Ukibe[1]*, Solomon Nwabueze Ukibe[2], Emmanuel Ikechukwu Onwubuya[3], Chinedum Charles Onyenekwe[1], Ifeoma Nwamaka Monago[4], Obiageli Fidelia Emelumadu[5], Ifeoma Chinwe Ikegwuonu[6], Innocent Nwabueze Okonkwo[6], Joseph Eberendu Ahaneku[7]

[1]Department of Medical Laboratory Science, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, PMB 5025, Nnewi, Anambra State, Nigeria.
[2]Department of Prosthesis and Orthosis, Federal University of Technology Owerri, Imo State Nigeria.
[3]Department of Internal Medicine, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, PMB 5025, Nnewi, Anambra State, Nigeria.
[4]Department of Medical Services, Federal Polytechnic, Oko, Anambra State, Nigeria.
[5]Depatment of Community Medicine, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, PMB 5025, Nnewi, Anambra State, Nigeria.
[6]Department of Medical Laboratory Science, Faculty of Health Sciences and Technology, College of Medicine, University of Nigeria, Enugu Campus, Enugu, Nigeria.
[7]Department of Chemical Pathology, Faculty of Medicine, College of Medicine, Nnamdi Azikiwe University, Nnewi Campus, PMB 5025, Nnewi, Anambra State, Nigeria.
*Author for correspondence:
E-mail: nr.ukibe@unizik.edu.ng

Introduction

Tuberculosis, whether pulmonary or extra pulmonary has been known to affect the menstrual cycle. Hassan and Darwish reported a prevalence rate of 66% menstrual abnormalities in women with pulmonary tuberculosis [1]. Most of these women (76%) reverted to normal menstruation

following anti TB treatment. Of all the menstrual abnormalities reported, secondary amenorrhoea constituted 26.5%, while hypomenorrhoea was 20%. Similar reports have been documented [2,3]. When menstrual abnormalities associated with tuberculosis fail to disappear after anti-TB treatment the possibility of having genital TB becomes very high. Female genital TB is usually an asymptomatic or latent infection and often undiagnosed unless specifically searched for in the course of investigating female infertility and other reproductive problems [4].

Mycobacterium tuberculosis is one of the chronic infections which solely depend on cell mediated immunity (CMI) for their control. The major cells involved are CD4+ and CD8+ T cells. Those cells quickly migrate to the site of infection and interact with antigen presenting cells (APCs). They are also found within the tuberculous granulomas where they restrict the organism and prevent reactivation [5]. The depletion of CD4+ T cells is associated with increased susceptibility to both acute and reactivation TB [6]. CD4 T cells produce IFN-Y and other cytokines which help in macrophage activation. CD4 T cells also induce apoptosis and lysis of infected cells [7]. CD8+ T cells are capable of secreting cytokines such as IFN-Y and IL-4 and thus may play a role in regulating the balance of TH1 and TH2 cells in the lungs of patients with pulmonary TB.

Reports have shown that a wide range of immune components are involved in an effective immune response against M. tuberculosis including cytokines and CD4+ T cells produced by the immune cells [8-10]. The most important among these are CD4+ T cells and the cytokine IFN-γ. The CD4+ T cells carry out several functions that are important to control infection in the granuloma. These include apoptosis of infected macrophages through Fas/Fas ligand interaction, production of other cytokines (such as IL-2 and TNF-α), induction of other immune cells (macrophages or dendritic cells) to produce other immune-regulatory cytokines such as IL-10, IL-12, and IL-15, and activation of macrophages through direct contact via CD40 ligand [11,9,12]. The CD4+ T cells also appear to be critical for the cytotoxic function of CD8+ T cells that is mediated by IL-15 [13,12]. It has also been shown that CD4+ T cells can control the intracellular growth of M. tuberculosis by a nitric oxide-dependent mechanism that is independent of IFN-γ production [14,12]. Thus, CD4+ T cells, in addition to early production of IFN-γ appear to have several other secondary functions that are critical in the control of M. tuberculosis infection.

A major effector mechanism responsible for the antimicrobial activity of IFN-γ in association with TNF-α is the induction of the production of nitric oxide and other reactive nitrogen intermediates (RNI) by macrophages via iNOS [15]. However, some M. tuberculosis factor(s), such as the 19-kDa lipoprotein, have the potential to attenuate the response of macrophages to IFN-γ by blocking the transcription of a subset of IFN-γ-responsive genes [16-18].

TNF-α, produced by macrophages, dendritic cells, and T-cells, has a major protective role against M. tuberculosis infection both in mice and humans [19,20]. It also contributes significantly to the development of immunopathology associated with TB [21]. This cytokine is involved in both immune and immunomodulatory responses and acts in synergy with IFN-γ to enhance the expression of iNOS and the antimycobacterial activity of macrophages [15]. TNF-α also initiates cell migration and formation of microbicidal granulomas while disruption of TNF-α responses leads to overgrowth of the mycobacterial pathogens [9,12]. The TNF-α produced by the infected macrophages induces the expression of chemokines, such as IL-8, MCP-1, and RANTES which provide signals for migration of immune cells to the sites of M. tuberculosis infection [22]. The phenolic glycolipid, a virulence factor in the cell wall of a hypervirulent strain of M. tuberculosis (W-Beijing family) inhibits the release of pro-inflammatory cytokines TNF-α, IL-6 and IL-12 by macrophages [23].

The advent of anti-tuberculosis therapy (ATT) has revolutionized TB management thereby raising the hope for effective management of the infection. Since the reproductive function is not immune to TB infection. The present study sets to evaluate the possible impact of variation in some cytokines during menstrual cycle among women of reproductive age infected with pulmonary Tuberculosis.

Materials and Methods

Subjects

This was a prospective study designed to assess the possible impact of variation in some cytokines during menstrual cycle among women of reproductive age infected with Pulmonary Tuberculosis at NAUTH, Nnewi, Nigeria. A total of 90 premenopausal females aged between 18 and 45 years were recruited from Dec 2014 – Nov 2015 for the study. The participants consist of 30 apparently healthy females recruited amongst the hospital staff which served as Control

group. These participants were general hospital staff who had neither any contact with tuberculosis patients or work in the direct observed therapy clinic or TB laboratory. They were all screened for tuberculosis infection. The remaining participants were randomly recruited at Direct Observed Therapy (DOT) clinic of NnamdiAzikiwe University Teaching Hospital Nnewi which served as Test subjects. All the participants were screened to exclude HIV subjects, TB infected subjects were classified using WHO and CDC criteria for TB staging as Symptomatic TB infected participants (n=30); Symptomatic TB infected participants on ATT (n=30); these participants had been placed on category 1 anti-tuberculosis therapy for six months

A well-structured questionnaire was administered to each participant to ascertain the history of their menstrual cycle, reproductive history and other biodata. Routine investigations for Mycobacterium tuberculosis were done using concentrated sputum for microscopy and Zielh Neelson staining techniques for AFB. Chest x-ray examination results of participants were obtained from their respective EPI data files for confirmation of pulmonary tuberculosis and other data.

Neurotrophins are diffusible peptides secreted from neurons and neuron-supporting cells. They serve as growth factors for the development, maintenance, repair, and survival of specific neuronal populations. During development, NTFs promote neuronal survival, stimulate axonal growth, and play a key role in the construction of the normal synaptic network [8]. Therefore, any alterations in their local synthesis transport or signaling (e.g. binding, internalization, receptor synthesis etc.) due to local damage, aging, mutation, or polymorphism could adversely affect neuronal survival and lead to neuronal death. Indeed studies shows that alteration of neurotrophins for selective neuronal population might correlate with the neurodegeneration. Recently, researchers have directed their attention to the identification of those conditions promoting human neuronal survival and repair in neurodegenerative diseases.

Blood sample collection

Ten ml of blood sample was collected from each participant at follicular (7-13th day) and at luteal (21-23rd day) phases of menstrual cycle. The phases of menstrual cycle was confirmed using structured questionnaire and the participant recruited were women who had the same menstrual cycle pattern (28 days cycle). The blood sample was collected between 8 to 10 am by venepuncture. Six ml was dispensed into dry plain

bottles and allowed to clot, retracted and centrifuged. Five ml of blood was collected dispensed into EDTA bottles and was used immediately for screening to exclude malaria parasite and HIV infected subjects, CD4+ T-Cell count and absolute lymphocyte count. The plasma content was separated and stored at -200°C for determination of cytokines levels (Interleukin-8, Interleukin-6, Interleukin-4, Tumor Necrosis alpha).

Ethical clearance and informed consent

The Ethics Committee of Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra state, Nigeria approved the study design according to the principles expressed in the declaration of Helsinki. The participants were informed about the study design and only those who gave their consent were recruited for the study. The informed consent form was written and approved along with the ethical clearance obtained from the ethics committee board of NAUTH Nnewi. The consent form was issued with the questionnaire during recruitment which only those who agreed and volunteered to participate signed before their blood samples were collected. All the participants recruited were assured that information obtained from them would be treated with uttermost confidentiality and they had the full right not to participate in or withdraw from the study at any point they desired to.

Exclusion and inclusion criteria

Participants with HIV infection were excluded from the study. Participants with malaria parasite infection as at the time of study were also excluded. Participant with relapse, those that failed to respond to drug regimen and that extra pulmonary tuberculosis were excluded and subjects with known fertility problems before contracting TB infections were also excluded. Hence the female participants used were those newly diagnosed for TB and those who conformed and completed their category 1 treatment with no prior fertility problems until the existence of TB infection.

Drug administration for TB Infection

Only the antituberculosis therapy was given to Tuberculosis subjects as follows: The drug regimen comprised of (1) two months intensive therapy and 4 months continuation therapy and the combination of drugs were as follows (RHZE): Rifampicin (R) 150 mg + isoniazide (H) 75 mg + pyrazinamide (Z) 400 mg + ethambutol (E) 275 mg orally once daily for 2 months at dosage according to the patient's body weight. For continuation therapy, rifampicine and isonizide (RH) were given orally once daily for 4 months.

Methods

Examination of Sputum for AFB by ZiehlNeelson Method as described by Cheesbrough (2005) and Culture Methods for the determination of Mycobacterium Tuberculosis using Lowestein Jensen medium technique as described by chessbough 24.

Determination of CD4+ T-Cells Count using Cyflow SL Green cytometer

Determination of Full blood count (FBC) using Sysmex K21N HematologyAnalyzer

Determination of Tumour Necrosis factor Alpha and Interleukin-4, 6, 8 Assay as described by Enzyme Linked Immunosorbent Assay ELISA kits (Glory Science Laboratory USA)

Statistical analysis

The version 16 of SPSS package was used in statistical analysis. The variables were expressed as mean (± SD). The Student independent t-test and analysis of variance (ANOVA) and post-hoc (LSD) were used to assess significant mean differences. The Pearson correlation coefficient was used to assess the level of association between two variables. The level of significance was considered at $P<0.05$.

Results

Levels of cytokines (IL-8, IL-6, IL-4 and TNFα) at follicular and luteal phases of menstrual cycle

The mean values of IL-8, IL-6 and TNFα (pg/ml) in Symptomatic TB females were not significantly different between follicular (530.9 ± 189.3, 533.3 ± 302.6, 610.9 ± 260.2) and luteal (547.9 ± 214.2, 507.9 ± 273.0, 674.6 ± 233.8) phases of menstrual cycle ($P>0.05$). Similar result was observed in same parameters in SymptomaticTB females on ATT between follicular (628.5 ± 262.3, 427.4 ± 145.7, 719.9 ± 122.8) and luteal (533.1 ± 273.1, 388.3 ± 170.8, 568.0 ± 276.3) phases of menstrual cycle ($P>0.05$). Furthermore, no significant difference was observed in the mean IL-6 value between follicular (217.6 ± 64.9) and luteal (204.6 ± 36.7) phases of menstrual cycle in Control female subjects ($P>0.05$). Conversely, the mean TNFα value (pg/ml) dropped significantly at follicular phase (211.8 ± 57.6) compared to luteal phase (333.0 ± 72.2) of menstrual cycle in Control female subjects ($P<0.05$).

The mean IL-4 value (pg/ml) was significantly lower in Symptomatic TB female and Symptomatic TB females subjects on ATT between follicular (508.2 ± 250.5, 510.0. ± 228.5) and luteal (403.8 ± 166.6,

666.9 ± 259.2) of menstrual cycle ($P<0.05$ respectively). IL-4 value (pg/ml) was significantly lower at follicular phase (210.7 ± 71.2) compared with luteal phase of menstrual cycle (334.8 ± 76.5) in Control female subjects ($P<0.05$).

When the mean IL-8, IL-6, IL-4 and TNFα values (pg/ml) at follicular phase of menstrual were compared between the Control group and Test groups, the mean IL-8, IL-6, IL-4 and TNFα were significantly higher in Symptomatic TB females (530.9 ± 189.3, 427.4 ± 145.7, 610.9 ± 260.2, 403.8 ± 166.6) and Symptomatic TB females on ATT (628.5 ± 262.3, 553.3 ± 302.6, 719.9 ± 122.8, 510.0. ± 228.5) compared with follicular value in the Control female subjects (280.1 ± 47.7, 217.6 ± 64.9, 211.8 ± 57.6, 210.70 ± 71.2) ($P<0.05$ respectively). The mean IL-8, IL-6, IL-4 and TNFα values (pg/ml) at luteal phase of menstrual cycle were significantly higher in Symptomatic TB females (547.9 ± 214.2, 507.9 ± 273.0, 674.6 ± 233.8, 508.2 ± 250.5) and Symptomatic TB females on ATT (533.1 ± 273.1, 388.3 ± 170.8, 568.0 ± 276.3, 508.2 ± 250.5) compared with luteal value in the Control female subjects (276.9 ± 56.3, 204.6 ± 36.7, 333.0 ± 72.2, 334.8 ± 76.5) ($P<0.05$ respectively). The mean TNFα concentration (pg/ml) at luteal phase of menstrual cycle was significantly higher in Symptomatic TB females (674.6 ± 233.8) and Symptomatic TB females on ATT (568.0 ± 276.3) compared with luteal value in the Control female subjects (333.0 ± 72.2) ($P<0.05$ respectively).

The post hoc analysis also showed significantly lower mean IL-6 (pg/ml) at follicular phase of menstrual cycle in Symptomatic TB females on ATT (427.4 ± 145.7) compared with follicular value in the Symptomatic TB females (533.3 ± 302.6) ($P<0.05$). Contrastingly, The post hoc analysis showed significantly higher mean IL-4 value (pg/ml) at follicular phase of menstrual cycle in Symptomatic TB females on ATT (510.0. ± 228.5) compared with follicular value in Symptomatic TB female subjects (403.8 ± 166.6) ($P<0.05$). IL-6 (pg/ml) was significantly lower in Symptomatic TB females on ATT (388.3 ± 170.8) compared with the value observed in Symptomatic TB females (507.9 ± 273.0) at luteal phase of menstrual cycle ($P>0.05$) However, The post hoc analysis showed significant higher mean IL-4 value (pg/ml) at luteal phase of menstrual cycle in Symptomatic TB females on ATT (666.9 ± 259.2) compared with luteal value in Symptomatic TB female subjects (508.2 ± 250.5) ($P<0.05$) (See **Table 1**).

Parameters	IL-8 (pg/ml)			IL-6(pg/ml)			IL-4(pg/ml)			TNFα(pg/ml)		
Groups	Follicular	Luteal	P-value	Follicular	Luteal	P-value	Follicular	Luteal	P-value	Follicular	Luteal	P-value
Symptomatic TB (A) (n=30)	530.9 ± 189.3	547.9 ± 214.2	0.850 (ns)	553.3 ± 302.6	507.9 ± 173.0	0.120 (ns)	433.8 ± 166.6	508.2 ± 250.5	0.032	610.9 ± 260.2	674.6 ± 233.8	0.421(ns)
Symptomatic TB on Drugs (B) (n=30)	628.5 ± 262.3	533.1 ± 273.1	0.267 (ns)	427.4 ± 145.7	388.3 ± 170.8	0.077 (ns)	510.0 ± 228.5	666.9 ± 259.2	0.006	719.9 ± 122.8	568.0 ± 276.3	0.064(ns)
Control (D) (n=30)	280.1 ± 47.7	276.9 ± 56.3	0.822 (ns)	217.6 ± 64.9	204.0 ± 36.7	0.349 (ns)	310.7 ± 71.2	232.0 ± 76.5	0.020	341.8 ± 77.6	333.5 ± 70.9	0.682(ns)
F-value	10.948	10.441		14.137	7.266		6.730	3.565		12.362	7.784	
P-value	0.000	0.000		0.000	0.001		0.000	0.033		0.000	0.001	
AvsB	0.214	0.809		0.030	0.029		0.367	0.091		0.141	0.174	
Avs C	0.000	0.000		0.000	0.000		0.002	0.000		0.000	0.000	
BvsC	0.000	0.000		0.000	0.000		0.000	0.004		0.000	0.002	

Table 1: Serum levels of some Cytokines in Symptomatic TB, Symptomatic TB on ATT and Control female subjects at follicular and luteal Phases of menstrual cycle

Blood concentrations of CD4+ T-Cells and Absolute Lymphocyte counts at Follicular and Luteal Phases of Menstrual cycle

The mean CD4+ T- cell count (/μl) and Absolute Lymphocyte count (x103/μl) in Symptomatic TB females were not significantly different between follicular (217 ± 93, 2.21 ± 1.13) and luteal (212 ± 97, 2.24 ± 1.43) phases of menstrual cycle (P>0.05). Similar observation was made in in same parameters Symptomatic TB females on ATT between follicular (387 ± 114, 2.33 ± 1.00) and luteal (367 ± 136, 2.61 ± 1.65) phases of menstrual cycle (P>0.05). The mean CD4+ T-cell count (/μl) was not significantly different between follicular (689 ± 172) and luteal (660 ± 157) phases of menstrual cycle in Control female subjects (P>0.05). However, the mean Absolute Lymphocyte count (x103/μl) was significantly lower at follicular phase (5.39 ± 1.59) compared with luteal phase (6.19 ± 1.69) in Control female subjects (P<0.05)

When the mean CD4+ T-cell (/μl) and Absolute Lymphocyte count at follicular phase of menstrual cycle were compared between the Control group and Test groups, the mean CD4+ T-cell and Absolute Lymphocyte count were significantly lower in Symptomatic TB (217 ± 93, 2.21 ± 1.13) and Symptomatic TB females on ATT (387 ± 114, 2.33 ± 1.00) compared with follicular value in the Control female subjects (689 ± 172, 5.39 ± 1.59) (P<0.05). Similar observation were made with same parameters at luteal phase of menstrual cycle in Symptomatic TB (212 ± 97, 2.24 ± 1.43) and Symptomatic TB females on ATT (367 ± 136, 2.61 ± 1.65) compared with Control female subjects (660 ± 157, 6.19 ± 1.69) (P<0.05 respectively).

The post hoc analysis showed significantly higher mean CD4+ T-cell count (/μl) in Symptomatic TB females on ATT (387 ± 114367 ± 136) compared with Symptomatic TB females (217 ± 93, 212 ± 97) at follicular and luteal phases of menstrual cycle (P<0.05 respectively) (See **Table 2**).

Pattern of gonadal function, proinflammatory cytokines (IL8 and IL-6, TNFα) and anti-inflammatory cytokins (IL-4) in Symptomatic TB female subjects at follicular phase of the menstrual cycle

The result shows that in Symptomatic TB females 23(77%) had hypogonadism and high plasma levels of proinflammatory cytokines (IL-8 and IL-6) while 7(23%) of Symptomatic TB females had normogonadism and normal or low proinflammatory cytokines (IL-8 and IL-6) levels at follicular phase of menstrual cycle (X2 = 20.000, P = 0.000). There was significant positive correlation between hypogonadism and proinflammatrory cytokines (IL-8 and IL-6) at follicular phase of menstrual cycle (r=1.000, P=0.000). Similarly, the result shows that in Symptomatic TB females 20(67%) had hypogonadism and high plasma levels of proinflammatory cytokines (IL-8 and IL-6) while 6(20%) of Symptomatic TB females had normogonadism and high plasma levels of proinflammatory cytokines (IL-8 and IL-6) but 4(13%) of Symptomatic TB females had normogonadism and normal or low levels of proinflammatory cytokines at luteal phase of menstrual cycle (X2 = 6.555, P = 0.010), There was also significant positive correlation between hypogonadism and proinflammatory cytokines (IL-8 and IL-6) at luteal phase of menstrual cycle (r=0.572, P=0.008).

Furthermore, 23(77%) of Symptomatic TB females had hypogonadism and high plasma levels of anti-inflammatory cytokines (IL-4) while 1(3%) had normogonadism with high levels of anti-inflammatory cytokines but 6(20%) of Symptomatic TB females had

Table 2: Blood concentrations of CD4+ T-cell and Absolute Lymphocyte Count in Symptomatic TB, Symptomatic TB on ATT and Control female subjects at follicular and luteal Phases of menstrual cycle

Parameters	CD4 T- cell Count (/µl)			Abs Lym Count (x10³/µl)		
Groups	**Follicular**	**Luteal**	**P-value**	**Follicular**	**Luteal**	**P-value**
Symptomatic TB (A) (n=30)	217.0 ± 93.0	212.0 ± 97.0	0.772(ns)	2.23 ± 1.05	2.24 ± 1.43	0.990(ns)
Symptomatic TB on Drugs (B) (n=30)	387.5 ± 114.0	367.1 ± 136.0	0.879(ns)	2.33 ± 1.03	2.61 ± 1.65	0.538(ns)
Control (D)(n=30)	689.0 ± 172.0	660..0 ± 157.0	0.425(ns)	5.39 ± 1.59	6.19 ± 1.69	0.169(ns)
F-value	23.561	24.653		11.788	7.084	
P-value	0.000	0.000		0.000	0.001	
AvsB	0.000	0.000		0.811(ns)	0.000	
Avs C	0.000	0.000		0.000	0.000	
BvsC	0.000	0.000		0.000	0.000	

normogonadism and normal or low antinflammatory cytokines (IL-4) level at follicular phase of menstrual cycle (X^2= 15.000, P= 0.000). Hypogonadism significantly correlated positively with antinflammatory at follicular phase of menstrual cycle (r=0.866, P=0.000). Similarly, 20(67%) of Symptomatic TB female had hypogonadism and high plasma levels of anti-inflammatory cytokines (IL-4) while 6(20%) of Symptomatic TB females had normogonadism with high levels of anti-inflammatory cytokines but 4(13%) of Symptomatic TB females had normogonadism and normal or low antinflammatory cytokines (IL-4) level at luteal phase of menstrual cycle (X^2= 6.555, P= 0.010). Hypogonadism significantly correlated positively with antinflammatory at luteal phase of menstrual cycle (r=0.572, P=0.008). In addition, 23(77%) of Symptomatic TB females had hypogonadism with high plasma levels of TNFα while 1(3%) of Symptomatic TB females had normogonadism with high levels of TNFα and 6(20%) of Symptomatic TB females had normogonadism and normal or low TNFα levels at follicular phase of menstrual cycle (X^2= 15.000, P=0.000). Hypogonadism significantly correlated positively with TNFα at follicular phase of menstrual cycle (r=0.866, P=0.000). Similarly, 20(67%) of Symptomatic TB females had hypogonadism with high levels of TNFα while 8(27%) of Symptomatic TB females had normogonadism with high plasma levels of TNFα and 2(7%) had normogonadism and normal or low TNFα level at luteal phase of menstrual cycle (X^2 = 4.127, P = 0.041). Hypogonadism significantly correlated positively with TNFα at follicular phase of menstrual cycle (r = 0.454, P=0.044) (See **Table 3**).

Pattern of gonadal function and proinflammatory cytokines (IL-8, IL-6, TNFα) and anti-inflammatory cytokines (IL-4) in Symptomatic TB female

participants on ATT at follicular and luteal phases of menstrual cycle

The result shows that in Symptomatic TB females on ATT 15(50%) had hypogonadism and high plasma levels of proinflammatory cytokines (IL-8 and IL-6) while 8(27%) of Symptomatic TB females on ATT had normogonadism and high levels of proinflammatory cytokines (IL-8 and IL-6) and 7(23%) had normogonadism with normal or low proinflammatory cytokines at follicular phase of menstrual cycle ($X2$ = 6.607, P = 0.011).

There was significant positive correlation between hypogonadism and proinflammatory cytokines (IL-8 and IL-6) at follicular phase of menstrual cycle (r=0.577, P=0.008). Similarly, 18(60%) of Symptomatic TB females on ATT had hypogonadism and high plasma levels of proinflammatory cytokines (IL-8 and IL-6) while 8(27%) of Symptomatic TB females on ATT had hypogonadism and normal or low proinflammatory cytokines (IL-8 and IL-6) levels but 4(13%) of Symptomatic TB females on ATT had normogonadism and normal or low proinflammatory cytokines at luteal phase of menstrual cycle ($X2$ = 5.294, P = 0.021). There was significant positive correlation between hypogonadism and proinflammatory cytokines (IL-8 and Il-6) at luteal phase of menstrual cycle (r=0.0514, P= 0.020).

Furthermore, 15(50%) of Symptomatic TB female subjects on ATT had hypogonaism and high plasma levels of anti-inflammatory cytokines (IL-4) while 10(33%) had normogonadism with high levels of anti-inflammatory cytokines but 5(17%) of Symptomatic TB females on ATT had normogonadism and normal or low anti inflammatory cytokines (IL-4) level at follicular phase of menstrual cycle ($X2$= 3.529, P= 0.060). There was significant positive correlation

Table 3: Pattern of gonadal function, proinflammatory cytokines (IL-8 and IL-6, TNFα) and anti-inflammatory cytokines (IL-4) in Symptomatic TB female participants at follicular and luteal phases of the menstrual cycle						
	Follicular phase			**Luteal phase**		
	Proinflammatory cytokines(IL8 and IL-6)					
Gonadal status	High proinflammatory cytokine (%)	Normal/low (%)	Total (n)	High proinflammatory cytokines (%)	Normal/low (%)	Total
Hypogonadism	23(77)	0(0)	23	20(67)	0(0)	20
Normogonadfism	0(0)	7(25)	7	6(20)	4(13)	10
Total	23	7	30	26	4	30
	X^2 = 20.000, P = 0.000 Pearson correlation r=1.000, P=0.000			X^2 = 6.555, P = 0.010 Pearson correlation r=0.572, P=0.008		
	Antiiflammatory cytokines (IL-4)					
Gonadal status	High antiinflammatory cytokines	Normal/low	Total	High antiinflammatorry cytokines (%)	Normal/low (%)	Total
Hypogonadism	23(77)	0(0)	23	20(67)	0(0)	20
Normogonadism	1(3)	6(20)	7	6(20)	4(13)	10
Total	24	6	30	26	4	30
	X^2= 15.000, P= 0.000 Pearson correlation r=0.866, P=0.000			X^2= 6.555, P= 0.010 Pearson correlation r=0.572, P=0.008		
	TNFα					
Gonadal status	High TNFα (%)	Normal/low (%)	Total	(High TNFα (%)	Normal/low (%)	Total
Hypogonadism	23(77)	0(0)	23	20(67)	0(0)	20
Normogonadism	1(3)	6(20)	7	8(27)	2(10)	10
Total	24	6	30	28	2	30
	X^2= 15.000, P= 0.000 **Pearson correlation** r=0.866, P=0.000			X^2= 4.127, P= 0.041 **Pearson correlation** r=0.454, P=0.044		

between hypogonadism and antiinflammatory cytokines (IL-4) at follicular phase of menstrual cycle (r=0.420, P=0.065). Similarly, 18(60%) of Symptomatic TB females on ATT had hypogonadism and high levels of anti-inflammatory cytokines (IL-4) while 8(27%) of Symptomatic TB females on ATT had normogonadism with high levels of anti-inflammatory cytokines but 4(13%) of Symptomatic TB females on ATT had normogonadism and normal or low antinflammatory cytokines (IL-4) level at luteal phase of menstrual cycle (X^2=5.294, P=0.021). There was significant positive correlation between hypogonadism and antiinflammatory cytokines (IL-4) at luteal phase of menstrual cycle (r=0.514, P=0.020).

However, there was no significant association between hypogonadism and TNFα in Symptomatic TB females on ATT at follicular phase of menstrual cycle (X^2=1.053, P=0.305, r=0.229, P=0.331). On the other hand, 20(67%) of Symptomatic TB females on ATT had hypogonadism and high plasma levels of TNFα while 6(20%) of Symptomatic TB females on ATT had hypogonadism and normal or low levels of TNFα but 4(13%) of Symptomatic TB females on ATT had normogonadism and high levels of TNFα at luteal phase of menstrual cycle (X^2=6.555, P=0.010).

There was significant positive correlation between hypogonadism and TNFα at luteal phase of menstrual cycle (r=0.572, P=0.008) (See **Table 4**).

Discussion

The significantly higher levels of IL-8, IL-6, IL-4 and TNFα in Symptomatic TB females and Symptomatic TB females on ATT compared to Control females at both follicular and luteal phases of menstrual cycle showed higher degree of inflammation in diseased subjects when compared to healthy people. This is consistent with the report of Verthelyi et al. [25]. Significant elevation of some cytokines including TNF-α in the lungs of women with pulmonary TB have also been reported [26]. This was also attributed to sex steroid hormones changes with indication that testosterone impairs macrophage activation and pro-inflammatory cytokines production, while estrogens are pro-inflammatory mediators inducer. Previous study has also reported that pulmonary TB had marked reversible effect on the menstrual cycle in women of reproductive aged1. Locksley et al. reported that excessive production of cytokines especially TNFα has been associated with fever and tumor formation [27]. Hypogonadism has been associated with this

Table 4: Pattern of gonadal function and proinflammatory cytokines (IL-8, IL-6, TNFα) and antiiflammatory cytokines (IL-4) in Symptomatic TB female participants on ATT at follicular phase of the menstrual cycle						
Follicular phase				**Luteal phase**		
Proinflammatory cytokines (IL-8 and IL-6)						
Gonadal status	**High proinflammatory cytokines (%)**	**Normal/low (%)**	**Total**	**High proinflammatory cytokines (%)**	**Normal/low (%)**	**Total**
Hypogonadism	15(50)	0(0)	15	18(60)	8(27)	26
Normogonadism	8(27)	7(23)	15	0(0)	4(13)	4
Total	23	7	30	18	12	30
$X^2 = 6.607$, P = 0.011 Pearson correlation r=0.577, P=0.008				$X^2 = 5.294$, P = 0.021 Pearson correlation r=0.0514, P= 0.020		
Antiinflammatory cytokines (IL-4)						
Gonadal status	High Antinflammatory cytokines (%)	Normal/low (%)	Total	High Antiinflammatory cytokines (%)	Normal/low (%)	Total
Hypogonadism	15(50)	0(0)	15	18(60)	8(27)	26
Normogonadism	10(33)	5(17)	15	0(0)	4(13)	4
Total	25	5	30	18	12	30
$X^2 = 3.529$, P= 0.060 Pearson correlation r=0.420, P=0.065				$X^2 = 5.294$, P= 0.021 Pearson correlation r=0.514, P=0.020		
TNFα						
Gonadal status	High TNFα (%)	Normal/low (%)	Total	High TNFα (%)	Normal/low (%)	Total
Hypogonadism	15(50)	0(0)	15	20(67)	6(20)	26
Normogonadism	14(47)	1(3)	15	0(0)	4(13)	4
Total	29	1	30	20	10	30
$X^2 = 1.053$, P= 0.305 Pearson correlation r=0.229, P=0.331				$X^2 = 6.555$, P= 0.010 Pearson correlation r=0.572, P=0.008		

disease stateand may be responsible for the increase in pro-inflammatory cytokines such as TNFα and IL-6 as observed in this study [28]. Previous reports have shown higher serum levels of TNF-α in pulmonary TB patients than control subjects [29-33]. TNFα is essential for initiation and co-ordination of cellular responses although its excessive production can lead to tissue damage [34]. TNF-α is produced at the site of disease in tuberculosis patients [35]. Early clinical deterioration in treatment is associated with a selective increase of TNF-α in plasma [36]. PTB patients accompanied by systemic manifestations showed increased TNF-α and IL-10 and decreased IL-12 levels compared with controls [37]. An earlier study has also identified elevated blood plasma levels of IL-6 in TB patients with developed lung lesions [38].

The significantly higher levels of IL-4 in TB infected subjects may be due to anti-inflammatory effects of estrogens. This is known to promote type 2 immune responses. Even though tuberculosis as a chronic inflammatory disease is known to promote type 1 type of immune responses which is characterized by the induction of pro-inflammatory cytokines [39].

The present study also showed that between 70-85%

of Symptomatic TB females had hypogonadism and high serum levels of pro and anti - inflammatory cytokines (IL-8, IL-6, IL-4 and TNFα) at both follicular and luteal phases and hypogonadism significantly correlated positively with pro- and anti- inflammatory cytokines at both phases of the menstrual cycle. Previous reports have highlighted the implication of elevated cytokines on endocrine and metabolic functions [40,41]. This has a far reaching effect on menstrual and reproductive functions of the affected women [42]. Evidence suggests that serum IL-1 increases while IL-6 decreases during the luteal phase, whereas serum IL-10 levels do not fluctuate with the menstrual cycle [43-45]. However, the effect of estrogen levels on TNF-α production has been controversial [46-48]. Some previous studies have also shown increased production of IL-4 in human TB patients, especially those with cavitory disease [49,50]. This is consistent with the present study; however, it is still not clear if IL-4 causes or reflects disease activity in human TB [51,52]. Some studies showed no detectable IL-4 in any TB patient and no significant variation in IL-4 level between TB subjects and controls [53-56].

However, the significantly reduced levels of IL-8, IL-6, IL-4 and TNFα in Symptomatic TB females on ATT compared to their counterparts without treatment

showed significant reduction of inflammation. This shows some level of improvement in the reproductive function in these subjects which may be due to clearing of TB bacilli from blood [57]. This signifies beneficial effects of treatment which results in significant restoration of the cellular immunity and reduction of inflammation in these patients. Significant increase in IL-4 has been observed in the plasma of TB patients compared to household contacts but did not differentiate between HIV patients and non-HIV patients with TB [58,59]. However, anti-TB treatment is associated with decreased plasma IL-4 levels [60]. This is consistent with the present study.

The significantly higher level IL-4 in TB subjects on ATT especially at the luteal phase was consistent with the value observed in Control females. This shows a reduction in inflammatory activities and humoral immunity which is a Th2 type response. It should be noted that IL-4 is also secreted by activated immune cells such as monocytes. Previous report has shown some variations in progesterone and estrogen which is thought to be induced by HIV/TB infections [60]. Reduced ovarian function (hypogonadism) is responsible for the reduction of estrogen and progesterone which accounts for menstrual changes that can lead to infertility 28.

The insignificant difference in the level of CD4+ T-cell observed in Symptomatic TB females and Symptomatic TB females on ATT between follicular and luteal phases of menstrual cycle is consistent with normal physiological findings.

On the contrary, some other studies carried out in apparently healthy individuals indicated that Absolute lymphocyte count was significantly lower at the follicular phase than the luteal phase [61,60]. It was also reported that in healthy women with normal menstrual cycle, the luteal phase of the ovarian cycle was associated with increased WBC count and Absolute lymphocyte counts and the immune response shifted towards a Th2-type response with increased production of progesterone and estrogen and increased levels of anti-inflammatory cytokines (IL-4). The Absolute lymphocyte count has been reported to reach a maximum at mid cycle coinciding with maximum level of estrogen peak in normal women [61]. These reports from normal women seem to contrast the findings in the present study suggesting that HIV and TB infections altered the normal immune response associated with menstruation by inducing hypogonadism. Some evidence however, has also shown that sex hormones can affect the number and/or activation state of lymphocytes. For example, disease severity in patients with immune-mediated disorders is influenced by serum estrogen, progesterone and/or androgen levels [62,63].

The significantly reduced level of CD4 T-cell count and Absolute lymphocyte counts in Symptomatic TB females and Symptomatic TB females on ATT compared to Control females at both follicular and luteal phases of the menstrual cycle signify a reduction in cellular immunity which is the hallmark of HIV and TB infections. This has been previously reported [64-67]. Cellular immunity involving CD4+T-cells plays a major role in tuberculosis infection and loss of CD4+ T-cells was associated with increased susceptibility to TB [68-70]. However, it has been postulated that CD4 T-cells could promote rather than control tuberculosis in the absence of PD-1 (protein derivative mediated inhibition) [71]. It has also been reported that changes in CD4 T-cell counts correlated significantly with progesterone and estrogen concentration [72].

The hypogonadism which has been associated with TB infections has been reported to cause a significant reduction in cellular immunity and severity of the infections in the affected individuals [72,73].

The significantly high levels of CD4+ T- cell count in Symptomatic TB females on ATT compared to their counterparts without treatment indicates improvement in immune functions showing the benefits of the treatment and some levels of restoration in cellular immunity in these patients. This may be probably due to significantly reduced bacilli in the affected subjects. The same situation applies in the case of absolute lymphocyte count which appreciates once the subjects have been placed on therapy. TB infections exert significant alteration in cytokine levels of the TB infected which may affect the reproductive potentials of these women.

Conclusions

The study revealed significant cytokine variations which suggest active inflammatory process while CD4 T-cells and Abs Lym dropped showing some degree of derangement in cell mediated immunity at both phases of menstrual cycle. This tends to normalize on treatment. Hypogonadism significantly correlated positively with pro- and anti- inflammatory cytokines at both phases of the menstrual cycle. These may explain some of the hypogonadism seen in these women. Evaluation of cytokines may be useful for evaluating the activity of TB disease in reproductive immunity and monitoring of the clinical effect of anti-tuberculous treatment. Further studies on the role of inflammatory cytokines in endocrine and immune response are advocated.

Executive summary

Objective: The second leading cause of human mortality from infectious diseases worldwide is Mycobacterium tuberculosis (Mtb). This chronic infection is accompanied by prolonged cytokine production, which might affect the immuno-reproductive communication and favour the establishment of an adverse state. This was a prospective study designed to evaluate possible impact of some cytokine variations on menstrual cycle in TB infected females.

Methods: A total of 90 premenopausal females aged (18-45) years were randomly recruited and grouped into 30 Symptomatic TB, 30 Symptomatic TB females on ATT and 30 Control females. Blood samples were collected at follicular (Fp) and luteal phases (Lp) of menstrual cycle for determination of IL-8, IL-6, TNFα, IL-4, CD4+ T-cells, and Absolute Lymphocytes counts using enzyme-linked immunosorbent assay (ELISA), Cyflow SL Green Cytometer and Sysmex K21N Hematology Analyzer respectively.

Results: There was significantly higher IL-6, IL-8, IL-4 and TNFα with lower CD4 T-cells and Abs Lym counts in TB and TB on ATT compared to Control females at both phases of menstrual cycle (P<0.05). All the cytokines were significantly lowered with higher CD4 T-cells in TB on ATT compared to TB females at both phases (P<0.05). Hypogonadism correlated positively with pro and anti-inflammatory cytokines

Conclusion: The study revealed significant cytokine alterations which suggest active inflammatory process while CD4 T-cells and Abs Lym dropped showing some degree of derangement in cellular immunity at both phases of menstrual cycle; which tends to normalize on treatment. This may affect the reproductive potentials in these women.

References

1. Hassan WA, Darwish AM. Impact of pulmonary Tuberculosis on Menstrual pattern and fertility. *J Clin Resp* 4: 157-161 (2010).

2. Sharma S. Menstrual Dysfunction in Non-genital tuberculosis. *Inter J Gynaecol Obstet* 79: 245-247 (2000).

3. Fallahian M, IIkhani M. Menstrual Disorders in Nongenital Tuberculosis. *Infect Dis Obstet Gynaecol* 10: 18452 (2006).

4. Bateman BG, Nunley NC, Kilchen RE. Genital tuberculosis in reproductive age women. *J Reprod Med* 31: 287-290 (1986).

5. Randhawa PS. Lymphocyte subsets in granulomas of human tuberculosis: an in situ Immunoflourescence study using monoclonal antibodies. *Pathol* 22: 153-155 (1990).

6. Selwyn PA, Harel D, Lewis VA, Schoenbaum EE, Vermund SH, Klein RS. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. *New Eng J Med* 320: 545-550 (1989).

7. Keane J, Balcewwicfz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ. Infection by Mycobacterium tuberculosis promotes human alveolar microphage apoptosis. *Infect Immun* 65: 298-304 (1997)

8. Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *The Lancet Infect Dis* 3: 578-590 (2003).

9. Chan J, Flynn J. The immunological aspects of latency in tuberculosis. *Clin Immunol* 110: 2-12 (2004).

10. Beetz S, Wesch D, Marischen L, Welte S, Oberg HH, Kabelitz D. Innate immune functions of human   T cells. *Immunobiol* 213: 173-182 (2008).

11. Oddo M, Renno T, Attinger A, Bakker T, MacDonald HR, Meylan PRA. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. *J Immunol* 160: 5448–5454 (1998).

12. Cooper AM. Cell-mediated immune responses in tuberculosis. *Ann Rev Immunol* 27: 393-422 (2009).

13. Serbina NV, Lazarevic V, Flynn JL. CD4+ T cells are required for the development of cytotoxic CD8+ T cells during Mycobacterium tuberculosis infection. *J Immuno* 167(12): 6991-7000 (2001).

14. Cowley SC, Elkins K. CD4+ T Cells Mediate IFN- - Independent Control of Mycobacterium tuberculosis Infection Both in Vitro and in Vivo. *J Immunol* 171: 4689-4699 (2003).

15. Scanga CA, Mohan VP, Tanaka K, Alland D, Flynn JL, Chan J. The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of Mycobacterium tuberculosis in mice. *Infect Immun* 69: 7711-7717 (2001).

16. Gehring AJ, Rojas RE, Canaday DH, et al. The Mycobacterium tuberculosis 19-kilodalton lipoprotein inhibits gamma interferon-regulated HLA-DR and Fc R1 on human macrophages through toll-like receptor 2. *Infect Immun* 71: 4487-4497 (2003).

17. Pai RK, Convery M, Hamilton TA, Henry Boom W, Harding CV. Inhibition of IFN- -induced class II transactivator expression by a 19-kDa lipoprotein from Mycobacterium tuberculosis: a potential mechanism for immune evasion. *J Immunol* 171: 175-184 (2003).

18. Pai PK, Pennini ME, Tobian AAR, Canaday DH, Boom WH, Harding CV. Prolonged toll-like receptor signaling by Mycobacterium tuberculosis and its 19-kilodalton lipoprotein inhibits gamma interferon-induced regulation of selected genes in macrophage. *Infect Immun* 72: 6603-6614 (2004).

19. Bean AGD, Roach DR, Briscoe H. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *J Immunol* 162: 3504-3511 (1999).

20. Keane J. TNF-blocking agents and tuberculosis: new drugs illuminate an old topic. *Rheumatol* 44: 714-720 (2005).

21. Flynn JL, Chan J. What's good for the host is good for the bug. *Trends in Microbio* 13: 98-102 (2005).

22. Algood HMS, Chan J, Flynn JL. Chemokines and tuberculosis. *Cytokine Growth Factor Rev* 14: 467-477 (2003).

23. Reed MB, Domenech P, Manca C. A glycolipid of hyper virulent tuberculosis strains that inhibits the innate immune response. *Nature* 430: 84-87 (2003).

24. Cheesbrough M. District Laboratory Practice in Tropical Countries. Part 2, 2nd edition. Chembridge University press, New York, USA pp 279-298 (2005).

25. Verthelyi D, Klinman DM. Sex hormone levels correlate with the activity of cytokine-secreting cells in vivo. *Immunol* 100: 384-390 (2000).

26. Bini EI, Mata Espinosa D, Marquina Castillo B, et al. The Influence of Sex Steroid Hormones in the Immunopathology of Experimental Pulmonary Tuberculosis. *PLoS ONE* 9: e93831 (2014).

27. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor super families: integrating mammalian biology. *Cell* 104: 487-501 (2001).

28. Ukibe NR, Onyenekwe CC, Ahaneku JE, et al. Evaluation of hormonal changes in menstrual cycle of women infected with pulmonary tuberculosis in NNEWI, south eastern Nigeria. *Indian J tuber* 61:152-158 (2014).

29. Kart L, Buyukoglan H, Tekin IO, et al. Correlation of serum tumor necrosis factor-alpha, interleukin-4 and soluble interleukin-2 receptor levels with radiologic and clinical manifestations in active pulmonary tuberculosis. *Mediators Inflamm* 12: 9-14 (2003).

30. Ameglio F, Casarini M, Capoluongo E, Mattia P, Puglisi G, Giosue S. Post-treatment changes of six cytokines in active pulmonary tuberculosis: differences between patients with stable or increased fibrosis. *Int J Tuberc Lung Dis* 9: 98-104 (2005).

31. Nakaya M, Yoneda T, Yoshikawa M, et al. The evaluation of interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-alpha) level in peripheral blood of patients with active pulmonary tuberculosis. *Kekkaku* 70: 461-466 (1995).

32. Kawaguchi H, Ina Y, Ito S, et al. Serum levels of soluble tumor necrosis factor (TNF) receptors in patients with pulmonary tuberculosis. *Kekkaku* 71: 259-265 (1996).

33. Tang S, Xiao H, Fan Y, et al. Changes of proinflammatory cytokines and their receptors in serum from patients with pulmonary tuberculosis. *Zhonghua Jie He He Hu Xi Za Zhi* 25: 325-329 (2002).

34. Domingo-Gonzalez R, Prince O, Cooper A, Khader S. Cytokines and Chemokines in Mycobacterium tuberculosis infection Microbiol Spectra 4:10 (2016).

35. Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL. Cytokine production at the site of disease in human tuberculosis. *Infect Immun* 61: 3482-3489 (1993).

36. Bekker LG, Maartens G, Steyn L, Kaplan G. Selective increase in plasma tumor necrosis factor-alpha and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. *J Infect Dis* 1998; 178: 580-584.

37. Pereira CB, Palaci M, Leite OH, Duarte AJ, Benard G. Monocyte cytokine secretion in patients with pulmonary tuberculosis differs from that of healthy infected subjects and correlates with clinical manifestations. *Microbes Infect* 6: 25-33 (2004).

38. el-Ahmady O, Mansour M, Zoeir H, Mansour O. Elevated concentrations of interleukins and leukotriene in response to Mycobacterium tuberculosis infection. *Ann Clin Biochem* 34: 160-164 (1997).

39. Chowdhury RG, Pain SK, Bhattachariee B, Chatterjee S. Infestation of Endometrium by Mycobacterium Tuberculosis Bacilli-cause of Reproductive Failure. *Al Meen J Med Sci* 34: 322-331 (2010).

40. Hashimoto H, Igarashi N, Yachie A, Miyawaki T, Sato T. The relationship between serum levels of interleukin-6 and thyroid hormone in children with acute respiratory infection. *J Clin Endocrinol Metab* 78: 288-291 (1994).

41. Gatner R. Selenium and thyroid hormone axis in critical ill states: an overview of conflicting viewpoints. *J Trace Elem Med Biol* 23: 71-74 (2009).

42. O' Brien SM, Fitzerald P, Scully P, Landers A, Lucinda V, Scott Dinan TG. Impact of Gender and Menstrual cycle phase on Plasma cytokines concentrations. *Neuroimmunomodulation* 14: 84-90 (2007).

43. Polan ML. Gonadal steroids modulate human monocyte interleukin-1 (IL-1) activity. *Fertil Steril* 49: 964 (1988).

44. Angstwurm MWA, Gartner R, Loms Ziegler-Heitbrock HW. Cyclic plasma IL-6 levels during normal menstrual cycle. *Cytokine* 9: 370 (1997).

45. Maskill JK, Laird SM, Okon M, Li TC, Blakemore AIF. Stability of serum IL-10 levels during the menstrual cycle. *AJRI* 38: 339 (1997).

46. Lahita R. The basis for gender effects in the connective tissue diseases. *Ann Med Interne* 147: 241 (1996).

47. Cannon JG, Dinarello CA. Increased plasma interleukin-1 activity in women after ovulation. *Science* 227: 1247 (1985).

48. Gilmore W, Weiner LP, Correale J. Effect of estradiol on cytokine secretion by proteolipid protein specific T cell clones isolated from multiple sclerosis patients and normal control subjects. *J Immunol* 158: 446 (1997).

49. Surcel HM, Troye-Blomberg M, Paulie S, et al. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Immunol* 81: 171-176.

50. Crevel R, Karyadi E, Preyers F, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. *J Infect Dis* 181: 1194-1197 (2000).

51. Lai CK, Ho S, Chan CH, et al. Cytokine gene expression profile of circulating CD4 + T cells in active pulmonary tuberculosis. *Chest* 111: 606-611 (1997).

52. Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF. Absence of a prominent Th2 cytokine response in human tuberculosis. *Infect Immun* 1996; 64(4): 1351–1356.

53. Zhang M, Lin Y, Iyer DV, Gong J, Abrams JS, Barnes PF. T-cell cytokine responses in human infection with mycobacterium tuberculosis. *Infect Immun* 63: 3231-3234 (1995).

54. Verbon A, Juffermans N, Van Deventer SJ, Speelman P, Van Deutekom H, Van Der Poll T. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol* 115: 110-113 (1999).

55. Condos R, Rom WN, Liu YM, Schluger NW. Local immune responses correlate with presentation and outcome in tuberculosis. *Am J Respir Crit Care Med* 157: 729-735 (1998).

56. Zhong D, Dong L, Liang Q. Alteration of interferon-gamma and interleukin-12 released by bronchoalveolar lavage cells from pulmonary tuberculosis. *Zhonghua Jie He He Hu Xi Za Zhi* 23: 552-555.

57. Al-Harthi L (2010) A menstrual cycle pattern for cytokine levels exists in HIV-positive women: implication for HIV vaginal and plasma shedding. *AIDS* 15: 1535-1543 (2010).

58. Mihret A, Bekele Y, Bobosha K, Kidd M, Aseffa A, Howe R, Walzl G. Plasma cytokines and chemokines differentiate between active disease and non-active tuberculosis infection. *J Infect* 66: 357-365 (2013).

59. Mihret A, Abebe M, Bekele Y, Aseffa A, Walzl G, Howe R. Impact of HIV co-infection on plasma level of cytokines and chemokines of pulmonary tuberculosis patients. *BMC Infect Dis* 14: 125 (2014).

60. Marijke F, Annechien B, Henk M, Maas JH, Loe L, Gerard S. The immune response during the luteal phase of the ovarian cycle: a Th2-type response? *Fert Ster* 74: 1008-1013 (2000).

61. Subbi M, Rajesh SM, Jean MGH, Oliver W, Hugh H. Cyclic variations in white cell subpopulation in the human menstrual cycle. Correlation with progesterone and estradiol. *Clin immunol immunopathol* 13: 246-253 (1979).

62. Steinberg AD, Steinberg BJ. Lupus disease activity associated with menstrual cycle. *J Rheumatol* 12: 816 (1985).

63. Lahita RG. Sex hormones and the immune system – part 1. Human data. *Baillière's Clin Rheumatol* 4:1 (1990).

64. Levine AM, Karim R, Mack W, Gravink J, Anastos K. Neutropenia in Human immunodeficiency Virus Infection. *Archive J Inter Med* 166: 405-410 (2006).

65. Ifeanyichukwu M, Onyenekwe CC, Elee PU, Ukibe NK, Meludu SC, Ezechukwu CC, Okeregwo AA. Evaluation of CD4 T-cell count, Interleukin 6 (IL-6) and Interferon Gamma in HIV infected Symptomatic and Asymptomatic Individuals. *J Biomed Inves* 5: 70-73 (2007).

66. Ukibe NR, Ukibe SN, Monago IN, Onyenekwe CC, Ahaneku JE Ilika AL. Possible Impact of Thyroid Dysfunction on Menstrual Cycle in HIV/TB Co-infected Females in NAUTH, Nnewi, Nigeria. *JAMMR* 22: 1-13 (2017).

67. Amilo GI, Meludu SC, Onyenekwe CC, Elee PU, Ezechukwu C, Ekejindu GOC, Ifeanyichukwu MO, Nwobodo E. Evaluation of CD4+ and CD8+ T-lymphocytes counts and serum levels of immunoglobulin in pulmonary tuberculosis (PTB) patients with or without HIV Co-infection in South Eastern Nigeria. *African J Pharm Pharmacol* 6: 1639-1643 (2012).

68. Orme IM, Collins FM. Adoptive protection of the Mycobacterium tuberculosis-infected Lung. *Cell-Immunol* 84: 113-120 (1984).

69. Muller I, Cobbold SP, Walmann H, Kaufrmann SH. Impaired resistance to Mycobacterium tuberculosis infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. *Infect Immun* 55:2037-2041 (1987).

70. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol* 162: 5407-5416 (1999).

71. Barber DL, Mayer-Barber KD, Feng CG, Sharpe AH, Sher A. CD4 T-cells promote rather than control tuberculosis in the absence of PD-1-mediated inhibition. *J Immunol* 186: 1598-1607 (2011).

72. Weinberg A, Enomoto L, Marcus R, Canniff J. Effect of menstrual cycle variation in female sex hormones on cellular immunity and regulation. *J Repro Immunol* 89:70-77 (2011).

73. Oertelt –Prigione S. Immunology and menstrual cycle. A486-A492 beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Autoimmun Rev* 11: A486-A492 (2012).