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A Study of the Natural Immune Reaction to Mycobacterial Isolates of Human

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Abstract - Mycobacterium tuberculosis (M. tb) causes TB and kills millions worldwide. Immune-dependent tissue-damaging inflammation promotes aerosol transmission, making this disease ubiquitous. Since Type I IFNs play a debatable role in TB and their induction precedes clinical tuberculosis in patients, this study sought to identify a pattern (absolute levels and relative ratios) in interferon and regulatory molecule expression levels that correspond with TB patients and pulmonary versus extra pulmonary TB. Using an in vitro M. tb THP-1 infection model and antibody-based neutralization of specific interferons, we found that modulating interferon production promotes intracellular bacterial burden or clearance.

Real-time PCR was performed to compare IFN alpha, beta, and gamma fold mRNA expression in 123 untreated TB patients (PTB - 56; EPTB - 67) and 86 healthy family contacts clinically clear of TB. EPTB and PTB patients' three IFN expression levels were compared. ELISA for Interleukin-1β, -1α, and Prostaglandin E2 was done on sera from 62 TB patients and 18 healthy contacts from the same residence.

TB patients expressed more IFN-α than counterparts. IFN-α had the highest median fold mRNA expression (0.80) in untreated TB patients. PTB patients had a median fold mRNA expression level of IFN- (0.87) that was higher than EPTB patients'. EPTB patients expressed 0.81 times more IFN-α than PTB patients. The three IFNs were measured in matched samples from each patient at enrolment and after treatment. IFN-β and IFN- reduced considerably after therapy. IFN-α high or IFN-β low patients and contacts were then identified. All samples tested by ELISA had IFN- levels below detection. Every sample has PGE2. IL-1 detection vary. PGE2 and IFN- levels were found inversely correlated. These data suggest measuring type I IFNs may help monitor therapy response. The pattern of IFNs in patients (host factors) and the identification of acid-fast bacilli (AFB) by conventional smear microscopy (mycobacterial factors) are expected to occur earlier and precede mycobacterial load reduction.

Keywords - Mycobacterium tuberculosis, Patients, Human, Immune Response

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INTRODUCTION

The TB-causing agent, *Mycobacterium tuberculosis (M. tb*) claims millions of lives annually throughout the world. The disease's ability to spread by aerosol transmission, which is facilitated by immune-dependent tissue-damaging inflammation, accounts for its widespread nature (North and Jung, 2004). In 2019, 7.1 million new TB cases were anticipated. Since 2013, there has been an increase in newly diagnosed cases across many countries. In India, there were 2.2 million cases in 2019, up from 1.2 million in 2013. Despite this increase, there is still a substantial gap between the estimated number of TB cases in 2019 (10 million) and the number of patients who have been identified and reported (2.9 million) (WHO TB Report, 2019). Under diagnosis (when people with TB cannot access care or are not diagnosed when they do) and underreporting of TB patients both contribute to this gap. Governments are working to close the gap by tracking down and reporting notified cases that have been bacteriologically verified so that appropriate treatment can start as soon as possible. By identifying early indicators, we have concentrated on the role of host cytokines in the diagnosis of TB.

One-third of the world's population is infected with *M. tb*, however this infection rarely leads to an active illness. Pulmonary tuberculosis (PTB) is the most common condition caused by *M. tb,* which is usually spread through the respiratory system. It can affect a number of organs, but PTB is the most common. An infection that manifests extra-pulmonary (EPTB) affects the lungs. The main problem with the disease is that the bacterium is paucibacillary, which makes it challenging to diagnose it early and consequently affects the start of the patient's actual treatment. The majority of patients do not display these symptoms, particularly in the case of EPTB, which delays diagnosis. Fever, cough, loss of appetite, and weight

loss are frequent signs of tuberculosis. Additionally, the processes for obtaining specimens are arduous and intrusive, and repeating the sample collection if the results are negative is painful for the subject. Therefore, it is urgently necessary from a therapeutic standpoint to create a less invasive method of TB detection.

The amount of illnesses present and the immunity a person produces are always in balance. If the person has some immunodeficiency, this equilibrium is broken when the illness or active disease starts (Figure 1). In order to prevent latent TB from developing, host immunity is essential.

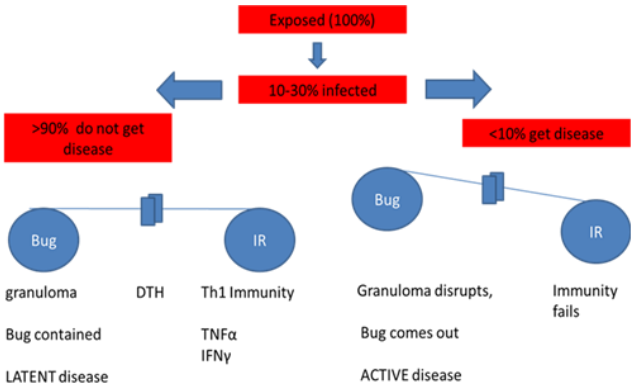

Figure 1: Mycobacterium-host immunity balance.

After infection, *M. tb* triggers an innate immune response that appears as type I interferons (IFN-α/β) and IL-12. The TH1 branch of cell-mediated immunity, however, is preferable for protection, in which IL-12 primed Ag specific CD4+ T cells generate typeII IFN, leading to the overexpression of other crucial cytokines like TNF-α (Zeng et al., 2018). Contrary to IFN-γ's (type II IFN) well-known protective impact, type I IFNs' (extracellular and intracellular) activity in bacterial infections may be either harmful or beneficial (Trinchieri et al., 2010). A dedicated cytokine family called type I IFNs, which includes numerous IFN-α subtypes and just one IFN-β, typically provides protection against viral infections. Although its function in the setting of tuberculosis is widely disputed, IFN-α/β has the ability to directly stimulate IFN-γ from T and NK cells (TH1 boosting, protective), as well as to directly suppress IL-12 and TH1 immunity (detrimental effect) (Byrnes et al., 2001). (Freudenberg et al., 2002). Furthermore, *M. tb*-infected IFNAR mice outlast wild type infected animals (Manca et al., 2005) because IFN-α/β inhibits the bacteriostatic activity and antigen presentation capability of *M. tb*-infected monocytes and macrophages (Bouchonnet et al., 2002). In contrast, type I IFNs employed as a therapeutic agent have been shown to be successful in the treatment of individuals with pulmonary TB who are multidrug resistant (Giouse et al., 1998; Palmero et al., 1999). The objective of this study was to first identify a pattern (absolute levels and relative ratios) in the expression levels of interferons and their receptors because Type I IFNs have a contentious role in tuberculosis and their induction occurs before the onset of clinical tuberculosis in patients (Remoli et al., 2002; Novikov et al., 2011; Ottenhoff et al., 2012). Second, using an in vitro *M.tb* THP-1 infection model and antibody-based neutralisation of certain interferons, we showed how altering interferon expression patterns enhance intracellular bacterial burden or clearance. Because of their great affinity, thermal stability, and specificity, aptamers have become more and more popular in the last ten years in the fields of diagnosis and therapy. Aptamers can also be made very easily and don't need to know the target molecule's structural history.

Why is Type I Interferons used?

The majority of viral infections have been found to be protected against by type I interferons, which are potent antiviral immune-modulators. A natural immune response is initially sparked by the majority of microbial infections by producing type I IFN. Type I IFNs have been shown in numerous studies to have both beneficial and detrimental effects in bacterial infections.

Aptamers.

A special family of small substances known as aptamers can be utilised for both treatment and diagnosis. Many of these tiny molecules are nucleic acids, such as DNA/RNA aptamers, but there are also a few peptide aptamers that have been identified. Single-stranded RNA or DNA nucleotide sequences known as nucleotide aptamers cling to their target with high affinity and selectivity (Market, 2013). Because aptamers' binding kinetics are so similar to those of antibody-antigen interactions, they are sometimes referred to as "chemical antibodies" (Sun et al., 2014). Aptamers can generate a variety of configurations during target binding, including a pseudoknot, hairpin, stem-loop, and G-quadruplex (Dhiman et al., 2019; Kalra et al., 2018). The chemical biology method known as Systematic Evolution of Ligands through Exponential Enrichment (SELEX) is used to produce aptamers (Sharma et al., 2017). A customised in vitro oligonucleotide selection gives aptamers an edge over antibodies. Aptamers are often affordable, reliable, non-toxic, readily available, and flexible (FAM, Biotin, and conjugates with nanomaterial). Aside from that, aptamers have revolutionised diagnostics and treatments since they can be produced more quickly, have more functionalization options, and have better stability than antibodies (Lavania et al., 2017; Dhiman et al., 2018; Kumari et al., 2019; Kaur et al., 2018; Shigdar, 2019; Belleperche et al., 2018).

OBJECTIVES OF STUDY

- To assess the levels of circulating Type I and Type II interferon at the mRNA and protein levels in individuals with active

tuberculosis, as well as in healthy volunteers and household contacts of such individuals.

- to investigate the temporal dynamics and expression of interferon type I and type II in THP1 molecules that have been co-cultured with live and heat-killed H37Rv mycobacteria.
- To examine the effect that Type I and Type II interferons have on the growth or inhibition of mycobacterial in vitro.
- To examine expression of Interleukin-1β, Interleukin-1α, Prostaglandin E2 in tuberculosis patients, healthy family contacts and healthy volunteers

RESEARCH METHODOLOGY

Materials

- **List of Primers used in the Study:**

Table 1: list of primers used

Target	Primer	
	Forward	Reverse
IFN -α	5'- GCTGAATGACCTGGAAGCCTGTG -3'	5'-GGGAGGTTGTCAGAGCAGAAATC-3'
IFN -β	5'-AAGGAGGACGCCG CATTGAC-3'	5'-ATAGACATTAGCCAGGAGGTTC-3'
IFN -γ	5'- TCGTTTTGGGTTCTCTTGGC-3'	5'-TCCGCTACATCTGAATGACC-3'

- **E. coli strains and plasmid constructs list: Plasmids:** pTZ57R/T vector, pTZ57R/T – IFNA, pET 28a vector, pET 28a – IFNA were used.
- **M. tb Strain and Antibodies used in the Study:** M. tb. H37Rv strain was used in the assays in both live and heat-killed forms. The different antibodies utilised in the study were Anti-his, Anti IFN-α, Anti IFN-β, Anti IFN-γ.
- **Cell lines used in the study:** A human THP1 monocytic cell line from ATCC, USA, was provided by Dr. S. S. Chauhan's lab at the AIIMS in New Delhi's Department of Biochemistry.
- **List of Software and Computational Tools**: Several software programmes and computational tools were used in this study for a number of tasks, including the creation of primers, sequence analysis, database access, alignment, binding site and structure prediction, etc.
- **The following equipment was used in the study:** Blue light trans-illuminator, centrifuge, ELISA microplate reader, image capture and analysis system, CO2 incubator, PCR thermal cycler, refrigeration bath, real-time PCR cycler, semi-dry transfer apparatus, shaker incubators, dot blot apparatus, vacuum manifold assembly, gel dryer, and spectro-polarimeter. *M. tb* handling and cultivation studies was conducted at the AIIMS BSL3 Facility.
- **Chemicals and Reagents List:** Monoclonal antibody against histone, Kanamycin sulphate, Potassium chloride, Sodium chloride, Rabbit anti-goat IgG HRP conjugate, Bovine Serum Albumin (BSA), Middlebrook OADC supplement, Middlebrook 7H9 media and Middlebrook 7H11 media, RPMI-1640 culture media, heparin Sodium bicarbonate, foetal calf serum (FCS), Trizol, DNA polymerases such as Taq, Pfu, Milex syringe filters, 0.45 membrane made of nitrocellulose, specific primers, dNTPS, T4 DNA ligase, tissue culture-grade plastic ware, restriction enzymes, limiting enzymes, Wizard® genomic DNA isolation kit, Wizard® PCR-Gel extraction kit, Wizard® plasmid DNA purification kit QIAamp RNA blood micro kit, DNAse, Agarose, Tryptone, Yeast extract, LB broth, LB agar a marker for medium-range protein molecular weight, Ampicillin, Diaminobenzidine (DAB), Glycine, Magnesium chloride, N, N, N', N'- tetramethylenediamine (TEMED), Polyoxyethylene sorbitan monooleate (Tween - 80), Sodium Dodecyl Sulfate (SDS), tris base, Ficoll (Histopaque 1077), Protease Inhibitor Cocktail, Phorbol Myristoyl Acetate. All extra chemicals used in this investigation were of the AnalaR or ExcelaR grade and were obtained from the Bombay, India-based companies E-Merck, Glaxo Laboratories, and Spectrochem Pvt. Ltd.

Method- The method includes following steps

1. **Ethics Clearance:** The study has received the approval of AIIMS's Ethics Committee (IEC/NP-196/2013).
2. Patients with tuberculosis (TB) who visited the All India Institute of Medical Sciences (AIIMS), New Delhi, Out-patient Department (OPD), Pulmonary Critical Care and Sleep Disorders, as well as their healthy family connections, were recruited for the study. The AIIMS Department of Biotechnology in New Delhi used a variety of healthy lab volunteers as controls. Pulmonary tuberculosis, extra-pulmonary tuberculosis (EPTB), healthy family contacts (HHC), and healthy volunteers (HV) were the four groups.
3. Newly diagnosed TB patients (n=123) who visited the Pulmonary Medicine OPD at AIIMS in New Delhi, their household contacts (n=86), and healthy volunteers (n=55) were included in the study. Depending on the inclusion criteria, such as microbiologically validated or clinically diagnosed TB, patients were recruited for PTB. Each TB patient's demographic details, BCG vaccination history, baseline clinical information, including signs and symptoms, type of TB, and diagnostic criteria, as well as co-morbidities and pre-existing conditions, including diabetes, hypertension, cardiovascular disease, renal disease, and blood tests like the complete hemogram,

liver function tests, kidney function tests, chest radiograph findings, and sputum reports (if available), were recorded.

4. Blood was drawn from each person via a venepuncture, and 7 ml of blood (typically containing 28 000–50 000 leukocytes per l) was collected in two tubes: serum separation tubes (SST), used to separate serum from blood, and EDTA vacutainers, used to extract RNA.
5. Utilizing cDNA as a template, real-time PCR was carried out. Maxima® SYBR Green / ROX qPCR Master Mix, 0.5 M of the appropriate primer (forward and reverse), and nuclease-free water were used to set up four distinct PCR master reactions for the four genes. This master mix was divided into 23ul and placed in each of the 96 wells of a Real Time plate (BIORAD). It is necessary to collect the fluorescence signals during the extension process. The bands were seen using the Gel Documentation system, and the PCR results will be resolved using agarose gel electrophoresis.
6. In order to determine the concentrations of interleukin-1, interleukin-1, and prostaglandin E2 in blood samples from 62 TB patients and 18 household healthy contacts, an enzyme-linked immunosorbent assay (ELISA) was conducted.

RESULT AND ANALYSIS

- The recruited patients ranged in age from 18 to 80 years old, with a median age of 28. 46 (37.4%) women and 77 (62.6%) males made up the TB patients. The demographic information of TB patients is shown in table.

Table 2: Analysis of age of the sample.

Age (yrs.)	PTB	EPTB	Total
18-30 yrs. (males)	15	25	40
18-30 yrs. (females)	13	19	32
31-45 yrs. (males)	13	10	23
31-45 yrs. (females)	5	1	6
46-60 yrs. (males)	7	6	13
46-60 yrs. (female)	0	6	6
61-75 (male)	1	0	1
61-75 (female)	1	0	1
>75	1	0	1
	56	**67**	**123**

- Of the 123 patients with newly diagnosed TB who were recruited, 67 (54.5%) were EPTB and 47 (83.9%) had sputum/BAL - Acid Fast Bacilli positive results. 30 (46.8%) of the 64 extra pulmonary specimens were cervical lymph nodes.
- **Result of initial blood tests.**

Table 3: Result analysis of initial blood test

Variable	Value- median (range)	Mean ± SD	Normal values
Hemoglobin (gm/dl)	11.9 (6.9-16.6)	11.8 ± 1.83	
Total leucocyte count (cells/mm³)	7750 (4400 -1600)	8485 ± 2672	4000-11000
Platelet count (no./ml)	244500 (95000 - 450000)	251869 ± 80715	1.5 lac to 4.0 lac
ESR mm/1st hour	45 (4-116)	51 ± 29	< 15
RBS (mg/dl)	105 (64 - 157)	107.5 ± 20.56	60-140
T. Bilirubin (mg/dl)	0.7 (0.2-2.1)	0.6 ± 0.3	0.8-1.0
SGOT (I.U/L)	24 (3-125)	28 ± 15	Upto 50
SGPT (I.U/L)	26 (6-139)	28 ± 17.1	Upto 50
Alkaline phosphatase	216 (64-548)	219 ± 91	80-240
Corrected calcium (mg%)	-	8.8 ± 1.29	8.5-10.2
S. Phosphate (mg/dl)	4 (2.2-6.9)	3.8 ± 0.8	2.5-4.5
Total protein gm%	7.4 (6.3-10.3)	7.5 ± 0.63	6.6-8.7
S. Albumin gm%	4.1 (2.3-5.8)	4 ± 0.5	4.0-5.5

- **Comparative analysis of the fold mRNA expression by Real Time PCR** for IFN-α, IFN-β and IFN-γ in 123 untreated TB patients. IFN-expression was higher in TB patients than in healthy controls (p 0.0001). Between TB patients and HHC, there were no appreciable changes in the fold mRNA expression of IFN-β and IFN-.γ.
The 25th and 75th percentiles are shown by the boxes, the lowest and maximum values of the data are indicated by the whiskers, and the median value for mRNA in each group is represented by the bar. Log10 scale has been used to plot the data. Mann Whitney test, ***p < 0.0001, IFN-α expression in TBP Vs HHC. P- TB patients; C- Healthy household contacts

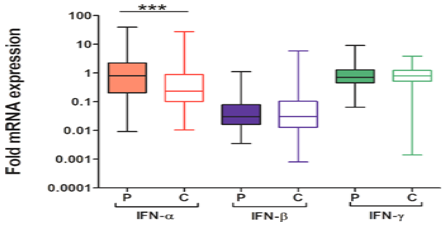

Figure 2: mRNA expression by RTPCR in untreated sample

- Among TB patients, the highest fold expression was observed with respect to IFN-α (0.80), followed by IFN-γ (0.7) and IFN-β (0.03) being the lowest (IFN-α > γ > β); whereas among HHC, IFN-γ expression (0.8) was the highest, followed by IFN-α (0.2) and then IFN-β (0.01), (IFN-γ > α > β).

Table 4: Analysis of fold mRNA expression in healthy and TB infected patients.

Group	Fold mRNA expression of Interferon								
	IFN-α			IFN-β			IFN-γ		
	Lower limit	Median	Higher limit	Lower limit	Median	Higher limit	Lower limit	Median	Higher limit
TB Patients (n=123)	0.207@	**0.804***	2.242	0.016	0.030	0.079	0.459	0.710	1.298
Healthy Contacts (n=86)	0.102	**0.235**	0.893	0.012	0.031	0.103	0.515	0.793	1.230

- **Expression of IFN-α, IFN-β and IFN-γ mRNA in EPTB vs PTB patients:**

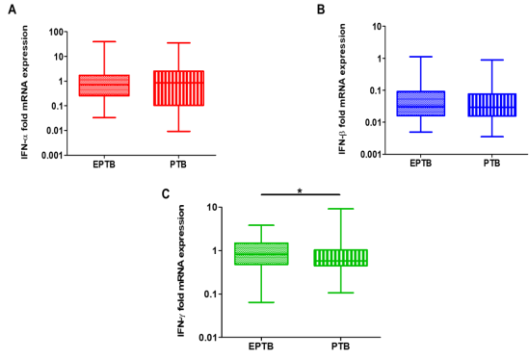

Figure 3: Analysis of expression of IFN in EPTB vs PTB

Panel A: Fold mRNA expression of IFN-α among EPTB and PTB patients.

Panel B: Fold mRNA expression of IFN-β among EPTB and PTB patients.

Panel C: Fold mRNA expression of IFN-γ among EPTB and PTB patients.

Bar represents the median value for mRNA in each group; the boxes represent the 25th and 75th percentile; and ⊥ & ┬ the whiskers represent the minimum and maximum values of the data, respectively. Data has been plotted in log10 scale.

Mann Whitney test, *-p < 0.02- IFN-γ expression EPTB Vs PTB.

Table 5: Analysis of expression of IFN in EPTB vs PTB

Patients	Fold mRNA expression of Interferon								
	IFN-α			IFN-β			IFN-γ		
	Lower limit	Median	Higher limit	Lower limit	Median	Higher limit	Lower limit	Median	Higher limit
EPTB (n=67)	0.256@	0.719	1.723	0.016	0.030	0.090	0.481	**0.810**	1.491
PTB (n=56)	0.106	0.867	2.505	0.015	0.029	0.075	0.449	**0.575***	1.032

Among EPTB patients, the highest fold expression was observed with respect to IFN-γ (0.81), followed by IFN-α (0.719) and IFN-β (0.03) being the lowest (IFN-γ> α > β); whereas among PTB, IFN-α expression (0.86) was the highest, followed by IFN-γ (0.575) and then IFN-β (0.029), (IFN-α > γ> β).

- **Fold mRNA expression of IFN-α, IFN-β and IFN-γ** detected in TBP samples obtained at the time of recruitment and after completion of treatment from 36 patients.

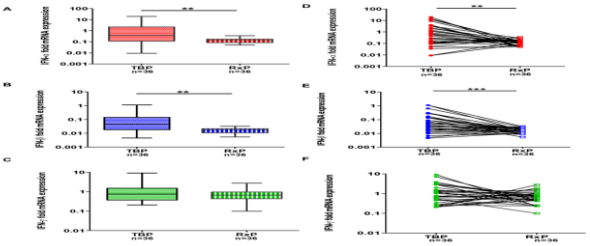

Figure 4: Analysis of fold mRNA expression of interferon -before and after having treatment

Table 6: Analysis of fold mRNA expression of interferon -before and after having treatment

Follow-up Patients (n=36)	Fold mRNA expression of interferon								
	IFN-α			IFN-β			IFN-γ		
	Lower limit	Median	Higher limit	Lower limit	Median	Higher limit	Lower limit	Median	Higher limit
Baseline	0.114@	**0.376**	2.186	0.018	**0.045**	0.141	0.369	0.746	1.507
After Treatment	0.084	**0.133****	0.170	0.010	**0.017***	0.021	0.434	0.672	0.960

Among baseline, the highest fold expression was observed with respect to IFN-γ (0.74), followed by IFN-α (0.376) and IFN-β (0.04) being the lowest (IFN-γ> α > β); whereas after treatment, IFN-γ expression (0.67) was the highest, followed by IFN-α (0.13) and then IFN-β (0.017), (IFN-γ > α> β).

- **Expression of Interleukin-1β, Interleukin-1α, Prostaglandin E2 in tuberculosis patients, healthy family contacts and healthy volunteers using ELISA:**

PGE2 was found in every sample. The detection of IL-1 and IL-1 varied, however. Low expressers (1) of IFN-α (either TB patients or contacts) had significantly higher concentrations of PGE2 than high expressers (>1) of IFN-α. The median concentration of PGE2 was 2972 pg/ml in patients and 3094 pg/ml in healthy contacts among IFN-α low expresser (1), respectively. Patients had a median PGE2 concentration of 418.9 pg/ml, while healthy contacts had a median concentration of 363.9 pg/ml (Figure, Panel A and B; Table). The inverse relationship between PGE2 and IFN-α level was statistically significant.

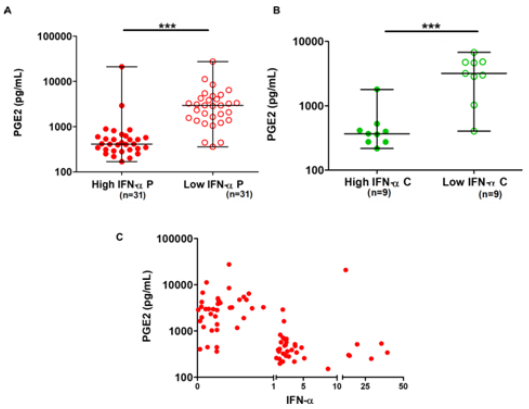

Figure 5: Circulating levels of PGE2 among high and low IFN-α expressers

Panel A: Circulating levels of PGE2 among high and low IFN-α expressers among TBP. - High IFN-α expressers,- Low IFN-α expressers. Mann Whitney test, ***- p<0.0001, TB patients expressing IFN-α High Vs Low expressers.

Panel B: Circulating levels of PGE2 among high and low IFN-α expressers among HHC. - High IFN-α expressers, -Low IFN-α expressers. Non-parametric Mann Whitney test, ***- p < 0.0001, Family Contacts expressing IFN-α High Vs Low expressers.

Panel C: Correlation plot showing negative correlation between IFN-α expression and PGE2 levels (Spearman's r = -0.56, p < 0.0001).

CONCLUSION

IFN-α expression was elevated among TB patients compared to household contacts, (p < 0.0001). Fifty-six of the 123 (45.5 %) TB patients exhibited >1- fold enhanced mRNA expression of IFN-α, whereas identical levels of mRNA expression for IFN-α was limited to 22 of the 86 household contacts, (25.6%). The IFN-α expression level detected among untreated TB patients was highest (median fold mRNA expression 0.80) compared to expression levels of IFN-β (0.03) and IFN-γ (0.7; IFN-α>IFN-γ>IFN-β). The pattern seen among the household contacts differed, in that the highest expression levels of IFN-γ (0.8) were detected compared to IFN-α (0.2) and IFN-β (0.01), (IFN- γ>IFN-α>IFN-β). It must be noted that the expression level of IFN-α was significantly lower (0.23) among the healthy family contacts compared to that detected among TB patients, (0.80). When the expression levels of the three IFNs were compared between EPTB and PTB patients, it was found that PTB patients had higher median fold mRNA expression levels of IFN- than EPTB patients (0.87 to 0.87) did (0.72, not significant). When compared to PTB patients (0.57, p=0.02), EPTB patients had the highest levels of IFN- γ expression (0.81, p=0.02). There were no changes between EPTB and PTB patients' IFN-β expression levels. After that, the patients and contacts were divided into high (IFN alpha mRNA >1-fold; EPTB-19; PTB-12) and low (IFN alpha mRNA 1-fold; EPTB-19; PTB-12) IFN- expressers. All samples analysed by ELISA had IFN- levels below the detection threshold. PGE2 was found in every sample. The detection of IL-1 and IL-1, however, differed. There was a substantial inverse association between PGE2 and IFN- levels (Spearman's rho = - 0.56, p>0.0001).

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