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Genetic Polymorphism of *NOD2 (Arg702Trp) G*ene and its Association with Tuberculosis in Vindhyan Population

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ARTICLE DETAILS

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ABSTRACT

Tuberculosis (TB) is an infectious disease caused by various strains of mycobacteria, usually *mycobacterium tuberculosis (Mtb)* in humans. According to World Health Organization (WHO) report, about one-third of the current global population is infected asymptomatically with Mtb, of whom 10% will develop clinical disease during their lifetime. The difference between infection rate and incidence rate may be caused by physical condition, genetic factor, socio-economic factor, and health resource allocation factor. Significant difference in the incidence of TB in different regions, ethnic groups, and populations also proved that genetic factor is responsible for TB susceptibility. Significant level of change has been seen in overall distribution of *NOD2 (Arg702Trp)* genotypes in healthy control group as compared to disease group although healthy control group showed little increase in 'CC' genotype as compared to Patients of Tuberculosis (83.68% *vs* 71.87%). Similarly, mutant type 'TT' genotype was present in low frequency in Tuberculosis patients group 02.50% and also in control group 01.57% (χ^2 =7.139, P=0.0282*). Carriage rate of allele 'C' was also high in Tuberculosis group as compared to healthy control and disease group (85.77% Vs 77.61%). The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests *NOD2 (Arg702Trp)* is significantly associated with Tuberculosis in Vidhyan population.

1. Introduction

Tuberculosis (TB) disease caused by *Mycobacterium tuberculosis* accounts for 1.7 million deaths worldwide each year and for decades has been the most deadly infectious disease. Alternative strategies for TB treatment and prevention are eagerly needed, and they depend in part on a better understanding of the molecular mechanisms of the interaction between human immunity and *M. tuberculosis*. *M. tuberculosis* is an intracellular pathogen capable of surviving and persisting within host immune cells [5]. In most cases, human adaptive immunity can confine the infection to a latent state (disease free). Successful eradication of *M. tuberculosis* infection depends on rapid activation of macrophages and T lymphocytes. Macrophages are activated through the recognition of mycobacterial components by pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) and nucleotide binding oligomerization domains are considered to be major extracellular and intracellular PRRs for recognition of *M. tuberculosis* and its components.

The occurrence of this infection at different rates across countries and ethnicities indicates that genetic determinants may underlay the risk of developing diseases caused by Mtb infection such as pulmonary or extrapulmonary TB [2,4]. Work to date has highlighted notable gaps in factors that influence the risk of Mtb diseases. For example, the associations of host genetic factors with Mtb infection have not been validated in multiple populations, and some study findings are inconsistent. The immune system has a fundamental role in response to Mtb [1,6]. Thus, it is expected that polymorphisms in immune-related genes may directly affect the capacity of a host exposed to Mtb to control infection. Indeed, many

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studies have reported relationships between SNPs of immune-related genes and risk of Mtb diseases, such as the association between SNPs in TLR4, TNFA, and increased risk of active TB among highly exposed individuals [1-6].

Human NOD2, together with its signaling pathway, is an independent recognition mechanism against *mycobacterial infection*. Macrophages from NOD2 knockout mice produced a significantly lower level of cytokines than did control cells. In line with this finding, macrophages isolated from human subjects homozygous for the 3020insC mutation of NOD2 produced 65%– 80% fewer cytokines after stimulation with *M. tuberculosis* than did individuals heterozygous or homozygous for the wild genotype of this variant [8]. More interestingly, the NOD2 ligand muramyl dipeptide (MDP) had a strong synergistic effect on tumor necrosis factor production induced by TLR2 ligand, the 19-kDa lipoprotein of M. tuberculosis. This synergism was lost in individuals homozygous for the NOD2 3020insC mutation or macrophages harvested from Tlr2 knockout mice [9,10]. This finding suggests that cross-talk occurs between the 2 PPRs systems during recognition of M. tuberculosis. In light of these findings, we hypothesize that amino acid–altering polymorphisms in NOD2 may contribute to human genetic susceptibility or resistance to TB disease [7-10].

The nucleotide-binding oligomerization Domain-Containing protein 2 (NOD2) genes are frequently studied in this setting, as these genes account for proteins that act in the recognition of mycobacterial molecular patterns and lead to immune activation against Mtb [12]. While prior studies reported on the role of NOD2 many have disparate results, and often are restricted to certain populations. NOD2 is expressed in numerous cell types of the immune system, including macrophages, neutrophils, and eosinophils [13]. It encodes a specialized protein that functions as an intracellular PRR of peptidoglycan through the recognition of muramyl dipeptide (MDP), a motif common to all bacteria, with a stimulating signal towards activation of immune responses [8]. When NOD2 is activated by specific substances produced by bacteria, it turns on a protein complex named nuclear factor kappa-B (NFkB), resulting in transcription of pro-inflammatory mediators [14]. As such, there is mounting evidence that deregulation of NOD2 signaling causes or contributes to a variety of human diseases, including asthma, cancer, inflammatory bowel disease, and TB. Of note, studies have reported conflicting results on the relationship between NOD2 SNPs and TB infection, finding mutations in the NOD2 gene that may lead to both the increased and decreased risk of Mtb diseases [12,15]. Notwithstanding, like studies of CD14 SNPs, most results diverge depending on the investigated population, leaving several knowledge gaps for a complete understanding of these relationships. The present study aimed to evaluate work published to date on the influence of polymorphisms of the abovementioned PRRs on risk of Mtb diseases [12,14]. We performed a systematic review to evaluate the association between all reported polymorphisms of CD14 and NOD2 and occurrence of Mtb diseases, and how such association may differ in distinct ethnic populations [11-15].

2. Materials and Methods:

2.1 Study population

The study population consisted of 350 unrelated subjects comprising of 160 Tuberculosis patients and 190 ethnically matched controls of central Indian population were included in this study. Blood sample of case and control group included consecutive patients who attended the Department of Medicine, Shyam Shah Medical College and Sanjay Gandhi Memorial Hospital, Rewa, Ayurveda Medical College, Rewa, Ranbaxy pathology Regional collection centre Rewa, District hospital Satna, Shahdol, Sidhi. Tuberculosis was diagnosed in accordance with World Health Organization criteria. Pregnant women, children under age of 18 years and any patients with Tuberculosis were excluded from the study. Blood sample of control group composed of non- Tuberculosis healthy individuals that were collected during "Tuberculosis Awareness Camps" organized in urban regions in and around SSMC Rewa and many volunteers were also included to collect control sample. The control subjects were recruited from the regions that from homogenous cluster in Vindhyan region India in accordance with a recent report of genetic landscape of the people of India.

2.2 Anthropometric and Biochemical Measurements:

Height and Weight were measured in light clothes and without shoes in standing position as per standard guidelines. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured in standing position midway between iliac crest and lower costal margin and hip circumference was measured at its maximum waist to hip ratio (WHR) was calculated using waist and hip circumferences. Systolic and diastolic blood pressures were measured twice in the right arm in sitting position after resting for at least 5 minute using a standard sphygmomanometer and the average of the two reading was used. Biochemical parameters related to type 2 diabetes were estimated for both cases and controls subjects. Measurement of Serum levels of Total cholesterol (TC), Triglycerides (TG), HbA1c, High density lipoprotein-cholesterol (HDL-C), Low density lipoprotein-cholesterol (LDL-C), Urea, Uric acid, C-reactive protein (CRP) and Creatinine were measured based on spectrophotometric method using automated clinical chemistry analyzer Cobas Integra 350 plus (Roche Diagnostics, Mannheim, Germany).

2.3 Blood collection and plasma/serum separation:

Venous blood samples were obtained from the subjects after 12 hours of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min. at 4˚C.

2.4 DNA isolation:

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988). Frozen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was than allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was re-suspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete, 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nucleic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantization.

2.5 Detection of Genetic Polymorphism in NOD2 (Arg702Trp) gene via PCR-RFLP:

The ***NOD2 (Arg702Trp)*** (rs2066844, 16p21.1) polymorphic site at Exon4 containing fragment was amplified by PCR. The C>T transversion at the polymorphic site of *NOD2 (Arg702Trp)* gene creates a *MspI* restriction site. Primer sequence used to amplify the *NOD2 (Arg702Trp)* gene are given as follows.

Forward primer- 5`- AGATCACAGCAGCCTTCCTG -3`
Reverse primer- 5`- CACGCTCTTGGCCTCACC -3`.

2.6 PCR Mix

The PCR was carried out in a final volume of 25 µl, containing 100 ng of genomic DNA(4-5 µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd. India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of forward and reverse primers specific for and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalre genei).

2.7 PCR Thermal Program

After an initial denaturation of 5 min at 94˚C, the samples were subjected to 35 cycles at 94˚C for 1 min, at 65˚C for 40 s, and 72˚C for 40 s, with a final extension of 10 min at 72˚C in a thermal cycler. A 100bp ladder with amplified product has been run under 1 % agarose gel electrophoresis. 83 bp product will be generated after PCR.

2.8 Restriction Digestion

Restriction Digestion The 83-bp product was digested with *BamHI* enzyme (New England Biolabs, overly, MA) for 16 h at 37˚C. The wild-type genotype (GG) was not digested, whereas the mutated homozygous genotype (CC) was cut as a doublet of 56 and 27 bp. The heterozygous genotype (GC) was represented as 3 fragments of 83, 56, and 27 bp. Samples were analyzed by electrophoresis using 2.5% agarose gels to analyze the genotype pattern of the gene.

3. Results:

3.1 Anthropometric results:

We selected anthropometric parameters like age, sex, BMI, WHR were the parameters for association analysis between healthy control and Tuberculosis patient. Our statistical data suggest BMI are associated with Tuberculosis whereas WHR are not associated. As expected the Tuberculosis patients had markedly lower weight of women (P<0.0001***), Men (P<0.0001***), and BMI also lower in Women (P<0.0001***) and Men (P<0.0001***). Thus Waist circumference of women (P=0.0219*) and men (P=0.0239*) were associated with tuberculosis whereas WHR in Women (P=0.1689ns) and Men (P=0.1116ns) was not significantly different between case and control.

3.2 Biochemical and clinical findings:

Biochemical and clinical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t test and p value obtained suggests the level of significant changes here. The descriptive data and comparison of biochemical parameters of Tuberculosis patients versus controls are presented in Table No. 2, As expected the Tuberculosis patients had markedly higher levels of Cholesterol (P<0.0001)

and Triglycerides (P=0.0013**). In addition, HDL-C (P<0.0001) and LDL-C (P=0.0043**) were compared to that of control subject whereas Systolic BP (P=0.4259ns), Diastolic BP (P=0.8721ns), Blood Urea (P=0.7185ns), Creatinine (P=0.1050ns) and Post-Prandial Glucose (P=0.0977ns) were not associated with Tuberculosis.

Table . 1. Comparison of anthropometric parameters of Tuberculosis patients and controls:

Characteristics	Cases	Controls	P-value
n(Men/Women)	**160(92/68)**	**190(114/76)**	
Age(years)	52.5±12.7	52.2±13.2	0.8295ns
Height(m)	162.5±13.20	162.2± 12.6	0.6128ns
Weight (Kg)			
Women	56.5 ±2.70	61.4 ± 4.50	P<0.0001**
Men	58.8±6.60	67.2±5.1	P<0.0001**
BMI (kg/m²)			
Women	22.9±3.6	25.2 ± 2.3	P<0.0001**
Men	20.3±7.3	23.2± 2.1	P<0.0001**
Waist circumference (cm)			
Women	90.2±6.2	91.8±6.7	0.0219*
Men	88.1±7.2	89.8±6.8	0.0239*
Hip (cm)			
Women	95.8±5.1	96.2±3.2	0.3727ns
Men	91.9±4.8	92.7±5.1	0.1341ns
WHR			
Women	0.94±0.09	0.95±0.04	0.1689ns
Men	0.96±0.08	0.97±0.03	0.1116ns

* *Denotes level of significant change between case and control.*

Table. 2 Comparison of Biochemical and clinical findings of Tuberculosis patients and controls**:**

Characteristics	Cases	Controls	P-value
n(Men/Women)	**160(92/68)**	**190(114/76)**	
Post-Prandial Glucose (mg/Dl)	117.4±11.4	119.5±12.1	0.0977ns
HDL-C(mmol/L)	96.2±13.8	108.8±12.6	P<0.0001***
LDL-C (mg/dL)	40.1±4.3	41.3±3.5	0.0043**
Cholesterol (mg/dL)	42.1±4.5	46.3±3.7	P<0.0001***
Triglycerides (mg/dL)	122.1±13.2	126.9±14.2	0.0013**
Systolic BP (mmHg)	126.20±8.1	126.8±7.7	0.4259ns
Diastolic BP (mmHg)	86.1±4.8	86.2±6.5	0.8721ns
Blood Urea(mg/dL)	8.8±2.3	8.9±2.8	0.7185ns
Creatinine(mg/dL)	1.05±0.12	1.07±0.11	0.1050ns

** denotes the level of significant change between case and control*

3.3 Cytokine Test:

TNF-α is a pro-inflammatory cytokine associated with increased inflammatory response elevated during Tuberculosis infection. TNF-α level in serum reveals strong association with Tuberculosis infection. Concentration of TNF-α level in Tuberculosis patient (case) and healthy population (control) is depicted in table no.-3, showing elevated level during infection and statistically significant (P<0.0001***).

Table. 3 Comparison of TNF-α Cytokine Level in serum (pg/mL) between Tuberculosis patients and controls group:

Biochemical Factor	Cases(160)	Controls(190)	P-value
TNF-α Cytokine Level in serum(pg/mL)	23.7 ± 9.52	11.2 ± 6.41	(P<0.0001)***

3.4 Detection of Genetic Polymorphism in NOD2 (Arg702Trp) gene:

PCR amplification with specific primers gave 185-bp product which was digested with *MspI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 130 and 55bp. The heterozygous genotype (CT) was represented as 3 fragments of 165, 130, and 55bp as depicted. The distribution of the polymorphisms of *NOD2* was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for *NOD2 (Arg702Trp)* polymorphism are depicted in table 5 and table 6 and Graph 2,3, 4.

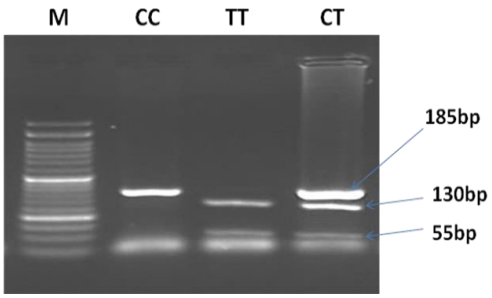


Fig 1: Representative gel picture of NOD2(Arg702Trp) polymorphism. Lane M represents 50 bp molecular marker, Lane GG Wild type genotype, Lane GC heterozygous genotype and Lane CC variant genotype**.**

Significant level of change has been seen in overall distribution of *NOD2 (Arg702Trp)* genotypes in healthy control group as compared to disease group although healthy control group showed little increase in 'CC' genotype as compared to Patients of Tuberculosis (83.68% *vs* 71.87%). Similarly, mutant type 'TT' genotype was present in low frequency in Tuberculosis patients group 02.50% and also in control group 01.57% (χ^2 =7.139, P=0.0282*). Overall allele 'T' was found little lower frequency in disease group as compared to Healthy control group whereas allele '*C*' was present in high frequency in the disease group and the difference was significant (χ^2 = 6.734, P=0.0095 **). Carriage rate of allele 'C' was also high in Tuberculosis group as compared to healthy control (85.77% Vs 77.61%) and disease group. and significant level of change (χ^2 = 4.699, P= 0.0302 *) has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests *NOD2 (Arg702Trp)* is significantly associated with Tuberculosis in our population.

Table 5. Frequency distribution and association of Genotype, allele frequency and carriage rate of *NOD2 (Arg702Trp)* polymorphism in population of Vindhyan region using Chi Square Test

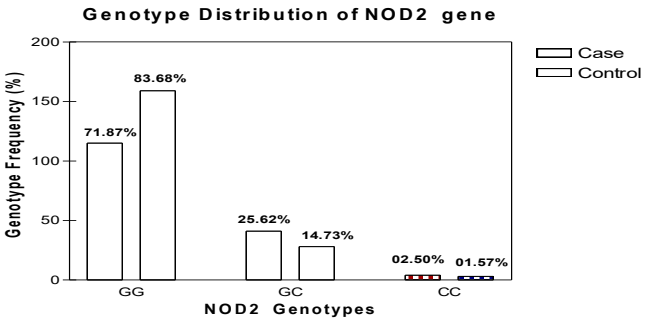
***NOD2* GENOTYPE**	**CASE N= 160**		**CONTROL N=190**		**CHI SQUARE VALUE χ^2 (P Value)**
	N	**%**	**N**	**%**	
CC	115	71.87	159	83.68	
CT	41	25.62	28	14.73	7.139 (0.0282*)
TT	04	02.50	03	01.57	DF:2
Allele					
C	271	84.68	346	91.05	6.734 (0.0095 **)
T	49	15.31	34	08.94	DF:1
Carriage Rate					
C	156	77.61	187	85.77	4.699 (0.0302 *)
T	45	22.38	31	14.22	DF:1

*N – Number of individuals in study group; %- Genotype allele frequency and carriage rate expressed in percentage; * denotes the level of significant association between case and control*

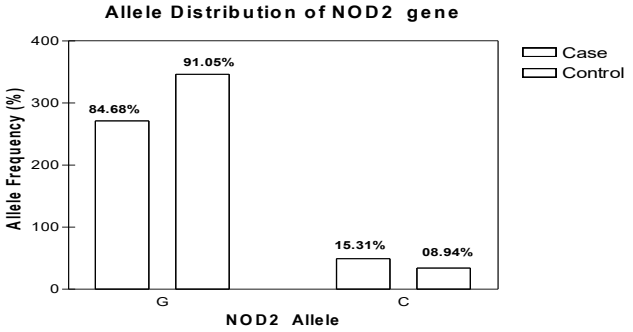
Table 6 Fisher Exact Test values of *NOD2 (Arg702Trp)* polymorphism

***NOD2* GENOTYPE**	**CASE N= 160**		**CONTROL N=190**		**P Value**	**Odds Ratio (CI)**
	N	**%**	**N**	**%**		
CC	115	71.87	159	83.68	0.0091**	0.4983 (0.2972 to 0.8353)
CT	41	25.62	28	14.73	0.0148*	1.993 (1.167 to 3.406)
TT	04	02.50	03	01.57	0.7068 ns	1.598 (0.3523 to 7.252)
Allele						
C	271	84.68	346	91.05	0.0100**	0.5435 (0.3412 to 0.8657)
T	49	15.31	34	08.94		1.840 (1.155 to 2.931)
Carriage Rate						
C	156	77.61	187	85.77	0.0317*	0.5747 (0.3470 to 0.9518)
T	45	22.38	31	14.22		1.740 (1.051 to 2.882)

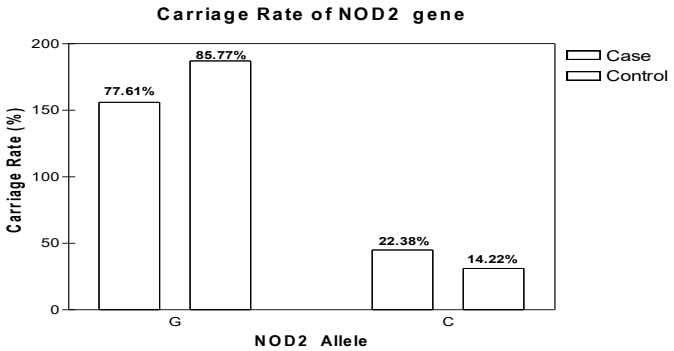
** denotes the level of significant association between case and control; N – Number of individuals in study group; %- Genotype allele frequency and carriage rate expressed in percentage*



Graph 2: Genotype Frequency of *NOD2*



Graph 3: *NOD2* Allele Frequency Percentages



Graph 4: *NOD2* Carriage rate percentages

4. Discussion:

Anthropometric measurements were made and serum iron and ferritin levels determined in a group of Gambian children at the beginning of the rainy season and these findings were related to the Tuberculosis experience of the children during the following Tuberculosis transmission season [16]. Susceptibility to Tuberculosis was not correlated with prior weight-for-age, height-for-age, weight-for-height or serum albumin, or with serum iron, serum iron binding capacity nor serum ferritin [17]. Thus, our findings do not provide any support for the view that poor nutritional status, as assessed by anthropometric measurements, or iron deficiency protect against Tuberculosis infection. Children who developed a clinical attack of Tuberculosis accompanied by a high level of parasitaemia tended to have a higher mean weight-for-age at the beginning of the rainy season than did children who had a clinical attack accompanied by a low level of parasitaemia, but the difference between groups was statistically significant. However, they had a significantly higher mean serum TNF-α level [16-19].

The association with nutrition with Tuberculosis infection revealed how anthropometric parameter influenced Tuberculosisl infection. The complex relationship between malnutrition and Tuberculosis affects morbidity and mortality in children younger than 5 years, particularly in parts of sub-Saharan Africa where these conditions occur together seasonally. Previous research on this relationship has been inconclusive [19,20]. This cross-sectional study is a secondary analysis of a cluster-randomized trial comparing treatment strategies for trachoma in Niger. The children aged 6–60 months residing in the 48 communities enrolled in the trial who completed anthropometric and Tuberculosis infection assessments at the final study visit The association between anthropometric indicators, including height-for-age z-score (HAZ) and weight-for-age z-score (WAZ) and indicators of Tuberculosis infection, including Tuberculosis parasitemia and clinical Tuberculosis [21]. Data trends are inferred from the patient's physical examination, and a food questionnaire detailing the daily diet of the patient. The average age in this sample was 34.66 years; the average BMI was 20.05 kg/m2 and the prevalence of Tuberculosis was approximately 8.5%. Data indicates that among those who had the most diverse daily diet, only 5% had Tuberculosis, while 9% of those who did not eat a daily diverse diet had Tuberculosis [23]. Patients with a severely thin Body Mass Index (BMI) were found to be at a higher risk (12.8%) of having Tuberculosis, whereas the pre-obese and obese had no (0%) Tuberculosis. Data also indicated that with the average prevalence of worms being 7% in the sample, the severely thin manifested a proportion of 17%, with the pre-obese and obese manifesting no diagnoses of worms [20-23].

Our statistical data suggest BMI are associated with Tuberculosis whereas WHR are not associated. As expected the Tuberculosis patients had markedly lower weight of women (P<0.0001***), Men (P<0.0001***), and BMI also lower in Women (P<0.0001***) and Men (P<0.0001***). Thus Waist circumference of women (P=0.0219*) and men (P=0.0239*) were associated with tuberculosis whereas WHR in Women (P=0.1689ns) and Men (P=0.1116ns) was not significantly different between case and control. TNF-α is a pro-inflammatory cytokine associated with increased inflammatory response elevated during Tuberculosis infection. TNF-α level in serum reveals strong association with Tuberculosis infection. Concentration of TNF-α level in Tuberculosis patient (case) and healthy population (control) is depicted in table no.-3, showing elevated level during infection and statistically significant (P<0.0001***).

5. Conclusion

In this study, we found that *NOD2* SNPs show different effects on the incidence of TB. The meta- analysis revealed an association between the Arg702Trp T allele and TB protection. At the same time, different ethnicity groups contribute to different TB susceptibility, such as Arg587Arg G allele is a TB risk SNP in Chinese Han while having no association with Kazak and Uygur. However, the Gly908Arg polymorphism did not show significant association with TB. NOD2 is a kind of PRRs, which can identify Mtb invasion in cells [24,23]. A recent report indicates that NOD2 and TLR pathways are non-redundant in the recognition of Mtb, but can synergize to induce a robust pro-inflammatory response. Arg587Arg G allele was associated with significantly better lung function in pulmonary sarcoidosis. In addition, Arg587Arg G allele has also been found to be a protective site of inflammatory bowel disease [4,25]. Several studies have revealed a substantial contribution of synonymous SNPs to human disease risk. We suggested that Arg587Arg may be associated with susceptibility to TB in the Chinese Han population [26]. However, in our meta-analysis, Arg587Arg GT genotype is a TB risk factor in Chinese Han, but not in Kazak and Uygur, with the fact that this SNP even do not exist in African Americans. The reasons for the difference were unclear, but the differences in the polymorphisms frequency and the differences in the infected Mtb strains might contribute to this heterogeneity [24-26].

Our data suggested that Significant level of change has been seen in overall distribution of *NOD2 (Arg702Trp)* genotypes in healthy control group as compared to disease group although healthy control group showed little increase in 'CC' genotype as compared to Patients of Tuberculosis (83.68% *vs* 71.87%). Similarly, mutant type 'TT' genotype was present in low frequency in Tuberculosis patients group 02.50% and also in control group 01.57% (χ^2 =7.139, P=0.0282*). Overall allele 'T' was found little lower frequency in disease group as compared to Healthy control group whereas allele '*C*' was present in high frequency in the disease group and the difference was significant (χ^2 = 6.734, P=0.0095 **). Carriage rate of allele 'C' was also high in Tuberculosis group as compared to healthy control (85.77% Vs 77.61%) and disease group. and significant level of change (χ^2 = 4.699, P= 0.0302 *) has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests *NOD2 (Arg702Trp)* is significantly associated with Tuberculosis in our population.

This SNP can lead to an amino acid changes, from a low hydrophobic arginine to a high hydrophobic tryptophan. Articles showed Arg702Trp did not change the membrane localization function of NOD2 protein with the normal MDP-induced NF-κB activation [25]. Because of the location of Arg702Trp, we speculated that Arg702Trp could reinforce the LRR combination with MDP, making it easier to clear intracellular Mtb. Gly908Arg is also a missense mutation coding for LRR, which is located in the exon 8. Literature showed that Gly908Arg expressed in HEK293T cells reveals defects in NF-κB transcription, leading to a broadly impaired transcriptional response and impaired cytokine, and co-stimulatory protein induction with MDP stimulation, thus affecting bacterial diseases. Existing literature suggest that Gly908Arg is associated with TB [27]. In conclusion, this systematic review summarized the association between *NOD2* polymorphisms and TB susceptibility. Our results indicated that *NOD2* Arg702Trp polymorphisms might be the genetic risk factors for TB susceptibility in vindhyan population.

6. References:

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