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Association of Ficolin-2 Serum Levels and *FCN2* Genetic Variants with Indian Visceral Leishmaniasis

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Abstract

Background

Visceral leishmaniasis (VL), one of the neglected tropical diseases, is endemic in the Indian subcontinent. Ficolins are circulating serum proteins of the lectin complement system and involved in innate immunity.

Methods

We have estimated ficolin-2 serum levels and analyzed the functional variants of the encoding gene *FCN2* in 218 cases of VL and in 225 controls from an endemic region of India.

Results

Elevated levels of serum ficolin-2 were observed in VL cases compared to the controls (adjusted $P<0.0001$). The genetic analysis revealed that the *FCN2*structural variant +6359 C>T (p.T236M) was associated with VL (OR=2.2, 95% CI=1.23-7.25, $P=0.008$) and with high ficolin-2 serum levels. We also found that the *FCN2*AAAC* haplotype occurred more frequently among healthy controls when compared to cases (OR=0.59, 95%CI=0.37-0.94, $P=0.023$).

Conclusions

Our findings indicate that the *FCN2* variant +6359C>T is associated with the occurrence of VL and that ficolin-2 serum levels are elevated in *Leishmania* infections.

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Introduction

Visceral leishmaniasis (VL; Kala-Azar), a neglected tropical disease strongly associated with poverty, claims 400.000 new cases and 40.000 deaths annually [1]. VL leads to a loss of about 2 million disability adjusted life years (DALYs) every year [2]. The vector-borne infection occurs in the four distinct clinical manifestations as cutaneous leishmaniasis, muco-cutaneous leishmaniasis, VL and post-kala-azar dermal leishmaniasis [3]. VL is the severest form and severely affects visceral organs including the spleen, liver and lymph nodes [4]. Although transmission of VL has been reported in 66 countries, more than 90% of the disease burden are observed in six countries only, viz. Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil [3]. Among these countries, the Indian sub-continent (India, Nepal and Bangladesh) harbours 67% of the global VL disease burden [5]. In particular, the Bihar state of India shares 50% of VL and is considered a "hot spot" of VL [6]. Inadequate vector control practice and disease management have been claimed to be responsible for the increased incidence of VL and associated mortality in India [7].

Leishmania donovani is the causative agent of VL in India. The organism is transmitted to mammalian hosts by infective bites of the sandfly *Phlebotomus argentipes*. *L. donovani* is a unicellular trypanosomatid protozoan parasite with a dimorphic life cycle between the sandfly vector (extracellular promastigotes) and the human host (intracellular amastigotes) [8]. Both developmental stages of *L. donovani* are coated with various secreted and membrane bound phosphoglycans. During the promastigote stage, abundant lipohosphoglycan (LPG) and gp63 are expressed, which aid immune evasion of the parasite by inhibiting the phagolysosome biogenesis in phagocytes [9]. Further, these glycoconjugates facilitate the parasite´s survival in the hostile macrophage environment [10]. However, LPG and gp36 may also serve as pathogen-associated molecular patterns (PAMPs) which are recognized by pattern recognition molecules (PRMs) of the innate system such as complement serum proteins, mannose-binding lectin (MBL), ficolins (FCN), other soluble C-type lectins and toll-like receptors [11]. Serum complement activating pattern recognition molecules act in a first-line innate defense against promastigotes inoculated by the sandfly bite. *Leishmania* parasites have developed various evasion strategies to avoid the lytic action of the complement system. The parasites use host complement proteins to escape the immune attack by entering into macrophages [12]. Mannose-binding lectin (MBL), a circulating serum protein, recognizes the carbohydrate domain of *L. major*, *L. mexicana*, and *L. braziliensis*. MBL binds to the surface of *Leishmania* promastigotes to opsonize the parasites. Upon binding to parasites, MBL initiates the complement cascade and provides an additional uptake mechanism of parasites by enhancing opsonophagocytosis and protects them from the immune attack [13,14] and, thus, modulates the clinical outcome of VL [15].

Ficolins are serum complement lectins that are structurally and functionally analogous to MBL [16] and, hence, expected to modify the clinical outcome of VL due to their involvement in innate immunity. Interestingly, a significant association of a distinct *FCN2* haplotype with cutaneous leishmaniasis has been reported from a Syrian population [17]. Ficolins are a group of complement activating pattern recognition molecules consisting of a collagen-like tail region and a fibrinogen-like domain (FBG) [18]. Three types of ficolins (Ficolin-1, -2, -3) of similar structure exist in humans. These types have differential tissue expression patterns and functions [19]. The role of ficolin-2, as an innate immunity component, has been studied in several infectious diseases including Hepatitis B, schistosomiasis, Chagas disease and others [16,20–22]. Ficolin-2 recognizes superficial acetylated compounds of invading pathogens by their FBG domain and initiates the lectin complement cascade [23]. The *FCN2* gene localizes to chromosome 9q34.3 (OMIM 601624) and hepatic cells predominantly express the corresponding

protein. The variants in the promoter region of *FCN2* gene at positions -986A>G, -602G>A and -4A>G have been observed to modulate the circulating ficolin-2 concentration in a dose-dependent manner. The non-synonymous exon-8 variant alleles at positions +6359C>T and +6424G>T were shown to exhibit differential binding affinities to acetylated compounds when compared to the wildtype reference alleles [24]. Studies have shown that inter-individual variation of circulating ficolin-2 concentration are correlated with polymorphisms in the promoter and exon-8 regions [25].

Although it has been showed that *FCN2* gene polymorphisms and haplotypes are associated with cutaneous leishmaniasis [17], no investigations of ficolins have so far focused on VL. Moreover, we recently observed that functional *MBL2* polymorphisms and lower MBL levels confer relative protection against VL (unpublished). As ficolin-2 shares similarities both in structure and function with MBL [26], we aimed to explore the role of potentially important *FCN2* gene variants and circulating ficolin-2 levels in VL in our Indian study group. Three promoter SNPs (-986A>G, -602G>A and -4A>G) and two structural SNPs in exon 8 (+6359C>T and +6424G>T) were genotyped and studied.

Materials and Methods

Ethics statement

Informed written consent was obtained either from the participating individual or from the parents/guardians if an individual was less than 18 years old. The study was approved by the Institutional Ethical Committee (IEC) of the CSIR-Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. Permission was also sought for and obtained from district government hospitals.

Study design and sample collection

This is a case-control study matched for ethnicity, sex and geographical location. All cases and controls were recruited through multiple field visits from villages located within a radius of ~120 km from the city of Muzaffarpur in the Bihar state of India. Previous epidemiological studies of VL have indicated that the Bihar state is a hot spot for VL with an average annual incidence of 2.49/1000 individuals [6]. The sample size was calculated prior to recruitment using the Open Epi platform (http://www.openepi.com/) based on the incidence rate and the risk of VL in the study area. A total of 443 unrelated subjects (218 cases and 225 healthy controls) were recruited. The mean age of VL cases was 28.7±16.7 and 35.3±16.2 in healthy controls ($P = 0.001$). No significant difference in the male/female ratio was observed in cases (125:95) and controls (122:93). The cases were determined based on the clinical features of VL in medical records issued by government hospitals in the study region. Typical clinical features of the cases included fever with rigors and chills and significant splenomegaly. Cases were tested with the rk39 leishmanin antigen by nitrocellulose dipstick tests (InBios International, Seattle, USA). The control subjects were free of any relevant infectious disease. Pregnant women, cases with other infections, healthy controls with a family history of VL and relatives of cases were excluded from the study. About 5.0 mls of full venous blood were collected from study subjects for serological and genetic studies. The samples were immediately transported to the lab and the serum samples were separated from whole blood and stored in the same type of tubes at -20°C until further use.

DNA isolation and *FCN2* genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the protocol described previously [27]. The reference genomic sequence was retrieved from the Ensembl database (www.ensembl.org). The five *FCN2* variants studied were PCR amplified from two genomic regions. The three promoter variants -986A>G, -602G>A and -4A>G were amplified by the primer pairs `PromF-5'-ATTGAAGGAAAATCCGATGGG-3'` and `PromR-5'-GAAGCCACC AATCACGAAG-3'`, and the two exon-8 variants +6359 C>T and +6424 G>T were amplified using the primer pairs `Exon8F-5'-CCAGCTCCCATGTCTAAAGG-3'` and `Exon8R-5'-TTACAAACCGTAGGGCCAAG-3'`. Primers were designed by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized commercially (Eurofins, Bangalore, India). The target regions were amplified using an Emerald PCR master mix (TaKaRa, Shiga, Japan) and reactions were carried out in the ABI GeneAmp PCR system 9700. The thermal cycling parameters for both amplicons were: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 30 seconds and elongation at 72°C for 1 minute. PCR products were purified using Exo-SAP-IT (USB-Affymetrix, Santa Clara, USA) and 1.0 μl of the products were directly used as templates for sequencing using the BigDye terminator (v.3.1) cycle sequencing kit (Applied Biosystems, Texas, USA) on an ABI 3730XL DNA Analyzer. Variations were identified by assembling DNA sequences with the reference sequence using AutoAssembler software (Applied Biosystems, Texas, USA) and were reconfirmed visually from their electropherograms.

Ficolin-2 serological assay

Ficolin-2 levels were measured in the sera of VL cases (n = 166) and healthy controls (n = 85) using the human Ficolin-2 ELISA kit following manufacturer's instructions (Hycult Biotech, Uden, The Netherlands). The detection limit of the assay was 16 ng/mL.

Statistical analysis

Data were analyzed using the STATA software (Intercooled STATA, STATA Corp., College Station, TX, USA) and the level of significance was set to a P value of <0.05. Genotype or haplotype frequencies were calculated by simple gene counting and by expectation-maximum (EM) algorithm and the deviations from Hardy-Weinberg equilibrium were tested using the random-permutation procedure as implemented in the Arlequin v.3.5.1.2 software (http://lgb.unige.ch/arlequin). The linkage disequilibrium (LD) analysis was performed using Haploview v.3.2 (http://broadinstitute.org/haploview). Multivariate analysis was performed after adjustment with the confounding factors such as age, ethnicity and gender using the STATA software. In all comparisons, P values <0.05 were considered significant. Kruskal-Wallis or Wilcoxon-Mann-Whitney rank sum tests were applied wherever appropriate to analyze the correlation of serum ficolin-2 levels with *FCN2* variants and haplotypes by using the Kaleidagraph software (www.synergy.com).

Results

Association of *FCN2* variants with the risk of VL

The genotype and allele frequencies for the variants -986G>A, -602A>G, -4A>G and +-6359C>T) in VL cases and controls were in Hardy-Weinberg equilibrium ($P>0.05$). This did not apply to the variant +6424G>T in VL cases. This variant was excluded from further analyses. The LD patterns of the *FCN2* variants are given in Fig 1. The LD plot indicates that the promoter variants -986G>A, -4A>G and the exon 8 variant +6359C>T were in strong LD with

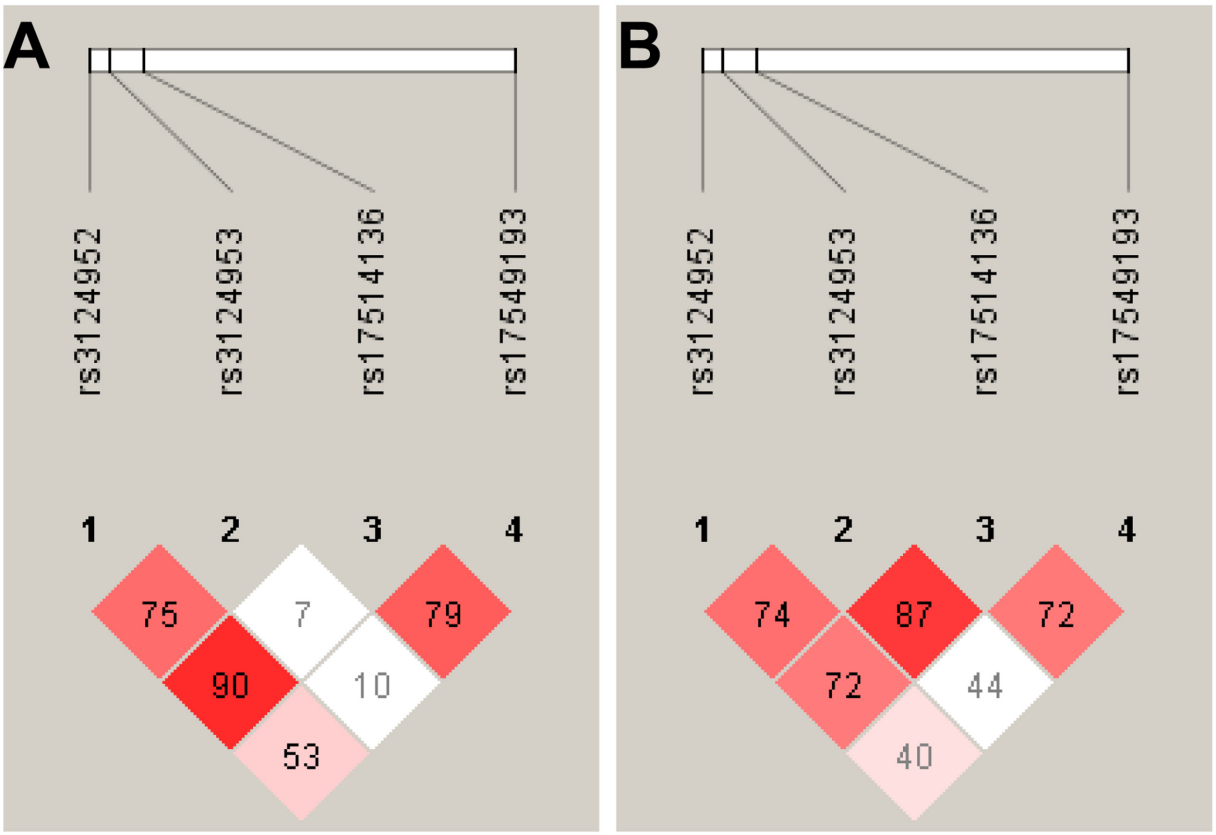

Fig 1. Linkage disequilibrium (LD) pattern of SNPs studied. (A): LD pattern of *FCN2* variants in visceral leishmaniasis cases and (B): LD pattern of *FCN2* variants in healthy controls. Numbers indicate the D' value expressed as percentile. Open squares indicate the high degree of LD (LD coefficient D' = 1) between pairs of variants. The red squares indicate pairs in strong LD with LOD scores.

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each other both in cases and controls. The variant -602A>G was in LD with +6359C>T only in controls. Significant differences were observed both in genotype and allele distributions between cases and controls for the non-synonymous variant +6359C>T (p.Thr236Met). The homozygous genotype *+6359TT* occurred more frequently among VL cases compared to controls after adjusting for age, sex and ethnicity (OR = 2.2, 95%CI = 1.23–7.25, $P = 0.008$), indicating that this variant was associated with an increased risk for *L. donovani* infection (Table 1). We observed a similar effect of the *+6359T* variant, when different genetic models are employed [Allelic: OR = 1.4, 95%CI = 1.02–1.94, $P = 0.03$; Recessive: OR = 2.2, 95%CI = 1.23–7.25, $P = 0.008$] (Table 1). The different genetic models indicate that the *+6359T* minor allele increases the susceptibility of *L. donovani* infection. The other investigated *FCN2* variants were not significantly associated with VL.

The distribution of reconstructed *FCN2* haplotypes including variants -986G>A, -602A>G, -4A>G and +6359C>T are summarized in Table 2. Fifteen secretor haplotypes were observed. The four haplotypes *FCN2*GGAC*, **AGGT*, **AAAC* and **GGAT* occurred at frequencies >10%. The reconstructed haplotype *FCN2*AAAC* was found more frequently in healthy controls compared to VL cases (OR = 0.59, 95%CI = 0.37–0.94, $P = 0.023$).

Table 1. Distribution of *FCN2* genotypes and alleles among visceral leishmaniasis cases and healthy controls.

rs17549193 (+6359C>T) (p.T236M)	VL Cases n = 204 (%)	Controls n = 223 (%)	OR (95% CI)	$P^{\#}$ value
Genotype				
CC	110 (53.9)	134 (60.1)	1	Reference
CT	72 (35.2)	80 (35.8)	NA	NS
TT	22 (10.7)	9 (4.1)	2.2 (1.23–7.25)	0.008
Allele				
C	292 (71.5)	348 (78)	1	Reference
T	116 (28.5)	98 (22)	1.4 (1.02–1.94)	0.03
Dominant				
CC	110 (53.9)	134 (60.1)	1	Reference
CT+TT	94 (46.1)	89 (39.9)	NA	NS
Recessive				
CC+CT	182 (89.3)	214 (95.9)	1	Reference
TT	22 (10.7)	9 (4.1)	2.2 (1.23–7.25)	0.008

Note: CI, confidence interval; OR, odds ratio; NS, not significant; NA, not applicable. Percentage may not add up to 100 due to rounding errors

[#] Adjusted *P* values for age, gender and ethnicity

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Table 2. Association of functional *FCN2* haplotypes and visceral leishmaniasis.

FCN2 Haplotypes (-986/-602/ -4/+6359)	VL Cases n = 408(%)	Controls n = 446(%)	OR (95% CI)	$P^{\#}$ value
GGAC	225 (55.1)	236 (52.9)	NA	NS
AGGT	54 (13.2)	53 (11.8)	NA	NS
AAAC	**36 (8.8)**	**62 (13.9)**	**0.59 (0.37–0.94)**	**0.023**
GGAT	24 (5.8)	20 (4.4)	NA	NS
AAAT	16 (3.9)	12 (2.6)	NA	NS
AGAC	14 (3.4)	24 (5.3)	NA	NS
AGGC	9 (2.2)	13 (2.9)	NA	NS
AAGT	8 (1.9)	0	NA	NA
AGAT	8 (1.9)	0	NA	NA
GAAC	7 (1.7)	10 (2.2)	NA	NS
GGGT	4 (0.9)	12 (2.6)	NA	NA
GAAT	2 (0.5)	0	NA	NA
AAGC	1 (0.2)	0	NA	NA
GGGC	0	3 (0.6)	NA	NA
GAGT	0	1 (0.2)	NA	NA

Note: CI, confidence interval; OR, odds ratio; NS, not significant; NA, not applicable. Percentage may not add up to 100 due to rounding errors

[#]Adjusted *P* values for age, gender and ethnicity

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Ficolin-2 serum levels and risk of VL

Ficolin-2 serum levels were significantly higher in VL cases (mean 2.77 μg/ml) compared to healthy controls (mean 1.94 μg/ml) (adjusted $P<0.0001$ for age, sex and ethnicity; Fig 2). Ficolin-2 levels are significantly distributed across different +6359 genotypes in controls ($P = 0.03$; Fig 3). Serum ficolin-2 levels in cases with the reconstructed *FCN2*AAAC* haplotypes were significantly higher than those measured in individuals of the control group ($P = 0.01$; Fig 4).

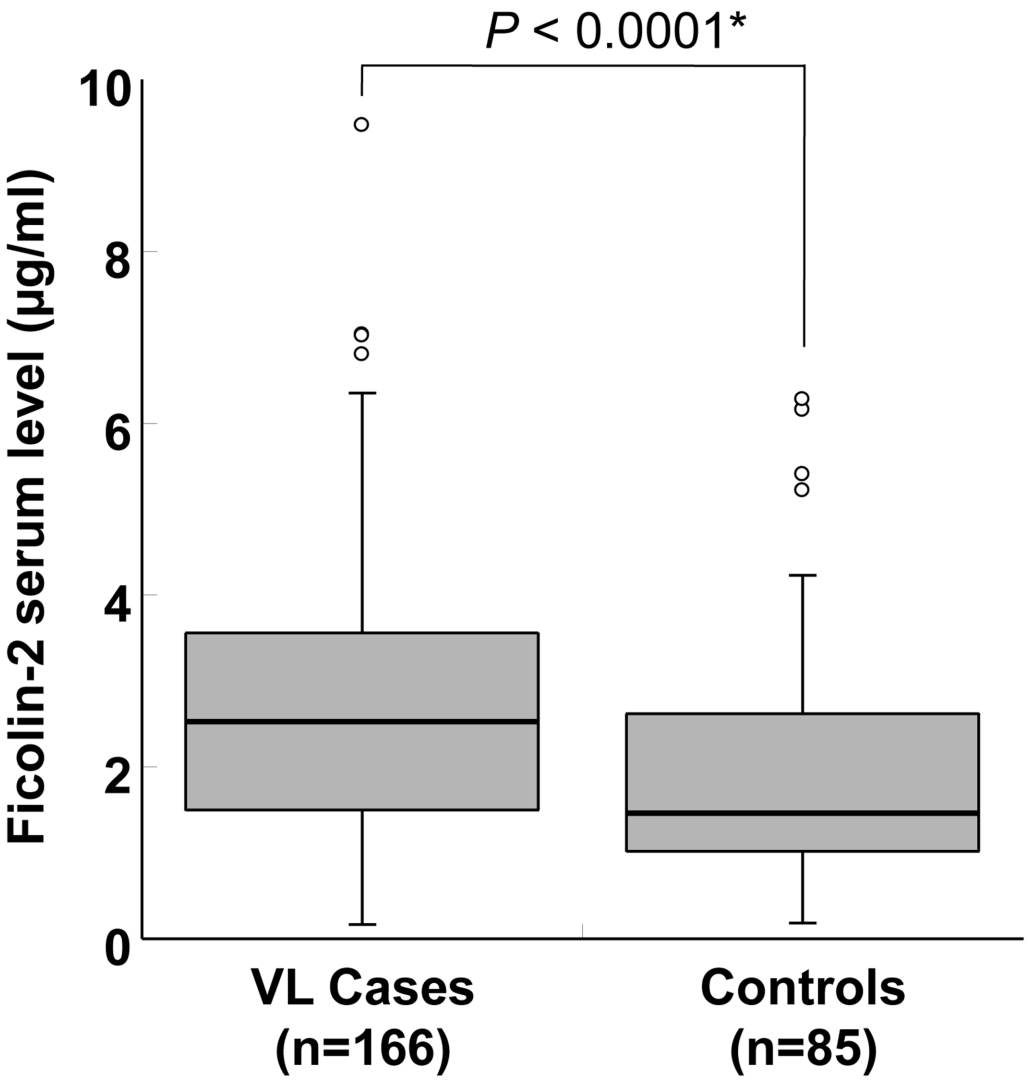

Fig 2. Distribution of ficolin-2 serum levels in visceral leishmaniasis cases and healthy controls. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles. **P* values were calculated by multivariate analysis adjusted for age, gender and ethnicity. Numbers in parenthesis represent the number of samples.

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Discussion

Visceral leishmaniasis develops when *L. donovani* parasites are successfully inoculated and survive the first-line attack of innate immune components such as phagocytes and the complement system. Indeed, these innate immune components play a major role both in the control and establishment of *L. donovani* infections [28]. Complement components including lectins are the primary molecules of the innate immune system to encounter inoculated metacyclic promastigotes. The early activation of the complement system during pathogen invasion occurs predominantly by the lectin pathway, as it is independent of a specific antibody response. Moreover, it prompts the activation of the alternative pathway [29]. The lectin pathway protein MBL induces opsonophagocytosis by depositing C3b on the surface of *Leishmania* which is crucial for parasite survival and multiplication [13,14]. We assume that, as ficolins are functionally similar to MBL, they equally influence the outcome of VL. No study so far, however,

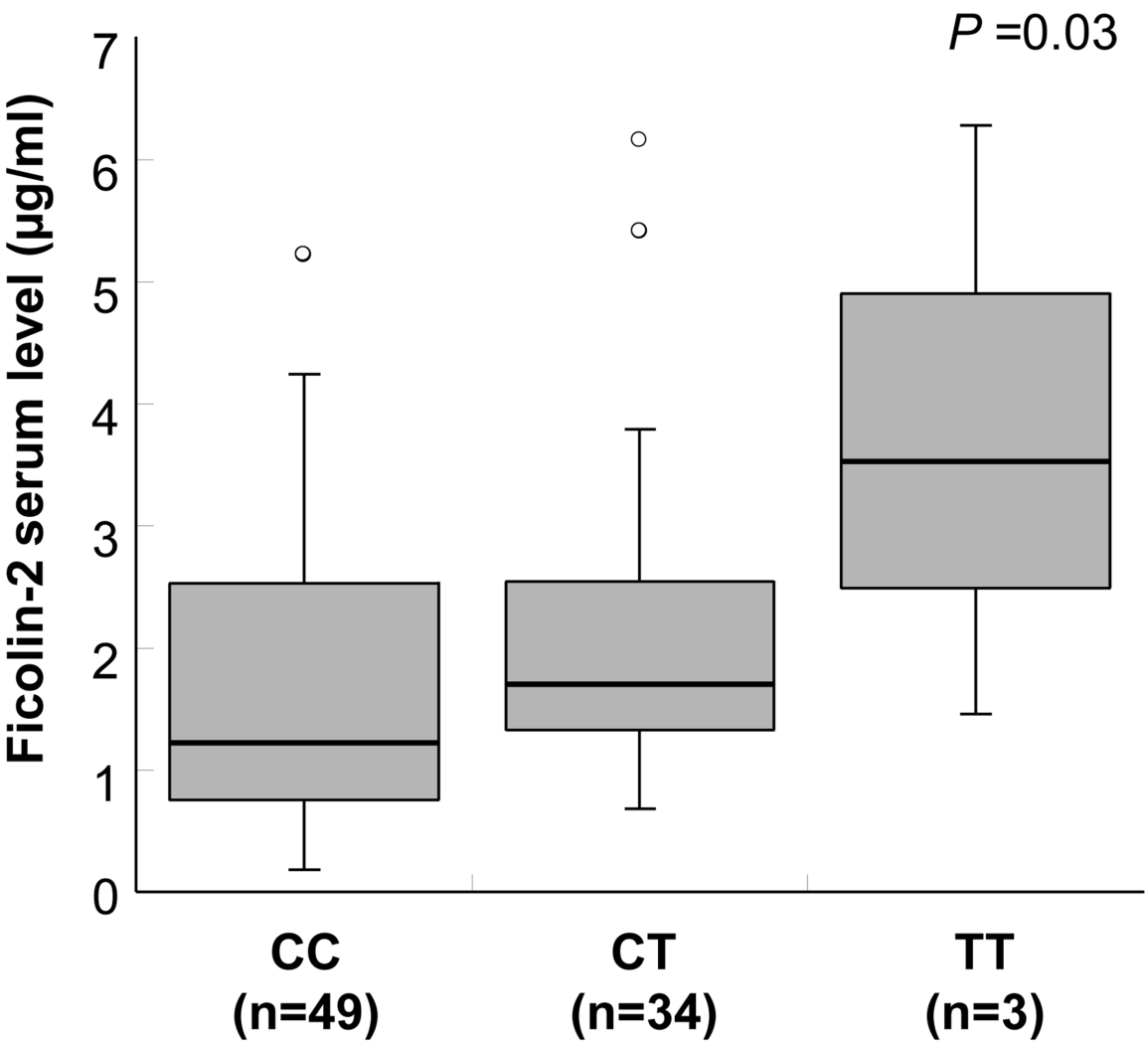

Fig 3. Distribution of ficolin-2 serum levels with +6359C>T variant in controls. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles. Ficolin-2 serum levels were measured and separated based on different genotypes of *FCN2* variant +6359C>T. *P* = 0.03 illustrated in the figure is calculated by Kruskal-Wallis rank sum test.

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has focused on the role of ficolin-2 in VL. We studied the contribution of ficolin-2 serum levels and of *FCN2* functional variants in VL.

The structural variant +6359C>T (p.T236M) in the fibrinogen-like domain of the *FCN2* gene confers relative susceptibility to VL. The finding remains consistent in recessive and allelic genetic models. The computational prediction revealed that the T236M substitution has a major impact on the physiochemical property of ficolin-2 [30]. In addition, the *+6359T* allele was found associated with higher ficolin-2 serum levels [31] and the observation was reconfirmed in a cohort of neonates [32]. We also observed a similar effect of the *+6359T* allele in controls, but not in cases. Our results inferred that ficolin-2 serum levels were modulated significantly by the infection in VL cases rather than by *FCN2* variants. Moreover, the ficolin-2 protein with T236M substitution had a markedly decreased binding capacity to acetylated agarose beads. Therefore, this structural variant is believed to alter the binding properties of the protein to recognize invading pathogens [24,33,34]. These reports indicate that individuals with higher ficolin-2 serum levels and altered binding capacities might favor *L. donovani*

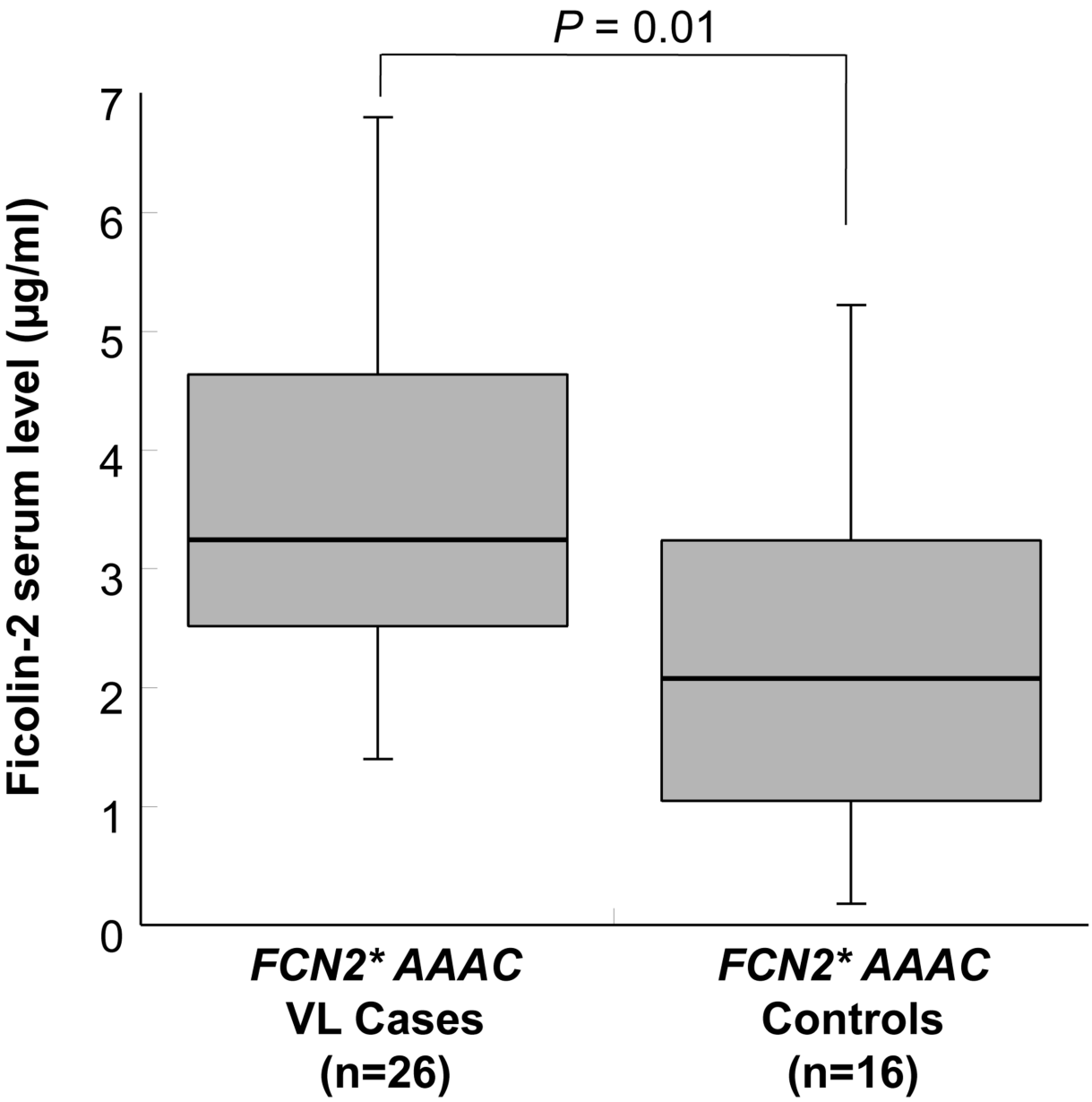

Fig 4. Distribution of ficolin-2 serum levels with *FCN2*AAAC* in VL cases and control. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles. *P* = 0.01 illustrated in the figure is calculated by Wilcoxon-Mann-Whitney rank sum test.

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invasion into macrophages and the development of VL. Previous studies have also reported that +6359C>T in the *FCN2* gene is a risk factor for staphylococcal peritonitis in continuous ambulatory peritoneal dialysis cases [35] and for bloodstream infections in kidney transplant recipients [36].

In *FCN2* gene-association studies, haplotype analyses should be taken into account as they may influence disease susceptibility [37]. Our *FCN2* haplotypes revealed that the *FCN2*AAAC* haplotype frequency was higher among controls than in VL cases, indicating that individuals with this haplotype had a diminished probability to develop VL. The *FCN2*AAAC* haplotype harbors the *+6359C* major allele, which accounts for reduced ficolin-2 levels [31,32]. In light of

these observations, it is evident that the *FCN2* genetic factors that contribute to low ficolin-2 level decrease the risk of VL. *FCN2* promoter haplotypes did not show any differences among groups, suggesting the relative contribution of the +6359C>T genotype in Indian VL.

Ficolin-2 serum levels were elevated in VL cases compared to controls, indicating that ficolin-2 is a susceptibility factor. The result is in accordance with a study published previously [15]. Corresponding results were also observed in infections with *Mycobacterium* spp., where higher MBL serum levels increased the risk of infection [38–40]. The proposed mechanism may be that intracellular parasites abuse C3 opsonization and enhance opsonaophagocytosis by monocytes/macrophages to avoid complement attacks. Any increase in the MBL and ficolin levels in turn may enhance complement activation and, thus, the probability of parasitization by depositing C3b on parasite surfaces [41]. Our observation supports this notion as cases with VL had higher ficolin-2 levels than uninfected controls. Nevertheless, discordant results were reported for ficolin-2 in tuberculosis and Chagas disease, where cases presented lower ficolin-2 plasma levels than did controls [22,42]. No clear mechanism is proposed to address the conflictive observations of functionally similar proteins in intracellular habitant infections. In addition, the recognition and interaction of mannose binding lectin (MBL) with *Leishmania* parasites are well established [13,14] and ficolins were shown to be functional analogous to MBL [16]. However, a limitation of our study is that there is a lack of data showing the interaction of ficolin-2 with *L. donovani*. Nevertheless, our earlier study demonstrated the genetic association of *FCN2* polymorphism with cutaneous leishmaniasis in Syrian population [17].

In conclusion, our results show that the *FCN2* +6359C>T variant is associated with increased susceptibility to VL and that the *FCN2*AAAC* haplotype is associated with relative protection. Higher serum ficolin-2 levels were observed in cases with VL than among controls.

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Author Contributions

Conceived and designed the experiments: KT TPV. Performed the experiments: AM JSA PS RDJ. Analyzed the data: JSA HVT TPV. Contributed reagents/materials/analysis tools: AM KT TPV. Wrote the paper: JSA CGM TPV KT.

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