Divergent Pro-Inflammatory Cytokine Response Induced by GB Virus C/ Hepatitis G virus (GBV-C/HGV) and Torque Teno Virus (TTV) Co-Infections in Hepatitis C Virus (HCV) Infected Thalassemia Patients

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Abstract

Despite common incidence of GB virus C/Hepatitis G virus (GBV-C/HGV) and Torque Teno Virus (TTV) co-infections in subjects with Hepatitis C virus (HCV) infection, pathogenic role of these co-infections in HCV infected subjects has not been clear since studies have mostly been based on liver enzyme profile yielding variable results. Pro-inflammatory cytokines that participate in host immune response against viruses are generated as a consequence of triggering of related genes by Nuclear Factor Kappa B (NF-kB) that is a member of transcription family. Study of three pro-inflammatory cytokines e.g. interleukin 1 beta (IL-1β), Interleukin-6 (IL-6), interleukin-8 (IL-8) in a group of multi-transfused thalassemic subjects in relation to positivity for HCV, HGV and TTV infections showed elevated levels of these cytokines in serum as well as in supernatant of peripheral blood mononuclear cell (PBMCs) cultures in HCV-GBV-C/HGV co-infected subgroup compared to HCV mono-infected subgroup (p <0.05 for comparison of all three cytokines) while HCV-TTV co-infected subgroup showed lowering of these levels compared to HCV mono-infected subgroup (p <0.05 for comparison of all three cytokines). Levels of p65 component of NF-kB i.e.NF-kB p65 in nuclear extracts of lipopolysaccharide stimulated PBMCs correlated positively with the levels of pro-inflammatory cytokines in serum as well as with that in supernatants of PBMC cultures in both HCV- GBV-C/HGV co-infected and HCV-TTV co-infected subgroups (p values ranging from <0.001 to 0.004). Levels of NF-kB p65 in nuclear extracts and levels of pro-inflammatory cytokines in serum as well as in supernatants of PBMC culture did not show any alteration in thalassemic subjects with HGV or TTV infections alone compared to healthy non-transfused subjects. Based on the inhibitory role of TTV on activation of NF-kB in TTV-HCV co-infected cases observed in the present study and the reported contribution of NF-kB towards development of hepatocellular carcinoma (HCC) due to establishment of chronicity, it may be worth evaluating if TTV or any component of TTV can be utilized as therapeutic vaccine against development of HCC in HCV infected subjects.

Keywords: GBV-C/HGV; TTV; HCV; IL-1β; IL-6; IL-8; NF-kB p65

1. Introduction

Co-infections of GB Virus C/ Hepatitis G virus (GBV-C/HGV) and Torque Teno virus (TTV) have been frequently reported to be associated with Hepatitis C virus (HCV) infection in multi-transfused individuals (Prati et al., 1999; Sehgal & Sharma 2002; Sampietro et al., 1997; Alavi et al., 2011). However, role of GBV-C/HGV or TTV co-infections in the pathogenesis of HCV infection has not been clear since these studies have been predominantly based on liver enzyme profile yielding conflicting results (Chen et al., 1999; Asim, Singla, Gupta & kar, 2010; Jeon, Shin, Suh, Lin & Ryang, 2003).

Relatively recently, alteration in levels of pro-inflammatory cytokinese. eg. interleukin 1 beta (IL-1β), interleukin-6 (IL-6) and Tumor necrosis factor alpha (TNF- α) have been considered to be associated with hepatic inflammation and hepatocellular carcinoma (HCC) in HCV infection (Par et al., 2013; Nishitsuji et al., 2013;

Falasca et al., 2006). Activation of these pro-inflammatory cytokines is triggered by Nuclear factor kappa beta (NF-κB), a transcription factor present in the cytoplasm of all eukaryotic cells. A component of NF-κB with molecular weight of 65 kd (NF-κB p65) is translocated into the nucleus in response to various stimuli including viral infections triggering activation of the genes for pro-inflammatory cytokines (Tripathi & Aggarwal 2006; Baldwin, 1996).

A study was carried out in HCV infected children of beta thalassemia major co-infected with GBV-C/HGV or TTV to measure levels of IL-1β, IL-6 and TNF-α in serum as well as in culture of peripheral blood mononuclear cells (PBMCs). Level of NF-κB p65 were also estimated in nuclear extracts of cultured PBMCs as a measure of activation of pro-inflammatory genes to find out their correlation with the levels of pro-inflammatory cytokines secreted by PBMC culture.

2. Materials and Methods

2.1. Subjects

A total of 200 clinically diagnosed cases of thalassemia major from the cities in northern India including Delhi, were selected for the study over a period of 2013-2017 at NTWS, Thalassemia Centre, Tilak Nagar, New Delhi. A total of 50 healthy age, sex and socio-economically matched local young adults and children without any history of blood transfusion were selected as controls. The study was approved by the institutional review board as well as by ethical committee. Consent was obtained from the thalassemic adults and parents of the thalassemic children for the study, although the samples were blindly coded with complete anonymity. Information regarding the age at onset of transfusion was collected from the records available with the NTWS centre, while information regarding number of blood transfusions received could not be calculated due to lack of reliable records of all subsequent blood transfusions. Brief information on major clinical symptoms was obtained from the study subjects at the time of enrollment in the present study while clinical examination was limited to evidence of hepatomegaly and splenomegaly. History was collected regarding vaccination against Hepatitis B virus (HBV), *Streptococcus pneumonae*, *Neisseria meningities* and *Haemophilus influenzae type b*.

2.2 Collection of Samples

Fifteen ml of blood was collected from each subject, at least three weeks following last blood transfusion, out of which 5 ml was distributed in plain sterile tube to yield serum for biochemical studies viz. estimation of liver enzymes, bilirubin and ferritin levels, estimation of cytokines and molecular detection of TTV, GBV-C/HGV (referred as HGV subsequently in the paper), HCV and HBV. The remaining blood (10ml) was distributed in EDTA containing tube for isolation of PBMCs to be utilized for extraction of DNA for molecular detection of HIV-1 as well as for setting up PBMC culture for estimation of pro-inflammatory cytokine production and for estimation of NF-kB p65 level in nuclear extracts of cultured PBMCs.

2.3 Estimation of Liver Enzymes, Bilirubin and Ferritin Level in Serum

Estimation of three enzymes as indices of liver function viz. alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) as well as total bilirubin in serum were carried out using auto-analyzer (Hitachi 912, Hitachi Ltd, Japan). Reference upper limit of serum ALT, AST, ALP and bilirubin were accepted as 55 U/L, 48 U/L, 115 U/L and 1.2 mg/dL respectively (Liver function test reference, Mayo Clinics, 2015). Serum ferritin level was estimated by commercial enzyme linked immunosorbent assay (Calbiotech, California, USA, FR248T) with sensitivity of 10 ng/ml.

2.4 Estimation of Serum Levels of Pro-Inflammatory Cytokines

Serum levels of IL-1β, IL-6 and TNF-α were measured using commercial ELISA kits (R&D Systems, Minneapolis, MI, USA) with detection limits of 1 pg/ml, 0.7 pg/ml and 0.38 pg/ml respectively. Any sample showing undetectable level of cytokine was assigned a value of 0 pg/ml (Chattopadhya & Baveja, 2018). Inter-assay and intra-assay variation for all of these assays were found to be less than 10%.

2.5 Molecular Detection of HBV, TTV, HGV, HCV and HIV-1

(i) Extraction of DNA for HBV and TTV from serum

DNA for HBV and TTV was extracted from serum samples by Proteinase K extraction method using manufacturer's protocol (DNA Sure Blood Mini Kit, Nucleopore, Genetix Biotech Asia Pvt. Ltd.). Briefly, 200 μl of serum was mixed with the same volume of Proteinase K and lysis buffer provided with the kit. After an incubation period of 10 minutes at room temperature, the mixture was centrifuged, washed twice with wash buffer and final elution was made in 100 μl of elution buffer provided with the kit. Quality of the genomic DNA was

checked by 1% agarose gel electrophoresis run on 0.5X Tris-acetate EDTA (TAE) buffer. The bands were visualized in UV-transilluminator.

(ii) Extraction of RNA for HGV and HCV from serum

Viral RNA for HCV and HGV was extracted from serum sample using the QIAmp Viral RNA Kit (Qiagen, Hildon, Germany) according to the manufacturer's protocol. Briefly, 140 µl of serum was mixed with viral lysis buffer (200 mM NaOH, 1% SDS) and was further treated with proteinase K and carrier RNA (provided with the kit). After 10 minutes of incubation at room temperature the RNA was precipitated with ethanol, washed twice with washing buffer and finally eluted in 60 µl of elution buffer (1.25M NaCl, 50 mM Tris-HCl) provided with the kit.

(iii) Extraction of DNA of HIV-1 from PBMC

(a) Isolation of PBMCs

PBMC was isolated from the blood samples using Ficoll-Hypaque density gradient centrifugation method. Briefly, Ficoll-Hypaque solution, density 1.077 (Histopaque, Sigma chemicals, USA) was layered over blood containing EDTA, ratio 1:1 in a 15 ml Falcon tube. The tube was centrifuged at 2500 rpm for 20 minutes. After centrifugation, the middle layer of buffy coat containing PBMCs was aspirated from the tube, mixed with 1X PBS (5 times the quantity of PBMCs) followed by centrifugation at 5000 rpm for 10 minutes. Supernatant was discarded and the pellet was rewashed twice in PBS. The PBMC suspension was found to contain > 95% of viable cells by trypan blue exclusion method and >97% pure PBMCs by flow cytometric analysis using monoclonal anti-CD3, anti-CD19 and anti-CD14 antibodies. The PBMC suspension was adjusted in 10 ml of PBS to yield ~ 1×10^6 cells/ml. Two ml of PBMC suspension (containing ~ 2×10^6 PBMC) was utilized for extraction of pro-viral DNA for molecular detection of HIV-1, using same procedure carried out for HBV and TTV.

(iv) PCR Amplifications

The extracted DNA was amplified by PCR for *pre-core/core* and partial *pol* genes of HBV (amplicon size 1078 bp) and conserved 5' end region of TTV (amplicon size 199 bp). The extracted RNA was amplified by nested RT-PCR for conserved 5' UTR gene of HCV (amplicon size 250 bp) and 5' NTR gene of HGV (amplicon size 204 bp), HIV-1 DNA was also amplified by nested PCR for p24 gene (amplicon size 717p). The outer PCRs (RT-PCR) for HGV and HCV were carried out using GeneAmp Gold RNA PCR Core Kit while all the DNA PCRs of TTV, HIV-1, HBV and inner PCRs of HCV and HGV were carried out using Go Taq Green Master Mix (Promega, Madison, USA) kit using the manufacturer's instructions. Concentration of primers used in all the rounds of PCR was 10 pmol. The polymerase chain reaction was performed in GeneAmp PCR system 9700 (Applied Biosystems) in 200 µl MicroAmp reaction tubes. The amplified product was analyzed on 2% agarose gel under UV light after staining with ethidium bromide (PCR-EB). In every PCR run two positive and two negative samples were included and run in duplicate until congruent results, only were accepted. The primer sequences and PCR cycling conditions for each virus studied is shown in Table 1.

2.6 Estimation of NF-κB in Nuclear Extract and Secreted Pro-Inflammatory Cytokines in Supernatant of LPS Stimulated PBMC Culture

Remaining 8 ml of PBMC (~ 8×10^6 cells) suspension was equally divided into two portions, 4 ml each, for stimulation by mitogen LPS, one portion for shorter duration i.e. 30 minutes followed by estimation of NF- κB p65 in nuclear extracts of stimulated PBMCs, the other portion for longer duration i.e. 48 hours followed by estimation of pro-inflammatory cytokines secreted by LPS stimulated PBMCs.

2.6.1 Estimation of NF- κB in Nuclear Extracts of LPS Stimulated PBMCs

This was carried out following broadly the original protocol by Osborn et al., (1989) with minor modifications adapted by Yu et al., (1995). The protocol involved three steps viz. (i) treatment of PBMCs with mitogen LPS to induce translocation of NF-kB from cytoplasm into the nucleus (ii) extraction of nuclear proteins from LPS stimulated PBMCs (iii) estimation of NF-kB in nuclear protein extracts of LPS stimulated PBMCs.

(i) Treatment of PBMC with LPS

Out of the first 4 ml portion of the PBMC suspension, 3 ml was subjected to treatment with LPS (derived from *Escherichia coli*, serotype 0111:B4, Sigma chemicals, USA),100 ng/ml for 30 minutes followed by distribution in triplicate tubes 1 ml each. The triplicate tubes were incubated at 37^0C in a humidified CO_2 chamber to facilitate translocation of NF-kB from cytoplasm into nucleus. A fourth tube containing one ml of PBMC suspension was included without any mitogen treatments control and incubated under identical condition.

Table 1. Details of primer sequences and cycling conditions for various viruses

Virus	Primer sequences	Cycling conditions
HBV	F- GGAGTTGGGGGAGGAGATTA R- AGGCGCTACGTGTTGTTTCT	Initial denaturation at 95^0C for 5 min. followed by amplification comprising of 37 cycles of alternate 94^0C for 1 min; 55^0C for 1 min; 72^0C for 2 min; final extension at 72^0C for 10 min.(Khare et al.,2012)
TTV	F-GCTACGTCACTAACCACGTG R-CTBCGGTGTGTAAACTCACC (B= G,C OR T)	Initial denaturation at 95^0C for 5 min. followed by amplification comprising of 55 cycles of alternate 94^0C for 15 sec; 60^0C for 15 sec; 72^0C for 20 sec; final extension at 72^0C for 7 min. (Inami, Konomi,Yasuyuki & Abe 2000).
HCV	Outer PCR F- CTGTGAGGAACTACTGTCTT R-ATACTCGAGGTGCACGGTCTACGAGACCT Inner PCR F-TTCACGCAGAAAGCGTCTAG R-ACTCTCGAGCACCCTATCAGGCAGT	Outer PCR: c DNA synthesis at 45 °C for 45 min. initial denaturation at 95 °C for 10 min. followed by amplification comprising of 35 cycles of alternate 95 °C for 30 sec; 48 °C for 30 sec; 72 °C for 30 sec; and a final extension at 72 °C for 7 min. Inner PCR: initial denaturation at 95 °C for 5 min followed by amplification comprising of 35 cycles of alternate 94 °C for 30 sec; 50 °C for 30 sec; 72 °C for 30 sec; and a final extension at 72 °C for 7 min. (Asim, Singla, Gupta & Kar, 2010).
HGV	Outer PCR F-AAGCCCCAGAAACCGACGCC R-TGAAGGGCGACGTGGACCGT Inner PCR F-CGGCCAAAAGGTGGTGGATG R-GTAACGGGCTCGGTTTAACG	Outer PCR: c DNA synthesis at 45 °C for 45 min. initial denaturation at 95^0C for 10 min followed by amplification comprising of 35 cycles of alternate 95^0C for 30 sec; 65^0C for 30 sec; 72^0C for 30 sec; final extension at 72^0C for 7 min. Inner PCR: initial denaturation at 95^0C for 5 min. followed by amplification 30 cycles of 60^0C for 15 sec; 56^0C for 20 sec; 72^0C for 25 sec; final extension at 72^0C for 7 min (Souza et al.,2006).
HIV-1	Outer PCR G00- GACTAGCGGAGGCTAGAAG G01- AGGGGTCGTTGCCAAAGA Inner PCR G60-CAGCCAAAATTACCCTATAGTGCAG G25-ATTGCTTCAGCCAAAACTCTTGC	Outer PCR: initial denaturation at 95^0C for 2 min. followed by amplification comprising of 30 cycles of alternate 92^0C for 10 sec; 55^0C for 30 sec; 72^0C for 1min; final extension at 72^0C for 7 min. Inner PCR: initial denaturation at 95^0C for 2 min followed by amplification comprising of 35 cycles of alternate 92^0C for 10 sec; 56^0C for 20 sec; 72^0C for 1min; final extension at 72^0C for 7 min. (Sanders-Buell, Salminen & McCutchan, 1995).

(ii) Extraction of nuclear proteins from LPS stimulated and control PBMC nuclei

This procedure was carried out in presence of protease inhibitor cocktail to prevent loss of extracted protein.

(a) Reagents used

Reagents for extraction of proteins viz. Nonidet P-40 and for preparation of buffers viz. HEPES, DTT (DL-Dithiothreitol), $MgCl_2$, KCL, Tris-HCl, Na-EDTA and Glycerol were all procured from local supplier (Hi-Media Laboratories, India).

The reagents for preparation of protease inhibitor cocktail were all procured commercially (Sigma chemicals, USA) viz. Phenyl-methyl-sulfonyl fluoride (PMSF) (Cat No P7626) 0.5 mM

Leupeptine (Cat No L8511) 1mg/ml, Pepstatin (Cat No P 4625) 0.5 mg/ml, Aprotinin (Cat No A1153) 1mg/ml, EGTA (Cat No E8145) 1mM, Sodium orthovanadate (Na_3VO_4, Cat No S6508) 1mM. PMSF was prepared fresh each time before use.

(b) Extraction of nuclear protein from LPS stimulated and unstimulated (control) PBMCs

LPS treated PBMC suspensions were centrifuged at 750 g at 4^0C. The pellets were washed twice with cold HEPES buffer followed by suspension in 20-40 µl ice cold hypotonic HEPES buffer composed of HEPES (10 mM, pH 7.8), $MgCl_2$ (1.5 mM), KCL (10 mM), DTT (0.5 mM) containing 0.1% Nonidet P-40.The suspensions were kept on ice for 10 minutes followed by centrifugation at 10,000 g for 10 minutes to settle cellular debris. The pellets representing nuclear fractions were re-suspended in 35-40 µl/ tube of ice cold high salt buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5mM $MgCl_2$, 0.2 mM EDTA and 25 % Glycerol) containing 10 µl of protease inhibitor cocktail, followed by incubation at 4^0C for 15 minutes with intermittent shaking. The suspensions containing lysed nuclei were vortexed and centrifuged at 10,000 g at 4^0C for 10 minutes for settling the nuclear debris. The supernatants representing the nuclear extract were collected and stored at 4^0C with addition of 75-100 µl of HEPES buffer (pH 7.9) in each tube.

The protein concentrations of the extracts were determined by Bradford's method in 96-well microplate using Coomassie dye reagent with bovine serum albumin as calibration standards (Bradford, 1976). The concentration of protein in nuclear extract suspension in each tube was adjusted to 100 μg /ml.

(iii) Estimation of NF-kB p65 in nuclear fraction

This procedure was carried out by commercial ELISA kit as per manufacturer's protocol (Cusabio, Wuhan, China; sensitivity 0.078 ng/ml; intra-assay and inter-assay coefficient of variation <10%). Briefly, 100 μl (10μg) of protein extracts from triplicate LPS treated tubes as well as from untreated tube and 100 μl of dilutions of standards (supplied with the kit) were charged in the ELISA plate wells coated with monoclonal anti- NF-kB p65 antibody followed by incubation at 37^0C for 2 hr. The liquid was aspirated from the wells and 100 μl of biotin-conjugated anti-NF-kB antibody was added to each well. The plate was incubated at 37^0C for 1 hr followed by washing 3 times. One hundred microliter of HRP-avidin conjugate was added on to the wells and the plate was incubated at 37^0C for 1 hr. The plate was then washed 5 times followed by addition of 90 μl of TMB and incubated at 37^0C for 15-30 minutes in dark followed by addition of 100 μl of 0.18M H_2SO_4 as stop solution. The plate was read at 450 nm with correction at wavelength of 540 nm. Concentration of NF-kB p65 in the nuclear extract was expressed as nanogram (ng) of NF-kB p65 per microgram (μg) of nuclear protein extract after taking the average of three wells (Garbin et al., 2009).

2.6.2 Estimation of Pro-Inflammatory Cytokines viz. IL-1β, IL-6 and TNF-α Secreted by LPS Stimulated PBMCs

The second 4 ml portion of PBMC suspension i.e. (~$1x10^6$cells/ml) was utilized for this purpose. Three ml of the suspension was treated with LPS 1 μg/ml and distributed in triplicate tubes, 1ml each, while a fourth tube containing 1ml of PBMC suspension without any LPS treatment served as control. The tubes were incubated in humidified 5% CO_2 cell culture incubator for 48 hrs. At the end of incubation, the suspension was centrifuged at 350 g for 5 min at 4^0C. The supernatant was collected for estimation of three pro-inflammatory cytokines viz. IL-1β, IL-6 and TNF-α by commercial ELISA (R&D systems, Minneapolis, MI, USA). The supernatant from the unstimulated PBMC culture in the control tube was also subjected to estimation of IL-1β, IL-6 and TNF-α to check spontaneous secretion of these cytokines.

2.6.3 Statistical Analysis

Statistical analysis was carried out employing SPSS package version 20. Risk for acquisition of viral infections in multi-transfused children was evaluated in comparison to control children by Odd's ratio. Other characteristics were compared using χ^2 test for categorical variables and student's t-test or one way ANOVA followed by post-hoc test with Bonferroni adjustment for continuous variables with normal distribution characteristics while continuous variables not following normal distribution was compared by Kruskal Wallis test followed by multiple comparison using Mann Whitney test with Bonferroni adjustment. P value <0.05 was considered as statistically significant and it was adjusted for multiple comparisons. Fisher's exact test was used in which any of the expected cell frequency was less than five.

4. Results

The thalassemia subjects belonged to age group of 4 to 36 years (mean ± SD,17 ± 8.6 years) with mean male to female ratio as 1.2 vs 1. Abdominal pain was the commonest clinical presentation at the point of collection of samples from the thalassemia children with more than half of the study children having hepatomegaly or splenomegaly on clinical examination. Most of the thalassemia children were vaccinated for HBV, while more than half of the children including all of those splenectomised, were covered by meningococcal, pneumococcal and *H. influenzae* type b vaccines (Table 2).

Out of 200 samples tested for HIV, HBV, HCV, TTV and HGV infection by molecular analysis, no sample was positive for HIV infection while only two samples were positive for HBV (HBsAg positive) alone that were excluded from analysis for the purpose of the present study. There were 76 (38%) samples positive for HCV infection comprising of 36 (18%) co-infected with TTV, 20 (10%) co-infected with HGV and 20 (10%) with HCV alone. There were 36 (18 %) samples positive for TTV infection alone and 14 (7%) for HGV infection alone (Fig 1, 2 and 3).Remaining 72 samples were negative for HCV, HGV or TTV infection (referred subsequently as viral marker negative group for the purpose of present study). The age distributions of subgroups of thalassemic subjects positive for various markers were comparable. At first blood transfusion also, age were comparable between the various subgroups except the viral marker negative subgroup, that showed relatively higher age distribution. Out of 50 control subjects, TTV and HGV infections could be detected in 12 and 2 cases respectively without positivity for any of other virus studied (Table 3).

Table 2. Clinical characteristics and vaccination status of the thalassemic subjects (n=200)

Clinical presentation		No (%)
	Abdominal pain	77 (38.5)
	Fever	25 (12.5)
	Generalised weakness	76 (38)
	Anorexia/loss of apatite	49 (24.5)
	Hepatomegaly	106 (53)
	Splenomegaly	102 (51)
Vaccination status		
	HBV	192 (96)
	Pneumococcal	110 (55)
	Meningococcal	108 (54)
	H.influenzae type b	132(66)
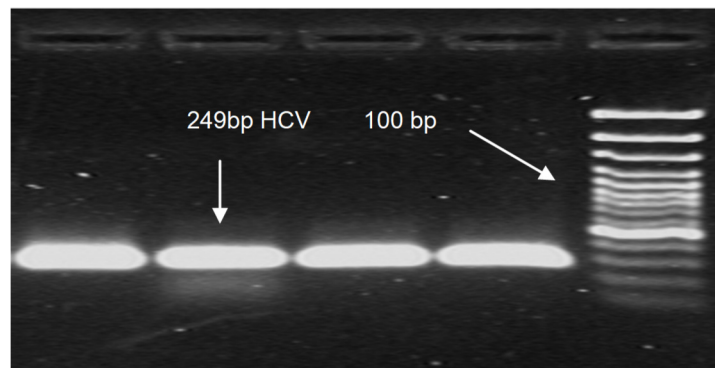
Figure 1. Gel picture showing bands of amplified HCV PCR products
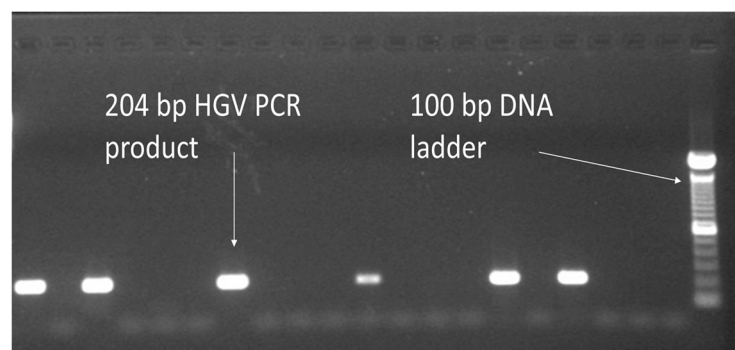

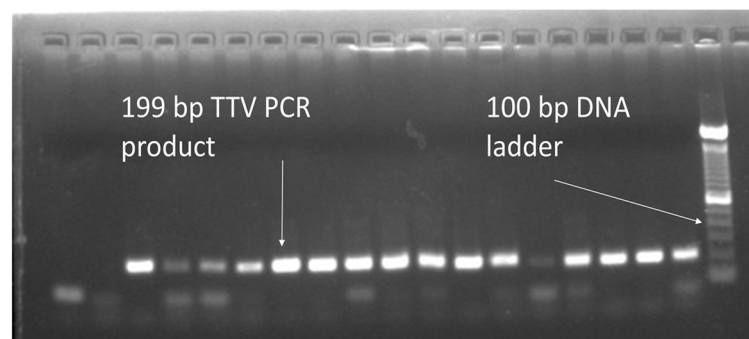Figure 2. Gel picture showing bands of amplified HGV PCR products

Figure 3. Gel picture showing bands of amplified TTV PCR products

Table 3. Prevalence of HCV, TTV and HGV in thalassemic subjects and controls

Viral markers	Thalassemia cases (n=200) No (%)	Age (yrs) (mean $\pm$ SD)	Age (months) at first blood transfusion
HCV + TTV	36 (18)	18.3 $\pm$ 9.4	7.75 $\pm$ 1.76
HCV + HGV	20 (10)	16.0 $\pm$ 8.5	8.6 $\pm$ 1.85
HCV only	20 (10)	17.2 $\pm$ 6.8	7.7 $\pm$ 1.49
TTV only	36 (18)	19.5 $\pm$ 8.8	7.6 $\pm$ 1.44
HGV only	14 (7)	16.9 $\pm$ 6.8	7.2 $\pm$ 1.12
Negative for HCV/TTV/HGV	72 (36)	17.8 $\pm$ 7.7	10.3 $\pm$ 1.76

Note:

1) 2 cases in thalassemia group showing HBs Ag positivity only are not included in the table and were excluded for further analysis for the purpose of the present study.

2) In the control group n (50), there were 12 (24%) subjects positive for TTV only and 2 (4%) positive for HGV only.

Serum levels of liver enzymes and bilirubin were elevated in thalassemic subjects with positivity for HCV infection, alone or co-infected with TTV compared to non-transfused community controls. However levels of liver enzymes and bilirubin were comparable to controls in HCV infected group with HGV co-infection. Serum ferritin levels were elevated in all the subgroups regardless of presence or absence of viral marker although among the cases with HCV infection, those with HGV co-infection showed significantly lower level of ferritin in serum compared to cases with HCV infection alone or co-infected with TTV (Table 4).

Table 4. Serum levels (mean $\pm$ SD) of liver enzymes, bilirubin and ferritin in various subgroups of thalassemic subjects

Biochemical Parameters	HCV+TTV (n= 36)	HCV+HGV (n=20)	HCV only (n =20)	TTV only (n=36)	HGV only (n= 14)	Negative for HCV/HGV/ TTV (n=72)	Controls (n= 50)
ALT (U/L)	110$\pm$32[a]	41 $\pm$15[a,b]	102$\pm$28[a]	20 $\pm$7	18$\pm$6	24 $\pm$13	21 $\pm$8
AST (U/L)	92 $\pm$22[a]	32 $\pm$12[a,b]	87 $\pm$25[a]	21 $\pm$7	23 $\pm$9	20 $\pm$8	17$\pm$6
ALP (U/L)	331 $\pm$103[a]	191$\pm$61[a,b]	293 $\pm$109[a]	188 $\pm$69	170 $\pm$41	162$\pm$53	156$\pm$24
Bilirubin (mg/dL)	2.54 $\pm$0.89 [a]	0.90$\pm$0.29 [a,b]	2.82 $\pm$1.09[a]	0.69$\pm$0.31	0.76 $\pm$0.40	0.65$\pm$0.25	0.63 $\pm$0.17
Ferritin (ng/ml)	4036$\pm$1329 [a]	2681 $\pm$704[a,b]	4174$\pm$923[a]	2622$\pm$1077[a]	2763$\pm$1255[a]	2428 $\pm$704[a]	68$\pm$15

Statistical comparisons between the subgroups:

Over all: $p < 0.05$ for all the biochemical parameters in all subgroups

Individual:

a) Indicates significant elevation compared to controls

b) Indicates significant depression compared to HCV infection, alone or co-infected with TT

Serum levels of the three pro-inflammatory cytokines studied viz. IL-1β, IL-6 and TNF-α in cases with TTV infection or HGV infection individually showed no difference with viral marker negative or control group while the subgroup with HCV infection alone showed higher levels of enzymes compared to viral marker negative or control group. The subgroups of HCV infection co-infected with HGV showed elevated levels of the pro-inflammatory cytokines compared to cases with HCV infection alone while subgroup of HCV infection co-infected with TTV showed level of pro-inflammatory cytokines lower compared to HCV infection alone (Table 5).

The levels of pro-inflammatory cytokines i.e. IL-1β, IL-6 and TNF- α secreted by cultured PBMCs in various subgroups reflected the pattern observed in serum i.e. higher levels in the subgroup with HCV-HGV co-infection and lower levels in the subgroup with HCV-TTV co-infection compared to HCV infection alone and unchanged levels in isolated HGV or TTV infections compared to viral marker negative or control group (Table 6).

Table 5. Serum levels (pg/ml) of cytokines in subgroups of thalassemic subjects

	Subgroups of thalassemic subjects viral infections based on positivity for various markers studied						
Cytokines	HCV+TTV n =36)	HCV+HGV (n= 20)	HCV alone (n =20)	TTV alone n= 36)	HGV alone (n=14)	Negative for all the Viral markers (n=72)	Controls (n= 50)
IL-1β	1.2 $\pm$ 1.1[a,b]	22.6$\pm$ 11.4[c,d]	8.2 $\pm$ 3.6[c]	4.6 $\pm$ 0.9	4.2 $\pm$ 2.2	5.1$\pm$ 3.6	3.2 $\pm$ 2.1
IL-6	3.2 $\pm$2.3[a,b]	37.6$\pm$18.6[c,d]	17.2 $\pm$ 7.6[c]	8.6 $\pm$ 2.1	9.2 $\pm$ 4.2	8.2 $\pm$ 3.6	7.2 $\pm$ 4.5
TNF-α	2.2$\pm$ 1.8[a,b]	30.2 $\pm$ 7.2[c,d]	16.3 $\pm$ 4.6[c]	6.6 $\pm$ 3.8	7.2 $\pm$ 4.6	8.2 $\pm$ 3.6	5.1 $\pm$ 3.3

Note: Mean + SD levels (pg/ml) of IL-1β, IL-6 and TNF-α in serum in control group (n=50) were 3.2 $\pm$ 2.1, 7.2 $\pm$ 4.5 and 5.1 $\pm$ 3.3 respectively.

Statistical comparisons:

Over all: <0.05 for IL-1β, IL-6, TNF-α in various subgroups.

Individual:

a) Indicates significant depression compared to controls

b) Indicates significant depression compared to HCV infection alone

c) Indicates significant elevation compared to controls

d) Indicates significant elevation compared to HCV infection alone

Table 6. Levels of pro-inflammatory cytokines secreted by PBMC culture and NF-kB content in nuclear extract following LPS stimulation

Activation Markers	HCV+TTV (n =36)	HCV+HGV (n= 20)	HCV alone (n =20)	TTV alone (n= 36)	HGV alone n=14)	Negative For HCV/ HGV/TTV (n=72)	Controls (n= 50)
IL-1β (pg/ml)*	3.6$\pm$2.4 [a,b]	34.6 $\pm$16.6 [c,d]	18.8$\pm$8.7 [c]	8.6 $\pm$4.1	7.6$\pm$3.5	6.9 $\pm$2.9	7.4 $\pm$3.2
IL-6 (pg/ml)*	2.7$\pm$1.5[a,b]	36.1 $\pm$21.4[c,d]	16.5$\pm$8.6[c]	5.9 $\pm$2.8	5.2$\pm$2.8	5.4$\pm$2.9	4.2$\pm$3.6
TNF-α (pg/ml)*	3.9 $\pm$2.6[a,b]	45.5$\pm$23.8[c,d]	25.4$\pm$13.0[c]	13.8$\pm$6.9	12.9 $\pm$7.6	11.9 $\pm$7.6	12.3 $\pm$5.4
NF-kB (ng/μg of protein)**	0.69$\pm$0.28[a,b]	5.5$\pm$2.5[c,d]	2.21$\pm$1.14[c]	1.28$\pm$ 0.65	1.19 $\pm$ 0.66	1.10$\pm$0.45	1.14 $\pm$0.18

*Indicates quantity in culture supernatant

**Indicates protein content of nuclear extract from cultured PBMC

a. Indicates significant depression compared to controls

b. Indicates significant depression compared to HCV infection alone

c. Indicates significant elevation compared to controls

d. Indicates significant elevation compared to HCV infection alone

The NF-κB p65 levels in nuclear extracts of PBMCs from various subgroups showed unaltered levels in the subgroups positive for TTV or HGV. The subgroups with HCV infection alone or with co-infection by HGV showed levels higher than uninfected or control group while the subgroup of HCV infection co-infected with TTV showed unaltered level. Alterations of NF-kB p65 levels in the three subgroups with HCV infection showed positive correlations with the levels of IL-1β, IL-6 and TNF-α secreted by cultured PBMCs in the corresponding subgroups (Supplementary Figures 1(a-c), 2 (a-c) and 3 (a-c)).

5. Discussion

A perceptible percentage (38%) of thalassemic subjects in the present study were positive for HCV infection that could be due to acquisition of HCV infection prior to introduction of mandatory HCV screening in blood banks in the country coupled with persistent nature of HCV infection (Rosen, 2011). Reports on prevalence of TTV and HGV infections in multi-transfused children from various countries have been extremely variable, possibly due to varying incidence of these infections in blood donors (Kondili et al., 2001; Sampietro et al., 2001; Kar et al.,2000;

Ahmed, 2011). However, TTV and HGV infections were also recorded in as many as 24% and 4 % respectively of control subjects indicating involvement of non-parenteral routes of transmission (Schrotter et al., 2000; Okamoto et al., 1998). In the present study, prevalence of TTV and HGV co-infections with HCV infection among thalassemic subjects were found to be comparable to that reported in studies from India (Panigrahi, Saxena, Acharya & Panda, 1998; Asim, Potukuchi, Arora, Singh & Kar, 2008). Several studies based on liver enzyme profile concluded that neither HGV nor TTV has any pathogenic role in liver damage (Sampeitro et al.,1997; Irshad, Joshi, Sharma & Dhar 2006; Matsumoto et al.,1999). However few studies have postulated a beneficial role of HGV co-infection in the pathogenesis of HIV infections, based on study of liver enzyme profile, short term clinical observation and have included HIV/HGV co-infected patients as study subjects (Feng et al., 2014; Stapleton, Williams & Xiang, 2004).

However, although apparently neither HGV nor TTV infections individually showed any evidence of liver enzyme alterations, there was distinct divergence in pro-inflammatory cytokine response induced by these two agents when co-infected with HCV as reflected in their levels in serum as well as in PBMC culture supernatant that corroborated with the levels of NF kB activation in these co-infections. Increased levels of IL-1β, IL-6 and TNF-α in serum as well as in culture supernatant of LPS stimulated PBMCs in HCV-HGV co-infection compared to that in HCV infection alone indicate a perceptible contribution of pro-inflammatory response induced by HGV on HCV infection that further corroborated with the elevated level of NF-κB p65 in PBMCs in the same subgroup. On the other hand lowered levels of the pro-inflammatory cytokines in serum as well as in supernatant of PBMC culture, both correlating with NF-kB p65 level in cultured PBMC, in the subgroup with HCV-TTV co-infection compared to the subgroup with HCV infection alone suggest a possible down regulatory role played by TTV on the pro –inflammatory response induced by HCV.

Nuclear-kappa beta (NF-kB) is a transcription factor that resides in the cytoplasm of many cell types including T-lymphocytes, monocytes, macrophages, endothelial cells and smooth muscle cells. In unstimulated state, NF-kB is bound to its inhibitor i.e. the inhibitor kappa B (IkB) protein and remains in an inactive form. Following stimulation by agents e.g. virus/viral proteins, IkB is first phosphorylated by IkB kinase (IKK) and then rapidly degraded by the proteiosome releasing the NF-kB in activated form followed by its translocation into the nucleus where it binds to the DNA regulatory site to activate the genes for production of pro-inflammatory cytokines (Baldwin et al., 1996).

Activation of NF-kB is reported to be one of the key events in HCV infection towards establishment of chronicity due to its anti-apoptotic properties resulting in a chronically activated persistent state in hepatocytes eventually leading to hepatocellular carcinoma (HCC) (Hiscott, Kwon & Genin, 2001). Wu et al., (2009), in experimental rat model observed aberrantly over expression of hepatic NF-kB mRNA during the course of hepatocarcinogenesis that also corroborated with the finding of Yokoo et al., (2011) reported a constitutive activation of NF-kB in human HCC tissue samples compared to surrounding liver tissues. These studies provide collective evidence that level of NF-kB activation could be considered as a prognostic marker to predict development of HCC in HCV infection (Villanueva & Leudde, 2016). On the contrary, lowered levels of IL-1β, IL-6 and TNF-α in serum and in PBMC culture supernatant that correlated with the level of NF-kBp65 in PBMC on activation in vitro in the HCV-TTV group compared to HCV infection alone suggest a possible role played by TTV in down regulating NF-kB mediated pathway in HCV co-infected cases. It has been shown by Zheng et al., (2007) in, in-vitro experiment that TTV ORF2 (open reading frame) protein suppresses the NF-kB pathway. Incidence of HCC in thalassemics has been reported to be approximately 2 % although report in this regard is scanty possibly due relatively rapid disease course and mortality in thalassemia resulting in non-availability of subjects with long term period of follow up (Mancuso, 2010).

However, it is expected that commensurate with advancement in early diagnosis, management and prophylaxis against commonly offending organisms e.g. *S. pneumonae, H. influenza* and *N. menigities* the life expectancy of the thalassemia children will increase over the years and therefore HCC may emerge out to be one of the major causes of mortality in them. Thus, should it be the case, long term follow up study in HCV-TTV infected cases will provide information whether TTV co-infection may have any protective role in development of HCC in cases infected with HCV. There has been considerable interest evaluating inhibition of NF-kB as a promising approach for anti-cancer therapy (Godwin et al., 2013; Baud & Michael 2009).

6. Conclusion

Based on the inhibitory effect of TTV on NF-kB as evident by lowered level of NF-kB p65 in nuclear extract of LPS stimulated PBMCs in HCV-TTV infection compared to HCV monoinfection, it may be worth evaluating

various subunits of TTV as pharmacological agents for prevention or eradication of HCC as a long term sequelae in HCV infection.

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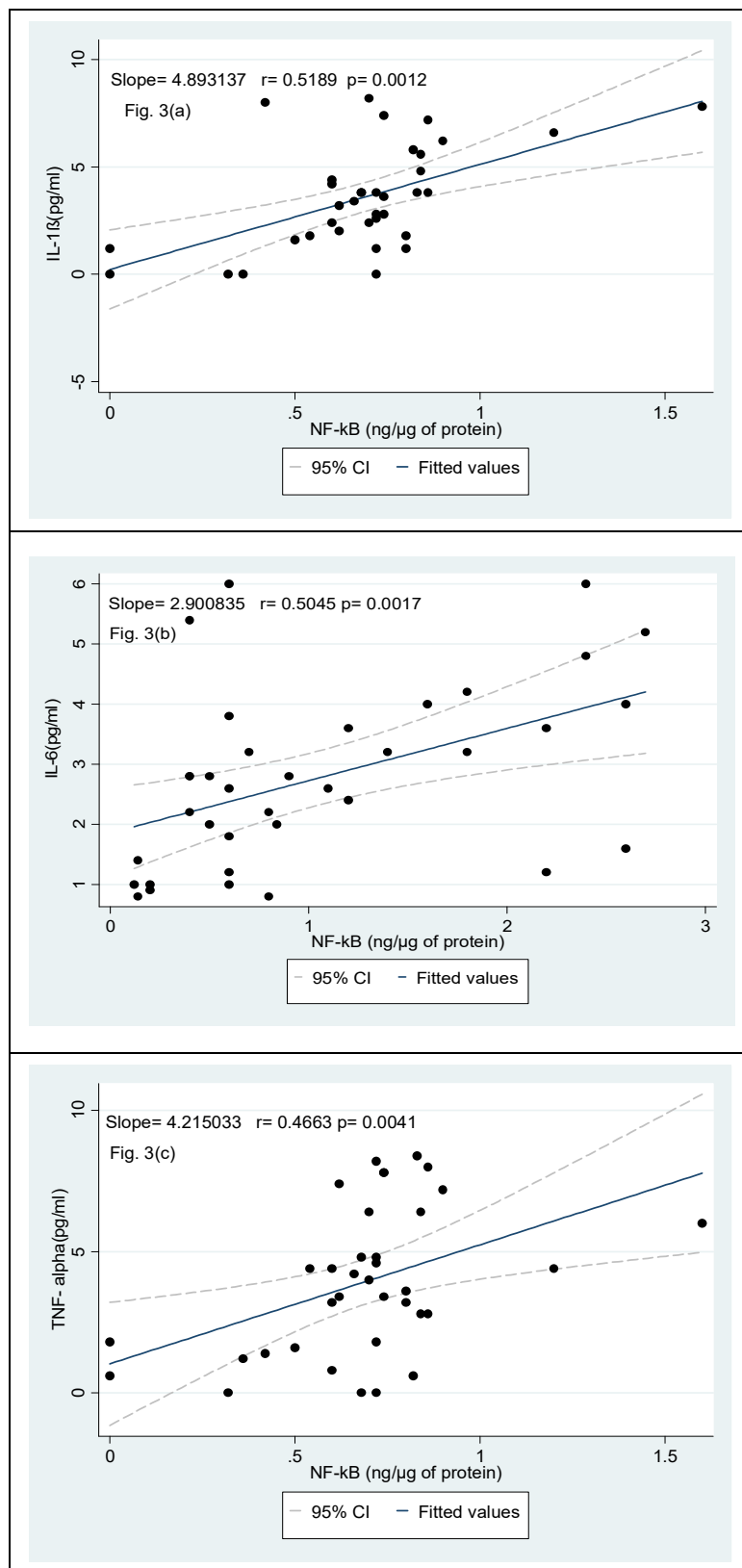
Appendix

Supplementary Figures 1(a, b and c)
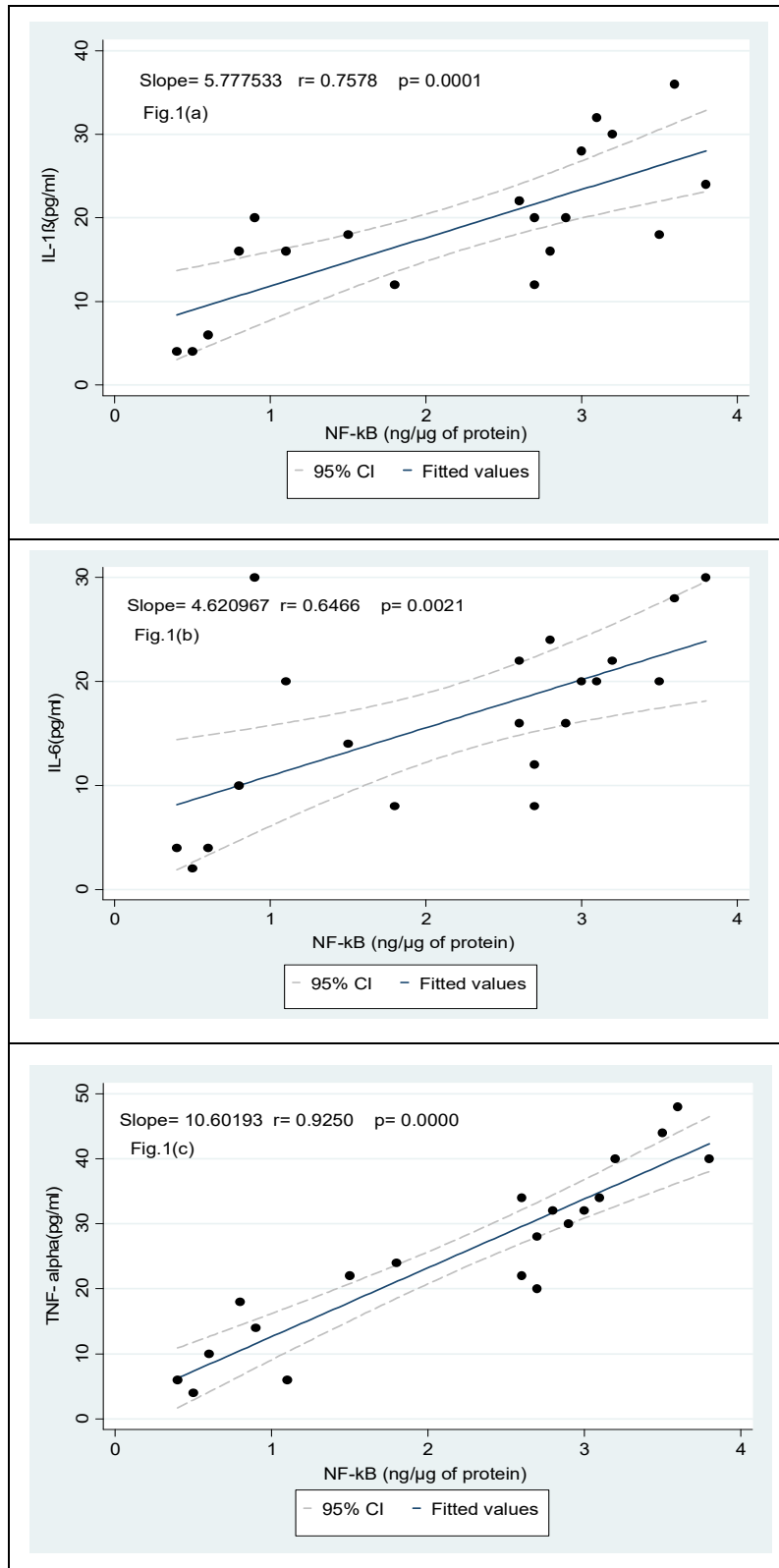
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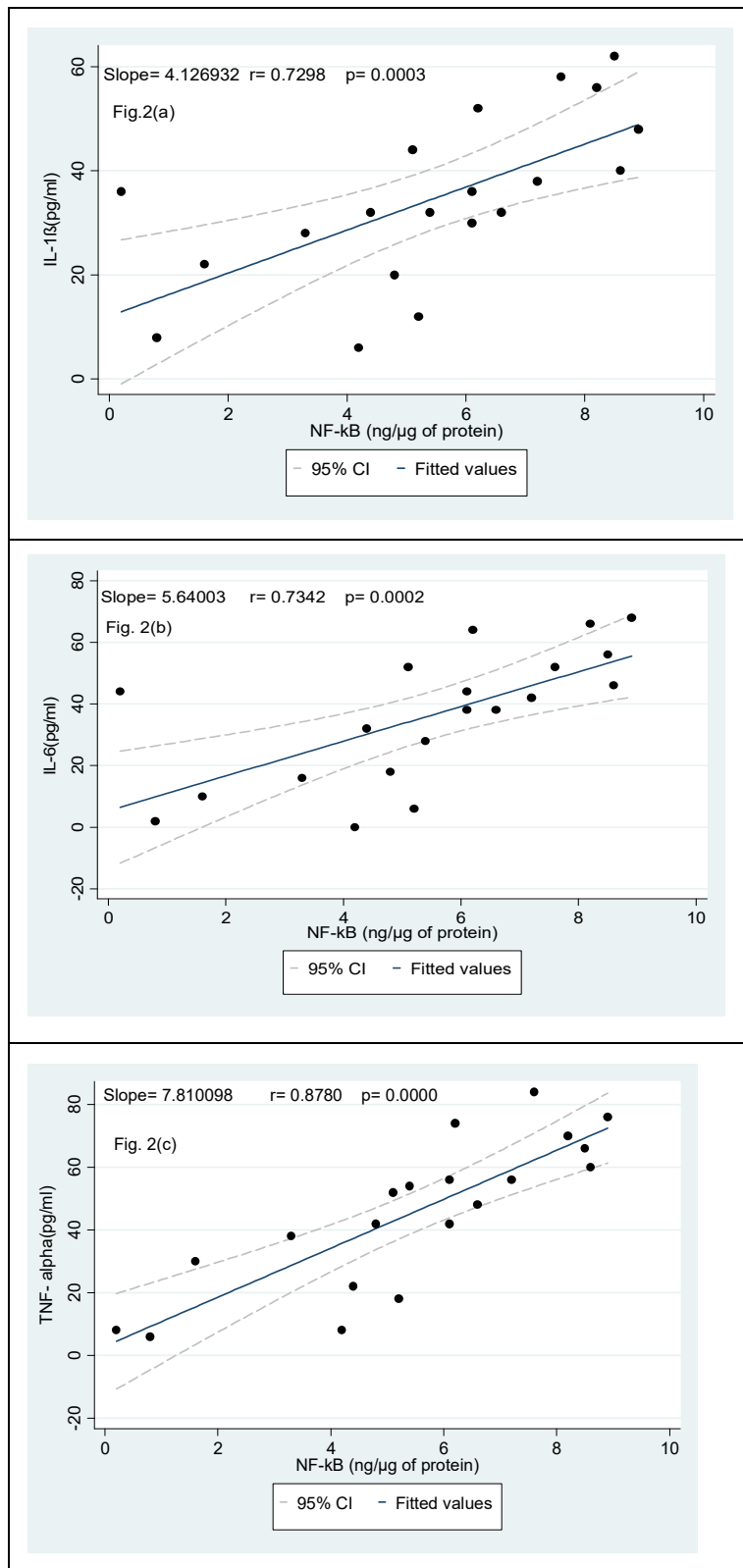
Supplementary Figures 3(a, b and c)

Correlations between levels of NF-kB p65 in nuclear extracts and levels of pro-inflammatory cytokines viz. IL-1β, IL-6 and TNF-α in supernatants of LPS stimulated PBMC cultures from subgroups of thalassemic subjects positive for various viral markers.

1 (a) to 1(c): positive for HCV only (n=20); 2(a) to 2(c) positive for HCV and HGV (n=20) ; 3(a) to 3 (c) positive for HCV and TTV (n=36)