

# Association of Toll-like receptor (TLR) 3 & 9 genes' polymorphism with Hepatitis C virus-specific cell-mediated immunity outcome among Egyptian healthcare workers

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## Abstract

Immune response variations could define successful resistance to Hepatitis C Virus (HCV) infection. Toll-like receptors (TLR)-3 are innate detectors of dsRNA viruses while bacterial and viral unmethylated CpG motifs are recognized by TLR9. We previously reported that TLR3.rs3775290 "CC" genotype was associated with HCV chronicity, while TLR9 gene played no major role in this infection. This study identified the role of TLR3.rs3775290 (c.1377C/T), TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) gene polymorphisms in predicting the outcome of HCV-specific cell-mediated immunity (CMI) among Egyptian healthcare workers (HCWs) and patients. We enrolled 546 subjects (409 HCWs and 137 patients) divided into four groups. Group1: 265 seronegative, aviraemic subjects; group2: 25 seronegative, viraemic subjects; group3: 87 subjects with spontaneously resolved HCV infection; and group4: 169 chronic HCV HCWs and patients. All subjects were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis for the TLR3.rs3775290, TLR9.rs5743836 and TLR9.rs352140 SNPs. We, also, quantified HCV-specific CMI in 265 HCWs distributed among the four groups using an interferon gamma (IFN- $\gamma$ ) enzyme-linked immunospot (ELISpot) assay in response to nine HCV genotype 4a overlapping 15mer peptide pools covering the whole viral genome. No statistically significant difference was found between CMI responding subjects with different HCV states and TLR3.rs3775290 genotype or TLR9.rs352140. However, there was a significant relationship between the outcome of the HCV-specific CMI and the TLR9.rs5743836 genotype among the responding subjects ( $p=0.005$ ) and the chronic HCV patients ( $p=0.044$ ). In conclusion, TLR9.rs5743836 SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

**Association of Toll-like receptor (TLR) 3 & 9 genes' polymorphism with Hepatitis C virus-specific cell-mediated immunity outcome among Egyptian healthcare workers**

**Running title : TLR3&9 SNPs in HCV-infected Egyptians**

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**Abbreviations:** CMI, Cell-mediated immunity; HCV, hepatitis C virus; HCWs, healthcare workers; TLR, toll-like receptor

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### Summary:

Immune response variations could define successful resistance to Hepatitis C Virus (HCV) infection. Toll-like receptors (TLR)-3 are innate detectors of dsRNA viruses while bacterial and viral unmethylated CpG motifs are recognized by TLR9. We previously reported that TLR3.rs3775290 “CC” genotype was associated with HCV chronicity, while TLR9 gene played no major role in this infection. This study identified the role of TLR3.rs3775290 (c.1377C/T), TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) gene polymorphisms in predicting the outcome of HCV-specific cell-mediated immunity (CMI) among Egyptian healthcare workers (HCWs) and patients. We enrolled 546 subjects (409 HCWs and 137 patients) divided into four groups. Group1: 265 seronegative, aviraemic subjects; group2: 25 seronegative, *viraemic* subjects; group3: 87 subjects with spontaneously resolved HCV infection; and group4: 169 chronic HCV HCWs and patients. All subjects were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis for the TLR3.rs3775290, TLR9.rs5743836 and TLR9.rs352140 SNPs. We, also, quantified HCV-specific CMI in 265 HCWs distributed among the four groups using an interferon gamma (IFN- $\gamma$ ) enzyme-linked immunospot (ELISpot) assay in response to nine HCV genotype 4a overlapping 15mer peptide pools covering the whole viral genome. No statistically significant difference was found between CMI responding subjects with different HCV states and TLR3.rs3775290 genotype or TLR9.rs352140. However, there was a significant relationship between the outcome of the HCV-specific CMI and the TLR9.rs5743836 genotype among the responding subjects ( $p = 0.005$ ) and the chronic HCV patients ( $p = 0.044$ ). In conclusion, TLR9.rs5743836

SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

## Introduction

Immune response variations may help define successful resistance to Hepatitis C virus (HCV) infection particularly among seronegative healthcare workers, reviewed elsewhere [1]. Toll-like receptors (TLR)-3 are innate detectors of dsRNA of viruses while TLR9 recognizes bacterial and viral unmethylated CpG motifs. HCV virions bind to the cell surface and get into the cell through receptor-mediated endocytosis. The structure of HCV with different parts are recognized by different TLRs. The core and non-structural (NS) proteins are vital sequences recognized by pattern recognition receptors (PRRs), including TLRs. They are, also, important inhibitors of TLR signalling [2, 3]. HCV core and NS proteins are important pathogen-associated molecular patterns (PAMPs) for TLR2, TLR3, TLR4, TLR7, 8, and 9. TLR3 is critical for its antiviral immune actions, as it stimulates non-parenchymal liver to regulate HCV replication through the synthesis of interferon (IFN)- $\beta$  [4, 5]. In chronic HCV infections, TLR3 mRNA is significantly increased in monocytes [6]. An IFN-responsive element has been identified in the TLR3 gene promoter region and, so, it appears likely that expression TLR3 is responsive to IFN treatment in HCV infection [7]. Myeloid dendritic cells (mDCs) have ordinary operative TLR3 and are capable of producing interleukin (IL)-12, IL-6, IL-10, IFN- $\gamma$ , and tumour necrosis factor (TNF)- $\alpha$  with TLR3 stimulation regardless of HCV infection [8].

HCV viral proteins activate TLR signalling, which plays a significant role in viral immune clearance. However, HCV can at the same time evade immune clearance by targeting and ruining TLR signal transduction via numerous mechanisms. First, HCV intervenes with signalling via the TIR-domain-containing adapter-inducing IFN $\beta$ /TANK-binding kinase (TRIF)/TBK1-Interferon regulatory factor 3 (IRF3) pathway. The HCV NS3 protein induces degradation of TRIF, while the NS3/4A protein prevents IRF3 and NF $\kappa$ B activation by downgrading the amount of TRIF in serum and by producing cleavage products with dominant-negative action [4, 9]. NS3/4A, also, interacts directly with TBK1 to reduce the interaction between TBK1 and IRF3 and, so inhibit IRF3 activation [10]. HCV, also, interferes with the TLR-MyD88 (Myeloid differentiation primary response 88) pathway through NS5A interaction with MyD88 to prevent Interleukin-1 receptor associated kinase 1 (IRAK1) recruitment and cytokine production in response to ligands for TLR2, TLR4, TLR7, and TLR9 [11].

HCV has been shown to regulate the expression of TLR9 through the transcription factor (Elk-1), which is an essential signal integration point between T-Cell Receptor (TCR) and CD28 in T helper 1 (Th1) cell activation [12]. HCV, also, impedes the production of IFN- $\alpha$  and IFN- $\beta$  through TLR9 and decreases human leukocyte antigen DR (HLA-DR) expression by pDCs, linked to diminished activation of naïve T cells [13]. TLR9 signalling in mDCs is unchanged [8, 13]. Consequently, it is clear that compartmentalization of the outcomes on TLR function is a crucial strategy by which HCV can escape immune surveillance yet can lead to chronic inflammatory hepatic injury and fibrosis.

We previously showed that TLR3.rs3775290 “CC” genotype was associated with HCV chronicity, while TLR9 gene played no major role in HCV infection [14]. This study identified the role of TLR3.rs3775290 (c.1377C/T), TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) single nucleotide polymorphisms (SNP) in predicting the outcome of HCV-specific cell-mediated immunity (CMI) among Egyptian healthcare workers (HCWs) and patients. We show that TLR9.rs5743836 SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

## Subjects and Methods

### Study subjects

Two hundred sixty-five HCWs were selected for the evaluation of IFN- $\gamma$  producing T-cells by an Enzyme Linked Immuno-Spot (ELISpot) assay in response to nine HCV genotype 4a overlapping 15mer peptide pools covering the whole viral genome. The NLI-Institutional Review Board (IRB) approved the study protocol,

and all subjects had given informed consent prior to enrolment. The demographic characteristics of the participants were previously reported [14].

## Laboratory testing

### HCV molecular diagnosis and quantification of HCV-RNA

HCV diagnosis was determined by using the Murex third generation anti-HCV ELISA assay (version 4.0 USA) that utilizes antigens from the core, NS3, NS4 and NS5 regions of the virus. All the study subjects were tested by quantitative real-time RT-PCR (qPCR) for detection of current HCV infection and quantification of the HCV viral load using QIAamp® Viral RNA purification kit (QIAGEN, USA). The amplification step was conducted using AgPath-ID one-step assay as instructed by the manufacturer (Applied Biosystems-Life Technologies Corporation, CA).

### HCV-genotyping and genotyping for TLR3.rs3775290 (c.1377C/T) SNP

PCR-RFLP was used for HCV genotyping after reverse transcription as described [15].

PCR-RFLP was, also, used for the identification of the TLR3.rs3775290 (c.1377 C/T) gene SNPs. PCR was conducted in a total of 25  $\mu$ l reaction volume with 20 pmol of each of the forward and reverse primers as described [16].

### Genotyping for TLR9.rs5743836 (-1237T-C) and TLR9.rs352140 (G2848A) SNPs

The total reaction volume of TLR9.rs5743836 (-1237TTBC) PCR was 25  $\mu$ l with 20 pmol of each of the forward and reverse primers as described [17]. Also, the total reaction volume of TLR9.rs352140 (G2848A) PCR was 25  $\mu$ l with 20 pmol of each of the forward and reverse primers as reported [16].

### Εαλυατιον οφ ΙΦΝ-γ προδουσιγγ σελλε υσιγγ ΕΛΙΣΠΟΤ ασσαψ

An ELISPOT assay was used to quantify IFN- $\gamma$  synthesis by PBMC separated from the study subjects in response to nine HCV genotype 4a isolate ED43 peptide antigen pools consisting of 15 (15mer) and overlapping by 10 amino acids. These were 600 peptides combined in nine pools corresponding to all the HCV proteins. These synthetic peptides were specially manufactured by Mimotopes (Australia). About 15ml of blood was collected in EDTA vacutainer tubes (Becton Dickinson Biosciences, NJ, USA). PBMC were separated from whole blood using Ficoll-Hypaque density gradient centrifugation. Cellular viability was determined by trypan blue exclusion method. Briefly,  $2 \times 10^5$  PBMC (200 $\mu$ l/well) were incubated in triplicate cultures in the ELISpot plates (Whatman Unifilter, USA) coated with anti-human IFN $\gamma$  antibody and incubated for ~16 hours with or without recombinant HCV antigens at 3 $\mu$ g/ml of each single peptide in complete RPMI-1640 medium. A medium containing DMSO alone and 0.1 $\mu$ g / ml of SEB (or other polyclonal stimuli) represented the negative and positive controls, respectively. The assay was developed at the end of the incubation period until the spots appeared. The wells were, then, rinsed with tap water to stop the reaction. The number of spots/well was calculated using an automated ELISpot reader (Cellular Technology Ltd., Cleveland, USA) as reported [18]. The mean number of spot forming cells (SFC) in control wells were subtracted from the mean number of peptide-stimulated wells to correct for background cytokine production and are expressed as SFC/million PBMC. A positive antigen-specific HCV response was considered if the SFC in the presence of antigen was at least three times the number of SFCs in the medium control and > 55 SFC / million PBMC were present, as previously reported [19].

### Statistical analysis

Allele and genotype frequencies of each SNP were calculated by direct counting. Genotype and allele frequencies between groups were compared using the  $\chi^2$  test and Fisher's exact test when appropriate. For allelic associations, p values with Yates correction and odds ratio (OR) with 95% confidence intervals (CI) were calculated using Statcalc program (Epiinfo version 6.0.4, CDC, Atlanta, GA). Pair-wise LD ( $D'$ ,  $r^2$ ) was estimated by use of Haploview software version 4.2. For categorical data, chi-square tests were performed, while the student's  $t$  -test (or Mann Whitney U test where appropriate) was performed to compare the

continuous data. Differences in mean with a  $p$  value  $<0.05$  were deemed significant. Standard errors of the means (and/or deviations) were also calculated

## Results

### Ρελατιονσηπ βετωεεν της ΤΑΡ3.ρσ3775290 (ς.1377<sup>α</sup>/Τ) γενοτυπε ανδ της Η<sup>α</sup>-σπεσιφικς ΙΦΝ-γ ΕΛΙΣποτ ρεσπονσε

All subjects enrolled into the study were tested for IFN- $\gamma$  production by PBMCs using ELISpot assay to evaluate CMI response against nine HCV genotype 4a isolate ED43 peptide antigen pools composed of 15 (15mer) and overlapping by 10 amino acids. Only 140 (53%), 20 (80%), 35 (40%) and 70 (41.4%) samples had valid CMI data among the four study groups (seronegative, aviraemic subjects; seronegative, *viraemics* subjects; spontaneously resolved subjects and chronic HCV patients groups, respectively).

There was no association between the results of the HCV-specific immune response and the TLR3.rs3775290 genotype among the 263 HCWs with valid CMI responses and TLR3.rs3775290 genotyping tests ( $p = 0.222$ ). Also, there was a significantly ( $p = 0.004$ ) higher proportion of the CC genotype (73.3%) in responding subjects than in non-responding seronegative, aviraemic individuals (65.1%). In addition, there was a differential distribution of the three TLR3.rs3775290 genotypes among the total study participants with and without HCV-specific CMI (Table 1).

The average totals ( $\pm$ SEM) of IFN- $\gamma$  responses measured in SFC per million PBMCs in the *responding* total subjects were  $455 \pm 90$ ,  $507 \pm 122$ , and  $317 \pm 45$  with the TLR3.rs3775290 genotype CC (n=37), CT (n=16) and TT (n=6). On the other hand, among the *non-responding* subjects, the average totals ( $\pm$ SEM) of IFN- $\gamma$  SFC per million PBMCs in total subjects were  $29 \pm 5$ ,  $23 \pm 5$ , and  $49 \pm 19$  with the TLR3.rs3775290 genotype CC (n=130), CT (n=65) and TT (n=9), respectively. There was no significant difference between the responding total subjects with different TLR3.rs3775290 genotypes ( $p = 0.89$ ; Figure 1A).

For the *seronegative*, *aviraemic* subjects, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the responding subjects were  $404 \pm 97.7$ ,  $1058 \pm 807.5$ , and  $249 \pm 21.9$ , among those with the TLR3.rs3775290 CC genotype (n=22), CT (n=4) and TT (n=4) genotypes, respectively. Differences were not statistically significant ( $p = 0.88$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$  SEM) of the non-responding subjects were  $26.7 \pm 5.7$ ,  $16.7 \pm 4$ , and  $13.3 \pm 13.3$ , among those with the TLR3.rs3775290 CC (n=71), CT (n=36) and TT (n=2) genotypes, respectively (Figure 1B).

Regarding *seronegative*, *viraemic* subjects, the average totals ( $\pm$ SEM) of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs for *responding* subjects were  $657 \pm 443$ , and 163, among those with the TLR3.rs3775290 genotype CC (n=3) and CT (n=1) genotypes, respectively. There were no responding subjects with the TT genotype. Differences were not statistically significant ( $p = 0.18$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the non-responding subjects were  $42 \pm 22$ ,  $30 \pm 11$  and  $8 \pm 8$ , among those with the TLR3.rs3775290 CC (n=9), CT (n=5) and TT (n=2) genotypes, respectively (Figure 1C).

For *spontaneously resolved subjects*, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the *responding* subjects were  $793 \pm 605.8$ , and  $325 \pm 121$ , among those with the TLR3.rs3775290 CC (n=2), CT (n=5) genotypes, respectively. No TT genotype was recorded in this category. Differences were not statistically significant ( $p = 0.698$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the non-responding subjects were  $51 \pm 21$ ,  $15 \pm 12$  and 58, among those with the TLR3.rs3775290 CC (n=14), CT (n=12) and TT (n=1) genotypes, respectively (Figure 1D).

For *chronic HCV patients*, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the *responding* subjects were  $439 \pm 214$ ,  $349 \pm 181$ , and  $453 \pm 9$ , among those with the TLR3.rs3775290 CC (n=10), CT (n=6) and TT (n=2) genotypes, respectively. Differences were not statistically significant ( $p = 0.228$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the non-responding chronic HCV patients were  $26 \pm 8$ ,  $38 \pm 13$ , and  $84 \pm 36$ , among those with the TLR3.rs3775290 CC (n=36), CT (n=13) and TT (n=4) genotypes, respectively (Figure 1E).

## Relationship between the TLR9.rs5743836 (-1237T-C) genotype and the HCV-specific IFN ELISpot response

There was no association between the results of the HCV-specific CMI response and the TLR9.rs5743836 genotype among the 265 HCWs with valid CMI responses and TLR9.rs5743836 genotyping tests (Table 2,  $p=0.12$ ). However, there was a statistically significant ( $p=0.019$ ) lower proportion of the TT genotype in responding spontaneously resolved individuals compared to non-responding spontaneously resolved subjects (20% Vs 80%, respectively, Table 2).

The average totals ( $\pm$ SEM) of IFN- $\gamma$  responses measured in SFC per million PBMCs in the responding total subjects were  $624\pm125$ , and  $381\pm100$  with the TLR9.rs5743836 CT ( $n=18$ ) and TT ( $n=41$ ) genotypes, respectively. There were no responding subjects with the CC genotype. Additionally, among the non-responding subjects, the average totals ( $\pm$ SEM) of IFN- $\gamma$  SFC per million PBMCs in total subjects were  $40\pm23$ ,  $40\pm10$ , and  $26\pm4$ , among those with the TLR9.rs5743836 CC ( $n=4$ ), CT ( $n=40$ ) and TT ( $n=162$ ), respectively. There was a significant difference between the responding total subjects with different TLR9.rs5743836 genotypes ( $p=0.005$ ; Figure 2A).

For the *seronegative, aviraemic* subjects, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the *responding* subjects were  $617\pm192$ , and  $398\pm164$ , among those with the TLR9.rs5743836 CT ( $n=10$ ) and TT ( $n=20$ ) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were not statistically significant ( $p=0.159$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the non-responding seronegative, aviraemic subjects were  $32\pm31$ ,  $44\pm14$ , and  $18\pm3$ , among those with the TLR9.rs5743836 CC ( $n=3$ ), CT ( $n=25$ ) and TT ( $n=82$ ) genotypes, respectively (Figure 2B).

For *seronegative, viraemic* subjects, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the *responding* subjects were 238, and  $632\pm455$ , among those with the TLR9.rs5743836 CT ( $n=1$ ) and TT ( $n=3$ ) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were not statistically significant ( $p=0.654$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the non-responding subjects were 65,  $18\pm14$ , and  $38\pm18$ , among those with the TLR9.rs5743836 CC ( $n=1$ ), CT ( $n=4$ ) and TT ( $n=11$ ) genotypes, respectively (Figure 2C).

For *spontaneously resolved subjects*, the average total HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ ) of the *responding* subjects was  $795\pm345$ , and  $206\pm49$ , among those with the TLR9.rs5743836 CT ( $n=3$ ) and TT ( $n=4$ ) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were not statistically significant ( $p=0.077$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ ) of *non-responding subjects* were 1, and  $36\pm12$ , among those with the TLR9.rs5743836 CT ( $n=1$ ) and TT ( $n=27$ ) genotypes, respectively. Also, there were no subjects with the CC genotype among non-responding subjects (Figure 2D).

For *chronic HCV patients*, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$  SEM) of the *responding* subjects were  $610\pm212$ , and  $354\pm156$ , among those with the TLR9.rs5743836, CT ( $n=4$ ) and TT ( $n=14$ ) genotypes, respectively. There were no responding subjects with the CC genotype. Differences were statistically significant ( $p=0.044$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the *non-responding chronic* HCV subjects were  $43\pm19$  and  $32\pm8$ , among those with the TLR9.rs5743836 CT ( $n=10$ ) and TT ( $n=42$ ) genotypes, respectively. There were no subjects with the CC genotype among the non-responding chronic patients (Figure 2E).

## Ρελατιονσηη βετωεεη ηηε ΤΑΡ9.ρσ352140 (Γ2848Α) γεηοηψπε αηδ ηηε Η<sup>η</sup>-σπεεεεεε ΙΦΝ-γ ΕΛΙΣποη ρεεποηεε

There was no association between the results of the HCV-specific CMI response and the TLR9.rs352140 (G2848A) genotype among the 258 HCWs with valid CMI responses and TLR9.rs352140 genotyping tests ( $p=0.356$ ). Also, there was no significant difference ( $p=0.299$ ) in the proportion of the GG genotype in responding (46.4 %) and in the non-responding seronegative aviraemic subjects (31.4 %; Table 3).

The average totals ( $\pm$ SEM) of IFN- $\gamma$  responses measured in SFC per million PBMCs in the responding total

subjects were  $587 \pm 176$ ,  $390 \pm 85$ , and  $327 \pm 45$ , among those with the TLR9.rs352140 GG (n=24), GA (n=23) and AA (n=10) genotypes, respectively. On the other hand, among the non-responding subjects, the average totals ( $\pm$ SEM) of IFN- $\gamma$  SFC per million PBMCs in the total subjects were  $30 \pm 6$ ,  $31 \pm 6$ , and  $26 \pm 7$ , among those with the TLR9.rs352140 (G2848A) GG (n=66), GA (n=86) and AA (n=49) genotypes, respectively. There was no significant difference between the responders of the total subjects with the TLR9.rs352140 genotypes ( $p = 0.652$ ; Figure 3A).

For the *seronegative, aviraemic* subjects, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the *responding* subjects was  $672 \pm 278.8$ ,  $307 \pm 86$ , and  $357 \pm 68$ , among those with the TLR9.rs352140 GG (n=13), GA (n=10) and AA (n=5) genotypes, respectively. Differences were not statistically significant ( $p = 0.594$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) among the *non-responding seronegative, aviraemic* subjects were  $23 \pm 7$ ,  $31 \pm 8$ , and  $17 \pm 5$ , among those with the TLR9.rs352140 GG (n=33), GA (n=43) and AA (n=29) genotypes, respectively (Figure 3B).

For *seronegative, viraemic* subjects, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the responding subjects were  $214 \pm 24.2$ , and  $853 \pm 689$ , among those with the TLR9.rs352140 GG (n=2) and GA (n=2) genotypes, respectively. There were no responding subjects with the AA genotype. Differences were not statistically significant ( $p = 1.000$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) were  $12 \pm 2.22$ ,  $30 \pm 10.4$ , and  $60 \pm 47.6$ , among the *non-responding seronegative, viraemic* subjects with the TLR9.rs352140 (G2848A) GG (n=3), GA (n=9) and AA (n=4) genotypes, respectively (Figure 3C).

For *spontaneously resolved subjects*, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the *responding* subjects were  $244 \pm 48.2$ , and  $619 \pm 301$ , among those with the TLR9.rs352140 GG (n=3) and GA (n=4) genotypes, respectively. There were no responding subjects with the AA genotype. Differences were not statistically significant ( $p = 1.000$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) were  $26 \pm 14.1$ ,  $60 \pm 26.9$ , and  $10 \pm 10$ , among the *non-responding spontaneously resolved subjects* with the TLR9.rs352140 GG (n=11), GA (n=10) and AA (n=7) genotypes, respectively (Figure 3D).

For *chronic HCV patients*, the average totals of HCV-specific IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of the responding subjects were  $698 \pm 357.2$ ,  $245 \pm 51.5$ , and  $296 \pm 57.8$ , among those with the TLR9.rs352140 GG (n=6), GA (n=7) and AA (n=5) genotypes, respectively. Differences were not statistically significant ( $p = 0.775$ ). On the other hand, the average totals of IFN- $\gamma$  SFC/ $10^6$  PBMCs ( $\pm$ SEM) of *non-responding chronic HCV subjects* were  $46 \pm 13.8$ ,  $18 \pm 7.9$ , and  $50.9 \pm 23.1$ , among those with the TLR9.rs352140 GG (n=19), GA (n=24) and AA (n=9) genotypes, respectively (Figure 3E).

## Discussion

In this study, there was no statistically significant difference between CMI responding individuals in Egyptian HCWs with diverse HCV states with different TLR3.rs3775290 (c.1377C/T) genotypes. Also, there was no association between the results of the HCV-specific CMI response and the TLR3.rs3775290 (c.1377C/T) genotype among the 263 subjects tested. This agrees with another report [20]. TLR3's function was investigated in four different viral models of infection in TLR3-/- mice, including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), Murine Cytomegalovirus (MCMV), and reovirus. The researchers found that TLR3 is not always required for effective antiviral responses to be generated, as the absence of TLR3 did not alter either viral pathogenesis or the generation of adaptive antiviral responses to these viruses by the host. Remarkably, intracellular transduction of poly I: C signals begins TLR3-independent activation of an IFN response, therefore, restricting the role of TLR3 in the IFN pathway [21]. Other reports [22, 23] indicate that HCV may use TLR3 pathway to escape immune scrutiny through HCV NS3/4A protease-mediated cleavage of the TLR3 adaptor protein TRIF. Also, HCV NS3/4A impedes RIG-I, which is a key factor in TRIF-independent signalling [21]. In addition, it was suggested that several polymorphisms that alter TLR3 amino acids initiate resulting protein changes and that gene expression may be down-regulated while TLR3 activities required for proper signalling may be reduced. [24]. Lower TLR3 activity results in inability to identify the invading microorganisms and consequently insufficient immune responses. This increases

the risk of infections and infectious diseases [25].

Our results provide strong indirect evidence that TLR9 could play a greater role than previously expected in HCV infection. We identified an association between the TLR9.rs5743836 polymorphism within the TLR9 gene and the natural history of HCV infection. In this study, there was a strong association between the results of the HCV-specific CMI response and the TLR9.rs5743836 (-1237T-C) genotype among the 265 subjects with valid CMI responses in total responders of individual HCWs ( $p=0.005$ ). Also, there was a statistically significant difference between responding individuals in chronic HCV patients with distinct TLR9.rs5743836 genotypes ( $p = 0.044$ ).

We show that the TLR9-1237T allele was significantly associated with HCV-specific CMI response. In this regard, the TT genotype is transcribed more effectively than the CC genotype as reported by others [26] who showed that the wild-type construct stimulates higher transcriptional activity compared to the CC allele. The data showed that individuals with the “favourable” TT allele would have HCV-specific CMI response when compared to those with the “unfavourable” CC allele. Also, several reports found that the mutant allele “T” imparts the immunity against the various pathogens. However, statistically inconsiderable, the mutant allele was declared to be linked with the depressed microbial load in Africans [27-29]. In addition, the mutant allele T manipulate immunity against establishing the infections, which is convincing that the TLR9 gene had bear the influence of genetic assortment to cope with the infections [30]. Functional analyses of the impact of TLR9 polymorphisms on basal promoter activity, on the other hand, revealed that the rs5743836 SNP causes higher gene expression than the wild type promoter. The findings of others [31] could explain the increased promoter activity of TLR9.rs573836. That study [31] showed that the variant C allele at rs5743836 establishes a potential NF- $\kappa$ B-binding site that increases the gene’s transcriptional activity . The presence of this extra putative NF- $\kappa$ B-binding site promotes transcription of TLR9 more effectively than the wild type TLR9.rs5743836 T sequence in response to different stimuli. This finding agrees with another report [32], where TLR9 promoter SNPs were associated with the natural course of HCV infection and exhibit higher transcriptional activities and imply the role of the DNA sensor TLR9 in natural immunity against HCV. Our data are analogous to other studies [1, 33], where the rs5743836 TT variant was reported to have a greater promoter activity than the CC genotype. That study suggested that this activity could lead to increased pro-inflammatory cytokine production during malaria infection, leading to successful malaria control and elimination [33].

There was no association between the results of the HCV-specific immune response and the TLR9.rs352140 (G2848A) genotype among the 258 Egyptian HCWs with valid CMI responses and TLR9.rs352140 genotyping. There have been no studies to date that indicate an association between TLR9.rs352140 (G2848A) and HCV infection. The region around 2848 is the major coding region of the TLR9 protein [34, 35] and a TLR9 2848 GA genotype polymorphism has been reported at the transcriptional level to reduce TLR9 expression [35, 36]. Down regulated TLR9 could reduce the functions of the innate immune response against HCV infection. Our results are related to another study that indicates that the TLR9 gene may play a role in cervical carcinogenesis but has a minor (or no) role in tumour progression where individuals with the favourable” GG allele were reported to be more frequent than the “unfavourable” AA allele [37]. In addition, a recent meta-analysis showed that there is no relationship between TLR9.rs352140 (G2848A) and cervical cancer vulnerability [38]. Our findings suggest that the TLR9.rs352140 (G2848A) genotype does not affect the natural course of HCV infection.

In conclusion, this study shows that TLR9.rs5743836 SNP; but not TLR3.rs3775290 or TLR9.rs352140 genotypes; could predict the outcome of HCV-specific CMI responses among genotype-4-infected Egyptians.

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**Author contributions** :

Conceptualization and design of experiments: SFA, SH, AO, ZZ, ER, and IW; Data collection, experimentation, analysis, and investigation: SH, SFA, MA, ZZ, IG, MS, WA; Funding acquisition and resources: SFA,

ER, and IW; Writing-original draft (SH and SFA); Writing-review and editing (SFA, AO, MS, MH, WA, MA, ER, and IW). All authors approved the final version of the manuscript.

*Compliance with Ethical Standards :*

**Conflict of Interest:** None declared.

**Data Sharing and Data Accessibility:** All data pertinent to this article are included herein.

**Research involving human participants and/or animals :**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments.

**Informed Consent :** Informed consent was obtained from all enrolled study participants.

**Table (1):** Relationship between TLR3.rs3775290 (c.1377C/T) and HCV-specific cell mediated immune (CMI) response among NLI HCWs in terms of CMI responders frequencies.

<i>p</i> -value	Total	TLR3.rs3775290 genotype		
		TT	CT	CC
0.004	30 (21.6)	4 (13.3)	4 (13.3)	22 (73.3)
	109 (78.4)	2 (1.8)	36 (33)	71 (65.1)
0.694	4 (20)	0(0)	1 (25)	3 (75)
	16 (80)	2 (12.5)	5 (31.3)	9 (56.3)
0.425	7 (20.6)	0(0)	5 (71.4)	2 (28.6)
	27 (79.4)	1 (3.7)	12 (44.4)	14 (51.9)
0.57	18 (25.7)	2 (11.1)	6 (33.3)	10 (55.5)
	52 (74.3)	4 (7.7)	12 (23.1)	36 (69.2)
<b>0.222</b>	<b>59 (22.3)</b>	<b>6 (10.2)</b>	<b>16 (27.1)</b>	<b>37 (62.7)</b>
	<b>204 (77.7)</b>	<b>9 (4.4)</b>	<b>65 (31.9)</b>	<b>130 (63.7)</b>

**Table (2) :** Relationship between TLR9.rs5743836 (-1237T-C) and HCV-specific cell mediated immune (CMI) response among NLI HCWs in terms of CMI responders frequencies.

<i>p</i> -value	Total	TLR9.rs5743836 genotype		
		TT	CT	CC
0.353	30 (21.4)	20 (66.7)	10 (33.3)	0 (0)
	110 (78.5)	82 (74.5)	25 (22.7)	3 (2.7)
0.87	4 (20)	3 (75)	1 (25)	0 (0)
	16 (80)	11 (68.8)	4 (25)	1 (6.3)
<b>0.019</b>	7 (20)	4 (57.1)	3 (42.9)	0 (0)
	28 (80)	27 (96.4)	1 (3.6)	0 (0)
0.74	18 (25.7)	14 (77.8)	4 (22.2)	0 (0)
	52 (74.3)	42 (80.8)	10 (19.2)	0 (0)
<b>0.12</b>	<b>59 (22.3)</b>	<b>41 (69.5)</b>	<b>18 (30.5)</b>	<b>0 (0)</b>
	<b>206 (77.7)</b>	<b>162 (78.6)</b>	<b>40 (19.4)</b>	<b>4 (1.9)</b>

**Table (3) :** Relationship between TLR9.rs352140 (G2848A) and HCV-specific cell mediated immune (CMI) response among NLI HCWs in terms of CMI responders frequencies.

<i>p</i> -value	Total	TLR9.rs352140 genotype	TLR9.rs352140 genotype	TLR9.rs352140 genotype	GRO
		<b>AA</b>	<b>GA</b>	<b>GG</b>	
0.299	28 (21.1)	5 (17.9)	10 (35.7)	13 (46.4)	Respo
	105 (78.9)	29 (27.6)	43 (41)	33 (31.4)	Non-r
0.32	4 (20)	0 (0)	2 (50)	2 (50)	Respo
	16 (80)	4 (25)	9 (56.3)	3 (18.8)	Non-r
0.299	7 (20)	0 (0)	4 (57.1)	3 (42.9)	Respo
	28 (80)	7 (25)	10 (35.7)	11 (39.3)	Non-r
0.627	18 (25.7)	5 (27.8)	7 (38.9)	6 (33.3)	Respo
	52 (74.3)	9 (17.3)	24 (46.2)	19 (36.5)	Non-r
<b>0.35</b>	<b>57 (22.1)</b>	<b>10 (17.5)</b>	<b>23 (40.4)</b>	<b>24 (42.1)</b>	<b>Respo</b>
	<b>201(77.9)</b>	<b>49 (24.4)</b>	<b>86 (42.8)</b>	<b>66 (32.8)</b>	<b>Non-</b>

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#### Figure legends :

**Figure (1) : Relation between TLR3.rs3775290 (c.1377C/T) genotype and total HCV-specific CMI response among total subjects (A), seronegative, aviraemic subjects (B), seronegativeviraemic subjects (C), spontaneously resolved subjects (D),and chronic HCV patients (E).** Data are shown as scatter plots. For each subject, the total cumulative number of HCV-specific IFN- $\gamma$  spot-forming cells (SFC) per  $10^6$ PBMCs is shown for responding and non-responding healthcare workers (HCWs) and patients. Error bars represent the SEM.

**Figure (2) : Relationship betweenTLR9.rs5743836 (-1237T-C) genotype and total HCV-specific CMI response among the total subjects (A), seronegative aviraemic subjects (B), seronega-**

tive *viraemic* subjects (C), spontaneously resolved subjects (D), and chronic HCV patients (E). Data are shown as scatter plots. For each subject, the total cumulative number of HCV-specific IFN- $\gamma$  spot-forming cells (SFC) per  $10^6$  PBMCs is shown for responding and non-responding healthcare workers (HCWs) and patients. Error bars represent the SEM.

**Figure (3): Relationship between TLR9.rs352140 (G2848A) genotype and total HCV-specific CMI response among the total subjects (A) seronegative, *aviraemic* subjects (B) seronegative, *viraemic* subjects (C) spontaneously resolved subjects (D) and chronic HCV patients (E).** Data are shown as scatter plots. For each subject, the total cumulative number of HCV-specific IFN- $\gamma$  spot-forming cells (SFC) per  $10^6$  PBMCs is shown for responding and non-responding HCWs and patients. Error bars represent the SEM.

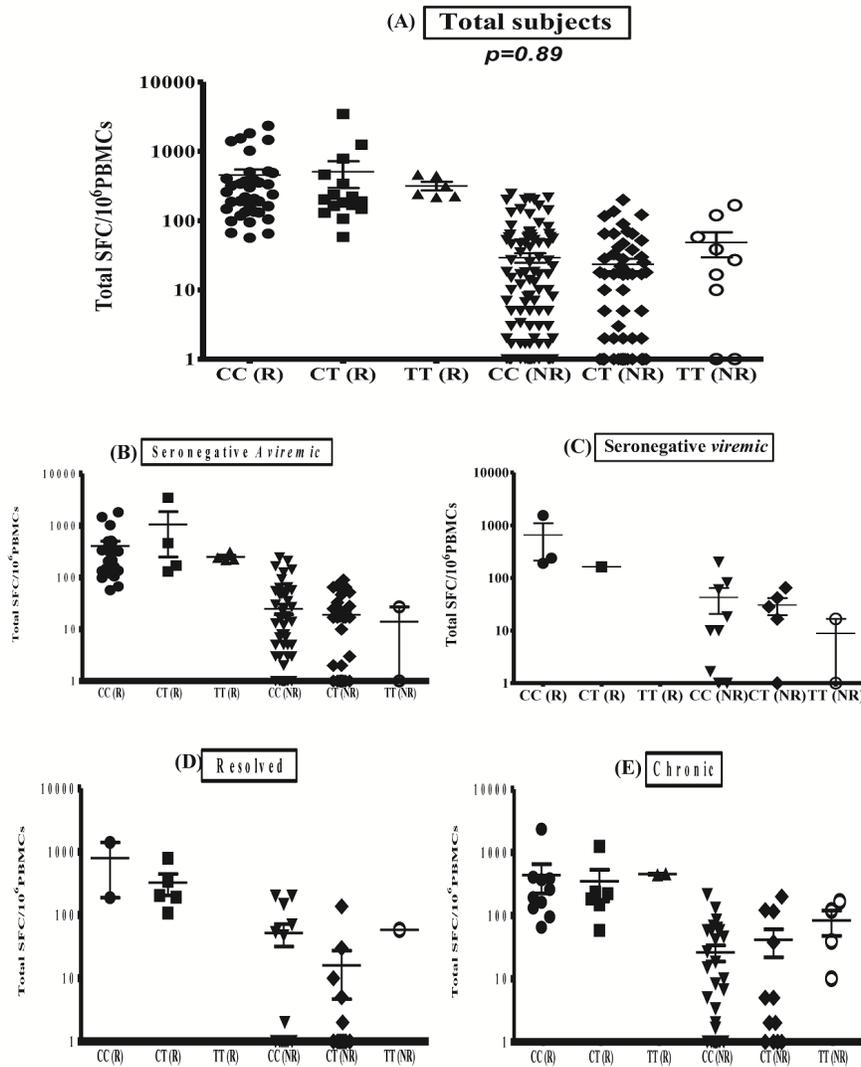


Figure 1:

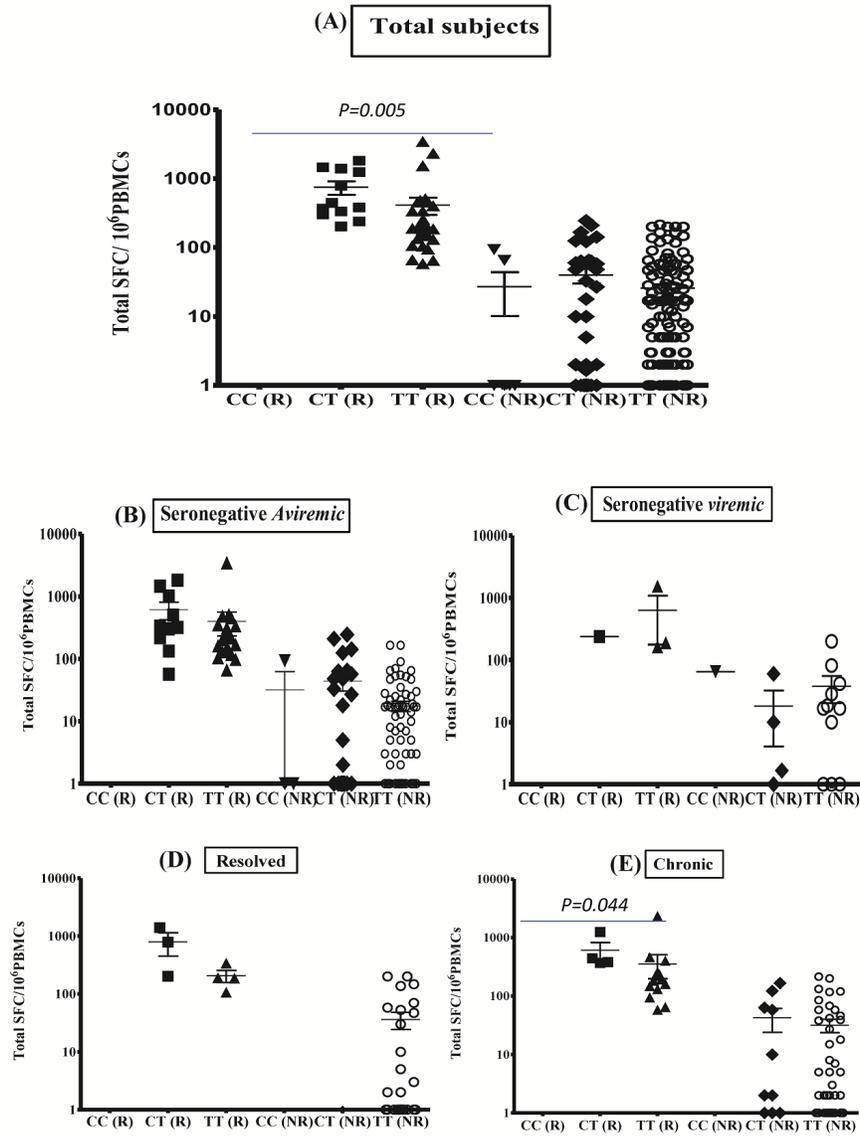


Figure 2:

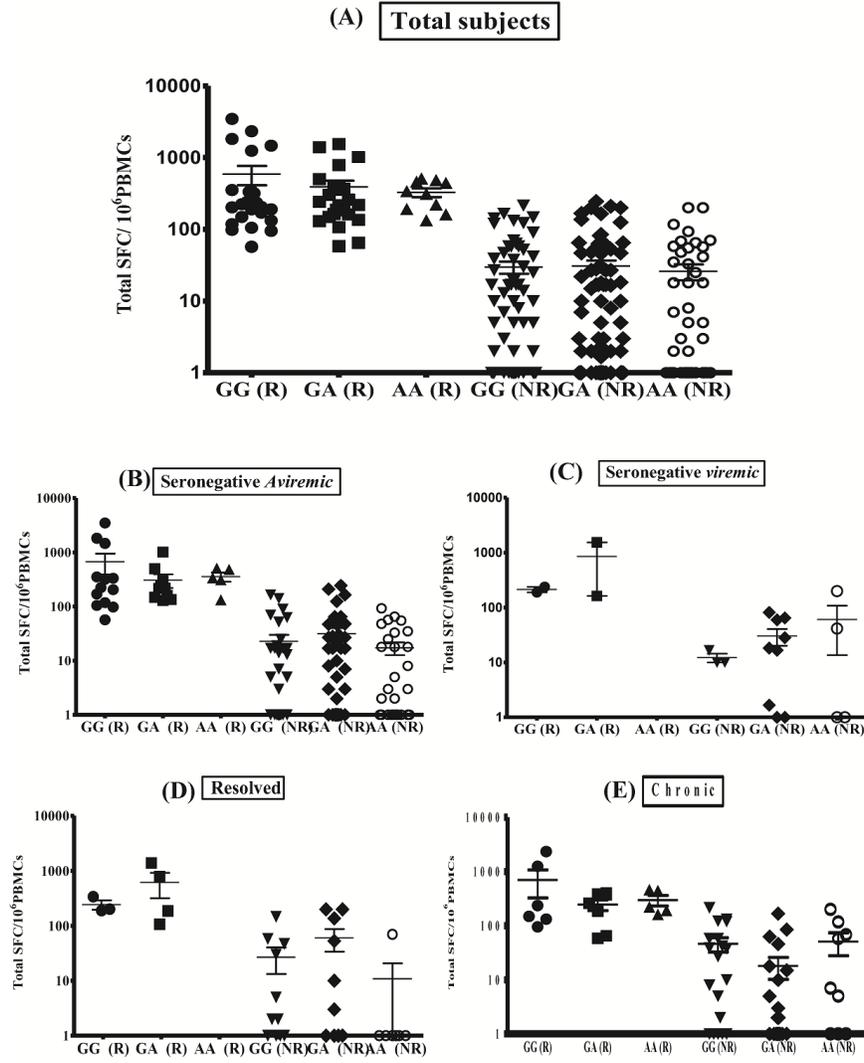


Figure 3: