

## Bacterial pathogens associated with severity of HCV infection

Nanis Gamal Allam<sup>1</sup>, Mohamed Labib Salem<sup>2</sup>, Hassan Elbataa<sup>3</sup>, Maii Moustafa Nabieh<sup>1</sup>

<sup>1</sup>Microbiology section, Botany Department, Faculty of Science, Tanta University, Tanta, Egypt.

<sup>2</sup>Immunology section, Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt.

<sup>3</sup>Hepatology, Tropical diseases medicine, Dean of Medicine faculty, Kafr elsheikh University, Egypt.

**Abstract:** Hepatitis C virus (HCV) is a serious pathogen with epidemic  $\geq 170$  millions and Egypt considered the highest, while immunocompromised chronic HCV patients suffer from serious microbial infections especially bacteria. **Aim:** investigation of common bacterial infections in patients with chronic HCV in Egypt and its association with HCV severity. **Methods:** This clinical study was conducted on chronic HCV patients and healthy individuals, while urine and blood samples were collected under optimized conditions. Bacteria were isolated from urine samples and identified using VETIK2 system. Flow cytometry was used for CD56<sup>+</sup> and CD3<sup>+</sup> levels detection. Alanine/Aspartate aminotransferase (ALT, AST) levels were detected biochemically, enzyme-linked immunosorbent assay (ELISA) was used for diacylglycerol acyltransferase-1 (DGAT1) level detection and HCV PCR was performed for viral load detection. **The results:** showed that *Staphylococcus lentus*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Eschirechia coli* were the common bacterial infections. The slight reduction in CD3<sup>+</sup> and CD56<sup>+</sup> levels were coincident with the increase of Alt, AST and DGAT1 levels as patients suffered from high HCV load. **Conclusion:** HCV patients are immunocompromised and suffer from serious secondary bacterial infections which increased the severity of HCV infection.

**Key words:** Hepatitis C HCV; *Lactobacillus acidophilus* and *Bifidobacterium bifidum*; CD3<sup>+</sup>; CD56<sup>+</sup>; IFN- $\alpha$  and ribavirin.

### Introduction:

Hepatitis C virus is a member of Flaviviridae family, a major human virus that infects approximately >170 million individuals worldwide (Dore, 2014) with impact sever progression (Sy et al., 2006). Chronic hepatitis HCV infection is characterized by slow developing to liver cirrhosis, and liver cancer worldwide (Shrivastava et al., 2013). In Egypt at >10% of the general population were recorded with the highest epidemic HCV (Shepard et al., 2005; Mohamoud et al., 2013). Transmission of Hepatitis C virus has been tightly associated with intravenous and percutaneous drug and needle use, blood transfusions, hemodialysis patients, tattooing and the role of sexual activity in the transmission of HCV remains unclear (Alter, 2011). Another severity factor of HCV infection is the seven genotypes of HCV particle with numerous subtypes. these genotypes can differ up to 30% from each other in nucleotide sequence. Depending on the HCV genotype, length of treatment can differ. Genotype 1b is less responsive to alpha-interferon therapy compared to genotypes 2 and 3. Genotype 3 is most common on the Indian subcontinent, while genotype 4 is the most common genotype in Africa and the Middle East (Ohno et al., 1997). Disease Severity is mainly related to human immunity and how it works against the viral infection (Georgel et al., 2010)

as patients with chronic HCV are immunocompromised and may be infected with bacteria as a secondary infection, The most common pathogenic bacteria in patients with chronic HCV were Enterobacteriaceae species, *E. coli*, and *Klebsiella*, while the most Gram-positive species bacteria including *Staphylococcus aureus* (Carrion et al., 2009; Jalan et al., 2014; Kawano et al., 2015). Cytotoxic cells CD3- CD56 (+dim) natural killer (NK) against virally infected cells showed that cell-enriched expansion strongly inhibited replication of HCV and HCC (Dorskali et al., 2011). DGAT1 is an enzyme that catalyzes the synthesis of triglycerides and maturation of luminal LD. In active HCV replication, it targets core to lipid droplets LDs and bounded with NS5B (Huang et al., 2013) while it served as the main host factor for HCV infection (Herker et al., 2010). Absence or inhibition of DGAT1 leads to inhibition of viral replication (Kapadia et al., 2005; Counihan et al., 2011) Additionally, higher levels of serum aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) were an indicators for abnormal liver functions (Wanachiwanawin et al., 2003).

## Material and methods:

**Patients:** this study was based on selection of patients with chronic hepatitis C in mean age of  $47 \pm 5$  and this was by a specialist physician while they were selected to be free from liver cirrhoses, hepatocellular carcinoma (HCC), HBV, renal impairment and diabetes. While samples were collected in time ranged from October 2013 up to April 2014 at the virology department, El-Obour hospital, Kafr El-sheikh, Egypt. This clinical study was approved by the ethics committee, Faculty of Medicine, Tanta University.

**Samples collection:** Blood and urine samples were collected from two groups: **Group A** control, while ten samples were collected from healthy individuals. **Group B**, twenty samples were collected from patients with chronic hepatitis C before the application of the treatment protocol using INF- $\alpha$  and ribavirin for 12 weeks.

## Materials:

### Quantitative RNA PCR assay

It was performed using Artus HCV RG RT- PCR kit (Qiagen GmbH, Qiagen Strasse, Germany) with lower detection limit of 34 IU/ml, automated instrument (HVD Auto Q server).

## Media:

- 1. Nutrient agar Difco Manual (1977) ingredients g/l:** Peptone: 5.00, NaCl: 5.00, beef extract: 3.00, agar: 20.00, PH was adjusted to 5.0. **2. Mannitol salt agar (Oxoid product) Lally et al., (1985) ingredients g/l:** enzymatic digest of casein: 5, beef extract: 1, animal tissue enzymatic digest: 5, D-Mannitol: 10, Phenol Red: 0.025, sodium Chloride: 75, agar: 15 Final, pH:  $7.4 \pm 0.2$  at  $25^\circ \text{C}$ . **3. MacConkey Agar (Oxoid product) :** Peptone: 20.2, sodium chloride: 5.0, lactose: 10.0, bile salts: 5.0, neutral red: 0.075, agar: 12, pH  $7.4 \pm 0.2$  at  $25^\circ \text{C}$ .

**Gram stain:** Crystal violet, Gram's iodine solution, acetone/ethanol (50:50 v: v) and 0.1% basic fuchsin solution.

**VITEK 2 system (bioMerieux Inc., Hazelwood, MO) Funke (1998):** VITEK 2 Cards, Suspension Tubes and the Automatic Transport System were used.

**CD3<sup>+</sup> and CD56<sup>+</sup> monoclonal antibodies (Coulter, 1953):**

(BD Biosciences, San Jose, CA, USA) CD3<sup>+</sup> and CD56<sup>+</sup> monoclonal antibodies (mAbs) which used for immunophenotype analysis, PBS buffer solution for washing and suspension and RBCs were degraded using lysing BD FACS (Fast Access to Critical Solutions) solution or ACK (Ammonium-Chloride-Potassium) Lysing buffer.

Anticoagulant EDTA tubes for blood collections sterilized Petri dishes and sterilized swabs.

**ALT and AST:** used reagents (ELITECH) Tris buffer: 125 mmol/L / pH: 7.4, L-Alanine or Aspartate: 680

mmol/l, LDH: 2000 U/L, NADH: 1.1 mmol/L, A-ketoglutarate: 97mmol/l, IFCC method without pyridoxal phosphate, kinetic.UV.

**DGAT1:** Enzyme-linked Immunosorbent Assay Kit for Diacylglycerol-O-Acyltransferase Homolog 1 (DGAT1). Organism Species: Homo sapiens (Human). This assay has high sensitivity and excellent specificity for detection of DGA T1. Reagents: Pre-coated, ready to use 96-well strip plate, Standard Detection, Reagent A Detection, Reagent B, TMB Substrate, Wash Buffer (30×concentrate), sealer for 96wells Standard Diluent, Assay Diluent A, Assay Diluent B, Stop Solution.

## Methods:

### -Real Time Polymerase Chain Reaction (PCR) HCV Assay:

The investigated blood samples were processed using automated instrument (HVD Auto Q server) at the virology lab, El-Obour hospital, Kafr El-sheikh to check the quantitative RNA copies per milliliter of blood plasma while viral load often correlates with the severity of an active viral infection **Higuhi et al. (1992 and 1993).**

### Isolation and counting of bacteria:

Isolation of bacteria from urine samples was performed on nutrient agar that was inoculated with 100  $\mu\text{l}$  of urine sample then incubated for 18-24 hours at  $37^\circ\text{C}$ . The obtained bacterial isolates were investigated by Gram reaction. Subsequently, G+ve bacteria were subcultured on Mannitol salt agar while, G-ve bacteria were grown on MacConkey agar. Bacterial counts in the investigated samples were determined according to the following equation:  $\text{CFU/ML} = (\text{no. of colonies} \times \text{dilution factor}) / \text{volume of culture plate}$ . Dilution factor was zero as there was no dilution was performed.

### Identification of bacteria:

BioMerieux VITEK® 2 systems version: 06.01 was used for identification of isolated bacteria while 64 identification tests were processed, as enzyme hydrolysis acidification, alkalization and growth under inhibition conditions like lactase, oxidase test. Transfer a sufficient number of pure colonies in 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0). then, the suspended bacterial cells were placed into a special rack (cassette) and the identification card was placed in the contiguous slot then moved to the optical system where readings were observed and data were collected at each 15-minute.

### Detection of CD3<sup>+</sup> and CD56<sup>+</sup> cells count: Flow cytometric analysis (Coulter, 1953):

Fresh venous blood samples were collected on EDTA tubes for identification of CD3<sup>+</sup>, CD56<sup>+</sup> cells. On brief, fresh blood about 100  $\mu\text{l}$  were transferred into staining tubes which was stained by human mAbs, using recommended concentrations by the manufactures. Incubation of the stained tubes for 20 minutes in particular cold and dark conditions, and then RBCs were lysed by adding ACK lysing solution (1x) or BD FACS

for 15 minutes then 5 minutes centrifugation at 1250 rpm, supernatant contained lysed RBCs was discarded. PBS buffer solution was added for washing to complete removing for any debris or RBCs and for re-suspend the pellet too, acquisition was by FACS Calibur or FACS Canto II (BD Biosciences, SanJose, CA, USA). FACS Diva, CellQuest (BD Biosciences) and data were analysed by Flowjo software.

**Biochemical measurements of ALT and AST (Bruns et al., 1981):**

**International federation of clinical chemistry and laboratory medicine (IFCC)** method without pyridoxal phosphate, kinetic UV., adjust the wave length at 340 nm, add sample volume of 20  $\mu$ L to reagent volume 200  $\mu$ L and incubate at temperature 37°C. The set tubes mixed and the absorbance of the sample was measured after 50, 120 and 180 seconds. The mean absorbance change per minute was determined. Calculations were achieved by using the equation  $U/L = 1746 \times A / \text{min}$ , while Reference values: - Men:  $\leq 45$ , Women:  $\leq 45$ .

**DGAT1 enzyme ELISA Engvall and (Perlmann, 1971):**

Prepare all reagents, samples and standards; Add 100  $\mu$ L standard or sample to each well. Incubate 2 hours at 37°C. Aspirate and add 100  $\mu$ L prepared Detection Reagent A. Incubate 1 hour at 37°C, Aspirate and wash 3 times. Add 100  $\mu$ L prepared Detection Reagent B. Incubate 30 minutes at 37°C. Aspirate and wash 5 times. Add 90  $\mu$ L Substrate Solution. Incubate 15-25 minutes at 37°C. Add 50  $\mu$ L Stop Solution. Read at 450 nm immediately. Calculations:- The calculation, we plot the O.D. value of the standard (X-axis) against the known concentration of the standard (Y-axis).

#### Statistical analysis:

The data of present study were conducted using the mean, standard deviation, Chi-square test by SPSS V.20. Standard student "t test" test of significance of the differences between means  $*P \leq 0.05$ ,  $**P \leq 0.01$ . These clinical data were recorded and analyzed along the study for each patient.

#### Results:

##### Hepatitis C Viral load by PCR:

Viral load of healthy individuals (group A) was zero which is negative result as they are healthy individuals but its load in patients with chronic hepatitis C (group B) was a highly positive load. Subsequently, their susceptibility to the secondary bacterial infections increased (Table 1).

##### Isolated pathogenic bacteria and its load that associated with chronic HCV infection:

The microscopic investigation of Gram reaction revealed the presence of all G+ve bacterial isolates in coccoid form especially staphylococci, while G-ve isolates were bacilli. The secondary bacterial infection in patients with chronic HCV (gp B) were compared to that in healthy people (gp A). It was found that investigated

patients with chronic HCV who infected with both G+ve and G-ve bacteria were 30% and who infected with only G+ve bacteria were 37.5% and others infected with only G-ve were 7.5%. Number of chronic HCV patients who revealed no bacterial infections were 25%. Moreover, the load of isolated Gram positive bacteria (75%) on Mannitol medium was more than the Gram negative load on MacConkey (25%).

##### The Identified pathogenic bacteria associated with chronic HCV infection by VITEK 2 System:

It was found that *Eschirechia coli*, *Staphylococcus lentus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia* were five common bacterial pathogens which were identified by VITEK 2 system in patients with chronic HCV.

##### Reduction of CD3<sup>+</sup> and CD56<sup>+</sup> populations during HCV infection.

It was found that populations of CD3<sup>+</sup> and CD56<sup>+</sup> in healthy individuals (gp A) were more than their populations in patients with chronic hepatitis c (gp B) who infected with high viral load and this another evidence of being patients immunocompromised that due to viral infection (Table 2 (A,B) and Figure 1 (A,B))

**Increasing of liver enzymes ALT and AST levels:** they increased as a result of chronic HCV infection as shown in (Table 3).

**Increasing of DGAT1 enzyme level in patients with chronic HCV:** it increased compared to its level in healthy individuals as a result of the viral infection (Table 4).

#### Discussion:

This clinical study demonstrated that the most common secondary bacterial infection associated with HCV infection especially chronic hepatitis C were *Staphylococcus* spp. in Egypt where genotype 3 and 4 which are dominant. Bacterial load of G+ve on Mannitol medium (75%) was more than that of G-ve on MacConkey (25%). The identification by VITEK 2 system as a precise automated technique was used to identify the isolated pathogens and the results revealed the presence of *S. lentus*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*. Moreover, the group of healthy people showed no bacterial growth. Those obtained by Carrion et al. (2009), Jalan et al. (2014) and Kawano et al. (2015) that *E. coli*, *Klebsiella*, and Enterobacteriaceae species were the most common pathogenic bacteria in patients with chronic hepatitis C which was called secondary bacterial infections and recently, Gram-positive bacteria including *S. aureus*.

Subsequently, hepatitis C patient is one of the immunocompromised patients while viral infection followed by several symptoms by time related to infection severity and development of the disease while it began as acute infection then chronic infection, fibroses, cirrhosis and developed to hepatocellular carcinoma that followed by death (Tang, 2009).

biochemical changes had been occurred as a result of these stages like decreasing of albumin, respiration difficulties, digestion disorders, increasing of liver enzymes, liver dysfunction, diarrhea, immune dysfunction (Park, 2014). Side effects of several HCV medications strategies and others related to secondary bacterial infection (Jalan et al., 2014). According to current study, reduction of CD3<sup>+</sup> and CD56<sup>+</sup> levels and increased levels of liver enzymes ALT and AST act as other evidences of immune commpromisation and liver dysfunction during HCV infections.

DGAT1 enzyme involved in lipoprotein coat structure of HCV particle which protects the virus so it can escape from the immune defense. There is a balance between the amount of core on lipid droplet (LD) surface and virus production. However, recent reports showed a critical involvement of lipid droplets (LDs) in infectious virion production, and viral budding requires the lipoprotein secretion pathway. This active process is mediated by the interaction between the diacylglycerol acyltransferase-1 (DGAT-1) and HCV core (Camus et al., 2013). Both RNA replication and infectious particle assembly are thought to take place at ER membranes (Tardif et al., 2005), and these previous studies support the current study result, while DGAT1 enzyme level in chronic HCV Egyptian patients was increased by HCV infection in a comparison to its level in healthy individuals in Egypt where genotype 3, 4 and this new evidence proved that previous evidences which were performed outside Egypt provided DGAT1 enzyme as a Key of HCV infection.

#### Conclusion:

The levels of DGAT1 enzyme, ALT, AST, CD3<sup>+</sup> and CD56<sup>+</sup> could be used as indicators for development of HCV infection. Bacterial infections especially *Staphylococcus* spp are associated with hepatitis C viral infection in Egypt. Moreover, a light spot on the importance of finding new HCV treatment strategy that provides both antibacterial and antiviral responses. DGAT1 enzyme may play a role in development of new antiviral treatment as it plays a role in HCV infection.

Table1. PCR results of HCV patients

| Patient's code number | Sex | Age/ years | PCR result IU/ml |
|-----------------------|-----|------------|------------------|
| p1                    | F   | 48         | 23,030,498       |
| p2                    | M   | 43         | 1,005,317        |
| p3                    | M   | 45         | 1,016,250        |
| p4                    | M   | 44         | 1,581,138        |
| p5                    | M   | 56         | 1,787,737        |
| p6                    | M   | 50         | 804,704          |
| p7                    | M   | 48         | 199,35           |
| p8                    | F   | 53         | 214,933          |
| p9                    | M   | 50         | 367,821          |

|     |   |    |            |
|-----|---|----|------------|
| p10 | M | 45 | 138,772    |
| p11 | M | 50 | 193,035    |
| p12 | F | 48 | 7,684      |
| p13 | M | 45 | 13,561,362 |
| p14 | M | 55 | 23,207     |
| p15 | F | 45 | 1,190,245  |
| p16 | F | 43 | 170,727    |
| p17 | M | 40 | 711,710    |
| p18 | F | 53 | 1,356,13   |
| p19 | M | 52 | 1,250,23   |
| p20 | F | 50 | 1,150,13   |

M=Male, F=Female. P1 = patient number 1

Table 2: CD3<sup>+</sup> and CD56<sup>+</sup> cell counts in both healthy individuals (group A, n=10) and patients with chronic HCV (group B, n=15).

A: Populations of CD3<sup>+</sup> in healthy individuals and patients with chronic HCV

| CD 3 <sup>+</sup> | Group A         | Group B          |
|-------------------|-----------------|------------------|
| Range             | 18 – 27         | 12 – 25          |
| Mean $\pm$ SD     | 23.0 $\pm$ 4.58 | 18.53 $\pm$ 4.26 |
| t. test           | 2.699           |                  |
| P. value          | 0.120           |                  |

P.value= 0.01-0.05 showed significant result, gpB = chronic HCV patients. As shown slightly increased the populations of CD3<sup>+</sup> (gpA) than gpB that introduced by Mean  $\pm$  SD

B: Populations of CD56<sup>+</sup> in healthy individuals and in chronic HCV patients

| CD 56 <sup>+</sup> | Group A       | Group B         |
|--------------------|---------------|-----------------|
| Range              | 24 – 36       | 22 – 38         |
| Mean $\pm$ SD      | 30 $\pm$ 6.11 | 29.0 $\pm$ 4.06 |
| t. test            | 0.002         |                 |
| P. value           | 0.962         |                 |

P.value= 0.01-0.05 showed significant result, gpB = chronic HCV patients. As shown a slightly high of the populations of CD56<sup>+</sup>(gpA than gpB) that introduced by Mean  $\pm$  SD

**Table 3: liver enzymes SGPT, SGOT levels in both group A and group B:**

| Enzyme/IU/ML                    | SGPT             |                   | SGOT             |                   |
|---------------------------------|------------------|-------------------|------------------|-------------------|
|                                 | Gp A             | Gp B              | Gp A             | Gp B              |
| <b>Range</b>                    | 15.00-25.00      | 19.00 – 90.00     | 15.20 – 28.00    | 23.00-84.00       |
| <b>Mean <math>\pm</math> SD</b> | 17.80 $\pm$ 3.08 | 49.00 $\pm$ 19.74 | 19.83 $\pm$ 3.55 | 49.76 $\pm$ 17.04 |
| <b>t. test</b>                  | 24.246           |                   | 29.644           |                   |
| <b>P. value</b>                 | 0.001*           |                   | 0.001*           |                   |

P.value \* = significant result 0.001\*

**Table 4:DGAT1 levels in both healthy individuals (group A) and patients with chronic HCV (group B).**

| DGAT1                           |                   |                   |
|---------------------------------|-------------------|-------------------|
| IU/ML                           | Group A           | Group B           |
| <b>Before</b>                   |                   |                   |
| <b>Range</b>                    | 68.9 – 1011       | 42.3 – 1819       |
| <b>Mean <math>\pm</math> SD</b> | 302.1 $\pm$ 264.2 | 528.4 $\pm$ 438.7 |
| <b>t. test</b>                  | 2.214             |                   |
| <b>P. value</b>                 | 0.148             |                   |

DGAT1 (diacylglycerol acyltransferase-1), P.value= 0.01-0.05 showed significant result, gpB = chronic HCV patients, gp A (healthy individual)

**Figures ligands:-**

- Figure1. (A,B) Flow Jo software analysis of CD3<sup>+</sup> and CD56<sup>+</sup> counts in healthy individuals are more than that in HCV patients.**

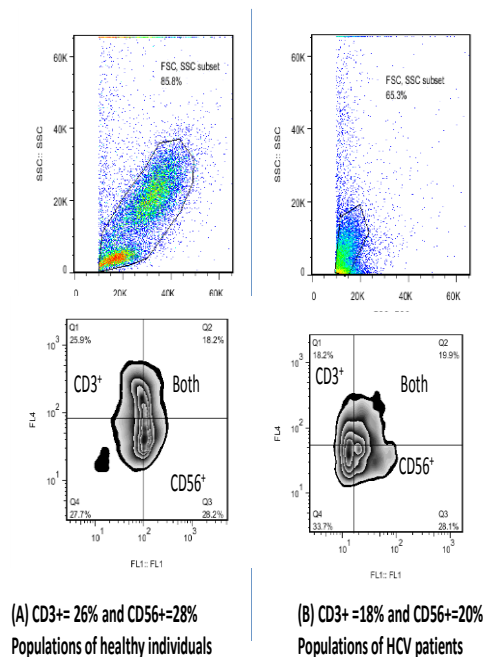


Fig1.(FlowJo analysis B), figures A,B, CD3+ (FL4)and CD56+ (FL1)populations in healthy individuals are 86% more than in HCV patients 65.3%.

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## الملخص العربي

يصيب التهاب الكبد الوبائي المزمن فيروس سي أكثر من ١٧٠% ملايين من المرضى عالميا وتعتبر مصر أعلى نسبة مصابين, حيث يعتبر مرضى التهاب الكبد الوبائي المزمن فيروس سي يعانون من نقص المناعة مما يعرضهم للإصابة بالميكروبيه وبالأخص البكتيريا.

تم إجراء هذه الدراسة على مرضى التهاب الكبد الوبائي المزمن فيروس سي والافراد الاصحاء ككنترول, وتم تجميع عينات البول والدم لإجراء التجارب, حيث تم عزل البكتريا من البول وتعريفها باستخدام تقنيتي VTEK2system وكذلك تقنية 16srRNA وتم تعيين عدد الخلايا المناعية cd3+,cd56+ كتعبير عن الاستجابة المناعية بواسطة تقنية التدفق الخلوي, ايضا تعيين نسبة انزيمات الكبد وكذلك انزيم DGAT1 بواسطة تقنية ELISA وكذلك نسبة الدهون بواسطة تقنيات البيوكيميائية وتعيين نسبة العبء الفيروسي باستخدام HCV PCR.

واظهرت النتائج أن المكورات لنتس ، المكورات العنقودية الذهبية، كليبسيلا الرئوية، الزائفة الزنجارية والقولونية كانت العدوى البكتيرية الشائعة. وكان انخفاض طفيف في مستويات CD3<sup>+</sup> و CD56<sup>+</sup> تتزامن مع زيادة مستويات انزيمات AST, ALT ، DGAT1 ، كما عانى المرضى من عبء فيروسي HCV عالي. الخلاصة: مرضى التهاب الكبد الوبائي المزمن يعانون من نقص المناعة و من الالتهابات البكتيرية الثانوية الخطيرة مما يزيد من شدة التهاب الكبد الوبائي فيروس سي .