

# Evaluation of Iron Biomarkers and Haematological Variables in Asymptomatic Individuals Infected by Hepatitis C Virus in Ebonyi State, Nigeria

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*Abstract- Alteration in iron biomarkers is a common finding in viral hepatitis. The knowledge of the frequency of the haematological abnormalities in patients with hepatitis C virus (HCV) infection will support the need for this indispensable tool in the investigation of infectious disease. This work was designed to investigate the changes in iron biomarkers and haematological variables of asymptomatic HCV infected individuals. Ethical clearance was obtained from Health Research and Ethical Committee of the University of Nigeria Teaching Hospital, Ituku/Ozalla. One hundred and thirty individuals with asymptomatic HCV infection were drawn from thirteen Local Government Areas of Ebonyi State, consisting of 60 males and 70 females. Informed consent were obtained from them after pretest counseling. Blood sample (8.0ml) was collected aseptically from each individual using a sterile syringe and needle and 3.0ml was placed in ethylene diamine tetra-acetic acid and 5.0ml in plain container. The analysis of the samples were done in Marylmas Research and Diagnostic Laboratory in Abakaliki using standard operating procedure and automation using automated machine, sysmex XT4000i (United State of America) for complete blood count. The significant ( $p < 0.05$ ) results of male asymptomatic HCV infected individuals were increased ferritin ( $644.35 \pm 98.42\mu\text{g/L}$ ), increased total iron binding capacity (TIBC) ( $88.51 \pm 4.68\mu\text{mol/L}$ ), increased transferrin ( $3.54 \pm 0.91\text{g/L}$ ), decreased total iron ( $15.81 \pm 8.94\mu\text{mol/L}$ ) and decreased transferrin saturation ( $17.82 \pm 10.21\%$ ) compared to control: ferritin ( $227.67 \pm 43.95\mu\text{g/L}$ ), TIBC ( $67.00 \pm 4.10\mu\text{mol/L}$ ), transferrin ( $2.68 \pm 0.16\text{g/L}$ ), total iron ( $28.33 \pm 1.03\mu\text{mol/L}$ ) and transferrin saturation ( $42.67 \pm 1.37\%$ ). Their significant ( $p < 0.05$ ) results for complete blood*

*count were increased total white blood Cell (TWBC) ( $4.78 \pm 1.54 \times 10^9/\text{L}$ ), increased red blood cell (RBC) ( $5.23 \pm 0.75 \times 10^{12}/\text{L}$ ), increased haemoglobin ( $15.34 \pm 2.30\text{g/dL}$ ), increased haematocrit ( $42.34 \pm 5.25\%$ ) compared to control: TWBC ( $3.33 \pm 0.21 \times 10^9/\text{L}$ ), RBC ( $4.33 \pm 0.41 \times 10^{12}/\text{L}$ ), haemoglobin ( $13.33 \pm 0.49\text{g/dL}$ ) and haematocrit ( $35.80 \pm 2.06\%$ ). The significant ( $p < 0.05$ ) results of female asymptomatic HCV infected individuals were increased ferritin ( $502.78 \pm 350.14\mu\text{g/L}$ ), increased TIBC ( $89.30 \pm 11.07\mu\text{mol/L}$ ), increased transferrin ( $3.57 \pm 0.44\%$ ), decreased total iron ( $13.43 \pm 7.66\mu\text{mol/L}$ ), decreased transferrin saturation ( $14.78 \pm 7.79\%$ ) compared to control: ferritin ( $82.67 \pm 19.13\mu\text{g/L}$ ), TIBC ( $53.33 \pm 2.73\mu\text{mol/L}$ ), transferrin ( $2.13 \pm 0.11\text{g/L}$ ), total iron ( $21.33 \pm 1.37\mu\text{mol/L}$ ), transferrin saturation ( $40.00 \pm 0.89\%$ ). Their significant ( $p < 0.05$ ) results for complete blood count were increased TWBC ( $5.53 \pm 1.83 \times 10^9/\text{L}$ ), increased RBC ( $5.28 \pm 1.12 \times 10^{12}/\text{L}$ ), increased haemoglobin ( $15.20 \pm 3.36\text{g/dL}$ ), increased haematocrit ( $41.39 \pm 8.23\%$ ) compared to control TWBC ( $3.40 \pm 0.32 \times 10^9/\text{L}$ ), RBC ( $3.81 \pm 0.77 \times 10^{12}/\text{L}$ ), haemoglobin ( $11.33 \pm 0.40\text{g/dL}$ ), haematocrit ( $30.87 \pm 1.36\%$ ). The results suggested that HCV infection modulates iron metabolism and homeostasis in host hepatic cell. The elevated serum ferritin acts as a marker of inflammation and infection in these individuals. The severity and progression of liver diseases depends on the levels of serum ferritin and transferrin. The HCV infection appears to have little effect on the haematological variables in the study.*

## I. INTRODUCTION

### 1.1 BACKGROUND OF THE STUDY

The harms associated with hepatitis C virus (HCV) constitute a major public health challenge globally. It is estimated that 71 million people are living with chronic HCV infection, with a significant proportion who are at high risk of developing advanced liver disease, cirrhosis or liver cancer (Lianping *et al.*, 2018). Viral hepatitis was estimated to be the 7<sup>th</sup> leading cause of mortality globally (Silva *et al.*, 2018). About half of this mortality is attributed to hepatitis C virus (HCV), a primary cause of liver fibrosis, cirrhosis and cancer (Hiemet *et al.*, 2018). In fact, a review of the literature revealed that the risk of hepatocellular carcinoma, (HCC) increases up to 17-fold in patients living with chronic HCV compared with their HCV-negative counterparts, and this may persist even after achieving a treatment-induced sustained virological response (SVR) (Conti *et al.*, 2016). If left untreated, approximately 399,000 people die annually from consequences associated with HCV, mostly from advanced liver cancer and HCC (WHO, 2016).

Information on the epidemiology of this virus is limited. Nevertheless, it has long been suspected that it may be endemic. A pilot study done in Nigeria adults and children using second generation of enzyme immunoassay (EIA) kit gave an average sero-prevalence of 8% (Oni and Harrison, 1996), while another study on adult blood donors in Nigeria using EIA kit of an unspecified generation reported a prevalence rate 12.3% (Halim and Ajayi, 2000). However, a study of adolescent and adult patients with sickle cell anaemia (SCA) in Benin by Mutimer *et al.* using second generation EIA kit showed 20% prevalence rate (Mutimer *et al.*, 1994). Ejiofor *et al.* in Enugu using second generation EIA kit reported 6.6% and 5.3% prevalence rates respectively among transfused and non-transfused children with SCA.

Hepatitis C virus is a ribonucleic acid (RNA) blood-borne virus of the Flaviviridae family (Bo *et al.*, 2015). Hepatitis C virus particles are enveloped by covalently linked envelope glycoproteins 1 and 2 (E<sub>1</sub>

and E<sub>2</sub>) (Bartenschlager *et al.*, 2011). There are six genotypes of the HCV (NPS, 2018). The treatment decision of the disease depends on the knowledge of genotype and the degree of liver damage (Edwards *et al.*, 2015). The incubation period of HCV is between 2 weeks to 6 months (NJDHSS, 2010). History of blood transfusion, intravenous drug use, inadequate sterilization of medical apparatus such as dental instruments, tattoos, and history of medical injection are all common modes of HCV transmission (Nwankiti *et al.*, 2009). It can also be transmitted by sexual contact, and from an infected mother to her baby, yet such modes of transmission are less common (WHO, 2016). The symptoms of acute hepatitis C infection include decreased appetite, fatigue, abdominal pain, jaundice, itching and flu-like symptoms (Tsang *et al.*, 2008). Different screening strategies have been implemented in different regions, based on the local epidemiology (EASL, 2016). Groups at higher risk of HCV infection can be identified and should be tested. In regions where the majority of patients belong to a well-defined age group, birth cohort testing has proven efficacious, with limitations (Pawlotsky, 2016). The diagnosis of acute and chronic HCV infection is based on the detection of HCV RNA in serum or plasma by a sensitive, exclusively qualitative, or both qualitative and quantitative, molecular method (Terrault *et al.*, 2005). The primary goal of HCV therapy is to cure the infection (EASL, 2018) i.e. to achieve a sustained virological response (SVR) defined as undetectable HCV RNA 12 weeks (SVR 12) or 24 weeks (SVR 24) after treatment completion (WHO, 2016). There is no vaccine for HCV till date, yet treatment comprises antiviral therapy plus interferon and ribavirin, which are effective against all genotypes of hepatitis C (WHO, 2016). The liver is the main organ responsible for iron homeostasis and the status of the liver is closely related to the distribution of iron within the body (Pietrangelo, 2007). This organ has incontrovertible influence on several functions of many other organs in the body, the haematopoietic system inclusive. Besides its role as an extravascular haematopoietic organ in early foetal life and bone marrow infiltrative disease, the liver forms and stores many of the elements and proteins necessary in blood productions (Fasola *et al.*, 2009).

## 1.2 STATEMENT OF THE PROBLEM

Various stages of HCV infection including the acute and chronic stages may cause liver disease of varying severity, evidenced in the levels of serum iron indices at these stages. In acute HCV infection, there is usually slight increase in iron markers especially Total Iron Binding Capacity whilst chronic HCV infection usually present with moderate to an astronomical increase in about 90% of patients (Shan *et al.*, 2005). The varying severity of liver disease associated with acute and chronic HCV infections may consequently result in varying degree of abnormal haematological parameters in HCV infected persons. However, research in this area is scanty and little is known on the likely haematological picture and status of iron markers that may present at different asymptomatic stages of the HCV infection in Ebonyi State, Nigeria.

Despite the tremendous progress in the field of hepatitis C virus and its related consequences, knowledge about individuals infected with hepatitis C that are still asymptomatic in relation to iron bio-markers and haematological variables are very limited in Ebonyi State, Nigeria.

## 1.3 RESEARCH QUESTIONS

- Can level of ferritin increase in hepatitis C virus infection?
- Does hepatitis C virus influences the level of iron?
- What happens on the level of transferrin during hepatitis C virus infection?
- Does hepatitis C virus effect any changes in haematological variables?

## 1.4 AIM OF THE STUDY

To evaluate iron bio-markers and haematological variables in asymptomatic Hepatitis C virus infected individuals in Ebonyi State, Nigeria.

## 1.5 OBJECTIVES

1. To determine the levels of some iron bio-markers in HCV infected asymptomatic individuals.

2. To assess the complete blood count (CBC) of HCV infected asymptomatic individuals.

## II. LITERATURE REVIEW

### 2.1 CONCEPTUAL FRAMEWORK OF THE STUDY

The Study used a conceptual framework adapted and modified to suit the present study from the Vertical Transmission of Hepatitis C Virus: Systematic Review and Meta-analysis (Benova *et al.*, 2014). Hepatitis C virus (HCV) is a serious liver disease that results from infection with the HCV (CDC, 2017). Hepatitis C has been called “a silent disease” because people can get infected without knowing it. Early diagnosis through targeted symptoms screening of key population will ensure early referral and treatment, reducing the chances of onward transmission, and the onset of complications (Duffell *et al.*, 2017). Haematological abnormalities are regularly seen among infected viral hepatitis and haemolysis is considered to be one of the most common causes (Arjun and Chaitali, 2015). A full haematological examination of the infected viral hepatitis include, red blood cell count, haemoglobin concentration, packed cell volume, mean cell volume, mean cell haemoglobin concentration, white cell count, and platelet count. Iron is an essential element for several metabolic pathways and physiological processes (Elsayed and Stack, 2016). Among these are serum ferritin, iron, transferrin, transferrin saturation, total iron binding capacity. Process-of-Care defines what is being delivered and includes three categories of care: pretreatment, preventative, and treatment (Urbano *et al.*, 2016). Pretreatment measure (risk factor assessment, and anti-HCV antibody test), preventative measure (counseling about alcohol use), and treatment (antiviral treatment) (Mayer, 2013).

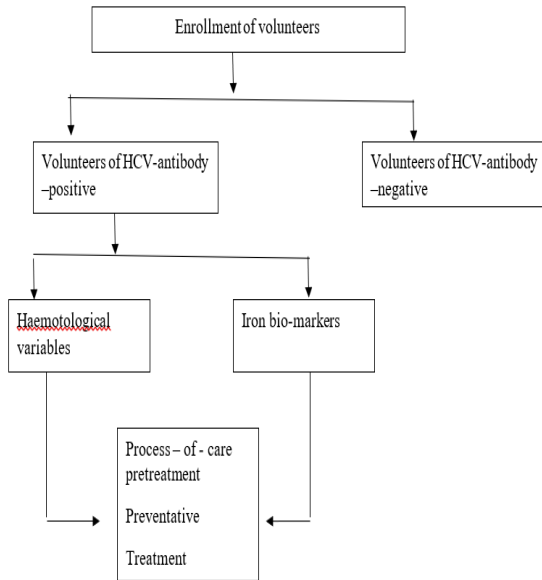


Figure 2.1 conceptual framework on evaluation of haematological variables and iron bio-markers in hepatitis C infection

Source: Adapted from the vertical transmission of Hepatitis C virus: Systematic Review and meta-analysis (Benova *et al.*, 2014).

## 2.2 THEORETICAL REVIEW

The definite underlying pathogenesis of haematological manifestations in hepatitis C virus (HCV) infection is unclear, but several theories have been proposed. It has been proposed that the chronic antigenic stimulation of the immune system by the virus leads to clonal B – cell expansion. This is supported by the following findings: most of the non-Hodgkin lymphoma (NHL) cells in HCV infected patients is typical of germinal Centre and post germinal Centre B cells (De Re *et al.*, 2000) and immunoglobulin variable region genes expressed by B – cell NHL from HCV positive patients show certain somatic mutation, which indicates antigenic selection process (Marasca *et al.*, 2001). The antigenic stimulation is induced by a viral enveloped protein, known as E2 which bind to a specific receptor, CD81 present on the hepatocytes as well as the T- and B- lymphocytes. Cluster of differentiation 81 (CD81) along with CD19 and CD21 present on the B-cell provides stimulatory signals that lower the threshold required for B-cell to respond to antigen

(Maecker *et al.*, 1997). The other theories propose that HCV infection enhances deoxyribonucleic acid (DNA) damage and gene mutations as well as inhibits apoptosis of the infected lymphocytes. The viral core and NS3 proteins activate the gene for inducible nitric oxide synthase and hence stimulate production of nitric oxide, which can cause double-stranded DNA breaks and DNA mutation (Machida *et al.*, 2004). In fact, HCV infection has been shown to induce error-prone DNA polymerase and activation – induced cytidine deaminase. These enzymatic alterations result in the formation of double-stranded DNA breaks and an increase in the mutation of immunoglobulin heavy chains as well as tumour-suppressor genes and proto-oncogenes such as myc, bcl-6, p-53 and beta-catenin genes in HCV-infected B cell lines. The mutated proto-oncogenes are found to be amplified in HCV-associated lymphomas. The mutation of immunoglobulin heavy chains may reduce the immune response to the viral infection (Machida *et al.*, 2004). Hepatitis C virus infected lymphocytes also have chromosomal translocation (18;14) resulting in over-expression of bcl-2 oncogene, which inhibit apoptosis (Zignego *et al.*, 2000). Finally, not all HCV infection causes lymphocyte abnormality, which indicates that the interaction of environmental and genetic factors may influence the manifestation of various HCV-related B-cell lymphoproliferative diseases (Mazzaro *et al.*, 2005).

## 2.3 EMPIRICAL REVIEW

### 2.3.1 HISTORICAL REVIEW OF HCV

With the development of specific diagnostics for hepatitis A virus (HAV) and the hepatitis B virus (HBV) in the 1970s, it become clear that most cases of hepatitis arising from blood transfusion were not caused by infections with these or other known viral agents (Knodel *et al.*, 1975).

There is evidence that one blood-borne non-A, non-B hepatitis (NANBH) agent may be a small, enveloped virus that is readily transmissible to chimpanzees (Bradley *et al.*, 1985).

Although, this failure could be interpreted in terms of a lack of viral antibody. Therefore, in order to

increase viral antigen concentrations, a complement DNA (cDNA) library derived from infections material was constructed in the bacteriophage  $\lambda$  *gt11*. This vector allows the efficient expression of cDNA-encoded polypeptides and was designed originally to facilitate the isolation of cDNA clones by means of well-characterized antibodies that bind to clones synthesizing the polypeptides of interest (Young and Davis, 1983). This library was then screened for rare clones expressing viral antigen with serum from a chronic NANBH patient as a presumed source of viral antibodies.

The serum was subject to extensive ultracentrifugation in order to ensure the pelleting of a small virus, and nucleic acid was recovered from the pellet. Since the nature of the genome was unknown, the recovered nucleic acid was completely denatured before synthesizing cDNA from both RNA and DNA with random primers of reverse transcriptase.

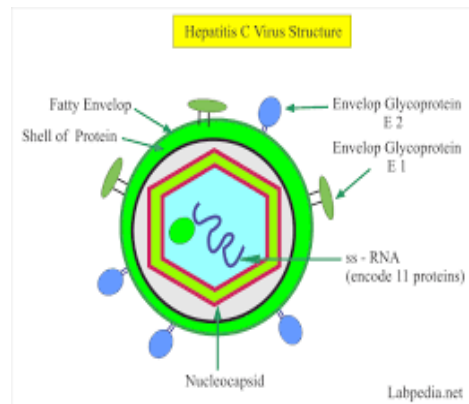
Screening  $\sim 10^6$  of the resulting recombinant  $\lambda$  *gt11* 5-1-1. To investigate its potential viral origin, a larger overlapping clone (clone 81) was first isolated from the same library, and the cDNA was hybridized to human and chimpanzee DNA by southern blot analysis. Four chimpanzees experimentally infected with the NANBH agent all sera converted to PS5 antibody after acute infection, whereas seven animals infected with HAV or HBV showed no such sera conversion. Sera from these animals were also assayed with a radio immunoassay containing purified PS5 to capture and measure reactive antibodies. The data along with results from a large study of well-characterized patients demonstrate that the polypeptide encoded by the clone 5-1-1 ORF is closely associated with NANBH infections. Thus, the data indicate that clones 5-1-1 and 81 are derived from the genome of a blood-borne NANBH virus that we now term the hepatitis C virus (HCV) (Choo *et al.*, 1989). The virus is less than 80nm in diameter and from its proven sensitivity to organic solvents, it would appear to possess an envelope made up of essential lipid (He *et al.*, 1987). These observations led to the suggestions that the agent may be togavirus-like (Bradley, 1985).

### 2.3.2 VIROLOGY OF HCV

The hepatitis C virus (HCV) is an RNA virus that belongs to the family flaviviridae (Lauer and Walker, 2001). HepatitisC virus replicates in the cytoplasm of hepatocytes, but is not directly cytopathic. Persistent infection appears to rely on rapid production of virus and continuous to cell-to-cell spread, along with a lack of vigorous T-cell immune response to HCV antigens. The HCV turnover rate can be quite high with replication ranging between  $10^{10}$  to  $10^{12}$  virions per day, and a predicted viral half-life of 2 to 3 hours (Neumann, 1988). Therefore, the virology of HCV could be summarized as:

1. Hepatitis C virus is an enveloped RNA virus belonging to the genus hepacivirus of the flaviviridae family
2. Hepatitis C virus has a spherical shape, 50nm in diameter.
3. It has smooth outer surface and spike projections formed of E<sub>1</sub> and E<sub>2</sub> proteins.
4. The outer larger surrounds the spherical nucleocapsid consisting of the HCV core.

### 2.3.3 STRUCTURE OF HCV



(Lauer and Walker, 2001)

### 2.3.4 GENETIC DIVERSITY OF THE HCV GENOME

Although both the 5' and 3' NC of the HCV genome possess highly conserved terminal sequences (Tanaka *et al.*, 1995). Hepatitis C virus is characterized by a high degree of genetic heterogeneity (Choo *et al.*, 1991) and as such it is similar to other RNA viruses.

The most heterogeneous regions of the genome are the genes encoding the two envelope proteins, E<sub>1</sub> and E<sub>2</sub>. The N terminus of the E<sub>2</sub> gene contains the most variable region of the entire genome and has been referred to as the first hypervariable region (HVR<sub>1</sub>) (Weiner *et al.*, 1991). A second hypervariable region, referred to as HVR<sub>2</sub>, is located just 3' of HVR<sub>1</sub> (Hijikata *et al.*, 1991). This genetic heterogeneity of HCV has been classified into four hierarchical strata: genotypes, sub genotypes, isolates and quasi-species. The degree of nucleotide sequence variation for each stratum (expressed in percentage) is 30-50%, 15-30% and 15% respectively (Nguyen and Kneeffe, 2004). A total of eleven major genotypes, designated according to the order of their discovery (Seeff, 1995) have been identified (Tokira *et al.*, 1995) and more than 90 subtypes have been described. An increasing number of novel subtypes are emerging and more detailed phylogenetic analysis of these isolates is recommended (Mizokami *et al.*, 1996).

2.3.5 HCV GENOTYPES

There are six known genotypes (NPS, 2018) (numbered 1 through 6) and more than 50 subtypes (e.g. 1a, 1b, 2a ...). Frequent HCV mutations and numerous subtypes have made the search for an HCV vaccine challenging. The worldwide distribution of HCV genotypes and their subtypes is shown in table below:

Region	Genotype/subtype(most common)	Less common
North America	1a	1b,2b,3a
Europe	1a	1b,2b,3a
North Europe	1a	1b,2, 3a
Southern and Eastern Europe	1b	1a, 2, 3, 2c
Japan	1b	1a, 2, 3, 2c
African	1b	a, 2b, 3b
North Africa	1b	a, 2b, 3b
Tunisia	1b	2a, 2b, 3b
Morocco	1b	a, 2c, 1a
Central	1b	a, 2c, 1a

African		
Gabon	4	1a, 1b, a, 2b
Nigeria	1, 4	2
Cameroon	4	1, 2
South African	5	1,2,3 and 4
South East Asia	5	1,2,3 and 4
Throughout the region	1	6,7,8,9,2
Vietnam, Thailand and Myanmar	7,8,9	10, 11
Indonesia	10, 11	1a
Philippines	1a	1b, a, 2b

(Chamberlain *et al.*, 1997)

Distribution of HCV genotypes and subtypes in the Middle East (Chamberlain *et al.*, 1997) is shown in table above. Surprisingly, the accumulated data show that there are two main patterns for the distribution of HCV genotypes: one is peculiar to the Arab countries (except for Jordan) where genotype 4 predominates, while the other pattern is characteristic of the non-Arab countries (Turkey, Israel, and Iran) where genotype 1 have been reported from the Arab countries, it is noteworthy that genotype 4 is quasi-exclusive (Fakeeh and Zaki, 1999).

The significance of HCV genotyping as an epidemiological marker has been clearly substantiated, particularly in tracing the sources of infection and elucidating the possible modes of transmission (McTutchison *et al.*, 1998 ).

2.3.6 EPIDEMIOLOGY OF HCV

World Health Organization (WHO, 2016) has reported data on the worldwide prevalence of HCV infection, based on both published studies and submitted data. Although, HCV is endemic worldwide, there is a large degree of geographic variability in its distribution. Countries with the highest reported prevalence rates are located in African and Asia; areas with lower prevalence include the industrialized nations in North America, Northern and Western Europe, and Australia.

Populous nations in the developed world and relatively low rates of HCV seroprevalence include Germany (0.6%) (Palitzsch *et al.*, 1999), Canada (0.8%) (Zou *et al.*, 2000), France (1.1%) (Desenchos, 2000), and Australia (1.1%) (Law *et al.*, 2002).

Low, but slightly higher seroprevalence rates have been reported in the USA (1.8%) (Alter *et al.*, 1999), Japan (1.5 – 2.3%) (Ohshima *et al.*, 2000) and Italy (2.2%) (Puro *et al.*, 1995).

Information on the epidemiology of this HCV in Nigeria is limited. Nevertheless, it has long been suspected that it may be endemic. A pilot study done in Nigerian adults and children using second generation of enzyme immunosorbent assays (EIAs) Kit gave an average sero-prevalence of 8% (Oni and Harrison, 1996) while another study on adult blood donors in Nigeria using EIAs kit of an unspecified generation reported a prevalence rate 12.3% (Halim and Ajayi, 2000).

### 2.3.7 GLOBAL HCV DISTRIBUTION

The estimated global prevalence of HCV is 2-3% (Shepard *et al.*, 2005). Countries with the highest reported prevalence rates are located in African and Asia. China has reported seroprevalence of 3.2%. One community based survey in India reported an overall rate of 0.9%. Indonesia's rate is 2.1% in sero surveys of voluntary blood donors. The sero prevalence in Pakistan is reported to range from 2.4% to 6.5%. Egypt has the highest reported sero prevalence rate, 22% (CDC, 2012). The distribution of HCV genotypes vary according to the geographical region. Genotypes 1-3 are widely distributed throughout the world (Esfahani *et al.*, 2010). Subtype 1a is prevalent in North and South America, Europe and Australia (Rivas-Estilla *et al.*, 2008). Subtype 1b is common in North America and Europe (Lopez-Labrador *et al.*, 1997), and is also found in part of Asia.

Genotype 2 is present in most developed countries (Pouillot *et al.*, 2008), but is less common than genotype 1. While there are no country wide surveys on the epidemiology of HCV in Nigeria, HCV prevalence appears to be increasing (Godwin *et al.*, 2013). Studies of HCV rates among blood donors in Nigeria show prevalence ranging from 5.4% in

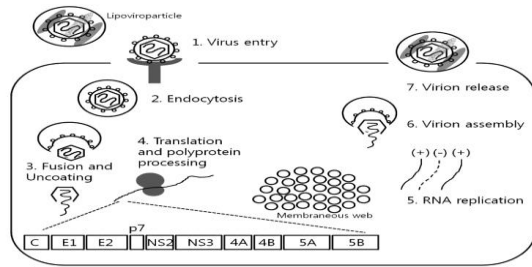
Makurdi (Alao *et al.*, 2010), C 8.4% in Lagos (Ayolabi *et al.*, 2006), 12.3% in Benin City (Halim and Ajayi, 2000). Hepatitis C virus rates among pregnant women in Nigeria appear to be lower ranging from 1.8%-3.6% in Benin city (Ugbeabor *et al.*, 2009), 4.3% in Port Harcourt (Ojule *et al.*, 2008), 4.5% in Kaduna (Sheyinet *et al.*, 2012), 4.7% in Enugu (Obieniu *et al.*, 2011).

### 2.3.8 HCV LIFE CYCLE

Viral attachment involves the two envelope glycoproteins, E1 and E2, apolipoproteins present at the surface of the lipovirions and several cell surface molecules. Glycosaminoglycans and the LDL receptor seem to be involved in low-affinity initial cell binding. Then, E1 – E2 interacts with CD81 and scavenger receptor class B member 1, whereas claudin 1, occludin and possibly other molecules, such as Claudin 6 or Claudin 9, epidermal growth receptor or ephrin receptor type A2, are required for cell entry (Zeisel *et al.*, 2013). This multi-receptor complex mediates uptake and defines organ and species specificity. The E2 envelope glycoprotein contains hypervariable regions that play the part of immunodominant neutralization epitopes.

Antibodies against these hypervariable regions in patients' sera are protective. However, the high variability of HCV with different viral quasi-species in the same patient has so far prevented the development a successful prophylactic vaccine based on these viral proteins. Hepatitis C virus - complexes are seemingly associated with tight junctions, which enable direct cell transmission (Timpe *et al.*, 2008). After attachment, Hepatitis C virus entry into cells result in clathrin-mediated endocytosis, followed by fusions between viral and endosomal membranes, which leads to the release of the nucleocapsid into the cytoplasm. The E1 envelope glycoprotein is believed to be the fusogen (the glycoprotein that facilitates cell fusion).

Schematic representation of HCV life cycle



(Chang and Kyong, 2013)

The hepatitis C virus must attach to and infect liver cells in order to carry out its life cycle and reproduce - this is why it is associated with liver disease. While various details remain unknown about the exact natural processes of hepatitis C, like other viruses, it must complete eight key steps to carry out its life cycle:

1. The virus locates and attaches itself to a liver cell. Hepatitis C uses particular proteins present on its protective lipid coat to attach to a receptor site (a recognizable structure on the surface of the liver cell).
2. The virus'protein core penetrates the plasma membrane and enters the cell. To accomplish this, hepatitis C utilizes its protective lipid (fatty) coat, merging its lipid coat with the cells outer membrane (the coat is in fact composed of a fragment of another liver cell's plasma membrane). Once the lipid coat has successfully fused to the plasma membrane, the membrane engulfs the virus - and the viral core is inside the cell.
3. The protein coat dissolves to release the viral RNA in the cell. This may be accomplished during penetration of the cell membrane (it is broken open when it is released into the cytoplasm), or special enzymes present in liver cells may be used to dissolve the casing.
4. The viral RNA then coopts the cell's ribosomes, and begins the production of materials necessary for viral reproduction. Because hepatitis C stores its information in a "sense" strand of RNA, the viral RNA itself can be directly read by the host cell's ribosomes, functioning like the normal RNA present in the cell. As it begins producing the materials coded in its RNA, the virus also possibly shuts down most of the normal functions of the cell, conserving its energy for the

production of viral material, although it occasionally appears that hepatitis C will stimulate the cell to reproduce (presumably to create more cells that can produce viruses), which is why hepatitis C is often associated with liver cancer. The viral RNA first synthesizes the RNA transcriptase it will need for reproduction.

5. Once there is adequate RNA transcriptase, the viral RNA creates an antisense version (the paired opposite) of itself as a template for the creation of new viral RNA. The viral RNA is now copied hundreds or thousands of times, making the genetic material for new viruses. Some of this new RNA will contain mutations.
6. Viral RNA then directs the production of protein-based capsomeres (the building blocks for the virus'protective protein coat). Ribosomes create the proteins and release them for use.
7. The completed capsomeres assemble around the new viral RNA into new viral particles. The capsomeres are designed to attract each other and fit together in a certain way. When enough capsomeres are brought together, they self-assemble to form a spherical shell, called a capsid that fully encapsulates the virus's RNA. The completed particle is called a nucleocapsid.
8. The newly formed viruses travel to the inside portion of the plasma membrane and attach to it, creating a bud. The plasma membrane encircles the virus and then releases it - providing the virus with its protective lipid coat, which it will later use to attach to another liver cell. This process of budding and release of new viruses continues for hours at the cell surface until the cell dies from exhaustion.

Each surviving virus - those which are not destroyed by the immune system or other environmental factors - can produce hundreds or thousands of offspring. Over time, this endless cycle of reproduction results in significant damage to the liver, as millions upon millions of cells are destroyed by viral reproduction or by the immune system's attacks on infected cells.

Uncoating of the viral nucleocapsid liberates the positive strand genomic RNA into the cytosol, where it serves as mRNA for synthesis of the HCV polyprotein. The hepatitis C virus 5' untranslated regions contain an internal ribosome entry site which

controls HCV open reading frame translation (Honda *et al.*, 1996).

The large precursor polyprotein generated is translated at the endoplasmic reticulum membrane where the processing events take place, resulting in the generation of the three structural proteins and the seven non-structural proteins (Niepmann, 2013). At least two host cellular particles (Signalase and Signal peptide peptidase) are required for the processing of the HCV structural proteins, whereas two viral peptidases (NS2 and NS3/4A) are involved in the processing of the HCV non-structural proteins. The viral proteins remain associated with intracellular membranes after processing (Moradpour and Penin, 2013). Replication is categorized by the NS5B protein. The NS5A protein and the helicase NTPase domain of NS3 play an important regulatory part in virus replication (Lohmann, 2013).

NS5A acts as a dimer with a basic channel involved in RNA binding. Domain I and domain II of the NS5A protein are required for HCV replication in the replication complex. The phosphorylation state of NS5A modulates the balance between replication and later stages of the HCV life cycle. The NS3 helicase has an important role in separating nascent and template RNA strands, unwinding RNA secondary structures and displacing RNA-binding proteins (Schoogins and Rice, 2013). Non-structural 4B is an integral membranes protein with a role in membrane rearrangements that are induced by HCV proteins, leading to the formation of the “membranous web” or replication complex that supports and compartmentalizes HCV replication. The positive strand genome RNA serves as a template for the synthesis of a negative strand intermediate of replication.

Then, negative-strand RNAs serve as templates to produce numerous strands of positive polarity that are subsequently used for polyprotein translation, the synthesis of new intermediates of replication or packaging into new virus particles (Lohmann, 2013). Various host factors have also been shown to have important functional roles in the HCV life cycle. Cyclophilin A (also known as Peptidylprolyl isomerase A) binds to both NS5B and NS5B, thereby

inducing the conformational changes that are required for efficient HCV replication.

### 2.3.9 HCV LANDING

Hepatitis C virus is a blood-borne infections agent that only infects human and chimpanzees naturally. Within the host, though HCV can replicate to low levels in non-hepatic cells, such as cells derived from brain tissue (Forton, 2016), polarized hepatic cells are the primary platform for HCV landing and, unsurprisingly, express the full complement of entry receptors. The liver comprises mainly of hepatocytes, and also contains non-parenchymal cells such as endothelial, kuppfer, stellate cells, and lymphocytes (Meredith *et al.*, 2012). Hepatocytes are structurally and functionally polarized with three distinct membrane domains: the sinusoidal (basal), lateral, and canalicular (apical) surfaces (Mee *et al.*, 2009). The sinusoidal endothelium is highly fenestrated and closely associated with hepatocytes and stellate cells in the space of Disse.

Further DC-SIGN is expressed on some dendrite cells (DCs), while L-SIGN is abundantly expressed in sinusoidal endothelial cells. Many previous studies have shown that DCs may bind multiple pathogens via DC-SIGN at a site of mucosal exposure and carry the virus to target cells within the draining lymph node, thereby facilitating establishment of an infection (Geijtenbeck *et al.*, 2000).

Other studies showed that L-SIGN and DC-SIGN specifically bind HCV envelope glycoproteins E<sub>2</sub> (Gardner *et al.*, 2003), and this binding of DC-SIGN interactions (Pohlmann *et al.*, 2003).

This suggests that L-SIGN and DC-SIGN initially interact with hcv and deliver the virus to the target cells, which may explain hcv tissue tropism and contribute to the establishment or persistence of infection.

#### 2.3.9.1 CAPTURE OF VIRAL PARTICLES

Once HCV lipoviro particles (LVP) are present in the space of Disse, where the basolateral membrane of hepatocytes are exposed, LVPs are captured HSPG and LDLR on the surface of hepatocytes in a

spatiotemporally regulated manner. HSPG, namely heparin/heparan (HSGAG), is a particular glycosaminoglycan (GAG) that plays an important role during HCV entry. HSPG is dominant in the space of Disse and can mediate the metabolism of remnant lipoproteins (Mahley and Ji, 1999).

### 2.3 9.2 VIRAL PARTICLE ATTACHMENT

Structural ribosomal - B1 is a cell surface receptor in high density lipoproteins (HDL) found in various cell types (Muraio *et al.*, 1997). It is expressed in high levels in the liver and steroidogenic tissues (Douam *et al.*, 2015). Structural ribosomal - B1 (SR-B1) mediates lipid metabolism particularly the uptake and recycling of HDL particles and binds to different classes of lipoproteins, such as very low density lipoproteins (VLDL), low density lipoprotein (LDL) and HDL (Eck, 2008).

Hepatitis C virus -SR-BI interaction alters the conformation of HCV virions (Catanese *et al.*, 2010). Taken together, the attachment of HCV virus particles is mediated by E<sub>2</sub> and requires the lipid transfer activity of SR-BI, leading to E<sub>2</sub> conformational change.

### 2.3.10 TRANSMISSION OF HEPATITIS C VIRUS

The HCV is transmitted by parental routes, of which blood transfusion and intravenous administration of drugs are the most frequent (Mercy *et al.*, 2013). Before 1992, when screening of blood donors for hepatitis was introduced, transfusions with blood or products derived from blood increased the risk of transmission of HCV (Nishioka *et al.*, 2002).

Other potential routes by which the HCV may be transmitted include tattooing, the use of intranasal cocaine, body piercing, and accidental injuries with infected needles. Tattoos require special attention because the risk of HCV infection seems to be dependent on the size, number and site of the tattoos (Kao *et al.*, 1996).

Common risk factors for hepatitis C are:

1. Intravenous drug use.
- (2) Blood transfusions conducted before 1992
- (3) Accidental puncture with infected needles
- (4) Perinatal transmission (vertical

transmission) (5) Haemophilia (6) Haemodialysis (7) Transplant conducted before 1992

Uncommon risk factors for hepatitis C are;

1. Intranasal cocaine use,
- (2) Body piercing
- (3) Tattoos
- (4) Shared shaving equipment,
- (5) Sexual activity
- (6) Transmission from health workers to patients

Transmission of hepatitis C from health workers to patients in their care has been reported but is rare (Valtuile *et al.*, 1988). It is more likely that hepatitis will be transmitted from health workers during invasive procedures such as colonoscopy or cardiothoracic surgery (Bronowicki *et al.*, 1997). The virus may be transmitted to health workers by accidental puncture of the skin, the rate of transmission of hepatitis C by this route is lower than that of type B hepatitis but higher than that of HIV. Sexual transmission of hepatitis C occurs in less than 5% of cases (McQuillan *et al.*, 1999), but the transmission rate is increased in instances of multiple sexual partners, sex with prostitutes, anal sex, traumatic sex, sex during menstruation, or sex without suitable vaginal lubrication. In married couples, the risk of transmission increase with time (Bronowicki *et al.*, 1997), but the effect of common shaving blades has not been determined (Puro, *et al.*, 1995). As the HCV is rarely present in serum or vaginal fluid, the risk of sexual transmission of the HCV is very small. Oral sex does not constitute a risk except when mouth ulcers or gingival bleeding is present (Jonas, 2002). Perinatal transmission of hepatitis occurs in 3% to 5% of infants born to mothers infected with HCV. The risk of perinatal transmission is greater in mothers with high viral loads, anti-HCV IgM at the time of delivery, or HCV (Mansell and Locarnimi, 1995). Studies have shown that the risk of perinatal transmission is less when delivery is via caesarian section (CS) than when delivery is via the vaginal channel. Infants born to mothers infected with the HCV may acquire anti-HCV antibodies through transplacental transfer. Such antibodies persist during the first year of life and then disappear. Therefore, detection of HCV RNA in the serum of newborns is necessary to make a positive diagnosis of HCV. As some studies have shown that infants who were breast fed by HCV-infected

mothers did not become infected, nursing should not be prevented (Briggs *et al.*, 2001).

### 2.3.11 PATHOGENESIS OF HEPATITIS C VIRUS

Hepatitis C virus is a non-cytopathic virus (Irshad and Dhar, 2006) that enters the liver cell and undergoes replication simultaneously causing cell necrosis by several mechanisms including immune – mediated cytolysis in addition to various other phenomena such as hepatic steatosis, oxidative stress and insulin resistance. The proteins/peptides encoded by different sub-genomic regions of the HCV genome and their quasi species influence the above mechanism, and thus, have a significant role in HCV pathogenesis and disease causation.

#### 2.3.11.1 VIRAL ENTRY

Hepatitis C virus is a blood -transmitted virus that reaches the liver via circulation. The entry of HCV isolates requires at least 4 host-derived factors including scavenger receptor class B type 1, occluding, claudin-1 (CLDN1) and CD81. In addition, CLDN6 and CLDN9 have been shown to substitute for CLDN1 as HCV entry factors in human non-liver cells (Haid *et al.*, 2013). The CD81 mole on host cell surfaces acts as a viral receptor, which binds with the viral particle and facilitates its entry in the liver cell (Masciopinto *et al.*, 2002 and Zeisel *et al.*, 2013).

Cluster of differentiation 81 is expressed on the surface of almost all nucleated cells and complexes with a variety of other cell-surface receptors such as CD19 and CD21 on B cells, and sends a costimulatory signal to the cells (Maeker *et al.*, 1997). The viral envelope protein, E2, binds to the major extracellular loop of CD8 (Flint *et al.*, 1999). HepatitisC virus shows multi-site binding and can also bind to several other molecules such as the receptor for low density lipoprotein (LDL), the dendritic cell (DC) – specific intercellular adhesion molecule 3 – grabbing non-integrin (DC-SIGN) and its liver counterpart (Lozach *et al.*, 2003 and Scarselli *et al.*, 2002). The envelope protein, E2 is the most variable viral proteins and therefore, its interactions with CD81 have been reported to be strain specific (Roccasecca *et al.*, 2003). It has two hyper variable

regions, HVR-1 and HVR-2 which undergo frequent mutations, possibly due to virus-neutralizing antibodies and HCV-specific cytolytic T lymphocytes (CTLs). HepatitisC virus also has a high mutation rate due to the lack of proof reading ability of its RNA-dependent RNA polymerase. Therefore, HCV exist in several distinct, but closely related virus species within an infected individual. These species are called HCV quasi species.

#### 2.3.11.2 Host immunity

##### 2.3.11.2.1 Innate Immunity

Innate immunity presents a first line defense for the control of HCV infection as it does for several other viral infections. During HCV infection, cells produce type I interferon which prepares and induces the cells to resist infection, check viral replication, promote adaptive immunity and activate natural killer (NK) cells, DCs and Kupffer cells etc. Once inside the cell, the innate immunity verse HCV is triggered through host recognition of viral macromolecular motifs, known as pathogen-associated molecular patterns (PAMPS) as non-self by cellular pathogen recognition receptors. These receptors include toll-like receptors (TLRs) and retinoic acid inducible gene-1 (RIG-1) like receptors (RLRs) (Saito *et al.*, 2008). The retinoic acid inducible gene, RIG-1 binds PAMP on HCV-RNA and activates interferon regulating factor-3 (IRF-3) for expression of interferon (IFN) and anti-viral/interferon stimulated genes (ISGs) (Liu and Gale, 2010). The secreted IFN and cytokines then activate NKs, DCs, and Kupffer cells. These cells also play a significant role in mounting T/B cell-based immunity (Saito and Gale, 2008).

Hepatitis C virus can effectively evade innate immunity resulting in persistent viral infection. This occurs because HCV has evolved to counteract the RIG-1 pathway (Schoggins and Rice, 2013) and thus evade immune challenge. This phenomenon is the reason for chronicity in the majority of HCV infected patients. For this, the non-structural proteins of HCV i.e. NS3 and NS4A form a complex which activates the NS protease domain to target cleavage of IPS-1. After cleavage, IPS-1 can no longer signal downstream to activate IRF-3 and Necrosis factor B

and the infected cells no longer produce IFN or express ISGS (Loo *et al.*, 2006).

Natural killer cells (Nk) are major arm of innate immunity, play an important role in eradication of HCV. The liver is enriched in NK cells that are usually activated in an early phase of HCV infection. The activated NK cells recruit virus-specific T cells and induce antiviral immunity in their liver. They also eliminate virus infected hepatocytes directly by cytolytic mechanisms and indirectly by secreting cytokines including IFN and TNF. These cytokines induce an antiviral state in host cells. Surprisingly, hepatitis C virus has evolved multiple strategies to counter the host's NK cell response. It is interesting that activated Nk cells contribute toward liver injury, while inactive or compromised Nk cells permit the virus to continue invasion (Golden-mason and Rosen, 2013).

#### 2.3.11.2.2 ADAPTIVE IMMUNITY

After entry and replication of the virus inside liver cells, the viral molecules are transferred to the endoplasmic reticulum and associate with major histocompatibility complex (MHC) molecules, which are finally transported to the cells surface.

These molecules on the cell surface are recognized by T cells for their immune action. The majority of CTLs and CD8<sup>+</sup> and recognize antigen presented on MHC class I molecules. Approximately 10% of CTLs and CD8<sup>+</sup> which recognize antigen presented on MHC II molecules. These CTLs eliminates cells infected with virus. However, hepatitis C virus is reported to have evolved mechanisms to avoid recognition by CTLs. They either reduce the expression of MHC molecules or prevent the viral peptide from presentation at the cell surface. Thus, CTLs play a major role in viral eradication (Zinkernagel *et al.*, 1986) and immunopathogenesis of HCV infection (Neumann-Haefelin and Thimme, 2013).

#### 2.3.11.3 METABOLIC CONDITIONS AFFECTING HCV PATHOGENESIS

In addition to immune mediated HCV pathogenesis, there are several other clinical and metabolic

conditions that have a strong association with HCV pathogenesis. These include HCV induced insulin resistance, oxidative stress and hepatic steatosis. The following is a brief description of the conditions affecting HCV pathogens.

#### 2.3.11.4 HCV-INDUCED INSULIN RESISTANCE

Hepatitis C virus infection influences overall metabolism leading to increased steatosis, fibrosis, inflammation, apoptosis and insulin resistance (IR) (Arrese *et al.*, 2010) during the course of the disease. The resulting insulin resistance shows modulating impact on liver pathogenesis by HCV infection (Bieche *et al.*, 2005). Insulin resistance increase the de novo lipogenesis i.e., fatty acid (FA) synthesis via over expression and maturation of SREBP-1C. This in turn, increases the activities of lipogenic enzymes including Acetyl CoA carboxylase and FA synthase. At the same time, intermediates of triglycerides biosynthesis also activate inhibitors of insulin signaling. For example, activation of protein kinase C- $\epsilon$  by phosphorylating insulin receptor substrate and thus inhibiting phosphatidylinositol 3,4,5 triphosphate (Foster, 2007) inhibits translocation by ceramides etc. (Holland and Summers, 2008). Hepatitis C virus -core protein, either directly or via increased secretion of TNF causes insulin resistance, (Shintani *et al.*, 2004 and Pazienza *et al.*, 2007).

#### 2.3.11.5 HCV-ASSOCIATED OXIDATIVE STRESS:

Oxidative stress is reported to be an important part of HCV-induced liver damage. Hepatitis C virus-core proteins present within the outer membrane of mitochondria induces oxidation of glutathione and promote Ca<sup>2+</sup> uptake into mitochondria. The presence of oxidative stress has been noted in different types of hepatitis including hepatitis B. However, there is a marked increase in oxidation stress (OS) in HCV infection (Fujita *et al.*, 2008). Several studies have shown that structural components of HCV induce effective oxidative stress (Fujita *et al.*, 2008).

#### 2.3.11.6 HCV-INDUCED STEATOSIS

Hepatitis C virus infection is reported to have a strong association with hepatic steatosis. There are

several other factors also responsible for steatosis, which include alcohol consumption, obesity, and diabetes (Kan *et al.*, 2010). Studies on steatosis in relation to hepatotropic viruses demonstrated that HCV infection directly causes steatosis in some patients (Rubbia-Brandt *et al.*, 2001). This report concluded that HCV causes steatosis in three different ways:

1. Impaired secretion of lipids from hepatocytes; (2) Increased de novo synthesis of free fatty acids (FFAs) and; (3) Impaired Fatty acid degradation. The first aspect of HCV induced steatosis was proposed due to the impaired secretion of very low density lipoprotein (VLDL). To substantiate this, report from different studies demonstrated a decreased level of apolipoprotein B and cholesterol in chronic HCV infected patients (Shi *et al.*, 2002 and Serfaty *et al.*, 2001). These low levels pointed to HCV disturbing the assembly and secretion of VLDL from the liver (Perlemuter *et al.*, 2002).

### 2.3.12 COMORBIDITIES AND MORTALITY

Chronic HCV infection can result in hepatic fibrosis, cirrhosis and hepatocellular carcinoma. The progression through these stages is a function of time since infection and age of initial infection. Japan, which has the oldest HCV infected population, is already seeing a drop in hepatocellular carcinoma (Osaki and Nishikawa, 2015), as infected individuals are dying because of other causes before progressing to hepatocellular carcinoma. However, in nearly every other country, the projected number of hepatocellular carcinoma and decompensated cirrhosis cases as a result of HCV infection has been increasing and will continue to increase in the absence of treatment and universal screening programmes and interventions (Alfaleh, 2015). A recent study showed that, worldwide, the number of hepatitis C related deaths as a result of hepatocellular carcinoma and cirrhosis increased from 895, 000 deaths in 1990 to 1,454, 000 deaths in 2017; the proportion of deaths that were attributed to hepatitis C without hepatocellular carcinoma also increased in the same period from 23.8% in 1990 to 48.4% in 2015 (Stanaway, 2016).

In addition to liver-related complications, HCV infection is associated with numerous extrahepatic manifestations (Negro *et al.*, 2015). Individuals with chronic HCV infections are more likely to develop cryoglobulinaemia and non-Hodgkin lymphoma (Negro *et al.*, 2015).

Finally, fatigue is more common among those with chronic HCV infection and in patients with lower health-related Quality of life, QOL (HRQOL); fatigue and HRQOL scores improve after achieving a sustained virological response (SVR).

### 2.3.13 DIAGNOSTIC TEST FOR HEPATITIS C VIRUS

Diagnostic tests for HCV infection are divided into serologic assays for antibodies and molecular tests for viral particles. Screening assays based on antibody detection have markedly reduced the risk of transfusion related infection, and once persons seroconvert, they usually remain positive for HCV antibodies. However, recent data indicate that the level of HCV antibodies decreases gradually over time in the few patients in whom infection spontaneously resolves (Takaki *et al.*, 2000).

The primary serologic screening assay for HCV infection is the enzyme immunoassay, for which there have been three consecutive versions with a resultant progressive increase in sensitivity. The currently used second and third generation enzyme immunoassays contain core protein as well as nonstructural proteins 3 and 4 (the third generation assay also contains non-structural protein 5) and can detect antibodies within 4 to 10 weeks after infection. In low risk populations, the test misses only 0.5 to 1 percent of cases (Vrieling *et al.*, 1997). It can be falsely positive, especially in persons without risk factors and without sign of liver disease, such as blood donors or health care workers, and therefore, other tests must be used to confirm infection in these persons. Furthermore, false negative tests can occur in persons with immune compromise, such as HIV-1 infection (Cribier *et al.*, 1995), patients with renal failure; and those with HCV associated essential mixed cryoglobulinaemia (Agnello *et al.*, 1992).

The recombinant immunoblot assay has been used to confirm positive enzyme immunoassays. It uses antigens similar to those for the enzyme immunoassay but in an immunoblot format, so that the responses to the individual proteins can be identified. A positive assay is defined by the detection of antibodies against two or more antigens, and an indeterminate assay by the detection of antibodies against a single antigen. In the past few years new assays based on the molecular detection of HCV RNA have been introduced. These tests can be categorized as qualitative and quantitative. Since viral RNA is unstable, the appropriate processing of samples is critical to minimize the risk of false negative results; samples to be tested should be separated and frozen within three hours after phlebotomy (Busch *et al.*, 1992). Qualitative HCV RNA tests are based on the PCR technique and have a lower limit of detection of fewer than 100 copies of HCV RNA per milliliter (Belden *et al.*, 2000). These are the tests of choice for the conformation of viraemia and the assessment of treatment response.

The viral load has been shown to be relevant to the outcome of anti-HCV therapy (Poynard *et al.*, 1998), but not to predicting the likelihood of disease progression. Three commercial tests are currently available to quantitative the degree of viraemia: a branched-chain DNA assay (Quantiplex hcv RNA, version 20) and two assays involving reverse transcription PCR. All systems deliver reliable, but not easily comparable results (Martinot-Peignoux *et al.*, 2000), since no standard system of expressing the viral load has been established.

Histologic evaluation of a liver-biopsy specimen remains the gold standard for determining the activity of HCV-related liver disease, and histologic staging remains the only reliable predictor of progression (Yano, 1996). A biopsy may also help to rule out other, concurrent causes of liver disease. Therefore, biopsy is generally recommended for the initial assessment of persons with chronic HCV infection (ICC, 1999).

### 2.3.14 PREVENTIVE STRATEGIES FOR HEPATITIS C VIRUS

Primary prevention activities include: Screening and testing of blood, plasma, tissue, organ and semen donors; virus inactivation of plasma derived products, risk reduction counseling services and inflammation of interferon control practices.

### 2.3.15 TREATMENT OF HEPATITIS C VIRUS

All patients with chronic hepatitis C infection should be considered potential candidate for drug therapy (Kim and Saah, 2005). Treatment is recommended for patients who are at risk of developing cirrhosis, generally defined by a measurable hepatitis C RNA level and liver biopsy showing portal or bridging fibrosis along with moderate inflammation and necrosis (Ghany *et al.*, 2009).

A pretreatment liver biopsy is not mandatory but may be helpful in patients with normal transaminase levels, particularly those with a history of alcohol dependence, in whom little correlation may exist between liver enzyme levels and histologic findings (NIH, 1997). Spontaneous resolution of hepatitis C virus is common and waiting 2-4 months before initiation of therapy is recommended (NIH, 1997). The objective of therapy is to eradicate the virus and prevent potential complications from chronic HCV infection. If detected early, progression of chronic hepatitis to severe liver disease can be prevented in 54 – 63% of patients through antiviral treatment (Friedrich –Rustet *et al.*, 2007). Efficacy of treatment is assessed by measuring Hepatitis C RNA Viral Load. The goal is to achieve a Sustained Virological Rate (SVR), defined by the continued absence of hepatitis C RNA for six months after the completion of treatment (Ghany, *et al.*, 2009). Treatment for chronic HCV infection has evolved from interferon monotherapy, which results in an SVR of 10 to 20% (Mcttutchison *et al.*, 2001) to combination therapy with interferon plus ribavirin, which is associated with a higher SVR rate of nearly 40% (Poynardet *et al.*, 1998).

The duration of standard interferon plus ribavirin therapy has been based on the viral genotype and the pretreatment viral load (EASL, 1999). The sustained

virology response (SVR) rates for patients infected with genotype 2 or 3 are essentially the same for 24 and 48 weeks of therapy, showing no benefit for the longer course of therapy (Lee *et al.*, 2002). For patients infected with genotype I isolates, 48 weeks of interferon plus ribavirin therapy is recommended for those with a high viral load (> 800,000 iu/ml) and only 24 weeks of therapy for patients with those with as low pretreatment viral load (< 800,000 iu/ml)(Pawlowsky *et al.*, 2000).

### 2.3.16 IRON METABOLISM

Iron is essential for life, but both severe iron deficiency and iron overload pose significant and potentially fatal health risks (Dicky, 2002). In human body, iron is crucial for maintaining the fundamental function of many proteins (Paganelli *et al.*, 2016 and He *et al.*, 2016). Iron in the diet is absorbed through divalent metal transporter I (DMT I) (Winter *et al.*, 2014), a multi-trans membrane protein, or heme carrier protein (Winter *et al.*, 2014). It is then exported by the ferroportion (FPN) to bind to transferrin in the blood stream (Chen *et al.*, 2009) and taken to the erythroblasts for erythropoiesis. Iron that is not utilized can also be stored as ferritin or haemosiderin (Winter *et al.*, 2014), in enterocytes, macrophages, and hepatocytes. There are no efficient physiologic mechanisms to excrete iron, a little iron (about 2mg per day) is lost by sloughing of intestinal epithelial cells, desquamation of skin and urinary cells, blood loss, and sweat (Zou and Sun, 2017).

### 2.3.17 IRON HOMEOSTASIS

Iron homeostasis in the human body is mainly regulated by the hepcidin/ferroportin and iron-regulatory protein/iron-response element (IRP/IRE) systems. Hepcidin is a peptide hormone mainly secreted by the liver (Winter *et al.*, 2014). When iron levels increase, hepcidin negatively regulate iron levels by binding to the ferroportin and promoting the internalization and degradation of ferroportin (Nemeth *et al.*, 2004). This reduces the amount of iron absorbed by enterocytes, released by hepatocytes, and recycled from macrophages, and finally reduces transferrin levels. Iron homeostasis may affect the clinical course of HCV infection. The findings of some studies have suggested that excess iron in the

liver may predispose a patient to persistent viral infection and could have a negative effect on the response to interferon (IFN) therapy (Fargion *et al.*, 2002).

Iron may thus modulate the course of HCV infection via 2 mechanisms. First, iron interacts directly with cell mediated effector immune pathways, thereby weakening Th1-mediated effector mechanisms, such as the formation of nitric oxide and the production of TNF- $\alpha$  (Recalcati *et al.*, 1998).

Second, iron may worsen the clinical course of HCV infection by causing oxidant stress in nonparenchymal cells, which appear to cause irreversible mitochondria derangements associated with the onset of hepatic fibrosis (Pietrangelo *et al.*, 2002).

### 2.3.18 FERRITIN

Ferritin provides the major storage form of iron, whereas transferrin is the major iron transport protein in humans. Ferritin is a ubiquitous protein and is found in all cells (Bell *et al.*, 1994). Ferritin is an acute phase protein, and its levels are increased in response to iron overload and systemic inflammation (Blumberg *et al.*, 1984). Ferritin is the primary tissue iron storage protein in the liver, where most extra body iron is stored, and its expression in this organ is induced in primary or secondary iron overload disorders, resulting in increased hepatitis and circulating ferritin levels (Bell *et al.*, 1994). Hepatic accumulation of excess iron can be toxic, initiating harmful free radical reactions (Bonkovsky, 2002), which may result in liver damage. Iron may be important for the progression of liver fibrosis in viral hepatitis, and serum iron parameters, especially ferritin levels, may reflect hepatic iron accumulation (Bonkovsky, 2002). Several studies have shown that serum ferritin can be increased in chronic liver diseases due to hepatitis C or B and alcohol (Chandok *et al.*, 2012). Some authors have evaluated serum iron parameters in hepatitis C virus (HCV)– or HBV- related cirrhosis (D'Souza *et al.*, 2005).

The iron content of the human body may be divided into three classes: iron in storage, iron in use, and iron in transport. Iron in storage is reserved iron

contained with the cells. Iron in use contained in haemoglobin, various enzymes, and several other types of proteins. Iron in transport is being moved to storage or is being removed from storage to be utilized in the formation of haemoglobin, etc.

Iron in a free state is not only relatively insoluble, but it is toxic. Therefore, nearly all iron in the body is attached to some type of protein. It is of fundamental importance to note that a specimen should be analyzed for both values in the differential diagnosis of various types of anaemia and liver disease. For this reason, the current procedure is designed for the simultaneous determination of iron and iron binding capacity (Henry, 1984 and Tietz, 1976).

Serum iron assays measure transport iron bound to the protein transferrin. Increase in serum iron levels may indicate increase erythrocyte destructive, decreased erythrocyte formation, increased absorption, or defects in storage capabilities. Decrease in serum iron levels may indicate iron deficiency or inability to retrieve storage iron. Iron binding capacity is usually increased to iron deficient anaemia and decreased in haemochromatosis, malignancies, rheumatic fever, Hodgkin's disease, and collagen vascular disease (Henry, 1984).

The expected value of total iron is 60 – 150ug/dl and total iron binding capacity, TIBC is 250 – 400ug/dl (Henry, 1984).

### 2.3.19 HAEMATOLOGICAL VARIABLES

These are variables that are related to blood and blood forming organs. These haematological variables are influenced by various factors like age, ethnicity, diet, genetic and gender differences and hence it is important to define the specific reference values with regards to the age, gender and region.

The haematological variables include red blood cells (RBC), white blood cells (WBC), platelet, haemoglobin concentration, haematocrit, red cell indices etc.

#### 2.3.19.1 RED BLOOD CELLS

Red blood cell also known as red blood corpuscles, hematids, erythroids cells or erythrocytes (Vinar *et al.*, 2007) (from Greek erythros for “red” and kytos

for “hollow vessel” with - cyte translated as “cell” in modern usage), are the most common type of blood cell and vertebrates principal means of delivering oxygen (O<sub>2</sub>) to the body tissues – via blood flow through the circulatory system (Richard *et al.*, 2007). Red blood cells (RBCs) take up oxygen in the lungs and release it into tissues while squeezing through the body's capillaries. The red blood cells measures 6-8 µm in diameter. The cytoplasm of erythrocytes is rich in haemoglobin, an iron-containing biomolecule that can bind oxygen and is responsible for the red color of the cells. The cell membrane is composed of proteins and lipids, and this structure provides properties essential for physiological cell function such as deformability and stability while traversing the circulatory system and specifically the capillary network. In humans, mature red blood cells are flexible and oval beconcave discs. They lack a cell nucleus and most organelles in order to accommodate maximum space for haemoglobin, they can be viewed as sacks of haemoglobin, with a plasma membrane as the sack.

Approximately 2.4 million new erythrocytes are produced per second in human adults (Erich and Sackmann, 1995). The cells develop in the bone marrow and circulate for about 100-120 days in the body before their components are recycled by macrophages. Each circulation takes about 60 seconds (Bloom, 2003). Approximately, a quarter of the cells in the human body are red blood cells (Laura, 2003).

Nearly half of the blood's volume (40% to 45%) is red blood cells. The red blood cells of an average adult male stored collectively about 2.5 grams of iron, representing about 65% of the total iron contained in the body (Synder *et al.*, 1999). These cells have a volume of about 90 femptolitre (90fl) and can swell up to a sphere shaped containing 150fl, without membrane distension. An adult humans have roughly  $5 \times 10^{12}$  (50 trillion) red blood cells for litre of blood at any given time, comprising approximately one quarter of the total human body cell number (women have about 4-5 million erythrocytes per microlitre of blood and men about 5-6 million, people living at high altitudes with low oxygen tension will have more).

### 2.3.19.2 WHITE BLOOD CELLS

White blood cells also known as leukocytes are the cells of immune system that are involved in protecting the body against both infectious disease and foreign invaders (Maton *et al.*, 1997). All white blood cells are produced and derived from multi potent cells in the bone marrow known as haematopoietic stem cells. Leukocytes are found through the body, including the blood and lymphatic system (Maton *et al.*, 1997). All white cells have nuclei, which distinguishes them from the other blood cells, the anucleated red blood cells (RBCs) and platelets. The number of leukocytes in the blood is often an indicator of disease, and thus the white blood cell count is an important subset of the complete blood count. The normal white cell count is usually between  $4 \times 10^9/L$  and  $11 \times 10^9/L$  of blood making up approximately 1% of blood in a healthy adult (Alberts *et al.*, 2002), making them substantially less numerous than the red blood cells at 40% to 45%. However, this 1% of the cells at 40% to 45%. However, this 1% of the blood makes a large difference to health, because immunity depends on it. Types of white blood cells can be classified in standard ways. Two parts of broadest categories classify them either by structure (granulocytes or agranulocytes) or by cell lineage (Myeloid cells or lymphoid cells). These broadest categories can be further divided into the five main types: neutrophils, eosinophils (acidophiles), basophils, lymphocytes, and monocytes (Laufleur-Brooks, 2008). These types are distinguished by their physical and functional characteristics. Monocytes and neutrophils are phagocytic.

Further subtypes can be classified, for example, among lymphocytes, there are B cells, T cells and natural killer (NK) cells. Further subtypes can be classified. Granulocytes are distinguished from agranulocytes by their nucleus shape (lobed versus round, that is, polymorphonuclear versus mononuclear) and by their cytoplasm granules (present or absent, or more precisely, visible on light microscopy or not thus visible).

#### 2.3.19.2.1 NEUTROPHILS

The fully matured neutrophil measures  $12 \mu\text{m}$  in diameter, has a nucleus with 3-5 lobes, separated by thin strands of chromatin. These cells, constitute about 60-70% of the circulating leukocytes. (Albert *et al.*, 2000). The cytoplasm appear light pink to blue, and the numerous, small granules, pinkish in a Romanosky stained blood film, a characteristics nuclear appendage may be seen in 2-3% of neutrophils, in a stained blood films of normal female individual (Abayomi, 2007).

Neutrophils defend against bacteria or fungal infections and other inflammatory processes. They are usually first responders to microbial infection, their activity and death in large numbers form pus. The neutrophilic granules are divided into primary, which appear at the promyelocytes stage, and secondary (specific) which appear at the myelocyte stage and predominate in the mature neutrophil (Hoffbrand and Moss, 2011). Both types of granules are lysosomal in origin: the primary contains myeloperoxidase, acid phosphate and other acid hydrolases, the secondary contains collagenase, lactoferrin and lysozyme. The life span of neutrophil in the blood is only 6-10 hours (Hoffbrand and Moss, 2011).

#### 2.3.19.2.2 EOSINOPHILS

These cells are similar to neutrophils, except that the cytoplasmic granules are coarser and more deeply red staining, with two nuclear lobes. Eosinophils myelocytes can be recognized but earlier stages are indistinguishable from neutrophil precursors (Brito-Babapulle, 2003). The blood transit time for eosinophils is longer than for neutrophils. They measures  $12 \mu\text{m}$  in diameter and constitutes 2-4% of the total leukocytes in normal blood. They enter inflammatory exudates and have a special role in allergic response, defense against parasites and removal of fibrin formed during inflammation (Abayomi, 2007).

#### 2.3.19.2.3 BASOPHILS

These are only occasionally seen in normal peripheral blood. They have many dark cytoplasmic granules

which overlie the nucleus and contain heparin and histamine (Hoffbrand and Moss, 2011). Basophils, measuring 9  $\mu\text{m}$  in diameter has a large nucleus with an irregular twisted shape. It constitutes 0-1% of the total blood leukocytes. They have immunoglobulin E (IgE) attachment sites and their degranulation is associated with histamine release.

#### 2.3.19.2.4 LYMPHOCYTES

Lymphocytes are spherical cells, of two types: large, 8-16  $\mu\text{m}$  and small 6-8  $\mu\text{m}$ . The nucleus is usually spherical or round in shape, eccentrically placed. In small lymphocytes, the cytoplasm is scanty, appearing as a thin rim around the nucleus (Abayomi, 2007).

The small lymphocytes, being the more mature form, is more frequently seen in adult blood. Nucleoli are not generally seen. The cytoplasm appears as a dark blue band around the nucleus and azure dust is usually absent.

The large lymphocyte has a slightly larger nucleus and more abundant cytoplasm. The nucleus stains lightly as compared to that of small lymphocyte. The cytoplasm is pale blue in color. The lymphocyte count of normal blood may range between 20-45% (1000-4500/  $\mu\text{l}$ ) (Abayomi, 2007). There are two subpopulations of lymphocytes, T cells and B cells, which are morphologically indistinguishable in the peripheral blood smear. Both T and B lymphocytes are immune competent cells which act to direct and effect the immune defense system of the body. The cells migrate to various sites in the body to await antigenic stimulus. When activated, they respond to antigenic challenges. B-lymphocytes produce antibodies whereas T-lymphocytes are responsible for the cell mediated immunity (Abayomi, 2007).

#### 2.3.19.2.5 MONOCYTES

Monocytes, the largest type of white blood cells, share the "vacuum cleaner" (phagocytosis) function of neutrophils, but are much longer lived as they have an extra role: the present pieces of pathogens to T cells so that the pathogens may be recognized again and killed. This causes an antibody response to be mounted. Monocytes eventually leave the

bloodstream and become tissue macrophages, which remove dead cell debris as well as attack microorganisms.

Neither dead cells debris nor attacking microorganisms can be dealt with effectively by the neutrophils. Unlike neutrophils, monocytes are able to replace their liposomal contents and are thought to have a much longer active life. They have the kidney shaped nucleus and are typically agranulated. They also possess abundant cytoplasm.

#### 2.3.19.3 PLATELETS

The platelets, also known as thrombocytes are the smallest cells in the peripheral blood, are minute discs, 2-4  $\mu\text{m}$  in diameter. The normal range is between 150,000 to 400,000 per microlitre of blood. The platelets produced from the fragmentation of the megakaryocytes in the bone marrow and blood as minute bodies, are non-nucleated and can therefore, not reproduce. Platelets have a life span of 7-10 days in the blood and are eliminated by the spleen and to a lesser extent, the liver.

### 2. 4 SUMMARY OF REVIEW

Hepatitis C is a liver disease caused by the hepatitis C virus (HCV). The virus can cause both acute and chronic hepatitis, ranging in severity from a mild illness lasting a few weeks to a serious lifelong illness. The hepatitis C virus is a ribonucleic acid (RNA) blood-borne virus of the Flaviviridae family. The most common modes of infection of HCV are through exposure to small quantities of blood. This may happen through injection drug use, unsafe injection practices, unsafe health care, and the transfusion of unscreened blood and blood products. It can also occur through sex. All these modes are referred to as "horizontal mode of transmission". It has also been noted that it can be transmitted via vertical transmission, i.e. from an infected mother to the baby. Globally, an estimated of 71 million people have chronic hepatitis C infection. A significant number of those who are chronically infected will develop cirrhosis or liver cancer. Approximately, 399000 people die each year from hepatitis C, mostly from cirrhosis and hepatocellular carcinoma (HCC). The incubation period for hepatitis C is two weeks to

six months. Following initial infection, approximately 80% of people do not exhibit any symptoms. Those who are acutely symptomatic may exhibit fever, fatigue, decreased appetite, nausea, vomiting, abdominal pain, dark urine, grey-coloured faeces, joint pain and jaundice (yellowing of the skin and the whites of the eyes). Diagnosis of HCV infection is of two steps; screening for anti-HCV antibodies with a serological test identifies people who have been infected with the virus. Secondly, if the test is positive for anti-HCV antibodies, a nucleic acid test for HCV RNA is needed to confirm chronic infection because about 30% of people infected with HCV spontaneously clear the infection by a strong immune response without the need for treatment. Antiviral medicines such as sofosbuvir, daclatasvir, and the ledipasvir in combination can cure more than 95% of persons with hepatitis C infection, thereby reducing the risk of death from liver cancer and cirrhosis. There is currently no vaccine for hepatitis C; however, research in this area is ongoing.

### III. MATERIALS AND METHOD

#### 3.1 AREA OF STUDY

The study was conducted in Ebonyi State. Ebonyi State is in the South-East geo-political zone of Nigeria. It is located at 6°N of Equator, and 8°E of latitude. It shares a border with Benue State to the North, Enugu State to the West, Imo and Abia States to the South, and Cross River State to the East. It covers an area of 5,533 km<sup>2</sup>. The state has an estimated population of 1,339,136 (2014 updated). The state is divided into thirteen local government areas. The people of the state are predominantly igbo people and are farmers and traders. Some tertiary institutions are located in the state such as Alex Ekwueme Federal University Ndufu-Alike Ikwo (AE-FUNAI), Ebonyi State University (EBSU) and others. It has health facilities like Federal Teaching Hospital Abakaliki (FETHA), Mile 4 hospital and others (FECAI, 2014).

#### 3.2 SUBJECTS

A total of 130 volunteers aged, 20 to 40 years were recruited from all the 13 local governments of Ebonyi State. The number of males were 60 while females

were 70. All their serum samples were tested for anti-HCV antibody. All the tests were carried out strictly according to manufacturer's instructions. Doubtful results were repeated and inconclusive ones excluded.

#### 3.3 SAMPLE SIZE CALCULATION

The sample size, n used for the study was calculated using the formula below:

$$n = \frac{Z^2(p)(1-p)}{d^2} \quad (\text{Araoye, 2004})$$

where

n = the desired sample size when the population is more than 10,000

Z = Standard variation usually set at 1.96 (which corresponds to 95% confidence interval)

p = the proportion in the target population estimated to have a particular characteristic (prevalence of HCV infection in children reported in Ibadan, South West Nigeria, by (Okonkwo *et al.*, 2015) was 9% which is equal to 0.09

d = degree of accuracy desired; set at 5% (i.e. tolerated error of 5% which is equal to 0.05

Therefore, the minimum sample size,

$$\frac{(1.96)^2(0.09)(1-0.09)}{(0.05)^2} = \frac{3.8416 \times 0.09 \times 0.91}{0.0025} = 126$$

The minimum sample size, n is 126 subjects.

#### 3.4 STUDY DESIGN

This work adopted survey-based study.

#### 3.5 ETHICAL APPROVAL

Ethical approval was obtained from the Health Research and Ethical Committee of the Federal Teaching Hospital, Ituku/Ozalla, Enugu State.

### 3.6 SCREENING INTERVENTION

We conducted screening activities of Hepatitis C Virus infection in thirteen local governments ( Abakaliki, Afikpo North, Afikpo South, Ebonyi, Ezza North, Ezza South, Ikwo, Ishielu, Ivo, Izzi, Ohaozara, Ohaukwu, and Onicha) of the state from January to July, 2018. All the residents participated in the screening activities were voluntarily and signed informed written consent. Items of screening activities included questionnaire, and blood sampling for viral hepatitis C marker (anti-HCV antibody). The first stage included recording baseline characteristics of participants, questionnaire (supporting information) and blood sampling for viral marker, haematological tests, and iron bio-markers tests. In questionnaire, current co-morbid diseases, knowledge of chronic liver diseases, detail history of alcohol and viral hepatitis, history of occurrence of complications of liver diseases, and family history of liver diseases were asked and recorded. The second stage held three weeks after the first stage. In this second stage, we explained all of the examination reports to participants and referred those who required further evaluation or treatment to hospital.

### 3.7 INFORMED CONSENT

Blood samples were collected only to those who have duly signed informed written consent.

### 3.8 SAMPLE COLLECTION

Approximately 8.0ml of blood were collected aseptically from each volunteer using a sterile syringe and needle from a prominent vein situated at the antecubital fossa and placed 3.0ml in EDTA container and 5.0ml in a plain container for assessments of haematological parameters and serum iron markers respectively. Blood samples were transported in insulated containers containing ice packs to Marylmas Diagnostic and Research Laboratory Ltd, opposite G-hostel Ntezi-aba, Abakaliki Ebonyi state, where the complete blood count (CBC) were carried out immediately and the serum of each sample in a plain container were separated from the whole blood and placed in fresh plain tubes and stored in the deep freezer for

preservation and hereafter tested for iron bio-markers.

### 3.9 ASSAYS

#### 3.9.1 TOTAL IRON

Principle: The iron in serum is dissociated from its fe(III)-transferrin complex by the addition of an acidic containing hydroxylamine. This addition reduces the fe (III) to fe (II)-complex that is measured photometrically at 560nm.

#### Reagents

1. Iron buffer reagent (2) Iron color reagent (3)Iron standard

#### Procedure

1. Three test tubes were labeled test, blank and standard
2. 2.5ml of iron buffer reagent were added in all the tubes.
3. 0.5ml (500 µl) of sample were added in the tube labeled test
4. 0.5 (500 µl) of standard were added in the tube labeled standard
5. 0.5ml (500 µl) of iron-free water (deionized water) were added in the tube labeled blank.
6. The spectrophotometer were zero at 500nm with the reagent blank
7. The absorbance of all the tubes were read and recorded as A<sub>1</sub> reading
8. 0.05ml (50 µl) of iron color reagent were added in all the tubes.
9. It was mixed properly
10. All the tubes were incubated in a water bath at 37<sup>0</sup>C for 10 minutes
11. The instrument was also zero at 560nm with the reagent blank
12. The absorbance of all the tubes were read and recorded as A<sub>2</sub> reading.

#### Calculations

A = Absorbance, Std = Standard

$A_{2test} - A_{1test}$

$\frac{A_{2std} - A_{1std}}{\text{The concentration of st}}$  x conc. of std = Total iron (µg/dl)

### 3.9.2 UNSATURATED IRON BINDING CAPACITY (UIBC)

Principle: The unsaturated iron binding capacity (UIBC) is determined by adding Fe(II) ion to serum so that they bind to the unsaturated iron binding sites on transferrin. The excess fe(II) ions are reacted with ferrozine to form the color complex, which are measured spectrophotometrically at 560nm. The total iron binding capacity (TIBC) is determined by adding the serum iron value to the UIBC value.

#### Reagents

1. UIBC buffer reagent (2) Iron color reagent (3)Iron standard

#### Procedure:

- Three test tubes were labeled test, standard and blank
1. 2ml of UIBC buffer reagent were added in all the tubes
  2. 1ml of iron-free water were added in tube labeled blank and was mixed properly.
  3. 0.5 ml (500µl) of iron-free water plus 0.5ml (500µl) of standard were added in tube labeled standard and was mixed properly.
  4. 0.5ml (500µl) of sample plus 0.5ml (500µl) of standard were added in tube labeled test and was mixed very well.
  5. The spectrophotometer were zero at 560nm with reagent blank
  6. The absorbance of all the tubes were read and recorded as A<sub>1</sub> reading
  7. 0.05ml (50µl) of iron color reagent were added in all the tubes and was mixed properly.
  8. All the tubes were incubated in a water bath at 370° for 10 minutes
  9. The spectrophotometer were zero at 560nm with reagent blank
  10. The absorbance of all the tubes were read and recorded as A<sub>2</sub> reading.

#### UIBC CALCULATIONS

Conc. Of std -

$$\frac{A_{2test} - A_{1test}}{A_{2std} - A_{1std}} \times \text{conc. of std} = \text{UIBC } (\mu\text{g/dl})$$

The concentration of standard = 500µg/dl

### CALCULATIONS

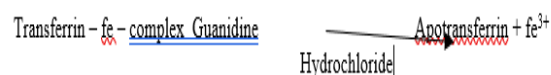
#### 3.9.3 TIBC (Total Iron Binding Capacity):

Iron level + UIBC = TIBC (µg/dl), SI Unit  
Conversion: Ug/dl x 0.179 = µmol/l

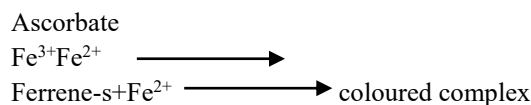
#### 3.9.4 SERUM FERRITIN

##### Principle:

Under acidic conditions, the iron is liberated from transferrin as fe<sup>3+</sup>



In the second step, the fe<sup>3+</sup> is reduced by Ascorbate to Fe<sup>2+</sup> then react with ferene-s to a color complex



The color intensity is directly proportional to the iron concentration and can be measured spectrophotometrically at 580nm

METHOD: Colorimetric determination of ferritin in serum by the Ferene-s method.

##### Working solution

Reaction mixture: 100ml of buffer + 1 bottle of colour reagent (iron ferene-s)

Sample/ standard blank reagent: 50ml of buffer +2 scoops of bottle, iron- ferene-s.

##### Procedure:

1. five test tubes were labeled sample, sample blank, standard, standard blank and reagent blank
2. 1ml of reagent mixture were added to the tube labeled sample, standard and reagent blank
3. 1ml of sample /standard blank reagent were added to the tubes labeled sample blank and standard blank.
4. 0.2ml of sample were added tue labeled samle and sample blank
5. 0.2ml of iron- free water were added in tube labeled reagent blank

The whole tube were mixed properly and read within 10-30 minutes at 580nm

Spedrophotometrically.

Protocol:

	Sample	Sample blank	Standard	Standard blank	Reagent blank
Reaction mixture	1000µl		1000µl		1000µl
Sample/std blank		1000 µl		1000 µl	
Sample	200µl	200µl			
Water					200µl

Calculations

$$fA (s) = A(\text{sample}) - A(\text{sample blank}) - A (\text{reagent blank})$$

$$fA(\text{Std}) = A(\text{Standard}) - A(\text{standard blank}) - A (\text{reagent blank})$$

$$\text{Concentration of serum ferritin} = \frac{30 \times fA(s)}{fA(\text{Std})} \times \text{Conc of Std} \quad (\mu\text{mol/l})$$

The concentration of standard = 30 µmol /l

### 3.9.5 TRANSFERRIN SATURATION (Calculation method)

$$\frac{\text{Total iron} \times 100}{\text{TIBC}} \quad (\%)$$

### 3.9.6 COMPLETEBLOODCOUNT(AUTOMATIO)

A fully automated machine, sysmex XT4000i, was used for the analysis of the complete blood count (CBC) of the study subjects. The EDTA anticoagulated blood samples were well mixed and introduced to the probe of the machine, after the subjects ID, age, sex and the appropriate test had been keyed into the machine. The machine then analysed and generated a printed report for the samples. The machine was well calibrated and an external quality control material /sample consisting of high values, normal values and low values were run before batches of subjects' samples were analysed.

### 3.10 STATISTICAL ANALYSIS

Data were subjected to descriptive statistics and analyzed using analysis of variance and student's t-test. The probability value less than 0.05 were considered statistically significant.

## IV. RESULTS

The results of this study were expressed in table 4.1, 4.2, 4.3, and 4.4

Parameters	Male HCV (+)	Control	P – value
Total iron (µmol/L)	15.81 ± 8.94	28.33± 1.03	P<0.05
Total Iron Binding Capacity (µmol/L)	88.53 ± 4.68	67.00 + 4.10	P<0.05
Ferritin (µg/L)	644.35 + 98.42	227.67 + 43.95	P<0.05
Transferrin saturation (%)	17.82 + 10.21	42.67 + 1.37	P<0.05
Transferrin (g/l)	3.54 + 0.91	2.68 + 0.16	P<0.05

Table 4.1 above showed the Mean and Standard Deviation (Mean±SD) of Iron biomarkers in asymptomatic males HCV infected individuals and apparent healthy males as control. The results revealed a significantly increased in ferritin, Total Iron Binding Capacity (TIBC) and Transferrin, also a significantly decreased in Total Iron and Transferrin Saturation (TS), compare to control (P<0.05)

Parameters	Female HCV (+)	Control	P – value
Total iron (µmol/L)	13.43 ± 7.66	21.33± 1.37	P<0.05
Total Iron Binding Capacity (µmol/L)	89.30 ± 11.07	53.33 + 2.73	P<0.05
Ferritin (µg/L)	502.78 + 350.14	82.67 + 19.13	P<0.05
Transferrin saturation (%)	14.78 + 7.79	40.00 + 0.89	P<0.05
Transferrin (g/l)	3.57 + 0.44	2.13 + 0.11	P<0.05

Table 4.2 above showed Mean and Standard Deviation (Mean±SD) of Iron biomarkers in asymptomatic Females HCV infected individuals and apparent healthy Females as control. The results revealed a significantly increased in ferritin, Total Iron Binding Capacity (TIBC) and transferrin, also, a significantly decreased in Total Iron, and Transferrin Saturation, when compared to control (P<0.05).

Variables	Male HCV (+)	Control	P value
TWBC(x10 <sup>9</sup> /L)	4.78 +1.54_	3.33 ± 0.21	P<0.05
RBC(x10 <sup>12</sup> /L)	5.23 + 0.75 _	4.33 + 0.41 _	P<0.05
Platelet(x10 <sup>9</sup> /L)	167.65 + 60.18 _	226.33 + 80.49 _	P>0.05
Haemoglobin(g/dl)	15.34 + 2.30 _	13.33 + 0.49 _	P<0.05
Haematocrit(%)	42.34 + 5.25 _	35.80 + 2.06 _	P<0.05
MCV(FL)	81.51 + 7.45 _	80.30 + 2.36 _	P>0.05
MCH(pg)	29.40 + 2.85 _	30.91 + 1.81 _	P>0.05
MCHC(g/dl)	36.15 + 1.79 _	37.28 + 0.78 _	P>0.05
Neutrophilic (%)	39.61 + 16.48 _	44.90 + 10.59 _	P>0.05
Mid (%)	10.31 + 1.54 _	7.67 + 1.86 _	P<0.05
Lymphocyte (%)	49.91 + 16.11 _	48.07 + 12.38 _	P>0.05

Table 4.3 above showed the Mean and Standard Deviation (Mean±SD) of Haematological variables of asymptomatic Males HCV infected individuals and apparent healthy males as control. The results revealed a significantly increased in Total white blood cell count (TWBC), Red blood cell count (RBC), Haemoglobin (HB) and Haematocrit (HCT) when compared to control (P<0.05).

Variables	Female HCV (+)	Control	P value
TWBC(x10 <sup>9</sup> /L)	5.53 +1.83_	3.40 + 0.32 _	P<0.05
RBC(x10 <sup>12</sup> /L)	5.28 +	3.81 +	P<0.05

	1.12	0.77	
Platelet(x10 <sup>9</sup> /L)	153.96 + 70.51 _	239.67 + 93.68 _	P>0.05
Haemoglobin(g/dl)	15.20 + 3.36 _	11.33 + 0.40 _	P<0.05
Haematocrit(%)	41.39 + 8.23 _	30.87 + 1.36 _	P<0.05
MCV(FL)	78.65 + 7.37 _	82.00 + 10.21 _	P>0.05
MCH(pg)	28.85 + 2.37 _	30.87 + 6.52 _	P>0.05
MCHC(g/dl)	36.60 + 1.61 _	36.95 + 0.57 _	P>0.05
Neutrophil (%)	46.24 + 14.07 _	53.57 + 2.44 _	P>0.05
Mid (%)	9.60 + 2.97 _	7.97 + 3.46 _	P>0.05
Lymphocyte (%)	44.09 + 14.23 _	38.47 + 1.49 _	P>0.05

Table 4.4 above showed the Mean and Standard Deviation (Mean±SD) of Haematological variables of asymptomatic Females HCV infected individuals and apparent healthy Females as control. The results revealed a significantly increased in TWBC, RBC, HB, and HCT when compared to control (P<0.05).

## V. DISCUSSION

In the study, we observed a significant increase in serum ferritin levels of male and female hepatitis C virus (HCV) infected individuals. Serum ferritin is one of the positive acute phase protein produced in the liver (Margaretha and Alida, 2014). Its level rises in response to inflammation and infection. Pathogens require iron for survival and proliferation. Whenever the human body is infected, the pathogen takes advantage of the host's iron pool, for its own needs. The hypoferraemia commonly associated with the infection is one of the several innate and adaptive responses of the immune system (Nemeth *et al.*, 2004). This suggested the reason why the individuals used in the study were asymptomatic and has not progressed to chronic stage of the disease. Elevated serum ferritin concentration may reflect systemic inflammation (Sehmus *et al.*, 2016). The serum ferritin concentration is also frequently increased during infection, systemic inflammatory conditions

and malignant conditions (Yoon *et al.*, 2018). This is in line with Ohkoshi *et al.*, who demonstrated that Hepatitis C Virus (HCV) – related liver damage is characterised by increased iron storage, which may influence the patient's response to interferon therapy. We also observed with respect to significant increased in serum ferritin that males have higher values than females. This was confirmed by Rushton *et al.*, who observed that ferritin levels vary considerably between genders because of poor diet and menstrual loss in females.

There was a significant decreased level of serum iron when compared to control. This is probably due to stage of the liver disease among subjects in the study. By lowering the iron levels, the organism tries to arrest the proliferation of iron – dependent pathogens avoiding a fast septicaemia outcome and allowing the effective elimination of the infection. The induction of the hypoferraemia during inflammation/infection is mediated by hepcidin (Nemeth *et al.*, 2003). As circulatory hepcidin levels increase, ferroportin is internalized and degraded prompting the organism to absorb lower amounts of iron from the diet and to arrest the body iron pool in macrophages and storing cells. During infection, pathogens are recognized as foreign elements by several cell types, such as macrophages. This recognition triggers the expression and secretion of the pro-inflammatory cytokines interleukin (IL) - 6, 22 and type I interferon (INF) (Lee *et al.*, 2005). These cytokines are recognized by surface Toll-like receptors expressed by hepatocytes and leukocytes, triggering the Janus associated kinase (Jak) – signal transducer and activating the transcription 3 (STAT 3) pathway. The translocation of factor stat 3 to the nucleus and its binding to the stat 3 binding sites in the HAMP promoter trigger the expression of this gene (Wrighting and Andrews, 2006). Therefore, extracellular pathogens are deprived of iron. This was in agreement with the result of Buyukasik *et al.*,. However, other authors did not observed alterations in serum iron levels (Boige *et al.*, 2013), or they reported an increased in liver injury (Mao *et al.*, 2015). These discrepancies may be because of the differences in stages of liver diseases among the patients in the various studies.

Increased levels of transferrin and total iron binding capacity (TIBC) were observed when compared to control in the study. Transferrin mainly functions to regulate iron transport and to prevent excessive iron deposition. The reason for an increased in transferrin and TIBC observed in the study is likely to be that subjects used in the study have not developed liver damage as liver is the organ responsible for the secretion of transferrin. This was in contrasts to what Viveiros *et al.*, observed in HCV patients that have developed cirrhosis.

The result of the study also revealed a significant decrease in transferrin saturation in relation to control. Acute infection, menses, recent blood donation may temporarily reduce transferrin saturation concentrations in persons with excessive hepatocyte iron deposition (Adams and Barton, 2011). Transferrin saturation <16% is poor specific, as pregnancy, oral contraceptive use, and chronic illness can result in low transferrin saturation without iron deficiency (Stein and Dignass, 2013). High serum transferrin and TIBC, and low total iron and transferrin saturation as observed in this study indicated that the individuals used in this study have uncomplicated iron deficiency.

In the assessment of Complete Blood Count in the study, the observed significant increase in total white blood cells count (TWBC) in both sexes might probably be due to infection and inflammation. Healthy blood cells which, as part of the body's immune system, help the body fight infection (Health Library, 2017). Leukocytosis is one of the hallmark of infection (Mayo Clinic, 2017). The observed increase in red blood cell count, haemoglobin concentration and haematocrit values in both sexes might probably be due to the fact that liver is not the major site for erythropoietin production and the stage of the disease is not yet chronic. Shih *et al.*, demonstrated that liver is not the major site for the secretion of erythropoietin – a circulating hormone that governs the rate of red cells production. The study also revealed that males have higher values than the females in red blood cells, haemoglobin and haematocrit. This is attributed that these values are inversely proportional to body fat, and females have higher percentage of body fat than males (an adaptation to preparedness for pregnancy and

nursing) and in their reproductive years, women periodically lose blood by menstruation (Health Library, 2017). Correlating the results of iron bio-markers and that of the haematological variables, it showed that the individuals used in the study have iron depletion without anaemia. This is likely to be the reason why they are still asymptomatic.

#### CONCLUSION

In conclusion, our findings in the study revealed that hepatitis C virus infection modulates iron metabolism and homeostasis in host hepatic cells. The elevated serum ferritin is a marker of infection and inflammation. Also, the serum ferritin and transferrin levels serve as an indicator in determining the severity and progression of liver diseases. The varying values of iron bio-markers and haematological variables seen in this study deduced iron depletion without anaemia. The increased or decreased values of some iron bio-markers and haematological variables in both sexes as observed in this study falls within their physiological ranges. We then, deduced that hepatitis C virus infection do not vary in pathophysiology in both sexes.

#### RECOMMENDATION

Further studies need to be carried out in asymptomatic individuals at molecular level using polymerase Chain Reaction (PCR) in order to explore the progression of the disease, fate of inflammation, and the state of the liver.

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