INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the World. It is the main type of primary liver cancers and the third most common cause of cancer mortality worldwide **(Maillard, 2011)**.

The epidemiology of HCC varies according to the geographic area because of differences in the repartition of major causative factors. In countries where hepatitis C virus (HCV) infection is endemic, such as Egypt, high prevalence of HCV infection is reported among people with HCC. HCC is closely associated with liver cirrhosis, which is a true precancerous state **(Morgan et al., 2013)**.

Hepatitis C virus is currently the most significant public health problem in Egypt, with an overall prevalence of 17.4% in males and 12.2% in females, which further increases with age to 39.4% in 55 to 59 years old subjects **(Sievert et al., 2011)**.

The current recommendations and most commonly used screening method for patients at risk for HCC is measurement of serum alpha-fetoprotein (AFP), along with ultrasound every 6 to 12 months. However, this strategy has been shown to be far from perfect **(Chung et al., 2009)**. The clinical use of AFP has been shown to present some important limitations in sensitivity and specificity, (41–65%) and (80–94%), respectively, and hence, it is ineffective for diagnosis of early stages of the disease **(Bertino et al., 2011)**.

Ultrasound surveillance, even when performed every three month, cannot improve detection of small HCC (less than 2 cm in diameter) because of limitations in the procedure, the stage when surgical ressection can greatly improve the survival rates **(Radoslav et al., 2009)**. This makes early detection of HCC an active area of research, where several new candidate markers were reported within the last few years **(Trinchet et al., 2011)**.

Glutamine synthetase (GS), also called glutamine ammonia ligase, is a 42 KDa molecule composed of twelve identical subunits, each of which has an active site for production of glutamine **(Krajewski et al., 2008)**. Glutamine synthetase is present predominantly in the liver, kidneys and brain, it is a member of an enzyme family that catalyzes the synthesis of glutamine from glutamate and ammonia. It plays an important role in ammonia detoxification, nitrogen balance and pH regulation in the liver **(Bioulac-Sage and Rullier, 2009)**.

Glutamine is the major energy source for tumor cells. Accumulation of GS was first found through analyzing increased ubiquitinated protein in HCC and its stepwise increase in expression from precancerous lesions to early HCC (**Di-Tommaso et al., 2009)**. These data have proposed GS as a useful biomarker for early diagnosis and prognosis in HCC patients **(Long et al., 2011)**.

AIM OF THE WORK

The aim of this work is to study the clinical utility of serum glutamine synthetase in hepatocellular carcinoma patients with HCV infection, and to compare its diagnostic performance with that of serum AFP.

I. HEPATOCELLULAR CARCINOMA

A. Epidemiology:

1. Incidence & Geographic Distribution:

Hepatocellular carcinoma (HCC) is the most common primary liver cancer which accounts for 85-90 % of all primary liver tumors **(Kew, 2010)**. The annual number of new cases of HCC Worldwide is over one million, making it the 5th most common cancer Worldwide and the 3rd leading cause of cancer-related death; with about 600,000 patients dying from the disease annually, preceded only by the lung and stomach cancers. The global distribution varies by region due to factors at the origin of the disease **(El-Serag and Davila , 2011)**.

Among the main risk factors for HCC, chronic infections of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most important in humans, accounting for more than 70% of HCC cases Worldwide. The geographical variability in the incidence of HCC has been attributed to the changing distribution and the natural history of HBV and HCV infections **(Poon et al., 2009)**. In high risk areas, as most Asian and African countries, where more than 80% of HCCs develop, HBV is the primary cause of HCC. China especially comprises more than half the rate of new cases recorded **(Yang and Roberts, 2010)**, whereas in developed countries as United States and European countries, HCV plays a more dominant

role. Currently, there are an estimated 3 million people in United States with chronic hepatitis C; these patients are estimated to develop HCC at a rate of 0.5% to 5% per year **(Ferenci et al., 2010)**.

In Egypt, HCV is the most leading cause of HCC. Egypt is known for being the country in the World with the highest prevalence of HCV, where about 24% of the people are estimated to carry HCV and more than 50% of blood donors have anti-HCV in some towns **(Anwar et al., 2008)**. It has been estimated that 20% of HCV-infected patients develop liver cirrhosis and that approximately 40% of these patients develop HCC within 10–15 years **(Sherman, 2010)**. HCC contributes to 14.8% of all cancer mortality in Egypt, with a higher incidence in males (17.3%) than in females (11.5%). It is the second most frequent cancer type in Egyptian males after bladder cancer and the eighth most frequent in Egyptian females **(Sievert et al., 2011)**.

2. Demographic Factors:

a. Age:

Age varies widely according to geographic distribution. In low incidence regions, the median age at diagnosis is 65 years. HCC is rarely diagnosed before age 40 years except in patients with cirrhosis. In high incidence areas, age at diagnosis is substantially younger, occurring in the fourth and fifth decades of life. Diagnosis at a younger age is thought to reflect

the natural history of hepatitis B virus and hepatitis C virus related HCC **(Deli et al., 2008)**.

b. Sex:

In almost all countries, men are more likely to be affected with HCC than women. This trend is more prominentin European countries, where in Switzerland (male : female) (4 : 1), in Italy (5 : 1) and in France (5 : 1), than in the developing states, where in China (3 : 1), in (Gambia 2.8 : 1) and in Zimbabwe (2 : 1) **(Shariff et al., 2009)**. The reasons of these trends are not well understood but several factors may explain that. Males are more likely to be infected with HBV and HCV, in addition cigarettes smoker, and alcohol consumer have a higher risk for developing HCC. Testosterone level has been shown to correlate with HCC, indicating a probable role for the sex hormones in the development of HCC. Interleukin 6 (IL-6) is also thought to be implicated, as IL-6 disruption abolishes the gender differences in hepatocarcinogenesis **(Nordenstedt et al., 2010)**.

3. Mortality and Morbidity:

Most patients with HCC die within 1 year after diagnosis. Surgical cure is possible in less than 5% of patients. The median survival in unresectable cases is less than 4 months and under a year for untreated patients with less advanced disease **(Llovet et al., 2008)**. The prognosis is affected by both the tumor severity and the degree of pre-existing liver damage **(Kuwaki et al., 2011)**.

B. Risk Factors:

Known risk factors for HCC include chronic viral hepatitis, cirrhosis, heavy alcoholism, non-alcoholic fatty liver disease and certain inherited metabolic conditions such as hemochromatosis. The proportion of cases of HCC associated with these risk factors has been estimated. In Africa and East Asia, the largest attributable fraction is due to hepatitis B (60%), whereas in the developed western world, only 20% of cases can be attributed to HBV infection **(Parkin, 2006)**.

1. Hepatitis B Viral Infection:

Hepatitis B virus (HBV) is the most common risk factor for HCC identified on a worldwide basis. There are two possible pathways for the involvement of HBV in the development of liver cancer. In the first, the HBV genome may directly participate in causing genetic changes in the host genome that lead to hepatocarcinogenesis. In the second, HCC develops due to HBV-related cirrhosis. HBV DNA has been shown to become integrated within the chromosomes of infected hepatocytes, the integration of viral genetic material may occur in a critical location within the cellular genome **(Guangyu and Wei, 2013)**.

Moreover, HBV genome encodes a regulatory element, the hepatitis B x protein (HBx) which is believed to contribute

to the development of HCC. HBx is a multifunctional regulator that is essential for viral replication and plays an important role in regulating gene transcription, participating in cell signaling, and controlling cell proliferation and apoptosis **(Muroyama et al., 2006)**. The HBx expression is also associated with activation of the mitogen-activated protein kinase (MAPK) pathway, an important cellular pathway that has been implicated in hepatocarcinogenesis. In addition, HBx has been found to interact with p53, interfering with its function as a tumor suppressor. Also, experimental observations suggest that HBx protein increases telomerase reverse transcriptase (TERT) and telomerase activity, prolonging the lifespan of hepatocytes and contributing to malignant transformation **(Ha and Yu, 2010)**.

It is not possible, on clinical grounds, to differentiate hepatitis B from hepatitis caused by other viral agents and, hence, laboratory confirmation of the diagnosis is essential. A number of blood tests are available to diagnose and monitor people with hepatitis B. They can be used to distinguish acute and chronic infections. Tests, for detection of hepatitis B virus infection involve serum or blood tests that detect either viral antigens (proteins produced by the virus) or antibodies produced by the host **(Liaw et al., 2010)**.

The hepatitis B surface antigen (HBsAg) is most frequently used to screen for the presence of this infection. It is the first detectable viral antigen to appear during infection.

Acute HBV infection is characterized by the presence of HBsAg and immunoglobulin M (IgM) antibody to the core antigen, (HBcAg). During the initial phase of infection, patients are also seropositive for hepatitis B e antigen, (HBeAg). Chronic infection is characterized by the persistence (>6 months) of HBsAg (with or without concurrent HBeAg). Persistence of HBsAg is the principal marker of risk for developing chronic liver disease and HCC later in life. The presence of HBeAg indicates that the blood and body fluids of the infected individual are highly contagious. A person negative for HBsAg but positive for anti-HBs either has cleared an infection or has been vaccinated previously **(Awadalla et al., 2011)**.

Also, polymerase chain reaction tests (PCR) have been developed to detect and measure the amount of HBV DNA, called the viral load, in clinical specimens. These tests are used to assess a person's infection status and to monitor treatment **(Aspinall et al., 2011)**.

2. Hepatitis B and D Viruses, Co-Infection:

Hepatitis D virus (HDV) co-infection with HBV is associated with increased liver damage. Some studies showed that HBsAg positive patients with HDV super-infection develop cirrhosis and HCC at an earlier stage compared to HBsAg carriers without HDV infection **(Shariff et al., 2009)**.

3. Hepatitis C Viral Infection:

Hepatitis C virus is the most common cause of chronic liver disease and cirrhosis in the world, and represents the main cause of liver transplantation in the United States of America, Australia and Europe **(Imperial, 2010).** The exact pathogenesis of HCC associated with HCV remains controversial, whether the virus plays a direct or indirect role. There is currently no evidence that HCV by itself is oncogenic, this is due to absence of reverse transcription activity of the HCV RNA virus so its viral genome unlike HBV is not able to integrate into the genome of the infected cell. However, HCC may rarely develop in non-cirrhotic HCV-infected individuals. It is possible that certain viral proteins may interact with oncogenes or tumor suppressor genes that regulate the cell cycle, so a direct oncogenic effect cannot be excluded. HCV causes HCC via an indirect pathway by causing chronic inflammation, cell death, proliferation and cirrhosis **(Nakano et al., 2011)**.

Diagnosis of Hepatitis C involves confirmation of the diagnosis of HCV infection and assessment of the severity of liver disease. In addition, evaluation of patients with Hepatitis C should include determination of the patients' suitability for treatment **(Houghton, 2009)**.

Two classes of assays are used in the diagnosis and management of HCV infection: serologic assays that detect specific antibody to HCV (anti-HCV) and molecular assays that detect viral nucleic acid. These assays have no role in the

assessment of disease severity or prognosis **(Wilkins et al., 2010)**.

i. Serologic assays:

Tests that detect anti-HCV are used both to screen for and to diagnose HCV infection. Anti-HCV can be detected in the serum or plasma. They include three different types of assays: enzyme immunoassay (EIA), chemiluminescent assay (CIA) and recombinant immunoblot assay (RIBA). The third generation EIA test is now the dominant HCV screening test used in clinical practice because of its high sensitivity and relatively low cost. The specificity and sensitivity of the third generation EIAs in patients with chronic liver disease due to HCV infection are >98% and >97%, respectively. False negative results may occur in patients on long-term hemodialysis and immunosuppressed persons such as those with HIV infection, solid organ transplant recipients. False positive results are more likely to occur in some patients who have autoimmune liver disease or hypergammaglobulinemia **(De Leuwet al., 2011)**.

The recombinant immunoblot assay (RIBA) originally was developed as a high-specificity confirmatory test for patients with a positive EIA result, but the importance of the RIBA has diminished with the marked improvement in specificity of the third generation EIA tests and with the more widespread use of molecular assays **(De Leuwet al., 2011)**.

One disadvantage of the antibody-based tests is that they cannot distinguish acute from chronic infection. The third generation EIAs generally become positive about 6 to 8 weeks after acquisition of infection. Therefore, anti-HCV immunoglobulin G (IgG) antibodies are usually negative during the acute phase and HCV infection during this time frame can only be determined by serum HCV RNA. Also, Anti-HCV immunoglobulin M (IgM) is not useful for distinguishing between acute and chronic HCV infection and measuring HCV IgM is not recommended **(Mukherjee et al., 2011)**.

Another antibody-based test of interest, the OraQuick HCV rapid antibody test, was approved by the Food and Drug Administration (FDA) in 2010. It uses whole blood samples obtained by venipuncture. The major advantage of this test is that it allows point-of-care testing in that it is portable and easy to use; it provides an answer within 40 minutes and can therefore be used for HCV screening for persons who are at risk for hepatitis C infection. Further testing is necessary to confirm HCV infection if the test result is positive **(Shivkumar et al., 2012)**.

ii. Molecular assays:

The molecular HCV RNA tests, also called nucleic acid tests (NAT) or nucleic acid amplification tests (NAAT), provide an advantage over the antibody-based tests, since a positive HCV RNA test both verifies the HCV EIA and establishes the presence of chronic HCV infection. HCV RNA tests can be detected in serum or plasma as early as 1 to 2

weeks after exposure to the virus and weeks before the antibody tests become positive or the liver enzymes become elevated. The HCV RNA tests used for diagnostic purposes include the qualitative and quantitative assays. Previously, the qualitative HCV RNA test was the most common molecular test used for HCV diagnosis, primarily because of the very high sensitivity. The qualitative assay, however, only determines the presence or absence of HCV RNA **(Gruppioni et al., 2009)**. In contrast, a quantitative HCV RNA test can determine whether chronic HCV infection is present and generate an HCV RNA level, which can provide prognostic information for treatment. Further, in recent years, real-time polymerase chain reaction technology for quantitative HCV RNA testing has become widely available and real-time PCR has been reported to be as sensitive as qualitative assays, providing a very low limit of detection and a broad dynamic quantitative range across the different HCV gentoypes. Most experts now recommend using quantitative HCV RNA assays (typically real-time PCR assays) as the preferred confirmatory test for persons with a positive HCV antibody screening test **(Mukherjee et al., 2011)**.

iii. Assessment of the severity of liver disease:

Severity of liver disease is best assessed by liver biopsy; however, there are risks from the procedure. In general, there is a poor correlation between liver enzymes and activity of liver disease. More importantly, several recent studies found that significant liver disease can be found in anti-HCV-positive

patients despite normal liver enzymes levels **(Wilkins et al., 2010)**. This may be related to the fluctuating course of chronic HCV infection with intermittently normal liver enzymes levels and undetectable levels of viremia. It may also reflect variations in sensitivities of "home-made" real-time PCR assays for HCV RNA. It is unlikely that quantitative tests for HCV RNA will replace liver biopsy in the determination of activity or stage of liver disease. HCV genotype I b has been shown to be associated with more advanced liver disease. Nevertheless, there is a wide spread in severity of liver disease associated with each genotype. Thus, genotyping cannot be used to determine severity of liver disease **(Senadhi, 2011)**.

4. Alcoholism:

Chronic alcohol consumption has long been associated with progressive liver disease because the liver is the major site of ethanol metabolism and thus, sustains the most injury from alcohol consumption. No agreement exists on the dose-effect relationship between alcohol intake and risk of HCC, but some experts defined it as a proven risk factor for HCC when the daily consumption is estimated at 50 to 75 g per day **(Chagas et al., 2009)**. It is not known whether alcohol is directly carcinogenic or acts indirectly through repeated injuries progressing to extensive fibrosis and cirrhosis. Some mechanisms, as chromosomal loss, oxidative stress, a decreased retinoic acid level in the liver, altered DNA methylation, and genetic susceptibility, can be regarded as causes of alcohol

leading to HCC. Alcohol, because of its increasing intake in many countries, may continue to be an obvious cause of HCC development in the World **(Hassan and Kaseb, 2011)**.

5. Aflatoxin:

Aflatoxin is believed to be a major causative agent in the high incidence of primary liver cancer seen in certain regions of the World. In some African and Asian regions especially, it is known to be a key risk factors of HCC. Aflatoxin is a mycotoxin released by *Aspergillus flavus* and related fungi that grow on improperly stored foods, as corn, rice, and peanuts. Aflatoxins are a group of approximately 20 related fungal metabolites with four major known types (B1, B2, G1, and G2). Among them, B1 is the most potent, naturally occurring chemical liver disease carcinogen **(Polychronaki et al., 2008)**. Aflatoxin carcinogen results in p53 tumor suppressor gene mutations. Generally, in more than 50% of human cancers, p53 is mutated and these mutations occur at the third position of codon 249. Associated with HBV, the dietary exposure to aflatoxin increases the HCC risk factors due to presence of synergetic effect of B1 **(Liu and Wu, 2010)**.

6. Disturbed Iron Metabolism:

Hemochromatosis is characterized by an excessive accumulation of body iron, most of which is deposited in parenchymal organs such as the liver and pancreas**.** Iron is potentially mutagenic through a number of mechanisms related