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Vitamin B12: A Non-invasive Biomarker for Monitoring Hepatocellular Carcinoma Development among Egyptian HCV-Infected Patients

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Abstract: Several studies have revealed an association between the high serum levels of vitamin B12 (vit B12) and the stage of chronic liver diseases. This study analyzes serum vit B12 levels among Egyptian hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) patients. The serum levels of vit B12 were examined in HCV patients without cirrhosis (HCV group, n=30), with cirrhosis (HCV+cirrhosis group, n=24), HCC patients (HCC group, n=30), and healthy individuals (control group, n=16). Serum vit B12 levels increased significantly in HCV+cirrhosis and HCC groups compared with the control group. HCC patients showed a significant increase in vit B12 levels compared with HCV patients with cirrhosis. Moreover, HCV patients without cirrhosis showed no significant increase in vit B12 level than the control group. Also, patients with fibrosis scores (F) from F2 to F4 showed a significant increase in serum vit B12 levels compared with the control group. Regarding correlations with liver functions, serum vit B12 was negatively correlated with serum albumin level and positively correlated with total bilirubin level. Interestingly, serum vit B12 was also positively correlated with serum AFP level. This clinical study revealed that the level of vit B12 could be used as a non-invasive biomarker to monitor liver fibrosis and HCC development among HCV-infected patients.

Keywords: biomarker; cirrhosis; hepatocellular carcinoma; hepatitis C virus; vitamin B12.

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1. Introduction

Hepatitis C virus (HCV) represents a worldwide infection that threatens many people in different countries, especially Egypt, where people suffer from the highest incidence of HCV infection [1,2]. HCV infection is a slowly progressive disease, and untreated patients are at the risk of developing hepatic damage disorders such as fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [3-6]. Therefore, different studies have focused on discovering the mechanism of HCC progression in patients infected with HCV and the characterization of novel treatment strategies for HCC that enhance cancer cell sensitivity [7-9].

HCV infection induces inflammations in hepatocytes that trigger ROS production and lead to hepatic cell death induction [10]. The produced ROS and the released inflammatory mediators induce a wound healing/fibrogenesis process *via* activated hepatic stellate cells

leading to fibrosis [10-12]. Indeed, the fibrosis process leads to gradual scarring on the liver and increases the incidence of cirrhosis and HCC progression [2,10,13]. The development of HCC after HCV infection is a long-term process that may take 40 years [14]. It was discovered that the possibility of HCC development in HCV patients might be attributed to the interfering effect of HCV viral proteins on cell cycle checkpoints and tumor suppressor genes such as p53 [15]. Moreover, HCC risk increases among HCV patients co-infected with HIV or HBV [16]. HIV infection induces immune response suppression in HCV-infected patients, leading to an increase in HCV replication and an increased chance of cirrhosis development [17].

Vitamin B12 (vit B12) is considered one of the essential elements in the body stored in hepatocytes and participates in one carbon fragment metabolism [18]. Moreover, vit B12 functions involve red blood synthesis and maintenance of the nervous system functionality. In addition, vitB12 has a role in DNA synthesis and repair mechanisms and, thus, is necessary for cell division [19]. Therefore, it is highly recommended to have a daily intake of vit B12 of 2-5 µg to maintain health [18]. It is worth mentioning that a low vit B12 level is most likely associated with many health disorders such as anemia, some neurological disorders, impairing memory function, and increased cardiometabolic risk [20-23]. On the other hand, high vit B12 level is correlated with serious human diseases such as cancer. Indeed, vit B12 plays a crucial role in methionine synthesis from homocysteine by methionine synthase enzyme [19,24]. Interestingly, hyperhomocysteinemia is recently correlated to the risk of developing different human diseases such as cancer [25,26]. Based on the vital role of vit B12 in homocysteine metabolism, alteration in homocysteine level leads to alteration in vit B12 level that may explain the detection of high vit B12 levels in some malignancies [27-29]. Although high vit B12 levels are reported in some malignancies, some nonmalignant cases reported high vit B12 levels, such as liver diseases, kidney diseases, and some infectious diseases [30,31].

The liver is considered the main site for vit B12 accumulation and metabolism. Therefore, several studies detected an increase in vit B12 levels in patients suffering from a chronic or acute liver disease, but it is poorly known [32,33]. Therefore, the current study aimed to analyze serum vit B12 levels in HCV and HCC Egyptian patients and evaluate its validity as a biomarker for the progression of liver damage.

2. Materials and Methods

2.1. Patients criteria and classification.

This study was conducted after the approval of the medical research ethics committee for human studies in the faculty of pharmacy (code number: 2020-7). Moreover, all the rights, subjects, and interests of patients were protected in this research via consent for each participant.

Inclusion criteria: Patients (males and females) from the clinics of the University hospitals were confirmed for HCV infection using RT-PCR technique (mentioned below), and they had the age range from 39±9.5 to 56±7.5 years old. HCC incidence was detected by measuring serum AFP level using ELISA method according to manufacturer protocol (Calbiotech company, USA). HCC is detected in patients with AFP levels more than 400 ng/ml [34,35] and was further confirmed by ultrasound examination.

Exclusion criteria: Patients with acute HCV infection or chronic illness such as diabetes, inflammatory diseases, chronic renal failure, chronic heart disease, mixed HBV-HCV infection, and patients receiving exogenous vit B12 supplements were excluded from the study.

Fibrosis grading: Fibrosis score (F) was determined in the University hospitals according to Metavir score [36] and mentioned in the clinical reports of patients.

Patients and individuals used in the study were classified into the following groups:

a. Thirty HCV patients without incidence of cirrhosis or HCC development (HCV group). The fibrosis score was F0.
b. Twenty four HCV patients with detected cirrhosis and without HCC development (HCV+cirrhosis). The fibrosis score was from F1 to F4.
c. Thirty HCV patients with detected cirrhosis and incidence of HCC development (HCC group). The fibrosis score was from F1 to F4.
d. Sixteen healthy individuals (control group).

2.2. Blood collection and analysis.

A blood sample was isolated from each individual that was further used to isolate whole blood samples using anticoagulant and to isolate serum samples by allowing blood to clot for 20-30 min at room temperature before centrifugation of clotted blood (1500 rpm) for 10 minutes. Serum samples were either freshly used or stored at - 80°C till further analysis.

2.3. RT-PCR analysis of HCV and quantification of viral load.

HCV RNA was extracted from all samples using QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). HCV RNA was quantified using a Taqman probe-based technique by Artus kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). All tests were performed on Step one RT-PCR machine (Thermo Scientific, Waltham, Massachusetts, United States).

2.4. Biochemical analysis.

Analysis of liver enzymes (ALT and AST), total bilirubin, and albumin were done in serum samples using the corresponding kits following the manufacturer's instructions (ELITech Clinical Systems, France).

2.5. Determination of vit B12 serum levels.

Serum vit B12 levels in different subjects were analyzed using immulite 2000 vit B12 detection kit according to the manufacturer's protocol (Siemens Healthcare company, for Diagnostics Products, United Kingdom).

2.6. Statistical analyses.

GraphPad Prism 5.0 program was used to analyze results and data (GraphPad Software, Inc.). Comparison between different groups were done using one way ANOVA test (ns; nonsignificant, ** $P < 0,01$ and *** $P < 0,001$). ROC curve that was done using SPSS program (IBM SPSS statistics 23).

3. Results and Discussion

3.1. Biochemical markers and characteristics of patients.

All characteristics and biochemical parameters of all groups of patients and control group are listed in Table 1 and represented as mean ± SD.

Table 1. Patients' characteristics and clinical, biochemical parameters.

Characteristics	Control group (n= 16)	groups of patients		
		HCV (n= 30)	HCV+cirrhosis (n= 24)	HCC (n= 30)
Age (years)	38±9.2	39±9.5	50±8.3	56±7.5
HB (g/dl)	14.1±1	14.3±1.1	13.4±1.5	10.9±1.5
Platelet count (10^3/µl)	220±55.8	214±60.9	86±41.7	72±41.9
ALT (U/l)	33±10.4	80±49.2	81±48.6	89±53.5
AST (U/l)	36±7.2	80±47.1	71±46.3	80±48
Albumin (g/dl)	4.5±0.6	4.5±0.5	3.4±0.3	3.2±0.4
Total bilirubin (mg/dl)	0.8±0.2	0.9±0.3	1.6±0.8	1.6±0.7
AFP (ng/ml)	3±1.3	3±2.5	22±16	8388±9000
INR	1±0.06	1±0.08	1.5±0.33	1.7±0.4
Viral load (IU/ml)	-	$3x10^5$±$3.8x10^5$	$1.3x10^6$±$2x10^6$	$2.4x10^6$±$3x10^6$

AFP, α-fetoprotein; ALT, alanine transaminase; AST, aspartate transaminase; HB, Haemoglobin; INR, international normalized ratio. Data are represented as mean ± SD.

3.2. Analysis of serum vit B12 levels in the different patients' groups and the control group.

Serum vit B12 levels were analyzed in control (n=16), HCV (n=30), HCV+cirrhosis (n=24) and HCC (n=30) groups. As indicated in figure 1, the highest serum vit B12 level was detected in the HCC group, which was significantly higher than control and HCV+cirrhosis groups (P < 0,001). Both HCV and HCV+cirrhosis groups showed an increase in serum vit B12 that was significantly high only in the HCV+cirrhosis group compared with the control group (P < 0, 01). Moreover, serum vit B12 was significantly higher in the HCV+cirrhosis group than in the HCV group (P < 0, 01) (Figure 1).

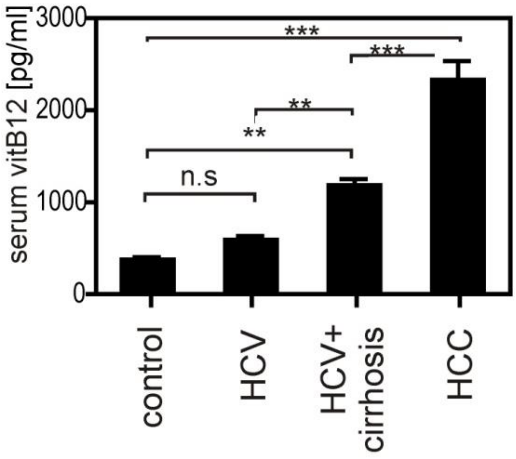


Figure 1. Analysis of serum vit B12 levels in the different patients' groups and the control group: Serum vit B12 level was analyzed in HCV (n=30), HCV+cirrhosis (n=24), HCC (n=30), and control (n=16) groups. Results were represented as mean±SE. One way ANOVA test was used for statistical analysis and comparison between groups (ns; nonsignificant, ** P < 0,01 and *** P < 0,001).

3.3. Analysis of fibrosis score (F) impact on serum vit B12 level.

Patients in HCV, HCV+cirrhosis, and HCC groups were classified according to their F into 5 groups starting from F0 till F4 groups, and serum vit B12 level was compared in each group with the control group (Figure 2). According to our results, F2-F4 groups showed a significant increase in serum vit B12 levels compared with the control group, and interestingly,

F3 and F4 groups showed the highest vit B12 levels. On the other hand, F0 and F1 groups showed no significant increase in vit B12 levels compared with the control group.

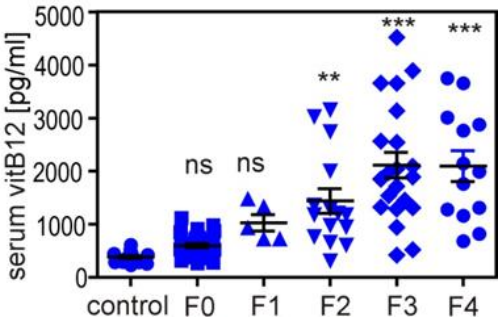


Figure 2. Analysis of F impact on serum vit B12 level: Patients were divided into different groups according to F of each patient (F0, n=30; F1, n=5; F2, n=15; F3, n=21; and F4, n=13). Results were represented as mean±SE. Serum level of vit B12 was determined in each group and compared with control group using one way ANOVA test (ns; nonsignificant, ** P < 0,01 and *** P < 0,001).

3.4. Correlation between serum vit B12 levels and hematological parameters and viral load in patients' groups.

As indicated in figure 1, HCC and HCV+cirrhosis groups showed the highest measured vit B12 levels among the other groups. Therefore, it was interesting to see whether serum vit B12 levels were correlated with clinical, hematological parameters, and viral load in these patients. We correlated serum vit B12 level and platelet count, HB, INR, and viral load in HCV, HCV+cirrhosis, and HCC groups (Figure 3). According to our results, serum vit B12 levels correlated positively with INR (r^2= 0.31, p<0.0001) and viral load (r^2= 0.26, p<0.0001) and correlated negatively with platelet count (r^2= 0.25, p<0.0001) and HB concentration (r^2= 0.3, p<0.0001).

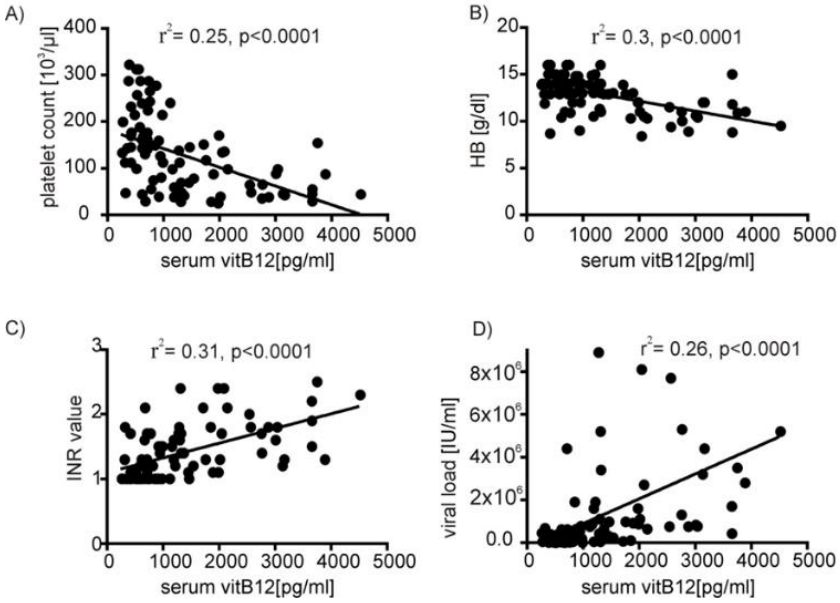


Figure 3. Correlation between serum vit B12 levels and hematological parameters and viral load in patients' groups: Serum vit B12 was correlated with different hematological parameters (platelet count, HB, and INR) and viral load (HCV RNA) in HCV, HCV+cirrhosis, and HCC groups. Correlations and calculations of correlation coefficient (r^2) were done using Graphpad Prism 5.0 program.

3.5. Correlations between serum vit B12 levels and clinical indicators of liver functions in patients' groups.

To explore the correlation between the increase in serum vit B12 level and liver damage in HCV-infected patients, we analyzed correlations between serum vit B12 level and biomarkers of liver functions indicated in table 1. We selected albumin and total bilirubin as markers of liver functions for correlations analysis with serum vit B12 level because there were higher in HCC and HCV+cirrhosis groups than in the HCV group, as indicated in table 1 (Figure 4). According to our results, serum vit B12 level correlated negatively with serum albumin level (r^2= 0.35, p<0.0001) and correlated positively with total bilirubin level (r^2= 0.15, p=0.0003).

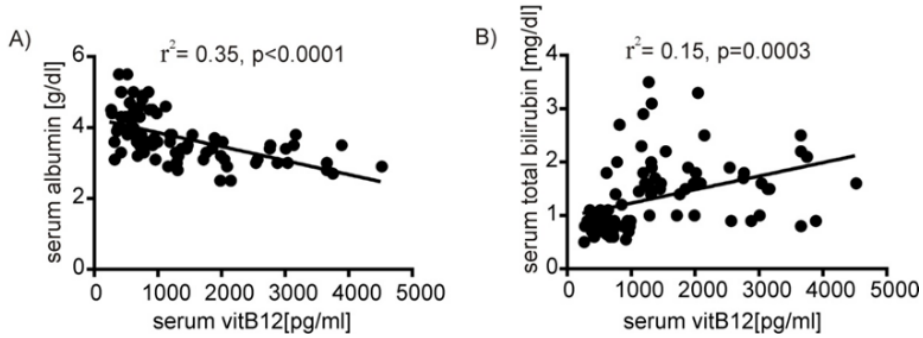
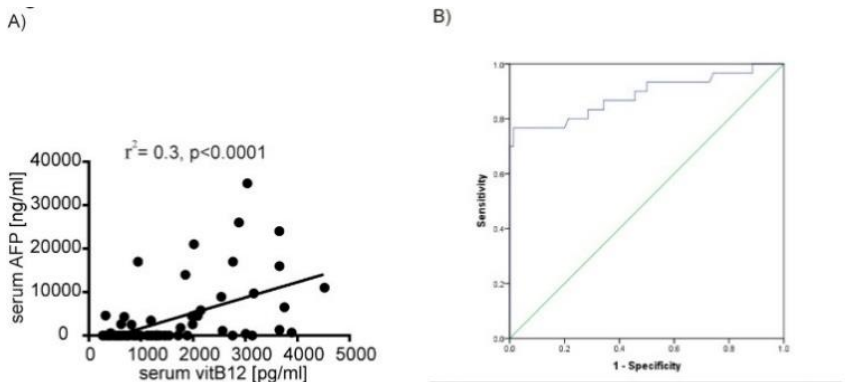


Figure 4. Correlations between serum vit B12 levels and clinical indicators of liver functions in patients' groups: Serum vit B12 was correlated with serum albumin and total bilirubin levels in HCV, HCV+cirrhosis, and HCC groups. Correlations and correlation coefficient calculations (r^2) were done using Graphpad Prism 5.0 program.

3.6. Correlation between serum vit B12 and HCC development.

Serum AFP is the well-known tumor marker for HCC detection (32). As far as HCC group showed the highest serum vit B12 levels (Figure 1), we were motivated to analyze the correlation between serum vit B12 levels and serum AFP levels in HCV, HCV+cirrhosis, and HCC groups (Figure 5A). Our results indicated a significant positive correlation between serum vit B12 and serum AFP levels (r^2= 0.3, p<0.0001). Also, we analyzed serum vit B12 levels as a prospective biomarker for HCC development using ROC curve. According to our results, the AUC was 0.885, and 95% confidence interval was from 0.799 to 0.971, indicating the validity of vit B12 as a prospective biomarker for HCC development (Figure 5B).



Area	0.885
Std. error	0.044
95% confidenece interval	0.799 to 0.971

Figure 5. Correlation between serum vit B12 and HCC development: (A) Serum vit B12 was correlated with serum AFP level in HCV, HCV+cirrhosis, and HCC groups. Correlations and correlation coefficient calculation (r^2) were done using Graphpad Prism 5.0 program. (B) ROC curve analysis of serum vit B12 levels in all groups as a biomarker for HCC development using SPSS program (IBM SPSS statistics 23).

3.7. Discussion.

Although HCC develops due to several factors, HCV infection represents the most common cause of HCC development in Egypt [37,38]. Thus, there is no wonder that recent research trials have focused on evaluating biomarkers that can help in detecting HCC development in HCV-infected patients at early stages [39-41]. The liver is considered a store of vit B12 and provides the human need for a long time [42]. Therefore, elevated vit B12 is correlated with several liver diseases such as acute hepatitis and HCC, and several types of malignancy [33,42-46]. Thus, this research aimed to analyze vit B12 serum levels in HCV patients without cirrhosis, HCV patients with cirrhosis, HCC patients, and healthy individuals.

Regarding our results, HCC patients showed the highest vit B12 level compared to the other HCV patients. Moreover, HCV patients with detected cirrhosis showed a significant increase in vit B12 level than HCV patients without any detected cirrhosis. Interestingly, these results are reinforced by previous studies that detected elevated vit B12 levels in HCC patients [47-49]. However, to the best of our knowledge, this is the first study to investigate vit B12 levels in Egyptian HCV patients with and without HCC development.

Indeed, it was reported previously that acute and chronic liver diseases are associated with elevated serum vit B12 [50]. In addition, vit B12 was high in patients with HCC and used as a prognostic marker [51,52]. However, the exact molecular mechanism for elevated vit B12 in chronic liver diseases is still unclear. It may be attributed to the decrease in its liver uptake, as proved by biopsy studies [42,50,53]. It may also be related to increased vit B12 released from damaged hepatocytes or decreased haptocorrin clearance by the liver [30, 51].

Regarding our results, vit B12 level was correlated to the type and degree of liver disease. Vit B12 level was significantly high in HCC and HCV patients with detected cirrhosis. On the other hand, vit B12 level was not significantly high in HCV patients without cirrhosis compared with the control group (Figure 1). Moreover, vit B12 level increased significantly as the degree of fibrosis increased (Figure 2). Indeed, HCV utilizes materials that are present in adequate amounts in liver cells, and it was previously detected that vit B12 plays a vital role in HCV replication and, therefore, HCV replication and further hepatic complications correlated directly with serum vit B12 level that may explain our findings [54]. This previous finding can be reinforced by the detected significant positive correlation of serum vit B12 level with viral load and significant correlations with subsequent disturbance in hematological parameters (Figure 3).

Moreover, our results revealed a significant negative correlation between vit B12 and serum albumin levels and a significant positive correlation between vit B12 and total bilirubin and AFP levels. Thus, this research indicates that vit B12 level can be a prospective biomarker for liver function status, especially in HCV patients who are highly susceptible to HCC development.

Interestingly, several research studies indicate that vit B12 supplementation is required to enhance anti-viral therapy for HCV and as a supplement for chemotherapy for HCC patients [55-58]. However, regarding our results, further research studies may be required to decide whether patients with chronic liver diseases need vit B12 supplementation or not.

4. Conclusions

This research proves that vit B12 can be used as a non-invasive biomarker to follow up liver functions status in HCV-infected patients who are susceptible candidates for HCC

development. Moreover, this preliminary study may raise an issue in the hepatology research about the usefulness of vit B12 supplementations, whether they are necessary for HCV and HCC patients or not.

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Conflicts of Interest

The authors declare no conflict of interest.

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