# AFP-L3 Snap Shot

**Sites Participating:** All sites and all patients in the HALT-C Trial

**Principal Investigator:** Richard K. Sterling (Virginia Commonwealth University)

**Co-Investigators:**

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|---|---|
| Marc G. Ghany | NIDDK-LDS |
| Gregory T. Everson | University of Colorado School of Medicine |
| Leonard B. Seeff | NIDDK |

**PARTICIPATING INSTITUTIONS:**

All HALT-C Clinical sites.
Wako Pure Chemicals, Ltd. Tonya Mallory

**Study Name:** Clinical Utilities of AFP-L# Determination in Early Recognition, Diagnosis and Prognosis of Hepatocellular Carcinoma (HCC) in patients with chronic Hepatitis C

**Separate Consent Form:** NO

**Withdrawal Form:** NO

**Eligible Patients**: Lead-in, Responder, and Randomized patients

## Visit Schedule (additional data/specimen and forms for AS)

Note: "**X**" means all participating sites take part. No additional specimens are collected for this study. Tests are performed on specimens stored for the main trial (seq.#111 or seq.#123). Results are maintained in a separate data file and are not included in the main HALT-C database.

### Lead-In Phase or Responder Phase

| Visit Number → | S00 | W00 | W02 | W04 | W08 | W12 | W16 | W20 | W24 | W48 | W72 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Stored sample (#111 or #123) | | X | | | | | | | X | X | X |

### Randomized Phase

| Visit Number → | R00 | M09 | M12 | M15 | M18 | M21 | M24 | M27 | M30 | M33 | M36 | M39 | M42 | M45 | M48 | M54 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stored sample (#111 or #123) | X | X | X | X | X | X | X | X | X | X | X | X | X | X | X | |

# Clinical Utilities of AFP-L3 Determination in Early Recognition, Diagnosis and Prognosis of Hepatocellular Carcinoma in Patients with Chronic Hepatitis C

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**Outside Collaborators:** Wako Pure Chemicals, Ltd, Osaka, Japan
Wako Chemicals USA, Richmond, Virginia

## INTRODUCTION

Alpha fetoprotein (AFP) is currently utilized to screen for hepatocellular carcinoma (HCC). Unfortunately, it is neither sensitive nor specific for detection of small tumors and levels are often elevated in patients with chronic hepatitis without HCC [1-5]. AFP can be fractionated by affinity electrophoresis into three different glycoforms (L1-L3) based upon its reactivity with *Lens culinaris* agglutinin (LCA). In HCC, the activity of 1-6 fucosyltransferase in tumor tissue is consistently higher than that from the adjacent cirrhotic nodules. As a result, the AFP L3 glycoform has an additional 1-6 fructose allowing it to bind to LCA. It is through this mechanism that AFP L3 may be a better marker for HCC than total AFP. Recently, an automated assay for AFP L3 was developed and is in use in Japan. However, prospective studies on the clinical utility of AFP L3 in North American patients at risk for HCC is lacking.

## AIMS

1. To determine whether AFP-L3 is useful in discriminating between HCC and a non-specific elevation in total AFP in the setting of chronic hepatitis C virus (HCV).
2. To determine whether AFP-L3 determination is useful in early detection of HCC in patients with chronic HCV.
3. To determine the usefulness of AFP-L3 in the prognosis of patients who develop HCC.

## BACKGROUND

Hepatocellular carcinoma (HCC) has become a public health concern largely due to the worldwide epidemic of hepatitis B and hepatitis C viruses [5-7]. Ninety percent of patients with HCC are infected with HBV in China while HCV infection was detected in 76% of patients with HCC in Japan [6]. There is also an increase in the number of cases of HCC in the US over the past two decades. The incidence rose from 1.4 per 100,000 persons during the period from 1976 to 1980 to 2.4 per 100,000 persons during the period 1991 to 1995 [8], and to 7/100,000 persons between 1996 and 1998[7]. Similar trends in HCC incidence have been observed in European countries such as Italy, France, and in the UK, as well as in Japan and Australia [1]. In 1997, chronic liver disease ranked as the tenth most frequent cause of disease according to US Center for Disease Control and Prevention (CDC) [9].

Screening for early detection of HCC presents a challenge. Currently, periodic ultrasound combined with alpha-fetoprotein (AFP) measurement is recommended to screen for HCC in patients at risk [10,11]. Unfortunately, serum AFP is neither sensitive nor specific for HCC[1,3-

5]. In small HCC, the sensitivity of AFP is lower ranging from 33-65%[2]. Slight but significant elevation of AFP (20-200 ng/mL) is also frequently seen in a considerable number of patients with chronic liver diseases without HCC. It has been reported that 15-58% of patients with chronic hepatitis and 11-47% of patients with cirrhosis had elevated serum AFP[2]. Therefore, it is not uncommon that serum AFP levels in patients with HCC and cirrhosis overlap. This confounds interpretation of assay result of AFP in patients with chronic liver diseases. In addition, macro-regenerative nodules account for up to 30% of solid nodular lesions that are less than 3 cm in diameter in cirrhotic liver making their differentiation from HCC paramount [1]. Therefore, a more specific test for HCC is needed. Des--carboxy prothrombin (DCP), a protein induced by vitamin K absence or antagonist II (PIVKA-II), has also been used as an adjuvant marker for AFP negative tumors and is under study in the HALT-C trial.

AFP is synthesized in large quantities during embryonic development by the fetal yolk sac and by the liver[12]. AFP concentration decreases gradually after birth to less than 10 ng/mL by 12-18 months[2,12]. AFP reappears in maternal serum during pregnancy. Pathologically, it is associated with HCC, gastric, lung, pancreatic and biliary tract cancer, and testicular carcinoma.[2]. AFP is a glycoprotein with a single asparagine-linked complex-type carbohydrate chain on each molecule. Total AFP consists of three different glycoforms, L1-L3, which can be separated by affinity electrophoresis based on its reactivity to *Lens culinaris agglutinin* (LCA)[2]. AFP-L1 does not bind to LCA and constitutes the major fraction of AFP in adults. It can be present in benign chronic liver diseases such as chronic hepatitis and liver cirrhosis. AFP-L2 is mostly derived from yolk sac tumors and shows intermediate binding to LCA. The AFP-L3 glycoform has an additional 1-6 fructose which allows it to bind to LCA. In HCC, the activity of 1-6 fucosyltransferase in tumor tissue is consistently higher than that formed in the adjacent cirrhotic nodules. AFP-L3 is only produced by malignant hepatocytes which have increased activity of 1-6 fucosyltransferase. AFP-L3 may therefore be a better marker for HCC than total AFP.

AFP-L3 may also be a biologic marker of malignant potential. Liver cancer cells with expression of AFP-L3 glycoform have a tendency for early vascular invasion and intra-hepatic metastasis. AFP-L3-producing liver cancer cells were more likely to stain positive with Ki67 in the nucleus, an indicator for malignant potential of the cancer cells, and had decreased staining for alpha-catenin. The absence of alpha-catenin was reported to correlate with distant metastasis of HCC [13]. Imaging studies show that AFP-L3-positive HCC were hypervascular with blood supplies derived from hepatic artery, and had shorter tumor doubling times [14],indicating that AFP-L3-positive HCC had potential for rapid growth and early distant metastasis [14,15]. Therefore, a high AFP-L3% in small HCC could suggest that the cancer is more aggressive.

Screening for HCC is not cost-effective unless conducted in a well-targeted patient population. There is evidence that early identification of HCC presents the best chance for successful treatment [16]. In this regard, AFP-L3 could be useful. In clinical studies, AFP-L3 could detect HCC of less than 2 cm in diameter in high-risk patients such as those with chronic hepatitis B, chronic hepatitis C, as well as in patients with liver cirrhosis [17,18]. Furthermore, elevated AFP L3 yielded up to 12 months of lead-time in early recognition of HCC compared to imaging techniques [18,19] that may provide more treatment options for HCC, such as surgical resection or hepatic transplantation.

Data on the clinical utility of AFP L3% has been derived mostly from studies in Japan. Elevation of AFP-L3% was found to be independent of the elevation of total AFP in HCC[14] and has been felt to be a better marker for HCC than total AFP with *a* specificity of greater than 95% [2]. The sensitivity of AFP L3 has been related to the clinical stage of the tumor. Although the

overall sensitivity is moderate (50-60%), it approaches 80-90% for tumors ≥ 5cm. In small HCC (< 2cm), the sensitivity is only 35-45%. However, small HCC can be aggressive with potential for rapid growth and early metastasis. In studies of ALP L3 in small tumors, those with low fractions had a better prognosis after therapy compared to tumors with high AFP L3[20-25]. These data suggest that not only is AFP L3 superior to total AFP for screening for HCC, it can also be used as a biologic marker of tumor aggressiveness in those with small cancers. Furthermore, AFP L3 is useful in following patients after treatment for HCC for disease recurrence [25].

An automated assay for AFP L3 has been developed and introduced for clinical use in Japan. The new automated method for measurement of AFP-L3 is an immunoassay based on liquid phase binding of L3 subspecies of AFP with two specific monoclonal antibodies labeled with peroxidase and polysulfated tyrosine peptide, respectively. The assay simultaneously determines concentrations of AFP-L3 and total AFP. The bound and free AFP isoforms are separated by affinity liquid chromatography. The concentration of bound AFP-L3 then is determined fluorophotometrically. The ratio of concentrations of AFP-L3 and total AFP is calculated. Results are reported as percentage ratio of AFP-L3 to total AFP [26-28]. The cut-off value for positive a test for this assay is currently set at 10%, which was derived from ROC analysis [18]. Inter-assay CV for AFP-L3 ranged from 2.8-5.8%. Recovery was from 92.6-105.6%. The automated assay showed good correlation with lectin-affinity electrophoresis (r=0.98) and endogenous substances did not interfere with the assay.

**RELATION TO HALT-C**

The HALT-C study has been designed to determine if continuing interferon long term over several years will suppress HCV, prevent progression to cirrhosis, prevent liver cancer and reduce the need for liver transplantation. As HCC is a major complication of chronic HCV, a reliable serum-based test like AFP-L3 is needed. It has been demonstrated that AFP-L3 is able to compensate for the relatively low specificity of total AFP providing a cost-effective way for monitoring for the development of HCC. However, the clinical data have been collected from retrospective studies outside the United States. A systemic evaluation of the AFP-L3 in a large-scale prospective study like HALT-C would be valuable to further verify the clinical utility of this cancer marker. The HALT-C trial offers unparalleled opportunity to compare AFP-L3 with results of other serum markers (total AFP, DCP or PIVKA-II), liver biopsy as well as with imaging modalities like ultrasound MRI or CT that are being collected in the HALT-C trial. The aims of this proposed ancillary study on AFP-L3 in this proposal are consistent with the major aims of HALT-C trial.

**DESIGN AND METHODS**

All patients enrolled in HALT-C will be eligible. All testing will be done on stored sera available in the repository (BBI). The AFP-L3/AFP test will be performed on LiBASys automated analyzer of Wako Pure Chemicals of Osaka, Japan. Each assay run will require 120 □l of serum. The actual assay will use 20 □l and the remaining 100 □l will be returned to BBI. Testing will be performed as follows: at baseline, at the end of the lead in phase, and every 3 months during the randomized phase for those patients who do not respond (total 16 tests/patient over 48 months). For those who achieve virologic response, the assay will be formed at the end of 48 weeks of treatment and then at week 72 (total 4 tests/patient). For those patients who develop HCC during the randomization phase of the trial, AFP-L3 will be performed at time of diagnosis and then every 3 months until liver transplantation, or death. All test will be performed from serum that is already being collected at specific time points in the HALT-C trial. No additional visits or blood draws are needed. Furthermore, a change in the consent or additional consent is not needed.

All test procedures including the LiBASys analyzer, chemicals and reagents, controls and calibrators as well as shipping costs will be provided by Wako Pure Chemicals. No additional HALT-C funds will be required for this proposal. The AFP-L3 assay will be performed by Wako in Richmond, VA. The specimens will be batched and sent from each participating institution to BBI every 3 months during the study; unused sera will be returned to BBI. Specimens will be shipped to Wako at BioSafety Level 2 on dry ice using Federal Express with no more than 4000 ml of serum shipped in one container. Wako will provide NERI with adequate information on quality control and storage of samples. The results from this study will not be applied to direct patient care.

**ANTICIPATED RESULTS**

Given the annual incidence for HCC of 1-3%/per year in patients with advanced HCV, it is anticipated that the number of patients who are likely develop HCC during the HALT-C trial will be 30-60. AFP-L3 should be able to detect HCC in relatively small size (less than 2 cm in diameter) tumors with 35% sensitivity, and in large tumors ($\geq$5 cm in diameter) with 80% sensitivity. Of the remaining patients with chronic HCV who will not develop HCC, the AFP-L3 would remain negative (95% specificity).

**ANALYSIS**

The AFP-L3 assay will be compared to total AFP, DCP-PIVKA II (performed not as part of the current ancillary study), and ultrasound screening. Patients with HCC will be assessed for tumor size (small: $\leq$ 2cm; medium: >2 and <5 cm; and large: $\geq$5 cm), vascular invasion, uni- or multilobular characteristics. Sensitivity, specificity, positive and negative predictive values of AFP-L3 will be calculated. Univariate and multivariate analysis of factors will be assessed by standard statistical criteria.

**PROBLEMS AND PITFALLS**

*Use and maintenance of LiBASys automated analyzer*: Wako Japan and Wako USA will provide technical support for the instrument. The LiBASys is currently in phase III clinical trial at six medical centers in North American. The AFP-L3 is available for research only, not for clinical diagnostic purposes.

*Interpretation of AFP-L3 results:* Total AFP and AFP-L3 provide different information on liver cancer. The total AFP may be an indicator of tumor mass in liver cancer while AFP-L3 measures malignant potential of liver cancer cells. While sensitivity of total AFP-L3 may relate to clinical stage, mainly to tumor differentiation, the sensitivity of AFP-L3 relates to biologic behavior (aggressiveness) of liver cancer. Therefore, sensitivity of AFP-L3 is largely related to the tumor characteristics. From clinical data derived from Japan, the aggressive liver cancers with rapid doubling time usually accounts for about 30% of small liver cancers (less than 2cm in size). The 35-45% sensitivity of AFP-L3 assay in small liver cancer is in line with the percentage of aggressive tumors in small size category. Although sensitivities of total AFP and AFP-L3 are comparable in small liver cancer, the meanings of positive AFP and AFP-L3 are obviously different. Total AFP and AFP-L3 are measured simultaneously in order to estimate percentage ratio of AFP-L3 in total AFP. AFP-L3 provides complementary information to total AFP for early recognition of malignant liver tumor and follow-up patients after therapies.

Logistics of testing: All testing will be done on serum collected as part of the HALT-C trial. Ideally, AFP-L3 should be done at the center that performs both total AFP and DCP-PIVKA-II, using the same sample for all three assays. However, if the DCP-PIVKA-II assay is performed at a separate center outside of this study, the results will be retrospectively compared by the

data coordinating center on all available and comparable specimens. If serum AFP measurements for clinical use during the trial are done at the individual centers, the AFP-L3 should be done by a single center. For this study, the AFP-L3 will be performed by Wako in Richmond, VA.

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