Viral Infection and Hepatocellular Carcinoma

Juan Li

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Virale infectie en hepatocellulair carcinoom

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Juan Li

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Doctoral Committee

Promoter:

Prof.dr. M. P. Peppelenbosch

Inner Committee:

Prof.dr. R.A. de Man

Prof.dr.C.A.Spek

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Chapter 1 General introduction

Part One: Virus

- Hepatitis virus

Hepatitis B virus (HBV), discovered in 1966, is a major public health problem which affects more than 350 million people worldwide.[1] The distribution of HBV infection varies worldwide. More than half of the population living in areas such as Southeast Asia, China, and Africa have been infected by HBV. Modes of disease transmission can be either through neonatal transmission (vertical) or transmission from one child to another (horizontal). As for the developed countries, which include North America, Western Europe, and Australia and are characterized by low levels of endemicity, most HBV infections result from horizontal transmission among adults, such as sexual activity, injection-drug use (IDU), or occupational exposure.[1, 2] The life cycle of HBV is characterized into four stages present in all infected patients. The first stage is characterized by immune tolerance. During the second stage an immunological response develops or increases in strength. When the host is able to eliminate HBV-infected cells or diminishes their number considerably, then active viral replication ends, which is followed by the third stage. Most HBV infected individuals eventually become negative for HBsAg and positive for anti-HBsAg, marking the fourth stage in the HBV life cycle.[1] For those getting rid of the infection, the short-term (six-month) infection is called acute hepatitis B. In other words, if HBV infection lasts longer than six months, it is considered a chronic HBV infection (CHB). Chronic hepatitis B is associated with some severe liver diseases, such as hepatic steatosis, inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC).[3] Especially for the life-threatening conditions like cirrhosis and HCC, it is estimated that, of the patients with CHB, there are 20% developing cirrhosis,[4] and moreover, they are 100 times more likely to develop HCC than non-CHB.[5] With respect to the underlying mechanism of the development of chronic infection with HBV, HLA phenotype is one the factors influencing the response to infection with this virus.[6] In a clinical

setting, histological examination is considered an integral part when evaluating CHB patients, and the evaluation of the CHB associated liver damage is critically important for making decisions in antiviral therapy and need for further surveillance.[7] Thus liver biopsy remains the golden standard in assessing the severity of inflammation and fibrosis.[7, 8] However, the procedure is costly, sensitive to sampling errors, and a potential cause for complications.[9] Therefore, a reliable non-invasive diagnostic tool is urgently needed.

Two strategies – vaccination and antiviral treatment – are traditionally implemented to manage hepatitis B infection. HBsAg remains the basis for the different HBV vaccines globally available. Antibody development to HBsAg, which is detectable in patients who have recovered from an acute hepatitis B infection and also those in people immunized with HBV vaccine, confers protective immunity. Being highly cost-effective, HBV vaccination has now been introduced into many nation-wide vaccine programs in a variety of countries. Based on the great success of this vaccination, it is estimated that universal childhood hepatitis B vaccination will eventually prevent more than 80 percent of HBV-associated mortality globally.[11] The second important strategy is the implementation of antiviral therapy, the goal of which is to decrease the morbidity and mortality related to CHB.[8] One best predictor to assess the success of current therapy, HBsAg loss, however, is infrequently observed in current therapy.

Mirroring the situation associated with HBV also the Hepatitis C virus (HCV), another important and prevalent hepatotropic virus, is also a critical public health problem affecting 130 million people worldwide.[12] Prevalence of HCV shows significant geographical variation in Europe. IDU appears to be the major risk factor for HCV infection in this continent.[13] HCV is related to development of HCC, and this appears to be mainly through fibrosis development.[6,14]

- <u>Human immunodeficiency virus (HIV)</u>

HIV affects 40 million people worldwide.[12] As estimated by the Chinese health authority,[15] there were 501,000 people living with human

immunodeficiency virus (HIV/AIDS) by the end of 2014 in China, accounting for 0.037% of the total population. Despite of a low national prevalence rate, the HIV infection is reaching epidemic rates in some areas of the Southwest China, in particular the Yunnan, Sichuan, and Guangxi provinces. Yunnan is the area most affected by HIV/AIDS in China. The epidemic has spread from high-risk groups including drug users, sex workers and unsafe blood recipients to the general population. Within a population of 44 million, officials estimate that this province has 80,000 HIV infected individuals.

Antiretroviral drugs in applied in combination therapy consisting of three or more drugs hailing from more than one class of biological mechanism, often called “highly active antiretroviral therapy (HAART)”, are very effective in suppressing HIV, although this does not eradicate the virus. Since the first introduction of HAART in the mid-1990s, it has led to an unprecedented decline of mortality caused by HIV/AIDS both in the USA and Europe.[16,17] Because of the great success of HAART in treating HIV/AIDS in the developed countries, the World Health Organization (WHO) has promoted a public health approach to scale up the access of antiretroviral therapy in resource-limited setting since 2002.[18] As an emergency response to save and improve the lives of AIDS patients in China, the China National Free Antiretroviral Treatment Program (NFATP) was piloted in 2002 and scaled up in 2003 national wide.[19] Until 2014, a total of 295,358 patients in China have received HAART, and thus a large proportion of HIV patients have benefited from this program.

Still, it is of importance to identify prognostic factors for survival among HIV-infected patients receiving HAART. Based on previous studies, the main risk factors for death include baseline low CD4 cell count, old age and advanced WHO stage. With respect to these earlier results, especially the prognostic value of CD4 cell counts seems unequivocal, even if being an imperfect measure of prognosis.[20-25] A recent study suggested that patients with low baseline CD4 cell counts only display increased mortality risk for up to 5 years after HAART.[26,27] This indicated the effect of CD4 cell count on the long-term survival is temporally speaking dynamic; however, this observation requires validation in other studies to substantiate this notion.

- Coinfection

As pointed out above, HBV and HCV are both important global health problems, and can cause severe liver disease. Many intravenous drug users have detectable antibodies to HBcAg and HCV in their circulation and injection needles are considered an important source of disease transmission and coinfection with hepatitis virus among HIV infected patients. Coinfection of HIV with hepatitis B virus (HBV) or hepatitis C virus (HCV) frequently occurs. Approximately 5 to 20% of the HIV-infected population is estimated to be co-infected with HBV and for HCV this figure is estimated be around 5 to 15%. Of note, coinfection rates differ significantly according geographic regions for different types of hepatitis viruses.[28]

However, it remains controversial to which extent coinfection with hepatitis virus impacts on the clinical outcome for patients with HIV infection receiving HAART. For instance, one study reported that HBV infection neither alters the clinical outcome of HIV infection nor could an effect be detected the other way around,[29]. In apparent disagreement, however, another study demonstrated that HBV is significantly related to high mortality in HIV patients.[30] Similarly, inconsistent data have been reported for HCV coinfection as well.[31,32] Therefore, further study is required to characterize the relationship between hepatitis virus coinfection and mortality in HIV infected patients.

Part Two: Cancer

- Global burden of HCC attributable to virus

Hepatocellular Carcinoma (HCC) is the fifth most common malignancy and the second leading cause of cancer-related death worldwide.[33] Prognosis of patients with advanced stage HCC is poor, with median overall survival below 20 months.[34] Importantly, cancer in the liver can often develop as a consequence of an earlier infection and thus their relation is important subject for study. The attributable fraction (AF) is commonly used in epidemiological studies to quantify the burden of the relevant factor poses in causing a

particular disease. AF, in this study, means the proportion of new cancer incidences that would have been theoretically be avoided if exposure to a specific viral factor was modified or removed.[35] AFs estimates for all cancers worldwide and viral-associated cancers worldwide in 2012 are displayed in Figure 1.

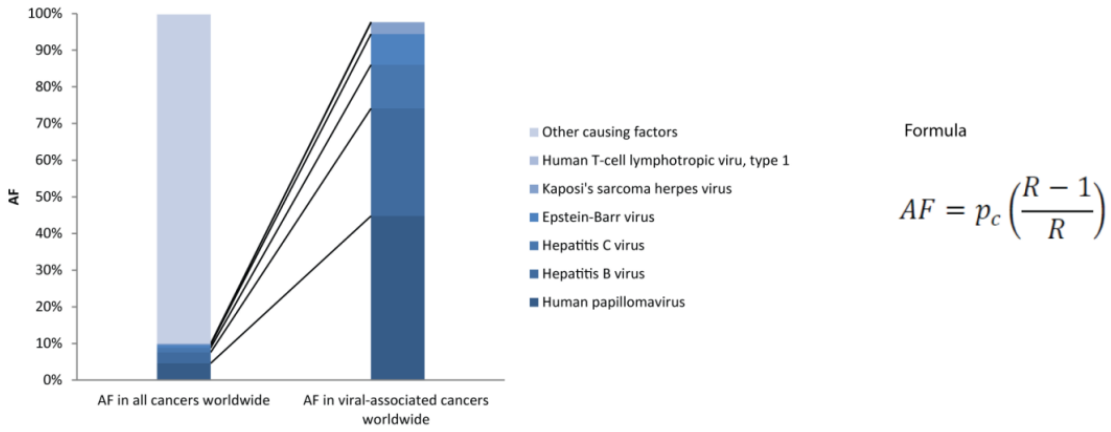

Figure 1 AFs for all cancers (left stacked bar) and viral-associated cancers worldwide in 2012 (right stacked bar). In the formula, R indicates the relative risk associated with exposure. p_c indicates the prevalence of cases. Data is calculated from the original data in a previous study (reference No.35),

HBV and HCV are two important factors of HCC development. HBV and HCV caused 420,000 and 170,000 new cancers in 2012.[35] The incidence of HCC is much higher among HIV patients co-infected by hepatitis virus than in patients infected only by hepatitis virus.

- <u>Prognostic factors in HCC natural history of disease</u>

  Racial disparity in survival among American population with HCC has been identified in several studies. In particular, poor survival of African Americans has been noted. Ethnicity-dependent natural history of disease has been documented demonstrated for HCC in the United States (U.S.) in several studies.[36-38] To improve the quality of health care for HCC patients, it is important to identify the factors affecting this ethnical disparity in overall

survival rates (OS), and to compare their impact. As a matter of fact, several factors associated with tumor presentation at time of diagnosis, type of surgical treatment, and socioeconomic status (SES), have previously been studied with regard to ethnic disparity in OS for HCC patients, but many questions remain and require urgent clarification.

- Therapeutic treatment of HCC
  Surgical resection and liver transplantation are the only potentially curative treatment for a small proportion of early stage patients of HCC. Sorafenib was an only FDA approved systematic chemotherapy to treat patients with advanced HCC, which could only increase overall survival with 2-3 months.[39] Therefore, new strategies to improve the prognosis of HCC patients are necessary.

Scope and aims of this thesis

Much of liver pathology is related to infection with HBV and HCV and it is important to define factors associated with clinical behavior of disease following infection with these viruses. Thus in this thesis I first focus on the natural history of chronic viral diseases associated with HBV or HCV infection, also in relation to HIV coinfection. Problem is that disease progression and its effects on liver physiology can be difficult to monitor and often reliable determination of liver health currently involves highly invasive liver biopsies. Evidently, serum based markers would bring disease monitoring forward and thus allow more precise analysis of disease course and thus also aids delineation of risk factors associated with alternative outcome. To address this void I first explore a potential clinically useful biomarker, cytokeratin-18. Its potential in monitoring chronic hepatitis is documented in **Chapter 2**. Concomitantly, however, I also tried to characterize the general beneficial effects of antiretroviral therapy in patients with HIV infection. To this end a big cohort from Yunnan province in China was analyzed for identification of the factors associated with overall survival and the results, described in **Chapter 3**, show that especially CD4 counts are important, but only in the early course of disease. This highlights the care that should be taken when interpreting biological information in chronic disease and highlights the importance of defining novel markers specifically aimed at assessing disease course in chronic patients (as I attempted in **chapter 2**) but also in general show that

careful of epidemiological data is necessary and one should be careful not to over-extrapolate results obtained, a theme that will also emerge from other parts of this thesis. Indeed. prevalence of coinfection with hepatitis virus and its impact on clinical outcome of patients with HIV infection were explored in **chapter 4** and also show this: no negative impact of coinfection was found on the overall survival of these patients treated with HAART and thus the notion that more infection means more disease is simplistic. Due to the fact that chronic HBV/HCV infection is associated with developing HCC, I subsequently focus in this thesis is on the clinical outcome of patients with HCC. In particular, because of the existing racial disparity in overall survival for American patients with HCC, I aimed to identify the actual contributors to such disparity based on the Surveillance, Epidemiology, and End Results (SEER) database (**Chapter 5**). Again the result show a surprising correlation to the time domain: even many years after diagnosis racial disparity is maintained. The notion that racial disparity is driven access to health care thus appears an oversimplification and highlights the importance of studies exactly dissecting the contribution of different factors to disease course and the necessity of careful study of their relationship to continuous variables in the study cohort, especially the time domain. These lessons were taken into account in the last experimental chapter (**Chapter 6**) in which I study a potentially effective anti-HCC treatment, metformin, which was systematically analyzed via the meta-analysis method. The relationship between these findings and their place in the contemporary body of literature are presented in a general discussion on this thesis (**Chapter 7**). In conjunction I hope my studies will contribute to better clinical management of liver disease.

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Chapter 2 Serum levels of caspase-cleaved cytokeratin 18 (CK18-Asp396) predict severity of liver disease in chronic hepatitis B

Juan Li, Auke P. Verhaar, Qiuwei Pan, Rob de Knegt, Maikel P. Peppelenbosch*

Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands.

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Abstract

Caspase-cleaved cytokeratin 18 (CK18-Asp396) is a potential clinically useful biomarker in liver disease as it is released from hepatocytes during apoptosis. Here we investigate serum CK18-Asp396 levels in chronic hepatitis B (CHB). Overall 163 patients with CHB were included. Serum CK18-Asp396 were determined by elisa and results were related to steatosis grade, histological activity index (HAI), inflammation score, and METAVIR fibrosis grade in parallel obtained liver biopsies as well as to viral load, serum levels of liver enzymes and albumin. Receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic performance of serum CK18-Asp396 levels for assessing disease activity. A higher level of serum CK-18 concentrations was found in patients with significant inflammation vs no significant inflammation (378.5 [IQR:173.2-629.6] vs 137.3 [87.5-197.7], $P < 0.05$) and in patients with significant fibrosis vs no significant fibrosis (177.8 [IQR:120.8-519.1] vs 142.7 [IQR:88.8-214.4], $P < 0.05$). There was no differential CK-18 level by degree of steatosis. CK-18 was an independent predictor for significant inflammation with an 82% specificity and a 94% negative predictive value. We found the strongest correlation of CK-18 with ALT and AST(both r = 0.52; $P < 0.001$), less with Albumin (r = -0.24; $P < 0.05$) and viral load (log) (r = 0.19; $P < 0.05$). CHB is accompanied by continuous high levels of hepatocyte apoptosis, suggesting that elimination of the infected compartment constitutes a defensive strategy against disease. Accordingly, CK-18 works as an independent predictor for significant inflammation with a high specificity.

Plain language summary

In recent years it has become clear that serum levels of a fragment of cytokeratin 18 (so-called caspase-cleaved cytokeratin 18) reflects the amount of programmed cell death in the liver. It remains, however, obscure whether programmed cell death is in general important during hepatitis B infection and specifically whether serum levels of caspase-cleaved cytokeratin 18 can be a marker for disease severity in hepatitis B. Here we show that liver inflammation of hepatitis B is characterized by high levels of called caspase-cleaved cytokeratin 18 and this novel marker for hepatitis B-associated inflammation is potentially superior to currently used serum markers.

Introduction

Chronic hepatitis B (CHB) infection is a major public health problem, which affects more than 350 million people worldwide[1]. It is well known that CHB is associated with hepatic steatosis, inflammation, and fibrosis[2-5]. Hence, histological examination is an integral part of evaluating CHB patients. Evaluation of the CHB associated liver damage is critically important for making decisions in antiviral therapy and need for surveillance[6]. Liver biopsy remains the golden standard in assessing the severity of inflammation and fibrosis [6, 7]. However, the procedure is costly, sensitive to sampling errors, and a potential cause for complications[8]. Therefore, a reliable non-invasive diagnostic tool is urgently needed.

An increasing number of studies have emerged to explore potential non-invasive tools. Given the importance of apoptosis in development of chronic liver disease in general[9-11], the importance of apoptosis in chronic hepatitis B remains unclear, and studies are hampered by the difficulty of distinguishing apoptosis in the hepatocyte compartment and the lymphocyte compartment. The former may represent efforts of the body to limit the size of the infected compartment and may thus be associated with antiviral responses *in lieu* of viral clearance and consequently with aggravated inflammation[12] and progression to HCC[13]. Apoptosis in the lymphocyte compartment is however associated with reduced inflammatory activity[14]. Hence studies looking specifically at hepatocyte apoptosis are urgently needed to characterize the role of programmed cell death in CHB and hepatocyte-specific apoptosis markers may also have substantial diagnostic value.

In this context cytokeratin-18 (CK-18) is interesting. Expression of CK-18 is limited to certain endodermal derivatives, but is most prominently expressed in hepatocytes[15]. During hepatocyte apoptosis CK-18 is subject to specific cleavage by caspases, resulting in the release of neo-epitope (CK18-Asp396), which is not detectable in necrotic or vital cells[16]. Cytokeratin-18 (CK-18) is the best described hepatocyte-specific apoptosis marker[17], and many studies investigated the potential value of CK-18 as a non-invasive marker in predicting severity of steatosis, inflammation, and fibrosis. Previous studies have shown that increased levels of CK18-Asp396 are associated with CHB [18, 19] and relates to the severity of steatosis[20]. In addition, CK18-Asp396 can be a predictive marker for distinguishing between inactive carrier and HBeAg-negative CHB [21] and CK-18 correlate with the

presence of significant fibrosis in chronic hepatitis C (CHC)[22], non-alcoholic fatty liver disease (NAFLD)[23], as well as cirrhosis associated with CHB [24]. Thus CK-18 appears useful to measure hepatocyte apoptosis in CHB and its measurement would allow making correlations between hepatocyte programmed cell death, inflammatory responses and viral load, allowing assessment as to the extent to which hepatocyte apoptosis contributes to anti-viral defense.

Here we measured CK18-Asp396 in a group of CHB patients in a well characterized Dutch cohort which includes patients across all grades of steatosis, inflammation and fibrosis. The results show that CK18-Asp396 is associated with hepatic inflammation and diagnostically useful for clinical determination of this condition. Furthermore the results support the notion that hepatocyte apoptosis may contribute to limiting the size of the virus-infected compartment.

Results

Patient characteristics

The characteristics of the patients are described in **Table 1**. The mean age of the 163 patients was 40 years old. In terms of inflammation, there were 22 patients with significant inflammation (grade ≥ 7), which accounted for 13% of total patients. As for fibrosis, there were 33 cases with significant fibrosis (advanced fibrosis or cirrhosis), accounting for 20% of patients. Finally the grade of steatosis was considered as normal in 2 patients (1%), mild in 104 patients (64%), moderate in 44 patients (27%) and severe in 13 patients (8%).

Elevated serum CK-18 levels in patients with significant inflammation and fibrosis

Figure 1 displays the serum CK-18 levels across patients when differentiated to either steatosis grade, severity of inflammation or to fibrosis grade. When comparing steatosis grade the difference in CK-18 serum levels was not significant ($P > 0.05$). However, there were significant differences in CK-18 levels among different grades of inflammation and fibrosis. Patients with significant inflammation had a higher CK-18 serum level than those with no significant inflammation (378.5 [IQR:173.2-629.6] vs 137.3 [87.5-197.7], $P < 0.05$). Similarly, patients that presented with significant

fibrosis had a higher CK-18 level than those with no significant fibrosis (177.8 [IQR:120.8-519.1] vs 142.7 [IQR:88.8-214.4], $P < 0.05$). Correlation of CK-18 serum levels was analyzed with several other clinical parameters recorded in this cohort (**Table 2**). In terms of biochemical parameters, the strongest correlation was with ALT and AST(both r=0.52; $P < 0.001$), followed by a negative correlation with Albumin (r = -0.24, $P < 0.05$) and a positive correlation with viral load (log) (r = 0.19, $P < 0.05$). No correlation with either age or BMI was found ($P > 0.05$). Among the histological parameters, CK-18 correlated best with the grade of inflammation (r = 0.37; $P < 0.001$), followed by the grade of fibrosis (r = 0.18; $P < 0.05$). No correlation with steatosis was found($P > 0.05$). Through multivariate analyses, adjusting for age, BMI, viral load (log), ALT level, ASL level and Albumin level, it was demonstrated that CK-18 serum levels can act as an independent predictor for the presence of significant inflammation ($P < 0.05$), but not for significant fibrosis ($P > 0.05$).

Predictive value of CK-18 for significant inflammation

We then assessed the potential value of CK-18 as a diagnostic tool by estimating AUROC, sensitivity and specificity. The AUROC of CK-18 for predicting significant inflammation was 0.81 (95%CI: 0.72-0.91) (**Figure 2**). Sensitivity, specificity, positive/negative predictive values, and positivity/negative likelihood ratios are summarized in **Table 3**. Of note, all these values were calculated according to the optimal cut-off points of CK-18 levels: 243.0 U/L. Although CK-18 had a low sensitivity of 68% and a very low positive predictive value of 37%, it showed a high specificity of 82% and a high negative predictive value up to 94%.

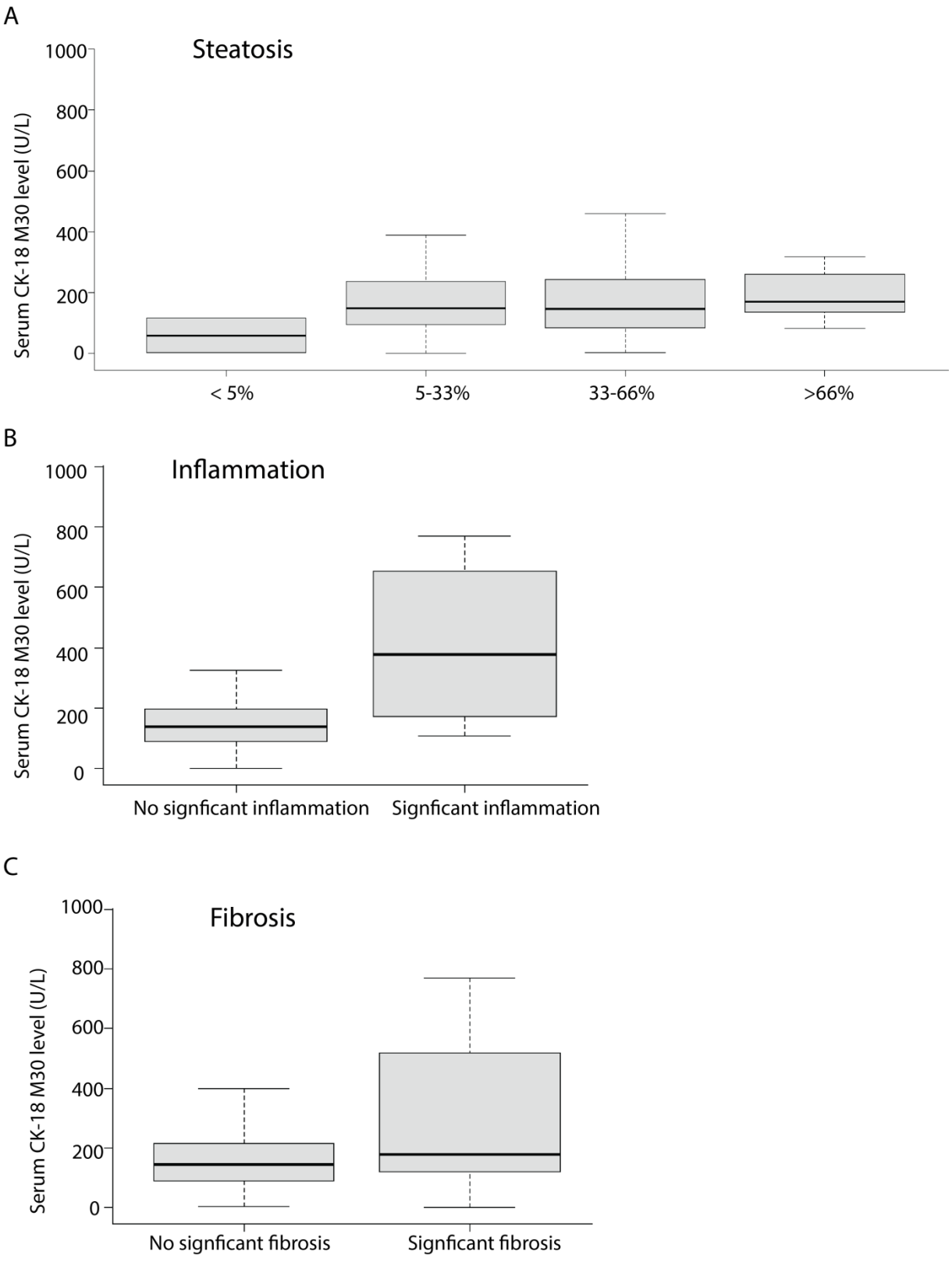

Figure 1 Boxplots of serum CK 18 levels in relation with steatosis, inflammation, and fibrosis in CHB patients.

Notes: (**A**) No difference across grades of steatosis ($P > 0.05$); (**B**) significant differences between significant and no significant inflammation ($P < 0.05$); (**C**) significant differences between significant and no significant fibrosis ($P < 0.05$).

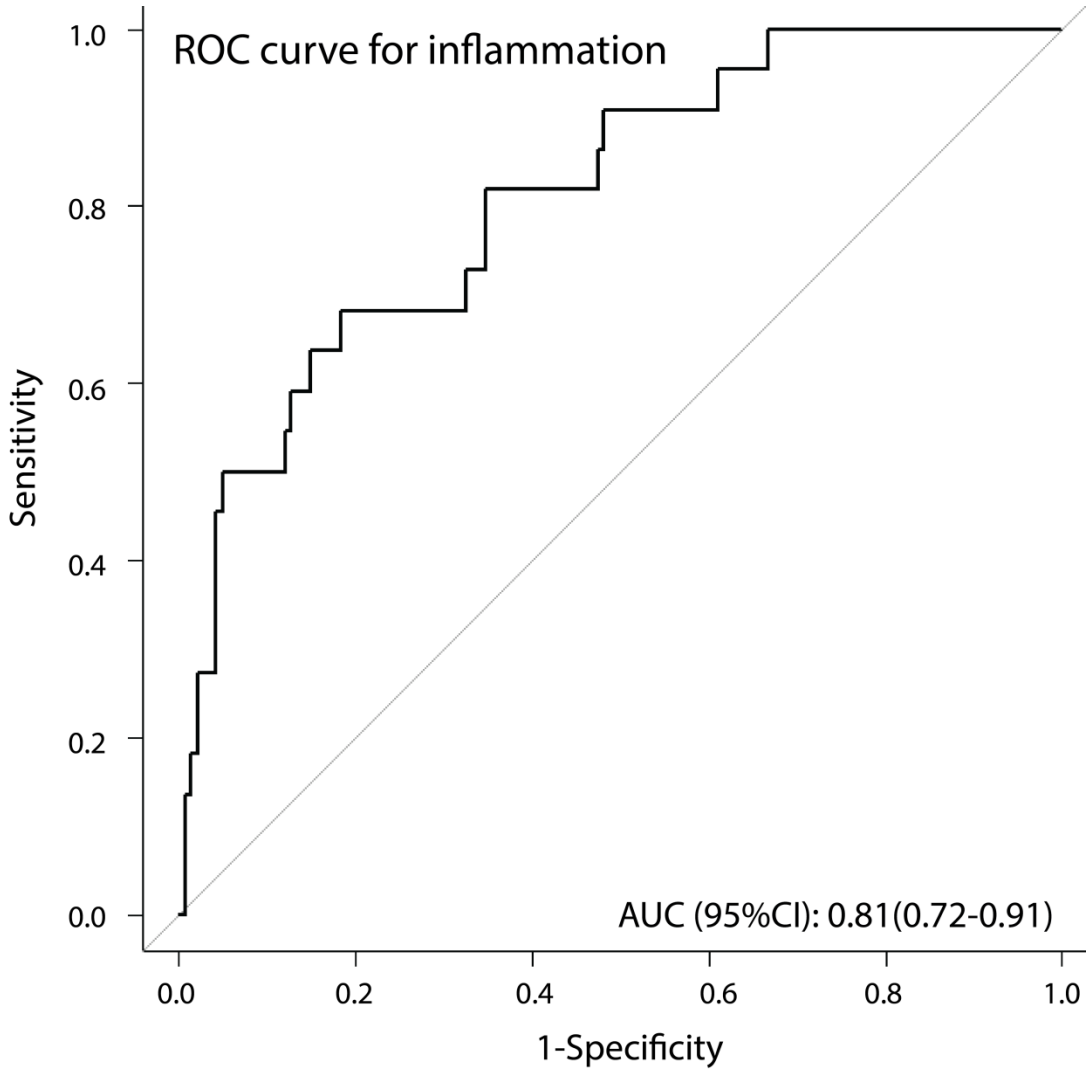

Figure 2 Predictive value of CK 18 for significant inflammation.

Notes: We assessed the potential value of CK 18 as a diagnostic tool by estimating AUC, sensitivity, and specificity. The AUC of CK18 for predicting significant inflammation was 0.81 (95%CI: 0.72-0.91).

Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic.

Discussion

The contemporary of biomedical literature supports the notion that CK18-Asp396 serum levels adequately reflect hepatocyte apoptosis[32]. In our study we exploited this notion to evaluate hepatocyte apoptosis in CHB. The study demonstrated that CHB is indeed associated with hepatocyte apoptosis, showing strong correlations with significant inflammation and fibrosis, but not with steatosis, and especially demonstrating a diagnostic value of CK-18 serum levels for identification of significant inflammation of the liver in CHB patients. The strong correlation with inflammatory responses suggests that hepatocyte apoptosis may serve as a strategy to limit the size of the HBV-infected compartment, a notion possibly supported by the observation that viral load shows only weak correlation with CK18-Asp396 serum levels, possibly reflecting functional effects of compartment size reduction. Future studies are necessary to substantiate this idea.

A previously published study suggested a combination of routine tests as non-invasive markers for liver inflammation and fibrosis [7]. In that report, a prediction model was suggested for identification of significant liver inflammation combining age, HBV DNA levels, AST, and albumin. However, in the current study, CK-18 serum level was the single independent predictor based on our multivariate analysis. In terms of significant inflammation, CK-18 serum level was regarded as a good predictor with an 82% specificity and 68% sensitivity. The apparent superiority of CK18-Asp396 serum level determination over other non-invasive markers further highlights the intimate connection between CHB-associated inflammation and hepatocyte apoptosis.

The diagnostic value of CK-18 serum levels for identification of significant inflammation in viral infection-associated chronic liver disease corresponds well with the findings of Bae et al. [21]. This study reports a high specificity of 89% for CK-18 serum levels and a lower sensitivity of 45% for CHB patients, but also reported that the combination of CK-18 with AST yielded a much higher specificity up to 96% in their study, although simultaneously decreasing sensitivity to 38% [21]. As a liver enzyme, AST is more representative of hepatocyte death in general rather as indicating hepatocyte apoptosis per se. In spite of a strong correlation between CK-

18 and AST observed in our study, AST was not an independent predictor, suggesting that hepatocyte apoptosis and necrosis is a defining property in CHB.

Considering significant fibrosis, CK-18 serum levels were previously reported as a useful predictor of this process in NAFLD patients [10, 23] and a correlation of CK-18 serum levels with fibrosis stage in CHB is supported by the observations of Sumer et al. [24]. Similar to our findings, these authors detect increased CK-18 serum levels when significant fibrosis is present. Our data, however, indicate that CK-18 serum level cannot predict significant fibrosis independently, which also contrasts a recent study showing that CK-18 serum levels work independently as a predictor for the presence of significant fibrosis (F≥3)[10]. Generally speaking we feel that hepatocyte apoptosis is unlikely to have a causal relationship with the fibrosis process and in potential agreement the Rosso study presented a low AUROC value (0.61) for fibrosis with a sensitivity of 88% and a specificity of 38%. As a matter of fact, the beneficial role of apoptosis was ever reported in pancreatitis disease[33]. Indeed, transient elastography[34], measuring liver stiffness, was found to be a better predictor than CK-18 serum levels. To obtain a more accurate prediction for the presence of significant fibrosis, both markers, transient elastography and CK-18 serum levels, were combined. Unfortunately, the combination performed not better than the transient elastogrpahy alone. In conclusion, we feel that CK-18 may have only limited value for identification of significant fibrosis.

In summary, our study has further demonstrated the predictive value of CK-18 serum levels as a non-invasive marker for the presence of significant liver inflammation in CHB. Additionally, the potential combination with other non-invasive markers might improve the total performance, especially the sensitivity. With regard to the identification of significant fibrosis, we consider the limited diagnostic value of CK-18. Further study is required to identify more reliable and efficient non-invasive tool. Finally, our results support the notion that hepatocyte apoptosis has an important functionality in CHB pathogenesis.

Methods

Patient selection

This retrospective study includes 163 consecutive patients with CHB from 1985 to 2012 at the Erasmus University Medical Center in Rotterdam, the Netherlands. Three indications were defined as an endpoint in this study: steatosis grade, inflammation, and fibrosis. Steatosis was classified into the following groups according to Brunt score[25]: <5%, normal; 5-33%, mild steatosis; 33-66%, moderate steatosis; >66%, severe steatosis. The inflammatory activity (grade) and the degree of hepatic fibrosis (stage) were assessed according to modified histological activity index (HAI) of Ishak system[26] and Metavir system (nil fibrosis F0, mild fibrosis F1, moderate fibrosis F2, advanced fibrosis F3, and cirrhosis F4), respectively[27]. For the purpose of this study, inflammation was divided into two groups: significant inflammation (grade ≥ 7) and non-significant inflammation (grade < 7). Similarly, fibrosis staging was divided in subgroups: significant fibrosis(stage ≥ 3) and non-significant fibrosis (stage < 3)[7].

This study was performed in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. Due to the retrospective nature of this study, the informed consent was not obtained from patients. Patients' identities were not revealed in our study. This study was approved by the ethical review board of Erasmus Medical Center (Rotterdam, The Netherlands)[28].

Laboratory test

Serum level of CK-18 was measured with the M30-Apoptosense enzyme-linked immunosorbent assay(ELISA) kit (VLVbio (PEVIVA), Nacka, Sweden) according to manufacturer’s instructions, which have been well established to accurately measure CK18-Asp396[29]. Each patient sample was measured in triplicate and the absorbance value was determined by microplate reader (FLUOstar Omega, BMG Labtech, De Meern, The Netherlands), according to routine procedures[30]. In short, the ELISA measures apoptosis in CK-18 positive cells, such as hepatocytes, with an antibody that specifically recognizes soluble caspase cleaved CK-18.

Statistical analysis

Continuous variables were reported as mean (SD) or median(interquartile range[IQR]) according to data distribution, and categorical variables as percent. Quantitative variables were analyzed using t test for normal distribution data or Mann-Whitney U test for highly skewed data. The eventual diagnostic predictor value was calculated by receiver-operating curve (ROC). Youden index was used to determine the optimal cut-off value of CK-18. The probabilities of true positive (sensitivity) and true negative (specificity) were determined according to the calculated optimal cut-off value. The positive/negative likelihood ratio (LR+/LR-) were calculated by the following formula: LR+ = sensitivity / (1-specificity); LR- = (1-sensitivity) / specificity. The area under the ROC curve (AUROC) was generated to assess the diagnostic performance of each independent predictor. ROC relevant analyses were done by using pROC package[31]. Correlation was calculated using Spearman's rank correlation coefficient. All statistical analyses were performed in R software(version 3.2.0), and statistical significance was set at $P < 0.05$ (two-tailed).

Table 1 Clinical characteristics of patients with chronic hepatitis B-virus infection (CHB)

Characteristics	All Patients (n=163)	Significant Inflammation (n= 22)	No Significant Inflammation (n=141)	Significant Fibrosis (n=33)	No Significant Fibrosis (n=130)
Gender, n(%)					
Male	136 (83)	18 (18)	118 (84)	28 (85)	108 (83)
Female	27 (17)	4 (82)	23 (16)	5 (15)	22 (17)
Age, years					
Mean [SD]	40.0 [11.5]	45.5 (13.1)	39.1 [11.0]	47.2 [12.1]	38.1 [10.6]
Median [IQR]	41.0 [31.0-48.0]	47.5 [35.3-55.8]	39.0 [31.0-46.0]	48.0 [39.0-56.0]	37.0 [30.3-45.8]
Race, n(%)					
Caucasian	84 (52)	12 (55)	72 (51)	18 (54)	66 (51)
Asian	51 (31)	8 (36)	43 (30)	11 (33)	40 (31)
African/Black	23 (14)	1 (5)	22 (16)	4 (12)	19 (15)
Other Race	5 (3)	1 (5)	4 (3)	0 (0)	5 (4)
BMI					
Mean [SD]	27.2 [4.1]	27.5 [4.0]	27.2 [4.2]	27.1 [3.3]	27.3 [4.3]
Median [IQR]	27.0 [24.8-29.8]	28.6 [25.6-30.1]	27.0 [24.8-29.8]	27.2 [24.8-29.2]	27.0 [24.8-29.8]
HBeAg Status, n(%)					
Positive	58 (36)	13 (59)	45 (32)	11 (33)	47 (36)
Negative	105 (64)	9 (41)	96 (68)	22 (67)	83 (64)

ALT, xULN, U/L					
Mean [SD]	2.3 (3.9)	3.2 [1.8]	2.2 [4.1]	2.4 [1.8]	2.3 [4.3]
Median [IQR]	1.5 [1.1-2.2]	2.8 [1.9 -4.6]	1.4 [1.1-1.9]	1.8 [1.0-3.4]	1.5 [1.1-2.0]
AST, xULN,U/L					
Mean [SD]	1.4 [1.2]	2.1 [0.9]	1.2 [1.2]	1.8 [1.1]	1.3 [1.2]
Median [IQR]	1.0 [0.8-1.4]	1.9 [1.5-2.6]	1.0 [0.8-1.2]	1.5 [1.0 -2.1]	1.0 [0.9-1.2]
ALK					
Mean [SD]	79.9 [64.5]	85.6 [33.8]	78.9 [68.6]	82.4 [28.5]	79.2 [71.2]
Median [IQR]	72 [60.0-89.0]	72.0 [63.0-97.0]	71.0 [59.0-86.0]	75.0 [62.8-90.5	71.0 [59.0-84.0]
Albumin, g/L					
Mean [SD]	45.0 [3.5]	42.4 [3.1]	45.5 [3.4]	42.8 [4.0]	45.7 [3.1]
Median [IQR]	45.0 [43.0-47.0]	42.5 [40.0-45.0]	46.0 [44.0-48.0]	44.0 [40.0-45.3]	46.0 [44.0-48.0]
Viral Load (log)					
Mean [SD]	5.0 [2.9]	7.2 [1.9]	4.8 [2.9]	5.8 [2.4]	4.9 [3.0]
CK-18 Serum Level, U/L					
Mean [SD]	206.6 [190.5]	404.8 [236.6]	175.7 [162.6]	321.3 [304.1]	177.5 [135.7]
Median [IQR]	150.6 [95.8-242.9]	378.5 [173.2-620.6]	137.3 [87.5-197.7]	177.8 [120.8-519.1]	142.7 [88.8-214.4]
Significant Inflammation, n(%)					
Yes (Grade ≥ 7)	22 (13%)	NA	NA	NA	NA
No (Grade < 7)	141 (87%)	NA	NA	NA	NA
Significant Fibrosis,					

n(%)					
Yes (F ≥ 3)	33 (20%)	NA	NA	NA	NA
No (F < 3)	130 (80%)	NA	NA	NA	NA

ALT, alanine aminotransferase; AST, aspartate aminotransferase

Table 2 Correlation of Clinical Parameters with CK-18

Parameters	**rho**	***P* Value**
Age	0.04	0.603
BMI	0.11	0.183
ALT	0.52	<.0001
AST	0.52	<.0001
Albumin	-0.24	0.015
Viral Load (log)	0.19	0.017
Steatosis	0.07	0.368
Inflammation	0.37	<.0001
Fibrosis	0.18	0.020

Table 3 Performance of serum CK-18 levels for the diagnosis of significant inflammation and significant fibrosis

	Cut-off value (U/L)	**Sensitivity (%)**	**Specificity (%)**	**PPV[a]**	**NPV[a]**	**LR+[a]**	**LR-[a]**
Significant inflammation	243.0	68	82	37	94	3.9	0.4

[a]PPV indicates positive predictive value; NPV indicates negative predictive value; LR+ indicates positive likelihood ratio; LR- indicates negative likelihood ratio.

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Chapter 3 Prognosis of HIV Patients Receiving Antiretroviral Therapy According to CD4 Counts: A Long-term Follow-up study in Yunnan, China

Li Ren[1,2,3#], Juan Li[4#], Shiyi Zhou[5], Xueshan Xia[6], Zhenrong Xie[5], Pan Liu[7], Yu Xu[5], Yuan Qian[8], Huifeng Zhang[2], , Litang Ma[8], Qiuwei Pan[4], Kunhua Wang[1,5*]

[1]Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Kunming, 650093, Yunnan Province, China;

[2]The First People's Hospital of Yunnan Province, Kunming, 650031, Yunnan Province, China;

[3]Medical faculty of Kunming University of Science and Technology, Kunming, 650093, Yunnan Province, China;

[4]Department of Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam, The Netherlands;

[5]Yunnan Institute of Digestive Disease, the First Affiliated Hospital of Kunming Medical University, Kunming, 650032, Yunnan Province, China;

[6]Faculty of Life Science and Technology, Center for Molecular Medicine in Yunnan province, Kunming University of Science and Technology, Kunming, 650093, Yunnan Province, China;

[7]Yan'an Hospital of Kunming Chenggong hospital, Kunming, 650501, Yunnan Province, China;

[8]The First People's Hospital of Zhaotong City, Zhaotong, 657000, Yunnan Province, China.

#These authors contributed equally.

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Abstract

We aim to evaluate the overall survival and associated risk factors for HIV-infected Chinese patients on antiretroviral therapy (ART). 2517 patients receiving ART between 2006 and 2016 were prospectively enrolled in Yunnan province. Kaplan-Meier analyses and Cox proportional hazard regression analyses were performed. 216/2517 patients died during a median 17.5 (interquartile range [IQR] 6.8-33.2) months of follow-up. 82/216 occurred within 6 months of starting ART. Adjusted hazard ratios were10.69 (95%CI 2.38-48.02, p = .002) for old age, 1.94 (95%CI 1.40-2.69, p < .0001) for advanced WHO stage, and 0.42 (95%CI 0.27-0.63, p < .0001) for heterosexual transmission compared to injecting drug users. Surprisingly, adjusted hazard ratios comparing low CD4 counts group (<50 cells/μl) with high CD4 counts group (≥500 cells/μl) within six months after starting ART was 20.17 (95%CI 4.62-87.95, p <.0001) and it declined to 3.57 (95%CI 1.10-11.58, p=.034) afterwards. Age, WHO stage, transmission route are significantly independent risk factors for ART treated HIV patients. Importantly, baseline CD4 counts is strongly inversely associated with survival in the first six months; whereas it becomes a weak prognostic factor after six months of starting ART.

Introduction

As estimated by the Chinese health authority [1], there were 501,000 people living with human immunodeficiency virus (HIV/AIDS) by the end of 2014 in China, accounting for 0.037% of the total population. Despite of a low national prevalence rate, the HIV epidemic is severe in some areas of the Southwest China, in particular the Yunnan, Sichuan, and Guangxi provinces. Yunnan is the area most affected by HIV/AIDS in China. The epidemic has spread from high-risk groups including drug users, sex workers and unsafe blood recipients to the general population. With a population of 44 million, officials estimate that this province has 80,000 HIV infected individuals.

Antiretroviral drugs in combination of three or more drugs from more than one class, often called “highly active antiretroviral therapy (HAART)”, are very effective in suppressing HIV, although do not eradicate the virus. Since the first introducing of HAART in the mid-1990s, it has led to an unprecedented decline of mortality caused by HIV/AIDS both in the USA [2] and Europe [3]. Because of the great success of HAART in treating HIV/AIDS in the developed countries, the World Health Organization (WHO) has promoted a public health approach to scale up the access of antiretroviral therapy in resource-limited setting since 2002 [4,5]. As an emergency response to save and improve the lives of AIDS patients in China, the China National Free Antiretroviral Treatment Program (NFATP) was piloted in 2002 and scaled up in 2003 national wide [6,7]. Until 2014, a total of 295,358 patients in China have received HAART [1], and thus a large proportion of HIV patients have benefited from this program. Based on China national HIV database as well as a few local studies, an increasing coverage of antiretroviral treatment has significantly decreased HIV/AIDS-related mortality [8-12].

It is important to identify prognostic factors for survival among HIV-infected patients receiving ART. Based on previous studies, the main risk factors for death include baseline low CD4 cell count, old age and advanced WHO stage. Among them, CD4 cell count was suggested as the most important prognostic factor based on the estimated hazard ratio values [9-11,13-21]. According to those results, the prognostic value of CD4 cell count seems to be well established. However, a recent study, examining European and North American patients, suggested that patients with low baseline CD4 cell count only carry the burden of increased risk of death up to 5 years after ART 16. This indicated the impersistence of the CD4 cell count on

the increased mortality, although no other study has reported similar observation. In order to better understand the treatment outcome of HIV patients, we have carried out a large prospective cohort study with long-term follow up in China, enrolling patients from Zhaotong, a prefecture-level city located in the northeast corner of Yunnan province. In this study, we aim to evaluate the overall survival and associated risk factors for the HIV-infected patients on ART in this cohort, with particular focusing on the prognostic value of CD4 cell count.

Results

Baseline Characteristics

A total of 2517 patients (adult) before starting ART were enrolled between July 2006 and April 2016 in this study. The baseline characteristics of the study population at start of ART are summarized in **Table 1**. Patients had a median age of 39 (interquartile range 31-50) years. The number of male patients accounted for 59.9% of all patients. A total of 76.2% patients were married. As for the HIV transmission routes, more than two-thirds (71.9%) were infected through heterosexual; whereas 10.5% through injecting drug use (IDU). In terms of WHO clinical stage, 78.9% received ART at stage I/II and 20.9% at stage III/IV. When measuring CD4 cell count on continuous scale, CD4 cell count had an overall median 281 (IQR 177-388) per μl. By dividing CD4 cell count into subgroups, there are 5% of patients presenting low CD4 cell count (< 50 per μl), and 66.9% presenting high CD4 cell count (≥ 200 per μl).

Cumulative mortality in study population

The crude cumulative mortality for study population is displayed in **Figure 1**. Their median follow-up time was 17.5 (IQR 6.8-33.2) months. The cumulative probabilities for mortality were 9%, 13%, 19%, and 26% at 2 years, 4 years, 6 years, and 8 years, respectively. 8.6% (216/2517) of patients died during the follow-up period. Within these patients, 38.0% (82/216) died in their first 6 months. Patients who died at their first six months of follow-up period had a median age of 42.0 (IQR 34.3-60.0), 70.7% were male, and 57.3% were in advanced WHO clinical stage of III/IV. The continuous CD4 cell count had a baseline median 140 (IQR 59-236) cells/μl. 25.6% (21/82) patients were in low CD4 cell count stratum (lower than 50 per μl); while

26.8% (22/82) in high CD4 cell count stratum (200/µl or greater) (**supplementary Table S1**).

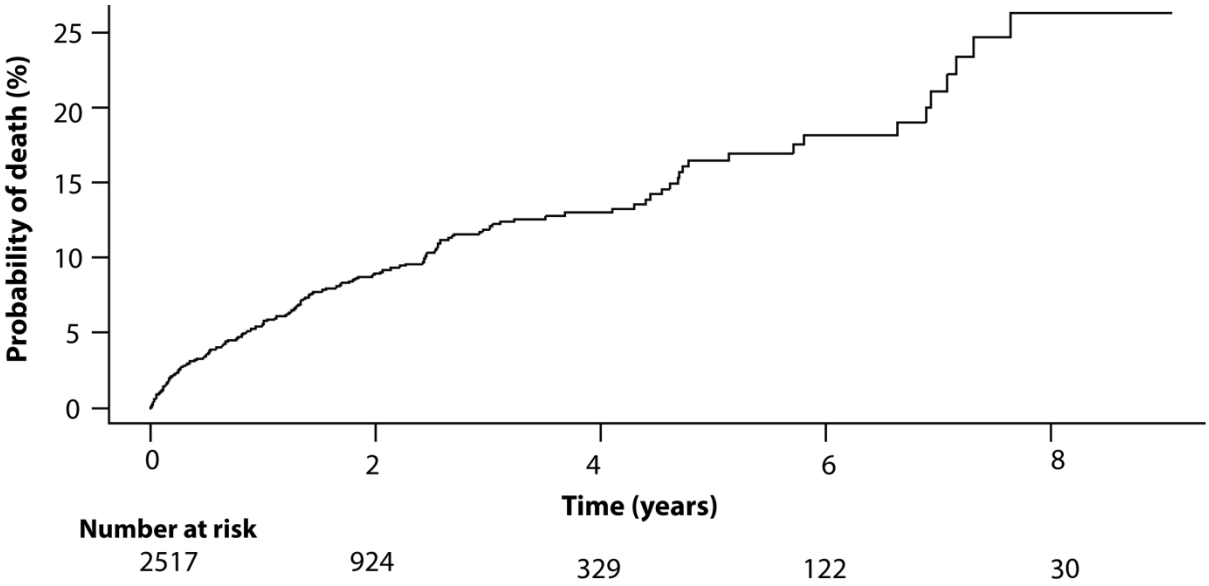
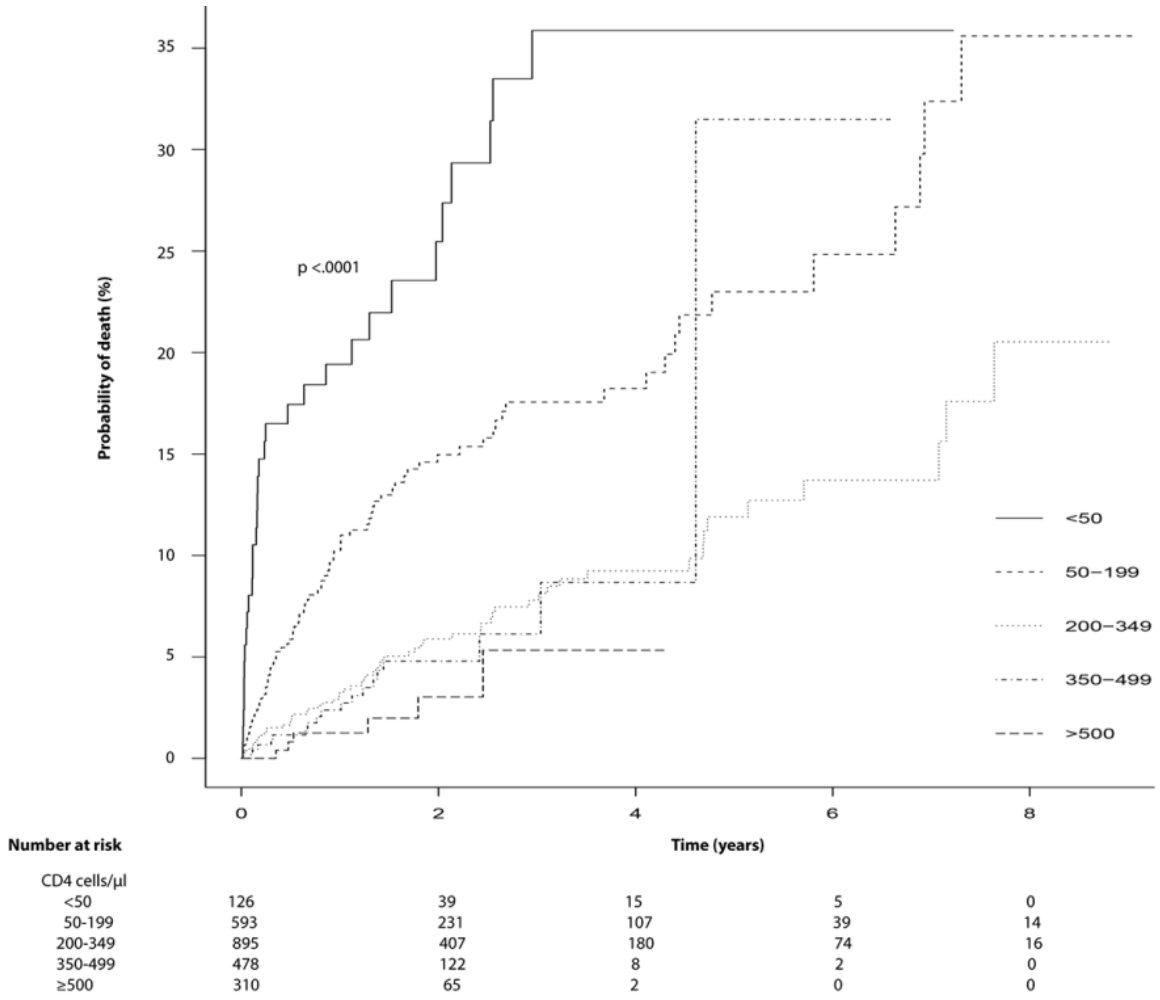


Figure 1 Cumulative morality from all-cause mortality for study population with 290 human immunodeficiency virus (HIV) infection receiving antiretroviral therapy (ART). 291 Corresponding numbers at risk at different time-points have been indicated below 292 the graph.

Prognostic values of baseline factors

The associations of baseline factors at start of ART with mortality, estimated from crude and adjusted Cox models with time-dependent coefficients for CD4 cell count are shown in **Table 2**. Age and CD4 cell count were significant prognostic factors for survival (p <.0001 by log-rank test) based on crude survival analysis as shown in **Figure 2** and **Supplementary Figure S1**. Of note, patients with low CD4 cell count (<50 cells/µl) had drastically increased risk for mortality at early follow-up time. In multivariate model, advanced WHO clinical stage (HR 1.94, 95%CI 1.40-2.69, p < .0001) and old age (>75 years) (HR 10.69, 95%CI 2.38-48.02, p = .002) were

significantly associated with worse survival; heterosexual mode was associated with better survival (HR 0.42, 95%CI 0.27-0.63, p < .0001) than injecting drug users. Varying hazard ratios for baseline CD4 cell count were observed in analysis for different time intervals since the start of ART. For instance, the adjusted HRs comparing low CD4 cell count group (<50 cells/μl) with high CD4 cell count group (≥ 500 cells/μl) during the first six months was 20.17 (95%CI 4.62-87.95, p <.0001), but it became 3.57 (95%CI 1.10-11.58, p=.034) after the first six months. By comparing those with CD4 cell count (50-199 cells/μl) and high CD4 cell count (≥500 cells/μl), significant HRs were found in the two intervals: 5.06 (95%CI 1.20-21.32, p = .027) ≤0.5 year and 2.89 (95%CI 1.02-8.04, p = .045) > 0.5 year (**Table 2**). With respect to the model accuracy, concordance with 0.791 suggested good predictive performance of the Cox model.



Number at risk

CD4 cells/µl					
<50	126	39	15	5	0
50-199	593	231	107	39	14
200-349	895	407	180	74	16
350-499	478	122	8	2	0
≥500	310	65	2	0	0

Figure 2 Cumulative morality from all-cause mortality for study population infected 294 by human immunodeficiency virus (HIV) receiving antiretroviral therapy (ART) 295 according to CD4 cell count (p <.0001). Corresponding numbers at risk at different 296 time-points split by CD4 cell count have been indicated below the graph.

Table 1 Baseline characteristics of the patients according to whether lost to follow up or not

Characteristics	**Overall (n=2517)**	**LTFU (n=199)**	**Non-LTFU (n=2318)**	**p-value[d]**
Gender, no. (%)				.512[a]
Male	1507/2517 (60)	124/199 (62)	1383/2318 (60)	
Female	1010/2517 (40)	75/199 (38)	935/2318 (40)	
Marital status, no. (%)				.004[a]
Unmarried	296/2501 (12)	39/197 (20)	257/2304 (11)	
Married	1919/2501 (76)	137/197 (70)	1782/2304 (77)	
Divorced	156/2501 (6)	10/197 (5)	146/2304 (6)	
Widowed	130/2501 (5)	11/197 (6)	119/2304 (5)	
Age at ART initiation, year				.137[b]
Mean (SD)	42 (14)	40 (15)	42 (14)	
Median (IQR)	39 (31-50)	36 (29-49)	39 (31-50)	
WHO HIV Clinical Stage, no. (%)				.004[a]
Stage I/II	1986/2511 (79)	158/197 (80)	1828/2314 (80)	
Stage III/IV	525/2511 (21)	39/197 (20)	486/2314 (21)	
CD4, cells/µl				.010[a]
Median (IQR)	281(177-388)	330 (220-455)	278 (173-383)	
<50	126/2402 (5)	5/179 (3)	121/2223 (1)	

50-199	593/2402 (24)	31/179 (17)	562/2223 (25)	
200-349	895/2402 (37)	67/179 (37)	828/2223 (37)	
350-499	478/2402 (20)	44/179 (25)	434/2223(20)	
≥500	310/2402 (13)	32/179 (18)	278/2223 (13)	
Transmission category, no. (%)				
Injecting drug users (IDU)	263/2517 (11)	42/199 (21)	221/2318 (10)	<.0001[a]
Homosexual	54/2517 (2)	3/199 (2)	51/2318 (2)	
Heterosexual	1809/2517 (72)	136/199 (68)	1673/2318 (72)	
Others/unknown[c]	391/2517 (16)	18/199 (9)	373/2318 (16)	

Abbreviations: LTFU=lost to follow up; Non-LTFU=not lost to follow up; ART indicates antiretroviral treatment; SD=standard deviation; IQR=interquartile range.
[a]Indicates Pearson's Chi-squared test;
[b]Indicates Welch Two Sample t-test;
[c]Include blood transfusion, mother-to-Child, and others;
[d]Baseline characteristics were compared between patients of LTFU and patients without LTFU.

Table 2 Cox proportional hazard regression analyses analyzing all-cause mortality after starting ART

	Univariate analysis		Multivariate analysis	
Baseline characteristics	Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value
Gender				
Female	1	(Reference)	1	(Reference)
Male	1.74 (1.30-2.34)	<.001	1.26 (0.90-1.75)	.167
Marital status				
Married	1	(Reference)	1	(Reference)
Unmarried	1.14 (0.78-1.68)	.506	-	-
Divorced	1.11 (0.64-1.92)	.706	-	-
Widowed	1.81 (1.05-3.14)	.033	-	-
Age at ART initiation, year[a]				
(0,20]	1	(Reference)	1	(Reference)
(20,25]	1.07 (0.23-4.97)	.929	0.96 (0.20-4.47)	.961
(25,30]	1.47 (0.35-6.21)	.604	0.83 (0.20-3.63)	.818
(30,35]	1.99 (0.48-8.28)	.344	0.88 (0.21-3.79)	.872
(35,40]	2.10 (0.50-8.78)	.308	1.05 (0.25-4.47)	.948
(40,45]	2.26 (0.53-9.59)	.271	1.18 (0.27-5.09)	.828
(45,50]	1.81 (0.41-8.11)	.435	0.77 (0.16-3.60)	.739

(50,55]	2.23 (0.49-10.17)	.302	1.45 (0.31-6.66)	.636
(55,60]	4.40 (1.02-18.91)	.046	2.92 (0.67-12.66)	.153
(60,65]	3.71 (0.82-16.57)	.087	1.84 (0.40-8.51)	.436
(65,70]	5.42 (1.20-24.49)	.028	2.96 (0.64-13.70)	.165
(70,75]	6.73 (1.49-30.40)	.013	2.45 (0.51-11.74)	.264
(75,90]	16.25 (3.73-70.85)	.000	10.69 (2.38-48.02)	.002
WHO HIV Clinical Stage				
Stage I/II	1	(Reference)	1	(Reference)
Stage III/IV	3.15 (2.40-4.13)	<.0001	1.92 (1.38-2.66)	<.0001
Transmission				
Injecting drug users (IDU)	1	(Reference)	1	(Reference)
Homosexual	0.34 (0.08-1.41)	.137	0.38 (0.09-1.59)	.184
Heterosexual	0.60 (0.43-0.84)	.004	0.42 (0.28-0.64)	<.0001
Others/unknown[b]	1.06 (0.71-1.59)	.771	0.62 (0.38-1.02)	.060
Baseline CD4, cells/µl				
≤0.5 years				
≥500	1	(Reference)	1	(Reference)
350-499	1.61 (0.31-8.30)	.569	1.40 (0.27-7.25)	.685
200-349	2.53 (0.58-11.07)	.217	2.14 (0.49-9.39)	.312
50-199	8.41 (2.01-35.07)	.003	5.06 (1.20-21.32)	.027

<50	28.51 (6.68-121.60)	<.0001	20.17 (4.62-87.95)	<.0001
>0.5 years				
≥500	1	(Reference)	1	(Reference)
350-499	1.89 (0.62-5.79)	.267	1.73 (0.56-5.32)	.338
200-349	1.91 (0.70-5.35)	.220	1.71 (0.61-4.81)	.310
50-199	3.93 (1.41-10.94)	.009	2.89 (1.02-8.04)	.045
<50	4.94 (1.57-15.57)	.006	3.57 (1.10-11.58)	.034

Abbreviations: 95% CI =95% confidence interval; ART = antiretroviral therapy.

[a]The patient number in each age subgroup were the following:

(0,20] 50; (20,25] 185; (25,30] 369; (30,35] 383; (35,40] 389; (40,45] 295; (45,50] 226; (50,55] 158; (55,60] 147; (60,65] 122; (65,70] 80; (70,75] 67; and (75,90] 45.

[b]Includes blood transfusion, mother-to-Child, and others/unknown.

Discussion

This is a large prospective study pioneering the assessment of patient survival and associated risk factors in Chinese population with HIV infection receiving ART. We described the treatment outcome and identified baseline age, WHO clinical stage as independent predictors for patients survival for all time. The low CD4 cell count at baseline had a strong inverse association with survival at first six months of starting ART.

The survival benefit from ART have been demonstrated intensively among the HIV infected patients[2,3]. A high tolerance of ART regimen and fewer regimen switches were found among our patients (**Supplementary figure S2**). Of the patients who died during the follow-up, more than two thirds occurred within the first six months since ART initiation, similar to previous studies[11,14,22]. The patient characteristics between those dead at first six months and the entire study population were clearly described in this study. The former group had a higher percentage of patients with old age, advanced WHO clinical stage (stage III/IV) and low CD4 cell count compared with the entire study population. Thus, poor baseline patient characteristics seem to contribute largely to the worse survival, supporting the importance of early diagnosis and treatment[11,14,15,23-25]. Overall, we feel that the first six months of ART is critical for improving survival outcome in HIV-infected patients.

The varying coefficients for CD4 cell count was demonstrated among our patients. Many previous studies only reported CD4 cell count as a strong prognostic factor for all time of their study period, but the potential varying effects of CD4 cell count on survival were hardly discussed in their studies[9,11,21,26,27]. A potential reason could be the lack of testing the proportional hazard assumption for Cox model, or such assumption was met in those studies. Another potential explanation could be the relatively short follow-up time in many previous studies. Taking this into consideration, a recent retrospective study with long-term follow-up (more than ten years) from the Antiretroviral Therapy Cohort Collaboration (ART-CC) have suggested that the baseline CD4 cell count is less prognostic after five years since starting ART[16]. In other words, the patients with low baseline CD4 cell count, who survived the first five years since ART, may expect similar mortality to that of patients

with high baseline CD4 cell count. In our prospective cohort study, a strong inverse association between baseline CD4 cell count and risk for mortality was also observed, but only for the first half year after starting ART. We observed a 10-fold increased mortality for low CD4 cell count, which was much higher than the 2.8-fold increase in the ART-CC study[16]. Of note, there are several differences between these two studies, in particular European Americans vs Chinese population. Another important difference to be noticed is the anti-HIV drugs used in those studies. The patients engaged in the ART-CC study received ART between 1996 and 2001; however, our patients have been treated since 2006 with the newer anti-HIV drugs. Given the fact that new drugs introduced since 2002 provide a better immunological response[28,29], our data might reflect more the treatment effect on HIV infected patients nowadays. These factors may contribute to the discrepancies observed between our study and the previous ones. Besides, the CD4 cell count at six months after starting ART was also examined in our study for assessing its potential association with survival after six months of starting ART. In contrast to values at baseline, the six-month CD4 cell count was strongly associated with the worse survival for the period after six months of ART initiation. Consistently, the prognostic value of six-month CD4 cell count has been demonstrated previously[30]. To be noticed, due to the missing values of six-month CD4 cell count among the patients who had survived for six months (displayed in Supplementary Figure S3), these data were not shown in this study.

A potential limitation of this study is the lacking of baseline viral load. Although the primary aim of ART is to inhibit the viral replication and reduce viral load, the resulted increase of CD4 cell count is the main goal as it serves as the most important indicator of immune function in HIV-infected patients. Despite this, WHO clinical stage and particularly CD4 cell counts were analyzed. Therefore, the potential bias from not analyzing viral load have been largely circumvented by including the analysis of WHO clinical stage and particularly CD4 cell counts. Besides, the present study only analyzed the data which were collected at start of ART initiation. Although baseline CD4 cell count has been proven to be an important predictor for the long-term outcome of ART and patient survival[19,31,32], the dynamics of CD4 cell count across follow-up period could also be very important, deserving further investigation.

In conclusion, this is a large prospective study with long-term follow-up investigating the treatment outcome and prognostic factors for the HIV-infected patients receiving ART. Baseline characteristics including gender, age and WHO clinical stage are significantly associated with all-cause mortality. Importantly, we reported the time-dependent coefficients for CD4 cell count over different time intervals among Chinese population. The strong inverse association between CD4 cell count and risk for mortality has been demonstrated for the first half year after starting ART. However, CD4 cell count at six month has a strong inverse association with survival after six months of starting ART.

Methods

Study population

A prospective cohort study was conducted by enrolling HIV patients at start of ART between 2006 July to 2016 April in Zhaotong, Yunnan province, China. Patients were enrolled from different areas of Zhaotong (a prefecture-level city), including Zhaoyang District, Ludian County, Qiaojia County, Yanjin County, Daguan County, Yongshan County, Suijiang County, Zhenxiong County, Yiliang County, Weixin County and Shuifu County. All patients were treated based on the criteria of the "National AIDS Free Antiviral Treatment Manual", HIV drug resistance, individual health, and other factors. We collected data on demographics (age, sex, marital status, transmission category), histological parameter (WHO clinical stage), and laboratory maker (CD4 cell count) at baseline when patients initiated ART.

The institutional ethical committee of the First Affiliated Hospital of Kunming Medical University has approved this study. Informed consent was obtained from all participants in this study. No personally identifiable information was seen and used in our data analysis. All the methods of this study were performed in accordance with the guideline and regulation of the institutional ethical committee of the First Affiliated Hospital of Kunming Medical University.

Statistical Analysis

All patients were followed up from the date since starting ART until the date of death, loss to follow-up, or the end of follow-up. Patients who were lost to follow-up or the event did not occur within the study duration were considered as censored cases. For the patients with treatment changes or interruptions, we analyzed the data as their intent to continue treatment, same as the other patients.

In descriptive statistics, continuous variables were reported as mean with standardized deviation (SD) and median with interquartile range (IQR). Categorical variables were reported as number with percentage. A crude survival analysis (Kaplan-Meier curve) was utilized to analyze the patients' survival on ART during 10-year follow up. An adjusted survival analysis, Cox proportional hazard regression analysis, was used to evaluate the factors related to survival outcome. Variables with

a p-value below 0.20 on univariate analysis were included in multivariate analysis[33-35]. All statistical analyses were performed in R (version 3.3.1)[36]. Particularly, "survival" package[37] was used for survival analyses. The loss to follow up and not loss to follow up against survival time were plotted to depict the their patterns (**Supplementary Figure S4**). The assumption of proportional hazards was tested both statistically (**Supplementary Table S2**) and graphically (**Supplementary Figure S5**) using function *cox.zph*. Besides, Concordance was calculated to assess the Cox model accuracy. One of the strengths of Cox model is its ability to encompass the time-varying coefficients. A step function was used to analyze the time-dependent effect of baseline CD4 cell count over different time intervals after breaking the data set into time dependent parts using *survSplit* function[38]. $P < .05$ (two-tailed sides) was considered as significant. The R code for performing Cox proportional regression analyses in this study were shown in **supplementary Figure S6**.

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Supplementary data

Table S1 Baseline Characteristics of the Patients who Died at First 6 Months of Follow-Up Period

Characteristics	Descriptive statistics
Total	
no.	82
Gender, no. (%)	
Male	58 (70.7)
Female	24 (29.3)
Marital status, no. (%)	
Unmarried	11 (13.4)
Married	61 (74.4)
Divorced	3 (3.7)
Widowed	7 (8.5)
Age at ART initiation, year	
Mean (SD)	47.6 (15.8)
Median (IQR)	42.0 (34.3-60.0)
WHO HIV Clinical Stage, no. (%)	
Stage I/II	35 (42.7)
Stage III/IV	47 (57.3)
CD4, cells/μl	
Mean (SD)	152.7 (128.9)
Median (IQR)	129.0 (44.5-215.0)
<50	21 (25.6)
50-199	32 (39.0)
≥200	22 (26.8)
Transmission category, no. (%)	
Injecting drug users (IDU)	12 (14.6)
Homosexual	0 (0)
Heterosexual	53 (64.6)
Others/unknown	17 (20.7)

ART indicates antiretroviral treatment

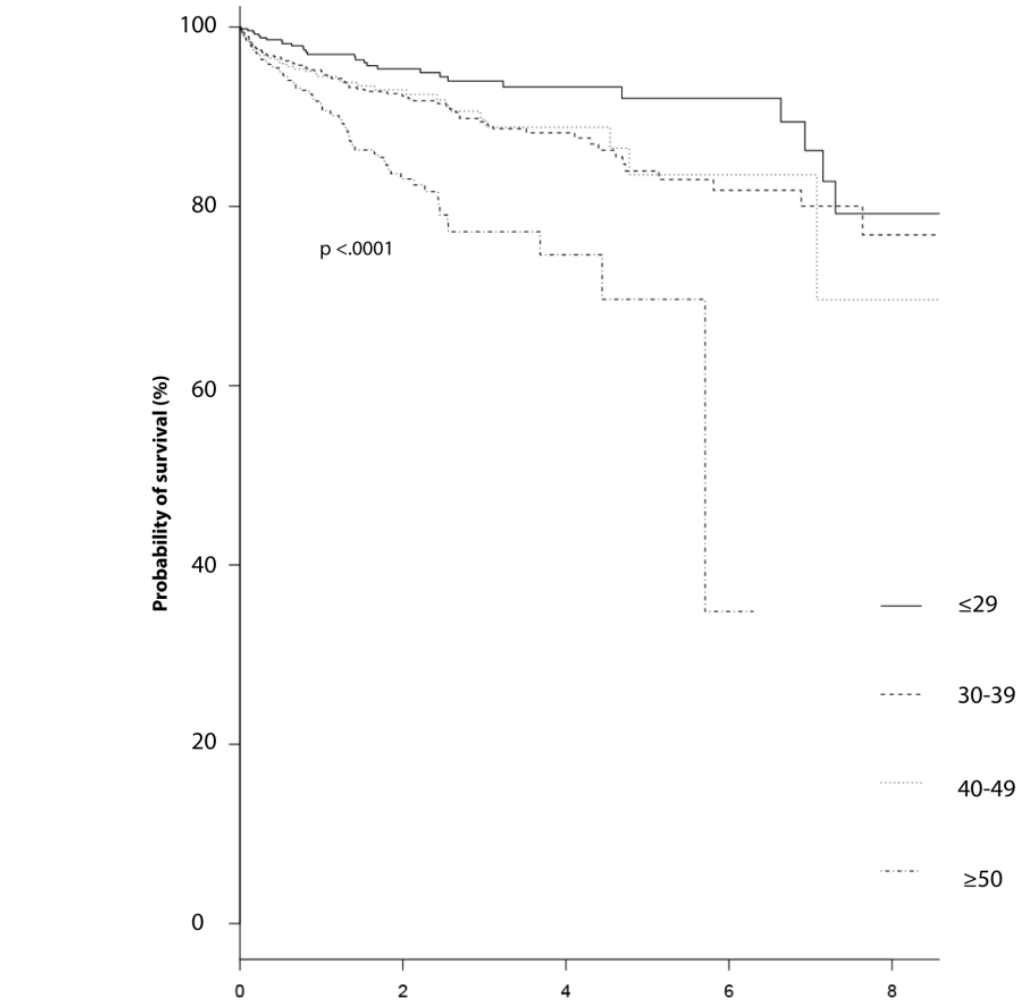
Table S2 Testing the Proportional Hazards Assumption of a Cox Regression Model Fit[a]

	rho	chisq	p
Gender			
Female	-	-	(Reference)
Male	-0.014	0.040	.841
Marital status			
Married	-	-	(Reference)
Unmarried	-0.071	1.073	.300
Divorced	0.107	2.25	.134
Widowed	0.066	0.991	.320
Age at ART initiation, year			
WHO HIV Clinical Stage			
Stage I/II	-	-	(Reference)
Stage III/IV	-0.081	1.536	.215
CD4, cells/µl			
≥200	-	-	(Reference)
50-199	-0.104	2.425	.119
<50	-0.150	5.029	.025
Transmission category			
Blood transfusion	-	-	(Reference)
Injecting drug users (IDU)	-0.031	0.192	.661
Homosexual	0.003	0.002	.957
Heterosexual	-0.039	0.296	.586
Mother-to-Child	-0.013	0.036	.850
Others/unknown	-0.022	0.093	.760
GLOBAL[b]	NA	19.958	.096

[a]Compuated by cox.zph() function in package of "survival" from R

[b]Indicates the global test

rho = Spearman's rank correlation coefficient



Number at risk

Age, years					
≤29	527	250	93	43	10
30-39	770	355	155	66	16
40-49	551	179	61	12	4
≥50	668	140	20	1	0

Figure S1 Cumulative morality from all-cause mortality for study population infected by human immunodeficiency virus (HIV) receiving antiretroviral therapy (ART) according to age (*p* <.0001). Corresponding numbers at risk at different time-points split by age categories have been indicated below the graph.

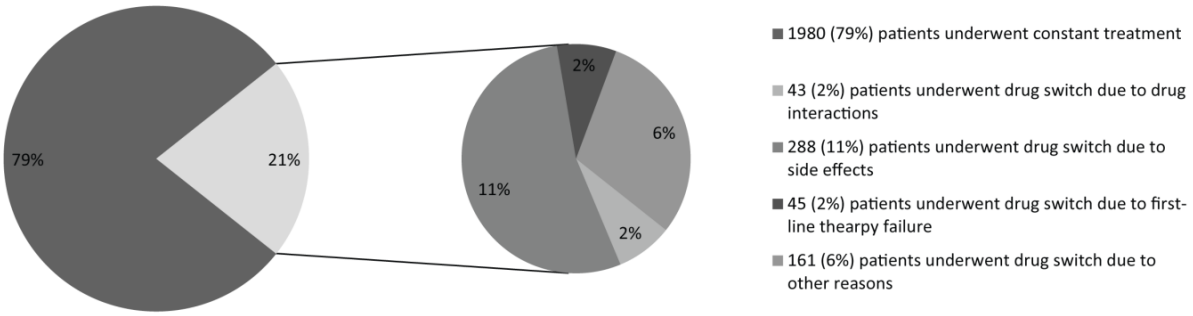


Figure S2 The Pie Chart showing the percentage of patients who switched drugs during Antiretroviral Therapy.

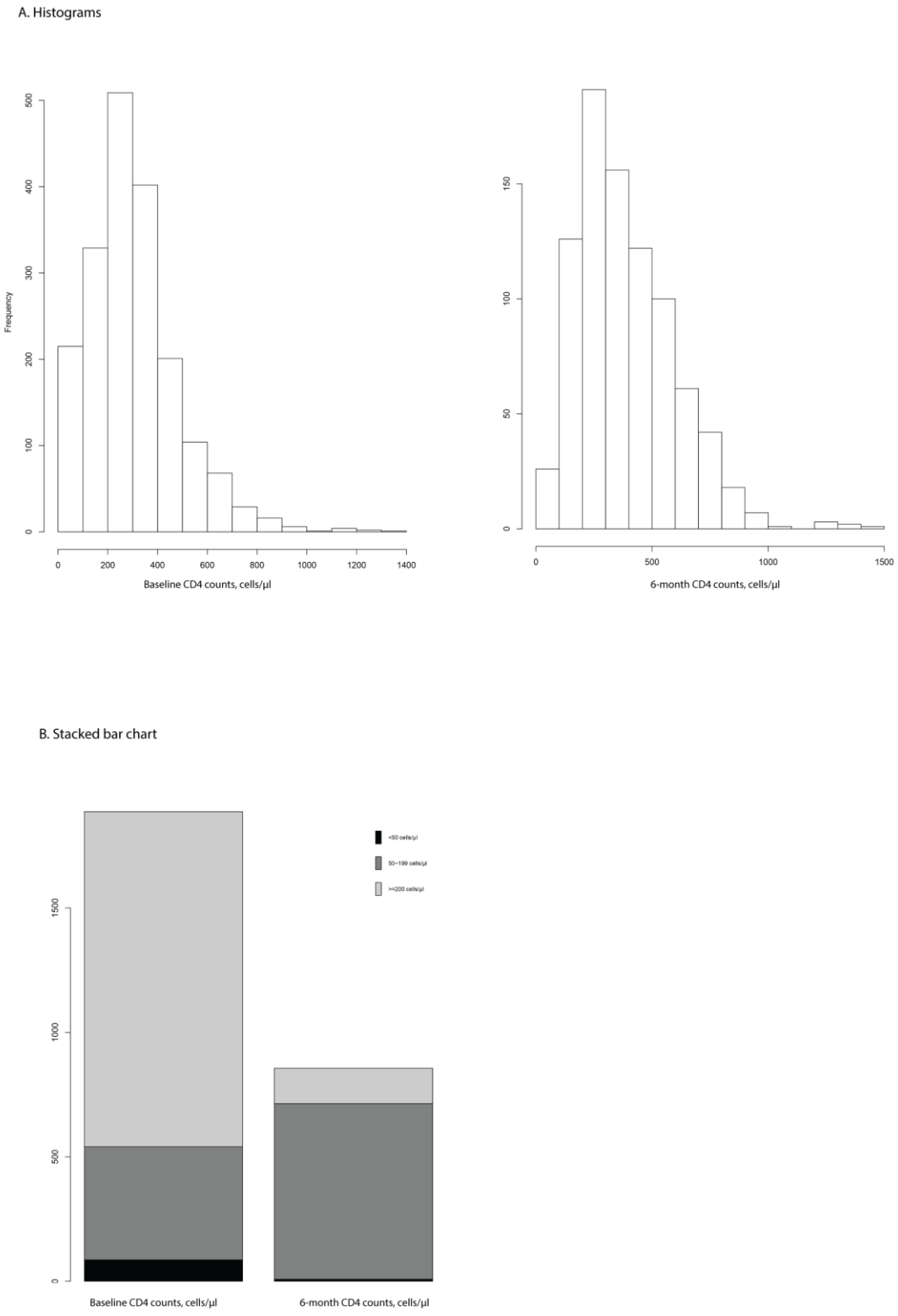


Figure S3 Plots presenting the values of baseline CD4 counts and 6-month CD4 counts for the patients who had survived 6 months after antiretroviral therapy. (A): histograms for continuous data of CD4 counts; (B): stacked bar chart for categorical data of CD4 counts: <50, 50-199, and ≥200 (unit: cells/µl).

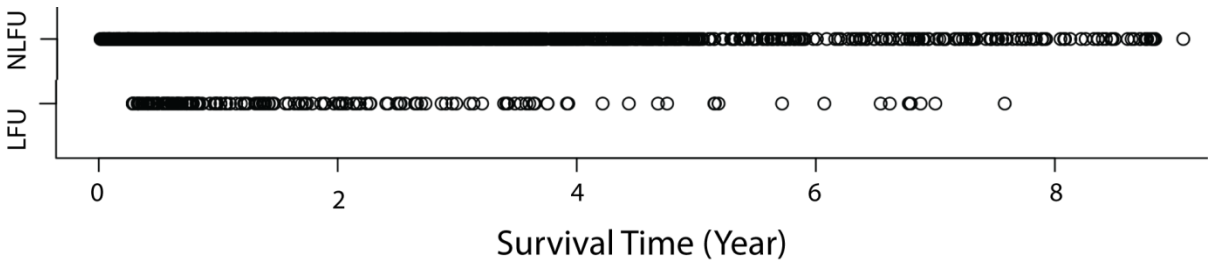


Figure S4 Plot of loss to follow up or not loss to follow up against survival time. LFU = loss to follow up; NLFU = not loss to follow up.

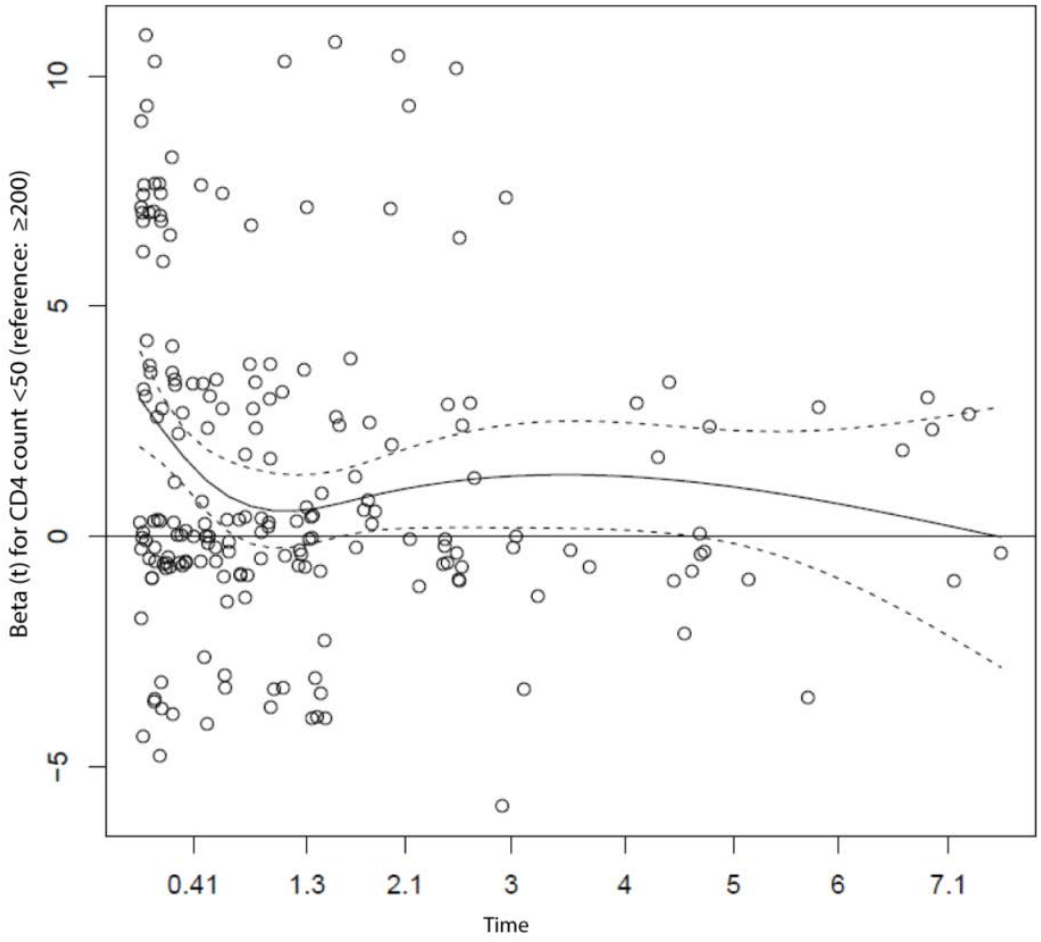


Figure S5 Plot of scaled Schoenfeld residuals against transformed time for covariate CD4 count in Cox model fit. The upper solid line is a smoothing spline fit to the plot, with the broken lines representing a ± 2-standard-error band around the fit; and the bottom solid line is a horizontal line.

```
# Import data.....

data1 <- read.csv ('HIV_data.csv')

# Split dataset into two time intervals: <=0.5 year; >0.5 year.....

data2<-survival::survSplit(Surv(time,event)~.,data1,cut=c(0.5),

            episode = "tgroup",

            id="id")

# Cox model.....

# 1.Univariate analysis

## CD4 for two time intervals

summmry(coxph(Surv(tstart,time,event)~CD4:strata(tgroup),data=data2)

## Other factors

variables<-c('sex','marriage','transmission','WHO_stage','age')

lapply (variables, function(x)summary(coxph(Surv(time,event)~data2[,x],data=data2)))

# 2.Multivariate analysis

summary(coxph(Surv(tstart,Time_m,Event)~CD4:strata(tgroup)+sex+WHO_stage+age, data=base2))
```

Figure S6 R code for performing Cox proportional regression analyses

Chapter 4 The prevalence and impact of hepatitis virus coinfection in HIV patients: a large cohort study in Yunnan province of China

Juan Li[1#], Li Ren[2,3#], Shiyi Zhou[4], Xueshan Xia[5], Zhenrong Xie[4], Pan Liu[6], Yu Xu[4], Yuan Qian[7], Litang Ma[7], Maikel P. Peppelenbosch[1], Qiuwei Pan[1], Kunhua Wang[4*]

1Department of Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam, The Netherlands;

[2]The First People's Hospital of Yunnan Province, Kunming, 650031, Yunnan Province, China;

[3]Medical faculty of Kunming University of Science and Technology, Kunming, 650093, Yunnan Province, China;

[4]Yunnan Institute of Digestive Disease, the First Affiliated Hospital of Kunming Medical University, Kunming, 650032, Yunnan Province, China;

[5]Faculty of Life Science and Technology, Center for Molecular Medicine in Yunnan province, Kunming University of Science and Technology, Kunming, 650093, Yunnan Province, China;

[6]Yan'an Hospital of Kunming Chenggong hospital, Kunming, 650501, Yunnan Province, China;

[7]The First People's Hospital of Zhaotong City, Zhaotong, 657000, Yunnan Province, China.

[#]These authors contributed equally.

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Abstract

This study aimed to evaluate the prevalence and impact of coinfection with hepatitis virus on the overall survival among HIV patients treated with antiretroviral therapy (ART). A total 2517 patients receiving ART between 2006 and 2016 were enrolled in Yunnan, China. The prevalence and characteristics of hepatitis virus coinfection was determined for four groups: HIV only, HBV-HIV, HCV-HIV, and triple infection. The independent effects of coinfection on long-term overall survival were evaluated by both conventional and extended Cox proportional hazard models. Finally, potential correlation of coinfection with longitudinal changes of CD4 cell count was examined. 1868 patients of the entire population have available data on coinfection with hepatitis virus. HIV mono-infection accounts for 82% (1537/1868). HBV or HCV coinfection are 8% (149/1868) and 8% (157/1868), respectively. There are 1% (25/1868) of patients having triple infection. We found that hepatitis virus coinfection has no significant negative effect on long-term overall survival: HBV-HIV (HR 1.32, 95%CI 0.73-2.40, *p*=.359), HCV-HIV (HR, 0.71, 95%CI 0.32-1.57, *p* =.399), and triple infection (HR, 0.37, 95%CI 0.05-2.84, *p*=.342). Interestingly, within 24-month of ART, a remarkable increase of median CD4 cell count was observed among patients with HIV mono-infection, from a median of 294 (IQR: 180-412) cells/μl at baseline to 401 (IQR:297-556) cells/μl at 24 months. We have determined the rates of hepatitis virus coinfection in a large HIV cohort from Yunan province of China. In our cohort, no negative impact of coinfection was found on the overall survival of these patients treated with ART. Interestingly, patients with HIV mono-infection have a better recovery of CD4 cell count.

Introduction

By the end of 2014, there were approximately 501,000 people living with human immunodeficiency virus (HIV/AIDS) in China [1], representing 0.037% of the total population. Despite the relatively low prevalence rate national-wide, high prevalence of HIV infection is found mainly in Southwest China, among which Yunnan is the most affected area, with up to 80,000 infected individuals.

Coinfection of HIV with hepatitis B virus (HBV) or hepatitis C virus (HCV) frequently occurs. In respect to the burden of chronic viral infections worldwide, HBV is estimated to accounting for 370 million, HCV for 130 million, and HIV for 40 million [2]. 5~20% of HIV-infected population are estimated to be co-infected with HBV and 5-15% with HCV [3]. Of note, the burden of coinfection differs significantly by geographic regions for different types of hepatitis viruses, among different countries[3], and across different provinces in China [4-8]. Although previous studies have investigated the coinfection of HIV with hepatitis virus in China [5], those estimates were mainly based on relatively small population of particular provinces.

The impact of coinfection on the mortality among HIV-infected patients receiving antiretroviral therapy (ART) has been highlighted [9]. Giving the significant improvement of patient survival by initiating ART [1, 10, 11], several important prognostic factors have been identified, including CD4 cell count, baseline age on ART and advanced stage [12-14]. However, it remains controversial regarding the association of coinfection and patient morality. A US cohort reported that HCV coinfection did not substantially impact the survival outcome [15], while a Chinese study indicated significant roles of both HCV coinfection and triple infection (HBV-HCV-HIV) [7].

This study aimed to evaluate the prevalence, patient/disease characteristics, and the impact of coinfection on mortality of HIV patients in a large cohort from Yunnan province in China.

Results

Patient characteristics by HIV and hepatitis virus infection

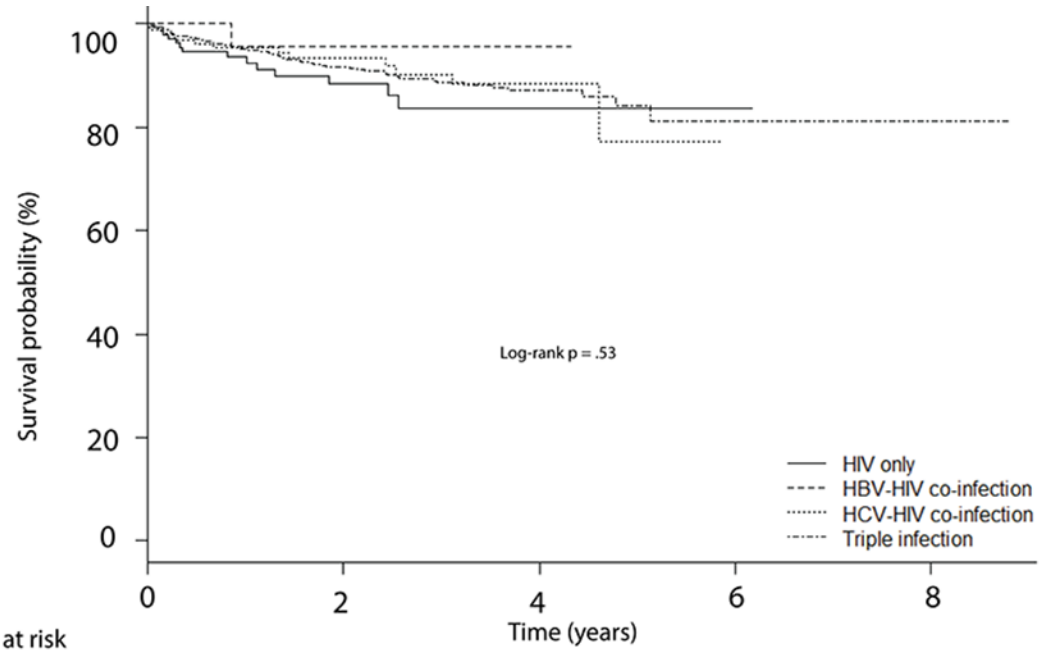
There were 2517 patients on ART enrolled between 2006 to 2016 in Zhaotong, Yunnan province of China. Hepatitis coinfection data were available for a total of 1868 patients. Characteristics for all patients according to hepatitis virus status are summarized in **Table 1**. 149 patients had HBV co-infection, 157 patients had HCV co-infection, 25 patients had triple infection, and 1537 had HIV infection only. A median age for entire population was 39 with interquartile range (IQR) between 31 and 50. Male patients accounted for 60%. 79% of patients presented early stage of HIV infection (WHO Clinical Stage I/II). The median of CD4 cell count was 281 (IQR: 177-388) cells/μl for the entire population.

Prognostic value of hepatitis virus infection for HIV-infected patients

Figure 1 displays the crude survival analysis by Kaplan-Meier curve by coinfection type for the overall population who had followed up to nearly ten years. As shown in the figure, there was no significant difference of survival probability among different coinfection groups (log-rank p = .53).

The potential association of baseline factors on ART initiation with overall survival was evaluated as well in Cox models. Results from a conventional Cox model and an extended Cox model with time-by-covariate interaction term were summarized in **Table 2.** With conventional Cox model, assuming all covariates meet proportional hazard assumption, it turned out that sexual transmission (HR 0.33, 95%CI 0.17-0.65, p < .01), baseline age on ART (HR 1.04, 95%CI 1.02-1.05, p <.0001), advanced WHO Stage (stage III/IV) (HR 2.13, 95%CI 1.44-3.17, p <.001), CD4 cell count (HR 1.00, 95%CI 1.00-1.00, p <.0001), AST (HR 1.01, 95%CI 1.01-1.02, p <.0001), and ALT (HR 0.99, 95%CI 0.98-1.00, p =.049) are significant factors associated with overall survival. In contrast, hepatitis virus coinfection has no significant role for long-term overall survival, HBV-HIV (HR 1.32, 95%CI 0.73-2.40, p=.359), HCV-HIV(HR, 0.71, 95%CI 0.32-1.57, p =.399), and triple infection(HR, 0.37, 95%CI 0.05-2.84, p=.342*)*. Furthermore, testing proportional hazards assumption (**Supplementary Table S1**) revealed time-dependent coefficient for covariate of CD4 cell count. Thus, an extended Cox model was carried out to include

time-by-covariate interaction term. A significant interaction term between CD4 cell count and follow up time (log) was observed in this model. As shown in **Table 2**, statistical estimates for covariates were similar between conventional Cox model and extended Cox model.



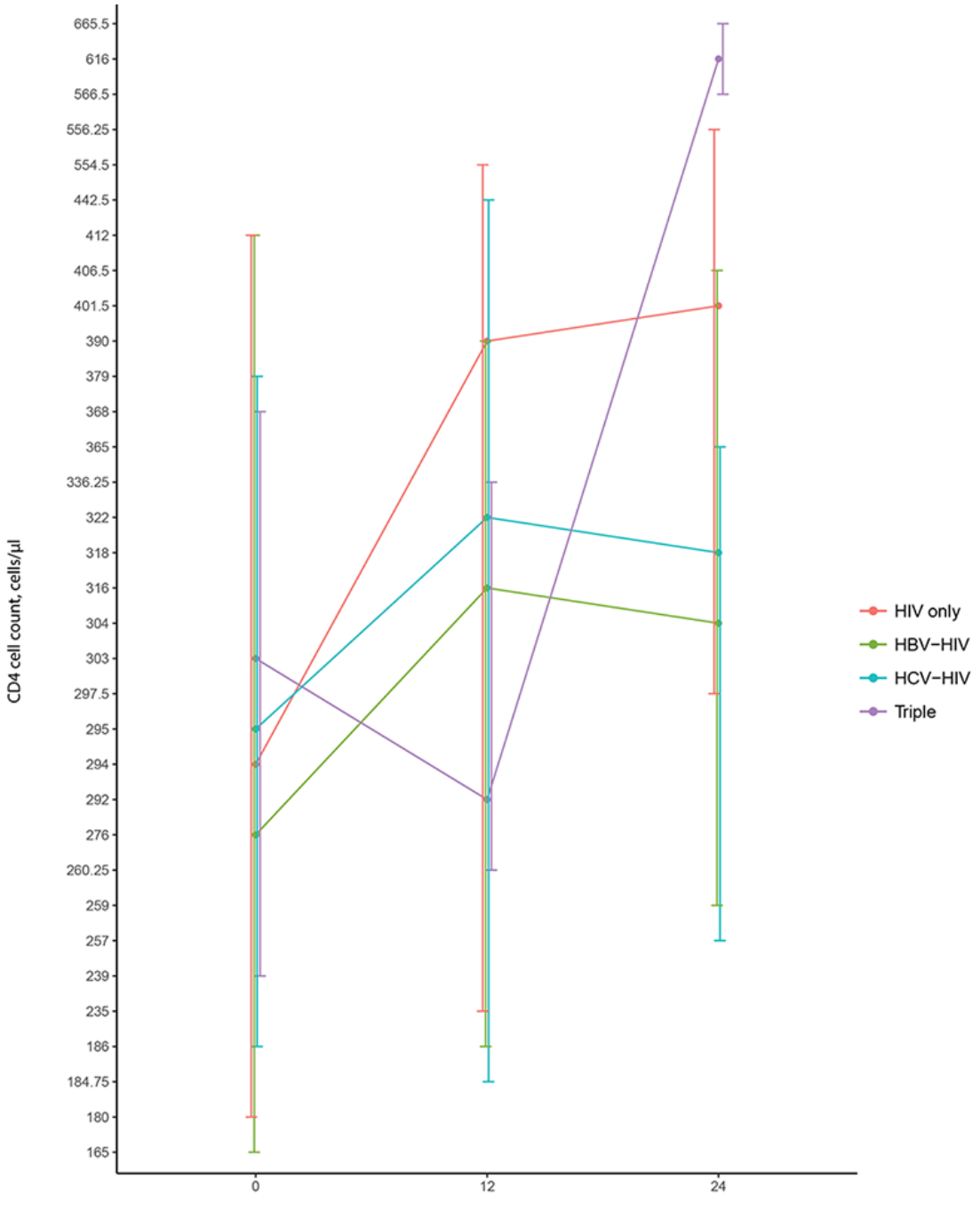
Number at risk

HIV only	1537	457	103	5	2
HBV-HIV co-infection	149	50	10	2	0
HCV-HIV co-infection	157	78	21	0	0
Triple infection	25	12	2	0	0

Figure 1 Cumulative probability of overall survival by hepatitis virus infection status: HIV only, HBV-HIV co-infection, HCV-HIV co-infection, and triple infection.

Impact of coinfection on response to antiretroviral therapy

Next, the potential role of coinfection of hepatitis virus on the longitudinal changes of CD4 cell count was evaluated. According to **Supplementary Figure S5**, majority of patients remained in the cohort for analysis until the first 24 months after ART initiation. As shown in **Figure 2**, baseline median CD4 cell counts were 294 (IQR: 180-412) cells/μl, 276 (IQR: 165-412) cells/μl, 295 (IQR: 186-379) cells/μl, and 303 (IQR: 239-368) cells/μl for groups of HIV only, HBV-HIV coinfection, HCV-HIV coinfection, and triple infection, respectively. For patients with HIV infection only, CD4 cell count kept increasing during 24-month treatment period, with median of 401 (IQR:297-556) cells/μl. With coinfection of HBV or HCV, increased median CD4 cell count was observed in HBV-HIV(316; IQR: 186-390), and HCV-HIV (322; IQR: 184-442) at 12 months, but it started decreasing afterwards. As for triple infection, a clear decrease of CD4 cell count (292 cells/μl; IQR: 260-336) was found since ART initiation till the 12 months. Although a significant increase of CD4 cell count was observed at 24 months, the data should be taken into caution due to small population remained by that time (only 4 patients).



Number of patients

HIV only	1537	457	224
HBV-HIV	149	48	22
HCV-HIV	157	26	19
Triple infection	25	8	4

Figure 2 The distribution of CD4 cell count at different time points of follow up for different patient groups. The midpoints of the vertical bars represent the medians, and the error bars represent the interquartile range of CD4 cell count. The x axe shows the time points of follow up, and y axe shows the CD4 cell count (cells/µl).

Table 1 Patient characteristics by HIV mono-infection, HIV-HBV or HCV co-infection status

Characteristics	Overall N = 2517	HIV monoinfection no. (%)	HBV coinfection no. (%)	HCV coinfection no. (%)	Triple infection no. (%)
		1868/2517 (74)			
Total		1537/1868 (82)	149/1868 (8)	157/1868 (8)	25/1868 (1)
Gender, no. (%)					
Male	1507 (60)	872/1537 (57)	108/149 (73)	114/157 (736)	18/25 (72)
Female	1010 (40)	665/1537 (43)	41/149 (28)	43/157 (27)	7/25 (28)
Marital status, no. (%)					
Unmarried[a]	582/2501 (23)	323/1530 (21)	40/149 (27)	53/156 (34)	9/25(36)
Married	1919/2501 (77)	1207/1530 (79)	109/149 (73)	103/156 (66)	16/25 (64)
Transmission					
Injecting drug users (IDU)	263/2517 (10)	39/1537 (3)	5/149 (3)	86/157 (55)	16/25 (64)
Sexual[b]	1863/2517 (74)	1270/1537 (83)	123/149 (83)	58/157 (37)	6/25 (24)
Others/unknown[c]	391/2517 (16)	228/1537 (15)	21/149 (14)	13/157 (8)	3/25 (12)
Age at ART initiation, year					
Mean (SD)	42 (14)	43 (14)	40 (13)	38 (8)	36 (9)
Median (IQR)	39 (31-50)	40 (31-52)	38 (29-48)	37 (32-41)	37 (31-38)
WHO HIV Clinical Stage, no. (%)					
Stage I/II	1986/2511 (79)	1215/1537 (79)	113/149 (76)	131/157 (83)	18/25 (72)
Stage III/IV	525/2511 (21)	322/1537 (21)	36/149 (24)	26/157 (17)	7/25 (28)
CD4, cells/µl					
Mean (SD)	302 (186)	316 (193)	308 (194)	309 (162)	306 (126)
Median (IQR)	281 (177-388)	294 (180-412)	276 (165-412)	295 (186-379)	303 (239-368)
<200	719/2402 (30)	420/1490 (28)	43/147 (29)	43/154 (28)	4/24 (17)
≥200	1683/2402 (70)	1070/1490 (72)	104/147 (71)	111/154 (72)	20/24 (83)
AST, U/L					
Mean (SD)	33 (27)	30 (24)	39 (31)	52 (38)	42 (24)
Median (IQR)	27 (21-37)	26 (21-34)	30 (24-40)	42 (30-61)	36 (23-47)

ALT, U/L					
Mean (SD)	30 (27)	27 (21)	33 (22)	50 (42)	42 (18)
Median (IQR)	23(16-35)	22 (15-32)	28 (18-38)	37 (23-63)	36 (33-54)

Abbreviations: HBV = hepatitis B virus; HCV = hepatitis C virus; HIV = human immunodeficiency; AST = asparate aminotransferase; ALT = alanine transaminase; SD = standard deviation; IQR = interquartile range.

[a]Includes unmarried, divorced, and widowed.

[b]Includes heterosexual and homosexual categories.

Table 2 All-cause mortality since starting ART analyed both in a conventional Cox model and an extended Cox model

	Conventional Cox model				Extended Cox model			
	Original data		Imputed datasets		Original data		Imputed datasets	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Gender								
Female	1	(Ref)	1	(Ref)	1	(Ref)	1	(Ref)
Male	1.35 (0.88-2.06)	.169	1.25(0.91-1.71)	.175	1.34 (0.88-2.05)	.171	1.25(0.91-1.71)	<.173
Marital status							1.00(1.00-1.00)	
Married	1	(Ref)	1.00(1.00-1.00)	(Ref)	1	(Ref)	1.00(1.00-1.00)	(Ref)
Unmarried[a]	0.92 (0.60-1.42)	.718	1.08(0.78-1.48)	0.647	0.91 (0.59-1.40)	.666	1.06(0.78-1.46)	<.700
Transmission			1.00(1.00-1.00)				1.00(1.00-1.00)	
IDU	1	(Ref)	1.00(1.00-1.00)	(Ref)	1	(Ref)	1.00(1.00-1.00)	(Ref)
Sexual[b]	0.33 (0.17-0.65)	.001	0.48(0.27-0.84)	.011	0.33 (0.17-0.64)	<.001	0.47(0.00-0.83)	.010
Others/unknown[c]	0.54 (0.25-1.14)	.107	0.68(0.37-1.26)	.214	0.52 (0.24-1.11)	.092	0.68(0.00-1.25)	.208
Baseline age	1.04 (1.02-1.05)	<.0001	1.03(1.02-1.05)	<.0001	1.04 (1.03-1.05)	<.0001	1.04(1.02-1.05)	<.0001
Stage			1.00(1.00-1.00)				1.00(1.00-1.00)	
Stage I/II	1	(Ref)	1.00(1.00-1.00)	(Ref)	1	(Ref)	1.00(1.00-1.00)	(Ref)
Stage III/IV	2.13 (1.44 -3.17)	<.001	1.97(1.45-2.67)	<.0001	2.08 (1.40-3.11)	<.001	1.94(1.42-2.63)	<.0001
CD4, cells/μl	1.00 (1.00-1.00)	<.0001	1.00(1.00-1.00)	<.0001	1.00 (1.00-1.00)	.006	1.00(1.00-1.00)	<.001
Infection			1.00(1.00-1.00)				1.00(1.00-1.00)	
HIV only	1	(Ref)	1.00(1.00-1.00)	(Ref)	1	(Ref)	1.00(1.00-1.00)	(Ref)
HBV-HIV	1.32 (0.73-2.40)	.359	1.16(0.61-2.22)	.644	1.33 (0.73-2.41)	.355	1.15(0.61-2.2)	.653
HCV-HIV	0.71 (0.32-1.57)	.399	1.00(0.43-2.32)	.991	0.69 (0.32-1.52)	.352	0.99(0.44-2.24)	.977
Triple infection	0.37 (0.05-2.84)	.342	0.59(0.00-2.21)	.426	0.35 (0.05-2.66)	.311	0.57(0.15-2.20)	.410
AST, U/L	1.01 (1.01-1.02)	<.0001	1.01(1.01-1.01)	<.0001	1.01 (1.01-1.02)	<.0001	1.01(1.01-1.01)	<.0001
ALT, U/L	0.99 (0.98-1.00)	.049	0.99(0.98-1.00)	.027	0.99 (0.98-1.00)	.045	0.99(0.98-1.00)	.022
CD4*log(time)	-	-	-	-	1.00 (1.00-1.00)	<.001	1.00(1.00-1.00)	<.001

Abbreviations: HR= hazard ratio; 95%CI = 95% Confidence Interval; HBV = hepatitis B virus; HCV = hepatitis C virus; HIV = human immunodeficiency; AST = asparate aminotransferase; IDU=Injecting drug users; ALT = alanine transaminase; SD = standard deviation; IQR = interquartile range.
[a]Includes unmarried, divorced, and widowed.
[b]Includes heterosexual and homosexual.
[c]Includes blood transfusion, mother-to-Child, and others/unknown.

Discussion

This is a large cohort study evaluating the coinfection of hepatitis virus with HIV from Yunnan province in China. Globally, it has been estimated that, of HIV-infected population, 5-20% have HBV and 5-15% have HCV infection [3]. We found 8% of HBV and 8%of HCV) coinfection, that both are within this range. As a matter of fact, the prevalence of HBV and HCV coinfection among Chinese HIV patients varies remarkably among different studies. The prevalence of HBV or HCV infection in our study was close to that reported in previous studies (HBV: 8.7%; HCV: 10.4%) [7, 21], but much lower than other reports (HBV: 14.6%; HCV: 40%) [6, 22]. These phenomenon are probably attributed to the mode of acquisition, including childhood acquisition and adult transmission mode such as injecting drug practice and unprotected sex [8]. Consistently, our data (**Table 1)** have shown that injecting drug users (IDU) are associated with high prevalence of HCV infection and triple coinfection, and sexual transmission is related to a high prevalence of HBV infection in our cohort.

Whether coinfection affects HIV patient survival remains under debating. In particular, there are substantial controversial data concerning coinfection of hepatitis virus on the overall survival of HIV patients receiving ART. By multivariate Cox analysis, we have demonstrated that coinfection has no significant relation to overall survival, consistent with the results of several previous studies [15, 21, 23, 24]. However, other studies have reported significant association between coinfection and patient survival, *e.g.* worse survival in patients with triple or HCV-HIV coinfection [7, 25-27]. These discrepancies are likely related to several factors, such as patient population, follow up time, and adjusted confounders.

Finally, we assessed the correlation between coinfection and longitudinal changes of CD4 cell count receiving ART. Within 24 months of ART, patients with HBV or HCV coinfection and in particular with HIV mono-infection have increased CD4 cell count. Within 12 months of ART, patients with triple infection have a dramatic decline of CD4 cell count. However, the change after 12 months in this group is difficult to predict, because of limited patients by that time. In contrary, a previous study has reported no significant difference of CD4 cell count after ART between hepatitis virus coinfection and HIV only [7]. Of note, categorical variable of

CD4 (increase ≤30% and > 30%) was used rather than continuous CD4 values in their study.

Nevertheless, there are some limitations in our study. First, we were not able to evaluate the longitudinal changes of viral load after long-term ART, because of the lacking of data. However, CD4 cell count, which serves as the most important indicator of immune function in patients with HIV infection, was analyzed throughout the study. Second, no data regarding specific liver diseases is available for these patients. Thus, the association between coinfection of hepatitis virus and liver disease among HIV-positive patients was not attainable. In other words, no significant association with overall survival observed in this study does not necessarily exclude the possible relation between coinfection and liver diseases.

In conclusion, we found that sexual transmission, WHO clinical stage, age, CD4 count, AST, and ALT are independent prognostic factors for overall survival in this large HIV cohort Yunnan province in China. Yet, coinfection with hepatitis virus has no significant negative effect on the survival of HIV patients treated with antiretroviral therapy. Interestingly, constant increase of CD4 cell counts was observed among patients without coinfection of hepatitis virus receiving ART. These findings bear important implications for better management of hepatitis virus co-infected HIV patients.

Methods

Participants

A cohort study was conducted by enrolling HIV patients at start of ART between 2006 to 2016 in Zhaotong, Yunnan province, China. Patients were included from different areas of Zhaotong (a prefecture-level city), including Zhaoyang District, Ludian County, Qiaojia County, Yanjin County, Daguan County, Yongshan County, Suijiang County, Zhenxiong County, Yiliang County, Weixin County and Shuifu County. All patients were treated according to the national AIDS treatment criterial, ART drug resistance, individual health, and other relevant factors. Data were collected on demographic variables such as age, sex, marital status, and transmission category; and pathological variables such as liver enzyme indicators (AST and ALT), WHO clinical stage at baseline of ART initiation for each individual.

The institutional ethical committee of the First Affiliated Hospital of Kunming Medical University has approved this study. Informed consent was obtained from all individuals in this study. No personally identifiable information was seen and used in our analysis. All the methods of this study were performed in accordance with the guideline and regulation of the institutional ethical committee of the First Affiliated Hospital of Kunming Medical University.

Laboratory tests

Serum samples were used to detect HIV-1 using SD BIOLINE HIV Ag/Ab Combo Rapid (Standard Diagnostics, INC.; Korea) at the HIV testing outreach lab in the first people's hospital of Zhaotong. HIV-1-positive samples were screening for HIV antibodies with an enzyme-linked immunosorbent assay (Beijing WANTAI Biological Pharmacy Enterprise Co., Ltd.; China). Positive samples were subsequently transported to the central lab in Kunming within 12 hr. Western blot analysis (MP Diagnostics Co., Ltd.; Singapore) was performed for confirmation. Both HBV and HCV infections were tested by enzyme-linked immunosorbent assay (Xiamen Yingke Xinchuang Technology Co., Ltd.; China; Beijing WANTAI Biological Pharmacy Enterprise Co., Ltd.; China).

Statistical Analysis

Standardized deviation (SD) and median with interquartile range (IQR) were computed for continuous variables and number with percentage for categorical variables. All of descriptive statistics were completed by *dplyr* package [16]. Missing values were firstly explored using *Visualization and Imputation of Missing Values (VIM)* package [17], which was shown in **Supplementary Figures S1 and S2**, and for the purpose of checking the sensitivity of inferences from Cox model, missing values were handled by *Multiple Imputation (five complete datasets were constructed) by Chained Equations (MICE)* package [18]. Survival analyses were done using *survival* package [19]. Kaplan-Meier survival curve was used for examining overall survival stratified by hepatitis virus status, with the statistical difference among groups assessed by Log-rank test. Furthermore, the assumption of proportional hazards was tested both statistically and graphically using function *cox.zph* from package of *survival* (**Supplementary Table S1 and Figures S3**). Prognostic factors for overall survival were further evaluated both in the conventional Cox model and the extended Cox model (addressing time-varying coefficients) for each original data and multiple imputed datasets. To be noted, covariates for inclusion in statistical model were based on prior research and biomedical importance [7, 13]. All statistical analyses were performed in R (version 3.4.0) [20]. Two sided $P < .05$ was considered significant. Finally, R code for performing statistical analyses in this study was provided in **supplementary Figure S1.**

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Supplementary data

Table S1 Testing proportional hazards assumption of conventional Cox model

	rho	chisq	p
Gender			
Female	-	-	(Reference)
Male	-0.024	0.071	0.790
Age	0.115	1.995	0.157
Marital status			
Married	-	-	(Reference)
Unmarried[a]	0.062	0.445	0.505
Transmission			
Injecting drug users (IDU)	-	-	(Reference)
Sexual[b]	-0.145	2.334	0.127
Others/unknown[c]	-0.077	0.658	0.417
WHO HIV Clinical Stage			
Stage I/II	-	-	(Reference)
Stage III/IV	-0.063	0.497	0.481
CD4	0.262	10.775	0.001
Infection			
HIV only	-	-	(Reference)
HBV-HIV co-infection	0.015	0.027	0.870
HCV-HIV co-infection	-0.186	4.565	0.033
Triple infection	-0.015	0.029	0.864
AST, U/L	-0.166	1.962	0.161
ALT, U/L	0.099	1.411	0.235
GLOBAL[b]	NA	23.739	0.022

Abbreviations:HBV = hepatitis B virus; HCV = hepatitis C virus; HIV = human immunodeficiency; AST = asparate aminotransferase; ALT = alanine transaminase; SD = standard deviation; IQR = interquartile range.
[a]Includes unmarried, divorced, and widowed.
[b]Includes heterosexual and homosexual.
[c]Includes blood transfusion, mother-to-Child, and others/unknown.

rho indicates Pearson's correlation between the scaled Shoenfeld residuals and g(t) where g is a function of time, where transform= function(time) log (time) is used in cox.zph() function.

A. Missing data exploration

```
library(mice)
library(VIM)

mice::md.pattern(data)
VIM::matrixplot(data)
VIM::aggr(data,prop=TRUE,numbers=TRUE)

mi.df <- mice (data,m=5,seed=12,printFlag=F)
summary(mi.df)
```

B. Build Cox model

```
library(survival)

# Conventional Cox model

coxph_conventional <-
coxph(Surv(time,event)~gender+age+marital_status+transmission+WHO_stage+CD4+infection+AST
+ ALT, data)
summary(coxph_conventional)


# Testing proportional hazards assumption of conventional Cox model

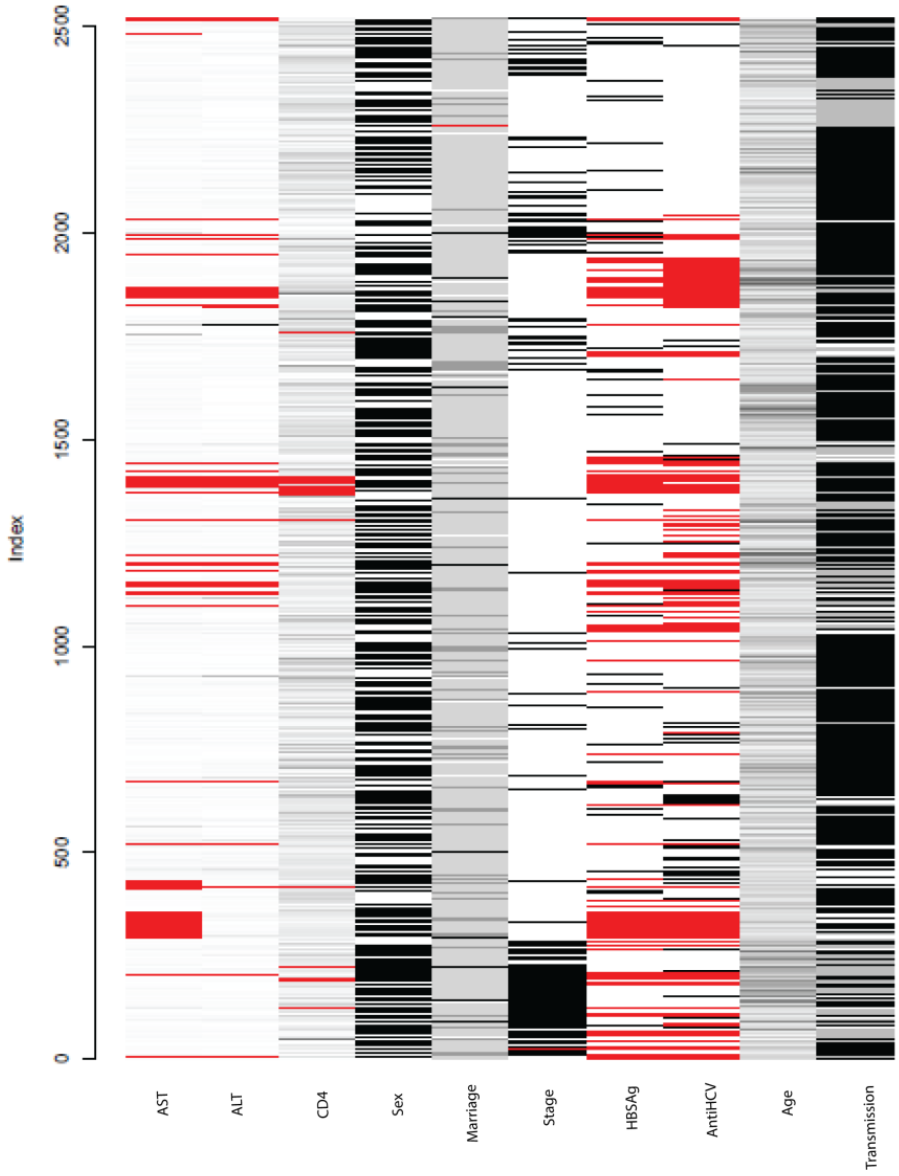
zp<- (cox.zph(coxph_conventional, transform=function(time) log(time+20))

# Ploting scaled schoenfeld residuals against transformed time for covariate of CD4 count in
conventional Cox model fit
plot (zp[7])
abline (0,0)


# Adding time-by-covariate interaction to Cox model

coxph_timeByCovariate <-
 coxph (Surv(time,event)~
CD4+tt(CD4)+gender+marital_status+transmission+WHO_stage+age+infection+ALT+
AST, data, tt=function(x,t,… x*log(t+20))
summary(coxph_timeByCovariate)
```

Figure S1 R code used for performing missing value exploration & multiple imputation, and fitting Cox model

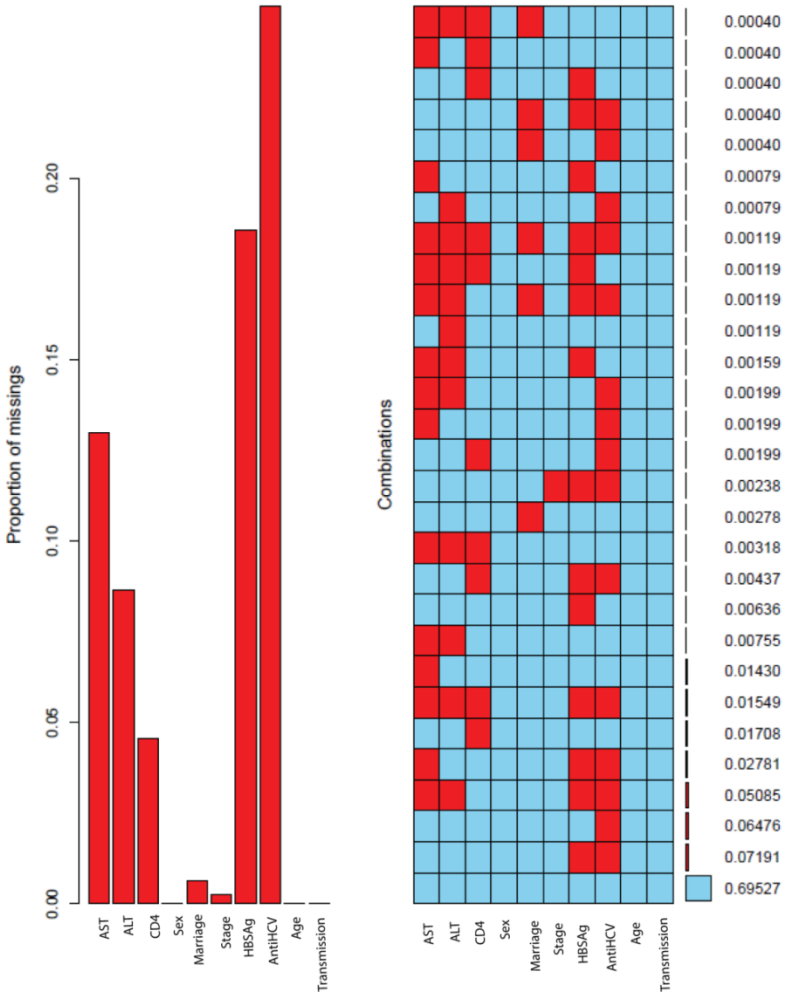


Supplementary Figure S2 Matrix plot of missing and non-missing values for all included patients.

A.

	Sex	Age	Transmission	Stage	Marriage	CD4	ALT	AST	HBSAg	AntiHCV	
1750	1	1	1	1	1	1	1	1	1	1	0
36	1	1	1	1	1	1	1	0	1	1	1
3	1	1	1	1	1	1	0	1	1	1	1
7	1	1	1	1	0	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	0	1	1
163	1	1	1	1	1	1	1	1	1	0	1
19	1	1	1	1	1	1	0	0	1	1	2
2	1	1	1	1	1	1	1	0	0	1	2
5	1	1	1	1	1	1	1	0	1	0	2
2	1	1	1	1	1	1	0	1	1	0	2
1	1	1	1	1	0	1	1	1	1	0	2
181	1	1	1	1	1	1	1	1	0	0	2
43	1	1	1	1	1	0	1	1	1	1	2
4	1	1	1	1	1	1	0	0	0	1	3
5	1	1	1	1	1	1	0	0	1	0	3
70	1	1	1	1	1	1	1	0	0	0	3
1	1	1	1	1	0	1	1	1	0	0	3
6	1	1	1	0	1	1	1	1	0	0	3
1	1	1	1	1	1	0	1	0	1	1	3
1	1	1	1	1	1	0	1	1	0	1	3
5	1	1	1	1	1	0	1	1	1	0	3
128	1	1	1	1	1	1	0	0	0	0	4
8	1	1	1	1	1	0	0	0	1	1	4
11	1	1	1	1	1	0	1	1	0	0	4
3	1	1	1	1	0	1	0	0	0	0	5
1	1	1	1	1	0	0	0	0	1	1	5
3	1	1	1	1	1	0	0	0	0	1	5
39	1	1	1	1	1	0	0	0	0	0	6
3	1	1	1	1	0	0	0	0	0	0	7
	0	0	0	6	16	115	218	327	468	623	1888

B.



Supplementary Figure S3 Missing data pattern presented visually: (**A**) In the main body of text, "1" indicates non-missing value and "0" indicates missing value. pattern.

The first column displays the number of patients with unique missing value pattern.

For instance, 1750 patients have non-missing values, 36 patients have missing value for AST, etc..

The last column shows the number of missing variables in the corresponding missing value. (**B**) The left panel displays proportion of missing values for each variable. And the right panel the same information as (**A**).

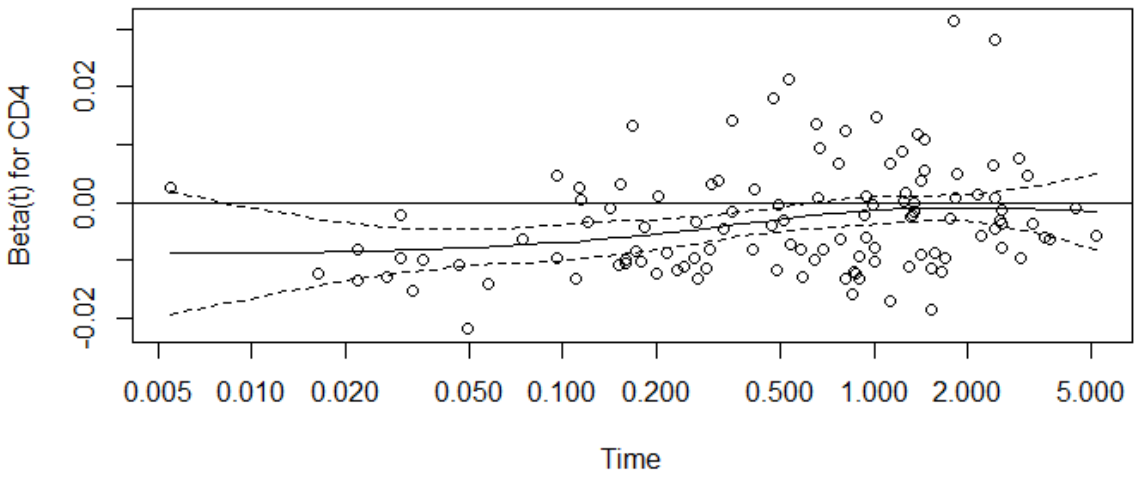


Figure S4 Plots of scaled Schoenfeld residuals against time for covariate of CD4 count in Cox model fit. The solid curve is a smoothing spline fit to the plot, with the broken lines representing a ± 2-standard-error band around the fit; and the solid line is a horizontal line.

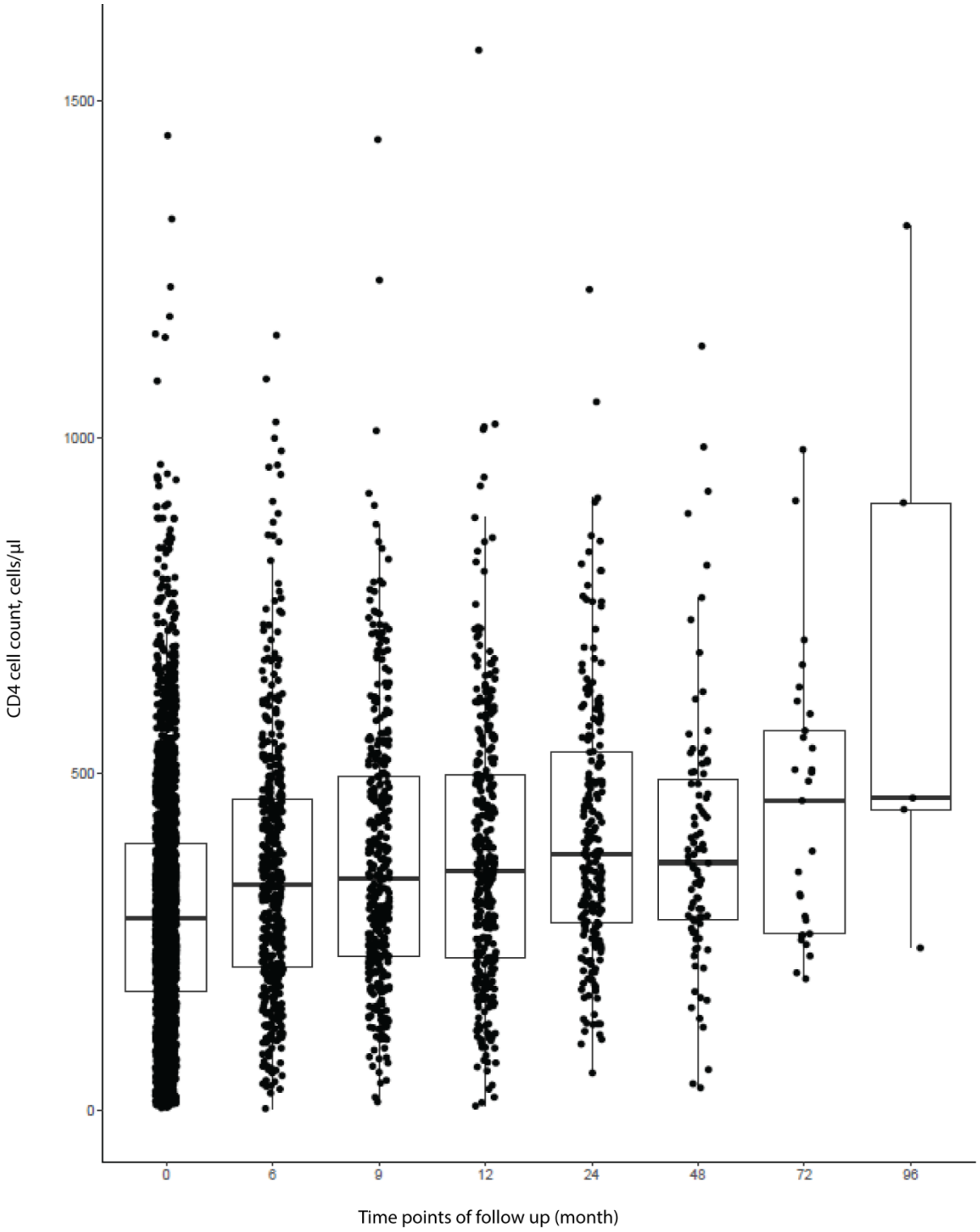


Figure S5 Scatterplot displaying the longitudinal changes of CD4 cell count at various time points of follow up. The boxplots represent interquartile range and medians.

Chapter 5 Factors Associated with Ethnical Disparity in Overall Survival for Patients with Hepatocellular Carcinoma

A Population-Based Study from the National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) Program in the United States, 2004-2012

Juan Li[1], Bettina E Hansen[1], Maikel P. Peppelenbosch[1], Robert. A. De Man[1], Qiuwei Pan[1#], Dave Sprengers[1*#]

[1]Department of Gastroenterology and Hepatology, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands.

Oncotarget 2017

Abstract

Hepatocellular carcinoma (HCC) is an important cause of cancer-related death worldwide. Ethnical disparity in overall survival has been demonstrated for HCC patients in the United States (U.S.). We aimed to evaluate the contributors to this survival disparity. The SEER database was used to identify HCC patients from 2004 to 2012. Kaplan-Meier curves and Cox proportional hazard models were used to evaluate overall survival by ethnicity and the contributors to ethnical survival disparity. A total of 33 062 patients were included: 15 986 Non-Hispanic Whites, 6535 Hispanic Whites, 4842 African Americans, and 5699 Asians. Compared to Non-Hispanic Whites, African Americans had worse survival (HR, 1.18; 95%CI, 1.14–1.23), while Asians had a better survival (HR, 0.85; 95%CI, 0.82–0.89), and Hispanic Whites had a similar survival (HR, 1.01; 95%CI, 0.97–1.05). Multivariate Cox analysis identified that tumor presentation- and treatment-related factors significantly contributed to the ethnical survival disparity. Especially, tumor size was the most important contributor (HR, 1.11; 95%CI, 1.07–1.16). There is no ethnical survival disparity in patients undergoing liver transplantation and sub-analysis of patients within the Milan criteria for liver transplantation demonstrated no significant survival disparity between African Americans and non-Hispanic Whites in transplantation adjustment analysis (HR, 1.23; 95%CI, 1.11–1.35 in non-adjustment analysis to HR, 1.05; 95%CI, 0.95–1.15 after adjustment). Finally, no important contributor to the superior overall survival in Asians was identified. In conclusion, poor tumor presentation at diagnosis, limited benefit from resection and restricted utilization of liver transplantation are important contributors to poorer survival of African Americans with HCC.

Introduction

Hepatocellular Carcinoma (HCC) is the fifth most common malignancy and the second leading cause of cancer-related death worldwide [1]. Ethnicity has been demonstrated to be related to the prognosis of HCC patients in the United States (U.S.) [2–5]. In general, African American ethnicity is associated with the poorest overall survival rate; whereas Asian ethnicity is associated with the best overall survival [3–5]. To improve the quality of health care for HCC patients, it is important to identify the factors affecting this ethnical disparity in overall survival rates (OS), and to compare their impacts.

Several factors associated with tumor presentation at time of diagnosis, type of surgical treatment, and socioeconomic status (SES), have previously been studied with regard to ethnic disparity in OS for HCC patients. Previous literature has reported that African American patients have a more advanced, and Asian patients a less advanced tumor stage at the moment of diagnosis, compared to non-Hispanic white patients [6, 7]. Obviously, more advanced disease at time of diagnosis may affect OS. However, access to curative treatment options may also play a role. African American patients have been demonstrated to have less surgical treatment (resection or liver transplantation (LT)) than non- Hispanic white patients, and Asian patients are less likely to have transplantation but more likely to have hepatectomy than non-Hispanic white patients [6–8]. However, it should be noted that there have been inconsistent reports about treatment effects. Mathur et al [9] reported that after tumor ablation and hepatic resection, African American and Hispanic patients had the worst survival. Asian patients had better survival than white patients after ablation and similar survival after hepatectomy. After liver transplantation, there was no significant difference in survival by race/ethnicity [9]. On the other hand, several other studies have reported that African American patients have worse OS after liver transplantation than non-Hispanic white patients [3, 10]. Finally, socioeconomic status (SES) could be the potential driving factor for ethnical survival disparity as it can affect healthcare-utilization (early detection, treatment, and post-treatment quality of life) in cancer patients [11]. However, several studies have found that SES does not explain ethnic disparity in OS for HCC patients [11, 12].

Inconsistencies in study results might be due to differences between the populations and study designs. Nevertheless, there are many socioeconomic and tumor-and treatment-related factors that may impact racial disparity in survival of HCC patients more or less, and as far as we know their influence on OS has not been compared [4]. In this study we describe the relative contributions of these factors to the ethnical disparity in OS for HCC patients, using The Surveillance, Epidemiology, and End Results (SEER) database.

Results

Population

Based on inclusion criteria, this study included a total of 33 062 patients who were diagnosed with HCC from 2004 to 2012 (**Supplementary Figure 1**). The population characteristics are summarized in Table 1. Among them, 15 986 (48%) were Non-Hispanic Whites, 6535 (20%) were Hispanic Whites, 4842 (15%) were African Americans, and 5699 (17%) were Asians (Asian or Pacific Islander). Although not statistically significant, in comparison to other ethnicities, Asians had a higher average age (mean age: 63 years, IQR:55–73) than Non-Hispanic Whites (mean age: 62; IQR: 55–72) and African Americans (mean age: 59; IQR: 54–64). Additionally, African Americans were more likely to be diagnosed with large tumor (tumor size > 5cm) than Non-Hispanic Whites, and vice versa. For example, among African Americans 38% had large tumor and 17% had small tumor; among Non-Hispanic Whites 34% had large tumor and 22% had small tumor ($p < .0001$).

Ethnical disparity in overall survival in overall HCC population

Figure 1 displays the overall survival (OS) rates among different ethnical populations. The median survival was 8 months (95%CI: 7.6–8.4), 9 months (95%CI: 8.4–9.6), 6 months (95%CI:5.5–6.5), and 13 months (95%CI: 12.0–14.0) for Non-Hispanic White, Hispanic White, African American, and Asian patients, respectively. 1-year and 3-year survival rates were 44% and 24%, 45% and 23%, 38% and 18%, and 51% and 31% for Non- Hispanic White, Hispanic White, African American, and Asian patients, respectively. Therefore, Hispanic White and Non-Hispanic White patients had similar survival rates. Asian patients displayed the best OS, and African American patients had the poorest OS. Specifically, there was significant “negative” survival disparity between African American and Non-Hispanic White patients ($P < .0001$), and “positive” survival disparity between Asian and White patients ($P < .0001$).

Figure 1.

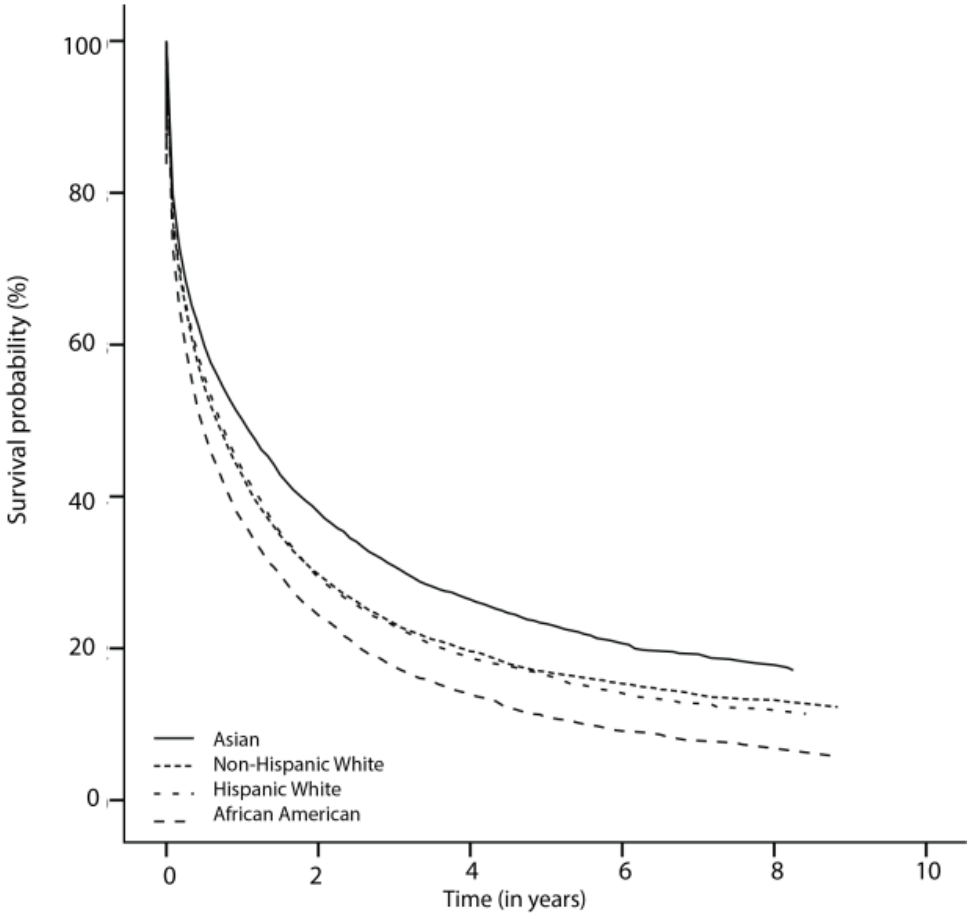


Figure 1 The Kaplan-Meier survival curves showing ethnical survival disparities

To determine the importance of several demographic-, tumor- and treatment-related factors for ethnical survival disparity, we performed multivariate analyses, and then observed the change of hazard ratios (HRs). **Figure 2** shows a forest plot presenting results from multivariate Cox models for all ethnical groups in the overall population (reference: Non-Hispanic White). No significant difference was observed between Hispanic White and Non-Hispanic White both in univariate analysis (HR, 1.01; 95%CI, 0.97–1.05) and multivariate analysis (HR, 0.98; 95%CI, 0.95–1.02). However, with respect to African American patients, we noticed some remarkable changes in survival disparity in Cox models. The initial survival disparity between African American and Non-Hispanic White (HR, 1.18; 95%CI, 1.14–1.23) did not change much when we adjusted demography-related variables. However, it was

affected by tumor size (HR, 1.11; 95%CI, 1.07–1.16), which indicated that the increased occurrence of large tumor in African Americans was associated with their poor survival. The other tumor-related variables that we studied did not significantly change the survival disparity any further. After additional adjustment for treatment-related factors, the significant survival disparity between African Americans and Non-Hispanic Whites became non-significant (HR, 1.03; 95%CI, 0.99–1.07). Therefore, we conclude that tumor size and treatment contributed largely to the survival disparity between African American and Non-Hispanic White patients. When comparing Non-Hispanic Whites to Asian patients, the latter population displayed a significantly better survival (HR, 0.85; 95%CI, 0.82–0.89), which remained constant from univariate analysis (HR, 0.85; 95%CI, 0.82–0.89) to multivariate analysis (HR, 0.86; 95%CI, 0.83–0.90). In other words, we did not identify the contributors to superior survival in Asian patients.

Since, for a large group of patients fibrosis scores were unavailable in the full SEER dataset, which may present a bias with respect to the survival data, we further analyzed a subset of patients for which this fibrosis score was available (n = 7070, characteristics in **Supplementary Table 1**). **Supplementary Figure 3** demonstrates that this subpopulation African Americans also had a poorer survival than Non-Hispanic Whites (HR, 1.19; 95%CI, 1.08–1.31). This survival disparity in multivariate analysis was again affected by tumor size; the factor large tumor size was associated with poor survival (HR, 1.10, 95%CI, 1.00–1.22).

Ethnical disparity in overall survival in patients stratified by treatment

We further explored the survival patterns among ethnicities in subgroups stratified by treatment: patients treated with tumor destruction (radiofrequent ablation / percutaneous ethanol injection (PEI) etc.) (9% of total), those that had surgical resection (9% of total), and those that have had liver transplantation (6% of total) (**Table 1**). As for the patients who underwent tumor destruction, both African Americans and Hispanic Whites showed non-significant survival difference compared to Non-Hispanic Whites. Asians had a much higher survival rate than Non- Hispanic Whites (HR, 0.71; 95%CI, 0.61–0.82) and no specific reason was

found for this disparity (**Figure 3A**). For patients receiving surgical resection, we found significantly lower survival rates in Hispanic Whites (HR, 1.20; 95%CI, 1.00–1.42) and African Americans (HR, 1.27; 95%CI, 1.08–1.50) than in Non-Hispanic Whites. Demographic and tumor-related factors negatively influenced survival in Hispanic Whites but for African Americans no such contribution could be identified among the various Cox models. Of interest, after surgical resection Asians did not have significantly higher survival rates than Non-Hispanic Whites (**Figure 3B**). Interestingly, no significant ethnical difference in survival was detected in patients after liver transplantation (**Figure 3C**).

Ethnical survival disparity in patients eligible for liver transplantation

To further explore the impact of liver transplantation on ethnical survival disparity, we performed survival analyses in a subgroup of patients who met the Milan criteria for liver transplantation (1 nodule ≤ 5 cm and max 3 nodules ≤ 3cm and no signs of vascular invasion/ extrahepatic spread). **Table 2** describes the characteristics for those patients. The patients receiving liver transplantation accounted for 15% of total patients “within Milan”; 19% of Non-Hispanic Whites (n = 802), 11% of African Americans (n = 111), 13% of Hispanic Whites (n = 232), and 10% of Asians (n = 137) (P < .0001). The survival rates are displayed in **Figure 4**. Compared to Non-Hispanic Whites, Hispanic White patients exhibited a poorer survival (HR, 1.12; 95%CI, 1.03–1.22), which was improved when adjusting for tumor-related factors but became not significant after adjusting for liver transplantation. Also, African American patients displayed a poorer survival (HR, 1.23; 95%CI, 1.11–1.35), and their outcome was improved when adjusting tumor-related variables. But especially when transplantation status was adjusted, the survival discrepancy disappeared (HR, 1.05; 95%CI, 0.95–1.15). Finally, the superior survival remained constant for Asian patients from crude analysis to adjustment analyses.

Figure 2.

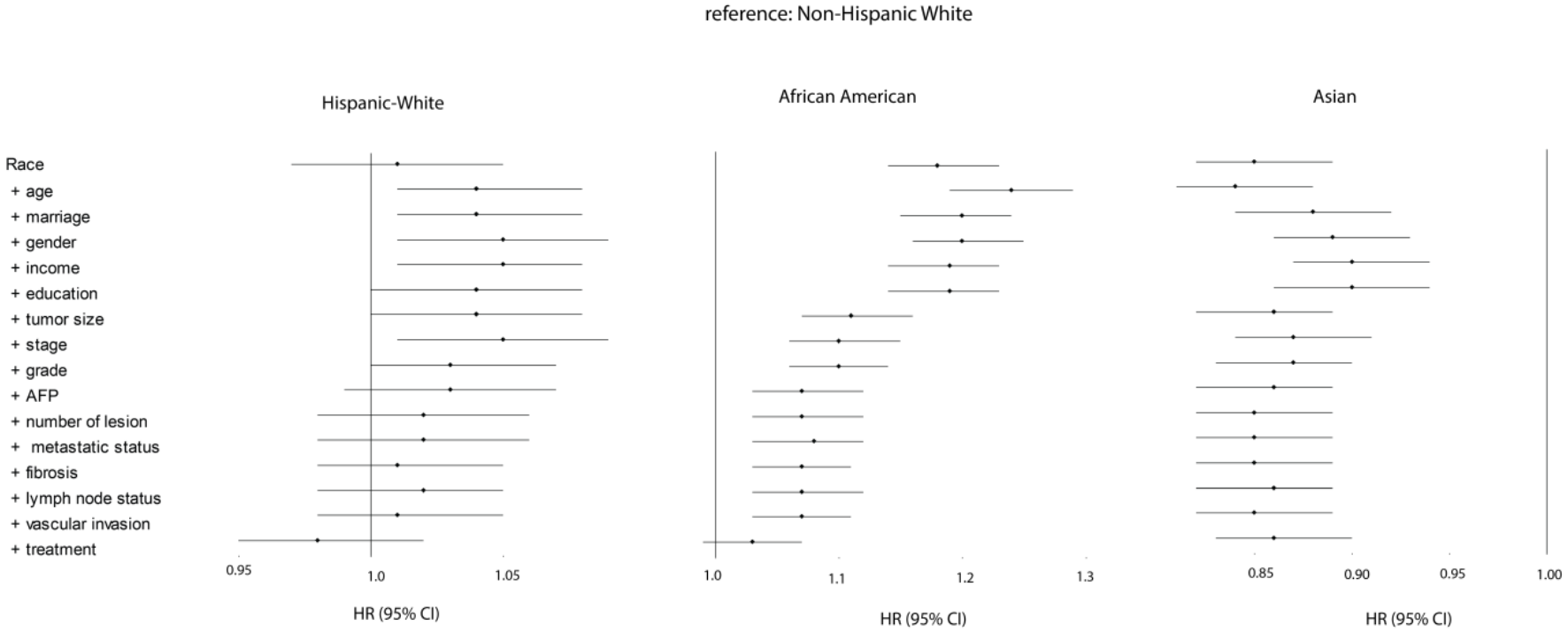


Figure 2 Forest plot presenting the estimated HR's of ethnicity on overall survival from multivariate Cox models for all ethnical groups (reference: Non-Hispanic White). The first HR is the crude effect followed by HR after adjustment entering covariates in a forward stepwise manner (LR): age, marriage, gender, income, education, tumor size, stage, grade, AFP, number of lesion, metastatic status, fibrosis, lymph node status, vascular invasion, and treatment. Block 1 included race, block 2 included age, gender, marital status, education, income, poverty, residence, block 3 included grade, stage, number of lesion, tumor size, lymph node status, vascular invasion, metastatic status, AFP, and fibrosis, and block 4 included treatment.

Figure 3

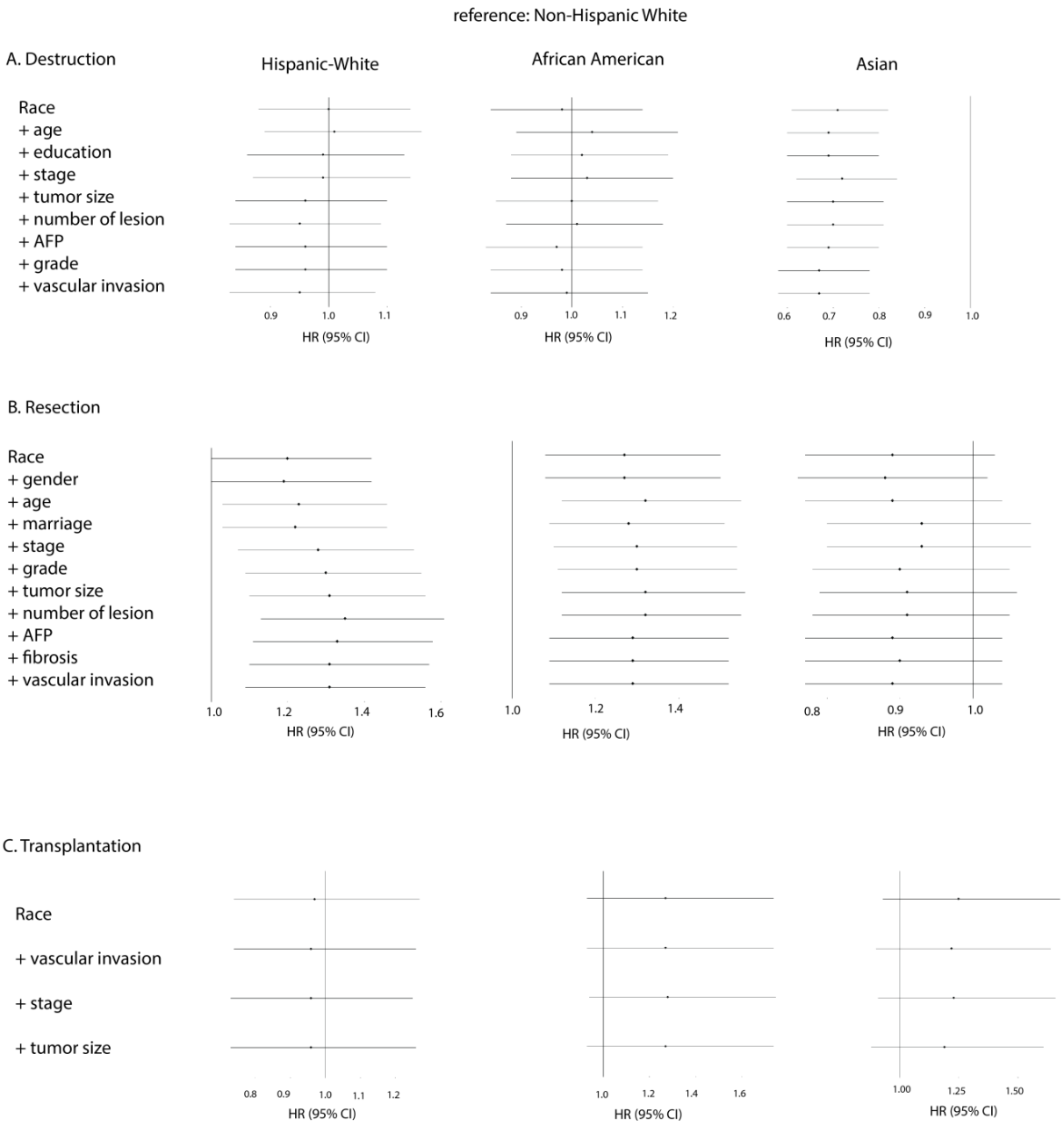


Figure 3 Forest plot presenting the estimated HR's of ethnicity on overall survival from multivariate Cox models for all ethnical groups stratified by treatment (reference: Non-Hispanic White). Forward stepwise method was used to study the changes of HR of ethnicity after entering the following covariates: in the stratum A. No treatment: age race, marriage, gender, income, tumor size, stage, grade, AFP, number of lesion, metastatic status, fibrosis, vascular invasion, and lymph node involvement. In the stratum B. Destruction: race, age, education, stage, tumor size, number of lesion, AFP, grade, and vascular invasion. In the stratum C. Resection: race, gender, age, marriage, stage, grade, tumor size, number of lesion, AFP, fibrosis, and vascular invasion. And in the stratum D. Transplantation: race, vascular invasion, stage, and tumor size.

Figure 4

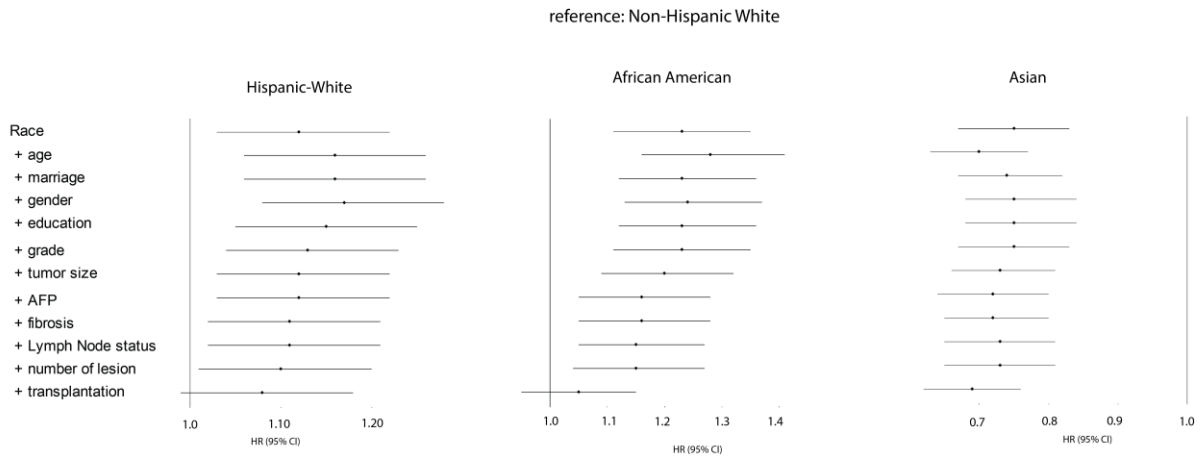


Figure 4 Forest plot presenting the estimated HR's of ethnicity on overall survival from multivariate Cox models for all ethnical groups who met Milan criteria (reference: Non-Hispanic white). The first HR is the crude effect followed by HR after adjustment entering covariates in a forward stepwise manner (LR): age, marriage, gender, education, grade, tumor size, AFP, fibrosis, lymph node status, number of lesion, and transplantation. Block 1 included race, block 2 included age, gender, marital status, education, income, poverty, residence, block 3 included grade, stage, number of lesion, tumor size, lymph node status, vascular invasion, metastatic status, AFP, and fibrosis, and block 4 included treatment.

Table 1 Characteristics of all patients by ethnicity

Characteristics	Total	Ethnicity				*P* Value
		Non-Hispanic White	Hispanic White	African American	Asian	
Patients						
No. %	33 062	15 986 (48)	6535 (20)	4842 (15)	5699 (17)	
Age						<.0001[a]
Mean [SD],y	63 [12]	63 [11]	62 [12]	60 [10]	63 [13]	
Median [IQR],y	61 [55-71]	62 [55-72]	60 [53-70]	59 [54-64]	63 [55-73]	
Gender (%)						<.0001[b]
Male	25 728 (78)	12 698 (79)	5061 (77)	3779 (78)	4190 (74)	
Female	7334 (22)	3288 (21)	1474 (23)	1063 (22)	1509 (27)	
Marital status (%)						<.0001[a]
Married	16937(51)	8151(51)	3285(50)	1607(33)	3894(68)	
Unmarried	14775(45)	7158(45)	3003(46)	2999(62)	1615(28)	
Unknown	1350(4)	677(4)	247(4)	236(5)	190(3)	
Education (%)[c]						<.0001[b]
Mean [SD]	16 [6]	15 [6]	19 [6]	16 [5]	16 [5]	
Median [IQR]	15 [12-22]	14 [11-20]	20 [14-23]	15 [12-18]	14 [12-23]	
Poverty (%)						<.0001[a]
Mean [SD]	16 [5]	15 [5]	17 [4]	18 [6]	14 [4]	
Median [IQR]	16 [12-18]	14 [12-18]	18 [13-18]	18 [13-23]	13 [10-18]	
Income (%)						<.0001[a]

Mean [SD]	59619 [14401]	58 600 [14690]	59478 [12533]	53924 [13423]	67477 [13132]	
Median [IQR]	55910 [50588-69710]	56490 [48260-66520]	55910 [54090-62960]	55060 [41180-61260]	67180 [55910-75600]	
Residence (%)						<.0001[b]
Rural	2592(8)	1957(12)	255(4)	265(5)	115(2)	
Urban	30470(92)	14029(88)	6280(96)	4577(95)	5584(98)	
Lesion number (%)						.002[b]
Single	32015(97)	15438(97)	6373(98)	4697(97)	5507(97)	
Multiple	1047(3)	548(3)	162(2)	145(3)	192(3)	
Grade (%)						<.0001[b]
Well differentiated	4325(13)	2209(14)	883(14)	598(12)	635(11)	
Moderately differentiated	5377(16)	2664(17)	896(14)	797(16)	1020(18)	
Poorly differentiated	2854(9)	1358(8)	466(7)	438(9)	592(10)	
Undifferentiated	281(1)	140(1)	43(1)	39(1)	59(1)	
Unknown	20225(61)	9615(60)	4247(65)	2970(61)	3393(60)	
Stage (%)						<.0001[b]
Localized	16143(49)	7822(49)	3304(51)	2175(45)	2842(50)	
Regional	9618(29)	4573(29)	1849(28)	1473(30)	1723(30)	
Distant	5201(16)	2466(15)	984(15)	906(19)	845(15)	
Unstaged	2100(6)	1125(7)	398(6)	288(6)	289(5)	
Tumor size (cm),%						<.0001[b]
<3	6693(20)	3499(22)	1335(20)	808(17)	1051(18)	

3-5	7519(23)	3613(23)	1601(24)	1030(21)	1275(22)	
>5	12184(37)	5497(34)	2370(36)	1847(38)	2470(43)	
Unknown	6666(20)	3377(21)	1229(19)	1157(24)	903(16)	
Lymph node involvement (%)						<.0001[b]
No lymph node	25833(78)	12380(77)	5091(78)	3753(78)	4609(81)	
Lymph node	2272(7)	1239(8)	351(5)	401(8)	281(5)	
Unknown	4957(15)	2367(15)	1093(17)	688(14)	809(14)	
Vascular Invasion (%)						<.0001[b]
No Vascular Invasion	16749(51)	8187(51)	3412(52)	2297(47)	2853(50)	
Vascular Invasion	13467(41)	6269(39)	2569(39)	2137(44)	2492(44)	
Unknown	2846(9)	1530(10)	554(8)	408(8)	354(6)	
Metastatic status (%)						<.0001[b]
No metastasis	25038(76)	12119(76)	4937(76)	3579(74)	4403(77)	
Metastasis	5154(16)	2469(15)	989(15)	882(18)	814(14)	
Unknown	2870(9)	1398(9)	609(9)	381(8)	482(8)	
AFP[d] (%)						<.0001[b]
Positive	19366(59)	8672(54)	3928(60)	3221(67)	3545(62)	
Negative	5705(17)	3002(19)	1186(18)	546(11)	971(17)	
Borderline	74(0)	48(0)	8(0)	10(0)	8(0)	
Unknown	7917(24)	4264(27)	1413(22)	1065(22)	1175(21)	
Fibrosis (%)						<.0001[b]
None to moderate fibrosis	1622(5)	723(5)	236(4)	220(5)	443(8)	

Severe fibrosis or cirrhosis	5448(16)	2678(17)	1209(19)	709(15)	852(15)	
Unknown	25992(79)	12585(79)	5090(78)	3913(81)	4404(77)	
Treatment (%)						<.0001[b]
None	24646(75)	11672(73)	5178(79)	3850(80)	3946(69)	
Tumor destruction	3102(9)	1544(10)	596(9)	368(8)	594(10)	
Surgical resection	3002(9)	1364(9)	349(5)	406(8)	883(15)	
Liver transplantation	2016(6)	1221(8)	374(6)	178(4)	243(4)	
Unknown	296(1)	185(1)	38(1)	40(1)	33(1)	

[a] one-way ANOVA test

[b] Pearson Chi-Square

[c] Indicates the percentage of adults aged ≥ 25 years who had < 12 years of education.

[d] AFP positive indicates AFP >15 ng/ml.

Table 2 Characteristics of the patients within Milan criteria

Characteristics	Total	Ethnicity				*P* Value
		Non-Hispanic White	Hispanic White	African American	Asian	
Patients						
No. %	8 524	4290 (50)	1779 (21)	1056 (12)	1399 (16)	
Age						<.0001
Mean [SD],y	61 [10]	61 [10]	60 [10]	60 [9]	64 [11]	
Median [IQR],y	60 [54-68]	60 [55-67]	59 [53-67]	59 [55-64]	63 [55-72]	
Gender (%)						<.0001
Male	6425 (75)	3380(79)	1319(74)	791(75)	935(67)	
Female	2099 (25)	910(21)	460(26)	265(25)	464(33)	
Marital status (%)						<.0001
Married	4471(52)	2223(52)	896(50)	379(36)	973(70)	
Unmarried	3732(44)	1893(44)	825(46)	625(59)	389(28)	
Unknown	321(4)	174(4)	58(3)	52(5)	37(3)	
Education						<.0001
Mean [SD]	16 [6]	15 [6]	19 [6]	16 [5]	16 [5]	
Median [IQR]	15 [12-22]	14 [11-19]	18 [14-23]	15 [12-16]	14 [13-20]	
Poverty						<.0001
Mean [SD]	15 [5]	15 [5]	16 [5]	18 [6]	14 [4]	
Median [IQR]	15 [12-18]	14 [12-18]	18[13-18]	18 [13-21]	13 [10-18]	
Income						<.0001

Mean [SD]	60176 [14217]	58775 [14214]	60168 [12703]	54675 [13422]	68633 [13069]	
Median [IQR]	56530 [51380-72110]	56530 [48510-65590]	55910 [54090-63360]	55060 [41180-62000]	72110 [55910-75600]	
Residence (%)						<.0001
Rural	585(7)	468(11)	60(3)	41(4)	16(1)	
Urban	7939(93)	3822(89)	1719(97)	1015(96)	1383(99)	
Lesion number (%)						.100
Single	8309(97)	4170(97)	1747(98)	1033(98)	1359(97)	
Multiple	215(3)	120(3)	32(2)	23(2)	40(3)	
Grade (%)						<.0001
Well differentiated	1457(17)	766(18)	293(16)	176(17)	222(16)	
Moderately differentiated	1582(19)	807(19)	270(15)	213(20)	292(21)	
Poorly differentiated	461(5)	230(5)	79(4)	49(5)	103(7)	
Undifferentiated	39(0)	20(0)	7(0)	3(0)	9(1)	
Unknown	4985(58)	2467(58)	1130(64)	615(58)	773(55)	
Stage (%)						.013
Localized	8228(97)	4128(96)	1728(97)	1009(96)	1363(97)	
Regional	295(3)	162(4)	51(3)	46(4)	36(3)	
Distant	1(0)	0(0)	0(0)	1(0)	0(0)	
Tumor size (cm),%						< .001
<3	4465(52)	2348(55)	901(51)	524(50)	692(49)	

3-5	4059(48)	1942(45)	878(49)	532(50)	707(51)	
Lymph node involvement (%)						.001
No lymph node	8173(96)	4099(96)	1706(96)	1004(95)	1364(97)	
Lymph node	149(2)	92(2)	19(1)	25(2)	13(1)	
Unknown	202(2)	99(2)	54(3)	27(3)	22(2)	
Vascular Invasion (%)						NA
No Vascular Invasion	8524(100)	4290(100)	1779 (100)	1056 (100)	1399 (100)	
Metastatic status (%)						NA
No metastasis	8524(100)	4290(100)	1779 (100)	1056 (100)	1399 (100)	
AFP (%)						<.0001
Positive	4629(54)	2126(50)	985(55)	682(65)	836(60)	
Negative	2176(26)	1196(28)	482(27)	175(17)	323(23)	
Borderline	21(0)	15(0)	2(0)	2(0)	2(0)	
Unknown	1698(20)	953(22)	310(17)	197(19)	238(17)	
Fibrosis (%)						<.0001
None to moderate fibrosis	523(6)	253(6)	59(3)	68(6)	143(10)	
Severe fibrosis or cirrhosis	2139(25)	1081(25)	469(26)	261(25)	328(23)	
Unknown	5862(69)	2956(69)	1251(70)	727(69)	928(66)	
Therapy (%)						<.0001
None	4478(53)	2167(51)	1103(62)	604(57)	604(43)	

Tumor destruction	1746(20)	864(20)	320(18)	202(19)	360(26)
Surgical resection	980(11)	432(10)	120(7)	133(13)	295(21)
Liver transplantation	1282(15)	802(19)	232(13)	111(11)	137(10)
Unknown	38(0)	25(1)	4(0)	6(1)	3(0)

Discussion

Since HCC related mortality continues to increase in the US, the ethnical disparities in overall survival has attracted attention [13]. Many efforts have been devoted to exploring the reasons behind this phenomenon [4, 14] for a better understanding of its contributors, which shall help us to determine which interventions could reduce this disparity.

We have confirmed the ethnical survival disparity in overall survival that has previously been reported by others [3–6, 9]. We demonstrated how tumor-related and treatment-related factors contribute strongly to survival disparity between African American and Non-Hispanic White patients.

As demonstrated in previous studies, compared to non-Hispanic Whites we found a poor survival in African Americans and a good survival in Asian patients [4, 7, 9, 12, 15, 16]. Our results demonstrate that increased presence of large tumor size was associated with poor survival in African Americans. Tumor size is considered as an important prognostic determinant in several HCC staging systems such as the TNM classification [17], the Barcelona Clinic Liver Cancer (BCLC) staging system [18], and the Hong Kong Liver Cancer (HKLC) classification [19]. Although previous studies have reported the significant differences in tumor size in HCC patients stratified by race and proposed it as a predictor of prognosis for HCC patients [4, 7, 9, 12, 15, 16], our study has demonstrated that tumor presentation indeed is the dominant contributor to the poor OS of African Americans. Such a clear dominant factor could not be demonstrated for Hispanic-Whites and Asians. In the former population demographic factors contributed to OS to some extent, but again presentation-related factors were shown to be the dominant contributors. Stage of liver disease as represented by fibrosis score did not significantly impact the observed survival disparities, as in a sub-analysis of patients of which these data were available tumor size remained a major confounder.

Consistent with previously studies, we found superior OS in Asians. Marital status affected HRs to some extent, and in our population Asians showed the highest percentage of marriage: up to 70% ($P < .0001$). Multiple studies have shown that being married is associated with more favorable survival for various cancer types [20–23] and this also appears to be the case in Asian HCC patients.

Treatment related factors also contributed to ethnical survival differences. As reported by others [9] African Americans displayed the poorest response to resection. We cannot explain this finding based on our data. African Americans were previously reported to have a longer waiting time period before surgery [12], which may affect the severity of their liver disease and consequently the chance on complications after surgery. However, this information was not available for us to study.

We speculate that there is an impact of the etiology of liver disease on both the observed overall survival disparities and the discrepancies found in relation to treatment modality. The cause of liver disease in the majority of Asian HCC patients is chronic HBV infection; whereas in African Americans and non-Hispanic Whites chronic HCV infection, non-alcoholic fatty liver disease (NAFLD) and alcohol abuse are more common [24]. HBV infection is well manageable whereas HCV infection (before the DAA era) would have been progressive after resection or destruction. NAFLD is associated with obesity and diabetes and both can potentially lead to serious comorbidity [25], and alcohol abuse may have continued or recurred. Differences in etiology of underlying liver disease or (their impact on) comorbidity as contributors to the observed survival disparities after resection could not be studied since these data were not present in our database.

Interestingly, no significant ethnical survival difference was observed after liver transplantation. These results do not match the study by Ananthakrishnan et al. [10], who reported that African American patients benefitted less from transplantation than Non-Hispanic Whites, using a United Network for Organ Sharing database [10]. However, our findings are in line with the data reported by Mathur et al [9], who also studied the SEER-database [9]. Therefore, these inconsistencies may be due to different patient populations analyzed. Although, Artinyan et al also used SEER data to report poorer survival after transplantation for African Americans [3], we believe that the population described in this particular study which included patients with diagnostic year as early as 1973 until 2004, is significantly different from our study population since implementation of the Milan criteria for liver transplantation in clinical practice only occurred after year of 1996 [26]. Therefore, differences in

eligibility criteria for liver transplantation may explain the differences in results between our and their study.

We next explored a potential role for receiving liver transplantation on ethnical survival disparity. This issue has been discussed extensively over the years [6–8]. Several studies have demonstrated limited access to transplantation for African American patients, and indeed also in our study African Americans eligible for liver transplantation received this potentially curative treatment less frequently than non-Hispanic Whites. But as also reported before, so did Asian HCC patients [8–10, 12, 27–30]. Some studies have suggested that disparity in receiving transplantation may have contributed to ethnical disparity in survival [4, 9]. To determine the impact of undergoing liver transplantation on ethnical differences in survival, we analyzed the subgroup of patients who met the Milan criteria. Compared to Non-Hispanic Whites, Hispanic White and African American patients exhibited a poorer survival, and indeed their survival discrepancy disappeared after adjusting transplantation status. Asians “within Milan” on the other hand have better outcome which is unaffected by the factor liver transplantation. These patients have been shown to receive resection more often than liver transplantation and more often than any other race [7], and this is confirmed in our study. Since most of the Asian cases are likely HBV related [24] and may therefore have relatively preserved underlying liver function, more Asians can tolerate liver resection. It probably explains the small impact of transplantation on their survival. Of note, we could not identify a significant contributing role for socioeconomic or demographic factors to the ethnic survival discrepancy in this subgroup, suggesting that these factors may not determine access to transplantation. Indeed, whether or not to transplant is a complex decision making process that involves evaluation of etiology of liver disease, comorbidity, social context etc. and as said, these factors could not all be analyzed in our study.

Our work has some limitations. Firstly, since our study is retrospective in nature, it holds the known biases associated with this type of study. Secondly, as mentioned the level of clinical detail available to us does not capture significant details that may affect the use of surgical therapy or survival, such as medical comorbidities, presence of chronic liver disease and its etiology, and information on the details of all treatments received. Thirdly, the county-level socioeconomic data may not fully capture the economic, educational, and social factors for individual

patients. Lack of social support, density of specialists within a region, hospital volume, distance to care, and other unmeasured confounders may have influenced access to therapies. Lastly, the effects of sorafenib or TACE on ethnical survival difference could not be studied since SEER has no specific coding for these treatment modalities. Nevertheless, to our knowledge, our analysis represents the most comprehensive study on ethnic differences in survival for HCC patients in the US.

In conclusion, we have confirmed the ethnical disparities in overall survival of HCC patients in the US. Poor tumor-presentation at diagnosis, poor response to resection, and limited utilization of transplantation all play essential roles in the poorer survival of African Americans compared to other races. Asian patients have superior survival, but after liver transplantation ethnic disparity in survival is absent.

Methods

Patients selection

This study was performed using data from the Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database (version 8.2.1). The procedure for selecting the patients for the cohort is shown in Supplementary Figure 1. Briefly, these patients were diagnosed between 2004 and 2012. We included the following ethnicities: Non-Hispanic White, Hispanic White, African American, and Asian (Asian/Pacific Islander). Among the Asian population in this study, 41.4% (2360/5699) were East Asians, 3.8% (219/5699) were South Asians, 39.2% (2233/5699) were Southeast Asians, and 15.6% were other Asians (887/5699). Native American (American Indian/Alska native) were excluded from our study. SEER Vital status recode (study cutoff used) variable was used to define the status of patients after the last follow-up date: death and alive. The survival time months variable, starting from diagnosis to last follow-up, was used for extracting information on patients' survival time. The follow-up cut-off date was December 31, 2012. Among the overall population, we selected patients within Milan criteria: one lesion ≤ 5 cm or up to 3 lesions each with diameter ≤ 3 cm; no extra-hepatic involvement; and no vascular invasion.

Definition

SEER Staging (also called Summary Staging) was used to define HCC stage: localized, regional, and distant. SEER Staging is the most basic way of categorizing how far a cancer has spread from its point of origin, as it combines the most precise clinical and pathological documentation of the extent of disease (http://training. seer.cancer.gov/ss2k/staging/). The detailed SEER Staging for HCC is documented in the "SEER Summary Staging Manual". For example, localized HCC indicates the cancer confined to one lobe with or without vascular invasion, or multiple nodules/tumors confined to one lobe.

HCC therapies were categorized into groups based on data available in SEER database: none, local tumor destruction, surgical resection, and liver transplantation (LT). None indicated: without any intervention such as local tumor destruction, surgical resection, or liver transplantation. Local tumor destruction included:

photodynamic therapy (PDT), electrocautery, cryosurgery, laser, PEI, heat-radio-frequency ablation (RFA). Since SEER has no specific coding for chemotherapy (sorafenib) or chemoembolization (TACE), they were not specified as such in SEER database. Resection included wedge, segmental resection, and lobectomy. “Unknown” means uncertainty about whether surgery was performed or what type of surgery was done.

The following SES related variables were included: education (the percentage of adults aged ≥ 25 years who < 12 years of education), poverty (the percentage of individuals living below the poverty line), and income (median annual household income). These variables were used as continuous variables in this study. According to the definitions of Country Attributes in SEER data, the higher values of the variables of education and poverty are, the lower the values of SES are. Please see “http:// seer.cancer.gov/seerstat/variables/countyattribs/#08–12” for details.

Statistical analysis

Standardized deviation (SD), and median with interquartile range (IQR) for continuous variables, and whole number with percentage for categorical variables. One-way ANOVA was used to compare groups for continuous variables. Pearson Chi-Square was used for comparing groups for categorical variables. Crude (non-adjustment) survival analysis (Kaplan-Meier curve) was first used to display the overall observed ethnical survival differences. Hazard ratio (HRs) and 95% CIs were calculated to evaluate the prognostic power of variables in survival. The included variables were divided into three categories:1) demographic variables, including race, age, SEER site, gender, marital status, education, income, poverty, residence; 2) presentation-related variables, including grade, stage, number of lesion, tumor size, lymph node involvement (yes or no), vascular invasion (yes or no), metastatic status (yes or no), AFP (Alpha-fetoprotein), and fibrosis degree (none to moderate fibrosis; several fibrosis or cirrhosis); and 3) treatment-related variables, including treatment presenting with categories — no treatment, tumor destruction, resection, and transplantation. For the procedure of multivariate cox model, race was entered as block 1, the remaining demographic variables were entered as block 2 (including age, gender, marital status, education, income, poverty, residence), presentation-related variables were entered as block 3 (including tumor size, stage, grade,

number of lesion, lymph node status, vascular invasion status, metastatic status, AFP, and fibrosis staging), and finally treatment-related variable was entered as block 4. All analysis were stratified on SEER site to adjust for any heterogeneity between sites. Regarding block 2 and 3, the covariates were entered in a forward stepwise manner using the Likelihood Ratio test (LR) to describe their impact on survival. The changes in the HR's of African Americans, Hispanic White and Asian versus Non-Hispanic White after the stepwise adjustment of the covariates are shown in forest plots. There were some missing values for several categorical variables in our study. As we did not find any significant differences from the analysis of all cases and the cases with known values, we treated the missing values (with "unknown" label shown in **Tables 1–2**) as a separate subcategory. Data preparation and forest plot were done in R (version 3.3.1). Statistical analyses was performed in SPSS (version 21); syntax shown in **Supplementary Figure 2**. $P < .05$ (two tailed sides) was considered as significant.

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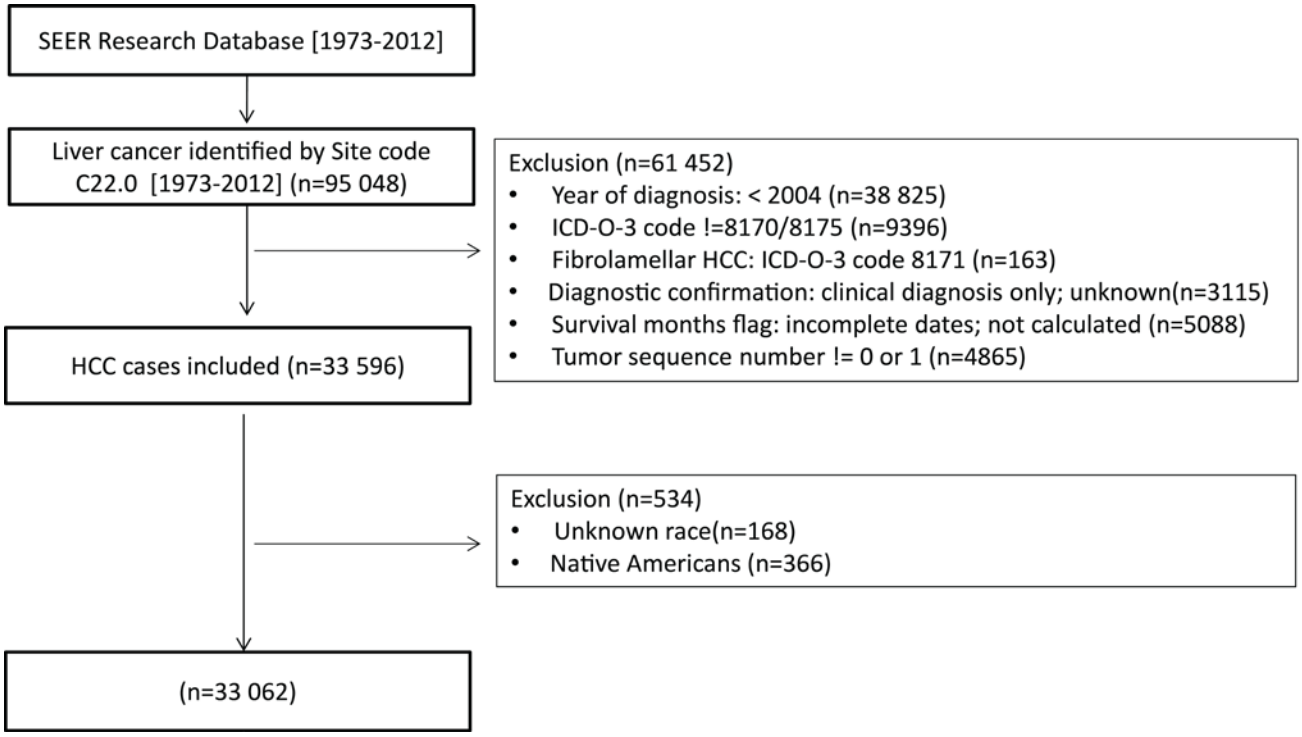
Supplementary Data



Supplementary Figure S1 Flowchart displaying the selection procedure of HCC cases in SEER database.

```
COXREG Time

/STATUS=Vital(1)

/STRATA SEER_Site (SEER site was stratified for all analysis)

/CONTRAST (Race)=INDICATOR(1)

/CONTRAST (Gender)=INDICATOR(1)

/CONTRAST (Marriage)=INDICATOR(1)

/CONTRAST (TumorSize)=INDICATOR(1)

/CONTRAST (Stage)=INDICATOR(1)

/CONTRAST (Grade)=INDICATOR(1)

/CONTRAST (LesionNumber)=INDICATOR(1)

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/CONTRAST (Vascular)=INDICATOR(1)

/CONTRAST (Metastasis)=INDICATOR(1)

/CONTRAST (AFP)=INDICATOR(1)

/CONTRAST (Fibrosis)=INDICATOR(1)

/CONTRAST (Surgery)=INDICATOR(1)

/METHOD=ENTER  Race

/METHOD=FSTEP(lr)  Age Gender Marriage Education Income Poverty Residence

/METHOD =FSTEP(lr) TumorSize Stage Grade LesionNumber  LymphNode  Vascular Metastasis
AFP Fibrosis

/METHOD=FSTEP(lr) Treatment

/PRINT=CI(95)

/CRITERIA=PIN(.05) POUT(.10) ITERATE(20).
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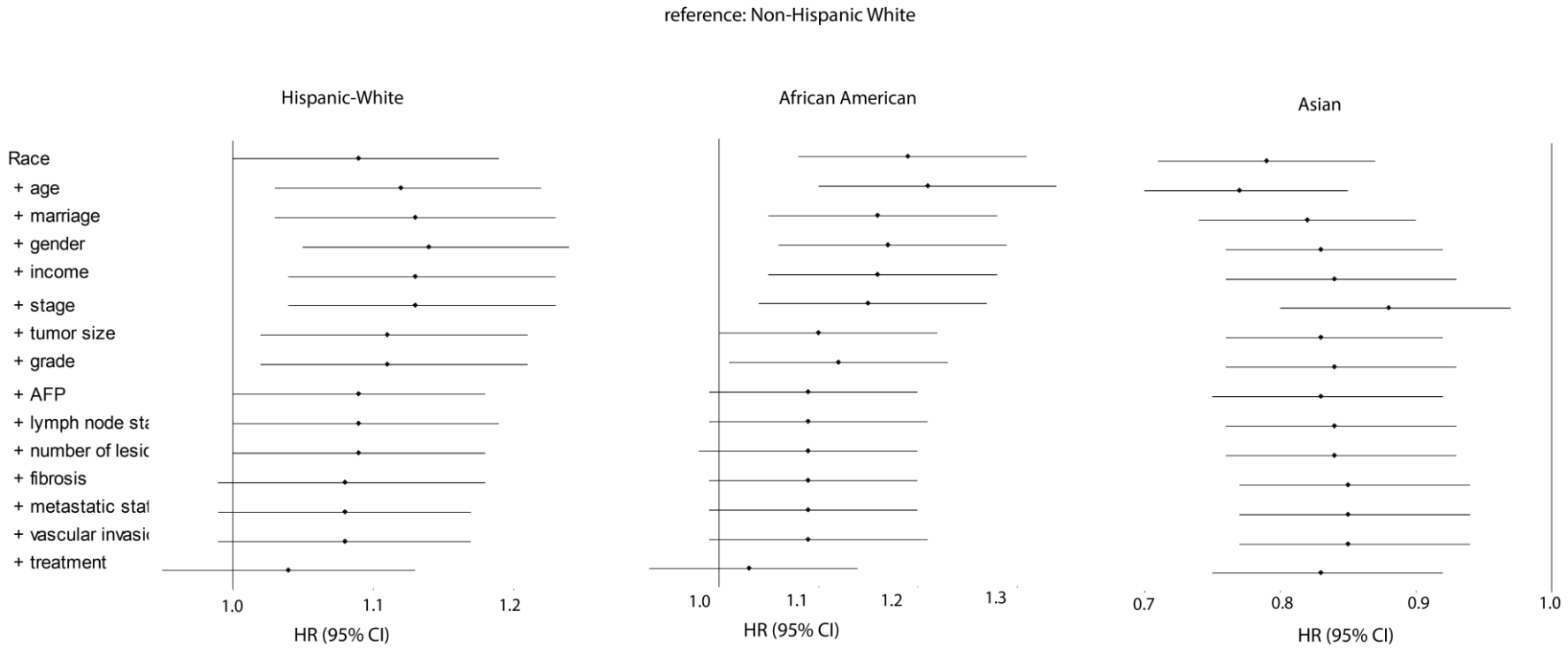
Supplementary Figure S2 SPSS syntax for performing multivariate analysis in this study



Supplementary Figure S3 Forest plot presenting the estimated HR's of ethnicity on overall survival from multivariate Cox models for all ethnical groups with available fibrosis score (reference: Non-Hispanic white). The first HR is the crude effect followed by HR after adjustment entering covariates in a forward stepwise manner (LR) : age, marriage, gender, education, grade, tumor size, AFP, fibrosis, lymph node status, number of lesion, and transplantation. Block 1 included race, block 2 included age, gender, marital status, education, income, poverty, residence, block 3 included grade, stage, number of lesion, tumor size, lymph node status, vascular invasion, metastatic status, AFP, and fibrosis, and block 4 included treatment.

Supplemental Table S1 Characteristics of the patients with known fibrosis value stratified by ethnicity.

		Ethnicity				
Characteristics	**Total**	**Non-Hispanic White**	**Hispanic White**	**African American**	**Asian**	***P* Value**
Patients						
No. %	7070	3401 (48)	1445(20)	929 (13)	1295(18)	
Age						<.0001[a]
Mean [SD],y	61[10]	61 [10]	60 [10]	60 [8]	62 [12]	
Median [IQR],y	60[55-67]	60 [55-67]	59 [53-66]	59 [55-64]	62 [54-71]	
Gender (%)						<.0001[b]
Male	5620 (80)	2783	1141	723	973	
Female	1450 (20)	618	304	206	322	
Marital status (%)						<.0001[a]
Married	3723 (53)	1743	747	318	915	
Unmarried	3153 (45)	1574	655	578	346	
Unknown	194(3)	84	43	33	34	
Education (%)						<.0001[b]
Mean [SD]	16 [6]	15 [6]	18 [7]	15 [5]	15 [5]	
Median [IQR]	14 [12-20]	14 [11-18]	16 [13-23]	15 [12-16]	14 [12-23]	
Poverty (%)						<.0001[a]
Mean [SD]	15 [5]	15 [5]	16[5]	18 [5]	13 [4]	
Median [IQR]	14[12-20]	14 [12-18]	16 [13-18]	18 [13-23]	13 [10 - 14]	
Income (%)						<.0001[a]
Mean [SD]	61885 [14989]	59674 [14246]	62087 [14426]	55815 [13994]	71822 [13537]	
Median [IQR]	59720 [53380-	57650 [49058-	56530 [54090-	55100 [41180 -	72760 [60450 -	

	72760]	67180]	72110]	6390]	78760]	
Residence (%)						<.0001[b]
Rural	469 (7)	370	48	35	16	
Urban	6601 (93)	3031	1397	894	1279	
Lesion number (%)						.091[b]
Single	6822 (97)	3264	1407	900	1251	
Multiple	248 (4)	137	38	29	44	
Grade (%)						<.0001[b]
Well differentiated	1017 (14)	532	188	135	162	
Moderately differentiated	1284 (18)	625	222	176	261	
Poorly differentiated	503 (7)	223	85	62	133	
Undifferentiated	45 (1)	18	4	4	19	
Unknown	4221 (60)	2003	946	552	720	
Stage (%)						.013[b]
Localized	4066 (58)	1947	822	511	786	
Regional	2163 (31)	1059	442	279	383	
Distant	715 (10)	327	158	122	108	
Unstaged	126 (2)	68	23	17	18	
Tumor size (cm),%						<.0001[b]
<3	2123 (30)	1091	421	255	356	
3-5	2034 (29)	965	424	262	383	
>5	2159 (31)	955	450	303	451	
Unknown	754 (11)	390	150	109	105	
Lymph node						.010[b]

involvement (%)						
No lymph node	6822 (88)	2955	1270	809	1168	
Lymph node	390 (6)	215	69	58	48	
Unknown	478 (7)	231	106	62	79	
Vascular Invasion (%)						.235[b]
No Vascular Invasion	3945 (56)	1914	802	506	723	
Vascular Invasion	2948 (42)	1392	612	394	550	
Unknown	177 (3)	95	31	29	22	
Metastatic status (%)						.002[b]
No metastasis	6122 (86)	2941	1238	787	1146	
Metastasis	708 (10)	326	158	120	104	
Unknown	250 (4)	134	49	22	45	
AFP[c] (%)						<.0001[b]
Positive	4552 (64)	2061	968	678	845	
Negative	1545 (22)	821	289	137	298	
Borderline	17 (0.2)	10	2	2	3	
Unknown	956 (14)	509	186	112	149	
Fibrosis (%)						<.0001[b]
None to moderate fibrosis	1622 (23)	723	236	220	443	
Severe fibrosis or cirrhosis	5448 (77)	2678	1209	709	852	
Therapy (%)						<.0001[b]
None	4590 (65)	2172	1052	651	715	
Tumor destruction	787 (11)	391	141	96	159	

Surgical resection	889 (13)	374	89	117	309
Liver transplantation	773 (11)	443	159	61	110
Unknown	31 (0.4)	21	4	4	2

[a] one-way ANOVA test

[b] Pearson Chi-Square

[c] AFP positive indicates AFP >15 g/ml.

Racial disparity in overall survival in patients with hepatocellular carcinoma

Chapter 6 Anti-Tumor Effects of Metformin in Animal Models of Hepatocellular Carcinoma: A Systematic Review and Meta-Analysis

Juan Li[1], Pratika Y. Hernanda[2], Wichor M. Bramer[3], Maikel P. Peppelenbosch[1],

Judith van Luijk[4], Qiuwei Pan[1]

[1] Department of Gastroenterology and Hepatology, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands. [2] Laboratory of Medical Genetics, Biomolecular Research Centre, Wijaya Kusuma University, Surabaya, Indonesia, [3] Medical Library, Erasmus University Medical Center, Rotterdam, The Netherlands. 4 SYRCLE at Central Animal Laboratory, Radboud University Medical Centre, Nijmegen, The Netherlands

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Abstract

Several studies have reported that metformin can reduce the risk of hepatocellular carcinoma (HCC) in diabetes patients. However, the direct anti-HCC effects of metformin have hardly been studied in patients, but have been extensively investigated in animal models of HCC. We therefore performed a systematic review and meta-analysis of animal studies evaluating the effects of metformin on HCC. We collected the relevant studies by searching EMBASE, Medline (OvidSP), Web of Science, Scopus, PubMed Publisher, and Google Scholar. Studies were included according to the following inclusion criteria: HCC, animal study, and metformin intervention. Study quality was assessed using SYRCLE's risk of bias tool. A meta-analysis was performed for the outcome measures: tumor growth (tumor volume, weight and size), tumor number and incidence. The search resulted in 573 references, of which 13 could be included in the review and 12 included in the meta-analysis. The study characteristics of the included studies varied considerably. Two studies used rats, while the others used mice. Only one study used female animals, nine used male, and three studies didn't mention the gender of animals in their experiments. The quality of the included studies was low to moderate based on the assessment of their risk of bias. The meta-analysis showed that metformin significantly inhibited the growth of HCC tumour (SMD -2.20[-2.96,-1.43]; n=16), but no significant effect on the number of tumors (SMD-1.05[-2.13,0.03]; n=5) or the incidence of HCC was observed (RR 0.62[0.33,1.16]; n=6). To investigate the potential sources of significant heterogeneities found in outcome of tumor growth (I^2=81%), subgroup analyses of scales of growth measures and of types of animal models used were performed. Metformin appears to have a direct anti-HCC effect in animal models. Although the intrinsic limitations of animal studies, this systematic review could provide an important reference for future preclinical animal trials of good quality and clinical development.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide and the third leading cause of death from cancer. Surgical resection and liver transplantation are the only potentially curative treatment for a small proportion of the patients. However, disease recurrence hampers the ultimate success of the treatment [1]. Sorafenib, an oral multikinase inhibitor, was approved to treat advanced HCC. This however only increases patient survival with approximately 2-3 months [2,3].Therefore, it is necessary to explore new strategies to improve the management of HCC.

Metformin is an oral drug widely used for treatment of type II diabetes. Interestingly, several studies, including observational studies and some randomized controlled trials (RCT), have reported that metformin can affect the risk of hepatocellular carcinoma (HCC) in diabetic patients [4-6]. Although these studies have suggested a preventive effect of metformin on the risk of HCC in these diabetic patients, there is still lacking of investigation whether metformin has direct anti-tumor effect in HCC patients. Nevertheless, substantial research has been performed in animal models of HCC, although the data are still inconclusive. Meta-analyses on data from animal studies can be used to explain clinical observation and to inform clinical trial design.

To better understand the direct effects of metformin on HCC and to pave the way for further prospective clinical study, we performed a systematic review and meta-analysis of currently available data from HCC animal models treated with metformin.

Results

Description of the included studies

The comprehensive search strategy on the effects of metformin on HCC in animal models resulted in 573 records. After duplicates were removed, 340 studies were left. After title and abstract screening, 21 studies were screened full text. Ultimately, 13 studies were included in our systematic review [9-21], of which 12 studies (total 29 animal experiments and 311 animals involved) could be included in the meta-analysis (**Figure 1**) [9-20].

The characteristics of all included studies are described in Table 1. Since the investigation of metformin on HCC using animal models has only been started in recent years, the publication dates of the included studies ranged from 2012 to 2014. Apart from these, the characteristics among these studies varied considerably. The characteristics of animals themselves differed substantially between the studies. Seven of the studies used BALB/c nude mice, and others used C57BL/6J mice, NOD/SCID mice, HBxTg mice, and Wistar rat. Among these studies, seven used a subcutaneous xenograft model, while others used oncogenic compound inducing models. Only half of the studies used the same dosage of metformin (250 mg/kg). Administration timing and duration of metformin varied greatly. Besides, various time-points for outcome measurements were mentioned in the studies.

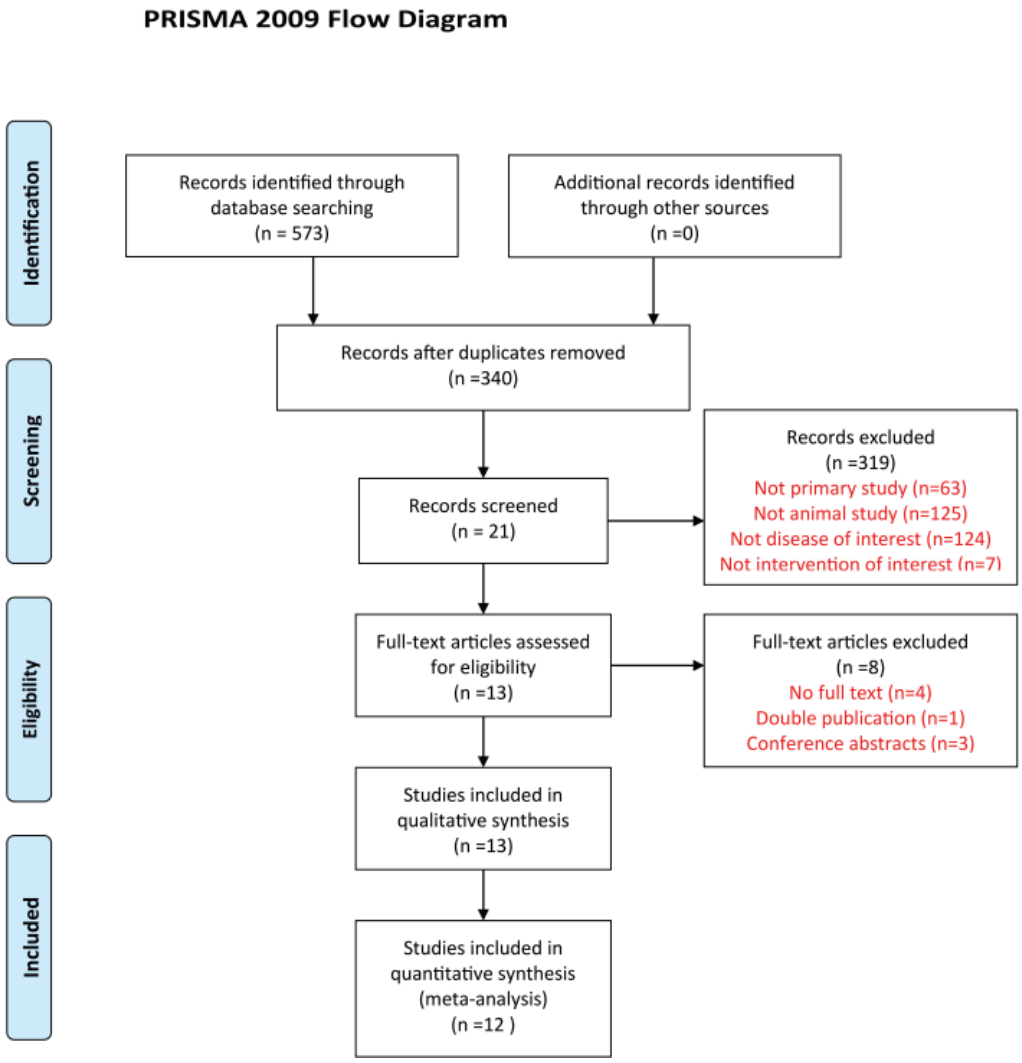


Figure 1 Flow diagram showing literature search and selection results

Risk of bias and quality of included studies

Figure 2 shows the results of the risk of bias assessment of the 13 studies included in this systematic review. Based on this assessment, 7 (54%) of the studies stated that the allocation was randomized. Since the background of animals were essentially homogenous, most of the studies didn't describe the method of randomization. Besides, none of the studies mentioned whether the allocation was adequately concealed. As shown clearly in Fig. 2, many items were scored as "unclear", which indicates that reporting – and presumably experimental design - of these animal studies can be improved.

Table 1 Characteristics of the included animal studies

Study	Language	strain/Species	Experimental group	control group	Gender	Age	Weight	animal number: c/exp	Type of animal model	HCC Number/ animal	Dosage	Timing of metformin	Duration of metformin	Adminstration Route	Outcome measures
Chen 2013[9]	English	BALB/c nude mice	HCC+MET	HCC	F	5-6 week	*	5/5.	subcutaneous xenograft	1/1	200 mg/ml in drinking water	10 days after implanation	30 days	orally	TW
Qu 2012[10]	English	BALB/c nude mice	HCC+MET	HCC+saline	M	3-4 week	14.0-16.0 g	12/12 ; 12/12	orthotopic xenograft	1/1	125& 250 mg/kg /day	10 days after implanation	30 days	i.p.	TV
Miyoshi 2014[11]	English	BALB/c-nu/nu mice	HCC+MET	HCC+PBS	M	8 weeks	20-25 g	10/10; 10/10	subcutaneous xenograft	1/1	1& 2 mg/body/day	after an identifiable mass > 6 mm	14 days	i.p.	TV; cell cycle regulators; angiogenesis
Bhalla 2012[12]	English	C57BL/6J mice	HCC+MET	HCC	M	2 week	*	7/7.	DEN	1/1	250mg/kg/day	2 weeks after DEN	168 days; 252 days	orally	TS, TN; AMPK activation
Kim 2013[13]	English	HBxTg mice	HCC+MET	HBx Tg mice	M	*	*	20/26	HBx Transgenic	1/1	250mg/kg/day	6 weeks of age	462 days	orally	TN; Hepatic CRBP-1 protein level, Akt
DePeralta 2013**[14]	English	Rat (Wistar)	HCC+MET	DEN induction	M	0 week	*	9/9.	DEN	1/1	250 mg/kg/day	8 weeks of age; 12 weeks of age	70 days; 42 days	orally	TI
Cai 2013[15]	English	BALB/c-nu mice	HCC+MET	HCC+PBS	M	6-8 week	*	10/10.	subcutaneous xenograft	4/1	250 mg/kg/day	1 week after transplantation	49 days	i.p.	TV; cell cycle regulators; p-AMPK
Saito 2013[16]	English	NOD/SCID mice	HCC+MET	HCC	*	*	*	5/5.	subcutaneous xenograft	1/1	250 mg/kg/day	just after the transplantation	56 days	i.p.	TV; ki-67, casp3.
Afzal 2012[21]	English	Wistar albino rat	MET+DENA; DENA+MET	DENA induction	M	Adult	100-125g	6/6.	DENA	1/1	125mg/kg/day	Day 1; Day 7.	*	i.p.	animal weight, SGPT/ALT, SGOT/AST
Tajima 2013[17]	English	C57B1/6 mice	non-NAFLD+M	HFD-HFD	M	8 week	*	6/4; 17/16;4/7;4/	HFD	1/1	250 mg/kg/day	30 weeks after HFD	210 days	orally	TS, TN; AMPK/mTOR/

			ET; NAFLD+MET					8							S6k
Cheng 2014[18]	English	BALB/c-nu mice	HCC+MET	HCC	M	5 week	*	28/10[#]	subcutaeneous xenograft	4/1	30 Ag/g body weight	1 week after transplantation	49 days	i.p.	TW, TI; activity of AMPK; Ki-67
Zheng 2013[19]	English	BALB/C nude mice	HCC+MET	HCC+vehicle	*	*	*	8/8; 8/8.	subcutaeneous xenograft[§]	1/1	*	*	49-56 days	*	TV; AMPK activation
Xiong 2012[20]	English	BALB/c nude mice	HCC+MET	HCC+PBS	*	*	size of ~100mm^3	5/5.	subcutaeneous xenograft	1/1	40 & 200mg/kg/day	7 days after transplantation	126 days	*	TV, TW

*=not mentioned; **=only abstract available; #= indicate tumor number; [§] = include two different xenograft model (HCC-LM3 and SMMC7721); MET=metformin; DEN= diethylnitrosamine (liver-specific carcinogen); FBS=fasting blood glucose; HFD= high-fat diet; TV=tumor volume; TW=tumor weight; TS=tumor size; TI=tumor incidence; TN=tumor number.

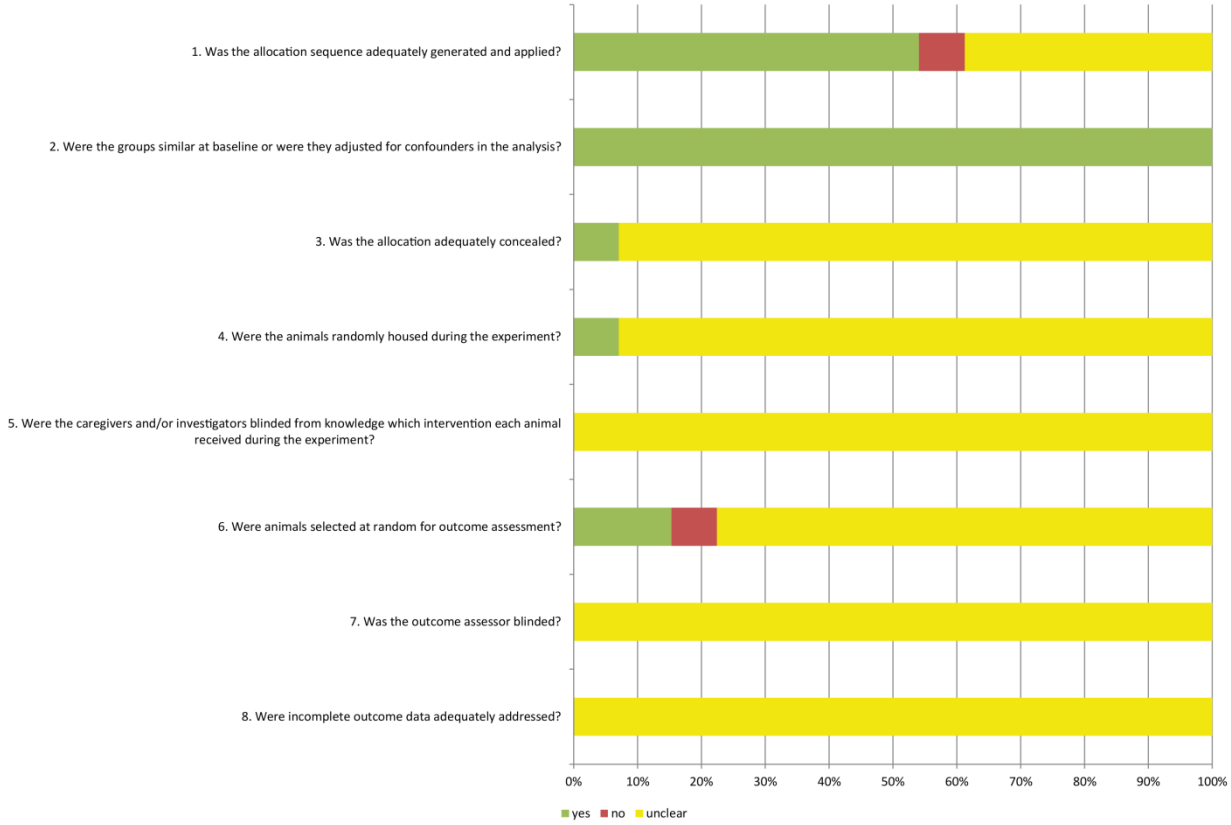


Figure 2 Risk of bias, score (%) per risk of bias item. Yes=low risk of bias, no=high risk bias, ?=unclear risk of bias

Overall analysis of the effects of metformin on HCC growth

Ten out of the twelve studies reported outcomes related to tumor growth (tumor volume, tumor size or tumor weight). These 10 studies contained 18 independent experiments [9-12,15-20]. Of these 18 experiments, 12 showed a significant decrease of tumor growth. None of the experiments showed a significant increase of tumor growth. Meta-analysis of these experiments revealed that metformin intervention had a significant inhibiting effect on HCC growth (SMD -2.20[-2.96, -1.43]; n=18) (**Figure 3A**). However, heterogeneity was quite high (I^2 = 81%).

Subgroup analysis

To determine whether the effects differed per scale of measurement, the clinically relevant outcome measures including “tumor volume”, “tumor weight” and “tumor size” were analyzed separately in subgroups. As displayed in **Figure 3B**, even

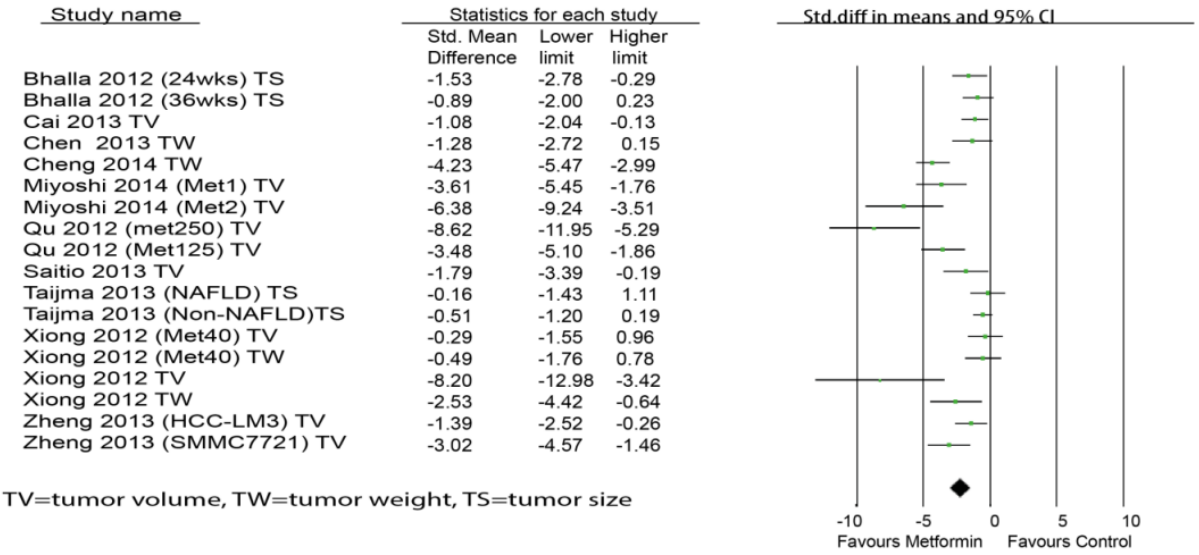
though all three subgroups showed a statistically significant inhibition of growth, the effect on volume (SMD -3.11[-4.36, -1.87]; n = 10) seemed to be larger than on size (SMD -0.69[-1.18, -0.20]; n = 4). Besides, heterogeneity levels significantly decreased in the subgroup analysis of "tumor size"(I^2 = 0.0%) while still high heterogeneity level were observed in subgroups of "tumor volume" (I^2 = 82%) and "tumor weight" (I^2 = 84%).

In addition, a subgroup analysis was performed for the types of HCC model used. This analysis demonstrated that metformin had significant effect on both xenograft (SMD -2.77[-3.74, -1.79]; n = 14) and non-xenograft model (SMD -0.69[-1.18, -0.20]; n=4), but the former group seemed to be affected by metformin more than the latter group. Subgroup analysis of "non-xenograft model" clearly reduced heterogeneity (I^2=0.0%), while "xenograft model" subgroup analysis did not change high heterogeneity level.

Effects of metformin on HCC number

In addition to the analysis of the effect on tumor growth, we also did analysis on HCC number in five animal experiments. Of these five experiments, four showed a significant decrease of HCC number and none showed a significant increase. Although it was unclear how the data were presented in DePeralta's study [14], we assumed they were presented as mean ± SE. The result didn't show significant inhibitory effect on HCC tumor number by metformin (SMD -1.05[-2.13, 0.03]; n = 5) (**Figure 4**). We found high heterogeneity (I^2 = 78%).

A.



B.

HCC growth	SMD	LL	HL	n	I^2	p
Overall	-2.20	-2.96	-1.43	18	81%	<0.00001
Tumor volume	-3.11	-4.36	-1.87	10	82%	<0.00001
Tumor weight	-2.13	-3.93	-0.34	4	84%	0.02
Tumor size	-0.69	-1.18	-0.20	4	0%	0.006
Xenograft model	-2.77	-3.74	-1.79	14	81%	<0.00001
Non-xenograft model	-0.69	-1.18	-0.20	4	0%	0.006

Figure 3 Effects of metformin on tumor growth in HCC animal models. (A) Forest plot and (B) subgroup analysis of the 16 included studies. The forest plot displays the SMD, confidence interval, and effect weight for each study, plus the pooled effect estimate & confidence interval.

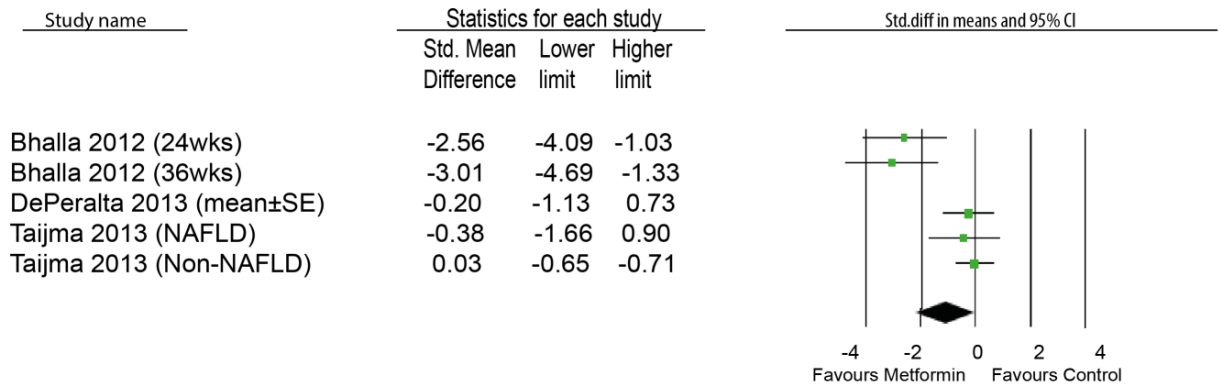


Figure 4 Effects of metformin on tumor number in HCC animal models.

Effects of metformin on HCC incidence

The effects of metformin on the incidence of HCC in animal models was evaluated. Only two studies showed a significant decrease of HCC incidence; whereas the others showed trend of decrease but one showed a trend of increase. However, the meta-analysis didn't show a significant effect of metformin on the incidence of HCC in comparison with non-treatment group (RR 0.62[0.33,1.16]; n=6) (**Figure 5**). The heterogeneity was high (I^2 = 83%).

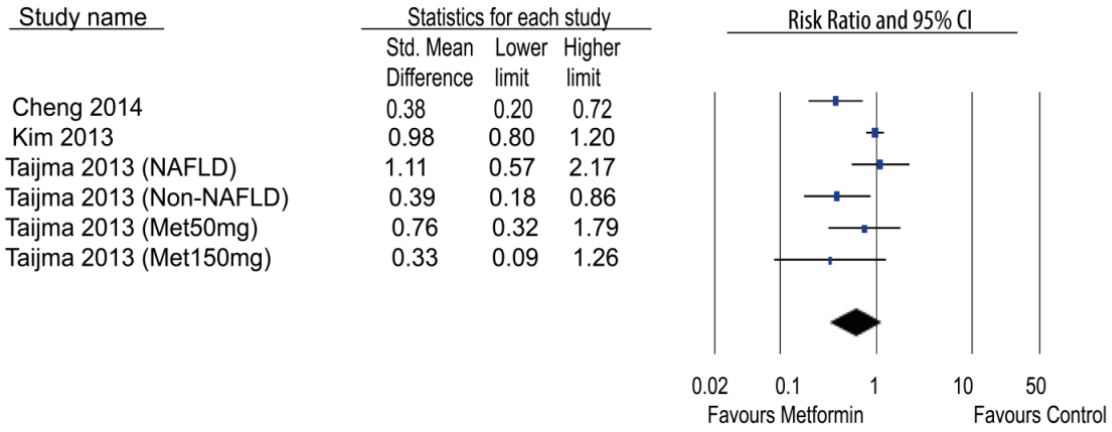


Figure 5 Effects of metformin on tumor incidence in HCC animal models

Sensitivity analysis

We performed sensitivity analysis to assess the robustness of our results on the effects of metformin on HCC number. In the subgroup analysis of “tumor number”, we assumed that DePeralta [14]presented the data as mean ± SE. For this sensitivity analysis we test the assumption that the data were presented as mean ± SD. With this assumption the meta-analysis showed that metformin could significantly reduce HCC tumor number (SMD -1.21[-2.29, -0.13];n=5). This result differs from our previous findings in the subgroup analysis. Interpretation of this outcome measure should be done with extreme caution, as current available evidence is still inconclusive.

Publication bias

Publication bias was assessed for the outcome of overall tumor growth, since the analysis of this outcome included the highest number of studies. On visual inspection of the funnel plot (**Figure 6**), small studies with no or a negative effect seem to be missing. This asymmetry might indicate the presence of publication bias.

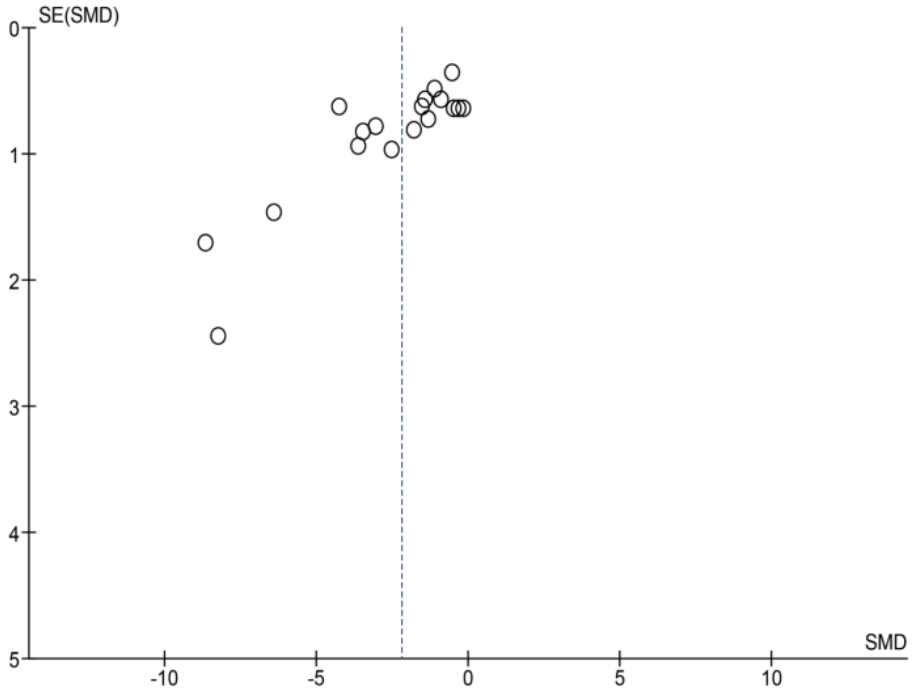


Figure 6 Funnel plot overseeing publication bias of included studies

Discussion

Although there were already several clinical studies evaluating the effects of metformin on HCC risk in human population, all of them were on the chemo-preventive effect by metformin rather than the therapeutic effect and, moreover, the population were all diabetic patients. A meta-analysis of the effects of anti-diabetic medications on the HCC risk, in which both observational and RCT studies were included, has suggested a half reduction in HCC incidence when using metformin treatment [22]. However, our meta-analysis systematically analyzed all relevant animal studies to assess the therapeutic potential of metformin against HCC.

In this comprehensive systematic review and meta-analysis, we analyzed and described the effects of metformin on HCC growth and incidence. The overall analysis of the effect of growth showed that use of metformin was associated with a significant inhibitory effect (SMD= -2.20 ± 0.76) on HCC growth, compared to untreated group. This therapeutic effect remained stable across subgroups of tumor volume, tumor weight and tumor size. It was most pronounced in “tumor volume” measurement while least in “tumor size” measurement. Besides, we performed subgroup analysis under the “tumor growth”. Both xenograft and non-xenograft studies showed significant inhibitory effect of metformin on HCC growth.

Mechanism underlying metformin anti-HCC

The anti-cancer effect of metformin was speculated to be associated with the activation of adenosine monophosphate-activated protein kinase (AMPK). An important upstream kinase of AMPK is LKB1, an very important tumor suppressor [23,24]. This signaling pathway was also discussed and explored in the included HCC animal trials. The coexistence of AMPK-dependent and AMPK-independent mechanisms for the effects of metformin on cancer was proposed[9,10,12,15]. But we should keep in mind of different study methodologies. For instance, two studies used cell lines *in vitro*[9,15]; one study used tumor harvested from xenograft[10]; whereas one study demonstrated their result based on observing AMPK level in liver[12]. In addition, metformin may also inhibit HCC cell growth by regulating cell-cycle regulatory proteins, such as cyclin D1 and cyclin E [10,14]. Of particular note, c-myc was suggested as a critical mediator in hepatocarcinogenesis [25]. Metformin treatment has been shown to inhibit c-myc expression by up-regulating let-7 family

(tumor suppressor) [11]. However, no study among the included animal studies discussed such mechanism underlying specific HCC stage. Although we found one human study relevant to early stage of HCC, no clear mechanistic insight of metformin on early stage HCC was described[26].

Side effects of metformin in animal models

Being a classic antidiabetic medication, metformin is widely used among patients because of its relatively low cost and high safety profile. 10 out of 13 studies included in this systematic review mentioned tolerability of metformin treatment. These studies consistently showed that metformin didn't change body weight and serum glucose level of animals. However, it should also be taken into consideration of further investigation on the appropriate therapeutic dosage rang of metformin for anti-cancer treatment. Evaluation of safety with these particular dosages is very necessary, especially when metformin is applied in non-diabetic patients.

Limitations

By using the risk of bias tool, we found out that reporting is poor and therefore the methodological quality of many studies is unclear. It shows that there is much room for improvement, since many items were shown "unclear" and only a few items were shown low risk only in very few studies. Besides, by visualizing the funnel plot, publication bias seems also to be present, probably due to the missing of studies with no or negative effects. Actually, in total, only 12 studies were included in the meta-analysis. Both the unclear methodological quality and publication bias might lead to under or overestimation of the overall effect size of metformin effect on anti-HCC, which is an additional threat to the robustness of the data especially the ones that are already inconclusive. What's more, high heterogeneity was also seen among the studies, which is common in animal studies, although we tried to explore the potential factors contributing the heterogeneity. All of these potential limitations might influence us to draw concrete conclusions on the anti-HCC effect of metformin.

Besides, there were some methodological issues which might influence the translation of animal results to human trials. Firstly, the literature is unclear about which animal method is most representative for patient HCC. We found various HCC methods used in these studies: xenograft, DEN-induction, transgenic and dietary models. Secondly, there are two different administration routes (oral or i.p.) of

metformin in the studies. However, metformin is usually an orally administrated drug in clinic, raising the question whether administration method could also affect the effect of metformin on tumor.

HCC invasion and metastasis are crucial factors related to poor prognosis. However, none of the animal studies described if metformin had potential effect on HCC metastasis, except one study indirectly mentioned correlation of metformin/p-AMPK with distant metastasis in human HCC cohort study [19].

Another limitation is that these animal studies did not study the HCC stage indicated for metformin. HCC stage could be an important factor for the therapeutic efficacy of metformin and has implication for selecting appropriate candidates for metformin treatment. Therefore, it's very necessary to take the stage of HCC into account in the experimental design. As for xenograft animal model, which were used in most of the studies, however, it's hard to define the cancer stage which didn't discuss in the studies. In addition, although it's possible to identify tumor stage in spontaneous animal tumor model, the authors did not report any information about HCC stage in their animal study[12,17].

Implications for practice

Based on the results of this meta-analysis, metformin could potentially have a therapeutic effect on HCC. Besides, the maximal dose of metformin used in all the included animal studies were consistent with human therapeutic dose in diabetics according to the calculation [10]. This furthermore supported the reliable and applicable results from animal models. Although several clinical studies reported that metformin could negatively modify the risk of HCC in diabetic patients [4-6], there are not yet any clinical trial investigating the therapeutic effect of metformin on HCC. Currently, several ongoing clinical trials (www.clinicaltrials.gov) are evaluating the effects of metformin on different cancers (breast cancer, colorectal cancer, pancreatic cancer, etc.), But not for HCC. Thus, the results of this systematic review provide an important reference for the future preclinical animal trials with high quality to aid the development of metformin for anti-HCC treatment in clinical trials. Considering the dominant risk factor for HCC in many western countries in particular United States--obesity and metabolic syndrome, metformin would be an appropriate choice for such a high-risk of population of HCC.

CONCLUSION

In summary, this systematic review of animal studies suggests that metformin potentially has a direct inhibitory effect on HCC growth, although the effects on tumor number and incidence are inconclusive. It supports the clinical observation that metformin is associated with lower risk of HCC in diabetic patients. Although these animal studies have some intrinsic limitations, these results do provide an important reference for future high-quality preclinical animal trials and potential clinical development.

Methods

Review protocol

A protocol for this systematic review was prepared using SYRCLE's protocol format (https://www.radboudumc.nl/Research/Organisationofresearch/Departments/cdl/SYRCLE/Pages/Protocols.aspx)[7].

Literature search

A systematic search (conducted on July 2014, without any restrictions on publication data or language) was conducted in Medline (OvidSP), Embase.com, Web of Science, and Scopus. Additional referenes were retrieved from Google Scholar, and unindexed references from PubMed. The searches were designed and executed by an experience information specialist (WB). The search strategy consisted of two main components: hepatocellular carcinoma, and metformin, and results were limited to animal studies. For each element multiple synonyms were searched in title and/or abstract, and when available thesaurus terms (Mesh for medline and Emtree for embase). The full strategy is available in supplementary S1 Table.

Study selection and inclusion criteria

The selection procedure was performed by two independent reviewers (J.L. and P.H.). The exclusion criteria for the title and abstract screening phase include: 1). not primary study; 2). not animal study; 3). not disease of interest (HCC), 4). not intervention of interest (metformin). The following additional criteria were used for full-text screening: 1). full-text not available; 2). double publication; 3). conference abstracts. In case of disagreement between the reviewers, consensus was reached.

Study characteristics and data extraction

Data was extracted from the full-text papers of the studies. The following items were extracted: author, year, language, species/strain, description of control group, animal gender, age and weight, number of animals in control and experimental group, the method for the establishment of the animal models, metformin dosage, timing, duration and route of metformin administration, and outcome measures (Table 1).

The outcome measures including HCC growth, number and incidence were included in the meta-analysis. Mean value, standard deviation (SD) and the number of animals per group were extracted. If relevant data were not available in the text but only presented in graphic form, obtaining the data by measuring the graphs using Universal Desktop Ruler (Universal On-screen Digitizer, AVPSoft.).

Quality assessment of included studies

The SYRCLE's Risk of Bias tool was used to assess the risk of bias of all included studies [8]. Two independent investigators (J.L.&P.H.) performed quality assessment of all included studies. Disagreements were resolved by discussion.

Data synthesis and statistical analysis

For the outcome measures of HCC growth and number, the standardized mean difference (SMD) was used as the effect measure. For the outcome measure of HCC incidence, Risk Ratio (RR) was used. If studies contained multiple independent groups (e.g. different animal models or different time points), they were treated as separate experiments. Because of expected heterogeneity, the statistical model of analysis used in this meta-analysis was a random effects model. I^2 was used as a measure of heterogeneity. In order to explore potential causes of heterogeneity, predefined subgroup analyses were conducted for tumor volume, weight and size. With assistance of RevMan5.3 (Cochrane Library) software, Forest Plots were established. In addition, sensitivity analyses were conducted as to evaluate whether the findings were robust enough to the decisions made. Visual inspection of funnel plots was used to detect publication bias. Our procedures accorded with PRISMA guidelines for reporting systematic review/meta-analysis(supplementary S2 Table).

Supplementary Data

Table S1 Search Strategy

embase.com	**133**
Component 1: hepatocellular carcinoma	('liver cell carcinoma'/de OR 'hepatocellular carcinoma cell line'/exp OR 'hepatoma cell'/de OR (((liver OR hepat*) NEAR/6 (carcino*)) OR hepatoma* OR (hepatocell* NEAR/3 cancer*) OR hepatocarcinom* OR hepatoma OR 'AH 109a' OR AH109a OR 'AH 130' OR AH130 OR 'AH 272' OR AH272 OR 'AH 66' OR AH66 OR HepG2 OR 'Hep G2' OR hcc):ab,ti)
Component 2: metformin	AND (metformin/de OR (metformin* OR metphormin* OR methformin* OR metaformin* OR dimethylbiguanide OR 'dimethyl biguanide' OR dimethyldiguanide OR dimethylguanylguanidine OR apophage OR aron OR benofomin OR dabex OR denkaform OR deson OR dextin OR diabetase OR diabetformin OR diabetmin OR diabetosan OR diabex OR diafat OR diaformin OR diaformina OR diametin OR diamin OR diformin OR dimefor OR dimethylbiguanide OR dimethyldiguanide OR dmgg OR dybis OR eraphage OR espa-formin OR 'euform retard' OR fluamine OR flumamine OR fornidd OR fortamet OR glafornil OR glibudon OR glifage OR gliguanid OR glucaminol OR glucofage OR glucofago OR glucoform OR glucoformin OR glucohexal OR glucoless OR glucomet OR glucomin OR glucomine OR gluconil OR glucophage OR glucotika OR gludepatic OR glufor OR gluformin OR glumeformin OR glumet OR glumetza OR glupa OR glustress OR glyciphage OR glycomet OR glycon OR glycoran OR glyformin OR glymet OR haurymellin OR hipoglucin OR i-max OR islotin OR juformin OR 'la 6023' OR la6023 OR maformin OR meglucon OR meguan OR melbin OR melformin OR mellittin OR mescorit OR metaformin OR metfogamma OR metforal OR metformin* OR methformin OR metiguanide OR metomin OR metphormin OR miformin OR neoform OR nndg OR reglus-500 OR riomet OR siamformet OR siofor OR thiabet OR vimetrol OR walaphage):ab,ti)
Component 3: animal	NOT ([humans]/lim NOT [animals]/lim)
Medline (OvidSP)	**82**
Component 1: hepatocellular carcinoma	("Carcinoma, Hepatocellular"/ OR (((liver OR hepat*) ADJ6 (carcino*)) OR hepatoma* OR (hepatocell* ADJ3 cancer*) OR hepatocarcinom* OR "AH 109a" OR AH109a OR "AH 130" OR AH130 OR "AH 272" OR AH272 OR "AH 66" OR AH66 OR HepG2 OR "Hep G2" OR hcc).ab,ti.)
Component 2: metformin	AND (metformin/ OR (metformin* OR metphormin* OR methformin* OR metaformin* OR dimethylbiguanide OR "dimethyl biguanide" OR dimethyldiguanide OR dimethylguanylguanidine OR apophage OR aron OR

	benofomin OR dabex OR denkaform OR deson OR dextin OR diabetase OR diabetformin OR diabetmin OR diabetosan OR diabex OR diafat OR diaformin OR diaformina OR diametin OR diamin OR diformin OR dimefor OR dimethylbiguanide OR dimethyldiguanide OR dmgg OR dybis OR eraphage OR espa-formin OR "euform retard" OR fluamine OR flumamine OR fornidd OR fortamet OR glafornil OR glibudon OR glifage OR gliguanid OR glucaminol OR glucofage OR glucofago OR glucoform OR glucoformin OR glucohexal OR glucoless OR glucomet OR glucomin OR glucomine OR gluconil OR glucophage OR glucotika OR gludepatic OR glufor OR gluformin OR glumeformin OR glumet OR glumetza OR glupa OR glustress OR glyciphage OR glycomet OR glycon OR glycoran OR glyformin OR glymet OR haurymellin OR hipoglucin OR i-max OR islotin OR juformin OR "la 6023" OR la6023 OR maformin OR meglucon OR meguan OR melbin OR melformin OR mellittin OR mescorit OR metaformin OR metfogamma OR metforal OR metformin* OR methformin OR metiguanide OR metomin OR metphormin OR miformin OR neoform OR nndg OR reglus-500 OR riomet OR siamformet OR siofor OR thiabet OR vimetrol OR walaphage).ab,ti.)
Component 3: animal	NOT (humans/ NOT animals/)
Web-of-science	**100**
Component 1: hepatocellular carcinoma	TS=(((((liver OR hepat*) NEAR/6 (carcino*)) OR hepatoma* OR (hepatocell* NEAR/3 cancer*) OR hepatocarcinom* OR hepatoma OR "AH 109a" OR AH109a OR "AH 130" OR AH130 OR "AH 272" OR AH272 OR "AH 66" OR AH66 OR HepG2 OR "Hep G2" OR hcc))
Component 2: metformin	AND ((metformin* OR metphormin* OR methformin* OR metaformin* OR dimethylbiguanide OR "dimethyl biguanide" OR dimethyldiguanide OR dimethylguanylguanidine OR apophage OR aron OR benofomin OR dabex OR denkaform OR deson OR dextin OR diabetase OR diabetformin OR diabetmin OR diabetosan OR diabex OR diafat OR diaformin OR diaformina OR diametin OR diamin OR diformin OR dimefor OR dimethylbiguanide OR dimethyldiguanide OR dmgg OR dybis OR eraphage OR espa-formin OR "euform retard" OR fluamine OR flumamine OR fornidd OR fortamet OR glafornil OR glibudon OR glifage OR gliguanid OR glucaminol OR glucofage OR glucofago OR glucoform OR glucoformin OR glucohexal OR glucoless OR glucomet OR glucomin OR glucomine OR gluconil OR glucophage OR glucotika OR gludepatic OR glufor OR gluformin OR glumeformin OR glumet OR glumetza OR glupa OR glustress OR glyciphage OR glycomet OR glycon OR glycoran OR glyformin OR glymet OR haurymellin OR hipoglucin OR i-max OR islotin OR juformin OR "la 6023" OR la6023 OR maformin OR meglucon OR meguan OR melbin OR melformin OR mellittin OR mescorit OR

	metaformin OR metfogamma OR metforal OR metformin* OR methformin OR metiguanide OR metomin OR metphormin OR miformin OR neoform OR nndg OR reglus-500 OR riomet OR siamformet OR siofor OR thiabet OR vimetrol OR walaphage))
Component 3: animal	AND (mice OR mouse OR rat OR rats))
Scopus	**106**
Component 1: hepatocellular carcinoma	TITLE-ABS-KEY(((((liver OR hepat*) W/6 (carcino*)) OR hepatoma* OR (hepatocell* W/3 cancer*) OR hepatocarcinom* OR hepatoma OR "AH 109a" OR AH109a OR "AH 130" OR AH130 OR "AH 272" OR AH272 OR "AH 66" OR AH66 OR HepG2 OR "Hep G2" OR hcc))
Component 2: metformin	AND ((metformin* OR metphormin* OR methformin* OR metaformin* OR dimethylbiguanide OR "dimethyl biguanide" OR dimethyldiguanide OR dimethylguanylguanidine OR apophage OR aron OR benofomin OR dabex OR denkaform OR deson OR dextin OR diabetase OR diabetformin OR diabetmin OR diabetosan OR diabex OR diafat OR diaformin OR diaformina OR diametin OR diamin OR diformin OR dimefor OR dimethylbiguanide OR dimethyldiguanide OR dmgg OR dybis OR eraphage OR espa-formin OR "euform retard" OR fluamine OR flumamine OR fornidd OR fortamet OR glafornil OR glibudon OR glifage OR gliguanid OR glucaminol OR glucofage OR glucofago OR glucoform OR glucoformin OR glucohexal OR glucoless OR glucomet OR glucomin OR glucomine OR gluconil OR glucophage OR glucotika OR gludepatic OR glufor OR gluformin OR glumeformin OR glumet OR glumetza OR glupa OR glustress OR glyciphage OR glycomet OR glycon OR glycoran OR glyformin OR glymet OR haurymellin OR hipoglucin OR i-max OR islotin OR juformin OR "la 6023" OR la6023 OR maformin OR meglucon OR meguan OR melbin OR melformin OR mellittin OR mescorit OR metaformin OR metfogamma OR metforal OR metformin* OR methformin OR metiguanide OR metomin OR metphormin OR miformin OR neoform OR nndg OR reglus-500 OR riomet OR siamformet OR siofor OR thiabet OR vimetrol OR walaphage))
Component 3: animal	AND (mice OR mouse OR rat OR rats OR animal*))
PubMed Publisher	**2**
Component 1: hepatocellular carcinoma	((((liver OR hepat*[tiab]) AND (carcino*[tiab])) OR hepatoma*[tiab] OR (hepatocell*[tiab] AND cancer*[tiab]) OR hepatocarcinom*[tiab] OR AH 109a OR AH109a OR AH 130 OR AH130 OR AH 272 OR AH272 OR AH 66 OR AH66 OR HepG2 OR Hep G2 OR hcc))
Component 2: metformin	AND ((metformin*[tiab] OR metphormin*[tiab] OR methformin*[tiab] OR metaformin*[tiab] OR dimethylbiguanide OR dimethyl biguanide OR

	dimethyldiguanide OR dimethylguanylguanidine OR apophage OR aron OR benofomin OR dabex OR denkaform OR deson OR dextin OR diabetase OR diabetformin OR diabetmin OR diabetosan OR diabex OR diafat OR diaformin OR diaformina OR diametin OR diamin OR diformin OR dimefor OR dimethylbiguanide OR dimethyldiguanide OR dmgg OR dybis OR eraphage OR espa-formin OR euform retard OR fluamine OR flumamine OR fornidd OR fortamet OR glafornil OR glibudon OR glifage OR gliguanid OR glucaminol OR glucofage OR glucofago OR glucoform OR glucoformin OR glucohexal OR glucoless OR glucomet OR glucomin OR glucomine OR gluconil OR glucophage OR glucotika OR gludepatic OR glufor OR gluformin OR glumeformin OR glumet OR glumetza OR glupa OR glustress OR glyciphage OR glycomet OR glycon OR glycoran OR glyformin OR glymet OR haurymellin OR hipoglucin OR i-max OR islotin OR juformin OR la 6023 OR la6023 OR maformin OR meglucon OR meguan OR melbin OR melformin OR mellittin OR mescorit OR metaformin OR metfogamma OR metforal OR metformin*[tiab] OR methformin OR metiguanide OR metomin OR metphormin OR miformin OR neoform OR nndg OR reglus-500 OR riomet OR siamformet OR siofor OR thiabet OR vimetrol OR walaphage))
Component 3: animal	AND (mice OR mouse OR rat OR rats OR animal*) AND publisher[sb]
Google Scholar	**150**
Component 1: hepatocellular carcinoma	"liver cell carcinoma"|"hepatocellular carcinoma|cancer"|hepatoma|hepatocarcinoma
Component 2: metformin	Metformin
Component 3: animal	mice|mouse|rat|rats|animal|animals

Table S2 PRISMA Checklist for the Systematic Review and Meta-analysis to Estimate the anti-HCC effect of metformin in animal studies

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it	S1 table

		could be repeated.	
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5-6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6-7
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6-7
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	7
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8+fig1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table1

Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Fig2
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Fig3-5
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	9-10
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	11
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	9-11
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12,13, 15
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	14
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	16
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	17

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Chapter 7 General discussion, summary and conclusion

The liver is an organ of vital importance as it supports almost every other organ in the human body. Because of its strategic location with respect to the intestine and its plethora of functions, the liver is also prone to many diseases. Especially viral hepatitis is a common condition of inflammation of the liver. Among both the most usual causes of viral infection and also clinically potentially most serious are the hepatitis B and C viruses. Some of these infections are sexually transmitted or by dirty needles among intravenous drug users and thus co-infection with HIV is depressingly common. Infection with hepatitis B virus or hepatitis C virus is the main cause of liver cancer. At present reliable assessment of liver condition in chronic viral hepatitis is problematic and relies histological assessment of invasive liver biopsies. This situation prompts investigation into more convenient biomarkers. This consideration prompted me to investigate caspase-cleaved cytokeratin 18 (CK18-Asp396) as a serum marker for disease activity. I considered this a potential clinically useful biomarker in liver disease as it is released from hepatocytes during apoptosis. In **Chapter 2**, I investigate serum CK18-Asp396 levels in a well characterized Dutch cohort consisting of patients with chronic hepatitis B infection across all grades of steatosis, inflammation and fibrosis. We exploited this notion to evaluate hepatocyte apoptosis in CHB. The study demonstrated that CHB is indeed associated with hepatocyte apoptosis, showing strong correlations with significant inflammation and fibrosis, but not with steatosis, and especially demonstrating a diagnostic value of CK-18 serum levels for identification of significant inflammation of the liver in CHB patients with a high specificity. The availability of such a biomarker should substantially aid future studies in disease course modifying parameters.

As said, chronic viral infection of the liver is often associated with HIV infection. Hence understanding response of therapy to anti-HIV drugs is also relevant in the context of the present dissertation. The factors associated of overall survival in patients with HIV that receive ART initiation was analyzed in **Chapter 3**. Consistent with previous studies, a male sex, increasing age, advanced WHO clinical stage were demonstrated to be related to worse overall survival. But interestingly, our result revealed the time-dependent effect of CD4 cell counts on the long-term outcome of patients. We found that, although the baseline CD4 cell count is

positively associated with overall survival in the first six months of ART initiation, it becomes weak after six months. These results reveal the importance of the time domain when analyzing this type of epidemiological data. Furthermore, coinfection with hepatitis virus was also analyzed for its prevalence and impact on clinical outcome of HIV infected patients. Our data suggest no significant relation between coinfection with hepatitis virus and patients' outcome. As extensively discussed in **Chapter 4**, data on coinfection affecting HIV patient survival remains under debate. The surprising nature of our results could be possibly related to several factors, such as patient population, follow up time, and adjusted confounders, etc.. I feel, however, that it shows the importance of performing this type of study, as it seems that we often take a too simplistic look at our data. This notion is also highlighted by the results in the remainder of my thesis, in which I look at liver cancer, a feared complication of chronic viral hepatitis.

Liver cancer, known also as hepatic cancer or primary hepatic cancer, is cancer that starts in the liver, in contrast to cancer that has spread from elsewhere to the liver, which we know as liver metastasis. Symptoms of liver cancer may include a lump or pain in the right side below the rib cage, swelling of the abdomen, yellowish skin, easy bruising, weight loss, and weakness. The leading cause of liver cancer is cirrhosis due to hepatitis B, hepatitis C, or alcohol. Other causes include aflatoxin and non-alcoholic fatty liver disease. The most common types are hepatocellular carcinoma (HCC), which makes up 80% of cases. Following the established poor survival of HCC among African Americans, many studies were carried out to provide evidence on this survival disparity.[1-3] Although several factors have been identified as contributors to such disparity including advanced tumor presentation at time of diagnosis, restricted access to liver transplantation, and lower SES, none of previous studies have analyzed the actual impact of each contributor to patients' survival. Therefore, based on SEER database, we aimed to identify contributors to the racial disparity in overall survival through a strategic modeling analysis. Our data suggest that tumor presentation at diagnosis, limited benefit from resection and restricted utilization of liver transplantation are important contributors to poorer survival of African Americans with hepatocellular carcinoma (**Chapter 5**). It would be interesting to investigate the same question in European cohorts. The Erasmus MC being an important referral center for this disease would be a good place for such studies. Health care is equally accessible to the whole population in the Netherlands

and thus it would be interesting to study the effect of socioeconomic status in the Dutch context. Both patient-related and physician-related factors may still influence treatment choices and thus results.

Indeed, treatment is important in predicting outcome for hepatocellular carcinoma. In very early and early stage HCC, surgical therapy such as resection and liver transplantation constitute curative treatment. In advanced stage HCC, systematic chemotherapy with sorafenib is the only one FDA-approved first line treatment available.[4] However, this therapy can only increase overall median survival with 2-3 months.[5] Therefore, new strategies to improve the management of HCC are very necessary. Metformin, an oral drug widely used for treating type II diabetes, were reported to be associated with low risk of HCC. However, there is a lack of investigations assessing whether metformin has direct anti-tumor effects in HCC patients. Giving substantial research having been performed in animal models of HCC, I performed a systematic review and meta-analysis on those data from previous animal studies to better understand the direct effects of metformin on HCC, the results have been shown in **Chapter 6**. This study suggests that metformin can potentially have a direct inhibitory effect on HCC growth. Although these animal studies have some intrinsic limitations, our results do provide an important reference for future high-quality preclinical animal trials and potential clinical development. Moreover, considering the dominant risk factor for HCC in many western countries in particular United States--obesity and metabolic syndrome, metformin would be an appropriate choice for such a high-risk of population of HCC.

In conjunction, I feel my studies add to the framework necessary for improved analysis of liver disease, for instance by identifying novel biomarkers (**chapter 2**), but also by showing the use of meta-analysis as a strategy towards understanding of preclinical studies of liver disease (**chapter 6**) or by identifying the importance of the time domain in analyzing epidemiological results on the natural history of disease (**chapter 3** and **chapter 5**). Simultaneously, I have employed these concepts to obtain insight in factors in contributing or not contributing to alternative disease course (chapter 4 or chapter 6, for instance). Thus I hope with this work to have added to the efforts to come to better management of liver disease).

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Nederlandse samenvatting voor niet ingewijden

De lever is een belangrijk en veelzijdig orgaan bij de mens. Ze speelt een belangrijke rol in het metabolisme en vrijwel alle andere organen zijn op één of andere wijze wel afhankelijk van de lever. In het menselijke lichaam is de lever beveiligd door de onderste ribben van de borstkas, rechtsboven in de buikholte. Met een gewicht van anderhalve kilogram is het na de huid het zwaarste orgaan en wellicht ook het veelzijdigste orgaan**.** Ziekte aan de lever heeft dan ook ernstige consequenties voor de betrokken patiënten. In **hoofdstuk één** van dit proefschrift zet ik uiteen welke vragen ik denk dat beantwoord moeten worden om dit veld verder te brengen

In dit proefschrift concentreer ik me dan in eerste instantie mij op leverontsteking of hepatitis Het woord hepatitis komt uit het Grieks en Latijn en bestaat uit twee delen: 'ἡπαρ' (hèpar) en '-itis'. Hepar betekent lever en het achtervoegsel -itis betekent ontsteking. Hepatitis betekent dus een ontsteking van de lever. Dit kan verschillende oorzaken hebben, maar met name virale hepatitis kan verstrekkende gevolgen hebben.Er worden nog steeds nieuwe hepatitisvirussen ontdekt die een ontsteking van de lever kunnen geven maar in het kader van dit proefschrift zijn met name hepatitis B virus en hepatitis C virus belangrijk. Chronische hepatitis B kan na een infectie met het hepatitis B-virus ontstaan als het lichaam niet in staat is het virus te klaren. Een chronische infectie met het hepatitis B-virus kan zowel asymptomatisch zijn (in het merendeel van de gevallen) als gekoppeld zijn aan een chronische ontsteking van de lever (chronische hepatitis). Ook een hepatitis C-infectie verloopt in het begin meestal asymptomatisch. De infectie gaat in de meerderheid van de gevallen (70%) ongemerkt over in een chronische vorm.De chronische ontsteking kan na meerdere jaren leiden tot cirrose en leverkanker. Het is belangrijk de reactie van lever op een virale hepatitis goed te kunnen volgen. Nu gebeurt dat vaak door leverbiopten te laten beoordelen onder de microscoop door de patholoog. Leverbiopten nemen is echter invasief, pijnlijk en niet zonder risico. Er is dus grote behoefte aan een betere methode. In **hoofdstuk twee** van dit proefschrift exploreer ik cytokeratine 18 fragmenten als een nieuwe methode. Cytokeratine 18 is een taai, onoplosbaar eiwit dat voorkomt in de lever. Als levercellen doodgaan door geprogrammeerde celdood, komen er echter oplosbare fragmenten in de bloedsomloop terecht. Omdat virale infectie gepaard gaat met geprogrammeerde celdood in de lever redeneerde ik dat cytokeratine 18 fragment in de bloedsomloop een mooie maat zouden zijn voor virale infectie van de lever. In dit proefschrift laat ik zien dat deze redenering klopt.

Intraveneus drugsgebruik en seksueel contact zijn belangrijke bronnen van transmissie van hepatitis virussen, maar dezelfde risicofactoren zijn ook gerelateerd aan HIV infectie. Er is dan ook vaak co-infectie van hepatitis virus en HIV. Gelukkig kan HIV vaak behandeld worden met antivirale therapie. In **hoofdstuk drie** laat ik zien dat de grootte van een populatie van witte bloedcellen, de CD4 T cellen een

goede voorspeller vormt van het succes van antivirale therapie, maar alleen vroeg in het natuurlijk verloop van de ziekte. Het is belangrijk het tijdsdomein mee te nemen in analyse van factoren die gerelateerd zijn aan het verloop van ziekte. Ook verrassend was de uitkomst van de analyse in **hoofdstuk vier**, hier laat ik zien dat co-infectie niet van invloed is op therapiesucces in patiënten geïnfecteerd met zowel hepatis C als HIV. Mijn resultaten laten dus zien dat interpretatie van epidemiologische data met grootste zorgvuldigheid dient te gebeuren.

Gewapend met deze kennis stortte ik mij vervolgens op leverkanker. Hiermee bedoelt men een primaire kwaadaardige tumor die in de lever is ontstaan. Primaire leverkanker komt slechts zelden voor in Nederland, maar elders maken de frequente besmettingen met hepatitis (vooral hepatitis B), en het gebruik van voedsel dat is bedorven door de schimmel Aspergillus flavus, die aflatoxine produceert dit een groot probleem. In mijn onderzoek, beschreven in **hoofdstuk vijf**, maakte ik gebruik van een Amerikaanse database en analyseer het effect van ras op de uitkomst van leverkanker. Het blijkt dat vooral mensen van Afrikaanse afkomst het slecht doen en ik probeer de oorzaken te achterhalen. In **hoofdstuk zeven** speculeer ik over de noodzaak om een dergelijke studie ook in Nederland uit te voeren. De belangrijkste reden voor de relatieve overstrefte in patiënten van Afrikaanse afkomst lijkt te liggen in verschillen in de behandeling. Dit brengt mij op het punt van dat het noodzakelijk is om nieuwe behandelingen te vinden. Zulk onderzoek vindt vaak eerst plaats in pre-klinische modellen. Interpretatie van de resultaten van zulk pre-klinisch onderzoek is vaak lastig. In **hoofdstuk zes** laat ik een nieuwe aanpak zien hoe de literatuur te onderzoeken. Het succes van deze aanpak blijkt uit mijn bevinding dat metformine effectief uit zulke modellen. Verdere interpretatie en integratie van bevindingen alsmede speculatie over toekomstig onderzoek kan men vinden in **hoofdstuk zeven**. Samen hoop ik, dat ik met deze dissertatie een bijdrage heb kunnen leveren aan toekomstig onderzoek gericht op de betere behandeling van leverziekte,

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I also want to extend my thanks to all nice colleagues who ever helped me without any hesitation, which really surprises me quite a bit. Being a boring and a little odd person, it is hard for someone to approach me. Therefore, everyone who ever talked to me with my study and my personal happy life even are highly credited for everything offered to me so generously.

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Curriculum Vitae

Juan Li was born in June 25, 1985, in China. From 2003 to 2008, she studied clinical medicine in Anhui University of Chinese Medicine. After that, she moved to Shanghai for her three-year master program in Internal Medicine in Tongji University until 2011. During her resident training in Shang Hai Chang Zheng Hospital, she realized that a clinician job might not be well suited for her. Compared to facing so many untreatable diseases, she dreamed of making breakthrough in clinical treatment for those disease. After a short period doing a medical assistant job in a company, she finally went to Holland for an interesting master program in Leiden University. She is lucky as China Scholarship Council (CSC) offered her a four-year scholarship to pursue a PhD study in Erasmus MC.

PhD Portfolio

Name PhD Student	Juan Li
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	October 2013 - November 2017
Promoter	Prof. Dr. Maikel P. Peppelenbosch
Copromoters	Dr. Dave Sprengers and Dr. Qiuwei Pan

Major Training

Courses in biostatistics

- Regression analysis, NIHES institute, Erasmus MC, Rotterdam (2016)
- Introduction to Bayesian Methods in Clinical Research, NIHES institute, Erasmus MC, Rotterdam (2015)
- Topics in Meta-analysis, NIHES institute, Erasmus MC, Rotterdam (2015)
- Hands-on training in Synthesis of evidence in animal experimentation, Nijmegen, Radboud University Medical Center (2014)

Courses in computer programming

- The course on Programming with Python, Molecular Medicine Postgraduate School, Erasmus MC, Rotterdam (2015)
- Course on R, Molecular Medicine Postgraduate School, Erasmus MC, Rotterdam (2015)

Attended national and international conferences

- Annual Day of the Molecular Medicine Postgraduated School, Rotterdam, the Netherlands (2014, 2015)

- European Association for the Study of the Liver (EASL), poster presentation (2016)

Reviewing for scientific journals_____

- Two papers for *Scientific Reports*
- One paper for *Plos One*
- One paper for *Cancer Causes & Control*

Academic awards_____

- Travel award from Erasmus TRUSTFONDS (2016)
- China Scholarship Council (CSC) for funding PhD fellowship (No. 201307720054)

Publications

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[#]: These authors contributed equally as first author.

*: These authors contributed equally as corresponding authors.