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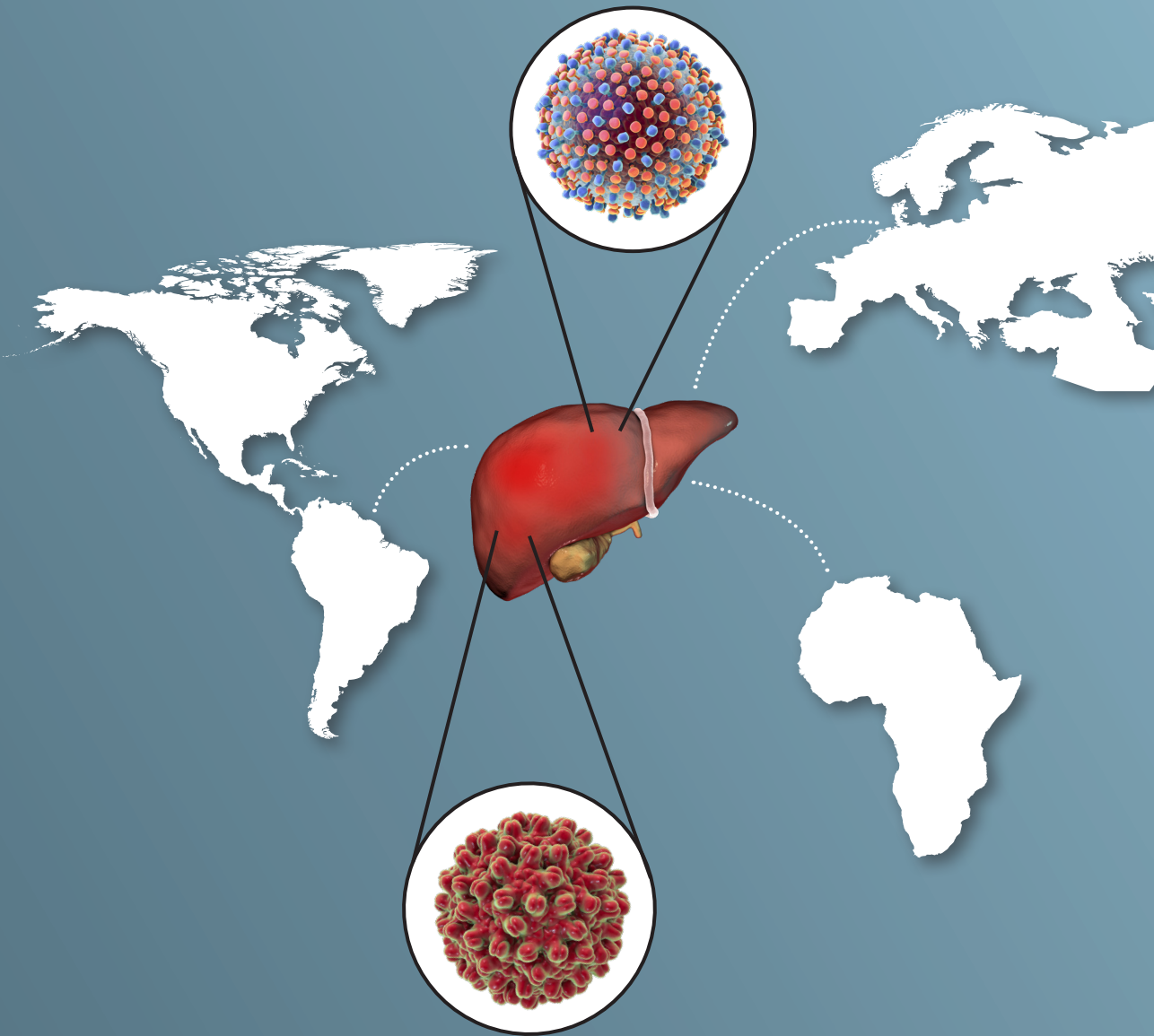
The interconnection of epidemiology, immunopathogenesis and treatment

Jose Daniel Debes

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Hepatocellulair Carcinoom:
De verbinding tussen epidemiologie,
immuunpathogenese en behandeling.

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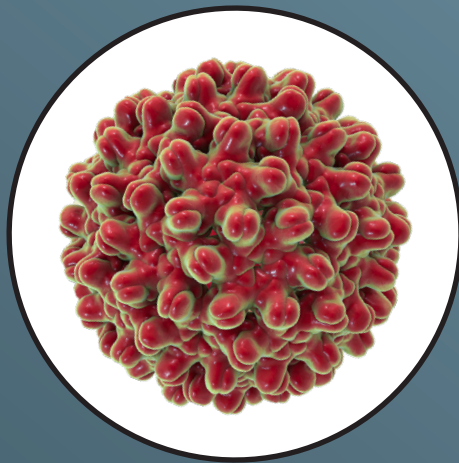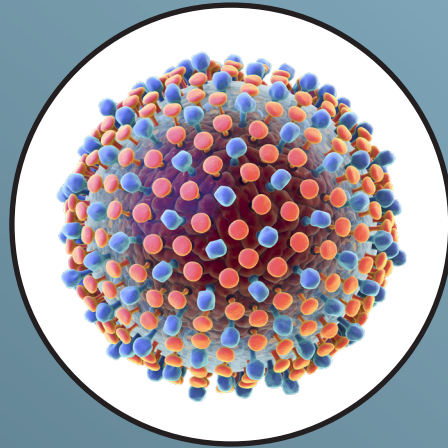
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Introduction

CHAPTER 1A

General introduction

Hepatocellular carcinoma (HCC), also known as liver cancer, is the most common primary malignancy of the liver and second most common cause of cancer-related death worldwide. HCC occurs mainly in individuals with chronic liver disease. Therefore, the epidemiology related to causes of liver disease in a particular region, or area, will have a significant impact in the development, progression and treatment of HCC [1]. In this regard, the risk and prognosis of liver cancer will be different for an individual with hepatitis B in Africa than for an individual with non-alcoholic fatty liver disease in Europe, and this difference will stand beyond that of the richness or resources in the region.

HCC does present a particular aspect unique to its formation, as it does rarely develop in the setting of a normal liver physiology. The great majority of HCCs (over 95%) will develop in the setting of liver disease, with 90% of these tumors developing in the setting of liver cirrhosis [2]. This represents a distinctive characteristic of the tumor as the presence of certain underlying liver disease will dictate the frequency, age of diagnosis and even prognosis of HCC. In this regard, it is thought that a continuous injury to the liver, caused by culprits such as viral hepatitis B or C, or alcohol use disorder will lead to a state of inflammation which will eventually lead to different stages of fibrosis, culminating in liver cirrhosis, the ultimate advanced state of liver fibrosis. It is the milieu of cirrhosis and the prolonged transition (over years) to this stage that produces a series of changes involving genetic modifications, telomerase alterations and chromosomal instability that eventually leads to the formation of a liver nodule and HCC [3]. Epidemiologically speaking, the interconnection between underlying liver disease and formation of HCC is important as the frequency of certain liver diseases (i.e. hepatitis B) will dictate the frequency, and to an extent, prognosis of HCC in a region. **Section #2** of this thesis explores new aspects on HCC epidemiology related to South America and provides new insights on age of occurrence of HCC based on regional patterns of underlying liver disease [4]. Moreover, it exposes new associations between Hepatitis B and HCC in the region, and evaluates clinical characteristics of HCC [5].

> *Section 2: a) Focuses on the epidemiology of HCC in South America; b) Describes for the first time early occurrence of HCC in individuals infected with hepatitis B in the region*

Among all the causes of underling liver disease that leads to HCC, the most common globally is chronic viral hepatitis B (HBV) infection [6, 7]. HBV not only leads to the higher number of HCC cases, but also affects resource-limited settings disproportionately compared to resource-rich ones [6]. This, combined with the silent progress of the disease makes hepatitis B infection one of the most lethal causes of HCC worldwide. As I will show in **Section #3** of this thesis, HBV infection represents a challenge but also an opportunity to provide preventive measures against HCC. As HBV infection is preventable with vaccination, increasing awareness and implementation of vaccination worldwide can lead to an eventual decrease of HCC cases related to HBV [8, 9]. As I will also show that HBV prevention via increase in awareness and testing and vaccinating in novel settings will be critical to achieve this [10-12].

> *Section #3: a) Aims to validate of rapid tests for hepatitis B detection in different continents and resource settings; b) Assesses implementation of these tests to screen for hepatitis B in individuals that present to Emergency departments in Argentina in a novel approach; c) Evaluates new tactics for effective education of healthcare workers regarding hepatitis B and the public in general with the objective of increasing awareness all of ultimately can lead to reduction in hepatitis B-related HCC.*

Many questions however remain on the initial changes that leads to a carcinogenesis background before cirrhosis occurs. It is thus unclear if epigenetic or genetic changes before cirrhosis lead to a higher risk for HCC in certain individuals. **Section #4** of this thesis focuses on new discoveries on single cell sequencing of hepatitis C infected livers that suggest an early induction of oncogenes and decrease in tumor suppressors in individuals with no cirrhosis. These findings will propose a concept in which early infection with viral hepatitis can induce genetic changes that ultimately lead to HCC formation. This section also assess how early changes in immune signaling from HCC formation can be used to detect early HCC before is clinically evident [13]. This is a critical issue as currently there is a lack of easy-to-measure and reliable biomarkers for HCC.

> *Section #4: a) Uses single cell sequencing of hepatocytes from hepatitis C-infected individuals to understand early changes induced by the virus that can lead to hepatocarcinogenesis; b) Evaluates the measurement of immune markers in blood to predict the development of HCC before it is clinically evident in individuals infected with hepatitis C.*

Interestingly, once the tumor is formed, the continuous presence of inflammatory signals such as in untreated viral hepatitis or fatty liver disease (both alcoholic and non-alcoholic) can lead to a more rapid extension of the tumor and further invasion of distant tissues or internal liver vessels. In this setting, local therapy is unlikely to improve outcomes, and only systemic therapy can provide an increase in survival time [14, 15]. Response to systemic therapy is, however, partially dependent on the triggering cause of HCC as well as on parameters of liver function, as I will show in **section #5** of this thesis.

Section #5: a) Describes the different treatments used for HCC in South America and in regions of Africa (Ethiopia) and evaluates survival under these therapies; b) It describes the comprehensive use of systemic therapy for HCC (sorafenib) in South America and in Africa delineating survival based on underlying liver diseases; c) It proposes a novel and simple score to predict response to sorafenib in HCC.

This doctoral thesis aims to:

A) Understand the epidemiological impact of liver diseases in the development of HCC in different geographical regions of the world.

B) Address genetic and immune patterns that interconnect viral hepatitis with the formation of HCC and how to evaluate these patterns for the early diagnosis of HCC

C) Define implementation of simple measures that can help prevent liver disease and therefore decrease the incidence of HCC in areas with limited-resources

D) Identify treatment options that best adapt to treat HCC, based on socio-economical and resource-related needs

In the quest to define a broad set of aspects related to HCC, this research work makes use of a diverse set of approaches. These include: multinational retrospective studies that establish the underlying epidemiology of viral hepatitis and HCC; cross-sectional translational studies addressing immune variables that predict HCC; translational studies involving single cell hepatocyte sequencing to further define the initial factors leading to HCC formation; prospective single center studies addressing better practices to improve vaccination that leads to prevention of HCC; cross-sectional and retrospective evaluation of HCC treatment outcomes in different settings; and modeling studies to identify scores that predict treatment response in HCC. In addition, this thesis incorporates a series of review articles and personal opinions that bring together the objective research findings with their applicability in the real world, as well as the needs and gaps that future research in the field should address.

In summary, this thesis will drive the reader to the multiple and complex aspects of HCC across the globe. It will focus on the changing epidemiology of liver disease and its implications on regional HCC incidence; basic new aspects that lead to HCC formation related to viral hepatitis and the use of immune-analytes to predict early HCC; the use of new modalities with region-specific components that can increase awareness and vaccination of hepatitis B with the ultimate goal of preventing HCC; and the complex aspects of HCC therapy interfacing response to therapy with underlying liver disease and resources of specific geographic regions.

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CHAPTER 1B

The path to cancer, and back: Immune modulation during hepatitis C virus infection, progression to fibrosis and cancer, and unexpected roles of new antivirals

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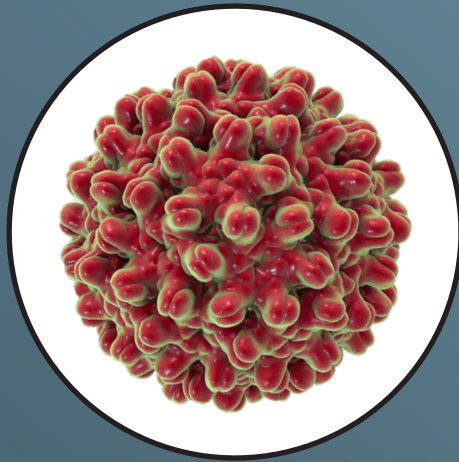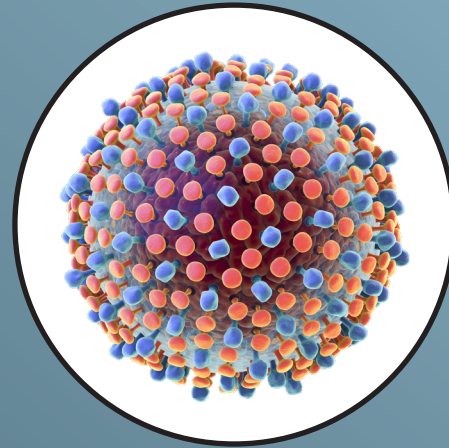
Abstract

Hepatitis C virus (HCV) infection affects over 130 million individuals worldwide, and it is the number one reason for liver transplantation in the United States. HCV infection progresses in a slow chronic fashion eliciting a strong but ineffective immune response, mainly characterized by NK cell dysfunction and T cell exhaustion. The chronic hepatic inflammation leads to liver fibrosis, cirrhosis and cancer in a significant number of patients. In recent years, groundbreaking research has led to the discovery of new HCV-specific direct acting antivirals (DAAs), which have an unprecedented efficacy to clear the virus, and establish a sustained virological response. Indeed, curing HCV infection with an oral medication is now reality. The effects of DAAs in mitigating the HCV-related complications of liver fibrosis and cancer are yet largely unknown. Nonetheless, recent controversial reports suggest a potential increase in liver cancer recurrence upon use of DAAs. In the current manuscript we review the most important immune-mediated mechanisms underlying HCV chronicity and the development of liver fibrosis and cancer. Furthermore, we discuss recent concern on use of the new agents.

Introduction

Viral hepatitis as a consequence of a chronic infection with the hepatitis C virus (HCV) is one of the leading causes of liver inflammation worldwide. There are an estimated 130-150 million individuals chronically infected with HCV [World Health Organization (WHO), Hepatitis C Fact Sheet, 2016]. Following acute HCV infection only 15-25% of adults infected spontaneously clear the virus without treatment and the majority progress to chronicity[5,6]. Prolonged infection with HCV results in a state of chronic liver inflammation, and consequently a significantly increased risk of developing liver cirrhosis and/or hepatocellular carcinoma. Unprecedented progress has been recently made with development of novel targeted therapies for HCV, making what was once a difficult to treat infection, into a curable disease. In this review we describe the role of the immune system in response to HCV infection, what we believe are the most important mechanisms involved in disease progression, as well as the changes exerted by new antiviral therapy.

The immune response during chronic HCV infection

The majority of individuals infected with HCV develop a chronic infection in which the virus persists in the liver with minimal immunopathology. HCV is a non-cytolytic virus and consequently the high rates of viral replication in the liver do not result in immediate apoptosis of infected hepatocytes. Importantly, the continuous production of 10^{12} virions per day by infected hepatocytes triggers certain antiviral immune responses, without resulting in substantial killing of hepatocytes, as evidenced by low levels of serum alanine transaminases (ALT) during the course of persistent infection [1,2]. Following acute infection with HCV and during the persistent phase of infection, antiviral cytokines including interferon (IFN)-α, IFN-β and IFN-λ, are induced upon recognition of virus components, such as HCV RNA, by intracellular pathogen recognition receptors in infected cells. Interferons are responsible for the up-regulation of multiple IFN-stimulated genes (ISG) with a wide range of antiviral effects as well as activation and recruitment of leukocytes, such as natural killer (NK) cells. These NK cells are a major component of the innate immune system that control viral infections by the release of the cytotoxic enzymes usually resulting in apoptosis and lysis of the infected cells. Activated NK cells can also release cytokines, such as IFN-γ, that result in triggering of antiviral responses leading to inhibition of viral replication in infected cells. Numerous studies have reported on interference mechanisms of HCV to evade IFN-mediated events, and impairment of the function of circulating and intrahepatic NK cells during persistent HCV infection [3]. Also, other components of the innate immune system, such as monocytes and the more recently described mucosal associated invariant T (MAIT) cells are functionally impaired in chronic HCV [4,5].

In patients that develop a persistent HCV infection, the initial phase of the immune response to the virus seems to develop normally. However, the HCV-specific CD4^{+} and CD8^{+} T cell responses are not sustained during the chronic phases of infection, and these cells become functionally impaired and difficult to detect in blood and liver. The functional impairment of the T cell compartment as observed in chronic HCV patients has also been described for other persistent viral infections with a high viral load, such as HIV and HBV. Detailed studies have demonstrated that the dysfunctional T cell responses in chronic HIV, HBV as well as HCV infections are caused by multiple mechanisms including viral escape mutations, inappropriate activation of T cells by dendritic cells, the activity of regulatory T cells, and exhaustion of the T cell compartment, which clearly illustrates that similar mechanisms are responsible for the initiation and maintenance of persistence in HIV, HBV and HCV infections [6,7]. During these persistent infections, as a consequence of the continuous exposure to high levels of viral antigens, a gradual loss of effector functions is described (CD4^{+} help, cytotoxicity) accompanied by loss of IL-2, IFN-γ and TNF production following antigenic stimulation [8]. This phenomenon is called T cell exhaustion, and virus-specific exhausted T cells have been shown to overexpress multiple inhibitory receptors on their surface, such as PD-1, Tim-3, CTLA-4 and CD244 [9]. The importance of these molecules in T cell exhaustion during chronic viral infection was shown by the observation that *in vitro* blockade of the inhibitory pathways restores virus-specific T cell function in cells from chronic HCV patients. Currently, numerous studies are ongoing to evaluate various therapeutic strategies involving neutralization of exhaustion markers to restore impaired T cell responses against viral infections as an attractive approach for complete viral eradication [10].

Besides T cell exhaustion, increased activity of the immunosuppressive cytokines TGF-β and IL-10 or by regulatory T cells impair HCV-specific T cell proliferation and IFN-γ production in blood. Importantly, substantial numbers of regulatory T cells are present in the liver, the site of HCV viral replication, which are likely to inhibit T cell proliferation and effector function, thereby suppressing HCV-specific T cell responses. These findings are observed in HCV-infected livers, but not in those of healthy individuals [6].

As a consequence of the inability of the patient's immune system to mount a strong and lasting T cell response during the chronic phase of HCV infection the virus persists and replicates in hepatocytes in a non-cytolytic manner, and immune cells control the infection, but do not cause an immediate but a rather slow-progressing immune-mediated damage to the infected liver. The prolonged coexistence of HCV with the host is reflected by the absence of very elevated transaminases or bilirubin levels and the slow progression of liver disease in chronically infected patients. However, because of the chronic nature of the HCV infection, the continuous low grade immune-mediated inflammation may lead to the development of liver fibrosis, which may further progress towards cirrhosis and hepatocellular carcinoma (HCC) [11,12].

HCV infection leading to liver fibrosis and cancer

HCV and liver fibrosis

Liver fibrosis is a dynamic process initiated by hepatic injury resulting in an abundance of deposition of extracellular matrix proteins in the space of Disse, the area in between the hepatocytes and the liver sinusoids [13,14]. Accumulation of extracellular matrix proteins promotes a progressive replacement of the liver parenchyma by scar tissue, leading to liver fibrosis and its complications [15]. Activation of hepatic stellate cells (HSC) is a key event in the process leading to excessive deposition of extracellular matrix proteins and subsequent fibrosis.

One of the mediators that are able to activate HSC is reactive oxygen species (ROS) [16]. ROS can be induced in HCV-infected hepatocytes by its core protein [17], and by the HCV non-structural protein NS5A, which modulates cytosolic calcium levels leading to production of ROS [18]. This increase in ROS results in fat deposition in the liver sinusoids, leading to steatosis, which eventually will progress to liver fibrosis (Figure 1). HSC are also activated by TNF and IL-1, released by HCV-infected hepatocytes, which augments the imbalance between matrix metalloproteinases and tissue-inhibitor metalloproteinases [19]. Moreover, once HCV infection is established, the virus can also modulate chemokine gradients to the liver, which attracts immune cells into the hepatic parenchyma [20,21]. Chemokines, such as CCR2 can promote recruitment of inflammatory macrophages into the sinusoids, thus increasing progression to liver injury [22]. Also, cytotoxic T lymphocytes (CTL) recruited to the liver, induce liver injury from CTL-directed apoptosis upon recognition of viral antigens on hepatocytes [23]. Livers infected with HCV exhibit an increased expression of TNF-related apoptosis inducing ligand receptor 2 (TRAIL-R2) [24]. TRAIL-R2 modulates an important apoptosis-related pathway that can regulate HSC activity thus increasing liver fibrosis [23].

During HCV infection, Kupffer cells increase expression and release of tumor growth factor-β (TGF-β), one of the best-characterized fibrogenic cytokines [25]. TGF-β stimulates collagen-I and alpha-smooth muscle actin, both of which are important components of the extracellular matrix [26]. Kupffer cells which intake apoptotic bodies of HCV-infected hepatocytes express TNF and the death receptor component, TRAIL, both of which worsens liver injury [23,27].

Overall, infection with HCV orchestrates a conundrum of direct HCV-mediated as well as immune-related effects that lead to liver fibrosis and cirrhosis in infected patients.
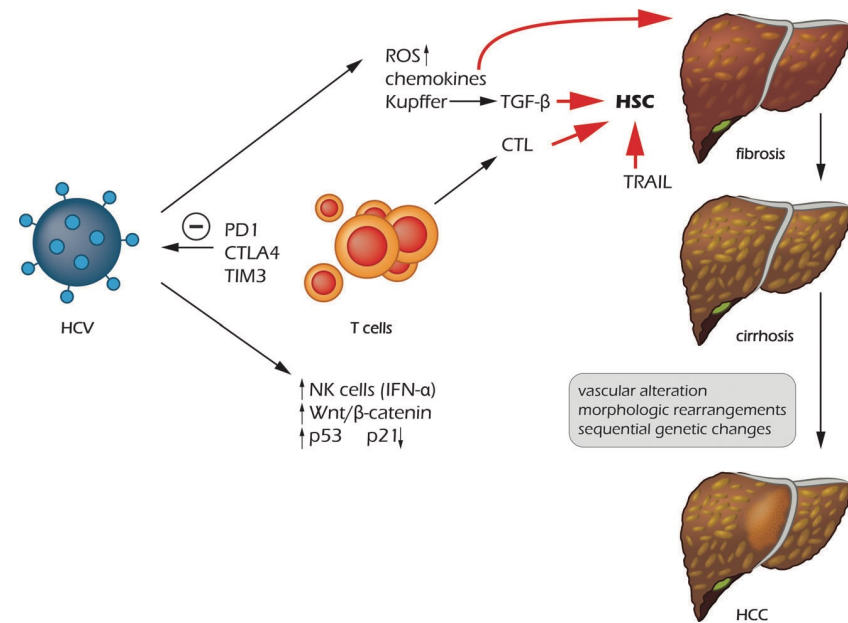
Figure 1. HCV increases ROS, leading to liver steatosis and fibrosis. It attracts chemokines as well as CTL to the liver, thereby inducing inflammation. During HCV infection Kupffer cells produce TGF-β, a profibrogenic cytokine. TRAIL receptor and its apoptotic activity are increased in the liver. T cells aim to stop the virus, but they face an exhausted pattern with increased expression of PD1, CTLA4 and TIM3. HCV proteins activate Wnt/β-catenin and p53 both of which lead to HCC transformation in a setting of cirrhosis. Modulation of NK cells and endogenous IFN-γ production can affect progression to HCC as well as control of HCC.

HCV leading to hepatocellular carcinoma

Chronic HCV patients are at increased risk of HCC, especially those with liver cirrhosis who have a 4-7% per-year risk of developing HCC [28]. Progression of liver fibrosis to cirrhosis will generate a local milieu that predisposes to HCC [29]. In this environment, dramatic changes occur to the hepatic parenchyma, with hepatocyte injury, cellular morphologic rearrangement and changes in vascularization patterns, all of which contribute to sequential genetic hits that culminate in malignant transformation [29]. One of the cell types involved in cancer are NK cells, which play a role in immunosurveillance for various tumors [30,31]. Moreover, NK cells from HCC patients are dysfunctional and exhibit an impaired production of IFN-γ and cytotoxic capabilities [32]. In mice, endogenously produced IFN-γ plays a protective role against transplanted tumors, and it is likely that low IFN-γ activity decreases immunosurveillance leading to malignant cell proliferation, although this hypothesis has not been tested in humans [33,34].

Several studies have implicated a more direct role of HCV in hepatocellular carcinogenesis[12]. Transgenic mice expressing the HCV polyprotein can develop liver cancer in the absence

of inflammation, hepatic cirrhosis or immune recognition of the transgene [35]. These mice initially develop adenomas with subsequent transformation to HCC [35]. The HCV core protein is also thought to play a role in the development of HCC via the activation of the Wnt/β-catenin cascade, and 50-70% of HCCs exhibit abnormal nuclear accumulation of β-catenin [36,37]. Finally, multiple studies suggest that the HCV protein NS5A promotes liver carcinogenesis by binding to p53, which inhibits its transcriptional activity, thereby down-regulating endogenous p21/waf1 expression [38]. As a result, p53-induced apoptosis can be abrogated by NS5A, which contributes to the carcinogenic potential of HCV[39]. Importantly, NS5A can also increase the expression of TGF-β in HCV-expressing cells [40,41], which not only promotes liver fibrosis, but also carcinogenesis in cellular models and correlates with poor prognosis in patients with HCC [41-43].

Direct acting antivirals and HCV

New HCV treatments

The initial armamentarium to fight HCV infection was based on IFN-α -containing therapies. These treatments were prolonged, burdensome and had a rather low efficacy [44]. There is limited evidence of the role of IFN-α -based therapy in the prophylaxis of HCC in patients with chronic HCV, as most studies were primarily designed to assess the antiviral effect of treatment and not the long-term impact on the natural history of the disease [45]. An early study found that treatment with IFN-α/ribavirin provided a 75% HCC risk reduction based on the level of fibrosis in the affected liver, while another study in Italy found no risk reduction for HCC in cirrhotic patients [46].

New treatments for HCV with direct-acting antivirals (DAAs) are now available [44,47]. These treatments provide an extraordinary rate of sustained virological response (suppression of HCV blood levels for a period of 6 months) with over 95% cure rates reported in most trials involving all but type 3 HCV genotypes [48]. However, it is yet unknown whether HCV treatment with DAAs reduces HCV-related complications such as HCC. A recent study which followed patients up to one year, found a minimal to no decrease in HCC risk post HCV treatment with DAAs [49].

DAA effect in the immune system

As described above HCV-specific immune responses are weak or absent as a consequence of the continuous antigenic pressure resulting from high concentrations of viral antigens. The introduction of new DAA treatments has allowed studies to determine whether the dysfunctional immune response in chronic HCV can be restored upon successful therapy-induced viral load decline; an answer that previous pegylated IFN-α-based treatments were unable to provide since IFN-α itself is a potent immunomodulatory agent. These

studies showed a rapid down-regulation of IFN-stimulated genes in liver and blood of successfully treated patients as well as a reduced serum level of IP-10, IL-12p40 and IL-18, comparable to levels observed in healthy individuals [50,51]. Importantly, enhanced HCV-specific CD8+ T cell responses were observed in blood of patients successfully treated with DAAs [52,53]. Also, normalization of the impaired NK cell response was observed as evidenced by reduced expression of NK cell specific receptors and TRAIL on NK cells [53-55]. The only leukocyte population in blood whose numbers and activity has shown not been restored after DAA-induced HCV viral load clearance are MAIT cells, which remain impaired after successful DAA therapy [5,55]. Thus, with the exception on MAIT cells, the immune system seems to normalize and becomes less activated after successful DAA treatment.

DAA and hepatocellular carcinoma

Recent studies with limited number of patients have suggested a more aggressive and early form of recurrent HCC in patients following successful treatment with DAAs [56]. These studies have sparked controversy as they have exposed a potential "side effect" of the new HCV treatment. Two European studies showed 27% and 29% recurrence of HCC within 6 months of treatment with DAAs, respectively [57,58]. Importantly, a small study from the United States found a 27% recurrence of HCC in patients that underwent liver transplantation following treatment with DAAs, compared to 9% recurrence in those with no DAA treatment in the same period of time (6 months post liver transplant) [59]. In contrast, a pool of three studies from France failed to show an additional increase in HCC recurrence post DAA treatment [59]. All of the above-mentioned studies are small, and larger studies are necessary before reaching conclusions that could dramatically affect clinical practice. Moreover, there were different HCC treatments before recurrences, with different initiation points and duration of HCV-therapy, making it difficult to compare these studies or integrate them into one conclusion. Also concern has been raised about the statistical methods to analyze some of the data that could provide a different interpretation of the results, such as reporting of crude instead of entire Kaplan-Meier curves [60,61]. The definition of complete remission of HCC before DAA treatment was also incompletely clear, which would cloud the definition of recurrence of HCC. Nonetheless, much speculation has arisen about the consequences of blunting HCV activity with IFN-free treatment in immunosurveillance to counteract tumorigenesis. The DAA-induced viral load decline in HCV patients is accompanied by a fast reduction and normalization of serum transaminase levels, indicating that immune-mediated responses in the liver are contracting (Figure 2). However, as mentioned above, the studies from our group and others have shown a "normalization" and decrease of NK cell activity following treatment with DAAs [51]. Together with the reduced presence of the NK cell activating cytokines IL-12 and IL-18 in serum, one can speculate that a possible explanation for enhanced HCC recurrence upon DAA treatment is a reduced NK cell-mediated immunosurveillance.

The importance for NK cells in tumorigenesis is supported by numerous experimental models in which NK cell-derived IFN-γ is shown to be protective to tumorigenesis. This notion is further supported by the finding that peripheral NK cell numbers are reduced in patients with HCC [62]. Reduced activation of NK cells will likely decrease "immune-editing" of HCC leading to cancer recurrence.
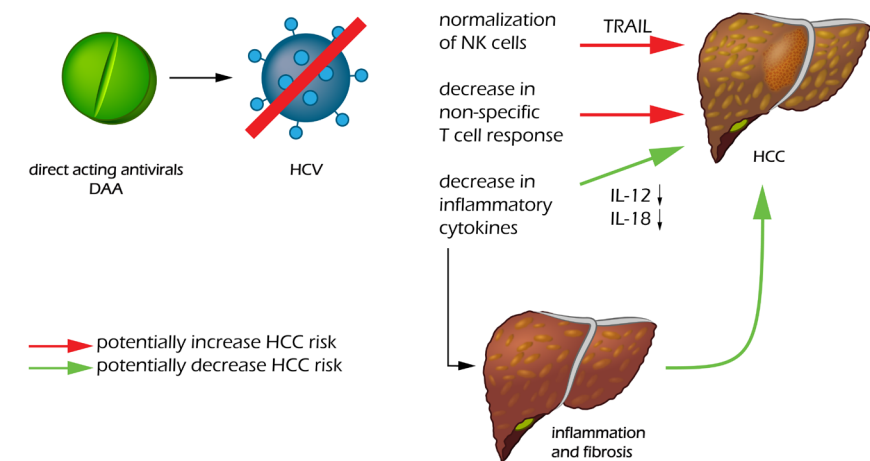

Figure 2. A schematic presentation of proposed mechanisms of how DAA therapy may increase the risk of HCC. DAA-induced viral clearance leads to improvement of inflammation and fibrosis. However, reduced immune surveillance and normalization of NK cell activity in highly selected patients may result in accelerated HCC recurrence.

However, since NK cells obtained from chronic HCV infected patients prior to DAA treatment are considered to be not fully functional NK cells, more detailed studies need to be conducted to prove or reject the immunosurveillance hypothesis. Interestingly, the relatively high expression of TRAIL-R2 on intrahepatic NK cells during HCV infection may also play a role [63]: DAA-induced normalization of the NK-cell compartment could decrease TRAIL-R2 expression, allowing for an "anti-apoptotic" environment friendly to HCC development. This hypothesis still needs to be tested in humans, but it is important to note that polymorphisms in TRAIL receptor gene are associated with increased risk for HCC in HCV-infected cohorts [64]. Another important factor that may influence immunosurveillance is reduced recruitment of inflammatory cells to the liver, as suggested on the basis of reduced serum levels of chemokines following successful DAA treatment of chronic HCV patients [53,54]. During the chronic phase of infection, relatively high numbers of HCV-specific and non-HCV-specific T cells are present in the inflamed liver establishing an equilibrium between ongoing viral replication and weak T cell activity, as evidenced by the presence of exhausted T cells and active

immunoregulation [6,65]. It is reasonable to suspect that following treatment of HCC, a low number of malignant cells could remain present in the liver (or circulating), and these cells are kept "suppressed" by this non-specific T cell response. Once HCV infection is rapidly removed with DAAs, the HCV-specific but also general T cell response decreases dramatically. In this setting, a few "orphan" malignant cells could proliferate to develop a rapid recurrence of HCC. It is important to emphasize that experimental evidence to support these speculations is not available, and research is needed to better understand possible underlying mechanisms. However, the unexpected correlation of DAAs and HCC is of concern, and it is important to determine whether the observations are genuine or biased due to selection of specific clinical cohorts. A joint multicenter effort is urgently needed to identify all cases of HCC recurrence and determine whether DAA treatment promotes this process.

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2

Epidemiology of HCC

CHAPTER 2A

Hepatobiliary cancers in South America, where disparity strikes

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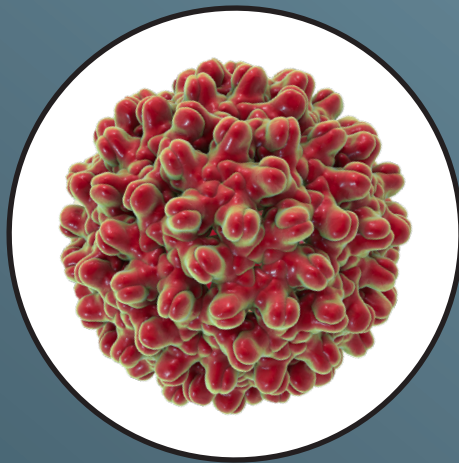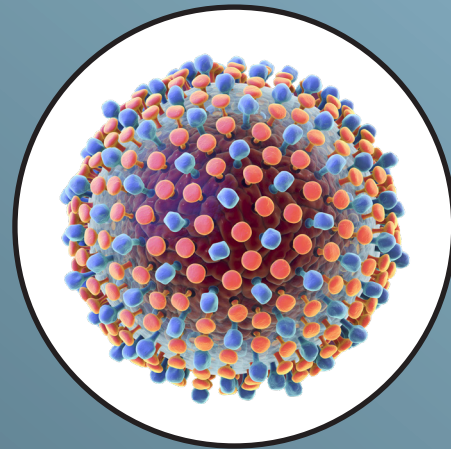
Hepatobiliary malignancies represent an important cause of death worldwide [1]. Mortality from these tumors occurs primarily due to late detection, which precludes potentially curative interventions. Currently, there are no reliable markers for early recognition and screening of hepatobiliary malignancies, leading to these tumors being detected at a late stage. This is more evident in regions of the world with scarce access to modern diagnostic technology for the entire population. In this regard, South America exhibits a rather unique geo-political dichotomy with advanced health centers in major cities, capable of providing most diagnostic and therapeutic options, including liver transplantation; but pitiable resources to deal with complex diagnostics in smaller cities and towns. It is in this setting where the need for biomarkers for early detection becomes critical, as they can help stratify patients to be shuttled from areas with poor resources to larger cities when necessary. Interestingly, the majority of hepatobiliary cancers occur in individuals with known risk factors (i.e. cirrhosis, gallstones, IBD), making it easier to identify those that should be screened. In South America, these tumors have unique epidemiologic characteristics compared to other parts of the world: hepatocellular carcinoma (HCC) associated to hepatitis B occurs more frequently at an earlier age, and gallbladder cancer (GBC) disproportionally affects Amerindians in Chile and Peru [2,3]. Indeed, Chile has the highest incidence of GBC in the world [4]. Cholangiocarcinoma (CCA) is an epidemiological mystery in the region, yet with the high local frequency of liver flukes it is likely to represent a high burden of disease.

Currently, the diagnosis of hepatobiliary cancers relies on advanced imaging or invasive instrumentation. However, recent advances that permitted identification of exosomes in blood as well as identification of circulating tumor cells has led to the concept of "liquid biopsy", the detection in blood of organ-specific markers originating from particular tumors [5]. It is with this in mind that investigators from Europe, North America and South America formed ESCALON (European South American Consortium to Assess Liver-Originated Neoplasia), funded by the European Union mechanism Horizon 2020. The main objective of this initiative is to develop a large South American biobank to identify biomarkers for early detection and diagnosis of hepatobiliary cancers. Specifically, looking at immune-markers for HCC (a highly immunogenic tumor) and exosomes for CCA and GBC. The final aim? to create a "liquid" link between high-risk individuals in small South American towns and the resources available for them in larger cities.

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CHAPTER   2B

Hepatocellular Carcinoma in South America: Evaluation of Risk Factors, Demographics, and Therapy

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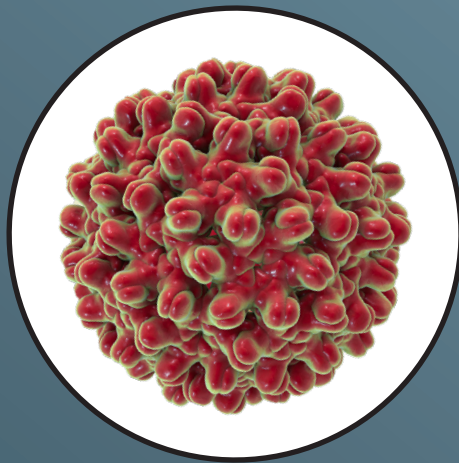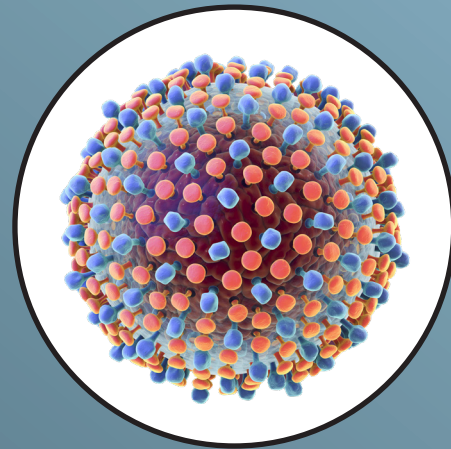
Abstract

Background and Aims
Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide. Most studies addressing the epidemiology of HCC originate from developed countries. This study reports the preliminary findings of a multinational approach to characterize HCC in South America.

Methods
We evaluated 1336 HCC patients seen at 14 centers in six South American countries using a retrospective study design with participating centers completing a template chart of patient characteristics. The diagnosis of HCC was made radiographically or histologically for all cases according to institutional standards. Methodology of surveillance for each center was following AASLD or EASL recommendations.

Results

Sixty-eight percent of individuals were male with a median age of 64 years at time of diagnosis. The most common risk factor for HCC was hepatitis C infection (HCV, 48%), followed by alcoholic cirrhosis (22%), Hepatitis B infection (HBV, 14%), and NAFLD (9%). We found that among individuals with HBV-related HCC, 38% were diagnosed before age 50. The most commonly provided therapy was Trans-arterial chemoembolization (35% of HCCs) with few individuals being considered for liver transplant (<20%). Only 47% of HCCs were diagnosed during surveillance and there was no difference in age of diagnosis between those diagnosed incidentally versus by surveillance. Nonetheless, being diagnosed during surveillance was associated with improved overall survival (p=0.01).

Conclusions
Our study represents the largest cohort to date reporting characteristics, and outcomes of HCC across South America. We found an important number of HCCs diagnosed outside of surveillance programs, with associated increased mortality in those patients.

Keywords
Hepatocellular Carcinoma; South America; Risk Factors; Demographics

Key Points Box
- Our manuscript provides the most comprehensive study on liver cancer in South America. We found most common risk factor for liver cancer to be hepatitis C virus infection.
- A significant number of patients with hepatitis B virus infection had liver cancers diagnosed earlier than the recommended screening age.
- Over half of patients were diagnosed with liver cancer outside of surveillance programs highlighting deficiencies in surveillance.
- The majority of individuals received TACE.

Abbreviations
HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; NAFLD, Non-alcoholic Fatty Liver Disease; AFP, Alpha-fetoprotein; AASLD, American Association for the Study of Liver Diseases; EASL, European Society for the Study of the Liver; OR, Odds ratio; HR, Hazard ratio; CI, Confidence interval; RFA, Radiofrequency ablation; PEI, Percutaneous alcohol injection; TACE, Trans-arterial chemoembolization; NASH, Non-alcoholic steatohepatitis.

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Introduction

Hepatocellular carcinoma (HCC) is the most common cancer originating from the liver and it is universally associated with chronic liver disease and cirrhosis (1). HCC is the fifth most common cancer in men and the second overall cause of cancer death accounting for 746,000 deaths worldwide (2). Moreover, a recent study showed that in the United States, during the last 10 years, liver cancer diagnosis increased at a higher rate than any other cancer (3). Risk factors for HCC vary by geographic region (4, 5). For instance, hepatitis B (HBV) and exposure to dietary aflatoxins have been identified as major risk factors in sub-Saharan Africa, while hepatitis C (HCV) has been recognized as a major risk factor in North America and Japan (4, 6, 7). Furthermore, alcohol consumption is thought to play a major role in the development of HCC and related mortality in Eastern Europe and Russia (5). There are also differences in age-based HCC surveillance protocols depending on the geographic origin of a person, with recommendations to screen earlier in patients of African or Asian origin with non-cirrhotic chronic hepatitis B infection (8). The largest burden of HCC is in the developing world, with most cases occurring in Asia and Africa (2, 9, 10). To date, studies have focused on characterizing patients with HCC in Europe, North America, Asia and, to a lesser extent, Africa (11, 12). Moreover, most of the information about clinical outcomes from curative or palliative therapy originates from clinical trials, most of which are conducted in the United States, Europe or Asia (12-14). The largest study to date describing the demographics of HCC around the globe is the BRIDGE study (12). This study showed the average age of HCC to be 61 years, and the most common associated risk factor to be HCV infection in all areas except for China, South Korea and Taiwan, where HBV was more common. However, this study did not include data from the regions of Africa and South America. A recent study by Yang *et al* described the demographics of HCC in Africa (14). This study showed a much earlier age of HCC diagnosis in sub-Saharan Africa, mean of 47 years, with the most common associated risk factor being HBV infection. To date, little is known about the underlying demographic characteristics, risk factors and use of surveillance for HCC in South America, Moreover, there is a paucity of data, from clinical trials or daily clinical practice, about therapeutic options for HCC in this region. In this manuscript we describe for the first time the demographics of HCC in South America in a comprehensive fashion including data from six different countries in the region. This study represents the early results of a multinational effort to characterize HCC in South America.

Patients and Methods

Data collection

We designed a retrospective cohort study aimed at identifying the demographics and risk factors associated with HCC in South America. A concerted effort was made to identify characteristics of HCC at the time of diagnosis. Overall, fourteen medical centers from six countries in South America participated. Each center was responsible for adhering to their respective institutional review policies, and ethical approval was obtained from participating centers and Hennepin County Medical Center (supplementary data). No informed consent was obtained due to the retrospective nature of the study. Ten participating centers were considered academic (provided information on 1185 patients) and four centers were non-academic (provided information on 151 patients). The primary objective was to assess the epidemiology of HCC in different countries in South America, focusing mainly on risk factors, age and gender and associated clinical outcomes. Secondary objectives included assessment of therapies offered and evaluation of survival based on the presence of multiple variables.

Participating centers completed a standardized, retrospective chart review of patient characteristics at the time of HCC diagnosis, obtained from each center's database. Data was then de-identified and placed into a composite database. Diagnosis of HCC was made radiographically or histologically for all cases as defined by institutional standards. Radiographically diagnosed cases were requested in accordance with AASLD or EASL guidelines, or comparable local guidelines (8, 15, 16). Variables abstracted included age, gender, chronic infection with viral hepatitis C (HCV) or hepatitis B (HBV), presence of alcohol abuse, evidence of nonalcoholic fatty liver disease spectrum or cryptogenic cirrhosis, and evidence of other underlying liver disease including cirrhosis. Select centers also provided additional data on HCC surveillance and alpha-fetoprotein (AFP) levels at the time of diagnosis, and treatment. When more than one treatment was offered, only the first treatment was included, unless the treatment was in combination with liver transplantation, in that case liver transplantation was considered the main treatment. Diagnosis of HCC under surveillance program in each center was defined when a patient was undergoing systematic screening for HCC following either AASLD or EASL guidelines.

Statistical analyses

Continuous variables were summarized as means or as medians (interquartile range-IQR or range) according to their homogeneity. Categorical variables were compared with the χ^2 test or Fisher's exact test when appropriate. Continuous variables were compared with the Mann-Whitney U test or Student T test. For regression models, we included predictors with $P < 0.05$ in the univariate analysis. Analyses of factors independently

associated with cirrhosis at the time of HCC diagnosis and with administration of curative therapy for HCC were performed using logistic regression. An analysis of factors independently associated with survival after HCC diagnosis was performed using the Cox regression model.

Survival rates after HCC diagnosis were computed from the day of diagnosis until death or the last follow-up visit using the Kaplan–Meier method and compared by using the log-rank test. Survival was corrected for lead-time bias using a similar approach as Cucchetti *et al* and Mourad *et al* (17, 18). Briefly, because there were no tumor size data in our database we considered a lead-time of 7 months according to previous studies (17, 18). Then, a probabilistic analysis (Monte-Carlo simulation) was applied to estimate the lead-time bias. A theoretical cohort of 1000 patients undergoing HCC screening was compared to a theoretical cohort of 1000 patients with a symptomatic diagnosis. Survival rates in relationship with surveillance programs were properly calculated and reported in 10-years life expectancy before and after adjustment for lead-time bias, subtracting the lead-time from life expectancy.

Associations are reported as odds ratios (OR) or hazard ratios (HR) with 95% confidence intervals (CI). A 2-sided probability value < 0.05 was considered to be significant. Statistical analysis was performed using the SPSS v 24.0 statistical package (IBM Corp., Armonk, NY).

Results

Fourteen centers from six countries across South America contributed data for an aggregate 1,336 patients. Brazil accounted for 40% (*n*=540) of patients, Argentina 19% (*n*=251), Colombia 18% (*n*=239), Peru 16% (*n*=220), Ecuador 5% (*n*=65) and Uruguay 2% (*n*=21) (Figure 1A). Each center provided information on HCC from a period between 5 and 10 years (depending on the center) retrospectively, with the earliest report being from April 2005 and the latest from May 2015. Of the 1,336 patients, 68% were male and the overall median age of both males and females was 64 years. A total of 1,153 (86%) patients had complete data on risk factors for HCC (Table 1). The most common risk factor for HCC was HCV infection (48%), followed by alcoholic cirrhosis (22%), HBV infection (14%), NAFLD (9%) and other causes (8%) (Figure 1B). Twenty-nine percent of HCV and 18% of HBV patients also had alcohol consumption as a second risk factor. Comprising the "other causes" risk factor group were 44 patients with cryptogenic cirrhosis, 17 patients with hemochromatosis, 12 patients with autoimmune related liver disease, 12 patients with HBV/HCV co-infection, 4 patients with primary biliary cirrhosis, 4 patients with schistosomiasis, 2 patients with drug related liver injury and 2 with reported vascular complications. The distribution of risk factors was relatively homogeneous through out

the countries, with HCV and Alcohol use being the two most common risk factors for HCC in all countries except Peru (Figure 2). Interestingly, in this country HBV infection was the most important risk factor for HCC accounting for 34% of cases. Of those patients infected with HCV, 64% were males compared to 74% males in those infected with HBV, 89% of individuals with alcohol related liver disease, and 56% of those with NAFLD associated HCC. Age at time of diagnosis differed by independent risk factor as shown in Figure 2. When evaluating all new cases of HCC in individuals infected with HBV, we found that 38% (*n*=48) of HCCs occurred before age 50, with a median age at diagnosis of 58 years, while in those infected with HCV, only 6% (*n*=24) were diagnosed with HCC before age 50 (*p*<0.001) and the median age of diagnosis was 63 years (percentage of cases per age group described in Figure 3). Even larger differences were observed when HBV-induced HCC was compared with NAFLD, median age at diagnosis 67 years (*p*<0.001) and alcohol-induced HCC, median age at diagnosis 68 years (*p*<0.001).

Table 1. Demographic and clinical characteristics of HCC per country

Country	Age*	% Males	% Cirrhotics**	AFP#	%AFP >20#	% Curative treatment#
Argentina	65	74	86	76	60	23
Brazil	61	74	92	51	58	27
Colombia	67	64	95	20	46	49
Ecuador	56	48	72	4	27	32
Peru	67	59	85	90	63	4
Uruguay	65	86	100	11	50	32

*Age: Median age in years
**Number of patients with data about cirrhosis: Argentina: 112, Brazil: 380, Colombia: 125, Uruguay: 21, Peru: 220, Ecuador: 65
#Median ng/ml , number of patients with AFP data: Argentina: 112, Brazil: 370, Colombia: 115, Uruguay: 18, Peru: 130, Ecuador: 65
##Number of patients with data on treatment: Argentina: 110, Brazil: 379, Colombia: 125, Uruguay: 20, Peru: 137, Ecuador: 65
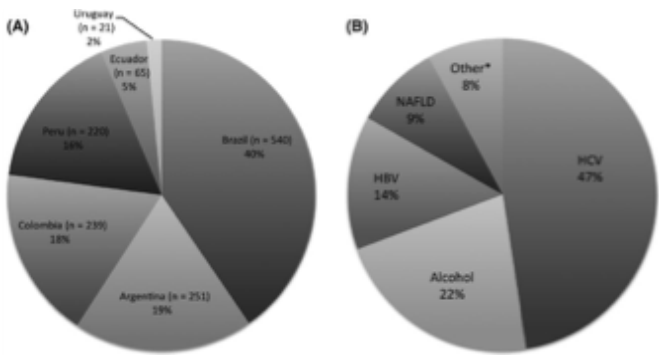

Figure 1. Distribution of hepatocellular carcinoma in South America. *A)* Number of cases of hepatocellular carcinoma (HCC) by contributing countries. *B)* HCC risk factor distribution by country (HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic liver disease; alcohol, current alcohol consumption). Percentage reflects the percentage within the entire cohort.
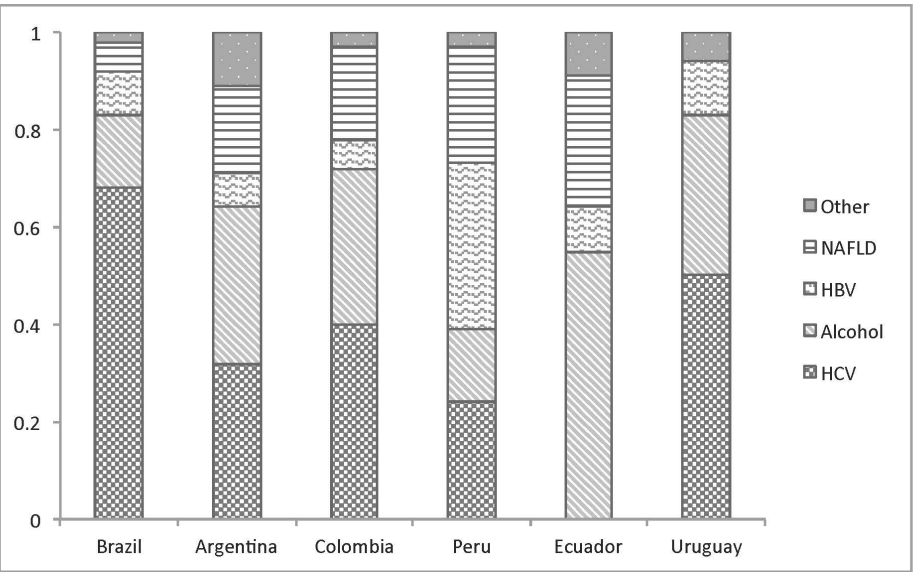
Figure 2. Distribution of risk factors for hepatocellular carcinoma within each country. Y-axis represents percentage of the cases per each country (1 represents 100%); X-axis represents each country. Filling of bar correlates with description of each risk factor, corresponding to percentage of risk factor within the total of each country. NAFLD, non-alcoholic liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus.
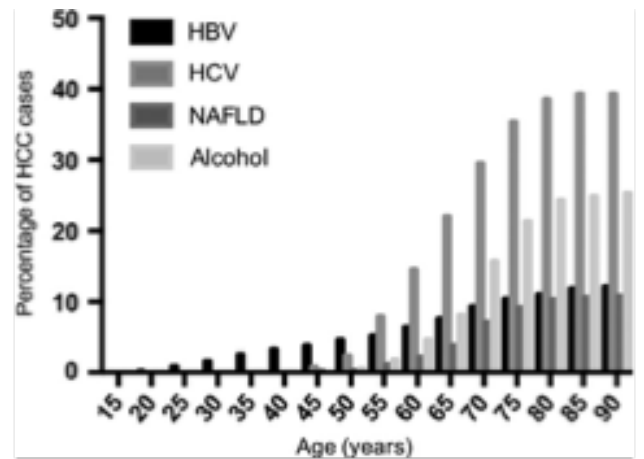
Extended data was provided by eight centers from the six countries (Figure 4). Descriptive statistics were calculated to determine the proportion of HCC diagnoses that were made during regular surveillance for HCC, as well as to evaluate AFP levels at the time of diagnosis. Of 732 patients for whom screening information was available, we found that only 343 (47%) were diagnosed with HCC during surveillance as defined by institutional standards. The median ages of those diagnosed during surveillance vs. incidental or symptom-based diagnosis were not significantly different, both being 64 years (*p*=0.967). Median time of follow-up was 49, months (IQR: 27-65 months).
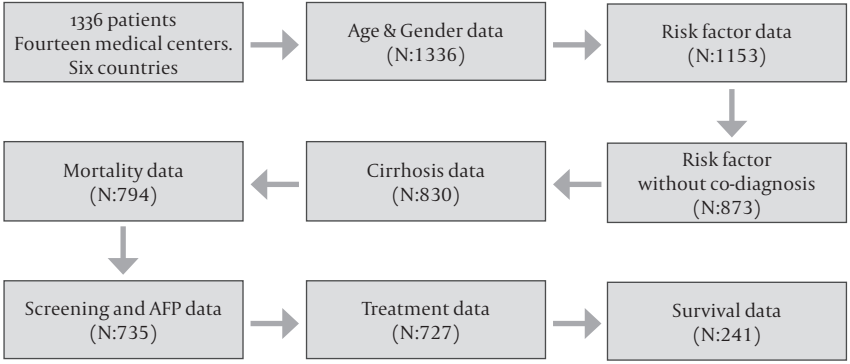
Figure 4. Flow chart of patients with specific data based on collection from all centers.

Using logistic regression models we found that HCV (OR 3.25, 95% CI 1.63-7.08, *p*=0.001) and alcoholic liver disease (OR 3.82, 95% CI 1.46-13.17, *p*=0.005) were significantly associated with the presence of cirrhosis at the time of HCC diagnosis, while other risk factors were not. We also examined the risk factors associated with a higher likelihood of receiving curative therapy, defined as resection, transplantation, radiofrequency ablation (RFA) or percutaneous ethanol ablation (PEA). We found significant associations of cirrhosis (OR 3.96, 95% CI 2.09-7.44, *p* <0.001), having been diagnosed during surveillance (OR 2.22, 95% CI 1.43-3.48, *p* <0.001) and AFP >200ng/ml (OR 2.29, 95% CI 1.25-4.24, *p*=0.007) with receipt of curative therapy. A proportional hazards model was then used to evaluate survival. Having underlying HCV (HR: 0.74, 95% CI 0.56-0.98, *p*=0.042), and being diagnosed under surveillance (HR: 0.62 (95% CI 0.48-0.78, *p*<0.001) were both significantly associated with decreased mortality (Table 2). Survival rates after HCC diagnosis were higher in patients that underwent screening after lead-time correction (log rank P = 0.001). The median life expectancy in the screening group before and after lead-time correction was 47.3 months (IQR 34.3-66.1 months) and 40.4 months (IQR 27.3-59.1 months) respectively (Table 3). In the symptomatic diagnosis group the median life expectancy was 24.6 months (IQR 14.4-45.9 months).

Figure 3. Percentage of cases hepatocellular carcinoma per age group and associated risk factor. Y-axis represents precedence of HCCs per risk factor, X-axis represents age in 5-year intervals. NAFLD, non-alcoholic liver disease; HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 2. Analysis of survival of HCC patients within different variables

Variable	Mean Survival (months)	Standard Deviation	p=	RR
HBV	7.3	7.4	0.063	1.43 (95% CI 0.98-2.07)
HCV	13.6	12	0.042*	0.74 (95% CI 0.56-0.98)
Alcohol	11.3	9	0.169	0.79 (95% CI 0.56-1.10)
NAFLD	10	9.2	0.383	1.21 (95% CI 0.78-1.83)
Cirrhosis	11	10.1	0.128	1.44 (95% CI 0.90-2.42)
Screening	14.4	11	<0.001*	0.62 (95% CI 0.48-0.78)
AFP >20mg/ml	10.1	9.8	0.466	1.12 (95% CI 0.82-1.54)
AFP >200ng/ml	9.5	10.2	0.501	1.10 (95% CI 0.83-1.49)
Curative therapy	17.3	10.1	0.222	0.79 (95% CI 0.53-1.15)

*Denotes statistically significant value. CI: Confidence interval. RR: Risk ratio.

AFP: Alpha-fetoprotein. HBV: Hepatitis B virus. HCV: Hepatitis C virus. NAFLD: Non-alcoholic steatohepatitis.

Table 3. Overall survival according to surveillance (N: 739)

Follow-up	Surveillance	Symptomatic diagnosis	P value
1-year, % (CI 95%)	78.7 (74.5-82.9)	59.8 (54.8-64.9)	<0.001
3-year, % (CI 95%)	52.2 (46.6-57.7)	36.3 (30.8-41.8)	<0.001
5-year, % (CI 95%)	30.6 (24.7-36.4)	25.7 (20.3-31.4)	0.22

We assessed treatment data on 727 patients from the six countries (all centers that provided such information). Specific treatment modalities for each country varied widely per country and are described in Figure 5. Trans-arterial chemoembolization (TACE) was the most commonly used method as first therapeutic approach (269 patients) regardless of whether tumors were found incidentally or during screening (37% and 35% of HCCs, respectively). Palliative treatment (aside from TACE) was the second most often used modality (218 patients), with an overall of 30% of patients being offered such approach. Only 50 patients (7%) underwent radiofrequency ablation (RFA) as first approach, although it should be noted that 78% of those tumors treated by RFA were found during screening. Only eight participating centers performed liver transplantation, and 120 patients underwent a liver transplant as a therapeutic option for HCC. The great majority of tumors (95%) that were treated with liver transplantation were found during screening, but across the board, with the exception of Colombia (43%), the option of liver transplantation was available to less than 20% of the patients, and as low as 4% in some cases as Brazil, likely reflecting the advanced stage of presentation.
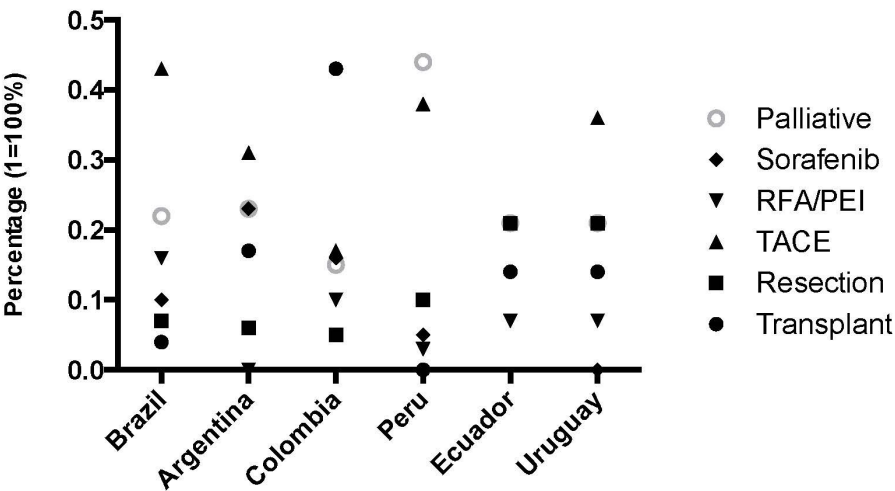

Figure 5. Treatment for HCC distribution within each country. Symbol in the figure correlates with specific treatment on the side legend. Information about transplantation as treatment was included only in those centers that perform liver transplantation. Upper limit of 0.5 in the Y-axis represents 50% of the total number of cases treated in each country. X-axis represents each country. When more than one treatment was offered per patient, only the first treatment was included, unless the treatment was in combination with liver transplantation in which the later one was used.

Discussion

This study provides the first comprehensive description of risk factors, demographics, data on surveillance, and therapy offered for HCC in the continental region of South America.

When comparing our data from South America to results from the BRIDGE Study by Park *et al*, the overall demographics and age at the time of diagnosis in our South American cohort were similar to the results from North America, Europe and Japan (7). However, we found that 38% of HCCs in HBV-infected individuals occurred before age 50. This finding is concerning and questions whether current guidelines are appropriate for the region. Moreover, our results suggest the presence of unknown environmental factors in the region that could predispose to HCC. The early-age diagnosis of HBV-related HCC has usually been reported from Africa and in association to aflatoxin exposure (19). Exposure to aflatoxins affects the TP53 gene and this is thought to lead to a rapid occurrence of HBV-induced HCC (19, 20). Aflatoxins have not been thought to represent a major risk factor in South America, although aflatoxin-associated p53 mutations have been found in HBV-related HCCs in a small Brazilian study (21).

Similar to other populations, we found that HCV and alcoholic liver disease were associated with the presence of cirrhosis at the time of HCC diagnosis (22, 23). This reflects the observation that both HBV and NASH induced HCC have a higher propensity to develop in individuals without cirrhosis. Interestingly, the presence of NALFD was not a major single risk factor associated to HCC. This was a rather surprising finding since the NAFLD prevalence is higher in South America than in other regions of the world (24). Although we did not specifically address the presence of NAFLD within other risk factors, such as HCV or HBV infection, it is probable that the underlying presence of fatty liver contributed to HCC when combined with other risk factors (25, 26). Nonetheless, it is possible that the clinical presentation of NAFLD in the region differs from that of other parts of the world with a lower propensity to HCC (25). More research in this area will be needed with studies addressing biopsy-proven NAFLD or NASH as well as detailed risk factor exposure, in order to address this question.

We found that the presence of cirrhosis, a diagnosis of HCC during surveillance, or an AFP >200ng/dl were associated with receiving curative therapies. AFP levels varied quite significantly between countries, with Argentina and Peru showing higher medians of AFP on HCC diagnosis (>50 ng/ml) and Ecuador showing a lower median AFP of 4 ng/ml. This finding is similar to that of the BRIDGE study, which found large differences between regions (25 ng/ml in Europe and 219 ng/ml in China) (12). It is unclear whether these differences represent lack of standardization of AFP testing or variable clinical behavior of the tumors. We did not find, however a significant correlation with AFP levels and survival. The presence of cirrhosis in our cohort correlated with receipt of curative therapy, but did not correlate positively with survival. Edenvik *et al* in Europe did not study the association between cirrhosis and survival. However, the authors found a significant association between cirrhosis and missed surveillance, which did indeed correlate with higher mortality (27). Improved survival was seen in patients with HCV and in those who were diagnosed while under regular surveillance. Survival rates for those on HCC surveillance were significant at 1 and 3 years on the lead-time analysis, but were not significant at 5 years. However our follow up period was shorter than in other studies likely leading to a non-significant difference at 5 years. Interestingly, the receipt of curative therapy was not associated with a survival benefit, however, one could postulate that this is due to selection bias as patients who are still living at the time of the study were unlikely to have a date of death included in the data set and were thus excluded from the analysis. It is concerning that over half of the individuals in our study had HCCs diagnosed outside of surveillance programs. However, a low rate of HCC diagnosis through surveillance programs represents a well-known deficiency around the world (28, 29). In this regard, Edenvik *et al* reported a 30% HCC diagnosis through surveillance in Sweden and Singal *et al* reported 24% surveillance screening, which improved to 47% after outreach (27, 30). Although our data is preliminary, we found a survival advantage

for those diagnosed during surveillance and therefore more emphasis on adherence to surveillance programs should be encouraged in the region. Edenvik *et al* had similar findings in a European cohort. Interestingly, in that study although the rate of curative treatment was similar in HCCs diagnosed through surveillance or not, those diagnosed under surveillance programs had a significant survival benefit (27).

In those patients from whom we had treatment information, the majority was offered either curative therapy or disease modifying approach with only 30% being offered a palliative option. TACE was the most frequently first used therapeutic modality with liver transplantation being the less used modality and this remained constant throughout the different countries. These findings are similar to those the United States, China, Japan and Europe and likely reflect diagnosis of HCC at late stages (12). The use of resection, RFA/PEI, liver transplantation and sorafenib did vary among the countries, and likely reflects regional differences in clinical practice due to access and cost. Despite liver transplantation being the most efficacious treatment, very few patients received this therapy. In South America, this is likely related to an inadequate availability of donors, but also possibly affected but a less efficient system to identify suitable candidates. A high percentage of individuals (30%) were offered palliative approach, only, as therapy. This is concerning, and likely related to a high percentage of patients diagnosed with HCC by symptom presentation and not during surveillance. Much improvement is needed in health-related infrastructure and organization, to detect HCCs earlier in order for a possible referral to a transplant center and a curative approach being possible. Interestingly, 116 patients (16%) underwent treatment with sorafenib. The frequency of use and success rate of the multi-kinase inhibitor has been evaluated in multiple regions, but only one study has addressed its efficacy in Hispanic individuals within the context of a larger cohort of patients (31).

The limitations of this study include its retrospective nature and the absence of validated information on the standards used for determining NAFLD spectrum or alcohol misuse disorders. There was also selection bias as site participation was voluntary and some countries were only represented by one center. However, it is likely that the large sample size partially overcomes such confounding. On the other hand, due to the size of the studied population and difference in centers, some interactions between variables are difficult to account for and therefore results should be interpreted with some caution. Moreover, due to a large proportion of data collected through paper charts we did not properly addressed Barcelona Clinic Liver Cancer Stage in all patients. As our research network develops a prospective assessment of HCC in South America we expect to address these variables.

In summary, our study represents the largest cohort to date addressing HCC in South

America. Similar to North America, Europe and Japan, HCC in South America is most often diagnosed in older males, with HCV being the predominant risk factor. We also highlight a large number of patients with HBV in South America diagnosed at an early age. The most commonly used treatment modality was TACE with liver transplantation being the less frequently used. As in many other countries and continents around the globe, HCC surveillance is under-utilized in South America.

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CHAPTER 2C

Early-age hepatocellular carcinoma associated with hepatitis B infection in South America

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Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and is almost universally associated with chronic liver disease and cirrhosis. Risk factors for HCC generally vary by geographic region. To date, studies have focused on characterizing patients with HCC in Europe, North America, Asia and, to a lesser extent, Africa [1, 2]. However, little is known about the underlying demographic characteristics and risk factors for HCC in South America, particularly the association between viral hepatitis and HCC. In this study we describe the early results of a multinational effort to characterize HBV-related HCC in South America.

Methods

We designed a retrospective cohort study aimed at identifying the demographics and risk factors associated with HCC in South America. Overall, fourteen medical centers from six countries in South America participated. Each center was responsible for adhering to their respective institutional review policies. Participating centers completed a standardized, retrospective chart review of patient characteristics at the time of HCC diagnosis. Data was then de-identified and placed into a composite database. Diagnosis of HCC was made radiographically or histologically for all cases as defined by institutional standards. Continuous variables were summarized as means or as medians (IQR) according to their homogeneity. Statistical analysis was performed using the SPSS v 22.0 statistical package.

Results

Fourteen centers from six countries across South America contributed data for an aggregate 1,336 patients. Brazil accounted for 540 patients, Argentina 251, Colombia 239, Peru 220, Ecuador 65 and Uruguay 21. Of the 1,336 patients 68% were male and the overall median age of both males and females was 64 years. A total of 1,153 (86%) patients had complete data on risk factors for HCC. HBV infection represented the main risk factor for HCC in 131 subjects (11% of the those with complete data), of which 74% were males. Centers from Peru and Brazil contributed for the majority of HBV patients (34 and 38% respectively), followed by Argentina (16%), Colombia (7%), Ecuador (3%) and Uruguay (2%). The median Alpha-fetoprotein level on diagnosis was 161 ng/ml and 86% of HBV-infected individuals had evidence of cirrhosis (in those the provided that information, N=81). When evaluating HCC in individuals infected with HBV, we found that 38% (n=48) of cases occurred before age 50, with a median age at diagnosis of 58 years, while in those

infected with HCV, only 6% (*n*=24) were diagnosed with HCC before age 50 (*p*<0.001) and the median age of diagnosis was 63 years (Figure 1). Even larger differences were observed when HBV-induced HCC were compared with NAFLD (median age at diagnosis 67 years, *p*<0.001) and alcohol-induced HCC (median age at diagnosis 68 years, *p*<0.001). We did analyze inter-country variability for HBV-related HCC and age of incidence and found a larger number of HCC diagnosed below age 50 from Peru (43%) compared to other countries (25%) but the difference was not significant (p=0.09).
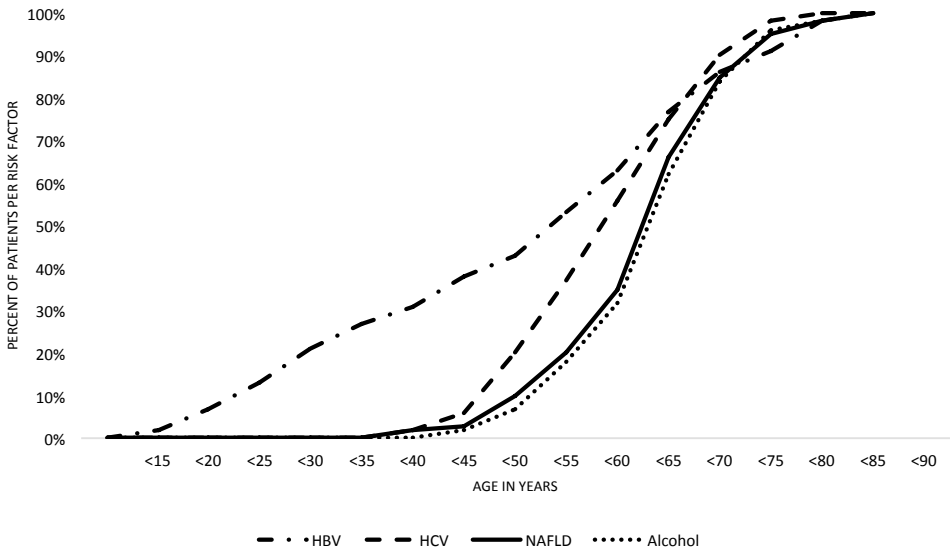
Figure 1. Cumulative percent of HCC per independent risk factors based on age. There was a significant difference between age of diagnosis for HBV-related HCC versus others (p<0.001).

Discussion

Our study unexpectedly found that nearly 40% of HCCs in HBV-infected individuals occurred before age 50. This finding raises the question of whether surveillance at earlier ages should be considered in this group. We did not obtain information about cirrhosis in all HBV-infected patients with HCC before age 50, but less than half of those with such information had cirrhosis (15/34). Peru contained the highest rate of HBV-related HCC (35%), making it the most common risk factor for HCC in the country. Of those individuals from Peru with specific information about area of origin (N=24), 45% were from the Amazonian region which has a higher prevalence of HBV [3]. Mode of transmission could also play a role in early HCC, but this was not assessed in our study.

Interestingly, the most frequent HBV genotype in South America is F and a significant association between HBV genotype F and early HCC occurrence has been found in Alaska natives [3, 4]. It is possible that the viral genotype had a role in early HBV-associated HCC in our cohort. However, our centers did not perform HBV genotype and sequence-specific studies should be performed addressing this question.

The diagnosis of HCC at an early age in individuals infected with HBV in Africa has been attributed to a synergy between HBV and dietary aflatoxins, which is thought to induce mutations in the TP53 gene. However, aflatoxins have not been thought to play a role in South America, although one study found aflatoxin-associated p53 mutations in HBV-related HCCs [5]. Other factors such as insertional mutagenesis or family history could play a role in early HCC [6]. However, we could not assess for these variables in our study. A larger, comprehensive study is needed to further understand the clinical implications of HBV infection in HCC development in South America.

Key Words
Hepatocellular Carcinoma; South America; Hepatitis B

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3

Prevention of HCC

CHAPTER 3A

Evaluation of Rapid Diagnostic Tests for Assessment of Hepatitis B in Resource-Limited Settings

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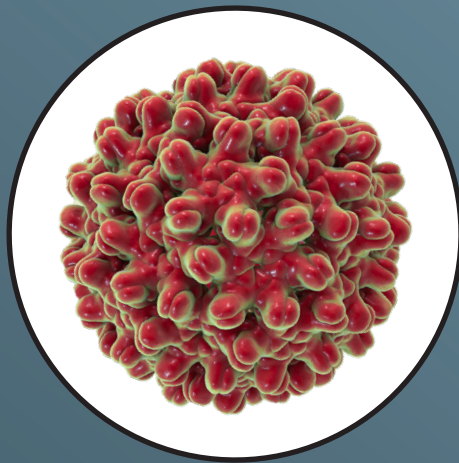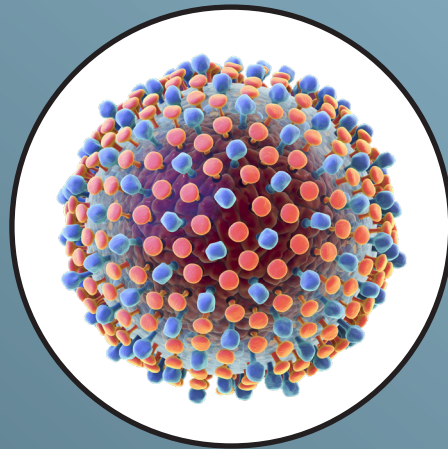
Chronic Hepatitis B virus (HBV) infection is the most frequent cause of liver disease and hepatocellular carcinoma (HCC) worldwide, with most cases occurring in resource-limited settings [3]. Early diagnosis is critical in reducing hepatitis-related morbidity and mortality. Ironically, regions of the world with the largest HBV burden (i.e. Africa) are the regions with suboptimal laboratory infrastructure, leading to lack of diagnosis [4]. Past studies of HBV rapid tests have shown highly variable results and elevated costs for these tests make them often un-affordable in resource-limited regions [1, 2]. We evaluated the efficacy of low-cost rapid diagnostic tests (RDTs) for HBV, in Europe, Africa and South America.

We performed an external validation of RDTs designed for the detection of various HBV serological markers (PRECHEK Bio. Inc., Korea). These RDTs were selected because of their low-cost (approximately 1–2% of the cost of WHO recommended RDTs) and their ability to be used for point-of-care diagnostics. These RDTs are immunochromatographic assays in which monoclonal antibodies against specific antigens or antibodies are immobilized on the test line of a nitrocellulose membrane pad. In positive tests, as serum/blood is added, the antigen-antibody complex migrates towards the test zone (T) where it is captured by immobilized antibodies, forming a visible line. In negative tests, the antigen or antibody is absent and there is no visible line.

HBV serological markers tested included HBV-surface antigen (HBsAg) HBV-surface antigen antibody (anti-HBsAb), HBV E antigen (HBeAg) and HBV E antibody (anti-HBeAb). Serum and whole blood samples used for testing were obtained from repositories (stored at -80C) in hospitals in the Netherlands, Argentina and Ethiopia. Testing was discontinued in RDTs that performed poorly during initial assessment. The performance of RDTs was assessed by ROC curve analysis, using the local diagnostic standard as the reference test (Argentina: ARCHITECT Reagent kits [Abbott, Germany]; Netherlands: LIAISON XL system [Diasorin, Italy]; Ethiopia: Onsite Rapid Test [CTK Biotech, USA]. Statistical analyses were performed using STATA v15.1 (Statacorp, College Station, TX.).

A total of 200 unique serum and whole-blood samples were tested using RDTs. The median age of patients was 40 years (IQR 31-50) and 67% were male. HBV genotypes A-F were tested. The HBsAg serum strip had a sensitivity and specificity of 100%. The median HBsAg level of tested samples was 2800 IU (range: 150-110,000). The anti-HBeAb serum cassette had a sensitivity of 80% and a specificity of 100%. The HBsAg whole-blood cassette and strip had specificities of 100%, but sensitivities of 56% and 45%, respectively. The anti-HBsAb serum cassette had a sensitivity of 57% and a specificity of 93%. The anti-HBsAb serum strip had a sensitivity of 20% and a specificity of 100%. The HBeAg serum strip had a sensitivity of 81% and a specificity of 67%. The median HBeAg level of tested samples was

2806 IU/ml (range: 1952-3149). Specific RDT performance is available in **Table 1**.

Table 1. Rapid Diagnostic Test Performance

Test Type (Catalog Number)	Number[1] Tested (T/P/N)	Test Site[2] (A/E/N)	Age[3]	Male	Sensitivity	Specificity
HBsAg Serum Strip (HBV 211)	81/55/26	A/E/N	39	74%	100%	100%
HBsAg WB Cass. (HBV 214)	23/16/7	A/N	43	70%	56%	100%
HBsAg WB Strip (HBV 213)	13/11/2	A/N	42	54%	45%	100%
Anti-HBsAb Serum Cass. (HBV 222)	38/23/15	N	52	58%	57%	93%
Anti-HBsAb Serum Strip (HBV 221)	46/20/26	N	38	80%	20%	100%
Anti-HBeAb Serum Cass. (HBV 232)	64/20/44	N	37	63%	80%	100%
HBeAg Serum Strip (HBV 242)	27/16/11	A/N	39	81%	82%	67%

[1]T=total, P=known positive, N=known negative; [2]A=Argentina, E=Ethiopia, N=Netherlands; [3]Median age. HBsAg, hepatitis B surface antigen; anti-HBsAb, hepatitis B surface antibody; HBeAg, hepatitis B e antigen; Cass., cassette; WB, whole-blood.

The HBsAg serum strip RDT demonstrated optimal sensitivity and specificity in the three different continents, indicating that it can reliably diagnose HBV in various populations with different genotypes. The anti-HBeAb RDT showed acceptable sensitivity and excellent specificity, making it useful to differentiate HBeAb status. Overall, whole-blood HBsAg and serum anti-HBsAb kits performed poorly, as they were specific but insufficiently sensitive to be clinically useful for screening. The serum HBeAg kits demonstrated acceptable sensitivity, but poor specificity, making them unlikely to be useful in the clinical setting. Our results suggest that HBsAg and anti-HBeAb serum RDTS are reliable, and in conjunction with alanine aminotransferase levels (ALTs), can be useful for diagnosis, as well as informing the need for treatment in resource-limited settings.

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CHAPTER 3B

Hepatitis B Screening in an Argentine Emergency Department: A Pilot Study to Increase Vaccination in a Resource-limited Setting

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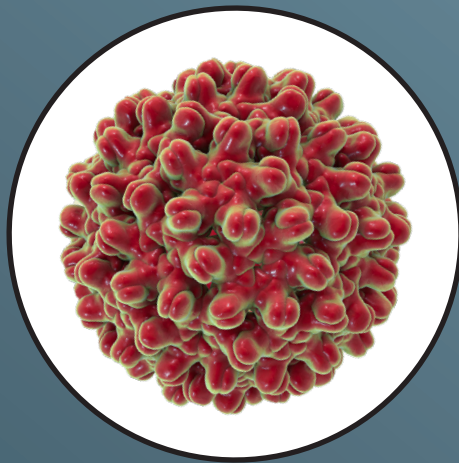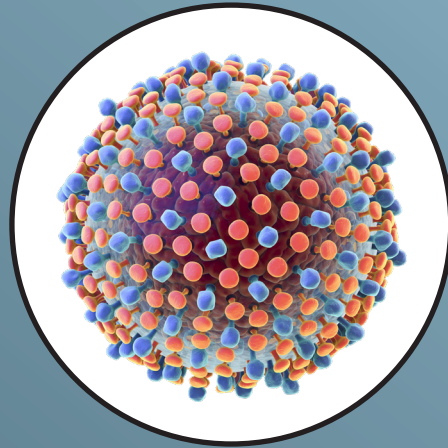
Abstract

Background
There is limited data regarding the use of emergency departments (EDs) for infectious disease screening and vaccination in resource-limited regions. In these settings, EDs are often the only contact that patients have with the healthcare system, turning an ED visit into an opportune time to deliver preventative health services.

Methods
In this pilot study, patients that met inclusion criteria were prospectively tested for hepatitis B surface antigen test (HBsAg). Previously unvaccinated patients who tested negative for HBsAg were offered HBV vaccination. The study setting was a public infectious disease hospital in Cordoba, Argentina. The primary outcomes were new HBV diagnoses, as well as vaccination completion between screening modalities (Point-of-Care-Testing-POCT vs. laboratory testing) and same vs. different day vaccination.

Results
We screened 100 patients for HBV (75 POCT & 25 laboratory). The median age of participants was 35 years (IQR 24-52) and 55% were male. No patients tested positive for HBsAg. All patients who completed first dose vaccination were initially screened with the POCT. No patients screened with laboratory testing returned for vaccination. Patients who were scheduled for vaccination the same day were more likely to complete vaccination compared to those scheduled for another day (75% vs. 14%, p<.001).

Conclusion
Our study supports the use of HBV POCTs in the ED in conjunction with vaccination of HBV-negative individuals. In regions with low HBV endemicity, direct vaccination without HBsAg testing may be more cost effective. We believe that this acute-care screening model is applicable to other resource-limited settings.

Key words
hepatitis B virus, rapid diagnostic test, point-of-care test, hepatitis B vaccination

BRIEF REPORT

Introduction

Background
Screening for infectious diseases during emergency department (ED) visits has been studied in resource-rich countries (1-4). However, data about this approach in resource-limited settings is scarce, with very few studies exploring the use of ED visits as a setting to link patients to preventative services (5-7). In Argentina, EDs are often the first, and only contact that patients have with the healthcare system, as many patients do not seek primary care services and instead seek acute-care services only when they become ill. This is of particular importance in young populations who are at high-risk for sexually transmitted infections (STIs), such as hepatitis B virus (HBV), that would benefit from vaccination. Testing recommendations for HBV are similar between the Argentine Ministry of Health and the United States Centers for Disease Control and Prevention (CDC) (8, 9). We provide a comparative summary of these recommendations in Table 1.

Importance
The sequelae of untreated chronic HBV include cirrhosis and the development of hepatocellular carcinoma, both of which present insidiously and often do not manifest symptoms until they are irreversible or incurable (10). Similar to many countries, HBV was not added to the national Argentine vaccination schedule until the year 2000, leaving a large at-risk adult population susceptible to infection upon exposure (11). There have been subsequent efforts by the Argentine federal government to vaccinate unvaccinated adults, however, acute HBV incidence remained unchanged between 2007 and 2016, underlying the difficulty of vaccinating a young adult population (11, 12). While vaccination against the influenza virus in the ED has gained popularity in recent years, to our knowledge, there are no published data regarding vaccination for other infectious diseases, such as HBV, in an ED setting (13). As there is a significant time delay between when blood is drawn for HBV laboratory testing and a result being ready, we thought it was important to evaluate if the use of a HBV POCT test would provide superior linkage-to-care rates when compared to laboratory testing. Previous studies employing point-of-care testing for HBV in resource-limited settings have targeted linkage-to-care for treatment of HBV-positive persons, but have not addressed vaccination of HBV-negative persons (14). We believe that HBV testing in acute-care settings, with linkage to treatment if positive and vaccination if negative, can be an effective model of healthcare delivery in clinical settings with insufficient primary care services.

Goals of This Investigation
We aimed to explore the concept of HBV screening and vaccination during emergency

department visits. The primary outcomes were new HBV diagnoses, as well as vaccination completion rates between screening modalities (POCT vs. laboratory) and timing of vaccination (same vs. different day).

Methods

Study Design and Setting
In this prospective pilot study, we recruited patients who presented to the ED of Hospital Rawson between April and May of 2018. This is a public infectious disease hospital located in Cordoba, Argentina that services a population of approximately 3.3 million people (15). This ED evaluates approximately 120 individuals a day with an admission rate of 6%. The median age of patients seen in the ED at the study institution is 45 years, and 51% of patients seen are women. This study was approved by the medical education committee of Hospital Rawson.

Selection of Participants
To meet inclusion for the HBV screening portion of our study, individuals had to be ≥18 years and deemed clinically stable by ED staff. As many patients had no documentation of previous vaccination, self-reported previous vaccination was not considered an exclusion criteria, and patients were still offered HBV testing. To meet inclusion for the vaccination portion of our study, individuals had to meet inclusion criteria for the HBV screening portion of the study, have either a negative HBV test result (laboratory or POCT) or no previous history of vaccination (including self-reported), and have no contraindications to vaccination. Following work-up and clearance by the ED provider, the research assistant approached individuals to offer participation in the study. All patients interested in participation underwent informed consent. Patients were recruited on weekdays between the hours of 8am and 5pm.

Interventions
In order to not disrupt ED workflow and efficiency, patients were screened for HBV using either HBV laboratory or a point-of-care test (POCT) using a practical sampling method. In patients who had a clinical indication for a blood-draw (as determined by the ED provider), the research assistant added HBV serology to their lab orders. In patients who did not have an indication for a blood-draw, the research assistant performed the POCT. This sampling method was chosen in favor of randomization because it preserved ED workflow by preventing the extension of ED patient wait times for patients who did not require blood draws. Point-of-care testing was performed using the PRECHEK Bio. Inc. HBsAg serum strip (Cat. NO. HBV 213, South Korea).

Patients who tested negative for HBsAg using the POCT and who were seen during the vaccination center's operating hours (8am-2pm) were offered same-day vaccination. The vaccination center was located within the same facility as the ED. Patients who tested negative for HBsAg who were seen outside the vaccination center's operating hours and patients who tested negative for HBsAg with laboratory testing were offered different-day vaccination. Since the result of HBV laboratory testing typically took one week to return, patients who tested negative for HBV were called one week after testing with instructions to return for vaccination. Patients who did not respond after daily phone calls on three separate days were considered lost to follow-up. A conceptual map of HBV screening and vaccination in the ED is provided in **Figure 1**.
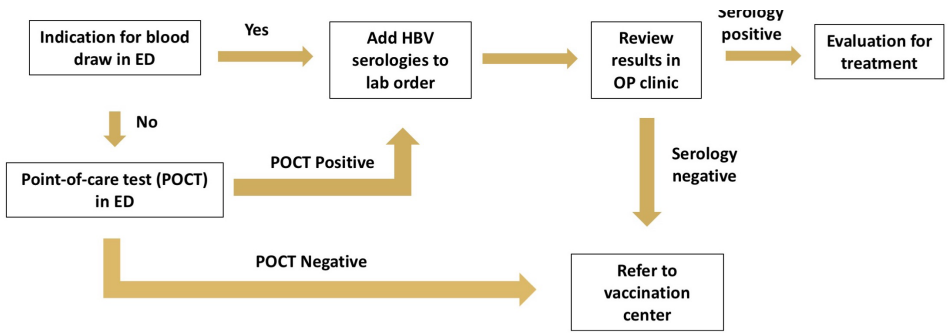
Figure 1. Model for hepatitis B virus screening in the emergency department. ED, Emergency Department; HBV, hepatitis B virus; POCT, point-of-care test; OP, outpatient.

Measurements
Demographic and clinical data were collected using standardized questionnaires at the time of patient enrollment. As the POCTs used in our study gave a result in 10 minutes, this data was recorded immediately by the research assistant. The results of the traditional laboratory testing were recorded by the research assistant when they became available (~typically one week later). The vaccination completion data were acquired by the research assistant after consulting the vaccination center's digital vaccination registry.

Outcomes
The primary outcomes were new HBV diagnoses, as well as vaccination completion rates between screening modalities (POCT vs. laboratory) and timing of vaccination (same vs. different day).

Analysis

Statistical analyses were performed using STATA v15.1 (Statacorp, College Station, TX.).

Results

Characteristics of Study Subjects

We screened 100 patients for HBV. The median age of participants was 35 years (IQR 24-52) and 55% were male. Forty-one percent of individuals reported having no health insurance and 77% reported having no primary care provider. Fifty-six percent of patients reported having a doctor's visit within the last six months.

Table 1. Summary and comparison of hepatitis B virus testing recommendations from the Argentine Ministry of Health and the United States Centers for Disease Control and Prevention†

Overlapping Recommendations
• Individuals with elevated AST/AST of unknown etiology*
• Infants born to HBsAg positive mothers
• Household or have sex contacts of persons known to be HBsAg positive**
• HIV positive persons
• Individuals with a history of intravenous drug use***
• Individuals with a history of hemodialysis
• Pregnant women

Additional Recommendations	
Argentina	**United States**
• Individuals with occupational exposure to HBV (i.e. needle stick injury)	• Individuals who are the source of blood or body fluid exposure (i.e. sexual assault, needlesticks)
• Individuals with a history of another parenterally-transmittable hepatitis virus	• Individuals born in intermediate (HBsAg prevalence ≥2%) and high-endemicity (≥8%) countries
• Individuals with a history of another sexually-transmitted infection or with history of sexual intercourse without barrier contraception	• US-born individuals whose parents were born in high-endemicity countries
• Individuals who received a blood product transfusion prior to 1993	• Men who have sex with men (MSM)
	• Individuals requiring immunosuppressive therapy
• Individuals with a history of recent invasive medical or surgical procedures within the last 6 months	• Donors of blood products, organs, tissues or semen

Hepatitis Risk Factors

Twenty-two percent of individuals reported previous HBV testing (all negative), and 13 patients reported previous vaccination. Thirteen individuals presented to the ED for STI testing due to the presence of concerning symptoms or a recent high-risk exposure. Nine individuals reported having surgery or blood transfusions before 1992 [the year Argentina began testing for hepatitis C]. Two individuals reported using intravenous drugs. Thirteen patients had a previous diagnosis of an STI and no subjects had a known history of HBV.

Screening and Vaccination

Seventy-five individuals were screened for HBV (HBsAg) using the POCT, and 25 were screened with standard HBV testing. None tested positive for HBsAg. Thirteen subjects reported previous vaccination, seven were lost to follow-up and five had a contraindication to vaccination (i.e. active fever). Of the 75 individuals who were eligible for vaccination and/or still engaged in care, 59 (79%) agreed to HBV vaccination. Of those who agreed to vaccination, 16 were scheduled for same-day vaccination and 43 were scheduled for a future date. Those who were scheduled for vaccination the same day were significantly more likely to complete vaccination compared to those scheduled for another day (75% vs. 14%, p<.001, **Table 2**). All of the subjects that completed vaccination had been screened using the POCT. No patient screened with traditional laboratory testing went on to complete vaccination.

Table 2. Hepatitis B virus screening and vaccination outcomes

Outcome	POC Testing (n=75)	Standard Testing (n=25)	All (n=100)
Positive Test Result	0/75 (0%)	0/25 (0%)	0/100 (0%)
Vaccination Status			
Previous vacc.	7/75 (13%)	6/25 (24%)	13/100 (13%)
Contraindication to vacc.	4/75 (5%)	1/25 (4%)	5/100 (5%)
Refused vacc.	13/75 (17%)	3/25 (12%)	16/100 (16%)
Lost to follow-up	0/75 (0%)	7/25 (28%)	7/100 (7%)
Agreed to vacc.	51/75 (68%)	8/25 (32%)	59/100 (59%)
Completed 1st vacc.	18/51 (35%)	0/8 (0%)	18/59 (31%)
Date of Vaccine Appointment			
Same day as test	16/51 (31%)	0/8 (0%)	16/59 (21%)
Completed 1st vacc.	12/16 (75%)	-	12/16 (75%)
Different day as test	35/51 (69%)	8/8 (0%)	43/59 (73%)
Completed 1st vacc.	6/35 (17%)	0/8 (0%)	6/43 (14%)

POC, point-of-care; Vacc., vaccination

Discussion

In our study, we did not diagnose any new cases of HBV. However, as Argentina is considered a country with low HBV endemicity (~0.7%), this is not surprising (16, 17). The median age of our study population was slightly younger than the median age of the whole ED population (35 vs. 45 years). One explanation is that older patients were more likely to present with unstable conditions, and thus were less likely to fit inclusion criteria for our study. The study population sex ratio was similar to the ED population. Interestingly, while most patients did not have established primary care providers, over half of the study population had seen a physician (non-PCP) within the last 6 months, indicating that most patients seek treatment in acute-care settings. This suggests that

ED visits may be an ideal setting in which to provide this population with preventative health services (18).

It is notable that of the 25 patients who were screened using traditional laboratory testing, none completed the first dose of the HBV vaccine. Since the result of laboratory testing took approximately one week, these patients had to be called with the result of their test, and were required to return to the hospital for vaccination. This led to a high rate of loss to follow-up, as scheduling providers were often unable to contact the patient following discharge from the ED. At our institution, the vaccine center was only open from 8am to 2pm, which proved to be a major obstacle, as this was during normal work hours and patients were often unable to take time off from work. Patients who were screened with POCT after the vaccine center closed needed to return at a later date to receive the first dose of their HBV vaccine, leading to a decreased rate of completion. Unsurprisingly, individuals with same day appointments were significantly more likely to complete vaccination. This observation is consistent with non-ED, community-based programs, which have demonstrated that POC infectious disease testing can significantly improve linkage to care (19, 20). Offering subsequent HBV vaccinations (2nd and/or 3rd doses) through the ED could be envisioned if patient volumes and resources allowed, but the feasibility of such a practice would likely be site-specific. Our results, albeit from a small cohort, are important in designing public health programs that target young patient populations, as difficulties with follow-up will require innovative strategies to effectively deliver preventive services.

We believe that the use of POCTs for diseases like HBV facilitates improved linkage-to-care because of the immediacy and actionability of the result. We found that non-use of POCT and limited vaccine center hours were barriers to initiation of vaccination. We believe that even higher rates of linkage-to-care can be achieved if an ED were to stock its own HBV vaccines, rather than directing patients to a separate vaccination area. While this study was performed in a country with a low HBsAg prevalence, we hypothesize that the potential preventative impact of ED-based vaccination programs would be substantially greater in high prevalence areas, where the risk of HBV infection of non-infected individuals is much larger.

As there was often a single research assistant available to enroll patients in the study, and the assistant only worked daytime hours (8am-5pm) on weekdays, ED patient volumes and staff scheduling inhibited our capacity to include all eligible and interested patients beyond these times. Our study did not assess for HBV surface antibody (HBsAb) to reflect HBV immunity, due to poor reliability of commercial rapid tests available in the country. This assessment would have led to a better understanding of those in need of vaccination. It is important to note that HBsAg screening may be negative in patients with resolved infections and those with previous vaccination. While our methodology may result in the vaccination of patients who are already immunized, we believe these patients represent a minority of those screened, and this issue is outweighed by the benefit of vaccinating at-risk, unvaccinated persons. Nonetheless, future studies should include HBsAb if reliable testing is available. Patients with new HBV infections in the "window" period, those with HBsAg mutants and some with chronic HBV infections may have negative HBsAg tests. However, the frequency of individuals in this category is likely to be low.

Our study supports the use of HBV POCTs in the ED in conjunction with vaccination of HBV-negative persons. However, in low-endemicity regions, direct vaccination without HBsAg screening may be more cost effective in high-risk patients who do not present with signs, or symptoms of viral hepatitis or liver disease. Future steps include performing a regional cost-analysis study to determine whether POC testing with subsequent vaccination or direct vaccination without testing, is most practical and affordable. While we believe that the simplicity of our approach makes it transferable and applicable to other resource-limited settings, due to income and resource differences worldwide, our results should be interpreted with caution when implemented in different geographical areas. Larger studies will be needed to further identify barriers for vaccine program development and effectiveness. Lastly, this acute-care screening and linkage-to-care model could be useful in screening for non-communicable diseases such as diabetes (POC HgA1c) and anemia (POC hemoglobin).

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Conflict of Interest Statement

The authors of this manuscript do not have any conflicts of interest to disclose.

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C H A P T E R   3C

Hepatitis B Awareness and Vaccination Patterns among Healthcare Workers in Africa

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Abstract

Hepatitis B virus (HBV) vaccination patterns and the understanding of its risks among health care workers (HCWs) is a critical step to decrease transmission. However, the depth of this understanding is under-studied. We distributed surveys to HCWs in 12 countries in Africa. Surveys had nine multiple-choice questions that assessed HCWs awareness and understanding of HBV. Participants included consultants, medical trainees, nurses, students, laboratory personnel, and other hospital workers. Surveys were completed anonymously. Fisher's exact test was used for analysis with a p-value of <0.05 considered significant. 1044 surveys were collected from Kenya, Egypt, Sudan, Tanzania, Ethiopia, Uganda, Malawi, Madagascar, Nigeria, Cameroon, Ghana, and Sierra Leone. HBV serostatus awareness, vaccination rate, and vaccination of HCWs children were 65%, 61%, and 48%, respectively. Medical trainees had higher serostatus awareness, vaccination rate, and vaccination of their children than HCWs in other occupations (79% vs 62%, p<0.001; 74% vs 58%, p<0.001; and 62% vs 45%, p=0.006, respectively). Cost was cited as the most frequent reason for non-vaccination. West African countries were more aware of their serostatus but less often vaccinated than East African countries (79% vs 59%, p<0.0001 and 52% vs 60%, p=0.03, respectively). West African countries cited cost as the reason for non-vaccination more than East African countries (59% vs 40%, p=0.0003). Our study shows low HBV serostatus awareness and vaccination rate among HCWs in Africa and reveals gaps in the perception and understanding of HBV prevention that should be addressed to protect HCWs and improve their capacity to control HBV infection.

Keywords
Hepatitis B, Africa, healthcare workers, vaccination, awareness

Introduction

Infection with Hepatitis B virus (HBV) is a leading cause of liver disease worldwide, affecting an estimated 240 million people.[1,2] Chronic HBV infection is an important cause of cirrhosis and hepatocellular cancer leading to significant morbidity and mortality across the globe. Recent data from the Global Burden of Disease Study shows that mortality attributable to HBV has risen from 1990 to 2013.[3] This is particularly concerning in sub-Saharan Africa where the seroprevalence of HBV is among the highest, with rates of infected individuals above 8%.[4] Although several countries are beginning to develop protocols to manage the hidden HBV pandemic, overall access to diagnostics and effective treatment remains poor.[5,6] Most African countries have implemented early-life vaccination to prevent complications of HBV,[7,8] however prevention of infection and vaccination of adults, particularly those at risk, remains inadequate.

Health care workers (HCWs) are known to have a higher risk of contracting HBV than the general population due to needle stick injuries and continuous contact with seropositive patients.[6] Transmission of HBV via a needle stick injury is thought to be two percent from HBV e antigen negative blood and 19% from e antigen positive blood,[9] and this leads to an estimated 66,000 infections among HCWs every year.[10] Standard precautions to mitigate this risk are present in many countries. However, full implementation of safe practices is still lacking in low and middle-income countries, such as those in sub-Saharan Africa,[11-15] with estimates showing that about one-third of HCWs are exposed to body fluids every year.[16] As such, awareness of this elevated risk of infection and prophylaxis against it are key to minimizing risk of infection.

Few studies have examined HCWs perception of HBV.[17-19] This is important as awareness and attitudes of HCWs towards HBV represent a double impact: prevention of HBV infection for HCWs themselves since they are a high-risk population, and understanding the overall impact of HBV in order to properly advise patients in terms of prophylaxis and transmission mitigation. A study from our group in northern Tanzania noted that about 90% of HCWs were not aware of their HBV serostatus and had not been vaccinated for HBV, with these results varying significantly depending on the type of medical occupation.[17] A group out of Adama, Ethiopia obtained survey data from their hospital staff and found that while 75% were aware that working in the hospital would put them at a higher risk of infection, their vaccination rate was only 25%.[18] In Yaoundé, Cameroon, 47% of HCWs showed awareness of HBV with significant differences between gender and an overall vaccination rate of 19%.[19] All these studies focused on specific populations and a continent-wide assessment of the understanding of HBV among HCWs in Africa is lacking. Meta-analyses have been done on studies performed in individual countries, but differing methodologies and survey questions preclude firm conclusions.[20,21] In this

study, we aimed to address this gap by performing the first pan-African assessment of HBV awareness among HCWs in the continent.

Materials and Methods

Development of a HBV awareness network

Multiple institutions throughout Africa were approached based on previous publications in the field; identification of interest in the field due to personal conversation or previous email contact; or discussion with trainees in scientific meetings. Individual participants from each institution were contacted via email following identification of interested subjects. Each email was personalized to an individual institution and contained information regarding the purpose of the survey and a specific timeline. Following responses to the initial email by interested institutions, further communication was via email or telephone and surveys were submitted electronically (to be printed) in the preferred language of the participating institution.

Surveys

We formed a research network aimed at understanding HBV awareness among HCWs and distributed paper surveys across hospitals in 12 countries in Africa as described above (Figure 1). All hospitals surveyed are considered teaching institutions with a varying number of clinical residents as well as medical and nursing students. Inclusion to the network was achieved by email invitation to senior employees in hospitals from capital and non-capital cities across the continent. A minimum of 20 surveys and more than two occupations per hospital (physician, nurse, laboratory technician, and so forth) were requested. The survey consisted of nine multiple choice questions, assessing knowledge of modes of HBV transmission and existing treatment as well as the participants' awareness of their serostatus and increased risk of disease, vaccination status, reasons for non-vaccination, children's vaccination status, family members with HBV, occupation, age, and sex (Figure 1, supplement). To indicate their occupation, participants chose from either intern, resident, registrar, consultant, assistant medical officer (AMO), nurse, AMO student, nursing student, laboratory technician, and other occupation not listed. An intern is defined as a person who is in his or her first year of training after finishing medical school. A registrar is defined as someone who has finished intern year and works in a specific specialty but is not a consultant in that specialty. A resident is defined as someone who has finished intern year and is training to be a consultant in a specialty. Henceforth, interns, residents, and registrars are collectively referred to as medical trainees. An AMO is similar to a physician assistant, having completed advanced medical training after working as a clinical officer and then working independently.[22] The surveys were written in English, Swahili (regional language of East Africa), and

French (one of the official languages of Madagascar). To maximize participation, surveys used simple language and easy-to-follow formatting. They were distributed to medical and non-medical support staff with anonymous collection arranged either by drop box or to a staff member collecting surveys.

Egypt: 106
Sudan: 100
Ethiopia: 161
Kenya: 14
Uganda: 130
Tanzania: 102
Malawi: 66
Madagascar: 27
Nigeria: 151
Sierra Leone: 98
Ghana: 48
Cameroon: 41

Figure 1. Survey distribution by country. Countries included in the study and the number of surveys obtained per country.

Ethical approval

Participation in the study was voluntary and non-paid. The study was approved by the ethics committee of Hennepin Healthcare in Minneapolis, USA, and each center obtained their respective institutional research board approval.

Statistical analysis

Analyses was performed using Fisher's exact test with a p-value of <0.05 considered

significant. For the analysis, Kenya, Sudan, Tanzania, Ethiopia, Uganda, Malawi, and Madagascar were considered part of East Africa, and Nigeria, Cameroon, Ghana, and Sierra Leone were considered part of West Africa. Comparisons were made between the various populations of interest using Statistical Analysis System Enterprise Guide 4.3.

Results

Demographics

A total of 1044 surveys were collected from 12 African countries, which included Kenya, Egypt, Sudan, Tanzania, Ethiopia, Uganda, Malawi, Madagascar, Nigeria, Cameroon, Ghana, and Sierra Leone (Table 1). Of all participants, 92% (n=959) completed the survey in English, 6% (n=58) in Swahili, and 2% (n=27) in French. Female participants accounted for 54% (n=558) of the responders, and the median age of the participants was 30 years (IQR 25-37). Twenty-four percent (n=248) of participants were nurses, 18% (n=189) medical trainees, 11% (n=112) laboratory personnel, 16% (n=162) nursing students and AMO students, 6% (n=60) consultants, and 4% (n=38) were AMO's. The remaining 22% (n=225) were a combination of staff performing different duties in the hospital (janitor, midwife, medical technician, pharmacy technician, and housekeeping). Overall responses to the survey questions by all participants are presented in Table 2.

Table 1. Demographics of survey participants

Surveys	1044
Completed in English	959 (92%)
Completed in Swahili	58 (6%)
Completed in French	27 (2%)
Female responders	558 (54%)
Male responders	483 (46%)
Median age in years (IQR)	30 (25-37)
Nurses	248 (24%)
Medical trainees	189 (18%)
Laboratory personnel	112 (11%)
Nursing students	82 (8%)
Assistant Medical Officer (AMO) students	80 (8%)
Consultants	60 (6%)
Assistant Medical Officers (AMO)	38 (4%)
Other (janitor, midwife, medical technician, pharmacy technician, housekeeping)	225 (22%)

Results are expressed as n (%) or median (IQR)

**Table 2.** Participant responses regarding HBV

HBV serostatus aware	673 (65%)
Vaccinated against HBV	627 (61%)
Cost as a reason for non-vaccination	167 (46%)
Lack of awareness as a reason for non-vaccination	81 (22%)
Other reasons for non-vaccination	119 (32%)
Correct understanding of HBV transmission routes	881 (84%)
Family member with HBV	45 (4%)
Aware of increased risk of HBV	988 (95%)
Children vaccinated	221 (48%)
Aware of available treatment for HBV	689 (66%)

Results are expressed as n (%)

HBV serostatus awareness

Participant awareness of their HBV serostatus was relatively low at 65% (n=673) with considerable variability among occupations and geographical location. Medical trainees had the highest awareness and were significantly more aware of their HBV serostatus than that of the other occupations combined (79% vs 62%; p<0.0001, Figure 2). Awareness among students (AMOs and nursing) was significantly lower than that of other occupations combined (56% vs 67%; p=0.01). Laboratory personnel's awareness of HBV serostatus was not significantly different than the other occupations (73% vs 64%; p=0.06). Serostatus awareness among nurses was not significantly different than the rest of the occupations (65% vs 65%; p=1.0). Upon performing country-specific analyses we found a significant variation among countries with regards to HBV serostatus awareness, with the lowest being in Madagascar at 30% and highest in Cameroon at 95% (Figure 3). When this was analyzed geographically, we found that participants in West Africa had a significantly higher serostatus awareness than those from East Africa (79% vs 59%; p<0.0001, Figure 4).

HBV vaccination rate

Vaccination rate among participants averaged 61% (n=627), with medical trainees having a significantly higher rate of HBV vaccination compared to other occupations (74% vs 58%; p<0.001, Figure 2). Vaccination rate among students was significantly lower than that of other occupations (50% vs 63%; p<0.01), and laboratory personnel also had a significantly lower rate of vaccination compared to the rest of the population (49% vs 62%; p=0.01). Nurses' vaccination rate was not significantly different than that of the rest of the occupations (61% vs 61%; p=1.0). Country-specific analyses showed that the vaccination rate was the lowest in Madagascar at 15% and highest in Kenya at 93% (Figure 3). Interestingly, geographical analysis showed that West African countries had a lower rate of vaccination than East African ones (52% vs 60%; p=0.03, Figure 4).
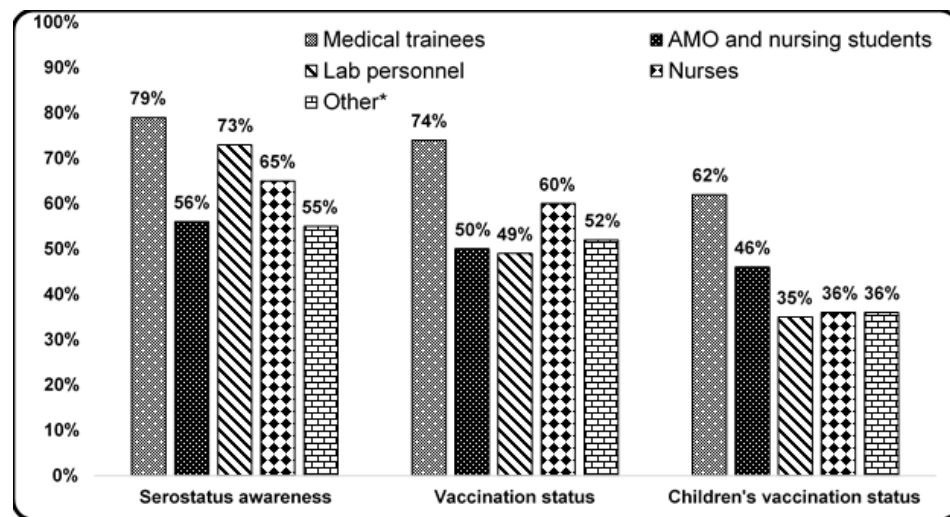
*Janitor, midwife, medical technician, pharmacy technician, housekeeping

Figure 2. Awareness of serostatus, vaccination, and children's vaccination by occupation. *Responses to survey questions of serostatus, vaccination, and children's vaccination by occupation.*
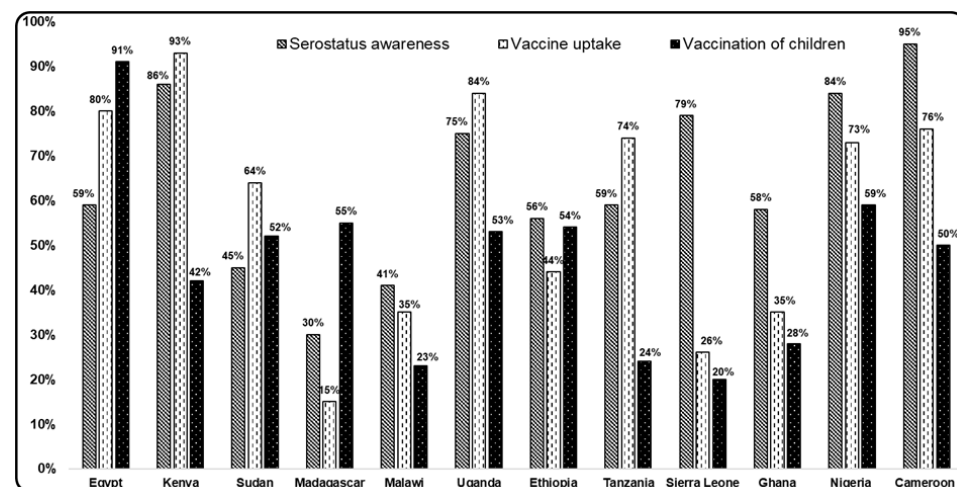

Figure 3. Participant responses by country. *Responses to survey questions on serostatus, vaccine uptake, and vaccination of children by country.*

Reasons for non-vaccination

Among the participants who provided a reason for non-vaccination, cost was the most frequently cited at 46% (n=167), while lack of awareness was indicated by 22% (n=81) of the participants (Table 2). Other reasons such as unavailability, negligence, too busy, procrastination, and complacency accounted for the remaining 32% (n=119). When

analyzed by occupation, medical trainees reported vaccine unawareness significantly less often in comparison to the rest of the occupations combined (5% vs 22%; p=0.007, Figure 5), but they reported "other reasons" significantly more than the other occupations (52% vs 32%; p=0.01). None of the occupations reported cost or "other reasons" as a reason for non-vaccination more often than the other occupations combined (Figure 5). When reasons for non-vaccination were compared between West and East Africa, unawareness was not significantly different (22% vs 23%; p=0.9, Figure 4), however West Africans cited cost as a reason for non-vaccination more than East Africans (59% vs 40%; p=0.0003, Figure 5). East Africans were significantly more likely to report "other reasons" for non-vaccination than West Africans (37% vs 18%; p=0.0002, Figure 4).

HBV vaccination of children

Among all participants, 50% (n=516) reported having children, with the vaccination rate among their children being 48% (n=221) (Table 2). When this was analyzed by occupation, children of medical trainees had a significantly higher rate of vaccination than the rest of the children (62% vs 45%; p=0.006, Figure 2). The vaccination rate of children of laboratory personnel and nurses was lower than that of children of the other professions, but the difference was not significant (35% vs 50%; p=0.07 and 41% vs 51%; p=0.06, respectively). Country-specific analysis showed that children's vaccination rates varied from a low of 15% in Sierra Leone to a high of 91% in Egypt (Figure 3). Regional analysis showed that the vaccination rate of children of West African HCWs was not significantly different than that of children of East African HCWs (40% vs 38%; p=0.7, Figure 4).
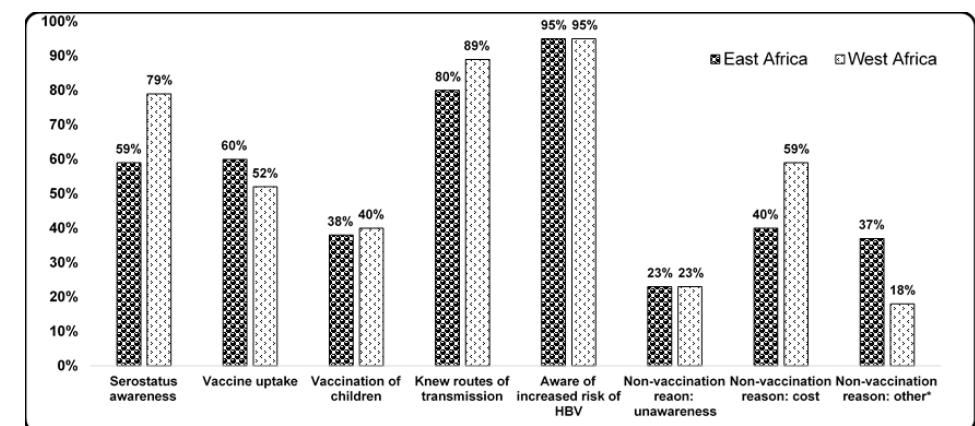
*Unavailability, negligence, busy, procrastination, complacency

Figure 4. Comparison of responses between East African and West African countries. *Responses to survey questions by countries in East Africa (Kenya, Sudan, Tanzania, Ethiopia, Uganda, Malawi, and Madagascar) and West Africa (Nigeria, Cameroon, Ghana, and Sierra Leone)*
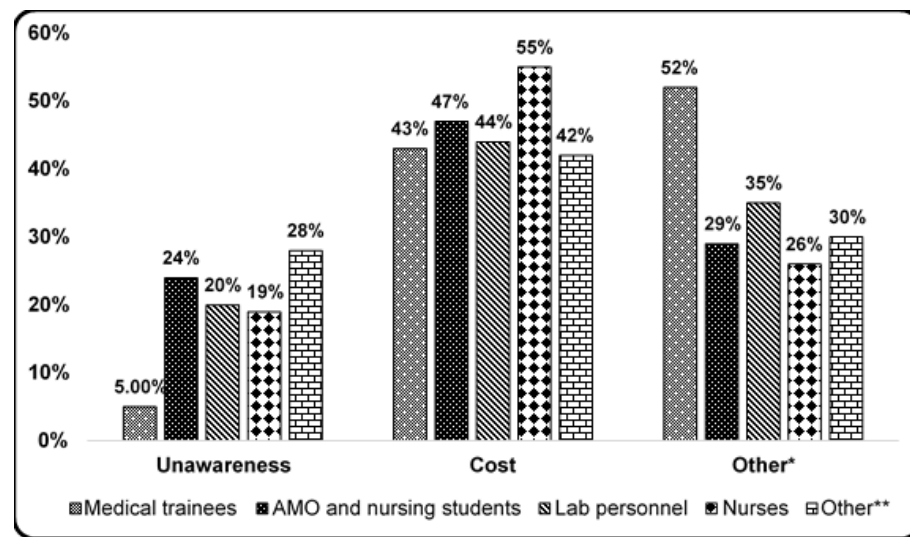
*Unavailability, negligence, busy, procrastination, complacency
**Janitor, midwife, medical technician, pharmacy technician, housekeeping

Figure 5. Reasons for not vaccinating based on occupation. *Survey participants' stated reasons for not vaccinating for HBV.*

*Janitor, midwife, medical technician, pharmacy technician, housekeeping

Figure 6. Awareness of transmission routes and risk of HBV by occupation. *Responses by occupation to survey questions on routes of transmission of HBV and increased risk of HBV.*
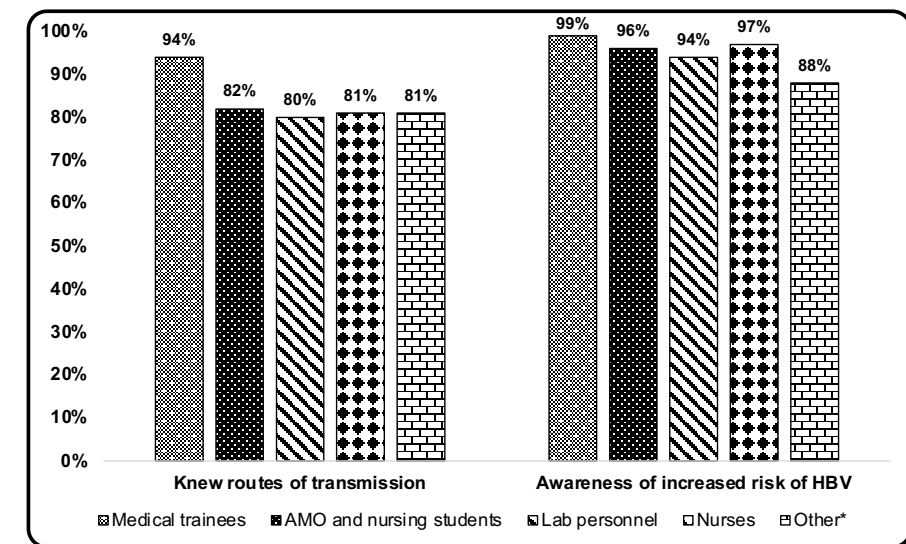
Understanding of HBV transmission route

Overall, 84% (n=881) of the participants correctly identified all routes of transmission (percutaneous, intercourse, and vertical; Table 2). When analyzed by occupation, medical trainees accurately identified all transmission routes significantly more often than the rest of the occupations (94% vs 83%; p<0.0001, Figure 6). Students' knowledge of transmission routes was not significantly different than the rest of the occupations (82% vs 85%; p=0.34), just as there was no significant difference between knowledge of transmission routes for laboratory personnel and nurses (80% vs 85%; p=0.13 and 81% vs 85%; p=0.13, respectively). Geographical analysis showed that West African countries properly identified all the routes of transmission significantly more often than East African countries (89% vs 80%; p=0.0002, Figure 4).

Awareness of increased risk of HBV due to hospital work

Of all responders, 95% (n=988) were aware of their increased risk of contracting HBV due to working in a hospital. Medical trainees and nurses were significantly more aware of that possibility compared to the rest of the occupations (99% vs 95%; p=0·001 and 98% vs 94%; p=0.02, respectively, Figure 6). Students' and laboratory personnel's awareness rate was not significantly different than the other occupations (96% vs 96%; p=1.0 and 94% vs 96%; p=0.32, Figure 6). There were no significant differences between West African and East African countries related to awareness of increased risk (95% vs 95%, p=0.9, Figure 4).

Discussion

Chronic Hepatitis B infection is a silent disease until it reaches advanced stages (cirrhosis and hepatocellular carcinoma) when therapeutic approaches are limited. This situation is aggravated in many regions of the African continent where resources are limited, HBV rates are high, and access to advanced health care such as liver transplantation is low.[23,24] HCWs are at increased risk of contracting HBV and are also in a strategic position to educate patients and raise awareness about the risks of HBV. Since it is a vaccine-preventable disease, understanding the risk factors for HBV and prioritizing vaccination of HCWs in resource-poor areas is critical.

Our study is the first pan-African assessment of HBV awareness among HCWs, and the results are striking. Analyses show that the overall HBV serostatus awareness and vaccination rate among HCWs is low, with significant variability among occupations. Even though over 95% of participants expressed understanding that working in a hospital increased their risk for HBV infection, there was a surprisingly low degree of HBV serostatus awareness. Our study found that physicians were more likely to be tested and vaccinated for HBV compared to the rest of the occupations (students, nurses, and laboratory personnel). Indeed, physicians were also more likely than other occupations to be aware of their increased risk of HBV transmission, to properly know all the routes

of transmission, and to be aware of the vaccine, likely explaining their higher serostatus awareness and vaccination rate. These results are consistent with a study conducted in Nigeria which found a higher level of knowledge, serostatus awareness, and vaccine uptake among physicians.[25] Similar results were obtained at a tertiary care hospital in Dar es Salaam, Tanzania and in Gondar, Ethiopia where physicians were more likely to be vaccinated than medical attendants and laboratory personnel.[26,27] Taken together, these findings are particularly concerning since outside of surgical specialties, needle stick injuries and contaminated fluid exposure are more likely to occur to nurses and laboratory personnel than physicians,[15,28,29] and thus, those populations are at a higher risk of occupational HBV exposure. Since adults may develop HBV immunity from natural exposure, one reasonable approach could be to withhold HBV vaccination in HCWs until serostatus is determined. However, most hospitals in Africa do not offer HBV screening to HCWs as a standard policy.[17] Given this inconsistency and the higher likelihood of needle stick injuries in Africa than elsewhere (due to unsafe needle practices),[29] primary prevention alone is not enough. Universal vaccination of HCWs, as the World Health Organization recommends,[30] should be thoroughly implemented.

Among participants who provided a reason for non-vaccination, cost was cited most often. Likewise, a study from Yaoundé, Cameroon revealed cost as one of the leading reasons for non-vaccination among HCWs.[19] However, this differs from a study conducted in Gondar, Ethiopia where participants stated vaccine unavailability as the major hurdle to vaccination.[27] A likely explanation for this trend could be related to geographical variation in HBV vaccine price. As such, it is important to note that our analysis showed that participants from West African countries cited cost as a reason for non-vaccination significantly more than East African countries. Thus, higher HBV vaccine prices may have led to the lower rate of vaccination observed in West African participants, despite their higher serostatus awareness and knowledge of routes of transmission. The price of the HBV vaccine, when provided by the Global Alliance for Vaccines and Immunization, has dropped steadily and in 2010 the cost for one monovalent dose of HBV vaccine was $0.18 U.S. cents.[31] However, this is usually the amount paid by health ministries to include the HBV vaccine in childhood immunization programs and not the cost paid by HCWs to obtain the vaccine. In general, vaccine costs vary significantly between countries (and depend on government-pharmaceutical company agreements), and HCWs who are not provided with vaccination by their employer and seek to purchase the vaccine in a private pharmacy, face much higher costs.

Our study found a low rate of vaccination among the children of HCWs, with children of physicians again demonstrating a higher rate of vaccination compared to the rest of the occupations. This is concerning since the likelihood of developing chronic HBV is much higher when individuals are exposed at an earlier age,[32] and our analysis

exposes a generational decrease in HBV vaccination rate from parent to child. Having baseline vaccination rates of children in the countries studied would be useful, but an explanation for the low rate of vaccination in children may be the lack of the birth dose of HBV vaccine. It has been well documented that administration of the first HBV vaccine dose at birth, instead of waiting several weeks after birth (which would increase the number of concurrent injections during future healthcare encounters), decreases parental anxiety and therefore increases the likelihood of completing the HBV vaccine series.[33] This policy is lacking in most sub-Saharan countries surveyed except Nigeria,[34] whose vaccination rate was higher than that of the rest of the sub-Saharan countries (59% vs 40%; p=0.006). However, even in countries where this policy is in place, there are significant barriers to the administration of the birth dose vaccine, such as unavailability of the monovalent HBV vaccine and a high proportion of home births.[34] It is possible, however, that a lack of specific understanding of vaccine administration by HCWs could have contributed to a negative answer (i.e. children have been vaccinated but the HCW does not know if it was specifically for HBV since the vaccine was grouped with those against other pathogens during the same visit). Since we did not request vaccination cards for children during the survey, it was not possible to clarify this variable.

Our study has several limitations. Among them is the fact that the majority of participating centers are medical institutions in major cities which may skew the data since knowledge and awareness are likely lower in smaller and rural institutions.[35] Moreover, we did not keep track of the number of surveys distributed and there were differences in the number of surveys received from each institution. This prevented us from calculating a response rate and it could have lead to misinterpretation of data from one center that provided a small number of surveys. Also, our study relies on survey data that is self-reported and is thus subject to recall and observational bias by participants. Moreover, the distributed surveys were brief and did not examine in detail the barriers for lack of awareness or vaccination. We did not perform analysis by age groups which could have some utility as well since it is possible that medical trainees, who are younger, may have a higher likelihood of being vaccinated. Finally, in most of the surveyed countries (apart from Egypt, Uganda, Nigeria, and Tanzania), we received surveys from only one institution. It is unlikely that one institution represents the current situation in the entire country (or city), however we expect the overall large number of surveys and wide distribution to compensate for this and provide a comprehensive outlook of the continent.

Our study shows low HBV vaccination rates and serostatus awareness among HCWs across Africa, with geographic variations and differences among occupations. Increasing parity across the continent by raising awareness of HBV and increased utilization of the vaccine among those at risk is vital. However, it is difficult for healthcare providers

to educate patients and promote HBV awareness and vaccination if they themselves do not completely understand the risks of HBV and benefits of vaccination. Ample evidence shows that increasing knowledge about the HBV vaccine improves vaccination rates by conveying its efficacy and allaying fears about safety and adverse effects.[26,36,37] In fact, participants in our center in Arusha, Tanzania requested the HBV vaccine from hospital administration after completing the survey and taking part in a HBV awareness seminar. Adding the HBV vaccine to mandatory onboarding requirements when hiring new HCWs would substantially reduce the rate of unvaccinated HCWs. This could be performed within existing hospital infrastructure or by extending the Expanded Program of Immunization, as shown by a study done in Kenya.[38] Lastly, awareness should be raised to make standard precautions universal to help prevent needle stick injuries and thus transmission of blood borne diseases such as HBV. In conclusion, our study provides valuable information by revealing gaps in knowledge, awareness, and vaccine availability that should be addressed to improve HCWs ability to prevent HBV infection.

Acknowledgements

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Supplementary files

Hepatitis B
-Please answer the following questions by placing an x in the lines
-This is anonymous, don't write your name on the paper

1) Have you been tested for Hepatitis B? Yes__ No__

2) Have you been vaccinated against Hepatitis B? Yes__ No__ I don't know__
   If not vaccinated, why not?
       -Did not know about a vaccine ___
       -Vaccine is expensive ___
       -Other reason ___

3) Do you know how Hepatitis B is transmitted?
   -Blood__
   -Sex__
   -Mother to child___
   -All of the above (blood, sex and mother to child)__

4) Do you have a family member with Hepatitis B at home? Yes__ No__

5) Are you aware that working in hospitals increases your risk of contracting Hepatitis B?
   Yes__ No__

6) What is your work?
   -Intern/Resident/Registrar ___
   -Consultant ___
   -AMO__
   -AMO student__
   -Nurse__
   -Nurse student__
   -Laboratory__
   -Other__

7) Do you have children?   Yes__ No__
   If you have children: Have they been vaccinated for Hepatitis B? Yes__ No__

8) Do you know if there is treatment for Hepatitis B?   Yes__ No__

9) Your age___

10) Male__ Female ___

Figure 1 Supplement. Hepatitis B survey in English. *Hepatitis B survey in English that was distributed to the healthcare workers.*

CHAPTER 3D

Promoting Hepatitis B Awareness: Evaluating an Educational Approach through Health Care Workers in Tanzania

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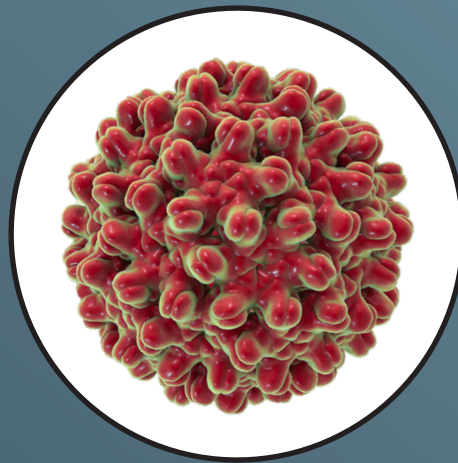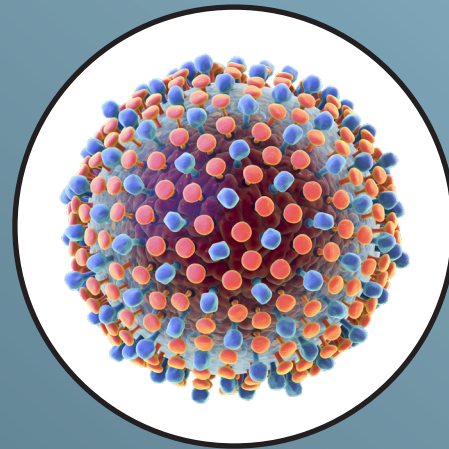
Abstract

Background
Hepatitis B virus (HBV) infection disproportionally affects populations in sub-Saharan Africa. Lack of HBV awareness perpetuates disease burden in Africa.

Objective
To promote HBV awareness in Tanzania using a systematic, measurable, and expandable approach to educating health care workers (HCW)

Methods
We designed and implemented an HBV knowledge and teaching skills session in southern Tanzania to empower HCWs in leading education to promote disease awareness in their communities. Training was divided into two sessions: didactic and practical. A five-question, anonymous survey was distributed in person immediately before and after the practical portion of the training to evaluate HBV knowledge as well as specific skills for teaching. Differences between responses before and after the sessions were evaluated by Chi-Square analysis. A sub-group of questions were further analyzed for differences based on HCW self-report of HBV serostatus awareness.

Findings
130 HCWs participated in the didactic lecture and 30 HCWs participated in both portions. A pre-post training five-question survey showed an increase in correct answers for all questions, with two showing statistical significance: HBV is silent (7% pre vs. 87% post; p < 0.0001), and repetition as key to promote awareness (63% pre vs. 100% post; p = 0.0002).

Conclusions
Our low-cost intervention is applicable to increase HBV awareness in low resource settings across Africa.

Background

Despite the introduction of universal vaccination and effective antiviral therapies, hepatitis B infection (HBV) still causes a high burden of disease in sub-Saharan Africa.[1] Unlike other chronic infectious diseases (i.e. HIV), there is a lack of awareness about HBV in communities across the continent. Indeed, data from our studies and others have found gaps in African health care workers' (HCW) knowledge about HBV.[2] Awareness about HBV among the general public is even lower; globally, roughly 10.5% of people with chronic hepatitis infection know their HBV status.[3] In chronic HBV, awareness is critical as the majority of infected individuals are asymptomatic until they develop cirrhosis or hepatocellular carcinoma (HCC), at which point those living in resource-limited settings are presented with very few options to prolong survival or improve quality of life. Community education is an essential, durable and sustainable solution to tackle the needs of preventing HBV and its complications; education can lead to vaccination and testing with appropriate linkage of patients to health care before irreversible sequelae of HBV occur.[1] However, promoting disease awareness in low resource settings is challenging. Barriers include low perception of personal risk, stigma and priority of emergency over preventive health-seeking behavior.[4] Even among screened populations with self-reported awareness of HBV-positive status, 43.4% did not follow up with a health care professional for monitoring or treatment.[5] We approached this challenge by designing a workshop to empower HCWs with high yield information about HBV while highlighting skills for teaching and engagement at the community level. Our approach relied on empowering HCWs at every level with specific HBV knowledge and teaching skills. HCWs are trusted members of society and can play an important role in increasing awareness in their respective communities.

Methods

Training session and setting

We designed and implemented a training session consisting of two portions: an initial didactic lecture on the current state of HBV globally and a practical "training the trainer" approach strategically scheduled after the didactic lecture. The session was implemented in Iringa, southern Tanzania, in the setting of an annual conference with 130 multi-disciplinary attendees from several regions of the country. The first portion described multiple aspects of HBV and was performed in a one-hour period. It focused, as described below, on expanding knowledge regarding the disease, intended for a professional audient of HCW with use of medically accepted language. The didactic lecture reviewed HBV epidemiology as well as the impact of its complications: cirrhosis and hepatocellular carcinoma, emphasizing that 25% of HBV-related deaths worldwide

occur in Africa. We briefly covered diagnosis using specific testing modalities and indications for treatment and therapy options, particularly those available in the region. We emphasized both how to protect the patient living with HBV, promoting simple cares to minimize progression of disease, and how to protect close contacts. We counseled on specific interpersonal activities that do not transmit the virus and we countered misinformation about how the virus is spread amongst individuals. We further focused on preventable targets (screening for HCC, decrease in alcohol intake, etc) sharing evidence-based tools that could be of use in resource-limited areas.

The second portion, divided participants into small groups, focused on approaches to teaching about HBV using common language to engage the community. This portion was interactive and used adaptive teaching methods. The specific objective was to provide visual tools for HCWs to increase HBV awareness in their settings. Four major health messages about HBV were taught: hepatitis B is common, silent, preventable and complications can be manageable. Further specifics emphasizing each point included: a) A visual of the HBV prevalence in Tanzania displayed 8% or 8 in 100 Tanzanians has HBV; b) description of how the virus typically does not cause symptoms until it has progressed late in the course with sequelae of cirrhosis or hepatocellular carcinoma; c) Prevention described in two-fold: knowledge about transmission and universal vaccinations for newborns, health care professionals and ideally the large majority of the population; d) Management of HBV, focusing on prevention of complications including avoidance of smoking, alcohol and aflatoxins. Skills shared about teaching included speaking clearly, intentionally using short sentences, emphasizing through repetition, engaging participants in active learning and practicing teaching techniques frequently. An example short 3-minute video of a physician teaching about HBV was shown during the workshop with a corresponding outline of talking points to emulate. Participants were encouraged to disseminate information in clinic or dispensary waiting rooms where patients in sub-Saharan Africa already spend large periods of time awaiting medical consultation or assistance. The remainder of the workshop was utilized to address participant questions and distribution of health education handouts to be used in their individual health systems (Figure 1).

Survey design and analysis
To evaluate immediate effectiveness of the teaching session, a 5-question multiple choice survey covering HBV knowledge and teaching techniques was administered before and after the workshop. Demographic information gathered on the survey included age, sex, profession and awareness of personal HBV serostatus. The survey consisted of five questions examining different aspects of HBV teaching and knowledge including amplifying the message through repetition, importance of serostatus awareness, approach to teaching, common HBV complications in Africa, and vaccination. The

surveys were distributed in-person and filled out on paper immediately before and after the second portion of the teaching session, kept anonymous and collected in bulk to ensure the lack of visual identification of respondents. Differences between responses before and after the sessions were evaluated by Chi-Square analysis. A sub-group of questions were further analyzed for differences in comparison to HCW self-report of HBV serostatus awareness.
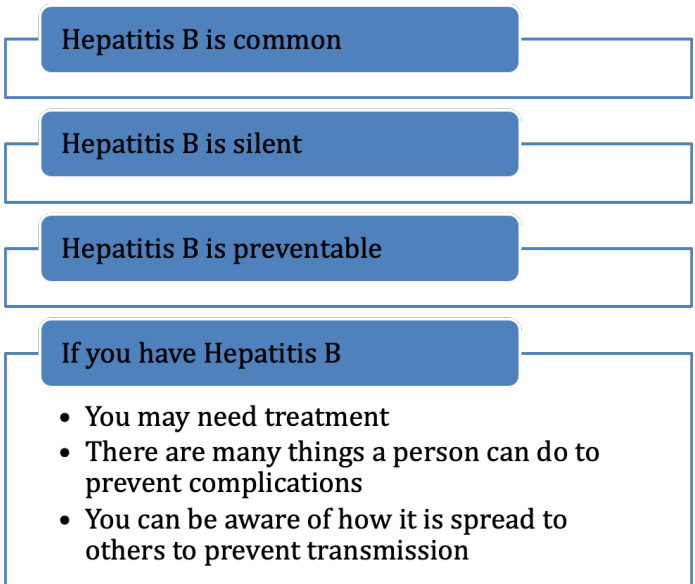
Figure 1: Workshop Messaging

Results

Survey analysis
A total of 130 multidisciplinary HCWs were involved in the first portion of the teaching session and 30 HCWs were involved in both the didactic and practical portions. The latter cohort completed the written survey and consisted of 25 nurses, 3 doctors, and 2 pharmacists. As expected, all respondents performed better on the post-workshop test (Table 1). In two of the survey questions, the improvement was statistically significant. This included evaluating that HBV is silent (7% pre-course vs. 87% post-course; $p < 0.0001$), and repetition as key to promote awareness (63% pre-course vs. 100% post-course; p = 0.0002). 100% of doctors, 60% of nurses and 50% of pharmacists self-reported awareness of personal HBV serostatus. Interestingly, after correction for self-reported awareness of HBV status, the differences remained significant for both of those questions (11% pre-

course vs. 86% post-course, p<0.0001) and (63% pre-course vs. 100% post-course, p=0.009), respectively. Four health care workers corresponded with questions and updates about educational sessions in their setting after an email was sent to all workshop participants one month later.

Table 1: Survey results evaluating hepatitis B knowledge and teaching skills

	Pre-Workshop Correct (%)	Post-Workshop Correct (%)	P Value
Question #1[a]	7%	83%	p<0.001
Question #2[a]	83%	90%	NS[b]
Question #3[a]	63%	100%	p=0.0002
Question #4[a]	57%	80%	NS[b]
Question #5[a]	77%	90%	NS[b]

[a]Which one of the following messages about hepatitis B is critical to tell the patient when counseling? (HBV is silent). 2-The importance of a person knowing about their hepatitis B status (prevent complications). 3-A simple and useful approach to teach about hepatitis B (repetition). 4-The most feared complication of hepatitis B in Africa (liver cancer). 5-Which of the following is NOT true (there is no vaccine for HBV)
[b]NS – Not Significant

Discussion

Our approach provides a low-cost HCW-led model that allows scalability and sustainability to promote HBV awareness ensuring that knowledge and tools for dissemination can reach a wide array of communities in sub-Saharan Africa. HCWs are familiar with their communities from their intimate work alongside patients and families in their individual settings and have gained the respect of their communities. The trust, language compatibility and familiarity with the landscape all make local HCW the ideal leaders for sharing educational materials. Moreover, they also have a familiarity of medical facilities in the community for specialty referral, knowledge of supply for vaccinations in the region and appropriate laboratories for testing referrals. We believe that resources centered on education will enable adoption of all other strategies for hepatitis B elimination such as universal vaccination, HBV screening, linkage to care for diagnosis and treatment, prevention of mother to child transmission and prevention of adult acquisition, similar to other infectious diseases like HIV.[6] This low-cost, one-time intervention methodology is easily applicable to other regions of the continent particularly in low resource settings with the ultimate goal of tackling morbidity and mortality from HBV in sub-Saharan Africa.

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4

HCC mechanisms and early detection

CHAPTER 4A

Single hepatocyte RNA sequencing in HCV-infected individuals evidence tumor-related transcriptomes early into the infection

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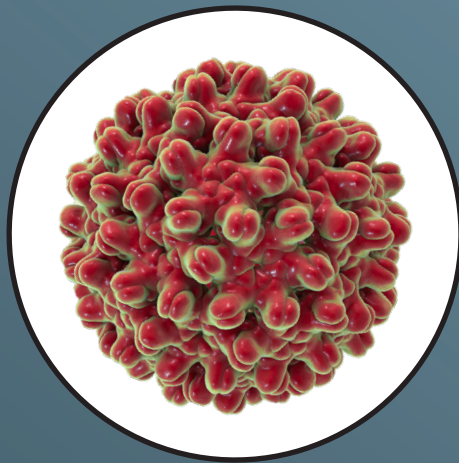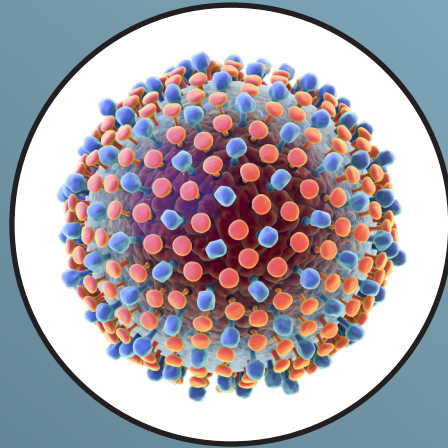
Hepatocellular carcinoma (HCC) related to hepatitis C infection (HCV) occurs almost exclusively in the setting of cirrhosis. HCV-infected individuals with cirrhosis have a 4% to 6% per-year risk of developing HCC[1]. The specific mechanisms leading to this process of viral-carcinogenesis are poorly understood and thought to occur once cirrhosis has become evident. Indeed, it is hypothesized that the progression from liver fibrosis to cirrhosis generates a local milieu that leads to HCC development, with hepatocyte injury and cellular rearrangement contributing to genetic hits that culminate in malignant transformation[2]. Nonetheless, several studies performed in mice have shown a more direct role of HCV in hepatocellular carcinogenesis in the absence of inflammation and hepatic cirrhosis[3]. Similar findings in humans have not been reported and are difficult to perform due to the challenges of hepatocyte isolation from patients. However, several reports of HCC in non-cirrhotic HCV-infected subjects exist[4]. To assess whether carcinogenic events take place in pre-cirrhotic livers, we performed single-cell RNA sequencing of primary human hepatocytes from HCV-infected individuals, applying a novel technique to differentiate HCV-infected and –uninfected hepatocytes within a patient.

Material from core needle liver biopsies was obtained from four treatment naïve HCV genotype 1-infected patients with F0-F1 fibrosis as evidenced by liver elastography and biopsy. All patients were negative for human immunodeficiency virus and hepatitis B virus. Hepatocytes were sorted as individual cells in 96-well plates by flow cytometry (BD FACS Aria) on the basis of their forward and sideward scatter, negativity for CD45 and CD3 using optimized in-house protocols. Single-cell RNA-seq libraries were generated using an adapted SMART-seq2 protocol, and raw RNA-seq data was processed using a Drop-seq analytic pipeline. In short, the adapter trimmed raw data files were aligned to reference genomes using STAR/2.5.1b-foss-2015b. Before gene quantification picard/2.2.2-foss-2015b-Java-1.8.0_74 was used to sort the aligned reads. Dropseq tools/1.12-Java-1.7.0_80 was used for flagging individual cells and unique molecular identifiers (UMI's). Confirmation of cells as hepatocytes was achieved by quantification of albumin expression in each cell. To evaluate infection of individual hepatocytes, sequences were aligned to that of the HCV genome (G1, NC004102)[5]. Ranking of the expression levels of HCV allowed for stratification into 3 categories: hepatocytes with low, medium or high probability of HCV infection based on <30%, 30-70% and >70% HCV expression level percentile. Absence of matching to the hepatitis B genome was used as negative control. Transcript-pathway identification was performed via ingenuity pathway analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG).

A total of 320 sorted hepatocytes from liver biopsies of four different HCV-infected patients underwent single-cell RNA sequencing. The number of detected genes, defined as UMI>0, ranged from 75 to >2200 (median 1008, IQR 518-1861) between individual cells from each patient. A total of 38 (12%) hepatocytes showed no evidence of HCV infection while 108

(33%) hepatocytes fitted criteria for definitive HCV infection (a ratio similar to that found by others via in-situ hybridization)[6]. Across all patients, HCV-infected cells showed a significantly more active transcriptome than uninfected cells, evidenced by the number of cells with more than 1000 genes expressed (95% in infected cells vs. 17% in non-infected cells). There was enough degree of heterogeneity in hepatocyte transcriptomes observed between patients to clearly identify each subject. In addition, the average expression level of genes in infected cells was substantially higher than levels in non-infected cells. Canonical pathway analysis exposed alterations in transcripts related to HCC among the top five most affected pathways in HCV-infected hepatocytes as compared to uninfected hepatocytes (the other pathways included: cardiac inflammation, lipid rearrangement, renal damage, liver cholestasis). When specifically assessing tumor-related transcripts, we observed an increased expression of HCC-related genes including TP-53, EGFR, AKT-2, PTEN and HRAS, among others, in HCV-infected hepatocytes, as well as a decreased expression of the tumor suppressor genes MAP2K4, RB1 and BCL11B (Figure 1). Transcripts related to the TP-53 pathway, such as BBC3, CCND1 E2F1 and PPMD1, were particularly up-regulated in cells with highest expression of HCV.
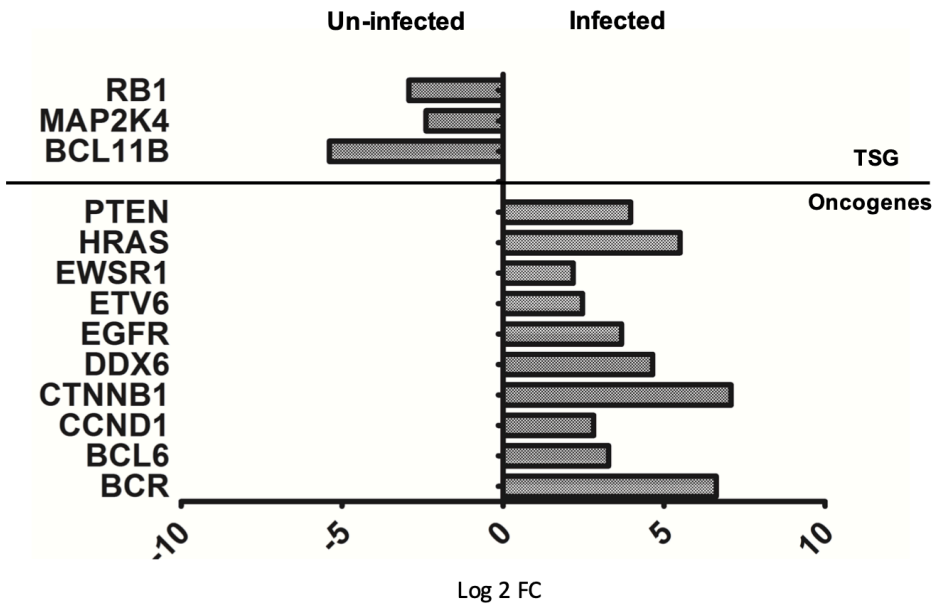
Figure 1. Transformed log-expression of tumor suppressor genes (TSG, left upper panel) and oncogenes in (right lower panel) in HCV-infected hepatocytes

Our findings show a differential expression of tumor-related and tumor-suppressor transcripts in single hepatocytes infected with HCV compared to those uninfected.

Since all four individuals in the study had F0-F1 fibrosis, our findings suggest that the mechanisms related to HCC development during HCV infection arise earlier than the development of cirrhosis or advanced fibrosis. Further studies will be necessary in larger numbers of subjects and cells. However, our data exposes novel insights into pre-cirrhosis carcinogenic mechanisms in individuals infected with HCV.

Keywords
single cell, hepatocyte, HCV, cancer

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C H A P T E R   4B

Serum biomarkers for the prediction of hepatocellular carcinoma

Review

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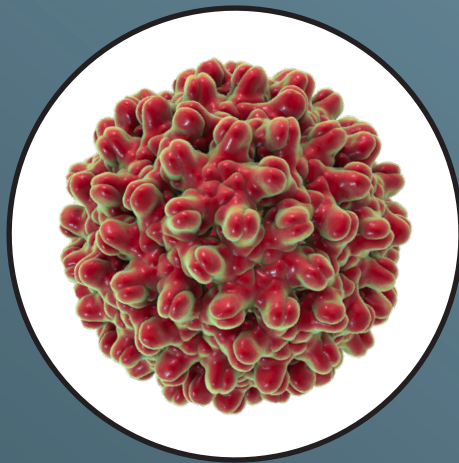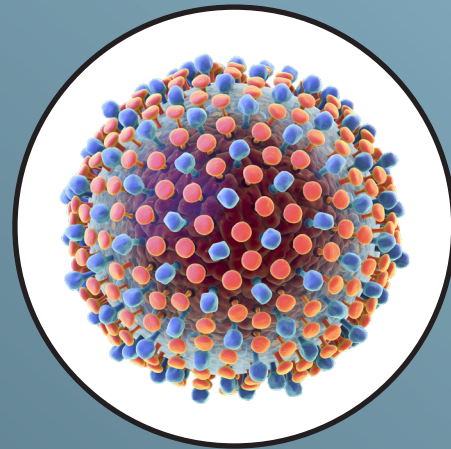
Abstract

Hepatocellular carcinoma (HCC) is a leading cause of global cancer death. Major etiologies of HCC relate to chronic viral infections as well as metabolic conditions. The survival rate of people with HCC is very low and has been attributed to late diagnosis with limited treatment options. The combination of ultrasound and the biomarker alpha-fetoprotein (AFP) is currently one of the most widely-used screening combinations for HCC. However, the clinical utility of AFP is controversial and the frequency and operator-dependence of ultrasound leads to a variable degree of sensitivity and specificity across the globe. In this review, we summarize recent developments in the search for non-invasive serum biomarkers for early detection of HCC in order to improve prognosis and outcome for patients. We focus on tumor-associated protein markers, immune mediators (cytokines and chemokines), and micro-RNAs in serum or circulating extracellular vesicles and examine their potential for clinical application.

Keywords
Hepatocellular carcinoma, biomarker, cytokines, microRNA, tumor marker

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and, according to current epidemiological data, is the fourth leading cause of cancer mortality worldwide ranking among the most commonly diagnosed cancer in both genders [1, 2]. HCC generally develops in the context of liver cirrhosis of any cause but it is particularly linked to hepatitis B virus (HBV), hepatitis C virus (HCV) infection, alcoholic and non-alcoholic fatty liver disease (NAFLD), which are the most common underlying etiologies [2]. Thus, although cirrhosis is considered a predisposing condition for HCC, diverse and disease-specific mechanisms of HCC development may be at play in viral and metabolic dysfunction-related liver disease [3, 4]. Indeed, the observation that HCC can also surge in a non-cirrhotic livers in the specific context of HBV infection or NAFLD suggests that these diseases are etiologically linked to HCC development regardless of their intersection with liver cirrhosis [1].

HCC-related mortality has steadily increased and almost tripled in the United States since 1980's, where it is the fastest rising cause of cancer-related deaths with more than 39,000 cases and 29,000 deaths in 2018 [5]. Importantly, according to the World Health Organization estimates, globally more than 1 million patients will die from liver cancer in 2030 [6]. These data underscore the magnitude of the HCC-associated disease burden, which in spite of the advances made in its surveillance and diagnosis, is still often diagnosed at advanced stages precluding timely and eventually curative therapeutic intervention resulting in poor prognosis. Thus, early HCC diagnosis is critical in order to improve patient outcomes. Proposed strategies for early detection of HCC include adherence to surveillance programs in populations at risk and the development of sensitive and specific diagnostic biomarkers [7, 8]. HCC surveillance comprising of ultrasound screening every 6 months is recommended for all patients with cirrhosis but, as mentioned above, tailoring HCC surveillance programs may be necessary in certain diseases (i.e. HBV and NAFLD) so to include at-risk non-cirrhotic patients [9]. IN this regard, non-cirrhotic HBV-infected individuals are advised to undergo surveillance in a range of ages that is based on geographic location across the globe. Participation in surveillance in HCC in cirrhotic individuals has been regarded as suboptimal, with some studies in the United States and Europe showing that less that 40% of patients with cirrhosis undergo proper HCC surveillance [10, 11]. A large study from our group involving over 1300 HCC cases in South America found that less than 50% of HCCs were diagnosed via surveillance[12]. Although there are several issues that contribute to such low participation in surveillance, the lack of a reliable blood biomarker which is easy to detect and that provides high degree of sensitivity and specificity is certainly among the most important contributing factors. In this regard, measurement of serum alpha-fetoprotein (AFP) levels is most widely used but the biomarker has limited sensitivity for detection of

early disease and it is recommended to be used only in addition to ultrasound. A multitude of biomarkers ranging from proteins detected in serum, to in situ genetic testing in liver biopsies and standardization of imaging via artificial intelligence have been studied in an attempt to reliably identified early HCC. Due to the diverse genetic nature of HCC and the existence of different underlying liver diseases, none have progressed to the point of implementation. Underlying liver disease is indeed a point of contention as in processes like viral hepatitis there is an ongoing inflammatory cascade that obscures the approach to identifying immune or inflammatory markers that could be related to the tumor. With new advancing technologies which allow us to detect low expressing proteins, RNAs and genetic material in endovesicles, a number of new circulating biomarkers are currently under study [8, 13]. In this review, we aim to summarize the available information and recent developments on HCC biomarkers detected in serum, classified as protein, miRNA and immune biomarkers (Figure 1), which may impact early HCC diagnosis and therefore implementation of appropriate management that can optimize prognosis in HCC. We aim to present what are considered to be the most advanced, important or novel biomarkers and not to present an exhaustive review.
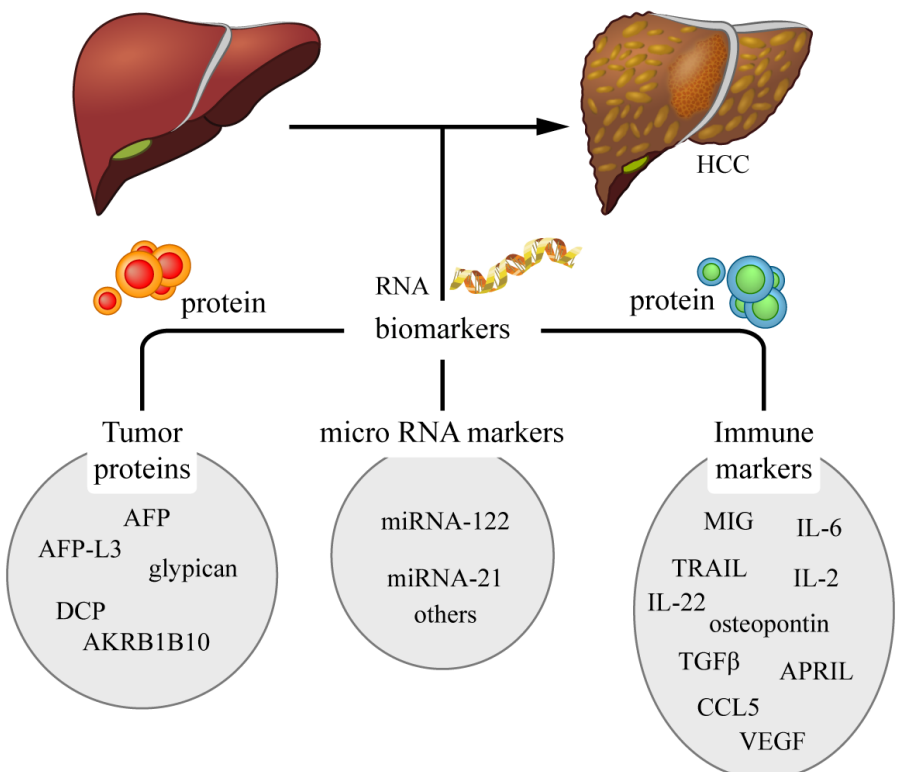

Figure 1. Graph summarizing different classifications of biomarkers discussed in the review.

Protein biomarkers

Alpha-fetoprotein (AFP) is the most commonly used biomarker for surveillance and diagnosis of HCC. Nevertheless, current guidelines either do not recommend its use or make it optional in addition to ultrasound [14, 15]. The main reasons that prevent the widespread use of AFP are concerning the specificity and limited sensitivity it has in the detection of early stage HCC, with a considerable number of HCC that do not have elevated serum AFP levels. Elevated AFP serum levels are also observed in some patients with viral hepatitis, cholangiocarcinoma and other tumors, leading to false-positive results for HCC diagnosis[16]. However, a meta-analysis on HCC surveillance has demonstrated that ultrasound alone is less sensitive than ultrasound associated with AFP (sensitivity of 45% versus 63%, relative risk of 0.81 for early-stage HCC in patients with cirrhosis) [17]. Also, a model based on the pattern of increase of AFP over time identified patients at high-risk of developing HCC as early as 15 years before the diagnosis, so that these individuals could be monitored more intensively than their counterparts. The area under the receiver operating characteristic curve (AUROC) for AFP ranged from 0.73 to 0.83 in different cohorts [18].

As the performance of AFP is suboptimal, many other serum biomarkers are under investigation. Two markers that received much attention are a glycoform of AFP, lectin-binding AFP-3 (AFP-L3), and des-gamma-carboxyprothrombin (DCP), also known as prothrombin induced by vitamin K absence-II (PIVKA-II), which is a non-functional protein produced by HCC. A randomized controlled trial evaluating HCC surveillance through ultrasound with or without AFP, AFP-L3 and DCP has demonstrated that the association of these biomarkers with ultrasound increases sensitivity while decreasing specificity [19]. In another study, in which samples from four prospective Korean cohorts were analyzed, AFP performed better than AFP-L3 and DCP up to 12 months prior to the diagnosis of HCC. When biomarkers were combined, the association between AFP and AFP-L3 had the best performance both at 12 and at 6 months before the diagnosis. At the moment of the diagnosis, AFP combined to AFP-L3 performed comparable as the combination of all three biomarkers. Regarding early-stage HCC, results were also favorable to AFP combined to AFP-L3. Finally, adding AFP and AFP-L3 to ultrasound improved the sensitivity (94.3%), despite decreasing specificity (82.7%) [20].

A score which includes these biomarkers is the GALAD score, an acronym for gender, age, AFP-L3, AFP and DCP, which resulted in AUROC values of more than 0.88 irrespective of the HCC disease stage [21]. In a North-American validation cohort, the score had an AUROC of 0.95 for HCC detection, while ultrasound had an AUROC of 0.82, a superiority that remained for early-stage as well as very early-stage HCC. In the same study, the GALADUS score was described, adding ultrasound findings to the GALAD score. The GALADUS score

had AUROCs of 0.98 for any-stage HCC and 0.97 for early-stage HCC [22]. In patients with nonalcoholic steatohepatitis, the GALAD score demonstrated sensitivity and specificity with an AUROC of 0.96 to identify patients with any stage HCC Importantly, these high AUROC values were observed in NASH patients with and without cirrhosis (0.93 and 0.98, respectively). The GALAD score also identified individuals who would develop HCC as early as 1.5 years before the diagnosis. When early-stage HCC was concerned, the score had a sensitivity of 86.2% and a specificity of 90.9% [23].

Also, different classes of serum protein biomarkers have been described. The LCR1 model identifies patients without cirrhosis who are at risk of developing primary liver cancer. The parameters that make up LCR1 include serum apolipoprotein A1, haptoglobin, gamma-glutamyltranspeptidase, alpha2-macroglobulin, age and gender. Also, a second model had been described, the LCR2 model, which is used to follow individuals identified by LCR1 as well as patients with cirrhosis; LCR2 includes the same variables combined with AFP. AUROCs were 0.78 for LCR1 and 0.87 for LCR2, and LCR2 performed better than AFP (AUROC=0.72) [24].

Another panel of serum biomarkers for early diagnosis of HBV-related HCC consists of five plasma proteins (P5). The P5 panel includes osteopontin, growth and differentiation factor 15 (GDF15), neuron-specific enolase, thrombin receptor activator for peptide 5 and osteoprotegerin. The panel outperformed AFP in the diagnosis of early-stage HCC (AUROC=0.85-0.91 for P5 and 0.54-0.59 for AFP, according to different cutoff values and different validation cohorts). Furthermore, P5 predicted HCC development approximately one year prior to being clinically diagnosed [25]. Regarding osteopontin alone as a marker, it was demonstrated in a different study that it performs better than AFP (AUROCs of 0.85 and 0.68, respectively), a benefit that remained for early-stage HCC, this is further discussed below [26].

An interesting study from Asia evaluated serum levels of aldo-keto reductase family 1 member B10 (AKR1B10) as a putative HCC biomarker. The biomarker detected early stage HCC with a sensitivity of 61% and a specificity of 86%, and performed better than AFP alone. However, the highest performance was achieved by the combination of both biomarkers (AUROC=0.94). Such findings were similar in a validation cohort [27].

Finally, glypican-3 is a biomarker that has been evaluated in many studies. Glypican-3 is a transmembrane proteoglycan anchored to the cell membrane, highly expressed by some HCC tumors and can be detected in serum. Two recent meta-analyses have been published in which the value of serum glypican-3 levels in the diagnosis of HCC was evaluated. In the first study, glypican-3 performed similar to AFP (AUROCs of 0.78 and 0.79, respectively), while their combination had a good accuracy (AUROC=0.94) [28] in discriminating HCC

from liver cirrhosis. The second meta-analysis evaluated the performance of glypican-3, Golgi protein 73 and AFP levels. These 3 biomarkers combined had an AUROC of 0.95 and performed better than any biomarker alone or any pair of biomarkers [29].

MicroRNA biomarkers

Micro RNAs (miRNAs) are small non-coding RNA molecules of approximately 22-24 nucleotides in length that regulate gene expression and are critically involved in the processes of liver development during embryogenesis, liver homeostasis and liver pathophysiology [30]. miRNAs can be secreted into the extracellular space and are found circulating in various body fluids as either part of extracellular vesicles or exosomes (exo) or associated to circulating proteins [31]. Dysregulated expression of miRNAs has been demonstrated in various tumors, including the most common, such as lung, prostate, colon, breast and also liver cancers, and has been shown to affect the regulation of the activity of oncogenes and tuumor suppressor genes, thereby directly influencing carcinogenesis [32]. As a consequence of their dysregulated expression, circulating miRNAs have been studied as potential biomarkers for cancer, including for HCC, detected using non-invasive techniques in serum or plasma [33]. miRNAs can be measured by molecular biology methods, like quantitative polymerase chain reaction (PCR), microarray or RNASeq analysis. Moreover, because miRNAs are small molecules, have a high sequence homology among family members and low abundance, new methods, such as those based on nanomaterials, are being developed for highly sensitive detection of miRNAs [34].

In a study from Korea, the expression levels of circulating miRNA were determined in serum of patients with HCC and in controls individuals with chronic HBV or liver cirrhosis. In this study, four exosome derived miRNAs were found to be of interest (exo-miR-10b-5p, exo-miR-18a-5p, exo-miR-215-5p, and exo-miR-940) when examining serum samples from 90 patients with HCC and 60 controls with chronic liver disease. In particular, exo-miR-10b-5p appeared as a promising biomarker for early-stage HCC with an AUROC of 0.93, a sensitivity of 90.7% and specificity of 75.0% [35]. Another set of miRNAs, exo-miR-25-3p, exo-miR-1269a, exo-miR-4661-5p, and exo-miR-4746-5p with increased expression in HCC were found by selecting driver oncogenic miRNAs using analysis of miRNAs expression profiles from three different RNA sequencing datasets of human HCC [36]. In particular, serum exo-miR-4661-5p was able to detect HCC at all stages with AUROC of 0.92; even at early-stage HCC the AUROC remained at 0.92. Furthermore, a panel composed of both exo-miR-4661-5p and exo-miR-4746-5p was able to detect early-stage HCC with an AUROC of 0.95, a sensitivity of 81.8% and a specificity of 91.7%. A retrospective study from China identified a miRNA classifier containing seven circulating miRNAs (mIR-29a, mIR-29c, mIR-133a, mIR-143, mIR-145, mIR-192 and

mIR-505) in serum that could detect HBV-induced HCC. This classifier showed a higher accurancy than AFP (when using the 20 ng/ml cut-off) to distinguish individuals with HCC from individuals with chronic HBV or liver cirrhosis [37]. Interestingly, this study also established the ability of the miRNA classifier to predict preclinical HCC before diagnosis in a surveillance programme with HBV. The miRNA panel detected 8 cases of HCC at 12 months before diagnosis (8 out of 27), whereas AFP was able to detect only 2 cases. A recent study evaluated the use of circulating miRNAs to identify HCC by analyzing serum samples from 353 HCC patients, 46 chronic hepatitis patients, and 93 patients with liver cirrhosis [38]. This study found that a combination of 8 miRNAs, miR-320b, miR-663a, miR-4448, miR-4651, miR-4749-5p, miR-6724-5p, miR-6877-5p, and miR-6885-5p could discriminate HCC from at-risk control samples (chronic hepatitis and/or cirrhosis) with a diagnostic value AUC of 0.99, a sensitivity of 97.7% and specificity of 94.7%. This model is proposed to detect 98% of stage I HCC cases [38]. The diagnostic value of this miRNA panel is superior to the diagnostic values achieved by either serum AFP, the GALAD score and the GALADUS score [20-22].

Since the various studies that examined the value of miRNA as an HCC biomarker examined either cell-free or exosomal derived miRNAs, a study involving 72 patients with HCC, 72 cirrhotic controls and 72 patients with HBV compared the diagnostic value and showed that a microRNA panel (miRNA-26a, miRNA-29c, and miRNA-21) in exosomes provided better diagnostic value for patients with HCC than circulating cell-free miRNAs among different groups [39]. Along the same line, it has been reported that the number of detected miRNA in plasma outnumbers the number detected in paired serum samples, and that the expression levels in plasma were found to be higher than in serum, indicating that standardization of the biological material is crucial[40]. These obervations may also, at least partially, explain that no research group has found the same discriminating set of miRNAs, with the exception of known dysregulated miRNAs in liver disease like miRNA-122 [41, 42] or miRNA-21 [43, 44] as candidates for early-stage HCC detection. Also, Table 1, which summarizes a series of microRNAs that are not further discussed in the review, clearly shows that most studies that evaluate circulating miRNAs as potential biomarkers for HCC detection were conducted using Asian cohorts which are generally characterized by a high frequency of HBV-induced HCC, and no or small numbers of HCC induced by HCV, alcohol or NAFLD. Also, these studies were conducted involving small groups of patients and have yet to be validated within large cohort of patients [35, 36, 43-47].

Overall, circulating miRNAs appear to be promising biomarkers to detect early stage HCC. To move this field further, longitudinal studies should be carried out including larger cohorts to validate the diagnostic performance obtained by different sets of miRNAs in cross-sectional studies across different standardized detection platforms and disease etiologies.

Table 1. Summary of published literature on miRNA evaluation as biomarker for HCC

miRNA	Etiology	Country	Source	Study subjects	Reference
miR-21, miR-106b, miR-125b, miR-182, miR-224	Multiple	China	Serum	66 HCC patients and 82 healthy controls	Liu et al. Oncotarget. 2017 Dec 12; 8(65):108810-108824.
miR-21, miR-26a, miR-101	Multiple	China	Serum	52 HCC patients, 42 chronic hepatitis patients and 43 healthy controls	Zhuang at al. Clin Res Hepatol Gastroenterol. 2016 Sep;40(4):386-96.
miR-88	HBV	China	Serum	80 HCC patients, 45 liver cirrhosis patients, 45 chronic HBV patients and 43 healthy controls	Long et al. Aging (Albany NY). 2017 Jun 27; 9 (6):1565-1584.
miR-375, miR-10a, miR-122, miR-423	Multiple	China	Serum	149 HCC patients and 149 controls	An et al. World J Gastroenterol. 2018 Jun 28; 24(24):2596-2604.
miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, miR-106a	Multiple	China	Serum exosomes	80 HCC patients and 30 healthy controls	Xue et al. J Cell Biochem. 2019 Jan; 120(1):135-142.
miR-122, miR-224	HCV	Egypt	Plasma	40 HCC patients related to HCV, 40 chronic HCV patients and 20 healthy volunteers	Amr et al. Genes Dis. 2017, 4:215-221.
miR-21, miR-122	Multiple	India	Serum	50 HCC patients, 25 chronic hepatitis patients, 25 liver cirrhosis patients and 10 healthy individuals	Bharali et al. J Clin Exp Hepatol. 2019;9(3):294-301.
miR-122, miR-148a, miR-1246	Multiple	China	Serum exosomes	68 HCC patients, 53 liver cirrhosis patients, 50 chronic hepatitis patients, 64 controls	Wang et al. Cancer Med. 2018 May; 7(5):1670-1679.
miR-486, miR-584					
miR-19a, miR-296, miR-195, miR-192, miR-34a, miR-146a	HCV	Egypt	Serum	112 HCC patients related to HCV, 125 chronic HCV patients and 42 healthy controls	Motawi et al. PLOS One. 2015; 10:e0137706.
miR-375	Multiple	China	Serum	78 HCC patients and 156 healthy controls	Yin et al. Tumor Biol. 2015, 36, 4501-4507.
miR-199a-3p					
miR-214-5p, miR125b, miR-1269	HCV	Egypt	Serum	224 HCC patients related to HCV, 250 chronic HCV patients and 84 healthy controls	Elemeery et al. World J Gastroenterol. 2017;23:3864-3875.
miR-375					
miR-4463	HBV	China	Serum	45 HCC patients and 45 controls	Hu et al. Genes Dis. 2017 Jun; 4(2):116-122.
miR-106b	Not mentioned	China	Serum	335 HCC patients	Shi et al. World J Gastroenterol. 2017 May 28; 23(20):3713-3720.
miR-10b, miR-106b, miR-18a	Multiple	China	Serum	27 HCC patients, 31 chronic liver disease patients and 50 healthy controls	Jiang et al. Medicine (Baltimore). 2015 Mar; 94(10):e603;
miR-23a	Not mentioned	Egypt	Serum	57 HCC patients, 57 liver cirrhosis patients and 57 healthy controls	Mohamed et al. World J Hepatol. 2017; 9:1001–1007.
miR-10b-5p, miR-215-5p	HBV	Korea	Serum exosomes	90 HCC patients, 60 chronic liver disease patients and 28 healthy controls	Cho et al. J. Clin. Med. 2020, 9, 281; doi:10.3390/jcm9010281

Table 1 continued.

miRNA	Etiology	Country	Source	Study subjects	Reference
miR-301	HCV	Egypt	Plasma	42 HCC patients related to HCV, 48 chronic HCV patients (all with liver cirrhosis) and 40 healthy controls	El-Hamouly at al. Mol Biol Rep. 2019.46(6):5759-5765.
miR-1246, miR-101-3p miR-106b-3p	Multiple	Italy	Plasma	cohort 1: 7 HCC patients, 10 liver cirrhosis patients and 7 controls; cohort 2: 9 HCC patients and 6 liver cirrhosis patients cohort 3: 22 HCC patients and 11 healthy controls	Moshiri et al. Oncotarget. 2018. 9(20):15350-15364.
miR-224	HBV	China	Serum	182 HCC patients	Zhuang et al. Biomed Res Int. 2015. 737781.
miR-150 miR-182	HCV	Egypt	Serum	40 HCC patients, 40 chronic HCV patients (20 cirrhotic and 20 non-cirrhotic) and 40 healthy controls.	Shaheen et al. Virus Res. 2018. 255:77-84.
miR-422; miR-221; miR-222 miR-224	HBV	China	Serum exosomes	20 HCC patients, 20 chronic HBV patients, and 20 liver cirrhosis patients	Sohn et al. Exp Mol Med 2015. 47, e184

Immune biomarkers

Immunosurveillance, the recognition of tumor cells by leukocytes, has been well-described for many tumors and has changed the way to interpret the role of circulating immune markers in the setting of oncogenesis [48]. Hence, cytokines and chemokines induced upon recognition of cancerous lesions can be detected in serum or plasma of individuals at risk. This fact is highlighted in the formation of HCC, as the tumor usually arises in the setting of chronic hepatitis where a hyper-immune environment due to the continuous presence of an inflammatory response in the liver could lead to further alterations in measurable immune analytes during the transition from a liver nodule to HCC [49, 50]. Our group recently identified a series of immune markers in serum of patients with hepatitis C that were associated with the future development of HCC, even when the cancer occurred up to two years later [49]. These markers include, among others, soluble proteins such as MIG, interleukin (IL)-22 and IL-3, which are highly immunogenic, as well as vascular endothelial growth factor (VEGF) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which are related to vascular formation and apoptosis modulation. The c-statistic for correct prediction of HCC was >0.90 in four of these markers (MIG, IL-2, TRAIL, APRIL), which is defined as extraordinary and >0.80 in the rest. However, this study was performed in a small number of samples (13 subjects per group) and all samples were from HCV-infected individuals. It is likely nonetheless, that in other chronic infections, such as HBV, these immune markers would play a role in early HCC detection as well.

Tumor growth factor β (TGF-β), a polyfunctional growth factor that has been shown to interact literally on all cell types, modulating cell-proliferation, cell differentiation and even cell survival, also has potential as a biomarker for HCC. Mutations and differential expresison of TGF-β has been found to be altered in a variety of tumor tyopes. Previous studies have shown that serum levels of TGF-β are associated with HCC development, mainly in HCV-infected individuals [51]. However, other studies have addressed TGF-β role as a biomarker in HCC in combination with expression of other proteins or mRNA and not as a stand alone biomarker [52]. Moreover, several of these studies have been performed in single-country populations without confirmation in other settings.

Osteopontin (OPN), a versatile cytokine which mediates a wide array of biological functions in the immune and vascular system has been reviewed before as a marker for a variety of tumors [53]. Several studies have shown increased serum and plasma levels of OPN in individuals with HCC compared to those with liver cirrhosis and/or chronic liver disease controls [26, 54, 55]. The majority of these studies were carried out in Asian cohorts, with a large multi-center study conducted using West-African and European cohorts replicating these findings [56]. In most studies, OPN has shown an AUROC of

no less than 0.75 for HCC prediction. In contrast, diagnostic efficacy of OPN in detecting early stage HCC vs non-HCC patients varied considerably depending on the study. Ge et al and Vongsuvanh et al reported an AUROC of 0.57 and 0.78, respectively, whereas Shang et al reported an AUC of 0.73 [55]. Interestingly, a prospective evaluation in an Asian cohort of 115 patients with chronic liver disease at risk of HCC showed increased plasma OPN levels 24 months prior to diagnosis in 21 subjects who developed HCC [56].

Recently, serum pentraxin 3 has also been suggested as a candidate biomarker of HBV-induced HCC in a study from China [57]. Pentraxin 3 is a protein produced by multiple cell types, such as macrophages, monocytes, fibroblasts and endothelial cells in response to inflammatory signals (such as bacterial components or cytokines, such as TNF or IL-1), as such pentraxins behaves as an acute phase protein. Pentraxin 3 may also be involved in cancer development although the underlying mechanisms are not well understood. Elevated pentraxin 3 levels have been reported in patients with acute liver injury, NASH and HCV, amongst others. Evaluating the serum pentraxin 3 levels in 107 patients with HCC in comparison to 159 chronic HBV and 99 cirrhotic patients demonstrated that pentraxin 3 was highly discriminitive of AFP-negative and early-stage HCC, and the dignostic performance of pentraxin 3 was superior to AFP. In fact the AUC for pentraxin to discriminate early HCC from cirrhosis was 0.90, while it was 0.68 for AFP, clearly suggesting thee potential of pentraxin 3 as a biomarker for early HCC.

Chemokines play an important role as mediators of immune responses since they are instrumnetal in the recruitment and activation of leukocytes at the inflammed or injured location.The chemokines C-C motif ligand 4 (CCL4) and CCL5 bind to the same receptor, C-C receptor 5, which is expressed in effector and memory T cells, making this interaction critical in controlling chronic viral infections [58]. Only one study to date has evaluated serum levels of various chemokines in the context of HCC detection, and multivariate regression analysis found that serum CCL4 and CCL5 levels were higher in cirrhotics with HCC than cirrhotics without HCC (n=78), making them interesting candidate diagnostic markers for HCC. The performance of CCL4 and CCL5 was comparable for HCC detection, with an AUROC for CCL5 of 0.72, and a relatively high sensitivity of 71% and specificity of 68% [56].

VEGF, an angiogenic factor for vascular endothelial cells, is produced by many cell types, including tumor cells [60]. A Japanese study involving HCV-infected individuals with HCC demonstrated that the AUROC for detection of HCC of VEGF was superior to that of AFP (AUROC of 0.98 versus 0.71, respectively) [61]. However, a study performed in Egypt, similarly in HCV-infected individuals (all genotype 4), did not detect serum VEGF differences among HCV patients who developed HCC and control HCV patients [62]. A recent longitudinal study from our group, mentioned above, identified serum VEGF

as one out 12 immune mediators to be increased in HCV-induced HCC [49]. Despite a promising status, VEGF requires further investigation as a stand-alone marker for early or late HCC detection.

Multiple studies have shown a role for IL-6 in inflammation leading to liver cancer, and even gender disparities in HCC have been explained by the interrelation between estrogen and IL-6 [63]. Serum IL-6 has been shown to be increased in HCC patients compared to patients with chronic liver disease [64]. However, most studies have evaluated the performance of IL-6 in the setting of advanced HCC [65], but not during the early stages. Of note, IL-6 pretreatment levels have not associated with macrovascular invasion or extrahepatic spread, but some studies show a potential for this cytokine as a predictor to response to systemic therapy [65, 66].

Growth differentiation factor 15 (GDF15) a member of the TGF- β superfamily has been shown to be elevated in HCC compared to controls is HBV- and HCV-positive Chinese cohorts [67]. Although this study initially raised promise about the perspectives of GDF15, a later study found increased serum levels of the immune analyte in HBV-related HCC and HCV-related HCC compared to either chronic viral infection, but no statistical difference when compared to cirrhotic patients [68]. Further studies performed in prospective fashion are needed to assess the role of GDF15 in HCC as well as in non-viral hepatitis related HCC.

Conclusions

One of the main factors related to high mortality in HCC is the frequency of late diagnosis of this tumor. Despite attempts of implementation of surveillance strategies, the adherence to surveillance programs has been reported to be low across the globe [69]. Although several factors are involved in this poor performance, the cumbersome approach of q6 months ultrasonogrpahy, the cornerstone of HCC surveillance, has undoubtley play a role in the lack of adherence to surveillance. Peripheral biomarkers that are easily measured in serum or plasma are of critical need in the field of HCC. However, the genetic diversity of the tumor has blunted efforts to discover reliable and specific proteins that could aid in early detection of HCC. Moreover, the conundrum of liver diseases underling the development of liver cirrhosis and hence HCC, such as viral hepatitis, create an immune imbalance that further obscures the ability to identify immune markers or peripherally expressed proteins that could assist in early detection. Moreover, the diffrrential epideimology o funderlying liver disease in HCC in different regions of the world, make a one-fits-all biomakrer approach even more difficult to attain. However, recent advances in techniques allowing the detection of multiple immune analytes, as well as the progress

in quantifying microRNAs specific to the liver has contributed to a change in balance that favors proper implementation of biomarkers to easily and effectively predict or diagnose early HCC. Moreover, new technologies that allow for exosome (endovesicles) assessment in relation to HCC detection will bring this field to new territories. It should be expected that in the next 5-10 years the hepato-oncology community will have a broader spectrum of tools to predict HCC with a simple serum assessment.

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CHAPTER 4C

Serum immune signatures associate with HCC development in DAA-treated HCV patients

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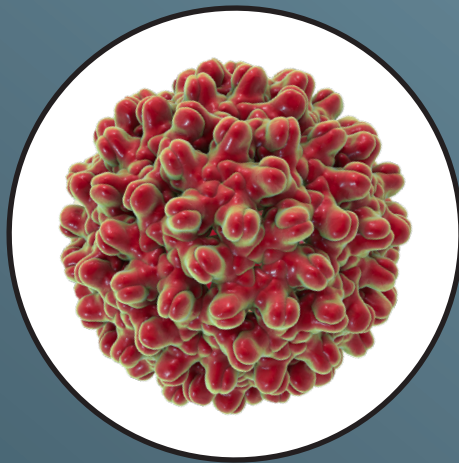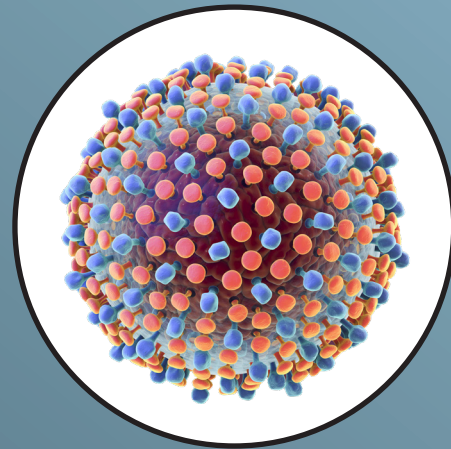
Abstract

Concern has arisen about development of hepatocellular carcinoma (HCC) in hepatitis C (HCV) infected patients treated with direct-acting antivirals (DAA). We evaluated for the first time expression of serum immune mediators before and during treatment of HCV with DAAs in a group of 13 patients that developed HCC post DAA treatment, and 10 controls without HCC on follow up. We identified a novel immune signature of 12 cytokines that showed significantly higher expression at baseline in those individuals that developed *de novo* HCC compared to controls, with 9 markers showing an AUROC above 0.8. We also found modulation of inflammatory cytokines throughout treatment that provides insight into HCV-related carcinogenesis in the context of DAAs.

Manuscript

New treatments with direct acting antivirals (DAAs) are highly effective in targeting hepatitis C virus (HCV) infection, with an extraordinary rate of patients achieving cure [1]. This has led to extensive use of DAAs for the treatment of HCV around the globe, regardless of patient's liver fibrosis stage, co-morbidities, or history of liver cancer, and this trend is likely to increase.

Recent studies have reported an unexpected high rate of early hepatocellular carcinoma (HCC) recurrence in HCV-infected patients treated with DAAs. Two European studies showed 27% and 29% recurrence rate of HCC within 6 months of treatment initiation, respectively [2, 3]. These recurrence rates were higher than expected, and higher than those with no history of HCC [2, 3]. In addition, a study from the United States found a 27% recurrence of HCC in DAA-treated patients who underwent liver transplantation, which was higher than the 9% recurrence in those with no DAA treatment in a similar follow up period [4]. Interestingly, a recent report, presented in abstract form, suggested a more aggressive pattern of HCC in those treated with DAAs without history of HCC (*de novo* HCC) [5]. In contrast, a pull of prospective studies from France failed to show an increased HCC recurrence following HCV treatment with DAAs [6]. Most of these studies presented confounding factors such as follow up of the cohorts, starting point of the analyses, association between time of DAA initiation and HCC recurrence, and statistical limitations [7, 8]. However, those findings are concerning and have sparked speculations of a potential risk for HCC in DAA-treated patients due to an effect in tumor immune-surveillance and normalization of the immune milieu following the elimination of the virus [9].

In this study, we aimed to address immune-related changes, by measuring soluble immune mediators, in a cohort of 13 patients who developed HCC following HCV DAA treatment and compared them to 10 matched controls who did not develop HCC during the follow up period (Supplementary Table 1). Twenty-two different immune mediators were measured in serum samples that were originally obtained at baseline before starting treatment with DAAs and at different time points during and after treatment. From the 13 samples of patients who developed HCC, three of them had recurrence of the tumor and 10 developed *de novo* HCC, all within 18 months of DAA treatment initiation. Controls were followed for at least 24 months (median 26 months, IQR 25-30). The median age of patients who developed HCC was 59 years (IQR 57-63) with an average model of end stage liver disease (MELD) score of 8 and median ALT levels of 51 IU/L (IQR 36-96) at the start of DAA, and 56 years (IQR 53-57) with an average MELD score of 8 and median ALT of 48 IU/L (IQR 28-102) for controls who did not develop HCC. All patients had abdominal ultrasound within 3 months prior to starting antiviral treatment.

We identified a set of 12 immune mediators, comprised of cytokines, growth factors and apoptosis markers, that showed statistically significant higher serum expression levels before treatment in patients that eventually developed *de novo* HCC compared to controls (Figure 1). Moreover, 8 out of these 12 markers had an AUROC value above 0.80 (4 of them above 0.9) for those that developed *de novo* HCC (Table 1). The cytokine SCF showed a significant AUROC but was not significant on ANOVA. There were no significant differences between the performance of these markers. These results potentially suggest that before immune changes occur, due to the targeting of HCV virus by DAAs, individuals who developed HCC already expressed a differential pattern of immune mediators, possibly induced by ongoing carcinogenic or precarcinogenic activity. Interestingly, this differential expression was not affected by how early or late HCC occurred following DAA treatment, since some of those with the highest serum levels of immune mediators at baseline were diagnosed with HCC almost a year later (Supplementary Table 1). Some of the differentially expressed markers have been shown before by others to be associated to HCC development in humans (TRAIL, VEGF, and IL-22)[10]. However, none of these or the other immune mediators have been evaluated in the setting of DAA treatment during HCC development.

We analyzed a further 44 cytokines in a subgroup of 7 cases (4 *de novo* and 3 recurrences) and controls of whom sequential blood samples during DAA treatment were available. In this subset serum levels of TNFα decreased after 4 weeks of DAA treatment in controls, likely indicating normalization of innate immunity after successful elimination of the virus as we reported before[11]. However, in patients who later developed HCC, levels of TNFα remained stable or trended up 4 weeks after start of treatment, with the virus being undetectable in serum and ALT levels normalized (Supplementary figure 1A). This suggests that sustained TNFα release during DAA treatment in the absence of HCV is either mechanistically involved in the induction of HCC development, or a response to the presence of occult HCC in the liver. The apoptosis marker TRAIL has been proposed to be implicated in HCC development after immune-modulation of HCV[9]. In this subset, the levels of TRAIL remained stable in controls 4 weeks after DAA treatment, but trended down (not significantly) in those that developed HCC, and this effect seemed to persist at 24 weeks of the start of treatment in those with available samples (N=4) (Supplementary figure 1B).

Also for IL-6 an association with HCC development has been reported by many groups by promoting hepatic carcinogenesis via the β-catenin pathway[10, 12,13]. Interestingly, in the cohort of 7 cases we observed that IL-6 levels in patients with HCC recurrence were lower at baseline compared to patients with recurrent HCC and increased at the end of treatment, whereas in patients with *de novo* HCC, IL-6 levels trended down (Supplementary figure 2). This suggests alternative mechanisms for tumorigenesis

during recurrence of HCC versus *de novo* HCC following DAA treatment.

Our results, albeit from a small cohort, point towards a potential pre-treatment modification of the immune-milieu, suggesting that immune-modulation by DAA-mediated targeting of HCV probably does not trigger the development of HCC, but rather that the immune background could be already affected before DAA treatment. However, on the basis of our data we cannot exclude that DAA treatment accelerates HCC development in those treated with DAAs. Our study does not include none-treated controls or interferon-treated patients. This poses limitations to the findings, since we cannot specifically address the impact of DAA-therapy on HCC development compared to other or no treatment. Interestingly, Zhu et al found low baseline levels of several immune markers addressed in our study before treatment with pegylated-interferon in patients that did not develop HCC upon follow up[14]. Our study identified a group of 12 immune mediators that showed significantly higher serum expression levels before DAA treatment in patients that later developed HCC. Our study is based on a reduced number of samples, and we believe larger multicenter studies will be necessary to further address these findings.
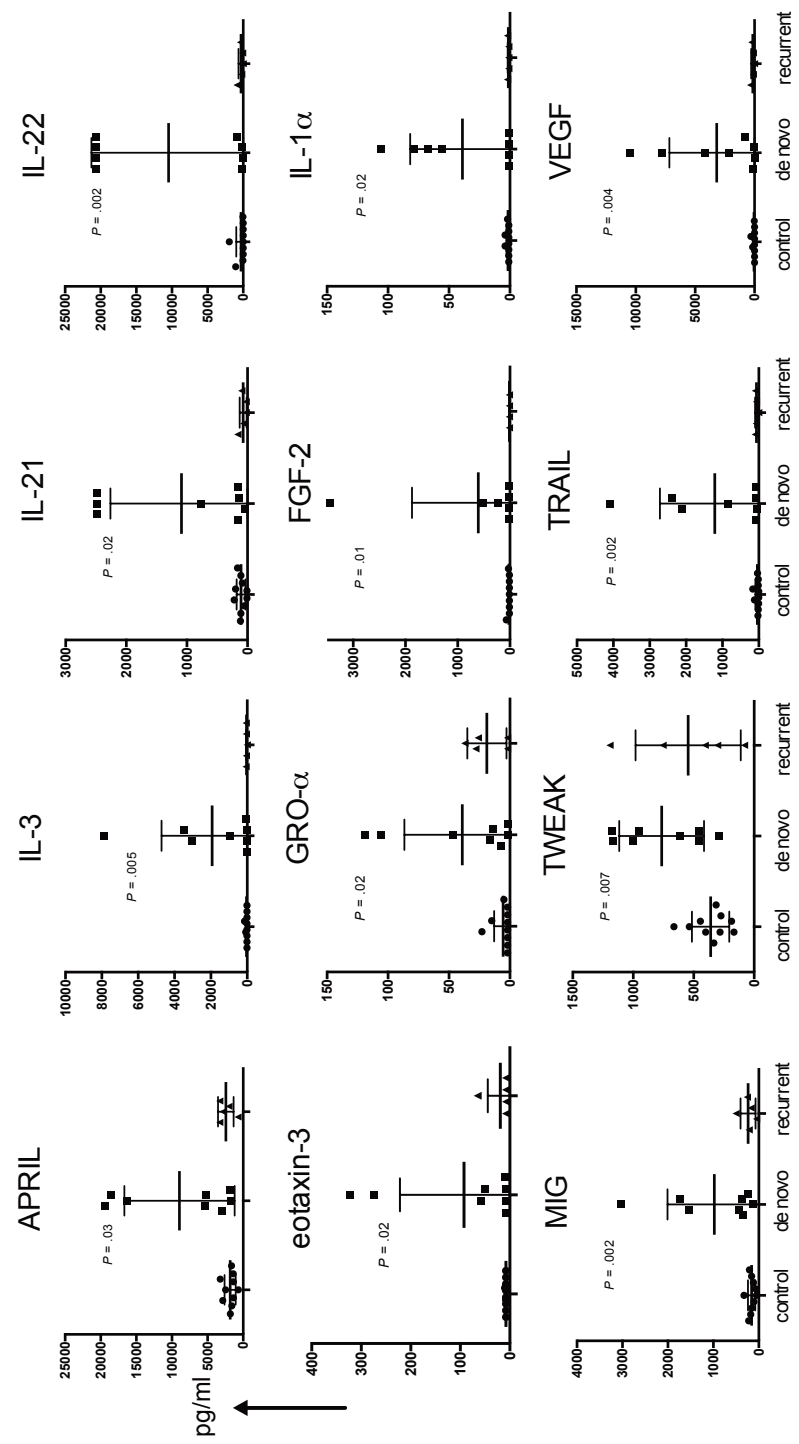
Figure 1. Expression levels of cytokines in *de novo* hepatocellular carcinoma (HCC), recurrent HCC and controls in serum at baseline, before treatment with direct acting antivirals. P-values represent cytokines with statistically significant difference at baseline in those who developed *de novo* HCC compared to controls (based on ANOVA analysis with Bonferroni assessment). Y-axis of each cytokine represents pg/ml; X-axis represents *de novo* HCC, recurrent HCC and control groups. *P* < .05 were considered significant between *de novo* HCC and controls.

IL-22 P = .002
IL-21 P = .02
IL-3 P = .005
APRIL P = .03
IL-1α P = .02
FGF-2 P = .01
GRO-α P = .02
eotaxin-3 P = .02
VEGF P = .004
TRAIL P = .002
TWEAK P = .007
MIG P = .002

pg/ml

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Supplementary files

A

B

C

Supplementary figure 1.

A

IL-6

P = .05

Baseline before DAA

B

IL-6 in HCC

- – – de novo
- · · · · recurrence
- ——— de novo
- · · · · recurrence
- ——— de novo
- · · · · recurrence
- ——— de novo

HCC IL-6
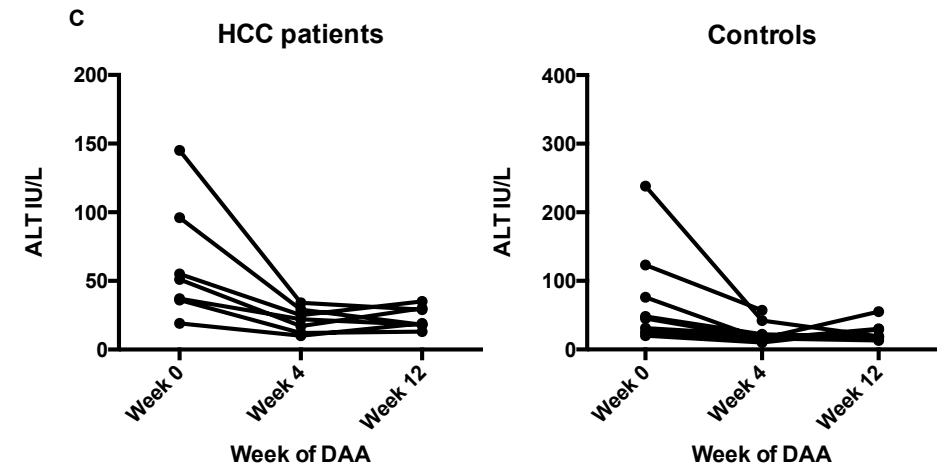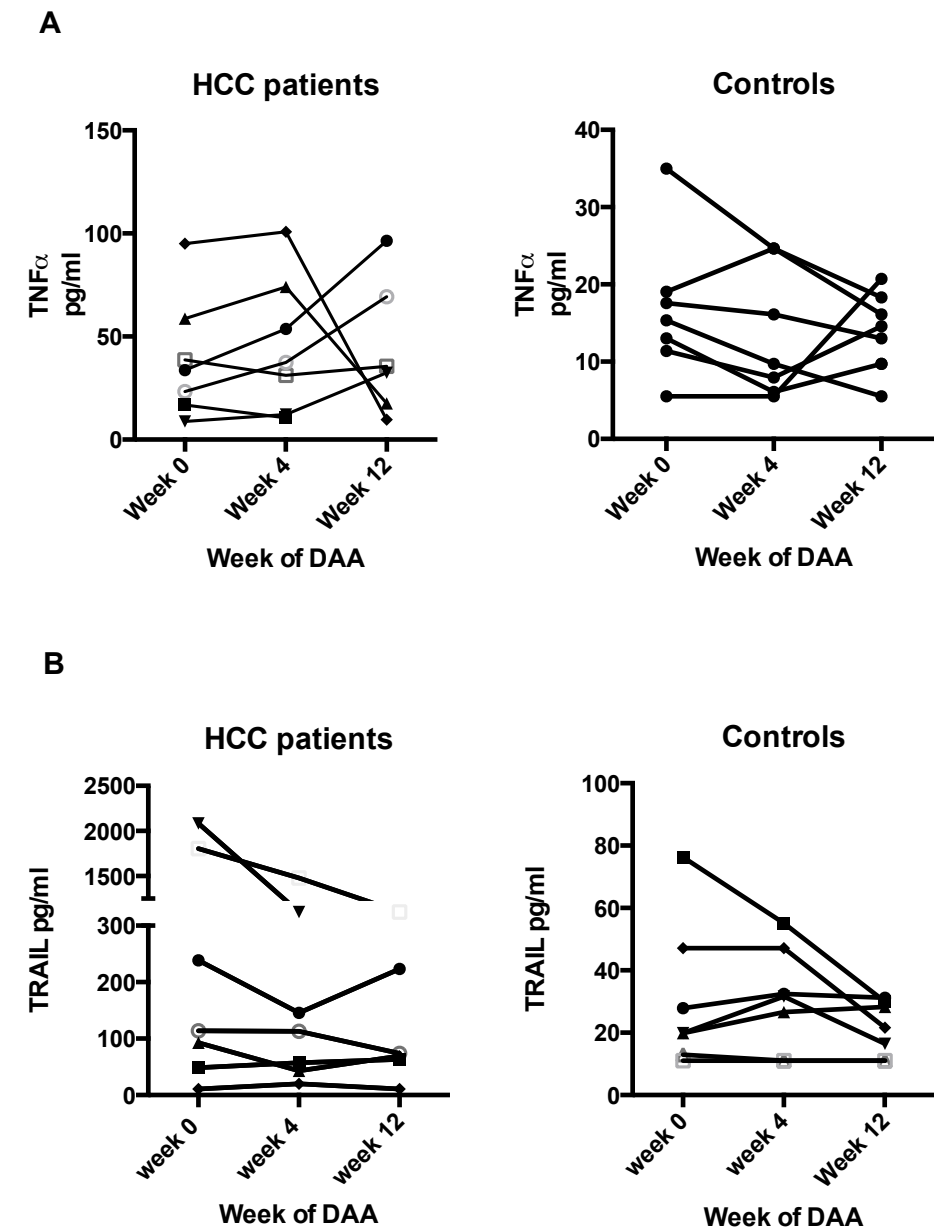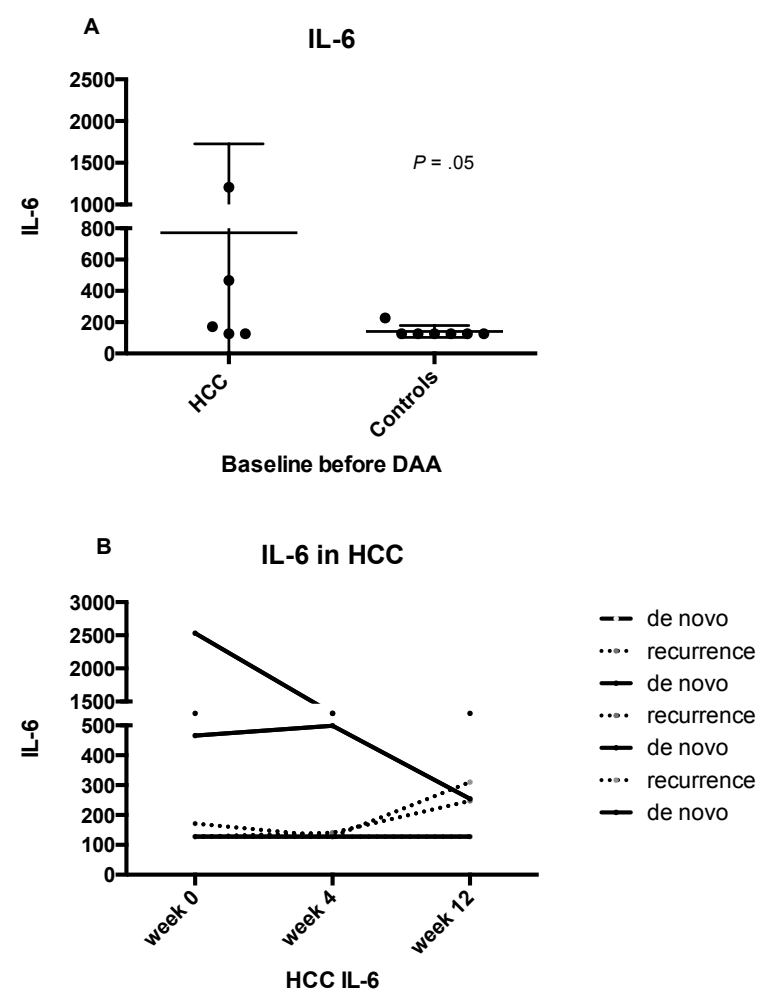
Supplementary figure 2.

5

Treatment of HCC

CHAPTER 5A

Sorafenib for Treatment of Hepatocellular Carcinoma: A Survival Analysis from the South American Liver Research Network

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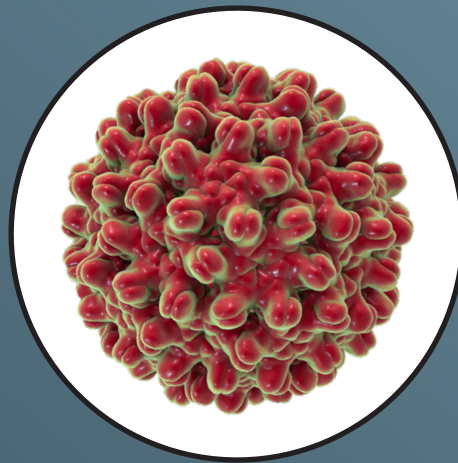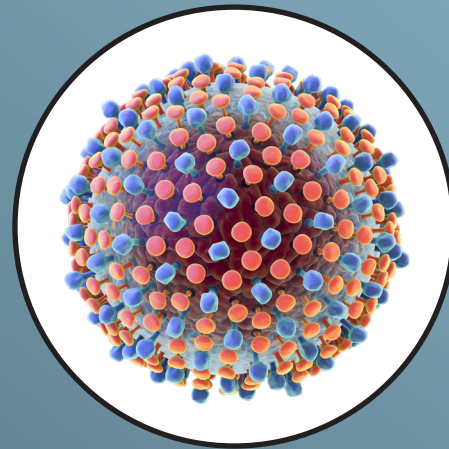
Abstract

Goals
We aim to describe the efficacy, safety profile and variables associated with survival, in patients with hepatocellular carcinoma (HCC) treated with sorafenib in South America.

Background
Sorafenib has been shown to improve survival in patients with advanced HCC. There are little data on sorafenib use for HCC in South America.

Study
We performed a retrospective analysis of HCC cases treated with sorafenib from eight medical centers in five South American countries, between January 2010 and June 2017. The primary endpoint was overall survival (OS), which was defined as time from sorafenib initiation to death or last follow-up. Risk factors for decreased OS were assessed using Cox proportional hazard regression and log-rank tests.

Results
Of 1336 evaluated patients, 127 were treated with sorafenib and were included in the study. The median age of individuals was 65 years (IQR 55-71) and 70% were male. Median OS in all patients was 8 months (IQR 2-17). Variables associated with survival on multivariate analysis were platelets >/< 250.000 mm³ (2 vs. 8 months, P = 0.01) and Barcelona Clinic Liver Cancer (BCLC) stage (A/B, 13 vs. C/D, 6 months; P = 0.04). In a sub-analysis of patients with BCLC stage C, platelets >/< 250.000 mm³ were also independently associated with survival (2 vs. 5.5 months, P = 0.03). Patients lived longer if they experienced any side effects from sorafenib use (11 vs. 2 months, p = 0.009). Patients who stopped sorafenib because of side effects had shorter survival compared to patients who were able to tolerate side effects and continue treatment (7.5 vs. 13 months, p = .01).

Conclusions
Pre-treatment elevation of platelets and advanced BCLC stage were independently associated with poor survival on sorafenib in a South American cohort.

Key Words
hepatocellular carcinoma, South America, sorafenib, survival

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide, with an estimated 745,000 deaths annually [1,2]. Depending on the region of the world, approximately 25–70% of patients with HCC are diagnosed with advanced-stage disease, which is regarded as incurable [3-6]. The landmark multicenter European Sorafenib Hepatocellular Carcinoma Assessment Randomised Protocol (SHARP) trial was the first to assess the efficacy of sorafenib, a multi-targeted orally-active tyrosine kinase inhibitor, in patients with advanced HCC. For the first time, a systemic treatment provided a survival benefit for these patients [7]. Throughout most studies, sorafenib has shown improved survival when compared to placebo regardless of underlying liver disease [8]. However, recent evidence suggests a variable response to sorafenib in different patient populations, especially among different etiologies of HCC, and in different geographical areas [9-11]. Indeed, a sub-analysis of two large phase III studies favored the use of sorafenib in patients infected with hepatitis C (HCV) compared to other risk factors for HCC [10]. It is unclear why patients with HCV would have a more favorable outcome, but some studies suggest that the baseline inflammatory environment induced by HCV plays a role (15-17). This differential effect, based primarily on the etiology of liver disease, suggests a complex relationship between tumor behavior and the molecular characterization of HCC [12,13]. Because of the changing epidemiology of HCC across the globe, the use of systemic treatment for HCC, as well as the response to treatment, will invariably differ among different regions. To date there are no comprehensive data regarding the use of sorafenib in patients with advanced HCC from South America, with only one study from a single tertiary center in Brazil reporting no survival benefit with the use of sorafenib [14]. Recently, we addressed the epidemiology of HCC in over 1300 cases from the South American region, through the creation of the South American Liver Research Network [15,16]. In the current study, we describe for the first time the efficacy and safety profile of sorafenib in patients from South America.

Materials and Methods:

Study Design
We performed a retrospective cohort study of HCC cases treated with sorafenib in South America. We included patients from eight medical centers in five different countries. We evaluated the medical records of all patients diagnosed with HCC between January 2010 and June 2017, in the South American Liver Research Network. Details about the patient population and diagnosis of HCC have been previously described [15].

Data Collected

Variables collected included demographic information, age, sex, etiology of liver disease, cirrhosis status, diagnostic methodology for HCC, date of diagnosis, date of death or last follow-up, laboratory data, clinical data, prognostic data, HCC characteristics based on imaging or clinical evaluation, treatment regimen and treatment side effects. No information on sorafenib dosing was collected. The primary endpoint was overall survival (OS), which we defined as time from sorafenib initiation to death or last follow-up. Secondary analyses included assessment of side effect profiles and comparisons of patient characteristics between Barcelona Clinic Liver Cancer (BCLC) stages. As previous clinical trials of sorafenib use for HCC included only patients with BCLC stage C, a sub-analysis of this cohort was performed to better assess the broad applicability of our findings.

Statistical Analysis

Summary statistics were performed and stratified by BCLC stage. Continuous variables were expressed as medians (Q1-Q3), and categorical variables were expressed as proportions (%). Due to relatively small sample sizes, the Fischer's Exact test was used to compare categorical variables. Due to the non-parametric distribution of certain variables, the Mann-Whitney U test was used to compare continuous variables. Kaplan-Meyer curves for OS were constructed using Cox proportional hazard regression, using the Breslow method for ties. The log-rank test was used to assess the equality of survivor functions. Variables trending with a p-value < 0.1 on univariate survival analysis underwent multivariate analysis. A p-value ≤ .05 was taken as the criterion for statistical significance. A biostatistician was consulted for review of our data analysis. Statistical analysis was performed using STATA v14.2 (Statacorp, College Station, TX.). All authors had access to study data and approved the final version of the manuscript.

Ethical Considerations

This study was approved by the Institutional Review Board (IRB) of Hennepin County Medical Center. In addition, each center was responsible for obtaining appropriate IRB approval.

Results

Patient Characteristics

Of a total of 1336 patients from the South American Liver Research Network, 127 were treated with sorafenib and were included in the study. In six patients (5%) we had no follow-up information. A full distribution of study patients by country is available in **Table 1.** The median age of the study population was 65 years (IQR 55-71) and 70% of

patients were male. The most common etiology of HCC was hepatitis C (HCV) infection (38%) and 25 (52%) patients were diagnosed with HCC outside of screening programs. Sorafenib was the first line treatment in 79% of all patients.

Table 1. Patient characteristics at time of diagnosis

Parameter	Value
Age	65.0 (55-71)(n=127)
Gender	
Male	70.1% (89/127)
Female	29.9% (38/128)
Etiology of HCC	
Infectious	51.2% (65/127)
HBV +	15.0% (19/127)
HCV +	37.8% (48/127)
ASH	32.3% (41/127)
NASH	16.5% (21/127)
Diagnostic Method	
Screening	36.2% (46/127)
Incidental	63.8% (81/127)
Clinical Data	
Cirrhosis	92.9% (118/127)
Ascites	42.1% (53/126)
Vascular Invasion	33.3% (42/126)
Sorafenib as 1st Treatment	78.5% (84/107)
Country	
Argentina	48.8% (62/127)
Brazil	27.6% (35/127)
Colombia	16.5% (21/127)
Ecuador	2.4% (3/127)
Peru	4.7% (6/127)

Continuous variables expressed as medians (Q1-Q3)(n) and categorical variables as % (proportion). HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ASH, alcoholic steatohepatitis; NASH, non-alcoholic steatohepatitis.

There were 3 (2%) patients with BCLC stage A, 30 (24%) with stage B, 73 (57%) with stage C, 1 with stage D (1%) and 20 (16%) with unknown stage. All BCLC stage A patients were from a single institution in Argentina. Most patients with BCLC stage B were from Argentina (n = 23) or Colombia (n = 9), and there was a single patient from Ecuador. Patients with BCLC stage A/B were more likely to be older than those with BCLC stage C/D (67 vs. 61 years respectively, P = 0.04). An infectious etiology (HBV or HCV) of HCC was more common in patients with BCLC stage C/D, compared to patients with BCLC stage A/B (63% vs. 33%, respectively, P = 0.006). An etiology of NASH was more common in patients diagnosed with BCLC stage A/B, compared to patients with BCLC stage C/D (33% vs. 9%, P = 0.004). Sorafenib was the first treatment in 45% (n=15) of patients with BCLC stage A/B and 96% (n=63) of patients with BCLC stage C/D.

Survival Analysis

Eighty-nine patients (73%) died during the study period. Eleven patients who died did not have the date-of-death recorded and were excluded from survival analysis. Overall survival in all patients was 8 months (IQR 2-17) after sorafenib initiation. Patients with pre-treatment BCLC stage A/B were more likely to live longer than those with BCLC stage C/D (13 vs. 6 months, P = 0.008, **Table 3**). HBV negative patients lived twice as long as HBV positive patients (8 vs. 4 months, P = 0.5), and HCV negative patients lived longer than HCV positive patients (9 vs. 7 months, P = 0.2), however, these results did not reach statistical significance. There was also a trend for longer survival in those with alcoholic steatohepatitis (12 vs. 6 months, P = 0.1), but again, this result did not reach statistical significance.

Table 2. Summary Statistics Stratified by BCLC Staging

Parameter	A-B	C-D	P
Age (years)	67.0 (49-80)(n=33)	61.5 (21-83)(n=74)	**0.05**
Male Gender	69.7% (23/33)	70.3% (52/74)	1
Etiology of HCC			
Infectious	33.3% (11/33)	63.5% (47/74)	**0.01**
HBV +	9.1% (3/33)	18.9% (14/74)	0.26
HCV +	24.2% (8/33)	47.3% (35/74)	**0.03**
ASH	33.3% (11/33)	33.8% (25/74)	1
NASH	33.3% (11/33)	9.5% (7/74)	**<0.01**
Diagnostic Method			
Screening	45.5% (15/33)	27.0% (20/74)	0.08
Incidental	54.5% (18/33)	27.0% (20/74)	0.08
Clinical Data			
Cirrhosis	90.9% (30/33)	93.2% (69/74)	0.7
Ascites	24.2% (8/33)	47.3% (35/74)	**0.03**
Vascular Invasion	12.1% (4/33)	50.0% (37/74)	**<0.01**
Sorafenib as 1st Treatment	45.5% (15/33)	95.7% (67/70)	**<0.01**
MELD	10.0 (7-14)(n=33)	10.0 (8-13)(n=73)	**0.6**
Laboratory data			
AFP	61 (6.4-713)(n=31)	267.1 (19-8699)(n=73)	0.07
Bilirubin (Total)	1.18 (0.8-2.04)(n=32)	1.14 (0.8-2.21)(n=69)	0.74
Creatinine	0.84 (0.74-0.99)(n=33)	0.88 (0.71-1.0)(n=68)	0.84
INR	1.14 (1-1.29)(n=32)	1.17 (1.1-1.4)(n=68)	0.20
Platelets	117 (82-160)(n=33)	134 (100-201)(n=69)	0.09

Continuous variables expressed as medians $(Q_1-Q_3)(n)$ and categorical variables as % (proportion). Statistical comparisons are made between Barcelona Clinic Liver Cancer (BCLC) stages A/B and C/D. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ASH, alcoholic steatohepatitis; NASH, non-alcoholic steatohepatitis; MELD, Model for End-stage Liver Disease; AFP, alpha-fetoprotein; INR, international normalized ratio.

Pre-treatment laboratory variables identified as risk factors for decreased OS were total bilirubin > 3mg/dl (2 vs. 8.5 months, P = 0.001), INR > 1.6 IU (5 vs. 8 months, P = 0.01) and platelets > 250,000 mm³ (2 vs. 8 months, P = 0.017, **Figure 1**). Multivariate analysis identified pre-treatment platelets > 250,000 mm³ and pre-treatment BCLC stage C/D as statistically significant independent risk factors for decreased survival (P = 0.01 and P = 0.04, respectively, **Table 4**).

Table 3. Univariate Analysis of Prognostic Factors Associated with Overall Survival

	Median Time (months)	HR (95% CI)	P
Age			
>69	9.5 (1-61)(n=26)	0.82 (0.48, 1.43)	0.48
≤69	7.5 (1-58)(n=68)		
Gender			
Male	8.0 (3-19)(n=64)	1.37 (0.82, 2.28)	0.22
Female	6.5 (2-12)(n=30)		
Etiology			
Viral	6.0 (2-17)(n=51)	1.39 (0.86, 2.24)	0.16
Non-viral	9.0 (2-18)(n=43)		
HBV			
HBV +	4.0 (2-15)(n=15)	1.23 (0.62, 2.43)	0.55
HBV -	8.0 (2-18)(n=79)		
HCV			
HCV+	7.0 (2-18)(n=37)	1.34 (0.83, 2.16)	0.21
HCV-	9.0 (2-17)(n=57)		
ASH			
Present	12.0 (4-22)(n=31)	0.69 (0.42, 1.15)	0.14
Absent	6.0 (2-13)(n=63)		
NASH			
Present	8.0 (3-14)(n=13)	0.80 (0.38, 0.167)	0.53
Absent	8.0 (2-17)(n=68)		
Diagnosis			
Screening	10.5 (5-19)(n=30)	0.82 (0.50, 1.36)	0.44
Non-screening	6.5 (2-14.5)(n=64)		
AFP			
≥200 ng/mL	8.0 (2-18)(n=45)	1.21 (0.75, 1.95)	0.46
<200 ng/mL	8.0 (2-17)(n=49)		
Bilirubin			
>3mg/dl	2.0 (2-7)(n=9)	3.28 (1.50, 7.19)	**<0.01**
≤3mg/dl	8.5 (2-18)(n=78)		
INR			
>1.7 IU	5.0 (2-8)(n=9)	2.40 (1.17, 4.93)	**0.01**
≤1.7 IU	8.0 (2-18)(n=78)		
Platelets			
>250.000 mm³	2.0 (2-10.5)(n=12)	2.21 (1.10, 4.45)	**0.02**
≤250.000 mm³	8.0 (2.5-18.5)(n=76)		
Cirrhosis			
Present	8.0 (2-18)(n=86)	0.45 (0.20, 1.00)	**0.04**
Absent	2.0 (2-9)(n=8)		
Ascites			
Present	7.0 (2-11)(n=37)	1.44 (0.90, 2.33)	0.12
Absent	10.0 (3-18)(n=57)		
Vascular Invasion			
Present	7.5 (2-17.5)(n=36)	0.93 (0.57, 1.53)	0.76
Absent	8.5 (2-17)(n=58)		
BCLC			
A-B	13.0 (6-20)(n=29)	2.03 (1.17, 3.54)	**0.01**
C-D	6.0 (2-12)(n=64)		

Hazard ratios derived using Cox proportional regression. P-values for the equality of survivor function derived using the log-rank test). HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ASH, alcoholic steatohepatitis; NASH, non-alcoholic steatohepatitis; AFP, alpha-fetoprotein; INR, international normalized ratio.
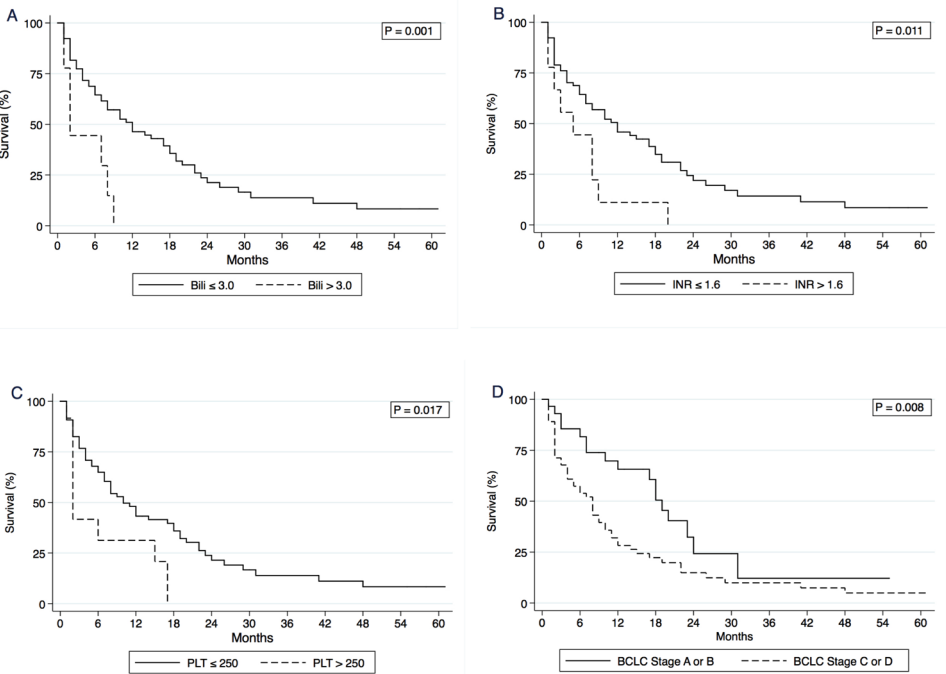
Figure 1. Kaplan-Meyer survival functions for pre-treatment variables: A. Pre-treatment bilirubin; B. Pre-treatment International Normalized Ratio; C. Pre-treatment platelets (PLT); D. Pre-treatment Barcelona Clinic Liver Cancer (BCLC) stage. Y-axis represents survival (%). X-axis represents survival time in months. Kaplan Meyer survival functions were performed using Cox proportional hazard regression and p-values were derived using the log-rank test.

Table 4. Multivariate Analysis of Prognostic Factors Associated with Overall Survival

Parameter	HR (95% CI)	P
Bilirubin ≤ 3.0 mg/dl	2.30 (0.86, 6.17)	0.09
INR ≤ 1.7 IU	1.81 (0.71, 4.59)	0.22
Platelets ≤ 250.000 mm³	2.50 (1.20, 5.20)	**0.01**
BCLC A or B	1.68 (0.77, 3.69)	**0.04**

Variables analyzed by Cox proportional hazard regression. INR, international normalized ratio; BCLC, Barcelona Clinic Liver Cancer.

Side Effects Related to Sorafenib Treatment

Sixty-six percent of patients had side effects related to sorafenib, with diarrhea (30%) and skin toxicity (25%) being the most common. The presence of sorafenib-associated arterial hypertension was relatively rare (4%). Side effect scores were calculated using the methodology described by Di Costanzo *et. al* 54% of individuals had a score of 0, 35% a score of 1, 10% a score of 2 and 1% a score of 3 [17]. Thirty-one percent of patients had to stop or lower the sorafenib dose due to side effects. Patients were more likely to live longer if

they experienced diarrhea (13 vs. 6 months, P = 0.02), skin toxicity (15.5 vs. 6 months, P = 0.034) or any side effects from sorafenib use (11 vs. 2 months, P = 0.008). Patients with a side effect score of 0 had a lower survival than those with side effect scores between 1-3 (4 vs. 12 months, P = 0.004). Lastly, among patients who experienced side effects, those who had to stop sorafenib because of these side effects had shorter survival compared to patients who were able to continue treatment (7.5 vs. 13 months, p = .01).

BCLC Stage C Sub-Analysis

Of the 73 patients within the BCLC stage C sub-cohort, 63 (86%) had sufficient survival data to be included in the analysis. The median age was 61 years (IQR 54-69) and 71% were men. Macrovascular invasion was present in 37 patients (51%). The median survival was 6 months (IQR 2-12). Multivariate analysis within this sub-cohort identified pre-treatment platelets >/< 250,000 mm³ as an independent risk factor for decreased survival (2 vs. 5.5 months, P = 0.03).

Discussion

Until recently and for over 10 years, sorafenib has been the only available systemic chemotherapy for the treatment of advanced HCC. Currently, it is the most widely used medication for HCC worldwide. However, due to its modest survival benefit in some studies, further analyses were necessary to delineate its efficacy in different etiologies of liver disease. In the current study, we provide the first multi-country analysis of sorafenib use in the treatment of HCC, in South America.

The survival benefit of sorafenib in the treatment of HCC, compared to placebo, was demonstrated by two large phase III clinical trials (SHARP: 10.7 vs 7.9 months; Asia-Pacific: 6.5 vs. 4.2 months, respectively, favoring sorafenib use) [7, 11]. As our study represents real-world use of sorafenib, we do not have a placebo-group for comparison. In our study, the median overall survival was 8 months, a value approximating the placebo-arm of the SHARP trial. However, in a sub-analysis of BCLC stage C patients, the median survival was 6 months. Notably, macrovascular invasion was more common in the BCLC stage C patients in our study (51%) than in the SHARP cohort (36-41%)[7]. While this sub-cohort was similar to the SHARP trial cohort with respect to gender and age, this higher proportion of macrovascular spread, in addition to socioeconomic and geographical differences, may help explain the difference in survival between the studies[9-11, 18].

Stratification of HCC by underlying liver disease led to small sub-groups, and as such, our analyses were unable to detect statistical differences in survival by HCC etiology. In the SHARP trial, the predominant etiology of patients treated with sorafenib was HCV

(29%), and in this subgroup, individuals taking sorafenib lived longer compared to those with placebo (14 vs. 7 months, HR: 0.5 [0.32-0.77]) [8]. However, in the Asia-Pacific trial, the predominant etiology of patients treated with sorafenib was HBV (71%), and in this subgroup, individuals taking sorafenib had no survival benefit compared to placebo (5.9 vs. 4.1 months, HR: 0.74 [0.51, 1.06]) [19]. In our study, 38% of HCCs were related to HCV-infection and only 15% to HBV-infection, a distribution similar to most trials performed in Europe and the United States [7, 20-22]. A sub-analysis of the SHARP trial showed that in patients receiving sorafenib, survival was over four months longer in HCV+ patients when compared to HBV+ patients, suggesting that HBV positivity was a risk factor for poor survival [8]. Moreover, a recent study from Europe shows that sorafenib has a substantial survival benefit in patients with HCV-related tumors [10]. Our data shows a similar trend in regards to HBV, as patients who were HBV positive lived half as long as HBV negative patients, although this finding did not reach statistical significance. Interestingly, in our study, positivity for HCV was also associated with slightly lower survival, but this trend also failed to meet statistical significance. These results support the hypothesis that the underlying liver disease in HCC affects the systemic response, particularly the presence of viral hepatitis, since the degree of inflammation and immune response within the liver vary dramatically in the setting of HCV, HBV, or other liver diseases [10, 23]. Remarkably, a recent study from our group revealed that immune markers in serum can predict HCC development in HCV-infected patients, suggesting a stronger role of the immune system in virus-associated HCC [24]. Further studies should investigate immune markers that can help predict treatment response to sorafenib and other systemic treatments for HCC. We were surprised to find that patients with a diagnosis of NASH lived twice as long as those without NASH (although the difference did not reach statistical significance). This could be partially explained by the fact that patients with NASH were more likely to have an earlier BCLC stage, which was found to be an independent predictor of survival. Alternatively a different path of hepatocarcinogenesis (independent of inflammation) could be implicated in the survival difference.

While sorafenib is typically reserved for patients with BCLC Stage C, 33% of our study cohort received sorafenib with a BCLC Stage A or B [25]. Interestingly, sorafenib was the first treatment in almost half of patients (45%) with BCLC stage A/B, whereas it was the first treatment in most patients (96%) with BCLC stage C/D. The differences in treatment patterns by BCLC stage are likely due to variations in clinical practice between countries. These variations include delayed access to transplant lists or TACE, which lead to providers starting immediate therapy with sorafenib.

Univariate survival analysis revealed that elevated pre-treatment bilirubin, INR and platelets, were risk factors for decreased OS. International Normalized Ratio (INR) <1.7IU and total bilirubin >3.0mg/dL are prognostic cut-off points used in the Child-Turcotte-Pugh (CTP) scoring system [26]. While we were unable to calculate CTP scores in our study population due to lack of data on certain variables (albumin and hepatic encephalopathy status), these results suggest that CTP scores may have a role in predicting post-sorafenib survival, which is consistent with previous studies [27-30]. The Model for End-Stage Liver Disease (MELD) score, which also incorporates INR and total bilirubin, has also been associated with post-sorafenib survival [31]. Past studies have found elevated bilirubin to be independently associated with decreased survival, which likely represents either biliary obstruction in the context of mass effect from tumor progression or a decreased synthetic liver function [10, 32]. While INR is a component of the CTP and MELD scores, the isolated effect of INR on survival is not identifiable from past studies [27-30]. It is unclear if these variables reflect more advanced tumor invasion of the liver parenchyma or are a result of more advanced liver disease associated with etiology and not tumor invasion.

Among pre-treatment laboratories trending with decreased survival, only platelets > 250,000 mm^3 remained an independent risk factor. While thrombocytopenia is traditionally considered a hallmark of advanced liver disease, the unique inflammatory environment of HCC may paradoxically promote platelet production, given that platelets are a well-known acute phase reactant. While no previous studies have reported elevated platelets as an independent risk factor for decreased survival in patients on sorafenib, elevated platelets have been associated with increased HCC tumor size and an increased risk of HCC recurrence after liver transplant [33, 34]. Thus, it is possible that elevation or pseudo-normalization of platelets in patients treated with sorafenib, may correlate with a resurging inflammatory environment that leads to treatment failure. Alternatively, the degree of portal hypertension (independent of the presence of cirrhosis) could also play a role in treatment response by affecting drug distribution within the liver parenchyma. Interestingly, our study found an inverse association between the presence of cirrhosis and survival, although this is likely related to HCC development rather than the inflammatory milieu.

The association of sorafenib treatment side effects with improved survival has been well-described [17, 35-37]. In particular, the presence of diarrhea, skin toxicity and arterial hypertension were shown in one study to be independently associated with both overall survival and time to progression of tumor [17]. Our study confirms that diarrhea and skin toxicity are associated with improved survival. Arterial hypertension trended with increased survival, but as it was a relatively rare side effect in our study population, this trend did not reach statistical significance.

Our study is the first of its kind in South America. However, the results of our study should be interpreted in the context of its limitations. As our study did not have a control group, and was performed retrospectively rather than as a randomized, placebo-

controlled clinical trial, it is difficult to compare survival data with past studies. While we conducted a multi-country, multi-center study, data were unequally distributed between participating hospitals, with a large portion of the data coming from Argentina and Brazil. While the size of our study cohort is comparable to prior retrospective studies, missing data in key variables limited the statistical power of our analyses.

Our study indicates that the etiology of HCC may play a role in determining survival outcomes in patients taking sorafenib. In particular, a negative HBV or HCV status and the presence of NASH, may be positive prognostic indicators of survival. Our study identified elevated platelets and advanced BCLC stage as independent risk factors for decreased survival, and confirmed post-treatment side effects are associated with improved survival.

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CHAPTER 5B

PIB: A score to select sorafenib treatment candidates for hepatocellular carcinoma in resource-limited settings

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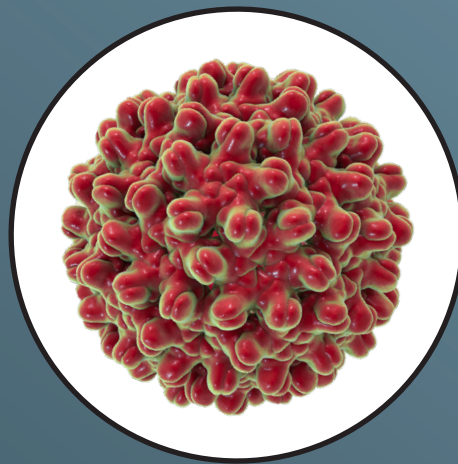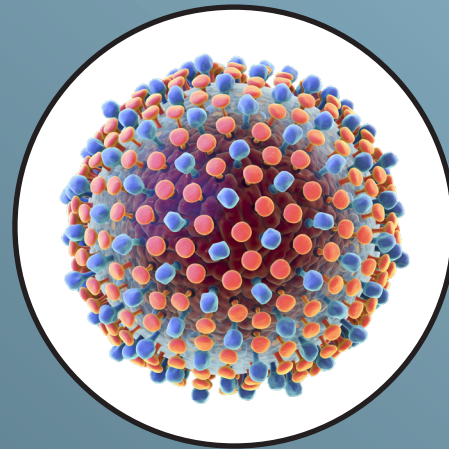
Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death globally [1]. Sorafenib, a multi-tyrosine kinase inhibitor, remains the standard of care for patients with inoperable or advanced-stage HCC. In resource-limited settings without access to surgical or locoregional therapy, sorafenib may be the only option for treating HCC. However, due to a modest survival benefit, as well as the limiting cost of sorafenib in certain regions, appropriate selection of patients for treatment is essential. Evaluation of Barcelona Clinic Liver Cancer (BCLC) criteria in resource-limited settings is frequently unachievable due to a variety of reasons. Using a cohort from the South American Liver Research Network (1336 HCC cases), we created a cost-effective prognostic scoring system to help identify patients likely to have a survival benefit on sorafenib treatment, using simple laboratory variables [2].

Methods

In order to design the Platelet-INR-Bilirubin (PIB) Score, we assigned each patient in the sorafenib cohort, with available laboratory and survival data, one point for each of the following: 1) total bilirubin ≤ 3.0 mg/dL, 2) platelets ≤ 250 x 10^9/L, 3) INR ≤ 1.6, following the methodology previously described by di Constanzo et. Al [6]. Each of these variables showed a similar significant difference in predicting survival and therefore were chosen for this score. Our group previously identified these variables as prognostic factors for improved survival on sorafenib in a South American population [3]. The PIB score has a hypothetical score range of 0 to 3. Measures of central tendency were expressed as medians (Q1-Q3). Kaplan-Meyer survival curves were constructed to graphically compare scores. Hazard ratios were derived using Cox proportional hazard regression with the Breslow method for ties. The log-rank test was used to assess the equality of survivor functions. A level of evidence of $p \leq 0.05$ was taken as the criterion for significance. A biostatistician was consulted for review of our data analysis. Statistical analysis was performed using STATA v14.2 (Statacorp, College Station, TX). This study was approved by the Institutional Review Board (IRB) of Hennepin County Medical Center. In addition, each center was responsible for obtaining appropriate IRB approval.

Results

Of the total 1336 patients with HCC, 127 patients were treated with sorafenib. Of these, 86 had complete laboratory and survival data. Patient characteristics stratified by data completeness are available in **table 1.** The median age of this sub-cohort was 64 years

(IQR: 55-71) and 67% of subjects were male. Hepatitis C infection was the most common etiology of HCC (42%). Sixty-three patients (76%) died during the designated study period. The median survival time after initiation of sorafenib treatment was 7.5 months (IQR 2-17) in all subjects. There were no patients with a PIB score of "0", five patients with a score of "1", 21 patients with a score of "2" and 61 patients with a score of "3". Patients with a PIB score of "1" or "2" had a median survival of 2 months (IQR 1-8 and 2-7, respectively). Patients with a PIB score of "3" had a median survival of 10.5 months (IQR 4-21). Increasing PIB score was significantly associated with improved survival (HR 0.44 [0.29, 0.65], p < 0.001). Kaplan-Meyer survival curves by score are displayed in **Figure 1**.

Table 1. Patient characteristics of Patients with HCC on Sorafenib

Parameter	All Patients (n=127)	Complete Survival Data (n=86)	P
Age	65 (55-71)(n=127)	64 (55-71)(n=86)	.3
Gender			
Male	70% (89/127)	67% (58/86)	.4
Female	30% (38/127)	33% (28/86)	
Etiology of HCC			
Infectious	51% (65/127)	54% (47/86)	.3
HBV +	15% (19/127)	15% (12/86)	.8
HCV +	38% (48/127)	42% (36/86)	.2
ASH	32% (41/127)	36% (31/86)	.08
NASH	17% (21/127)	10% (9/86)	.01
BCLC Stage			
A/B	31% (33/107)	33% (28/86)	.6
B/C	69% (74/107)	67% (58/86)	

Continuous variables expressed as medians (Q1-Q3)(n) and categorical variables as % (proportion). HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; ASH, alcoholic steatohepatitis; NASH, non-alcoholic steatohepatitis.
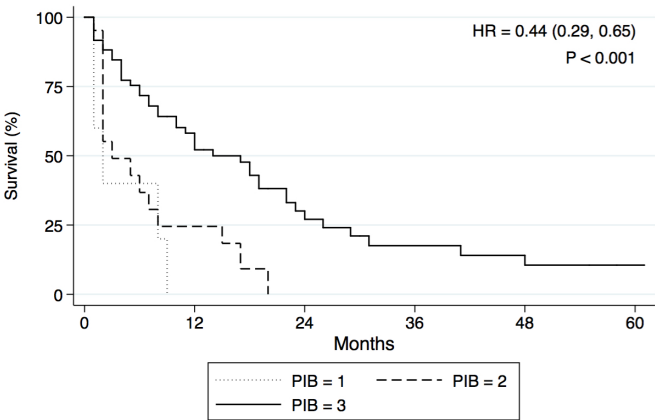
Figure 1. Kaplan-Meyer survival function for Platelet-INR-Bilirubin (PIB) score. Kaplan Meyer functions were performed using Cox proportional hazard regression and p-values were derived using the log-rank test.

Discussion

Our study describes a simple, cost-effective scoring system associated with improved survival in patients with HCC treated with sorafenib. While many new scoring systems are available for this purpose, they tend to include variables that are too financially prohibitive or too administratively cumbersome for under-staffed, resource-limited settings [4-6]. Many physicians rely on Barcelona Clinic Liver Cancer (BCLC) staging to determine sorafenib eligibility, but as this clinical tool is dependent on advanced imaging modalities, its utility is severely limited. Moreover, as HCC in areas such as Africa is generally treated by oncologists rather than hepatologists, addressing BCLC criteria represents an additional burden. Other prognostic scores include sorafenib-associated side effects, making them useful for decision-making related to continuing therapy, but unhelpful for determining which patients should receive initial treatment [6].

Due to the retrospective design, data completeness limited the size of the cohort used to design the PIB score. However, groups stratified by data completeness were similar with regards to baseline characteristics, with the exception of non-alcoholic steatohepatitis (NASH) status, which was less common in patients whose data was used to make the PIB score. As NASH status was not a statistically significant factor associated with survival in our previous analysis, we do not feel that this impacts the reliability of the PIB score[2].

The simplicity and affordability of using the PIB score makes it useful in developing settings with limited access to laboratory and imaging resources. Further validation in a larger prospective study will be of benefit.

Acknowledgement

Previous presentation

Study presented in abstract form at the Global Hepatitis Summit in Toronto 2018.

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CHAPTER 5C

Single center analysis of therapy and outcomes of Hepatocellular carcinoma in Sub-Saharan Africa: An Ethiopian Experience

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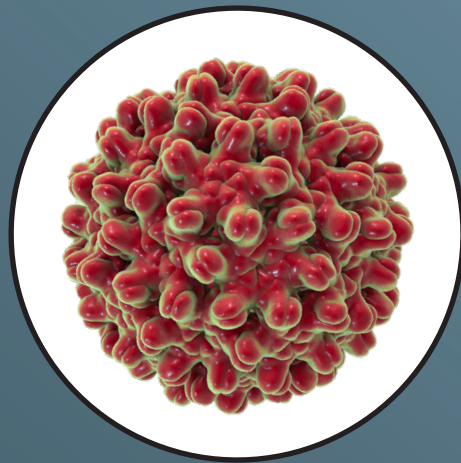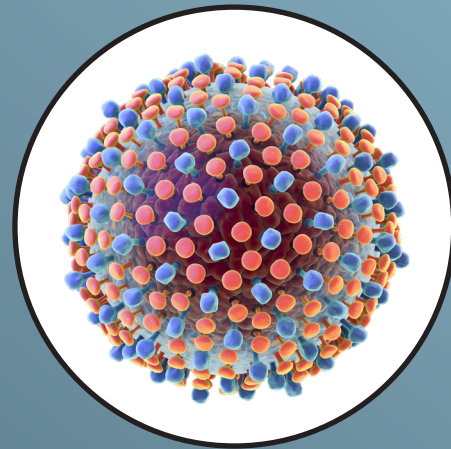
Abstract

Purpose
To evaluate the characteristics and response to therapy for HCC in the sub-Saharan Africa. Patients and methods: We retrospectively evaluated demographic, clinical and outcome variables of individuals diagnosed with HCC in a referral clinic in Ethiopia from 2016 to 2018. Survival assessment was performed using the Mann-Whitney test. Associations between categorical variables was assessed using Pearson Chi-square test. A P-value ≤ 0.05 determined statistical significance.

Results
Forty-six HCC cases were reported, with a median age of 54 years (IQR 45 - 62) and half of them being female. Viral hepatitis was the most common underlying etiology of liver disease, with 41% of subjects infected with hepatitis B virus (HBV) and 45% with hepatitis C. The median MELD was 12 (IQR 8-17) and we found no association between survival and a MELD score </> 15, regardless of underlying disease (pr=0.61, p>0.05). Thirty-one percent of individuals underwent supportive treatment with a median survival of 27 days (IQR 19-181), 18% used Sorafenib (median survival of 94 days, IQR 24-121), and trans-arterial chemoembolization (TACE) with curative intent was utilized in 16% (median survival of 352 days, IQR 30-436). In those treated with sorafenib, a neutrophil-to-lymphocyte ratio (NLR) of <2.5 correlated with better survival (109 vs 68 days for NLR >2.5, p=0.01). HBV cases were diagnosed younger (31% before the age of 40) and those on Tenofovir had a longer median survival than those off Tenofovir (121 vs 34 days).

Conclusion
Our study found that antiviral treatment of HBV infection was associated with longer survival in HCC. Furthermore, Sorafenib was beneficial in patients that used this modality of treatment and the NLR was a good prognostic factor in those patients.

Keywords
Hepatocellular carcinoma, Ethiopia, Sorafenib, Outcomes

Introduction

Hepatocellular carcinoma (HCC) remains one of the leading causes of cancer-related mortality in the world. In 2018 alone, it accounted for approximately 782 000 deaths worldwide, according to reports from the World Health Organization (WHO)[1]. Despite the advancements in technology that enable rapid and accurate diagnosis as well as development of multiple locoregional and systemic treatment strategies, it still accounts for increasing number of hospitalizations with rising inpatient charges and significant financial burden[2,3]. Hepatitis C (HCV) infection remains the most common etiological factor in the development of HCC in the United States and in most parts of Europe. In contrast, globally, Hepatitis B (HBV) infection is recognized as the most common risk factor for HCC. Prior studies have documented the significant healthcare burden associated with HCC in Sub-Saharan Africa[4]. Due to a variety of factors, including viral and environmental ones, HCC tends to affect younger populations in the continent[5]. Moreover, lack of support infrastructure and surveillance programs lead to late presentation of HCC with limited therapeutic options and a dismal prognosis. A recent report from the Africa Liver Cancer Consortium, across nine countries in the continent, showed that outside Egypt, less than 3% of individuals diagnosed with HCC received any form of treatment[4]. Overall, this increased mortality of HCC in Africa has been attributed primarily to lack of resources for early diagnosis and treatment, low rates of childhood immunization against HBV, presentation at late stage, and a more aggressive clinical behavior likely secondary to aflatoxin exposure[6]. Although prognosis and approach to management may differ among these regions, there is a paucity of knowledge on potential treatment approach and survival from these treatments in the African region. Moreover, most studies reporting treatment response originate from areas outside of Africa. In this study, we evaluated a single center in Ethiopia to describe clinical characteristics, survival and therapeutic response to HCC.

Methodology

Study design

We retrospectively evaluated the medical records of individuals that presented with a diagnosis of hepatocellular carcinoma (HCC) to a major referral clinic (Adera Clinic) located in Addis Ababa, Ethiopia from 2016 to 2018. The diagnosis of HCC was made via liver biopsy or radiological imaging criteria as established by the guidelines from the American Association for the Study of Liver Diseases (AASLD)[7].

Data collected

We collected basic demographic data on individuals, including that of the underlying

etiology of liver disease (viral hepatitis testing, alcohol intake, other), presence of cirrhosis and jaundice, as well as other liver and tumor variables such as Model for End Stage Liver Disease (MELD) score, size of tumor at time of diagnosis, regular laboratories including complete blood count and basic metabolic panel as well as serum aminotransferases (AST & ALT), bilirubin and alkaline phosphatase levels and serum Alpha Feto-Protein (AFP). In those individuals with a positive result for viral hepatitis B or C, pertinent information about each virus was obtained (genotype of HCV and viral load when available; e antigen status for hepatitis B, HBV DNA when available) as well as treatment status.

Analysis

Median survival time was calculated from the date of last visit to the date of the initial diagnosis visit. Continuous variables were expressed as medians (Interquartile range Q_1 to Q_3), and categorical variables were expressed as proportions (%). Individuals with date of last visit to demise of less than seven days were excluded from the final survival analysis. Survival assessment was done using the Mann-Whitney test. Associations between categorical variables was assessed using Pearson Chi-square test. A P-value ≤ 0.05 was taken as the criterion for statistical significance

Ethical Considerations

This study was approved by the Ethics committee of Adera Clinic, Addis Ababa, Ethiopia.

Results

Individual and tumor characteristics

The baseline characteristics of the individuals diagnosed with hepatocellular carcinoma (HCC) are represented in Table 1. A total of 46 cases of HCC were reported and collated with a median age of 54 years (IQR 45 - 62). Exactly half of these patients were female. The regions of origin of all the reported cases are depicted in Figure 1. Most cases were from the Addis-Ababa region (67%), whilst the Tigray and Harari regions were represented by 7% of the cases each. The most common presenting symptoms were abdominal pain in 96% of patients and weight loss in 76% of patients. Sixty-five percent of patients had evidence of ascites at time of presentation. Viral Hepatitis accounted for the underlying etiology of liver disease in 87% of cases; with the viral origin somewhat evenly divided between Hepatitis B virus (HBV) infection in 41.3% of cases and Hepatitis C virus (HCV) infection in 45.7% of cases. Thirteen percent of individuals had no specific diagnosis of etiology of liver disease, and there were no documented cases of alcohol-related cirrhosis or non-alcoholic fatty liver disease. We found no association between region of origin and etiology of liver disease (pr=0.126, p>0.05). At time of diagnosis of HCC, 85% of

the patients had cirrhosis with 17.4% of them presenting concomitant jaundice. Review of laboratory data revealed a median AST level of 91 U/L (IQR 67 - 113) and calculated median MELD was 12 (IQR 8 - 17). We found no association between survival and MELD score </> 15 regardless of underlying liver disease (pr=0.61, p>0.05). The median tumor size in these cases was noted to be 6.5 cm (IQR 4.5 - 6.9), and serum AFP was found to be > 400 ng/ml in about half of the reported cases. About a third of patients had evidence of vessel invasion and 37% of patients were Barcelona Clinic Liver Cancer Stage C or D.

Table 1. Baseline characteristics

| Variables	
Demographics	
Age, in years (IQR)	54 (45 - 62)
Female sex (%)	50
Black Ethnicity, N (%)	46 (100%)
Underlying liver disease	
Hepatitis B infection, N (%)	19 (41.3)
Hepatitis C infection, N (%)	21 (45.7)
Alcoholic liver disease, N (%)	0 (0)
Non-alcoholic fatty liver disease, N (%)	0 (0)
Other etiology, N (%)	6 (13)
Tumor variables	
Evidence of cirrhosis, N (%)	39 (84.8)
Presence of jaundice, N (%)	8 (17.4)
Median MELD score (IQR)	12 (8 – 17)
Median serum AST, U/L (IQR)	91 (67 – 113)
Median Tumor size, cm (IQR)	6.5 (4.5 – 6.9)
Serum Alpha Feto-Protein >400 ng/ml, N (%)	24 (52.2)
Major vessel invasion, N (%)	15 (32.6%)
Child-Turcotte-Pugh B or C, N (%)	31 (67.4%)
Barcelona Clinic Liver Cancer Stage A/B, N (%)	22 (47.8%)
Barcelona Clinic Liver Cancer Stage C, N (%)	13 (28.3%)
Barcelona Clinic Liver Cancer Stage D, N (%)	4 (8.7%)
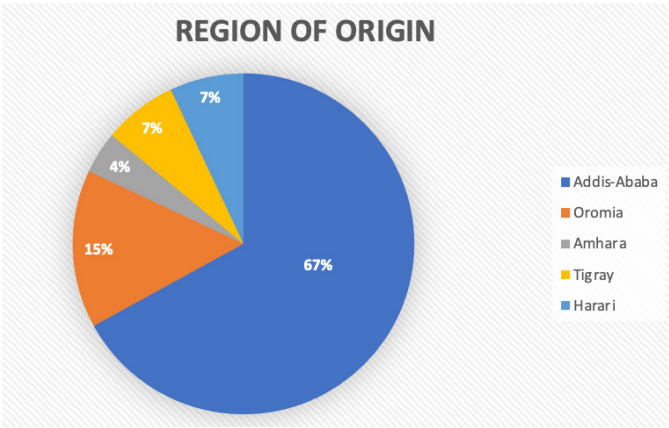
Figure 1. Distribution of HCC cases based on region of origin

Treatment and survival

Treatment strategies varied widely (Table 2). About a third of the individuals underwent supportive treatment (32.6%), and median survival in these cases was 27 days (IQR 19 - 181). Sorafenib was used in 19.6% of patients, whereas locoregional therapy with trans-arterial chemoembolization (TACE) with curative intent using Doxorubicin, Cisplatin and Lipidiol, was utilized in 8 patients (17.4%). There was a trend towards improved survival in the first 6 months among patients treated with TACE, but this was not statistically significant (p 0.55). Among the patients treated with Sorafenib 55% had advanced HCC with an estimated Barcelona Clinic Liver Cancer (BCLC) stage C to D. Two patients (22%) had BCLC stage C and one patient each (11%) had BCLC stage A and stage B. Overall, Sorafenib was well tolerated as a medication and only one patient (11%) stopped the treatment, reporting significant nausea and vomiting. The median survival in patients treated with Sorafenib or TACE was 94 days (IQR 24 - 121) and 352 days (IQR 30 - 436) respectively. A lower neutrophil-to-lymphocyte ratio (NLR) of <2.5 was found to correlate with better survival (109 days vs 68 days for those with NLR >2.5, p=0.01). From a total of 9 individuals treated with Sorafenib, 5 were infected with HBV and all of these patients had an AFP level >400ng/ml (Table 3). Although proper evaluation of survival was not possible due to low numbers (2 cases of HBV-Sorafenib had less that 7-day follow up visit), the median survival in the remaining 3 was comparable to that of non-HBV HCCs treated with Sorafenib (122 vs 94 days). In the HBV subgroup of nineteen (41%) patients, the median age of diagnosis was 48 years, (IQR 19 – 38), with 31.6% of patients diagnosed before the age of 40. Serum AFP was higher than 400 ng/ml in 57.9% of these HBV infected patients and the median survival was 83 days (IQR 18 – 381). Among those on Tenofovir, the median survival was 121 days, whereas it was 34 days in patients who were off antiviral therapy despite having larger tumor-size (median 7.3cm vs 6cm).

Table 2. Treatment regimen and survival

Variables	
Treatment Regimen, N (%)	
Surgery	1 (2.2)
Trans-arterial chemoembolization	8 (17.4)
Sorafenib	9 (19.6)
Palliative	15 (32.6)
Others (Lost to follow up, no access to therapy)	13 (28.3)
Survival, days (IQR)	
General median survival	68 (17 – 334)
Trans-arterial chemoembolization	351 (30 – 436)
Sorafenib	94 (24 – 121)
Palliative	27 (19 – 181)
Overall survival in HBV infected patients	83 (18 – 381)
HBV patients on anti-viral treatment	121 (19-387)
HBV patients not on anti-viral treatment	34 (15-294)

Table 3. Characteristics of HCC-HEPATITIS B SUBGROUP

Variables	
Number of patients, N (%)	19 (41.3)
Median age at diagnosis, years (IQR)	49 (19 – 38)
Diagnosis <40 years, N (%)	6 (31.6)
AFP >400 ng/ml, N (%)	11 (57.9)
Median survival on antiviral therapy	121 days
Median survival off antiviral therapy	34 days
NLR >2.5	68 days

Table 4. Characteristics of individuals treated with SORAFENIB

Variables	
Patients on Sorafenib, N (%)	9 (19.6)
Median survival, days (IQR)	94 (24 – 121)
Alpha Feto-Protein >400 ng/ml, N (%)	9 (100)
HBV infection, N (%)	5 (55.6)
Neutrophil-to-lymphocyte ratio (NLR) and survival	
NLR < 2.5	109 days
NLR > 2.5	68 days

Discussion

The burden of HCC cannot be overemphasized as it is associated with significant morbidity, mortality and financial repercussions world-wide [1,8]. In Africa, the incidence rates of HCC range from 20 – 40 cases per 100,000 population, but the true incidence of HCC in this region is underestimated, mostly due to paucity of records, absence of cancer registries in some countries and unclear diagnosis[9,10]. Viral hepatitis remain the predominant risk factors for development of HCC, with HBV sustaining a major burden in Sub-Saharan Africa[4]. Although HCC can occur in patients with chronic HBV infection, cirrhosis has been established as a very important risk factor for the development of HCC with studies estimating about 90% of patients having a diagnosis of cirrhosis at the time of HCC diagnosis [11-13]. We found that 85% of the cases of HCC had a diagnosis of cirrhosis irrespective of etiology of liver disease. Hence, routine HCC surveillance of these high-risk groups will promote the detection of early stage HCC and hence increase the chances of curative therapy[11,14]. In our cohort, only a minority of individuals received any attempt at HCC cure. It should be noted, that in the region, the characterization of "palliative approach" mainly applies to "best supportive therapy". Under the strict definition of non-curative approach, over 80% of individuals in our study received palliative care, with only 19.6% of them receiving a potentially curative therapy.

Treatment using TACE had a trend towards improved 6-month survival, though this was not statistically significant (Figure 2). In these patients, the median survival was 351 days. However, only a small number of patients (17.4%) were treated with this modality.

In addition, most of the patients treated with sorafenib (55%) had advanced stage HCC, underscoring the prevalence of late diagnosis of HCC in the region. Due to the study's retrospective nature, the Barcelona Clinic Liver Cancer (BCLC) staging of HCC was estimated in a limited number of patients, and performance status estimation was deemed inaccurate with the available information. Nevertheless, using the Child-Turcotte-Pugh (CTP) score, size and number of tumors and evidence of portal invasion, we estimated the HCC stage in these patients.
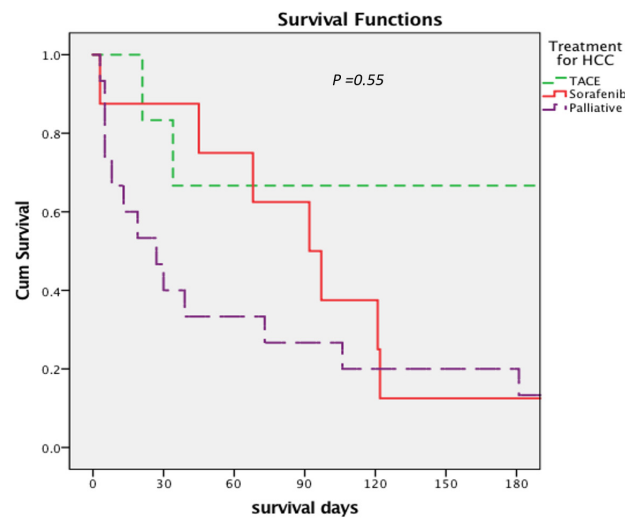

Figure 2. Kaplan-Meier survival curve showing survival for Sorafenib, TACE and Palliative care

Late diagnosis of HCC, probably from paucity of resources, poor access to healthcare as well as late presentation of patients, is likely responsible for our findings. These factors stymie adequate healthcare in many regions of Sub-Saharan Africa, so effective health policies and early testing for viral hepatitis will help improve survival in these high-risk group of individuals[6,15]. In this region, vaccination rates among healthcare workers were low, and range from 4 – 28.7%, while vaccine coverage among children was as high as 85.1%[16-18]. Improved education of people at high risk of infection, such as HCW, as well as improving childhood vaccination programs, will also be beneficial.

Our results show an increase in median survival in patients with HBV who were on antivirals (121 days) compared to those who were not (34 days). Active HBV DNA replication and integration in hepatocytes induces genetic damage, predating the formation of abnormal cells and development of HCC[19]. It is therefore not inconceivable that

suppression of viral replication will be beneficial to the survival of patients with HBV-related HCC, and this has been echoed in multiple studies[20-22]. Though these findings have been clearly described in resource-rich settings, the information from HBV-endemic areas with low resources is not readily available. In regions of Sub-Saharan Africa where access to locoregional or systemic therapy is limited, consideration should be given to starting antiviral therapy for HBV, when available, in individuals with HBV-HCC as this could impact their survival.

Finally, our study found a better-than-expected survival in those individuals treated with Sorafenib. The use of Sorafenib for HCC as not been described before in Africa. Moreover, previous studies have shown a preferential response to Sorafenib in individuals infected with HCV[23]. Our results, albeit from a very small number of patients, suggest that even in the setting of HBV infection, Sorafenib could have a role in prolonging survival of HCC-affected patients. Furthermore, Sorafenib was well tolerated in these patients. This is significant, because, in areas with derisory infrastructure required to establish advanced loco-regional therapy, the administration of oral medications is, at times, the only available therapy. Furthermore, cell-mediated inflammatory response is increasingly being recognized as having an important role in tumorigenesis and a the Neutrophil-to-lymphocyte ratio (NLR) has been reported to be a prognostic factor in many solid tumors with high levels suggesting a worse overall survival[24,25]. In patients with HCC treated with Sorafenib, high NLR values have been associated with worse outcomes[26]. We found a worse median survival in those with NLR >2.5. As technology yielding automated cell counts is becoming increasingly popular, NLR values could be implemented as a tool to further stratify the cost-benefit of treating individuals with Sorafenib.

We aimed to assess the characteristics, prognosis and management of HCC in a large center in Ethiopia to give an insight to the burden of disease in Sub-Saharan Africa. Despite the uniqueness of our study, it has several limitations. Firstly, we reported a limited number of cases with HCC and this may not be entirely representative of the population in Sub-Saharan Africa, however representative it might be from Ethiopia as patients presented from different regions of the country. Secondly, the retrospective nature of the study limits our collated variables to those available in the patients' medical records. As such, it is difficult to evaluate the effect of additional patient variables on median survival. Finally, the assessment of Barcelona Clinic Liver Cancer (BCLC) score, was not assessed at the time.

Nonetheless, our report provides important insight on HCC outcomes in a single center in Sub-Saharan Africa. Our study found that antiviral treatment of HBV infection was associated with higher median survival in our cohort of cases with HCC; the use of

sorafenib was beneficial in the limited number of patients that used this modality of treatment, and the Neutrophil-to-lymphocyte ratio showed to be a good prognostic factor in those patients. Larger prospective studies are needed to evaluate its specific role in the management of HCC in the region.

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ANNOTATIONS

* Of interest
** Of considerable interest

Prior presentations

AORTIC Meeting 2019, Maputo, Mozambique (poster).

6

Discussion

This thesis deals with different aspects of hepatocellular carcinoma (HCC) from epidemiology, prevention, mechanisms of cancer formation and treatment. It approaches however, each of these issues in a global fashion with emphasis on the differential geo-epidemiological aspects of HCC as well as the prevention and treatment based on resource levels in different regions of the world.

In this discussion, I summarize the findings of these studies and their implications to specific individuals suffering from HCC as well as their potential influence in global policy.

The epidemiology of hepatocellular carcinoma in South America

The risk factors for HCC express considerable variation based on the geographical region of the world, with hepatitis C recognized as a major risk factor in North America and Europe, hepatitis B in Asia and Africa, and alcohol user disorder in Eastern Europe (1-3). There are indeed differences in recommendations for age-based HCC surveillance protocols depending on the country or continent of origin of a person, with recommendations to screen earlier in patients of African and Asian descent (4). Most of what is known from the epidemiology of HCC is based on the BRIDGE study, a large collaboration of 42 sites across the globe involving over 18.000 patients with HCC (5). This study did not include data from the regions of Africa and South America.

Our study (Debes et al Liv Int) evaluated 1300 patients with HCC in 6 countries in South America and found that, similar to other populations in the Americas, HCV and alcoholic liver disease were the most common risk factors for HCC in the region.

More importantly, our study in CGH (Chan et al CGH) unexpectedly found that almost 40% of HBV-associated HCC in South America occurs before the age of 50 years. The early-age diagnosis of HBV-related HCC has previously been reported in African studies and in association to aflatoxin exposure (6). Exposure to aflatoxins is known to affect modulation of the TP53 gene, and this is thought to lead to an early occurrence of HCC (6). Aflatoxins are not known to present a major issue in South America, although aflatoxin-associated p53 mutations have been found in HBV-related HCCs in a small Brazilian study (7). This finding, never reported before in the area, is of high significance, as it questions whether individuals in South America, or from South American origin infected with hepatitis B, should begin surveillance for HCC before the recommended age of 50 years. In this regard, Peru as a single country accounted for 35% of these cases and this should be taken into account. Moreover, the great majority of these cases originated from the Amazonian Basin, thus suggesting that a micro-geographic approach to screening could be used in certain patients. What seems clear is that a "blanket" screening approach for HCC is incompatible with reality and that even "continent general" recommendations might not be adequate for proper screening. A "targeted" approach specific for certain regions, based on epidemiological studies, might provide the best methodology with the most benefit for patients at risk for HCC.

Interestingly, the presence of NALFD was not a major single risk factor associated with HCC in our study, which was a rather surprising finding since the NAFLD prevalence is higher in South America than in other regions of the world (8). As the dynamics of HCC risk factors evolve it is likely that an updated study (ongoing through our –South American Liver Research Network-SALRN) might show a different picture. Indeed, the global epidemic of obesity and lean NAFLD will represent a new challenge for HCC surveillance, as many of these patients develop HCC before cirrhosis and it will be difficult to ascertain who to screen on a regular basis. The approach of HCC surveillance in NAFLD will need specific developments in which individuals with NAFLD and certain unique characteristics that puts them at higher risk for HCC are recruited into screening programs. It is likely that for the success of these programs they will be run in conjunction with other specialties such as primary care or endocrinology in a multidisciplinary fashion to maximize adherence.

Hepatitis B and HCC: awareness and diagnosis as cancer prevention

Hepatitis B infection (HBV) is the most common risk factor for HCC worldwide by numbers, with a specific high incidence in Asia and Africa (3). Hence, prevention of HBV via vaccination and early diagnosis so to implement proper screening have a direct impact in HCC-related morbidity and mortality. It is impossible to talk about prevention of a disease without talking about awareness of a disease. Populations will rarely take action to "prevent" a problem if they are not "aware" of it. This represents a major challenge in vaccination campaigns in sub-Saharan Africa. Although programs aiming at implementation of HBV vaccine are present, they mainly serve larger metropolitan areas. In rural areas, where over 60% of the African population lives, awareness of hepatitis B is scarce, and therefore efforts to obtain the vaccine are low (9, 10). Boye *et al* reported that two thirds of a rural population interviewed in Senegal recognized the sequelae of chronic hepatitis B but only one third was familiar with the term "hepatitis B" used by medical personnel (11). In our study (Debes et al AJTMH) assessing HBV knowledge and vaccination uptake among healthcare workers in Tanzania, we found that over 50% of healthcare workers did not know whether they were vaccinated or not for HBV and the

great majority were not tested for hepatitis B. Moreover, a larger study from our group covering a dozen countries in Africa (Shah et al AJTMH) found that a large number of healthcare workers in the continent were not aware whether their children received or not the vaccine against HBV. In this regard, the requirements to promote disease awareness are relatively simple: spreading of a message and simplicity of a message. In our efforts, we proposed a term-program called "The waiting room project" in which providers can teach about hepatitis B to patients waiting to be seen in clinic. We applied this approach (Quadri et al AJTMH) in two hospitals in rural Tanzania, first surveying patients about hepatitis B and later providing a 3-minute discussion about the disease. A post teaching survey, showed significant differences in HBV-focused knowledge. An important aspect of this work is the emphasis on teaching healthcare workers to teach the community about hepatitis B. We have now engaged into developing an app-platform that can provide information about hepatitis B to healthcare workers, and directions in how to communicate and increase awareness about the disease to different communities in Africa. This is a critical point, as academia tends to focus on more advanced scientific details, forgetting at times that an increase in population awareness of hepatitis B can have a dramatic impact on vaccine uptake, particularly a push for birth-dose, thus preventing the precise risk factor that leads to liver cancer as well as advanced liver disease.

The second aspect addressed in this section is that of diagnosis of hepatitis B. Almost 70% of individuals with hepatitis B in the United States are not aware of their infection, and this number is likely to be much higher in resource-limited areas (12). We evaluated a novel systematic approach of using rapid tests to diagnose hepatitis B in Argentina. First, we validated point-of-care (POC) tests for HBV in a collaboration between the Netherlands, Argentina and Ethiopia (Leathers et al AofGH). We found that rapids tests measuring HBsAg test had 100% sensitivity and specificity and a HBeAb test had over 80% sensitivity with 100% specificity, allowing us to not only detect HBsAg but also to assess patients with HBeAg/Ab status. Later we used these tests to offer hepatitis B to individuals that presented with non-life threating complaints to an Emergency room in Argentina (Leathers et al AJEM). The study, albeit small, showed that over 55% of individuals that tested negative for HBsAg underwent a first dose of the hepatitis B vaccine. Therefore, it can be speculated that a cost-effective approach such as this can not only improve hepatitis B diagnosis at a population level but also provide an opportunity to vaccinate those at risk. Similar studies have been performed to link individuals to preventative services during emergency room visits, but none for hepatitis B (13). This approach represents a unique opportunity as in most cases of young individuals (at higher risk for HBV) living in resource-limited settings a visit to the emergency room is frequently the only time they visit a healthcare provider, thus allowing for a one-time intervention that could prevent HCC.

Overall, this section brings a different perspective to the scientific approach to HCC prevention, and that is one that focuses on detection of a risk factor (HBV in this case, although this could be applied to HCV), so to either implement vaccination and prevention of disease, or so to implement early detection for proper follow up and treatment. In addition, this style of approach can undoubtedly lead to broader awareness of risk factors for HCC (such as hepatitis B, C and excessive alcohol intake) which in turn will increase public interest and promote better preventative health dynamics. Sadly, most the work in prevention and awareness has fallen on the shoulders of general providers or epidemiologists, while specialists in liver disease have tended to focus on HCC treatments or hepatitis elimination. It will likely be more productive in terms of lives saved, if liver specialists become more involved in the awareness and prevention aspects of viral hepatitis so to maximize mitigation factors that lead to HCC. This involvement will benefit patients even in a case-by-case of single hepatologists, but the impact will be more robust in a team-approach with liver specialist work in conjunction with other providers.

Molecular mechanisms of HCV-related carcinogenesis

Hepatitis C infection leads to liver cancer through the process of cirrhosis. Hence, the great majority of individuals with HCV and HCC will develop cirrhosis before developing HCC (14). One would assume then that the only aspect of viral-induced cancer in HCV is that of inflammation-mediated cirrhosis. There is, however, data from mice that suggests a more direct role for the hepatitis C virus into liver cancer formation. Indeed, mice expressing the HCV polyprotein can develop liver cancer in the absence of inflammation, hepatic cirrhosis or immune recognition of the transgene(15). The HCV core protein can also activate the Wnt/b-catenin cascade, which is thought to play a role in the development of HCC, as 50-70% of HCCs showing nuclear accumulation of b-catenin (16).

To assess whether carcinogenic events take place in pre-cirrhotic livers, we performed single-hepatocyte RNA sequencing of hepatocytes obtained from core needle liver biopsies (single cell sequencing) from individuals infected with HCV, in a fashion that allowed us to differentiate gene expression of hepatocytes infected or not with HCV within the same patient (Debes *et al* submitted). We found that tumor-related and tumor-suppressor transcripts are differentially modulated in hepatocytes with HCV compared to those without HCV, within the same patient. Several of these cancer-related transcripts are known to be differentially expressed in HCC (i.e. p53, E2F, CCN), suggesting that this initial genetic hit towards hepatocarcinogesis could be HCV-mediated (17). Although this study was small (4 patients), it was significant in the fact that all four individuals

had little to no fibrosis (F0-F1) suggesting that mechanisms related to HCC during HCV infection can occur before the development of liver cirrhosis. Several lines of research are likely appropriate and necessary next steps in this area: a) further confirmation and characterization of this finding should be performed in a larger cohort of patients (this could be difficult as in Europe the majority of individuals with HCV are treated without the need for biopsy); b) assessment of whether this process is unique to HCV infection or if it also applies to individuals infected with hepatitis B and; c) Evaluation of these transcripts as potential biomarkers to predict the future development of HCC in those infected with viral hepatitis.

Liver cancer detection through immune evaluation

Mortality from hepatocellular carcinoma (HCC) occurs primarily due to late detection, which precludes potentially curative surgical or minimally invasive interventions. Indeed, there are currently no reliable markers for early recognition and screening of HCC, and the tumor rarely elicits symptoms, which leads to liver cancer being diagnosed at a late stage (18). Ultrasonography is currently used every 6 months for surveillance of HCC in those at risk. However, the sensitivity of ultrasonography for HCC detection is low (65-80%), it is operator dependent, and cumbersome for patients, as they need to fast and attend the hospital twice a year just for screening (19). It is not surprising then that adherence to surveillance programs for HCC is quite low, <50% in Europe and the United States (20, 21). Moreover, our study in South America (Debes et al Liv Int) found that only 50% of HCC diagnosed in referral centers in the region were due to participation in surveillance programs. There is indeed a clear need for the development of biomarkers to early and easily identify HCC in individuals at risk, which will lead to more efficient and cost-effective therapies against the tumor. However, the genetic diversity of HCC leads to unreliable production of tumor proteins therefore impairing this approach to find useful and precise biomarkers in blood.

In this thesis, we describe a 12-marker immune-signature that proved reliable to predict HCC development over the next 2-years at one single baseline point of measurement (Debes *et al* Gastroenterology). The immune-signature was born out of a concept speculating that immunogenic markers would be elevated in peripheral blood before a tumor is "visible" under imaging techniques in individuals that eventually develop HCC. This novel concept is particularly critical in the case of HCC related to chronic viral hepatitis and HCC arises from a setting of liver disease with a highly immunogenic and inflammatory background (22). Therefore, investigating the immune reaction of the body elicited by HCC as a predictive marker of tumoral presence is a logical, yet unexplored, approach. This published study, nonetheless, was performed in a small

number of samples (13 HCC cases) and of HCV-infected individuals. The ramifications have been important, as this paper led to a larger study that will analyze immune signatures for HCC in a cohort in South America and Europe (ESCALON). The obvious question here is whether this signature will apply to other HCC secondary to other liver diseases such as hepatitis B or even HCC related to alcohol use disorder. It is likely that as different diseases promote differential expression of immune analytes, such signature would vary based on the underlying immune trigger. It is however likely that biomarkers for HCC will have to be based specifically on the underlying liver disease, as more data (from our group and others) suggest that the formation of HCC as well as prognosis and its response to therapy does vary according to the underlying liver risk factor (17, 23-25). It is nonetheless, clear that the current approach to HCC surveillance is suboptimal. When one compares HCC surveillance to that of colon cancer surveillance via colonoscopy (71% participation), breast cancer screening via mammography (83% participation), the approximately 50% participation for HCC surveillance described worldwide is quite demoralizing. Although the specific population of individuals with liver disease might play a role, there is a lack of adaptation of surveillance methods from specific health programs to that specific population, evident by the low levels of adherence. One change of focus by the scientific community, brought up in the section above, would be that of awareness: improve the knowledge and awareness of those at liver about HCC risk. Another change, which is already happening is the quest of easy to measure biomarkers in blood. Our model of immune-markers, as well as others using microRNAs, exosomes and Single-nucleotide polymorphism (SNPs) will likely bring to light new and easy ways to predict HCC. This will be critical in implementing proper surveillance not only in large referral centers, but also in more isolated areas of African and South America, where the problem is even larger.

Treatment for HCC: the impact of underlying liver disease and geo-epidemiology in the response to systemic therapy

Until 2007 there was really no systemic therapy effective for HCC. From 2008 until 2017 the only systematic therapy available was sorafenib, a multikinase inhibitor that is taken orally two times a day (24). Since 2017 until present day, we have witnessed a dramatic increase in systemic therapy options for HCC, including a variety of multikinase inhibitors, targeted antibodies and immunotherapy options that have become available this year (26).

However, due to its wide availability and simplicity of administration, sorafenib still plays a major role in systemic therapy for HCC, particularly in resource-limited settings. The survival benefit of sorafenib in the treatment of HCC, compared with

placebo, was demonstrated by 2 large clinical trials, the SHARP trial: showing survival of 10.7 vs 7.9 months and the Asia-Pacific trial: showing survival of 6.5 vs. 4.2 months; respectively, favoring sorafenib use (24, 27). Later studies suggested that sorafenib can have a differential (better) response in HCC secondary to HCV infection compared to other etiologies (24). The basis of this differential effect are unclear, but the immune mechanisms related to HCC in different primary liver diseases might play a role in this response. In this thesis, we provided the first multicountry analysis of sorafenib use in the treatment of HCC in South America, addressing the real-world use of sorafenib (Leathers *et al* J Clin Gastroenterol). In our study, the median survival of HCC under sorafenib was 8 months, a value approximating the placebo arm of the SHARP trial. However, in a subanalysis of BCLC stage C patients, the median survival was 6 months. Interestingly, sorafenib was the first therapy in the great majority of those with BCLC C or D. This suggests that in real-world practice sorafenib is used in individuals with a more advanced BCLC stage than those used in the trials, likely showing a real-world lower survival. Surprisingly, we found that patients on sorafenib with a diagnosis of NASH lived twice as long as those without NASH, but this could be explained by the fact that patients with NASH were more likely to have an earlier BCLC stage, which was found to be an independent predictor of survival. Alternatively, a different path of hepatocarcinogenesis (independent of inflammation) could be implicated in the survival difference. These results should be taken overall with caution as we had no placebo randomization, but rather analyzed observational data. In this work, we also proposed a simple score to predict response to sorafenib therapy in HCC (the PIB score, based on platelets, INR and bilirubin). In our study (Leathers *et al* Hep Month), this simple score correlated well with response to sorafenib. Although such a score should be validated prospectively and in other cohorts, it provides a reasonable alternative to be used in resource limited settings, were at times a hepatologist is not the person to start therapy and proper use of the BCLC criteria is not standard.

Our studies on HCC in a single center in Ethiopia (Anugwom *et al* Exp Rev Gastroenterol Hepatol) showed an even more complex picture in terms of systemic therapy for HCC. The most commonly used treatment (almost 20%) was sorafenib. However, survival in this group was rather dismal, approximately 3 months. Certain positive aspects were taken from these study: A) in those with HBV as underlying liver disease treatment with antivirals against HBV improved survival. Active HBV DNA replication in hepatocytes induces genetic damage, promoting the formation of abnormal cells and development of HCC (28). It is therefore reasonable to assume that suppression of HBV replication will be beneficial to the survival of patients with HBV-related HCC. This has been shown in other studies in the past (29, 30); B) The NLR (Neutrophil to Lymphocyte Ratio) did correlate with survival in those treated with sorafenib. This ratio has been proposed in the past to predict survival in HCC treated with sorafenib, but only in Europe (31).

Our study is the first to show this correlation in an African cohort, and although the difference in survival was not statistically significant (likely due to low number of patients) those with NLR<2.5 had almost double the survival as others. Although these studies (our published paper and an additional published letter, Aby et al EJGH) are the first of its kind in the region, they are small and the need for larger studies involving sorafenib and other simple to administer therapies in resource-limited areas is quite clear. From a cost-effective point of view, it is likely that medications like sorafenib will decrease in price as other therapies for HCC become available in the field. Hence, it might become a reasonably priced option for those in areas with low resources. However, the use of cost-effective medications should be seen as temporary approaches and not abrogate the implementation of best therapies in the region. In an ideal world, proper training of providers in resource-limited areas for the use or radio-frequency ablation or transarterial embolization seems a reasonable and reachable short-term goal. It is likely that "centers of Excellence" in treatment of cancer, will be the path to follow, so that patients at least have a place to be referred to for proper treatment. The ultimate cure, which would be liver transplantation, although present in some resource-limited countries, is likely a be part of a long-term horizon. The complex surgical and medical dynamics as well as prohibitive costs for years of life makes this approach rather unattainable until a region would become plainly rich. This, of course requires changes not only in healthcare but in educational, economic and political policies and goes far beyond the speculations addressed in this thesis.

Our studies emphasize the point that although HCC is one disease it is quite variable in its behavior and response to therapy depending on the underlying liver etiology and the epigenetic effects of the regional area where it occurs.

Sadly, the most important remark of these studies is that HCC is diagnosed too late in too many patients, leading to a struggle in providing appropriate therapy that could lead to meaningful survival and decent quality of life. Despite the fact that, as mentioned above, the field has now been improved with new therapies, the most important factor still remains appropriate surveillance of HCC in those at risk, so to achieve early diagnosis and accomplish therapy directed to cure the tumor. This "factor" involves identifying who is at risk and developing and implementing simple tools for surveillance, while also focusing on prevention by vaccination when possible. Until this relatively simple but incredibly difficult to implement factor becomes a standard reality, providers around the globe will continue to provide too little-too late to individuals diagnosed with liver cancer.

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7

Summary

This thesis argues that as underlying liver disease defines occurrence and prognosis of HCC, the area of the world where a patient is born or lives is of importance when considering screening or treatment. Indeed, the thesis shows that hepatitis B-related HCC can occur at early ages in South America, exposing consideration for early screening in this region. It also argues that even in a relatively homogenous area like South America, the main risk factors for HCC do vary country by country. The prevention section focuses of hepatitis B-related HCC (the most common risk factor worldwide) and explores new rapid tests for hepatitis B, arguing that easy diagnosis of the disease is the ultimate approach to implement screening for HCC in this population. Moreover, it proposes a novel approach of using emergency department visits as platforms to test young people for hepatitis B, a concept that could have considerable success in resource-limited areas where primary care establishment is not as strong as in resource-rich areas.

Mechanistically wise, this thesis shows for the first time that a differential gene expression in sorted hepatocytes of the liver (increase in oncogenes and decrease in tumor suppressor genes) is present in individuals infected with hepatitis C, the most frequent risk factor for HCC in western countries, even at very early fibrosis levels. Suggesting that the path to hepatocarcinogenesis in these individuals is already present before the development of liver cirrhosis. This section also provides evidence that immune markers detected in peripheral blood can predict the development of HCC in individuals with hepatitis C. A certainly novel approach to screening. Whether this is applicable or not to HCC from other causes is discussed below. Finally, the thesis explores the different treatments used for HCC in areas of South America and Africa. It brings to light the low number of individuals that get treated via liver transplantation, the ultimate treatment for HCC and highlights the large number patients that can only receive palliative therapy. It also assesses the use of sorafenib, one of the few systemic therapies for HCC, in these parts of the world, providing insight into survival in different HCC groups as well as proposing a new score to predict survival, that could benefit resource-limited areas.

Chapter 1 is a general introduction that describes the basic aspects of hepatocellular carcinoma (HCC) and elaborates into the mechanisms underlying the progression of viral hepatitis to HCC formation (*Transplantation 2017*).

Chapter 2 discusses the epidemiology of HCC. It contains an opinion describing the inequalities on HCC screening in South America (*Lancet Gastroenterol 2019*), and describes the largest study assessing the epidemiology of HCC in South America (*Liv Int 2017)*. We also show for the first time that hepatitis B-related HCC occurs at much younger ages in South America than HCC related to other causes (*Clin Gastro and Hepatol 2018*).

Chapter 3 focuses on prevention of HCC. With the understanding that viral hepatitis is the main underlying factor for HCC worldwide, this chapter describes how mass diagnosis of viral hepatitis can lead linkage to care and early detection of HCC, and evaluated point-of-care rapid testing for hepatitis B in different continents (*Ann of Glob Health 2020*). We also describe a novel approach to test for hepatitis B in emergency rooms, which can lead to further linkage to care in resource-limited areas (*Am J of Emerg Med 2019*). Finally, we describe the limited knowledge about hepatitis B in health care workers across Africa, and provide innovative insights into a methodology to increase awareness regarding hepatitis B across resource-limited settings (*Am J Trop Med Hyg 2020 x2*).

Chapter 4 provides novel findings related to mechanism of viral hepatocarcinogeneiss as well as immune-related biomarkers for HCC. First showing via single cell RNA sequencing in hepatocytes in HCV-positive individuals that cancer-related genes are differentially expressed and suggesting novel pathways for HCC development (*Submitted*). Later providing detailed introduction to the need for new biomarkers for HCC (*Cancers 2020*) and finally, evaluating how levels of cytokines measured peripherally can predict the development of HCC in individuals infected with HCV post treatment (*Gastroenterology 2018*).

Chapter 5 evaluates responses to therapy for HCC in different parts of the world. It provides important insights on the use of sorafenib in South America (J Clin Gastroenterol 2018), and proposing a novel easy-to-measure score to predict response to sorafenib, that could be of use in resource-constrained areas (Hep Month 2018). It also evaluates therapy for HCC in a single center in sub-Saharan Africa (Exp Rev Gastro & Hepatol 2020) and reports for the first time the use sorafenib in sub-Saharan Africa and its performance related to overall survival.

A

Samenvatting
PhD portfolio
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Samenvatting

Dit proefschrift stelt dat de geboorteplaats en/of leefomgeving van een patiënt van belang is bij het overwegen van screening of behandeling, aangezien onderliggende leverziekte het ontstaan en de prognose van HCC bepaalt.

Inderdaad, laat dit proefschrift zien dat hepatitis B-gerelateerd HCC op jonge leeftijd kan voorkomen in Zuid-Amerika, waardoor vroege screening in deze regio mogelijk is. Het stelt ook dat zelfs in een relatief homogeen gebied als Zuid-Amerika de belangrijkste risicofactoren voor HCC van land tot land verschillen. Het preventiegedeelte richt zich op hepatitis B-gerelateerd HCC (de meest voorkomende risicofactor wereldwijd) en onderzoekt nieuwe snelle tests voor hepatitis B, met het argument dat een eenvoudige diagnose van de ziekte de ultieme benadering is om screening op HCC in deze populatie te implementeren. Bovendien stelt het een nieuwe benadering voor om bezoeken aan de spoedeisende hulp te gebruiken als platform om jongeren te testen op hepatitis B, een concept dat aanzienlijk succes zou kunnen hebben in gebieden met beperkte middelen waar de eerstelijnszorg niet zo sterk is als in gebieden met veel middelen.

Mechanistisch gezien toont dit proefschrift voor het eerst aan dat een differentiële genexpressie in gesorteerde hepatocyten van de lever (toename van oncogenen en afname van tumoronderdrukkingsgenen) aanwezig is bij individuen die zijn geïnfecteerd met hepatitis C, de meest voorkomende risicofactor voor HCC in westerse landen, zelfs bij zeer vroege fibrose stadia. Dit suggereert dat het pad naar hepatocarcinogenese bij deze personen al aanwezig is vóór de ontwikkeling van levercirrose. Deze sectie levert ook het bewijs dat immuunmarkers die in perifeer bloed worden gedetecteerd, de ontwikkeling van HCC bij personen met hepatitis C kunnen voorspellen. Dit is met zekerheid een nieuwe benadering van screening. Of dit al dan niet van toepassing is op HCC vanwege andere oorzaken, wordt hieronder besproken. Ten slotte onderzoekt het proefschrift de verschillende behandelingen die voor HCC worden gebruikt in gebieden in Zuid-Amerika en Afrika. Het brengt het kleine aantal individuen aan het licht dat wordt behandeld via levertransplantatie, de ultieme behandeling voor HCC en benadrukt het grote aantal patiënten dat alleen palliatieve therapie kan krijgen. Het beoordeelt ook het gebruik van sorafenib, een van de weinige systemische therapieën voor HCC, in deze delen van de wereld, waardoor inzicht wordt verkregen in de overleving in verschillende HCC-groepen en er wordt een nieuwe score voorgesteld om de overleving te voorspellen, waar gebieden met beperkte middelen van kunnen profiteren.

Hoofdstuk 1 is een algemene inleiding die de basisaspecten van hepatocellulair carcinoom (HCC) beschrijft en dieper ingaat op de mechanismen die ten grondslag liggen aan de progressie van virale hepatitis tot HCC-vorming (Transplantation 2017).

Hoofdstuk 2 behandelt de epidemiologie van HCC. Het bevat een advies waarin de ongelijkheden van HCC-screening in Zuid-Amerika wordt beschreven (Lancet Gastroenterol 2019), en beschrijft de grootste studie naar de epidemiologie van HCC in Zuid-Amerika (Liv Int 2017). We laten ook voor het eerst zien dat hepatitis B-gerelateerd HCC op veel jongere leeftijd voorkomt in Zuid-Amerika dan HCC gerelateerd aan andere oorzaken (Clin Gastro and Hepatol 2018).

Hoofdstuk 3 richt zich op preventie van HCC. Met het besef dat virale hepatitis de belangrijkste onderliggende factor is voor HCC wereldwijd, beschrijft dit hoofdstuk hoe massadiagnostiek van virale hepatitis kan leiden tot koppeling met zorg en vroege detectie van HCC, en evalueerde het point-of-care sneltesten voor hepatitis B op verschillende continenten. (Ann of Glob Health 2020). We beschrijven ook een nieuwe benadering om te testen op hepatitis B binnen eerstehulpafdelingen, die kan leiden tot een verdere koppeling met zorg in gebieden met beperkte middelen (Am J van Emerg Med 2019). Tot slot beschrijven we de beperkte kennis over hepatitis B bij gezondheidswerkers in heel Afrika en bieden we innovatieve inzichten in een methodologie om het bewustzijn over hepatitis B te vergroten in omgevingen met beperkte middelen (Am J Trop Med Hyg 2020 x2).

Hoofdstuk 4 bevat nieuwe bevindingen met betrekking tot het mechanisme van virale hepatocarcinogeniteit en immuungerelateerde biomarkers voor HCC. Eerst tonen we via single cell RNA-sequentiebepaling in hepatocyten bij HCV-positieve individuen aan dat kankergerelateerde genen differentieel tot expressie worden gebracht en suggereren we nieuwe routes voor HCC-ontwikkeling (submitted). Verder geven we een gedetailleerde inleiding over de behoefte aan nieuwe biomarkers voor HCC (Cancers 2020) en ten slotte is geëvalueerd hoe niveaus van perifeer gemeten cytokinen de ontwikkeling van HCC kunnen voorspellen bij HCV geïnfecteerde personen na behandeling (Gastroenterology 2018).

Hoofdstuk 5 evalueert respons op therapie voor HCC in verschillende delen van de wereld. Het biedt belangrijke inzichten over het gebruik van sorafenib in Zuid-Amerika (J Clin Gastroenterol 2018), en stelt een nieuwe, eenvoudig te meten score voor om de respons op sorafenib te voorspellen, die nuttig zou kunnen zijn in gebieden met beperkte middelen (Hep Month 2018) . Het evalueert ook de therapie voor HCC in een enkel centrum in Afrika ten zuiden van de Sahara (Exp Rev Gastro & Hepatol 2020) en rapporteert voor het eerst het gebruik van sorafenib in Afrika ten zuiden van de Sahara en de prestaties ervan met betrekking tot de algehele overleving.

PhD portfolio

Courses

2016   Postgraduate course in Gastroenterology, ACG
2018   Molecular Medicine PRISM
2018   Molecular Medicine Genetics course
2018   Molecular Medicine SPSS
2020   Molecular Medicine Flow Cytometry course
2020   Molecular Medicine Research Integrity course
2020   Analysis of qualitative data, University of Minnesota
2020   Advanced Immunology Course, American Association for the Study
           of Immunology

Other conferences

2016   American Association for the Study of Liver Disease
2016   American College of Gastroenterology
2016   American Society of Tropical Medicine and Hygiene
2016   HIV&Liver Disease Meeting
2017   American Association for the Study of Liver Disease
2017   European Society for the Study of the Liver
2017   International Liver Cancer Association
2017   American College of Gastroenterology
2018   European Society for the Study of the Liver
2018   European HCC summit
2018   Global Hepatitis Summit
2018   American Association for the Study of Liver Disease
2019   International Liver Cancer Association
2019   American College of Gastroenterology
2019   American Association for the Study of Liver Disease
2020   American College of Gastroenterology

Teaching and Supervising

PhD student, University of Minnesota, L. Guo 2016-2018
Doris Duke Fellow, Vanderbilt University, J. Leathers 2017-2018
PhD student, University of Minnesota, C. Campbell 2017-2019
NPGH Fogarty Fellow, Chiang Mai University, S. Hongyasee 2019-2020
Research Fellow, University of Minnesota, E. Aby, 2019-2020
PhD student, Erasmus MC, N. Rico-Montanari 2019-2021
Research Fellow, University of Minnesota, C. Anugwom 2018-2021

Curriculum Vitae

Jose Debes was born in San Juan, Argentina. He attended high school in San Francisco de Asis, and later Medical School at Universidad Nacional de Cordoba, graduating in the year 2000. He then became a postdoctoral research fellow in molecular biology at the Mayo Clinic in Rochester MN, USA, where he completed in parallel a Masters in tumor biology, focusing on the transcriptional activation of steroid receptors in prostate cancer. In his last year at Mayo he became an instructor in Molecular Biology. In 2005 he started residency in Internal Medicine at the University of Minnesota in Minneapolis, MN, completing his medicine training in 2008. He then spent 2 years working as an Instructor in Medicine at the University while doing research on viral hepatitis in Tanzania, living part of the year in Tanzania and part in the United States. Between 2010 and 2013 he completed a clinical fellowship in Gastroenterology and Hepatology at the University of Minnesota, funded by an Individual National Research Service Award (NRSA F32) from the National Institutes of Health, to focus his research on the role of the NS5A protein in Hepatitis C infection. In 2013 he became a Fogarty Fellow in infectious diseases at the University of Minnesota, to continue his research studies in viral hepatitis and liver cancer. In 2014 he became Junior faculty at the University of Minnesota and over the following years he combined his academic career between the United States and the Netherlands, performing research at Erasmus MC in Rotterdam. In 2015 he was named Assistant Professor in Medicine at the University of Minnesota and in 2020 was promoted to Associate Professor.

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My friends, in all continents of the world, for believing in me even when they don't understand what I do.

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My colleagues in the research, clinical and public health world for making tough times OK and good times better.

My students and trainees from whom I believe I learn more than I teach, and who keep me motivated.

My patients who through their questions and stories of life teach me something new every day

To every scientist and health care worker out there who, despite the challenges of these professions, wake up every day to try to make the world a better place.

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List of Publications

Debes JD, de Knegt R, Boonstra A. The Path to Cancer and Back: Immune Modulation During Hepatitis C Virus Infection, Progression to fibrosis and Cancer, and Unexpected Roles of New Antivirals. <u>Transplantation</u>, 2017 May;101(5):910-915

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