Molecular epidemiology of human liver cancer: Insights into etiology, pathogenesis and prevention from The Gambia, West Africa

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Abbreviations: anti-HBc, antibody to HBV core antigen; anti-HCV, antibody to HCV; AFB1, aflatoxin B1; AFP, α-fetoprotein; CI, confidence interval; EIA, enzyme immunoassay; GHIS, Gambia Hepatitis Intervention Study; GLCS, Gambia Liver Cancer Study; hepatitis B virus, HBV; IARC, International Agency for Research on Cancer; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B “e” antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; OR, odds ratio; qSOMA, quantitative SOMA; RIBA, recombinant immunoblot assay; SOMA, Short Oligonucleotide Mass Analysis.
Abstract

Human liver cancer, primarily hepatocellular carcinoma (HCC), is both common and lethal. Notable variation in HCC incidence rates worldwide corresponds to the prevalence and pattern of the primary etiologic factors. In summary of decades of collaborative research centered in The Gambia, West Africa, this review explores the independent and combined effects of hepatitis B virus (HBV), hepatitis C virus (HCV) and dietary aflatoxin exposure in the etiology of HCC. Through population surveys, field trials and a series of HCC case-control studies, the patterns and natural history of HBV, HCV and aflatoxin exposures have been defined within this population. These investigations have paralleled and informed the development of molecular biomarkers of these etiologic agents and contributed to understanding the complex mechanisms involved in hepatocarcinogenesis. We discuss preventive approaches to reduce the global burden of HCC, emphasizing the Gambia Hepatitis Intervention Study, a country-wide randomized controlled trial designed to document the efficacy of HB vaccination in preventing HBV infections and HBV-related HCC. By recognizing the synergy of applying molecular techniques to population-based epidemiological studies, the portfolio of Gambian research projects presented provides a model for partnering etiologic and mechanistic investigations with applied research.
Primary human liver cancer, of which hepatocellular carcinoma (HCC) is by far the predominant type, is a major cause of cancer death worldwide, accounting for over half a million deaths per year [1]. The number of new HCC cases occurring each year is roughly equivalent to the number of deaths (Figure 1). Chronic infection with hepatitis B or C viruses (HBV, HCV) have both been recognized as human liver carcinogens with a combined attributable fraction of at least 75% of all HCC cases (Table I). The global epidemiology of HCC is striking, with both geographic and temporal patterns of incidence paralleling exposure to these viral etiologic factors. The highest HCC incidence rates occur in sub-Saharan Africa and parts of Asia, areas endemic for chronic infection with HBV [1].

However, human liver carcinogenesis is much more complex than simply reflecting the presence or absence of an antecedent viral infection. The natural history of infection with HBV or HCV and the subsequent development of serious sequelae including liver cirrhosis and HCC notably varies depending on factors as disparate as the age or gender of the infected person to the genetic characteristics of the virus. Given chronic HBV or HCV infection, there are marked geographical differences in risk for HCC which remain unexplained. Although the clinical presentation of HCC is uniformly advanced with a generally dismal prognosis irrespective of etiology, there is significant variation in the pathologic, radiographic and natural history of the disease. The pathways by which HCC develop are heterogeneous and influenced by a variety of environmental and host factors. The role of other carcinogens, namely aflatoxin exposure and alcohol consumption, are clearly recognized in HCC, but the
mechanisms of these factors, both individually and in conjunction with viral infection are not well defined.

In order to tease out the individual and combined effects of known etiologic factors in the complex, multifactorial process of hepatocarcinogenesis, an integrated approach is required. Systematic evaluation of molecular markers of viral (HBV, HCV), environmental (aflatoxin) and genetic factors in well-characterized HCC cases and appropriate control groups may provide an improved mechanistic understanding of hepatocarcinogenesis. In-depth knowledge of the pattern and distribution of exposure to etiologic factors in a given population is a requisite for estimating the burden of exposures, for understanding the natural history of HCC and for development of preventive interventions. In this paper, we summarize decades of molecular and epidemiological research on viral hepatitis, aflatoxin and HCC carried out in The Gambia in West Africa. Within this focused context, we attempt to gain insight into the current understanding and address unanswered research questions regarding the etiology, pathogenesis and prevention of HCC.

**Etiology of HCC in The Gambia**

The Gambia is the smallest country in continental Africa (Figure 2), with a population of almost 1.5 million people and an economy historically based on subsistence agriculture and increasingly supported through tourism. Overall, despite a good basic primary health care system, health indicators are poor relative to many more-developed countries. The Gambia has a high HCC incidence [2], endemic chronic
HBV infection [3,4], low but present HCV infection [5], and near ubiquitous aflatoxin exposure [6,7]. Over 95% of the population is Muslim and rates of alcohol and cigarette consumption are low.

There is a long history of collaborative research between the Gambian Government Department of State for Health and international groups, starting with the establishment of the Medical Research Council-UK (MRC) field station in 1947. Early MRC research efforts focused on a variety of tropical diseases, including HBV infection [4]. In 1986, the International Agency for Research on Cancer (IARC) in collaboration with the above partners initiated the Gambia Hepatitis Intervention Study [8], the first programme in Africa designed to assess the efficacy of HB vaccination in prevention of chronic liver disease and HCC [8]. Also, a series of case-control studies were implemented to assess the role of aflatoxin exposure and HCV infection in addition to HBV, in the etiopathogenesis of HCC [9,10] (A. Hall, unpublished data).

Concurrent with these efforts, a population-based National Cancer Registry (NCR), the first country-wide cancer registry with substantial rural coverage in Africa [11], was established as part of national research efforts for two main reasons. First, NCR was to provide for the final evaluation of the public health strategy of the GHIS program. Second, NCR was to provide data for studying the occurrence of all cancers locally and contribute to international studies on cancer. NCR data confirm the high incidence of HCC in The Gambia (age standardized incidence rates [ASR] were 35.7 and 11.2 per 100,000 for males and females, respectively) the early onset of HCC (median age at presentation is 45 years) and male predominance (overall gender ratio
of 3.4 males per female) of HCC cases [2]. Similar demographic patterns were observed in HCC case-control studies conducted in The Gambia in 1981-82, 1988-89, and more recently in 1997-2001 [9,10] (A. Hall, unpublished data) (Table II). Onset of HCC in the 4th or 5th decade of life is much more commonly seen in areas, such as sub-Saharan Africa and Asia, where HBV is the primary etiologic factor. The earlier onset of HCC is largely attributed to the high prevalence and earlier age of HBV exposure in these endemic regions, and perhaps also mediated by concomitant exposure to aflatoxin. Similarly, the gender ratios display a notably higher male predominance in HBV endemic regions, where ratios of up to 8 males per female may occur compared to ratios of only around 2:1 in North America and Europe [1].

In comparison of the earliest (1981-82) to most recent HCC case-control study (1997-2001), the mean age of HCC cases (48 years) and proportion of HCC attributable to HBV (57% vs. 53%, respectively) has remained stable over the intervening 2 decades (Table II). Although testing methods for HCV infection were not widely available until the early 1990’s, evaluation for antibodies to HCV has been performed on preserved samples from 1988-89. While the HCV prevalence was expectedly low among the control groups (1% and 3%, respectively), HCC cases from both the 1988-89 and 1997-2001 studies displayed a 15% and 19% HCV prevalence, respectively (Table II). Notable differences in the age at presentation were observed by viral etiology with HBV-related cancers diagnosed on average, around a decade younger than HCV-related cases [9]. From the two more recent case-control studies, we estimated the fraction of HCC attributable to HBV and HCV respectively, to be 65% and 15% in 1988-89 and to be 57% and 20% in 1997-2001.
While the predominance of HBV has persisted, there is the suggestion that the burden of HCC resulting from HCV infection may be increasing. The relative contribution of decreasing HBV versus increasing HCV prevalence on these proportional estimates is unclear. Additionally, there could be other limitations to this interpretation, including differences in recruitment procedures, case and control definitions, and most notably, differences in HCV testing methods. However, these studies were conducted with very similar study designs, including sharing two of three study sites. The earlier generation anti-HCV tests generally suffered from a lack of specificity which may have over-estimated the prevalence of HCV among HCC cases in 1988-89. In this situation, the actual temporal increase in HCV-related HCC may be even greater. These attributable risk estimates of the burden of HCC resulting from hepatitis viruses in The Gambia are comparable to IARC estimates of 60% and 20% of HCC cases in sub-Saharan Africa attributable to HBV and HCV, respectively [12]. This suggests that The Gambia shares common HCC risk factors with the rest of Africa and supports the generalizability of our research findings.

**Natural history of hepatitis B infection**

Chronic HBV infection is endemic in The Gambia with population surrogate prevalences of HBV surface antigenemia (HBsAg) ranging from 11-16% [4,9,13]. Contrary to the common modes of transmission in most Western countries (parenteral, drug use, sexual) and in many Asian populations (perinatal), most HBV
infections in The Gambia are a result of horizontal transmission occurring during young childhood. HBV seroconversions were uncommon during the first year of life, generally occurring after 1 year and prior to 5 years of age [3]. Despite various investigations, the exact mechanisms of horizontal HBV transmission remain unclear. Risk for HBsAg seroconversion among Gambian children is increased with an older sibling or mother who is an HBV carrier and with the presence of non-healing chronic skin lesions [3]. Similarly, in a nearby West African country, household transmission, rather than within the larger domestic compound, was identified as the primary setting for HBV transmission [14]. Although HBV may be isolated from insects and preliminary data suggested an association with bedbug exposure, a randomized community intervention trial of insecticide spraying did not impact HBV infection rates despite being highly effective in reducing exposure to bedbugs [13,15].

Irrespective of the exact mechanisms of transmission, it is clear that a major determinant of an individual’s response to primary HBV infection is the age at exposure. Using data from rural Gambian children followed longitudinally in the 1980’s [3,4], the strong association between age of initial infection and chronic carriage was delineated. Combining this cohort data with that from other populations [16,17], Edmunds and colleagues clearly identified that delaying HBV exposure from the perinatal period into the first few years of childhood resulted in a dramatic decline in subsequent carriage rates (Figure 3). European and North American populations, where HBV transmission generally occurs in young adulthood, subsequently have a much lower risk for carriage.
In either developed or developing country settings, HBV transmission is also highly dependent on the infectivity of the ‘donor’. Infected mothers with active HBV replication (manifested by HBeAg or HBV DNA production) are much more likely to transmit HBV perinatally and for their children to be chronically infected. Transmission from an HBsAg-positive ‘donor’ through contaminated blood transfusions, organ transplants, occupational needle-sticks, or through shared syringes among drug users is greatly increased if active HBV replication is present in the HBV carrier. Even in the setting of horizontal transmission among Gambian children, HBV chronic infections are associated with active HBV replication among the older siblings and parents in the household [4].

The natural history of chronic HBV infection involves high levels of viral replication following initial infection with a progressive waning in viral levels with time. Interestingly, there are major differences in patterns of HBV replication decay between populations. Among Gambian chronic carriers, the prevalence of HBV DNA detection (by dot-blot hybridization) declines dramatically with increasing age after adolescence, with almost no persistent HBV replication detected in individuals over 25 years of age [18]. This is in notable contrast to Asian populations where active replication often persists into advanced age. Evans and colleagues compared HBV DNA detection and quantification among two populations of male HBV carriers [19], Chinese residents of Haiman county and military personnel from Senegal (which shares borders with The Gambia and has similar ethnic populations and HBV endemicity) (Figure 4). While the quantitative levels of HBV DNA when detected were roughly equivalent, the two groups displayed very different rates of decline in
HBV DNA detection with age. These data should be replicated and further explored using more sensitive PCR tests.

Although some data are inconsistent, the levels and duration of HBV viral replication appear to be a primary determinant of HCC incidence [20-25]. Consequently, it is of interest to note that in parallel to this prolonged HBV replication among older Asians, higher HCC incidence rates are also observed into much older ages among Asians while there is a relative plateau of age-specific HCC incidence seen among older Gambians (Figure 5). Thus, these observed population differences in the natural history of HBV infection and of the incidence of HCC may be simply the consequence of the different HBV replication-infection cycle. In Asian populations, HBV replication persists well beyond the normal child-bearing years, resulting in most HBV-infected mothers delivering infants with a high likelihood for chronic, active infection. These infants, subsequent to having early perinatal infection, are extremely likely to develop chronic HBV infection and appear prone to persistent replicative HBV infection well into their adult years. While in Gambians, because so few adolescent or adult mothers have replicative infection, HBV transmission is delayed from the perinatal period into early childhood, which reduces the likelihood of chronic infection and also may accelerate the HBV replication decay process if infection does occur. The critical event in this model therefore is the timing of initial infection which results in different trajectories for the duration of active HBV replication. The differing natural history of HBV infection in these two populations may also have a direct bearing on the efficacy of HBV vaccination in newborns (see below).
An alternative or co-existing explanation for these differences in the natural history of HBV infection may be in the genetic characteristics of the virus itself. HBV can be grouped into 8 genotypes based on genetic variation in the HBsAg component; globally, there is significant geographical variation in the prevalence of HBV genotypes [26]. Asian populations are commonly genotype B or C, while African populations are commonly A, D, or E [26]. The distribution of genotypes in the US generally represents the ethnic origin of the populations infected [26,27]. There is growing evidence that different HBV genotypes may result in different clinical outcomes, including variation in persistent infection, viral replication, and HCC risk [28,29]. Increased risk for HCC has been associated with genotype A compared to non-A and with genotype C compared to non-C within African and Asian populations, respectively [25,30,31], although some data have been inconsistent [32]. Different genotype distributions patterns worldwide can be hypothesized to explain geographical variation in HBV replication duration and HCC risk [26]. However, comparative data on outcomes by HBV genotype between populations from different geographic regions are limited not only by differences in the circulating HBV genotypes but also by many potential confounders (e.g., variable HBV transmission patterns, HBV therapy options, socioeconomic factors, exposure to other carcinogens).

It has been recognized for some time that the prevalence of pre-core stop mutations varies by genotype; these mutations can result in the loss of HBeAg expression despite continued viral replication and may be associated with HCC [33,34]. More recently, other specific mutations of the HBV genome, including the
basic core promoter region have been associated with HCC [35-37], although further study with appropriate controls is needed. In The Gambia, most HBV infections (~85%) are genotype A, among which precore stop codon mutations were not identified. Only 15% of infections were genotype E with a low 15% prevalence of these with the precore mutation [38]. It is likely, as with other chronic viral infections, that further characterization of the genetic diversity of HBV will be important in predicting pathogenesis.

**Natural history of hepatitis C infection**

While HBV is the predominant virus in sub-Saharan Africa and Asia, HCV remains an important HCC etiologic factor, with an estimated attributable fraction of 23% [12]. There is significant geographic variability in the population estimates of HCV prevalence within the African continent. A recent review estimated the HCV prevalence in sub-Saharan Africa at 3% but with gradients from north to south and urban vs. rural [39]. However, much of the HCV prevalence data from Africa are limited by variability in and selectivity of the populations studied, inconsistent HCV testing methods, and a lack of data regarding mode of transmission.

Worldwide, most incident HCV infection can be attributed to intravenous drug use [40]; however, in much of sub-Saharan Africa, this is rare and less likely to be a primary mode of HCV transmission. There is strong epidemiological evidence that the 20-25% HCV prevalence observed in Egypt is explained by widespread iatrogenic transmission through a parenteral anti-schistosomiasis campaign conducted
in the 1960’s [41]. Other sporadic communities or isolated populations with higher HCV prevalence and possible iatrogenic sources for HCV transmission have been identified, such as rural Chinese villages where blood selling was common [42]. Similar to horizontal HBV transmission, the exact mechanisms by which HCV is transmitted in The Gambia and within Africa remain unclear. Although blood donations are not generally screened for HCV, the rarity of transfusions in The Gambia make it unlikely to account for most HCV infections. WHO provides historical estimates that up to 40% of medically-related injections given in sub-Saharan Africa may be unsterile [43]. Subsequently, HCV transmission through reuse of contaminated syringes and medical equipment, both in routine medical and traditional care settings, appears the most plausible hypothesis [44]. There is surprisingly little data supportive of this, however. Because of the significant burden of HCV (and perhaps HBV) related to the use of contaminated syringes and medical equipment in the health care delivery system [43], WHO has initiated a global campaign to ensure the safety of injections [43,45].

In The Gambia, there is a notable absence of HCV infection in younger individuals. Among 199 healthy control participants in GLCS younger than 40 years of age, none were found to be HCV positive (using a third generation EIA with confirmatory RIBA testing) [9]. The explanation for this age effect could be that HCV exposures are delayed until adulthood or that repeated exposures are generally required prior to HCV transmission. However, medically related injections are common at young ages and may actually decrease slightly with increasing age. Data from occupational exposures and from injection drug users suggest that the risk for
HCV transmission per single exposure to an HCV contaminated syringe is around 1 in 15-50 [46]. WHO reports that injections in sub-Saharan Africa are extremely frequent even in childhood, averaging around 1.5 injections per year [43]. The likely exposure to potentially contaminated injections at ages prior to any observed HCV infection in The Gambia suggests either that transmission per needlestick is more infrequent than hypothesized or that alternative modes of transmission predominate. Another alternative explanation is that there is a cohort effect of HCV in The Gambia. That is, that circulating HCV infection was present in The Gambia during prior decades, but now is rare. General improvements have been made in routine health care over the past several decades, especially provision of sterile equipment within the national immunization program. There is no clear historical program similar to Egypt’s anti-schistosomiasis campaign that would explain an HCV cohort effect. Comparing age-specific HCV prevalence data from similar control groups a decade apart (Table II), we can see a suggestion of this cohort effect with a drop from 3% to 1% over time in those controls under 50 years but a stable prevalence of 6% among those over 50 years of age.

**Aflatoxin exposure and HCC**

While HBV and HCV may account for the majority of HCC in Africa, there is strong evidence for an etiologic role of aflatoxin in hepatocarcinogenesis. Aflatoxins are toxic and carcinogenic metabolites of molds, mainly *Aspergillus flavus* and *parasiticum*, that contaminate a variety of agricultural commodities, particularly groundnuts (or peanuts), maize and cottonseed, in countries with hot and humid
climates. Aflatoxin B1 (AFB1) is the major metabolite produced by these molds. In The Gambia, groundnuts are the staple food and primary cash crop, and their consumption results in high and prolonged exposure to AFB1 (see below) [47,48]. AFB1 has been shown to be a very potent liver carcinogen in various animal species [49,50]. Early epidemiological studies showed a clear association between exposure to AFB1 contaminated foods and risk of HCC in Sub-Sahara Africa and South East Asia (reviewed in [51]). However, these studies suffered from important limitations in determining a causal association, mainly the poor assessment of exposure to AFB1 and the lack of information on the contribution of HBV infection. The development of individual biomarkers of AFB1 exposure and their integration into epidemiological studies has permitted a better understanding of AFB1 in HCC etiopathogenesis [52-59]. IARC subsequently labeled AFB1 as a carcinogen in humans [48].

Extensive research conducted since the 1980’s in The Gambia has pioneered the development and field validation of aflatoxin biomarkers and resulted in a simpler methodology to measure AFB1 exposure. Studies conducted in The Gambia [47,60,61] showed that AFB1 contaminates a variety of foodstuffs with the major determinant of human exposure being dietary consumption of contaminated groundnuts. Because of the monotony and frequency of groundnut consumption in the population, high levels of exposure are present in almost all Gambians. Exposure to this carcinogen occurs during the entire lifespan, starting during the perinatal period as shown by the detection of in utero transfer of aflatoxin-albumin adducts [62] and by the presence of AFM1, a metabolite of AFB1, in breast milk [63,64]. Recent data show that AFB1 exposure in childhood may affect growth [65] and immune function
Rural Gambians generally have higher levels of AFB1 adducts than urban dwellers [47]; this difference probably reflects the higher contamination of groundnuts in rural areas and more diversified diet in urban areas. There is a strong seasonal variation in AFB1 exposure which correlates with the availability of groundnuts in the diet [47,67]. Withstanding these seasonal variations, it should be noted that the prevalence and quantitative levels of AFB1 exposure in The Gambia are among the highest in the world [52,61]. Despite recent economic advances, high aflatoxin exposures remain a major health issue in many sub-Saharan countries; evidence of continued dangerous levels of exposure includes a recent epidemic of acute aflatoxin poisoning resulting in the death of hundreds of Kenyans [68].

Aflatoxin exposure and p53 mutations in HCC

Since the initial observation by Hsu et. al. and Bressac et. al. [69,70] that a specific transversion mutation (Arg -> Ser) in the third nucleotide of codon 249 of the p53 tumor suppressor gene (249\textsuperscript{ser}) was highly associated with HCC from regions of the world with heavy AFB1 exposure, considerable data to substantiate this observation have accumulated [61,71]. Experimental studies in bacteria, in cultured cells, and in animals support this association (see [61,71-74]). Data derived from the IARC p53 mutation database comprised of around 1000 HCC tumors from various countries show a clear association between 249\textsuperscript{ser} mutation prevalence, exposure to AFB1, and incidence of HCC [75]. These data suggest that the 249\textsuperscript{ser} mutation may be a durable indicator of past dietary exposure to AFB1. Subsequently, we detected the 249\textsuperscript{ser} p53 mutation in cell-free DNA isolated from the plasma of Gambian HCC patients (35%)
but not in HCC cases from Europe [76]. After this initial report, we further validated a strong concordance (~90%) between plasma and liver tissue detection of the mutation [77] and identified a dramatic multiplicative effect on HCC risk with combined exposure to HBV and 249ser p53 mutations [78]. These findings have been replicated with consistent observation of higher 249ser mutation prevalence among HCC cases in populations with higher AFB1 exposures, including Qidong County in China, Northern Thailand, and South Africa [37,79-82].

The timing of aflatoxin exposure and development of p53 mutations in relation to HCC risk remains undefined. In this context, previous investigations have shown the presence of the 249ser p53 mutation in non-tumorous liver tissue [83]. We and others demonstrated that this plasma marker could be detected, albeit at lower prevalence than in HCC cases, among persons with cirrhosis or without overt clinical liver disease [76,78,79,81]. In longitudinal analyses from Qidong, 249ser mutations were detectable in nested sera samples 1 to 5 years prior to detection of the mutation in tumors at HCC diagnosis [84]. These data suggest that the 249ser mutation may occur as an early event in aflatoxin-related hepatocarcinogenesis; this could have applications in developing markers for earlier HCC detection or for targeting preventive interventions. These prior studies of plasma 249ser mutations were carried out among adult populations; it should be noted that 249ser mutations were not detected among aflatoxin-exposed healthy children from Guinea [85]. As discussed by the authors, an adequate period of time may not have elapsed for expansion of clonal cells containing the 249ser mutation at low copy numbers.
Building on the more sensitive SOMA techniques for plasma 249ser detection [82], we subsequently developed a novel plasmid standard which allows quantitative estimation of the copies of the mutation per ml of blood (qSOMA); a dose-response increase in HCC risk was observed [86]. This quantitative marker could prove useful in epidemiological studies for determining levels of aflatoxin exposure, for predicting individual HCC risk thereby allowing targeted preventive interventions, or as an intermediate endpoint in chemoprevention trials.

**Interaction of HCC etiologic factors in The Gambia**

The availability of serum-based markers of HBV and HCV infection and of AFB1 exposure allows molecular epidemiological studies to examine the relative individual contribution and interaction of these risk factors in the etiopathogenesis of HCC. The outcome of such studies carried out in The Gambia are discussed here.

*HBV and HCV coinfection*

In The Gambia, HBV and HCV infection have clearly different patterns of transmission with marked variances in prevalence but the estimates of HCC risk from either infection were similar (ORs of 17 for both) [9]. It is difficult to differentiate HCC originating from HBV in contrast to HCV infection by clinical or radiographic criteria. The chronic process of hepatocyte destruction, regeneration and development of fibrosis, cirrhosis and eventually cancer requires decades of chronic infection with
either virus [87-90]. This biological model of viral hepatocarcinogenesis would suggest that either infection should act through a common pathway. Data from GLCS demonstrates an OR with combined HBV/HCV infection of 35, roughly equivalent to the sum of the individual ORs and approximating an additive model of interaction (17 + 17 -1=33) [9].

It is important to recognize the difficulties in assessing, quantifying and interpreting the interactive effects of joint exposures to HBV and HCV infections, evidenced by the inconsistencies in the reported literature [91]. We find some evidence that HBsAg clearance (or diminished detection) may be increased among HCV co-infected persons, supporting others reported findings of reciprocal inhibition of viral replication. In another study, Zarski and colleagues suggested that generally only one infection may be replicative at any given time [92]. In the presence of reciprocal inhibition, estimates of the risk from each individual infection or from dual infection may be underestimated. The cellular effects of either chronic infection may be acting through the same pathway but rarely at concurrent times. In this model, the observed risk from co-infection may result in a sub-additive estimate of interaction. These difficulties were manifest in a recent meta-analysis looking at HBV/HCV coinfection in 21 HCC case-control studies [91]. Only seven studies had any co-infected controls; six studies with one and one study with two. Based on this limited data, they estimated the HCC risk with co-infection to be somewhere between additive and multiplicative. Recently data have emphasized the need to examine for ‘occult HBV’ infection [93] by testing for HBV DNA in the sera of HCC patients that are HBsAg negative or HCV positive [87]. Obviously, full ascertainment even of
low-level HBV infection will be important in understanding the effect of HBV/HCV co-infection.

*Combined HBV infection and aflatoxin exposure*

Another major research question remains the effect of combined exposure to aflatoxin and HBV infection. Regions of the world with endemic HBV are also frequently the regions with highest aflatoxin exposure making epidemiological studies which can address potential confounding difficult [94]. Prior research had suggested a multiplicative interaction between HBV and AFB1 in animal models and in some epidemiologic studies from Asia [95-97]. In The Gambia, the effect of HBV infection on the level of AFB1-albumin adducts was examined. In children with serological markers of acute HBV infection, a higher prevalence and level of AFB1-adducts was detected [60]. In adults, no such association was observed [47]. In comparison to studies of AFB1-adducts, the 249<sup>ser</sup> p53 mutation may represent a more biologically effective marker of past aflatoxin exposure. However, extensive data on 249<sup>ser</sup> p53 mutations in tumors from around the world did not show any difference in the prevalence of HBsAg among HCC cases by p53 status or by AFB1 exposure level [71].

In GLCS, over 25% of HCC cases were both HBsAg and 249<sup>ser</sup> mutation positive compared to less than 1% of controls. The combined effect of chronic HBV infection and 249<sup>ser</sup> p53 mutations approximated a multiplicative model of interaction with a risk estimate for HCC of around 400. The prevalence of 249<sup>ser</sup> p53 mutations tended to be higher among HBeAg-positive persons, suggesting an association of the
mutational effect of aflatoxin exposure with HBV viral replication. These findings are supportive of previous work that suggested a multiplicative interaction between HBV infection and shorter term biomarkers of aflatoxin exposure [96]. Simultaneous quantification of HBV DNA viral load and qSOMA determination of 249ser p53 copies will allow more precise evaluation of this observed interaction. A multiplicative model of interaction with combined exposure may be interpreted at the biological level to represent HBV and AFB1 acting through independent steps in a shared causal pathway. Specific biological mechanisms underlying the interaction between HBV infection and aflatoxin exposure are discussed by Kew [98] and Essigman [72].

**Host genetic determinants of HCC**

With high HBV carriage rates and heavy aflatoxin exposure, it remains unclear why only a small proportion of exposed Gambians actually develop HCC. In order to determine if individual genetic differences in aflatoxin metabolism are associated with susceptibility to HCC, we investigated polymorphisms in enzyme systems which potentially either bioactivate or detoxify aflatoxins to their active and carcinogenic metabolites or which may repair any induced DNA damage. In addition to identifying persons that may have increased susceptibility to HCC, genetic studies allow evaluation of enzyme pathways potentially involved in hepatocarcinogenesis.

Multivariable analysis of GLCS data showed a modest increase in HCC risk associated with the *GSTM1*-null genotype, one of the primary enzymes responsible
for AFB1 detoxification, but no strong evidence for effects of other carcinogen-
metabolizing enzymes [99]. Individuals who were homozygous or heterozygous for
the G allele in the 399 locus of the \textit{XRCC1} DNA repair enzyme, previously
associated with increased AFB1-DNA adduct levels [100], also displayed an
increased HCC risk [99].

Gene effects may often be variable depending on the levels of exposures,
requiring evaluation of gene-environment effects. The extremely high level of
aflatoxin exposure in The Gambia may act to saturate metabolism or DNA repair
pathways, obscuring relatively minor modulating effects conferred by genetic
polymorphisms. Alternatively, gene effects may only be observed when a threshold
level of exposure occurs. Studies in different populations with less pronounced
aflatoxin exposure have resulted in different findings. In Asia, associations of
carcinogen-metabolizing polymorphisms and HCC have generally been absent or
weak; positive findings have generally been limited to the sub-set of participants with
the highest estimated aflatoxin exposure [101,102]. Similarly, we also observed that
increased HCC risk with \textit{GSTM1}-null genotypes was most prominent among those
with the highest groundnut intake [99].

In a recent report from Taiwan, a trend of increased HCC risk was seen with
\textit{XRCC1}-399G polymorphisms; significant associations were observed among subjects
with both \textit{XRCC1} variants and the null \textit{GSTT1} genotype [103]. While we observed an
independent effect on HCC risk with \textit{XRCC1}-399G, we also identified a greater than
additive effect on HCC risk with the DNA repair variant in combination with
polymorphisms in several aflatoxin detoxification enzymes including \textit{GSTM1} and
HYL1. Gene-gene effects with combined variants of the metabolic enzymes were largely not observed or less than the predicted effect. In summary, these data support a possible synergy between bioactivation of aflatoxin and repair of DNA damage in the process of hepatocarcinogenesis.

A major limitation in analysis of genetic polymorphisms is commonly a lack of power; the confidence intervals for both polymorphisms associated with HCC in our study were close to 1.0. Despite the larger sample size compared to many HCC studies, GLCS still had small numbers of participants with many of the variant polymorphisms, accentuated by the lower prevalence of susceptible genotypes in Gambians compared to other populations. Larger scale genetic studies in populations with varied exposures should provide further understanding of important pathways leading to HCC.

**Approaches to HCC prevention**

With recognized etiologic factors and improved mechanistic understanding, rationale interventions to reduce the incidence and morbidity associated with HCC can be designed and implemented. Vaccination against HBV in infancy is the most effective approach to prevent HCC, particularly in developing countries (see[29]). In parallel, reduction of exposure to AFB1, although a difficult task, may also prove an effective primary prevention measure. However, vaccines for prevention of HCV are only just reaching early phase clinical trials. Anti-viral treatment against HBV or HCV may interrupt or delay progression to HCC. The lengthy asymptomatic period prior to HCC diagnosis at advanced stages provides opportunities for earlier HCC detection.
Key to the success of these secondary preventive interventions will be identifying the target groups for which antivirals or screening are appropriate. The quantitative plasma 249\textsuperscript{ser} p53 mutations is a prototypical example of a marker of both exposure and HCC risk; screening and prevention strategies could be tailored to individuals based on 249\textsuperscript{ser} p53 levels. Similarly, recent data on the predictiveness of HBV DNA levels for HCC suggests that appropriate management should be tailored to the specific levels and persistence of HBV viral load. Analysis of multiple markers can result in a “molecular profile” of each stage of progressive liver disease from chronic viral infection to significant fibrosis / cirrhosis to HCC. These “molecular profiles” may further delineate individuals for whom aggressive monitoring may most beneficial. Unfortunately, once advanced HCC is diagnosed, limited options exist for limiting complications or reducing mortality from HCC. Further discussion of secondary HCC prevention approaches are discussed elsewhere [104]; our discussion focuses on the available primary preventive measures.

**Vaccination against HBV**

Recent press reports have heralded a new human papillomavirus vaccine as the ‘first’ vaccine against cancer [105]. However, as it was evident early in the 1980’s that HBV infection was a major etiologic factor in HCC [106], an HB anti-cancer vaccine was developed with good efficacy and safety in preliminary trials [107,108]. Prior to wide-scale implementation, it was of great importance to provide evidence that HB vaccination of infants could be safely and efficiently introduced into countries with a high prevalence of HBV infection and at high risk of HCC. The natural choice for
efficient delivery of HB vaccine in HBV endemic countries was to be incorporated into the routine childhood immunization programs. In 1986, IARC partnering with MRC and the Gambia Government initiated GHIS, a population-based randomized controlled trial of vaccination against HBV infection to prevent chronic liver disease and HCC [8].

GHIS HB vaccine efficacy results reported in children at 9 years [109] confirmed those reported earlier among children at 4 years of age [110]. After almost a decade of follow-up, the HBV carriage prevalence was 10% among unvaccinated compared to <1% among vaccinated children. Protection against HBV infection (measured by antibodies to HBV core [anti-HBc]), was over 80% and protection against chronic carriage (measured as repeated HBsAg detection) was well over 90% (Table III). The high efficacy against infection and carriage was equally present in boys and girls and there were no statistically significant variations among the different zones of the country. In a separate HB vaccination study carried out in two Gambian villages (Keneba and Manduar) [111], results after 14 years of follow-up show similar efficacy estimates of 82% against infection and 94% against carriage [112].

During similar time periods, other HB vaccination programs were initiated in South-East Asia and China. HB vaccination in infancy has consistently proven highly efficacious in reducing the prevalence of HBV infection and of chronic carriers among the vaccinated populations [29]. Only limited data from randomized controlled trials of HB vaccination are available; most evidence consists of comparisons of chronic HBV prevalence before vs. after vaccine introduction (see
While results from different regions are largely comparable, it should be noted that the efficacy of HB vaccine in Asia appears somewhat lower than observed in The Gambia [114,115] (Table III). This may be related to differences in HBV replication and the more prevalent vertical HBV transmission among Asian populations, whereas in The Gambia horizontal HBV transmission predominates (see above).

Through continued ascertainment of HCC cases through the population-based NCR, it will be possible to establish at the individual level, the effect of HB vaccination on HCC incidence in The Gambia. At a population level rather than in a randomized controlled trial, early data on the impact of HB vaccination on HCC rates have been reported. The introduction of HB vaccine in Taiwan has been associated with a significant reduction in childhood HCC incidence [116]. With further surveillance, HCC rates declined among boys born after 1984 (time of inception of HB vaccination) compared to boys born before 1978; however, no such reduction in early HCC was observed among girls [117]. Gender differences in HBV carriage rates, HCC rates and HB vaccine responses have been observed but mechanisms underlying these findings remain largely unexplained.

HB vaccine has now been given to millions of persons worldwide and is one of the most safe and efficacious vaccines in use [118]. An important question related to evaluation of HB vaccine effectiveness is to determine the durability of protection, particularly as the levels of protective antibody diminish fairly soon after vaccination [119]. Despite the lower rates of chronicity among adolescents and adults exposed to HBV, the increasing exposure risk that occurs with initiation of sexual activity has
prompted the question of whether children previously vaccinated require booster doses in adolescence to ensure protection. Studies carried out in The Gambia and other HBV endemic areas indicate that protective antibody levels do tend to wane and some data suggests that vaccine efficacy against primary infection decreases over time; however, efficacy against chronic infection remains high over a period of up to 15 years [112,120-122]. However, the number of individuals in these studies is relatively small and protection during the higher risk period following initiation of sexual activity has not been fully assessed. Although there has been legitimate concern for HBV breakthrough infections with vaccine-escape mutants [123,124], further investigation has suggested that these variants are not likely to greatly impact HB vaccination programmes [125,126] although continued surveillance may be appropriate [127]. It remains unclear whether the presence of other infections, particularly HIV infection with impairment of the immune system, will significantly impact HB vaccine effectiveness.

*Interventions to reduce aflatoxin exposure*

Until other lower cost crops are shown to be viable and culturally acceptable alternatives to groundnuts, heavy lifetime exposures to aflatoxin will continue in West Africa. In high exposure environments, community interventions aimed at modifying post-harvest practices may have the greatest impact on lowering aflatoxin contamination levels in groundnuts and other staple crops [128]. The economic necessity of groundnuts as the primary income source for many Gambians means that the practice of selling the best quality, least visibly-contaminated groundnuts while
keeping the heavier contaminated ones for personal consumption is likely to continue. Easier methods for field detection and quantification of aflatoxin contamination of foodstuffs could aid epidemiologic studies of exposure and behavioral intervention trials aimed at reducing contamination [129].

Chemoprevention trials have provided ‘proof of principle’ that agents which modulate the effective level of aflatoxin exposure by increasing metabolic detoxification (as with oltipraz) or by reducing the bioavailability (as with chlorophyllin) may reduce levels of aflatoxin exposure [130]. Incorporation of aflatoxin-albumin adducts and 249ser p53 mutations as intermediate endpoints may improve the efficacy and power of these trials [131,132]. The inconsistent data on the effect of genetic polymorphisms in carcinogen-metabolizing enzymes with known functionality in aflatoxin metabolism suggests that these approaches may need to be targeted to specific geographic or ethnic populations with certain levels of aflatoxin exposure and/or susceptibility. In addition, the direct costs of purchasing chemopreventive agents and lifelong adherence to taking a pill are significant barriers. Development of food-based alternatives would greatly facilitate both the feasibility and acceptance of chemopreventive efforts [130].

Behavioral interventions, such as modification of storage practices, would require minimal direct expenditures [128]; however, the difficulty in changing cultural practices also must be recognized. A recent community intervention study in rural Guinea, West Africa of low-technology post-harvest interventions demonstrated a reduction in aflatoxin contamination of food stores and in aflatoxin-albumin adduct detection prevalence and levels during a single harvest cycle [133]. With lifetime
exposures occurring in many regions, the appropriate time for initiation and the
duration of aflatoxin-reduction interventions will need to be further investigated. At
the other end of the technology spectrum, genetic engineering could produce aflatoxin
resistant crops; although theoretically promising, many scientific as well as ethical
and environmental barriers to this approach remain (see [128]).

**Conclusions and public health implications**

In The Gambia, extensive research efforts have documented high HCC incidence
resulting from childhood HBV infection, lifetime dietary aflatoxin exposure and
chronic HCV infection. Around 57% of HCC cases in The Gambia are attributable to
chronic HBV infection. GHIS has clearly shown that HB vaccination can be
implemented in the national immunization programs of developing countries and that
immunization is highly effective in the preventing chronic HBV infection and likely,
HCC. Cost-effectiveness analysis of GHIS data suggests that prevention of one HBV
carrier by HB vaccination costs less than US$30 and prevention of one HCC case
costs around US$130 [134,135], making HB vaccination one of the more cost-
effective interventions HBV-endemic countries could implement.

Globally, the burden of disease attributed to HBV infection is enormous, with
some 2 billion persons having serological markers of infection, around 350 million
persons chronically infected and around 65 million of these likely to die of HBV-
related liver disease. Despite WHO recommendations that all countries incorporate
HB vaccine into their routine EPI program by 1997, only the wealthiest 25-30% of
the 132 million children born in 2000 had access to the vaccine (see [113]). Despite
significant reductions in the cost (now <US $0.50 per dose), the greatest barrier to widespread HB vaccine in endemic regions remains vaccine expense [118,136]. Economic and other barriers to widespread HB vaccination are discussed by Kao and Chen [29]. Through coordinated efforts of many international partners, namely the Global Alliance on Vaccines and Immunizations, many HBV endemic countries with limited resources have now begun or are preparing to introduce the HB vaccine into their routine EPI [113]. Universal HB vaccination of all the world's children is nearing a reality, with potential to save an estimated 1 million lives per year. Continued advocacy and funding will be required to maintain and ensure the long-term availability of HB vaccine for resource-limited countries.

Although less common than HBV infection, HCV accounts for around 20% of HCC cases in The Gambia. Based on the additive model of interaction we observed with HBV and HCV co-infection, HB vaccination is unlikely to have any impact on the burden of HCC related to HCV. However, because of the predominance of HCV infection among older Gambians, it is unclear if the impact of HCV will wane among future cohorts. Further investigation of the risk factors and modes of HCV transmission of HCV in sub-Saharan Africa is required to assess the likelihood of continued transmission and predict future contribution to HCC rates.

The multiplicative interaction between markers of HBV infection and AFB1 exposure suggests that reduction in either exposure may have significant effects on reducing the HCC burden related to the other. That is, the high efficacy of HB vaccination could decrease the impact of continuing aflatoxin exposure. Conversely, low tech interventions to reduce aflatoxin exposure may be associated with decreases
in HBV-related HCC; this may provide a reasonable HCC prevention strategy in regions without the resources or infrastructure to deliver HB vaccine. Using these models of interaction, population-based sampling of markers of HBV, HCV and AFB1 exposure could provide useful data in formulating reasoned, cost-effective and country-specific intervention strategies to reduce HCC.

Despite these optimistic trends for primary HCC prevention, millions of HBV or HCV infected individuals remain at high risk for developing HCC. The advanced presentation and limited effectiveness of therapy for HCC is markedly evident in The Gambia. Aflatoxin reduction interventions may benefit chronically infected carriers who will not be helped by vaccination. Antiviral therapies hold some promise for interrupting or slowing progression to HCC. Further, the development of early detection markers is a vital missing component in strategies to reduce HCC mortality. With the growth in discovery driven technologies, including genomics and proteomics, systematic evaluation of biomarkers representing the complex process of hepatocarcinogenesis appear promising. As in many human cancers, molecular epidemiology studies may provide a better understanding of the temporal occurrence of genetic and epigenetic alterations in the natural history of liver carcinogenesis [61,74,137]. Biomarkers provide a link to evaluation of the mechanisms involved in hepatocarcinogenesis and to reasoned development of preventive interventions. Intensive and systematic evaluation of well-characterized human subjects, such as reported from studies carried out in The Gambia, provide an invaluable resource for investigating and validating these molecular epidemiology approaches.
Acknowledgments

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References


LEGEND FOR FIGURES

Fig. 1. Worldwide primary liver cancer incidence and mortality for the year 2000. The estimated annual number of new cases approximated the number of deaths (in thousands). Adapted from reference [1].

Fig. 2. The Gambia, West Africa.

Fig. 3. Younger age at infection increases the risk to become an HBV carrier. Adapted from reference [16].

Fig. 4. Age-related differences in HBV DNA persistence between Asian and African HBV carriers. Adapted from reference [19].

Fig. 5. Comparison of age-specific HCC incidence rates among males for selected countries with endemic HBV infection (per 100,000 persons). Data obtained from International Cancer Registry Data available through IARC GloboCan, 2001.
Table I. Major etiologic factors and global statistics for primary liver cancer.\(^a\)

<table>
<thead>
<tr>
<th>Major Etiologic Factors</th>
<th>Incidence Data</th>
<th>Mortality Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Hepatitis B infection (&gt;50% (^b))</td>
<td>➢ 551,000 cases/year worldwide</td>
<td>➢ 529,000 deaths/year worldwide</td>
</tr>
<tr>
<td>➢ Hepatitis C infection (&gt;25% (^b))</td>
<td>➢ 5(^{th}) most common cancer</td>
<td>➢ 3(^{rd}) most frequent cause of cancer death</td>
</tr>
<tr>
<td>➢ Alcohol consumption</td>
<td>➢ 83% of all cases in developing countries</td>
<td>➢ 8.8% of total cancer deaths</td>
</tr>
<tr>
<td>➢ Aflatoxins</td>
<td>➢ 54% of the total cases in China</td>
<td></td>
</tr>
<tr>
<td>➢ Tobacco smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>➢ Obesity / Diabetes / Fatty Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>➢ Iron overload</td>
<td></td>
<td></td>
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</tbody>
</table>

\(^a\) Adapted from reference [1]. \(^b\) These percentages represent the estimated fraction of worldwide cases attributable to hepatitis B or C infections.
<table>
<thead>
<tr>
<th></th>
<th>1981-1982&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1988-1989&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1997-2001&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Ctrls</em></td>
<td><em>HCC</em></td>
<td><em>Ctrls</em></td>
</tr>
<tr>
<td>No.</td>
<td>70</td>
<td>70</td>
<td>212</td>
</tr>
<tr>
<td>Male</td>
<td>87</td>
<td>87</td>
<td>66</td>
</tr>
<tr>
<td>Mean / median age, in years</td>
<td>48</td>
<td>48</td>
<td>40</td>
</tr>
<tr>
<td><em>Viral Serology</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg +</td>
<td>21</td>
<td>63</td>
<td>14</td>
</tr>
<tr>
<td>HBeAg +&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Anti-HCV +&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td><em>Age &lt;50</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg +</td>
<td>24</td>
<td>87</td>
<td>18</td>
</tr>
<tr>
<td>HBeAg +</td>
<td>0</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>Anti-HCV +</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Age &gt;50</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg +</td>
<td>14</td>
<td>41</td>
<td>6</td>
</tr>
<tr>
<td>HBeAg +</td>
<td>0</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Anti-HCV +</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
</tbody>
</table>

Note: All values expressed as percentages unless indicated otherwise.

<sup>a</sup> Adapted from reference [10]. HCC cases recruited from 4 hospital sites with neighbor controls.

<sup>b</sup> Hall AJ, unpublished data. HCC cases recruited from 2 hospital sites with inpatient hospital-based controls.

<sup>c</sup> Adapted from reference [9]. HCC cases recruited from 3 hospital sites with outpatient hospital-based controls.

<sup>d</sup> HBeAg status determined as percentage of persons who were HBsAg positive.
HCV testing not available for 1981-82 study samples, anti-HCV status determined for 1988 and for 1997-2001 samples by 2nd generation EIA and by 3rd generation EIA with RIBA confirmation, respectively.
Table III. Status of hepatitis B vaccination efforts in The Gambia, Qidong, China and Taiwan.

<table>
<thead>
<tr>
<th>Study Site (N in each arm)</th>
<th>Recruitment / Vaccination Start Dates (yrs of follow-up)</th>
<th>HBsAg Prevalence (Unvaccinated &gt; Vaccinated)</th>
<th>Vaccine Efficacy (against chronic infection)</th>
<th>Decline in HCC Incidence (per 100,000 / yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population-based, randomized control trials</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The Gambia</td>
<td>&gt;60,000</td>
<td>1986-1990 (9 yrs)</td>
<td>10.0% &gt; 0.6%</td>
<td>94%</td>
</tr>
<tr>
<td>Qidong, China</td>
<td>&gt;38,000</td>
<td>1984-1990 (11 yrs)</td>
<td>7.1% &gt; 1.7%</td>
<td>75%</td>
</tr>
<tr>
<td>National universal vaccination programme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taiwan</td>
<td>1984</td>
<td>9.8% in 1984 4.8% in 1989 1.3% in 1994 0.7% in 1999</td>
<td>84%</td>
<td>Children 6 to 14: 0.7 (1981-1986) 0.57 (1986-1990) 0.36 (1990-1994)</td>
</tr>
</tbody>
</table>

\[a\] adapted from reference [109].  
\[b\] adapted from reference [115].  
\[c\] adapted from references [114,116,117].
The Gambia
Uganda
Hong Kong

HCC incidence / 100,000

Age group, in years

0-14 15-44 45-54 55-64 65+