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Miryam Müller[1], Thomas G Bird[1, 2, 3], Jean-Charles Nault[4, 5, 6]

1. Cancer Research UK Beatson Institute, Garscube Estate, Switchback Road, Glasgow, G61 1BD, UK.
2. MRC Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh, EH164TJ, UK
3. Institute of Cancer Sciences, University of Glasgow, Garscube Estate, Switchback Road, Glasgow, G61 1QH, UK;
4. Service d'Hépatologie, Hôpital Jean Verdier, Hôpitaux universitaires Paris-Seine-Saint-Denis, Assistance publique Hôpitaux de Paris, Bondy, France
5. Unité mixte de Recherche 1162, Génomique fonctionnelle des Tumeurs solides, Institut national de la Santé et de la Recherche médicale, Paris, France
6. Unité de Formation et de Recherche Santé Médecine et Biologie humaine, Université Paris 13, Communauté d'Universités et Etablissements Sorbonne Paris Cité, Paris, France

Corresponding authors

Thomas G. Bird
Cancer Research UK Beatson Institute,
Switchback Road,
Glasgow, G61 1BD, UK
TEL: +44 (0)14133019222
FAX: +44 (0)1419426521
Email: t.bird@beatson.gla.ac.uk

Jean-Charles Nault
Service d'hépatologie, hôpital Jean Verdier
Avenue du 14 juillet
93140 Bondy
TEL: +33 6 10 67 94 61
FAX: +33 1 53 72 51 92
Email: naultjc@gmail.com

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- Liver damage and aging promote the establishment of mutant clones
- Cirrhotic nodules are formed from clonal subpopulations of hepatocytes
- Mutations in specific driver genes promote malignant transformation of hepatocytes
- Understanding the mutational patterns of individual HCCs may aid diagnosis and treatment
- Studying specific mutations in preclinical models may highlight subtype specific treatment vulnerabilities

List of Abbreviations

AAV Adeno associated virus, ALD alcoholic liver disease, cfDNA cell free DNA, CLD chronic liver disease, CTC circulating tumour cell, DNA deoxyribonucleic acid, HBV Hepatitis B virus, HCA hepatocellular adenoma, HCC hepatocellular carcinoma, InDel Insertion/Deletion, NAFLD non-alcoholic fatty liver disease, RNA ribonucleic acid, SNP single nucleotide polymorphism, TGF transforming growth factor.

Abstract

Chronic liver disease and primary liver cancer are a massive global problem, with a future increase in incidences predicted. The most prevalent form of primary liver cancer, Hepatocellular carcinoma (HCC), occurs after years of chronic liver disease. Mutations in the genome are a causative and defining feature of all cancers. Chronic liver disease, mostly at the cirrhotic stage, causes the accumulation of progressive mutations which can drive cancer development. Within the liver a Darwinian process selects out dominant clones with selected driver mutations but also leaves a trail of passenger mutations which can be followed allowing tracking of this evolution. Understanding what causes specific mutations and how they combine with one another to form cancer is a question at the heart of understanding, preventing and tackling liver cancer. Here we review the landscape of gene mutations in cirrhosis, especially those paving the path toward HCC development characterised by recent studies capitalising on technological advances in genomic sequencing. With these insights we are beginning to understand how cancers form in the liver, particularly on the background of chronic liver disease. This knowledge may soon lead to breakthroughs in the way we detect, diagnose and treat this devastating disease.

Introduction

Liver disease and liver cancer, specifically hepatocellular cancer (HCC), are an increasing global pandemic. Chronic liver disease (CLD) is responsible for approximately 2 million deaths annually worldwide with liver cancer responsible for nearly 800,000 deaths [1-3]. HCC mostly forms after years of chronic liver disease, against a background of severe liver scarring and typically cirrhosis. Even HCC related to non-alcoholic fatty liver disease (NAFLD) in individuals without liver cirrhosis is a process occurring over many years with the establishment occurring as early as childhood [4]. Mutations are ubiquitous in cancer, and the link between somatic mutations and cancer has been clearly appreciated for many years [5, 6]. Whilst mutations are necessary they are not always sufficient to drive cancer [7]. Instead, we are now appreciating that combinations of mutations are required, the amount dependent on the type of cancer [8]. In the last decade sequencing of cancer genomes and related non-cancerous tissue has revolutionised our understanding of how cancers form in many tissues. Here, we will summarize how sequencing studies in the liver have shed light on the genetic progression from a healthy liver to HCC through the key 'breeding ground' of chronic liver disease. The hepatocyte is the principle cell of origin of HCC. However, it is now appreciated that there is plasticity between biliary epithelial cells and hepatocytes [9-15]. An intermediate population may represent a form of adult stem cell in the liver and is associated with severity of liver disease [16]. None the less, little is known about mutations within a putative liver stem cell compartment and their role in subsequent HCC development. For these reasons we will focus on mutations affecting the hepatocellular liver parenchyma and their role in the early steps of HCC development.

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General concepts about the mutational events leading to HCC development

The development of HCC is almost never a sporadic event. It can be viewed as a Darwinian progression through a spectrum, ranging from an entirely healthy liver to a clonal aggregation of cells able to escape both intrinsic programmes regulating and enforcing normal cell behaviour and the exogenous restraints on cell proliferation imposed by the environment (Figure 1). Once these processes controlling equitable hepatocellular proliferation are evaded a dominant hepatocyte clone may become established. Additional mutations may then cause further escape of restriction, with subclones becoming capable of unlimited and unregulated proliferation. However, at each stage of

this process, further mutation and selection still continue. In response to liver damage all hepatocytes are capable of regeneration (reviewed in [17]), but are kept under a tight control in a healthy liver. Yet it only takes a small advantage to circumvent the restrictions and outgrow the surrounding cells.

Although cirrhosis may be regarded as a premalignant stage, the development of HCC in cirrhosis requires additional and progressive pre-neoplastic stages as additional stepping stones towards HCC [18]. This spectrum of development can be crudely broken down into distinct stepwise progression events from injured, often cirrhotic, liver to focal patches of low grade dysplasic nodule, then through high grade dysplasic nodule onto HCC [19] (Figure 1). Risk of malignant transformation increases incrementally at each stage. Whilst these lesions may progress to frank HCC, each can be seen as a stage along a continuum from normal to cancer. At each stage, competition occurs with successful clones outcompeting their neighbours, increasing their number. This deregulated growth results in microscopic nodules and they may progress to nodules visible either macroscopically or radiologically. If the overall nodule enlarges significantly within a fibrotic lobule then it compresses the surrounding tissue. Though clonal nodules are not necessarily visible by clinical imaging methods they have become identifiable thanks to our ability to visualise them by their genetic profile.

The human genome, spread across 23 chromosome pairs, comprises approximately 3 billion base pairs in a diploid cell. Hepatocytes can be both multinucleate and polyploid [20], meaning any one usually has up to 12 billion base pairs. The presence of multiple gene copies in hepatocytes impacts the probability of genetic mutations affecting all copies of a specific gene required for a loss of function phenotype. Therefore, gain-of-function mutations as drivers of disease are more prominent in the liver. Thus, polyploidy in the liver likely acts to protect against loss of tumour suppressing genes in the same way that losing function is more difficult when a cell has multiple copies of a gene [21-23]. In contrast to polyploidy being protective aneuploidy, an imbalance in the copy number of the 23 chromosome pairs, is associated with higher risk of cancer development including HCC [24]. Aneuploidy is also associated with chronic liver disease and aging [25] as well as shortened telomers [26]. As aneuploidy results from mistakes in chromosomal separation during cell division this is consistent with the concept that repeated division, both in healthy aging or through forced regeneration during chronic liver disease, promotes both aneuploidy in hepatocytes and subsequent HCC.

Aside from changes in chromosome number the genetic code of chromosomes may be altered by mutations. Most mutations affecting the genome do not confer a selection advantage and can be

termed passenger mutations. However, they can be utilized by mankind, both as potential therapeutic targets [27] and as markers of cellular heritage. Conversely, driver mutations do instil a selective advantage and thus are selected over time. This may be, but is not necessarily, linked to malignant transformation. To understand how cancers form we have to understand not only how the mutated genes make cells cancerous what but causes these mutations.

Constitutional predisposition to cirrhosis and HCC

Mutations are either inherited or arise within an individual cell. Inherited mutations, or those occurring in gametes, are known as germline, whilst those which cannot be inherited are somatic. Somatic mutations are then passed to descendants of the original cell in which the mutation formed. They can have multi-organ effects if they occur during development. However, if they occur after early development, when cellular differentiation and geography are more restricted, their descendants are more local. This is particularly the case in the liver. We are now appreciating that mutations occurring are restricted, particularly during chronic liver disease states, within individual lobules of the liver. On the other hand germline mutations will, by definition, be present throughout the liver being disseminated during the organ's development.

Several germline mutations predispose to chronic liver disease. This is mainly due to iron (hemochromatosis due to C282Y/C282Y mutations of *HFE*) or copper overload (Wilson disease due to *ATP7B* germline mutations), protein deficiency (alpha 1 anti-trypsin deficiency due to *SERPINA1* mutations) or metabolic disorders (tyrosinemia due to *FAH* mutations) [28]. With the exception of germline *HNF1A* mutations or glycogenosis type 1A (due to *G6PC* inactivating mutations) which both predispose to benign liver tumours with potential malignant transformation in a non-fibrotic liver, all these inherited mutations foster HCC development on a background of cirrhosis [29, 30].

Single nucleotide polymorphisms (SNPs) are frequent constitutional variants in the general population (generally at a frequency of more than 5%) and some of them modulate the risk to develop various human diseases [31]. Several SNPs, belonging to different pathways such as inflammation, DNA repair, cell cycle regulation, oxidative stress, iron metabolism and growth factors, have been involved in HCC development but few of them have been robustly validated in the literature [31] (Table 1). *PNPLA3* and *TM6SF2* genes encode for proteins involved in lipid metabolism and in the composition of lipid droplet [32-38]. Moreover, recent studies have identified a SNP of rs72613567 *HSD17B13* leading to

a truncated protein and loss of function associated with a reduced risk of cirrhosis occurrence in alcoholic liver disease (ALD) and NAFLD and a decreased HCC occurrence in ALD [39, 40]. The exact function of this gene and the consequence of the loss of function in the liver remains to be explored.

In contrast to germline mutations that cause chronic liver diseases, some single nucleotide polymorphisms are not pathogenic per se, but require an additional cause of chronic liver disease. These SNPs are associated with only a slight increase in cirrhosis and HCC risks, with an odds ratio below 2 in most of the studies [41]. Consequently, these SNPs only marginally increase our ability to predict HCC compared to the combination of classical clinical features and are not currently used in clinical practice [42]. Interestingly, some constitutional variants predispose to a specific molecular subtype of liver tumours. For example, germline *HNF1A* mutations predisposes to liver adenomatosis composed of *HNF1A* inactivated hepatocellular adenoma (HCA). Conversely HCA developed after glycogenosis are never *HNF1A* inactivated. This is likely due to the similar metabolic defects observed with *HNF1A* inactivation and *G6PC* deficiency [43-45]. In contrast, *PNPLA3*, *TM6SF2* or *HSD17B13* SNPs were not associated with development of a specific molecular subgroup of HCC.

Clonal and sub-clonal evolution of cirrhotic hepatocytes

Recently somatic mutations and clonal evolution have come more into focus as we aim to better understand disease development affecting the majority of patients with HCC. Early studies examining clonal selection and dominance needed to overcome the limitations of polyclonality in tissue. Bulk-sequencing approaches at low read depth are insensitive and unable to capture clonal and subclonal dynamics. Instead investigators made use of genetic marker of clones detected through immunohistochemistry in patients with cirrhosis. These showed clonal spatial restriction within cirrhotic nodules sequestered by the extracellular matrix. Two different approaches were used to identify these clones, using either X-Chromosome inactivation or mitochondrial DNA mutations as markers [46-48]. These early reports have been reinforced by more recent microbiopsy sequencing studies [49, 50], including a landmark study from Peter Campbell's group, where Brunner *et al.* used mapped microbiopsies within cirrhotic nodules [51]. All approaches showed that the majority of regenerative nodules are monoclonal in nature. Some nodules are oligoclonal, but all contain

genetically mutant hepatocytes. In every case, clonality is restricted to each individual nodule (Figure 2). This finding that many cirrhotic nodules are monoclonal or oligoclonal suggests a non-neutral drift towards mutations conferring a selective advantage. Whilst oligoclonality may be an intermediate stage of one clone displacing another we are, as yet, unable to predict whether, or indeed which, one will become dominant. Driver mutations are not necessarily stepping stones to cancer, however, and in some instances may actually protect nodules from the liver injury driving chronic disease as well as promoting regeneration of these clones [49]. Overall, in cirrhosis, as a nodule develops over time it is formed from a single clone possibly with a selective advantage over its original cohabitants within the lobule.

While some data also suggest clonal patches in healthy liver [52], this is harder to confirm. The lack of physical restriction enforced by cirrhotic fibrous bands makes microdissection of related patches challenging. Additionally, without high cell turnover in healthy liver compared to chronic disease the forces driving clonal selection may be lessened. Additional studies utilising either microdissection or spatially-registered single cell analysis are needed to confirm whether or not clonal expansions are typical in the healthy liver. Assuming that clonal expansions become more prevalent in chronic disease an interesting concept arises; the potential role of fibrous bridging acting to restrict the spread of clonal populations (Figure 2). This may have important implications for reducing the risk of future cancer by preventing the spread of clones with harmful mutations to more distant sites in the liver and limit clonal size.

Healthy livers steadily accumulate mutations over time, with approximately 33-40 per year per diploid genome, with only moderate variation between individuals [53]. Insertion/Deletions (InDels) and substitutions are heterogeneous between and within individuals, but structural variants and copy number alterations are more often found in patients with chronic liver disease. Also Chromothripsis, a localized massive chromosomal rearrangement probably due to a catastrophic event during cell proliferation, is more often found in patients with chronic liver disease [51]. Mutational rates and patterns are influenced by the causative factors of chronic liver disease as well as the change in microenvironment, such as inflammation [54-56]. Overall, the mutational burden correlates with both fibrosis stage and tissue damage and increases during malignant transformation.

It is plausible that the increased risk of progressing to hepatocellular carcinoma on a background of chronic liver disease is more driven by the constant evolution of countless numbers of clones that can independently acquire sufficient driver mutations than the simple presence of one specific driver

mutation. Consistent with this it may be more informative to evaluate gene-expression in non-tumoural adjacent tissue in regards to the risk of *de novo* HCC recurrence [57].

Mutations in the *TERT* promoter, while not found in healthy or cirrhotic tissue, are one of the first indicators of malignant transformation, since they arise already in dysplastic nodules [50, 58]. Usually only 2 to 6 driver mutations are found in HCC, but they are not restricted in the order in which they appear [8, 54]. Interestingly, it has been suggested that not all mutations found in cirrhotic tissue are necessarily linked to malignant transformation and some might be beneficial for regeneration without carrying the risk of progression to cancer [49]. Conversely some mutations, well described as cancer drivers, are found in non-cancerous cirrhotic nodules and even non cirrhotic liver. These appear to be present only in a very small minority (<5%) of nodules or hepatocytes respectively [49, 51].

Mutational signatures and viral insertional mutagenesis in HCC development

Mutational signatures are the consequences of endogenous and/or exogenous processes on the genome [59, 60]. As a fingerprint of early and late events occurring in the cell, the analysis of mutational signatures could be used to understand the mechanisms of transformation of normal cells into malignant cells and to identify new risk factors for tumour development [61]. In each tumour, the mutational spectrum observed is due to a combination of several mutational processes operating at different times during the life of a cell. The different mutational signatures in one HCC can be derived from mathematical analysis of the type of substitution, taking into account the trinucleotide context (single base substitution signature) as well as the type of larger rearrangement in the genome (rearrangement signature) [62]. In HCC a number of common and unique signatures have been observed (Figure 3 and Table 2). Ubiquitous mutational signatures related to age (signature 1 and 5) as well as others considered as liver specific (signature 12 and 16) have been identified in most HCC genomes [54, 63, 64]. In contrast, sporadic signatures were identified with a high intra- and inter-patient variability and were related to mismatch repair deficiency (signature 6), exposure to aflatoxin B1 (signature 24), tobacco (signature 4) or aristolochic acid (signature 22) [54, 65, 66]. Importantly, the mechanisms explaining several sporadic signatures (such as signature 23) remain to be identified. These studies also suggest that some mutational signatures are dependent on the aetiology of the underlying liver diseases and exposure to carcinogens.

Moreover, the link between mutational signatures with specific mutational processes and carcinogens could explain the association observed between mutations in driver genes and a specific aetiology. For example, the increased likelihood of *TP53* mutations in aflatoxin B1 related HCC is mainly due to R249S *TP53* mutations, a result of C to A substitutions in the GCC trinucleotide context (signature 24) induced by aflatoxin B1 [67, 68]. We also recently showed that signature 16, linked with alcohol consumption, is the most important contributor to mutations in *CTNNB1*, explaining the fact that *CTNNB1* mutations were more frequent in alcohol related HCC [54].

The mutational signatures in cirrhosis are subtly different to those found in HCC. Based on the analysis of different human adult stem cells, a study showed that liver adult stem-like cells harboured a predominant signature with T:A to C:G transitions with a transcriptional bias close to the features of signature 5 (age-related) observed likewise in HCC [29]. However, the significance of the enrichment of this mutational signature in adult liver stem cells remains still unknown. In the adult liver, a recent study has described the mutational signatures operative at subclonal and clonal level in cirrhotic hepatocytes [51]. They reported that signature 5, related to aging, and signature A, described in haematopoietic stem cells [69], account for most of the mutations observed in cirrhotic nodules. Interestingly, although both signatures were conserved in HCC, signature A was present in HCC at a lower proportion than other mutational signatures, suggesting that mutations related to signature A are probably surpassed by other mutational processes during progression from cirrhosis to HCC. Signature 1, related to age, and signatures 12 and 16, specific to liver tumours, were identified in cirrhosis but account for a lower rate of the mutations in cirrhotic hepatocytes compared to the higher frequencies observed in HCC. This observation suggests an active role for the agents causing these mutational signatures during malignant transformation. Brunner *et al.* also identified mutational signatures related to exposure to aflatoxin B1 and aristolochic acid in cirrhotic liver with regional variability in term of mutagen exposure [51]. Importantly, this study was able to predict the clinical risk factors from the mutational spectrum. For example, all cases of smoking signatures were found in known smokers and rarer signatures, resulting from environmental factors, were found in patients with known risk of exposure to these factors. Therefore, mutational signatures in cirrhosis are not only indicative of the drivers of mutational processes and may reflect the underlying disease but also are subtly different to those in HCC.

Another imprint in the liver and tumour genome is due to viral insertional mutagenesis [70]. Hepatitis B virus is one of the most frequent causes of HCC worldwide. A subset of HCCs, particularly in some sub-Saharan African and Asian populations [71], develop in minimally fibrotic livers of young patients

infected by hepatitis B virus (HBV) suggesting a direct oncogenic effect of the virus. HBV is able to insert in the host genome in the liver and deep sequencing data identified non-clonal HBV insertion that occurs randomly in all chromosomes in a subset of hepatocytes. Yet, most of the detected HBV breakpoints are located in the X gene and the viral enhancer [72]. In some cases, the insertion of HBV genome near a cancer gene modifies its expression and function and promotes malignant transformation of hepatocytes [73, 74]. In HCC a subset of HBV-related tumours harbour clonal insertion of HBV in major driver genes such as *TERT*, *MLL4*, *CCNE1* or *CCNA2* whereas insertion in non-tumour liver are subclonal and inserted in various places of the genome [75, 76].

A similar mechanism is observed in a subset of HCC developed in normal liver that harboured clonal insertion of adeno associated virus type 2 (AAV2) [77]. AAV2 and AAV2/AAV13 strand insertions have been observed in the hepatocyte genomes of approximately 20% of patients [78]. Most of these insertions were subclonal and randomly inserted into the genome of normal hepatocytes. However, a subset of HCC harboured clonal insertion of the 3' inverse tandem repeat region of AAV2 in driver genes such as *TERT*, *CCNA2*, *CCNE1*, *TNFSF10* and *GLI1* underlining a role of AAV2-insertional mutagenesis in liver carcinogenesis on a healthy liver background [77-79]. The reasons for the rare occurrence of AAV2-related HCC in the context of the high incidence of AAV2 strand insertion in the general population remains to be better understood. In contrast, the direct oncogenic role of hepatitis C virus outside the background of cirrhosis remains controversial [80]

Driver mutations involved in tumour initiation and malignant transformation on cirrhosis

Next generation sequencing (RNA sequencing, whole exome sequencing and whole genome sequencing) has described the genetic landscape of hepatocellular carcinoma (HCC) (Table 1). The most frequent alterations in driver genes were mutations in *TERT* promoter (40-60%), *TP53* (15-40%), *CTNNB1* (10-35%), *ARID1A* (5-17%), *ARID2* (3-18%), *AXIN1* (5-15%), *RPS6KA3* (2-9%), *NFE2L2* (3-6%), *KEAP1* (2-8%), *RB1* (3-8%) and *VEGFA* (5%) and *FGF19* (5-10%) amplifications [63, 64, 81-83]. Beyond the most frequent somatic mutations there is also a long list of rarer mutations in other cancer driver genes. The Transforming Growth Factor β (TGFβ)~~B~~ pathway has been also involved in the pathogenesis of HCC with a dual role in liver carcinogenesis with an aberrant activation of this pathway observed in

some HCC whereas others HCC harboured inactivating mutations of genes belonging to the pathway such as *ACVR2A* (5%) [63, 84, 85] . Moreover, frequent activation of the Ras/Raf/Map-kinase pathway at the protein level has been described in human HCC [86] as well as rare somatic mutations leading to the constitutive activation of the pathway such as *RPS6KA3* mutations (2-9%) and *Kras* mutations (1%) [63, 87]. Interestingly, this pathway could be targeted using MEK inhibitors such as trametinib or refametinib [88] . Altogether, each HCC is a unique combination of somatic alterations with 40 to 60 non-synonymous mutations in the coding sequence per tumour and with around 2 to 6 mutations in driver genes [63, 64, 89]. A recent study has highlighted an enrichment of mutations in *TP53*, *RB1* and *SF3B1* in advanced HCC and in patients with poor prognosis proposing an important role of mutations in genes controlling the cell cycle (*TP53, RB1*) and the spliceosome machinery (*SF3B1*) for tumour progression [90].

Even though the genetic landscape of HCC is relatively well characterised, the early mechanisms of transformation of hepatocytes into cancer cells remains only partially understood, particularly the key steps from cirrhosis to HCC. Studying progressive mutations during dysplastic nodule formation has given us some insights into this process, however. Senescence, a state of permanent cell cycle arrest, together with short telomeres in hepatocytes are hallmarks of cirrhosis [91]. Telomeres are shortened in chronic liver injury due to a combination of lack of expression of telomerase in the adult liver and long-standing regenerative proliferation [92]. One of the key mechanisms involved in malignant transformation of cirrhotic hepatocytes is reactivation of telomerase, which is observed in most HCCs and occurs progressively from low to high grade dysplasia [93] (**Figure 1**). *TERT* promoter mutations are observed in around to 10 to 20% of low grade and high-grade dysplastic nodules and in up to 60% of early HCC [58, 82]. No other recurrent genetic alterations in other driver genes have been observed in premalignant nodules in cirrhosis to date. Whilst *TERT* promoter mutations are considered a key event in HCC occurrence, currently no study has found sub-clonal *TERT* promoter mutations in cirrhosis. These data suggest that the *TERT* promoter is a gatekeeper involved in tumour initiation and malignant transformation of hepatocytes through reactivation of telomerase [63]. Other studies highlighted that transcriptomic dysregulation of signalling pathway such as TGFβ, WNT, NOTCH and extensive epigenetic modifications occurred after malignant transformation mainly in progressed HCC confirming that genomic and epigenomic diversity occurred in the late stages of liver carcinogenesis [94-96].

Although recently our understanding of mutations and mutational signatures in human patients at different stages of liver disease has improved greatly, there remain key questions of how a particular

cirrhotic nodule is most likely to become a cancer depending on its current genetic profile. One question is if every nodule holds the potential for malignant transformation? If not, are dysplastic nodules required as an intermediary for HCC? If HCC may arise independently of a dysplastic nodule precursor this would be a non-linear model for HCC development. This model has been proposed [97], however, it is difficult to trace clonal evolution longitudinally in patients. The lack of inter-relationships between dysplastic nodules and separate HCCs in this study would be predicted given that each cirrhotic nodule is distinct by high resolution whole genome sequencing [49, 51]. An important study in human tissue will be to examine nodule in nodule disease in which dysplastic nodules contain early forms of HCC. Even though dysplastic nodules and HCC may be different at the time of the study it is impossible to determine what status they had in the past or how they will progress in the future. It would therefore be ideal to investigate these questions in various model systems in addition to humans.

How to model early genomic events of liver carcinogenesis?

Characterising the role of driver genes and distinct mutational progression from health, through fibrosis, to HCC may be possible using large scale human tissue sequencing, however, to mechanistically unravel these we will require manipulation using preclinical model systems. *In vitro* systems exist for human hepatocyte culture both in 2D and as 3D organoids [53, 98-104] with the potential for overlaying chronic liver disease conditions. However, modelling of HCC formation over prolonged time, especially given the role of both disease and aging in the mutational landscape, within the complex multicellular environment will likely require complex *in vivo* modelling systems. Preclinical *in vivo* models offer an opportunity to monitor and manipulate hepatocytes as they progress from native state to cancer, including studying both the complex interactions within the multicellular liver environment. Mouse models have proven to be widely used particularly in view of their flexibility for genetic targeting, immunomodulation (including immunodeficiency) and combinations with transplantation or xenotransplantation [105]. None the less, all the models have limitations when it comes to recapitulation of the progression to HCC in man. Murine models vary greatly in the rapidity of tumour formation, with rapid modelling being attractive from a practical standpoint. Yet, a balance needs to be found between quick models, that are still able to capture the mutational processes developed over decades in human disease, and models that accurately represent human disease. Unfortunately, there is still no consensus model that reproduces human cirrhosis closely and often damage is induced by chemicals very different to the exogenous toxins

causing liver disease in humans. This leads to the dilemma that tumours induced by chemical agents in mice differ greatly from human HCC, whereas long-term spontaneous tumourigenesis in mice more closely resembles mutational patterns found in human HCC [106]. Another study analysing four different mouse models of HCC at the molecular level confirmed that chemically-induced mouse models are considerably different to human HCC at a molecular level, whereas genetic models mimic them more closely [107]. Therefore, it will be crucial to investigate a broader array of genetic models of liver disease in detail, particularly those modelling stages from genetic predispositions to chronic liver disease, through to early carcinogenesis. This may allow dissection of the natural history of HCC as they form in these model systems. The development of mouse models where chronic liver disease and subsequent HCC development is driven by exogenous factors relevant to human disease, like ALD or NAFLD, will be crucial. Modelling of disease states including fibrosis [108] and NAFLD [109] is possible in the mouse and could be combined with genetic modelling of HCC.

More recently specific mutations have been modelled in the mouse liver and have driven HCC-like tumour expansions. An early observation from these targeted genetically engineered mouse models is that therapeutic responses may be dependent upon the genetic makeup of a tumour [110-112]. Whether or not these will be applicable to human HCC subtypes based on the mutational drivers remains to be seen. However, it is promising that the lack of responsiveness to immunotherapy observed in murine tumours models driven by βB-catenin mutations [111] is also reported in some relatively early observational studies in man [113]. Model systems therefore offer the opportunity to understand and develop treatments for these early stages of disease which could then be applied to the clinic with implications for the way that tumours are detected, monitored, diagnosed as well as treated.

Implications for disease and therapy

Currently there are many shortcomings in our clinical approach to patients at risk of and with HCC. For disease detection we generally apply a 'one size fits all' surveillance approach. Early detection of HCC through surveillance is recommended but notoriously inaccurate and cost inefficient [114-118]. Disease prevention is centred upon avoiding or treating the underlying aetiology. Diagnosis of HCC disease is typically radiological and gives little information about tumour biology, patient prognosis or the likely response to therapy. Each of these areas could theoretically be improved by appreciating the mutational steps leading to HCC development (Figure 4). Profiling the mutational landscape of the

liver could be achieved either by liver biopsy (targeted and/or untargeted) or using a liquid biopsy using either cells or DNA from the liver present in the blood steam. The source and fidelity of this material will be critical for clinical decision making.

At the population level identifying exogenous mutational signatures can link specific risk factors to HCC development. The causative agents of a number of current mutational signatures remain unidentified. Should further causative agents become apparent then this would have major public health implications, like the initial linkage between aflatoxin and HCC.

At the patient level characterising mutational signatures could be used clinically. As the mutational signature is relatively conserved across the liver [51], an untargeted biopsy can still give information on particular exogenous risk factors driving mutagenesis. This could then be used to focus prevention measures specific to the individual. However, the absence of driver mutations in one nodule does not predict absence in other nodules. Therefore, cautious interpretation will be required for untargeted biopsies. A potential utility for overall mutational rate might be to stratify HCC risk. Mutational load could be used to target HCC surveillance. Similarly, this could be used to predict risk of recurrent disease, as has already been suggested for epigenetic signatures [57].

Targeted biopsies can provide information on specific mutations within a nodule. For example, the presence of *TERT* promoter mutation in low or high grade dysplastic nodules could be useful to identify the premalignant lesions at high risk of malignant transformation [58]. This could be used in a similar fashion to our current risk stratification of hepatic adenoma based on high risk β-catenin mutations [119].

Additionally, treatment decisions for other conditions could be tailored to the mutational load of the liver. It is becoming apparent that systemic chemotherapy results in the accumulation of higher mutational load in other organs [120] and the same is likely to be true in the liver also. Therefore, the use of non-selective chemotherapy, or even repeated irradiation may prove to be disadvantageous for patients with high mutational burden in a cirrhotic liver.

As a characterised handful of mutations drive the majority of HCC, detecting these may aid HCC surveillance. Through analysis of circulating tumour cells (CTCs) or tumour cell-free DNA (cfDNA) it is possible that tumours could be detected and even phenotyped using liquid biopsy from a blood sample [121, 122]. There are well described limitations to analysis of CTCs and cfDNA however. These

include difficulty in differentiating potential tumours within a mixed liquid biopsy due to the low allelic frequency using either ctDNA or bulk sequencing from CTCs [123]. The co-occurrence of relevant driver mutations together might increase this yield but would require single cell analysis and be outside the availability and budget of most healthcare providers. CTCs are associated with larger tumours and hence this approach may not be applicable to early stage disease. Crucially, genetic drivers of HCC are also more rarely found in the myriad of non-tumoural cirrhotic nodules (approximately half a million in the average liver) [49-51], as well as other non-malignant tissues outside the liver [53, 120, 124-126]. Detection of HCC cancer drivers does therefore not equate to the presence of HCC.

The most obvious role for genetic analysis in HCC is for treatment stratification. Although identifying driver mutations may deliver effective therapies, the present potential for druggable targets in HCC based on current knowledge and pharmacotherapy remains poor [127]. None the less, as we begin to examine the role for precision medicine in HCC treatment decisions will become increasingly dependent upon genetic stratification. It is becoming apparent that tumour biology [128] and treatment responses, even for immunotherapy [113], may be predictable based on mutational subtypes. However, inter and intratumour genomic and epigenomic heterogeneity have been described in HCC with a potential impact in clinical practice. A better understanding of genetic heterogeneity will be helpful to dissect the mechanisms of primary and secondary resistance to systemic treatments [129]. Another important application could be using our knowledge about mutations which predict extrahepatic spread. This could, for example, have profound implications upon the decision to proceed to liver transplantation.

Unmet needs and conclusion

Understanding how normal hepatocytes and cirrhotic hepatocytes becomes cancer is central to tackling the HCC pandemic and managing patients with chronic liver disease. Whilst we already known a lot about the genomic defects shaping hepatocellular carcinoma, the early events occurring in the genome of normal and cirrhotic livers need to be better described. It is not clear how the subclonal or clonal events observed in cirrhosis are involved in malignant transformation, if they provide plasticity and survival advantages in a context of chronic liver disease or could be sometimes a dead-end street for the cell. More data are required to confirm the robustness and reproducibility of the observation of sub-clonal and clonal mutations in cirrhosis using a larger number of samples. More data are also

warranted to understand the relationship between the different aetiologies of chronic liver diseases, exposure to carcinogens and the early modifications of the hepatocyte genome as well as the effect of the treatment of underlying liver disease on genomic dysregulation observed in cirrhosis. New model systems to help understand the effects of underlying aetiology and subclonal mutations on liver carcinogenesis and that better mimic the human diseases are essential. Finally, a better understanding of early changes in cirrhotic hepatocytes, including both driver genes and overall mutational signature and burden, will be helpful to perform clinical weaponisation of this knowledge, develop preventive strategies and adapt treatment of patients with chronic liver disease and early HCC.

Table 1: Driver mutations associated with HCC.

Summary of the most frequent driver mutations associated with HCC

Table 2: Mutations signatures associated with HCC.

Summary of key mutational signatures described in chronic liver disease and HCC. A comprehensive list of signatures can be found at https://cancer.sanger.ac.uk/cosmic/signatures.

Figure 1: Early genomic events in cirrhotic hepatocytes and their role in malignant transformation

Schematic of the main somatic genetic alterations responsible for the malignant transformation of premalignant lesions developed from chronic liver disease, with examples given of transition through low-grade dysplastic and high-grade dysplastic nodules. This stepwise transition is typical but not exclusive. The establishment of cirrhosis with fibrotic scar separating nodules is associated with mutations and establishment of mutant clones. Coloured hepatocytes depict those of a distinct lineage. Over time and with progression to Hepatocellular Carcinoma (HCC) the overall mutational rate and burden increases. Specific driver mutations are associated with progression to HCC. Some are associated with pre-malignant stages of the disease e.g. TERT whilst others are associated with HCC; particularly *TP53* in late stage disease.

Figure 2: Clonality and subclonality within cirrhotic nodules

Within cirrhotic nodules, each separated by fibrosis, clones of hepatocytes form and are selected for by a process of natural selection. Over time, clones (represented by colours – brown with passenger mutations; yellow, pink and green for driver mutations) become selected. Within nodule 1 a driver mutation (yellow) grows to replace the other hepatocytes in the nodules. Expansion between nodules is limited by the fibrotic boundaries. A neoplastic subclone (red) then forms within this area. Most nodules however do not progress to malignancy e.g. nodule 2 where a stable clone expands to repopulate the nodule. Other nodules are oligoclonal e.g. nodule 3.

Figure 3: mutational processes in liver carcinogenesis

Summary of the main mutational processes operating in hepatocellular carcinoma and chronic liver disease and their relationship to specific risk factors and carcinogens. In one tumour, several mutational processes may operate synchronously or at different times. Some mutational signatures are ubiquitous whereas other mutational signatures are identified only in a subset of tumours and are considered as sporadic. Some have been associated with specific environmental pathogens, including smoking and alcohol.

Figure 4: Implications for diseases and therapies

Pathways to translate improved understanding of the mutational landscape of cirrhosis and HCC to impact clinical practice. Profiling can be achieved from blood as a liquid biopsy (sequencing either cell free DNA or circulating tumour cells). Alternatively, a liver biopsy sample can be sequenced from either an untargeted area of the liver or directly from nodules targeted based on imaging characteristics. At the population level, primary prevention requires identification of risk factors which may be identified by their mutational signatures. For individual patients at risk of HCC their genetic profile could inform both surveillance and targeted prevention. For individuals with HCC, genetic tumour profiling may aid precision medicine for the tumour itself and guide tertiary prevention and surveillance for recurrence after tumour treatment.

Table 1

Mutations associated with HCC	Stage	References in text
Constitutional mutations/single nucleotide polymorphisms		
ATP7B	Wilson disease: Cirrhosis/HCC-predisposition	28
FAH	Tyrosinemia: Cirrhosis/HCC-predisposition	28
G6PC	Glycogenosis 1a: HCA-HCC-predisposition	45
HFE	Hemochromatosis: Cirrhosis/HCC-predisposition	28
HNF1A	MODY 3 diabetes and HCA-predisposition	29
HSD17B13 rs72613567	Cirrhosis/HCC-predisposition (SNP)	39, 40
PNPLA3 rs738409	Cirrhosis/HCC-predisposition (SNP)	32-38
SERPINA1	α-1 anti trypsine deficiency: Cirrhosis/HCC-predisposition	28
TM6SF2 rs58542926	Cirrhosis/HCC-predisposition (SNP)	35-37
Somatic mutations		
TERT promoter	Tumour (early) (40-60%)	63,64,75-79, 82, 89, 90
ACVR2A	Tumour (5%)	63
ARID1A	Tumour (5-15%)	63, 64, 83, 89, 90
ARID2	Tumour (3-15%)	63, 64, 83, 89, 90
AXIN1	Tumour (5-15%)	63, 64, 83, 89, 90
CTNNB1	Tumour (15-35%)	63, 64, 81, 83, 89, 90
FGF19	Tumour (4-6%)	63, 64, 83, 89, 90
KEAP1	Tumour (2-8%)	63, 64, 83, 89, 90
KRAS	Tumour (1%)	63, 87, 90
MLL4	Tumour (5%)	75, 89
NFE2L2	Tumour (3-6%)	63, 64, 83, 89, 90
RB1	Tumour (3-8%)	63, 64, 89, 90
RPS6KA3	Tumour (2-9%)	63, 64, 83, 89, 90
SF3B1	Tumour (3%)	89, 90

TP53	Tumour (15-45%)	63,64, 82, 83, 89, 90
VEGFA	Tumour (3-5%)	63, 89, 90

Table 2

Mutational Signature	Stage	Occurrence in HCC	Aetiology	Mutational features
Signature A	Cirrhosis/HCC^{low}	Ubiquitous	Endogenous mutational process	T>C substitutions
Viral Insertion	Cirrhosis/HCC	Sporadic	Virus infection `(AAV2, HBV)	Insertional mutagenesis
Signature 1	Cirrhosis^{low}/HCC	Ubiquitous	Endogenous mutational process (Age)	Deamination of 5-methylcytosine
Signature 4	HCC	Sporadic	Exposure to tobacco mutagens	C>A mutations
Signature 5	Cirrhosis/HCC	Ubiquitous	Endogenous mutational process (Age)	T>C substitutions in ATN trinucleotides
Signature 6	HCC	Sporadic	Defective DNA mismatch repair	C>T substitutions, small insertions and deletions
Signature 12	Cirrhosis^{low}/HCC	Ubiquitous	Unknown, hallmark of liver cancer	T>C substitutions
Signature 16	Cirrhosis^{low}/HCC	Ubiquitous	Exposure to Alcohol, hallmark of liver cancer	T>C mutations in ATN trinucleotides
Signature 22	HCC	Sporadic	Exposure to Aristolochic Acid	T>A mutations in CTG trinucleotides
Signature 24	HCC	Sporadic	Exposure to Aflatoxin	C>A mutations in GCC trinucleotides

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Figure 1

Figure 1

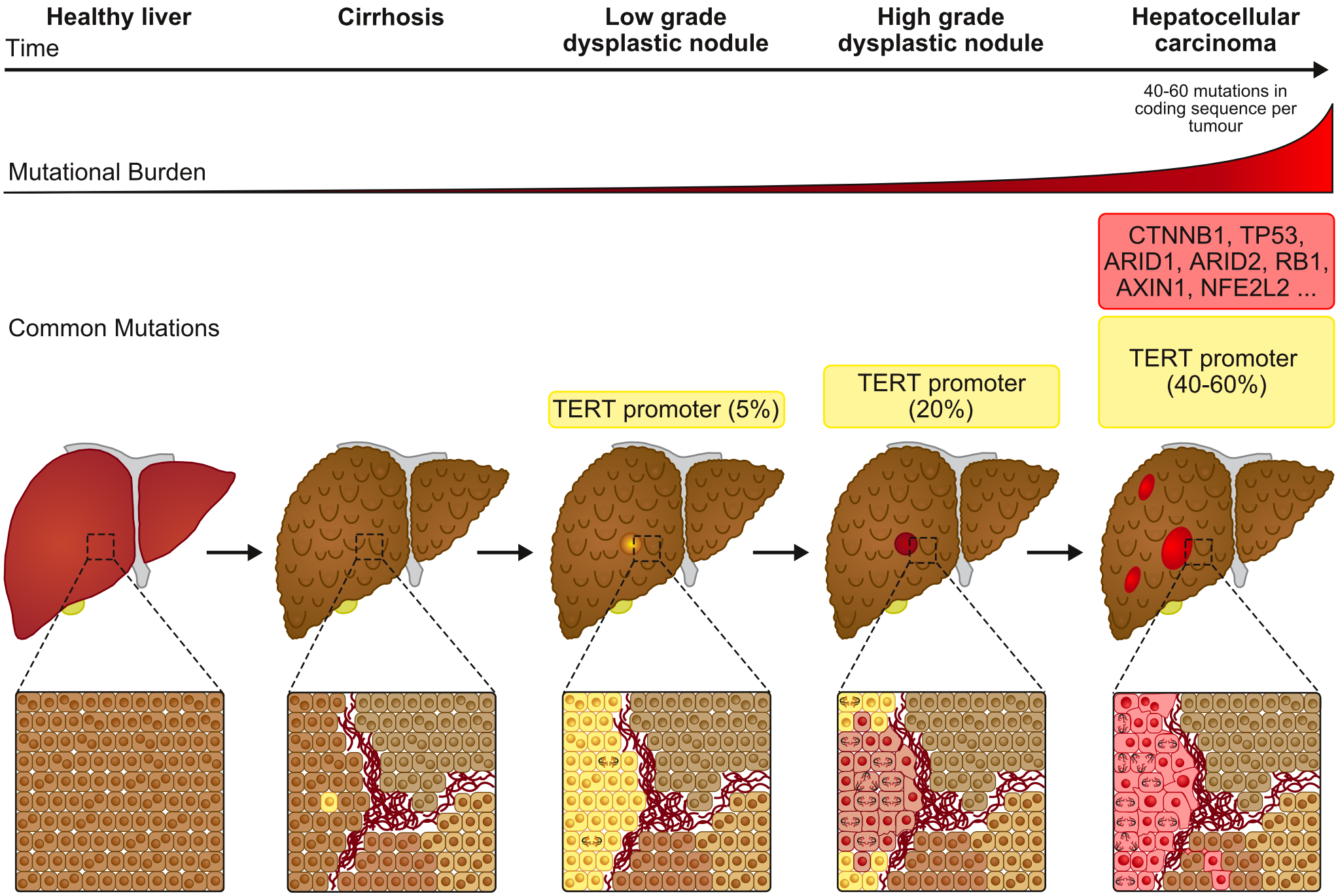
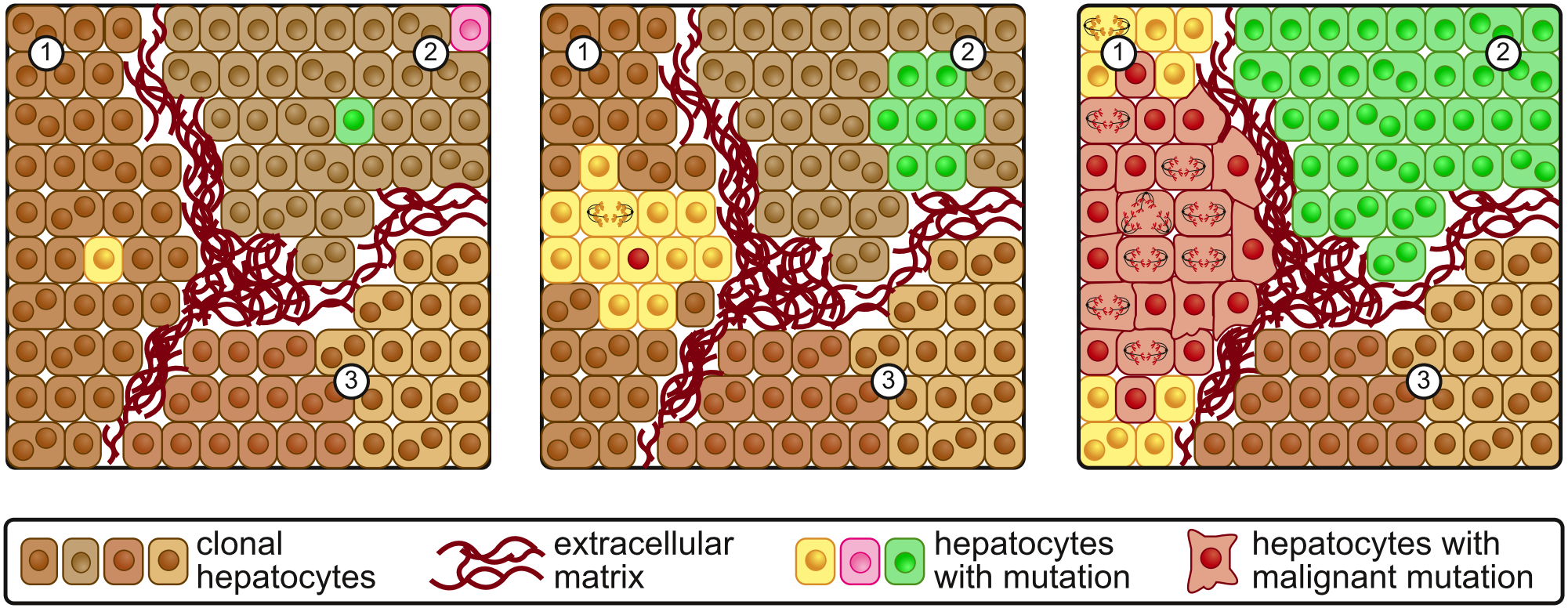


Figure 2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1
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1
2
3
clonal hepatocytes
extracellular matrix
hepatocytes with mutation
hepatocytes with malignant mutation

Figure 4

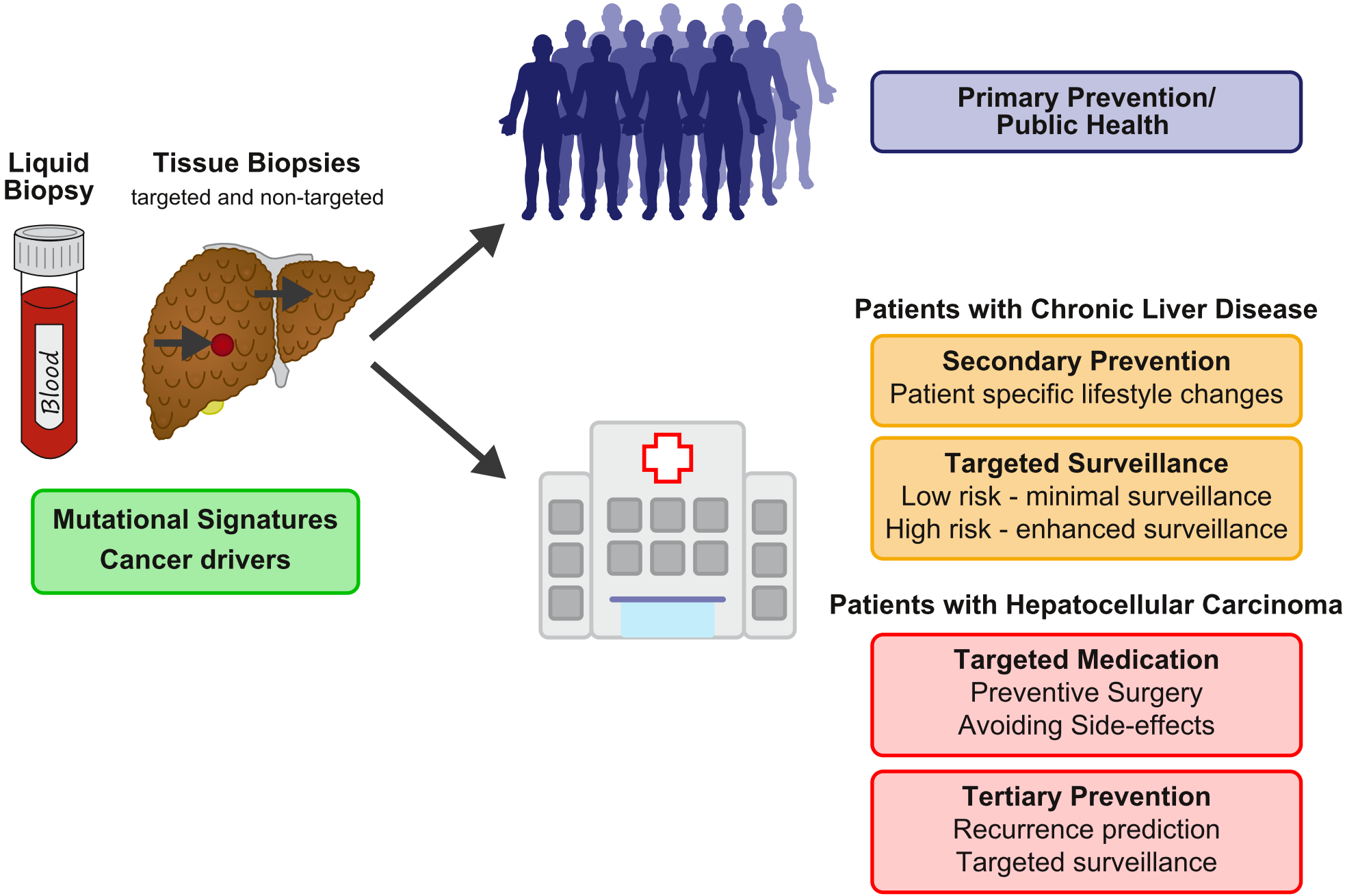
Liquid Biopsy
Tissue Biopsies
targeted and non-targeted
Blood
Mutational Signatures
Cancer drivers
Primary Prevention/
Public Health
Patients with Chronic Liver Disease
Secondary Prevention
Patient specific lifestyle changes
Targeted Surveillance
Low risk - minimal surveillance
High risk - enhanced surveillance
Patients with Hepatocellular Carcinoma
Targeted Medication
Preventive Surgery
Avoiding Side-effects
Tertiary Prevention
Recurrence prediction
Targeted surveillance

Figure 3

Figure 3