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Roadmap

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Towards a reference cell atlas of liver diversity over the human lifespan

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Abstract

The goal of the Human Liver Cell Atlas (HLiCA) is to create a comprehensive map that defines the normal functions of diverse liver cell types and their spatial relationships over the human lifespan. This project fits within the goals of the Human Cell Atlas to create comprehensive reference maps of all human cells as a basis for both understanding human health and diagnosing, monitoring and treating disease. Through collection of samples from diverse individuals, data integration across technologies and overcoming liver-specific challenges for experimental methods, the HLiCA will map as many cell types and states as possible in healthy human livers from individuals across all ages and many ancestries. Establishing this HLiCA of healthy livers is a critical step to begin to understand perturbations in disease. The HLiCA will be available on an open-access platform to facilitate data sharing and dissemination. We expect that creation of the HLiCA will help to lay the foundation for new research initiatives to advance our understanding of liver disease, improve methods of tissue engineering, and identify novel prognostic biomarkers and therapies to improve patient outcomes. We describe key experimental and computational challenges to overcome in building the atlas and the potential impact of the atlas on disease research.

Sections

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Key points

- Building the Human Liver Cell Atlas requires collaborative effort within the liver single-cell genomics community.
- Characterization of the normal human liver must account for sample-to-sample variability due to age, gender, ancestry, lifestyle, microbiome, environmental factors and experimental approaches, among other factors.
- Collecting standardized metadata and optimizing data integration is critical to generate a useful and comprehensive cell atlas across multiple laboratories and institutions.
- The Human Liver Cell Atlas will provide the foundation for understanding disease-specific perturbations and hopefully identify cell-type-specific therapeutic strategies to reduce the global burden of liver disease.

Introduction

Approximately 1.5 billion people in the world have chronic liver disease[1], and cirrhosis is the 11th leading cause of death worldwide[2,3]. A review of studies published from 1989 to 2015 found that ~25% of adults worldwide had metabolic dysfunction-associated steatotic liver disease (MASLD), with a subsequent meta-analysis including data through May 2021 showing a worldwide prevalence of ~32% in adults[4]. Alcohol-related liver disease is also a rising cause of mortality in developed countries[5] and was the most common reason patients were listed for liver transplantation in the USA between 2014 and 2019 (ref. 6). Furthermore, hepatitis B and C affect an estimated 350 million people worldwide (most of whom live in developing countries) and, collectively, are responsible for over 1 million deaths annually[7]. Although there is a substantial burden from liver disease, the liver is also highly resilient, with tremendous capacity for regeneration. Living donor liver transplantation is possible because the donor's remaining liver and the lobe transferred to the recipient can both regenerate and reach up to 90% of the original organ's size and function[8]. In addition, the liver performs numerous essential functions for health at baseline, including coordinating metabolism, supporting digestion, synthesizing blood products, breaking down drugs and cellular by-products, and interfacing with the immune system[9,10]. Understanding how the liver performs these unique functions at single-cell resolution is necessary to discover and interpret the importance of changes that occur in disease.

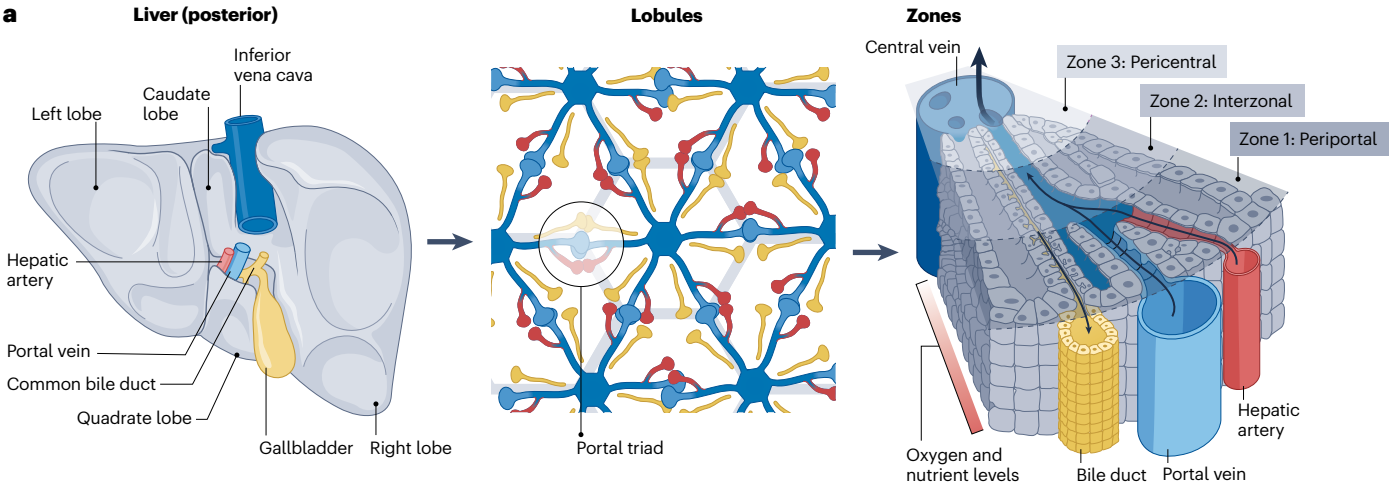
The liver is composed of four lobes (right, left, quadrate and caudate) that contain smaller, repeated structural units called lobules[11] (Fig. 1). Cells within the liver are organized into approximately 1 million lobules, each about 0.5–1.0 mm in diameter[12]. Lobules are defined anatomically by the sites where blood enters via branches of the portal vein and hepatic artery at intervals along the lobule circumference. Blood empties into sinusoids that provide radially convergent blood flow to the central vein. This structure creates a gradient whereby there are relatively high levels of oxygen and nutrients in the periphery of the lobule and lower levels towards the centre, a phenomenon termed 'liver zonation'. Zonation is responsible for specialization of many hepatic cell types (Fig. 1b), corresponding to their position along this gradient[13,14]. Although we know much about liver physiology and anatomy, there remains a lot to learn about how cells work together to perform normal functions and how regeneration, inflammation, fibrosis and liver failure are mediated by these interactions. The Human Liver Cell Atlas (HLiCA) aims to fill this knowledge gap, ultimately creating a next-generation reference map for the benefit of the liver research community. In line with the Human Cell Atlas (HCA), critical milestones to achieve this aim include profiling over 1 million total healthy human liver cells followed by obtaining over 1 million cells from subpopulations of patients by age (for example, paediatric patients) and ancestry. This Roadmap highlights current progress in developing the comprehensive HLiCA, ongoing areas of unmet need, and liver-specific wet laboratory and computational challenges. We expect that, when complete, the HLiCA will improve our understanding of the healthy human liver and enable translational studies to identify new therapies for disease states.

Building the healthy liver atlas

Assembling the HLiCA requires the input of a diverse community of basic scientists, computational biologists and clinicians with expertise spanning cell biology, technology development, microscopy, pathology, immunology, bioinformatics, machine learning, genomics, adult and paediatric hepatology, and liver transplantation. The community of experts participating in single-cell analysis of the liver was originally formed at the grassroots level by individuals who developed their expertise at multiple centres worldwide[15–18]. These experts met at HCA meetings, and collaborations grew, leading to large multicentre projects supported by funding from the Chan Zuckerberg Initiative and national funding agencies. The HLiCA community is committed to the principles defined by the HCA, including diversity, inclusivity and equity in terms of membership and liver samples analysed, and welcomes opportunities to expand the community and the populations that will benefit from the HLiCA. We encourage all interested researchers to join the HCA community and HLiCA bionetwork, to share their single-cell studies of the human liver on open-access platforms, and to contribute data to the HLiCA through publication and dissemination. The geographical distribution of active HLiCA members mirrors that of the HCA as a whole, with an initial bias towards North American and European researchers, highlighting the ongoing need to increase member diversity as one strategy to improve sample diversity. Continuing growth of the HCA community, especially via regional networks in Latin America, Asia and the Middle East, is addressing this issue.

The goal in creating HLiCA version 1.0 is to confidently map as many cell types and states as possible from samples of healthy human livers from individuals of all ages and multiple ancestries (Table 1 and Fig. 2). This step will incorporate single-cell RNA sequencing (scRNA-seq), including droplet and plate-based methods, single-nucleus RNA sequencing (snRNA-seq), cellular indexing, assessment of the chromatin landscape and multimodal techniques from a range of healthy human liver tissue sources (such as transplantation, surgical resection and biopsy)[19]. As of 2024, RNA profiles from over 1 million cells from more than 150 male and female donors without liver disease, ranging from 5 weeks after conception to >65 years of age have been published or mapped (Supplementary Table 1). Most samples are from adults and were originally analysed in different laboratories; these samples are the focus of initial integration efforts. Samples from healthy children are currently being profiled to develop a paediatric reference atlas. Ongoing efforts will increase genetic ancestry diversity among samples. Future atlas versions will integrate these data with

a



b **Cellular level**

Hepatic cell type	Primary function in homeostasis
Cholangiocytes	Bile acid transport and secretion
Hepatocytes	Metabolism, detoxification, bile acid and protein synthesis, innate immune function
Infiltrating immune cells (higher in disease)	Antigen clearance, regulation of inflammation, tissue repair and liver regeneration (variable disease-specific functions)
Mesenchymal cells	Tissue remodelling and repair (hepatic stellate cells are included within this category)
Sinusoidal endothelial cells	Maintenance of vascular tone, filtration and nutrient transport
Stellate cells	Regulation of sinusoidal blood flow, extracellular matrix production and vitamin A storage
Tissue-resident macrophages (i.e. Kupffer cells)	Phagocytosis, tissue repair and immunity

Fig. 1 | Overview of human liver anatomy. a, The human liver is composed of four lobes. Blood flows into the liver through the portal vein and hepatic artery and exits through the inferior vena cava for transport back to the heart. Bile produced by hepatocytes travels through the common bile duct and is stored in the gallbladder (left). Each lobe is composed of lobules (0.5 mm to 1.0 mm diameter, centre). Blood from the portal vein (light blue) and hepatic artery (red) flows through the sinusoids (darker blue) to the central vein (dark blue circles) before exiting the liver through the inferior vena cava. Bile produced by hepatocytes travels through the bile ducts (yellow) to the common bile duct. Each lobule is divided into zones (right). Blood from the hepatic artery mixes with blood from the portal vein in sinusoids in zone 1. As the blood flows through the sinusoids to the central vein, oxygen and nutrient levels decrease, and reach their lowest levels in zone 3. **b**, Multiple cell types are present within the liver lobule in homeostasis and disease. Cell function is specialized by the gradient of oxygen and nutrients within the lobule.

spatial transcriptomics to place each cell type within the structure of the liver lobule. Eventual integration with 3D imaging methods, such as hierarchical phase-contrast tomography (microscale) and volume electron microscopy (nanoscale) will provide a high-resolution and multiscale visual map of cell interactions across the lobule and ultimately the entire liver anatomy[20–22]. Through these approaches, the HLiCA will be a comprehensive representation of the liver across a diverse, global population.

Scientific challenges in adult liver biology

The atlas as a healthy control in disease studies

Characterizing the healthy liver at the single-cell level will require consideration of many factors (Table 1), including sample-to-sample variability between donors with a normal liver, as a result of age, sex, ancestry, lifestyle, microbiome and other environmental factors, as well as the different technical approaches used to process individual samples. In addition, the spatial context of cells within the lobule influences their phenotype[23]. Defining the diversity of the normal liver across these variables will enable HLiCA to support a more complete understanding of patient-specific perturbations that occur in disease (Fig. 2).

Sexual dimorphism is an important component in determining inter-individual liver diversity, which also affects disease susceptibility. For example, growth hormone regulates the expression of various hepatic genes[24] and is secreted in a pulsatile manner in men, whereas levels remain more constant in women[25]. Diseases influenced by sexual dimorphism include immune-mediated disorders, such as primary biliary cirrhosis, which occurs more frequently in women[26], and liver cancer, which occurs more frequently in men[27].

Differences in disease frequency by geographical region, ancestry and environmental exposures suggest that there are diverse susceptibility factors in specific populations. The interaction between genetics, diet and environment greatly influences the risk of MASLD and its complications across diverse populations[28]. For example, individuals from South Asian regions have a higher prevalence of MASLD without obesity than individuals in high-income countries[29], and in South Asia MASLD without obesity remains an independent risk factor for coronary artery disease similar to the risk in individuals with obesity and MASLD[30]. Metabolites derived from the diet differ between individuals, which in turn might influence the composition of upregulated immune metabolic inflammatory pathways that

Table 1 | Overview of the HLiCA: status and proposed strategies

Unmet need	Current status	Goals	Proposed strategies
Technical			
Establish a comprehensive single-cell atlas of normal liver	Multiple independent studies using different experimental methods and technologies that can affect cell type frequencies	Improve data integration strategies to account for different sources of variation during analyses	Expert consensus on optimal data integration strategies and technologies to benchmark
	Limited spatial transcriptomic data compared with single-cell and single-nucleus data	Incorporate new technologies that enable spatial transcriptomics to be performed at single-cell resolution	Increase access to newer technologies (lower cost)
	Identification of new cell types and cell states	Establish consistent nomenclature of cell types based on single-cell data and actively update as new datasets become available	Expert consensus on criteria for cell nomenclature across organ systems
Integrate current and emerging single-cell technologies	scRNA-seq and snRNA-seq are being integrated to generate HLiCA v1.0	Share data and analysis pipelines developed and adapted to analyse data for HLiCA v1.0 and continue to develop approaches to integrate new single-cell modalities	Evaluate existing integration methods in the context of human liver data to identify those that work well to integrate across modalities, including spatial, ATAC-seq and metabolomics
Map cell perturbations in disease to help translate findings from single-cell maps to new medical therapies	Independent single-cell analyses that vary by disease aetiology, model and/or species, patient diversity, collection of metadata	Increase sample size of single-cell datasets with standardized metadata to determine if findings are disease-specific or due to an unrelated covariate	Increase global access to technologies and disseminate recommendations on standardized metadata collection
		Integrate findings across single-cell projects into disease models for therapeutic testing and future clinical trials	Disseminate open-access liver atlas data to enable integration into independent basic and translational research studies
		Perform cross-species single-cell comparisons to help understand outcomes of mechanistic studies in model systems of disease	
Wide use of the HLiCA	First human liver atlas in preparation	Share integrated atlas of normal and diseased liver widely available via multiple online platforms	Complete HLiCA version 1.0 and disseminate via open-access platforms
Diverse analyses applied to generate new hypotheses and discoveries	Initial analysis to identify new cell types and states, as well as covariate effects, such as age and genetic ancestry	Engage a wide community to share data analysis effort, including understanding how different types of cells work together to carry out liver functions, what pathways are involved in liver regeneration, and how the liver develops from fetal to adult stage	Increase access and member diversity of the HLiCA
Sample diversity			
Broad sample diversity	The data currently available are often enriched in adult donors of northern European ancestry	Expand diversity of collection sites and investigators involved in single-cell analysis	Increase access and member diversity of the HLiCA
	Adult datasets are currently larger than paediatric datasets	Overcome barriers of sample acquisition from children to improve collection over the lifespan	Expert panel to establish age-specific ethical guidelines
Metadata			
Metadata linked to single-cell data from healthy and diseased liver	Limited metadata are being included in more datasets while balancing what can be included in open-access platforms; protected access platform available to store sensitive metadata; basic (tier 1) and more detailed (tier 2) metadata are under development by HCA and HLiCA	Establish guidelines for the types of metadata that should be collected	Global expert panel to establish and disseminate recommendations for metadata collection and data sharing, and ethical guidelines
		Establish guidelines for metadata that can be linked to sequence data in open-access platforms and those that should have restricted access	
		Establish platforms to share metadata linked to single-cell sequencing data that can be released as open access or restricted access	

ATAC-seq, assay for transposase-accessible chromatin using sequencing; HCA, Human Cell Atlas; HLiCA, Human Liver Cell Atlas; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single-nucleus RNA sequencing.

have been identified in human studies and are linked to disease[31,32]. Ancestry and geography also influence susceptibility to specific liver diseases as shown by the higher incidence of biliary atresia in individuals of East Asian descent (1 in 5,000–10,000 live births) than in those from Europe and North America (1 in 15,000–20,000 live births)[33]. The role of ancestral epigenetic imprinting of specific hepatic cell populations and its contribution to inter-patient heterogeneity remains poorly defined[34].

Various dynamic factors also affect the hepatic transcriptome. Changes in liver function and gene expression occur in response to circadian rhythms and gut-derived and diet-derived factors[35]. For example, bile acid synthesis exhibits a diurnal rhythm in humans[36], and

in vitro cultures of human hepatocytes can develop circadian cycles that impact drug toxicity[37]. Although the populations of some immune cell types, such as T cells and neutrophils, are present in low number in homeostasis, their spatially driven transcriptional polarization is important to prime an effector response in disease[38].

Each of these factors, and others, are likely to influence measurement of single-cell and spatial transcriptomes. It will be important to collect information about these factors with each sample to better understand the effects of these covariates on liver cellular function. Initial data collection will enable us to evaluate readily collected variables, including age, sex, genetic ancestry and anatomical sampling location in HLiCA version 1.0, whereas other variables will need to be addressed with expanded data collection strategies. The HCA effort is working to standardize metadata formats and naming conventions to ease collection and sharing of this information (Fig. 3). For example, basic (tier 1) metadata are defined across all HCA projects, and more detailed (tier 2) metadata are under development by the HCA, along with those specialized for liver studies by the HLiCA.

Paediatric and developing liver

The liver changes greatly over the course of development. The liver is the site of haematopoiesis during fetal development, and it then progressively matures from infancy through adolescence to take on adult functions[39]. Single-cell analysis of fetal liver has highlighted stage-specific transcriptional differences that occur during human liver development in utero[40]. Although the magnitude of these changes decreases after birth, an infant's liver remains less mature in terms of metabolism and detoxification than the liver in older children and adults[41,42]. For example, jaundice commonly affects newborns, as their liver does not have the same capacity to process bilirubin as that in older children and adults[43]. A single-cell map derived from liver samples from healthy donors aged 2–17 years showed differences in myeloid

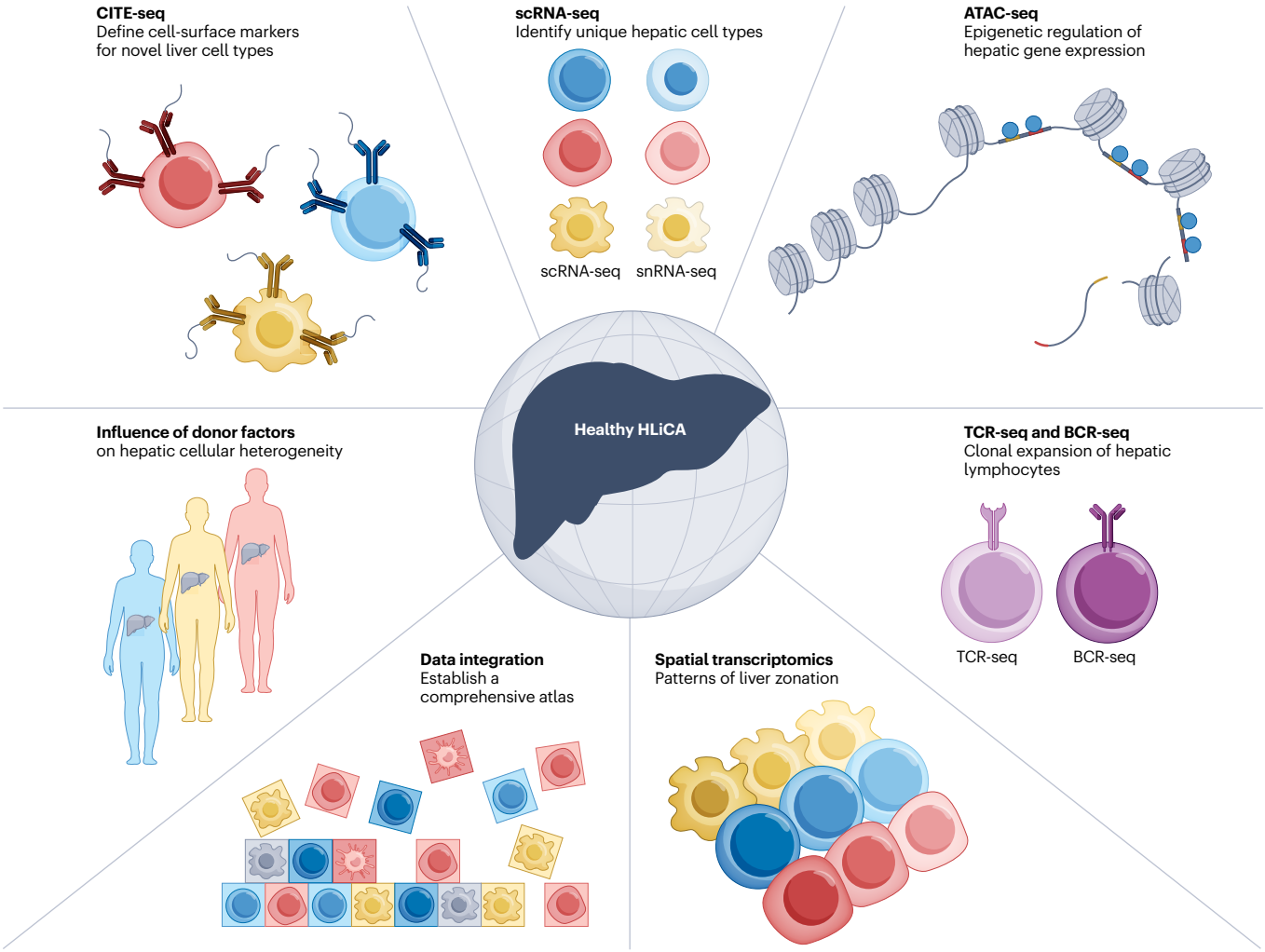


Fig. 2 | Methods for single-cell analyses to build an atlas for healthy human liver cells. The Human Liver Cell Atlas (HLiCA) for healthy liver will include data from a range of experimental types, measuring multiple types of molecular information, including transcript (single-cell RNA sequencing (scRNA-seq) and single-nucleus RNA sequencing (snRNA-seq)) and protein (cellular indexing of transcriptomes and epitomes by sequencing (CITE-seq)) expression levels, chromatin state (assay for transposase-accessible chromatin using sequencing (ATAC-seq), and immune cell activity (T cell receptor sequencing (TCR-seq) and B cell receptor sequencing (BCR-seq) of dissociated cells and cells in situ (for example, spatial transcriptomics). Computational tools will be applied and developed for integration of all data into a comprehensive human liver cell atlas.

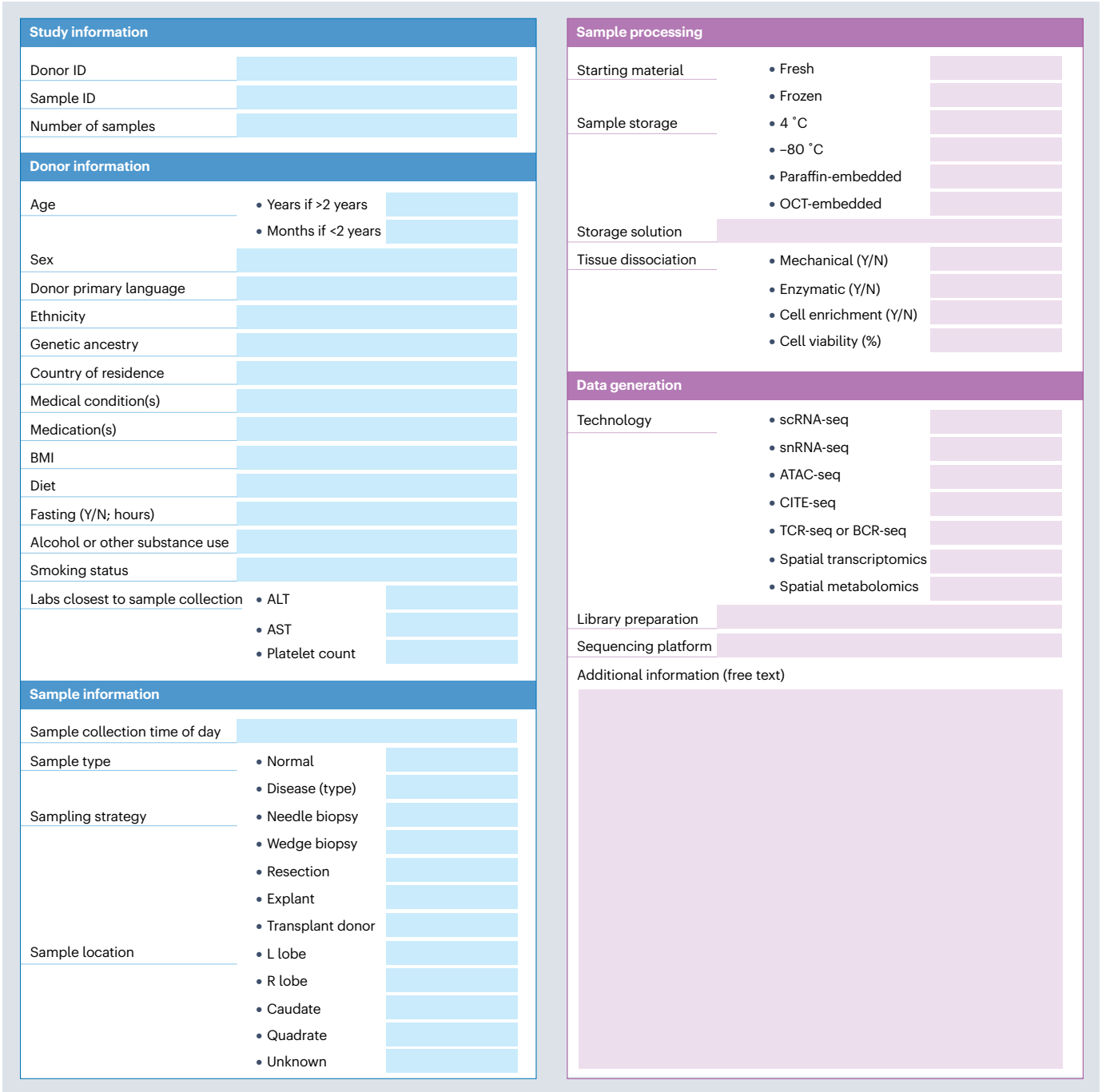


Fig. 3 | Proposed metadata to generate a comprehensive liver cell atlas. Our team will follow Human Cell Atlas recommendations for collection of metadata to capture donor diversity. Based on the study design and age of the donor population, additional specific fields on patient medical conditions and comorbidities, and laboratory data may be added. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATAC-seq, assay for transposase-accessible chromatin using sequencing; BCR-seq, B cell receptor sequencing; CITE-seq, cellular indexing of transcriptomes and epitomes by sequencing; L, left; N, no; OCT, optimal cutting temperature compound; R, right; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single-nucleus RNA sequencing; TCR-seq, T cell receptor sequencing; Y, yes.

populations compared with liver samples from adults[44]. Spatial gene expression patterns also vary with age as demonstrated by the higher incidence of portal steatosis and fibrosis in children with MASLD than in adults, who tend to present with pericentral and pericellular localized disease[45,46]. Furthermore, environmental exposures, such as diet, differ between age groups. In infants, breastfeeding modulates the gut–liver

axis and might protect against future MASLD[47,48]. Nutritional diversity, including geographically distinct diets, affects liver metabolic and immune cell programming, as described in MASLD[31,49,50]. Lastly, certain liver diseases, such as biliary atresia, are more prevalent in children than in adults[51], whereas common disease processes might exhibit age-specific phenotypic differences. In particular, recent research has helped to characterize the single-cell transcriptome in paediatric cholestatic liver disease[52,53]. Including fetal and paediatric samples in the liver map will help us to better understand the cell-type-specific differences between children and adults, identify pathways and chromatin accessibilities critical for liver regeneration, and provide a healthy liver reference for paediatric liver disease studies.

Mapping liver cell types

Liver zonation is responsible for much of the spatial heterogeneity within the lobule[13] and has been extensively studied in mice, using spatially resolved scRNA-seq technologies[14,46]. Hepatocytes, the primary liver parenchymal cell type, exhibit strongly zonated gene expression patterns[14,46]. Venous and arterial blood mix as they enter the liver, resulting in greater oxygen availability in periportal regions than in pericentral regions[54,55]. Energy-demanding tasks, such as gluconeogenesis, lipid metabolism and protein secretion, are allocated to the relatively oxygen-rich periportal regions where hepatocytes can obtain more ATP through respiration, whereas pericentral hepatocytes specialize in xenobiotic metabolism, glycolysis and glutamine synthesis[54]. Some biological pathways exhibit 'production-line patterns' whereby enzymes in a metabolic cascade are expressed in sequential lobule layers[56]. Hepatocytes also allow recycling of metabolites between distinct zones. For example, glutamate and glucose are produced in periportal layers and reused in pericentral layers[57]. In addition to gradients in oxygen and nutrients, approximately 75% of afferent lobule blood flow is venous, with only 25% perfusion from highly oxygenated arterial blood, imposing limits in oxygen-dependent hepatocyte functions[46].

Extrinsic signals influencing hepatocyte zonation include blood-borne molecules such as oxygen[56] and glucagon[58], as well as morphogens produced by non-parenchymal cells. About one-third of zonated hepatocyte genes are regulated by canonical Wnt signalling[56]. Broad zonation programmes specifically define endothelial cell[59] and hepatic stellate cells (HSC) subtypes[60]. Studies in mice have shown that endothelial cells and HSCs residing around the central vein constitute a localized WNT signalling niche, and pericentral endothelial cells produce WNT2, WNT9B and RSPO3 to jointly shape hepatocyte zonation[61–63].

Liver-resident macrophages are the dominant immune cell type present in homeostasis, and are another non-parenchymal cell type influenced by zonation. Kupffer cells (KCs) constitute a major component of this macrophage population that is exposed to distinct levels of oxygen, hormones and morphogens based on their location within the hepatic lobule. KCs are present in high numbers in periportal lobule layers, presumably to provide a first line of defence against bacteria that have infiltrated from the gut, as this preferential localization is lost in germ-free animals[64]. Moreover, KC identity is tightly regulated by signals, including DLL4–Notch and BMP9–ALK1 signalling[65,66], provided by other liver cells in close proximity, namely hepatocytes, sinusoidal endothelial cells and HSCs[65–67]. Disruption of these signals is thought to result in a loss of KCs in diseased states such as MASLD and, in mouse models, in the recruitment of other non-KC macrophage populations; however, the precise function of KCs remains unclear[68,69]. Gaining insights into the transcriptional programmes of healthy KCs and the cell–cell communication axes governing these cells will enable a better understanding of alterations in disease.

Although patterns of liver zonation have been identified in mouse models, especially for hepatocytes, such patterns remain to be resolved in the healthy human liver. Moreover, less is known about how macroscopic differences in liver anatomy affect cell-subset-specific transcriptional programmes. In particular, portal blood flow is critical in hepatic clearance of gut-derived toxins and microbiota that have entered the systemic circulation[70]. However, the distribution of portal blood flow is not uniform across the liver and might differentially influence hepatic, immune and parenchymal cell transcriptional programmes at homeostasis. Through adequate tissue sampling across the liver, the HLiCA effort will better define the effects of anatomical differences at the single-cell level.

Liver regeneration: from repair to disease

Insights into the plasticity of liver cell types will advance our understanding of liver injury, repair, regeneration and disease. Single-cell genomic approaches have markedly expanded our understanding of how intrahepatic cell lineages interact to regulate these processes. Studies of human liver regeneration have identified mesenchymal cells and a novel migratory hepatocyte subpopulation critical in mediating successful regeneration and reconstitution of normal hepatic architecture following liver injury[71,72]. In addition, plasticity between hepatocytes and cholangiocytes has been observed in MASLD in snRNA-seq studies in humans, which seems to be independent of bipotent progenitors[73].

Fibrosis develops as a result of chronic liver injury when liver regeneration fails to restore normal liver morphology. Single-cell approaches in humans and mouse models have defined distinct populations of macrophages, endothelial cells and mesenchymal cells that reside within the spatially distinct fibrotic niche and interact to promote scar formation[16,60]. For example, a population of monocyte-derived macrophages is present during early stages of MASLD and expands during fibrosis progression to promote mesenchymal cell activation and extracellular matrix (ECM) deposition[16]. Expansion of distinct endothelial and mesenchymal cell populations also occurs within the human fibrotic niche during repair, with subsequent interactome remodelling of ligand–receptor pairs occurring between subpopulations of macrophages, endothelial cells and mesenchymal cells[74]. Single-cell studies have further demonstrated impaired macrophage differentiation during late stages of fibrosis[75].

Aberrations in regenerative pathways are also linked to cancer and have been further elucidated by single-cell studies. Changes observed in hepatocellular carcinoma (HCC) result in an immunosuppressive ecosystem that shares features with the fetal liver[18]. Non-parenchymal cells involved in the injury response also influence the development of cancer, as hepatocyte growth factor produced by subpopulations of HSCs can inhibit the development of HCC in mouse models of hepatocarcinogenesis[76], and the desmoplastic reaction characteristic of intrahepatic cholangiocarcinoma produces distinct populations of cancer-associated fibroblasts that further support the tumour environment[77–79].

The features of injury and disease that are common across organs are not yet fully understood but will become clearer as more single-cell datasets are generated across healthy and injured organs. Many immune cell types share transcriptional features across human organs, such as $CD9^{+}TREM2^{+}$ macrophages associated with the fibrotic niche in both the liver and the lung[80]. Immune cell populations with shared signatures have also been identified across organs with disease. For

example, in the setting of HCC, LAMP3$^+$ dendritic cells are present in tumour and draining lymph nodes as well as in breast and lung cancer, and tumour-associated macrophages identified in HCC share gene expression signatures with those in lung cancer[81]. Additionally, populations of human fibroblasts have been defined that emerge across multiple organs with injury[82]. Although transcriptional signatures are also shared by fibroblasts across healthy organs[82], murine studies that include the liver have also highlighted the fibroblast heterogeneity driven by ECM gene expression[83]. Ultimately, expanding single-cell maps unified across tissues will continue to improve our understanding of the spectrum of cell states as well as the shared and unique features of injury and repair responses in the liver.

Liver-specific challenges in single-cell atlas assembly

Wet laboratory challenges

A major experimental challenge in generating the HLiCA is tissue access, as the invasive nature of liver biopsy and surgical resection limits tissue availability for research. As a result, multiple approaches have been applied to collect tissue samples, including excess healthy donor tissue collected at the time of liver transplantation (Fig. 1), excess healthy tissue adjacent to liver lesions removed during surgical resection, core-needle liver biopsies, fine-needle aspirates[84] and, occasionally, whole organs from deceased individuals. Not all sample types are comprehensive, highlighting the need for complementary tissue sampling strategies. For example, fine-needle aspiration can efficiently capture most immune cells, but hepatocytes, KCs and mesenchymal cells are under-represented in samples obtained by this technique[84].

Distinct hepatic cell populations possess varying sensitivity to cell stress caused by tissue dissociation, and a combination of single-cell experimental strategies is required for a robust atlas representing the greatest diversity of hepatic cell types. For example, hepatocytes are very sensitive to dissociation-related damage[15,17], and mesenchymal cells are particularly difficult to dissociate from tissues[67]. snRNA-seq minimizes dissociation bias and captures a more accurate snapshot of the frequency of certain cell types in tissues[67,85]. Side-by-side comparisons of hepatic scRNA-seq and snRNA-seq studies have shown that snRNA-seq leads to better capture of hepatocytes, cholangiocytes and mesenchymal cells[19,67]. By contrast, enzymatic digestion followed by scRNA-seq yields an enrichment of immune cells[19,67], and using the seqWell platform captures neutrophils, whereas droplet-based platforms such as Chromium (10× Genomics) do not[84]. A main advantage of scRNA-seq over snRNA-seq is that it can be combined with cellular indexing of transcriptomes and epitomes by sequencing (CITE-seq) to simultaneously detect hundreds of cell-surface proteins using barcoded antibodies that might not be well represented at the RNA level and are well-established for identification of immune cell subsets[86,87]. In addition to differences in the abundance of cell populations captured by scRNA-seq versus snRNA-seq, these methods also lead to enrichment of different genes[67].

Spatial transcriptomics bypasses the need for enzymatic dissociation while also mapping cell localizations within tissues, leading to less biased cellular representation[18,19,67]. CITE-seq-barcoded antibodies can also be used on tissue sections to identify cells by surface protein expression and to define suitable antibodies for histologic studies[67]. However, there is currently a trade-off among the multiple spatial transcriptomic technologies between transcriptome coverage and spatial resolution, and the number of tissue slices that can be analysed at once[88].

To help overcome these challenges, the HLiCA coordinates the activity across multiple sites to increase the number of samples collected using complementary methods. Many factors contribute to differences in sample processing protocols worldwide, such as different clinical procedures used to collect data. Although these factors prevent uniform sample processing across sites, common guidelines for timely sharing of data and sample processing details help in enabling the development of improved computational approaches to address batch effects during data analysis.

Computational challenges

To comprehensively define the distribution of normal liver gene expression across diverse human populations, it is necessary to integrate large datasets across multiple institutions. Computational integration of single-cell datasets remains a challenge despite the large number of algorithms available[89]. The experimental challenges discussed above introduce systematic differences in amplification rates and cell type frequencies that can confound data scaling and normalization. Ambient RNA released during tissue handling is also captured non-uniformly across individual cells in a sample, thereby violating the assumptions underlying most integration algorithms[90]. Although current state-of-the-art tools enable integration of datasets in reduced dimensions, accounting for different sources of variation during differential analyses remains an open challenge[91,92]. Integration of single-cell and spatial transcriptomic technologies promises to enable comprehensive identification of cells and their transcriptomic signatures.

Inferring accurate cell–cell interactions in primary liver tissue from single-cell transcriptomics will enable the study of how liver cells work together to coordinate processes such as regeneration, and how these processes fail in disease. However, these methods only measure mRNA, whereas cell–cell communication relies on protein–protein interactions, and RNA abundance does not always correlate with protein abundance[93]. Integration of scRNA-seq and spatial transcriptomic data with the addition of protein expression data (for example, CITE-seq) can provide a starting point for more accurate cell–cell communication inferences[67]. However, this approach can result in reduced sensitivity for detecting both transcriptome and protein levels, compared with independent measurements. Alternatively, integration followed by imputation might be used to infer protein-level information from complementary protein measurements from, for example, multiplexed imaging technologies or cytometry by time of flight[94–97]. Despite these challenges, integration of single-cell data with spatial transcriptomics and protein expression will better enable validation and interpretation of scRNA-seq findings. Finally, integration of data from multiple modalities to build a dynamic, 3D view of the liver at molecular resolution will require newer machine learning methods[98].

Data interpretation challenges

Although some of the interpretation challenges are common across organs, others are unique to liver single-cell data. The classic view of cell types has been one of strict, fixed classes with distinct functions. However, both scRNA-seq and stem cell research have revolutionized this view by demonstrating greater continuity and plasticity between cell types, and more overlap in cell type-specific functions than previously known[73,99–101]. This shift in understanding has been especially pronounced in the liver, as a large portion of functional specialization is driven by smooth spatial gradients across liver lobules[102]. Interpreting more subtle changes related to cell plasticity and spatial gradients must

be disentangled from non-biological factors including ambient RNA contamination and the presence of doublets. Doublets can also give rise to false clusters that can be misinterpreted as novel cell types[103], and about one-third of hepatocytes are multinucleated[104], which could affect interpretation of snRNA-seq in which each nucleus is considered a single cell. Ambient RNA and doublets are particularly difficult factors to control for and distinguish from true biological signals, owing to their prevalence. Careful data interpretation is therefore critical to accurately identify new cell types and spatially distinct transcriptional patterns.

Historically, cells have been characterized by their location, function and structure based on expression of canonical cell-specific proteins. Single-cell technologies have increased our understanding of cellular heterogeneity, but these new technologies have also introduced new challenges associated with data interpretation and accurate cell annotation. There is a lack of uniform standards to define new cell types, and there is no generally accepted approach to choose a clustering resolution or validate computationally identified clusters[105]. Although identification of differentially expressed genes is the most common approach, current single-cell analysis algorithms may introduce biases and prevent the definition of a rigorous standard[106]. Alternative approaches have sought confirmation of cell type identity using spatial transcriptomics or proteomics that enables in situ visualization of transcriptional signatures to confirm cell identity independently of potential processing artefacts. Epigenetic changes, such as DNA methylation, are typically more stable than transcriptomic shifts[107], whereas proteomic information is more directly relevant to cellular function than mRNA expression[108]. Other potential complementary methods to confirm novel cellular functions include secretome and exosome analyses, and metabolic modelling. Innovative computational approaches will be necessary to integrate these diverse data types with current and future human cell atlases.

Once a group of cells has been determined to represent a cell type or cell state, it is critical to establish rigorous and unambiguous naming of that cell type or cell state. Frequently, different researchers will annotate their data using different names for the same cell type (for example, liver monocyte-derived macrophages versus pro-inflammatory macrophages) or use different sets of marker genes to define the same cell type. Centralized databases such as CELL×GENE, Cell Annotation Platform or CellMarker[109], combined with standardized terminology such as Cell Ontology[110], can help to address these issues, although these approaches will need to be extended to capture the complete multidimensional and dynamic nature of cell function. Historically, however, such nomenclature challenges have been addressed by international scientific meetings and large-scale data integration. We anticipate that, as the amount of human liver and human cell atlas data grows, collaborative efforts within and across organ systems will help to reach a consensus to establish new cell-subset-specific nomenclature for future studies.

Lastly, a major challenge for all tissue application studies is to identify information specific to the studied context unrelated to covariates, such as age, sex or ancestry. Liver samples are diverse among individuals, especially in relation to hepatocyte populations[15], which emphasizes the value of a large, diverse, comprehensive healthy reference atlas to help to reduce false-positive and false-negative results when comparing disease samples with healthy control samples. Furthermore, a temporal healthy liver atlas that includes fetal, neonatal, paediatric and adult liver will need to be useful to the widest range of individuals and diseases.

Influence and outlook of the HLiCA

Clinical and research influences

Establishing the HLiCA is essential to identify perturbations from homeostasis and to define disease-specific cell states that might serve as therapeutic targets. To test the utility of this approach, HLiCA will include a disease map integrated with the healthy map, but it will be left open to the community to continue this effort as more data from human liver diseases become available. We expect that over time, this effort will help to address many unanswered questions on disease pathogenesis. For example, there are no effective anti-fibrotic therapies to reverse cirrhosis, many major liver diseases have no targeted therapy, and many rare liver diseases have no identified mechanism, leaving liver transplantation as the only option for many patients with end-stage liver disease. Unravelling how hepatocytes and non-parenchymal cells work together at the molecular, cellular and tissue levels in health and to promote liver regeneration will help to identify treatments for chronic liver diseases and liver failure as alternatives to liver transplantation (Fig. 4).

Single-cell studies of liver disease have identified diverse dysregulated transcriptomic signatures. For example, HSCs expressing myofibroblast markers and matrix remodelling factors are present in MASLD and in fibrosis and cirrhosis of other aetiologies, whereas HSCs in healthy liver or non-fibrotic liver injury have greater expression of genes encoding cytokines and growth factors[76,111]. Distinct immune and mesenchymal cell subsets are associated with specific disease states such as MASLD[112], primary sclerosing cholangitis[113], biliary atresia[114], intrahepatic cholangiocarcinoma[115] and hepatoblastoma[116]. Data obtained from the HLiCA could help to explain some of the most fundamental questions in the pathogenesis of liver diseases, including how hepatitis B evades immune surveillance[38], how alcohol causes cholestasis in alcohol-induced hepatitis, what are the drivers of autoimmunity in autoimmune hepatitis and primary biliary cholangitis, and what is the precise mechanism of biliary atresia. Findings from single-cell studies could lead to new cell-subset-specific therapeutics to prevent or slow disease progression or reduce the burden of complications from end-stage liver disease.

A reference atlas of the healthy liver will also establish a basis for assessing the physiological relevance of human liver disease models. Cross-species comparisons using the HLiCA will be critical to identify transcriptional explanations for the failure of novel therapeutic agents developed using animal models. For example, human fetal hepatocytes demonstrate greater heterogeneity in the expression of genes involved in metabolism than mouse fetal hepatocytes[117]. Furthermore, lipogenesis genes are enriched in periportal hepatocytes in adult mice but in pericentral hepatocytes in humans[118], a difference that could explain species-specific patterns of lipid accumulation, and might influence translation of findings from mouse models to human disease[119,120]. The liver atlas will also provide an excellent benchmark for refining organoids and other engineered human tissues. Many similarities and differences exist in single-cell transcriptomes comparing human liver organoids derived from pluripotent stem cells, patient-derived xenograft mouse models, and cells directly isolated from liver tissue[17]. A robust healthy liver cell atlas will help to improve tissue engineering strategies to more closely model human disease, provide the basis for preclinical therapeutic studies, and ultimately provide engineered liver tissue for whole-organ transplantation.

Future directions

Optimization of current techniques as well as development of new assays will continue to increase access to single-cell technologies and

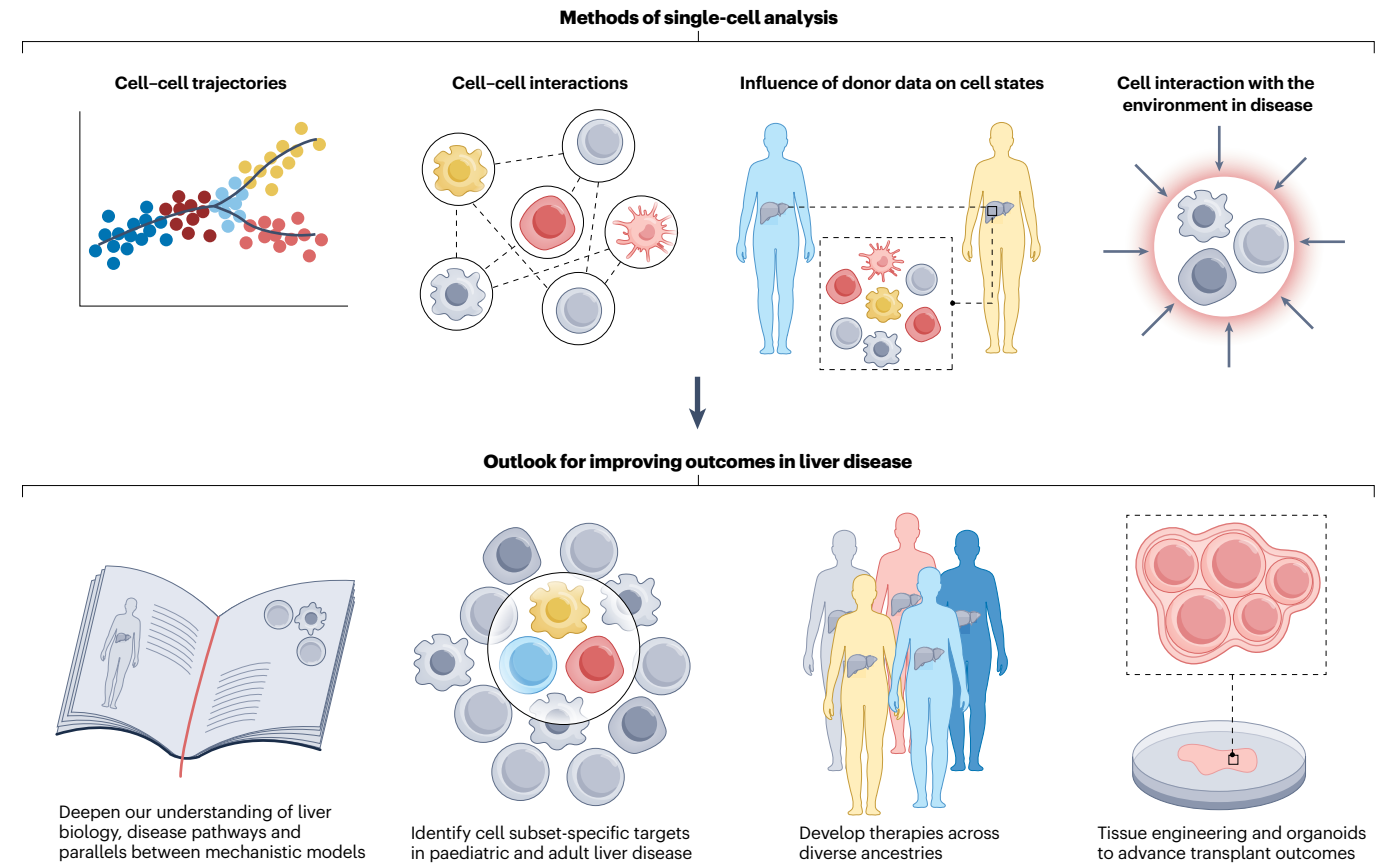


Fig. 4 | Single-cell technology can help to improve outcomes in human liver disease. Incorporating diverse approaches to the analysis of single-cell data and integrating these data with information about donors and disease states (top) will hopefully lead to new understanding of biology and pathology, identify cell types most responsible for disease, determine how cell types are influenced by ancestry and help to direct targeted therapies for individual patients (bottom). Analysis of single-cell data from healthy individuals might also help to guide more efficient approaches in engineering liver tissue for therapeutic purposes.

lead to even greater sample and data acquisition to build the globally diverse HLiCA (Table 1). Although disease states might increase wet laboratory challenges in achieving uniform cell isolation, we expect that ongoing improvements in technologies, acquisition of large numbers of samples and data integration across multiple modalities will help to overcome the limitations currently faced when analysing diseased tissue. We further anticipate that future integration of transcriptomics into the multiomics data framework, including genetics, epigenetics, metabolomics, lipidomics and proteomics will deepen our understanding of the complex networks governing the physiology of healthy liver and diseased liver (Fig. 4). For example, spatial assay for transposase-accessible chromatin using sequencing (ATAC-seq) and spatial metabolomic technologies have recently emerged[121,122] but still face technical limitations, including how to perform multiple analyses on the same tissue slice or cells. Although the evaluation of these datasets in parallel with spatial transcriptomics and scRNA-seq is currently limited, the integration of large datasets across these modalities will lead to maps that incorporate the metabolic and proteomic networks that contribute to cell identity and cell fate at the single-cell level. Ongoing efforts are also needed to evaluate the ability of deep-learning-based 3D reconstruction algorithms to integrate current and future data into single-cell maps[123,124].

State-of-the-art machine learning techniques, such as deep neural networks, will also facilitate cell type annotation of disease datasets and identify cell-subset-specific transitional states between normal and disease states[125–127]. Through acquisition of this knowledge, single-cell and multiomics technologies will help to drive precision medicine to predict patients' responses to targeted therapies across liver diseases of different aetiologies. For example, understanding the immune–tumour cellular landscape in liver cancer can help to predict chemotherapy sensitivity, an effort already underway in the treatment of paediatric hepatoblastoma[128].

The primary focus of the HLiCA version 1.0 is to confidently identify as many cell types and cell states as possible across the human lifespan and diverse ancestry, define gene expression signatures that separate these cell types and states, and present these data on an open-access platform for interactive visualization and analysis (including the HCA data portal and CELL×GENE)[129]. HLiCA is not meant to be a static reference, but a consensus from which these metrics can continue to evolve. Over time, we also hope to develop standardized guidelines and recommended wet bench protocols for tissue preparation and isolation tailored to specific questions in the liver (for example, scRNA-seq rather than snRNA-seq for immune cell profiling). Our goal is also to share computational approaches developed and refined for

human liver data integration to create the HLiCA version 1.0 and continue to apply computational advances to improve both integration of new datasets and further refine the data incorporated into HLiCA.

Conclusions

In summary, we review the status of the HLiCA and describe the ongoing impact that this comprehensive liver cell atlas will have on deepening our understanding of healthy human liver heterogeneity across the human lifespan. We highlight specific challenges and propose solutions including improvements in the standardization of metadata collection, expanding sample collection across geographically diverse sites, and optimizing data integration techniques to limit the impact of technical variables. Accomplishing these goals for the healthy liver will enable researchers to more precisely define perturbations in disease states and ultimately lessen the burden of liver disease through improved preventative and treatment strategies.

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Competing interests

S. A. Taylor serves as a consultant for Ipsen Pharmaceutical. A.C.M. receives funding from GSK and Boehringer Ingelheim for unrelated projects. A.J.G. receives research funding from Aligos Therapeutics, Bluejay Therapeutics, GSK, Roche and Vir Biotechnology, and performs scientific advisory services for Aligos Therapeutics, Arbutus Biopharma, Assembly Biosciences, Bluejay Therapeutics, Gilead Sciences, GSK, Janssen Pharmaceuticals, Roche, Vir Biotechnology, Virion Therapeutics and VBI for unrelated projects. D.G. serves as a consultant for Gordian Biotechnology. M.G. receives funding from the Sanofi iTech Award programme for an unrelated project. N.C.H. has received research funding from AbbVie, Pfizer, Gilead and Boehringer-Ingelheim, and is an adviser or consultant for Astra-Zeneca, GSK and MSD. S.S.H serves as a consultant for ARNATAR Therapeutics. Z.G.J. serves on the advisory board of Olix Pharmaceuticals, and received grants from Pfizer and Gilead Sciences. A. Ricciuto receives a stipend for academic lectures from Janssen and Organon. C.L.S. receives funding from Novo Nordisk for unrelated projects. M.M.T has served as a consultant for Merck for an unrelated project. L.V. is a shareholder of Definigen, BiliTech and Bit.bio. I.S.V. consults for Guidepoint Global, Cowen and Mosaic. B.W. serves as a consultant for Alnylam Pharmaceuticals, Disc Medicine, Mitsubishi Tanabe Pharma and Recordati Rare Diseases. G.D.B. advises Adela Bio and BioRender. The other authors declare no competing interests.

Additional information

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