Synthetic biomarkers: a twenty-first century path to early cancer detection

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Abstract | Detection of cancer at an early stage when it is still localized improves patient response to medical interventions for most cancer types. The success of screening tools such as cervical cytology to reduce mortality has spurred significant interest in new methods for early detection (for example, using non-invasive blood-based or biofluid-based biomarkers). Yet biomarkers shed from early lesions are limited by fundamental biological and mass transport barriers — such as short circulation times and blood dilution — that limit early detection. To address this issue, synthetic biomarkers are being developed. These represent an emerging class of diagnostics that deploy bioengineered sensors inside the body to query early-stage tumours and amplify disease signals to levels that could potentially exceed those of shed biomarkers. These strategies leverage design principles and advances from chemistry, synthetic biology and cell engineering. In this Review, we discuss the rationale for development of biofluid-based synthetic biomarkers. We examine how these strategies harness dysregulated features of tumours to amplify detection signals, use tumour-selective activation to increase specificity and leverage natural processing of bodily fluids (for example, blood, urine and proximal fluids) for easy detection. Finally, we highlight the challenges that exist for preclinical development and clinical translation of synthetic biomarker diagnostics.

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https://doi.org/10.1038/s41568-021-00389-3

Dedication: This article is dedicated to the late Dr Sanjiv Sam Gambhir, a visionary pioneer and thought leader in bioengineering who devoted his career to developing methods for early disease detection.

The earliest stages of cancer detection are when our existing clinical interventions can be more successful. Detecting pre-invasive tumours before clinical symptoms appear is likely to enhance the effect of medical interventions such as surgical resection, which can be curative for most types of localized cancers that have not metastasized[1]. When accurate tests are available, risk-based cancer screening of populations is recommended by regulatory agencies, and contributes to lowering cancer deaths. Examples include mammography for breast cancer, colonoscopy for colorectal cancer, Papanicolaou test (Pap smear) for cervical cancer and low-dose chest computed tomography for those at high risk of lung cancer[2–7]. However, accurate tests based on imaging and/or non-invasive analysis of patient fluids such as blood are not available for the vast majority of cancer types, and the diagnostic specificity of current tests is insufficient to allow routine screening of asymptomatic segments of the population where the cancer prevalence is low. A test with low positive predictive value would lead to an unacceptably high percentage of false positives and unnecessary medical interventions, precluding broad deployment. The continuing debate over whether the only widely used blood biomarker test, the prostate-specific antigen (PSA) test, is useful for reducing prostate cancer mortality despite its drawbacks (such as unnecessary treatments, patient morbidity and costs) serves as an important lesson for future tests[8].

There are several ongoing efforts towards detecting other endogenous biomarkers (for example, cell-free nucleic acids, proteins, lipids and metabolites) via analysis of blood and other biofluids[9–17]. Significant strides have been made with sequencing of cancer genes from circulating tumour DNA (ctDNA), as evidenced by the recent success of a multianalyte, multicancer test in a prospective study of women without a history of cancer in which the feasibility of using a blood test to detect multiple cancers was established[9,14]. However, biological and technical challenges remain obstacles to the early detection of cancer, especially before symptoms are apparent; a test with high sensitivity would be required to detect very low signal levels, but such a test must not contribute substantially to the overdiagnosis of inconsequential cancers. The expression or release of biomarkers is variable and compounded by interpatient

Multicompartment models
A mathematical modelling technique whereby distinct compartments are used to represent organs, tissues, blood or lymph to predict how an administered drug is absorbed, distributed, metabolized or excreted.

variation, tumour heterogeneity, comorbidities and background secretion by healthy cells. Moreover, individual biomarkers often lack specificity because their levels can be elevated in non-cancerous conditions, as in the case of DNA mutations from non-malignant clonal haematopoiesis of indeterminate potential (CHIP)[18] or PSA level increase from benign prostatic hyperplasia[19], or they are shed across many types of cancer, as is the case for carcinoembryonic antigen (CEA), the level of which is elevated in cancers of the colon, breast, lung and other organs[20]. This necessitates identifying multianalyte panels that combine different classes of biomarkers into a single predictive score to assess the presence of disease and localize the cancer to anatomical sites[9,14,21].

These lessons learned are informing the design of an emerging class of diagnostics based on the design of bioengineered sensors — such as molecular probes or genetically encoded vectors — that exploit dysregulated features of early-stage tumours or their precursors, which have the potential to become lethal, to produce an amplified signal that cancer cells would otherwise not produce or would produce at undetectable levels. These exogenously administered sensors harness tumour-dependent activation mechanisms such as enzymatic amplification to drive the production and amplification of synthetic biomarkers. Cancers can also be detected by imaging systems that may share essential features of a synthetic biomarker approach, such as reporter gene imaging, whereby an exogenous molecular tracer (for example, a positron-emitting probe) is systemically infused[22,23]. Imaging will also play an essential role in detecting the location of the tumour following confirmation of a detectable synthetic biomarker signal. However, as advances in cancer imaging have been extensively reviewed elsewhere[22,24], this Review focuses on synthetic biomarkers detectable from biofluids such as blood and urine. First, we highlight the challenges associated with early cancer detection that have motivated research into synthetic biomarkers. We then review advances in activity-based and genetically encoded sensors, which are the two major strategies being used for synthetic biomarker development. Finally, we discuss the challenges that exist for this growing field in the setting of preclinical studies and strategies for clinical translation.

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The challenge of early detection

The rationale for synthetic biomarker development comes from the biological, physiological and mathematical limitations of endogenous biomarkers (FIG. 1). For continuously shed biomarkers such as proteins, patient tumours are not universally biomarker positive, and secretion rates can vary by as much as four orders of magnitude, even for cells of the same tumour type[25]. Moreover, biomarkers that are released only by dead or dying cells are shed just once, and their detection is confounded by background shedding from healthy tissues. Cell-free DNA (cfDNA), for example, is released from non-cancerous cells throughout the body, which makes the proportion of somatic mutations in malignant cells versus normal cells, or the variant allele frequency (VAF), increasingly difficult to detect at low tumour burdens. Analysis of the Tracking Non-Small-Cell Lung Cancer Evolution Through Therapy (TRACERx) study predicted that primary tumour burdens of 1 cm^3, 10 cm^3 or 100 cm^3 would result in average clonal plasma VAFs of 0.006%, 0.1% or 1.3%, respectively[26]. For a typical 4 ml of plasma from a 10-ml blood draw and a VAF of 0.1%, it has been estimated that there would be an average of just six molecules per tube carrying the respective somatic mutation[27]. Further compounding the technical challenge, shed biomarkers are diluted by a large pool of blood (~5 l) and circulate for short periods owing to degradation or clearance; ctDNA, for example, has a circulation half-life of less than 1.5 h in blood[28,29]. By comparison, the resolution of clinical positron emission tomography (PET)-based molecular imaging (specifically using Jaszczak phantoms and fluorine-18) has been reported[30] as ~200 mm^3, which is equivalent to a tumour diameter of ~7 mm.

Despite the technical challenges associated with detecting shed biomarkers, mathematical model predictions and genomic timeline studies consistently estimate a window of opportunity for early cancer detection that may span at least a decade. Work on multicompartment models[31–34] to understand the relationship between tumour volumes and shed biomarker levels has resulted in predictions that tumours could remain undetectable for more than 10 years following initiation of tumorigenesis[32]. Genomic studies on cancer progression timelines have estimated periods of ~7 years or more from the birth of a founder carcinoma cell to macrometastatic tumours, given the inherent inefficiency of individual tumour cells to seed and survive in distant organs[35–38]. It is important to keep in mind, however, that cancers that are present for a decade or more are likely to be of indolent nature and eventually detectable at some point by existing screening modalities that are not based on biofluids. By contrast, fast-growing and highly aggressive cancers — including interval cancers that are diagnosed during the time between a regular screening that appears normal and the next screening — may rapidly progress within a relatively narrow window of months to years and are associated with poor clinical outcomes. Examples include triple-negative breast

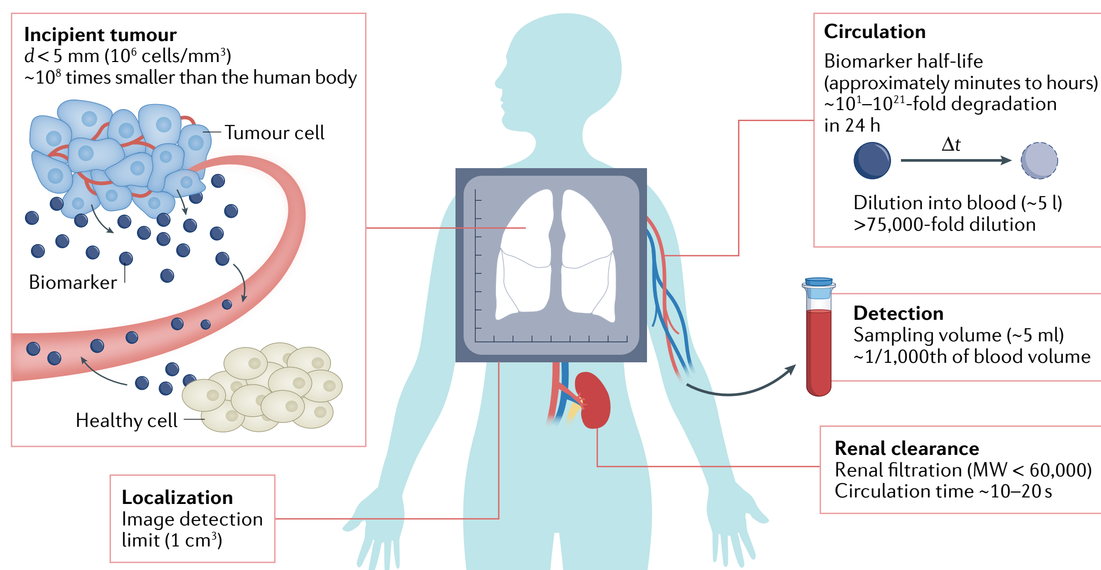

Fig. 1 | **Challenges associated with detecting early-stage tumours.** An early-stage tumour (smaller than 5 mm in diameter) is on average eight orders of magnitude smaller in volume than the human body. Several factors hinder the ability to detect biomarkers shed from tumours, including transport challenges from the tumour microenvironment (TME) into the circulation, an approximately five orders of magnitude-fold dilution into blood and short circulation times owing to degradation and renal filtration. These factors decrease the number of tumour-associated biomarkers (for example, cell-free nucleic acids, proteins, metabolites and circulating tumour cells) that can be found in a typical 5–10 ml blood draw, which represents only ~1/1,000th of the total circulation volume. *d*, diameter; MW, molecular weight; Δ*t*, change in time.

cancer and high-grade serous ovarian carcinoma (HGSOC) in women whose tumours have *BRCA1* or *BRCA2* mutations, or homologous recombination deficiency[39,40]. Detecting such aggressive cancers at an early stage would likely require identification of cancer precursors (such as serous tubal intraepithelial carcinoma for HGSOC) and the development of new ultrasensitive approaches that permit increased frequency of testing. Advances that are occurring in the field of synthetic biomarker research aim to address these challenges, with the main approaches being those that leverage activity-based or genetically encoded mechanisms for early detection.

Activity-based synthetic biomarkers

The systemic administration of exogenous agents to assess biological function in vivo has a long clinical history. Examples include infusion of patients with inulin, which is an inert polysaccharide that is not digestible or absorbed, to measure kidney function[41] and indocyanine green, a fluorescent dye, to quantify liver dysfunction[42]. These biomarkers and other similar tests target known features of human physiology (for example, plasma clearance via hepatocytes) or established disease mechanisms with a biologically inert probe to produce a readout that is not normally found in the body, thereby maximizing the signal-to-noise ratio. Activity-based synthetic biomarkers are based on this paradigm but include sensor components that are activated by enzymes in the tumour or its microenvironment to provide a mechanism for molecular amplification of tumour biomarkers. Here we discuss the key design considerations for activity-based synthetic biomarkers, with a particular focus on protease-activated sensors and small-molecule probes.

Protease-activated synthetic biomarkers. The human genome encodes more than 550 proteases, and their dysregulation has broad implications at the molecular level (for example, protein activation and matrix degradation), cellular level (for example, immune cell cytotoxicity and apoptosis) and systems level (for example, cancer-induced hypercoagulable state) in cancer[43]. For example, matrix metalloproteinases (MMPs) are overexpressed across the vast majority of cancer types[44] as one of their key functions is to regulate the bioavailability of vascular endothelial growth factor (VEGF) during the angiogenic 'switch', a process that occurs when nascent tumours reach 1–2 mm in diameter and require increased access to blood nutrients to overcome diffusion-limited growth[45,46]. Recent studies have also highlighted that dysregulated protease expression can be used for predictive cancer classification, for example, separating prostate cancer into aggressive and indolent phenotypes[47] using machine learning algorithms. Another study showed that protease transcript signatures can differentiate lung adenocarcinoma from interstitial lung disease or chronic obstructive pulmonary disease[48], demonstrating the potential of protease-based classifiers for differential diagnosis.

Protease-activated synthetic biomarkers comprise peptide substrates conjugated to the surface of an inert carrier[25,47–51] that upon enzymatic cleavage by tumour proteases release reporters into the blood or urine for detection (FIG. 2). Proteases are particularly potent molecular amplifiers because hydrolysis of peptide bonds is irreversible and proteases are not consumed during peptidolysis, thereby allowing a single copy to turn over thousands of substrates[52]. In addition to molecular amplification, another key strategy to attain the limit of

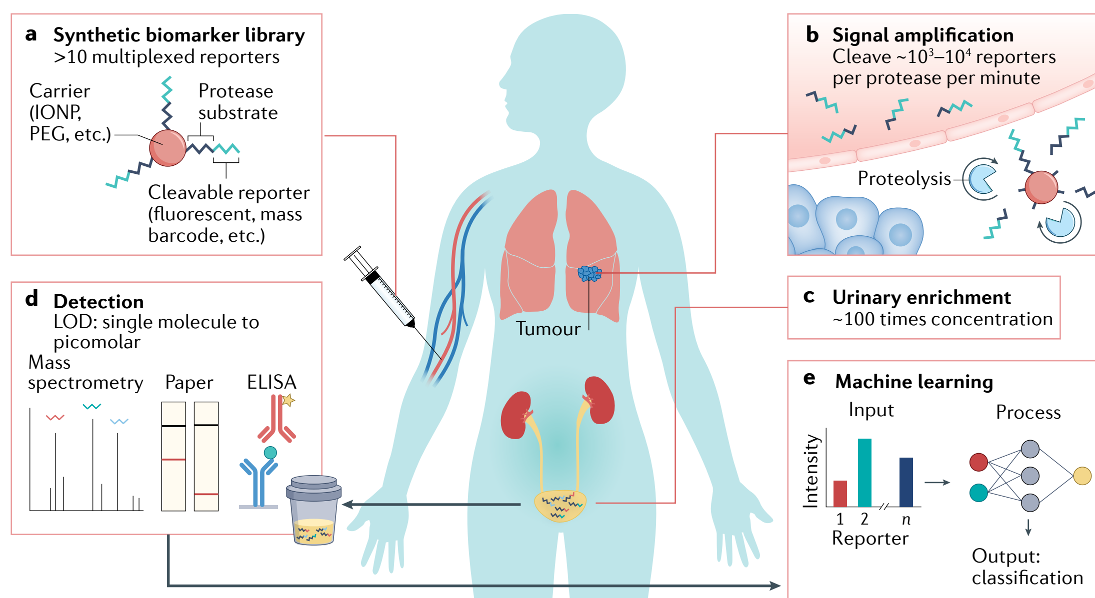

Fig. 2 | **Activity-based synthetic biomarkers enrich tumour protease signatures. a** | Synthetic biomarkers are activity-based sensors that comprise a biocompatible carrier (for example, iron oxide nanoparticles (IONPs), polyethylene glycol (PEG) or iron oxide nanoworms) coupled to peptide substrates for dysregulated proteases and a cleavable reporter (for example, mass-barcoded or fluorescent peptides). Peptide substrate libraries can be multiplexed by using orthogonal reporters. **b** | Following non-invasive delivery of a synthetic biomarker library (for example, by intravenous or intranasal administration), protease signatures are amplified by enzymatic turnover, resulting in the release of multiple reporters from each sensor upon proteolytic cleavage at the tumour site. **c**,**d** | The cleaved reporters are shed into the circulation, where they are further enriched by renal filtration (panel **c**) and detected in urine samples by several analytical platforms, including mass spectrometry, enzyme-linked immunosorbent assay (ELISA) and paper tests (panel **d**). **e** | Diagnosis is performed using machine learning-based classification algorithms. LOD, limit of detection; *n*, number.

detection (LOD) required for early detection involves harnessing features of human physiology to increase synthetic biomarker concentration in biofluids. One approach is to take advantage of size filtration by the kidneys by selecting a carrier with a hydrodynamic radius larger than the ~5-nm size cut-off of the glomerular filtration barrier[53] to prevent surface-conjugated peptides from being cleared into urine. Production of detection signals occurs after intravenous administration when the peptides are cleaved from the surface of the carrier by tumour proteases, releasing synthetic biomarkers into the circulation that are then rapidly cleared into urine for detection based on their reduced hydrodynamic diameters[25,47–49,51,54]. Although the use of a nanoparticle carrier increases the circulation time of surface-conjugated peptides, one limitation is the reliance on passive delivery to tumour sites. Approaches that use carriers with smaller hydrodynamic diameters, such as polyethylene glycol (PEG) polymers, which are characterized by higher passive diffusion rates than larger, iron oxide nanoparticles (IONPs)[55], could increase delivery to tumours[51]. Another approach is to functionalize sensors with tumour-penetrating ligands that engage active trafficking pathways to the tumour microenvironment[44].

Proteases are promiscuous enzymes capable of cleaving a variety of substrate sequences, which limits the detection specificity of a single sensor. Therefore, another key design principle is to design a multiplexed library of sensors to detect cancer by signature analysis. This approach requires each synthetic biomarker within a cocktail to be labelled with a unique molecular barcode. Various strategies have been developed, including mass barcodes[25,47,48,56], whereby reporters are differentially enriched with stable isotopes such as ^{13}C to generate a unique mass detectable by tandem mass spectrometry; DNA barcodes[57], whereby each reporter is labelled with a unique DNA sequence for detection by sequencing, PCR or CRISPR–Cas; ligand-encoded reporters[50,58,59], which are labelled with small molecules for detection by antibodies; volatile organic compounds[60] that are emitted as gases after cleavage; and ultrasmall gold nanoclusters[61] to catalyse a colorimetric readout. The most densely multiplexed cocktails of sensors reported to date are mass-barcoded 14-plex[48,56] or 19-plex[47] systems. These densely multiplexed approaches allow classifiers to be trained on the basis of multivariate machine learning algorithms that have the potential to indicate disease with increased diagnostic sensitivity and specificity compared with univariate classifiers trained on a single biomarker. Indeed, development and utilization of machine learning approaches have great potential for expediting the development of synthetic biomarkers for a variety of clinical applications. Machine learning or deep learning methods (BOX 1), when powered correctly along with considerations for comorbid conditions and other confounders, can help to increase the signal-to-noise ratio. In addition, deconvolution of complex signatures also has the potential to reveal new biological insights.

Hydrodynamic radius
For a macromolecule in solution, the radius of an equivalent hard sphere diffusing at the same rate as the macromolecule.

Deuterated metabolite
A compound in which one or more hydrogen atoms have been replaced by the stable isotope deuterium to distinguish it from its unmodified counterpart.

Bio-orthogonal reporters
Non-native reporters that do not interfere with biological functions.

Small-molecule probes. In light of the increasing number of tumour-specific antigens, cell surface markers and metabolic pathways that are targetable with small molecules, a number of studies are emerging that focus on engineered molecular probes to generate synthetic biomarkers for cancer detection (FIG. 3). Nishihara and colleagues[62] reported a strategy to generate synthetic biomarkers by targeting cancer cell-surface lectins using a two-step strategy. First, they labelled LoVo human colorectal carcinoma cells with a protein conjugate composed of the enzyme β-galactosidase conjugated to avidin. Avidin is a positively charged protein that contains terminal *N*-acetylglucosamine and mannose residues that bind to lectins overexpressed by tumour cells. In a second step, they administered a substrate for β-galactosidase called 'β-galactosidase-responsive acetaminophen' that is converted into acetaminophen (also known as paracetamol) by exogenous β-galactosidase on the surface of tumour cells. They found that acetaminophen plasma levels generated in this two-step process were elevated within 60 minutes in tumour-bearing mice. A similar approach was reported to quantify H_2O_2 activity using H_2O_2-responsive acetaminophen[63].

Small molecules labelled with stable isotopes have been widely used as diagnostic probes in research laboratories for more than 30 years[64]. The advantages of stable isotope labelling include the lack of radiation risk to patients, indistinguishable metabolism compared with their unmodified counterparts and high signal-to-noise ratio owing to the lack of background signal. Among the first clinically approved tests was the ^{13}C-urea test for *Helicobacter pylori* that detects urease activity central to *H. pylori* metabolism and virulence based on the level of $^{13}CO_2$ released in the breath[65]. Several isotope-labelled probes, including ^{13}C-methacetin[66] and ^{13}C-cholate[67], which measure hepatic cytochrome P450 activity and liver shunting, respectively, are approved by the FDA for measurement of liver dysfunction in the context of liver fibrosis, which is an important risk factor for hepatocellular carcinoma.

Natural volatile organic compounds (VOCs) present in patient breath samples have also been investigated for cancer diagnosis[68,69]. However, identifying a VOC signature for cancer is not trivial because of the high variability and low concentration of natural VOCs in breath. Lange and colleagues[70] used an isotope-labelled synthetic VOC called 'D_5-ethyl-β-D-glucuronide' (EtGlu), which is a deuterated metabolite of ethanol. Following intravenous administration, EtGlu is enzymatically converted by β-glucuronidase, an extracellular enzyme secreted by solid tumours, into D_5-ethanol, which is then detected from the breath by gas chromatography coupled with high-resolution mass spectrometry. In various tumour models, including a transgenic mouse model of mammary tumours, Lange and colleagues found significantly increased D_5-ethanol levels in breath following a single injection of EtGlu, and used D_5-ethanol levels to monitor the response to chemotherapy. In the future, isotope-labelled probes have the potential to be expanded into synthetic agents that sense different classes of tumour enzymes, including proteases[60]; they could also be densely multiplexed by mass to allow rapid analysis of breath samples for synthetic biomarkers.

Genetically encoded synthetic biomarkers

Design-driven advances in mammalian synthetic biology are pushing the boundaries for biological sensing. In addition to activity-based probes, genetically encoded constructs form the other major group of strategies that use engineered components or cells to amplify the release of synthetic biomarkers. These methods focus on strategies that drive resident cells or infiltrated cells within the tumour microenvironment to produce or secrete bio-orthogonal reporters[71–77]. The main advantage of these approaches is the ability to transcriptionally target synthetic biomarker production to cells of a particular phenotype, thereby potentially reducing the number of false positives caused by background production in healthy tissues. Here, we review advances in three main classes of genetically encoded systems for producing synthetic biomarkers, namely vector-based, mammalian cell-based and bacterial cell-based systems (FIG. 4).

Vector-based synthetic biomarkers. Transcriptional targeting with gene vectors is a powerful method to restrict transgene expression in target tissues and has been extensively explored for cancer imaging and therapy[71,78]. Building upon this foundation, vector-based systems rely on two key design components: a tissue-selective or cancer-selective promoter to drive transcription and a synthetic biomarker designed to be secreted into blood or urine for detection[79,80]. Tissue-selective promoters provide the first level of specificity — for example, the promoter for the gene pulmonary surfactant-associated protein B (*SFTPB*) restricts transgene expression to

Box 1 | **The application of machine learning to cancer**

- Machine learning is a branch of artificial intelligence based on the theory that computers can learn from prior examples to perform tasks and predict outcomes rather than being explicitly programmed with rules to make decisions[157,158]. A key advantage of machine learning compared with human learning is that computers can learn from complex and massive amounts of data. For example, machine learning is being applied to wide-ranging areas in medicine from pathology for automated detection of cancer in digitized histology slides[159,160] to prediction of disease aggressiveness and patient outcomes from -omic datasets[161–166].
- Supervised learning is a type of machine learning algorithm whereby the model learns from prior examples by training on a range of input features (for example, biomarker levels, height and weight) associated with a known output label (for example, cancer)[157,158]. The trained model can then generalize the input-to-output mapping to predict the assignment of never-before-seen inputs to an output label. These predictions can result in discrete categories (for example, benign or malignant) or a continuous range (for example, a score from 0 to 100).
- A classifier is a supervised learning method that categorizes unlabelled data into one or more discrete categories, also referred to as 'classes', such as cancer stages. For example, a random forest classifier is a collection of a large number of randomly created decision trees in which each node in the decision tree works on a random subset of features to calculate the output. The predicted output class is based on the most popular prediction among individual decision trees[167].
- In contrast to supervised learning, unsupervised learning is a type of machine learning algorithm that draws inferences from unlabelled data without prior knowledge. Clustering tumour specimens based on RNA transcript levels by *t*-distributed stochastic neighbour embedding (*t*-SNE), for example, is a form of unsupervised learning since the data are categorized without the use of predefined labels[168].

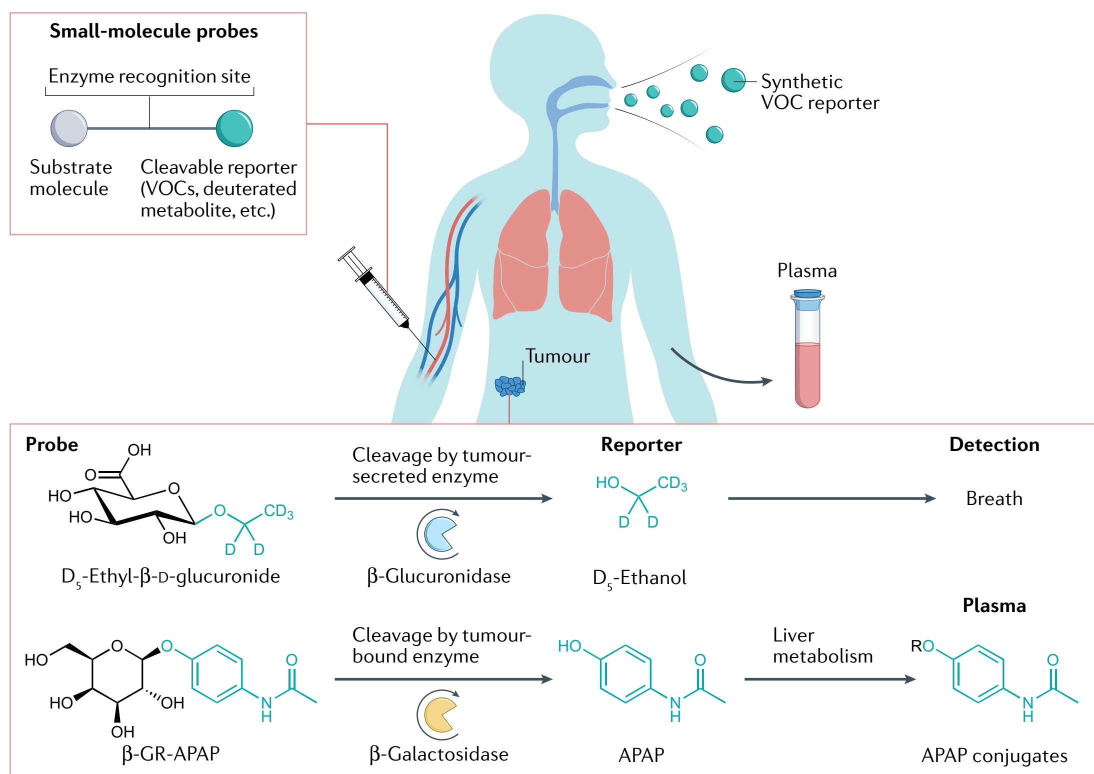

Fig. 3 | **Small-molecule probes sense tumour-associated enzymatic activity.** Small-molecule probes comprise an enzyme recognition site linked to a synthetic cleavable reporter, such as volatile organic compounds (VOCs) or stable isotope labels (for example, ^{13}C-methacetin or ^{13}C-cholate). Following systemic administration, tumour-associated enzymes convert the probes into synthetic biomarkers (for example, D_5-ethanol or acetaminophen (APAP) conjugates) whose abundance is detectable in breath or plasma samples. β-GR-APAP, β-galactosidase-responsive acetaminophen.

alveolar type II cells and Clara cells of the lung[81], and similarly, use of the promoter for the gene glial fibrillary acidic protein (*GFAP*) restricts expression almost exclusively to astrocytes[82]. However, with this approach, systemic delivery will result in transgene production by normal as well as tumour tissue derived from the same cell type, thereby increasing the background signal. By comparison, cancer-selective promoters increase the precision of transcriptional targeting as these are driven primarily by cancer cells but have limited activity in normal cells. One example is the promoter for the normally silent human telomerase reverse transcriptase (*TERT*), which encodes telomerase, which is frequently activated in cancer cells to achieve proliferative immortality[83], one of the hallmarks of cancer. As *TERT* is expressed at high levels in ~90% of human cancers but silenced in almost all somatic cells, the *TERT* promoter has been used to drive expression of genes in a wide variety of tumour cells[84–86].

The second component of vector-based strategies is a secreted reporter that acts as synthetic biomarker and can be detected in blood or urine. Secreted embryonic alkaline phosphatase (SEAP) was among the first reporters to be engineered for applications in vivo. SEAP is an engineered form of human placental alkaline phosphatase that contains a termination codon at the membrane-anchoring domain to convert it into a truncated but fully active secreted reporter[87,88]. In xenograft tumour models, production of SEAP by cancer cells allowed early and long-term measurement of tumour growth and response to drug treatment as SEAP levels directly correlated with tumour size and cell numbers[89–91]. The limitations associated with SEAP include its high molecular mass (64 kDa), which limits its use to a synthetic blood biomarker as it is not normally excreted in urine[92]. Moreover, alkaline phosphatases are naturally expressed by major organs and may leak into the bloodstream through tissue injury and interfere with SEAP measurements. Another commonly used reporter is a luciferase cloned from the marine copepod *Gaussia princeps* (Gluc)[93–98]. Unlike earlier luciferases such as *Photinus pyralis* luciferase (Fluc) and *Renilla reniformis* luciferase (Rluc), Gluc is naturally secreted and is among the smallest luciferases at 19.9 kDa, and its initial activity per mole is about 100–1,000 times higher than that of Rluc or Fluc and it is more than 20,000-fold more sensitive than SEAP[93]. On the basis of these favourable properties, Wurdinger and colleagues[94] demonstrated that Gluc could detect as few as 1,000 Gli36 human glioma cells in vivo compared with a LOD of ~500,000 cells with use of SEAP.

One limitation of vector-based strategies is the requirement for efficient tumour delivery without the use of viral vectors, given concerns regarding immunogenicity and insertion mutagenesis, particularly for early detection applications that will require longitudinal assessment and repeated administrations.

Fang and colleagues[99] designed plasmid vectors that were charge-complexed with cationic polyethylenimine for detection of bladder cancer. These constructs used cancer-selective promoters from cyclooxygenase 2 (*Cox2*) and osteopontin (*Opn*) to drive production of Gluc for detection from urine samples in mice. Although plasmids have a superior safety profile compared with viral vectors, they are limited by low gene transfer rates and transient expression profiles. By contrast, DNA 'minicircles'[100], which are minimal vectors free of prokaryotic components that conform to regulatory principles for plasmids free of antibiotic resistance genes (pFAR)[101], have increased delivery efficiency, enhanced expression and reduced transcriptional silencing compared with plasmids. For cancer detection, Ronald and colleagues[77] designed minicircles that encoded SEAP driven by the cancer-selective survivin promoter. Systemic delivery of these in a melanoma lung metastasis mouse model led to detectable elevations of SEAP levels in plasma that correlated with tumour burden. With this approach, it would be possible to create bespoke vectors for particular cancer types by designing minicircles with alternative promoters, such as the mucin 1 promoter for breast cancer[102]. In addition, their application could be extended beyond early cancer detection, for example, to assess the aggressiveness of prostate cancer[103].

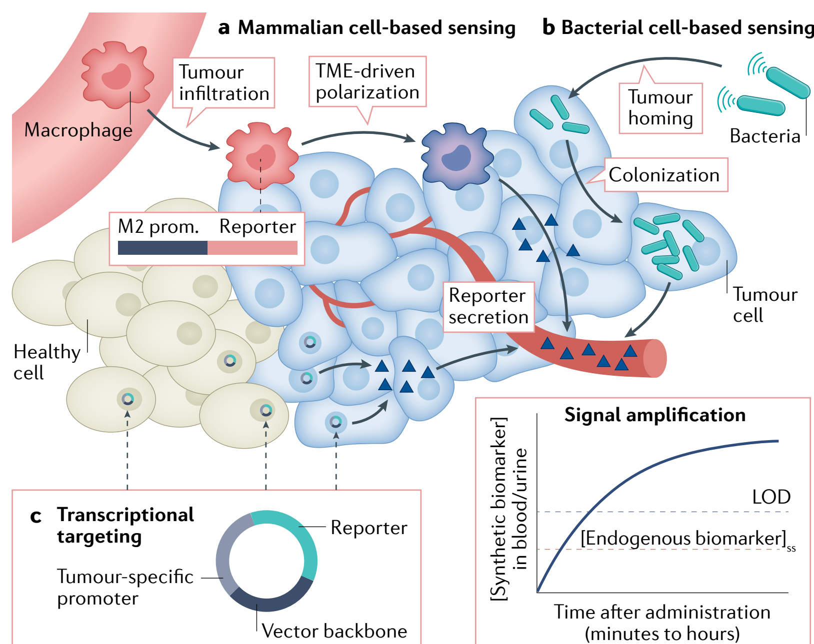

Fig. 4 | **Genetically encoded synthetic biomarkers leverage tumour-specific cues to achieve detectable signals.** Cells engineered with genetically encoded synthetic biomarkers exploit key features of the tumour microenvironment (TME) to trigger the secretion of detectable reporters. Secreted reporters can be detected in blood to indicate the presence of disease or they can be imaged to provide spatial information on tumour location or immune cell activation. **a** | Mammalian cell-based 'immunodiagnostics' exploit the metabolic alterations of tumour-infiltrating macrophages to trigger the production of a secreted biomarker by engineered macrophages. **b** | Bacteria, which colonize tumours owing to suppressed immunosurveillance and increased availability of nutrients in the necrotic tumour core, release programmed reporters at the site of the tumour. **c** | DNA vectors leverage tumour-associated gene expression patterns by encoding a secretable reporter transcriptionally targeted to cancer cells using tumour-specific promoters. LOD, limit of detection; prom., promoter; ss, steady state.

Mammalian cell-based synthetic biomarkers. The recent clinical successes of adoptive cell therapies have inspired the idea of engineered mammalian cells as living biosensors (FIG. 4). A clear advantage of cells as diagnostic vehicles is that some are capable of homing to and infiltrating cancer sites, in contrast to molecular probes, which are limited by their reliance on passive diffusion from the vasculature to accumulate in tumours. Mesenchymal stem cells (MSCs) are adult multipotent stem cells that possess regenerative and immunomodulatory properties, and systemically infused MSCs selectively home to primary and metastatic tumours[104]. Liu and colleagues[105] used a mouse model to demonstrate the use of engineered MSCs for detection of cancer metastasis from blood. First, MSCs were engineered to secrete humanized Gluc; upon intravenous administration, engineered MSCs persisted longer in mice with MDA-MB-231 breast cancer lung metastases than in tumour-free mice, resulting in higher blood levels of humanized Gluc. However, as MSCs exhibit tropism to sites of inflammation and injury[106], or may themselves participate in cancer progression[107], additional studies are needed to understand these potential limitations.

Aalipour and colleagues[76] further developed the concept of cell-based diagnostics using engineered macrophages as living cellular sensors. Within the tumour microenvironment, a subset of macrophages is polarized to an M2 tumour-associated metabolic profile that promotes an immunosuppressive microenvironment. Aalipour and colleagues found that M2 reprogramming led to striking changes in the levels of arginase 1 (encoded by *ARG1*), which was upregulated by as much as 200-fold by adoptively transferred macrophages in solid tumours. On the basis of this finding, they used the *ARG1* promoter to drive production of Gluc upon macrophage M2 polarization. This study laid the foundation for the concept of cellular immunodiagnostics, and considering that a number of other immune cells likewise modulate expression of metabolic genes in the context of the tumour microenvironment, this approach could also be extended to T cells[108], B cells[109] and natural killer cells[110]. Several limitations are worth noting, including observations that macrophage sensors did not detect visibly necrotic tumours in high tumour burden settings, which could be attributed to poor infiltration. Another limitation is the high cost of adoptive cell transfer and the complex pipeline for good manufacturing practice (GMP) cell manufacture that would prevent this approach from being a routine screening tool. However, numerous efforts are under way to reduce the time and cost, including in situ reprogramming of circulating cells[111], which circumvents the need for ex vivo cell isolation, and allogeneic 'off-the-shelf' immune cells[112].

Bacterial cell-based synthetic biomarkers. Certain types of bacteria infiltrate and selectively grow in tumours, which has been attributed to suppressed immunosurveillance and increased levels of nutrients released by necrotic cells within the core of solid tumours[113–116]. This has prompted the use of engineered tumour-targeting bacteria as programmable vehicles for cancer detection. Panteli and colleagues[117,118] genetically modified an attenuated strain

of *Salmonella enterica* that is 10,000-fold less toxic than its wild-type counterpart to release ZsGreen as a fluorescent biomarker, or 'fluoromarker'. Following intravenous administration in tumour-bearing mice, fluoromarker levels in serum were dependent on tumour mass and were predicted by mathematical modelling to have the capacity to detect tumours as small as 120 mg. Danino and colleagues[119] showed that the nonvirulent probiotic bacterium *Escherichia coli* Nissle 1917, genetically engineered with a *lacZ* reporter, selectively colonizes colorectal cancer liver metastases following oral delivery in recipient mice. One limitation of gene circuits constructed on intracellular plasmids is that they lose stability and function over time and under environmental conditions that disrupt cellular homeostasis. Therefore, the team engineered a dual-maintenance vector including a toxin–antitoxin system that simultaneously produces a long-lived host-killing (hok) toxin and a short-lived suppression of killing (sok) antitoxin, such that in the event of plasmid loss, the cell will be killed by the long-lived toxin. To detect the presence of liver metastases, the team showed that a LacZ substrate could be administered to produce a colorimetric reporter in urine. A demonstrated advantage of this approach is the ability of tumour-targeting bacteria to expand by more than 10^6-fold after colonization, providing yet another mechanism to amplify detection signals beyond enzymatic turnover and urinary enrichment.

Moving forward, several challenges need to be addressed for bacteria to be used for early cancer detection. Although engineered strains, including *Clostridium*, *E. coli* Nissle and *Salmonella*, have been shown to be non-pathogenic in animals and humans[113], the inherent toxicity of bacterial components and the potential to revert to virulence pose safety concerns. It is also not clear whether all tumour types and nascent lesions that lack a necrotic core can be colonized by systemically delivered bacteria. Advances in synthetic biology could offer solutions to mitigate these challenges as well as providing the opportunity to engineer 'smart' microorganisms with specified and controlled behaviour[120]. For instance, bacteria engineered with quorum-sensing biocircuits can be used for bacterial communication to synchronize activity[121,122] and produce emergent behaviour such as timed release of therapeutic cargo after a threshold population density has been reached to either kill tumours[123] or promote systemic antitumour immunity[124,125]. Applied to the field of early cancer detection, these biocircuits have the potential to increase specificity by reducing background activity from healthy tissues, since off-target bacteria would not reach a quorum and therefore not falsely produce a reporter. In the future, these genetically programmable vehicles may have the potential to be developed into safe and regularly ingested food products (for example, yogurt) to allow routine cancer screening or cancer chemoprevention[126].

Preclinical studies

A number of preclinical studies have been reported that demonstrate the potential of activity-based synthetic biomarkers to achieve the LOD required for earlier detection (FIG. 5). In a xenograft mouse model, an activity-based sensor composed of IONPs conjugated with mass-barcoded peptide substrates was able to discriminate LS174T colorectal tumours that were 60% smaller in volume than those detected by the shed serum biomarker CEA (130 mm^3 versus 330 mm^3 on average, respectively) with an area under the receiver operating characteristic curve (AUROC) of 0.94 (REF.[25]). By contrast, a separate study by Aalipour et al.[76] that also used the LS174T colorectal cancer model found that ctDNA was detectable from blood only when tumour volumes reached ~1,000 mm^3. Kwon and colleagues[44] reported a formulation of activity-based sensors that incorporated tumour-penetrating peptides to target and increase their delivery to metastatic nodules in an orthotopic ovarian cancer model to further lower the LOD. By quantifying cleaved synthetic biomarkers enriched in urine, they reported the ability to detect disseminated disease with near-perfect accuracy (AUROC of 0.99) when the median nodule diameter was less than 2 mm and the average total tumour burden was 36 mm^3. By comparison, the shed human epididymis protein 4 (HE4) serum biomarker was able to indicate disease only when the average tumour burden reached 88 mm^3. This 59% reduction in tumour burden LOD was an important demonstration considering that current transvaginal ultrasonography can reliably resolve individual tumour nodules only when they are larger than 5 mm in diameter (equivalent to 65 mm^3 per nodule), and estimates indicate that decreasing serous ovarian cancer mortality by 50% would require a test capable of detecting nodules smaller than 5 mm in diameter[127].

Depending on the cancer type, use of different delivery routes also provides another approach to reduce the LOD by reducing sensor activation by off-target organs. Kirkpatrick and colleagues[48] showed that intrapulmonary delivery of a 14-plex cocktail of sensors, composed of mass-barcoded peptides conjugated to an eight-arm PEG carrier, could be used to query the lungs for early tumours by producing cleaved synthetic biomarkers detectable in urine. In the *Kras*- and *Trp53*-mutant genetically engineered mouse model of lung adenocarcinoma, they reported the ability to detect a total average tumour burden of 2.8 mm^3. This LOD compared favourably with that in an independent publication by Rakhit and colleagues[128], where they showed that ctDNA from an autochthonous $Kras^{G12D}$-mutant lung cancer model was detectable only when average tumour volumes were 7.1 mm^3. Kirkpatrick and colleagues further showed that a random forest machine learning classifier (BOX 1) trained on the 14-plex synthetic biomarker signature predicted lung cancer progression with high accuracy (AUROC greater than 0.90) and distinguished lung cancer-bearing mice from mice with benign lung inflammation (AUROC greater than 0.97)[48].

In vivo LOD studies have also been reported for genetically encoded synthetic biomarkers. Aalipour and colleagues[76] showed that the adoptive transfer of engineered macrophage sensors detected moderately sized CT26 colorectal tumours (volume 50–250 mm^3) with 100% sensitivity and specificity, while small tumours (25–50 mm^3) were also discriminated, with an AUROC of ~0.85, compared with healthy animals. The team further demonstrated the potential for translation by using

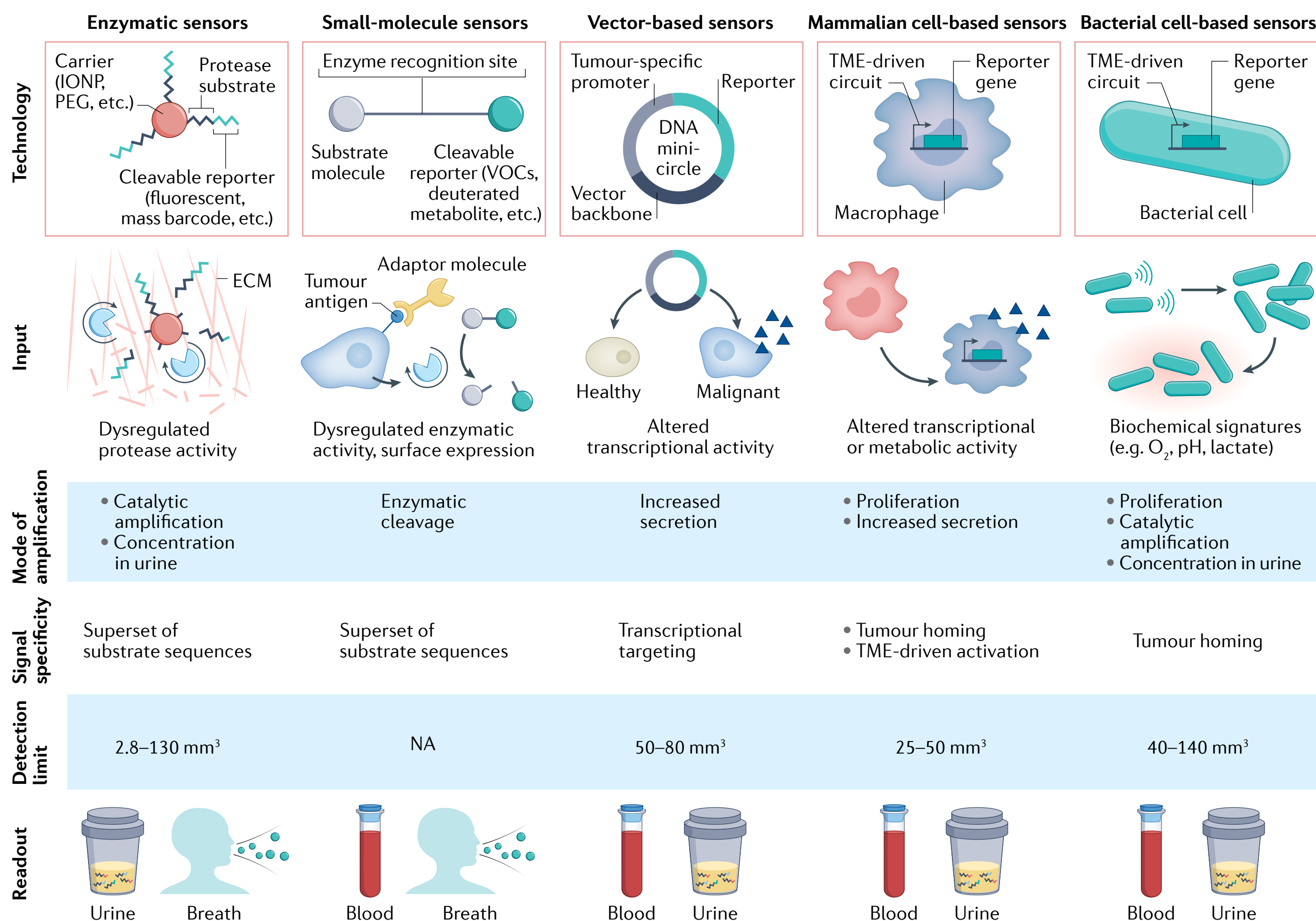

Fig. 5 | **Characteristics of synthetic biomarkers for early-stage cancer detection.** Enzymatic, small-molecule, DNA-based, mammalian cell-based and bacterial cell-based sensors leverage synthetic biomarkers to enhance early cancer detection. Each technology senses dysregulated activity (that is, the 'input') associated with the tumour microenvironment (TME), such as protease activity, metabolic activity or biophysical features. Through diverse modes of amplification and strategies for improving signal specificity, these approaches lower the limit of detection below current clinical thresholds (~1 cm^3). ECM, extracellular matrix; IONP, iron oxide nanoparticle; PEG, polyethylene glycol; VOCs, volatile organic compounds; NA, not available.

primary bone marrow-derived macrophages in addition to the RAW264.7 macrophage cell line. They found that engineered bone marrow-derived macrophage sensors detected CT26 tumours with a volume of 60–75 mm^3 with an AUROC of 0.81. In benchmarking studies comparing the performance of their macrophage sensors with either plasma CEA secreted by LS174T tumours or cfDNA released by CT26 tumours, they reported a lower LOD; tumours ~136 mm^3 in volume were detectable by CEA, while tumours larger than 1,500 mm^3 were detectable by cfDNA.

Challenges for clinical translation

Preclinical limitations. Significant sources of noise for synthetic biomarker strategies include off-target and on-target, off-tumour activation. For activity-based synthetic biomarkers, most published protease substrates were identified through in vitro selection; therefore, these substrates were not selected against background activity arising from circulating blood (for example, coagulation and complement proteases) or organ-associated proteases in vivo. Therefore, it will be important to develop screening strategies that permit substrate discovery by negative selection under healthy as well as comorbid conditions. Ideally, the substrate development pipeline would include steps conducted in vivo or, at a minimum, with appropriate control plasma samples in vitro that account for the anticoagulant used during sample collection and the classes of proteases it inhibits. Developing peptide display technologies that permit sequence selection based on on-target and off-target protease activity in vivo would significantly advance the design of peptide-based protease sensors[129,130].

Standardized and better preclinical models are needed to accurately recapitulate pre-invasive and early-stage cancer. The vast majority of immortalized cancer cell lines were derived from patients with advanced metastatic disease, which do not fully reflect early or precancerous conditions. Moreover, the rate at which endogenous biomarkers are produced by these cancer cell lines can vary by as much as four orders of

magnitude[25], which makes benchmarking studies difficult to compare across laboratories unless the same cell lines are used. Additional methods need to be developed to increase the information we are able to collect from patients, including the 'age' of a tumour[131–134], the relationship between tumour sizes and secreted biomarker levels, and the permeability of tumours. This increased understanding will provide important clinical data to support the development and validation of predictive mathematical models and to optimize formulations of synthetic biomarkers[51]. Genetically engineered animal models that recapitulate pre-invasive conditions, such as prostatic intraepithelial neoplasia (PIN)[135], would provide a rich test bed for future synthetic biomarker studies geared towards early detection of cancers.

Allometric scaling. It is likely that several key system parameters will be linearly proportional between preclinical rodent models and humans. For example, for protease-activated synthetic biomarkers, it is estimated that more than 500 of 628 mouse proteases are considered true orthologues[136] of the ~550 proteases encoded by the human genome. Thus, the efficiency with which a protease cleaves a substrate sequence (that is, the catalytic efficiency k_{cat}/K_m, where k_{cat} is the catalytic rate constant and K_m is the Michaelis–Menten constant[137]) would likely be similar between rodent and human orthologues, especially for proteases that perform conserved functions. For others that are substantially different, substrate screening technologies could be used to identify target substrates with similar Michaelis–Menten constants between species. Therefore, it is likely that the kinetics of protease cleavage and signal amplification observed in mouse models would be reflected in humans. Similar assumptions could be drawn for other parameters, such as tumour transfection efficiencies, biomarker secretion and degradation rates, and safety and clearance from the body.

However, there are significant physiological differences between mice and humans, including blood pool volume (2 ml versus 5 l), urine volume (500 μl versus 500 ml) and glomerular filtration rates, such that allometric scaling across species would likely be non-linear. For example, Kwong and co-authors[51] developed a physiologically based pharmacokinetic model to understand how probe and physiological parameters affect the performance of activity-based synthetic biomarkers. Their model revealed a number of intuitive relationships (for example, signal is proportional to sensor delivery) but also predicted relationships that were non-linear and non-intuitive. Several of these non-linear relationships (for example, signal-to-noise ratios are largely independent of the dose of the administered sensor) have been experimentally validated in mice[44,51,58] but have yet to be shown in humans. Moreover, synthetic biomarkers shed by genetically encoded vectors into the circulation will be diluted by ~3,500-fold in humans over mice if scaling is calculated linearly on the basis of only blood volume. Yet this does not imply that a synthetic biomarker that can discriminate ~5-mm^3 tumours in mice can discriminate only tumours that are ~3,500-fold larger in volume in humans, as both clinical data and mathematical modelling support that smaller tumour sizes are detectable even by shed endogenous blood biomarkers[31,32]. Clearly, biological factors other than tumour burden affect detection sensitivity.

Tumour localization. For cancer screening applications, a blood or urine synthetic biomarker has limited utility unless it also reports on which organ should be followed up for tumour localization. One approach could involve signal normalizers such as a probe that reports on organ-specific proteases (for example, liver hepsin) or, alternatively, normalization against a synthetic biomarker released by a tissue-specific promoter. Another potential approach is to combine synthetic biomarkers with different classes of endogenous analytes and clinical variables such that a multianalyte classifier can be trained to predict potential tumour sites. This strategy was recently demonstrated by CancerSEEK[9], a blood test designed to detect eight common cancer types by ctDNA sequencing. The test included 31 proteins and the patient's sex to generate a score that correctly localized the tumour to one of the two top predicted anatomical sites in 83% of patients. A similar approach was reported for a stool-based test for colon cancer screening that included a haemoglobin immunoassay and was able to detect significantly more cancers than a faecal immunochemical test alone[21].

Imaging will play a critical role in determining the location of the tumour following confirmation of a synthetic biomarker signal. Molecular imaging with reporter genes is a rich area of research for platforms such as single photon emission computed tomography (SPECT) and PET that are not limited by depth or tumour site within the body compared with optical modalities[138]. Genetically encoded synthetic biomarkers are most amenable to these approaches, which essentially involve exchanging the secreted synthetic biomarker for a reporter gene. For example, macrophage sensors could be engineered to express the herpes simplex virus 1 thymidine kinase (HSV1-TK) reporter gene to allow tumour site-induced M2 polarization of macrophages to be detected by PET[22]. Similar approaches have also been demonstrated with vector-based strategies; for example, the tumour-specific progression elevated gene 3 (*Peg3*) promoter (PEG-Prom) has been used to drive HSV1-TK expression, enabling tumour-specific imaging of lung metastasis[71]. This approach showed the ability to detect small lesions that were missed by fluorodeoxyglucose PET in preclinical studies. Imaging strategies that integrate concepts from synthetic biology have the potential to further increase the sensitivity and specificity of cancer imaging, as illustrated by Widen and colleagues[139], who described an AND gate optical probe requiring two cleavage events by multiple tumour proteases to produce a signal.

Strategies for clinical testing. A densely multiplexed cocktail of synthetic probes would likely be necessary to achieve the selectivity required to handle the expected tumour heterogeneity, interpatient variations and comorbidities in the human population. Importantly, in the reported preclinical studies with activity-based

Herpes simplex virus 1 thymidine kinase
(HSV1-TK). The enzyme expressed by the reporter gene phosphorylates radiolabelled purine and pyrimidine nucleoside analogues to trap the probe within cells and thereby allow visualization by positron emission tomography (PET).

AND gate
A Boolean logic gate operation that outputs a value of 1 if and only if both inputs are 1; otherwise it outputs 0.

synthetic biomarkers[25,47,48], the study authors showed that the diagnostic performance of two or three probes was sufficient to attain the sensitivity and specificity of the entire panel (more than ten probes). Moreover, when the same multiplexed probe set was used, different subsets of probes distinguished different disease states, including liver fibrosis progression from regression[25], lung cancer from benign lung inflammation[48] and response from resistance to checkpoint blockade immunotherapy[56]. While the ability to distinguish disease states in mice with low-dimensional data can be attributed partially to the lack of variation in isogenic tumour models, these observations also highlight a potential strategy for clinical trial design that makes use of a 'superset' of probes. This approach may provide the ability to capture high-dimensional data for classifier training (BOX 1), while allowing the possibility of down-selection after classifier validation. In addition, once the safety and immunogenicity of a superset of probes have been demonstrated, it could potentially be applied to different clinical use cases without changing its composition, which could reduce the amount of resources required and regulatory burden. Similar specificity challenges lie ahead for genetically encoded sensors, which thus far have been designed to produce a single synthetic biomarker. One approach to increase the specificity of tumour detection would be to multiplex several metabolic gene reporters. This could be accomplished with different promoters paired with different secreted reporters, such as artificial microRNAs[140]. Cell-based sensors could also be engineered with synthetic circuitry to endow them with the capacity to perform logic-based computations. This could take the form of biocircuits that require the presence of multiple environmental inputs before a single output reporter is produced, for example, using AND-gated sensing[141–143] to increase tumour-selective activation or analogue-to-digital conversion[143] to reduce background noise.

As the field is in its infancy, human testing of synthetic biomarkers has yet to proceed to pivotal trials to determine their use for early cancer detection. To the best of the authors' knowledge, the synthetic biomarker formulation that has advanced the furthest in clinical testing is mass-barcoded PEGylated peptides[48], which were found to be well tolerated and safe in healthy human volunteers on the basis of preliminary data from a recent phase I study[144,145]. The first several clinical use cases for synthetic biomarkers need to be carefully considered, as an early failure may set the field back. Screening asymptomatic patients for early cancer is highly challenging and may present ethical challenges in clinical trial validation studies; for example, patients with a positive synthetic biomarker test result may need to wait for confirmation by imaging (that is, allow tumours to grow) before therapeutic intervention. Potential clinical entry points such as pharmacodynamic assessment of treatment response[56] or monitoring for recurrence following primary resection could show the utility of a synthetic biomarker approach before transitioning to early detection applications. As the field advances towards human testing, it should be noted that a number of components and vehicles that form the 'parts' for synthetic biomarker generation are undergoing clinical evaluation, have a demonstrated safety record in humans or are approved by the FDA. Examples include protease-activated substrates used in imaging probes for intraoperative detection of tumour margins[146–148], linkers for masked antibodies (NCT03993379 and NCT03013491)[149–151], activation domains for T cell engagers[152,153] (NCT03577028) and antibody–drug conjugates[154]. Similarly, for genetically encoded synthetic biomarkers, numerous clinical trials have highlighted the safety and utility of attenuated bacteria as vehicles for targeting tumours and delivering therapy[155]. These precedents provide a broader understanding of the embodiments of synthetic biomarkers that will be safe and well tolerated by humans, and anticipate the types of substrate sequences that will be selective for different types of human tumours.

Charting the course ahead

Although the nascent field of synthetic biomarkers is exciting and full of promise, there are gaps in our current knowledge of cancer pathogenesis that need to be filled alongside addressing technical challenges to guide future advances. In particular, there is limited understanding of the biology of early lesions and when and how a precursor lesion transitions into malignancy, yet such information is needed to guide sensor engineering strategies. This highlights a challenge in the synthetic biomarker field for early detection of cancers. The Cancer Genome Atlas (TCGA) has generated a tremendous knowledge base for the biomedical community but there is a bias towards advanced and locally advanced tumours. Therefore, there is a need for extensive profiling of early-stage and in situ tumours as well as lethal precursors that have a high propensity for malignancy. There is also an urgent unmet clinical need to detect aggressive cancers, and early detection efforts would be greatly bolstered by the ability to predict tumour aggressiveness and lethality. In addition, one has to be mindful of the fact that tumours are heterogeneous, and the biology can be very complex. For translation of some of these synthetic biomarker technologies into humans, one has to select the tumour system carefully to avoid a potentially harmful combination of unknown biology and an agent for which information on pharmacokinetics and long-term safety in a particular clinical setting is limited. The US National Cancer Institute (NCI) initiated the Human Tumor Atlas Network (HTAN) to create detailed molecular, cellular and spatial maps of a variety of precancers, in situ cancers and advanced cancers as a function of time[156]. This will lead to a profound understanding of how precancers transition to malignancy for those cancer types studied by the HTAN, and how invasive cancers progress, metastasize and respond to or develop resistance to treatment. The knowledge generated from in situ and early lesions will generate testable hypotheses and biological information that could be leveraged to develop synthetic diagnostic tools, while also unearthing new candidate endogenous biomarkers.

Multidisciplinary teams of bioengineers, biologists and clinicians should work together strategically and in an integrated manner to find the answers to a number of

questions that include, but are not limited to, the following. For which early-stage tumours or precancer lesions of lethal potential are the biology and pathogenesis sufficiently understood to drive the engineering of sensors? How can machine learning support identification of key features within complex biological datasets to achieve the required predictive power for synthetic biomarkers? Which populations will benefit the most from early detection? What are the short-term or long-term tolerability profiles of bioengineered sensors? How often can patients be screened? What additional complications can result from patient comorbidities? Under what situations can the same probes be used for detecting cancer recurrence? How expensive will long-term surveillance of 'at risk' patients be compared with the current standard of care? How can patient and tumour heterogeneity be overcome to ensure diagnostic accuracy? Which proof-of-principle studies in humans are worth consideration? How can mathematical models assist in such endeavours? Although there may seem to be more unknowns than definitive answers at this time, we anticipate that solutions will emerge at an increasingly rapid pace through collective, multidisciplinary efforts, and the audacious and innovative visions of scientists.

Published online: 06 September 2021

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Acknowledgements
The US National Cancer Institute organized the Synthetic Biomarkers for Detection of Cancers at Incipient and Early Stages (SYNDICATE) Think Tank Meeting in 2019, where bio-engineers, biologists and clinicians discussed the promises and challenges of synthetic biomarkers from development to preclinical models to scale up to meeting the ultimate goal of safe use in the clinic. The authors express their gratitude to all SYNDICATE meeting participants for their thoughts, expertise and insightful comments. They thank T. Danino (Columbia University) and L. Chan (Georgia Tech & Emory University) for insightful discussions. This work was funded by the US NIH Director's New Innovator Award (DP2HD091793) and the National Cancer Institute R01 grant 5R01CA237210 to G.A.K. S.N.B. is a Howard Hughes Medical Institute investigator. L.G. was supported by the US National Science Foundation Graduate Research Fellowship Program (DGE-1451512). This work was performed in part at the Georgia Tech Institute for Electronics and Nanotechnology, a member of the National Nanotechnology Coordinated Infrastructure, which is supported by the National Science Foundation (grant ECCS-1542174). This content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Author contributions
G.A.K., S.G., L.G., S.S. and S.N.B. researched the data for the article, provided substantial contributions to discussions of the content and contributed to writing the article. G.A.K., S.G., L.G., C.P., S.S. and S.N.B. reviewed and/or edited the manuscript before submission.

Competing interests
G.A.K. is a co-founder of Glympse Bio, and consults for Glympse Bio and Satellite Bio. S.N.B. is a director of Vertex, is a co-founder of and consultant for Glympse Bio, Satellite Bio and CEND Therapeutics, is a consultant for Moderna, and receives sponsored research funds from Johnson & Johnson. S.G., C.P., S.S. and L.G. declare no conflicts of interest.

Peer review information
Nature Reviews Cancer thanks the anonymous reviewers for their contribution to the peer review of this work.

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