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Detection and analysis of chiral molecules as disease biomarkers

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Abstract

The chirality of small metabolic molecules is important in controlling physiological processes and indicating the health status of humans. Abnormal enantiomeric ratios of chiral molecules in biofluids and tissues occur in many diseases, including cancers and kidney and brain diseases. Thus, chiral small molecules are promising biomarkers for disease diagnosis, prognosis, adverse drug-effect monitoring, pharmacodynamic studies and personalized medicine. However, it remains difficult to achieve cost-effective and reliable analysis of small chiral molecules in clinical procedures, in part owing to their large variety and low concentration. In this Review, we describe current and emerging techniques that detect and quantify small-molecule enantiomers and their biological importance.

Graphical Abstract

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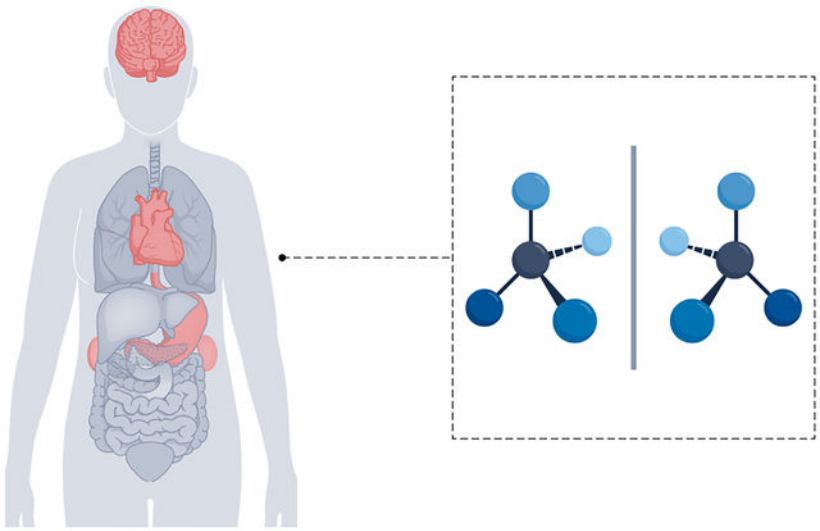
Author contributions

All authors collected data, wrote and edited the manuscript. Y.L. drafted the manuscript. D.W.A and H.W. provided substantial content. Y. L., Z. W. and Y. Z. conceived the content, and edited and finalized the manuscript.

Competing interests

The authors declare no competing interests.



Introduction

The identification and detection of distinct biomarkers is important for the diagnosis of disease, evaluating disease progression and even the development of therapeutics[1–3]. Small molecules (<1,000 Da) — for example, sugars, fatty acids and amino acids — are gaining prominence as novel biomarkers[4], largely owing to the ‘omics’ revolution in the past decade[5]. In contrast to other biomarkers, such as proteins and genetic materials, small molecules can reflect not only downstream results of the genome but also upstream inputs from drugs. This allows scientists to monitor the onset and progress of diseases, as well as responses to drugs[6].

An advantage of small-molecule biomarkers is that many of them are `chiral`[7]. Such molecules are referred to as `enantiomers`, which can be said to exist in ‘right-handed’ and ‘left-handed’ forms. Common chiral small molecules in humans (and other species) include carbohydrates (for example, glucose), organic acids (for example, lactate and 2-hydroxyglutarate (2-HG)) and amino acids (for example, serine, aspartate and alanine)[8]. Normally, chiral molecules with specific types of ‘handedness’ will dominate over the other types in biological systems[9]. For example, chiral amino acids exist mainly as L-enantiomers, whereas the D-enantiomers of carbohydrates are dominant in all living things[10]. Such basic chemical components are fundamental for stereoselective protein formation. The chirality of these small molecules also affects biochemical processes, such as molecular binding, molecular synthesis and signalling in cell communication[11]. Interestingly, the chirality of small molecules depends on the physiological status of animals, including humans. Internal or external influential factors (Fig. 1), such as toxins, mutations, racemization, enzyme deficiencies, infection and radiation, can induce enantioselective changes, leading to abnormal chirality of molecules in biofluids or tissues[12–14].

Because of the strong correlation between molecular chirality and pathological processes, abnormal concentrations of chiral small molecules are increasingly being identified as biomarkers for disease monitoring and treatment[15,16]. For example, many types of cancer

cells have a greater enantiomeric excess of D-2-HG than healthy cells. These small molecules can be used for disease prognosis and can be used to inform the most effective treatment approach[17]. Similarly, the determination of the D-enantiomers of glutamine and isoleucine may help identify patients with early gastric cancer for surgery or specific chemotherapies[16,17]. Diabetic complications and sepsis are linked to enantiomers of lactate, the measurement of which has the potential to identify patients who are candidates for intensive therapies[18]. The other main disease area in which chiral small molecules are implicated is brain disease. For example, D-serine (D-Ser) accounts for one third of the total serine in the brain[19,20] and works as a physiological ligand of a key neurotransmitter receptor, *N*-methyl-D-aspartate receptor (NMDAR)[21–24]. This D-amino acid is synthesized by serine racemase[25] and has release mechanisms[26,27] and catabolic pathways[28,29]. D-Ser is associated with the pathophysiology of several diseases, including traumatic brain injury[30,31], stroke damage[32], neuropsychiatric disorders[33,34], epilepsy[35,36] and amyotrophic lateral sclerosis[37,38], and is a potential biomarker for renal disease[39]. These are just a few examples that demonstrate the importance of measuring the enantiomeric composition of specific molecules in the diagnosis and prognosis of diseases, monitoring of drug effects and prediction of patient responses to treatments[40].

This Review focuses on how chiral biomarkers are identified and analysed using the different molecular measurement techniques available. First, we describe the identification of chiral biomarkers and their correlations with diseases, followed by the difficulties in measuring low enantiomeric concentrations in clinical settings. We then discuss the working principles, strengths and limitations of the different analytical methods. We conclude by discussing the challenges and future perspectives of existing methods in achieving cost-effective and accurate detection and identification of enantiomers for biomedical research and clinical applications.

Chiral molecules in diseases

There are two major strategies for discovering and identifying chiral biomarkers. One is to hypothesize the existing potential chiral biomarkers based on chirality-dependent physiological pathways and then to validate the hypothesis through targeted biomarker detection[41]. The other strategy is to identify metabolites (a subset of which are chiral small-molecule biomarkers) in human bodies in an untargeted way[42]. Chiral biomarkers can then be identified by establishing correlations between metabolite profiles and disease status[43,44]. In this section, we discuss established correlations between chiral biomarkers and diseases for three categories of disease (brain diseases, kidney disease and diabetes, and cancer), and how these biomarkers were identified.

Brain physiology and pathology

NMDARs are major excitatory receptors in the brain and are activated by the binding of glutamate to GluN2 and a co-agonist to the GluN1 subunit[45]. The binding events between endogenous neurotransmitters and NMDARs in synapses are pivotal for neurotransmission[23,45,46]. Overstimulation or hypofunction of NMDARs by endogenous modulators can lead to synaptic dysfunction and cognitive impairments[47]. Recent studies

have shown that certain amino acids and chiral organic acids can enantioselectively bind to NMDARs. In this section, we review the most important of these: D-Ser, D-aspartate (D-Asp), D-alanine (D-Ala), D-lactate and L-2-HG and D-2-HG.

D-Serine.—D-Ser, but not L-Ser, binds to the co-agonist site of NMDARs, resulting in activation of the channel[21,22,48]. Targeted deletion of serine racemase — the biosynthetic enzyme for D-Ser — leads to NMDAR hypofunction and behavioural abnormalities[49–51], indicating that maintaining a proper concentration of D-Ser in the brain is critical to modulate neurotransmission. However, overstimulation of the NMDARs through excessive D-Ser and glutamate leads to massive calcium influx into the cells and promotes neurotoxicity[52,53]. D-Ser-mediated cell death through NMDAR activation has been associated with several brain diseases, including stroke, epilepsy, chronic pain, amyotrophic lateral sclerosis and Alzheimer disease[54,55]. By contrast, NMDAR hypofunction caused by low D-Ser concentrations can lead to cognitive impairments, reminiscent of diseases such as schizophrenia[33,50,56] or normal ageing[57–59]. Low D-Ser concentrations have also been reported in disorders of serine biosynthesis, such as 3-phosphoglycerate dehydrogenase deficiency and phosphoserine phosphatase deficiency[60]. The understanding of the role of D-Ser in such biochemical processes gave researchers the impetus to detect it in human biosamples.

D-Ser has been tested as a therapeutic agent for the treatment of schizophrenia and has been shown in murine models and human patients to improve several schizophrenia symptoms when used in combination with conventional antipsychotics[61–66]. For example, the administration of D-Ser to mice improves recognition tasks and working memory[64]. Moreover, in patients with schizophrenia, the serum concentrations of D-Ser are positively associated with cognitive gains induced by intensive cognitive training[65].

D-Ser has also proved effective in the treatment of other neurological diseases and conditions. For example, a pilot randomized controlled trial found improvement in symptoms of post-traumatic stress disorder in patients who were treated with D-Ser[67]. Another pilot study indicated that the basal plasma concentrations of D-Ser correlate with response to the antidepressant ketamine[68]. In line with the decrease in brain D-Ser in ageing mice[57–59], administration of D-Ser reverses the age-related decrease in cognitive flexibility and restores functional brain connectivity and neuronal morphology[69].

D-Ser was proposed to be a suitable biomarker in Alzheimer disease, but the results are inconsistent. Concentrations of D-Ser in the cerebrospinal fluid (CSF) of patients were reported to increase the sensitivity and specificity of the diagnosis of Alzheimer disease[70]. However, much smaller or no changes were observed in another cohort of patients[71]. Recently, Sachi and co-workers found a correlation in a larger number of subjects between serum concentrations of D-Ser and patients with Alzheimer disease[72]. As an internal control, they showed that D-Asp concentrations do not change in the patients. Studies in larger cohorts and with more standardized techniques are still needed to address some of the inconsistencies in the use of D-Ser, as well as other D-amino acids, as biomarkers of Alzheimer disease.

D-Aspartate.—D-Asp is also found in the brain and is synthesized, at least in part, by the serine racemase[73]. D-Asp is mostly present in neurons and decreases rapidly in the neonatal period[74] owing to the expression of its catabolic enzyme D-Asp oxidase[75]. D-Asp also binds to the glutamate-binding site of NMDARs and, when in excess, may mediate NMDAR neurotoxicity[76]. Transgenic mice engineered to overexpress D-Asp oxidase during the prenatal period exhibit lower brain D-Asp and changes in brain morphology[77], indicating that D-Asp has a role in normal brain development. Similar to D-Ser, people with schizophrenia have lower concentrations of D-Asp[78,79], which might contribute to the NMDAR hypofunction in disease development[80]. In Parkinson disease, D-Asp concentrations decrease in the substantia nigra of patients[81], which may reflect the loss of dopaminergic neurons in this region. Although these findings are encouraging, the use of D-Asp as a biomarker requires further research in larger patient cohorts.

D-Alanine and other D-amino acids.—D-Ala is a co-agonist of the glycine and D-Ser site of NMDARs[82]. However, physiological concentrations of D-Ala in the brain are at least one order of magnitude lower than those of D-Ser[82], making it unlikely that this D-amino acid interacts physiologically with NMDARs. D-Ala has been tested as a therapeutic agent for schizophrenia but requires much higher dosages than D-Ser to achieve the same therapeutic effect[83]. Higher concentrations of D-Ala were detected in the white matter of brains among patients with Alzheimer disease[84]. However, in a rat model of Alzheimer disease, plasma D-Ala concentrations were lower than in controls. In this study, concentrations of D-Ser, D-Asp, D-leucine (D-Leu) and D-proline (D-Pro) were also lower than in controls, whereas D-phenylalanine (D-Phe) concentrations increased[85]. Although these observations suggest that D-amino acids may be biomarkers for Alzheimer disease, these changes are not consistent across disease models and need to be confirmed in further studies in animals and humans. The research on D-amino acid biomarkers will likely involve investigating D-cysteine (D-Cys), recently identified as a major product of serine racemase enzyme activity towards L-Cys. This endogenous D-amino acid is very high in neonatal brains and is a negative regulator of cell proliferation; its potential as a biomarker is yet to be investigated[86]. Despite the progress made in recent years regarding the physiological and pathological roles of D-amino acids in the nervous system, much remains to be learned about their suitability as biomarkers, in part because of the difficulties in detecting small amounts of enantiomers in various body fluids.

L-2-Hydroxyglutarate and D-2-hydroxyglutarate.—A strong correlation between chiral organic acids and inherited neurometabolic diseases was found in the neonatal period[87]. In particular, accumulation of L-2-HG and D-2-HG in body fluids can cause L-2-hydroxyglutaric aciduria and D-2-hydroxyglutaric aciduria, respectively, which are accompanied by many neurological symptoms, such as psychomotor retardation, hypotonia, ataxia and seizures[17,88]. In early studies, despite unclear physiological pathways, untargeted metabolic profiling in human tissues led to the discovery of correlations between L-2-HG or D-2-HG and brain diseases[17]. Recently, in vitro studies revealed that increased D-2-HG enhances the glutamate uptake in synaptosomes and, therefore, potentially affects the excitatory neurotransmission in the central nervous system[89]. Studies in transfected cells indicate that D-2-HG exerts its harmful effect by activating NMDARs and causing

dysregulation of intracellular calcium and mitochondrial dysfunction[90]. For L-2-HG, the pathophysiology can be similar to that of D-2-HG[17]. However, characteristic patterns of brain magnetic resonance imaging abnormalities and neurologic symptoms suggest that L-2-hydroxyglutaric aciduria affects different brain regions than D-2-HG. Despite intensive research on the aetiology, the pathophysiology is still under investigation[17,91].

Kidney disease and diabetes

The kidneys regulate the chemical balance of vertebrate animals by selectively filtering and reabsorbing metabolites from blood. Estimation of the glomerular filtration rate (GFR) as an indicator of kidney function is crucial for early diagnosis and follow-up of chronic kidney disease[92]. However, routine laboratory techniques (for example, creatinine clearance) for estimating GFR are inaccurate at high GFR values and depend on muscular mass[92]. Normally, L-amino acids are reabsorbed much more efficiently than D-amino acids at the proximal tubules[93]. D-Ser accumulates in high concentrations in urine because L-Ser displaces D-Ser during amino acid reabsorption[94].

Increases in plasma D-Ser are found in renal failure caused by IgA nephritis[95], diabetic nephropathy[95], hypertensive nephropathy[96] and systemic lupus erythematosus[97]. For example, in one case of rapidly progressive glomerulonephritis due to systemic lupus erythematosus, the concentration of D-Ser in the patient's plasma was extremely high and comprised 19% of whole blood serine in the acute phase[97]. The concentration of D-Ser in the plasma better correlates with the actual GFR, which is decreased in these diseases, and is independent of common clinical factors that affect routine laboratory techniques, such as muscular mass, age and sex[98]. Monitoring the urinary D-Ser to L-Ser ratio rather than plasma has advantages in detecting renal failure. In an acute model of kidney ischaemia, measurement of the urinary D-Ser to L-Ser ratio was more sensitive than the serum D-Ser to L-Ser ratio and all other commonly used laboratory tests to detect renal dysfunction[99]. Thus, for kidney ischaemia, the measurement of D-Ser could provide a useful clinical marker that outperforms the usual assays, provided that techniques for enantiomeric detection in body fluids are widely available. It is noteworthy that the concentrations of other D-amino acids, such as D-Phe, D-tyrosine (D-Tyr), D-asparagine (D-Asn) and D-Pro, also increase in the plasma in chronic kidney injury and may represent new disease biomarkers[100].

Untargeted metabolic profiling has revealed an excess of D-amino acids in patients with diabetes mellitus. In particular, the ratios between D and L-enantiomer concentrations of four amino acids (that is, alanine, valine, isoleucine and leucine) in the nails of patients with diabetes were higher than those of healthy volunteers[101]. One study also found increased urinary D-Phe concentrations in patients with gestational diabetes[102]. However, further studies are needed to confirm this finding. Given that the gut microbiome is also involved in the development of obesity and obesity-related complications, such as type 2 diabetes mellitus, it is tempting to speculate that microbiome-derived D-amino acids may have a role in the diabetic pathogenesis[103]. Despite this potential pathophysiological pathway, further work is needed to evaluate the utility of D-amino acids as biomarkers in diabetes mellitus. In addition, higher concentrations of two D-carboxylic acids (that is, D-lactate and L/D-2-HG) were also found in the saliva, urine and plasma of patients with diabetes[104].

Moreover, elevated D-lactate — a product of methylglyoxal metabolism — can contribute to acidosis and a high anion gap (a medical term that describes the gap between negatively and positively charged electrolytes in serum) in diabetic ketoacidosis[105]. Interestingly, L/D-2-HG not only modulates intracellular communication, as mentioned in the previous section, but also serves as a metabolic by-product to control cell growth[106,107]. Given the structural similarity between 2-HG and α-ketoglutarate, 2-HG is a potent competitor of α-ketoglutarate at the catalytic site of metabolic enzymes[108]. One example is its inhibition of α-ketoglutarate-dependent dioxygenases, which are critical for chromatin modifications required for normal gene expression[104,109].

Cancer

Cancer cell growth is highly influenced by the intracellular and extracellular environment. Metabolic profiling revealed abnormal concentrations of certain D-amino acids and their potential use as biomarkers for different cancer types[110]. For example, elevated concentrations of D-amino acids, such as D-Ala and D-Pro, in gastric juice are associated with early gastric cancer[111]. By contrast, the concentrations of other D-amino acids, such as D-glutamic acid (D-Glu) and D-glutamine (D-Gln), decrease in the serum of patients with hepatocellular carcinoma[112]. Metabolic profiling of human breast cancer cells (MCF-7) and non-tumorigenic human breast epithelial cells (MCF-10) showed upregulation of some D-amino acids in MCF-7, including D-Asp and D-Ser[113].

The concentrations of D-Asp and D-Ser in some cancer cells are thought to be controlled by their synthesis by serine racemase and uptake from the extracellular medium[73]. Serine racemase is upregulated in colorectal adenoma and adenocarcinoma, where it produces D-Ser and also promotes cancer cell growth by dehydrating serine to pyruvate[114]. Many other cancer types upregulate serine racemase mRNA, but the role of serine racemase and D-Ser as biomarkers or in the pathology of these tumours remains to be investigated[115]. Cancer cells also overexpress neutral amino acid transporters, such as ASCT2 (also known as SLC1A5), SNAT1 (also known as SLC38A1) and SNAT2 (also known as SLC38A2)[116]. In addition to L-Gln, which is required for cancer growth, these transporters also use D-Ser as a substrate and can transport D-Ser into cells[117] and, in the case of ASCT2, exchange intracellular D-Ser for extracellular amino acids[118]. At high intracellular concentrations, neutral D-amino acids are degraded by D-amino acid oxidase (DAAO) into hydrogen peroxide (H_2O_2), ammonia and the corresponding α-keto acid[119,120]. Given the cytotoxicity of H_2O_2, treatment of cancer cells with D-amino acids has been proposed as a strategy to inhibit cancer growth[121]. However, this enzyme is mainly expressed in the kidneys, liver and cerebellum, and there is little information on its enzymatic activity in different cancer cells[122]. The accumulation of D-amino acids in cancer cells suggests low endogenous degradation by DAAO in cancer tissue[110].

Although NMDARs are highly expressed in the central nervous system, their upregulation also occurs in tumours and cancer cells outside the brain[123,124]. Blockade of NMDARs decreases cell proliferation in a wide variety of cancer cells[125]. Consequently, binding of D-Ser or D-Asp (either produced by cancer cells or present in the extracellular medium) to non-neuronal NMDARs could influence cancer cell proliferation[126]. Collectively, the

concentrations of D-amino acids may increase or decrease in different cancers, and further research is needed to evaluate their effectiveness as biomarkers and in anticancer therapy[113,119,124,126–128].

The growing understanding of the biochemical consequences of protein mutations has also led to the discovery of novel chiral biomarkers. In particular, mutations of isocitrate dehydrogenase 1/2 (IDH1/2), which controls cellular metabolism, are frequently observed in various cancers such as glioma, cholangiocarcinoma, acute myeloid leukaemia, lung cancer, colorectal cancer and nasopharyngeal carcinoma[129]. Mutations in IDH1/2 prevent the catalytic conversion of isocitrate to α-ketoglutarate, resulting in D-2-HG accumulation[130]. Moreover, D-2-HG inhibits α-ketoglutarate-related diooxygenases, including several histone demethylases, leading to changes in DNA methylation and gene expression[108,130–133].

Summary

Identification of novel chiral biomarkers is important for developing new avenues for disease diagnosis and prognosis, as well as pharmacological studies and therapeutic innovations. The correlation between chiral biomarkers and diseases discovered by untargeted metabolic profiling may also accelerate our understanding of pathological pathways in various diseases. Table 1 summarizes many of the identified disease-related chiral biomarkers and their concentrations in various tissues and fluids. As discussed in the following sections, there are no standardized instruments and protocols for chiral measurements and data analysis. Therefore, the concentrations in different studies may vary owing to differences in sample preparation and detection methods[56,72,134].

Difficulties in clinical detection

The detection of low-concentration chiral biomarkers for diseases in clinical settings is challenging[135]. Herein, we discuss the main factors that make it difficult to discover new chiral biomarkers and complicate the clinical application of chiral separation techniques for known chiral biomarkers.

Variation among chiral small molecules

There are thousands of different chiral small molecules in the human body. A technique that can specifically and accurately detect as many of these as possible is desirable for `multiplexed` chiral analysis to improve disease diagnosis and prognosis. However, the large variation in their molecular properties (for example, charge, mass and functional groups) makes it challenging for a single technique to be universally applied to all relevant species[136,137]. Further, depending on the species and the detection method, analytes may be analysed in native or derivatized forms[138]. There are many possible derivatization agents available, each of which has advantages and disadvantages in terms of extraction and pre-concentration, chiral separation and compatible detection approach(es). Szökö et al. provide an extensive table focusing on how the molecular properties of chiral small molecules affect the requirements and utility of the available detection techniques[137]. For example, different derivatization approaches in mass spectrometry are required for compounds with amine groups and those with carboxylate groups. Grossman and Colburn indicate that different

chiral selectors are required for compounds with different charge, functionality and size in capillary electrophoresis (CE)[139]. The effective dynamic range of cutting-edge metabolomics techniques is nearly five orders of magnitude[140]. However, this is still insufficient for quantification of some small chiral molecules, the concentration range of which can exceed five orders of magnitude (Table 1).

Low concentration in clinical samples

Many chiral small molecules are present at micromolar or lower concentration in human biofluids, which necessitates the use of a highly sensitive detection technique[141]. Moreover, the changes in chiral biomarkers between normal and disease states may be small, especially at the onset of a disease. As shown in Table 1, the average concentration of L-2-HG in the plasma of healthy subjects and patients with cancer may differ by only 0.2 μM. Therefore, detecting changes of 0.2 μM or even less in enantiomeric concentration is essential in such cases for early diagnosis and prognosis of diseases. This detection limit is challenging for some existing techniques (for example, polarimetry[142,143], which can barely quantify concentrations below 1 μM (ref.[144]), and circular dichroism (CD) spectroscopy[145]).

The presence of salts in high concentration and other constituents (for example, proteins, viruses, cells, L-amino acids and D-sugars) in clinical samples can interfere in the detection of low-concentration chiral small molecules[146]. For example, the 22 most abundant proteins account for more than 99% of the dry mass of human plasma, with chiral small molecules making up the remaining 1% of the mass[147]. Therefore, it is necessary to conduct pre-detection sample preparation steps (for example, separation and filtration) to remove interfering constituents. However, poor sample preparation and pre-concentration techniques can result in the loss of low-concentration metabolites or changes in their relative concentrations, resulting in low detection sensitivities and/or inaccurate determination of the targeted metabolites in biological samples[148,149].

Clinical samples collected from the human body are often limited in quantity. With the exception of urine, of which up to 1 l can be collected per day, the collection of biosamples (for example, CSF, brain tissue and blood samples) is limited in volume, and can involve pain and sometimes life-threatening risks. Specifically, because of human health-risk considerations, the maximum quantity of CSF from the brain and blood from a healthy adult human body that can be collected within a 24-h period should be below 20 ml (refs.[150,151]). For paediatric patients and patients in critical conditions, the acquirable sample volume is much smaller. Therefore, it is important for an effective technique to use as little biosample as possible to achieve accurate enantioselective detection. Unfortunately, some of the prevailing analytical techniques (for example, polarimetry[142,143] and CD spectroscopy[145]) cannot meet the quantity requirement for paediatric patients and patients in critical conditions.

Frequency of testing and high cost

For some diseases, diagnosis, treatment monitoring and prognosis via detection of chiral biomarkers will require long duration and frequent testing. For example, in a mouse study, routine chiral testing over 40 h led to a deeper understanding of the pathophysiology and

biochemical consequences of renal failure[99]. However, comparable routine chiral testing has not been conducted on human subjects, and there is no consensus on the standardized frequency and methods of such testing. Considering the frequency of conventional chronic kidney disease and cancer testing, the potential frequency of chiral testing can vary between 0.5 and 1 year, or more often, depending on the disease severity[147].

To detect chiral molecules at low concentrations (for example, <1 μM), costly instrumentation (for example, liquid chromatography–tandem mass spectrometry) might be necessary to achieve high sensitivity (down to picomoles) and selectivity (up to 18 chiral amino acids)[152]. The number of samples to be analysed per individual can be large when chronic diseases such as certain cancers and diabetes are involved, given the long duration of treatments and potential for relapse. This results in high testing costs. Tremendous efforts are required to develop high-throughput techniques that can routinely and accurately detect chiral biomarkers at lower cost to reduce the financial burden on patients and healthcare systems while increasing patient access.

Summary

Owing to these existing challenges, there is still no standardized chiral detection technique for disease applications. Usually, there are tradeoffs in several parameters (for example, sensitivity, accuracy, speed of analysis, cost). For example, certain aromatic tagging agents can render amino acids and carboxylates fluorescent or amenable to chiral analysis[137]. Therefore, several chiral detection techniques have been developed to satisfy specific parameters.

Detection techniques

In this section, we examine the existing techniques for identifying and measuring chiral biomarkers. We focus on the working principles and clinical applications of the most common of these techniques and highlight their unique features. A brief description of sample preparation is also provided because this is necessary to reduce the complexity of clinical samples for analytical chirally selective techniques.

Sample preparation

It is often necessary to include sample preparation steps prior to the detection and analysis of small chiral molecules. Human biofluids contain millions of small and large molecules. To specifically detect the often low-concentration chiral molecules of interest, it is important to purify the sample and separate specific targeted molecules from the biofluid (Fig. 2).

Sample collection.—Sample collection is the first step in the detection and analysis of chiral biomarkers. The reliability of the testing depends on several environmental and human factors during collection, minor variations of which can change metabolite concentrations. Consistent collection times and fasting status are vital to maintain similar conditions for all samples. It has been shown that at least 19% of the metabolites in human plasma — especially amino acids, corticosteroids and bilirubin — exhibit substantial time-of-day variation[153]. The time-of-day variations in urine metabolites can be even greater than those

of plasma[154]. Therefore, sample collection for chiral metabolites should typically be done in the morning after 8–12 h of fasting[155].

Another important factor is contamination during sample collection. Sample collection should be conducted quickly to avoid airborne contamination. Furthermore, all sample collection tubes must be kept sterile and dust-free. This is because microbial contamination can rapidly alter concentrations of small-molecule metabolites by adsorption and metabolism. In addition, dust particles are well-known sources of amino acids and adsorb many small-molecule metabolites[156]. Sample tubes containing plasticizers and polymers have been found to be the major sources of contamination in mass spectrometry analysis[157]. Depending on sample type, ethylenediaminetetraacetic acid (EDTA)-containing, heparin-containing and citrate-containing tubes are most commonly used for serum to prevent coagulation of blood in a clinical setting. In particular, EDTA sample tubes were found to present a higher content of amino acids (for example, aspartate, histidine and glutamine) than citrate ones[158]. Unlike serum containers, which require specialized materials, urine sample containers can be simple polypropylene tubes[159].

Sample pre-processing.—After sample collection, pre-processing steps must be performed on samples before chiral analysis, while ensuring that the original chiral metabolite concentrations are preserved. In general, this involves centrifugation, filtration and addition of pre-servatives. The aim of centrifugation or filtration is to remove the cells, bacteria and large proteins. Typically, cells and cell debris are removed from biofluid by centrifuging at low speed (~1,000–3,000 *g*) for between 15 and 30 min (refs.[160,161]). Some studies showed that the centrifugation speed and time affect the metabolomic profiles of plasma samples[162], whereas others showed no significant differences based on these centrifugation parameters[163]. It should be noted that the speed and time of centrifugation should not be too high or too long to prevent the rupturing of blood cells, which can alter the concentrations of metabolites[164]. After the first centrifugation, the supernatant liquid is taken. To further extract small chiral metabolites (for example, amino acids), 5–15% sulfosalicylic acid and trichloroacetic acid (TCA) should be added to the supernatant to help precipitate and remove proteins[165]. This is followed by a second high-speed centrifugation (~10,000–15,000 *g*)[160,166]. This second supernatant layer contains mostly small molecules. Further purification can be conducted through cation or anion exchangers by derivatizing polar small molecules; or using solid-phase extraction with a more hydrophobic solid phase; or by liquid–liquid extraction[167].

The preparation processes for serum and plasma are similar, except that an additional coagulation process is required for serum prior to centrifugation, which has been shown to affect metabolite concentrations[168]. Higher lactate and amino acid amounts and lower glucose concentrations have been found in serum than in plasma[169]. However, there is still no general selection rule for the most suitable matrix. The bottom line is to maintain consistency of sample type throughout a study. The pre-processing procedures for urine and saliva are similar to those of serum and plasma, and also require centrifugation and TCA precipitation[170–172]. For tissue samples and cell cultures, extra steps before centrifugation are required to collect the desired small molecules. These steps involve the breakdown of

the tissue matrices into liquid suspensions through homogenization[173]. For more details, additional discussion about serum and plasma can be found elsewhere[174,175].

In summary, sample preparation steps are essential for measuring chiral biomarkers. Several factors in the sample preparation, including small-molecule `recovery rates`, filtration efficiency and procedural complexity, need to be balanced to achieve accurate, reproducible and cost-effective chiral detection[176]. The addition of an internal standard greatly improves accuracy and reproducibility. Standardization of pre-processing approaches is still under debate and can depend on the nature of the sample and the diseases of interest.

Chromatography

Liquid chromatography.—Liquid chromatography is a commonly used separation technique for small molecules[177]. When different small molecules in a solution migrate in a chromatography column, they are separated — owing to their different affinities towards the solid phase — and have molecule-dependent retention times. The detection and quantification of specific molecules can then be achieved by coupling the chromatography to a mass spectrometry detector or a fluorescence detector. Conventional liquid chromatography–mass spectrometry can achieve separation of molecules in a mixture based on their mass to charge ratio, but cannot distinguish between enantiomers, which have identical mass. Different separation strategies can be used to achieve enantiomeric separation and detection of small chiral molecules (Fig. 3).

The oldest approach to achieve enantiomeric separation and detection is to pre-derivatize the clinical analytes of interest with an enantiomerically pure chiral derivatization reagent (CDR) before chromatography[152,178]. The resulting diastereomeric products from the enantiomers can be separated based on their different affinities with the achiral stationary phase in a chromatography column. For example, a chiral *o*-phthalaldehyde-*N*-acetyl-l-cysteine CDR can react with the primary amine groups of most amino acids[179]. With this approach, 15 pairs of enantiomers of chiral amino acids were resolved using high-performance liquid chromatography (HPLC). Liquid chromatography with CDRs has led to the discovery of fivefold and threefold increases in the concentrations of D-Ser and D-Asp, respectively, in CSF samples from patients with Alzheimer disease, compared with healthy control groups, whereas the concentrations of L-amino acids remained almost the same[180]. Chiral organic acids, such as 2-HG, can be separated using *O*-acetyl-di-(*o*)-2-butyl esters as the CDR in liquid chromatography. Their use for chiral detection in urine, plasma and CSF from patients with aciduria led to the observation of a 150–200-fold increase of the measured concentration of D-2-HG, which is related to aciduria[181]. Liquid chromatography with a CDR also has the advantage of being low cost. Disadvantages include the requirement for the CDR to be enantiomerically pure, which is rarely the case. Reagent chiral impurities result in spurious diastereomeric peaks and poor quantification[182]. Incomplete derivation, for kinetic reasons, can result in altered diastereomeric ratios, and diastereomers do not have to have identical detector responses[183]. Moreover, not all molecules of interest have appropriate functional groups to derivatize. In addition, different CDRs need to be developed for different types of analytes. Finally, unwanted side reactions might lead to strong

background noise and interfering spectra during data acquisition. For these reasons, this approach is rarely used today.

The other strategy is to use chromatography columns with a chiral stationary phase or, less commonly, to use a chiral mobile-phase additive with an achiral stationary phase[152,177]. Chiral mobile phases must not interfere with detection (for example, they must be non-UV absorbing and volatile if using mass spectrometry detection) and be enantiomerically pure, as is the case for CDRs[183]. Moreover, a considerable amount (several grams) of the chiral mobile-phase additive may be needed for separations. Nevertheless, it has been shown that liquid chromatography with a chiral mobile phase allowed the separation and subsequent detection of 18 chiral amino acids in plasma at picomolar concentrations. Such amino acid profiling enabled the observation of clear association between Alzheimer disease and plasma concentrations of D-Ser, D-Asn, D-Asp, D-Pro, D-Leu and D-Phe[85].

Liquid chromatography with a chiral stationary phase is applicable to a greater variety of small molecules than liquid chromatography with a chiral mobile-phase additive, and is often used for untargeted metabolite profiling[183]. Moreover, without the requirement for chiral derivatization, there are fewer sample preparation steps. In one example application of liquid chromatography with a chiral stationary phase, chiral organic acids were quantified, leading to the detection of elevated concentrations of D-2-HG in urine samples from patients with D-2-hydroxyglutaric aciduria[184]. It has been noted that chiral columns are also usually more expensive than achiral columns with chiral derivation. However, because individual chromatography columns are typically used for hundreds or even thousands of analyses, the cost per separation between chiral and ‘derivatized achiral’ separations is negligible.

To improve the selectivity and peak capacity of chiral separation, 2D and 3D chiral liquid chromatography techniques have been developed, in which two or three sequential columns are applied[126,185–188]. For example, a 2D chiral liquid chromatography that combined an achiral column and a chiral column was used to detect trace amounts of chiral metabolites, including those with enantiomeric excess below 1%[187]. In another example, four D-amino acids (phenylalanine, tyrosine, tryptophan and leucine), with concentrations ranging from 0.01% to 1% of the corresponding L-amino acids, were found in human urine through 2D chiral liquid chromatography that combined an octadecylsilyl column (achiral) and a Crownpak CR(+) (chiral) column[188]. 3D-HPLC methods have shown improved selectivity for the clinical samples over 2D-HPLC. For example, a 3D-HPLC method was developed for the determination of trace concentrations of D-Asn, D-Ser, D-Ala and D-Pro in human serum[186]. In this case, the extra anion-exchange column was needed as the second dimension to separate the interfering compounds from the derivatized amino acid analytes.

Gas chromatography.—Gas chromatography operates on an analogous principle to liquid chromatography, but requires analytes to be vapourized before they are delivered to, and separated in, a gaseous mobile phase travelling through a capillary column[189]. Either a CDR or a chiral stationary phase is needed for gas chromatography to achieve separation and subsequent detection of enantiomers. For example, gas chromatography with *N,O*-pentafluoropropionyl isopropyl derivatives has been applied to enantioselectively separate serine and alanine, and to determine their enantiomeric excess in blood and brain

tissues from rats[20]. Because gas chromatography requires volatile compounds, it is often used for compounds that contain derivatized hydroxyl, amine or carboxylate groups[190]. Amino acids must be doubly derivatized (that is, both the carboxylate group and the amine group must be sequentially derivatized) to obtain a sufficiently volatile product[191]. Typically, an ester is formed from the carboxy group and a small amide is formed from the amine functional group. When using an achiral column, one of the two derivatizing agents must be chiral. In contrast with liquid chromatography, gas chromatography often requires an additional derivation step for volatilizing the analytes into the gaseous phase.

Capillary electrophoresis

CE separates migrating molecules of different size to charge ratios in a capillary or channel across which a high voltage is applied[139] (Fig. 3). After separation, a mass spectrometry or fluorescence detector is applied at the end of the channel to obtain sufficiently sensitive detection of the analytes. CE requires chiral mobile-phase additives in the running buffer in the capillary or channel to achieve enantiomeric separation and detection. CE often provides high efficiency separations and relatively short migration times, but these vary widely with the chiral mobile-phase additive used, the nature of the running buffer and the separation voltage. In one example study, the separation of ten chiral and three achiral amino acids using chiral CE was obtained in ~25 min[192]. CE has also been used to detect chiral amino acids and chiral drug molecules in human biofluids (for example, urine and plasma) with nanomolar sensitivity[193]. Chiral CE coupled with laser-induced fluorescence (CE–LIF) detection also allowed the simultaneous determination of the D-Ser and L-Ser concentrations in the midbrain tissues of a Parkinson disease mouse model[194]. CE–LIF has been used to detect high concentrations of D-Ser and L-Glu in single glial vesicles, indicating that these vesicles share similar biochemical features to neuronal synaptic vesicles[195]. CE–LIF has also been used to detect D-Asp and D-Glu in individual neurons isolated from the central nervous system[196]. The main advantage of chiral CE is its small sample size requirements and relatively short separation times[197]. However, chiral CE has relatively low sensitivity compared with chromatography and difficulties in coupling chiral CE and mass spectrometry, which result from contamination of the mass spectrometry by chiral selectors, such as cyclodextrins[198]. For these reasons, chiral CE is usually coupled with fluorescence detectors and used for large-molecule separations (such as proteins, peptides and oligonucleotides), and is used far less commonly than liquid chromatography for small-molecule separations in clinical settings[199].

Nuclear magnetic resonance spectroscopy

Conventional nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for detecting molecular fingerprints and determining molecular structures[200,201]. For example, NMR spectroscopy was applied to determine the absolute configurations (that is, the chirality) of 2-HG and 5-oxoproline in human urine samples, which had strong correlations with independently diagnosed D-2-hydroxyglutaric aciduria and L-5-oxoprolinuria[202]. However, enantiomers of small molecules have indistinguishable conventional NMR spectra. To achieve enantiomeric detection, NMR requires either CDRs or the addition of chiral shift reagents to convert enantiomers to diastereomeric derivatives or association complexes,

respectively[203] (Fig. 4a). Moreover, without proper sample pre-separation, the NMR spectra are noisy, which limits the number of detectable metabolites to ~10, far from the 20 detectable with sample pre-separation and sensitivity to ~50 nM (refs.[202,204]). Chromatographic or electrophoretic separation as an extra sample preparation step can be coupled with chiral NMR to reduce noise and improve resolution for the detection of chiral small molecules in complex biosamples. However, if such pre-separation steps are necessary, NMR is superfluous unless it also can be used to determine the absolute configuration. Hence, there are few reports involving the use of NMR to detect and identify low-concentration chiral biomarkers of clinical importance.

Enzymatic assays

Enzymatic assays with chiral reagents can detect targeted enantiomers of specific molecules by catalysing the chemical reaction of a specific enantiomer to a by-product[205]. For example, the glucose oxidase enzyme catalyses the oxidation of D-glucose, but not L-glucose, to H_2O_2 and D-gluconolactone[206]. Fluorescent imaging of the by-products can be applied to determine the concentrations of the specific enantiomers (Fig. 4b). Many common chiral small molecules, including amino acids and organic acids, have well-developed enzymatic assay kits for their measurements in biofluids[207]. For example, a DAAO-based chemiluminescent assay was used to assess the concentrations of D-Ser in brain tissues[21] and to monitor online its release from living neural cells[208]. In another study, using purified DAAO coupled to a tyramine-based fluorescent assay, the total concentration of 14 D-amino acids in CSF was profiled and was found to be 1.48 times higher in people with Alzheimer disease than in healthy controls[209]. In the context of diabetes, enzymatic detection of D-Lactate in human plasma and urine uncovered a twofold increase in the D-Lactate concentrations in urine samples from people with diabetes when compared with healthy controls[18]. Conventional transition metals can be used to enhance the catalysis reaction without chiral selectivity[210]. However, by engineering chiral monodentate or multidentate ligands within the coordination sphere of the metal catalyst, it can be used to enhance the reaction for the asymmetric synthesis of nonracemic chiral compounds[211], and their products can also be measured by current flow using amperometric biosensors. For example, an amperometric chiral biosensor was used to detect several D-amino acids in serum[212] and urine[213] with a better response time (<50 s) than fluorescence imaging (~ 30 min).

Because of the high selectivity, low cost, minimal sample preparation requirements and short detection times (<50 s)[212,213], enzyme-based chiral sensing is widely used in measuring concentrations of chiral small molecules in biofluids and tissues for early diagnosis and prognosis of diseases, such as cancers, kidney diseases and cardiovascular diseases. However, enzyme assays usually have limited sensitivity at ~1 μM, which is above the concentration of some chiral small molecules (Table 1). In addition, the specific enzymes required are not readily available for all chiral biomarkers. For example, no L-glucose oxidase is commercially available. Moreover, enzymes often display cross-selectivity for other related molecules. For example, DAAO can react with more than one type of D-amino acid. This limits specificity and accurate quantification and makes it difficult to measure enantiomeric excess using enzymatic assays in these cases.

Chiroptical spectroscopy

Polarimetry.—Polarimetry can determine the overall chirality of molecules in a solution by monitoring the degree of rotation of plane-polarized light passing through the sample[144] (Fig. 5). However, polarimetry has low sensitivity and additional separation or fractionation steps (for example, using chromatography and centrifugation) are required to achieve the detection of targeted chiral small molecules in complex biosamples. For example, conventional liquid chromatography with an achiral column was coupled with a polarimeter for determining the enantiomeric purity in a standard solution with a sample loading of 50 μg and claimed enantiomeric excess accuracies of ±1%[142]. However, if a certain chiral biomarker has a much higher concentration than other biomarkers in a sample, polarimetry can also be used to approximately quantify the enantiomeric purity without the sample separation[214]. For example, in vivo, real-time, non-invasive monitoring of blood chirality was demonstrated by measuring the polarimetric signals from the anterior chambers of rabbits' eyes, which showed a strong linear correlation with blood glucose (which exists in the D-form in living bodies) concentrations that were measured using glucose meters[143]. Overall, polarimetry has the advantages of being capable of monitoring chirality changes with good speed (<1 min) and having simple sample preparation steps. Moreover, it can be used detect analytes that do not absorb UV–visible light, if they are in sufficiently high concentrations. However, because of its limited capability of detecting enantiomeric concentrations and its limited sensitivity above 1 mM, it is rarely used to measure disease-related chiral biomarkers.

Circular dichroism spectroscopy.—CD spectroscopy is another `chiroptical` technique, which can quantify the enantiomeric excess of chiral molecules by measuring the difference in molecular absorption between left and right-handed circularly polarized light[215] (Fig. 5). The signs and amplitudes of the spectral peaks and troughs of the CD spectra can provide information about the enantiomeric purity of chiral molecules in a sample, although standard sample compounds and curves are required. Moreover, additional separation steps are required to detect specific chiral biomarkers[216]. For example, CD spectroscopy coupled with HPLC has been developed to quantify the enantiomeric excess of phenylalanine in a mixture of 15 amino acids with a detection limit of ~5 μM (ref.[217]).

Although it can detect the enantiomeric purity of chiral metabolites at a similar enantiomeric excess to polarimetry, CD spectroscopy is mainly used to determine the secondary and tertiary structures of chiral macromolecules, such as proteins[145,218–220]. For example, disease-related secondary structures of amyloid-β-associated and amyloid light chain-associated amyloidosis can be quantified in the far-UV region[221]. However, as with polarimetry, extra separation and concentration steps are necessary to achieve targeted amyloidosis measurements. For example, CD spectroscopy has been used to detect amyloidosis in biofluids and tissues for brain, kidney and cancer diseases[145,218,219]; the lowest reported detection limit for protein in these studies was 0.1 mg ml^{-1} (ref.[145]).

CD spectroscopy provides more molecular information than polarimetry and has greater sensitivity for analytes that contain chromophores. However, it has poorer sensitivity and specificity than chiral liquid chromatography coupled with mass spectrometry or

fluorescence detectors. Moreover, a relatively large volume of biofluid (~1 ml) is required for CD spectroscopy[145].

Raman optical activity spectroscopy.—Raman spectroscopy typically detects vibrational modes of molecules based on the inelastic scattering of light. The high specificity of Raman spectral shifts has allowed these shifts to be used as molecular ‘fingerprints’ to detect amino acids, protein and cells in human biofluids for diagnostic applications[222]. Raman optical activity (ROA) arises from the difference between the Raman spectra of molecules excited by left and right-handed circularly polarized light. The ROA spectra can reveal the absolute configuration and conformational dynamics of chiral molecules with high specificity[222,223] (Fig. 5). ROA spectroscopy has been used to successfully detect a wide range of chiral biomolecules, including amino acids, carbohydrates, polypeptides and nucleic acids[224,225]. ROA spectra provide more stereochemical information than polarimetry and CD spectroscopy. However, for clinical measurements, ROA spectroscopy requires substantial analyte enrichment because its sensitivity is limited to ~0.2 mM (ref.[226]). The long signal acquisition time also limits ROA’s applicability in measuring chiral biomarkers for diseases.

Vibrational circular dichroism spectroscopy.—Vibrational circular dichroism (VCD) spectroscopy distinguishes enantiomers by their chirality-dependent optical absorption of circularly polarized light upon the excitation of their vibrational modes[227]. By analysing the *g* factors (that is, asymmetry in circularly polarized light absorption) across a broad frequency range, one can quantify the enantiomeric concentrations of chiral molecules with high resolution (Fig. 5). For example, the enantiomeric excess of chiral camphor and borneol was determined by VCD spectroscopy with a resolution of 1%[228]. By comparing the different vibrational modes in the VCD spectra with ab initio calculations, the absolute configuration of chiral molecules can also be determined, leading to high molecular specificity[228,229]. However, similar to ROA, VCD has limited sensitivity (~5 mM) and long signal acquisition times (up to several hours)[230]. Methods for analyte enrichment or signal enhancement are required for VCD spectroscopy to routinely detect chiral biomarkers in clinical samples.

Surface-enhanced chiroptical sensing

Chiroptical spectroscopic techniques have unique advantages in the enantiomeric detection of chiral small molecules and can often be used without the need for chiral derivations or added chiral selectors. However, most of them, including polarimetry, CD spectroscopy, ROA spectroscopy and VCD spectroscopy, have limited sensitivity and often require large quantities of sample, which limits their applicability in detecting low concentrations of disease-related chiral biomarkers in low-volume clinical samples. To overcome these limitations, surface-enhanced chiroptical spectroscopy has been developed.

Surface-enhanced CD spectroscopy.—Chiral plasmonic nanostructures, which are systems containing nanostructured metallic components that allow exploitation of surface plasmon resonances, have been used to enhance the sensitivity of CD spectroscopy for detecting small chiral molecules adsorbed to appropriate nanostructures. The enhancement

is enabled by the strong chiral electrical fields that exist near optically excited plasmonic nanostructures[231,232]. For example, gammadion-shaped metasurfaces, twisted nanorod metasurfaces and moiré chiral metamaterials with strong intrinsic CD signals have been applied to achieve sensitive detection of chiral molecules[233–236]. When chiral molecules approach metasurfaces or metamaterials, the CD spectral shifts of the metasurfaces or metamaterials with opposite handedness respond differently. The spectral shifts of left-handed and right-handed metasurfaces or metamaterials are typically represented as λ_{LH} and λ_{RH}, respectively. Depending on the absolute value of the dissymmetry factor ($\Delta\Delta\lambda = \lambda_{RH} - \lambda_{LH}$), the handedness and chiral purity of the chiral molecules can be determined. Because of the enhanced sensitivity, the application of chiral metasurfaces or metamaterials for chiral molecule detection can greatly reduce the sample consumption from millilitre to microlitre amounts. Moreover, with the integration of plasmon-enhanced molecular accumulation, moiré chiral metamaterials[234,235] have been used to detect enantiomeric purity of chiral biomarkers, including glucose and lactate, with a sensitivity of ~100 pM[237]. This higher sensitivity enabled the detection of a diabetes-induced abnormal dextrorotatory shift in the chirality of urine metabolite with a good diagnostic accuracy of 84%[237]. Achiral plasmonic nanostructures have also been demonstrated to enhance the CD spectroscopy of chiral small molecules[238]. Once chiral small molecules are adsorbed on the surfaces of achiral plasmonic nanostructures, strong CD signals are induced near the plasmonic-resonance wavelengths, leading to higher sensitivity than conventional CD[231,232].

Another strategy for surface-enhanced CD spectroscopy is to use the self-assembly of plasmonic nanoparticles and chiral small molecules, whereby two or more colloidal plasmonic nanoparticles spontaneously bind with chiral molecules to form chiral metamolecules[239]. Such self-assembled metamolecules can exhibit strong CD signals near the plasmonic-resonance wavelengths, improving the sensitivity of CD spectroscopy. For example, cysteine was bound with gold nanoparticles to form chiral metamolecules, which enabled the enantiomeric detection of cysteine with a sensitivity of 20 pM (ref.[240]). Dielectric nanostructures and metasurfaces have also been demonstrated for surface-enhanced CD spectroscopy with a potentially higher figure of merit and better sensitivity than their plasmonic counterparts[241].

With strongly enhanced sensitivity and drastically reduced sample consumption, surface-enhanced CD spectroscopy is a promising technique for detecting abnormal chiral biomarkers for disease diagnosis[242,243]. Further, the small size of plasmonic and dielectric nanostructures may lead to the development of point-of-care biomedical devices[244]. However, similar to conventional CD spectroscopy, proper sample separation and extraction procedures are required for surface-enhanced CD spectroscopy to be used to specifically measure a targeted chiral biomarker in complex clinical samples.

Surface-enhanced ROA spectroscopy.—The Raman signals of molecules can also be enhanced by plasmonic nanostructures[245]. This enhancement strategy has been applied to increase the intrinsically weak ROA signals of chiral small molecules. For example, L-ribose and D-ribose attached to silver–silica nanotags via covalent binding led to enhanced ROA spectra with mirror symmetry for the ribose enantiomers[246]. The enhancements can be up to three to six orders of magnitude greater than those of conventional ROA. In addition,

the acquisition time of surface-enhanced ROA spectroscopy is ten times shorter than that of conventional ROA spectroscopy[246]. Therefore, this may become a powerful tool for the detection of chiral biomarkers with high specificity. However, the reliability and the ease of spectral interpretation of surface-enhanced ROA spectroscopy need to be improved before it can be widely adopted for clinical applications.

Challenges and opportunities

Each technique for the detection and quantification of disease-related chiral biomarkers has advantages and limitations in biomedical research and for clinical applications (Table 2). Currently, chiral liquid chromatography and 2D liquid chromatography–mass spectrometry are the most widely used approaches because of their universality of application, sensitivity and reproducibility of qualitative and quantitative data. Many new approaches, especially chiroptical spectroscopy methods, could be advantageous in terms of speed, cost per analysis and sensitivity. However, they are currently not as common as chiral liquid chromatography or 2D liquid chromatography–mass spectrometry in clinical applications. In addition, every method requires some degree of sample preparation, which is often the ‘rate limiting step’ of the analysis. In this section, we discuss the challenges and opportunities for the development of reliable analytical techniques that can be widely adopted for chiral biomarker detection in disease diagnosis and monitoring.

Sample preparation

Proper sample preparation that can extract targeted chiral biomarkers from complex biosamples with a high recovery rate is crucial for accurate chiral detection[247]. This often requires the use of sophisticated sample preparation procedures[248]. Moreover, the large compositional, chemical and physical differences among different types of biosamples, such as urine, blood, CSF and tissues, require the sample-specific procedures[249]. Finally, many laboratories develop their own sample preparation procedures independently. These factors contribute to the high cost, high time consumption and low reliability of chiral biomarker detection. Therefore, standardization and automation of the sample preparation procedures will facilitate the advancement the field[250]. Automation is dependent on progress in the fields of microfluidics, machine learning and robotics.

Data validation and reproducibility

Many of the spectroscopic analytical techniques for detecting chiral biomarkers in clinical samples are still in the early stages of development. In all techniques, there is a lack of standardized instruments and protocols for measurements and data analysis. Further, sample complexity and variability can lead to changeable and sometimes contradicting measurement results[251]. These problems need be addressed to advance the detection of chiral biomarkers into clinical practices[252].

Cost-effective and rapid detection

Because the concentrations of chiral biomarkers are strongly related to the identification and progression of many diseases, it is essential to routinely detect the variation of chiral biomarkers during diagnosis, prognosis and treatment monitoring[253]. Devices that can

operate at the point of care will be especially beneficial to patients with chronic diseases, including some cancers and diabetes, by reducing the cost and increasing accessibility[254]. Rapid detection (that is, with results produced within 1 h of sample collection) is desirable for physicians to identify diseases, identify the potential for relapse and determine treatment strategies at the earliest time to improve patient outcomes[255]. However, the most widely adopted techniques, such as chiral chromatography coupled with a mass spectrometer, are expensive and can be time-consuming (that is, hours to days) unless multiplexed. Moreover, they are often limited to use by experienced technicians or scientists in centralized laboratories. We expect that tremendous development in the field of point-of-care devices will lead to cost-effective and rapid detection of chiral biomarkers in the future[256].

Multiplexed detection of chiral biomarkers

Multiplexed detection (that is, simultaneous characterization and quantification of multiple biomarkers) is becoming increasingly important owing to the growing demand for untargeted profiling[257]. Simultaneous measurement of several chiral biomarkers is also desirable in clinical practices to increase disease specificity when the detection of a single biomarker is insufficiently sensitive. It is also desired for ruling out factors that could drive changes of a specific biomarker[258]. Currently, most chiroptical techniques have not achieved multiplexed detection. However, chromatography and CE can be used to analyse multiple chiral small molecules in a single sample analysis[259]. A challenge in simultaneously quantifying various amino acids and organic acids arises from the large amount of complex data generated by different chiral molecules within the low m/z area where there is also strong background noise[260]. To address the challenge for multiplexed detection, novel approaches and sensor designs are needed[261]. Machine learning has proved promising in complex data analysis. Its implementation in chiral detection will increase the detection accuracy and accelerate the identification of multiplexing chiral biomarker detection[262].

Sensitivity improvement

Many of the techniques for the detection of small chiral molecules have limited sensitivity, which is typically at a micromolar or milli-molar concentration (Table 2). The limited sensitivity prevents their clinical application, for which it is necessary to detect much lower concentrations of targeted chiral biomarkers to distinguish between patient groups and control groups[263]. The development of analytical techniques with improved sensitivity even up to the single-molecule concentration will broaden the biomedical applications of small-molecule chiral biomarkers in both scientific research and clinical settings[264].

Conclusions

‘Abnormal’ chirality and trace concentrations of enantiomeric small molecules are emerging as potential biomarkers for various diseases. For example, strong correlations have been observed between chiral biomarkers and life-threating and/or chronic diseases, including brain diseases, cancers, kidney disease and diabetes, as well as for osteoporosis, inflammatory bowel disease, atherosclerosis and hypertension[265,266]. However, the pathophysiological mechanism of many of these chiral molecules is still unclear.

Nevertheless, these studies have provided a valuable framework for the future study of new chiral biomarkers in other diseases.

Despite considerable efforts in developing techniques for detecting chiral molecules, some are still at proof-of-concept stages. Improving the sensitivity, increasing the sample throughput and lowering the cost per analysis of these techniques are necessary for both clinical and commercial viability. These improvements will enable the discovery, verification and pathological understanding of new chiral biomarkers, as well as the clinical diagnosis and prognosis of diseases, monitoring of adverse drug effects, companion diagnosis for personalized (precision) medicine and acceleration of treatment decisions.

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Glossary

Chiral	The geometric property of a molecule that precludes it from being superimposable on its mirror image
Chiral selectors	Chiral components in the chiral separation system that interact enantioselectively with the target chiral molecule
Chiroptical	The optical properties related to the interaction of chirality
Enantiomeric excess	The excess of one enantiomer over the other in a mixture of enantiomers
Enantiomers	A pair of compounds that are not superposable onto their own mirror image
Metabolites	Substances produced after metabolism (food, drugs or chemical digestion)
Metabolomics	A systematic study of chemical processes involving metabolites, the small-molecule substrates, intermediates and products of cell metabolism
Metamaterials	Materials engineered to have a property that is not found in the equivalent naturally occurring materials
Metasurfaces	2D thin film materials that can modulate the propagation of electromagnetic waves
Multiplexed	A way of measuring the concentration of multiple biomarkers

Recovery rates	The percentages of molecular concentration that can be recovered from the total concentration after physical or chemical processes

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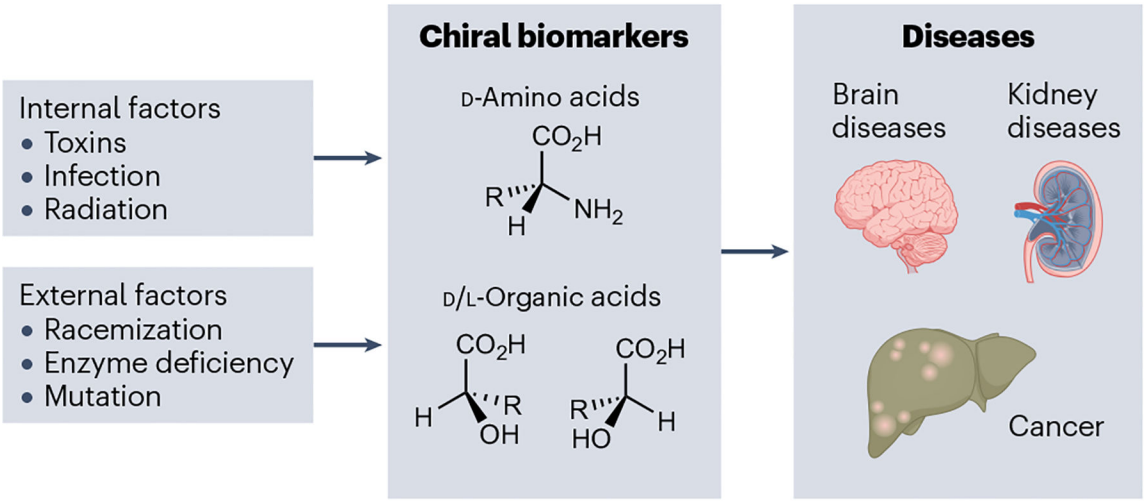

Fig. 1 |. Influential factors for 'abnormal' chirality, common chiral biomarkers and associated diseases.

Internal and external factors can induce abnormal concentrations of chiral biomarkers in the human body, which are associated with various diseases.

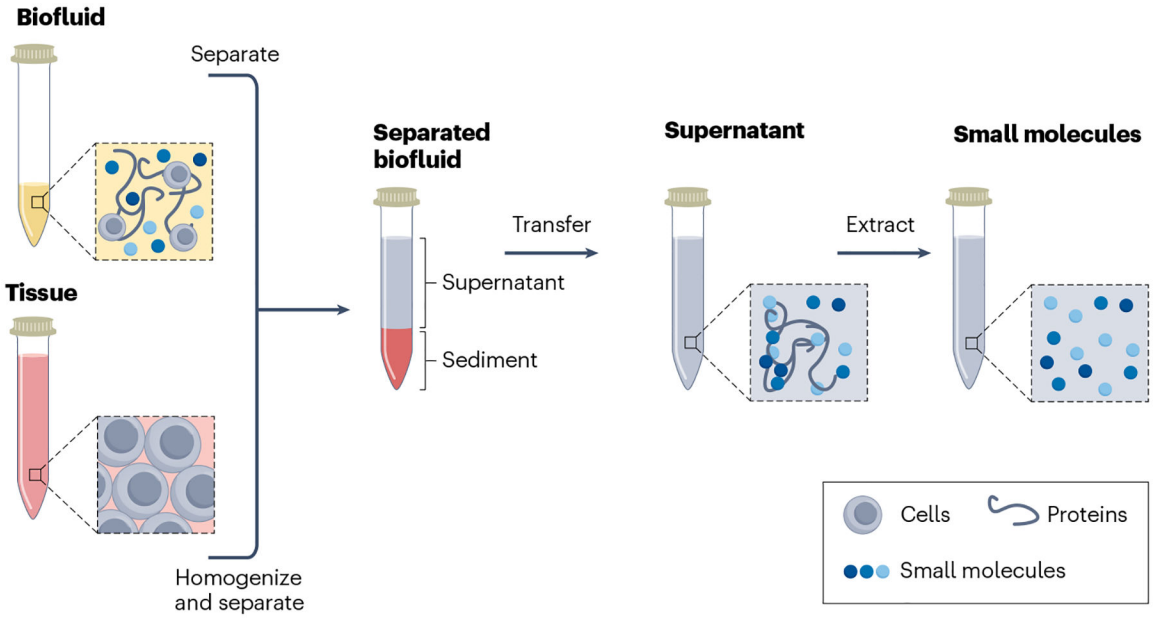

Fig. 2 |. Sample preparation before chiral detection.
Filtration and extraction of chiral small molecules from biofluids and tissues.

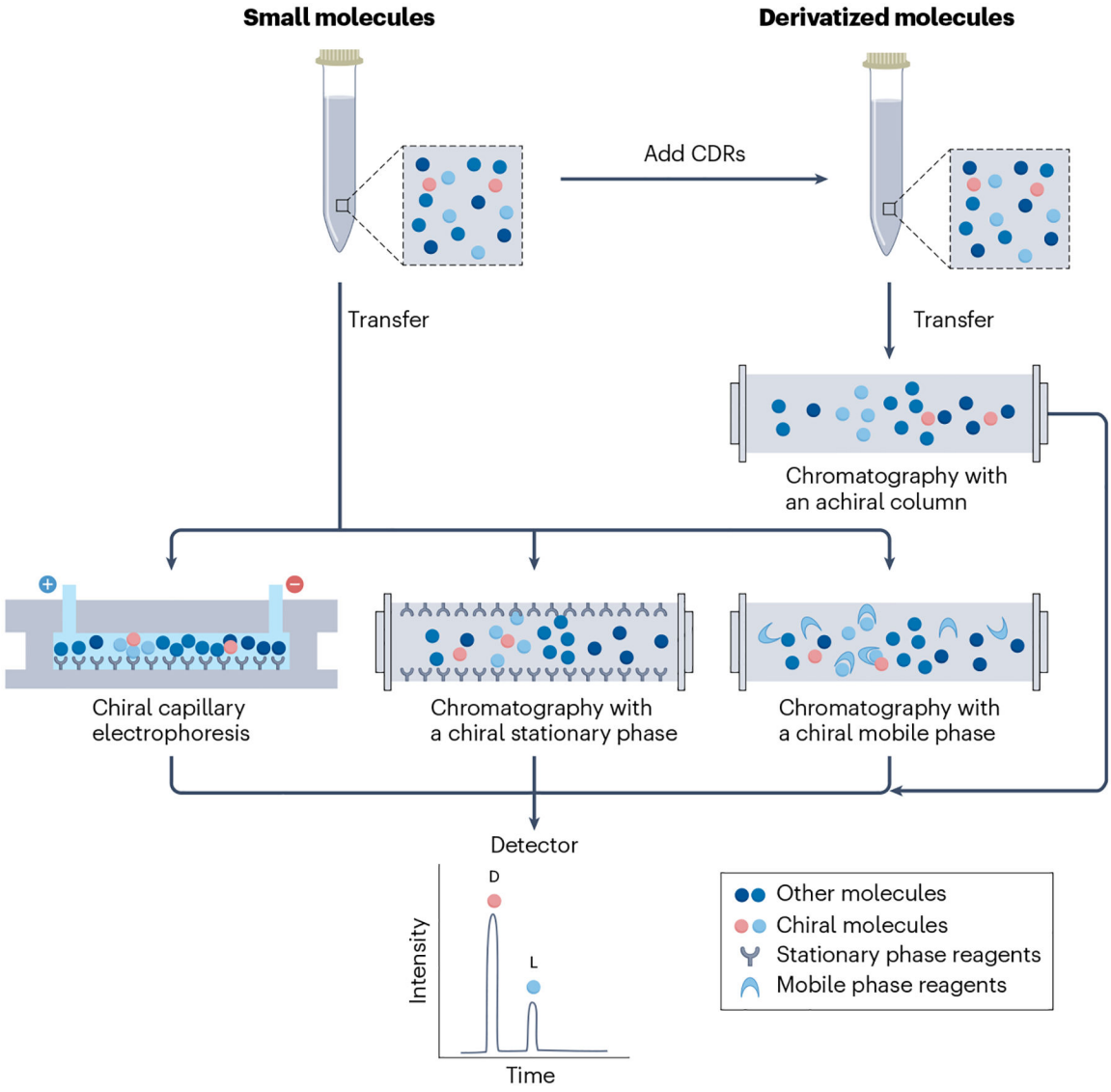

Fig. 3 |. Chiral detection of small molecules via chromatography and capillary electrophoresis coupled to mass spectrometry.

After sample preparation, small molecules can be enantiomerically separated using a chiral selector, such as a chiral derivatization reagent (CDR), a chiral mobile phase or a chiral stationary phase during chromatography. The small molecules can also be enantioselectively separated using chiral capillary electrophoresis. The enantiomers, which have different retention times, are separated and resolved by mass spectrometry.

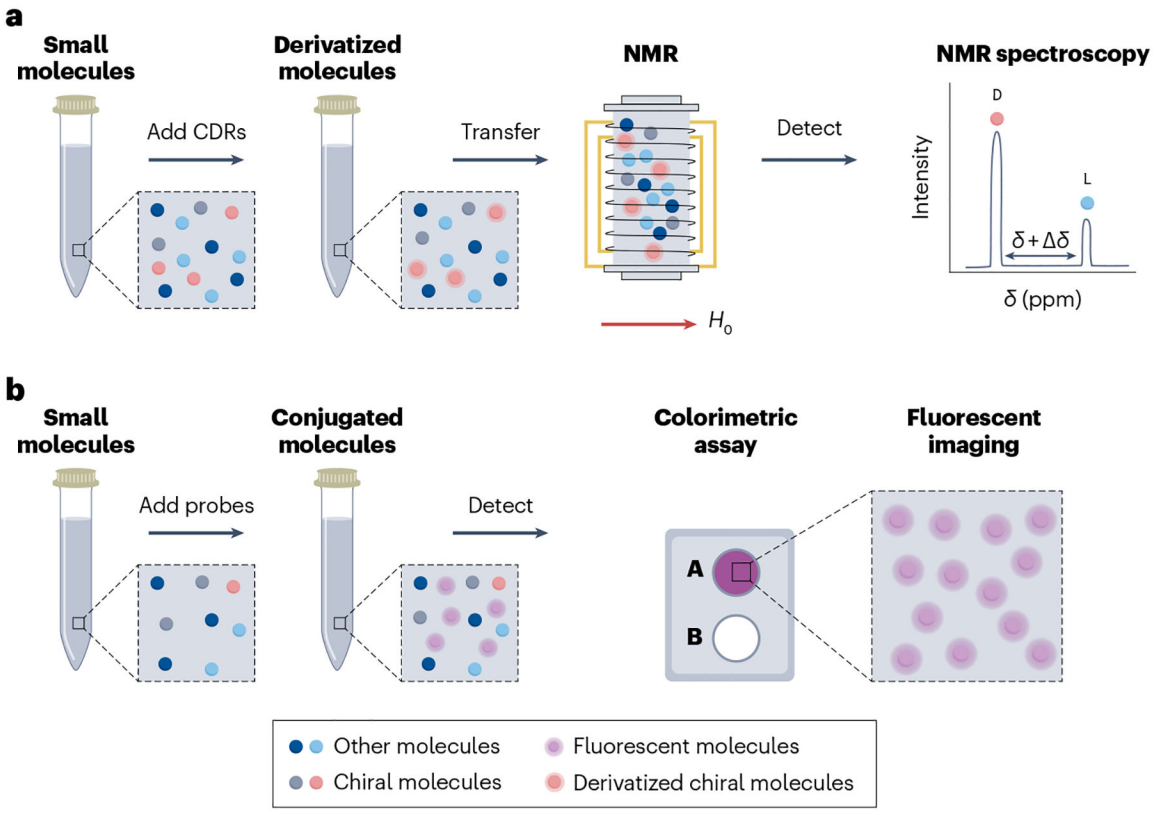

Fig. 4 |. Chiral detection of small molecules with NMR spectroscopy and an enzymatic assay. **a**, After sample preparation, small molecules, with the addition of chiral derivatization reagents (CDRs), can be enantiomerically resolved using nuclear magnetic resonance (NMR) spectroscopy. The intensity between D and L peaks on the NMR spectrum reflects the enantiomeric excess. **b**, After sample preparation, enzymatic probes with enantiomeric selectivity are added to samples to form conjugated molecules. Fluorescent imaging of a colorimetric assay is used to determine the concentration of the targeted enantiomers. H_0, magnetic field.

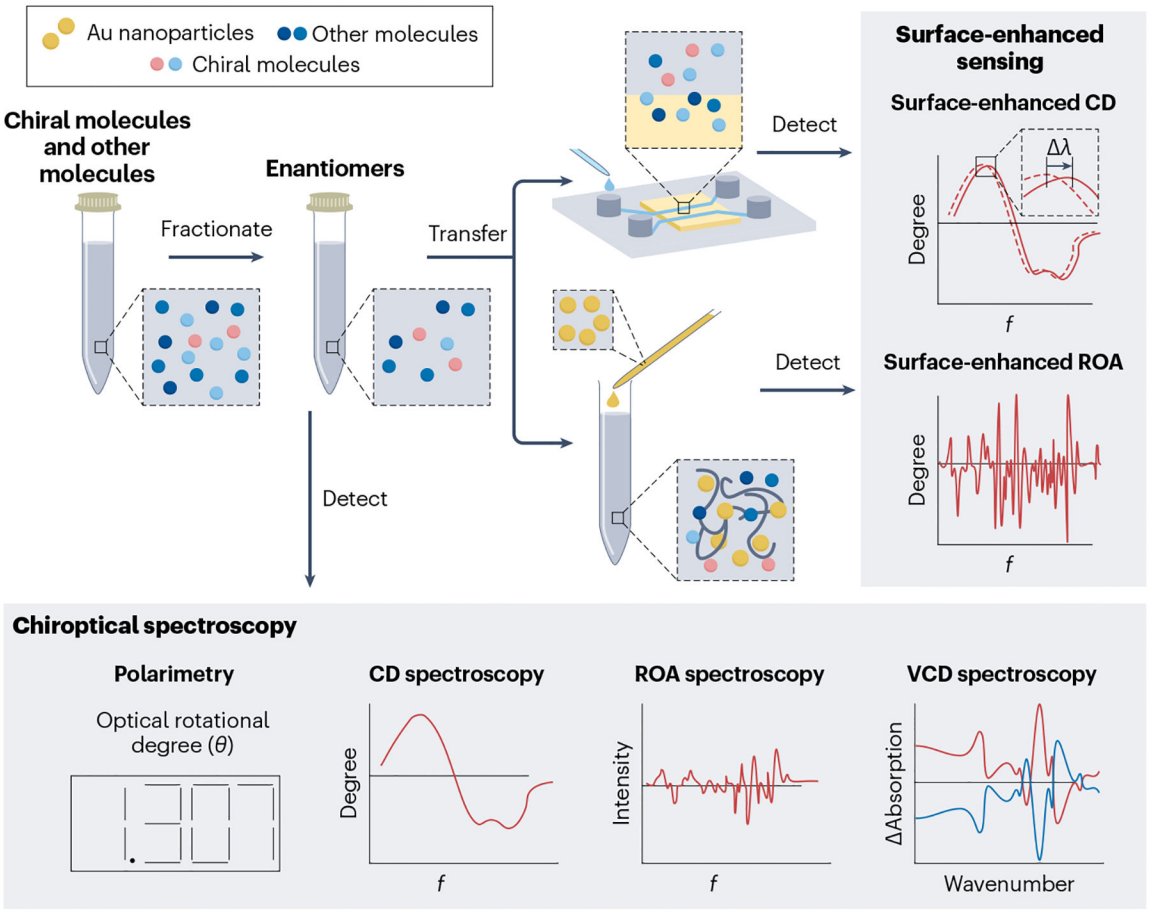

Fig. 5 |. Chiral detection using chiroptical spectroscopy.

Several chiroptical methods and surface-enhanced strategies can be used to detect the chirality and stereochemical purity of small molecules. λ, wavelength; CD, circular dichroism; *f*, frequency; ROA, Raman optical activity; VCD, vibrational circular dichroism.

Table 1 |

Diseases and their associated abnormal chiral biomarkers

Disease	Biomarkers		Location	Concentrations in healthy subjects	Concentrations in patients
Cancer	Amino acids	D-Pro	Gastric juice	6–30 μM (ref.[111])[a]	2–110 μM (ref.[111])[a]
			Saliva	0–11.3 μM (ref.[207])[b]	22.5–112.6 μM (ref.[207])[b]
		D-Ala	Gastric juice	5–24 μM (ref.[111])[a]	20–280 μM (ref.[111])[a]
			Saliva	0–25.3 μM (ref.[207])[b]	50.6–253.2 μM (ref.[207])[b]
		D-GLu	Gastric juice	0–0.8 μM (ref.[111])[a]	0.4–1.7μM (ref.[111])[a]
			Serum	0.003–0.008 μM (ref.[112])[c]	0.002–0.004 μM (ref.[112])[c]
		D-Ser	Gastric juice	0.3–5.7 μM (ref.[111])[a]	2–34 μM (ref.[111])[a]
		D-Gln	Serum	0.013–0.03 μM (ref.[112])[c]	0.005–0.019 μM (ref.[112])[c]
	Organic acids	D-2-HG	Urine	2.8–17 $mmolmol^{-1}$ (ref.[181])[d] 2.5–12 $mmolmol^{-1}$ (ref.[104])[c]	4.1–2,668 $mmolmol^{-1}$ (ref.[181])[d] 146–995 $mmolmol^{-1}$ (ref.[267])[c]
			Serum	1–1.2 μM (ref.[268])[d] 0.5–0.8 μM (ref.[269])[c]	0.7–4.4 μM (ref.[268])[d] 2.3–28 μM (ref.[269])[c]
			Plasma	0.3–0.9 μM (ref.[181])[d] 0–26 μM (ref.[270])[d] 0.1–0.4 μM (ref.[109])[c]	0.3–73 μM (ref.[181])[d]
		L-2-HG	Urine	2.4–14 $mmolmol^{-1}$ (ref.[104])[c] 1.3–19 $mmolmol^{-1}$ (ref.[271])[d] 0–52 $mmolmol^{-1}$ (ref.[181])[d]	2–22 $mmolmol^{-1}$ (ref.[104])[c] 4.1–2,742 $mmolmol^{-1}$ (ref.[271])[d] 41–158 $mmolmol^{-1}$ (ref.[267])[c] 121–471 $mmolmol^{-1}$ (ref.[272])[c]
			Serum	1–1.22 μM (ref.[268])[d] 0.4–0.8 μM (ref.[269])[c] 0–1.2 μM (ref.[269])[d]	1.2–5.8 μM (ref.[268])[d] 0.4–2.1 μM (ref.[269])[c]
			Plasma	0.5–1.0 μM (ref.[181])[d]	0.3–1.7 μM (ref.[181])[d]
Brain diseases	Amino acids	D-Asp	Grey matter	15–22.2 $nmolg^{-1}$ (ref.[84])[b]	8.9–20.7 $nmolg^{-1}$ (ref.[84])[b]

Disease	Biomarkers	Location	Concentrations in healthy subjects	Concentrations in patients
		White matter	17.8–27 nmolg^{-1} (ref.[84])[b]	3.9–17.1 nmolg^{-1} (ref.[84])[b]
			0.5–2.0 μM (ref.[180])[b] 0.5–1.6 μM (ref.[273])[e]	1.2–5.4 μM (ref.[180])[b]
	D-Ala	Grey matter	16–26 nmolg^{-1} (ref.[84])[b]	7–12 nmolg^{-1} (ref.[84])[b]
		White matter	2–23 nmolg^{-1} (ref.[84])[b]	6–21 nmolg^{-1} (ref.[84])[b]
	D-Ser	Serum	1.7–2.9 μM (ref.[56])[c] 0.9–2.2 μM (ref.[72])[a] 1.0–2.9 μM (ref.[134])[d] 1.5–2.8 μM (ref.[274])[c]	1.3–2.4 μM (ref.[56])[c] 0.8–3.2 μM (ref.[72])[a] 1.4–2.4 μM (ref.[274])[c]
		Plasma	1.4–2.5 μM (ref.[275])[a] 1.7–2.9 μM (ref.[276])[a] 0.6–1.3 μM (ref.[277])[c]	1.2–4.2 μM (ref.[275])[a]
		CSF	0.6–3.0 μM (ref.[180])[b] 1.8–11.7 μM (ref.[278])[d] 1.1–1.6 μM (ref.[71])[c] 1.4–2.2 μM (ref.[33])[b]	4.9–13.1 μM (ref.[180])[b] 1.3–1.9 μM (ref.[71])[c] 0.9–1.8 μM (ref.[33])[b]
		Total[f]	CSF	12.5–23.3 μM (ref.[180])[b]	18.8–34 μM (ref.[180])[b]
	Organic acids	D-2-HG	CSF	0.07–0.3 μM (ref.[17])[d]	0.42–6 μM (ref.[17])[d]
			Plasma	0.3–0.9 μM (ref.[181])[d] 0–26 μM (ref.[270])[d] 0.1–0.4 μM (ref.[109])[c]	2.2–26 μM (ref.[17])[d] 2.5–17 μM (ref.[91])[d]
			Urine	2.8–17 mmolmol^{-1} (ref.[181])[d] 2.5–12 mmolmol^{-1} (ref.[104])[c]	228–750 mmolmol^{-1} (ref.[17])[d] 18–1,185 mmolmol^{-1} (ref.[91])[d]
		L-2-HG	Plasma	0.5–1.0 μM (ref.[181])[d]	1.1–3.0 μM (ref.[17])[d] 2.2–3.7 μM (ref.[91])[d]
			Urine	2.4–14 mmolmol^{-1} (ref.[104])[c] 1.3–19 mmolmol^{-1} (ref.[271])[d] 0–52 mmolmol^{-1} (ref.[181])[d]	630–1,420 mmolmol^{-1} (ref.[279])[d] 1,000–5,520 mmolmol^{-1} (ref.[280])[c]

Disease	Biomarkers		Location	Concentrations in healthy subjects	Concentrations in patients
					25.2–430 mmol mol^{-1} (ref.[91])[d] 671–3,392 mmol mol^{-1} (ref.[281])[d]
Kidney diseases and diabetes	Amino acids	D-Asn	Plasma	0–0.12 μM (ref.[277])[c] 0.02–0.18 μM (ref.[277])[c]	0.14–0.36 μM (ref.[186])[c]
		D-Ser	Plasma	1.4–2.5 μM (ref.[275])[a] 1.7–2.9 μM (ref.[276])[a] 0.6–1.3 μM (ref.[277])[c]	1.76–3.13 μM (ref.[186])[c]
		D-Ala	Plasma	0–0.14 μM (ref.[277])[c] 1.21–2.43 μM (ref.[282])[e]	1.18–4.73 μM (ref.[186])[c]
			Nail	0–14.6 pmolmg^{-1} (ref.[101])[c]	3.6–49.5 pmolmg^{-1} (ref.[101])[c]
		D-Pro	Plasma	0–0.7 μM (ref.[186])[c] 0.15–0.56 μM (ref.[277])[c]	0.80–0.88 μM (ref.[186])[c]
			Nail	0.3–2.3 pmolmg^{-1} (ref.[101])[c]	0–7.8 pmolmg^{-1} (ref.[101])[c]
		D-Tyr	Plasma	0.7–1.1 μM (ref.[100])[b] 0–0.12 μM (ref.[277])[c]	0.8–5.6 μM (ref.[100])[b]
		D-Phe	Plasma	0.3–0.7 μM (ref.[100])[b] 0.10–0.30 μM (ref.[277])[c]	0.1–3.5 μM (ref.[100])[b]
		D-Val	Nail	0.1–0.4 pmolmg^{-1} (ref.[101])[c]	0–14.8 pmolmg^{-1} (ref.[101])[c]
		D-Ile	Nail	0.1–0.4 pmolmg^{-1} (ref.[101])[c]	0–6.4 pmolmg^{-1} (ref.[101])[c]
		D-Leu	Nail	1.3–3.8 pmolmg^{-1} (ref.[101])[c]	0–35.6 pmolmg^{-1} (ref.[101])[c]
	Organic acids	D-Lac	Plasma	7.4–8.6 μM (ref.[283])[c] 21.0–25.0 μM (ref.[284])[b]	9.5–11.9 μM (ref.[283])[c] 15.9–63.3 μM (ref.[18])[b]
			Urine	0.9–1.3 mmolmol^{-1} (ref.[283])[c] 6.2–30.2 mmolmol^{-1} (ref.[18])[b]	2.3–3.9 mmolmol^{-1} (ref.[283])[c] 11.7–60.1 mmolmol^{-1} (ref.[18])[b]
			Saliva	0.02–0.11 μM (ref.[285])[c]	0.00–0.96 μM (ref.[285])[c]
		D-HA	Saliva	0.02–0.10 μM (ref.[285])[c]	0.13–0.34 μM (ref.[285])[c]

Disease	Biomarkers		Location	Concentrations in healthy subjects	Concentrations in patients
		D-2-HG	Urine	2.8–17 mmolmol^{-1} (ref.[181])[d] 2.5–12 mmolmol^{-1} (ref.[104])[c]	0.2–16.5 mmolmol^{-1} (ref.[104])[c]
		L-2-HG	Urine	2.4–14 mmolmol^{-1} (ref.[104])[c] 1.3–19 mmolmol^{-1} (ref.[271])[d] 0–52 mmolmol^{-1} (ref.[181])[d]	0.1–22.5 mmolmol^{-1} (ref.[104])[c]

2-HG, 2-hydroxyglutarate; CSF, cerebrospinal fluid; D-HA, D-3-hydroxybutyric acid; D-Lac, D-lactate.

[a]Concentrations determined by liquid chromatography–fluorescence.

[b]Concentrations determined by enzymatic assay.

[c]Concentrations determined by liquid chromatography–mass spectrometry.

[d]Concentrations determined by gas chromatography–mass spectrometry.

[e]Concentrations determined by capillary electrophoresis–fluorescence.

[f]Total concentration of D-amino acids.

Table 2 |

Comparison of chiral detection techniques

Method	Limit of detection	Sample quantity	Clinical sample type	Number of measurable metabolites per run	Cost	Diseases	Level of development
Chromatography[152,177–179,185,189,286,287]	100 pM (refs.[185,287])	10 μL-100 ml	Serum, plasma, urine, CSF, saliva, tissue	1–20	Medium	Cancer, brain diseases, kidney disease, diabetes	Well developed
Capillary electrophoresis[139,192–195,288]	5nM (refs.[193–288])	100 nl-1 ml	Serum, plasma, urine, glial vesicles, tissue	1–20	Low	Brain diseases	Well developed
NMR spectroscopy[200–202,204]	10 nM (refs.[202,204])	10 μl-100 ml	Urine	1–20	High	Brain diseases	Under development
Enzymatic assay[21,205–208]	200 nM (ref.[289])	10 μl-10 ml	Serum, CSF, plasma, urine, saliva, tissue	1	Low	Cancer, brain diseases, kidney disease, diabetes	Well developed
Polarimetry[142–144,214]	1 mM (refs.[143,214])	10 ml	Blood	1	Low	Diabetes	Under development
CD spectroscopy[145,217]	5 μM (refs.[145,217])	100 μl-100 ml	CSF, tissue	1	Low	Brain diseases	Under development
ROA spectroscopy[222–226]	0.2 mM (ref.[226])	100 μl-100 ml	–	1	Medium	–	Under development
VCD spectroscopy[227–230]	5 mM (ref.[230])	100 μl-100 ml	–	1	Medium	–	Under development
Surface-enhanced CD spectroscopy[233–236,240,244]	20 pM (refs.[240,244])	10 μl-100 μl	Tissue, urine	1	Low	Brain diseases, diabetes	Under development
Surface-enhanced ROA spectroscopy[245,246]	100 nM (ref.[246])	10 μl-1 ml	–	1	Low	–	Under development

CD, circular dichroism; CSF, cerebrospinal fluid; NMR, nuclear magnetic resonance; ROA, Raman optical activity; VCD, vibrational circular dichroism.