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BioEssays

REVIEW

Prospects & Overviews

The researcher's guide to selecting biomarkers in mental health studies

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Abstract

Clinical mental health researchers may understandably struggle with how to incorporate biological assessments in clinical research. The options are numerous and are described in a vast and complex body of literature. Here we provide guidelines to assist mental health researchers seeking to include biological measures in their studies. Apart from a focus on behavioral outcomes as measured via interviews or questionnaires, we advocate for a focus on biological pathways in clinical trials and epidemiological studies that may help clarify pathophysiology and mechanisms of action, delineate biological subgroups of participants, mediate treatment effects, and inform personalized treatment strategies. With this paper we aim to bridge the gap between clinical and biological mental health research by (1) discussing the clinical relevance, measurement reliability, and feasibility of relevant peripheral biomarkers; (2) addressing five types of biological tissues, namely blood, saliva, urine, stool and hair; and (3) providing information on how to control sources of measurement variability.

KEYWORDS
biomarker, guideline, mental health, peripheral, psychiatric disorders

INTRODUCTION

Despite the high prevalence and burden of mental disorders, knowledge of the underlying biological pathways remains limited. A clear understanding of the pathoetiology of these conditions is important for improved prevention, diagnosis, and treatment strategies. There is a large amount of evidence showing mental disorders are accompanied by a variety of physiological dysregulations, that in turn may be associated with societal determinants such as poverty and discrimination. Moreover, mental disorders are no longer considered to solely reflect perturbations of the "mind" or the brain, but, in many cases, are associated with systemic conditions that affect the whole body. Despite this progress, the lack of sensitive and specific biological tests reflecting underlying pathological processes limits our current diagnostic system of phenomenology, with little grounding in biology.[1] Consequently, the fields of clinical and biological psychiatry still largely operate in separate worlds—in both research and clinical practice—limiting progress in each domain.

Several lines of research demonstrate a strong interplay between mind and body processes. Several mental disorders have been linked to systemic physiological dysregulations including altered functioning of the immune system,[2] the hypothalamic–pituitary–adrenal (HPA) axis,[3] mitochondrial biology, and the microbiome,[4] although most of these dysregulations are not specific for specific mental disorders. It has also become clear that genetics and epigenetic mechanisms (which can "switch" genes on and off) play an important role in psychiatric conditions.[5] Further, mental disorders frequently co-occur with each other, but also with several somatic illnesses, such as cardiovascular disease, diabetes, and cancer,[6,7] and individuals with severe mental disorders have lower life expectancy.[8]

Biological measurements can advance clinical and epidemiological mental health research for several reasons. First, biological measurements are necessary to understand the pathophysiology of mental disorders. Second, tracking biological changes over time allows a better understanding of responses to interventions and their mechanisms of action. Finally, this work holds the promise of better understanding why person A responds to a specific intervention when person B does not. Mapping biological profiles will allow the development of prediction models that forecast, for example, who is at risk for an unfavorable course or who will respond to which treatment. Such knowledge is essential for the development of precision treatment approaches in psychology and psychiatry, allowing us to identify and deliver the right treatment, at the right time, for the right person.

In the current paper, we evaluate a selection of biological indicators based on their clinical relevance in mental health research and their measurement reliability and feasibility. Further, we provide an overview on the collection and storage of different types of biological specimens, including blood, saliva, urine, stool and hair, and discuss potential benefits, challenges and solutions for each type of material.

BIOLOGICAL MARKERS RELEVANT IN MENTAL HEALTH RESEARCH

Genetics

Clinical relevance

Complex traits such as psychiatric disorders are typically polygenic, namely they are characterized by hundreds or thousands of genetic variants (single nucleotide polymorphisms, SNPs) of small effects scattered across the genome. Genome-Wide Association Studies (GWAS) interrogate SNPs that differ between individuals and have identified > 10 000 SNPs associated with human traits and disorders.[9] Due to the small genetic effects and various methodological standards, GWAS require large samples—usually achieved through data sharing and/or large biobanks. Downstream applications of GWAS results, such as polygenic risk scores (PRS), can be applied to smaller datasets and their potential clinical relevance is currently a major research topic.[10,11] With PRS, the number of risk variants for a certain trait is summed and weighted for the effects identified in a GWAS, in order to index an individual's lifetime genetic risk for a disorder.[12] Promising evidence of clinical relevance comes from cardiovascular diseases[13] and breast cancers,[14] where PRS provide information above traditional risk factors, with potential for stratified screening and targeted interventions. Clinical relevance of PRS in mental health is less clear: PRS derived from large-scale GWAS can significantly predict psychiatric traits in independent samples but the proportion of disorder liability explained (~ 7% for schizophrenia[15] and ~ 6% for major depression[16]) is still limited for direct clinical implementation.[17] Yet new research investigating the predictive or prognostic relevance of PRS is underway.[18]

Measurement reliability and feasibility

Genotype data can be reliably measured with various GWAS array chips (currently < 100 USD per sample) in DNA extracted from blood or saliva samples, both providing genotyping call rates and reproducibility frequencies of > 97%.[19] Open computational resources[20] and pipelines[21] for data generation and quality control make genetic research methodology available to all researchers while ensuring reliable and standardized data.

PRS are obtained by leveraging GWAS summary statistics for a given trait from repository data (e.g., GWAS Catalogue https://www.ebi.ac.uk/gwas/, UK Biobank http://www.nealelab.is/uk-biobank). Standardization and reproducibility of PRS are obtained using PRS Reporting Standards[22] and the Polygenic Score Catalog.[23] PRS research considerations include sample size as well as statistical power, which is impacted by genetic architecture of the trait and the size of the discovery GWAS.[24] Most GWAS have been performed in European ancestry samples, and PRS accuracy declines when target sample ancestry differs from that of the discovery GWAS.[25] Ongoing trans-ancestral GWAS are aimed at improving the predictiveness and representativeness of PRS research.

Epigenetics

Clinical relevance

Epigenetics encompasses molecular mechanisms that regulate gene function without changing the genetic code itself. In addition to directing genomic and cellular function, epigenetic mechanisms respond to environmental factors associated with mental health, including psychological trauma and stressors,[26] but also air pollution and chemical exposures.[27,28]. Epigenetic mechanisms can thereby act as a molecular interface that can transduce the environmental impact on brain function and mental health. Unlike genetic mutations, epigenetic modifications can evolve over time, be inherited across multiple generations,[29] and are potentially preventable and reversible. Epigenetic markers thus hold promise as indicators that can track the course of mental disorders and their response to treatment.

Epigenetic changes can be studied through three mechanisms: (1) DNA methylation, (2) post-translational histone modification, and (3) post-transcriptional RNA interference through MicroRNAs. While histone modification and MicroRNAs have also been studied in, for example, schizophrenia and major depression,[30,31] DNA methylation is the most commonly studied form to date and will be further discussed in the context of mental health. Promising examples include the potential of DNA methylation to predict depressive course over time,[32] the response of individuals with posttraumatic stress disorder to psychotherapeutic interventions,[33] and the vulnerability of individuals to stressful and traumatic events.[34] Such work has employed both hypothesis-driven and unbiased epigenome-wide approaches.[32–35] Epigenetic "clocks" are composite markers that predict chronological age[36,37] or mortality-associated phenotypes[38,39] from DNA methylation at multiple genomic sites, and accelerations of epigenetic age have been shown in those with mental disorders[5,40,41] and can predict medical illness risk and mortality.[39,42]

Measurement reliability and feasibility

Epigenetic changes exhibit substantial cell and tissue specificity and are ideally measured in the primary tissue implicated in the phenotype of interest. As the brain is not accessible in (live) humans, most studies to date have been conducted in blood and saliva. However, epigenetic alterations in such tissues can be similar to patterns observed in the brain.[37,43] The right tissue for potential biomarker assessment remains an important open question. For example, in contrast to blood, saliva has been found to follow more similar epigenetic patterns to brain tissue for some methylation sites.[44,45] Epigenetic changes can be dynamic and reversible, studies would therefore benefit from

employing longitudinal assessments at different stages of disease course and different time points during treatment interventions.

Researchers in epigenetic studies may choose a DNA methylation profiling platform based on various factors, including, but not limited to, the study's objectives, sample size, DNA sample quantity and quality, and the availability of bioinformatics software for analysis and interpretation.[46] The human genome comprises 28 million sites where cytosines are found in the cytosine-guanine dinucleotide (CpG context and can undergo methylation. The choice of platform often hinges on whether the goal is to explore unknown DNA methylation changes (i.e., optimizing methylome coverage) or to assess methylation within specific regions of interest. Next-generation sequencing methods enable the analysis of approximately 3 million CpG sites, making them suitable for large-scale methylome-wide association studies (MWAS) and less hypothesized data mining. These methods have trade-offs in costs, sample size required, and reliability.[47] On the other hand, many epigenetic studies favor commercially available array-based technologies, which cover a smaller portion of the methylome (2%–3% of CpG sites). An investigation into the reliability of BeadChip arrays showed variable intraclass correlation between repeat measurements of individual probe beta-values across two different chips (i.e., Illumina 450K and EPIC).[48] Reliably measured probes demonstrated higher heritability, and more consistent associations with environmental exposures, gene expression, and greater cross-tissue concordance.[49] These BeadChip-wide differential reliabilities appear reproducible and systematic in pattern. Once CpG sites are identified as biomarker candidates, relatively low-cost targeted sequencing at single-base resolution can be used to validate and further characterize key findings. It is crucial to acknowledge that, similar to genetic assays, epigenetic assay technologies are constantly improving, becoming more reliable and cost-effective over time.

Hypothalamic-pituitary adrenal (HPA) axis

Clinical relevance

The hypothalamic-pituitary adrenal (HPA) axis and its end product cortisol have been proposed to play a key role in mediating the impact of stress on health.[50] Cortisol coordinates bio-behavioral stress responses[51] and follows a diurnal pattern (morning rise and gradual diurnal decline). Decades of research have documented inconsistent effects of HPA axis dysregulation in psychiatric disorders.[52] Heterogeneous findings may result from sample variations in clinical subtypes,[53] comorbidity,[54] and trauma exposure and its timing.[52,55] Recent evidence suggests that HPA axis alterations may reflect the impact of underlying genetic and environmental (e.g., childhood trauma) vulnerability factors that can alter both adult HPA axis functioning and increase risk for mental disorders.[56] Incorporating measures of HPA axis activity, which may also include other hormones such as ACTH and dehydroepiandrosterone (DHEA), into clinical studies will contribute to our understanding of the pathogenesis of clinical disorders and its role in treatment.

Measurement reliability and feasibility

Cortisol can be measured in blood, saliva, urine, and hair. The fluid/tissue of choice depends on the research question and practical considerations (see Table 1). Cortisol is most commonly measured in saliva given its numerous advantages (Table 1). Excellent reviews are available that discuss salivary cortisol collection and storage, measurement of relevant covariates, and data analytic approaches for epidemiological[57] as well as ambulatory and stress reactivity studies,[58–61] but a brief overview is provided here. The most frequent diurnal cortisol measures include the cortisol awakening response (CAR; cortisol rise within 30–45 min after awakening), the diurnal cortisol slope (change across the day from morning to evening),[62,63] and the magnitude of cortisol across the day.[57,61] There is substantial intra-individual day-to-day variation in salivary cortisol levels, so collecting samples over multiple (2–6) days is necessary.[64] Acute stress activation of the HPA axis has also been studied using laboratory psychosocial stress paradigms, such as the Trier Social Stress Test, which involves repeated cortisol sampling (10–15 min intervals) before, during, and after the stressor.[65] Cortisol collection in saliva using Salivettes is also described in detail on the Biomarker Network website (https://gero.usc.edu/cbph/network/protocol/saliva). Salivary cortisol levels can be influenced by collection timing (e.g., weekend/weekday, wake time), health behaviors (e.g., smoking, caffeine, alcohol use, recent food intake, recent tooth brushing), as well as sociodemographic (e.g., gender, age, race) and health-related factors (e.g., medical diagnosis, BMI, medication, menstrual timing).[57,60,61,64] Samples are mostly assayed using immunoassays (currently $5–10 per sample).

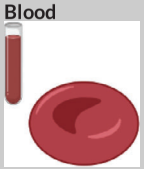
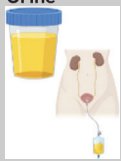
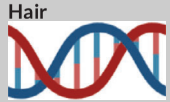
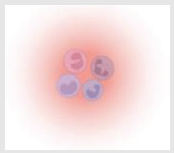
Cortisol reliability varies based on collection time, collection method, subject factors, and metrics used.[66,67] The Cortisol Assessment List provides a comprehensive and systematic way to plan cortisol assessments to improve reliability.[68] A meta-analysis of diurnal salivary cortisol metrics reported that area-under-the-curve (AUC) has fair-good reliability.[69] Studies suggest that, outside of adrenal insufficiency testing in children,[70] serum and salivary or urine cortisol measurements are highly correlated.[71,72] Assessment of hair cortisol concentrations (HCC) in human scalp hair shows high test–retest reliability.[73,74] Russell et al. (2015) found that HCC values measured for the same samples by several international laboratories using four different immunoassay methods were highly correlated ($r2 > 0.91$).[75] Reference ranges for healthy (i.e., non-clinical) HCC in adults have just recently been published.[76]

Inflammation

Clinical relevance

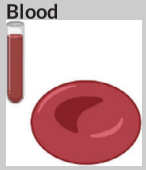
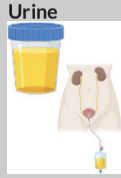
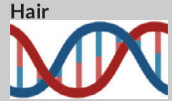
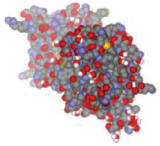
The role of the immune/inflammatory system in the pathophysiology of mental disorders has been well established. Pillars of the evidence are meta-analyses consistently showing significant associations between inflammatory markers, including cytokines and acute phase protein C-reactive protein (CRP), and a wide range of mental disorders including

TABLE 1 Strengths and limitations per biomarker for measurement in each tissue type.

	Tissue				
	Blood 	Saliva 	Urine 	Stool 	Hair 
General strengths	• Ease of collection • Low cost • If venous blood collection is not possible, finger-prick capillary blood sampling/dried blood spot can be done at home by participant	• Ease of collection • Low cost • Non-invasive • Not requiring professional assistance • Can be stored at higher temperatures	• Non-invasive • Not requiring professional assistance • Participant can conserve the sample in their freezer[77] • Easy to measure certain biomarkers in already collected samples	• Relatively easy to collect • Relatively cheap depending on method	• Ease of collection • Non-invasive • Not requiring professional assistance • Storage at room temperature • Less susceptible to confounds (e.g., timing or oral contraceptive usage)
General limitations	• Participants may have to be on the research site • May require trained phlebotomist • Minimally invasive and could be scary for children	• Saliva donors should avoid food, drinks and tooth-brushing within 60 min prior sampling	• Collected data may include breakdown products from foods, drinks, drugs, and environmental exposures • Concentrations may depend on time of day • Should be stored at 4°C before freezing	• Contamination • Storage bias • Collection bias • Strong associations with drugs, diet, and sleep patterns • Results may depend on time of day	• Difficult when participant has short hair or no hair
Genetics 	DNA extracted from blood and saliva samples is comparable (except in the case of mosaicism).**Strengths**: • Commonly used	DNA extracted from blood and saliva samples is comparable (except in the case of mosaicism).**Strengths**: • Commonly used (e.g., children) • The potential to increase remote recruitment, as you can ship the saliva kit to the participants, and they can return it via mail	N/A	N/A	N/A
Epigenetics 	**Strengths**: • Good proxy for immune cell epigenetic states, relevant for multiple diseases • There are well established markers of epigenetic aging and methods for estimating blood cell-type proportions	**Strengths**: • Salivary DNA methylation patterns may correlate more with brain than blood DNA methylation[44] • For a review see Wren et al.[78]	N/A	N/A	N/A

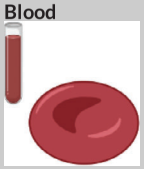
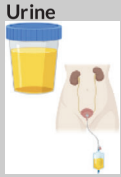
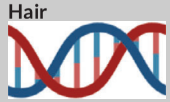
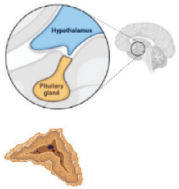
(Continues)

TABLE 1 (Continued)

	Tissue				
	Blood 	Saliva 	Urine 	Stool 	Hair 
Inflammation/immune activity 	**Strengths**: • Established assay **Limitations**: • Some markers less stable when stored over longer periods of time • Aliquoting of samples needed upon sampling as many cytokines are less stable when freeze-thawed and stored over longer periods of time	Applicable for measuring immune activity status in the oral cavity.	N/A	N/A	N/A
BDNF 	**Strengths**: • Established assays **Limitations**: • Large variability of results due to inconsistent methodologies • Consider pre-analytic variables and subject-level factors in experimental design • Earlier studies using commercial ELISA kits cannot distinguish mature BDNF from proBDNF	N/A	N/A	N/A	N/A

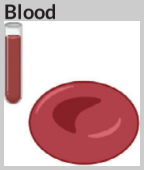
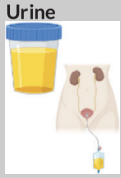
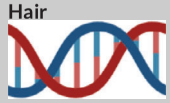
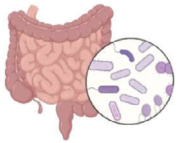
(Continues)

TABLE 1 (Continued)

	Tissue				
	Blood 	Saliva 	Urine 	Stool 	Hair 
Insulin resistance 	**Strengths**: • Established collection methods that have been well-validated to the gold standard (euglycemic clamp test) • Clinically accessible measures **Limitations**: • Gold standard can be expensive and time-consuming • Certain proxy measures of insulin resistance may have limited correlation to gold standard	N/A	N/A	N/A	N/A
HPA axis 	**Strengths**: • Reflects short-term HPA axis activity (minutes)	**Strengths**: • Reflects short-term HPA axis activity (minutes)	**Strengths**: • Reflects short-term HPA axis activity (several hours)	N/A	**Strengths**: • Reflects long-term HPA axis activity (several months) • Provides cumulative, retrospective measure of long-term cortisol secretion **Limitations**: • Does not inform about HPA axis dynamics • Covariates that influence hair cortisol concentrations or hair growth rates are not fully understood • How cortisol is incorporated into the hair is not fully understood yet • No normal reference ranges established yet

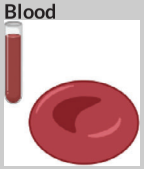
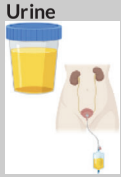
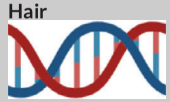
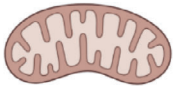
(Continues)

TABLE 1 (Continued)

	Tissue				
	Blood 	Saliva 	Urine 	Stool 	Hair 
Telomere length (TL) & Telomerase (TA) 	TL is measured in DNA (see Genetics above)**Strengths**: • Most commonly used • The only option for robust telomerase activity • Dried blood spots stored at room temperature can provide DNA, but may not provide enough material for telomere length quantification[79] **Limitations**: • −80°C freezer storage required for TA, and generally better for TL, though bulk TL can be measured in dried blood spots • Cannot rule out possibility of pseudo lengthening without cell sorting • Different cell types (monocytes versus granulocytes) have different TL, thus, correction for cell type is preferable	**Strengths**: • Buccal cells can be stored at room temperature **Limitations**: • Applicable for TL • Need to differentiate between buccal swabs (buccal cells) and saliva samples (mainly blood-derived cells) • Salivary TL seems to have different relations with outcome measures than blood telomere length, which may be due to different proportions mononuclear vs polynuclear cells[80] • Cells (DNA) from eating animal meat may contaminate the TL measurement.	N/A	N/A	N/A
Microbiome 	Gut microbiota metabolites can be measured.	**Limitations**: • Reflects a different habitat of microbiota • Hard to compare results to fecal microbiota • Less studied	Gut microbiota metabolites can be measured.	**Limitations**: • Doesn't reflect specific parts of the gut microbiota • Potential dietary influenced variability • Participants may find this distasteful	N/A

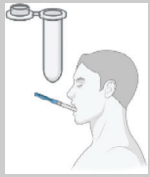
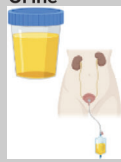
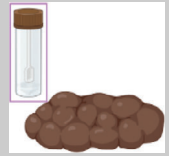
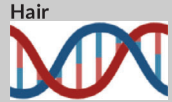
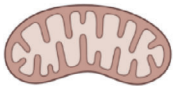
(Continues)

TABLE 1 (Continued)

	Tissue				
	Blood 	Saliva 	Urine 	Stool 	Hair 
Mitochondria **1) cf-mtDNA** 	**Strengths**: • Easy to measure in already collected samples **Limitations**: • cf-mtDNA is highly dynamic, multiple timepoints should be used to assess within- and between- person variations • Higher cf-mtDNA levels were found in inflammatory diseases and is a predictor of mortality in critically-ill patients, but clinical significance of individual variation in healthy subjects remains to be determined (for a review, see Trumff et al.[81]) • Should be measured in plasma or serum after a centrifugation to remove cellular debris. For guidelines on cf-mtDNA detection see Michelson et al.[82]	Cf-mtDNA is detectable in saliva.[77] **Strengths**: • Participants can collect samples at home and conserve the sample in their freezer [77] • Easy to measure in already collected samples **Limitations**: • Same as for blood. • Physiological significance may differ from blood, and is currently unknown • Should be measured after a centrifugation to remove cellular debris	Cf-mtDNA is detectable in urine.[83] **Limitation**: • Physiological significance may differ from blood • Should be measured after a centrifugation to remove cellular debris	N/A	N/A

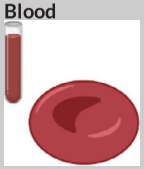
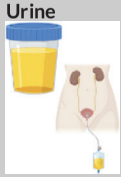
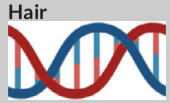
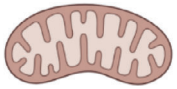
(Continues)

TABLE 1 (Continued)

	Tissue				
	Blood 	Saliva 	Urine 	Stool 	Hair 
Mitochondria **2) mtDNAcn** 	**Strengths**: • High-throughput relative to direct measure of respiratory chain activity **Limitations**: • Not interpretable on its own[84] • Measurements require the normalization of mtDNA copies to copies of the nuclear genome in the cell nucleus (i.e., mtDNA to nDNA ratio). In blood, the circulating platelets do not contain a nucleus so have no nDNA, but contain mtDNA. This makes mtDNAcn measured in whole blood[85] or in PBMCs contaminated with sticky platelets,[86] highly skewed by platelet abundance • Need to be measured in specific immune cells[86]	**Strengths**: • High-throughput relative to direct measure of respiratory chain activity **Limitations**: • mtDNAcn on its own is not interpretable[84]	**Strengths**: • High-throughput relative to direct measure of respiratory chain activity **Limitations**: • mtDNAcn on its own is not interpretable[84]	N/A	N/A
Mitochondria **3) Mitochondrial enzymatic activities** 	**Strengths**: • Higher throughput than respirometry **Limitations**: • Indirect assessment of maximal mitochondrial respiratory capacity • Need to be collected in specific immune cells,[86] cells should be cryopreserved in liquid nitrogen (−170°C) as enzymatic activities may degrade over time.[87]	N/A	N/A	N/A	N/A

(Continues)

TABLE 1 (Continued)

	Tissue				
	Blood 	Saliva 	Urine 	Stool 	Hair 
Mitochondria **4) Respirometry** 	**Strengths**: • Direct assessment of maximal mitochondrial respiratory capacity **Limitations**: • Low throughput • Need to be measured in specific immune cells,[86] preferably on fresh cells to avoid potential artifacts of cryopreservation.	N/A	N/A	N/A	N/A
Protein and other soluble markers (e.g., GDF15, lactate)[88]	**Strengths**: • Easily measured by ELISA, by proteomics, or other methods **Limitations**: • Lack of specificity • Labile and acutely responsive to mental states within seconds to minutes (lactate) • Uncertain interpretation	**Strengths**: • Easily measured by ELISA, by proteomics, or other methods **Limitations**: • Lack of specificity • Labile and acutely responsive to mental states within seconds to minutes (lactate) • Uncertain interpretation	**Strengths**: • Easily measured by ELISA, by proteomics, or other methods **Limitations**: • Lack of specificity • Uncertain interpretation	N/A	N/A

Abbreviations: N/A, not applicable.

major depressive disorder, bipolar disorder, and psychosis,[89–93] the observation that persons with auto-immune disease or a higher infection load are more at risk of developing psychiatric disorders,[94,95] and the fact that endotoxin, interferon-alpha and typhoid vaccine administration yield so-called sickness behavior that is overlapping with mood symptoms.[96,97] Inflammatory signals in the periphery can reach the brain via cellular, neural and humoral routes where they can influence pathways involved in onset of psychiatric symptoms and behaviors.[98] Both from the prevention and treatment perspective, the inflammatory system is of importance, but it should be noted that low-grade inflammation does not occur in all psychiatric patients. A recent meta-analysis demonstrated that the prevalence of elevated C-reactive protein (CRP > 3 mg/L) was only 27% in depressed persons,[99] and also in schizophrenia, it has been posed there may be distinct immune subgroups within patient groups.[100,101] Such patient groups could benefit from personalized medicine approaches.[102,103]

Measurement reliability and feasibility

Inflammatory markers can be measured in blood and cerebrospinal fluid (CSF), with blood measurements being most widely used. Dried blood spots may also be an easy, cheap and feasible way to measure low-grade inflammation.[104] To capture low-grade inflammation, high sensitivity assays are required. A comprehensive overview on current methods for cytokine analysis has been provided by Keustermans et al.[105] For studies planning on storing samples for a longer time before assay, it is important to note that most cytokines remain stable for 2 years at −80°C, but that several cytokine concentrations decrease with longer storage times, sometimes quite substantially.[105,106] Most cytokines are resilient to one, or possibly two, freeze/thaw cycles, but additional cycles should be avoided, as they may increase or decrease values[106,107] see also the review by Simpsom et al.[108] on thermal stability properties of many individual cytokines. Studies suggest that reliability of inflammatory markers is high within patients up to 6 months,[109] and between measures outside of IL-6.[110]

Insulin resistance

Clinical relevance

Insulin resistance (IR) is a modifiable, metabolic, inflammatory condition that occurs in approximately a 40% of otherwise-healthy young adults in the United States.[111] It is associated with a decreased ability to utilize blood glucose as an energy source for cellular activity and a diminished ability to dispose of excess glucose. There is considerable evidence linking depression with IR. Depressed individuals with higher IR experience greater depression severity and chronicity.[112] In addition, individuals with depression and IR are more resistant to antidepressant and lithium treatments, suggesting that IR may exacerbate the neurobiological dysfunction related to depression or hinder the body's ability to contest it.[113] IR is associated with a current state of MDD, but not with remitted MDD, suggesting IR is a state, rather than a trait, marker for depression. This modifiability of IR is promising for potential interventions and treatments.[114] One promising pathway supporting the IR-depression link is peroxisome proliferator-activated receptor gamma (PPAR-γ) and PPAR-γ agonist (e.g., pioglitazone). Pioglitazone has been shown to improve mood in patients with non-remitted depressive disorders and IR. While more research is necessary to understand pioglitazone and other PPAR-γ-related interventions, this is a promising area of study.[115]

While insulin resistance (IR) is linked to major depressive disorder (MDD), it is also common in various non-psychiatric diseases such as type 2 diabetes, obesity, cardiovascular disease, and metabolic syndrome. Research is progressing with promising developments like neurally-enriched exosomes that measure biomarkers of insulin activity in neural tissue. This approach aims to provide more specific insights into the relationship between IR and brain function, potentially enhancing its diagnostic value for psychiatric disorders.[116]

Measurement reliability and feasibility

The gold standard in IR research is the euglycemic clamp technique and has high reproducibility.[117] However, this technique is time and resource-intensive. The next best technique is the steady-state plasma glucose concentration (SSPG)[118] derived from an insulin suppression test, which along with euglycemic clamp is one of the most reliable tests.[119] Other tests may be less time or resource-intensive, including the oral glucose tolerance test, which has variable reliability depending on the derived measure, timepoint, and fasting versus non-fasting state,[120] and various single-blood measures that can be obtained relatively easily—these include triglyceride/HDL ratio,[121] perhaps the most clinically accessible measure. The Triglyceride/HCL ratio reliability varies by race and ethnicity.[122,123] Finally, fasting glucose and fasting insulin can be combined to calculate the homeostatic model of insulin resistance (HOMA-IR)[124] While these are not as sensitive or specific as the euglycemic clamp technique or SSPG, they can be used to approximate IR when the above tests would be expensive or time-consuming.

Microbiome

Clinical relevance

Microbiota-gut-brain communications and their potential relevance for mental disorders have largely been explored in animal studies, with critical human research lagging behind.[125] The specific mechanisms putatively linking dysbiosis (disruption to the microbiome resulting in an imbalance in the microbiota) to certain mental disorders, such as MDD, Autism, and Schizophrenia, as well as somatic disorders such as Cardiovascular disease, Diabetes, and Inflammatory Bowel Disorder are not fully understood.[126] Candidate mechanisms

include vagal signaling, neuroendocrine pathways, and chemical signaling through bacterial metabolites.[127] Increased gut permeability may induce chronic low-grade inflammation and gut microbiota alterations regulate microglial development[128,129] which could lead to neuroinflammation which in turn may give rise to depressive or other symptoms.[130] As reviewed elsewhere,[131] the antidepressant effects of probiotics have been tested in several clinical studies, but so far results have not been robust enough for clinical prime time.[132] A more recent meta-analysis, however, suggested that the antidepressant effect of probiotics is greater when given as an adjunctive versus monotherapy.[133] Given the potential anti-inflammatory effect of probiotics, it is possible that this intervention is more efficacious in a subgroup of depressed patients with systemic low-grade inflammation.[134]

Measurement, reliability, and feasibility

Analyses of the human microbiota are complex and the various different assay methods, protocols, and software hamper the comparison of results across studies. Gut microbiota composition and function are influenced by genetics, age, sex, diet, disease, medication, and environment.[126,135–137] The intra-individual changes of the gut microbiota over time seems to be smaller than differences between individuals, which can differ up to 90%,[138,139] making it a suitable marker for monitoring therapeutic improvement and other longitudinal clinical outcomes.[140–145] The gut brain-axis can be analyzed through microbiota analysis, or indirectly through various metabolites. Following prescribed best practices can optimize reliability.[146]

Bioinformatics has made it possible for compositional and functional analysis of the gut microbiota. Microbiome profiling utilizes two major approaches, 16S rRNA sequencing and shotgun metagenomic sequencing, each with their own costs/benefits that must be considered in designing a study. 16S rRNA profiling generally requires significantly lower read depth than the alternative method (costs are currently $60 per sample).[126,145] In contrast, shotgun metagenomics sequences all DNA in a sample providing both taxonomic information (down to the strain level) as well as allowing for functional profiling of the gut microbiome. Despite these advantages, shotgun sequencing requires significantly greater read depths to identify organisms, resulting in significant cost (currently $200 per sample).[126,145,147–150] Given these aspects, the choice of technique should be based on the research question. See review by Claesson and colleagues for information about designing studies with microbiome analysis.[145]

Mitochondria

Clinical relevance

Mitochondria are organelles with their own DNA that perform dozens of functions,[151] one of which is energy (ATP) production. Mitochondrial energy transformation by oxidative phosphorylation (OxPhos) inside the cell powers most cellular activities that enable brain and body functions.[152–154] Other important functions include ion homeostasis, steroid hormone biosynthesis, proinflammatory signaling, apoptosis, and reactive oxygen species production.[155] Alterations in different domains of mitochondrial biology likely contribute to the development of mental disorders (reviewed in[156–161]), and can produce anxiety-like and depression-like behavior (e.g.,[162,163]) as well as altered stress reactivity in animals.[164] In cross-sectional human studies, variation in immune cell mitochondrial OxPhos capacity and mitochondrial DNA copy number (mtDNAcn) measures has been associated with mood,[165] childhood adversity[166,167] and major depression,[168] antidepressant treatment responsiveness.[168,169], among others.[170]

In recent years, mitochondria have emerged as signaling organelles,[171,172] which open new avenues to monitor their behavior from biofluids.[82] Cell-free mitochondrial DNA (cf-mtDNA) is an emerging biomarker of psychobiological stress levels[81] which predict mortality in critically-ill patients and inflammation.[173,174] Blood cf-mtDNA is elevated in response to acute psychological stress,[81,175,176] after suicide attempt,[177] and in mental disorders including depression ([81], e.g.,[168,178]), but unresolved technical and psychobiological factors contribute to heterogeneity within this literature.[82,159]

Measurement reliability and feasibility

The spectrum of methods available to assess mitochondrial biology in human studies has been presented previously in some detail.[179] Here we focus on blood and saliva-based measurements. Mitochondria possess their own genome, the mitochondrial DNA (mtDNA) and the number of mtDNA copies per cell (mtDNAcn) has been proposed to reflect mitochondrial health/function, since mtDNA is needed for the expression of mitochondrial respiratory chain proteins. mtDNA cn reliability can be assayed using optimized real-time PCR methods and standard inter- and intra-assay coefficients of variability.[180] However, both an increase or a decrease in mtDNAcn can reflect abnormal mitochondrial energetics; mtDNAcn on its own is difficult to interpret.[84] This calls for direct biochemical measurements of mitochondrial content or respiratory chain function in parallel with mtDNAcn and their integration into composite indices (e.g., Mitochondrial Health Index, MHI)[165] to enable biological interpretation.

Respirometry[166] directly measures oxygen consumption and OxPhos-derived energy production capacity in live cells and tissues. Measurements of enzymatic activities involved in mitochondrial respiration and ATP synthesis is a proxy measure to quantify energy production capacity from frozen cells and tissues (e.g.,[86,165]). In general, enzymatic activities or respirometry quantify the *maximal capacity* of mitochondria, which reflects the capacity of the system, rather than its acute state. Reliability depends on the method used, with phosphorous magnetic resonance spectroscopy (^{31}P-MRS) showing high reliability.[181]

Multiple aspects of mitochondrial biology differ by cell subtypes.[86,182] Combinations of mitochondrial markers, as opposed to single markers, can increase robustness and interpretability of

mitochondrial recalibrations in relation to stress exposure[179] and psychopathology.[183] It is also possible that mitochondrial energy production capacity may change within days to weeks,[86,165] calling for repeated-measures design to capture stable inter-individual differences.

Finally, biofluids such as blood-derived plasma and serum,[81] cerebrospinal fluid,[184] urine,[83] and saliva[77] can be used to measure cell-free mitochondrial DNA (cf-mtDNA) (for technical recommendation see Michelson et al.[82] This area of research is rapidly expanding, and questions remain about the interpretation of cf-mtDNA in different biofluids.[82] Rapid changes in cf-mtDNA within minutes have been reported in blood[175,176] and saliva,[77] positioning cf-mtDNA as a potential state responsive marker of mitochondrial signaling.[81] Therefore, within-person study designs might be needed to accurately assess inter-individual variation in relation to study outcomes. Overall, developing scalable and biologically robust biomarkers of mitochondrial health in accessible human tissues remains an active area of research for mitochondrial psychobiology and mental health research in general.

Brain-derived neurotrophic factor (BDNF)

Clinical relevance

BDNF is a protein and a member of the neurotrophin family of growth factors. BDNF plays a crucial role in neuronal survival, growth, differentiation and synapse formation. Accumulating evidence suggests that BDNF is associated with the pathophysiology of stress and mood related disorders.[185,186] Since the first reports on BDNF in depression in the early 2000's, there have been conflicting reports on the pre-treatment peripheral levels of BDNF in MDD samples. Some showed decreased levels of serum BDNF in individuals with MDD,[187–191] while several studies did not find any significant associations.[192,193] Variability in results was also shown in posttraumatic stress disorder (PTSD),[194–198] bipolar disorder and anxiety disorders across studies.[197,199]

Several treatment studies linked pre-treatment serum BDNF levels and antidepressant treatment response and found a positive association: high BDNF pre-treatment levels predicted better response to treatment with SSRI's.[200,201], but this has not been universally replicated. Pre-treatment serum BDNF may be a potential biomarker to predict response to antidepressant treatment. Currently, the majority of the literature supports the effect of antidepressants on BDNF levels.[202]

Measurement reliability and feasibility

There have been no studies directly comparing blood BDNF levels to central nervous system BDNF levels in humans, although there have been animal models showing the blood levels of BDNF reflect brain tissue BDNF levels across species.[203,204] ELISA is the common method used to assay for BDNF. Different assays show varying reproducibility.[205] Studies in the literature use either plasma or serum, but these are not interchangeable and their inter-correlations are low, suggesting that they represent separate pools of BDNF.[206–208]. Peripheral BDNF is mostly stored in platelets,[209] and since the serum preparation process involves clotting, platelet degranulation releases platelet BDNF stores into the circulation, resulting in substantially higher BDNF levels in serum than in plasma.

Prominent sources of methodological variability in the BDNF literature are preparation and storage conditions. The duration and temperature of clotting, as well as centrifugation techniques, could all affect the measurement of BDNF levels.[210] Pre-analytic factors largely influence BDNF levels by altering platelet degranulation. For serum, a minimum of 1 h of room temperature clotting time is recommended prior to centrifugation to allow full degranulation of the platelets. For plasma, centrifugation of cold samples should be done promptly; a second centrifugation within 30 min is ideal, if practical, to remove most platelets that survived the first centrifugation. With regards to assay specificity, BDNF is synthesized in cells as a precursor molecule called proBDNF that is proteolytically cleaved to become mature BDNF (mBDNF). Some studies questioned the specificity of the current ELISA technique to differentiate mBDNF from pro-BDNF,[211] although circulating pro-BDNF levels are very low. Important "controllable" covariates to consider at the data analysis stage include age, sex, BMI, smoking, alcohol consumption, exercise, platelet number, time of day, fasted status, and concurrent illnesses and medication.

Telomere biology

Clinical relevance

Telomeres are DNA-protein complexes that flank the ends of eukaryotic chromosomes to protect coding regions of DNA from instability and degradation. Telomeres shorten incrementally as cells divide and replicate, making them a marker of cell aging and function.[212] Telomerase is the specialized ribonucleic enzyme capable of elongating telomeres by adding telomeric DNA.[213] Shorter telomeres and altered patterns of telomerase expression have been linked to several psychiatric disorders[214] and risk factors.[215,216] The leading hypothesis linking telomere biology to psychiatric disorders supposes that these disorders stem in part from, or are exacerbated by, accelerated aging due to stress exposure—although these associations are likely multidirectional.[213,217–219]

Given the broad range of factors that influence telomere length (TL) and telomerase activity (TA), these biomarkers are unlikely to serve as useful diagnostic markers for psychiatric disorders.[219] However, measuring telomere markers along with other measures could help to elucidate common mechanistic pathways linking mental and physical health outcomes.[183] Indeed, several lifestyle interventions have been associated with changes in telomere biology,[213] and telomerase is hypothesized to mediate the therapeutic effects of psychiatric

medications.[220] These measures may also be useful predictors or moderators of behavioral outcomes[221] and responsiveness to various treatments, as seen in several smaller studies (e.g.,[220,222–226]).

Measurement reliability and feasibility

Several assays exist to measure TL and TA,[227] which vary greatly in cost, reliability, and amount of tissue required. The strengths and limitations of these methodologies are discussed in a number of topical reviews[217,227] and methodological papers.[213,228–232] There are presently no established population standards for human TL and no gold standard assay. The Telomere Research Network was formed in 2019 to address these issues, and new TL researchers are encouraged to work with the latest guidelines (e.g.,[233–235]) and established labs.

Other factors to consider include tissue source, sample processing, and the temporal dynamics of telomere measures in relation to intervention and study timelines. TL is most often measured in whole blood or peripheral blood mononuclear cells (PBMC), but can also be measured in saliva and buccal cells, whereas TA can only be measured in PMBCs. DNA extraction methods are a critical contributor to TL assay variability, and the effects of prolonged storage are unknown.[231] Lin and colleagues (2019) discuss strategies for accounting for these factors, and we advocate for freezing and storing samples from baseline and follow-up so that DNA can be extracted under the same conditions. Both TL[236,237] and TA[238] vary by cell type, so changes in cell type distributions can obscure true changes in TL[239] and TA. Cell sorting methods can account for these shifting distributions but require fresh blood or PBMCs cryopreserved in liquid nitrogen, making them more logistically challenging. TL also varies within cells, and research suggests that it is the shortest telomere, rather than average TL, that initiates cellular senescence.[240–242] Meanwhile, evidence suggests average TL tends to shorten at a rate of 25–50 base pairs/year,[243] which is smaller than most assays can reliably detect.[232] Therefore, measuring changes in TL is presently recommended for multi-year longitudinal studies only. TA, on the other hand, has been shown to change in the course of an hour in response to acute social stress[244] (though not all studies find this, e.g.,[213]). This lability makes telomerase a more appealing target for shorter term interventions, but can also make it more challenging to interpret.

COLLECTING BIOLOGICAL material

The use of biological samples in research raises a number of ethical issues in relation to consent, privacy, storage, export, and re-use of samples. Ethical consent will always be required for projects involving the collection of human biological samples. Therefore, permission should be obtained by local Research Ethics Committees and Biobanks. Acquiring ethical approval is particularly important when collecting genetic data, as it contains highly personal and identifiable information.

Pre-analytic sources of variability in biomarker assessment

Half or more of all errors or inconsistencies in assay results arise from "pre-analytic" variables, including those that are "uncontrollable" (e.g., participant demographics like age, sex, BMI, fasting status and fasting duration, and others)[245–248] and those that are "controllable" (reviewed in[245–248]). These sources of variability likely contribute to the large percentage of findings that fail to replicate.[249] Despite this, there are few standardized protocols for pre-analytic procedures for researchers to use.[247] Here, we recommend literature on "controllable" pre-analytic sources of variability, such as: type of biofluid obtained, type of collection tube used, volume of blood and concentration of the analyte, temperature at each stage of sample preparation and storage, time from venipuncture to freezing, centrifugation procedures, length of freezing, number of freeze/thaw cycles and others. Although many questions remain as to optimal procedures, several studies have experimentally evaluated some of these variables, and excellent resources provide recommendations and examples of protocols. Ones that we recommend include: Andreazza et al. (2019), with recommendations of the "Biomarkers Task Force of the World Federation of Societies of Biological Psychiatry"[245]; O'Bryant et al. (2015),[247] with recommendations of an International Working Group for Alzheimer's Disease blood-based biomarkers, and Dale et al. (2018),[248] which details specific analytes that are affected by many of these pre-analytic variables; and the Women's Health Initiative (WHI) Long Life Study, which provides an example of a carefully designed biomarker protocol.

Blood

Whole blood consists of red blood cells (erythrocytes, RBCs), white blood cells (leukocytes, WBCs), platelets (thrombocytes), and plasma (serum is the plasma without coagulation factors and cells). Depending on the analytes-of-interest to be measured, serum or plasma may be preferable, and various sampling techniques may need to be considered for blood collection and storage, as extensively detailed elsewhere.[245] For certain compounds, plasma and serum may be used interchangeably but for others they should not.[250] Certain advantages of plasma are time-saving (samples can be centrifuged immediately after blood collection) and a higher fluid yield. Certain advantages of serum are less cellular contamination and the absence of anticoagulants. It is important to use the same tube type for all participants that will be compared in a study. EDTA-tubes, which yield plasma, are often used for collection of WBCs and for hematology measures. However, while EDTA is useful for DNA extraction from WBCs, RNA collection should be done in specific blood collection tubes containing RNA stabilizing agents. For cellular biomarkers that differ between subtypes of WBCs, such as epigenetic and telomere biology markers, separation of WBC subtypes is advisable within 2 h of sampling (e.g., using fluorescence activated cell sorting (FACS) or immune-magnetic cell sorting). If separation of mononuclear WBCs from granulocytes

is all that is required, cell separation tubes with centrifugation are useful.

To analyze molecules in plasma, whole blood is collected in tubes precoated with anticoagulants (e.g., EDTA, sodium citrate, sodium heparin or lithium heparin), and the plasma is separated from the cells and platelets by centrifugation. While most plasma markers are measurable in plasma collected using any anticoagulant, some anticoagulants may interfere with particular analytes or the analysis reaction. For instance, heparin can inhibit enzymes in PCR. To analyze molecules in serum, collection tubes specific for serum are used. Most analytes can be measured in either serum or in EDTA- or heparin-plasma, although different absolute values may be obtained. Moreover, analytes influenced by the coagulation process, such as analytes secreted by activated platelets during clotting, for example, BDNF, will differ in level between serum and plasma.[207] Tubes with gel are commonly used in clinics as the gel allows pouring the plasma/serum after centrifugation. However, the gel could absorb hydrophobic substances, and be unstable at extreme temperatures.

The handling and storage of the blood samples need to be similar for all samples to be grouped or compared, since key parameters, such as temperature and time until freezing or analysis, can affect analyte levels, for example, being dependent on cell secretion and the analyte's stability.[251] Thus, time to centrifugation (preferably within 30 min but always less than 2 h), speed and time of centrifugation (commonly 2000 $\times g$ at 15 min) and the subsequent time and temperature until freezing or direct analysis (as soon as possible, preferably within 2 h) are critical parameters. Serum/plasma should be physically separated from contact with cells in less than 2 h. Moreover, the process time from sampling to freezing should be less than 4 h, and samples should be frozen at below −70°C for stability (although small molecules and DNA can be stable at −20°C for shorter storage times). Repeated freeze-thawing cycles should be minimized to avoid analyte break-down. Moreover, functional analyses of, for example, enzymes, may require fresh non-frozen samples. Importantly, since different blood biomarkers have different requirements for adequate measurements, and since detailed stability characteristics of specific biomarkers are rarely known, each study needs to design dedicated sampling protocols, preferably based on published guidelines, which take into consideration sample handling and storage conditions, as well as other parameters such as sampling tools, fasting, and resting status of participants (see also,[245,247,252]).

In addition to traditional blood biomarkers, emerging research emphasizes the potential of blood-derived extracellular vesicles (EVs) as novel biomarkers in mental health studies.[253] EVs are lipid bilayer-enclosed structures released by cells into the bloodstream, carrying proteins, lipids, and nucleic acids that reflect the physiological state of their originating cells. Critically, EVs are capable of crossing the blood-brain barrier, potentially offering direct insights into central nervous system pathologies without invasive procedures. This property makes them particularly intriguing for mental health research, where they could provide molecular insights into psychiatric disorders.[254] Analyzing EVs involves specific collection and processing techniques to ensure their integrity and representative sampling. In addition, as with other biomarkers, the isolation and characterization of EVs require careful consideration of collection methods and sample handling to prevent degradation and ensure reliable analysis.[254]

Saliva

Saliva is produced and secreted by the salivary glands in the mouth. It is a complex, heterogenous biospecimen containing white blood cells (WBCs), epithelial cells, enzymes, hormones, mucus, immune-activity cytokines, immunoglobulin (Ig) A and electrolytes. The non-invasive nature of saliva sampling, including the use of self-administered saliva collection kits, can prove beneficial compared to blood sampling, for example in studies with children or in non-clinical settings. Common clinical assessments using saliva include measurements of illicit drugs, nicotine, certain microorganisms, IgA, and cortisol. As saliva samples contain cells, they are commonly collected for DNA analysis. In research settings, other hormones are also commonly measured in saliva, including the steroid hormone precursor Dehydroepiandrosterone (DHEA) and DHEA-sulfate (S), estrogen, progesterone, testosterone, and alpha-amylase.

To achieve high-quality reproducible data, proper saliva collection and handling procedures are necessary. The collection time is also critical since many hormones, like salivary cortisol,[61] have a circadian rhythm, which needs to be considered during sampling. Saliva donors should avoid food, drinks, and tooth-brushing within 60 min prior sampling. Use of alcohol, caffeine, nicotine, medications, and vigorous physical exercise within 12 h prior to sampling should be documented. The collection procedure and device can also influence the analyte levels. Passive drool is commonly used and provides saliva that is representative of all salivary glands. Oral saliva stimulants should not be used unless known to not affect levels of the analyte of interest (opt instead for olfactory or visual stimulants, if needed). Saliva can be collected through a straw-like device or can be spit directly into a tube. Alternatively, a swab can be used; however, the position of the swab can influence which salivary gland gets stimulated and overrepresented in the sample. Thus, it is advisable to only use swabs that have been recommended for the analyte-of-interest. Detailed sampling advice is found in Saliva Collection Handbook by Salimetrics (https://salimetrics.com/saliva-collection-handbook/).

Following saliva collection, it is important to consider the stability of analytes-of-interest, which may depend on the material of the collection container, temperature, time, and freeze-thaw cycles. In general, it is advisable to use polypropylene tubes, to avoid unnecessary freeze-thaw cycles, to aliquot samples, and to store aliquots at −20C or −80C depending on the analyte stability. Moreover, since the viscosity of saliva differs between samples and there are particles (e.g., cells) in the saliva, it is recommended to centrifuge and then vortex the supernatant prior to aliquoting. The DNA from the saliva cells contains the same DNA sequence as that in other cells of the body (except in the case of local mutations/mosaicism). However, epigenetic modifications, such as DNA methylation, can differ considerably between cell types.[255] It is, therefore, important to take into consideration the origin of the cell types that are present in samples. Finally, the

proportion of non-human DNA in saliva-derived DNA is important to consider, which has been reported to be on average 32%,[256] with most of the non-human DNA being bacterial in origin (food-derived DNA can also represent a considerable fraction). Thus, human-specific analyses of saliva DNA should be performed (e.g., using human-specific primer pairs in PCR).

Urine

Urine contains salts, urea, creatinine, organic ammonium salts, as well as break-down products (i.e., metabolites) of hormones and other molecules. Common routine urine tests in the clinic include measurements of pH, glucose, albumin, creatinine, ketones, drugs, infections, bilirubin, cytology, and metabolites from hormones such as serotonin, cortisol, androgens, aldosterone, and human chorionic gonadotropin (HCG).

In research settings, urine has also traditionally been used for measuring levels of hundreds to thousands of endogenous metabolites (e.g., amino acids, lipids, organic acids, sugars, and nucleotides), which constitute the metabolome. Although the metabolome can also be assessed in blood serum or plasma, urine presents with the advantage of having higher concentrations of metabolites and is often easier to prepare since protein concentration in normal urine is very low.[257] Thus, the urine metabolome constitutes an important research tool in the study of biochemical patterns of health and disease.[258,259] However, studies of the metabolome are complicated since the collected data also include breakdown products from foods, drinks, drugs, and environmental chemical exposures, as well as metabolites from hosted microorganisms such as intestinal bacteria (a list of > 4500 human metabolites in urine or other body fluids can be found in the Human Metabolome Database, hmdb.ca;[260]).

The non-invasive nature of sampling urine, in combination with the fact that urine metabolites are generally more stable (e.g., compared to proteins and peptides), simplifies sample handling and storage. Nonetheless, analyses of multiple metabolites often require expensive laboratory equipment such as chromatography coupled to mass spectroscopy or nuclear magnetic resonance (NMR).[261] Moreover, similar to blood and saliva, urine samples need to be properly stored to ensure that the metabolite profile at sampling is stable until analysis, and changes in the metabolite profile (e.g., by bacterial activity, can occur unless the urine is frozen). Urine samples should, therefore, be frozen as soon as possible, preferably at −80°C (although −20°C is enough for most metabolites) and should be kept at 4°C until freezing.[262–264] Although less sensitive than many protein/peptide analytes, some urine metabolites are affected by repeated freeze-thaw cycles;[262,263] hence aliquoting urine samples is recommended. The protocol for preprocessing of urine samples and detection of metabolites depends on the metabolites of interest. When specific metabolites are of interest, so-called targeted analysis is employed, and the preprocessing is often extensive to improve signal-to-noise ratio. By contrast, for untargeted metabolomics the preprocessing is simpler. The different amounts of water in the urine of individuals also need to be taken into consideration during analyses (e.g., by normalization of the metabolite concentrations, often using creatinine levels).

Collection parameters also need to be considered when sampling urine since they can affect downstream measurements of analytes-of-interest. For instance, time of urine collection should optimally be similar for all samples to be compared in a study. Moreover, urine metabolite levels can – similar to other analytes – show a circadian pattern. The first urine in the morning is often more concentrated and may therefore be preferable (although random day time is also often employed). For some clinical routine assays (e.g., urinary free cortisol), urine is collected over 24 h to get the total amount of analyte over a day and night. Taken together, and regardless of the tissue to be sampled, the sampling protocol for each study should be tailored based on which analytes are to be assessed. Moreover, the sampling protocols must also take into consideration the ability of the study participants to adhere to the sampling procedures. For home-sampling protocols, difficulties related to fasting, timings of sampling and storing of samples need additional attention.

Stool

Stool samples are by far the most widely used medium to study the gut microbiome. This method has clear advantages over other methods such as mucosal biopsies, being non-invasive, inexpensive, and useful for large-scale studies.[145] Several factors and possible biases need to be taken into account for stool sampling. Storage procedures vary between studies, making it difficult to compare and pool data.[265] Microbial growth usually continues after sample collection, which makes proper handling, to avoid biased microbiomes essential. This includes minimizing room temperature exposure, and time of transport to laboratory for freezing at −80°.[147,266] More details regarding this and alternative collection methods have been reviewed elsewhere.[147] The current gold standard is immediate freezing at −20°,[267] using regular fecal tubes without preservatives, for both microbiome and metabolomics analyses. The choice of method for stool sampling and storage should carefully be considered in light of these discrepancies across studies in order to optimize generalizability of results.[267,268]

Diet and feeding patterns are important factors for the composition and function of our gut microbiota. However, circadian rhythms have also been demonstrated to influence alterations in gut microbiota, which can only partly be explained by dietary factors.[269] Differences have been shown, based on when we eat and when we sleep, with change of up to 20% in relative abundance of commensal bacteria,[270] hence collecting information about diet is crucial when studying the gut microbiota. The importance of diet also reflects the diurnal rhythmicity of our metabolic system. The majority of human studies rely on Food Frequency Questionnaires for the collection of dietary data.[271] These findings show the importance of taking into account the time of day stool samples are collected. It might therefore be recommended that stool samples are collected at the same time of the day, preferably in the morning, to minimize the effect of diurnal changes.

Hair

Clinical and epidemiological studies are increasingly collecting cortisol concentrations in human scalp hair. This method takes advantage of the fact that cortisol is incorporated in the hair as it grows.[272] Because scalp hair grows about 1 cm per month,[272–274] the scalp-close 1 cm hair segment reflects the total cortisol secretion in the past month, the second 1 cm segment reflects cortisol production in the month before that and so on. As such, measurement of cortisol levels within a specific hair segment reflects the integrated, cumulative cortisol production within that hair growth period, providing a retrospective marker of longer-term hypothalamic-pituitary-adrenal (HPA) activity.[275] HCC can be reliably measured in scalp-close samples of up to 4–6 cm of hair length.[276,277] Several excellent reviews on the hair cortisol methodology and its validation, including in stress-exposed and psychiatric populations, have been published.[275,276,278–281] Below is a brief summary on hair sample collection, storage, assays, and confounding factors.

Hair sample collection is a non-invasive, painless, field-friendly and quick (approximately 10 min) method. Hair strands from 2–3 different spots are cut close to the scalp with scissors from the posterior vertex region of the head. The total amount of strands should be about half the diameter of a pencil (50–100 hair strands). After sample collection, hair strands are wrapped in aluminum foil to protect them from light exposure, humidity, and contamination. The scalp-close end of the hair sample is marked on the aluminum foil. All samples should be stored at room temperature and can be shipped through the mail.[278] Detailed descriptions and videos of supply materials, collection protocols, and academic and commercial laboratories are available on The Biomarker Network (https://gero.usc.edu/cbph/network/protocol/hair/) and the Stress Measurement Network (https://www.stressmeasurement.org/hair-cortisol). Information on how to obtain hair samples from African American participants, including sample collection for short hair (1–3 cm), has also been published.[282] To extract cortisol, scalp hair samples are cut into segments. The number and length of segments depends on the research question, though 1 cm hair segments have been the shortest used and hair segments greater than 4–6 cm might be affected by wash-out effects.[277] An immunosorbent assay (ELISA) is the most common method,[278] which requires about 5–50 mg of hair (per segment). The coefficient of variation is $< 10\%$.[278] Costs are currently about $40 per hair segment. Confounding factors may include sex, race, anthropometry, hair washing frequency, hair dyes, medication (e.g., steroid), stress exposure, and medical conditions.[275,283,284]

CONCLUSION AND PROSPECTS

Mental disorders are among the most common illnesses in the world, representing a leading cause of disability. Yet, core pathophysiological "targets" remain obscure, hindering the development of more effective and personalized treatments. Further, as mental disorders are increasingly seen as having whole-body concomitants, a broadening of the search for biological mechanisms is required. The search for biomarkers requires meticulous careful laboratory investigation alongside diligent careful clinical assessments, with attention to important covariates and an eye towards identifying homogeneous subtypes within heterogeneous psychiatric diagnoses. This brief review article describes a relevant yet non-exhaustive selection of biomarkers and their methodologies, and new leads and approaches that are under continuous development. It should be noted that in this manuscript we have focused on individual biomarkers, but it is likely that consideration of more than one biomarker together may prove more informative. Thus, while we discover individual biological factors involved in psychiatric illnesses and treatment response, we must also investigate integrated, multidimensional traits. To further advance our understanding and validation of biomarkers for psychiatric disorders, we emphasize a promising avenue for approaches that integrate multi-omics platforms towards predictive, preventative, and personalized psychiatry.[285] By harnessing machine learning approaches that take high-dimensional multi-omics data as input, we may uncover complex biological interactions, identify novel biomarkers, and develop personalized treatment strategies.[286–289] In addition, the integration of brain organoid technology also presents a promising pathway. Brain organoids, which are three-dimensional cultures derived from human pluripotent stem cells, offer a unique model for studying the complexity of the human brain in vitro. These organoids can replicate key aspects of brain development and disease, providing a dynamic system for testing the functionality and efficacy of identified biomarkers under controlled, yet biologically relevant conditions. Brain organoids facilitate detailed studies of neuronal processes and interactions that are otherwise inaccessible, making them invaluable tools for elucidating the biological underpinnings of psychiatric conditions and potentially guiding the development of targeted therapies.[290]

The most important key take-home messages of the present review, are therefore, broad: (1) Consider adding hypothesis-based biological assessments to well-designed clinical studies; (2) Ideally, this includes collection and proper preservation of biological samples for subsequent hypothesis-testing and exploratory analysis (e.g., for *post hoc* identification of individual subgroups or for testing novel biomarkers); (3) Pay particular attention to "controllable" and "uncontrollable" design features to achieve valid and reproducible results; including often-neglected pre-analytic laboratory procedures; (4) Since most psychiatric biomarker studies are exploratory, replication, and validation of results and combination of data across studies are paramount, given sufficient methodological similarity and adequate detailing of methods; (5) While we discover individual biological factors involved in psychiatric illnesses and treatment response, we must also investigate integrated, multidimensional traits.[291] With broader and more informed inclusion of biomarker assessment in rigorous clinical studies, and with greater uniformity and standardization of procedures, we anticipate great advances in the discovery of the biological mechanisms involved in psychiatric illnesses and treatment.

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All authors wrote and edited the manuscript and the content of the table. Figures in the table were prepared by Dr Han. The graphical abstract was prepared by Dr Månsson.

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CONFLICT OF INTEREST STATEMENT

Dr Milaneschi is paid consultant for Noema Pharma and Karla Therapeutics and dr. Lamers is paid consultant for Noema Pharma and has received speaking fees from Novo Nordisk.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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