

# Chapter 2

## The Application of Multiplex Biomarker Techniques for Improved Stratification and Treatment of Schizophrenia Patients

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### Abstract

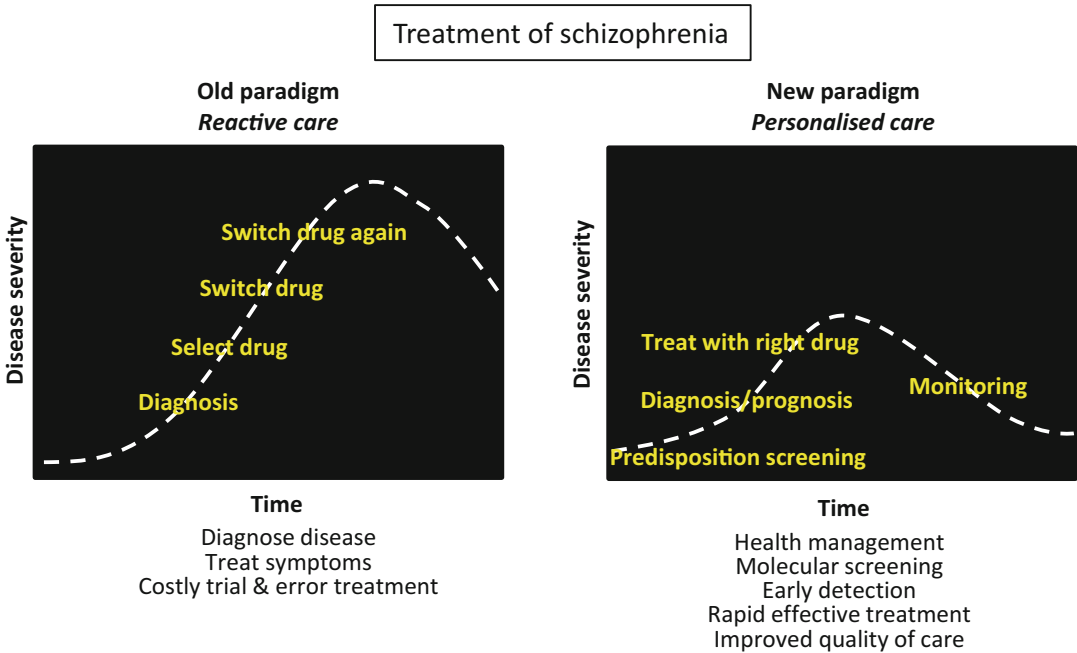
In the case of major psychiatric disorders such as schizophrenia, shortcomings in the conversion of scientific discoveries into newer and safer treatment options has led to a loss of confidence and precipitated a crisis for large pharmaceutical companies. This chapter describes how incorporation of multiplex biomarker approaches into the clinical pipeline can lead to better patient characterization, delivery of novel treatment approaches and help to renew efforts in this important area. The development of specific biomarker test panels for disease prediction should facilitate early intervention strategies, which may help to slow disease development or progression. Furthermore, the development of such tests using lab-on-a-chip and smart-phone platforms will help to shift diagnosis and treatment of this major disorder into a point-of-care setting for improved patient outcomes.

**Key words** Schizophrenia, Blood-based biomarkers, Proteomics, Multiplex immunoassay, Mass spectrometry, Point-of-care, Lab-on-a-chip, Smartphone apps

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### 1 Introduction

Schizophrenia is a debilitating, mental health disorder which can strike individuals in their late teens or early adulthood and seriously impair medical health, quality of life, social well-being and productivity [1]. Clinical presentation usually occurs with symptoms such as hallucinations, delusions, anhedonia, social retreat, disorganized thinking and cognition impairment. At present, diagnosis is still based on expression of symptoms and is dependent on communications between the affected individual and the attending clinician or psychiatrist. This is usually achieved in an interview-like format using the Diagnostic and Statistical Manual of Mental Disorders (DSM) [2] or the International Classification of Diseases (ICD-10) [3] criteria as guidelines. However, these texts can only detail



**Fig. 1** Comparison of the old and new treatment paradigms involving schizophrenia patients, distinguished by the use of biomarkers for improved stratification

the symptoms of schizophrenia without pointing to the underlying molecular physiological pathways that may be affected. Furthermore, classification of a person as having schizophrenia can be confounded by the fact that individuals with other psychiatric disorders can share many of the same symptoms. For this reason, there are now concerted efforts to identify specific multiplex biomarker fingerprints that can potentially predict the onset of schizophrenia, improve diagnostic accuracy, monitor disease progression, and guide treatment options. The availability of such tests for use in blood serum or plasma would be ideal as this would facilitate use in clinical settings. This is because blood-based biomarkers would have high accessibility in clinical practice due to the low invasiveness of the sampling procedure and the low associated costs.

The application of biomarker-based diagnostic tests that can accurately classify patients according to the type of disorder or even disease subtype will help to reduce duration of untreated mental illness and improve individual responses by placing the right patients on the right treatments as early as possible. This is because there is a direct correlation between longer periods without treatment and poor outcomes [4]. It is thought that this will change the overall paradigm from reactive psychiatric care to a more optimized personalized treatment approach in the field of psychiatry as well as in other areas of medicine (Fig. 1). Also, implementation of earlier effective treatment should help to reduce patient referral to

secondary services such as hospitals, community groups, and crisis teams. Any reduction in the use of these expensive services will help to reduce the overall financial burden of psychiatric disorders, which surpassed 60 billion dollars per year in the 1990s in the USA alone [5]. More importantly, an early successful intervention will help to curb symptom severity. This is because schizophrenia may lead to decades of life disability [6], more than double that of cardiovascular disorders [7].

The discovery of validated biomarker tests that reflect the correlation between the patient clinical and molecular readouts, would also enhance future mental healthcare significantly if the resulting tests can be incorporated into standard operating systems and clinical decision making, as well as being deployed as fast, cost-effective, user friendly, point-of-care devices. The strictest classification of newly developed biomarker tests requires that results must be replicated in different laboratories and in different sites. In the case of psychiatric disorders like schizophrenia, this will be difficult to achieve. The major reason for this is that these conditions are poorly understood at the molecular level and there is high heterogeneity in the way that they are manifested in the affected persons [8]. In this chapter, we discuss the challenges and requirements of developing and rolling out molecular biomarker tests for schizophrenia. In addition, the chapter focuses on the use of biomarkers for improved classification and management of patients with schizophrenia for improved point-of-care treatment and as a means of rekindling drug discovery efforts within the pharmaceutical industry in the area of psychiatric disorders.

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## 2 Current Diagnosis of Schizophrenia

Most psychiatrists and clinicians agree that schizophrenia is a general term for a mixture of mental conditions that present with similar symptoms, in the same way that most people with acute infectious disorders present with an elevated body temperature [8]. It should be emphasized that the enormous variety of psychopathological alterations summarized under the term “schizophrenia” do not represent a disease entity, but rather a hypothetical construct that was created many decades ago by leading authorities in the field and is now defined by international classifications committees, which have inclusion criteria that are changed from issue to issue. However, this crossover can lead to misdiagnosis in psychiatric practice. As an example, one investigation found that more than 30% of patients who actually had bipolar disorder were initially diagnosed as having schizophrenia [9]. Another study challenged the basis of the current classifications systems by pointing out that there are no current methods to validate the basic concepts which are independent of the same concepts [10]. In any event, psychiatrists do not always use these classification systems

for making a diagnosis. In many cases, diagnosis may be made based on experience and personal views in a more heuristic manner. Again, this is not ideal as it can result in errors based on misconceptions, biases or selective memories.

Aside from these issues, the DSM and ICD-10 classification systems work based on the framework that mental disorders such as schizophrenia are distinct diseases with common etiologies which can be defined by criteria based on signs and symptoms. In reality, it is often not the case that specific symptoms are linked to defined diseases. For example, individuals with traumatic disorders, infectious diseases, metabolic conditions or even those under the influence of certain substances can present with symptoms that occur in schizophrenia [11, 12]. In addition, it is not uncommon for a diagnosis to change over time. A long-term study found significant changes in diagnosis from major depressive disorder to bipolar disorder and schizophrenia [13] and another found that only half of the patients stayed on their initial diagnosis [14].

## **2.1 The Importance of Early Diagnosis**

The concordance rate for a diagnosis of schizophrenia in identical twins ranges from 10 to 70 % [15–17]. Although this provides evidence that there can be a genetic predisposition for schizophrenia, it also indicates that an individual will not necessarily develop schizophrenia even when a potential genetic effect is present. In fact environmental and other nongenetic factors are also important. Factors which could precipitate schizophrenia include pregnancy or delivery complications, such as infections, hypoxia or malnutrition [18, 19], as well as nonbiological factors, including social stressors such as experiencing a natural disaster, loss of a family member, or the chronic experiences of an unbearable environment such as an intolerable work situation, a dysfunctional family life, or an abusive relationship [20]. On a positive note, the presence of an environmental component also suggests that disease prevention or minimization might be possible if the responsible factors can be identified and avoided.

It is not difficult to imagine that certain environmental factors such as poor nutrition, social stress, and physical trauma can affect a person's physiological state. Several research groups have now shown that metabolic abnormalities such as insulin resistance occur in 20–50 % of schizophrenia patients at their first clinical presentation [21–23]. Furthermore, multiple research groups have found alterations in circulating inflammatory and immune response markers in first onset schizophrenia patients [24, 25]. Two studies have now shown that such changes can occur months to years before full clinical manifestation of schizophrenia symptoms, suggesting that perturbations in these molecular pathways may play a role in the disease etiology [26, 27]. This also gives hope for identifying individuals at risk of developing the disease at the earliest

possible phase, as described above. This is important as numerous reports have now described importance of early intervention therapeutics for individuals at high risk of developing schizophrenia [28–30]. Any delay in diagnosis can have detrimental effects on the lives of the patients, such as the patient experiencing a full blown psychosis leading to other problems including substance abuse, alienation from family and friends, increased accidents, and the potential of self-harm [31, 32]. There is the problem of misdiagnosis which can lead to inappropriate treatments, which can either be ineffective or even harmful to the patient. In addition, misdiagnosis followed by inappropriate treatment can have a number of socioeconomic consequences, such as inflated medical costs, work absence, and harmful effects on family and relationships [33].

## ***2.2 The Development of Biomarker Assays for Diagnosis of Schizophrenia***

The European health authorities have lent support to the development and implementation of biomarkers through agencies such as the Innovative Medicines Initiative [34, 35]. This began as a partnership between the European Commission and the major pharmaceutical companies with the overall objective of promoting more efficient discovery and development of better medicines. A key objective is the discovery of translational biomarkers which, in this case, means incorporating them into drug discovery pipelines for use in human clinical studies. The European Commission contributed one billion Euros to this project and this has been matched in kind by contributions from the participating companies.

Diagnostic biomarker tests in the USA are regulated by the Clinical Laboratory Improved Amendments (CLIA) agency [36]. These imposed regulatory standards govern any tests that are performed in a clinical setting on human samples for the purpose of diagnosis, disease prevention, treatment or assessment of health. Commercially available tests marketed under CLIA are categorized by the FDA depending on the potential risks for health. The development of diagnostic biomarker tests for all diseases including psychiatric disorders requires repeated demonstrations of precise performance characteristics including scores such as sensitivity and especially specificity, given the symptomatic and molecular overlap among all psychiatric disorders. This is an absolute requirement since biomarker measurements can be affected by many factors including biological, ethnicity, gender, environmental, sample collection, and analytical variables. For example, development of multiplexed immunoassays requires the testing and validation of each component immunoassay as well as the combination of assays used in each multiplex to maximize repeatability, precision and accuracy. This includes selection and immobilization of capture ligands on microbeads, calibration steps, testing for reagent–antibody compatibility, and ensuring each individual assay has sufficient dynamic range and the required limits of detection [37].

Another criterion of biomarker tests that is often overlooked is that they must be in a format that is high throughput, accurate and user friendly to allow use by clinicians, hospital staff and scientists. Along these lines, we suggest that the discovery and implementation platforms should be different to maximize development of tests with the highest performance. Thus, although they are powerful discovery tools, mass spectrometry and two-dimensional gel electrophoresis techniques may be too cumbersome in their larger formats and may require a high level of expertise to be considered as realistic options for clinical use. In contrast, an automated platform based on multiplexed immunoassay is a more likely candidate as a clinically friendly platform as it has already shown some promise in this area. However, even this would be too slow in its discovery format.

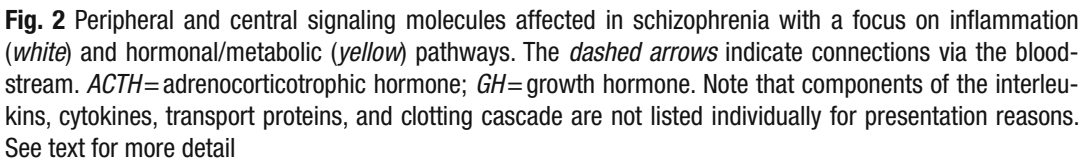
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### 3 Biomarkers Identified for Schizophrenia

Although genomic studies are able to identify genes conferring susceptibility to a particular disease, the functional abnormalities of most disorders are reflected ultimately in the proteome and metabolome. This is because proteins and metabolites represent the molecular phenotype of a disease in parallel with the clinical manifestation. Recent years have seen the increasing use of proteomics as a tool for the discovery of biomarkers for diagnosis, monitoring disease progression, treatment response and for the identification of novel therapeutic targets. It is also important to remember that analysis of central nervous system (CNS) disorders is difficult as the brain is not readily accessible for invasive diagnostic purposes. Thus, sources such as serum and plasma have been undergoing increasing scrutiny as they have a higher utility in the clinic.

#### **3.1 Inflammation Biomarkers in Schizophrenia**

A multiplex immunoassay profiling study which used cytokine arrays identified increased levels of interleukin (IL)-1 $\beta$  in cerebrospinal fluid from first onset schizophrenia patients, suggesting that the inflammation response may be perturbed in the brains of some patients [38]. This is consistent with other studies which demonstrated that brain development can be disturbed by changes in the balance of pro-inflammatory and anti-inflammatory cytokines [39, 40]. In addition, altered inflammation has been linked to changes in the glutamate system, the main excitatory neurotransmitter in the brain. Transcriptomic and proteomic profiling studies of post mortem brains from schizophrenia patients have identified increased levels of inflammation-related gene products in oligodendrocytes and endothelial cells in comparison to non-psychiatric control subjects [41, 42]. However, it is possible that this is a confounding factor of prolonged drug treatment or an unhealthy lifestyle, as often occurs in the chronic or latter stages of individuals suffering from this disorder (Fig. 2) [43].



The finding of circulating changes in molecules such as inflammatory factors in psychiatric disorders like schizophrenia is what makes biomarker testing for psychiatric disorders feasible [44, 45]. In addition, these factors may be informative as either trait or state biomarkers. A meta analysis of circulating inflammation-related changes in schizophrenia patients showed that cytokines including IL-12, soluble IL-2 receptor, interferon- $\gamma$ , and tumor necrosis factor- $\alpha$  may be useful as trait biomarkers, giving a stable indication that the disease is present [44]. In contrast, cytokines such as IL-1 $\beta$ , IL-6, and transforming growth factor- $\beta$  may represent state



biomarkers, which means that these may be used as readouts for acute changes in the disease. In addition, there have been many reports on the discovery of blood-based biomarker signatures comprised of a large number of inflammation-related proteins including some components of the clotting cascade and transport proteins in first onset schizophrenia patients [46, 47].

Inflammation in the periphery can affect brain function through effects on the hypothalamic–pituitary–adrenal (HPA) axis (see below) [48, 49]. Activation of inflammatory pathways stimulates release of corticotrophin releasing factor from the hypothalamic region of the brain and this initiates a cycle causing adrenocorticotrophic hormone (ACTH) to be released from the pituitary, which in turn drives cortisol release from the adrenal cortex [50]. Along with several other effects in the body, cortisol also exerts a negative feedback control on the HPA axis by binding to specific receptors in the brain and pituitary [51]. The link to psychiatric disorders comes from the fact that the HPA cycle also alters neurotransmitter systems throughout the brain, which are involved in regulation of mood and behavior. Given this link, it is not surprising that some investigators have tested the use of anti-inflammatory drugs such as aspirin or cyclooxygenase-2 inhibitors in combination with traditional antipsychotics as a novel treatment approach to relieve some symptoms of schizophrenia, with some success [52–55]. However, these findings require validation in further studies involving larger cohorts.

### **3.2 Neuroendocrine-Related Biomarkers**

Several studies have now shown effects on a number of hormonal systems related to metabolic homeostasis in schizophrenia. A number of studies over the past decade have shown that impaired fasting glucose tolerance, high insulin levels and insulin resistance occurs in both first onset [21, 22] and chronic schizophrenia patients [55, 57], as can occur in type 2 diabetes patients. One study showed the presence of hepatic insulin resistance in schizophrenia patients using a hyperinsulinemic clamp [58]. In terms of biomarkers, two studies found that first onset schizophrenia patients had increased levels of circulating insulin-related peptides and high levels of chromogranin A, pancreatic polypeptide, prolactin, progesterone and cortisol, along with lower levels of growth hormone, in comparison to controls [23, 59]. This indicated altered secretion from several neuroendocrine glands including pancreatic  $\beta$  cells, pancreatic PP cells, the anterior pituitary, the sex organs and adrenal glands (Fig. 2). This could have important implications since chronically high insulin levels can have disruptive effects on brain function such as inducing increased brain inflammation, aberrant phosphorylation of filamentous structural proteins and increased deposition of amyloid plaques [60–62]. High insulin levels have also been found to lead to altered function of neurotransmitter pathways [63] and perturb synaptic plasticity



in brain regions such as the hippocampus [64]. The increased cortisol secretion is indicative of an activation of the HPA axis, as described above, which has been identified as a risk factor for schizophrenia in adolescents [65]. Another study showed gender-specific changes in the sex hormones estradiol and testosterone in schizophrenia patients, suggesting effects on the gonadal tissues [66]. More recent studies found decreased serum levels of thyroxine, triiodothyronine, and thyroid-stimulating hormone in schizophrenia patients [67], which may be tied in with the metabolism-related hormone changes described above. Another factor to consider is that many hormones are influenced by circadian rhythms and it is likely that some of those described above are co-regulated as part of an oscillatory feedforward–feedback mechanism between pancreatic islet cells, the pituitary and other components of the diffuse neuroendocrine system. For example, high insulin secretion has been associated with increased prolactin levels [68] and disrupted pulsatile release of growth hormone [69].

The repeated finding that hyperinsulinemia occurs in some first onset schizophrenia patients suggests that drugs which alleviate insulin resistance may offer a novel treatment approach. Furthermore, chronically treated patients can also exhibit high insulin levels since antipsychotic drugs can induce metabolic side effects such as insulin resistance and weight gain. Interestingly, the weight gain appears to be linked to antipsychotic therapeutic efficacy. One investigation showed that changes in body weight, blood glucose, and leptin levels were associated with improvement in both positive and negative symptoms of schizophrenia [70]. However these effects may not be an absolute requirement for improvement as studies that used the insulinsensitizing agents metformin and rosiglitazone to treat the antipsychotic-induced insulin resistance did so without disrupting the psycho-therapeutic benefits [71]. Therefore, the relationship between metabolism and psychiatric symptoms requires further scrutiny. It is possible that insulin-sensitizing agents may have a direct effect on alleviating some symptoms, such as the cognitive deficits. In support of this possibility, one study found that patients with mild Alzheimer's disease who were given pioglitazone showed improvements in cognition along with increased regional cerebral blood flow [72].

Drugs which target other hormone systems have also been tested as a novel means of treating schizophrenia symptoms. Dehydroepiandrosterone (DHEA), an adrenal steroid-like compound, has been tested as a potential add-on therapeutic with antipsychotics and this led to improvements in depression and anxiety symptoms in some schizophrenia patients [73]. Furthermore, treatment with the selective estrogen receptor modulator raloxifene resulted in reduced negative symptoms in postmenopausal females with schizophrenia compared to controls [74].

### **3.3 Biomarkers for Prediction of Treatment Response**

Biomarker tests that can be used for better classification of schizophrenia patients opens up the possibility of better treatment options. For example, biomarkers that can be used to predict response of schizophrenia patients to treatment would be an important step forward for the well-being of the patients and it will assist the prescribing physicians, as well as pharmaceutical companies conducting clinical trials. Genetic studies have shown that polymorphisms in the histamine 2 receptor (*HRH2*) gene can be used to predict response to clozapine treatment in 76% of schizophrenia cases [75]. Other studies have shown that variants in genes for dopamine receptors, serotonin receptors and enzymes involved in drug metabolism or neurotransmitter turnover can have influence of patient response to treatment including the propensity to develop certain side effects [76]. Another way of predicting response is through the use of physiometric measurements such as waist circumference, adiposity, body mass index (BMI), which have already been used to predict the development of side effects such as metabolic syndrome or other insulin resistance with good sensitivity and specificity [77, 78]. As for blood-based proteomic biomarkers, one study showed that schizophrenia patients with higher levels of serum prolactin have a better outcome following 5 years of antipsychotic treatment [79]. Two multiplex immunoassay serum profiling studies found that the levels of insulin were predictive of improvement in negative symptoms [80] and those of specific apolipoproteins, growth factors, hormones and interleukins could be used to predict weight gain [81] in first-onset schizophrenia patients after 6 weeks of antipsychotic treatment (Table 1). Another study showed that the levels of fatty acid binding protein could be used to predict response to olanzapine [82]. It should be kept in mind that these three investigations involved study of first or recent onset patients and biomarker profiles may be different for more chronic patients. Further studies aimed at retesting these prototype biomarker panels may lead to development of validated molecular tests that can be used to identify those patients who are more likely to respond to particular antipsychotic medications as well as those who are likely to benefit from add-on compound that target either the inflammatory or metabolic symptoms. This could also lead to the opportunity for clinicians to take actions such as patient assessment, counseling, or even readjusting treatments in accordance with measured biomarker readouts.

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## **4 Point-of-Care Methods for Use in Schizophrenia**

For decades psychiatrists and have acted on the assumption that psychiatric disorders such as schizophrenia are caused by defects in brain. However, developments over recent years have resulted in a new concept involving the whole body in the precipitation and

**Table 1**

**Significant associations between molecular levels at baseline and changes in either (A) psychopathology scores (positive and negative syndrome scale—PANSS) or (B) body mass indices (BMI) after 6 weeks of antipsychotic treatment. *R* = Spearman correlation coefficient. *NS* = not significant. *ANCOVA* = analysis of covariance [80, 81]**

<b>A</b>				
<b>Positive symptoms</b>			<b>Negative symptoms</b>	
<b>Protein</b>	<b><i>P</i>-value</b>	<b><i>R</i></b>	<b><i>P</i>-value</b>	<b><i>R</i></b>
Insulin	NS	–	0.005	–0.37

■

<b>B</b>		
<b>Protein</b>	<b><i>ANCOVA</i></b>	<b><i>R</i></b>
Apolipoprotein CIII	0.019	–0.33
Apolipoprotein H	0.005	–0.33
Epidermal growth factor	0.025	–0.28
Follicle-stimulating hormone	0.043	–0.28
Interleukin 18	0.015	0.24
Interleukin 25	0.024	–0.26
Interleukin 6 receptor	0.031	–0.30
Matrix metalloproteinase 1	0.011	–0.24
Placental growth factor	0.016	–0.24
Thyroid-stimulating hormone	0.026	–0.23

progression of these conditions. This is because the brain is holistically integrated in most fundamental biological functions of the body and therefore the functioning of this organ can be monitored by examining changes in the molecular composition of the blood. This is the basis for the use of blood serum or plasma in the study of psychiatric diseases [46, 47, 83]. This is useful since blood can be taken from living patients at different stages of the disease or throughout a treatment course. In the foreseeable future, it is likely that increased biomarker testing by clinicians will lead to more extensive “bio-” signatures in individuals that reflect the physiological status occurring in health or disease. Blood serum and plasma samples contain many molecules such as hormones, growth factors and cytokines which can only be detected using methods

that are highly sensitive. One of the best methods to achieve this is the sandwich format of immunoassay [84, 85] and this is the basis for the multiplex immunoassay platform described above.

#### ***4.1 Credit Card-Sized Devices and Mobile Phone Apps for User Friendly Rapid Testing***

Multiplex immunoassay biomarker tests have now been available for more than a decade on medium-sized laboratory equipment and with typical turnaround times of around 1 week from the sample preparation stages to the final results analysis. More recently, multiplex methods have been developed using microfluidic approaches to yield a devices which are approximately the size of a credit card [86]. This offers the possibility of inexpensive analysis using either electrochemical or optical read-outs. Most importantly, this approach is user friendly as no expertise is required for operation. The protocol involves application of a blood drop to the card followed by insertion of the card into a book-sized reader/analyzer and then a diagnosis score can be read out in less than 15 min. The major benefit of this approach is the rapid turnover time and this will help to minimize waiting times for lab test results, which can often take several days or even weeks using standard methods. Furthermore, these devices can connect to a computer for transmission of data to a smartphone device. Large consumer market companies like Apple and Google are now showing interest in the diagnostic market and are exploiting the potential of linking diagnostic test results with an app driven by smart software. This would allow testing using real-time, multiplexed sensors, linked with artificial intelligence through mobile communication systems. This is of particular relevance to mental disorders such as schizophrenia, since it is generally a long term disease that requires constant monitoring and treatment. A recent review of trials involving medical care interventions facilitated by smartphones showed that patient outcomes were improved more than 60% of the time [87]. Recently, multiplex immunoassay based tests have been developed on a handheld smartphone-based colorimetric reader using a 3D-printed optomechanical interface [88]. To date, this approach has been tested successfully in a clinical microbiology laboratory using mumps, measles and herpes simplex I and II virus immunoglobulin tests. It is not hard to imagine that similar tests for other diseases such as psychiatric disorders will be available in the not so distant future.

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## **5 Conclusions**

This chapter describes recent advances using biomarker tests which can be used for improved diagnosis and classification of individuals with schizophrenia. The ultimate goal is to provide more informed treatment options for improved patient outcomes. The use of the multiplex biomarker approach provides a way of unraveling the

convoluted array of molecular pathways affected in this disorder and potentially facilitate identification of disease subtypes which require different treatment approaches. For example, many patients show distinct patterns of blood-based molecules which suggest the presence of perturbed inflammation- or metabolism-related pathways as described in this chapter. Thus, improved classification of such patients based on biomarker profiling would enable selection of better treatment options such as the potential of including add-on therapeutics targeting these pathways. Finally, the use of multiplex tests on handheld devices capable of distinguishing schizophrenia patients who are most likely to respond to specific psychiatric medications would be an important breakthrough for point-of-care applications. This could help to improve the lives of individuals suffering from this debilitating disorder and have beneficial effects on society as well as significant cost savings for the healthcare services in general.

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