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Schizophrenia biomarkers: translating the descriptive into the diagnostic

Benjamin S. Pickard

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow, G4 0RE, Scotland, UK

Email: benjamin.pickard@strath.ac.uk

Tel: +44 141 548 4572

Abstract

While schizophrenia and mental health are qualitatively distinct at the level of clinical presentation, the specific molecular signatures that underlie, or associate with, illness are not. Biomarker identification in schizophrenia is intended to offer a number of important benefits to patient well-being including prediction of future illness, diagnostic clarity and a level of disease description that would guide treatment choice. However, the choice of sample and form of analysis used to produce useful biomarkers is still uncertain. In this review, advances from recent studies spanning the technical spectrum are presented together with comment on their comparative strengths and weaknesses. To date, these studies have aided our understanding of the pathological processes associated with illness much more than they have provided robust biomarkers. A number of reasons for this observation are suggested, as are new strategies for the extraction of biomarkers from large '-omics' datasets.

Keywords

Schizophrenia, biomarker, epigenetics, methylomics, microarray, transcriptomics, metabolomics, neurodevelopment, inflammation, metabolism, oxidative stress.

Introduction

One of the goals of schizophrenia research is to define the disorder in clear and simple biological terms and, in so doing, relegate it to the catalogue of mundane – and more tractable - human disorders. The current pace of developments in large-scale biological data acquisition and processing might make the task appear eminently achievable. However, even the most fervent optimists among us would have to admit that, despite this effort, we are still piecing together the edge of the jigsaw

puzzle rather than seeing the full picture. Contrast this situation with that of Parkinson's disease – a cousin-by-neurotransmitter of schizophrenia. A cross-disciplinary understanding of environmental influences, genetic risk, cellular pathology, anatomical pathology, network dysfunction, and outward symptomatology have converged to inform the development of novel therapies. Multiple explanations have been put forward for this qualitative difference in disorder complexity, with most focused on the idea that schizophrenia is not a unitary disorder but rather the common symptomatic end-point of a great variety of brain dysfunctions and insults. In support of this, schizophrenia now belongs to a wide family of disorders that have demonstrated interrelationships at the level of genetics. These include bipolar disorder, depression, epilepsy, intellectual disability, autism spectrum disorders and, recently, multiple sclerosis [Consortium, 2013, Lee *et al.*, 2013, Andreassen *et al.*, 2014, Mccarthy *et al.*, 2014, Ruderfer *et al.*, 2014]. Hence, the aetiopathology of one individual's schizophrenia is unlikely to be the same as that of the next individual; and two people with similar aetiologies may diverge at the level of diagnosis [Corvin, 2012]. These features should actively shape any analytical approaches: both in terms of the necessity for increased sample sizes in any form of a case-control study, and the need for new approaches to tease out pathologies relevant to the individual as well as the population. Biomarker identification is one such field of research that will have to meet these demands.

Biomarkers are the molecular, functional, anatomical, or physiological signs that can be translationally harnessed to predict or confirm a particular diagnosis, or suggest or monitor a particular treatment strategy. A recent review [Weickert *et al.*, 2013] has succinctly described the importance of these applications of biomarkers in the context of schizophrenia diagnosis and treatment. The progress in development of commercial schizophrenia biomarker tests depends on economic models, regulation and perceived healthcare need, just as much as bioinformatics. Hence, there may be a more pragmatic short-term goal for biomarkers: to help sub-divide and classify schizophrenia according to its principal molecular aetiopathologies.

Biomarkers could be considered to extend all the way to include our fixed genomic attributes. However, it is perhaps more useful to consider only those quantifiable features that display a potential for response and variation in the lifetime. Schizophrenia researchers have searched for biomarkers in altered morphology and representation of specific CNS cell types in post mortem tissue, structural and functional brain imaging, externally measured electrophysiological properties, cognitive performance, and various dimensions of the psychiatric phenotype. At the sub-cellular and tissue levels, the search has queried the transcriptomic, proteomic, metabolomic, immunological, and epigenetic levels of biology. It is this second group of potential biomarkers that will be covered in this review. Particular attention will be paid to new methods (e.g. epigenetics) and the larger-scale

studies which avoid sample size issues. Additionally, the current state of biomarker discovery in other disorders will be briefly mentioned in order to highlight the distance that schizophrenia has left to travel.

Transcriptomics

The search for gene expression changes associated with schizophrenia is the most worn research path towards biomarker identification. However, despite the efforts of many laboratories, the transcriptomics of cancer, for example, is still considerably further progressed. A commercially and clinically viable test, Oncotype DX (Genomic Health, US), can now generate a risk value for the re-occurrence of breast cancer (and thus guide follow-on monitoring and treatment) based on the analysis of 21 genes [Paik *et al.*, 2004]. Schizophrenia has some way to go to match this, but the field has generally appreciated the need for large sample sizes to compensate for its complex and heterogeneous nature. Transcriptomics studies in schizophrenia have either focused on the ease of access to blood samples - and their downstream translational potential – or the pathological relevance offered by post mortem brain tissue. The use of blood transcriptomics for the purpose of disease diagnosis and patient stratifications has been recently reviewed [Kumarasinghe *et al.*, 2012, Mamdani *et al.*, 2013] and so here only particularly illuminating examples will be discussed. These are followed by the latest individual and meta-analyses from post mortem cortex samples.

In one study, whole blood was obtained from 52 antipsychotic-naive schizophrenia patients and 49 healthy controls [Takahashi *et al.*]. 792 differentially expressed genes were discovered through microarray analysis and the process of cell adhesion identified as a significantly over-represented gene ontology term within. A neural network approach was then adopted in order to define a diagnostic set of genes. This set comprised *CINP*, *TDRD9*, *DAOA*, *PGRMC1*, *NAF1*, *LIPH*, *MAP1D*, and *INSL3*, and had the power to correctly diagnose samples with 88% accuracy. In another study, blood gene expression was profiled in patients diagnosed with schizophrenia that were being treated with clozapine (n=10), risperidone (n=10) or haloperidol (n=8), and compared to gene expression profiles of healthy controls (n=10) [Maschietto *et al.*, 2012]. Six genes that were identified on the basis of disease-specific gene expression changes (*HERPUD1*, *HOXA13*, *CTNNA1*, *SULT1A1*, *PIK3R3* and *MALAT1*) were used as the basis for a diagnostic test. This was able to correctly identify disease status in 89.3% of cases of schizophrenia and 70% of healthy controls. However, this assessment was carried out in the same set of samples and controls - meaning its use is still to be truly tested. Next generation RNA-seq was used to analyse differential gene expression differences in blood from 36 drug-naive schizophrenia patients and 40 healthy controls [Sainz *et al.*, 2013]. Of the 200 genes

identified, some overlapped with genome-wide association studies of the disorder (including *CSMD1*, *EHF*, and *RFX2*), and others had been previously described in schizophrenia literature (*GRIK3*, *LPL*, *S100B*, *SNCA*, *SYN2*, *TUBB2A*, and *SELENBP1*). Gene ontology enrichment in the differentially expressed genes suggested a key role for wounding, and acute inflammatory and innate immune response. A larger study assessed gene expression changes in peripheral blood mononuclear cells taken from 114 cases of schizophrenia or schizoaffective disorder and 80 healthy controls [Gardiner *et al.*, 2013]. The genes *EIF2C2*, *EVL*, *DEFA4*, *S100A12*, *PI3*, and *MEF2D* all showed validated expression changes in this cohort and, overall, a strong indication of a role for immune gene involvement in schizophrenia was described. Transcriptomic analysis of a large set of blood samples from 121 individuals diagnosed with schizophrenia (29 of whom were anti-psychotic treatment free) and 118 healthy controls was recently published [De Jong *et al.*]. Their analysis differed from the standard 'ranked' or 'fold-change' approaches, and instead used Weighted Gene Co-expression Network Analysis in which 'modules' of genes are identified that co-vary in expression between cases and controls. Two identified modules strongly associated with the disease state were replicated in an antipsychotic-free dataset, and were highly enriched in brain genes. They included the genes *ABCF1*, *SLC2A6*, *SDHA*, *DHRS1*, *sep-06*, *CNDP2*, *SIGIRR*, *FBXL5*, and *DHX58*. These data possessed several interesting features. Firstly, there was a profound effect of medication on gene expression, indicating that treatment-response related biomarkers may be readily achievable, but at the cost of the identification of disease-specific biomarkers. Secondly, the fact that the two replicated modules were significantly enriched for brain genes confirms the relevance of blood analysis for the investigation and diagnosis of brain dysfunction. In fact, independent studies have confirmed this point [Harris *et al.*, 2012] . Lastly, one module contained numerous circadian genes that most likely relate to the time of blood sample collection [Whitney *et al.*, 2003]. This is a technical issue that should be factored into any clinical biomarker study.

A parallel study also used co-expression modules [Chen *et al.*, 2012]. However, the authors studied post mortem prefrontal cortex samples from those diagnosed with schizophrenia (n=45) and healthy controls (n=46) and identified two modules centred around the *NOTCH2* and *MT1X* genes, respectively, that were associated with the diagnosis of schizophrenia. Ontology analysis of the two modules revealed significant signatures for neuronal differentiation/development and metallothioneins/metal-binding proteins, respectively. A meta-analysis was conducted on data from microarray studies of the dorsolateral prefrontal cortex comprising 107 patients with schizophrenia and 118 healthy subjects [Perez-Santiago *et al.*, 2013]. The expression of genes *BAG3*, *C4B*, *EGR1*, *MT1X*, *NEUROD6*, *SST*, and *S100A8* was found to be significantly altered in schizophrenia. Note that the gene *MT1X* is shared between this study and that of Chen *et al.* described above: but also note

that *MT1X* and *S100A8* were pinpointed in this study as genes whose regulation probably reflects anti-psychotic treatment rather than disease status. A second meta-analysis combined gene expression studies of prefrontal cortex from 153 patients diagnosed with schizophrenia and 153 healthy controls. It also used co-expression analysis models to identify gene ontologies contributing to disease pathology [Mistry *et al.*, 2013]. Upregulated genes included *SMG1*, *PLOD2*, *LPL*, *RHOBTB3*, *BBX*, *EIF2C2*, *FTL* and *P4HA1*. Downregulated genes included *NECAB3*, *RFTN1*, *HBQ1*, *GNAL*, *PPA2*, *KCNK1*, *OPCML*, *OPN9*, *FBXO9*, and *RGS7*. Analysis of the full set of significant genes indicated that altered energy metabolism and immune response were probable key pathologies. The co-expression analysis picked up similar ontology themes but, based on the location of certain transcripts, was also able to assign pathologies to particular cell types. For example, oxidative phosphorylation, synaptic and ubiquitination deficits were assigned to neurons, immune and glutamine metabolism deficits to astrocytes, and myelination deficits to oligodendrocytes – thus increasing our cell-level understanding of pathologies.

Epigenetics

Epigenetics is the study of long-lasting modification of nuclear DNA (for example, methylation or nucleosome modification) that is often influenced by the environment and manifests itself as changes in gene expression. Recently reviewed [Nishioka *et al.*, 2013], the study of global methylation changes associated with illness is the newest source of potential biomarkers for schizophrenia. As with genomics, transcriptomics and metabolomics, new analytical platforms (e.g. the Illumina Infinium HumanMethylation27 BeadChip array) as well as databases and resources are now available to aid biomarker discovery. Of particular interest is the brain-centric MethylomeDB which includes data from psychiatric disorders [Xin *et al.*].

The methylation state of genomic DNA from the white blood cell genomes of 177 individuals diagnosed with schizophrenia and 171 healthy controls was recently studied [Melas *et al.*]. Global hypomethylation was observed in schizophrenia and was exacerbated in early-onset cases. Treatment with haloperidol, alone among the antipsychotics, was observed to return methylation to levels approaching normal. However, this analysis was carried out using methylation-sensitive restriction enzymes. Most studies, including those included below, apply bisulphite analysis and assess specific loci. Therefore, the general significance of global methylation, as opposed to locus-specific methylation, has yet to be fully clarified.

One study looked at methylation changes in DNA from post mortem frontal cortex samples from 35 individuals diagnosed with bipolar disorder, 35 with schizophrenia and 35 healthy controls [Mill *et*

al., 2008]. Among their findings, hypermethylation was observed in the genomic loci associated with *RPP21* (tRNA maturation pathway) and *KEL* (blood group). Hypomethylation was observed near *WDR18* and *GRIA2* (a glutamate receptor gene that has also been frequently linked with age-dependent hypermethylation). In another study, 5 individuals diagnosed with schizophrenia, 7 with bipolar disorder and 6 healthy controls were assessed [Xiao *et al.*]. Over one thousand differentially methylated regions were identified, including overlaps with the data from Mill *et al.*. The authors also showed that local methylation changes clearly correlated with local gene expression changes. The identified methylation changes were over-represented in genes involved in synaptic transmission and axon guidance. A third study described the methylation analysis of white blood cell genomic DNA from 18 patients diagnosed with first-episode schizophrenia and from 15 healthy controls [Nishioka *et al.*, 2013]. Methylation changes were largely associated with CpG islands in genes which were enriched for functions linked to the nuclear lumen, transcription factor binding, and nucleotide binding. Specific changes were observed for the catecholamine neurotransmission genes *HTR1E* and *COMTD1*. In another example, the analysis of 24 patients diagnosed with schizophrenia and 24 unaffected controls was described. Again, many thousands of differentially methylated regions were identified (~50% were hypermethylated, and often in the CpG island regions of gene promoters) [Wockner *et al.*, 2014]. Significant overlaps with other genetic and 'methylomics' studies were reported, for example *DRD2*, *NOS1*, *AKT1*, *HTR2A*, *SOX10*, *FOXP2*, *DTNBP1*, *NRG1*, *PPP3CC*, *BDNF*, *ZNF804A*, *NRGN*, *DRD4*, *MGST1*, *COMTD1* and *GABRB2*. Importantly, the multidimensional scaling employed to cluster individuals was not only able to partially resolve schizophrenia from control samples but also subdivided schizophrenia into two distinct groups. The clinical presentation of the two groups offered no clues as to the pathological basis of this separation but methylation levels at the schizophrenia candidate disease genes *DTNBP1*, *COMT* and *DRD2* were distinct between the two. Finally, next generation sequencing was used to profile methylation-enriched blood genomic DNA taken from very large cohorts of 750 individuals diagnosed with schizophrenia and 750 healthy controls [Aberg *et al.*, 2013]. With such a large sample set it might be expected that the results would be clearer, but in fact they were more conservative than those described above. Particularly evident was how the age of the individual and various lifetime/environmental effects (e.g. smoking) appear to act as significant confounders to the interpretation of such data. However, replicated evidence was indeed found for altered methylation at the genes *FNDC3B* (involved in cell mobility) and *DCTN2* (encoding a cytoskeleton remodelling protein that is known to interact with psychiatric illness candidate proteins GSK3β and DISC1).

Metabolomics

Metabolomics is the global study of body tissue or fluid constituents. Genomic variation, transcriptomic regulation, and environmental influence (e.g. drug treatment or life exerperience) all converge on metabolomic profile changes. Therefore, it offers a pragmatic means to interrogate state- and disease-dependent changes in patient samples. A very recent example of metabolomics being successfully applied to blood samples comes from a study of Alzheimer's disease (AD) [Mapstone *et al.*, 2014]. 525 elderly individuals were followed up over a 2-3 year period and, of this cohort, 74 showed progression to mild cognitive impairment (MCI) or AD. From the metabolomic profiling of blood from 53 of the 'converters' and 53 healthy non-converters, a simple logistic classifier model - based on phospatidylinositol, proline-asparagine dipeptide, glycoursodeoxycholic acid, and malic acid- was created and verified as 90% accurate in its prediction of conversion to an AD phenotype in a second validation sample set. In a related study, a proteomics test for AD using multiplexed bead-based bioassays has recently identified 10 plasma proteins (including transthyretin, clusterin, cystatin C, CC4 and ApoE) that have an 87% accuracy to predict transition to AD from MCI [Hye *et al.*, 2014]. These two studies show that predictive tests based on metabolomic/proteomic analysis of blood can be a powerful clinical tool facilitating a rapid and tailored therapeutic response for neurological disorders.

A number of high-quality studies have now queried the schizophrenia metabolome. They have demonstrably outperformed the transcriptomic studies in terms of consistency and biological informativeness. For example, in one study, blood plasma analysis was carried out on 103 lipid, amino acids and carnitine metabolites from 216 healthy controls and 265 schizophrenic patients [He *et al.*, 2012]. Significant changes in this limited panel were observed for the amino acids arginine (decreased), glutamine (decr.), histidine (decr.) and ornithine (increased)) as well as the lipid phosphatidylcholine (C38:6) (decr.). This phosphatidylcholine decrease was replicated in a second study in which a wide range of phosphatidylethanolamines and phosphatidylcholines were found to be reduced in drug naïve (n=20) and relapsed patients (n=20) compared to matched heathy controls (n=17) [Kaddurah-Daouk *et al.*, 2012]. The authors suggest these changes might reflect the way in which schizophrenia alters phospholipase-mediated signal transduction pathway activity or an oxidative stress environment increasing lipid peroxidation.

Gas chromatography-mass spectrometry was applied to identify metabolite marker profiles before and after treatment with the anti-psychotic risperidone in a cohort of patients newly diagnosed with schizophrenia (n=18), as well as untreated healthy controls (n=18) [Xuan *et al.*, 2012]. Energy metabolism deficits in the form of increased glucose concentrations and decreased TCA pathway intermediates were present in drug-naïve patients. This has previously been proposed to be indicative of a metabolic syndrome/diabetes-like state in schizophrenia [Pickard, 2011, Harris *et al.*,

2013]. The study also showed that monitoring changes in citrate (decr.), palmitic acid (decr.), myo-inositol (incr.) and allantoin (incr.) offered the greatest discrimination between those with schizophrenia and healthy controls. Furthermore, myo-inositol, uric acid and tryptophan levels were perturbed after the commencement of risperidone treatment. The presence of uric acid and allantoin in these profiles may reflect a state of cellular oxidative stress. Interestingly, those patients who exhibited a therapeutic response to risperidone showed an increase in patient uric acid levels to those present in healthy controls. This may be worthy of follow-up as a means to monitor or predict treatment outcome. The involvement of oxidative stress in schizophrenia is not as clearly defined as that for inflammation [Flatow *et al.*] but is strengthened by observations such as these.

Another metabolomic study assessed serum samples from those with diagnoses of primary psychotic disorder (n=45), other non-affective psychosis (n=57), affective psychosis (n=37) and matched healthy controls [Oresic *et al.*, 2011]. Increases in saturated tryglycerides, proline, glutamate, and lactate were observed – with a highly significant finding for proline seemingly restricted to a diagnosis of schizophrenia. The lipid/glutamate profile fits with an energy metabolism dysfunction in schizophrenia with compensatory up-regulation of fatty acid/ketone body metabolism.

Still further support for this pathology comes from a metabolomic study of 112 schizophrenic patients and 110 healthy subjects, [Yang *et al.*]. Training and test sets identified glycerate (incr.), pyruvate (incr.), glutamate (incre.), 2-hydroxybutyrate (incr.), and myo-inostiol (incr.). However, citrate was shown to be increased in schizophrenia, in contrast to the findings of Xuan *et al.* described above. A combined classifier set of glycerate, eicosenoic acid, 2-hydroxybutyrate, pyruvate and cysteine profiles was found to be 90% accurate in diagnosing schizophrenia in the test set.

Inflammation markers

The findings detailed above suggest that schizophrenia has a biomarker profile that not only impacts of energy metabolism but is associated with dysfunction in the innate immune system and oxidative stress processes. The inflammatory component of schizophrenia has been recently reviewed [Michel *et al.*, 2013] and, interestingly, can often show improvement with antipsychotic treatment. This has prompted studies that directly query inflammatory biomarker changes.

A novel approach used a suite of biochemical and molecular assays to identify inflammatory pathologies in plasma and peripheral blood mononuclear cell samples from 117 patients recently diagnosed with schizophrenia and 106 matched controls applied [Garcia-Bueno *et al.*, 2013]. For NFκβ (incr.), iNOS (incr.), COX2 (incr.), Iκβα (decr.), and PPAR (decr.), changes were all indicative of

an active inflammatory response. A study of blood samples from 180 individuals with untreated schizophrenia and 380 matched healthy controls queried a panel of immunological and growth factor/hormone markers [Schwarz *et al.*, 2014]. The case population showed a replicated division between those with altered immunological profiles and those with growth factor/hormone changes. This raises the prospect of pathological discrimination with potential for tailored treatments (see below).From the same laboratory, a recent survey of cytokine profiles in serum from 180 antipsychotic-naïve first-episode schizophrenia patients and 350 healthy controls revealed increases in IL-1-RA , IL-10, and IL-15 cytokines [De Witte *et al.*, 2014]. A meta-analysis of inflammatory cytokine production in first episode psychosis confirmed that there are significant increases for IL-6, IL-12, TNF-α, IL-Iβ, IL-8, TGF-β, IL-1 RA. IFN-γ, ad sIL-2R, whereas the IL-10 cytokine is significantly reduced [Miller *et al.*, 2011].

Summary and future needs

Despite evident methodological improvements, the transcriptomics studies have rarely found consistent specific gene biomarkers for illness. In that light, they can be considered more 'descriptive' than 'diagnostic' in outcome. That aspect can be credited to the use of gene ontology enrichment statistics which, although limited by our relatively poor understanding of gene function, has repeatedly identified a set of aetiopathologies associated with schizophrenia that include energy metabolism, immune response and synaptic function. There is, however, still uncertainty whether these represent causes or consequences of the schizophrenia pathology. For example, the neurodevelopmental consequences of maternal immunological activation appear quite causative in nature, but it is much harder to categorise the adult chronic inflammatory state that is associated with schizophrenia (and neurodegenerative diseases). In order to consistently identify a set of biomarkers for schizophrenia, transcriptomics may need to approach the scale of sample numbers used in genomics [Schizophrenia Working Group of the Psychiatric Genomics, 2014]. This has a downside in that the need for large cohorts reflects small effect sizes; and thus a requirement for diagnostics to be highly sensitive. Alternatively, new ways to disentangle environmentally-generated noise from disease state may be required. It is also vital that the public deposition and availability of microarray datasets continues, allowing researchers to take their particular panels of genes that discriminate disease status in their studies, and test them in a wider context. As with transcriptomics, there is perhaps an inevitability that the methylation field will also have to adopt genomics-level sample sizes – it is sobering to see results becoming less striking as sample numbers increase.

In this era of personalised medicine, it is important that biomarker findings from populations are verified and pursued at the level of the individual. An example where this has been successfully applied is the FINRISK study in which 17,345 individuals were assessed for blood biomarkers predicting short-term risk of mortality [Fischer *et al.*, 2014]. Importantly, the four most powerful biomarkers; alpha-1-acid glycoprotein, albumin, very-low-density lipoprotein particle size, and citrate; could be combined to form a 'summary score' that had significant power to predict the risk of an individual's death within five years. It is reasonable to expect that schizophrenia sub-classification and diagnosis will require similar combinatorial assessment of markers. However, it is probably fair to say that most current '-omics' analysis is carried out with a view to discern the 'horizontal' population average differences between cases and healthy controls – marker by marker. The opposite, 'vertical', approach demands new ways of analysing data in order to determine the set of genes that are contributing to an individual's risk and expression of illness. These two analytical paradigms are presented in Figure 1. The occasional analytical approach [Wockner *et al.*] has attempted to define biomarker profiles for the individual and this may be a direction that more studies would profit from. One strategy to subtype individuals might be the targeted analysis of specific pathologies (for example, inflammation and energy metabolism) on order to guide selection of the best therapy. The development of mathematical strategies to identify and apply vertical bio-/genomic marker subsets for schizophrenia characterisation, at the level of the individual, should be a research priority.

[Figure 1 near here]

Legend

Figure 1 Two opposing schemes for biomarker analysis. In the more common horizontal approach the comparison is made between the case and control populations for each biomarker separately. This has statistical power to identify association but is confounded by heterogeneous intra-cohort disease structure. Our current knowledge of schizophrenia would suggest that a simple case-control split would not be possible due to the variable and combinatorial nature of aetiology and pathology in cases of schizophrenia. Therefore, a vertical approach might be preferable, which attempts to combine data from a subset of markers in an individual and compare this with similar subsets in other individuals. For example, the pairs of markers highlighted in A and B might define pathological aspects of an individual's schizophrenia, or potential response to treatment. The issue with the vertical approach is that discovering the marker subsets is non-trivial as they have to expose

differences between schizophrenia and healthy controls as well as the internal discontinuities within schizophrenia. The horizontal approach can provide some aid in marker selection for the vertical approach but cannot realistically inform the more complex structure of individual risk.

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