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Trends in Molecular Medicine

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Review

Strengthening Causal Inference for Complex Disease Using Molecular Quantitative Trait Loci

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Large genome-wide association studies (GWAS) have identified loci that are associated with complex traits and diseases, but index variants are often not causal and reside in non-coding regions of the genome. To gain a better understanding of the relevant biological mechanisms, intermediate traits such as gene expression and protein levels are increasingly being investigated because these are likely mediators between genetic variants and disease outcome. Genetic variants associated with intermediate traits, termed molecular quantitative trait loci (molQTLs), can then be used as instrumental variables in a Mendelian randomization (MR) approach to identify the causal features and mechanisms of complex traits. Challenges such as pleiotropy and the non-specificity of molQTLs remain, and further approaches and methods need to be developed.

Highlights

GWAS using large sample sizes have allowed the identification of many DNA sequence variants associated with molecular traits such as gene expression, DNA methylation, and protein levels that could be mediators between disease-associated genetic variants and the disease.

Quantitative trait loci (QTLs), genetic variants influencing molecular traits, are increasingly used to identify causal features of complex traits.

MR, a method using genetic variants as instrumental variables for a modifiable exposure, is employed to evaluate whether a molecular trait has an influence on a complex trait.

Many challenges remain, such as linkage disequilibrium between causal variants of different complex traits, pleiotropy, and the non-specificity of molQTLs, or molQTLs being reverse causally influenced by a complex trait; methods to address them are being developed.

Genome-Wide Association Studies

GWAS (Box 1) have identified thousands of sequence variants that contribute to the genetic architecture of complex diseases and medically-relevant quantitative traits. This endeavor has been fuelled by two major ambitions: creating genetic predictors for disease, and identifying the genomic regions responsible for the disease to gain a better understanding of the relevant biological mechanisms [1,2]. The latter objective is the focus of this review.

Typically, associated variants individually account for a very small proportion of phenotypic variation. This is common for quantitative or 'complex' traits which are usually influenced by a large number of genes with small effects on the trait [3]. There is no simple Mendelian inheritance pattern, but random sampling of alleles at each associated gene results in a normally distributed phenotype in the population [4]. Functional information on the underlying mechanisms of genetic variants identified by GWAS is often unclear, in other words it is challenging to identify effector genes based only on the observed association summary statistics [3,5]. The majority of complex trait variants reside in noncoding regions of the genome [6,7], and it is possible that they confer their effects through modulating gene expression levels [8]. In their second decade of existence, GWAS are showing signs of maturity, with increasing diversity in the populations studied [9], the inclusion of low-frequency and rare variants, and finer definition of phenotypic traits examined.

In this review we describe how molecular traits are also being assayed and analyzed for genetic associations, and how the understanding of complex disease etiology is improving through combining genetic analysis of both the disease and molecular traits. The presiding manner in which these relationships are constructed is by using a causal inference method, MR, which capitalizes on the abundance of GWAS results now available. In the following we describe MR in terms of both its current implementation and the future developments that will be necessary to address known limitations.

MolQTLs

The influence of a genetic variant associated with a disease is likely to be mediated via molecular traits (Figure 1), which themselves are often complex. Quantitative molecular traits, such as gene expression or protein abundance, are frequently dysregulated in disease and can act as intermediate phenotypes, affording greater power to detect association compared to the dichotomous definition of a disease endpoint, which is the culmination of multiple biological processes being perturbed [10].

Multiple studies have investigated mRNA levels combined with genome-wide genotype information to identify expression (e)QTLs, in other words genetic variants associated with gene expression levels [11]. The first studies to investigate molQTLs started out with small sample sizes. Given the challenges

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Box 1. Genome-wide Association Studies

GWAS compare large numbers of affected with unaffected individuals to identify sequence variants that are associated with the risk of complex diseases, or at the population level to identify associations with quantitative traits. The foundation for GWAS was laid by the sequencing of the human genome [72], the characterization of correlation patterns between pairs of variants genome-wide [73], the development of high-throughput genotyping platforms, and the availability of large-scale sample sizes. Millions of SNPs have been mapped [74]. For several reasons it has been difficult to elucidate the underlying mechanism linking an associated genetic variant with a disease trait. One reason is the coinheritance of many genetic variants with the disease-associated variant (linkage disequilibrium, LD) [75]. Because of the complex correlation structure of the human genome, the most strongly associated GWAS signal (index variant) is often not causal [76]. Similarly, compounded by complex regulatory mechanisms, the nearest gene to the top GWAS signal is not necessarily the causal gene [11].

associated with collecting human biospecimens using invasive procedures, analyses initially focused on using the most accessible tissues [12]. Today, sample sizes used for molQTL investigation in blood have become very large [13]. MolQTLs are generally classified into *cis*-acting, which is typically defined as the regulation of genes within 1 Mb, or *trans*-acting, defined as molQTLs affecting genes further away or on different chromosomes [14]. Whereas the *cis* effects detected have generally been large and are easily found using small sample sizes, *trans* effects tend to be much smaller and larger sample sizes are required. Large studies such as the eQTLGen Consortium [13] or GoDMC (www.godmc.org.uk/) are emerging to identify these small effects that might play central roles in disease etiology. Molecular trait loci seem to be highly tissue-dependent [15,16]. However, tissue-sharing of *cis*-eQTLs seems to be bimodal. Either *cis*-eQTLs seem to be shared across many tissues or they are very specific to only a small subset of tissues [17]. To provide a resource which enables the systematic study of the effects of genetic variation on gene expression regulation in multiple human tissues, the Genotype-Tissue Expression (GTEx) project was initiated a decade ago [18]. The current GTEx

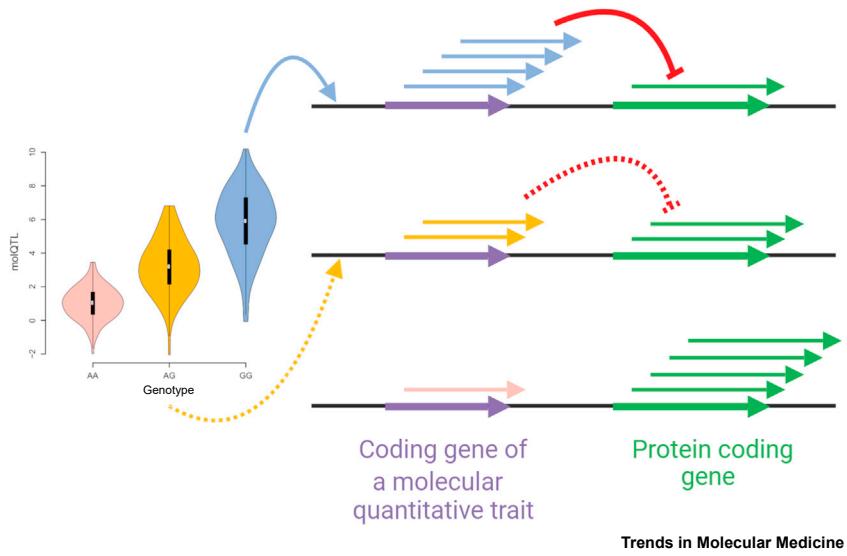

Figure 1. Molecular Quantitative Trait Loci (molQTLs) Influencing Intermediate Traits.
(Left) molQTLs are genetic variants associated with a molecular trait that have an influence on intermediate traits (genotypes AA, AG, GG). (Right) The GG genotype (blue) is associated with higher expression levels of the molecular quantitative trait compared to the AG (yellow) and AA (pink) genotypes. These molecular traits can modulate the expression of further target genes (green).

Glossary

1p13 locus: GWAS analysis in humans demonstrated that this locus on chromosome 1 is strongly associated with plasma low-density lipoprotein cholesterol (LDL-C) levels, which in turn is a major risk factor for myocardial infarction. SNPs (see below) in this locus have also been linked to coronary artery disease. This locus alters the expression of *SORT1* (see below) in the liver.
CELSR2: the gene encoding cadherin EGF LAG seven-pass G-type receptor 2, a receptor with a possible role in cell–cell signaling during nervous system formation. *CELSR2* is physically linked to the 1p13 locus. Because of this, *CELSR2* expression was thought to be controlled by the 1p13 locus until expression (e)QTL analysis showed that this was not the case.
Linkage disequilibrium (LD): the non-random association of alleles at different loci. Based on the assumption that, over time, recombination events will result in a random association of alleles at two loci, linkage disequilibrium is defined as the difference between the observed frequency of a particular combination of alleles at two loci compared to the frequency expected at random. When analyzing causal SNPs in GWAS analysis, special care must be taken to not wrongly interpret a non-causal SNP that is in LD with a causal SNP.
Single-nucleotide polymorphism (SNP): a DNA sequence variant within a population. SNPs can be linked to disease development and response to pathogens or medication in humans, which makes them invaluable in personalized medicine. Comparison of SNP composition in genomic regions between different cohorts (e.g., with and without disease) is of great importance in biomedical research on a larger scale (e.g., GWAS).
SORT1: the gene encoding sortilin, which is localized in intracellular compartments, notably the Golgi apparatus. Sortilin is involved in endocytosis and functions as a sorting receptor in the Golgi compartment and as a clearance receptor at the cell surface. SORT1 expression is

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release provides a total of 11 688 samples and 53 tissues across 714 donors (current release V7, dbGaP accession phs000424.v7.p2). Sample sizes of other studies have also largely increased [19–21] and a variety of tissues have been studied. The picture is far from complete, but has been massively enhanced since the inception of these studies.

The first expression phenotypes to be studied were gene transcript levels. These are highly heritable [22]. It is estimated that ~88% of all genes have at least one eQTL [13]. To date, many different molecular traits with a potential influence on gene regulation have been investigated [23]. These range from traits that influence the epigenome, such as DNA methylation (meQTL), histone modification (hQTL), and chromatin accessibility (caQTL), to alternative splicing (sQTL), protein levels (pQTL), microRNA expression (mirQTL), and ribosome occupancy (rQTL) [23]. In addition, higher-level intermediate phenotypes such as metabolites have been investigated, and QTLs for metabolites such as carbohydrates, amino acids, or fatty acids have been identified [24].

In an effort to find the molecular pathways that connect genetic variants to complex traits, overlapping/colocalization methods between GWAS and molQTL signals have been developed. Colocalization of an eQTL with a GWAS signal suggests that the eQTL target gene could be involved in the molecular pathway underlying the complex disease under investigation [25]. Several studies have already discovered GWAS signals enriched for molQTLs in a tissue-dependent manner [26]. For example, the myocardial infarction and high LDL cholesterol-associated **1p13 locus** (see Glossary) had been fine-mapped to the ***CELSR2*** gene. Using eQTL analyses, it was discovered that the expression of ***SORT1*** was influenced by this variant [27].

MolQTLs are being used as instrumental variables for molecular traits in a variety of ways: to infer the relative effects of different classes of molecular features on variation in complex traits, to identify the causal gene for a particular complex trait [23], to identify the causal tissue for a complex trait [28], and to estimate causal relationships between different molecular traits [29]. In this review we focus on their use for identifying the causal features of complex traits.

modulated by the 1p13 locus (see above). In liver cells of mouse models, LDL-C levels are significantly decreased by SORT1 overexpression, whereas SORT1 knockdown resulted in increased LDL-C levels.

Mendelian Randomization (MR) Studies Strengthen Causal Inference

MR (Box 2) studies use genetic variants as proxies for modifiable risk factors to test whether the risk factor is causally relevant to an outcome of interest [30,31]. The advantage of such an approach is that unmeasured confounding, an issue of observational studies, and reverse causation can be minimized. It is therefore possible to use genetic information to draw causal inferences.

Early MR studies mainly used one-sample approaches, where the exposure and outcome phenotypes, as well as the genetic variants that were used to instrument the exposure, were available for all samples in a single dataset. Currently, when many large-scale GWAS are conducted, it is much more powerful to use published **single-nucleotide polymorphism** (SNP)–trait associations from large consortia. It is therefore common to use two-sample MR approaches where SNP–exposure and SNP–outcome associations are estimated in different studies and are subsequently combined [32]. When using genome-wide significant SNPs as instrumental variables for an exposure, the first MR assumption should be verified.

Box 2. Mendelian Randomization Studies

The laws of Mendelian inheritance assign alleles at conception to individuals independently of environmental risk factors and confounders. To obtain valid estimates using MR, three assumptions must be met: first, the genetic variants must be sufficiently associated with the exposure of interest; second, the genetic variants should not be associated with any confounder of the risk factor–outcome relationship; finally, there should not be any other pathway leading from genetic variants to outcome except through the exposure of interest. Except for the first assumption, which can be tested, the other two assumptions can only be addressed by sensitivity analyses [77].

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For two-sample MR methods, only summary statistics are required (per-allele regression coefficients, standard errors, and effect alleles) which are typically obtained from published GWAS of the largest possible datasets [33]. The causal effect can be estimated using the Wald ratio estimate, which is the ratio of SNP–outcome association and SNP–exposure association.

SNP–exposure and SNP–outcome association statistics should ideally be obtained from studies of non-overlapping individuals (two-sample MR). When using summary statistics from only one sample or from partially overlapping samples, results might be biased in the direction of the observational estimate, especially if the genetic effects on the exposure are weak [34]. When several independent genetic variants are known to be associated with the exposure of interest, these can be combined into a single MR estimate using inverse variance weighted meta-analysis of the single Wald ratio estimates [32]. In doing so, the MR framework can then be viewed as a meta-analysis problem which itself has a rich set of tools to evaluate and correct for bias [35]. One issue that has been of particular concern in MR is in proving that violation of the third assumption, in other words that the genetic instrument influences the outcome only through the exposure, does not induce bias [36]. A suite of sensitivity analyses [37–41] are now routinely implemented in MR studies that use multiple independent instruments to model pleiotropy [42].

MR Studies Using Molecular QTLs as Instrumental Variables

Whole-genome approaches have indicated that the causal variants influencing complex traits are over-represented in those that are also associated with eQTLs [43,44]. This supports the notion that disease biology may be unraveled by mapping the causal path from genetic variant through the use of intermediate molQTLs [45]. In its most basic implementation, an MR framework for evaluating the causal influence of a molecular trait on a complex trait would be to test if a known molQTL is also associated with the complex trait (Figure 2, Key Figure). The Wald ratio of SNP–complex trait and SNP–molecular trait effects can then be obtained as an estimate of the causal effect. This simple method suffers from several potential pitfalls and is often performed as an initial screen to find, from among many molecular phenotypes (e.g., hundreds of thousands of DNA methylation levels), a few putative causal molecular phenotypes for more detailed follow-up and sensitivity analysis [46–48]. We describe some of these approaches below.

Linkage Disequilibrium Links a Causal Variant for One Trait with a Different Causal Variant for Another Trait

A major lesson from GWAS analysis is that complex traits follow a polygenic architecture [49,50]. As a consequence, finding that a chosen SNP happens to show an association with a complex trait might not be surprising because many non-causal common variants are likely to be in **linkage disequilibrium** (LD) with a causal variant for a complex trait (Figure 3A). Colocalization techniques seek to analyze specific genomic regions, determining whether the pattern of test statistics for one trait is concordant with the pattern from another, often with respect to the underlying LD structure. Evidence for shared causal variants at a locus is determined by the extent to which the test statistic patterns are shared between the two traits. An important recent finding is that the majority of genes that colocalize with a trait are not the genes that are closest to the biggest signal for the trait [11].

Typically, the proportion of overlapping signals between molecular and complex traits that appear to be due to LD is high. For example, in [29] it was shown that two thirds of putative expression–trait MR relationships were due to LD, with a similar proportion being found for DNA methylation–trait MR relationships. Nevertheless, when assessed across hundreds of complex traits, there are now tens of thousands of examples of colocalization between gene expression levels and complex traits [51]. It is important to note that there are many colocalization techniques [11,52–54],but there is not always strong agreement between them [54].

The Association Is Reverse Causal

One of the purported advantages of MR is that it protects against reverse causation. This is true to the extent that the instrument is known to primarily influence the hypothesized exposure. However, it is

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Key Figure

Schematic Representation of a Mendelian Randomization Study Using Quantitative Trait Loci as Instrumental Variables

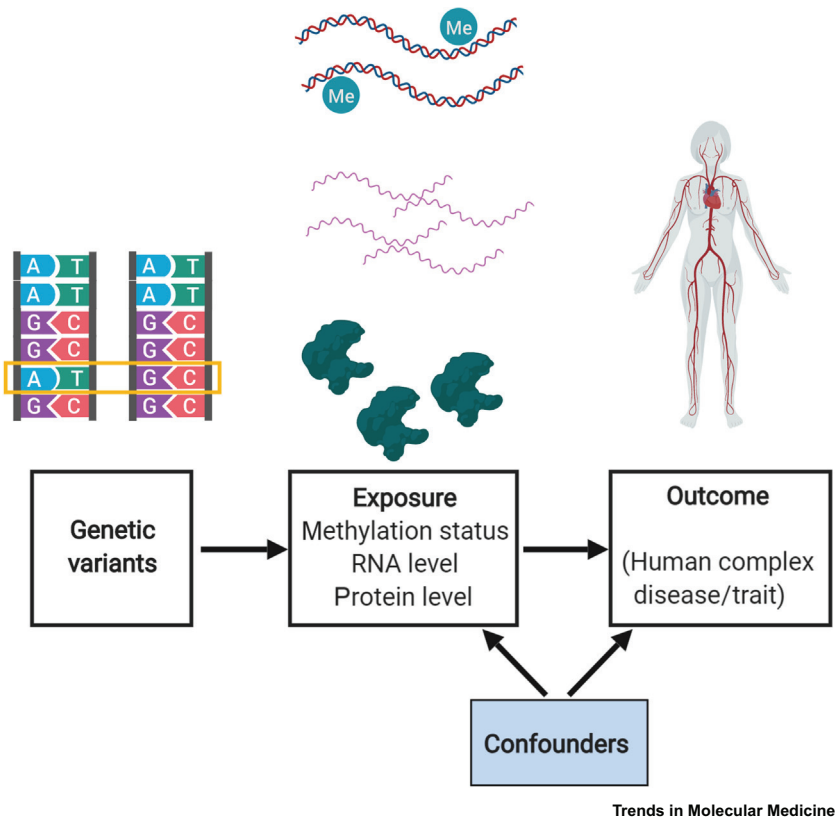

Figure 2. Owing to the random distribution of alleles at conception, genetic variants are unrelated to environmental confounders. If genetic variants are sufficiently associated with the modifiable exposure of interest [in this case levels of methylation (Me), RNA expression levels, or protein levels], and are not associated with the outcome via a different pathway, they can then be used as instrumental variables for the exposure.

conceivable that a molQTL arises because a complex trait influences it. Mediation-based methods exist that require individual-level data to orient the causal direction [55–57], but these are susceptible to making the wrong orientation under specific patterns of confounding or measurement error [58]. An alternative approach is to perform MR in the reverse direction [47], identifying SNPs that instrument the complex trait and testing for its association with the molecular trait. Typically, however, one would not expect reverse causal relationships to explain a molQTL associated with a complex trait because, for the molQTL to have been detected in a small sample size, it will necessarily be a large effect, which is impossible if it is mediated through a polygenic trait [29].

The Instrumenting SNP Is Non-Specific to the Hypothesized Exposure

Often a single SNP is detected as an instrument for multiple molecular phenotypes. For example, a SNP could be strongly associated with more than one gene expression level, or the same gene expression level in different tissues or at different timepoints, or both a gene expression level and

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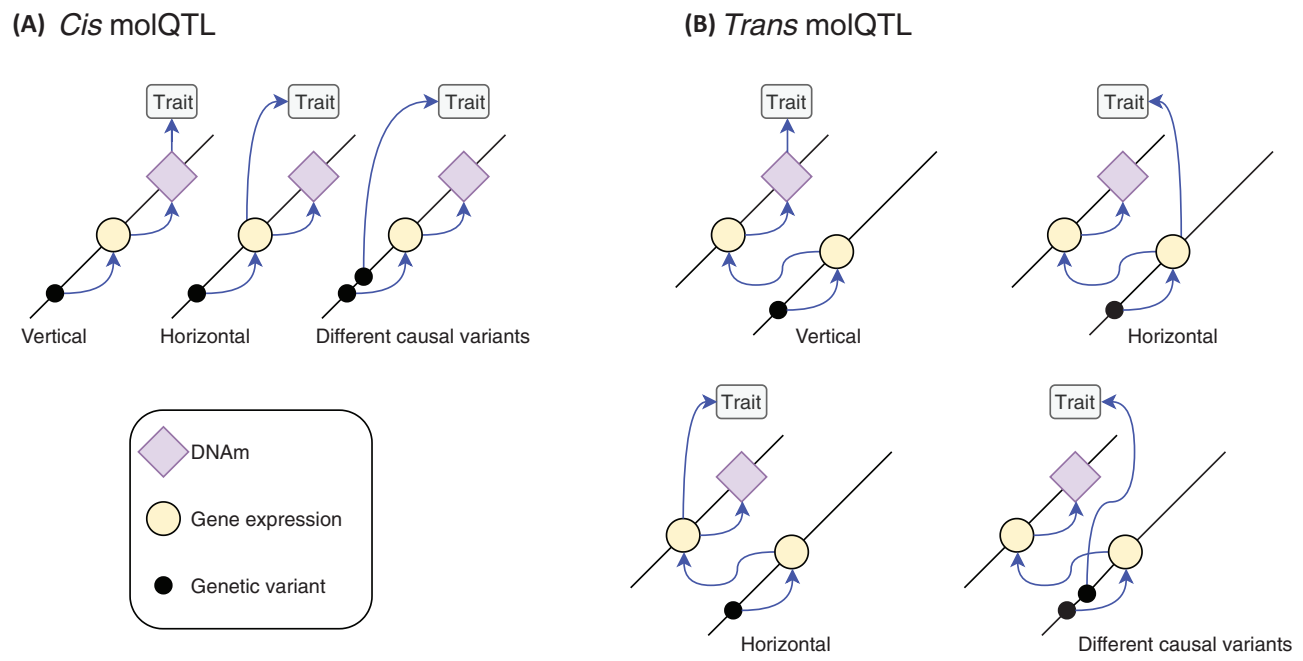

Figure 3. Simplified Directed Acyclic Graphs of Possible Systems That Would Lead to an Apparent Causal Effect of Gene Expression on a Trait. Gene regulation may be regulated by several elements. In all the situations depicted, a naïve Mendelian randomization (MR) analysis would return a causal signal for any of the regulatory elements, even though they are usually not on the causal pathway. (A) Three scenarios for *cis* molecular quantitative trait locus (molQTL) regulation are presented. (Vertical) Both gene expression and DNA methylation (DNAm) are on the causal pathway, hence MR using the *cis* genetic variant will give valid causal estimates if it is used to instrument either of these elements. (Horizontal) Using the instrument for DNAm will be invalid because of horizontal pleiotropy. (Different causal variants) The molQTL is in linkage disequilibrium (LD) with another variant that influences the trait, hence neither regulatory element is causally influenced, although naïve MR could indicate otherwise. (B) Four scenarios where molQTL regulation is similar to (A) except that the molQTL for DNAm is on a different chromosome. There are now more opportunities for horizontal pleiotropy because there will be a longer path from the *trans* chromosome to the DNA methylation level.

a DNA methylation level (Figure 3). This is not necessarily a problem because all the molecular phenotypes that are associated with the trait could be on the same causal pathway to the disease, and indeed it could be advantageous because it presents us with multiple points of intervention. Non-specificity of genetic associations is classically known as pleiotropy, although care should be taken in using the term. MR assumes a 'vertically' pleiotropic relationship in which the genetic instrument is associated with the outcome because it is mediated by the exposure. By contrast, 'horizontal' pleiotropy is a source of problems in MR, inducing bias or false causal inference if the SNP influences the outcome through a pathway other than the hypothesized exposure [59]. Proving that a putative MR finding is due to vertical and not horizontal pleiotropy is far from trivial [36].

There are vastly more molecular phenotypes than there are independent genetic regions, especially when temporal- and tissue-specific measurements are possible [60]. By definition, it is expected that many molQTL will not be specific for a particular molecular trait. Therefore, it is difficult to prove which, from among the set of molecular traits that are influenced by the molQTL, is the causal factor [51].

One approach is to focus on the use of *cis*-acting molQTLs, with the rationale that they are biologically 'closer' to the intended molecular trait. *Trans*-acting QTLs are likely to only influence the molecular trait because they are mediated by other molecular traits, opening up a greater possibility that

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the instrument is not specific to the intended target (Figure 3B). Testing explicitly if the molQTL is associated with other molecular traits is also sensible because this can be used to (de-)prioritize a putative association depending on how much evidence there is for (non-)specificity [2]. Methods are now emerging that attempt to model the MR estimates of multiple molecular exposures simultaneously, thereby adjusting for potential horizontal pleiotropy [61]. Although a useful tool, interpretation remains difficult because the use of multivariable MR [62] requires that there are marked differences in the genetic signatures across the exposures [63]. It also requires measurement of all possible exposures that could be inducing the pleiotropy, which is a similar assumption to observational study designs that prompted the development of MR in the first place.

There are more standard MR sensitivity analyses that can be applied in the event that multiple independent causal variants are available [42]. However, this typically requires introducing *trans*-QTLs into the analysis, and this may not bring clarity because they could have systematically different properties from *cis*-QTLs. At this stage, if a molecular trait colocalizes with a complex trait, and does not appear to be reverse causal, it is still extremely difficult to prove that it is causal, and is not simply one of many traits that are all influenced by the same molQTL.

In the GoDMC study, which used 30 000 samples to discover instruments for DNA methylation levels, multiple *cis* and *trans* instruments were used to model causal relationships between DNA methylation levels and complex traits. It was found that, although there were many putative colocalizing signals with complex traits, there was almost no agreement between the causal effect estimated using primary and secondary molQTLs, implying that the majority of colocalizing signals were due to horizontal pleiotropy.

Current Challenges and Issues

The prospect of finding new drug targets has propelled the development of data acquisition and methodological techniques for mapping the pathways between molecular and complex traits.

Genetic variation is finite, and, although molecular traits are often polygenic, the use of more than the *cis* region for instrumentation is currently not fully understood. This incurs a limit on the extent to which current tools designed to protect against incorrect causal inference due horizontal pleiotropy can be used. Conceptually, we use genetic instruments here as a proxy for molecular phenotypes. However, molecular phenotypic variation dwarfs the *cis* genetic resource that is available for instrumentation. Hence, the ubiquitous non-specificity of any molQTL makes it very difficult to determine which molecular feature is actually mediating the genetic effect on a trait. This could be because inference is for the wrong developmental timepoint (e.g., genetic effects are very consistent over time [64] for DNA methylation) or the wrong tissue (*cis*-QTLs are strongly shared across tissues [17]). Alternatively, it could be that it was an entirely different molecular feature (e.g., gene expression, DNA methylation, and histone variation often share similar *cis*-regulatory features [65]).

Coupled with this problem of non-specificity is emerging evidence supporting a model of ubiquitous horizontal pleiotropy [40,66], in which a given genetic variant potentially influences a particular complex trait through multiple independent pathways. The omnigenic model offers an extreme viewpoint on this problem, in which polygenic architecture arises because every gene is related to every trait through an underlying dense gene regulatory network [3].

Making meaningful inferences from such an under-specified model requires a departure from current practices of treating molecular features singly, and reliably incorporating *trans* instruments, which may exhibit tissue specificity [17]. Although any one instrument might be non-specific, it is seldom the case that the genetic correlation of complex traits is unity [67], meaning that there are potentially combinations of instruments that together provide some specificity. Large-scale pleiotropy maps are beginning to be produced [40,68,69], and may provide an avenue for constructing instrument combinations conditional on a background of complex pleiotropy.

Clinician's Corner

Poor efficacy and poor safety are the two major reasons for the very high failure rate of drug trials, ultimately driving up the cost of drugs and their development times. This can be partly framed as a causal inference problem, where the objective is to identify which molecular targets are causal for the disease of interest, and to filter out those that are likely to fail before initiating trials.

Randomized controlled trials (RCTs) are ideal for making causal inferences but are expensive, slow, and often impracticable for a particular causal enquiry. The Mendelian randomization (MR) statistical framework leverages genetic associations to mimic randomized control trials. The potential of this strategy is increasingly being exploited given the ready availability of data to quickly and cheaply evaluate the causal importance of thousands of molecular features in complex diseases.

To interrogate the causal influence of a particular molecular trait on a particular disease, knowledge of robust genetic factors for the molecular trait, and the corresponding effect of those factors on the disease, are both required. Owing to more than a decade of genome-wide association studies and the recent emergence of national genetic biobanks, genome-wide genetic associations from large sample sizes have been made publicly available for most complex diseases. In addition, the genetic influences on a range of molecular features such as protein levels, gene expression levels, DNA methylation levels, and metabolite profiles are being mapped and made publicly available.

Although it is impossible to mimic an RCT perfectly using such observational data, statistical techniques and data continue to improve, and MR is poised to further help in making causal claims about a link between a molecular trait and complex disease.

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Concluding Remarks

Many genetic variants associated with complex traits and diseases have been discovered, but there is often a lack of knowledge about the mechanisms involved. Investigation of intermediate traits and associated molQTLs has been very helpful because these better explain how genetic variants influence complex traits. Using molQTLs combined with an MR approach, causal features of a complex trait can be revealed. Challenges remain, such as the model of ubiquitous horizontal pleiotropy and, therefore, a non-specificity of molQTLs to a particular molecular trait (see Outstanding Questions). Therefore, new methods need to be developed, including for example those that reliably incorporate *trans*-molQTLs, which have a greater possibility for non-specificity of the instrument.

Despite our growing understanding of the limitations of MR, current data resources and statistical frameworks for MR can be viewed as a resource with tremendous utility. Most directly, using MR to support a negative association could be less prone to some of the issues described. Of growing importance in causal inference is the concept of triangulation, where data from orthogonal experimental designs are integrated together to obtain a more reliable conclusion [70]. There are now open-source data and software repositories (including those that can be used in web browsers [42]) that automate MR analyses. The inclusion of genetic evidence through MR should be a natural part of any causal inquiry [71].

Outstanding Questions

How can *trans*-acting molQTLs be used to draw reliable causal inference?

How can challenges such as the non-specificity of molQTLs be overcome?

Considering all limitations, are molQTLs still a good way to investigate the causal features of complex diseases?

Are the currently available methods for MR and sensitivity analyses sufficient to interpret results as causal, given that there could still be bias due to pleiotropy?

What alternative study designs would complement the molQTL approach, and aid with the triangulation of evidence?

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