

Celiac Disease Triggers

Using Mendelian Randomization to infer causality

The largest conducted Mendelian Randomization screening

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PREFACE

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ABSTRACT

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ABBREVIATIONS

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INTRODUCTION

Celiac disease is a complex autoimmune disorder of the small intestine characterized by gluten-sensitivity. When people with celiac disease digest gluten their body triggers an immune response that attacks the small intestine. This causes damage to the villi that are lined up in the small intestine for nutrient uptake.^[1] Celiac disorder affects up to two percent of the European population, and it is hereditary. People with a first-degree relative with celiac disease have 30 times higher risk of developing celiac disease. The inheritance model of celiac disease is oligogenic, which means that a phenotypic outcome is determined by multiple genes, with one having a much stronger impact than others. In this model, HLA-DQ2 is a genetic variant in the major histocompatibility complex (MHC) region that accounts for 40% of the inheritable risk. But, this genetic variant alone is not sufficient enough to develop celiac disease. 30% of the Europeans are carriers of the variant, however 3% express celiac disease.^[2]

The aim of this study is the identification of factors that cause or protect against celiac disease, and to quantify the impact of the causal or protective relationship. To determine potential causal or protective relationships more than 500.000 public available clinical parameters and molecular mechanisms -- like Crohn's disease, Inflammatory bowel disease, Hemoglobin concentration, and Eosinophil percentage of granulocytes -- will be screened with the Mendelian Randomization (MR) approach. MR is an approach that uses genetic variants associated with a phenotype of interest to estimate a causal or protective relationship between this phenotype (exposure) and a relevant medical condition (outcome).^[3] This study uses two-samples MR^[4] methods: Inverse-Variance Weighted^[5] (IVW) method and MR-Egger method^[5]. Such methods can infer causality links by statistical methods that combine summary statistics data from Genome-Wide Association Studies (GWAS), and thus have a great potential to be applied today in the era of data sharing.

We expect to find multiple causal or protective clinical parameters or molecular mechanisms that will be quantified to understand the impact of the causal or protective relationship, that can potentially explain what is triggering celiac disease in the 3% from the 30% of the Europeans that are carriers of HLA-DQ2.

THEORY

CELIAC DISEASE

Celiac disease is an autoimmune disorder that has the possibility to occur genetically in predisposed people where the ingestion of gluten cause damage. When people with celiac disease digest gluten their body triggers an immune response that attacks the small intestine.^[1] This is characterized by small intestinal damage with the loss of function from the villi to take up nutrients, which leads to malabsorption.^[6] **! NEED TO CHANGE !**

MENDELIAN RANDOMIZATION

MR refers to the process of random segregation and assortment of alleles from an ancestor to offspring, that occur during gamete formation, in meiosis.^[3, 4, 5]

This process gives us the advantage of using these genetic variants in observational settings to predict causal or protective relationships between exposure and outcome.^[3, 4, 5] This process is comparable to Randomized Controlled Trials (RCT). In RCTs, the process of random and evenly distributing the sample into two arms ensures that known and unknown confounders are distributed evenly across both arms, see figure 1, stage (A). In MR, the random segregation and assortment of alleles is analogous the process of randomization in RCTs. In stage (B) for RCT one arm will be the case group, where some form of experiment will be conducted, and the other is the control group. At this stage for MR, both arms will represent different genotypes which in stage (C) will lead to

different products. For RTC at this stage, in the case arm there will take place on-off-target effects. At the final stage (D), differences between the two arms from both RCT, and MR can be studied and should only reflect the differences between the two arms.

The MR approach uses genetic variants as Instrumental Variables (IVs) to infer a causal or protective relationship between an exposure and outcome. IVs are variables that are associated with the exposure phenotype of interest and does not suffer from reverse causality or confounding factors.^[3, 4, 5] Reverse causality is the phenomena where it is expected that for example X causes changes in Y, but it can be that Y causes a change in X. Take this example: an individual who is characterized by being a heavy alcohol consumer, and because of the over consumption is that individual depressed. But, it is possible that this individual is a heavy alcohol consumer due to being depressed. Confounding is the variable that is not accounted for and can bias the analysis by suggesting that there is correlation when that is not the case.

MR uses genetic variant for a variety of reasons. (I) genetics variants are less amenable for confounding factors. As denoted by Mendel's first law, the law of segregation; genetic variants segregate randomly and independently from environmental factors, and Mendel's second law, the law of independent assortment; genetic variants segregate independently from other traits. (II) reverse causality does not affect the genetic instruments because an individuals germline genotype precedes the medically

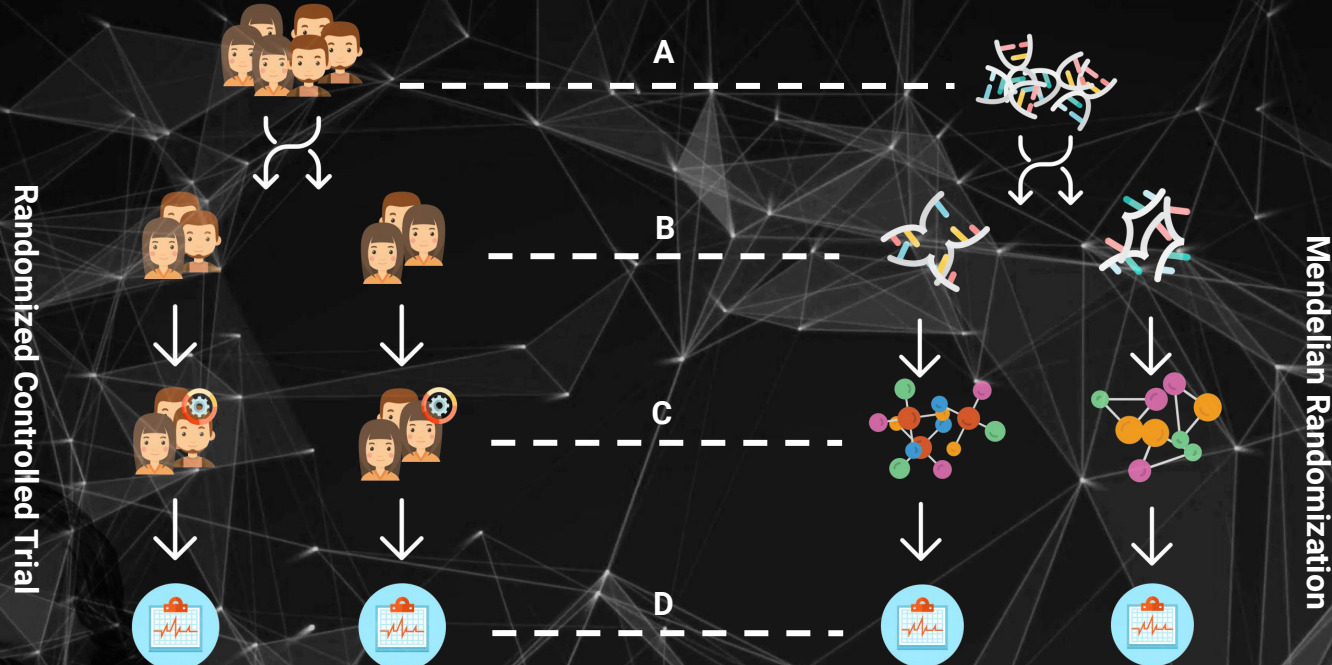


Figure 1: RCT in comparison to MR. The left diagram is an overview of a RCT, and the right diagram is an overview of a MR. (A) describes the random allocation and segregation of alleles in MR, and in RCT the random distribution of the population into two arms. At stage (B); here in RCT there will be a case group and a control group, and for MR there will be two different genotypes. After that, stage (C); for RCT here will take place different target effects for one of the arms, for MR there will be two different products from the genotype which in turn in stage (D) give rise to a different effect, as does this take place for RCT. And these differences in arms can be studied.

relevant disease.^[3, 4, 5] (III) genetic variants are subjected to little measurement error or bias. (IV) MR does not force to use the actual causal variant but is satisfied with a marker that is in Linkage Disequilibrium (LD) with the causal genetic variant. (V) MR gives us the advantage to use the genetic variants from GWAS summary statistics, which are routinely available on large well-phenotyped scale.^[7, 8]

Linkage Disequilibrium is the association of alleles at different loci in a population that are not random. Loci are in LD when the frequency of alleles of the associations is higher or lower than is expected to occur when the loci were independent and associated at random.^[11]

For a genetic variable to be used as an IV, the variant must meet three core assumptions. (I) the genetic variant must be associated with the exposure, (II) the genetic variant must not be associated with confounders, (III) the genetic variable can only be associated with the outcome through the exposure^[3, 4, 5], see figure 2.

The first assumption can be assessed by investigation of the strength of the association between the genetic instrument and the exposure. The second assumption can be assessed by examining the relationship between the genetic variant and potential confounders. Final, the third assumption can be addressed by examining the biological pathways of the genetic instruments.^[3]

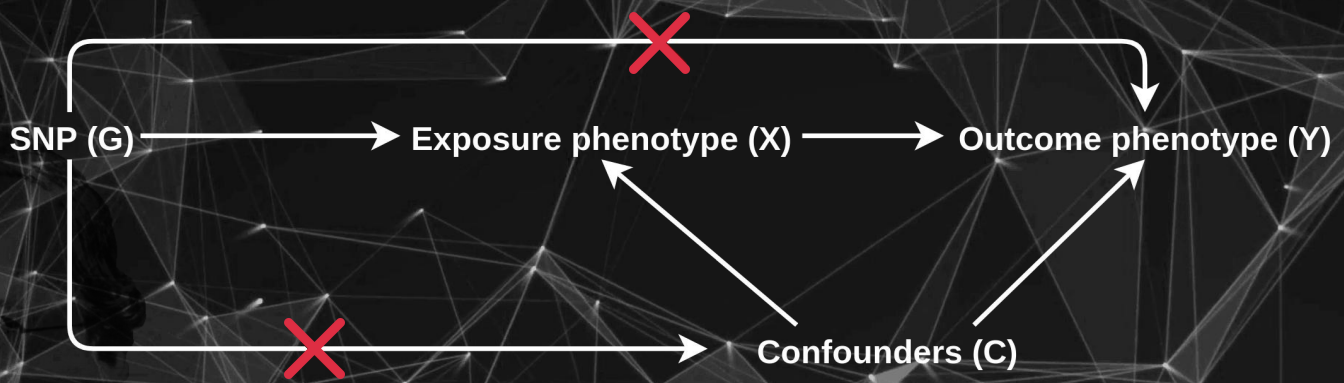


Figure 2: graph showing the assumptions made by MR. The nodes in this graph represent the genetic instrument: Single-nucleotide polymorphism (SNP), the exposure, the outcome, and confounders. This graphs shows the relationships between variables that meet the three core assumptions. (I) Genetic variant must be associated with exposure, (II) no association between genetic variant and confounders, (III) only association with outcome through exposure. The red crosses show the relationships that are not allowed in the MR framework.

ONE-SAMPLE MENDELIAN RANDOMIZATION

One-sample MR is the basic implementation of MR on SNPs, exposure, and outcome from a single data set, with the addition of genetic data from participants. The causal effect of the exposure on the outcome can be estimated by a two-step regression analysis. In the first step, a linear regression is fitted on the genetic variants from the exposure. In the second step, the genetic variants from the outcome are regressed over the predicted values from step one by linear or logistic regression. The coefficient from the second stage can be interpreted as the change in the outcome per unit increase in the exposure. If this coefficient is significant, than the exposure is causal (or protective, depending on the sign of the coefficient) for the disease.^[9]

TWO-SAMPLES MENDELIAN RANDOMIZATION

Two-samples MR takes advantage of the fact that it is not needed to obtain both, the exposure and outcome from the same

population sample, nor the addition of genetic data. The two-sample approach method makes it possible to use publicly available GWAS.

To calculate the causal estimate between the outcome (Y) and exposure (X) one needs to take the ratio of the effect from the genetic variant (G) on outcome divided by the effect from the genetic variant on the exposure.^[4] This is referred to as the Wald method ($\hat{\beta}_{IV}$), see equation 1. Where $\hat{\beta}_{Y|G}$ is the effect from the genetic variant on outcome, and $\hat{\beta}_{X|G}$ is the effect from the genetic variant on the exposure.^[3, 4]

$$\hat{\beta}_{IV} = \frac{\hat{\beta}_{Y|G}}{\hat{\beta}_{X|G}} \quad (1)$$

In addition, the standard error of $\hat{\beta}_{IV}$ ($\sigma_{\hat{\beta}_{IV}}$) can be calculated as follow (equation 2):

$$\sigma_{\hat{\beta}_{IV}} = \sqrt{\hat{\beta}_{IV}^2 / \frac{Z_{\hat{\beta}_{Y|G}}^2 Z_{\hat{\beta}_{X|G}}^2}{Z_{\hat{\beta}_{Y|G}}^2 + Z_{\hat{\beta}_{X|G}}^2}} \quad (2)$$

where $Z_{\hat{\beta}_{Y|G}}$, and $Z_{\hat{\beta}_{X|G}}$ are the number of standard deviations from the mean of the effect from the genetic variant on outcome or exposure.^[3, 5]

The use of one single variant in two-samples MR has however several limitations. The first is lack of power: one single variant in fact is often not sufficient to represent the entire variation in exposure, thus limiting the ability to assess causality: the link between exposure variation and outcome. One single variant is also highly prone to biased estimates. In fact, one variant may be associated with (or maybe in LD with another variant associated with) another exposure (this phenomena is known as pleiotropy), and thus the estimated causal effect can be due to both or only one of the two exposure phenotypes. Distinguish which exposure is causal is not possible with only one single variant, this will be significant because of the causal link of the other exposure. To overcome those limitations, other methods have been introduced that can combine multiple genetic variants to increase power and disentangle the issue of pleiotropy.^[3]

INVERSE-VARIANCE WEIGHTED MENDELIAN RANDOMIZATION

With multiple genetic variants, the causal estimate ($\hat{\beta}_{IV}$) for each variant can be averaged by using an inverse-variance weighted approach to compute an overall causal estimate, which is denoted as the inverse-variance weighted (*IVW*) estimate ($\hat{\beta}_{IVW}$). This method assumes that all genetic variants bring independent evidence, that there is no correlation between ratio estimates.

The pooled estimate can be calculated as follow (equation 3)^[3, 4, 5]:

$$\hat{\beta}_{IVW} = \frac{\sum_G \hat{\beta}_{Y|G} \hat{\beta}_{X|G} \sigma_{\hat{\beta}_{Y|G}}^{-2}}{\sum_G \hat{\beta}_{X|G}^2 \sigma_{\hat{\beta}_{Y|G}}^{-2}} \quad (3)$$

This pooled estimate can also be calculated by deploying a weighted linear regression of the genetic variants from the outcome on the genetic variants from the exposure with using inverse-variance weights ($\sigma_{\hat{\beta}_{Y|G}}^{-2}$), this method does not contain an intercept term (equation 4)^[3, 4, 5]:

$$\hat{\beta}_{Y|G} = \hat{\beta}_{IVW} \hat{\beta}_{X|G} + \epsilon_G; \quad \epsilon_G \sim \mathcal{N}(0, \sigma_{\epsilon_G}^2 \sigma_{\hat{\beta}_{Y|X}}^2) \quad (4)$$

Here $\hat{\beta}_{IVW}$ is the parameter, ϵ_G is the residual term, and σ_{ϵ_G} is the residual standard error.^[3, 4, 5]

In addition, to compute the standard error of the pooled estimate ($\sigma_{\hat{\beta}_{IVW}}$) see equation 5:

$$\sigma_{\hat{\beta}_{IVW}} = \frac{1}{\sqrt{\sum_G \hat{\beta}_{X|G}^2 \sigma_{\hat{\beta}_{Y|G}}^{-2}}} \quad (5)$$

MENDELIAN RANDOMIZATION-EGGER METHOD

MR-Egger is a method which can be deployed when the core assumptions do not hold, because it uses a weighted regression with an unconstrained intercept to regress the outcome on the exposure. The unconstrained intercept from this does not take into account the assumption that all genetic variants meet the assumptions, due to this, this method is

less biased by potential confounding. MR-egger contains three parts: (I) a test for violations of the core assumptions, (II) a test for testing the causal effect, and (III) an estimate of the causal effect.^[5]

MR-Egger is similar to IVW with respect to a simple modification of the weighted linear regression. Instead of setting the intercept term zero, it is estimated as part of the analysis (equation 6)^[3, 4, 5]:

$$\hat{\beta}_{Y|G} = \hat{\beta}_{0E} + \hat{\beta}_{1E} \hat{\beta}_{X|G} + \epsilon_G; \quad \epsilon_G \sim \mathcal{N}(0, \sigma_{\epsilon_G}^2 \sigma_{\hat{\beta}_{Y|X}}^2) \quad (6)$$

Here $\hat{\beta}_{0E}$ is the intercept, and $\hat{\beta}_{1E}$ is the slope, that is the causal estimate of the method. The standard deviation from the MR-Egger estimate can be calculated by dividing the slope ($\hat{\beta}_{1E}$) by the residuals standard deviation (σ_{ϵ_G}) from the weighted linear regression.

The MR-Egger method contains a test for the estimate, which is a test to determine if the estimate differs from zero (MR-Egger causal test). The intercept is interpreted as the overall pleiotropic effect. If the computed intercept is zero, then the estimate will be the same as the estimate from IVW. But with a non-zero intercept it is indicated that the IVW estimate can not be trusted, this is known as the MR-Egger intercept test.^[5]

COCHRAN'S Q TEST

To test if the results from MR are sufficiently similar to warrant their combined result, the homogeneity (measure of the similarity between studies) of the study is assessed by the Cochran's Q statistic (Q). This Q statistic is

applied to determine if there is a subset of the data that differs from the rest of the pool, which has the potential to mask the true identity of the direction of the causality or reflect causality when it is not present.^[12, 15, 16]

To apply Q statistic it needs estimates of the causal effect ($\hat{\beta}_{IV}$) and the estimated standard errors of those effects ($\sigma_{\hat{\beta}_{IV}}$). Which are the the causal estimates calculated by the Wald method. Cochran's Q measures heterogeneity of $\hat{\beta}_{IV}$ by inspecting the variation around the estimate from the IVW method ($\hat{\beta}_{IVW}$), where the weight for $\hat{\beta}_{IV}$ is the estimated variance ($1/\sigma_{\hat{\beta}_{IV}}$), see equation 7.^[12]

$$Q = \sum_G \frac{1}{\sigma_{\hat{\beta}_{IV|G}}} (\hat{\beta}_{IV|G} - \hat{\beta}_{IVW})^2 \quad (7)$$

Here, G is the amount of causal estimates between exposure and outcome. Q is a test for homogeneity, which under the null hypothesis follows a chi-squared distribution. The null hypothesis is rejected when it does not follow this distribution, then heterogeneity is present.^[12, 13, 14, 15, 16]

PATHWAY SCORING ALGORITHM

The Cochran's Q method, when significant will identify a subset of variants that deviates from the average causal estimate, and another subset of variants that lead to homogeneous estimates. Both subsets are variants associated with the same exposure, but they may act through different biological pathways. Understanding the biological difference identified by the two subsets is key to better understand the link between the exposure and outcome.

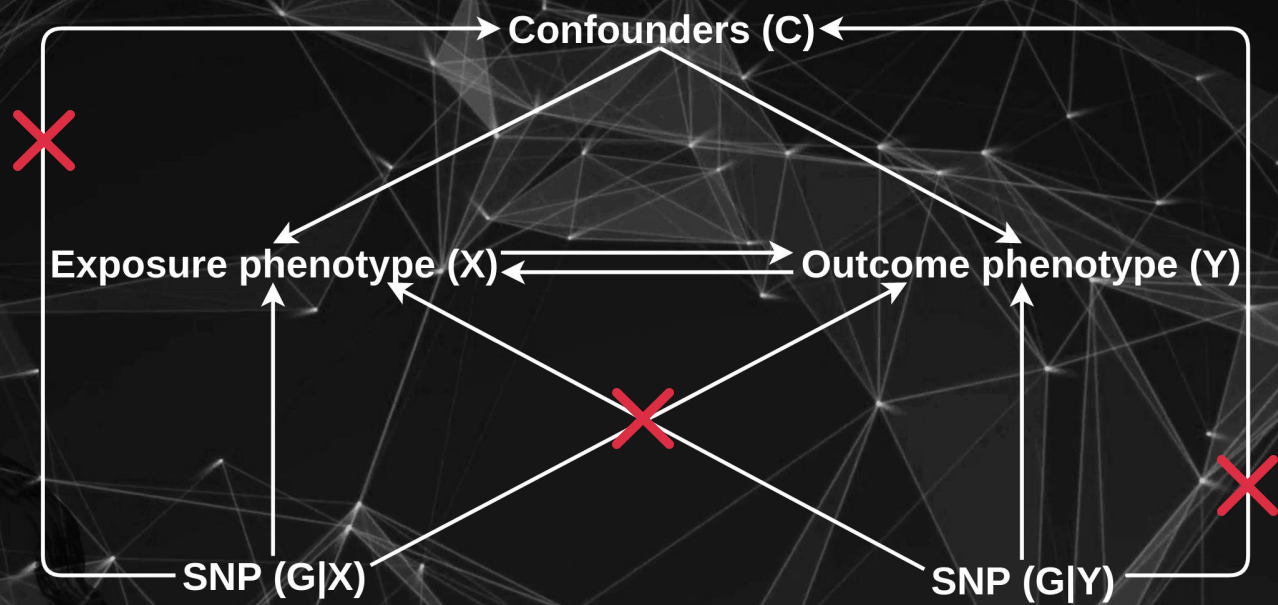


Figure 3: this graph shows the framework of a two-sample bidirectional MR. This framework is comparable to that of two-samples MR, but in addition the relationship between an exposure phenotype and outcome phenotype is exploited in both directions to gain insight in the link between two phenotypes. As in the MR framework there are assumptions that must be met, which are also applied in this framework. (I) genetic variants must be associated with the exposure, (II) no association between genetic variants and confounders, and (III) only association with outcome through exposure. This is here exactly the same, but only if the direction changes, the phenotypes for exposure and outcome will be flipped. The red crosses represent the relationships that violate the core assumptions.

To gain biological insight a tool for computing gene and pathway scores from SNP-phenotype summary statistics can be used: Pathway scoring algorithm (Pascal). This tool allows gene and pathway-level analysis of GWAS without the restriction to access original genotypic data.^[17]

TWO-SAMPLES BIDIRECTIONAL MENDELIAN RANDOMIZATION

To investigate the direction of the causal or protective relationship between a phenotype of interest and a relevant medical outcome the two-samples MR does not suffice. Two-samples bidirectional MR (figure 3) can be used to clarify the nature of the direction, whether the exposure causes or protects for the outcome phenotype or the outcome causes or protects for the exposure phenotype.^[18]

MATERIAL & METHODS

The aim of this study is to find factors that protect or cause celiac disease, and to quantify the protective or causal relationship.

More than 500.000 public available clinical and molecular mechanisms have been analyzed with the MR approach in both directions to find these factors, which makes this study the largest of its kind by having performed over two million MR analysis.

In order to perform a MR analysis at this scale several steps have been made. The first step is data acquisition (public available GWAS summary statistics), (II) pre-processing of the data, (III) clumping of SNPs that are in LD, (IV) harmonization of the outcome on exposure or vice versa if direction is to be changed, (V) performing two-samples MR, (VI) testing for heterogeneity in the data set, (VII) perform pathway analysis to understand the biological difference identified by the previous step to understand the link between the exposure and outcome, (VIII) interpreted results by plotting the outcome against the exposure, (IX) quantification of the causal or protective impact from exposure on outcome, or vice versa.

R

R is a language and environment for statistical computing and graphics.^[19] For which an open-source package has been written to enable easy MR analysis.

DATA

The first step is to define the genetic variants for the outcome and the exposures. For the outcome, which is celiac disease two cohorts have been used: a second-generation genome-wide association study of 4533 individuals with celiac disease^[20], and a densely genotyped cohort in 12,041 cases and 12.228 controls.^[21]

The exposures are a variety of clinical parameters and molecular mechanisms like Crohn's disease, Inflammatory bowel disease, Hemoglobin concentration, and Eosinophil percentage of granulocytes. Which were acquired by public available databases: UK Biobank^[22, 23], GWAS Catalog^[24], GTExPortal^[25], mQTL Database^[26]. And additional downloaded GWAS summary statistics from publicly available publications.

HUMAN LEUKOCYTE ANTIGEN REGION

The Major Histocompatibility Complex (MHC), also defined as the Human Leukocyte Antigen (HLA) region encodes several key roles in the immune system. This region encompasses 7.6Mb on chromosome 6p21, and is within the human genome the most dense one, and exhibits the most dense LD.^[35]

This region has been removed from both outcome cohorts (celiac disease^[20, 21]) for a variety of reasons: (I) to suppress potential pleiotropic effects introduced by LD, (II) the lack of specific genotype data that is needed for computing LD, (III) this region is too complex to accurately determine the genetic effect on the phenotype of interest.

PRE-PROCESSING

In addition to defining genetic variants to use for a MR analysis, these variants should be chosen appropriately, meaning that the study should not violate the core assumptions. In this step the first core assumption was addressed (the genetic variant must be associated with the exposure^[3, 4, 5]) by selecting for genome-wide significance, only SNPs with a p-value of 5e-8 (the conventional threshold for GWAS statistical significance^[27]) or smaller were used.

Furthermore, genetic variants were removed if it was not possible to calculate their effect size or Odds Ratio (OR), when there was allelic information missing and it was not possible to query them by the 1000 Genomes phase 3 reference panel^[28], when a genetic variant shared a duplicate, when it was not possible to infer the direction of the allele, that the SNP is palindromic.

LINKAGE DISEQUILIBRIUM CLUMPING

LD is defined as the difference between the observed frequency of an allele at two loci and the frequency expected from random association.^[11] Gene loci are presumed to be independent of one another, however LD suggests otherwise, it describes the situation in which they are not, and genetic variants could be co-inherited. This creates a situation where LD is introducing pleiotropy. This leads to the associations of SNPs related to more than one post-transcriptional process.^[29]

Clearly this violates the second core assumption: the genetic variant must not be associated with confounders.^[3, 4, 5] To counter

attack SNPs that are in LD which can introduce pleiotropy, these SNPs have been clumped with PLINK 1.9^[30].

Clumping was performed on the exposure data in order to find LD structures and to disentangle pleiotropy. With PLINK LD is estimated between SNP-SNP pairs based on a reference genotype data set, where a LD score is calculated (the squared correlation based on genotypic allele counts: r^2).^[31] And only one SNP per region of LD is used, and this SNP must also be present in the outcome data set. The clumping analysis was performed using the following PLINK command:

```
./PLINK
--bfile EUR_1000G_phase3_vcf
--clump exposure.data
--clump-p1 5*10^-8
--clump-p2 5*10^-8
--clump-r2 .1
--clump-kb 1000
--clump-verbose
--out exposure.clump.data
```

Clumps are formed around index variants which by default have a p-value not greater than 1e-4, this threshold can be adjusted with --clump-p1. Sites that are n kilobase (kb) from an index variant, and have r^2 larger than m , and have association p-value lesser than given are assigned to that index variant, if they have not been previously assigned to another LD structure. These parameters can be adjusted with --clump-p2, --clump-r2, and --clump-kb. The --bfile parameter expects the reference genotype data. The --clump-verbose parameter is an option for returning an extended report. Last, --out is used for

defining the output file.^[31]

As stated earlier, if MR wants to access the causal or protective relationship between an exposure and outcome, the genetic variants from the exposure must be present in the outcome, however, this is not always true. This is where LD has been used in advantage of the study. Namely, MR does not force to use the actual causal variant but is satisfied with a marker that is in LD. When a certain SNP in the exposure is not present in the outcome, a proxy SNP has been used that is in LD if its r^2 is equal or greater than .8 , with respect to the given parameters.^[32]

HARMONIZATION

When using two or more independently generated data sets data harmonization is an essential step for two-samples MR, because seldom GWAS results have harmonized alleles.^[33]

In the harmonization from the exposure on the outcome, several steps have been made. (I) all the signs from genetic variants from the exposure where flipped if they where negative, and if the effect was negative, the major allele and minor allele also have been flipped if true. This has been done because this study wants to measure the positive effect from the exposure on the outcome, (II) all variants from the outcome must be associated with the exposure in the same direction. When a variant is not coded in the same direction, then the variant has been flipped, which means that in the outcome the alleles, and genetic effect are flipped :

outcome		
effect allele	other allele	effect size
A	C	0.5

exposure		
effect allele	other allele	effect size
C	A	0.5

↓

outcome		
effect allele	other allele	effect size
C	A	- 0.5

exposure		
effect allele	other allele	effect size
C	A	0.5

(III) When the variant in exposure and outcome do not share the same allelic information, they where discarded from the data set:

outcome		
effect allele	other allele	effect size
A	G	0.5

exposure		
effect allele	other allele	effect size
C	A	0.5

TWO-SAMPLES MENDELIAN RANDOMIZATION

The basic principle that is deployed in MR is that genetic variants either amend the level of, or mirror the biological effects of, an exposure that in turn alters the disease risk, should be associated with the disease risk.^[34]

Two-samples MR is applied to identify the

factors that cause or protect for celiac disease, to determine potential causal or protective relationships more than 500,000 clinical parameters and molecular mechanisms have been screened with the MR approach.

The two-samples MR Wald methods has been applied to calculate causal estimate between the variants in exposure and outcome (equation 1). To combine the these causal estimates from multiple genetic variants to increase power both IWV (equation 3), and MR-Egger (equation 6) have been deployed to accesses an overall causal estimate between exposure and outcome.

COCHRAN'S Q TEST

To warrant the combined causal estimate from the IVW method the Cochran's Q test was used. Cochran's Q assesses the homogeneity by testing the chi-square distribution from the association between exposure and outcome by computing a Q term for every SNP-SNP association between exposure and outcome.

The sum of the Q terms is taken and tested by the chi-square (χ^2) test, to estimate how similar the observed distribution is to an expected distribution. If the null hypothesis is not rejected, then the max Q term will be removed, and the IVW causal estimate will be re-calculated with the remaining genetic variants. This is recursively executed until the alternative hypothesis is accepted (no chi-square distribution), then heterogeneity is present.^[36]

When Cochran's Q method was significant a subset of variants that deviates from the

average causal estimate, and another subset of variants that identifies homogeneous variants, where identified. On the subset that deviates from the average causal estimate, the IVW, and MR-Egger methods have been executed again.

PATHWAY SCORING ALGORITHM

TWO-SAMPLES BIDIRECTIONAL MENDELIAN RANDOMIZATION

RESULTS

R

mendelianRandomization is a software package for the R open-source environment that performs MR analyses using summarized data.

The mendelianRandomization package can be downloaded from the web-based hosting service Bitbucket:

<https://bitbucket.org/MatthijsKnigge/mendelianRandomization>.

Or can be directly installed into R by using libraries that are described in the tutorial on the source page.

This package provides the user with several options to perform a MR analysis. The core functionality that is implemented are the two-samples MR methods: Wald ratio, IVW, and MR-Egger. However, there is also the functionality to pre-process the data, clump the data, harmonization of the data, a test for assessing the homogeneity, and a function for predicting biological pathways, and other functionality which is discussed in detail on the source page of the package.

DATA

DISCUSSION

CONSLUSION

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APPENDIX

Appendix A. Additional downloaded GWAS summary statistics from publicly available publications

just	for	show	at	the	moment			

