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Article

Genetic and biomechanics insights into cathepsins and non-cancerous digestive diseases: A bidirectional two-sample Mendelian randomization study

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Abstract: Background: Digestive diseases have high incidence and mortality rates, posing a significant threat to global health. However, research on these disorders is uneven, while digestive cancers are well-studied and non-cancerous digestive diseases, despite their considerable health impact, have received less attention. Although cathepsins (CTSs), proteases that regulate extracellular matrix (ECM) turnover and cellular stiffness, have been implicated in digestive disorders, their role in disease-specific mechanical perturbations remains unclear. This study bridges this gap by integrating genetic causation with organ-level biomechanics. **Methods:** To overcome the constraints of conventional epidemiological methods, we employed a dual-sample bidirectional Mendelian randomization (MR) analysis leveraging genome-wide association study (GWAS) data to explore the causal relationships among 9 cathepsins and 23 non-cancerous digestive diseases. We conducted inverse variance weighted (IVW), weighted median (WM), MR-Egger, MR-PRESSO, Cochran's *Q*, and sensitivity analyses for thorough evaluation. We also performed correlation analyses to link the biomechanical data with the genetic and disease outcomes, aiming to identify the relationships between mechanical factors, CTSs, and non-cancerous digestive diseases. **Results:** Forward MR analysis indicated that CTSB promotes both cholecystitis and cholelithiasis and CTSZ promotes chronic gastritis and diverticulosis. Higher CTSL2 levels promote non-alcoholic fatty liver disease (NAFLD) and liver cirrhosis, whereas upregulated CTSG reduces NAFLD risk. Reverse MR analyses indicated that gastroesophageal reflux, gastric ulcer, NAFLD, and cholecystitis elevated CTSE, G, Z, and B levels, respectively; non-alcoholic steatohepatitis elevates CTSB and H levels. Liver cirrhosis increases CTSB, S, and Z; Barrett's esophagus, celiac disease, and diverticulosis downregulate CTSO, F, and H respectively; chronic pancreatitis lowers CTSE, F, and L2. Multivariable MR analyses revealed the independent effects of individual CTSs on specific diseases: CTSZ as a promoter for diverticulosis, CTSG as a protective factor for NAFLD, and CTSB as a promoter for cholecystitis and cholelithiasis. **Conclusion:** This study confirmed the causal relationships between cathepsins, mechanical factors in the digestive system, and non-cancerous digestive diseases. By integrating genetic and biomechanical analyses, we have provided a more in-depth understanding of how mechanical forces interact with biological molecules during the development of non-cancerous digestive diseases. Moreover, they may lead to the establishment of novel clinical practice approaches that take into account both the mechanical and biological aspects of digestive diseases, ultimately improving the diagnosis, treatment, and management of these conditions.

Keywords: Mendelian randomization; biomechanics; genome-wide association; single nucleotide polymorphisms; cathepsins; non-cancerous digestive diseases; causal relationship

1. Introduction

Non-cancerous digestive system diseases include a wide array of conditions that affect the digestive tract and accessory organs, such as the liver, gallbladder, and pancreas. The high prevalence of non-cancerous digestive diseases such as gastritis, peptic ulcers, enteritis, and liver diseases significantly affects public health worldwide [1,2]. These conditions have led to a decrease in quality of life and substantial healthcare burdens [1,3]. According to a study focusing on the global burden of digestive diseases, they accounted for over 7.3 billion cases in 2019 alone. The global age-standardized incidence and mortality rates of these conditions have not shown a downward trend from 1990 to 2019, with digestive disorders constituting more than one-third of the overall disease burden [4,5]. This situation highlights the pervasive impact of digestive disorders on well-being worldwide and the critical need for a wider understanding of the factors contributing to the development of these diseases [4].

Cathepsins are a critical subset of the protease enzyme family [6], mainly including B(CTSB), E(CTSE), F(CTSF), G(CTSG), H(CTSH), L2(CTSL2), O(CTSO), S(CTSS), and Z(CTSZ) [7], among others, serve as vital components in maintaining cellular homeostasis through their roles in lysosomal degradation, immune regulation, autophagy, protein metabolism, and extracellular matrix remodeling [8–11]. These functions form the basis of mechanisms implicated in various digestive diseases, with each cathepsin contributing distinctly to the progression of various disorders [11,12]. For instance, elevated levels of CTSB [13,14], CTSS [13], and CTSZ [15,16] in Helicobacter pylori-infected gastritis tissues drive local immune and inflammatory responses, thereby influencing the development of peptic ulcers. In pancreatitis, heightened CTSB and CTSS activity is observed in macrophages and pancreatic acinar cells, emphasizing their roles in the inflammatory response [17]. CTSD, expressed in pancreatic acinar and inflammatory cells, intensifies acute pancreatitis by amplifying CTSB, which subsequently triggers intracellular trypsinogen activation, further aggravating the condition [18,19]. On the other hand, CTSE is broadly expressed in immune cells, underscoring its diverse roles in immune regulation [10,20]. The relationship between CTSG and inflammatory bowel diseases such as ulcerative colitis (UC) remains complex. While a 1992 study by Mayet et al. detected no antibodies against CTSG in patients with UC [21], a later 2000 study by Kuwana et al. reported significantly higher CTSG antibody levels in patients with active UC than in healthy individuals [22], suggesting a potential but unresolved connection between CTSG and inflammatory bowel disease. CTSH contributes to fibrotic progression in cirrhotic livers by modulating mesenchymal cell plasticity and extracellular matrix responsiveness, although its effects diminish in advanced fibrosis due to its downregulation [23]. In pancreatic β-cells, high CTSH levels protect against cytokine-induced apoptosis, helping to preserve cellular function amidst immune-mediated damage [24]. CTSS activity, which can be upregulated by pathobionts, drives T cell-mediated colonic inflammation, whereas cystatin C-dependent regulation of CTSS activity contributes to the generation of tolerant intestinal dendritic cells, thereby reducing inflammatory responses [25]. Additional studies are required to clarify the distinct roles of CTSF, CTSL2, and CTSO in noncancerous digestive disorders.

Despite these findings, the causal relationships between specific cathepsins and non-cancerous digestive diseases remain unclear. Conventional observational studies frequently encounter challenges arising from confounding variables and the possibility of reverse causation [26], making it challenging to establish definitive causal links. To address these limitations, our research employs Mendelian randomization (MR), which employs genetic variants as instrumental variables (IVs) to determine causality between exposures (nine cathepsins) and outcomes (23 non-cancerous digestive diseases) [27,28]. Because genetic variants are essentially randomized at the time of conception, this approach reduces bias from acquired factors, providing a more robust framework for causal inference. Furthermore, MR extends causal inference to domains where traditional randomized controlled trials (RCTs) are unlikely to be conducted, such as studying the effects of long-term exposure or those involving ethical risks. Using existing genetic data, MR avoids the high costs and complications of primary data collection in large-scale RCTs, making MR a cost-effective solution for evidence generation in modern epidemiological research [29]. Compared with previous MR analyses, guided by the concept of benefiting a wider range of people, we expanded the types and scope of digestive diseases, covering as many common non-cancerous digestive diseases as possible, such as gastroesophageal reflux disease, gastritis, peptic ulcer, inflammatory bowel disease, liver cirrhosis and other chronic liver diseases, gallbladder diseases, pancreatic diseases, etc. [4].

Based on this situation, we aimed to employ MR to investigate the potential causal links between more non-cancerous digestive diseases and nine cathepsins (B, E, F, G, H, O, S, L2, Z) to provide a focused and comprehensive analysis of their potential causal impacts on digestive health. The insights gained from this research could revolutionize our understanding and management of these conditions, ultimately leading to better patient outcomes and advancements in digestive health care.

2. Materials and methods

2.1. Study design and selection of IVs

To ensure that the selected IVs are valid for MR analysis, they must fulfill three core assumptions: exclusion restriction, relevance, and independence [30] (**Figure 1**). (1) The relevance assumption: The selected IVs should have a strong correlation with the exposure variable; (2) the exclusion restriction assumption: The IVs must be unrelated to any potential confounding factors, meaning that they must not be linked to any confounding factors that could affect the outcome; (3) the independence assumption: There is no direct association between the IVs and the outcome variable; the IVs can modulate the outcome variable exclusively by exposure.

Therefore, strict criteria were applied for the genome-wide association study (GWAS) data selection of cathepsin-related IVs. We ensured low LD with $r^2 < 0.001$ within a 10,000 kb interval and a significant correlation with each phenotype with p-values $< 5 \times 10^{-6}$. All single-nucleotide polymorphisms (SNPs) of cathepsins selected had an F-value > 10 to maintain the validity and effectiveness of the IVs (Supplementary **Table S1**). The same rigorous standards were applied to non-cancerous digestive disease analyses. We confirmed that the selected SNPs had no

association with confounding factors that could interfere with the relationship between exposure and outcome. This process involved excluding SNPs linked to the outcome, managing LD, correcting for pleiotropy using the MR pleiotropy residual sum and outlier (MR-PRESSO), and filtering IVs according to strength and association, thereby guaranteeing the reliability of causal inference in our MR study (**Figure 1**).

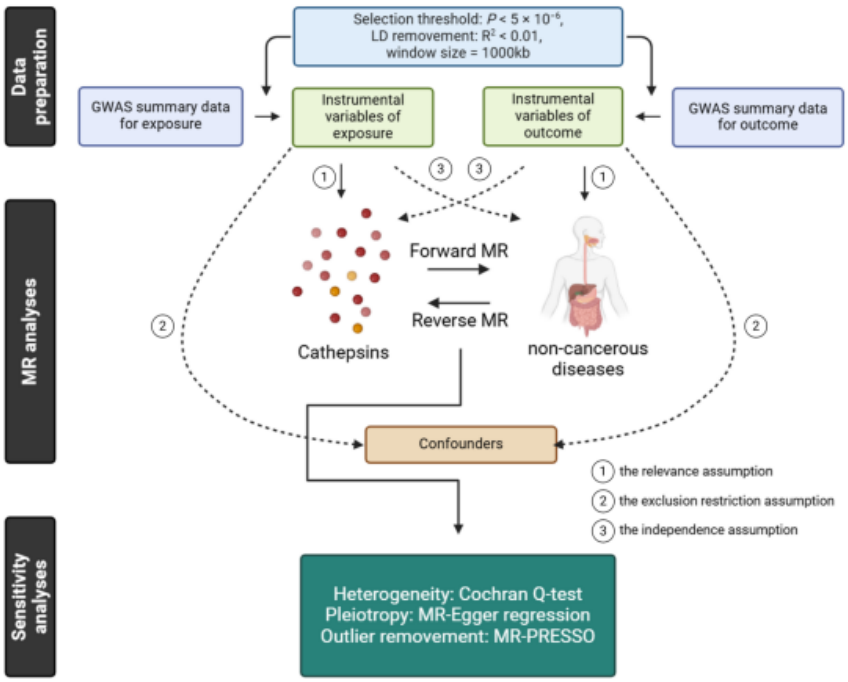

Figure 1. Study design and workflow.

2.2. Data source of cathepsins

Genetic summary statistics of nine cathepsins (B, E, F, G, H, L2, O, S, and Z) were sourced from the INTERVAL study, a comprehensive genetic research program that recruited 3301 European participants (**Table 1**). The IEU OpenGWAS project website for accessing the data is https://www.ebi.ac.uk. Notably, the study was conducted under ethical standards and each participant provided informed consent to ensure voluntary and informed participation [31].

Table 1. GWAS data of 9 cathepsins.

GWAS ID	Year	Trait	Sample size	Number of SNPs
prot-a-718	2018	Cathepsin B	3301	10,265,264
prot-a-720	2018	Cathepsin E	3301	10,265,264
prot-a-722	2018	Cathepsin F	3301	10,265,264
prot-a-723	2018	Cathepsin G	3301	10,265,264
prot-a-725	2018	Cathepsin H	3301	10,265,264
prot-a-728	2018	Cathepsin L2	3301	10,265,264
prot-a-726	2018	Cathepsin O	3301	10,265,264
prot-a-727	2018	Cathepsin S	3301	10,265,264
prot-a-729	2018	Cathepsin Z	3301	10,265,264

2.3. Genetic association of SNPs with non-cancerous digestive diseases

Statistics on 23 non-cancerous digestive diseases were obtained from the FinnGen database following the coding of the International Classification of Diseases Tenth Revision (ICD-10). The latest summary statistics of 23 non-cancerous digestive diseases were obtained from the FinnGen Consortium (data freeze 11), including gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), gastric ulcer, duodenal ulcer (DU), acute gastritis (AG), chronic gastritis (CG), irritable bowel syndrome, celiac disease (CD), diverticulosis, Crohn's disease of the small intestine, Crohn's disease of the large intestine, ulcerative colitis (UC), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcoholic liver disease, liver cirrhosis, cholangitis, cholecystitis, cholelithiasis, acute pancreatitis, chronic pancreatitis (CP), acute appendicitis, and other appendicitis. Importantly, informed written consent was obtained from each participant, and relevant institutional ethical review boards reviewed and approved all studies. Therefore, no further ethical approval or license was necessary for this MR study. **Table 2** provides details on the sample size, number of cases, and control groups.

Table 2. GWAS data of 23 non-cancerous digestive diseases.

Phenotype	Number of SNPs	Cases	Controls	Sample size	Ancestry	Year	Website
Gastroesophageal reflux disease (GERD)	21,306,231	32,232	385,082	417,314	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_REFLUX.gz
Barrett's esophagus (BE)	21,305,544	1486	385,082	386,568	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_BARRET.gz
Gastric ulcer	21,305,720	7012	385,082	392,094	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_GULC.gz
Duodenal ulcer (DU)	21,305,607	4115	385,082	389,197	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_DULC.gz
Acute gastritis (AG)	21,305,600	2770	385,082	387,852	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_ACUTGASTR.gz
Chronic gastritis (CG)	21,305,758	11,226	385,082	396,308	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_CHRONGASTR.gz
Irritable bowel syndrome	21,305,149	11,742	360,393	372,135	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_IBS.gz
Celiac disease (CD)	21,306,572	4568	433,899	438,467	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_COELIAC.gz
Diverticular disease	21,305,864	37,886	360,393	398,279	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_DIVERTIC.gz
Crohn's disease of small intestine	21,306,471	2457	432,380	434,837	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_CHRONSMALL.gz
Crohn's disease of large intestine	21,306,421	1870	432,380	434,250	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_CHRONLARGE.gz
Ulcerative colitis (UC)	21,306,469	6435	446,419	452,854	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_UC_STRICT2.gz
Non-alcoholic fatty liver disease (NAFLD)	21,306,794	3006	450,727	453,733	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_NAFLD.gz

Table 2. (*Continued*).

Phenotype	Number of SNPs	Cases	Controls	Sample size	Ancestry	Year	Website
Nonalcoholic steatohepatitis (NASH)	21,306,788	218	453,515	453,733	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_NASH.gz
Alcoholic liver disease	21,306,599	3330	440,301	443,631	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_ALCOLIV.gz
Cirrhosis of liver	21,306,741	1470	448,864	450,334	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_CHIRHEP_NAS.gz
Cholangitis	21,305,815	2068	397,583	399,651	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_CHOLANGI.gz
Cholecystitis	21,305,892	5237	397,583	402,820	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_CHOLECYST.gz
Cholelithiasis	21,306,603	44,582	397,583	442,165	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_CHOLELITH.gz
Acute pancreatitis	21,305,964	7562	397,583	405,145	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_ACUTPANC.gz
Chronic pancreatitis (CP)	21,305,846	4222	397,583	401,805	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_CHRONPANC.gz
Acute appendicitis	21,306,750	35,180	415,845	451,025	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_APPENDACUT.gz
Other appendicitis	21,306,290	3101	415,845	418,946	European	2024	https://storage.googleapis.com/finngen-public-data-r11/summary_stats/finngen_R11_K11_APPENDOTH.gz

2.4. MR analysis

We aimed to understand the potential forward and reverse causations between a single cathepsin and single diseases, as well as the effects of multiple cathepsins in a more physiological state on specific diseases. To this end, we performed forward MR, reverse MR, and multivariate MR analyses. First, we performed forward MR analysis. Three analysis methods were employed: inverse variance weighting (IVW), MR-Egger, and weighted median (WM). In our study, IVW was used as the main method for estimating the bidirectional effect, owing to its stability. Widely considered the primary approach in MR studies for assessing causality, IVW uses the Wald ratio to weigh the impact of each variant on disease risk relative to its effect on exposure [29]. It provides a total estimated effect of exposure on outcome, accounting for both fixed and random influences, enabling subsequent adjustments to achieve more reliable outcomes in the face of result heterogeneity [32]. Additionally, MR-Egger and weighted median analyses were performed as complementary approaches to validate the robustness of our MR analysis.

To further explore bidirectional causality by assessing reverse causality, we performed reverse MR analyses using the same GWAS dataset, data selection threshold, and analytical methods described above, with non-cancer digestive diseases as the exposure and cathepsins as the outcome.

Multivariable MR (MVMR) was designed to analyze the causal impact of multiple exposure factors on every outcome, making it particularly suitable for exploring the interactions between the nine cathepsins and their independent and comprehensive effects on a single non-cancerous digestive disease. When

investigating the effect of a specific cathepsin, we controlled for the other eight cathepsins within the same disease context. Consequently, we can confidently conclude that our results reveal that the effect of several cathepsins on digestive diseases is independent and not mediated by other factors.

Causality was assessed using odds ratios (OR) and 95% confidence intervals (CIs). An OR < 1 indicates a protective effect, whereas an OR > 1 suggests a risk factor.

2.5. Sensitivity analyses

We utilized multiple sensitivity analyses to bolster our study's rigor and reliability. The Cochrane *Q* Test identified heterogeneity among SNPs at a significance level of *Q*-*p*-value < 0.05. In the presence of heterogeneity, we switched to inverse variance weighting with a random-effects model for a more robust analysis, replacing the initial findings.

To examine the potential for pleiotropy in MR results, which could arise if IVs directly affect the outcome via pathways other than the exposure of interest, we utilized MR-PRESSO (including globe and outlier tests) and the MR-Egger intercept test [29,33]. Outliers were detected by the MR-PRESSO global test, where a *p*-value threshold of less than 0.05 was set to indicate significant pleiotropy, and the specific SNPs were pinpointed for removal using the MR-PRESSO outlier test [34]. By excluding outliers and reconducting the MR analysis, we were able to significantly minimize pleiotropy. Subsequently, the MR-Egger intercept test was employed to detect any remaining horizontal pleiotropy [29]; a *p*-value greater than 0.05 suggested the absence of significant pleiotropy.

By individually removing each IV and analyzing the effects of the remaining variables, the leave-one-out approach can evaluate the impact of each IV on the overall estimation. If there is little change in the results after removing a certain variable, it indicates that the model's results are robust.

Implementing these stringent methods and conducting sensitivity analyses helped bolster our research's scrutiny and stability, safeguarding against potential biases, and affirming the reliability of our findings.

2.6. Software and packages

All analyses were conducted using R version 4.4.1. The following R packages were utilized: Two-sample MR version 0.6.8 [35] for the IVW, MR-Egger, and weighted median methods; MR-PRESSO version 1.0 [36] for the MR-PRESSO global test and outlier removal, and Mendelian randomization version 0.10.0 [32] for Multivariable MR.

It is important to add that this study adheres to the STROBE-MR checklist for reporting Mendelian randomization research [37], and to enhance the reproducibility of our work.

3. Results

3.1. IV selection

In our study, we sourced genetic variants for analyzing nine cathepsins from the INTERVAL study, whereas data on 23 digestive diseases were gathered from the FinnGen GWAS databases. We identified multiple SNPs as IVs for each cathepsin and disease in the bidirectional MR analysis by applying a genome-wide significance threshold of $P < 5 \times 10^{-6}$. Each IV demonstrated an *F*-statistic > 10, indicating no weak bias. Detailed information on the SNPs is provided in Supplementary **Table S1**.

3.2. MR analysis results

3.2.1. Forward MR analysis

First, we performed 2-sample MR analysis. Causal relationships indicating potential horizontal pleiotropy, as identified by the MR-Egger intercept, were excluded to ensure the robustness of the findings. The following findings (**Figures 2–4**) after this exclusion revealed that elevated CTSB levels were linked to a higher risk of developing cholecystitis (OR = 1.074, 95% CI: 1.007–1.145, IVW: $P = 0.029$) and cholelithiasis (OR = 1.047, 95% CI: 1.017–1.078, IVW: $P = 0.002$). Furthermore, a significant positive association was found, indicating that higher CTSL2 levels were associated with an increased risk of liver cirrhosis (OR = 1.221, 95% CI, 1.011–1.473; IVW: $P = 0.038$). Similarly, CTSL2 levels predict an increased risk of NAFLD, highlighting its role as a potential predictor (OR = 1.147; 95% CI, 1.006–1.308; IVW: $P = 0.041$). A positive correlation was observed between CTSZ levels and the incidence of CG (OR = 1.063, 95% CI: 1.004–1.125, IVW: $P = 0.036$), and heightened CTSZ levels were associated with a greater risk of diverticulosis (OR = 1.040; 95% CI, 1.006–1.075; IVW: $P = 0.021$). In addition, heterogeneity in the results between CTSZ and diverticulosis was detected after the removal of outliers, indicating that the findings are not robust and may require further experimental validation. Conversely, a notable decrease in the risk of NAFLD was associated with higher CTSG levels (OR = 0.897, 95% CI, 0.806–0.999; $P = 0.047$). These findings emphasize the crucial role of cathepsin level in disease risk assessment and may have implications for future diagnostic and therapeutic strategies. The full forward MR results, including detailed statistics and methods, are available in Supplementary **Table S2**.

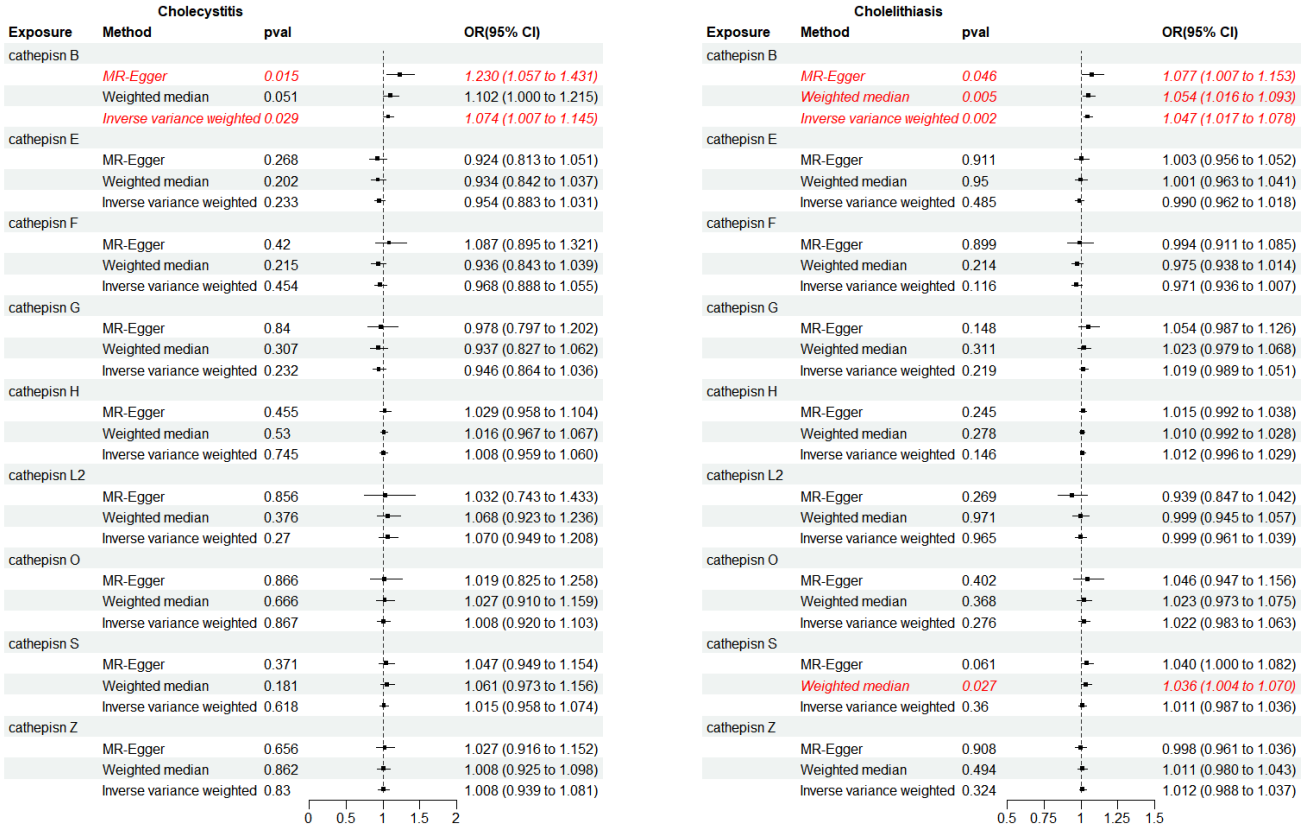

Figure 2. Positive result of forward MR analysis: Cholecystitis and cholelithiasis.

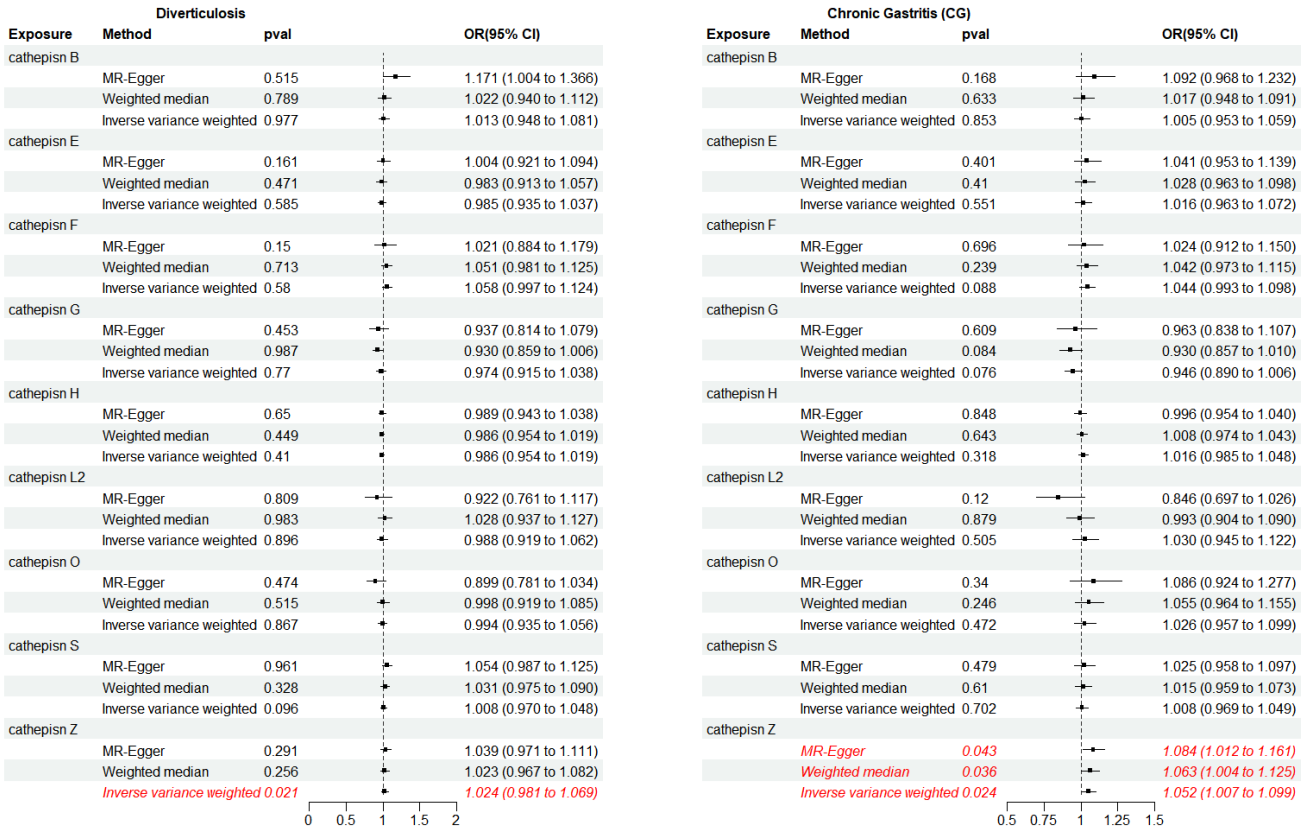

Figure 3. Positive result of forward MR analysis: Diverticular disease and chronic gastritis (CG).

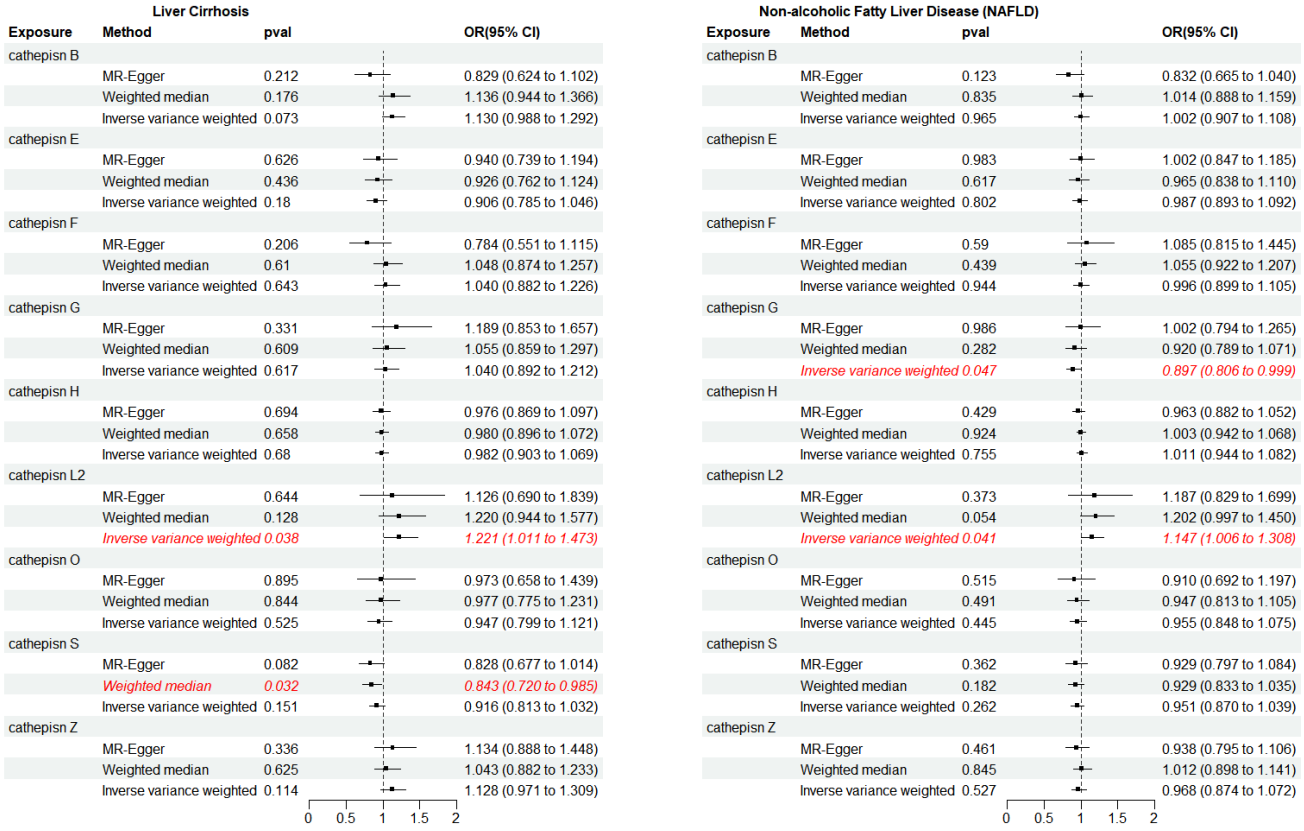

Figure 4. Positive result of forward MR analysis: Cirrhosis of liver and non-alcoholic fatty liver disease (NAFLD).

3.2.2. Reverse MR analysis

We used reverse MR analysis to investigate the impact of diseases on cathepsin levels. Specifically, a promoting effect on cathepsin levels was observed between CTSB and NASH (OR = 1.084, 95% CI: 1.001–1.175, IVW: P = 0.047) and liver cirrhosis (OR = 1.070, 95% CI: 1.014–1.129, IVW: P = 0.014); CTSH and NASH (OR = 1.078, 95% CI: 1.013–1.147, IVW: P = 0.017), CTSS, and liver cirrhosis (OR = 1.071, 95% CI: 1.014–1.130, IVW: P = 0.013); CTSZ and both NAFLD (OR = 1.119, 95% CI: 1.044–1.198, IVW: P = 0.001) and liver cirrhosis (OR = 1.101, 95% CI: 1.028–1.179, IVW: P = 0.006). In contrast, BE lowered the levels of CTSO (95% CI: 0.850–0.997, IVW: P = 0.043), while diverticulosis reduced CTSH levels (OR = 0.914; 95% CI, 0.841–0.992; IVW: P = 0.031). Additionally, CP showed a negative correlation with CTSE, F, and L2 (OR = 0.891, 95% CI: 0.806–0.985, IVW: P = 0.024; OR = 0.882, 95% CI: 0.786–0.988, IVW: P = 0.031; OR = 0.885, 95% CI: 0.800–0.978, IVW: P = 0.017) (**Figures 5–7**). Notably, the reverse and forward MR analyses yielded no overlapping results, indicating that no bidirectional causal relationships were identified between any cathepsin-disease pairs. The full-reserve MR results are presented in Supplementary **Table S3**.

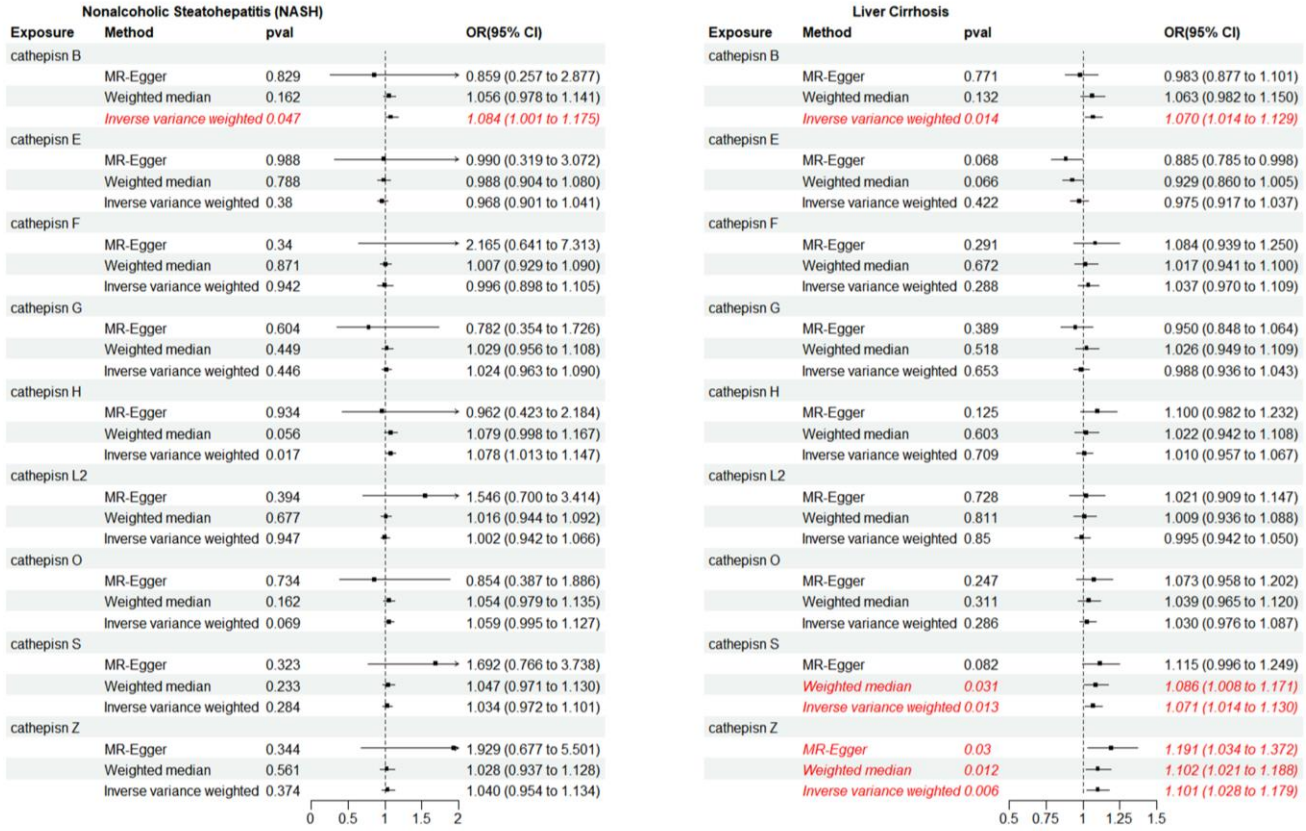

Figure 5. Positive result of reverse MR analysis: Nonalcoholic steatohepatitis (NASH) and cirrhosis of liver.

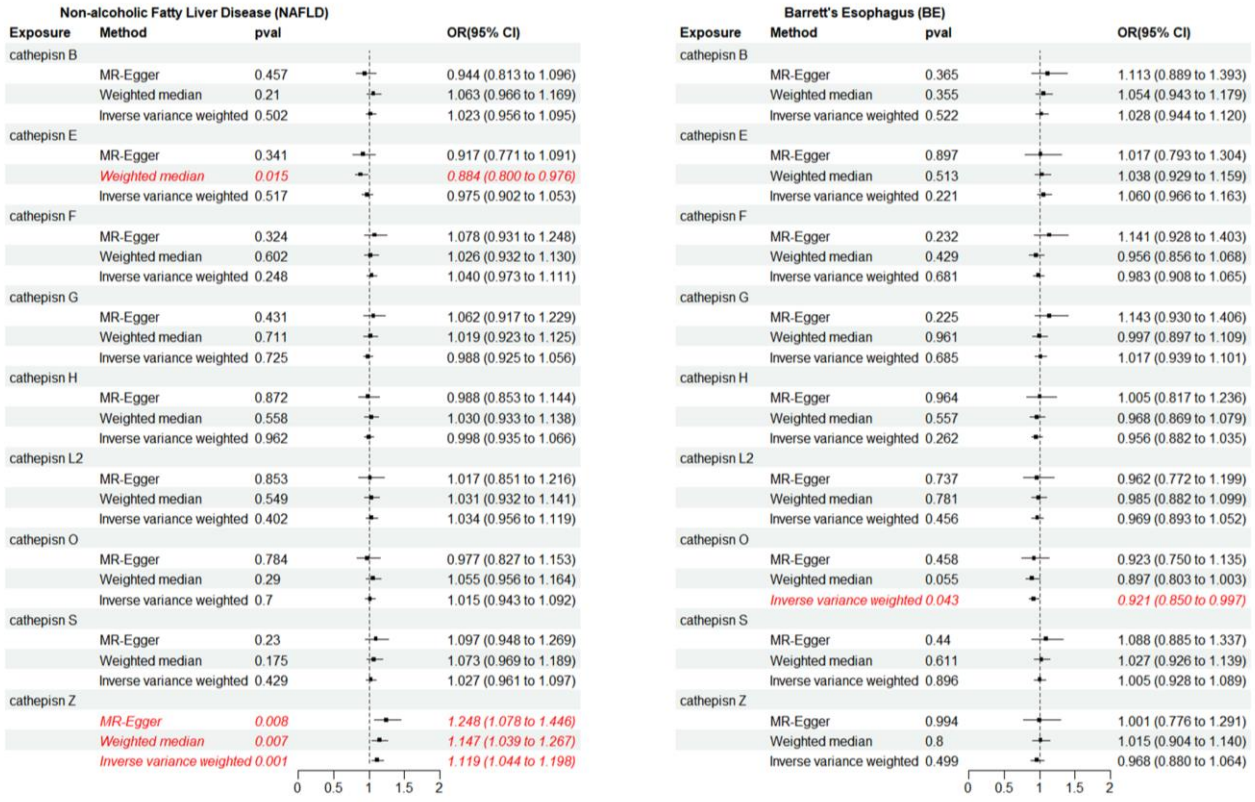

Figure 6. Positive result of reverse MR analysis: Non-alcoholic fatty liver disease (NAFLD) and Barrett's esophagus (BE).

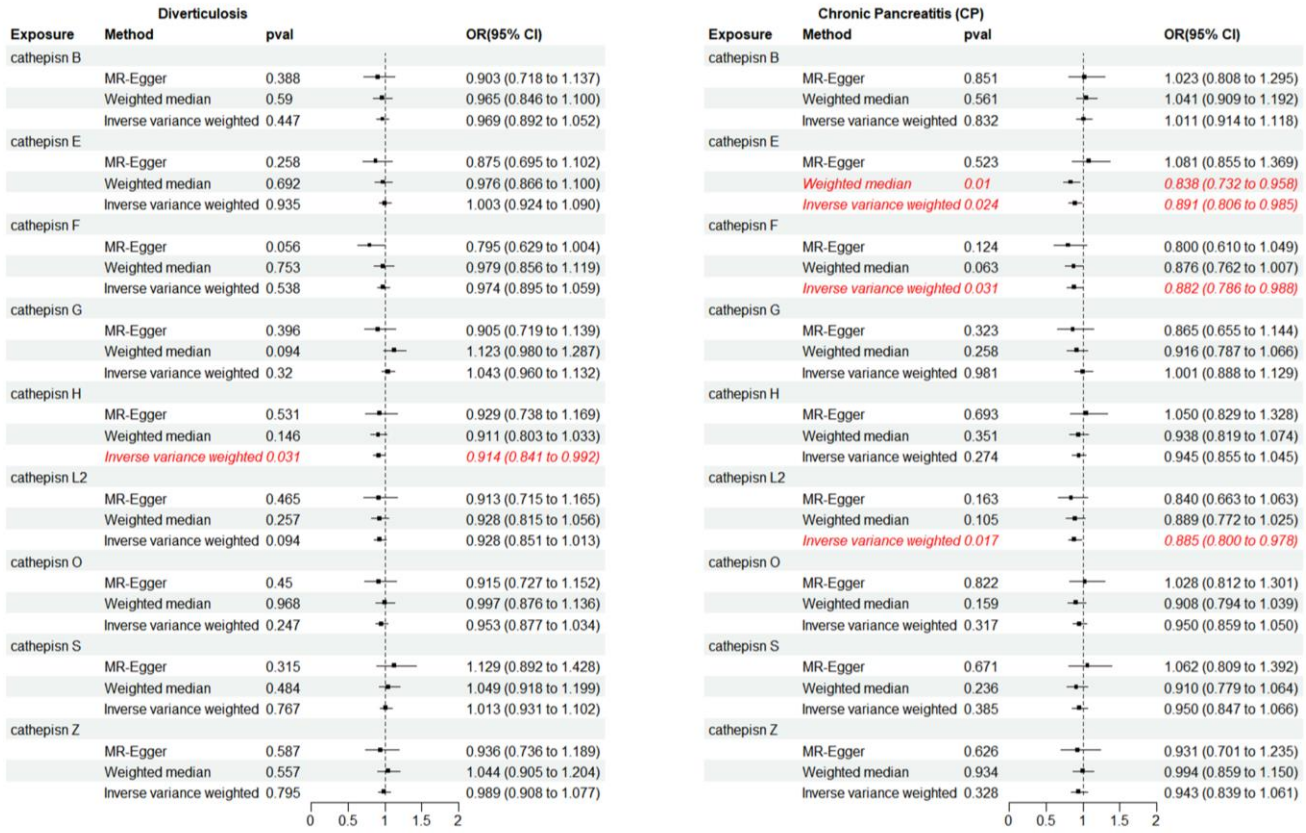

Figure 7. Positive result of reverse MR analysis: Diverticular disease and chronic pancreatitis (CP).

3.2.3. Multivariable MR analysis

By employing the MVMR method, we conducted a more in-depth examination of the 9 cathepsins linked to each of the 23 digestive diseases. Our results indicate that after controlling for the expression of other cathepsins within the same disease context, the impact of specific cathepsins on digestive diseases is independent and not influenced by other exposures. Specifically, CTSG was associated with a higher risk of GERD (OR = 1.034, 95% CI: 1.000–1.069, P = 0.047) and showed a protective trend against NAFLD (OR = 0.856, 95% CI: 0.744–0.984, P = 0.029). CTSL2 was associated with a higher likelihood of DU (OR = 1.201, 95% CI: 1.074–1.344, P = 0.001) than CTSO, which indicated a protective effect against DU (OR = 0.873, 95% CI: 0.770–0.990, P = 0.035). CTSE promoted AG (OR = 1.111, 95% CI: 1.006–1.227, P = 0.038). CTSH and S protected against CD (OR = 0.946, 95% CI: 0.900–0.995, P = 0.033; OR = 0.933, 95% CI: 0.874–0.994, P = 0.033), whereas CTSO was associated with an increased risk for CD (OR = 1.150, 95% CI: 1.020–1.298, P = 0.023). CTSZ independently promotes diverticulosis (OR = 1.045, 95% CI, 1.009–1.083; P = 0.014). CTSB was independently associated with an increased risk of liver cirrhosis (OR = 1.149, 95% CI: 1.011–1.305, P = 0.034) and cholecystitis (OR = 1.082, 95% CI: 1.008–1.161, P = 0.028), while CTSS showed an independent protective effect in liver cirrhosis (OR = 0.870; 95% CI, 0.772–0.979; P = 0.021). For cholelithiasis, CTSB and CTSZ promoted disease risk (OR = 1.048, 95% CI: 1.007–1.091, P = 0.021; OR = 1.052, 95% CI: 1.002–1.105, P = 0.040), while CTSF offered protection (OR = 0.934, 95% CI: 0.888–0.983, P = 0.009). Positive results are shown in **Figure 8**. The causal

relationships between certain cathepsins and relevant diseases that match the forward MR results were as follows: CTSG and NAFLD, CTSZ and diverticulosis, CTSB and cholecystitis, and CTSB and cholelithiasis.

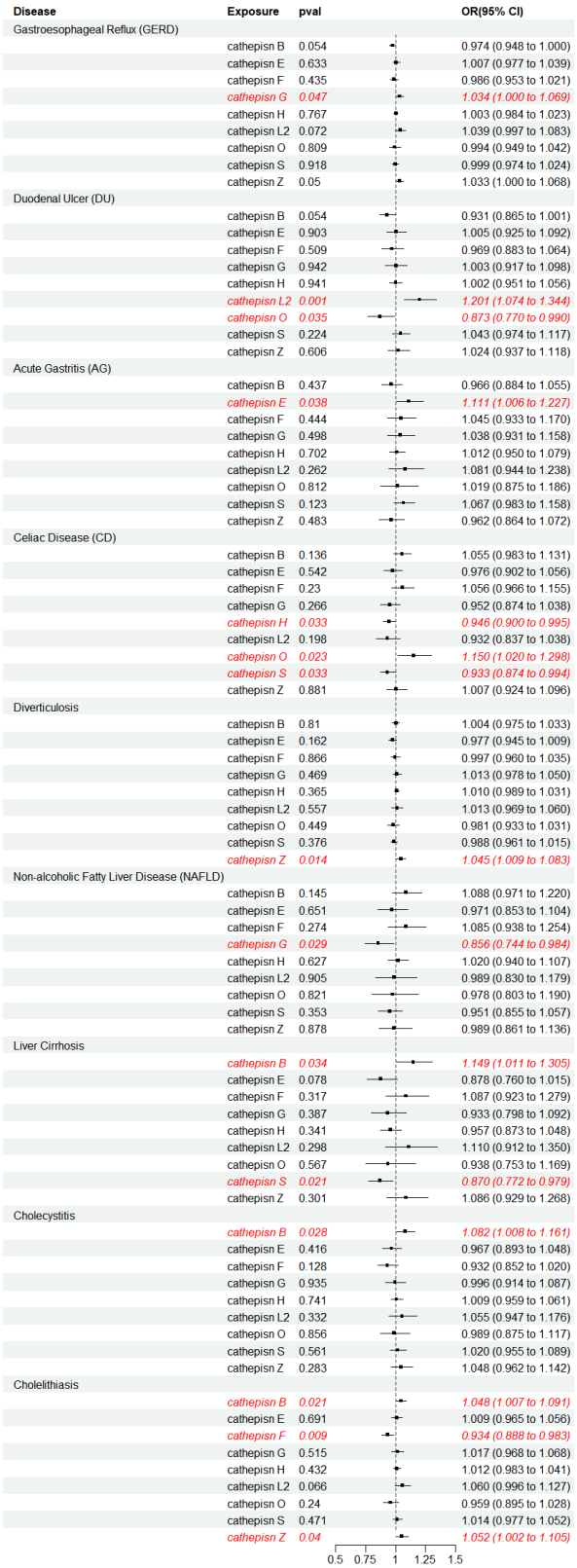

Figure 8. Positive result of Multivariable MR (MVMR) analysis.

3.3. Heterogeneity testing, outlier removal and sensitivity analysis

The Cochrane *Q* Heterogeneity Test was employed to detect potential heterogeneity among SNPs, defined as a *Q*-pval < 0.05. In cases where heterogeneity was detected, we adopted inverse variance weighting (using a random-effects model with multiplication) as a more robust analytical approach and replaced the original results (Supplementary **Table S2**). The MR-PRESSO global test and MR-PRESSO outlier test were employed to identify and adjust for potential outliers in our dataset, thus mitigating the impact of horizontal pleiotropy.

After identifying these outliers using MR-PRESSO, we excluded them and repeated our MR analysis to ensure the stability of our results, thereby mitigating the influence of any single SNP on our findings. In addition, the MR-Egger intercept test was used to identify signs of horizontal pleiotropic effects, which manifested as intercept values close to 0 ($P < 0.05$). The results showing pleiotropy were excluded from the positive conclusions (Supplementary **Tables S2** and **S3**). However, after the removal of outliers, there was heterogeneity ($P < 0.05$) in the MR analysis of the CTSZ levels and diverticulosis. Supplementary **Table S2**, indicating that the findings were not robust and may necessitate further experimental validation.

The robustness of the findings was underscored by the "leave-one-out" analysis, which showed that the results remained unchanged when any individual SNP was removed (Supplementary **Table S2**).

4. Discussion

This study revealed previously unrecognized associations between specific cathepsins and non-cancerous digestive diseases, providing new insights into their roles in pathophysiology and disease progression.

Using 2-sample bidirectional MR analysis, we identified specific cathepsins as potential causal factors in these diseases. We established seven pairs of causal relationships between cathepsins and diseases, four of which remained independently causal after controlling for other cathepsins. Specifically, CTSZ emerged as an independent risk factor for diverticulosis, CTSB for cholecystitis and cholelithiasis, and CTSG as a protective factor against NAFLD. These findings broaden our understanding of the complex roles that cathepsins play in non-cancerous digestive diseases, and point to new avenues for further exploration.

To further our discussion on each positive outcome in the forward MR study:

The study revealed a positive association between CTSZ and diverticulosis, and that CTSB was associated with an increased risk of cholecystitis and cholelithiasis. We hypothesize that in the gallbladder, cholesterol crystals can cause the destabilization of lysosomal membranes, leading to the leakage of CTSB from lysosomes into the cytoplasm. Once in the cytoplasm, CTSB activates the NLRP3 inflammasome. The activated NLRP3 inflammasome recruits and activates caspase-1 through the adaptor protein ASC (apoptosis-associated speck-like protein). The activated caspase-1 promotes the processing and secretion of IL-1β and IL-18. These cytokines are then released extracellularly, triggering an inflammatory response. This inflammatory response is likely to be involved in the development of cholecystitis and cholelithiasis [38–41]. To the best of our knowledge, the relationship between CTSZ

and diverticulosis has not been extensively documented in the current literature, underscoring the novelty of our findings and the potential for these associations to provide new insights for clinical studies.

We also observed a positive correlation between CTSZ and CG, aligning with previous findings by Teller et al., which reported a significant upregulation of CTSZ in CG patients infected with Helicobacter pylori, a condition that may contribute to further disease progression, potentially culminating in gastric cancer [42]. It is hypothesized that CTSZ does not participate in the degradation of the extracellular matrix. Instead [43], it is involved in the immune response induced by Helicobacter pylori, as well as subsequent inflammatory responses. In the gastric mucosa during the late stage of Helicobacter pylori infection, ribosomal protein P0 (RPLP0) and CTSZ are strongly colocalized and highly expressed, with distinct distribution selectivity in epithelial and inflammatory cells. Their interaction weakens G1-phase arrest, disrupts the cell apoptosis pathway, and promotes cell proliferation [42]. Among the cells mentioned above, H. pylori upregulates CTSZ in macrophages using the extracellular signal-regulated kinase 1/2 signaling pathway and in N87 cells via the JUN N-terminal Kinase pathway pathway [15]. Therefore, in the pathological process of chronic gastritis, CTSZ is not only involved in the immuno-inflammatory mechanisms but also related to cell apoptosis dysregulation. Overall, CTSZ emerges as a key factor in the intricate mechanism of chronic gastritis development induced by H. pylori.

Furthermore, we found that CTSL2 levels promote NAFLD. We hypothesize that CTSL2 might exert effects similar to cathepsin L (CTSL) in the context of NAFLD, potentially owing to their genetic proximity and functional similarities. Specifically, a positive association was observed between CTSL2 expression and NAFLD. While CTSL2 is predominantly expressed in the cornea, thymus, heart, brain, and skin [44], its relationship with liver-related diseases in humans has been less explored than that of CTSL, which has been proven to be linked to NAFLD [45]. We hypothesized that the similar functions of CTSL2 and CTSL in liver disease may be related to their highly adjacent gene loci on chromosomes, as well as their remarkably high homology of up to 78% [46]. Furthermore, it is noteworthy that despite the close relationship between human CTSL2 and human CTSL, mouse CTSL is the functional equivalent of human CTSL2, instead of human CTSL [44]. Thus, Manchanda M's mouse experiment may corroborate our findings, as it observed specific upregulation of CTSL in an induced liver fibrosis model, with diffuse and strong cytoplasmic staining of CTSL in hepatocytes and fibroblasts [47]. At present, there are relatively few papers on the direct report of the relationship between CTSL2 and NAFLD in humans, and the mechanism is not clear, and our findings need to be further explored and verified.

Interestingly, our results suggest that CTSG may play a protective role against NAFLD. In contrast, Toonen et al. observed no CTSG overexpression in NAFLD-affected mouse livers and noted that NAFLD mice with neutrophil elastase/CTSG double knockout showed improved metabolic profiles [48]. These findings suggest that CTSG is a potential risk factor rather than a protective factor, which is inconsistent with our results. This situation may be due to the fact that mice cannot fully mimic humans and it is difficult to simulate the in vivo physiological changes of humans as a whole. Perhaps it is necessary to pay attention to relevant clinical cases and conduct research. Traditionally, CTSG has been associated with intestinal inflammatory

processes, such as Crohn's disease [21] and UC [22]. However, recent findings indicate that it is also biosynthesized in non-inflamed intestinal mucosa, particularly within specialized epithelial cells [49]. This might explain the lack of a causal association between CTSG and intestinal inflammatory diseases in our study.

The primary strength of our study lies in the use of genetic markers as instrumental variables, which reduces confounding and reverse causality [50] and overcomes some of the limitations of RCTs. Genetic variants, which are inherited and unaffected by environmental factors [51], offer a more reliable proxy for exposure than traditional observational data. Moreover, while previous MR analyses were mainly occupied with cancerous digestive diseases, understanding non-cancerous digestive diseases is of utmost importance for public health. Our study rises to this challenge. This MR study has expanded the disease research scope allowing us to explore the relationships between cathepsins and a wider range of non-cancerous digestive diseases. It provides a more thorough understanding of the complex associations in this field. Besides, we conducted a MVMR analysis, which helps control the interference from other exposure factors when multiple exposure factors are incorporated simultaneously and more accurately assess the causal relationships between a single cathepsin and different diseases. However, there were several limitations to our study. First, the analysis mainly included individuals with European heritage, which limited the generalizability of the findings. Additionally, although we selected relatively reasonable instrumental variables based on the selection norms in existing literature, this does not completely rule out the impact of selection bias. Genetic data complexity and resource constraints may have led to the exclusion of potentially effective instrumental variables. If these overlooked variables are related to exposure and outcome variables, it could bias the estimated causal link between cathepsins and non-cancerous digestive diseases [27,52].

In addition, horizontal pleiotropy is another issue that cannot be ignored in MR studies. In this study, we adopted multiple methods to detect horizontal pleiotropy. When pleiotropy was detected, we re-ran MR analysis by excluding relevant SNPs. If pleiotropy persisted, we discarded the data, even if positive results were obtained. Although the methods we used attempted to correct the biases caused by pleiotropy to a certain extent, due to the complexity of the underlying mechanisms of horizontal pleiotropy, we were unable to completely eliminate its impact [29]. MR studies typically require larger sample sizes owing to their reduced statistical power [53]. Independent replication and experimental validation are essential for strengthening our findings.

5. Conclusion

In conclusion, our study sheds light on the potential roles of specific cathepsins in non-cancerous digestive diseases and identifies several novel associations that could inform future research:

Our research findings indicate that CTSB seemingly promotes the occurrence of both cholecystitis and cholelithiasis, which may be linked to the activation of the NLRP3 inflammasome. CTSZ has been observed to promote chronic gastritis and diverticulosis. The underlying mechanism is associated with immune-inflammatory

processes. Diverticulosis is a novel discovery, as there has been no previous report on it. Higher levels of CTSL2 have been found to promote NAFLD and liver cirrhosis. Although we can assume that, in terms of function, CTSL in mice is equivalent to CTSL2 in humans, thus finding some support for our conclusion. However, considering the differences between mice and humans, further research is required to demonstrate the direct causal effect of CTSL2 on NAFLD in humans. In addition, we have discovered that CTSG reduces the risk of NAFLD, which contradicts the existing literature on mouse models. This discrepancy may also be attributed to the differences between mice and humans, suggesting that targeted research in this area is essential. Overall, these results highlight the intricate relationships between genes and digestive diseases, as well as the significance of further investigation, especially taking into account the limitations of animal models in mimicking human physiological conditions.

The current two-sample Mendelian randomization study has revealed some potential associations. However, to further confirm the causal relationships, future experimental validations are indispensable. In vitro experiments, like culturing relevant cell lines to manipulate gene expressions, can offer initial insights into the underlying mechanisms. Meanwhile, in vivo animal models, such as genetically modified mice, will help us assess these associations in a more physiological context. These additional experimental studies are expected to strengthen the reliability of our findings and fill the gap between genetic evidence and biological reality.

Supplementary materials: Supplementary **Table S1**: Information of selected SNPs of cathepsins. Supplementary **Table S2**: The results of forward MR analysis between various cathepsins and digestive disorders. Supplementary **Table S3**: The results of reverse MR analysis between various cathepsins and digestive disorders.

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Data availability: The genetic summary statistics for the nine cathepsins analyzed in this study, derived from the INTERVAL study, are publicly available on the IEU OpenGWAS project website (https://www.ebi.ac.uk). Additionally, summary statistics for 23 non-cancerous digestive diseases were sourced from the FinnGen database, which can be accessed via https://www.finngen.fi/en. For data that required additional privacy protection, we adhered to all relevant data protection regulations and ensured participant anonymity. In compliance with these regulations, access to such data can be obtained by contacting project administrators.

Ethical approval: Not applicable.

Informed consent statement: Informed consent was obtained from all subjects involved in the study.

Conflict of interest: The authors declare no conflict of interest.

Abbreviations

AG	Acute gastritis	IVs	Instrumental variables
BE	Barrett's esophagus	IVW	Inverse variance weighting
CD	Celiac disease	MR	Mendelian randomization
CG	Chronic gastritis	NAFLD	Non-alcoholic fatty liver disease
CIs	Confidence intervals	NASH	Non-alcoholic steatohepatitis
CP	Chronic pancreatitis	OR	Odds ratios
CTS	Cathepsin	RCTs	Randomized controlled trials
DU	Duodenal ulcer	SNPs	Single-nucleotide polymorphisms
GERD	Gastroesophageal reflux	UC	Ulcerative colitis
GWAS	Genome-wide associated studies	WM	Weighted median

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