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Evaluation of causal links of gut microbiota and inflammatory cytokines with 10 fracture locations

A Mendelian randomization study

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Abstract

Recent investigations have revealed an association of variations in gut microbiota (GM) composition and inflammatory cytokine (IC) levels with fracture risk; however, the causal relationship of GM or inflammatory factors with fracture risk remains unelucidated. The study utilized Mendelian randomization (MR) analyses, utilizing aggregated data from the genome-wide association study of GM, ICs, and 10 fracture locations. The primary aim was to examine the causal associations between GM, ICs, and 10 fracture locations. Furthermore, mediational analyses and multivariate MR were conducted to explore the potential mediating role of ICs in this relationship. MR analysis identified 35 positive and 53 negative causal associations between GM and 10 fracture locations. ICs showed 22 positive and 24 negative correlations with 10 fracture locations. However, after false discovery rate correction, most associations lost significance, leaving only 1 IC significant for foot fractures. Moreover, our findings suggest that the ICs may be act as a mediating factor in the pathway from GM to 10 fracture locations. GM and ICs exhibited a significant causal relationship with the 10 fracture locations; furthermore, ICs may function as mediators in the pathway from GM to fracture risk.

Abbreviations: BMD = bone mineral density, CXCL10 levels = C-X-C motif chemokine ligand 10 levels, GM = gut microbiota, GWAS = genome-wide association study, ICs = inflammatory cytokine, IL-4 levels = interleukin-4 levels, IVW = inverse-variance weighting, MR = Mendelian randomization, MVMR = multivariate MR, OP = osteoporosis, OR = odds ratios, SCFAs = short-chain fatty acids, TRAIL levels = TNF-related apoptosis-inducing ligand levels, TSMR = two-sample MR.

Keywords: fracture risk, gut microbiota, inflammatory cytokines, mediation analysis, Mendelian randomization

1. Introduction

Fractures are a commonly occurring orthopedic injury that affects people worldwide, particularly the elderly populations.[1] Severe fractures may cause loss of physical function, reduce quality of life, pose a higher risk of rehospitalization, increase complication risk, and may even increase the likelihood of death.[2] As reported previously, approximately 3% of adults aged ≥ 50 years in the United States experience a fracture each year.[3] There has been an increase in the incidence of fracture occurrence worldwide because of the aging global population.[4] Fractures impose a huge economic burden on patient families, the healthcare system, and the overall society. Both environmental and genetic factors influence fracture occurrence.[5] Recent genetic studies have examined the genetic factors that influence bone mineral density (BMD). According to genome-wide association studies (GWAS), over 56 novel genome-wide significant loci associated with BMD as well as 14 loci related to fracture risk have been detected.[6] However, the complete genetic mechanism underlying fracture risk currently remains unknown.

Gut microbiota (GM), a diverse microbial community comprising bacteria, fungi, and viruses, inhabits the host's gastrointestinal tract.[7] As reported earlier, GM is involved in human health and disease development, including skeletal muscle health, obesity, and diabetes.[8] In the field of bone biology, the topic of GM-skeletal muscle interaction has received increasing attention, and GM has a crucial function in bone formation and development as well as bone metabolism by regulating multiple aspects of the host, including metabolism, endocrine system, immune status, and nutrient absorption. The interaction between GM and bone fracture risk may be

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The authors have no conflicts of interest to disclose.

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval was not required for this study that uses aggregate data from publicly available data sources.

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facilitated by the gut–bone axis, which involves various pathways such as immune system regulation, synthesis and catabolism of metabolic substances, and intestinal mucosal barrier maintenance.[9] Alterations in GM composition, including reduction in species diversity and increment in pathogen load, have been reported in fracture-related diseases. An imbalance in the gut flora may lead to osteoporosis (OP): a major risk factor for fracture development; this suggests the crucial role of GM in fracture occurrence.[10] Furthermore, abnormal levels of inflammatory cytokines (ICs) promote the development of OP and bone disease and exacerbate susceptibility to fracture development.[11] However, thus far, limited investigations have been conducted on the relationship of GM and ICs with fracture risk.

Mendelian randomization (MR) studies provide a novel approach to assess causality between exposures and outcomes by combining data collected from GWAS with genetic variations to generate exposure-related instrumental variables (IVs).[12] MR analysis decreases the likelihood of confounders, reverse causation, or other biases that affect the accuracy of results obtained in traditional observational studies.[13] MR studies are currently widely used for examining causal relationships between GM and diseases such as autoimmune diseases, metabolic disorders, and bone diseases.[14] Hence, in the current study, MR analysis was applied for determining causal relationships of GM and ICs with 10 fracture locations. Bidirectional MR and mediation analyses were carried out using GWAS data on human GM, ICs, and 10 fracture locations to examine the correlations between them. We expect that the study results could provide new perspectives for preventing and treating fractures.

2. Materials and methods

2.1. Study design

We investigated the genetic association of GM and ICs with 10 fracture locations by using publicly accessible GWAS data (Fig. 1). First, two-sample MR (TSMR) analysis was conducted to reveal bidirectional causality between GM, ICs, and 10 fracture locations. Subsequently, we used the multivariate MR (MVMR) method for quantitative analysis. We also explored whether ICs function as mediators in the association pathway between the GM and 10 fracture locations. We performed the MR analysis by following the STROBE-MR checklist. The study design is schematically represented in Figure 1. Single-nucleotide polymorphisms (SNPs) were defined as IVs. The following core assumptions were considered for the MR analysis: (1) IVs should exhibit an association with exposures; (2) no relationship should exist between IVs and confounders related to exposures and outcomes; and (3) IVs should affect the outcomes only through exposures.

2.2. Data source

In this study, GM abundance and ICs were defined as exposure factors, and the 10 fracture locations were defined as outcome factors. Summary statistics for GM abundance were extracted from a GWAS (Table S3, Supplemental Digital Content, https://links.lww.com/MD/O836); this study included 18,340 participants of mixed ancestry, and 85% of these participants were of European ancestry. The MiBioGen consortium served as a source for these data.[15] The 16S ribosomal RNA gene sequencing identified 211 bacterial taxa; of these taxa, we excluded 15 unclassified units, and the dataset finally included 196 bacterial taxa. Furthermore, 91 ICs were assessed in 11 cohorts containing 14,824 European participants by using GWAS data from the Olink Target Inflammation panel.[16] Fracture summary statistics from GWAS were sourced from the FinnGen Biobank (Round 10) (Table S3, Supplemental Digital Content, https://links.lww.com/MD/O836). In this study, the outcome variables were determined for the following 10 fracture locations in humans: fracture of foot, except ankle (383,369 controls and 8397 cases); fracture of lower leg, including ankle (352,617 controls and 22,027 cases); fracture of femur (394,217 controls and 9489 cases); fracture of lumbar spine and pelvis (398,057 controls and 6831 cases); fracture of rib(s), sternum and thoracic spine (395,649 controls and 9995 cases); fracture of shoulder and upper arm (376,853 controls and 12,920 cases); fracture of neck (404,306 controls and 1649 cases); fracture of skull and facial bones (358,857 controls and 7580 cases);

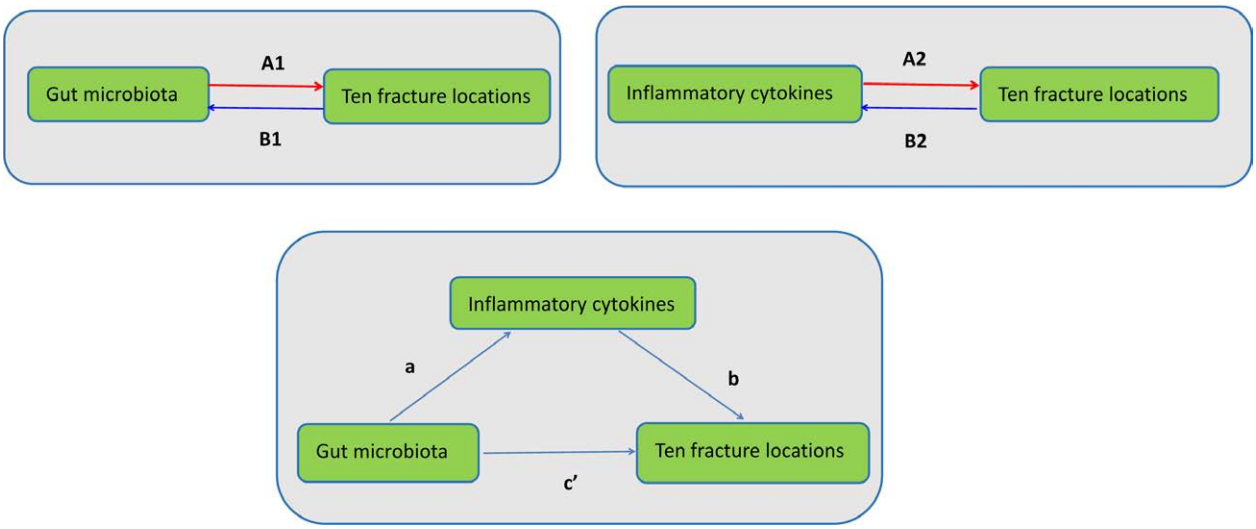

Figure 1. A flowchart presents the study design. A1 represents the causal effect of GM on 10 fracture locations. B1 represents the reverse causal effect of 10 fracture locations on GM. A2 represents the causal effect of ICs on 10 fracture locations; B2 represents the reverse causal effect between the 10 fracture locations and ICs. a, b, and c′ represent the mediation analysis of ICs in the path from GM to 10 fracture locations: c′ is the total effect of GM on 10 fracture locations; a represents the causal effect of GM on ICs; b represents the causal effect of ICs on the 10 fracture locations. The mediating effect was calculated as the product of "a" and "b" (a×b), and the mediating proportion was calculated as the ratio of the mediating effect product to the total effect [(a×b)/c′]. GM = gut microbiota, ICs = inflammatory cytokines.

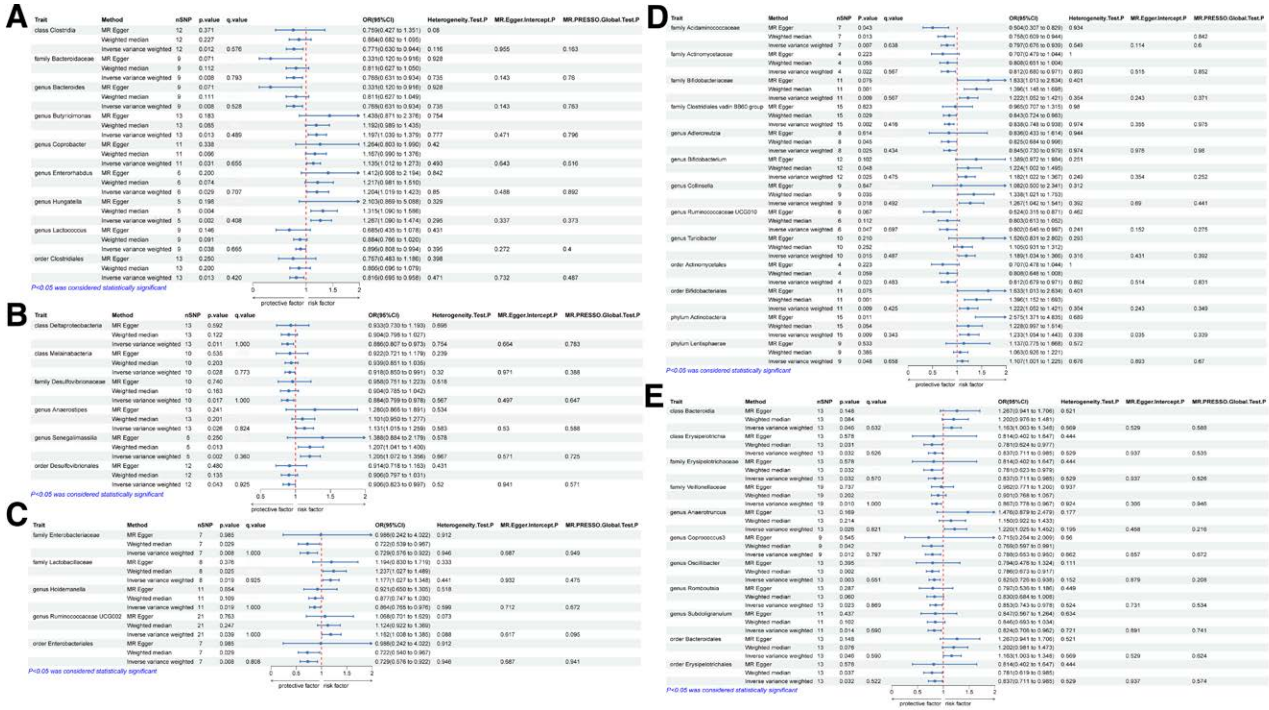

Figure 2. MR estimates for the association between GM and 10 fracture locations (A). GM = gut microbiota, ICs = inflammatory cytokines, IVW = inverse-variance weighting, MR = Mendelian randomization.

fracture of forearm (383,405 controls and 21,495 cases); and fracture at wrist and hand level (366,724 controls and 12,701 cases).

2.3. IVs selection and data harmonization

We used screening criteria to obtain the appropriate genetic factors. Initially, we implemented a strict significance threshold ($P < 5 \times 10^{-8}$) for selecting IVs linked with GM and ICs. However, because of very low number of appropriate IVs, according to previous studies, we then used mild statistical thresholds ($P < 1 \times 10^{-5}$ and $P < 5 \times 10^{-6}$) to screen for IVs associated with GM and ICs, respectively. Subsequently, a linkage disequilibrium approach ($r^2 = 0.001$, kb = 10000) was used to adhere to MR assumptions, and palindromic SNPs were ruled out to minimize the potential effect of alleles on causal outcomes. The F statistic was then calculated to confirm the robustness of the IVs through the following formula: $F = \text{beta}^2/\text{SE}^2$,[17] where F < 10 indicates avoidance of bias from weak instruments in MR studies.

2.4. Statistical analyses

First, we performed comprehensive bidirectional TSMR analyses to assess the bidirectional causality between GM, ICs, and the 10 fracture locations. Inverse-variance weighting (IVW) was employed as the primary method, together with weighted median and MR-Egger as supporting methods. All results are expressed as odds ratios (OR) and 95% confidence interval values (95% CI). We considered differences to be significant at 2 levels: (1) $P < .05$ for IVW method, and (2) the estimation directions of these 3 methods, IVW, MR-Egger, and weighted median, were consistent. The false discovery rate (FDR) correction was implemented using the q-value procedure, where a FDR q < 0.1 was deemed statistically significant, while values of $P < .05$ but q ≧ 0.1 were considered to indicate a suggestive association. Second, the MVMR analysis was performed for determining how ICs mediate the relationship between GM and the 10 fracture locations. We used various methods to confirm the robustness and reliability of the study. To determine potential heterogeneity, we assessed SNP-related heterogeneity by Cochran *Q* test; significant heterogeneity was represented by $P < .05$. If heterogeneity was present, the random-effects IVW approach was used. Potential horizontal multidirectionality effects were analyzed by the MR-Egger regression intercept and the MR-PRESSO global test, with $P < .05$ as the significance threshold; the presence of horizontal multidirectionality was indicated by a significant result. The statistical analyses were conducted utilizing the R (v4.3.2) software. MR analysis was executed with the R-based package "TwoSampleMR," while multiplicity tests were conducted using the "MR_PRESSO" package.

3. Results

3.1. Selection of IVs

The genetic association data for 196 GM and 91 ICs are summarized in Tables S1 and S2, Supplemental Digital Content, https://links.lww.com/MD/O836. The F-statistics for all SNPs are > 10, suggesting that the selected SNPs have sufficiently strong effects as IVs and unlikely have weak instrument bias.

3.2. Associations of GM, ICs, and the 10 fracture locations

Eleven bacterial taxa were associated with fracture of foot, except ankle. Specifically, 4 bacterial genera remarkably increased the risk of this fracture type (Fig. 2A), including *Butyricimonas*, *Coprobacter*, *Enterorhabdus*, and *Hungatella* (OR = 1.197, 1.135, 1.204, and 1.267, respectively; 95% CI values = [1.039–1.379], [1.012–1.273], [1.019–1.423], and [1.090–1.474], respectively; *P* values = .013, .031, .029, and .002, respectively). Additionally, the following taxa remarkably reduced the risk fracture of foot, except ankle: class Clostridia, family Bacteroidaceae, genus *Bacteroides*, genus *Lactococcus*, and order Clostridiales (OR = 0.771,

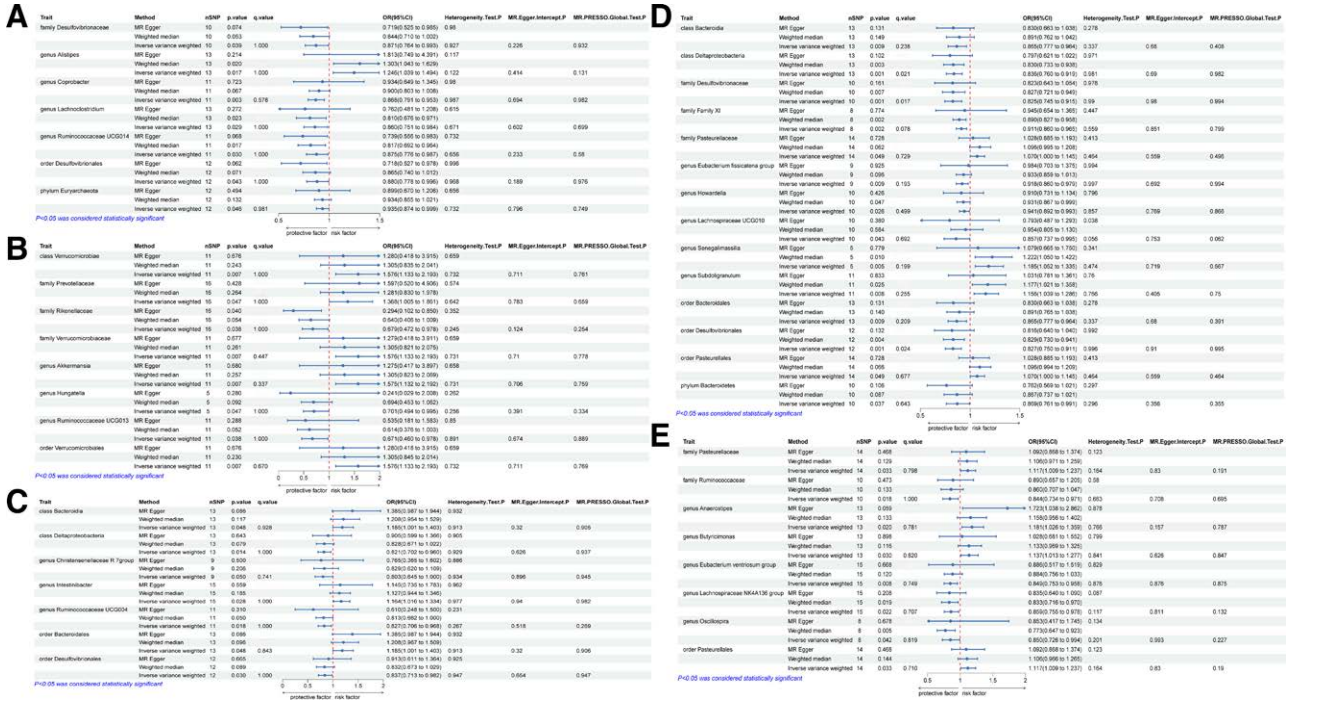

Figure 3. MR estimates for the association between ICs and 10 fracture locations (A). ICs = inflammatory cytokines, IVW = inverse-variance weighting, MR = Mendelian randomization, nSNP = number of single-nucleotide polymorphisms.

0.768, 0.768, 0.896, and 0.816, respectively; 95% CI values = [0.630–0.944], [0.631–0.934], [0.631–0.934], [0.808–0.994], and [-0.695 to 0.958], respectively; *P* values = .012, .008, .008, .038, and .013, respectively). However, none of these associations were consistent with FDR correction (q > 0.1). For genetically proxies calculating ICs, 6 ICs were associated with this fracture of foot, except ankle (Fig. 3A). The levels of T-cell surface glycoprotein CD5, C-X-C motif chemokine ligand 10 (CXCL10), TNF-related apoptosis-inducing ligand (TRAIL), and urokinase-type plasminogen activator were correlated positively with foot, except ankle, fracture and significantly increased the risk of this fracture type (OR = 1.117, 1.239, 1.075, and 1.109, respectively; 95% CI values = [1.018–1.226], [1.124–1.366], [1.013–1.140], and [1.017–1.210], respectively; *P* values = .019, .001, .018, and .020, respectively). In contrast, the levels of C-C motif chemokine 23 and Fms-related tyrosine kinase 3 ligand were correlated negatively with this fracture type and significantly reduced its risk (OR = 0.903 and 0.923, respectively; 95% CI values = [0.843–0.969] and [0.854–0.998], respectively; *P* values = .004 and .044, respectively). However, following FDR correction, only the correlation of CXCL10 levels remained significant (q < 0.1).

The IVW analysis found a correlation of 6 bacterial genera with the risk of fracture of lower leg, including ankle (Fig. 2B). Specifically, the genera *Anaerostipes* and *Senegalimassilia* were correlated positively with this fracture type and significantly increased its risk (OR = 1.131 and 1.205, respectively; 95% CI values = [1.015–1.259] and [1.072–1.356], respectively; *P* values = .026 and .002, respectively). Furthermore, the presence of the following taxa significantly reduced the risk of lower leg, including ankle, fracture: class Deltaproteobacteria, class Melainabacteria, family Desulfovibrionaceae, and order Desulfovibrionales (OR = 0.886, 0.918, 0.884, and 0.906, respectively; 95% CI values = [0.807–0.973], [0.850–0.991], [0.799–0.978], and [0.823–0.997], respectively; *P* values = .011, .028, .017, and .043, respectively). FDR correction, however, ceased to make these associations statistically significant (q > 0.1). The IVW analysis results showed that 4 ICs were associated with this fracture type (Fig. 3B). Among these ICs, interleukin-8 levels (OR = 1.097, 95% CI value = [1.008–1.193], *P* value = .032) showed a significant positive causal association with this fracture type and remarkably increased its risk. The levels of hepatocyte growth factor, interleukin-2, and interleukin-24 significantly decreased the risk of this fracture type (OR = 0.894, 0.854, and 0.892, respectively; 95% CI values = [0.816–0.978], [0.792–0.921], and [0.801–0.993], respectively; *P* values = .015, .001, and .036, respectively). However, after applying the FDR correction, it was found that only the correlation of Interleukin-2 levels remained statistically significant (q < 0.1).

We identified 5 GM taxa associated with fracture of lumbar spine and pelvis (Fig. 2C). Specifically, family Lactobacillaceae and genus *Sellimonas* exhibited a link with the risk for this fracture type (OR = 1.177 and 1.182, respectively; 95% CI values = [1.027–1.348] and [1.008–1.385], respectively; *P* values = .019 and .039, respectively). In contrast, family Enterobacteriaceae, genus *Holdemanella*, and order Enterobacteriales were protective factors for the risk of this fracture type (OR = 0.729, 0.864, and 0.729, respectively; 95% CI values = [0.576–0.922], [0.765–0.976], and [0.576–0.922], respectively; *P* values = .008, .019, and .008, respectively). These associations were not significant after FDR correction (q > 0.1). Four ICs were significantly associated with fracture of the lumbar spine and pelvis (Fig. 3C). Among these ICs, the levels of CUB domain-containing protein 1, CXCL10, and monocyte chemoattractant protein-4 were the risk factors for fracture of the lumbar spine and pelvis (OR = 1.082, 1.148, and 1.143, respectively; 95% CI values = [1.003–1.167], [1.003–1.314], and [1.021–1.280], respectively; *P* values = .041, .045, and .020, respectively). However, artemin level was a protective factor for this fracture type (OR = 0.871, 95% CI value = [0.767–0.990], *P* value = .034). Similarly, these associations lost statistical significance after applying the FDR correction (q > 0.1).

TSMR analyses revealed a causal relationship of 13 GM taxa with fracture of femur (Fig. 2D). Among these taxa, family Bifidobacteriaceae, genus *Bifidobacterium*, genus *Collinsella*, genus *Turicibacter*, order Bifidobacteriales, phylum

Actinobacteria, and phylum Lentisphaerae significantly increased the risk of femur fracture (OR = 1.222, 1.182, 1.267, 1.189, 1.222, 1.233, and 1.107, respectively; 95% CI values = [1.052–1.421], [1.022–1.367], [1.042–1.541], [1.034–1.366], [1.052–1.421], [1.054–1.443], and [1.001–1.225], respectively; *P* values = .009, .025, .018, .015, .009, .009, and .048, respectively). In contrast, the following taxa significantly reduced the risk of this fracture type: family Acidaminococcaceae, family Actinomycetaceae, family Clostridiales vadin BB60 group, genus *Adlercreutzia*, genus Ruminococcaceae UCG-010, and order Actinomycetales (OR = 0.797, 0.812, 0.838, 0.845, 0.802, and 0.812, respectively; 95% CI values = [0.676–0.939], [0.680–0.971], [0.748–0.938], [0.730–0.979], [0.646–0.997], and [0.679–0.971], respectively; *P* values = .007, .022, .002, .025, .047, and .023, respectively). When the FDR correction is applied (q > 0.1), these associations cease to be statistically significant. Six ICs were associated with femur fracture (Fig. 3D). Specifically, the levels of sulfotransferase 1A1 (OR = 1.090, 95% CI value = 1.012–1.174, *P* value = .022) exhibited a positive association with femur fracture and remarkably increased the risk of this fracture type. In contrast, the levels of axin-1, C-C motif chemokine 4, interleukin-2, monocyte chemoattractant protein-3, and TNF-related activation-induced cytokine significantly attenuated the risk of femur fracture (OR = 0.842, 0.903, 0.888, 0.889, and 0.925, respectively; 95% CI values = [0.715–0.993], [0.856–0.952], [0.791–0.996], [0.811–0.974], and [0.862–0.992], respectively; *P* values = .041, .001, .042, .012, and .029, respectively). Nevertheless, after FDR correction, only the correlation of C-C motif chemokine 4 levels remained significant (q < 0.1).

Eleven bacterial taxa were associated with fracture of the rib(s), sternum, and thoracic spine (Fig. 2E). Among these taxa, the following taxa were the risk factors for this fracture type: class Bacteroidia, genus *Anaerotruncus*, and order Bacteroidales (OR = 1.163, 1.220, 1.163, respectively; 95% CI values = [1.003–1.348], [1.025–1.452], [1.003–1.348], respectively; *P* = .046, .026, .046, respectively. The class Erysipelotrichia, family Erysipelotrichaceae, family Veillonellaceae, genus *Coprococcus*, genus *Oscillibacter*, genus *Romboutsia*, genus *Subdoligranulum*, and order Erysipelotrichales were protective factors for the risk of this fracture type. (OR = 0.837, 0.837, 0.867, 0.788, 0.825, 0.853, 0.824, and 0.837, respectively; 95% CI values = [0.778–0.967], [0.711–0.985], [0.711–0.985], [0.653–0.950], [0.726–0.938], [0.743–0.978], [0.706–0.960], and [0.711–0.985], respectively; *P* = .032, .032, .010, .012, .003, .023, .014, and .032, respectively. However, associations were not significant after FDR correction (q > 0.1). Three ICs were associated with this fracture type (Fig. 3E). Furthermore, the levels of CXCL10 (OR = 1.103, 95% CI value = [1.010–1.204], *P* value = .030) and glial cell line-derived neurotrophic factor levels (OR = 1.120, 95% CI value = [1.018–1.232], *P* value = .020) were risk factors for this fracture type. In contrast, delta and Notch-like epidermal growth factor-related receptor levels (OR = 0.889, 95% CI value = [0.802–0.986], *P* value = .026) were protective factors for fracture of the rib(s), sternum, and thoracic spine. In a similar way, these associations became statistically nonsignificant once the FDR correction was applied (q > 0.1).

The IVW analysis found a correlation of 7 bacterial taxa with fracture of the shoulder and upper arm (Fig. 4A). Specifically, genus *Alistipes* (OR = 1.246, 95% CI value = [1.039–1.494], *P* value = .017) was a risk factor for this fracture type. Moreover, the following taxa were protective factors for this fracture type: family Desulfovibrionaceae, genus *Coprobacter*, genus *Lachnoclostridium*, genus Ruminococcaceae UCG-014, order Desulfovibrionales, and phylum Euryarchaeota (OR = 0.886, 0.868, 0.860, 0.875, 0.880, and 0.935, respectively; 95% CI values: [0.807–0.973], [0.791–0.953], [0.751–0.984], [0.776–0.987], [0.778–0.996], and [0.874–0.999], respectively; *P* values = .039, .003, .029, .030, .043, and .046, respectively). However, these associations ceased to be statistically significant following the application of the FDR correction (q > 0.1). The IVW analysis results showed that 7 ICs were associated with fracture of the shoulder and upper arm (Fig. 5B). Among these ICs, the levels of eotaxin, C-C motif chemokine 23, and C-X-C motif chemokine 9 were protective factors for this fracture type (OR = 1.092, 1.079, and 1.099, respectively; 95% CI values = [1.008–1.182], [1.016–1.147], and [1.014–1.192], respectively; *P* values = .031, .014, and .021, respectively). The levels of fibroblast growth factor 19, monocyte chemoattractant protein-4, interleukin-6, and stem cell factor were protective factors for this fracture type (OR = 0.909, 0.882, 0.911, and 0.934, respectively; 95% CI values = [0.840–0.984], [0.778–0.999], [0.858–0.967], and [0.883–0.986], respectively; *P* values = .019, .048, .002, and .014, respectively). In a similar way, these associations became statistically nonsignificant once the FDR correction was applied (q > 0.1).

TSMR analyses showed a causal relationship of 8 GM taxa with fracture of neck (Fig. 4B). Among these, the following taxa were risk factors for neck fracture: class Verrucomicrobiae, family Prevotellaceae, family Verrucomicrobiaceae, genus *Akkermansia*, and order Verrucomicrobiales (OR = 1.576, 1.368, 1.576, 1.575, and 1.576, respectively; 95% CI values = [1.133–2.193], [1.005–1.861], [1.133–2.193], [1.132–2.192], and [1.133–2.193], respectively; *P* values = .007, .047, .007, .007, and .007, respectively). In contrast, family Rikenellaceae, genus *Hungatella*, and genus Ruminococcaceae UCG-013 were protective factors for neck fracture (OR = 0.679, 0.701, and 0.671, respectively; 95% CI values = [0.472–0.978], [0.494–0.995], and [0.460–0.978], respectively; *P* values = .038, .047, and .038, respectively). In a similar way, these associations became statistically nonsignificant once the FDR correction was applied (q > 0.1). Furthermore, 4 ICs were associated

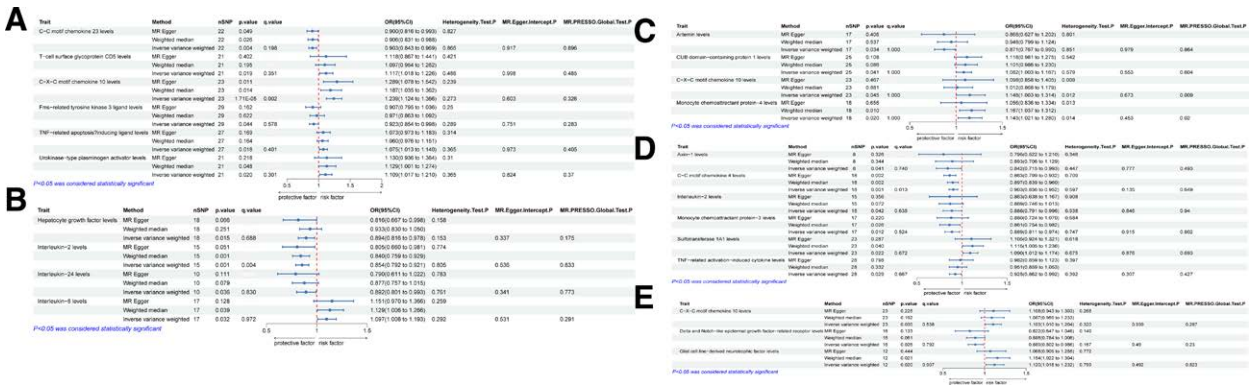

Figure 4. MR estimates for the association between GM and 10 fracture locations (B). GM = gut microbiota, IVW = inverse-variance weighting, MR = Mendelian randomization, nSNP = number of single-nucleotide polymorphisms.

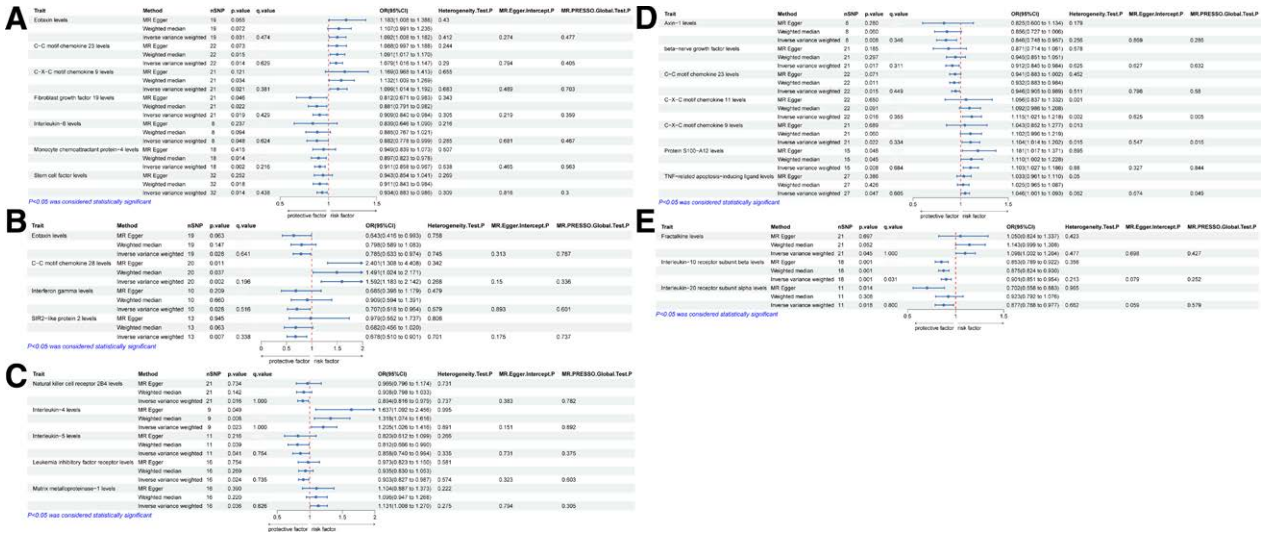

Figure 5. MR estimates for the association between ICs and 10 fracture locations (B). ICs = inflammatory cytokines, IVW = inverse-variance weighting, MR = Mendelian randomization, nSNP = number of single-nucleotide polymorphisms.

with fracture of neck (Fig. 5B). C-C motif chemokine 28 levels (OR = 1.592, 95% CI value = [1.183–2.142], *P* value = .022) were a risk factor for neck fracture. The levels of eotaxin, interferon-gamma, and SIR2-like protein 2 were protective factors for neck fracture (OR = 0.785, 0.707, and 0.678, respectively; 95% CI values = [0.633–0.974], [0.518–0.964], and [0.510–0.901], respectively; *P* values = .028, .028, and .007, respectively). The statistical significance of these associations was also lost after applying the FDR correction (q > 0.1).

Seven GM taxa associated with fracture of skull and facial bones (Fig. 4C). Class Bacteroidia, genus *Intestinibacter*, and order Bacteroidales were risk factors for fracture of the skull and facial bones (OR = 1.185, 1.164, and 1.185, respectively; 95% CI values = [1.001–1.403], [1.027–1.348], and [1.001–1.403], respectively; *P* = .048, .028, and .048, respectively). Furthermore, the following taxa were protective factors for this fracture type: class Deltaproteobacteria, genus Christensenellaceae *R*-7 group, genus Ruminococcaceae UCG-004, and order Desulfovibrionales (OR = 0.821, 0.803, 0.827, and 0.837, respectively; 95% CI values = [0.702–0.960], [0.645 to 1.000], [0.706–0.968], and [0.713–0.982], respectively; *P* = .014, .050, .018, and .030, respectively. These associations were not significant after FDR correction (q > 0.1). Five ICs were significantly associated with this fracture type (Fig. 5C). Among these ICs, the levels of interleukin-4 (IL-4) and matrix metalloproteinase-1 were risk factors for this fracture type (OR = 1.205 and 1.131, respectively; 95% CI values = [1.026–1.416] and [1.008–1.270], respectively; *P* values = .023 and .036, respectively). However, the levels of natural killer cell receptor 2B4, interleukin-5, and leukemia inhibitory factor receptor were protective factors for this fracture type (OR = 0.894, 0.858, and 0.903, respectively; 95% CI values = [0.816–0.979], [0.740–0.994], and [0.827–0.987], respectively; *P* values = .016, .041, and .024, respectively). Similarly, these associations lost statistical significance after applying the FDR correction (q > 0.1).

A significant association was observed between 14 GM taxa and fracture of forearm (Fig. 4D). Four of these taxa showed a positive association with this fracture type, including family Pasteurellaceae, genus *Senegalimassilia*, genus *Subdoligranulum*, and order Pasteurellales (OR = 1.070, 1.185, 1.156, and 1.070, respectively; 95% CI values = [1.000–1.145], [1.052–1.335], [1.039–1.286], and [1.000–1.145], respectively; *P* = .049, .005, .008, and .049, respectively). Moreover, the following taxa were negatively correlated with this fracture type: class Bacteroidia, class Deltaproteobacteria, family Desulfovibrionaceae, family Family XI, genus *Eubacterium fissicatena* group, genus *Howardella*, genus Lachnospiraceae UCG-010, order Bacteroidales, order Desulfovibrionales, and phylum Bacteroidetes (OR = 0.865, 0.836, 0.825, 0.911, 0.865, 0.941, 0.857, 0.865, 0.827, and 0.869, respectively; 95% CI values = [0.777–0.964], [0.760–0.919], [0.745–0.915], [0.860–0.965], [0.777–0.964], [0.892–0.993], [0.737–0.995], [0.777–0.964], [0.750–0.911], and [0.761–0.991], respectively; *P* values = .009, .001, .001, .002, .009, .026, .043, .009, .001, and .037, respectively). Following FDR correction, the associations between class Deltaproteobacteria, family Desulfovibrionaceae, family Family XI, order Desulfovibrionales, and fracture of the forearm remained statistically significant (q < 0.1). Additionally, 7 ICs exhibited a significant relationship with this fracture type (Fig. 5D). Among these ICs, the levels of C-X-C motif chemokine 11, C-X-C motif chemokine 9, protein S100-A12, and TRAIL were correlated positively with this fracture type (OR = 1.115, 1.104, 1.103, and 1.046, respectively; 95% CI values = [1.021–1.218], [1.014–1.202], [1.027–1.186], and [1.001–1.093], respectively; *P* values = .016, .022, .008, and .047, respectively). In contrast, the levels of Axin-1, beta-nerve growth factor, and C-C motif chemokine 23 were correlated negatively with this fracture type (OR = 0.846, 0.912, and 0.946, respectively; 95% CI values = [0.748–0.957], [0.845–0.984], and [0.905–0.989], respectively; *P* values = .008, .017, and .015, respectively). Nevertheless, these associations lost statistical significance after applying the FDR correction (q > 0.1).

Eight bacterial taxa exhibited an association with fracture of wrist and hand (Fig. 4E). Among these taxa, family Pasteurellaceae, genus *Anaerostipes*, genus *Butyricimonas*, and order Pasteurellales were risk factors for this fracture type (ORs = 1.117, 1.181, 1.137, and 1.117, respectively; 95% CI values = [1.009–1.237], [1.026–1.359], [1.013–1.277], and [1.009–1.237], respectively; *P* values = .033, .020, .030, and .033, respectively). Additionally, family Ruminococcaceae, genus *Eubacterium ventriosum* group, genus Lachnospiraceae NK4A136 group, and genus *Oscillospira* were risk factors for this fracture type (OR = 0.844, 0.849, 0.859, and 0.850, respectively; 95% CI values = [0.734–0.971], [0.753–0.958], [0.755–0.978], and [0.726–0.994], respectively; *P* values = .018, .008, .022, and .042, respectively). The observed associations did not reach statistical significance following FDR correction (q > 0.1). Three ICs were associated with this

fracture type (Fig. 5E). Specifically, the levels of fractalkine (OR = 1.098, 95% CI value = [1.002–1.204], *P* value = .045) were a risk factor for this fracture type. In contrast, the levels of interleukin-10 receptor subunit beta and interleukin-20 receptor subunit alpha were protective factors for this fracture type (OR = 0.901 and 0.877, respectively; 95% CI values = [0.851–0.954] and [0.788–0.977], respectively; *P* values = .001 and .018, respectively). In a similar way, these associations became statistically nonsignificant once the FDR correction was applied (q > 0.1).

3.3. Reverse MR analysis and sensitivity analysis

In the opposite pathway, as shown in Tables S4 to S23, Supplemental Digital Content, https://links.lww.com/MD/O836, we also found reverse causality between 10 fracture locations and a variety of GM and ICs. As shown in the graphs (Figs. 2–5), the results for IVW analyses (*P* > .05) and MR-Egger analyses (*P* > .05) showed no heterogeneity; moreover, the random-effects IVW method was performed for any observed heterogeneity. Additionally, multiple effects were not observed in the MR-Egger regression intercept test (*P* > .05) and the MR-PRESSO global test (*P* > .05).

3.4. Mediation analysis of potential ICs

We utilized the MVMR method to assess specific GM and ICs that simultaneously exhibited a causal association with the 10 fracture locations. Some of the results remained significant even after correction for the GM (Table 1). Specifically, TRAIL levels mediated the association of genus *Coprobacter* with foot, except ankle, fracture, with a mediating effect of 3.937% of the total effect. CXCL10 levels mediated the association of genus Lactococcus with foot, except ankle, fracture, with a mediating effect of 15.455% of the total effect. Monocyte chemoattractant protein-3 levels mediated the association of genus Turicibacter with Fracture of femur, with a mediating effect of 10.983 of the total effect. Furthermore, IL-4 levels mediated the causal relationship between class Bacteroidia and fracture of the skull and facial bones, with a mediating effect of 10.588% of the total effect. IL-4 levels also mediated the causal relationship of order Bacteroidales and fracture of the skull and facial bones, with the mediating effect accounting for 10.588% of the total effect.

4. Discussion

Our study is a pioneering approach to utilize MR for examining bidirectional causality between GM, ICs, and 10 fracture locations and to analyze the plausible mediating function of ICs between GM and 10 fracture locations. We found a causal relationship between a variety of GM, ICs, and 10 fracture locations. Furthermore, by using the MVMR method, we also noted that ICs functioned as mediators of the pathway from GM to fracture risk.

The gut microbiome can exert powerful regulatory effects on distant organs such as bone and brain. GM residing in the gastrointestinal tract comprises trillions of bacteria; it promotes intestinal permeability, reduces inflammation, and regulates skeletal immune responses, which is called the "gut–bone axis."[18] As reported by studies in the field of bone health and orthopedics, the gut–bone axis is crucial for maintaining bone mass.[19] Postprandial release of intestinal hormones, including glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1, may facilitate bone resorption at physiological concentrations, thereby contributing significantly to the gut–bone axis.[20] Microbiota dysbiosis may affect bone metabolism through the GM, making many metabolites, cytokines, and ICs transported to the bone marrow and affecting the overall inflammatory state of patients, which is a common manifestation of imbalance of the "gut–bone axis."[21] Both GM and the immune system are critically involved in sustaining bone homeostasis.[22] Recent studies have shown that intestinal homeostasis is maintained through a range of complex interactions of the host immune system with commensal microorganisms.[23] A study in mice found that segmented filamentous bacteria increase the production of IFN-γ and IL-17, which are involved in bone formation in vivo, and alleviate OP after ovariectomy,[24–26] preservation of skeletal muscle mass and function,[27] and skeletal disease prevention, thereby influencing fracture development.[28] Second, GM functions as a key component of the host immune system,[29] host metabolism, calcium absorption, and the endocrine system for regulating bone homeostasis, which subsequently affects fracture.[10]

Our study noted causal relationships of multiple GM taxa with 10 fracture locations. Family Bifidobacteriaceae and order Bifidobacteriales were risk factors for femur fracture. Family Lactobacillaceae was a risk factor for lumbar spine and pelvis fracture. Bifidobacteria positively regulates systemic leptin levels. This released leptin binds to leptin receptor expressed in brainstem neurons, thereby reducing the release of serotonin in the brain; this affects bone homeostasis by influencing the synthesis of peripheral and central serotonin and regulating the metabolism of tryptophan (a serotonin precursor).[23] Bifidobacteria may also affect bone health by altering body calcium levels. Bifidobacteria also promotes calcium absorption.[30] According to a double-blind, randomized controlled trial, *Lactobacillus reuteri* attenuates bone loss in older women with low BMD and promotes better bone formation in OP patients.[25] The current study suggests that reduction in Bacteroidales abundance may increase fractures, thus confirming that intestinal flora may influence fracture risk through pathways that affect BMD.[31] A Japanese cohort study involving 38 postmenopausal women found higher fracture risk in women with lower Bacteroidales abundance.[10] Our results confirmed these previous findings.

Table 1

Mediating effects of ICs on the causal pathway from GM to 10 fracture locations.

Exposure	Mediator	Outcome	Mediation effect, β (95% CI)	Total effect	Mediation proportion (%)
Genus Coprobacter	TRAIL levels	Fracture of foot, except ankle	0.005 (0.000–0.015)	0.127	3.937
Genus Lactococcus	CXCL10 levels	Fracture of foot, except ankle	-0.017 (-0.041 to 0.000)	-0.110	15.455
Genus Turicibacter	MCP-3 levels	Fracture of femur	0.019 (0.001–0.047)	0.173	10.983
Class Bacteroidia	IL-4 levels	Fracture of skull and facial bones	0.018 (0.001–0.045)	0.170	10.588
Order Bacteroidales	IL-4 levels	Fracture of skull and facial bones	0.018 (0.001–0.045)	0.170	10.588

The mediation proportion represents the ratio of the product of the mediation effect effects to total effects [(a*b/c)].

CXCL10 levels = C-X-C motif chemokine 10 levels, GM = gut microbiota, ICs = inflammatory cytokine, IL-4 levels = interleukin-4 levels, MCP-3 levels = monocyte chemoattractant protein-3 levels, TRAIL levels = TNF-related apoptosis-inducing ligand levels.

We find phylum Bacteroidetes and genus *Bacteroides* were protective factors for fracture of the forearm, and Ruminococcacea was a protective factor for fractures of the femur, shoulder, upper arm, neck, skull, facial bones, wrist, and hand. Recent studies have found that Bacteroidetes decompose lignocellulose into short-chain fatty acids (SCFAs).[32] SCFAs can maintain bone homeostasis, contribute to bone nutrient absorption, increase bone mass, and stimulate bone anabolism. A mouse study showed that SCFAs influence T regulatory cell development and promote bone metabolism through Wnt family member 10b.[33] SCFAs are also possible regulators of insulin secretion, leading to increased serum insulin-like growth factor 1 levels, which indirectly regulates anabolic stimulation of bone and increases bone density.[34] Furthermore, SCFAs have been reported to have anti-inflammatory properties, which can further support bone health by reducing inflammation-induced bone loss.[35] Genus *Bacteroides* synthesizes polyamines by metabolizing proteins.[36] A rat study revealed that an adequate amount of polyamines protects the trabecular structure of the tibia and that genus *Bacteroides* could indirectly participate in bone metabolism through polyamine synthesis.[37] In another study, the inclusion of Ruminococcacea in the microbiota of malnourished donors enhanced BMD and femoral cortical bone volume in recipient mice. A possible reason for this observation is that a nutrient-poor diet impairs bone growth in mice and leads to decreased bone length. Supplementation with Ruminococcacea improved bone growth in mice.[10] The findings of our study are in line with those of previous studies.

This study has several strengths. First, according to our knowledge, this study is the first investigation to examine causal relationships of human GM with 10 fracture locations in a comprehensive MR analysis and to consider the potential mediating role of ICs. Although previous studies have suggested that human GM has an association with fractures, the causal relationship remains unelucidated due to study design limitations and ethical issues. Second, we considered the potential mediating role of ICs in these causal associations by MVMR analysis. Moreover, in multiple comparisons, we used the FDR correction. However, there are also some limitations of this study. First, when the GWAS data were analyzed with the MR Approach, most participants were of European ancestry. This feature may to some extent limit the generalizability and applicability of the findings to other populations. Second, the SNPs associated with GM and ICs did not meet the traditional GWAS significant threshold ($P < 1 \times 10^{-8}$) because of small GM sample size. Third, the results of the MR study can only establish a causal relationship between exposures and outcomes; however, they do not allow to further investigate the biological mechanisms underlying the association of gut flora with fractures. Hence, more basic experimental and clinical studies are required to establish causal relationships between GM and fractures and to comprehend the long-term effects of GM interventions on fractures. Fourth, 196 GM and 91 ICs were tested at 10 fracture locations, and most associations lost significance after FDR correction, with only 1 IC retaining significance for foot fractures. Most of the associations await further investigation. Finally, the MVMR mediation analysis assumes that there are no unmeasured confounders between ICs and fractures, which has not been clearly tested and needs to be verified by further studies.

5. Conclusions

We comprehensively explored causal relationships of GM and ICs with 10 fracture locations. Our combined MR analysis identified 35 positive and 53 negative causal associations between genetic predisposition to GM and 10 fracture locations. ICs exhibited 22 positive and 27 negative associations with 10 fracture locations. We also found reverse causality between 10 fracture locations and a variety of GM and ICs. Furthermore, ICs were observed to function as mediators in the pathway from GM to fracture. Finally, we proposed innovative concepts to treat fractures based on GM- and ICs-based approaches.

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