Mendelian randomization: evaluation of causality between risk factors and outcomes

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GRAPHICAL ABSTRACT

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ABSTRACT
Mendelian randomization (MR) is a powerful approach that evaluates the causal association between a risk factor and an outcome. It makes use of the random allocation of genetic variants to mimic randomizers in randomized controlled trials (RCTs), providing quality evidence that is less susceptible to unmeasured confounding and reverse causality, when compared to conventional observational studies. Currently, MR has been applied in osteoporosis-related research to begin to unravel the causal risk factors that predispose to low bone mineral density (BMD) and increased susceptibility of fracture. Some MR studies made use of serum level measurement as a surrogate to mimic the role of supplementation, such as vitamin D and calcium, and evaluate the effects of the supplements in bone metabolism. From clinical perspective, MR studies enable identification of diagnostic markers and therapeutic targets. They provide evidence on the efficacy and adverse effects of drugs, contributing to discovery and repurposing of drugs.

Introduction
Mendelian randomization (MR) is an emerging and powerful approach to evaluate whether a risk factor is likely to be causally associated with an outcome. It employs genetic variants as instrument variables for the risk factor under investigation. The principle of MR is explained briefly below. At conception, individuals are randomly inherited with genetic variants. These genetic variants may be associated, or not associated with a particular risk factor. Individuals who are inherited with the risk-associated variants have life-long exposure to the risk factor, and they are followed up on the outcome.

For a MR study to be valid, three key assumptions must hold. Firstly, the genetic instruments are associated with the exposure under investigation. Secondly, the genetic instruments are not associated with any confounders of the exposure-outcome association. Thirdly, the genetic instruments affect the outcome only via the exposure under investigation. Violation of the third assumption is known as horizontal pleiotropy.

Due to the random assignment of genetic variants at conception, MR studies are less susceptible to bias arising from confounding and reverse causation when compared to conventional observational studies. In particular, reverse causality can be evaluated using bi-directional MR approach, which is infeasible in observational studies. With the use of summary statistics from genome-wide association studies (GWAS), MR enables testing of hypothesis that cannot be ethically and practically tested in randomized controlled trials (RCT), such as testing the causal effects of a harmful exposure (e.g. alcohol drinking) on an outcome. There is often a long lead time between the exposure and the outcome but RCT can only examine the effects of short-term intervention. Meanwhile, genetic variants adopted in MR studies represent life-long exposure. As long as GWAS data is available, MR studies can be conducted in an economical way in comparison with the expensive and resource-intensive RCT. Taking into
account its limitation (as detailed in later paragraph), MR approach is reported to be at the interface between traditional observational studies and RCT, with improved reliability in the presence of evidences obtained from different study designs².

The advances in genotyping technology have made GWAS of large sample size feasible at a lower cost, enhancing the availability of summary statistics from large-scale GWAS/GWAS meta-analysis and making the relevant MR studies possible. Using "Mendelian Randomization" AND ("bone mineral density" OR "fracture") as keyword search in PubMed, there are around 50 published MR studies in osteoporosis-related research as at March 2020. A sharp increase has been observed in the number of publications since 2017. In particular, MR approach has been employed to identify causal risk factors of osteoporosis-related traits, including bone mineral density (BMD) derived by dual X-ray absorptiometry (DXA) at various skeletal sites (such as total body, lumbar spine, femoral neck, etc), estimated BMD (eBMD) at the heel derived by ultrasound, as well as fracture. In this perspective, we will briefly discuss how MR approach has been applied in osteoporosis research.

**MR studies with BMD as outcome**

Majority of the osteoporosis-related MR studies examined BMD as the health outcome due to the availability of GWAS data, which are deposited on the website of the GEnetic Factors for OSteoporosis Consortium ([http://www.gefos.org/](http://www.gefos.org/)). Most of these studies aimed to determine the causal relationship between a risk factor and DXA-derived BMD, while some assessed the causality with eBMD as well. Researchers also explored the potential causal roles of circulating proteins⁴⁻⁷, metabolites⁸⁻⁹ and phospholipids¹⁰ in bone metabolism by utilizing their serum level measurement as exposures in MR studies. In case causality is confirmed, they may be developed as diagnostic markers or potential therapeutic targets. Conversely, MR studies may also provide insight on whether commonly used drugs or supplementations are beneficial to the health outcome as expected. Examples include the widespread use of vitamin D and calcium supplementations which are anticipated to improve bone health. The causality of serum vitamin D¹¹⁻¹³ and calcium¹⁴,¹⁵ levels on BMD were investigated using MR approach, although it is unclear if the life-long increase in serum level implied in the MR approach fully mimics the short-term increase in serum level due to supplementation. Consistently, three MR studies did not support the causal association of serum vitamin D with DXA-derived BMD at total body¹¹, femoral neck¹²,¹³, lumbar spine¹²,¹³, total hip¹³, as well as eBMD¹². This is partially in line with a meta-analysis of randomized trials that compared interventions differing in vitamin D supplementation only, which demonstrated the supplementation had a small beneficial effect on BMD at femoral neck, but not other skeletal sites at lumbar spine, total hip, trochanter, total body and forearm¹⁶. Both MR studies and clinical trials suggested the extensive use of vitamin D supplementation in the general healthy population may not have big impact on bone health¹²,¹⁶. Regarding

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calcium, one MR study revealed that genetic predisposition to increased serum level per se was inversely associated with TB-BMD in multivariable analyses adjusted for serum parathyroid hormone, vitamin D and phosphate\textsuperscript{14}. Meanwhile, another study could not provide evidence on the causal relationship of serum calcium with eBMD and fracture\textsuperscript{15}. Whereas, a meta-analysis of RCTs showed that increase in calcium intake from supplementation had non-progressive and small beneficial effect on BMD, with approximately 0.8-1.8\% increase in BMD with supplements at 1 to >2.5 years\textsuperscript{17}. Clinically, the key message brought out by the MR studies and RCT meta-analysis is that the calcium supplementations unlikely have significant impact on bone health\textsuperscript{15,17}. These examples showed how MR studies could provide complementary evidence to the gold standard RCT.

On the other hand, five studies investigated the levels of serum lipids [including low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides] as potential risk factors of DXA-derived BMD at total body (TB-BMD), lumbar spine, femoral neck, forearm and/or eBMD\textsuperscript{18-22}. In view of the close correlation between the lipid fractions, multivariable MR analyses should be performed to account for the confounding effects of other lipids and assess each lipid fraction’s independent role on BMD. Only two of five studies had performed multivariable MR analyses of lipids on BMD\textsuperscript{13,22}. Both these two studies consistently demonstrated that genetically predicted decrease in serum level of LDL-C was causally associated with increased TB-BMD\textsuperscript{21,22}, forearm BMD\textsuperscript{22} and eBMD\textsuperscript{21,22} after adjustment for beta estimates of HDL-C and triglycerides. While both studies did not support the presence of causal relationship between HDL-C and eBMD\textsuperscript{21,22}, null\textsuperscript{21} and inverse\textsuperscript{22} causal association was observed between HDL-C and TB-BMD in multivariable analyses adjusted for LDL-C and triglycerides\textsuperscript{22}. One study additionally revealed the inverse relationship between HDL-C and lumbar spine BMD\textsuperscript{22}. After adjustment for LDL-C and HDL-C, triglycerides was shown to have null and inverse causal association with eBMD and TB-BMD respectively in both studies\textsuperscript{21,22}.

The discrepant findings among studies can be explained by statistical power of the MR analyses, which mainly depends on the proportion of variance explained by the genetic instruments on the exposure, as well as the sample size of the outcome dataset. It is not uncommon that different studies used different definition to select independent genome-wide significant instruments from different GWAS datasets, resulting in different statistical power of MR studies although they are investigating on the same pair of exposure and outcome. A MR study utilizing a few genetic instruments could only explain a low proportion of variance on exposure. If the outcome dataset is of small sample size, the study may have low power in detecting genuine causality. Moreover, the discrepancy may also be explained by the intrinsic difference between the BMD phenotypes. DXA is the gold standard of BMD measurement and DXA-derived BMD is more relevant to the clinical diagnosis of osteoporosis. In particular, DXA-derived femoral neck

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BMD is a stronger predictor of hip fracture than eBMD\textsuperscript{23}. eBMD measurement does not include the most critical diagnostic skeletal sites at lumbar spine or femoral neck that are more prone to fracture. While DXA-derived BMD is a measurement of both trabecular and cortical bone, eBMD mainly measures the trabecular bone\textsuperscript{24}. Discordant results between eBMD and DXA-measured BMD are often observed\textsuperscript{25}. Notably, six reported eBMD-associated loci had opposite directions of effects when compared with GWAS of DXA-measured BMD\textsuperscript{24}. Only moderate correlation was observed between eBMD and DXA-measured BMD at sites prone to fracture (femoral neck and lumbar spine) (r=0.5-0.6)\textsuperscript{24}. DXA-derived BMD and eBMD (forearm BMD: -0.81; femoral neck BMD: -0.47; lumbar spine BMD: -0.36; TB-BMD: r=-0.24; eBMD: r=-0.47)\textsuperscript{24} have different strength of correlation with fracture. Although eBMD is a quick and relatively inexpensive estimate of BMD for a large number of study participants, findings related to eBMD require cautious interpretation.

Among the serum biomarkers tested by MR approach, some causal risk factors have potential to be therapeutic targets of osteoporosis. MR studies have confirmed the beneficial role of estradiol on BMD\textsuperscript{26,27}, while estrogen therapy has already been adopted to prevent bone loss in post-menopausal women for decades. In addition, significant positive causal association was observed for serum growth differentiation factor 15 (GDF15) level with eBMD\textsuperscript{4}. Whereas, genetical predisposition to increased serum sclerostin\textsuperscript{5} and leptin\textsuperscript{6} levels were causally linked to lower BMD at different skeletal sites. These findings might suggest that GDF15 treatment, reduction of sclerostin or leptin levels may have therapeutic potential in osteoporosis treatment. Notably, RCT has provided evidence that 12 months of treatment with romosozumab, a monoclonal antibody targeting sclerostin, could improve the hip areal BMD of patients by 2.6%, which has better bone-forming effects than teriparatide\textsuperscript{28}. However, further investigations are warranted to consider their possible adverse effects on other disease outcomes. For example, a MR study suggested that genetic predisposition to lower sclerostin level was causally associated with higher risk of cardiovascular events\textsuperscript{29}.

**MR studies with fracture as outcome**

Clinically, fracture is the most relevant and direct outcome of osteoporosis. Evidences were also presented by a MR analysis that genetically decreased femoral neck and lumbar spine BMD were causally linked to increased risk of fracture\textsuperscript{30}. However, relatively small number of MR studies examined the causal risk factors of fracture, probably because large-scale GWAS / GWAS meta-analysis of fracture were only available until 2018.

Only a few exposures were investigated for their potential causal roles on both BMD and fracture. In line with the null causal association between vitamin D and BMD suggested by three MR studies\textsuperscript{11-13}, genetic predisposition to increased vitamin D level was not causally linked to fracture risk\textsuperscript{30}. Among the two studies...
which adopted multivariable MR analyses to evaluate the causal relationship of lipids with both BMD and fracture, one study did not support LDL-C’s causal role on fracture, unlike the observed inverse causal association between LDL-C level and BMD\textsuperscript{21}. Another study revealed inverse causation of LDL-C and HDL-C level on DXA-derived BMD, while it showed suggestive evidence that genetically determined levels of LDL-C and HDL-C were positively associated with fracture risk\textsuperscript{22}. However, the weak results disabled conclusions on whether the lipids may affect fracture risk\textsuperscript{22}. The different causal effects of lipid levels on BMD and fracture may be explained by the multifactorial nature of fracture. In addition to BMD, a wide range of factors, such as muscle strength and risk of falls, may contribute to fracture. For example, hand grip strength may act as a proxy of muscle strength and motor responses, and it was reported that increased hand grip strength might causally reduce the risk of fracture\textsuperscript{30}. Future studies may explore taking these factors into account in evaluating the risk of fracture. Amid the potential therapeutic targets causally linked to BMD, only sclerostin showed consistent causal association with fracture: genetically determined increased sclerostin level was found to increase fracture risk\textsuperscript{5}. This finding is also in line with a RCT that individuals receiving subcutaneous injections of romosozumab for 12 months had lower risk of both vertebral and nonvertebral fracture\textsuperscript{31}.

**Limitations**

As MR studies can be easily performed with the use of publicly available platform\textsuperscript{32} and summary statistics from GWAS, there is a sharp increase in the number of MR studies. However, MR approach has several limitations that need to be carefully considered before valid findings can be obtained. These limitations were extensively discussed previously\textsuperscript{33} and the major ones were summarized below. Firstly, the genetic association between the instrument variables and exposure / outcome may not be accurately obtained due to genotyping errors, population stratification in the genetic association studies, etc. Secondly, there may be confounder(s) in the genotype – exposure – outcome association. For example, the exposure-associated genetic instruments may influence some behavioral factors (confounder, such as smoking) which in turn affect the outcome, or the exposure-associated variants may be in linkage disequilibrium with another risk locus of exposure that act on the same pathway. Thirdly, the genetic instruments may be associated with the outcome via pathways other than the exposure under investigation (horizontal pleiotropy). Fourthly, the exposure may not have suitable genetic instruments even if the instruments lie on loci involved in the disease process. For example, the exposure-associated genetic variant is not a common SNP. In view of the above, poor design of MR studies may lead to invalidation of the analysis and hence misinterpretation of the findings. Interested readers may refer to the “Guidelines for performing Mendelian Randomization investigations\textsuperscript{34}” for suggested procedures in performing a MR study.
**Future directions**

Currently, most of the MR studies were conducted in Europeans, mainly attributed to the availability of large-scale well-powered GWAS in the population, which would in turn identify strong genetic instruments that represent the exposure in MR studies. In future, when GWAS of sufficient sample size becomes available, it may be worthy to re-visit the MR studies in independent populations to explore if the causality differs among populations. In addition, new MR designs and methodologies with different assumptions and features are being developed and become available. These MR methodologies can be applied in osteoporosis-related research if the questions in mind could meet the criterion specified by the respective MR method.

As mentioned above, applications of MR include discovery of diagnostic markers and development of novel therapeutic targets. MR can be applied to predict adverse side-effects of drugs by using genetic proxies of drugs in the target gene as the instruments, and performing a phenome-wide MR scan. In fact, MR can also form basis for drug repurposing before costly and lengthy RCTs are performed. For example, genetic proxies mimicking the LDL-C-lowering effects of statin therapy were shown to increase TB-BMD and eBMD but have null effects on fracture risk. The finding was in line with a meta-analysis of RCTs that statin use was significantly associated with increased BMD while it had null association with fracture.

**Conclusion**

In conclusion, MR studies are not just applicable in the research field to identify causal risk factors and unravel the underlying mechanisms of health outcomes. From the clinical perspective, findings from MR studies may identify adverse side-effects of drugs, provide insight and enhance the process of drug discovery and repurposing before expensive and lengthy RCTs take place.
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