RECENT ADVANCES IN THE GENETICS OF OSTEOPOROSIS

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RECENT ADVANCES IN THE GENETICS OF OSTEOPOROSIS

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EDITORIAL
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Editorial: Recent Advances in the Genetics of Osteoporosis

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Editorial on the Research Topic

Recent Advances in the Genetics of Osteoporosis

INTRODUCTION

The last few years have seen considerable advances in our understanding of the genetic factors influencing osteoporosis, driven by a range of break-throughs. The seven papers comprising this Research Topic together provide a timely update, describing new insights into the genetic architecture of osteoporosis, application of genetic findings to study causal inference, and state-of-the-art approaches to functional genomics, paving the road for multi-omic applications.

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GENETIC ARCHITECTURE OF BONE MASS AND BONE FRAGILITY

The genetic architecture of osteoporosis comprises mutations affecting single genes responsible for rare monogenic causes of osteoporosis, and common genetic variants representing genetic susceptibility factors for osteoporosis in the wider population. Makitie et al. discuss how recent advances in genetic methodology have led to a rapid increase in identification of monogenic causes of osteoporosis and related conditions associated with low bone mass and/or increased bone fragility. Osteogenesis Imperfecta (OI), the most common monogenic cause of bone fragility, is due to a defect in bone extracellular matrix, with 85% of cases harboring a mutation in type I collagen. Many other genes are now recognized to cause an OI-like skeletal disorder. Some of these perturbate type I collagen function, such as cartilage-associated protein (*CRTAP*), while others may act by impairing bone mineralization, as proposed for Plastin 3 (*PLS3*). There has also been considerable interest in the discovery of mutations which impair osteoblast differentiation and function, not the least since these may also prove useful therapeutic targets for osteoporosis in the wider population. These include genes involved in WNT signaling, which has an important role in skeletal homeostasis, such as *WNT1*, and the WNT inhibitor frizzled-related protein 4 (*SFRP4*); mutations in both these genes being implicated in rare monogenic cases of osteoporosis.

The paper by Gregson and Duncan provides a comprehensive review of disorders associated with high bone mass (HBM). Even having excluded secondary causes such as degenerative changes, unexplained HBM is not uncommon (prevalence approximately 0.2%). Several very rare mutations underlying HBM have been described, which cause a generalized increase in bone mass as a result of

increased bone formation, and a reduction in bone fragility and fracture risk. These mainly arise from mutations leading to increased activation of the WNT/β-catenin signaling pathway. Sclerosteosis, van Buchem's disease, *LRP4* HBM, *LRP5* HBM, and *LRP6* HBM are all thought to involve this mechanism. Other signaling pathways may also contribute to HBM, as exemplified by HBM arising from a mutation in *SMAD9*, part of the TGF-β superfamily. Several monogenic HBM conditions are also recognized where increased bone mass arises from defective bone resorption, and is associated with increased bone fragility, such as osteopetrosis. In the great majority of HBM cases, no underlying monogenic disorder is evident. Although cases of unexplained HBM were found to be enriched for common high BMD alleles identified in GWASs, this does not exclude a role of rarer mutations yet to be discovered. Given the successful translation from identification of the genetic basis of sclerosteosis to new therapy in the case of Romosozumab (1, 2), further genetic dissection of HBM cases may provide additional benefits for human health.

In terms of polygenic disease risk in osteoporosis, the paper by Koromani et al. describes recent advances in our understanding of the genetic architecture of fracture risk, focusing on common variants as identified from genome wide association studies (GWAS). The article reviews genetic factors found to be associated with fracture risk, after comparing individuals with and without a history of fracture, as well as endophenotypes related to fracture risk including bone mineral density (BMD) measured by DXA scans, and BMD estimated from heel ultrasound (eBMD). To date, the majority of susceptibility loci for fracture have also been implicated in BMD. The authors anticipate that further progress will be helped by improved phenotyping. For example, whereas the great majority of GWAS fracture studies combine all fracture types, fractures at specific sites, like the spine, may have a specific genetic architecture. Progress may also be achieved in extending GWAS to endophenotypes other than BMD. A number of advanced imaging methods have been developed which are capable of ascertaining detailed components of bone structure, which may also have a specific genetic architecture, though a challenge is to assemble large enough study samples to conduct well powered GWASs.

STUDIES OF CAUSAL INFERENCE

The paper by Zheng et al. discusses the application of common susceptibility loci to examine causal inference using Mendelian Randomization (MR). It provides a number of examples where MR has been applied to examine risk factors for osteoporosis, using either BMD or fracture risk as the outcome. This paper also reviews the main limitations and assumptions involved in MR, such as horizontal pleiotropy, whereby the genetic instrument is related to the outcome *via* a separate pathway to the exposure. A number of methods are discussed to exclude pleiotropy, including the use of bi-directional analyses. If bi-directional causal effects are observed, these usually reflect pleiotropy, for example as a result of genetic correlation between the exposure and outcome due to shared genetic instruments resulting from common biological pathways. That said, instances of true bi-directional pathways may exist, such as a proposed positive effect of BMD on sclerostin, whereas sclerostin also exerts a negative effect on BMD, possibly as part of a negative feedback loop. As well as examining causal relationships for associations initially identified in conventional epidemiological studies, more recent applications of MR are also discussed, such as hypothesis-free approaches to examine causal effects of a given risk factor on a range of outcomes, and application of MR for target validation and drug discovery.

FUNCTIONAL GENOMICS STUDIES

Three articles in this Research Topic discuss recent advances in functional studies aiming to provide a basis for translation of human genetic studies into new treatments for osteoporosis. Maynard and Ackert-Bicknell discuss the availability of data from mouse models, as well as other online resources such as tissue expression panels and expression quantitative trait loci (eQTLs). The authors point out that over 500 susceptibility loci have been identified for osteoporosis, however these causative variants nearly all act to alter gene expression rather than representing the actual causative gene. Identifying the genes responsible for mediating the effects of genetic susceptibility loci on BMD is a prerequisite for identifying potential drug targets for new osteoporosis treatments, remains a major goal of functional genomic studies. Mouse models have proven enormously helpful in this regard, by providing a means of characterizing the function of candidate genes through studying the skeletal phenotype of mice where these have been deleted. The repertoire of mice with specific gene knockouts has increased massively over recent years, due to the work of the International Mouse Phenotyping Consortium (IMPC), which ultimately aims to making embryonic stem cells carrying a knockout allele for all protein coding genes. Mice generated from this program undergo phenotypic screens including limited assessment of skeletal phenotype, with more detailed skeletal phenotyping undertaken by BoneBase, and the Origins of Bone and Cartilage Disease (OBCD) projects, based in the US and UK respectively.

Bergen et al. review the emerging use of zebrafish as an animal model in functional follow up studies of osteoporosis. Zebrafish are vertebrates which show strong similarities in their skeletal physiology to mammals, and are highly suited to genetic studies since constructs which modify the genome can be directly injected into embryos at the single cell stage. Bone formed in zebrafish has the same skeletal cell types and modes of regulation as higher vertebrates, making them suitable for studying processes involved in osteoporosis, which can be carried out dynamically and imaged *in vivo*, for which a range of fluorescent reporter constructs are available. Several imaging methods have been applied to zebrafish which together enable highly detailed assessment of skeletal morphology. A number of zebrafish with

mutations in skeletally relevant genes have been studied which re-capitulate a range of skeletal disorders, including osteoporosis and OI. As well as helping to identify causative genes by evaluating the effect of their deletion on the skeleton using methods such as CRISPR/Cas9 editing, zebrafish can be applied to osteoporosis genetics research by providing high throughput assays for compounds which target these genes, based on harvesting and culture of osteoblasts from elasmoid scales.

The final article covers proceedings from a workshop held by the Royal Osteoporosis Society in the UK to review opportunities and challenges in functional genomics research in osteoporosis (Tobias et al.). One of the main conclusions from the workshop is that whereas many promising genetic signals have been identified for osteoporosis, to date it has only been possible to interrogate a small fraction of them in functional studies. Whereas financial and manpower resources remain an important limiting factor, functional studies in osteoporosis genetics will benefit from an expanding repertoire of on-line resources, such as the IFRMS knowledge portal which aims to bring together all relevant functional data (https://msk.hugeamp.org/). In addition, a number of multi-omic resources are available which, with the application of causal inference methods described above, can be applied to identify causative genes responsible for genetic association signals. The paper concludes that a roadmap of functional assessments needs to be established, aiming to integrate multi-omic resources with datasets from human genetics and animal models, in order to translate the wealth of genetic discoveries into new therapies for osteoporosis.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Zebrafish as an Emerging Model for Osteoporosis: A Primary Testing Platform for Screening New Osteo-Active Compounds

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Osteoporosis is metabolic bone disease caused by an altered balance between bone anabolism and catabolism. This dysregulated balance is responsible for fragile bones that fracture easily after minor falls. With an aging population, the incidence is rising and as yet pharmaceutical options to restore this imbalance is limited, especially stimulating osteoblast bone-building activity. Excitingly, output from large genetic studies on people with high bone mass (HBM) cases and genome wide association studies (GWAS) on the population, yielded new insights into pathways containing osteo-anabolic players that have potential for drug target development. However, a bottleneck in development of new treatments targeting these putative osteo-anabolic genes is the lack of animal models for rapid and affordable testing to generate functional data and that simultaneously can be used as a compound testing platform. Zebrafish, a small teleost fish, are increasingly used in functional genomics and drug screening assays which resulted in new treatments in the clinic for other diseases. In this review we outline the zebrafish as a powerful model for osteoporosis research to validate potential therapeutic candidates, describe the tools and assays that can be used to study bone homeostasis, and affordable (semi-)high-throughput compound testing.

Keywords: zebrafish, screening, genetic mutants, osteoblast, osteoclast, osteoporosis, drug development, animal model

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INTRODUCTION

Osteoporosis (OP) is a degenerative bone disease that affects around 27.6 million people over the age of 50 in the 27 European Union (EU27) countries alone (1). As average life expectancies increase, it is predicted that the annual cost of treating OP in the EU will rise from €37 billion in 2010 to €46.5 billion by 2025 (2). OP is characterized by a reduction in bone mineral density (BMD), reduction of bone mass (BM), and a decrease in the trabecular volume of long bones; resulting in brittle bones that are more prone to fracture (3). The underlying mechanism behind OP is a dysregulation of bone homeostasis; with decreased bone anabolism (decreased activity of osteoblasts and osteocytes) and increased catabolism (enhanced osteoclast activity). Successful treatment of OP should therefore increase bone anabolism and decrease catabolism to reinstate the equilibrium in bone homeostasis (4, 5). While therapeutic options are increasing, all but one available therapies aim to reduce bone resorption. However, as osteoclast and osteoblast activity

are coupled, anti-resorptives can negatively affect anabolic osteoblast activity and may not fully restore bone architecture (6). The only injectable osteoanabolic compound, teriparatide, is an analog of the parathyroid hormone (7). However, it is not an ideal long-term therapy option as, not only is it expensive, long term exposures in rat increase susceptibility to osteosarcoma (8, 9) limiting treatment duration (currently 2-years) in OP patients (10). Thus, an ideal treatment plan should focus on both strengthening bones using an osteoanabolic compound, combined with use of an anti-resorptive treatment (also ideally non-invasive) to maintain bone integrity (5), few such options exist. Currently, a major bottleneck in the development of new pharmaceuticals is the collection of primary functional data on new biological drug targets with osteo-anabolic capacities.

The twinning of genetic information with mechanistic data is key for development of new treatments. For example, familial studies on high bone mass (HBM) cases led to the discovery of mutations in *SOST* (*Sclerostin*). Further mechanistic data generated in model systems showed that SOST acts negatively on the WNT signaling pathway and led to the development of a novel antibody treatment Romosozumab (approved in 2018 for clinical use), which blocks SOST activity (11–13). With the advent of genome-wide association studies (GWAS), and efficient whole-genome/exome sequencing (WGS/WES) data mapping there has been a sizeable increase in availability of human genetic data from cohort studies for musculoskeletal conditions including OP, high bone mass (HBM), and osteoarthritis (OA) (14–20). Recent large cohort studies, such as UK-Biobank, have identified many new loci that contain novel osteogenic factors. For example, the UK-Biobank (21) data yielded 518 loci associated with changes in BMD using heel ultrasound data (16, 19). Currently, there is a substantial gap in translating these human genetic findings to model systems (22) in which the mechanism by which these genes act on the skeleton can be defined, where hypotheses can be tested, and ultimately define new putative drug targets that can be assessed with pharmacological agents. Because the skeletal system involves complex interactions between different cell and tissue types, genes and mechanical stimuli it is difficult to recapitulate features of OP in a petri dish. However, traditional rodent models are expensive to genetically manipulate. Zebrafish (*Danio rerio*) could therefore bridge this gap by offering fast genetic manipulation and complex tissue interactions required to model complex diseases such as OP.

Zebrafish are vertebrates and show strong similarities in their skeletal physiology to mammals (23). They are highly fecund and a single pair of fish can lay up to 300 eggs a week, which develop externally and are translucent (24). They show conservation of 70% of all genes and 85% of disease genes with humans (25, 26). However, the main advantage of zebrafish for functional genetic studies is their genetic tractability, as constructs that modify the genome can be injected directly into embryos at the single cell stage. This has allowed the generation of transgenic lines that allow dynamic imaging of all the cells of the developing skeletal system in live larvae (27–29) (**Table 1**) and in more recent years allowed genome editing strategies to be employed. In this review we set-out these different approaches and how developing and adult zebrafish can be used to study bone mineralization, bone content formation, and osteoblast-osteoclast interactions in a whole animal context. We also discuss future prospects for drug screening pipelines in zebrafish which may confer advantages over other pre-clinical model systems.

FLEXIBLE GENETIC MANIPULATION IN THE ZEBRAFISH

Zebrafish are genetically high amenable and new ways to manipulate the genome are constantly being added to the zebrafish genetic toolbox, which includes knockout, knock-down and, DNA insertion strategies. The external development of the embryos allows tools targeting genes of interest to be microinjected directly in embryos at the 1-cell stage and hundreds of embryos can readily be injected in a morning. Acute knockdown of gene expression can be achieved either by targeting mRNA with antisense RNA morpholino (MO) molecules that stably bind the target mRNA to block translation or splicing through steric hindrance (41). MOs offer a rapid method to assess the phenotype of a gene of interest during early development. However, they can only be used to study developmental processes occurring over the first 4 or 5 days of development, which limits their utility in skeletal studies as mineralization occurs from 4 days of development. While concerns have been raised about MO veracity as morphants frequently show more severe phenotypes than stables mutants generated for the same gene (42, 43). This is due to a transcriptional compensation response for chronic loss of a gene as has been shown in mouse, cultured human cell lines, plants, and zebrafish models (44–51). Thus, while MOs have a role, their use has been largely supplanted by use of genome editing strategies.

Traditionally, zebrafish mutant lines have been generated by forward genetic screening; using mutagens [e.g., N-ethyl-N-nitroso urea (ENU)] to induce random point mutations in offspring that were then screened for phenotypes of interest (52–56). The expansion of the zebrafish genetic toolkit with zinc-finger nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN) (57, 58), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (59) reverse genetic strategies, which, in combination with a fully sequenced genome (25), allow tailored gene-specific mutagenesis in the zebrafish. Gene function can be studied in genetic knockouts by generating insertion/deletion (indel) mutations leading to premature stop codons, deleting whole exons containing important protein domains and generate new stable mutant lines (**Figure 1A**). Moreover, the CRISPR/Cas9 protocol is so efficient that the F0 injected fish (crispants) can be used to study loss of gene function in these crispants, despite them carrying mosaic mutations (i.e., not every cell carries a mutation and more than one mutation may be present) (23, 60) (**Figure 1B**). Single base gene editing (knock ins) using modified Cas9 enzymes or supplying a DNA template for the endogenous homologous recombination machinery initiated by a double stranded break allows to introduce specific genetic changes to

TABLE 1 | Common transgenic lines to study musculoskeletal system in small teleostei.

Gene/pathway	Cell type(s)	Description	Transgenic line	Citation
BMP pathway	BMP transcriptionally activated cells	Reporter—21 BMP responsive elements (BMPRE) from *X. laevis*	*Tg(5xBMPRE-Xla.Id3:GFP)*	(30)
collagen10a1a	Osteoblasts (juvenile)	Reporter—BAC containing zebrafish collagen10a1a promoter	*TgBAC(col10a1a:Citrine)*	(29)
collagen2a1	Chondrocytes	Reporter—BAC containing zebrafish *collagen2a1* promoter	*Tg(Col2a1aBAC:mCherry)*	(29)
ctsk	Osteoclasts	Reporter—BAC containing zebrafish *ctsk* promoter	*TgBAC(ctsk:Citrine)*	(27)
entpd5a	Mineralizing osteoblasts	Reporter—BAC containing zebrafish *entpd5a* promoter	*TgBAC(entpd5a:Citrine/YFP)*	(27)
fli1a	Vasculature/neural crest	Reporter —BAC containing *fli1a* promoter	*Tg(fli1a:EGFP)*	(31)
Hedgehog pathway	Gli transcriptionally activated cells	Reporter—8 Gli responsive elements driving *egfp* or *mCherry*	*Tg(Gli-d:gfp/mCherry)*	(32)
Osteocalcin	Osteoblasts (mature)	Reporter—3.7 kb upstream osteocalcin promoter from Medaka driving *gfp* expression	*Tg(Ola.osteocalcin:EGFP)*	(33)
rankl	Osteoclast-osteoblast interaction	Conditional—Heat shock inducible (HSE) ubiquitous simultaneous expression of *rankl* and *cfp* in medaka	*Tg(rankl:HSE:CFP)*	(34)
runx2	Osteoblasts (juvenile) forming new bone	Reporter—557 bp intronic human *RUNX2* enhancer (Hsa), regulating *RUNX2*, conserved in multiple species, driving *gfp* expression	*Tg(Hsa.RUNX2-Mmu.Fos:EGFP)*	(33)
sox10	Mesenchymal chondrocytes	Reporter—4.9 kb of *sox10* promoter driving *egfp*	*Tg(−4.9Sox10:EGFP)*	(35)
sp7 (osx)	Osteoblasts	Reporter—BAC containing zebrafish *sp7* promoter	*Tg(sp7:EGFP)*	(36)
sp7 (osx)	Osteoblasts	Reporter—Medaka *sp7* regulatory elements driving *nls-gfp* or *mCherry*	*Tg(sp7:nuGFP/mCherry) or Tg(Ola.sp7:NLS-GFP)*	(37)
sp7 (osx)	Osteoblasts	Reporter—BAC *sp7* promoter driving *luciferase* expression	*Tg(Ola.sp7:luciferase)*	(38)
sp7 (osx)	Osteoblasts (ablation)	Conditional—Chemical ablation of osteoblasts by *E. coli* enzyme Nitroreductase (NTRo) activity	*Tg(osterix:mCherry-NTRo)pd46*	(39)
WNT - β-catenin pathway	β-catenin activated cells	Reporter—T-cell factor enhancer (TCF) promoter containing 7 beta-catenin binding sites	*Tg(7xTCF.XlaSiam:nlsGFP)*	(40)

BAC, bacterial artificial chromosome; bp, base pair; kb, kilobase.

model specific human disease mutations in zebrafish orthologs (62, 63).

SIMPLE ASSESSMENT OF ZEBRAFISH BONES DURING DEVELOPMENT AND ADULTHOOD

Zebrafish in common with higher vertebrates, have both dermal/intramembranous ossification, in which bone is formed *de novo* directly by osteoblasts, and chondral/endochondral ossification in which bone forms by progressively replacing a cartilaginous template. Although zebrafish have thinner bones than terrestrial vertebrates, with fewer embedded osteocytes and little trabeculation, all of the relevant skeletal cell types and modes of regulation are conserved between zebrafish and higher vertebrates. This, importantly for the study of OP, includes osteoblast and osteoclast coupling and regulation of bone remodeling (64, 65).

A major advantage of using zebrafish to probe the mechanism of bone homeostasis is that cell behavior can be visualized dynamically *in vivo*. Zebrafish larvae are translucent and develop rapidly (24), and early skeletal processes can be dynamically visualized in the living fish through use of fluorescent transgenic reporter lines marking these cell types (see **Table 1** for examples). Formation of the craniofacial skeleton occurs early, with the first cartilaginous structures of the jaw forming by 2 days post fertilization (dpf) (66), the first skeletal joints are formed and mobile by 3 dpf (60), by 5 dpf, hypertrophic chondrocytes, marked by *col10a1a*, are seen in some elements from 5 dpf (29), and first osteoblasts surrounding the cartilage and forming bone matrix by 7 dpf (67). The first intramembranous bones, such as the cleithrum, anterior notochord, and operculum, are visible in the craniofacial skeleton from 72 hpf (66). While skeletal development occurs early, true remodeling through the combined activity of osteoblasts and osteoclasts does not commence until the second week of development as osteoclasts (marked by Cathepsin-K (Ctsk) or TRAP) are not formed until day 10–12. Unlike mammals, mononucleated osteoclasts as well as multinucleated cells are present and actively resorb bone (65, 67).

There are many transgenic lines available to mark musculoskeletal tissues, these include reporter lines which label cells or signaling pathway activation by driving expression of proteins in the cytoplasm, targeted to the nucleus, or plasma membrane, and lines that tag proteins (28, 68). Reporter lines mark cell types by using a tissue specific promotor, responsive elements from a signaling pathway, or transcription factor binding sites controlling expression of a fluorescent protein (**Table 1**). For example, to study bone homeostasis, osteoblasts

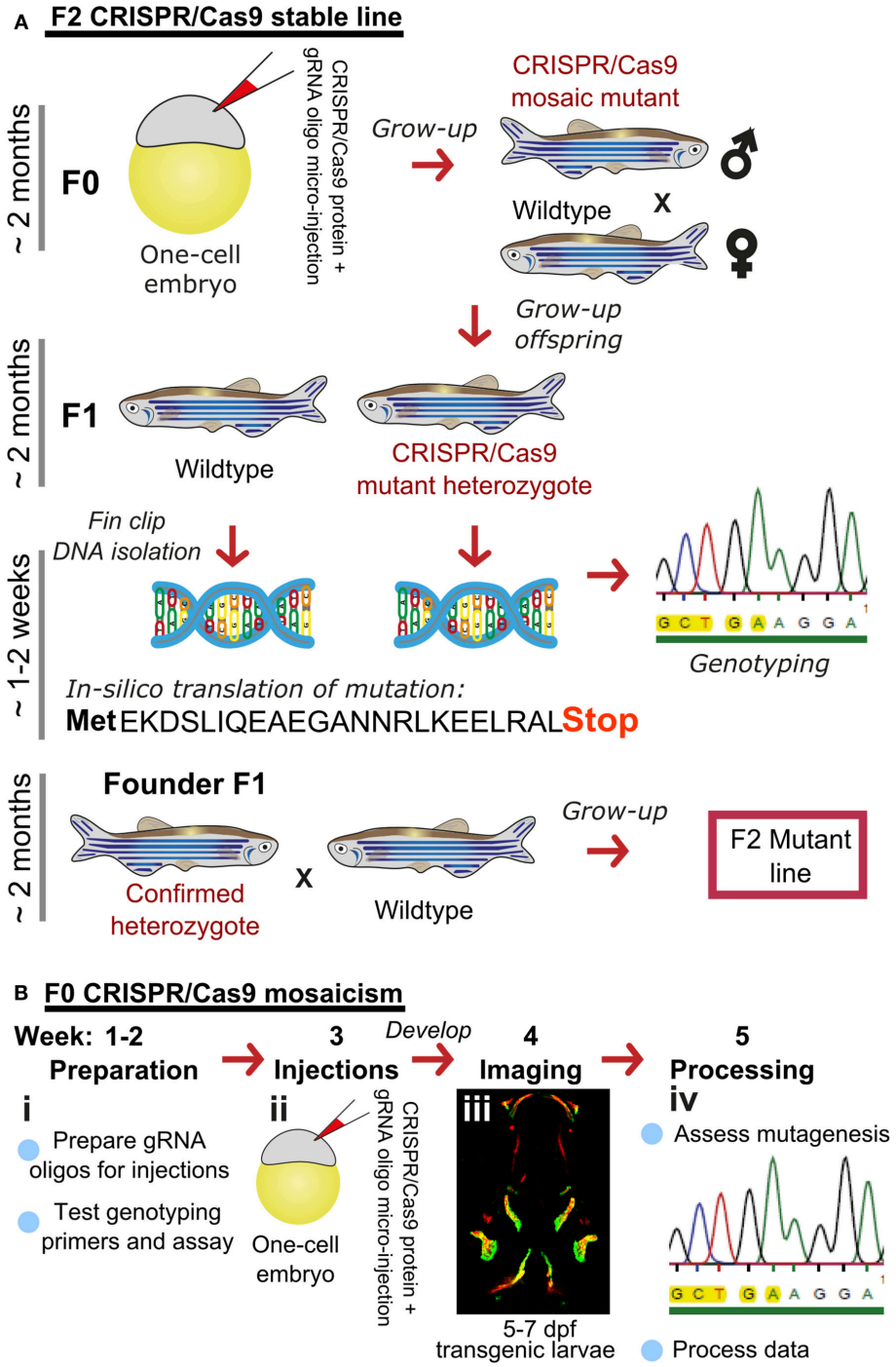

FIGURE 1 | Rapid and efficient mutagenesis using CRISPR/Cas9 genome editing in zebrafish. **(A)** To generate a stable mutant line, F0 CRISPR/Cas9 injected individuals carrying mosaic mutations (defined by fin-clipping, **B**) should be outcrossed to wildtype fish to allow selection of a single germline mutation. Out-crossing the founder to wildtype will establish a stable F2 mutant line. Note that the F1 can have multiple founders with damaging mutations, incrossing these will result in F2 homozygotes (for recessive alleles) for functional analysis. When performing incrosses from F2, it will take another 2 months of breeding time. **(B)** This rapid protocol can be used to generate mutations in a gene of interest using CRISPR/Cas9 RNA or protein with gRNAs targeted against the gene from custom made gRNA oligos (i). Micro-injection of CRISPR/Cas9 RNA or protein and gRNAs specific to gene of interest into embryos at the single cell stage (ii) generating double stranded breaks during the first few rounds of cell divisions. The repair machinery is prone to errors and those cells will carry a different type of mutation giving a range of insertion and deletion (indel) mutations (spectrum of mutations, mosaicism). The overall mutagenic efficiency is typically high (around 80% with fragment analysis) allowing larval skeletal phenotypes to be assessed in the injected (F0) population (60). After imaging an Alizarin Red S (AR) stained individual in a transgenic background (here osteoblast marker *sp7:gfp*)(iii), mutagenesis assessment such as fragment analysis will determine a quantified mutagenesis rate (61) which can be correlated to a phenotype (iv). Note that mosaic mutants (crispants) can also be grown up to see the effect on the adult skeleton.

and osteoclasts can both be labeled *in vivo*, using osteoblast reporters such as *sp7*, and osteoclast reporters such as *ctsk*, so that their numbers, location and activity monitored in living bone tissue either longitudinally, in response to drug treatment, genetic mutation, or environmental stimuli (**Table 1**). Relevant to research into OP, osteoclasts can be specifically temporally activated by use of a heat shock promoter driving RANK ligand (*rankl*) expression; such that following a period of immersion in water at 39°C, osteoclast activity, labeled with the blue fluorescent protein (CFP), is increased resulting degradation of the bone matrix and in an osteoporotic phenotype of low BMD (34). A simple Alizarin Red S (AR) staining, which marks calcium phosphate crystals and fluoresces strongly in the red channel (580 nm wavelength), allows a rapid assessment of ossified elements in live or fixed fish. In combination with transgenic lines, endochondral ossification in the lower jaw (**Figure 2A**) and intramembranous bone formation in the operculum (**Figure 2B**) can be easily visualized compared to traditional rodent models.

IMAGING THE ADULT SKELETON FOR ASSESSING MINERALIZATION

The zebrafish adult skeleton is relatively complex and once fully formed by around 2 months is composed of 74 ossified cranial elements (compared with 22 in humans), 28–31 vertebrae; 4 cervical, 10–11 thoracic vertebrae, and 15–16 separated vertebrae in the tail region and fins (pectoral, dorsal, anal (ventral), and caudal) (69). As in larvae, live AR and Calcein staining, or use of transgenic lines, allows easy detection of superficially located calcified elements in the skull, elasmoid scales, and fins using a simple fluorescent microscope. Deeper tissues can be imaged by multiphoton microscopy in small juveniles. However, bones located more internally (e.g., vertebrae and ribs) in large adults are difficult to visualize using this method. Post-mortem staining of bone (AR) and cartilage [Alcian blue (AB)] is a cost-effective way to analyse these structures for adult skeletal abnormalities (**Figure 3A**) and has been used in forward genetic screens to obtain detailed skeletal morphology information (56, 70, 71).

Recent advances in X-ray based imaging: radiographs, micro-computed tomography (μCT), and synchrotron equipped μCT technologies (SR-μCT), and their subsequent downstream imaging processing, opened avenues to assess the adult zebrafish skeleton. The major advantage of using these X-ray imaging techniques is that they are non-destructive and can be used in the intact fish, allowing the samples to be used for other purposes, such as histology. Radiographs give two-dimensional (2D) images of the zebrafish skeleton at relatively low resolution (**Figure 3B**), permitting the visualization of bone elements and a broad evaluation of changes in the skeleton, radiographs can

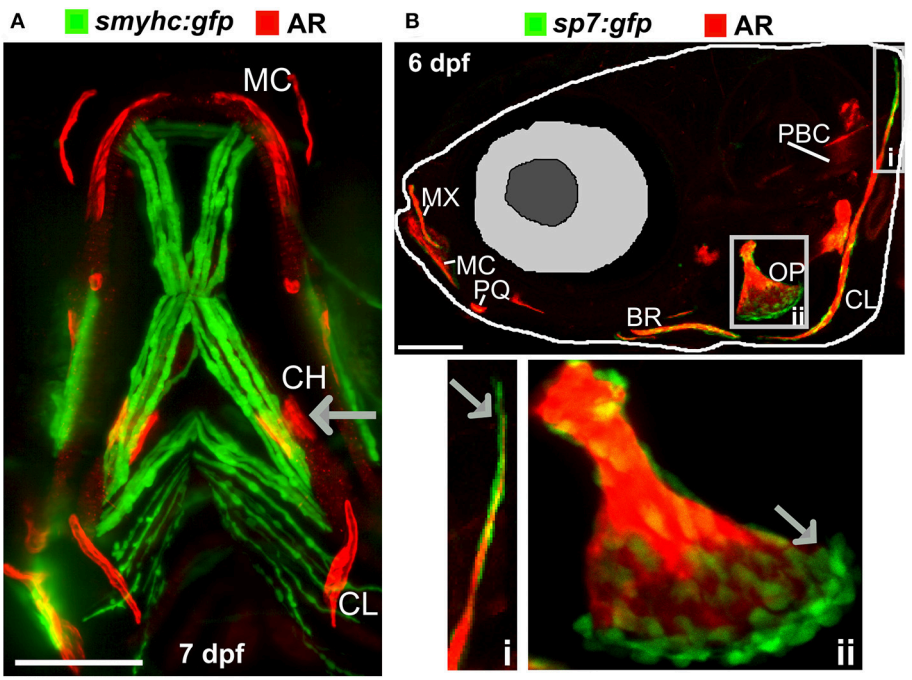

FIGURE 2 | Ossified elements in the cranial region during early development. **(A)** Ventral view of a 7 days live Alizarin Red S (AR) labeled larval jaw showing dermal ossification of cleithrum (CL), and ossification of the cartilaginous ceratohyal (CH). Arrow indicates the CH which undergoes endochondral ossification. Slow muscle transgene reporter in green (*smych:gfp*). Image taken on a Leica lightsheet microscope. **(B)** Lateral view of a 6 days old larva live labeled with Alizarin Red S (red) and carrying GFP under the control of the osteoblast promoter s7/osterix (green; *sp7:gfp*) allowing visualization of mineralized elements (red) and osteoblasts (green) in a living individual. Insets show the cleithrum (i) and operculum (ii) with osteoblast enrichment at the distal ends of these elements (gray arrows). Image taken on a confocal microscope. Wildtype strains AB/TL in both panels. Ossified elements: BR, branchiostegal ray; CH, ceratohyal; CL, cleithrum; MC, Meckel's cartilage; MX, maxilla; OP, operculum; PBC, posterior basicranial commissure; PQ, palatoquadrate. Scale bars = 100 μm.

be used to image live anesthetized fish permitting longitudinal analysis of the skeleton over time (70). Higher resolution (2 μm voxel size) and three-dimensional (3D) assessment of the zebrafish skeleton can be achieved by using μCT (**Figures 3C–E**). As fish bone, like that of mammals, is composed of hydroxyapatite crystals, quantification of BMD can be performed by comparison to phantoms, which are samples of known hydroxyapatite content (72). Additionally, treatment with agents to improve contrast such as silver nitrate ($AgNO_3$) or iodine, allow detection of juvenile (less dense) bone and of soft tissues such as muscle and cartilage (72).

Very detailed data on bone micro-architecture can be achieved with SR-μCT (73, 74). This technique can yield a spatial resolution of 100 nanometres on tissue samples and visualize fine bone structures at a cellular level including the vasculature in mineralized bone, osteoclast resorption pits, and osteocyte lacunae (75). As the size and resolution of data sets increase, the bottleneck in the process is frequently data analysis. Commercially available software packages such as "boneJ" are tailored for CT data analysis, and recently open source user friendly software have become available to process μCT data from zebrafish scans. For example, the "FishCut" software processes whole-body μCT scan datasets and applies semi-automated analysis algorithms. The current version segments the axial skeleton, then generates values for the surface area of vertebrae and centrae, and calculates BMD and mineralized thickness in a semi-automated fashion (76).

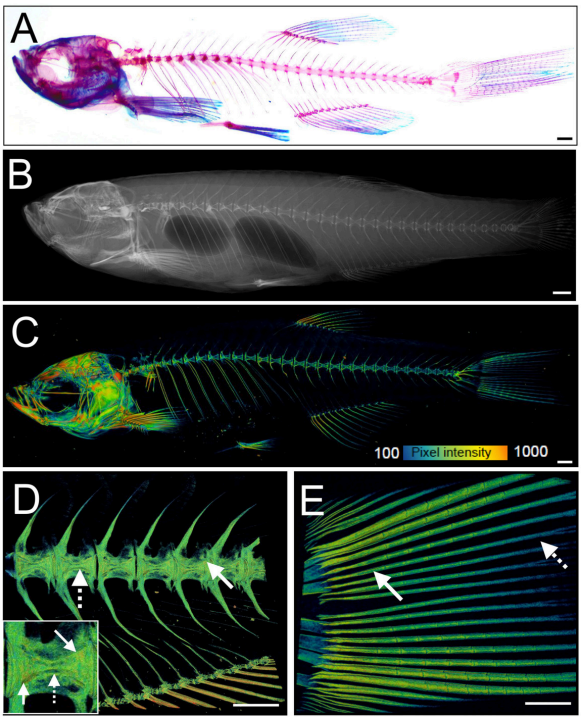

FIGURE 3 | Examples of visualization and quantification of mineralized bone in zebrafish. **(A)** Wholemount Alizarin Red S (AR) and Alcian Blue staining of 3 months fixed fish. **(B)** Radiograph of 1-year old live fish showing whole body: endo- and exoskeleton. **(C)** Low resolution μCT images acquired with a 20 μm voxel size of a 3 months old fish. Note that pixel intensity can be used to determine BMD; represented on the color coded pixel intensity bar. **(D,E)** High resolution (5 μm voxel size) μCT images of vertebral column with anal fin rays **(D)** and caudal fin rays **(E)**. Vertebral centrae have higher density at their edges (solid arrow) than the center (dashed arrow). In the fin rays, a higher density (solid arrow) is observed in older segments within the proximity to the body in comparison to younger segments located more caudally showing lower pixel intensity (dashed arrow). The same pixel intensity color coding as **(C)** applies. All fish and their insets are depicted from a lateral view in an anterior-posterior (left-right) orientation. Scale bars = 50 μm in **(A,B)**; and 100 μm in **(C–E)**.

ZEBRAFISH MUTANTS OF BRITTLE OR THIN BONES

An increasing number of zebrafish genetic mutants in skeletally relevant genes have been shown to recapitulate human bone disease. These have provided insight into the dynamic regulation of bone formation, mineralization, and remodeling. We have included a list of zebrafish skeletal mutants in **Table 2**. While there are currently few models for OP, there are various zebrafish mutant lines that accurately model human skeletal dysplasias, including collagenopathies and forms of osteogenesis imperfecta, which are characterized by brittle bones and frequent low-impact bone fractures. Autosomal dominant mutations *COL1A1* and *COL1A2* genes predominantly affect glycine-X-Y (Gly-X-Y) repeat domains that result in collagen α1(I) and α2(I) heterotrimer maturation defects (119), causing fragile bone matrix and insufficient mineralization (120). The Gly-X-Y mutations lead to impaired hydroxylation and defects in collagen maturation in the endoplasmic reticulum (ER), which is also conserved in zebrafish (121–123). The autosomal dominant *chihuahua* (*chi*) zebrafish mutant, was identified in a forward genetic screen using radiography (70). Linkage mapping identified a mutation causing a glycine to aspartate amino acid substitution in a conserved Gly-X-Y repeat of *col1a1a* (zebrafish *col1a1* is duplicated). Note that in contrast to mammals, zebrafish type-I collagen is constituted by three different α chains [α1 (*col1a1a*), α3 (*col1a1b*), α2 (*col1a2*)] due to duplication (124). *chi*/+ zebrafish display phenotypes resembling those seen in humans, including a shortened axial skeleton, with irregular radiodensity, uneven mineralization, and brittle bones that fracture easily (especially ribs). Transmission electron microscopy revealed that *chi*/+ fish show signs of ER stress (70). The ER trapping of insufficiently hydroxylated oligotrimerized α1(I)/α2(I)/α3(I) collagen leads to lower extra-cellular collagen maturity, abnormally shaped and thinner vertebrae bodies, areas of higher calcium content, different local mechanical properties, and reduced osteocyte number (84). Osteogenesis imperfecta has a broad disease spectrum in the clinic, and recent comparative studies of multiple mutant alleles for *col1a1a, col1a1b, col1a2,* and also *bmp1a* (described later) and *plod2* described a diversity of skeletal phenotypes (**Table 2**) with brittle bones as the common feature (85).

The zebrafish *sp7/osterix* mutant has been shown to model human osteogenesis imperfecta caused by recessive damaging mutations in *SP7* (125). This mutant showed uneven mineralization, severe fractures caused by minimal impact, and

TABLE 2 | Zebrafish mutants, transgene insertion mutants, and morphants showing altered skeletal mineralization.

Human gene	Zebrafish name	BMD effect	Primary defect/effect	Fish modeling	Human skeletal phenotype	Citation
ABCC6	*gräte*	+	ATP hydrolysis defects causing (ectopic) increased mineralization in spine and soft tissues	N/A	Pseudoxan-thoma elasticum	(77)
ATP6V1H	*atp6v1h*	−	Increased osteoclast activity by upregulated *mmp9* and *mmp13*	Osteoporosis	Familial osteoporosis with short stature	(78)
BMP1	*frilly fins, welded*	−	Fibrillar collagen processing affecting bone matrix integrity	Osteogenesis imperfecta	Osteogenesis imperfecta; high BMD (in vertebrae) but weak bones	(72, 79, 80)
C-FMS (CSF1R/CD115)	*panther, csfr1a*	+	Reduced osteoclast number and immune cell mobility causing stenosis	Osteopetrosis	N/A	(72, 81, 82)
COL11A2	*col11a2*	+	Collagen triple helical stability; dominant effect	OA: Stickler syndrome	Stickler Syndrome	(83)
COL1A1	*chihuahua, microwaved, dmh13, dmh14, dmh15, dmh29*	−	Collagen triple helix stability; dominant effect leading to brittle bones in axial and fin skeleton.	Osteogenesis imperfecta and Ehlers-Danlos syndrome (*chuhuahua* and *microwaved*)	Osteogenesis imperfecta and Ehlers-Danlos Syndrome	(56, 70, 79, 84–86)
COL2A1	*dmh21 (?), dmh27, dmh28, dmh30 (?)*	=	Collagen triple helical stability; dominant effect. Notochord and vertebra deformations.	Spinal deformations	Stickler syndrome	(56)
CTSK	*ctsk* *¥	+	Depletion of pre and mature osteoclasts	Osteopetrosis	Osteopetrosis	(87)
CX43 (GJA1)	*stoepsel, short-of-fin*	− (?)	Brittle vertebrae anomalies due to loss of function hemichannel (Ca^{2+}) activity	N/A	Oculodento-digital dysplasia	(88, 89)
CYP26B1	*stocksteif, dolphin, cyp26b1*	+	Hyper-mineralization and fusion of the vertebrae and joints due to altered intracellular retonic acid metabolism	Retonic acid processing	Craniosynostosis, craniofacial anomalies, fusions of long bones	(37, 90, 91)
DKK1 (DICKKOPF)	*hs:dkk**	−	When heat-shocked, Dkk1 is expressed and blocks Wnt/Beta-catenin signaling. Impaired elasmoid scale and ray fin outgrowth.	N/A	Osteolytic bone lesions in multiple myeloma patients	(92)
EDA and EDAR	*nackt (eda), finless (edar), fang (edar), topless (edar)*	−	Absence and deformation of dermal bone structures such as lepidotrichia, elasmoid scales, and skull	Ectodermal dysplasia, impaired teeth	Hypohidrotic ectodermal dysplasia 1 (X-linked); Tooth agenesis	(93)
ENPP1	*dragonfish*	+	Ectopic hyper-mineralization in axial skeleton due to altered phosphate metabolism	Arterial calcification of infancy	Arterial calcification /hypophosphatemic rickets	(94, 95)
ENTPD5	*no bone*	−	Does not mineralize bone due to altered phosphate metabolism	N/A	N/A	(94)
GBA1	*gba1*	−	Impaired osteoblast differentiation due to altered Wnt signaling	Osteoporosis, Gaucher disease	Osteoporosis, Gaucher disease	(96)
GLI2	*hs:gli2-DR**	−	Heat-shock (hs) initiates expression of dominant repressive Gli2. Impaired scale calcification.	N/A	Culler-Jones syndrome; holoprosencephaly	(92)
GOLGB1 (giantin)	*golgb1*	+	Ectopic mineralization in spine and soft tissues by transcriptionally down regulating *galnt3* and changed cilia morphology	N/A	*GOLGB1* unknown–*GALNT3* mutations cause tumoral calcinosis	(49, 50)
IHH	*ihha*	−	Loss of mineralization due to blocked osteoblast differentiation in endochondral bone. Irregular operculum and scale morphology with reduced AR stain	Endochondral bone repair and dermal ossification	Acrocapitofemoral Dysplasia, Brachydactyly Type A1	(67, 97–99)
ITGA10 *ITGBL1 #*	*itga10 ($)* *itgbl1 ($)*	−	Focal adhesion Integrin A/B subunits. Downregulated in prednisolone larvae.	Osteoporosis	N/A	(100)

(Continued)

TABLE 2 | Continued

Human gene	Zebrafish name	BMD effect	Primary defect/effect	Fish modeling	Human skeletal phenotype	Citation
LGMN	*lgmn ($)*	+	Legumain (secreted cysteine protease) inhibits osteoblast activity by degradation of fibronectin	Osteoporosis	Osteoporotic–upregulated in OP bone	(101)
LRP4	*lrp4 MO*	– (?)	Malformed pectoral and tail fin and deformed craniofacial skeleton with kidney cysts	Cenani-Lenz syndactyly	Cenani-Lenz syndactyly, osteoporosis, Sclerosteosis	(102)
MEF2C	*mef2ca*	+	Ectopic bone formation of neural crest derived ligament due to altered DNA methylation	N/A	Unknown	(103, 104)
N/A	*bone calcification slow*	–	Non-mapped mutation causing delayed ossification and increased Cyp26b1 expression	N/A	Unknown	(105)
PANX3	*panx3 MO*	–	Altered Ca2+ channel activity reducing endochondral ossification	N/A	N/A	(106)
PLS3	*pls3 MO*	–	Reduced larval operculum mineralization	Osteoporosis	X-linked osteoporosis	(107)
PTCH1, PTCH2	*ptch1 (ptc2), ptch2 (ptc1)*	+	Increased mineralization in endochondral bone	N/A	Holoprosencephaly	(67)
PTH4 #	*pth4**	–	Neuronal regulation of phosphate metabolism	N/A	PTH4 is absent in terrestrial animals	(108)
PTHrP / PTHLH / PTH3	*pthlha/pthlhb MOs*	+	Premature ossification during larval stage under control of sox9	N/A	Brachydactyly; mutation in promoter	(109)
RANKL	*rankl ¥**	–	Induces osteoclast activity	Osteoporosis	Osteoporosis	(34)
RPZ #	*rapunzel*	+	Increased BMD in craniofacial and spinal column elements	N/A	None–Teleost specific gene	(110)
SLC10A7	*slc10a7 MO*	–	Secretory pathway defect	N/A	Decreased BMD; skeletal dysplasia	(111)
SP7 (OSX, osterix)	*sp7 (osx, osterix)*	–	Decreased mineralization, skull sutures defects, impaired teeth formation, increased BMP signaling, and reduced differentiation, but increased proliferation, of osteoblasts. Homozygous mutant adults are viable	Osteogenesis imperfecta, osteoporosis (?)	Osteogenesis imperfecta	(112, 113)
SP7 (OSX, osterix)	*sp7 (osx, osterix) ¥*	–	Decreased mineralization of endochondral bone and vertebrae. Reduced osteoblast number. Homozygous lethal at 14 dpf	Osteogenesis imperfecta	Osteogenesis imperfecta	(114, 115)
SPP1	*spp1 (osteopontin)$*	–	Reduced AR staining in 5 dpf craniofacial skeleton. Absent in whale shark genome	N/A	N/A	(116)
TGFB3	*tgfb3 MO*	–	Reduced calcification of juvenile bone	N/A	Oral clefting	(117)
TSHR	*opallus*	+	Mutation causes a constitutive active Tshr leading to hyperthyroidism causing high BMD	Hyperthyroidism	Hyperthyroidism	(76)
TWIST and TCF12	*twist1b and tcf12*	+/=	Frontal skull sutures due to increased osteoblast proliferation. Mineralization normal.	Saethre-Chotzen syndrome	Saethre-Chotzen syndrome	(118)

*$Mosaicism; MO, Morpholino; #No clear ortholog; (?)Indicated / implied; *Transgene affecting gene; ¥ Medaka.*

misshapen bones. Moreover, rare craniofacial characteristics caused by impaired SP7 function, such as wormian bones, reported in human patients carrying mutations in *SP7* were also observed in zebrafish (112).

Another example of a zebrafish mutant that recapitulates patient phenotype is the *bmp1a* mutant *frilly fins* (*frf*). In humans a damaging missense mutation in the BMP1 signal peptide causes brittle bones in an osteogenesis imperfecta pedigree (79). *frf* mutants showed normal osteoblast number, but pericellular pro-collagen processing (C-pro-peptide removal) defect leading to mineralization defects in the axial skeleton and fin rays (79).

Collagenopathies, such as Stickler Syndrome, have also been successfully modeled in zebrafish. We have recently reported a *col11a2* zebrafish mutant showing specific traits of the human disease which include thicker collagen fibers and degradation of

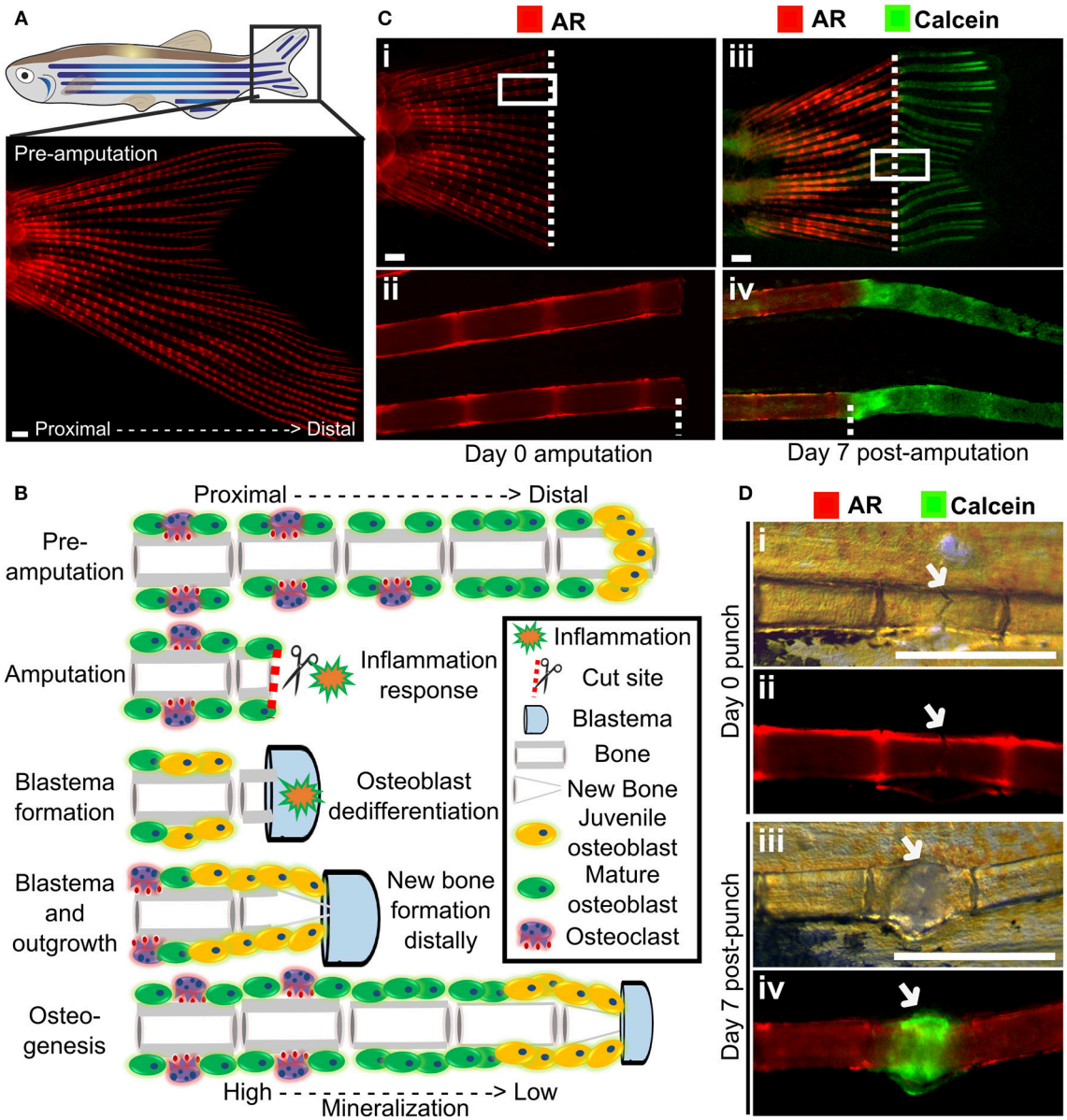

FIGURE 4 | Fin regeneration and fracture assay to visualize and quantify live bone formation and repair. **(A)** Schematic representation of a zebrafish with a standard fluorescent stereomicroscope image of a live Alizarin red S (AR) pre-amputation caudal fin (inset). **(B)** Schematic representation of bone regeneration after fin amputation showing the (simplified) cascade of events that follow after fin amputation to regenerate bone (a single ray depicted here). This allows studying *de novo* bone formation by newly formed osteoblasts (orange cells) and differentiated osteoblasts (green cells) and subsequent remodeling by osteoblasts and osteoclasts (purple cells) in an adult fish. Note that during osteogenesis that there is a gradient of mineralization. **(C)** Live images of the tail fin labeled with Alizarin red (red) prior to amputation (i, ii) and Calcein (green) post-amputation (iii, iv) taken on a fluorescent dissecting microscope. All images in panel come from the same fish. Seven days post-amputation showing regrowth of new bone (green). Note that intense Calcein staining is visible distally from the amputation site (white dotted line). **(D)** The fracture healing assay involves applying pressure on a fin ray bone element to induce a small fracture to one segment of the fin ray (i), which is visible with life AR staining (ii). Green Calcein labels the new bone formed in the fracture callus by 7 days (iii and iv). The white arrow indicates the fracture site. Scale bars = 500 μm, 3 months old wildtype TL/EKK females.

type-II collagen in zebrafish larvae leading to compromised jaw shape, mechanical properties and movement of the jaw leading to premature OA (83). In many skeletal dysplasias zebrafish not only model the human condition but allow mechanistic insight into how genetic changes lead to the cellular changes that underpin the disease symptoms. As such zebrafish offer exciting prospects for delivering functional studies in new osteoporotic genetic loci.

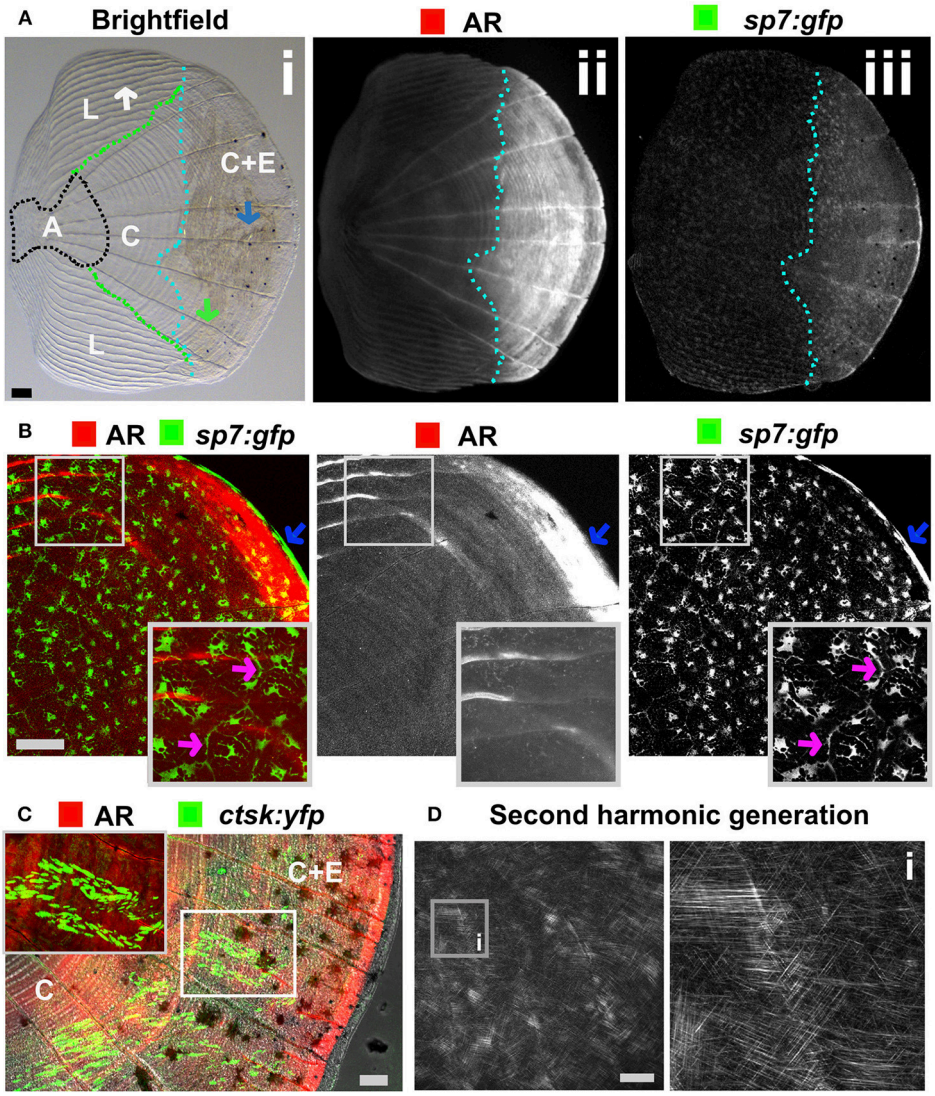

FIGURE 5 | Zebrafish elasmoid scale structure and bone cell types. **(A)** Single scale from the flank of a 3 months old fish carrying the *sp7:gfp* osteoblast reporter transgene (green) and stained for Alizarin Red S (AR, red). Whole scale is shown in bright field (i) and gray scale images for AR (ii) and GFP (iii) in the top panels. The brightfield image (i) depicts the anterior anchor region (A, black dotted line boundaries), the lateral circuli (L, green dotted line boundaries, white arrow), central region (C, surrounded by black, green, and light blue dotted lines), and central region covered by epidermis (C+E, light blue dotted line, with grooves by green arrow) with enhanced mineralization. **(B)** Confocal images showing a merge image of osteoblasts (*sp7:gfp* transgenic fish, green) abundantly distributed over the freshly harvested scale and AR staining (red). Individual channels are depicted in gray scale images. Note increased mineralization at the edge of the scale corresponding increased GFP presence (blue arrows). Insets focus on the lateral circulus and note osteoblast cytoplasmic protrusions (pink arrows). **(C)** Confocal images visualizing osteoclasts with cathepsin K (ctsk) YFP reporter expression (green), mineralization by AR (red), and brightfield (gray). Note that YFP positive cells were predominantly seen in the central region with epidermis (C+E) and distal edges of the central region **(C)**. **(D)** Multiphoton forward scattering (second harmonic generation (SHG), 880 nm wavelength) visualizes collagen fibrils in an ethanol fixed scale. Inset (i) shows the organization of collagen fibrils in a plywood structure. Wildtype strains (panel): TL/EKK **(A)**, TL **(B)**, AB/TL **(C)**. Scale bars 100 μm.

ASSAYS OF CAUDAL FIN REGENERATION AND FRACTURE REPAIR TO ASSES *DE NOVO* BONE MATRIX FORMATION

Zebrafish are capable of regeneration many tissues and organs including the heart, lens, and pancreas. They also show regeneration of skeletal tissues following amputation of the tail fin (lepidotrichia) or removal of elasmoid scales (126, 127). As the fins and scales are translucent, and readily imaged they allow cells and their calcified matrix to be visualized in detail using standard fluorescent microscopes (**Figure 4A**). After amputation of a ray fin (typically a caudal fin), a wound healing response results in the formation of an epimorphic blastema which regenerates all affected tissues of the amputated organ, including bone, in a controlled fashion (128). Following this inflammation response, osteoblasts undergo dedifferentiation and proliferate to

contribute to the blastema (33, 129). These juvenile osteoblasts then secrete matrix with intermediate properties between cartilage and bone and are later remodeled as mature bone by matured osteoblasts and recruited osteoclasts (**Figure 4B**) (33, 128). These fins can also be injured via cryo-injury by placing a −196°C knife perpendicularly to the caudal fin rays allowing to study the bone resorption response (130). These techniques offer great perspectives to compare bone formation and bone remodeling, in an adult context.

Using transgenic lines and *in vivo* staining methods, such as AR (fluoresces red, 545 nm excitation, 580 nm emission) and Calcein (fluoresces green, 495 nm excitation, 515 nm emission), which binds to calcified matrix, the dynamics of bone formation can be visualized by using a fluorescent stereomicroscope in a regenerating caudal fin of a living fish. This allows longitudinal analysis by following regeneration rate and volume, since AR stains fully mineralized bone and Calcein binds to newly deposited bone matrix (**Figure 4C**).

The utility of fin regeneration assays to test bioactive compounds has been demonstrated by treating regenerating fins with the glucocorticoid prednisolone. Following treatment bone formation was reduced, and furthermore, both osteoblast number and subsequent bone deposition and osteoclast recruitment was reduced in these fins (131). Interestingly, skull injury repair is less affected following prednisolone treatment (131), this is similar to mammals. Treatment of fins with *Botulinum toxin* (Botox) leads to a reduction in bone mineralization and regeneration following amputation (132), comparable to the situation in mammals where fracture repair is impaired following Botox induced paralysis (133, 134).

A major issue with OP is increased fracture risk due to weaker bone structure, and therefore identification of therapeutics that can improve fracture healing is desirable. Zebrafish show a fracture healing response, including callus formation (**Figure 4D**), with strong similarities to that of mammals. Fractures can be induced in zebrafish fins using simple pressure applied externally to the fin (131, 135). As the fin has around 300 bony rays, multiple fractures can be induced in a single fin. A fracture callus is formed and *de novo* bone formation is initiated 2 days post-injury accompanied by an increased expression of osteoblast genes such as *runx2* and *sp7/osx* (131, 135). As the fin is flat, the fracture repair process can be dynamically tracked at cellular resolution using transgenic lines (**Table 1**) or by labeling bone formation with AR and Calcein (**Figure 4D**). As for regeneration, it is possible to add pharmacological agents to the regenerating tissue (131), allowing potential osteoanabolic compounds to be tested for beneficial effects in fracture repair *in vivo* (136).

SKELETAL ASSAYS USING ELASMOID SCALES

The body of zebrafish is covered with elasmoid scales made of calcified dermal bone harboring osteoblasts and osteoclasts (**Figures 5A–C**). The calcified matrix is composed of a plywood structure of collagen fibrils (137), which are easily visualized with second harmonics generation microscopy (**Figure 5D**). Scales are embedded in, and grow from, the dermis and shed and replace naturally throughout life of the fish (138). As scales are part of the exoskeleton they are easy to collect from an anesthetized fish. Each flat scale is subdivided in four regions by its morphology: anterior, lateral, central, and central with epidermis (**Figure 5A**) (139). The anterior region is attached to the skin and does not grow or form new bone. The lateral area is characterized by its curved ridges (circuli), whereas the central area has linear trenches. Within the lateral circuli and central grooves newly mineralized matrix is formed by osteoblasts (139) and degraded by osteoclasts (140). The posterior area has increased osteoblast number and bone is continuously deposited (**Figures 5A,B**). Osteoblasts in different regions of the scale express different markers of maturity (97). As the scale contains living cells, including nerve and vascular endothelial cells, their use offers an opportunity to study bone cell behavior in a mature context.

PHARMACOLOGICAL MANIPULATION OF BONE TISSUE AND CHEMICAL GENETIC SCREENING

As larvae are small and develop in water, it is possible to grow larvae in multi-well format with the addition of water-soluble compounds to their growth media for easy uptake. Zebrafish have been used extensively for high-throughput screening using larvae and now drugs are used in clinical studies that were first identified in zebrafish. A great example is the identification of the kinase inhibitor dorsomorphin (BMP type-1 receptor (BMP1R) antagonist) to treat lymphoma which was discovered in an early embryogenesis phenotype screen using 7,500 small-molecules (141, 142). Another example used semi-automated imaging strategy of Calcein stained larvae exposed to a small-compound library identifying 6 catabolic and 2 anabolic compounds that alter notochord mineralization (143) (**Table 3**). Thus, when fluorescent compounds are twinned with fluorescent reporters for osteoblasts (e.g., *sp7:gfp* with AR) (**Figure 2B**), it will allow assessment of osteoblast number and activity in a semi-high content setting using plate imaging microscopy (162). When these assays are combined with high efficiency CRISPR/Cas9 genome engineering strategies, it will open avenues to test compounds of interest that could alter disease causing mutations deteriorating effects. Thus, this comprehensive approach will also offer opportunities to develop compounds for personalized medicine. For OP research it may be more advantageous to focus on adult skeletal assays to allow assessment of osteoclast activity (bone catabolism) simultaneously with an assessment of osteoblasts (bone anabolism). An example of pharmaco-genetics improving brittle bones, is when type-I collagen secretion in the bone matrix is ameliorated by treating *chi/+* mutants with 4 phenyl butyrate (4PBA) compound (86).

Zebrafish elasmoid scales are bony plates that are small and contain bioactive osteoblasts and osteoclasts (**Figures 5B,C**). These therefore offer huge potential as a primary pharmacological screening tool for skeletal compounds. The scales can be cultured for 72-h post-harvesting during which they

TABLE 3 | List of compounds, diets, and exercise that alter ossification in zebrafish larvae, adults, and/or adult elasmoid scales.

Treatment	Gene/pathway	BMD effect	Primary effect	Part of compound screen?	Life stage	Citation
4PBA	HSP47–ER protein/fibrillar collagen folding	+	Increased mineralization in both WT and chi/+ fish due to better clearing of type-I collagen from ER	No	Adult larval	(86)
Alendronate / etidronate	Alendronate/etidronate therapies (bisphosphonates)	+	Counteracts the negative effects of GIOP on scales. Reduced TRAP and increased AL activities.	No	Adult larval	(144, 145)
BGJ398	FGF-receptor kinase inhibitor	–	Reduced sp7 positive osteoblasts in elasmoid scales resulting in impaired scale growth	No	Adult	(92)
BML-2832 library	Alkaline phosphatase inhibitors	+/–	Six catabolic and two anabolic compounds affect larval mineralization of the vertebral region.	Yes	Larval	(143)
BMP-2a	BMP pathway	+	Increased *sp7:luciferase* activity on cultured scales	No	Adult	(38)
Botulinum toxin	Botox muscle paralyzes	–	Lower BMD and bone deposition in fin ray bones due to muscle paralysis. Impaired osteoblast differentiation.	No	Adult	(132)
Cobalt chloride	Down-regulation of stem cell markers	–	Reduced number of osteoblasts and subsequent mineralization of the operculum, without affecting its size.	No	Larval	(146)
Cyclopamine and BMS-833923	Hedgehog pathway	–	Smaller scales and fins during regeneration. Scales show a lower number of osteoblasts.	No	Adult	(97, 147)
Dexamethasone	Glucocorticoids	–	Glucocorticoid pathway inducing osteoporosis (GIOP) by inhibiting osteoblast activity	No	Adult larval	(148)
DMP-PYT	BMPII-R–SMAD1/5/9	+	Increased BMP (pSMAD1/5/8(9)) and WNT signaling in 6–7 dpf larvae exposed for 4 days.	Yes, C2C12 cells	Larval	(149)
Dorsomorphin	BMPI-R–SMAD1/5/9	–	Reduced BMP (pSMAD1/5/8(9)) and ALK activity, reducing osteogenesis by inhibiting osteoblast activity.	Yes, compound libraries	Embryo Larval	(141)
Ferric ammonium citrate	Radical Oxygen Species	–	Iron overload down regulating osteogenic markers which can be rescued with *hepcidin1* overexpression	No	Adult larval	(150, 151)
High fat diet	Obesity risk factor for OP	–	Increased osteoclast activity in elasmoid scales	No	Adult	(152)
High glucose diet	Hyperglycemia OP risk factor	–	Increased osteoclast activity and peripheral bone degradation in elasmoid scales	No	Adult	(153)
Hyper-gravity	Increased loading	+	Enhanced mineralization after exposure to 3 g in a large diameter centrifuge	No	Larval	(154)
Niclosamide, Riluzole, Genistein	WNT pathway	+	Increased *sp7:luciferase* activity on cultured scales	Yes, WNT compound library	Adult	(38)
N-LLEL and anandamide	Long-chain fatty acids binding cannabinoid type receptors	+	Higher alkaline phosphatase activity and protecting effect on the alteration of bone markers induced by GIOP	Yes, on scales	Adult	(155)
Oligosaccharides	*A. bidentata* oligosaccharides	+	Dried root extract of Asian medicinal herb reducing osteoclast and increasing osteoblast activities	No	Larval	(156)
Omega-6 Arachidonic acid	Omega-6 derivative	–	Stimulating matrix metalloproteinase activity Enhanced bone turnover by increased osteoclast activity in the scale.	No	Adult	(157)
Prednisolone	Glucocorticoids	–	Glucocorticoid pathway inducing osteoporosis by inhibiting osteoblast activity	Yes, used as OP control	Adult larval	(100, 140)
R115866	Cyp26 antagonist–retonic acid metabolism	+	Hyper-mineralization of axial skeleton and phenocopying of *stockteif* mutant phenotype	No	Larval	(37)

(Continued)

TABLE 3 | Continued

Treatment	Gene/pathway	BMD effect	Primary effect	Part of compound screen?	Life stage	Citation
Retonic acid	Cyp26b1 and collagen deposition	+	Altered collagen deposition due to increased activity of Cyp26b1	No	Larval	(37, 158)
RU486	Glucocorticoid receptor antagonist	+	Used as prednisolone specificity/toxicity control–reverses its catabolic effect	No	Larval	(145)
SD-134	Inhibits legumain (LGMN) protease domain	+	Increase in larval vertebrae mineralization after 4 days of exposure (7 dpf)	No	Larval	(101)
Sodium metasilicate	Silicate ion	+	Silicate ion stimulating osteoblast function	No	Larval	(159)
SU5402	FGF-1 receptor antagonist	–	Impaired osteoblast proliferation in amputated fins	No	Adult	(33)
Swimming exercise	Bone loading	+	Zebrafish performed controlled exercise in a tunnel have a higher vertebrae BMD compared to non-exercising fish	No	Adult	(160)
Tanshinol	D(þ)b-3,4-dihydroxyphenyl lactic acid	+	Herbal extract reducing oxidative stress and reduction of glucocorticoid induced osteoporosis phenotype.	No	Larval	(148)
Teriparatide	Teriparatide (parathyroid hormone)	+	Human osteoporosis treatment increases mineralization in GIOP fish.	No	Larval	(161)
Vitamin D3	Cholecalciferol and calcitriol	+	Enhanced mineralization in prechordal sheet and cleithrum due to altered calcium uptake.	No	Larval	(146, 161)

can arrayed in multi-well plates and exposed to pharmacological compounds. As the scale is thin, osteoblasts are accessible to osteogenic factors, and have been demonstrated to react in a dose dependent manner to BMP-2 (38). To model an OP-like phenotype, individuals can easily be exposed to prednisolone / dexamethasone (glucocorticoid pathway) (140, 148), ferric ammonium citrate (150, 151), or metabolically with a high fat or glucose diet (152, 153), see also **Table 3**. In the context of glucocorticoid induced OP (GIOP), the bisphosphonate Alendronate reverses the effects of prednisolone on *ex vivo* cultured elasmoid scale bone, which showed a reduction in osteoclast activity (measured by TRAP) and an increase in bone anabolism (measured by alkaline phosphatase activity) (144); the same response as in mammals (163, 164). As fat metabolism has been implicated with OP, a small fatty acid derivative library was used on GIOP adult fish. Biochemical assays on scales derived from these fish showed that cannaboid receptor 2 binding anandamide and N-linoleoylethanolamine (N-LLEL) fatty acids drive osteogenesis by stimulating alkaline phosphatase (ALK) activity (155).

A WNT-pathway compound library was tested to identify new osteo-anabolic compounds using an assay in which luciferase was expressed under control of the *sp7* promoter allowing a quantitative readout of osteoblast activity (**Figure 6**). This screen identified three osteo-anabolic (**Table 3**) and 15 osteo-catabolic compounds from 85 trial compounds (38). This library contained five previously published compounds tested *in vivo*, and nine tested *in vitro* mammalian bone progenitor cell lines. Strikingly, this scale luciferase assay was able to reproduce the effect of all *in vivo* tested compounds and about half of all *in vitro* tested compounds (38). These studies demonstrate the exciting potential that scale assays represent for testing of skeletal compounds relevant to OP in a cost-effective manner.

POTENTIAL DRUG DISCOVERY PIPELINE FOR OSTEOPOROSIS

Recently, there has been a substantial expansion in the quantity of high-quality genetic data from large-scale human genomic and transcriptomic studies that contain potential osteo-anabolic factors. Here we describe a potential screening pipeline that makes use of the genetic tractability and imaging in zebrafish to offer a relatively low cost, high-throughput option compared to traditional *in vitro* and *in vivo* models (**Figure 7**).

After identification of several candidate genes/drug targets from human genetic studies, the pipeline consists of two experimental arms that can be carried out simultaneously to generate primary pre-clinical data to validate the putative drug targets. Using genome editing, loss-of function studies can be performed in transgenic backgrounds to test the effect of the gene of interest on the developing skeleton or on mineralization, and simultaneously allowing safety testing for deleterious effects on other tissues or organs. For example, using CRISPR/Cas9 editing, it is possible to generate hundreds of mosaic zebrafish mutants within 3–4 weeks (includes the generation of the targeting reagents), which is difficult to achieve in other available systems, such as cultured chondrocytes and osteoblasts (differentiation of

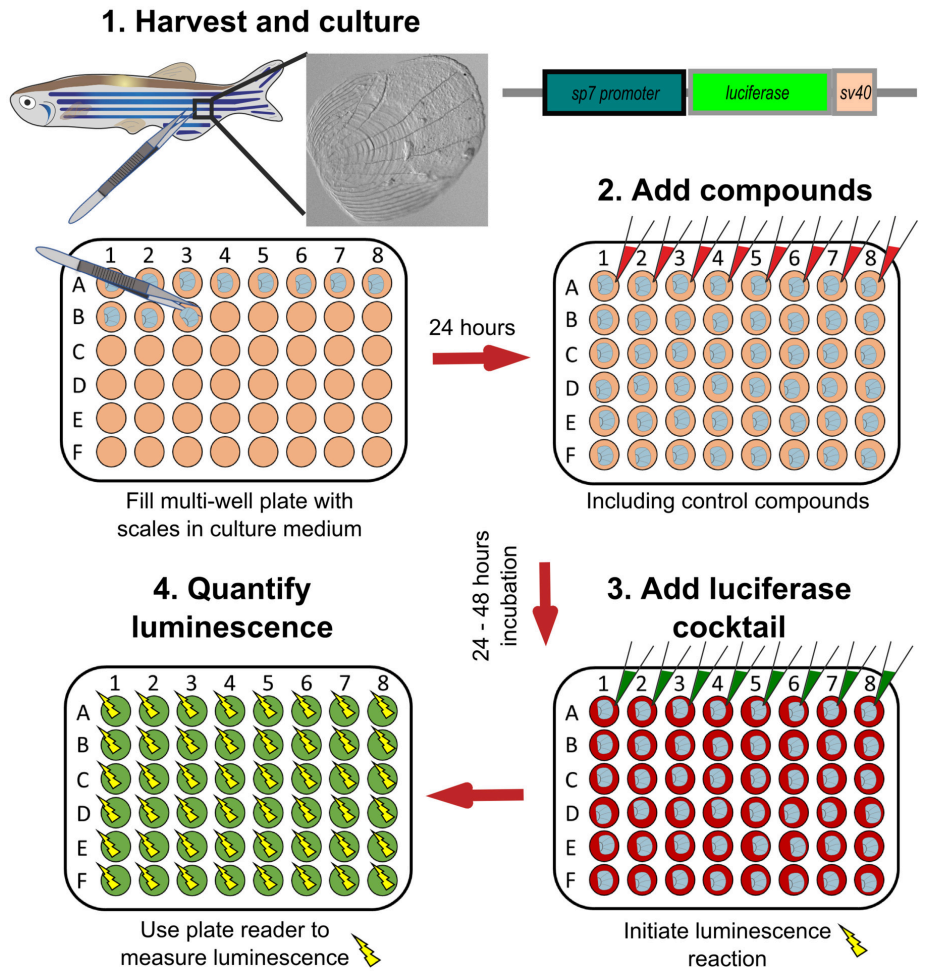

FIGURE 6 | Schematic representation to show how osteoblast activity can be quantified from scales**.** Scales from *sp7:luciferase* transgenic reporter fish are harvested from the lateral flanks of a fish, then cultured in multi-well plates with DMEM culture medium (orange wells) at 28°C for 24 h. Compounds of interest can then be added (red wells) to the scales and incubated prior addition of a luciferin cocktail (green wells) and measurement of luciferase activity with a luminescent (yellow sparks) plate reader. Based on text from de Vrieze et al. (38).

these takes multiple weeks). With CRISPR/Cas9 editing it is also feasible to study the specific human disease mutation in zebrafish, as long as it is in a conserved coding region. These fish can be grown to adulthood and germline mutations identified allowing more detailed studies on the mature skeleton to be performed (**Figure 7**).

In addition to genetic studies, pharmacological assessment of the identified putative drug target can be performed. By using water-soluble compounds, or lipid soluble compounds dissolved in DMSO, screens in a multi-well format can be performed using *ex vivo* culture of elasmoid scales. As a single adult fish has around 200 scales (138), this assay allows testing of many compounds, including control compounds (e.g., osteo-anabolic (alendronate) and -catabolic (prednisolone), on scales harvested from a single individual, reducing intra-individual variation (38). Therefore, this technique offers a platform to generate a primary read-out of novel osteo-active compounds in the context of homeostasis in a mature tissue. Additionally, this *ex vivo* technique will reduce the number of (potentially harmful) compounds being exposed to living fish, therefore contributing to ethical refinement and reduction of experimental animal use, but also reducing associated costs. As this scale assay reduces the number of putative osteogenic compounds substantially, these positive compounds can be further validated (along with safety testing) on developing transgenic larvae. These larvae would be plated out at 3 larvae per well and the compounds added from 3 days of development, with high-content imaging used for preliminary assessment of the effects of each compound and more detailed analysis including dose response followed up for validated positive hits. Further downstream tests, such as fin regeneration or fracture assays, can further reduce the number of compounds as such that only high-confidence compounds will be assessed in tetrapod pre-clinical studies (**Figure 7**).

If desired, the two experimental arms can be performed simultaneously, so that stable mutants are being generated during the compound testing phase. This opens the possibility to perform pharmacogenetic experiments in a relatively short time frame to validate the effects of putative drugs on specific disease mutations to see if they can "rescue" the disease phenotype (**Figure 7**). Together, zebrafish offer the potential in future to

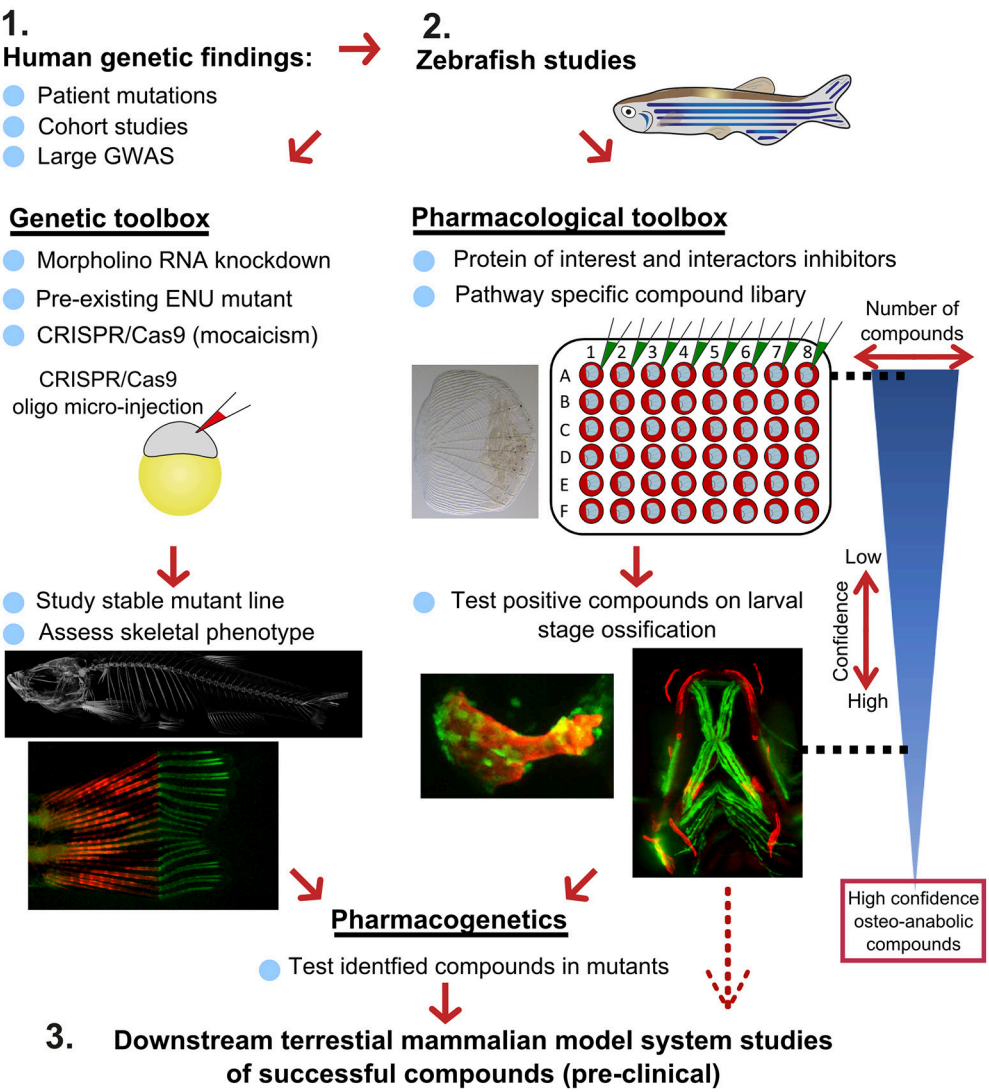

FIGURE 7 | Proposed pipeline using zebrafish as a primary testing platform to address bottleneck for fast and affordable translation of human genetic findings. Two experimental arms using the genetic and pharmacological toolboxes allow simultaneous drug target validation. The blue reversed triangle depicts the reduction in number of putative osteo-anabolic compounds (along with an increase in confidence) when testing the compounds using the skeletal assays available.

bridge the gap between human genetic hits, and fast functional validation.

PROSPECTS FOR ZEBRAFISH IN OSTEOPOROSIS RESEARCH

The zebrafish is a well-established increasingly used animal model for studying various diseases including (congenital) metabolic bone diseases (165). As zebrafish have historically been mainly used for its fast-embryonic development properties to better understand disease onset, zebrafish aging studies have only recently been conducted to model age-related diseases such as OA and OP. OP is an emerging field in zebrafish modeling and more research is needed to fully establish an OP-like phenotype as it was previously determined in its teleost cousin medaka (34). The advantageous properties as set-out in this review should be further exploited to benefit drug development for OP. Zebrafish show the appropriate response to increased mechanical loading, where the cellular (transcriptional) response initiates increased bone formation and mineralization in the loaded bone elements that are easily quantified (154, 160). However, since zebrafish and mammalian bone morphology show some differences (64), a pharmacological assay should particularly focus on the complex tissue and osteoblast-osteoclast interactions that underpin OP pathology. As traditional rodent and *in vitro* co-culture both have limitations to pursue large-scale drug discovery in a genetic context, zebrafish can take the place as a primary testing platform and therefore opening avenues to work toward gene specific compound discovery that have been identified as risk factors

in human genetic studies. After primary safety testing, these identified compounds can be further tested in mammalian OP models to determine the effect on BMD, bone strength, and trabeculation. Fully exploiting these opportunities by using zebrafish as a primary screening model will open exciting avenues to perform pharmacogenetics for OP on a larger scale.

ETHICS STATEMENT

Zebrafish procedures were approved by the University of Bristol Animal Welfare and Ethical Review Body (AWERB) and performed in accordance with a UK Home Office project license.

AUTHOR CONTRIBUTIONS

DB and EK generated the figures for the manuscript. DB, EK, and CH researched and drafted the manuscript together.

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REVIEW
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New Insights Into Monogenic Causes of Osteoporosis

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Osteoporosis, characterized by deteriorated bone microarchitecture and low bone mineral density, is a chronic skeletal disease with high worldwide prevalence. Osteoporosis related to aging is the most common form and causes significant morbidity and mortality. Rare, monogenic forms of osteoporosis have their onset usually in childhood or young adulthood and have specific phenotypic features and clinical course depending on the underlying cause. The most common form is osteogenesis imperfecta linked to mutations in *COL1A1* and *COL1A2*, the two genes encoding type I collagen. However, in the past years, remarkable advancements in bone research have expanded our understanding of the intricacies behind bone metabolism and identified novel molecular mechanisms contributing to skeletal health and disease. Especially high-throughput sequencing techniques have made family-based studies an efficient way to identify single genes causative of rare monogenic forms of osteoporosis and these have yielded several novel genes that encode proteins partaking in type I collagen modification or regulating bone cell function directly. New forms of monogenic osteoporosis, such as autosomal dominant osteoporosis caused by *WNT1* mutations or X-linked osteoporosis due to *PLS3* mutations, have revealed previously unidentified bone-regulating proteins and clarified specific roles of bone cells, expanded our understanding of possible inheritance mechanisms and paces of disease progression, and highlighted the potential of monogenic bone diseases to extend beyond the skeletal tissue. The novel gene discoveries have introduced new challenges to the classification and diagnosis of monogenic osteoporosis, but also provided promising new molecular targets for development of pharmacotherapies. In this article we give an overview of the recent discoveries in the area of monogenic forms of osteoporosis, describing the key cellular mechanisms leading to skeletal fragility, the major recent research findings and the essential challenges and avenues in future diagnostics and treatments.

Keywords: early-onset osteoporosis, Wnt signaling, osteogenesis imperfecta, PLS3, bone metabolism

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INTRODUCTION

Bone Health

Bone is a rigid connective tissue composed mainly of organic components (90% type I collagen, the rest other non-collagenous structural proteins and cells) and inorganic minerals (mostly calcium hydroxyapatite). These combined give bones their sturdiness to withstand an individual's weight and the elasticity to enable movement and resist fractures. Bone comprises dense and compact cortical bone and cancellous, loosely-webbed trabecular bone, and serves as a reservoir for minerals, growth factors, cytokines, and fat. Bone also functions as an endocrine organ by secreting several systemic hormonal factors (1).

Bone is all but a quiescent tissue—it undergoes active renewing and remodeling throughout life. By coupled, successive processes of bone resorption and bone formation, together called "bone turnover," old or damaged bone is eroded and replaced by new bone to maintain healthy and strong bone tissue. Throughout childhood and adolescent growth, the period of bone mass accrual, bone turnover is formation-favoring, until the highest amount of bone mass, termed "peak bone mass," is attained by young adulthood. Thereafter, bone mass remains fairly constant until bone resorption begins to dominate by the age of menopause and bone mass slowly declines.

Factors that impede skeletal growth in childhood or accelerate bone loss later in adulthood, such as long-term or chronic illnesses, glucocorticoid treatment and other medications, hypogonadism and menopause, other endocrine disorders and cancers, impose a great risk for low bone mass and osteoporosis (1–3). In childhood, especially glucocorticoids play a major role in secondary osteoporosis. Studies on patients receiving systemic steroids for acute lymphoblastic leukemia (4), juvenile idiopathic arthritis (5, 6), Duchenne muscular dystrophy (7) or asthma (8) all indicate increased peripheral and vertebral fracture rates.

Osteoporosis

Osteoporosis is a chronic skeletal disease with high prevalence and mortality worldwide. It is characterized by low bone mass and bone mineral density (BMD), and by destructed bone microarchitecture that often results from imbalanced bone formation and resorption or from abnormal matrix. Impaired bone quality leads to compromised bone strength and high propensity to low-energy fractures in long bones and vertebrae (9). Osteoporosis, with frequent fractures, pain and physical limitations, causes significant human suffering and burdens the health care system (9). BMD is considered to define osteoporosis and risk of fractures. It is assessed using dual-energy X-ray absorptiometry (DXA), where reduction of more than 2.5 standard deviations from the normal mean for young adults (T-score) is diagnostic of osteoporosis. Of note, osteopenia (T-score 1.0 to −2.5) together with a high probability of fractures, or a fragility fracture without another metabolic bone disease and independent of BMD are also clinically indicative of osteoporosis. Pediatric osteoporosis requires more than mere DXA-determined low BMD, as variation in growth and pubertal maturation make interpretation of BMD values challenging. Therefore, age-, gender-, and body size–adjusted DXA measurements (Z-scores) must be considered together with fracture history. A pathologic fracture history entails (i) $\geq$2 clinically significant long bone fractures by age 10 years, (ii) $\geq$3 clinically significant long bone fractures by 19 years, or (iii) one or more vertebral compression fractures in the absence of high-energy trauma, meaning a $\geq$20% loss in vertebral anterior, middle or posterior height. However, a vertebral compression fracture alone suffices for the diagnosis of pediatric osteoporosis even in the presence of normal BMD (2, 9–12).

GENETICS IN BONE HEALTH

Genetics in Bone Health

Genetics play a substantial role in determining an individual's skeletal strength, bone microarchitectural properties and risk of osteoporosis. BMD is known to be a highly heritable trait and twin studies have shown genetic factors to determine up to 80% of its variance (13, 14). Genetic factors influence bone health in a polygenic manner and multiple gene variants, or single nucleotide polymorphisms (SNPs), in several different genes each contribute to the overall risk for compromised bone health. Recent research, especially large-scale genome-wide association studies in large cohorts, has elucidated the complexity of genetic networks that are important for bone metabolism but also evidenced limitations in our current knowledge. On the other hand, significant scientific advances have been made by studying rare monogenic forms of osteoporosis in which one mutation in a single gene with a major role in bone metabolism dominates and is alone sufficient to cause osteoporosis. Technical advancements in research methods, especially high through-put sequencing techniques, have made family-based studies an efficient way to identify new genes relevant to osteoporosis. Such studies have enabled recognition of novel molecular mechanisms and given leeway to understanding the intricacies behind bone metabolism (13, 14). In this article we only briefly summarize GWAS methodology and recent advancements while the main focus is on discoveries made from family-based research on patients and families with monogenic forms of osteoporosis.

Genome Wide Studies to Identify Contributing Genetic Factors

Genome wide association studies (GWASs) have proven successful and robust in deciphering the genetic mechanisms underlying complex diseases, including osteoporosis (14, 15). As mentioned, single nucleotide variants (SNVs) in several different genomic sites all contribute to bone quality and strength and risk of osteoporosis but are often very common in the general population and, by themselves, have only a minor effect (16). The current GWAS catalog, released in September 2018, comprises 55 separate studies focusing on bone properties, fractures or osteoporosis. Together they report 425 different lead SNVs, in 118 different genomic regions, that associate with some aspect of bone on a genome-wide significant level (**Figure 1**). From these, altogether 144 different genes are reported to be directly linked to, or plausible candidate effectors, for the identified signals. Of note, this catalog is not entirely up to date due to the extensive curation required

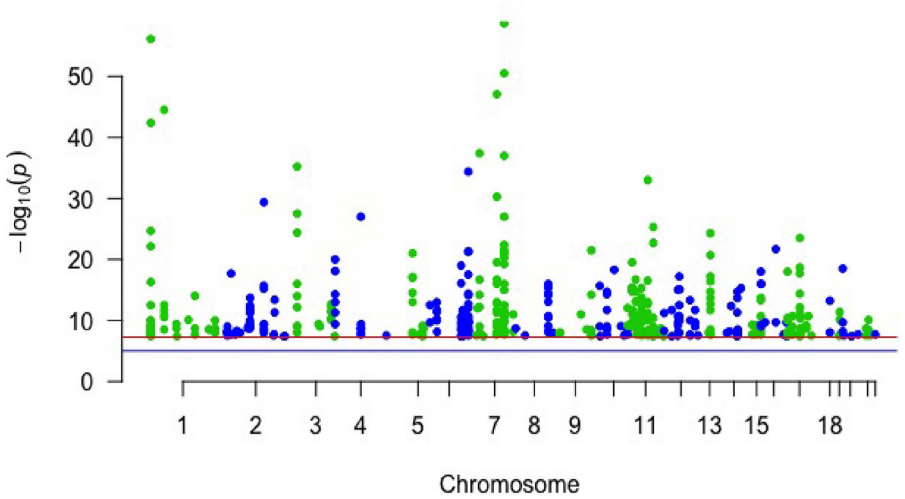

FIGURE 1 | Manhattan plot displaying all lead SNPs independently associated with bone-related traits reported to the GWAS catalog as of September 2018. The associated SNPs highlight genomic regions important to bone. However, they each have only a minor effect on an individual's skeletal qualities and risk of osteoporosis and hence have limited use in clinical practice.

before publication in the GWAS catalog. Recently, Kemp et al. undertook a colossal genome-wide search for genetic factors correlating with BMD, estimated from quantitative ultrasound of the heel (eBMD) (17). The GWAS is the largest to date, encompassing a total of 142,487 individuals from the UK Biobank. The authors were able to identify 203 loci, of which 153 were novel, to be associated with eBMD. These together explained about one third of the total variance in eBMD (17). Although highly successful, none of the previous GWA-studies with DXA-derived BMD have been as successful as the study by Kemp et al.

Despite these great advances, thus far, only <10% of the total estimated genetic variance in BMD can be explained by the results of the performed GWA-studies (18–21). Further, GWASs have predominantly been successful in identifying common variants with a small effect size (**Figure 1**), which, while giving insight into bone biology, have no clear or direct clinical relevance. However, recent GWASs utilizing whole genome sequencing (WGS) data have been able to identify variants with larger effect sizes. In the largest DXA-derived GWAS to date, Zheng et al. showed that rs11692564, a non-coding SNP around 50 kb downstream of *EN1*, had an estimated effect size of +0.20 SD for lumbar spine BMD (19). Leveraging the Icelandic sequencing initiative with WGS data for >2,000 individuals, rare variants can be imputed and assessed. These data have enabled low frequency variants with large effect sizes for BMD in *COL1A2* and *LGR4* to be identified (22, 23). Several genomic loci, identified through common genetic variation, have also been linked to genes known to underlie monogenic forms of skeletal pathology. In a large meta-analysis on BMD conducted by Estrada et al. (18), the authors were able to identify 60 genes likely to underlie the association signals. Of these, 13 genes (22%) had been implicated in monogenic skeletal disorders and 27 genes (45%) had a corresponding knockout mouse with a skeletal phenotype (14, 18). This demonstrates that even though the signals picked up by GWASs might indicate a weak effect from the measured variation, it is likely that rare and more damaging genetic variations in the same genomic locus might have a large effect. The genomic areas implicated in these GWASs are therefore likely to be of greater importance than the individual signal divulges (24).

While considering the great success of GWASs, the results need to be interpreted in light of the studied trait. Fracture is the most clinically relevant outcome measured, while BMD represents perhaps the best proxy as it is still considered the main determinant for bone strength, and the main diagnostic measurement for osteoporosis (10, 25). BMD measured by quantitative ultrasound (QUS) of the heel (eBMD) can be used as a cost-effective alternative for BMD and is also independently associated with fractures (ISCD Official positions, 2015). The correlation between BMD and eBMD is, however, not very strong (17, 26). Even the DXA-derived BMD is a blunt measurement for bone health and fracture prediction and needs to be considered with other diagnostic parameters when clinically evaluating a patient's skeletal health (27).

Recent Advances in Genetic Research

As mentioned, several monogenic forms of osteoporosis have been described. Osteogenesis imperfecta (OI) is the best-known form of monogenic osteoporosis and comprises a

heterogeneous family of different heritable bone dysplasias with skeletal fragility (28). Parallel to new developments in genetic methodology, new gene discoveries in variable forms of monogenic osteoporosis have been made and, to date, the list of genetic causes of OI and monogenic primary osteoporosis comprises altogether 19 genes (**Table 1**). The novel genetic findings have considerably enhanced our understanding of the complexities of bone metabolism and uncovered new molecular pathways that regulate bone metabolism and contribute to skeletal pathology. They span beyond the collagen-related pathways to include signaling cascades regulating bone cell function and the extracellular matrix, as described in detail below. The great variability in clinical features and inheritance patterns emphasize the importance of a molecular diagnosis in these patients.

PATHS TO MONOGENIC OSTEOPOROSIS

Defects in Bone Cell Function and Bone Remodeling

Normal osteoblast and osteoclast functions are key to sustaining healthy bone tissue. Bone resorption by osteoclasts and formation by osteoblasts are tightly linked in successive repetitive cycles at specific bone sites and the processes are meticulously controlled by several locally produced and circulating systemic factors (29). Communication between the

TABLE 1 | Different molecular mechanisms and genes underlying osteogenesis imperfecta.

Pathophysiological mechanism	Gene	Protein	Inheritance	Number of known mutations	OMIM (Phenotype MIM number)
Defects in collagen type I synthesis, structure, folding, post-translational modification, processing and cross-linking	*COL1A1*	Collagen alpha-1(I) chain	AD	>1,000*	166200; 166210; 259420; 166220
	COL1A2	Collagen alpha-2(I) chain	AD; AR°	>600*	259420; 166210; 166220
	CRTAP	Cartilage-associated protein	AR	32*	610682
	PPIB	Peptidyl-prolyl cis-trans isomerase B; cyclophilin B	AR	17*	259440
	P3H1	Prolyl 3-hydroxylase 1	AR	69*	610915
	FKBP10	Peptidyl-prolyl cis-trans isomerase FKBP10	AR	38*	610968
	PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	AR	10*	609220
	SERPINH1	Serpin H1	AR	9*	613848
	BMP1	Bone morphogenetic protein 1	AR	11*	614856
Defects in other proteins leading to abnormal bone mineralization	*SPARC*	SPARC; osteonectin	AR	2*	616507
	SERPINF1	Pigment epithelium-derived factor (PEDF)	AR	38*	613982
	IFITM5	Interferon induced transmembrane protein 5	AD	2*	610967
	PLS3	Plastin 3	XLD	17	300910
Defects in osteoblast differentiation and function	*TMEM38B*	Trimeric intracellular cation channel type B	AR	6*	615066
	WNT1	Proto-oncogene Wnt-1	AR	35*	615220
	SP7	Transcription factor Sp7; osterix	AR	2*	613849
	CREB3L1	Cyclic AMP-responsive element-binding protein 3-like protein 1	AR	3*	616229
	MBTPS2	Membrane-bound transcription factor site-2 protease	XLR	2	301014
Unknown	*TENT5A* (also known as *FAM46A*)	Terminal nucleotidyltransferase 5A	AR	3	617952

AD, autosomal dominant; AR, autosomal recessive; XLD, X-linked dominant; XLR, X-linked recessive.

°*Seen only in a few consanguineous families.*

**Information taken from the Osteogenesis imperfecta & Ehlers-Danlos syndrome variant databases.*

osteoclast and osteoblast is crucial for balanced bone turnover and defects in either cell's function can jeopardize bone health. Osteoblasts express the receptor activator of nuclear factor kappa-B ligand (RANKL), which binds to its conjugate receptor RANK on osteoclast cell surface (**Figure 2**) (30, 31). This activates osteoclastogenesis and osteoclastic bone resorption. Osteoblasts also secrete osteoprotegerin (OPG) that serves as a decoy receptor for RANKL to inhibit RANKL-RANK–binding, therefore downplaying RANKL's osteoclastogenesis-promoting effect and, as its name implies, protecting bone from over-resorption (**Figure 2**) (30, 31). Recently, RANK was also noted to relay back by vesicular trafficking from mature osteoclasts to osteoblasts to promote bone formation by reverse signaling (32). The significance of the RANK-RANKL–communication is portrayed in several monogenic conditions with abnormal bone mass resulting from defective RANK-RANKL-OPG–axis: osteoclast-poor osteopetrosis with excessive bone formation due to mutated RANKL, juvenile Paget's disease with osteopenia and progressive skeletal deformity from mutated OPG, and familial expansile osteolysis (FEO) with osteolytic lesions and increased bone remodeling from mutated RANK (33–35).

Alongside osteoblasts and osteoclasts, osteocytes have emerged as key regulators of bone turnover, mineral homeostasis and hematopoiesis (36). Osteocytes are terminally differentiated osteoblasts embedded throughout the mineralized matrix. They communicate with each other and other cells through an extensive network of long cytoplasmic dendritic processes and are thought to orchestrate the interplay between osteoblasts and osteoclasts in bone modeling and remodeling by sensing mechanical loading and responding to endocrine factors, and blood calcium and phosphate concentrations (37). Osteocytes express a range of proteins, such as dentin matric protein 1 (DMP1), phosphate-regulating neutral endopeptidase on chromosome X (PHEX), and matrix extracellular phosphoglycoprotein (MEPE), that are crucial for local matrix mineralization (38). Osteocytes are the primary source of sclerostin, RANKL, and fibroblast growth factor 23 (FGF23), through which osteocytes exert their endocrine functions in bone (**Figure 2**) (36, 38).

The WNT pathway has a key role in all aspects of bone health—from fetal skeletal development to childhood bone mass accrual to adult bone homeostasis and microarchitectural sustenance (39). WNTs act locally by activating adjacent cells' WNT signaling in a paracrine manner: in developmental stages to partake in the cross-talk between osteoblasts and hematopoietic stem cells (HSCs) in bone marrow and promote bone cell development, differentiation and proliferation, and later in mature adult bone, to induce osteoblastic bone formation (39). WNTs can also act by autocrine means by regulating cells of the same osteoblast or osteoclast lineage (40). The activated pathway is anabolic to bone, leading to increased bone formation and decreased bone resorption. Three different WNT pathways are recognized: the canonical pathway (WNT/β-catenin pathway), the non-canonical planar cell polarity pathway, and the non-canonical WNT/Ca^{2+} pathway. While the latter two, also known as the β-catenin-independent pathways, participate in a range of development process and in bone metabolism, the canonical WNT/β-catenin pathway is considered the predominant pathway maintaining skeletal health (41).

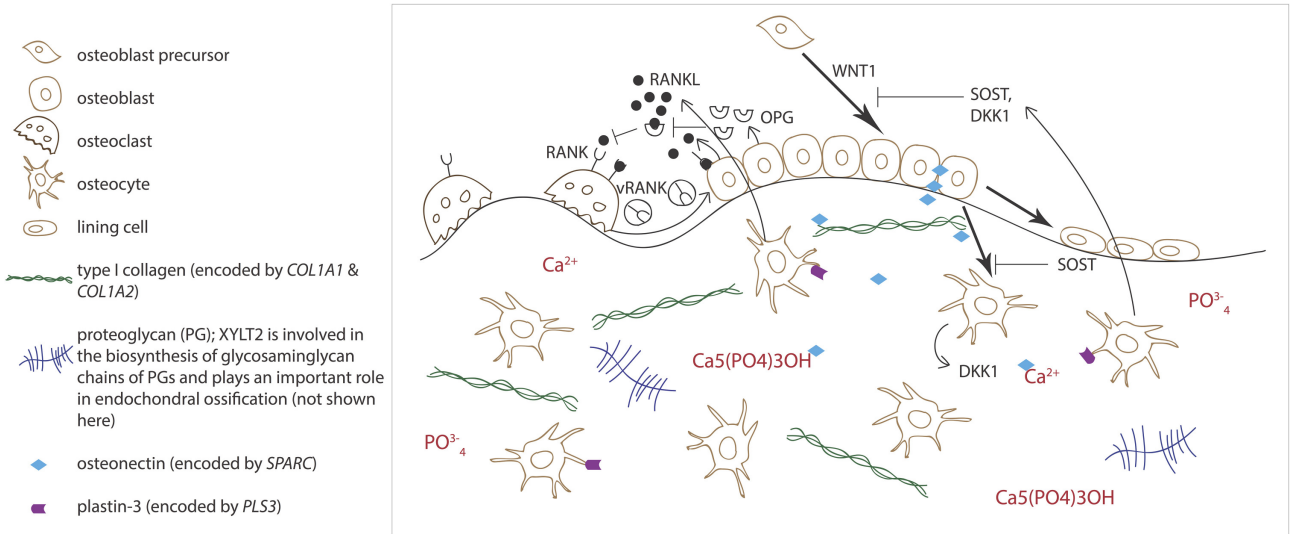

FIGURE 2 | Schematic overview of bone cells and extracellular matrix components involved in regulating bone homeostasis. Receptor activator of nuclear factor kappa-B ligand (RANKL) binds to its conjugate receptor RANK on osteoclast cell surface to stimulate osteoclast differentiation and activity. Osteoprotegerin (OPG) inhibits RANK/RANKL-binding to inhibit bone resorption. WNT signaling pathway stimulates osteoblast function and bone formation. Sclerostin (SOST) and dickkopfs (DKK1), produced by the osteocytes, are two WNT antagonists that promote osteoclasts differentiation. Osteonectin, produced by the osteoblasts, binds calcium, hydroxyapatite and collagen type I and thus regulates bone mineralization. Plastin-3 (PLS3), expressed by the osteocytes, may also be involved in the mineralization of the extracellular matrix but its role in osteoprogenitors and other bone cells is yet to be confirmed.

Dysregulated WNT/β-catenin signaling leads to various skeletal disorders of both high and low bone mass. This was first recognized in 2001 when mutations in low-density lipoprotein 5 (*LRP5)*, encoding a coreceptor for WNT ligands, were found to lead to low bone mass in the autosomal recessive osteoporosis pseudoglioma syndrome (OPPG, MIM 259770), characterized by early-onset severe osteoporosis and blindness (42, 43). The *LRP5* mutations inhibit normal WNT signaling and lead to reduced osteoblast proliferation and function and subsequently decreased bone formation (43). Since then, many other mutations in *LRP5* have been shown to cause OPPG (44). In addition, functionally significant SNPs in *LRP5* have been linked to adolescent bone mass accrual and peak bone mass (45, 46), and genome-wide searches have found common *LRP5* polymorphisms that contribute to population-based variance in BMD, confirming its significant role in osteoporosis risk also in the general population (14, 18). The molecular mechanisms by which these missense mutations in *LRP5* decrease WNT signaling, however, remain largely unknown (46, 47). Conversely, inadequate WNT inhibition from mutations or deletions in the sclerostin-encoding *SOST* results in high bone mass phenotypes sclerosteosis (MIM 269500) and van Buchem disease (MIM 239100), respectively (48, 49). In the absence of sufficient sclerostin, WNT signaling is unrestrained, leading to continuous bone formation.

All in all, 19 different WNT proteins are known and together they initiate several intracellular signaling cascades to regulate organogenesis, cell fate determination, primary axis formation, and stem cell renewal (39). Several of the WNT proteins are expressed in bone tissue and regulate bone health at various phases during skeletal growth, development, and e.g., osteoporosis pathogenesis (50). For example, WNT16 is considered an important ligand in bone WNT signaling and has been shown to mediate its bone-specific actions via both canonical and non-canonical WNT pathways (51). Although the specifics behind its mechanisms are unclear, GWASs show that polymorphisms of the *WNT16* locus associate with cortical bone thickness, BMD, and osteoporotic fracture risk in large observational studies and variations in *WNT16* may also impact individual peak bone mass (18, 52, 53). These findings are echoed in *in vivo* studies as *Wnt16* KO mice have reduced cortical thickness and bone strength leading to spontaneous peripheral fractures (54).

In 2013, several groups identified WNT1 as a key ligand to the WNT pathway in bone; heterozygous *WNT1* mutations were reported to cause autosomal dominant osteoporosis, and homozygous mutations, a more severe osteogenesis imperfecta (55). Since then, various other mutations have been found worldwide, all reporting skeletal morbidity with frequent and childhood-onset peripheral and vertebral compression fractures and successive changes in spinal stature (55–61). In our comprehensive clinical analyses of a large cohort of 25 *WNT1* mutation-positive subjects with the same heterozygous missense mutation p.C218G, the aberrant WNT1 signaling results in a severe skeletal pathology (62). In addition to prevalent fractures, long bone modeling is altered and BMD low in affected children, while vertebral compression fractures are very common later in adulthood and result in severe kyphotic deformity and loss of adult height soon after the age of 50 years (**Figure 3**). Bone biopsy histomorphometry demonstrated low-turnover osteoporosis with scarce and inactive bone cells and stagnant bone turnover. Noted extra-skeletal traits included changes in spinal cartilaginous structures, namely vertebral endplate

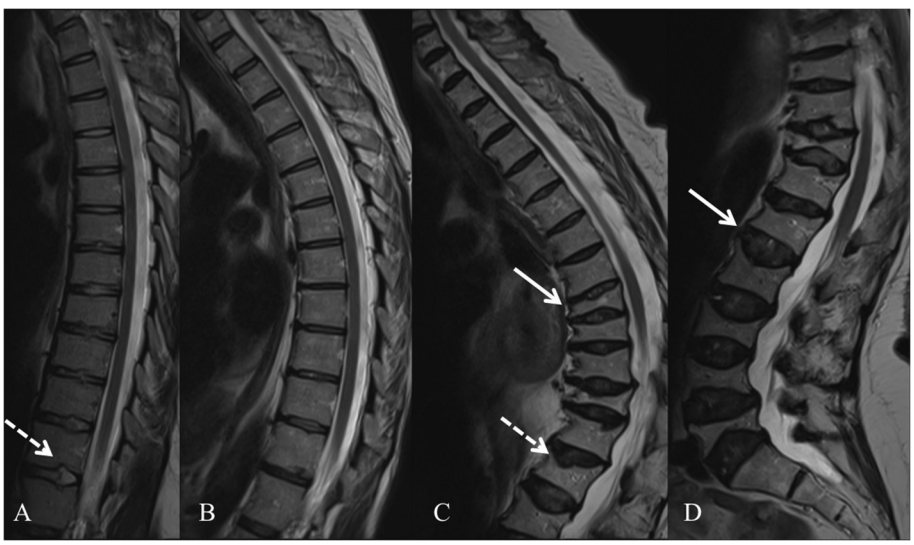

FIGURE 3 | Spinal magnetic resonance images of four *WNT1* p.C218G mutation-positive subjects. **(A)** Thoracic spine of a 17-years-old female showing multiple Schmorl nodes (arrow). **(B)** Thoracic spine of a 44-years-old female showing exaggerated thoracic kyphosis. **(C)** Thoracic spine of a 76-years-old male showing several compressed vertebrae, kyphotic stature, and Schmorl hernia (arrow). **(D)** Lumbar spine of a 74-years-old female showing several compressed vertebrae and enlarged intervertebral discs (arrows). Reprinted from Mäkitie et al. (63) with permission from Elsevier.

deterioration and frequent Schmorl nodes, and increased reticulin and early-phase–shifted granulopoiesis as signs of abnormal bone marrow function (63, 64).

The latest finding of dysregulated WNT signaling in monogenic osteoporosis is *SFRP4* mutations in Pyle's disease (65). Frizzled-related protein 4 (SFRP4) acts as an WNT inhibitor and biallelic, truncating mutations in its encoding gene *SFRP4* result in aberrant regulation of WNT signaling, osteoblasts and osteoclast function and bone remodeling (65). The patients' clinical phenotype is predominated by cortical-bone thinning and fragility and expanded metaphyseal trabecular bone, resulting in limb deformity and high propensity to fracture. Correspondingly, *Sfrp4*-null mice present with increased trabecular bone, decreased cortical bone and failure in bone modeling (65).

Despite their important functions, known monogenic forms of bone diseases stemming from osteocyte defects are rare and often relate to defective mineral metabolism, especially hypophosphatemia due to disturbed FGF23 regulation. One of the most recently identified monogenic forms of osteoporosis is caused by mutations in the PLS3 gene (66–70), encoding the actin binding, actin bundling protein plastin 3. This X-linked form of primary early-onset osteoporosis is characterized by low BMD, frequent peripheral fractures and vertebral compression fractures, and subsequent severe thoracic kyphosis. Due to its X-chromosomal inheritance, male patients are more severely affected, usually presenting with severe childhood-onset osteoporosis. Clinical manifestations in females with heterozygous *PLS3* mutations are variable ranging from subclinical osteopenia to a more severe phenotype resembling that of males' (68). The total number of diagnosed patients is still scarce and hence the comprehension of the clinical and genetic spectrum, the disease progression and appropriate treatment is limited.

While the role of PLS3 in bone fragility is yet unknown, one theory presumes PLS3 to alter osteocyte function through abnormal cytoskeletal microarchitecture. Plastins, in general, are Ca-dependent actin binding and bundling proteins and as such, are involved in cytoskeletal arrangements and partake in regulating cellular morphology, motion, and adherence (71). Despite lack of systematic studies, plastin 3 (also called T-plastin) is supposedly expressed in all solid tissues and through indicated functions in other tissues, such as spinal muscle, inner ear stereocilia, and periodontal ligaments, is suggested to be involved in bone mechano-transduction (72–74). This is supported by the high expression of plastin 3 in chicken osteocyte dendrites, especially during dendrite formation (**Figure 2**) (75–77). Although this is supported by clinical investigations from biochemical and bone biopsy findings indicating that osteocytes appear affected in *PLS3* mutation-positive subjects (78), the observation remains mostly theoretical.

Another suggested role for PLS3 in bone is involvement in mineralization. This is collectively supported by the patients' low BMD and their bone biopsies' histology. We have reported accumulation of non-mineralized osteoid in trabecular bone in patient biopsies (69, 70, 78, 79) and shown that biochemical markers of bone turnover, although not directly echoing the mineralization process, are normal despite altered bone formation (68). The detailed mechanisms of bone tissue mineralization are still debated, but extracellular mineral deposition through budding off of intracellular microvesicles has emerged as one part of the process (80). This process requires dramatic changes in the cell membrane through a complex and well-orchestrated process involving the actin cytoskeleton. Thouverey et al. (81) and Piehl et al. (82) have demonstrated congruently that plastin 3 is involved in the formation of extracellular vesicles. It can thereby be speculated that *PLS3* mutations could have deleterious effects on the mineralization process in bone through defective microvesicle formation, although the details behind this too remain undisclosed.

Lastly, a recent experimental animal study presented new findings suggesting involvement of osteoclast malfunction as part of pathophysiology in PLS3 osteoporosis (83). *In vivo* and *in vitro* studies using *Pls3* knockout and overexpressing mice confirmed the osteoporotic phenotype in the former and thickening cortical bone in the latter. *In vitro* studies of osteoclasts derived from the animals demonstrated a regulatory role of PLS3 in osteoclastogenesis. Additionally, a dysregulation of osteoclast activity was found in cells from *Pls3* knockouts, likely connected to impaired podosome organization due to decreased actin regulation (83). These findings are yet to be confirmed in humans.

Defects in Bone Extracellular Matrix

In addition to bone cells, reduced bone strength and various skeletal disorders can also stem from defects in the extracellular matrix (ECM). The ECM is primarily composed of different collagenous proteins, non-collagenous proteins (in particular glycoproteins and proteoglycans), lipids, minerals and water (84, 85). The most abundant protein is the type I collagen, made of two alpha-1 and one alpha-2 chains intertwined in a triple helical structure. Mutations in the encoding genes, *COL1A1* and *COL1A2*, respectively, lead to qualitative or quantitative defects in the protein and give rise to osteogenesis imperfecta (OI), a skeletal dysplasia characterized by low BMD and enhanced bone fragility, and often extra-skeletal features, such as blue sclerae, dentinogenesis imperfect, and hearing loss (86, 87). Heterozygous glycine substitutions that affect the Gly-Xaa-Yaa pattern in the triple helix are the most common mutations and can cause mild to lethal OI (87). However, multiexonic deletions or deletion of an entire allele have been sporadically found (88–91). Interestingly, mutations that lead to a reduced amount of normal protein give rise to a milder phenotype than missense mutations affecting the primary structure of the triple helix (dominant negative effect) (87). Furthermore, homozygous glycine substitutions in *COL1A2* have been identified in a handful of consanguineous families (92–95). Surprisingly, the patients harboring biallelic *COL1A2* mutations have a moderate to severe phenotype whereas the mutation carriers are only mildly affected or free from any obvious skeletal impairment. On the other hand, homozygous *COL1A1* mutations are likely to be lethal since they have never been reported in humans. Furthermore, some previous

reports have indicated that when the *COL1A1* or *COL1A2* mutation involves the C-propeptide cleavage site, the phenotypic manifestations may include high BMD and mild skeletal fragility (96). A recent study on such cleavage site variants showed that the mutations lead to a distinctive OI phenotype with variable expression, mild to moderate disease severity, moderate fracture rate, high bone mass and increased bone mineral density (97).

Although *COL1A1* or *COL1A2* mutations are detected in ∼85% of OI cases, to date, mutations in altogether 17 other genes are also known to cause OI-like skeletal disorders (**Table 1**). Some of these genes play a role in the post-translational modification of type I collagen while some are key regulators of osteoblast differentiation and function and/or lead to abnormal bone mineralization (**Table 1**). One example of severe autosomal recessive OI caused by a mineralization defect is linked to mutations in *SPARC* (98). The encoded protein Secreted Protein Acidic and Cysteine Rich, better known as osteonectin, is a glycoprotein that is mainly expressed by osteoblasts during bone formation and binds calcium, hydroxyapatite and collagen type I and other proteins in the ECM (**Figure 2**). Null mutations in *SPARC* lead to reduced accumulation of type I collagen in the ECM (99). Furthermore, the osteonectin-type I collagen complex is suggested to sequestrate calcium and phosphate in order to initiate bone mineralization (100). An impairment of two other proteins expressed by the osteoblasts, the pigment epithelium-derived factor (encoded by *SERPINF1*) and the interferon-induced transmembrane protein 5 (encoded by *IFITM5*), respectively, can also compromise bone mineralization and lead to OI (86, 87, 101, 102). Most recently, mutations in *FAM46A*, encoding the terminal nucleotidyltransferase 5A, have been detected in four patients with OI. However, the molecular function of this protein and the pathophysiological mechanism by which the mutations lead to OI are not yet known (103).

Besides OI, there are several other skeletal syndromes that feature osteoporosis and are caused by defects in the ECM. For example, mutations in *XYLT2* lead to spondyloocular syndrome characterized by childhood-onset osteoporosis, cataract, cardiac defects and hearing impairment (104–106). The mutated protein xylosyltransferase 2 is involved in the biosynthesis of glycosaminoglycan chains and plays an important role in endochondral ossification and chondrocyte differentiation and maturation. Proteoglycans are also important for other tissues and organs, including brain, heart, and retina, which could explain why the clinical manifestations of spondyloocular syndrome are not only restricted to the skeleton (106).

In addition to causing autosomal recessive OI, inadequate folding and post-translational modification of type I collagen can result in another skeletal syndrome characterized by congenital contractures, named Bruck syndrome. Homozygous mutations in *FKBP10* and *PLOD2* result in Bruck syndrome 1 and 2, respectively (107–110). *FKBP10* encodes the immunophilin FKBP65, a molecular chaperon of type I collagen and *PLOD2* encodes the procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2, which catalyzes the hydroxylation of lysyl residues in type I collagen. Mutations in both *FKBP10* and *PLOD2* can also cause autosomal recessive OI (**Table 1**).

TOOLS FOR DIAGNOSING MONOGENIC OSTEOPOROSIS

Uncovering the Genetics

As discussed, to make the diagnosis of osteoporosis in children two criteria need to be met; (1) low BMD or BMC (Z-score ≤ -2.0 SD) and (2) a clinically significant fracture history. A vertebral fracture indicates severely compromised bone strength and suffices alone for the diagnosis (12). The diagnosis of primary osteoporosis in children can be made when potential causes of secondary osteoporosis, such as other underlying illnesses or medical treatments, have been excluded (2). Most forms of childhood-onset primary osteoporosis are termed osteogenesis imperfecta, although the diagnosis is vague and merely appoints the disease to belong to a heterogeneous group of skeletal disorders with diverse clinical presentation (86, 87). As indicated earlier, the genetic background of OI is heterogeneous and the phenotypic and genetic variability have complicated OI classification. As of yet, there is no consensus indicating which genotype-phenotype combinations should be classified under the umbrella of OI and which should not. The current classification of OI is based on phenotypic features, but the molecular cause is often the key factor determining clinical prognosis, appropriate treatment approach and recurrence risk in the family, and should therefore be emphasized (28). A molecular diagnosis also facilitates the refinement of future treatment and clinical care protocols (87, 111). Although more than 85% of OI cases can still be traced to pathogenic variants in either of the two collagen type I–coding genes *COL1A1* or *COL1A2* (112, 113), the several other genes identified over the past 12 years in OI or monogenic forms of primary osteoporosis need to be kept in mind (92, 114, 115).

While most clinicians begin by screening *COL1A1* and *COL1A2* possibly in combination with MLPA, proceeding to a full OI gene panel using massive parallel sequencing is recommended (87). A sequencing-based gene panel will not only capture sequence variants but also possible structural variations including larger deletions and duplications. Although the surge of new genetic findings has facilitated interpretation of sequence variants, deep intronic splice variants or splice variants masked as synonymous variants are still difficult to correctly annotate. Transcriptome analysis using RNA sequencing together with DNA sequencing has proven successful in increasing the diagnostic yield and assessing functional impact of variants that are otherwise hard to interpret (116). This, however, requires that the disease in focus has a readily accessible proxy tissue, where the gene expression reflects the expression in the affected tissue. Unfortunately, tissue accessibility is very difficult in bone diseases and the method cost-restricted in clinical settings.

Regarding structural variants, WGS has provided an advantage in assessing structural variants compared to exome sequencing or other capture-based protocols. However, all short-read sequencing technologies have shortcomings in their ability to detect and identify structural variants, and, as concluded by Telenti et al. (117), after sequencing 10 000 human genomes the interpretation of structural variants on an individual level still remains challenging. Older methods to indirectly detect

structural variations, such as array-based comparative genomic hybridization (array-CGH), are still applicable in specific cases and can help clinicians in their search for a molecular diagnosis (91).

Clinical Characterization

Owning to the wide spectrum of genetic causes, the clinical presentation of different OI and primary osteoporosis forms is unsurprisingly miscellaneous (87). The diseases vary in their primary skeletal traits, age-at-onset, natural progression, sensitivity to treatment, and presence and spectrum of extra-skeletal characteristics. Although severely compromised bone strength is usually a unifying finding, the DXA-derived BMD, bone biopsy findings, prevalence and type of fractures, and radiographic findings are inconsistent. The phenotypic severity can vary from mild to severe and disease onset from childhood to early adulthood—at times provoked by pregnancy-related calcium loss. Presentation may vary between patients with different mutations and even between family members with identical mutations (87). Classical OI-related extra-skeletal findings include blue sclerae, increased joint laxity, dentinogenesis imperfecta and impaired hearing (28, 87, 118). Mutations in proteins affecting the collagen-related pathways all seem to exhibit similar traits; only the severity and array of affected skeletal sites vary. Some typical presentations include popcorn epiphyseal plates in CRTAP, calcifications of interosseous membranes and hyperplastic callus formation in IFITM5, and skull ossification defects in SEC24D-related OI (**Table 1**) (86, 87, 118). The extra-skeletal manifestations of bone cell-related forms are still incompletely defined; with monoallelic *WNT1* mutations patients have changes in spinal cartilaginous structures (63) and mild abnormalities in bone marrow hematopoiesis and reticulin formation (119), while in biallelic mutations the phenotype is more severe and OI-like but no bone marrow defects have been reported (55). However, central nervous system manifestations have been reported in some patients with homozygous *WNT1* mutations (55, 61). Patients with *PLS3* mutations do not exhibit any apparent extra-skeletal traits, though this is still scantily explored.

Novel Biomarkers

In addition to DXA and plain radiography, several factors can be measured from systemic circulation and urine when diagnosing and monitoring patients' disease state, progression and treatment response. The conventional metabolic markers reflect bone turnover and consist of enzymatic and proteinaceous by-products; the most widely used resorption markers include mainly by-products of collagen breakdown, [urinary collagen type 1 cross-linked N-telopeptide (NTX), urinary/serum collagen type 1 cross-linked C-telopeptide (CTX), and collagen fragments from matrix-metalloproteases (ICTP)], and formation markers procollagens from collagen synthesis [serum amino-terminal propeptide (PINP) and carboxyl-terminal propeptide (PICP)] or osteoblast-related proteins (serum osteocalcin (OC) and serum bone isoenzyme of alkaline phosphatase (ALP) (120, 121). While these markers are commonly used and easily analyzed in automated routine laboratories, they do lack specificity and are easily confounded by other patient-related (e.g., body adiposity, inflammation, blood glucose level, time of sampling) and analytical factors. Furthermore, they often respond inadequately to bisphosphonate treatment and correlate poorly with BMD and bone histomorphometric parameters (55, 120–125). None of the monogenic forms of osteoporosis have a specific biomarker profile and these conventional markers are of little value in differentiating between the various genetic forms of osteoporosis.

The limitations of the conventional bone markers have fueled a field-wide search for new potential biomarkers. Zooming into smaller cell-released particles, small microRNAs (miRNAs), as one, have attained much attention and are proposed to hold promise in future diagnostic and treatment in skeletal disorders. These small, non-coding fragments of RNA are highly conserved and comprise, on estimate, 1% of our genome (126, 127). They alter gene expression by RNA silencing and post-transcriptional regulation; each miRNA is predicted to regulate hundreds of different target genes, thus serving important functions in many tissues and biological processes (127, 128). While their exact function in gene regulation is still largely unknown, miRNAs are thought to mediate intercellular communications in various metabolic processes and diseases and a unique imprint of differentially expressed miRNAs is observed in e.g., certain cancers, metabolic diseases and viral infections. In bone, miRNAs contribute to homeostasis and their dysfunctional expression relays to progression of skeletal disorders (129, 130). Their expressions change in result of low BMD, frequent fractures, or menopausal osteoporosis (129, 130).

These findings have encouraged researchers to explore the clinical potential of miRNAs in disease diagnostics and follow-up. Several clinical studies have evaluated miRNA expression in osteoporotic patients and distinguished specific miRNAs correlating with the degree of osteoporosis (131). miR-133a was significantly elevated in postmenopausal Caucasian women with low BMD (132), and miR-194-5p and miR-21-5p negatively correlated with BMD in Chinese osteoporotic women (133, 134). Seeliger et al. (135) also identified miR-21-5p, in addition to four other miRNAs (miR-23a-3p, miR-24-3p, miR-100-5p, and miR-125b-5p) to be differentially expressed in serum and upregulated in bone tissue in patients with osteoporotic fractures. *In vitro* studies have observed miRNAs that interact with known key regulators of bone metabolism, such as miR-152-3p and miR-335-3p with Dickkopf-1 (136, 137), miR-30e-5p with Lrp6 (138), and the aforementioned miR-133 with Runx2 (139). Furthermore, Anastasilakis et al. (140) reported that serum levels of miRNAs changed in response to anti-osteoporotic treatment. While different studies pinpoint to varying miRNAs depending on cohort size, demographic or other factors, a clear congruency is echoed that a unique miRNA signature is observed in osteoporosis.

We have reported altered miRNA pattern in patients with WNT1 osteoporosis, with two upregulated and six downregulated miRNAs, as compared with age and sex-matched mutation-negative controls from the same family (119). While specific miRNA alterations may be recognized in certain monogenic forms of osteoporosis, the role of miRNAs in complementing or substituting genetic testing remains to be

explored in future studies. Further, the utilization of miRNA assessments in clinical practice demands further methodological development but based on present data, they hold great potential for future diagnosis and follow-up, including monogenic forms of osteoporosis.

OPTIONS FOR TREATMENT

Conventional Osteoporosis Drugs and Implications for Treatment

Conventional osteoporosis drugs, namely bisphosphonates, have been the mainstay of pharmacological treatment in classical, type I collagen-related OI forms. These typically have high bone turnover and thus the osteoclast-targeting and resorption-decreasing bisphosphonates have proven effective in increasing BMD, reducing fractures, and improving VCFs in patients (141–143). Contrary to collagen I-related OI, bisphosphonates have proven insufficient in improving BMD or fracture tendency in several new forms of primary osteoporosis (55, 57, 60). These OI forms often present with low-turnover osteoporosis and hence the benefits of anti-catabolic treatment are not optimal. We have also shown that patients with prior bisphosphonate treatment have abnormal and apoptotic osteocytes, suggesting adverse effects of bisphosphonates in WNT1 osteoporosis (63). However, our longitudinal study on the effects of teriparatide-treatment in WNT1 osteoporosis indicated that exogenous PTH may be efficient in increasing bone formation and BMD during a 24-months-long treatment in adults; however, there may be simultaneous increase in bone marrow adiposity (79). Thus far, the efficacy of anti-sclerostin antibodies have been experimented in mice only; subcutaneous administration of Scl-Ab to the murine model of WNT1 OI *Wnt1*$^{sw/sw}$ mice significantly improved fracture rate and increased bone mass that seemed to result from increased osteoblast activity (144).

Besides WNT1-related skeletal pathologies, even less is known about the optimal treatments in other new forms of primary osteoporosis and OI, such as PLS3 and XYLT2 (105, 145). Efficacy of bisphosphonates in PLS3 osteoporosis has been evaluated in a handful of cases and indicate positive response (66, 67, 70). Our above-mentioned clinical study on teriparatide also included *PLS3* mutation-positive subjects and they showed congruent, although slightly lesser, improvement in bone parameters in 24-months follow-up, as compared with patients with WNT1 osteoporosis (79). Patients with *XYLT2* mutations seem to benefit from pamidronate treatment with increase in BMD and improvement in vertebral morphology (104, 105).

Clinical care of OI patients, including both classical and newer forms of OI and monogenic osteoporosis, is often complex and challenging. Means of treatment and pace of clinical follow-up are dependent on the patient's age, clinical manifestations, and degree of impairment, and should be individually tailored and regularly evaluated. Bisphosphonates are still the main treatment option for pediatric patients and are often used to prevent greater decrease in BMD and enable maximum yield in bone mineral throughout childhood and adolescent bone mass accrual. The overall benefits of bisphosphonate treatment in most cases of OI are non-negligible (146). Variable treatment protocols exist. Clinical care and follow-up are advised to be centered in special health care units with abilities to provide multidisciplinary care and expertise.

Novel Target-Drugs

Discoveries through rare, monogenic forms of skeletal disorders have provided new information on the biology of bone health and revealed previously unidentified proteins that take part in key regulatory pathways. Naturally, these proteins also present as appealing target molecules for development of new treatment modalities. In early 2000s, inhibition of RANKL by a monoclonal antibody denosumab brought a novel approach for treatment of osteoporosis (147). The drug has been used to improve skeletal health in some forms of OI. Particularly patients with *SERPINF1* mutations show a modest increase in BMD in response to denosumab whereas treatment outcomes with bisphosphonates are poor (148). Due to the coupled nature of osteoblast-osteoclast–activity, blocking osteoclastogenesis through RANKL is also unfavorably accompanied by reduced osteoblast function. The previously mentioned discovery of RANKL reverse signaling could offer a novel solution to avoid this problem (32). Also, inhibition of cathepsin K, an osteoclast-derived lysosomal enzyme, seemed promising due to its coupled bone formation-favoring action, but its development was later discontinued due to increased risk of cardiovascular complications (149). As of recently, the effects of anti-TGF-β antibodies have been studied in *Crtap*$^{-/-}$ and *Col1a1*$^{frt/-}$ mice with varying results; while the *Crtap*$^{-/-}$ showed great improvements in bone mass and biochemical qualities, *Col1a1*$^{frt/-}$ mice did not show significant changes in bone quality or strength (150).

Along with the discovery of van Buchem disease and sclerosteosis, two human models of sclerostin inhibition, fueled the development of a new anabolic target drug named romosozumab—a monoclonal anti-sclerostin antibody targeting the WNT pathway (151, 152). Its efficacy has been evaluated in several clinical trials with promising results; a placebo-controlled, multicenter, phase II study on 419 postmenopausal women with osteoporosis treated with subcutaneous injections of romosozumab at 3-months intervals showed significant, and superior to those attained by alendronate and teriparatide, increase in areal BMD and a tilt in BTMs reflective of increased bone formation (151), and another phase III study reported a reduction in fracture risk in 7,180 postmenopausal osteoporotic women (153). Anti-DKK1 antibodies act similarly to oppose WNT signaling and are potent as osteoanabolic agents. However, administration of anti-DKK1 is only mildly efficacious as the WNT-neutralizing effect is compensated by upregulation of sclerostin, although the opposite is not seen when given only anti-sclerostin antibodies. Thus, the benefits of anti-DKK1 antibodies manifest only when given in conjunction with anti-sclerostin (154).

Another target of interest for new drug development is Notum. It is a secreted enzyme that inhibits WNTs by removing the palmitoleic acid group that is essential for binding of WNTs to Frizzled receptors, thereby inhibiting WNT signaling. Interestingly, experimental studies in rodents have shown that inhibiting Notum through either knockout, or by oral

administration of molecular inhibitors or neutralizing antibodies increase cortical bone formation and strength, but do not affect trabecular bone mass (155, 156).

Possible undesired adverse and extra-skeletal effects of new drugs are inevitable as many of the targeted proteins have tissue-wide expression and key roles in various biological processes. Side effects can be latent and subtle but also challenging and life-threatening. Knowing the WNT pathway's fundamental role in embryonic development, tumorigenesis and pathogenesis of other systemic or chronic diseases, romosozumab has been under careful scrutiny for its clinical safety. In mice receiving different doses, no malignancies were noted over a 98-weeks follow up (157). However, along with the robust and positive skeletal effects, use of romosozumab has been associated with cardiovascular and cerebrovascular events, and the drug is currently under FDA review (Amgen and UCB).

MicroRNAs

Recently, researchers have acknowledged the opportunities in targeting miRNA pathways to develop new therapeutic means and genome editing approaches (128, 158). A few groups have pursued clinical trials to evaluate efficacy of miRNAs in disease target treatment: an on-going clinical trial evaluates the anticancer effect of miRNA lethal-7 in binding to Kirsten rat sarcoma viral oncogene homolog (KRAS) gene in patients suffering from stage III colon cancer, and miR-122 in hepatitis C (159, 160). Bone-specific miRNAs have not been evaluated clinically, but analyses have shown that for example *in vitro* miR-21 could promote osteogenesis in bone marrow stem cells, and systemic administration of miR-214 induced BMD increase and miR-92a enhance fracture healing in mice (161–163). In fracture healing, also angiogenesis is vital to the repair process and Li et al. (164) were able to demonstrate that implantation of MSCs transfected with an angiogenesis-involved anti-miR-26a showed good bone repair. Further, anti-miR-31-transfected MCSs efficiently repaired bone defects by increasing BMD and new bone volume (165). These findings and the efficacy, safety and possible side effects need to be confirmed and carefully evaluated in clinical settings *in vivo*.

CONCLUSIONS

Recent advances in genetic methodology have resulted in several new discoveries relating to the genetic architecture of bone homeostasis. Not only have the basic clinical and genetic pillars of classical OI been refined, but several new forms of monogenic osteoporosis have also been identified that have pinpointed novel molecular mechanisms contributing to skeletal health and disease. The clinical presentation, inheritance mode, natural course and response to conventional osteoporosis drugs are diverse, often variable and logically dependent on the affected protein. Although uncovering the limitations in our current diagnostic and treatment modalities, they have also provided new signaling pathways that hold promise in new targeted drug development. Future research will hopefully continue expanding the genetics and molecular mechanisms behind bone metabolism and increasing our understanding of the specific skeletal and extra-skeletal characteristics of monogenic osteoporosis, while finding new avenues for improved diagnosis and treatment of patients with severe bone diseases.

AUTHOR CONTRIBUTIONS

RM and OM initiated the manuscript. RM wrote the first draft. All authors contributed to the writing and approved the final manuscript.

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Mouse Models and Online Resources for Functional Analysis of Osteoporosis Genome-Wide Association Studies

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Osteoporosis is a complex genetic disease in which the number of loci associated with the bone mineral density, a clinical risk factor for fracture, has increased at an exponential rate in the last decade. The identification of the causative variants and candidate genes underlying these loci has not been able to keep pace with the rate of locus discovery. A large number of tools and data resources have been built around the use of the mouse as model of human genetic disease. Herein, we describe resources available for functional validation of human Genome Wide Association Study (GWAS) loci using mouse models. We specifically focus on large-scale phenotyping efforts focused on bone relevant phenotypes and repositories of genotype-phenotype data that exist for transgenic and mutant mice, which can be readily mined as a first step toward more targeted efforts designed to deeply characterize the role of a gene in bone biology.

Keywords: osteoporosis, mouse models, genetics, bone, functional validation

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INTRODUCTION

The NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy defined this disease as, "*a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture*" wherein bone strength was defined as the combination of bone mineral density (BMD) and quality (1). In the year 2000 it was estimated that there were 8.9 million osteoporotic fractures worldwide (2) and existing data suggests that, on average, half of all women and 20% of all men will experience a facture in their adult life (3). The economic burden of osteoporosis is immense, resulting in up to $22 billion in direct health care costs per year in the U.S (4) and €37 billion annually in the European Union (3). Further, osteoporotic fractures are associated with increased morbidity and mortality (5). Bone mineral density, as measured by Dual X-Ray Absorptiometry (DXA), is inversely correlated with fracture risk. For this reason, BMD remains the method used to diagnose this disease clinically. It is estimated that over 50% of the variation in BMD is attributed to genetic factors (6), but importantly in humans, fracture risk is also heritable (7).

Since the first genome wide association study (GWAS) for BMD in 2007 (8), there has been an explosion in the number of loci found to be associated with BMD, bone structure and fracture risk. The largest GWAS conducted to date suggests that there are over 1,000 conditionally independent genetic signals in 515 discrete loci associated with the phenotype of estimated bone mineral density (eBMD) (9). Fracture is inherently a more complicated phenotype, and 14 significant loci were

identified in this same study (9). In a second large meta-analysis GWAS, 15 loci were identified as associated with fracture incidence, but all of these loci had been previously found to be linked to traditional DXA derived BMD (10). These data highlight the incredible complexity involved in the genetic regulation of BMD and the difficulties associated with accounting for the genetic regulation of clinically important phenotypes such as fracture incidence.

THE CAUSAL VARIANT VS. THE CANDIDATE GENE

Despite the identification of this astonishing number of loci, these 515 eBMD loci only account for only 18% of the trait variance (9), suggesting that there may yet be more loci to be discovered. Further, one must remain cognizant of the fact that a locus does not equal a mechanism of action. Much of the focus of the so-called "post-GWAS era" is on identifying the underlying gene or genes, pin pointing the causative variant(s) and determining the hows, the whats, the whys, and the whens by which these loci act and interact to cause a phenotype (11). Ideally, every nucleotide in every person would be examined in a GWAS for association between genotype and phenotype. In practice, this is rarely possible due to cost, and fortunately, it is not completely necessary. Over short distances, single nucleotide polymorphisms (SNPs) are often in linkage disequilibrium (LD) with other nearby SNPs (12). It is common practice in GWAS to select representative SNPs or "tagging SNPs" for genotyping, which in turn are used to represent a haplotype (13–15). This tagging genotyped SNP is a proxy for the causative variant and may or not have any functional role in disease.

Overwhelmingly, the causative variant for a given genetic locus is not located within the coding region of a gene, and even more rarely is the causative variant one that leads to an altered protein product. Rather, causative variants are often located in intergenic regions and are thought to modify the expression of one or multiple genes (16). Thus, the term "causative variant" is not to be confused with, nor is it synonymous with, the term "causative gene" (11). Understanding the nature and mechanism of action of the causative variant is critical for understanding the etiology of disease. A case in point is the comparison of two Mendelian conditions: Van Buchem disease and Sclerosteosis Type I. In both of these conditions, a thickening of the cortical bone, narrowing of the medullary canal of the long bones and thickening of the mandible are observed (17, 18). However, gigantism is seen in Sclerosteosis (17), but not in Van Buchem disease. In Van Buchem disease, a 52 Kb deletion occurs in an intergenic region on human Chromosome (Chr) 17q21.3 (19) and putatively impacts expression of two genes: *MEOX1* and *SOST* (20). For Sclerosteosis, up to 10 homozygous loss-of-function mutations in the coding region of the *SOST* gene have been identified (21). Thus, in both of these diseases, there is a common gene impacted, but clinically the presentation is different, in part because the causative variant(s) leads to disease in differing ways.

Following this same theme, functional validation of GWAS candidate genes is not to be confused with the identification of the functional variant(s). The functional validation of a candidate gene means to determine if that gene could plausibly be associated with the phenotype of interest. Both functional validation of a candidate gene and determination of the causative variant are of value for understanding human disease especially when there are one or more uncharacterized genes in the locus (22). To be a candidate, a gene must fulfill two straightforward criteria. First, the gene must be expressed in the appropriate tissue(s) and at an appropriate time point to influence the phenotype of interest. Second, the gene must play a role in a biological process relevant to the phenotype of interest (11). For many diseases, the first criteria can be used to remove a surprising number of candidate genes and is therefore an easy first pass filter to narrow down to genes of interest. However, for bone, what constitutes an appropriate tissue or appropriate time point is less easy to define, yet is critical for the design of experiments to determine function (11). The reasons for this are that bone turnover, bone size and geometry, BMD and even fracture risk, are impacted indirectly by a number of other organ systems such as the digestive tract (23), brain (24), kidney (25), and skeletal muscle (26), and processes occurring during development that have lasting impacts on the adult skeleton (27). That said, the majority of validated GWAS genes impacting BMD appear to be expressed in bone tissue (9, 28). The second criteria, namely that the candidate gene plays a role in a relevant biological system, can be a little harder to ascertain, especially for uncharacterized or understudied genes for which there is little known about function. It is here that the mouse has proven to be invaluable (22), and indeed, the bulk of functional validation has been accomplished by so called reverse genetic approaches in mice.

THE GENOME OF MICE AND MAN

Mice have been used for over 100 years to study the genetic regulation of physiology, development and disease (29). Like other animal models, mice fill two specific needs particularly well: they can be used to collect phenotype data that cannot be collected from human subjects, and they can be used to study single factors (i.e., diets, alleles, ages) in isolation. The mouse genome, while smaller than the human genome, is highly conserved for protein coding genes (22). At the gene level, ∼17,094 mouse protein coding genes have a known direct human ortholog (http://www.informatics.jax.org, accessed Oct 2018), and overall organization of the mouse and human genomes is remarkably syntenic despite 75 million years of evolutional distance between the two species (30). Thus, genetic findings in mice are often concordant with genetic findings in humans (31). However, with the refinement of GWAS and improved annotation of the human genome, data is accumulating to suggest that long non-coding RNA genes also play a role in human disease (32) and not surprisingly, these non-coding genes have been found at GWAS loci for bone phenotypes (33). While homologs for long non-coding RNA genes have been found in mice for human genes (34), generally, these genes are poorly conserved (35).

DIFFERENCES BETWEEN MOUSE AND MAN IN BONE

The physiologic and anatomic similarity between mice and humans has long been appreciated, and, given the high degree of genome homology, is not surprising (31). Regardless, there are differences in the skeletal system that should be considered in a functional validation experiment. In mammals other than mice, lamellar bone is organized into Haversian systems or secondary osteons in which lamellar bone is arranged in concentric rings around a central cavity (36) whereas in mice, a Haversian system of organization is not seen (37). There are also subtle differences between mouse and human bone growth during aging. In humans, the epiphyses fuse shortly after puberty resulting in a halt in long bone growth. In mice, epiphyseal fusion either never completes or is delayed until old age (depending on the strain), and thus some strains of mice can continue to experience some degree of long bone growth to at least 2 years of age (37). In both men and women, cortical bone gain has essentially stopped in early adulthood, and a steep loss in cortical bone volume begins at menopause in women and after the age of 75 years old in men. In contrast, trabecular bone loss begins in early adulthood, irrespective of sex (38). In mice, cortical bone volume increases out to at least 7 and possibly 12 months of age (depending on strain), in part due to the increases in skeletal size that arise from continued growth (39, 40). Decreases in trabecular bone amount occur far sooner in mice than in humans. In fact by weaning, inbred mice have already begun to lose trabecular bone (41) and in outbred mice, a complete lack of trabecular bone volume in the distal femur was observed as young as 6 months of age (42). In comparison to humans, this would be the equivalence of bone loss beginning in toddlers to the point of complete loss of trabecular bone in some anatomic sites as young adults. The aggregate peak femoral volumetric BMD in mice (cortical and trabecular) is generally accepted to happen at about 16 weeks of age, but this varies by mouse strain (39). This does not mean that mice are inappropriate for the functional validation of human bone GWAS loci, but rather that experiments must be designed to ensure that appropriate comparisons in bone are being made. Justifying an age for mice in a functional validation experiment is not as simple as scaling chronological age relative to lifespan and calling it equivalent.

REVERSE GENETICS

Reverse genetics simply means to reverse engineer the function of a gene in a biological system. In contrast, forward genetics approaches such as GWAS move from a disease or phenotype to find the genetic cause. Thus, reverse and forward genetics are inseparable for the study of human disease (22). The mouse genome is easily modified, and 62,025 targeted alleles in 16,947 genes are listed in the Mouse Genome Database (http://www.informatics.jax.org/, accessed October 2018). This means that for some genes, multiple targeted alleles have been constructed and at least partially characterized but for a fraction of protein coding genes, we as of yet have no direct evidence of function gleaned from genetically engineered mouse models. The methods for generating these targeted mouse models are described in detail elsewhere (43), but what these models are and where to find both the mice and the phenotype data available for these mice is described in greater detail in the sections below.

Both global and tissue specific models have been used to functionally validate bone GWAS loci. An elegant example using both global and cell type specific models in mice is the work conducted to confirm the *WNT16* gene as a candidate gene for bone mass and fracture risk (44). In this study, the authors used both global and cell lineage specific knockout mouse models to show that WNT16, a secreted factor, is produced by the osteoblast and acts on the osteoclast precursor to inhibit osteoclastogenesis. In addition, this WNT also acts on the osteoblast to inhibit the formation of the osteoclastogenesis inhibitor Osteoprotogerin (OPG). As a result, the loss of the *Wnt16* gene globally in mice or in the osteoblast lineage only results in an increase in osteoclast-mediated bone resorption leading to reductions in cortical bone mass, but interestingly not loss of trabecular bone. Further, these mice present with spontaneous fractures of the long bones, a phenotype rarely seen in laboratory mice. Thus, this study confirmed that *WNT16* is indeed a bona fide bone gene and was able to demonstrate the mechanism of action by which fracture risk was increased.

A global knock out may not be desirable or plausible for the study a gene in adult bone biology. A case in point is the global *Runx2* knockout mouse, which dies shortly after birth presumably due to breathing difficulties (45). As is outlined in the *Wnt16* example, conditional knockouts and inducible knockouts allow one to restrict gene loss to a cell type of interest and/or after, critical development milestones have been met. Such studies require an appropriate Cre-diver strain wherein expression of Cre-recombinase is restricted to a desired cell type and/or time point in cell maturation. Ideally, this allows excision of the gene of interest in only the cell type of interest. Some of these Cre-drivers are inducible, meaning that the timing of Cre-induction can be carefully controlled. A summary of many of the bone relevant Cre-Driver strains for musculoskeletal tissues and cells that have been described in the literature are summarized here [reviewed in Elefteriou and Yang (43)]. In addition, several Cre-databases are available online which provide more up to date information about where the Cre-driver is expressed (**Table 2**). It is important to remember that Cre-drivers may be expressed in undesired tissues as well as the desired location.

PHENOTYPED MOUSE MODELS

The nascent stages of the identification of mouse models of human disease relied on the identification of outliers in a colony of mice, followed by breeding to determine heritability of the observed phenotype (46). With advances in technology, the process of finding the mutation(s) causing the phenotype has changed, but finding spontaneous mutations in mice remains a valuable source of human disease models. Many spontaneous mutations are not gene ablation models and may more closely mimic human disease than a knockout mouse (46). Relevant

to bone biology are models such as the *oim* mouse, which was discovered in a breeding colony at the Jackson Laboratory in 1985. In this mouse, a single base pair deletion in the *Col2a1* gene results in a truncated protein product (47), and phenotypically this mouse mimics aspects of Osteogenesis Imperfecta Type III (48).

A second method to generate mouse models of human disease is chemically induced mutagenesis via delivery of compounds such as Ethylnitrosourea (ENU) (46). This forward genetics approach, while successful in that many models for various diseases were generated, is laborious and inefficient as the location of the mutation(s) is random in the genome and therefore genes impacting the phenotype of interest will not be specifically targeted. While it is possible to identify recessive traits in an ENU protocol, it is much faster to restrict a screen to find traits acting in a dominant fashion. Typically, so-called Generation-0 (G0) male mice are treated with ENU to induce mutations. The G0 males are bred to wildtype females to generate so called G1 offspring, which are then screened for phenotypes of interest. Approximately 2–4% of these G1 mice will carry mutations yielding a phenotype (49). Several models relevant to bone biology have been identified this way (50–54). For example, we recently described the *tvrm111B* mutant mouse strain wherein an inactivating mutation in *Lrp5* was identified. As expected, these mice have mild decreases in bone mass, abnormalities in the retinal vasculature and other eye phenotypes, and are a model of osteoporosis pseudoglioma (OPPG) (55).

With the completion of the first draft sequence of the mouse genome in 2002 (30), sights were set on determining the function of all of the known and newly discovered genes. By this time, generating genetically engineered mice was common practice and becoming increasingly more efficient (49). This resulted in the development of two "mouse clinics" pilot programs to make new models of human disease: the Mouse Genetics Project (MGP) at Sanger in the UK and the multi-site European Mouse Disease Clinic (EUMODIC) program (56). In short, *de novo* transgenic knockout mouse models were generated, and this was coupled with the employment of high throughput, comprehensive and cost effective phenotyping pipelines to characterize these new strains. These projects largely were designed to be hypothesis free in that the genes of interest were not pre-screened to be involved in a specific disease. The goal of these clinics was 2-fold: (1) to identify new models of human disease and (2) catalog the function of protein coding genes in the mouse. These mouse clinics enjoyed economy of scale allowing for more phenotypes to be captured per animal than was previously possible in a single laboratory working in isolation (57).

The mouse clinic method identified weaknesses in the gene-by-gene study approach that had been the mainstay of determining mammalian gene function. From these preliminary proof-of-concept mouse clinics it became apparent that pleiotropy is very common, yet commonly new mouse models were only being phenotyped for traits relevant to the interests of the group making the model. This observation of pleiotropy led to the concern that incomplete information was being generated in the historical gene-by-gene approach. Further, there was concern that that inconsistent data was being collected, as the gene-by-gene approach was not held to any standardized methods for data collection (56). In contrast, the application of a systematic and high-throughput phenotyping pipeline overcame these issues wherein only "some" types of data were collected per strain and allowed enforcement of data collection standard operating procedures (SOPs) (56). Another major issue with the gene-by-gene approach is that mouse models were generated and/or maintained on a wide variety of genetic background strains, precluding straightforward comparison of one model to another because of strain background differences. Further, breeding of one model to another created the risk of passenger mutation effects (58). With all of this in mind, the "second generation mouse clinics" were carefully designed with standardized and validated SOPs developed for both animal model generation and for capturing the phenotype data. The phenotyping pipelines and the SOPs for collecting data are reviewed extensively elsewhere (59), but below some of the pros and cons of the largest of these data collections are described in the context of validating GWAS loci for bone phenotypes and disease. **Table 1** summarizes these data collections.

International Mouse Phenotyping Consortium (IMPC)

The International Knockout Mouse Consortium (IMKC) began in 2003 with the goal of making embryonic stem cells carrying a knockout allele for all protein coding genes. Indeed, embryonic stem cell (ESC) lines carrying mutant alleles were generated for 18,500 genes (60). This effort was conducted by numerous sites and programs internationally, including the Knockout Mouse Program (KOMP) in the United States. The vast majority of these mutant alleles are knockout–first and conditional-ready, meaning that by employing appropriate breeding strategies, both global gene ablation can be achieved or genes can be knocked out in a temporal or cell/tissue specific manner (61). It must be noted, though, that not all genes were knocked out in this fashion. A smaller fraction of the ESC cell lines are knockout-only (22). There many impressive aspects of this ambitious and highly successful project, but the one that is perhaps not as well appreciated by non-mouse geneticists is that all of these cell lines were created on a single genetic background, C57BL/6N (60). As a result, when animated into live mice, double- and triple-knockouts can be generated without the time consuming and costly step of breeding all lines onto a uniform genetic background before interbreeding (58). In 2011, the International Mouse Phenotyping Consortium (IMPC) was formed to conduct high-throughput, multi-systems phenotyping on the IMKC generated mice. In 2015, the efforts of the IKMC were folded into that of the IMPC, and, under the umbrella of the IMPC, mouse model generation continues. It should be noted that the use of CRISPr/Cas9 is becoming more widely adopted by the IMPC, producing global gene disruption including conditional and lacZ reporter lines. However, like the previous mutant alleles, these new models are being made on the C57BL/6N background (59).

Currently, the IMPC is comprised of 19 research institutions located in 11 countries and was funded by five national funding organizations. For the 10 year span that the IMPC was funded

TABLE 1 | Selected repositories of phenotyping data for mouse genetic models.

Title	URL	Content
BoneBase	http://bonebase.org	In-depth bone specific phenotype data for selected IMPC generated mice.
International Mouse Phenotyping Consortium	http://www.mousephenotype.org/	The website of the IMPC, including SOPs, data, and resources for ordering IMPC mice and targeted ES cells.
Origins of Bone and Cartilage Disease	http://www.boneandcartilage.com/	In-depth bone specific phenotype data for selected IMPC generated mice.
Mouse Genome Database	http://www.informatics.jax.org/	The international resource database for the mouse. Includes genomic, phenomic and gene function information.
Infrafrontier	https://www.infrafrontier.eu	Access to mouse models and data collected by mouse clinics in Europe and Canada. House the European Mouse Mutant Archive (EMMA).

to operate (2011 to 2021), five goals were laid out: (1) create a consortium capable of generating targeted mutations for 20,000 mouse genes, (2) conduct high-throughput, standardized phenotyping of these knockout lines, (3) determine the biological function of these genes, (4) create a network of secondary phenotyping consortia that can conduct additional phenotyping to enrich the primary data set, and (5) provide the means and support for free and unrestricted data disseminations for all IMPC generated data (56).

At the heart of the IMPC is the phenotyping pipeline (https://www.mousephenotype.org/impress/). This pipeline can be divided into four sections. In the first part, lines are assessed for viability and fertility in the homozygous global knockout state. Approximately one third of all IMPC knockout lines generated to date were found to be embryonically lethal (no homozygous knockout mice found after screening 28 pups from a heterozygous by heterozygous mating) or sub-viable (less than half of the homozygous knockout mice survive to weaning) (62). In recognition of this high number of non-viable lines, an embryonic pipeline is currently in development. This pipeline is envisioned to collect the duration of viability post fertilization, and histopathology and gross morphology data at multiple time points during development. In the third part of the pipeline, a robust set of phenotype data is collected covering most body systems. This adult phenotyping pipeline has been applied largely, but not exclusively, to homozygous knockout mice. This pipeline is conducted using a rigid schedule of tests starting when the mouse is 9 weeks of age and extends until the animal is euthanized at 16 weeks of age. This test battery consists of a core set of 15 tests that are conducted at all phenotyping sites using carefully developed SOPs, as well as a set of optional tests that are collected at some, but not all, of the phenotyping centers. Lastly, at euthanasia, biological specimens are collected and analyzed. Like for the *in vivo* testing, there is a core of data collected on all mice as well as optional collection SOPs (56, 59). For example, all sites must collect data regarding heart weight at death, but only some of the sites bank tissues and embed them for histopathology (59).

There are two sets of data collected on mice in the IMPC pipeline that are of primary interest to bone biologists: body composition and skeletal dismorphology (56). Body composition traits, including bone mineral content (BMC), bone area (BA), bone mineral density (BMD), lean mass and fat mass, are collected. All of these phenotypes are collected on the whole body sans the head via Dual X-ray Absorptiometry (DXA) on male and female mice at 11 weeks of age. At the same time, a simple 2D whole-body X-ray is collected, and a very comprehensive list of bone sites are examined for malformations and dismorphologies (57). The way this pipeline is set up, data is collected on each line until 7 males and 7 females per line have been examined for body composition and at least 5 males and 5 females have had X-ray images captured (https://www.mousephenotype.org). Control mice of the C57BL/6N line are run through the pipeline such that a new cohort of control mice is started through the pipeline every week and therefore, there is always concurrent control data collected for every mutant strain. The data for each mutant strain is compared to the aggregate collection of control data using a statistical analysis protocol designed to be robust to the imbalance of group sizes between the cases (mutants) and controls (63). The data is presented on the IMPC web-portal and can be screened in a number of ways. For example, an investigator can look specifically for the BMC data for their favorite strain only, search for all lines with significantly higher BMD and they can download the raw data for their own analyses.

There are many advantages of using these data for functional validation of GWAS loci. As of data release 8.0, which was announced on July 16th of 2018, phenotype data for 5,115 genes were available, which is just over 20% of all known protein coding genes in the mouse genome (https://www.mousephenotype.org). This data is freely available for use by anyone at any time and is presented in an easy-to-interpret format on the IMPC website. Further, this data can be downloaded and queried in bulk allowing one to quickly search their list of GWAS candidate genes for those with a known bone mass phenotype. At the time of writing this review, just under 300 lines (6.4% of all those tested) were annotated to have an abnormal BMD or BMC phenotype (http://www.mousephenotype.org, accessed October, 2018). Equally important, this list can be screened to eliminate genes that were tested and found not to impact any of the bone phenotypes examined. This latter step can be critical when more than one candidate exists for a single locus. Lastly, the mouse can be ordered from the IMPC to conduct additional phenotyping should an investigator choose.

There are many caveats and cautions that must be considered when using this data for functional validation. In the 7 years since the start of the IMPC, technology has advanced. There is now data available in the IMPC database from multiple different DXA scanners that range in resolution from

∼180 μm spatial resolution for the older (and now no longer commercially available) PIXImus scanners made by GE-Lunar® to ∼50 μm spatial resolution for the newer instruments from Faxitron®. While both instruments have been validated against bone ash weight standards, the superior resolution of the newer instruments may provide increased fidelity in BMD via refinement in accuracy of projected area measured (64). As a result, there will be less noise in measures such as bone area, which may or may not affect achievement of statistical significance for any mutant line.

DXA BMD in a mouse is different than that collected usually for GWAS purposes in humans. Even with the superior resolution of the newer DXA machines, in mice, these instruments are not able to discriminate between cortical and trabecular regions of interest without specialized analysis (65). It has been estimated for long bones that three quarters of the bone mass is contributed by the cortical compartment primarily in the diaphyses (66). The majority of the attenuation of the X-ray in DXA imaging for the whole body of a mouse is achieved by the cortical compartments (65). However, BMD for clinical purposes is measured in the lumbar spine, which is largely trabecular, as well as in the hip, which is proportionally more trabecular than the femoral diaphysis. While anatomic site-specific region of interest (ROI) data can be captured on the mouse DXA instruments, this data is not typically available in the IMPC database. Phenotypes that impact bone in subtle ways, such as only in the trabecular compartment or in only one anatomic site, may be missed by the IMPC screen. In this scenario, the mouse line could be mistakenly annotated as having no abnormality in bone mass.

All of the bone phenotyping in the IMPC pipeline is collected on 11 week old animals. From a sexual maturity point of view, this represents an adult animal, but from a skeletal growth point of view, these mice are still in the bone acquisition phase. As was outlined earlier, cortical bone volume can increase far past this 11 week age point (40). Thus, these 11 week old mice would likely be the equivalent of an adolescent human. It could reasonably be argued that the trends leading to lower/higher adult BMD or smaller/larger adult skeletal size will be well established by 11 weeks of age, but one should remain cognizant of what these data represent when using it to interpret and functionally validate a human GWAS locus.

While Quantitative Ultrasound (QUS) phenotypes do moderately correlate with areal BMD (67), bone architecture, and mechanical properties in humans and large animals (68), there is no directly measurable equivalent phenotype in mice for speed of sound (SOS) or Broad Ultrasound Attenuation (BUA). Because of the relationship between estimated BMD (eBMD) as determined from ultrasound measures and areal BMD from DXA (67), it is assumed that the same advantages and caveats for using IMPC data for functional validation of areal BMD GWAS loci also apply to eBMD loci. Similarly, IMPC does not contain equivalent data such as trabecular bone volume and other compartment-specific phenotypes like those captured by the ultra-high-resolution CT machines. Therefore, while the IMPC is a rich source of data, it may not have utility for functional validation for some GWAS.

As mentioned previously, the IMPC mice are generated on a C57BL/6N (N) genetic background (62). This strain is related to, but is not genetically the same as the more commonly used C57BL/6J (J) strain or other C57BL/6 strains available from other vendors. Over 200 generations of breeding have occurred since the J and the N lines were originally separated in 1951 and during that time, almost 700,000 genetic differences have accumulated between these two strains including 51 coding variants (69). It is not surprising that phenotypic differences between these strains have been observed including differences in behavior, blood pressure, metabolism and immune function. In direct comparisons of the two, it was observed that the N males and females have higher body fat whereas the J strain has increased lean body mass. The male J mice trend toward increases in whole-body BMD, but no differences in trabecular bone mass or bone turnover markers were observed for either sex (69). It is well understood that changing genetic background can modify the phenotype of knockout and transgenic mice due to modifier genes, complicating the interpretation of the impact of a gene on a phenotype (70). Direct comparisons on a uniform genetic background takes multiple generations of backcrossing to avoid effects from segregating modifiers (71). This matters for functional validation of GWAS loci as segregating modifiers present on a mixed genetic background can mask or alter the phenotypic presentation of the allele of interest, leading to inappropriate conclusions about a gene's involvement in a biological system of interest.

BoneBase

Two programs are expanding the skeletal phenotypic data available for IMPC mice. The first of these, BoneBase, is located in the US. This program received live breeder mice from The Jackson Laboratory IMPC production site to conduct in-depth skeletal phenotyping. It should be noted that this program was not part of the IMPC and received independent funding but did work with the IMPC data coordinators. Like the IMPC, this program was designed to be hypothesis-free in that lines were not a priori selected because of evidence suggesting a role in bone biology. All lines that were viable, fertile and free of profound pathologies (i.e., early renal failure, spontaneous early cancers, etc.) were accepted for this program. This program was designed to add on to, but not replicate, the data generated by the IMPC (72).

Like the IMPC, homozygous animals were used for phenotyping. Group sizes of at least 8 male and 8 female mice were phenotyped at 12 weeks of age. Two main phenotyping mechanisms were used: microCT analysis of the lumbar vertebrae and the femur, and dynamic cryo-histomorphometry (73) of adjacent lumber vertebrae and the contralateral femur. The pipeline (**Figure 1**) was set up such that if a phenotype at either anatomic site or in either sex was found, cryo-histomorphometry was conducted and this data is not available for all lines. In this manner, a wide-ranging set of data was collected capturing information on cortical bone size and shape, trabecular bone mass and architecture, bone formation, and osteoblast and osteoclast number. Also like the IMPC, rigorous SOPs were implemented at all stages of animal breeding, tissue collection, and analysis to ensure that data was collected in an unbiased

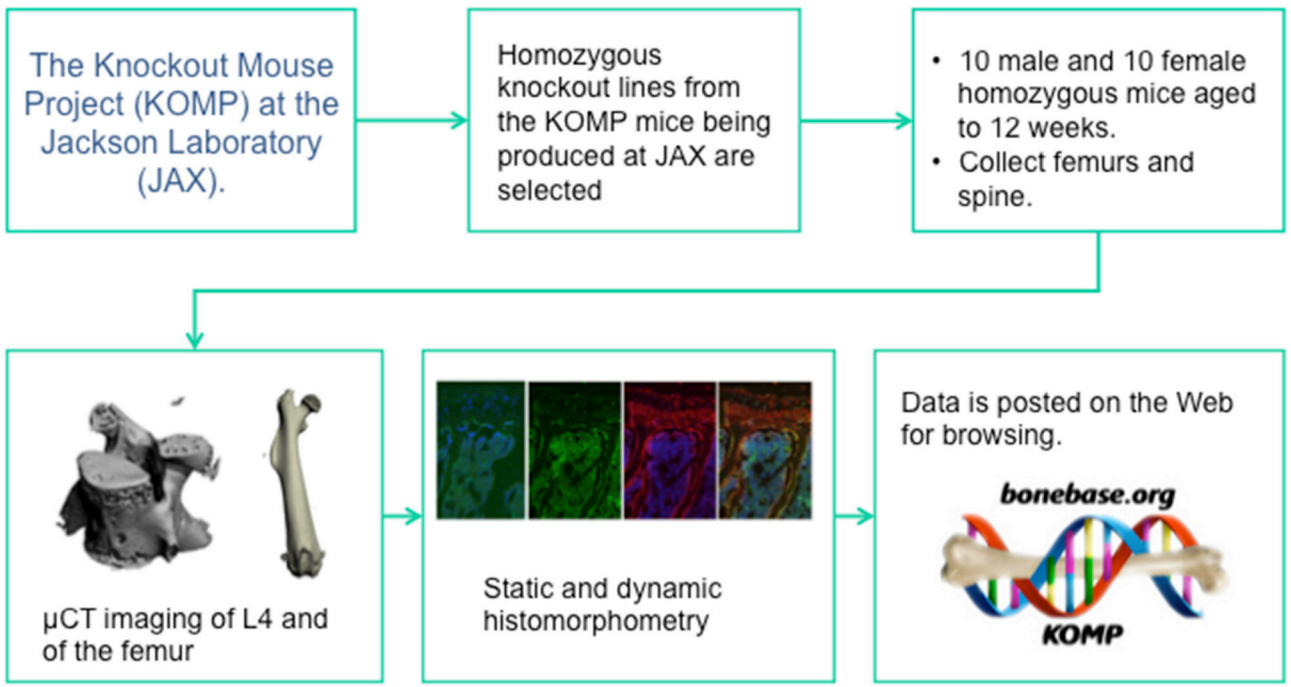

FIGURE 1 | The data collection pipeline for the BoneBase.org phenotyping project. This is one of two specialized high throughput phenotyping pipelines that is conducting auxiliary, bone specific phenotyping of mice generated by the IMPC. The Bonebase.org logo is used with permission from the database owners.

and rigorous fashion. Also like the IMPC protocol, this group collected data from C57BL/6N mice at regular intervals to ensure that concurrent controls existed for every line, however, these controls were collected monthly, not weekly (72).

For illustration purposes only, the data for a single gene examined in the BoneBase pipeline (**Figure 2**). The data presented here, which is freely available at the BoneBase web portal (Bonebase.org, accessed Oct, 2018), is for the gene *Osteoclast stimulatory transmembrane protein* (*Ocstamp*), which is not a known GWAS candidate gene. This gene is part of a growing list of genes shown to be required for the fusion of pre-osteoclasts into mature multinucleated and functional osteoclasts (74). A substantial increase in bone volume fraction (BV/TV) in the femur (**Figures 2A,B**) was observed in the female but not male mice (data not shown). A substantial increase in the amount of TRAP staining per unit bone surface (TRAP/BS, **Figures 2C,D**) but no change in bone formation rate (BFR, **Figure 2E**) was noted. Collectively, these data suggest an involvement of the osteoclast, but not the osteoblast. From this simple example, it is readily apparently how this is a valuable resource for functional validation of GWAS loci as information is available to provide confirmation that a gene impacts bone biology. In addition, putative mechanistic information is also available to provide a first tier of evidence about how a candidate gene at a locus acts to impact bone biology without the costly investment in *de novo* model construction.

It is interesting to note that while the IMPC found that 6.4% of lines presented with a bone phenotype by DXA alone (www.mousephenotype.org), the Bonebase protocol found that ∼15% of all lines presented with an increase or decrease in bone mass as determined by microCT. There was little overlap between those determined to be bone genes by the IMPC and those found by the BoneBase protocol (72). Given that whole body areal BMD obtained from DXA is largely a cortical and bone size driven phenotype (65) and that microCT can be used specifically to look only at the trabecular bone, this is not an unexpected finding. This is further supported by evidence that suggests that trabecular and cortical bone are controlled by independent genetic signals (75–77).

All of the data generated by the Bonebase project can be queried at any time via a webportal (www.bonebase.org). To date, 220 lines have been analyzed and the data for these lines is available both as summary statistics for a line (separated by sex) and as raw data available for each individual mouse (as is presented in **Figure 2**). It is interesting to note that far more anatomic site-specific effects (i.e., only in the spine or only in the femur) and sexually dimorphic effects (i.e., only in males or only in females) were found than that which has been noted for genetic loci in GWAS. This may reflect differences in mice vs. humans, or may reflect that subtle effects could be more easily detected in this repeated measures study design.

Origins of Bone and Cartilage Disease Project

The Origins of Bone and Cartilage Disease (OBCD) project is the second of two programs expanding skeletal phenotype data (57, 78) and is very similar in philosophy to that of the BoneBase project. Like BoneBase, this project is designed to

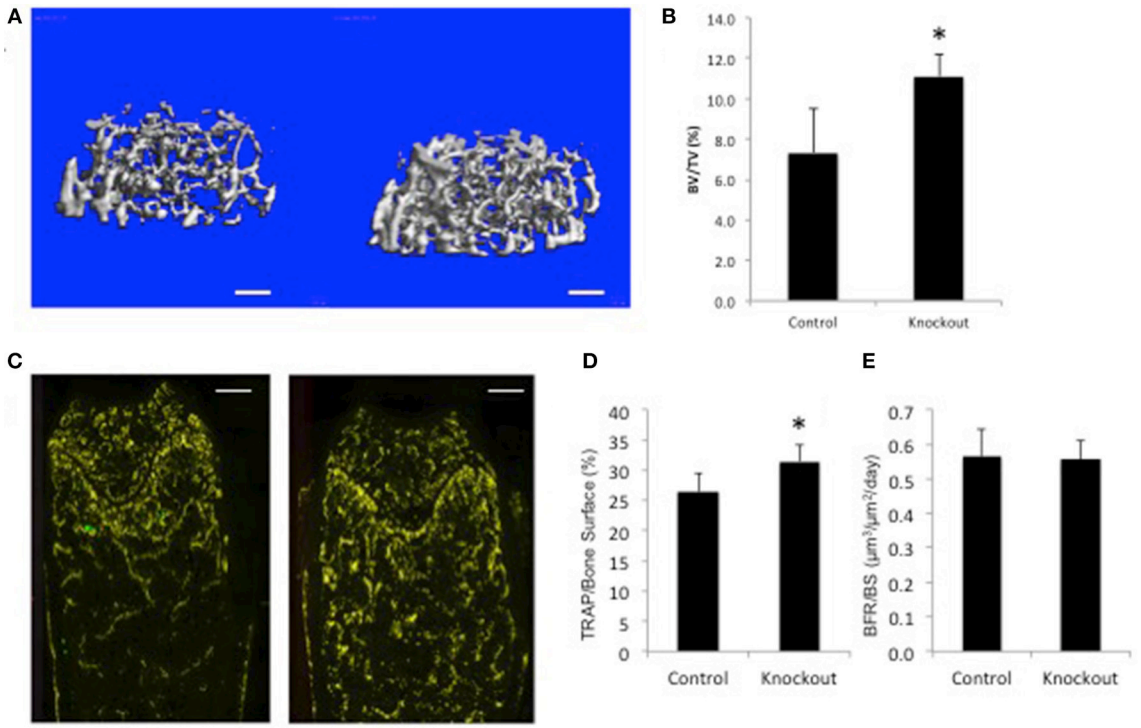

FIGURE 2 | Bone Phenotype of female mice lacking *Ocstamp*. Representative data as collected via the BoneBase pipeline for Ocstamp null female mice. **(A)** Reconstructions of the distal femoral trabecular compartment for *Ocstamp*$^{+/+}$ (left) and *Ocstamp*$^{-/-}$ mice. **(B)** A significant increase in bone volume over total volume (BV/TV) was observed in the null vs. control animals (*$p < 0.001$). **(C)** Staining for tartrate resistant acid phosphatase (TRAP), a marker of osteoclast cells (yellow) in *Ocstamp*$^{+/+}$ (left) and *Ocstamp*$^{-/-}$ mice. **(D)** An increase in the number of TRAP positive cells per unit bone surface observed in the null vs. control animals (*$P = 0.001$), but no difference in bone formation rate (BFR) was seen **(E)**.

expand on the phenotype data collected on IMPC generated mice. This project uses mice generated by the Welcome Trust Sanger Institute (WTSI) IMPC phenotyping pipeline and, to date, data is available for 733 lines. The summary data is available for all lines examined by the OBCD and is provided in a straightforward web portal (http://www.boneandcartilage.com/index.html). Unlike the Bonebase project, OBCD was able to collect bone samples from the same mice that went through the primary IMPC phenotyping pipeline and these mice are 16 weeks of age at phenotyping. A primary difference between these two programs is that only data from female mice are available for the traits of interest for bone and osteoporosis research in the OBCD, but data exists for males and females in Bonebase. Since the OBCD collected samples directly from the WTSI pipeline, data is available for some lines in a heterozygous state (57). This may be a better reflection of what is captured by GWAS, as many GWAS loci do not negate expression or alter protein function. The haploinsufficient state may more closely mimic what is expected to be the consequence of many GWAS loci.

There is some overlap in the types of data collected by these two projects, but each project has a different focus with regards to the kind and purpose of the data collected. Both groups conducted microCT-based imaging of the distal femur and femoral midshaft and both groups report data on trabecular bone mass and architecture, as well as cortical size and geometry (57, 72). However, the OBCD group collects two types of data that are unique to this program. First, they collect digital X-ray microradiography on the femur and caudal vertebrae to collect bone mineral content (BMC) data. This method overcomes some of the limitations already outlined regarding the IMPC DXA data (57). This method is site specific, has higher resolution than the older DXA machines, and there are no concerns about artifacts arising from extra-osseous calcification. Second, measures of bone strength and stiffness are collected by the OBCD via mechanical testing of the femur (via three point bending) and the caudal vertebrae (via compression). In addition to the bone data described above, this group has plans to collect a plethora of data related to the knee joint which may be informative for osteoarthritis (79). This arm of the project uses the male mice generated by the WTSI pipeline, but data for only 29 strains is currently available.

Lexicon Pharmaceuticals Inc.

Between 2000 and 2008, Lexicon Pharmaceuticals Inc. embarked on an ambitious project to generate and phenotype ∼5,000 knockout mice via a high throughput pipeline. The overarching goal of this project was to identify novel avenues of therapeutic intervention for a wide variety of diseases. The choice of genes for interrogation was enriched for enzymes, receptors, and secreted proteins (80). To find genes of interest, their

phenotyping protocol was designed to capture information on behavior, cardiology, immunology, metabolism, oncology, and ophthalmology. Of note for bone biology, three types of data were collected: (1) DXA imaging, including whole body and region of interest (ROI) analysis of the femur and spine, (2) microCT imaging of the fifth lumbar vertebrae and femoral midshaft and (3) static histological analysis of the long bones (80). In total, bone relevant data was collected for 3,762 genes; however, the complete DXA and microCT analysis was not conducted on all lines. This program did identify and name 10 novel genes that are involved in the regulation of bone. An additional three genes were identified as having a role in bone biology, but as of yet the names of these genes are being withheld. Lastly, they confirmed the role in bone biology for an additional 23 genes (80). A subset of the data generated by this project can be found on the MGI webpage (http://www.informatics.jax.org/knockout_mice/).

Mouse Genome Database

The mouse genome database (MGD) is maintained at the Jackson Laboratory (http://www.informatics.jax.org (81), and is a central part of the larger Mouse Genome Informatics (MGI) consortium. The MGD is an incredible resource for the study of the mouse as a model of human disease and serves as the repository for information regarding mouse genes, gene function(s), and mouse strain information. At present, it contains a summary of the phenotype(s) associated with over 50,000 mutant alleles in over 12,000 genes (http://www.informatics.jax.org, accessed, Oct, 2018) Unlike the resources listed above, the MGD is not, in and of itself, making and phenotyping new mice. Rather, the data contained in the MGD comes primarily from the literature and is entered by expert curators. However, data from other sources such as the IMPC is captured. All of the data presented in the MGD is linked to the primary references and to other mouse model resources. A summary page for each mouse gene is provided and included on this page are: the human homolog, any human diseases associated with that gene, a brief synopsis of the phenotype of knockout mice or mice carrying mutations in that gene, and a visual presentation of the physiological systems affected by mutations in this genes (81). In addition, the MGD and the parent MGI project (82) have built an ever-increasing toolbox for mining this data collection. While it is possible to bulk query this data set for terms such as "decreased trabecular bone mass," more complete information is obtained by searching for each gene individually when looking to validate GWAS candidate genes. In **Table 2**, the links for selected search engines and databases useful for finding mouse strains are provided.

TABLE 2 | Selected resources for locating inbred, transgenic and mutant mouse strains and targeted ES cells.

Name	URL
International Mouse Strain Resource	http://www.findmice.org/index.jsp
Australian Phenomics Facility	http://pb.apf.edu.au/phenbank/homePage.html
Canadian Mouse Mutant Repository	http://www.cmmr.ca/
European Mouse Mutant Archive	https://www.infrafrontier.eu/
International Mouse Phenotyping Consortium	http://www.mousephenotype.org/
Riken Bioresource	http://mus.brc.riken.jp/en/
Charles River	https://www.criver.com/
The Jackson Laboratory	www.jax.org
Taconic Bioscience	https://www.taconic.com/
Envigo	https://www.envigo.com/
NIH Aged Rodent Colonies	https://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook
International Gene Trap Consortium	https://igtc.org/
MGI Cre portal	http://www.informatics.jax.org/home/recombinase
NCBI guide to mouse genome resources	https://www.ncbi.nlm.nih.gov/genome/guide/mouse/

TRANSCRIPTOMICS

The integration of –omics data such as transcriptomics has been highly successful in many areas of research for identification of the causative variant(s) as well as for interpreting the role of a causative variant and/or candidate gene in the disease process. It is difficult to collect large numbers of specimens from humans for bone research, which limits the number of sizeable expression resources available for human tissue (57). Further, extracting quality RNA from bone and cartilage is laborious and technically challenging (83). A large number of databases containing raw and processed gene expression data exist. The largest of these are described below and summarized in **Table 3**.

Tissue Expression Panels

Expression data can provide information about when and where a gene is expressed. Fortunately for bone, there are excellent resources from mouse that can be used to assess tissue distribution and cell type expression. BioGPS is a gene-annotation portal that houses such data for 8 different species (84). Like many web portals, BioGPS contains a plethora of tools for easy access of the data featured. Included therein is a tissue expression panel collected by the Novartis Research Foundation. In this panel, expression of protein coding genes was assessed in a large number of primary mouse tissues from male and female C57BL/6 mice, and also from selected mouse cell lines (85), GEO Series: GSE10246). All samples were run on the Affymetrix mouse MOE430 microarray chip (GEO platform accession: GPL1261), and this data is freely available for download. Relevant to bone biology, this data set includes expression in the following cultured mouse cells from three time points post differentiation (days 5, 14, and 21), primary calvarial osteoblasts, primary cultured osteoclasts, the MC 3T3 pre-osteoblast cell line (86), the C3H10T1/2 pluripotent mouse embryonic fibroblast line, and the RAW264.7 macrophage cell line. The C3H10T1/2 cell line is considered to have mesenchymal stem cell characteristics and, if appropriately treated, these cells can be induced to become osteoblast-like, chondrocyte-like or adipocyte-like cells (87). The RAW264.7 cell line can be induced to form multinucleated, TRAP positive osteoclast-like cells (88). Thus, these three cell lines may model some features of bone stem cells. The caveat with this data is that it is microarray data,

TABLE 3 | Selected resources for gene expression and localization in the mouse.

Title	URL	Description
BioGPS	biogps.org	Gene portal containing tissue distribution and eQTL data for 8 species
Gene Expression Ominbus	www.ncbi.nlm.nih.gov/geo/	NCBI repository for microarray and RNAseq data
Gene Paint	www.genepaint.org/	Tissue distribution of gene expression in the mouse embryo as determined by in situ hybridization. This includes data from the Eurexpress project.
Gene Expression Database (GXD)	www.informatics.jax.org/expression.shtml	Repository of gene expression in the mouse collected via a variety of methods
EMBL-EBI Expression atlas	https://www.ebi.ac.uk/gxa/home	Gene expression abundance and localization in multiple species including human and mouse
GeneNetwork	www.genenetwork.org/	A web service for systems genetics that includes mouse bone eQTL data and phenotype data from a large number of inbred mouse strains.

and differentiating between a lack of expression and a lack of sensitivity by the probe on the array is difficult (89). In addition to establishing that a gene is putatively expressed in bone, these data can be used to differentiate between systemic expression that would be expected for a housekeeping gene, and tissue enriched expression. Housekeeping genes are defined as genes that control basal cellular functions in most tissues and are less likely to be disease-causing genes (90). Conversely, tissue enriched genes may be informative for disease and the patterns of tissue-enriched expression may be helpful in establishing the biological role of a poorly characterized gene (91).

Newer resources for bone include data collected via next generation RNA sequencing (RNAseq). Both whole-tissue and cell type-specific expression data sets have been deposited in the public domain. Two of these data sets have been used for functional validation of GWAS loci. In the first, gene expression across osteoblastogenesis was profiled by RNAseq. In this study, primary calvarial cells were isolated from neonatal C57BL/6J mice carrying an allele whereby cyan florescent protein (CFP) expression was driven by the Col3.6 promoter (92). These cells were then sorted by FACS to remove the cells not expressing CFP and were therefore considered non-osteoblast-like. The remaining cells were placed into culture and differentiated into osteoblasts using standard protocols (93). Gene expression was measured in this osteoblast-enriched population in a dense time-course series from the pre-osteoblast to mature osteoblast stages of maturation. This is a valuable dataset for determining if a candidate gene plays a role in osteoblast maturation. Indeed this data was used to show that Engrailed 1 (*EN1*), a candidate gene for a bone mass GWAS locus, is expressed in a relevant cell type and at an appropriate time point to impact the phenotype of interest [Zheng et al. (93), GEO Series: GSE54461]. This data has been subsequently used in a number of GWAS to screen putative candidate genes (28, 33, 94, 95). The second data set was not originally created for the purpose of functionally validating GWAS loci. RNAseq data for cultured bone marrow derived mouse osteoclasts has been deposited in the Gene Expression Onmibus (GEO Accession Number: GSM1873361), and this data has been used in concert with the above osteoblast data to determine if GWAS candidate genes are expressed in relevant bone cells [28. 33]. The most abundant cell in bone tissue is the osteocyte (96) and a variety of gene expression data sets profiling expression in the osteocyte have been collected. Much of these data have not been used extensively as of yet for functional validation of human GWAS loci. Some of these data sets are so called "enrichment signature" meaning that expression is not necessarily unique to the osteocyte, but rather is higher in cells sorted based on a known osteocyte marker (97), or in a tissue type known to contain largely osteocytes (9). Using one of these data sets, Morris *et al* showed that eBMD GWAS candidate genes were highly enriched among genes showing a 4-fold higher expression in tissues high in osteocyte number as compared to bone marrow, suggesting that genes expressed in the osteocyte play a significant role in the genetic regulation of bone mass (9).

Expression QTLs

QWAS loci overwhelmingly are thought to be caused by variants in non-coding regions (16) and may be involved in the regulation of gene expression. These variants may affect the level of transcription of the gene(s) leading to the phenotype of interest (98), or impact the post-transcriptional processing of one or more genes (99). The expression level of a gene can be used as a phenotypic trait for genetic mapping to determine if there are local alleles controlling expression. Such a locus is referred to as a *cis* expression Quantitative Trait Locus [eQTL, (100)]. Limited eQTL data exists for isolated human osteoblasts (101) and for iliac crest biopsy samples (102), but both of these data sets have low power for mapping. Use of data from other tissue types as a surrogate for expression in bone for eQTLs has yielded mixed results. This is not a unique problem for bone and, indeed, analysis of the 44 tissues collected as part of the GTEX project suggested that the distribution of the number of tissues in which a *cis*-eQTL is found is bimodal. Namely, there are a large number of eQTL found in nearly all tissues and there is an equally large number showing a high degree of selectivity in that they are found in one to three tissues only (103).

There is accumulating evidence suggesting a high degree of evolutionary conservation of patterns of gene co-expression between mice and humans in many tissues. In particular, the degree of conservation in bone is among the highest (104). Further, co-expression of pathways associated with metabolic disease, cell adhesion, and the cell cycle are also highly conserved between the species (104), suggesting conserved mechanisms of regulation. In other diseases and tissues, strong concordance for

eQTL identified in mice and humans has been observed (105, 106). Collectively, this suggests that, in bone, the examination of eQTL and gene co-expression in mice would be highly informative for human bone disease and provide valuable information toward functional validation of GWAS loci. One set of data exists in the public domain that can be used for eQTL mapping in mice (GEO series number: GSE27483). This data set is comprised of long bone (sans marrow) gene expression as obtained by microarray from male mice from the Hybrid Mouse Diversity Panel (HMDP) (107). This panel of mice, which has been described in detail elsewhere (108), is comprised of 29 inbred strains, as well as 71 recombinant inbred strains of mice wherein each strain is genetically distinct. Whole body, femoral, and spinal BMD data are also available for these same strains of mice. By leveraging the genetic diversity present in this panel, loci can be mapped for both traditional and expression phenotypes (107). These phenotypic and expression data for the HMDP have been deposited in the GeneNetwork repository (http://www.genenetwork.org/). GeneNetwork is a toolbox for facilitating systems genetics (109). Deposited in the GeneNetwork repository are collections of phenotype, expression and genotype data for a number of species including mouse, rat, non-human primates, and humans. This repository is coupled to tools that facilitate analyses within a single data set or across multiple datasets. Built into GeneNetworks is the ability to conduct correlation analyses on the HMDP phenotype and genotype data, map eQTLs, and to conduct pair-scans to look for gene-gene interactions (109).

Co-expression Networks

Network-assisted analysis of GWAS data has proven to be a powerful way to select candidate genes and provide possible mechanisms of biological action (110). The principal behind this approach is the understanding that genes function as part of larger pathways and that the allelic differences leading to complex genetic disease act on members of these pathways to mediate biological function (111). In other words, genes important for a complex disease are functionally related at some level (112). In practice, an unbiased biological network is constructed, such as a gene co-expression network (113), and the genes found in GWAS loci are mapped onto this network to identify pathways of interest and causal genes (107, 114–116). For example, bone resorption by the osteoclast is a biological function that may be perturbed in osteoporosis. There are multiple signaling pathways that control the formation and function of the osteoclast. By creating a co-expression network from bone, gene expression modules associated with this biological function of bone resorption can be identified. All genes in GWAS loci can then be overlaid to find the subset of genes that are members of these biologically relevant modules. In this manner, causal genes can be pinpointed, and biological mechanism of action is putatively determined. The important part of this method is that the networks are created in an unbiased manner as opposed to a curated or directed manner, and therefore novel discoveries can be made. Because of the conservation of co-expression between mouse and human for bone (104), network-assisted analysis of GWAS is an powerful way to augment and direct functional validation efforts for bone disease. This was elegantly demonstrated by Calbrese et al. (111). In this paper, the authors examined the 64 loci identified in the GEFOSII meta-analysis GWAS published in 2012 (117). By integrating all genes located in these 64 human loci with a gene co-expression network constructed using femoral expression data from the mouse (118), these authors were able to predict the causal gene and infer their function in bone biology for 30 of these loci. They then went on to use traditional experimental approaches to validate that two of these genes were involved in the predicted biological process and were indeed bone genes. In total, network-assisted analysis of GWAS loci is a powerful and efficient method to prioritize genes for functional validation and direct functional validation experimental design.

CONCLUSIONS

The power of the mouse to elucidate the cause of human disease has been recognized for over 100 years. Data on gene function is being collected using mouse models at a pace and in a scope that could only be dreamed of a decade ago. In the not so distant future, a transgenic or mutant mouse model will exist for every protein coding gene in the mouse genome and with a few key strokes, any researcher, anywhere will have access to reliably collected data regarding what loss of function of that gene does to the bone and many other physiological systems. By marrying this functional data with GWAS, an unparalleled level of understanding of human disease is not over the horizon, but rather practically on our doorsteps.

The challenge moving forward will be to make sense of the function of each gene in the context of all other genes and all of the various physiological systems. Very soon it will not be adequate to write out a cell-signaling pathway as if it acted in isolation and was the sole driver of disease. As we develop new tools and methods for network analyses we are better able to comprehend and define the complex interactions leading to skeletal development, maintenance and decline. Our ultimate goal must be to determine how to leverage this new-found knowledge in the context of each person's physiology to predict, prevent or treat skeletal disease in manner that is safe and effective for that patient.

AUTHOR CONTRIBUTIONS

This work was written and edited by CA-B with significant writing contributions and editorial assistance from RM.

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REVIEW
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Recent Advances in the Genetics of Fractures in Osteoporosis

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Genetic susceptibility, together with old age, female sex, and low bone mineral density (BMD) are amongst the strongest determinants of fracture risk. Tmost recent large-scale genome-wide association study (GWAS) meta-analysis has yielded fifteen loci. This review focuses on the advances in the research of genetic determinants of fracture risk. We first discuss the genetic architecture of fracture risk, touching upon different methods and overall findings. We then discuss in a second paragraph the most recent advances in the field and focus on the genetics of fracture risk and also of other endophenotypes closely related to fracture risk such as bone mineral density (BMD). Application of state-of-the-art methodology such as Mendelian randzation in fracture GWAS are reviewed. The final part of this review touches upon potential future directions in genetic research of osteoporotic fractures.

Keywords: genetics, osteoporosis, fracture risk, genome-wide association studies, review, family, single nucleotide polymorphism, copy number variation

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GENETIC ARCHITECTURE OF FRACTURE RISK

Bone fractures are considered the most relevant clinical sequelae of osteoporosis. Genetic susceptibility, together with old age, female sex, falls (1) and low BMD are amongst the strongest determinants of fracture risk. A positive family history is a risk factor for osteoporosis and fractures thus reinforcing the role of genetics in the basis of liability to osteoporotic fractures (2). Moreover, parental hip fracture has been incorporated as a risk factor in the FRAX clinical assessment algorithm in the last decade. Heritability studies have reported estimates for bone mineral density (BMD) and fractures of up to 66 and 46%, respectively (3, 4). A parental history of fracture has been related to any-type of fracture risk (risk ratio (RR) for any-type of fracture 1.17, 95% CI 1.07-1.21), and hip fracture (RR 1.49, 95% CI: 1.17-1.89) (5). These previous findings are at the background of further genetic investigations.

Different types of genetic changes may underlie diseases; structural variations, including deletions or base pair changes, vary from mutations of larger stretches of genetic material to single nucide polymorphisms (SNPs) and mutations affecting 1 base pair together with structural variation comprising insertions and deletions of different size across the genome. As discussed elsewhere in this journal issue, there are a multitude of genetic mutations known to cause relatively infrequent monogenic conditions presenting with bone fragility including familial forms of osteoporosis, osteogenesis imperfecta and other bone disorders, for example: *COL1A1* (6), *COL1A2*, *LRP5* (7), *WNT1* (8), *LGR4* (9), *PLS3* (10), *CRTAP*, *FKBP10*, *LEPRE1*, *PLOD2*, *PPIB*, *SERPINF1*, *SERPINH1* and *SP7* (11), summarized in **Table 1**. One human genome contains roughly 3 billion (3,000,000,000) nucleotides, which are the building blocks of the genome in the form of the letters A, T, G,

TABLE 1 | An overview of monogenic bone disorders and the genes involved in their pathology.

Disease	Gene	Locus	References
Autosomal dominant Osteopetrosis type II	*CLCN7*	16p13	(12)
Autosomal dominant hypophosphataemic rickets	*FGF23*	12p13.32	(13)
Early-onset osteoporosis	*WNT1*	12q13.12	(8)
Familial hypocalciuric hypercalcaemia (FHH)	*CASR*	3q21.1	(14)
	GNA11	19p13.3	(15)
	AP2S1	19q13.3	(16)
Hereditary hypophosphataemic rickets with hypercalciuria	*SLC34A3*	9q34.3	(17)
Hypophosphatasia	*TNS/ALPL*	1p36.12	(18)
Juvenile Paget disease	*TNFRSF11B*	8q24.12	(19)
Osteogenesis imperfecta (OI)	*COL1A1*	17q21.33	(6)
	COL1A2	7q21.3	(7)
	IFITM5	11p15.5	(20)
	SERPINF1	17p13.3	(11)
	CRTAP	3p22.3	(11)
	PRH1/LEPRE1	1p34.2	(11)
	WNT1	12q13.12	(8)
Pseudohypoparathyroidism	*GNAS*	20q13.3	(21)
Sclerostosis	*SOST*	17q21.31	(22)
	LRP4	11p11.2	(23)
Vitamin D-dependent rickets	*CYP3A4*	7q22.1	(24)
	CYP27B1	12q14.1	(24)
	VDR	12q13.11	(25)
X-linked hypophosphatemic (XLH) rickets	*PHEX*	Xp22.11	(11)
X-linked osteoporosis	*PLS3*	Xq23	(11)

and C. When a SNP in the sequence is swapped for another letter, this is called a mutation and considered a SNP when occurring relatively frequent, i.e., with a minor allele frequency (MAF) >0.5% in the population). Technologies for SNP genotyping include enzyme-based methods (e.g., polymerase chain reaction [PCR]-based), hybridization-based methods (e.g., microarrays) and next-generation sequencing.

Genome-wide screening, as applied in genome-wide association studies (GWAS), tests for associations between genetic markers (SNPs and traits of interests in a hypothesis -free manner. This approach can add onto *a priori* knowledge about the physiological, biochemical or functional aspects of possible candidates (26). On the other hand, genome-wide genotyping is unbiased in the sense that by surveying the whole genome in a hypothesis-free manner, involvement of unexpected candidates or even loci with unknown function could be revealed (27). Meta-analyses are an appropriate way for follow-up in candidate gene studies of top loci and genes prioritized by GWAS, and use of existent GWAS for look-ups of functional biological hypotheses.

It has been shown that SNPs underlie differences between people, including the variability in disease susceptibility, and recent GWAS have vastly expanded our knowledge in this area (28). Apart from developing our understanding of disease etiology, expectations are that these genetic markers will be useful in disease diagnostics and prediction, form potential drug targets and potentially modulate treatment response (9).

Fracture is the most clinically relevant endpoint of osteoporosis and its etiology is complex. Similarly to other traits strongly related with old age, the heritability of fracture risk decreases with age. Studying correlated endophenotypes that are associated with fracture risk, such as BMD, lean mass and hand grip strength might be a good alternative to study the genetic basis of fracture risk. GWAS for various osteoporosis-related traits have shown that targeting these quantitative endophenotypes with excellent measurement properties (root mean square standard deviation expressed as coefficient of variation of 1.0–1.2% for the spine and 1.1–2.2% for the femoral neck by DXA)(29) is efficient in the number of loci discovered. The earliest GWAS of DXA-BMD identified 24 loci that influence DXA-BMD variation explaining ∼3% of trait variance (30–36) of which several variants have also been nominally associated with fracture risk (37, 38). A breakthrough was the meta-analysis by the genetic factors for osteoporosis (GEFOS) and genetic markers for osteoporosis (GENOMOS) consortia (39), where the top-associated BMD markers explaining ∼6% of BMD variance were also tested for fracture risk (31,016 cases and 102,444 controls), where 14 out of 56 BMD loci were associated at Bonferroni corrected significance level with fractures, of which six loci at genome-wide significant level. An alternative measurement method for DXA is total body BMD, as is more commonly applied in childhood and adolescence, where GWAS recently reported more than 80 loci explaining 10% of the variance (40). This same publication examined these SNPs in an independent fracture study, where a decrease of one standard deviation in genetically determined total body BMD resulted in 56% higher odds of fracture. Another endophenotype is BMD estimated from quantitative heel ultrasound, where in this GWAS 12 out of the associated 307 SNPs were also associated with fracture risk, newly adding the *AQP1* and *SLC8A1* loci as potential fracture genetic determinants (41).

BMD is among the quantitative traits for which GWAS have been effective in discovering high numbers of loci (42, 43). On the other end, GWAS for dichotomous disease as a direct outcome have yielded relatively lower numbers of loci discovered (42), probably due to study power issues. This might concern the studies for osteoporotic fractures as well. Further, identifying the specific genetic determinants contributing to the risk of fracture has been difficult due to its multifactorial nature and occurrence late in life. High phenotype heterogeneity and ascertainment bias reduce the power to detect association, making the genetic studies even more difficult. Endophenotypes may be nearer to the coding DNA in the chain of events at the basis of multifactorial diseases, and, homogeneous determination of endophenotypes may be simpler than defining certain diseases. Indeed, hypothesis-free genome-wide screens have shown that the most prominent and consistently replicating genetic loci associated with fracture risk are also associated with BMD, which serves as proof of BMD being a very powerful endophenotype for fracture prediction (44). This also implies that an underlying fragility component

mediated through genetic predisposition seems to form a major part of the basis for fracture risk.

At the beginning of the GWAS era, the genomics field was dominated by the common disease-common variant hypothesis, which states that common diseases are caused by common genetic variants (45). Yet, the list of rare genetic variants influencing common disease is growing (46). In between these two categories are SNPs with minor allele frequency (MAF) of 0.5–5%.

RECENT ADVANCES IN THE GENETICS OF OSTEOPOROTIC FRACTURES

Several GWAS specifically aimed at fracture risk, have been performed to date, as discussed below and summarized in **Table 2** and **Figure 1**.

GWAS for Fracture Risk and DXA-BMD

With regard to the allele frequencies, osteoporotic fracture risk has been shown to be associated with common, uncommon and rare variants. In a study of structural variation in relation to fracture risk (5,178 Dutch individuals of which 809 fracture cases), the proportion of fracture cases with at least one deletion was significantly higher compared to controls and a 210 kb deletion located on chromosome 6p25.1 was associated with fracture risk (OR=32.58, 95% CI 3.95 to 1488.89). An *in silico* meta-analysis in four studies with copy number variation microarray data found similar results for the association with fracture risk (OR 3.11, 95% CI 1.01 to 8.22). Notably, this variant was absent in samples from several countries; indicating geographic diversity.

Nevertheless, this study indicates that the study of rare CNVs deserves follow-up (49). Also, another effort in the GEFOS and GENOMOS consortium encompassing for the first time a sequencing-based GWAS meta-analysis has discovered *EN1* as a determinant of bone density and fracture (rs11692564(C) allele OR = 1.18) (52). Further, deCODE investigators have discovered common sequence variants in *PTCH1* (53) (MAF = 11.4–22.6%) and less frequent (MAF = 0.14%−0.18%) variants in *LGR4* (9) associated with BMD and fractures (OR = 1.09 and OR = 3.12).

The first two published GWAS for fracture risk identified the *SVIL* gene locus in African American populations (50) and the MECOM gene locus in Korean and Japanese populations (48), respectively. It should however be noted that access to larger sample sizes is still limited for samples of non-European descent, as reflected in a lack of a replication meta-analysis for the African American fracture GWAS. The second GEFOS GWAS meta-analysis for BMD assessed the identified loci for their relation with fracture (39). The recently published large scale GWAS meta-analysis for fracture in 25 cohorts from all over the world with genome wide genotyping and fracture data (discovery in 37,857 fracture cases and 227,116 controls; replication in up to 147,200 fracture cases and 150,085 controls) identified 15 loci (44), of which all were also associated with bone mineral density. Relative to the previous DXA-BMD GWAS–fracture association study (39), we confirmed the 2p16.2 (*SPTBN1*), 7q21.3 (*SHFM1*), 10q21.1 (*MBL2*/*DKK1*), 11q13.2 (*LRP5*), and 18p11.21 (*FAM210A*) loci, and observed an increased signal at *SOST*, *CPED1*/*WNT16*, *FUPB3*, *DCDC5*, *RPS6KA5*, *STARD3NL*, and *CTNNB1*. Additionally, we added the 6q22.33 (*RSPO3*), 6q25.1 (*ESR1*), 7p12.1 (*GRB10*/*COBL*), and 21q22.2 (*ETS2*) loci to the list of novel fracture loci. The signals mapped to genes clustering in pathways known to be critical to bone biology (e.g., *SOST*, *WNT16*, and *ESR1*) or novel pathways (*FAM210A*, *GRB10*, and *ETS2*). These variants explain approximately 2% of variance in fracture risk (unpublished data).

As reviewed elsewhere (54), several Mendelian randomization (MR) studies in relation to fracture risk have been published. One of the first publications in this field was an exploration of the association between C-reactive protein levels and increased fracture risk, where we did not find evidence for a causal effect (55). Nevertheless, particularly for proving negative associations well-powered meta-analyses are required. The largest MR study to date was conducted on behalf of the GEFOS/GENOMOS consortium and the 23andMe research teams (44). In this study, SNPs that had been previously reported in GWAS were used as instrumental variables, representing 15 risk factors for fracture including: BMD (femoral neck and lumbar spine), age of puberty, age at menopause, grip strength, vitamin D, homocysteine, thyroid stimulating hormone level, fasting glucose, type 1 diabetes, type 2 diabetes, rheumatoid arthritis, inflammatory bowel disease, coronary artery disease, and the lactose intolerance marker (rs4988235) as a surrogate to assess long term differences in dairy derived calcium intake. SNPs influencing BMD were strongly and inversely correlated with odds of fracture (for femoral neck BMD SNPs genetic correlation −0.59; and for lumbar spine BMD SNPs genetic correlation −0.53). By contrast, of the remaining clinical risk factors evaluated, only homocysteine was shown to be genetically correlated with fracture risk (genetic correlation >0.2 or <-0.2, and surpassing the threshold for statistical significance for multiple testing), but this should be interpreted with caution as the confidence interval is wide. In the subsequent Mendelian randomization analysis, again, only the BMD SNPs were significantly associated with fracture risk. This implies a causal effect of these SNPs through BMD on fracture risk, without any evidence for pleiotropic effects as the Mendelian randomization-Egger regression intercepts centered around zero. By contrast, despite high statistical power, none of the other tested and well-accepted risk factors had evidence for a major causal effect on fracture risk. These results should be interpreted with caution as reviewed elsewhere (56). Still study power is limited in spite of the large sample sizes and the LD score regression method used. Potentially existing pleiotropy or non-linear relationships (e.g., threshold effects and extremes of the population) may be subjects of future research. Another very recent study (57) extensively assessed genetic determinants of osteoporosis, combining the UK Biobank and 23andMe cohorts (57). The authors, first identified 518 genome-wide significant loci (of which 301 novel) associated with heel BMD and then identified 13 loci associated with fractures across 1.2 million individuals (all also associated with heel BMD). Furthermore, they identified target genes known to influence bone density and

TABLE 2 | Findings of fracture risk genome wide association studies.

Sample size fracture cases vs. controls	Type of fracture	Ethnicity	Type of genetic variation	References
A. PUBLISHED FRACTURE RISK GENOME WIDE ASSOCIATION STUDIES				
329 vs. 2,666	Vertebral (radiographic)	Caucasian	Single nucleotide polymorphism	Oei et al. (47)
288 vs. 1,139	Any	Asian	Single nucleotide polymorphism	Hwang et al. (48)
809 vs. 4,369	Any	Caucasian	Copy number variation	Oei et al. (49)
540 vs. 10,305	Any	African-American	Single nucleotide polymorphism	Taylor et al. (50)
1,553 vs. 4,340	Vertebral (clinical)	Caucasian	Single nucleotide polymorphism	Alonso et al. (51)
37,857 vs. 227,116	Any	Caucasian	Single nucleotide polymorphism	Trajanoska et al. (44)

References	Variant	Effect allele	Effect allele frequency	Alternate allele	Odds ratio	95% Confidence interval	Locus	Candidate gene
B. GENETIC VARIANTS FOUND ASSOCIATED IN THE FRACTURE RISK GENOME WIDE ASSOCIATION STUDIES								
Oei et al. (47)	rs11645938	C	9.65%	T	1.06	0.98–1.14	6p25.1	*FOXC2*
Hwang et al. (48)	rs784288	A	25%	G	1.39	1.24–1.56	3q26.2	*MECOM*
Oei et al. (49)	210 kb deletion	N.A.	0.14%	N.A.	3.11	1.01–8.22	6p25.1	*PECI*
Taylor et al. (50)	rs12775980	A	3%	C	2.12	1.61–2.79	10p11.23	*SVIL*
Alonso et al. (51)	rs10190845	A	4.9%	C	1.74	1.06–2.06	2q13	*FBLN7*
Trajanoska et al. (44)	rs4233949	G	61%	C	1.03	1.02–−1.04	2p16.2	*SPTBN1*
	rs430727	T	45%	C	1.03	1.02–1.04	3p22.1	*CTNNB1*
	rs10457487	C	51%	A	1.05	1.04–1.06	6q22.33	*RSPO3*
	rs2982570	C	58%	T	1.04	1.03–1.05	6q25.1	*ESR1*
	rs2908007	A	60%	G	1.06	1.05–1.07	7q31.31	*WNT16*
	rs6465508	G	34%	A	1.04	1.03–1.05	7q21.3	*C7orf76*
	rs6959212	T	34%	C	1.03	1.02–1.04	7p14.1	*STARD3NL*
	rs1548607	G	32%	A	1.03	1.02–1.05	7p12.1	*GRB10*
	rs7851693	G	35%	C	1.04	1.03–1.05	9q34.11	*FUBP3*
	rs11003047	G	11%	T	1.09	1.07–1.10	10q21.1	*MBL2*
	rs3736228	T	15%	C	1.06	1.05–1.08	11q13.2	*LRP5*
	rs1286083	T	82%	C	1.05	1.04–1.07	14q32.11	*RPS6KA5*
	rs2741856	G	92%	C	1.10	1.07–1.11	17q21.31	*SOST*
	rs4635400	A	36%	G	1.04	1.03–1.05	18p11.21	*FAM210A*
	rs9980072	G	73%	A	1.04	1.03–1.05	21q22.2	*ETS2*

strength and performed a rapid throughput skeletal phenotyping of 126 knockout mice with disruptions in predicted target genes. They found an increased abnormal skeletal phenotype frequency compared to unselected lines and a further in depth analysis on gene DAAM2 showed a disproportionate decrease in bone strength relative to mineralization.

Another Mendelian randomization study is the report on a causal effect of serum estradiol concentrations (interestingly in men) and an increased risk of any fracture (OR 1.35, 95% CI, 1.18-1.55), non-vertebral major osteoporotic fractures (OR 1.75, 95% CI, 1.35-2.27) and wrist fractures (OR 2.27, 95% CI, 1.62-3.16) (58).

Although most genetic studies on fracture risk have pulled together fracture information of any type, without discrimination of site, there are two major efforts on vertebral fracture GWAS that have been published. The first genome-wide association study for radiographic vertebral fractures in the Rotterdam Study, found a marker on chromosome 16q24 as genome-wide significantly associated (59). Although the 16q24 locus was found associated with BMD and vertebral defects at birth before, the association with vertebral fracture risk could not be replicated by *de-novo* genotyping across 15 studies worldwide, likely due to the heterogeneity underlying the different fracture definitions. A subsequent publication focusing on clinical vertebral fractures (i.e., those presenting with clinical manifestations) identified and replicated a locus tagged by rs10190845 on chromosome 2q13 where differential expression of the positional candidate genes TTL and SLC20A1 was shown (51).

Recent GWAS for Heel BMD and Other Endophenotypes

The most recent study by Morris et al. (57) identified 518 genome wide significant loci (of which 301 novel) across 426,824 individuals of UK-Biobank which altogether explain around 20% in heel BMD variance. Earlier in 2017, Kemp et al. had identified

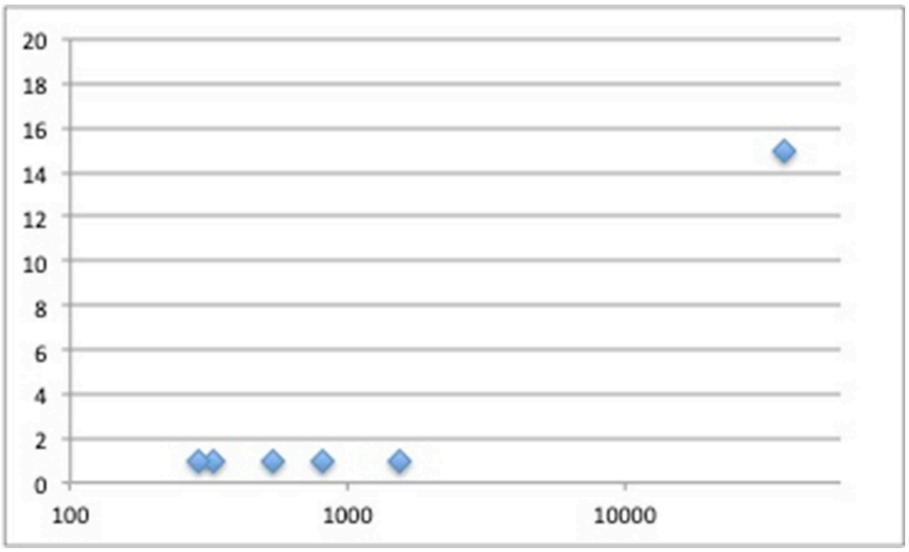

FIGURE 1 | Number of loci discovered in fracture genome-wide association studies (Y-axis) plotted by fracture cases sample size (X-axis).

across a subsample of UK-Biobank ($N = 142{,}487$) 203 loci, of which 153 novel at the time of publication (41).

Lean mass and hand grip strength have been associated with fracture risk (60) and may provide a possible endophenotype for potential genetic studies to elucidate fracture risk. It is thought that this relationship may be because of an inverse relationship between muscle strength and balance and thus fall risk. A study by Zillikens et al. (61) found five SNPs in/near HSD17B11, VCAN, ADAMTSL3, IRS1, and FTO for total body lean mass across 101767 individuals and three SNPs in/near VCAN, ADAMTSL3, and IRS1 for appendicular lean mass among 73,420 individuals. Karasik et al. (62) additionally identified a novel LM locus (TNRC6B).

Hand grip strength GWAS by Willems et al. was associated with 16 new loci. Furthermore, in the same study, the authors found evidence of shared genetic etiology of BMD and lean mass with grip strength and moreover a suggestive causal role for higher grip strength and lower risk of fracture (63). Similar results were found for the potential causal relationship between hand grip strength and fracture risk, but could not be replicated with a multiple testing significance threshold in the study by Trajanoska et al. (44).

POTENTIAL FUTURE DIRECTIONS IN GENETIC RESEARCH OF OSTEOPOROTIC FRACTURES

Increasing Sample Size

A minimum sample-size threshold needs to be reached in GWAS, from where the number of discovered loci increases along with growing sample sizes as study power improves (42). Mega-sized biobanks, such as 23andMe and UK Biobank, including hundreds of thousands of participants with GWAS are increasingly becoming available (64, 65). A drawback from such Mega-GWAS is that phenotype data tends to be of variable quality and less accurate. However, there is a trade-off where the huge numbers may boost study power tremendously and overcome measurement error to a certain extent. In addition, the success rate of unraveling underlying genetic mechanisms may be influenced by the complexity of the genetic architecture of the trait of interest, including imperfect penetrance, allelic heterogeneity, and gene-environment and epigenetic effects (42, 43). The discovery of rare variants is hindered by the large sample sizes required to attain sufficient study power, where research consortia and Mega-GWAS with even larger sample sizes prove their worth through ever-increasing sized meta-analyses. Larger imputation reference panels and sequencing-based genotyping are becoming progressively available, facilitating more accurate examination of lower-frequency SNPs and other type of genetic variants such as indels and larger deletions (66).

Furthermore, it has been proposed that the missing heritability for human height and body mass index is likely to be small after estimating the genetic variance from all imputed variants (67); this will likely be the case for a (quantitative) trait such as BMD as well. Until now, rare variant association studies have found variants with larger effects where each explains only a tiny proportion of the phenotypic variance, because the heritability explained is dependent on the effect size and allele frequency (68). Therefore, arguments can be found to study both common and rare variants in the occurrence of common diseases (68), as also confirmed by our experiences in the bone field.

Increasing Phenotyping Quality

More detailed phenotyping is believed to be of value for scrutinizing skeletal-site specific effects for fracture risk, for example cortical vs. trabecular bone, which justifies separate GWAS efforts for specific fracture types. This thinking comes

from the observations that heritability of BMD varies across skeletal sites due to a mixture of shared and specific genetic and environmental influences as quantified by the genetic correlations (69), which supports the findings that some genetic loci display skeletal-site specific effects (32). Furthermore, it has been hypothesized that using stricter phenotype definitions and taking into account fracture mechanisms may increase study power. Yet, a major drawback is the decreasing sample size. Results for radiographic (59) and clinical vertebral fractures (51) have been published, as described above, efforts for hip and wrist fractures are underway, but struggle with attaining sufficient study samples to enable discoveries. Therefore, the all-type of fracture GWAS approach seems the starting point to attain maximum sample size for power to perform the first screening for genetic variants that contribute to osteoporotic fracture risk in general. Other even more specific subjects of clinical studies could be atypical (femoral) fractures or fracture healing, which could yield insight into differences in natural healing mechanisms and efficacy of medical treatment between patients.

The tough start of the fracture GWAS may be rooted in the complex phenotype definition and heterogeneity of the trait and its underlying genetics. A better understanding of the genetic architecture seems necessary. More clarity is needed which fracture phenotypes should be studied together because they have a joint genetic etiology, and which do not and thus should be analyzed separately; for example vertebral vs. non-vertebral fractures are distinguished clinically and probably also genetically. Then robust selection criteria should be defined for an optimal fracture phenotype definition of interest. Research ideas include data enrichment for cases that have a known family history for osteoporosis, having fractured at relatively young age or having sustained multiple fractures. This because the heritability of osteoporotic fractures at younger age is higher (4). Nonetheless, the osteoporotic fracture incidence at young age is lower, which may limit study sample sizes. Theoretically, it has been speculated that perhaps further exclusion criteria need to be established for cases that are thought to be caused by arguably non-genetic mechanisms (e.g., non-genetic secondary osteoporosis, high-trauma, old age, malnourishment, etc.), where refinement and automatization of measurements may enhance the richness, quality and quantity of research data available. However, until now in practice, bigger seems better to efficiently identify genes; then one should take these discoveries and bring them in a candidate-gene context and look across rich sets of detailed phenotypes that help understand the underlying biology. Combination into multivariate GWAS of multiple disease-related traits could further exploit the detection of pleiotropic effects (70) and novel statistical methods may be able to better utilize the richer phenotype information that will become available (71, 72).

Additionally, richer phenotyping of endophenotypes may yield more insight. Dual energy X-ray absorptiometry still misses 80% of patients who will fracture (73). One of the underlying reasons is that it generates two-dimensional scans and does not sufficiently appreciate bone microarchitecture, an important determinant of bone strength (74). Areal BMD does appreciate bone size and in part the internal architecture; the trabecular bone score (TBS) which can also be derived from DXA data will be worth further investigations (75). Further improvements require more advanced imaging than dual energy X-ray absorptiometry, principally by direct three-dimensional radiological imaging investigations, such as computed tomography or magnetic resonance imaging, to directly visualize microstructure, differentiate cortical and trabecular bone, and model bone strength biomechanically (76). Second, the contribution of the mineral phase to bone's mechanical properties has dominated scientific thinking, while bone is composed of three different phases (by volume: mineral 42%, collagen matrix 35%, and water 23%) (77). Novel imaging techniques that can quantify this bone composition are coming up (78), and genetic studies into these endophenotypes are yet to come.

Finally, it could be argued that bone geometry and its genetics should be studied. Intriguingly, taller persons are at increased risk of fractures in spite of having larger bones with more mass (79, 80). This may be caused by a different distribution of bone mass by periosteal apposition (81). Further, loci implicated in the GWAS of human stature are enriched for genes important for skeletal growth (82). And more specifically, a GWAS meta-analysis for hip shape was published very recently and found 17q24.3 and ASTN2 as associated in lookups in hip fracture GWAS (unpublished data) (83).

Richer Genotyping

However, some of the measurement methods with respect to both genotyping and phenotyping currently available are simply too expensive or invasive to apply on a population level at present. Yet, current limits are being challenged, with the very first successful large-scale applications of whole-genome sequencing and deep imputation using sequencing-based reference panels in the osteoporosis research field (52). The Haplotype Reference Consortium (HRC) and the Trans-Omics for Precision Medicine (TOPMed) Program have created large reference panels of human haplotypes by combining together sequencing data from multiple cohorts. Further studies of copy number and structural variations should be performed. However, the genome may be too distant in the cascade from the disease of interest to detect clinically relevant patterns, therefore, screening the transcriptome, epigenome, metabolome, proteome and even microbiome at perhaps multiple time points may prove necessary. This may be applied to clinical fracture patient studies as well as population-based cohorts, where subgroups could be studied including for example individuals with multiple fractures, persons with fractures at young age, and elderly individuals free of fractures. The osteoporosis field has started to explore epigenetic regulation for instance: microRNA (84, 85), long non-coding RNA (86), gene expression (87), and DNA methylation (88).

Functional Follow-Up

Oftentimes the function of genes contained in the associated loci are not (completely) known. Functional follow-up studies are needed, yet, the development of animal knock-out-models may take years. Establishment of multi-disciplinary research consortia worldwide may be beneficial to efficiently take GWAS discoveries

to functional follow-up in a harmonized research pipeline. Also, publicly available databases are being launched to enhance interpretation of genomic sequence information, promoting mutual data sharing between expert consortia, professional organizations, health care providers, and patients. An inventory of the GWAS catalog in 2009 revealed that 88% of the GWAS associations are in either intergenic or intronic regions (28), regions of the genome we still understand little about, but to which GWAS has contributed by indicating regulatory sites (89). Moreover, the GWAS association signal in the radiographic vertebral fracture GWAS did not lie within a gene (59), and the same was true for some of the signals in the BMD and all-type of fracture GWAS (44). The Encyclopedia of DNA Elements (ENCODE) project, aiming to identify all functional elements in the human genome, has drastically enriched our comprehension about regions outside of the exome and showed that many GWAS SNPs overlap transcription-factor-occupied regions or DNase I hypersensitive sites and are particularly enriched in the segmentation classes associated with enhancers and transcription start sites (90). A striking finding is that obesity-associated noncoding sequences within the *FTO* locus are associated with expression of the homeobox gene *IRX3* at megabase distances, but not with expression of *FTO* itself; (91) this association seems to be driven by a topologically associated domain (TAD) structure encompassing the *FTO* and *IRXB* genes cluster (92). Such genomic explorations remain to be performed for osteoporosis-related traits.

Pharmacogenomics

So far, therapies used to increase bone strength in individuals with osteoporosis are mainly based on antiresorptives (93). Bisphosphonates are the most widely used first-line because of their effectiveness, reasonable safety, and a low cost price (94). However, in practice, no single antiresorptive therapy is currently appropriate for all patients, as a subgroup of patients on anti-fracture medication responds suboptimally, e.g., small gain in bone mass or new fractures occur in spite of treatment, or negative side-effects such as osteonecrosis of the jaw or atypical femoral fractures (AFF) among others (95). To our knowledge no large-scale pharmacogenetic GWAS studies examining these phenomena in osteoporosis have been published to date, though initial case studies on the genetics of AFF and an accompanying systematic review have been published (96). In the future, results from pharmacogenomic studies may aid in assigning the most effective therapy to specific patient groups and it has been hypothesized that genetic biomarkers can be identified to pinpoint those patients most vulnerable to side-effects of certain agents. Nevertheless, because interaction studies tend to involve more parameters, up to four times as many subjects are needed (97); unless extremely large effects are in place, as we have witnessed for a few pharmacogenomic successes, such as anticoagulant dosing according to *VKORC1* haplotypes and HLA-B*5701 screening for the risk of hypersensitivity reaction to abacavir in HIV (98). Until now in genetic osteoporosis research, solely candidate gene studies have been performed investigating genetically-based variation in treatment response to raloxifene, teriparatide, and bisphosphonates (99). One of the reasons for this is that the coverage of pharmacogenomics variants was limited on GWAS genotyping platforms (100, 101), but this is improving with novel microarrays becoming available.

CONCLUSION

GWAS is the study design necessary to further investigate the complex phenotypic and genetic architecture of osteoporotic fracture risk. Although fractures can be considered a complex trait, so far, the majority of susceptibility loci for fractures are also associated with bone mineral density. Hopefully, novel discoveries in the genetics of fracture risk will increasingly be translated clinical practice, with genotyping increasingly being successfully applied providing access to previously unknown information that may change the diagnostics and treatment of patients with bone diseases including osteoporosis with increased fracture risk in the future.

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FK, KT, FR, and LO have written and revised the manuscript.

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REVIEW
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Use of Mendelian Randomization to Examine Causal Inference in Osteoporosis

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Epidemiological studies have identified many risk factors for osteoporosis, however it is unclear whether these observational associations reflect true causal effects, or the effects of latent confounding or reverse causality. Mendelian randomization (MR) enables causal relationships to be evaluated, by examining the relationship between genetic susceptibility to the risk factor in question, and the disease outcome of interest. This has been facilitated by the development of two-sample MR analysis, where the exposure and outcome are measured in different studies, and by exploiting summary result statistics from large well-powered genome-wide association studies that are available for thousands of traits. Though MR has several inherent limitations, the field is rapidly evolving and at least 14 methodological extensions have been developed to overcome these. The present paper aims to discuss some of the limitations in the MR analytical framework, and how this method has been applied to the osteoporosis field, helping to reinforce conclusions about causality, and discovering potential new regulatory pathways, exemplified by our recent MR study of sclerostin.

Keywords: bone mineral density (BMD), fractures - bone, pleiotropy, sclerostin, GWAS - genome-wide association study

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INTRODUCTION

Osteoporosis is a common disorder leading to skeletal fragility and increased fracture risk. This condition is strongly influenced by age and sex, as well as genetic factors. Establishing which risk factors play a causal role in osteoporosis is helpful in unraveling pathogenic mechanisms, and in identifying potential new preventative and treatment strategies. Epidemiology studies in the osteoporosis field have examined relationships between putative risk factors and fracture risk, the main clinical consequence of osteoporosis. Investigations have also studied risk factors for bone mineral density (BMD) as measured by DXA, which is a strong predictor of fracture risk (1). Traditional observational studies have reported that a range of potentially modifiable risk factors, including sex-steroid deficiency, low body mass index (BMI), physical inactivity, smoking, heavy alcohol consumption, and low calcium and vitamin D, are related to BMD and fractures. However, studies of this type suffer from confounding and reverse causality (2, 3). Randomized controlled trials (RCTs) are the gold standard for inferring causality, because they are unaffected by these issues if performed correctly. However, RCTs are expensive, resource-intensive, time consuming, and may have important ethical limitations.

MR is a statistical method for inferring causality which is analogous to an RCT, except that genotypes are used to randomize participants into different levels of the exposure/treatment. MR can be implemented as a form of instrumental variables analysis, where genetic variants, normally single nucleotide polymorphisms (SNPs), are used as proxies ("instruments") for the exposure of interest (see **Figure 1**) (4, 5). According to Mendel's Laws of Inheritance, alleles segregate randomly when passed from parents to offspring. According to his (second) Law of Independent Assortment, which forms the foundation of MR, the inheritance of one pair of factors (genes) is independent of the inheritance of the other pair. Thus, offspring genotypes are unlikely to be associated with confounders in the population. In addition, since germ-line genotypes are determined at conception, they precede outcomes being investigated, and so observed associations cannot be explained by reverse causation. However, unlike RCTs which generally involve relatively short term interventions, genetic influences exert their effects from conception onwards, and so causal effects estimated from MR represent life-long exposures.

MR was initially developed in the form of one-sample MR, which relies on access to individual level data (**Figure 1A**). One limitation of this method is that most individual cohorts do not have many traits measured simultaneously. Two sample MR was subsequently developed to overcome this issue by using summary level data derived from independent cohorts that collectively have many exposures and outcomes measured (**Figure 1B**). GWAS data is now available on thousands of plausible osteoporosis risk factors which can be leveraged by two sample MR. The extensive opportunities to explore causal influences on bone phenotypes using the MR approach is summarized in two recent reviews (2, 5).

Though previous MR studies have contributed to our understanding of causal factors involved in the etiology of osteoporosis, as discussed below, MR has a number of inherent assumptions and limitations, for which a range of sensitivity analyses have been developed (6–8). MR analyses may also be subject to several sources of bias (9). For example, if individuals with a certain disease outcome are drawn from a population with distinct ancestry to disease-free controls, this may lead to differences in frequency of genetic variants between those with and without disease, and hence spurious associations with genotypes related to putative risk factors. Furthermore, dynastic effects need to be considered for many traits, including BMD, whereby effects of genetic variation in the offspring are partly mediated by shared parental genetic influences acting via early life environment (10). The present paper aims to discuss how MR has been applied to the osteoporosis field, including the approaches taken to address limitations in the MR analytical framework.

OSTEOPOROSIS OUTCOMES

Consistent with epidemiology studies of osteoporosis in general, the majority of MR studies in osteoporosis have utilized DXA-measured BMD as the outcome, which is widely used clinically as the gold standard for diagnosing osteoporosis. As well as being predictive of the clinical consequence of osteoporosis, namely fractures (1), BMD has a major heritable component, making it a highly suitable outcome for MR analyses (11, 12). Initial studies utilized summary statistics from GWASs, such as those based on the GEnetic Factors for OSteoporosis Consortium (GEFOS, http://www.gefos.org/), which made GWAS findings for DXA-measured BMD publicly available for a range of sites including the lumbar spine (LS) and femoral neck (FN) (13). Whereas, GEFOS has the advantage of providing BMD GWAS data for multiple skeletal sites, a potential disadvantage is that the latter GWAS adjusted for weight. The justification for this is that areal BMD measured by DXA is influenced by body size, which is partly accounted for by adjusting for weight. However, this can have unintended consequences, such as the introduction of spurious genetic associations as a consequence of collider bias (14). In addition, by using BMD summary statistics corrected for weight, subsequent MR analysis may be biased as effects on BMD mediated by weight may not be accurately estimated. Moreover, as described below, use of GWAS outputs that were adjusted for weight and/or height may complicate interpretation when applying these data in MR studies examining relationships between BMI and BMD. The latter relationship is thought to be relatively complex, involving both a mass effect leading to greater loading acting via the mechanosensor, and shared endocrine pathways.

Recently published GWASs for estimated BMD (eBMD) derived from heel ultrasound in UK Biobank (11, 12) have the advantage that both unadjusted and adjusted summary statistics are available on request, enabling sensitivity analyses to be performed. Moreover, the large sample size provides a significant advance in terms of power, which is one of the major limitations of MR studies. Ultrasound derived BMD does not involve radiation, is quick and cheap, and is therefore well-suited to population studies involving hundreds of thousands of people. The limitation is that estimated BMD is not well-understood and we are not entirely sure how well it proxies BMD. That said, eBMD and DXA-BMD measures are reasonably highly correlated genetically ($r = \sim 0.5$), as are eBMD and fractures ($r = \sim 0.5$) (11), and ultrasound-derived measures have previously been reported to predict subsequent fractures with similar accuracy to DXA BMD (15–18).

BMD is an intermediary phenotype; low BMD is only of pathological significance as a result of its causal relationship with fracture. However, since many risk factors for osteoporosis act via BMD, their relationship with BMD is somewhat stronger than that with fracture, with the result that MR studies using fracture as the outcome tend to be underpowered. That said, in a large MR study of fractures based on discovery set of 37,857 fracture cases and 227,116 controls, with replication in 147,200 fracture cases and 150,085 controls, Trajanoska et al. found that higher BMD had an expected causal effect in reducing fractures (19). Moreover, Morris et al. identified 13 bone fracture loci in approximately 1.2 million individuals, all of which were associated with eBMD (12). It may also be possible to extend MR studies in osteoporosis

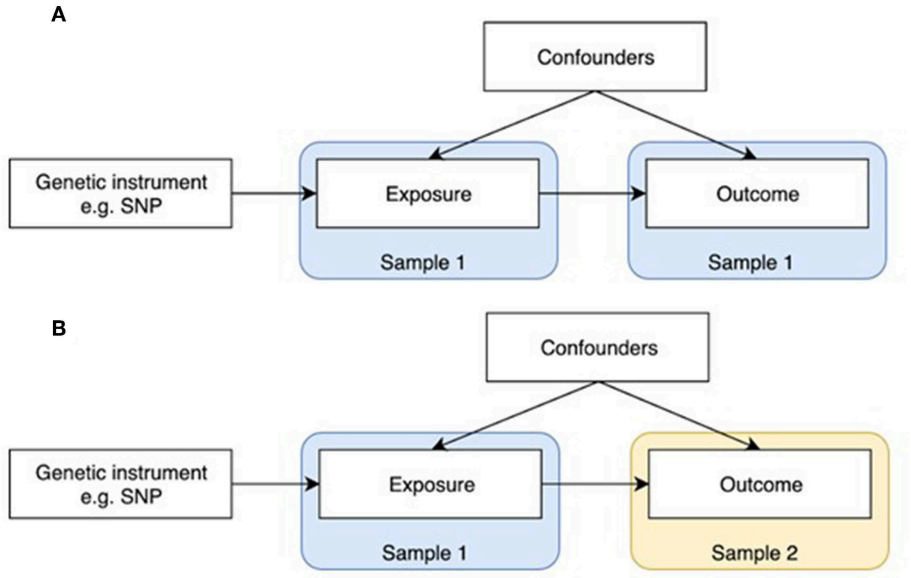

FIGURE 1 | One-sample and two-sample Mendelian randomization study designs. **(A)** One-sample Mendelian randomization is based on a population where both exposure and outcome have been measured. **(B)** In two-sample Mendelian randomization, exposures and outcomes are measured in non-overlapping populations. SNP-exposure is derived in Sample 1, and SNP-outcome in Sample 2.

to examine causal effects on other phenotypes relevant to osteoporosis. BMD is not the sole causal determinant of fracture, and GWAS signals have recently been identified for several geometric parameters derived from hip DXA, which are also thought to be related to fracture risk (20). GWAS efforts are also underway for osteocalcin and CTX, offering opportunities for MR studies to examine causal pathways for other outcomes contributing to fracture risk, such as bone turnover.

OSTEOPOROSIS RISK FACTORS

A range of risk factors for osteoporosis identified in epidemiological studies have been examined in MR studies intended to explore their causal effects, using BMD as the outcome, the majority of which have yielded no or weak evidence of causality (see **Table 1**). For example, studies using BMD as an outcome did not find support for a causal effect of vitamin D (21–23) or genetically determined calcium intake as reflected by lactase persistence genotype (24). Guo et al. found no evidence to suggest a causal effect of alcohol consumption on BMD whereas smoking status was found to be causally related to lower BMD (25); however, it should be noted that smoking (exposure) and eBMD (outcome) instruments were derived from the same sample population which could result in biased estimates (8). In a subsequent study, genetic predisposition to smoking initiation was associated with fracture risk, but not eBMD; genetic liability to alcohol dependence was also associated with fracture and lower eBMD, whereas no association was seen for genetically predicted alcohol intake (26). However, this study also included UK Biobank participants in exposure and outcome instruments which could lead to bias. Other studies exploring the effect of serum urate (27, 28), inflammatory markers (29) and thyroid stimulating hormone (30) found no evidence for association with FN or LS BMD. Rather than a risk factor, MR analysis suggests that lowering LDL-C levels and statin therapy improve BMD (31).

In terms of constitutive factors, a causal association was observed between later age at menarche and reduced FN and LS BMD in adults (32), and reduced LS BMD in adolescents (33). A study in children found a causal association between BMI/adiposity and BMD (34). A previous study in adults using summary data from GWASs in Europeans found no evidence of a causal effect of BMI (based on 77 SNPs) on FN or LS BMD, however since FN and LS BMD were corrected for weight prior to the GWAS, the variation in BMD attributable to BMI may not be adequately captured by MR analysis (35). In contrast, a one-sample MR in Koreans using 13 BMI-associated SNPs identified in a GWAS of east Asians was suggestive of a causal effect of BMI on BMD on weight bearing sites in men and pre-menopausal women (36). Observation studies have implicated several diseases in the development of osteoporosis, however MR has subsequently found no causal effect of type 2 diabetes (T2D) and coronary heart disease (CHD) on eBMD (37). Another study reported a weak association between increased T2D risk and increased FN BMD, whereas no association was seen with LS BMD (35). In terms of other constitutional factors,

TABLE 1 | Examples of studies investigating causal associations between risk factors and BMD.

Exposure	Sample source for exposure	Genetic variants (*n*)	Outcome	Sample size and data source for outcome	Method	Evidence of causal effect (Yes/No)	References
Vitamin D	Chinese populations	10	LS BMD	Postmenopausal Chinese women, *N* = 1,824	One-sample	No	(21)
		10	FN BMD				
		10	Total hip BMD				
		10	LS BMD				
		10	FN BMD				
		10	Total hip BMD				
Vitamin D	Europeans, *N* = 79,366	6	TB BMD	Individuals from Europe (86%), America (2%) and Australia (14%), *N* = 66,628	Two-sample (IVW)	No	(22)
Vitamin D	Europeans, *N* = 42,274 (SUNLIGHT consortium)	5	DXA FN BMD	Europeans, *N* = 32,965 (GEFOS Consortium)	Two-sample (weighted median)	No	(23)
		5	DXA LS BMD				
		5	eBMD	Europeans, *N* = 142,487 (UK Biobank)			
Milk intake	Lactase persistence SNP in the *MCM6* gene, based on previous studies	1	Forearm BMD	Europeans, *N* = 53,236 (GEFOS Consortium)	Two-sample (Wald estimate)	No	(24)
		1	FN BMD				
		1	LS BMD				
Alcohol consumption	Europeans	6	FN BMD	Europeans, *N* = 32,735 (GEFOS Consortium)	Two-sample (IVW)	No	(25)
		6	LS BMD	Europeans, *N* = 28,498 (GEFOS Consortium)			
		6	Forearm BMD	Europeans, *N* = 8,143 (GEFOS Consortium)			
		5	Heel BMD	Europeans, *N* = 445,921 (UK Biobank)			
Smoking status	Europeans (including UKBB results)	142	FN BMD	Europeans, *N* = 32,735 (GEFOS Consortium)	Two-sample (IVW)	No	
		142	LS BMD	Europeans, *N* = 28,498 (GEFOS Consortium)			
		139	Forearm BMD	Europeans, *N* = 8,143 (GEFOS Consortium)			
		142	Heel BMD	Europeans, *N* = 445,921 (UK Biobank)	Two-sample (IVW)	Some evidence but could be biased	
Smoking initiation	Europeans (including UKBB results)	1	FN BMD	Europeans, *N* = 32,735 (GEFOS Consortium)	Two-sample (IVW)	No	
		1	LS BMD	Europeans, *N* = 28,498 (GEFOS Consortium)			
		1	Forearm BMD	Europeans, *N* = 8,143 (GEFOS Consortium)			
		1	Heel BMD	Europeans, *N* = 445,921 (UK Biobank)	Two-sample (IVW)	Some evidence but could be biased	
No. of cigarettes smoked per day (CPD)	Europeans (Tobacco and Genetics Consortium)	3	FN BMD	Europeans, *N* = 32,735 (GEFOS Consortium)	Two-sample (IVW)	Very weak evidence	
		3	LS BMD	Europeans, *N* = 28,498 (GEFOS Consortium)	Two-sample (IVW)	No	
		3	Forearm BMD	Europeans, *N* = 8,143 (GEFOS Consortium)		No	
		3	Heel BMD	Europeans, *N* = 445,921 (UK Biobank)		No	

(Continued)

TABLE 1 | Continued

Exposure	Sample source for exposure	Genetic variants (*n*)	Outcome	Sample size and data source for outcome	Method	Evidence of causal effect (Yes/No)	References
Smoking initiation	Europeans *N* = 1,232,091 (including UK Biobank)	376	eBMD	Europeans, *N* = 426,824 (UK Biobank)	Two-sample (IVW)	No	(26)
			DXA derived BMD	Europeans *N* = 32,965 (GEFOS Consortium)	Two-sample (IVW)	No	
Genetically predicted alcohol intake	Europeans *N* = 941,280 (including UK Biobank)	96	eBMD	Europeans, *N* = 426,824 (UK Biobank)	Two-sample (IVW)	No	
			DXA derived BMD	Europeans *N* = 32,965 (GEFOS Consortium)	Two-sample (IVW)	No	
Genetic liability to alcohol dependence	Europeans *N* = 46,568 (11,569 cases and 34,999 controls)	1	eBMD	Europeans, *N* = 426,824 (UK Biobank)	Two-sample (IVW)	Yes	
		1	DXA derived BMD	Europeans *N* = 32,965 (GEFOS Consortium)	Two-sample (IVW)	No	
Serum urate	Europeans	5	LS BMD	1,322 postmenopausal women and elderly men from Shanghai	One-sample	No	(27)
		5	FN BMD				
		5	Total hip BMD				
Serum urate	Europeans	3	Total hip BMD	Generation 3 cohort in the Framingham Heart Study (*N* = 2,501)	One-sample	No	(28)
		3	FN BMD				
		3	LS BMD				
Inflammatory markers - hsCRP	Europeans	16	Forearm BMD	Europeans, *N* = 32,965 (GEFOS Consortium)	Two-sample (IVW)	No	(29)
		16	FN BMD				
		16	LS BMD				
Thyroid Stimulating Hormone	Europeans, *N* = 26,420	20	FN BMD	Europeans, *N* = 28,498 (GEFOS Consortium)	Two-sample (IVW)	No	(30)
		20	LS BMD				
Low LDL-C levels	Global Lipids Genetics Consortium *N* = 188,577	76	TB BMD	Populations from America, Europe and Australia *N* = 66,628	Two-sample (IVW)	Some evidence	(31)
					Multivariable IVW	No	
		76	eBMD	Europeans, *N* = 142,487 (UK Biobank)	Two-sample (IVW)	Yes	
					Multivariable IVW	Yes	
Gene encoding molecular target of LDL-C-lowering therapy (HMGCR)	Global Lipids Genetics Consortium *N* = 188,577	3	TB BMD	Populations from America, Europe and Australia *N* = 66,628	Two-sample (IVW)	Yes	
		3	eBMD	Europeans, *N* = 142,487 (UK Biobank)	Two-sample (IVW)	Yes	
AAM	European women ReproGen Consortium *N* = 182,416	116	LS BMD	GEFOS Consortium (*N* = 53,236) (both males and females)	Two-sample (IVW)	Yes	(32)
		116	FN BMD				
AAM on aBMD in adolescent girls	ReproGen Consortium	331	LS BMD	aBMD in childhood/ adolescence (BMDCS)	Two-sample (FE meta-analysis)	Yes	(33)
		331	FN BMD			No	
		331	Distal radius			No	
AAM on aBMD in adult women	ReproGen Consortium	309	LS BMD	GEFOS Consortium	Two-sample (FE meta-analysis)	Yes	
		309	FN BMD			Yes	
		309	Distal radius			No	
AVB on aBMD in adolescent boys	ReproGen Consortium	43	LS BMD	aBMD in childhood/ adolescence (BMDCS)	Two-sample (FE meta-analysis)	No	
		43	FN BMD			No	
		43	Distal radius			No	
AVB on aBMD in adult men	ReproGen Consortium	42	LS BMD	GEFOS Consortium	Two-sample (FE meta-analysis)	Yes	
		42	FN BMD			Yes	
		42	Distal radius			No	

(Continued)

TABLE 1 | Continued

Exposure	Sample source for exposure	Genetic variants (*n*)	Outcome	Sample size and data source for outcome	Method	Evidence of causal effect (Yes/No)	References
BMI	Europeans	32	SK-BMD	Europeans, *N* = 5,221 (ALSPAC cohort) **N* = 4,223 for SK-BMD	One-sample	No	(34)
		32	UL-BMD			Yes	
		32	LL-BMD			Yes	
		32	SP-BMD			Yes	
		32	PE-BMD			Yes	
Fat mass	Europeans	32	SK-BMD	Europeans, *N* = 5,221 (ALSPAC cohort) **N* = 4,223 for SK-BMD	One-sample	No	
		32	UL-BMD			Yes	
		32	LL-BMD			Yes	
		32	SP-BMD			Yes	
		32	PE-BMD			Yes	
Fat mass	Europeans	32	SK-BMD	Europeans, *N* = 5,221 (ALSPAC cohort) **N* = 4,223 for SK-BMD	One-sample multivariable MR	No	
		32	UL-BMD			No	
		32	LL-BMD			Yes	
		32	SP-BMD			Yes	
		32	PE-BMD			Yes	
Lean mass	Europeans	32	SK-BMD	Europeans, *N* = 5,221 (ALSPAC cohort) **N* = 4,223 for SK-BMD	One-sample multivariable MR	No	
		32	UL-BMD			Yes	
		32	LL-BMD			Yes	
		32	SP-BMD			No	
		32	PE-BMD			Yes	
BMI	GIANT consortium	77	FN BMD	Europeans, GEFOS 2012	Two-sample (IVW)	No	(35)
		77	LS BMD			No	
BMI	East Asian populations	13	Weight-bearing bones	Men, *N* = 1,110	One-sample	Yes	(36)
		13	Non–weight-bearing bones			Yes	
		13	Skull			No	
		13	Weight-bearing bones	Premenopausal women, *N* = 1,015	One-sample	Yes	
		13	Non–weight-bearing bones			No	
		13	Skull			No	
		13	Weight-bearing bones	Postmenopausal women, *N* = 32	One-sample	No	
		13	Non–weight-bearing bones			No	
		13	Skull			No	
T2D	DIAGRAM: 26,676 T2D cases and 132,532 controls	94	eBMD	∼150,000 UK Biobank participants	Two-sample (IVW)	No	(37)
CHD	CARDIoGRAMplusC4D	52	eBMD	∼150,000 UK Biobank participants	Two-sample (IVW)	No	
T2D	DIAGRAM consortium	32	FN BMD	GEFOS, *N* = 83,894	Two-sample (IVW)	Weak evidence	(35)
		32	LS BMD			No	
Metabolites	Europeans	481 blood metabolites	Hip BMD	2,286 unrelated white subjects for the discovery samples	Pearson correlation	Associations between BMD and 54 blood metabolites	(38)
Total serum calcium	Europeans (discovery cohort *N* = 39,400, replication cohort *N* = 21,676)	7	eBMD	Europeans, *N* = 426,824 (UK Biobank)	Two-sample (IVW)	No	(39)

LS, lumbar spine; FN, femoral neck; TB, total body; eBMD, estimated bone mineral density; AAM, age at menarche; AVB, age at voice break; UL, upper limbs; LL, lower limbs; SP, spine; PE, pelvis; IVW, inverse-variance weighted; T2D, type 2 diabetes; CHD, coronary heart disease; BMI, body mass index; FE, fixed-effects.

genetic predisposition to increased calcium levels was recently found to be unrelated either to eBMD or fracture risk in UK Biobank (39).

Several MR studies have examined osteoporosis risk factors with fracture as the outcome (**Table 2**). In the study based on UK Biobank from Trajanoska et al. (19), while confirming the expected protective effect of higher BMD on fractures, there was little evidence to suggest a causal effect of dietary factors (vitamin D levels and calcium intake), early menopause, late puberty and range of diseases (including type 1 and 2 diabetes, CHD and inflammatory bowel disease) on risk of fracture. These findings are consistent with results from the above MR studies based on BMD. However, the study did provide some evidence for causal effect of decreased grip strength on fracture risk. A study in 97,811 Danish individuals failed to provide evidence for a relationship between calcium intake and hip fracture (40). Interestingly, an MR study investigating the causal effect of height with 50 diseases reported that one SD increase in genetically determined height was associated with increased risk of hip fracture (41). In terms of the effect of serum hormones, a previous MR study in men reported lower levels of estradiol to be causally related to increased risk of fracture (including all self-reported fractures, major non-vertebral osteoporotic fractures and wrist fractures), whilst there was no evidence for causal association between serum testosterone and fracture risk (42). Furthermore, using a genetic risk score for CRP levels in a Rotterdam study, there was no evidence to support a causal effect of CRP on fracture risk (43).

Some MR studies have set out to test hypothesized causal effects of BMD on other outcomes. For example, in a study which used summary statistics from the first release of the UK Biobank data ($N = 11{,}650$), the authors reported some evidence for a causal effect of eBMD on T2D, CHD, HDL-c, and HOMA-IR, testing reciprocal associations for two traits (T2D and CHD), for which there was no evidence of a causal effect on BMD (37). The most recent study in 426,824 UK Biobank participants identified 518 loci associated with eBMD, explaining 20% of its variance (12), meaning that many powerful and robust instruments for MR analyses examining causal effects of eBMD will be available.

ADDRESSING PLEIOTROPY

Key points:-

1. Vertical pleiotropy, when the genetic variant has an effect on two or more traits that both influence the outcome via the same biological pathway, is usually not problematic for MR analyses
2. In contrast, horizontal pleiotropy, when a genetic variant is associated with two traits which influence the outcome via independent biological pathways, violates one of the key MR assumptions
3. Several methods have been developed that relax the strict requirement that genetic instruments exhibit no horizontal pleiotropy yet still produce consistent causal effect estimates
4. Where genetic instruments are known to be pleiotropically associated with multiple correlated phenotypes, it may be possible to examine independent effects through exclusion of certain SNPs, or use of multivariable MR.

One of the main assumptions of MR is that genetic instruments are only associated with the interest via the exposure being tested. This is known as the "no pleiotropy" assumption or the "exclusion restriction criterion." When performing an MR study, it is usually unclear whether such an assumption holds. Therefore, various sensitivity analyses are applied to detect the existence of pleiotropy, and to estimate the un-biased causal effect of the exposure on the outcome. Vertical pleiotropy (i.e., a genetic variant has an effect on two or more traits that both influence the outcome via the same biological pathway) is not generally an issue for MR analysis (**Figure 2A**) (8). However, this can be this can be problematic in situations where the exposure variable is mis-specified i.e., the genetic instrument is biologically related to an intermediate or outcome, but has been identified as being related to the exposure by virtue of the latter's correlation with the biologically related trait (4), termed correlated pleiotropy (44). For example, although a locus in *FTO* was initially identified in relation to type II diabetes, this was subsequently found to primarily influence BMI with secondary effects on type II diabetes (45), leading to difficulties in interpreting MR studies where *FTO* variation is used as instrumental variable for type 2 diabetes.

In contrast, horizontal pleiotropy (i.e., a genetic variant is associated with two traits which influence the outcome via independent biological pathways) violates the exclusion restriction criterion (**Figure 2B**). GWAS identify genetic instruments purely on statistical grounds. Even if instrumental variables used in MR studies intersect genes with plausible pathways to the exposure it's not possible to be sure whether they mediate the causal effect being evaluated. Therefore, potential horizontal pleiotropy as a result of unknown pathways needs to be excluded if MR studies are to reach robust conclusions about causality. One simple method to limit the impact of horizontal pleiotropy is leave-one-out as a sensitivity analysis to ensure that the causal effect is not mediated by an outlier effect of one specific locus (46).

Over the last few years, several methods have been developed that relax the strict requirement that genetic instruments exhibit no horizontal pleiotropy yet still produce consistent causal effect estimates (7). One such approach is MR-Egger regression (47), where given a set of genetic variants that proxy an exposure variable of interest, a regression is performed between estimates of the SNP-outcome association and SNP-exposure association (this can be performed in both one and two-sample MR analyses). Unfortunately, Egger regression is limited by very poor power. Weighted median and weighted mode approaches have since been developed to derive causal estimates based on the relationship between the strength of the association between the SNP and the outcome, and the strength of the association of the SNP with the exposure, which are more robust to violation of horizontal pleiotropy by a substantial proportion of instruments (48, 49). Several additional methods now exist which assume

TABLE 2 | Examples of MR studies using fracture as an outcome.

Exposure	Sample source for exposure data	Genetic variants (*n*)	Outcome	Sample size and data sources for the outcome data	MR method	Evidence of causal effect (Yes/No)	References
Decreased FN BMD	Europeans	43	Fractures at any skeletal site confirmed by medical, radiological, or questionnaire reports	147,200 cases and 150,085 controls (primarily of European ancestry)	Two-sample (IVW)	Yes	(19)
Decreased LS BMD		40				Yes	
Earlier menopause		54				No	
Rheumatoid arthritis		30				No	
Inflammatory bowel disease		19				No	
Type 1 diabetes		151				No	
Decreased THS		20				No	
Homocysteine		13				No	
Decreased Grip strength		15				Yes	
Late puberty		106				Some evidence	
Fasting glucose		35				No	
Coronary heart disease		38				No	
Type 2 diabetes		38				No	
Vitamin D		4				No	
Dairy calcium intake		1				No	
Lactase persistence LCT-13910 C/T genetic variant	Northern Europeans	1	Hip fracture	97,811 Danish individuals	Fixed effects meta-analysis	No	(39)
Height	Europeans, *N* = 253,288 (GIANT)	697	Hip fracture	2,451 fracture cases of 417,434 individuals from UK Biobank	Two-sample (IVW)	Yes	(40)
Serum estradiol	Europeans	2	All self-reported fractures	Europeans, *N* = 17,650 (UK Biobank)	Two-sample (IVW)	Yes	(41)
		2	Major nonvertebral osteoporotic fractures	(*N* = 4,379; wrist, arm, and hip)			
		2	Wrist fractures	(*N* = 2,637)			
Testosterone	Europeans	3	All self-reported fractures	(*N* = 17,650)		No	
		3	Major nonvertebral osteoporotic fractures	(*N* = 4,379; wrist, arm, and hip)			
		3	Wrist fractures	(*N* = 2,637)			
Serum CRP levels	Europeans	29	Any fracture	6,386 participants (59% women), of whom 1,561 sustained a fracture	One-sample	No	(42)
Smoking initiation	Europeans *N* = 1,232,091 (including UK Biobank)	377	Any fracture (excluding skull, face, hands and feet, pathological fractures due to malignancy, atypical femoral fractures, periprosthetic, and healed fracture) and any self-reported fractures	Europeans *N* = 426,795 (53,184 cases and 373,611 non-cases) (UK Biobank)	Two-sample (IVW)	Yes	(26)
Genetically predicted alcohol intake	Europeans *N* = 941,280 (including UK Biobank)	99	Any fracture (excluding skull, face, hands and feet, pathological fractures due to malignancy, atypical femoral fractures, periprosthetic, and healed fracture) and any self-reported fractures	Europeans *N* = 426,795 (53,184 cases and 373,611 non-cases) (UK Biobank)	Two-sample (IVW)	No	

(Continued)

TABLE 2 | Continued

Exposure	Sample source for exposure data	Genetic variants (*n*)	Outcome	Sample size and data sources for the outcome data	MR method	Evidence of causal effect (Yes/No)	References
Genetic liability to alcohol dependence	Europeans *N* = 46,568 (11,569 cases and 34,999 controls)	2	Any fracture (excluding skull, face, hands and feet, pathological fractures due to malignancy, atypical femoral fractures, periprosthetic, and healed fracture) and any self-reported fractures	Europeans *N* = 426,795 (53,184 cases and 373,611 non-cases) (UK Biobank)	Two-sample (IVW)	Some evidence	
LDL-C levels	*N* = 188,577 (GLSC)	76	Fractures at any skeletal site confirmed by medical, radiological, or questionnaire reports	147,200 cases and 150,085 controls (primarily of European ancestry)	Two-sample (IVW)	No	(32)
Gene encoding molecular target of LDL-C-lowering therapy (HMGCR)	*N* = 188,577 (GLSC)	76	Fractures at any skeletal site confirmed by medical, radiological, or questionnaire reports	147,200 cases and 150,085 controls (primarily of European ancestry)		No	
Total serum calcium	Europeans (discovery cohort *N* = 39,400, replication cohort *N* = 21,676)	6	Fracture	76,549 cases and 470,164 controls from GEFOS, EPIC-Norfolk study and UK Biobank	Two-sample (IVW)	No	(38)

OR, Odds ratio; IVW, inverse-variance weighted; HR, hazard ratio; THS, thyroid stimulating hormone; LS, lumbar spine; FN, femoral neck.

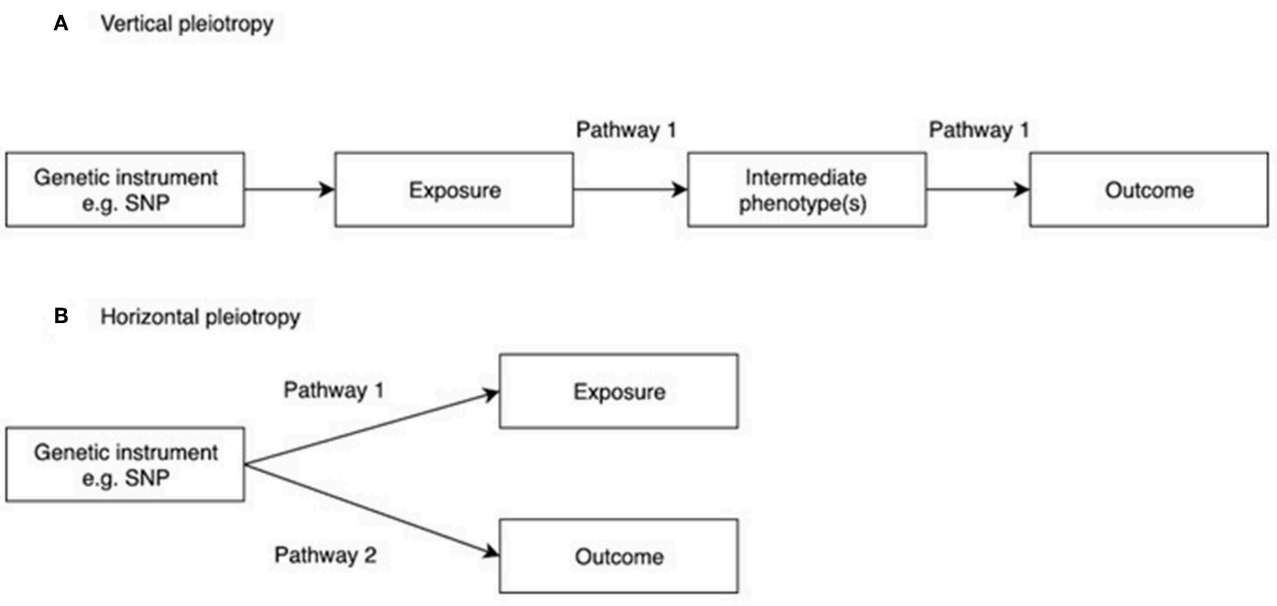

FIGURE 2 | Vertical and horizontal pleiotropy. **(A)** Vertical pleiotropy, which does not violate the MR assumption; **(B)** Horizontal pleiotropy, which violates the MR assumption.

that only a certain proportion of the genetic instruments have a horizontal pleiotropic effect. These methods aim to reduce heterogeneity by removing SNPs that contribute to heterogeneity disproportionately, based on the standard errors of the Wald ratios. Such outlier removal strategies are applied in the MR-PRESSO (50), and generalized summary MR (GSMR) approaches (51). One of the issues in applying MR methods that are robust to pleiotropy is that in order to detect causal effects these require large sample sizes (49). Another issue concerns the number of SNPs used as IVs; significant numbers of SNPs are required to provide sufficient data points for meaningful analysis. However, an advantage is that these approaches often rely on different

sets of assumptions, and if consistent, conclusions can be drawn regarding causality with reasonable confidence.

The recent review by Lawlor et al. provides a useful summary of the various methods and extensions of Mendelian Randomization (52). Some of these more advanced MR analysis methods have been applied in relatively recent studies examining causal inference in osteoporosis. For example, in their study examining causal relationships between blood lipids and eBMD, Cherny et al. found broadly similar inverse associations between LDL-C and eBMD as assessed by inverse-variance weighted (IVW), MR-Egger, weighted median and weighted mode estimates (53). Similarly, in our recent study, MR-Egger, IVW and weighted median estimates showed similar causal effects of BMD on sclerostin (54). That said, the statistical evidence against the null for the MR-Egger estimates was somewhat lower, in both these papers, reflecting the lower statistical power of this test (48, 49). However, these types of sensitivity analyses have been lacking in many of the MR analyses in osteoporosis, including those analyzing a range of metabolites for which genetic influences could well-exert pleiotropic effects via unknown pathways (38).

It may be hard to exclude pleiotropy where the genetic instruments comprise just one or two SNPs, however there are exceptions to this. For example, we recently performed an MR study to examine the causal relationship between sclerostin levels and eBMD, based on results of a sclerostin GWAS where we identified just two loci. However, we were able to establish a causal relationship between sclerostin and eBMD using co-localization analysis, which interrogates LD structure at a single locus, in this case the gene encoding B4GALNT3 (54).

Several analysis methods have been developed to explore causal pathways in those situations where genetic instruments are pleiotropically associated with multiple correlated phenotypes. For example, in studying the causal relationship between genetically determined BMD (reflected by eBMD) and OA, Funck-Brentano et al. observed a strong causal effect of BMI on knee and hip OA, suggesting that if any eBMD SNPs are shared with BMI, this may influence OA via pathways other than BMD, which will violate the 3rd MR assumption (horizontal pleiotropy). The authors established that genetically determined BMD also has a causal effect on OA after excluding pleiotropic pathways involving BMI, by removing SNPs from their eBMD polygenic risk score that were related to BMI (55). Similarly, Cousminer et al. excluded SNPs for height and BMI in their MR analysis of the causal role of pubertal age on BMD (33).

An alternative method of accounting for pleiotropy where genetic variants are pleiotropically associated with multiple correlated phenotypes, is to perform multivariable MR. The latter aims to address this limitation by using instruments associated with multiple exposures to jointly estimate the separate causal effect of individual risk factors on the outcome (34, 56–58). For example, Kemp et al. used a one-sample multivariable method to show that BMI SNPs acted via both lean and fat mass to increase BMD (34). In an MR analysis of relationships between plasma lipids and BMD, we observed a strong inverse association between LDL cholesterol and forearm BMD in multivariate MR analyses adjusting for HDL cholesterol and triglycerides, which was not evident in univariate analyses involving only LDL cholesterol (59). This indicates that complex relationships may exist between the causal effects of different lipids and BMD, which MR analyses need to account for, and may help to explain the conflicting results from different MR analyses examining relationships between lipid levels and eBMD in the UK Biobank (53, 59, 60).

DISTINGUISHING GENETIC CORRELATION FROM CAUSALITY

Key points:-

1. Traits which are correlated as a result of shared underlying biology are likely to have shared genetic influences, leading to a positive signal in MR studies
2. MR signals arising from genetic correlation between two traits are expected to be bidirectional; true causal effects generally produce a positive MR signal in one direction only (i.e., exposure to outcome as opposed to outcome to exposure)
3. In bidirectional MR, it may be helpful to use methods such as Steiger filtering to restrict SNPs to those which have strongest effects on the outcome as opposed to the exposure being tested
4. Though rarer, bidirectional causal effects may exist, exemplified by a positive causal effect of BMD on sclerostin levels, and a negative causal effect of sclerostin on BMD.

It's common for two related traits to share a proportion of their heritability, as quantified by genetic correlation, implying some form of shared underlying biology. Bidirectional MR can help distinguish causality from correlation by first testing the associations in one direction (i.e., "exposure" to "outcome"), and then performing these in the opposite direction (i.e., "outcome" to "exposure"), using SNPs found to be associated with each trait in different GWASs. In those instances where certain SNPs are common to GWASs for both the exposure and outcome, methods such as Steiger filtering are recommended to remove these SNPs to ensure they are used correctly as instruments for analyses in one direction only (61). Bidirectional MR assumes that the underlying causal association works in a single direction. Where there is evidence for "bidirectional causality," this may simply reflect genetic correlation arising from a common genetic pathway affecting both the exposure and outcome. That said, bidirectional Mendelian randomization can identify causal effects that do work in both directions; for example, smoking reduces BMI and higher BMI increases smoking (62). In the case of bidirectional causality where evidence is stronger in one direction, although the main causal pathway may be in this direction, findings may reflect misspecification of the exposure variable as described above. Alternative strategies to MR, such as latent causal variable analysis, have been developed to distinguish correlation from causality (63).

Certain biomarkers and risk factors for osteoporosis may be unlikely to show strong genetic correlations with BMD, and to be influenced by common biological pathways, nevertheless it's still good practice to perform bidirectional MR. For example,

in the case of factors such as smoking, which was found to be genetically related to lower heel BMD (25), in the absence of bidirectional MR, it's not possible to exclude reverse causality, which is not inconceivable given the casual effect of BMI (which is known to influence BMD) on smoking (62). In recent studies examining relationships between panels of blood metabolites and BMD, where the direction of the causal effect is unclear, whereas one study reported findings from bidirectional analysis (64), a further one did not (38). In addition, genetic correlation as a consequence of shared biological pathways could conceivably explain relationships between BMD and other disease phenotypes such as osteoarthritis (OA). For example, a previous study revealed significant genetic correlation between LS BMD and hip and/or knee OA, suggesting common genetic influences, exemplified by the *SMAD3* locus found to affect both OA risk and BMD (65). Shared genetic influences on BMD and OA could also explain recent findings that genetic instruments for eBMD are associated OA (55); whereas the authors interpreted this as indicative of a causal effect of eBMD on OA, bidirectional MR is required to prove such a causal pathway exists, as opposed to common biological mechanisms contributing equally to both traits.

One of the challenges in performing bidirectional MR between two variables which are highly correlated genetically is that the two traits are likely to share one or more SNPs in common. This is particularly an issue when using results derived from large GWAS studies that generate many signals. For example, in our recent study of relationships between eBMD and lipids, a bidirectional effect for eBMD on LDL-C was investigated using 404 eBMD associated SNPs as genetic instruments (12). Steiger filtering was used to identify SNPs that had stronger effects on the outcome (LDL-C) compared to the exposure (BMD). This analysis suggested that 394 of 404 SNPs exerted their primary effect on BMD as opposed to LDL-C levels. IVW MR, weighted median MR and MR-Egger regression results showed some evidence that eBMD might influence LDL-C, and the association remained unchanged after Steiger filtering to remove those SNPs that primary affected LDL-C levels (59).

As well as selectively removing SNPs to assist interpretation of bidirectional analyses, this approach may also be helpful in examining the role of specific biological pathways involved in mediating causal effects. For example, in the above MR analysis of the effects of plasma lipids on eBMD, we were able to confirm that the inhibitory effect of LDL cholesterol on eBMD which we observed was not solely mediated by SNPs intersecting the *HMGR* locus which is the target for statin therapy, since similar results were obtained when SNPs at this locus were removed from the polygenic risk score. Similarly, SNPs can be stratified into relevant/specific biological pathways and their association with outcomes of interest tested. For example, although not a formal MR analysis, Warrington et al. used genetic risk scores constructed from SNPs belonging to specific biological pathways, and showed that genetic risk scores comprising variants that belonged to the RANK-RANKL-OPG pathway, the mesenchymal stem cell differentiation functional pathway and the WNT signaling function pathway were associated with bone measures at age 13, but only mesenchymal stem cell differentiation and the WNT pathway SNPs showed associations with rate of change in BMD between 9 and 17 years (66).

It's a reasonable assumption that correlated variables as a result of shared biology show equivalent "causal" effects on bidirectional MR, whereas for a true causal relationship, an effect is just observed in one direction. However, we recently observed a further pattern in our study exploring the relationship between circulating sclerostin levels on eBMD, namely bidirectional causal pathways in opposite directions (54). We found that higher levels of serum sclerostin were causally related to lower FN BMD, lower eBMD and higher fracture risk. In contrast, greater BMD was causally related to higher sclerostin levels, using BMD SNPs identified in the GEFOS BMD GWAS (54). This finding aligned with the observational relationship between BMD and sclerostin we reported in the same paper and may be a reflection of a previously unsuspected negative control feedback mechanism for BMD (see **Figure 3**). However, the exact mechanisms involved remain unclear and functional validation of such a pathway is still needed.

POWER CONSIDERATIONS

In contrast to conventional epidemiological studies where the exposure variable comprises the population variance of the trait of interest, in MR, genetic instruments only capture a small proportion of trait variance (not infrequently <1%). As a consequence, the strength of the relationship between an instrumental variable used for MR, and the outcome of interest, will only be of a small fraction of that seen for the measured exposure variable. Therefore, limited power is a common problem for MR analyses, and a frequent explanation of null findings, and needs to be an important consideration particularly when the findings fail to support other well-established lines of evidence. Limited power is even more problematic for some of the more recent extensions to MR, such as multivariable MR.

In any given MR study, the major factors governing the power are the sample size, the strength of the genetic instruments available, the strength of the underlying causal relationship being evaluated, and the type I error rate. Recent availability of very large datasets, such as the UK Biobank, have facilitated well powered MR studies, as have the increasing number of GWAS signals available for any given trait. Nonetheless, even where a large sample is available, MR may be uninformative where available instruments are lacking, or if there is a weak underlying relationship. These considerations are particularly important when null associations are obtained, where it is helpful to report power calculations to illustrate the strength of any underlying relationship which would have been detectable, and how this compares with that seen in observational studies (67).

A related issue is weak instrument bias. In one-sample MR, using weak instruments may bias the causal association toward the observational association between the exposure and outcome, whereas in two-sample MR, weak instruments may bias MR estimates toward the null (68). Therefore, it is important to avoid such bias by evaluating instrument strength at the outset of the study. For most human phenotypes, common genetic

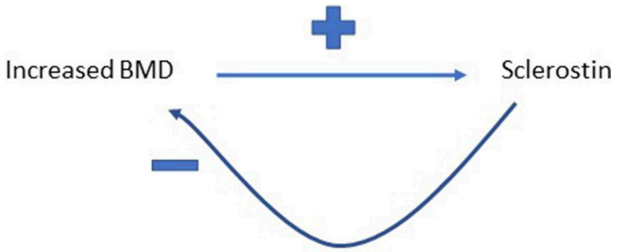

FIGURE 3 | Proposed feedback pathway between BMD and sclerostin. Greater BMD is proposed to increase circulating levels of sclerostin, which then feeds back to inhibit bone formation, and hence limit further gains in BMD.

variants only explain a limited proportion of the variance of the phenotypes; combining small effects across these common variants into a score (known as the polygenic risk score) may increase instrument strength (69). Another relevant concept is the "No Measurement Error" (NOME) assumption of MR, which assumes the association between a given genetic instrument and the exposure is estimated without measurement error (70). This is particularly important when weak instruments are estimated from GWAS with small sample sizes. In an IVW setting, the mean F-statistic can be used to assess whether instruments violate the NOME assumption, a value below 10 implying a high likelihood of weak instrument bias (71). In an MR-Egger setting, the I2 statistic (between 0 and 1) can be used to quantify violation of the NOME assumption, a lower value indicating greater likelihood that this assumption has been violated (48).

In the majority of instances of null findings reported in recent MR studies of osteoporosis risk factors, genetic instruments have been identified based on genome-wide significant associations from large scale GWASs, and although instrument strength is not universally reported, weak instrument bias is less likely to be an issue under these circumstances (70). However, weak instrument bias may be an issue in those instances where instruments have been identified from relatively small GWAS studies. For example, in a two-sample MR study examining causal relationships between inflammation and BMD reporting null findings, three of the genetic instruments for IL-6 were derived from a population of 1,664 individuals, and had F-statistics ranging from 3 to 8, indicating high likelihood of weak instrument bias (29).

FUTURE DIRECTIONS

It may be possible to extend MR to identify novel risk factors for osteoporosis using a hypothesis-free approach. For example, centralized databases such as MR-Base (46) and UK Biobank (http://www.nealelab.is/uk-biobank) have harmonized GWAS summary results for more than 20,000 complex human traits. Such resources make it feasible to conduct a phenome-wide MR for osteoporosis, aimed at identifying novel causal effects on BMD from screening a comprehensive range of complex traits. In many cases, mega biobanks such as UK Biobank, provide the richest source of GWAS-linked exposure or outcome data. Consequently, the issue of overlapping samples for generating genetic instruments and providing outcome data in a two-sample MR framework, potentially providing biased estimates (8), is becoming increasingly problematic. With the burgeoning opportunities for performing MR analyses, there also comes the need to ensure these are performed and reported comprehensively, with thorough exploration of issues such as pleiotropy, reverse causality and power, to ensure appropriate conclusions are drawn. STROBE-MR guidelines, intended to improve the quality of reporting of MR studies, have recently been produced (72).

MR was initially developed to examine the causal role of environmental exposures on the outcome of interest. This method has since been applied to a wide range of research areas, including drug target validation and prioritization, and the interpretation of multi-dimensional omics data. Large-scale GWASs of omics data, such as metabolites, DNA methylation, gene expression and protein expression provide a timely opportunity to identify the causal relationship of thousands of molecular phenotypes with osteoporosis in a MR framework. Automated tools such as summary-data-based MR (SMR), Generalized Summary-data-based MR (GSMR) and the two-sample MR R package make it possible to conduct such large-scale analyses effectively (46, 51, 73).

For omics studies of osteoporosis, one of the issues that needs further consideration is tissue specificity. Most molecular phenotypes to date have been measured in whole blood, for which the sample size of expression QTLs and methylation QTLs studies exceeds 30,000 (74) (http://www.godmc.org.uk/) and protein QTLs studies exceed 6,000 (75, 76). In contrast, the QTLs measured in bone tissues are limited to several hundreds of individuals. Whether molecular phenotypes measured in blood can be used as a proxy for those measured in bone tissues remains unclear, particularly methylation which shows a high degree of tissue specificity, in line with emerging trends in tissue specific MR (77), implying an urgent need for osteoblasts, osteoclasts and osteocytes and other skeletal cell types to be sufficiently well-represented in omics resources.

CONCLUSIONS

MR is being increasingly applied to examine causal inference in osteoporosis, reflecting the increasing availability of large datasets such as the UK Biobank, and multiple GWASs for potential risk factors. To date, the most important findings have been around the lack of causal role of traditional risk factors such as vitamin D in determining variation within the normal range of BMD/fracture risk. High-dimensional omics studies, based on GWASs of metabolites, gene expression and DNA methylation, offer exciting opportunities for future discovery, with the emergence of the first MR studies of metabolites in osteoporosis. However, an important caveat is that MR studies can be complicated by a number of issues including horizontal pleiotropy, reverse causality, and lack of power. Several extended MR methods have been developed to explore these aspects, and while not always applied consistently, r STROBE-MR guidelines have recently been produced, intended to support the quality with which MR studies are reported.

AUTHOR CONTRIBUTIONS

JZ and MF helped to write the manuscript. JK, DE, and GS reviewed the manuscript and provided critical comments. JT conceived and helped to write the manuscript.

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REVIEW
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The Genetic Architecture of High Bone Mass

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The phenotypic trait of high bone mass (HBM) is an excellent example of the nexus between common and rare disease genetics. HBM may arise from carriage of many 'high bone mineral density [BMD]'-associated alleles, and certainly the genetic architecture of individuals with HBM is enriched with high BMD variants identified through genome-wide association studies of BMD. HBM may also arise as a monogenic skeletal disorder, due to abnormalities in bone formation, bone resorption, and/or bone turnover. Individuals with monogenic disorders of HBM usually, though not invariably, have other skeletal abnormalities (such as mandible enlargement) and thus are best regarded as having a skeletal dysplasia rather than just isolated high BMD. A binary etiological division of HBM into polygenic *vs*. monogenic, however, would be excessively simplistic: the phenotype of individuals carrying rare variants of large effect can still be modified by their common variant polygenic background, and by the environment. HBM disorders—whether predominantly polygenic or monogenic in origin—are not only interesting clinically and genetically: they provide insights into bone processes that can be exploited therapeutically, with benefits both for individuals with these rare bone disorders and importantly for the many people affected by the commonest bone disease worldwide—*i.e*., osteoporosis. In this review we detail the genetic architecture of HBM; we provide a conceptual framework for considering HBM in the clinical context; and we discuss monogenic and polygenic causes of HBM with particular emphasis on anabolic causes of HBM.

Keywords: high bone mass (HBM), osteopetrosis, *SOST*, *LRP5*, dual-energy X-ray absorptiometry (DXA), bone mineral density (BMD), genome-wide association studies (GWAS)

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INTRODUCTION

Most people are first introduced to genetics through the gardening career of Gregor Mendel and his observations regarding various features of the pea plant (flower color, pod shape, etc.). Mendel's studies led him to conclude that individual characteristics (*i.e*., phenotypes) were determined by discrete units of information (*i.e*., genes) that came in pairs (*i.e*., alleles), with one of each pair inherited by each offspring randomly and independently of the genes determining other characteristics (1). He also concluded that at any particular locus one allele would be dominant and the other recessive.

Mendel's laws certainly explained the phenotypes observed in his multigenerational plant breeding experiments; and they provided an explanation for the inheritance of autosomal monogenic disorders (2). However, they appeared not to explain the inheritance of many traits that exhibit continuous distribution in the population (*e.g.*, height, weight). Initial attempts at reconciliation proposed that continuously distributed phenotypes might still be determined by a single locus but with a 'blending' of each parent's characteristics rather than a pure dominant/recessive model of inheritance (*e.g.*, a tall mother and a short father would have children of average height); but ultimately this question was resolved by the demonstration that continuously distributed (or quantitative) traits arise from the effect of multiple genetic loci, each of which individually exhibits Mendelian inheritance (3), which combine, both additively and interactively, and within a given environment, to produce the final phenotype.

These concepts are not just of historical interest but highly relevant when considering the genetic architecture of high bone mass (HBM)—or indeed any other heritable disease.

WHAT IS GENETIC ARCHITECTURE?

To quote Gratten et al., "genetic architecture refers to the number of genomic loci contributing to risk, the distribution of their allelic frequencies and effect sizes, and the interactions of alleles in and between genes, all of which contribute to the relationship between genotype and phenotype. Understanding genetic architecture is the foundation on which progress in dissecting etiology is built because it dictates which study designs for identifying risk variants are likely to be most successful." (4) It is hard to improve upon this elegant definition and its clear consequences regarding gene mapping strategies [for an in-depth discussion of this topic, the reader is referred to an excellent recent review (5)].

In considering the genetic architecture of HBM specifically, the simplest question that can be asked is whether HBM is monogenic (due to carriage of a rare variant of large phenotypic effect) or polygenic (arising from the cumulative effect of multiple variants, each individually of small effect). However, even answering this apparently simple question is not straightforward, as these are not necessarily mutually exclusive options, whether considering either the HBM population as a whole or a particular affected individual.

Monogenic diseases, whether dominant or recessive, autosomal or X-linked, are due to rare highly penetrant alleles affecting a single gene. Monogenic diseases generally follow classical Mendelian inheritance such that the presence or absence of disease is mathematically predictable, with some leeway for variable penetrance and expressivity from genetic and/or environmental modifiers (6). Although individually rare, the World Health Organization (WHO) estimates that monogenic disease affect 1% of the worldwide population (7); and there are many skeletal dysplasias that display classic Mendelian inheritance, with either high (*e.g.* osteopetroses) or low (*e.g.* osteogenesis imperfecta) bone mineral density (BMD) (8).

However, this does not mean that all heritable dichotomous disease states are monogenic. Many common diseases (*e.g.*, ankylosing spondylitis, osteoarthritis, breast cancer) are defined as present or absent according to particular characteristics, whereas other common diseases (*e.g.*, hypertension, type 2 diabetes) are defined using a threshold value along a continuously distributed phenotype (*i.e.*, blood pressure and glycemia). It is perhaps easier to understand how quantitative disease states may be polygenic in inheritance (3), compared with qualitative (*i.e.*, dichotomous) common disease states. However, qualitative diseases may also be polygenic: it is the underlying risk of disease that is quantitative, with disease manifest once a particular genetic threshold is reached (3, 9). Indeed, *a priori* even diseases that might appear monogenic are more likely to be polygenic (10). The validity of this concept has been demonstrated comprehensively by the enormous success of genome-wide association studies (GWAS), which have identified thousands of variants associated with a host of common quantitative and qualitative diseases as diverse as type 1 diabetes to schizophrenia to prostate cancer (11). The polygenic common variant 'background' can also modify the phenotype of persons carrying rare highly penetrant monogenic variants—such as *BRCA1* mutations in breast cancer or carriage of HLA-B27 in ankylosing spondylitis (12–14). Here, it is worth highlighting that extreme HBM populations are enriched with common variant 'high BMD' alleles (discussed further later in this article) (15).

In considering the translational applications of GWAS, it would be fair to say that at least initially the clinical utility of polygenic (or genomic) risk scores (PRS) calculated using genome-wide associated SNPs was underwhelming—certainly in bone disease. At the time of publication of the second GEnetic Factors in Osteoporosis Study [GEFOS-2], a study involving tens of thousands of cases and controls, the PRS derived from variants associated with femoral neck BMD at genome-wide significance (*i.e.*, $p < 5 \times 10^{-8}$) performed less well in predicting BMD than age and weight alone (area under receiver-operator characteristics curve: 0.59 *vs.* 0.75) (16). This was not really surprising: despite the large sample size, the identified variants still explained only a small proportion (<6%) of overall BMD heritability (16). Over time, ever larger GWAS have been performed (17, 18); and certainly increasing GWAS population size strongly correlates (on a log scale) with the number of SNPs identified at genome-wide significance to be associated with disease (11), capturing a greater proportion of heritability, and improving PRS utility. Additionally, adopting a less stringent threshold for SNP inclusion in PRS also increases the proportion of genetic variance captured—at the cost of more noise and inclusion of more false-positive results. There is no fixed formula for the sweet spot between sensitivity and specificity for PRS (*i.e.*, maximizing AUCs in ROC analyses or similar statistic). It is disease-specific (19); and for maximal clinical utility the PRS must also be interpreted in the context of other disease-specific factors including disease heritability, prevalence, and prior probability (19–23). [For further discussion on the calculation and clinical utility of PRS, the reader is referred to two recent review articles

(19, 23)]. However, despite these caveats, PRS have reached the point whereby, to quote Khera et al., "for a number of common diseases, polygenic risk scores can now identify a substantially larger fraction of the population than is found by rare monogenic mutations, at comparable or greater disease risk" (24). For example, at a population level, the proportion of individuals who carry a sufficient burden of common variants to place them at three-fold risk of coronary artery disease is twenty-fold that of individuals carrying rare highly penetrant LDL-R mutations of equivalent risk (24). Moreover, common variants can be easily and cheaply genotyped, without needing whole population whole-genome sequencing, noting that the choice of technology in this area is sometimes a political rather than a strictly scientific decision.

USE OF BMD TO DEFINE DICHOTOMOUS DISEASE STATES OF OSTEOPOROSIS AND HIGH BONE MASS

Defining a disease state by use of a particular threshold value within the normal population distribution of a quantitative trait is a concept extremely familiar to the bone community. The most commonly employed measure of bone strength is BMD, usually assessed using dual-energy X-ray absorptiometry (DXA). The result is then compared against an age, ethnicity and sex-specific reference population, allowing calculation of T- and Z-scores (the number of standard deviations (SDs) by which the result differs from the mean BMD of a young adult or age-matched population, respectively). Individuals with lower BMD are at higher risk of fracture, particularly low trauma fractures (25). Reflecting this risk, in 1999 the WHO used DXA BMD to define osteoporosis and osteopenia (for osteoporosis, a T-score of ≤−2.5; for osteopenia, a T-score between −1 and −2.5) (26). These threshold definitions do not account for other major risk factors for fracture—such as age and prior fragility fracture, both of which independently increase future fracture risk (27)—and use of BMD in isolation to define the real clinical issue (*i.e.*, bone fragility and fracture risk) can lead to apparent paradoxes, *e.g.*, a woman with osteopenia (according to BMD) and a previous low trauma fracture is at higher risk of a fracture than a women with BMD-defined osteoporosis who has not yet fractured (28). Nevertheless, such thresholds are useful in identifying a high-risk group of clinical relevance, in whom intervention might be most clinically- and cost-effective. Further, fracture risk calculators [*e.g.*, FRAX, Garven (29)] have been developed to account for key clinical risk factors, as well as BMD, to circumvent the limitations of BMD alone.

At the other end of the normal distribution for BMD are individuals with HBM. It is tempting to regard these individuals simply as phenotypic outliers, with their BMD results of little serious clinical consequences unless such individuals unexpectedly find themselves in deep water (non-metaphorically). However, studying individuals with HBM is of relevance both for their own sake and for the community more broadly. Firstly, HBM may indicate an underlying and hitherto-unsuspected skeletal dysplasia with specific clinical needs (*e.g.*, monitoring of cranial nerve function, therapeutic choices, and genetic counseling) (30). Second, these individuals provide novel insights into the regulation of bone mass: such discoveries may inform not only therapeutic approaches to their own HBM condition but also for the opposite, and more prevalent, bone phenotype of osteoporosis. In considering this last point, an important caveat applies. While low BMD is closely related to increased fracture risk (25), the converse is not necessarily true (31). For example, individuals with high BMD due to disorders of bone resorption (*e.g.*, osteopetroses) or disturbed bone turnover (*e.g.*, Paget's disease), can manifest high fracture rates.

HOW HIGH IS HIGH BONE DENSITY?

Epidemiological studies of high BMD are few and definition thresholds are variable (32, 33). Indeed, the absence of an upper limit to define 'normal' BMD risks those with a pathological cause for high BMD being missed and labeled as "normal." In 2005, Michael Whyte proposed a high BMD definition of a Z-score >+2.5, to alert clinicians to this issue (30). However, until more recently publications around high BMD were still the purview of case reports and small case series.

To address this question, we conducted the first systematic analysis of patients undergoing routine clinical DXA scanning, encompassing 335,115 DXA scans across 15 UK centers. We first used a screening threshold T or Z-score ≥+4 at any lumbar/hip site to identify those with extreme high BMD, in whom we investigated the potential underlying causes for a high BMD, trying to identify within this heterogeneous population with high BMD, a sub-group with unexplained generalized HBM (identified using a Z-score threshold ≥+3.2) (discussed in detail later) (34). Overall, within this UK population, scanned by DXA over a retrospective 20 year period for a wide variety of more-or-less clinically justifiable indications, we found the prevalence a T or Z-score ≥+4 to be 0.5%, and within that of unexplained generalized HBM with Z-score ≥+3.2 to be 0.18% (34). Interestingly, reflecting on the mathematics of a normal distribution, four standard deviations (SDs) would be expected to identify just 0.003% of a population, while 3.2 SDs equates to 0.069% (35). Taken together, it seems BMD might have a marginally bimodal distribution at the upper tail of its distribution.

While this study was the first to assess the prevalence of high BMD within the general population, the—albeit large—population composed of individuals referred for DXA scanning for clinical reasons, rather than selected to represent the general population. Thus, selection bias is possible. However, as most individuals are referred for DXA due to a pre-test suspicion of low BMD and/or osteoporosis (*e.g.*, a history of steroid use), if anything the true prevalence of high BMD may have been underestimated to date. Thus, this study provides a minimal prevalence for this condition.

DETECTING HIGH BMD IN CLINICAL PRACTICE

Incidental high BMD results in clinical practice are relatively common (34), and we have previously published an approach to

guide their assessment and investigation (36). The commonest causes for a high BMD are artefactual, with osteoarthritic degeneration explaining half of all high BMD measurements (34) (see **Table 1** for list of artefacts). Importantly, identifying the presence of artefact in someone with apparently high BMD on DXA does not mean fracture risk is necessarily low; and artefact is important to recognize as it may mask osteoporosis. For example, an osteoporotic vertebral fracture with vertebral collapse will reduce measured bone area while maintaining bone mineral content, and thus increase calculated BMD.

Interestingly, many artefactual causes of high BMD are themselves heritable. The most common example is spinal osteoarthritis with osteophytosis (**Table 1**). The heritability of osteoarthritis is approximately 50%, and two large GWAS published in the last two years have identified association with 96 loci (37, 87). As another example, ankylosing spondylitis [AS] is highly (>90%) heritable (88), and associated with over 100 loci, in addition to HLA-B27 (42). AS artefactually elevates BMD through syndesmophyte formation at vertebral margins, anterior longitudinal ligament ossification, and scoliosis (43). It is also associated with increased fracture risk (89), which may be due to the rigidity of the axial skeleton, the presence of inflammation, or a combination of both. A further example is diffuse idiopathic skeletal hyperostosis (DISH), most commonly seen in older men and characterized by widespread spinal calcification (38), which is also heritable (39), though as yet no causative variants have been published (90). The relationship between DISH and abnormal phosphate handling, may also carry implications for bone mineralization, bone strength, and fracture risk, though this has not been formally assessed. The closely related disease ossification of the posterior longitudinal ligament (OPLL) is also heritable (91), with both common and rare susceptibility variants identified (92, 93)—though as this condition most commonly affects the cervical spine, a site not routinely screened by DXA, OPLL is less likely to cause clinical conundrum as an artefactual cause of high BMD in daily practice.

TABLE 1 | Causes of a high BMD measurement on a DXA scan.

Artefactual causes of raised BMD—no true increase in bone mass			**Genetic Contribution**		**Refs.**
			Monogenic	**Polygenic**	
Osteoarthritis			[a]	Yes	(34, 37)
DISH: Diffuse idiopathic skeletal hyperostosis			Yes	Yes	(38–41)
Ankylosing spondylitis				Yes[b]	(42, 43)
Vertebral fractures			Yes[b]	Yes	(17, 34)
Vascular calcification			Yes	Yes	(44–48)
Thalassemia major			Yes		(49, 50)
Gaucher's disease (splenomegaly overlies the lumbar spine DXA field)			Yes		(34, 51)
Abdominal abscesses					(52)
Gallstones					(53, 54)
Renal calculi			Yes		(54, 55)
Gluteal silicon implants					(56)
Intestinal barium					
Surgical metalwork					(34)
Laminectomy					(57)
Vertebroplasty & kyphoplasty					
Acquired causes of true increased bone mass and/or density					
Localised	Tumors	Primary malignancies*e.g. osteoblastoma, Ewing's sarcoma, carcoinoid, hemangioma, plasmocytoma, Hodgkin's disease*Secondary osteosclerotic metastases*e.g. prostate, breast, gastric, colonic, cervical carcinoma*	Yes	Yes	(58)
	Chronic infective osteomyelitis				
	SAPHO (Synovitis Acne Pustulosis Hyperostosis and Osteitis) syndrome			Yes	(59–61)
	CKD-MBD (Chronic Kidney Disease-Metabolic Bone Disorder)[c]		Yes	Yes	(62–64)
	Paget's disease of Bone (PDB)		Yes	Yes	(34, 65, 66)
	Early onset Paget's like syndromes		Yes		(67)
	X-linked hypophosphatemia (XLH)		Yes		(68)
	Osteogenesis imperfecta associated with mutations affecting the carboxy-terminal-propeptide cleavage site of the type 1 procollagen chain		Yes		(69)
	Gnathodiaphyseal dysplasia		Yes		(70, 71)
Generalized	Fluorosis				(72–74),
	Acromegaly		Yes	Yes	(75, 76)
	Hepatitis C-associated osteosclerosis				(77–79),
	Myelofibrosis		Yes	Yes	(80–82),
	Mastocytosis		Yes		(83–85),
	Oestrogen replacement implants				(86)

[a]*While there are no forms of monogenic OA, there are many monogenic skeletal dysplasias with degenerative joint disease—e.g. spondyloepiphyseal dysplasia tarda, achondroplasias.*
[b]*vertebral fractures occur in osteogenesis imperfecta.*
[c]*CKD-MBD increases in BMD can also be generalized.*

Calcification of structures anterior to the spine but within the DXA field can artefactually elevate BMD measurements (**Table 1**). Although vascular calcification of the abdominal aorta is common, reported in 43% of patients having lumbar DXA assessment (mean age 68 years), it is surprising how little evidence there is regarding the effect of this on lumbar spine BMD measures (94–97). The relationship between vascular calcification and low BMD is of particular interest (98), most evident (though not exclusively) in the chronic kidney disease population. Abdominal aortic calcification is associated with lower BMD and vertebral fractures (99); and the extent to which genetic pleiotropy underpins vascular calcification [itself heritable (44)] and osteoporosis is the source of active investigation. There are also monogenic forms of vascular calcification (for example, pathogenic variants in *ABCC8* causing pseudoxanthoma elasticum (MIM264800) [45–47)].

Beyond artefact, there are a number of other conditions, usually acquired through life, that cause true increases in bone mass and density, which may be localized or generalized.

LOCALIZED INCREASES IN BMD

From a clinical perspective, the most important question when faced with a localized increase in BMD is whether this might represent a tumor. Tumors causing local increases in BMD may be benign or malignant, primary or secondary (**Table 1**); in this context special mention must be made of breast and prostate cancer, both of which are associated with osteosclerotic bony metastases.

Paget's disease of the bone (PDB) explains 1.4% of incidental high BMD results (34)—though this figure may fall given the declining population prevalence of PDB (current UK age-adjusted prevalence of 2.5% and 1.6% for men and women respectively) (100). Excessive and disorganized woven and lamellar bone expands bone size and raises density, causing focal increases in BMD but also increasing deformity and risk of fracture. PDB commonly affects the lumbar spine and hips [after the pelvis, the commonest sites of involvement are lower lumbar vertebrae (101)] and may be monostotic (*e.g.*, affecting an isolated vertebra) or polyostotic. PDB is often asymptomatic and may be present for years before diagnosis. PDB also displays both monogenic and polygenic inheritance. Mutations in *SQSTM1* (p62) account for 40% of familial and 10% of sporadic PDB (MIM167250) (65); and other monogenic forms of PDB include the more severe and/or early onset PDB caused by mutations in *ZNF687*, *FKBP5*, and *TNFRSF11A* (which codes for RANK). Common variants in loci harboring the genes *CSF1*, *OPTN*, *TM7SF4*, and *RIN3* have also been implicated (65). It is thought that environmental triggers interact with this genetic architecture to predispose to disease, with one hypothesized environmental trigger being zoonotic infections (102).

In additional to classical PDB, a number of rarer Paget's-like syndromes have been described with onset early in life that can also cause localized increases in measured BMD. These include expansile skeletal hyperphosphatasia, familial expansile osteolysis (FEO) (MIM174810), Juvenile Paget's disease (MIM239000), early-onset familial Paget's disease (MIM602080), and panostotic expansile bone disease (67). Children present with deafness, dental disorders and on occasion, active focal bone lesions; and as in PDB alkaline phosphatase levels tend to be raised. These conditions are due to genetic mutations in the RANK-NFkappaB signaling pathway [comprehensively reviewed in (103)].

Mutations affecting the carboxy-terminal-propeptide cleavage site of the type 1 procollagen chain (*COL1A1*) cause an unusual form of osteogenesis imperfecta in which individuals manifest marked bone fragility while having high BMD, due to hyperosteoidosis and hypermineralization. Patchy sclerotic lesions are often evident in the spine and elsewhere; in particular, these individuals develop unusual fibro-osseous lesions in the jaw ("cementoma") (69). There is a clinical overlap of this condition with gnathodiaphyseal dysplasia (70), which features also include bone fragility, irregular sclerotic BMD, and fibro-osseus lesions in the skull and jaw. Gnathodiaphyseal dysplasia is associated with mutations in *ANO5 (71)*, a gene not known to be involved in collagen production or processing; and the overlap in phenotype between these conditions is not fully understood. However, recent studies have suggested that ANO5 may be involved in osteoclast regulation (104).

SAPHO syndrome (Synovitis, Acne, Pustulosis, Hyperostosis and Osteitis) is a rare and poorly understood condition, in which about half the cases manifest spinal involvement including patchy osteosclerosis, hyperostosis, and para-vertebral ossification (59, 60). Clustering within families is reported and a genetic etiology (including an HLA contribution) has been suggested (61).

Chronic kidney disease-mineral bone disorder (CKD-MBD, previously referred to as renal osteodystrophy) causes osteomalacia, secondary hyperparathyroidism, and fracture. Radiological features of CKD-MBD include bony sclerosis, particularly of the vertebral body endplates, leading to a 'rugger-jersey' spine appearance (an appearance distinctive for hyperparathyroidism); or it can be more diffuse (62–64). CKD-MBD is associated with markedly increased fracture risk (64).

GENERALIZED HIGH BMD

A number of causes of generalized high BMD may be acquired through life (**Table 1**). For example, fluoride causes diffuse axial osteosclerosis with ligamentous calcification, periostitis and vertebral osteophytosis, and has been associated with excessive tea and toothpaste consumption (72–74). The increase in BMD led to fluoride being historically trialed as an osteoporosis therapy—but it resulted in a higher fracture risk, emphasizing that high BMD *per se* does not necessarily equate to stronger bones (105, 106). Other rare acquired causes of generalized high BMD are listed in **Table 1**.

However, rarer still, but fascinating are the monogenic causes of generalized high bone density, known as high bone mass (HBM) syndromes; these we discuss next.

MONOGENIC CAUSES OF GENERALIZED HIGH BMD

Several rare genetic disorders with skeletal effects, collectively termed osteopetroses and sclerosing bone dysplasias, are associated with generalized increased BMD. The most recent (10^{th}) edition of the Nosology and Classification of Genetic Skeletal Disorders (2019 revision) lists 462 genetic disorders of the skeleton among which are 45 conditions characterized by osteosclerosis or osteopetrosis, with the underlying gene(s) identified in 40 conditions at the time of going to press (8). As suggested previously (36), and per a recent review paper of de Ridder et al. (107), an intuitive biological separation can be made into disorders in which bone formation is enhanced, those in which bone resorption is depressed, and those with a disturbed balance between bone formation and resorption. Importantly, the associated changes in bone structure and quantity in the various sclerosing bone disorders can have quite different—indeed, completely opposite—effects on fracture risk (8).

It is not our intention to discuss all 45 osteosclerotic and osteopetrotic conditions listed in the current edition of the Nosology (8). Rather, we will focus on cases illustrative of the differences between types of monogenic high BMD, with a particular focus on anabolic HBM.

GENETIC CAUSES OF INCREASED BONE FORMATION AND HIGH BMD

A common feature of anabolic HBM is activation of the Wnt/β-catenin signaling pathway, with increased signaling through this pathway underlying the phenotype of sclerosteosis, van Buchem's disease, *LRP4* HBM, *LRP5* HBM, and *LRP6* HBM (all discussed below). For a detailed discussion of Wnt signaling in bone, the reader is referred to the excellent review of Baron and Kneissel (108). A brief—and, acknowledged, simplistic—description of the canonical Wnt/β-catenin signaling pathway is provided here. Wnt ligands bind to the dual receptor complex comprising Frizzled and LRP5 or LPR6 [LRP5/6], resulting in β-catenin escaping phosphorylation by being released from a multiprotein β-catenin "destruction complex", leading to β-catenin accumulation in the cytoplasm and ultimately translocation to the nucleus to activate target genes. In the absence of Wnt binding, β-catenin is phosphorylated by GSK-3β (a component of the "destruction complex") leading to its degradation and, consequently, loss of downstream signaling. Sclerostin inhibits Wnt signaling, by binding to LRP5/6 and preventing LRP5/6 from forming the dual receptor complex with Frizzled. LRP4 anchors sclerostin, enhancing sclerostin's interaction with LRP5/6, thus facilitating sclerostin's inhibition of Wnt/β-catenin signaling (108, 109).

Several human diseases characterized by HBM are associated with mutations of components of the Wnt/β-catenin signaling pathway (see **Figure 1** and text below). As a corollary, mutations of other components of this pathway may also cause HBM, with several such examples evident from mouse genetic studies (108). Thus, sequencing efforts in human populations may lead to the identification of other anabolic HBM conditions in humans.

Sclerosteosis and van Buchem's Disease

Sclerosteosis (MIM269500) and van Buchem's disease (MIM239100) are rare, clinically similar conditions of excessive bone growth. Loss-of-function *SOST* mutations cause sclerosteosis, generally thought the more severe of the two disorders; in contrast, a 52-kb intronic deletion downstream of *SOST*, thought to disrupt post-transcriptional sclerostin processing, results in the milder phenotype of van Buchem's disease (110, 111). In both disorders, reduced osteocytic production of sclerostin permits activation of osteoblastic Wnt signaling, leading to enhanced bone formation, increased bone strength, and resistance to fracture (110, 112) (**Table 2**). Understanding the molecular biology of sclerosteosis and van Buchem's disease has led to the development of monoclonal antibodies against sclerostin, which act to suppress the inhibitory action of sclerostin on Wnt signaling, allowing gains in bone formation (147, 148). Thus, anti-sclerostin antibodies represent a new class of anti-osteoporosis therapy; and recently the first-in-class agent (romosozumab) was approved by the United States Food and Drug Administration and the European Medicine Agency (discussed further below).

Sclerosteosis causes a large skeleton (sometimes termed 'gigantism', though this term is more usually reserved for children with excess long bone growth due to growth hormone excess prior to epiphyseal closure), mandible enlargement, and torus palatinus and mandibularis which can complicate tooth extractions (113, 149). Calvarial overgrowth compresses cranial nerves, particularly facial nerves, sometimes from infancy; in one series 83% of 63 adults had recurrent facial nerve palsies (113). Hearing loss and headaches are common; as is raised intracranial pressure—to the point that craniotomy may be required to prevent sudden death by coning (113, 150). Cutaneous syndactyly of fingers (present in 76%) and toes is an important defining feature, often accompanying dysplastic or absent nails and camptodactaly (113, 150, 151). Sclerosteosis is progressive, which may cause bone and back pain, with bony overgrowth requiring spinal and cranial decompression (113).

Van Buchem's disease is milder than sclerosteosis, importantly without syndactyly or "gigantism" (110, 150); however, cranial nerve impingements and hearing loss remain common (152). Management is generally limited to surgical bone removal. However, as tried in Camurati-Engelmann disease (progressive diaphyseal dysplasia, MIM131300) glucocorticoids have been used with the aim of reducing high bone turnover, in an isolated case report (153).

LRP5 High Bone Mass

In 1997, a family with HBM but an otherwise normal phenotype was reported with the genetic abnormality localized by linkage analysis to chromosome 11q12–13 (120). The 18-year-old proband had presented following a road traffic accident, without bone injury, but with consequent back pain. In the initial publication, 28 family members were phenotyped, aged 18 to 86 years. Inheritance of the HBM phenotype was autosomal dominant. Affected individuals were asymptomatic and had never

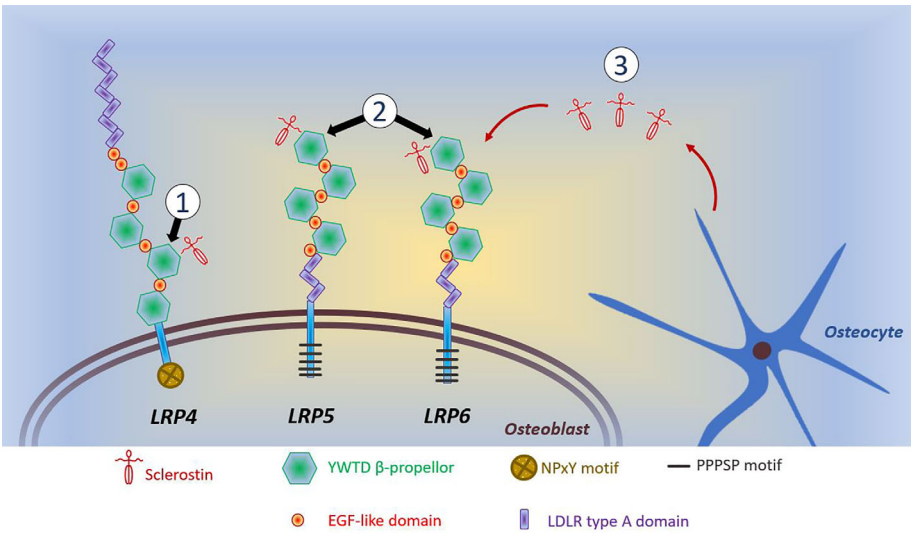

FIGURE 1 | Schematic diagram of reported mutations affecting osteoblastic Wnt signaling. (1) *LRP4* mutations coding for the 3rd β-propellor impair sclerostin binding; (2) *LRP5* and *LRP6* mutations coding for the 1st β-propellor impair sclerostin binding; (3) *SOST* mutations inhibit sclerostin production by osteocytes. Reductions in the inhibitory effects of sclerostin allows LRP5/6 to interact with Wnt and its co-receptor Frizzled, which prevents phosphorylation of β-catenin allowing it to accumulate in the cytoplasm of the osteoblast. Translocation of β-catenin to the nucleus activates transcription of target genes. This activation of canonical Wnt/β-catenin signaling increases osteoblastic bone formation. The intracellular consequences of LRP4-sclerostin binding are less well characterized; however, reductions in LRP4-sclerostin binding have a similar effect to increase osteoblastic bone formation. LDLR, low-density-lipoprotein receptor. LRP, LDLR related proteins; PPPSP, Proline, Proline, Proline, Serine, Proline; EGF, epidermal growth factor; NPxY, Aspartate, Proline, any amino acid, Tyrosine; YWTD, Tyrosine, Tryptophan, Threonine, Aspartate.

fractured; their biochemistry was normal (measured in a subset of 5 affected individuals); and their radiology showed dense bones with thick cortices and reduced medullary cavity but without consequent reduction in hemopoietic capacity. Affected individuals had spinal BMD Z-scores ranging from approximately +3.2 to +7.9; authors used a case definition threshold of Z-score > +3.0. They concluded that as HBM affected individuals aged as young as 18 years of age, the mutation has a role to play in the acquisition of peak bone mass; similarly, the clinical evaluation of older members of the pedigree supported a persistent influence throughout life without consequent disability (120, 121).

Interestingly, around the same time, osteoporosis pseudoglioma syndrome (OPPG) (MIM259770), was mapped to the same region (154). OPPG is characterized by osteoporosis, extreme bone fragility, fracture, and deformity; and although initially considered autosomal recessive, obligate carrier parents usually have low BMD. OPPG is due to inactivating mutations in *LRP5* (155). OPPG also leads to visual deterioration at birth or soon after, due to vitreoretinal degeneration, with multiple consequences including retrolental masses, retinal detachment, cataract, phthisis bulbi, microphthalmia, vitreous hemorrhage, secondary glaucoma and blindness (156). Inactivating *LRP5* mutations have also been associated with familial exudative vitreoretinopathy type 4 (FEVR-4) (MIM601813), with low BMD a common feature of affected individuals also (157); and it is now recognized that FEVR-4 and OPPG are allelic disorders with overlapping phenotypes. Notably, other forms of familial exudative retinopathy are associated with mutations in other genes that affect Wnt signaling, including *LRP4* and *FZD4*.

Further genetic analysis of the original HBM family, with extension of the pedigree to 38 members, identified a mis-sense *LRP5* mutation (c.512G>T, p.Gly171Val), in exon 3. All affected individuals were heterozygous for this mutation, consistent with autosomal dominant inheritance (121). HBM affection status in this kindred-based study was then defined as sum of hip and spine Z-score > +4.

In contrast to inactivating *LRP5* mutations associated with OPPG, the HBM phenotype results from activating *LRP5* mutations which stimulate osteoblastic bone formation (158). *LRP5* has 23 exons, coding for a 1615 amino acid protein, an essential cell membrane co-receptor key to the Wnt signaling pathway which regulates osteoblastic bone formation (122). The majority of the protein constitutes the extracellular β-propellor which has four domains (1180 amino acids in length). All HBM-associated *LRP5* mutations identified to date lie in exons 2, 3 and 4, which collectively code for the 1st β-propellor domain (**Figure 1**; **Table 3**); and protein modeling suggests they all lie at the top/central region of the extracellular protein (163). It is thought that these mutations reduce binding affinity with sclerostin and Dkk1, negative regulators of LRP5 signaling (163, 164). No inactivating mutations in this 1st β-propellor domain have been identified to date; instead, OPPG-associated mutations have been located within the 2nd and 3rd β-propellor domains, the binding domain, and the terminal signaling peptide (123, 165, 166). A hallmark of increased Wnt signaling in many organs, other than bone, is the development of malignant tumors (167, 168). However, fortunately this has not been reported as a feature of *LRP5* HBM.

TABLE 2 | Inherited HBM conditions due to enhanced bone formation: gene defects, function, and clinical characteristics.

Condition	MIM	Inheritance	Gene	Mutation	Protein	Function	Clinical Features	Ref
Increased bone formation								
Sclerosteosis	269500	AR	*SOST*	Loss of function	Sclerostin	Osteoblast Wnt signaling inhibitor	Cutaneous digital syndactyly excessive height. Skull/mandible thickening, tori[a], CN palsies (incl. neonatal),. Headaches, raised ICP, coning. Back/bone pain. Fracture resistance	(110, 113–115)
Van Buchem's Disease[b]	239100	AR	*SOST*[c]	Reduced function	Sclerostin	Osteoblast Wnt signaling inhibitor	No syndactyly, no excess height. Skull/mandible thickening, tori[a], CN palsies. Headaches, back/bone pain. Fracture resistance	(110, 116, 117)
***LRP4* HBM**	604270	AD & AR	*LRP4*	Loss of function	LRP4	Impaired sclerostin-LRP4 interaction	Syndactyly, dysplastic nails, gait disturbance, facial nerve palsy, deafness	(118, 119)
***LRP5* HBM**	603506	AD	*LRP5*	Gain of function	LRP5	Osteoblast cell membrane co-receptor regulating Wnt signaling	Asymptomatic or tori[a], skull/mandible thickening, CN palsies, neuropathy, neuralgia, headaches, back/bone pain, spinal stenosis, reduced buoyancy, craniosyntosis, increased height. Fracture resistance	(120–139),
***LRP6* HBM**	awaited	AD	*LRP6*	Gain of function	LRP6	Osteoblast cell membrane co-receptor regulating Wnt signaling	Mandible thickening, torus palatinus, teeth encased in bone, absence of adult maxillary lateral incisors, inability to float. Fracture resistance. Increased height	(138)
***SMAD9* HBM**	awaited	AD	*SMAD9*	Loss of function	SMAD9	Inhibits BMP dependent targetgene transcription to reduce osteoblast activity	Mandible enlargement, broad frame, torus palitinus/mandibularis, pes planus, increased shoe size, inability to float	(140)
Cranio-metaphyseal dysplasia	123000218400	AD	*ANKH*	Gain of function	Homolog of mouse ANK	Osteoclast-reactive vacuolar proton pump	Macrocephaly, cranial hyperostosis CN palsies, wide nasal bridge, dental overcrowding, craniofacial hyperostosis & sclerosis, metaphyseal flaring, and high BMD	(141–143),
		AR	*GJA1*	Loss of function	Gap junction protein alph-1			
Lenz-Majewski hyperostotic dysplasia	151050	SP	*PTDSS1*	Gain of function	Phosphatidylserine synthase 1	Phospholipid biosynthesis	Mandible enlargement, generalized hyperostosis, proximal symphalangism, syndactyly, brachydactyly, cutis laxa, developmental delay, hip dislocation, marked hypertelorism, and enamel hypoplasia	(144, 145)

MIM®, Online Mendelian Inheritance in Man; CN, Cranial Nerve; ICP, Intracranial pressure; BMP, bone morphogenetic protein.

[a]*Tori: Oral exostoses which include torus palatinus & mandibularis; found in approximately 25% of a general Caucasian population (146).*

[b]*Initially known as hyperostosis corticalis generalisata familiaris (116, 117).*

[c]*A 52-kb intronic deletion downstream of SOST.*

TABLE 3 | *LRP5* mutations and associated clinical and radiological characteristics reported to date.

No. of reported individuals[a]	*LRP5* base change	Amino acid change	Exon	Country	Ethnicity	Tori TP & TM	Mandible	Neurological complications	Fracture History (#)	Clinical Features other than HBM (reported in at least one person in the kindred)	Radiology	Biochemistry	Refs
1	*c.266A>G*	p.Gln89Arg	2	UK	Caucasian	No	No	Carpel tunnel syndrome	None	Osteoarthritis	NDG	NDG	(159)
1	*c.331G>T*	p.Asp111Tyr	2	Argentina	NDG	NDG	Enlarged mandible. Mandibular pain	Headaches	NDG	Severe headaches, extremity pain	Dense cranium, loss of diploe, enlarged mandible, increased cortical thickness of long bones	NDG	(127)
6 of 15	*c.461G>T*	p.Arg154Met	2	USA from Lithuania	Caucasian	Yes[b]	Enlarged mandible	None	None	Pain in right hip after prolonged standing in the index case	Increased density of calvarium, mandible & endosteal surface of long bones	Calcium, PO_4, bALP normal	(123)
2 of 2	*c.509_514dupGGGGTG*	p.G171_E172insGG	3	Austria	NDG	No	NDG	Congenital deafness, VII palsy	None	Cochlear implant, migraine. Removal of occipital bone in one individual. Hypergonadotropic hypogonadism.	Increased calvarial and cortical thickness. Foramen magnum stenosis	Osteocalcin normal. CTX normal in mother, raised in daughter	(160)
3	*c.511G>C*	p.Gly171Arg	3	Belgium	NDG	NDG	NDG	Headaches	NDG	Severe headaches in one affected individual	Dense skull bones, cortical thickening of the vertebrae and long bones with normal development	NDG	(127)
1	*c.511_516delGGTGAG*	g.69547_69552delGGTGAG	3	NDG	Caucasian	NDG	Thickened mandible	Hearing impairment. Sudden sight loss aged 16	None	Generalized bone pain, headaches	Calvarial thickening. Restriction of auditory and optic canals	Calcium, PO_4, ALP normal. PTH mildly raised.	(161)
19 of 38	*c.512G>T*	p.Gly171Val	3	USA	Caucasian	NDG	NDG	None	Resistant to #	Asymptomatic	All bones of skeleton radiologically dense, thick cortices, reduced medullary cavity, normal shape	bALP, osteocalcin, deoxy- & pyridinoline X-links normal in subgroup of 5 affected	(120, 121)
7 of 16	*c.512G>T*	p.Gly171Val	3	Connecti-cutUSA	Caucasian	Yes	Wide, deep mandible, decreased mandibular angle	None	None	Asymptomatic other than difficulty floating	Thickened mandibular rami, marked cortical thickening of long bones, dense vertebrae but shape normal	bALP Ca, PO_4, PTH, OPG, RANKL, & urinary NTX normal.Osteocalcin, elevated. All in subgroup of 4 affected	(124)
1	*c.512G>T*	p.Gly171Val	3	Colorado USA	NDG	Yes	Wide deep mandible	Strabismus, Bells' palsy, trigeminal neuralgia, headaches, paraesthesias	NDG	Bone painPseudotumor cerebri, type 1 Chiari malformation	Dense skeleton, marked thickening of skull, and skull base, cortical widening that narrowed medullary cavity of the long bones	bALP and osteocalcin normal	(125)
6 of 13	*c.512G>T*	p.Gly171Val	3	NDG	NDG	TP in all but 1 case	Wide deep mandible	Deafness, sensorimotor neuropathy, dysphonia, spinal stenosis	None	One affected individual required surgery for spinal stenosis, another underwent hip replacement, with difficult surgery attributed to unusual hardness of bone	NDG	NDG	(126)
2 of 2	*c.512G>T*	p.Gly171Val	3	NDG	NDG	Yes	Wide deep mandible	No detail given	None	One individual had hydromyelia (a complication of type 1 Chiari malformation)	NDG	NDG	(126)
1	*c.518C>T*	p.Thr173Met	3	UK	Caucasian	No	No	Ulna nerve decompression	2 high impact	OsteoarthritisPalpable enthesophyte at tibial tubercle	NDG	NDG	(159)
1 of 4	*c.592A>T*	p.Asn198Tyr	3	NDG	NDG	Yes	NDG	No	None	HBM despite steroids. Ear canal decompression surgery. Headaches, back pain	Severe cortical thickening of cranial and long bones	P1NP increased. Calcium, PO_4, ALP, PTH normal	(162)
2 of 6	*c.593A>G*	p.Asn198Ser	3	NDG	NDG	Yes	Wide deep mandible	Deafness, sensorimotor neuropathy, & spinal stenosis	None	NDG	NDG	NDG	(126)

(Continued)

TABLE 3 | Continued

No. of reported individuals[a]	*LRP5* base change	Amino acid change	Exon	Country	Ethnicity	Tori TP & TM	Mandible	Neurological complications	Fracture History (#)	Clinical Features other than HBM (reported in at least one person in the kindred)	Radiology	Biochemistry	Refs
3	*c.593A>G*	p.Asn198Ser	3	UK	Afro-Caribbean	Yes	Enlarged mandible	None	None	Individuals affected differently Chest wall prominence Fixed flexion at elbows Osteotomy of tibial tubercle (for tendonitis)..:. HBM despite steroids	Increased calvarial thickness	Low bone turnover on Alendronic acid	(159)
?	*c.640G>A*	p.Ala214Thr	3	Portland USA	NDG	Yes	Elongated mandible	NDG	Resistant to #	Similar phenotype to that described by Boyden et al., 2002.	Increased density of calvarium, mandible and endosteal surface of long bones	NDG	(127, 134)
13 of 24	*c.640G>A*	p.Ala214Thr	3	Holland	NDG	None	Prominent mandible	CN VII palsy in 2 cases	None	Craniosynostosis, developmental delay, tinnitus, headaches, prominent forehead present in >1 member of pedigree	Increased density of calvarium, mandible, pelvis & endosteal surface of long bones	Calcium, PO_4, bALP normal	(129)
1	*c.641C>T*	p.Ala214Val	3	UK	NDG	NDG	Enlarged mandible	NDG	NDG	Similar phenotype to that described by Boyden et al., 2002	Similar to Boyden et al., 2002.	NDG	(127, 130)
Family 1:1 of 1Family 2 & 3 unknown	*c.724G>A*	p.Ala242Thr	4	Portland USA, & Sardinia	NDG	Yes	Enlarged mandible	None in one case (393). No detail given in 2 families	Resistant to #	NDG	Increased density of calvarium. Mandible and endosteal surfaces of long bones	NDG	(127, 133–135)
?	*c.724G>A*	p.Ala242Thr	4	France	NDG	Yes	Enlarged mandible	NDG	Resistant to #	Osteomyelitis of the jaw, hearing difficulties due to small auditory canals in 2 affected individuals	Increased density of calvarium, enlargement of cranial vault	NDG	(136)
2 of 10	*c.724G>A*	p.Ala242Thr	4	UK	Caucasian	Yes	Enlarged mandible	None	None	Renal calculi (in one individual) Dental overcrowding	NDG	Increased bone turnover at age 21	(159)
1	*c.724G>A*	p.Ala242Thr	4	UK	Caucasian	Yes	Enlarged mandible	CN V & VII mildly impaired	None	Widespread arthralgia, shin pain & headaches	Increased calvarial thickness with tightly packed brain gyri on MRI. Anterior lumbar syndesmophytes	NDG	(159)
2	*c.724G>A*	p.Ala242Thr	4	UK	Caucasian	Yes	Enlarged mandible	Conductive deafness	None	Osteoarthritis	NDG	NDG	(159)
Family 1:13 of 32Family 2:7 of 16	*c.758C>T*	p.Thr253Ile	4	Fyn, Denmark	NDG	NDG	NDG	NDG	No increased # rate	NDG	Generalized sclerosis including calvarium (obliteration of frontal sinuses & mastoids), pelvis & long bones.Enlargement of cranial vault	NDG	(127, 128, 131, 132)
1	*c.796C>T*	p.Arg266Cys	4	UK	Caucasian	Yes	Enlarged mandible	None	None	HBM despite steroids	NDG	Normal bone turnover	(159)
1	*c.844A>G*	p.Met282Val	4	Belgium	NDG	Yes	None	None	NDG	Knee pain, chondrocalcinosis & OA. Cervical spine pain. Developed breast cancer	Thickened skull and long bones on MRI and phalanges on X-ray. Increased density of vertebral bodies without OA	Calcium, PO_4, bALP, CTX all normal	(137)

 # fracture CN Cranial nerve; TP, torus palitinus; TM, torus mandibularis; bALP, bone specific alkaline phosphatise; PO_4, phosphate; PTH, parathyroid hormone; CTX, NTX, C and N-telopeptides cross-links of bone Type I collagen; UK, United Kingdom; NDG, No detail given.

[a]*No. of reported individuals (with pedigree size where reported).*

[b]*3 requiring surgical debulking of TP & TM.*

Comprehensive description of the clinical phenotype of *LRP5* HBM was reported by Boyden et al., in 2002 (124). Seven individuals from a family of 16 were analyzed after routine DXA screening detected two related individuals with high BMD, again due to *LRP5* c.512G>T, p.Gly171Val. Again, radiographic thickening of cortices of otherwise normal bones was reported, without history of fracture. However, all cases had deep wide mandibles and torus palitinus. Two of 7 cases reported difficulty floating; the first-time buoyancy was reported in association with bone density in humans. Serum calcium, phosphate, ALP, nf-kB and osteoprotegerin (OPG) were normal, as was urinary NTX-1. However, unlike the first family, osteocalcin was elevated threefold (mean 32.3 (SD 7.4) *vs*. 9.8 (1.8) ng/mL). This finding was suggestive of a stimulatory effect on periosteal bone formation, leading to an increase in cortical thickness, as has been previously reported in transgenic mice expressing the p.Gly171Val mutation (169).

While Boyden et al.'s large family were asymptomatic of their HBM trait, in a letter of response two years later Whyte et al. reported a 37 year old woman with the same pathogenic variant (*LRP5* c.512G>T, p.Gly171Val) but a more severe phenotype, including congenital strabismus, childhood Bells' palsy, trigeminal neuralgia, life-long headaches, bone pain, and paresthesias (125). She also had a widened and enlarged mandible; and her osseous tori had required dental intervention and removal as they had encased her teeth from an early age. Interestingly, this more extreme case had a normal osteocalcin level.

In their letter of response Boyden et al. detailed a further three unrelated families with *LRP5* HBM, two with the same *LRP5* c.512G>T, p.Gly171Val variant and one with a novel variant in *LRP5* c.593A>G (p.Asn198Ser), also in exon 3 (126) (**Table 3**). Among these families, some affected individuals had also reported deafness, sensorimotor neuropathy and spinal stenosis, all likely due to bone overgrowth and nerve compression. Contemporaneously, van Wesenbeeck et al. identified a further six novel *LRP5* mutations spread through exons 2, 3 and 4 (127). Cases generally had similar features of mandible enlargement, fracture resistance, increased skull thickness and oral tori.

Sixteen activating *LRP5* mutations affecting 29 families have now been reported globally, all in exons 2, 3 and 4 and affecting the 1st β-propeller domain (**Table 3**) (120, 121, 123–137, 159–162). Almost all are missense mutations, with two indels reported. Half of all cases report osseous tori (see below); and only one case has experienced fractures (notably, after high impact). As increasing numbers of individuals are reported, it is apparent that *LRP5* HBM may not be as benign as first thought, and indeed shares many features with sclerosteosis and van Buchem's disease (**Table 2**)—which is not particularly surprising given they share a common pathway. In addition to the adverse clinical features reported by Whyte et al. (detailed above) (125), Kwee et al. reported a multi-generation family with an *LRP5* c.640G>A, p.Ala214Thr mutation, in whom the phenotype extended beyond HBM to include craniosynostosis, developmental delay, and multiple dysmorphic features including macrocephaly and hypertelorism (129). Premature closure of cranial sutures in one infant reportedly caused raised intracranial pressure, optic nerve atrophy and visual impairment (this child also manifest a ventriculoseptal defect); two other individuals had required craniotomy. Foramen magnum stenosis, in one case ultimately requiring craniotomy, has also been reported in association with the only HBM-associated *LRP5* insertion reported to date (c.509_514dupGGGGTG, p.G171_E172insGG) (160). The p.Gly171Val mutation has been reported twice in association with a type 1 Chiari malformation (125, 126). Headaches and bone pain are common; bony compression of the optic and auditory nerves causing loss of sight and hearing respectively are frequently reported; and mandibular osteomyelitis, renal calculi and spinal stenosis have also been reported (126, 136, 159). However, joint disease does not appear to be a common feature, with osteoarthritis reported only in some older individuals (159).

Certainly, there will be a bias toward identification of more severe HBM phenotypes associated with *LRP5* mutations, as these are more likely to be identified clinically and thus reported. In our own study mentioned earlier, in which we screened 335,115 DXA scans across 15 UK centers, we identified seven families with *LRP5* HBM; two with novel and five with previously reported mutations (159). The two novel mutations had milder phenotypes than those reported previously, and arguably would have been less likely to have been identified without gene screening. Our findings suggested that the clinical variability in *LRP5* HBM cases may arise from genotype-phenotype correlation, with our protein modeling suggesting the severity of high BMD corresponds to the degree of predicted LRP5 protein disruption (159).

LRP4 High Bone Mass

To date, four cases of HBM associated with pathogenic variants in *LRP4* have been published, with both autosomal dominant and recessive inheritance reported (109, 118, 119, 170, 171). Mutations causing *LRP4* HBM occur in the central cavity of the third β-propeller domain of the LRP4 protein, impairing the interaction between sclerostin and LRP4 (119) (of note mutations elsewhere in *LRP4* are associated with Cenani-Lenz Syndactyly Syndrome, MIM212780) (**Figure 1**). In addition to HBM, clinical features of *LRP4* HBM include syndactyly, dysplastic nails, gait disturbance, facial nerve palsy and hearing loss (of note, no osseous tori have been reported to date) (**Table 2**) (119). As the phenotype of *LRP4* HBM is very similar to sclerosteosis, it has been termed sclerosteosis type 2 (118)—though the clinical course is less severe and arguably more similar to van Buchem's disease.

LRP6 High Bone Mass

In 2019 Whyte et al. reported two multi-generational families with *LRP6*-associated HBM, identifying two different heterozygous missense mutations both affecting the first β-propeller of LRP6 (homologous to *LRP5* HBM mutations) (**Figure 1**) (138).

The clinical features of *LRP6* HBM are highly reminiscent of *LRP5* HBM: generalized osteosclerosis and hyperostosis,

mandible enlargement, torus palitinus, teeth encased in excessive bone, resistance to fracture, and an inability to float in water (**Table 2**), with an additional phenotypic feature of absence of adult maxillary lateral incisors in some individuals (observed in both families). Of note, no signs of osteoarthritis were detected.

Interestingly, not only was *LRP6* HBM associated with above-average height, this paper also highlighted increased height in individuals with *LRP5* HBM (who were studied for comparison with the *LRP6* families) (138). Taken together, the phenotypes suggest increased Wnt signaling seen in all three conditions affects not only bone density but also skeletal growth in childhood and adolescence.

Other Forms of High BMD With Increased Bone Formation, Not Associated With Wnt Signaling Pathways

SMAD9 High Bone Mass

In 2019 we reported the first pedigree with a segregating *SMAD9* mutation, with replication in two further unrelated individuals with HBM. Based our population size (34), we estimated the prevalence of *SMAD9* HBM as approximately 1 in 100,000, less common than *LRP5* HBM (159). As with *LRP5* HBM, the clinical phenotype included mandible enlargement, a broad frame and tall stature, torus palatinus, and a tendency to sink when swimming; and no adult fractures were reported (**Table 2**). A further characteristic, not reported in *LRP4, LRP5*, or *LRP6* HBM, was pes planus. Reassuringly, unlike sclerosteosis and some cases of *LRP5* HBM, nerve compression was not seen (159).

SMAD9 (also known as *SMAD8, MADH6*, and *MADH9*) encodes a downstream modulator of the BMP signaling pathway. BMPs are members of the TGF-β superfamily, and induce both bone and cartilage formation (172). Our *in-silico* protein modeling predicted the mutation severely disrupted the structure of the MH1 DNA binding domain of *SMAD9*, leading to loss-of-function, such that this inhibitory SMAD could no longer repress BMP receptor activation and downstream signaling (173). Our novel findings support the SMAD9-dependent BMP signaling pathway as a potential novel anabolic target for future osteoporosis therapeutics.

Craniometaphyseal Dysplasia

Craniometaphyseal dysplasia (MIM123000), which may be autosomal dominant or recessive, is caused by a mis-sense mutation in *ANKH*, which encodes the inorganic pyrophosphate channel ANK. The phenotype includes macrocephaly, cranio-facial hyperostosis and sclerosis with cranial nerve palsies, wide nasal bridge, dental overcrowding, metaphyseal flaring and marked HBM (the latter predominantly in AR disease) (141–143).

Lenz-Majewski Hyperostotic Dysplasia

Autosomal dominant gain of function mutations in *PTDSS1* are responsible for Lenz–Majewski syndrome (LMS) (MIM 151050) (174). This very rare syndrome is characterized cutis laxa, facial dysmorphism, severe short stature, brachydactyly, intellectual disability and hyperostotic skeletal dysplasia. Skeletal characteristics include calvarial thickening, marked sclerosis of the skull base and facial bones, a markedly enlarged mandible (much more so than is seen in the Wnt signaling HBM syndromes), dense vertebral bodies, shortened broad ribs, hyperostotic clavicles, scapulae and iliac wings (144, 145). Progressive osteosclerosis with "massive thickening of long tubular bones" is described by the age of 30 years (144). Bilateral hip dislocation has been reported. *PTDSS1* codes for phosphatidylserine synthase 1 (PSS1), an enzyme involved in phospholipid biosynthesis, although the mechanism by which this affects bone metabolism is not yet fully understood (174).

Osseous Tori

Oral exostoses include torus palatinus (TP), torus manibularis (TM) and, less commonly, torus maxillaris. Their site determines their nomenclature (with TP lying in the midline of the hard palate and TM usually in the premolar region of the lingual side of the mandible). The size and number of tori an individual may have is highly variable. They are each made up of dense cancellous bone with a surrounding rim of cortical bone; occasionally they contain hemopoietic tissue (175). The only apparent clinical problem associated with tori *per se* is obstruction to dentition (including denture fitting); and tori rarely require surgical de-bulking. Notably, tori are not present in all cases of *LRP5 or LRP6* HBM (**Table 3**).

Although prevalence estimates have varied widely (between 1 and 64% depending upon the study, definition and population), overall tori appear to be relatively common (approximately 25% of a Caucasian population, clearly much higher than the prevalence of HBM) and appears similar across all ages (146). Interestingly, two separate studies (one among US (90% Caucasian) postmenopausal women and another in elderly Japanese women) have found an association between tori and higher BMD (176, 177). The US study graded tori size (0 to 5) and found a strong correlation with BMD Z-score among 469 women; however, they did not find a similar correlation between age and torus size (176).

Taken together, these data suggesting that torus may reflect acquisition of peak bone mass in early adult life rather than a progressive skeletal change. Moreover, tori do not appear to be sensitive or specific indicators of a monogenic form of HBM but may simply reflect a general association with higher peak bone mass.

UNEXPLAINED HBM—A NEW ENTITY?

Mutations in the genes mentioned above are extremely rare within the general population, and the vast majority of HBM cases (~97%) remain genetically unexplained (159). Based on our UK study, *LRP5* HBM mutations have an estimated prevalence of approximately 5 per 100,000 (159). We identified only one sclerosteosis carrier, who manifested moderately high BMD due to a novel heterozygous nonsense *SOST* mutation predicted to either prematurely truncate sclerostin or cause nonsense-mediated decay (159). No cases of autosomal recessive sclerosteosis, *LRP4* HBM or *LRP6* HBM have been identified in the UK to date (159).

Thus there remains a population with generalized raised BMD (Z-score ≥+3.2 at either L1 or hip), usually identified incidentally on routine DXA scanning (34, 159), in whom fracture risk is not increased, with clinical characteristics suggestive of a mild skeletal dysplasia (associated features of mandible enlargement, extra bone at the site of tendon and ligament insertions, broad skeletal frame and larger shoe size, poor buoyancy, as well as an increased BMI) (34). The population is characterized by increased trabecular BMD and by alterations in cortical bone density and structure, leading to substantial increments in predicted cortical bone strength (178). Neither trabecular nor cortical BMD appear to decline with age in the tibia of HBM individuals, suggesting resistance to age-related bone loss in weight-bearing limbs may contribute to their bone phenotype (178). Furthermore, body composition assessment suggests that HBM is associated with a marked increase in fat mass, particularly android fat, in women but not men (179). This clinical appearance of a mild skeletal dysplasia explains 35% of incidental identified high BMD on routine DXA scanning, such that unexplained generalized HBM has a prevalence of 0.18% among a UK DXA-scanned adult population (34).

Within our cohort with unexplained HBM, 41% have a first-degree relative with a similar phenotype; thus unexplained HBM appears to be heritable though this figure has not been formally calculated (34). As mentioned above, mutations of other components of the Wnt/β-catenin pathway have been associated with HBM in murine genetic studies; and it may be these HBM individuals carry rare variants in genes yet to be identified through further sequencing efforts. However, this population with unexplained HBM is enriched for 'high BMD alleles' of loci identified through BMD GWAS in the general population. Thus, the genetic architecture of unexplained HBM is, at least in part, explained by common variants (15, 16). This does not exclude the possibility of rare variants of large effect in other genes in some (or all) of this cohort; rather, the effect of such variants with large effect may be modified by their background polygenic architecture.

As higher (*i.e.*, non-artefactually elevated) BMD is associated with prevalent osteoarthritis in the general population (180–183), it is perhaps not surprising that individuals with unexplained HBM have a greater prevalence of radiographic osteoarthritis than their unaffected family members and general population controls, along with a higher incidence of joint replacement (184–186). Interesting, when assessing the individual radiographic sub-phenotypes of osteoarthritis, be it at the hip, knee or hand, osteophytes predominate, with some increased subchondral sclerosis, rather than joint space narrowing (185–187). Taken together this suggests HBM might be associated with a hypertrophic 'bone-forming' osteoarthritis phenotype (188). While increased adiposity is also a clinical feature of HBM (with weight a major contribution to the development of degenerative joint disease), there remains an association between BMD and osteophytes, even after adjusting for BMI, at both weight-bearing (*e.g.* knee) and non-weight-bearing (*e.g.* distal interphalangeal [DIP] and carpometacarpal [CMC] joints of the hand) joints (186, 187).

More recently, we have been able to follow-up a proportion of our original HBM cohort eight years after initial assessment. We have observed increases in knee osteophytes and joint space narrowing, as well as knee pain and functional limitation (189); findings at the hip are similar (A Hartley et al., submitted for publication). Taken together, these insights from the study of an extreme HBM population suggest that raised BMD may contribute to pathogenesis of osteoarthritis.

OSTEOPETROSES AND OSTEOSCLEROTIC CONDITIONS WITH DISTURBED FORMATION AND RESORPTION

High BMD due to Osteoclast Dysfunction

The osteopetroses (Greek etymology: "petro"—to turn to stone) are rare genetic conditions of reduced osteoclastic bone resorption. Defective bone remodeling during growth induces skeletal sclerosis and abnormally dense but brittle bones. First described by the German radiologist Albers-Schönberg as "marble bone disease," (190, 191) osteopetrosis is now classified by clinical severity (**Table 4**). The worst prognosis is seen in severe neonatal or infantile forms; a number of intermediate forms have been identified; and later-onset forms characterise the other end of the clinical spectrum (8, 237). Autosomal dominant osteopetrosis (ADO) was historically subdivided into ADO type I and type II. However, ADO type I was subsequently identified as high bone mass due to *LRP5* (low-density lipoprotein receptor-related protein 5) mutations (128) (discussed earlier). As *LRP5* HBM is not primarily a disease of osteoclasts, and is not characterized by bone fragility, we agree with the most recent edition of the Nosology [compared with the 2015 Nosology (237)] that *LRP5* HBM should not be considered an osteopetroses. *LRP5* HBM has now been reclassified within the group of "other sclerosing bone dysplasias" (8).

Osteopetrosis, Late-Onset Form Type 2 (OPTA2), Previously Known as Autosomal Dominant Osteopetrosis II (ADOII)

OPTA2 (MIM166600, eponymously known as Albers-Schönberg disease) is caused by *CLCN7* mutations. CLCN7 functions as a voltage-gated Cl-/H+ ion channel, and is found in lysosomes and on the ruffled boarder of osteoclasts. By acid efflux, it facilitates inorganic bone matrix dissolution (238). Mutations in *CLCN7*, therefore, result in decreased osteoclastic bone resorption. Multiple mutations have been identified throughout the gene, in association with a range of osteopetrotic phenotypes (239–241). The prevalence of OPTA2 is estimated between 0.2 and 5.5/100,000 (242, 243); however, it exhibits both variable penetrance (60–80%) and expressivity, results in a varied clinical phenotype including detection as an incidental radiographic finding (244). The phenotype can include facial nerve palsy, visual loss (in 5–25%), carpal tunnel syndrome, hip osteoarthritis (in 7%), increased fracture risk and delayed fracture healing, osteomyelitis (in 10–13%, particularly in the mandible), dental abscesses (10%) and deep decay (36%) and, in extreme cases, bone marrow failure (≈3%) (192, 203–206).

TABLE 4 | Osteopetrotic conditions and osteosclerotic conditions with disturbed formation and resorption.

Condition	MIM	Inheritance*	Gene	Mutation	Protein	Function	Symptoms	Ref
Severe/neonatal/ infantile. autosomal recessive osteopetrosis[a]	259700604592	AR(OPTB1)	*TCIRG1*	Loss of function	T-cell, immune regulator 1, H+ transporting, lysosomal subunit A3 of V-ATPase pump	Acidification of the resorption lacuna	Fractures, infections (*e.g.* osteomyelitis), macrocephaly, frontal bossing, neurologic symptoms, CN compression, blindness, deafness, delayed tooth eruption, hemopoietic failure, death (usually before aged 10)	(190, 192, 193)
	602727611490	AR (OPTB4)	*CLCN7*	Loss of function	Chloride Channel	Acidification of the resorption lacuna		
	259720 607649	AR (OPTB5)	*OSTM1*	Loss of function	Osteopetrosis associated transmembrane protein 1	β-subunit for CLC-7		
	615085	AR (OPTB8)	*SNX10*	Loss of function	Sorting Nexin 10	Acidification of the resorption lacuna	Macrocephaly, broad open fontanelle, frontal bossing, small chin, and splenomegaly, severe optic atrophy with blindness, anemia, thrombocytopenia	(194, 195)
	602642259710	AR (OPTB2)	*RANKL/ TNFSF11*	Loss of function	Receptor Activator for Nuclear Factor κ B ligand/ tumor necrosis factor (ligand) superfamily, member 11	Osteoclastogenesis, resorption, survival	Osteoclast poor osteopetrosis. Fractures, hydrocephalus, nystagmus, seizures, hypersplenism, less severe course than *TCIRG1*, *CLCN7*, *OSTN1, SNX10* mutations	(196)
	603499612302	AR (OPTB7)	*RANK/ TNFRSF11A*	Loss of function	Receptor Activator for Nuclear Factor κ B[b]	Osteoclastogenesis, resorption, survival		
Intermediate autosomal recessive osteopetrosis	259710	AR (OPTA2)	*CLCN7*	Partial loss of function	Chloride Channel	Acidification of the resorption lacuna	Onset in childhood, fractures, short stature, cranial nerve compression	(192, 197)
Osteopetrosis with renal tubular acidosis	259700, 611497	AR (OPTB6)	*PLEKHM1*	Loss of function	Pleckstrin homology domain containing family M (with RUN domain), member 1	Vesicular trafficking	Osteopetrosis of the skull only (L2-L4 T-score -2.3). Fractures. Raised osteocalcin	(198)
	259730, 611492	AR (OPTB3)	*CA2*	Loss of function	Carbonic anhydrase II	Intracellular acidification	Developmental delay, short stature, CN compression, blindness, dental complications, fractures, maintained hemopoietic function.	(190, 192)
Osteopetrosis with ectodermal dysplasia and immune defect	300301	XL (OLEDAID)	*IKBKG*	Loss of function	Inhibitor of kappa light polypeptide gene enhancer in B-cells kinase gamma (NEMO),	Unknown	Lymphoedema, severe infections, no teeth, skin abnormalities, early death	(193)
Leucocyte adhesion deficiency syndrome and osteopetrosis	612840	AR (LAD-3)	*KIND3/FERMT3*	Loss of function	Kindlin-3/Fermitin-3	Cell adhesion	Bacterial infections, bleeding, osteopetrosis, hepatosplenomegaly	(199)
	612840	AR	*CalDAG-GEF1/ RASGRP2*	Loss of function	Calcium and diaclyglycerol-regulated guanine nucleotide exchange factor 1			(200)
Osteosclerotic metaphyseal dysplasia (OSMD)	615198	AR	*LRRK1*	Loss of function	Leucine-rich repeat kinase 1	Osteoclast function; sealing zone formation	Developmental delay, seizures, metaphyseal osteosclerosis, diaphyseal osteopenia of long bones. Recurrent fractures. Skull unaffected.	(201, 202)
Late onset osteopetrosis (Albers-Schönberg disease) ADOII	166600	AD (OPTA2)	*CLCN7*	Dominant negative effect	Chloride Channel	Acidification of the resorption lacuna	Classic radiographic features, fractures, nerve compression, osteomyelitis, dental complications.	(192, 203–206)
Pycnodysostosis	265800, 601105	AR	*CTSK*	Loss of function	Cathepsin K	Collagen degradation	Delayed cranial suture closure, short stature and phalanges, dental abnormalities, fractures	(207–209)
Osteopoikilosis	166700	AD	*LEMD3*	Loss of function	LEM domain-containing 3	Disrupted BMP and TGFβ signaling pathways	Benign incidental osteosclerotic foci (can mimic metastases),[c]	(192, 210–214)
Melorheostosis	155950	AD	*LEMD3/ MAP2K1SMAD3*	Loss of function	LEM domain-containing 3Mitogen-Activated Protein		Characteristic radiographic features asymmetric 'flowing hyperostosis' or 'dripping candle wax'. Soft tissue	

(Continued)

TABLE 4 | Continued

Condition	MIM	Inheritance*	Gene	Mutation	Protein	Function	Symptoms	Ref
					Kinase Kinase 1SMAD Family Member 3		changes (hypertrichosis, fibromas, hemangiomas and pain): associated with radiographic features in sclerotome. Contractures can develop	
Osteopathia striata[d] **with cranial stenosis**	300373	XL (OSCS)	*WTX/AMER1*	Loss of function	Wilms tumor gene on the X chromosome/APC Membrane Recruitment Protein 1	Wnt signaling suppression	Macrocephaly, CN compression, cleft palate, skull/long bone sclerosis in females. Usually lethal in males	(162, 215)
Dysosteosclerosis	224300	AR	*SLC29A3CSF1R*	Loss of function	Solute carrier family 29 (nucleoside transporter) Colony Stimulating Factor 1 Receptor	Osteoclast differentiation and function	Neurodevelopmental deterioration, platyspondyly, cranial nerve compression, abnormal dentition	(216–219)
Diaphyseal dysplasia Camurati-Engelmann[e]	131300	AD	*TGFβ1*	Probable gain of function	TGFβ	Cell proliferation, differentiation, migration and apoptosis	Variable phenotype. Thickened diaphyseal cortices, limb pain, fatigability, muscle weakness, waddling gait. Variably raised ALP, hypocalcemia & anemia	(220–226)
Ghosal hematodiaphyseal syndrome	274180	AR	*TBXAS1*	Loss of function	Thromboxane synthase	Modulates RANKL & OPG expression	Impaired platelet aggregation (steroid-sensitive), anemia. Similar to Camurati-Engelmann syndrome but metaphyses also involved	(227, 228)
Trichodentoosseous dysplasia	190320	AD	*DLX3*	Loss of function	Distal-less homeobox 3	Ectodermal development	Sparse curly hair, severe dental abnormalities, defective tooth enamel. Sclerosis of calvaria and/or long bones	(229)

MIM® Online Mendelian Inheritance in Man CN, Cranial Nerve; RANKL, Receptor Activator of Nuclear Factor-κβ Ligand; OPG, Osteoprotegerin; XL, X-linked; ADOII, Autosomal dominant type 2 osteopetrosis.

[a]*ARO incidence is 1/200,000–300,000 live births (193).*

[b]*As well as an osteoclast poor ARO phenotype, RANK mutations have also been linked to the Paget's-like diseases (familial expansile osteolysis, expansile skeletal hyperphosphatasia and early-onset Paget's disease); (230, 231)*

[c]*When associated with connective tissue naevi, dermatofibrosis lenticularis disseminata then termed Buschke-Ollendorff syndrome (192, 210, 232)*

[d]*can occur in combination with focal dermal hypoplasia, skin pigmentation, hypoplastic teeth, syndactyly, ocular defects and fat herniation through skin and is known as Goltz Syndrome (233–236).*

[e]*Also known as progressive diaphyseal dysplasia.*

Radiographs feature (a) vertebral end-plate thickening (another cause of 'rugger-jersey spine'), (b) 'bone-within-bone' particularly in the pelvis, and (c) transverse sclerotic bands within the distal femorae (203, 206). However, the radiological phenotype is not ubiquitous (≈60–90%) (233, 242). DXA BMD Z-score ranges from +3 to +15 (203, 205).

OPTA2 highlights that high BMD does not necessarily equate to lower fracture risk. In one case series of 94 *CLCN7* mutation cases, almost every adult (98%) had experienced a fracture (including, in half of carriers, their hip), with a third having fractured more than once (five had >15 fractures) (205). Among another 42 cases from 10 families, age range 7 to 70 years, the mean number of fractures per person was 4.4 (205). However, these case series are not performed systematically; thus, patterns are difficult to generalise.

Pycnodysostosis

First described in 1962 and said to be the malady of both Toulouse-Lautrec and Aesop (known for his fables) (234–236), pycnodysostosis (MIM265800) is caused by defective enzymatic degradation of organic bone matrix, due to an autosomal recessive mutation in *CATK* (coding for cathepsin K) (207). To date approximately 30 mutations have been reported among fewer than 200 cases globally (207–209). Secreted by osteoclasts, cathepsin K cleaves type I collagen (245). The characteristic bone dysplasia includes skull deformities, under-developed facial bones with micrognathia, beaked nose, short stature and phalanges, dental caries, persistence of deciduous teeth and abnormally dense but brittle bones (192, 207–209, 246). Affected individuals may also manifest hip fractures indistinguishable clinically from atypical femoral fractures associated with antiresorptive therapy (247). Interestingly, particularly in light of the previous statement, the molecular understanding of pycnodysostosis underpinned development of a novel class of anti-resorptive therapy (248), although ultimately this agent did not make it to market (see below).

β3-Integrin Disorders Associated With Platelet Dysfunction and Osteopetrosis

β3-integrins act with filamentous actin to facilitate podosome attachment of osteoclasts to bone. β3-integrin double knock-out mice develop osteosclerosis, with increased cortical and trabecular mass, as well as hypocalcemia, due to defective osteoclast function (249). *ITGB3* encodes glycoprotein IIIa which is the β subunit of the glycoprotein IIb/IIIa cell adhesion complex. Interestingly this IIb/IIIa complex acts as a fibrinogen receptor and mediates platelet aggregation; it is this complex which the widely used cardiological drugs tirofiban and abciximab target in their anti-platelet action as glycoprotein IIb/IIIa inhibitors, used at the time of percutaneous coronary interventions. Hence unsurprisingly, dysfunction of β-integrins appears to cause defective platelet aggregation and HBM in mice models (250). Autosomal recessive mutations in *ITGA2B* lead to reduced production of either glycoprotein IIb or IIIa, resulting in Glanzmann thrombasthenia (MIM273800) which is characterized by excessive bleeding (251). Only one case of Glanzmann thrombasthenia has been reported with a bone phenotype, with generalized and skull base osteosclerosis observed on plain radiographs of a 5 day old baby (252), termed osteopetrosis and thought due to impaired osteoclast function (253). A similar platelet phenotype has been associated with osteopetrosis (reported in three cases) in the presence of mutations in *Kindlin-3* (MIM612840), coding for Kindlin-3 which also interacts with β-integrins. The resulting condition is termed leukocyte adhesion deficiency-3 (LED-3) and predisposes to bacterial infections and bleeding despite normal platelet counts, as well as a bony phenotype (199).

OTHER OSTEOSCLEROTIC DISORDERS

Osteopoikilosis and Melorheostosis

Osteopoikilosis (MIM166700; Greek etymology: poikilos-various) is benign, usually incidental finding characterized radiographically by multiple small round osteosclerotic foci, which can cause concern for metastases. When associated with connective tissue naevi, (dermatofibrosis lenticularis disseminate) it is termed Buschke-Ollendorff syndrome (BOS) (192, 210, 232). Melorheostosis, an asymmetric radiographic appearance of 'flowing hyperostosis' described as 'dripping candle wax' down the bone, can co-occur with osteopoikilosis. Approximately 200 cases have been described to date. Soft tissue signs and symptoms (see below) are associated with the radiographic features in a sclerotome distribution. Hypertrichosis, fibromas, hemangiomas and pain are sometimes a feature; and contractures and deformity can develop if limbs can become unequal in length (192, 210–212).

Osteopathia Striata

Osteopathia striata can occur in combination with cranial sclerosis (MIM300373) or focal dermal hypoplasia (known as Goltz Syndrome; MIM305600)); both are X-linked dominant diseases and cause striations visible on bone radiographs, together with learning difficulties. In the former, which is due to mutations in *AMER1*, cranial osteosclerosis can lead to cranial nerve compression (215). In Goltz syndrome, caused by mutations in *PORCN*, the bone features are associated with skin pigmentation, hypoplastic teeth, syndactyly, ocular defects, and fat herniation through skin (254–256).

Camurati-Engelmann Disease

Camurati-Engelmann disease (progressive diaphyseal dysplasia) (MIM131300) results from a gain-of-function mutation in *Transforming Growth Factor Beta-1* (*TGFB1*), resulting in thickened diaphyseal cortices, increased BMD, limb pain, fatigability, muscle weakness and a waddling gait (220). TGF-β controls cell differentiation, proliferation and apoptosis in many tissues; and its pivotal role in bone regulation is highlighted by the number of skeletal diseases associated with abnormal TGF-β signaling, which include Marfan's syndrome, Loey-Dietz syndrome, acromesomelic and geleophysic dysplasias and even osteogenesis imperfecta (257).

Ghosal Syndrome

Ghosal syndrome (MIM231095) is a rare autosomal recessive disorder caused by a mutation in *TBXAS1*, which encodes thromboxane synthase, resulting in HBM, impaired platelet aggregation, and anemia. The phenotype is not dissimilar from Camurati-Engelmann disease; however, here metaphyses are also affected (227, 228). This condition has linked platelet function with the RANKL/OPG pathway *in vitro* as thromboxane synthase modulates both RANKL and OPG expression in osteoblasts (228).

X-Linked Hypophosphatemia

X-linked hypophosphatemia (XLH) (MIM307800), caused by phosphate-regulating endopeptidase homolog (*PHEX*) mutations, has also been reported as a cause of modestly elevated axial, though not appendicular, BMD, in both children (258) and adults (68). However, given the high prevalence of ligamentous calcification and degenerative joint disease in adults with XLH, interpreting a DXA BMD result is complex. Individuals with XLH have a high prevalence of pseudo- and complete fractures, with mean age at first fracture of 26 years (259). However, pertinent to the point regarding fracture *vs*. BMD, these fractures typically affect the lower limbs, noting that appendicular BMD is not usually increased in XLH; and are usually attributed to the combination of osteomalacia and mechanical stress (from rickets and joint mal-alignment).

Neonatal Osteosclerotic Dysplasias

A handful of rare mutations can cause osteosclerosis in the neonate. Caffey disease (MIM114000), also known as infantile cortical hyperostosis, is a highly unusual bone disease causing excessive bone overgrowth (two-three times normal width—to the point of bone fusion with neighboring bones (*e.g.* ribs, radius and ulna)), along with joint and soft tissue swelling—which then resolves over the following months. To date all cases carry a single point mutation in *COL1A1* (c.3040C>T; p.Arg836Cys) (260). Mutations in *COL1A1* usually cause osteogenesis imperfecta; and the reason for the differing phenotype in Caffey's disease is not known – nor is it known why this condition settles down over time. Interestingly, a *COL1A1* mutation has been identified in an Australian terrier with canine hyperostosis (261), and mutations in various solute carrier genes have been described in other cases of canine calvarial hyperostosis and craniomandibular osteopathies (which are likely overlapping conditions) (261). Whether mutations in these genes contribute to human diseases similarly is unknown.

Other forms of neonatal osteosclerosis include Blomstrand dysplasia (MIM215045), due to autosomal recessive inactivating mutations in *PTHR1* which codes for the PTH/PTHrP receptor 1, and which is usually lethal; desmosterolosis (MIM602398), due to autosomal recessive mutations in *DHCR24* which codes for 3-beta-hydroxysterol delta-24-reductase, with mutations resulting in impaired sterol-metabolism; and Raine dysplasia (MIM259775), due to autosomal recessive *FAM20C* mutations coding for Dentin matrix protein 4, which can also be lethal.

POLYGENIC INHERITANCE OF HIGH BMD

GWAS in populations selected due to their high BMD have identified novel BMD-determining loci relevant not only in the extreme population but also in the general population. In 2011, we performed the first extreme truncate selection GWAS of BMD (33), as the use of extreme cases and/or "super controls," drawn from opposite ends of the same population distribution, maximizes statistical power (262). This was one of the first such extreme truncate selection GWAS for any phenotype; and such augmentation of statistical power through analysis of extreme phenotypes has since been shown to be advantageous in a range of clinical phenotypes (263–266) and is now an established approach to investigate the genetic architecture of complex disease (262, 267). In addition to replicating associations for 21 of the then 26 known BMD loci (identified from analyses of populations with normally distributed BMD) (268), we identified six new genetic associations in loci near *CLCN7, GALNT3, IBSP, LTBP3, RSPO3*, and *SOX4* (33), which subsequently replicated in larger general population GWAS (17, 18). This project highlighted the efficiency of extreme-truncated selection for quantitative trait GWAS design (33).

More recently, we conducted a GWAS of arguably the most extreme BMD population to date, identifying further two genome-wide significant SNPs, rs9292469 (48.5kb 3′ of *NPR3* with the LD block including part of this gene) and rs2697825 (within an intron of *SPON1*) associated with lumbar spine and hip BMD respectively. *NRP3* regulates endochondral ossification and skeletal growth (269–272), while *SPON1* modulates TGF-β-regulated BMP-driven osteoblast differentiation (273). *SPON1*, coding for an extracellular matrix glycoprotein, had not previously been associated with a bone phenotype in humans; however interestingly, *Spon1* knockout mice have a skeletal HBM phenotype (274). These novel loci are now under active investigation as future therapeutic targets.

TRANSLATIONAL POTENTIAL OF DISSECTING THE GENETICS OF HBM

The working assumption underlying the efforts of ourselves and others in this field is that understanding the genetic architecture of skeletal diseases characterized by HBM will elucidate critical pathways involved in bone growth and regulation, and aid development of novel therapeutics to increase bone mass (275). Successful drug targets (*i.e.*, those for whom drugs have successfully passed through all development steps to an approved drug indication) are enriched with genes known to be involved in human disease, whether identified through common or rare variant analysis (276). Inspiringly for those of us who study bone, the concordance between disease indication and disease/pathway association (whether identified through rare or common variant studies) is strongest for drugs targeting the musculoskeletal system, compared with all other systems (including diabetes, autoimmunity, cardiovascular disease and oncology). Importantly, there is no relationship between genomic effect size and approved drug status, emphasizing the

role of studying both rare variants of large effect and common variants of small effect (276).

The definitive proof-of-concept for this working hypothesis has been the development of antibodies to sclerostin, a protein only identified through analysis of HBM families with sclerosteosis and van Buchem's disease (110–112), with completion of phase 3 clinical trials (147, 277) and the first-in-class agent (romosozumab) approved for clinical use by the US Food and Drugs Administration. Similarly, genetic dissection of pycnodysostosis led to the development of Cathepsin K inhibitors and the first-in-class agent (odanocatib) (207), successful in phase 3 and extension trials but disappointingly not taken forward into clinical practice (248). Although we acknowledge wholeheartedly that many medications currently used in osteoporosis, were not developed as a direct consequence of genetic studies, it is interesting to reflect that bisphosphonates, selective estrogen receptor modulators, estrogen, cathepsin K inhibitors, denosumab, anti-sclerostin antibodies and PTH and its analogs all target proteins associated with a monogenic bone condition; and, with the exception of bisphosphonates and cathepsin-K inhibitors [but with the potential addition of DKK-1 inhibitors, which have shown promise in murine models (278, 279)], all target genes in loci with common variant association with BMD. We await news of further Wnt pathway agonists, also in development, as novel anabolic treatments for osteoporosis (278–281).

CONCLUDING COMMENTS: THE VALUE OF STUDYING EXTREME PHENOTYPES

In the 17th century William Harvey acknowledged the potential benefits of studying the natural, but rarely occurring, extreme cases, in order that they might elucidate systems pertinent to the general population: "Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of Nature by careful investigation of cases of rare forms of disease" (282).

These words summarise the rationale that we (and others) have used in considering and investigating individuals with high BMD (15, 33). The genetic revolution—both sequencing and high-throughput microarray genotyping—has contributed greatly to the understanding of both common (17, 18) and rare (8) bone pathologies, with identification of multiple genes and critical pathways, leading already to the development of novel therapeutics. We would particularly like to highlight that progress in this field has been greatly enabled by collaboration and co-operation between centers and within consortia around the globe. However, as discussed above, the most common form of sclerosing dysplasia appears to be the currently unexplained HBM phenotype, with features suggestive of a mild skeletal dysplasia. Given past history in this field, it is highly likely that further genetic dissection of HBM cases will yield further novel insights into bone regulation; and it is our hope that this work will contribute to improved health for individuals with HBM and for other individuals with metabolic bone diseases.

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All authors contributed to the article and approved the submitted version.

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Opportunities and Challenges in Functional Genomics Research in Osteoporosis: Report From a Workshop Held by the Causes Working Group of the Osteoporosis and Bone Research Academy of the Royal Osteoporosis Society on October 5th 2020

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The discovery that sclerostin is the defective protein underlying the rare heritable bone mass disorder, sclerosteosis, ultimately led to development of anti-sclerostin antibodies as a new treatment for osteoporosis. In the era of large scale GWAS, many additional genetic signals associated with bone mass and related traits have since been reported. However, how best to interrogate these signals in order to identify the underlying gene responsible for these genetic associations, a prerequisite for identifying drug targets for further treatments, remains a challenge. The resources available for supporting functional genomics research continues to expand, exemplified by "multi-omics" database resources, with improved availability of datasets derived from bone tissues. These

databases provide information about potential molecular mediators such as mRNA expression, protein expression, and DNA methylation levels, which can be interrogated to map genetic signals to specific genes based on identification of causal pathways between the genetic signal and the phenotype being studied. Functional evaluation of potential causative genes has been facilitated by characterization of the "osteocyte signature", by broad phenotyping of knockout mice with deletions of over 7,000 genes, in which more detailed skeletal phenotyping is currently being undertaken, and by development of zebrafish as a highly efficient additional *in vivo* model for functional studies of the skeleton. Looking to the future, this expanding repertoire of tools offers the hope of accurately defining the major genetic signals which contribute to osteoporosis. This may in turn lead to the identification of additional therapeutic targets, and ultimately new treatments for osteoporosis.

Keywords: genome-wide association study, bone mineral density, mouse model, zebrafish, "omics" data

INTRODUCTION

This perspective article provides a viewpoint on the opportunities and challenges in functional genomics research in osteoporosis, synthesizing the content of a recent workshop of invited experts. This was held to provide a blueprint for research and funding proposals in this area, with the ultimate aim of translating discoveries from human genetic studies into new therapies for patients with osteoporosis.

The Need for New Osteoporosis Therapies

Anti-resorptive drugs are the mainstay of treatment in osteoporosis. Despite being widely used, adherence rates in the US (1) and UK (2) are decreasing, and these agents have several limitations including poor tolerability in the case of oral bisphosphonates, and risk of rare adverse effects including osteonecrosis of the jaw and atypical femoral fractures. Anabolic therapies for osteoporosis may offer certain advantages, including greater efficacy than some anti-resorptives, and lack of the adverse effects associated with suppression of bone resorption. However, currently available anabolic drugs are costly, and need to be given by injection, limiting their use to a small fraction of patients with osteoporosis. Thus, there is an urgent need for a low cost, ideally orally active, anabolic therapy for osteoporosis.

The Potential of Human Genetic Studies for Drug Discovery in Osteoporosis

Rare Bone Diseases

The heritable condition of increased bone fragility, Osteogenesis Imperfecta (OI), was the first bone disorder to have the underlying genetic mutation identified. Linkage analysis identified the *COLIA1* and *COLIA2* genes as candidate loci for the disease and soon after this, various mutations were identified in both genes as a common cause of OI (3, 4). Many other mutations underlying OI have since been identified. Though most of these affect genes which are involved with post-translational modifications of type 1 collagen, some affect osteoblast differentiation and function (5). However, findings from OI genetics studies are yet to provide tangible opportunities for developing new osteoporosis treatments.

In contrast, studies of rare bone diseases associated with low or high bone mass (HBM) have provided the basis for a new osteoporosis therapy in the form of Romosozumab, following the discovery that sclerostin – LRP5 regulation of the Wnt signaling pathway plays a major role in bone biology. Romosozumab is an anti-sclerostin antibody, which has recently been developed as anabolic treatment for osteoporosis, and is now widely available. Sclerostin, encoded by *SOST*, was initially identified from the study of patients with the heritable HBM disorder, sclerosteosis (6). Several other genes underlying HBM have also been identified, representing possible therapeutic targets for additional anabolic therapies. These include a recently identified inactivating mutation in *SMAD9*, which encodes an inhibitor of BMP signaling (7). Further analysis of existing case collections of familial HBM may offer the opportunity to identify further possible drug targets.

Advances in the understanding of other rare bone diseases with a genetic component, such as pregnancy associated osteoporosis (8), might yield targets for new drug design or re-purposing existing drugs, which if successful might also apply to treating osteoporosis (9). In considering the pipeline for similar discoveries, the classification of genetic skeletal disorders lists 437 genes for 425 different diseases (10). For example, achondroplasia caused by a mutation in the fibroblast growth factor receptor 3 gene, a negative regulator of bone growth, now has an natriuretic peptide receptor 2 (NPR2) agonist (Vosoritide) developed as treatment (11). Of interest, factors from the C-type natriuretic peptide signaling pathway have previously been associated with human stature (12), among which a locus within the gene encoding NPR3, with which NPR2 complexes, was also recently identified in a genome wide association study (GWAS) of extreme high bone mass (13). Rare disorders associated with impaired osteoclastic bone resorption may also have utility in treating osteoporosis, exemplified by pycnodysostosis caused by cathepsin K deficiency (14), for which the inhibitor

Odanocatib was developed as a new anti-resorptive treatment for osteoporosis. In addition, drug-repurposing may provide novel means of treating rare bone disorders. For example, palovarotene, a retinoic acid receptor gamma (RAR-γ) agonist developed for use in emphysema, was found to be efficacious in an animal model of fibrodysplasia ossificans progressiva (FOP), and is now in phase 3 clinical trials (15). In addition, Fresolimumab, a human monoclonal antibody directed against transforming growth factor B2, developed for treating idiopathic pulmonary fibrosis, is currently being examined to treat OI (ClinicalTrials.gov identifier NCT03064074).

Genome Wide Association Studies (GWAS)

Many GWAS have been performed for endpoints related to osteoporosis-related phenotypes, including fractures (16), bone mineral density (BMD) as measured by DXA (17–19), as well as estimated by calcaneal ultrasound (eBMD) (20–22). These were undertaken by the GEnetic Factors for OSteoporosis Consortium (GEFOS), representing over 30 countries (http://www.gefos.org/). Whereas BMD represents an overall measure of bone quantity, GWAS have also been performed of endophenotypes related to cortical and trabecular bone as measured by peripheral quantitative computed tomography (pQCT) (23–26), and more recently high resolution (HR)-pQCT (27). Fracture risk can also reflect other characteristics such as bone shape and geometry, which have similarly been examined by GWAS (28, 29). To date, in contrast to the study of rare monogenic disorders, no GWAS of common variation in bone phenotypes has led to a new treatment for osteoporosis. That said, well powered GWAS have only been available in the relatively recent past, and the above GWAS have found genome-wide significant variants in genes coding for existing osteoporosis drug targets, e.g., romosozumab (*SOST*), denosumab (*RANKL*) and raloxifene (*ESR1*).

GWAS findings can also be helpful in predicting side-effects arising from the drug target in question having actions outside the skeleton. For example, a BMD GWAS signal related to *SOST* was used to examine potential cardiovascular toxicity of romosozumab (30). Several open-source data and analytical platforms, using published and unpublished GWAS summary datasets, have been developed to interrogate genetic correlations/causal effects in relation to thousands of traits and diseases, thereby predicting co-morbidities and extra-skeletal effects using a hypothesis-free approach. Examples include MR-Base (31), the polygenic risk score atlas (32), and LD-hub (33). Multiple risk factors for osteoporosis have also been scrutinized for causal associations using a Mendelian randomization (MR) framework (34, 35). For example, the GEFOS GWAS for fracture risk leveraged the MR approach to demonstrate, that among the recognized clinical risk factors, BMD is a "causally-related" determinant of fracture risk implying that targeting to increase BMD or prevent its loss is likely to be successful in decreasing fracture risk (16). This MR study also found that genetically-determined vitamin D levels are not causally related to fracture risk, supporting conclusions from clinical trials that vitamin D supplementation in "sufficient" individuals is ineffective in preventing fractures. Likewise, calcium intake was found to have no causal effect on fracture risk, suggesting an adverse risk/benefit ratio when the associated increased risk of coronary artery disease is taken into account (36, 37). GWAS findings may also have potential application as clinical risk prediction tools, as exemplified by a recent study examining implementation of a polygenic fracture risk score in combination with FRAX (38).

GWAS have been helpful in predicting the effectiveness of new drug therapies (39, 40). GWAS have also identified potential new drug targets in other musculoskeletal conditions. For example, GWAS in ankylosing spondylitis (AS) and psoriatic arthritis (PsA) identified IL23R, IL12B, and IL17A as associated loci, facilitating the development of ustekinumab, an IL12/23 inhibitor used in PsA, and secukinumab, an IL17A inhibitor used in both AS and PsA (41, 42).

Large well-powered GWAS often yield a multitude of genetic signals, but a major challenge is to map the association signals to the causal gene due to the correlated structure of the genome and to follow up those genetic signals from the point of view of functional studies. For example, the most recent eBMD GWAS from the UK Biobank Study, based on the whole cohort of around 425,000 individuals, identified 1103 independent association signals mapping to 515 loci (22). Combined with an earlier eBMD GWAS performed on a subset of the UK Biobank Study (20), these two studies investigated, and functionally annotated over 160 mouse lines with deletions of orthologous genes corresponding to associated GWAS loci, demonstrating the power of integrating disparate datasets from human GWAS and animal studies. Nevertheless, little functional information was obtained in the case of many of the genetic signals identified, due to lack of an available mouse model. For example, a signal associated with the *SMAD9* locus, described above, was initially discovered, but not interrogated further, in the original eBMD GWAS on 150,000 UK participants (20). The latter GWAS also identified a further signal, *B4GALNT3*, which was only later found to influence BMD by altering sclerostin levels, following a separate GWAS of serum sclerostin (43).

FUNCTIONAL GENOMICS: *IN SILICO* STUDIES

As stated above, a major challenge in analyzing outputs of genetics studies is to identify the gene underlying the genetic association observed. Several platforms are available to interrogate outputs from GWAS studies, aiming to identify causal SNPs and the genes they affect. Different methods are often applied in parallel to identify potential functional effects of SNPs, map these to genes and investigate the function of candidate genes identified in this way. A range of sources of omic information used for interrogating genetic signals in relation to skeletal disorders have recently been integrated within the IFMRS knowledge portal [https://msk.hugeamp.org/ (44)].

SNP-Based Analyses

Independent signals and lead variants are initially fine mapped across an identified locus using methods such as FINEMAP (45).

Subsequently, identified SNPs are annotated according to their likelihood of exerting a functional effect. In the case of monogenic disorders, the underlying genetic variant is expected to alter protein function, for example as a consequence of a non-synonymous exon variant. In contrast, in GWAS, the variant is likely to affect gene expression, for example due to a base change affecting DNA binding of a transcriptional activating factor within the promoter region. Several different approaches for SNP annotation have been developed. For example, ENCODE, the encyclopedia of DNA elements (https://www.encodeproject.org/) provides a range of features which can be used to evaluate potential functional SNPs. Machine learning approaches have been used to predict functional effects using the most discerning features from ENCODE and other databases. These algorithms are trained on disease-causing mutations and assumed neutral variants, enabling the algorithms to classify SNPs as potentially deleterious or neutral. In non-coding regions, where most GWAS SNPs are located, sequence conservation has been found to be by far the most informative feature (46). However, such algorithms are not disease-specific, so whether this also applies to bone related conditions remains to be established. Besides ENCODE, several other strategies for SNP annotation have been developed. These include ATAC-seq to study intersections between SNPs and sites of open chromatin as previously identified in osteoblast cell lines (22) and mouse bone tissue (43), and Hi-C to interrogate 3D DNA interactions as previously characterized in osteoblasts (22).

Gene-Based Analyses

Having identified lead SNP(s), the function of closest protein coding genes is explored to guide further follow up. Since bone-specific pathways are thought most likely to underlie skeletal phenotypes, if a gene is found to be expressed in bone, this is assumed to increase the likelihood that it underlies a given osteoporosis genetic association signal. This approach has been facilitated by description of the "osteocyte signature" (47), referring to the set of genes expressed preferentially in osteocytes, which was used to interrogate the genetic signals identified by Morris et al. (22). As described below, information from skeletal phenotyping of mouse lines can also help to identify genes which are likely to underlie genetic association signals relevant to osteoporosis, as are previous reports that the gene in question is related to a skeletal disorder in humans. In the case of genes not previously known to play a role in bone, methods such as DEPICT can be used to predict function based on relationships with known pathways (48).

"Omics" Approaches to Map GWAS Signals to Specific Genes

One approach to mapping genetic signals to specific genes is to examine causal pathways between the genetic signal and the phenotype being studied, involving potential molecular mediators such as mRNA expression, protein expression, and DNA methylation levels. These molecular quantitative trait loci (QTLs) are generally classified into *cis*-acting, where the SNP is located nearby a gene or site or *trans*-acting, where the site or gene is located more distantly or on another chromosome. *Cis*-acting molecular QTLs tend to have larger effect sizes whereas *trans* effects have smaller effect sizes and require larger sample sizes to detect these associations. Large scale initiatives such as GTEX, eQTLGen (49), Genetics of DNA Methylation Consortium (GoDMC) (50) and SCALLOP (51) have been established to identify these small effects that might play a role in disease etiology.

Co-localization studies across a range of disorders and phenotypes have been conducted to examine whether molecular QTLs share genetic variation with GWA signals, thereby linking a given genetic signal with the function of a specific gene. Evidence for a number of shared genetic factors between BMD GWA loci and protein quantitative trait loci (pQTLs) have been found in blood (52). However, whereas approaches such as co-localization analyses can be used to examine shared relationships between a given genetic locus, phenotype, and intermediary signal, these may not necessarily represent a causal pathway from the genetic signal to the phenotype. Approaches such as MR can be used to estimate causal effects (**Figure 1**), but require bi-directional analyses to exclude reverse causation, and in many cases will not exclude horizontal pleiotropy as an alternative explanation of co-localization (i.e., the genetic signal influences the phenotype being studied *via* an independent pathway to the gene showing related changes in expression). To improve reliability of MR, multiple independent genetic variants influencing a molecular trait can be employed, which should exhibit consistent causal effects (53). In a recent analysis combining MR and co-localization analysis to examine GWA with the plasma proteome, only a minority of associations were found to be causal, and mainly restricted to *cis*-associations (52). Similarly, the GoDMC study estimated the causal relationship between DNA methylation in blood and 116 complex traits. This study used multiple *cis* and *trans* instruments to evaluate whether the MR estimates based on the co-localizing signals corresponded amongst multiple independent methylation quantitative trait loci (mQTLs). Although many co-localizing putative signals were found including for BMD traits, the agreement between the independent mQTLs was very low (50). These results imply that many of the co-localizing signals were due to horizontal pleiotropy. Alternatively, other regions or proteins that are currently not captured by the technology may still have a causal effect.

There is growing evidence that the same gene expression level might have many different *cis* and *trans* expression quantitative trait loci (eQTLs) in different cell types and contexts. However, only a subset of those are active in the disease-relevant cell type or context and contribute to disease etiology (54). The resources used to curate eQTLs, pQTLs and mQTLs generally comprise bulk tissue and exclude bone tissue and cell types. Large scale QTL datasets derived from blood may still be useful, as osteoclasts and macrophages/monocytes originate from a common precursor. That said, the only EWAS study of BMD performed to date, based on whole blood samples, revealed negative findings (55). Novel methods to infer cell type specific

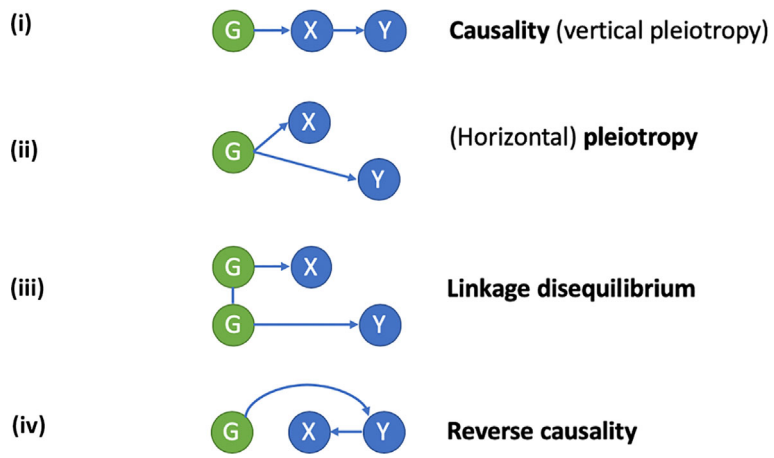

FIGURE 1 | Applying a Mendelian randomization (MR) framework to study causal inferences in "omics" data. In conventional MR, a genetic instrument (G) is used as a proxy for an exposure (X), to study its relationship with a disease outcome (Y). A causal relationship of X on Y exists, if G is related to Y *via* its effects on X. An example is the use of genetic polymorphisms related to bone mineral density (BMD) to study the causal relationship between low BMD (X) and fracture risk (Y). When applied to "omics" data, X represents an intermediate molecular trait (i.e., mRNA, DNA methylation, or protein level) mediating the relationship between genotype (G) and disease outcome (Y). Since the intermediate trait is gene-specific, finding of a causal relationship is helpful in defining which gene (or regulatory element in the case of DNA methylation) underlies the association between G and Y. Causal inference using MR relies on the exclusion of horizontal pleiotropy, confounding by linkage disequilibrium and reverse causality. (i) Causality/vertical pleiotropy: G has a causal effect on intermediate molecular trait X, which in turn has a causal effect on Y. (ii) Horizontal pleiotropy: G has a causal effect on both X and Y *via* independent pathways. (iii) Linkage disequilibrium: G has a causal effect on X, but its relationship with Y is a consequence of linkage disequilibrium with a separate genetic variant causal for Y. (iv) Reverse causality: G has a causal effect on Y which subsequently alters X.

DNA methylation or gene expression from bulk tissue are currently being developed (56), which may help to identify molecular signatures related to osteoporosis phenotypes. To date, osteoblast, and bone tissue eQTL datasets have only been generated in small sample sizes. However, these are currently being expanded upon, and the IFRMS knowledge portal described above is due to be updated with osteoblast-specific "omics" data in the near future. Alternatively, to gain an understanding of whether other cell types or tissues underlie GWAS signals, functional enrichment analyses across cell-type specific elements on the GWAS summary statistics can be performed (57).

FUNCTIONAL GENOMICS: *IN VITRO* STUDIES

Modeling the functional impact of osteoporosis *in vitro* offers a complementary approach to GWAS and *in vivo* studies, validating targets and revealing new modes of action on a cellular and molecular level. Typically, osteoblast and osteoclast cultures are employed (or precursor cell populations, e.g., mesenchymal stem cells, monocytes, respectively) to study the effect of a specific gene on cell formation and function in osteoporosis. This is achieved for example, by examining the cellular effect of gene deletion through CRISPR–Cas9 editing of candidates identified through human GWAS or *in silico* studies (58–60). Similarly, the direct use of bone cell screening assays can be used, recording evidence related to growth phenotypes, live-dead readouts, or bone cell activity and differentiation which are commonly dysregulated in human osteoporosis. This includes alkaline phosphatase levels, mineralization rates, tartrate-resistant acid phosphatase (TRAP) production, dentine resorption or alterations in key molecular markers such as Runx2, BMP2, OCN, RANKL, OPG gene expression, quantified through the use of fluorescent reporter assays, qPCR or gene array. Combination approaches assessing multiple readouts simultaneously are being explored to deliver high-throughput assessments of thousands of potential gene variants in a single experiment (61).

A major difficulty in modeling human skeletal responses and disease pathogenesis *in vitro* centres upon the cellular heterogeneity of this micro-environment. While bone-forming osteoblasts and bone-resorbing osteoclasts are most often targeted in such efforts, the complex multi-cellular bone niche consists of many more cell types including adipocytes, osteocytes, fibroblasts, stem cells and a large immune cell component. Importantly, many cell types have been linked to the onset and progression of bone disease, including osteoporosis. More accurate model systems are needed, capable of mimicking the multicellular bone environment and where the combined contribution of specific cell types and impact of genomic alterations can be more fully explored. Cellular heterogeneity within individual cell populations is being effectively probed through single cell genomic approaches, where distinct features captured at the resolution of individual cells have allowed for a more efficient isolation

and characterization of cell types within the normal and osteoporotic bone marrow niche (62, 63).

In vitro studies also allow examination of the impact of multiple causative genomic targets operating in networks within bone cell populations, which is only beginning to be explored. A clearer understanding of the intersecting relationships between genes, and how this may contribute to a cellular osteoporotic phenotype is necessary. This may be achieved for example, by systematically analyzing a defined cellular output (e.g., growth, alkaline phosphatase) of multiple gene-pair combinations, and where gene interactions are identified by quantifying the deviation from the expected phenotype of a single-gene alteration when combined with a second (64). This allows for clusters of related genes to be characterized which may act collectively as a genomic circuit in the prevention of aberrant bone cell biology or trigger for osteoporosis pathogenesis.

FUNCTIONAL GENOMICS: *IN VIVO* STUDIES

Mice

Mice are the most widely used animal model to investigate the functional role of genes identified in human genetic studies. The International Mouse Phenotyping Consortium (IMPC) aims to generate knockout mice harbouring deletions of all protein-encoding genes in a single C57BL/6N genetic background. To date, broad phenotyping of knockout mice with deletions of over 7,000 genes has been completed using the International Mouse Phenotyping Resource of Standardised Screens (IMPReSS; www.mousephenotype.org/impress/). Nevertheless, IMPReSS lacks both in depth and functional analysis of the skeleton and the IMPC thus collaborates with the Origins of Bone and Cartilage Disease (OBCD) Programme (65, 66) and the Bonebase Consortium (67) to undertake bespoke and detailed skeletal phenotyping.

The OBCD Programme uses digital X-ray microradiography, micro-CT and biomechanical testing in a rapid-throughput skeletal phenotyping pipeline to determine 19 parameters of cortical and trabecular bone structure, mineralization and strength in knockout mice compared to reference ranges obtained from >350 wild-type C57BL/6N mice. Knockout mice with abnormal parameters of both bone structure and strength (defined as >2 standard deviations away from the wild-type reference mean) are defined as having an outlier phenotype. Preliminary analysis of 1,000 knockout mice using this pipeline indicates approximately 10% display outlier phenotypes, a percentage that is broadly consistent with the >500 independent loci associated with eBMD in the recent UK Biobank GWAS (22). About 50% of mice with outlier phenotypes have deletions of genes that have not been functionally annotated to the skeleton and are not known to be related to human skeletal disease. Integration of large scale mouse phenotype data with GWAS (20, 22) and other cross-species multi-"omic" datasets (47) thus provide a rich resource to identify new genes and mechanisms involved in the pathogenesis of osteoporosis and monogenic human skeletal disorders (65, 68, 69).

Zebrafish

More recently, zebrafish have been developed as an animal model for functional evaluation of genes linked to the skeleton (70, 71). As well as showing changes in bone density and microarchitecture (72), gene deletion can lead to bone fragility as recognized by the accumulation of fractures in the fin (73–75), and the ribs (76). Zebrafish have several advantages over mice, making experiments quicker and less expensive: they are highly fecund, laying up to 300 eggs a week; phenotypes may be evident at the larval stage (skeletal elements develop by four days); embryos develop externally, enabling genetic manipulation at the single cell stage. In addition, larvae are translucent, allowing dynamic visualization of skeletal cell behavior, for which several transgenic reporter lines are available (77, 78). Embryonic lethality is rare with fish able to survive despite mutations leading to a complete absence of bone tissue, as they are supported by water as they swim, which limits loading of malformed skeletal elements (79). As well as generating knockouts, the CRISPR/Cas9 system has proven highly efficient in zebrafish, such that a homozygous null phenotype is already detected in G0s (mosaics, crispants) in larval and adult skeleton, therefore allowing rapid screening of candidate genes (80, 81). In addition, fish scales represent a good model for performing subsequent organ cultures for drug screening (82).

OBSTACLES AND OPPORTUNITIES

GWAS Data Sets

Several historical obstacles to functional evaluation of genetic signals related to osteoporosis have now been overcome. For example, we now have well-powered GWAS, through which hundreds of genetic loci have been robustly identified. That said, only relatively small GWAS datasets are available relating to endophenotypes obtained using methods such as HR-pQCT, which are helpful in determining the mechanisms by which genetic pathways influence overall bone strength as reflected by BMD/eBMD. In addition, genetic studies in osteoporosis have largely been confined to cross sectional analyses, with only limited studies examining associations with longitudinal changes, exemplified by a previous look up of adult GWAS hits in BMD acquisition in adolescents (83), and a recent GWAS of pediatric bone accrual (84).

Resources to Support Functional Studies

Osteoblast eQTL datasets based on larger samples are being generated, which will improve the accuracy of, for example, co-localization studies. The IFMRS knowledge portal is bringing together all relevant functional data, making it easier to perform functional annotation of large gene sets. Functional annotation has been advanced by characterization of the transcriptome of different cell types including osteocytes, the generation of over 7,000 knockout mouse lines, and the development of zebrafish as

a rapid-throughput screening tool. However, although homology across human/mouse/zebrafish is good, there are still gaps, and difficulty in accessing bone samples to characterise expression in human tissue remains a challenge. In addition, it is technically challenging to obtain single cells from mineralized tissues, hindering evaluation of bone cell transcriptomics *in vivo*. A further limitation is that datasets available for *in silico* analysis have an inherent bias because it is only possible to analyze and interrogate genes that have already been annotated or gene functions/pathways that are already known. Furthermore, it is difficult to "quality control" the data that is interrogated. For example, there are several papers that assign different activities to PLS3 but no clear function for the protein has yet emerged and it is still unclear whether its major role is in osteoblasts or osteoclasts or both (85).

Funding

The funding underpinning many of the resources used to support functional analyses of genetics data is finite, such as the IMPC consortium and IFMRS knowledge portal (44). In addition, given the myriad of tools available, and the range of scientific disciplines involved, the different research groups working in this area tend to pursue varying approaches. Functional follow-up of genetic signals is often performed in the context of specific projects, with the result that analyses are time- and resource limited. Funding models are generally in the form of fellowships, PhD studentships or project grants focussed on initial data collection; in contrast, it can be relatively difficult to obtain funding to support functional follow-up studies of previously collected GWAS data. Nevertheless, given the current pause in new human data collections due to the COVID-19 pandemic, arguably, greater priority should now be given to analyzing outputs of previous data collections.

FUTURE DIRECTIONS

Given the success of genetic discovery in delivering new therapies, including anabolic treatments for osteoporosis, there is a strong case for harnessing this expanding repertoire of tools to functionally annotate the array of genetic signals for osteoporosis-related phenotypes that have already been identified. However, new strategies need to be developed to fully integrate multi-"omic" datasets with those relating to human monogenic and complex diseases, and equivalent datasets from zebrafish and mice, and potentially other species. Furthermore, it will be essential to establish large collaborative groups of experts with the necessary skillsets to harness these. Ultimately, a roadmap of functional assessments needs to be established as a coordinated effort, if the emerging wealth of genetic discoveries is to be successfully translated into new therapies for osteoporosis. The Genomics of Musculoskeletal Traits Translational network (GEMSTONE www.cost-gemstone.eu/) is a leading example of how investigators in the field from a range of different disciplines can come together to coordinate functional evaluation across genes and pathways, promote interactions between experts from different fields, and limit duplication of efforts across teams. Whereas the initial focus of GEMSTONE has been to educate and disseminate through publications and meetings, a similar approach is needed to construct an effective, multi-disciplinary, research collaboration, in order to fully exploit the exciting opportunities for pursuing functional genomics studies in osteoporosis.

AUTHOR CONTRIBUTIONS

All authors contributed to the paper by participating in the workshop, providing written material and/or suggestions which were incorporated into the paper, and critically appraising the scientific content. All authors contributed to the article and approved the submitted version.

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GLOSSARY

EWAS	epigenome-wide association study
eQTL	expression quantitative trait locus; a genetic locus associated with gene expression (transcript) levels in a particular tissue
GWAS	genome wide association study
HR-pQCT	High resolution peripheral quantitative computed tomography
MicroCT	Micro computed tomography
mQTL	methylation quantitative trait locus; a genetic locus associated with DNA methylation levels in a particular tissue
pQCT	peripheral quantitative computed tomography
pQTL	protein quantitative trait locus; a genetic locus associated with protein levels in a particular tissue

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