Chapter 14: **Genetics of Skeletal Disorders**

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Abstract

Bone and mineral diseases encompass a variety of conditions that involve altered skeletal homeostasis, and are frequently associated with changes in circulating calcium, phosphate, or vitamin D metabolites. These disorders often have a genetic etiology and comprise monogenic disorders caused by a single gene mutation, which may be germline or somatic; or an oliogenic or polygenic condition involving multiple genetic variants. Single gene mutations causing Mendelian diseases are usually highly penetrant, whereas the gene variants contributing to oliogenic or polygenic disorders are each associated with smaller effects with additional contributions from environmental factors. The detection of monogenic disorders is clinically important, and facilitates timely assessment and management of the patient and their affected relatives. The diagnosis of monogenic metabolic bone disorders requires detailed clinical assessment of the wide variety of symptoms and signs associated with these diseases. Thus, clinicians should undertake a systematic approach commencing with careful history taking and physical examination, followed by appropriate laboratory and skeletal imaging investigations. Finally, clinicians should be familiar with the range of molecular genetic tests available to ensure their appropriate use and interpretation. These considerations are reviewed in this chapter.

Keywords

Bone, osteoporosis, genetic, variant, next generation sequencing

Introduction

Bone and mineral disorders commonly have a genetic basis (Table 1), which may be due to a pathogenic germline single gene variant (i.e. Mendelian or monogenic disorder), a somatic single gene variant (i.e. post-zygotic mosaic disorder), or involve multiple genetic variants (i.e. oliogenic or polygenic disorders) (Hannan et al. 2019; Mortier et al. 2019). Genetic variants causing Mendelian diseases typically have a large effect (i.e. penetrance), whereas oliogenic or polygenic disorders are caused by multiple genetic variants, each of which have smaller effects, in combination with environmental factors (Newey et al. 2018). Although most monogenic disorders arise from rare coding-region pathogenic variants, the majority of common genetic variants associated with polygenic traits are located in non-coding regions, usually in proximity to candidate genes implicated in the respective phenotype (Ward and Kellis 2012). Considerable overlap exists between genes causing monogenic bone disorders and those contributing to polygenic skeletal phenotypes. The identification of these loci has improved our understanding of the molecular basis of metabolic bone disorders, and provided targets for novel therapeutic agents (Karasik et al. 2016; Richards et al. 2012; Rivadeneira and Makitie 2016). In this chapter, the genetics of metabolic bone and mineral disorders, and the clinical and molecular diagnostic approaches required to investigate these typically heritable disorders, are reviewed.

Genetics of Bone and Mineral Disorders

Inheritance

Skeletal diseases are often single-gene monogenic disorders or polygenic complex traits (Mortier et al. 2019; Newey et al. 2018). Monogenic bone and mineral metabolic disturbances may be inherited as one of six traits (Table 1): autosomal dominant (e.g. osteogenesis

imperfecta (OI) due to type 1 collagen alpha-1 and alpha-2 chain (*COL1A1* and *COL1A2)* mutations (Marini et al. 2017); autosomal recessive (e.g. vitamin D dependent rickets types I and II due to mutations of the renal 1α-hydroxylase (*CYP27B1*) and vitamin D receptor (*VDR*) genes, respectively (Carpenter et al. 2017)); X-linked recessive (e.g. X-linked osteoporosis due to mutations of the plastin 3 (*PLS3*) gene (van Dijk et al. 2013)); X-linked dominant (e.g. X-linked hypophosphatemia (XLH) due to mutations of the phosphate endopeptidase on the X chromosome (*PHEX*) gene (Carpenter et al. 2017; Dixon et al. 1998)); Y-linked (e.g. azoopermia and oligospermia due to deletions of regions of the Y-chromosome (Hannan et al. 2019)); and non-Mendelian mitochondrial disorders (e.g. hypoparathyroidism associated with the Kearns-Sayre syndrome and mitochondrial encephalopathy, lactic acidosis, and stroke (MELAS) syndrome (Tengan et al. 1998; Wilichowski et al. 1997)). The pattern of transmission of polygenic disorders may be especially complex because of environmental factors. Examples of polygenic skeletal and mineral disorders include osteoporosis (Estrada et al. 2012; Kemp et al. 2017) and hypercalciuric nephrolithiasis (Stechman et al. 2009). Furthermore, polygenic traits such as osteoporosis can also occur as monogenic disorders, yet be overlooked, e.g. X-linked osteoporosis due to *PLS3* mutations, or early-onset osteoporosis due to heterozygous mutations of the Wnt family member 1 (*WNT1*) or sphingomyelin synthase (*SGMS2*) genes (Laine et al. 2013; Pekkinen et al. 2019; van Dijk et al. 2013). In addition to 'classical' Mendelian modes of inheritance, some kindreds exhibit apparent inherited disease due to alternate mechanisms. Thus, germline mosaicism (in which a post-zygotic mutation occurs during or prior to gametogenesis in a parent) may cause a seemingly autosomal recessive mode of inheritance with multiple affected children of apparently unaffected parents (Biesecker and Spinner 2013). Disease traits may also be conditioned by epigenetic mechanisms, which cause parent-of-origin effects. For example, germline mutations and epigenetic changes at the *GNAS* complex locus cause distinct pseudohypoparathyroidism

phenotypes (Lemos and Thakker 2015). Thus, maternally inherited inactivating coding region mutations in *GNAS* give rise to pseudohypoparathyroidism type 1A (PHP1A), which is characterised by parathyroid hormone (PTH) resistance together with Albright's hereditary osteodystrophy (AHO), whilst the identical paternally inherited mutations instead give rise to pseudopseudohypoparathyroidism (PPHP), which is characterised by AHO in the absence of PTH resistance (Table 1) (Lemos and Thakker 2015).

Genetic Heterogeneity

Clinical assessment, investigation, and treatment of patients with bone and mineral disorders requires familiarity with the diversity of phenotypes caused by underlying hereditary disease. Thus, establishing the correct diagnosis requires careful clinical work-up as well as appropriate laboratory investigation and genetic testing. Whilst the diagnosis of some skeletal disorders may be apparent from characteristic clinical or radiographic features, many diseases share overlapping phenotypes such as decreased bone mineral density (BMD), skeletal fragility, and altered mineralization (Newey et al. 2018). Similarly, whilst certain disorders are caused by mutation(s) in a single culpable gene, other diseases are a consequence of marked genetic heterogeneity with mutation(s) occurring in one of many candidate genes. Thus, in some situations a broad genetic differential diagnosis persists. For example, although most cases of OI are caused by mutations in the *COL1A1* and *COL1A2* type-I collagen genes (Marini et al. 2017), >15 additional genes, which are mainly involved in post-translational processing of collagen, account for a small percentage of OI cases (Cho et al. 2012; Laine et al. 2013; Morello et al. 2006; Pyott et al. 2013; Semler et al. 2012). Similarly, hypophosphatemic rickets is caused by mutations of genes encoding phosphatonins like fibroblast growth factor-23 (FGF-23) or bone cell proteins that regulate the expression and secretion of FGF-23 (e.g. PHEX, dentin matrix protein 1 (DMP1), and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1))

(Consortium 2000; Dixon et al. 1998; Feng et al. 2006; Levy-Litan et al. 2010), or by mutations affecting renal sodium phosphate co-transporters (e.g. SLC34A3*)* (Table 1) (Bergwitz et al. 2006; Lorenz-Depiereux et al. 2006). In addition, familial hypocalciuric hypercalcemia types 1-3 (FHH1-3), which are disorders of calcium homeostasis, have been shown to involve germline loss-of-function mutations affecting the calcium-sensing receptor (CaSR), G-protein subunit-α11 ($G\alpha_{11}$), and adaptor-related protein complex-2 σ-subunit (AP2σ), respectively (Table 1) (Hannan et al. 2012; Nesbit et al. 2013a; Nesbit et al. 2013b).

In contrast to when phenotypically similar disorders are caused by mutations in one of many different genes, mutations in the same gene may underlie a range of clinical phenotypes, with some considered distinctive diseases. For example, mutations in the signal peptide of receptor activator of NF-κB (RANK) can cause familial expansile osteolysis (FEO), expansile skeletal hyperphosphatasia (ESH), and early-onset familial Paget's disease of bone (PDB)], which arise as a consequence of rapid remodeling (Ralston 2008; Whyte 2018a). Disease severity can also be determined by whether a mutation is harbored in the heterozygous or homozygous state. For example, the severe perinatal and infantile forms of hypophosphatasia, an inborn-error-of-metabolism characterized by low serum alkaline phosphatase (ALP) activity, have an autosomal recessive mode of inheritance, whereas more mild and later-onset forms are usually inherited in an autosomal dominant manner (Table 1) (Whyte 2018b). In addition, some disorders are caused by loss- or gain-of-function mutations affecting the same gene. Thus, loss-of-function CaSR mutations cause FHH1 or neonatal severe hyperparathyroidism (NSHPT), whereas gain-of-function CaSR mutations cause autosomal dominant hypocalcemia type 1 (ADH1) or Bartter syndrome type V (Hannan et al. 2012; Pearce et al. 1996; Watanabe et al. 2002). Given this complex relationship between patient phenotype and the underlying

genotype, establishing the genetic diagnosis may be challenging yet crucial for the evaluation of patients and kindreds with skeletal diseases.

Molecular insights from monogenic disorders and polygenic traits

Genetic investigation of monogenic diseases has provided fundamental insights into the molecular regulation of bone biology and mineral homeostasis. Thus, utilization of classical gene-discovery approaches, which involve studying affected kindreds for co-segregation with polymorphic genetic markers to define the chromosomal location, followed by DNA sequence analysis of genes located within the candidate region (Newey et al. 2018), have identified several Wnt pathway components, which regulate bone mass (Figure 1). Indeed, autosomal recessive loss-of-function mutations of the LDL receptor related protein 5 (*LRP5)* gene, which encodes a key Wnt co-receptor (Figure 1), have been shown to cause osteoporosis-pseudoglioma syndrome, which is characterized by juvenile osteoporosis and congenital or childhood-onset blindness (Ai et al. 2005). Whereas, heterozygous gain-of-function mutations in *LRP5* (Little et al. 2002) and *LRP6* (Whyte et al. 2019), which encode the LRP5 and LRP6 cognate co-receptors, respectively, cause autosomal dominant high bone mass. In addition, autosomal recessive loss-of-function mutations of sclerostin (*SOST*), a Wnt-β-catenin inhibitor, develop sclerosteosis, type 1, which is associated with progressive bone overgrowth (Brunkow et al. 2001; Whyte et al. 2018); whilst individuals harboring a homozygous 52kb deletion containing an enhancer element downstream of the *SOST* gene manifest van Buchem disease, which has a milder but similar skeletal phenotype compared to sclerosteosis, type 1 (Balemans et al. 2002; van Lierop et al. 2013). Furthermore, bi-allelic truncating mutations in secreted frizzled-related protein 4 (*SFRP4),* which encodes a soluble Wnt inhibitor (Figure 1), have been reported in patients with Pyle's disease, a disorder characterized by cortical bone loss, fracture, and limb deformity (Kiper et al. 2016). Moreover, next generation sequencing (NGS)

approaches (Shendure et al. 2017), which include whole exome sequencing (WES) and whole genome sequencing (WGS), are being increasingly utilized to investigate monogenic diseases. Such approaches have demonstrated that heterozygous loss-of-function mutations of *WNT1* cause autosomal dominant early-onset osteoporosis, whereas biallelic loss-of-function *WNT1* mutations lead to an autosomal recessive form of OI (Laine et al. 2013). In addition, NGS has revealed that alterations in non-protein encoding genes can cause skeletal diseases (Mortier et al. 2019). Thus, a heterozygous germline microRNA mutation has been shown to cause a novel autosomal dominant skeletal dysplasia in two unrelated families (Grigelioniene et al. 2019). This point mutation increased expression of the chondrocyte-specific microRNA-140 (miR-140), and led to derepression of wild-type miR-140 targets whilst repressing mutant miR-140 targets, consistent with both loss- and gain-of-function effects (Grigelioniene et al. 2019).

Genetic investigation of polygenic disorders such as osteoporosis has utilized genome-wide association studies (GWAS), which require large populations of cases and controls (Estrada et al. 2012; Karasik et al. 2016; Kemp et al. 2017; Richards et al. 2012). Such studies generally involve direct or imputed genotyping of large numbers of common (e.g. minor allele frequency >5%) and infrequent (e.g. minor allele frequency 1-5%) single nucleotide polymorphisms/variants (SNPs/SNVs) to identify genetic loci enriched for the trait (Kruglyak 2008; Newey et al. 2018). GWAS has further highlighted the pivotal role of Wnt signalling in bone biology, and shown that many Wnt pathway components (>15 genes), including *LRP5* and *SOST*, are candidate genes for bone mineral density (BMD) (Estrada et al. 2012; Kemp et al. 2017), and that *WNT16* is a key determinant of cortical bone strength (Moverare-Skrtic et al. 2014; Zheng et al. 2012). Moreover, a GWAS of BMD estimated by quantitative heel ultrasound has reported that the dishevelled associated activator of morphogenesis 2 (*DAAM2*) gene, which likely modulates canonical Wnt signalling, has a major influence on bone

composition, strength, and mineralisation (Morris et al. 2019). Furthermore, osteoporosis GWAS variants have been mapped to target gene promoters using chromatin conformation capture methods (Chesi et al. 2019). Such studies, which involved human mesenchymal stem cell derived-osteoblasts, have identified the inhibitor of growth family member 3 (*ING3)* and ependymin related 1 (*EPDR1)* genes as potential osteoblastic regulators of BMD (Chesi et al. 2019).

In addition to investigating the influence of common variants in polygenic traits, some studies have highlighted a role for low-frequency and rare variants with larger effect sizes, which provide further insight into skeletal biology. For example, in Icelandic people, a rare truncating variant of the leucine-rich receptor containing G-protein-coupled receptor (*LGR4*) gene was shown to be associated with phenotypic traits that included low BMD and osteoporosis (Styrkarsdottir et al. 2013), whilst two rare *COL1A2* coding region variants were also detected in this population in association with low BMD (Styrkarsdottir et al. 2013). A further study of individuals of European ancestry reported a low–frequency, non-coding variant in close proximity to the Engrailed homeobox-1 (*EN1*) gene, which was associated with ~4-fold and ~3 fold greater effect size on BMD and fracture risk, respectively, compared to reported common variants (Zheng et al. 2015). This and other studies indicate a role for *EN1* in bone biology (Adamska et al. 2004; Mitchell et al. 2016), likely through an interaction with Wnt factors, and regulation of bone turnover, thereby highlighting the utility of these genetic approaches for investigating the molecular pathogenesis of bone and mineral metabolic diseases.

Genes and pathways as therapeutic targets

A key purpose of characterizing the genetic basis for bone and mineral disorders is the identification of genes and cellular pathways that may be targeted therapeutically. Indeed, many treatments now target key components identified from these studies. For example, the discovery that low skeletal ALP activity causes hypophosphatasia led to the multinational approval in 2015 of the bone-targeted enzyme-replacement biologic asfotase-alfa (Whyte 2016). This is further highlighted by the identification of FGF-23 as a key promoter of renal tubular phosphate excretion, which led to the approval in 2018 of burosumab, an anti-FGF-23 monoclonal antibody, for the treatment of XLH (Collins 2018). A phase 3 trial has demonstrated that children with XLH, who received subcutaneous burosumab injections every 2 weeks, achieved normal serum phosphate concentrations, better growth, and reduction in rickets severity compared to those who continued treatment with conventional therapy comprising oral phosphate and active vitamin D (Imel et al. 2019). Understanding the molecular basis of bone cell function has resulted in the development of the monoclonal antibody denosumab for the treatment of osteoporosis. Denosumab blocks RANKL within the OPG/RANKL/RANK/NF-κB signaling pathway to inhibit the formation and activity of osteoclasts, thereby inhibiting bone resorption (Karasik et al. 2016). Treatment with denosumab significantly reduced fracture risk in women with postmenopausal osteoporosis (Cummings et al. 2009). Osteoporosis therapies are now also directed at the Wnt pathway. This includes romosozumab, an anti-sclerostin monoclonal antibody that received FDA approval in 2019 for the treatment of post-menopausal osteoporosis. Romosozumab causes a rapid and marked increase in bone formation, primarily through modeling-based mechanisms, whilst simultaneously decreasing bone resorption (Ferrari 2018). The Fracture Study in Postmenopausal Women with Osteoporosis (FRAME) clinical trial showed that once-monthly

subcutaneous administration of romosozumab over 12 months reduced new vertebral fractures and clinical fractures by >70% and >35%, respectively (Cosman et al. 2016).

Approach to The Patient With Genetic Bone and Mineral Disease

Clinical approach

Bone and mineral disorders can manifest a variety of clinical symptoms and signs and an awareness of the range of potential phenotypes is central to appropriate investigation and treatment (Newey et al. 2018). Clinicians have at their disposal many biochemical and radiological tools to aid diagnosis, but they should be used judiciously and following the acquisition of a detailed personal and family history and the undertaking of a careful clinical examination (Newey et al. 2018). As the range and complexity of molecular genetic testing increases, selecting appropriate investigations may be challenging. In addition, the interpretation of genetic test results requires the clinician to recognize and assess potential uncertainties and limitations. Finally, it is important to include the patient in decision-making processes and ensure that appropriate informed consent is acquired before testing (Newey et al. 2018).

Medical history and physical examination

The history of present illness provides key information regarding disease etiology, pathogenesis, and prognosis and is important for guiding diagnosis and therapy. Determining whether signs and symptoms have begun recently or been longstanding can prompt different diagnostic considerations and interventions. It is also important to review prior medical records, radiographs, and laboratory tests to aid diagnosis and prognostication (Newey et al. 2018). Physical examination can reveal a range of findings for diagnosis including skeletal

deformities, which are common and perhaps unique in children. Diagnosis may emanate from recognition of a single physical finding; e.g. large café-au-lait spots (McCune-Albright syndrome), premature loss of deciduous teeth (hypophosphatasia), blue or gray sclerae (osteogenesis imperfecta), hallux valgus (fibrodysplasia ossificans progressiva), alopecia (vitamin D-dependent rickets, type II), brachydactyly (PHP1a and PPHP), syndactyly (sclerosteosis types 1 and 2), torus palatinus (high bone mass due to activating *LRP5* or *LRP6* mutations), or numerous surgical scars (multiple endocrine neoplasia (MEN) syndromes) (Carpenter et al. 2017; Collins et al. 2012; Lemos and Thakker 2015; Marini et al. 2017; Pignolo et al. 2011; Thakker et al. 2012; Whyte 2016; Whyte et al. 2018; Whyte et al. 2019). For some genetic diseases, an amalgam of physical findings suggests the diagnosis; e.g. rickets with craniotabes at birth, a rachitic rosary (enlargement of the costochondral junctions) developing during the first year of life, and leg deformities and short stature occurring in infancy or childhood (Carpenter et al. 2017). In adults, skeletal deformation arising in childhood can cause much of the morbidity from metabolic bone disease. Bowing of the lower limbs can lead to osteoarthritis, especially in the knees. Without a comprehensive physical examination, these issues may go undetected.

Family history and mode of disease inheritance

A variety of metabolic bone disorders have a monogenic etiology, and this may be suspected because of an early age of onset, occurrence of clinical features consistent with a syndromic disease, or a family history of the condition (Newey et al. 2018). The family history is required to establish the mode of disease inheritance. Thus, in autosomal dominant disorders such as FHH (Table 1) (Hannan et al. 2018), the proband typically has one affected parent, and the disease affects both sexes and is transmitted by either the father or mother. An autosomal dominant mode of inheritance is therefore disclosed by prior or prospective study of the

relatives' affected status. In contrast, in autosomal recessive diseases, which can affect both sexes, the proband is born to parents who may be related, and are usually asymptomatic "carriers". Therefore, parental consanguinity, which can be apparent, or inapparent involving endogamy within a specific geographical location and a "founder" mutation, can provide an important clue for autosomal recessive inheritance. In X-linked recessive diseases such as early-onset osteoporosis with fractures due to *PLS3* mutations (Table 1) (van Dijk et al. 2013), only males are typically affected, who are born to often unaffected parents yet the mother is an asymptomatic carrier with affected male relatives. There is no male-to-male transmission. In X-linked dominant diseases such as XLH (Table 1) (Carpenter et al. 2017; Dixon et al. 1998), both males and females are affected. However, females are usually more mildly and variably affected than males, and 50% of children (boys and girls) from an affected woman will have the disease, and all of the daughters, but none of the sons, of an affected man will have the disease (Hannan et al. 2019). In Y-linked diseases such as azoopermia and oligospermia, only males are affected and unless representing a sporadic case they have an affected father (patrilineal inheritance) and all sons of an affected man will have the disease (Hannan et al. 2019). In contrast, mitochondrial inherited disorders such as Kearns-Sayre syndrome and MELAS (Table 1) (Tengan et al. 1998; Wilichowski et al. 1997) can affect both sexes, and these disorders are only transmitted by an affected mother (matrilineal inheritance) from her egg mitochondrial DNA (Hannan et al. 2019). These modes of inheritance can be influenced by: i) non-penetrance or variable expression in autosomal dominant disorders such as MEN1 (Thakker et al. 2012); ii) imprinting, whereby expression of an autosomal dominant disorder is conditioned by whether it is maternally or paternally transmitted (e.g. PHP1a versus PPHP) (Lemos and Thakker 2015); iii) anticipation, whereby some dominant disorders become increasingly severe, or have earlier onset, in successive generations; iv) pseudo-dominant inheritance of autosomal recessive disorders reflecting repeated consanguineous marriages in

successive generations; and v) mosaicism in which an individual has two or more populations of cells with different genotypes because of post-zygotic mutations occurring during their development from a single fertilized egg (e.g. McCune-Albright syndrome) (Boyce and Collins 2019). In the particular circumstance of germline mosaicism arising from somatic mutation during gametogenesis, there may be confusion regarding diagnosis and recurrence risk because of apparently unaffected parents having multiple affected offspring that would suggest autosomal recessive inheritance, but actually reflect an autosomal dominant disorder such as OI type II (Biesecker and Spinner 2013). Hence, characterization of the family history can establish the mode of inheritance, help diagnose a genetic disorder, and also identify individuals at risk (Newey et al. 2018)

Overview of Genetic Tests

Clinical value of genetic testing

Identifying the genetic basis for a patient's bone and mineral disorder facilitates appropriate treatment and assessment of the patient for associated clinical features that may not have been apparent at initial evaluation. A genetic diagnosis can also provide prognostic information and facilitate genetic counseling as well as the testing of relatives who may be asymptomatic "carriers". Furthermore, family members found not at risk of having or transmitting the disease can be reassured. For parents with children affected with severe skeletal disease, the identification of a genetic cause may lead to pre-natal counseling and/or pre-conception genetic testing for future pregnancies. Genetic testing may also aid risk profiling. For example, SNPs associated with osteoporosis have been reported to predict fracture risk in bisphosphonate-treated patients (Lee et al. 2016), and potential genetic markers of anti-resorptive-induced osteonecrosis of the jaw have also been identified (Yang et al. 2018).

Pre-test considerations

For individuals with a possible genetic metabolic bone disease, several factors require consideration before arranging genetic analysis (Figure 2). These include the clinical phenotype, mode of inheritance, and availability of additional pedigree members to aid the diagnosis. For example, DNA sequencing of 'trios' (i.e. the affected proband and both parents) may facilitate detection of compound heterozygous or *de novo* mutations (Goldstein et al. 2013). Moreover, the type of underlying genetic abnormality will influence the likelihood of an investigation achieving the correct genetic diagnosis. For example, direct DNA sequencing methods are suitable for detecting coding region single nucleotide variants (SNVs) or small insertions or deletions ('indels') which cause the majority of monogenic bone and mineral disorders. However, these sequencing techniques may not detect whole or partial gene deletions that are associated with some monogenic syndromes, or be suitable for identifying large chromosomal abnormalities (e.g. 22q11.2 microdeletion in DiGeorge syndrome), whose detection requires alternative approaches (Figure 2 and Table 2) (Gijsbers and Ruivenkamp 2011). For some monogenic disorders, gene panel analysis should be considered if genetic heterogeneity is likely (e.g. in OI or FHH) (Hannan et al. 2018; Marini et al. 2017). Thus, genetic testing that fails to detect an abnormality does not necessarily exclude a genetic disease, but instead may reflect: i) the limitations of the utilized genetic methodology (e.g. inadequate resolution or coverage); ii) incorrect assumptions regarding the clinical phenotype or mode of inheritance; or iii) an alternative genetic cause to the one being tested (Newey et al. 2018). Consequently, sequential or simultaneous genetic tests can be required to ensure comprehensive evaluation of the underlying etiology, although such testing may be limited by local availability and cost (Hannan et al. 2019).

Detection of chromosomal abnormalities, copy number variations (cnvs), and mutations causing disease

Karyotype

Conventional karyotyping is the initial test for assessing major chromosomal abnormalities such as aneuploidy (i.e. abnormal number of chromosomes), or large insertions, deletions, duplications, inversions, or reciprocal translocations (Table 2) (Dave and Sanger 2007; Gijsbers and Ruivenkamp 2011). Such abnormalities may be suspected in the presence of major congenital abnormalities, marked developmental delay, or features of a specific chromosomal abnormality disorder such as Klinefelter and Turner syndromes, both of which may be associated with osteoporosis (Faienza et al. 2016; Ferlin et al. 2010). Karyotyping is usually performed using peripheral blood leucocytes, which are cultured, prior to evaluation by high resolution G-banding (Giemsa-staining) of at least 20 metaphase nuclei. Evaluation of multiple cells allows a reliable analysis of each chromosome, and also facilitates identification of mosaicism (Dave and Sanger 2007; Gijsbers and Ruivenkamp 2011). High resolution G-band karyotype analysis identifies the most major chromosomal defects, however, its resolution is limited to ~5-10Mb of DNA, and will therefore not identify smaller abnormalities such as CNVs) (Table 2) (Dave and Sanger 2007; Gijsbers and Ruivenkamp 2011).

Fluorescence In Situ Hybridization (FISH)

FISH utilizes DNA probes, which hybridize to specific target regions on metaphase chromosomes, thereby enabling visualization by fluorescence microscopy (Dave and Sanger 2007; Gijsbers and Ruivenkamp 2011). A range of chromosomal abnormalities can thus be identified resulting from: absence of probe binding (e.g. due to a deletion), additional probe binding (e.g. due to a duplication), or probes binding to an aberrant chromosomal region (e.g.

due to a translocation or inversion). Molecular resolution of FISH is typically 50kb-2Mb (Table 2). The major limitation is that it does not allow genome wide analysis, but is limited to detecting abnormalities covered by the probe sets (e.g. detection of 22q11.2 deletion in DiGeorge syndrome). Alternate FISH-based methods that allow the simultaneous evaluation of several regions of interest have been developed, which include: whole-chromosome painting probes (termed multiplex FISH (M-FISH) and spectral karyotyping (SKY)), with each chromosome labeled a different color (Dave and Sanger 2007; Gijsbers and Ruivenkamp 2011). Whilst such techniques can identify interchromosomal abnormalities such as translocations, they do not identify small deletions, duplications, or inversions (Dave and Sanger 2007; Gijsbers and Ruivenkamp 2011).

Multiplex-Ligation Dependent Probe Amplification (MLPA)

MLPA utilizes a pool of custom-designed probes to amplify specific genomic regions, and is used to detect small chromosomal abnormalities such as single or partial gene deletions (Gijsbers and Ruivenkamp 2011). Adjacent probes hybridize to each other and undergo ligation followed by polymerase chain reaction (PCR) amplification of the ligated product, with products separated by electrophoresis (Table 2). The probes are generally designed to detect partial or complete gene deletions. In addition, MLPA can evaluate alterations in methylation, for example pseudohypoparathyroidism 1b (PHP1b), which is associated with deletion of one or more of four differentially methylated regions (Tafaj and Juppner 2017).

Array-based screening

Array comparative genomic hybridization (aCGH) is used for the genome-wide detection of small chromosomal abnormalities (Table 2) and commonly represents first-line investigation for multiple congenital abnormalities, which include skeletal manifestations and/or

neurodevelopmental delay (Gandomi et al. 2015; Koczkowska et al. 2017). The aCGH technique involves mixing the patient's DNA sample (labeled green) with a reference DNA sample (labeled red), prior to applying the sample to the array platform for competitive hybridization with a set of immobilized reference DNA fragments. Automated analysis of the array measuring red-green fluorescence facilitates identification of deletions and duplications in the patient's sample (Gijsbers and Ruivenkamp 2011; Kharbanda et al. 2015). The aCGH methodology is frequently used to detect copy number variants (CNVs). However, all individuals harbor small CNVs without apparent adverse health effects, whilst several potentially pathogenic CNVs have reduced penetrance and do not always cause disease (Newey et al. 2018).

SNP arrays may also detect CNVs and be used for genome-wide genotyping (Table 2). For example, deletions or uniparental disomy spanning several adjacent SNPs on the array can reveal loss of heterozygosity (LOH), whilst copy number gains (e.g. duplication) may be indicated by increased numbers of different genotypes (Gijsbers and Ruivenkamp 2011). SNP arrays can also be used for homozygosity mapping to localize recessive disorders in the offspring of consanguineous parents (Caparros-Martin et al. 2017).

DNA sequence analysis

Sanger DNA sequencing remains the gold standard for detecting DNA sequence variants due to the high fidelity of the DNA polymerase used during DNA amplification (base accuracy of >99.99%) (Falardeau et al. 2017; Lazarus et al. 2014). However, it is labor intensive and generally reserved for disorders with limited genetic heterogeneity such as single gene disorders e.g. hypophosphatasia caused only by *TNSALP/ALPL* mutations (Tenorio et al. 2017). Sanger sequencing is increasingly being replaced by NGS approaches, which allow the

massively parallel sequencing of large amounts of genetic material. The three major uses of NGS are: WGS; WES; and disease-targeted gene panel sequencing (Table 2). WGS analyses the entire genome DNA sequence including coding and non-coding regions, and can detect SNVs, small insertions or deletions, and CNVs (Newey et al. 2018). In contrast, WES analyses the ~20,000 protein-coding genes (i.e. the 'exome'), which comprise 1-2% of the genome, and is expected to harbor most disease-causing mutations (Newey et al. 2018). Consistent with this, WES has represented the mainstay of disease-gene discovery studies over the past decade, and led to the identification of the genetic cause for a range of metabolic bone diseases (e.g. *AP2S1* mutations in FHH type 3 (Nesbit et al. 2013b); *BMP1* mutations causing increased BMD and recurrent fractures (Asharani et al. 2012); *SFRP4* mutations in Pyle's disease (Kiper et al. 2016); and *CYP3A4* mutations in vitamin D-dependent rickets, type 3 (Roizen et al. 2018)). Disease-targeted sequencing represents the principal NGS method used in clinical practice, and is used to analyze small or large numbers of genes (e.g. <10 to >150 genes) associated with a specific disease (Falardeau et al. 2017; Lazarus et al. 2014; Rehm 2013). Indeed, NGS disease-targeted panels are established for genetically heterogeneous disorders, which include OI, disorders of calcium-sensing, and hypophosphatemic rickets (Arvai et al. 2016; Bardai et al. 2017; Polla et al. 2015).

Assessment of variant pathogenicity

A major challenge arising from increasing utilization of genetic testing is ascribing pathogenicity to individual variants. Several international bodies have published guidelines recommending that variant classification systems utilize the terms 'pathogenic', 'variant of unknown significance (VUS)', and 'not pathogenic or benign', and that evidence for pathogenicity is gathered from multiple sources (Group et al. 2017; Richards et al. 2015). The initial analysis of non-synonymous variants should report the frequency of the variant in large-

scale population level databases such as GnomAD and the Exome Aggregation Consortium (ExAC) (GnomAD ; Lek et al. 2016). A frequency of >1% in such databases indicates that the variant is likely a benign polymorphism. However, the presence of variants at low frequencies (<1%) does not exclude their pathogenicity as the databases may contain representative individuals from the disease population (e.g. The Cancer Genome Atlas) (Richards et al. 2015). In addition, clinical variant and disease databases (e.g. ClinVar, Human Gene Mutation Database) should be consulted to determine if the gene has previously been linked with the condition (Johnston and Biesecker 2013). Further investigation of variants involves using *in silico* algorithms such as SIFT, Polyphen-2, and MutationTaster to predict whether amino acid changes are likely to affect protein function (Adzhubei et al. 2013; Schwarz et al. 2010; Vaser et al. 2016). In addition, demonstration of evolutionary conservation of the variant residue or location of the variant residue within a critical structural domain of the affected protein, using *in silico* tools such as Clustal Omega and Pymol, respectively, can indicate that a variant may be pathogenic (Newey et al. 2018). Once a variant has been assessed by these bioinformatics methods and still remains a plausible candidate, then determining whether the variant segregates only with affected family members is key to attributing disease-association (Newey et al. 2018). Functional analysis should also be considered and the choice of experimental technique will depend on the type of variant (e.g. splice site variants are assessed by measurement of RNA and protein expression, whereas missense mutations may require functional cellular assays); the tissue affected in the disease; and the availability of samples (Newey et al. 2018). Ideally, assessments should be made using patient tissue where possible, for example in cell-lines derived from lymphoblastoids or skin fibroblasts, or histological sections of tissues removed by biopsy or surgery. In choosing an *in vitro* assay the heritability of the variant should also be considered as overexpression systems may exaggerate the effect of a variant (Newey et al. 2018).

Special circumstances for genetic testing

Genetic tests for mosaicism

Genetic bone disorders can arise from somatic mosaicism (e.g. *GNAS* mutations in McCune-Albright syndrome) (Collins et al. 2012), and may also rarely occur as germline mosaicism arising from somatic mutation during gametogenesis in seemingly unaffected parents (e.g. OI type II). This may cause diagnostic confusion, as in this setting, one parent is carrying the mutation in their gametes, and may give rise to more than one affected offspring, suggesting possible autosomal recessive inheritance, in contrast to the underlying autosomal dominant inheritance pattern (Cohen et al. 2015). Detection of mosaicism has been improved by sensitive genome-wide testing strategies (e.g. aCGH, SNP arrays, and NGS), which can detect low-level mosaicism (e.g. 5% for SNP array) (Biesecker and Spinner 2013). However, selecting the appropriate test depends on the clinical phenotype, the type of mutation suspected (e.g. SNV, CNV, aneuploidy), the likely extent of mosaicism, and its tissue distribution. In general, blood lymphocyte DNA will suffice, but analysis of other affected tissues may be required (e.g. fibroblasts or bone) (Kang et al. 2018; Lindhurst et al. 2011).

Genetic tests for prenatal diagnosis

Genetic testing can be performed at pre-implantation or pre-natal stages, and is used to detect severe disorders such as perinatal lethal OI (Pyott et al. 2011). Pre-implantation diagnosis involves a single cell being obtained from the embryo several days after *in vitro* fertilization to identify single gene defects or chromosomal abnormalities, thereby allowing selection of an unaffected embryos for uterine implantation (Vermeesch et al. 2016), whereas pre-natal genetic testing is undertaken once pregnancy is established (Vermeesch et al. 2016). Typically, detection of fetuses at risk from genetic disease involves cells being obtained for DNA analysis

using invasive methods such as chorionic villous sampling or amniocentesis (Vermeesch et al. 2016). However, progress made in the detection of cell-free circulating fetal DNA in the maternal circulation (e.g. after ~10 weeks gestation) now offers the potential for non-invasive prenatal testing (NIPT) (van den Veyver and Eng 2015). Thus, a maternal blood sample may facilitate fetal sex determination, which is important for X-linked disorders, and allow screening for aneuploidy (Breveglieri et al. 2019). Monogenic skeletal disorders such as OI, achondroplasia, and craniosynostosis have also been diagnosed by NIPT (Zhang et al. 2019). However, this approach is limited to autosomal dominant disorders caused by *de novo* or paternally inherited mutations, whereas maternally inherited or autosomal recessive disorders are more challenging to diagnose given the similarity between maternal DNA and the maternally inherited region of the fetal genome (Breveglieri et al. 2019).

Informed consent and ethical considerations

Genetic evaluation of skeletal disorders may clearly benefit the patient and family, however, such testing also poses clinical and ethical challenges. For example, NGS approaches frequently identify variants of uncertain significance (VUS) (Richards et al. 2015), and communicating the relevance of such variants to the patient remains a major challenge. It is clear that explaining the possibility of such ambiguous results should comprise part of informed consent prior to genetic testing (Figure 2) (Newey et al. 2018). Studies have also indicated that variants reported as pathogenic could instead be benign (Manrai et al. 2016) or far less penetrant than previously recognized, and such diagnostic misclassification could potentially lead to inappropriate investigation or treatment. Thus, caution is required in data interpretation and both the clinician and patient must understand the genetic test result and its potential limitations. Genetic testing may also lead to incidental findings, which are abnormalities unrelated to the clinical question but of potential significance to the patient and family. For

example, the additional identification of penetrant pathogenic mutations in hereditary cancer genes (e.g. *BRCA1*, *BRCA2*) could result in complex ethical considerations, and plans for managing such findings should be in place beginning with the informed consent pathway (Rigter et al. 2014).

Conclusions

Bone and mineral diseases commonly have a genetic basis, and represent either a monogenic disorder due a germline or somatic single gene mutation, or an oliogenic or polygenic disorder involving several genetic variants. Recognition of these heritable disorders is fundamental for appropriate investigations and treatment for patients and families. The advent of high-content genetic testing employing NGS approaches has revolutionized investigation and diagnosis of genetic disease. The clinician must now acquire an understanding of these complex tests to combine with his/her fundamental skills of history taking and physical examination to ensure their judicious use to benefit patients.

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Table 1. Examples of monogenic metabolic bone disorders, modes of inheritance and genetic etiology

Mode of inheritance/ Disease	Gene(s)	Chromosomal location	References
Autosomal Dominant			
Osteogenesis imperfecta (OI), types I-IV	*COL1A1, COL1A2*	17q21.33, 7q21.3	(Marini et al. 2017)
Osteogenesis imperfecta (OI), type V	*IFITM5*	11p15.5	(Cho et al. 2012; Semler et al. 2012)
Autosomal dominant hypophosphatemic rickets	*FGF23*	12p13.32	(Consortium 2000)
Autosomal dominant high bone mass, type 1	*LRP5*	11q13.2	(Little et al. 2002)
Autosomal dominant high bone mass, type 2	*LRP6*	12p13.2	(Whyte et al. 2019)
Early-onset osteoporosis	*WNT1*	12q13.12	(Laine et al. 2013)
Osteoporosis and skeletal dysplasia	*SGMS2*	4q25	(Pekkinen et al. 2019)
Familial hypocalciuric hypercalcemia (FHH), types 1-3	*CASR, GNA11, AP2S1*	3q21.1, 19p13.3, 19q13.3	(Hannan et al. 2012; Nesbit et al. 2013a; Nesbit et al. 2013b)
Autosomal dominant hypocalcemia (ADH), types 1-2	*CASR, GNA11*	3q21.1, 19p13.3	(Nesbit et al. 2013a; Pearce et al. 1996)
Familial expansile osteolysis	*TNFRSF11A*	18q21.33	(Ralston 2008; Whyte 2018a)
Hypophosphatasia	*TNSALP/ALPL*	1p36.12	(Whyte 2016)
Vitamin D-dependent rickets, type 3	*CYP3A4*	7q22.1	(Roizen et al. 2018)
Pseudohypoparathyroidism, type 1a (PHP1a)*	*GNAS*	20q13.3	(Lemos and Thakker 2015)
Pseudopseudohypoparathyroidism (PPHP)*	*GNAS*	20q13.3	(Lemos and Thakker 2015)
Pseudohypoparathyroidism, type 1b (PHP1b)*	*GNAS, NESP55, STX16*	20q13.3	(Lemos and Thakker 2015)
Autosomal Recessive			
Osteogenesis imperfecta (OI), type VI	*SERPINF1*	17p13.3	(Becker et al. 2011)
Osteogenesis imperfecta (OI), type VII	*CRTAP*	3p22.3	(Morello et al. 2006)
Osteogenesis imperfecta (OI), type VIII	*P3H1/LEPRE1*	1p34.2	(Baldridge et al. 2008)
Osteogenesis imperfecta (OI), type XV	*WNT1*	12q13.12	(Laine et al. 2013)
Hypophosphatasia	*TNSALP/ALPL*	1p36.12	(Whyte 2016)
Neonatal severe hyperparathyroidism (NSHPT)	*CASR*	3q21.1	(Hannan et al. 2012)
Vitamin D-dependent rickets, type 1	*CYP27B1*	12q14.1	(Carpenter et al. 2017)
Vitamin D-dependent rickets, type 2	*VDR*	12q13.11	(Carpenter et al. 2017)
Autosomal recessive hypophosphatemic rickets	*DMP1, ENPP1*	4q22.1, 6q23.2	(Feng et al. 2006; Levy-Litan et al. 2010)
Hereditary hypophosphatemic rickets with hypercalciuria	*SLC34A3*	9q34.3	(Bergwitz et al. 2006; Lorenz-Depiereux et al. 2006)
Osteoporosis-pseudoglioma syndrome	*LRP5*	11q13.2	(Ai et al. 2005)
Sclerosteosis, type 1	*SOST*	17q21.31	(Brunkow et al. 2001)
Sclerosteosis, type 2	*LRP4*	11p11.2	(Whyte et al. 2018)
Pyle's disease	*SFRP4*	7p14.1	(Kiper et al. 2016)
Juvenile Paget disease	*TNFRSF11B*	8q24.12	(Whyte et al. 2002)
X-linked Dominant			
X-linked hypophosphatemia (XLH)	*PHEX*	Xp22.11	(Dixon et al. 1998)
X-linked recessive			

X-linked osteoporosis	*PLS3*	Xq23	(van Dijk et al. 2013)
Dent disease, type 1	*CLCN5*	Xp11.23	(Devuyst and Thakker 2010)
Mitochondrial			
Mitochondrial encephalomyopathy with lactic acidosis and stoke-like episodes (MELAS)	Mitochondrial genome	-	(Tengan et al. 1998)
Kearns-Sayre syndrome	Mitochondrial genome	-	(Wilichowski et al. 1997)
Mosaicism			
McCune-Albright syndrome (polyostotic fibrous dysplasia)	*GNAS*	20q13.3	(Boyce and Collins 2019)
Osteogenesis imperfecta (OI)§	*COL1A1*/*COL1A2*	17q21.33, 7q21.3	

*Parentally imprinted.
§Autosomal disorder manifesting as post-zygotic somatic mosaicism in the developing fetus, or arising from germline mosaicism in an apparently unaffected parent.
Adapted from *Hannan FM et al. Br J Clin Pharmacol 2019; 85: 1147-1160*.

Table 2: Examples of genetic tests, their molecular resolution and utility

Genetic Test	Resolution	Abnormalities detected	Additional Notes
Detection of chromosomal abnormalities including copy number variations (CNVs)			
Karyotype: G-banding (trypsin-Giemsa staining)	5-10Mb	Aneuploidy Large chromosomal deletions, duplications, translocations, inversions, insertions	Limited resolution Requirement to study many cells to detect mosaicism
Fluorescence in situ hybridization (FISH)	50kb - 2Mb (dependent on size of probes employed)	Structural chromosomal abnormalities (e.g. microdeletions, translocations)	Labour-intensive Low resolution limits its use Unsuitable where unknown genetic aetiology
Multiplex-ligation probe amplification	Probe dependent 50-70 nucleotides Single exon deletion or duplication possible	Copy number variations (CNVs) including (partial) gene deletions or duplications	Low cost, technically simple method Simultaneous evaluation of multiple genomic regions Not suitable for genome-wide approaches Not suitable for analysis of single cells
Array Comparative genomic hybridization (aCGH)	10Kb (high resolution) 1Mb (low resolution) (Dependent on probes set)	Genome-wide copy number variations (CNVs)	Inability to detect balanced translocations Useful for detection of low level mosaicism
Single Nucleotide Polymorphism (SNP) array	~50-400Kb (Dependent on probe set)	Genome-wide detection of SNP genotypes Copy Number Variations (CNVs)	Inability to detect balanced translocation Useful for detection of low level mosaicism Detection of copy number neutral regions or absence of heterozygosity (i.e. due to uniparental disomy)
Detection of monogenic disorders (and copy number variations (CNVs))			
First generation sequencing (Sanger)			
Single gene test	Single nucleotide (exonic regions and intron/exon boundaries of candidate gene)	Single nucleotide variants (SNVs) Small insertions or deletions ('indels')	Relative high cost/base May miss large deletions/duplications Unsuitable where unknown genetic aetiology
Next generation sequencing			
Disease-targeted gene panels	Single nucleotide (exonic regions and intron/exon boundaries of candidate genes)	Single nucleotide variants (SNVs) Small insertions or deletions ('indels')	May lack complete coverage of exomic regions (may require Sanger sequencing to fill in 'gaps') Increased likelihood of identifying variants of uncertain significance (VUS) as number of genes increases Unsuitable where unknown genetic aetiology
Whole exome sequencing (WES)	Single nucleotide (all exonic regions and intron/exon boundaries)	Single nucleotide variants (SNVs) *Small insertions or deletions ('indels') Copy Number Variations (CNVs)	Not all exons may be covered/captured Difficulties with GC-rich regions and presence of homologous regions/pseudogenes *Small indels may not be captured Bioinformatic expertise required for data analysis High likelihood of incidental findings and VUSs

			Detection of CNVs requires additional data analysis (i.e. loss of heterozygosity mapping across exonic regions) Suitable for disease associated gene-discovery
Whole Genome Sequencing (WGS)	Single nucleotide	Single nucleotide variants (SNVs) Small insertions or deletions ('indels') Copy Number Variations (CNVs) (Translocations/rearrangements)	Relative high cost Large data sets generated and complex data analysis requiring bioinformatic expertise High likelihood of incidental findings and VUSs CNV analysis possible but may present specific challenges Suitable for disease associated gene-discovery

From *Hannan FM et al. Br J Clin Pharmacol 2019; 85: 1147-1160.*

Figures

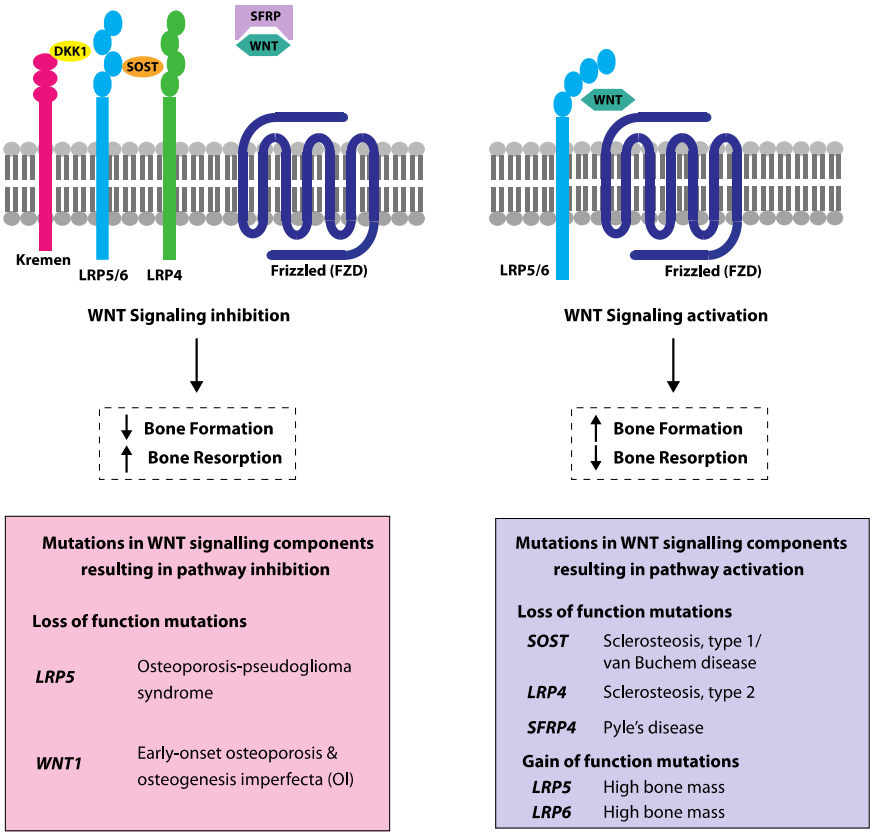

Fig. 1. Schematic representation of Wnt signalling pathway components reported to be mutated in disorders of bone development and skeletal homeostasis. Activation of the canonical Wnt pathway increases bone mass, and this is mediated by the binding of extracellular Wnt ligands (dark green) to a transmembrane receptor complex comprising the Wnt co-receptor LRP5 or LRP6 (LRP5/6, light blue) and a member of the frizzled (FZD) family (dark blue). In contrast, inhibition of the canonical Wnt pathway decreases bone mass (Baron and Kneissel 2013; Krishnan et al. 2006). This inhibition is mediated by extracellular factors such as sclerostin (SOST, orange) and Dickkopf-related protein 1 (DKK1, yellow), which bind to the LRP5/6 co-receptor thereby preventing activation by Wnt ligands, as well as recruiting inhibitory transmembrane proteins such as: LRP4, which is a SOST-interacting protein (light green); and the Kremen proteins (pink), which are high-affinity DKK1 receptors that functionally cooperate with DKK1 to decrease Wnt signalling (Mao et al. 2002). Secreted-frizzled-related proteins (SFRPs, purple) also inhibit the canonical Wnt pathway by sequestering Wnt ligands. The importance of the canonical Wnt pathway for the regulation of bone mass has been highlighted by loss-of-function mutations affecting SOST and LRP4, and by gain-of-function mutations of LRP5 and LRP6, which lead to the disorder called high bone mass (Balemans et al. 2002; Brunkow et al. 2001; Fijalkowski et al. 2016; Little et al. 2002); and also by loss-of-function mutations of LRP5 and the Wnt1 ligand, which lead to monogenic osteoporosis disorders (Ai et al. 2005; Laine et al. 2013). From *Hannan FM et al. Br J Clin Pharmacol 2019; 85: 1147-1160.*

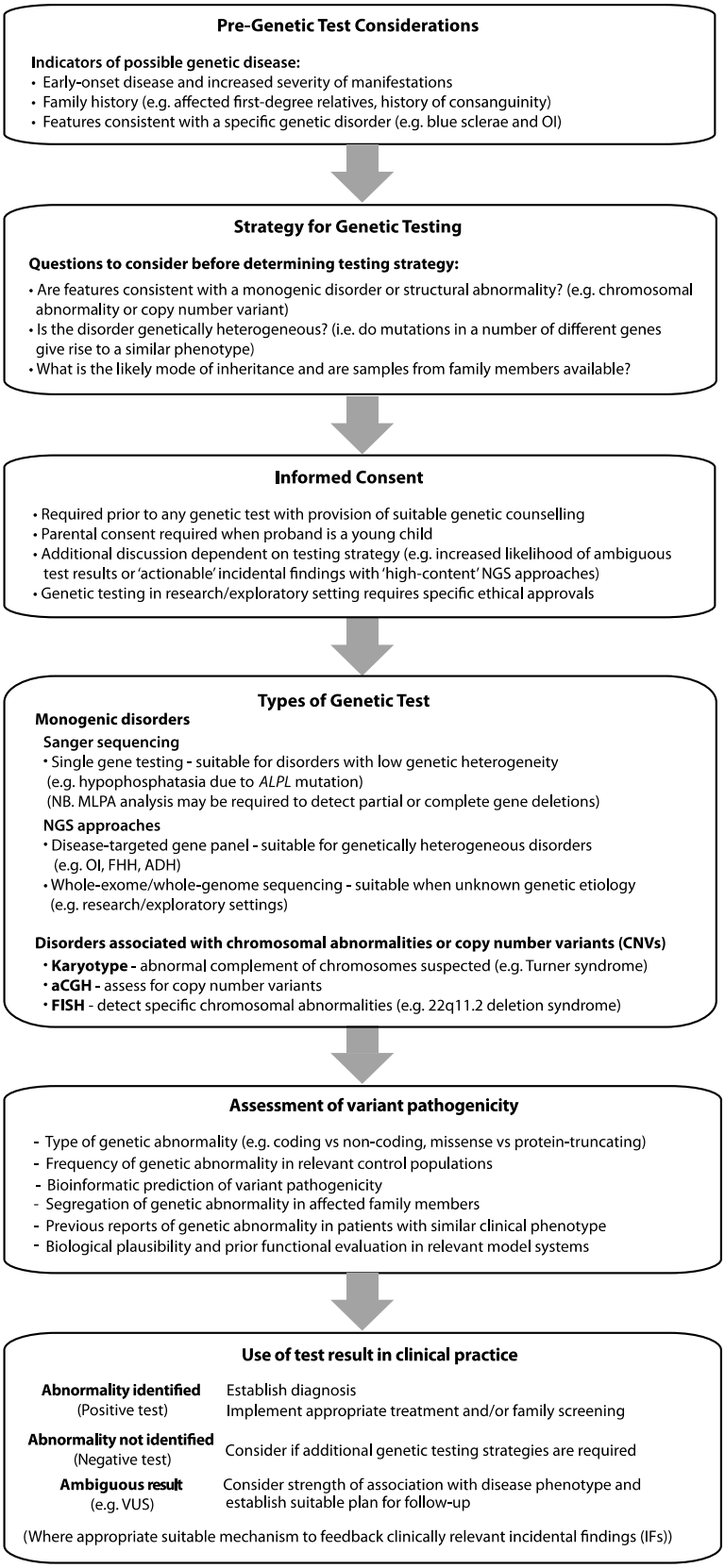

Fig. 2. Flowchart outlining considerations for genetic testing in patients with metabolic bone disease. From *Hannan FM et al. Br J Clin Pharmacol 2019; 85: 1147-1160.*