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# Systems Genetics: A Novel Approach to Dissect the Genetic Basis of Osteoporosis

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## Abstract

From the early 1990s to the middle of the last decade, the search for genes influencing osteoporosis proved difficult with few successes. However, over the last 5 years this has begun to change with the introduction of genome-wide association (GWA) studies. In this short period of time, GWA studies have significantly accelerated the pace of gene discovery, leading to the identification of nearly 100 independent associations for osteoporosis-related traits. However, GWA does not specifically pinpoint causal genes or provide functional context for associations. Thus, there is a need for approaches that provide systems-level insight on how associated variants influence cellular function, downstream gene networks, and ultimately disease. In this review we discuss the emerging field of "systems genetics" and how it is being used in combination with and independent of GWA to improve our understanding of the molecular mechanisms involved in bone fragility.

### Keywords

Systems genetics; Systems biology; Coexpression network; Causality modeling; Genetics of osteoporosis; Genome-wide association study

## Introduction

Osteoporosis is a disease of weak and fracture-prone bones affecting approximately 200 million people worldwide [1]. It is characterized by low bone mineral density (BMD) and the microarchitectural deterioration of bone, which together significantly increase the risk of fracture [2]. As a result of its high prevalence, osteoporosis is directly responsible for ~1.5 million fractures annually, with an estimated health care cost of $17 billion in the United States alone [3].

Many intrinsic characteristics of the skeleton contribute to its strength including the mass, size, morphology, and material properties of bone [4]. Each of these traits is determined by a complex combination of environmental and genetic factors. However, heritability (percent of total variance due to genetics) estimates for osteoporosis-related quantitative traits generally exceed 50%, indicating that most of the variation in risk of fracture is determined by genetics [5]. For example, a number of epidemiological studies have shown that the heritability of BMD is between 60% and 80% [5]. The fact that complex bone phenotypes

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are highly heritable indicates that a thorough understanding of osteoporosis necessitates the comprehensive genetic dissection of its component traits.

From the mid 1990s to 2007, numerous attempts were made to identify the individual genes that explained the heritable component of osteoporosis [6]. These early gene discovery attempts relied on linkage analyses in families (or experimental crosses in rodents) and candidate gene association studies. Genes underlying the quantitative trait loci (QTL) identified by linkage proved difficult, if not impossible, to positionally clone, while the reproducibility of candidate gene associations was low. As a result, very few osteoporosis genes were discovered [6].

This began to change in 2007 with publication of the first genome-wide association (GWA) study for BMD and bone morphology traits in the Framingham Osteoporosis Study [7••]. In a GWA, hundreds of thousands of common single nucleotide polymorphisms (SNPs) are genotyped in tens of thousands of individuals and then tested for their effect on quantitative phenotypes, such as BMD, or for differences in allele frequencies between disease cases (eg, osteoporotic individuals) and controls (eg, non-osteoporotic individuals) [8]. Since this initial paper, there have been over 25 GWA studies for osteoporosis-related phenotypes that have identified nearly 100 independent associations [9-13]. An up-to-date summary of GWA results for BMD and other osteoporosis-related traits can be found by searching the "Catalog of Published Genome-Wide Association Studies" (http://www.genome.gov/gwastudies/).

GWA is designed to identify high-resolution associations across the genome in an unbiased fashion. However, GWA does not intrinsically identify individual causal genes, nor does it provide functional information. This is an important point since translating genetic discoveries into new therapies relies on both gene discovery and the elucidation of their function. In addition, it is clear that complex bone phenotypes, such as BMD, are not exclusively determined by the cumulative effects of individual genetic influences, but instead are the result of emergent properties of biological networks [14]. This makes it necessary for new approaches that can extend, complement, and enhance GWA by generating a systems-level view of disease [15, 16]. Systems genetics is one such approach and below we discuss examples of how it is being used to advance our understanding of osteoporosis.

## Systems Genetics Defined

Systems genetics is an emerging approach that can be used to investigate cell function and disease from a systems-level perspective (Fig. 1) [16]. Systems genetics relies on the principles and methods of systems biology, but focuses on determining how naturally occurring genetic variation perturbs cellular systems and ultimately disease [15]. In a systems genetics study, data are collected from multiple biological "components" [17]. Components include the genome, transcriptome, proteome, metabolome, and phenome, among many others. The components can be assayed in a high-throughput and massively parallel manner with technologies such as microarrays (genomes, epigenomes, and transcriptomes), next-generation sequencing (genomes, epigenomes, and transcriptomes), mass spectrometry (proteomes and metabolomes), and clinical imaging (phenome) [17]. These data are analyzed using a suite of analytical approaches that include GWA, expression quantitative trait locus (eQTL) discovery, causality modeling, and network analysis [17].

To illustrate the multifaceted nature of a systems genetics analysis consider the following human dataset: 500 individuals with microarray gene expression profiles generated from bone biopsies, genotype information on 1 million SNPs and BMD levels. In terms of the analysis, one could begin by identifying eQTLs regulating the transcript levels of genes

expressed in bone. This list of eQTL SNPs could then be cross-referenced with a list of BMD GWA SNPs from prior GWA studies. SNPs found in this list would implicate a specific gene in the regulation of BMD (the gene whose expression it regulated) as well as its mechanism of action (difference in expression). The relationships between these SNPs, gene expression, and BMD could also be formally tested using modeling approaches such as causality prediction. This would be especially useful to identify BMD-associated SNPs that were suggestive (eg, $P<0.001$) in a GWA, but did not reach genome-wide significance. Additionally, the transcriptomic data could be used to reconstruct a coexpression network for bone. The network could then be mined to identify groups of interacting genes that were associated with changes in BMD or for changes in network connections between individuals with low and high BMD. Together, these analyses would provide a much more comprehensive systems-level perspective on BMD than GWA alone.

Systems genetics is a novel approach that is only starting to be applied to complex diseases and this is particularly true in the bone field where there have only been a small number of studies performed to date. In addition, the vast majority of studies involve the integration of transcriptomic data and not any other type of molecular data. Here, we attempt to summarize this work. We specifically focus on systems genetics studies that identify and utilize eQTL, causality modeling, and network analysis in the context of complex bone phenotypes.

## Using eQTL Data to Enhance GWAS

Gene expression can be measured in large populations of humans or rodents using genome-wide gene expression microarrays or RNA-seq [18-20]. Both techniques have their advantages and disadvantages. Microarrays are relatively cheap, provide coverage of most major transcript isoforms, and are high-throughput [17]; however, they have a low dynamic range, are prone to artifacts due to overlapping SNPs [21], and do not provide comprehensive quantitation of entire transcriptomes. In contrast, RNA-seq provides a digital readout of gene expression and comprehensive quantitative information on isoform abundance; however, it is currently costly and requires significant computational expertise and infrastructure for data analysis [22]. Independent of the technique used, however, quantitative measures of transcript or isoform expression can be mapped using association [23]. Genetic loci that regulate the transcript levels of a particular gene are referred to as eQTL [17, 23, 24]. Associated SNPs within an eQTL are referred to as expression SNPs (eSNPs). There are two types of eQTL, local and distant (the terms cis and trans have also been used in the eQTL literature) [17, 23]. Local eQTLs are located in close proximity to the gene they regulate. A number of different mechanisms can manifest as a local eQTL, including promoter polymorphisms altering gene transcription in an allele-specific manner or polymorphisms that alter mRNA stability. In contrast, distant eQTLs are typically not located close to the gene they regulate (often on a separate chromosome). An example of a distant eQTL would be a polymorphic transcription factor located on chromosome 3 affecting the transcription of a gene located on chromosome 10.

A series of recent papers by Grundberg et al. [25••] and Kwan et al. [26] nicely demonstrate how eQTLs can inform BMD GWA studies. For this work, high-density genotyping and microarray-based gene expression data were generated on primary human osteoblasts (hOBs). In the first study, the authors identified several hundred genes regulated by local eSNPs in hOBs ($N=95$) [25••]. They then cross-referenced the list of eSNPs with the top SNPs identified in a separate BMD GWA [10]. Two key observations were made. First, there was a significant enrichment of hOB eSNPs among those that were also associated with BMD. A parallel analysis using lymphoblastoid cell lines (LCLs) did not reveal this enrichment. Although somewhat obvious, this suggested that it is advantageous to use primary bone cells (or bone tissue) for systems genetic studies of osteoporosis compared to

the more assessable cells or cells lines such as LCLs. Second, of the top 10 local eSNPs that were correlated with BMD, a variant in the serine racemase (*SRR*) gene was found to be associated with BMD in two independent studies, providing strong support for the hypothesis that differences in its expression lead to alterations in BMD. A similar approach was used in a second study of hOBs ($N$=60) that were profiled using Affymetrix exon arrays [26]. Instead of probing the expression of a gene with one or a small number of probes, exon arrays consist of probes for the majority of characterized exons in the human genome [27]. Having data on all exons allows one to identify differentially expressed transcript isoforms. In this particular study, the data were used to identify a novel transcript isoform of the *FAM118A* gene whose expression was regulated by a local eSNP [26]. This eSNP was also found to be associated with BMD in two independent studies. Together, these two analyses demonstrate how eQTL discovery can be used to identify novel BMD genes that were not detected by GWA alone. In addition, gene discovery in this way also identified the functional mechanism (ie, alteration in expression) through which the associated variants are influencing BMD.

It is also possible to perform the reciprocal experiment in which GWA SNPs are queried to determine if they are also eSNPs. A number of osteoporosis GWA studies have used this approach. For example, Richards et al. [12] identified a SNP (rs4355801) located upstream of tumor necrosis factor receptor superfamily, member 11b (*TNFRSF11B*; Osteoprotegerin) that was associated with BMD and risk of osteoporosis. Using microarray data from LCLs from 55 unrelated HapMap individuals, it was determined that rs4355801 was also a local eSNP regulating the expression of *TNFRSF11B*. These data are consistent with the observation that perturbing the expression of *TNFRSF11B* in knockout and transgenic mice alters bone mass [28, 29]. Similarly, in a large meta-analysis of five BMD GWA studies, Rivadeneira et al. [9] identified 20 loci affecting BMD, of which 13 were newly identified. Using microarray data on primary hOBs, SNPs within 3 of the 13 novel loci were found to regulate the expression of the genes (G protein-coupled receptor 177 [*GPR177*], myocyte enhancer factor 2C [*MEF2C*], and forkhead box C2 [*FOXC2*]) nearest the most significant SNPs. Kung et al. [30] used quantitative real-time polymerase chain reaction to determine that the SNP rs2273061, which was strongly associated with BMD and located in the third intron of the *JAG1* gene, regulated the expression of *JAG1* in human-derived bone cells and peripheral mononuclear blood cells. Additionally, Hsu et al. [31••] recently identified seven loci affecting BMD and/or femoral neck geometry. Three of the top seven SNPs were also found to be eSNPs that regulated the genes (RAS-related protein-1a [*RAP1A*], TBC1 domain family, member 8 [with GRAM domain] [*TBC1D8*] and *TNFRSF11B*) nearest the trait associated SNPs. As demonstrated by these studies, the identification of GWA SNPs that are also eSNPs provides two key pieces of information, 1) it assists in identifying the causal gene(s) responsible for a specific association, and 2) it defines how the associated variants are influencing the trait.

## eQTL and Causality Modeling Enhances Gene Discovery in the Mouse

Since the work of Beamer et al. [32] demonstrating that inbred strains of mice displayed strikingly different BMD, mice have played a major role in the genetic analysis of osteoporosis [33]. Mice also have a number of advantages for systems genetics, including the ability to control the environment and access to tissues that would be difficult to obtain in large human cohorts [34]. High-resolution GWA approaches are only starting to be used in mouse populations [35, 36••, 37]. As a result, most studies have used linkage analysis in experimental crosses to map BMD QTL [38••]. Similar to human QTL, it has proven difficult to positionally clone the underlying genes [34]. However, there have been a number of recent studies demonstrating that in this setting the use of systems genetics techniques can significantly assist in gene discovery. Mehrabian et al. [39] provided one of the first

successful examples of this approach for BMD. In this study, the authors mapped a QTL affecting a number of metabolic traits (obesity, plasma lipid levels, etc.) and BMD to chromosome (Chr.) 6 in an F2 cross between the C57BL6/J and DBA/2J mouse strains [39]. Using microarray data from F2 mice, it was found that this locus also regulated the expression of over ~2000 transcripts (identified as distant eQTL linked to the Chr. 6 QTL). These data suggested that the underlying gene affected BMD and the metabolic traits through a major impact on the transcriptome. Of the 172 positional candidates within the Chr. 6 BMD locus, none were regulated by local eQTL that might explain the association and 5-lipoxygenase (*Alox5*) was the only gene that harbored a missense mutation between the two strains. The list of ~2000 genes with distant eQTL mapping to this locus was then cross-referred with genes showing a significant difference in expression between mice deficient in *Alox5* (*Alox5*$^{-/-}$) and controls. There was a highly significant ($P$=3.3 × 10$^{-17}$) overlap between the two gene lists. This suggested that the *Alox5* missense mutation disrupted the expression of a network of genes, which in turn perturbed a wide range of physiological systems including bone. Consistent with this hypothesis, it was shown that mice deficient in *Alox5* (*Alox5*$^{-/-}$) have reduced BMD [39, 40].

Systems genetics can also be used to identify candidate genes for BMD in mice on a genome-wide scale. We recently reported the genome-wide systems genetics analysis of BMD in an $F_2$ cross between the C57BL6/J and C3H/HeJ mouse strains [38••]. A total of nine BMD QTL were identified. We used microarray data from adipose on all ~300 $F_2$ mice to identify genes that were regulated by local eQTL that overlapped one of the nine BMD QTL. We did this to identify likely candidates under the assumption that local eQTLs would be responsible for a subset of BMD QTL. Although we did not have access to bone-relevant expression data, it has been shown that local eQTLs are generally conserved across tissues [41]. This analysis identified 148 genes whose expression was regulated by local eQTL and correlated with BMD. We then used a causality prediction method to formally “orient” the relationships between the most significantly linked SNP at each QTL, the expression of eQTL genes overlapping each locus and BMD. To illustrate how causality prediction works consider the example of a SNP that is associated with both differences in a gene’s expression and BMD. We know that the flow of information has to begin with the SNP (ie, genetic variation can alter a gene’s expression and/or BMD, but changes in expression or BMD do not alter primary DNA sequences); therefore, the possible relationships can be modeled as: 1) causal (SNP→Gene→BMD), 2) reactive (SNP→BMD→Gene), 3) independent (Gene←SNP→BMD), or 4) confounded (SNP→Gene←BMD or SNP→BMD←Gene). Probabilities for each model can be calculated using likelihood-based approaches or structural equation models [42, 43]. Hypotheses are then drawn based on relative model probabilities. Typically, the causal model is the one we are most interested in because it links a gene’s expression to a change in a clinical trait; however, the approach can also be used to identify “reactive” genes downstream of other genes, such as key transcriptional regulators [43]. Using this approach, 18 of the 148 genes with eQTL were predicted to be associated with variation in BMD. Four of the 18 (*Twist2, Mmp14, Grem2*, and *Wnt9a*), have been previously implicated in skeletal development or bone cell activities [44-48].

It is also possible to use causality prediction methods in the mouse to prioritize genes located within human GWA signals. In the BMD and bone morphology GWA by Hsu et al. [31••] discussed above, four genome-wide significant and 16 suggestive BMD/morphology associations were identified. For the four genome-wide significant hits, causality prediction in an F2 mouse cross was able to identify at least one putatively causal gene for three of the four associations. Additionally, the gene nearest the most significant SNP for 12 of the 16 suggestive GWA signals was predicted to be causal in mice, suggesting that many of these associations represent true signals.

## Generating a Systems-Level View of Osteoporosis Using Network Analysis

Complex traits, such as BMD, are influenced by thousands of functionally polymorphic genes, which act in concert with environmental stimuli to generate specific network “states” [14]. Network states are defined as the entire set of interactions between molecular components within cells and across cells and tissues at a particular time [49••]. The network state of an individual determines where on the spectrum of quantitative variation that individual lies. In the case of BMD, a network state that leads to high values protects against fracture, whereas a network state that leads to values at the opposite end of the distribution will promote fracture. Given that complex non-additive interactions are responsible for setting a specific network state, it is unlikely that states can be inferred based solely on the knowledge of all the individual genetic variants that contribute to a trait or disease. Therefore, it is not feasible for a reductionist approach, such as GWA, to provide a comprehensive view of the genetic basis of variation in osteoporosis-related traits. On the contrary, systems genetics-based network analysis can begin to identify and characterize these interactions by determining how genetic information is coordinated, processed, and transmitted through cellular networks [15].

Networks consist of nodes connected by edges. In a biological network nodes are the entities of cellular components, such as transcripts, proteins, and metabolites. The edges are any one of several different types of interactions including genetic, transcription-factor binding, protein-protein, and coexpression [50]. In systems genetics research, coexpression networks are the most common and are generated using global expression data (generated using microarrays or RNA-seq) collected across many genetically unique individuals [51]. The patterns of gene expression that result from each unique genetic background are then used to quantify correlational relationships among genes on a transcriptome-wide scale. The edges then reflect the strength of the correlation between two transcripts. As with most types of networks, coexpression networks are highly modular with distinct modules representing dense clusters of highly correlated and connected nodes [52]. Coexpression modules can be associated with a particular phenotype by determining their enrichment for genes predicted to be causal [49••] or correlating their behavior with the phenotype [53]. Modules have been shown to typically be enriched for genes sharing a similar function [54-58]. Thus, the identification of modules associated with disease provides insight into the pathways that are important for that disease and module membership can be used to predict the function of novel genes [36••]. One of the most popular algorithms for the generation of coexpression networks is Weighted Gene Co-expression Network Analysis (WGCNA) [59]. WGNCA networks can be easily created using an R package [60].

In the first systems genetics analysis of chondrocytes, Suwanwela et al. [61••] generated a WGCNA gene coexpression network consisting of 14 modules. The network was generated using microarray data from chondrocytes isolated from 27 mouse strains from two recombinant inbred panels, C57BL6/J and DBA/2J (BXD) and C57BL6/J × C3H/HeJ (BXH). One of the coexpression modules was correlated with bone morphology and BMD in the strains. Twenty-eight of the most highly connected genes from this module were knocked down using RNA interference in the ATDC5 chondrogenic cell line. Knockdown of five (*Hspd1, Cdkn1a, Bhlhb9, Cugbp1*, and *Spcs3*) of the 28 significantly altered chondrocyte differentiation. This systems genetic study identified five new regulators of chondrogenesis and suggested that the module of genes they belonged to played an important role in cartilage and/or bone development.

We also recently generated a coexpression network for circulating monocytes from individuals with low (BMD *Z*-score of -1.72 ± 0.60) and high (BMD *Z*-score of 1.57 ± 0.57) BMD [55]. The network consisted of 11 coexpression modules. One module, referred to as

module 9, was found to be more highly expressed in individuals with high BMD, relative to the low BMD group. This module was significantly ($P$=7.6 × $10^{-11}$) enriched in genes that were involved in the immune response. This included a large number of genes that were induced by the pro-resorptive cytokine interferon-γ [62]. Importantly, it was found that the most highly connected "hub" genes in the network were more likely to be genetically associated with BMD in two independent GWA studies [7••, 10], than genes that were more lowly connected [55].

One of the other ways network analysis can be used is to provide information on gene function. This was highlighted in a recent mouse systems genetics study in which GWAS was used in a new genetic reference population referred to as the Hybrid Mouse Diversity Panel (HMDP) to identify BMD associations [36••]. The HMDP consists of ~100 inbred strains that have been genotyped at high density (actual or imputed genotypes for millions of SNPs are available on the HMDP) [35]. We identified an association on Chr. 12, which harbored six genes. Of the six, none were regulated by local eQTL. However, the most significant association was with a non-synonymous SNP in the additional sex combs-like 2 (*Asxl2*) gene that was predicted to have deleterious effects on protein function. BMD in *Asxl2* knockout (*Asxl2*$^{-/-}$) mice was found to be lower, indicating that *Asxl2* was a regulator of BMD. The GWAS had identified *Asxl2* as a regulator of BMD; however, it was unclear how *Asxl2* altered BMD. To begin to uncover its function we generated a WGCNA bone coexpression network using microarray data from the HMDP. In the network, *Asxl2* was the most closely connected to genes involved in myeloid cell differentiation. In bone, osteoclasts are bone-resorbing cells of myeloid origin [63]. Additionally, in a human protein–protein interaction network, ASXL2 interacted with TRAF6, a key component of the major signaling pathway regulating osteoclastogenesis [63]. Thus, we predicted that *Asxl2* was involved in the differentiation of osteoclasts. Consistent with this hypothesis, an ~50% knockdown of *Asxl2* in osteoclast precursors significantly inhibited their ability to form mature osteoclasts. These data suggested that *Asxl2* influences BMD, at least in part, through its regulation of osteoclastogenesis. This study demonstrates the utility of coexpression networks to provide functional context for genes associated with complex bone phenotypes.

## Conclusions

Approaches such as GWA are rapidly improving our ability to develop lists of genes that influence traits such as BMD; however, it is unlikely that strictly correlating genotype with phenotype will provide a complete picture of osteoporosis. Systems genetics is an approach that has the potential to complement and enhance GWA through techniques such as eQTL identification, multivariable modeling, and network reconstruction. These studies improve gene discovery, and more importantly, they allow one to approach disease genetics from a systems perspective instead of through the eyes of reductionism. It is expected that in the years to come systems genetics will provide a much clearer understanding of the nature and composition of the genetic architecture of osteoporosis.

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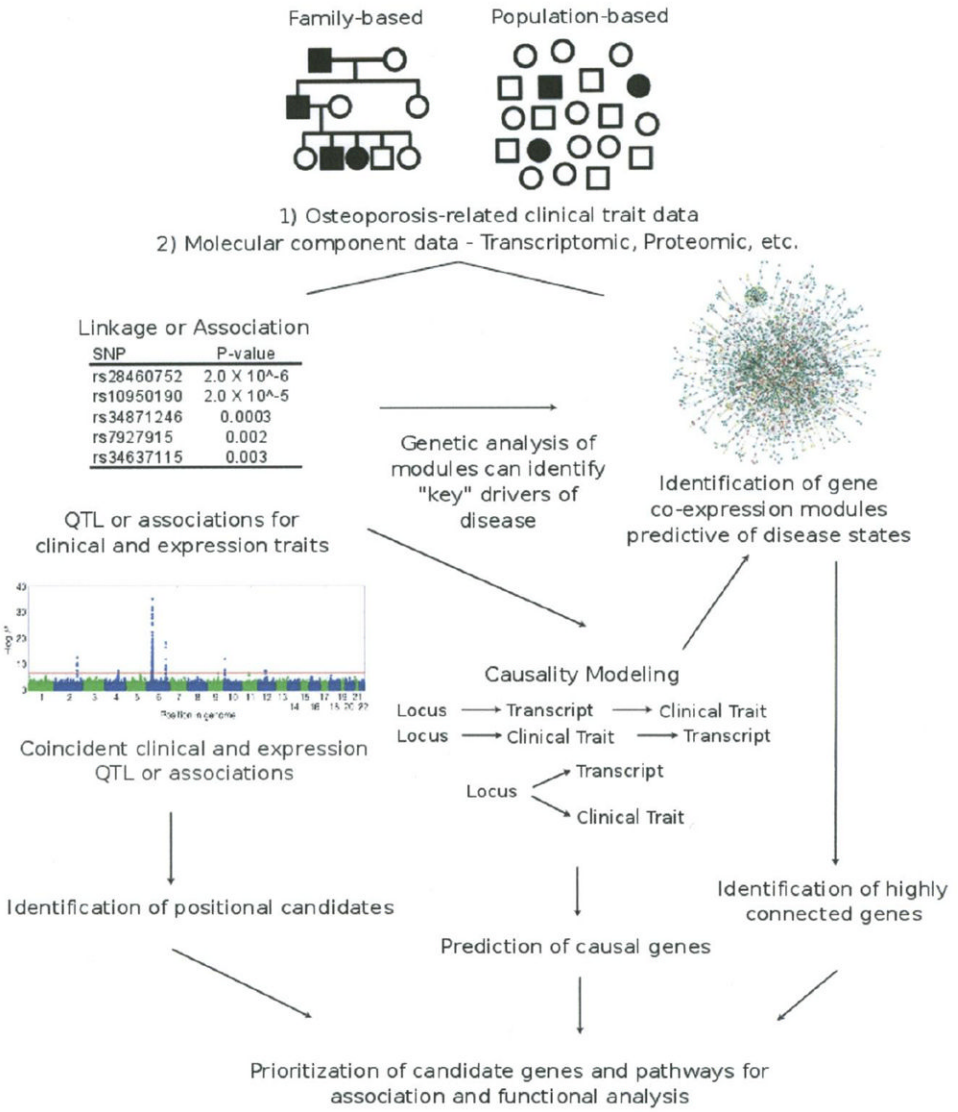


**Figure 1.**

Systems genetics schema for the investigation of complex osteoporosis-related phenotypes. The approach begins by collecting clinical, global gene expression, and genotype data from family- or population-based samples. QTL or association analysis, depending on the population type, can be used to identify correlations between genotype and clinical/gene expression traits. These data can then be used to prioritize genes based on coincidence between gene expression and clinical QTL or associations and causality modeling. Additionally, network data on highly connected genes or genes belonging to a module correlated with a clinical trait can also be used to screen candidates. High priority genes and pathways can be validated in large populations using association analysis and/or in animal models using transgenic mice. QTL—quantitative trait loci; SNP—single nucleotide polymorphism.