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GENETIC CAUSES AND UNDERLYING DISEASE MECHANISMS IN EARLY-ONSET OSTEOPOROSIS

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Genetic causes and underlying disease mechanisms in early-onset osteoporosis
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ABSTRACT

Adult-onset osteoporosis is a disorder that affects a significant proportion of the elderly population worldwide and entails a substantial disease burden for the affected individuals. Childhood-onset osteoporosis is a rare condition often associating with a severe bone disease and recurrent fractures already in early childhood. Both childhood-onset and adult-onset osteoporosis have a large genetic component, but in children the disorder is usually genetically less complex and often caused by a single gene variant. This makes genetic studies a well-suited approach to explore primary osteoporosis in children, which is the focus of this thesis. Genetic studies can also be used to study bone metabolism in healthy children because of the dynamic stage of the skeleton during growth. Studies in children also have the advantage of involving less confounding environmental factors and other co-morbidities than studies in adults.

Our genetic studies had two main goals. First of all, for individuals affected with a severe bone phenotype, a molecular diagnosis is important for several reasons, but particularly for prognostic purposes and for decisions related to treatment strategy. Secondly, the hope is that uncovering genetic regulators of bone metabolism in severely affected children will reveal universal mechanisms that are important also for the adult osteoporosis population. **Paper I** and **Paper II** had a monogenic focus and investigated individuals with childhood-onset osteoporosis or fracture propensity. In **Paper I** we identified two novel disease-causing variants in the *PLS3* (Plastin 3) gene. The findings allowed us to conclude that *PLS3* screening should be recommended in children with primary osteoporosis, especially if vertebral compression fractures are a dominant feature. In **Paper II** we showed for the first time that *PLS3* gene deletions can cause osteoporosis in children. We also found evidence suggesting that *PLS3* has an important role in bone matrix mineralization. **Paper III** and **Paper IV** approached bone health as a polygenic trait. In **Paper III** we explored, for the first time, the polygenic contribution to osteoporosis in children with presumed monogenic bone phenotypes. The study findings suggest that a proportion of the children with severe bone phenotypes and a suspected monogenic etiology for osteoporosis instead may have a polygenic cause underlying the disorder. Finally, in **Paper IV** we show that the genes *GC* and *CYP2R1* are important determinants of the 25(OH)D concentration in 24-month-old healthy children. Using a Mendelian randomization approach, we also provide support for a causal relationship between 25(OH)D and bone strength in these 24-month-old children.

LIST OF SCIENTIFIC PAPERS

I. **Kämpe A**, Costantini A, Mäkitie RE, Jäntti N, Valta H, Mäyränpää M, Kröger H, Pekkinen M, Taylan F, Jiao H, Mäkitie O. *PLS3* sequencing in childhood-onset primary osteoporosis identifies two novel disease-causing variants. Osteoporos Int. 2017;28(10):3023-32.

II. **Kämpe A**, Costantini A, Levy-Shraga Y, Zeitlin L, Roschger P, Taylan F, Lindstrand A, Paschalis EP, Gamsjaeger S, Raas-Rothschild A, Hövel M, Jiao H, Klaushofer K, Grasemann C, Mäkitie O. *PLS3* Deletions Lead to Severe Spinal Osteoporosis and Disturbed Bone Matrix Mineralization. J Bone Miner Res. 2017;32(12):2394-404.

III. Manousaki D, **Kämpe A**, Forgetta V, Makitie RE, Bardai G, Belisle A, Li R, Andersson S, Makitie O, Rauch F, Richards JB. Increased Burden of Common Risk Alleles in Children With a Significant Fracture History. J Bone Miner Res. 2020;35(5):875-82.

IV. **Kämpe A**, Enlund-Cerullo M, Valkama S, Holmlund-Suila E, Rosendahl J, Hauta-Alus H, Pekkinen M, Andersson S, Mäkitie O. Genetic variation in *GC* and *CYP2R1* affects 25-hydroxyvitamin D concentration and skeletal parameters: A genome-wide association study in 24-month-old Finnish children. PLoS Genet. 2019 15;12 e1008530-

ADDITIONAL PUBLICATIONS

I. **Kämpe A**, Makitie RE, Makitie O. New Genetic Forms of Childhood-Onset Primary Osteoporosis. Horm Res Paediatr. 2015;84(6):361-9

II. Makitie RE, **Kämpe A**, Costantini A, Alm JJ, Magnusson P, Makitie O. Biomarkers in *WNT1* and *PLS3* Osteoporosis: Altered Concentrations of DKK1 and FGF23. J Bone Miner Res. 2020;35(5):901-12.

III. Makitie RE, Costantini A, **Kämpe A**, Alm JJ, Makitie O. New Insights Into Monogenic Causes of Osteoporosis. Front Endocrinol (Lausanne). 2019;10.

IV. Costantini A, Krallis P, **Kämpe A**, Karavitakis EM, Taylan F, Makitie O, et al. A novel frameshift deletion in *PLS3* causing severe primary osteoporosis. J Hum Genet. 2018;63(8):923-6.

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VII. Costantini A, Vuorimies I, Makitie R, Mayranpaa MK, Becker J, Pekkinen M, Valta H, Netzer C, **Kämpe A**, et al. *CRTAP* variants in early-onset osteoporosis and recurrent fractures. Am J Med Genet A. 2017;173(3):806-8.

VIII. Makitie RE, **Kämpe A**, Taylan F, Makitie O. Recent Discoveries in Monogenic Disorders of Childhood Bone Fragility. Current osteoporosis reports. 2017;15(4):303-10.

IX. Taylan F, Costantini A, Coles N, Pekkinen M, Heon E, Siklar Z, Berberoğlu M, **Kämpe A**, et al. Spondyloocular Syndrome: Novel Mutations in *XYLT2* Gene and Expansion of the Phenotypic Spectrum. J Bone Miner Res. 2016;31(8):1577-85.

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CONTENTS

LIST OF ABBREVIATIONS

25(OH)D	25-hydroxyvitamin D
ACMG	American Collage of Medical Genetics
Array-CGH	Array comparative genomic hybridization
BMD	Bone mineral density
BMDD	Bone mineralization density distribution
BUA	Broadband ultrasound attenuation
CLSM	Confocal laser scanning microscope
CYP2R1	Vitamin D 25-hydroxylase (gene)
ECM	Extra cellular matrix
eQTL	Expression quantitative trait locus
ExAC	Exome Aggregation Consortium
DNA	Deoxyribonucleic acid
DXA	Dual-energy x-ray absorptiometry
gnomAD	Genome Aggregation Consortium
gSOS	Genetically predicted heel ultrasound derived speed of sound
GC	Vitamin D bindning protein (gene)
GTEx	The Genotype-Tissue Expression project
GWAS	Genome-wide association study
ISCD	The International Society for Clinical Densitometry
LD	Linkage disequilibrium
LRP5	LDL Receptor Related Protein 5 (*LRP5*)
OI	Osteogenesis imperfecta
pQCT	Peripheral quantitative computed tomography
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant

SOS	Speed of sound (heel quantitative ultrasound derived)
SOST	Sclerostin
PLS3	Plastin3
qBEI	Quantitative backscattered electron imaging
QUI	Quantitative ultrasound index
WES	Whole exome sequencing
WGS	Whole genome sequencing

1 INTRODUCTION

1.1 Osteoporosis

1.1.1. What is osteoporosis?

Osteoporosis is defined as a "systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture" [1]. This explanatory, or soft, definition of osteoporosis reflects the true nature of osteoporosis as a metabolic bone disease. The unhealthy bone is weaker than healthy bone and this increases the risk of fractures. Bone fragility in turn is a severe condition that has significant implications both on the individual level and for the society. Osteoporotic fractures, or fragility fractures, of the hip and vertebrae are associated with a high and immediate mortality risk, especially in the elderly population, and lead to great costs for the healthcare system [2]. Fragility fractures of the hip and vertebrae are the most hazardous fractures, but studies have shown increased mortality also for non-vertebral, non-hip fractures. The increase in mortality is the highest during the first year after the fracture, but remains elevated for up to 10 years [2-4]. Osteoporosis and the fragility fractures caused by the disease are thus a major threat to the affected individuals and to public health and a major economic issue for our healthcare system. It is therefore of great importance to better understand the pathogenesis and underlying biology of osteoporosis. This will hopefully enable more precise predictions for an individual's fracture risk and development of new treatment strategies. Improved osteoporosis care will become even more relevant in the future, as fragility fractures are predicted to steadily increase due to population aging [5].

1.1.2 Diagnosis of osteoporosis

Research initiatives to understand the underlying mechanisms and biology of osteoporosis face many difficulties. One fundamental difficulty is the definition of the disease – how to decide which patients should be given the diagnosis of osteoporosis. The most encompassing and true definition of osteoporosis would be a definition that could precisely describe the metabolic disturbance in the bone tissue that eventually leads to the clinically most significant

event, the fracture. However, like many other diseases, the clinical definition of a condition needs to be standardized, non-arbitrary and not too difficult to measure. Because of that, a proxy measurement is often used to make the diagnosis, and for osteoporosis that proxy measurement is bone mineral density (BMD). For the last 30 years osteoporosis has been defined as a dual-energy x-ray absorptiometry (DXA) derived BMD (measured at the hip) of equal or more than -2.5 standard deviations below the average BMD in young white women [6]. In practice however, the -2.5 SD cutoff is more widely used, and is not always confined to hip DXA measurements or the original reference population. This diagnostic criterion of a DXA-derived BMD $\leq$ -2.5 SD for osteoporosis was established by the WHO study group in 1992 because it was viewed as the best indicator for the disease [7]. However, it is likely that the cutoff of a BMD $\leq$ -2.5 SD was chosen semi-arbitrarily.

Although widely used, this definition has also been criticized for its lack of specificity when it comes to discriminating patients with true osteoporosis and increased risk of fractures from individuals without osteoporosis or increased fracture risk [8]. Further, BMD displays a normal distribution in the population, and no "hump" in the curve can be observed for individuals in the lower BMD ranges, suggesting that BMD does not capture or separate any specific subgroup of individuals with a specific condition [8]. Although BMD is arguably still the best single measurement for estimation of bone strength and prediction of fracture risk, an individual's age supersedes BMD by far as a fracture risk predictor [9, 10]. Taken together BMD should be considered a relevant, but somewhat blunt, measurement to identify individuals with a metabolic bone disease and an increased fracture risk. Individuals with a diagnosis of osteoporosis thus represent a very heterogenous group with various underlying causes for low BMD. Furthermore, although the risk of fractures is higher in individuals with very low BMD, the majority of fractures occur in the group which the WHO definition describes as having 'osteopenia', or a BMD above -2.5 SD, but below -1.0 SD [11, 12].

1.1.3 Multifactorial etiology of low BMD

Normally peak bone mass is reached early in adulthood when growth ceases. An individual's bone mass then slowly starts to decline with age, and in women this decline is especially fast after menopause [13]. A low bone mass, resulting in a diagnosis of osteopenia or osteoporosis, can thus be due to either an accelerated bone loss later in life or a decelerated bone growth earlier in life. Both scenarios can lead to a diagnosis of osteoporosis, but the underlying mechanisms for the disease will likely be different.

Various factors affect bone mass accrual and skeletal health during childhood. Especially for children living in the northern latitudes, such as in Sweden or Finland, vitamin D is an important factor. Severe vitamin D deficiency can lead to poorly mineralized bone, a disorder called rickets in children and osteomalacia in adults [14, 15]. A Finnish study showed that vitamin D concentration is a major determinant for BMD in adolescents [16]. Several other factors can also affect bone mass accrual or maintenance, including a variety of underlying medical conditions, medications, ethnicity, physical activity and other life-style factors (for example smoking and diet) [6]. Since there are so many different reasons for low bone mass, there are most probably also several different biological and cellular mechanisms leading to a low BMD. This lack of homogeneity in patients with a DXA-derived diagnosis of osteoporosis constitutes a major challenge for the research field. Because scientific conclusions often are based on group comparisons, it is imperative that the biological (not only measured) phenotype of the individuals in each group used for comparisons is correct, meaningful and also somewhat similar. Unfortunately, BMD only captures part of the osteoporosis phenotype and does not separate the mechanisms leading to low bone mass.

1.1.4 Pediatric osteoporosis

Early-onset/childhood-onset osteoporosis is a special entity within osteoporosis. The term childhood-onset osteoporosis describes fragile bone with a propensity to fracture in childhood. The most recent (2019) Official Position by The International Society for Clinical Densitometry (ISCD) defines pediatric osteoporosis as: A) a BMD Z-score < -2.0 AND B) a clinically significant fracture history defined as 1) two or more long bone fractures by age 10 years OR 2) three or more long bone fractures at any age up to age 19 years. The fracture criterion (B) is important because BMD in children is largely affected by the child's bone size / height and pubertal development [17]. Because children grow and develop in different rates it can be difficult to compare children's BMD, even if they are the same age. Therefore, the diagnosis of osteoporosis also requires that the child has an abnormal fracture history. An exception is vertebral compression fractures, which are considered more powerful in predicting osteoporosis in children [18]. The diagnosis of osteoporosis in children can therefore be made if vertebral fractures are present even if BMD is normal. The requirement of a clinically significant fracture history is a necessity in children for biological reasons, but it also adds a functional component to the diagnosis. This functional criterion, which is lacking for adults, might lead to better identification of children with 'true' osteoporosis.

1.2 Genetics of osteoporosis

1.2.1 Genetics underlying osteoporosis in children

The distinction between primary and secondary osteoporosis is important, especially when the diagnosis of osteoporosis is considered in a child. Primary osteoporosis in children is a term used to emphasize that the bone condition is primary, or inherent, and cannot be explained by any other underlying disease, medication or other external factors. In contrast, in secondary osteoporosis exogeneous factors lead to osteoporosis [18, 19]. Primary osteoporosis in children is a rare and often severe disease. It often has a more direct genetic component with familial clustering, and in many cases a single gene variant can explain the full phenotype seen in the child [20]. The Mendelian, or monogenic, nature of primary osteoporosis in children opens up the possibility to conduct family studies, in which heterogeneity of a disorder is no longer a problem. Family studies also have a great advantage in the aspect that they are not bound by the strict definitions of a disease. Phenotyping of a family can be much more specific and detailed than what the standard definition of childhood-onset osteoporosis (or adult-onset osteoporosis) can capture. In family studies, experienced clinicians thus have the possibility to identify and delineate rare bone conditions in a specific and detailed manner. Uncovering the underlying genetics in these rare families can reveal novel genetic mechanisms directly involved in bone metabolism. Since some families have a very rare or perhaps even private bone condition, genetic causes underlying such disorders cannot be identified in cohort studies, where the methodology is based on comparisons between groups. In such group comparisons, these rare causes and disease mechanisms become too diluted and will escape detection. Using a family-based approach for genetic studies therefore has the possibility to uncover mechanisms that cannot be detected when studying larger patient cohorts of unrelated individuals.

1.2.2 Monogenic osteoporosis in children

The largest subgroup of children with primary osteoporosis will get the diagnosis Osteogenesis imperfecta (OI) [19, 21] OI is originally a clinical diagnosis and defined as a connective tissue disorder that is primarily characterized by susceptibility to fractures throughout life [22, 23]. This wide definition of OI will include a fairly large and very heterogenous group of patients and distinction from other forms of primary osteoporosis is

often challenging. However, in practice, the diagnosis of OI is most often confined to individuals with a clinically more severe bone fragility phenotype associated with typical extra-skeletal features such as blue sclerae and where a monogenic cause almost always can be confirmed [24, 25]. Yet, even if using a narrow definition of OI and only considering patients with a known monogenic cause, the group as a whole is still very heterogenous. The osteogenesis imperfecta database [26], contained within the Leiden open variation database, today reports pathogenic variants in 20 genes as the underlying cause of OI. However, not all genetic forms of primary osteoporosis are included [22, 27, 28].

1.2.3 Classification of Osteogenesis imperfecta

The classification of Osteogenesis imperfecta has traditionally been based on the Sillence classification from 1979. In the Sillence classification patients with OI were divided into 4 subgroups (OI I-IV), with the addition of a fifth subgroup in 2000 (OI V with very specific radiologically findings) [23, 29]. The Sillence classification is a clinical classification that relies on the phenotypic presentation and severity, radiological findings and inheritance pattern of the disorder [22]. The traditional classification is preferred because with discovery of more and more genes underlying OI, it has become a practice to add a new subtype of OI to the classification for each new gene discovery. This newer type of classification, which first seemed logical, has evolved on its own and has become confusing. Today OMIM reports 20 different OI types (OI I-XX), but this long list of different subtypes has only little clinical relevance. When the traditional OI classification was developed in 1979 the underlying genetic causes of OI were not known, but it had already been shown that defects in collagen production and collagen crosslinking were involved in the disorder [30, 31]. Today we know that OI is predominantly a type I collagen related disorder [24]. Pathogenic variants (including both single nucleotide variants (SNVs) and structural variations (SVs)) in the genes *COL1A1* and *COL1A2* explain the great majority (about 90%) of OI cases [32]. It has also been suggested that the diagnosis of OI should be used only for bone disorders caused by type I collagen defects, but presently no clear consensus exists [22, 33].

1.2.4 Osteogenesis imperfecta and type I collagen

COL1A1 and *COL1A2* are the genes that produce the pro-α1 and pro-α2 chains that after post translational modifications, cleavage and added stabilizing crosslinks will come to form

mature collagen type I, the most abundant protein in bone [34]. Mature collagen type I consists of two α1-chains and one α2-chain that assemble into a triple helix, many triple helices are then crosslinked together to form fibrils, which in turn can bundle into thicker fibers [35]. The collagen fibrils in bone are often compared to iron bars used to reinforce concrete, but they also make up the scaffold in the extra cellular matrix (ECM) that helps the bone to mineralize. Collagen fibrils in the ECM take active part in the mineralization process, both in the nucleation and in the orientation of hydroxyapatite crystals that eventually will make up the largest proportion of any component in bone [36]. Because collagen has such an important role in bone, providing strength and elasticity to the mature bone and actively taking part in its formation, it is understandable that genetic aberrations affecting the two genes encoding type I collagen can cause bone disorders. However, since mature collagen is the result of a very complex post-translational processing, also pathogenetic variants in genes involved in the maturation steps of type I collagen can give rise to type I collagen related OI. The majority of the 20 genes underlying OI are known to affect collagen, either in a direct or indirect fashion. Yet, there are OI cases that are due to pathogenic variants in genes not related to collagen production or processing, and instead other pathways are affected. This has led to the suggestion that the OI classification should rely on the underlying metabolic pathway that is compromised in each specific case. In a fairly recent review article, *Forlino et al*. suggested to divide OI forms into five functional groups (A-E) [Table 1] to emphasize the biological aspect of each different sub-type [24]. This type of newer classification system, which utilizes recent advances in understanding the molecular cause of a genetic disorder and places each form into a biological context depending on which pathway is affected, is much more informative than classifications that solely rely on clinical or genetic features. Such a classification will probably allow clinicians to give more accurate estimates of prognosis and enable identification of patient groups that might respond similarly to treatment strategies. This type of classification would also circumvent some of the inconsistencies seen in current classifications, where pathogenic mutations in *WNT1* are classified as OI, while pathogenic variants in *LRP5* causing primary osteoporosis are not [28, 37]. *WNT1* and *LRP5* does, however, affect the same signaling-pathway and should mechanistically be grouped together [38, 39].

Table 1. Proposed functional classification of Osteogenesis imperfecta

Gene symbol	Severity	Inheritance
Defects in collagen synthesis, structure, or processing (group A)		
COL1A1	mild to severe	AD
COL1A2	mild to severe	AD
BMP1	mild to severe	AR
Defects in collagen modification (group B)		
CRTAP	severe rhizomelia	AR
LEPRE1/*P3H1*	severe rhizomelia	AR
PPIB	severe	AR
TMEM38B	severe	AR
Defects in collagen folding and cross-linking (group C)		
SERPINH1	severe	AR
FKBP10	mild to severe	AR
PLOD2	moderate to severe	AR
Defects in bone mineralization (group D)		
IFITM5	mild to severe	AD
SERPINF1	moderate to severe	AR
*PLS3**	mild to moderate	XL
Defects in osteoblast development with collagen insufficiency (group E)		
SP7	severe	AR
WNT1	moderate-severe	AD/AR
CREB3L1	severe	AR

**PLS3* was not included in the original classification, but has in this thesis been placed in group D. [Autosomal dominant (AD), Autosomal recessive (AR), X-linked (XL)].

1.3 Approaches to decipher underlying mechanisms in osteoporosis

1.3.1 Different angles to approach bone biology in research

The higher purpose for all medical research focusing on human disorders is to enhance understanding of the underlying biology. Increasing knowledge of a disorder facilitates improvement of clinical management and treatment strategies, and thus helps the affected patient. Also, the hope is often (or always) to provide tools and ideas for the development of new pharmaceutical drugs to further improve the affected patients' health, or in a best-case scenario, to cure the disorder. What constitutes a disease or disorder can however be debated – especially when it comes to a disorder like osteoporosis. It can be questioned whether a low BMD in a 90-year-old individual can be considered a disorder or just normal aging. In many ways, these questions may not be important. Instead the important question is whether a treatment (or similar) would benefit a person with a specific condition, regardless of whether that condition is normal or not. This means that the full breadth of the disease – from defective bone accrual in children to age-related bone loss in the elderly – needs to be investigated further. The quest to uncover the underlying bone biology can be approached from different angles, where each angle will have its own pros and cons.

1.3.2 The genetic approach

One way to tackle the important questions in bone biology is to use genetics as the angle of attack. To improve clarity and to minimize the effect of confounding environmental factors and co-morbidities frequently present in adult or elderly cases, it can be advantageous to study osteoporosis in children. In children, the bone tissue is also in a much more critical and dynamic stage than in adults, because during growth a large quantity of bone needs to be accumulated in a short period of time. In such a situation, a defect in bone metabolism might be more visible, and findings might have more direct relevance for the actual trait (bone) under scrutiny. To study a disorder like osteoporosis in an elderly population is more complicated because it may be difficult to understand which factors possibly can confound the results. Also factors that seem to have a true causal effect on adult osteoporosis can just as well be the result of reversed causality. The interpretation of study results can therefore be difficult, even if methods do exist to address these issues [40].

1.3.3 Rare phenotype approach

As discussed above, disorders with a childhood onset are often less multifactorial than disorders with an adult onset. From a genetic perspective this means that undertaking genetic studies on individuals/families with a rare and severe phenotype that presents already in childhood will have a greater chance for success than studying less clear phenotypes where environmental factors are likely to explain a larger proportion of the cases. This is best done using carefully selected families where the inheritance pattern suggests, or confirms, that the disorder is inherited. The rarity of the phenotype (in this thesis primary osteoporosis in children) is not important in itself and is most often just a consequence of the severity of the disorder. The fact that the disorder is severe does, however, imply that a central mechanism is affected and can, if identified, confidently be assumed to be of direct importance. This type of approach has been proven successful in a variety of human disorders [41], but also in the field of bone metabolism. In 2001 several different research groups identified the cause of (1) Osteoporosis-pseudoglioma syndrome (OPPG), a low bone mass syndrome with extra-skeletal features and (2) Sclerosteosis, a high bone mass disorder [42-44]. Biallelic pathogenetic variants in the gene LDL Receptor Related Protein 5 (*LRP5*) were found to cause OPPG, and one year later in 2002, a pathogenic variant in a specific domain of the *LRP5* gene was shown to cause an autosomal dominant high bone mass disorder [45]. For Sclerosteosis biallelic null-variants in the gene sclerostin (*SOST*) were discovered to be the cause of the high bone mass syndrome and in 2002 a homozygous large deletion affecting the regulation of *SOST* was shown to cause the phenotypically similar disorder Van Buchem disease [46]. These genetic discoveries, made possible by genetic studies in families with rare phenotypic presentations, led to the understanding that WNT signaling is one of the most important pathways for bone tissue homeostasis [39]. A promising new osteoporosis drug, an anti-sclerostin antibody, that blocks the WNT-signaling inhibitor sclerostin was also developed as a result of the studies in these families [47]. After its discovery, anti-sclerostin antibody treatment was anticipated to become a powerful tool in osteoporosis care. Treatment trials have shown convincing results [47], but suspected adverse cardiovascular events have limited the popularity first foreseen [48]. However, despite this drawback, the story of how WNT signaling was found to be one of the most import signaling pathways in bone elegantly shows the full strength of a family-based rare phenotype approach. The benefits are twofold, because the results were important both for the individuals affected by these rare disorders and for the general population. The individuals studied received a molecular diagnosis and much knowledge about their condition was gained. The results were also generally applicable, because the genetic findings revealed a universally important pathway for bone

homeostasis, and manipulation of this pathway could in the future help patients with a variety of low bone mass disorders. The story thus displays that one of the basic ideas of the rare phenotype approach can work – that severely affected individuals with suspected (mono)genic disorders will have aberrations in fundamental mechanisms or pathways for the affected tissue(s). New pharmaceutical drugs developed to manipulate these fundamental mechanisms or pathways can in turn provide a treatment option also for other more common, but similar, disorders.

1.3.4 Approaching the polygenicity of osteoporosis

Adult-onset osteoporosis is a complex trait with a large heritable component [49, 50]. This statement is most certainly true for a subgroup of children with primary osteoporosis as well, but because of the rarity of the disorder, the polygenetic contribution is more difficult to study. To decipher the underlying polygenetic component of osteoporosis, one approach (as already discussed) is to study rare phenotypes and build the puzzle one piece at a time. However, not all results from studies of monogenic osteoporosis can be extrapolated to the more common form of complex osteoporosis [51]. Instead, another approach is to directly study the polygenic structure of a trait using a genome-wide association study (GWAS) approach. The basic principle of a GWAS relies on the idea of comparing genotype frequencies between or within groups. To understand the concept of a GWAS, the case-control study using a dichotomous trait (osteoporosis vs not having osteoporosis) is intuitively easiest to understand, but a GWAS often performs better if the studied trait is quantitative [52]. In a basic case-control GWAS common biallelic single nucleotide variants/polymorphisms (often referred to as SNVs/SNPs) are genotyped at certain intervals throughout the genome while taking linkage disequilibrium (LD) structure into account. The genotypes for each location in the genome are then compared between individuals with the trait (osteoporosis) and individuals without the trait (not having osteoporosis). If the genotypic distribution at a certain genomic position differs between the cases and controls, and the observed difference cannot statistically be explained by coincidence, then the SNP at that certain genomic position can be regarded as being associated with the trait (osteoporosis). The results cannot tell if the associated SNP has an effect in itself on the studied trait, but it can tell that the cause for the association should exist somewhere in the genomic vicinity of that SNP (i.e. the genomic region in LD with that SNP). A GWAS can therefore pinpoint genomic regions of importance for a trait, even though it cannot identify the actual genetic cause behind the association signal. The hope is that the underlying genetic cause behind the

association signal is directly linked with the trait's biology, here osteoporosis, meaning something that would have a direct biological impact on bone metabolism. However, the genetic cause for an association signal in a GWAS does not necessarily have a direct impact on the trait being studied. If we consider a situation where a genetic variant increases an individual's risk to become addicted to smoking, and smoking in turn affects another trait (for example cardiovascular disease), then the same variant could give rise to an association signal in a cardiovascular GWAS. In this example the variant does increase an individual's risk for cardiovascular disease, but only if that individual actually starts to smoke, otherwise it does not. Although GWASes lack the ability to identify underlying causative genetic aberrations, GWASes have been a success story from the beginning, also for the trait osteoporosis [51, 53, 54].

1.3.5 GWAS - Strength and weakness

The major caveat with a GWAS design is that of power. GWASes suffer from power problems mainly because of two reasons: 1) The target traits in GWASes are polygenic in nature, but the study design essentially only allows separate assessment of each variant. Because of that, the study needs to be powered to find variants with a very small effect size. 2) In a GWAS millions of variants are separately assessed, or at least semi-separately assessed (depending on LD structure), meaning that adjustment for multiple testing will have a major impact. However, large international collaborations and consortiums have been developed through the years to overcome these power problems and GWASes nowadays can include even up to a million individuals [55-58]. In 2012 *Estrada et al.* performed a large meta-analysis on 17 GWASes including more than 30,000 individuals, which at that time was the largest DXA-derived GWAS performed. The study was able to identify 56 BMD-associated loci, explaining around 5% of the total variance in BMD [49]. In 2017, utilizing the UK biobank [56], a GWAS on estimated BMD (eBMD) derived from quantitative ultrasound of the heel including 142,000 individuals was able to identify 203 genetic loci associated with eBMD; these together explained 12% of the variance seen in the trait. More recently an even larger GWAS on eBMD that included >400,000 individuals identified 518 genetic loci that together explained 20% of the variance [57]. This illustrates a fantastic progress in the field of bone genetics and the studies have provided vast new insight into bone biology. However, all GWAS findings need to be interpreted in relation to the trait being studied. Estimated BMD derived from quantitative ultrasound of the heel is a cost-effective alternative for DXA-derived BMD that enables the collection of very large cohorts,

which is important in association studies. eBMD is independently associated with fractures, suggesting that it is a meaningful bone parameter to measure [59]. On the other hand, it is known that eBMD is not a very precise predictor of DXA-derived BMD, which in turn is quite a blunt measurement for 'true' osteoporosis [8, 60]. It can therefore be wise to interpret GWAS-derived genetic findings from eBMD keeping that in mind, because it is not certain that they fully reflect the biology underlying the disease osteoporosis.

1.3.6 Monogenic vs polygenic approaches

Comparing the rare phenotype approach (monogenic approach) to the GWAS approach (polygenic approach) in a simplified manner, the rare phenotype approach can identify rare variants with large effect sizes while GWASes are able to identify common variants with small effect sizes. Although recent GWASes, by leveraging WGS-data, are closing the gap and have now been able to identify a rarer variants with larger effect sizes [61, 62]. However, there is reason to believe that the rare phenotype approach more precisely targets what was intended. Pharmaceutical companies have for a long time tried to improve means to predict which new drugs will survive the clinical testing stage and eventually come out on the market. For pharmaceutical companies this is a pressing issue because of the high costs of drug development. A systematic review recently showed that if there are (human) genetic evidence that a new drug targets a biologically meaningful mechanism, the chances for that drug to come out on the market are better. The study showed that if the evidence is based on findings from GWASes, the evidence is good, but if it is based on findings from studies on monogenic disorders, that evidence is better [63].

1.4 Heritability of osteoporosis and fractures

1.4.1 Heritable traits are suited for genetic studies

The heritability of a trait tells about how large the inherited (i.e genetic) component of a trait is. Traits like the disease Phenylketonuria (PKU), which is monogenic in nature, have an extremely large heritability that approaches 100%. In contrast, an individual's tea or coffee consumption has a low heritability and probably is the result of environmental influence [64]. This also means that if one was to study the biology of a trait using genetics as an angle of attack, the approach would be more successful in PKU than in tea drinking preference. The heritability of a trait will also give a hint of how much more unknown genetic information there is to discover beyond to what is already known.

1.4.2 Heritability of osteoporosis

Because the definition of osteoporosis in adults relies on BMD, and because BMD can be relatively easily and precisely measured, it is widely used in research. Genetic studies on the disease osteoporosis can therefore better be described as genetic studies on BMD. To estimate the heritability of a trait (here BMD), twin studies are often used. Comparing monozygotic twins with dizygotic twins for a specific trait or disease is an approach that aims to omit environmental factors from the equation and indirectly estimate the genetic component of the studied trait. Twin studies can estimate heritability without needing to identify the specific genetics underlying the trait. Twin studies focusing on bone have shown that bone parameters are highly heritable, especially BMD, where estimates as high as 85% have been proposed [50]. Twin studies are a good and relatively simple approach to circumvent unknown confounding factors that might obscure heritability estimates, although suspicions are strong that twin-studies overestimate the heritability [65]. Family based studies on extended pedigrees using data from the Genetic Analysis of Osteoporosis Project have resulted in lower heritability estimates in BMD (up to 50%) [66].

1.4.3 LD score regression, and unbiased approach to estimate heritability

Another approach to estimate the heritability of a trait is called LD score regression. LD score regression can estimate the SNP-heritably, which is the sum of the genetic effect that can be attributed to single nucleotide variants/polymorphisms. When performing an association analysis, it is unlikely that the causal genetic variant underlying the association signal actually is included in the dataset (either directly genotyped by the applied array or imputed). Instead the associations between the genetic markers and the trait arise because some genotyped SNPs will be in close proximity (genomic distance) to the causal genetic variant (that actually causes the association signal). Variants in close genomic proximity will most likely be inherited together, because they are unlikely to be separated by meiotic recombination and can thus be used as proxies for each other. SNPs that are inherited together are said to be in LD, and can be said to belong to the same haplotype. Haplotype estimation is also the basis for imputation, where the genotype of a SNP can be inferred even though it has not been directly genotyped by the array used [67]. LD score regression is based on the fact that the average association strength of a genetic variant (most often a SNP) to a trait will be dependent on the size of the genomic region that the genetic variant tags (i.e is in LD with). Because SNPs that tag a larger genomic region will have a greater chance to also tag a causative variant underlying an association signal, those SNPs will on average also have a stronger association to the trait. The greater the average difference in association strength between SNPs that tag a large genetic region and SNPs that tag a small genetic region, the greater is the heritability of the trait. Looking at LD regression score estimates, the heritability of BMD is around 30% for lumbar and femoral neck BMD, as well as for ultrasound estimated heel BMD [68, 69]. LD score regression has the advantage that it is not affected by population stratification, but does underestimate the true heritability because it only accounts for the heritability explained by the sum of commonly occurring SNPs, not all genetic factors [70]. However, even if it is not possible to precisely estimate the true influence genetics has on bone metabolism, it can be confidently said that BMD, and therefore by definition osteoporosis, is a highly heritable trait. This is an important conclusion, because this means that performing genetic studies on bone disorders or bone parameters is a good and meaningful approach to decipher bone metabolism and discover underlying molecular mechanism important for bone homeostasis. The SNP heritability from LD-score regression also gives a quantified estimate of how well a GWAS can perform.

1.4.4 The etiology of fractures is more complex

What about the genetics underlying fractures? It is important to understand if genetic studies on BMD or osteoporosis can lead to new insights to the clinically most important feature of osteoporosis, the osteoporotic fracture. Some even argue that osteoporosis should not be seen as a disease at all, instead it should merely be regarded as a risk factor for the real disease – the fracture [71]. However, only a very narrow view of what constitutes osteoporosis can make such an argument viable. Nonetheless, the fracture is what causes the greatest cost for the society, and the greatest morbidity and mortality for the affected individuals [6, 72]. A large Swedish twin study investigated the fracture heritability in almost 25000 twins, of which 6021 had sustained a fracture. The study concluded that fractures have a substantial genetic component, and this component explains on average around 20% of the variance in fracture rates. However, the heritability was strongly influenced by fracture site and age at fracture. The highest heritability estimate was for hip-fractures before the age of 69, where around 2/3 of all fractures could be explained by genetic liability. However, in individuals ≥80 years of age the genetic composition did not seem to have a strong effect [73]. In a twin study conducted in Finland the authors concluded that they found little evidence for genetic influence on the risk of osteoporotic fractures, however, the study did only include a total of 786 fractures [74]. SNP heritability calculated from LD score regression also suggests that fractures have a less strong genetic component than bone parameters [**Fig. 1**].

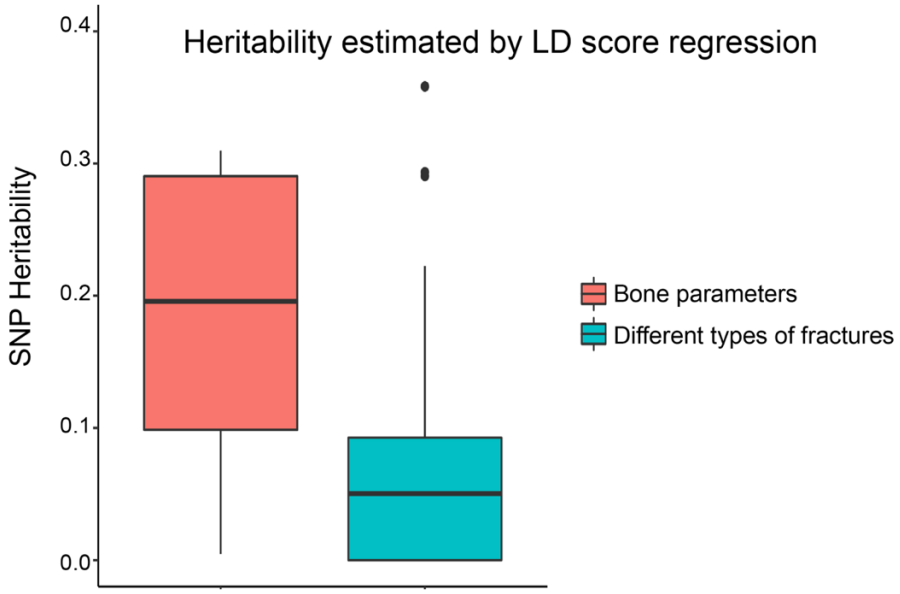

Fig. 1. SNP heritability estimated from LD score regression. The average SNP heritability of bone parameters, such as BMD, is fairly high while the SNP heritability of fractures is lower and more dependent on the specific fracture trait. (Data from http://ldsc.broadinstitute.org/ and https://nealelab.github.io/UKBB_ldsc/.)

A more recent study looking at >3000 individuals from the Framingham Heart Study was able to estimate the heritability of vertebral compression fractures to somewhere in the range of 43-69%, suggesting that the risk for vertebral compression fractures has a larger genetic component [75]. These results are interesting, because as discussed earlier, vertebral compression fractures are a good indicator of osteoporosis, and could possibly identify a specific sub-group of osteoporosis individuals especially suited for genetic studies.

1.5 Genetic correlation between traits

1.5.1 How much does proxy variables like BMD tell us?

Can genetic studies on bone parameters tell us anything about fracture risk? It is evident, that a fracture is the most important feature of osteoporosis in both adults and children. We also know that osteoporosis increases the risk of fractures, simply because fractures are encompassed in the (broader) definition of osteoporosis for both children and adults [1]. Deciphering the underlying mechanisms in osteoporosis should therefore also reveal underlying mechanisms for fractures. However, since BMD is the basis for the diagnosis of osteoporosis, the question is whether deciphering the underlying mechanisms for BMD can help to decipher the underlying mechanisms for fractures? A recent article using the FRISBEE cohort (Fracture Risk Brussels Epidemiological Enquiry), consisting of 3560 post-menopausal women, investigated the risk of fractures in relation to BMD and other clinical risk factors. The authors could conclude that the best independent predictors of a future fracture were a diagnosis of osteoporosis, BMD (measured at several sites) and previous fractures [76]. Also, *Kanis et al.* performed a meta-analysis of 9 different population-based studies and the authors could clearly show that BMD, as a single predictor, was superior for fracture prediction compared to other clinical risk factors [77]. However, even if BMD is a better predictor for fractures than other risk factors or measurements, this does not mean that BMD fully explains why fractures occur. The above-mentioned study from *Kanis et al.* shows that their model for fracture prediction improves when BMD is combined with other clinically relevant risk factors. Professor Kanis is also the father of FRAX, the most widely used tool in clinical practice for fracture risk assessment [78]. The FRAX algorithm predicts fractures better than BMD alone, and it does this by using 11 different variables in addition to BMD. Although the FRAX algorithm has been criticized, it clearly shows that fracture models can be improved by adding additional information beyond BMD [79]. The fracture rate in the elderly population is especially high in the Scandinavian countries, particularly in Sweden. In fact, Sweden has the highest rate of osteoporotic fractures in the world. *Eklund et al*. performed a study in a large Swedish cohort, mainly consisting of at-risk individuals, to investigate this phenomenon [80]. The authors showed that despite the high fracture prevalence in the Swedish cohort, the Swedish participants did not have a lower BMD than at-risk individuals belonging to similar cohorts from other countries where the fracture rates are lower. The results highlight that BMD, although informative, cannot alone explain all

fractures. But taken together there is evidence that a proportion of the underlying mechanisms for BMD and fractures could be the same.

1.5.2 Shared genetics between BMD and fractures

Despite the acknowledged shortcomings, BMD is still a good indicator of bone health, and insights into the biology of BMD can provide useful information on bone metabolism and mechanisms leading to bone fragility and fractures. We know that BMD has a high heritability, and although the heritability seen in fractures is lower, fractures still have a substantial genetic component. As discussed above, there is reason to believe that some of the underlying mechanisms for BMD and fractures are shared. From a genetic perspective the interesting question is whether also the genetic mechanisms underlying BMD variability and fracture risk are shared, i.e. can the results from genetic studies on BMD be extrapolated to fractures. Benjamin Neale and his lab (http://www.nealelab.is) have done extensive work on calculating heritability of different traits using LD score regression from UK biobank data [69, 70]. However, LD score regression can also be used to assess genetic correlation (rg) between traits, a measurement of how similar the underlying genetics of two different traits are [81]. In short, genetic correlation can be estimated by quantifying the similarity of the association signals from two different GWASes that focus on separate traits. Identifying genetic correlations between traits can thus be used to mechanistically group traits together and help to shed light on relationships between traits that otherwise could be hard to foresee. Genetic correlations can also help to understand if results from genetic studies on proxy measurements such as BMD can be used to gain information on traits like fractures. The UKBB browser (https://ukbb-rg.hail.is) provides publicly accessible results from genetic correlations between hundreds of different traits within the UK biobank. Data from the UKBB browser is presented in **Fig. 2** to visualize how BMD and fractures are genetically correlated, both to each other as well as to other traits. Specifically, the BMD trait in **Fig. 2** is 'ultrasound derived heel BMD' and the fracture trait is defined as 'fractures within the past 5 years'. Because of the large number of traits being compared (≈700), the results have been adjusted for multiple testing and only genetic correlations with a false discovery rate (FDR) ≤0.05 are shown. Of all traits tested, ultrasound derived heel BMD correlates best with the diagnosis of osteoporosis (DXA-derived diagnosis), which was expected. However, more interesting is that it also genetically correlates quite well with fractures, indicating that ultrasound-derived heel BMD can be a good proxy measurement for genetic bone studies. **Fig. 2** also visualizes that the genetic structure underlying fractures is very complex.

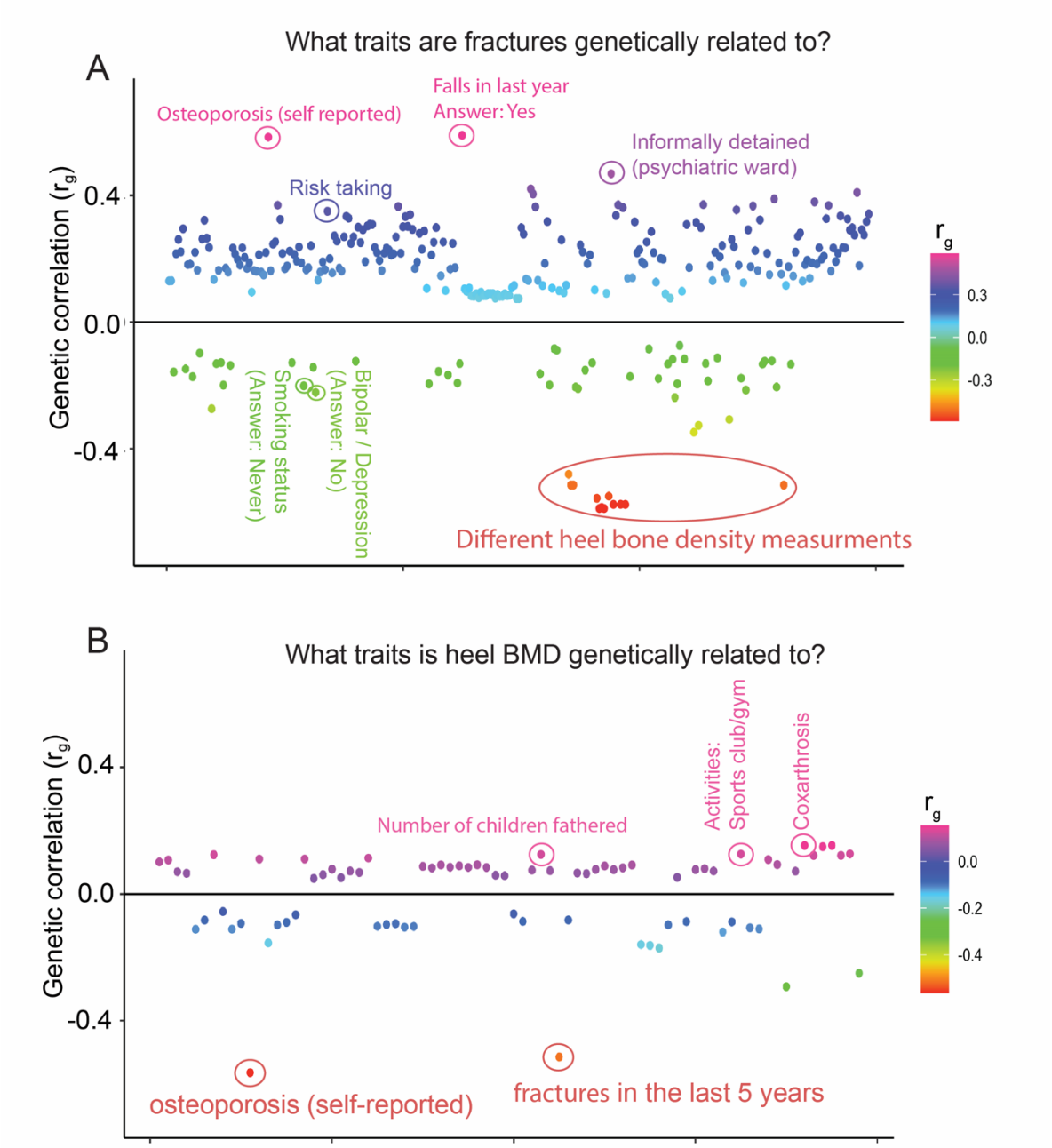

Fig.2. Genetic correlation. The genetic correlation, representing shared underlying genetics, between fractures and BMD is large, suggesting that genetic results from studies on BMD can be extrapolated to fracture genetics. However, it is clear that the genetic component underlying fractures is more complex than for BMD. **A)** Significant genetic correlations between fractures and 297 different traits. Fractures are here defined as 'fractures within the past 5 years'. **B)** Significant genetic correlations between BMD and 78 different traits. BMD is here defined as 'quantitative ultrasound-derived BMD'. Data have been downloaded from the UKBB browser (https://ukbb-rg.hail.is).

Although fractures are genetically highly correlated to different types of heel BMD measurements, fractures also show shared genetics with 'mental illness', 'risk taking', 'smoking' and 'risk of falls'. It is important to understand that genetic correlation results do not necessarily allow for a straight forward interpretation. As an example, the genetic

correlation observed between 'risk of falls' and 'fractures' could be the result of a genetic variant giving rise to a neurological disease that impairs balance, which then in turn leads to both falls and fractures. Thus, shared genetics does not always mean that a close relationship exists between the traits A and B. Instead a genetic variant increasing the risk for trait C, where trait A and trait B are consequences of trait C, could actually be the best explanation for the results seen.

The results from the genetic correlation analysis provide support for heel BMD measurements as useful and meaningful parameters in genetic studies focused on osteoporosis and/or fractures. However, it cannot be assumed that all genetic findings from heel BMD measurements will be biologically relevant for osteoporosis. Even less information can be expected to be gained for fractures, also because the fracture is a less heritable trait. Due to the reasons above, there is a limit for how much information a proxy measurement can yield.

1.5.3 Other proxy measurements

So far, I have been somewhat critical to the use of proxy measurements. However, this is merely to point out that there are limitations in using such an approach, but if these limitations are recognized, using proxy measurements is an easy, good and valid approach. Using a more targeted proxy measurement to investigative bone metabolism, for example serum calcium level, vitamin D status or PTH dynamics, can also reveal new insights. The use of such narrow proxy measurements could be compared to using a monogenic approach in the sense that perhaps only one, or at best a few, underlying mechanisms can be anticipated to be revealed by the study. In analogy with the monogenic approach, such studies would help to build the puzzle one piece at a time. If 25-hydroxyvitamin D (25(OH)D) can be considered a narrow proxy measurement, BMD would be considered a broad measurement, but as long as there is a good reason to believe that the chosen study measurement is biologically related to the trait of interest (bone metabolism), new insights can be gained. In this thesis we have directed special attention, in addition to BMD and fractures, also to 25(OH)D, a substance that has been implicated in various health outcomes and continues to be extensively studied [82]. The biological link between vitamin D and bone is clear, but many of the positive skeletal effects seen for vitamin D have been hard to replicate [83-85].

2 AIM OF THESIS

As the title of the thesis "Genetic causes and underlying disease mechanisms in early-onset osteoporosis" implies, the overall aim of our studies was to better understand the biology of bone metabolism by using a genetic approach.

The specific aims were:

1) To identify novel genetic causes of early-onset osteoporosis in affected individuals, families or cohorts.
2) To link the genetic findings to underlying biological mechanisms in order to expand the knowledge on how bone is formed, lost and maintained.

3 PATIENT COHORTS

3.1. Rare Phenotype Cohort

As part of an ongoing research program, we have recruited single individuals and families with bone fragility disorders of unknown etiology, but where an underlying genetic cause is suspected. The recruited individuals and families all had bone-related disorders and the great majority of the affected individuals fulfilled the ISCD criteria for primary osteoporosis in children. In patients not fulfilling the strict ISCD criteria, individual assessment of radiographs and fracture history still strongly suggested increased bone fragility and primary osteoporosis. The individuals were extensively phenotyped and a careful clinical and biochemical investigation excluded secondary osteoporosis. The most common monogenic causes of osteoporosis had been genetically excluded in the majority of the patients. Bone biopsies were performed when possible, either as part of the clinical investigation or because of research interest. Individuals from this research effort are included in Paper I-III. In Paper III a phenotypically similar cohort from Canada was also included.

Aim of genetic studies in the Rare Phenotype Cohort

The main aim of conducting genetic studies in this cohort / type of cohort, is to find novel monogenic causes of disease, including both the discovery of novel variants in known disease genes and discovery of novel disease genes. Naturally, implicating novel genes in a disease often reveals more interesting biological information than the discovery of novel variants in known genes. However, in monogenic osteoporosis, as in many other genetic disorders, exploring the allelic heterogeneity can yield relevant information to explain the often-seen variable expressivity. In OI glycine substitutions in *COL1A1* often are lethal, while nonsense variants in the same gene cause a much milder phenotype. Furthermore, pathogenic variants at certain specific locations in the genes *COL1A1* and *COL1A2* lead to Ehlers-Danlos syndrome rather than OI [86]. Genetic studies in this kind of rare phenotype cohorts can also be carried out to validate new scientific findings using a candidate gene approach, to screen for the presence of pathogenetic variants in recently discovered genes. The first approach was used in Paper II and the latter, the candidate-gene approach, was used in Paper I.

3.2. Fracture-Prone Children Cohort

This cohort consisted of 64 healthy children, without prior diagnosis or suspicion of primary osteoporosis, but with unusual propensity to fracture. This cohort was collected as part of a prospective epidemiological study at the Children's Hospital, Helsinki, Finland [87]. All children aged 4-15 years (n=1412) who during a 12-month period had been treated for an acute fracture were also questioned about previous fracture history. All children meeting the criteria for a clinically significant fracture history (n=71) were invited to take part in this research study. The inclusion criteria were: (1) age 4-15 years; (2) ≥ 2 low-energy long bone fractures before age 10 years; or (3) ≥ 3 low-energy long bone fractures before age 16 years; or (4) ≥ 1 low-energy vertebral fracture (loss of ≥ 20% vertebral height). Children with suspected secondary osteoporosis were excluded, as were children with a previous diagnosis of OI or suspicion of OI. The current ISCD criteria for a pathological fracture history in children are largely based on this study. The Fracture-Prone Children Cohort was included in Paper I and Paper III.

Aim of genetic studies in the Fracture-Prone Children Cohort

The distribution of childhood fracture in Finland is best represented by a Poisson distribution with a low mean [88]. The children in the Fracture Prone Children Cohort represent the tail of this distribution. However, a simple simulation exercise (unpublished) suggests that a slightly greater number of children meet the inclusion fracture criteria than what would be expected from Finnish age-adjusted fracture frequencies [88]. Since this cohort includes otherwise healthy children with a less severe bone phenotype, the underlying causes for their fractures are probably more heterogeneous. It is reasonable to believe that environmental or random factors will explain some (or perhaps a large proportion) of cases, while the sum of polygenetic contributions could explain another proportion. We also hypothesized that a minor proportion could be explained by single monogenic variants, which might have a less damaging effect than those present in severe forms of osteoporosis, or monoallelic variants with a damaging effect in genes usually associated with a recessive disease. This cohort was studied in Papers I and III: Paper I focuses on a monogenic approach, while Paper III aims to study the polygenic contribution to the fracture phenotype.

3.3. The VIDI Cohort

Vitamin D and its effect on health outcomes in general, and on skeletal outcomes in particular, have over the years drawn a large research interest. *In vitro* and *in vivo* studies have suggested vitamin D to be an important substance, in particular for bone, but not all randomized clinical trials have been able to replicate vitamin D's proposed positive effects [89]. However, more research is needed, especially to determine how geographical location and age affect response to vitamin D and what is the optimal serum 25(OH)D concentration for bone health.

The VIDI Cohort was recruited to evaluate the effects of different supplemental vitamin D dosages in Finnish infants [90, 91]. In short, the VIDI study was a randomized clinical trial that included 975 new-born healthy infants, born to healthy mothers at the Kätilöopisto Helsinki Maternity Hospital in Finland. All included children were born at term and had a birth weight within the normal range [92]. The original study investigated whether a daily dose of 30 µg of vitamin D_3 (intervention dose) compared to a daily dose of 10 µg (standard dose), initiated at the age of 2 weeks, had an impact on bone strength, measured by peripheral quantitative computed tomography (pQCT), and on infectious outcomes at age 24 months. However, one objective was also to study genetic variation in relation to serum 25(OH)D concentrations, vitamin D supplemental response, and skeletal and infectious outcomes. The VIDI Cohort's polygenic structure is studied in Paper III and Paper IV.

Aim of genetic studies in the VIDI Cohort

This cohort consist of healthy children, all with healthy mothers of Northern European decent, and who were all recruited at the same medical center. Also, by study design, all children have the exact same age at each point of measurement. Based on how this cohort was collected and how the follow-up visits were performed, it can be argued that it has a very homogeneous composition for a population-based cohort, both when it comes to background genetics and obtained study measurements. Both 25(OH)D concentrations and pQCT parameters, which were the focus of the study, are complex traits with high heritability estimates [49, 93]. To study the underlying genetics of these traits, an approach that can investigate the polygenic structure in this cohort would therefore be suitable.

4 METHODS

4.1 Genetic methods

Sanger sequencing (Paper I). Genomic DNA was extracted from peripheral blood. Sanger sequencing was sequentially performed using BigDye® technology on a 3730 ABI sequencer. Primer3Plus was used for PCR primer construction.

Massive parallel sequencing (Paper I and II). Genomic DNA was extracted from peripheral blood. Whole exome sequencing was performed both at Science for Life Laboratory, Stockholm, Sweden and Oxford Gene Technologies, Oxfordshire, UK using Illumina technology. Agilent SureSelect enrichment kits were used for whole-exome capture. The Speedseq framework [94] was used for alignment and variant calling and variant exploration was primarily performed using GEMINI (0.18.3) [95] and BEDTools [96]. All computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX)[97].

Array comparative genomic hybridization (array-CGH) (Paper II). A custom designed 2x400K array from Agilent Technologies that was enriched with probes in over 300 genes known to underlie skeletal disorders. The Agilent Genomic Workbench 7.0 was used to analyze the results.

Genome wide genotyping (Papers III and IV). Individuals from the Rare Phenotype Cohort, the Fracture-Prone Cohort and the VIDI Cohort were genotyped using the Illumina Infinium Global Screening Array v1.0 at the Human Genomics Facility (HuGe-F) at Erasmus MC, Netherlands. In total 686,085 different genomic positions were genotyped. Reference populations from the Haplotype reference consortium and 1000 Genomes phase 3 were used for haplotype estimation and imputation [98, 99]. Association tests were conducted using Plink (v 1.9) [100], FlashPCA was used to conduct principal component analyses [101] and the LASER suite was used for ancestry inference [101, 103]

4.2 Measurement of skeletal characteristics

Bone densitometry (Papers I and II). Dual-energy X-ray absorptiometry (DXA) measurements were performed using GE Lunar Prodigy (Madison, WI, USA) and Hologic QDR Discovery device (Hologic Inc., Waltham, MA, USA). Z-scores were calculated using either published or equipment-specific references accounting for age- and sex.

Peripheral quantitative computed tomography (pQCT) (Paper IV). pQCT measurements, using Stratec XCT2000LResearch+; Stratec Medizintechnik GmbH, were obtained at the 20% distal site of the left tibia [90]. Three pQCT parameters (bone mineral density, bone mineral content and cross-sectional area) for both total and cortical bone were used for analyses.

4.3 Evaluation of bone tissue characteristics

Bone histomorphometry (Papers I and II). Transiliac bone biopsies were obtained after a double course of oral tetracycline. In Paper I bone biopsies were analyzed using a semiautomatic image analyzer (Bioquant Osteo; Bioquant Image Analysis Corp., Nashville, TN, USA). In Paper II bone biopsies were analyzed using an Axiophot light microscope combined with a confocal laser scanning microscope (CLSM) (Leica TCS_SP5, Leica Microsystems, Wetzlar, Germany). Double tetracycline labels were used to assess bone turnover and bone tissue dynamics.

Quantitative Backscattered Electron Imaging (qBEI) (Paper II):

The qBEI analysis of the transiliac bone biopsy in Paper II was performed using a Zeiss Supra 40 scanning electron microscope that had a spatial resolution of 1.8 µm/pixel. Bone mineralization density distribution was compared to previously published reference data [104].

Raman microspectroscopy (Paper II):. For the Raman microspectroscopy a Senterra instrument was used (Bruker Optics GmbH Ettlingen, Germany).

For a detail description of the methods concerning **bone tissue characteristics**, please see Supplemental appendix in Paper II.

4.4 Biochemical measurements (Paper IV)

Vitamin D measurements. The 25(OH)D in serum was measured at 12 and 24 months. At birth 25(OH)D was taken from umbilical cord blood. The 25(OH)D measurement at 24 months was used for the association analysis because it was assessed as the measurement best reflecting the children's inherent 25(OH)D concentrations.

Biochemical analyses in Papers I-III were performed during the clinical assessment to exclude secondary osteoporosis.

4.5 Statistical analyses

Statistics (Paper I-IV). The software R (version 3.3.1 – 3.3.5) was used for statistics, high level data processing and graphics.

5 RESULTS AND DISCUSSION

5.1 Paper I

Analyzed cohorts: 1) Rare Phenotype Cohort and 2) Fracture-Prone Children Cohort.

PLS3 sequencing in childhood-onset primary osteoporosis identifies two novel disease-causing variants. Paper I had a simple and straight forward approach. At the time of the study, pathogenetic variants in *PLS3* had just recently been shown to be the cause of an X-linked form of early-onset primary osteoporosis [105, 106]. The aim of the study was to investigate whether we can identify novel *PLS3* variants in the Rare Phenotype Cohort and/or in the Fracture-Prone Children Cohort. The results aimed to clarify weather *PLS3* screening should be recommended in clinical practice for patients with primary osteoporosis or an OI-like bone phenotype. Further, the study aimed to investigate weather variants in the X-chromosomal gene *PLS3* could explain milder bone phenotypes, such recurrent fractures in childhood, because it is well known that boys have a higher fracture rate than girls [107, 108]. For this purpose, all coding exons and flanking intronic regions were screened for pathogenic variants in *PLS3* for 31 patients in the Rare Phenotype Cohort and 64 children in the Fracture-Prone Children Cohort.

Novel *PLS3* variants in children with primary osteoporosis. In the Rare Phenotype Cohort, the sequencing results revealed that two individuals harbored novel *PLS3* variants that was deemed to be the cause of their bone phenotypes. The two individuals with damaging *PLS3* variants both had severe vertebral compression fractures and very low BMD, even in relation to other children with primary osteoporosis [**Fig. 3**]. The Rare Phenotype Cohort comprised 31 patients who had been investigated at the Metabolic Bone Clinic, Children's Hospital, Helsinki because of primary osteoporosis, but without an identified molecular cause. Most of the 31 patients fulfill the ISCD pediatric criteria for primary osteoporosis, but 6 individuals did not. However, we believe that this type of inclusion criteria, which are not only built on static parameters but also on careful individual clinical assessments, are more likely to identify individuals suited for genetic studies. The individuals in this cohort often had a family history that suggested an inherited disorder, even though the inheritance pattern seldom provided indisputable evidence for a monogenic cause.

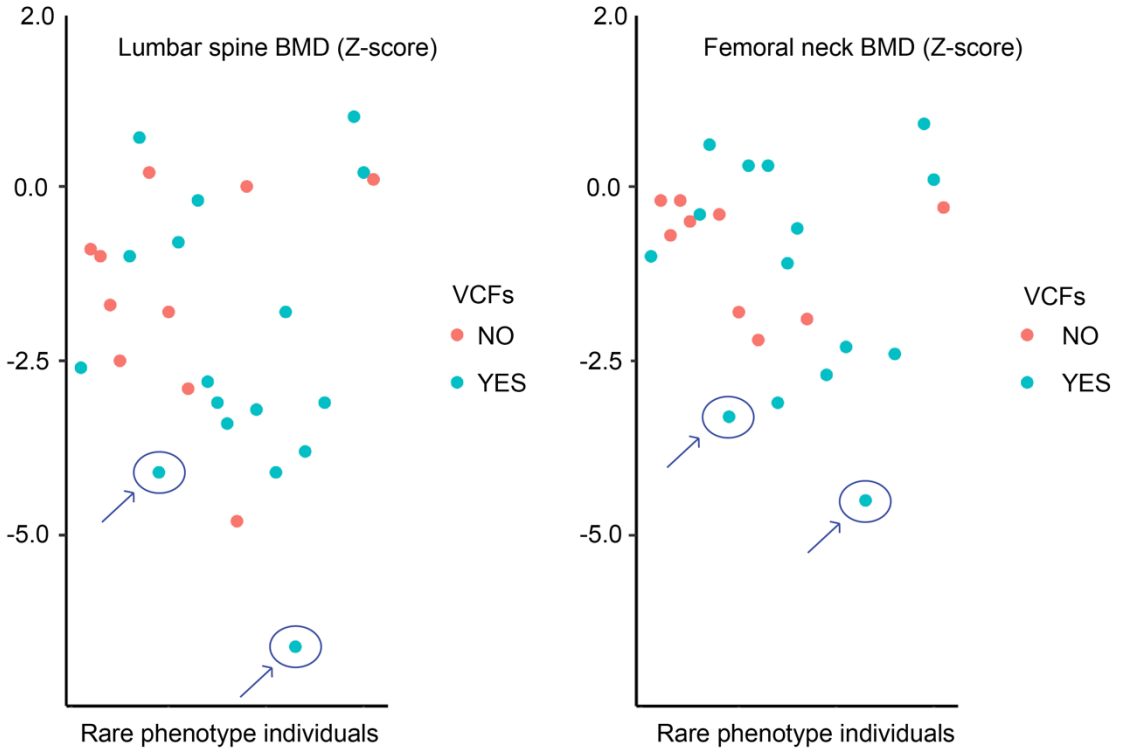

Fig. 3. BMD measurements in the Rare Phenotype Cohort. DXA-derived BMD measurements for the 31 individuals in the Rare Phenotype Cohort included in Paper I. The two individuals with novel disease-causing *PLS3* variants (circled) have an exceptionally low BMD both at the lumbar spine and at the femoral neck. [Vertebral compression fractures (VCFs), please also note that not all BMD measurements in this cohort are bisphosphonate treatment naïve.]

Patient I was diagnosed with multiple vertebral compression fractures at the age of 10 years and had before that broken both his femurs at two separate occasions. He was discovered to have a novel nonsense variant (p.Arg256*) in *PLS3,* classified as pathogenic (class V) according to the ACMG criteria [109]. The variant was inherited from his healthy mother. *Patient II*, at the time 10 years of age, also had a fracture history that included multiple peripheral and vertebral compression fractures. She also had an exceptionally low BMD, with a lumbar spine DXA-derived Z-score of -6.6. *Patient II* was discovered to have a heterozygous *de novo* missense variant in *PLS3*, which was consistent with her parents' lack of fracture history. The variant (p.Asn446Ser) could be classified as likely pathogenic (class IV) according to the ACMG criteria, which is regarded as clinically actionable. Because the significance of a heterozygous variant in the X-chromosomal *PLS3* is more difficult to interpret in girls, we also performed an array-CGH in the girl and WES of her nuclear family without being able to find another plausible genetic explanation. However, it is known that the female disease spectrum in PLS3 osteoporosis is wide, and females also within the same

family can have mild to severe disease [106]. The results in Paper I indicate that screening for *PLS3* sequence variants is indicated in boys and girls with primary osteoporosis of unknown cause, especially if spinal fractures are a dominant feature.

***PLS3* variants are not common in children with mild bone phenotypes.** The majority of children in the Fracture-Prone Children Cohort were, as expected, boys (43 boys vs 21 girls). Although there are a number of reasons why boys might be more prone to fractures than girls, genetic explanations related to the X-chromosome are possible. However, in this cohort we identified no rare or damaging variants in *PLS3*, suggesting that *PLS3* is not a common cause for milder bone phenotypes with recurrent fractures but normal BMD and that *PLS3* variants cannot explain the observed gender differences in fracture rates. From inclusion design and from what we know about fracture rates in children [88], random accidents and environmental factors will probably explain a large proportion of fractures, also in this cohort. Based on the way fractures are distributed, a genetic cause in this cohort would be more likely to be polygenic in nature, and not monogenic. In that perspective our negative results were expected since only a minor proportion of the Facture-Prone Children Cohort could be suspected to have an underlying monogenic cause for their fractures. Still, it can be argued that this type of candidate-gene approach is valid, also in a cohort such as this one. The genetic causes for increased fracture tendency in childhood, and for male predominance in those with multiple fractures, remain inadequately understood and in that regard our study provided novel information, despite the negative findings. An oligogenic scenario is also possible, in which some of the fracture-prone individuals would have functional and relatively rare variants in *PLS3* that would confer a moderate fracture risk. In such a scenario, positive results would be possible to obtain also in a cohort such as this one. Previous studies have also indicated that common variants in *PLS3* could confer a slightly increased risk of fractures and a low BMD [105].

5.2 Paper II

Analyzed cohorts: 1) Rare Phenotype Cohort

PLS3 deletions lead to severe spinal osteoporosis and disturbed bone matrix mineralization. In this study we identified two large *PLS3* deletions in patients from two different families. At the time of the study, no gene deletions in *PLS3* had been reported. *Family I* consisted of two siblings, both boys, with severe and almost identical bone phenotype that differed from classical OI. The parents were healthy. Secondary causes had been excluded and because of the specific bone phenotype in the two brothers, a monogenic condition was strongly suspected. Whole exome sequencing was performed for the entire nuclear family. No sequence variants were found in our in-house OI gene panel, and no other plausible causative variants consistent with the inheritance pattern could be found in the exome-wide analysis. However, a read-depth analysis for all OI-genes included in the gene panel revealed a large deletion in *PLS3* spanning exons 4-16 in hemizygous form in both boys. The deletion was inherited from their heterozygous, but healthy, mother [**Fig. 4**].

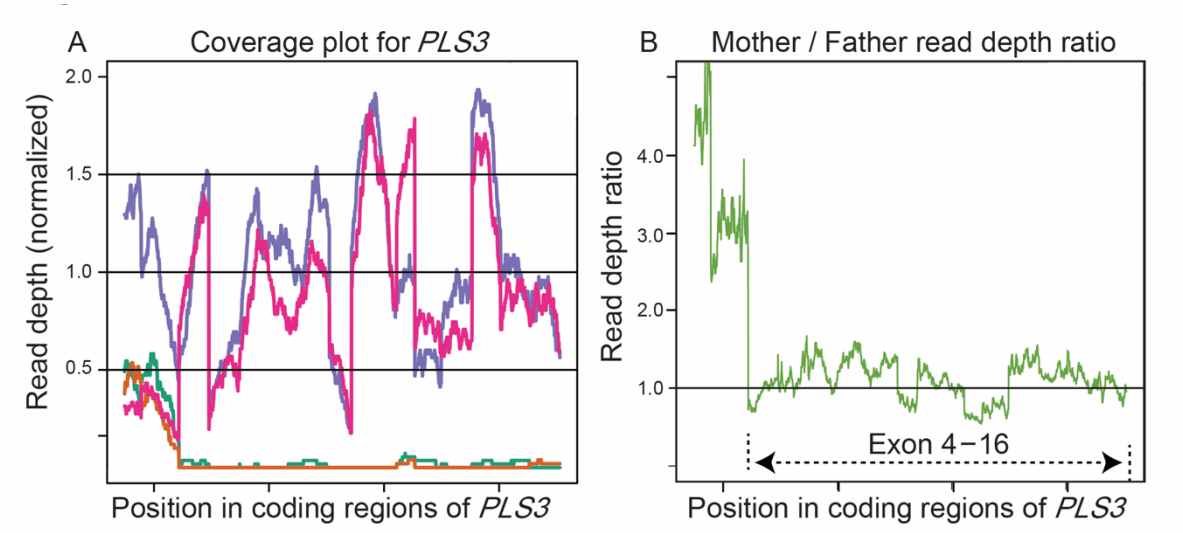

Fig.4. A large deletion in *PLS3*. Read depth plots for all coding exons of *PLS3* in *Family I*. **A)** The two brothers (orange and green) have no reads mapping to exon 4-16 in *PLS3*, indicating a hemizygous deletion. **B)** Comparing the read depth ratio between the father and the mother (pink and purple) show that the they have an equal amount of reads mapping to exon 4-16 in *PLS3*, indicating a heterozygous deletion in the mother for the same genomic region.

Normally whole exome sequencing (WES) is not a good method for analyzing structural variants because of very uneven read-depths and an improbability of capturing SV break points. Instead, whole genome sequencing (WGS) is preferred [110]. **Fig. 5** visualizes the variability in read depth between the two methods (unpublished data). However, WES still has the possibility to identify larger deletions in preselected genes, but for duplications this possibility is limited.

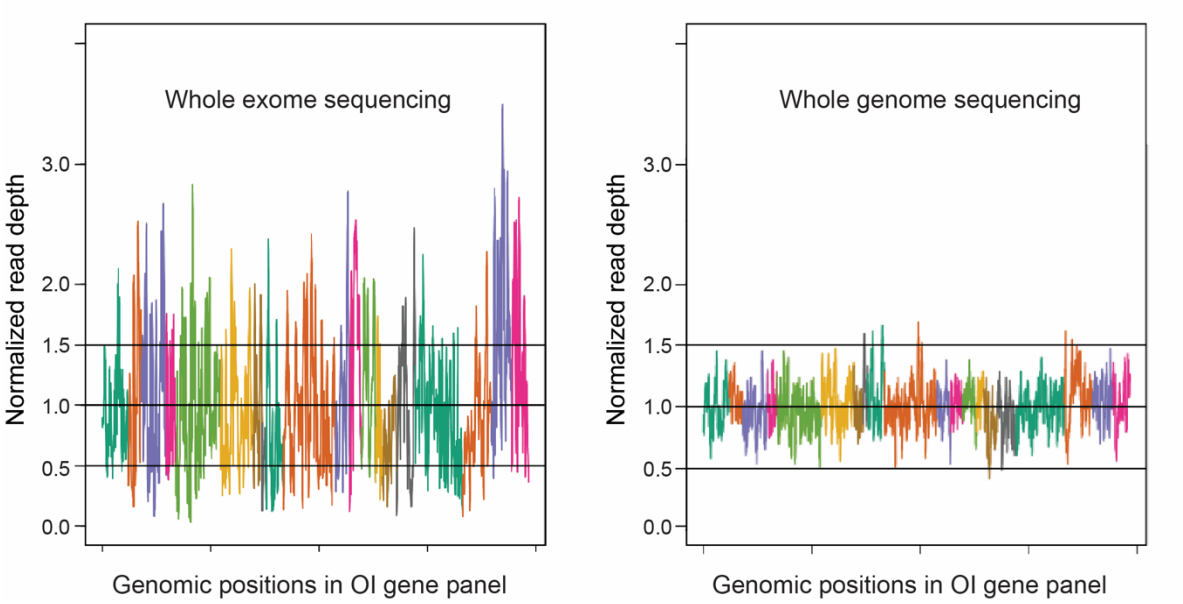

Fig. 5. Read depth variability between WES and WGS for an in-house OI gene panel. As can be seen, WGS provides more stable and less variable data and therefore perform much better when analyzing structural variants (SVs). However, homozygous or hemizygous deletions can be detected also by WES, at least for pre-specified genes.

In *Family II* an array-CGH identified a complete gene deletion in *PLS3* in a 12-year-old boy with a very low BMD and vertebral compression fractures. This study thus identified two novel *PLS3* deletions. Shortly after our publication, another group also published a case report on a young boy with a notably low BMD and multiple vertebral compression fractures, who also harbored a large deletion in *PLS3* [111]. In all described individuals with *PLS3* deletions the affected individuals (boys) have had a severe skeletal phenotype, including a very low BMD, but particularly, severe spinal osteoporosis with multiple vertebral compression fractures.

***PLS3* gene deletions lead to disturbed bone matrix mineralization.** Large, or complete, deletions of a gene can with certainty be regarded as functional null variants and can therefore be used to study how different tissues react to the absence of a protein. As

previously discussed, careful and correct phenotyping to select the appropriate patients for genetic studies is very important in order to be successfully able to discover underlying genetic causes. However, when an underlying genetic cause has been identified, extensive tissue phenotyping needs to be performed to uncover the mechanisms affected by that same genetic aberration. In this study, we were able to confirm previous reports that spinal osteoporosis, and in particular, vertebral compression fractures should be considered a hallmark of PLS3 osteoporosis. A bone biopsy was also taken from the index case in *Family I* and extensively analyzed. The results from the bone histomorphometry showed a clear increase in mineralizing lag time as well as an increase in osteoid volume, surface and thickness. The quantitative backscattered electron imaging (qBEI) showed a strong hypomineralization of the bone matrix and a broadened bone mineral density distribution peak, suggesting that the mineralization was spatially heterogenous. The hypomineralization could also be detected by Raman microspectroscopy which showed a markedly low mineral/matrix ratio and a decrease in glycosaminoglycans, proteoglycans and pyridinoline, suggesting that the bone composition was also altered. Because of double tetracycline labels the dynamics in bone deposition could be assessed by analyzing altogether four different tissue ages. Collectively the results indicated that the *PLS3* deletion led to a disturbed bone matrix mineralization, which also was supported also by the results by *Wesseling-Perry et al.* [112].

During bone matrix mineralization hydroxyapatite crystals are formed within the lumen of matrix vesicles released from chondrocytes into the extra cellular matrix (ECM) [113]. Because *PLS3* has been shown to be expressed in these released matrix vesicles from mineralizing cells, and because PLS3 is known to be involved in the regulation of the actin cytoskeleton, it is possible that PLS3 plays an important role for bone matrix mineralization [114-116]. Although our study could not pinpoint the exact role of PLS3 in bone matrix mineralization, our results give strong support for the suggestion that *PLS3* is directly involved in the process. At the time of the study, and even presently, very little was known about PLS3's function in bone. Our results provided evidence that *PLS3* has a major role in primary mineralization of the bone matrix, which has also recently received support from mouse studies [117]. The results highlight the strength of careful and precise phenotyping, because the study was able to produce meaningful novel results while using standard methods on very few individuals.

5.3 Paper III

Analyzed cohorts: 1) Rare Phenotype Cohort; 2) Fracture-Prone Children Cohort and 3) The VIDI Cohort

Increased burden of common risk alleles in children with a significant fracture history. In Paper III the aim was to assess the polygenetic contribution to rare and severe bone phenotypes in children. As discussed throughout this thesis, we often suspect that children presenting with primary osteoporosis have a monogenic cause for their disease. In children whose standard gene panel has been negative, it is common to reason that an expanded genetic investigation using WES or WGS could identify novel genetic causes for primary osteoporosis. In our Rare Phenotype Cohort, although monogenic causes are primarily suspected, the observed inheritance pattern seldom excluded other causes. For this purpose, we used a polygenic risk score derived from genetic association data from the UK biobank on heel quantitative ultrasound speed of sound. The polygenic risk score, called gSOS, has been validated and shown to predict fractures in a large cohort of adult individuals, also from the UK biobank [118].

A polygenic risk score derived from quantitative ultrasound of the heel. Findings from GWASes can be used to identify genomic loci important for a trait and help to decipher underlying mechanisms for that trait. However, a single SNP associated with a trait only explains a very small fraction of the genetic variance of the associated trait, but the sum of all associated SNPs might explain a larger fraction. A polygenic risk score is a way to quantify all available genetic information, to measure the sum of the genetic effects. As discussed in the introduction, BMD can be estimated from quantitative ultrasound of the heel (eBMD), which is a good proxy measurement for DXA-derived BMD and has a fair correlation with fractures [57, 119]. The results from genetic correlation analyses [**Fig. 2**] also suggest shared inheritance between ultrasound derived heel BMD, osteoporosis and fractures. To be precise, eBMD is derived from quantitative ultrasound index (QUI) which is a combination of the two quantitative ultrasound variables 1) speed of sound (SOS) and 2) broadband ultrasound attenuation (BUA). In this study the polygenic risk score was developed only from heel quantitative ultrasound SOS, but the correlation between SOS and QUI is very high ($r>0.9$) and SOS is also independently associated with fractures [118-121]. The polygenic risk score

was developed, using machine learning algorithms, from an underlying GWAS that included 341,449 individuals and validated for fracture prediction in a set of 80,027 individuals. The final polygenic risk score (gSOS) included 21,717 genomic markers and could predict fractures just as well as measured SOS and DXA-derived hip BMD [118], suggesting that the score has the capability to assess bone health. In our study, the polygenic risk score was not used to predict fractures. Instead we hypothesized that children with a polygenic cause of their bone phenotypes would have a lower gSOS score compared with a normal population. However, for children with bone phenotypes due to monogenic variants, the gSOS score should not be impacted. The aim was therefore to use gSOS scores to assess etiology at the cohort level.

Evidence for polygenic contribution in children with primary osteoporosis. This study comprised four different types of cohorts: The Rare Phenotype Cohort from Finland, A Rare Phenotype Cohort from Canada, The Fracture-Prone Children Cohort and the VIDI Cohort. The two Rare Phenotype Cohorts included 18 individuals from Finland and 60 individuals from Canada, all with a suspected monogenic bone disorder with childhood-onset, for which a molecular cause could not be identified. The majority of the children in these two cohorts would qualify for the diagnosis primary osteoporosis. The results showed that the polygenic risk score was significantly lower in these two rare phenotype cohorts than the gSOS score in the normal reference population from the UK biobank. Because the gSOS score was derived from an adult population, as well as the UK biobank reference used for comparison, we also included another reference cohort consisting of 898 healthy Finnish children (the VIDI Cohort), but the results remained the same [**Table 2**]. The VIDI Cohort had a very similar gSOS score as the UK biobank reference, suggesting that the gSOS score is valid also for pediatric cohorts and that the study results can be trusted. The results provide evidence that a proportion of children with primary osteoporosis and a clinical suspicion of a monogenic cause actually have a polygenic cause of their disease. The gSOS score was developed from heel quantitative ultrasound speed of sound, which is a legitimate proxy measurement for bone health, but it cannot serve as a replacement of a more careful phenotype assessment. It is therefore reasonable to believe that the gSOS score only captures a part of the polygenic contribution to osteoporosis in these children, meaning that the polygenic contribution in childhood primary osteoporosis is likely to be larger than what we can show in this study. However, because of the small sample sizes in our study, the results would need to be replicated before far-reaching conclusions can be drawn.

Table 2. Mean gSOS score per cohort compared to the UK biobank normal reference.

Cohort	gSOS score (SD)	p-value (vs UK Biobank reference)
Reference datasets		
UK Biobank (n = 80,027)	0.00 (1.00)	NA
VIDI Cohort (n = 898)	0.01 (1.00)	0.69
More severe bone phenotypes		
Rare Phenotype Cohort (Canada, n = 60)	−0.82 (0.90)	3.7×10^{-9}
Rare Phenotype Cohort (Finland, n = 18)	−0.54 (1.01)	0.04
Rare Phenotype Cohorts combined (n = 78)	−0.76 (1.06)	5.3×10^{-10}
Milder bone phenotypes		
Fracture-Prone Cohort (Finland, n = 53)	−0.04 (1.06)	0.77
Combined analysis		
All study Cohorts combined (n = 131)	−0.47 (1.00)	1.1×10^{-5}

No evidence for a polygenetic etiology in milder phenotypes. The last cohort evaluated with the gSOS score was the Fracture-Prone Children Cohort, in which the children had a milder bone phenotype. We recognize that this cohort is heterogeneous, but due to reasons previously stated, we hypothesized that polygenic causes could explain a sizable proportion of the fracture susceptibility in this cohort. However, the gSOS score for the entire cohort was not significantly different from the UK biobank reference. Analyzing the cohort as a whole, the results suggest that polygenic factors do not explain this milder bone phenotype in children. Instead, it might be likely that the presumed fracture susceptibility could be explained largely by exogenous or environmental factors. Interestingly though, when stratifying the Fracture-Prone Children Cohort based on whether or not they meet the criteria for primary osteoporosis, interpretation of the results changes (unpublished sub-analysis) [**Fig. 6**]. Using the healthy VIDI Cohort as the reference, which might be more appropriate when separately assessing the Finnish cohorts, the Rare Phenotype Cohort (n=18) had a significantly lower gSOS score than the VIDI Cohort (t-test, p=0.026). For the Fracture-Prone Children Cohort as a whole, no difference in gSOS score compared to the VIDI Cohort was seen (t-test, p=0.83). However, when dividing the Fracture-Prone Children Cohort into two subgroups: 1) children who fulfill the ISCD criteria for primary osteoporosis (n=15); 2) children who do not fulfill the ISCD criteria (N=40), the results change. The 15 children fulfilling the diagnosis of primary osteoporosis had a significantly lower gSOS score than the VIDI cohort (t-test, p=0.048). Looking at the density plot [Fig 7B] it is also clear that the gSOS score is completely normally distributed in the VIDI Cohort as well as in the sub-group of the Fracture-Prone Children Cohort that did not fulfill the criteria for osteoporosis. For the children belonging to the Rare Phenotype Cohort and the children from the Fracture-Prone Children Cohort who did fulfill the criteria for osteoporosis, the density plots display two peaks. This suggest different underlying etiology, where a proportion of these children do have a polygenic cause for their osteoporosis, while the proportion with a normal gSOS score have a non-polygenic cause (note that the cause could still be monogenic). In summary, in children with fractures, support for a polygenic cause was only found for children with a more severe bone phenotype, but not for children with non-vertebral fractures and a normal BMD. Further, even though gSOS score cannot discriminate children with a polygenic bone disorder from those with a monogenic bone disorder on the individual level, the results strongly suggest that gSOS can suggest etiology on a group level.

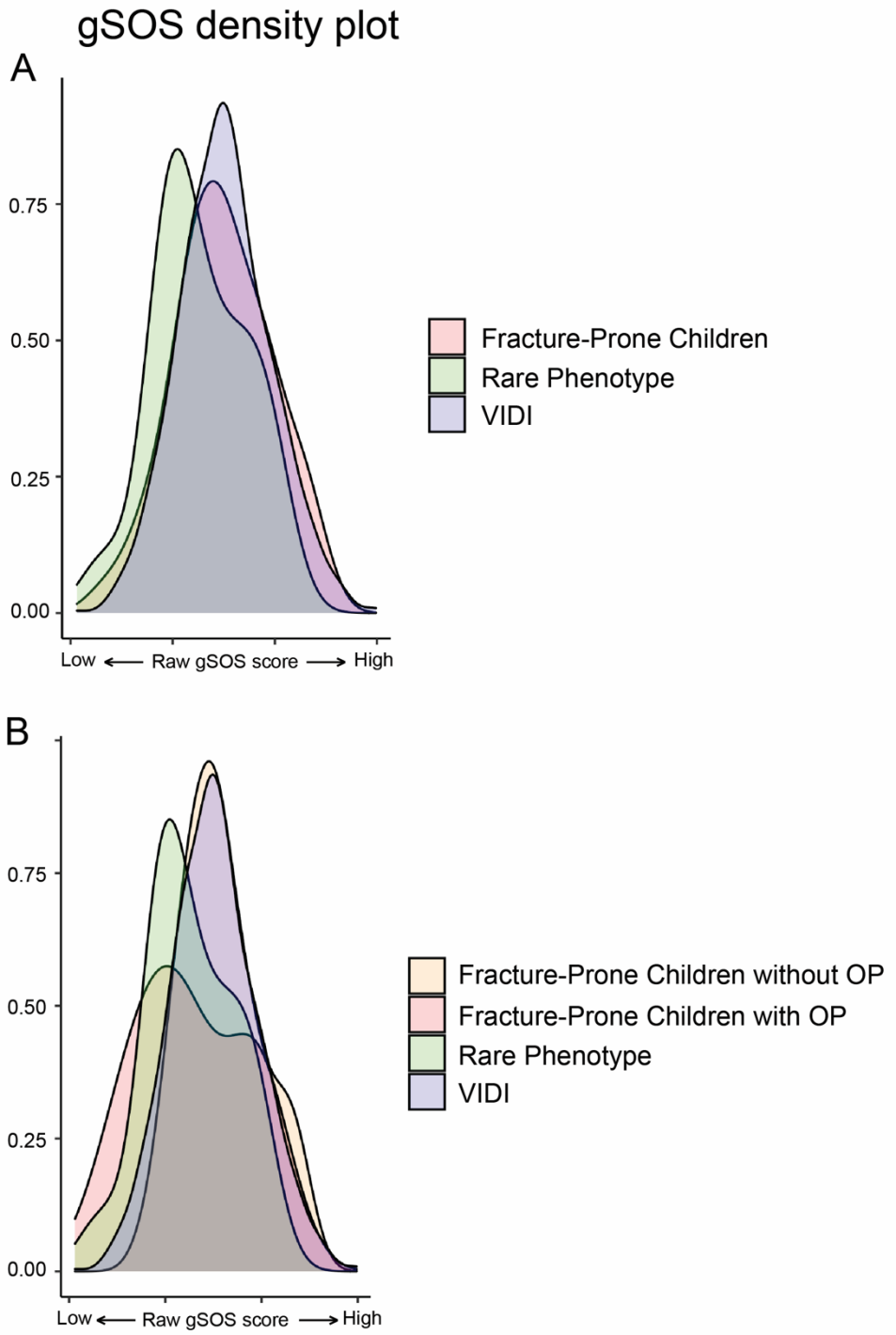

Fig. 6. ***Density plot of the gSOS score distribution in the three Finnish cohorts. A)*** The Rare Phenotype Cohort had a significantly lower gSOS score compared to the healthy VIDI Cohort, while the Fracture-Prone Children Cohort had a similar gSOS score compared to the VIDI Cohort. ***B)*** Dividing the Fracture-Prone Children Cohort into two groups depending of whether they fulfill the criteria for osteoporosis (OP) changes the results. The subgroup of Fracture-Prone Children Cohort with osteoporosis have a significantly lower gSOS score than the VIDI Cohort. The double peaks in the density plot for the Rare Phenotype Cohort and for the subgroup of the Fracture-Prone Children Cohort with osteoporosis suggests that theses cohorts consist of two different populations, one that has a polygenic cause of disease and one that has a non-polygenic cause of disease.

5.4 Paper IV

Analyzed Cohorts: 1) The VIDI Cohort

Genetic variation in *GC* and *CYP2R1* affects 25-hydroxyvitamin D concentration and skeletal parameters: A genome-wide association study in 24-month-old Finnish children. In Paper IV we performed a GWAS focused on 25(OH)D concentrations in 24-month-old children. We were able to provide strong support that the genes *GC* (Vitamin D binding protein) and *CYP2R1* (Vitamin D 25-hydroxylase) affect 25(OH)D concentration in 24-month-old children, and that the gene *GC* might be important for vitamin D supplementation response. Furthermore, we could show that children with genetic constellations associating with lower vitamin D had lower BMD.

Vitamin D is a prohormone important for both calcium and phosphate homeostasis and it has caught the interest of many research groups [122]. That vitamin D deficiency can cause rickets in children and osteomalacia in adults has been known for a long time. Both rickets and osteomalacia are disorders characterized by poor bone matrix mineralization. These disorders can also be regarded as evidence for the importance of vitamin D sufficiency for bone health [14, 15]. Positive effects of high concentrations of vitamin D have also been associated with several different health outcomes, including all-cause mortality, but the prohormone's causal effect has been hard to prove [83-85]. However, because of the geographic location of the Nordic countries, having winters with long periods of little sunlight, low vitamin D concentrations have been a concern [123]. In Finland, vitamin D concentration has also been shown to be associated to BMD in adolescents [16]. The aim of Paper IV was to investigate genetic variation associated with serum 25(OH)D levels in 24-month-old children. Because of the study design, also genetic variants associated with vitamin D supplementation response could be assessed. The study also set out to investigate vitamin D concentrations in relation to skeletal outcomes in these 24 month-old-children. Apart from severe vitamin D deficiency, it has been difficult to confidently prove positive skeletal effects of vitamin D. However, we hypothesized that because of Helsinki's northern location and reduced cutaneous UV exposure, a genetic constellation that helps to ensure vitamin D sufficiency might be proportionally more important and genetic liabilities might thus be more easily revealed. Also, the extraordinary rapid skeletal growth rate

occurring in children from birth to 24 months could render the skeleton more vulnerable to small effects that might go undetected in adults.

Small cohorts are usually not suitable for GWASes. Our GWAS on 25(OH)D, which is the metabolite used to determine vitamin D status, could be considered underpowered and very small for this type of approach. In comparison, a GWAS that was very recently published looked at genetic variation associated with 25(OH)D in more than 400,000 adult individuals [124]. However, we argue that the genetic homogeneity of the study cohort partly compensates for the small sample size. Furthermore, no GWAS on vitamin D has previously been performed in this age group. Moreover, because of the participants young age we believe that our study is less confounded by environmental and behavioral factors affecting an individual's vitamin D status. As discussed above, because of the biologically dynamic situation during early childhood, the importance of genetic regions regulating vitamin D concentration might be easier to detect. Also, because the skeletal measurements were taken with high precision, using pQCT, when the children had exactly same age (24 months), we believe that our study has the possibility to reveal relationships and associations that are hard to detect in adults, despite our small samples size. Finland can also be regarded as a genetic isolate in Europe where founder effects have had a strong effect [125, 126] Therefore, it is possible that in an association study certain loci affecting vitamin D metabolism may be less difficult to identify in the Finnish population.

***GC* and *CYP2R1* are likely to affect 25(OH)D concentrations in children.** The GWAS identified two strong association signals near the genes *GC* and *CYP2R1* [**Fig. 7**]. To map these signals, we compared, and quantified, the similarities between the association results in our study to publicly available expression quantitative trait locus (eQTL) data from the GTEx project [**Fig. 8**] [127]. This allowed us to confidently map our two identified association signals to the genes *GC* and *CYP2R1*.

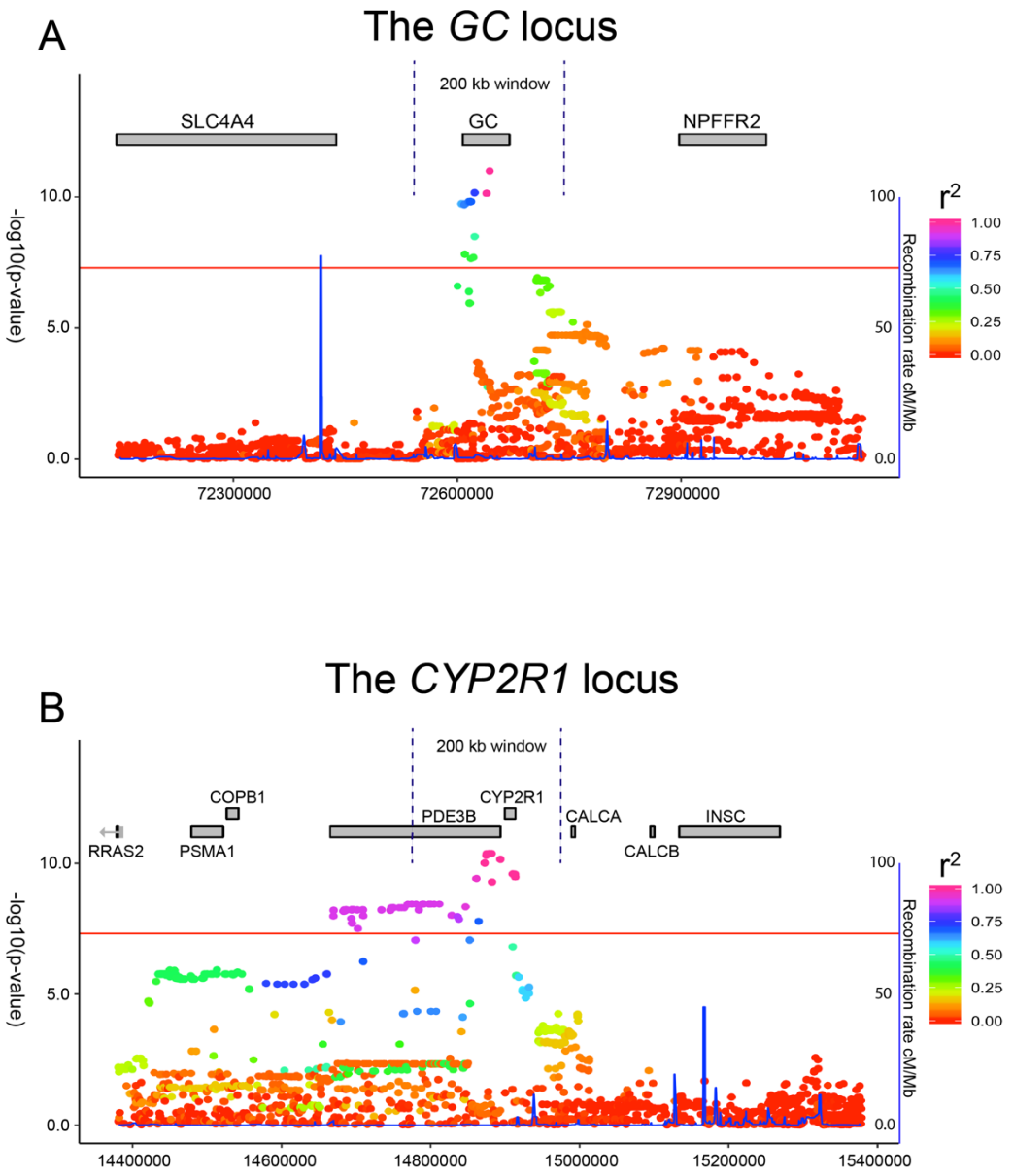

Fig. 7. The *GC* and *CYP2R1* loci. A view of the genomic regions surrounding the strongest associated SNPs in both loci. Based on simulation studies by Wu et al [128], a 200 kb target window was used as our presumed resolution. **A)** The *GC* gene is the only gene within this 200 kb target window on chromosome 4 and therefore our best candidate to underlie the signal. B) For the *CYP2R1* locus, the genes *PDE3B* and *CYP2R1* are both located within our target window, and cannot be separated by this proximity analysis.

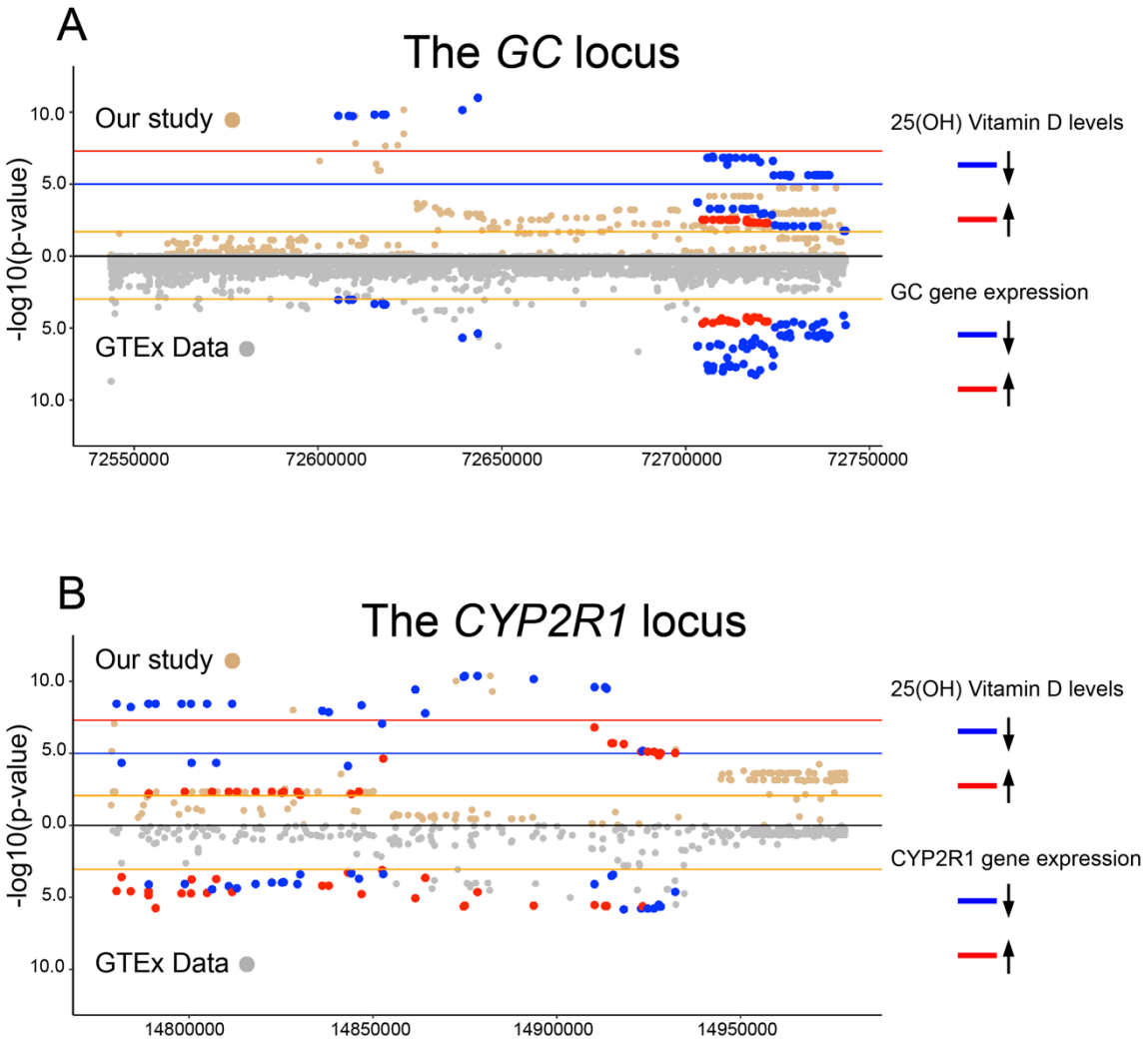

Fig 8. Mapping our association signals using GTEx eQTL data. Association results from our GWAS on 25(OH)D in 24-month-old children compared to eQTL data from the GTEx project. All significant SNPs (FDR ≤0.05), simultaneously present in both datasets, and within the 200 kb target window are displayed. Significant SNPs are color-coded dependent on association direction. As shown, all significant SNPs present in both datasets have a concordant direction of association, suggesting a shared underlying genetic cause for the signals in our study and the GTEx datasets. **A)** *GC* locus; tissue analyzed: Stomach. **B)** *CYP2R1* locus; tissue analyzed: Thyroid.

The *GC* and *CYP2R1* genes have previously been shown to be associated to 25(HO)D in several GWASes [93, 129-133] and their functional importance for vitamin D metabolism is known [134, 135]. **Fig. 9** illustrates how the association results from our study compare with all other GWASes on 25(OH)D that have been reported to the GWAS Catalog. Because we had umbilical cord 25(OH)D measurements, we were also able to assess genetic variation in

relation to vitamin D supplementation response, defined as the change in 25(OH)D from birth to 24 months. The study showed that the *GC* locus associated with the magnitude of 25(OH)D change during intervention. Because the results strongly suggested that the association signal could be mapped to the gene *GC*, it is thus likely that the gene *GC* also underlies the association seen in how the children responded to vitamin D supplementation.

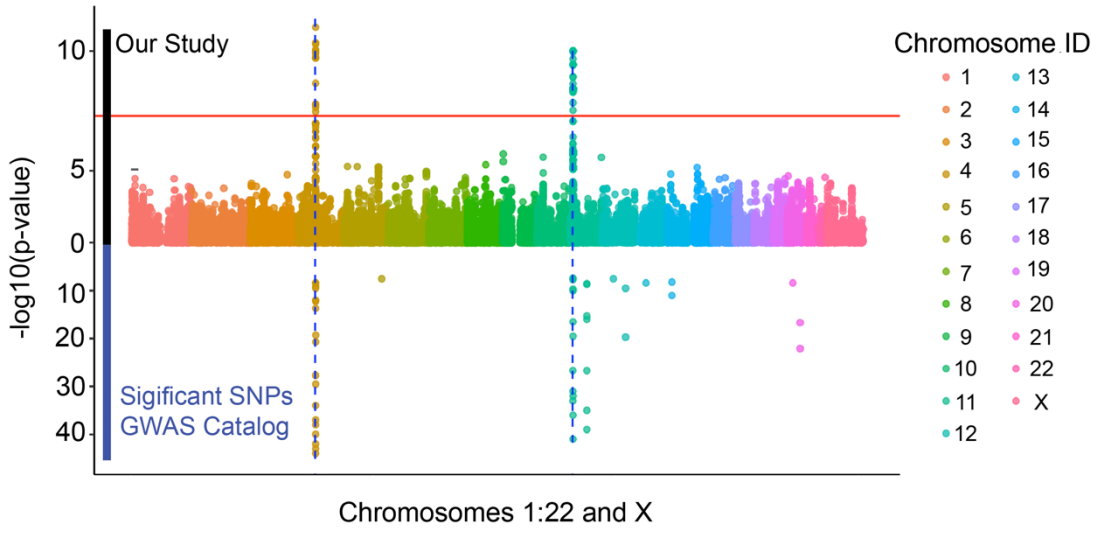

Fig. 9. Comparison of significant associations in our study vs the GWAS catalog. The GWAS results from our study on 25(OH)D are presented above the line y=0, while all genome-wide significant SNPs reported to the GWAS Catalog are presented below the same line. As shown, the two genome-wide significant loci, near the genes *GC* and *CYP2R1*, identified in our study on 25(OH)D in 24-month-old children are located within the two strongest loci previously reported to associate with 25(OH)D.

Support for causal positive effect of 25(OH)D on skeletal outcomes. We could observe from our data that the 25(OH)D concentration was significantly associated with several pQCT parameters. We also observed that the lead SNPs in the *GC* locus and the *CYP2R1* locus also associated with pQCT parameters in the same manner as 25(OH)D, but these associations did not reach statistical significance. In an effort to gain power, we identified the major haplotypes for the two genome-wide significant loci in the hope that we would capture more of the underlying genetic information. We could also show that the haplotypes associated more strongly to 25(OH)D than the lead SNPs alone, suggesting that the haplotypes indeed captured more information. When looking at combinatory effects we could show that the combination of the two loci's risk haplotypes was additive, approximately doubling the effect seen on 25(OH)D. Using a single sample mendelian randomization approach we stratified the children based on their combination of 25(OH)D haplotypes and

associated these new haplotype groups to pQCT parameters. The results showed a strong association between a child's genetic vitamin D constellation and pQCT parameters [**Table 3**], suggesting that low 25(OH)D has a true negative impact on bone in 24-month-old children. Or, in other words, 25(OH)D is not only a marker for skeletal outcomes in 24-month-old children, but a mediator of the actual effect.

Table 3. Haplotypes associated with low 25(OH)D also associates to skeletal parameters when combined.

pQCT parameter (Measured at the Tibia)	Beta coefficient for haplotype status*	Std. Error	p-value
Total bone			
Bone mineral density	-31.8998	10.2872	0.00224**
Bone mineral content	-1.3693	0.9583	0.154736
Cross-sectional area	9.9368	3.6646	0.00734**
Cortical bone			
Cortical density	-27.681	7.881	0.00056***
Cortical content	-3.7949	1.1646	0.00134**
Cortical area	-3.1087	1.1568	0.007869**

*The major haplotypes at both significant loci were identified and denoted [A] and [a] for the GC locus and [B] and [b] for the CYP2R1 locus. Because of high haplotype frequency we could assessed the combinatory effects by creating 3 new haplotype groups that included individuals with the haplotype status of either: [AABB] or [AaBb] or [aabb]. In total 193 children matched one of these 3 haplotype combinations and had available pQCT measurements.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The field of bone genetics has rapidly grown in the 21st century. The widespread use of WES and WGS has revealed many new genes underlying monogenic bone disorders, which also has led to the discovery of new signaling pathways important for bone. GWASes have quickly evolved from merely being a method that could prove its own principle to a method providing clinically relevant information. In this thesis we have used different genetic methods to uncover underlying causes for primary bone disorders in children, with the hope to also learn more about the underlying biology of bone. Today's DNA sequencing methods allow for a precise assessment for SNVs and short indels, but the addition of RNA sequencing data has proven useful for variant interpretation. Analysis of structural variants in the human genome is still a difficult task, but the technique has matured and public reference datasets are growing. I do therefore believe that there still are interesting genetic findings to be discovered in families and individuals with monogenic osteoporosis and that these additional methods will improve the success rate. Regarding GWASes I believe that the current methods will continue to reveal underlying genetics causes for bone disorders. The steady increase in sample size together with the combination of sequencing data to assess rare variants and more sophisticated statistical models will make the GWAS approach successful also in the future. I do believe that there is a sample size limit, and when that limit is exceeded, additional results will no longer be biologically relevant. However, we are not there yet. The precision in how the studied GWAS trait is measured can also be improved, and in my opinion, a refinement of the trait being studied could be a better way forward than sample size increase.

To conclude, it is evident that much information is still to be extracted only using current methods. However, I also believe that the techniques in molecular biology are now mature enough for a more integrative approach. To understand the full picture, data must be combined. The combination of omics data, including genomics, proteomics, metabolomics, interactomics etc. will help us to understand how biology really works. This integrative approach is being adapted by researchers around the world, and I do believe that it is the way forward although we have probably not even seen the beginning of it.

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