Osteogenesis Imperfecta

Genetic and Therapeutic Studies

KATARINA LINDAHL
Osteogenesis imperfecta (OI) is a heterogeneous disease of connective tissue, the cardinal symptom being fractures and severity ranging from mild to lethal. Dominant mutations in collagen I, encoded by COL1A1 and COL1A2, cause >90% of cases.

To delineate genotype-phenotype correlations and pharmaco-genetic response, collagen I was sequenced in 150 unrelated Swedish families and clinical data were collected in Paper I. Mutation type, gene affected, and N- to C-terminal location correlated with phenotype and severity. Bisphosphonate response assessed by calculated yearly change in lumbar spine bone mineral density (BMD) was inversely related to age and BMD at treatment initiation. Mutations associated with a more severe phenotype exhibited an increased response after 2 years; however, all types of OI responded well.

To investigate the effect of naturally occurring variations in collagen I, the only common coding single nucleotide polymorphism (rs42524 in COL1A2) was genotyped in 2004 healthy men in Paper II. Heterozygous genotype was associated with decreased BMD and an increased risk of stroke.

An adolescent with repeated fractures despite a markedly high BMD harbored a unique C-terminal procollagen cleavage-site mutation in COL1A1, which motivated extensive investigations in concert with a similar COL1A2 case in Paper III. The probands were found to have impaired procollagen processing, incorporation of collagen with retained C-propeptide in matrix and increased mineral to matrix ratio, which demonstrates that C-propeptide cleavage is crucial to normal bone mineralization and structure.

Bisphosphonate therapy has insufficient effect in OI, and as classical OI is a dominant disorder severe cases would benefit from silencing of the mutated allele. In Paper IV and V small interfering RNAs (siRNAs) were used to allele-specifically target primary human bone cells heterozygous for I) a coding polymorphism in COL1A2 and II) insertion/deletions in the 3’UTR of COL1A1 and COL1A2. Results were promising with altered allele ratios and decreased mRNA levels in the predicted fashion.

To summarize, this thesis found that collagen I is crucial to bone and connective tissue and that collagen I mutations create markedly diverse phenotypes. Age, BMD and pharmaco-genetic effects influence the response to bisphosphonate therapy in individuals with OI; however, novel approaches are needed. Utilizing allele-specific siRNAs may be a way forward in the treatment of severe OI.

Keywords: OI, BMD, Genotype, Phenotype, Pharmaco-genetics, Bisphosphonate, Therapy, Gene-therapy, Mutation, Collagen, Collagen type I, Allele-specific silencing, siRNA, RNAi, COL1A1, COL1A2, Stroke, C-propeptide, Mineralization, Heterozygous disadvantage

Katarina Lindahl, Uppsala University, Department of Medical Sciences, Metabolic Bone Diseases, Akademiska sjukhuset, SE-751 85 Uppsala, Sweden.

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ISSN 1651-6206
ISBN 978-91-554-8772-0
urn:nbn:se:uu:diva-208942 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-208942)
For my three sparkling angels
Filippa, Alexander and Eleonora
You light my way
Cover: Fluorescence microscope image of Cy3-labeled COL1A1 insertion targeting siRNAs in primary bone cells overlaid with silenced vs. non-silenced COL1A1 3’UTR indel sequence chromatograms from a heterozygous individual and the sequence of a heterozygous COL1A1 mutation causing OI type III, a potential candidate for siRNA treatment. The blue ovals are DAPI-stained cell nuclei and the pink specks are Cy3-labeled siRNAs in the cytosol of MATra transfected cells.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I) **Lindahl K**, Åström E, Rubin C-J, Söderhäll S, Malmgren B, Kindmark A, Ljunggren Ö. Genotype-Phenotype Correlations, Response to Bisphosphonate Treatment and Pharmaco-genetics in 150 Swedish Families with Osteogenesis Imperfecta (Type I, IV and III). *Manuscript*


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## Abbreviations

### General abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>OI</td>
<td>Osteogenesis imperfecta</td>
</tr>
<tr>
<td>DI</td>
<td>Dentinogenesis imperfecta</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual X-ray absorptiometry, a way to measure BMD</td>
</tr>
<tr>
<td>T-score</td>
<td>DXA result/BMD measurement, stated as +/- standard deviations compared to a young healthy reference population. Used to define osteoporosis.</td>
</tr>
<tr>
<td>Z-score</td>
<td>DXA result/BMD measurement, stated as +/- standard deviations compared to an age-, gender- and ethnicity-matched cohort. Used e.g. for children or individuals with OI.</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>Osteoblast</td>
<td>Cell responsible for bone formation</td>
</tr>
<tr>
<td>Osteoclast</td>
<td>Cell responsible for bone resorption</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA (synthesized from mRNA)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism, common genetic variation</td>
</tr>
<tr>
<td>Indel</td>
<td>Insertion/deletion in a gene or gene region</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction, increases amount of a defined DNA sequence</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR, yields information on initial amount of DNA</td>
</tr>
</tbody>
</table>
### Abbreviations for genes and proteins associated with osteogenesis imperfecta

<table>
<thead>
<tr>
<th>Protein/Chain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I/Collagen I</td>
<td>A heterotrimer consisting of two α1-chains and one α2-chain (classic OI type I-IV)</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Gene coding for collagen type 1 alpha 1</td>
</tr>
<tr>
<td>COL1A2</td>
<td>Gene coding for collagen type 1 alpha 2</td>
</tr>
<tr>
<td>Coll1α1/Col1a1/α1(I)/α1-chain</td>
<td>Protein: collagen type 1 alpha 1</td>
</tr>
<tr>
<td>Col1α2/Col1a2/α2(I)/α2-chain</td>
<td>Protein: collagen type 1 alpha 2</td>
</tr>
<tr>
<td>IFITM5</td>
<td>Gene coding for interferon induced transmembrane protein 5, involved in bone formation (OI type V)</td>
</tr>
<tr>
<td>SERPINF1</td>
<td>Gene coding for pigment epithelium-derived factor, involved in bone mineralization (OI type VI)</td>
</tr>
<tr>
<td>CRTAP</td>
<td>Gene coding for cartilage-associated protein (CRTAP), involved in hydroxylation of certain collagen proline residues (OI type VII)</td>
</tr>
<tr>
<td>LEPRE</td>
<td>Gene coding for prolyl 3-hydroxylase (P3H1), involved in hydroxylation of certain collagen proline residues (OI type VIII)</td>
</tr>
<tr>
<td>PPIB</td>
<td>Gene coding for cyclophilin B (CyPB), involved in C-terminal folding, trimer and helix formation and hydroxylation (OI type IX)</td>
</tr>
<tr>
<td>SERPINH1</td>
<td>Gene coding for heat shock protein 47 (HSP47), a collagen chaperone (OI type X)</td>
</tr>
<tr>
<td>FKBP10</td>
<td>Gene coding for 65kDa FK-binding protein (FKBP65), a collagen chaperone (OI type XI)</td>
</tr>
<tr>
<td>SP7/OSX</td>
<td>Gene coding for osteoblast transcription factor, osterix (OI type XII)</td>
</tr>
<tr>
<td>BMP1</td>
<td>Gene coding for bone morphogenetic protein-1 (BMP1), responsible for C-terminal cleavage of collagen (OI type XIII)</td>
</tr>
<tr>
<td>TMEM38B</td>
<td>Gene coding for a channel involved in Ca^{2+} release (OI type XIV)</td>
</tr>
<tr>
<td>WNT1</td>
<td>Gene coding for Wnt1, inducer of a major pathway in bone formation (OI type XV)</td>
</tr>
<tr>
<td>CREB3L1</td>
<td>Gene coding for OASIS, a regulator of type I procollagen (proposed OI type XVI)</td>
</tr>
<tr>
<td>PLS3</td>
<td>Gene coding for plastin 3, may influence mechanosensing properties of osteocytes (proposed OI type XVII)</td>
</tr>
</tbody>
</table>
Collagen I - One α-chain (left), triple helix of three α-chains (middle) and glycine residues, colored red, facing confined center of triple helix (left).
Introduction

The earliest known studies of osteogenesis imperfecta (OI) began with the Swedish army surgeon Olof Jakob Ekman (1764-1839) who studied at Uppsala University under the supervision of Johan Gustaf Acrel (1741-1801). Ekman defended his thesis “Dissertatio medica - Descriptionem et causus aliquot osteomalaciae sistens” (“Medical dissertation - Describing some cases with softening of bone tissue”) [1] on May 10th, 1788 in the still standing anatomical theatre Gustavianum in Uppsala, Sweden (Figure 1). In this thesis he described the condition and mentioned cases of OI from 1678. Subsequently one of many historic names for this disorder is Ekman syndrome; others are Ekman-Lobstein syndrome, Vrolik syndrome, and glass-bone and brittle bone disease. Evidence of OI was found as early as 1000 B.C. in an ancient Egyptian mummy [2], and it is believed that the Viking Ivar the Boneless (approximately 830 A.D.) may also have suffered from this disorder.

Figure 1 The front page of the first thesis on OI (left) and the still standing anatomical theatre “Gustavianum” in Uppsala, Sweden (right) where the thesis was defended in 1788. (Picture is a scan of the original thesis, courtesy of Uppsala University Library)

OI is a rare heterogeneous genetically inherited disease, with severity spanning from subclinical to perinatal lethal and an incidence between 1/10 000-1/20 000 [3-6]. It is a generalized disorder of connective tissue since it affects collagen type I, the most abundant collagen in the human body, found
in e.g. bone, skin, blood vessels and tendons. However, the cardinal symptom is bone fragility and to understand OI it is necessary to be acquainted with the anatomy, physiology, and genetics of bone.
Bone

More than half a century ago Dr. A. M. Cooke vividly depicted the skeleton as a lively tissue in the medical journal “The Lancet”, an image that is still contrary to common belief. He wrote “The skeleton, out of sight and often out of mind, is a formidable mass of tissue occupying about 9% of the body by bulk and no less than 17% by weight. The stability and immutability of dry bones and their persistence for centuries, and even millions of years after the soft tissues have turned to dust, give us a false impression of bone during life. Its fixity after death is in sharp contrast to its ceaseless activity during life.” [7]

The 206 bones of the adult human skeleton are highly active tissues, responding every second to circulating levels of Ca\(^{2+}\) and phosphate, repairing microfractures, and constantly remodeling to meet the needs of the organism. The bones house hematopoietic tissue and act as levers for the muscles controlling our extremities. Furthermore, the skeletal framework gives us the ability to stand straight and it protects vital body parts such as the brain, spinal cord, and our thoracic organs.

The content of bone

Skeletal tissue consists of approximately 50-70% mineral, 20-40% organic matrix, 5-10% water, and <3% lipids [8]. Collagen type I comprises 80-90% of the organic matrix [9,10] on which mineral, hydroxyapatite (Ca\(_{10}(PO_4)_6(OH)_2\)), can crystallize. In the skeleton the Ca\(^{2+}\) is sometimes substituted for other ions such as magnesium (Mg\(^{2+}\)), and the mineral can contain impurities such as carbonate, its extent depending on environment and diet [8,11]. These imperfect crystals are smaller and more soluble than apatite in nature and the minerals are thus rapidly accessible for the body’s essential ion homeostasis. Collagen I to bone is like steel-reinforcing bars to a concrete building, it permits resilience to torsion and bending powers and renders bone toughness [12,13], whereas hydroxyapatite crystals give bone the strength to withstand compression and the mechanical rigidity to protect vital organs and act as levers for muscles [14,15].
Cortical and trabecular bone

Cortical (80%) and cancellous/trabecular (20%) bone are the two types of mature bone tissue in human beings. Cortical bone, found in the shafts of our long bones and in the outer layer of most bones, is dense and solid. Cancellous bone is honeycomb-like, houses the bone marrow, and is found in our vertebrae and in the meta- and epiphyses of long bones. Trabecular bone has a high turnover rate, about 25% yearly, and thus has an important role in mineral metabolism, whereas cortical bone has a low turnover rate, less than 5% yearly, and primarily serves a mechanical purpose [16].

Both trabecular and cortical mature bone have similar organization microscopically (Figure 2). The collagen I is highly organized in parallel fibrils which make up thin sheets, lamellae, where the direction of each lamella is oblique to the layer above and below in cross-section. These lamellae can be organized in a porous fashion, oriented along the lines of stress as is the case for the coral-like trabecular bone or organized in bundles in a straw-bunch like fashion, where each straw is denoted “osteon”, as is the case for cortical bone. The center of an osteon, also called “Haversian system”, hosts vessels and nerves and is referred to as a “Haversian canal”. In cortical bone the Haversian systems generally follow the long axis of the bone and in cancellous bone the lamellae follow the direction of each trabecula.

Figure 2. Illustration of the organization of compact (cortical) and cancellous bone with arrows pointing out important structures. (Picture from Wikipedia)
**Bone cells**

There are four main cell types in bone: osteoclasts, osteoblasts, bone lining cells, and osteocytes. The osteoblast and osteoclast are tightly coupled in bone remodeling, which is described below. Bone lining cells, residing on the bone surface, are differentiated osteoblasts, and it has been suggested that these cells can be reactivated to osteoblasts by parathyroid hormone (PTH) [17]. Bone lining cells may potentially function as a barrier for inappropriate preosteoclast interaction with the bone surface, whereas under the appropriate circumstances they may orchestrate osteoclast activation [18]. The osteocyte is the terminal differentiation stage of the osteoblast and it supports bone structure and has a metabolic function. It resides in lacunae embedded deep in bone tissue and has numerous cellular extensions called filapodia that lie in canaliculi. These extensions are in contact with other osteocytes and create a network that ensures contact with the surface bone lining cells and nutrients as the bone mineralizes; moreover, they enable communication between cells. The osteocyte is thought to act as a mechanosensor, signaling the need of repair and affecting bone remodeling [19, 20], and it is the exclusive producer of sclerostin, an inhibitor of bone formation described below [21].

**Bone remodeling and modulators thereof**

**The osteoclast**

From fetal life until death bone is constantly being remodeled; old bone is broken down and new bone is formed by a group of cells denoted the bone remodeling unit (Figure 3). Bone remodeling is initiated by the recruitment of mononucleated osteoclast precursor cells from the monocyte macrophage lineage in the circulation. These cells attach to bone and form a giant multinucleated osteoclast (Figure 4) that seals off a section of bone through a “ruffled border”. Proton pumps are activated to acidify the sealed space and enzymes, namely cathepsin K [22], are secreted and dissolve the underlying bone. This resorptive phase ends with osteoclast apoptosis.
The bone remodeling unit - Osteoclasts are recruited and seal off an area of bone through a “ruffled border”, creating an acidic environment and releasing enzymes that dissolve the underlying bone; a resorption lacuna is formed. Osteoblasts are then attracted and activated to produce osteoid, which is subsequently mineralized and new bone is formed. Some of the osteoblasts will be terminally differentiated and embedded deep in bone tissue as osteocytes. (Picture courtesy of Navya Laxman PhD-student)

The differentiation and activation of multinucleated osteoclasts depends mainly on the binding of receptor activator of NF-κB ligand (RANKL) produced by osteoblasts, bone marrow stromal cells and lymphocytes, to its receptor RANK [23,24]. RANKL has a natural decoy receptor, osteoprotegerin (OPG). In e.g. menopause or other conditions with sex hormone decrease [25] or during treatment with glucocorticoids, bone resorption is increased by a decrease of OPG in relation to RANKL, which stimulates the differentiation and activation of osteoclasts. Denosumab is a fully human monoclonal antibody against RANKL and a relatively new osteoporosis drug, which inhibits osteoclasts formation and function, leading to increased bone mineral density (BMD) and decreased fractures [26]. Odanacatib, a cathepsin K inhibitor, is another potential osteoporosis therapeutic, still being evaluated in clinical trials [27]. (Figure 4)
**Figure 4. The osteoclast** - The main signaling pathway of the osteoclast is stimulated by RANKL binding to its receptor RANK, a signaling cascade will ensue that orchestrates osteoclast differentiation and activation. The balance between RANKL and the decoy receptor osteoprotegerin (OPG) determines degree of stimulation. The relatively new osteoporosis therapeutic denosumab is a fully human monoclonal antibody that acts as OPG and inhibits RANK signaling and thus inhibits the osteoclast. Another potential new osteoporosis drug is odanacatib, which blocks the action of cathepsin K, an enzyme active in bone resorption.

The osteoblast

The released cytokines from osteoclast resorption attract cells from the vicinity of the dissolved bone, which populate the resulting lacuna, and bone formation is initialized by not fully understood coupling signals [28,29]. Osteoblasts are recruited and activated, produce unmineralized osteoid (collagen I and bone matrix proteins), and then regulate matrix mineralization in concert with osteocytes [30,31], the terminally differentiated osteoblasts embedded in matrix. Some of the remaining osteoblasts become bone lining cells, while the fate of the majority (50-70%) is apoptosis [32]. Osteoblast function is stimulated by vitamin D, pulsatile PTH, and Wnt signaling, which is the major anabolic pathway in osteoblasts (Figure 5). When Wnt binds to its receptor LRP5/6 the amount of the transcription factor β-catenin ultimately increases and upregulates transcription of anabolic osteoblast genes, and bone formation ensues. Sclerostin and dickkopf-1 (Dkk-1) are Wnt inhibitors, which act through blocking Wnt from binding its receptor LRP5/6. Sclerostin is produced by the bones’ mechanosensors osteocytes; microcracks or need of bone remodeling decrease osteocyte production of
sclerostin and subsequently increase Wnt-signaling and bone formation. In a young and healthy organism, the bone formation in the osteoclast-created lacuna proceeds until there is no net bone loss; however, after the plateau following peak bone mass or in certain diseases, net bone loss may lead to osteoporosis. It is believed that this bone remodeling cycle has several functions including calcium and phosphate homeostasis, microfracture repair and maintenance of bone health to prevent injury. There are ongoing trials with sclerostin antibodies as a new anabolic osteoporosis therapy, and results in a phase-1 study were promising [33]. There are also preclinical trials with an antibody neutralizing Dkk-1. (Figure 5)

Figure 5. The osteoblast – Wnt signaling is the main anabolic pathway in the osteoblast. When Wnt binds to its receptor LRP5/6 the level of B-catenin ultimately increases and enhances transcription of anabolic osteoblastic genes, promoting bone formation. Signaling can be inhibited by dickkopf-1 and sclerostin, produced by osteocytes, and new osteoporosis therapeutics are being developed to target these inhibitors of the Wnt pathway.
Bisphosphonates affect bone remodeling

Bisphosphonates are the standard of care for treating osteoclast-mediated bone loss in osteoporosis, metastatic and glucocorticoid mediated bone loss, and Paget’s disease; moreover, they are the main pharmacological agent used in osteogenesis imperfecta. Bisphosphonates are derivatives of pyrophosphate (PPi) (Figure 6), which is naturally released in many of the body’s biochemical reactions and has a high affinity for hydroxyapatite. The extremely high affinity of bisphosphonates for bone causes them to be deposited in the skeleton in a high concentration or secreted in urine, whereas other tissues remain essentially unaffected. Modern bisphosphonates have a nitrogen-containing side group in position R1, while the side group in the R2 position determines the specific characteristics of the compound, such as antiresorptive potency [34].

![Figure 6. Schematic molecular structure of bisphosphonates compared to pyrophosphate. The two side groups (R’ and R”) determine the specific characteristics of the particular bisphosphonate, such as potency and mechanism of osteoclast inhibition.](image)

Osteoclasts internalize bisphosphonates on the bone surface by endocytosis, and once intracellular the compound inhibits a key regulatory enzyme, farnesyli pyrophosphate synthase, in the mevalonate pathway. Important cellular mechanisms are disrupted, e.g. formation of the ruffled border and stress fiber assembly, and ultimately this disturbs the action of the osteoclast and may lead to apoptosis [34-36]. Osteoblast activity is not suppressed, at least initially, and therefore bone formation proceeds with a net bone gain. Oral and intravenous bisphosphonates have been shown to increase BMD and decrease osteoporotic fractures in vertebrae, hip, and forearm [37-39]. Prevalent adverse effects for oral administration are indigestion and, if not taken in upright position with enough fluid, esophageal inflammation or even erosion. Intravenous administration often causes a flu-like acute phase reaction after the first dose; however, the risk lessens for every successive administration [39]. Serious adverse effects are few and uncommon and include osteonecrosis of the jaw, atypical femur fractures, and ocular inflammation.
Bone and calcium homeostasis

Skeletal tissue is the largest reservoir of Ca\(^{2+}\) in the body. Regulating the serum and cellular level of calcium is of utmost importance to the organism because this ion regulates many biochemical reactions. Low levels of Ca\(^{2+}\) stimulate the release of PTH from the parathyroid, and PTH subsequently influences kidney, intestine, and bone to elevate the serum level of Ca\(^{2+}\) through decreased loss and increased uptake and release from skeletal tissue. In bone continuously elevated PTH stimulates the production of RANKL, which as described above binds to and activates the osteoclast. The ensuing bone resorption increases the serum level of Ca\(^{2+}\).

Osteoporosis

Osteoporosis is a major health problem, causing peripheral fragility fractures and vertebral compression fractures in individuals with too little or fracture prone bone. It is a disease primarily of the elderly and has only recently received due attention; osteoporosis was not even recognized as a disease by the WHO until 1994 [40]. Common osteoporosis associated fractures are hip, vertebral, and distal forearm fractures. Hip and vertebral fractures have been found to increase mortality; 5-year survival rate was found to be only 80% of expected [41]. All types of osteoporotic fractures have been shown to affect 7% of survivors, leading to some degree of permanent disability [42]. The major predictors of primary osteoporosis are age and gender, whereas those of secondary osteoporosis are various diseases, medications, life-style factors, and nutritional deficiencies. Osteoporosis is a socioeconomically costly disease; approximately one-third of women and one-fifth of men between the ages of 70-79 suffer from osteoporosis [43-46] and fracture patients often need surgical care, long hospital stays, and rehabilitation [47].

Bone mass and bone loss is generally estimated by dual X-ray absorptiometry (DXA), which measures areal BMD. BMD accounts for about 60-70% of the variation in bone strength [48-52]. WHO criteria for postmenopausal osteoporosis is a BMD less than -2.5 standard deviations (SD) compared to a young, healthy, and gender matched control group (denoted T-score), while osteopenia is defined as a BMD T-score between -1.0 to -2.5 SD. When comparing individuals with an age, gender, and ethnically matched population the term Z-score is used, and this measure is the most useful for e.g. assessments of pediatric populations and individuals with OI.
Figure 7. DXA scan of whole body (left), lumbar spine (L1-L4) (middle), and total hip (right). Lumbar spine alongside total hip are two of the most common measuring sites for an osteoporosis diagnosis.
DNA and the genetics of bone

DNA

Deoxyribonucleic acid (DNA) is the backbone of life. It resides in the nuclei of our cells and can be compared to an enormous library, storing all of the information needed to create and maintain a living organism. Some regions of our DNA encode messenger ribonucleic acid (mRNA), which are translated into proteins that build and produce substances creating and sustaining life, while other parts orchestrate protein production or regulate the genetic information.

In the cell nucleus, DNA is organized in structures called chromosomes, of which we have 46 in most cells. Chromosomal DNA is an enormous, double-stranded molecule consisting of four different nucleotides abbreviated A, T, C, and G, which are bound together by a backbone of sugar and phosphate in seemingly endless combinations creating the genetic code (Figure 8). Some parts of the DNA encode distinct genes, which translate into proteins such as collagen. Every triplet of coding nucleotides, e.g. ATC, GGC or GTA, is denoted a codon and determines which amino acids the cell machinery should incorporate into the protein under translation.

Figure 8. DNA is an enormous double-stranded molecule consisting of combinations of four nucleotides bound together by a backbone of sugar and phosphate. The nucleotides (abbreviated A, T, C, and G) create the genetic code. (Picture from Internet)
Single nucleotide polymorphisms – Normal variation

Substantial genetic differences, such as having or not having a Y-chromosome, create considerable contrasts between individuals. However, slight variations in the DNA code between individuals act in concert with the environment to produce normal variation in different traits, for example physical appearance, personality, and disease susceptibility. The most common type of these subtle interindividual DNA variations are called single nucleotide polymorphisms (SNPs), and act through e.g. variation in regulating elements, affecting amount or function of a protein produced from a certain gene or have no known impact at all. Two human chromosomes differ due to an SNP on average every 1000-2000 base pairs [53], which is often when considering the length of the haploid human genome (23 chromosomes) is thought to be around 3 billion base pairs [54]. These positions are being thoroughly investigated because they explain many differences in human traits in sickness and health and may influence the response to for example medical treatment. The definition of an SNP is often stated as a minor allele frequency of >1%, whereas sequence variations with frequencies <1% are defined as mutations, and although this concept is often associated with disease the opposite is possible. Mutations are what have propelled evolution; mutations associated with a benefit for the organism will have been favorable for survival and reproduction.

SNPs and mutations in collagen type I often have bone phenotypes

Family and twin studies have found that BMD is under strong genetic control [55-59]. Genetic variants that predispose to interindividual differences in bone traits may reveal so far unknown cellular pathways affecting bone and could lead to novel therapeutics for bone disease, or to the possibility of earlier identification of individuals at risk of developing osteoporosis, or both. Collagen type I is a highly conserved, repetitive protein and known monogenetic diseases due to mutations in this protein include Ehler-Danlos disease and OI. Because the organic matrix of bone consists mainly of collagen I, investigating SNPs in the two coding genes COL1A1 and COL1A2 has received due interest, and these are two of the most extensively studied genes in relation to bone phenotypes. The collagen type I SNP that has received the most attention so far is commonly referred to as Sp1, a binding site in the promotor region of COL1A1. Sp1 genotype is believed to influence the binding affinity of a transcription factor which determines amount of COL1A1 mRNA transcribed and can predict BMD [60] in healthy individuals, as well as osteoporotic fracture independently of BMD [61-64].
Osteogenesis imperfecta – A brittle bone disease

OI is a heterogeneous connective tissue disorder with a point prevalence at birth estimated to 1/10 000 - 1/20 000 [3-6] and disease severity spanning from subclinical osteoporosis to intrauterine lethality. The most common cause (>90%) is a dominant mutation in one of the two genes encoding collagen type I (COL1A1 and COL1A2) [65]. However, in recent years the molecular background of one X-linked, one non-collagen dominant, and eleven recessive forms of OI has been described [66-87]. The cardinal symptom of OI is bone fragility with subsequent fractures, bone deformity, and growth retardation. Nonetheless, collagen type I is the most abundant collagen and present in substantial amounts also in blood vessels, tendons, and skin rendering OI a generalized connective tissue disorder. Thus, individuals affected may have joint hypermobility, increased bruising and bleeding, scoliosis, and cardiac valvular disorders [88]. Other common features are dentinogenesis imperfecta (DI), blue sclerae, triangular facies, basilar impression, wormian bones, and hearing impairment [88] (Figure 9). Low BMD is typically found and to a certain extent negatively correlated to severity [89]; mild OI often leads to BMD Z-scores between -1 to -2, whereas severe OI generally leads to Z-scores between -3 to -6.

OI classification

OI has traditionally been classified according to Sillence (type I-IV) based on clinical, radiological, and hereditary findings [88,90-92], and this system is still attractive in the clinical setting and used worldwide. However, the International Skeletal Dysplasia Society stated in 2010 that it is not reasonable to maintain tight correlations between “Sillence types” and their molecular cause; rather they recommended preserving the essence of the Sillence classification to describe the phenotypic severity in OI [93]. As molecular diagnosis has become more readily available and the genetic panorama has extended to recessive, X-linked, and noncollagenous dominant variants of OI, it is currently debated how OI would best be classified in the future. To date, Roman numerals have been added to the original four classes for every new gene shown to cause an OI phenotype. Certain types were categorized
based on distinct clinical or histomorphometric phenotypes or both prior to the discovery of their genetic background, which is why the chronological order of discovered genes is not in strict accordance with the Roman numerals.

Silence classification (type I-IV)
The dominant collagen cases together comprise approximately 90% of OI.

OI type I
The mildest and most common (60-80%) form of OI [94]. Fractures often decrease after puberty and may increase again at an older age, especially in postmenopausal women. Blue sclerae are the rule and hearing impairment in adulthood is commonly found. DI may be present and a division can be made between IA (normal teeth) and IB (DI). These individuals reach near normal stature and have an average life-span expectancy [95]. This form is predominantly caused by a null allele mutation in COL1A1 and some advocate this should be a requirement for type I [66]; however, a mild helical glycine mutation may have the same phenotype clinically.

OI type II
Type II is a lethal form of OI and represents about 5-10% of cases [3,92,96]; however, incidence is difficult to accurately state because these cases may lead to spontaneous abortion or planned termination and might never be recorded in a register. Infants are stillborn or die within days or weeks from respiratory failure due to a small thorax and rib fractures affecting breathing or causing pneumonia. Tissues are extremely fragile and the head or a limb may be torn during delivery [97]. Qualitative collagen mutations are generally the cause; however, recessive mutation can also have a lethal phenotype [98] that is difficult to distinguish clinically.

OI type III
Type III is severely deforming and the most serious form of OI compatible with surviving the neonatal period and accounts for about 10% of OI [3,96]. An individual with OI type III may suffer hundreds of fractures and often has extremely short stature, blue/gray sclerae, severe scoliosis, DI, and a triangular facies. Ambulation is not always achieved and an electric wheelchair is a necessity. These patients have a decreased life expectancy, generally caused by increased pulmonary and cardiovascular mortality or less frequently due to basilar invagination [95,99]. As for type II, qualitative colla-
gen defects have traditionally been considered the cause although the recently discovered recessive and noncollagenous dominant types may historically have been denoted type III and still may be in the clinical setting.

**OI type IV**

Type IV OI spans between types I and III in severity and represents about 10% of OI [3,96]; however, distinguishing between types I and IV and III and IV may be difficult and this is a very heterogeneous group. Scleral hue is variable in childhood, but according to the original definition should be normal in adulthood. DI is often present and as for type I subdivides type IV into A (no DI) and B (DI). Individuals often have a short stature but attain ambulation. Life expectancy is near normal [95].

**Additional classification (type V-XV)**

The noncollagen cases together comprise approximately 10% of OI.

**OI type V**

Individuals with OI type V would previously have been categorized as type IV, but are distinguished by hypertrophic callus formation, radio-opaque bands adjacent to growth plates on X-ray, calcification of the radio-ulnar interosseous membranes, and a typical mesh-like appearance on microscopy. It was recently discovered that this phenotype is caused by a dominant mutation in the 5′ untranslated region of *IFITM5*, coding for interferon-induced transmembrane protein 5, restricted to skeletal tissue and involved in bone formation [68].

**OI type VI**

OI type VI causes moderate to severe disease and is distinguished from patients with type IV by "fish-scale" bone appearance when examined histologically. A recent publication described this to be the cause of recessive mutations in *SERPINF1* that encodes pigment epithelium-derived factor, involved in bone mineralization [100].

**OI type VII**

Type VII is a severe to lethal recessive form of OI, overlapping with Sillence types II-III clinically. This type is caused by mutations in *CRTAP*, encoding cartilage associated protein (CRTAP), belonging to a collagen-modifying
complex that hydroxylates specific proline residues, namely Pro986 in the α1-chain [72,101]. The biological effect of this is uncertain.

OI type VIII
OI type VIII is clinically indistinguishable from type VII, but is caused by recessive mutations in another protein, P3H1 (encoded by LEPRE) that is part of the same complex as CRTAP (above) [73,102].

OI type IX
Individuals with OI type IX, due to recessive mutations in cyclophilin B (CyPB) (encoded by PP1B) [103,104], have multiple long-bone fractures but often attain ambulation. Type IX is moderate to lethal, and thus encompasses a less severe spectrum than types VII and VIII [105] despite CyPB being part of the same collagen-modifying complex as CRTAP and P3H1.

OI type X
Type X is a severe to lethal form of recessive OI that is caused by mutations in SERPINH1, encoding the collagen chaperone heat shock protein 47 (HSP47), and has been described to be associated with lung disease and renal stones [76]. HSP47 may form an ER-complex with FKBP65 (below); however, this has not been determined with certainty.

OI type XI
Individuals with OI type XI have a progressively deforming disorder and recessive mutations have been localized to FKBP10 coding for FKBP65, a collagen chaperone important for telopeptide lysyl hydroxylation [75,79,83]. The phenotype is overlapping with mutations in PLOD2 that can cause an OI-Bruck syndrome phenotypic spectrum [81].

OI type XII
Type XII is a moderate to severe recessive form of OI due to mutations in the gene SP7/OSX, which encodes an osteoblast specific transcription factor, osterix [77]. Clinical features include recurrent fractures, mild bone deformities, delayed tooth eruption, and normal hearing.

OI type XIII
OI type XIII is caused by recessive mutations in bone morphogenetic protein-1 (encoded by BMP1) that cleaves the C-terminal globular end off pro-
collagen in the pericellular space. The phenotype is severe [80], in contrast to the herein described patients with heterozygous C-terminal cleavage site mutations (Paper III).

**OI type XIV**
Type XIV is a moderate to severe OI type due to recessive mutations in the gene *TMEM38B*, which encodes a channel involved in Ca\(^{2+}\) release from intracellular stores that has been shown to act in cell differentiation. The molecular mechanisms leading to an OI phenotype are unknown. Individuals have osteopenia and fractures, normal sclerae and teeth, and no progressive hearing loss or other organ involvement [84,85].

**OI type XV**
OI type XV is moderately to progressively deforming and induced by recessive mutations in *WNT1*, encoding Wnt-1 that is a key player in the major pathway of bone formation and maintenance. Blue sclerae can be seen and this type may be associated with brain anomalies. Teeth and hearing are reported to be normal. Heterozygous *WNT1*-mutation carriers have been shown to be affected by early onset osteoporosis [70,71].

**Proposed OI type XVI**
Homozygous genomic deletion of the ER-stress transducer OASIS (encoded by *CREB3L1*) was shown to cause severe OI in two out of three children in a Turkish family from neighboring villages in a recent publication [86]. OASIS is a regulator of type I procollagen and facilitates secretion of matrix proteins. Heterozygous carriers were described to exhibit certain features of type I OI, however no fractures. This mutation has not been defined to cause an OI type in OMIM to date; however, OI XVI is rational based on publication date.

**Proposed OI type XVII**
Hemizygous mutations in *PLS3* (coding for plastin 3, involved in F-actin formation) were found in males with childhood osteoporosis and in males with suspected OI type I without mutations in *COL1A1* or *COL1A2* [87]. The clinical picture in heterozygous women ranged from normal to early onset osteoporosis. The exact mechanism through which mutations in *PLS3* cause bone fragility is not known; however, F-actin may influence the mechanosensing properties of osteocytes. This mutation has not been defined to cause an OI type in OMIM to date and would be the first X-linked type of OI. OI XVII is rational based on publication date.
Figure 9. Clinical characteristics of OI – (A) An individual with OI type I caused by a *COL1A1* null allele. (B) Blue sclerae in OI type I caused by a *COL1A1* null allele. (C) OI type III in a patient who was treated in utero with mesenchymal stem cells after multiple intra uterine fractures were detected during prenatal ultrasound screening. (D) Hyperextensibility in individual with OI caused by C-terminal cleavage site mutation. (E) Dentinogenesis imperfecta in primary teeth, OI type IV. (F) Dentinogenesis imperfecta in permanent teeth, OI type III. (All pictures were kindly approved by patients and several were courtesy of Eva Åström MD and Barbro Malmgren DDS.)

**Diagnosis**

Severe forms of OI are sometimes detected prenatally by ultrasound or are apparent at birth, whereas milder variants generally are not discovered until birth or in the first years of life. Very mild forms may go undetected. Diagnosis is traditionally made through typical clinical characteristics in combination with increased fracture susceptibility, radiology, and BMD measurements. In typical familial dominant cases the diagnosis can be straightforward; however, many cases are *de novo* mutations and phenotype can vary between generations complicating matters [106]. About 60% of types I and IV, and close to 100% of types II and III have been reported to be *de novo* mutations. It is estimated that parental gonadal mosaicism causes dominant
OI in about 3-5% of cases, which may explain recurrence in siblings [107,108]. Sequencing of genes known to cause OI was previously performed predominantly for research purposes; however, molecular analysis is being performed with increasing frequency in many clinical settings.

Collagen type I – A crucial protein for bone

Collagen type I is the most abundant protein in connective tissue and constitutes 80-90% of the organic bone matrix [9,10]. It is a heterotrimer comprising two $\alpha_1$- and one $\alpha_2$-chains, encoded by two large genes both composed of 52 exons, $COL1A1$ and $COL1A2$ respectively (Figure 10). $COL1A1$ measures 18 kb and is located on chromosome 17, whereas $COL1A2$ measures 37 kb and is located on chromosome 7. Each chain has globular N- and C-terminal sections flanking a highly repetitive helical center of 1014 amino acid residues with triplicate repeats of Gly-X-Y, where X and Y often are proline or lysine residues. The C-terminal ends of the three chains will spontaneously congregate and in a zipper-like fashion a triple helix will form from the C- to N-terminal. The only amino acid small enough to sterically fit inside the tightly packed helix is glycine and therefore the correct positioning of a glycine residue on every third helical amino acid position is a prerequisite for correct folding. Once the helix is formed and posttranslational modifications such as hydroxylation and glycosylation of certain residues are completed, the procollagen molecule is transported to the pericellular space where the N- and C-terminal ends are cleaved off by specific proteases. The remaining fibrillar molecule is incorporated into the highly structured extracellular matrix, covalently linking to neighboring collagen molecules in adjacent and telopeptide regions. This organic frame is subsequently mineralized by hydroxyapatite, making the structure hard and resilient to compression.

Collagen defects in OI

There are two main categories of dominant collagen type I associated OI: quantitative and qualitative collagen defects. The most common cause of OI type I is a quantitative defect with reduced but structurally normal collagen I due to a $COL1A1$ null allele. This can be caused by a premature termination codon or a frame-shift mutation after an insertion, deletion or splice site mutation, all variants leading to nonsense mediated decay (NMD) of $COL1A1$ mRNA [109,110]. Qualitative defects are generally due to the substitution of a helical glycine for a bulkier amino acid residue (80%) or a splice site mutation (20%) that may create e.g. exon skipping or a cryptic splice site [65].
Figure 10. The highly structured architecture of collagen type I - Collagen type I is a helical heterotrimer (2 α1-chains and 1 α2-chain). Several cross-linked helices form collagen fibrils which organized in parallel bundles are denoted collagen fibers. (Picture courtesy of Andreas Kindmark MD and PhD.)

Exchange of glycine for any other amino acid will delay helix formation and lead to chain over modifications [65,111], pathological extracellular matrix structure [65,112,113], compromised cell-matrix [114] and cell-cell interactions [115] and intracellular stress [116]. Mineralization is affected by the primary organic matrix pathology and subsequently OI is associated with changes in the mineral phase of bone as well, with e.g. mineral crystals of abnormal shape, size, composition, and alignment [117-122]. Qualitative collagen mutations due to splicing defects lead to similar dysfunctional pathways. Unusual qualitative variants caused by X- or Y-position mutations and N- and C-terminal mutations have also been described [123-130].

Is there a clear genotype-phenotype relationship?

COL1A1 and COL1A2 are large genes and to date over 1200 mutations have been reported in the collagen data base [131]; however, according to the last large scale OI consortium only about 10% of theoretically possible glycine substitutions have been described [65]. Reported mutations are not randomly distributed, rather there appear to be mutational “hotspots”, and it has been shown that these are often associated with CpG nucleotides where a methylated cytosine is spontaneously deaminated and mutated to thymine [132]. By definition little is known of the nature of the “missing mutations” that should be present under neutral naturally occurring mutation frequencies. By
chance, some mutations may simply not have been present in OI patients referred for sequencing. Furthermore, several ascertainment bias scenarios are possible, e.g. subclinically mild phenotypes or on the other hand phenotypes so severe that they cause early miscarriage and thus are not assessed.

The mutations reported so far delineate a complex relationship between genotype and phenotype, e.g. the proposed “increased severity gradient from N- to C-terminal” theory initially presented has been modified to lethal regions in both chains while it still holds true that N-terminal mutations are generally nonlethal [65]. Identical mutations often have similar phenotypes; however, certain glycine substitutions are reported to cause both lethal and nonlethal phenotypes [65] and severity can vary from mild to severe for the same mutation even within the same family due to unknown modifying elements [106,133]. It has been concluded that structural mutations are more often lethal in COL1A1 than COL1A2 (36% vs. 20%), that zones that align with major ligand binding regions (COL1A1) or proteoglycan binding sites (COL1A2) are predominantly lethal, and that certain amino acid residues (branched and charged) are more likely than others to cause a severe phenotype [65]. There is also evidence that the gene affected and chain location relative to N- to C-terminal influence phenotypes such as DI, blue sclerae and severity of clinical phenotype [134,135]. More genotype-phenotype studies with detailed clinical information are needed to saturate the map of clinical outcome because many previously published studies and reported mutations only have information on OI type.

**Treatment of OI**

**Pharmaceutical agents**

Despite decades of research, there is no satisfactory treatment for severe OI. Stem-cell transplantation [136-138] and various gene therapies [139-143] have been studied yet there are no current clinically available applications. Most centers treat individuals with mild OI conservatively. In moderate to severe cases, with multiple long-bone fractures and/or vertebral compression fractures, bisphosphonate treatment is the standard of care for children and often initiated at a young age, even in infancy. However, less is known about how to best to treat adults with moderate to severe disease.

It has been shown that bisphosphonates increase lumbar spine BMD, ameliorate bone phenotype and improve vertebral geometry concerning height and areal measurements [144-149]. Nonetheless, the euphoric results of initial reports on decreased pain and improved ambulation regrettably have not been possible to support in later controlled trials and data on fracture reduction is conflicting [144,150,151]. Recently a randomised, double-blind, placebo-controlled trial of oral Risedronate in children with predomi-
nant mild OI demonstrated a reduction of clinical fractures [152], yet to date this type of trial has not been performed with intravenous Pamidronate in severe OI and thus there is no confirmed data on fracture reduction. Considering that intravenous bisphosphonates now are the standard of care for severe OI, this type of study may be difficult to perform.

There are more studies of bisphosphonate use in pediatric than adult OI populations and some positive outcomes in children have been difficult to demonstrate in adults [153]. BMD treatment response in relation to BMD at onset and age at initiation has not been thoroughly studied in OI patients; however, there are reports supporting a negative correlation to BMD at onset [147,153,154]. Infants as young as two months have been treated with promising results and safety data [148,149,155,156] and according to one study younger children did not gain as much bone compared to older children, explained by the fact that the deficit in BMD was smaller in younger children [147]. Another study considered the response in infants to be faster and more pronounced than in older children [156]. Furthermore, although the majority of studies of bisphosphonate treatment are on children older than 3 years of age, there is support in observational trials with historical controls for increased BMD, improved vertebral shape and attainment of motor milestones at an earlier age when treating severely affected infants with Pamidronate [148,149].

In Sweden, clinical guidelines recommend severely affected children and milder cases with vertebral compression fractures to be treated at earliest from 3 months of age with monthly infusions of Pamidronate, whereas adults often follow the same regimes as patients with idiopathic osteoporosis. Studies have not shown serious adverse effects of bisphosphonate treatment in children [156-160], but pediatric safety data are inadequate and the use of bisphosphonates is debated [161] due to the concern of inducing e.g. osteomalacia [162], osteopetrosis [163], or osteonecrosis of the jaw. Until more is known of potential benefits of treating mild OI these patients are usually treated conservatively and followed clinically.

Data on the influence of genotype on response to bisphosphonate treatment are scarce. Nevertheless, a study from 2002 concluded that the histomorphometric response to Pamidronate was not influenced by genotype [164], whereas another study from 2006 reported a greater response in growth and increase of BMD in children with mild OI on Pamidronate or Alendronate treatment [165].

Over the years many different treatments have been studied without robust clinical effect despite initially positive publications, e.g. cortisone, vitamin A, vitamin D, fluoride and strontium and hormones such as calcitonin, thyroxine, estrogens and androgens. Combining recombinant growth hormone (rGH) and bisphosphonates is still under investigation and may be beneficial.
for OI types I, IV and III for increased linear growth although these patients are not endogenously GH deficient [166].

Very little is known about benefits of other osteoporosis therapies for OI patients. There are a few published [167] and unpublished studies [168,169] on the effects of Teriparatide, which suggest a positive effect on BMD but no effect on fracture rate and the BMD increase may be lower than in primary osteoporosis. The RANKL antibody denosumab was well tolerated in a small scale study in recessive OI [170] and sclerostin antibodies, an emerging osteoporosis therapeutic, have been shown to act as an anabolic agent in the type III OI murine model Brtl+/− [171].

Physical therapy and orthopedic surgery

Physical therapy from infancy and orthopedic care in the hands of an experienced surgeon is of utmost importance for more severe forms of OI. Protected ambulation is a goal for all individuals because weight bearing is extremely important for bone strengthening. Intramedullary rodding with telescoping rods is often performed and can protect long weight-bearing bones from fracturing, prevent severe deformities and consequently facilitate ambulation [172]. Scoliosis is rarely responsive to bracing [173] and corrective surgery may be needed although optimal technique remains to be established [174]. Stabilization of basilar impression is necessary for some individuals, but rarely progresses to clinically significant compression [175].

Therapeutic vision for OI – Gene therapy using siRNAs

A therapeutic vision is to treat severe dominant OI by silencing the mutated allele with gene therapy. In the case of a COL1A1 mutation the consequence of silencing of the mutated allele would be a COL1A1 null allele and thus a phenotypically mild OI (type I). Heterozygous COL1A2 null alleles are phenotypically normal, and even homozygous COL1A2 null alleles caused by NMD with α1-homotrimers often exhibit only a mild Ehler-Danlos phenotype with hyperextensibility and a propensity to cardiac valvular abnormalities [176,177]. The first steps toward allele-specific silencing in OI were taken in a study utilizing RNA interference (RNAi) to silence COL1A1 in mesenchymal progenitor cells [178] and in a recent publication allele-specific silencing of COL1A1 using short hairpin RNAs (shRNAs) reduced the amount of mutant collagen in Brtl+/− mice, a murine model for classical dominant OI [143]. Silencing by RNAi is studied further in this thesis (Paper IV and V).
RNAi

The attractiveness of RNAi emerges from a high inhibitory activity in concert with specific inhibition [179]. RNAi is the process by which cellular RNA is targeted for degradation on exposure to complementary RNA. Small interfering RNAs (siRNAs) were discovered as endogenous molecules mediating RNAi in plants in 1999 [180], and in 2001 it was shown that exogenous double stranded siRNAs introduced into animal cells efficiently reduced mRNA levels in vitro [181]. Since these original discoveries the siRNA technology has been extensively developed, and siRNAs are now essential tools for creating partial gene knockout in vitro as well as in vivo [182]. The key steps of gene silencing utilizing siRNAs are that a double stranded siRNA is introduced into the cell and is incorporated into a protein complex, the RNA induced silencing complex (RISC) (Figure 11). The passenger strand of the siRNA is subsequently cleaved, while the guide strand remains intact and controls incorporation of complementary target mRNA. This mRNA is cleaved, leaving less intact coding gene mRNA and subsequently protein, rendering the gene “silenced”.

Figure 11. Schematic overview of gene silencing by RNAi via siRNAs – A double stranded siRNA is introduced into the cell and incorporated into the RNA induced silencing complex (RISC). The passenger strand is cleaved, while the guide strand remains in RISC and aids incorporation of complementary target mRNA. Target mRNA is cleaved, thus not expressed, and as a result the coding gene/allele is “silenced”. (Picture adapted from Navya Laxman, PhD-student)
Allele specific silencing

Recent publications report successful allele-specific gene silencing by siRNAs that discriminate between single nucleotide variants within mRNAs [183-191]. These studies suggest that siRNAs may be interesting to explore as therapeutics in dominant monogenic disorders such as vascular Ehler-Danlos, epidermolysis bullosa, and Huntington’s disorder, where the dysfunctional allele could be targeted specifically. The siRNA approach should thus be possible in dominant OI as well. The actual delivery is a challenge and it will be necessary to guide the siRNAs specifically to the target cells in sufficient quantity. Avenues that are being investigated are viral vectors expressing target tissue specific short hairpin RNAs (shRNAs) as well as aptamer-shRNA chimeras [143,192]. Atelocollagen-bound siRNAs may be promising [193] because this complex is resistant to nucleases and is transduced efficiently into cells, thereby allowing long-term gene silencing [194], while showing neither antigenicity nor toxicity in animals [195]. This approach may also avoid the risk of malignant transformation associated with viral vectors.

![Figure 12. Polymorphisms in cis as a handle for RNAi](image)

**Figure 12. Polymorphisms in cis as a handle for RNAi** - Schematic illustration of mutation independent, allele-specific gene silencing utilizing heterozygous polymorphisms in cis position as a handle for RNAi. The SNP in this picture could also be an insertion/deletion polymorphism, as was targeted in Paper V in this thesis. Another siRNA would be developed and used for heterozygous individuals with a mutation on the “A”-allele of the SNP and with these two siRNAs to choose from all heterozygotes for this SNP could be treated.
Target sites for a mutation independent approach in OI

More than 800 qualitative mutations have been described in COL1A1 and COL1A2 [65,131] and thus it would be labor intensive to create mutation unique siRNAs. A mutation independent approach is desirable, and targeting a heterozygous SNP or insertion/deletion polymorphism (indel) in the mutation-affected gene may be the way forward. By allele-specifically targeting both alleles of a common heterozygous position all heterozygous individuals carrying a mutation further away on the corresponding allele (cis-mutation) could be treated, and development of a limited number of siRNAs would potentially treat a majority of patients (Figure 12). This is the approach that has been investigated further in this thesis.

Alternative approaches – Gene and cell-based treatment strategies

Gene therapy

RNAi is the gene therapy approach that has been studied in this thesis; however, other treatment strategies may be alternative or complementary ways forward in different types of OI. For OI type I due to a quantitative collagen defect, gene correction of the mutated allele or enhanced activity of the functioning allele would be necessary. Increased gene activity of a healthy allele in combination with allele-specific silencing of the mutated allele would also avoid a type I phenotype in the case of allele-specific silencing utilizing siRNAs described previously. Moreover, for many recessive disorders even a moderate increase in gene product can have a crucial effect on biological activity and thus may rescue some of the recessive OI phenotypes. The most common approach for this is utilizing a viral vector introducing a cDNA copy of the missing allele, with the largest conferred risk being turning on an oncogene or turning off a tumor suppressor gene. Several studies using viral vectors are ongoing [196]; however, collagen I is a large gene with high expression levels and this approach will most likely not be optimal for classical dominant OI. The most attractive avenue for dominant OI would likely be correction of the mutant allele and subsequent return of the corrected cells to the affected individual, and steps in this direction are beginning to be taken through utilizing e.g. zinc-finger nucleases [197] and TALEN systems [198].

Cell based therapy

Parental somatic mosaicism is thought to cause about 5% of classical OI, and the observation that these mosaic parents are phenotypically normal has sup-
ported different cell-based therapies. It is proposed that the normal osteoblasts of these individuals have an advantage over the osteoblasts producing inferior collagen, and that the normal cells consequently suppress the mutant-carrying ones. Thus, if normal osteoblasts could be introduced to an OI patient, the notion is that they would mimic the situation in a mosaic carrier of OI, with the normal cells outnumbering and out conquering the mutation harboring cells [199,200]. Bone marrow transplantation has been performed in OI patients in studies, aiming at introducing normal osteoblasts through differentiation of mesenchymal stem cells, and there are a few positive reports despite a low number of engrafted cells [136-138]. However, preclinical data and animal studies are lacking, and this is not yet a reasonable clinical approach. The exception may be intrauterine transfusions, where fewer infused cells are filtered out and the immune response is more tolerant, potentially leading to a more beneficial clinical outcome [201,202]. Another field of research is induced pluripotent cells [203], which can be engineered to produce any tissue. In an individual with OI, such cells induced towards the osteoblast lineage would be optimized for bone formation.
Aims of this thesis

The general aim
The aim of this thesis was to describe the spectrum of mutations in collagen type I in individuals with OI in Sweden, and apply this information to genotype-phenotype correlations and pharmaco-genetic studies. Unusual clinical phenotypes or mutations found would be considered for further investigations. As OI is primarily a collagen type I disorder, a study on the effect of the only common coding polymorphism in these genes on healthy subjects was included. Lastly, the goal was to develop a novel treatment approach in OI, through investigating allele-specific gene silencing utilizing siRNA technology in a mutation independent way, targeting heterozygous SNP and indel positions.

The specific aims
Paper I - Genotype-phenotype correlations, response to bisphosphonate treatment and pharmaco-genetics in OI
• To describe the spectrum of mutations in collagen type I in individuals with OI in Sweden.
• To investigate the relationship between genotype and phenotype in this cohort.
• To evaluate bisphosphonate response and pharmaco-genetics in this cohort.
• To extensively study unusual clinical phenotypes or genotypes found in order to further the understanding of pathogenetic mechanisms.

Paper II - Effects of common variation in collagen type I
To investigate the impact of the only common coding polymorphism in collagen type I (rs42524 in COL1A2) on BMD, stroke, and myocardial infarction in healthy subjects, on the grounds that this SNP has previously been associated with intracranial aneurysms.
Paper III - Consequences of inhibited C-propeptide cleavage on bone mineralization and phenotype
To delineate the effect of collagen type I C-propeptide cleavage on bone mineralization and phenotype through clinical, radiological, biochemical, histomorphometrical, and electron microscope conducted studies on two adolescent children, with repeated fractures despite a normal to high BMD, and mutations in the C-propeptide cleavage site of COL1A1 and COL1A2.

Paper IV - Novel treatment approach through mutation independent gene silencing targeting a heterozygous SNP
To develop the concept of gene silencing in primary bone cell cultures, utilizing allele-specific siRNAs in a mutation independent approach by designing siRNAs targeting a heterozygous SNP in COL1A2.

Paper V - Novel treatment approach through mutation independent gene silencing targeting heterozygous indels
To develop the concept of gene silencing in primary bone cell cultures, utilizing allele-specific siRNAs in a mutation independent approach by designing siRNAs targeting heterozygous indels in COL1A1 and COL1A2. Targeting heterozygous indel positions is a completely novel approach, which has not been studied in OI or other dominant diseases.
Subjects and methods

Subjects

Paper I

A total of 202 individuals with OI from 150 unrelated families were recruited mainly from the Uppsala University Hospital Osteoporosis unit, the Astrid Lindgren Children’s Hospital at Karolinska University Hospital, Stockholm and the Eastman Institute, Stockholm. Detailed clinical information was collected on phenotype, heredity, medical history, treatment, and treatment response. The Swedish regional ethics committee at Uppsala University (Ups 06-212) approved the study and written consent was obtained from all participants, a parent or legal guardian signing for minors.

Paper II

The MrOS study is an international multicenter prospective fracture study involving elderly men from Sweden, the United States and Hong Kong. A subsample of 2004 individuals, all participants from the Swedish centers in Uppsala and Malmö, was selected for this study. DNA from 1991 participants was available and these individuals were included in the study. The Swedish regional ethics committee at Uppsala University (Ups 01-057) approved the study and written consent was acquired from all participants.

Paper III

Two children (13-year-old Swedish girl and 14-year-old American boy) with mild OI, normal to high BMD and C-terminal cleavage site mutations in collagen type I were included in the study. The Swedish regional ethics committee at Uppsala University (Ups 06-212) approved the study (Swedish case) and written consent was obtained from parents.
Materials and methods

DNA isolation (Paper I-V)

DNA from peripheral blood was isolated from leucocytes using Wizard® Genomic DNA Purification Kit (Promega) in accordance with the manufacturer’s protocol. DNA from bone and peripheral blood from infants (small amount) was isolated using GenElute™ Mammalian Genomic DNA mini-prep Kit (Sigma) in accordance with the manufacturer’s protocol. DNA concentrations were measured using Nanodrop ND-1000 (NanoDrop Technologies, USA).

Polymerase chain reaction (PCR) and DNA sequencing (Paper I-V)

Polymerase chain reaction (PCR) was used to amplify DNA sequences in preparation for sequencing, primers, and temperatures are listed in the relevant manuscripts. PCR reactions were run on a GeneAmp PCR system 9800 using AmpliTaq® Gold kits and standard reagents. The sequencing reactions were performed with an adjusted BigDye® Terminator 3.1 sequencing protocol. The products were run on a 16-capillary ABI 3130xl Genetic Analyzer automated sequencer and analyzed in software SeqScape® v2.5. All reagents, instruments, and software were purchased from Applied Biosystems, USA.

Illumina sequencing (Paper I)

To reduce sequencing costs and time-consuming Sanger sequencing, an approach involving sequencing of DNA pools using the Illumina Genome Analyzer was developed. The entire sequences of COL1A1 and COL1A2 were amplified using long-range PCR on pooled DNA from patients with OI and subsequently PCR amplicons were gel purified and subjected to nebulization, from which fragments corresponding to approximately 250 base pairs were excised. “Paired-end” sequencing was performed and thereby information was retained concerning sequence 250 base pairs away from any stretch of sequenced nucleotides. This spatial context is highly valuable not only for correct inference of repetitive stretches of DNA, but also to infer structural variations, such as inversions and small and large insertions/deletions.
Multiplex ligation-dependent probe amplification (MLPA) (Paper I)

Multiplex ligation-dependent probe amplification (MLPA) analysis of COL1A1 and COL1A2 was performed on individuals with no mutations found utilizing Sanger sequencing, aiming to detect large deletions and insertions that could have been missed by previous methods. Prefabricated SALSA®-MLPA® Kits P271 COL1A1 and P272 COL1A2 were utilized (MRC-Holland, Holland), and manufacturer’s protocol was followed.

Nomenclature (Paper I)

Sequence variations were described as recommended by the Nomenclature Committee of the Human Genome Variation Society (HGVS) (www.hgvs.org/mutnomen/recs-DNA.html). In brief, mutation numbering was based on cDNA sequences, where “A” of the ATG translation initiation codon was denoted nucleotide “+1” and methionine of the signal peptide was designated amino acid “1”. For introns, numbering was positive (starting at “+1”) from 5' of intron to approximate midpoint and negative (starting at “-1”) from 3' of intron to approximate midpoint. Protein substitution variants were described using the format Xyz1234Xyz.

Dual-energy X-ray absorptiometry (DXA) (Paper I-III)

BMD of the lumbar spine (L1–L4), total hip, femoral neck, trochanter, and total body was measured using a Lunar prodigy dual-energy X-ray absorptiometry (DXA) machine (Uppsala) and a Hologic QDR 4500 system (Stockholm). The T- and Z-scores supplied by the DXA softwares were used for subsequent calculations.

Fluorescence polarization (FP) genotyping (Paper II)

PCR reactions were run as described above. A detection primer was designed and genotyping was performed with a modified protocol of template-directed dye-terminator incorporation fluorescence polarization (FP) [16,17]. The AcycloPrime™-FP SNP Detection Kit (PerkinElmer, USA) was used with R110 and TAMRA-labeled nucleotide terminators. Detection of fluorescence polarization was done on a Criterion Analyst HT System (Molecular Devices, USA). Genotype calling was carried out using clustering analysis, where individual DNA samples were grouped into four categories (homozygous GG, heterozygous GC, homozygous CC or failed/negative samples).
Cell culture (Paper III-V)

Cultures of primary bone-derived cells were obtained from patients undergoing hip and knee replacement surgery or through bone biopsy of the iliac crest. Trabecular bone specimens were cut with a scalpel into small pieces that were rinsed in phosphate buffered saline (PBS), and subsequently placed in culture flasks, allowing primary bone derived cells to migrate from the specimens. The bone pieces were incubated in α-modification of Eagle’s medium (α-MEM) to which calf serum (10%), penicillin, streptomycin, amphotericin-B, and L-glutamine were added. At 75-90% confluence cells were trypsinized and reseeded for amplification or used in experiments. Cultures of skin fibroblasts were obtained from skin punch biopsies, cut into minute pieces, placed in culture flasks and treated as described above.

Cultures of skin fibroblasts were obtained from skin punch biopsies, cut into minute pieces, placed in culture flasks and treated as described above.

Collagen biochemical studies (Paper III)

Steady-state collagen analysis and pericellular processing were conducted as previously described [129]. Briefly, cells were incubated with tritiated proline for 16-18 or 24 h for the steady-state and processing assays, respectively. Collagens were then precipitated and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

For the chain incorporation assay the method of Chessler et al was used [130]. In short, procollagen alpha chains were labeled with a pulse of tritiated proline for 80 min and then chased with DMEM containing 10% serum, 50 µg/ml ascorbic acid, and 10 mM proline. Samples were subsequently ethanol precipitated and procollagens were separated by SDS-Urea-PAGE.

Differential scanning calorimetry (DSC) was analyzed from 10 to 55 °C at 0.125°C/min heating rate in a Nano III DSC instrument (Calorimetry Sciences Corporation, USA), as previously described [204].

*In vitro* assays of procollagen C-proteinase activity (Paper III)

Procollagen C-proteinase (BMP1) activity was determined by measuring the amount of radioactively labeled C-propeptide released from control and patient procollagens, as previously described [205]. Briefly, after incubation of procollagen with BMP1 the free radioactively marked C-propeptide was measured. In a subsequent experiment, procollagen was incubated with BMP1 with or without the enhancer protein PCPE1. Recombinant BMP1
and PCPE1 used in the *in vitro* assay were expressed in HEK-293 cells and purified as described [206].

**Transmission electron microscopy of dermal fibrils (Paper III)**
Dermal punch biopsies, 600-800 Å in thickness, from both patients and an age-matched control were analyzed and photographed by transmission electron microscopy, as described [129].

**Bone histomorphometry (Paper III)**
Iliac crest bone biopsies were obtained after tetracycline double labeling for kinetic analysis. Bone histomorphometry analysis was performed according to Parfitt et al [207] on the whole area of the bone sections.

**Peripheral quantitative computed tomography (pQCT) (Paper III)**
Peripheral quantitative computed tomography (pQCT) was performed using an XCT 2000, Stratec Medizintechnik GmbH, Pforzheim, Germany. Previously published data were used as reference to calculate Z-score [208].

**Fourier transform infrared spectroscopy (FT-IR) (Paper III)**
Fourier transform infrared spectroscopy (FT-IR) spectral images of iliac crest biopsies were recorded on a SpotLight 300 infrared imaging system (Perkin Elmer Instruments, Shelton, CT, USA). The infrared spectrum of an organic compound provides a unique fingerprint, which is readily distinguished from the absorption patterns of other compounds. Cortical and cancellous bone was examined separately in each section. The parameters calculated for each image were as follows: mineral-to-matrix ratio (related to mineral content), carbonate-to-phosphate ratio (level of carbonate substitution in the hydroxyapatite crystal), crystallinity (related to crystallite size/perfection), and collagen maturity.

**Bone mineralization density distribution (BMDD) (Paper III)**
Bone mineralization density distribution (BMDD) was determined in the trabecular bone compartment of iliac crest biopsies using quantitative backscattered electron imaging (qBEI) [209,210]. The qBEI method makes use of the fact that the intensity of electrons backscattered from a depth of 1.5 microns from the surface-layer of a sectioned bone area is proportional to the weight concentration of calcium and thus mineral (hydroxyapatite) in
bone. A digital electron microscope (DSM 962, Zeiss, Oberkochen, Germany) equipped with a four-quadrant semiconductor backscattered electron BE detector was used. Five parameters were deduced from the BMDD curve [211]: CalciumMean (the weighted mean calcium concentration of the mineralized bone), CalciumPeak (indicates the most frequently measured calcium concentration), CalciumWidth (describes the heterogeneity of mineralization), CalciumLow (corresponds to the portion of bone area undergoing primary mineralization), and CalciumHigh (represents the portion of highly mineralized bone matrix).

SiRNA design (Paper IV-V)
SiRNAs were designed according to general guidelines based on empirical evidence [212] and were purchased from Ambion (USA) as double stranded RNA molecules. Each strand of siRNA had a two-basepair overhang in the 3'-end (always UU for sense strand). Negative control siRNAs were prefabricated and purchased from Invitrogen.

Allele-specific silencing by magnet-assisted transfection (MATra) (Paper IV-V)
Human bone derived cells were genotyped for a polymorphic position (position depending on experiment), and 24 h prior to transfection heterozygous cells from one individual were seeded and cultured at 37°C in the presence of 5% CO₂ overnight. Transfection was carried out by magnet-assisted transfection (MATra) (Promokine, Germany), a technique where siRNAs are coupled to magnetic beads and the created complex is magnetically drawn into adherent cells upon being placed on a magnet for 15 min. Dose of siRNA differed between studies and amount of magnetic MATra-beads was determined by the dose of siRNA. Posttransfection, cells were incubated for a varying number of hours depending on study and finally RNA was prepared.

RNA preparation and cDNA synthesis (Paper IV-V)
RNA was prepared using the QiaShredder™ kit and the RNeasy® mini kit (Qiagen, Germany). Each individual RNA sample was subjected to DNase treatment using TURBO-DNAfree™ (Ambion, USA), and equal amounts of RNA were then reversely transcribed with the High Capacity cDNA reverse transcription kit (Applied Biosystems, USA). RNA concentrations were measured using Nanodrop ND-1000 (NanoDrop Technologies, USA)
Cy3 labeling of siRNAs (Paper IV and V)
To verify the delivery of siRNAs to the cytosol of cells, and to determine the transfection efficiency the Silencer® siRNA Labeling Kit from Invitrogen (USA) was used to label siRNAs with Cy3. Transfection efficiency was determined by ocular examination of photographs taken with a fluorescent light microscope, where visible granules in the cytosol counted as a positive cell. The actual efficiency of each individual siRNA was assessed by monitoring the end result (i.e. allele ratio and quantitative sequencing), rather than by determining uptake of Cy3–labeled siRNA for all concentrations.

Quantitative PCR (Paper IV and V)
Quantitative PCR reactions were performed using 2x TaqMan® Universal PCR Master Mix, No AmpErase® UNG mixed with diluted cDNA and gene specific primers, and probes (all from Applied Biosystems, USA), which were subjected to PCR using the 7500 Fast Real-Time PCR System instrument (Applied Biosystems, USA). \(\text{COL1A1/COL1A2}\) mRNA abundance was normalized relative to Glyceraldehyde 3-phosphate dehydrogenase (\(\text{GAPDH}\)) and beta-actin (\(\text{ACTB}\)) mRNA levels.

PeakPicker (Paper IV and V)
To assess the relative allele abundance of \(\text{COL1A1}\) vs. \(\text{COL1A2}\) mRNA the software PeakPicker [213] was used to quantify the ratios of the heterozygote position for all cDNA samples. Briefly, for each individual cDNA-Sanger sequence chromatogram, SNP-peak heights were compared to peak heights of adjacent nonpolymorphic positions. For all siRNA treatments, allele ratios of the heterozygote positions were normalized in relation to the equivalent peak heights of negative control siRNAs, which were defined to have an allele ratio of 1.

Cell proliferation assay (Paper V)
MG-63 cells (osteosarcoma derived cell line) were cultured for 24 h in a humidified chamber at 37°C in the presence of 5% CO₂. Transfection was carried out under identical conditions described above with \(\text{COL1A1}\) or \(\text{COL1A2}\) indel targeting siRNAs, negative control siRNAs, or MATra particles only. Posttransfection, cells were incubated for 72 h at 37°C, after which the level of cell respiration was evaluated by MTS assay, using CellTiter 96®AQueousOne Solution Cell Proliferation Assay (Promega, USA) according to the manufacturer’s protocol.
Results and discussion

Paper I

Genotype-Phenotype Correlations, Response to Bisphosphonate Treatment and Pharmaco-genetics in 150 Swedish Families with Osteogenesis Imperfecta (Type I, IV and III)
K Lindahl et al. Manuscript

In this study the collagen type I genes (COL1A1 and COL1A2) were sequenced in 202 individuals belonging to 150 unrelated Swedish families with OI in order to describe the mutational spectrum of dominant collagen I mutations in Sweden. Extensive clinical data on phenotype, heredity, treatment, and treatment response were collected and genotype-phenotype correlations, response to bisphosphonate treatment, and pharmaco-genetic effects were subsequently studied. Typical OI-causing mutations were found in 119/150 families and out of these 52 were interpreted as COL1A1 null allele causing, whereas 35 vs. 32 were qualitative in the α1- and α2-chain respectively. Only 15 of the discovered qualitative mutations were novel, whereas some mutations with the same position and substitution were recurrent in two or three unrelated families. This distribution supports the idea of mutational “hotspots” that cause mutations at certain locations to appear more often than expected by chance. Some families (8%) harbored an additional mutation or coding polymorphism apart from the designated disease causing mutation, which may act as a phenotypic modifying element. Genotype-phenotype analysis confirmed that mutations situated in the α1-chain were associated with a more severe phenotype, blue sclerae were associated with COL1A1 null alleles, and qualitative mutations were associated with DI. N-to C-terminal chain location was correlated to presence of DI and blue sclerae in the case of qualitative mutations. A few new mutations with unconventional locations were found and described in the C-terminal propeptide of COL1A1 and in the predominantly lethal clusters of COL1A2. Bisphosphonate treatment response assessed by yearly change in lumbar spine BMD after two years was inversely correlated to age (p=<0.0001) (Figure 13) and lumbar spine BMD at treatment initiation (p=0.006). Furthermore, improved response to treatment was associated with mutations causing a more severe phenotype; mutations in COL1A1 vs. COL1A2 (p=0.03), qualitative mutations in COL1A1 vs. COL1A2 (p=0.006), serine substitutions in COL1A1 vs.
COL1A2 (p=0.007) and qualitative vs. quantitative mutations in COL1A1 (p=0.02) all displayed this pattern. There was no association between bisphosphonate response and OI type; type I, IV and III responded well with an overall average yearly lumbar spine BMD increase of 1.3 SD and 0.7 SD for the first two years and total treatment time respectively. In summary this study shows that there are clear genotype-phenotype correlations, that bisphosphonate treatment has an unequivocal effect on BMD in individuals with OI, and that the BMD response is influenced by age at treatment initiation, BMD at onset and by mutation type but not influenced by OI type. These result support bisphosphonate treatment of severely affected individuals in infancy; however, additional treatment endpoints need to be evaluated in future studies.

Figure 13. Bivariate regression analysis of age at treatment initiation vs. yearly change in lumbar spine BMD after two years of treatment shows a clear inverse correlation with age explaining 28% (R²) of the increase in two-year data (p<0.0001).
Family and twin studies have demonstrated that genetic variation plays an important role in osteoporosis [55-59]; moreover, given that the organic matrix of bone consists primarily of collagen type I, prime candidate genes are \textit{COL1A1} and \textit{COL1A2}. The only common coding SNP in these genes, rs42524 (\textit{COL1A2}), has previously been associated with intracranial aneurysms [214]. In this study, the effects of this polymorphism on BMD and prevalences of stroke and myocardial infarction (MI) were investigated in a population-based cohort. Rs42524 was genotyped in elderly men (n = 2004) from the Swedish MrOS cohort and genotypes were subsequently analyzed for association to BMD and certain health parameters. Significant associations (overall p < 0.05) were observed between rs42524 genotype and BMD at several skeletal sites (Figure 14). Surprisingly, the heterozygote genotype class exhibited lower BMD than either homozygote group. When subjects were classified as heterozygotes or homozygotes, the heterozygous genotype was found to confer a lower BMD at total hip, femoral neck, and trochanter (p=0.0098, 0.0270 and 0.0096 respectively). Furthermore, the heterozygote genotype had an increased risk of stroke and a trend for association to increased risk of MI, with population attributable risks being 0.12 and 0.08, respectively. In summary this study shows that common variation in collagen type I can affect the bone and vascular phenotype of healthy individuals.

\textit{Figure 14. Heterozygous disadvantage} - Heterozygous rs42524 genotype is associated with lower BMD; both homozygotes have significantly higher BMD compared to the heterozygote. Bars have been split in the range 0–0.97 for ease of viewing.
In bone, procollagen N- and C-propeptide cleavage is performed pericellularly by specific proteinases to produce mature collagen fibrils that associate with and covalently link to neighboring fibrils to create a framework for mineral deposition. A mutation in the \( \text{COL1A1} \) C-propeptide cleavage site was identified (P1: Asp1219Asn) while sequencing the cohort described in Paper I and the phenotype surprisingly combined high bone mass and bone fragility. This case was therefore investigated thoroughly in collaboration with another research group who had discovered a \( \text{COL1A2} \) C-terminal cleavage site mutation (P2: Ala1119Thr).

Both cases were adolescents with mild OI; P1 L1-L4 DXA Z-score and pQCT volumetric BMD Z-scores were +3.9 and +3.1 respectively, whereas P2 had a L1-L4 DXA Z-score of 0 and pQCT volumetric BMD Z-score of -1.8. Normal to elevated BMD was in contrast to radiographic osteopenia (described as coarse trabeculation for P1) and histomorphometry that showed near normal bone volume and no signs of osteosclerosis. Histomorphometry revealed generally markedly increased osteoid for P1, whereas P2 exhibited this pattern in smaller areas and mineral apposition rate was increased in both individuals. Dermal collagen fibrils had irregular borders when examined by transmission electron microscopy (Figure 15) compared to control. Patient procollagen processing was delayed in pericellular assays and \textit{in vitro} purified C-proteinase cleaved only 30% vs. 46% of procollagen C-propeptide trimers in the \( \alpha_1 \)- and \( \alpha_2 \)-cases respectively (Figure 16).

\[ \text{P1:Asp1219Asn (}\alpha_1\text{)} \quad \text{P2:Ala1119Thr (}\alpha_2\text{)} \quad \text{Control} \]

\textit{Figure 15.} Dermal collagen fibrils from skin punch biopsies were examined by transmission electron microscopy. Both P1 and P2 had irregular cross-sections with some blebs, whereas P1 fibrils were normal in diameter and P2 fibrils were decreased in size. Longitudinal cross-sections were normal with no appearance of branching or barbs.
Patients have delayed C-propeptide processing

(A) Pericellular processing of type I collagen from P1 shows delayed processing kinetics, increased pro-α1(I) and much less mature collagen formation than control. P2 has slightly delayed processing kinetics, increased pro-α1(I), and slightly increased pro-α2(I) forms with less mature α2(I) collagen formed than in control fibroblasts.

(B) Autoradiograms show that processing of the pro-α chains from P1 and P2 by BMP1 (C-proteinase) is markedly reduced (left panel). The enhancing activity of PCPE1 is reduced only toward P1 procollagen. Processing of the mutant pro-α chain (pro-α1(I) in P1 and pro-α2(I) in P2) appears to be preferentially affected, as evident in the right panel, showing the C-propeptide subunit bands, C1 and C2.

Analysis of the mineral phase of bone revealed that the incorporation of collagen with retained C-propeptide into matrix leads to increased bone mineralization. FT-IR imaging of both cortical and trabecular bone confirmed increased mineral/matrix ratios in both patients, compared to both normal controls and classical OI (figure 17). BMDD revealed a marked shift toward increased mineralization density for both patients compared to controls. Whereas P1 had matrix areas of higher and lower mineralization than normal or classical OI bone, the bone matrix of P2 had a mineral content predominantly exceeding even classical OI bone.

In conclusion, these patients show that defects in procollagen C-propeptide processing lead to a phenotype of mild OI with normal to high BMD, and that procollagen C-propeptide cleavage is crucial for normal bone mineralization.
Figure 17. Analysis of iliac crest biopsies by FT-IR reveals an increased mineral to matrix ratio for both cases compared to both control and classical OI bone in cortical and trabecular bone. The pictures to the right are images where color towards red end of spectrum represents high mineral to matrix content.

Paper IV

Allele Dependent Silencing of COL1A2 Using Small Interfering RNAs


Severe OI is generally caused by a dominant mutation in collagen type I, and hence an attractive therapeutic approach is allele-specific gene silencing of the mutated allele. This could convert a severe phenotype to an OI type I in the case of a COL1A1 mutation or to a phenotypically normal patient in the case of a COL1A2 mutation. Allele-specific silencing has been achieved in alleles differing by one nucleotide utilizing siRNAs; however, more than 800 qualitative OI-causing mutations have been reported and mutation-unique drug development would be costly and labor intensive. Targeting heterozygous SNP positions located within the same gene on the mutated allele could be a way to circumvent this (Figure 12). A small number of siRNAs complementary to a few common SNPs could be used for a majority of individuals with OI. In this study seven tiled siRNAs were created against a common SNP (rs1800222) in exon 6 of COL1A2. The siRNAs were transfected into cultured heterozygous primary human bone derived cells from healthy subjects undergoing hip or knee surgery. Magnet assisted transfection (MATra) was used and 48 h posttransfection RNA was prepared, cDNA synthesized,
and sequenced. The software PeakPicker [213] was used to calculate the relative abundance of mRNA from each allele. In brief this program calculates the peak heights in the polymorphic position in Sanger sequencing chromatograms by comparing them to adjacent non-polymorphic peaks (Figure 18).

![Allele Ratio Chart](image)

*Figure 18.* Bar chart with allele ratios for the seven tiled siRNAs targeting the T-allele of the COL1A2 exon 6 SNP rs1800222. Shown are mean ratios and standard deviations, derived from PeakPicker analysis. Allele ratios are presented relative to mRNA levels of COL1A2 in negative control (NC) treated samples, which were defined to have an allele ratio of 1.

The most effective of the seven tiled siRNAs, siRNA3 (Figure 18), was chosen for a second study where three different concentrations (0.3, 0.45 and 0.6µg) were studied. The 0.3µg dose of siRNA3 rendered a mean rs1800222 T/C allele ratio of 0.33, and the corresponding ratios for 0.45 µg and 0.6 µg were 0.30 and 0.35, respectively (Figure 19). These results were verified by PeakPicker analysis of cDNA sequence chromatograms of another heterozygous SNP in exon 25 of COL1A2 (rs412777). Quantitative PCR analysis revealed that with increasing siRNA3 dosage, COL1A2 abundance was decreased by 71%, 77% and 82% of which 75%, 75% and 73% could be attributed to silencing of the targeted T-allele (Figure 19).

In summary, this study demonstrates that allele-specific silencing is possible in COL1A2 and that heterozygous SNP positions can be used as tools for mutation independent allele-specific silencing, the next step being the design of more specific siRNAs for this and other polymorphic positions in the collagen I genes.
Figure 19. (A) Allele ratios of the two COL1A2 mRNA alleles 72 h post-transfection with three different concentrations of siRNA3. Colors of bars indicate the SNP used to calculate allele ratios from cDNA chromatograms in the software PeakPicker and error bars indicate standard deviations. (B) Relative overall COL1A2 mRNA levels after siRNA treatment quantified by real-time PCR. Expression levels were normalized for GAPDH levels and are presented relative to COL1A2 mRNA levels in cells treated with the negative control siRNAs. Error bars indicate standard deviations.

Paper V

Allele Dependent Silencing of Collagen Type I Using Small Interfering RNAs Targeting 3’UTR Indels – a Novel Therapeutic Approach in Osteogenesis Imperfecta


As described in Paper IV, allele-specific gene silencing utilizing siRNAs is an attractive therapeutic avenue for study in OI. However, satisfactory allele specificity was difficult to achieve for the studied polymorphism because the two alleles differ by only one nucleotide in an individual heterozygous for a SNP, and COL1A1 and COL1A2 are repetitive genes. Furthermore, there are almost no common exonic SNPs in COL1A1 to target. A common indel polymorphism was observed in the 3’untranslated regions (3’-UTR) of both
collagen I genes, and targeting indels is a theoretically enticing approach because here the two alleles of a heterozygote differ by several nucleotides, which should increase the chance of true allele-specificity.

Allele frequencies of the 3’UTR indels (rs3840870 and rs3917) were determined in 96 healthy individuals, in 96 patients with OI and have also previously been genotyped in various populations. Heterozygous frequencies were close to 50% in several populations and hence this location could be targeted in almost half of all individuals with severe OI caused by collagen I mutations. Four siRNAs targeting each allele of the two indels were designed and tested in various concentrations in cultures of primary bone derived cells using magnet-assisted transfection. RNA was prepared 72 h post-transfection and used as template for cDNA, which was sequenced and used for quantitative PCR. A time-course study found 72 h to be the allele ratio nadir for the targeted alleles, and this duration was chosen for further study. The software PeakPicker (described in Paper IV) was used for calculations of insertion/non-insertion allele ratios, and siRNA results were normalized to cell populations treated with negative control siRNAs (Figure 20). Quantitative PCR was used to determine the collagen type I mRNA abundances in treated and negative control treated cell populations.

![Figure 20. Representative chromatograms from sequencing of cDNA samples derived from RNA isolated 72 h posttransfection with: (I) 0.6 µg negative control siRNA and (II) 0.4 µg si_A1_i from the time-course study. Arrows mark the position for the COL1A1 rs3840780 insertion and the asterisks mark the peak pairs used to determine allele ratios using PeakPicker software.](image-url)
Successful allele-dependent silencing was observed, with promising results for siRNAs complementary to both the insertion and non-insertion harboring alleles. In *COL1A1* cDNA the indel allele ratios were shifted from 1 to 0.09 and 0.19 for the insertion and non-insertion allele respectively, while the equivalent resulting ratios for *COL1A2* were 0.05 and 0.01. Reductions in mRNA abundance were also found; in cells treated with siRNAs targeting the *COL1A1* alleles the average *COL1A1* mRNA levels were reduced by 65% and 78% compared to negative control levels and in cells treated with *COL1A2* siRNAs the average *COL1A2* mRNA levels were decreased by 26% and 49% of those observed in the corresponding negative controls (Figure 21).

Several challenges need to be overcome before this technique can be used *in vivo* inOI; administration and uptake of the siRNA by bone tissue is a hurdle and limiting off target effects, immune response and achieving absolute allele specificity may prove difficult. However, in conclusion this study provides proof of concept and demonstrates that utilizing 3’UTR indels common in the general population constitutes a promising mutation independent therapeutic approach for severe dominant osteogenesis imperfecta.
Figure 21. (A) Ratio of insertion (i)/non-insertion (ni) alleles for cell populations treated with either of the siRNAs targeting COL1A1 indel rs3840870 or COL1A2 indel rs3917 72 h posttransfection. A range of siRNA concentrations was used: 0.025 µg, 0.05 µg 0.1 µg, 0.2 µg, 0.4 µg and 0.6 µg for indel-targeting siRNAs and for negative controls (NC) 0.6 µg was used. The software PeakPicker was used to calculate the heights of the same three peak pairs in all cDNA chromatograms and values were normalized to negative control treated cells, with the mean defined as 1. Error bars indicate standard deviation. The allele ratios for non-insertion treated siRNAs have been inverted to enable presentation of results in the same chart. Color of bars denotes category of siRNA (NC-red, i-green and ni-blue). A two-tailed T-test comparing negative controls with each type and dose of siRNA was performed. * = p < 0.05; ** = p < 0.001 and n.s. = not significant B)

(B) Average COL1A1 and COL1A2 mRNA levels calculated from the two probes used per collagen gene in relation to levels of control genes (GAPDH and ACTB) measured by quantitative PCR in negative controls (NC) and siRNA treated wells. The mean ratio of COL1A and COL1A2 to either reference gene in negative control wells was defined as 1. Error bars indicate standard deviation. Pattern of bars denote reference gene (solid for GAPDH and striped for ACTB) used in quantitative PCR and color denotes the category of used siRNA (NC-red, i-green and ni-blue).
Conclusions

From the papers included in this thesis it can be concluded that:

Paper I

OI genotype is correlated to phenotype such that gene affected (COL1A1 vs. COL1A2), intrachain location and mutation type influences severity, and presence of clinical characteristics such as blue sclerae and DI.

The recurrence of mutations with the same substitution at the same α1- or α2-chain position in unrelated families more often than would be expected by chance supports theories of mutational “hotspots”.

A second mutation/coding polymorphism apart from the designated disease causing mutation was observed in 8% of families.

Bisphosphonate treatment response assessed by yearly change in lumbar spine BMD (Z-score) is inversely correlated to age and lumbar spine BMD at onset. Treatment response is not influenced by Sillence type (I, IV or III) or gender.

Pharmaco-genetic analyses support a mutation dependent response to bisphosphonate treatment after 2 years of treatment, such that mutations associated with a more severe phenotype have an improved response; however, this effect is attenuated over time.

Paper II

Polymorphisms in collagen type I can affect bone and vascular phenotype in healthy individuals; heterozygotes for the coding SNP rs42524 in COL1A2 have a lower BMD in hip and an increased risk of stroke compared to homozygous individuals.
Paper III
High BMD can be associated with bone fragility; defects in procollagen C-propeptide cleavage caused by mutations in either the *COL1A1* or *COL1A2* cleavage site leads to a phenotype of normal to high BMD with increased mineral to matrix ratio and bone fragility in childhood and adolescence.

Procollagen C-propeptide cleavage is crucial for a normal bone mineralization process.

Paper IV and V
Allele-specific gene silencing, with the goal of treating severe dominant OI, can be achieved in collagen type I in a mutation independent approach utilizing:

I) siRNAs targeting a coding SNP in exon 6 of *COL1A2*
II) siRNAs targeting an indel polymorphism in the 3’UTR of both *COL1A1* and *COL1A2*

Allele-specific silencing is best achieved (concerning allele ratio and mRNA abundances) when two alleles differ by several nucleotides, as is the case in heterozygotes for indel polymorphisms.

Allele-specific gene silencing targeting indel polymorphisms may thus be a way forward in gene therapy for OI.
General discussion and future perspectives

Collagen type I, a protein vital for connective tissues

Mutations in collagen type I exhibit a wide range of severity, from no overt phenotype to miscarriage due to fatal disease. Furthermore, it is noteworthy that different collagen mutations have such divergent effects on phenotype; from the hyperlaxity observed in Ehler-Danlos and in some OI cases, to the skeletal hypermineralization due to retained C-propeptides presented here (Paper III), to the extreme spectrum caused by helical glycine substitutions. This illuminates the importance and versatile roles of this common protein for connective tissues and indicates collagen is generally not altered with impunity. In support of this, there are few coding polymorphisms in collagen I, and the only common one was shown here (Paper II) to affect BMD and vascular phenotype.

The recurrence of certain mutations with identical substitution and position at a higher frequency than expected by chance has been explained by e.g. mutational hotspots associated with CpG-nucleotides [132,215]; however, it is interesting to speculate about the nature of the “missing” mutations on which there are no reports available to date. Some are most likely so mild that no sequencing has been performed, especially not in an era prior to the present-day when sequencing was exceedingly costly and predominantly performed within research projects or in severe cases. On the other hand, others may cause fatal disease and early miscarriage, and are therefore not as likely to be detected. Conceivably some alterations cause phenotypes that to date have not been associated with collagen type I mutations, such as the high BMD-phenotype described here (Paper III). As sequencing becomes more widely accessible, there may be an increase in both the number of individuals diagnosed with OI and in the number of patients with OI being molecularly analyzed, which could broaden the clinical spectrum and the knowledge of genotype-phenotype correlations.

OI classification revisited

The expanded spectrum of collagenopathies and clinical OI caused by non-collagen mutations renders categorization difficult and raises the question of what should be the definition of OI? Should it be: Fragile bones and typical
clinical signs irrespective of mutation type? Is a typical mutation sufficient? Should a collagen type I mutation be a prerequisite for a traditional OI diagnosis? Should clinical characteristics or molecular alteration dictate OI type? The distinction between the classical dominant types of OI can be difficult as the qualitative mutations can be seen as a continuous spectrum, the mild end overlapping with \textit{COLIA1} null alleles phenotypically [66,67,93] (Figure 22). Furthermore, haploinsufficiency of \textit{COLIA1} also has a range of severity due to modifying elements and can sometimes be associated with deformities and troublesome scoliosis, whereas in other cases it causes subclinical disease (Figure 22). Moreover, how should the new noncollagen I types of OI be categorized? At the moment, Roman numerals keep being added to the original Sillence types I-IV [86,87,90-92] for every new gene discovered to cause OI. For the clinician and the affected individual a simple classification is often preferable, while the complexity of the molecular background is important in a genetic counseling setting and may influence e.g. future treatment choices and response. Finally, because identical mutations can cause such diverse phenotype, it is important to consider that occasionally modifying elements are of equal importance but without impact in a strictly mutation-based classification system.

\textbf{Figure 22.} Whole body DXA scans of seven individuals with OI illustrate the continuum of severity and the overlapping phenotypes of Sillence classification, rendering distinct clinical and molecular classification challenging.

An important point when it comes to all genetics is the line between pathology and normal variation, and because sequencing is beginning to be performed to a larger extent in the clinical setting, the genotypic and phenotypic panoramas of many disorders will expand. If a proportion of the “missing
mutations” described above are very mild cases, is it important to diagnose these as OI and to distinguish them from ordinary osteoporosis with a complex genetic inheritance pattern? One could argue that even for individuals with sub-clinical OI a correct diagnosis is important because this may influence follow-up, heredity, and in the future treatment choices. With advanced knowledge of genotype-phenotype interaction it may be possible to better predict risk of fractures and guide management of other symptoms such as e.g. hearing impairment and hyperlaxity that are not typical in osteoporosis. Even in very mild qualitative cases, there is an important distinction to postmenopausal osteoporosis; OI is a generalized connective tissue disorder and tissues are weak due to an intrinsic qualitative collagen defect, which causes issues particular for OI. Moreover, although the collagen structure may be adequate in quantitative defects, this is also a generalized connective tissue disorder, with signs and symptoms accordingly.

To date OI type I is generally assumed to be the cause of a COL1A1 null allele or a mild helical glycine mutation, even though a collagen I mutation actually frequently is not found when sequencing is performed. A collagen I mutations was not found in 17% of individuals (15 families) classified as definite OI type I in this cohort. Approximately as many additional families were excluded from the study because no mutation was found and the bone fragility was not definitely associated with an OI phenotype. Notably, in the past year three publications have described noncollagen mutations that in heterozygous or hemizygous form have features of mild OI [70,86,87] and it is likely additional such mutations will be described in the future, expanding the understanding of genotype-phenotype correlations in OI.

It is intriguing that the same mutation can cause a marked variable phenotype, even within the same family [65,106]. Here, several individuals were found to have a second coding SNP or mutation, and these may act as modifying elements in concert with other collagen SNPs such as Sp1 in COL1A1[61] or rs42524 in COL1A2 studied here (Paper II) or with noncollagen type I polymorphisms affecting bone and other connective tissues. It is plausible that e.g. polymorphisms in the genes causing non-classical OI may also influence the bone phenotype of patients with collagen I mutations. Information on the reason for the described phenotypic variability is scarce and designing studies is challenging due to the low incidence of OI and the magnitude of reported causative mutations. It is important to be aware that to date it is almost impossible to accurately predict phenotype outcome in a prenatal screen situation or in an infant, despite the parental phenotype although recurrent mutations generally have similar outcome. In several instances in this Swedish cohort parents were diagnosed as a result of children coming to medical attention, perhaps a combined effect of more observant modern healthcare and variable phenotype severity.
Current therapeutic approaches

The treatment of OI with bisphosphonate therapy has been extensively studied since the first report in a 12-year-old child in 1987 [216]. Bisphosphonates unequivocally have a substantial effect on BMD and are positive for vertebral configuration concerning height and areal scores and some publications support fracture reduction [144,146,149,150,152]; however, this is not sufficient. Despite the 26 years that have passed an indisputable effect has not been possible to show on fracture frequency, pain, or ambulation. This is partially explained by the low incidence of OI, which complicates performing randomized, double-blind, placebo-controlled trials, and future larger studies may be able to confirm an impact on these parameters. However, arguably if the effect was satisfactory the relatively comprehensive studies that have been published should already have shown a clear benefit. Furthermore, optimal age for treatment initiation, dosing, and treatment length need to be studied in more detail. In osteoporosis a substantial increase of BMD is often observed on anabolic treatment with Teriparatide [217,218], and there are positive reports also in OI [167-169]. However, treatment response has been modest and not necessarily superior to bisphosphonates. Additionally, the study of pharmaco-genetics for available drugs will arguably become more established in the future in many disorders and needs to be evaluated also in OI, as was performed here (Paper I). Nevertheless, bisphosphonates as monotherapy will never constitute a cure or an adequate treatment for patients with severe OI. In these cases the disease mechanism is in the actual architecture of connective tissue and consequently this aspect will need to be targeted, as simply producing more bone will not solve the issue. New treatment avenues that are able to alter the origin of the pathology are necessary.

Novel therapeutic approaches

Explained simply, available osteoporosis drugs increase the amount of bone through boosting the action of osteoblasts or inhibiting osteoclasts, which is not necessarily the most optimal approach in bone with structural abnormalities and mineralization errors. The two cases with retained C-propeptides presented here (Paper III) are interesting because they demonstrate that the organic matrix structure is crucial for proper mineralization and subsequently that the mineral component is important for fracture resistance. Glycine mutations also generate pathological mineralization, which may be equally important to the collagen defect for bone fragility [117-121]. In as early as 1985 it was determined that collagen type I was overmodified in OI type II, most likely due to inhibited helix formation [219]. Soon after several cases were shown to be the result of helical glycine mutations [220,221] and it was
initially thought that the severity of disease was correlated to N- to C-terminal chain location, by influencing the degree of overmodification. Since those original discoveries the complexity has increased. To date it is known that in addition to the mineralization defects mentioned above, a myriad of consequences explain the bone fragility seen in classical dominant OI: chain overmodifications [65,111], pathological extracellular matrix structure [65,112,113], compromised cell-matrix [114] and cell-cell interactions [115] and intracellular stress [116]. The importance of other issues than actual mutated protein in the extracellular space can be illustrated by the wide divergence in phenotype between COL1A2 null alleles. Mutations that simply lead to NMD of COL1A2 mRNA and α1-homotrimer formation cause a mild Ehler-Danlos phenotype [177], whereas translation of mutated COL1A2 mRNA to protein that cannot be incorporated into collagen such as in the oim/oim mouse and in a published human case [222] causes a severe phenotype. Possibly the intracellular accumulation of mutated protein and the ER stress caused by this may be responsible for the OI phenotype because α1-homotrimers alone do not have this effect. Thus, an agent e.g. reducing intracellular stress or normalizing the mineralization process may be a way forward in developing an improved or complementary treatment for OI.

Are siRNAs a way forward?

siRNAs are an attractive approach because they target the root of the pathology in severe dominant OI, the qualitative defect. However, several hurdles remain before this therapy can be prescribed. Allele-specifcity is difficult but it has been achieved to a certain extent here (Paper IV and V) and previously [143,183,184,186,189,190]. Delivery in proper amount and to desired tissue is a challenge, and so far no single method has been found to be superior. One approach is atelocollagen [194,223], which compared to a viral vector has the advantage of transitory silencing of the mutant allele, avoiding the risks of permanent incorporation in the genome such as malignant transformation and ever-lasting off-target effects. Furthermore, concentration is most likely of utter importance for allele-specifcity because spill over on the nontargeted allele according to results presented here (Paper IV and V) is increased with elevated concentration, and simply silencing collagen type I would be devastating to any organism. Correct dosing may be found to be easier to achieve with a transitory vehicle carrying siRNAs injected intravenously, but naturally administration would need to be repeated regularly. The initial goal should be addressing the bone phenotype and directing siRNAs to skeletal tissue. Nonetheless, OI is a connective tissue disorder and as this technique is fine-tuned it will hopefully be possible to ameliorate the phenotype in all tissues expressing collagen type I because e.g. vascular and pulmonary phenotypes may otherwise cause morbidity [224,225]. A short-
coming of the siRNA gene therapy approach is that even if delivery, sequence-specificity and off-target effects are optimized, the patient is still not cured, and in the case of a COL1A1 mutation will still have OI type I. Furthermore, to avoid deformities in severe cases siRNA delivery may need to begin in utero. Nonetheless, siRNAs are a promising approach and the next steps need to be optimizing the sequences, studying off-target effects, and optimizing delivery before proceeding to studies in murine models of OI.

Of the alternate approaches being studied to date, gene therapy with correction of the mutated allele or cell therapy with pluripotent stem cells are promising. Nonetheless, these approaches may be even further away from a clinical reality, considering siRNA therapy already being in clinical trials for certain disorders, e.g. age-related macular degeneration [226] and transthyretin amyloidosis [227]. Arguably, individuals with mild OI will not be offered any type of gene therapy in the near future because the risk-benefit ratio is too high concerning for example the knock-in gene therapy that would be needed to rescue a COL1A1 null allele phenotype. Mild OI will presumably be managed by ordinary osteoporosis therapy for many years to come because these agents have been tested previously in large cohorts in osteoporosis trials and, although not efficient enough, are safe compared to gene therapy.

Future perspectives

All considered, the future perspectives in this field of research need to expand the genotype-phenotype understanding and saturate the map of collagen type I mutations to include all possible substitution outcomes for all positions and their resulting phenotype. Extended understanding of molecular disease mechanisms such as intracellular stress and mineralization errors could potentially be used as handles for drug therapy and furthermore may explain the interindividual phenotypic variability and help predict mutation outcome. Understanding modifying elements may also be of use to tailor treatment.

To date bisphosphonate therapy is the standard of care; however, future research needs to delineate optimal age of treatment initiation, dosing regimens and treatment length. Furthermore treatment endpoints such as fracture reduction, pain, bone deformation and long-time safety should be more extensively studied in all age groups. Parallel to this, gene and cell-based therapies need to be developed for severe cases and siRNAs have definite advantages and should be investigated further.

The recessive and noncollagenous dominant mutations need to be further researched concerning e.g. treatment response, phenotypic variation and possibilities for gene-therapy. Many of these OI types are based on case
reports on a few individuals and future studies need to deepen the knowledge of these disorders, which may also broaden the phenotypic spectrum.

Finally, in cases still negative for mutations in genes known to cause OI, whole genome or exome sequencing will help determine new genes with OI phenotypes.
Acknowledgments

First and foremost a special thank you to the participating individuals with OI and their families – without you this thesis would not have been possible!

Funding

Her Majesty Queen Silvia of Sweden jubilee fund for support and a memorable mingle and drink with Her Majesty Queen Silvia at the Royal Castle in Stockholm
Fondkistan fund for support and a wonderful dinner at Kolbäcks Gästgivaregård
The ASBMR young investigator award committee for support and an unforgettable evening including dinner and a ceremony in Honolulu, Oahu
Akademiska sjukhuset for funding in the form of a research residency and “FoU” funding
The Jerring fund for supporting costs associated with the International OI conference in Dubrovnik
The Swedish Research Council for funding of this project
The Swedish Endocrine Society for funding of this project

Special people

Professor Östen Ljunggren – supervisor
A real research group leader with all of the mandatory attributes: cellphone (!), enthusiasm, knowledge, generosity and connections everywhere. Every time we have had a meeting I feel like absolutely everything is possible. You promised me we would have a lobster feast after our first OI publication and shame on me for not taking you seriously! What a feast we had, champagne and all! You have a large heart, a terrific sense of humor and a very popular calendar ;-) ! Thank you for these years!
Helena Brändström – my first co-supervisor
Offered a small but very strong and confident hand to hold when I started my research career at the bone lab in November 2005. You taught me everything about PCR, sequencing and copy pasting in excel. We had so much fun I didn’t even consider giving up, although it took me over a year to manage sequencing of collagen type I and I was about ready to throw primers, reagents and the PCR machine out the window! Ever since you left for new adventures, I have missed you at the lab and I am so happy to have kept you as a close friend. I know you have dreamt of dance troops performing my publications at the dissertation and I hope you will not be too disappointed…

Andreas Kindmark – co-supervisor
In your pleasantly patient and calm way you taught me all about genes, fonts, the importance of a positive control, aerodynamics and the difference between right and left (no success on the last point however, so you’ve also kindly been my GPS in foreign cities, such as Stockholm). When you first told me about your two-year move to Addis Abeba I was quite sure I would lose my second co-supervisor, however you stayed in touch with a monster-router and Skype. Thank you for your time between sunbathing at the Sheraton pool and diplomat parties at different embassies; it has meant a lot to me!

Carl-Johan Rubin – co-supervisor
I think you think faster than lightning, or maybe just about as fast as your Mac. Always overflowing with new smart ideas that get you involved and enthusiastic, and when you start working these ideas on your computer it goes so fast it makes me dizzy. You are highly linguistically talented as well, and I have really appreciated your feedback on my manuscripts, which has always been amazingly accurate concerning both scientific content and language. Thank you for staying in touch during your post doc!

Elin Grundberg you were at the end of your time as a PhD student at the bone lab when I started mine. Thank you for all of your help with practicalities when I was a novice in the lab, your company over many cups of coffee and for introducing me to how to attend a conference. I will never forget the narrow bed we shared in Philadelphia, thankfully we were both silent and motionless sleepers! Thank you also to many other friends that have shared my bone-lab experience both in and outside of the lab, especially: Tijana Krajisnik, Eva Ribom-Ljunggren, Johanna Westerlund and Richard Marsell. Gry Hulsart-Billström, thank you for being the lab’s organizer of interesting lunches, social events and for always being so positive! A special thank you to Britt-Marie Andersson who has always helpfully answered questions concerning the wheres and hows in the lab when I needed help. Navya Laxman, thank you for your company during travels to Athens,
Minneapolis, Lisbon and Marstrand, for your help and input on our paper on silencing of collagen and for kindly sharing some of your artwork for my thesis! You always take the time to ask about how I am doing and about my children and I really appreciate your thoughtfulness.

A special thank you to Anna-Lena Johansson who has done an enormous amount of work on collagen sequencing. You have always been friendly and helped me with all favors I asked for, from small things such as purchasing a stamp to large things such as helping me ship cells to the United States. Thank you also Elin Carlsson, for taking over after Anna-Lena in such an enthusiastic way! You have already impressed me with your attitude and knowledge. And thank you Jessica Pettersson for the time you were at the lab and worked on sequencing of collagen. I know it is hard work!

A large part of the administration and the Uppsala-end DXA measurements of this study were organized and performed by the staff at the osteoporosis unit, Akademiska sjukhuset. Ann-Louise Jacobsson, you are wonderful to work with! You are positive, reply within hours and always have things in order! Ann-Charlotte Adolfsson, thank you for always helping me with a DXA-scan time when I have needed one! Thank you also to Marja Gustafsson, Joanna Apelgren and Klara Lindgren.

Eva Åström – collaborator and responsible for the pediatric OI center at Astrid Lindgren children’s hospital. In the beginning of this project it was very difficult to recruit individuals with OI for our studies. However after we met and planned our collaboration in 2007 there was a new referral for collagen sequencing in an individual with OI waiting for me almost every week. I am incredibly grateful for your enthusiasm, your tireless work extracting data from patient charts and your generosity in sharing time, materials and knowledge in the field.

Barbro Malmgren, I am very thankful that you sent your husband on the train from Stockholm to Uppsala with a cooler bag packed with frozen DNA samples from your study patients with OI at a time when I was starting to doubt we would be able to include more than a handful of individuals in our study.

Thank you also to other collaborators and clinicians who sent me referrals to be included in our study: Giedre Grigelioniene, Ylva Pernow and Dan Mellström. And I am grateful that Hans Mallmin helped me with the bone biopsies for Paper III.
When I found the C-terminal cleavage site mutation described in Paper III, I was very excited and anxious to investigate the effects and pathophysiology of this mutation further. A special thank you to Joan Marini who was curious to hear about my case and was interested in a collaboration when I approached you at the ASBMR meeting in Honolulu in 2007. Aileen Barnes, my co-author in this publication, thank you for your efforts, swift email replies and very nice company at conferences.

Thank you to all of my colleagues at the department of endocrinology for a friendly and warm atmosphere whenever I have had the opportunity to spend time at my “home-clinic” these past years. A special thank you to Britt Edén Engström, boss of the department of endocrinology, you have always supported me, been enthusiastic and emphasized how much you look forward to my return to clinical work.

Tobias Larsson Agervald, my brother-in-law who defended his own thesis at the bone lab in 2004 and recommended me to contact Östen Ljunggren for a fun PhD project, thank you! I was uncertain of which road I should choose; I had just received my M.D. license and was temporarily working at the department of infectious diseases. You coached me over coffee and cinnamon rolls at Café Linné, and here I am! And thank you Erik Lindahl, my brother and number one Mac and picture editing support, who helped me morph and tweak my front-page picture to perfect color and size in several sittings! I hope printing services are on our side!

Thank you Sofia Cars and Tina Ohlander for always being just a phone call away, waiting with a warm cup of tea and a cosy corner in the couch, being interested to hear what I have to say. You know every twist and turn of my past, keep track of details small and large in my present and our conversations guide my choices for the future. I honestly do not know what I do without you two. Your friendship is priceless.

Thank you Barbara Selvastrand, my mother who has babysat her three grandchildren from a few weeks of age, enabling me to attend meetings and do research work. Over the years you have always welcomed me and the increasing number of grandchildren for dinners and play when I have been tired and in need of an excursion. You are fantastic!

Thank you Lars Lindahl, my father who defended his own thesis the year I was born and who is the one who made me believe I can do whatever I want to do. You are one of the people who I think will actually read this book front to back ;-) When you come to babysit, the children get so excited and
say “Komnej mojfaj!? Då fäl vi glass!” and you always joke with them until they giggle, which reminds me so much of my own childhood. Thank you!

And finally my family, Andreas thank you for always believing in me and cheering me on! Ever since we were both students in medical school you have often fondly told me that I am much smarter than you and that the sky is my limit. It has meant the world to me that you have filled my thoughts with confidence, inspiration and dreams of a brilliant future. When I see you with our fantastic children, it is touching to see how you fill them with the same kind of love, self-confidence and sense of indisputable importance. Thank you for your love and support!

Filippa, Alexander and Eleonora…my three twinkling little stars…you fill my life with light, laughter and meaning. When you read this one day, thank you for being such lovely children despite a Mother who hasn’t played enough “Princesses”, “Dinosaurs” or “Peek a boo” with you. And I hope you keep telling me all those lovely, insightful thoughts that lift my spirit every day, such as: “Mamma, när du säger att du tycker om mig känns det som att du är en ängel” (Mom, when you say that you love me, it feels like you are an angel”) and “Mamma, när man pussas blir det hjärtan” (“Mom, when you kiss hearts are made”) and adoringly in a soon-to-be-two-year-old fashion “Tycker OM!” ( Meaning “I love you”). You three are exquisitely sweet.
Sammanfattning av avhandlingen

Osteogenesis imperfekta – Genetiska och terapeutiska studier

Liten ordlista

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>OI</td>
<td>Osteogenesis imperfekta, extrem benskörhet.</td>
</tr>
<tr>
<td>BMD</td>
<td>Bentäthet, ett mått på mängden benvävnad.</td>
</tr>
<tr>
<td>Osteoporos</td>
<td>Benskörhet på grund av för lite benvävnad och/eller ben av nedsatt kvalitet. Vanligast hos äldre kvinnor. Ökar risken för frakturer i bland annat höft, handled och kotor.</td>
</tr>
<tr>
<td>Bisfosfonat</td>
<td>Vanlig läkemedelsklasse vid osteoporos och även standardbehandling vid svårare former av OI.</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonuklein syra – enorm molekyl som bildar kromosomer. I kromosomerna finns praktiskt taget alla gener.</td>
</tr>
<tr>
<td>Gen</td>
<td>Kodande region i DNA. Grundläggande enhet i arvsmassan som bär ärtlig information och utgörs av en sekvens nukleotider. Genen kodar för ett visst protein, exempelvis kollagen.</td>
</tr>
<tr>
<td>Nukleotid</td>
<td>DNA består av fyra olika nukleotider (förkortas A, T, C och G) och kombinationen av dessa utgör den genetiska koden. Varje tripplett av nukleotider kodar för en viss aminosyra och aminosyrorna bygger upp våra proteiner.</td>
</tr>
<tr>
<td>Exon</td>
<td>De kodande delarna av våra gener. Mellan exonen finns introner och de kodar inte för ett protein.</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonukleinsyra – ”arbetskopia” av våra gener som används vid bland annat proteinsyntes i kroppens celler.</td>
</tr>
<tr>
<td>mRNA</td>
<td>Den typ av RNA som är ett recept för proteinproduktion (messenger RNA).</td>
</tr>
<tr>
<td>siRNA</td>
<td>Små interfererande RNA - korta sekvenser av RNA som har förmågan att tysta gener genom att orsaka spjällning av mRNA.</td>
</tr>
<tr>
<td>Genotyp</td>
<td>En individs genetiska uppsättning.</td>
</tr>
<tr>
<td>Fenotyp</td>
<td>En individs faktiska utseende/egenskaper.</td>
</tr>
<tr>
<td>Allel</td>
<td>En variant av en gen. Man ärver en allele av mamma och en av pappa.</td>
</tr>
</tbody>
</table>
Homozygot  Någon som ärvt samma genetiska variant från båda föräldrar, det vill säga alleleerna är likadana.

Heterozygot  Någon som ärvt olika genetiska varianter från sina föräldrar, det vill säga alleleerna är olika.

Sekvensering  Analys av en persons DNA för att hitta eventuella förändringar, t ex mutationer eller SNP:ar.

SNP  ”Single nucleotide polymorphism” - vanligt förekommande förändringar i DNA. Förekommer definitionsmässigt hos mer än 1/100 allele. En nukleotid lång.

Polymorfism  Som en SNP, men kan även vara längre än en nukleotid lång.

Mutation  Ovanliga förändringar i DNA, leder ofta till sjukdom. Förekommer definitionsmässigt hos mindre än 1/100 allele.

Kollagen typ I  Protein som är mycket vanligt i skelett, bindväv och blodkärl. Kollagen typ I utgör 80-90% av det organiska materialet i skelettet.

**COL1A1 & COL1A2**  De två generna som kodar för kollagen typ I. Generns namn skrivs vanligen i kursiv stil.

Kvantitativ kollagen-defekt  För lite men normalt kollagen.

Kvalitativ kollagen-defekt  Kollagen som är muterat/förändrat.

Kollagen-Fibriller  Buntar av parallella kollagenfibrer som utgör den organiska stommen som mineraliseras i benvävnad.

**Bakgrund**

Osteogenesis imperfecta (OI) är en ovanlig ärftlig sjukdom i skelett och bindväv där det mest framträdande symtomet är frakturbenägenhet. Cirka 1 på 15 000 föds med denna sjukdom och runt 90% av alla drabbade har en dominant mutation i en av de två generna som kodar för kollagen typ I (**COL1A1** och **COL1A2**). De mildaste fallen av OI tolkas ibland som vanlig osteoporos (benskörhet), medan de svårast sjuka dör under fosterlivet eller under nyföddhetsperioden.

Traditionellt klassificeras OI i Sillence typ I-IV, där typ I är den vanligaste och mildaste varianten. Dessa patienter har en normal förväntad överlevnad, men får ofta frakturer under uppväxten. Frakturbenägenheten kan avta efter puberteten för att sedan återkomma på äldre dagar. OI typ II ärrevis mot ger så skör benvävnad att förändringen inte är förenlig med liv; fostret
dör vanligen under graviditeten eller inom några dagar efter födseln till följd av multipla frakturer i bröstkorgen med andningspåverkan. Typ III är den svåraste formen som är förenlig med liv. Dessa patienter drabbas vanligen av hundratalt frakturer, sitter ofta i permobil och har en sänkt förväntad överlevnad. Typ IV är ett mellanting mellan typ I och typ III i svårighetsgrad och symtom.

Mild OI (typ I) orsakas ofta av för lite men normalt kollagen till följd av att en av de två alleleerna för en av de två kollagengenerna (COL1A1) är avstängd, en kvantitativ kollagen defekt. Vid de svårare formerna av OI (typ II-IV) är kollagen typ I istället kvalitativt påverkat. Kollagen typ I är ett mycket repetitivt protein som består av tre stycken långa proteinkedjor som bildar en helix. En person med en svårare form av OI har vanligen en mutationstyp som rubbar kollagenets helixformation, vilket i sin tur stör proteinets funktion och leder till en mängd konsekvenser för skelett och bindväv. Kollagendefekten kan ge typiska symptom även från andra organ som innehåller detta viktiga protein, exempelvis sköra tänder (dentinogenesis imperfekta/DI), blå ögonvitor (sklera), överrörliga leder, ökad blödningsbenägenhet och tidig hörnmisslättning/dövhet.

Det finns idag ingen medicinsk behandling av svår OI som har tillfredsställande effekt. Man behandlar vanligen svårare former med bisfosfonater, som är den vanligaste typen av läkemedel vid osteoporos. Man har sett positiva effekter av detta på kotornas form och skelettets bensäthet, men man har inte säkert kunnat påvisa en minskad frakturbenägenhet eller förbättrad livskvalitet för alla typer av OI. Det är också väldigt viktigt med god ortopedisk kompetens och väl utformad sjukgymnastik.

Avhandlingens målsättning

Målsättningen med denna avhandling var att beskriva det spektrum av mutationer i kollagen typ I som orsakar OI i Sverige och att sedan använda denna information i studier av relationen mellan genotyp och fenotyp samt mellan genotyp och hur patienterna svarar på behandling (studie I). Patienter med ovanliga kliniska fenotyper eller mutationer planerades för fördjupade separata studier (studie III). Med tanke på att OI är en sjukdom i kollagen typ I, inkluderades även en studie av effekterna på skelett och kärl av den enda vanligt förekommande kodande förändringen i de två kollagen typ I-generna (polymorfrismen rs42524 i COL1A2) hos en kohort friska män (studie II). Slutligen var målsättningen att studera ett potentiellt nytt behandlingsalternativ vid OI, genterapi med allelspecifika siRNA (studie IV och V).
Ingående vetenskapliga studier

Studie I

DNA från 202 individer tillhörande 150 obesläktade familjer samlades in till denna studie med målsättningen att tydliggöra förhållandet mellan genotyp och fenotyp samt studera effekten av bisfosfonatbehandling vid OI. Mutationsanalys gjordes av generna som kodar för kollagen typ I (COL1A1 och COL1A2) och kliniska data hämtades ur patientjournaler. En mutation hittades i 119 av de 150 familjerna. Korrelationer kunde ses mellan genotyp och fenotyp; svårare sjukdom var associerat med mutation i COL1A1, sköra tänder (DI) sågs oftare vid kvalitativ mutation, blå ögonvittor var associerat till kvantitativa defekter och förekomsten av DI och blå ögonvitor var kopplat till lokalisering av kvalitativa mutationer i kollagenkedjorna. Vidare hittades och beskrevs ett antal mutationer med ovanlig lokalisering och typ. Bisfosfonatbehandling tycks ha bäst effekt på bentäthet när de administreras i låg ålder och vid lågt utgångsvärde på bentäthet. Behandlingssvaret är initialt störst vid svår fenotyp, men alla typer av OI har en god respons. Det är viktigt att förstå sambandet mellan genotyp och fenotyp för att kunna förutspå hur en viss mutation kommer påverka en individ. Att förstå vem som svarar bra på en behandling och varför (ålder, bentäthet, mutationstyp och så vidare) är viktigt för att förutspå vem som har nytta av en viss behandling och när den bör sättas in.

Studie II

I denna studie analyserades vilken effekt den enda vanligt förekommande DNA-förändringen (polymorfismen rs42524 i COL1A2) i kollagen typ I har på benvävnad och kärl. En kohort friska män (2004 st) klassificerades efter genotyp och det konstaterades att heterozygot genotyp (olika allelvarianter) var associerad med sänkt bentäthet på flera mätpunkter i höft och dessutom medförde en ökad risk för att drabbas av stroke. Även normal genetisk variation i kollagen typ I kan således påverka ben- och kärlfenotyp. Att förstå vanligt förekommande DNA-förändringars påverkan på fenotypen är viktigt för insikten i hur olika proteiner inverkar på kroppliga funktioner och reaktioner. Man studerar även denna typ av samband för att kunna uttala sig om risken för att drabbas av en viss sjukdom och man hoppas även i framtiden kunna förutspå effekten av olika läkemedel utifrån en persons genetik.

Studie III

En av patienterna i studie I var en flicka utan klassiska tecken på OI, men med upprepade frakturer trots en mycket hög bentäthet (+3.9 standardavvi-

Studie IV och V
Nästa steg i utvecklingen av en siRNA-baserad genterapi för OI är finjustering av siRNA-sekvenser, sedan att studera oönskade effekter på andra gener och slutligen hitta ett sätt att leverera siRNA till skelettet.

Sammanfattning

Sammanaget visar den här avhandlingen betydelsen av normalt kollagen typ I för skelettets, bindvävens och blodkärlens konstruktion och styrka. Förändringar i detta protein kan ha en mängd skiftande effekter på kroppens vävnader, från den variation som ses hos friska människor, till hög bentäthet med frakturbanägenhet på grund av en spjälningsmutation av prokollagen, till den dödliga fenotypen som ses vid OI typ II. Bisfosfonatbehandling av dessa patienter har en tydlig, men långt ifrån tillräcklig, effekt som påverkas av ålder och bentäthet vid behandlingsstart. Vidare har studieresultat presenteres här som stödjer att gentystning av kvalitativt påverkat kollagen typ I med allelspecifika siRNA kan vara ett lovande spår att följa för att utveckla framtida genterapi av svår OI.
References


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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine.