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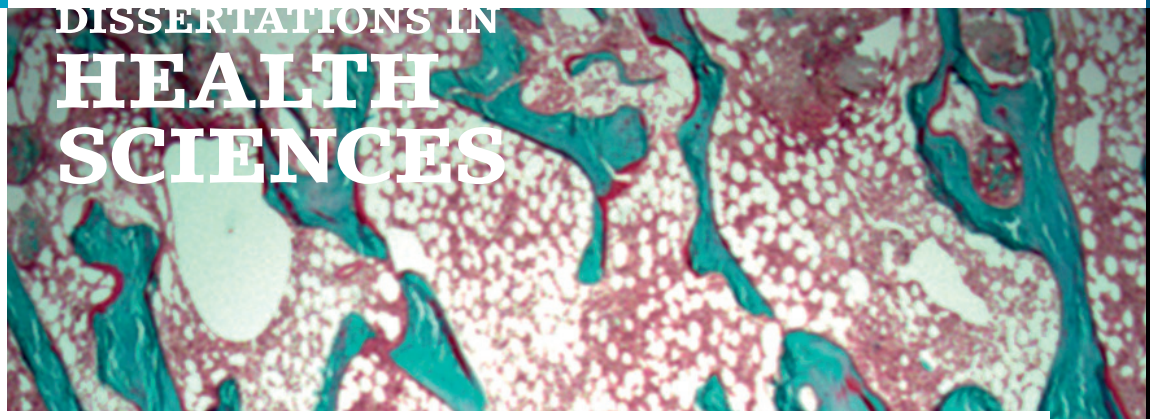
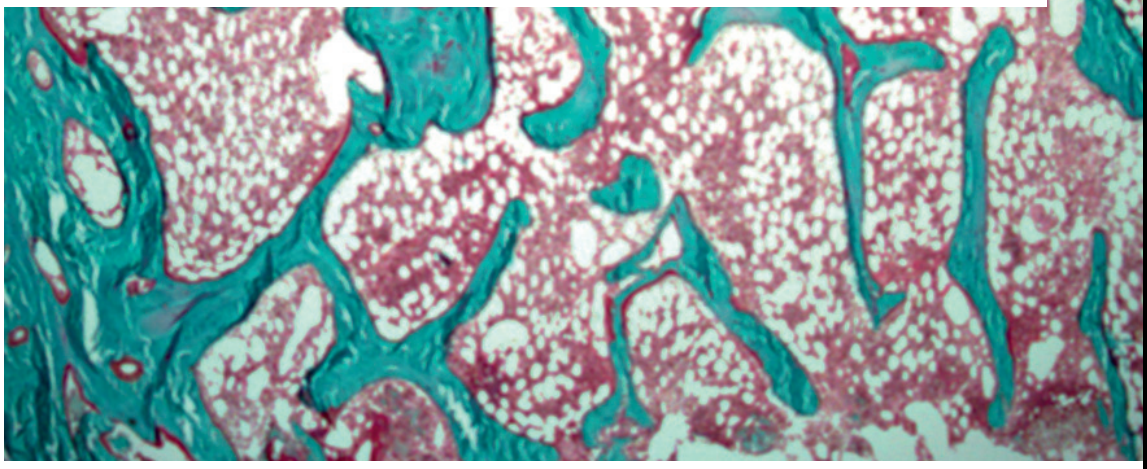


Inari Tamminen

Assessment of Bone Quality in Pediatric and Adult Patients with Osteoporosis



Publications of the University of Eastern Finland
Dissertations in Health Sciences

UNIVERSITY OF
EASTERN FINLAND

INARI TAMMINEN

Assessment of Bone Quality in Pediatric and Adult Patients with Osteoporosis

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Publications of the University of Eastern Finland
Dissertations in Health Sciences
Number 173

Bone and Cartilage Research Unit, School of Medicine, University of Eastern Finland;
Department of Applied Physics, University of Eastern Finland;
Department of Orthopaedics, Traumatology, and Hand Surgery,
Kuopio University Hospital
Kuopio
2013

Kopijyvä Oy
Kuopio, 2013

Distributor:
University of Eastern Finland
Kuopio Campus Library
P.O.Box 1627
FI-70211 Kuopio, Finland
http://www.uef.fi/kirjasto

ISBN (print): 978-952-61-1143-8
ISBN (pdf): 978-952-61-1144-5
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

Author's address: Bone and Cartilage Research Unit, Clinical Research Center
School of Medicine, Faculty of Health Sciences, University of Eastern Finland
P.O.Box 1627, 70211 KUOPIO
FINLAND

Supervisors: Professor Heikki Kröger, M.D., Ph.D.
Department of Orthopaedics, Traumatology, and Hand Surgery
Kuopio University Hospital and
Bone and Cartilage Research Unit, Clinical Research Center
School of Medicine, Faculty of Health Sciences, University of Eastern Finland
KUOPIO
FINLAND

Professor Jukka Jurvelin, Ph.D.
Department of Applied Physics, Faculty of Science and Forestry
University of Eastern Finland
KUOPIO
FINLAND

Docent Hanna Isaksson, Ph.D.
Department of Applied Physics, Faculty of Science and Forestry
University of Eastern Finland
KUOPIO, FINLAND and
Division of Solid Mechanics, Department of Orthopaedics, Lund University
LUND, SWEDEN

Reviewers: Professor Hannu Aro, M.D., Ph.D.
Department of Orthopaedic Surgery and Traumatology
University of Turku and Turku University Central Hospital
TURKU
FINLAND

Professor Karl Michaëlsson, Ph.D.
Department of Surgical Sciences, Orthopaedics
Uppsala University
UPPSALA
SWEDEN

Opponent: Dr Nigel Loveridge, Ph.D.
Orthopaedic Research Unit, School of Clinical Medicine
University of Cambridge
CAMBRIDGE
UNITED KINGDOM

Tamminen, Inari
Assessment of Bone Quality in Pediatric and Adult Patients with Osteoporosis
University of Eastern Finland, Faculty of Health Sciences, 2013
Publications of the University of Eastern Finland. Dissertations in Health Sciences Number 173. 2013. 160 pp.

ISBN (print): 978-952-61-1143-8
ISBN (pdf): 978-952-61-1144-5
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

ABSTRACT

Osteoporosis is the most common metabolic bone disease characterized by low bone mass and altered material properties. Compromised bone strength predisposes the individual to fractures. Dual-energy X-ray absorptiometry (DXA) is commonly used to measure bone mineral density (BMD). However, BMD accounts for only 60% of bone strength, i.e., changes in bone quality as well as density may increase the fracture risk. This study aimed to characterize bone quality in different cohorts of patients using bone histomorphometry, microcomputed tomography (micro-CT), and Fourier transform infrared spectroscopic imaging (FTIRI).

Iliac crest biopsies were collected from different cohorts: i) patients with osteoporosis (OP, *n*=15) or renal osteodystrophy (ROD, *n*=11) or healthy controls (*n*=10), ii) patients with atypical femoral fractures (AFFs, *n*=4), ii) children with suspected primary osteoporosis (*n*=24), and iv) children after solid organ transplantation (*n*=19). Bone histomorphometry was performed to examine bone remodeling and turnover, micro-CT to study bone microarchitecture, and FTIRI to study bone composition. The results were correlated with fracture history, clinical characteristics, densitometry, and biochemistry when appropriate. The reproducibility of bone histomorphometry and the agreement between bone histomorphometry and micro-CT were studied. Further, the incidence of AFFs was investigated within our hospital catchment area.

Repeated measurements for structural bone parameters revealed linear correlation coefficients (ρ) of 0.87-0.92 (CV 8.3-27.2%) for histomorphometry and of 0.66-0.94 (CV 4.4-23.4%) for micro-CT. There were no significant differences in reproducibilities in the samples obtained from the different study groups (OP, ROD, healthy controls). When comparing the techniques, cancellous bone volume (BV/TV), trabecular thickness, and trabecular number displayed moderate correlations (ρ=0.39-0.62, $p<0.05$), and the agreement for BV/TV was highest in OP samples. The incidence of AFFs among patients receiving biphosphonates was 0.61 fractures/1000 patients per year within the catchment area of Kuopio University Hospital, compared to 0.0067/1000 per year among untreated subjects. Histomorphometry in 4 patients revealed a low cancellous bone volume. Bone formation and resorption parameters were found to be low. Based on FTIRI, a high phosphate-to-amide I ratio and a high collagen maturity were detected in comparison with normal samples. Children with vertebral fracture (*n*=14) were examined, in 36% of cases low cancellous bone volume (BV/TV <-1.0 SD) was detected by histomorphometry, and in 64% of the children the bone turnover rate was abnormal. Children with vertebral fractures had lower a carbonate-to-phosphate ratio, higher collagen maturity, and a narrower collagen distribution ($p<0.05$) than children without vertebral fracture. In children after solid organ transplantation (*n*=19), 21% had sustained peripheral fractures and 58% had suffered vertebral compression fractures. Histomorphometric analyses showed low cancellous bone volume (<-1.0 SD) in six children (32%) and decreased trabecular thickness in 14 children (74%). Seven children (37%) had a high bone turnover and six children (32 %) had low turnover.

There was moderate agreement between bone histomorphometry and micro-CT using clinical bone samples. The reproducibility was not affected by the health status of bone. The overall incidence of AFFs was low. The poor fracture resistance in some patients on long-term bisphosphonate-therapy could be explained by low bone formation, and changes in bone composition. Further, changes in bone composition were found among fracture-prone children who had sustained vertebral fracture. The changes in these children might contribute to their greater propensity to sustain vertebral fractures. A great heterogeneity in the histological findings was found in children after organ transplantation, and the results were unpredictable if only non-invasive methods were available. The observed changes in bone quality rather than the actual loss of cancellous bone, might explain the increased fracture risk in pediatric solid organ transplant recipients. Based on the present results, histomorphometry is needed in clinical practice to study remodeling balance in bone, with the other potential methods available to study bone quality being complementary.

National Library of Medical Classification: WE 200, WE 225, WE 250, WN 206
Medical Subject Headings: Adult; Bone and Bones/ anatomy & histology; Bone and Bones/metabolism; Bone Density; Child; Diphosphonates/adverse effects; Fractures, Bone; Fractures, Spontaneous; Osteoporosis; Spectroscopy, Fourier Transform Infrared; Transplantation/adverse effects; X-Ray Microtomography

Tamminen, Inari
Luun laatuominaisuudet osteoporoottisilla lapsilla ja aikuisilla
Itä-Suomen yliopisto, terveystieteiden tiedekunta, 2013
Publications of the University of Eastern Finland. Dissertations in Health Sciences Numero 173. 2013. 160 s.

ISBN (print): 978-952-61-1143-8
ISBN (pdf): 978-952-61-1144-5
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

TIIVISTELMÄ

Osteoporoosi eli luukato on yleisin metabolinen luustosairaus. Osteoporoosissa luun määrä ja laatuominaisuudet ovat alentuneet altistaen luumurtumille. Luuntiheysmittausta (DXA) käytetään luun mineraalitiheyden (BMD) mittaamiseen. BMD selittää kutenkin vain 60% luun lujuudesta ja näin ollen muut luun laatuominaisuudet voisivat selittää lisääntynyttä murtumaherkkyyttä. Tämän tutkimuksen tavoitteena oli määrittää luunäytteiden laatuominaisuuksia eri potilas-aineistoissa käyttäen tutkimusmenetelminä kvantitatiivista luun histomorfometriaa, mikrotietokonetomografiaa (mikro-TT) ja Fourier-muunnos-infrapunaspektrometriaa (FTIRI).

Luunäytteet kerättiin suoliluuharjusta eri aineistoista: i) osteoporoosia (OP, *n*=15) tai renaalista osteodystrofiaa sairastavilta potilailta (ROD, *n*=11) sekä terveiltä yksilöiltä (*n*=10), ii) epätyypillisiä reisiluumurtumia sairastaneilta potilailta (*n*=4), iii) lapsilta, joilla epäiltiin primaarista osteoporoosia (*n*=24) ja iv) elinsiirron saaneilta lapsilta (*n*=19). Histomorfometriaa käytettiin luun uudismuodostuksen ja vaihtuvuuden tarkasteluun, mikro-TT:aa luun mikroarkkitehtuurin tutkimiseen ja FTIRI:aa luun koostumuksen selvittämiseen. Tuloksia verrattiin potilaiden murtumahistoriaan, kliinisiin muuttujiin, luuntiheyteen ja veren laboratorioarvoihin. Histomorfometria-menetelmän toistettavuus määritettiin. Histomorfometriasta ja mikro-TT:sta saatujen tulosten välinen korrelaatio laskettiin. Lisäksi selvitettiin epätyypillisten reisiluun murtumien esiintyvyys sairaanhoitopiirimme alueella.

Toistettavuusmittauksissa luun rakenteellisten parametrien korrelaatiokerroin (ρ) oli histomorfometrialla 0.87-0.92 (CV 8.3-27.2%) ja mikro-TT:lla 0.66-0.94 (CV 4.4-23.4%). Toistettavuudessa ei ollut eroa eri tutkimusryhmien (OP, ROD, normaalinäytteet) välillä. Tilastollisesti merkitsevä korrelaatio (ρ=0.39-0.62, p<0.05) tekniikoita verrattaessa voitiin osoittaa hohkaluutilavuudelle (BV/TV), palkkien paksuudelle (Tb.Th) ja palkkien määrälle (Tb.N). Korrelaatiot olivat parhaimmat OP näytteille. Epätyypillisten reisiluumurtumien esiintyvyys vuodessa Kuopion yliopistollisen sairaalan sairaanhoitopiirin alueella bisfosfonaatteja käyttäneillä naispotilailla oli 0.61 murtumaa/1000 potilasta. Vastaavasti vuosittainen esiintyvyys naisilla, jotka eivät olleet saaneet bisfosfonaatteja, oli 0.0067/1000 potilasta. Neljälle potilaalle tehtiin luun histomorfometria ja heillä havaittiin matala hohkaluutilavuus. Luun muodostuminen ja hajotus olivat hidasta. FTIRI:lla havaittiin korkeampi fosfaatti/amidi I suhde ja korkeampi kollageenin kypsyysaste verrattuna normaalinäytteisiin. Nikamamurtumia sairastaneista lapsista (*n*=14) 36%:lla oli matala hohkaluutilavuus (BVTV <-1.0 SD) ja 64%:lla luun vaihtuvuusnopeus oli poikkeava. Nikamamurtumia sairastaneilla lapsilla oli matalampi karbonaatti/fosfaatti suhde, korkeampi kollageenin kypsyysaste ja kapeampi kollageenin jakautumisaste (p<0.05) kuin nikamamurtumia sairastamattomilla lapsilla. Elinsiirron saaneista lapsista (*n*=19) 21% oli sairastanut perifeerisiä murtumia ja 58% nikamamurtumia. Histomorfometria-tutkimuksen perusteella hohkaluun tilavuus oli matala (<-1 SD) kuudella lapsella (32%) ja palkkien paksuus oli alentunut 14 lapsella (74%). Luun vaihtuvuus oli nopeaa seitsemällä lapsella (37%) ja hidasta kuudella lapsella (32%).

Luun histomorfometrian ja mikro-TT:an välinen korrelaatio kliinisillä luunäytteillä oli kohtalainen. Menetelmien välinen toistettavuus ei ollut riippuvainen aineenvaihdunnan tilasta. Epätyypillisten reisiluumurtumien esiintyvyys oli alhainen. Lisääntynyt murtumaherkkyys näillä potilailla voi selittyä hitaalla luun muodostuksella ja muutoksilla luun koostumuksessa. Nikamamurtumia sairastaneilla lapsilla havaittiin poikkeavuuksia luun koostumuksessa, mikä voisi selittää lisääntynyttä herkkyyttä kokea kyseisiä murtumia. Elinsiirron saaneilla lapsilla luun histologiset löydökset olivat moninaiset ja tuloksia ei voitu ennustaa ei-kajoavilla menetelmillä. Osoitetut luun laadun muutokset, todennäköisimmin kuin luun määrän alentuminen, voisivat selittää lisääntynyttä murtumariskiä näillä lapsilla. Luun histomorfometriaa voidaan hyödyntää niin kliinisessä työssä kuin tutkimustyössä luun uudismuodostuksen tutkimiseen. Muut luun laadun tutkimiseen tarkoitetut menetelmät ovat täydentäviä.

Yleinen Suomalainen asiasanasto: aikuiset; elimensiirto; lapset; luunmurtumat; luuston sairaudet; osteoporoosi; tietokonetomografia;

Acknowledgements

This study was carried out in Bone and Cartilage Research Center (BCRU), University of Eastern Finland, Kuopio; Department of Applied Physics, University of Eastern Finland, Kuopio; and at the Department of Orthopaedics, Traumatology, and Hand Surgery, Kuopio University Hospital, Kuopio, during the years 2006 to 2013.

I would like to acknowledge my deepest gratitude for Professor Heikki Kröger. I am grateful for all the possibilities you have given me and for the faith you had in me. Your acknowledgement of everyone's individuality together with your flexibility had impressed me greatly. In addition, your positive attitude makes it a pleasure to work with you.

For the passion towards our projects and the flexibility in working hours I would like to thank my supervisor Docent Hanna Isaksson. I admire your productivity but also your ability to ensure things are good for us. Your help has been priceless. Further, I would like to thank my supervisor, Professor Jukka Jurvelin. Without your support and tricky, technical questioning I would not be here.

Overall, the collaboration with the Department of Applied Physics, University of Eastern Finland, has been excellent. I have learned a lot when working with PhD students with backgrounds other than the medical sciences. Thank you Antti Aula, Mikael Turunen, and Lassi Rieppo for your support, patience, and collaboration.

I am deeply grateful for the collaboration with Helsinki University Hospital. Therefore, I would like to acknowledge Docent Outi Mäkitie for her support in completing these studies. I admire your effectiveness and co-ordination. I am also thankful for Dr Mervi Mäyränpää and Dr Helena Valta for their huge input. Your accuracy has been priceless.

I would also like to thank Dr Tero Yli-Kyyny for his collaboration. Without you this study would have not been possible.

Many thanks for my reviewers, Professor Hannu Aro and Professor Karl Michaëlsson, for your valuable comments and your help in improving this thesis. I also would like to thank Ewen MacDonald for careful English revision of my thesis.

I am thankful to our excellent laboratory assistants, Ritva Sormunen, Eija Rahunen, and Aija Parkkinen, who have always been ready to try new ways to improve our methods. I am also thankful for the invaluable collaboration with Sib-Labs. Thank you Arto Koistinen for helping me with new techniques and equipment.

Many thanks also need to be expressed to our research group at Bone and Cartilage Research Unit. Thank you Toni, Matti, and Kari for great conversations and the good humour in between our work, but especially for the encouragement to keep me going on.

Thank you to my nearests and dearests, Suppa, Ulla, and Laura, with your families. You girls are amazing and without your friendship, my life would be empty. I enjoy our time together and thanks for keeping me active regardless of my busy schedules. Thank you Annariitta for being there when needed, for all our fruitful conversions, and for taking me to surf with you. Thank you Ulla M for providing excellent accommodation and company. Many thanks also belong to Afrouz, Lena, and Jana for giving me wider perspectives in life. You are wonderful. In addition, there are so many important people who have helped me, but unfortunately, I do not have the opportunity to mention everyone's name although not forgetting your importance.

I can never thank enough my dear parents, Eila and Antti, for their never-ending support and encouragement during this work. Thank you for listening and just being there when needed. I also respect the similar support from my brother Jyrki and his family Annika, Siiri, and Veikko. I enjoy our time together.

And finally, thank you Andrew. You have brought so much light into my life. Thank you for understanding, respecting me, and standing by my side. I have enjoyed our challenging experiences and our chances to grow.

I would like to acknowledge financial and academic support from the National Doctoral Programme of Musculoskeletal Disorders and Biomaterials (TBDP). I am grateful for the possibility to belong to this graduate school. I would also acknowledge financial support from Kuopio University Hospital, the Academy of Finland, the Finnish Cultural Foundation, the Päivikki and Sakari Sohlberg Foundation, the Sigrid Juselius Foundation, Emil Aaltonen Foundation, and the strategic funding of the University of Eastern Finland.

Jyväskylä June 2013

Inari Tamminen

List of the original publications

This dissertation is based on the following original publications:

I Tamminen I, Isaksson H, Aula AS, Jurvelin JS, Kröger H. Reproducibility and agreement of micro-CT and histomorphometry varies in human trabecular bone with different metabolic status. *J Bone Miner Metab. 29(4):442-448, 2011.*

II Tamminen IS, Yli-Kyyny T, Isaksson H, Turunen MJ, Tong X, Jurvelin JS, Kröger H. Incidence and bone biopsy findings of atypical femoral fractures. *J Bone Miner Metab, 2013 Apr 4 Epub ahead of print, DOI : 10.1007/s00774-013-0448-7.*

III Tamminen IS, Mäyränpää MK, Turunen MJ, Isaksson H, Jurvelin JS, Kröger H, Mäkitie O. Altered bone composition in fracture-prone children with vertebral fracture. *J Bone Miner Res. 26(9):2226-2234, 2011.*

IV Tamminen IS, Valta H, Jalanko H, Salminen S, Mäyränpää MK, Isaksson H, Kröger H, Mäkitie O. Pediatric solid organ transplantation and osteoporosis: characterization of bone histomorphometric findings. *Submitted.*

Contents

Abbreviations

1CTP	C-terminal telopeptide of type I collagen
1,25-OHD	1,25-hydroxyvitamin D, calcitriol
25-OHD	25-hydroxyvitamin D, calcidiol
ABD	Adynamic bone disease
Ac.F	Activation frequency
AFF(s)	Atypical femoral fracture(s)
ASBMR	American Society for Bone and Mineral Research
BFR/BS	Bone formation rate
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic protein
BMU	Bone multicellular unit
BP(s)	Bisphosphonate(s)
BRC	Bone remodeling compartment
BS/BV	Bone surface per bone volume
BS/TV	Bone surface per tissue volume
BSU	Bone structural unit
BV	Bone volume
BV/TV	Bone volume
$Ca_{10}(PO_4)(OH_2)$	Calcium hydroxyl apatite
c-Fms	Receptor for M-CSF
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease – metabolic bone disorder
CSF-1	Macrophage-colony stimulating factor, see M-CSF
CV	Coefficient of variation
deH-DHLNL	Dehydrodihydroxylysinonorleucine
deH-HHMD	Dehydrohistidinohydroxy-merodesmosine
deH-HLNL	Dehydrohydroxylysinonorleucine
D-Pyr	Deoxypyridinoline
DXA	Dual-energy X-ray absorptiometry
ECM	Extracellular matrix
ES/BS	Eroded (resorptive) surface
FGF	Fibroblast growth factor
FTIRI	Fourier transform infrared spectroscopic imaging
Fx	Fracture
GC	Glucocorticoid
GFR	Glomerular filtration rate
HHL	Histidinohydroxylysinonorleucine
HRT	Hormone replacement therapy
HSC	Hematopoietic stem cell
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin-10
ISCD	International Society for Clinical Densitometry
KDIGO	Kidney Disease: Improving Global Outcomes
MAR	Mineral apposition rate
M-CSF	Macrophage-colony stimulating factor
Md.V/TV	Mineralized bone volume
Micro-CT	Microcomputed tomography, μCT
MMA	Methylmetacrylate
MS/BS	Mineralizing surface
MSC	Mesenchymal stem cell
Mlt	Mineralization lag time
MS/OS	Percentage of osteoid mineralizing
Ob.S/BS	Osteoblast surface
Oc.S/BS	Osteoclast surface
OI	Osteogenesis imperfecta
OP	Osteoporosis
OPG	Osteoprotegerin

OS/BS	Osteoid surface
Omt	Osteoid maturation time
O.Th	Osteoid thickness
OV/BV	Osteoid volume
P1NP	N-terminal propeptide of type I procollagen
P-ALP	Plasma alkaline phosphatase
P-Ca-ion	Plasma ionized calcium
PMMA	Polymethylmetacrylate
P-Pi	Plasma phosphate
PTH	Parathyroid hormone
Pyr	Pyridinoline
QUS	Quantitative ultrasound
RANK	Receptor activator of nuclear factor-κB (NFκB)
RANKL	Receptor activator of nuclear factor-κB ligand (NFκB ligand)
ROD	Renal osteodystrophy
ROI	Region of interest
Runx2	Runt-related transcription factor 2
S1P	Sphingosine-1-phosphate
SD	Standard deviation
SERM(s)	Selective estrogen-receptor modulator(s)
SMI	Structural model index
SOST gene	A gene that encodes production of sclerostin
T-score	The number of standard deviations above or below the mean for a healthy 30-year-old adult of the same sex and ethnicity as the patient
Tb.N	Trabecular number
Tb.Pf	Trabecular pattern factor
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
TV	Tissue volume
Tx	Transplantation
U-Ca	Urine calcium
U-Crea	Urine creatinine
VDR	Vitamin D receptor
Vert Fx	Vertebral fracture
VOI	Volume of interest
WHO	World Health Organization
WNT	Wingless-int
W.Th	Wall thickness
Z-score	The number of standard deviations above or below the mean for the patients age, sex, and ethnicity

1 Introduction

Osteoporosis is the most common metabolic bone disease worldwide and it has emerged as a health concern for all age groups (Cooper 1989). The number of patients suffering from osteoporosis will increase in the future in conjunction with the aging of the population (Melton et al. 1992; Ray et al. 1997; Burge et al. 2007). The disease has a major impact on society, it exerts physical, psychosocial, and financial consequences. Osteoporosis is characterized by low bone mineral density (BMD) measured by dual-energy X-ray absorptiometry (DXA) and a deterioration in bone quality that predisposes the individual to fractures even after minimal trauma (National Institutes of Health Consensus 2001). The slow loss of bone is often asymptomatic and often the first sign of the disease is a fracture (Vestergaard et al. 2005; Unnanuntana et al. 2010). The progressive increase in the fracture risk occurs after accelerated bone loss especially after menopause in women. There is a relatively high morbidity and mortality related to the fractures (Reginster and Burlet 2006).

Peak bone mass is achieved in the age of early 20's. This is the major determinant for the risk of osteoporosis later in life (Hernandez et al. 2003). Thus, the childhood and adolescent periods are important for bone health. Osteoporosis is caused by an imbalance in bone remodeling, and thus, understanding of the cellular events is important in advancing the treatment. BMD is a good measure of bone density but its ability to predict fractures is only moderate (Greenspan 2004; Pasco et al. 2006). Hence, other bone properties, especially bone structure, composition, and metabolism, need to be studied to understand better the factors contributing to bone strength, and thus, to predict fracture risk more effectively (Seeman and Delmas 2006).

Bone quality is a term used to describe aspects of bone structure and composition independently of BMD (Compston 2006). Bone quality not only includes bone architecture, geometry, turnover, microdamage, and mineralization but also the composition of bone matrix and mineral (National Institutes of Health Consensus 2001). Bone properties are largely interdependent. Thus, impairment in one property leads often to another abnormality. Bone quality can be measured using several approaches (Compston 2006).

Quantitative bone histomorphometry has become the standard method to evaluate cellular events of bone modeling and remodeling. It is a valuable and well-established clinical and research tool for studying the effects of treatment, etiology, and pathogenesis of metabolic bone diseases (Recker et al. 2011). Alterations in bone remodeling contribute to impaired bone microstructure (Compston 2006). A three-dimensional measure of bone microarchitecture using microcomputed tomography (micro-CT) provides better visualization of bone structures than any of the two-dimensional methods (Compston 2006; Recker et al. 2011). In addition to analysis of the trabecular microarchitecture, micro-CT enables quantification of the spatial mineral density (Entezari et al. 2012). The organic matrix of bone consists mainly of collagen type I that confers flexibility on the bone structure whereas the inorganic matrix consists of mineral crystals that provide its stiffness (Currey 2001). Changes in collagen cross-linking and alterations in mineral crystal size and structure may have biomechanical implications (Paschalis et al. 2001; Vashishth et al. 2001; Wang et al. 2002). Bone matrix and mineral composition can be measured using Fourier transform infrared spectroscopic imaging (FTIRI) (Boskey and Mendelsohn 2005).

This study aimed to reveal the role of qualitative properties of bone beyond what can be determined with bone density measurements. Changes in bone quality were characterized by quantifying bone remodeling using bone histomorphometry in specific cohorts of adult and pediatric patients. This was combined with studies of the bone microarchitecture and composition using micro-CT and FTIRI.

2 Review of Literature

2.1 BONE BIOLOGY

The skeletal system consists of bone and cartilage. The skeleton supports and protects the internal organs and muscle attachment to enable locomotion. In addition to this structural function, the skeleton participates in crucial hormonal functions. For instance, it is important in the maintenance of serum homeostasis as a reserve of calcium and phosphate. The microstructure of bone is constantly changing in response to mechanical and hormonal stimuli. Bone remodeling is a process that helps to maintain optimal bone structure through the renewal of old structures (Buckwalter et al. 1996a; Hadjidakis and Androulakis 2006).

2.1.1 Structure of bone

Bone tissue is a complex structure that consists of cortical (compact) bone and cancellous (trabecular) bone (Seeman and Delmas 2006). The outer layer of cortical bone is called periosteum and the inner side is called endosteum (Buckwalter et al. 1996a). Cortical bone accounts for most of the skeletal mass, i.e., approximately 80% of the mature skeleton (Adler 2000). Fatty or hematopoietic bone marrow is not only important for the development of bone cells but also for the support of blood circulation in bone tissue. Cancellous bone is characterized by its high metabolic activity and modeling properties. Thus, it can respond more rapidly to changes in mechanical loading than can cortical bone (Buckwalter et al. 1996a). This is explained by high bone surface area (80%), originating from the network-like structure of cancellous bone (Adler 2000). The bone mass, however, is much higher in cortical bone than in cancellous bone and the porosity of the cortical bone has been found to be around 3-5% in young individuals (Burr 2010).

Both cortical and cancellous bone can consist of woven (fiber or primary) or lamellar (secondary) bone (Sevitt 1981; Currey 1984; Buckwalter 1994). The composition, organization, mechanical properties, and formation differ between these structures. Woven bone is typically embryonic bone and it is rarely present in the healthy skeleton after the age of 4-5 years (Buckwalter et al. 1996a). Soft-tissue injury, therapies stimulating bone formation, inflammation, and some metabolic or malignant diseases may trigger the formation of woven bone. Woven bone is characterized by an irregular collagen structure and a high osteocyte volume (Buckwalter et al. 1996a). In contrast, lamellar bone is formed by tightly organized collagen fibrils that vary less in diameter than those found in woven bone (Cooper et al. 1966; Currey 1984; Martin and Burr 1989). Due to its more balanced cell and water content in combination with the organized collagen structure and regular mineralization pattern, lamellar bone is less brittle than woven bone (Seeman and Delmas 2006). Lamellar bone forms structures of trabeculae in cancellous bone, and circumferential, interstitial, and osteonal lamellae (i.e., Haversian canals) in cortical bone (Buckwalter 1994).

The vascular system in bone transports nutrients to the bone marrow, bone tissue, and periosteum. Disruption of the blood supply caused by a disease, injury, or operation may lead to tissue necrosis and impaired bone healing (Buckwalter et al. 1996a). The vascular system of bone consists of a complex network which is necessary to transfer systemic signals such as hormonal effects to and from bone.

2.1.2 Bone cells

Bone cells are responsible for bone formation and resorption events, taking part to retain the mineral homeostasis, and fracture repair. The cells have two origins. Firstly, the hematopoietic cell-line gives rise to circulating or marrow monocytes in addition to preosteoclasts and osteoclasts. Secondly, the mesenchymal stem-cell line are the origins of

undifferentiated cells or preosteoblasts, osteoblasts, bone lining cells, and osteocytes (Buckwalter et al. 1996a).

Osteoclasts

Unlike other bone cells, bone resorbing cells, osteoclasts, have a hematopoietic stem-cell (HSC) precursor from the monocyte/macrophage family (Teitelbaum 2011). There are two principal cytokines that are essential for osteoclast differentiation; receptor activator of nuclear factor-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF, also CSF-1) (Khosla 2001). RANKL and M-CSF are produced by marrow stromal cells, osteoblast lineage cells, and T cells (Pixley and Stanley 2004). RANKL is the key cytokine in osteoclastogenesis and in osteoclast activation (Khosla 2001); it belongs to the tumor necrosis factor (TNF) super family. M-CSF is important in proliferation, survival, and differentiation of osteoclast precursors but also in the survival and cytoskeletal rearrangement in bone resorption. Osteoprotegerin (OPG) is a physiological RANKL inhibitor, i.e., an anti-osteoclastogenic protein (Kostenuik and Shalhoub 2001) produced by osteoblasts (Khosla 2001). To sum up, the RANKL-OPG ratio is the key regulator of the bone resorption process.

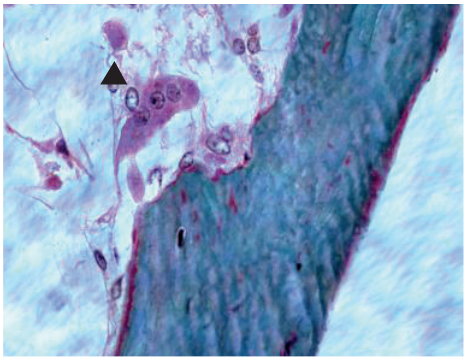

Figure 1. Histological image of bone resorbing cells, osteoclasts. Osteoclasts are identified as typically multinucleated, large to giant cells that are often irregular in shape (arrow). Osteoclasts are able to produce a microenvironment that degrades the bone matrix. Masson Goldner trichrome stain. Magn. 400×.

In the differentiation process of osteoclasts, HSCs express c-Fms and receptor activator of nuclear factor-κB (RANK) that are receptors for M-CSF and RANKL, respectively (Ross 2006; Mizoguchi et al. 2009). Mesenchymal stromal cells and osteoblastic lineage cells respond to signals that stimulate bone resorption, such as secretion of parathyroid hormone (PTH) and vitamin D_3 (Aubin and Bonnelye 2000; Zhang et al. 2001). A multitude of pro- and anti-osteoclastogenic proteins, such as RANKL, M-CSF, and OPG are secreted from stromal and osteoblastic cells. This enhances osteoclast differentiation leading to osteoclast progenitor cell fusion to become mature multinucleated giant osteoclasts under the influence of RANKL (Khosla 2001; Matsuo et al. 2004).

Bone resorption is the main function of osteoclasts by means of the degrading function of the lysosomal enzymes like tartrate-resistant acid phosphatase (TRAP) and cathepsin K (Väänänen et al. 2000). Osteoclasts are capable of forming a unique microenvironment that separates the cell from the general extracellular space (Boyle et al. 2003; Teitelbaum 2011).

Histologically osteoclasts are identified typically as multinucleated cells that range from large to giant in size (Figure 1). The cell is irregular in shape and stains positively for TRAP. Cytoplasm is foamy and acidophilic (Hancox 1972; Malluche and Faugere 1986) containing a large number of mitochondria and lysosomes (Buckwalter et al. 1996a). A perimeter zone can be detected on the attachment site to bone surface. The fluid cavity between the cell and

the mineral matrix, i.e., ruffled border can be observed (Hancox 1972). On trabecular and periosteal bone surfaces, osteoclasts form resorption depressions called Howship's lacunae. The resorption cavities in cortical bone are formed so that they follow the osteonal tunnels through the bone (Buckwalter et al. 1996a).

Osteoblasts

Osteoblasts constitute from mesenchymal precursor, i.e., osteo-chondrogenic precursor (Bianco et al. 2001). The differentiation from stem cells into mature osteoblasts has several phases. Mature osteoblasts account for bone formation by producing both collagenous and non-collagenous bone matrix at bone resorption site (Buckwalter et al. 1996a; Hadjidakis and Androulakis 2006). They also take part in bone mineralization (Marotti et al. 1972), and may play a role in electrolyte flow between extracellular and osseous fluid (Raisz and Kream 1983). Histologically, osteoblasts are identified as plump cells that lie in line on top of the unmineralized bone, osteoid (Figure 2). During the maturation process, the cells become less plump and lose their bone forming activity (Malluche and Faugere 1986; Hancox 1972).

Osteoblast differentiation can be divided into three phases: proliferation, maturation, and termination. Firstly, mesenchymal stem cells (MSCs) proliferate to osteo-chondogenic precursors. This is followed by maturation to pre-osteoblast, immature osteoblast, and mature osteoblasts (Eriksen 2010). The earliest osteoblastic marker called Runt-related transcription factor 2 (Runx2) has an important role in the stimulation of the progenitor cells to differentiate into osteoblast lineage cells (Komori et al. 1997). Osteoblast differentiation is also modulated by several autocrine, paracrine, and endocrine factors (Qin et al. 2003). Several pathways interact, and the balance between activators and repressors is crucial for adequate cell development and maturation. Some of the main pathways include bone morphogenetic proteins (BMPs), growth factors such as insulin-like growth factor 1 (IGF-1) and fibroblast growth factor (FGF), and systemic hormones like PTH (Qin et al. 2003; Westendorf et al. 2004).

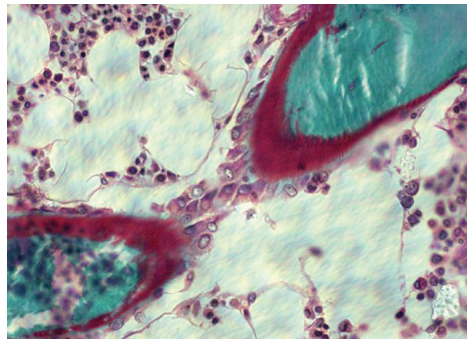

Figure 2. Histological image of bone forming cells, osteoblasts. Osteoblasts are identified as plump cells that lie in line on top of the unmineralized bone, osteoid, producing bone matrix material. Masson Goldner trichrome stain. Magn. 200×.

Another important regulation pathway in the osteoblast differentiation is so called Wingless-int, i.e., WNT signaling pathway. WNTs are glycoproteins (Eriksen 2010) that bind to their receptor complexes transducing their signals to β-catenin (Boyden et al. 2002; Little et al. 2002; Westendorf et al. 2004; Clevers 2006). WNT/β-catenin signaling is important in mechanotransduction, fracture repair, and osteoclast maturation (Robinson et al. 2006; Spencer et al. 2006; Chen et al. 2007) modulated by Runx2 and Osterix (Hill et al. 2005). Sclerostin is an osteocyte-derived WNT antagonist (a protein) that is encoded by SOST gene (Balemans et al. 2001; van Bezooijen et al. 2005). Sclerostin inhibits osteoblast

activity (van Bezooijen et al. 2007), and a monoclonal sclerostin antibody has been found to have anabolic skeletal effects in humans (Padhi et al. 2011).

Finally in the end of the bone formation cycle, the active osteoblast has three pathways to termination. The cells may remain on the bone surface and lose their formation activity thereby flattening and becoming bone lining cells. Some of the cells become surrounded by the matrix and become transformed into osteocytes whereas some of the cells undergo programmed cell death, apoptosis (Hadjidakis and Androulakis 2006).

Bone lining cells

Bone lining cells have a mesenchymal origin. Some of the osteoblasts differentiate as lining cells after completing bone formation (Hadjidakis and Androulakis 2006; Eriksen 2010). These flat cells are located in a line directly against the bone matrix on endocortical and cancellous bone surfaces (Bianco et al. 2001). Histologically, the lining cells are identified as flat and elongated cells (Hauge et al. 2001). The amount of cytoplasm and the number of organelles are lower than in mature osteoblasts (Buckwalter et al. 1996a).

Osteocytes

Osteocytes are non-proliferative bone cells originating from MSCs through osteoblast differentiation (Noble 2008; Bonewald 2011). They account for 90-95% of all bone cells in the skeleton (Bonewald 2011). Approximately 10-20% of the osteoblasts differentiate into osteocytes (Noble 2008) that can stay viable in bone for decades (Bonewald 2011). During osteocytogenesis, osteocytes become encased in the mineralized matrix, getting 'buried' in the bone matrix in lacunas (15-20 μm) as so called 'osteoid osteocytes' (Bonewald 2011). Osteocytes express long dendritic processes that extend through a canalicular structure (250-300 μm). These dendrites extend processes towards both the bone surface and blood vessels forming an elaborate canalicular structure with a large surface area (Bonewald 2011). Since they have a large surface area, osteocytes are thought to be capable of reacting to minor changes in the bone microenvironment thereby changing expression of growth factors and controlling mineralization (Frost 1960; Noble 2008). Thus, they could act as mechanosensory cells (Bonewald 2011). The mechanism triggering osteocytes to start to differentiate from osteoblasts to embedded cells in osteoid is unknown (Bonewald 2011). Sclerostin is a marker of mature osteocytes (Poole et al. 2005) and it is known to suppress bone formation (Balemans et al. 2001).

Histologically osteocytes can be identified as individual cells located in lacunae of the mineralized bone matrix. Osteocytes are relatively small cells with cytoplasmic processes that extend to narrow canaliculi forming an elaborate network. Hence, the osteocytic network interconnects throughout the living bone (Malluche and Faugere 1986).

2.1.3 Bone matrix

Bone consists of cells and matrix. The extracellular matrix (ECM) of the bone composite consists of mineral (50-70%), organic components (20-40%), and water (10%) (Dempster 2006). Both organic and inorganic matrix is necessary for metabolic and mechanical functions (Seeman and Delmas 2006). The organic matrix is responsible for tension resistance (viscoelasticity and tensile strength) whereas the inorganic matrix confers other properties such as compression resistance and stiffness (Buckwalter et al. 1996a; Currey 2005).

The organic matrix contains collagens (approximately 90%), non-collagenous proteins, and lipids (Dempster 2006). Type I collagen accounts for approximately 90% of the entire collagen content (Hadjidakis and Androulakis 2006). Type I collagen can be distinguished from other collagen types based on its unique amino acid content (Buckwalter et al. 1996a). Collagen forms intermolecular cross-links in tissue initiated by enzymes (Eyre et al. 1984a; Eyre et al. 1984b). Seven different cross-link patterns have been characterized: dehydrodihydroxylysinonorleucine (deH-DHLNL), dehydrohydroxylysinonorleucine

(deH-HLNL), dehydrohistidinohydroxymerodesmosine (deH-HHMD), pyridinoline (Pyr), deoxypyridinoline (d-Pyr), pyrrole, and histidinohydroxylysinonorleucine (HHL). The first three cross-links are reducible by sodium borohydride ($NaBH_4$) whereas the last four are non-reducible (Yamauchi 1996). With maturation, the number of DHLNL cross-links decreases whereas the number of Pyr cross-links increases partly due to the maturation of DHLNL to Pyr with time (Eyre et al. 1984b).

The inorganic matrix accounts for the mineral phase of bone that is a nanocrystalline, i.e., an analog of hydroxyl apatite [$Ca_{10}(PO_4)_6(OH)_2$]. Hydroxyl apatite may have carbonate, magnesium, or acid phosphate substitutes (Wopenka and Pasteris 2005). Inorganic matrix preserves a remarkable ion reservoir to control ion (calcium, phosphate, magnesium) homeostasis. Additionally, it is important for determining bone mechanical stiffness and strength (Buckwalter et al. 1996a). With increasing tissue age, changes in the mineral crystals may affect bone mechanical properties (Akkus et al. 2004). Therefore, both the age and the amount of mineral affects bone material properties (Buckwalter et al. 1996a; Faibish et al. 2006).

Water accounts for approximately 10% of the bone weight (Dempster 2006). The amount of water is dependent on tissue age (Robey and Boskey 2009). Lipids account for around 2% of the dry weight of bone. In addition, there are several growth factors (Buckwalter et al. 1996a) and enzymes in the bone matrix of which some are cell associated and some are located in the ECM (Robey and Boskey 2009).

2.1.4 Bone mineralization

Within the bone mineralization, solid calcium phosphate is formed from calcium and phosphate in the organic matrix (Buckwalter et al. 1996a). This transformation is a series of sequential events that occurs in two phases. The mineral deposition during the remodeling cycle is called primary mineralization, whereas secondary mineralization occurs after completion of the remodeling cycle (Meunier and Boivin 1997).

Firstly, a crystalline apatite, the early form of the calcium phosphate, is produced into the collagen network. Secondly, the amount of minerals increases in the hole zones followed by increase in their size. Hence, the mineral extends to fill the gaps between the holes (Glimcher 1992; Roberts et al. 1992). The mineralization increases and the crystals mature with time, thereby increasing the bone stiffness. Woven bone is replaced by lamellar bone (Torzilli et al. 1981). Bone turnover rate affects the degree of secondary mineralization. When the bone turnover rate is low, there is more time for the mineralization process to be completed in contrast to high turnover bone where removal of recently formed bone occurs prior to proper secondary mineralization (Meunier and Boivin 1997). If the mineralization fails, as is the case in osteomalacia and rickets, the high amount of unmineralized bone leads to increased fragility (Demiaux et al. 1992).

2.1.5 Bone formation, growth, and bone modeling

The formation of the skeleton starts in the embryo and continues after birth (Teti 2011). There are two types of bone formation: endochondral ossification and intramembranous ossification (Teti 2011). Peak bone mass is achieved in early adulthood, and heritable factors account for 60-80% of the variation in peak bone mass (Heaney et al. 2000).

Endochondral ossification, i.e., ossification via a cartilaginous model, accounts for the formation of long bones (not clavicle), short bones, and epiphyseal ossification centres. At epiphyseal sites, the ossification continues until the skeleton is mature. In intramembranous ossification, the bone formation occurs without a cartilage model (Buckwalter et al. 1996b). This is responsible for the embryonic development of the flat bones, frontal and parietal bones of the skull, and pelvis (Gentry and Bramblett 1988; Buckwalter et al. 1996b).

Bone modeling alters bone shape and size by adapting to the mechanical loading during growth, whereas bone remodeling accounts for bone turnover without causing any alterations in the bone shape mainly after growth (Seeman 2009). The adaptation to mechanical forces is also known as Wolff's law (Frost 1994). Initial ossification of the

embryonic skeleton requires simultaneous bone modeling and remodeling. Consequently, the collaboration between bone cells occurs in a specific order under multiple regulators (Seeman 2009). During growth, bone is removed and replaced rapidly (Buckwalter et al. 1996b; Teti 2011). Growth of the physes allows lengthening of the bone. Metaphyseal reshaping is necessary for maintaining the long shape of the bone. Without this, the periosteal bone formation would increase the bone diameter. The endosteal resorption enlarges the medullary cavity. The changes are mostly due to bone modeling but some remodeling occurs as well (Buckwalter et al. 1996b). In bone modeling, bone formation and resorption occur simultaneously even for long periods and over large regions (Seeman 2009).

2.1.6 Bone remodeling

Bone remodeling replaces the old damaged bone tissue with new competent bone tissue. The bone remodeling sites are located at cancellous bone and Haversian canal surfaces. The remodeling system is capable of responding to hormonal and nutritional influences as well as to the changing mechanical loads (Hadjidakis and Androulakis 2006). In general, if mechanical loads are redused as occurs during immobilization, all bone loss occurs through bone remodeling (Robling 2012). Bone remodeling can be differentiated into 5 stages: 1) quiescence, 2) activation, 3) resorption, 4) reversal, and 5) formation (Figure 3) (Seeman 2009, Teti 2011). The sequential remodeling events occur in bone multicellular units (BMUs). The length of the cycle can take up to 6 months (Recker et al. 1988) of which around 2 weeks is required for bone resorption, 4 to 5 weeks for the reversal phase, and finally bone formation may last for 4 months until the new bone structural unit (BSU) is completed. There are complex signaling pathways between the skeletal cells but also within each cell (Hadjidakis and Androulakis 2006).

As the key requirements for bone remodeling, both bone resorption and bone formation have to occur at the same site in combination with quantitative balance and precise timing. The first stage of remodeling, *quiescence*, is the phase when lining cells still cover the mineralized bone surface. It has been postulated that the bone remodeling site is anatomically separated by the surrounding environment by a bone remodeling compartment (BRC) that is formed either by osteoblast-lineage cells (Hauge et al. 2001; Andersen et al. 2009; Jensen et al. 2012) or by macrophage-lineage cells (OsteoMacs) (Pettit et al. 2008). The BRC has been proposed to form a sealing zone that critically controls bone remodeling (Andersen et al. 2009).

In the second stage, *activation*, osteoclast precursors are recruited into the remodeling site in response to multiple signals that also trigger the cells to undergo maturation (Seeman 2009). The pathways causing osteoclast recruitment before osteoclastogenesis are a topic of intense research (Teti 2011). Sphingosine-1-phosphate (S1P) and several other factors such as chemokines co-operate in osteoclast recruitment (Yu et al. 2003; Yu et al. 2004; Kim et al. 2005; Ishii et al. 2011). In addition, osteocyte apoptosis seems to trigger osteoclast activation although the exact details remain unclear (Verborgt et al. 2002). However, the osteoblast lineage cells secrete enzymes that digest the bone surface and express RANKL that is a member of the TNF superfamily. RANKL interacts with its receptor RANK on the surface of osteoclast precursors. These events then activate osteoclast differentiation and activation (Hsu et al. 1999). The effects of RANKL can be inhibited by OPG that is a glycoprotein belonging to the TNF superfamily and which is secreted mainly by osteoblast lineage cells (Simonet et al. 1997; Hofbauer and Schoppet 2004). OPG is a soluble receptor that acts as an antagonist for RANKL. It regulates bone resorption by inducing osteoclast apoptosis but also inhibits the final differentiation and activation. The effects of OPG are fully reversible (Hadjidakis and Androulakis 2006).

The third stage of remodeling, the *resorption phase*, is a short phase lasting for only from a few days to around two weeks. During the resorption phase, osteoclasts mature, polarize, adhere to the bone surface, and degrade the bone matrix (Recker 1988; Teti 2011). The

completion of resorption phase is caused by multiple signals that trigger osteoclast apoptosis (Del Fattore et al. 2008). However, the balance between pro-apoptotic and anti-apoptotic factors is crucial because the M-CSF and RANK/RANKL systems can intervene during extended osteoclast survival (George et al. 2010). As a results of osteoclast death, the remodeling site becomes free of osteoclasts (Del Fattore et al. 2008).

The next stage in the remodeling, *reversal,* is not fully understood. The cells involved and the biological importance of this stage are unknown, but typically macrophage-like mononuclear cells are thought to be involved. This might be a consequence of collagen degradation or to debris removal (Teti 2011). Additionally, proteoglycans, similar to those on the cement line, have been found at sites where the osteoclast activity has been reduced after the resorption phase suggesting that these reversal cells could contribute to the cement line cells. Further, the aim of the reversal cells could also be to stimulate the completion of the remodeling cycle (Raisz 1999).

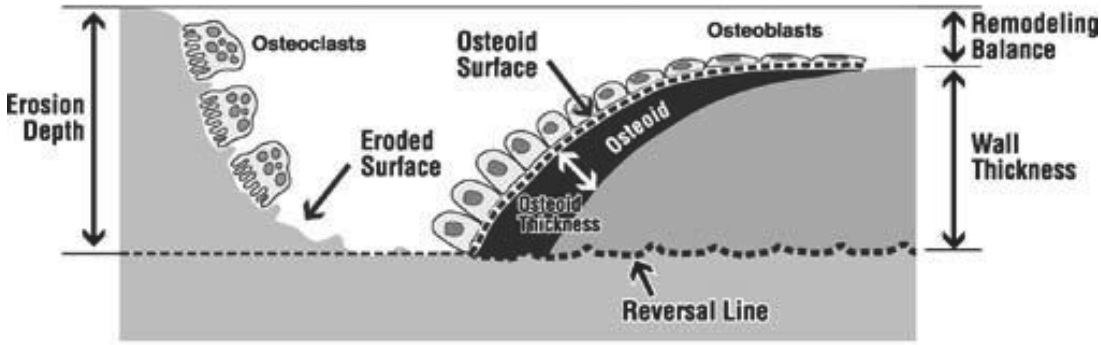

Figure 3. A bone remodeling site. The osteoblast precursors expressing RANKL on their surfaces interact with their receptor RANK on the osteoclast precursors. This enhances maturation and activation of the osteoclasts. After resorption of old bone by osteoclasts, the new bone matrix, osteoid, is formed by osteoblasts. Reprinted with the kind permission by Springer from Rauch 2006.

The last stage of remodeling cycle is the *bone matrix formation* (Hadjidakis and Androulakis 2006; Seeman 2009). Osteoblast precursors mature at the resorption site. The formation phase occurs slowly requiring 3 to 4 months before completion (Pevsner-Fischer et al. 2011). During this time, new unmineralized bone, osteoid, is formed and later mineralized (Hadjidakis and Androulakis 2006). The mechanisms involved in the recruitment of osteoblast precursors to the remodeling site are under investigation (Teti 2011). Osteoclasts could send signals to osteoblasts but also osteocytes are thought to co-ordinate the bone remodeling (Khosla 2001; Hofbauer and Schoppet 2004; Bonewald 2011). Several signals may interact to complete the formation phase (Teti 2011). Osteocytes have been believed to emit signals after they become trapped in the mineral matrix. Sclerostin has been thought to be one of the major factors accountable for this phenomenon. It is secreted by osteocytes and it blocks WNT signaling thereby blocking osteoblast induction (Moester et al. 2010). At the end of the formation phase in the healthy skeleton, approximately 5% of additional bone compared to resorption is formed in every remodeling cycle (Parfitt et al. 2000). After the fifth decade of life and in some metabolic bone diseases such as osteoporosis, the bone formation decreases with respect to bone resorption, leading to bone loss (Parfitt et al. 2000; Rachner et al. 2011). Further, altered mineralization may occur in metabolic bone disease such as in vitamin D deficiency (Demiaux et al. 1992).

2.1.7 Regulators of bone

Our understanding of the mechanisms controlling bone-cell activity and crosstalk has increased rapidly during the last years but many functions still remain a mystery. The discovery of the RANK/RANKL/OPG-pathway has increased the appreciation of the importance of regulatory factors (Khosla 2001). Several systemic and local factors, including genetic factors, systemic hormones, exercise, obesity, and local factors e.g. cytokines and prostaglandins, contribute to the regulation of the bone-cells and to the interactions between cells (Buckwalter et al. 1996b; Hadjidakis and Androulakis 2006).

Hereditability and local factors

The hereditability related to skeletal size with regards to height and other anthropometric variables has known for decades (Clark 1956). Genetic factors regulate peak bone mass and approximately 80% of the bone mass has been considered to be regulated by genes (Smith et al. 1973; Howard et al. 1998; Peacock et al. 2002). Genetic factors are associated with many bone diseases, for example osteogenesis imperfecta (OI) (Peacock et al. 2002; Rauch and Glorieux 2004). However, the hereditability of bone loss in advanced age is low (Moayyari et al. 2012). Similarly, the hereditability of fractures in the oldest age is low (Kannus et al. 1999; Michaëlsson et al. 2005).

Cytokines and prostaglandins exert direct effects on bone-cell activity (Buckwalter et al. 1996b). Several *in vitro* studies support the theory that cytokines, such as TNF-α and interleukin-10 (IL-10), affect bone metabolism including bone remodeling (Hofbauer and Schoppet 2004). Prostaglandins, which are fatty acid derivates, may have a local effect on bone cells, modulating cellular events e.g. inflammation and ion transport in other tissues (Buckwalter et al. 1996b).

Hormones

Systemic hormones mediate their effects throughout the skeleton and regulation of calcium homeostasis is one of their main functions. Nowadays, it is appreciated that the metabolic function of bone is an extremely vital phenomenon (Guntur and Rosen 2012).

PTH is a hormone secreted by the chief cells of the parathyroid gland. PTH is secreted in conditions of hypocalcaemia to ensure a normal serum calcium level (Figure 4). There is an integrated interaction between calcium regulation and phosphate metabolism. A high serum phosphate level stimulates PTH production because phosphate binds to free calcium thus leading to a reduction in the serum calcium level (Silver and Naveh-Many 2009). As a result of the elevated serum concentration of phosphate, FGF-23 is secreted from osteoblasts and osteocytes which leads to suppressed reabsorption of the phosphate in the kidneys (Fukumoto 2008). It has been speculated that FGF-23 could be a target for novel therapies (Shimada and Fukumoto 2012).

Calcitonin is a hormone that decreases serum calcium level. The C cells of the thyroid gland secrete this hormone. Calcitonin has been claimed to inhibit osteoclasts either by attenuating osteoclasts differentiation or by suppressing bone formation and activation of osteoclasts (Copp and Cameron 1961; Nicholson et al. 1986). The effects of calcitonin are very short-term, and its role for healthy bone physiology is unclear (Buckwalter et al. 1996b).

Age and sexual maturity affect the secretion of several systemic hormones that also regulate bone growth (Teti 2011). Before puberty, growth hormone stimulates pubertal bone growth thereby increasing the bone mass (Wang et al. 2004). Thyroid hormones have been shown to have a positive effect on skeletal growth due to increased energy production (Gogakos et al. 2010).

Estrogens, secreted from ovaries, have actions on PTH, calcitonin, and vitamin D thereby increasing calcium reabsorption in the kidney (Buckwalter et al. 1996b). Both osteoblasts and osteoclasts possess estrogen receptors. Estrogen deprivation, as occurs after menopause, accelerates bone loss in women due to increased remodeling activity and a

more negative bone multicellular unit (BMU) balance. The lifespan of osteoblasts is reduced whereas the lifespan of osteoclasts is prolonged. Further, the mineral content of bone decreases due to the high remodeling rate (Seeman 2003).

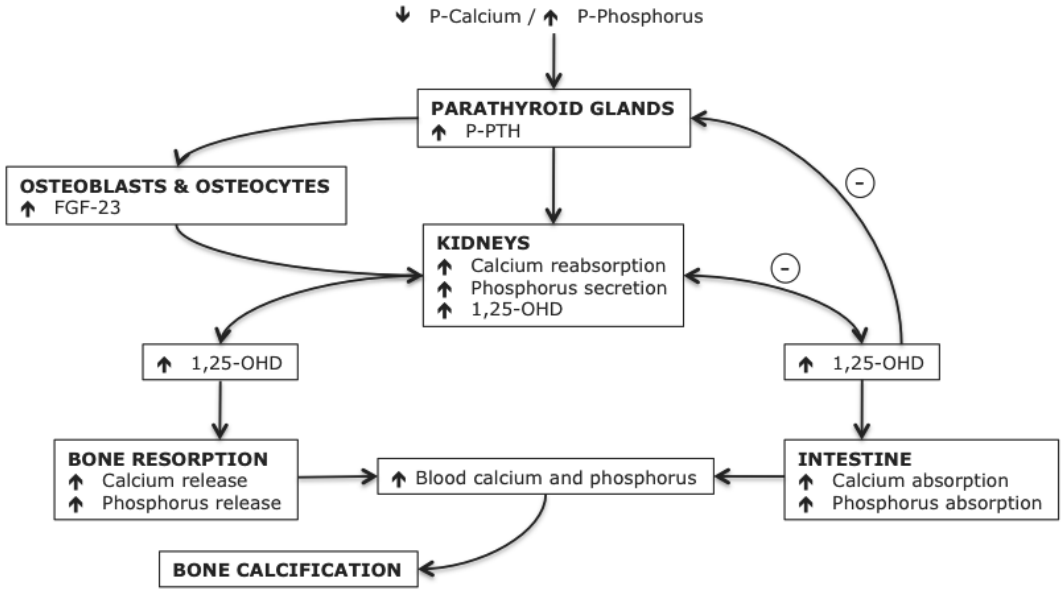

Figure 4. Regulation of calcium metabolism. In hypocalcaemia, parathyroid hormone (PTH) stimulates the release of calcium from bone, increases the reabsorption of calcium in the renal tubules, and also affects indirectly the reabsorption of calcium in the intestine by increasing the synthesis of calcitriol in the proximal tubules of kidneys to maintain normocalcaemia (Potts 2005). The regulation of phosphate metabolism is interrelated (Silver and Naveh-Many 2009). The increased phosphate level of blood stimulates production of FGF-23. This leads to decreased reabsorption of phosphorus in the kidneys (Fukumoto 2008). P=plasma, 1,25-OHD=1,25-dihydroxyvitamin D (calcitriol). Modified from Holick (2007) and Fukumoto (2008).

Vitamin D and calcium

Both vitamin D and calcium are vital for bone metabolism. Production of vitamin D_3 (cholecalsiferol) from the 7-dehydrocholesterol is promoted by ultraviolet B radiation in the skin. Calcium and vitamin D can also be obtained from food. Vitamin D_3 is further metabolized in the liver to 25-hydroxyvitamin D (25-OHD, calcidiol). The biologically active metabolite, 1,25-dihydroxyvitamin D ($1,25\text{-}(OH)_2D$, calcitriol), is formed by hydroxylation from calcidiol in the kidneys (DeLuca 2004; Holick 2006; Holick 2007). The effects of calcitriol on bone are complex. Calcitriol increases the serum concentration of calcium and phosphorus, and stimulates the absorption of calcium in the intestine. Although osteoclasts do not possess vitamin D receptors (VDR) (Yasuda et al. 1998), the increase in the serum concentration of calcium and phosphorus is mediated by the increased formation of osteoclasts by the activated RANK/RANKL pathway through osteoblasts (Raisz et al. 1972; Holick 2007). In contrast, calcitriol downregulates OPG production therefore stimulating osteoblast formation (Yasuda et al. 1998). Thus, calcitriol stimulates also mineralization of the bone matrix (Holick 2007).

PTH is the main stimulator of the production of calcitriol in the kidneys. There is a feedback mechanism through which calcitriol inhibits the synthesis and secretion of PTH (Martin and Gonzalez 2004). Low levels of serum phosphate, calcium, or FGF-23 and increased PTH level favor production of calcitriol (Holick 2007).

Mechanical loading
Previous studies have revealed that moderate regular physical exercise can help to maintain bone mass not only in young subjects but also in the elderly (Rubin et al. 1990; Callreus et al. 2012; Korhonen et al. 2012), or at least to decelerate bone loss in elderly (Rubin et al. 1992; Rikkonen et al. 2010). Regular exercise in children and adolescents improves gain in bone mass (Pitukcheewanont et al. 2010). Age and hormonal balance may change the responses of bone to mechanical loading (Bain and Rubin 1990).

2.2 BONE QUALITY

Bone density accounts for only 70% of bone strength but there are currently no better measures available for clinical practice (National Institutes of Health Consensus 2001). Bone strength can be defined by the composition and structure of bone material (Currey 2001). Bone has to be stiff in order to resist deformation, and thereby, to be able to resist fractures. In addition, bone must absorb energy and respond to forces with appropriate flexibility. Furthermore, the weight of bone should be light enough to enable locomotion. Therefore, numerous aspects of bone material and structural properties account for bone health (Seeman and Delmas 2006) and are able to respond to the bone's mechanical needs (Currey 2005).

Currently, the definition of osteoporosis includes both loss of bone mass and alteration in bone quality (National Institutes of Health Consensus 2001). Bone quality is the term used to describe aspects of bone structure and composition independently of BMD (Compston 2006). Bone quality includes bone architecture, geometry, turnover, microdamage, and mineralization but also there are components reflecting the composition of the bone matrix and mineral (National Institutes of Health Consensus 2001). Several techniques to measure bone quality have been developed to understand the pathogenesis of bone disease in more detail, to evaluate the effects of treatments, and especially to develop new therapeutic agents (Compston 2006).

2.2.1 Assessment of bone quality

Bone properties are strongly interdependent. Thus, an imbalance in one function typically leads to another abnormality. Bone quality can be measured using various approaches depending on the research question under investigation (Figure 5). Most of the techniques require a biopsy or an autopsy specimen, and are currently available only for research purposes. The improvements in research techniques have enhanced the understanding of the mechanisms that contribute to compromised bone strength, and thus to the mechanisms that can be targeted by therapeutic intervention (Compston 2006).

Bone turnover can be determined *in vivo* using biochemical bone turnover markers. These markers include osteocalcin, bone specific alkaline phosphatase, procollagen type I N propeptide (P1NP), C-terminal telopeptide of type I collagen (1CTP), and tartrate-resistant acid phosphatase type 5b (TRAP-5b). The markers are mainly serum-based, and therefore, reflect the turnover rate in the whole body. Hence, the information obtained mirrors more carefully changes in cortical bone due to higher proportion of the total skeleton (approximately 80-90%). Several factors, such as diet, intra- and inter-individual variation, and sampling time, can affect the results (Compston 2006). Quantitative histomorphometry of bone sections can provide information on bone volume and bone remodeling activity, e.g., cells, osteoid formation, resorption and mineralization (Compston 2004), but a bone biopsy is required for this kind of analysis. Bone histomorphometry will be discussed more in detail below (Chapter 2.2.2). New methods for measuring bone turnover have been developed. It has been claimed that the regional turnover rate can be measured by using technetium labeled bisphosphonates (BPs) detected by single photon emission computed tomography (SPECT) imaging (Compston 2006).

Bone microarchitecture can be analysed in two-dimensions by using quantitative histomorphometry (Parfitt et al. 1983) or in three dimensions by using high-resolution micro-CT (HR-CT), high-resolution magnetic resonance imaging (HR-MRI), and peripheral quantitative computed tomography (pQCT) (Järvinen et al. 2005; Entezari et al. 2012). The three-dimensional approaches provide better visualization of bone structures than the two-dimensional methods (Compston 2006; Recker et al. 2011).

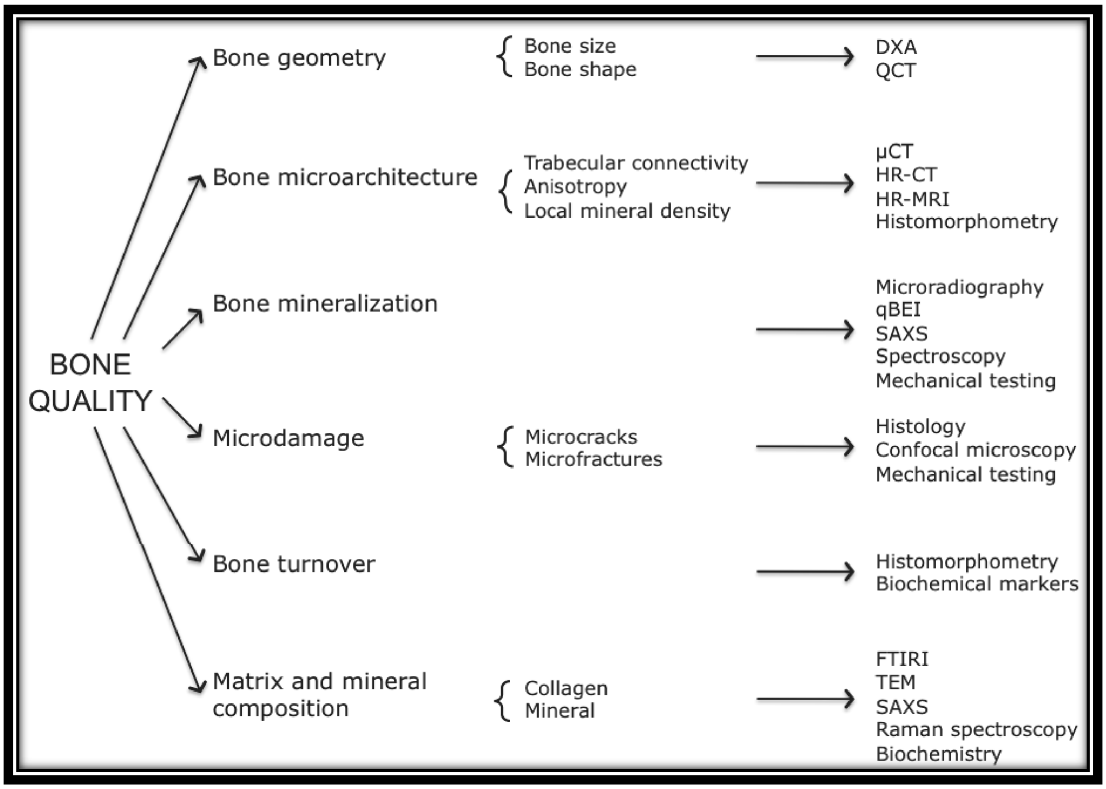

Figure 5. Assessment of different bone quality aspects using the various available techniques. Modified from Compston (2006). DXA = dual-energy X-ray absorptiometry, QCT = quantitative computed tomography, µCT = microcomputed tomography, HR-CT = high-resolution computed tomography, HR-MRI = high-resolution magnetic resonance imaging, qBEI = quantitative backscattered electron imaging, SAXS = small angle X-ray scattering, FTIRI = Fourier transform infrared spectroscopic imaging, TEM = transmission electron microscopy.

Bone mineralization can be quantified in several ways such as quantitative backscattered electron imaging (qBEI) and spectroscopic techniques. BMD measurements are able to detect the degree of mineralization. Mineralization of bone is biphasic, and especially the second phase, i.e., accumulation of mineral matrix after completed remodeling cycle, is highly dependent on bone turnover. In high turnover bone, the newly formed bone is removed even prior to the second phase of mineralization whereas prolonged mineralization occurs in low turnover bone (Compston 2006).

Bone matrix and mineral composition has been evaluated using variety of methods. Raman spectroscopy or FTIRI, transmission electron microscopy (TEM), or small angle X-ray scattering (SAXS) have been used for defining organic and inorganic composition of bone. However, only little is known about how the alterations in bone composition affect bone strength.

Histological techniques, including confocal and fluorescence microscopy, are used to detect microdamage, i.e., microcracks and microfractures, in bone. The amount of microdamage increases with age but its contribution to bone strength remains unclear (Burr 2002).

2.2.2 Bone histomorphometry

By developing a new technique to study unmineralized bone sections in the 1950s, the understanding of bone physiology accelerated. Prior to that, bone mineral had to be removed for the histological bone analysis (Recker et al. 2011). In the 1960s, Frost presented a new technique to measure bone formation using tetracycline labeling (Frost et al. 1960; Frost 1969). In 1987, the nomenclature, symbols, and units were standardized (Parfitt et al. 1987) and later updated (Dempster et al. 2013). Consequently, quantitative bone histomorphometry has become the standard method to evaluate cellular events of bone modeling and remodeling and it is a valuable and well-established clinical and research tool for studying the effects of treatment, etiology, and pathogenesis of metabolic bone diseases (Recker et al. 2011). Unfortunately, bone histomorphometry at one site, i.e., usually at the iliac crest, may not reflect changes in the whole skeleton since the turnover between the sites may vary (Eventov et al. 1991). In addition, due to differences in the technique together with variation in the remodeling activity even within one sample, relatively high inter- and intraobserver variations have been reported (de Vernejoul et al. 1981; Chavassieux et al. 1985; Compston et al. 1986; Wright et al. 1992).

Table 1. Some clinical situations in which bone histomorphometry may be useful (Alhava and Kröger 2002; Barger-Lux and Recker 2002; Trueba et al. 2003; Compston 2004; Recker 2005).

Clinical situation
Suspicion of metabolic bone disease
Osteomalacia
Renal osteodystrophy
Hyperparathyroidism
Increased bone resorption and bone loss
Osteosclerosis
Paget disease
Unexplained hypophosphataemia or hyperphosphataemia
Difficult osteoporosis
Management plan and follow-up
Vague bone pain
Unknown increase of bone specific alkaline phosphatase
Delayed bone healing after fracture
Low energy fractures in young patients
Atypical stress fractures in adults
Evaluation of bone specific therapies

Indications for bone biopsy

Bone biopsy is required for the histomorphometric analysis, and because of this, the information gained should be greater than the risk and discomfort to the patient, or the financial cost of the procedure. Bone histomorphometry can be used to establish diagnosis, estimate prognosis, or to evaluate response or adherence to treatment (Table 1). Furthermore, bone histomorphometry remains important in research since all new bone specific drugs should be tested using bone histomorphometry (Compston 2004; Recker 2005).

Bone biopsy

Prior to the biopsy, the bone is labeled *in vivo* using a fluorescent agent to study bone mineralization rate and bone formation. Labeling is currently performed using antibiotics

from the tetracycline family due to their adherence to forming bone surfaces, nontoxicity, and spontaneous fluorescence. Tetracycline fluorescence is light yellow, and optionally democycline, which emits a yellow-orange fluorescence, can be used. Patients with malabsorption syndrome may require a higher dose than the average. A double-labeling technique provides the optimal result, and thus, tetracycline is given in two courses (each 2 days) having a period of time between the regimen courses (usually 10 days). Bone biopsy is usually taken four days after the last dose of antibiotic (Trueba et al. 2003, Rauch 2009).

The anterior iliac crest is the site most frequently used for the biopsy specimen. If anterior iliac crest is not accessible, e.g., because of obesity, the posterior iliac crest immediately lateral to the sacral-iliac joint can be used (Trueba et al. 2003). Bone biopsy procedure can be performed either using outpatient surgery and local anesthetic agents (Hodgson et al. 1986) or day-surgery setting and general anesthesia (Trueba et al. 2003). The patient is placed in the supine position and a local anesthetic agent such as lidocaine is infiltrated around the biopsy site including the periosteum. The biopsy site is supported by the finger and a short longitudinal incision of 0.5-1 cm is made using a scalpel over the anterior iliac crest. Blunt dissection is used to achieve the correct biopsy site. Bone biopsy can be taken using either vertical or horizontal (i.e., through-and-through) technique. Vertical biopsy is taken by approaching the iliac crest superiorly and alongside the iliac crest so that only one cortex is included whereas the horizontal biopsy is taken through the iliac crest such that both cortices are included. Because of the iliac crest growth plate, the vertical approach should not be used in children (Rauch 2009). It is recommended that the investigator should use a funnel and electric drill when taking the specimen (Trueba et al. 2003). Bone biopsy needle set needed in the biopsy procedure is illustrated in Figure 6.

The sample should be carefully removed from the drill and placed in 70% ethanol solution (Hodgson et al. 1986; Recker 2008). A biopsy trephine with an inner diameter of at least 7.5 mm is recommended for adults and a smaller one with inner diameter of 5 mm for children (Dempster et al. 2013). If needed, 1-2 attempts can be made to obtain a proper specimen (Hodgson et al. 1986). The bony defect(s) are packed with sterile medical wax to minimize bleeding and prevent hematoma formation and the incision is closed in two layers. The total time required for the surgical procedure is usually 10-15 minutes. Appropriate wound care is necessary in order to avoid postoperative wound infections (Trueba et al. 2003).

Figure 6. The equipment needed for bone biopsy. From left: guide sleeve, trephine cutter, pointed obturator, and extractor (Medical Innovations International Inc., Rochester Bone Biopsy).

Complications after bone biopsy

Bone histomorphometry is considered to be a fairly safe method due to the low complication rate after the biopsy. Duncan et al. (1981) studied over 14,000 biopsies that were taken by numerous physicians using various biopsy techniques. The complication rate

was as low as 0.52% showing a higher rate of 0.63% for the horizontal approach compared to 0.36% for the vertical approach. The most common complications encountered were hemorrhage (in 35 patients), prolonged pain for over a week (in 17 patients), neuropathy (in 13 patients), wound infection (in 10 patients), fracture (in 6 patients), and osteomyelitis (in 1 patient) (Duncan et al. 1981). In contrast, Hodgson et al. (1986) showed that even severe pain was frequently suffered at the time of the biopsy (24% of the 76 patients) and 16 hours after the biopsy (8%). Seven days after the biopsy, almost 80% of the patients experienced no or mild pain whereas 9% of the patients still considered the pain being as severe. There were no major infections, and hemostasis was easily achieved for all patients (Hodgson et al. 1986).

Sample preparation

In the laboratory, the bone biopsy is prepared for the analysis through several steps: fixation, dehydration, embedding, sectioning, and staining. First, the biopsy is fixed in ethanol for 24-48 hours depending on the size of the sample. Then, the sample is dehydrated using increasing concentrations of ethanol because the embedding medium is not miscible with water. Further, the sample is embedded in methylmetacrylate (MMA). MMA is a non-toxic media, which penetrates into the sample quickly, and rarely evokes any artifacts such as bubbles. Prior to sectioning, the embedding medium should harden slowly, which can take up to a month. Then, a microtome is used to section the sample, and the sections are further stained. Different stains can be used depending on the investigation of interest (Trueba et al. 2003).

Quantitative histological analysis

Histological sections can be analyzed under bright light, polarized light, and fluorescence microscopy using automatic or semi-automatic software. The nomenclature, symbols, and units were standardized in 1987 by a committee of American Society for Bone and Mineral Research (ASBMR) (Parfitt et al. 1987) and later updated (Dempster et al. 2013). Based on primary parameters that can be quantified, several indices can be derived (Table 2). Parameters can be divided into four main categories: structural, static erosion, static formation, and dynamic formation variables (Rauch 2009). Quantitative results can be compared to reference values defined for healthy individuals in different age groups and races. However, for obvious ethical reasons only limited data is available especially for dynamic variables. Recker et al. (1988) have studied histomorphometric findings in healthy postmenopausal women (Recker et al. 1988). Rehman et al. (1994) have reported data in different age groups of both genders but because part of the study consisted of cadaver material, fewer cases could be included into the dynamic analysis (Rehman et al. 1994). Glorieux et al. (2000) have reported normative data in growing children in different age groups (Glorieux et al. 2000). Malluche et al. (1982) have also presented normal data but the terminology used differs somewhat from the later recommendations, and thus, the comparisons are complicated (Malluche et al. 1982). The Finnish reference values by Hoikka and Arnala have also been published and these included static histomorphometry data only (Hoikka and Arnala 1981).

To conclude, quantitative bone histomorphometry has developed remarkably during the last decades. The technique is only minimally invasive, and important information about bone remodeling activity and metabolism can be achieved in clinically challenging situations. As the gold standard, prior to development of a noninvasive method which would provide the same information, bone histomorphometry is a valuable and well-established clinical and research tool for evaluating the effects of treatment, etiology, and pathogenesis of metabolic bone diseases (Compston 2004; Recker 2005; Dempster et al. 2013).

Table 2. Frequently used bone histomorphometry parameters and their interpretation. Modified from Rauch (2009), Recker et al. (2011), and Dempster et al. (2013).

Variable	Symbol	Unit	Equation	Definition
STRUCTURAL PARAMETERS				
Bone volume	BV/TV	%	B.Ar/T.Ar	Percent of marrow space occupied by mineralized and unmineralized bone
Trabecular thickness	Tb.Th	µm	2/(BS/BV) (plate referent)	Average thickness of trabeculae
Trabecular number	Tb.N	N/mm	(BV/TV)/Tb.Th	Number of trabecular silhouettes
Trabecular separation	Tb.Sp	µm	(1/Tb.N)-Tb.Th	Average distance between trabeculae
STATIC EROSION PARAMETERS				
Eroded (resorptive) surface	ES/BS	%	(E.Pm/B.Pm)*100	Percent of bone surface presenting resorption surface
Osteoclast surface	Oc.S/BS	%	(Oc.Pm/B.Pm)*100	Percent of bone surface occupied by osteoclasts
STATIC FORMATION PARAMETERS				
Osteoid volume	OV/BV	%	(O.Ar/B.Ar)*100	Percent of bone volume consisting of unmineralized bone, i.e., osteoid
Osteoid surface	OS/BS	%	(O.Pm/B.Pm)*100	Percent of bone surface covered by osteoid
Osteoid thickness	O.Th	µm	(π*O.Wi)/(4*N.O.Wi)	Average thickness of osteoid seams
Osteoblast surface	Ob.S/BS	%	(Ob.Pm/B.Pm)*100	Percent of bone surface occupied by osteoblasts
Wall thickness	W.Th	µm	(π*W.Wi)/(4*N.W.Wi)	Average thickness of bone tissue that has been deposited at a remodeling site
DYNAMIC FORMATION PARAMETERS				
Mineralizing surface	MS/BS	%	[(dLS+½*sLS)/BS]*100	Percent of bone surface showing mineralizing activity
Mineralizing surface	MS/OS	%	[(dLS+½*sLS)/OS]*100	Percent of osteoid surface mineralizing
Mineral apposition rate	MAR	µm/d	Ir.L.Th/Ir.L.t	Distance between the two tetracycline courses divided by the length of the labeling interval
Mineralization lag time	Mlt	d	O.Th/Aj.AR	Time interval between deposition and mineralization of bone matrix
Bone formation rate (bone surface ref.)	BFR/BS	$\mu m^3/\mu m^2$/year	MAR*(MS/BS)*365.25	Amount of bone formed per year on a given bone surface
Activation frequency	Ac.F	N/year	[(BFR/BS)/W.Th]*0.001	Frequency of appearance of new remodeling units at one location per year

*B.Ar = bone area, T.Ar = tissue area, N = number, E.Pm = erosion perimeter, B.Pm = bone perimeter, Oc.Pm = osteoclastic perimeter, O.Ar = osteoid area, O.Pm = osteoid perimeter, π = an estimate for certain parameters (Parfitt et al. 1987), O.Wi = osteoid width, N.O.Wi = number of measured osteoid widths, Ob.Pm = osteoblast perimeter, W.Wi = wall width, N.W.Wi = number of measured lamellar structures, dLS = double labeled surface, sLS = single labeled surface, BS = bone surface, OS = osteoid surface, d = day, Ir.L.Th = interlabel thickness, Ir.L.t = interlabel time, Aj.AR = adjusted apposition rate = MAR*MS/OS*

2.2.3 Microcomputed tomography

High-resolution micro-CT enables three-dimensional analysis of trabecular micro-architecture and quantification of spatial mineral density of bone samples. The accuracy of micro-CT depends on the image resolution. Studies have shown that high resolution (10-20 µm voxel size) is necessary to accurately quantify the trabecular micro-architecture (Muller 2003; Isaksson et al. 2011; Entezari et al. 2012). Micro-CT has been used for detecting structural changes in bone (Uchiyama et al. 1997). It has also been used to assess the effects

of therapeutic agents such as bisphosphonates (Borah et al. 2004; Recker et al. 2005). *In vivo* high-resolution micro-CT is also commercially available, but so far only for use with small animals (Campbell et al. 2008).

Bone strength in cortical bone is mainly determined by cortical width, cortical porosity, and bone size. In cancellous bone, the main determinants of bone strength are the size and orientation of trabeculae together with the trabecular connectivity and orientation (anisotropy) (Compston 2006). In addition to the analysis of the trabecular microarchitecture, micro-CT enables quantification of the spatial mineral density (Entezari et al. 2012). Alterations in bone remodeling contribute to impaired bone microstructure (Compston 2006).

2.2.4 Fourier transform infrared spectroscopic imaging

Fourier transform infrared spectroscopy imaging (FTIRI) can be used to study the chemical composition of bone. The vibrations of the chemical bonds of the specimen are excited by incident light to visualize mineral and protein components of bone. Bone composition can be analyzed based on characteristic frequencies that correspond to absorption peaks on an infrared spectrum (Carden and Morris 2000; Boskey and Mendelsohn 2005).

The organic matrix of bone consists mainly of collagen type I that provides flexibility for bone structure. The inorganic matrix consists of mineral crystals that provide the necessary tissue stiffness (Figure 7) (Currey 2001). Thus, changes in collagen cross-linking and alterations in mineral crystal size and structure may have biomechanical implications (Paschalis et al. 2001; Vashishth et al. 2001; Wang et al. 2002).

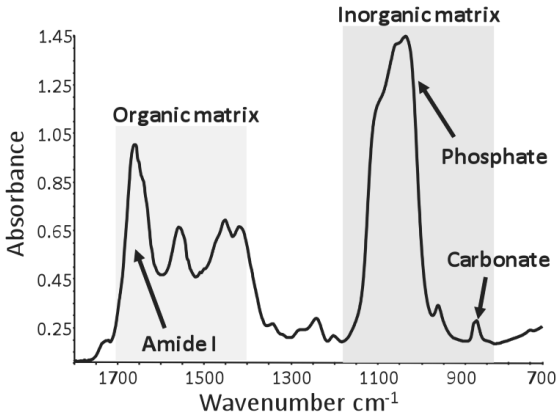

Figure 7. Fourier transform infrared spectroscopic imaging (FTIRI). The organic matrix of bone consists mainly of collagen type I that can be estimated by amide I peak C spectrum. The inorganic matrix is estimated by using the phosphate and carbonate peaks in FTIRI.

2.3 METABOLIC BONE DISORDERS

2.3.1 Osteoporosis

Osteoporosis has emerged as a health concern in all age groups. However, the disease is most common in white postmenopausal women. Osteoporosis is characterized by low bone mass measured by DXA and changes in bone quality that predispose individuals to fractures even after minimal trauma (National Institutes of Health Consensus 2001). There is a relatively high morbidity and mortality related to the fractures. Due to the advancing age of the population, the annual incidence of osteoporotic fractures and costs have been estimated to rise 50% by the year 2025 (Burge et al. 2007).

Peak bone mass, achieved in an individual's early 20's, is the major determinant for the risk of osteoporosis later in life (Hernandez et al. 2003). Thus, childhood and adolescence

are important for general bone health. Most importantly, osteoporosis is largely preventable due to the evolving understanding of the causes of the disease, and thus, the diagnosis and treatment of the disease have improved during the last decades (National Institutes of Health Consensus 2001). Osteoporosis is attributable to an imbalance in bone remodeling, and thus, the understanding of the cellular events is important to target the treatment.

2.3.1.1 Pathophysiology of osteoporosis

The compromised bone strength, as defined by decreased BMD measured by DXA and deterioration of bone quality, i.e., architecture, turnover, damage accumulation such as microdamage, and mineralization, predisposes individuals for fractures (National Institutes of Health Consensus 2001). After the fifth decade of life and in some metabolic bone diseases like osteoporosis, the bone formation decreases more than bone resorption, leading to bone loss (Parfitt et al. 2000; Rachner et al. 2011). Activation of the RANK-RANKL pathway, most commonly due to estrogen deficiency, leads to increased osteoclast activity that is further associated with increased bone resorption (Rachner et al. 2011). The progressive increase in the fracture risk occurs after accelerated bone loss especially in women after menopause. However, it is notable that individuals with compromised peak bone mass are more susceptible to develop osteoporosis even without accelerated bone loss (National Institutes of Health Consensus 2001). The cellular events play an important role in the pathogenesis of osteoporosis, and various signals mediated via the endocrinal, neuroendocrinal, inflammatory, and mechanical systems have been characterized (Rachner et al. 2011). In addition, genes seem to intervene with environmental factors (Ferrari et al. 1999) but also with some nutrients (Chevalley et al. 2008).

Table 3. The risk factors and/or causes of osteoporosis. Modified from Moulds et al. (2001) and National Institutes of Health Consensus (2001). BMI = body mass index.

Genetic and non-modifiable	Female
	Aging
	Thin build: low BMI <18
	Race: Asian, Caucasian
	Family history: Maternal hip fracture <75 years
	Premenopausal estrogen deficiency, e.g. amenorrhea
	Late menarche
	Early menopause <45 years (natural or surgical)
Lifestyle factors	Cigarette smoking
	High caffeine intake >4 cups a day
	Alcoholism and high alcohol intake >2 standard drinks a day
	Low calcium intake
	Lack of vitamin D or vitamin D deficiency
	Physical inactivity
Medical causes	Eating disorders, especially anorexia nervosa
	Malabsorption syndrome, e.g. celiac disease, inflammatory bowel disease
	Endocrine disorders: Cushing syndrome, diabetes mellitus, hyperparathyroidism, thyrotoxicosis, hypogonadism or sex hormone deficiency, acromegaly
	Connective tissue disorders, e.g. rheumatoid arthritis
	Inherited disorders of collagen metabolism, e.g. osteogenesis imperfecta
	Chronic organ failure, transplantation
	Iatrogenic causes: Corticosteroids, anti-epileptic drugs, thiazolidinediones, long-term heparin, excessive thyroid hormone, prostate cancer hormone therapy, breast cancer hormone therapy
	Prolonged immobilization

There are both primary and secondary causes for osteoporosis. Primary osteoporosis is most common in women after menopause due to the loss of estrogen but it can occur in both males and females at any age. Secondary osteoporosis, however, is caused by previously used medication such as the glucocorticoids (GCs), some diseases such as celiac disease, or other conditions (e.g. hypogonadism). Secondary causes account for 30-60% of osteoporosis in men and for over 50% of osteoporosis in perimenopausal women (National Institutes of Health Consensus 2001). There are several risk factors for osteoporosis (Table 3). Vitamin D deficiency is common all over the world and it also causes many extra-skeletal adverse effects, such as cardiovascular and metabolic diseases, malignancies, and predisposes individuals to falls (Holick 2007).

2.3.1.2 Diagnosis of osteoporosis

The diagnosis of osteoporosis is currently based on the measurement of BMD at the hip or lumbar spine using DXA. BMD accounts for approximately 70% of bone strength, however, there are currently no better measures available for clinical practice (National Institutes of Health Consensus 2001). The BMD value is converted to a T-score. According to the criteria devised by the World Health Organization (WHO), osteoporosis is defined as a BMD of 2.5 standard deviation units below the mean of young white adult women (T-score) (Table 4). Osteopenia is defined as a BMD T-score between -1.0 and -2.5 standard deviations (WHO 1994; Kanis et al. 2013). In addition, newer techniques such as quantitative ultrasound (QUS) have been introduced in the measurement of bone properties. Compared to DXA, QUS of the heel has been shown to predict both vertebral and non-vertebral fracture almost as well as DXA of the hip (National Institutes of Health Consensus 2001), and might improve the early diagnosis of osteoporosis (Liu et al. 2012).

Table 4. Interpretation of bone mineral density (BMD) T-scores according to WHO criteria (WHO 1994).

BMD T-score	Interpretation
≥ -1.0	Normal
-1.0 to -2.5	Osteopenia
≤ - 2.5	Osteoporosis
< -2.5 with fracture	Severe osteoporosis

2.3.1.3 Treatment of osteoporosis

Advances in understanding the pathogenesis of osteoporosis have lead to novel therapies that inhibit excessive bone resorption and increase bone formation. Lifestyle modifications including cessation of smoking, low alcohol intake, and regular exercise together with adequate calcium and vitamin D supplementation form an important baseline treatment of osteoporosis. In addition, various medical treatments are available (Rachner et al. 2011) that have been shown to decrease the fracture risk for both vertebral and non-vertebral fractures (Black et al. 1996; Cummings et al. 1998; Delmas et al. 2002; Reginster et al. 2005; Black et al. 2007; Greenspan et al. 2007). The drugs can be divided into those that decrease resorption, i.e., antiresorptive drugs and those that increase bone formation, i.e., anabolic drugs. Although long-term side effects are not completely clear, the new therapies combine better efficacy and convenient administration which may achieve better compliance to the therapy when compared to the older therapies (Rachner et al. 2011).

Calcium and vitamin D are a baseline treatment for every patient with osteoporosis. In the elderly, vitamin D deficiency has been associated with lower BMD, high turnover, and increased risk of falls and hip fractures (Sambrook and Cooper 2006). ASBMR recommends adequate dietary calcium uptake rather than calcium supplements or combined calcium

and vitamin D supplementation. This is because of the suspected cardiovascular adverse effects related to calcium only supplementation (Pentti et al. 2009; Bolland et al. 2010). There is a speculation that the increased risk of cardiovascular events might have been due to vitamin D deficiency (Holick 2007). The recommended 25-hydroxyvitamin D (25-OHD) level is extremely controversial. It has been concluded by the Institute of Medicine (2011) that the limit for insufficiency of serum 25-OHD levels is less than 50 nmol/L. To achieve a 25-OHD level 75 nmol/L, usually the required daily dose of vitamin D is 800 IU (Rachner et al. 2011). For adults, the recommended calcium intake is 800 mg daily (NNR 2012a). In a Finnish study, calcium and vitamin D supplementation (1000 mg/800IU) tended to reduce the fracture risk, however, no statistical significance was achieved (Salovaara et al. 2010).

Hormone replacement therapy (HRT) has been used in the prevention and treatment of osteoporosis in postmenopausal women. A reduced rate of osteoporotic fractures has been related to HRT due to its antiresorptive effects (Rossouw et al. 2002; Anderson et al. 2004). However, an increased rate of strokes has been associated with both combination therapy (estrogen and progestagen) (Rossouw et al. 2002) and estrogen alone therapy (Anderson et al. 2004). There has also been an increased rate of cardiovascular events related to the combination therapy. An increased risk of breast cancer has been related to combination therapy (Rossouw et al. 2002) whereas prolonged estrogen alone therapy has reduced the risk of breast cancer (Anderson et al. 2004). Women with osteoporosis and cardiovascular risk factors should be treated with an alternative medication for osteoporosis. However, symptomatic women with high fracture risk around the menopause can receive HRT but only for a short time (Rossouw et al. 2002; Pentti et al. 2006; Sambrook and Cooper 2006).

Selective estrogen-receptor modulators (SERMs) are diverse compounds that have a tertiary structure similar to estrogen to allow them to bind to the estrogen receptor, and therefore, they can exert antagonist or agonist effects selectively on various tissues with estrogen receptors (Sambrook and Cooper 2006). Raloxifene is the most studied compound having less effect than BPs on both bone turnover markers (reduction of 30-40%) and BMD (Delmas et al. 1997; Johnell et al. 2002; Sambrook et al. 2004). Raloxifene has reduced the vertebral fracture rate by almost 50% in patients with no fracture history (Ettinger et al. 1999; Delmas et al. 2002) but no effects to achieve a reduction of hip fractures have been found (Delmas et al. 2003). Raloxifene can cause vein thrombosis and exacerbate hot flushes (Cummings et al. 1999).

Bisphosphonates (BPs) are widely used as an antiresorptive treatment for osteoporosis. They have high affinity on bone and a long half-life, and thus, may remain in bone for decades (McClung et al. 2001). In clinical trials, BPs have been shown to reduce the risk of both vertebral fractures (40-50%) and non-vertebral fractures (20-40%, including hip fractures) (Cranney et al. 2002). BPs can be administered either orally or intravenously. The treatment is inexpensive and suitable for several types of osteoporosis such as postmenopausal, male, and GC-induced (Rachner et al. 2011). BPs can also be used in children (Chapter 2.3.2) (Papapoulos 2011). In postmenopausal women, other antiresorptive therapies, i.e., raloxifene, strontium renelate, and denosumab can be used as alternative therapies. The present antiresorptive therapies have impressive antifracture efficacy but their use is sometimes limited by adverse effects (mainly upper gastrointestinal), concomitant comorbidities, or poor long-term compliance (Siris et al. 2009). Bone turnover markers have been suppressed for as long as five years after discontinuation of treatment (Ensrud et al. 2004). A histomorphometric evaluation revealed a reduction in the bone forming surfaces by as much as 40-60% with usual BP doses (Chavassieux et al. 1997). It has also been postulated that the reduced bone turnover might lead to microdamage accumulation over time, and thus, to an impaired fracture repair (Sambrook and Cooper 2006). A rare but serious adverse effect, osteonecrosis of the jaw, has also been reported in some patients on BP therapy but these cases are mainly restricted to patients treated with high BP doses or many BPs for cancer (Marx 2003; Ruggiero et al.

2004). There are reports of atypical femoral fractures (AFFs) related to BPs (Shane et al. 2010; Schilcher et al. 2011; Dell et al. 2012, Meier et al. 2012, Schilcher et al. 2013).

A new antiresorptive agent, denosumab, has recently been approved in Europe for the treatment of osteoporosis and in USA for the treatment of osteoporosis and bone metastases (Rachner et al. 2011). Denosumab is a fully human monoclonal antibody against RANKL with high specificity and affinity for RANKL. Bone turnover markers decrease during the treatment with this agent (Bekker et al. 2004) and increase rapidly after discontinuation of the therapy (Miller et al. 2008). According to bone histomorphometry, over 50% of the patients given denosumab had no osteoclasts. Additionally, decreased bone formation and remodeling have been observed (Reid et al. 2010). The risk of new vertebral fractures was reduced by 58% over the period of three years, and similarly, the risk of hip fractures was reduced by 40% and other non-vertebral fractures by 20%. Osteonecrosis of the jaw and atypical femoral fractures are the greatest concern as a side effect of the treatment (Paparodis et al. 2013; Pichardo et al. 2013; Rachner et al. 2013). Denosumab can be considered as a first-line treatment or it can be given as an alternative to BPs, especially if BPs are not tolerated. Denosumab has certain benefits compared to BPs; it does not bind to bone, and thus, it is reversible, and because of the subcutaneous administration route, it does not evoke any gastrointestinal side effects. Better adherence to the therapy may be achieved by its administration twice a year compared with weekly or monthly treatments with BPs. Denosumab is not eliminated by the kidneys and is therefore safer in patients with impaired kidney function although dose adjustments may be necessary in those with lower creatinine clearance levels (<30 mL/min) (Rachner et al. 2011).

Bone anabolic drugs stimulate bone formation. The intact PTH (hPTH 1-48) or its N-terminal fragment (hPTH 1-34) called teriparatide are available at the moment and can be administered subcutaneously (Daddona et al. 2011). There is a report that teriparatide (dose 20 μg daily) can reduce the risk of new vertebral fractures by 65% and it can decrease non-vertebral fractures by 53%. The beneficial effects on BMD seem to vanish after discontinuation of the therapy (Neer et al. 2001). Intact PTH has reduced the rate of new vertebral fractures by 68% in patients with no previous fractures (Greenspan et al. 2005). The combination therapy with BPs does not seem to be more effective than PTH alone but further studies are needed to investigate these combined effects (Black et al. 2005). Continuous treatment with PTH leads to bone loss whereas intermittent PTH therapy enhances bone formation (Rubin and Bilezikian 2003; Keller and Kneissel 2005; Eriksen 2010).

Strontium renelate is a compound that both decreases bone resorption and increases bone formation although its exact mechanism of action remains unclear (Meunier et al. 2004). In postmenopausal women, the risk of clinical vertebral fracture has decreased 52% whereas the reduction in the risk of non-vertebral fractures has been lower (16%). Adverse effects include venous thrombosis for unknown reasons and gastrointestinal discomfort (Reginster et al. 2005).

There are also other promising targets for novel treatment of osteoporosis including cathepsin K, Dickkopf, and sclerostin inhibitor (Sambrook and Cooper 2006). The number of new approved bone therapies is expected to increase in the coming years (Rachner et al. 2011). For example, the novel cathepsin K inhibitor, odanacatib, targets mature osteoclasts inhibiting bone resorption while preserving osteoclast viability (Stoch et al. 2009; Bone et al. 2010). Some promising anabolic drugs also are under development. In the future, calcilytic drugs and antagonists of WNT inhibitors might provide an anabolic treatment in individuals with severe osteoporosis with excessive bone loss and slow bone healing (Rachner et al. 2011).

2.3.1.4 Atypical femoral fractures

During recent years, it has been proposed that the long-term use of BPs could be related to the development of atypical femoral fractures (AFFs) (Shane et al. 2010, Schilcher et al.

2011, Dell et al. 2012, Meier et al. 2012, Schilcher et al. 2013). Later, it has been shown that long-term BP therapy is not a prerequisite for the development of these fractures (Giusti et al. 2010). BPs have long half-life in bone and they inhibit bone resorption and formation, and this could evoke a major suppression of bone turnover (Jamal et al. 2011). There have been speculations that prolonged suppression of bone resorption could lead to the accumulation of microdamage, and thus, to increased bone fragility (Sellmeyer 2010). Patients with AFF may develop a further AFF on the contra-lateral site suggesting that there may be a systemic reason that predisposes certain patients to suffer these fractures (Giusti et al. 2010). Generally, AFFs are rare and the vast majority of patients on BPs do not develop this kind of fractures (Compston 2011, Meier 2012). Surprisingly, the BMD T-score was above -2.5 at the time of the fracture in more than 75% of the women with AFF. Further, 18% had a normal BMD T-score at that time. This indicates that patients treated with BPs without frank osteoporosis (BMD T-score <-2.5) might be at risk of developing AFF (Giusti et al. 2010).

AFFs are characterized by an atypical fracture morphology and occur always as insufficiency fractures. The criteria for AFFs have been defined by ASBMR Committee (Table 5) (Shane et al. 2010). Further, there are other findings that are commonly present but are not essential for the diagnosis. Prodromal pain has been common as well has several comorbid conditions and bilaterality of the fractures. Delayed fracture healing has often been reported. Cortical thickening has often been found, and the co-administration of some other drugs, such as proton-pump inhibitors or GCs, may have a role in the pathogenesis of AFFs (Shane et al. 2010). However, it is not known whether the cortical thickening is actually caused by BP therapy. There is one report of cortical thickening during alendronate treatment (Lenart et al. 2009) whereas another study detected thick cortices before the BP treatment (Kwek et al. 2008). Further, BP-naïve patients with AFF have thick cortices (Giusti et al. 2011), and another study found no significant differences in the cortical thickness before and at 7 years after BP-therapy (Unnanuntana et al. 2012). Thus, the data available does not support the theory that BPs would cause cortical thickening (Compston 2011). A recent study used different criteria for an AFF and found even higher association with bisphosphonate use (Schilcher 2013). The fracture angle from 75° to 125° and presence of callus reaction showed high specificity to detect bisphosphonate use.

Table 5. The major criteria of atypical femoral fractures (AFFs) as defined by ASBMR Committee. Modified from Shane et al. (2010).

Major features of AFFs
Fracture at subtrochanteric or diaphyseal region
Fracture associated with no trauma or minimal trauma, i.e., a fall from maximum a standing height
Transverse or short oblique configuration of the fracture without comminution
Incomplete fractures may involve only lateral cortex whereas complete fractures occur through both cortices
A medial spike may be associated

Epidemiological studies have revealed that BP-treated women with AFF have lower mean age (68 years) compared to those with subtrochanteric or femoral shaft fractures (73-79 years), and the patients seem to sustain AFFs at any age (Salminen et al. 1997; Salminen et al. 2000; Weiss et al. 2009; Nieves et al. 2010). The similar fracture morphology has also been identified in patients without a history of BP treatment or without a metabolic bone disease (Kumm et al. 1997; Niimi et al. 2008; Lenart et al. 2009; Schilcher and Aspenberg 2009). It has been postulated that BP treatment could cause increased risk for

subtrochanteric fractures whilst decreasing the risk for femoral neck fractures. Thus, a secondary analysis of large randomized controlled BP trials (FIT, FLEX, HORIZON) was performed (Black et al. 2010) which noted no increase in the incidence of subtrochanteric fractures compared to placebo but the X-rays were not available and the duration of the treatment was relatively short (Black et al. 2010). In contrast, Abrahamsen et al. suggested that there was an increased incidence of subtrochanteric fractures related to BP use but the risk was not dose-dependent (Abrahamsen et al. 2010). A similar trend was found by Wang and Bhattacharyya (Wang and Bhattacharyya 2011). The risk of AFFs has shown to increase with longer duration of the treatment (Schilcher 2011, Dell 2012, Meier 2012). AFFs have often been described to be related to the use of alendronate but there are also reports of AFFs on other BPs such as risendronate and ibandronate as well as denosumab in the literature. The duration of BP use before the AFF has varied from 3 months to 16 years (Giusti et al. 2010; Paparodis et al. 2013).

The pathophysiology of AFFs remains to be established (Giusti et al. 2010; Compston 2011). The suppression of bone turnover evoked by BPs may have adverse effects on bone material properties and strength. The underlying mechanism could be increased mineralization, decreased homogeneity of the mineralization, changes in collagen composition, and accumulation of microdamage (Compston 2011).

Only limited histological data is available and most studies have examined iliac crest biopsies rather than biopsies at the fracture site (Shane et al. 2010; Compston 2011). Bone formation has generally been decreased after BP treatment but some studies have found normal or even increased resorption parameters. Giusti et al. performed a review of the literature on BP treated patients and concluded that 8 of 27 patients had double labels and most of the patients had single labels in the biopsy. Thus, an oversuppression of the bone turnover seems to be an unlikely explanation (Giusti et al. 2010). Further, Jamal et al. have reported bone histomorphometry findings in a 55-year-old woman who had been receiving alendronate therapy for 9 years (Jamal et al. 2011). She suffered an atypical femoral fracture and a tetracycline labeled biopsy was taken near to the fracture site. In contrast with the previous presumptions of oversuppression of bone remodeling due to the BP treatment, this patient had normal lamellar bone structure, no cortical or endocortical adynamic bone disease, no evidence of mineralizing defect or osteomalacia, and the mineralization was normal. The decreased bone turnover could cause an increased incidence of non-vertebral fractures but this has not been reported related to the BP use (Bone et al. 2004; Black et al. 2006). Thus, the atypical femoral fractures may not be due to the oversuppression of bone turnover (Jamal et al. 2011).

2.3.2 Osteoporosis in children

Osteoporosis has been recognized as an increasing health problem among children and adolescents. This is because there is now a better appreciation of the importance of the bone mass laid down at the end of growth as a major determinant of the risk of osteoporosis later in life. Further, many even apparently healthy children may sustain repeated fractures during their childhood (Bianchi 2007; Mäyränpää et al. 2011). By the age 18, about 90% of the peak bone mass will have been acquired (Bailey et al. 1999) and adequate calcium and vitamin D uptake and regular exercise play important roles in this process (Bianchi 2007). The significance of low BMD and association with fractures is less well-established in children than in adults. The diagnostic criteria for osteoporosis in children were first established in 2007 by Pediatric Official Positions of the International Society for Clinical Densitometry (ISCD). It is clear that early diagnosis of juvenile osteoporosis is important for appropriate intervention (Mäyränpää et al. 2011).

As in adults, osteoporosis in children may be caused by primary or secondary causes (Table 6). Primary osteoporosis is relatively rare; multiple fractures are common in children with primary osteoporosis. Idiopathic juvenile osteoporosis has been thought to be a self-

limiting disease characterized by repeated fractures that spontaneously resolve after puberty (Bianchi 2007).

Table 6. Primary and secondary causes of osteoporosis in children (Rauch and Glorieux 2004; Bianchi 2007). GC = glucocorticoid.

Primary causes of osteoporosis	**Secondary causes of osteoporosis related to multiple chronic conditions**
Hereditary disorders of connective tissue e.g. osteogenesis imperfecta	Neuromuscular disorders e.g. Duchenne muscular dystrophy
Idiopathic osteoporosis	Chronic disorders e.g. cystic fibrosis, malabsorption, organ transplantation
	Endocrine disorders e.g. delayed puberty
	Inborn error of metabolism e.g. protein intolerance
	Iatrogenic causes such as GCs and radiotherapy

DXA has been most commonly used to measure bone mass in children as well as in adults. The good reproducibility, and precision are advantages of this technique. Since three-dimensional bone structure is measured by a two-dimensional approach using DXA, the method measures only areal BMD, not volumetric BMD. DXA also fails to distinguish between cortical and cancellous bone (Bianchi 2007). Falsely low BMD values might be observed in children of short stature or in those with delayed skeletal maturation because the reference values are obtained from healthy children (Bachrach and Sills 2011). Thus, it is essential to correct the BMD values in children for bone size, body height and/or bone age (Kröger et al. 1993; Rauch et al. 2008).

The diagnosis of osteoporosis in children is challenging. Biochemistry and bone turnover markers seem to provide little help in evaluating bone mass and the susceptibility to fractures (Mäyränpää et al. 2011). The diagnosis of osteoporosis in pediatric patients should include a significant fracture history in addition to a low bone mineral content (BMC) or BMD (Rauch et al. 2008). However, a fragility fracture in adults is usually considered as a complication of osteoporosis, and therefore, the diagnosis of osteoporosis in children might be delayed. For densitometry, BMD values are interpreted in terms of standard deviation scores which are compared with ethnicity-, age-, and gender matched healthy controls (Z-score) (Bianchi 2007) that can be further adjusted for bone size, body height and /or bone age (Kröger et al. 1993; Rauch et al. 2008). Z-score values below -2.0 are considered as a serious warning of osteoporosis. A significant fracture history is defined when at least one of the following criteria is included: 1) a vertebral compression fracture, 2) one lower extremity long bone fracture, or 3) two or more upper extremity long bone fractures (Rauch et al. 2008). Because of the difficulties in establishing the diagnosis of osteoporosis in children, bone biopsy and histomorphometric analysis may be considered in patients who have a high-risk for osteoporosis and other metabolic bone diseases, such as after organ transplantation (Helenius et al. 2006; Valta et al. 2008) or in children with repeated fractures (Mäyränpää et al. 2011). Nonetheless, the diagnosis of osteoporosis in children needs to be definite before starting bone medications, e.g., with BPs since these drugs may have adverse effects on mineral homeostasis and renal function.

In order to achieve optimal bone growth, sufficient dietary calcium intake is recommended for all children but it has been claimed that only 25% of boys and 10% of girls aged 9-17 years meet the recommendations (National Institutes of Health Consensus 2001). The children with diagnosed osteoporosis should be evaluated for adequate calcium and vitamin D levels and supplementation started when appropriate (Bianchi 2007). In Finland, a national recommendation for vitamin D supplementation in children has been given due to the northern location of the country: 10 μg (400 IU) in under 2-year-old children and 7.5 μg (300 IU) for children aged 2 to 18 years (NNR 2012b). Before starting

any bone specific drugs, the cause of osteoporosis should be identified and risk factors for the disease reduced or eliminated (Bianchi 2007). BPs have been shown to increase BMD, reduce the rate of fragility fractures, and relieve pain together with providing increased mobility in children with secondary osteoporosis (Glorieux et al. 1998; Bianchi et al. 2000; Shaw and Bishop 2005). They are the only antiresorptives that have been successfully used in pediatric patients (Bianchi 2007). Cyclical intravenous pamidronate or oral alendronate have been the most commonly provided treatments (Glorieux et al. 1998; Bianchi et al. 2000; Shaw and Bishop 2005).

2.3.3 Renal osteodystrophy

Renal osteodystrophy is the term used to describe the skeletal complications of end-stage renal disease, i.e., disturbances in bone metabolism and remodeling (Hruska and Teitelbaum 1995). Chronic kidney disease (CKD) is characterized by progressive loss of kidney function (Eknoyan et al. 2004), and there is increasing evidence indicating that CKD is associated with cardiovascular complications including vascular calcification, morbidity, and mortality (Block and Cunningham 2006). Renal failure leads to changes in mineral (calcium, phosphorus) and hormonal metabolism (PTH, vitamin D). These disturbances further cause skeletal abnormalities such as changes in bone structure, bone turnover, and cellular level. An increased bone fragility and fractures have been associated with renal osteodystrophy (Moe and Drueke 2004). Biochemical markers may provide information about the underlying bone disease but the definite diagnosis and classification of renal osteodystrophy is based on bone histomorphometric analysis. Plasma levels of PTH together with serum levels of calcium, phosphorus, and ALP, however, have been used to evaluate bone turnover, to diagnose, and to monitor the success of the treatment of renal osteodystrophy. According to the latest recommendations by a foundation called Kidney Disease: Improving Global Outcomes (KDIGO), renal osteodystrophy should be distinguished from chronic kidney disease – mineral and bone disorder (CKD-MBD) (Moe et al. 2006). CKD-MBD is defined as a systemic disorder of mineral and bone metabolism caused by CKD, and its manifestations may include biochemical, vascular, or skeletal abnormalities. Renal osteodystrophy, however, is one component of CKD-MBD accounting for altered bone morphology that is quantifiable by bone histomorphometry (Moe et al. 2006).

2.3.4 Solid organ transplantation and related bone complications in children

Solid organ transplantation is an established treatment for children with end-stage renal disease, acute or chronic liver failure, terminal lung or intestinal disease, and with dilated cardiomyopathy as well as other terminal heart diseases. While the overall long-term patient survival has improved, new health concerns have emerged, i.e., secondary osteoporosis and fragility fractures (Cohen and Shane 2003; Cohen et al. 2004). Metabolic abnormalities related to the underlying disease, life-long immunosuppressive medication, especially GCs, and disturbances in pubertal development, may affect normal bone metabolism and lead to low bone mass and an increased fracture risk in pediatric transplant patients (Hill et al. 1995; Acott et al. 2003; Helenius et al. 2006; Valta et al. 2008; Valta et al. 2009; Sachdeva et al. 2010). Furthermore, impaired kidney function and a subsequent metabolic bone disease may occur also in pediatric liver and heart transplant recipients due to the nephrotoxicity of calcineurin inhibitors (Bharat et al. 2009; Kivela et al. 2011).

A greatly increased fracture risk has been observed in pediatric patients after solid organ transplantation (Hill et al. 1995; Helenius et al. 2006; Valta et al. 2008; Valta et al. 2009). High doses of GCs are frequently needed immediately after transplantation and during rejection periods. The GC-related fracture rate has been at its highest during 3-12 months after transplantation (Ebeling 2009). Even small doses of GCs increase fracture risk significantly (Van Staa et al. 2000). In a Finnish population-based study, the incidence of all fractures was 6-fold higher in children after solid organ transplantation compared to

controls in a 5-year follow-up time. Further, the incidence of vertebral fractures was 160-fold higher in the study cohort compared to that in the control group. Approximately 50% of vertebral fractures were asymptomatic, and thus, a systematic screening of vertebral fractures at regular intervals has been recommended. Male sex, pre-transplant fractures, older age, and liver transplantation were found to be risk factors for post-transplantation fractures (Helenius et al. 2006).

Only one study has evaluated skeletal findings by bone histomorphometry in children after solid organ transplantation. Sanchez et al. (1998) performed bone biopsies on 47 pediatric renal transplant patients at 3.2 ± 1.7 months after transplantation. Thirty-one (66%) of the 47 patients had normal bone formation in histomorphometry, while 23% had high-turnover renal osteodystrophy, and 8% displayed adynamic bone lesions. In these subgroups, no difference was found between the time from transplantation, cumulative GC dose, or the time on dialysis. The serum PTH values did not differ between the patients with normal bone formation, high turnover or adynamic bone. Defective skeletal mineralization with increased osteoid volumes and decreased mineral apposition rates was found in a substantial proportion of the patients. In addition, this study revealed that after adjusting the DXA values for height and age, low bone mass and osteoporosis were not common in pediatric patients shortly after transplantation (Sanchez et al. 1998). In adults, increased bone resorption biochemical markers and decreased bone formation biochemical markers have been detected shortly after transplantation whereas with the reduction in the GC doses in the later post-transplantation time, bone formation begins to increase (Shane and Epstein 2001). Bone formation parameters, as measured by histomorphometry, have been shown to increase significantly in adult patients at 3 months after liver transplantation (Vedi et al. 1999).

3 *Aims of the Study*

This study aimed to resolve the role of qualitative properties of bone beyond bone density measurements. Changes in biological aspects of bone quality were characterized by quantifying bone remodeling using bone histomorphometry in specific cohorts of adult and pediatric patients. This was combined with studies of the bone microarchitecture and composition using micro-computed tomography and Fourier transform infrared spectroscopic imaging.

The specific aims of the study were:

1. To characterize bone properties using bone histomorphometry and micro-CT to resolve whether the agreement of histomorphometry and micro-CT would depend on the metabolic status of bone. A further objective was to determine whether micro-CT is able to detect the differences between diseased and healthy bone.

2. To resolve the incidence of atypical femoral fractures in Kuopio University Hospital district, Kuopio, Finland, and to add these histomorphometric findings of patients with atypical femoral fractures to the growing international database. Further aims were to characterize bone composition in these patients with atypical fractures.

3. To determine whether bone composition would differ between the fracture-prone children with and without vertebral fractures.

4. To characterize bone histomorphometric findings in pediatric kidney, liver, and heart transplant recipients who were being evaluated for suspected post-transplantation osteoporosis.

4 Materials and Methods

4.1 PATIENTS AND STUDY DESIGN

4.1.1 Patients (I-IV)

Study I comprised 36 patients (46.0 ± 18.5 years, 16 males) with iliac crest biopsies to study the reproducibility and agreement between micro-CT and bone histomorphometry. Iliac crest biopsies (n=36) were collected including either both cortices (*n*=15) or only one cortex (*n*=21). The patients were selected based on histomorphometric evaluation and diagnosis. The patients with osteoporosis had a history of fractures, kidney disease, familial osteoporosis, or organ transplantation. The patients with renal osteodystrophy had a history of diabetes, dialysis, nephrosis, kidney transplantation, fractures, or glomerulonephritis. Based on the histomorphometric diagnosis, 15 of the patients had osteoporosis, 11 had renal osteodystrophy, and 10 had normal bone biopsy findings. Some of the normal samples (*n*=6) were obtained from cadavers with no medical history known to have any effect on bone metabolism.

In **Study II**, all femoral fractures with the diagnosis code S72.2, S72.3, or S72.4 (ICD-10 clinical coding system), or patients aged >49 years with fractures treated with a femoral intramedullary nail (NOMESCO procedural code NFJ60), were identified from Kuopio University Hospital Patient Discharge Registry. Kuopio University Hospital is responsible for the care of all surgically treated trauma patients in its catchment area of 248 000 inhabitants in Eastern Finland. The reviewed time period extended from January 2007 to December 2009. All preoperative radiographs were reviewed to identify femoral diaphyseal fractures, defined as fractures in the region of the femur between 5 cm distal to the lesser trochanter and the junction of the middle and distal third of the femur. These patients who met the major criteria for AFF defined by ASBMR were identified (Shane et al. 2010), including one patient who had fallen from a 0.5 m high stack of wood. The assessment of radiographs was conducted blind to the patients' medication. Pathological fractures caused by underlying malignancy were excluded. Further, the records of the patients that underwent bone histomorphometric evaluation at the Kuopio University Hospital, Kuopio, Finland due to an AFF were identified (*n*=7). Three of these samples had to be excluded from the report (1 malignancy, 1 cortical bone sample, 1 with long time, i.e., 3.8 years from AFF to biopsy). Thus, the findings from the remaining patients (*n*=4) are reported.

The prevalence data on continuous treatment with BP medication (ATC codes M05BA: BPs and M05BB: BPs, combinations) in the catchment area of Kuopio University Hospital was provided by the Finnish Social Insurance Institution's prescription database (Study II) (Furu et al. 2010). Statistics Finland provided the population data for the area of interest. All clinical data were acquired from medical records with information on comorbidities, duration of BP treatment, surgical treatment of fracture, and outcome. All patients were contacted via telephone to verify the data. The daily dose of alendronate was 10 mg until 2008 when it was changed to a weekly dose of 70 mg. The monthly dose of ibandronate was 150 mg. BP treatment was discontinued at the time that the diagnosis of the AFF was made.

In **Study III**, children with suspected primary osteoporosis where evaluated to characterize underlying bone changes by FTIRI together with clinical characteristics. Iliac crest biopsies were obtained from 24 children (aged 6.7-16.6 years, 17 males) with suspected primary osteoporosis. Primary osteoporosis was suspected based on either (**i**) low BMD [an SD score (Z-score) ≤ -2.0], or (**ii**) history of increased fragility, i.e., several non-vertebral fractures and/or low-energy vertebral fracture(s), and (**iii**) exclusion of secondary causes of osteoporosis. All children had sustained fractures and there was no significant difference in children that had sustained peripheral fractures between the children with (*n*=14) and

without (*n*=10) vertebral fracture. However, the average number of peripheral fractures was 4.1 ± 1.1 for children without vertebral fractures and 1.9 ± 1.8 for children with additional history of vertebral fracture (Mäyränpää et al. 2011). Children who had sustained high-energy fractures, comparable to a fall from ≥ 3 meters, were not included in the study.

In **Study IV**, bone changes were characterized by bone histomorphometry as a part of clinical evaluation for suspected osteoporosis in pediatric patients after solid organ transplantation. Iliac crest bone biopsies were taken from 19 children and adolescents with a history of kidney (*n*=6), liver (*n*=9), or heart (*n*=4) transplantation (age range 7.6-18.8 years, 11 males). The suspicion of secondary osteoporosis was based on low areal BMD (aBMD) measured by DXA and/or fracture history. All children had received GCs since transplantation and were receiving low-dose alternate-day oral GCs (methylprednisolone). Total cumulative (mg) and weight-adjusted (mg/kg) GC doses as well as GC exposure (mg/kg/days) during previous three years were calculated as methylprednisolone equivalents for each patient. None of the children had been treated with aluminum-containing phosphate binders or BPs. All patients were clinically assessed by a pediatric nephrologist.

Table 7. Main characteristics for studies I-IV.

Study	Number of subjects (*n*)	Males/ Females (*n*)	Age mean ± SD (years)
I	36	16/20	46 ± 19
II	8	1/7	71.2 ± 11.8
III	24	17/7	12.0 ± 2.6
IV	19	11/8	15.3 ± 2.9

The patient characteristics are summarized in Table 7. The study protocol was approved by the local healthcare authorities and the local ethics committee (Research Ethics Board, Kuopio University Hospital, Kuopio, Finland in Studies I and II; and Ethics Committee, Children's Hospital, Helsinki University Central Hospital, Helsinki, Finland in Studies III and IV).

4.1.2 Growth and pubertal assessment (III, IV)

Height was reported as standard deviation (SD) units (Z-score) based on Finnish reference data. BMI was calculated as weight in kilograms divided by square of height in meters (kg/m^2) and transformed into Z-scores using age- and gender-specific reference data provided by WHO (WHO 2006). Pubertal maturation was recorded according to Tanner (Tanner 1962).

4.1.3 Radiology and biochemistry (II, III, IV)

Radiographs at the fracture site were available for all patients in **Study II**. The fractures were classified according to the major criteria defined by ASBMR (Shane et al. 2010) by two orthopedic surgeons.

In **Study III**, thoracic and lumbar radiographs were available for all children (standard anterior-posterior and lateral neutral radiographs in supine position). In **Study IV**, bone age was determined from a plain radiograph of the left hand according to Greulich and Pyle (Greulich and Pyle 1959). In **Study III**, the diagnosis of vertebral fracture was established by two observers, who worked independently and then reviewed the results to reach a consensus (digitized images, AGFA ImPacs System®). In **Studies III and IV**, the classification of vertebral changes was based on previous work by Mäkitie et al. (2005). A reduction of ≥ 20% in anterior, middle, or posterior vertebral height was considered significant (Mäkitie et al. 2005).

Bone densitometry (Lunar Prodigy device, General Electric Medical Systems, Lunar, Liegen, Belgium) had been previously performed for some of the patients in **Study II**. Based on BMD values, T-score values were calculated. In **Studies III and IV**, areal BMD for the lumbar spine (L1-L4) and total hip (Study IV) were measured within six months of the biopsy by DXA (Hologic Discovery A®, Bedford, USA) using pediatric software (version 12.4). Values were transformed into BMD Z-scores by using age- and sex-specific reference data; these reference values have been shown to be appropriate for Finnish children (Valta et al. 2009). In children with compressed vertebrae in the plain radiographs of the lumbar spine, the BMD result was re-analyzed excluding the affected vertebrae. The Z-scores were corrected for bone age, provided that skeletal maturity was delayed or advanced by more than one year.

In **Studies III and IV**, blood and urine samples were collected from all children before noon (fasting blood tests in Study IV). By using standard methods, plasma concentrations of ionized calcium (P-Ca-ion), phosphate (P-Pi), and creatinine (P-Crea) were determined. For alkaline phosphatase (P-ALP), age- and sex-dependent reference values were used (Saarinen et al. 2010). Glomerular filtration rate (GFR) was measured by 51-labeled chromium ethylenediaminetetraacetic acid clearance (Study IV). Kidney function was classified as normal (GFR > 90 ml/min/1.73 m^2; CKD stage 1), mild kidney dysfunction (GFR 60 - 89 ml/min/1.73m^2; CKD stage 2), and moderate kidney failure (GFR 30 - 59 ml/min/1.73m^2; CKD stage 3). Urine samples were analyzed for calcium and creatinine ratio (U-Ca/U-Crea ratio, normal reference value ≤ 0.7 mmol/mmol) (Study IV). Serum concentration of 25-hydroxyvitamin D (S-25-OHD) was determined by high performance liquid chromatography followed by UV detection (HP® 1100 Liquid Chromatograph). Hypovitaminosis D was defined as S-25-OHD below 50 nmol/L and vitamin D deficiency below 37.5 nmol/L (Misra et al. 2008). Plasma parathyroid hormone (P-PTH) was measured by a solid-phase enzyme-labeled chemiluminescent immunometric assay (IMMULITE® 2000, DPD, Diagnostic Products Corporation, Los Angeles, CA); the reference range was 8-73 ng/L. In **Study III**, the bone formation marker N-terminal propeptide of type I procollagen (P1NP) and the bone resorption marker C-terminal telopeptide of type I collagen (1CTP) were determined as previously described (Saarinen et al. 2010; Mäyränpää et al. 2011).

4.2 ASSESSMENT OF BONE QUALITY

Table 8. Methods used in each study to quantify bone quality.

Study	Methods used to evaluate bone quality
I	Bone histomorphometry Microcomputed tomography
II	Bone histomorphometry Fourier transform infrared spectroscopic imaging
III	Bone histomorphometry Fourier transform infrared spectroscopic imaging
IV	Bone histomorphometry

4.2.1 Bone biopsy and histomorphometry (I-IV)

Iliac crest bone samples were obtained from a standardized site located at 2 cm below and posterior to the anterior superior iliac spine. In **Studies I and II**, the biopsies were obtained with a Bordier bone biopsy trephine (Rochester Bone Biopsy®, Medical Innovations Incorporation Inc., USA). In **Studies III and IV**, the biopsies were taken with a 5 mm bone biopsy trephine (modified Bordier) (Study III) and/or a 7.5 mm bone biopsy trephine

(Rochester Bone Biopsy®, Medical Innovations Incorporation Inc., USA) (Studies III and IV). Fluorochrome double labeling was performed prior to biopsy in most cases; in **Study I** in 25 of the 36 patients, in **Study IV** in 18 of the 19 children, or for all patients in **Studies II and III**. Tetracycline was used as a fluorescent agent in two separate 2-day courses, at a dose of 1500 mg per day in adults (Studies I and II) and 15-20 mg/kg/day (maximum of 1000 mg/day) in children (Studies III and IV). The interval between the two tetracycline courses was 10 days and the medication was terminated four days before the biopsy. The biopsy samples were fixed in 70% ethanol for at least 48 hours before embedding in polymethyl metacrylate (PMMA). In **Study III**, the iliac crest biopsies were taken mainly within 12 months of the fractures (*n*=19), but in some cases up to 18 months had elapsed since the fracture (*n*=5). In **Study IV**, the median time interval from organ transplantation to the bone biopsy was 4.6 years (range 0.6-16.3 years).

Bone histomorphometry (Bioquant OsteoII, Bioquant Image Analysis Corporation, Nashville, TN, USA) was performed for all samples. In **Study I**, the analysis was repeated two times on different days. In the light microscopic evaluation, 3 μm (Studies I, III, and IV) or 5 μm (Study II) thick sections were cut with a microtome and stained with modified Masson Goldner trichrome stain. Unstained sections (thickness 7 μm in Studies I, III, and IV and thickness 5 μm in Study II) were used for polarized light and fluorescence microscopy. For each sample, one (Studies I, II, and IV) or two (Study II) sections were randomly selected and analyzed with bone histomorphometry. Regions of interest (ROI) were carefully selected to ensure that only cancellous bone was measured, i.e., no cortical or subcortical bone was included. The tissue area varied depending on the sample size. The magnification used in all measurements was 200x. The nomenclature, abbreviations, and units adhered to the recommendations by ASBMR (Parfitt et al. 1987; Dempster et al. 2013). In **Study II**, the mean values of two observers for each parameter are reported except for tissue value that is presented as a sum.

Tissue volume (TV, mm^2) was determined by using the whole field of view including both trabeculae and bone marrow. Bone volume (BV, mm^2) includes both mineralized and unmineralized bone volumes. Bone surface parameters (BS/TV, mm^{-1}; BS/BV, mm^{-1}) were measured (Study I). Osteoid surfaces (OS/BS, %) were recognized as unmineralized seams on the bone surfaces, osteoid thickness (O.Th, μm) was measured, and osteoid volume (OV/BV, %) was calculated. The mineralized bone volume was defined (Md.V/TV, %) (Study I). Bone cells, osteoblasts and osteoclasts, were determined as a fraction of bone surface (Ob.S/BS, %; Oc.S/BS, %). Trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm^{-1}), and trabecular separation (Tb.Sp, μm) were calculated. Wall thickness (W.Th, μm) was measured under polarized light. Dynamic indices were defined in tetracycline-labeled biopsies using fluorescence microscopy. Mineralizing surfaces (MS/BS, %) were measured and the mineral apposition rate (MAR, μm/day) was defined as the interlabel width on double labels divided by the number of days between fluorochrome labeling. Additionally, activation frequency (Ac.F), bone formation rate (BFR/BS), and osteoid maturation time (Omt) are reported in **Study IV**. Because of scarce labeling in ROI in some samples in **Studies II and IV**, a total of four unstained sections (300 μm apart) were analyzed for those samples under fluorescence microscopy in order to evaluate fluorochrome labeling (extended label search).

Histological classification based on bone histomorphometry (I, II, IV)

On the basis of histomorphometric findings in combination with the patient's history in **Study I**, the samples were grouped into three subgroups: healthy subjects (*n*=10), patients with diagnosed osteoporosis (OP, *n*=15) and patients suffering from chronic kidney disease (renal osteodystrophy, ROD, *n*=11). The osteoporotic samples were from patients with low or high turnover. The renal osteodystrophy samples came from patients with hyperparathyroid disease, i.e., high turnover including hyperosteoidosis and increased

resorption. Reference values for postmenopausal women were obtained from Recker et al. (1988) (mean ± standard deviation) and used for comparisons (Study II).

In **Studies III and IV**, the histomorphometric parameters were transformed into SD units by comparing them with the reference values to allow cross-sectional comparison of the findings. The pediatric age-specific data for transiliac bone histomorphometry was used as reference for all parameters (Glorieux et al. 2000) except for the assessment of eroded surface (ES/BS), which was determined with a method more commonly used in adults; values over 10% were considered as abnormal (Recker et al. 1988; Rehman et al. 1994). After quantitative analysis of histomorphometric parameters, the patients were divided into subgroups by two experienced readers. Based on the interpretation of cancellous bone volume for age, the samples were divided into low or normal cancellous bone volume groups. Low cancellous bone volume was defined as cancellous bone volume (BV/TV) Z-score below -1.0 and normal bone volume as BV/TV Z-score ≥-1.0 as compared with age-specific normal values (Glorieux et al. 2000).

Further, the samples were grouped based on the bone turnover rate (low, normal, or high turnover for age) in **Studies III** (Table 9) **and IV**. The turnover rates were estimated based on Z-score values of formation (osteoid surface [OS/BS], osteoid volume [OV/BV], osteoid thickness [O.Th], and osteoblast surface [Ob.S/BS]) and resorption (erosion surface [ES/BS] and osteoclast surface [Oc.S/BS]) parameters, as previously described (Mäyränpää et al. 2011). It should be noted that in most of the cases the observed changes were more than ± 1.0 SD. In **Study IV**, children with high and low turnover rate were identified; those with low turnover rate were re-evaluated in order to detect adynamic bone disease (ABD) (Figure 8). ABD was defined according to the previously described criteria, i.e., absence of remodeling activity (absence of tetracycline labeling in extended label search), dramatically decreased bone formation with either relatively increased or decreased bone resorption, and paucity or absence of osteoblasts (Hruska and Teitelbaum 1995; Malluche and Monier-Faugere 2006; Recker et al. 2011). Mineralization was evaluated and defined as osteomalacia if there was clear evidence of suppressed bone mineralization, i.e., no double labels in the extended label search (Parfitt 2005; Recker et al. 2011).

Table 9. Turnover rate classification based on bone histomorphometry in Study III.

Turnover rate	**Classification criteria**
High turnover	(i) both resorption parameters were elevated (ES/BS > 10%, Oc.S/BS > +1.0 SD) and/or (ii) both formation parameters were elevated (> +1.0 SD) or (iii) at least one formation and one resorption parameter were elevated
Low turnover	(i) both of the formation parameters were low (< -1.0 SD) or (ii) mineral apposition rate (MAR) was not measurable

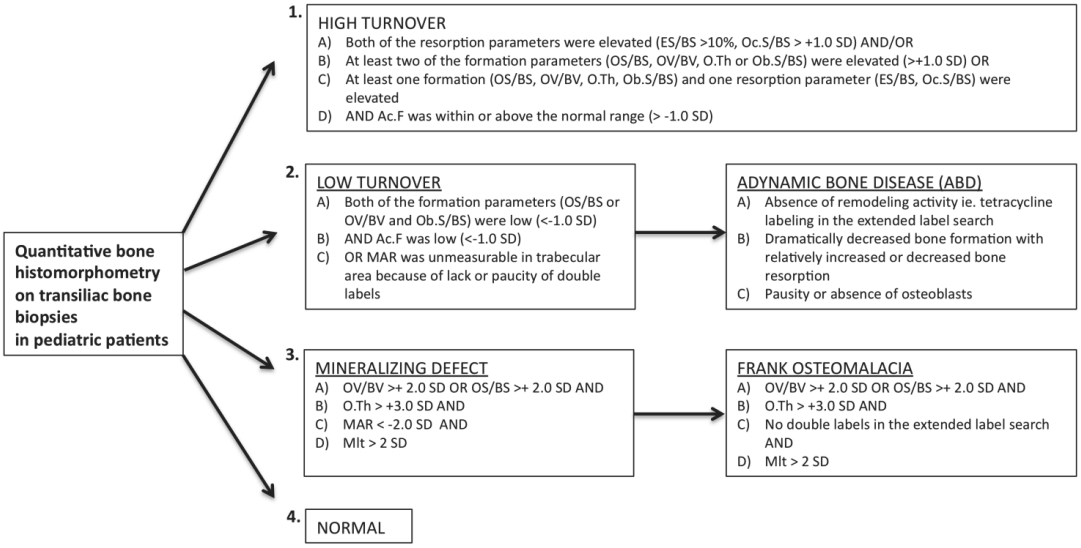

Figure 8. Histological bone turnover rate classification in Study IV. The turnover rates were classified based on Z-score values of formation (osteoid surface [OS/BS], osteoid volume [OV/BV], osteoid thickness [O.Th], and osteoblast surface [Ob.S/BS]) and resorption (erosion surface [ES/BS] and osteoclast surface [Oc.S/BS]) parameters. Mineralizing defects or osteomalacia were classified as described by Parfitt et al. for adults (Parfitt 2005) but modified for children so that ± 2.0 SDs were used as cut-off limits. For osteoid thickness, a different cut-off limit (+3.0 SD) was used because of the criteria used in adult population (osteomalacic O.Th >15 µm which is +2.7 SD) (Recker et al. 1988; Parfitt 2005).

4.2.2 Microcomputed tomography (micro-CT) (I)

PMMA-embedded bone cores were imaged twice on different days with a high-resolution micro-CT scanner (Skyscan 1172, Aartselaar, Belgium). The samples were scanned by the same operator with an isotropic voxel size of 14 µm after the histomorphometry section had been obtained. The X-ray tube voltage was 100 kV, current 100 µA, and the scanning time for each specimen was between 50 and 75 minutes. The rotation step was 0.40-0.56 degrees between each image acquisition, and the frame averaging was 5. A 0.5-mm aluminum filter was used for all measurements, and reconstruction of the 2D-cross-sections was performed with a modified Feldkamp cone-beam algorithm (NRecon, version 1.5.1.4, Skyscan, Aartselaar, Belgium). Volumes of interest (VOI) were carefully selected to exclude the cortical and subcortical bone area. An adaptive threshold was used to distinguish bone area from non-bone area, and this was kept constant for all samples (Waarsing et al. 2004). The adaptive threshold algorithm included a low pre-threshold (global threshold) value to binarise thin objects but not noise. After the pre-thresholding, despeckle (three-dimensional, 3D) was run twice; first to remove white speckles of <20 voxels, and then to remove artificial pores inside the bone. The adaptive threshold of the mean of minimum and maximum was selected for cancellous bone, using standard analysis methods in CTAn software (Skyscan, Aartselaar, Belgium).

The calculation of the three-dimensional bone parameters and structural indices followed the recommendations of American Society of Bone and Mineral Metabolism (Parfitt et al. 1987). Bone volume fraction was measured as the percentage of bone volume per tissue volume (BV/TV, %), and bone surface per tissue and bone volume were defined as bone surface density (BS/TV, μm^{-1}; BS/BV, μm^{-1}). Trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, μm^{-1}), and trabecular separation (Tb.Sp, µm) were also

quantified. The trabecular pattern factor (Tp.Pf, μm^{-1}) was calculated to describe bone connectedness. The structural model index (SMI) was quantified to provide information about the predominant shape of the trabeculae in the bone tissue, where values of zero and three corresponded to ideal plate and rod structures. The 3D-structural parameters were calculated with the CT Analyzer (version 1.6.1.1, Skyscan, Aartselaar, Belgium).

4.2.3 Fourier transform infrared spectroscopic imaging (FTIRI) (II, III)

In the FTIRI analysis, 3 μm sections were cut with a microtome (Polycut S®, Reichert-Jung, Germany). To ensure that there was no systematic error in section thickness, samples from all groups were randomly sectioned in the same session for each study. The samples were placed on ZnSe windows, and measurements were conducted with a PerkinElmer instrument in the transmission mode (Perkin Elmer Spotlight 300®, Waltham, MA, USA). A spatial resolution of 6.25 μm, a spectral resolution of 4 cm^{-1}, and 8 repeated scans were used for data collection. The absorption spectra were recorded between 2000-800 cm^{-1} wavenumbers. The background was corrected by measuring the spectrum from a clean site of the window with the same measurement parameters, but using 75 repeated scans. Based on the bright light microscope image, three to five trabeculae per sample were selected and imaged. The differences in section thickness were normalized using the absorbance of the PMMA (1728 cm^{-1}) on both sides of the trabeculae (Rieppo et al. 2004). The PMMA spectrum was subtracted from every bone spectrum to account for the differences in PMMA penetration into the trabeculae (Boskey and Mendelsohn 2005). The bone spectra were first normalized to the PMMA peak (1728 cm^{-1}), and subsequently the PMMA spectrum was subtracted from the bone spectra (Rieppo et al. 2004; Isaksson et al. 2010). The bone spectra were scaled using the same scaling factor back to the original absorbance values (Rieppo et al. 2004; Isaksson et al. 2010) (Studies II and III).

In **Study II**, age- and sex matched normal samples (*n*=4, aged 42-75 years, all females), which were part of the previous study, were selected for comparison (Isaksson et al. 2010) together with two additional samples (*n*=2, aged 51-58 years, both females). A total of six samples were used for comparisons (*n*=6, aged 42-75 years, mean age 59.0 years). The additional samples were obtained from cadavers with no history of metabolic bone disease. The cadavers were autopsied within seven days after death. The exclusion criteria included severe liver disease, kidney disease, diabetes, rheumatoid arthritis, Crohn disease, ulcerative colitis, stomach removal, hyper- or hypothyroid disease, myeloma, malignancy, alcoholism, hip prosthesis or similar, previous hormone replacement therapy, or clear pathological finding at the sample site. Only ethanol was used in the storage of the samples. Measurements were performed as above, with the exception that in the previous study four repeated scans were used.

Constant baseline correction was made for every IR spectrum (custom code, Matlab, version 7.6 Mathworks, Natick, MA, USA). The bone mineral content was assessed based on the peak areas of phosphate (900-1200 cm^{-1}) and carbonate (850-890 cm^{-1}) peaks (Boskey 2003). The collagen content was assessed based on the amide I peak area (1585-1720 cm^{-1}) (Boskey and Mendelsohn 2005). Collagen cross-linking ratio, i.e., collagen maturity, was determined as the ratio between the intensities at 1660 cm^{-1} and 1690 cm^{-1} (Donnelly et al. 2012). A linear baseline correction was performed for each peak before the analyses. The bone composition parameters were calculated using previously published methods (Table 10). In **Study III**, crystallinity was defined, using peak fitting of the second derivative spectrum, as a ratio between the sub-peaks at 1030 cm^{-1} and 1020 cm^{-1} (Pleshko et al. 1991). It has been shown to correlate with the mineral crystal size and perfection as determined by X-ray diffraction (Gadaleta et al. 1996). In **Studies II and III**, the spatial heterogeneity of each compositional parameter was assessed within each sample. The values for each pixel were calculated and used to create a histogram using Matlab® (version 7.6 Mathworks Inc.). Thereafter, a Gaussian curve was fitted to the histogram, and the full-width-at-half-

maximum (FWHM) of the Gaussian curve was determined as a measure of heterogeneity (Boskey et al. 2009; Turunen et al. 2012).

Table 10. Variables in bone composition based on Fourier transform infrared spectroscopic imaging (FTIRI).

Parameter	**Measure of**
Phosphate-to amide I ratio	Estimates the degree of mineralization, and has been shown to correlate with the ash content of the bone (Boskey and Mendelsohn 2005; Boskey and Pleshko Camacho 2007).
Carbonate-to-phosphate ratio	Has been suggested to reflect the level of carbonate substitution into the hydroxyapatite crystal (Huang et al. 2002; Isaksson et al. 2010).
Carbonate-to-amide I ratio	Reflects the carbonate content in bone. Carbonate accumulates slowly with age, and therefore, high turnover could lead to low carbonate-to-amide I and carbonate-to-phosphate ratios (Isaksson et al. 2010).
Collagen cross-linking ratio, i.e., collagen maturity	Has been suggested to reflect the degree of mature to immature collagen cross-links (Paschalis et al. 2001).
Crystallinity	Correlates with the mineral crystal size and perfection (Gadaleta et al. 1996).

4.3 STATISTICAL ANALYSIS (I, III, IV)

In **Study I,** the samples were analyzed both after pooling all samples as well as in groups based on the metabolic status of the bone. The nonparametric Mann-Whitney U-test was used to test differences for each parameter between the study groups. The nonparametric Wilcoxon signed rank test was used to confirm that there were no statistical differences between the two repeated measurements for each technique. The reproducibility between the two repeated measurements for both methods and each parameter was determined using the coefficient of variation (CV, %) as described by Glüer et al. (Glüer et al. 1995), as well as by calculation of Spearman's correlation coefficient (ρ). Statistical differences in the coefficient of variation between the study groups were analyzed using the Kruskal-Wallis test and Mann-Whitney U-test. The agreement between the histomorphometry and micro-CT measurements was analyzed by Spearman's correlation analysis using mean values of two measurements for both techniques.

In **Study III**, the age-specific reference values were used to calculate the bone histomorphometric data as SD units (Glorieux et al. 2000). Median values were calculated for parameters. The nonparametric Mann-Whitney U-test was used to test differences between two study groups for all compositional parameters. The differences between children with and without vertebral fractures were tested using Mann-Whitney U-test, as well as the differences between the normal and low cancellous bone volume samples. Additionally, differences between the turnover rates were defined. Spearman's correlation coefficients were calculated between biochemical and compositional bone parameters. Chi Square test was used to test differences when appropriate.

In **Study IV**, the bone histomorphometric data were calculated as SD units using the age-specific reference values (Glorieux et al. 2000). Median values were calculated for biochemical markers and bone densitometry; Spearman's correlation coefficients were calculated for various parameters. Samples were analyzed in subgroups based on transplantation history, i.e., kidney, liver, or heart transplant. For bone histomorphometric

parameters, the nonparametric Kruskal Wallis test was used to test differences between more than two subgroups of the samples, and Mann-Whitney U-test was used to test differences between two subgroups of the samples. Chi Square test was used to test differences when appropriate. Bonferroni correction was used when comparing several sample groups.

Statistical analyses were performed with the SPSS® Statistics software (version 19.0.0, SPSS Inc., Chicago, IL). A *p*-value below 0.05 was considered statistically significant.

5 Results

5.1 REPRODUCIBILITY AND AGREEMENT OF MICRO-CT AND HISTOMORPHOMETRY (I)

5.1.1 Histomorphometry and micro-CT parameters

Significant differences between the normal, osteoporotic (OP), and renal osteodystrophy (ROD) bone samples were found in some micro-CT parameters. When the values obtained with both techniques were assessed, OP samples had lower bone volume fraction (BV/TV) than normal and ROD samples (p<0.05) (Figure 9, Table 11)). In the micro-CT evalution, bone surface (BS/TV) was higher (p<0.05) in ROD samples than in OP samples. Micro-CT revealed that normal samples had the highest trabecular thickness (Tb.Th) (p<0.05), and trabecular number (Tb.N) was lower (p<0.05) in the OP group than in the normal and ROD groups. In OP samples, the trabecular pattern factor (Tb.Pf) was higher (p<0.05) than in normal and ROD samples and structure model index (SMI) was higher than in ROD samples.

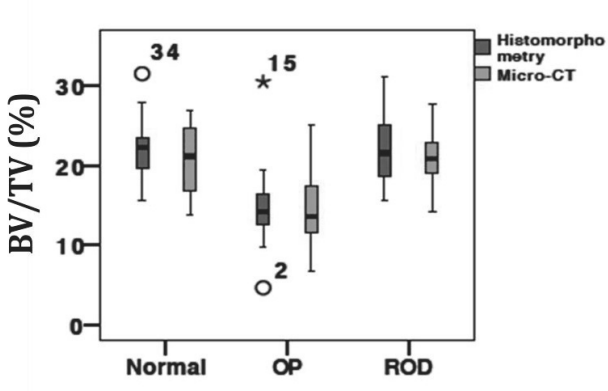

Figure 9. Bone volume (BV/TV) was lower in osteoporotic patients (OP, n=15) than in healthy subjects (n=10) or patients with renal osteodystrophy (ROD, n=11), as assessed by bone histomorphometry and microcomputed tomography (micro-CT). Outliers are depicted as a circle and an extreme outlier as a star.

5.1.2 Reproducibility

There were no differences between the two repeated measurements with either of the techniques (Wilcoxon signed rank test). The values of repeated bone histomorphometry measurements for structural parameters BV/TV, Tb.Th, Tb.N, and Tb.Sp exhibited correlation coefficients (ρ) between 0.87 and 0.92 (p<0.01), and the CV varied between 8.3 and 27.2%. The CV of micro-CT parameters for BV/TV, Tb.Th, Tb.N, and Tb.Sp was 4.4-23.4%. Moreover, Spearman's correlation coefficients (ρ) were significant (p=0.66-0.94, p<0.01). When the groups were compared, there were no significant differences in the reproducibility of bone histomorphometry or micro-CT (Kruskal-Wallis test of CV values) between the three study groups.

5.1.3 Agreement

When all samples were pooled, the correlation coefficients between the micro-CT and histomorphometry measurements were significant for BV/TV (ρ=0.54, p<0.01), BS/BV (ρ=0.51, p<0.01), Tb.Th (ρ=0.62, p<0.01), and Tb.N (ρ=0.39, p<0.05). Correlations between

BV/TV (micro-CT) and mineralized bone volume (Md.V/TV, histomorphometry) were slightly weaker (ρ=0.53, p<0.01) than between BV/TV (micro-CT) and BV/TV (histomorphometry) (ρ=0.54, p<0.01). When the groups were analyzed separately, OP samples showed significant correlations for BV/TV (ρ=0.58, p<0.05) and for Tb.Th (ρ=0.64, p<0.05).

Table 11. Bone histomorphometry and micro-CT parameters in different study groups.

Bone histomorphometry	**ALL**	**NORMAL**	**OP**	**ROD**
	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD
BV/TV (%)	19.1 ± 6.4	22.1 ± 5.0	14.8 ± 5.7	22.3 ± 4.8
OV/BV (%)	3.1 ± 2.4	2.8 ± 2.8	2.2 ± 1.4	4.7 ± 2.2
OS/BS (%)	23.3 ± 14.6	23.2 ± 16.9	16.3 ± 9.9	32.9 ± 12.5
ES/BS (%)	4.7 ± 4.4	3.2 ± 3.0	3.1 ± 2.3	8.2 ± 5.6
Ob.S/BS (%)	2.3 ± 3.0	0.4 ± 0.8	1.8 ± 2.8	5.0 ± 2.6
Oc.S/BS (%)	2.5 ± 3.3	1.6 ± 2.6	1.2 ± 1.4	5.2 ± 4.3
O.Th (µm)	6.5 ± 1.8	7.1 ± 2.1	5.7 ± 1.3	7.1 ± 1.6
Tb.Th (µm)	104 ± 28	121 ± 25	94 ± 31	103 ± 21
Tb.N (1/mm)	1.9 ± 0.5	1.9 ± 0.5	1.6 ± 0.5	2.2 ± 0.4
Tb.Sp (µm)	489 ± 238	446 ± 141	605 ± 306	370 ± 95
MS/BS (%)	5.4 ± 5.1	1.8 ± 2.1	4.7 ± 5.2	7.2 ± 4.9
MAR (µm/day)	0.36 ± 0.28	0.25 ± 0.29	0.38 ± 0.30	0.35 ± 0.25
W.Th (µm)	36.6 ± 7.3	34.9 ± 5.4	37.0 ± 8.8	36.4 ± 5.2
MicroCT-3D				
BV/TV (%)	18.2 ± 5.6	20.8 ± 4.7	14.7 ± 5.5	20.7 ± 3.9
BS/TV (1/mm)	4.3 ± 1.1	4.6 ± 1.1	3.8 ± 1.2	4.9 ± 0.8
SMI	1.3 ± 0.5	1.3 ± 0.5	1.6 ± 0.5	1.0 ± 0.3
Tb.Th (µm)	150 ± 25	164 ± 17	143 ± 27	145 ± 22
Tb.N (1/mm)	1.2 ± 0.4	1.3 ± 0.3	1.0 ± 0.3	1.4 ± 0.3
Tb.Sp (µm)	603 ± 160	602 ± 230	631 ± 141	567 ± 96

5.2 ATYPICAL FEMORAL FRACTURES (II)

5.2.1 Incidence

We identified eight patients with AFFs among the trauma patients in our hospital from January 2007 to December 2009. Two of these patients had bilateral fractures (not simultaneously), i.e., a total of 10 fractures. Six of these patients had been treated with BPs for several years prior to the AFF. Alendronate had been in use in five patients whereas only one patient, who had been on multiple BP treatments, was been treated recently with ibandronate. The mean age of the patients at the time of fracture was 71.2 years (range 55.5-89.9 years). The mean treatment duration with BPs before the fracture was 7 years (range 4-10 years). Only limited data for bone density was available. Patient #1 had BMD T-score -0.7 in lumbar spine, -1.2 in left femoral neck, and -0.4 in the upper part of the femoral diaphysis

on the left. Patient #7 had lumbar spine BMD T-score -2.8 and right femoral neck BMD T-score -0.4.

In the years 2007-2009, the mean population who were aged 50 years and over in the catchment area of Kuopio University Hospital was 103 932 inhabitants, of which 55 539 were women. During these years, the average number of patients on continuous BP therapy in the area was 4379. We calculated the annual occurrence of AFFs to be 3 per year. Two patients (with unilateral fractures) had not been receiving BP treatment. The incidence of fractures in BP-treated patients was 2.67 fractures per year and there were 4379 patients on BPs in the catchment area of our hospital. The incidence was therefore 2.67/4379 per year = 0.61/1000 per year for BP users (95% CI: 0.13/1000 - 0.92/1000), compared to 0.0067/1000 (95% CI: -0.0026/1000 - 0.016/1000) per year for untreated patients.

5.2.2 Histological bone findings

The patients (*n*=4), that underwent bone histomorphometry, were postmenopausal women (aged 55.5 – 81.1 years). None of the patients experienced any complications during or after the bone biopsy procedure. Cancellous bone volume (BV/TV) and trabecular thickness were low in 3 patients, osteoid surface (OS/BS) and osteoblasts surface (Ob.S/BS) were low in 2 and mineralizing surface (MS/BS) was low in all 4 cases (Figure 10). Further, erosion surface (ES/BS) was low in 3 cases. These findings suggest for low bone formation in 3 out of 4 patients. For patients #1 and #4, the tetracycline labeled biopsies were taken five and two months after the first unlabelled biopsy, respectively. By fluorescence microscopy, single labels were detected in the cancellous region of interest in only Patient #3 (MS/BS, 0.8%) who also had an increased amount of osteoid (OV/BV, 7.1%) and a higher fraction of osteoblasts of bone surface (Ob.S/BS 10.9%).

Since there were no labels in regular cancellous ROI, extended label search was performed. Patient #1 had one short single label in cortical bone but no cancellous bone labeling. Patient #2 had one double label in cortex together with one double label in a cancellous bone in one section. Another section had a cortical single label. Patient #3 had one double label in cancellous bone and a single label in cortex in one section. A cortical single label in two sections was found for Patient #4, and the other section of these included two short single labels in cancellous bone.

5.2.3 Bone composition findings

The bone composition measured by FTIRI showed higher degree of mineralization (phosphate-to-amide I ratio) in 3 of our 4 patients. Carbonate-to-phosphate ratio was within the normal range. Higher carbonate-to-amide I and collagen cross-link ratios were found in 3 of our 4 patients with AFFs compared with normals. Hence, the collagen cross-link maturity might be higher in patients with AFFs. The heterogeneity for phosphate-to-amide I ratio was lower for patients with AFFs when compared with normal samples, whereas the heterogeneity of carbonate-to-phosphate ratio and carbonate-to-amide I ratio were within the normal range.

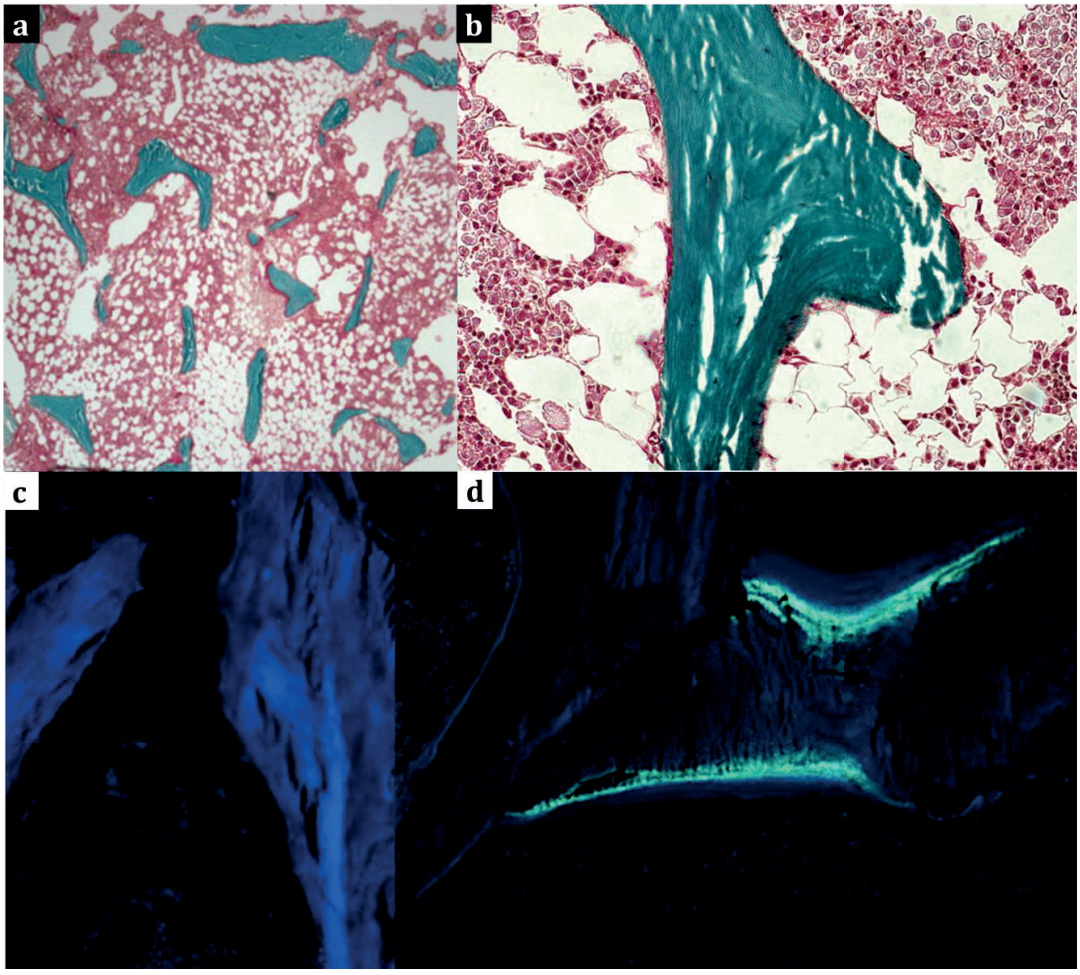

Figure 10. Bone histomorphometry findings in patients with atypical femoral fractures. Bone volume tended to be low (green areas, Masson Goldner stain) (**a**). Bone formation was low in 3 of our 4 patients (**b**). Under fluorescence microscopy, most patients showed no fluorochrome labeling (**c**). Double tetracycline labels were found in cancellous bone in two patients including the extended label search and single labels in the other two patients suggesting that mineralization of bone was obviously decreased but not absent (**d**). Magnification a) 20×, b-d) 200×.

5.3 VERTEBRAL FRACTURES AND BONE DISEASE AFTER SOLID ORGAN TRANSPLANTATION IN CHILDREN

5.3.1 Clinical characteristics (III, IV)

Bone characteristics were studied in two cohorts of children who were suspected for osteoporosis. The cohort of fracture-prone children included 24 children (17 males) who were suspected for primary osteoporosis (Study III). The median age was 12.0 years (range 6.7-16.6 years) (Table 12). Mäyränpää et al. (2011) has previously studied the correlation between bone biopsy findings and clinical, radiological, and biochemical parameters. The effect of age on compositional bone parameters was studied by dividing all samples into three sub-groups according to age. Increased carbonate substitution into hydroxyapatite crystals with age was indicated by the lower carbonate-to-phosphate ratio in the younger children (6.7-10.9 yrs, n=9) than in the older children (14.0-17.0 yrs, n=5). Carbonate-to-phosphate heterogeneity correlated positively with Tanner stage (ρ=0.46, p=0.023) (Figure

11a). Bone histomorphometry parameters were correlated with bone compositional heterogeneity variables. Collagen cross-link heterogeneity correlated positively with OV/BV absolute value (ρ=0.43, p=0.035) (Figure 11b) and O.Th Z-score (ρ=0.45, p=0.029). The heterogeneity of carbonate-to-phosphate ratio correlated positively with O.Th absolute value (ρ=0.48, p=0.019) (Figure 11c).

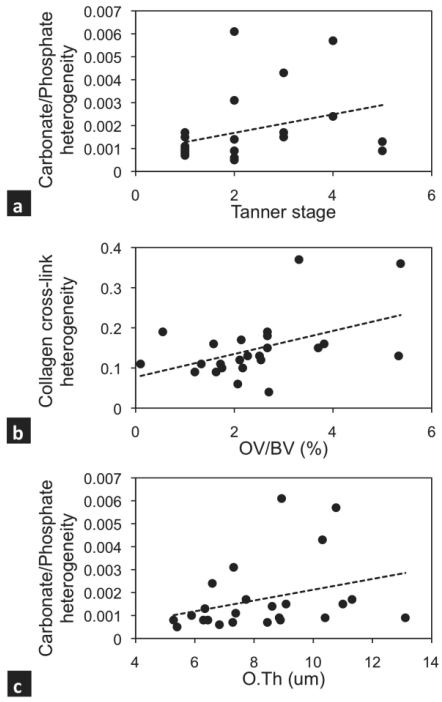

Figure 11. Significant correlations between bone composition parameters and other variables were found. The heterogeneity of carbonate-to-phosphate ratio correlated positively with Tanner stage, and thus, children prior to or in puberty might have a more uniform carbonate-to-phosphate content than children in more advanced puberty (ρ=0.46, p=0.023) (**a**). Further, the heterogeneity of collagen cross-links correlated positively with osteoid surface (OV/BV, ρ=0.43, p=0.035) suggesting narrower collagen cross-link ratio in children with lower amounts of osteoid (**b**). The heterogeneity of the carbonate-to-phosphate ratio correlated positively with osteoid thickness (O.Th, ρ=0.48, p=0.019) (**c**). Therefore, the children with low O.Th might have a more uniform carbonate-to-phosphate content.

The other cohort (Study IV) included 19 children (age range 7.6-18.8 years, 11 males) with a history of solid organ transplantation at 4.6 years (median, range 0.6-16.3 years) earlier (Table 12). The median height Z-score was -0.8 and the Z-score was below -2.0 in three children (16%); liver transplant recipients had milder growth deficit than kidney transplant recipients (p<0.05). BMI ranged from 14.0 kg/m^2 to 36.1 kg/m^2. The median BMI Z-score was -0.1 (range -2.2 to +3.3). Three children were overweight (BMI > +1.0 SD) and three children were obese (BMI > +2.0 SD). All patients were on triple-drug immunosuppressive medication including cyclosporine A (n=15), tacrolimus (n=4), azathioprine (n=11), or mycophenolate mofetil (n=8), and on low-dose alternate-day methylprednisolone (n=19). All children were receiving vitamin D substitution. One child had prolonged pain at the bone biopsy site for two weeks. None of the patients experienced any other complications during or after the bone biopsy procedure.

Table 12. Clinical characteristics of children with suspected osteoporosis. The median (range) or number of patients are presented.

	Fracture-prone children (*n*=24)	**Children after solid organ transplantation (*n*=19)**
Age (years)	12.0 (6.7-16.6)	15.5 (7.6 to 18.8)
Males / Females (*n*)	17 / 7	11 / 8
Height Z-score	0.4 (-1.4 to 2.1)	-0.8 (-2.4 to 0.9)
BMI Z-score	0.7 (-2.3 to 2.3)	-0.1 (-2.2 to 3.3)
Lumbar BMD Z-score	-1.2 (-3.1 to 1.0)	-2.0 (-3.9 to 0.4)
BV/TV < -1.0 SD in histomorphometry (*n*)	7	6
Children with non-vertebral fracture(s) (*n*)	22	4
Children with vertebral compression(s) (*n*)	14	11

5.3.2 Bone densitometry and fractures (III, IV)

The lumbar BMD Z-score tended to be low in both cohorts. Five fracture-prone children (21%), and similarly, nine transplant children (47%), including 7 liver and 2 heart transplant patients, had low lumbar spine BMD Z-score (<-2.0 SD) in DXA. A low total hip BMD Z-score was observed in five transplant children (26%), i.e, one kidney and four liver transplant recipients (Study IV). All but two fracture-prone children (92%) had sustained peripheral fractures and 14 children (58%) had suffered vertebral compressions. One fifth (21%) of the transplant children, all liver transplant recipients, had sustained peripheral fractures whereas vertebral compressions were found in more than half (58%) of the children. Vertebral compressions were found in all transplant groups.

5.3.3 Biochemistry (III, IV)

In fracture-prone children, the plasma PTH concentration (P-PTH, reference range 8-73 ng/L) correlated with the carbonate-to-phosphate ratio (Spearman's correlation coefficient, ρ=0.41, p<0.05) (Study III) (Figure 12). The other biochemical markers showed no correlation with the compositional bone parameters.

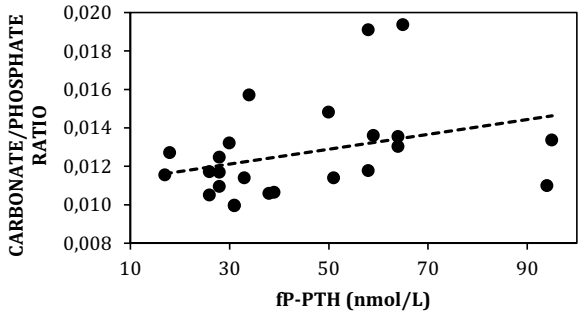

Figure 12. In fracture-prone children, plasma parathyroid hormone (PTH) level correlated positively with carbonate-to-phosphate ratio as assessed by Fourier transform infrared spectroscopic imaging (ρ=0.41, p<0.05). This pointed to an increased carbonate substitution into hydroxyapatite crystals among children with higher PTH level.

In the cohort of transplantation children, one child had mild hypovitaminosis D (S-25-OHD 43 nmol/L) whereas the other children were vitamin D sufficient (Study IV). The P-PTH level was slightly elevated in two children (74 ng/L and 77 ng/L). Ionized calcium levels were normal in all children. A low phosphate level was found in two kidney transplant children but hyperphosphatemia was not detected in any of the children. Mildly elevated P-ALP was found in two children. Creatinine was elevated (95-134 µm/L) in eight children from different transplant groups. Only one child had normal kidney function whereas all other of the children had mild (CKD stage 2, n=9) or moderate (CKD stage 3, n=9) kidney failure. The kidney transplant recipients had more severe kidney dysfunction than the liver transplant recipients (p<0.05).

5.3.4 Cancellous bone volume in biopsy (III, IV)

A low cancellous bone volume (BV/TV < -1.0 SD) was observed in 7 fracture-prone children (29%) and in 6 children (32%) after transplantation. There was no significant difference in cancellous bone volume between the transplant groups (Figure 13). In both cohorts, the children with low cancellous bone volume in biopsy (BV/TV < -1.0 SD) were compared to those with a normal bone volume (BV/TV ≥ -1.0 SD). In fracture-prone children, the carbonate-to-amide I and carbonate-to-phosphate ratios were lower for children with low BV/TV (n=7, p<0.05) when compared to children with normal bone volume (n=17) (Study III). This suggests that there is a lower level of carbonate substitution in crystals in children with low cancellous bone volume. In the transplantation cohort, children with low BV/TV (n=6) had thinner trabeculae (Tb.Th median -3.1 SD, range -5.0 to -2.2 SD) and higher trabecular separation (Tb.Sp median -0.5 SD, range -2.6 to +3.5 SD) than those with normal BV/TV (n=13, Tb.Th median -2.1 SD, range -3.7 to -0.7 SD; Tb.Sp median -2.2 SD, range -3.9 to -1.2 SD; p<0.05, Study IV). A trend was seen for lower trabecular number (Tb.N median 1.4 SD, range -1.9 to +6.3 SD, p=0.054) and higher mineral apposition rate higher (MAR median -0.7 SD, range -2.4 to +1.4 SD, p=0.053) in children with low BV/TV as compared to children with normal BV/TV but this was not statistically significant.

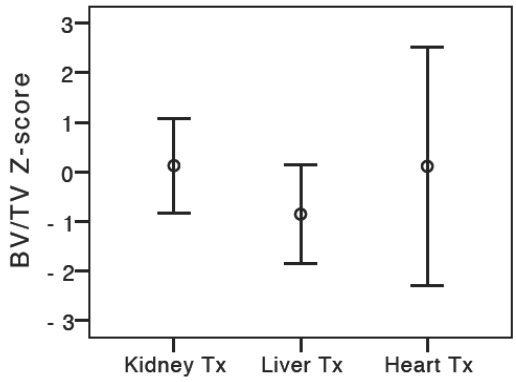

Figure 13. No significant differences between the different transplant groups (kidney, liver, and heart) were found for cancellous bone volume (BV/TV Z-score, p>0.05). Tx=transplantation.

5.3.5 Fracture-prone children with and without vertebral fractures (III)

Fracture-prone children with a previous a vertebral fracture (n=14) showed different bone composition compared with those without vertebral fractures (n=10). There was no significant difference in age between the study groups. There was no significant difference in the phosphate-to-amide I ratio, whereas the carbonate-to-phosphate ratio and the heterogeneity of carbonate-to-phosphate ratio were lower (p<0.05) in children with vertebral fracture. This is evidence of decreased and narrower carbonate substitution in

subjects with vertebral fracture compared to children without vertebral fracture. The collagen cross-link ratio (p<0.05) was higher and the heterogeneity of collagen cross-link ratio was lower (Figure 14) in children with vertebral fractures. Hence, the number of mature collagen cross-links might be higher and more uniform in children with vertebral fracture. There was no significant difference in crystallinity between the children with and without vertebral fracture.

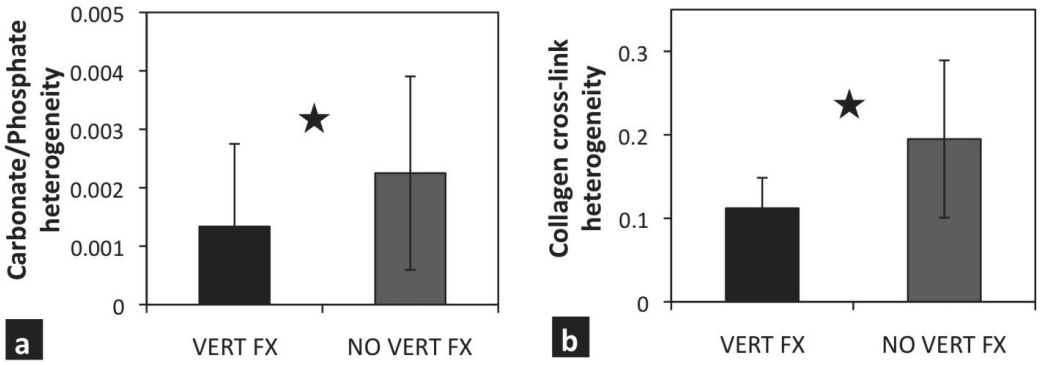

Figure 14. Based on FTIRI, the heterogeneity of carbonate-to-phosphate ratio and the heterogeneity of collagen cross-link ratio were lower in fracture-prone children with vertebral fracture compared to those without vertebral fracture (p<0.05, *). This points to more uniform collagen composition of bone in children with vertebral fracture than in children without vertebral fracture.

The histomorphometric evaluation revealed a low cancellous bone volume in 36% of the children with a vertebral fracture. The bone turnover rate was abnormal in 64% of the children who had sustained vertebral fracture. According to the biochemistry evaluation, children with and without vertebral fractures had no significant differences in biochemical data including levels of 25-hydroxyvitamin, PTH, ALP, P1NP, and 1CTP.

5.3.6 Histological findings in children after solid organ transplantation (IV)

Thin trabeculae and increased trabecular number were observed in 14 children (74%), i.e., increased trabecular separation was found in only one child (Table 13). Abnormal turnover findings were common in pediatric transplant patients: seven of the children (37%) had a high turnover whereas six children (32%) had a low turnover. Increased osteoid thickness (> +2.0 SD) was found in four children (21%) of whom one had received kidney and three liver transplants. Children with thick osteoid seams (O.Th > +3.0 SD, n=3) had normal labeling, i.e., no mineralizing defect nor any signs of osteomalacia were found. Two of the 18 children with appropriate tetracycline administration had no labels in the region of interest. In the extended label search the other patient had one subcortical double label and the histological classification remained as low bone turnover. The other patient had no labeling in the extended label search and the histological diagnosis was adynamic bone disease. In addition, in one child's labels were lacking because no tetracycline had been given prior the biopsy.

After excluding the children without labeling (n=3, extended label search), the dynamic bone histomorphometry parameters (MS/BS; MAR; BFR/BS, and Ac.F) tended to be low-normal; there were no significant differences between the different transplant groups. The different turnover rates were compared. Six children (32%) had normal turnover rate in the biopsy. These patients had a low lumbar BMD Z-score (median -2.3 SD, range -2.0 to -1.0 SD), and two of these children had low BV/TV (< -1.0 SD) by histomorphometry. Four of the

six children with normal turnover had vertebral compressions but none had sustained peripheral fractures.

Children with higher present GC dose (≥ 3mg/d) had lower Oc.S/BS and osteoid maturation time (Omt) than children with the lower GC dose (1-2 mg/d, $p<0.05$). No significant differences in absolute histomorphometry values were found between children with GC exposure exceeding 1000 days as compared to those with a shorter GC exposure. A negative correlation was found between the GC dose at the time of biopsy and Oc.S/BS Z-score (ρ=-0.55, p=0.016). The Oc.S/BS Z-score correlated positively with the time from transplantation to the biopsy (ρ=0.66, p=0.002) and lumbar BMD Z-score (ρ=0.47, p=0.043). CG exposure correlated negatively with lumbar BMD Z-score (ρ=-0.46, p=0.046).

No significant differences in any of the bone histomorphometry values (Z-scores) were detected between the subjects with normal and mild or moderate kidney insufficiency. Children with normal or mild kidney dysfunction were younger and more recently transplanted, had lower lumbar BMD Z-score, S-25-OHD, and P-Ca level, but possessed similar serum phosphate levels, as children with moderate kidney dysfunction ($p<0.05$). Children with compression fracture (n=11) had lower MS/BS, BFR/BS, and Ac.F than children without (n=8) vertebral compressions ($p<0.05$). The subjects with compression fractures also had higher present (median 2mg, range 2-5mg, p=0.022) and cumulative GC dose (4172mg, range 2188-10 133mg, p=0.023), and lower P-ALP (94 U/L, range 74-321 U/L, p=0.026) than those without compression fractures (present GC dose median 1.5 mg, range 1-4 mg; cumulative GC dose median 2080 mg, range 1095-3939 mg; and P-ALP 172 U/L, range 96-301 U/L).

Table 13. Bone histomorphometry findings in pediatric solid organ transplant recipients. Reference values for children were obtained from Glorieux et al. (2000), except for erosion surface (ES/BS, normal ≤10%). Table shows standard deviation Z-scores for each parameter. Trabecular bone volume (BV/TV) was considered normal when ≥1.0 SD.

				STRUCTURAL				FORMATION								RESORPTION		INTERPRETATION	
Patient #	Sex	Transplant organ	Age (yrs)	BV/TV	Tb.Th	Tb.N	Tb.Sp	OV/BV	OS/BS	O.Th	Ob.S/BS	MS/OS	BFR/BS	MAR	Ac.F	ES/BS (%)	Oc.S/BS	Bone volume	Turnover
1	M	Kidney	13.6	-0.7	-3.0	4.7	-2.2	0.0	-1.1	-0.4	-1.4	-3.0	-2.0	-4.3	-1.9	3.8	-0.7	Normal	Low TO
2	F	Kidney	14.7	0.6	-3.7	13.3	-3.9	3.6	0.6	1.1	-1.5	-1.5	-1.4	-2.1	-0.7	4.1	2.3	Normal	High TO
3	F	Kidney	17.1	0.9	-1.8	4.1	-2.2	-0.8	-2.2	3.8	-2.0	-3.1	-2.3	-3.4	-2.2	2.8	4.4	Normal	Low TO
4	M	Kidney	18.1	0.3	-2.6	5.1	-2.4	0.5	0.3	-1.1	-1.4	-3.9	-2.2	-2.1	-2.1	7.7	2.0	Normal	Normal
5	M	Kidney	18.2	-1.1	-2.3	1.7	-0.9	-0.1	-0.8	-0.7	-1.6	-1.1	-1.3	-2.4	-1.3	2.0	-1.0	Low	Normal
6	M	Kidney	18.8	1.0	-0.7	2.0	-1.5	2.2	1.5	0.7	3.3	-1.2	-0.3	-2.5	0.0	4.8	-0.2	Normal	High TO
7	M	Liver	14.1	-1.1	-2.2	1.1	-0.2	1.8	-0.5	2.9	2.9	-0.5	-1.2	-1.6	-0.7	4.6	2.0	Low	High TO
8	M	Liver	14.2	-3.7	-5.0	-1.9	3.5	3.3	-0.3	-1.6	-1.9	N/A	N/A	N/A	N/A	5.7	-1.5	Low	Low TO
9	F	Liver	14.8	-2.2	-3.1	-0.1	1.0	2.4	-0.8	4.6	-1.0	-0.5	-1.5	-1.5	-1.0	6.0	-0.5	Low	High TO
10	F	Liver	15.4	-0.1	-1.4	2.2	-1.2	-1.0	-1.5	0.4	-1.7	N/A**	N/A**	N/A**	N/A**	0.8	-1.5	Normal	Low TO*
11	M	Liver	15.5	0.2	-2.1	5.1	-2.3	0.5	-1.1	1.8	0.4	2.1	0.4	-0.1	0.6	5.4	-1.5	Normal	Normal
12	F	Liver	15.8	-0.5	-2.7	4.6	-1.9	1.9	-0.7	5.2	-0.8	-0.4	-1.3	-1.5	-0.8	3.5	1.1	Normal	High TO
13	F	Liver	16.9	0.1	-1.7	3.5	-1.8	-1.8	-2.6	-1.2	-1.8	2.2	-3.0	-4.2	-2.5	0.8	-0.9	Normal	Low TO
14	M	Liver	17.3	0.4	-1.6	2.7	-1.7	0.8	-0.3	1.7	1.4	2.7	1.1	-1.4	2.0	17.9	14.2	Normal	High TO
15	M	Liver	18.2	-0.3	-2.6	4.1	-2.0	0.1	-0.7	-0.6	-1.6	-3.2	-2.2	-4.2	-2.0	6.2	3.3	Normal	Normal
16	M	Heart	7.6	-1.2	-3.9	6.3	-2.6	-0.6	-1.5	0.2	-1.8	1.2	-1.4	1.4	-0.6	12.2	0.5	Low	Normal
17	F	Heart	10.4	0.9	-2.1	5.2	-2.7	0.5	-0.9	0.7	-1.2	-2.8	-1.7	-0.8	-1.8	5.3	1.5	Normal	Normal
18	F	Heart	11.7	1.9	-2.1	7.4	-3.3	2.7	1.2	0.7	2.0	0.2	0.2	-2.5	1.0	8.1	3.8	Normal	High TO
19	M	Heart	17.6	-1.1	-3.2	4.2	-1.9	-0.9	-1.9	-0.8	-2.0	N/A	N/A	N/A	N/A	4.8	-0.2	Low	Low TO - ABD
Mean			15.3	-0.3	-2.5	4.0	-1.6	0.8	-0.7	0.9	-0.6	-0.8	-1.2	-2.1	-0.9	5.6	1.4		
STD			2.9	1.3	1.0	3.2	1.6	1.6	1.1	2.0	1.7	2.1	1.1	1.5	1.3	4.0	3.6		
Median			15.5	-0.1	-2.3	4.1	-1.9	0.5	-0.8	0.7	-1.4	-0.8	-1.4	-2.1	-0.9	4.8	0.5		

yrs = years, M = male, F = female, N/A = not available, SD = standard deviation, ABD= adynamic bone disease. *A sub-cortical double label in the extended label search.

**No tetracycline labeling prior to biopsy, low turnover based on formation parameters. ***No tetracycline labels in the extended label search.

6 Discussion

In this study, the qualitative properties of bone were investigated using different techniques. Bone remodeling and mineralization were characterized using quantitative bone histomorphometry in certain cohorts of adult and pediatric patients. In specific cohorts, studies of the bone microarchitecture or composition were performed using micro-computed tomography or Fourier transform infrared imaging. Bone compositional changes in pediatric patients with vertebral fractures have not been reported previously, and thus, this study provides an insight into these changes. Bone histomorphometric findings in pediatric patients after solid organ transplantation have been described only in one previous report (Sanchez et al. 1998). That study reported findings in children after renal transplantation, whereas in this thesis, findings in pediatric patients after liver and heart transplantation are also presented.

6.1 DATA COLLECTION AND VALIDATION

In an attempt to decrease the relatively high inter-observer variations (de Vernejoul et al. 1981; Chavassieux et al. 1985; Compston et al. 1986; Wright et al. 1992), the samples were measured by the same observer in bone histomorphometry. Study II is one exception to this rule, since the mean value from two observers was used in the analysis.

This study combined different techniques to study bone quality and the invasive techniques were correlated with clinical characteristics. Furthermore, the PMMA embedded bone biopsies were suitable for all invasive techniques in this study. Based on the previous literature in FTIRI, the PMMA spectrum was subtracted from every bone spectrum to account for the differences in PMMA penetration into the trabeculae (Boskey and Mendelsohn 2005). In micro-CT, it is believed that embedding to PMMA had only insignificant effects on the micro-CT measurements. In fact, a previous study showed that the structural micro-CT parameters of the cancellous bone sample were not significantly affected by the surrounding medium (Perilli et al. 2007).

Bone histomorphometry has advanced our understanding of the cellular changes in bone diseases. For example, the rapid early bone loss related to GC-treatment and organ transplantation was detected by histological analysis instead of via biochemical markers of bone turnover (Vedi et al. 1999; Dalle Carbonare et al. 2005). Information about bone turnover and BMD may be acquired using serum bone turnover markers and densitometry. However, these methods cannot differentiate lamellar or woven bone structure, or detect a mineralizing defect or adynamic bone disease. Further, one cannot gather information about mechanisms involved in bone loss or gain (Compston 2004). In contrast, bone histomorphometry provides insight into these aspects.

Histomorphometry is a two-dimensional, invasive technique. It is time consuming and the technique requires high expertise and training (Compston 2004; Dempster et al. 2013). Nonetheless, many of the currently available techniques to measure bone quality, such as micro-CT or FTIRI, require an invasive bone biopsy. When studying bone microarchitecture, the three-dimensional approaches provide better resolution than two-dimensional bone histomorphometry (Compston 2004; Recker et al. 2011). When comparing the correlation of two repeated histomorphometry measurements on bone cell-counts, the correlation was lower among osteoporotic than among renal osteodystrophy samples. This may be explained by the lower number of bone cells in osteoporotic bone, and the consequently higher relative error of small miscalculations. The selection of the ROI is more critical in histomorphometry, since the

measured tissue area is smaller than the measured tissue volume in three-dimensional techniques. As regards to other limitations of bone histomorphometry, there may be considerable heterogeneity in bone remodeling and turnover between the different anatomical sites not only in diseased subjects but also in healthy individuals. In all of the studies, only iliac crest biopsies were investigated and the reference values for each sub-study were obtained from the same anatomic location (Recker et al. 1988; Glorieux et al. 2000). Histomorphometry cannot be repeated frequently for obvious ethical reasons but also because bone remodeling accelerates after the bone biopsy procedure. Thus, only one biopsy from each iliac crest can be used in one patient (Compston 2004).

The studies in this thesis are limited by their relatively low number of samples. In particular, the small size of the subgroups may have prevented from identifying other differences. Bone histomorphometry is a time-consuming technique and needs an invasive bone biopsy. Thus, the number of samples has been small also in previous studies (Compston et al. 1986; Jordan et al. 2003; Mäyränpää et al. 2011). Further, there was variation in the sample size due to sample collection and preparation problems. In each study, one to two sections were randomly selected for the histological analysis. Therefore, the results are based on a relatively small tissue area compared to three-dimensional techniques such as micro-CT. In Study I, micro-CT measurements were conducted after histomorphometry. Therefore, the exact same bone area could not be measured with both techniques. Additionally, mechanical testing of the samples would have improved the understanding of fracture resistance in these cohorts.

The limited data of biochemistry and bone densitometry in Study II was a shortcoming. Due to the retrospective nature of the data collection, it was not possible to collect further information at the time of the fracture. The BMD prior to the BP treatment was not available. Further, a relatively long time had elapsed since the fracture in one case of the patients with AFFs. Although one patient had fallen from a stack of wood that was 0.5 meters high, this was regarded as a low energy trauma, and she was included into the study because the other characteristics of AFF were present.

Ideally the histomorphometric findings should be compared with age- and gender-matched healthy control subjects whose biopsies are prepared and analyzed similarly to the patients' biopsies. In this study, this kind of control data was unavailable. The Finnish reference data includes only static histomorphometric values in adults, and therefore, was not suitable for this study (Hoikka and Arnala 1981). There is only a limited number of publications about histomorphometric normal values. In this study, the results were compared with the literature controls, using the normative data that have been published for both pediatric and adult patients (Recker et al. 1988; Glorieux et al. 2000). In Study II, a control group of patients with typical femoral fractures would have been of value. Access to a healthy control group for compositional bone variables would have improved Study III but unfortunately no such normative data in children is currently available. A positive control group might have improved the outcome.

The histological classifications used in this study differ somewhat from the previous studies because of the great variety of classifications (based on cut-off values and reference data) used earlier both in children and adults (Salusky et al. 1988; Hutchison et al. 1994; Alon 2001). We used age-specific reference data and standard deviations as cut-off limits which made it possible to have a classification subdivided by age groups. According to the recent recommendations for the classification of CKD in adults, specified criteria have been presented (Moe et al. 2006). However, osteoporosis, osteomalacia, CKD-MBD, and adynamic bone disease still remain poorly understood and inadequately defined in children. There is a need for accurate definitions for the histomorphometric diagnosis in pediatric patients.

6.2 ASSESSMENT OF BONE QUALITY

6.2.1 Bone histomorphometry

This study assessed histological findings in patients with AFFs and in children with suspected secondary osteoporosis after solid organ transplantation. The bone histomorphometry findings were heterogeneous and the clinical characteristics, e.g. biochemistry or densitometry, poorly predicted the histological findings.

It has previously been shown that fracture-prone children displayed heterogeneous findings by bone histomorphometry. The histomorphometric findings showed poor correlation with fracture history, serum bone turnover markers, and densitometry (Mäyränpää et al. 2011). In children with suspected secondary osteoporosis after organ transplantation, bone histomorphometry showed that abnormal bone biopsy findings in various parameters reflecting bone quantity, quality, or dynamics were common. All these children had been on GC treatment and were suspected to have secondary osteoporosis according to their fracture history and/or low BMD. The biochemical markers poorly predicted histological findings.

Only limited data of bone histomorphometry in patients with AFF is available. Among a few patients with AFFs, there have been reports of severely suppressed bone turnover rate both in the iliac crest and in the fracture site (Odvina et al. 2005; Shane et al. 2010). Bone histomorphometry revealed low cancellous bone volume in three of the four patients. Scarce tetracycline labeling was present as described previously (Odvina et al. 2005; Shane et al. 2010). However, similar findings have been observed even in untreated postmenopausal women (Whyte et al. 1982; Hauge et al. 1999). Due to existing labeling in the biopsy in ROI or outside it, mineralization although low does not seem to be totally absent. However, if only mineralization was low and bone formation was normal, thicker osteoid seams would follow, and this was not the case in our patients. This suggests that both low turnover and low mineralization may coexist in some of our cases. The low labeling by tetracycline without osteoid accumulation could be explained by the low bone formation rather than exclusively by bone mineralization defect.

Tetracycline labeling and related problems

The nomenclature, symbols, and units used in bone histomorphometry follow the standardized recommendations by ASBMR (Parfitt et al. 1987; Dempster et al. 2013). The anti-resorptive agents, such as BPs, have created new challenges for bone histomorphometry. The mechanism of these drugs may suppress bone resorption significantly leading to only a few or absent fluorochrome labels in the ROI. This may create difficulties in the interpretation in the dynamic indices and bone remodeling status (Recker et al. 2011).

Based on the recommendations, the variables describing bone remodeling were applied in this study. The mineralizing surface (MS/BS) is the best variable to describe the extent of remodeling surface in any sample. Based on MS/BS, other dynamic indices can be calculated. Mineral apposition rate (MAR) describes the rate at which the mineral accretion occurs during bone formation at the remodeling site. It is one of the fundamental parameters that reliably measure osteoblast function. In the case of scarce double labeling or even the absence of the double labels in the biopsy, MAR cannot be calculated, as was the case in some of the patients in this study. For reliable calculation of MAR, a minimum of two independent double labels including at least five measurements should be performed (Recker et al. 2011). In a human cohort, the lowest MAR reported has been 0.3 μm/day (Foldes et al. 1990). If MAR cannot be measured, this so-called 'imputed MAR' value could be used. Otherwise, the zero value of MAR should be reported as 'missing data'. Bone formation rate (BFR/BS) is a more complex variable describing the bone remodeling status. Activation frequency (Ac.F) is a further derived parameter that describes the probability (#/year) of the initiation of a new remodeling cycle at

any point on the trabecular surface. It represents the number of currently active BMUs. Ac.F is the best two-dimensional index currently available to measure the intensity of bone remodeling or turnover (Recker et al. 2011).

In cases of absent or insufficient double labels in ROI, a limited extended label search could be performed. The examined sections should be studied to identify labeling outside the ROI including cortical bone to assure that the patient has actually taken the fluorochrome labeling. A telephone contact to follow tetracycline labeling in research purposes is recommended. If labeling outside the ROI in the two examined sections (300 μm apart) is found, no further investigations are necessary. The absence of double labels in the standard ROI certainly indicates low bone remodeling (Recker et al. 2011). In this study, extended label search was carried out because of the possibility for oversuppression caused by BPs in patients with AFFs and to assure the extension of tetracycline labeling in the three children with absent labeling. In fact, one of these children had not taken the tetracycline tablets, and histological classification was made based on low static formation parameters. One patient had no trabecular labels but due to the subcortical double label and relatively high OV/BV, which were suggestive of the presence of osteoblast activity, this could not be classified as adynamic bone disease and the thin osteoid seams excluded the possibility of osteomalacia. The only patient with adynamic bone disease had no labels at all, low cancellous bone volume, generally low bone formation as well as low bone resorption.

Bone histomorphometry findings in children

There are no established cut-off values (e.g. SD-values) for the various histomorphometric parameters to define osteoporosis in children. In these studies, low cancellous bone volume was defined as a BV/TV Z-score below -1.0 compared to the age-matched reference values (Glorieux et al. 2000). Similar cut-off limits have been used before (Mäyränpää et al. 2011). In the histomorphometric evaluation, low cancellous bone volume was measured in 36% of the fracture-prone children with a vertebral fracture. However, low cancellous bone volume was also found in 20% of cases without vertebral fracture, and all these children had sustained peripheral fractures. In children with organ transplant, low cancellous bone volume was observed by histomorphometry in only one third (32%) of the patients whereas low lumbar BMD Z-score (below -2.0) was found in half (47%) of the children. Despite the normal cancellous bone volume, the trabecular architecture was often altered and was characterized by thin trabeculae and increased trabecular number. Since most of the patients had normal height and skeletal maturation, these changes are likely to reflect true alterations of bone quality having an adverse effect to bone strength and fracture resistance.

The bone turnover rate was abnormal in 64% of the fracture-prone children. Mäyränpää et al. has previously shown that vitamin D deficiency was associated with a high turnover in the biopsy in these children (Mäyränpää et al. 2011). Abnormal bone turnover findings were also frequent in children after a solid organ transplantation since only one third (32%) of the children had normal bone remodeling. A high turnover was present in 37% and low turnover in 32%, including one with an adynamic bone disease. The findings were heterogeneous, and the transplanted organ did not predict the histological findings. The prevalence of adynamic bone disease or mineralizing defect in children is highly dependent on the reference values being used, and the definitions vary greatly both in adult and pediatric patients (Salusky et al. 1988; Hutchison et al. 1994; Alon 2001; Edouard et al. 2011). The histological classification used in **Study III** (low, normal, or high turnover) was further improved in **Study IV** to also detect a mineralizing defect and adynamic bone disease. An abnormal turnover rate in combination with altered bone architecture rather than actual loss of cancellous bone volume might explain the increased fracture risk in children after solid organ transplant; true adynamic bone disease seems to be rare. In conclusion, there are no specific rules for classifying the turnover rates in children. There is a need for accurate definitions for the histomorphometric diagnosis in pediatric patients.

Complications after bone biopsy
The complication rate after the bone biopsy procedure in the current study was low. This concurs with previous studies (Duncan et al. 1981). The 4 patients assessed due to an AFF experienced no complications during or after the bone biopsy procedure. In the fracture-prone children, one superficial skin infection was reported, which was treated with oral antibiotics. In addition, one child suffered transient nerve symptoms during the operation (Mäyränpää 2012). In children after solid organ transplantation, one child felt prolonged pain at the biopsy site but no other complications were reported. All children received analgesics (usually a combination of paracetamol and ibuprofen) for three days post-operatively.

6.2.2 Micro-computed tomography (micro-CT)

Micro-CT enables relatively rapid analysis of bone microarchitecture in three-dimensions. Although invasive bone biopsy is needed for the analysis, the same PMMA embedded bone block used in bone histomorphometry can be used for the analysis. Some studies have shown highly significant correlations between bone histomorphometry and micro-CT for structural parameters (Uchiyama et al. 1997; Muller et al. 1998; Chappard et al. 2005) whereas in one other study only a weaker correlation was detected (Ito et al. 1998). In the present study, micro-CT showed slightly lower absolute values for BV/TV than those found with histomorphometry. The correlation between the techniques for these clinical bone samples, although significant, was weaker than has been described in the literature. The previous studies were conducted mostly on healthy normal and pathological human cancellous bone combined, whereas the present study also categorized the bone samples based on the metabolic status.

Micro-CT may be used to detect structural changes of the mineralized bone matrix. Micro-CT showed better intra-observer reproducibility of the structural parameters (Tb.Th and Tb.N) than histomorphometry measurements. The reproducibility was not affected by the health status of bone. Micro-CT was able to detect differences between the normal and OP, as well as between the OP and ROD groups, but it was less effective than bone histomorphometry in distinguishing renal osteodystrophy from normal samples. These results indicate that micro-CT can detect osteoid as bone tissue, i.e., separate osteoid from bone marrow with the used threshold levels but more studies will be needed to clarify its accuracy. The agreement between the techniques was highest in OP samples. Higher correlations are to be expected with a wider range of the measured variables. Therefore, the agreement between micro-CT and bone histomorphometry in OP patients might simply be a reflection of a greater exposure range in these patients compared to ROD or normal samples (Bland and Altman 1996). Micro-CT is a reproducible and effective tool for determining structural bone changes. Histomorphometry is still needed in clinical practice to study the remodeling balance in bone, and in fact, the methods are complementary in the assessment of bone quality.

6.2.3 Fourier transform infrared spectroscopic imaging (FTIRI)

FTIRI was used to assess the bone composition in children with suspected IOP with fractures and in patients with AFFs. In fracture-prone children, there was increased carbonate substitution in hydroxyapatite crystals with age. Fracture-prone children with a previous a vertebral fracture had a different bone composition than children without vertebral fractures. Children with vertebral fractures exhibited lower carbonate substitution, increased collagen cross-link ratio, and narrower heterogeneity of collagen cross-link ratio as compared to children without vertebral fractures. In the pooled sample set, the correlations between the biochemical and compositional bone parameters were mostly weak, showing only higher carbonate substitution in children with higher PTH levels. In patients with AFF, a higher degree of mineralization, increased collagen maturity, and decreased heterogeneity of the degree of mineralization were observed.

In this study, the mineral-to-matrix ratio did not change significantly with age although the age-dependent increases in the mineral content and in the mineral-to-matrix ratio have been described (Boskey and Pleshko Camacho 2007). The fracture history in all children included in this study might explain the findings. The younger children had lower carbonate-to-phosphate ratio than the older children, suggesting increased carbonate substitution in hydroxyapatite crystals with age. These findings are consistent with those reported in the literature (Paschalis et al. 1997; Gadeleta et al. 2000; Huang et al. 2003; Boskey et al. 2005; Faibish et al. 2006).

In the children with low cancellous bone volume in biopsy (BV/TV <-1.0 SD) the level of carbonate substitution in crystals was lower than in children with normal bone volume. Additionally, children with vertebral fracture had a lower level of carbonate substitution compared to children without vertebral fracture. Carbonate substitution in patients with AFFs was normal. The effect of the carbonate substitution on the biochemical and mechanical properties of bone is still poorly understood (Ruppel et al. 2006). Some studies have suggested that carbonate is an impurity, and therefore decreased substitution would be related to mature crystals. Others have suggested that carbonate substitution occurs randomly over time, and thus any increases in carbonate substitution would be related to increasing tissue age due to decreased turnover. Indeed, carbonate substitution has been shown to increase with age in rat and human bone (Akkus et al. 2004; Yerramshetty et al. 2006). Lower carbonate substitution may be linked to higher turnover rate or remodeling (Isaksson et al. 2010).

Previous studies have also indicated that collagen maturity increases with age (Paschalis et al. 1997; Huang et al. 2003; Boskey et al. 2005; Faibish et al. 2006). An increased collagen cross-link ratio and decreased heterogeneity of collagen cross-links were found in children with vertebral fractures, as compared to those without vertebral fractures. Similarly, an increased collagen cross-link ratio was found in patients with AFFs. Increased collagen maturity in cancellous bone has been shown to associate with an increased risk of fractures (Gourion-Arsiquaud et al. 2009). Increased collagen cross-link maturity has been found in osteoporotic bone (Paschalis et al. 2004) and in alendronate-treated postmenopausal women (Bala et al. 2012) whereas another study confirmed no difference in alendronate-treated women compared with non-treated controls (Boskey et al. 2009). BP-treated patients with femoral fractures have been postulated to exhibit a lower heterogeneity of collagen cross-links (Donnelly et al. 2012). Thus, altered collagen maturity or its more uniform distribution in the patients of this study may explain the greater propensity for developing fractures.

Lower heterogeneity of phosphate-to-amide I ratio was found among patients with AFFs and this coincides with previous findings among postmenopausal women treated with BPs (Boskey et al. 2009). The changes in the distribution of mineralization have been claimed to relate to the changes in bone remodeling, and therefore, caused by the antiresorptive therapy (Gourion-Arsiquaud et al. 2009; Donnelly et al. 2012). More homogenized mineral and collagen content of the bone may lead to more brittle bone (Gourion-Arsiquaud et al. 2009). Children with a vertebral fracture had also narrower distributions of collagen cross-links and carbonate-to-phosphate ratios. The more uniform bone composition found in the present study cohorts are in line with the previous studies and may explain the higher propensity to develop low energy or vertebral fractures.

6.3 CLINICAL CHARACTERISTICS

6.3.1 Atypical femoral fractures

Patients who had sustained AFFs in our healthcare district were studied. Eight patients with ten AFFs were identified among the patients older than 49 years treated for femoral fractures at Kuopio University Hospital during the time period from January 2007 to December 2009. Six of these patients were BP users. The annual incidence of these fractures in patients on BP in our

hospital's catchment area was estimated as 0.61/1000 patients. None of the patients suffered from diabetes or rheumatoid arthritis, and none of them were receiving GCs or proton pump inhibitors. The present results coincide with previous estimates as regards to the incidence of AFFs during the BP therapy. The method of our estimate is similar to Schilcher et al., who found the incidence of AFFs to be less than 1/1,000 among BP-treated patients in Sweden (Schilcher and Aspenberg 2009). In a secondary analysis of three large randomized trials, Black et al. proposed the incidence to be somewhat lower, 2.3/10,000 patient-years (Black et al. 2010). The patient material of those treatment-trials may differ from that found in everyday practice. Further, the radiographs were not reviewed. This may affect the estimate of incidence as it has been proposed that routine radiologic assessment does not detect AFFs sufficiently (Schilcher et al. 2011). In the report by Abrahamsen et al., the overall incidence of subtrochanteric femoral fractures was similar among alendronate users as among BP-naïve patients with hip fractures (Abrahamsen et al. 2009). It is concluded that the incidence of AFFs as the complication of the BP therapy seems to be very rare (Shane et al. 2010, Schilcher et al. 2011, Meier et al. 2012).

None of the patients in this study had malignancy. One patient was excluded from the study because of a malignant disease. One patient had suffered a pelvic fracture and bilateral diaphyseal femur fractures several years earlier. However, all metal implants had been removed years before she sustained an AFF.

Although the incidence of AFFs seems to remain low, these fractures have a specific morphology and occur especially as a result of low energy trauma. Bone formation was relatively low in 3 of the 4 patients, as judged by static bone histomorphometry parameters. This can be expected after BP treatment. Low mineralization was found due to the scarce labeling in the biopsy although double labels were not totally absent. The changes in bone composition, i.e., higher degree of mineralization, increased collagen maturity, and decreased heterogeneity of the degree of mineralization, could explain the atypically low fracture resistance among these patients. Although similar findings have been reported among non-BP-treated postmenopausal women, the patients with AFF most likely have some other concomitant factors that predispose to these fractures. More studies are needed to confirm these constituents, and to advance our understanding of AFFs.

6.3.2 Pediatric fracture patients

The diagnosis of osteoporosis in younger patients is often made based on low BMD and the presence of at least one fragility fracture (Ward and Glorieux 2003) which would be considered as a sign of osteoporosis in adults. Unfortunately, this means that the diagnosis is often made too late (Bianchi 2007). In a retrospective study of a fracture registry, the 5-year fracture risk increased markedly with a low BMD Z-score (below -2.0 SD) (Jones et al. 2006). Mäyränpää et al. has previously shown that a low lumbar BMD value was associated with high turnover in the biopsy in fracture-prone children (Mäyränpää et al. 2011). In the current study, normal BMD (within ± 2 SD) was found in 79% of 24 fracture-prone children. However, 92% of all fracture-prone children had sustained repeated fractures. Additionally, the lumbar BMD value was low (below -2 SD) for only four children who had sustained vertebral fracture. BMD as a fracture predictor in children has been somewhat controversial. Some studies propose that BMD alone seems not to be a good fracture predictor (National Institutes of Health Consensus 2001; Bianchi 2007) whereas others have found associations with increased fragility (Goulding et al. 2001, Goulding et al. 2005, Ferrari et al. 2006). Interestingly, no statistical differences in FTIRI parameters were observed between the fracture-prone children with low and normal BMD. However, this may be due to the small number of samples in each group. In addition, fractures were common in pediatric patients after solid organ transplantation; more than half (58%) had vertebral compression fractures and 21% had a history of peripheral fractures. A low BMD value was more often present among these children since 9 children (47%) had low lumbar BMD. This can be explained by GC use in all participants, i.e., high GC exposure was associated with a low lumbar BMD Z-score.

To avoid overestimation of BMD, bone age was adjusted in pediatric patients because delayed puberty and delayed skeletal maturity are common in children with chronic illnesses. Similarly, GC treatment could explain delayed bone age. In fracture-prone children, bone age was adjusted in 10 children; in four children it was delayed and in six children it was advanced for more than a year (Mäyränpää et al. 2011). Bone age was delayed over a year in six transplant children and these results were adjusted.

In the pediatric cohorts of this study, a vertebral fracture was defined as a loss of 20% or more in the vertebral height as used in previous studies (Genant et al. 1993; Mäkitie et al. 2005). Minor compressions from 5% to 20% have been measured in 70% of healthy children but no vertebral compression over 20% was found (Mäkitie et al. 2005). Spinal osteochondosis, i.e., Scheuermann's disease, is characterized by wedge-shaped vertebrae usually in the thoracic region of the spine in adolescents (Lowe et al. 2007; Masharawi et al. 2009). In this study, the timing of the injury could not been assessed by plain radiographs. It is not evident, whether the compressed vertebrae were caused by accidental crushing, growth disturbance, or normal variation. The preceding pain in younger children is not always reliably recorded. However, as shown previously, the loss of vertebral height over 20% was not found in healthy children (Mäkitie et al. 2005), and therefore, the reported findings in this study are likely to important.

The high-energy fractures in pediatric cohorts were justified as a falling height of 3 meters or more as well as all motor vehicle accidents (Landin 1983). Similar classification has been used in previous studies (Mäyränpää et al. 2010; Mäyränpää et al. 2012). In children, it is often quite complicated to find out the actual fracture energy. Therefore, low and moderate energy fractures were combined in this study.

The bone turnover markers were measured in pediatric fracture-prone patients. It has been shown in elderly women that bone turnover markers were affected by a recent fracture up to 12 months (Ivaska et al. 2007). Therefore, it remains unknown whether some differences in bone turnover markers are caused by recent trauma or whether they reflect true changes in bone turnover. In pediatric transplant recipients, the level of physical activity varied; some on them were very active but none of the children were so ill that their ability to exercise would have been limited because of their chronic illnesses.

6.3.3 Chronic kidney disease

Solid organ transplantation may lead to deterioration of kidney function. As one half of the children had normal or mild kidney insufficiency and the others had moderate (stage 3 CKD) disease, the role of CKD-MBD in skeletal pathology is likely to be less significant in this group compared to those with more severe kidney failure. Recent studies have indicated that alterations in skeletal mineralization and mineral homeostasis may be present already in the very early stages of CKD (Sabbagh et al. 2012; Wesseling-Perry et al. 2012). This study found no differences in histomorphometry between those children with mild or moderate kidney insufficiency. Although high turnover in the biopsy was quite common (37%), only one of these children had a mildly elevated P-PTH level and all had good P-Ca control. The other mildly elevated P-PTH level was found in a child with low turnover in the biopsy. Therefore, the biochemical markers do not seem to reliably reflect the bone turnover. All patients in this study were given vitamin D substitution and all but one had normal vitamin D status (Study IV). Hypovitaminosis D does not seem to be a major contributing factor in post-transplantation bone fragility.

6.3.4 Therapeutic agents

BPs are commonly used in the treatment of osteoporosis in adults. In selected cases, BPs can also be used in pediatric patients. BPs should, however, be avoided in osteomalacia and adynamic bone disease, both of which are relatively rare and can be ruled out by histomorphometry. This implies in this study that antiresorptive medication may be used in

selected pediatric patients for osteoporosis when there is a suspicion of primary osteoporosis or after transplantation.

The prevalence of osteoporosis in children after kidney transplantation despite the continuous GC treatment has been lower than expected (Valta et al. 2009), and the present findings support this result since only one third (32%) of the children had histologically confirmed low cancellous bone volume. It is known that GCs are a risk factor for adynamic bone disease (Cueto-Manzano et al. 2003; Freundlich et al. 2004). However, only one patient in this study was suffering from adynamic bone disease. Children after transplantation with vertebral compressions had higher present and cumulative GC doses than those without vertebral compressions. Similarly, high GC exposure associated with low lumbar BMD Z-score. Although extensive GC exposure did not result in reduced total bone volume or adynamic bone disease, GCs seems to have a role in the development of post-transplantation bone fragility.

6.4 FUTURE CONSIDERATIONS

In this study, bone histomorphometry and other sophisticated methods were applied to study bone quality. In patients with metabolic bone diseases such as osteoporosis, the definite analysis of bone remodeling is currently possible by bone histomorphometry. Serum biochemical markers and bone densitometry represent non-invasive approaches which can be utilized to assess bone health. Especially in clinically challenging scenarios where different treatment options are being considered, bone histomorphometry provides valuable information about bone metabolism. The other methods, such as micro-CT or FTIRI, are complementary and currently used in research. Based on this study, however, further studies in larger cohorts and with prospective follow-up will be needed to address the long-term consequences and clinical implications of the histological abnormalities in patients with AFFs, in fracture-prone pediatric patients, or in children after solid organ transplantation. Further, FTIRI reference values for healthy children should be established to enable proper correlations for findings in pediatric patients with suspected metabolic bone diseases. Thus, bone histomorphometry remains a valuable tool for studying histological bone changes and will remain such until an accurate non-invasive technique is developed.

7 Conclusions

Bone properties in various cohorts were measured using different techniques. The methods were complementary. Until progress is made in developing novel non-invasive techniques to quantify bone quality, the invasive methods including bone histomorphometry will remain important in characterizing the cellular changes in osteoporosis and other metabolic bone diseases.

The specific conclusions of the study were:

1. In this study, the agreement between bone histomorphometry and micro-CT using clinical bone samples was moderate. The reproducibility was not affected by the health status of bone. Micro-CT is a reproducible and effective tool for determining structural bone changes. However, it was less effective than bone histomorphometry in differentiating renal osteodystrophy from normal samples although osteoporotic changes in bone could be successfully detected. Similarly, the agreement between the techniques was better for OP than for ROD or normal samples. These results suggest that micro-CT can detect osteoid as bone tissue, i.e., separating osteoid from bone marrow with the threshold levels in use but more studies will be needed to clarify its accuracy. Thus, histomorphometry is still needed in clinical practice to study the remodeling balance in bone, and the methods are complementary.

2. The incidence of AFFs seems to be low. These fractures have a specific morphology and occur especially as a result of low energy trauma. Bone formation was relatively low in three of the four patients, as judged by static bone histomorphometry parameters. A low mineral apposition rate was found due to scarce labeling in the biopsy although double labels were not totally absent. The changes in bone composition, i.e., higher degree of mineralization, increased collagen maturity, and more uniform degree of mineralization, could explain the atypically low fracture resistance among these patients. Although similar findings have been reported among non-BP-treated postmenopausal women, the patients with AFF most likely have some other concomitant factors (e.g. different response to antiresorptive treatment) that predispose them to these fractures. More studies will be required to confirm these constituents, and to enhance our understanding of AFFs.

3. An attempt was made to determine whether bone composition differs between the fracture-prone children with and without vertebral fractures. The definition of osteoporosis among children and adolescents is complex and the changes in bone quality should be detected in the early stages to prevent fractures. The observed changes in bone composition, i.e., lower carbonate-to-phosphate ratio and increased collagen maturity in children with vertebral fractures might contribute to their greater propensity to sustain these fractures.

4. The bone histomorphometric findings in children who had undergone kidney, liver, or heart transplantation were characterized. Histologically reduced cancellous bone volume was found in one third (32%) of the patients. In addition, several other abnormal findings related to bone microarchitecture and turnover were observed in these children despite their relatively good kidney function and controlled P-PTH levels. The observed changes in bone quality (i.e., abnormal turnover rate and thin trabeculae) rather than the actual

loss of cancellous bone, might explain the increased bone fragility in pediatric solid organ transplant recipients. There was extensive heterogeneity in the histological findings in different transplant groups, and the results were not predictable by non-invasive methods. The degree of kidney insufficiency did not explain this variability but it is possible that some of the skeletal changes were caused by mild to moderate CKD. Thus, bone histomorphometry can provide important information in clinical practice with which to characterize the skeletal status in children after solid organ transplantation. However, further studies in larger cohorts and with prospective follow-up are needed to define the long-term consequences and clinical significance of these histomorphometric abnormalities.

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Inari Tamminen

Assessment of Bone Quality in Pediatric and Adult Patients with Osteoporosis

Dual-energy X-ray absorptiometry (DXA) is commonly used to measure bone mineral density (BMD) and to diagnose osteoporosis. However, BMD accounts for only 60% of bone strength, i.e., changes in bone quality as well as density may increase the fracture risk. This study aimed to characterize bone quality in different cohorts of patients using bone histomorphometry, microcomputed tomography (micro-CT), and Fourier transform infrared spectroscopic imaging (FTIRI).

UNIVERSITY OF EASTERN FINLAND

Publications of the University of Eastern Finland

Dissertations in Health Sciences

ISBN 978-952-61-1143-8