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BONE HEALTH, OSTEOPOROSIS AND FRACTURE RISK IN NEUROFIBROMATOSIS 1 – AN EMPHASIS ON OSTEOCLASTS

by

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ABSTRACT

Neurofibromatosis 1 (NF1) is an autosomal dominant hereditary syndrome, affecting skin, neural tissues and skeleton. Hallmarks of NF1 include benign cutaneous neurofibroma tumors, pigmentation lesions on the skin and in the iris, learning disabilities and predisposition to selected malignancies. Low bone mineral density (BMD) and osteopenia/osteoporosis are common in NF1.

Osteoporosis is a systemic disorder characterized by low bone mineral density and increased fracture risk. Treatment of osteoporosis aims to prevent falls and decrease fracture risk. Osteoporosis is diagnosed in adults by measuring BMD and evaluating clinical risk factors of the patient.

Bone turnover is a process of old bone resorbed by osteoclasts and new bone formed by osteoblasts. Multinuclear osteoclasts are derived from osteoclast progenitors, which can be isolated from peripheral blood. Osteoclast progenitors were isolated from 17 NF1 patients and healthy controls, and cultured *in vitro* to osteoclasts. NF1 osteoclasts are hyperactive, displaying increased differentiation and resorption capacity, abnormal morphology and tolerance to serum deprivation compared to control osteoclasts. These findings expanded the study to evaluate the effects of bisphosphonates, drugs designed to treat osteoporosis, in osteoclasts derived from blood samples of 20 NF1 and control persons. The number of control osteoclasts was expectedly reduced after bisphosphonate treatment. However, NF1 osteoclasts tolerated the apoptotic effect of alendronate, zoledronic acid and clodronate *in vitro* compared to controls.

NF1-related osteoporosis was found in ~20 % of the patients, and selected laboratory parameters were measured. Patients with NF1 have increased levels of serum CTX and PINP, reflecting increased bone turnover *in vivo*.

BMD decreases progressively in NF1 as evaluated in 19 NF1 patients 12 years after their initial BMD measurement. Patients with NF1-related osteopenia often progress to osteoporosis. This was found in patients aged 37-76.

Fracture risk in NF1 was evaluated in a controlled register-based study of 460 NF1 patients and 3988 control persons. Patients with NF1 aged ~40 or older have x5.2 risk ratio for fractures compared to controls, and NF1 children have x3.4 risk ratio compared to control children. NF1-related osteoporosis increased the fracture risk even further.

Keywords: Neurofibromatosis, osteoclast, bone turnover, bone mineral density, fracture

Eetu Heervä

LUUN TERVEYS, OSTEOPOROOSI JA MURTUMARISKI NEUROFIBROMATOOSI 1:SSÄ – ERITYISHUOMIO OSTEOKLASTEISSA

Biolääketieteen laitos, Solubiologia ja Anatomia, Turun Yliopisto
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YHTEENVETO

Neurofibromatoosi 1 (NF1) on vallitsevasti periytyvä oireyhtymä, joka vaikuttaa ihoon, hermostoon ja luustoon. Tyypillistä NF1 oireyhtymälle ovat hyvänlaatuiset ihon neurofibroomat, ihon ja silmän pigmenttimuutokset, sekä oppimisvaikeudet. NF1 altistaa tietyille pahanlaatuisille kasvaimille. Alentunut luuntiheys ja osteopenia/osteoporoosi (luukato) ovat yleisiä NF1:ssä.

Osteoporoosi on laaja-alainen luun häiriö, jossa on tyypillisesti matala luuntiheys ja suurentunut murtumariski. Osteoporoosin hoito on suunniteltu ehkäisemään kaatumista ja vähentämään murtumariskiä. Osteoporoosi todetaan aikuisilla mittaamalla luuntiheys ja arvioimalla kliinisiä riskitekijöitä murtumille.

Luun aineenvaihdunnassa osteoklastit eli luunsyöjäsolut hajottavat vanhaa luuta, ja osteoblastit eli luunrakentajasolut rakentavat uutta luuta. Monitumainen osteoklasti muodostuu osteoklastin esiastesoluista, joita voidaan eristää laskimoverinäytteestä. 17 NF1-potilaan sekä terveiden kontrollihenkilöiden verestä eristettyjä osteoklastin esiasteita viljeltiin soluviljelmässä osteoklasteiksi. NF1 osteoklastit ovat yliaktiivisia. Niillä on kiihtynyt muodostumisnopeus, epänormaali ulkomuoto ja ne hajottavat enemmän luuta verrattuna kontrollien osteoklasteihin. Lisäksi NF1 osteoklastit selviytyvät ilman seerumia. Näiden löydösten perusteella tutkimus laajeni arvioimaan bisfosfonaattien (osteoporoosilääke) tehoa NF1 osteoklasteissa, jotka eristettiin 20:stä NF1-potilaasta ja kontrollihenkilöstä. Kontrollien osteoklastien lukumäärä laski odotetusti bisfosfonaatti-käsittelyn jälkeen. NF1 osteoklastit sietivät soluviljelmässä alendronaatin, clodronaatin ja tsoledronaatin vaikutuksia kontrolleja paremmin.

Tässä tutkimuksessa osteoporoosi todettiin 20 %:lla NF1-potilaista, ja lisäksi näistä potilaista otettiin verikokeita. NF1 potilailla on korkeammat seerumin CTX ja PINP tasot verrattuna kontrolleihin, mikä viittaa nopeutuneeseen luun aineenvaihduntaan.

Luuntiheys laskee etenevästi NF1:ssä, kuten osoitimme 19 potilaalla, joiden luuntiheys mitattiin uudelleen 12 vuotta alkuperäisen mittauksen jälkeen. NF1-potilailla, joilla oli aikaisemmin osteopenia, olivat usein edenneet nyt osteoporoosiin saakka. Tätä havaittiin 37-76 vuoden ikäisissä potilaissa.

Murtumariskiä NF1:ssä arvioitiin kontrolloidussa rekisteritutkimuksessa, jossa oli 460 NF1-potilasta sekä 3988 kontrollihenkeä. NF1-potilailla jotka olivat 41-vuotiaita tai vanhempia, oli viisinkertainen riski saada luunmurtuma kontrolleihin verrattuna. NF1-lapsilla vastaava riski oli kolminkertainen. NF1-osteoporoosi suurensi vielä tätä riskiä.

Avainsanat: Neurofibromatoosi, Osteoklasti, Luun aineenvaihdunta, Luuntiheys, Murtuma

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ABBREVIATIONS

BMD	Bone mineral density
BMP	Bone morphogenetic protein
BP	Bisphosphonate
BTM	Bone turnover marker
CTX	Collagen type I C-terminal telopeptide
DXA	Dual energy X-ray absorptiometry
FTS	Farnesyl thiosalisylic acid
GAP	GTPase activating protein
HRT	Hormone replacement therapy
ICTP	Collagen type I telopeptide
IL	Interleukine
MCSF	Macrophage colony stimulating factor
MPNST	Malignant peripheral nerve sheath tumor
NF1	Neurofibromatosis type 1
NIH	National Institute of Health
NNT	Number needed to treat
NTX	Collagen type I N-terminal telopeptide
OPG	Osteoprotegrin
PA	Pseudarthrosis
PINP	Procollagen type I N-terminal propeptide
PKC	Protein kinase C
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa-beta
RANKL	Receptor activator of nuclear factor kappa-beta ligand (RANK-ligand)
SERM	Selective estrogen receptor modulator
TNF	Tumor necrosis factor
TRACP5b	Tartrate-resistant acid phosphatase 5b
WHO	World Health Organisation

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following communications, which are referred in the text by roman numerals I-V:

I Heervä E, Visnapuu V, Peltonen S, Pirttiniemi P, Happonen RP, Peltonen J: Short mandible, maxilla, and cranial base are common in neurofibromatosis type 1. *Eur J Oral Sci*. 2011. 119(2):121-7.

II Heervä E, Alanne M, Kuorilehto T, Peltonen S, Hentunen T, Väänänen K, Peltonen J: Osteoclasts in neurofibromatosis type 1 display enhanced resorption capacity, aberrant morphology, and resistance to serum deprivation. *Bone*. 2010. 47(3):583-90.

III Heervä E, Peltonen S, Svedström E, Aro HT, Väänänen K, Peltonen J. Osteoclasts derived from patients with neurofibromatosis 1 (NF1) display insensitivity to bisphosphonates in vitro. *Bone.* 2012. 50:798-803.

IV Heervä E, Leinonen P, Kuorilehto T, Peltonen S, Pöyhönen M, Väänänen K, Peltonen J. Bone mineral density remeasured in 19 neurofibromatosis 1 patients after 12 years: osteopenia often progresses to osteoporosis. Submitted manuscript.

V Heervä E, Koffert A, Jokinen E, Kuorilehto T, Peltonen S, Aro HT, Peltonen J. A controlled register based study of 460 neurofibromatosis 1 (NF1) patients: Increased fracture risk in children and adults over 41 years. Submitted manuscript.

1 INTRODUCTION

Neurofibromatosis type 1 (NF1) or von Recklinghausen's disease is a genetic syndrome affecting skin, neural tissues and skeleton. The incidence of NF1 is ~1/3000 and it is inherited in an autosomal dominant trait with variable phenotypic expression. The protein encoded by the *NF1* gene, neurofibromin, is a tumor suppressor protein that acts as Ras-GAP, thus inactivating Ras. In other words, neurofibromin deficiency may result in hyperactive Ras pathway.

Approximately half of the patients with NF1 have some form of skeletal disorder, either systemic or focal. Focal skeletal disorders include sphenoid wing dysplasia, lytic bone lesions, dystrophic scoliosis, and congenital bowing and pseudarthrosis of the tibia. Low bone mineral density (BMD) and osteopenia/osteoporosis are frequent findings of the NF1-syndrome.

Bone turnover is a process of bone being resorbed by osteoclasts, and new bone formed by osteoblasts. An imbalance in bone turnover may cause reduced bone mineral density and subsequent osteoporosis. Clinically osteoporosis is diagnosed by dual energy X-ray absorptiometry. Osteoporosis is a risk factor for fractures, but other factors such as risk to fall are equally important. Thus osteoporotic patients with high fracture risk profile may be considered for target population for medical intervention.

Bisphosphonates are drugs that are used to treat osteoporosis. These drugs bind to bone and are subsequently incorporated into bone-resorbing osteoclasts. This initiates a Ras-mediated molecular cascade leading ultimately to death of osteoclasts and slower bone remodeling. This in turn reduces the increased risk of fracture associated with osteoporosis.

The aim of this this study was to characterize bone health in NF1 and NF1-related osteoporosis. Specifically, the role of bone resorbing osteoclasts in NF1-related osteoporosis was poorly understood. In addition, low BMD in NF1 does not necessarily influence the fracture risk. It was also not known whether the frequency of NF1-related osteoporosis is age-dependent or not.

2 REVIEW OF THE LITERATURE

2.1 NEUROFIBROMATOSIS 1

2.1.1 Diagnosis of NF1

The clinical diagnosis of NF1 is most often based on café-au-lait macules and cutaneous neurofibromas. Mutation in the *NF1* gene has also been proposed as an absolute criterion for NF1 diagnosis [Huson 2008]. However, the diagnosis of NF1 may be complex in some patients. The NIH diagnostic criteria for NF1 are met, when two of the following are present [Stumpf et al. 1988, Gutmann et al. 1997, Williams 2009, Stevenson et al. 2011b]

- Six or more café-au-lait macules, diameter 5 mm before puberty, 15 mm after puberty
- Two or more neurofibromas, or one plexiform neurofibroma
- Freckling in inguinal or axillary region
- Optic glioma
- Two or more Lisch nodules in the eyes
- Skeletal abnormality typical for NF1, such as long bone dysplasia, pseudarthrosis, bone cysts, skoliosis, pectus excavatum or sphenoid wing dysplasia
- A close relative has NF1 (siblings, parents, or children)

2.1.2 Genetic background of neurofibromatosis 1

Neurofibromatosis type 1 (NF1) is a hereditary syndrome affecting skin, neural tissues and skeleton. NF1 is also known as von Recklinghausen's syndrome or disease, or peripheral neurofibromatosis. The hallmarks of NF1 include neurofibroma tumors, pigmented café-au-lait patches on the skin, osseous malformations, learning defects and predisposition to selected malignancies, such as malignant peripheral nerve sheath tumors [Riccardi et al. 1986, Huson 2008, Stumpf et al. 1988]. The incidence of NF1 is approximately 1/2600 – 1/3000 regardless of ethnical or geographical distribution [Friedman et al. 1997, Huson 2008, Jouhilahti et al. 2011a].

Approximately half of the patients with NF1 have negative familial background. In their case neurofibromatosis results from *de novo* mutation. Thus, the rate of mutations in the *NF1* gene is relatively high. In many countries, NF1 is considered as a rare syndrome, but the incidence makes NF1 "a common rare syndrome" [Gutmann et al. 1997, Jett et al. 2010].

Mutation in the *NF1* gene has 100 % penetrance in adults, but the severity of the NF1 syndrome is highly variable between the patients. Most of the mutations are

point mutations along the *NF1* gene. See also NF1 microdeletion (below) [Friedman et al. 1997, Riccardi et al. 1986, Szudek et al. 2003, Lammert et al. 2005a, Jouhilahti et al. 2011a]. NF1 is also considered as phakomatosis, a pathosis of neural crest cells (neurocristopathy), since the pathogenic cell types of neurofibromas and café-au-lait macules are derived from the neural crest cells [Ismat et al. 2006].

All cells in NF1 patients have $NF1^{+/-}$ genotype carrying inherited or sporadic mutation of the *NF1* gene. However, biallelic inactivation of the *NF1* gene has been shown in Schwann cells of neurofibromas, melanocytes of café-au-lait macules, tissues derived from some cases of congenital pseudarthrosis of tibia, and in certain MPNST, pheochromocytomas, astrocytomas, and in juvenile myelomonocytic leukemia. The "second hit" is speculated to be a result of either another *de novo* mutation in the originally healthy allele, or the loss of the healthy allele, defined as the loss of heterozygosity [DeRaedt et al. 2006, DeRaedt et al. 2008, Jouhilahti et al. 2011a].

The human *NF1* gene is located on chromosome 17q11.2, contains 61 exons, and codes for a tumor suppressor protein neurofibromin. Neurofibromin contains 2812 amino acids and includes a GTPase activating protein (GAP) domain, which converts active Ras-GTP to inactive Ras-GDP. In other words, NF1 protein is a Ras-GAP [Cawthon et al. 1990, Xu et al. 1990, Marchuk et al. 1991].

The Ras-signaling mechanism is described in **Figure 1.** Neurofibromin (NF1-protein) acts as a Ras-GTPase activating protein (GAP) inactivating Ras by enhancing the intrinsic GTPase activity of Ras. This leads to the switch of active Ras-GTP to inactive Ras-GDP. Consequently, NF1 functions as negative regulator of Ras signaling and cell growth [Binder 2009].

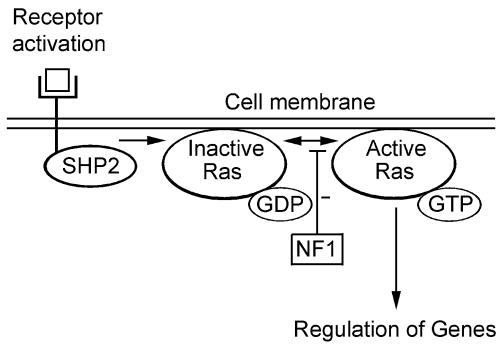

Figure 1. Activation of cell membrane receptor results in guanine nucleotide exchange, converting Ras-GDP to Ras-GTP, via SHP2 signaling. This activates molecular signaling leading to regulation of genes. Neurofibromin (NF1) inactivates Ras-GTP by enhancing its intrinsic GTPase activity [Modified from Binder 2009].

Three Ras isoforms are operative in man; H-Ras, N-Ras and K-Ras [Barbacid 1987]. Ras is a membrane bound G-protein which works as "a molecular switch" for cell signaling, transmitting signals from cell membrane to the nucleus [Valencia et al. 1991,

Adjei 2001]. All Ras proteins have a farnesyl group covalently attached to them, which allows the protein to anchor to the cell membrane. Prenylation or farnesylation means the process of covalent addition of either farnesyl-moeity (15-carbon) or geranyl-moeity (20-carbon) [Glomset et al. 1994].

The principal downstream signaling pathways of Ras include Raf/Mek/Erk and PI3K/Akt/mTOR, as shown in **Figure 2** [Le and Parada 2007, Binder 2009, Zhang et al. 2011]. Because these pathways are complex, they are only briefly discussed here. Calcium mediated protein kinase C (PKC) and NFAT pathways are also included because they may partially affect Ras.

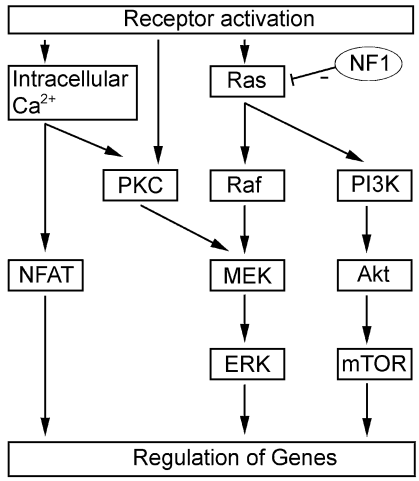

Figure 2. Selected signaling pathways relevant to the current study [Modified from Tiedemann et al. 2009, Zhang et al. 2011].

The pathways shown in **Figure 2** can be manipulated by selected pharmaceutical molecules designed to inhibit certain molecules, as described in **Table 1** [Tiedemann et al. 2009]. This may be important in NF1 as preclinical studies have shown that inhibition of Ras farnesylation may be used to treat defects in NF1-cells. Farnesylthiosalisylate (Salirasib ®, FTS) inhibits farnesylation of Ras, leading to dislodgement of all Ras isoforms from cell membrane. FTS has been shown to inhibit growth of NF1-associated MPNST cell lines, reducing their Ras activity and actin cytoskeleton to similar phenotype as seen in control non-NF1 cells [Barkan et al. 2006, Adjei 2001].

The upstream effector of Ras farnesylation is the mevalonate pathway. This pathway is regulated by enzyme hydroxymethylglutaryl-coenzyme A reductase, an enzyme in cholesterol synthesis. This enzyme can be inhibited by statins, widely-used medications for hyperlipidemia. Thus, it has been speculated that statins could inhibit farnesyl biosynthesis, leading to reduced Ras signaling, and thus could be used to treat symptoms of NF1 [Grasser et al. 2003, Kolanczyk et al. 2008].

Table 1. Selected inhibitors of cell signaling associated with Ras

Target	Molecule	Mechanism
Ras	Farnesylthiosalisylate (FTS)	Inhibition of farnesylation
Protein kinase C (PKC)	GÖ6976	Specific inhibitor
mTOR	Rapamycin	Specific inhibitor
Farnesyl biosynthesis	Statins	Reduced farnesyl biosynthesis

In addition to Ras there are other small GTPases, including the Rho-family GTPases. These include Rho, Rac and Cdc42 GTPases [Jaffe et al. 2005]. In rats, Rac has been shown to associate with Ras at least to some extent [Sun et al. 2005].

Rasopathies: Syndromes interfering with the Ras-pathway

Mutations in the Ras-pathway may result in several hereditary syndromes other than NF1. These include Noonan, Costello, LEOPARD and cardio-facio-cutaneous syndromes, which all are important in differential diagnosis of NF1. The detailed information about the genetic background of these syndromes is reviewed by Tidyman and Rauen [Tidyman et al. 2009] and is briefly summarized in **Table 2**. Ras-pathway can be inhibited by Spred-proteins. Therefore, genetic disruption of Spred leads to hyperactivation of Ras-pathway. In humans, mutation of the *Spred-1* gene causes the Legius-syndrome with NF1-like phenotype but without neurofibromas [Brems et al. 2007, Spurlock et al. 2009, Pasmant et al. 2009]. All these syndromes may include learning difficulties, cardiac defects, skin abnormalities, short stature and facial dysmorphism [Schubbert et al. 2007, Aoki et al. 2008, Pierpont et al. 2010, Jouhilahti et al. 2011a, Stevenson et al. 2011b]. Molecular diagnostics are available for differential diagnosis of these syndromes [Tidyman et al. 2009]. Since the focus of this thesis is in bone, the typical orthopedic manifestations of these syndromes are listed in **Table 2.**

Table 2. Syndromes of the Ras-pathway and associated orthopaedic manifestations [Tidyman et al. 2009, Stevenson et al. 2011B]. See **Figures 1 and 2** for details of the Ras-pathway.

Syndrome	Defective interaction located in	Selected orthopedic manifestations
NF1	Neurofibromin	short stature, low BMD, NF1-specific bone lesions (see 2.1.5)
LEOPARD	Raf and SHP2	short stature, chest wall abnormalities
Noonan	Ras, Raf and SHP2	short stature, scoliosis, chest wall abnormalities
Costello	Ras	short stature, scoliosis, chest wall abnormalities, reduced BMD
Cardio-facio-cutaneous	Ras, Raf and MEK	short stature, scoliosis, chest wall abnormalities, joint contractures
NF1-like (Legius)	SPRED1 (Ras inhibitor)	short stature, chest wall abnormalities

NF1 microdeletion

Approximately 5-10 % of NF1 patients have NF1 microdeletion, which means deletion of the entire *NF1* gene and variable number of flanking genes. The diagnosis of NF1 microdeletion can be verified by mutation analysis or multiplex ligation polymerase assay [DeRaedt et al. 2006, Pasmant et al. 2010]. NF1 microdeletion patients tend to have more severe clinical phenotype than the other NF1 patients. The NF1 microdeletion patients are prone to have high tumor burden, facial dysmorphism, learning disabilities, low intelligence quotient, cardiovascular malformations and tall stature (on contrary to short stature in NF1) [Pasmant et al. 2010].

2.1.3 Tumors in NF1

Neurofibromas

Neurofibromas can be divided into cutaneous, subcutaneous and plexiform neurofibromas based on their location in the body. Cutaneous neurofibromas are benign tumors traditionally considered to arise from the nerve sheaths and can be found nearly in all adult patients with NF1. Neurofibromas contain multiple cell types typical for peripheral nerves, but they are organized in a haphazard manner. These cells include Schwann cells, fibroblasts, mast cells, endothelial cells, perineurial cells, macrophages and axons. In addition, neurofibromas include cells which have multipotent ability to form Schwann cells, neurons, epithelial cells, and adipocytes *in vitro*. It is speculated whether these multipotent cells are derived from the cells of the hair root. The cell matrix of neurofibroma mainly consists of collagen [Peltonen et al. 1983, Riccardi et al. 1986, Jouhilahti et al. 2011a, Jouhilahti et al. 2011b]. Biallelic inactivation of both *NF1* genes has been demonstrated in Schwann cells cultured from neurofibroma tumors [Serra et al. 1997, DeRaedt et al. 2006].

Plexiform neurofibromas are well-vascularized, associated with nerve trunks or nerve plexuses, and harbor apparently the same cells as non-plexiform neurofibromas. Plexiform neurofibromas may cause severe neurological and cosmetic lesions according to their size and/or anatomical location. Plexiform neurofibromas may also progress into malignant peripheral nerve sheath tumors (MPNST) [Jett et al. 2010, Mautner et al. 2008, Jouhilahti et al. 2011a]. Plexiform neurofibromas are found in ~25 % of NF1 patients [Friedman et al. 1997].

MPNST

MPNST stands for malignant peripheral nerve sheath tumor, also referred to sarcomas of the peripheral nerve, neurofibrosarcomas or malignant schwannomas. Even though MPNST can be diagnosed in patients without NF1, NF1 is a marked risk factor for development of MPNST. In general population, the average age of MPNST diagnosis is 60 years, while the respective age in patients with NF1 is 20 years. Patients with NF1 have 8-13 % lifetime risk of developing MPNST [Evans et al. 2002]. MPNST

is usually derived from pre-existing plexiform neurofibroma, but only approximately 2-5 % of plexiform neurofibromas develop into MPNST [Ducatman et al. 1986, Evans et al. 2002]. Whole-body MRI is an effective method of detecting plexiform neurofibromas and MPNST, as they can be found in any anatomical location. However, positron emission tomography may be required to distinguish MPNST from plexiform neurofibroma [Ferner et al. 2007, Mautner et al. 2008]. MPNST has a poor prognosis and may metastasize. Histologically, MPNST resembles cell-rich sarcomatous tumor [Mautner et al. 2008, Upadhyaya et al. 2008].

Optic glioma

Optic glioma is classified as gradus I pilocytic astrocytoma according to WHO. Optic glioma is the most common type of brain tumor in children with NF1, and they appear before the age of 7 years. However, the optic glioma may remain asymptomatic and thus may be found in patients with NF1 at any age. Optic glioma is found in 15-20 % of the patients with NF1, but most are asymptomatic and remain so thorough the life. Symptomatic ones are found in ~4 % of NF1 patients. Children diagnosed with optic glioma may present with vision loss, disturbances in color vision and function of pupilla, and proptosis. In addition, optic gliomas may cause precocious puberty due to local pressure to pituitary gland [Listernick et al. 1989, 1997, 2007, Friedman et al. 1997].

The natural history and treatment of NF1-associated optic glioma is different from that of sporadic one in individuals without NF1 [Listernick et al. 2007]. Patients with NF1 who have received radiotherapy for optic gliomas are at significant risk of developing other central nervous system tumors later in their life. These tumors usually are malignant. Thus, radiation therapy is not recommended for NF1-related optic gliomas [Jett et al. 2008, Kleinerman 2009]. Chemotherapy of combination of vincristine and carboplatin is recommended for those patients with progressive symptomatic optic glioma. This therapy is especially effective in children aged 5 years or younger [Packer et al. 1997, Jett et al. 2008].

Other malignancies associated with NF1

NF1 is associated with a variety of different tumors and malignancies in addition to those mentioned above. These include:

- Central nervous system tumors, mainly medulloblastomas and astrocytomas, are associated with NF1 [Riccardi et al. 1986, Evans et al. 2002, Jett et al. 2008].
- Pheochromocytoma tumors are relative rare, but are associated with NF1 [Riccardi et al. 1986, Jett et al. 2008].
- Patients with NF1 are at marked risk for acute leukemia, especially juvenile myelomonocytic leukemia [Stiller et al. 1994, Side et al. 1997].
- Increased risk for breast cancer has been associated in women with NF1 and below 50 years of age [Walker et al. 2006, Sharif et al. 2007].

Mortality and life expectancy in NF1

The most common causes of death in NF1 are malignant peripheral nerve sheath tumors (MPNST) and gliomas. These two types of malignancy cause premature death and shorter life expectancy in NF1. Median survival of NF1 patients in the UK is 72 years for men and 79 for women. This is roughly 8 years less than in general population. Excess mortality was noted in NF1 population aged 10-40 years, mostly due to MPNSTs [Evans et al. 2011, Duong et al. 2011].

2.1.4 Pigmentation disorders in NF1

Café-au-lait macules and freckling

Café-au-lait macules are a common pigmentation disorder in NF1. The macules are usually oval, clear-bordered, unicolor and benign hyperpigmentation patches in the skin. Their average diameter is 2-5 cm. Café-au-lait patches can be found in any anatomical location, but are seldom present in face. They may be visible in patient's skin soon after birth, or appear during the first year. Six or more café-au-lait patches are found in 90 % of adult NF1 patients [Friedman et al. 1997, Jett et al. 2010, Jouhilahti et al. 2011a]. Melanocytes cultured from these café-au-lait patches have higher amounts of melanin compared to melanocytes derived from normally pigmented skin of NF1 patients. The number of melanocytes was equal between the skin samples from patients with NF1 and healthy controls. Thus, café-au-lait macules in NF1 appear to have normal number of melanocytes, but abnormally high levels of melanin [Kaufmann et al. 1991]. Double inactivation of the *NF1* gene has been demonstrated in melanocytes derived from café-au-lait macules of patients with NF1 [De Schepper et al. 2006].

Freckling in axillary or inguinal regions develops in almost 90 % of the patients with NF1 and usually by the age of 7 years. Freckling in these regions is rare in individuals without NF1 [Friedman et al. 1997, DeBella et al. 2000].

Lisch nodules

Lisch nodules are melanocytic pigmentation disorders of the iris, but their exact pathoethiology remains unclear. Lisch nodules are found in ~90 % of the postpubertal patients with NF1. In addition, the majority of these nodules are located inferiorly. It has been proposed that Lisch nodules are unique for NF1 [Lewis et al. 1981, Friedman et al. 1997, Richetta et al. 2004, Nichols et al. 2003].

2.1.5 Skeletal abnormalities associated with NF1

Overview

Approximately half of patients with NF1 have some form of clinically detectable skeletal abnormality [Crawford et al. 1986]. These abnormalities can be classified either as

focal or systemic. Focal lesions include long bone dysplasia, congenital pseudarthrosis, sphenoid wing dysplasia, bone cysts and scoliosis, and are rare compared to systemic ones, which include short stature and low BMD [Elefteriou et al. 2009]. It is suggested that NF1 patients with sphenoid wing dysplasia are more likely to have long bone dysplasia or vertebral dysplasia, compared other NF1 patients [Alwan et al. 2007].

In bone, the presence of neurofibromin mRNA and protein have been detected in human osteoclasts, osteocytes, osteoblasts and chondrocytes, and also in mouse mesenchymal stem cells. Since certain NF1-related skeletal lesions are congenital, NF1 protein is suggested to be essential for normal development of bone [Kuorilehto et al. 2004, Kolanzcyk et al. 2007, Leskelä et al. 2009].

Scoliosis

Scoliosis is evident in approximately 20-25 % of individuals with NF1. About 5 % of patients with NF1 and scoliosis will require some form of surgical intervention [Friedman et al. 1997, Young et al. 2002]. There are two types of scoliosis associated with NF1; dystrophic and idiopathic. The most common type is an idiopathic scoliosis with a long and mild spinal curve. The other type is specific for NF1, with a very sharp angle (kyphosis more than 50 degrees) and very rapid possibly life-threatening progression. The latter type is referred as dystrophic scoliosis and it usually affects up to five vertebrae [Crawford et al. 1986, Kim et al 1997, Crawford et al. 2007]. Patients with NF1 may also present a variety of dystrophic spinal features other than scoliosis. These include abnormally sized vertebral bodies, short vertebral segments, wide spinal canal, rotated ribs and the presence of paraspinal mass [Alwan et al. 2007].

Congenital bowing and pseudarthrosis of the long bones

Congenital bowing of long bones is also known as long bone dysplasia, and usually progresses to fracture and non-union of the bone, the pseudarthrosis (PA) [Stevenson et al. 2011b]. NF1-related congenital PA most often affects tibia, but it may affect ulna or other long bones. Congenital PA of tibia affects 2-4 % of patients with NF1 [Young et al. 2002, Friedman et al. 1997]. On the other hand, 50-80 % of all patients with congenital PA have NF1 [Stevenson et al. 1999]. The NF1-related congenital PA of tibia is typically unilateral and is clinically detected when the child begins to stand and walk, presenting with anterolateral bowing of the tibia [Stevenson et al. 1999, Young et al. 2002, Crawford and Schorry 2006]. Progressive deformity and atrophy caused by disused extremity in PA may lead to amputation of the affected limb [Ohnishi et al. 2005].

Tissue from the PA site has been characterized as osteoid rich, fibro-cartilaginous and well-vascularized tissue, with varying amounts of osteoclasts. Thickened walls of arteries and veins with narrowed lumens have been described in the vicinity of NF1 pseudarthrosis tissue, suggesting a vascular abnormality [Hermanns-Sachweh et al. 2005, Kuorilehto et al. 2006, Sakamoto et al. 2007]. Double inactivation of the *NF1*

gene in pseudarthrosis tissue has been shown in some, but not all, samples from patients with NF1 [Stevenson et al 2006, Sakamoto et al. 2007, Leskelä et al. 2009].

Congenital PA of tibia is difficult to treat. Both external fixation (Ilizarov method) and internal fixation (intramedullar nail) are used to treat the PA. Surgical fixation is often supported by cortical allocraft, ie. moving another healthy bone of the patient to support the PA site. The ossification process can be further supported by giving recombinant human bone morphogenetic proteins, which induce bone formation. The current treatment options are summarized by Schindeler et al. (2011).

Short stature and macrocephaly

The growth charts of children with NF1 follow the reference charts until the age of 7 (girls) and 12 (boys) in Italian and North American pediatric cohorts. The prepubertal growth charts do not necessarily predict the final height of NF1 patients, since height of adult NF1 patients is markedly shorter. In addition, growth rate of children with NF1 diminishes after the onset of puberty compared to predicted growth rate. True short stature (height ≤ -2 SD) is detected in 13-18 % of patients with NF1 [Clementi et al. 1999, Szudek et al. 2000, Virdis et al. 2003].

Macrocephaly, determined by occipito-frontal circumference, is common in NF1. Macrocephaly can be detected in ~25 % of North American patients with NF1. The brain volume has also been demonstrated to be larger in persons with NF1 compared to those without. This has been suggested as the reason for macrocephaly [Clementi et al. 1999, Szudek et al. 2000, Greenwood et al. 2005].

Craniofacial abnormalities and sphenoid wing dysplasia

A number of craniofacial findings in NF1 have been reported. These findings include enlarged mandibular foramen and canal, wide and branched inferior alveolar canal, and decrease in the mandibular angle. Neurofibromas can also reside in oral tissues, and plexiform neurofibromas have been reported to associate with supernumerary or missing teeth, retention of teeth, and deformed alveolar ridge [Shapiro et al. 1984, D'Ambrosio et al. 1988, Lee et al. 1996, Friedrich et al. 2003, Visnapuu et al. 2011]. Furthermore, a rare specific lytic bone lesion of the jaws, periapical cemental dysplasia, has been reported to be common in women with NF1 [Visnapuu et al. 2007]. Facial asymmetry afflicts ~10 % of patients with NF1, and is usually associated with sphenoid wing dysplasia or plexiform neurofibroma of the 5th cranial nerve [D'Ambrosio et al. 1988, Friedman et al. 1997].

Sphenoid wing dysplasia is estimated to be present in ~10 % of patients with NF1 [Friedman et al. 1997]. Dysplasia of the sphenoid wing is often associated with adjacent soft tissue tumor [Jacquemin et al. 2002]. These abnormalities are usually asymptomatic but are diagnosed in routine X-rays or tomography scans of the head [Elefteriou et al. 2009].

Chest wall abnormalities

Abnormalities of the chest wall are not well documented. Pectus excavatum, ie. impression of the anterior chest wall, is occasionally associated with NF1. The defect is usually cosmetic [Elefteriou et al. 2009].

Reduced bone mineral density

Reduced bone mineral density (BMD) is common in NF1. Specifically, lower BMD values in patients with NF1 compared to matched control individuals have been demonstrated in both sexes, all ages, and using both dual-energy X-ray absorptiometry (DXA) and ultrasound [Kuorilehto et al. 2005, Lammert et al. 2005b, Stevenson et al. 2007, Yilmaz et al. 2007, Dulai et al. 2007, Duman et al. 2008, Brunetti-Pierri et al. 2008, Tucker et al. 2009, Seitz et al. 2010, Petramala et al. 2011]. Also the frequency of osteoporosis and osteopenia is markedly higher in NF1 population compared to controls as summarized in **Table 3**. The lowest BMD values are clustered in load carrying parts of the body, such as the spine and legs, while this phenomenon was not evident in control persons. Kuorilehto et al. also demonstrated that total bone calcium and total bone mineral content were significantly lower in patients with NF1 than in the control persons [Kuorilehto et al. 2005].

Table 3. Osteoporosis and osteopenia in NF1.

Study	Patients	Osteoporosis	Osteopenia
Kuorilehto 2005	26 females, 14 males, aged 21-73	7 (27 %)	13 (50 %)
Stevenson 2007	39 females, 45 males, aged 5-18	BMD reported lower compared to controls	
Duman 2008	16 females, 16 males, aged 3-17	BMD reported lower compared to controls	
Seitz 2008	9 females, 5 males, aged 19-66	8 (57 %)	not reported
Brunetti-Pierri 2008	47 females, 26 males, aged 3-59	24 (32 %)*	41 (57 %)
Tucker 2009	43 females, 29 males, aged 18-72	14 (19 %)	36 (50 %)
Petramala 2011	33 females, 37 males, aged 27-55	13 (18 %)	31 (44 %)
Current study III	10 females, 10 males, aged 23-76	4 (20 %)	11 (55 %)
Current study IV	8 females, 11 males, aged 26-77	7 (37 %)	7 (37 %)
Current study V**	27 females, 23 males, aged 18-93	14 (28 %)	24 (48 %)

* in this study, osteoporosis was defined as Z-score -2.5 SD or less.
** in this study, a selected cohort was examined, and there is overlap with studies III and IV

A group of 16 adolescents with NF1 was treated with vitamin D supplementation and followed-up for two years. However, the age-matched BMD (Z-score) did not change [Brunetti-Pierri et al. 2008]. Correlation between whole-body subtotal age-matched BMD (Z-score), urine collagen degradation products and *in vitro* osteoclast differentiation capacity in 75 patients with NF1 aged 1-25 has been studied, but no significant correlations were found [Stevenson et al. 2011a].

Fracture risk in NF1 has been addressed in two previous studies [Brunetti-Pierri et al. 2008, Tucker et al. 2009]. Both studies used a questionnaire sent to the patients. Brunetti-Pierri et al. found a history of fractures in 35 of 67 (52 %) NF1 patients. Tucker et al. found 41 fractures verified by radiologist in 24 out of 72 (33 %) NF1 patients, and also reported that NF1 patients had increased fracture risk compared to their unaffected spouses and/or siblings.

Laboratory values reflecting bone dynamics

High levels of serum parathyroid hormone, and increased collagen degradation products in urine have been reported in patients with NF1 [Tucker et al. 2009, Brunetti-Pierri et al. 2008, Seitz et al. 2008, Stevenson et al. 2011A]. Low levels of serum vitamin D have been reported in NF1 patients, also in NF1 children [Lammert et al. 2006, Tucker et al. 2009, Brunetti-Pierri et al. 2008, Stevenson et al. 2011c]. It is also proposed that NF1 patients with high neurofibroma tumor burden have even lower vitamin D values than other NF1 patients [Lammert et al. 2006]. In the subpopulation of patients with NF1 and low BMD, low levels of serum osteocalcin [Duman et al. 2008], high levels of serum parathyroid hormone, calcium and TRACP5b have been reported [Brunetti-Pierri et al. 2008, Seitz et al. 2008].

Bone biopsies and histomorphometry in NF1

Bone biopsy histomorphometry from the patients with NF1 revealed increased volume of osteoid, and increased number of both osteoclasts and osteoblasts compared to controls [Brunetti-Pierri et al. 2008, Seitz et al. 2008]. Patients with NF1 have narrower tibial bones compared to healthy controls [Stevenson et al. 2009].

Osteoclasts and osteoblasts in patients with NF1

In bone, the presence of neurofibromin mRNA and protein have been detected in human chondrocytes, osteoblasts and osteoclasts [Leskelä et al. 2009]. Osteoclasts progenitors can be isolated from peripheral blood and cultured into osteoclasts *in vitro*. Osteoclasts derived from peripheral blood of the patients with NF1 are more numerous, resorb larger amounts of bone, and display increased ERK activity. Human NF1 osteoclasts progenitors from five patients also display enhanced migration, adhesion and proliferation capacity [Yang et al. 2006, Stevenson et al. 2011a].

Osteogenic potential of NF1-osteoblasts was estimated by levels of bone formation markers in the mesenchymal stem cell culture, including alkaline phosphatase and procollagen type I N-terminal propeptide (see 2.2.4). Levels of cell culture alkaline phosphatase and procollagen type I N-terminal propeptide were lower in mesenchymal stem cells derived from three patients with NF1 compared to healthy controls [Leskelä et al. 2009].

Modeling NF1-related skeletal lesions with murine models

$Nf1^{-/-}$ mouse embryos die in early embryonic state [Brannan et al. 1994, Lakkis and Epstein 1998]. $Nf1^{+/-}$ mice display defective osteoblast progenitors and osteoblasts, and hyperactive osteoclasts. However reduced bone mass or focal bone dysplasia was not found in this model [Yu et al. 2005, Yang et al. 2006].

Nf1-deficiency in committed osteoblasts was generated using Cre-recombinase in osteoblast-specific 2.3 kb type I collagen promoter ($Nf1^{-/-ob}$ mice). This produced mice with no apparent morphological defects, but with high bone turnover. The mice display increased collagen synthesis and defective mineralization causing increased osteoid volume. Also high numbers of osteoclasts were noted in these animals [Elefteriou et al. 2006]. $Nf1^{-/-ob}$ mice also display short vertebral segments, reduced vertebral bone mass, and enlarged intervertebral canal, in analogy to those findings seen in humans with NF1. Tissue sections of vertebrae of these mice display increased number of osteoclasts and decreased number of osteoblasts [Zhang et al. 2011].

Nf1-deficiency generated using Cre-recombinase under Prx promoter generates a $Nf1^{Prx-/-}$ mouse model, where cells derived from mesenchymal stem cells are affected. These cells include osteoblasts, adipocytes, chondrocytes, muscle cells and endothelial cells of the developing limb bud mesenchyme. These mice display growth retardation and grossly abnormal, stunted limbs, and bowing of the tibia similar to that seen in patients with NF1. Defects were also observed in osteoblasts [Kolanczyk et al. 2007, Kolanczyk et al. 2008].

Also the $Nf1^{+/-}$ mice have been used to study congenital pseudarthrosis of tibia. The tibia of these mice was fractured surgically and bone healing was found to be delayed [Schindeler et al. 2008]. Lovastatin has been shown to increase fracture healing rate in $Nf1^{-/-ob}$ and $Nf1^{Prx-/-}$ mice. Lovastatin inhibits mevalonate pathway, which in turn leads to reduced prenylation, ie. addition of farnesyl groups. Since Ras protein requires prenylation, it has been postulated that the effect of the drug is mediated through this mechanism [Kolanczyk et al. 2008, Wang et al. 2010].

Osteoclast-spesific knockout of the *Nf1* gene can be generated using Cre-recombinase under TRACP promoter. This generates a mouse model with Nf1-deficient osteoclasts in otherwise $Nf1^{+/+}$ background, the $Nf1^{Ocl}$ mice. These mice had normal BMD, but the perimeter of cortical bone and the bone marrow cavity were smaller compared to control mice. Osteoclasts derived from $Nf1^{Ocl-/-}$ mice showed a hyperactive phenotype, resorbing larger amounts of bone compared to control mice. In addition to osteoclast findings, these mice had enlarged spleen and narrower growth plates compared to control mice [Alanne et al. 2012].

Conditional inactivation of *Nf1* gene in type II collagen alfa-1 promoter results in inactivation of *Nf1* in chondrocytes, thus named $Nf1^{-/-col2}$ mice. However, for yet unknown reasons, the cre-recombinase also inactivates targeted genes (*Nf1* for instance)

in a subpopulation of bone marrow osteochondroprogenitors, and thus in some mature osteoblasts and osteocytes. $Nf1^{-/-col2}$ mice display a phenotype very similar to osseous malformations see in patients with NF1. The mice display scoliosis, kyphosis, bowing of tibia and abnormalities in the skull and chest wall. These mice also have increased number of osteoclasts and decreased number of osteoblast, and increased amount of osteoid compared to wild type mice [Wang et al. 2011].

Hyperactive osteoclasts are documented in $Nf1^{+/-}$, $Nf1^{-/-ob}$, $Nf1^{Ocl}$ and $Nf1^{-/-col2}$ murine models. The hyperactive Ras seen in osteoclasts of these mice has been shown to upregulate PI3K/Akt/mTOR and Raf/MEK/ERK pathway signaling [Yang et al. 2006, Ma et al. 2012]. In addition, Ras-regulated functions of rat osteoclasts have been shown to be mediated to some extent via the family of small Rho GTPases, including Rac1 GTPase [Sun et al. 2005]. In mice, genetic disruption of Rac1 function restored the hyperactive phenotype of $Nf1^{+/-}$ osteoclasts [Yan et al. 2008].

2.1.6 Other manifestations of NF1

Cardiovascular abnormalities and hypertension

A number of cardiovascular abnormalities have been reported to be associated with NF1. Most noted abnormalities are pulmonary stenosis, coarctation of aorta and fibrodysplasia of renal arteries. The latter usually results in stenosis of renal arteries. Some kind of cardiovascular abnormality has been found in 2-18 % of patients with NF1 [Friedman et al. 1997, Lin et al. 2000, Tedesco et al. 2004]. Hypertension is also associated with NF1 but the pathoethiology is poorly understood [Tedesco et al. 2005]. Hypertension occurs in 15-19 % in children and adolescents with NF1, and most of the hypertension cases are due renal artery stenosis [Fossali et al. 2000, Lama et al. 2004]. In addition, pheochromocytoma tumors are associated with NF1, and may be a cause of secondary hypertension. It is speculated that half of hypertensive patients with NF1 may have pheochromocytoma [Zinnamosca et al. 2011]. Hypertension in pediatric patients with NF1 is also associated with changes in the structures of heart, such as thickening of ventricular walls [Fossali et al. 2000, Tedesco et al. 2005, Lama et al. 2004].

Precocious puberty

Precocious puberty or pubertas praecox means premature beginning of puberty, and has been reported in 4-16 % of the patients with NF1 [Friedman et al. 1997, Virdis et al. 2003]. This incidence has been reported to be increased compared to patients' mothers [Virdis et al. 2003]. Optic glioma may cause precocious puberty due to local pressure to pituitary gland [Listernick et al. 2007].

Cognitive difficulties

Cognitive difficulties include for example learning and speech problems. Learning disabilities and social difficulties are associated with NF1, and are estimated to affect

30-60 % of patients with NF1 [Ferner et al. 2007, Huson 2008]. Speech characteristics in NF1 include deviations in voice quality, problems in regulating pitch, deviant nasality, misarticulation and disfluency [Alivuotila et al. 2010]. Delayed development of speech and articulation has also been reported in patients with NF1 [Thompson et al. 2010].

2.2 BONE DYNAMICS

2.2.1 Structure of bone

Overview

Bone consist of cortical (compact) and cancellous (spongious, trabecular) bone. Long bones have epiphyses (the growth plate), diaphyses (the shaft) and metaphyses (the between). The bone itself consists of the inorganic matrix, organic matrix, and bone cells. The organic matrix mainly consists of type I collagen and bone proteins. These proteins are for example osteocalcin, osteopontin, osteonectin and bone sialoprotein. The organic matrix is mineralized, and the inorganic matrix of bone is mostly calcium-rich hydroxyapatite [Shapiro 2008, Lefebvre et al. 2010]. The major bone cells are the bone-resorbing osteoclasts, bone-forming osteoblasts and osteocytes which lie within the bone. These cells are discussed in 2.2.2.

Osteons

Osteons are cylindrical structures formed around blood vessels in cortical bone. Osteons are formed during bone growth but also during the bone remodeling. Traditionally it is considered that old bone is first resorbed by osteoclasts, followed by mineralization of the resorbed space by osteoblasts, finally leading to formation of a new osteon. Osteon is pierced by multiple small canaculi, which are connecting the osteocytes to each other. The blood vessel is located in the centre of the osteon, in the Haversian canal, as depicted in **Figure 3**. However, alternating morphologies of the osteon are reported [Cooper et al. 2011]. Osteons are arranged to withstand the load-bearing stress on bone, usually parallel to the longitudinal axis of bone [Lin et al. 2011]

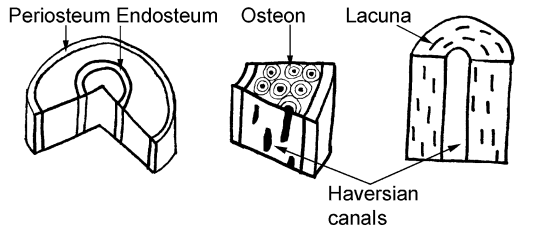

Figure 3. Structure of bone showing periosteum, endosteum, osteon, Haversian canal and lacunae [Modified from Lin et al. 2011].

Ossification

Bone is derived from three origins; paraxial mesoderm, lateral plate mesoderm and neural crest. Most bones are formed through endochondral ossification from paraxial and lateral plate origins. Most of facial bones are formed through intramembraneous ossification from neural crest cells.

- The endochondral ossification is a process of cartilaginous framework (the cartilage anlage) subsequently replaced by osteoblasts and mineralized into bone [Shapiro 2008, Lefebvre et al. 2010]. The chondrocytes undergo hypertrophy, and secrete factors that attract blood vessels. This in turn leads to invasion of osteoblasts into the cartilage. Thus the cartilage is transformed into bone [Zhao et al. 1997, Colnot et al. 2004].
- In intramembraneous ossification, mesenchymal stem cells within a fibrous plate differentiate directly into bone-forming osteoblasts [Shapiro 2008, Lefebvre et al. 2010].

Limbs are developed from limb bud mesenchyme derived from lateral mesoderm through endochondral ossification [Sadler 2006]. The structures of the skull develop in a complex fashion. The skull can be divided into neurocranium (cranial base and flat bones encasing the brain) and viscerocranium (the facial bones). Posterior cranial base is derived from paraxial mesoderm, and anterior cranial base from neural crest cells, through endochondral ossification. Flat bones encasing the brain are derived from paraxial mesoderm and neural crest cells through intramembraneous ossification. Viscerocranium is derived from neural crests cells of the first two pharyngeal arches through intramembraneous ossification [Nie 2005, Sadler 2006]. Craniofacial growth is generally considered to be regulated by interaction of genomic and functional factors. Cranial base exerts considerable influence on facial growth [Nie 2005].

Mandible develops from the 1st pharyngeal arch via intramembraneous ossification, as neural crest cells migrate and proliferate within the mandibular arch. Initial ossification of mandible occurs in the vicinity of a structure called Meckel's cartilage, and the bony plates spread out into anterior and posterior directions, following the outlines of Meckel's cartilage. Once the two mandibles fuse in the midline Meckel's cartilage becomes atrophic, and is subsequently resolved [Radlanski et al. 2003, Chai et al. 2006]. The cartilage of mandibular condyle develops separate from the mandible, and becomes attached to the membranous mandible during development, finally becoming articular cartilage [Morimoto et al. 1987, Visnapuu et al. 2000].

Fracture healing

Fracture healing is a process involving the different bone cells and a variety of cytokines. Both intramembraneous and endochondral ossification occurs during fracture healing. Fibrinous blood clot formed around the fracture site is essential for normal fracture healing. The healing process has three phases; inflammation, repair and remodeling.

During the inflammation phase, a fibrous external capsule is formed around the fracture site, the callus. Callus can be seen in radiographs. In repair phase, mesenchymal stem cells migrate into fracture site and differentiate into osteoblasts, and start forming new bone. Also chondrocytes are formed, and they take part in endochondral ossification. Finally, osteoclasts migrate to the site and start the bone remodeling phase, ultimately leading to "healed" bone [Shapiro 2008, Coulibaly et al. 2010].

2.2.2 Cells of bone and cartilage

Osteoblasts

Osteoblasts form new bone, produce proteins of extracellular matrix, play a crucial role in bone mineralization and fracture healing, and also regulate bone resorption through OPG/RANK/RANKL system (see 2.2.3). Osteoblasts are derived from mesenchymal stem cells, which differentiate into osteoblast progenitors, or osteoprogenitors, and finally to mature osteoblasts. Once embedded in mineralized bone matrix, mature osteoblasts may become osteocytes [Jensen et al. 2010].

Withdrawal of osteoblasts from trabecular or endosteal bone surface triggers the bone resorption by osteoclasts [Chambers et al. 1985]. Osteoblasts secrete MCSF, RANKL and OPG, and also several other signaling molecules, which are essential for bone dynamics and resorption. The OPG/RANK/RANKL pathway is the main regulator of bone resorption and is discussed in 2.2.3 [Yasuda et al. 1998, Katagiri et al. 2002].

Osteoblasts are characterized by their expression of type I collagen, alkaline phosphatase, osteopontin, osteonectin and osteocalcin in different stages of their differentiation. The three latter proteins are secreted and found within the bone matrix. *Runx2* is the master regulator gene in maturation of osteoblasts. Also multiple other factors influence positively on development of osteoblasts, including activation of *ATF4*, *NFAT1* and *AP-1* genes. Osteoblast maturation is enhanced by vitamin D as well as intermittent pulses of parathyroid hormone [Jensen et al. 2010, Rachner et al. 2010]. Bone morphogenetic proteins (BMPs) induce bone formation by affecting multiple bone cell types, but principally by affecting the OPG/RANK/RANKL pathway [Kamiya et al. 2011].

Osteocytes

Osteoblasts which are trapped inside bone may become osteocytes. They are stellate cells which are in contact with other osteocytes, but also with osteoblasts and bone lining cells. The osteocytes reside with structures called lacunae (**Figure 3**) and are connected to each other through structures called canaliculi. The actual osteocytes are interconnected by gap junctions [Ilvesaro et al. 2003, Shapiro 2008, Bonewald 2011].

Osteocytes secrete factors which stimulate osteoblast differentiation [Heino et al. 2002], as well as express sclerostin and fibroblast growth factor-23, which in turn affects bone dynamics. Sclerostin has been shown to cause bone lining cells to migrate, exposing

bone, and allowing osteoclasts to start remodeling cycle [Bonewald 2011, Agholme et al. 2008]. In addition, sclerostin downregulates Wnt signaling in osteoblasts, leading to decreased osteoblast activation [Miller et al. 2008].

Apoptotic osteocytes secrete RANKL, which promotes bone resorption through osteoclast activation (see below). This may be the mechanism how microdamaged bone is resorbed and subsequently replaced with new bone [Kogianni et al. 2008]. It is also speculated that osteocytes have mechanosensory functions in bone, due to their ability to sense changes in fluid flow through canalicular system [Shapiro 2008, Bonewald 2011, Klein-Nulend et al. 2003].

Bone lining cells and periosteal cells

Bone lining cells and periosteal cells are derived from osteoblastic lineage. They are flat cells which cover surfaces of bone not currently undergoing remodeling cycle. Removal of bone lining cells may trigger bone remodeling cycle as osteoclast may attach to the endosteal surface of bone [Miller et al. 1989, Everts et al. 2002, Agholme et al. 2008].

Osteoclasts

The bone-resorbing multinuclear osteoclasts belong to the monocyte/macrophage lineage and derive from haematopoietic CD14 positive progenitor cells. Mature multinuclear osteoclasts are formed by the fusion of mononuclear osteoclast progenitors [Scheven et al. 1986, Massey et al. 1999]. Dendritic-cell specific transmembrane protein is expressed in osteoclasts and macrophages, and is required for the fusion of osteoclast progenitors [Yagi et al. 2005]. During osteoclast differentiation, tartrate resistant acid phosphatase (TRACP5b) is expressed at earliest stage. This is followed by other proteins typical for osteoclasts, including calcitonin receptor and cathepsin K [Lee et al. 1995, Yuuki et al. 2010].

The two essential soluble factors required for osteoclast differentiation are receptor activator of nuclear factor kappa-beta ligand (RANKL) and macrophage colony stimulating factor (MCSF). They are mainly secreted by osteoblasts but also by other cell types [Walker 1975, Lacey et al. 1998, Yoshida et al. 1990]. The OPG/RANK/RANKL pathway is discussed in 2.2.3.

MCSF and RANKL signaling in osteoclasts involves multiple downstream signaling pathways. The most notable pathways are PKC, Raf/MEK/ERK, P38 and NFAT1, which all are anti-apoptotic and pro-osteoclastogenic signaling pathways. These pathways, excluding P38, are shown in **Figure 2**. Concerning the current study, Raf/MEK/ERK–pathway is also regulated by Ras and NF1 as shown in **Figure 2.** [Miyazaki et al. 2000, Tiedemann et al. 2009, Lee et al. 2009].

Osteoclasts have four distinct membrane domains as shown in **Figure 4** [Väänänen et al. 2000, Mulari et al. 2003].

- The ruffled border which is the bone dissolving organelle
- Sealing zone which separates the lytic domain from its surroundings
- Functional secretory domain, through which the trancytosed resorbed material is excreted
- Basolateral domain, the domain between sealing zone and functional secretory domain

Active osteoclasts resorb bone in the subosteoclastic space. Lysosomal enzymes are re-oriented within the osteoclast towards the bone surface. Thus, the ruffled border is formed. This results in release of hydrochloric acid by vacuolar H^+ATPase (the proton pump). In addition to acid, osteoclasts secrete enzymes such as cathepsin K and tartrate resistant acid phosphatase (TRACP5b). Cathepsin K degrades collagens, and TRACP5b dephosphorylates bone matrix proteins, such as osteopontin [Väänänen et al. 2000, Vasikaran et al. 2011]. It should also be noted, that extracellular acidosis stimulates osteoclast differentiation, which may be important in bone loss associated with hypoxia [Kato et al. 2011].

The sealing zone of osteoclasts contains bundles of F-actin, which follow the shape of the sealing zone. This forms the actin ring. Actin is associated with alfa-V-beta-3 integrins, and follows the double-ring structure of vinculin and talin. Alfa-V-beta-3 integrin is attached mostly to proteins present on bone containing RGD-sequence, such as vitronectin and osteopontin. Once the resorption at one site is over, the actin ring either moves as a kidney-shaped structure maintaining resorptive capacity of the cell, or the actin ring is dissociated [Väänänen et al. 2000, Schaffner et al. 2003].

The bone resorption in the subosteoclastic base is followed by internalization and transcytosis of degraded bone material through the osteoclast via functional secretory domain. This transport is partly regulated by Ras-related small GTPases of the rab-family [Mulari et al. 2003, Sun et al. 2005].

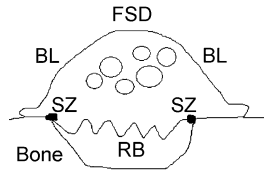

Figure 4. Osteoclast. BL: basolateral zone. FSD: functional secretory domain. RB: ruffled border. SZ: sealing zone.

Chondrocytes and cartilage

Chondrocytes are derived from mesenchymal stem cells. They express mainly collagen types II, IX and XI, but also types VI and X are found. Chondrocytes are present within cartilage and during endochondral bone formation [Zhao et al. 1997, Bertrand et al. 2010]. During endochondral bone formation, chondrocytes undergo hypertrophy, and

secrete factors that attract blood vessels. This in turn leads to invasion of osteoblasts into the cartilage. This is called endochondral bone formation where the cartilage is transformed into bone [Zhao et al. 1997, Colnot et al. 2004, Eyre et al. 2006].

There are three forms of cartilage; hyaline cartilage, elastic cartilage and fibrocartilage. Hyaline cartilage is found mainly in the growth plates of long bones and joints. Fibrocartilage is found in intervertebral discs, temporomandibular joint and in the menisci of the knee. Elastic cartilage is found in the epiglottis and in the Eustachian tube in the ear. Typical cartilage matrix contains type II collagen (type I also in the menisci), hyaluronan and different proteoglycans [Midura et al. 2003, Kirkham et al. 2009, Bertrand et al. 2010].

2.2.3 Regulation of bone mass

The coupled bone resorption and mineralization cycle

Bone mass is regulated by constant cross-talk between bone resorbing osteoclasts and bone forming osteoblasts. The modeling process is initiated when bone lining cells or osteoblasts uncover trabecular or endosteal bone surface, allowing osteoclasts to attach the bone. This is followed by bone resorption as described in the osteoclast section above. The resorption results in formation of resorption pits or lacunae (Howship's lacunae). Osteoblasts move towards the pit and start forming new bone. Freshly formed bone is called osteoid which is subsequently mineralized into mature bone [Novack et al. 2008, Martin et al. 2005]. Resorption part of the resorption cycle by osteoclasts takes in humans approximately 6-12 days, while formation of new bone in the same area 3-4 weeks [Eriksen et al. 1984a, Eriksen et al. 1984b]. This is traditionally considered to result in the formation of a new osteon.

Bone remodeling is coupled, which means that increased osteoclast activity (resorption) is followed by increased bone remodeling by osteoblasts, and vice versa [Miller et al. 2008]. This explains why osteoporotic patients usually have increased serum bone formation markers in addition to increased bone resorption markers, and why antiresorptive therapy results in decrease of bone formation markers.

Regulation of bone turnover

The main modulators of bone turnover are shown in **Figure 5**. The effects of parathyroid hormone, estrogens, glucocorticoids, vitamin D and sclerostin are mediated via the OPG/RANK/RANKL signaling through osteoblasts [Miller et al. 2008, Trouvin et al. 2010, Yuuki et al. 2010]. The inflammatory cytokines, including TNF-alfa, interleukine 1 beta and 6, affect osteoclastogenesis directly [Teitelbaum et al. 2007, Axmann et al. 2009, Trouvin et al. 2010].

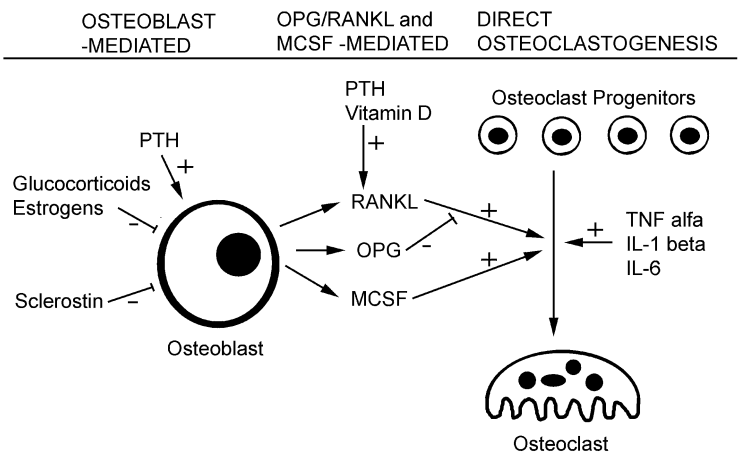

Figure 5. Summary of regulation of bone turnover, with emphasis on osteoblast – osteoclast mediated regulation.

OPG/RANK/RANKL –the main regulator of resorption cycle

The key regulator of the resorption cycle is the OPG/RANK/RANKL system. Soluble RANKL binds to its receptor RANK, which is present on the cell membrane of osteoclasts. This induces osteoclastogenesis. Osteoprotegerin (OPG) is soluble factor secreted mainly by osteoblasts, but also many other cell types. OPG is a decoy receptor for RANKL, thus reducing the concentration of soluble RANKL. Since RANKL is required for osteoclast maturation, OPG secretion reduces bone resorption [Trouvin et al. 2010].

Fas-receptors and Sclerostin

Fas-receptors are death receptors located on cell surface of many cell types, including osteoblasts and osteoclasts. Fas-receptor activation may trigger cell apoptosis [Yuuki et al. 2010]. This is particularly important in estrogen-mediated control of bone turnover (see below).

Sclerostin has been shown to cause bone lining cells to migrate, exposing bone, and allowing osteoclasts to start remodeling cycle [Bonewald 2011, Agholme et al. 2008]. In addition, sclerostin downregulates Wnt signaling in osteoblasts, leading to decreased osteoblast activation [Miller et al. 2008].

Serum calcium

Calcium does not regulate bone turnover, but bone turnover is an important part in maintaining the levels of serum calcium. Thus, abnormal serum calcium results in a variety of reactions concerning bone (**Figure 6**). Calcium is gained by absorption from the gut, and lost by excretion in the urine. Excess serum calcium is deposited in the bone, and may be subsequently released from the bone [Landry et al. 2010].

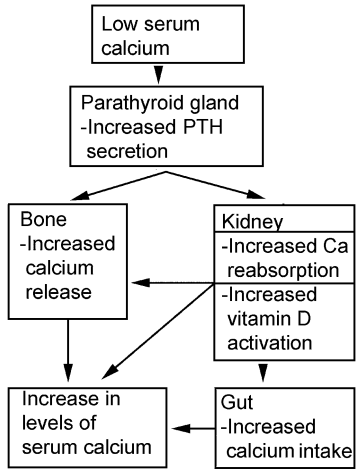

Figure 6. Regulation of serum calcium. The figure shows responses to low levels of serum calcium. Responses to high levels of serum calcium are the opposite.

Parathyroid hormone

Parathyroid hormone (PTH) has 84 aminoacids and is a bone anabolic hormone regardless of the fact that it stimulates bone resorption. PTH is secreted by parathyroid glands in response to low levels of serum calcium or vitamin D. Secondary hyperparathyreoidism is a response to low levels of vitamin D. In the kidneys PTH enhances reabsorption of calcium and improves activation of vitamin D [Landry et al. 2010, Rachner et al. 2010]. PTH decreases OPG expression and increases RANKL expression in osteoblasts and bone marrow stromal cells. Thus parathyroid hormone increases bone resorption and levels of serum calcium [Rachner et al. 2010, Trouvin et al. 2010]. However, PTH also stimulates bone formation, and PTH therapy results in increased bone mass and reduced fracture risk, as described in 2.3.5 [Compston et al. 2007, Gallacher et al. 2010].

Vitamin D

In this text vitamin D means vitamin D2 ergocalciferol (plant origin) or vitamin D3 cholecalciferol (animal origin). The structure of vitamin D is shown in **Figure 7.** Unhydroxylated form of vitamin D is synthesized from cholesterol in the skin as a result of exposure to ultraviolet radiation from the sun. Vitamin D is furthermore hydroxylated to 25OH-vitamin D, the storage form, in the liver and 1,25OH-vitamin D, the active form, in the kidneys. Vitamin D increases absorption of calcium from the intestine, enhances reabsorption of calcium in the kidneys and increases bone resorption rate. Thus vitamin D increases levels of serum calcium. Vitamin D is acquired from the diet but is also synthesized from cholesterol [Bouillon et al. 2006, Rachner et al. 2010, Landry et al. 2011].

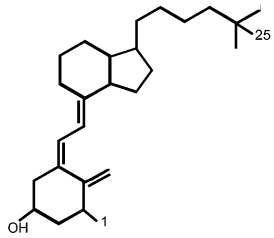

Figure 7. Structure of vitamin D. Carbons 1 and 25 are hydroxylated during activation of the molecule [Modified from Bouillon 2006].

Low levels of vitamin D leads to increased secretion of parathyroid hormone, leading to secondary hyperparathyroidism. Vitamin D also directly enhances osteoblast maturation and increase RANKL expression in osteoblasts [Jensen et al. 2010, Rachner et al. 2010]. In NF1, low levels of serum vitamin D have been reported, also in NF1 children [Lammert et al. 2006, Brunetti-Pierri et al. 2008, Tucker et al. 2009, Stevenson et al. 2011c].

Estrogens

In humans estrogens include estrone, the main estrogen estradiol, and estriol which is present during pregnancy. Estrogen deficiency is a well-known cause of reduced BMD and is characterized by high bone turnover, ie. high bone resorption and low bone formation [Yuuki et al. 2010, Nelson 2008]. Thus estrogens are considered as osteoprotective hormones.

This phenomenon can be modeled with ovariectomized rodents which develop osteoporotic phenotype. Estrogen binds to estrogen receptors alfa and beta, which are nuclear receptors. When estrogen receptor alfa is conditionally deleted in murine osteoclasts, both male and female mice have reduced bone mineral density. In addition it has been speculated that estrogen has an ability to increase Fas-ligand expression in both osteoclasts and osteoblasts, thus slowing the bone remodeling. Fas-receptors are death receptors that may trigger cell apoptosis. Estrogen receptor beta has more profound antagonist effects on bone than alfa [Yuuki et al. 2010]. However, female mice with all estrogen receptors genetically deleted do not display the expected osteoporotic phenotype, most likely due to over-production of testosterone [Yuuki et al. 2010].

In humans, estrogen is known to increase OPG expression and decrease RANKL expression in osteoblasts. Thus, estrogen decreases bone resorption to maintain the bone mass. During menopause, the reduced levels of estrogen leads to increased bone resorption and bone loss [Riggs 2000, Bord et al. 2003]. Similar findings are also noted in men aged 70 or more, since androgens are partially converted to estradiol [Ebeling 2010].

Androgens

The main androgen in humans is testosterone, which is further converted to dihydrotestosterone. Aromatase enzyme converts testosterone to estradiol, thus also

osteoprotective effects of estradiol are observed in men. Androgen receptors are expressed in osteocytes and osteoblasts, but not apparently in osteoclasts. It is known that reduced levels of serum androgens lead to reduced BMD. This mechanism is most likely mediated via osteoblasts. Low levels of testosterone are also associated with increased fracture risk [Ebeling 2010].

Calcitonin

Calcitonin is a peptide hormone secreted by parafollicular C cells within the thyroid gland. It is secreted in response to high levels of serum calcium. Calcitonin inhibits bone resorption by rapidly inhibiting osteoclast function through the calcitonin receptor. This results in dissociation of actin rings, representatives of active osteoclasts [Väänänen et al. 2000, Henriksen et al. 2010].

Glucocorticoids and glucocorticoid medication

Glucocorticoids are steroid hormones secreted by the adrenal cortex in response to various stimuli, such as stress. The most of glucocorticoid-mediated bone effects are seen in patients on glucocorticoid medication. The medication is often prescribed for example to rheumatoid diseases or lung diseases. Systemically taken glucocorticoid medication lasting over a month with any dose has an effect on bone turnover, while inhaled glucocorticoids seldom have any effect on bone turnover. Glucocorticoids are known to suppress osteoblast function, and increase bone resorption [Compston et al. 2009, Mazziotti et al. 2010, Rachner et al. 2010]. Glucocorticoid therapy also decrease levels of serum vitamin D. Taken together, glucocorticoid therapy leads to reduced BMD and increased risk of fractures in 30-50 % of patients taking these drugs [Mazziotti et al. 2010].

2.2.4 Bone turnover markers and their clinical use

Overview

Bone turnover markers (BTMs) are divided into bone resorption and formation markers, and several of them are described here. Bone resorption markers include C- and N-terminal cross-linking telopeptides of type I collagen (CTX, NTX), tartrate resistant acid phosphatase (TRACP5b), pyridinoline and deoxypyridinoline. Bone formation markers include alkaline phosphatase, osteocalcin and procollagen type 1 N-terminal propeptide (PINP). Like all laboratory measurements, these are affected by age, gender, medication and pregnancy of the patients. Additionally, BTMs are markedly affected by circadian rhythm and fasting status. Levels of BTMs are markedly reduced after food intake regardless of the type of food ingested. Thus all BTM samples should be taken in the morning after an overnight fast [Vasikaran et al. 2011]. Bone resorption and formation markers are coupled, ie. rise in bone resorption is usually followed by rise in levels of bone formation markers, and vice versa [Miller et al. 2008, Vasikaran et al. 2011].

Use of bone turnover markers in osteoporosis

The upper limit of premenopausal BTM values is used as a cut-off point for determining elevated BTM value. BTM levels have been shown to associate with the rate of bone loss. An increase in BTMs is associated with an increased risk of hip and other fractures in women aged 75 or more [Chopin et al. 2012].

Type I collagen synthesis in bone

To understand bone turnover markers it is crucial to understand type I collagen synthesis in bone. Procollagen type I is secreted by osteoblasts during bone formation (see PINP). The C- and N-terminal regions are cleaved from the procollagen type I, forming type I collagen. Type I collagen molecule is a triple helical protein, composed of two alfa1-polypeptide and one alfa2-polypeptide chains. The molecule contains gaps between collagen molecules, specifically their C- and N-terminal ends, respectively. Specific cross-links are found between these gaps, such as C- and N-terminal telopeptides (see CTX and NTX). These gaps are located within the collagen fibril. Cross-links are also found between alfa1 and alfa2 chains, such as pyridinoline and deoxypyridinoline which are described below [Civitelli et al. 2009, Coulibaly et al. 2010, Vasikaran et al. 2011].

CTX and NTX

CTX is C-terminal cross-linking beta-telopeptide of type I collagen, which is cleaved from type I collagen by cathepsin K, which is secreted by osteoclasts. It is used as an indicator of bone resorption, and is also in clinical use for the estimation of the catabolic rate of bone [Christgau et al. 1998, Vasikaran et al. 2011]. Food intake markedly decreases serum levels of CTX. Also the history of fractures affects the levels of serum CTX [Qvist et al. 2002, Ivaska et al. 2007]. CTX can also be measured from the urine. However, urine CTX must be adjusted for urine kreatinine, and the within subject variability is approximately twice as high compared to serum samples [Chopin et al. 2012]. Methods for the corresponding N-terminal telopeptide (NTX) are also available. The alfa-1 chain of type I collagen undergoes beta-isomerization as an age-dependent process. Thus, the ratio of alfa/beta-CTX can be used to estimate the amount of freshly formed and old bone, respectively [Civitelli et al. 2009].

ICTP

ICTP or MMP-CTX is a degradation product of type I collagen. ICTP is cleaved by matrix metalloproteinases, unlike CTX and NTX which are cleaved by protease cathepsin K. It appears that serum ICTP is not often affected by pharmaceutical therapy of osteoporosis, and thus is a poor bone turnover marker [Vasikaran et al. 2011].

Pyridinoline and deoxypyridinoline

Pyridinoline and deoxypyridinoline are produced during maturation of type I collagen, and subsequently released into circulation during bone resorption. Pyridinoline and

deoxypyridinoline are also released from cartilage. They can be measured from the urine and are used to estimate the catabolic rate of bone [Vasikaran et al. 2011].

TRACP5b

Tartrate resistant acid phosphatase (TRACP) has two isoforms. TRACP5a is found in platelets and red blood cells, while TRACP5b is secreted by osteoclasts in order to dephosphorylate bone proteins, such as osteopontin [Andersson et al. 2003, Civitelli et al. 2009]. Thus serum TRACP5b is a marker of osteoclast activity. It does not measure actual bone resorption [Vasikaran et al. 2011].

PINP

Serum procollagen type I N-terminal propeptide (PINP) is a marker of bone formation. PINP is formed through posttranslational cleavage of type I procollagen. Since type I procollagen is secreted by osteoblasts during bone formation, PINP is a marker of osteoblast activity [Civitelli et al. 2009, Vasikaran et al. 2011].

Alkaline phosphatase

Alkaline phosphatases are mainly derived from liver and bone in roughly equal amounts. Thus, bone-specific alkaline phosphatase assays have been developed. Bone specific alkaline phosphatase is expressed in osteoblasts, and the phosphatase is required for bone mineralization [Civitelli et al. 2009].

Osteocalcin

Osteocalcin is a bone matrix protein, which is produced by osteoblasts during bone formation. It can be measured from urine or serum. Osteocalcin is rapidly degraded, and thus methods are designed to measure both intact and fragmented osteocalcin. It is a bone formation marker [Vasikaran et al. 2011].

2.3 OSTEOPOROSIS

2.3.1 Pathoethiology of osteoporosis

Definition and prevalence of osteoporosis

WHO defines osteoporosis as a systemic skeletal disorder, which includes reduced bone mass, altered bone architecture and increased fracture risk. The diagnostic criteria of osteoporosis were revised by International Society for Clinical Densitometry in 2008 [NIH 2001, Lewiecki et al. 2008]. It is difficult to estimate prevalence of osteoporosis, as osteoporosis becomes more frequent in older age. In addition, people live longer lives, making estimations difficult. Approximately 40 % of postmenopausal Caucasian women have osteoporosis [Rachner et al. 2010]. In Netherlands age-adjusted prevalence

of osteoporosis is 12 % for men and 29 % for women, reaching up to 36 % in men and 57 % in women over 80 years of age [Schuit et al. 2003]. In Finland it is estimated that 6 % of women aged 40-50 have osteoporosis, and this increases to 9 % in 10 years of follow-up due to menopause [Alhava 2004]. In Europe, it has been estimated that 3.8 million osteoporotic fractures occurred in year 2000 [Reginster 2011].

Risk factors for low BMD

Genetics and gender

Peak bone mass is reached during puberty, and genetic factors determine roughly 70-80 % of the peak bone mass. Therefore a marked proportion of the risk for low BMD and osteoporosis are explained by genetic factors. In addition, genetic association in fracture risk has been demonstrated. This is partially explained by genetic association for low BMD [Bonjour et al. 1991, Eisman 1999]. For example, patients whose parents have suffered a hip fracture are at elevated risk of fracture themselves, suggesting genetic predisposition to fractures [Compston et al. 2009]. Men acquire higher peak bone mass. This is most likely due to higher levels of serum androgens. Also the estrogen induces earlier closure of the growth plates in girls compared to boys, leading to longer bones with higher bone mass in men [Ebeling 2010].

Age

In older age, the bone remodeling is slowed. This leads to slower resorption of bone, and even slower formation of new bone (the coupling effect). This leads to reduced BMD [Szulc et al. 2009]. Bone mass is even more dramatically lowered during menopause in women, since the levels of circulating estrogen decrease. Similar more rapid decrease in bone mass is observed in 70-year old men [Ebeling 2010].

Medical conditions and drug-induced osteoporosis

Many medical conditions and medications are associated with increased risk for osteoporosis (**Table 4**). Malabsorption diseases and bowel diseases, such as inflammatory bowel diseases and celiac disease, are associated with reduced calcium and vitamin D intake, and thus predispose to osteoporosis. Hypogonadism affect circulating levels of sex hormones, which are discussed in 2.2.3. Hyperthyroidism accelerates bone turnover leading to reduced BMD. In addition, rheumatoid arthritis, chronic obstructive pulmonary disease and chronic liver failure are associated with low BMD and increased fracture risk, partially due to medication of these conditions [Lane 2006, Compston et al. 2009].

Several medical conditions require medication, which may cause drug-induced osteoporosis. Multiple drugs are known to induce osteoporosis, especially glucocorticoids, hormonal therapy, some diabetes type 2 drugs, anticonvulsants, anticoagulants and loop diuretics [Mazziotti et al. 2010]. Effects of glucocorticoids, estrogens and androgens are described above. Drug-induced osteoporosis occurs also in premenopausal women

and in men, and fractures may occur at higher BMD values than in postmenopausal osteoporosis. Thus it is recommended that drug-induced osteoporosis, especially glucocorticoid-induced, should be treated when T-score is -1.5 SD or lower [Mazziotti et al. 2010].

Table 4. Selected known causes of secondary osteoporosis [Modified from Compston et al. 2009, Mazziotti et al. 2010].

Causes of secondary osteoporosis	Medication known to cause osteoporosis
Rheumatoid arthritis	Glucocorticoids
Hypogonadism	Anti-estrogen or anti-testosterone therapy
Diabetes type I	Anticonvulsants
Hyperthyroidism	Anticoagulants
Gastrointestinal diseases	Diabetes type II drugs
Chronic liver disease	Loop diuretics
Chronic obstructive pulmonary disease	
Malnutrition	
Anorexia	
Excess smoking and alcohol consumption	
Chronic kidney disease	

Behavior

Smoking is associated with accelerated bone loss, possibly due to direct toxic effects and reduced calcium absorption from the gut. High alcohol consumption is associated with reduced BMD. Certain types of physical activity have a small but important impact on BMD and fracture risk. Best types of physical exercise were walking, jogging, jumping and Tai Chi. Also improved muscle strength due to exercise reduces the risk of falling. Adequate dietary sources of both calcium and vitamin D are necessary for healthy skeleton [Lane 2006, Järvinen et al. 2008, Howe et al. 2011].

2.3.2 Diagnosis of osteoporosis

Clinically osteoporosis is diagnosed by measuring the bone mineral density (BMD) using dual energy X-ray absorptiometry (DXA). However, osteoporosis does not automatically mean medication, as described in 2.3.4. Therefore, DXA scans are recommended for patients who could benefit from the medication for osteoporosis. Indications for DXA scan are (see 2.3.4 for risk factors) [Lewiecki et al. 2008]:

- Women over 65 years with at least 1 risk factor
- Men over 70 years with at least 1 risk factor
- Patients with medical condition or medication known to affect bone

T- and Z-scores are calculated from DXA scans, which use a reference population. Currently used reference population is the 3rd national health and nutrition survey

(NHANES III) consisting of 20-29 year old women. T-scores are calculated in relation to this reference population, where persons with BMD lower than 2.5 SD from the average are considered to have osteoporosis. On the other hand, T-score -2.5 SD represents approximately a 25 % decrease in bone mass. Z-scores are furthermore adjusted to age and sex-matched subjects. Further adjustment can be made using database of the same nationality [Watts et al. 2004, Lewiecki et al. 2008].

DXA scans are affected by multiple factors. DXA equipment should be calibrated for correct sex, age and ethnicity. The X-ray beam and the patient must be positioned in standard position, and appropriate analysis software must be used. Diagnostic region of interest is the femoral neck. In patients older than 50 years also lumbar spine, total hip or distal radius BMD can be measured. BMD of distal radius should be measured from the non-dominant hand. Lumbar spine is measured from vertebrae L1 to L4, using the average value. Individual vertebrae may be excluded if marked deviation from average lumbar spine value is found [Watts et al. 2004, Lewiecki et al. 2008].

In adults, T-score -2.5 SD and below is considered as osteoporosis when measured from the locations described above. In adults younger than 50 years, also the Z-score must be -2.0 SD or less. T-scores between -1.0 SD and -2.5 SD are considered as osteopenia [NIH 2001, Lewiecki et al. 2008].

In children, age-matched Z-score -2.0 SD and history of marked fractures are considered as osteoporosis. Marked osteoporotic fractures in children are fractures of long bones (one in lower limbs or two in upper limbs), or a vertebral fracture. It is also suggested that the term osteopenia should not be used in children [Lewiecki et al. 2008].

Quantitative computer tomography and quantitative ultrasound are also used to assess bone quality. However, low BMD assessed by these methods should be verified by central DXA measurement. Quantitative ultrasound from the heel region is a good method for population screening as it doesn't involve the use of X-rays [Lewiecki et al. 2008].

2.3.3 Fracture risk in osteoporosis

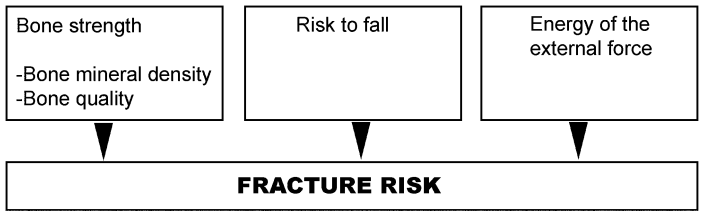

Figure 8. Determinants of fracture risk.

Fracture risk

Fracture risk is composed of bone strength, trauma energy and risk of fall (**Figure 8**). Trauma energy means for example motor vehicle accident versus a fall on level. Bone strength includes BMD and bone quality, while BMD is discussed above [Kanis et al. 2002, Järvinen et al. 2008, Reginster 2011].

Bone quality

Bone quality is determined by micro-architecture, mineralization, geometry and accumulation of damage to bone. Bone quality is an important factor in addition to bone mineral density (BMD), when the bone health is assessed. However, bone quality is clinically difficult to determine, while BMD is easy to measure [Gallacher et al. 2010]. Bone microarchtiecture is determined as width, number and spatial organization of bone trabeculae, and can be assessed by histomorphometry (bone biopsy) or modern computerized imaging methods [Dalle et al. 2005]. It is speculated that osteocytes maintain the microarchitectural organization of bone, which is arranged to withstand the load on the bone [Shapiro 2008, Bonewald 2011, Klein-Nulend et al. 2003].

Risk to fall

It is also important to remember that in addition to bone quality and BMD, fracture risk is affected by the risk to fall. Poor vision, balance and muscle strength, impaired cognition, and certain medical conditions predispose to falls and thus to fractures. These are especially important factors in the elderly people [Lane 2006, Järvinen et al. 2008].

Fracture risk and osteoporosis – the target of treatment

Osteoporotic fractures include fractures of wrist, spine and hip, but also trochanteric, humerus and rib fractures are considered as osteoporotic fractures [Rachner et al. 2010]. BMD alone is a poor predictor of fracture risk. It is estimated that from all types of fractures considered as osteoporotic fractures, such as wrist fractures, only a minority of fracture patients had osteoporosis, while approximately half of the fracture patients had osteopenia. In other words, majority of actual fractures occur in patients with osteopenia. However, the relative risk of having a fracture increases when BMD becomes lower, and therefore osteoporosis should be treated [Siris et al. 2004]. On the contrary, increase in BMD only is not associated with reduced risk of fracture [Roux et al. 2005]. Thus, other parameters in addition to BMD are required to assess fracture risk.

FRAX is a computer program designed to assess individual's risk for having a major osteoporotic fracture within the next 10 years, and thus guides clinician to select which patients to treat [Kanis et al. 2002, Rachner et al. 2010]. The calculation is based on femoral neck or total hip BMD and then a list of risk factors including age, body mass index, prior fragility fracture, parental history of hip fracture, smoking, alcohol, steroid usage and history of rheumatoid arthritis. For example, patient with T-score -2.5SD and aged 50 has a smaller risk of having an osteoporotic fracture than similar patient aged 80

(**Figure 9**). This risk is further modified by other risk factors for fracture. Fracture risk of 10 % is considered as a high risk. FRAX does not take into account the risk of falling (see above) and is not suitable for patients who are treated with antiresorptives or bone anabolic therapy [Kanis et al. 2002, Kanis et al. 2009].

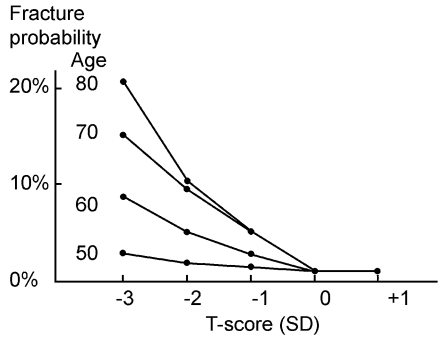

Figure 9. Ten-year hip fracture probability in Swedish women based on age and BMD [Modified from Kanis et al. 2002].

2.3.4 Treatment guidelines of osteoporosis

Treatment of osteoporosis should include fall prevention, lifestyle advice (smoking, alcohol), appropriate calcium and vitamin D supplementation and weight-bearing exercise [Compston et al. 2009, Reginster 2011]. In addition, antiresorptive therapy should be offered to patients with osteoporosis and additional risk factors. At least two risk factors for fracture support the start of osteoporosis medication. These risk factors include:

- Age 60 years or older
- Body mass index 19 kg/m^2 or less
- Previous osteoporotic fracture
- Parental history of hip fracture, reflecting genetic background of low BMD
- Current smoking, glucocorticoid treatment (any dose for at least 1 month) or high alcohol consumption
- High risk of fall
- Immobility
- Multiple other medical conditions known to affect bone, see **Table 4**

2.3.5 Current strategies to reduce the fracture risk

Non-pharmaceutical therapy of low BMD focuses on removing risk factors for fractures. This includes stopping of smoking, reduction of alcohol consumption, more physical exercise (see 2.3.1), and adequate supplementation of both calcium and vitamin D [Rachner et al. 10, Lane 2006]. Recommended daily doses for calcium for adolescents

are at least 500-1300mg and 600-800 IU (15-20 micrograms) for vitamin D [Compston et al. 2009, Rachner et al. 2010, Abrams et al. 2011]. Since most of osteoporotic fractures are caused by falls, it is important to reduce risk to fall, for example by improving muscle strength, vision and balance.

Pharmaceutical therapies have been designed to increase BMD. Patients with osteoporosis who are treated with osteoporosis drugs (**Table 5**) have usually their fracture risk reduced. However, BMD changes are not necessarily correlated with reduction in the risk of fracture. Thus the bone turnover markers are used. Generally, BMD correlates with bone turnover markers, such as serum CTX and PINP [Melton et al. 1997, Reginster et al. 2001, Vasikaran et al. 2011]. Bone turnover markers can be used to monitor efficacy of osteoporosis treatment and reduction in BTM is associated with reduced fracture risk. However, BTMs cannot be used to predict fractures in individual subjects [Brown et al. 2009, Vasikaran et al. 2011].

Table 5. Selected pharmaceuticals for osteoporosis in 2011 [Henriksen et al. 2010, Eastell et al. 2010, Rachner et al. 2011].

Antiresorptives	**Type**	**Administration**	**Dose**
Alendronate	N-Bisphoshonate	Oral	Weekly 70 mg
Risendronate	N-Bisphoshonate	Oral	Weekly 35 mg
Ibandronate	N-Bisphoshonate	Oral (also intravenous)	Monthly 150 mg
Zoledronic acid	N-Bisphoshonate	Intravenous	Annually 5 mg
Clodronate	Non-N-Bisphosphonate	Oral	not recommended
Etidronate	Non-N-Bisphosphonate	Oral	not recommended
Calcitonin	Hormone	Nasal spray	Daily 200 IU
Denosumab	Antibody	Subcutaneous injection	Every 6 months 60 mg
Hormone replacement therapy	Hormone	Varies	Varies
Raloxifene	SERM	Oral	Daily 60 mg
Lasofoxifene	SERM	Oral	In phase III
Tibolone	Hormone	Oral	Daily 1.25 mg
Bone anabolics			
Teriparatide	Hormone	Subcutaneous injection	Daily 20 μg
Strontium ranelate	Mineral	Oral	Daily 2 g

Bisphosphonates

Bisphosphonates (BPs) are designed to treat postmenopausal osteoporosis in women and also osteoporosis in men. Most commonly clinically used BPs are shown in **Table 5**. Non-amino-BPs are the older drugs, and amino-BPs the newer drugs with more available clinical data. Structure of bisphosphonates is shown in **Figure 10.** Recommended duration for BP treatment is 5 years, as the drug accumulates to bone and the suppression of bone turnover continues after stopping the therapy [Miller et al. 2008, Gallachan et al. 2010].

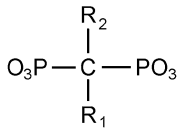

Figure 10. Structure of bisphosphonate. R1 is a hydroxyl group, except in clodronate where it is a chloride. R2 varies according to the drug [Modified from Halasy-Nagy et al. 2001].

Bisphosphonates listed in **Table 5** have number needed to treat (NNT) values between 14 and 21 for vertebral fractures in three years. This means that 14 to 21 patients should take the drug for three years to prevent one vertebral fracture from occurring. The respective NNT for hip fractures is 91 [Reginster 2011]. Alendronate therapy reduces the risk for vertebral and non-vertebral fractures in osteoporotic patients. In patients without osteoporosis, only vertebral fractures were prevented. Similar effects are seen with other BPs [Wells et al. 2008, Miller et al. 2008, Gallacher et al. 2010].

BP therapy reduces bone resorption markers, followed by reduction in bone formation markers due to coupling of bone remodeling. Average reduction in serum CTX in postmenopausal women is roughly 50 % after three-month therapy of oral alendronate 70 mg/week. This is though subject to a lot of variation [Rosen et al. 2004, Saag et al. 2007, Brown et al. 2009].

Patients treated with BPs gain more bone mass as estimated by bone biopsies [Gallacher et al. 2010]. This effect is also seen using DXA scans and the patients have their BMD increased. BMD increases using alendronate in postmenopausal women approximately +5 % in 12 months [Tonino et al. 2000, Rodan et al. 2004, Rosen et al. 2004]. However, BMD changes are not correlated with reduction in risk of fracture [Roux et al. 2004].

Children with osteogenesis imperfecta, a rare disease of fragile bones, may be treated with bisphosphonates, such as zoledronic acid. However, study cohorts of children with osteogenesis imperfecta are small, but zoledronic acid appears to be effective and safe drug in children. Growth curves of these children were not affected by zoledronic acid [Brown and Zacharin 2009].

When taken orally, only 1-2 % of the drug is absorbed from the gut. Thus BPs should be taken on empty stomach [Reginster 2011]. Bisphosphonates are designed to bind to bone, which is subsequently resorbed by osteoclasts. BPs enter the osteoclast killing these cells, leading to slower bone remodeling allowing the bone to mineralize properly. BPs inhibit bone resorption due their ability to bind to Ca^{2+} ions [Sato et al. 1991, Fujita et al. 2005]. Half of the drug binds instantly to bone and half is excreted in the urine [Miller et al. 2008]. Subsequently the bone is resorbed by osteoclasts, and the BPs enter the osteoclast via fluid-phase mediated endocytosis [Thompson et al. 2006]. In cell culture experiments, BPs reduce the number of both osteoclasts and resorption pits [Selander et al. 1994, Kellinsalmi et al. 2005, Coxon et al. 2008].

There are two types of BPs; amino and non-amino-BPs. Non-amino-BPs generate toxic analogues of ATP which disrupt the function of mitochondria, leading to osteoclast apoptosis [Reszka et al. 2003, Rogers 2003]. Amino-BPs inhibit farnesyldiphosphate syntase, interfering with posttranslational modification (isoprenylation) of small GTPases including Ras, Rac and Rho. This causes the membrane-bound small GTPases to relocate into cytosol. This in turn leads to reduced ERK-pathway signaling, which is a downstream mediator of Ras. This leads to mitochondrial membrane depolarization, activation of caspase cascade, and ultimately to osteoclast death [Fujita et al. 2005, Bergstrom et al. 2000, Dunford et al. 2006]. BPs inhibit bone resorption also through mechanisms independent of apoptosis, such as disruption of actin rings [Halasy-Nagy et al. 2001].

Adverse effects of BP therapy

Like all drugs, also BPs have adverse effects. Most common are gastrointestinal discomfort and influenza-like symptoms. Rare complications are the osteonecrosis of jaws and atypical fractures. Osteonecrosis of jaws is associated with intravenous zoledronic acid treatment, and is either sclerotic or lytic lesion. Bone microcracks and atypical fractures, such as subtrochanteric femoral fractures, are associated with alendronate therapy lasting over 5 years, but are rare compared to incidence of osteoporotic fractures [Abrahamsen 2010, Gallacher et al. 2010]. Atrial fibrillation is also a possible side-effect of zoledronic acid [Miller et al. 2008].

Bisphosphonates and other cell types

It has been shown, that in addition to osteoclasts, some cancer cells take up bisphosphonates. This triggers the expression of several phosphoantigens on the surface of certain cancer cells, especially in estrogen receptor-positive breast cancer cells. It has been postulated that bisphosphonates may have anti-tumor effects [Hamilton et al. 2011]. There is also some evidence that bisphosphonates may have anti-apoptotic effects in osteoblasts and osteocytes [Miller et al. 2008].

Denosumab

Denosumab is injected subcutaneously every six months. Denosumab is a human monoclonal antibody for RANKL. It thus reduces the amount of soluble RANKL mimicking the effects of OPG. This results in reduced activation of OPG/RANK/RANKL signaling between osteoclasts and osteoblasts, inhibiting development and activation of osteoclasts. This in turn leads to reduced bone resorption, increased BMD and reduction in levels of BTMs after denosumab therapy. These effects on bone are more profound with denosumab compared to bisphosphonates [Cummings et al. 2009, Eastell et al. 2011]. The effect on vertebral fracture prevention is similar between BPs and Denosumab (NNT 14-21). A slight but statistically significant preventive effect on hip fractures was observed (0.7 % versus 1.2 %), resulting in NNT value of ~300. Thus, the actual preventive effect of denosumab is currently under debate [Reginster 2011].

Calcitonin

Calcitonin is available as nasal spray, and salmon calcitonin is used. Calcitonin rapidly inhibits osteoclast function. Calcitonin therapy reduces levels of CTX, increases BMD of the patients, and reduces the risk for vertebral fractures. In addition, calcitonin has a small but notable analgesic effect [Miller et al. 2008, Henriksen et al. 2010].

Hormone replacement therapy and SERMs

The primary indication of hormone replacement therapy (HRT) is to treat menopausal symptoms, and HRT consists of supplemental estrogen and progestin therapy. Since estrogen is a bone-beneficial hormone and levels of serum estrogen decrease during menopause, HRT may be used to treat and prevent osteoporosis in menopausal women. However, HRT is associated with increased risk of breast and uterine cancer and deep venous thrombosis. Thus HRT is not licensed for prevention of osteoporosis as a first line-treatment, even though HRT has been shown to actually prevent osteoporosis and decrease fracture risk. Due to various adverse effects, the start of HRT must be considered carefully [Miller et al. 2007, Palacios 2008].

Because HRT is associated with severe adverse effects, selective estrogen receptor modulators (SERMs) are designed to mimic estrogen and cause either antagonist or agonist effects depending on the target tissue. Currently used SERMs are tamoxifen (no bone effects), raloxifene and lasofoxifene. SERMs are primarily designed to treat estrogen receptor positive breast cancer, but raloxifene and lasofoxifene are also approved for treatment of osteoporosis in postmenopausal women. Raloxifene therapy reduced levels of BTMs, increases BMD and reduces fracture risk, and is also beneficial in treatment of breast cancer [Miller et al. 2008, Bolognese 2010].

Tibolone

Tibolone is a progestin analogue which has also androgen effect on bone metabolism. It is licenced for prevention and treatment of postmenopausal osteoporosis. It increases BMD better than raloxifene [Miller et al. 2008].

Parathyroid hormone analogues

Teriparatide is a human recombinant parathyroid hormone analogue consisting amino acids 1-34 (PTH1-34). Also physiological PTH1-84 is used in some countries. Teriparatide is a bone anabolic drug, which results in increased bone remodeling, most likely due osteoblast activation. This is mediated via decreased OPG and increased RANKL secretion. This osteoblast modulation results in increased resorption, but rather surprisingly leads also to increased BMD. Both bone formation and bone resorption markers are increased during teriparatide therapy, and the therapy results in increased bone mass [Dobnig et al. 2005, Gallacher et al. 2010] and reduced fracture risk. Reduction in fracture risk is slightly better with Teriparatide (NNT 12) compared to bisphosphonates (NNT 14-21) [Neer et al. 2001, Reginster 2011]. The effects of teriparatide to bone

health are more profound than of alendronate [Gallacher et al. 2010]. However, if PTH is given continuously, it has catabolic effect on bone [Compston et al. 2007].

Strontium ranelate

Strontium ranelate has both bone anabolic and antiresorptive characteristics. The detailed mechanism of how strontium ranelate affects remains unclear. Strontium resembles calcium ions, and is conjugated to ranelic acid carrier. It has been speculated that the effects of strontium are mediated via reduced bone resorption and increased bone remodeling. Some studies show an increase in BTMs, while others show a decrease or no change at all. However, patients taking strontium have reduced risk of fractures compared to placebo [Gallacher et al. 2010, Reginster 2011].

3 AIMS OF THE CURRENT STUDY

The purpose of this study was to evaluate bone health and osteoporosis in patients with NF1. The specific aims were as follows:

1. To evaluate the dimensions of craniofacial bones between patients with NF1 and control persons using lateral X-rays of the skull

2. Characterize osteoclasts derived from peripheral blood samples of NF1 patients and healthy controls under laboratory conditions

3. The findings on osteoclasts in study II expanded this study to further evaluate how NF1-osteoclasts respond to bisphosphonates, drugs designed to cause osteoclast apoptosis

4. Assess the frequency of osteoporosis in Finnish NF1 patients

5. To evaluate possible changes in bone mineral density of NF1 patients, whose bone mineral density was remeasured 12 years after the initial study in 1999

6. To evaluate the fracture risk in NF1 by screening the medical records in a controlled register-based study

4 MATERIALS AND METHODS

4.1 RECRUITMENT OF PATIENTS WITH NF1 AND CONTROLS (I-V)

NF1 cephalometry study (I)

For study I the 85 patients were recruited from the NF clinic at the Department of Dermatology, Turku University Hospital, as well as among the members of the Finnish NF Society, during years 2005-2010. Ethical approval was obtained from the Ethics Committee of Southwest Finland Hospital District, Turku, Finland. Age and sex-matched controls were randomly selected from a group of healthy Finnish school children and adults from Oulu, Finland, who voluntarily took part in the cephalometric examination. An ethical approval to collect and use the control material for research was obtained from the Ethical Board of Oulu University Hospital, Oulu, Finland. The detailed information can be found in study I.

NF1 and bone health study (II,III)

For studies II and III the patients were recruited from patients attending the NF clinic at the Department of Dermatology, Turku University Hospital. Patients were enrolled to the study also via Finnish NF Society. The 17 patients for study II were recruited during years 2005-2009, and the 21 patients for study III between 2009 and 2011. There was some overlap between studies II and III. Age and sex-matched controls were healthy volunteers recruited from Turku University personnel. All participants gave their written consent. Ethical approval was obtained from the Ethics Committee of Southwest Finland Hospital District, Turku, Finland. The detailed information can be found in studies II and III.

BMD 12 years after study (IV)

Study IV was based on a cohort of 35 patients with NF1 who were examined by Kuorilehto et al. in 1999 [Kuorilehto et al. 2005]. Now the same group of 35 patients with NF1 was approached via a letter, and were invited to the current study. This resulted in 28 patients who took part in the study, and 19 of them visited Oulu University Hospital, Oulu, Finland. Patients were examined during the year 2011. Detailed information can be found on study IV.

Fracture risk in NF1 study (V)

A computerized search for all patients with ICD-10 diagnosis Q85.0 was performed in three University Hospitals (Turku, Tampere, and Oulu University Hospital). This included all ICD-10 subdiagnoses, including NF1 (Q85.00). The social security numbers of these patients were subsequently screened in the three University Hospitals. Patients

whose diagnostic criteria for NF1 were noted down in the hospital records were included. This resulted in 146 patients with NF1 in Turku region, 120 patients in Tampere region, and 196 patients in Oulu region (total 460). The study was approved by Ministry of Social Affairs and Health, Finland, and the Ethical Committee of Southwestern Hospital District, Turku, Finland. The detailed information can be found in study V.

4.2 SHORT INTRODUCTION TO CEPHALOMETRICS (I)

Cephalometric analysis is a method for evaluation of dimensions of cranial and facial bones. A number of computer-assisted analyses have been developed. The analysis is based on the lateral X-ray of the skull, and a cephalostat is used to position the participant's head in a fixed position. In this study, McNamara, Ricketts and Steiner analyses were used. McNamara analysis was used to measure dimensions and positions of maxilla and mandible. This analysis has been originally developed for planning of orthodontic and orthognathic treatment [McNamara 1984]. Steiner analysis was used to evaluate the anteroposterior position of mandible and maxilla [Steiner 1959]. Ricketts analysis was used in the current study to measure the dimensions of cranial base and jaws [Ricketts 1961, Ricketts 1981].

4.3 HISTOCHEMISTRY (II)

Histological analysis using TRACP and hematoxylin eosin-stainings was performed on samples from three patients [Kuorilehto et al. 2006, Leskelä et al. 2009] undergoing amputation for NF1-related pseudarthrosis. The detailed information can be found in study II.

4.4 OSTEOCLAST DIFFERENTIATION ASSAY (II,III)

The osteoclast differentiation assay is described here in whole.

Peripheral blood samples taken in lithium-heparin vials were diluted 1:1 in PBS immediately after taking the sample. Mononuclear cell fraction including osteoclast progenitor cells was isolated from blood samples using Ficoll gradient centrifugation, followed by PBS wash. Cell counting was carried out under light microscope with Bürker chamber. The cells were used either immediately for culturing or stored in liquid nitrogen. For storage in liquid nitrogen, the cells were suspended in 10 % dimethylsulfoxide and 90 % heat-inactivated fetal calf serum (Gibco, NY). Storaging of the progenitor cells in liquid nitrogen did not have any apparent effect on the results of following cell cultures.

Bone slices, size 5x10 mm, were cut from bovine bone using low-speed saw and rinsed in ethanol, followed by ultrasound sonication for 1 min. Cells of each patient/control pair were cultured on bone slices from the same bone of the same animal. Medium for culture

contained alpha-MEM (Gibco, NY) supplemented with 10 % heat-inactivated fetal calf serum (Gibco, NY), penicillin-streptomycin, and the essential osteoclastogenic factors RANKL (20 ng/ml, Peprotech, Rocky Hill, NJ) and MCSF (10 ng/ml, R&D systems, Minneapolis, MN) for 10 days.

Mononuclear cells stored in liquid nitrogen were melted and suspended in medium (50 µl, one million mononuclear cells or 0.1 million CD14 positive cells / bone slice). Six bone slices and one control slice were prepared for each patient/control pair. Cell suspension was placed on each bone slice. Control slices were covered with medium without cells. Cells were allowed to attach onto bone slices for two hours in cell culture incubator. After the incubation, the bone slices with the cells were placed into 24-well culture plates (Falcon, Franklin Lakes, NJ) with 1 ml of culture medium. The cells were cultured at 37°C in the presence of 5 % CO_2 for 10 days. Medium was changed once every four days by replacing half of old medium with fresh with double concentration of both RANKL and MCSF.

To collect CD14 positive osteoclast progenitor cells, magnetic cell separation method (MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany) was used. Cells were first filtered with MACS pre-separation filters, and then suspended into 80 µl of buffer and 20 µl MACS antibody to CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 million mononuclear cells. Suspension was kept in cold for 15 min, before applying into the magnetic separation column, followed by separation procedure according to the manufacturer's instructions.

Cell culture samples were fixed with 4 % paraformaldehyde in PBS for 20 min. Cells were then permeabilized using 0.1 % Triton X-100 in PBS on ice for 5 min. Bone slices were stained for tartrate resistant acid phosphatase staining (TRACP) for visualising osteoclasts in light microscopy. Leukocyte acid phosphatase kit (Sigma) was used. The staining was carried out according to manufacturer's instructions at 37°C for 60 min, followed by Hoechst-staining (1.25 µg / 1 ml PBS, Molecular Probes, Eugene, OR) for 3 min. To visualize actin rings, the slices were incubated with 3 nM rhodamine-conjugated phalloidin (Sigma-Aldrich, Steinheim, Germany) in dark at room temperature for 30 min. Finally, bone slices with cells were mounted with 90 % glycerol on microscope slides.

Micrographs were taken using Leica DMRB fluorescence microscope (Leica, Wetzlar, Germany) with Leica IM50 version 4.0 image analysis software using x10 or x20 objective depending on the density of cells. UV-micrography was chosen for better visualization of nuclei and for better contrast. Dark blue color represents TRACP-staining which looks purple in light microscopy. TRACP-positive cells having at least three nuclei were considered as osteoclasts. Due to the high number of cells per bone slice, twelve visual fields were photographed from each bone slice. Visual fields were always selected from the same areas on bone slices. Slices with notably small total amount of osteoclasts were excluded, such as those under 20 % of the average amount. The cell counts represent

averages of three to six bone slices per each person. Micrographs of actin rings for detailed analysis were taken with Leica DMR confocal argon-krypton laser scanning microscope (Leica, Heidelberg, Germany) with Leica Microsystems software version 2.5 for optimal image quality.

To analyze resorption pits, peroxidase-conjugated wheat germ agglutinin (WGA) -lectin staining (Sigma-Aldrich, Steinheim, Germany) was performed to locate the pits, as described in detail [Selander et al. 1994]. Pit edges appear as brown-stained areas. Bone slices were scanned with Olympus BX51 motorized microscope (Olympus, Tokyo, Japan) with Olympus DotSlide 1.2 software. Each pit on each slice was counted. A detailed analysis was performed on a smaller region of interest where pit density was highest, using NIH ImageJ software version 1.38x, and analysing a total of 50-200 pits per slice.

4.5 EVALUATION OF BISPHOSPHONATES AND FTS IN ISOLATED OSTEOCLASTS (III)

Cells were cultured for seven days as described above. This was followed by a two-day culture with normal medium with RANKL and MCSF, but different bisphosphonates were added, concentrations 10E-6 M, 10E-7 M and 10E-8 M. Amino-bisphosphonates zoledronic acid (Zometa ®, Novartis Finland, Finland) and alendronate (Sigma, dissolved in DMSO) were used. Also non-amino-bisphosphonate clodronate 10E-7 M (Leiras, Finland) was used. Ras-inhibitor farnesyl thiosalisylic acid (FTS) was purchased from Cayman chemicals (Ann Arbor, MI) and concentration 10E-6 M was used.

4.6 APOPTOSIS (III)

Cells were cultured on bone as described above, with and without 10E-7 M zoledronic acid. A caspase-3 colorimetric assay kit (PromoCell, Heidelberg, Germany) was used to measure enzymatic activity of caspase-3, a marker of apoptosis. Cells were lysed and treated according to manufacturer's instructions. Absorbance was measured using Hidex Chameleon 96-plate reader (Hidex, Turku, Finland) at 405 nm.

4.7 RADIOLOGICAL BMD MEASUREMENT (III-V)

BMD of the 21 patients with NF1 who took part in study III was measured using different densitometers. Detailed information can be found in study III. BMD of the 19 patients with NF1 who took part in study IV was measured in Oulu university hospital using Lunar densitometer. Detailed information can be found in study IV. BMD data shown in study V was acquired from medical records and thus were taken with different densitometers. The diagnosis of osteoporosis and osteopenia was made according to the current diagnostic criteria [NIH 2001, Lewiecki et al. 2008].

4.8 SERUM MEASUREMENTS (II-IV)

Serum sample measurements were performed in studies II, III and IV, and are listed below

Study II CTX

Study III Total calcium, ionized calcium, inorganic phosphate, parathyroid hormone, 25-D-vitamin, and alkaline phosphatase, including its isoenzymes. CTX, PINP and TRACP5b.

Study IV Total calcium, ionized calcium, inorganic phosphate, parathyroid hormone, 25-D-vitamin, alkaline phosphatase, and thyroid stimulating hormone. ICTP, PINP and TRACP5b.

The meaning and use of individual measurements are described in section 2.2.4. In study II, CTX measurement was purchased from TYKSLAB, Turku, Finland. In study III, the measurements were purchased from TYKSLAB, Turku, Finland, but control PINP samples were purchased from Oulu University Hospital, Oulu, Finland. In study IV, all serum measurements were purchased from Oulu University Hospital, Oulu, Finland. Detailed information can be found in the corresponding studies.

4.9 STATISTICS (I-V)

Detailed information about statistics can be found in each corresponding original publication.

5 RESULTS

5.1 SHORT JAWS AND CRANIAL BASE ARE COMMON IN NF1 (I)

Many developmental disorders are associated with craniofacial dysplasia. NF1 often displays with skeletal involvement, suggesting that there could be a disorder in craniofacial morphogenesis. The aim of study I was to characterize the craniofacial dimensions between NF1 patients and Finnish controls using lateral X-rays of the skull. A total of 85 patients with NF1 and their age and sex-matched controls were enrolled. The results showed that the patients with NF1 have short mandible, maxilla and cranial base compared to controls. Short jaws and cranial base were evident in males and females, but statistically significant only in adults, most likely due to limited number of children and adolescents. It should be noted that these NF1 patients did not require any orthodontic or orthognathic treatment, as marked malocclusion was not found.

5.2 OSTEOCLASTS IN NF1-RELATED PSEUDARTHROSIS (II)

Histological analysis of NF1-related pseudarthrosis tissues revealed large multinuclear TRACP-positive osteoclasts in contact with bone. Osteoclasts were also found without direct contact to bone, within the fibrous pseudarthrosis tissue, but without any signs of apoptosis. These findings were compared to adjacent areas of healthy-looking bone. Markedly higher amount of osteoclasts were present in the pseudarthrosis site compared to adjacent bone.

5.3 A NEW METHOD FOR STORING OSTEOCLAST PROGENITORS (II,III)

The methods for isolation of osteoclast progenitors in liquid nitrogen were optimized. Even after storage in liquid nitrogen, the osteoclast progenitors remained viable and we were able to culture them into multinuclear TRACP-positive cells which resorb bone, apparently osteoclasts. This method was fundamental in the studies included in this thesis.

5.4 NF1 OSTEOCLASTS ARE HYPERACTIVE AND APOPTOSIS-TOLERANT (II,III)

Osteoclasts derived from 17 patients with NF1 and controls were evaluated *in vitro*. The following parameters were assessed:

- Osteoclast number and number of nuclei per osteoclast, representing osteoclast formation capacity.

- Actin rings as a representative of active osteoclasts.
- Number of resorption pits on bone slice and CTX released into cell culture media, as representatives of osteoclast resorption capacity.
- Survival in serum-free conditions and under the *in vitro* effects of different bisphosphonates, as representatives of tolerance to apoptotic stimuli.

The results showed that NF1 osteoclast progenitors have increased osteoclast formation capacity compared to control osteoclast progenitors. Thus, more osteoclasts are generated from the same amount of progenitors. NF1 osteoclasts display more frequently an actin ring, representing that an increased proportion of osteoclasts is resorbing. The net result in bone resorption, estimated either by the number of resorption pits or CTX released into cell culture media, was increased approximately two-fold in NF1 samples. This two-fold increase was found using both measurement methods, indicating that these two methods give a comparable result. It was also shown that this NF1-osteoclast phenotype is independent of the presence of RANKL and MCSF. These findings were verified in study III which included 20 NF1 patients and 20 controls.

NF1 osteoclasts were shown to tolerate serum-free conditions better than the control osteoclasts in study II. This encouraged us to evaluate the effects of bisphosphonates on isolated NF1 osteoclasts. NF1 osteoclasts were demonstrated to be insensitive to apoptotic stimuli of alendronate, zoledronic acid and clodronate. The role of apoptosis was estimated by measuring the levels of caspase-3, a marker of apoptosis, in cell lysates with and without zoledronic acid. The increase in caspase-3 levels were lower in NF1 samples treated with zoledronic acid compared to controls. In addition, large multinuclear osteoclasts were noted in samples treated with bisphosphonates, supporting the view of apoptosis-insensitivity rather than increased proliferation of NF1 osteoclasts. Since clodronate causes apoptosis with a different mechanism compared to alendronate and zoledronic acid, it appears that NF1 osteoclast tolerate multiple types of apoptotic signals.

The addition of farnesyl thiosalicylic acid (FTS), an inhibitor of Ras, abolished the insensitivity to zoledronic acid observed in NF1 osteoclasts. This suggests that tolerance to apoptosis in NF1 osteoclasts could be mediated via Ras-pathway, which in turn is in agreement with neurofibromin functioning as a Ras-GAP.

5.5 SERUM VALUES ASSOCIATED WITH BONE HEALTH (III,IV)

Selected serum values were measured from 20 NF1 patients in study III and 19 NF1 patients in study IV, thus the results of 39 patients are summarized here. Reference values of University Hospitals of Turku and Oulu were used. Calcium and phosphorus levels were within reference ranges in these 39 patients with NF1, ruling out several secondary disorders of bone metabolism. Low levels of serum D-25 vitamin (below 40 nM) were observed in 7 (18 %) NF1 patients. High levels of serum parathyroid hormone (above 65 ng/ml) were found in 13 (33 %), high levels of CTX or ICTP (above 0.57

microgram/l for CTX) in 10 (26 %) and high levels of PINP (above 76 micorgram/l) in 6 (15 %) of NF1 patients. Alkaline phosphatase levels were normal. Four (10 %) patients had secondary hyperparathyreoidism (low vitamin D and high PTH).

5.6 NF1 PATIENTS SHOW BONE CATABOLISM BASED ON BONE TURNOVER MARKERS (III)

Serum CTX is considered as an indicator of bone resorption, and PINP as an indicator of bone formation. However, as these two events are coupled, increase in bone resorption (or CTX) is often followed by increase in bone formation (or PINP). CTX/PINP ratio is normal when the relative amount of resorption and formations is equal, even though the bone turnover rate is increased. However, increased ratio means that more bone is resorbed than gained [Chopin et al. 2012]. NF1 patients have higher levels of serum CTX, and also higher levels of PINP compared to controls, as estimated in 20 NF1 patients. The CTX/PINP ratio was also higher in NF1 patients compared to controls, suggesting bone catabolic status.

5.7 SERUM CTX CORRELATES WITH BONE RESORPTION *IN VITRO* (II,III,IV)

A considerable effort has been put to find a possible link between osteoclast activity *in vitro* and bone health *in vivo* by us and others [Stevenson et al. 2011a]. *In vitro* osteoclast activity was measured by quantitating number of osteoclasts in the cell culture, and the amount CTX released in the cell culture. These two parameters were correlated to serum laboratory values (see 4.8), BMD values, T- and Z-scores. Serum CTX levels correlated with levels of cell culture CTX, but other correlations were not found.

5.8 PATIENTS WITH NF1 OFTEN DISPLAY LOW BMD (III,IV,V)

The frequency of osteoporosis and osteopenia in NF1 are shown in **Table 3**, section 2.1.5. Frequency of osteoporosis and osteopenia in studies III, IV and V are in analogy with the summarized literature. It should be noted that patients in study V represent a selected population whose BMD was known, since the aim of the study V was not to evaluate the frequency of osteoporosis.

5.9 RISK FACTORS FOR LOW BMD ARE OFTEN ABSENT IN NF1 (III,IV)

Known risk factors for low BMD are high age, low peak bone mass, smoking, alcohol consumption, immobility, inadequate calcium and vitamin D supplementation and other medical conditions such as glucocorticoid use (see 2.3.1). Because NF1-related

osteoporosis was noted in young, non-smoking, physically active men and women, the NF1-related osteoporosis may not often be associated with traditional risk factors for low BMD. Approximately half of the NF1 patients with low BMD (osteopenia/osteoporosis) have one risk factor for low BMD, but seldom two or more. This is clinically important since physicians should screen for NF1-related osteoporosis based on the NF1 diagnosis, and not based on the clinical risk factors.

On the other hand, NF1-osteopenia often progresses to osteoporosis, which is also noted in the general population, suggesting that age is a significant risk factor for low BMD in NF1. A comprehensive risk factor analysis for both low BMD and fracture risk in NF1 is called for.

5.10 BMD DECREASES PROGRESSIVELY WITH AGING IN NF1 (IV)

BMD worsens progressively as the patients with NF1 become older, as shown in 19 NF1 patients whose BMD was remeasured 12 years after the initial measurement [Kuorilehto et al. 2005]. T-scores of all but one patient had decreased in 12 years. Clinically, four patients with NF1-related osteopenia now had osteoporosis. This was not explained by changes in physical activity, smoking habits, sunlight exposure, body mass index, or laboratory parameters, even though secondary hyperparathyreoidism was common. Thus, the decrease in BMD may represent the effect of aging.

This decrease in BMD often results in NF1-related osteopenia progressing to osteoporosis. This was noted in patients aged ~40-70 years. Clinicians should take this into account when a marked osteopenia is found, for example T-score -2.3 SD, because these patients may have osteoporosis in a few years. Since a slight drop in Z-scores was noted, it remains to be seen whether patients with NF1 lose bone faster than persons without NF1. However, study IV is limited due to small number of patients and due to fact that we were unable to reach controls for the study.

5.11 PATIENTS WITH NF1 ARE AT FRACTURE RISK (IV,V)

The fracture risk in 460 NF1 patients and 3988 controls persons was evaluated by screening the hospital medical records in a register-based study. Age and gender distribution was similar between NF1 and control cohorts, allowing the comparison of these two cohorts. In NF1 patients aged 41 years or older a risk ratio x5.2 for fractures was observed. NF1 children (age 3-16 yrs) had x3.4 risk ratio for fractures compared to control children. On the contrary, there was no difference in the fracture risk of patients with NF1 aged 17-40 years compared to controls. No gender-related differences were observed in any age cohort. Fractures of ribs, pelvis, fingers, toes and humerus were especially common in NF1, but skull fractures were rare.

The fracture incidence was equal in NF1 patients with cancer, compared to NF1 patients without cancer.

Study IV included a small cohort of six patients with NF1-related osteoporosis in 1999. During the 12-year follow-up, three osteoporotic elderly patients with NF1 had sustained fractures between years 1999 and 2011. These women were not treated with antiresorptives or bone anabolics before the fractures. This finding prompted us to screen for a subpopulation of NF1 patients, whose BMD was known. The search in study V resulted in 50 NF1 patients. Patients with NF1-related osteoporosis had higher risk for fractures compared to NF1 patients with normal BMD.

5.12 FRACTURE HEALING IN NF1 (V)

Fracture healing in NF1 was assessed in 60 fractures. One 16-year-old male patient who had fractured his ulna had subsequently developed an acquired pseudarthrosis. However, it may be possible that this pseudarthrosis was a NF1-related pre-existing lesion in bone, which had fractured.

6 DISCUSSION

6.1 CRANIOFACIAL MORPHOLOGY IN NF1

A novel finding was that craniofacial morphology of NF1 patients is clearly different compared to controls, displaying short mandible, maxilla and cranial base. This is in analogy with findings in Spred-1 knockout mice, which have hyperactive Ras signaling and display a short face [Inoue et al. 2005]. We speculate that Ras is required for normal growth and development of craniofacial structures, and *NF1* gene may thus regulate the development of the skull. Development and ossification of craniofacial bones includes both intramembraneous and endochondral ossification, and it may be possible that NF1 regulates either of them, or both. The short jaws and cranial base were evident in children, adolescents and adults, but statistically significant only in adults. Therefore, we can only speculate that the short jaws and cranial base are present at birth but become more prominent after puberty.

6.2 HYPERACTIVE OSTEOCLAST AND NF1-RELATED BONE CATABOLISM

The current study showed that NF1 osteoclasts isolated from bone microenvironment operative *in vivo* display hyperactive phenotype compared to controls. Specifically, NF1 osteoclasts have increased differentiation and resorption capacity, abnormal morphology and tolerate different apoptotic signals. The same number of osteoclasts derived from NF1 patients resorb bone roughly twice as much as osteoclasts isolated from control persons. The results show that the hyperactive NF1 osteoclast has this phenotype without the influence of osteoblasts or other factors. This NF1-osteoclast phenotype is similar to osteoclasts of different Nf1-mice models (see 2.1.5).

The *in vitro* results do not necessarily represent the situation in NF1 patients. Increased number of osteoclasts has been shown in bone biopsies from NF1 patients [Seitz et al. 2010], supporting the *in vitro* findings. Several multinuclear TRACP-positive osteoclasts were noted in samples from NF1-related pseudarthrosis tissue in study II, but the samples are from a specific NF1-related skeletal lesion site. However, our results show that patients with NF1 have increased levels of bone turnover markers CTX and PINP compared to controls, suggesting increased bone turnover. Taken together with this fact, and the high frequency of low BMD, it is likely that osteoclasts play a role in bone catabolism in NF1.

This study did not evaluate the effects of osteoblasts on osteoclasts. Osteoblasts regulate bone turnover through OPG/RANK/RANKL pathway, and it may be possible that this

signaling either suppresses or even enhances the bone catabolic phenotype in NF1. Defective osteoblast differentiation has been shown in osteoblasts derived from patients with NF1 [Leskelä et al. 2009] and in mouse models (see 2.1.5). Since NF1 osteoclasts are insensitive to apoptosis *in vitro* and are found within fibrous pseudarthrosis tissue, not adjacent to bone, it is feasible to speculate that NF1 osteoclasts do not respond to apoptotic signals, and thus continue resorption regardless of suppressive signals.

In addition, the effect of hormones on bone health in NF1 remains to be elucidated. The morphological changes observed in NF1 osteoclasts also suggest a disorder of osteoclast cytoskeleton, especially in actin cytoskeleton. However, since the osteoclast cytoskeleton changes during different phases of the resorption cycle [Väänänen et al. 2000], it may be difficult to assess cytoskeleton in NF1 osteoclasts. It also remains to be seen, whether NF1 osteoclast secrete acid and proteases more efficiently, explaining furthermore the increased osteoclast bone resorption rate in NF1. The role and health of bone marrow in NF1 remains also to be elucidated, since both osteoclast and osteoblast progenitors originate from the bone marrow.

6.3 SERUM MARKERS OF BONE HEALTH

One of our standout result was that patients with NF1 have higher levels of serum bone turnover markers CTX and PINP compared to controls (see 6.2). Serum calcium was within reference range in patients with NF1. However, NF1 patients with low BMD have higher serum calcium compared to NF1 patients with normal BMD. This may be due to increased bone loss in NF1 patients with low BMD. High levels of serum parathyroid hormone and low levels of serum vitamin D were observed in adult patients with NF1. In summary, the findings of the current study are in analogy to the previous literature [Lammert et al. 2006, Brunetti-Pierri et al. 2008, Tucker et al. 2009, Seitz et al. 2010]. Bone lysis due to adjacent tumors seems unlikely, since some osteoporotic patients have low tumor burden, and alkaline phosphatase levels were normal in NF1 patients of the current study.

Secondary hyperparathyroidism was common in NF1, and may contribute to bone health in some, but not all, patients with NF1. This should be assessed in the future studies. We also speculate that low levels of vitamin D may be an additional risk factor for low BMD in NF1.

6.4 *IN VIVO - IN VITRO* CORRELATION IN BONE RESORPTION

Preliminary results in study II showed that patients with NF1 who have high levels of serum CTX display also high bone resorption capacity in osteoclast samples derived from their peripheral blood. In study III, the levels of serum CTX correlated with CTX released in the osteoclast culture media in 20 patient/control pairs, providing a completely new approach to bone metabolism in NF1. This may suggest that osteoclast

behavior *in vitro* may reflect the bone resorption rate *in vivo*, since this correlation was seen in the NF1 cohort but also in the control cohort. We have identified serum CTX as an interesting candidate for future research searching for a marker of bone health in NF1, and also in the general population. However, the predictive value of serum or cell culture CTX remains to be elucidated.

6.5 BONE MINERAL DENSITY IN NF1

Our studies support the 20-50 % frequency of NF1-related osteoporosis, as summarized in **Table 3**, section 2.1.5. Osteoporosis is defined according to WHO by low BMD, altered bone architecture and increased fracture risk. Patients with NF1 have low BMD [Elefteriou et al. 2009], altered bone microarchitecture [Seitz et al. 2010] and increased fracture risk, as shown in study V. Thus, NF1-related osteoporosis in adults fills the strict WHO criteria for osteoporosis.

On the contrary to osteoporosis in common, our results suggest that NF1-related osteoporosis does not often display with traditional risk factors for low BMD. This is clinically important, since physicians should look for osteoporosis based on the NF1 diagnosis, instead of these traditional risk factors. However, we demonstrated that the BMD decreases progressively with aging in NF1, and thus age is an independent risk factor for low BMD also in NF1, similar to osteoporosis in general. This means that the 20-50 % frequency of NF1-related osteoporosis most likely increases among the older NF1 patients. In addition, the current literature on NF1-related osteoporosis lacks elderly patients. In the current study, the patients with NF1-related osteoporosis were aged 37-76 years. This is in analogy with study V, where increased fracture risk was observed in NF1 patients aged ~40 years and older, but not in the younger adults.

Increased levels of serum CTX and PINP compared to controls represent increased osteoclast and osteoblast activity, ie. bone turnover. This is typical for age-related osteoporosis in general. However, these markers do not describe the quality of the formed bone. The increase in levels of bone turnover markers may also suggest that NF1 patients lose slightly more bone over time compared to controls. This is supported by the slight drop in Z-scores of NF1 patients in 12 years, and may partially explain the increase in the frequency of NF1-related osteoporosis with aging.

6.6 FRACTURE RISK IN NF1

A key finding of the current study was to show that NF1 patients have increased fracture risk. Specifically, patients with NF1 aged ~40 years or more have x5.2 risk for having a fracture. This is in analogy to low BMD in NF1, and is in agreement with the previous questionnaire studies [Brunetti-Pierri et al. 2008, Tucker et al. 2009]. The age group of ~40 or older is in analogy to results of progressively worsening BMD in NF1 (see 6.5).

This is further supported by the fact that patients with NF1-related osteoporosis have increased fracture risk compared to NF1 patients with normal BMD.

Our results also showed that children with NF1 have x3.4 increased fracture risk compared to children without NF1. Low BMD has been reported in NF1 children [Stevenson et al. 2007, Duman et al. 2008], which supports this finding. Surprisingly, patients aged 17-40 years did not have an increased fracture risk compared to controls, even though low BMD had also been shown in this age cohort [Elefteriou et al. 2009]. NF1-related osteoporosis was also rare in patients aged 17-40 years in the current study. We can only speculate, that the bone strength is highest in NF1 patients aged 17-40 years, and is subsequently reduced, leading to increased fracture risk.

Fracture risk is a sum of bone strenght, risk to fall and trauma energy. Bone strength is usually evaluated by measuring BMD. In analogy to general population, BMD alone is a limited predictor of fractures [Järvinen et al. 2008, Compston et al. 2009, Rachner et al. 2011]. Low BMD is associated with NF1, but other factors such as bone macroscopic morphology, microarchitecture and qualitative changes of mineralized bone matrix could additionally affect bone strength in NF1. Also factors not related to bone directly should be taken into account. NF1 is associated with selected types of malignancy, which could weaken the bones [Evans et al. 2011], but no such a relationship was found in the current study. In addition, impaired motor proficiency has been described in NF1 children, possibly predisposing to falls [Thompson et al. 2010]

6.7 BONE DYNAMICS IN NF1

We speculate that hyperactive NF1 osteoclast and defective NF1 osteoblasts generate NF1-bone phenotype. In this phenotype the bone fails to mineralize and develop completely, supported by the increased amounts of osteoid in NF1 bone biopsies and low BMD noted in NF1 children [Stevenson et al. 2007, Duman et al. 2008, Leskelä et al. 2009, Seitz et al. 2010]. The peak bone mass in NF1 is possibly reached at age of 17-40 years, which is supported by the low fracture risk in patients of this age. Subsequently, the BMD worsens progressively after reaching adulthood as shown in study IV. The decreasing BMD is supported by the hyperactive NF1 osteoclasts and increased levels of bone turnover markers in NF1.

It is possible that other factors, such as mechanical loading, smoking, low levels of vitamin D and/or hormones, may further disturb this NF1-bone phenotype, leading ultimately to development of osteoporosis in some patients with NF1. The presence of other factors influencing the BMD in NF1 seems likely, since only approximately 20-50 % of patients with NF1 develop osteoporosis. However, majority of adult NF1 patients have low BMD.

6.8 NF1 CHILDREN WITH FRACTURES AND/OR OSTEOPOROSIS

We did not analyze BMD in NF1 children since the measurement involves the use of X-rays. However, there are currently no studies concerning BMD in NF1 children correlated with their fracture history. Therefore it is difficult to comment on possible osteoporosis in NF1 children. Increased fracture risk in NF1 children (aged 3-16 yrs) compared to control children was observed, fracture incidence reaching up to 12 per 1000 person-years. However, fracture incidences of up to 16 have been reported in healthy Finnish children [Mäyränpää et al. 2010]. Therefore, the pediatric fracture risk in NF1 should be evaluated in detail.

6.9 NEW RECOMMENDATION FOR DXA SCANS IN NF1

Based on the results of the current study, all patients with NF1, males and females, aged ~40 or more should be evaluated for fracture risk. This evaluation means assessment of clinical risk factors for fractures, such as previous fracture or medication. In addition it could include DXA scans, which are easily available in Finland.

6.10 TREATMENT RECOMMENDATION FOR NF1-RELATED OSTEOPENIA

The study IV shows that NF1-related osteopenia often progresses to osteoporosis in patients aged ~40-70. Therefore osteopenia in NF1-patients should not be ignored, but instead should be followed up and considered for prophylactic measures to prevent osteoporosis. These measures could include lifestyle advice, and correction of potentially low levels of vitamin D. It has been shown that vitamin D and calcium therapy restores the levels of vitamin D, but does not affect the BMD [Brunetti-Pierri et al. 2008, Petramala et al. 2011]. However, these studies do not evaluate the prevention of fractures or osteoporosis. It should be remembered that the majority of osteoporotic fractures occur actually in persons with osteopenia [Siris et al. 2004].

6.11 TREATMENT RECOMMENDATION FOR NF1-RELATED OSTEOPOROSIS

Our results showed that NF1 is an independent risk factor for fractures in patients aged ~40 or more. In addition, we showed that NF1-related osteoporosis increases this risk even further. Therefore, all patients with NF1-related osteoporosis should be evaluated for their risk factors for fractures. Patients with high risk for fractures should be considered for prophylactic measures to prevent fractures. These measures could include lifestyle advice, vitamin D supplementation and even pharmaceutical therapy for osteoporosis.

The study III evaluated the effects of bisphosphonates using a cell culture system. NF1-osteoclasts displayed insensitivity to alendronate, clodronate and zoledronic acid compared to osteoclasts derived from healthy controls. However, these *in vitro* results cannot be extrapolated to NF1 patients taking these drugs. It may be possible that as patients take these drugs for years, the cumulative exposure to bisphosphonates overcomes the NF1-related insensitivity to these drugs. Therefore, recommended first-line treatment of choice for NF1-related osteoporosis could be alendronate, similar to osteoporosis in general [Compston et al. 2009, Rachner et al. 2011].

6.12 FUTURE ASPECTS

In my opinion, the fracture prevention in NF1 should be the next step in research of this field. Due to research permits of the study V, we were not able to comprehensively characterize different risk factors for fractures in NF1. Also the trauma mechanisms for fractures should be evaluated in detail. Clinical data on bisphosphonate therapy in patients with NF1 should be collected, even in a small case series. Decrease in bone turnover markers and increase in BMD are the expected results of bisphosphonate therapy, and should be characterized in NF1. This would clarify the issue, whether bisphosphonates are effective in NF1.

7 CONCLUSIONS

- NF1 affects craniofacial morphogenesis. Patients with NF1 have short jaws and cranial base. The short jaws and cranial base did not require orthodontic/ orthognathic treatment in the NF1 patients enrolled in the current study.
- Osteoclasts derived from patients with NF1 display enhanced formation and resorption capacity, and tolerate apoptotic signals, compared to osteoclasts from healthy controls. This phenotype is independent of bone microenvironment operative *in vivo*, and may contribute to bone loss in NF1. Persons with high bone resorption as estimated by serum CTX usually have high bone resorption capacity *in vitro*. This suggests a possible link between osteoclast activity *in vitro* and *in vivo*.
- Osteoclasts derived from patients with NF1 display insensitivity to bisphosphonates. This tolerance to bisphosphonate-induced apoptosis is most likely due to hyperactive Ras, since Ras- inhibitor counteracted this NF1-related insensitivity.
- Low BMD and osteoporosis are common in adult patients with NF1. In addition, patients with NF1 have higher levels of serum CTX and PINP compared to controls, reflecting increased bone turnover.
- The BMD of patients with NF1 decreases progressively with aging. Osteopenia often progresses to osteoporosis in NF1, especially in patients aged ~40-70. This is in analogy with the increased fracture risk in the same age group of NF1 patients.
- Patients with NF1 aged ~40 or more have x5.2 risk ratio for fractures. Children with NF1 have x3.4 risk ratio for fractures.

Based on the findings of this thesis, we recommended the following:

- All patients with NF1 aged ~40 or more, males and females, should have their fracture risk evaluated and possibly BMD measured.
    - Patients with normal BMD do not require additional attention, unless indications for another DXA scan are present.
    - If osteopenia is found, we recommend follow-up of the BMD, since osteopenia may often progress to osteoporosis.
    - If osteoporosis is found, the patient thus has two clinical risk factors for fractures; osteoporosis and NF1. Physicians are advised to screen for other fracture risk factors to identify high-risk patients for fractures.
- High risk NF1 patients for fractures, as defined above, should be consider for prophylactic measures to prevent fractures from occurring. These measures could be lifestyle intervention, vitamin D supplementation and possibly medication for osteoporosis.

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