Blood and Bone

Epidemiological studies on the association between blood and bone

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Dedicated to Magnús, Sóley, Þórarinn and Birkir Orri

ABSTRACT

Introduction: Preclinical and clinical studies have suggested that blood- and bone cells are interconnected.
Aims: To study the association between hematological variables, specifically serotonin, erythropoietin (EPO), hemoglobin (Hb), neutrophil-, lymphocyte-, and platelet count, and bone mineral density (BMD), and/or risk for fractures (paper I-III). To study the risk for hip fractures in patients with lymphoma (paper IV).
Methods: In paper I-III data from MrOS (The Osteoporotic Fractures in Men Study), a prospective, population-based study, was used. Men in the ages 69-81 years were randomly selected from Gothenburg (n=1010), 2002-2004. In the second part of paper I, additional cohorts from Uppsala and Malmö were used. Baseline data included blood tests (serotonin, EPO, Hb, neutrophil-, lymphocyte-, and platelet count) and dual x-ray absorptiometry (DXA). Subjects were followed until the end of 2013. In paper IV adults ≥18 years diagnosed with lymphoma between 1995 and 2015 were identified in the Swedish Cancer Register. Data on the Swedish population and lymphoma patients was retrieved from Statistiska Centralbyrån, and hip fractures were identified via the Inpatient Register. The risk for hip fractures in patients with lymphoma was compared with that of the Swedish population.
Results: Serotonin was negatively associated with total hip BMD. Men with serotonin in quintile 5 had an increased risk for all fractures, nonvertebral osteoporotic fractures and hip fractures. In men with normal renal function EPO was positively associated with total hip BMD, inflammation, and comorbidities, as well as increased risk for all fractures and major osteoporotic fractures. Platelet- and neutrophil count, and not Hb and lymphocyte count, were negatively associated with total hip BMD. Women with lymphoma had increased risk for hip fracture compared with the Swedish population.
Conclusions: The results support the hypothesis that blood and bone are interconnected. Serotonin and EPO both predict for fractures in elderly men. Platelet- and neutrophil count are associated with BMD. Physicians treating lymphoma patients should be aware of the increased risk for hip fractures in women.
Keywords: blood, bone, fractures, osteoporosis
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SAMMANFATTNING PÅ SVENSKA

Blod- och benceller är lokaliserade nära varandra i benmärgen. Det finns ett flertal studier som tyder på att det existerar ett samband mellan blod- och bensystemet, detta är dock ett område som tämligen outforskat. Osteoporos (benskörhet) och benskörhetsfrakturer är vanliga i den äldre befolkningen och det är viktigt att kunna förutsäga vem som eventuellt kan drabbas för att på så sätt försöka förebygga frakturer. I delarbete I-III har vi använt en grupp av äldre män boende i Göteborg, Malmö och Uppsala. Män i åldrarna 69 till 81 år blev slumpmässigt utvalda under åren 2001 till 2004. Dessa män genomgick ett flertal undersökningar och analyser, bland annat blodprover, de svarade på frågeformulär gällande riskfaktorer för fraktur och genomgick bentäthetsmätning (BMD) med så kallad ”dual x-ray absorptiometry” (DXA). Männen följdes fram till 2013 avseende fraktur. I första delarbetet studerades serotonin, vilket är ett hormon som ofta är förknippat med hjärnan. Serotonin lagras dock i blodplättar och enstaka studier har visat att det finns ett samband mellan serotonin i blodet och bentäthet. Vi noterade att män med höga nivåer av serotonin i blodet hade lägre bentäthet och ökad risk för fraktur, framför allt höftfraktur. Vi mätte även erytropoietin (EPO), vilket också är ett hormon. EPO produceras i njurarna och stimulerar produktionen av röda blodkroppar. Vi fann att höga nivåer av EPO var associerat med högre bentäthet men även ökad risk för fraktur. Män med höga nivåer av EPO hade dock fler ”övriga” sjukdomar och lägre muskelstyrka. I tredje delarbetet fann vi att höga nivåer av blodplättar och neutrofiler, en typ av vita blodkroppar, hade ett samband med lägre bentäthet. I fjärde delarbetet undersöktes om patienter med lymfom, (lymfkörtelcancer), hade ökad risk för höftfraktur. Genom Svenska Cancer Registret identifierades patienter ≥18 år som diagnosticerats med lymfom under åren 1995 till 2015. Svenska slutenvårdsregistret användes för att undersöka förekomst av höftfraktur. Förekomsten av höftfraktur hos patienter med lymfom jämfördes med den svenska befolkningen. Vi noterade att kvinnor, framför allt yngre kvinnor, med lymfom hade ökad risk för fraktur jämfört med den svenska befolkningen. Risken minskade dock med åren om man jämförde patienter som diagnosticerades i början jämfört med slutet av perioden.

Vi har således noterat ett samband mellan blod och ben, huruvida detta samband kan appliceras på hela befolkningen är dock oklart då vi

endast studerat äldre män. Vårdpersonal som behandlar patienter med lymfom bör vara medvetna om att denna patientgrupp kan ha ökad risk för höftfraktur.

LIST OF PAPERS

This thesis is based on the following studies, hereafter referred to in the text by their Roman numerals (I-IV):

I. **Kristjansdottir H.L.**, Lewerin C., Lerner U.H., Waern E., Johansson H., Sundh D., Karlsson M., Cummings S.R., Zetterberg H., Lorentzon M., Ohlsson C, Mellström D. High Serum Serotonin Predicts Increased Risk for Hip Fracture and Nonvertebral Osteoporotic Fractures: The MrOS Sweden Study. J Bone Miner Res. 2018;33(9): 1560-1567.

II. **Kristjansdottir H.L.**, Lewerin C., Lerner U.H., Herlitz H., Johansson P., Johansson H., Karlsson M., Lorentzon M., Ohlsson C., Ljunggren Ö., Mellström D. High Plasma Erythropoietin Predicts Incident Fractures in Elderly Men with Normal Renal Function: The MrOS Sweden Cohort. J Bone Miner Res. 2020;35(2): 298-305.

III. **Kristjansdottir H.L.**, Mellström D., Johansson P., Karlsson M., Vandenput L., Lorentzon M., Herlitz H., Ohlsson C., Lerner U.H., Lewerin C. High platelet count is associated with low bone mineral density: The MrOS Sweden cohort. Osteoporos Int. 2020. Advance online publication. doi.org/10.1007/ s00198-020-05766-6

IV. Johansson P., **Lind Kristjansdottir H.**, Johansson H., Jakir A., Mellström D., Lewerin C. Increased Risk of Hip Fracture in Patients with Lymphoma, a Swedish Population Study of 37,236 Lymphoma Patients. Calcif Tissue Int. 2020;106(6): 591-598.

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TABLE OF CONTENTS

ABBREVIATIONS

Allo-tx	Allogeneic stem cell transplantation
ALP	Alkaline Phosphatase
Auto-tx	Autologous stem cell transplantation
BMD	Bone Mineral Density
BMI	Body Mass Index
cFGF23	cleaved Fibroblast Growth Factor 23
cGVHD	chronic Graft vs. Host Disease
CNS	Central Nervous System
CRP	C-Reactive Protein
CT	Computed Tomography
CV	Coefficient of Variation
DLBCL	Diffused Large B-Cell Lymphoma
DXA	Dual-energy X-ray Absorptiometry
ELISA	Enzyme-Linked Immunosorbent Assay
eGFR	estimated Glomerular Filtration Rate
EPO	Erythropoietin
FGF23	Fibroblast Growth Factor 23
FEV1	Forced Expiratory Volume in 1 second
FL	Follicular Lymphoma

FRAX	Fracture Assessment Tool
G-CSF	Granulocyte-Colony Stimulating Factor
Hb	Hemoglobin
HF	Hazard Function
HIF	Hypoxia Inducible Factor
HL	Hodgkin's Lymphoma
HR	Hazard Ratio
HRpQCT	High Resolution peripheral Quantitative Computed Tomography
HSC	Hematopoietic Stem Cell
ICD9 or 10	International Classification of Diseases and related health problems, 9th or 10th Revision
iFGF23	intact Fibroblast Growth Factor 23
LRP5	Low-density lipoprotein Receptor related Protein 5
M-CSF	Macrophage-Colony Stimulating Factor
MOF	Major Osteoporotic Fractures
MrOS	The Osteoporotic Fractures in Men Study
NHL	Non-Hodgkin's lymphoma
OPG	Osteoprotegerin
OR	Odds Ratio
PINP	Propeptide of type I collagen
PTH	Parathyroid Hormone

pQCT	peripheral Quantitative Computed Tomography
Q	Quintile
qCT	quantitative Computed Tomography
R	Rituximab
RANK	Receptor Activator of Nuclear factor-ß
RANKL	Receptor Activator of Nuclear factor-ß Ligand
R-CHOP	Rituximab-Cyclophosphamide, Doxorubicin, Vincristine, Prednisone
RTB	Register över totalbefolkningen
SCB	Statistiska Centralbyrån
SCR	Swedish Cancer Register
SD	Standard Deviation
SERT	Serotonin Transporter (same as 5-HTT)
SSRI	Selective Serotonin Reuptake Inhibitors
Tph1	Tryptophan hydroxylase 1 enzyme
Tx	Stem cell transplantation
VD	Vertebral Density
WHO	World Health Organization
5-HT	5-hydroxytryptamine (serotonin)
5-HTT	Serotonin Transporter (same as SERT)
25-OH-D	25-hydroxyvitamin D

THESIS AT A GLANCE

Paper	Aims	Method	Results/Conclusions
I	i) To assess an association between serum serotonin and bone mineral density (BMD) and fractures ii) To assess the risk for fractures in those using SSRI.	Prospective and cross-sectional cohort of elderly ambulatory men (MrOS) from i) Gothenburg n=917 and ii) Gothenburg, Malmö, Uppsala n=3014. Follow-up time for fractures 10.6 years.	i) Serotonin was negatively associated with total hip BMD. High serotonin increased the risk for all fractures, nonvertebral osteoporotic fractures, and hip fractures. ii) Ongoing use of SSRIs was not associated with increased risk of fractures.
II	To assess if there is an association between plasma erythropoietin (EPO) and BMD/fractures.	Prospective and cross-sectional cohort of elderly ambulatory men (MrOS) from Gothenburg n=999. Follow-up time for fractures 10.6 years.	In men with normal renal function EPO was positively associated with total hip BMD, inflammation, and comorbidities. High EPO increased the risk for all fractures and major osteoporotic fractures.
III	To assess if there is an association between Hemoglobin (Hb), neutrophil-, lymphocyte, and platelet count and BMD.	Cross-sectional data cohort of elderly ambulatory men (MrOS) from Gothenburg n=1005.	Platelet- and neutrophil count were associated with total hip BMD. Hb and lymphocyte count were not associated with BMD.
IV	To assess the risk for fractures in patients with lymphoma.	Patients with lymphoma identified from the Swedish Cancer Register. Risk for fractures compared with the total Swedish population. Fracture data collected from the Inpatient Register.	The risk for hip fracture was increased in women with lymphoma, especially younger women, compared with the Swedish population.

INTRODUCTION

Blood- and bone cells exists in close proximity to each other in the bone marrow, **(Figure 1)**. Hematopoietic stem cells (HSC) undertake self-renewal, differentiation, and proliferation in places in the bone marrow called the hematopoietic niche. The hematopoietic niche consists of HSC as well as a supportive microenvironment (1). HSC differentiate to either lymphoid cells or myeloid cells and myeloid cells further divide and differentiate into neutrophils, basophils, eosinophils, monocytes/macrophages, platelets, and red blood cells, **(Figure 1)**. Cells of the microenvironment of the hematopoietic niche are often called stromal cells and those differentiate from mesenchymal stem cells (1). Stromal cells include mesenchymal progenitor cells, fibroblasts, adipocytes, reticular cells, and osteoblasts (1). Osteoblasts **(Figure 2)**, one of three main bone cells, were the first bone cells to be identified as part of the supportive microenvironment of the

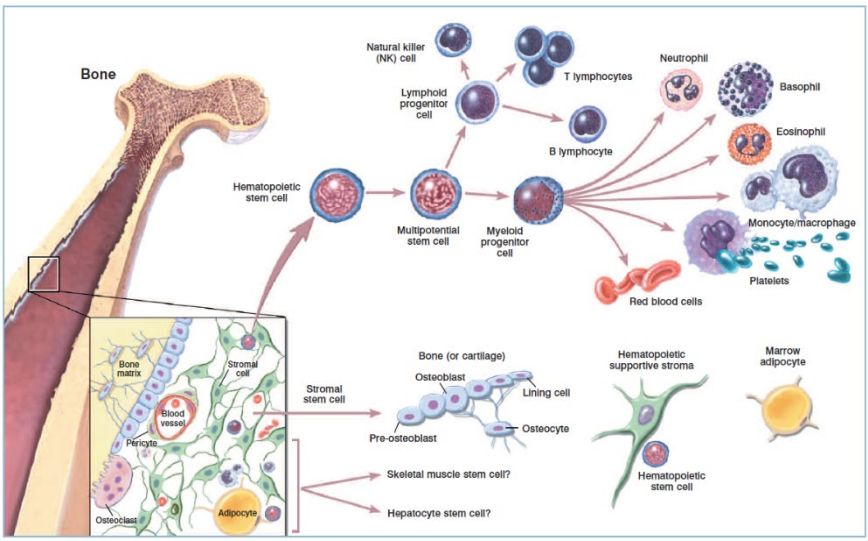

Figure 1. Hematopoietic- and stromal cell differentiation. (Reproduced with permission from Terese Winslow, © 2001, Terese Winslow LLC, U.S. Govt. has certain rights).

hematopoietic niche (2). Their role is to build and mineralize new bone (3). Bone absorbing osteoclasts directly differentiate from HSCs, the monocyte/macrophage line (1).

Due to the close proximity of hematopoietic cells and bone cells in the bone marrow, one can envision that a disruption in either cell formation system could affect the homeostasis of the other. The interaction can be direct, as well as through cytokines and other circulating factors. In this thesis I have used epidemiological data from both healthy individuals and patients with lymphoma to evaluate a possible association between blood and bone.

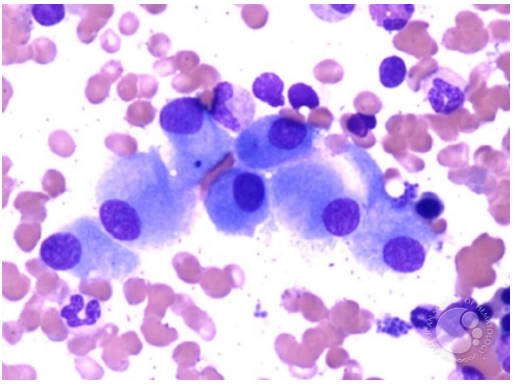

Figure 2. Normal osteoblasts from aspiration smear from bone marrow (This image was originally published in ASH Image Bank, Peter Maslak, Osteoblasts-1. ASH Image Bank, 2003, image number 000002116 © the American Society of Hematology).

BACKGROUND

Bone

Bone is a mineral connective tissue with three main functions in the human body: i) to provide structural support to the body and protection to vital organs, like the heart, lungs and brain, as well as providing support for muscles and tendons, ii) as a reservoir for minerals such as calcium and phosphorous and, iii) to provide a location for hematopoiesis (3). There are two histological bone types: cortical bone and trabecular bone. Cortical bone consists of dense calcified tissue and is the external portion of the bone. The interior part of the bone is made from a trabecular or sponge like network, called trabecular bone (3). The bone marrow, where hematopoeisis takes place, is located in between the trabecular network. The proportion of trabecular and cortical bone differs between different bone locations. The femoral neck is mostly made of cortical bone and the vertebras consist mostly of trabecular bone (3).

Bone metabolism

Bone constantly undergoes self-renewal and self-repair, a process called bone remodeling. The bone remodeling process begins with the recruitment and differentiation of osteoclasts that build resorption cavities. The cavities made by osteoclasts are then filled with osteoid secreted by osteoblasts, and subsequently mineralized into new bone (3). Osteocytes, the third and the most abundant of bone cells in the adult skeleton, develop from osteoblasts that have been trapped in the bone. Osteocytes reside in lacunas in the bone and are interconnected with each other through dentritic processes inside channels or canaliculi. They sense and mediate the effects of mechanical load (3).

The process of resorption by osteoclasts is initiated through activation of i) Macrophage Colony-Stimulating Factor (M-CSF), leading to clonal expansion of osteoclast macrophages and ii) binding of Receptor

Activator of Nuclear factor Ligand (RANKL) to Receptor Activator of Nuclear factor (RANK) on osteoclasts. RANKL is expressed by osteoblasts and partly responsible for osteoclast differentiation and bone resorption (3). Osteoprogetin (OPG), also produced by osteoblasts is a decoy receptor that competes with RANKL binding to RANK, and consequently inhibiting differentiation of osteoclasts and bone resorption. Dysregulation of the RANK/RANKL/OPG system plays a critical role in the pathophysiology of osteoporosis (3).

Propeptide of type I collagen (PINP), alkaline phosphatase (ALP) and osteocalcin, are markers of increased bone formation and their concentration can be measured in serum/plasma (4). PINP originates predominantly from proliferation of osteoblasts and fibroblasts and has been shown to be a sensitive and stable bone formation marker and for early detection of osteoporosis (4). ALP is an enzyme that degrades proteins in the body. ALP in bone is produced by osteoblasts but ALP is even produced in other organs such as the liver and the kidneys. In humans with normal liver function about half of the total ALP in serum is produced from bone (4). Osteocalcin is a matrix protein, produced exclusively by osteoblasts and is a marker of osteoblastic activity. Serum osteocalcin may be useful in assessing osteoporosis and fracture prediction (4). Increased bone turnover and high bone formation markers suggest a decline in structural integrity of bone, and deterioration of bone microarchitecture contributing to low BMD (4).

The skeleton has a central role in the regulation of calcium balance in the human body and bone serves as a reservoir for the body content of calcium. Vitamin D and Parathyroid Hormone (PTH) are important for calcium regulation (5). Vitamin D is mostly produced by ultraviolet irradiation in the skin when exposed to sunlight. It undergoes metabolization in the body to 25-hydroxyvitamin (25-(OH)-D) (5), which is used to assess vitamin D status. Vitamin D stimulates intestinal absorption of calcium and phosphate (5). PTH is closely related to 25-(OH)-D levels and increases calcium absorption and decreases calcium elimination. PTH inhibits bone resorption, but intermittent administration of PTH increases bone formation (5).

Dual X-ray Absorptiometry (DXA)

DXA is an x-ray method used for measuring bone densitometry and was introduced in the late 1980s. It is currently considered the gold standard for measuring bone mineral density (BMD) (6). It involves two x-ray beams with different energy levels. A low energy beam that is attenuated more by bone than soft tissue, and high energy beams that are attenuated equally by bone and soft tissue. By measuring how much each beam has passed through a certain area of the body, BMD can be calculated and is expressed in g/cm^2 (7). DXA is a two-dimensional method and does not differentiate between trabecular and cortical bone and does not account for three-dimensional aspects such as microarchitecture. One further limitation of the DXA scan is its susceptibility to spinal degeneration and aortic calcification, leading to falsely elevated BMD (7). BMD is commonly measured at the lumbar spine, total hip, and femoral neck. Total body BMD can be used to evaluated body composition and body fat (6).

Osteoporosis and osteoporotic fractures

The definition of osteoporosis has changed through the years and in 1991 the consensus development conference defined osteoporosis as "A disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk" (8). The current definition of osteoporosis by the World Health Organization (WHO) is from 1994 and was made for osteoporosis in post-menopausal women. It is based on DXA measurements where osteoporosis is defined as BMD equal to or more than 2.5 standard deviations (SD) below a young adult standard, also called a T-score of $\leq$-2.5 SD (9).

Osteoporosis is without symptoms until one suffers an osteoporotic fracture. The risk of osteoporotic fracture increases with age independent of BMD and the risk for fracture increases continuously and exponentially with a lower BMD. The same T-score at any one site has different significance with age and for any given T-score, the

absolute fracture risk is much higher in the elderly than in the young (10). The actual number of fractures that occur in patients with osteoporosis and in the very old are low, though the fracture rate is high, and the majority of individuals that suffer from osteoporotic fractures have normal BMD measurements on DXA (11), **Figure 3**. A T-score of ≤-2.5 SD will only find a minority of patients at risk for fractures and BMD as a predictor for fractures is of less value in individuals with low baseline fracture risk and has greater prediction capacity in those with multiple risk factors (12, 13). The same threshold for fracture prediction can be used for men as for women, since for any given BMD the age adjusted risk for fracture is about the same (10). For 70 years old men in Sweden with T-score -2.5, the absolute 10-year risk, or probability, of suffering hip fracture is 8.4% and the relative risk is 2.5, compared with men in the same age from the population. For 60 year old men the absolute and relative risk are 3.8% and 3.1, respectively, and for a 80 year old 13.0% and 1.8, respectively (13).

Major osteoporotic fractures (MOF) are typically defined as low energy fractures of the thoracic and lumbar spine, the hip, the wrist, proximal humerus and sometimes are pelvis fractures included (14, 15). However, almost all fractures, even those traditionally not considered osteoporotic are related to low BMD (16). Fracture of the hip and even vertebral fractures lead to increased mortality (17-19). Additionally, fractures are associated with pain, increased disability, and decreased quality of life (17, 19, 20). Around 50% of hip fracture survivors will not recover to the same level of mobility they had before the fracture (21).

The risk of osteoporosis and fractures increases with age in both genders, and in women the deterioration is faster after menopause, largely due to decreased estrogen levels leading to accelerated bone loss. Other known risk factors for osteoporosis and/or fractures in both genders are low body mass index (BMI), low serum levels of testosterone and estrogen, parent history of hip fracture, low physical activity, smoking, use of glucocorticoid steroids, and diseases such as rheumatoid arthritis and diabetes mellitus (13, 15, 22).

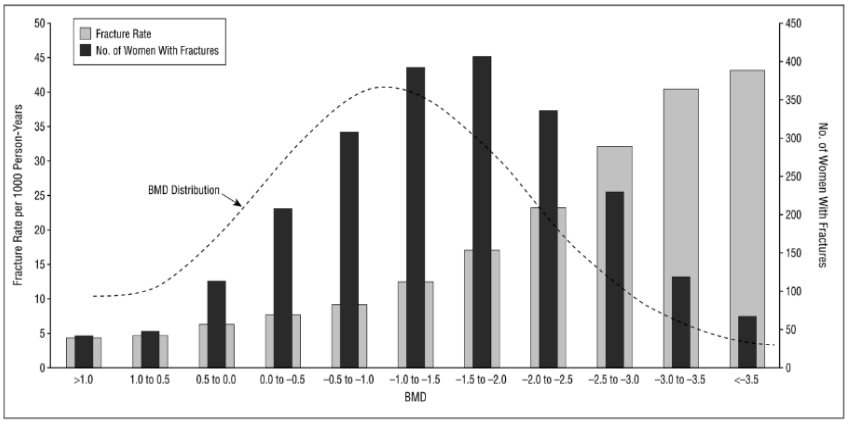

Figure 3. Number of fractures and fracture risk per BMD in post-menopausal women 50-104 years old (11) (Reproduced with permission from © 2004, American Medical Association).

Even socioeconomic factors like being unmarried, lower educational levels, and residency, such as high population density and higher latitude, have been associated with increased risk for osteoporotic fractures (23, 24).

Sweden and Norway have among the highest incidence of osteoporotic fractures in the world (25, 26). In Sweden the lifetime risk of suffering from a hip-, vertebral or forearm fracture after the age of 50 is 46.4% for women and 22.4% for men (26). Osteoporotic fractures lead to a huge financial burden for society (27). Early detection of osteoporosis is a key factor to enable initiating treatment to prevent osteoporotic fractures. Many of the known risk factors for fractures interact with each other and several predictive models for fractures have been developed, one such model being the Fracture Assessment Tool, FRAX® (22). FRAX is a web-based algorithm that calculates the total 10 years fracture risk of MOF and hip fracture. It is developed for both men and women and includes several known risk factors of fractures,

and can be used with or without femoral neck BMD (22). The results of FRAX are often used to guide treatment decisions.

Bone specific drugs are either anti-resorptive, meaning that they target osteoclastic mediated resorption, or anabolic, meaning that they stimulate osteoblasts to form new bone. Bisphosphonates, the most commonly used bone-specific medication, cause apoptosis of osteoclasts and are anti-resorptive. Another anti-resorptive drug is Denosumab, a human antibody against RANKL (28).

Not all osteoporotic fractures can be explained by currently known risk factors. Thus, it is of importance to identify new risk factors with the goal of increasing understanding of osteoporotic fractures and hopefully identify people who can be helped with preventive treatment against fractures.

Serotonin

The hormone serotonin (5-hydroxytryptamine; 5-HT), is a well-known neurotransmitter in the central nervous system (CNS). It is produced from the amino acid L-tryptophan and has several known roles in the human body, like mood control, regulation of sleep, cognition, emotion and appetite (reviewed in (29)). Serotonin is made both peripherally and centrally in the body and does not readily pass the blood brain barrier. Tryptophan hydroxylase 1 (Tph1), the rate limiting enzyme in the production of serotonin, is mainly expressed in the enterochromaffin cells in the gastrointenstinal tract, where majority of the body content of serotonin is found (reviewed in (29)). In the CNS another isoform, namely Tph2, is predominantly expressed (30).

Serotonin exerts its cellular effects via 15 different transmembrane receptors (5-HTr), belonging to seven different classes. Depending on the receptor, serotonin can produce opposing effects (31). Many of the serotonin receptors are drug targets, such as 5-HT3 receptor antagonists, like ondasetron, used to treat nausea, and 5-HT1B receptor

agonists, triptans, used to treat migraine (32). The activity of serotonin also depends on its extracellular availability which is controlled by specific membrane 5-HT transporters (5-HTT or SERT). 5-HTT removes secreted serotonin from the extracellular space, thus decreasing the concentration of serotonin without inducing intracellular signaling. Selective serotonin reuptake inhibitors (SSRIs) inhibit 5-HTT and in the CNS they increase the extracellular levels of serotonin and thereby decrease symptoms of depression (33).

In the beginning of the 20th century, gut derived serotonin was suggested to mediate the role of low-density lipoprotein receptor related protein 5 (LRP5) (34). LRP5 is an important regulator of bone remodeling and a pivotal study by Yadav *et al.* showed that LRP5 inhibits the expression of the *Tph1* gene in mice (34), thus leading to decreased levels of serotonin. These results have been challenged and other genetic studies in mice have shown that the important role of LRP5 for bone formation is due to its expression in osteoblasts (35-37). In humans, loss-of-function mutations of the *LRP5* gene are known to cause a rare disease called osteoporosis pseudoglioma, characterized by low bone mass and blindness (38). Consequently, gain-of-function mutation of the *LRP5* gene cause high bone mass syndrome (39). Tph1 has been shown to be expressed in all three major types of bone cells, that even can express some serotonin receptors and reuptake receptors. Both peripherally and centrally produced serotonin has been implicated to play a role in bone remodeling in a highly complex manner (reviewed in (31)). Additionally peripherally and centrally produced serotonin may have opposing effects on bone (40).

The first human study on the association between circulating serotonin and BMD was published in 2010 and found an inverse association between serotonin and BMD, in a cohort of 275 women from the USA (41). Subsequent studies on circulating serotonin and BMD in humans showed conflicting results (42-44). Conflicting results are even seen in patients with carcinoid syndrome, who have high circulating serotonin, and BMD (45-47). One study is published on serotonin and fracture risk in which an association between serum serotonin and fracture risk

was not seen (48). Several studies have shown that patients on SSRIs have increased risk for fractures (33, 49).

Serotonin, though not widely thought of as a hematological component, is stored in platelets where it serves as a weak platelet agonist (50). Serotonin has been shown to enhance megakaryocyte proliferation (51), and a long time ago it was shown that serotonin stimulates erythropoiesis by increasing erythropoietin (EPO) in mice (52). The suggested mechanism was that serotonin would induce local hypoxia in the kidneys leading to increased concentrations of circulating EPO (52). The Tph1 enzyme is found in erythrocyte progenitors in mice, and mice with decreased peripheral serotonin can develop macrocytic anemia (53, 54).

Erythropoietin

EPO, a hormone that in adults is produced principally in the kidneys, exerts its effect in the bone marrow where it stimulates the proliferation and differentiation of erythrocyte progenitor cells (55). Several studies imply that EPO affects numerous organs and cells beside the erythrocyte progenitor cells. Some human studies have implicated that EPO has a neuro- and cardioprotective effect, as well as increasing angiogenesis and improving wound healing, **Figure 4** (reviewed in (56)).

Osteoblasts have been shown to be able to produce EPO via Hypoxia Inducible Factor (HIF) dependent mechanism, and augmenting HIF activity in osteoblasts leads to increased erythropoiesis in the bone marrow (57). EPO receptors have been found on bone cells (58, 59), and it has been suggested that EPO is a link between osteoblasts and the hematopoietic niche.

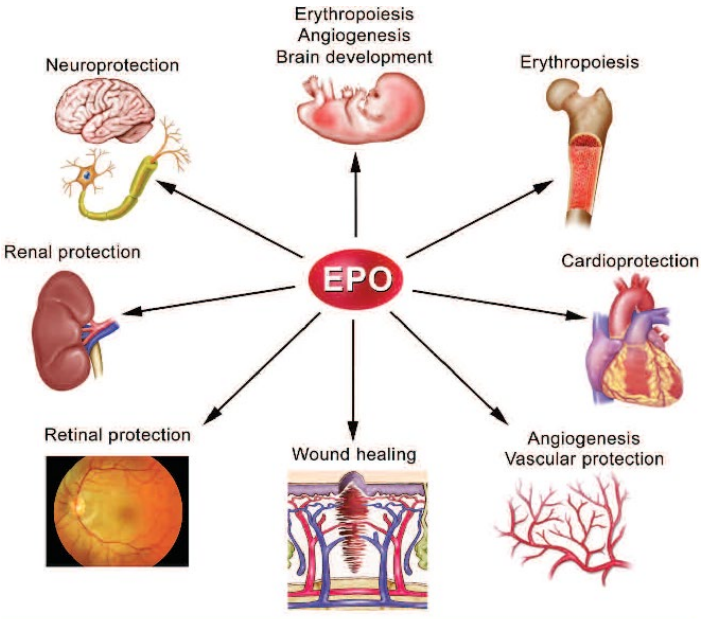

Figure 4. The non-erythroid effects of erythropoietin (Reproduced with permission from © Ferrata Storti Foundation, Pavia, Italy, (60)).

Various bone remodeling models in adult rodents show that EPO has a deleterious effect on bone mass (58, 61). On the other hand, traumatic bone models or those studying growing mice, have shown that EPO has an anabolic effect on bone regeneration, leading to increased callus formation and faster fracture healing (62-66). The same positive result on fracture healing has been seen in humans (67). EPO's positive effect on bone mass has been suggested to be through increased angiogenesis (62, 66, 68, 69), decreased inflammation (63), increased osteoblastic activity (59, 66) or decreased osteoclastic activity (65, 66).

Studies on the role of endogenous EPO and bone in humans are sparse. In a meta-analysis studying bone health and BMD in patients with hematological diseases, no correlation between EPO and bone mass was seen (70). A small prospective collected study on 41 patients receiving hemodialysis, where 33 patients received EPO injections and

eight controls, failed to show a correlation between peripherally measured EPO values and BMD (71).

Fibroblast Growth Factor 23 (FGF23)

FGF23 is a protein produced by osteoblasts and osteocytes (72, 73). It exerts its effect on the kidneys and intestines where it decreases reabsorption and increases excretion of phosphate. Additionally, it decreases vitamin D- and PTH synthesis (reviewed in (73)). Two forms of FGF23 are found in the circulation, the biologically active full length intact FGF23 (iFGF23) form, and the cleaved inactive form of C-terminal FGF23 (cFGF23) fragments. The cFGF23 can function as a competitive antagonist to iFGF23 (73). Levels of FGF23 increase with decreased renal function (74), and are correlated with iron deficiency and inflammation (75-77). Several groups have shown that EPO stimulates the production of FGF23 and suggest that EPO is a link between hypoxia, iron deficiency, inflammation, and FGF23 (78-80). If it is the intact or the cleaved form, or ratio of those two, that is important with regards to EPO, is unclear. Contradicting results have been published regarding the association between FGF23 and bone mass and/or fractures in humans (81-85).

Blood and bone - preclinical studies

Osteoblasts were the first bone cells to be identified as a part of the hematopoietic niche (2) and PTH signaling has been implicated as being important in regulating HSC numbers (86). Osteoblasts play a role in the regulation of maturation of lymphocytes, or more specifically B-lymphocytes (87, 88) and both lymphocytes (B-and T), and neutrophils are able to produce RANKL (89-91). As mentioned above, osteoblasts can modulate erythropoiesis by producing EPO (57, 92). This is supported by mouse models where induced osteoblast deficiency leads to decreased bone mass with a concordant loss of

lymphoid and erythroid progenitors (93). Osteocytes have been shown to regulate myelopoiesis through the $G_s\alpha$ signaling protein, but mice lacking $G_s\alpha$ in osteocytes have low bone mass accompanied by increase in neutrophil- and platelet count, and splenomegaly (94). The same study showed that osteocytes can produce granulocyte-colony stimulating factor (G-CSF) (94), a major stimulator of neutrophil differentiation. Other studies in mice have shown that severe lymphopenia is associated with ablation of osteocytes (95). Osteoclasts have been shown to be important for the establishment of the hematopoietic niche and mobilization of HSC by some (96-98), but not by others (99). Osteoclasts can act as antigen presenting cells and activate T-lymphocytes (100).

Thus, the association between bone- and hematopoietic cells is not restricted to the myeloid or lymphoid cell lines and several cytokines and growth factors have been implicated in their cross talk.

Blood and bone - normal population

Majority of previously published studies on the association between blood and bone variables in healthy individuals are summarized in **Table 1**. A study from the MrOS cohort in the USA found that hemoglobin (Hb)<120 g/L was not associated with cross sectional BMD values measured with DXA. However, Hb<120 was positively associated with rapid BMD loss, defined as >0.5% decrease in BMD per year (101). Two Italian cohort studies have shown an association between Hb measured as a continuous variable, and bone density measured with quantitative computed tomography (qCT) or ultrasound, respectively (102, 103). A newly published study evaluating Hb with regards to bone remodeling markers and ultrasound derived bone stiffness index, found that Hb is positively associated with stiffness index and negatively associated with bone remodeling markers in subjects older than 60 years old, but not in younger subjects (104). Several studies are available on the association between Hb and fracture risk. Jörgenssen *et al.* showed that anemia was associated with

nonvertebral fracture in men, but not in women, after adjustment for distal arm BMD (105). From MrOS USA, it has been reported that men with Hb<120 g/L have 67% increased risk for any fracture after adjustment for BMD (106).

Very few studies are available on the interaction between other blood cell counts such as neutrophils, lymphocytes, and platelets, and bone density. In previously mentioned study from MrOS USA no association was seen between cross-sectional BMD values and neutrophil-, lymphocyte- and platelet count, whereas high neutrophil- and low lymphocyte counts were associated with rapid annual decrease of BMD (101). One other study has shown that high neutrophils/lymphocyte ratio is positively associated with osteoporosis (107). A recent study has shown that high platelet count within the normal range is associated with osteoporosis and osteopenia (108).

Thus, few published studies are available on the relationship between blood variables and bone density but due to scarcity of available research, differences in study design and measurement of bone density it is difficult to draw overarching conclusions.

Table 1. Association between BMD and hemoglobin (Hb), neutrophil-, lymphocyte-and platelet count, table extensively modified from Valderrabano *et al.* (109).

Method of bone mass measurement	Hemoglobin	Findings	Neutrophils Lymphocytes Platelets	Findings	Study design	Reference
Men						
BMD by DXA	Hb<120 g/L	No association	Neutrophils Lymphocytes Platelets	No association No association No association	Prosp.observational	Valderrabano *et al.* (101)
>0.5% BMD decrease/year	Hb<120 g/L	Pos.association	Neutrophils Lymphocytes Platelets	Pos. association Neg. association No association	Prosp.observational	
BMD by DXA	Hb levels* and change in Hb	No association	NA	NA	Prosp.observational	Valderrabano *et al.* (110)
Low BMD (T-score <-1 at total hip)	Hb<130 g/L	No association	NA	NA	Prosp.observational	
Cortical bone density by qCT	Hb levels*	Pos.association	NA	NA	Cross sectional	Cesari *et al.* (102)
Trabecular bone density by pCT	Hb levels*	No association	NA	NA	Cross sectional	

Women						
Cortical bone density by qCT	Hb levels*	Pos. association	NA	NA	Cross sectional	Cesari *et al.* (102)
Trabecular bone density by pCT	Hb levels*	Pos. association	NA	NA	Cross sectional	
BMD by DXA	Hb levels* and changes in Hb	No association	NA	NA	Prosp.observational	Valderrabano *et al.* (110)
Low BMD (T-score <-1 at total hip)	Hb<130 g/L	No association	NA	NA	Prosp. observational	
Spine T-score <-1.0 SD by DXA	Hb<120 g/L	Increased odds of low spine T-score	NA	NA	Retrospective	Korkmaz *et al.* (111)
Not stratified by sex						
Ultrasound derived BMD and stiffness index	Hb levels*	Pos.association	NA	NA	Cross sectional	Laudisio *et al.* (103)
T-score by DXA (lumbar spine, total hip, and femoral neck)	NA	NA	Neutrophil/ lymphocyte ratio	Pos.association with osteoporosis	Cross sectional	Ozturk *et al.* (107)
Ultrasound derived stiffness index	Hb levels* Hb<120	Pos association Neg.association	NA	NA	Cross sectional	Hanneman *et al.* (104)
BMD by DXA	NA	NA	Platelets	Neg.associaton	Cross sectional	Kim *et al.* (108)
Quantitative ultrasound	NA	NA	Platelets	Neg.associaton	Prosp. observational	

qCT quantitative computed tomography, *Hb analyzed a continuous variable

Blood and bone - diseases in humans

The relationship between blood and bone has been observed in several bone related and hematological diseases. Increased risk for osteoporosis and fractures is well described in patients with thalassemia and sickle cell anemia, congenital diseases caused by absent or mutant hemoglobulin genes, resulting in hemolysis and ineffective erythropoiesis (112, 113). Multiple myeloma, a hematological neoplasm caused by malignant plasma cells, is the most frequent cancer involving bone. Myeloma associated bone disease is accompanied by increased osteoclast activity and preclinical studies support cell-to-cell contact between myeloma cells and osteoclasts. Myeloma cells secrete RANKL and suppress OPG, resulting in an imbalance in the normal RANKL/OPG ratio inducing osteoclast formation (reviewed in, (114)). Data has even shown that myeloma cells shift mesenchymal stem cells to differentiate into adipocytes instead of into osteoblasts (115). Patients with myeloproliferative neoplasm another group of hematological malignancies have, according to newly published study by our group, an increased risk for both vertebral- and hip fractures, compared to the general Swedish population (116).

Increased proliferation of hematopoietic cells in the bone marrow has been associated with lower bone density in patients with hematological diseases (70). The association between blood and bone is even seen in non-proliferative hematological diseases, such as Diamond-Blackfan anemia and Fanconi anemia (117). These are congenital diseases associated with anemia and pancytopenia as well as osteopenia and bone defects. Osteopetrosis, a congenital disease caused by dysfunctional or arrested osteoclast formation that leads to increased bone mass, can be associated with anemia and sometimes pancytopenia. The only cure for osteopetrosis is allogeneic stem cells transplantation (allo-tx) (117). Thus, the relationship between blood and bone diseases is seen both when the primary defect is in hematological cells as well as bone cells.

Lymphoma and bone

Lymphoma is a group of malignant tumors that originate in lymphocytes. Vast majority of lymphocytes are of the B-type, others being T-lymphocytes and NK-cells. Lymphoma, as a group, is the most common of hematological malignancies. The WHO has classified lymphoma into over 80 distinct entities, but a common division is made between Non-Hodgkin's lymphoma (NHL), which compromises up to 90% of all lymphomas, and Hodgkin's lymphoma (HL). NHL has several subtypes with two common ones being diffused large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) (118). Patients with DLBCL, classified as aggressive lymphoma, are almost always treated at diagnosis while patients with FL, an indolent lymphoma, do not receive treatment unless the disease is symptomatic (119). Indolent lymphoma can usually not be cured, in contrast to aggressive lymphomas (119). With improved treatment modalities, a high proportion of patients with lymphoma are cured or live many years with their disease. It is therefore important to identify potential complications like osteoporosis and fractures affecting quality of life of these patients.

Aggressive NHL, like DLBCL, are often treated with chemoimmunotherapy with rituximab, cyclophosphamide, doxorubicin, and vincristine, as well as glucocorticoids (R-CHOP) (119). When indicated, FL is treated with R only, R in combination with bendamustine (R-bendamustine) or sometimes R-CHOP (119). Rituximab is a monoclonal antibody against CD20, a membrane protein found on B-lymphocytes. In patients with rheumatoid arthritis, in whom rituximab also is used, it has been shown to decrease osteoclast activity (120, 121), and thus theoretically could decrease the risk for fractures, although this has not been studied. R-CHOP is given every second or third week, usually up to six courses. Cyclophosphamide is an alkylating agent that can cause hypogonadism and premature menopause, both known risk factors for osteoporosis. In male mice, cyclophosphamide has been shown to induce

osteoporosis attributed to its suppression of osteoblastogenesis, but even affecting osteoclastogenesis (122). It has also been demonstrated that doxorubicin inhibits the proliferation and differentiation of osteoblasts (reviewed in (123)). Vincristine can cause peripheral neuropathy and consequently increase risk for falls and fractures. The cumulative dose of prednisone (or other glucocorticoid) with six courses of R-CHOP is approximately 3000 mg (124). Glucocorticoids are well known to increase the risk for osteoporosis and fractures (reviewed in (125)), though the effect of intermittent use of high-dose oral glucocorticoids on osteoporosis and fractures is not well studied. G-CSF is often used for five to seven days in each cycle of R-CHOP and the long-term use of G-CSF has been associated with osteoporosis (126). In children with cancer, even short-term use of G-CSF has been shown to affect bone metabolism with decreased osteoblastic activity as well as increased osteoclastic activity (127).

R-bendamustine is often given every fourth week for up to six courses. The effect of bendamustine on bone metabolism or risk for osteoporosis is largely unknown. One preclinical study is published in a myeloma-mice model, indicating that bendamustine suppresses the growth of osteoclasts and maintains osteoblasts on the bone surface (128). Another study in male-mice showed that testosterone values were not affected by bendamustine treatment (129), but its effect on reproductive hormones in humans are unknown. In a course of R-bendamustine, the use of glucocorticoidsteroids and G-CSF, is usually limited.

Osteopenia (T-score between -1 and -2.5) and osteoporosis have been reported to be common in untreated patients with NHL (130, 131). Two recent large studies have addressed the risk for osteoporosis and fractures in lymphoma patients. A Danish case-control study showed a 61% increased risk for osteoporotic events (osteoporosis treatment or low-energy fracture) in patients with DLBCL or FL treated with a R-CHOP-like therapy compared with controls from the Danish population (132). A retrospectively collected UK study on patients ≥70 years treated with R-CHOP, showed a cumulative fracture incidence of

11.4% 18 months after treatment with R-CHOP (133). It is not clear whether it is the lymphoma in itself, the treatment, or other factors that affect the risk for fractures. Patients with NHL have been shown to have lower BMD and higher bone turnover markers after treatment compared with before treatment (134). Further attempts have been made to identify if it is the therapy or the lymphoma disease in itself that affects osteoporosis or fracture. In a study with over 13 thousand NHL patients identified from SEER-Medicare data, chemotherapy was associated with increased risk of osteoporosis and fractures compared to no chemotherapy (135). Some studies have indicated that the bone related changes seen with lymphoma treatment are temporary and recovery of BMD can be seen long term (136, 137).

AIM

The general aim of this thesis was to study the relationship between blood and bone in epidemiological studies. For this purpose, I used two separate cohorts, one with elderly men and another one with lymphoma patients.

The specific aims for each included paper were to:

I. Explore the potential association between serum serotonin and BMD and future fracture risk in elderly ambulatory men (paper I).

II. Explore the potential association between endogen plasma EPO and BMD and future fracture risk in elderly ambulatory men (paper II).

III. Analyze the association between Hb, neutrophil-, lymphocyte- and platelet count in blood, and BMD, in elderly ambulatory men (paper III).

IV. Compare the risk for hip fracture in patients with lymphoma with the risk for hip fracture in the entire Swedish population (paper IV).

SUBJECTS AND METHODS

This chapter summarizes the methods outlined in papers I-IV. Details of the analysis made in the four papers are presented in the respective papers.

Papers I-III

Study population

In papers I-III we used data from The Osteoporotic Fractures in Men Study, MrOS. MrOS is an international, prospective, population-based observational study with the primary aim to prospectively evaluate risk factors for osteoporosis and fractures in elderly men. The cohort from Sweden consists of 3,014 men aged 69-81 years at inclusion, recruited and investigated in 2001-2004 in the cities of Gothenburg (n=1,010) and Malmö (n=1,005) and the larger area of Uppsala (n=999). We foremost used the cohort from Gothenburg. Other countries participating in MrOS internationally, but not analyzed in our studies, were Hong Kong (n=2,000) and USA (n=5,995). At baseline, participants were randomly recruited through national registries and invited by letter to participate, followed by a telephone call. To be eligible, subjects had to be able to walk unassisted, be able to answer questionnaires and not having bilateral hip protheses or other osteosynthesis in the hips. No other exclusion criteria was applied. The acceptance rate was 45%. Written informed consent was obtained from all study participants.

Assessment of baseline variables

At baseline (October 2001–December 2004 in Uppsala and Malmö, and April 2002-December 2004 in Gothenburg), participants answered self-administered and interviewer-administered questionnaires, underwent physical examination and measurements, **Figure 5**.

Self-administered questionnaire

Information gathered in the self-administered questionnaires included questions regarding falls during the last 12 months preceding the baseline visit (yes/no), current or past history of smoking, previous fractures, and previous medical conditions (such as having been diagnosed with myocardial infarction, stroke, diabetes, chronic bronchitis, cancer, high blood pressure, yes/no). No verification of answers was done using medical records.

Interviewer-administered questionnaire

Information on amount of physical activity during the week before baseline and ongoing medications were collected with assistance from study personnel. Subjects were asked to bring both their current prescription and over the counter medications. The name and the dose of the drugs was registered by study personal.

Physical examination and tests

Height was measured with a standardized wall-mounted stadiometer. Weight was measured with the same scale for all participants. BMI was calculated as weight in kilograms divided by height (in meters) squared (kg/m^2). Hand grip strength was measured using a Jamar® (Sammons Preston Rolyan, Bolingbrook, IL, USA) dynamometer. Walking speed was assessed over 6 meters at usual pace, and measured in meters/sec. Lung function, including forced expiratory volume in one second (FEV1), was assessed with a standard spirometer used in clinical practice.

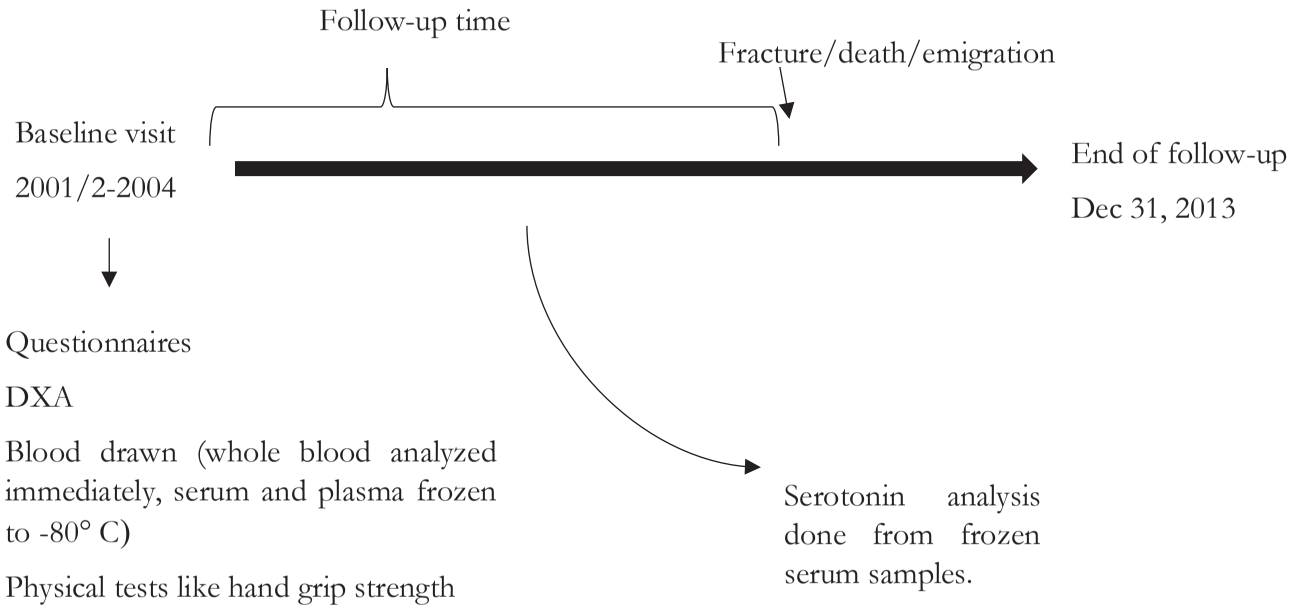

Figure 5. Timeline for baseline analysis and follow-up in MrOS cohort.

DXA

BMD was measured with DXA (Hologic DXA, Hologic QDR 4500/A-Delphi, Whaltman, MA, USA, in Gothenburg and with the Lunar Prodigy DXA, GE Lunar Corp., Madison, WI, USA, in Lund and Uppsala) in the whole body, total right hip (left hip was used if the right hip had a prothesis) and first to forth lumbar spine (L1-L4). The coefficients of variation (CVs) for the BMD measurements ranged from 0.5% to 3%, depending on which application was used. Results of Lunar devices are higher compared to those from Hologic devices thus a standardized BMD was calculated (6, 138). Body composition such as total body lean mass and total body fat mass was analyzed from the total body measurements with DXA.

Blood laboratory variables

Blood samples were collected around 8:00 a.m. after an over-night fast and abstinence from smoking. Serum and plasma samples were frozen within 1h and stored at -80°C until required for analysis. Some variables were analyzed immediately like blood cell count from whole blood and plasma glucose.

Serum serotonin was measured several years after baseline. Serum was available for analysis from 950 men. Serotonin was analyzed using a competitive enzyme-linked immunosorbent assay (ELISA); with an inter assay CV of 6%.

Plasma EPO concentrations were analyzed at baseline (from frozen samples) using an ELISA with a normal range of 3.1–14.9 IU/L in adults and total CV of 6.2% for a human serum control 11.2 IU/L.

Blood cell counts, including Hb, white blood cell- (neutrophils and lymphocytes), and platelet count, were analyzed using an automated cell counter at Sahlgrenska University Hospital, Gothenburg, Sweden (CV for blood count analysis are unavailable).

For analytic methods for other blood variables see published papers I-III (139-141).

Definition of comorbidities and comorbidity index

Prevalence of hypertension, myocardial infarction, cancer, chronic bronchitis, and stroke were assessed by the self-reported questionnaire. Diabetes was defined by self-reported questionnaire, and additionally the use of hypoglycemic medications or measured plasma-glucose >7.0 mmol/L. A comorbidity index was constructed using hypertension, myocardial infarction, diabetes, stroke, and in paper II FEV1 in the lowest quintile, and in paper III chronic bronchitis, respectively. Each disease rendered in one point that weighted equally. Subjects could thus have 0-5 points.

Identification of fractures and follow-up

Radiographic charts in respective health care region were screened on a regular basis. Date and type of fractures were registered for all new fractures and all answers were confirmed by the same physician's review of radiographic reports.

In paper I and II subjects were followed from the date of baseline visit until time of the first fracture, date of death, emigration, or end of study, whichever came first, **Figure 5**. Statistiska Central Byrån (SCB) provided information about death, dates of death and emigration. End of follow-up was December 31, 2013. At the end of the study period, 62% (n=621) of the men in the MrOS Gothenburg cohort had died and no one had emigrated. The median follow-up time for fracture was 10.6 years.

When a participant sustained a first fracture at different sites during the follow-up, the various fractures, and the corresponding follow-up times for each respective first fracture type were included in the analyses. The risk time (in days) for the first fracture in each fracture

group was calculated from the baseline date until the date of fracture, the date of death, date of emigration or the end of the follow-up time. Thus, the same person could be included in more than one fracture category rendering in different risk times, **Figure 6**.

The following International Classification of Diseases and Related Health Problems, 10th Revision (ICD-10) codes were used to define fractures.

- All fractures: S02, S12, S22, S32, S42, S52, S62, S72, S82, S92
- Nonvertebral osteoporotic fractures included hip, pelvis, proximal humerus and forearm: S32.1, S32.4-5, S42.2, S52.5, S56.6, S72.0-2
- MOF were defined as fracture of the hip, proximal humerus, pelvis, forearm and vertebral fractures: S22.0-1, S32.0-1, S32.4-5, S42.2, S52.5, S56.6, S72.0-2
- Hip fractures: S72.0-2
- Vertebral fractures: S22.0-1, S32.0

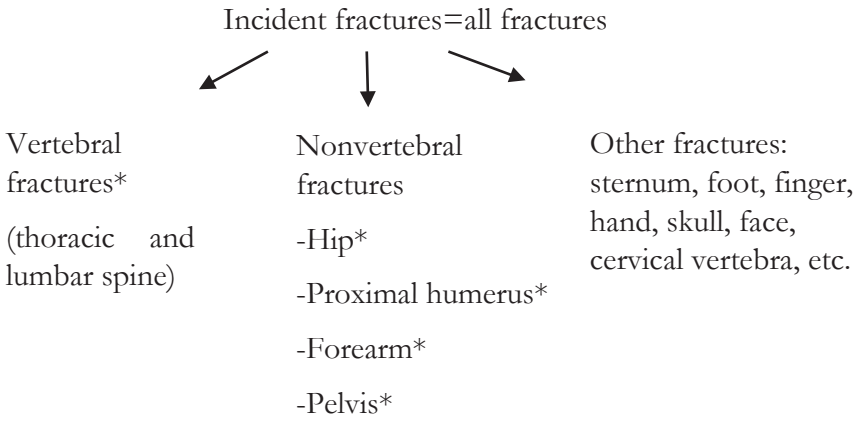

Figure 6. Classification of different types of fractures in paper I and II.

Specific cohorts

Paper I – The cohort of MrOS Gothenburg included 1,010 men of whom 950 had serum available for measurement of serotonin. Men taking SSRIs, n=33 were excluded after serotonin measurements. The final cohort included 917 men.

In the second part of the study an analysis of the effect of SSRIs on falls and fractures was performed. For this purpose, the whole MrOS *Sweden* cohort was used (n=3,014), including men with and without SSRIs.

Paper II – Of the 1,010 men included in the MrOS Gothenburg cohort 999 men had measurement of plasma EPO at baseline and were available for analysis.

Paper III - The cohort of MrOS Gothenburg included 1,010 men and 1,005 had measurement of at least one of blood cell count that is Hb, white blood cell-, or platelet count, and thus were included in the cohort in paper III.

Statistical analysis

A summary of statistical analysis used in papers I-III is provided in **Table 2**. Variables in a continuous scale are described by mean and SD or by median and interquartile range (IQR) if they had a markedly skewed distribution. When appropriate, skewed continuous variables were analyzed in the log scale. Differences in means between two groups were tested with t-test and in paper II a test of linear trend was used to compare difference in mean in ordinal scale over tertile groups. In paper I part 2, a Mann-Whitney u-test was used to compare non-parametric values. In paper I the odds for having a fall during the 12 months before baseline was computed by logistic regression. A Pearson correlation test was used for assessment of univariate correlations. In papers II-III multivariable linear regression was performed, adjusting for association of other covariates. In paper II results were stratified according to renal function. In paper III a covariate was considered as a confounding factor if associated with both blood variable (Hb, neutrophil-, or platelet count) and total hip BMD. We added other possible confounding factors separately, identified from previous studies. In papers I and II confounding factors were identified from previous studies. In papers I and II Poisson regression was used to calculate the rate of fracture per 1000 person years. In paper I and II the risk for fracture during the follow up from baseline until December 31 2013, was analyzed with Cox regression models adjusting for other covariates. In paper II, assumption of proportional hazard was tested from Schoenfeld residuals. Double sided tests were used throughout and a p-value <0.05 was regarded as statistically significant. The software used was SAS for Windows, version 9.3 (SAS Institute, Inc.

Cary, NC, USA), Stata version 15.1 (StataCorp LLC, College Station, Texas 77845 USA) and a database and statistics program package developed at the Department of Community Medicine and Public Health, Gothenburg University.

Table 2. Statistical methods used for each paper I-III.

Statistical methods used	P-I	P-II	P-III
Independent sample *t*-test	X		X
Mann-Whitney u-test	X		
Test of trend in ordinal scale		X	
Pearson correlation	X	X	X
Logistic regression	X		
Cox regression	X	X	
Poisson regression	X	X	
Linear regression		X	X
Schoenfeld residuals (Cox)		X	
Log transformation	X	X	X
Stratification		X	

Paper IV

Study population

Lymphoma population

Every new diagnosis of cancer is reported into the Swedish Cancer Register (SCR), which was started in 1958. This register includes diagnosis, date of birth, gender, date, and site of diagnosis. The reliability of the SCR is high with over 95% coverage (142). The SCR was used to identify lymphoma patients by searching for ICD-9 codes 200-202. Subjects ≥18 years old, diagnosed with lymphoma between the years 1995-2015 were included. Total of 37,236 patients were identified, 20,560 men and 16,676 women.

The following variables were collected through SCB from The Total Population Register (Registret över totalbefolkningen, RTB) and The Educational Register (Utbildningsregistret); date of birth, gender, population density, latitude, marital status (married, unmarried, widowed), and educational level (1-7 where higher figure indicates higher education level).

Swedish Population

All adults ≥18 years old living in Sweden during the period 1995-2015 were included, hereafter called "the Swedish population".

The same variables were collected by SCB for the Swedish population as were collected for lymphoma patients.

Identification of fractures and follow-up

Lymphoma patients as well as the Swedish population were observed until first hip fracture, death, emigration, or end of follow-up December 31, 2016.

The Inpatient Register (slutenvårdsregistret) from the National Board of Health and Welfare (Socialstyrelsen), in which all hospital admissions are documented, was used for identification of hip-fractures in both groups. Date of admission was registered as the date of fracture. The Inpatient Register started in 1964 and has national coverage from 1987. Every health care provider is legally obliged to report information about variables such as diagnosis and operations performed to this register (143) and its accuracy exceeds 90% for surgical admissions (144). The vast majority of patients who suffer from a hip fracture undergo surgery and the type of surgery is registered with an operation code. The operation code and hip fracture code are filed in the Inpatient Register.

The following ICD codes were used to define hip fractures:

ICD-9 820× and ICD-10 S72.0-2

The following operation code was used to identify surgical procedure for proximal femur fractures:

ICD-9 841 or 82× or NFJ 09-99 or NFB

SCB was responsible for the coordination of all the registers above for lymphoma patients and the Swedish population. Data was delivered without national identification numbers (personnummer), but with age, gender, and a study identification number.

Statistical Analysis

The Swedish population: The expected number of fractures in the Swedish population was calculated using hazard function (HF). A variant of Poisson regressions model was used to estimate HF to study the relationship between hip fracture and age, calendar year, population density, latitude, and seasonal variation. Only the first hip fracture was registered. The HF for hip fracture was piecewise linear in age and calendar year. The breakpoints for age were 40, 60 and 75 years, and for calendar year 2002 and 2009.

Lymphoma patients: A Poisson regression was used to assess the risk for hip fracture in lymphoma patients since the time of diagnosis, compared to the Swedish population. For lymphoma patients the breakpoints in age were 50 and 75 years and for calendar year was 2005. As the lymphoma population is much smaller than the Swedish population fewer breakpoints in age and calendar years where chosen. One fracture per person and time to first fracture was registered. Time at risk was censured when suffered from fracture, emigration, death, or end of follow-up.

The incidence of hip fracture for lymphoma patients and the Swedish population was calculated per 1000 person years as to be able to compare the incidence per age, gender, and calendar year between lymphoma patients and the Swedish population.

Hip fracture was used as a time-dependent variable when analyzing the risk of mortality. This means that when a person suffered a fracture, they "switched group" to the fractured group (from the non-fractured group). The time before suffering a fracture and the entire follow-up time for those who never suffered a fracture is counted towards the non-fracture group.

A determination of the number of patients needed to be included in the study was done as to be able to show a risk increase of 10%. The following assumptions were made: The total population has a 5% risk of suffering a hip fracture, power was set to 80%, and a double-sided

p-value at below 0.05. The desired size for each group calculated was at least 31,234 subjects and for this we had to include lymphoma patients for at least 12 years.

Ethical considerations

When conducting epidemiological studies, it is important to ensure personal privacy.

In MrOS all data was collected and handled by study personnel only. Every subject was signed a unique anonymous identification code and the identification list was stored in a locked room. Analysis and presentation of data was performed on group level and it is not possible to identify unique individuals by study results. The study participants were able to withdraw consent at any time.

The x-ray exposure from a DXA scan is low and the risk of serious complications because of venous blood sampling is extremely low.

In paper IV all data from the registers were coded, and we have no access to national identification numbers or names. Results were only available to the investigators.

Studies in the thesis were approved by the ethical review boards in Gothenburg (paper I-IV), Lund (paper I) and Uppsala (paper I).

RESULTS

This chapter summarizes the results outlined in papers I-IV. Details of the results are presented in the respective paper.

Paper I

Title: High Serum Serotonin Predicts Increased Risk for Hip Fracture and Nonvertebral Osteoporotic Fractures: The MrOS Sweden Study

Main results

The cohort included 950 men, with serum available for analysis of serotonin. Three percent, or 33 men, had ongoing treatment with SSRI medications, those subjects had significantly lower mean serotonin values (31.2 vs. 159.4 μg/L) compared with non-SSRI users and were excluded from further analysis (final cohort n=917). Mean age was 75.2±3.2 years.

Serotonin was negatively correlated with total hip BMD (r=-0.10, p=0.003). When analyzed per quintile, men in quintile 1 had higher odds (odds ratio (OR) 1.90 (95% CI 2.26-2.87)) of falls compared with men in quintile 2-5, after adjustment for age, BMI, smoking and total hip BMD.

An increased risk for all fractures, nonvertebral osteoporotic fractures, and hip fractures was seen in men with serotonin in quintile 5 compared with those in quintile 2-4, **Table 3**. In men with the highest serotonin the risk for hip fracture was 2.62 [hazard ratio (HR) of 2.62 (95% CI 1.43-4.79)], compared with men in quintile 2-4, in a multivariable Cox regression analysis adjusted for age, total hip BMD, BMI, smoking, and falls in the previous year. A trend to an increased risk for all fractures and nonvertebral fractures in men in serotonin quintile 1 compared with quintile 2-4 was seen, **Table 3** and **Figure 7**.

Table 3. Hazard ratio for all fractures, hip fractures, nonvertebral osteoporotic fractures and vertebral fractures (95% CI) in subjects in Serotonin Quintile (Q) 1 vs. Q2-4 and Serotonin Q 5 vs. Q1-4 and Q2-4. MrOS Gothenburg (From Kristjansdottir *et al.,* (140), reproduced with permission from © 2018 American Society for Bone and Mineral).

	Serotonin Q1 vs. Q2-4	Serotonin Q5 vs. Q2-4	Serotonin Q5 vs. Q1-4
All fractures, N=224			
Age adjusted	1.37 (0.98-1.91)	1.63 (1.20-2.23)	1.50 (1.12-2.02)
Multiv. analysis*	1.39 (0.98-1.96)	1.49 (1.08-2.08)	1.38 (1.01-1.89)
Hip fractures, N=57			
Age adjusted	1.48 (0.72-3.01)	2.92 (1.63-5.20)	2.62 (1.54-4.48)
Multiv. analysis*	1.65 (0.80-3.43)	2.62 (1.43-4.79)	2.30 (1.31-4.02)
Nonvertebral osteoporotic fractures, N=97			
Age adjusted	1.55 (0.92-2.60)	2.33 (1.48-3.68)	2.07 (1.35-3.16)
Multiv. analysis*	1.76 (1.03-2.99)	2.11 (1.31-3.41)	1.82 (1.17-2.85)
Vertebral fractures, N=86			
Age adjusted	1.33 (0.79-2.23)	1.15 (0.70-1.98)	1.07 (0.63-1.79)
Multiv. analysis*	1.33 (0.78-2.27)	0.95 (0.54-1.70)	1.35 (0.81-2.25)

*Multivariable analysis adjusted for age, BMI, total hip BMD, smoking, and falls previous year. Q Quintile

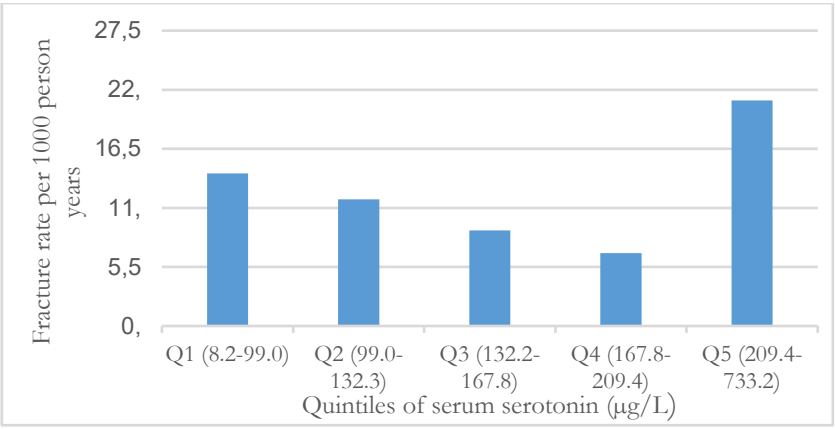

Figure 7. Risk per 1000 years for nonvertebral osteoporotic fractures by quintiles of serum serotonin in MrOS Gothenburg (From Kristjansdottir, *et al.* (116), reproduced with permission from © 2018 American Society for Bone and Mineral Research).

SSRIs; fractures in MrOS Sweden

When analyzing the association between SSRIs and fractures, data from the MrOS *Sweden* cohort (n=3,014) was used. Three percent (n=90) had treatment with SSRI at baseline. Subjects with ongoing SSRI medication had lower hip BMD, more falls, and decreased hand grip strength, compared with non-SSRI users. No adjustments were done for other covariates.

For SSRI users the risk for incident fractures (HR 1.62, 95% CI 1.11-2.35) and nonvertebral osteoporotic fractures (HR 1.87, 95% CI 1.13-3.09) was increased after adjustment for age and center of inclusion, compared to non-SSRI users. When adjusting for BMI, total hip BMD, smoking, hand grip strength and falls, the association was no longer significant.

Paper II

Title: High Plasma Erythropoietin Predicts Incident Fractures in Elderly Men with Normal Renal Function: The MrOS Sweden Cohort

Main results

The cohort included 999 men with available plasma EPO analysis. The mean age was 75.3±3.2 years and the median value of EPO was 10.0 IU/L (IQR 7.8-13.1 IU/L). EPO was analyzed per tertile and as a continuous variable. Data analysis was done for the whole group and stratified by renal function [estimated glomerular filtration rate (eGFR) 60 ml/min)].

Subjects with eGFR ≥60 ml/min (n=728)

EPO was associated with age (r=0.13, p<0.001), total hip BMD (r=0.14, p<0.001), iFGF23 (r=0.11, p=0.004) and PTH (r=0.14 p<0.001). EPO was negatively associated with osteocalcin (r=-0.09, p=0.022). No association was seen between EPO and PINP. The association between EPO and total hip BMD was independent of age, BMI, iFGF23 and Hb (β=0.019 per SD log(EPO), p<0.001).

EPO was associated with higher prevalence of hypertension (r=0.11, p=0.004), myocardial infarction (r=0.13, p<0.001), stroke (r=0.14, p<0.001), inflammation [C reactive protein (CRP), r=0.10, p=0.006 and interleukin 6 (IL-6), r=0.16, p<0.001], and more falls (r=0.08, p=0.034) in the previous year before baseline. EPO was associated with decreased lung function (lower FEV1, r=-0.14, p<0.001), and lower hand grip strength (r=-0.10, p=0.006).

The median follow-up time for fracture was 10.6 years and our results showed that EPO was associated with higher risk for all fractures (HR 1.43 per tertile EPO, 95% CI 1.15-1.79), MOF (HR 1.40 per tertile EPO, 95% CI 1.08-1.82) and vertebral fractures (HR 1.42 per tertile EPO, 95% CI 1.00-2.01) in a Cox regression model adjusted for age, BMI, iFGF23, PTH, CRP, eGFR, falls, hand grip strength and comorbidity index.

Subjects with eGFR <60 ml/min (n=267)

EPO was neither associated with total hip/lumbar spine BMD, nor PINP or osteocalcin in subjects with decreased renal function.

EPO was positively correlated with BMI (r=0.14, p=0.019), CRP (r=0.19, p=0.002), IL-6 (r=0.21, p<0.001), iFGF23 (r=0.16, p=0.010), PTH (r=0.18, p=0.004), and falls in the year prior to inclusion (r=0.15, p=0.014).

EPO was associated with higher prevalence of diabetes (r=0.14, p=0.027), stroke (r=0.17, p=0.006), and hypertension (r=0.14, p=0.019), at baseline.

No association was seen between EPO and fracture risk.

Paper III

Title: High platelet count is associated with low bone mineral density: The MrOS Sweden cohort

Main results

The cohort consisted of 1,005 men with a median age of 75.3 years (IQR 72.9–78.6).

Hemoglobin

Hb was positively correlated with total hip BMD (r=0.16, p<0.001), and negatively correlated with osteocalcin (r=−0.13, p<0.001). A statistically significant associations was not seen between Hb and the other bone remodeling markers, PINP and ALP.

Additionally, Hb was positively correlated with estradiol (r=0.26, p<0.001), eGFR (r=0.13, p<0.001) and hand grip strength (r=0.15, p<0.001) and negatively correlated with iFGF23 (r=-0.09, p=0.004), phosphate (r=-0.12, p<0.001) and CRP (r=-0.11, p<0.001).

Hb was associated with total hip BMD after adjustment for age and BMI but became statistically insignificant after adjustment with estradiol or osteocalcin in a multivariable linear regression.

Neutrophil count

Neutrophil count was negatively correlated with total hip BMD (r=−0.08, p=0.010) and total body BMD (r=−0.09, p=0.006). A positive association was seen between neutrophil count and ALP (r=0.15, p<0.001), but no association with osteocalcin and PINP was seen.

Additionally, neutrophil count was positively correlated with being a current smoker (r=0.23, p<0.001), CRP (r=0.32, p<0.001), phosphate (r=0.08, p=0.010), serotonin (r=0.10, p=0.001), iFGF23 (r=0.07, p=0.020), iron deficiency (r=0.19, p<0.001), and comorbidity index (r=0.19, p<0.001).

Neutrophil count was independently associated with total hip BMD in multivariable linear regressions analysis, β=−0.013 per SD neutrophils, p=0.008.

Lymphocyte count

Lymphocyte count was not associated with BMD.

Platelet count

Platelet count was associated with BMD at all sites. Platelet count had the strongest negative correlation with total hip BMD (r=-0.11, p=0.003). No association between platelet count and ALP, osteocalcin, or PINP, was seen.

Additionally, platelet count was positively associated with CRP (r=0.15, p<0.001), serotonin (r=0.27, p<0.001), phosphate (r=0.07, p=0.022) and iron deficiency (r=0.14, p<0.001). Platelet count was negatively associated with EPO (r=-0.18, p<0.001), PTH (r=-0.07, p=0.020), and iFGF23 (r=-0.08, p=0.018).

Platelet count was independently associated with total hip BMD in multivariable linear regression, after adjustment for other covariates, β=−0.013 per SD platelets, p=0.005.

Paper IV

Title: Increased Risk of Hip Fracture in Patients with Lymphoma, a Swedish Population Study of 37,236 Lymphoma Patients

Main results

The total number of lymphoma patients was 37,236, 20,560 men, (mean age at diagnosis of 65.5±15.9 years) and 16,676 women (mean age at diagnosis 67.3±16.7 years).

The mean follow-up time for hip fracture was 5.47±5.37 years, range 0-22 years. This corresponded to 203,681 person-years and we identified 460 fractures in men and 776 fractures in women during that period. A total of 3.3% (2.2% of men and 4.7% of women) of lymphoma patients developed a hip fracture, with a mean follow-up time of 4.87 ±4.24 years.

The ratio between observed/expected fractures in the lymphoma population, compared with the Swedish population was 1.19 (95% CI 1.11-1.28) for women and 1.06 (95% CI 0.97-1.17) for men.

Hip fracture incidence was higher in women than in men, increased with age, and decreased by calendar year in both lymphoma patients and the Swedish population, **Table 4**.

The results showed that 40-year-old women, diagnosed with lymphoma in 2012 have a risk of 2.8 (HR 2.80, 95% CI 1.20-6.53) of developing hip fracture four years after the diagnosis of lymphoma, compared with the Swedish population. In women diagnosed in the same year, 2012, but at the age of 70-years, the risk of developing hip fracture was not statistically increased, compared with women of the same age from the Swedish population. In 40-year-old men, diagnosed with lymphoma in 2012 there was a trend, however non-significant, for increased risk for hip fracture (HR 1.70, 95% CI 0.96-3.00), compared with the Swedish population.

The risk of death was more than double in lymphoma patients who developed a hip fracture compared to those who did not (HR 2.26, 95% CI 1.80–2.85 in men and HR 2.17, 95% CI 1.84–2.57 in women), after adjustment for age and time since lymphoma diagnosis.

Table 4. The Hazard ratio (HR) of hip fracture between subjects with lymphoma compared with the Swedish population (From Johansson *et al.* (145), reproduced with permission from © Springer Nature)

	Calendar years (4 years after diagnosis of lymphoma)		
Age (years)	2001	2009	2016
Men			
40	**2.47 (1.43-4.27)**	**1.86 (1.09-3.20)**	1.70 (0.96-3.00)
50	**1.85 (1.20-2.86)**	1.39 (0.91-1.13)	1.27 (0.80-2.01)
60	**1.72 (1.35-2.20)**	**1.30 (1.04-1.62)**	1.19 (0.90-1.57)
70	**1.21 (1.02-1.42)**	0.99 (0.84-1.18)	0.91 (0.71-1.15)
80	1.13 (0.88-1.46)	0.91 (0.80-1.03)	0.83 (0.68-1.02)
Women			
40	**3.20 (1.38-7.41)**	**2.89 (1.25-6.66)**	**2.80 (1.20-6.53)**
50	**2.32 (1.49-3.62)**	**2.10 (1.36-3.42)**	**2.04 (1.30-3.20)**
60	**1.78 (1.40-2.25)**	**1.61 (1.29-2.00)**	**1.56 (1.51-1.61)**
70	**1.23 (1.06-1.43)**	1.11 (0.97-1.27)	1.08 (0.89-1.30)
80	**1.22 (1.08-1.38)**	**1.11 (1.01-1.21)**	1.07 (0.92-1.25)

Bold indicates $p<0.05$. Age is presented *at* diagnoses of lymphoma, calendar years and HR are presented 4 years *after* diagnosis of lymphoma

DISCUSSION AND CLINICAL IMPLICATIONS

Serotonin

We have shown that high levels of serum serotonin are associated with increased risk of all fractures, nonvertebral osteoporotic fractures and hip fractures in ambulatory elderly men. Men in serotonin quintile 5 have a HR of 2.62 for hip fractures, compared with those in quintile 2-4, after adjustment for major confounding factors. A trend to a u-formed association between serotonin and fractures was seen. The explanation for trend to increased fracture risk in those with low levels of serum serotonin might be that low serotonin is associated with increased general weakness. Consequently, the reason for increased fracture risk associated with elevated serum serotonin levels could be that serotonin influences bone structure in itself as seen with decreased BMD and is supported by several preclinical studies (34, 146).

A negative association between serotonin and BMD shown in our study, is partly in agreement with population-based studies in humans (41, 48). Some studies have indicated that serotonin influences bone in a gender- and age specific manner (43, 147) and in one study a negative association between serum serotonin and BMD was seen only in postmenopausal women, and not men or premenopausal women (43). Even positive correlation between circulating serotonin and BMD has been seen (44). Thus, the inconsistency between studies on serotonin and BMD might partly be because of different cohorts with regards to gender and age. Differences in dietary habits also influences the results, as serum serotonin levels are highly affected by tryptophan intake, but tryptophan is found in various food products (148). Similarly, inconclusive results are seen when studying BMD in patients with carcinoid syndrome, a patient group that has chronically elevated circulating serotonin levels (45-47).

Our study is not in agreement with the only other human study on serum serotonin and fracture risk (48). However, that study is relatively

small with 18 reported hip fractures in 202 men (48), and due to the small sample size, it is more difficult to show a risk difference. Additionally, the mean age was lower, 64.8 years, and the follow-up time shorter, 3.7 years. Fractures were not x-ray verified in that study.

Using the whole cohort of MrOS *Sweden* we could assess if men taking SSRIs have increased risk for fractures. The number of men taking SSRIs in the whole group was 90, of whom 29 suffered a fracture, resulting in a relative risk of 1.60 (95% CI 1.06-2.32) for all fractures, in an unadjusted model. After adjusting for age, center of inclusion, BMI, total hip BMD, smoking, hand grip strength and falls we could not show a statistically significant increase in fracture risk. Several previous studies have shown that patients taking SSRIs have increased risk for fractures (49, 149, 150). We showed that subjects taking SSRIs have very low serum serotonin values, as supported by previous studies (151, 152). Men taking SSRIs had significantly lower total hip BMD in our study, in an unadjusted analysis. This has previously been shown in MrOS USA (n=5,995) where adjustments were made for major confounding variables (153).

Erythropoietin

We have shown that high EPO is independently associated with increased risk for all fractures and MOF in men with normal renal function. To the best of our knowledge, we were the first group to show this relationship in humans. In men with normal renal function, high EPO was also associated with increased risk for vertebral fractures in fully adjusted multivariable analysis, when EPO was analyzed per tertile. When EPO was analyzed as a continuous variable, and multiple adjustments were made, the risk increase of vertebral fractures was not statistically significant. The risk for hip fractures was consistently higher in men with high EPO, though not statistically significant. This might be because of lack of power or consequently lack of risk difference. Our findings are supported by preclinical research, but several studies on atraumatic bone models in mice have shown that

EPO is associated with worse bone health, mostly affecting trabecular bone (58, 59, 154, 155).

Paradoxically, and unexpectedly, we even found a positive linear association between EPO and BMD. The positive correlation between EPO and BMD is difficult to interpret together with the increased fracture risk seen in our study. This kind of paradoxical relationship is not unprecedented and is also seen in patients with type 2 diabetes (156). As previously mentioned, preclinical studies in atraumatic settings have shown that EPO has a degenerative effect on bone (58, 59, 154, 155). Nonetheless, studies in traumatic bone models and in growing rodents show that EPO has a positive effect on bone (62-65, 68). A meta-analysis on bone health in patients with hematological diseases, did not observe any correlation between blood values of EPO and bone density. However, increased bone marrow cellularity was correlated with decreased bone density (70).

Men with high EPO were older, had higher CRP, less muscle strength, more falls, and more comorbidities. This is in agreement with previous studies showing that EPO is associated with inflammation, increased morbidity, and age (157-161). We strived to adjust for comorbidities, age and inflammation when assessing the role of EPO in fracture risk. Nonetheless, residual confounding factors cannot be ruled out. EPO may merely be a proxy for poor health, which nonetheless does not preclude its value as a risk factor for fracture.

We showed a positive association between EPO and iFGF23 as well as CRP. This is supported by previous studies showing that EPO stimulates production of FGF23 and might be a link between iron deficiency, inflammation, anemia and FGF23 (78, 79, 162, 163). We found a stronger association between EPO and vertebral fractures than hip fractures, and this supports that EPO's effect on bone might, at least partly, be mediated by FGF23. Previous studies have shown that FGF23 expression in bone is mainly found in osteocytes peripherally in the trabecular space (164). Nonetheless the risk for vertebral

fractures with high EPO was seen even after adjustments for FGF23 suggesting that other mechanisms than FGF23 contribute.

Serotonin, EPO, and fracture prediction

Most fractures occur in people with normal BMD (11). Thus, it is important to find other predictors for fractures, and our findings that serotonin and EPO increase the risk for fracture may be clinically valuable. Most noticeable is the 2.62 times increased risk for hip fractures for men with the highest serotonin values, after adjustment for important confounders such as age, total hip BMD, BMI, smoking and prevalence of falls. Our results are based on a single cohort of less than 1000 men and 57 hip fractures and obviously need to be confirmed in other cohorts before serotonin can be considered as a predictor for hip fracture. Nevertheless, it would be interesting to further analyze how serotonin compares to other known risk factors for hip fracture, or in combination with them. Levels of serum serotonin are influenced by various foods and medications, but it is unclear if modifying them would change the fracture risk. Modifying serotonin values might have other clinically negative effects. If EPO indeed increases fracture risk, it might be clinically valuable information. But as for serotonin, this needs to be confirmed in more studies. Special notice is warranted for patients treated with exogenous recombinant human EPO, though we do not know if even exogenous EPO serves as a risk factor for fractures.

Hemoglobin, neutrophil- and platelet count

We showed that platelet count is negatively associated with BMD at all sites. Our results are in agreement with a recent Korean study that showed that platelet count is negatively associated with femoral neck- and lumbar spine BMD in both genders (108). Another study in patients with chronic graft vs. host disease (cGVHD), showed that platelet count was associated with osteoporosis, as well as the severity

of cGVHD (165). On the other hand, MrOS USA (101) that includes exclusively men, and the Cardiovascular Health study (110) that includes both sexes, did not show an association between platelet count and BMD. The inconsistency between different cohorts could be explained by the relatively weak association, or due to differences in characteristics of the study population, such as age, gender, medication, and underlying diseases. Even preclinical studies are contradictory on the association between platelets and bone cells (166-168). High serotonin is associated with low total hip BMD as seen in papers I and III. As serotonin is stored in platelets, the association between total hip BMD and platelets might partially be explained by serotonin, but not exclusively, as the association was independent of serotonin values in multivariable linear regression.

Neutrophil counts were negatively associated with total hip- and total-body BMD. This is consistent with some previous studies (101, 107), but not others (109). The inconsistency between different cohorts could be explained by the relatively weak association, different populations, and study design. In our cohort the association between total hip BMD and neutrophil count was consistent after adjustment for multiple covariates. Our findings are supported by preclinical studies that have shown that neutrophils are capable of expressing RANKL (90) and consequently activating osteoclasts (91). In mouse models of periodontitis, neutrophils can induce osteoclast formation (169). Further support of our findings can be seen in patients with COPD, in whom enhanced activation of RANKL in neutrophils in peripheral blood correlates with BMD (170).

Hb correlated with total hip BMD in univariate analysis. On the other hand, in multivariable linear regression analysis, the association between Hb and total hip BMD was not statistically significant, after adjustment for estradiol and osteocalcin. A recently published study from a German population based cohort did show that Hb was positively associated with ultrasound derived bone stiffness in the elderly (104). Two other population-based studies in elderly individuals from Italy, have shown significant correlation between Hb and bone

mass (102, 103). No adjustments were made for osteocalcin or estradiol in those studies (102-104). We have previously shown that in elderly men estradiol is positively and osteocalcin negatively correlated with Hb (171, 172). Additionally, in the Italian and German studies, DXA was not used for assessing bone density but rather peripheral quantitative computerized tomography (pQCT) and ultrasound, respectively (102-104). Thus, it is possible that Hb affects bone in a manner not captured by DXA. This would be supported by several studies showing that low Hb is related to increased risk for fractures (105, 106, 173, 174).

We found no association between Hb and serotonin, opposite to previously published preclinical studies (52-54). However, serotonin was positively associated with neutrophil- and lymphocyte count, and as previously mentioned, to platelet count.

The difference in total hip BMD per SD platelet-, and neutrophil count are small and unlikely to be clinically relevant. Nevertheless, the association is consistent and supports our hypothesis on the association between blood and bone, and hopefully will lead to further studies. Bone marrow aspiration and trephine biopsy may be interesting complements to assess this association. Peripheral blood can be influenced by factors outside the bone marrow, like in immune thrombocytopenia where the platelet counts are low, but the bone marrow is normal (175). Additionally, valuable information might be uncovered with high resolution peripheral quantitative computed tomography (HRpQCT), an imagine technique that even shows microstructural changes in bone (176).

Strengths and limitations papers I-III

MrOS is a large population-based cohort collected in a standardized way and we have a long follow-up time for fractures. The data on fractures, death, and emigration, are reliable.

There are, however, several limitations. The acceptance rate regarding inclusion in MrOS was 45%. We do not have information about how many declined and how many were excluded. It is likely that the men who accepted were healthier than those who declined. Nonetheless, it is possible, though unlikely, that those who accepted were in poorer health. Needing a walking-aid was an exclusion criterium, and this automatically excluded a, presumably large, group of men in poorer health. However, the aim of the study MrOS was not to find out the incidence of fractures in the entire population, rather to study risk factors for osteoporosis and fractures in ambulatory men. Another aspect is that the cohort only included men from the city of Gothenburg. It has been shown that men in densely populated places have increased risk of fractures compared to more rural areas (23). Thus, our results might not apply to subjects living in more rural areas. Our aim was to study the association between blood related variables and bone, and for this we have used the MrOS cohort. The metabolism of bone and blood changes with age (reviewed in (177, 178)) and consequently our results may not be generalized to men of other ages. That the cohort is relatively homogenous can be considered both as a strength and as a limitation. A limitation as it leads to decreased generalizability and a strength because there is decreased risk of residual confounding, although that cannot be excluded.

Hip fracture suffered outside the subject's home region could possibly have been missed if the participant had no follow up visits in their home region. We do not think that this would include many fractures.

Even though the cohort is relatively large, the rate of fractures in each subgroup is much lower, and we are at risk of making a type II error and missing a true association due to a lack of statistical power.

Patients with lymphoma

We showed that the risk for hip fracture in female lymphoma patients was increased compared to the Swedish population. Men with lymphoma had increased risk for hip fracture in the beginning of the study period. The risk increase was highest in younger women and with

time the incremented risk of fracture in lymphoma patients, at all ages and both sexes, decreased. The risk for hip fracture was consistently higher in women with lymphoma at all ages, though not statistically significant in women over 70 years old. Nevertheless, the actual number of fractures in the ages 18-49 years was low, reflected by the wide confidence interval in the youngest age group. It is possible that the risk increase in the younger female patients is true, but due to low incidence there is high amount of uncertainty. When assessing women 50 or 60 years old (in 2012) the results are more reliable and we can more safely say that they have a 50-100% increased risk for hip fractures four years after diagnosis, compared to the population. Overall, we expected the risk for hip fracture to be higher in lymphoma patients than we found it to be.

Our results are, to some extent, in agreement with other studies on osteoporosis and fractures in patients with hematologic malignancies and specifically lymphoma (132-134, 179). Most of the studies published on osteoporosis or fractures in lymphoma patients hitherto have been small. A large Danish nationwide cohort study from 2020 showed increased risk (10-years cumulative incidence 16.3% vs. 13.5%) for osteoporotic events (osteoporosis treatment or low-energy fracture) in over 2,500 FL and DLBCL patients with R-CHOP-like treatment compared to almost 13,000 matched controls from the Danish population. The risk increase was even seen after exclusion of vertebral fractures (132). However, women under 50 years of age had not increased fracture risk. The highest risk increase was seen in women (over 50 years) and older patients (132). Another large retrospective study from UK showed a cumulative incidence of fracture being 11.4%, 18 months after start of R-CHOP, in a cohort of 877 patients 70 years or older with DLBCL (133). No control group was provided but the authors assumed an annual risk of fracture being 2% in the population, based on UK register data (180). However, no evaluation was done of change in risk in the study period in this population. It is unclear why the risk increase for lymphoma patients was more pronounced in women than in men in our study. Another small prospective study in a

cohort in 61 patients with newly diagnosed NHL, reported high bone turnover (increase in bone resorption- and bone formation markers) and reduction of BMD foremost in men and patients over 55 years (134). Thus, other studies have reported increased risk in both men and women as well as in older patients. Our cohort was heterogenous, with a mixture of many lymphoma subtypes and included both treated and untreated patients. Additionally, we only included hip fractures while the studies from Denmark and UK included an "osteoporotic event" and all fracture types, respectively, which might partly explain the differences in our results (132, 133). In Sweden, the use of rituximab in first line therapy of lymphoma started between 2003 and 2007 (181). Additionally, first line treatment of FL has shifted from being often CHOP-like to being more often R monotherapy or R-bendamustine (181). Some studies have suggested that rituximab is bone protective (120, 121), though not supported by all (182). It is theoretically possible that bendamustine has a positive effect on bone metabolism (128), though not studied in humans. Patients receiving R or R-bendamustine are consequently spared CHOP with its potential negative effect on bone. Thus, it is possible that it is indeed the treatment with CHOP-like combination that leads to increased risk for fractures in lymphoma patients and not the lymphoma in itself. One might speculate if the untreated patients in our cohort, as well as those treated with R or R-bendamustine, dilute the risk increase in those who have received R-CHOP-like treatment in our cohort.

The risk for hip fractures decreased with calendar year in both men and women, as previously has been shown (183, 184). However, the risk decreased more in lymphoma patients than in the Swedish population. The reason for this is not clear. In recent years there has been increased awareness on exercise and rehabilitation in lymphoma patients which might explain why the risk for fracture has decreased proportionally more in patients with lymphoma. Another possibility is changes in lymphoma treatment over the years (119) as mentioned above.

DXA is the gold standard for the diagnosis of osteoporosis (6). However, radiodensity or attenuation of bone (as well as other tissue)

can be measured on computed tomography (CT). Studies have shown that attenuation measurements (measured in Hounsfield Units) at the lumbar spine on CT scans have good correlation with BMD values measured with DXA (185). On routine CT scans, taken pre- and post-treatment, patients with NHL have increased loss of density in vertebral bodies (vertebral density, VD), compared to controls (186). Low VD before treatment as well as loss of VD after treatment correlates with increased risk for incident fractures (186). In the future CT might be a useful method for physicians to identify which patients would benefit from DXA analysis, alternatively aiding in treatment decisions for preventive therapy against osteoporosis. Physicians treating lymphoma patients should be aware of other risk factors for osteoporotic fractures, such as previous fractures, parental fractures, BMI, and smoking.

Strengths and limitations paper IV

The main strength of paper IV is the large cohort of Swedish patients with lymphoma and the long follow-up time, as well as good reliability of fracture data. Almost all patients with hip fractures are admitted to hospital and get treated with surgical intervention and consequently registered in the Inpatient Register.

The main weakness of this study is the limited information we have about the lymphoma patients. We could not subclassify different types of lymphoma according to ICD-10, as was our intention. Additionally, we intended to analyze the use of corticosteroids and relate this to hip fractures. We received data from the Swedish Prescribed Drug Register ("Läkemedelsregistret"). A register that started in 2005 and shows medications picked up by prescription but not medications handed out or given in hospital. We could compare the use of corticosteroids in patients with DLBCL at different hospitals (from 2005), and we saw a great variation in prescription per diagnosis and found that many hospitals hand out corticosteroids. We could therefore not draw any conclusions based on the use of corticosteroids. We neither have

information about chemoimmunotherapy, nor information on risk factors for osteoporosis or fractures.

The lymphoma patients are also counted for in the Swedish population, but as they are extremely few in comparison, this would not influence our results.

CONCLUSIONS

- Serum serotonin is negatively associated with total hip BMD.
- Low serum serotonin is associated with somewhat lower hand grip strength and increased odds of falls.
- High serotonin predicts the risk for all fractures, nonvertebral osteoporotic fractures and hip fractures.
- Plasma EPO is positively associated with BMD (total hip, lumbar spine, and total body) in elderly men with normal renal function.
- Plasma EPO is associated with increased iFGF23, comorbidities and inflammation.
- High plasma EPO predicts the risk for all fractures and MOF, in elderly men with normal renal function.
- Platelet- and neutrophil count are negatively associated with total hip BMD.
- Women with lymphoma have increased risk for hip fractures compared with the Swedish population.

In summary our results support the hypothesis of interconnection between blood and bone. Our finding that serotonin and EPO predict the risk for fractures need to be validated in further studies. We do not know if our results apply in younger men and the relationship between blood and bone could indeed be different depending on age. Physicians treating lymphoma patients should be aware of the increased risk for hip fractures in women.

FUTURE PERSPECTIVE

Being a part of our research group on blood and bone has really piqued my interest and I am eager to explore this interaction further.

HEMOS

We have prospectively gathered a cohort of 110 patients from "Västra Götaland" planned for Allo-tx, and lymphoma patients planned for autologous stem cell transplantation (auto-tx) (HEMOS). The study started in 2015 and the last patient was recruited in the summer of 2019. Subjects have been investigated with DXA, HRpQCT, blood-, urine-, and feces samples have been collected and the patients have answered questionnaires at four time-points, before, and 6, 12 and 24 months after allo/auto-tx, respectively. 110 patients completed the first measurements, 90 the second (16 where too sick/diseased, two went off study, two did not receive the transplant), 80 the third (16 where too sick/diseased, two went off study, two did not receive the transplant and three were not able to because of logistical problems and six did not because of COVID19 restrictions) and 63 the fourth visit (21 where too sick/diseased, two went off study, two did not receive the transplant and 16 could not because of COVID19 restrictions). Data analysis started in quartile 2 2021 in collaboration with Department for Rheumatology and Inflammation at Gothenburg University.

Fractures in transplanted patients

We are now completing a register study on all patients in Sweden that have done auto- or allo-tx from 1995-2015 and comparing the fracture risk in this group to that of the Swedish population.

Bone health in patients with myeloproliferative neoplasm

We are planning a study, similar to HEMOS, in patients with newly diagnosed myeloproliferative neoplasm. We will prospectively collect a cohort of 100 patients from "Västra Götaland" preliminarily during a three-year period. We will measure bone densitometry by DXA and HRpQCT. These procedures are planned to be repeated after 2 and 5 years from the first investigation. Additionally, we will assess fracture risk with FRAX and collect blood samples for analysis of bone remodeling markers. As there is a deficiency of data as regards HRpQCT in the normal population there is a plan to randomly recruit an age-gender matched cohort by invitational letter.

Other

In Sweden we have an unique opportunity in cancer research thanks to **INCA**. INCA is a national web-based register on cancer, including lymphoma as well as other hematological malignancies. In this register detailed information is available beyond diagnosis, such as stage of the disease, risk factors (for survival) and several blood tests, as well as treatment received. This platform has information from 2007 and together with the Inpatient Register to detect fractures we might be able to answer some of still unanswered questions on fractures in lymphoma patients.

In recent years the importance of **tumor microenvironment** in lymphoma and other hematological malignancies has become increasingly appreciated. Measuring RANKL, RANK/OPG from biobank material in patients with hematological malignancies such as acute lymphatic- or myeloid leukemia and chronic lymphatic leukemia, and correlate with prognosis would be interesting. It is intriguing to wonder if hindering osteoclasts with bisphosphonates can have a role

in treating some hematological malignancies. Preclinical studies have indicated that they might in treating acute lymphatic leukemia (187).

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