

# **Estrogen and raloxifene in experimental arthritis and osteoporosis**

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## **ABSTRACT**

In postmenopausal rheumatoid arthritis (RA), both the estrogen deficiency and the inflammatory disease contribute to the development of generalized osteoporosis. This leads to an increased risk of fracture, with high morbidity and mortality. More than 50% of women with postmenopausal RA suffer from osteoporosis. Hormone replacement therapy (HRT) is used to treat postmenopausal osteoporosis. HRT has also been shown to ameliorate RA, with decreased joint destruction, reduced inflammation, increased bone density and better patient health assessment. Unfortunately, longterm hormonal treatment is associated with severe side effects, and is no longer recommended.

The aims of this thesis were to establish a murine model for studies of osteoporosis in postmenopausal RA. To investigate the relative contributions of estrogen deficiency and inflammation to osteoporosis development in arthritic disease. To examine whether treatment with raloxifene, a selective estrogen receptor modulator, would have the same beneficial anti-arthritic and anti-osteoporotic effects as estrogen. Furthermore, we wanted to compare the mechanisms for these effects between estrogen and raloxifene.

We found that lack of endogenous estrogen and arthritic disease contributed equally and additively to osteoporosis development in collagen-induced arthritis, a murine model of human RA. Arthritic ovariectomized mice lost 55% of their trabecular bone mineral density (BMD) compared with cycling healthy mice.

Raloxifene potently decreased the frequency and severity of arthritis, protected the joints from erosions, and preserved the BMD. These effects were sustained when treatment was given both as prophylaxis and in established disease, and during longterm treatment.

Raloxifene down-regulated the expression of TNF $\alpha$  and RANKL mRNA in the spleen. These molecules are important mediators of bone loss after menopause and in RA. In contrast to estrogen, raloxifene did not affect the effector phase of the disease, as demonstrated in collagen-antibody induced arthritis.

Many estrogenic effects are mediated via the classical estrogen receptors and binding to the estrogen response elements, which regulate gene transcription. We found that raloxifene activated this pathway at 1/4 of the intensity of estrogen.

In conclusion, our results show that estrogen deficiency and inflammation contribute equally to bone loss in arthritis. Furthermore, raloxifene has potent anti-arthritic and anti-osteoporotic effects, and is possibly a valuable addition to conventional treatment of postmenopausal RA.

**Key words:** rheumatoid arthritis, osteoporosis, estrogen, raloxifene, mice

## ORIGINAL PAPERS

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

- I. **Caroline Jochems, Ulrika Islander, Malin Erlandsson, Margaretha Verdrengh, Claes Ohlsson and Hans Carlsten.** Osteoporosis in experimental postmenopausal polyarthritis: the relative contributions of estrogen deficiency and inflammation.  
*Arthritis Research & Therapy* 2005, **7**:R837-R843
  
- II. **Caroline Jochems, Ulrika Islander, Anna Kallkopf, Marie Lagerquist, Claes Ohlsson and Hans Carlsten.** Role of raloxifene as a potent inhibitor of experimental postmenopausal polyarthritis and osteoporosis.  
*Arthritis & Rheumatism* 2007, **56**:3261-3270
  
- III. **Caroline Jochems, Marie Lagerquist, Cecilia Håkansson, Claes Ohlsson and Hans Carlsten.** Longterm anti-arthritic and anti-osteoporotic effects of raloxifene in established experimental postmenopausal polyarthritis.  
*Submitted for publication*
  
- IV. **Caroline Jochems, Cecilia Håkansson, Marie Lagerquist, Claes Ohlsson, Kutty Selva Nandakumar, Rikard Holmdahl and Hans Carlsten.** Effects of estradiol and raloxifene on collagen-antibody induced arthritis and osteoporosis.  
*In manuscript*

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## ABBREVIATIONS

BMD	Bone mineral density
CAIA	Collagen-antibody induced arthritis
CIA	Collagen induced arthritis
COMP	Cartilage oligomeric matrix protein
DHEA	Di-hydro-epi-androstendione
E1	Estrone
E2	17 $\beta$ -estradiol
E3	Estriol
ER	Estrogen receptor
ERE	Estrogen response element
HLA	Human leukocyte antigen
HRT	Hormone replacement therapy
IGF-1	Insulin-like growth factor 1
IL	Interleukin
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
OPG	Osteoprotegerin
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF $\kappa$ B ligand
SERM	Selective estrogen receptor modulator
TGF $\beta$	Transforming growth factor $\beta$
TNF $\alpha$	Tumour necrosis factor $\alpha$

## INTRODUCTION

The concept of osteoimmunology is a synthesis of research on the immune system and bone metabolism, and has evolved since many studies have highlighted the cellular and molecular common pathways in these two fields.

The immune system develops within the bone compartment, and cytokines produced by immune cells in turn control bone homeostasis. Also, precursor cells can develop both into inflammatory immune cells, and cells involved in bone remodeling. Autoinflammatory diseases, such as rheumatoid arthritis (RA), induce massive activation of the immune system, and simultaneously lead to bone loss.

It is well established that estrogen affects bone growth and skeletal maturation in both men and women, and that loss of estrogen results in osteoporosis. This occurs in women after menopause. Estrogen treatment compensates for the loss of natural hormones, but is no longer recommended for longterm therapy due to the risk of serious side effects. This has led to the development of other substances with estrogen-like benefits, but with less serious side effects. One such substance is raloxifene, a selective estrogen receptor modulator (SERM), which is approved for the treatment of postmenopausal osteoporosis.

Estrogen is also involved in the regulation of the immune system, suppressing T- and B-lymphopoiesis, while stimulating immunoglobulin production, and influencing the course of inflammatory diseases. RA has a female to male ratio of 3:1. During pregnancy (when estrogen levels are high) 75% of patients are ameliorated. The disease incidence increases after menopause, when ovarian estrogen production ceases. Hormone replacement therapy (HRT) reduced disease severity, joint destruction and bone loss. Anti-arthritic effects of estrogen have been shown in animal models as well. We therefore investigated if raloxifene exerts anti-arthritic and anti-osteoporotic effects, and if estrogen and raloxifene act via different molecular pathways.

This frame story aims to describe what is known today about the intricate relationship between the immune system and osteoporosis development during postmenopausal RA, and how increased knowledge of estrogen receptor modulation can help us find better therapies that regulate both autoimmune joint destruction and bone loss.

### OSTEOIMMUNOLOGY

The term "Osteoimmunology" was established in 2000 by Joseph Arron and Yongwon Choi[1], introducing a new way to view the interconnections between immunology and bone metabolism.

#### **The immune system**

The immune system has evolved to protect the body from infections caused by different microbes (bacteria, mycobacteria, viruses and prions). The immune system has developed two parts that work in concert with each other, the **innate** and the **adaptive immune system**.

The innate immune system is fast, non-specific and reacts in the same way each time it encounters a certain microbe or its products. The adaptive immune system is slower, and takes several days to become active the first time it confronts a pathogen. On the other hand, it becomes specifically designed to eradicate that microbe. It remembers and recognizes the microbe, and knows how to react the next time the body is infected.

The specificity of the immune cells is constantly checked during development, and faulty cells are destroyed. In autoimmune diseases the immune system becomes incorrectly activated, and develops an immune reaction that becomes directed towards the individual itself. This may result in disease development and tissue damage.

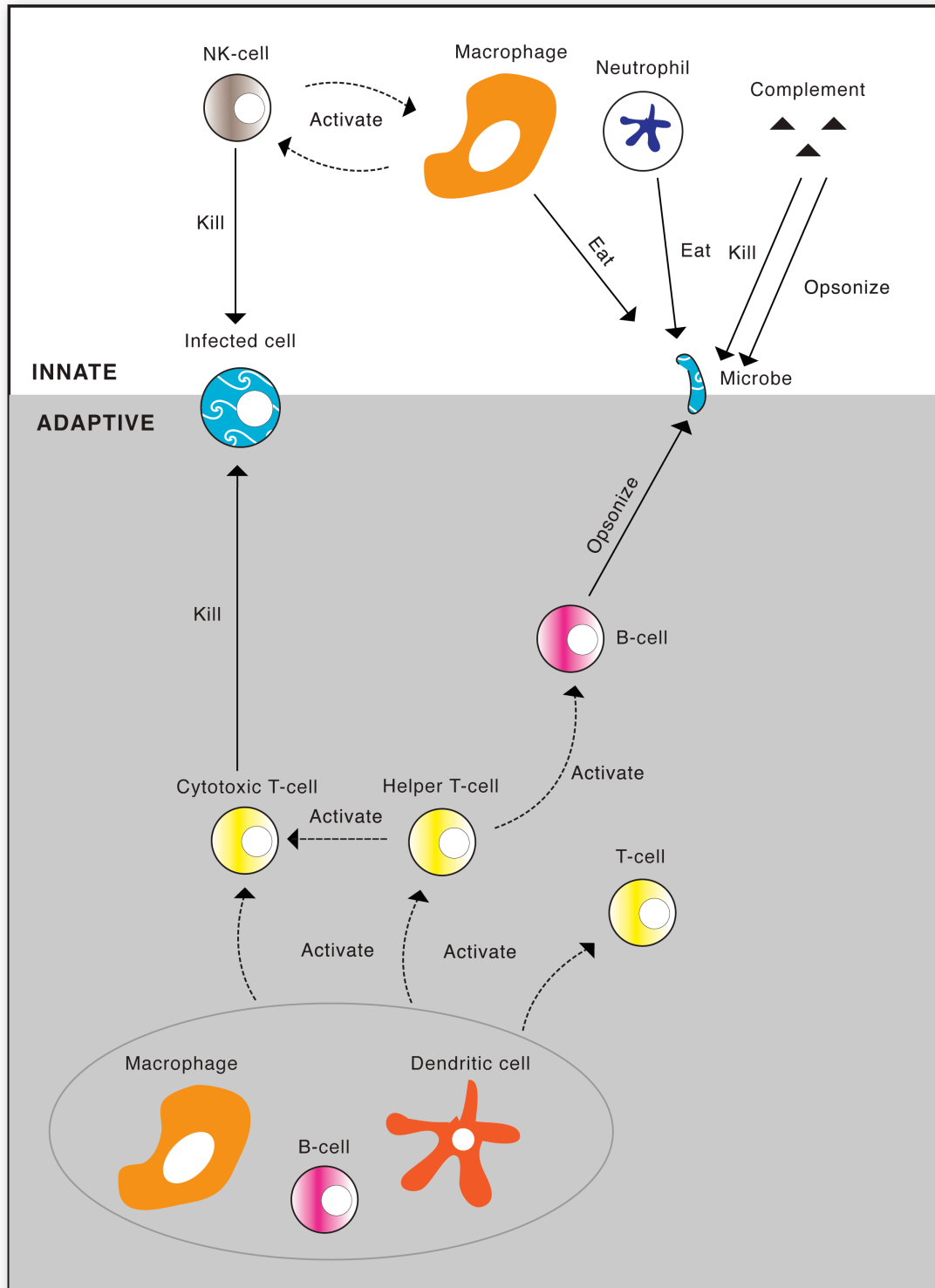
Hematopoietic stem cells develop inside the bone compartment, and are the precursors of all the cells of the immune system in mammals. The innate (native) immune system is composed of epithelial barriers, the complement system, cytokines, plasma proteins and monocytes, macrophages, neutrophils and natural killer cells.

**Monocytes** circulate in the blood, and are recruited to inflammatory sites. In the tissue they differentiate into macrophages. **Macrophages** become activated by microbes, T-cell cytokines and CD40-ligand, and when activated they phagocytose microbes, produce proinflammatory cytokines and present antigen to T-cells. **Neutrophils** are the most frequent white blood cells in the circulation, and are recruited to inflammatory sites, where they phagocytose and digest microbes. **Natural killer cells** are a special kind of lymphocytes, that kill tumour cells and cells infected with microbes, and produce interferon- $\gamma$  to activate phagocytes.



The adaptive (acquired) immune system consists of two parts, **humoral immunity** and **cell-mediated immunity**. Humoral immunity is directed at extracellular microbes. **B-lymphocytes** can mature into antibody-secreting **plasmacells**. Antibodies bind to microbes (or toxins) and stop them from entering cells and tissues, and make them more vulnerable to phagocytosis by macrophages. Cell-mediated immunity is directed at intracellular microbes. If microbes have been phagocytised, **helper T-lymphocytes** activate macrophages to kill them. In the case of intracellular microbes like viruses, **cytotoxic T-lymphocytes** kill the infected cells to eliminate the invader. Helper T-lymphocytes express CD4 on the surface, and recognize peptides displayed on MHCII, while cytotoxic T-lymphocytes express CD8 and recognize peptides on MHCI. A major difference between B-lymphocytes and T-lymphocytes is that B-cells recognize carbohydrates and lipids as well as proteins. B-cells develop in the bone marrow, and mature cells are mostly found in lymphoid follicles in secondary lymphoid tissues (spleen and lymph nodes) and in the bone marrow. T-cells mature in the thymus, and are found in lymphoid follicles, in the circulation and at sites of infection. Recently it was shown that naive antigen-specific T-cells also home to the bone marrow, where they can become activated by dendritic cells[2]. There are two more subsets of T-lymphocytes that influence autoimmune diseases, the pro-inflammatory **Th17-cells**, which produce IL-17[3], and the **regulatory T-cells**, which modulate the inflammatory response[4].

**Antigen-presenting cells** (APC) present peptides to T-cells. They are the dendritic cells, macrophages and B-cells, and they all express co-stimulatory molecules as well as MHC on their surface. **MHC**, the major histocompatibility complex, are molecules in which peptides are presented. MHCI is present on all nucleated cells, and presents intracellular peptides. MHCII is present on APCs and presents extracellular peptides (that have been endocytosed). The MHC in humans is called the human leukocyte antigen (HLA), and each individual expresses a specific repertoire of HLA molecules. The capacity of a certain HLA to present a specific peptide can influence the individual disposition for a disease. This is one mechanism for genetic susceptibility to RA.



**Figure 1. Overview of the immune system**

Several cytokines can function as mediators of immune reactions.

**TNF $\alpha$**  (tumour necrosis factor  $\alpha$ ) is a pro-inflammatory cytokine mainly produced by activated macrophages and T-cells. It helps activate and recruit neutrophils and monocytes to infection sites, induces chemokine secretion from macrophages, and stimulates endothelial cells to express adhesion molecules and produce chemokines. Large amounts of TNF $\alpha$  cause systemic effects (fever and acute phase protein production in the liver), and may cause septic shock. TNF $\alpha$  is highly involved in the pathogenesis of RA.

**IL-1 $\beta$**  (interleukin 1 $\beta$ ) is also a pro-inflammatory cytokine produced primarily by activated macrophages and endothelium. It has similar actions as TNF $\alpha$ . There are two isoforms of IL-1 ( $\alpha$  and  $\beta$ ), with the same biological activity.

**IL-6** is produced by many cell types, including activated macrophages, T-cells, fibroblasts and endothelium. It functions in both innate and adaptive immunity, stimulating synthesis of acute phase proteins and proliferation and differentiation of T-cells and B-cells in humans. It also has anti-inflammatory functions, for example preventing formation of autoreactive B-cells in mice[5]. It is involved in the pathogenesis of RA and bone loss (reviewed in [6]).

**IL-7** is produced by many cell types, including bone marrow stromal cells, macrophages, synovial fibroblasts and endothelium. It stimulates proliferation and survival of T- and B-cell precursors.

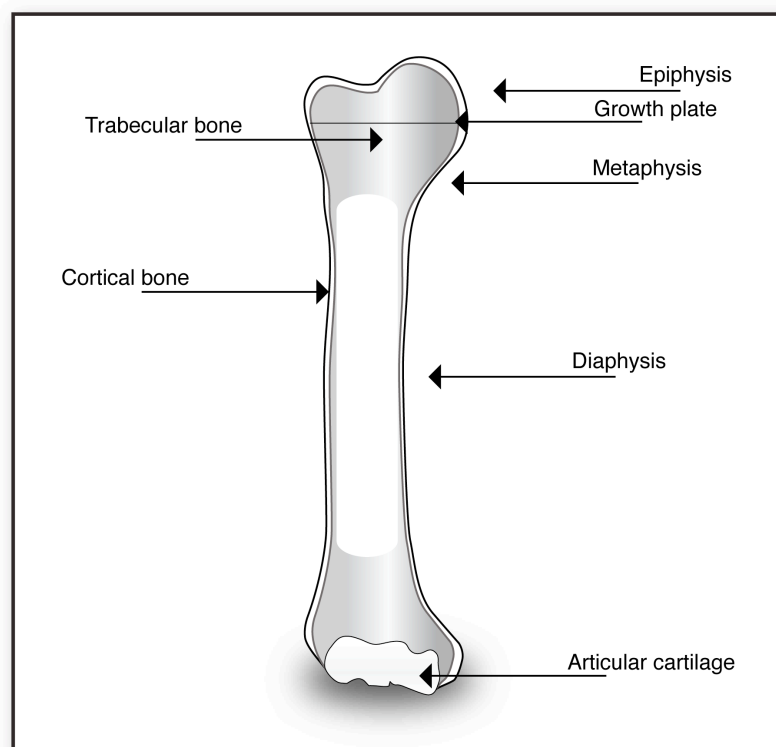
**IL-17** is a pro-inflammatory cytokine mainly produced by Th17 cells. Receptors for IL-17 are found on most cells. It induces production of TNF $\alpha$ , IL-1 and RANKL.

**TGF $\beta$**  (transforming growth factor  $\beta$ ) is an anti-inflammatory cytokine produced by activated T-cells, macrophages and other cells. It opposes the actions of pro-inflammatory cytokines, and inhibits T-cell proliferation and differentiation, and macrophage activation. It also stimulates the development of regulatory T-cells and osteoblasts.

**Bone**

The skeleton functions as support for the body and movement, protection for inner organs, production of blood cells and storage for minerals (calcium and phosphate).

Bone consists of inorganic matrix (mostly hydroxyapatite), organic matrix (collagen I, osteocalcin, bone sialoprotein and other bone proteins), and cells (osteoblasts, osteocytes, osteoclasts). There are two different types of bone, trabecular (=cancellous/spongy) bone and cortical (=compact/dense) bone. **Trabecular bone** contributes only to 20% of the total skeleton, but has 10 times the surface area of compact bone because of its porous appearance with much room for blood vessels and bone marrow. Due to this vast surface area, trabecular bone is metabolically more active. It is found in the metaphysis of long bones, vertebrae and pelvis. **Cortical bone** makes up 80% of the skeleton, and is the compact, hard outer layer of bones, with much less metabolic activity.



**Figure 2. Longitudinal section through femur**

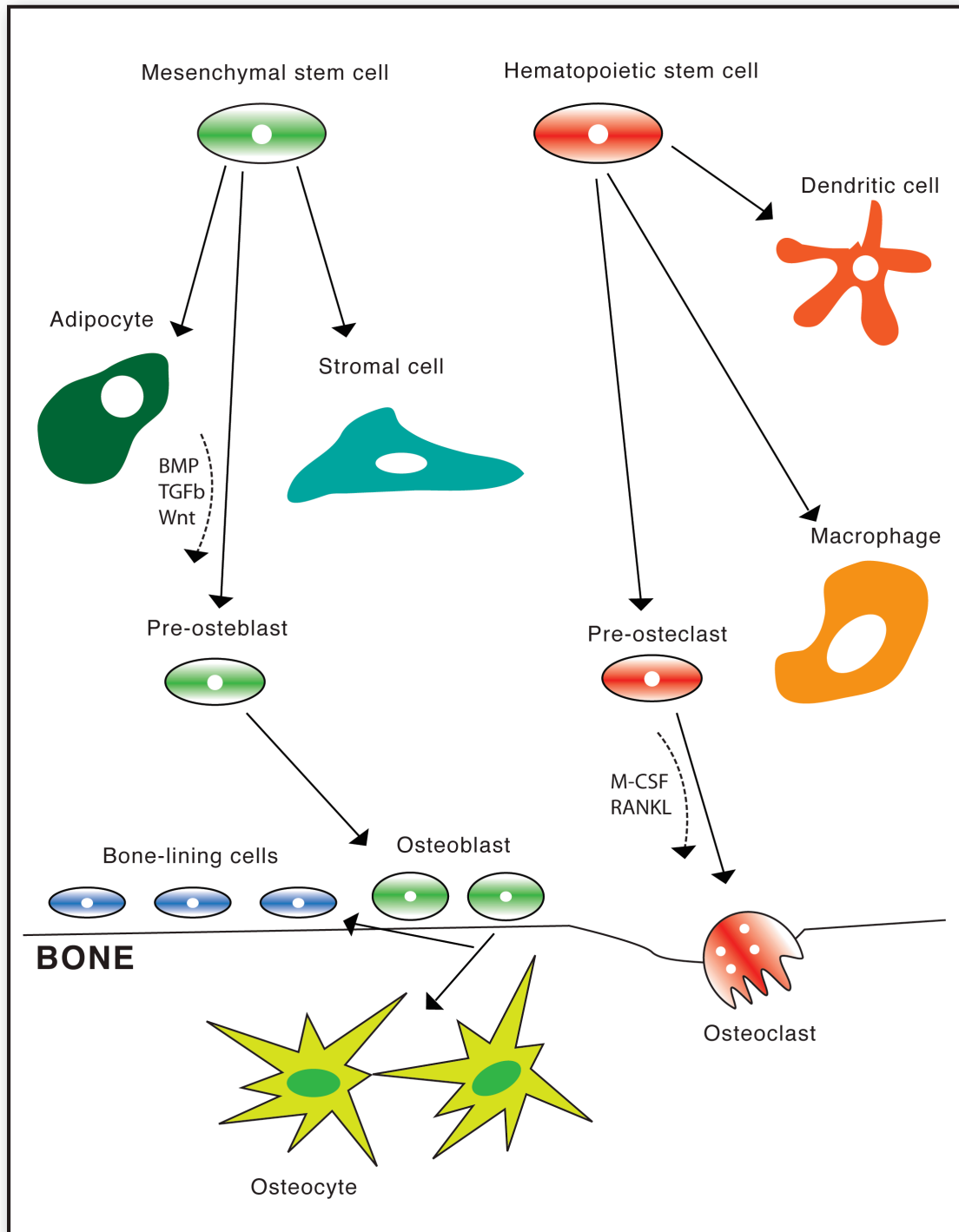
The cellular component of bone consists of osteoblasts, osteocytes, bone-lining cells and osteoclasts. **Osteoblasts** originate from pluripotent mesenchymal stem cells, that can also develop into adipocytes, myocytes and chondrocytes[7]. Important factors for the differentiation into osteoblasts are BMPs (bone morphogenetic proteins) and TGF $\beta$ , as well as signalling via Wnt (a family of proteins that initiate transcription factor formation)[8-10]. Osteoblasts are the cells responsible for bone formation. They secrete the bone proteins of the matrix, including osteocalcin, collagen type I and osteonectin. They are also responsible for the mineralization of the matrix, via ALP (alkaline phosphatase) expressed on their surface. Serum levels of osteocalcin is a marker of ongoing bone formation, since some osteocalcin leaks into the circulation, and its half-life in serum is only 5 minutes.

After the matrix (osteoid) is produced by the osteoblasts, it progressively hardens as calcium salts are deposited. Some osteoblasts become surrounded by the matrix, are trapped as the matrix hardens around them, and develop into **osteocytes**. In compact bone, the osteocytes lie in lacunae, concentrically arranged around a Haversian canal with blood vessels, nerves and lymphatic tissue, and communicate with each other via their processes, that lie in canaliculi. The osteocytes sense loading of the bone, and are important for regulation of bone remodeling, so that bone strength increases or decreases appropriately[11-14]. **Bone-lining cells** develop from mature osteoblasts, and lie on the bone surface. They produce several cytokines that help regulate bone remodeling.

**Osteoclasts** are responsible for bone resorption. They develop from hematopoietic stem cells, which can also become dendritic cells, monocytes and macrophages. In the presence of M-CSF (macrophage colony-stimulating factor) and RANKL (receptor activator of NF $\kappa$ B ligand), preosteoclasts fuse to form multinucleated osteoclasts, and then become activated. Osteoclasts can not be formed without both M-CSF and RANKL present, and mice deficient in either factor develop an osteopetrotic phenotype[15-17]. RANKL is also essential for osteoclast survival[18]. Mature osteoclasts express TRAP (tartrate-resistant acid phosphatase), cathepsin K,  $\beta$ 3-integrin and calcitonin receptor.

During resorption, collagen I is degraded, and some fragments (C-terminal telopeptides) are released into the circulation. Although type I collagen is not

restricted to bone, but is also found in skin, tendons, vessels and cornea, levels of C-terminal telopeptides in serum are a useful marker of bone resorption (CTX-I in humans, RatLaps in mice).



**Figure 3. Development of bone cells**

**Bone remodeling**

Bone remodeling is constantly going on, at a rate of total exchange of the skeleton in an adult about every 10 years.

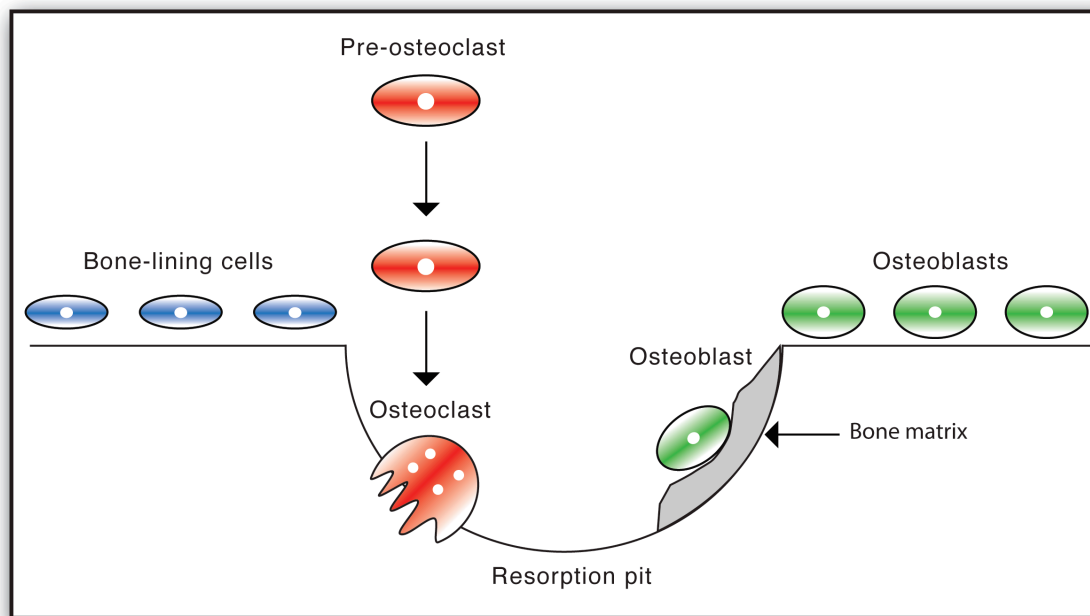
Bone-lining cells prepare a bone surface for degradation. Preosteoclasts are attracted to the site, fuse and mature into osteoclasts. Activated osteoclasts attach to the bone with their ruffled border, and seal off the area creating an acid microenvironment, ideal for bone resorption.

The osteoclasts form resorption pits on the surface of trabeculae in trabecular bone. In cortical bone a tunnel is formed. Osteoblasts produce new bone matrix to fill in the resulting gaps. The whole remodeling cycle takes about 90 days, 10 days for resorption and 80 days for bone formation.

In a healthy adult, the rate of bone resorption is balanced to the rate of bone formation, resulting in maintained bone strength. In a growing person there is a net increase in bone formation. The coupling of bone resorption and formation determines the bone mineral density, and hence the bone strength. A net increase in bone formation results in osteopetrosis (pathologically increased bone mass), while a net increase in resorption results in osteoporosis (low bone mass).

The rate of bone remodeling is controlled by several factors, including loading of the bone (sensed by osteocytes), parathyroid hormone, estrogen, growth hormone, and different cytokines.

The bone mineral density (BMD) increases until approximately 30 years of age, both in men and in women. At this point, the individual has reached peak bone mass. Men generally have higher peak bone mass than women, and this difference persists as the BMD declines. Women experience a period of rapid bone loss following menopause, but then the rate of bone loss slows again, and from 65 years the decline is equal in men and women (**figure 5**).



**Figure 4. Bone remodeling.** Mature osteoclasts resorb bone, forming a resorption pit. Osteoblasts fill in the pit with bone matrix that becomes calcified.

### Osteoporosis

Osteoporosis can develop when there is a net decrease in bone formation. This may be due to either increased bone resorption, decreased bone formation, or a combination of both. The result is decreased bone strength and increased risk of fracture.

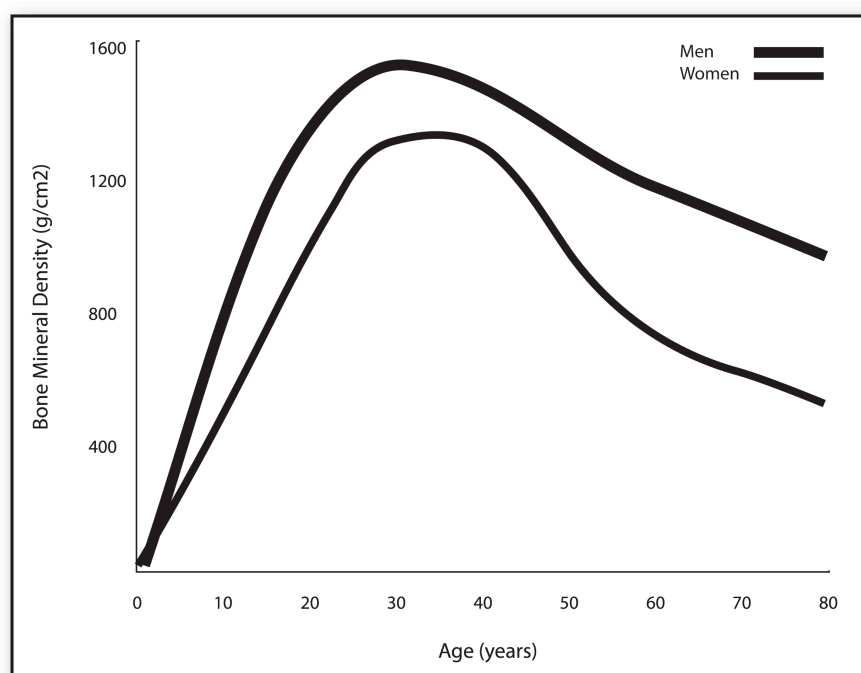
According to the WHO classification of 1994, osteoporosis is defined as BMD lower than 2.5 SD (standard deviations) below the young adults mean value (T-score)[19]. Osteopenia is a BMD value between 1 and 2.5 SD below the T-score. BMD is often measured by DXA (dual energy x-ray absorptiometry).

The prevalence of osteoporosis in Sweden is 2-3% among women in their 50's, and increases to approximately 50% in women over 80. Similar frequencies are found in other countries[20]. Age-related osteoporosis is due to decreased production of vitamin D, decreased uptake of calcium, and decreased concentrations of sex hormones and growth factors.

The risk of fracture also increases with age[20-22]. Other risk factors are low BMD, smoking, inactivity, low weight (BMI<22), earlier fracture and having a mother with a fracture[23]. Osteoporotic fractures are an important cause of morbidity and mortality[24], and the incidence of fractures is likely to rise due to longer life expectancy after the age of 50[25].



Anti-osteoporosis therapies today are directed at either stimulating bone formation (parathyroid hormone and strontium ranelate), or inhibiting bone resorption (bisphosphonates, strontium ranelate, hormone replacement therapy with estradiol (HRT) and selective estrogen receptor modulators (SERM))[26]. In addition, both bisphosphonates and estrogen inhibit osteocyte apoptosis[27]. All patients also receive a supplement of calcium and vitamin D3. HRT and SERM will be described later.



**Figure 5. Bone mineral density (BMD) in men and women**

### **Cartilage**

Articular cartilage is mainly composed of collagen fibers that give tensile strength, and proteoglycans that bind water to give compressive stiffness. The main collagen in articular cartilage is type II collagen, which is secreted by chondrocytes as a procollagen, and then cleaved. It makes up the major part of collagen fibrils. Several other proteins are found in cartilage. COMP (cartilage oligomeric matrix protein) is a pentameric protein that stabilizes the collagen network. It is found in cartilage, and is also secreted from synovial fibroblasts. Serum levels of COMP can be used as a marker of ongoing cartilage degradation[28-30].

**Interplay between the immune system and bone**

The location of bone marrow inside the trabecular bone creates the physical opportunity for interaction between immune cells, bone cells and their products. The first interactive molecule to be recognized was **RANKL** (receptor activator of NF $\kappa$ B ligand), also called TRANCE (TNF-related activation induced cytokine), or OPGL (osteoprotegerin-ligand)[16, 31].

RANKL is produced by activated T-lymphocytes[32], B-lymphocytes[33], osteoblasts[34], bone-lining cells[35], macrophages[36], synovial fibroblasts[37], chondrocytes[38], endothelium[39] and neutrophils[40], and is either soluble or bound to the cell membrane. RANKL regulates communication between T-cells and dendritic cells, dendritic cell survival, lymph node formation and formation of lactating mammary glands[41-43]. It promotes osteoclast differentiation and activation by binding to RANK, its receptor on pre-osteoclasts and osteoclasts[44]. It stimulates mature osteoclasts to resorb bone[45], and inhibits osteoclast apoptosis[18]. In addition to supporting osteoclastogenesis by RANKL expression, B-lymphocyte lineage cells can also serve as osteoclast precursors[33]. The proliferation and differentiation of B-cells are inhibited by the RANKL decoy receptor OPG[46]. Interestingly, both RANK- and RANKL-knock out mice develop grave osteopetrosis, since they have no osteoclasts[41, 44]. These mice can develop severe serum transfer induced arthritis without any bone destruction[47].

Several factors can induce RANKL expression on osteoblasts, including vitamin D3, PTH, IL-1, TNF $\alpha$ , estrogen deficiency and treatment with glucocorticoids[48]. The levels of IL-1 and TNF $\alpha$  are known to increase in many inflammatory conditions, thus providing a link between activation of the immune system and increased bone resorption.

In addition to RANKL, osteoblasts and bone marrow stromal cells also produce **OPG** (osteoprotegerin)[49]. OPG acts as a decoy receptor, binding and neutralizing soluble or membrane-bound RANKL, thus preventing osteoclastogenesis and bone resorption, and increasing apoptosis of osteoclasts. OPG-deficient mice develop early osteoporosis[50]. Estrogen induces OPG expression in human osteoblastic cells in vitro[51], and OPG-treatment counteracted the development of osteoporosis after ovariectomy in rats[49]. OPG also counteracted bone erosions in several murine

arthritis models[52-54]. The OPG/RANKL ratio determines the net degree of osteoclast activation.

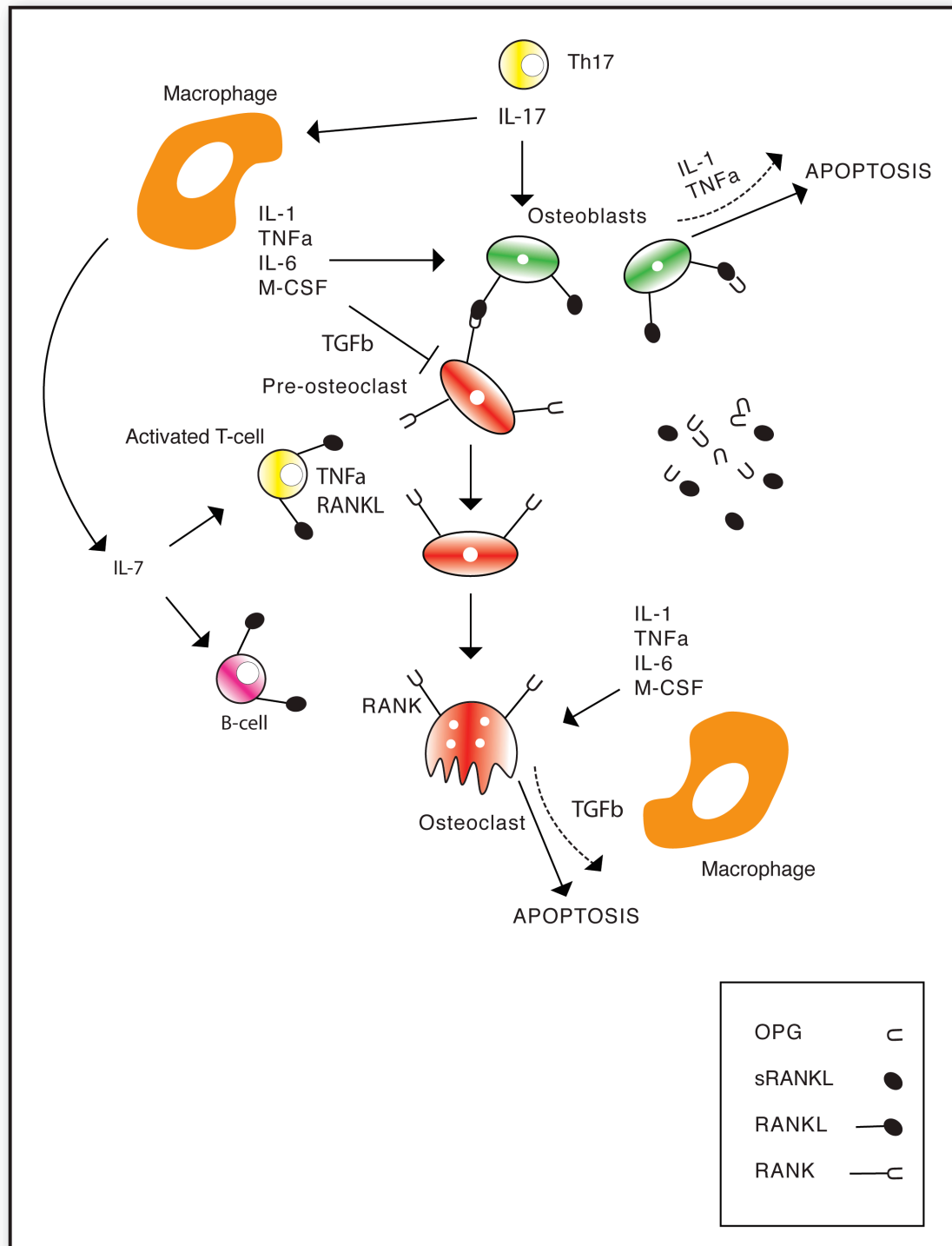
Regulatory T-cells have been demonstrated to suppress osteoclast formation *in vitro* via direct cell-cell contact[55].

Several cytokines and growth factors influence bone metabolism.

**TNF $\alpha$**  stimulates osteoporosis development by increasing RANKL production in bone-lining cells, leading to an increased number of osteoclasts[35, 56], by stimulating osteoclast activity[57], and by increasing the apoptosis of osteoblasts[58]. Production of TNF $\alpha$  is elevated during inflammatory diseases and after ovariectomy, increasing bone resorption[59]. Interestingly, treatment with monoclonal anti-TNF $\alpha$  antibodies has been shown to preserve the BMD in patients with RA[60-63]. **IL-1 $\beta$**  stimulates pre-osteoclast fusion[64], osteoclast activation and survival[65], and increases osteoblast apoptosis[58], thus contributing to bone loss. IL-1 receptor antagonist is used to hamper inflammation, and also inhibits osteoclast differentiation and bone resorption[66].

**IL-6** has pro-osteoporotic properties. It has been shown to increase after ovariectomy, and serum IL-6 levels can predict bone loss in postmenopausal women[67-69]. Soluble IL-6 receptor acts as an agonist, by binding to IL-6, and then interacting with the same signal-transduction pathways as the membrane bound receptor[70]. Soluble IL-6 receptor increases after menopause, and this increase can be prevented and reversed with HRT[71]. This prevention was recently also reported in women with postmenopausal RA[72]. Mice deficient in IL-6 did not develop ovariectomy-induced bone loss[73]. **IL-7** induces TNF $\alpha$  and RANKL secretion from T-cells, increased B-lymphopoiesis and bone loss[74, 75]. IL-7 knock out mice have increased bone volume and decreased B-lymphopoiesis[75]. **IL-17** stimulates differentiation of osteoblasts[76], and increases the RANKL/OPG ratio[77].

**TGF $\beta$**  is stored in an inactive form in the bone matrix[78]. Its effects are anti-osteoporotic, inhibiting bone resorption and fusion and proliferation of pre-osteoclasts, and increasing osteoclast apoptosis[79, 80]. It also stimulates osteoblast proliferation and differentiation[78].



**Figure 6. Interplay between the immune system and bone**

## RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a progressive systemic autoimmune disease with a prevalence of 0.5-1%[81, 82]. The first case report was published by Sydenham in 1676, but the disease was not recognized until 1859, when Garrod defined it. The disease is depicted in Dutch art from the 17th century, and examination of 5000 years old skeletons found in North America show characteristic rheumatoid changes[83].

RA is characterized by symmetrical polyarthritis with synovitis. The synovium, which lines the joints, is infiltrated by macrophages, T-cells and B-cells. Chronic inflammation leads to destruction of joint cartilage and bone.

The overall incidence of RA is 20-40/100 000/year in women and 10-20/100 000/year in men, based on studies from the United States, Europe and Asia[82, 84-86]. The female to male incidence ratio is 4-5:1 before 50 years of age, and 2:1 for patients with later onset[81, 87]. The peak incidence in women coincides with menopause, and the peak incidence for men occurs at 60-70 years of age[82, 88].

Genetic studies have found that the major genetic susceptibility for RA is associated with the HLA-DR4/shared epitope[89, 90]. Indeed, HLA-DR4 transgenic mice are susceptible to collagen induced arthritis[91]. Interestingly, the predisposing effect of gender is strongest in individuals who do not have the shared epitope, and virtually absent in homozygous individuals[92]. The proportion of disease-associated HLA-alleles in RA patients is not gender-specific[93].

### **Pathogenesis of RA**

The pathogenesis of RA is largely unknown, with genetic and environmental factors influencing disease development and progression. The clinical diagnosis is based upon certain criteria established in 1987, and may encompass several variations of arthritis.

From studies of animal models of RA it has been shown that mice expressing the H2q haplotype can develop arthritis upon immunization with collagen type II (CII) (collagen-induced arthritis). In humans it has been proposed that certain HLA-DR4 molecules present peptides of CII, which is present in joint cartilage, resulting in susceptibility to develop RA.

T-lymphocytes are important in the pathogenesis of arthritis as activators of B-lymphocytes and other cells, like synovial macrophages, via cytokine production

(IFN $\gamma$  and TNF $\alpha$ ). In one study on B10q mice (which are highly susceptible to CIA), lack of CD4<sup>+</sup> T-cells resulted in decreased susceptibility to disease and lower levels of CII antibodies, whereas lack of CD8<sup>+</sup> T-cells did not significantly affect the disease[94]. In contrast, another study in DBA/1 mice revealed that CD8<sup>+</sup> cells were necessary for disease development, while lack of CD4<sup>+</sup> cells did not decrease the susceptibility to CIA[95]. These data suggest that CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes may play differential roles in CIA depending on the genetic background of mouse strains.

IL-17-producing CD4<sup>+</sup> helper T-cells (Th17-cells) have been shown to be pathogenic in CIA. IL-17 enhances the development of CIA, and IL-17 deficiency protects against CIA development[96-98]. IL-17 also promotes bone erosion by disrupting the OPG/RANKL balance[99]. IL-17 in synovial fluid from RA patients was found to stimulate osteoclastogenesis[100].

B-lymphocytes are important in the pathogenesis of RA and CIA, by producing antibodies to CII, and for T-cell activation[101]. Indeed, B-lymphocyte deficient mice are resistant to CIA[102].

Anti-CII antibodies bind to the articular cartilage and initiate complement activation, which recruits inflammatory cells to the site[103]. First, neutrophils are recruited, and then monocytes and lymphocytes. Antibodies to CII have been detected in serum and synovial fluid of patients with RA[104, 105], and CII antibody-producing B-cells have been found in synovial fluid and synovial tissue[106, 107]. Transfer of CII-antibodies can induce arthritis in mice[103]. Administration of B-cell depleting anti-CD 20 antibodies is approved for treatment of RA[108, 109].

## **Murine models of RA**

Several different mouse models of RA are available. However, it has become clear that the human disease is much more complex than each of these models.

### **Systemic, erosive arthritis models:**

*Collagen II induced arthritis (CIA)* is a well-established murine model for human RA. It was first established in rats in 1977, and then in mice[110, 111]. It is similar to RA in several ways. MHCII molecules present similar peptides of CII in RA as in CIA, and B- and T-lymphocyte responses are directed to corresponding epitopes. The patterns of synovial infiltration and histological joint destruction are similar. One major difference is that while CIA is transient, and represents the acute phase of the disease, RA is chronic.

*Collagen-antibody induced arthritis (CAIA)* involves only the effector phase of the disease, bypassing the priming phase. It is induced by intravenous injection of a mixture of monoclonal antibodies directed towards different epitopes on CII. The arthritic disease can be aggravated by an intraperitoneal injection of LPS[112].

*B10q-ncf1<sup>-/-</sup> mice* develop chronic arthritis after immunization with heterologous CII, due to a defect in NADPH oxidase, resulting in reduced oxidative burst[113].

*K/BxN transgenic mice* spontaneously develop arthritis after 3 weeks of age[114]. Both T-cells and B-cells are involved in the pathogenesis. These mice produce antibodies against glucose-6-phosphate isomerase (GPI). The relevance of this molecule in RA is not yet clear. Transient arthritis can be induced by serum transfer[115].

*TNF $\alpha$  transgenic mice* express human TNF $\alpha$ , which leads to development of arthritis[116].

*HLA-DR4 transgenic mice* develop arthritis after immunization with CII[91].

*MRL/lpr mice* constitute a model for SLE (systemic lupus erythematosus), and spontaneously develop a milder form of arthritis[117].

### **One-joint, localized arthritis models:**

*Antigen-induced arthritis* is induced by intra-articular injection of an antigen after the animal has previously been sensitized to the antigen. Methylated bovine serum albumin or ovalbumin are often used, not joint-specific antigens like CII[118].

*CpG motifs* in bacterial DNA induce transient arthritis when injected intra-articularly[119].

### **Bone changes in RA**

RA is characterized by different skeletal manifestations including bone erosions[120], periarticular osteopenia[121] and generalized osteoporosis[122-126]. Joint inflammation causes production of pro-inflammatory cytokines that induce osteoclast-development and activation, leading to focal bone loss. In addition, the inflamed synovium acts like an endocrine organ, releasing these factors into the bloodstream and causing generalized bone loss. The prevalence of generalized osteoporosis in postmenopausal RA is more than 50%, resulting in increased risk of fractures[122-128]. The prevalence of osteoporosis is also elevated in men with RA, compared to a healthy reference population[129].

Osteoclasts were identified in subchondral bone in arthritic joints of RA patients in 1984[130], and have since been further characterized. They possess the phenotype of mature osteoclasts, expressing TRAP, cathepsin K and calcitonin receptor[131, 132]. They are also found in bone erosions of mice with collagen-induced arthritis[133].

Several factors enhance osteoclastogenesis and osteoclast function in arthritis:

**RANKL** is found at sites of bone erosion and in synovial tissue from RA patients[134]. The RANKL/OPG ratio is increased in active RA, and correlates with increased bone resorption[135]. Increased levels of RANKL were found in mouse and rat CIA[136-138], and RANKL knock-out mice were protected from bone erosions in serum-transfer induced arthritis[47]. Neutrophils are abundant in joints of RA patients, and express membrane-bound RANKL, RANK and OPG[40]. OPG is the naturally occurring decoy receptor for RANKL, and treatment with OPG has been found to reduce bone loss in experimental arthritis[52-54, 139], as well as in postmenopausal arthritis in women[140].

**TNF $\alpha$**  increases the number of pre-osteoclasts[141, 142], directly promotes osteoclast differentiation from precursors[143-145], increases the expression of RANK in pre-osteoclasts[146], and increases the RANKL expression in bone-lining cells and bone marrow stromal cells[147].

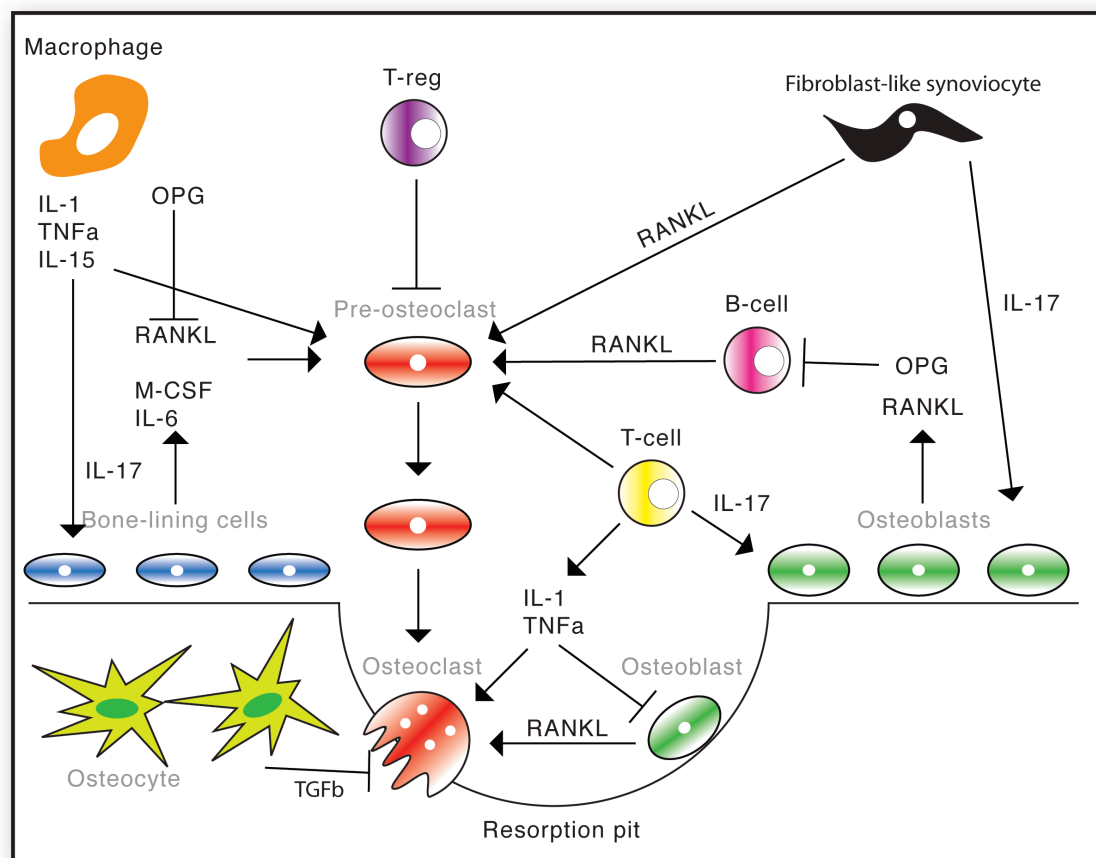
**IL-1 $\beta$**  stimulates pre-osteoclast fusion[64], and osteoclast activation and survival[65]. **IL-6** and the soluble IL-6 receptor are found at higher levels in serum and synovial fluid of patients with RA than healthy controls, and have been correlated with the degree of joint destruction[126, 148, 149].



**IL-7** is elevated in the joints of RA patients[150], and stimulates the production of new T-cells and B-cells, activation and differentiation of mature T-cells and increases the RANKL expression, thus enhancing osteoclastogenesis[74, 75, 151].

**IL-17** induces RANKL expression and decreases OPG expression in osteoblasts and increases RANKL, IL-1, IL-6 and TNF $\alpha$  expression in synoviocytes[100, 152]. It enhances the development of CIA, and IL-17 deficiency protects against CIA[96-98].

Osteoblasts are also affected by the inflammatory process: IL-1 $\beta$  and TNF $\alpha$  both induce osteoblast apoptosis, and other molecules influence their survival and function by inhibiting BMPs[9, 58, 153].



**Figure 7. Bone changes in RA**

## ESTROGEN

The female sex hormone estrogen has many physiological effects, affecting the development and maturation of the reproductive system, the skeleton, and the immune, nervous, and cardiovascular systems. There are 3 different estrogens in humans. **Estrone** (E1) is the least abundant. It is produced by the ovary and liver, and is the predominant estrogen after menopause. **17 $\beta$ -estradiol** (E2) is the most potent hormone. It is produced by the granulosa cells of the ovary, and to some degree by the adrenal cortex, adipose tissue and testicles via aromatization of testosterone. The ovarian production of E2 ceases after menopause. In serum E2 is bound to sex hormone binding globulin or albumin, and only the free hormone (2-3%) is biologically active. In premenstrual girls, the serum E2 level is <50 pg/ml, and after menopause <27 pg/ml. During the fertile period it varies between 27 and 460 pg/ml, depending on the menstrual phase. Men have serum estradiol levels <54 pg/ml. In mice the measured serum level varies between studies, but is about 50-400 pg/ml in fertile mice, 1000-2000 pg/ml during pregnancy and <30 pg/ml after ovariectomy. **Estriol** (E3) is produced by the placenta during pregnancy, but is otherwise present throughout life at a low concentration in both men and women. It is also the main estrogen metabolite in urine. Some metabolites of estrogen are excreted in the bile, and then reabsorbed in the intestine[154].

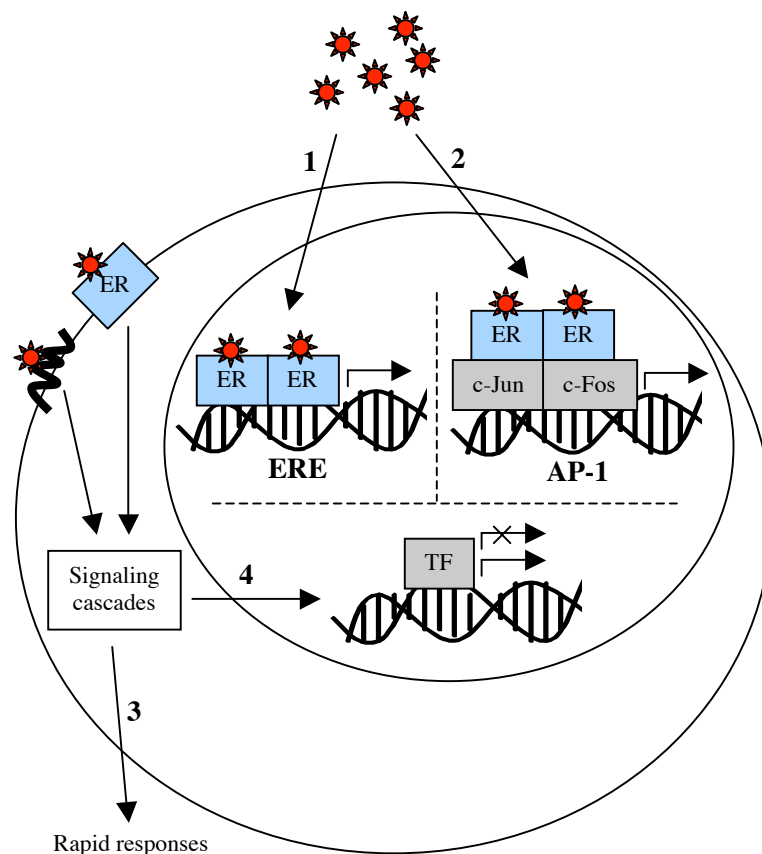
### **Estrogen receptors and signaling**

The classical estrogen receptors ER $\alpha$  and ER $\beta$  were cloned in 1986 and 1996, respectively[155, 156]. They are attached to receptor-associated proteins, and loosely bound in their locations in the cytosol or nucleus[157]. The distribution of ER $\alpha$  and ER $\beta$  varies in different tissues. After binding to estrogen, they form a receptor dimer and translocate into the cell's nucleus[158]. There, they form a complex with co-regulatory proteins and bind to the estrogen response element (ERE) to initiate transcription[159]. This is the classical transcription pathway. The EREs are located in the promoter regions of different genes that are regulated by estrogens[160].

The estrogen/ER-complex can also start transcription by binding to alternative transcription factors (AP-1, SP-1 and NF $\kappa$ B), which bind non-ERE sites. This is called non-classical transcription[161-163].

There are cell membrane associated estrogen receptors. GPR30 is a newly discovered G-protein-coupled receptor, and some studies have indicated that ER $\alpha$  may also be cell membrane associated[164-168]. Binding of these receptors leads to rapid activation or repression of intracellular signaling pathways (calcium mobilization and PI3K activation), leading either to non-genomic signaling or transcriptional activity via this indirect pathway.

In addition, estrogen receptors can be activated through phosphorylation, in the absence of estrogen, by dopamine, insulin-like growth factor-1, epidermal growth factor and cyclic AMP[169-172].



**Figure 8. Estrogen signaling.** 1) Classical transcription pathway 2) Non-classical transcription pathway 3) Membrane associated estrogen receptors, non-genomic response 4) Membrane associated estrogen receptors, indirect transcription pathway. (The drawing was a kind gift from Ulrika Islander)

### **Menopause and hormone replacement therapy**

At menopause, most of the ovarian production of sex hormones ceases, although some production of testosterone, androstendione, DHEA, estrone and estradiol has been shown 10 years after menopause. Ovariectomy of postmenopausal women significantly decreased serum levels of estrone and testosterone, revealing some remaining ovarian sex hormone production even after menopause[173]. After menopause estrone is the predominant estrogen in serum at 15-80 pg/ml, whereas estradiol is present at <27 pg/ml, and estriol at the same levels as throughout life, 3-11 pg/ml[154].

Mice do not lose the production of sex hormones with age. Therefore ovariectomy of mice is used to mimic menopause, to enable studies of the effects of estrogen deficiency.

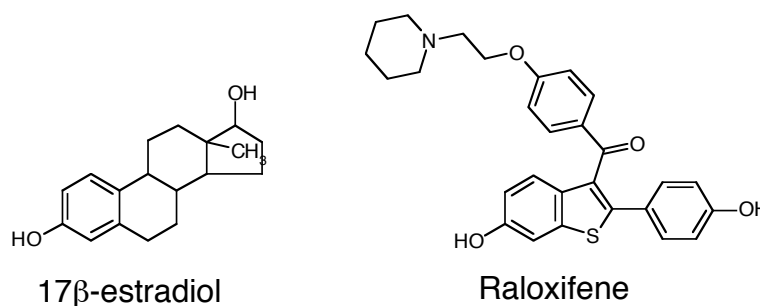
Hormone replacement therapy (HRT) with estradiol after menopause was first started in 1941, and was successful since the clinical symptoms from loss of estrogen could be abated. The use of estrogen further increased during the 60's and 70's, but in 1975 a study showed the relationship between estrogen treatment and endometrial cancer, which led to decreased use. The finding that addition of progesterone protects from endometrial cancer resulted in increased use once more. In 1984 HRT was recommended as treatment of postmenopausal osteoporosis. The pharmacological use of estrogens is reviewed in [174].

In 2002 the Women's Health Initiative study, which was the biggest study ever of the long-term effects of hormone replacement treatment, was prematurely interrupted due to severe side effects. The combination of conjugated equine estrogen and progesterone was shown to increase the risk of coronary heart disease, stroke and deep vein thrombosis, in addition to the previously known risk of breast and uterine cancer[175, 176]. One and a half years later the group taking only conjugated equine estrogen was also terminated due to increased risk of stroke and no evidence for cardiopulmonary benefits. The million women study found increased risk of breast cancer in women taking estrogen and progesterone in combination[177]. Since then, the use of HRT has decreased worldwide[174], and the search for other drugs with the beneficial effects of estrogen, but without the side effects, continues.

## SERM

Selective estrogen receptor modulators (SERM) are nonsteroidal molecules which bind to the estrogen receptors and display estrogen-like effects in some tissues, but antagonistic effects in other tissues. The tissue selectivity of a SERM depends on the relative amount of ER $\alpha$  and ER $\beta$  in that tissue, the affinity of the SERM, and upon the availability of co-activators and co-repressors.

Tamoxifene acts as an estrogen antagonist in breast tissue, and is approved for treatment of estrogen receptor positive breast cancer, but has agonistic effects on endometrium[178]. Raloxifene binds with high affinity to ER $\alpha$ , and acts estrogen-like in bone and on serum lipids[179-181], but as an antagonist in uterus and breast tissue[182, 183]. It is approved as treatment for postmenopausal osteoporosis[174]. ICI 182780 is a pure ER antagonist, without any known agonistic properties, used as adjuvant treatment for ER-positive breast cancer[184].



**Figure 9. Molecular structure of 17 $\beta$ -estradiol and raloxifene**

### Estrogen, raloxifene and the immune system

Estrogen affects the immune system in multiple ways. The estrogen receptors ER $\alpha$  and ER $\beta$  are found in cells of both the innate and the adaptive immune system, in both sexes[185].

Women have stronger humoral and cell-mediated immune responses to infections than men[186]. In contrast, women have 30% lower innate immune response, as measured *in vitro* by TNF $\alpha$  secretion after stimulation of whole blood with LPS[187]. Because of these dual effects on the immune system, estrogen may have an ameliorating or an enhancing influence in different autoimmune diseases. RA and multiple sclerosis (as well as their murine equivalents collagen-induced arthritis and experimental autoimmune encephalitis) are both ameliorated by endogenous and

## ESTROGEN

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exogenous estrogen[188-191]. In contrast, estrogen has been shown to aggravate systemic lupus erythematosus (SLE) in murine models, and to induce flares and increased antibody production in patients with SLE[192-196]. Interestingly, the arthritic disease is ameliorated, and the lupus-like disease aggravated, by estradiol in MRL/lpr mice that spontaneously develop SLE[197].

Estrogen inhibits neutrophil function and adhesion to endothelium, and the number of neutrophils in peripheral blood[198-201]. NK cell activity is decreased[202]. Estrogen induces apoptosis in human monocytes, and also modulates the proinflammatory cytokine release from activated monocytes and macrophages [203, 204]. Serum levels of IL-1, IL-6 and TNF $\alpha$  are increased after menopause, and decreased by HRT[205, 206].

The adaptive immune system is affected in differential ways by estrogen. Treatment with estradiol causes thymic involution, and reduces T-lymphopoiesis[207-209]. Contribution of the GPR30 membrane receptor to estrogen induced thymocyte apoptosis was recently shown[210].

The number of regulatory T-cells was found to be comparable between men and women, but it was found that the levels of estrogen present during pregnancy could stimulate proliferation and differentiation of regulatory T-cells[211, 212]. These effects were inhibited by ICI 182780, a specific inhibitor of estrogen receptors[212].

B-lymphopoiesis is down-regulated by estrogen, and both B- and T-lymphopoiesis are increased after ovariectomy[213]. In spite of this, estrogen induces increased antibody production from mature B-cells, and stimulates B-cell survival[202, 214-216].

Interestingly, raloxifene had the same effects as estradiol on B-lymphopoiesis, but did not stimulate immunoglobulin production in spleen cells[217].

The delayed type hypersensitivity reaction (DTH) is mediated by both T-lymphocytes and macrophages, and is reduced by estrogen[218, 219]. In contrast to estradiol, raloxifene did not affect DTH, and did not induce thymic involution[220].

Raloxifene decreased the serum levels of IL-6 in arthritic mice, but did not affect IL-6 in non-arthritic mice (paper II). Raloxifene, but not estrogen, decreased the expression of TNF $\alpha$  and RANKL mRNA in spleen from arthritic mice (paper II).

### **Estrogen, raloxifene and bone**

The classical estrogen receptors ER $\alpha$  and ER $\beta$  are present in osteoblasts, osteocytes, osteoclasts and chondrocytes, mediating estrogen effects on bone[221-223]. Indeed, the classical transcription pathway has been demonstrated to be activated in osteoblasts, osteocytes and chondrocytes exposed to estradiol[224].

Estrogen induces the longitudinal growth of bone during puberty in both men and women, and is also responsible for the closure of the growth plates when longitudinal growth ceases after puberty[225, 226]. In adults, estrogen has important influence on bone remodeling. The development of postmenopausal osteoporosis is to a large extent due to estrogen deficiency. At first there is a phase of rapid bone loss, dominated by increased bone resorption and trabecular thinning, leading to loss of connection between trabeculae. Then a slower rate of bone loss is sustained, dominated by decreased bone formation and trabecular thinning[227, 228]. Studies of the mechanisms behind postmenopausal osteoporosis are often conducted in ovariectomized mice. It is, however, important to remember that bone loss in estrogen deprivation is strain specific, and some mechanisms may not apply to humans[229]. Ovariectomy of female DBA/1 mice resulted in loss of 22% of the trabecular BMD, but did not affect cortical BMD (paper I).

Estrogenic effects on bone are likely to be mediated by both direct effects on the different cells, and changes of the cytokine milieu of the bone compartment.

The net effects of estrogen deprivation are increased bone resorption due to a higher number of activated osteoclasts[230], deeper resorption pits due to increased osteoclast survival[79], and increased bone formation that is not sufficient to compensate for the resorption. Estrogen deficiency augments the osteoblast formation, but simultaneously increases osteoblast apoptosis[231]. The serum levels of osteocalcin were still increased 8 weeks after ovariectomy of DBA/1 mice, revealing increased bone formation. The serum levels of RatLaps were not elevated, most likely due to a new steady state in bone resorption this long after ovariectomy (paper I).

Estradiol treatment of arthritic mice increased both trabecular and cortical BMD as compared to vehicle-treated controls (papers II and IV).

The response in bone to strain is decreased by estrogen deficiency, due to reduced ER $\alpha$  activity in osteocytes[232].

The serum levels of IL-1, IL-6, TNF $\alpha$  and M-CSF were found to be increased after natural or surgical menopause in women, and decreased upon hormone therapy[205, 233-235]. Ovariectomy also leads to an increase in proinflammatory cytokines in mice[59, 67, 236]. These cytokines reduce osteoblast activity[237, 238], increase osteoclast formation[35, 56, 151], and inhibit osteoclast apoptosis[65, 239].

In early menopausal women it was demonstrated that the expression of RANKL was upregulated on T-cells, B-cells and preosteoblastic marrow stromal cells[240].

The number of osteoclasts and their precursors have been shown to increase after ovariectomy[236]. Indeed, TNF $\alpha$ -knock out mice do not develop osteoporosis after ovariectomy[241], and anti-TNF $\alpha$  treatment has been shown to preserve BMD in RA patients[60-62], and in CIA in mice[242]. In contrast, mRNA levels of TNF $\alpha$  in the spleen of arthritic mice did not decrease in estradiol-treated mice (paper II).

In osteoblasts, estrogen has been shown to increase the expression of OPG, BMP-6, TGF $\beta$  and IGF-1, which results in osteoblast formation and increased osteoclast apoptosis[51, 79, 243-246]. In osteoclasts, estrogen directly decreases the secretion of lysosomal enzymes[247], and down-regulates the sensitivity to RANKL[248]. Estrogen stimulates proliferation and differentiation of regulatory T-cells, and these have been shown to suppress osteoclast formation[55, 211, 212]. In contrast, estrogen withdrawal in women is associated with increased osteocyte apoptosis[249]. Osteocytes inhibit osteoclast activity through TGF $\beta$ , and estrogen enhances this function[250].

Raloxifene is approved for treatment of postmenopausal osteoporosis. Raloxifene has been shown to influence the serum levels of IL-6, TNF $\alpha$ , TGF $\beta$ , as well as bone turnover markers in women with postmenopausal osteoporosis[251]. It inhibits IL-6 production by osteoblasts[252]. Serum OPG levels were found to be higher in postmenopausal women after raloxifene treatment[253], and the RANKL/OPG ratio was decreased by raloxifene treatment of osteoblastic cells *in vitro*[254]. Raloxifene also decreased osteocyte apoptosis both *in vivo* and *in vitro*[255-257]. Raloxifene treatment of arthritic mice resulted in increased trabecular and cortical BMD. These effects were also reflected in increased serum osteocalcin levels (bone formation) and decreased levels of RatLaps (bone resorption) (papers II, III and IV).



**Estrogen, raloxifene and RA**

The peak incidence of RA in women coincides with the time of menopause[88]. Mice subjected to ovariectomy display higher frequency and increased severity of collagen-induced arthritis, as compared to sham-operated mice[190 and paper I]. This could indicate a protective action of endogenous estrogen both in RA and in experimental arthritis. Several studies have shown that estrogen can affect both the incidence and the progression of RA in humans[188, 258], and in animal models[190, 259-261]. Exposure to oral contraceptives has been shown to reduce the risk of developing RA[262, 263].

In 75% of women with RA the disease activity diminishes during pregnancy, when the levels of female sex hormones are high[258, 264-266]. In contrast, the disease is often aggravated after delivery[264, 267]. Breastfeeding has been shown to increase the risk for RA, which may be due to pro-inflammatory effects of prolactin, the lactation hormone[268]. The mechanisms behind these effects are not fully established. The same effects have also been found in arthritic mice, with amelioration of arthritis during pregnancy and aggravation after delivery[269-273].

Serum levels of estrogen in male RA patients have been found normal in some studies, and increased in others, whereas the levels of testosterone were found to be lower than in controls[274, 275]. Increased levels of estradiol and decreased levels of androgens have been found in synovial fluid of both men and women with RA[276]. This could be due to increased peripheral conversion of androgens to estrogens, since pro-inflammatory cytokines have been shown to stimulate the peripheral aromatase activity[277, 278]. Increased estradiol decreased IL-6 production in synoviocytes from postmenopausal women[279].

Treatment with anti-TNF $\alpha$  antibodies in RA was shown not to influence the hormonal homeostasis, which was stable independently of the inflammatory level[280, 281]. In contrast, serum levels of DHEAS increased in patients treated for two years, which could be due to improved adrenal function[281].

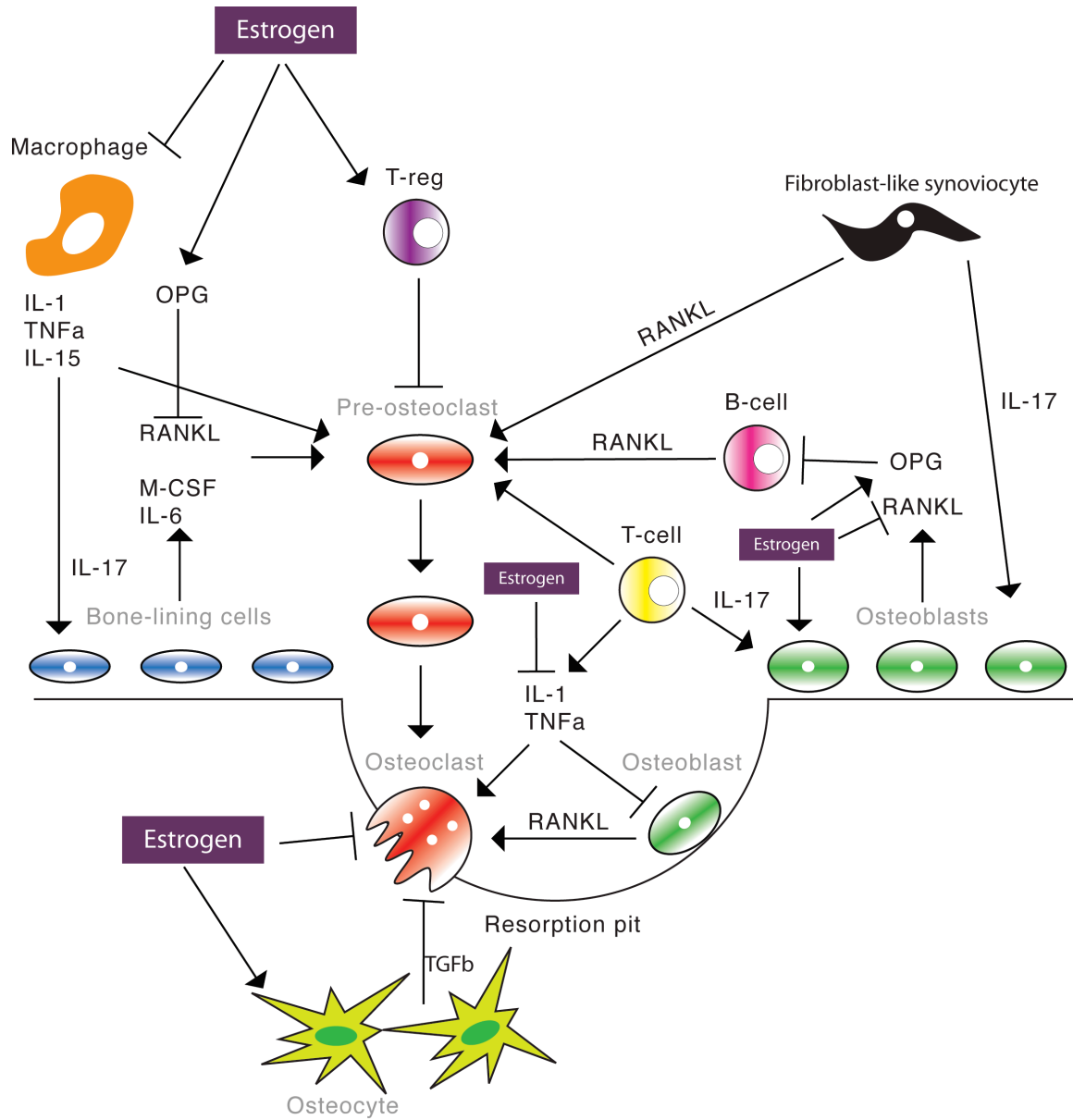
Use of non-contraceptive hormones in the perimenopausal period was negatively correlated to development of RA in a study of 490 women with RA and controls[282]. Some later studies failed to confirm this[262, 283-286], while others found an anti-arthritic effect of HRT[287-291]. A prospective two-year trial of 88 postmenopausal women with RA found that HRT (2mg estradiol and 1mg noretisterone) ameliorated

clinical disease, protected joints from destruction, and increased bone mineral density (BMD)[188]. Estradiol-treatment of CIA in mice also suppressed disease progression[190, 259, 292, papers II and IV], and blocking of the estrogen receptors enhanced the disease[293].

Because of the possible side effects of HRT treatment, longterm therapy is no longer recommended, and there is need to find other substances with the disease-modifying effects of estrogen, but without the side effects. Raloxifene-treatment of ovariectomized mice resulted in lower frequency of collagen-induced arthritis, suppressed disease severity and preserved joint histology (paper II). These effects were also seen during longterm treatment, when therapy was started in established disease (paper III).

Both estrogen deficiency and arthritic disease have deteriorating effects on bone density. HRT ameliorated both the arthritic disease and the development of osteoporosis in RA[188, 288, 290, 294]. Treatment with estrogen also counteracted osteoporosis development in arthritic mice and rats[260, 295]. Raloxifene treatment increased both trabecular and cortical BMD in CIA and CAIA in mice (papers II, III and IV).

**ACTIONS OF ESTROGEN IN ARTHRITIS AND OSTEOPOROSIS**



**Figure 10. Actions of estrogen on the bone changes in RA**

### CONCLUDING REMARKS

Osteoimmunology has become an established scientific area. It is necessary to study the interplay between the immune system and bone when developing new therapies, both for RA and osteoporosis. The role of estradiol in autoimmune diseases is very complex. It has stimulatory and inhibiting effects on different parts of the immune system, and some functions may not be the same *in vivo* as *in vitro*.

To increase our understanding of these intricate mechanisms many questions still need to be addressed.

The role of co-activators and co-repressors needs to be further studied. The conformational change of a hormone receptor when binding to a ligand, and the presence of diverse co-stimulatory molecules in the actual tissue, are what regulate transcription. Modulation of these molecules could provide new targets for treatment. Further understanding of target cells and transcription pathways for estrogen could enable the generation of tissue specific stimulators and inhibitors.

Our data shows that raloxifene activates the classical transcription pathway to a lesser extent than estradiol. This needs to be investigated more. Does raloxifene activate other pathways, and could these be specifically modulated?

The complex molecular mechanisms of osteoporosis development after menopause or ovariectomy need to be further investigated, to elucidate the contribution of T- and B-lymphocytes and cytokines. Identification of cytokines mediating bone loss could provide potent targets for therapy.

Increased knowledge of the RANKL/OPG pathway could open the possibility to regulate it to avoid excess bone resorption, while physiological bone remodeling could still take place.

We are currently conducting studies of immune modulation via the estrogen receptors in conjunction with other steroid receptors, such as the glucocorticoid receptor.

**MAIN CONCLUSIONS FROM THE THESIS****Paper I**

In this paper we investigated the relative contribution of inflammation and estrogen deficiency to the development of osteoporosis in experimental postmenopausal arthritis. We found that both arthritic disease and estrogen deficiency induced by ovariectomy contributed to a similar extent to osteoporosis, and that these effects were additive. We concluded that collagen-induced arthritis in ovariectomized DBA/1 mice is a relevant model for further studies of osteoporosis in postmenopausal RA.

**Paper II**

It is known that estradiol can ameliorate arthritic disease, and protect against osteoporosis, but longterm treatment is associated with serious side effects. Therefore, we investigated if the SERM (selective estrogen receptor modulator) raloxifene had the same effects. We found that raloxifene potently inhibited the frequency and severity of arthritis, joint destruction and loss of bone mineral density, as compared to controls. These results suggest that raloxifene could be a valuable addition to the treatment regimen of postmenopausal RA. In addition, raloxifene treatment down-regulated the expression of TNF $\alpha$  and RANKL mRNA in spleen cells from arthritic mice.

**Paper III**

This study was planned as a follow-up of the previous studies. We wanted to examine whether treatment with raloxifene would ameliorate arthritis and osteoporosis development in mice with an established arthritic disease, and if these effects would be sustained during longterm treatment. We found that raloxifene had a very potent anti-arthritic effect, even when treatment was started after 50% of the mice had developed arthritis. Indeed, in the raloxifene-treated group only 60% of the mice ever acquired disease. This study encourages the planning of a clinical trial with addition of raloxifene to the already established treatment of patients with postmenopausal RA.

### **Paper IV**

In this paper we found that in contrast to estradiol, raloxifene did not affect the effector phase of arthritic disease in collagen-antibody induced arthritis. Despite this, both raloxifene and estradiol treatment counteracted osteoporosis.

In addition, while estradiol potently activated the classical signaling pathway via ER and ERE, raloxifene only activated the ERE at about 1/4 of the intensity of estradiol. This indicates differential effector mechanisms between the two substances, and may explain how one substance can suppress a certain type of inflammation, while the other does not. This needs to be further addressed.

This paper will be extended with ongoing experiments on B10q-ncf1<sup>-/-</sup> mice, which develop chronic arthritis.

**POPULÄRVETENSKAPLIG SAMMANFATTNING**

Osteoimmunologi är ett nytt begrepp, som började användas år 2000 för att belysa de många samband som finns mellan immunsystemet och vårt skelett. Immunsystemets celler utvecklas inne i skelettet, och många processer som påverkar immunsystemet har även effekter på skelettet, och tvärtom. Att klargöra detta samröre ökar förståelsen för sjukdomsprocesser som drabbar skelettet och/eller immunsystemet. Med sådan insikt uppkommer nya möjligheter att utveckla effektiva läkemedel.

Immunsystemet har utvecklats för att skydda oss från olika typer av infektioner. För det mesta fungerar detta utmärkt, men ibland blir försvaret missriktat och angriper istället den egna kroppen. Dessa attacker kan leda till vävnadsskada och utveckling av så kallade autoimmuna sjukdomar.

Ledgångsreumatism (RA, reumatoid artrit) är en autoimmun sjukdom som drabbar ca 1% av världens befolkning. Sjukdomen orsakar ledförstörelse, och inflammationen bidrar till att benskörhet (osteoporos) utvecklas. Det är 3 gånger fler kvinnor än män som drabbas, och de flesta av dessa insjuknar i samband med eller efter klimakteriet.

Vid klimakteriet minskar kroppens produktion av könshormonet östrogen, vilket ofta leder till benskörhetsutveckling, även hos i övrigt friska kvinnor. Benskörhet ökar risken för benbrott, och behandling av osteoporos syftar till att minska denna risk.

Behandling med östrogen hämmar benskörhetsutvecklingen och gör att skelettets styrka ökar. Flera studier har visat att långtidsbehandling med östrogen tyvärr kan medföra risk för allvarliga biverkningar, och användandet av östrogen har därför minskat. Många forskningsgrupper arbetar med alternativa medel, som har östrogenets gynnsamma effekter men inte dess biverkningar. En sådan medicin är raloxifen (Evista®), som sedan 1997 är godkänd för behandling av osteoporos efter klimakteriet.

Vid ledgångsreumatism efter klimakteriet dominerar två faktorer som bidrar till benskörhetsutveckling, inflammation och brist på könshormoner.

Vi var intresserade av att undersöka till hur stor del dessa respektive faktorer bidrar, och undersökte därför benskörhetsutvecklingen vid ledgångsreumatism hos möss som antingen hade kvar eller saknade naturliga könshormoner. Vi fann att både

östrogenbrist och inflammation gav lika mycket förlust av bentätheten, och att de möss som hade båda faktorerna förlorade så mycket som 55% av sin bentäthet.

Det är tidigare känt att behandling med östrogen vid ledgångsreumatism efter klimakteriet mildrar sjukdomen. Efter två års östrogenbehandling sågs minskad sjukdomsaktivitet med lägre sänka, färre svullna leder, mindre ledförstörelse på röntgen och förbättrad bentäthet. Eftersom långtidsbehandling med östrogen inte längre rekommenderas på grund av biverkningar (tex bröstcancer, livmodercancer och stroke) ville vi undersöka om raloxifen kunde ha samma gynnsamma effekter som östrogen mot både ledgångsreumatism och benskörhet.

Möss med ledgångsreumatism behandlades med raloxifen eller östrogen löst i olja. Raloxifen var lika effektivt som östrogen mot sjukdomen, med färre sjuka djur, mildare sjukdom och mindre ledförstörelse. Dessutom hade de möss som fått behandling bibehållen hög bentäthet. Samma gynnsamma effekter mot ledgångsreumatism och benskörhet sågs även vid långtidsbehandling av djur med etablerad sjukdom.

Nedbrytningsprodukter från ben och brosk kan mätas i blodet. Mängden av dem minskade av raloxifen-behandling, vilket tyder på att mindre ben och brosk förstördes än i de obehandlade djuren. En markör för benupbyggnad mättes också, och den ökade av behandlingen.

Vi ville även undersöka om det finns några skillnader i hur östrogen respektive raloxifen utövar sina effekter. Därför mätte vi olika markörer för inflammation i blodet och i mjälten. Både östrogen och raloxifen minskade mängden IL-6 (en inflammationsmarkör) i blodet. Raloxifen-behandling minskade uttrycket i mjälte av två molekyler (TNF och RANKL) som kraftigt bidrar till både inflammation och benskörhetsutveckling, men det gjorde inte östrogen-behandling.

När östrogen kommer till en cell binder hormonet till östrogenreceptorn som vandrar in i cellkärnan, där produktionen av olika proteiner påverkas. Detta kallas för den klassiska signaleringsvägen. Vi kunde visa att även raloxifen delvis fungerar via den



här signaleringsvägen, genom att använda möss med en specialinsatt gen som aktiveras samtidigt som den klassiska signaleringsvägen, och då tillverkar ett protein som kan mätas.

Sammanfattningsvis visar de här studierna att både östrogenbrist och inflammation bidrar vardera lika mycket, och på ett additivt sätt, till benskörhetsutvecklingen i en djurmodell för ledgångsreumatism efter klimakteriet.

Behandling med raloxifen var mycket effektiv mot både ledgångsreumatism och benskörhetsutveckling, och skulle kunna vara ett värdefullt tillägg till den vanliga behandlingen av ledgångsreumatism efter klimakteriet.

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