# Effect of Estrogen and Dietary Loading on Condylar Cartilage

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***Aims:*** *To study the effect of estrogen deficiency and altered temporomandibular joint loading on the histomorphology of condylar cartilage and on the expression of types II and X collagen and matrix metalloproteinase-3 (MMP-3).* ***Methods:*** *Thirty-six female rats were divided into four groups: ovariectomized rats on a normal diet, nonovariectomized control rats on a normal diet, ovariectomized rats on a soft diet, and nonovariectomized control rats on a soft diet. Ovariectomy was performed at the age of 60 days. Repeated-measures ANOVA was used to analyze the data.* ***Results:*** *The condylar cartilage in the ovariectomized normal diet group showed a significantly higher number of cells than in the nonovariectomized control rats (*$P < .001$*). The proportional amount of MMP-3 expression was significantly higher in the ovariectomized rats than in the nonovariectomized control rats in both diet groups (*$P < .001$*). The area covered by types II and X collagen was significantly higher in the experimental groups than in the control groups (*$P < .01$*).* ***Conclusion:*** *Condylar cartilage is sensitive to both estrogen level and dietary loading.* J OROFAC PAIN 2012;26:328–336

**Key words:** estrogen, joint loading, mandibular condylar cartilage, MMP-3, type II collagen, type X collagen

Temporomandibular disorders (TMD) encompass a spectrum of clinical signs and symptoms involving the masticatory system, including the hard and soft tissue structures of the temporomandibular joint (TMJ).[1] According to epidemiologic studies, TMD are twice as prevalent in women compared with men.[2,3] TMD pain conditions are most prevalent among women of reproductive age and become less prevalent in the postmenopausal years.[3] This raises a question of the possible role of female reproductive hormones in the etiology of TMD.

Estrogen mediates its effect through estrogen receptors, and these receptors have also been found on the synovial membrane of the TMJ, the articular disc, and the cartilage layer of the mandibular condyle of rats, and the human TMJ.[4–6] It has been found that estrogen affects the metabolism of condylar cartilage, implying that it could play a role in TMJ pathophysiologic mechanisms. The enzyme aromatase, which is closely associated with the biosynthesis of estrogen, has been noted in chondrocytes of the mandibular condyle in animal experiments.[7] The use of exogenous hormones and the risk of TMD have been the focus of many studies. LeResche et al noted that the use of exogenous estrogen significantly increases the odds of having a TMD, and the risk increases with higher doses of estrogen.[8] In an animal experiment, the use of supplementary estrogen after ovariectomy was found to affect bone and cartilage formation,

thus taking part in TMJ remodeling.[9] In an organ culture study, supplementary estrogen reduced cartilage thickness; this may be directly related to the inhibitory effects of estrogen on chondrocyte proliferation, causing fewer cells to undergo differentiation and maturation.[10]

The condyle is an important growth site of the mandible.[11] In normal cartilage metabolism, there is a highly regulated balance between the synthesis and degradation of various components of the extracellular matrix, where collagens and proteoglycans are essential components.[12] Growth of the condylar cartilage depends on sufficient biomechanical stimuli. Previous studies have shown that reduced loading—for example, a soft diet, affects cartilage metabolism and has an impact on cartilage thickness.[13,14] Loading seems to be important for condylar cartilage growth, since it maintains both ideal proliferation and matrix production of chondrocytes.[13] A continuous soft diet has been found to reduce proliferative activity and proteoglycan synthesis in mandibular condylar cartilage, and these changes are associated with an increase in matrix metalloproteinase-3 (MMP-3) expression.[14] MMP-3 is an enzyme capable of degrading a wide variety of extracellular matrix proteins and activating other MMPs.[15,16]

Currently, there is little information of altered loading and estrogen deficiency affecting condylar cartilage, although the authors have recently shown[6] that they can affect estrogen receptor and MMP-8 expression in the rat condylar cartilage. Whether reduced loading could enhance or diminish the effect of estrogen deficiency in condylar cartilage metabolism remains uncertain. Wang et al discovered that female rats had more osteoarthritic changes in the condylar cartilage than male rats after an occlusal disorder was created by moving the first and third molars.[16] This raises the question of the role of estrogen and masticatory function in condylar cartilage metabolism. The purpose of this study was to investigate the effect of estrogen and dietary loading on rat condylar cartilage. The specific aim was to study the effect of estrogen deficiency and altered TMJ loading on the histomorphology of condylar cartilage and on the expression of types II and X collagen and MMP-3.

## Materials and Methods

### Animal and Tissue Preparation

Altogether, 36 female rats were divided into four groups: ovariectomized rats on a normal diet (pressed pellet, Landsmännen R36, Lactamin) (10 animals), nonovariectomized control rats on a normal diet (8 animals), ovariectomized rats on a soft diet (powder, Landsmännen R36, Lactamin) (10 animals), and nonovariectomized control rats on a soft diet (8 animals). The rats were weaned at the age of 21 days, and the experimental groups underwent ovariectomies at the age of 60 days. Seven days postoperation, the rats were sacrificed using carbon dioxide and decapitation. After the rat was decapitated, the cranium of each rat was placed in a 4% formalin solution for fixation. After 24 hours of fixation, the cranium was hemisectioned in the midsagittal direction, and the soft tissues, except for the articular capsule and surrounding muscles, were removed. The hemicranium was decalcified with 5% formic acid, heated in a microwave (Micromed T/T MEGA) at 37°C for 130 hours, and embedded in paraffin after laboratory procedures. Then, 6-µm sagittal sections were cut from the left and right TMJs for staining. The animal experiment was approved by the National Animal Experiment Committee of Finland and determined ethically acceptable considering the harm caused to the animals.

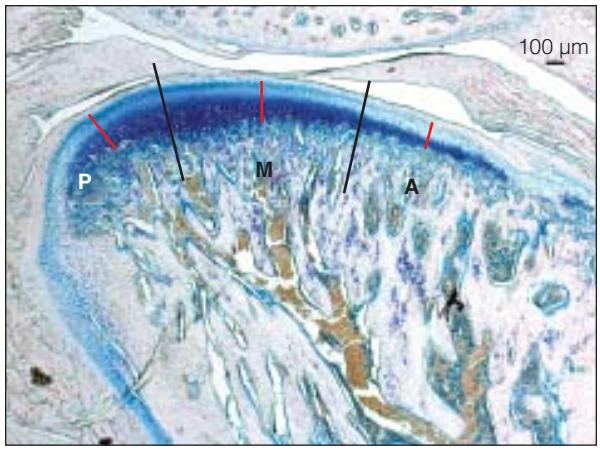
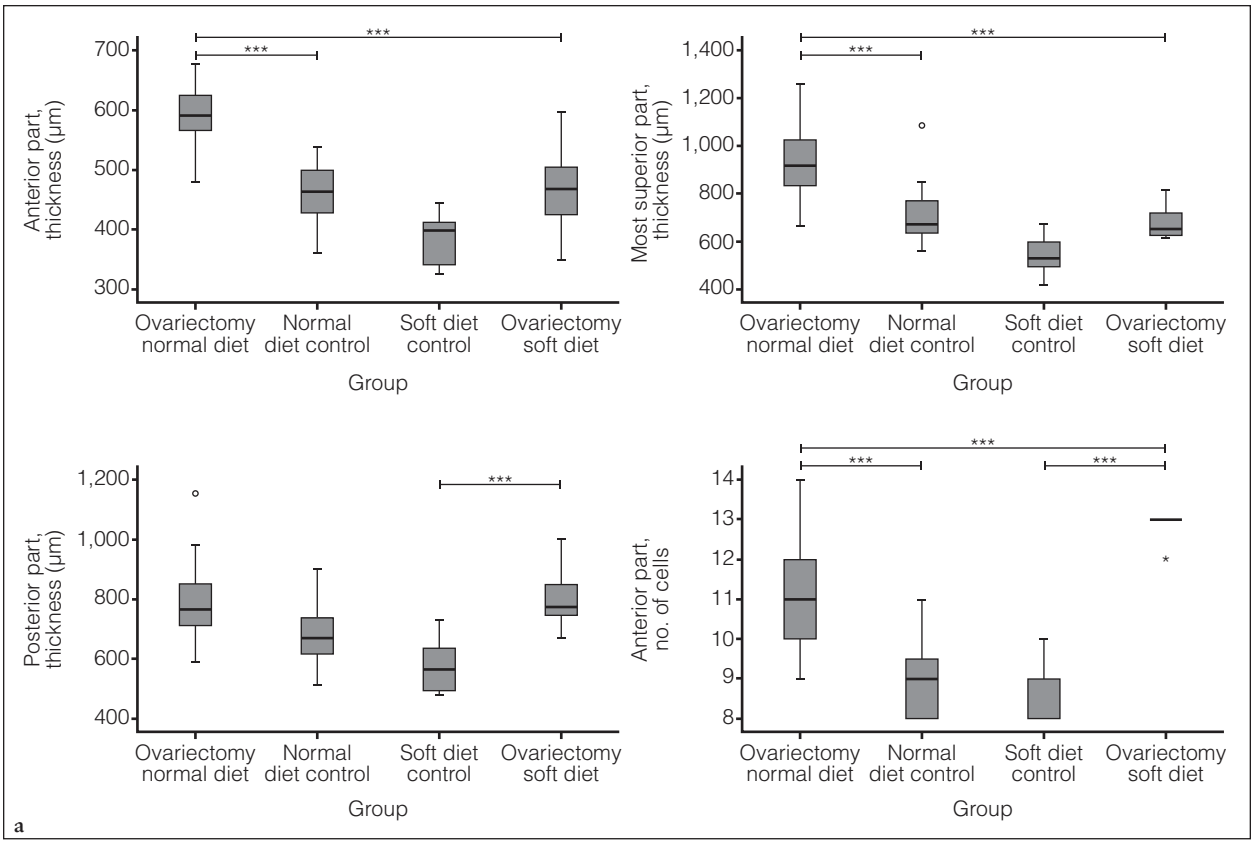



**Fig 1** Most central sagittal section of a rat's condylar head stained with toluidine blue. Black lines divide the head of the condyle into three sections: A = anterior, M = most superior, P = posterior. A red line was drawn in the center of each section, and the thickness of the cartilage and the number of cells were measured. The image was taken from the condylar head of an ovariectomized rat on a normal diet.

### Histomorphometric Analysis

Deparaffinized sections were stained with toluidine blue, and the most central sagittal sections of the condylar head were selected for histologic analysis. For the analysis, the condylar head was divided



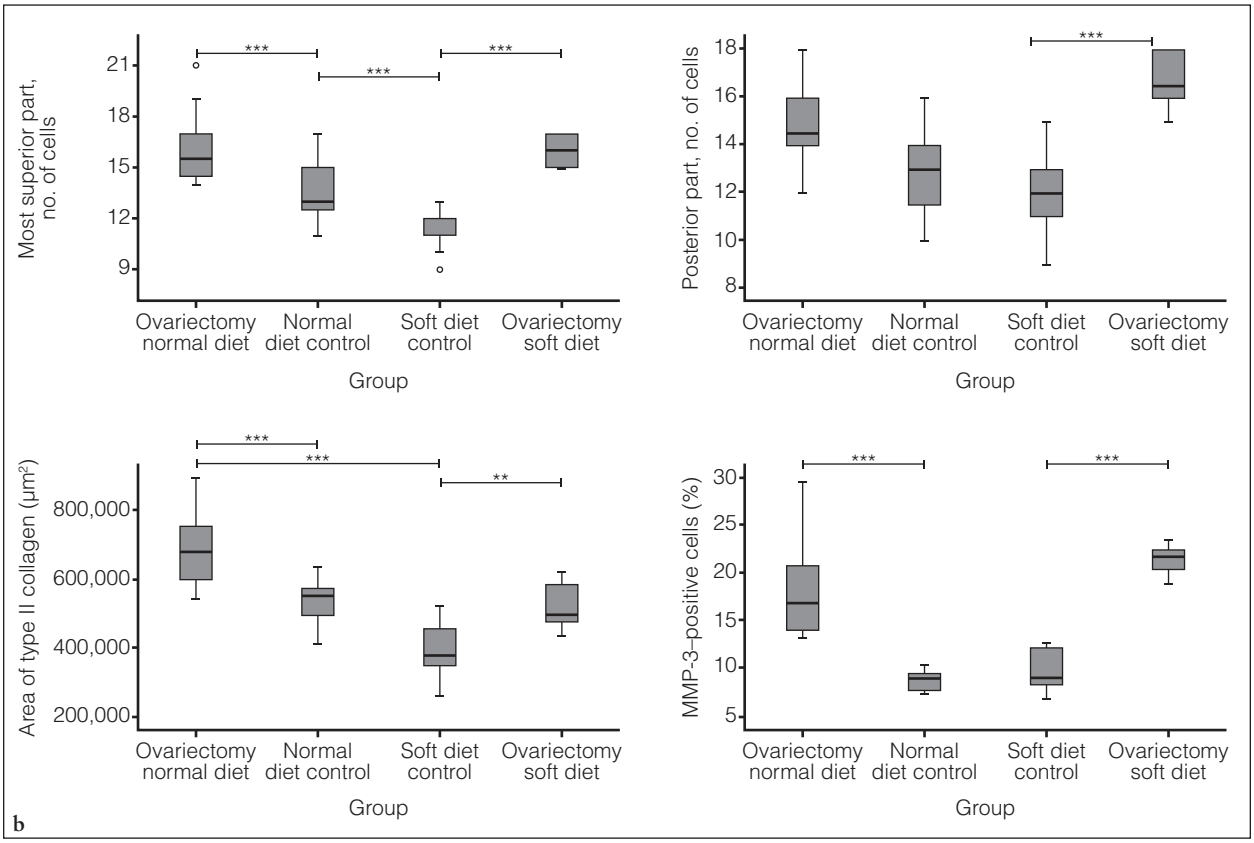




**Fig 2** Box plot diagrams illustrating the thickness of cartilage, number of cells, area of type II collagen, and MMP-3 expression in condylar cartilage. Open circles indicate outliers. Significant difference of: **P* < .05; ***P* < .01; ****P* < .001.

sagittally into anterior, most superior, and posterior segments of equal length (Fig 1). The center of the most superior segment was defined horizontally as the point of the condylar surface opposite the thinnest middle part of the articular disc. The same criteria for segmenting the condylar surface have previously been used.[6,13] The thickness of the cartilage and the number of cells were measured from each segment. A Leica DMRB camera (Leica Leitz) and a Leica Qwin V3 application were used.

### Immunohistochemical Analysis

The most central sagittal sections of the TMJs were chosen for the immunohistochemical analysis. Deparaffinized sections were treated with 0.4% pepsin for 60 minutes at 37°C for the types II and X collagen staining or with a citrate buffer for 25 minutes at 98°C in a microwave for the MMP-3 staining. Endogenous peroxidase was quenched by treatment with 0.2% hydrogen peroxide for 30 minutes. After each treatment, the sections were washed with phosphate buffered saline (PBS). Normal horse serum for types II and X collagen and MMP-3 staining (diluted 1:20 in PBS, 0.1% bovine serum albumin [BSA]) were used to block nonspecific binding of antibodies. The sections were incubated with monoclonal type II collagen antibody (US Bio Logical; diluted 1:1,000 in PBS, 0.1% BSA), monoclonal type X collagen antibody (Quartett Immunodiagnostika & Biotechnologie; diluted 1:100 in PBS, 0.1% BSA), and polyclonal MMP-3 antibody (Santa Cruz Biotechnology; diluted 1:100 in PBS, 0.1% BSA) overnight at 4°C. Negative controls without the primary antibody were also prepared. A secondary antibody antimouse/antirabbit immunoglobulin G was applied (1 hour at room temperature), followed by an avidin-biotin-peroxidase complex (Vector Elite Kit, 30 minutes at room temperature). The immunostaining was visualized with a Vectastain Elite Kit (Vector Laboratories) using peroxidase and a diaminobenzine substrate. The sections were counterstained with Mayer hematoxylin.

The samples were examined with an image analyzer in a light microscope (Leica Leitz). The area stained positively against the antibodies of type II and X collagen was measured with the Leica Qvin V3 application. In the analysis of the MMP-3–stained samples, the most central part of the condyle was defined analogously to the histomorphometric analysis. A box (width, 1,500 μm) was drawn at the superior point of the condyle. The height of the box was related to the thickness of the cartilage in that its upper border was at the surface of the cartilage, and the lower border was at the line of cartilage and subcondylar bone. Positively stained cells inside the box were counted and related to the total number of cells in the area.

### Statistical Analysis

A repeated-measures analysis of variance (ANOVA) was used to analyze the data. The side of the condyle (left versus right) was used as repeated measure. Values measured and tested for differences included thickness (μm) and the number of cells in three segments of cartilage, amount of types II and X collagen ($\mu m^2$), and proportional amount of MMP-3 positive cells in condylar cartilage (a difference of $P < .05$ was defined as significant). Intraclass correlation coefficients (ICCs) were 0.920 for the cell count, 0.810 for MMP-3, and 0.970 and 0.988 for types II and X collagen stained area, respectively. All measurements were performed blindly by one investigator (MO).

## Results

### Histomorphometric Analysis

The largest differences in condylar cartilage thickness were found in the anterior section: The condylar cartilage was significantly thicker in the ovariectomized normal diet group than in the ovariectomized soft diet group ($P < .001$) (Fig 2a). The anterior section of the condylar cartilage was significantly thicker in ovariectomized normal diet rats than in the control normal diet rats ($P < .001$) (Fig 2a). All three sections of the condylar cartilage in the ovariectomized rats on a normal diet showed a significantly higher number of cells than in the nonovariectomized control rats ($P < .001$) (Figs 2a, 2b, 3a, and 3b). In the soft diet groups, the ovariectomized rats had a larger number of cells in the condylar cartilage than did their controls ($P < .001$) (see Fig 2a and 2b). There was also a significant difference between the soft and normal diet control groups: The nonovariectomized normal diet group had a higher number of cells in the condylar cartilage than the soft diet control group ($P < .001$) in the most superior section of the condylar head (Figs 2b, 3b, and 3c).

### Immunohistochemical Analysis

The area of type II collagen was lowest in the control group on a soft diet and highest in the ovariectomized group on a normal diet (Fig 2b). The area of type II collagen was significantly higher in the ovariectomized group (Figs 2b and 3d) than in

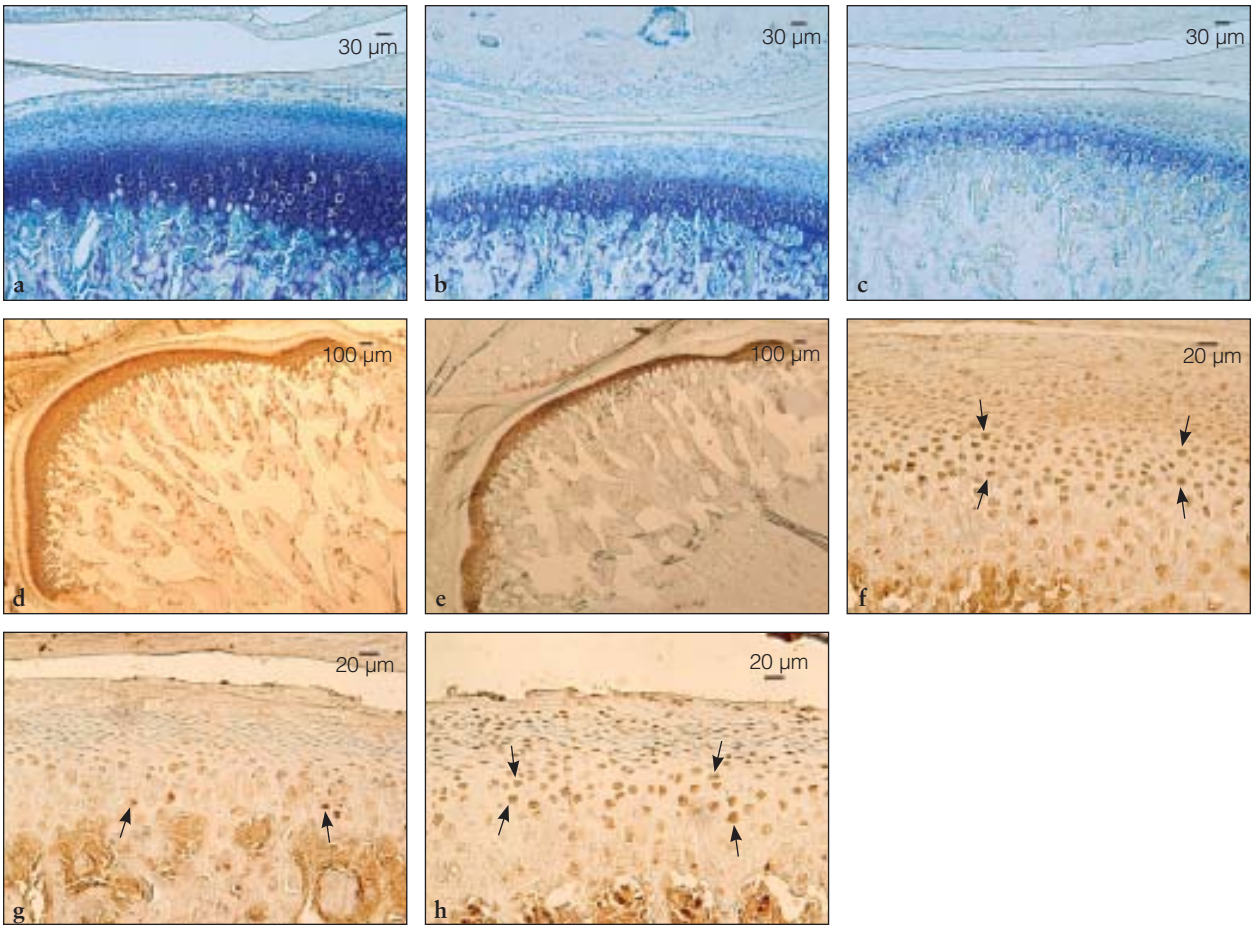


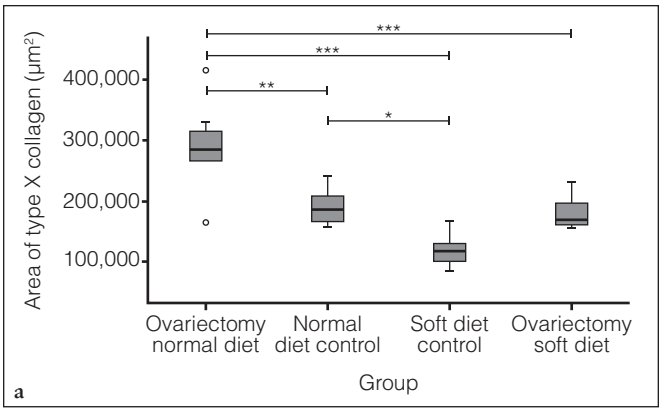

**Fig 3** Histologic samples on a light microscope (Leica Leitz). *(a)* Most superior segment of the condylar surface from the ovariectomized normal diet group stained with toluidine blue for histomorphometric analysis. *(b)* Middle section from the normal diet control group. *(c)* Middle section from the soft diet control group. *(d)* Immunohistochemical staining for type II collagen of the ovariectomized rat on a normal diet. *(e)* A control group rat on a normal diet. The area clearly stained brown expressed the amount of type II collagen. *(f)* Condylar cartilage of an ovariectomized rat on a normal diet was stained for MMP-3. *(g)* The same staining was used for a soft diet control rat and *(h)* an ovariectomized rat on a soft diet. Arrows indicate MMP-3–positive cells.

the control group of both the soft and normal diet groups ($P < .01$) (Fig 3e). The area of type X collagen was lowest in the control rats fed a soft diet and highest in the ovariectomized rats fed a normal diet (Figs 4a to 4c). The area of type X collagen was significantly higher in the ovariectomized normal diet group than in the normal diet control group ($P < .01$). The differences between diet groups were clearly seen; ovariectomized rats fed a normal diet had a larger area of type X collagen than ovariectomized rats fed a soft diet ($P < .001$). The normal diet control group had a larger area of type X collagen than the control soft diet group ($P < .05$).

The proportional number of MMP-3–positive cells was statistically significantly higher ($P < .001$) in the condylar cartilage of the ovariectomized rats than in the nonovariectomized control rats in both the normal and soft diet groups (Figs 2b, 3f, 3g, and 3h). No difference in MMP-3 expression was found between control rats fed a soft diet and control rats fed a normal diet.

## Discussion

This study has shown that the condylar cartilage is sensitive to both estrogen and loading changes, as well as the amount of types II and X collagen and the expression of MMP-3. This is in line with the authors' previous results, whereby the thickness of two distinctive condylar cartilage layers in the superior segment of the condyle was found to be sensitive to a lack of estrogen and altered loading caused by diet consistency.[6] The present study shows that decreased loading caused by a soft diet had a diminishing effect on the condylar cartilage by reducing the thickness, number of cells, and amounts of types II and X collagen. Nonetheless,





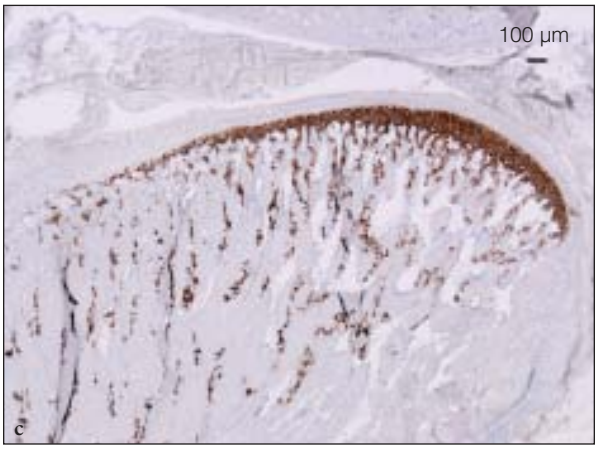



**Fig 4** *(a)* Box plot diagram illustrating the area of type X collagen in condylar cartilage. Open circles indicate outliers. Significant difference of: $*P < .05$; $**P < .01$; $***P < .001$. Immunohistochemical staining for type X collagen of *(b)* an ovariectomized rat on a normal diet and *(c)* a control group rat on a normal diet.

the ovariectomized soft diet group had the largest number of cartilage cells in all three condylar segments. It is possible that the ovariectomy combined with soft diet induced some increase in the proliferation of cartilage cells. The authors' previous study showed that the proliferative layer of condylar cartilage is thickest in ovariectomized rats fed a soft diet.[6]

The present results suggest that when estrogen is present, the condylar cartilage is thinner and has fewer cells compared with a condition in which there is no systemic estrogen at all. However, in the posterior segment of condylar head, the diet hardness did not have any significant effect on cartilage thickness in ovariectomized rats. From the analyses of the possible effects of estrogen on tissue response to loading, it seems that in ovariectomized rats, reduced loading leads to a slightly greater loss of cartilage thickness than the same diet change in nonovariectomized rats. To study the role of estrogen as an independent factor in cartilage metabolism is complicated because ovariectomy in rats increases food intake and therefore results in more chewing and loading of the condylar head.[17,18]

The present study showed that ovariectomy partially prevents the loss of thickness caused by a soft diet. It has been shown that there is a difference in chondrocyte maturation between sexes in response to reduced joint loading.[19] It seems that estrogen inhibits the maturation of chondrocytes in females but not in males.[19] Expression of type X collagen is often associated with mature cartilage, and it is synthesized by hypertrophic chondrocytes.[20] One of the possible functions of type X collagen is that it may act as an easily resorbable material for the deposition of bone matrix. Type X collagen expression therefore has a close correlation with endochondral ossification.[21] In the present study, the amount of type X collagen was highest in the ovariectomized rats fed a normal diet and lowest in the control rats fed a soft diet. It has been shown that estrogen increases the amount of type X collagen in hypertrophic cartilage in culture media and inhibits chondrocyte proliferation in cell culture,[22] suggesting that estrogen may induce the maturation of hypertrophic chondrocytes. This view partly conflicts with the results of the present study that the ovariectomized rats have the largest area of type X collagen. The present in vivo study was carried out with relatively young rats, so the possible changes in cartilage maturation could occur at a later stage of age, thus explaining the difference with previous studies.

It is as yet unknown by which mechanism estrogen might provoke the degradation of the condylar

cartilage. Estrogen modulates the expression of proinflammatory cytokines but does not participate in the production of anti-inflammatory cytokines. Yun et al suggested that TMJ symptoms may be caused by estrogen through an imbalance among cytokines such that proinflammatory cytokines are overproduced.[23] It has also been found that cytokines affect the synthesis of MMPs by fibroblasts and chondrocytes that participate in the degeneration of the extracellular matrix.[24] Previous studies have shown that estrogen and testosterone have opposing effects on inflammation-associated tissue damage in the TMJ, in that testosterone facilitates plasma extravasation in gonadectomized males.[25] In females, however, plasma extravasation is suppressed, increasing swelling and pain in the TMJ. This could partly explain the difference in the prevalence of TMD signs and symptoms between males and females. There are also suggestions that estrogen might regulate nociceptive neurotransmission, thus making the role of cartilage remodeling problematic in pain related to the TMD.[26]

Landi et al showed in human material that high serum estrogen levels might be implicated in the pathophysiology of TMD.[27] Luder studied human material and found that degenerative changes in the TMJ depended on age, loss of molar support, and articular disc position and load bearing (ie, altered loading changes the cartilage structure).[28] Degradation of condylar fibrocartilage in the TMJ is a complex process that involves many mediators, including different MMPs, especially in the osteoarthritic joint.[29] Matrix degradation caused by MMPs has been suggested as a primary event in joint disease.[30] It has been shown in cell cultures that reproductive hormones have a direct effect on modulating MMP expression, causing matrix degradation in fibrocartilage tissue.[31]

Degeneration of the TMJ structures may be affected by many factors. The tissue inhibitors of metalloproteinases are important regulatory factors of MMPs activity and may be involved in the cartilage metabolism in the early growth stage.[32] It has been shown that the condylar surface of human TMJs with internal derangement has a higher expression of MMP-3 and MMP-8.[33] Internal derangement of the TMJ is considered a risk factor in the development of osteoarthrosis. The authors of this study[33] also implied that the cells secreting matrix-degrading MMPs were not only chondrocytes, but also synoviocytes and inflammatory cells.

The staining for MMP-3 was localized mainly intracellularly to the early stage chondrocytes in the present study. Many previous studies have shown that the chondrocytes are responsible for secretion of MMPs.[34,35] MMP-3 immunoreactivity has previously been found in extracellular matrix and intracellular cytoplasm.[36] In the present study, the proportional number of cells expressing MMP-3 was higher in ovariectomized rats than in their controls, independent of the group's diet. Studies with chondrocyte cell cultures have shown that when estrogen is present in low doses, the expression of MMP-3 is reduced.[37] This is well in line with the present results, which showed that MMP-3 expression was highest in the ovariectomized groups. This result could be seen as a sign of active extracellular matrix remodeling. MMP-3 is thought to be one of the most important factors in the remodeling. However, the gene of MMP-3 is one of the few genes upregulated in the early stage of extracellular matrix degradation.[38] It has been shown that the amount of MMP-3 expression is a key in determining whether tissue goes through remodeling or excessive degradation.[15] It is not clear whether the present findings are showing the start of degradation or just normal extracellular matrix remodeling. Nevertheless, the role of MMP-3 in pathological cartilage degradation is not fully clear.[39] It has been shown that the incidence and prevalence of knee and hip osteoarthritis is larger in men than in women before the age of 50.[40] After age 50, women have a higher incidence and prevalence of osteoarthritis. Previoius studies have also shown that women receiving hormone replacement therapy have a lower risk of hip osteoarthrosis.[40] Kapila et al found that the TMJ disc and pubic symphysis have similar expression profiles of estrogen receptor-α, receptor-β, relaxin-1 receptor, and insulin-like peptide-3 receptor and that these profiles are different from the receptor profile of knee meniscus cells.[30] It is not clear which role estrogen has in the cartilage—proliferative or degenerative. It seems that physiologic estrogen level is important, protecting the cartilage from degradation, while higher doses induce degeneration.[40] The protective role of estrogen in osteoarthritis is proposed to be partially a consequence of the inhibition of subchondral bone remodelling, which is definitely one pathologic event in osteoarthritis.[41]

## Conclusions

Estrogen influences the development and metabolism of condylar cartilage. Condylar cartilage remodeling and a sufficient level of loading produced by masticatory function are especially needed to prevent the condylar cartilage from degrading. Further studies are needed to investigate the long-term effects of deficient estrogen level, decreased functional

loading, and especially the rate of early chondroblast proliferation and chondrocyte apoptosis on condylar cartilage.

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