

# Changes in Type I and Type II Collagen Expression in Rat Mandibular Condylar Cartilage Associated with Aging and Dietary Loading

## Marko Orajarvi, DDS, PhD

Researcher  
Department of Prosthetic Dentistry and  
Stomatognathic Physiology  
Institute of Dentistry

## Sakari Laaksonen, DVM, MSc

Veterinarian  
Laboratory Animal Centre

## Riikka Hauru, DDS

Doctoral Student  
Institute of Dentistry

## Eerika Mursu, PhD(c)

Doctoral Student  
Institute of Dentistry

## Egle Jonaviciute, DDS

Research Assistant  
Department of Prosthetic Dentistry and  
Stomatognathic Physiology  
Institute of Dentistry

## Hanna-Marja Voipio, DVM, PhD, DipECLAM

Director, Professor  
Laboratory Animal Centre

University of Oulu, Oulu, Finland

## Aune Raustia, DDS, PhD

Professor and Chairman  
Department of Prosthetic Dentistry and  
Stomatognathic Physiology  
Medical Research Center, Oulu

## Pertti Pirttiniemi, DDS, PhD

Professor and Chairman  
Department of Oral Development and  
Orthodontics  
Medical Research Center, Oulu

Oulu University Hospital; University of  
Oulu, Oulu, Finland

## Correspondence to:

Dr Marko Orajarvi  
Department of Prosthetic Dentistry and  
Stomatognathic Physiology  
Institute of Dentistry  
University of Oulu  
P.O. Box 5281  
FI-90014, Oulu, Finland  
Fax: +358-8-5375503  
Email: marko.orajarvi@oulu.fi

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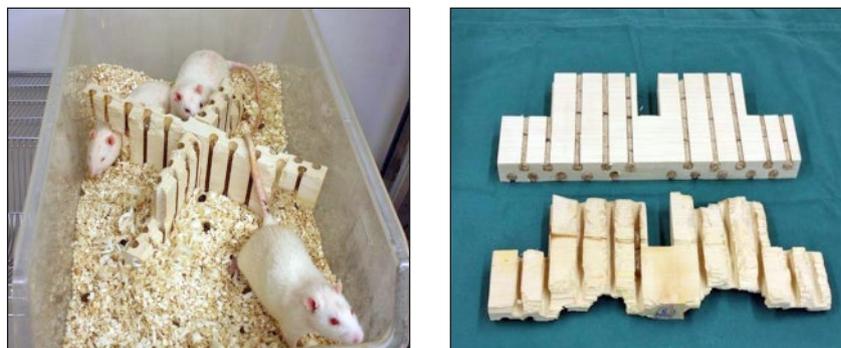
**Aims:** To evaluate the usefulness of diet board feeding as a model for temporomandibular joint (TMJ) research, characterize dietary loading-related morphometric changes in the mandibular condylar cartilage of aging rats, and investigate changes in type I and type II collagen expression in different age, sex, and diet groups. **Methods:** Material was collected from a study that examined the effects of 1-year and 2-year diet board feeding on rats. In diet board feeding, rats must gnaw wood to reach their food, leading to a higher masticatory workload. The material analyzed was comprised of 150 TMJ samples from 75 Hsd:Sprague Dawley rats grouped according to feeding method (diet board [experimental group] or ad libitum [control group]), sex, and experiment length (1 or 2 years). The rats were sacrificed at the age of 15 or 26 months (15-M rats or 26-M rats). From the TMJ samples, 5- $\mu$ m-thick sections were cut parallel to the sagittal plane of the mandibular condyle. Histomorphometric analysis of the thickness of the condylar cartilage and the number of cartilage cells was performed after toluidine blue staining. Immunohistochemical staining included type I and type II collagen antigens. Differences in the thickness of the cellular layer and the number of cells in the condylar cartilage were analyzed by means of a repeated-measures analysis of variance (ANOVA) model, and differences in the type of collagen with a one-way random-effects ANOVA model. **Results:** Condylar cartilage was significantly thicker in the 15-M diet board-fed rats than in the 15-M control rats and in the 26-M rats than in the 15-M rats. The number of cells was larger in the 26-M female rats than in the 26-M male rats. Type I collagen expression was significantly higher in the 15-M diet board-fed female rats than in the 15-M controls. Type II collagen showed increased expression in older rats compared to younger rats. **Conclusion:** Condylar cartilage is sensitive to the interplay between loading, aging, and sex of middle-aged and older rats. High loading of condylar cartilage increased the thickness of cartilage in younger rats. *J Oral Facial Pain Headache 2018;32:258–265. doi: 10.11607/ofph.1581*

**Keywords:** age-related changes, diet board, joint loading, mandibular condylar cartilage, rat, type I collagen, type II collagen

**A**ging affects all organs and tissues, including the temporomandibular joint (TMJ), making it prone to a higher risk of osteoarthritic changes.<sup>1</sup> Articular condylar cartilage is highly specialized tissue in which a single cell type—the chondrocyte—is widely spread throughout a large volume of extracellular matrix composed of water; type I, type II, and type X collagen; proteoglycan molecules; and hyaluronic acid.<sup>2</sup> Under normal loading conditions, chondrocytes help maintain tissue homeostasis. When normal mechanics are altered and a joint is loaded abnormally, chondrocytes can respond in such a way that the balance between matrix catabolism and anabolism is switched in favor of catabolism.<sup>3,4</sup> This may occur also as a consequence of the morphologic, structural, biomechanical, and biochemical changes taking place in the extracellular matrix and cells in aging condylar cartilage.<sup>3,5</sup>

Aging in the mandibular condyle is characterized histologically by a disappearance of hypertrophic cartilage, increased intercellular cartilage matrix, formation of calcified cartilage, and reduced bone marrow due to increased width of trabecular bone.<sup>6</sup> These changes seem to be closely associated with a process wherein, with advancing age,

**Fig 1** Diet board in a rat cage and boards before and after 1 week in a cage.



cartilage matrix is replaced by bone. Mechanical stress caused by masticatory function may be an important factor in these age-related changes.<sup>6</sup> There is evidence that pivotal regulatory transcription factors transmit the effect of loading on condylar cartilage.<sup>7</sup>

Rats are widely used as a model in studies investigating changes in TMJ morphology during normal growth and disease processes.<sup>8</sup> The average life span of laboratory rats is from 2 to 3.5 years, depending on the strain/stock and feeding.<sup>9–11</sup> In rats, the age period between 2 and 7 months may be regarded as equivalent to the young adult stage in human beings, although rats are still growing at that age.<sup>12</sup> The rats used in the present study were middle-aged or old at 15 or 26 months of age at sacrifice.

The study was based on diet board feeding, which forces the animals to gnaw wood to reach food.<sup>13–15</sup> It is a potentially useful model for studying the effects of increased dietary loading on the TMJ of rodents. Little research has been done on the long-term effects of different levels of dietary loading with full-grown, middle-aged, or old rats. Therefore, the aim of this study was to evaluate the usefulness of diet board feeding as a model for TMJ research, characterize dietary loading-related morphometric changes in the mandibular condylar cartilage of aging rats, and investigate changes in type I and type II collagen expression in different age, sex, and diet groups. The hypothesis was that the condylar cartilage benefits from diet board feeding (ie, the cartilage is thicker and has more cells; type I and II collagen expression is higher), which allows sufficient loading of the joint, and that the beneficial changes in the condylar cartilage remain as the rats grow older.

## Materials and Methods

The material was collected in collaboration with a group investigating long-term moderate dietary restriction of laboratory rats by diet board feeding. In diet boards, food pellets are firmly embedded in grooves cut into an aspen board, and rats have

to gnaw the wood in order to eat (Fig 1). Food is available all the time, but due to the workload, diet board-fed rats eat less than those fed ad libitum.<sup>13–15</sup> The aim of diet board feeding is to reduce energy intake moderately, consequently reducing adiposity, morbidity, and subclinical pathologic changes and lengthening life span. Presumably, diet board feeding also somewhat increases physical exercise and energy expenditure. In this study, the ad libitum-fed control group rats received the same food as those receiving diet board feeding (experimental group). Thereby, increased TMJ loading was due to gnawing wood instead of different consistency foods. The study involved no experimental treatments other than the feeding method.

The diet-board study protocol was reviewed and approved by the National Animal Experiment Board of Finland. The animal care and experimental procedures were in line with national and European legislation and recommendations.<sup>16–19</sup>

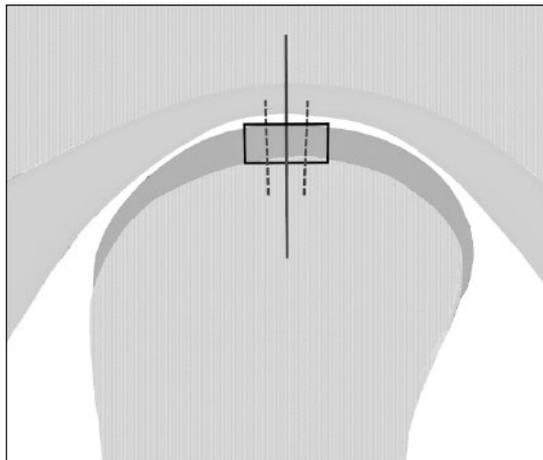
## Animals and Housing

Outbred specific pathogen-free Hsd:Sprague Dawley male and female rats born in the Laboratory Animal Centre, University of Oulu were used. At the beginning of the study, they were 9 weeks old, the males weighing 217 to 365 g (mean  $\pm$  standard deviation [SD] 303  $\pm$  24 g) and the females 167 to 243 g (mean  $\pm$  SD 202  $\pm$  11 g). The microbiologic health of the rats was monitored according to Federation of Laboratory Animal Science Associations recommendations.<sup>20</sup>

The rats were housed in single-sex groups of three in solid-bottom type IV (59.5  $\times$  38  $\times$  20 cm) polycarbonate cages with wire lids (Tecniplast 1354G) and supplied with aspen shavings (PM90L) as nesting material and 5  $\times$  5  $\times$  1 mm aspen chips (4HP) as bedding (Tapvei). The cages were changed twice a week. The room temperature was 21  $\pm$  1°C, and relative humidity was 40% to 60%. The ventilation rate was set at 15 air changes per hour. Room illumination followed a 12/12-hour cycle with a 1-hour gradual change; fluorescent tube lighting was on from 7:00 to 19:00.

**Table 1** Distribution of Rats (N = 75) into the Eight Study Groups According to Age, Sex, and Feeding Method

	15 mo old		26 mo old	
	Female	Male	Female	Male
Diet board	6	5	13	20
Ad libitum	6	5	7	13



**Fig 2** Schematic drawing of a rat condyle. The central point of the most superior segment was defined as the point where the articular disc is thinnest. Three different measurement points were used to assess cartilage thickness and number of cells: one in the middle, one 200 μm anteriorly, and one 200 μm posteriorly from the middle point.

**Feeding and Diet Boards**

Autoclaved natural ingredient food pellets (Lab For R 36) were available all the time in either diet boards (experimental groups) or cage lid hoppers (control groups). The food and boards were changed weekly, and the boards were turned upside down halfway through the week. The control cages were supplied with similar boards, but without food. Untreated tap water from the municipal pipeline was offered freely from polycarbonate bottles (Tecniplast).

The diet board consisted of two aspen boards (2.7 × 14 × 36 cm) joined in the middle to form a cross-shaped wall structure in the cage (Fig 1). There were 20 vertical, rounded grooves (diameter 12 mm) parallel to the 14-cm edge in each board, with 10 grooves opening to each board face at gaps of 6 to 7 mm. The grooves were filled with food pellets, fixed in place tightly by autoclaving for 45 minutes at 121°C.

**Study Design and Randomization**

A total of 168 rats were divided into 1-year (22 rats) and 2-year (146 rats) experiments.

A factorial block design was used. There were eight study groups defined by feeding method, sex, and experiment length (Table 1). In the 2-year experiment, the animals entered the study in four identical 36-rat birth cohorts at 1- to 2-month intervals. The rats in each cohort were derived from five litters born within 1 week: four pups of each sex from four litters, and two pups of each sex from one litter. The pups of each litter and sex were divided randomly into the feeding groups. Then the rats in each study group were randomized into single-sex cages of three rats so that siblings went into different cages. In the 1-year experiment, three litters of eight pups each formed one cohort that entered the study 1 year after the first cohort. Each cohort was housed in its own cage rack in the same room.

**Sample Size**

All the material available from the diet board study was used. All of the rats in the 1-year experiment and 53 rats in the 2-year experiment survived to the end. The different survival rates led to unequal group sizes in the 2-year experiment, as only clinically healthy rats that survived to the end were used. Thus, the total number of rats analyzed was 75. The distribution of the rats into study groups defined by sex, feeding method, and experiment length is presented in Table 1.

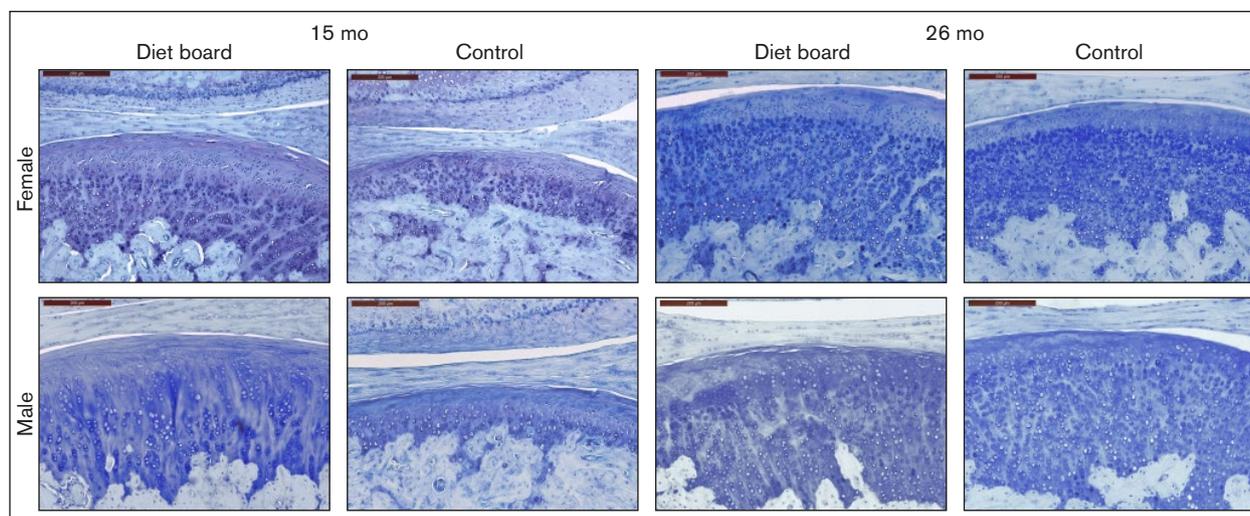
**Sampling and Tissue Preparation**

In the 1-year experiment, the rats were euthanized at the age of 15 months (15-M rats), and in the 2-year experiment, at the age of 26 months (26-M rats). They were sacrificed by collecting blood by heart puncture under isoflurane anesthesia, and then sacrificed in a CO<sub>2</sub> chamber.

Heads were collected in 10% buffered formalin, decalcified with ethylenediaminetetraacetic acid (EDTA), heated in a microwave oven at 37°C (Micromed T/T MEGA) for 100 hours, and then embedded in paraffin. Serial 5-μm-thick sections were cut parallel to the sagittal plane of the mandibular condyle. Deparaffinized sections were stained with toluidine blue.

**Histomorphometric Analysis**

For histomorphometric analysis, 5-μm-thick samples were stained with toluidine blue and examined with a light microscope image analyzer (Leica Leitz DMRB/E; software Leica Qwin V3). The thickness of the cartilage layer was measured in the central section of the condyle. The central point of the most superior segment was defined as the point where the articular disc was thinnest. Three different measurement points were used in the analyzed area—one in the middle, one 200 μm anteriorly, and one 200 μm posteriorly from the middle point (Fig 2). The mean of these three measurements was used to represent the condylar cartilage. The total thickness of the condylar cartilage, including the fibrous, proliferative, and hypertrophic layers, was measured by drawing a line at the three measurement points from the top of the cartilage (fibrous layer) to the



**Fig 3** Histologic view of the condylar cartilage ( $\times 10$  magnification) stained with toluidine blue. The scale bar corresponds to 200  $\mu\text{m}$ .

subcondylar bone. The number of cartilage cells was measured by counting the cells that intersected the line used to measure thickness.

### Immunohistochemistry

The most central sagittal 5- $\mu\text{m}$ -thick histologic sections of the condyles were selected for immunostaining. Antibody staining of type I and type II collagen was performed within 2 days in the same way. The primary antibody for type I collagen was polyclonal type I anti-COL (Sigma), concentration 0.17 mg/mL. The primary antibody for type II collagen was monoclonal type II anti-COL (US Biological), concentration 0.07  $\mu\text{g}/\mu\text{L}$  (diluted to 0.4  $\mu\text{g}/\text{mL}$ ), and NIS was Dako X0910 (0.4  $\mu\text{g}/\text{mL}$ ). The secondary antibody for both type I and type II collagen was Dako Kit (K5007, Dako Denmark A/S). On the first day, the staining samples were deparaffinized and the sections were pretreated with 0.4% pepsin for 1 hour at 37°C. Then they were washed twice with phosphate-buffered saline (PBS). Next, Dako real peroxidase blocking solution S2023 was applied to the samples for 30 minutes at room temperature, and again, the samples were washed twice with PBS. The sections were then incubated with a primary antibody for 30 minutes at 37°C and placed into a cold room overnight at 4°C. The next day the samples were washed twice with PBS, incubated with a labeled polymer (Dako's horseradish peroxidase [HRP] rabbit solution) for 30 minutes, and again washed twice with PBS. Finally, the specimens were incubated in diaminobenzadine (DAB) buffer (5 mL/20 glasses) and chromogen (20  $\mu\text{l}/\text{mL}$  of buffer) for 5 minutes and then washed with running water for 5 minutes. The sections were counterstained with Mayer's hematoxylin (Histolab Products AB).

The samples were examined with a light microscope image analyzer (Leica Leitz DMRB/E; software Leica Qwin V3) with  $\times 2.5$  magnification. After the central point was defined, a rectangular area in the most central third of the cartilage area with a thickness of 400  $\mu\text{m}$  and width of 800  $\mu\text{m}$  was analyzed. The stained area in relation to the total analyzed area (in percentage) was measured in three specimens of the same condyle.

### Statistical Analyses

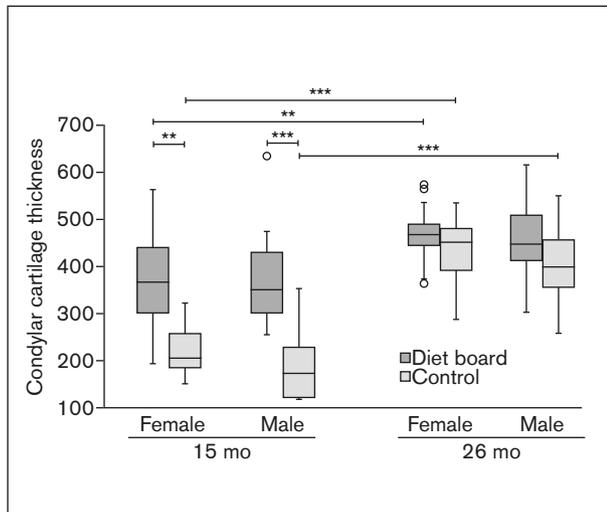
Differences in the thickness of the cellular layer and the number of cells in the condylar cartilage were analyzed by means of a repeated-measures analysis of variance (ANOVA) model where the side of the TMJ (left or right) was used as a repeated measure.<sup>21,22</sup> Differences in type I and type II collagen were determined by using a one-way random-effects ANOVA model. The model had two error terms: one measured the variation between different rats, and the second represented the variation between different specimens of the same rat. All the models were fitted to the data by using SAS Proc Mixed. Variation between different groups was examined with Tukey-Kramer multiple comparisons test. A difference of  $P < .05$  was considered significant.

The statistical software used in this study was SAS Enterprise Guide 4.3 (SAS Institute Inc) and SPSS 20 (IBM).

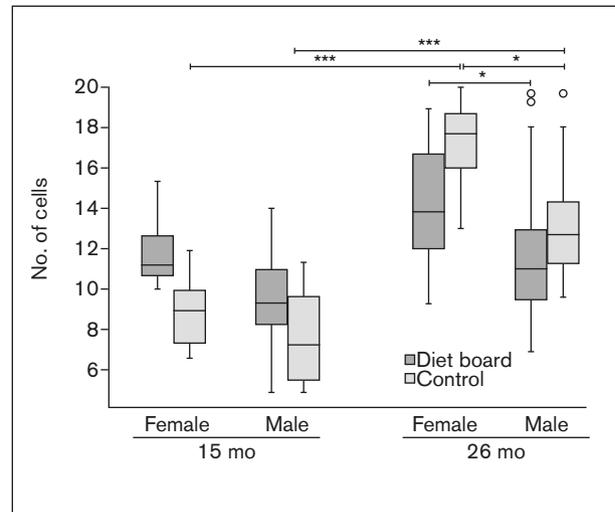
## Results

### Histomorphometric Analysis

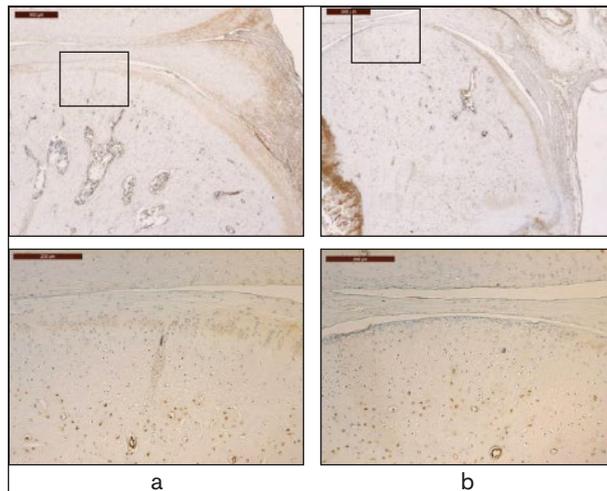
**Thickness of Condylar Cartilage.** The condylar cartilage of the 15-M diet board-fed rats in both



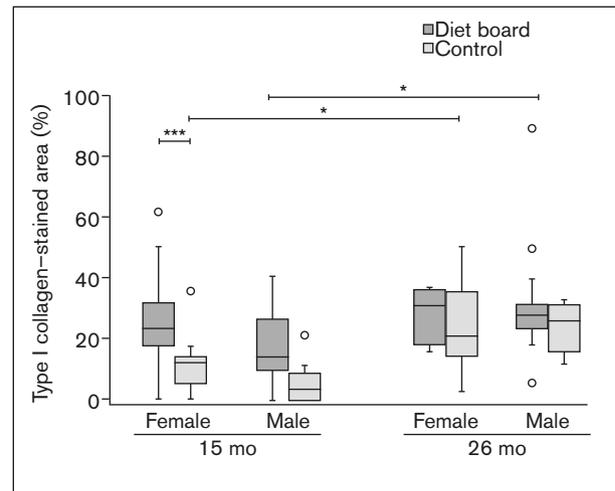
**Fig 4** Box plot diagram of condylar cartilage thickness. \* $P < .05$ . \*\* $P < .01$ . \*\*\* $P < .001$ .



**Fig 5** Box plot diagram of number of cells in condylar cartilage. \* $P < .05$ . \*\* $P < .01$ . \*\*\* $P < .001$ . The open circles represent outliers.



**Fig 6** Histologic view of the condylar cartilage stained with type I collagen antigen ( $\times 2.5$  magnification [scale: 500  $\mu\text{m}$ ] above,  $\times 10$  [scale: 200  $\mu\text{m}$ ] below). (a) 15-M diet board-fed female rat. (b) 15-M ad libitum-fed female rat.



**Fig 7** Box plot diagram of the total amount of type I collagen. \* $P < .05$ . \*\* $P < .01$ . \*\*\* $P < .001$ . The open circles represent outliers.

males and females was significantly thicker than that of the 15-M control rats ( $P < .001$ ). The 26-M control rats had a significantly thicker condylar cartilage than the 15-M control rats in both males and females ( $P < .001$ ) (Figs 3 and 4).

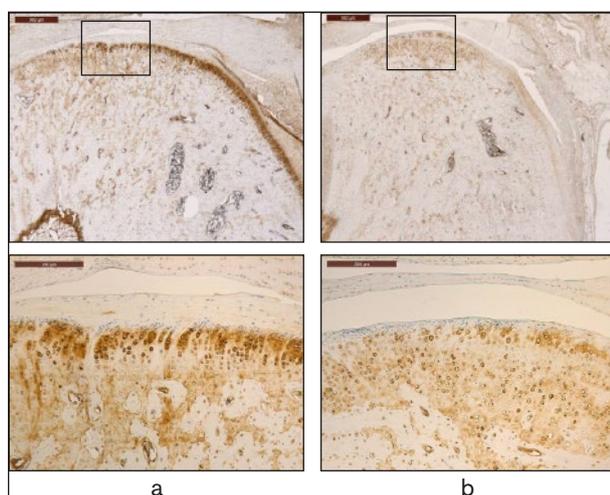
**Number of Cells in the Condylar Cartilage.**

The number of cartilage cells was significantly larger in the 26-M control rats than in the 15-M control rats in both males and females ( $P < .001$ ). In addition, the 26-M female rats had a larger number of cells than the 26-M male rats in both the diet board-fed group and the control group ( $P < .05$ ) (Figs 3 and 5).

**Immunohistochemical Analysis**

**Type I Collagen.** The type I collagen-stained area was larger in the 15-M female diet board-fed rats than in the 15-M female control rats ( $P < .001$ ), and the 26-M female control rats had a larger type I collagen-stained area than 15-M female control rats ( $P < .05$ ). The 26-M male diet board-fed rats had a larger type I collagen-stained area than 15-M male diet board-fed rats ( $P < .05$ ) (Figs 6 and 7).

**Type II Collagen.** The type II collagen-stained area was significantly larger in 26-M female control rats than in the 15-M female control rats ( $P < .05$ ). The 26-M male rats had a larger type II collagen-



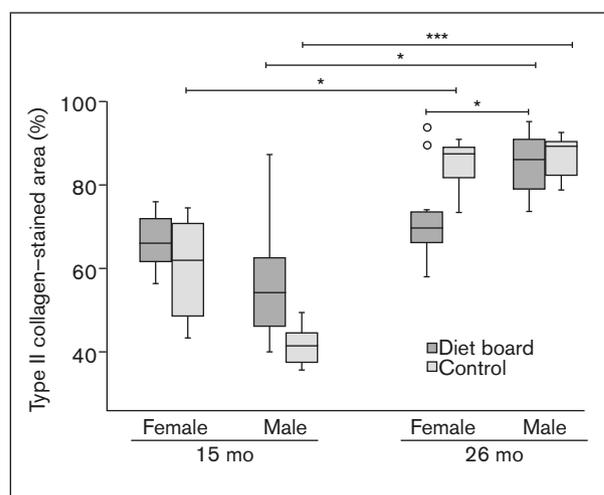
**Fig 8** Histologic view of the condylar cartilage stained with type II collagen antigen ( $\times 2.5$  magnification [scale: 500  $\mu\text{m}$ ] above,  $\times 10$  [scale: 200  $\mu\text{m}$ ] below). (a) 15-M diet board–fed female rat. (b) 15-M control ad libitum–fed female rat.

stained area than the 15-M male rats in both the diet board–fed group and the control group ( $P < .01$ ,  $P < .001$ , respectively). The 26-M diet board–fed female rats had significantly less type II collagen staining than the 26-M male experimental rats ( $P < .05$ ) (Figs 8 and 9).

## Discussion

Diet board feeding was used to evaluate the effects of different TMJ loading on the morphology and expression of type I and type II collagen of the mandibular condylar cartilage of aging male and female rats. Diet board–fed rats had to work harder for their food than the control rats, making this feeding method a good model for studying the effect of physiologic loading on the condylar cartilage. Indeed, as rodents have evolved to efficiently gnaw hard objects, it is likely that the TMJ loading was within a physiologic range with both an ad libitum hard pellet diet and a board-feeding diet. The masticatory sequence in rodents is divided into two phases: incision and mastication.<sup>23</sup> Incisors cut hard substances, and molars grind food.<sup>24</sup> Incision creates a greater load to the TMJ than mastication.<sup>25,26</sup> Therefore, it may be assumed that diet board feeding increased TMJ loading, especially during incision.

The condylar cartilage distributes loading stress and acts as a load-absorbing structure. While optimum condylar loading is considered essential for cartilage to maintain ideal growth and turnover,<sup>27–32</sup> excessive loading leads to degenerative changes in susceptible individuals by inducing production of



**Fig 9** Box plot diagram of the total amount of type II collagen. \* $P < .05$ . \*\* $P < .01$ . \*\*\* $P < .001$ .

cytokines, matrix-degrading enzymes, and other biomolecules associated with cartilage remodeling.<sup>33,34</sup> Old age, genetic factors, and female gender are considered risk factors.<sup>33</sup>

Mechanical loading increases chondrocyte proliferation in growing rats,<sup>32</sup> and the number of cartilage cells increases with the thickness of the condylar cartilage.<sup>22</sup> This is in accordance with the results of the present study, which showed that the number of cells was larger and the condylar cartilage thicker in the 26-M control rats than in the 15-M control rats and that condylar cartilage was thicker in the 15-M diet board–fed rats than in the 15-M control rats. On the other hand, within the 26-M rats, there was no significant difference in the thickness of the cartilage between the experimental and control groups. This finding may imply that the thickness of the condylar cartilage has reached its limit at the end of the second year of life in the rat, and the high loading in the diet board–fed group may suppress the proliferation of cartilage cells in the older age group, which was seen in the tendency for a lower cell count in the diet board–fed group compared to the control group.

Gender- and loading-related changes of the condylar cartilage thickness have been reported in previous studies.<sup>22,35,36</sup> It can be hypothesized that an appropriate thickness has the advantage of maintaining the functional structure of condylar cartilage because it has more strength and resiliency to withstand forces created by chewing. In this vein, an interesting question would be whether thinner condylar cartilage could be a risk factor for TMJ degeneration during a certain period of life, which in rats would be at the age of 1 year according to the present results concerning

the thickness of condylar cartilage. The 26-M female rats had a larger number of cells than the 26-M males in both the diet board-fed and control groups. This could point to the possibility that condylar cartilage has more proliferative activity in females than in males or that the catabolic activity is slower. It is noteworthy that there was no significant difference found in the thickness of condylar cartilage between female and male rats. One possible route of adaptation could be type II collagen expression regulation, because the 26-M diet board-fed female rats had significantly less type II collagen staining than the 26-M male diet board-fed rats. Another factor that may be affecting cartilage thickness is the effect of sex hormones, as estrogen receptors have been found in the condylar cartilage of both male and female rats and these receptors have been shown to react differently depending on the age and sex of the rat.<sup>1,37</sup>

Type I collagen fibers run parallel to the surface in the fibrous layer (the most superficial layer of condylar cartilage) and are the dominant extracellular component of this layer.<sup>38</sup> Type I collagen fibers anchor the condylar cartilage to the perichondrium, and expression and location of type I collagen has been associated with tensile forces acting on condylar cartilage.<sup>39</sup> The 15-M diet board-fed female rats had a significantly larger amount of type I collagen than the 15-M control female rats, but this difference was not seen in the 26-M rats. The turnover of condylar cartilage is slower in older rats, which could partly explain this lack of difference.<sup>40</sup>

In a chondrocyte cell culture, increased loading induces expression of type II collagen, which is a major biomolecule responsible for the mechanical structure of cartilage.<sup>41,42</sup> The breakdown of type II collagen is one of the most important elements of cartilage degeneration.<sup>3,43</sup> In the present study, the area of type II collagen was significantly larger in older rats. It is interesting to note that the 26-M diet board-fed female rats had significantly less type II collagen staining than the 26-M male diet board-fed rats, and the 26-M female control rats had significantly higher type II collagen staining than the 15-M female control rats. This might indicate that the cartilage of female rats is more load sensitive; ie, that the larger loading produced by diet board feeding depressed type II collagen expression in female rats, but not in males. Estrogen receptors (ER) have been found in the TMJ of various species,<sup>44</sup> indicating that estrogen may be involved in the metabolism of TMJ cartilage. Indeed, ovariectomized female rats have an increased cartilage thickness.<sup>22,36</sup> Moreover, ER $\beta$  knockout mice showed a decreased cartilage turnover.<sup>45</sup> These observations may explain the higher number of cartilage cells in female rats than in male rats found in this investigation.

In some comparisons, the small sample size may have precluded the statistical significance of real, biologically relevant differences. As there are only a few studies concerning the condylar cartilage of old rats, further investigations of the aging process and the effects of sex and dietary loading are needed.

## Conclusions

This study has shown that condylar cartilage is sensitive to the interplay between loading, aging, and sex of middle-aged and old rats. High loading of condylar cartilage increased the thickness of cartilage in younger rats.

## Acknowledgments

The authors acknowledge Ms Maritta Harjapää, Ms Tanja Kuusisto, and Ms Sanna Juntunen for their skillful assistance in the laboratory and MSc Paula Pesonen for helping with the statistical analysis. The authors report no conflicts of interest.

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