ACRYLAMIDE-INDUCED PRENATAL PROGRAMMING OF BONE STRUCTURE IN MAMMAL MODEL*

Ewa Tomaszewska*, Piotr Dobrowolski†, Iwona Puzio‡, Janine Donaldson‡, Siemowit Muszyński‡

†Department of Animal Physiology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Akademicka 12, 20-950 Lublin, Poland
‡Department of Functional Anatomy and Cytobiology, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland
¶School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road, Parktown, Johannesburg, 2193, South Africa
§Department of Biophysics, Faculty of Environmental Biology, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland

*Corresponding authors: ewaRST@interia.pl; siemowit.muszynski@up.lublin.pl

Abstract
Acrylamide (AA) is a chemical substance with a potentially carcinogenic effect. Its presence in food or animal food arises from its thermal processing. The experiment was conducted to evaluate the effect of AA exposure (3.0 mg/kg. b.w./day) of pregnant dams during the second half of the pregnancy on bone development in offspring. As an model animal, guinea pig was used. While term body weight of newborns was not influenced by maternal AA treatment, shorter bones with reduced bone diaphysis cross-sectional area were observed in experimental group. Numerous negative, offspring sex-dependent effects of maternal AA exposure were observed in femoral epiphysis and metaphysis as well as the articular and growth plate cartilages. These effects resulted from the AA-induced alterations in bone metabolism, as indicated by the changes in the expression of numerous proteins involved in bone development: receptor activator of nuclear factor kappa-B ligand (RANKL), tissue inhibitor of metalloproteinases 2 (TIMP-2), bone morphogenetic protein 2 (BMP-2), vascular endothelial growth factor (VEGF), and cartilage oligomeric matrix protein (COMP), all of whose expression was measured as well as distribution of immature collagen fibres was determined. Based on the results, it can be concluded that the exposure of pregnant dams to AA negatively affected the structure of compact bone in bone diaphysis, microarchitecture of trabecular bone in metaphysis and epiphysis as well as the structure of the articular and growth plate cartilages in their offspring. The AA-induced bone impairment increased osteoclast differentiation, as observed through the change in the RANKL/OPG ratio, which in turn inhibited osteoblast function by decreasing the expression of other proteins. The data of the present study suggests that maternal AA exposure can result in insufficient bone gain and even bone loss after the birth.

Key words: acrylamide, bone, guinea pig, prenatal programming

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Nutrition plays an important role in the prenatal structural development of mammals and has long-term effects that are evident later in life (Dauncey and Bicknell, 1999; Hulas-Stasiak et al., 2013). Humans are commonly exposed to toxic compounds through their intake with food, which is often done unknowingly. In addition to the nutrients essential for the proper development and functioning of each organism, substances that have a negative effect are sometimes also consumed. Among them is acrylamide (AA), which is formed at high temperatures in the Maillard reaction, when amino acids (mainly asparagine) react with reducing sugars (Koszucka et al., 2019). Elevated levels of AA are noted in numerous heat-treated carbohydrate-rich foods, for example, cereals and potatoes, which contain its (AA) precursors. For the same reason, AA is also present in thermally processed animal feed, particularly in potato- or wheat-based feeds, which are granulated by extrusion, with the highest concentrations of AA found in poultry feeds (Halle et al., 2006; Kienzle et al., 2005). Moreover, a possibility of a limited acrylamide carry-over from livestock animals to eggs or milk was reported (Blumenthal et al., 1995; Halle et al., 2006; Kienzle et al., 2005; Pabst et al., 2005).

The International Agency for Research on Cancer (IARC) has included AA as a group 2A carcinogen for humans. According to the Commission Regulation (EU) 2017/2158, AA is regarded as a chemical hazard in food and is considered to be a toxin with strong mutagenic properties (EC, 2017). Experimental mammalian in vivo and in vitro systems have also shown AA to be toxic with respect to both the nervous and reproductive systems (Hulas-Stasiak et al., 2015; Yu et al., 2019). Since AA is found in a wide range of everyday foods, it could potentially increase the risk of cancer development for consumers of all age groups. The WHO has estimated the average exposure to dietary AA to be between 0.3 and 0.8 µg/kg of body weight per day, for the general population (WHO, 2002). However, the intake of dietary AA is difficult to assess due to the lack of information concerning the level of AA in many food products, especially for homemade meals (EFSA, 2015; Sörgel et al., 2002).

Additionally, AA is able to cross the placental barrier and inhibits placental development (Nagata et al., 2019; Yu et al., 2018). Epidemiological studies have shown the presence of haemoglobin adducts of acrylamide, a biomarker used to assess exposure to AA, in the cord blood, as well as adverse effects of AA on human foetus birth weights (Pedersen et al., 2012; Duarte-Salles et al., 2013; Mojska et al., 2015; Nagata et al., 2019). Other in vivo studies have shown that gestational exposure to AA inhibits placentation and results in the lack of bone ossification centers in newborns (Yu et al., 2019).

The administration of AA to guinea pigs at doses equal to 3 mg/kg of body weight/day has also been found to play a role in prenatal AA-induced programming of postnatal development of gastrointestinal tract abnormalities, assessed on the basis of the structure and expression of protein in adherent type cell-cell junctions in the small intestine epithelium (Tomaszewska et al., 2014).

However, there are no studies to our knowledge that have investigated the effect of AA on prenatal bone development with regards to proteoglycan content in the articular cartilage and the distribution of mature and immature collagen fibres, as well as geometric and histomorphometrical analyses. There are also no studies showing
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the expression of proteins involved in bone metabolism. Therefore, the aim of this in vivo study was to determine the effects of maternal exposure to dietary AA on offspring long bone development. The experiment was performed on guinea pigs, therefore the obtained results may also be applicable in the area of animal sciences, where rodents as experimental animals are commonly used (Tomaszewska et al., 2017a, b; Rudyk et al. 2019).

Material and methods

The experimental procedures used throughout this study were approved by The Local Ethics Committee on Animal Experimentation of the University of Life Sciences in Lublin, Poland (45/2009).

Pregnant guinea pigs

Fourteen, randomly chosen, primiparous female Himalayan guinea pigs (Cavia porcellus), weighing about 420 g each, and their litters were used in this study. The guinea pigs were purchased from the Institute of Microbiology and Immunology of the University of Łódź, Łódź, Poland. The female guinea pigs were 90 days old upon arrival, mated naturally using an IV-block matching group system technique – outbreeding with healthy fertile males (at a ratio of 1 male to 2 females). This method produces accurately timed, pregnant guinea pigs. Vaginal smear examination was carried out to provide precise determination of the onset of gestation.

Guinea pigs were clinically healthy and individually housed in standardized cages for guinea pigs (Velaz, Praha, Czech Republic), providing them sufficient space, appropriate environmental enrichment equipment, and the possibility of social (sensory) contact between individuals. The study was conducted under vivarium conditions at the Faculty of Veterinary Medicine of the University of Life Sciences, Lublin, Poland. The guinea pigs were kept at a constant temperature of 20±2°C, humidity of 55–60%, and controlled 12 h/12 h light/dark cycle with an airflow speed not exceeding 0.3 m/s. Guinea pigs were fed standard laboratory rodents diet, formulated to meet nutritional requirements specified in AIN-93M directive (Reeves et al., 1993), ad libitum. The experimental period commenced at 32 days of gestation (term constituted approximately 67 days of gestation). Guinea pigs were randomly allocated to one of two dietary groups and weighed every two days. Seven guinea pigs (AA-dams) received AA-water solution during the last 35 days of pregnancy, while the other seven were control animals (C-dams). The body weight gain of pregnant guinea pigs was not different between the control and AA-treated groups.

Preparation and analysis of drinking solutions and food consumption

Commercially available AA for electrophoresis, in the form of powder (purity ≥ 99%) (Sigma-Aldrich, St. Louis, MO, USA), was used in the study. Diluted or powdered AA was kept in a freezer at –20°C.
Before beginning the experiment, water consumption during 24 h for both groups of pregnant guinea pigs was measured. The water volume left in the bottles was measured. The water consumption data, together with the body weight data were used to calculate the amount of AA (at a dose of 3.0 mg/kg b.w./day) to be administered in the tap water (as a drinking solution). The dose used in the current study was based on literature, in order to ensure that no characteristic signs of acrylamide-induced neurotoxicity, such as hind limb foot splaying or serious foetal abnormalities (Tyla and Friedman, 2003; Tyla et al., 2000), were induced. During the experimental period, the water and AA-water solution were provided ad libitum and were changed daily. The fluid volume remaining in the bottles was measured and the data was tabulated, in order to monitor consumption of the AA-water solution. Feed consumption was also measured daily in control animals and in those treated with AA. There were no differences in fluid intake or feed consumption between the groups.

**Guinea pig newborn offspring**

The gestation length did not differ between control and experimental females. All newborn guinea pigs were born naturally. The number of live-born offspring per litter from individual dams in both the AA and control groups were not different (no guinea pigs were stillborn). Pups born by control dams belonged to the control group (\(n=19\)) and pups born by AA-dams belonged to the AA-treated group (\(n=22\)). After weighing, \(n=7\) males and \(n=7\) females from each group, with a body weight close to that of the group average, were selected and euthanized by CO\(_2\) inhalation and cervical rupture. Blood samples were collected upon euthanasia, via cardiac puncture and then centrifuged at 2000x g for 15 min and the serum was collected and stored at \(-20^\circ\text{C}\) until further analysis. The activities of total alkaline phosphatase (TAP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum ionized and total calcium, and phosphorus in the blood serum were determined calorimetrically (Metrolab 2300 GL, Metrolab SA, Buenos Aires, Argentina) with commercial kits (BioMaxima, Lublin, Poland). The ratio of AST to ALT was calculated as the de Ritis coefficient (Śliwa et al., 2009).

**Bone geometric parameters**

The left and right femora from each guinea pig pup were carefully dissected out and then cleaned from adhering tissues. The left femora were then weighed and measured for length, after which the Seedor index (bone weight to length ratio, which can be used to describe the degree of bone mineralization) was calculated. They were then cut at the midpoint of the bone diaphysis with a diamond bandsaw (MBS 240/E, Proxxon GmbH, Foehren, Germany). Geometric properties of the bone diaphysis (cross-sectional area and mean relative wall thickness), as well as geometric indices of distribution of cortical bone mass (cortical index and cross-sectional moment of inertia) were calculated on the basis of measurements of the external and internal diameters of the bone diaphysis cross-section, assuming its elliptical shape (Tomaszewksa et al., 2018).
**Cartilage morphology and trabecular bone histomorphometry**

Five millimetre thick cylindrical samples, containing both cartilage and bone, were obtained from the middle part of the lateral condyle of the right femora from each guinea pig pup. Sagittal sections were cut perpendicularly to the articular surface and fixed in 10% neutral buffered formalin for 48 h. Following this initial fixation, a common histological and microscopic procedures were performed as previously described (Tomaszewska et al., 2013). Briefly, after decalcification in 10% EDTA solution (pH 7.4), four 5-µm thick slices were cut with a microtome and mounted onto a slide. In total, nine slides per guinea pig pup were prepared. Three slides from each guinea pig pup were stained with Goldner’s trichrome, Safranine O, and Picrosirus red (PSR) stains in order to assess basal morphology of the articular and growth plate cartilage, evaluate proteoglycan content in the articular cartilage and assess the distribution of thick (mature) and thin (immature) collagen fibres, respectively (Camplejohn and Allard, 1988; Rich and Whittaker, 2005; Suvara et al., 2013). Stained slides were observed in normal (Goldner’s, Safranine O) and polarized (PSR) light using a light microscope (CX43, Olympus, Tokyo, Japan).

Analysis of the recorded images of the growth plate cartilage and articular cartilage was performed using CellSens software (Olympus, Tokyo, Japan). For the growth plate cartilage, total thickness as well as the thickness of the main zones: the reserve zone (I), the proliferative zone (II), the hypertrophic zone (III), and the calcification zone (IV) were estimated (Tomaszewska et al., 2013). For the articular cartilage, total thickness and the thicknesses of the horizontal (I), transitional (II), and radial (III) zones were measured (Tomaszewska et al., 2012).

For trabecular bone, the relative bone volume (BV/TV), trabecular thickness (Tb. Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N) were calculated for both the metaphysis and epiphysis on the microscopic images using ImageJ software (Schneider et al., 2012; Tomaszewska et al., 2019).

The percentage of immature collagen as a proportion of total collagen content in PSR stained sections was calculated using the pixel counting method, using a color threshold tool in ImageJ software to calculate the area of red-orange (mature) and green (immature) collagen fibres in selected image sections (Muszyński et al., 2018).

**Immunohistochemistry**

Immunohistochemical staining of decalcified serial sections on the remaining microscopic slides was performed according to a previously described protocol (Tomaszewska et al., 2018). Briefly, after deparaffinization and rehydration with distilled water, antigen retrieval was achieved by 10-min enzymatic retrieval with proteinase K (Sigma-Aldrich, St. Louis, MO, USA) in 37°C. Endogenous peroxidase activity was blocked subsequently with a 3% solution of hydrogen peroxide in deionized water for 5 min. After blocking in normal goat serum, sections were incubated with the first antibody overnight at 4°C. Rabbit polyclonal to osteoprotegerin (OPG; Abcam, Cambridge, UK, dilution 1:100); rabbit polyclonal to receptor activator of nuclear factor kappa-B ligand (RANKL; Biorbyt, USA, dilution 1:50); rabbit monoclonal to tissue inhibitor of metalloproteinases 2 (TIMP-2; Abcam, Cambridge, UK, dilution 1:100); rabbit polyclonal to bone morphogenetic protein 2 (BMP-2; Abcam,
Cambridge, UK, dilution 1:250); rabbit polyclonal to vascular endothelial growth factor (VEGF; Biorbyt Ltd, Cambridge, UK, dilution 1:50) and rabbit polyclonal to cartilage oligomeric matrix protein (COMP; Elabscience, USA, dilution 1:100) antibodies were used as primary antibodies. The sections were then incubated (30 min) with secondary antibody (peroxidase conjugated goat anti rabbit, Rockland Immunochemicals, Inc. Limerick, USA, dilution 1:500). Negative control sections for each antibody were obtained by identical immunohistochemical staining, excluding the primary antibody. Then the sections were developed in 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) used as a chromogen for 15 min at room temperature. Counterstaining was performed with haematoxylin (Sigma-Aldrich, St. Louis, MO, USA) (Blicharski et al., 2017).

Microscopic images of immunohistochemistry reactions were further analysed. In trabecular and compact bone, as well in the articular cartilage the expression of following proteins was determined: osteoprotegrin (OPG), an osteoclastogenesis inhibitory factor; receptor activator of nuclear factor kappa-B ligand (RANKL), a binding partner of osteoprotegerin (OPG) expressed by mature osteoblast and their precursors, a key factor for osteoclast differentiation and activation; tissue inhibitor of metalloproteinases 2 (TIMP-2), a natural inhibitor of matrix metalloproteinases, a group of peptides involved in degradation of the extracellular matrix; bone morphogenetic protein 2 (BMP-2), which stimulates the development of bone and cartilage; vascular endothelial growth factor (VEGF), a signal protein produced by cells that stimulate the formation of blood vessels; cartilage oligomeric matrix protein (COMP), which plays a role in the structural integrity of cartilage via its interaction with other extracellular matrix proteins such as collagen and fibronectin via mediation in the interaction of chondrocytes with the cartilage extracellular matrix. In the growth plate cartilage the expression of RANKL, VEGF and BMP-2 was determined.

In the cells’ cytoplasm, the expression of each protein was described as positive (brown colour of cytoplasm) or negative (blue colour of cytoplasm) expression (Blicharski et al., 2017). The intensity of expression of the proteins in the osteocytes or matrix was measured by comparison of the pixel brightness value in the microscopic images converted to an 8-bit grayscale. The higher the pixel value, the lower the intensity of the immunoreaction. The analyses were done using ImageJ software, the data are presented in the bar graphs as mean ± standard deviation.

**Statistical analysis**

Normal distribution of data was examined using the Shapiro-Wilk W test and equality of variance tested by the Levene’s test. The biochemistry, bone geometry, histomorphometry and morphology data from the cartilage were analysed using a two-way analysis of variance (2x2 factorial design), with maternal acrylamide treatment and offspring sex as factors. The interaction between acrylamide treatment and offspring sex was added to the model. Whenever significant differences were found between treatment groups, treatment means were separated using a Tukey’s post-hoc test. The data concerning the intensity of the immunoreactions were analysed using a one-way ANOVA followed by a Tukey’s post-hoc test. In all analyses
a P-value of less than 0.05 was considered statistically significant. The data were analysed using Statistica 13 software (TIBCO Software Inc., Palo Alto, CA, USA).

Results

All newborn guinea pig pups were born alive. Newborns’ clinical features were checked by a qualified veterinarian and none of the newborn guinea pigs had any congenital abnormalities.

Body weight and general and geometric bone properties

The body weight of full-term female and male newborn guinea pigs was similar and not influenced by maternal AA treatment (Table 1). Femora weight was influenced by guinea pig sex, with female guinea pigs having significantly lighter femora than males, irrespective of treatment (Table 2). Female guinea pigs also had shorter femora, with lower cross-sectional areas, compared to males, although AA treatment resulted in shorter bones with lower cross-sectional area, irrespective of guinea pig sex. Cross-sectional moment of inertia was also lower in females, and AA treatment reduced this parameter, irrespective of guinea pig sex. The Seedor index, mean relative wall thickness and cortical index were unchanged in males and females after AA treatment (Table 2).

Table 1. Body weight of full-term newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item                     | Body weight (g) |
|--------------------------|-----------------|
| Main effect offspring sex |                 |
| male                     | 79.3            |
| female                   | 79.0            |
| Acrylamide               |                 |
| –                        | 79.5            |
| +                        | 78.8            |
| Treatment effect         |                 |
| male –                   | 80.4            |
| male +                   | 78.2            |
| female –                 | 78.5            |
| female +                 | 79.4            |
| Pooled SEM               | 1.0             |
| P-value                  |                 |
| offspring sex            | 0.717           |
| acrylamide               | 0.517           |
| (sex)×(AA)               | 0.122           |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

SEM = standard error of the means.

(sex)×(AA): interaction between offspring sex (sex) and acrylamide (AA).
Table 2. Osteometric properties of femora obtained from newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item | Bone weight (g) | Bone length (mm) | The Seedor index (g/mm) | Cross-sectional area (mm²) | MRWT (–) | Cortical index (%) | CSMI (mm⁴) |
|------|-----------------|------------------|------------------------|---------------------------|---------|------------------|----------|
| Main effect offspring sex | | | | | | | |
| male | 0.389 | 23.4 | 15.6 | 4.35 | 0.745 | 41.5 | 3.85 |
| female | 0.330 | 21.6 | 14.0 | 3.36 | 0.827 | 43.3 | 1.89 |
| Acrylamide | | | | | | | |
| – | 0.347 | 23.1 | 15.0 | 4.29 | 0.828 | 43.3 | 3.87 |
| + | 0.319 | 21.9 | 14.5 | 3.42 | 0.743 | 41.6 | 1.87 |
| Treatment effect | | | | | | | |
| male – | 0.367 | 23.7 | 15.6 | 4.94 | 0.779 | 42.5 | 5.13 |
| male + | 0.357 | 23.2 | 15.5 | 3.76 | 0.711 | 40.5 | 2.55 |
| female – | 0.326 | 22.5 | 14.5 | 3.63 | 0.877 | 44.1 | 2.60 |
| female + | 0.280 | 20.7 | 13.5 | 3.09 | 0.776 | 42.6 | 1.18 |
| Pooled SEM | 0.019 | 0.5 | 1.0 | 0.33 | 0.075 | 2.3 | 0.42 |
| P-value | | | | | | | |
| offspring sex | 0.004 | <0.001 | 0.125 | 0.007 | 0.292 | 0.435 | <0.001 |
| acrylamide | 0.145 | 0.008 | 0.587 | 0.017 | 0.275 | 0.459 | <0.001 |
| (sex)×(AA) | 0.345 | 0.126 | 0.659 | 0.339 | 0.832 | 0.908 | 0.182 |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

MRWT = mean relative wall thickness; CSMI = Cross-sectional moment of inertia.

SEM = standard error of the means.

(sex)×(AA): interaction between offspring sex (sex) and acrylamide (AA).

Trabecular bone histomorphometry

A significant interaction between maternal AA treatment and newborn guinea pig sex was observed in the relative volume of the femoral trabeculae from the epiphysis, their mean thickness, mean trabecular separation, and the relative volume of the femoral trabeculae from the metaphysis (Tables 3 and 4).

The administration of AA during pregnancy resulted in a decrease in relative bone volume in the femoral epiphysis, however the decrease was higher in males (46.15%) compared to the decrease observed in females (24.33%) (Table 3). A reduction in trabecular mean thickness was noted only in male guinea pigs following prenatal AA treatment. Trabecular maximal thickness and trabecular number were reduced, while maximal trabecular separation increased in both sexes after AA treatment. Treatment of dams with AA during pregnancy also resulted in an increase in mean trabecular separation in the femoral epiphysis of their newborn female guinea pigs.
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Table 3. Microarchitecture of distal epiphysis of femora obtained from newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item          | BV/TV (%) | Tb.Th mean (µm) | Tb.Th max (µm) | Tb.Sp mean (µm) | Tb.Sp max (µm) | Tb.N (mm⁻¹) |
|---------------|-----------|-----------------|----------------|-----------------|----------------|-------------|
| Main effect offspring sex |           |                 |                |                 |                |             |
| male          |           |                 |                |                 |                |             |
| female        |           |                 |                |                 |                |             |
| Acrylamide    |           |                 |                |                 |                |             |
| –             |           |                 |                |                 |                |             |
| +             |           |                 |                |                 |                |             |
| Treatment effect |           |                 |                |                 |                |             |
| male –        |           |                 |                |                 |                |             |
| male +        |           |                 |                |                 |                |             |
| female –      |           |                 |                |                 |                |             |
| female +      |           |                 |                |                 |                |             |
| Pooled SEM    |           |                 |                |                 |                |             |
| P-value       |           |                 |                |                 |                |             |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

BV/TV = relative bone volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number.

For the treatment effect, rows within a single column with different letters are significantly different (P<0.05).

SEM = standard error of the means.

(sex)×(AA): interaction between offspring sex (sex) and acrylamide (AA).

Table 4. Microarchitecture of distal metaphysis of femora obtained from newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item | BV/TV (%) | Tb.Th mean (µm) | Tb.Th max (µm) | Tb.Sp mean (µm) | Tb.Sp max (µm) | Tb.N (mm⁻¹) |
|------|-----------|-----------------|----------------|-----------------|----------------|-------------|
| 1    | 2         | 3               | 4              | 5               | 6              | 7           |
| Main effect offspring sex |           |                 |                |                 |                |             |
| male |           |                 |                |                 |                |             |
| female |           |                 |                |                 |                |             |
| Acrylamide |           |                 |                |                 |                |             |
| –    |           |                 |                |                 |                |             |
| +    |           |                 |                |                 |                |             |
Table 4 – contd.

| Treatment effect | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|------------------|---|---|---|---|---|---|---|
| male –           | 48.6 c | 54.6 c | 122 c | 62.6 | 151 | 9.4 |
| male +           | 33.2 a | 32.1 a | 71 a | 57.6 | 122 | 10.3 |
| female –         | 45.1 c | 46.4 bc | 111 bc | 59.5 | 137 | 9.8 |
| female +         | 38.7 b | 38.1 ab | 94 b | 50.0 | 121 | 9.9 |
| Pooled SEM       | 1.1 | 2.3 | 6 | 2.9 | 8 | 0.5 |

P-value

|          | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|----------|---|---|---|---|---|---|---|
| offspring sex | 0.411 | 0.633 | 0.294 | 0.078 | 0.337 | 0.969 |
| acrylamide | <0.001 | <0.001 | <0.001 | 0.020 | 0.012 | 0.337 |
| (sex)×(AA) | <0.001 | 0.005 | 0.005 | 0.439 | 0.450 | 0.351 |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

BV/TV = relative bone volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number.

For the treatment effect, rows within a single column with different letters are significantly different (P<0.05).

SEM = standard error of the means.

(sex)×(AA): interaction between offspring sex (sex) and acrylamide (AA).

Relative bone volume in the femoral metaphysis (Table 4) decreased after prenatal AA treatment and the decrease was higher in males (31.68%) than in females (14.19%). Mean and maximal trabecular thickness also decreased following prenatal AA treatment in male guinea pigs. This effect was not observed in females. The administration of AA during pregnancy resulted in an increase in the mean and maximal trabecular separation in both sexes. The trabecular number in femoral metaphysis remained unchanged in both sexes exposed to prenatal AA (Table 4).

**Morphology of articular and growth plate cartilage**

An interaction between maternal AA treatment and newborn guinea pig sex was observed in total thickness of the growth plate cartilage, the thickness of zone I, III and IV (Table 5). The thickest growth plate was observed in female newborn guinea pigs, who showed a 50% reduction in growth plate cartilage thickness after maternal AA treatment. Prenatal AA treatment has no effect on growth plate cartilage thickness in male guinea pigs. The reserve zone (I) was also thicker in females, and a greater reduction in zone I thickness was observed in females (83.55%) compared to males (20.3%) after AA treatment. Maternal administration of AA resulted in a reduction in the thickness of the proliferative zone (II), to a comparable extent in both sexes. The hypertrophic (III) and calcified zones (IV) were the thickest in the control group of female guinea pigs. The thickness of the hypertrophic and calcified zones decreased by 44.56% and 65.63%, respectively after AA treatment, only in females (Table 5, Figure 1).
Table 5. Morphology of the plate cartilage of femora obtained from newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item             | Total thickness (μm) | Zone I thickness (μm) | Zone II thickness (μm) | Zone III thickness (μm) | Zone IV thickness (μm) |
|------------------|----------------------|-----------------------|------------------------|------------------------|-----------------------|
|                  |                      | Zone I                | Zone II                | Zone III               | Zone IV               |
|                  |                      | (μm)                  | (μm)                   | (μm)                   | (μm)                  |
| Main effect offspring sex |                      |                       |                        |                        |                       |
| male             | 699                  | 119                   | 155                    | 130                    | 226                   |
| female           | 946                  | 259                   | 143                    | 143                    | 284                   |
| Acrylamide       |                      |                       |                        |                        |                       |
| –                | 1001                 | 289                   | 193                    | 162                    | 326                   |
| +                | 644                  | 90                    | 106                    | 112                    | 183                   |
| Treatment effect |                      |                       |                        |                        |                       |
| male –           | 713 b                | 133 b                 | 194                    | 140 b                  | 231 a                 |
| male +           | 685 ab               | 106 a                 | 116                    | 121 ab                 | 222 a                 |
| female –         | 1289 c               | 444 c                 | 192                    | 184 c                  | 422 b                 |
| female +         | 602 a                | 73 a                  | 95                     | 102 a                  | 145 a                 |
| Pooled SEM       | 25                   | 9                     | 9                      | 7                      | 31                    |
| P-value          |                      |                       |                        |                        |                       |
| offspring sex    | <0.001               | <0.001                | 0.184                  | 0.101                  | 0.806                 |
| acrylamide       | <0.001               | <0.001                | <0.001                 | <0.001                 | <0.001                |
| (sex) ×(AA)      | <0.001               | <0.001                | 0.306                  | <0.001                 | <0.001                |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

Zone I = reserve zone; Zone II = proliferative zone; Zone III = hypertrophic zone; Zone IV = calcification zone.

For the treatment effect, rows within a single column with different letters are significantly different (P<0.05).

SEM = standard error of the means.

(sex) ×(AA): interaction between offspring sex (sex) and acrylamide (AA).

Figure 1. Representative images of the femoral articular and growth plate cartilage stained with Safranine O, carried out on formaldehyde-fixed sections, from newborn guinea pigs exposed prenatally to acrylamide.
An interaction between maternal AA treatment and newborn guinea pig sex was observed in the total thickness of the articular cartilage and the thickness of all zones (Table 6). Total articular cartilage thickness was greatest in female guinea pigs from the control group. Total articular cartilage thickness decreased by 46.6% following prenatal AA treatment in females, while in males no changes were noted. Articular cartilage in zone I was the thickest in the control female group, and a decrease was observed following prenatal AA treatment in both females and males. The articular cartilage in zone II and zone III was thicker in females than in males, and maternal AA treatment resulted in a reduction in their thickness only in female newborns (Figure 1).

Table 6. Morphology of the articular cartilage of femora obtained from newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item                   | Total thickness (μm) | Zone I thickness (μm) | Zone I thickness (μm) | Zone III thickness (μm) |
|------------------------|----------------------|-----------------------|-----------------------|------------------------|
| Main effect offspring sex |                      |                       |                       |                        |
| male                   | 363                  | 28.7                  | 144                   | 132                    |
| female                 | 463                  | 43.9                  | 153                   | 185                    |
| Acrylamide             |                      |                       |                       |                        |
| –                      | 472                  | 49.9                  | 171                   | 191                    |
| +                      | 354                  | 22.7                  | 126                   | 127                    |
| Treatment effect       |                      |                       |                       |                        |
| male –                 | 340 a                | 35.7 b                | 139 ab                | 140 a                  |
| male +                 | 387 a                | 21.8 a                | 150 b                 | 125 a                  |
| female –               | 603 b                | 64.2 c                | 203 c                 | 242 b                  |
| female +               | 322 a                | 23.7 a                | 103 a                 | 129 a                  |
| Pooled SEM             | 19                   | 2.6                   | 11                    | 6                      |
| P-value                | <0.001               | <0.001                | 0.441                 | <0.001                 |
| offspring sex          | <0.001               | <0.001                | <0.001                | <0.001                 |
| acrylamide             | <0.001               | <0.001                | <0.001                | <0.001                 |
| (sex) × (AA)           | <0.001               | <0.001                | <0.001                | <0.001                 |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

Zone I = horizontal (superficial surface) zone; Zone II = transitional zone; Zone III = radial zone.

For the treatment effect, rows within a single column with different letters are significantly different (P<0.05).

SEM = standard error of the means.

(sex) × (AA): interaction between offspring sex (sex) and acrylamide (AA).

Distribution of thick and thin collagen fibres and proteoglycan content in articular cartilage

The structural information obtained from the PSR-stained sections revealed a difference between mature (red-orange), and immature (green) collagen fibres (Figur-
re 2). An interaction between maternal AA treatment and newborn guinea pig sex was observed in collagen distribution in both the femora epiphysis and metaphysis. The content of thin fibres in compact bone was higher in the control group, additionally their content was significantly higher in female offspring. Maternal AA treatment resulted in the reduction of thin collagen irrespective of the sex. However, in male offspring this reduction was more visible (almost five times) compared to in female offspring (almost two and a half times) (Table 7). The highest content of thin collagen fibres was observed in male offspring in the control group compared to other groups including female controls. Moreover, the reduction in thin collagen content after maternal AA treatment was dependent on the sex and more visible in male offspring. In female offspring it remained unchanged. In the metaphysis, the highest content of thin collagen fibres was observed in female offspring following prenatal treatment with AA. In male offspring, a reduction in the content of thin (green) collagen fibres was observed following prenatal AA treatment. No changes in articular cartilage was observed (Table 7, Figure 2).

Table 7. Percentage of immature (thin) collagen fibres in total collagen content (%) of femora obtained from newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item                        | Cancellous bone | Epiphyseal trabeculae | Metaphyseal trabeculae | Articular cartilage |
|-----------------------------|-----------------|------------------------|------------------------|--------------------|
| Main effect offspring sex   |                 |                        |                        |                    |
| male                        | 11.01           | 39.07                  | 7.05                   | 5.58               |
| female                      | 15.65           | 19.24                  | 17.06                  | 5.27               |
| Acrylamide                  |                 |                        |                        |                    |
| –                           | 20.19           | 36.07                  | 13.36                  | 4.87               |
| +                           | 6.47            | 22.25                  | 13.21                  | 5.97               |
| Treatment effect            |                 |                        |                        |                    |
| male –                      | 18.27           | 53.25 b                | 11.29 b                | 5.21               |
| male +                      | 3.74            | 24.90 a                | 2.82 a                 | 5.95               |
| female –                    | 22.10           | 18.89 a                | 10.51 b                | 4.54               |
| female +                    | 9.21            | 19.59 a                | 23.61 c                | 5.99               |
| Pooled SEM                  | 0.97            | 3.02                   | 1.07                   | 0.66               |
| P-value                     |                 |                        |                        |                    |
| offspring sex               | <0.001          | <0.001                 | <0.001                 | 0.639              |
| acrylamide                  | <0.001          | <0.001                 | 0.265                  | 0.112              |
| (sex)×(AA)                  | 0.407           | <0.001                 | <0.001                 | 0.591              |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

For the treatment effect, rows within a single column with different letters are significantly different (P<0.05).

SEM = standard error of the means.

P-value: interaction between offspring sex (sex) and acrylamide (AA).
Staining with Safranine O showed a difference in the content of proteoglycans (color intensity) in the articular cartilage of newborn guinea pigs, dependent on guinea pig sex and AA treatment (Figure 1). A higher proteoglycan content (very strong pink staining) was seen in the articular cartilage of control female and male newborns compared to those exposed to prenatal AA; however, the proteoglycan content appeared to be higher in male guinea pigs (Figure 1). In the control females there was no evident gradient in Safranine O staining, and their articular cartilage had a lower intensive red staining compared to that of the control males, showing gradual staining with the lowest intensity in the superficial zone (Figure 1). Moreover, both the male and female guinea pigs exhibited weak pink staining associated with a lower content of proteoglycans following maternal AA treatment, which was more evident in the females. The concentration of proteoglycans in the AA-treated groups, irrespective of newborn guinea pig sex, gradually increased along the distance from the periphery towards the deepest cartilage zone.

**Bone immunohistochemistry (OPG, RANKL, BMP-2, TIMP-2, VEGF, and COMP)**

RANKL and OPG are essential for the regulation of osteoclast functions, including proliferation, differentiation, fusion, activation and apoptosis (He et al., 2016).
Therefore, the protein expression and cellular localization of RANKL and OPG were determined by immunohistochemistry in trabecular bone. A more positive reaction for OPG was observed in the control groups compared to the weak OPG staining observed in the AA-treated groups, irrespective of newborn guinea pig sex (Figure 3). An opposite effect was observed for the expression of RANKL, where a very strong positive RANKL expression was observed in the AA treated groups compared to the weak expression observed in the control groups, with the expression in the control female group being lower than that in the control male group (Figure 3). Furthermore, the expression of BMP-2 – a protein which is involved in the differentiation of osteoblasts from progenitor cells and in inducing bone and cartilage formation during skeletogenesis and regeneration – was moderate in the majority of cells in trabecular bone in both control groups and in female AA-treated group. Although a weak reaction was observed in the cytoplasm of the control females, the periterritorial zone of osteocytes showed a well-marked reaction (Figure 3). Significantly stronger brown staining was visible in the cytoplasm of bone cells belonging to the AA males (Figure 3). A weak positive cytoplasmic TIMP-2 reaction – a natural inhibitor of the matrix metalloproteinases, a group of peptidases involved in degradation of the extracellular matrix, but well-marked in periterritorial zone of osteocytes – was detectable in the control females (Figure 3). A stronger positive cytoplasmic TIMP-2 reaction was observed in control males (Figure 3). Despite the differences in the position of the well-marked reaction for TIMP-2 in the control groups, the intensity of expression for this protein was similar in both control groups, irrespective of the sex (Figure 3). No positive staining or very weak positive staining for TIMP-2 was observed in the AA-treated females and a very weak cytoplasmic reaction in a very small number of the bone cells was observed in AA-treated males (Figure 3). The intensity of the TIMP-2 expression in both AA groups was similar (Figure 3). A very strong, well-marked reaction for VEGF, of the highest intensity, was noted in the control male group (Figure 3). A similar reaction was observed in the control female group, but the reaction was less intense. It was observed in the cytoplasm and periterritorial zone of osteocytes, as well as in the matrix (Figure 3). Although a weaker cytoplasmic reaction was observed in the majority of cells in the AA group of males, and very weak cytoplasmic signal was observed in a small number of cells in the AA group of females, the intensity of the reactions in both these groups was similar and of a lower intensity to that observed in the control groups (Figure 3). A very weak positive cytoplasmic COMP reaction was observed in the control females (with the lowest intensity; Figure 3). In the control males a weak reaction was in the periterritorial zone of osteocytes, and weak through moderate to strong cytoplasmic signal was observed. Very strong staining for COMP was observed in all osteocytes from guinea pigs in both the male and female groups prenatally treated with AA (Figure 3). Despite the fact that visually the intensity of the COMP staining differed in the cytoplasm versus in the periterritorial zone of osteocytes in the AA treated groups compared to the male control group, the intensity of staining in the male control group was not statistically different from the COMP intensity noted in both AA-treated groups (Figure 3).
Figure 3. The immunohistochemical analysis of the expression of bone morphogenetic protein 2 (BMP-2), osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), tissue inhibitor of metalloproteinases 2 (TIMP-2), vascular endothelial growth factor (VEGF), and cartilage oligomeric matrix protein (COMP) from the femoral trabeculae of newborn guinea pigs exposed prenatally to acrylamide.

Representative pictures of the immunohistochemical analysis of BMP-2, OPG, RANKL, TIMP-2, VEGF, and COMP carried out on formaldehyde-fixed sections from the femoral trabeculae of newborn guinea pigs from the control male (CM) and female group (CF), the AA-treated male (AM) and female group (AF).

The cytoplasm in the chondrocytes with negative protein expression is stained blue, while the positive protein expression is indicated in cells by brown staining. Moreover, secreted and released protein (BMP-2, RANKL, VEGF) into the matrix are also stained in a brown color.

Bar graphs show the intensity of expression of each protein in osteocytes measured by the comparison of the pixel brightness value in the microscopic images converted to 8-bit grayscale. The higher the pixel value, the lower the intensity of immunoreactions. Data are presented as mean ± standard deviation. Bars with different superscripts are significantly different (P<0.05)
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Figure 4. The intensity of expression of bone mineral protein 2 (BMP-2), receptor activator of nuclear factor kappa-B ligand (RANKL), and vascular endothelial growth factor (VEGF) in the matrix of femoral trabeculae of newborn guinea pigs exposed prenatally to acrylamide. The bar graphs show the intensity of expression of each protein in the bone matrix measured by the comparison of the pixel brightness value in the microscopic images converted to an 8-bit grayscale. The higher the pixel value, the lower the intensity of immunoreactions. Data are presented as mean ± standard deviation. Bars with different superscripts are significantly different (P<0.05)

Moderate brown BMP-2 staining, representing newly formed bone, was observed in the bone matrix of control females (Figure 3, 4). Significantly stronger brown staining was visible in the bone matrix belonging to the control males (Figure 3, 4). Significantly weaker positive bone matrix BMP-2 expression was observed in both AA-treated groups (Figure 3, 4). The strongest positive RANKL reaction in the bone matrix was also observed in both AA-treated groups compared to the lower expression observed in the control females and the near absent expression observed in the control males (Figure 3, 4). The highest positive VEGF reaction in the bone matrix of trabeculae was observed in the control female group, with a lower expression observed in the control male group, and the lowest expression observed in both AA-treated groups, irrespective of the sex (Figure 3, 4).

The expression of BMP-2 (Figure 5) was moderate or very strong in a large number of cells in the compact bone of both the control females and males. Despite the fact that this reaction was observed in a few cells in the AA-treated females, the cytoplasmic intensity was not significantly different compared to both control groups (Figure 5). A well-marked and significantly higher cytoplasmic BMP-2 reaction was observed in the AA-treated males, compared to that observed in the AA-treated female group (Figure 5). The strongest OPG staining reaction was observed in the control males, with a weaker reaction observed in the female control group, and the weakest positive signal observed in both AA-treated groups, irrespective of guinea pig sex (Figure 5). Furthermore, a weak RANKL staining reaction was observed in control females, the reaction was stronger in control males, with the strongest positive reaction observed in the compact bone of AA-treated groups, irrespective of guinea pig sex (Figure 5). A moderate and well-marked TIMP-2 staining reaction was observed in the compact bone of both control groups. A very weak and not well-marked positive signal was observed in a few osteocytes in the female AA-treated group (Figure 6). A more-marked positive TIMP-2 signal was observed in the AA-treated male group, however the signal was not statistically significant compared to other groups (Figure 6). A very strong and well-marked cytoplasmic VEGF staining
reaction was observed in all cells in both male groups, irrespective of the treatment. Although the intensity of the VEGF signal in the periterritorial zone of the cells was higher in the control male group compared to the AA-treated males, the total intensity of the protein expression in compact bone was significantly lower in the control males compared to the AA-treated males (Figure 6). In the AA-treated female group the VEGF reaction was significantly weaker than that observed in the AA-treated male group and the signal was only detected in single cells (Figure 6). Moderate COMP staining was observed in both control groups, compared to the very strong reaction observed in both AA-treated groups, with the strongest signal observed in all cells within the periosteal zone (Figure 6).

Figure 5. The immunohistochemical analysis of the expression of bone morphogenetic protein 2 (BMP-2), osteoprotegerin (OPG), and receptor activator of nuclear factor kappa-B ligand (RANKL) from femoral compact bone of newborn guinea pigs exposed prenatally to acrylamide. Representative pictures of the immunohistochemical analysis of BMP-2, OPG, and RANKL carried out on formaldehyde-fixed sections from the femoral compact bone of newborn guinea pigs from the control male (CM) and female group (CF), the AA-treated male (AM) and female group (AF). The cytoplasm in the chondrocytes with negative protein expression is stained blue, while the positive protein expression is indicated in cells by brown staining. Moreover, the protein secreted and released (BMP-2, RANKL, VEGF) into the matrix are also stained in a brown color. The bar graphs show the intensity of expression of each protein in osteocytes measured by comparison of the pixel brightness value in the microscopic images converted to an 8-bit grayscale. The higher the pixel value, the lower the intensity of immunoreactions. Data are presented as mean ± standard deviation. Bars with different superscripts are significantly different (P<0.05)
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Figure 6. The immunohistochemical analysis of the expression of tissue inhibitor of metalloproteinases 2 (TIMP-2), vascular endothelial growth factor (VEGF), and cartilage oligomeric matrix protein (COMP) from the femoral compact bone of newborn guinea pigs exposed prenatally to acrylamide. Representative pictures of the immunohistochemical analysis of TIMP-2, VEGF, and COMP carried out on formaldehyde-fixed sections from the femoral compact bone of newborn guinea pigs from the control male (CM) and female group (CF), the AA-treated male (AM) and female group (AF).

The cytoplasm in the chondrocytes with negative protein expression is stained blue, while the positive protein expression is indicated in cells by brown staining. The bar graphs show the intensity of expression of each protein in osteocytes measured by comparison of the pixel brightness value in the microscopic images converted to an 8-bit grayscale. The higher the pixel value, the lower the intensity of immunoreactions. Data are presented as mean ± standard deviation. Bars with different superscripts are significantly different (P<0.05)

The analysis of BMP-2 immunostaining in the articular cartilage showed a weak and moderate positive cytoplasmic signal in the control female and male group, respectively. Stronger brown staining was observed in the AA-treated female group, and a very strong signal was observed in the cytoplasm of the AA-treated males (Figure 7). Moreover, in the matrix of the articular cartilage, a weak BMP-2 positive signal was observed in the AA-treated females, while a stronger brown staining was observed in the other groups (Figure 7). The weakest OPG staining was observed in the control males, with a moderate signal observed in the control females, which was significantly higher compared to that observed in the control male group (Figure 7). Statistically stronger OPG staining was observed in all cells in the AA-treated
females, with a moderate to strong positive signal observed in the AA-treated males (Figure 7). The weakest RANKL staining was observed in the control males, with a moderate to strong (not in all cells) signal observed in control females. A very strong RANKL positive cytoplasmic reaction was observed in all cells of the AA-treated females, and a strong signal in the majority of cells was observed in the AA-treated males (Figure 7). A statistically stronger cytoplasmic TIMP-2 signal was observed in the majority of cells in the articular cartilage of both control groups, with a significantly lower and sometimes not even visible signal observed in the AA-treated groups (Figure 8). A strong VEGF signal was detected in both control groups compared to the AA-treated groups, irrespective of guinea pig sex (Figure 8). The strongest COMP cytoplasmic positive signal was observed in both AA-treated groups, compared to the control groups, irrespective of guinea pig sex (Figure 8).

Figure 7. The immunohistochemical analysis of bone morphogenetic protein 2 (BMP-2), osteoprotegerin (OPG), and receptor activator of nuclear factor kappa-B ligand (RANKL) from the femoral articular cartilage of newborn guinea pigs exposed prenatally to acrylamide. Representative pictures of the immunohistochemical analysis of BMP-2, OPG, and RANKL carried out on formaldehyde-fixed sections from the femoral articular cartilage of newborn guinea pigs from the control male (CM) and female group (CF), the AA-treated male (AM) and female group (AF). The cytoplasm in the chondrocytes with negative protein expression is stained blue, while the positive protein expression is indicated in cells by brown staining. Moreover, the BMP-2 secreted and released into the matrix is also stained in a brown color. The bar graphs show the intensity of expression of each protein in the osteocytes or matrix (BMP-2) measured by comparison of the pixel brightness value in the microscopic images converted to an 8-bit grayscale. The higher the pixel value, the lower the intensity of immunoreactions. Data are presented as mean ± standard deviation. Bars with different superscripts are significantly different (P<0.05).
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Either a moderate, well-marked brown cytoplasmic signal or very weak signal for RANKL was observed in the growth plate cartilage, mainly in the chondrocytes from the proliferative zone, in the control females and males, respectively (Figure 9). A stronger RANKL staining was observed in the AA-treated groups, however this signal was detected in a lower number of cells in males compared to females (Figure 9). No staining reaction was observed in the other zones of the growth plate cartilage, irrespective of the treatment. A very strong expression signal for VEGF was observed in the hypertrophic zone of the control females, with statistically less intensive staining observed in the control males. The maternal AA treatment resulted in very weak staining of many chondrocytes for VEGF in the growth plate cartilage,
irrespective of the sex (Figure 9). No staining reaction was observed in the reserve, proliferative or calcification zones. Moreover, moderate expression of BMP-2 was observed in the hypertrophic zone of the control males and females, however the reaction was observed in fewer cells in the control females (Figure 9). The maternal AA treatment resulted in very weak staining of many chondrocytes for BMP-2 with a similar intensity of staining observed in both sexes (Figure 9). No staining reaction was observed in the reserve, proliferative or calcification zones.

Figure 9. The immunohistochemical analysis of the expression of bone morphogenetic protein 2 (BMP-2), vascular endothelial growth factor (VEGF) and receptor activator of nuclear factor kappa-B ligand (RANKL) from the femoral growth plate cartilage of newborn guinea pigs exposed prenatally to acrylamide.

Representative pictures of the immunohistochemical analysis of BMP-2, VEGF, and RANKL carried out on formaldehyde-fixed sections from the femoral growth plate cartilage of newborn guinea piglets from the control male (CM) and female group (CF), the AA-treated male group (AM) and female group (AF). The cytoplasm in the chondrocytes with negative protein expression is stained blue, while the positive protein expression is indicated in cells by brown staining.

The bar graphs show the intensity of expression of each protein in osteocytes measured by comparison of the pixel brightness value in the microscopic images converted to 8-bit grayscale. The higher the pixel value, the lower the intensity of immunoreactions. Data are presented as mean ± standard deviation. Bars with different superscripts are significantly different (P<0.05)
Biochemical analysis

The activity of TAP decreased after AA treatment irrespective of the sex of the newborn guinea pigs, while the activity of AST and the de Ritis coefficient increased following AA treatment (Table 8). The concentration of ionized calcium was significantly lowered in AA-treated newborns of both sexes. Total serum calcium concentration was lower in females compared to males, with AA treatment decreasing total serum calcium irrespective of the sex of the newborn guinea pigs (Table 8). Although some changes were observed, values of all biochemical parameters assessed were still within physiological norms (Winnicka, 2015).

Table 8. Serum biochemical parameters measured in newborn guinea pigs exposed prenatally to acrylamide (AA)

| Item                     | TAP (U/L) | ALT (U/L) | AST (U/L) | de Ritis ratio | Ca (mmol/L) | Ca²⁺ (mmol/L) | P (mmol/L) |
|--------------------------|-----------|-----------|-----------|----------------|-------------|---------------|------------|
| Main effect offspring sex|           |           |           |                |             |               |            |
| male                     | 324       | 49.4      | 66.2      | 1.36           | 2.74        | 1.42          | 1.56       |
| female                   | 317       | 52.4      | 68.8      | 1.30           | 2.45        | 1.38          | 1.41       |
| Acrylamide               |           |           |           |                |             |               |            |
| –                        | 362       | 48.1      | 47.4      | 0.99           | 2.76        | 1.47          | 1.57       |
| +                        | 280       | 53.7      | 87.6      | 1.66           | 2.43        | 1.32          | 1.40       |
| Treatment effect         |           |           |           |                |             |               |            |
| male –                   | 363       | 47.0      | 46.5      | 1.01           | 2.93        | 1.49          | 1.66       |
| male +                   | 285       | 51.8      | 85.8      | 1.70           | 2.55        | 1.33          | 1.46       |
| female –                 | 360       | 49.2      | 48.3      | 0.97           | 2.59        | 1.44          | 1.48       |
| female +                 | 274       | 55.7      | 89.3      | 1.62           | 2.30        | 1.31          | 1.33       |
| Pooled SEM               | 29        | 3.8       | 5.7       | 0.12           | 0.12        | 0.05          | 0.11       |
| P-value                  |           |           |           |                |             |               |            |
| offspring sex            | 0.821     | 0.442     | 0.645     | 0.642          | 0.020       | 0.442         | 0.201      |
| acrylamide               | 0.011     | 0.154     | <0.001    | <0.001         | 0.010       | 0.005         | 0.134      |
| (sex) × (AA)             | 0.895     | 0.830     | 0.885     | 0.900          | 0.684       | 0.778         | 0.831      |

Number of observations included in the calculation of means for treatment effects are 7; number of observations included in the calculation of means for main effects are 14.

TAP = total alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase. SEM = standard error of the means.

Discussion

The pathophysiology of the effect of maternal AA exposure is more complex than direct AA exposure. Available data on the effects of maternal AA exposure relate to
the effects on organs like the spinal cord, medulla oblongata or liver of offspring obtained from pregnant rats treated with AA at a dose of 10 mg/kg b.w./day, and the effects on the ovaries of guinea pigs whose mothers were treated with AA at a dose of 3 mg/kg b.w./day. Moreover, AA is intensively studied in rodents and other animals in a very wide range of daily doses, ranging between 0.5–50 mg/kg b.w. (Al-lam et al., 2010, 2011; El-Bakry et al., 2013; Hułas-Stasiak et al., 2013). On the other hand, neurobehavioural studies are frequently conducted following AA-induced neurotoxicity (El-Bakry et al., 2013; Faria et al., 2018; Prats et al., 2017). In these studies very high doses of AA are used, e.g. 30 mg/kg b.w., p.o., daily in juvenile rat pups (Seale et al., 2012). Studies assessing bone development after AA exposure are limited, and studies assessing the effects of AA exposure prenatally (following maternal AA treatment) are very rare or non-existent. Sarocka et al. (2017) have presented the results of a study performed on 12-week-old male mice receiving AA orally at the dose of 1 mg/kg b.w. twice during 24 hours, or three doses of 1 mg/kg b.w. in a 48-h period, showing that the effect of AA is different on the microstructure of compact and trabecular bone tissues. Subacute exposure to AA significantly decreased the size of the primary osteon’s vascular canals. Moreover, bone volume, trabecular number, and bone surface significantly increased, while trabecular separation decreased in mice after AA treatment (Sarocka et al., 2017). In another study, they have shown that even a single administration to acrylamide had negative effects on cortical bone structure of mice after one remodeling cycle (Sarocka et al., 2019).

In the current study the guinea pig was used as a suitable model species for specific studies relating to maternal AA treatment. Among laboratory rodents, guinea pigs have the longest pregnancy duration (about 68 days). Therefore, in the present study guinea pigs were studied as a suitable rodent model for investigating nutritional maternal programming (Carter et al., 2005; McKendry et al., 2010; Palliser et al., 2010). The present study showed no effect of maternal AA treatment on the full-term body weight of offspring, with bone weight decreasing only in female offspring. Moreover, after maternal AA exposure to 3 mg/kg b.w. daily through the second half of the gestational period, the offspring, irrespective of the sex, had shorter bones with reduced cross-sectional area of bone diaphysis. This reduced development of the femora could have been caused by two independent influences. The first is the fact that AA reduced locomotor activity. Seale et al. (2012) have showed that AA-treated rats show a 52.36% reduction in locomotor activity after 21 days of AA treatment (30 mg/kg, p.o., daily) (Seale et al., 2012). It is known that physical activity is important for proper development including bone metabolism not only during the postnatal period, but also during the prenatal period. Prenatal exposure of the guinea pigs in the current study to AA could also have had a neurotoxic effect, influencing the developing nervous system and resulting in a reduction in locomotor activity within the uterus. Additionally, AA has been shown to increase oxidative stress, which in turn enhances bone resorption and osteoclastogenesis, leading to bone loss (Bai et al., 2005; Basu et al., 2001; Garrett et al., 1990; Raju et al., 2015).

Although indices of oxidative stress were not assessed in the current study, other biochemical parameters were determined. The decreased serum concentrations of
Dietary acrylamide alters bone structure in offspring. Calcium observed in male and female offspring following AA treatment, indicates that bone metabolism could be disturbed. However, calcium ions are also necessary for proper neuromotor activity and triggering the release of neurotransmitters within the neuromuscular junction. Moreover, TAP activity (biomarker of impaired bone metabolism) was also decreased. Even though the current study did not assess the presence of haemoglobin adducts of acrylamide, which serve as a biomarker for exposure to the toxic actions of AA, biochemical parameters such AST and ALT, which provide information regarding liver injury, were determined (Huang et al., 2006). The de Ritis coefficient, a marker of advanced hepatic fibrosis, was also calculated and can indicate increased release of AST from hepatocytes due to hepatocellular damage or death (Huang et al., 2006; Zoppini et al., 2016). All guinea pig offspring, irrespective of the sex, the de Ritis coefficient was significantly increased after maternal AA treatment. Exposure to AA during pregnancy at the dose used in the current study, did not induce the characteristic signs of AA-induced neurotoxicity, but it did result in disturbances in liver metabolism. Furthermore, the decreased bone length, reduced bone geometric parameters and the increase in basal biochemical parameters within the blood serum could be indications of the harmful actions of AA on foetus development, including on the skeletal system during the prenatal period. In addition, the histomorphometrical parameters of trabecular bone indicated bone loss after maternal AA treatment. However, maternal AA treatment was found to have a stronger influence on trabecular bone in the epiphysis than in the metaphysis. This could be a result of improved bone nutrition by better vascularisation, and the positive paracrine influence of growth factor released by the chondrocytes or osteoblasts. The epiphysis is separated by the articular and growth plate cartilage, thus nutritional factors or hormonal signalling might not have reached the bone tissue e.g. from the synovial fluid during movement or through blood vessels from the bone marrow. Thus the reduced locomotor activity observed in guinea pig offspring following AA treatment could result in reduced supply of nutritional factors to the bone, reducing the thickness of the growth plate cartilage and immunohistochemical analyses. Bone homeostasis seems to depend on the local RANKL/OPG ratio. The balance between OPG and RANKL is demonstrated to modulate bone formation and resorption, where OPG is an inhibitor of RANKL and a physiologically negative regulator of osteoclastogenesis (Blair et al., 2007). Osteoblasts, specialized bone-forming cells, express not only osteoclastogenic factors, but also synthesize bone matrix protein and play a main role in bone mineralization.
In the guinea pig offspring in the current study, the expression of OPG was reduced after maternal AA treatment in both parts of the bone – trabecular and cancellous bone. Simultaneously, an increased expression of RANKL was observed. This increased expression indicates impaired bone metabolism, since excess RANKL binds to RANK on the precursor cell of the osteoclast, allowing it to differentiate, mature and activate osteoclasts, and subsequently bone resorption can occur. When OPG is overexpressed, it can bind RANKL and block RANKL interaction with RANK, suppressing osteoclast action (Blair et al., 2007). Moreover, BMP-2 was also influenced by maternal AA treatment in our guinea pig offspring, irrespective of sex. Decreased BMP-2 expression impaired endochondral bone formation, the process which is responsible for skeleton development (Deng et al., 2018). For this reason, bone development in our offspring was disturbed during the prenatal period, decreasing bone metabolism and resulting in inhibited general growth and insufficient bone storage later in life, which in turn leads to earlier development of osteopenia or osteoporosis in these guinea pigs compared to control animals. On the other hand, an increased expression of BMP-2 in the matrix of articular cartilage in males after prenatal AA exposure was observed, which might indicate an intensive repair process. The reason why this was only observed in males needs further investigation.

BMPs signal is important for early bone and cartilage development, as well as in the maintenance of adult bone homeostasis and repairing processes (Deng et al., 2018; Wang et al., 2014). The most commonly studied osteogenic BMPs, based on their potent bone-inducing properties, include BMP 2, 4, 6, 7 and 9. BMP-2 has been shown to have the most endochondral bone activity. This protein plays an important role in endochondral bone development through its influence on chondrocyte proliferation and maturation. BMP-2 deficient mice have skeletal malformations due to disturbances in complex events in the growth plate cartilage, including proliferation, hypertrophic differentiation, and apoptosis (Lawson et al., 1999). The hypertrophic zone is especially important, since the chondrocytes in this part are invaded by blood vessels, osteoblasts, and osteoclasts in order to initiate the ossification process and build new cartilage matrix (Pan et al., 2008; Zehentner et al., 1999).

As mentioned above, when blood vessels reach the growth plate cartilage, bone cells like osteoblasts or another cell line, e.g. blood cells, can produce sufficient quantity of growth factors acting by paracrine or autocrine manner. This factor is vascular endothelial growth factor (VEGF), which is responsible for vasculogenesis and angiogenesis and is released under mechanical action on bone tissue. VEGF stimulates bone mineralization and osteoblast differentiation, and it is mainly released in the hypertrophic zone (Hu and Olsen, 2016).

In the guinea pigs in the current study, besides the reduction in total thickness of the growth plate cartilage and in the thickness of each zone, VEGF expression was also reduced. This was probably caused by increased oxidative stress after maternal AA treatment according to studies described by Bai et al. (2005), Basu et al. (2001), and Garrett et al. (1990). Raju et al. (2015) attributed an increase in oxidative stress to reduced locomotor activity. Decreased expression of VEGF resulted in impaired cartilage and bone nutrition, and inhibited bone development in the guinea pig offspring that were exposed to AA prenatally.Irrespective of sex, guinea pigs in the
current study also exhibited reduced TIMP-2 expression, a protein which is important for the maintenance of the balance between extracellular matrix deposition and degradation, as well as angiogenesis.

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors that block the extracellular matrix-degrading activity of matrix metalloproteinases. The family of TIMPs includes four members (1–4) that differ in their properties, such as expression patterns, regulation and ability to interact specifically with latent matrix metalloproteinases (Gomez et al., 1997). They play a key role in the maintenance of the balance between extracellular matrix deposition and degradation in different physiological and pathological processes (Deng et al., 2008).

Lastly, several cartilage-specific proteins act as adaptor proteins that connect collagen and proteoglycan networks. Among them is cartilage oligomeric matrix protein (COMP), a non-collagenous extracellular matrix protein, which is a marker of cartilage turnover. Its expression depends on the intensity of loading because chondrocytes have the ability to alter their metabolism in the response to mechanical loading. COMP production is important for skeletal growth, maturation, and the maintenance of cartilage homeostasis (Posey et al., 2017). Furthermore, articular cartilage shows topographical variations in morphology, matrix composition, and mechanical properties within an individual joint (Hamann et al., 2014).

Prenatal AA exposure affected not only the thickness of the articular cartilage in our guinea pigs, but it also increased COMP expression. Although Wong et al. (1999) have shown that COMP expression can be increased in the cartilage matrix after mechanical loading, we only observed an increase in COMP expression in the group of guinea pigs exposed to AA prenatally (Wong et al., 1999). The increase in COMP expression was mainly visible in the superficial zone, which is reflective of the role of this zone. The superficial zone reacts first to the mechanical load, disperses the force acting on the joint, and is influenced by the factors from synovial fluid. On the other hand, we had no increases in mechanical load and probably a higher physical activity level. Thus, COMP expression increased in the articular cartilage and bone in response to the AA exposure during the prenatal period, after which the composition of synovial fluid could be changed. It should be checked in other study. Moreover in our study, a decrease in glycoprotein content in the articular cartilage with a reduction in its thickness was observed. Each articular cartilage zone plays a specific role. For this reason, a reduction or change in cell metabolism, expressed as a change in collagen network or proteoglycan content, can result in increased risk of degradation, disability, pain, and reduced life quality as the physical properties of the articular cartilage (viscosity and elasticity) are determined by the diversity of the various components of the matrix. The degradation of proteoglycans can result in destabilization of the collagen network and influence the distribution of the load through the joint causing difficulties in movement.

In conclusion, the present study demonstrated that prenatal AA exposure induced a variety of bone impairments in a rodent model. Increased osteoclast differentiation was observed through the change in RANKL/OPG ratio, which in turn inhibited osteoblast function by decreasing the expression of other proteins like VEGF, BMP-2, and TIMP-2, with a simultaneous increase in COMP expression. Prenatal exposure
to AA, besides all the changes in bone cell metabolism, resulted in reduced thickness of growth plate cartilage leading to the loss of bone tissue, according to the histomorphometrical analysis of trabeculae. Data from the present study suggests that maternal AA exposure can influence the formation/differentiation of bone cells, therefore increasing the ability of osteoclasts to resorb bone, resulting in insufficient bone gain and even bone loss after the birth.

Conflict of Interests Statement
The authors declare that they have no conflict of interests regarding the publication of this article.

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