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Original Article

Intrauterine growth restriction affects bone mineral density of the mandible and the condyle in growing rats

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Abstract

Objectives: To investigate in growing rats the effect of intrauterine growth restriction (IUGR) on the bone mineral density of the mandible and tibia, as well as the quality of the mandibular and condylar bone. **Methods**: Twelve male rats were born IUGR by mothers sustaining 50% food restriction during pregnancy. Twelve control male rats were born by mothers fed *ad libitum*. Dual-energy X-ray absorptiometry (DEXA) of the tibia, proximal tibial metaphysis and the mandible, biochemical markers, histology and histomorphometrical analysis on the mandibular and subchondral bone of the condyle were performed. **Results**: IUGR significantly affected bone mineral density (BMD) of both tibial and mandibular bones. IUGR rats had significantly lower osteocalcin values (p=0.021) and phosphorus (p=0.028), but not 25-OH vitamin D (p=0.352). Bone area percentage in the mandible was significantly lower (51.21±5.54) in IUGR compared to controls (66.00±15.49), and for subchondral bone of the condyle for IUGR (47.01±6.82) compared to controls (68.27±13.37). IUGR had a significant reduction in the fibrous layer, but not the proliferating layer, with the hypertrophic layer significantly increased. **Conclusion**: Maternal restricted nutrition during gestation can affect BMD of the mandible and the tibia of the offspring animals.

Keywords: Bone Mineral Density, Condyle, IUGR, Mandible, Osteoporosis

Introduction

Physical growth and metabolism of an individual is regulated by the interplay between environmental and genetic factors. Epidemiological and experimental studies show that early-life events during gestation or lactation may play an important role in the development of disease in adult life[1-3]. Poor growth *in utero* may cause metabolic syndrome in adult life[2], defined by clustering of at least three of the following five medical conditions: abdominal obesity; high blood pressure; high blood sugar; high serum triglycerides; and low serum high-density lipoprotein (HDL). Metabolic syndrome is associated with the risk of developing cardiovascular disease that may be ischemic heart disease, arterial hypertension, lipid and glucose metabolism abnormalities, as well as type 2 diabetes[3-5]. Metabolic syndrome has been associated with intrauterine growth restriction (IUGR) that may also cause increased prenatal and postnatal morbidity and mortality, as well as a variety of maternal-fetal pathological conditions[3]. In addition, IUGR newborns of humans and experimental animals have low birth weight/poor growth from infancy to adolescence that is considered as a risk factor for bone diseases, such as osteoporosis[6-9]. IUGR modifies postnatal bone metabolism and skeletal growth, and it is associated with low bone mass in infancy, reduced bone mass and density in adulthood and increased risk for osteoporosis in adult life, suggesting that the lack of nutrients early in life may compromise the adult skeleton[10-11].

The craniofacial skeleton is critically affected by malnutrition[12-13]. In contrast to the neurocranium that grows

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with the expansion of the brain, growth of the viscerocranium is dependent on muscular loading exerted by feeding and breathing. Therefore, the viscerocranium appears to be more affected by epigenetic factors associated with feeding than the neurocranium[14-15]. However, there is lack of information on the impact of IUGR on the quality of mandibular bone.

The aim of the present study was to investigate the effect of IUGR caused by maternal food restriction on the tibia of growing and adult offspring rats, and mandible of adult offspring rats.

Materials and methods

The protocol of the present study was approved by the Athens Prefecture, Directorate of Veterinary Services (Project License No. 1061/19-03-2015) and the Ethical Committee of the Laboratory for Research of the Musculoskeletal System "Theodoros Garofalidis", School of Medicine, University of Athens, KAT Hospital, Athens, Greece (Establishment License No. EL 25 BIO 018). The guidelines of the Animal Research Committee, Ethical Committee and legislation of the Greek State and European Union (Directive 2010/63/EU) "on the protection of animals used for experimental purposes" were followed throughout the experiment. Every effort was undertaken to minimize pain or discomfort of the animals.

Six first-time pregnant Wistar rats at day 11 of gestation ("mothers") were purchased from the Hellenic Pasteur Institute, and the experiment was conducted at the Laboratory for Research of the Musculoskeletal System "Theodoros Garofalidis". The mothers were housed individually in cages measuring 65x47x35 cm and had free access to water. The room was maintained at constant temperature of 22°C, constant relative humidity of 65%, and controlled light cycle, consisting of 12 h light and 12 h darkness. The mothers were kept free of pathogens and treated in compliance with standardized institutional guidelines. All mothers were fed a standard laboratory rat food (4RF21, Mucedola, Milan, containing 18.5% protein, 53.5% carbohydrates and 3% fats, metabolizable energy 315.5 kcal/100 g).

For the experiment a model of rats that were either fed *ad libitum* or underwent 50% food restriction during pregnancy was used[16]. Three randomly selected mothers underwent 50% food restriction diet from day 12 of gestation until the end of pregnancy, at day 21. The amount of food intake was determined by calculating that the normal intake of the *ad libitum* fed pregnant rats was 40 g/day. Therefore, mothers of the 50% food restriction group were fed with 20 g/day. At birth, the offspring were culled to 8 (4 males and 4 females) per litter to normalize rearing.

Two offspring groups were assembled: Group A-IUGR, composed of 12 male rats born from mothers that underwent 50% food restriction, and Group B-control, composed of 12 male rats born from mothers that were fed *ad libitum*. Both offspring groups and all mothers after gestation were fed *ad libitum*. During the post lactation period from day 21 a standard diet was available *ad libitum* to all offspring. The experiment ended when the offspring reached day 150, they were sacrificed after being deeply anesthetized with injection of ketamine 100 mg/kg and dexmedetomidine 50 mg/kg.

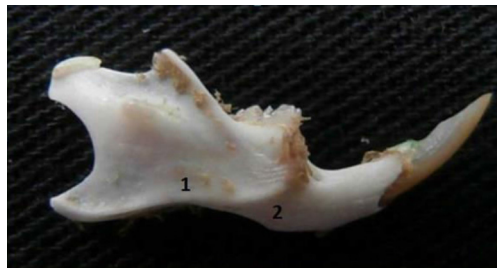

Figure 1. Area 1 above the antegonial notch and area 2 above Menton, the right hemi-mandible.

The rats were weighed on a digital precision scale immediately after birth, and at 30, 60 and 150 days.

DEXA

Bone mineral density (BMD) quantifies bone content per unit volume and was examined with the Dual-energy X-ray absorptiometry (DEXA) that is currently the gold standard in clinical practice. However, DEXA results represent areal BMD, that is, bone mineral content (BMC) per unit area of projected bone[17].

In our study we used the Advanced Densitometer (Lunar Prodigy, GE Healthcare, Diegem, Belgium) and applied the small-animal mode of the encore software (GE Healthcare, v. 13.40, Diegem, Belgium). The instrument was calibrated at each start.

The tibia and the proximal tibial metaphysis were examined at days 60 and 150, and the mandible at day 150, after the sacrifice of the offspring, as the superimposition of the bones of the craniofacial skeleton did not allow reliable measurements when the offspring were alive. For the DEXA of day 60, the offspring were anaesthetized with an intramuscular injection of ketamine hydrochloride and dexmedetomidine, ventrally positioned and scanned. For reversal of anesthesia atipamezole was injected. The same position was applied for day 150, measurements of the tibia and tibial metaphysis, after the sacrifice of the offspring.

For the examination of the mandible, after the sacrifice of the offspring the calvaria were excised using a surgical sawmill, skin and muscles were totally removed, and specimens were dissected and immediately fixed in 10% neutral buffered formalin (Formaldehyde solution, Sigma Aldrich, USA). DEXA was performed on the right hemi-sectioned mandibular specimen by placing it on the plate of the scanner.

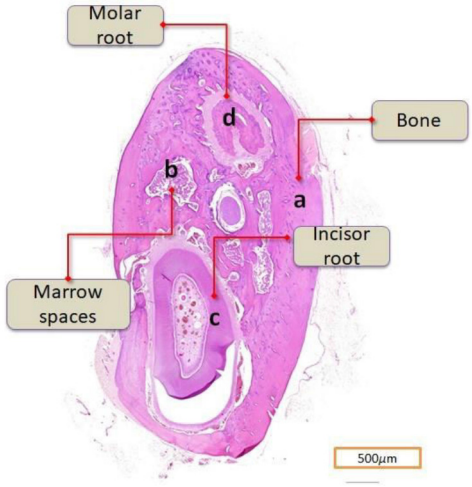

Figure 2. Transverse section of mandibular bone a) bone, b) marrow spaces, c) incisor root, d) molar root (hematoxylin-eosin staining). Scale bar 500 μm.

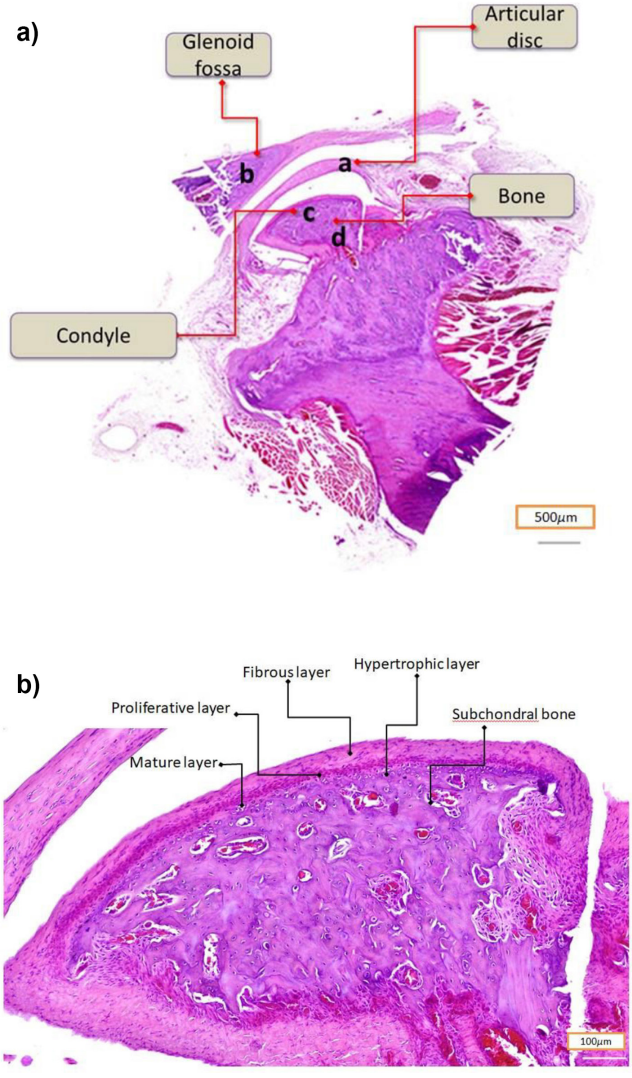

Figure 3. a) Section in the sagittal plane of the condyle. a. Articular disc, b. glenoid fossa, c. condyle, d. bone (hematoxylin-eosin staining). Scale bar 500 μm. **b)** Condylar cartilage layers (fibrous, proliferating and mature chondrocyte layer and the hypertrophic cartilage layer evaluated together) the subchondral bone is also shown (hematoxylin-eosin staining). Scale bar 100 μm.

For the tibia and tibial metaphysis two regions of interest (ROI) were checked, the whole tibia and the proximal tibial metaphysis. The ROI was an area of 2x2 mm. BMD was measured in g/cm^2. For the mandible, DEXA was performed at Area 1, 2 mm above the antegonial notch and the ROI was 2x2 mm; and Area 2, 2 mm above menton (ME), the most inferior point of the mandibular symphysis, and the ROI was 1.8x1.8 mm. Those areas were selected, as they do not have teeth, making BMD measurements reliable (Figure 1).

Biochemical markers

At the time of sacrifice 2-3 ml of blood were collected via the abdominal aorta by using a heparinised syringe with needle size 0.50x16 mm. The sample was centrifuged at 1600 rpm for 15 min, and plasma was stored at -80°C until analyzed. The levels of osteocalcin, 25-OH vitamin D and phosphorus in blood plasma were examined.

Histomorphometrical analysis

After being fixed in 10% neutral buffered formalin for 24 h the right hemi-sectioned mandibular specimen was decalcified in an EDTA-based solution (Micro Dec, Diapath, Italy) for approximately 7 days. For each animal, tissue blocks were prepared including: (a) the mandibular bone from the mesial end of the incisor tooth to the distal end of the first molar root and (b) the condylar process and its surrounding tissues extending approximately ½ cm peripherally to the condyle. The samples were embedded in paraffin following a standard procedure and 5μm thick sections were cut as follows: (a) for the bone specimen, a traverse section perpendicular to the middle of the incisor tooth (Figure 2) and (b) for the condyle, a sagittal section on its maximum anterio-posterior dimension (Figure 3a). Consequently, the sections were stained with a standard hematoxylin-eosin solution.

Histomorphometrical analysis was performed in one tissue section per animal and the investigator was blind to the intervention. The slides were digitized using an OLYMPUS CX 23LEDRFS2 microscope (Olympus Corporation, Tokyo, Japan) mounted with a Digital Camera OLYMPUS U-CMAD3 T7, U-TV1X-2 T7 (Olympus Corporation, Tokyo, Japan). A digital drawing and cropping tool were used to manually segment the ROI in Image Pro-Plus v6.0.0.260 (Media

Table 1. Weights for IUGR and Control pups (mean ± SD).

Body weight (g)	Control	IUGR	p-value
At birth	7.37±0.27	5.04±0.11	**<0.005**
30 days old	125.41±5.03	98.41±4.07	**<0.005**
60 days old	320.83±8.74	273.5±5.53	**<0.005**
150 days old	496±6.60	458.5±14.1	**<0.005**
Independent samples t-test.			

Table 2. Comparison between groups of BMD of tibia and proximal tibial in g/cm^2 during the observation period.

Total tibia	Control	IUGR	Comparison between groups
60 days	0.186±0.005	0.171±0.008	**p<0.005**
150 days	0.274±0.007	0.252±0.015	**p<0.005**
Comparison between time points	**p<0.005**	**p<0.005**	
Comparison between groups regardless of time	0.230±0.004	0.213±0.010	**p<0.005**
Interaction between "time"and "group" p=0.200			
Proximal tibia	**Control**	**IUGR**	**Comparison between groups**
60 days	0.266±0.029	0.237±0.022	**p=0.017**
150 days	0.404±0.021	0.360±0.018	**p<0.005**
Comparison between time points	**p<0.005**	**p<0.005**	
Comparison between groups regardless of time	0.355±0.022	0.298±0.016	**p<0.005**
Interaction between "time"and "group" p=0.200			
All values are presented as mean±SD. bg: between groups, independent samples t-test. bt: between time points, paired samples t-test. All variables are presented as mean±SD.			

Cybernetics, Rockville, MD, USA) software. The ROI of the mandibular bone (mROI) extended from the distal of the periodontal membrane of the incisor tooth to the most mesial point of the mesial root of the first molar tooth[19]. The ROI for the condylar bone (cROI) was defined by a 0.5x0.5 mm square[20] positioned beneath the interface of the cartilage and the subchondral bone and located at the center of the posterior third of the condylar cartilage. The parameters recorded for both ROI were (a) total area, (b) bone area and (c) marrow spaces, the latter two expressed as percentage (%) of the total bone area[21].

The ROI for condylar cartilage (IROI) included the fibrous, proliferative, mature, and hypertrophic layer of the condyle, with the mature and hypertrophic layers examined together[22-23] (Figure 3b). For the measurements of the condylar layers four lines were drawn along the length of the condyle in order to define the three layers (fibrous, proliferative, and hypertrophic cartilage layer). The system automatically calculates perpendicular to each region lines at 1 μm and gives the average thickness of each layer. The thickness for each layer, defined by the lines drawn by the operator, was expressed as a percentage. Finally, the average thickness for each layer was calculated.

Statistical analysis

Data were expressed as mean±SD for quantitative variables and as percentages for qualitative variables. The Kolmogorov-Smirnov test was used for normality analysis of the quantitative variables. Comparison of BMD measurements, biochemical markers and histomorphometrical results between IUGR vs. control group was performed using the independent samples t-test, or the Mann-Whitney test, in case of violation of normality. Comparison of BMD measurements between day 60 vs. 150 for each group separately was performed using the paired samples t-test, or the Wilcoxon test in case of violation of normality. All tests were two-sided, statistical significance was set at p<0.05. All analyses were carried out using the statistical package SPSS 21.00 (IBM Corporation, Somers, NY, USA)

Results

It was observed that even though the offspring number did not differ between the two groups the birth weight was lower for IUGR, compared to control offspring with the difference being statistically significant (p<0.005). Weight measurements which were taken at day 30, 60 and 150 also showed statistically significant differences (Table 1).

Table 3. Comparison between groups of mandibular BMD for Area 1 and Area 2.

	Control	IUGR	p-value
Area 1 (Antegonial notch)	0.290±0.021	0.233±0.031	**<0.005**
Area 2 (Menton)	0.350±0.029	0.308±0.029	**0.003**
All variables are presented as mean±SD, values in (g/cm²). Independent samples t-test.			

Table 4. Comparison between groups of biochemical markers (Osteocalcin, 25-OH vitamin D and Phosphorus).

	Control	IUGR	p-value
Osteocalcin (ng/ml) [1]	207.94±30.38	162.12±50.05	**0.021**
25-OH vitamin D (μg/ml) [1]	1098.07±180.53	1028.62±134.20	0.352
Phosphorus (mg/dL) [2]	27.77±6.01	22.40±3.27	**0.028**
All variables are presented as mean±SD. All variables are presented as mean±SD, [1] Independent samples t-test. [2] Mann-Whitney test.			

Table 5. Comparison of Histomorphometric results of the mandibular bone (mROI) between Control and IUGR group.

	Control	IUGR	p-value
(mROI) Bone Area % [2]	66.00±15.49	51.21±5.54	p=0.007
(mROI) Marrow spaces % [2]	34.01±15.49	48.79±5.54	p=0.007
All variables are presented as mean±SD, [2] Mann-Whitney test.			

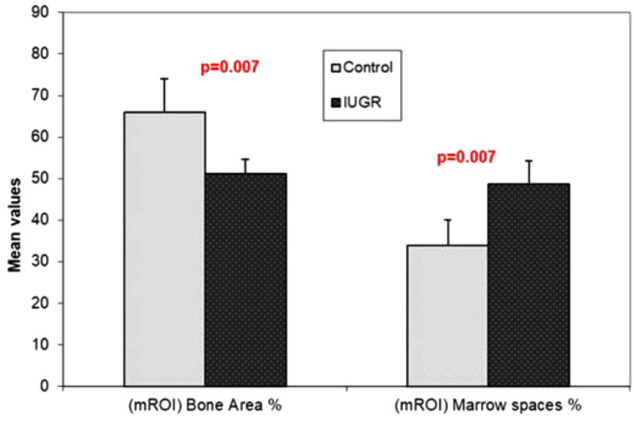

Figure 4. Comparison of Histomorphometrical results of the mandibular bone (mROI) between Control and IUGR group for Bone area and Marrow spaces.

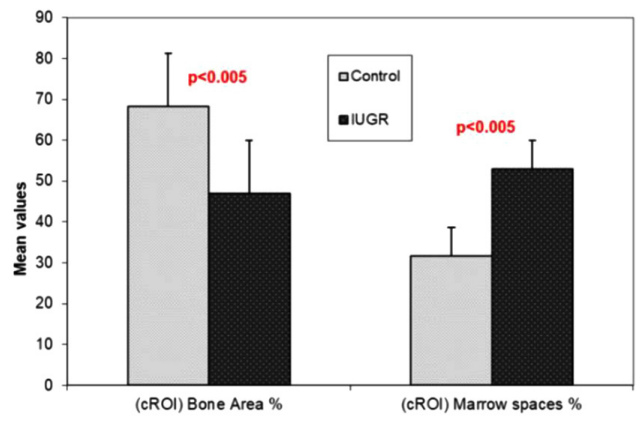

Figure 5. Comparison of Histomorphometrical results of the condylar bone (cROI) between Control and IUGR group for Bone area and Marrow spaces.

DEXA

There was a statistically significant increase from day 60 to 150 of tibial BMD for both the IUGR (0.171±0.008 vs 0.252±0.015, p<0.005) and the control group (0.186±0.005 vs 0.274±0.007, p<0.005). In particular, the IUGR group presented lower values of total tibia at days 60 (p<0.005) and 150 (p<0.005), and regardless of time (p<0.005) (Table 2).

There was a statistically significant increase from day 60 to 150 of proximal tibial metaphysis BMD for both the IUGR (0.237±0.022 vs 0.360±0.018, p<0.005) and the control group (0.266±0.0029 vs 0.404±0.021, p<0.005) (Table 2). The IUGR group presented lower values of proximal tibia at

Table 6. Comparison of Histomorphometric results of the condylar bone (cROI) between Control and IUGR group.

	Control	IUGR	p-value
(cROI) Bone Area %	68.27±13.37	47.00±6.82	p<0.005
(cROI) Marrow spaces %	31.73±13.37	53.00±6.82	p<0.005
All variables are presented as mean±SD. Independent samples t-test.			

Table 7. Comparison of Histomorphometric results for thickness of the condylar cartilage layers between Control and IUGR group.

Condylar cartilage layers	Control	IUGR	p-value
Fibrous layer%	26.21±7.79	20.48±3.45	**0.036**
Proliferative layer%	30.05±6.39	29.40±5.49	0.802
Hypertrophic layer%	43.74±7.93	50.12±6.31	0.053
All variables are presented as mean±SD. Independent samples t-test.			

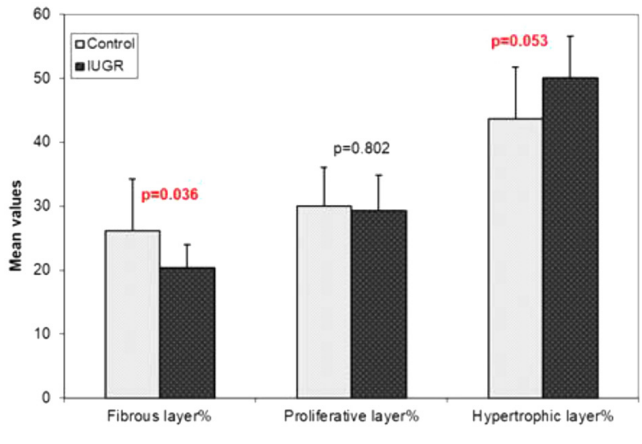

Figure 6. Comparison of Histomorphometrical results for thickness of the condylar cartilage layers between Control and IUGR group.

days 60 (p=0.017) and 150 (p<0.005), and regardless of time (p<0.005) (Table 2).

BMD of mandibular Area 1 at day 150 was 0.233±0.031 and 0.290±0.021 for IUGR and controls, respectively, revealing a statistically significant difference (p<0.0005). At Area 2, it was 0.308±0.029 and 0.350±0.029 for IUGR and controls, respectively, revealing statistically a significant difference (p=0.003) (Table 3).

Biochemical markers

The IUGR group compared to the control group presented at day 150 statistically significantly lower values for osteocalcin (162.12±50.05 ng/ml vs 207.94±30.38 ng/ml, p=0.021) and phosphorus (22.40±3.27 ng/ml vs 27.77±6.01 ng/ml, p=0.028), but not for 25-OH vitamin D (1028.62±134.20 ng/ml vs 1098.07±180.53 ng/ml, p=0.352) (Table 4).

Histomorphometrical analysis

Histomorphometrical analysis of the specimens revealed that the percentage of bone in mROI for the IUGRs was 51.21±5.54 vs 66.00±15.49 for the controls (p=0.007) (Table 5, Figure 4). The percentage of bone cROI for the IUGRs was 47.01±6.82 vs 68.27±13.37 for the controls, revealing statistically significant difference (p<0.005) (Table 6, Figure 5).

In the IUGR group, the thickness of the fibrous layer of the condylar cartilage was lower than in the control group, the difference being statistically significant (20.48±3.45 vs 26.21±7.79, p=0.036), for the hypertrophic layer higher, the difference being of marginal statistical significance (50.12±6.31 vs 43.74±7.93, p=0.053), while no difference was found for the for proliferative layer (29.40±5.49 vs 30.05±6.39, p=0.802) (Table 7, Figure 6).

Discussion

The present study showed that IUGR caused by maternal food restriction during gestation had a negative effect on the tibia of growing and adult offspring rats, and the mandible of adult offspring rats. Wistar rats are an appropriate animal model for studying the human skeletal system and IUGR caused by nutritional deficiencies[24-27]. *Ad libitum* feeding of the offspring after birth and till the end of the experiment was necessary as a recovery period. The length of the experiment was set to 150 days, since between days 100 and 200 the differences in bone growth are small and the growth of the rats maxillofacial skeleton ceases after day 150[28]. Finally, the dietary model of 50% food restriction of the *ad libitum* diet on day 12 of gestation has a great

effect on birth weight, without affecting litter size[29-33], as was also documented in our study. Body weight, of the IUGR offspring rats, was statistical significant lower than it was for control rats, from birth till the end of the experiment. It is interesting to note that the difference in weights at 30, 60 and 150 days is more or less constant (around 40 g), therefore the percentage of weight difference decreases as the rats grow. This indicates that nutrition promotes some catch-up growth in IUGR rats.This comes in agreement with the studies of Guimarey et al.[34-35]. In our study findings from DEXA and Histomorphometry identified that BMD is strictly affected by body weight which is also supported by Rexhepi et al.[36].

IUGR modifies postnatal bone metabolism and skeletal growth. Mehta et al.[37] in an animal study showed that maternal protein restriction reduced bone area and bone mineral content (BMC), but not BMD as measured by DEXA on the tibia, in late adulthood offspring. Romano et al.[17] found that IUGR caused by reduced maternal fetal blood flow during the last trimester of pregnancy resulted in a decrease in BMD in adult male rats, despite the restoration of nutrition immediately after birth. Femur length, dimensions, strength, mineral content, and bone density were measured by DEXA and peripheral quantitative computed tomography (pQCT) analysis[17]. Engelbregt et al.[38] suggested that food restriction in 6-month old male rats resulted in significantly lower total body bone mineral content, compared with controls. Chen et al.[39] concluded that IUGR decreases endochondral ossification responsiveness and in turn, postnatal linear skeletal growth, and strength in female offspring rats at days 21 and 120. They measured BMD with DEXA, and bone strength by three-point bending . Kimura et al.[40] supported that that maternal undernutrition during gestation alters the function of chondrocytes, resulting in reduced postnatal tibial growth in female offspring. In the present study IUGR male offspring rats had lower BMD at the tibia and the proximal tibial metaphysis at days 60 and 150 days compared to controls, even though a standard diet was available *ad libitum* to the offspring animals. Pillai et al.[41] could not confirm an association between maternal diet and mineral content or BMD and bone area in offspring but demonstrated decreased mesenchymal stem cell proliferation. Syggelos et al.[42] evaluated the impact of prenatal food restriction on the skeletal characteristics of 1-year old Wistar rats. They tested the tibia with pQCT and concluded that food restricted rats had lower values of total bone density and total/subcortical area compared to control fed.

In our study, DEXA showed a significant impact of IUGR on BMD both on the tibia and mandible. The mandibular BMD in IUGR offspring rats has not previously reported in the literature.

Osteocalcin is a bone protein synthesized only by osteoblasts and released in the serum during protein synthesis, therefore, it is considered a sensitive marker of the function of osteoblasts, compared to alkaline phosphatase that is produced by tissues other than bone[43-44]. Namgung et al.[44] found in Small Gestational Aged (SGA) neonates born smaller in size for gestational age, where osteocalcin values were lower compared to the corresponding according gestational age (AGA) animals. Reduced osteocalcin values at day 150 were also found by Lanham et al.[45] who studied intrauterine restriction by administering a low-protein diet in pregnant rats. In the present study the plasma concentration of osteocalcin in IUGR offspring rats was lower than in the control group, indicating that IUGR rats produced less bone formation and confirming DEXA results.

Vitamin D has a hormonal action and contributes to the homeostasis of calcium that promotes bone calcification. It is released in the liver, where it is hydroxylated to 25 hydroxy-vitamin D[46]. Minton et al.[47] and Namgung et al.[44] found in SGA infants smaller in size for gestational age that although they had reduced bone density compared to AGA, there was no difference in the concentrations of 25-hydroxy-vitamin D. Similar conclusions were reached by Lanham et al.[45] in IUGR rats following a low protein diet in pregnant rats, and Arden et al.[48] in an epidemiological study assessing whether birthweight and body weight at 1 year of age were associated with the calcium vitamin D axis. In line with all those studies, in the present one vitamin D was lower in IUGR compared to control rats, but not statistically significantly.

Plasma phosphorus levels were investigated as a marker of bone metabolism. Namgung et al.[44] found lower phosphorus values in neonates smaller in size for gestational age, but without statistical significance. In our study, phosphorus levels in the plasma of IUGR rats were lower and statistically significantly different from the levels of phosphorus in the control rats. Overall, lower levels of osteocalcin, vitamin D and phosphorus values in IUGR rats indicate lower bone metabolism, compared to normal rats, that could interpret the differences in bone maturation, shown by BMD.

Histomorphometrical measurements have not been previously performed on the maxillofacial skeleton of IUGR rats. Microstructural properties are well depicted with histomorphometry however cortical parameters can sometimes be underestimated by micro CT[49]. In the present study it was found that at day 150 in IUGR rats the percentage of bone in the mandible and subchondral bone in the condylar process was significantly lower, compared to controls. As the subchondral bone provides a structural support to the overlying articular cartilage that functions as a cushion during joint loading[50], the layers of the condyle were further evaluated.

Cavalli et al.[50] studied IUGR offspring rats due to maternal reduced protein supply, which followed a normal protein diet since day 100 after weaning. They found in IUGR rats reduced thickness of the fibrous and hypertrophic layers, compared to control, but no difference in the proliferative layer. In this study certain areas of the condyles were evaluated.

In contrast, in the present study where the full thickness of each layer was calculated by histomorphometry, analysis revealed that IUGR offspring in comparison to controls showed a statistically significant reduction in the thickness of the fibrous layer and a potential increase in the thickness of the hypertrophic layer, but not difference in the proliferative

layer. Therefore, IUGR does not seem to affect cellular proliferation in the condyle, but there is an adaptation of the condyle to the imposed nutritional conditions and the tissue preserves its organization to maintain tissue growth[51].

Conclusions

Our results provide original information regarding the quality of the tibial and mandibular bone in growing rats and indicate that maternal restricted nutrition during gestation can affect those bones. Normalization of the diet during the post lactation period in the IUGR offspring rats did not result in recovery of BMD. Differences in levels of osteocalcin, 25-OH vitamin D and phosphorus in blood plasma could be associated with these findings.

The fact that in the present study 4 male rat pups from the same mother were used might pose a limitation regarding genetic similarities affecting the results. Further analysis could consider cross-fostering the litters to investigate any possible differences.

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