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Changes in nutrition type between generations influence on bone structural changes in rat female offspring

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Abbreviations:

CD	– control diet
HFD	– high content of saturated fatty acid food
CD-CD	– control diet mothers and offspring
CD-HFD	– control diet mothers and high fat diet offspring
HFD-CD	– high fat diet mothers and control diet offspring
HFD-HFD	– high fat diet mothers and offspring
BV/TV	– trabecular bone volume
BS/TV	– trabecular bone surface density
Tb.Th	– trabecular thickness
Tb.N	– trabecular number
Tb.Sp	– trabecular separation
Ct.Th	– cortical bone thickness
CV	– cortical bone volume

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Abstract

Background and Purpose: *It is believed that changes in feeding protocol between generations have influence on the balance of the whole organism. Epidemiological studies suggest that skeletal growth is programmed during intrauterine and early postnatal life. The aim of the study was to determinate impact of maternal dietary fat excess and nutrition of female offspring on the bone structural changes in latter.*

Materials and Methods: *Ten female Sprague Dawley rats were randomly divided in two groups. One group was fed with high content of saturated fatty acid food (HFD) and the other with standard laboratory chow (CD). Offspring from both groups were randomly divided in two subgroups after coupling and lactation period, subsequently there were four groups of offspring (n=6 each) with different feeding protocol: a) CD-CD – control diet mothers and offspring, b) CD-HFD – control diet mothers and high fat diet offspring, c) HFD-CD – high fat diet mothers and control diet offspring and d) HFD-HFD – high fat diet mothers and offspring. At the age of 18 weeks in female offspring bone microstructure was analyzed in fifth lumbar vertebra using digital photographic images.*

Results and Conclusions: *The control diet female offspring of high fat fed mothers showed the highest values of trabecular thickness and trabecular number, while the CD-HFD offspring group had the highest values of trabecular separation and cortical thickness. Maternal nutritional status affects the future development of offspring.*

INTRODUCTION

The incidence of obesity and overweight almost double in Western societies and the trend is mirrored in developing nations. Disturbances in the metabolism of adipose tissue are the basis of the pathogenesis of many diseases *(1)*. Furthmore, it is widely accepted that the likelihood of offspring developing heart disease, stroke, diabetes or osteoporosis in later life, is influenced by their in utero environment and maternal nutrition. It is now well accepted that low-grade chronic systemic inflammation, also called sterile inflammation, is associated with high calorie diet in the absence of any systemic or local infection and that inflammation contributes to risk of insulin resistance and related disorders *(2)*. Numerous studies have shown elevated concentration of adipose tissue derived cytokines in obese humans, suggesting a concept that inflammation may be derived from the accumulation of activated macrophages surrounding enlarged adipocytes in obese subjects *(3)*, especially in visceral fat depots *(4)*.

Epidemiological and experimental evidence has shown that maternal malnutrition or overnutrition in critical periods of life (gestation and lactation) may cause several hormonal changes and programme obesity development in the adult offspring in rodent models *(5)*. Therefore offspring are extremely sensitive to nutritional, hormonal and environmental changes during the gestation and lactation periods, which may result in altered physiology of several systems throughout life. This phenomenon is named „programming", and its origins are related to epigenetic modifications which is usually explained as post-translational modification of the nucleic acids of offspring without changing the genetic sequence which are called epigenetic changes *(6)*. Therefore maternal nutrition has been shown to be very important in this hypothesis, with disease occurring when there is a mismatch between maternal and offspring diet.

There are many potential factors that could affect the balance between metabolism, fat, and bone. Adipose tissue produces adipokines, molecules that not only regulate food intake and energy metabolism, but are also involved in the complex interactions between fat tissue and bone *(7)*. Leptin is an important factor in regulation of bone remodeling. Thomas et. Al. *(8)* reported that leptin promote osteoblastogenesis and inhibits adipogenesis in human marrow stromal cells. The GH–insulin-like growth factor 1 (IGF1) via neuroendocrine axis stimulate proliferation of chondrocytes *(9)*. Higher levels of estrogen, producing by adipocytes, supress oceteoclastic bone resorption and stimulate osteoblastic bone formation *(10)*. Leptin is a particularly strong candidate mediator given its multiple roles in energetic, reproductive, and skeletal homeostasis. Moreover, maternal high fat diet is known to induce perinatal programming of leptin sensitivity in the ventromedial hypothalamus *(11)* which has the potential to alter the balance between cortical and trabecular bone mass. Furthermore, osteoblasts and adipocytes derive from a common mesenchymal stem cell progenitor, such that increased differentiation of one cell type might decrease differentiation of the other cell type *(11)*. Bone quantity in terms of bone mineral content and density has been observed to be lower in excised lumbar vertebrae as a result of high fat feeding *(12)*. Lanham et. al examined the bone development in a mouse model of high fat intake *(13)*. Their data demonstrated that excess nutrients during pregnancy can affect the bones of the offspring, by inducing excess deposition or storage of nutrients which can lead to permanent alterations of bone development and structure. Given that maternal diet appears to be able to programme bone growth in the offspring independently of offspring DNA sequence, manifestly an epigenetic mechanism would appear an important area for investigation. This confirms convincing evidence across mammalian species that skeletal growth is programmed during intrauterine and early postnatal life *(13)*.

Osteoporosis is a skeletal disorder characterized by low bone mass and micro-architectural deterioration, producing enhanced bone fragility and increasing susceptibility to fracture *(14)*. Epidemiological studies indicate that impaired growth during fetal life, infancy and early childhood is associated with reduced bone mass in the adult *(15)*. Quantitative analysis of trabecular bone structure and relationships between structural parameters of bones and bone strength are important topics of research in the area of osteoporosis *(16)*. It is also known that the amount of bone which is expressed through the percentage of trabecular tissue and referred to as trabecular bone volume, differs between patients with vertebral compression fractures and healthy subjects of the same age, being higher in healthy subjects *(17)*. Interestingly, bone is constantly remodeled throughout life *(18)* and, in particular, maternal nutrition appears to be important in determining skeletal size at maturity.

Additionally, we investigated whether a type of food consumption by the dams has differing effects compared to quality of food consummated by offspring and is there a potential link between these types of feeding and bone microstructure in female offspring. The aim of the study was to investigate the influence of obesity on bone microstructure and remodeling without the effect of mechanical loading, and therefore chose lumbar vertebra.

MATERIALS AND METHODS

Experimental design

Ten virgin female Sprague Dawley rats (Rattus Norvegicus) were randomly divided in two groups at the age of 9 weeks. One group was fed during 6 weeks period with standard laboratory chow (Mucedolla, Italy) (CD group; n=5) and the other one with high content of saturated fatty acid food (Žito d.o.o., Croatia) (HFD group; n=5) during the same period, see **Table 1**. At the age of 15 weeks, these female rats were mated by the same male rat in order to obtain similar paternal genetic background of the offspring and to allow independent study of the effects of intrauterine and postnatal nutritional environment. Following 3 weeks of coupling and further 3 weeks of lactation period their female litter was randomly divided into 4 subgroups (each n=6) with different feeding protocol: a) CD-CD – control diet mothers and offspring, b) CD-HFD – control diet mothers and high fat diet offspring, c) HFD-CD – high fat diet mothers and control diet offspring and d) HFD-HFD – high fat diet mothers and offspring. Rats were housed in separated cages, in a controlled temperature room (22 °C), with 12h light – 12h dark cycle, lights on at 6.00 a.m. Standard laboratory chow (CD) and tap water were available ad libidum. High content of saturated fatty acid food (HFD) group was given twice a day (at 9 a.m. and 4 p.m.). Experiments were approved by the Ethics Committee of the Faculty of Medicine University of Osijek and carried out according to Croatian 'Law for the Care and Use of Animals for Scientific Purposes'.

TABLE 1
Food content of CD and HFD diet used in the experiment.

	CD diet		HFD diet
%	< 14th week of life	> 14th week of life	
Cereals	53,7	66,5	30
Animal protein	4,7	3,5	17,2
Vegetable protein (soybean meal and yeast)	30,5	18,2	10
Fats (soy oil)	1,4	0,4	28§
Vitaminic and mineal mixture	4,1	3,2	3,9
Forage	3,0	7,5	9,7
Amino-acids	0,1	0,1	0,1

Food content: CD diet (control diet) and HFD diet (high fat diet); §fats in the form of palm oil

Tissue preparation and histopathologic examination

Fifth lumbar vertebral (L5) bodies (n=24) were isolated and soft tissue was carefully removed with a scalpel. Bone samples from male rats were fixed in 4% buffered paraformaldehyde and decalcified in osteodec (Bio-optica, Milano s.p.a., Italy). Six consecutive sections (5 μm thick) were cut using a Leica RM550 circular microtome (Leica, Vienna, Austria). The sections were stained with

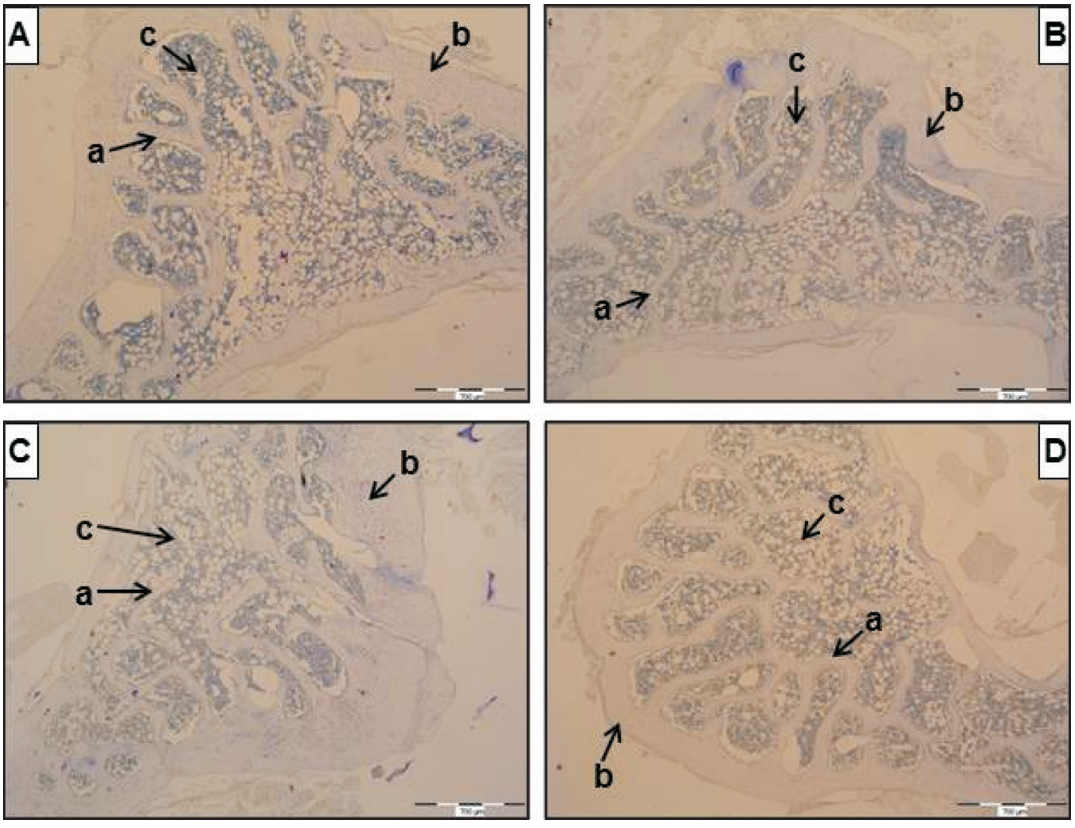


Figure 1. *Histological sections of 5th lumbar vertebra from four groups of female rat offspring.* **(A)** *CD-CD;* **(B)** *CD-HFD;* **(C)** *HFD-CD;* **(D)** *HFD-HFD. In CD-HFD group, trabecular bone is thin, and trabecular separation most pronounced, while in HFD-CD group, trabecular bone is thicker, and trabecular separation much less pronounced. Cortical bone is thinner in CD-CD than in CD-HFD group.* **a)** *trabecular bone;* **b)** *cortical bone;* **c)** *bone marrow. Staining: toluidine blue, magnification: 400x.*

TABLE 2
Structural data from 5th lumbal vertebra of offspring.

	CD-CD	CD-HFD	HFD-CD	HFD-HFD
BV/TV	17,66	15,59	24,57	20,10
BS/TV	2,08	2,76	2,20	2,01
Tb.Th	168,25	164,16	176,60	166,28
Tb.N	2,06	1,46	2,87	1,89
Tb.Sp	485,81	741,38	454,45	571,37
C.Th	1868,63	3456,59	2464,96	2135,70
CV	77,66	52,26	53,90	35,10

All values shown as median. Statistical significant results between groups are noted on graphs on Figure 2. ***BV/TV*** *= trabecular bone volume;* ***BS/TV*** *= trabecular bone surface density (%);* ***Tb.Th*** *= trabecular thickness (μm);* ***Tb.N*** *= trabecular number (/mm);* ***Tb.Sp*** *= trabecular separation (μm);* ***Ct.Th*** *= cortical bone thickness (μm);* ***CV*** *= cortical bone* volume (%).

toluidine blue, and slides were examined using Olympus® BX50 microscope, and photographs were taken with Olympus® C-5050 digital camera. QuickPHOTO PRO imaging software (Promicra s.r.o., Prague, Czech Republic) was used.

Bone histomorphometry

The system for microscopy of the bone sections is consisted of Olympus BHA microscope (Olympus, Tokyo, Japan) and Pulnix digital camera (Pulmix, Yokohama, Japan) connected to the personal computer. Digital images were captured under 40X magnification and stored until the measurements were performed. For histomorphometric analysis a semiautomatic image analysis system, equipped with Issa software (VAMS, Zagreb, Croatia) was used. According to the American Society of Bone and Mineral Research, trabecular bone volume (BV/TV, in %) corresponds to the amount of trabecular bone within the spongy space. It is derived from 2D measurements of bone area (B.Ar) and trabecular tissue area (T.Ar) using Parfitt's formula, as follows: BV/TV = 100 B.Ar/ T.Ar. Trabecular bone surface (BS/TV, in mm) was then calulated from the values of trabecular bone perimeter (B.Pm) and trabecular bone area (B.Ar): BS/TV= B.Pm/B.Ar. In addition, three more values were calculated to evaluate the architecture of trabecular bone: Trabecular thickness (Tb.Th, in mm) was derived from trabecular perimeter (B.Pm) and bone area (B.Ar) according to the Parfitt's formula: Tb.Th=(B.Ar/B.Pm) (π/2); Trabecular number (Tb.N, in mm) was derived from trabecular perimeter (B.Pm) and total tissue area (T.Ar) according to formula18: Tb.N=(B.Pm/T.Ar) * 10; Trabecular separation (Tb.Sp, in mm) was derived according to the formula18: Tb.Sp=(1000 * T.Ar–B.Ar)/B.Pm Cortical parameters included cortical thickness (Ct.Th, in mm) and cortical bone volume (CV, in %). They were calculated according to the following formulas: Ct.Th =2 Pm * p/4 (where Pm is a cortical bone perimeter), and: CV= (C.Ar/TV) * 100 (where C.Ar is cortical bone area and TV is total tissue area).

Statistical analysis

Data analysis was performed using SAS software (version 8.02, Cary, NC, USA). Variables were reported as median with interquartile range (Me [Q1-Q3]). Considering small sample size per group (because of the law limitations of animals use in scientific purposes), between – groups analysis was tested with non-parametric tests. Mann-Whitney test was used for testing two independent groups, which was extended with Kruskal-Wallis test for comparing three or more samples that are independent. Differences were declared to be statistically significant at $P < 0.05$.

RESULTS

Bone Histomorphometry

Mean trabecular bone volume was the highest in HFD-CD group (24,57) and the lowest in CD-HFD group (15,59) (**Table 2**). The proportion of trabecular bone volume (BV/TV) showed significant variation when comparing CD-HFD group with HFD-CD (P = 0,04) and HFD-HFD (P = 0,006) groups (**Figure 2**).

Trabecular bone surface (BS/TV) showed significant variation when comparing CD-CD group with CD-HFD group (P = 0,028), being higher in later one (2,76) (**Figure 2**). It was the lowest in HFD-HFD group (2,01).

Trabecular thickness (Tb.Th) showed statistically significant variation between CD-HFD and HFD-CD group (P = 0,009), being the highest in HFD-CD group (176,60). Trabecular number (Tb.N) showed significant variations comparing CD-CD and CD-HFD group (P = 0,016), and CD-HFD with groups HFD-CD and

HFD-HFD (**Figure 2**). Largest trabecular number was encountered in HFD-CD group (2,87), and the lowest in CD-HFD group (1,46) (**Table 2**). Trabecular separation (Tb.Sp) showed significant variations comparing CD-CD and HFD-HFD group (P = 0,045), and CD-HFD (741,38) with groups CD-CD and HFD-CD (454,45) (**Figure 2**).

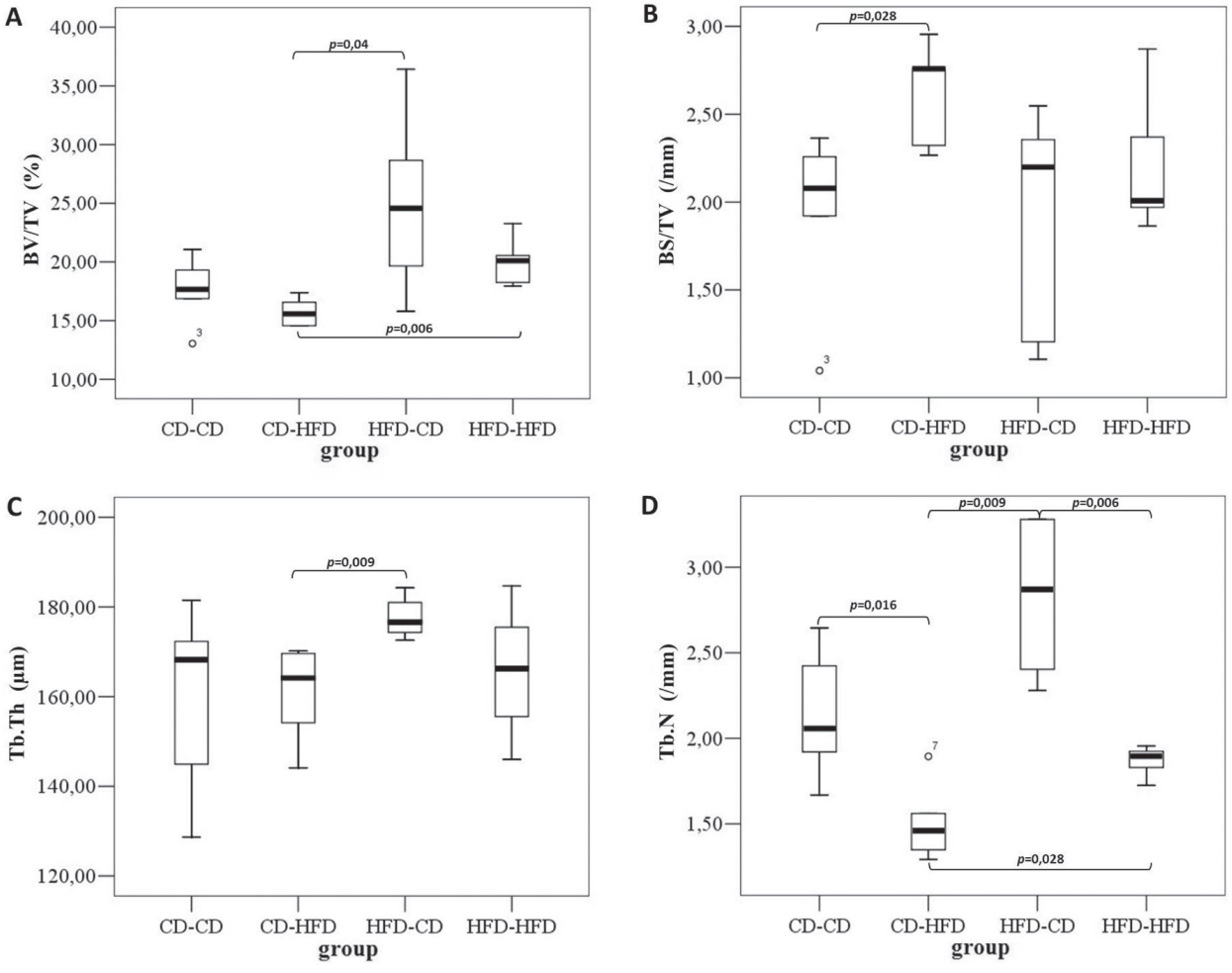
Cortical thickness (Ct.Th) showed statistically significant variation between CD-HFD group with CD-CD and HFD-CD groups, and comparing with HFD-HFD groups (**Figure 2**), being the highest in CD-HFD (3456,59), and lowest in CD-CD (1868,63). Cortical bone volume (CV) showed statistically significant variation between HFD-HFD and others groups, the mean value being 77,66 in CD-CD group and 35,10 in HFD-HFD group (**Table 2**).

DISCUSSION

According to increasing epidemiological evidence that osteoporosis in the offspring may also be influenced by the mother's nutrition during pregnancy, our results in general showed that mismatch between maternal and offspring diet influence on bone microstructure changes.

The HFD-CD and CD-HFD group appears to have altered the trabecular structure. The HFD-CD female offspring displayed increased bone volume, trabecular thickness and trabecular number compared to all other groups. In contrast, CD-HFD offspring showed increased in bone surface density and trabecular separation.

Exposure to the high fat environment in utero thus seems to induce a conservation of bone mass in the offspring, perhaps in prediction of exposure to an unbalanced diet postnatal in which micronutrients such as calcium might be limited, especially as a high fat diet reduces intestinal absorption of calcium *(19, 20)*. This was followed by a (re)modeling of bone structure to meet the weight-bearing demands on the skeleton. The lowest mean trabecular volume was in the CD-HFD group, which could be explained with thrifty phenotype hypothesis which says that the fetus makes physiological adaptations in response to changes in its environment to prepare itself for postnatal life and in his way prepares the organism for its likely adult environment in long term *(21)*.



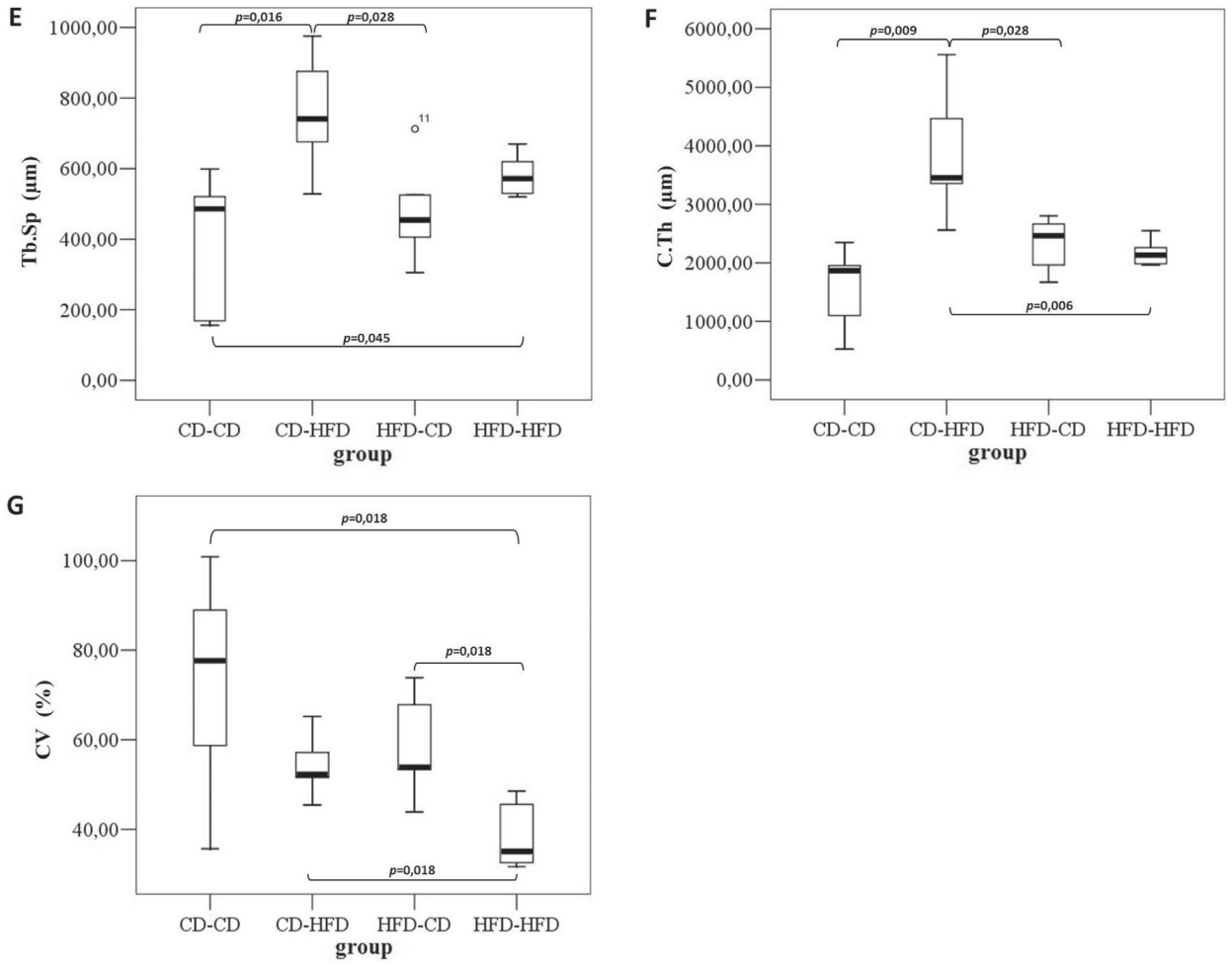


Figure 2. *Bone Histomorphometry in fifth lumbar vertebra.*

***(A)** BV/TV = trabecular bone volume; **(B)** BS/TV = trabecular bone surface density (%); **(C)** Tb.Th = trabecular thickness (µm); **(D)** Tb.N = trabecular number (/mm); **(E)** Tb.Sp = trabecular separation (µm); **(F)** Ct.Th = cortical bone thickness (µm); **(G)** CV = cortical bone volume (%). CD-CD – control diet mothers and offspring, CD-HFD – control diet mothers and high fat diet offspring, HFD-CD – high fat diet mothers and control diet offspring and HFD-HFD – high fat diet mothers and offspring. Dams were continued on their assigned diet in each dietary group during the whole lifetime. Data are presented using Whiskers bar graphs (mean, 5-95 percentile). For testing differences among two independent groups Mann-Whitney test was used, P<0.05 was considered significant and noted on graph.*

High-fat diet fed female offspring given birth by mothers fed with standard laboratory chow, showed a decrease in trabecular number. The same group of female offspring shows the largest trabecular separation. This, in combination with a low trabecular number and trabecular bone volume can indicate that individuals eating western diet are more prone to bone loss, osteopenia, osteoporosis and pathological fractures. The fact that control CD-CD group has higher bone surface density, trabecular thickness and trabecular number than the HFD-HFD group speaks in contribution of high fat diet induces cytokines with sterile inflammation and consequences mentioned in introduction of this article.

The lowest cortical volume was in HFD-HFD group, implying further bone deterioration on high-fat diet, whereas CD-CD showed the highest cortical volume. Interestingly, cortical thickness showed statistical differences in CD-HFD group compared to other three groups. This indicates that cortical bone was affected by the western-style diet. This finding is similar to that of several other groups that have demonstrated that a high-fat diet compromises the biomechanical properties of cortical as well as trabecular bone in developing female rats *(22)*. This research might spark more research in human subjects and help to clarify the effects of high content of saturated fatty acids in food, as well as add valuable insight into the effect of different nutritional habits of mothers and their offspring. Offspring who has received a positive maternal forecast (HFD-CD, HFD-HFD) will be adapted to good conditions and therefore better able

to cope with rich diets. In HFD-HFD group mother probably had protective effect on trabecular structure of offspring; respectively offspring are programmed by the hypercaloric HFD intrauterine mileu to be more susceptible to the deletiours effects of an adult HFD. Our results showed that better preparation for later life was given to HFD-CD group. These results can be correlated to the theory which says that populations who weren't used to western type of diet, evolved in higher rates of predisposition factors for metabolic diseases, which was most often ascribed to a genetically based susceptibility to the development of obesity and associated diseases on the adoption of a western way of life. An alternative explanation is that the susceptibility of these populations lies solely in the rapid changes of lifestyle they have experienced. Thus, it has been suggested that those born into a relatively poor environment may, if they later encounter a more western environment, be vulnerable to the development of obesity-related diseases. This latter explanation is sometimes referred to as the 'thrifty phenotype' hypothesis, to highlight its contrast with the thrifty genotype hypothesis *(23)*.

CONCLUSION

The offspring of the control diet dams fed with high content of saturated fatty acid food (CD-HFD) had the lowest values of the Tb.N and Tb.Th, but the highest values of the Tb.Sp of the lumbar vertebra. Although this animal model and therefore the effects of the diets cannot be directly transported to humans, these data demonstrate effects of high fat maternal diet during pregnancy, with or without a high fat diet in offspring post-weaning, on the bone quality of those offspring, which provide an indication as to the possible effects of such dietary manipulations on skeletal physiology which may suggest future avenues for research of underlying mechanisms. These studies indicate the importance of early life interventions that will be needed to promote the health of subsequent generations.

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