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Increase in Bone Mass Before Onset of Type 1 Diabetes Mellitus in Rats

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Abstract. *Background/Aim: The typical insulin deficiency in type 1 diabetes mellitus has general effects on metabolism and also affects bone quality. Materials and Methods: Two diabetic rat lines (BB/OK; BB.6KWR) and two non-diabetic rat strains (KWR and BB.14+18KWR), as control group, were included in the study. Bone mineral density, bone mineral content and body structure measurements were performed. The measurements took place before the onset of diabetes mellitus Results: A comparison of the groups showed increased bone density values of the diabetic rats in relation to the control groups. A new finding of increased bone density in the diabetic rats occurs. Conclusion: Diabetic rats showed no osteoporotic bone metabolism before the onset of clinically relevant type 1 diabetes mellitus, but rather increased bone metabolic activity.*

Type 1 diabetes mellitus (T1DM) is an immune-mediated disease that disrupts pancreatic beta cells and thus insulin production. T1DM is usually diagnosed as a chronic disease in children and young adults, who are then subjected to various long-term complications of metabolic imbalance. A severe complication is reduced bone mineral density (BMD), which occurs in over 50% of T1DM patients (1-3) compared to age-appropriate healthy individuals. In T1DM rodent models the animals show a significant bone loss with lower bone volume, osteoid surface and osseointegration of implants and distraction models (4-10). Furthermore, studies on T1DM-associated reduction of mineral apposition and expression of osteoblast markers as well as low serum osteocalcic levels suggest that bone loss is caused by inhibited osteoblast maturation (9, 10).

The relationship between T1DM and reduced BMD has also been reported in other studies. In children and adolescents, reduced bone mass in the radius was described, the reduced bone formation of which was attributed to skeletal growth (11). In adult T1DM patients, femoral BMD is significantly reduced, and lumbar spine BMD is slightly decreased (12-15). Furthermore, vascular complications such as retinopathy and neuropathy play a role in reduction of bone masses, while disease duration and impaired glycemic control are not associated (12-16).

A high number of T1DM patients already suffer from osteopenia at the time of diagnosis. This suggests that pathological mechanisms leading to a reduction in bone mass may already be effective before T1DM manifests itself (17). The present study was, therefore, conducted in a rat model to determine whether a reduction in bone mass was detectable prior to onset of T1DM. In BB/OK rats that develop T1DM at an age of 110±30 days with a frequency of 90% T1DM (18), BMD and body composition were determined and compared with T1DM-resistant wild rats.

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Key Words: Type 1 diabetes mellitus, diabetic rats, bone density.

Materials and Methods

The following rat lines were included in the study: 10 male animals BioBreeding/OttawaKarlsburg rats (BB/OK) and BB.6KWR (5 animals) as diabetes-endangered rat strains (18); as control group were used Kyoto Wistar rats (KWR, n=7 animals), and double congenic strain BB.14+18KWR (n=8 animals) both non-diabetic rat strains.

The strain BB.14+18KWR were established for this study by recombining segments of chromosome 14 (D14Rat142-D14Mgh3; 6.9 Mb-21.8mb) and chromosome 18 (D18Rat60-D18Rat15/D18Rat49-D18Rat81; 60.2Mb-69.4Mb/76.0-78.8Mb) into the genetic background of the BB/OK strain. With an incidence of <1% rats of this strain develop almost no T1DM.

The animals were kept in macrolon cages of the Animal Core Unit of the University Medicine Greifswald under specific pathogen-free conditions and 12 h light dark cycle (18). Food (Ssniff R, Soest, Germany) and water were available ad libitum. All experiments were performed according to the regulations for animal care of the Ministry of Food, Agriculture and Forestry of the State Government of Mecklenburg- Vorpommern (Germany) with the knowledge and approval of the competent veterinary authorities.

At the time of the examination all rats were 56±1 days old the measurements were obtained at the end of the animals' life and the organs were removed for scientific purposes, according to the current regulations for animal experiments in the state of Mecklenburg-Western Pomerania (§4 Abs.3 TierSchG.). Before measurement the animals were sacrificed by bleeding from the vena cava, after they were anaesthetised by intraperitoneal injection of 0.2 ml/kg Rompun® (Xylazin, Bayer, Leverkusen, Germany) and 0.4 ml/kg Ketanest (Ketamin, Sanofi, Berlin, Germany).

Bone mineral density (BMD) in g/cm^2 and bone mineral content (BMC) in g of right femur and right tibia were determined by dual energy X-ray absorption (DXA) measurement with a small animal suitable Lunar DPX (GE Healtcare, Lunar Prodigy Advance, Diegem, Belgium) in high resolution mode. The calibration was carried out according to the manufacturer's specifications. The animals were killed by bleeding from the vena cava, after they were anaesthetised by intraperitoneal injection of 0.2 ml/kg Rompun® (Xylazin, Bayer, Leverkusen, Germany) and 0.4 ml/kg Ketanest (Ketamin, Sanofi). The right femur and shin bone as well as the fourth and fifth lumbar vertebra were prepared free of soft tissue. The total length of the femur was measured three times with a caliper, and the mean value was taken as the length of the femur. To determine the BMC and BMD of the entire femur and the proximal third of the tibia, so the fourth and fifth lumbar vertebra, each bone was scanned three times DXA onto a 20 mm acrylic plate. The reproducibility of the data was evaluated using the coefficient of variation (CV%=100 SD/mean) of measurements on 5 animals within 24 h and was less than 2.0%.

The BMD of the entire femur and metadiaphyseal surface, consisting of trabecular bone 0.5 cm from the distal end of the femur, was determined. The femora were determined in 4 different regions of equal length. Region 1: Proximal femur, regions 2 and 3: Medial femur, and region 4: Distal femur. Regions 1 and 4 represented the proximal metaphysis and the distal metaphysis (with a high proportion of trabecular structures), regions 2 and 3 represented the diaphysis (mainly cortical structure) (Figure 1). Pilot tests showed that these areas allow optimal reproducibility of measurements.

Three replicates of each scan were averaged and expressed as average±SD. The differences were evaluated by a one-sided analysis of the variance corrected by Bonferroni-Holm using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA).

Results

Despite the comparable age of the rats, significant differences in body weight were found between diabetes-endangered (BB/OK; BB.6KWR) and non-diabetic (KWR; BB.14+18KWR) strains (Figure 2). The percentage of total body fat in the different strains was pretty similar: for BB/OK 13.07±1.79%, for BB.KWR6 11.78±0.7%, KWR 14.58±1.5% and for BB.14+18KWR 11.62±1.5%, so that there was a significant difference only between BB.KWR6 and KWR (p=0.05). The body fat measurement (in g) shows significant difference (p≤0.01) between diabetes-endangered (BB/OK 24.8±4.5 g; BB.6KWR 26.4±3.3 g) and non-diabetic strains (KWR 14.6±1.5 g; BB.14+18KWR 11.6±1.5 g). The difference by measurement of the fat free body between the groups was even higher (p≤0.001) with 170.3±25.9 g for BB/OK and 202.1±18.3 g for BB.6KWR *vs*. for KWR 99.2±23 g and 121.4±11.4 g for BB.14+18KWR. The separate consideration of the extremities or the trunk fat show clear tendencies. The diabetes-predisposed strains show scientifically more fat on legs (p≤0.001) than the non-diabetic one (BB/OK 5.8±1.3 g; BB.6KWR 7.2±1.2 g; KWR 4.1±1.4 g; BB.14+18KWR 4.0±0.8 g). Similar constellation has been found by the compering of the fat free tissue of the legs – the BB/OK (24.4±5.7 g) and BB.6KWR (29.8±2.7 g) shows significantly higher levels (p≤0.001) than KWR (14.1±4.5 g) and BB.14+18KWR (15.5±3.2 g) together.

The values of the trunk are similar to those of the legs, but not so pronounced. The trunk fat was increased (p≤0.05) by BB/OK (11.1±2.3 g) and BB.6KWR (11.3±1.9 g) rats *vs*. KWR (7.4±1.9 g) and BB.14+18KWR (6.5±1.3 g) rats. Also, the fat free trunk shows significant difference between the strains (p≤0.01): BB/OK (94.7±12.2 g); BB.6KWR (112.7±12.6 g); KWR (55.4±12.6 g) and BB.14+18KWR (65.9±4.8 g).

Comparison of bone mass density of the diabetes predisposed, and the non-diabetic rat populations shows significant elevation in diabetes risk animals (Figure 3). The BMD of the total scanned area shows high significant difference (p≤0.001) between the BB/OK (0.108±0.002 g/cm^2) and BB.6KWR (0.110±0.004 g/cm^2) rats on the one hand and KWR (0.102±0.004 g/cm^2) and BB.14+18KWR (0.099±0.004 g/cm^2) rats on the other hand. The MD of the right femur shows similar tendency (p≤0.01) (BB/OK 0.110±0.004 g/cm^2; BB.6KWR 0.111±0.008 g/cm^2; KWR 0.086±0.004 g/cm^2; BB.14+18KWR 0.090±0.005 g/cm^2). The BMD values of the fourth lumbar vertebra were also significant increased (p≤0.001) in the diabetes predisposed rats (BB/OK 0.105±0.002 g/cm^2; BB.6KWR 0.108±0.005

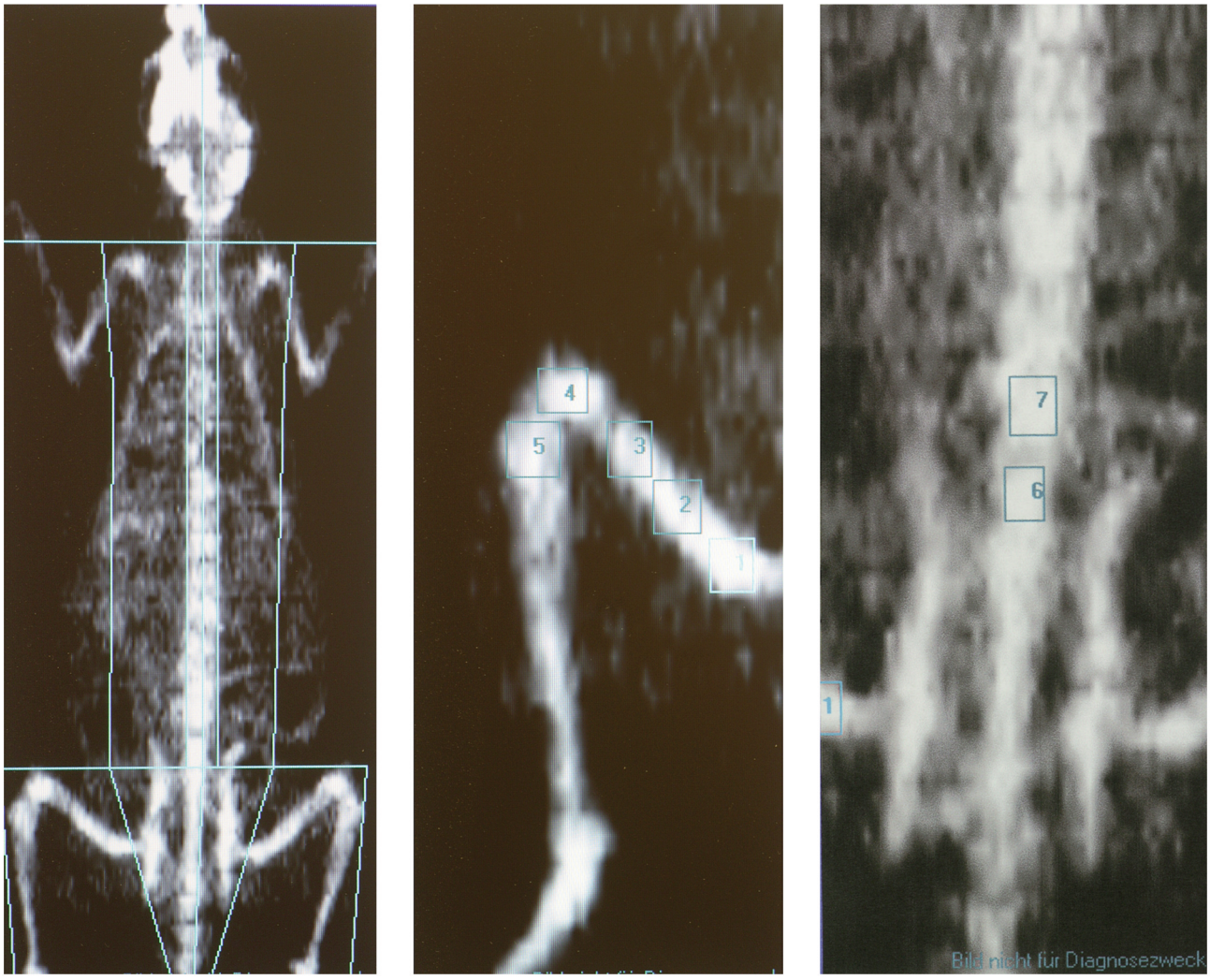

Figure 1. *Areas investigated for the determination for bone density measurement. (A) The standard scan measurement of the whole rat; (B) individual measurement of the femur (area 1-4) and proximal tibia (area 5). (C) Lumbar vertebra bone 4 (area 7) and lumbar vertebra bone 5 (area 6).*

g/cm^2) compared to non-diabetic rats (KWR 0.096±0.005 g/cm^2; BB.14+18KWR 0.094±0.005 g/cm^2). In the proximal tibia and fifth lumbar vertebra no difference was found between the different rat strains.

In addition, the decrease in BMD was greater in the metadiaphyseal area than in the overall area. These results indicate a trend towards a decrease in bone mass in advanced diabetes.

Discussion

It is known that in BB/OK rats spontaneously develop insulin-dependent type 1 diabetes at a mean age of 103±30 days (18, 19). The examination time chosen for the rat strains in this study was before the onset of diabetes, so that the measured BMD reflects the condition of the bones in the prediabetic phase. Klöting *et al*. investigated, in previous studies, the influence of spontaneous diabetes on bone gene expression in BB/OK rats, and their potential effect of bone formation and resorption (20, 21). T1DM is characterized by hypoinsulinemia and a lack of insulin-like growth factor (IGF-1). Both lead to impairment of osteoblast function and consequently to low bone turnover (22-24). Top bone mass is acquired during puberty and built up until the third year of life. Development of T1DM in childhood, therefore, leads to a reduced build-up of peak bone mass, which usually also results in reduced BMD in adulthood due to chronic hyperglycaemia (25, 26).

Pascual *et al*. could not prove a correlation between BMD and duration of the disease in children with T1DM (27).

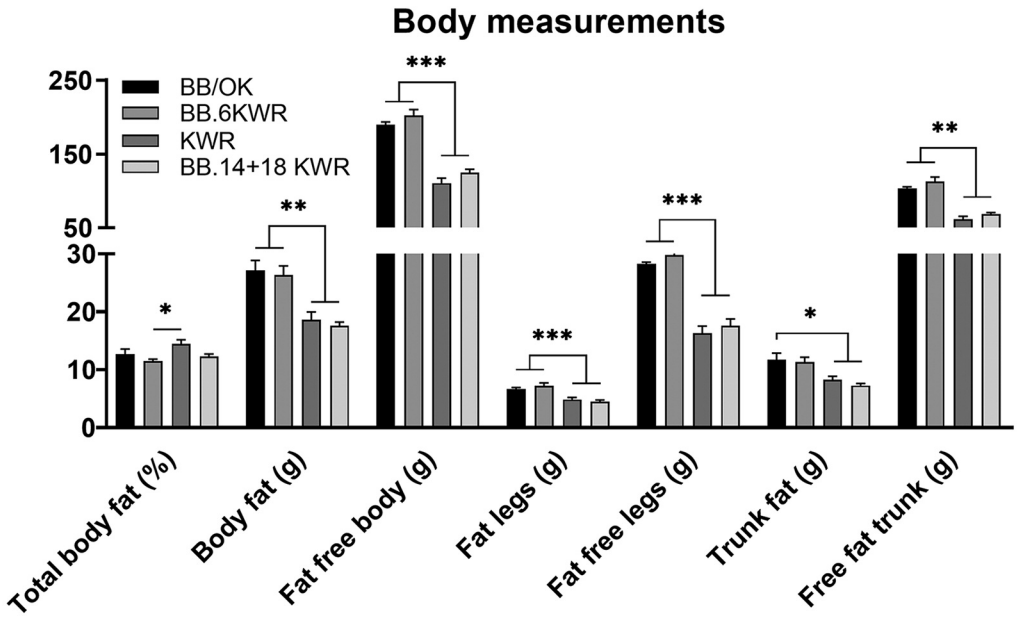

Figure 2. *Body measurements of the different rat populations. Comparison between diabetes-endangered (BB/OK; BB.6KWR) and non-diabetic (KWR; BB.14+18KWR) strains. Measurements of body fat and fat-free tissue in general and in the legs and in the trunk, shown separate. Significant differences were shown between the diabetes-endangered and the non-diabetic strains. *p≤0.05, **p≤0.01 and ***p≤0.001.*

Another study, however, determined the lumbar and total BMD in patients with an average disease duration of 5.4 years and concluded that children with T1DM have a reduced BMD (28). Further studies show that osteopenia and osteoporosis occur in 40-50% of T1DM patients before or shortly after disease manifestation and are therefore not late complications of T1DM (28). In our study, however, the analysis of spontaneously diabetic BB/OK rats showed no osteoporotic bone metabolism. In many cases the metabolic activity was even increased before manifestation of the clinically relevant T1DM.

Genetic studies on osteopenia/osteoporosis in T1DM patients or animal models are not available. However, studies in BB/OK rats indicated that in this model, which is very close to human T1DM (29), bone fragility was significantly higher compared to healthy animals. In gene expression studies, factors of autoimmune processes were identified that were localized not only in the pancreas but also in the bones and could explain the induction of osteopenia/osteoporosis (20).

Bouillon *et al.* referred to the difficult assessment of pathomorphological changes in bone in T1DM. Insulin deficiency leads to altered levels of hormones and growth factors and to a reduced number of osteoblasts, which influences bone metabolism (30). A study with 100 T1DM children found no differences in vitamin D, parathyroid hormone and metabolic control in children with normal and low BMD (31).

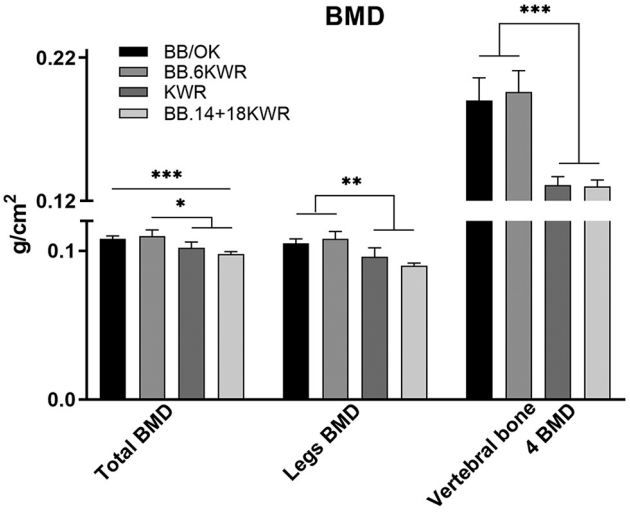

Figure 3. *Comparison of bone mass density (BMD) of different rat populations. TBB/OK and BB.6KWR diabetes-endangered strains; KWR and non-diabetic (KWR; BB.14+18KWR). The measurements of the total area, the right femur and the fourth lumbar vertebra shows significant elevation in diabetic predisposed animals. *p≤0.05, **p≤0.01 and ***p≤0.001.*

To explain the different bone metabolism in children with T1DM, the model of reduced activation of osteoblasts combined with more active osteoclasts was proposed (32, 33). In addition, insufficient glycaemic control of the

metabolism, decreased insulin growth factor I (IGF-I) levels, decreased physical activity and a lower body mass index were suggested (34, 35). Thrailkill *et al*. also see the absence of the anabolic effect of insulin as a possible factor for the clinical manifestation of T1DM (36).

Studies with type 2 diabetes mellitus patients have shown that BMD was elevated in the lumbar spine and hip areas (37, 38). This was confirmed in a meta-analysis (39). In adults, a study demonstrated that the entire spinal column was significantly thicker (40), which could potentially be a risk factor for the development of type 2 diabetes mellitus. Our study showed that the BMD in the lumbar region of T1DM rats was also significantly increased, but that the bones of the extremities of the animals showed no changes. Spinal BMD may be more affected than other bones and can be used to monitor T1DM.

Conclusion

Contrary to our hypothesis, diabetic rats showed no osteoporotic bone metabolism before the onset of clinically relevant T1DM, but rather increased bone metabolic activity. The role of an altered bone metabolism in the development of T1DM must be characterized by further mainly molecular-biological investigations.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization, J.L., M.B.S. and L.H.; Software, C.S.F., A.N. und A.L.; Data analysis, L.H., J.L. and M.B.S.; Investigation, L.H., J.L. and M.B.S.; Resources, L.H., A.L. and M.B.S.; Data curation, C.S.F., I.K., J.L., and A.N.; Writing—original draft preparation, L.H., A.N., and C.S.F.; Writing—review and editing, J.L., M.B.S., A.L., and A.E.; Visualization, A.N., I.K., and C.S.F.; Supervision, I.K. and A.E.; Project administration, I.K. A.E. and L.H.; Funding acquisition, A.L. and A.E.

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