**The interplay between the skeleton and whole-body metabolism and its clinical consequence in individuals with osteogenesis imperfecta type XIV**

**Scientific abstract:**

**Scientific background:**

Osteogenesis imperfecta (OI) also known as brittle bone disease is a heterogeneous group of bone dysplasias classified by severity of clinical symptoms and specific genetic alterations.

Individuals with osteogenesis imperfecta have low bone mass and increased bone fragility, resulting in susceptibility to fractures of the long bones, vertebral compressions, variable deformity of long bones, ribs and spine, and substantial growth deficiency. In addition to the skeleton in individuals with OI other tissues could be affected and secondary symptoms include hearing loss, dentinogenesis imperfecta, malocclusion, scoliosis, pulmonary complications, cardiovascular manifestations, ligamentous laxity and muscle weakness.

**There is no cure for osteogenesis imperfecta.** Treatment management is symptom based and depends on the type and severity of symptoms. **Therefore, identifying risk factors that may influence the skeleton condition is of extreme importance for management of patients with OI.**

Most patients with osteogenesis imperfecta (above 85%) carry mutations in the genes encoding collagen type I alpha chains COL1A1 and COL1A2 (ref). Collagen is the most abundant protein in the body and the major component of the fibers in the extracellular matrix of bone, skin and tendon. Mutations in *COL1A1* and *COL1A2* can result in a decrease in collagen amount or in the production of collagen molecules with altered structure(ref). Quantitative defects are usually associated with milder osteogenesis imperfecta, whereas structural collagen defects can cause the full range of moderate to lethal types of osteogenesis imperfecta. Initially osteogenesis imperfecta was classified to four types according to symptom severity as proposed by Sillence et al. (ref). According to this classification type I produced the milder form of the disease, type II is the most severe and lethal form of the disease, type III is second in severity but non lethal and type IV is more severe than type I but less severe than type II and III.

The biosynthesis of collagen type I is a complex process involving multiple stages controlled by a specialized group of proteins essential for collagen post translational modifications, folding, transport secretion, crosslinking and fiber deposition. We and others showed that mutations in genes which regulate collagen processing could affect bone composition and strength. Indeed, in the last two decades non-collagenous mutations have been found to cause osteogenesis imperfecta (Ref). Identification of these genes led to additional genetic based classification of the disease. Overall, according to both Sillence and the genetic factors, osteogenesis imperfecta is classified to 18 different types.

One of the recessive inherited forms of osteogenesis imperfecta, is classified as OI type XIV [ref]. Individuals with OI type XIV carry loss of function mutations in the gene TMEM38B encoding for the ER cation exchanger TRIC-B. These patients are characterized by osteopenia, and they show a great phenotypic variability with mild to severe bone deformities, none to frequent fractures, different degree of growth retardation and short stature [ref]. **However, the factors which determine this phenotypic variability in the severity of the disease in individuals with OI type XIV is unknown.**

OI type XIV was reported in patients from Albania, united Kingdom China and Saudia Arabia. OI type IX patients were also identified among the Bedouin community in Israel. These patients are the majority of OI treated patients in Soroka hospital, the largest hospital in the south of Israel and to the best of our knowledge consist of the largest group of patients with OI type XIV treated in a single medical center. Together with Dr Muhamad Eldada (see letter of collaboration below), an orthopedic surgeon treating these patients, we have identified 38 patients with OI type XIV. In agreement with other reports these patients exhibit clinical ranges from asymptomatic to severe skeletal symptoms.

Interestingly, many of the OI type XIV patients treated in Soroka medical center are overweight and obese especially after the age of 10 years old. However, how their obesity affect their clinical condition is currently unknown.

Several prior studies have shown that patients with OI often have significant alterations in both body weight and stature. OI-specific growth curves for 100 children with Types III and IV OI demonstrate both short stature and excessive weight gain. Moreover, in OI type III patients the BMI growth curves effectively demonstrated the presence of significant obesity (ref). In addition, a study of pediatric OI Types I, IV, and III caused by collagen defects found increased BMI, especially in Type III OI, and a good correlation between body fat calculated by dual-energy x-ray absorptiometry (DXA) and skin fold thickness and classified most Type III OI patients as having obesity (8). A recent study compared adiposity and resting energy expenditure (REE) in individuals with OI and age/ BMI-similar healthy controls and examined the correlation of OI genotype and clinical phenotype with adiposity and REE among several genetic subtypes of OI. This study have showed an overall trend of OI patients toward a high body fat percentage which became significant when individuals with non-collagenous mutations causing OI were compared to the healthy cohort suggesting that mutations in the collagen processing machinery in these types of OI predispose patients to fat gain (Ref Johan).

Weight gain in OI patients could be a result of limited mobility as a result of increased bone fragility and during recovery from fractures or surgery. In addition, there may be an intrinsic component to increased adipose tissue in OI. Osteoblasts and adipocytes share a common bone mesenchymal precursor and there is plasticity during differentiation between these cell types (12). Impaired differentiation of osteoblasts, as occurs in OI, may shunt precursors into other pathways, including adipocytes.

**Despite the evidence linking osteogenesis imperfecta to obesity, what are the factors that predispose these patients to obesity and more important what is the impact of obesity on the clinical condition of OI patients is unknown.**

Many studies point for a complex relationship between obesity and bone turnover. Traditionaly, Increased obesity was believed to promote anabolic signals which are derived from increased loading of bones (Cao, 2011). However, obesity often results in many derangements of whole-body metabolism including hyperglycemia and marked inflammatory responses. At the last two decades many studies established a strong connection between the increased prevalence of obesity to the staggering increase in cases of type 2 diabetes (T2D). At the beginning, studies designed to determine whether T2D influenced fracture risk and thus altered remodeling, were based solely on assessment of areal BMD. These studies reported mixed results, with the most of the evidence indicating that patients were not at increased risk (van Daele et al., 1995; Piepkorn et al., 1997; Rishaug et al., 1995; Stolk et al., 1996). However, subsequent studies with fracture and not BMD as the primary outcome variable have challenged these initial findings and show that patients with T2D have an increased risk of fracture, independent of BMD and fracture risk increases with increasing duration of T2D (Farr et al., 2014; Janghorbani et al.; de Liefde et al., 2005; Nicodemus et al., 2001; Schwartz, 2011; Schwartz et al., 2001).

Metabolic dearengments induced by obesity could act on the skeleton through different mechanisms affecting both bone formation by osteoblasts or bone resorption by osteoclasts. Osteoblasts and adipocytes share a common precursor. Different studies showed that increase obesity result in increased marrow adipocytes and decreased osteoblast numbers suggesting increased marrow adipocyte could come on the expense of osteoblasts negatively impacting bone formation. Moreover, a recent study showed that depletion of marrow adipocyte induces massive bone formation. This was attributed to a decrease in BMP inhibitors produced by marrow adipocytes. In addition, perturbations of the fat tissue in obesity is associated with chronic inflammation. The increased circulating and tissue proinflammatory cytokines in obesity may promote osteoclast activity and bone resorption. Furthermore, the excessive secretion of leptin and/or decreased production of adiponectin by adipocytes in obesity may either directly affect bone formation or indirectly affect bone resorption through up-regulated proinflammatory cytokine production. Finally, high-fat intake may interfere with intestinal calcium absorption and therefore decrease calcium availability for bone formation. Taken together, these determanentel effects of obesity on the skeleton could have a profound consequence on the skeleton of patients with osteogenesis imperfecta and increase the severity of their clinical symptoms. Indeed, a study conducted in pediatric patients found a significant higher BMI in patients with type III OI which was positively correlated with the number of fractures. In addition, in a recent study describing and comparing clinical symptomes in seven patients with OI, two patients were overweight and had higher fracture compared to the other patients.

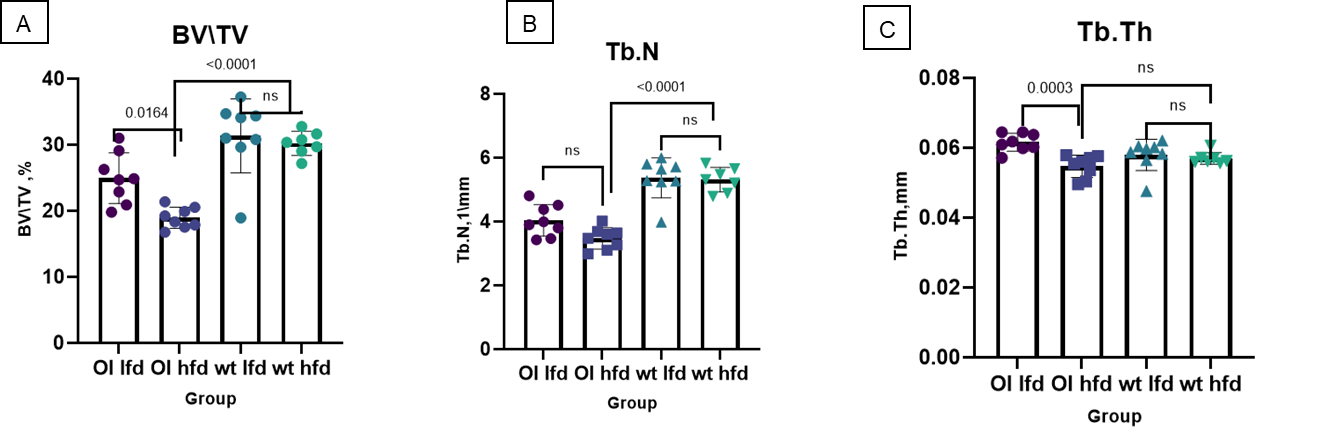
Moreover our preliminary data show an association between BMI and fracture numbers in type XIV OI patients. These observations raise several questions:

*Does loss of function of TMEM38b in the skeleton aggravate metabolic derangements induced by high weight gain. If so, is the alterations in metabolism a result of perturbations in skeletal homeostasis or effects of perturbed collagen processing in other tissues? Is there a vicious cycle in which perturbations in collagen processing increase the susceptibility to metabolic dearangments which in turn aggrevate skeletal condition of patients with OI type XIV?*

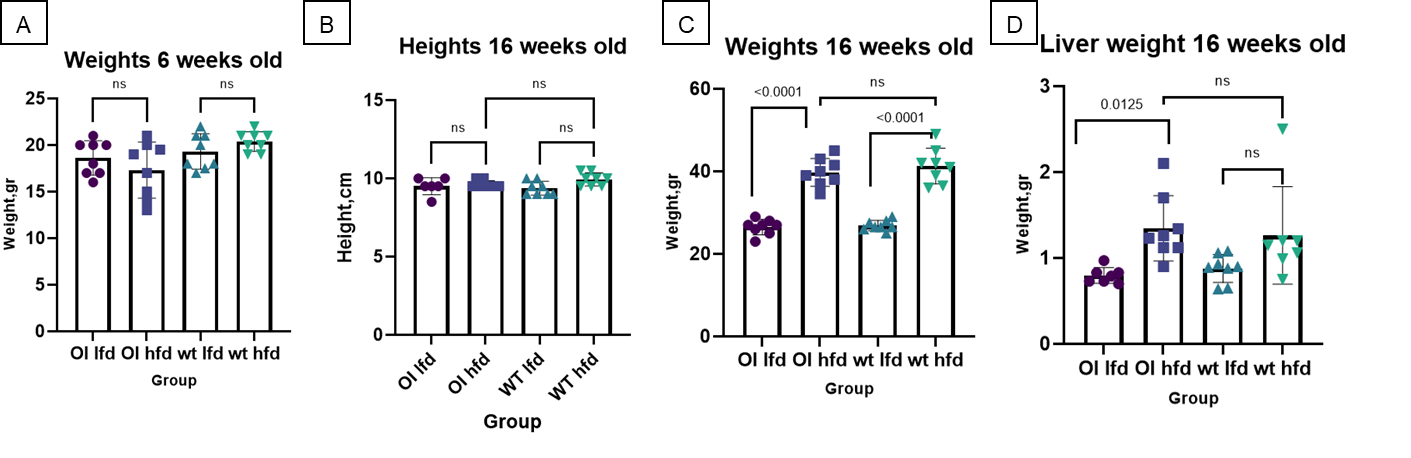
To test if loss of function of TMEM38 is associated with overweight in OI type XIV we compared the BMI and fracture numbers in patients treated in Soroka hospital (***Helsinki approval obtained***). Preliminary analysis of 16 patients that were treated in the orthopedic surgery department showed that 50% were overweight and 33% were obese (Figure 1 table). Five of the patients were six years and under and all had normal to low body mass index. When excluding these patients the rate of overweight or obese patients was 72% and the rate of obese patients was 54%. These data suggest that excessive weight gain initiate around puberty in type XIV OI patients. Many of the patients exhibited long bone deformities that resulted in frequent fractures. These patients had to undergo osteotomies and roding to straighten their bones. Retrospective analysis of these fracture cases showed that fracture rate was 30% higher in patients that were overweight or obese, compared to patients with normal weight but did not reach a statistically significant value likely because of the small cohort used in the preliminary analysis.

***Figure 1****: preliminary data describing some of the patients that were recruited for the study, for which body mass index (BMI, kg/m2) and fracture incidence data was already available. BMI was calculated and converted to age- and sex-specific z-scores (BMIz) based on reference data published by the Centers for Disease Control and Prevention (ref). For those with age >18 years, BMIz for age 18 years was used. The graph on the right shows the incidence of fractures in long bones that were not caused by trauma and required surgical treatment in the ortopaedic department.*

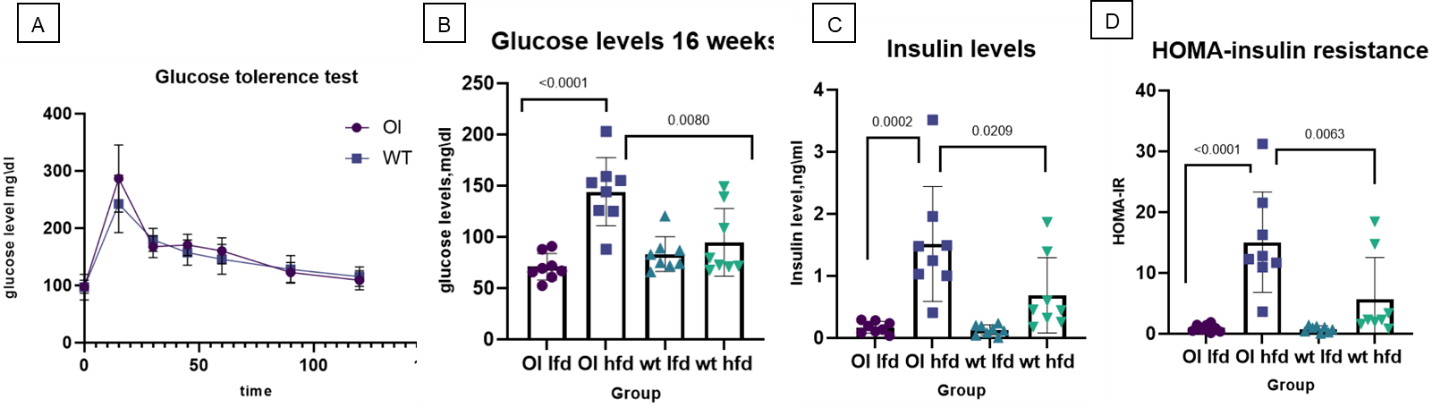
To determine how obesity effect the skeleton of patients with OI, we examined the effects of high fat diet on the skeletal structural parameters in, an established mice model of OI. These mice harbor a glycine to cysteine substitution in position 610 of Col1a2 alpha chain which was identified in the Amish population in the USA. Col1a2 tm1.1Mcbr mice. These mice exhibit a mild to moderate symptoms of osteogenesis imperfecta. Heterozygouse male mice were used in the study and are referred as OI mice. Age and sex matched wild type littermates (WT) were used as controls. Two different diets were used in the study, high fat diet consisting of 60% fat (HFD) and a control low fat diet (LFD) consisting of 10% fat. The mice were divided to four groups according to their genotype and their diet. At the age of six weeks all mice were given LFD for two weeks and then two groups were switched to HFD for additional eight weeks. Bone structural parameters in L5 vertabrae were analyzed using micro computed tomography. Regardless of the diet, the bone volume fraction of the OI mice was lower than its fraction in WT littermates (figure 2A). This difference was derived mainly by a reduction in trabecular numbers (figure 2B) and not trabecular thickness (figure 2C). HFD did not change vertebral structural parameters in wild type mice. In contrast, compared to LFD fed OI mice, HFD significantly lowered the bone volume fraction of OI mice (figure 4A). Interestingly trabecular thickness but not trabecular numbers were lower in mice heterozygouse for the G610C mutations fed HFD compared to mice fed LFD. Changes in trabecular thickness are often associated with alterations in bone formation while changes in trabecular numbers are associated with bone resorption. Therefore, our preliminary data suggest that obesity has a negative impact on the skeleton of OI mice, through a reduction in bone mass likely derived by a reduction in bone formation.

 *Figure 4: Vertebral trabecular bone structural parameters in Col1a2 tm1.1Mcbr mice and wild type littermates fed LFD or HFD. Mice were treated with the different diets for eight weeks and L5 vertebrae were scanned by microCT and analyzed. A. bone volume fraction (BV/TV). B. Trabecular number (Tb.N). C. Trabecular separation (Tb.S). N=8, p values < 0.05 was considered significant.*

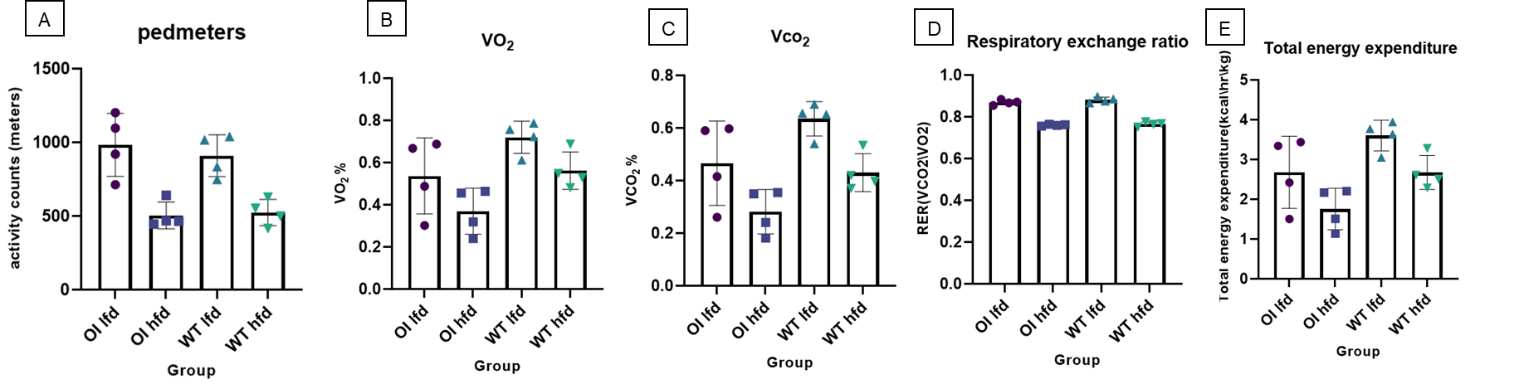
In order to determine if osteogenesis imperfecta increase the susceptibility to metabolic derangements induced by obesity, we examined the effects of high fat diet on whole body metabolic parameters in OI mice. Our preliminary data show that at the age of six weeks the weights of the mice were like the weight of their wild type counterparts (figure 3A). After eight weeks of diet the length of the mice in all groups was similar (figure 3B) but both the wild type and the OI groups that were fed high fat diet had higher total and liver weight compared to the mice fad low fat diet. No difference in weight between the WT and OI mice that were fed HFD was observed (figure 3C and D). These data suggest that OI mice did not tend to gain fat compared to normal mice.

***Figure 3:*** *Six-weeks-old male OI and WT littermates were fed low fat diet (LFD, 10% fat) for two weeks and then maintained on LFD or switched to high fat diet (HFD, 60% fat) for additional eight weeks. A. The weight of the mice before treatment with the different diets. B. The height of the mice at the end of the experiment. C. The weight of the mice at the end of the experiment. D. The weight of the mice livers at the end of the experiment.*

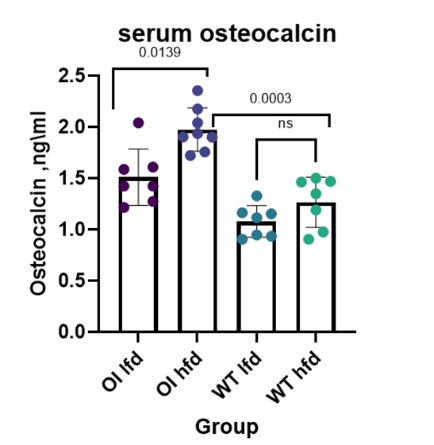
To determine the effects of high fat diet on metabolism we compared the glycemic parameters of the different groups of mice. At the age of eight weeks before the mice were switched to the different diets glucose tolerance tests showed significantly higher glucose pick in OI mice compared to wild type littermates (figure 4A). After eight weeks of diet fasting glucose and insulin levels were significantly higher in OI fed HFD compared to WT on the same diet (figure 4B and C). Homa-IR test confirmed a more significant insulin resistance induced by high fat diet in OI mice compared to their WT counterparts (figure 4D). Taken together these data suggest that mice with mutations in collagen alpha II chains are more susceptible to metabolic derangements induced by obesity.

***Figure 4:*** *Glycemic parameters in* *OI and WT littermates fed either low or high fat diet. A. Glucose tolerance test before initiation of the different diets. Fasting glucose B. and insulin C. levels after eight weeks of either HFD or LFD.*

In order to determine if this increased susceptibility to obesity-induced metabolic derangements is a result of reduced motility or lower energy expenditure of mice with OI, we used metabolic cage to measure indirect calorimetry parameters to characterize their metabolic phenotype (figure 5).

***Figure 5:*** *Locomotion, energy balance and fuel oxidation in OI and wild type mice fed low or high fat containing diets. Mice were separately housed in a metabolic cages system. The mice were allowed to acclimatize for two days before measurements were taken. A. Total walking distance. Oxigen consumption B and carbon dioxide production C. D. respiratory exchange rate and E total energy expenditure. n=4* *p values < 0.05 were considered significant.*

Our preliminary analysis showed a reduction in the distance travelled by mice fed on HFD compared to the mice fed LFD. However, no differences in the locomotion between OI mice to WT mice fed HFD were observed (figure 5A). We observed an overall reduction in total energy expenditure in the OI mice, which was associated with a reduction in oxygen consumption and carbon dioxide production, however these differences were not statistically significant likely because of the small cohort that was analyzed (figure 5 B,C and E). As expected, respiratory exchange rate was lower in the HFD fed mice pointing for a switch toward more fat oxidation as an energy source. However, no differences between the OI mice to their wild type counterparts were observed (figure 5D).

***Figure 6:*** *Serum osteocalcin levels in 16 weeks old OI mice and WT controls fed HFD or LFD. N=7-8, values < 0.05 were considered significant.*

Overall, our indirect calorimetry preliminary analysis suggests that the susceptibility to metabolic derangements induced by obesity is not a result of reduced activity of the OI mice. Although the cohort is currently to small and results are not statisticly significant, our preliminary data suggest there is lower volume of oxygen intake and carbon dioxide release in the OI mice compared to diet matched WT mice. These changes in gas exchange point for a lower, total energy consumption. Given that there is no difference in activity, and weights of the mice, the reduction in energy consumption could be a result of reduction in the ratio between lean body mass and fat mass. A recent study showed no difference between the lean body mass of Col1a2 tm1.1Mcbr and WT mice when they are 16 weeks old ,the same age which show differences in insulin resistance in our preliminary data. Therefore, the decrease in whole body energy expenditure is likely not a result of a reduction in lean mass (however this should be tested in a larger cohort, and after a high fat diet, see detailed research plan). If this is the case, the study of Gremminger et al. and our preliminary observation suggests that the high susceptibility to metabolic derangements induced by obesity in OI mice are not derived by reduced muscle function or decreased locomotion. Therefore, these metabolic perturbation could derive, at least in part from other tissues of the OI mice. Recent studies identified and showed that hormone derived from bones influence the body's overall energy metabolism (ref). Three of these hormones, osteocalcin, sclerostin and lipocalcin-2 has been shown to regulate insulin resistance. Increased circulating levels of osteocalcin (OCN) were previously shown in Brtl/+ mouse a different OI mouse model (Sinder et al. 2015) and in children with OI, in particular with OI type III and IV (Brunetti  et al. 2016). Osteocalcin deficient mice have increased blood glucose and decreased insulin circulating levels and are glucose intolerant compared to WT (Lee  et  al. 2007), whereas injection of OCN in male WT mice reduced blood glucose, increased insulin secretion and improved glucose tolerance (Ferron et al. 2008). Decrease in OCN was shown to be associated with decrease in VO2 and VCO2 as well as EE (Ferron et al. 2010a), whereas OCN increase resulted in increased EE (Ferron et al. 2012). OCN decreases adiposity and increases adiponectin production in white fat cells (Ferron  et  al. 2008). To determine if osteocalcin plays a role in the susceptibility to HFD induced insulin resistance in our OI mouse model, we measured the levels of undercarboxylated osteocalcin (the active form of the hormone) in the serum of the mice. Surprisingly, we observed a profound and significant higher level of osteocalcin in OI mice compared to WT mice. Moreover, HFD significantly increased osteocalcin levels in OI fed HFD compared to OI fed LFD (figure 6). Since osteocalcin serve as a protective factor, these data raise the possibility that osteocalcin levels increase to overcome insulin resistance in the OI mice. Interestingly, recent studies from the group of Komarova et al. showed that male mice with moderate to severe OI mediated by a deletion of 18 amino acids in col1a1 also have high levels of osteocalcin when they are four weeks old (prepuberty, but not 8 weeks old). These changes in osteocalcin were associated with higher levels of insulin and decreased adiposity. In the other study by this group they showed that OI male mice (but not female) were protected from HFD induced adiposity. However, despite reduced adiposity, these mice developed insulin resistance, and their fasting glucose levels were not significantly different than the HFD fed WT mice. Several studies in mice showed that undercarboxylated osteocalcin is released by osteoclasts. One of the differences between our model and the Jrt OI model used by Komarova’s group is the level of resorption. In male Jrt OI the resorption levels are very high as opposed to female JRT and both male and female Col1a2 tm1.1Mcbr . A reduction in resorption with age is associated with reduction in osteocalcin levels in the JRT mouse model. The female JRT mice were not protected from obesity as the male mice and developed similar insulin resistance when fed high fat diet, suggesting reduced resorption followed by a reduction in undercarboxylated osteocalcin increase the susceptibility of these female mice to gain fat and develop insulin resistance. Interestingly, pamindronate, a bisphosphonate, that inhibits bone resorption and is used to treat patients with OI has been associated with weight gain in some boys with OI (Zeitlin et al. 2003). However, what is the effect of bisphosphonates on osteocalcin and metabolism was not explored. Different types of OI are characterized by different rates of resorption and therefore may have different levels of osteocalcin. Importantly, osteogenesis imperfecta type XIV, present the OI type with the lowest bone turnover and bone resorption reported. However, what is the level of osteocalcin in these patients and whether it is associated with obesity and obesity induced metabolic derangements is not known.

**Hypothesis and research objectives**

We hypothesize that low bone turnover and low undercarboxylated osteocalcin levels in osteogenesis imperfecta type XIV increase the susceptibility to obesity induced metabolic derangements which in turn lowers bone formation and aggravate skeletal brittleness in these patients.

**Aim 1. Determine if there is a correlation between bone turnover, osteocalcin levels and obesity induced insulin resistance and skeleton deterioration in** Col1a2 tm1.1Mcbr **mice**

Studies by others together with our preliminary data show altered metabolism in patients and animals with OI. In this aim we will expand our analysis of the effects of OI on the susceptibility to obesity induced metabolic derangement. We will also include an analysis of female Col1a2 tm1.1Mcbr mice fed HFD and LFD and their WT counter parts. We hypothesize that following HFD metabolic challenge, the rise in insulin levels result in increased expression of osteocalcin. The effective undercarboxylated osteocalcin levels are then determined by osteoclast activity, protects against obesity induced metabolic derangements and the consequent bone deterioration. In pursuit of this aim, we will investigate the physiological mechanisms underlying the interplay between bone and metabolism in Col1a2 tm1.1Mcbrmice according to the following objectives.

1. Examine the skeletal and metabolic consequences of high fat diet in Col1a2 tm1.1Mcbr male and female mice
2. Determine how treatment with bisphosphonates which reduce bone turnover affect osteocalcin levels and what is the consequent skeletal and metabolic phenotype in HFD fed Col1a2 tm1.1Mcbrmice.

**Aim 2. Determine the effects of high fat diet on body composition, energy metabolism and skeletal homeostasis in mice with non-collagen mutations causing OI type XIV**

Our preliminary data show a high frequency of obesity in patients with OI type XIV. In this aim we will determine the effects of high fat diet on weight gain, glycemic control and skeletal homeostasis in mice with inducible global deletion of TMEM38b causing OI type XIV. To determine if the susceptibility to obesity induced metabolic derangements is derived by perturbations in the skeleton or perturbations in other tissues we will compare metabolic parameters in mice with germline deletion of TMEM38B to mice with osteoblast specific deletion of TMEM38B. The same parameters as in aim one above will be compared in the different mice.

A. Establish an inducible mouse model for osteogenesis imperfecta type XIV

B. Determine the effects of high fat diet on body composition, energy metabolism and skeletal homeostasis in mice with inducible global deletion or osteoblast specific deletion of TMEM38B

**Aim 3. Examine the association of bone turnover, undercarboxylated osteocalcin, glycemic parameters and clinical severity in patients with osteogenesis imperfecta type XIV.**

Soroka medical Center affiliated to our university treat a unique population of patients with type XIV OI. We have identified and recruited a cohort of 32 of these patients and a preliminary analysis of a group of these patients (for which the data was already obtained) show an increased rate of obesity and fracture incidence (figure 1).

In this aim we will determine if there is an association between bone turnover, osteocalcin, obesity, metabolic derangements, fracture incidence and fracture healing in these patients.

**Expected significance**

Our study will determine if the TMEM38B mutation in osteogenesis imperfecta type XIV increase the susceptibility for obesity and obesity induced metabolic derangements. Using a large group of patients, we will attempt to determine if there is an association between metabolic derangements and disease severity in OI type XIV. It will also be the first study to test if perturbations in bone drive obesity induced metabolic derangments in mice with OI type XIV.

Finaly our study will be the first to test the effects of bone turnover and osteocalcin supplementation on the skeleton and metabolism in mice with osteogenesis imperfecta on a fat rich “western diet”. The results of this study may shed light on novel mechanisms driving symptom severity in OI and will advance treatment management in OI type XIV and other types of OI.

Finally, this study will increase our understanding of the interplay between the bone niche and whole body metabolism.

**Detailed description of proposed research:**

**Aim 1. Determine if there is a correlation between bone turnover, osteocalcin levels and obesity induced insulin resistance and skeleton deterioration in** Col1a2 tm1.1Mcbr **mice**

We have chosen to evaluate the effects of obesity on OI using the Col1a2 tm1.1Mcbr mouse model as this is an established mouse model carrying mutations identified in humans. Individuals with OI derived by the G610C mutation develop OI with high clinical range from the mild type I to the moderate type IV which is like the clinical variability of patients with OI type XIV (ref).

**Aim1a: Examine the skeletal and metabolic consequences of high fat diet in male and female** **Col1a2 tm1.1Mcbr mice**

Male and female mice heterozygous for the G610C substitution will be used and age and sex matched wild type littermates (WT) will be used as controls. The mice will be divided to eight groups according to their gender, genotype and their diet (Heterozygous vs wild type and high fat diet, 60% fat vs low fat diet 10% fat). At the age of six weeks all mice will be given LFD for two weeks to acclimatize and then two groups will be switched to HFD for additional eight weeks.

Femora and vertebrae will be screened with a Skyscan 1174 system (Bruker, Belgium). Scans will be performed at a 7.6-μm voxel resolution. The mineralized tissues will be differentially segmented by a global thresholding procedure. Morphometric parameters will be determined according to the established guidelines for assessment of bone microstructure in rodents [57]. The parameters in the vertebrae will include: trabecular bone volume fraction (BV/TV), thickness (Tb.Th), number (Tb.N), separation (Tb.Sp) and connectivity density (Conn.D). In the femoral midshaft parameters will include: cortical periosteal and endosteal diameters, cortical thickness (Ct.th), cortical area fraction (Ct.Ar/T.Ar) and moment of inertia will be measured. Biomechanical testing will be carried out in collaboration with Dr. Bjorn Busse at the University Medical Center Hamburg-Eppendorf, an expert in bone biomechanics (see letter of support). Biomechanical testing will be performed on the contralateral femur. The bones will be horizontally mounted and centrally positioned, with the anterior surface facing downward, on a Bose® 5500 instrument (Bose Corporation, Eden Prairie, MN, USA), and destructive three-point bending will be carried out. A constant displacement rate of 1 mm/min will be applied, and load-displacement data will be recorded at 100 Hz until failure. Force and displacement will be recorded automatically, and resulting parameters will be calculated from the load-displacement curves. Moment of inertia (MOI) and bone area will be calculated from µCT data. Force-related data will be adjusted to the bone morphology using the MOI and the bone area to calculate the Young’s modulus and the stress. Additionally, the energy dissipation will be calculated as the area under the force displacement curve.

To determine the cellular mechanisms underlying selective bone loss in Col1a2 tm1.1Mcbr mice we will compare the distribution and function of osteoclasts and osteoblasts in the different genotype and diet groups described above. To determine osteoblast distribution and function, we will perform dynamic histomorphometric analysis on secondary spongiosa of vertebrae after sequential injection of calcein green (20mg/kg, Sigma). Mice will be injected two and five days before they will be sacrificed. vertebrae will be embedded in methyl methacrylate following the protocol of Sims et al. [58]. Undecalcified, unstained 5-μm sections will be imaged with a fluorescence microscope and analyzed. For osteoblast count, undecalcified sections will be stained with Goldner’s Trichrome (Electron Microscopy Sciences), sectioned with a Leica microtome to a thickness of 5 μm, and imaged with a Zeiss microscope. To identify osteoclasts, vertebrae will be fixed with 4% paraformaldehyde (PFA) for 48 hours and decalcified with EDTA. After decalcification, bones will be embedded in paraffin and sectioned with a Leica microtome to a thickness of 5 μm. Sections will be stained with tartrate-resistant acid phosphatase (TRAP) and counterstained with Mayer’s hematoxylin. The following parameters will be determined according to the 2012 convention of standardized nomenclature [59]: mineral apposition rate (MAR), mineralizing surface /bone surface (MS/BS), bone formation rate (BFR/BS) and osteoclast number (N.Oc/BS). Changes in bone resorption and formation will also be assessed by measuring the serum levels of carboxyterminal collagen crosslinks (CTX), a marker of bone resorption, and the amino-terminal propeptide of type I collagen (P1NP), a surrogate for bone formation, in samples obtained at the time of sacrifice using ELISA kits [5] (Immuno-Diagnostic Systems). Osteoid parameters will be assessed in undecalcified sections from mice that will be stained with Goldner’s Trichrome. In addition, Fourier transform infrared (FTIR) spectroscopy to assess the mineral to matrix ratio in bones will be carried out.

Changes in the bone derived hormones, undercarboxylated osteocalcin, sclerostin and lipocalcin-2 will be assessed using specific ELISA kits.

To determine if increased insulin resistance is not a consequence of reduced activity, we will expand our indirect calorimetry analysis and analyze additional mice using the metabolic cage system. We will also include an analysis of food consumption to determine if the mice with OI consume the same amount of food as their WT counterparts.

To determine if differences in body composition contribute to the differences in insulin resistance, we will compare body fat mass and lean mass in the four different experimental groups described in the former aims. Body composition will be determined by NMR spectroscopy using minispec LF50/mq7.5 analyzer (Bruker) in collaboration with Dr Amir \_\_\_\_\_.

**Aim1b: Determine how reduction in bone turnover affect osteocalcin levels and what is the consequent skeletal and metabolic phenotype in HFD fed Col1a2 tm1.1Mcbr mice.**

We hypothesize that elevated levels of undercarboxylated osteocalcin as a result of bone resorption increases the metabolic rate and protects against high fat diet induced weight gain and metabolic derangements. To test this hypothesis we will inhibit bone turnover, using pamidronate, a bisphosphonate that target osteoclasts and is used to treat patients with osteogenesis imperfecta including patients with OI type XIV in Soroka medical center(refs).

To spare animals and save time and resources male Col1a2 tm1.1Mcbr that exhibit weight gain and significant insulin resistance when fed high fat diet (see preliminary results) will be used in this aim. Only mice with OI will be used and divided according to treatment and diet to four groups including: LFD, LFD + pamidronate, HFD and HFD + pamidronate. At the age of six weeks, mice all mice will be fed a low fat diet, two weeks after the initiation of LFD, mice will be given an IP injection of either PBS (control) or pamidronate (3 mg/kg; Sigma Aldrich) and two groups will be switched to HFD. Ip injections of pamidronate will be repeated every 3 weeks till the end of the experiment. These dose was shown to effectively inhibit bone resorption in mice and is equivalent to the dose used for the treatment of children with OI ([25](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3959604/#B25)).

The mice will be treated and fed with the special diets for eight weeks as described above. Weight length will be recorded once a week. Fasting glucose will be recorded at the age of eight weeks before initiation of HFD and pamidronate and every two weeks thereafter. Glucose tolerance test will be performed at the middle of the treatment (After for weeks of HFD) and at the end of the experiment. Indirect calorimetric measurements will be performed in metabolic cages between the age of 12 to 14 weeks, the mice will be acclimatized for 48 hours and then parameters will be recorded for additional 72 hours. During the last week the mice will be injected with calcein green and the skeletal and metabolic phenotype will be performed as described in Aim1A.

**Expected results Pitfalls and alternative approaches Aim 1.** Given our preliminary results we expect to demonstrate we expect this aim to provide a mechanistic link between skeletal perturbation and obesity induced metabolic derangements. This aim is expected to provide evidence for the determinantal effect of obesity on the skeleton of OI and emphasize the importance of proper nutrition in the management of patients with OI.Aim1B is expected to show an increase in the susceptibility for obesity and metabolic derangements in mice treated with bisphosphonates presumably as a result of a reduction in bone resorption and a consequent reduction in osteocalcin. We are aware that bisphosphonate treatment increase bone volume and strength in treated mice. Therefore, as a result of improvement in their skeleton the activity of the mice could be changed which may mask the effects of reduction in bone turnover on HFD induced obesity, insulin resistance and the followed determinantal effects on the skeleton. Nevertheless, we didn’t observe a difference in the locomotion between WT and the OI mice (figure 5A) suggesting that activity is not expected to change as a result of improvements in skeletal mass of the treated mice but to confirm this assumption the movement of the treated mice will be monitored using the metabolic cage system. In any case, pamidronate is used in treatment of patients with OI and this aim is expected to show if pamidronate increase the susceptibility to obesity and metabolic derangements and the impact of these effects on the skeleton.

**Aim 2: Determine the effects of high fat diet on body composition, energy metabolism and skeletal homeostasis in mice with non-collagen mutations causing OI type XIV**

In this aim we will determine if osteogenesis imperfecta caused by mutations in TMEM38B increased the susceptibility for obesity and obesity induced metabolic derangements using a novel mouse model for osteogenesis imperfecta.For this purpose, we will utilize a mouse in which two CRE recombinase recognition sites have been introduced in the Flanks of Exon X in TMEM38B (TMEM38BFlox/Flox).

The mice have been generated and tested by Professor Antonella Forlino which have kindly agreed to provide them to us (See letter of support). The mice will be available to us at the beginning of the year and by the beginning of the funding period we expect to have a colony of these mice ready for crossing with the Cre expressing mice. Forlino group have showed that these mice develop a moderate to severe form of OI type XIV when crossed with mice expressing CRE recombinase driven by the osteoblast specific transcription factor RUNX2 (manuscript in preparation).

**Aim 2a: Establish an inducible mouse model for osteogenesis imperfecta type XIV**

When crossed with RUNX2 Cre the TMEM38BFlox/Flox develop severe osteogenesis imperfecta and die 3-4 weeks after birth. Therefore, in order to be able to assess the effects of high fat diet on body composition, energy metabolism and skeletal homeostasis we will deploy another cre driver in which the expression of cre recombinase is inducible. For this purpose, we will cross TMEM38BFlox/Flox with either UBC-Cre-ERT2 (Live stock are available from Jackson laboratories) or Col1a1 (2.3 kb)-CreERT2. UBC-Cre-ERT2 express a tamoxifen inducible functional CRE recombinase in all tissue tested to date, including Muscle, Bone and fat. The mice were selected based on a strong expression of Cre recombinase after a single dose of tamoxifen in all tested tissues (ref). Col1a1 (2.3 kb)-CreERT2 express a tamoxifen inducible functional CRE recombinase selectively in the osteoblast lineage.

To achieve effective deletion of TMEM38B and effective induction of OI different time points and frequency of tamoxifen injections will be tested.

Initially we will test the effectivity and persistence of TMEM38B deletion in bone, muscle and fat tissues. For this purpose TMEM38BFlox/Flox x UBC-Cre-ERT2 mice (TMEM38BKO) and TMEM38BFlox/Flox x Col1a1 (2.3 kb)-CreERT2 (TMEM38BOB-KO) will be injected ip for four consecutive days with 10mg/kg tamoxifen (Sigma Aldrich). This dose has been shown to effectively activate the cre recombinase with minimal non-specific anabolic effects on the skeleton (ref). Mice will be divided to four groups and injected when they are four weeks old. The mice will be sacrificed at two, four six and eight weeks after tamoxifen injection and Effective deletion of TMEM38B will be compared in bone, muscle and fat tissues using PCR and western-blot.

Next we will determine if inducible deletion of TMEM38B induces osteogenesis imperfecta. For this purpose TMEM38BKO will be injected with tamoxifen when they are four weeks old and injected again if necessary according to the results of the dosing experiment above. TMEM38BFlox/Flox injected with tamoxifen and UBC-Cre-ERT2 injected with vehicle will serve as controls. The mice will be sacrificed when they are sixteen weeks old (the end time point of the HFD treatment). Bone structural and mechanical parameters will be compared as described in Aim1A.

**Aim 2b:** **Determine the effects of high fat diet on body composition, energy metabolism and skeletal homeostasis in mice with inducible global deletion or osteoblast specific deletion of TMEM38B**

After optimal conditions for TMEM38B deletion and induction of OI are achieved, we will compare metabolic and bone parameters in TMEM38BKO andTMEM38BOB-KO fed eitherHFD or LFD. As controls we will use TMEM38BFlox/Flox injected with tamoxifen and and UBC-Cre-ERT2 injected with vehicle in case significant differences in skeletal parameters between these groups will be observed in aim2a. Four weeks old mice will be injected for four days with tamoxifen, at the age of six weeks the mice will be switched to LFD and thereafter treated with the different diets as described in Aim1. Skeletal and metabolic parameters will be measured as described in Aim1

**Expected results pitfalls and alternative approaches Aim 2.** Patients with OI type XIV are frequently obese. However, it is not known if the mutations in TMEM38B predisposes the patients to obesity. This aim is expected to answer these questions. Moreover, this aim will evaluate the contribution of perturbations in the skeleton to the susceptibility for obesity and metabolic derangements. Finally, this aim will reveal how obesity affect skeletal integrity in OI type XIV. To overcome the lethality induced by TMEM38B deletion we plan to use an inducible system based on activation of cre recombinase by tamoxifen. We plan to induce the deletion of TMEM38B when the mice are four weeks old presuming that the effects of the mutation on the skeleton is much more sever during early stages of skeletal development. In case the rate of death will be high till the mice reach 16 weeks of age we will try to overcome lethality by either using a lower dose of tamoxifen to reduce TMEM38B deletion or delet TMEM38B at an older age. Another possible pitfall is limited deletion of TMAM38B. In this case we will perform a second crossing of the TMEM38BKO andTMEM38BOB-KO and select for mice that have two copies of the CRE recombinase. This strategy has been utilized before to increase cre recombinase levels and increase the effectiveness of recombination (ref).

**Aim 3. Examine the association of bone turnover, undercarboxylated osteocalcin, glycemic parameters and clinical severity in patients with osteogenesis imperfecta type XIV.**

We hypothesize that patients with OI type XIV have high susceptibility to develop obesity and obesity induced metabolic derangements. Moreover, we hypothesize metabolic derangements in OI type XIV are associated with symptom severity. To execute this aim, we will rely on an existing collaboration with **Dr. Muhamad Eldada** from the Orthopedic Department of Soroka Medical Center (***Letter & Helsinki approval obtained***). Under this aim, 32 patients have bee identified and recruited at the ages ranging from 1-30. All recruited patients will have a genetic test confirming homozygosity for mutations in TMEM38B. All of the OI type XIV patients that are treated in Soroka medical center are form the Arab Beduin Community in the Negev. Most of these patients come from families in which consanguine marriage are common. To rule out other genetic and environmental factors that could affect symptom severity, BMI and metabolic derangments a group of 20 siblings within the same age ranges, from the same families will serve as a control group.

We have already obtained data and collected serum from 17 patients and our preliminary data suggest that overweight and obesity are common in these patients and are associated with higher fracture rate (figure 1). Here we will expand our analysis to include and compare the following information.

Genral information will include: Age, Height, Weight and current treatment regime.

Evaluation of clinical symptoms will be carried out by **Dr. Muhamad Eldada based on records of past treatments in the** Orthopedic Department and treatment that will take place during the funding period of the grant. Clinical symptomes will include number of fractures, fracture type, successful healing and fracture recurrence. Additional clinical symptoms will include hearing loss, dentinogenesis imperfecta, malocclusion, scoliosis, pulmonary complications, cardiovascular manifestations, ligamentous laxity and muscle weakness.

Serum will be obtained from all patients to evaluate metabolic derangements, inflammatory cytokines, bone derived hormones and bone turnover markers.

Metabolic derangements will be evaluated by measuring Fasting Glucose, Insulin and HbA1C.

To assess insulin resistance HOMA-IR and HOMA-B tests will be performed based on insulin and glucose levels.

To assess inflammation we will perform a blood count and the serum levels of CRP (MF) and IL6 will be measured.

The level of bone turnover markers will be evaluated by measuring P1NP and CTX and bone derived hormones will include total osteocalcin, undercarboxylated osteocalcin, sclerostin and lipocalcin-2.

The data from the whole OI cohort will be compared to the overall population and to the group of siblings. The OI cohort will be divided to overweight and healthy weight cohorts to determine how these groups are differed in respect of symptom severity and metabolic perturbations. Data will be adjusted for age and gender.

For the OI cohort partial correlations between biomarker levels and clinical outcomes, adjusted for age and other relevant background variables will be tested.

**Pitfalls and alternative approaches Aim 3.**

Aim 3a will provide clinical validation for Aim 2, and is expected to show that patients with OI type XIV are frequently obese. It will also test the association between obesity, bone turnover, insulin resistance and symptom severity in this very variable form of OI. We recognize that the cohort of patients with OI type XIV is relatively and the variability of the measured parameter of these patients could be high because of the variability in the severity of symptomes in this specific type of OI. Nevertheless, to the best of our knowledge this is the large group of OI type XIV to be reported and our preliminary data together with other reports and together with consistent unpublished observations of the medical staff in soroka medical center that treat these patients point for a strong correlation between OI type XIV and obesity. To overcome the challenge to obtain meaningful statistical data we plan to use different types of comparsions with the advice of X an experienced biostatistician (see letter of support).

**Available resources**

An established colonies of Col1a2 tm1.1Mcbr and Col1a1 (2.3 kb)-CreERT2 mice in the-BGU animal facility is managed by us and is available for the proposed research. UBC-Cre-ERT2 has been purchased from The Jackson Laboratory (Bar Harbor, ME, USA), and we are in the process of establishing colonies of these mice to be crossed with floxed TMEM38B mice. My lab is fully equipped for molecular biology, biochemistry and bone histology studies. The lab is equipped with a StepOne RTPCR, Leica automated motorized microtome, Olympus and Zeiss fluorescent inverted and upright microscopes. A skyscan 1174 microCT scanner in the Department of Biotechnology Engineering of Ben-Gurion University of the Negev is available for my use as needed. Metabolic cage system (Promethion High-Definition Behavioral Phenotyping System ,Sable Instruments, Inc., Las Vegas, NV) is available to us through BGU animal facility.

**References**