# Exercise as a mean to reverse the detrimental effect of high-fat diet on bone's fracture characteristics

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**ABSTRACT.** The aim of this study is to investigate whether exercise can reverse some of the adverse effects of high-fat-diet-induced obesity on lipid metabolism and bone biomechanical properties. A total of 26 adult male C57bl/6J mice were randomly assigned into three groups: (A) Control group (n=6), (B) High-fat diet group (n=10), (C) High-fat diet and exercise group (n=10). Body mass and relevant biochemical parameters were measured for the duration of the experimental protocol (37 weeks). Mechanical strength of both femurs of each animal was assessed in-vitro based on three point bending tests. It was revealed that exposure to high-fat diet led to significant increase of body mass and cholesterol levels and also to substantial changes in bone morphology and strength. Ultimate stress for the animals exposed to high-fat diet and those exposed to high-fat-diet and exercise was 25% and 24% lower compared to control, respectively. Exercise increased bone thickness by 15% compared to animals that were not exposed to exercise. It was concluded that high-fat-diet appears to have a detrimental effect on bone biomechanics and strength. Exercise reversed the reduction in bone thickness that appears to be induced by high-fat diet. However no statistically significant increase in bone strength was observed.

**KEYWORDS.** Bone biomechanics; Mice; Femur; High-fat-diet; Three-point bending; Bending strength.

## INTRODUCTION

Obesity's adverse effects on health include increased risk for diabetes (type-2), heart disease and certain types of cancer [1] leading to poor quality of life and ultimately to reduced life expectancy [2]. The continuous rise in its prevalence worldwide has highlighted obesity as one of the major epidemics of our time [3].
With regards to the risk for bone fracture, obesity has been traditionally believed to have a protective role [4,5]. Moreover a significant number of studies reported a positive relation between Body Mass Index (BMI) and bone density [6]. However, the aforementioned classic view on the effect of obesity has been put into question from findings that link obesity to the loss of bone mass and osteopenia [7, 8] and studies highlighting lean body mass as a stronger determinant of bone density in men than BMI [9, 10].
Currently adipose tissue (i.e. body fat) is considered to be hormonally active with a pivotal role with regards to energy homeostasis and metabolism and not just an organ for storing excess energy [11]. More specifically, adipose tissue has been found to produce and secrete numerous substances including the hormone adiponectin. Adiponectin is exclusively secreted by adipose tissue and appears to be linked to increased insulin sensitivity and to have anti-atherogenic and anti-inflammatory properties [12]. The levels of plasma adiponectin are strongly associated with BMI and appear to be higher in obese subjects compared to lean subjects [13, 14].
Animal models have been widely used for the investigation of the effect of obesity, nutrition and exercise. According to these models obesity is induced by subjecting the animals (mainly rats or mice) to a high-fat diet (HFD) [15].
According to literature one of possible ways to prevent bone mass loss is exercise [16, 17]. Besides of its overall positive effect on health, exercise is considered to positively influence bone microstructure [18] and improved strength [19, 20].
In this context the aim of this study is to assess the effect of HFD - induced obesity on bone biomechanics and biochemical measurements and investigate whether exercise can reverse its potentially negative effects.

## MATERIALS AND METHODS

### Selection and description of animals

A total of 26 male c57bl/6 mice, aged 10-11 weeks, were used. The mice were housed in groups of three in the Animal Housing Facility of the Laboratory of Experimental Surgery and Surgical Research "N.S. Christeas", National and Kapodistrian University of Athens, in a controlled environment. All conditions followed National and European legislation and standards, including cages (Tecniplast S.p.a., Italy) and the environment with 55% relative humidity, central ventilation (15 air changes/h), temperature of 20°C ± 2°C and artificial 12-h light-dark cycle. Access to food and water was ad libitum. The experimental protocol was approved by the Ethics Committee of the local Veterinary Directorate.
Following acclimatization, the rodents were randomized and allocated into three groups: Control group (Group A, n=6), which received a standard chow diet for 37 weeks; High Fat Diet (HFD) group (group B, n=10), which received a high fat diet (standard chow diet enriched with 45% fat) for 37 weeks; High Fat Diet and Exercise (HFDE) group (group C, n=10), which received the same diet as group B for 37 weeks and ran on a treadmill three times a week for the last nine weeks of the experimental protocol.

### Treadmill exercise

The duration of the exercise of group C was nine weeks in total. A specially designed treadmill was used (Columbus Instruments, USA, Model: Exer-3/6). An escalation of the vigorousness of exercise was followed. More specifically, the first two weeks were characterized as the adjustment period. Meanwhile, the mice began to run at speed of 5 m/min and gradually (additional 5 m/min per time) reached the speed of 30 m/min at the end of the second week. This was their final running speed until the end of the study. Each exercise session lasted precisely 30 minutes.

### Biochemical measurements

Blood samples were collected at baseline, at 12 weeks, at 28 weeks and at the end of the study (37 weeks) prior to euthanasia following a 12-h fast of the animals. Animals were anesthetized with ether and a quantity of approximately 500 μl of blood was collected from the ocular canthus of each mouse. Blood was collected in Vacutainer tubes (BD Diagnostics, NJ, USA). Serum was separated by centrifugation at 3000 rpm for 10 minutes and was stored at -20°C until analysis.

Total serum cholesterol (T-CHOL), high-density lipoprotein cholesterol (HDL-C) serum triglycerides (TG) and serum glucose concentrations were determined enzymatically with commercially available kits (Biosis Biotechnological Applications, Athens, Greece). Due to the nonconfirmed validity of the Friedewald formula for the calculation of low-density lipoprotein (LDL) in rodents this parameter was not included in our study. Moreover, serum adiponectin levels (ADIPO) were estimated with enzyme-linked immunosorbent assay (Mouse Adiponectin ELISA Kit, Intra-assay CV 5.3%, Inter-assay CV 9.9% ABCAM, Cambridge CB4 0FL UK).

*Mechanical testing*

After euthanasia, both left and right femur of each animal were resected and stored in gauze immersed into N/S 0.9%. The mechanical behaviour and the strength of the specimens was assessed from three point bending tests. All biomechanical tests were performed within four hours from the time the samples were harvested.

Mechanical testing was performed with the use of an electromechanical uniaxial load frame (INSTRON) which was equipped with a high accuracy tension-only load cell (50 N, INSTRON). Because of the use of a tension-only load cell, a custom device had to be used to transform tensional loading to three point bending (Fig. 1). This device comprises two main parts: Part A which was fixed to the load frame's base and B which was attached to its movable crosshead. Part A included the centrally placed cylindrical pin while Part B included the two support pins (Fig. 1). The diameter of all three pins was 2 mm and the distance between the two support pins was 14 mm. To improve the reliability of the testing procedure the distance between the stationary central pin and the movable support pins was directly measured using a lased micrometer (Keyence LS-3000) (Fig. 1). All samples were loaded with a displacement rate of 5 mm/min until failure. The sampling frequency for the distance between the central and the support pins as well as for the force was 3 Hz.
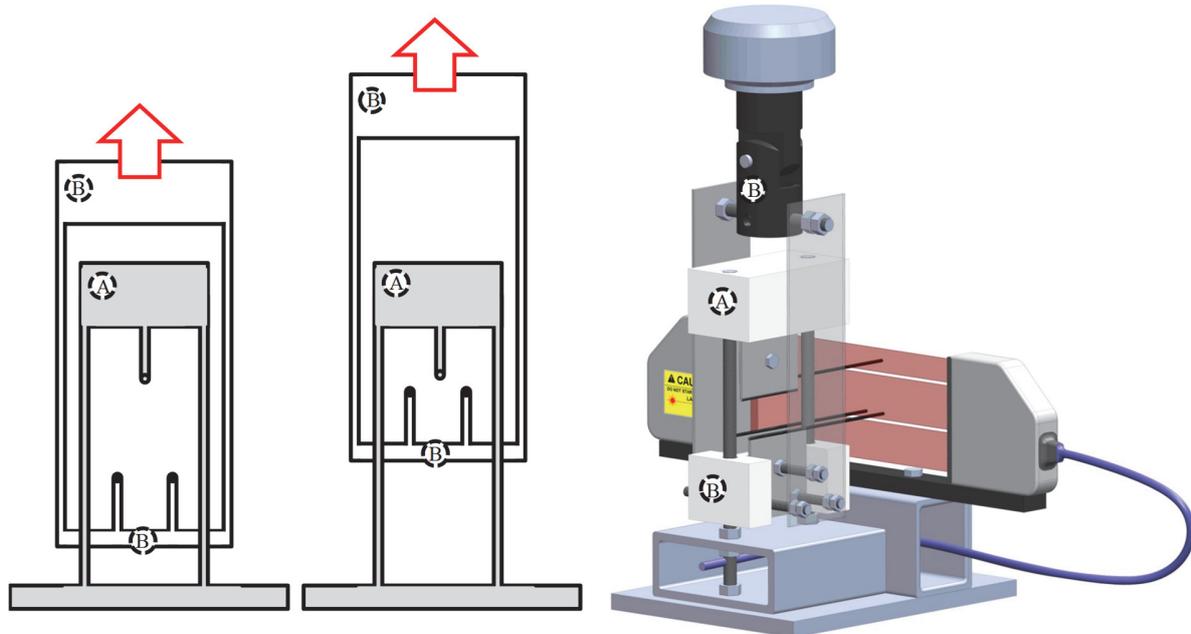


Figure 1: The custom device that was used to perform three point bending tests with a tension only load cell. The device comprises two parts: (A) which was fixed to the load frame's base and (B) which was attached to its movable crosshead.

Before testing, the maximum and minimum external thickness of each sample was measured at the central part of their diaphysis using a digital calliper. Assuming that the cross-section of the diaphysis is elliptical means that the aforementioned maximum and minimum external thickness correspond to the major axis (a) and minor axis (b) of the ellipse respectively (Fig. 2). After the end of the test the actual thickness of the bone cross-section was also measured on the surface of fracture. The measurement of wall thickness was repeated four times for each sample: two at opposite sides of the sample's major axis ($t_1, t_2$ in Fig. 2) and two at opposite sides of the sample's minor axis ($t_3, t_4$ in Fig. 2). In the end, these four measurements were used to calculate the average thickness of the sample (t) on the surface of failure.

The recorded data in terms of force were used to find the maximum force that was sustained by each sample, namely the fracture force. The force data combined with the measurements of the distance between the central and support pins were used to draw the force/deflection curve of each test and calculate the stiffness of each sample and also their energy to

failure. Sample stiffness was calculated as the slope of the linear part of the force/deflection curve while energy as the area below the curve. Ultimate stress was also calculated based on the assumption of elliptical cross-section with constant thickness (t) [21].
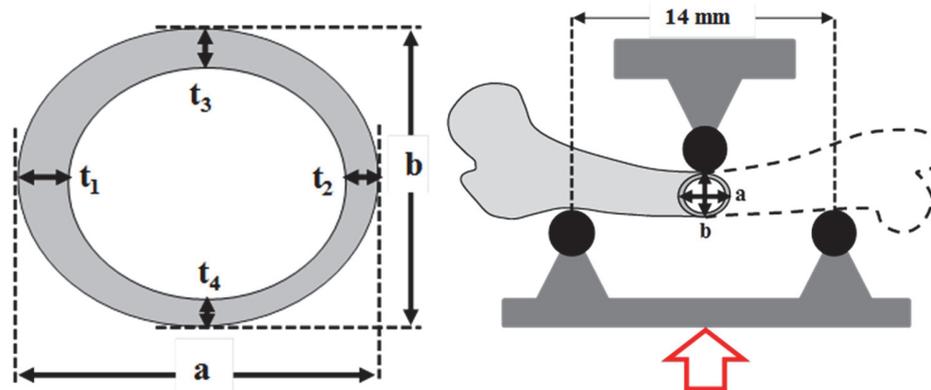


Figure 2: A schematic representation of the three point bending test. The measurement sites for thickness ($t_{1-4}$) and lengths of the major (right) and minor axis (left) of the samples are also presented.

## Statistical analysis

The results for the three groups were compared to each other and the statistical significance of the differences that were observed was evaluated following one way analysis of variance (ANOVA). The level of statistical significance was considered to be equal to 0.05.

The effect of exercise on the biochemical profile and body mass of group C was also investigated. For this purpose one way repeated measures ANOVA (statistical significance level = 0.05) with Bonferroni confidence interval adjustment was used to assess the statistical significance of differences between the measurements that were taken before the start and after the end of the exercise protocol (i.e. week 28 vs 37).

In order to assess the relationship between biomechanical and biochemical parameters and the effect of HFD, the correlation between the average biomechanical measures for each animal (i.e. average for left and right femur) and the biochemical measurements was investigated for groups B and C using Pearson correlation analysis. The correlation between biomechanical measures and body mass was also assessed. All data were tested for linearity, normality and homoscedasticity. The statistical analyses were performed using IBM® SPSS®v.21.

## RESULTS

### Biochemical measurements

At the beginning of the protocol differences between the three groups were non-significant (Fig. 3), with the exception of TG levels, which were somehow higher in group A (an issue that should be considered further). During week 12, the first changes that can be attributed to HFD are observed in the case of HDL-C, with groups B and C having significantly higher HDL-C levels compared to control (group A). Differences in biochemical parameters become clearer during week 28 when significant differences in terms of body mass are also observed. More specifically, groups B and C appear to have significantly higher body mass and higher levels of HDL-C and T-CHOL compared to control. Group C has significantly higher body mass than group B too (average (±STDEV) body mass for groups A, B and C is equal to 29.3kg (±2.4kg) 35.0kg (±4.2kg) and 39.2kg (±2.3kg), respectively). At the end of the experimental protocol (i.e. week 37) the difference in terms of body mass between control and groups B and C appears to crystallise (average (±STDEV) body mass for groups A, B and C is equal to 27.5kg (±1.4kg) 32.0kg (±3.0kg) and 31.8kg (±3.5kg) respectively). Moreover, groups B and C also have significantly higher levels of T-CHOL compared to control. At the end of the protocol, group C also appears to have significantly higher levels of TG compared to the other two groups (Fig. 3).

At this point it should be highlighted that group C was exposed to exercise only during the last nine weeks of the experimental protocol (i.e. weeks 28-37). Therefore any difference between groups B and C that is observed during the 28th week of the experimental protocol (or earlier than that) cannot be attributed to exercise (Fig. 3).

One way repeated measures ANOVA for group C before and after the introduction of exercise showed statistically significant:

- Decrease in body mass (Wilks' Lambda=0.276, F(1,9)= 23.649, p=0.001)
- Decrease in the levels of T-CHOL (Wilks' Lambda=0.357, F(1,9)= 16.97, p=0.003)
- Decrease in the levels of HDL-C (Wilks' Lambda=0.135, F(1,9)= 57.193, p<0.0005)
- Increase in the level of TG (Wilks' Lambda=0.068, F(1,9)= 124.149, p<0.0005)
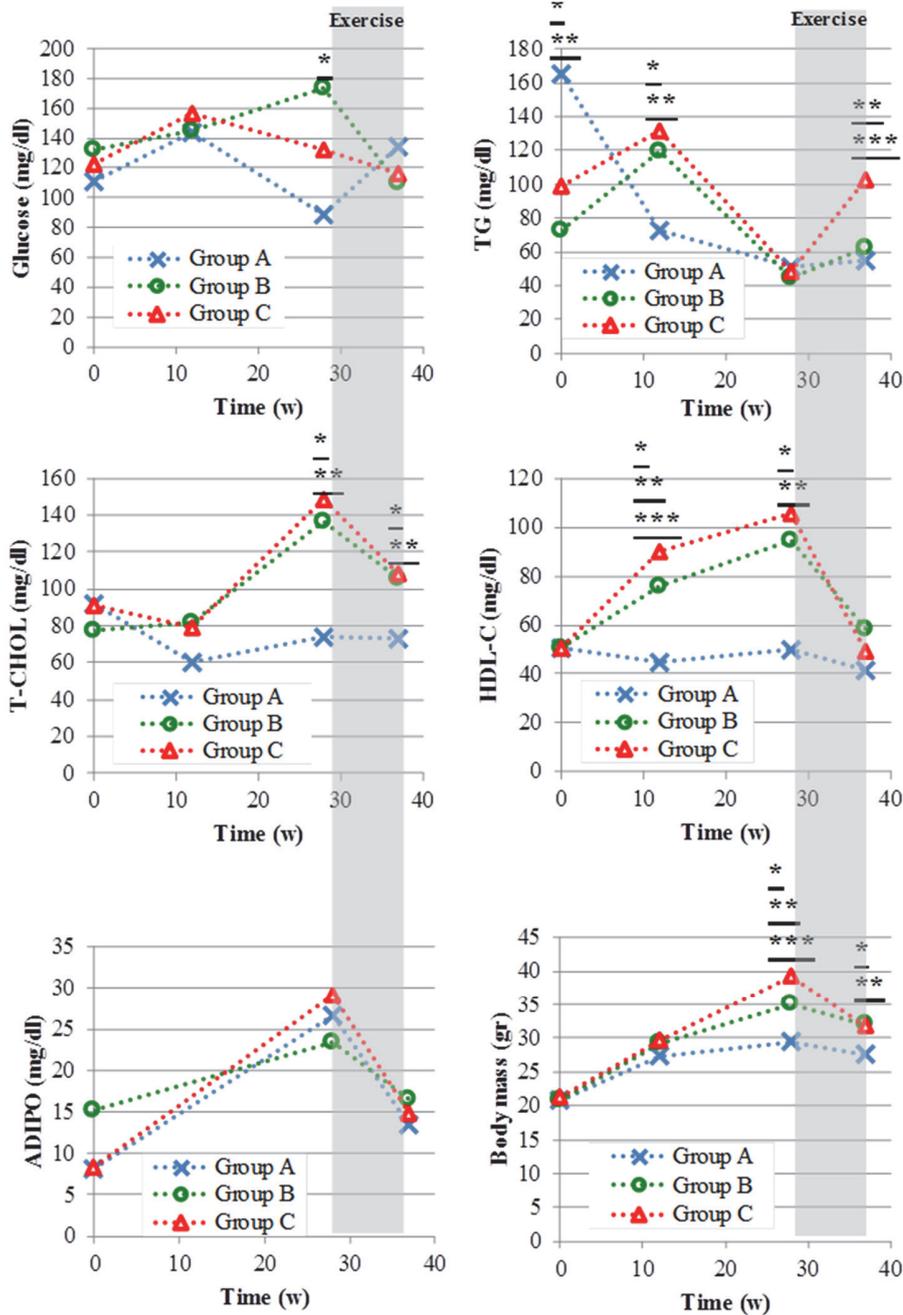- Decrease in the levels of ADIPO (Wilks' Lambda=0.244, F(1,9)= 27.816, p=0.001)



Figure 3: The change in biochemical measures and body mass for the duration of the study. More specifically, biochemical results for the levels of serum glucose, total serum cholesterol (T-CHOL), high-density lipoprotein cholesterol (HDL-C), serum triglycerides (TG) and serum adiponectin levels (ADIPO) are presented. Statistically significant differences (i.e. p<0.05) between groups A and B, A and C and between groups B and C are noted using *, ** or *** respectively. The time period when group C was exposed to exercise (i.e. weeks 28-37) is also highlighted.

*Mechanical testing*

Some of the samples were damaged before testing during harvesting. In the end, the number of sample pairs (i.e. left and right femurs) that were tested was 3, 6, and 10 for groups A, B and C respectively. The average values and standard deviations for all biomechanical parameters measured in the context of this study are presented in Tab. 1.

| Group: | A | B | C |
|---|---|---|---|
| Max Force (N) | 16.4 | 14.2 | 15.6 |
| | *(2.6)* | *(2.6)* | *(2.2)* |
| Stiffness (N/mm) | 77 | 90 | 89 |
| | *(25)* | *(34)* | *(28)* |
| Energy (N*mm) | 3.16 | 2.64 | 3.36 |
| | *(0.85)* | *(0.92)* | *(1.20)* |
| b (mm) | 1.42 | 1.59 | 1.53 |
| | *(0.06)* | *(0.07)* | *(0.11)* |
| a (mm) | 2.05 | 2.19 | 2.16 |
| | *(0.04)* | *(0.10)* | *(0.11)* |
| t (mm) | 0.24 | 0.22 | 0.25 |
| | *(0.03)* | *(0.03)* | *(0.04)* |
| Stress (MPa) | 58 | 43 | 44 |
| | *(12)* | *(7)* | *(11)* |

Table 1: The average values for the biomechanical parameters measured. The respective standard deviations are shown in brackets.

One way ANOVA revealed statistically significant differences between the three groups in terms of minor axis length (b), major axis length (a) and thickness of the cross-section (t) and also in terms of ultimate stress (Fig. 4). No statistically significant difference was found in terms of force, stiffness or energy to failure.
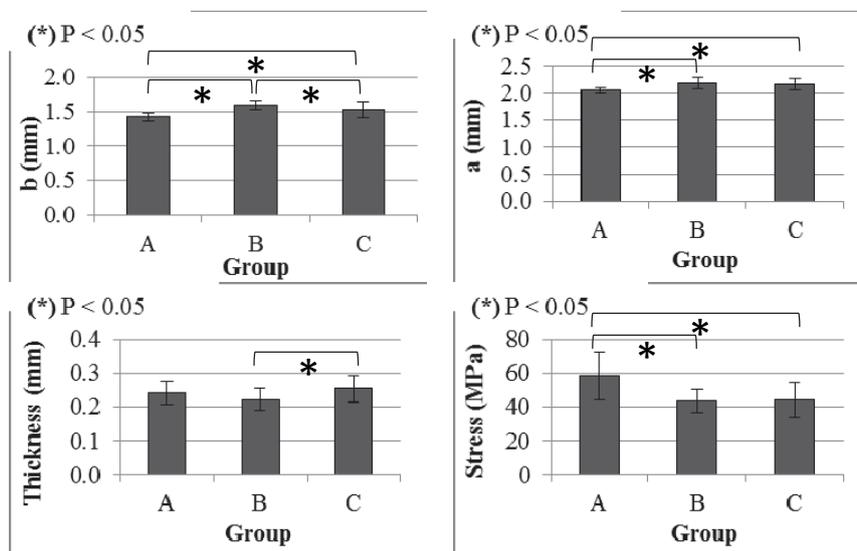


Figure 4: Comparative results in terms of bone morphology and mechanical strength. The lengths of the minor axis (b), of the major axis (a), of cortical shell thickness and of ultimate stress are presented.

More specifically the average minor axis (b) of group A was smaller than group's B and C by 12% (p=0.040) and 7% (p<0.001) respectively. The respective difference between groups B and C was 4% (p=0.041) with the minor axis of group

C being the smallest of the two groups. The major axis of group A was also smaller that group B and C by 7% (p=0.026) and 5% (p=0.004) respectively. The thickness of group C was bigger than group B by 15% (p=0.010). Finally the ultimate stress of group A was higher than B and C by 25% (p=0.021) and 24% (p=0.001) respectively (Fig. 4).

Pearson correlation analysis revealed a strong positive correlation between serum glucose and fracture force (r=0.560, N=18, p=0.016) and between glucose and energy (r=0.660, N=18, p=0.008) (Fig. 5a,b). Body mass was strongly and positively correlated to fracture force (r=0.606, N=18, p=0.008) and negatively correlated to minor axis length (b) (r=-0.644, N=18, p=0.004) (Fig. 5c,d).  T-CHOL was negatively correlated to minor axis length (r=-0.644, N=18, p=0.011) (Fig. 5e). The aforementioned correlations indicate that fracture force tends to be higher in animals with higher glucose levels and in animals with higher body mass. Moreover, fracture energy tends also to be higher in animals with higher glucose levels while the minor axis (b) appears to be smaller in animals with higher levels of T-CHOL and in animals with higher body mass.
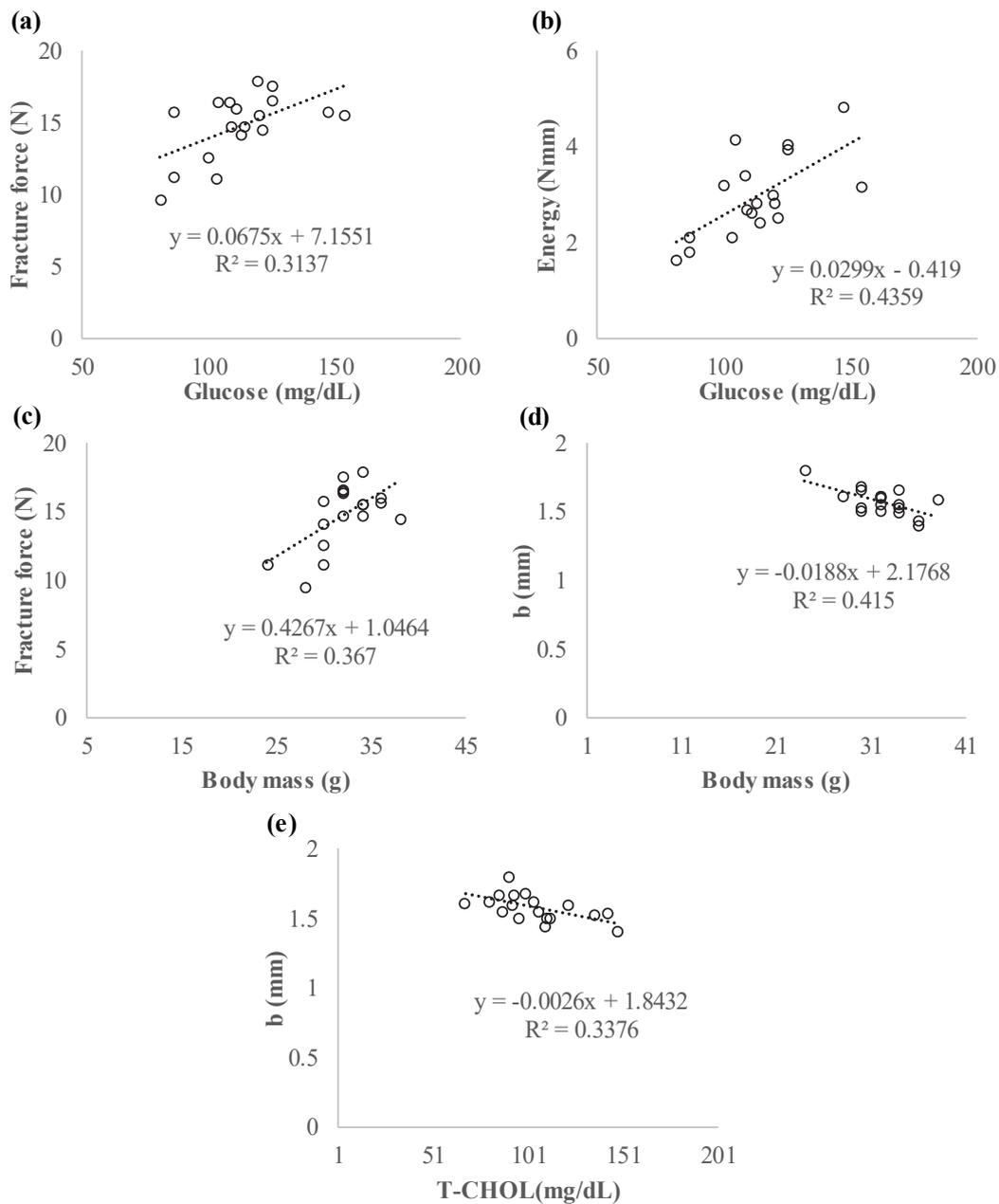


Figure 5: Correlations between biomechanical parameters, body mass and biochemical measurements.

## DISCUSSION AND CONCLUSIONS

This study aimed to assess the effect of obesity and exercise on bone biomechanics and biochemical measurements and investigate the potentially beneficial role of exercise. For this purpose, a well-established mouse model of HFD–induced obesity was used [7, 15]. More specifically, mice were randomly assigned in three groups, namely control (group A), HFD with no exercise (group B) and HFD with exercise (group C). Body mass and relevant biochemical parameters were measured for the duration of the study (i.e. 37 weeks).

During the first 28 weeks of the experimental protocol groups B and C were exposed to exactly the same conditions. Indeed, exercise was not introduced to the protocol until the end of the 28th week. This means that analysing the results for the first 28 weeks enables only the assessment of the effect of HFD on body mass and the biochemical profile of the animals. Any difference between groups B and C up to week 28 could be attributed to variations that are inherent in in-vivo testing.

As expected HFD had a significant effect on body mass [15]. More specifically, the animals that received HFD gradually increased their body mass relatively to control with statistically significant differences appearing during the 28th week of the study. With regards to the biochemical measurements, HFD appears to consistently lead to higher levels of cholesterol (T-CHOL and HDL-C).

One way repeated measures ANOVA for group C indicates that the introduction of exercise is followed by some changes, including a drop in body mass, increase in TG levels etc. However, in most cases these changes are not substantial enough to make group C significantly different compared to group B (Fig. 3). The fact that the introduction of exercise didn't appear to lead to substantial changes in body mass and the biochemical profile of group C could be attributed to the specific exercise protocol employed in this study and its relatively limited duration (i.e. 9 weeks). Indeed, there is evidence in literature that exercise of different intensity, frequency and duration can lead to different results in animal HDL-induced obesity models [20].

The levels of adiponectin were also not affected by HFD or exercise. Adiponectin has been reported in literature to have a positive effect on bone properties by activating osteoblastogenesis and suppressing osteoclastogenesis, thus leading to increased bone mass [22]. However, the fact that the results of the present study didn't reveal any statistically significant difference between groups with regards to adiponectin levels means that no relevant conclusion can be drawn.

So far, the effect of exercise and diet on bone strength has been either inferred based on non-invasive measurements and/or computer modelling [23] or directly measured through in-vitro testing [19, 20, 24, 25]. The most commonly used testing techniques are three point bending and torsion which are typically used to measure the maximum sustained force [25] or moment [20], respectively as a measure of strength. These studies have indicated that obesity and exercise can affect both the structure and also the mechanical characteristics of bones [19, 23, 24]. Assessing ultimate stress along with the maximum sustained force enables separating the effect of changes in geometry from changes in the actual material properties of bone tissue [19, 24].

In order to enable the calculation of ultimate stress from the measured fracture force, the cross-section of the specimens was considered to be elliptical with constant thickness [21]. This simplification was deemed to be necessary considering the small size of the samples. Indeed, the longest cross-sectional distance was smaller than 3 mm.

The results from biomechanical testing indicated that the morphology and mechanical strength of femurs was significantly affected by diet and exercise. In terms of morphology, HFD appears to increase the external dimensions of femur regardless of the exposure to exercise. However, the group that received HFD but was not exposed to exercise (group B) also appeared to have significantly lower bone thickness compared to the other two groups. These findings indicate that exercise tends to limit the HFD-induced loss of bone mass by increasing the thickness of the femurs' cortical shell. Interestingly though, this positive effect of exercise was not translated into increased ultimate stress. Hence, both groups that received HFD had significantly lower ultimate stress relatively to control. No statistically significant difference was found in terms of fracture force or total energy.

The aforementioned findings are in agreement with observations linking obesity to the loss of bone mass and osteopenia [7, 8]. High-fat diets in particular have been found to reduce the ability for calcium absorption with possible adverse effects on bone mineralization in growing animals [8].

An investigation of correlations between biomechanical parameters, body mass and biochemical measurements that was focused only on the mice that received HFD (i.e. groups B and C) revealed strong positive associations between fracture force and serum glucose and body mass. More specifically, the femurs of mice that, at the end of the protocol, had higher body mass or glucose levels were found to be stronger compared to mice with lower body mass or glucose levels.

One of the key limitations of the present study is the relatively small number of femurs that were included in the biomechanical testing. Due to the small size of the samples a significant number was damaged during harvesting making it impossible to use them for testing.

In conclusion, the results of this study suggest that exercise can partially reverse the detrimental effects of HFD on bone biomechanics by increasing the thickness of the femurs' cortical shell and thus limiting the HFD-induced loss of bone mass. However, the aforementioned positive effect of exercise was not translated to increased bone strength. Further in vitro/in vivo studies in experimental models and clinical trials are required to unveil the effect of HFD and exercise on bone metabolism and strength.

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