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"Effects of fiber enriched high carbohydrate diet (FEHC) on bone and muscle health in elderly obese subjects"

D.ssa ***Francesca Cannata***

Coordinatore

Chiar.mo Prof. Nicola Napoli

Tutor

Chiar.mo Prof. Paolo Pozzilli

Francesca Cannata

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ABBREVIATION

ACTH	Adrenocorticotropin Hormone
AEE	Activity Energy Expenditure
APC	Axin and adenomatous polyposis coli
BEE	Basal Energy Expenditure
BMD	Bone Mineral Density
BMI	Body Mass Index
BMP2	Bone Morphogenetic Protein 2
BMR	Basal Metabolic Rate
BSAP	Alcalin Phosphatase
CHD	Coronary Heart Disease
CRP	C-Reactive Protein
CTX	C-telopeptide
CVD	Cardiovascular disease
DAG	Diacylglycerol
DKK-1	Dickkpof-1
ECS	Endocannabinoid System
EEPA	Energy Expenditure linked to Physical Activity
ET	Endogenous Thermogenesis
FEHC	Fiber Enriched High Carbohydrate Diet
FFA	Free Fatty Acids
GLP-1	Glucagon-Like Peptide-1
GLUT4	Glucose Membrane Transporter 4
GSK3-β	Glycogen synthase kinase 3-β
HDL	High Density Lipoprotein
HR	Hazard Ratio
IGF-1	Insulin like Growth Factor-1
IGT	Impaired Glucose Tolerance
IL	Interleukin
IMCLs	Intromyocellular Lipids
IKKb	Inhibitor of nuclear fctor kappa-B kinase subunit beta
IR	Insulin Resistance
JNK	Jun N-terminal kinase
LDL	Low Density Lipoprotein

MCP	Monocytes Chemoattractant Protein
MSCs	Mesenchymal Stem Cells
mTOR	Mechanistic Target Of Rapamycin
NEAT	Non-exercise Activity Thermogenesis
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHANES	National Health and Nutrition Examination Survey
NTX	N-telopeptide
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OSAS	Obstructive Sleep Apnea Syndrome
PAI-1	Plasminogen Activating Inhibitor 1
PYY	YY Peptide
PTH	Parathyroid hormone
P1NP	Procollagen type 1 N-term
RANKL	Receptor Activator of NFκB Ligand
SCAT	Subcutaneous Adipose Tissue
SCFA	Short chain fatty acids
TDEE	Total Daily Energy Expenditure
TEF	Thermal Effect of the Food
TG	Triglycerides
TGFb/BMP	Transforming growth factor-beta /bone morphogenetic protein
TNFα	Tumor Necrosis Factor Alpha
TRACP5b	Tartrate-Resistant Acid Phosphatase 5b
T2D	Type 2 Diabetes
VAT	Visceral Adipose Tissue
WHO	World Health Organization

Abstract

Obesity is an increasing, global public health issue. Patients with obesity are at major risk for developing a range of comorbid conditions, including cardiovascular disease (CVD), gastrointestinal disorders, type 2 diabetes (T2D), respiratory problems, and psychological issues, which may significantly affect their daily lives as well as increasing mortality risks.

Obesity and aging have additive effects on chronic inflammation and thus may further contribute to the age-related obesity complications [1], including enhanced osteoclastic activity and sarcopenia [2-5]. In the last years, accumulating evidences suggest that obesity increased risk of fracture. Fractures in obese women are associated with greater morbidity [3] and postoperative complications.

A key role in these processes is played by the WNT signaling, which expression enhances osteoblastogenesis, myogenesis, while prevents adipogenesis. With aging, however, WNT expression is downregulated favoring adipogenic pathway and inflammatory state. New evidence supports also a link between Endocannabinoid system (ECS) dysregulation, and WNT signaling on determining inflammation associated with obesity. [6,7]

It has been shown that a fiber enriched high carbohydrate diet (FEHC) improved metabolic outcomes and inflammation parameters compared to a standard diet. [8,9] Furthermore, this diet approach modulated gut dysbiosis counteracting the increase of possible pro-inflammatory species. [10] However, previous data show that standard weight loss intervention increased bone resorption and in turn bone and muscle loss. [11,12]. The primary aim of this study was to test FEHC diet in elderly obese subjects

on improving obesity-related inflammation on bone, muscle and adipose tissue, through a positive modulation of WNT and ECS pathway.

Elderly subjects with obesity were enrolled prior total hip arthroplasty. Prior to procedure they were randomized to a 3-month diet intervention, according to 1) Control group 2) FEHC diet group. After the procedure, subjects were followed according to randomization for other 6 months with an isocaloric dietary regimen, different in macronutrients composition. Inflammation parameters was assessed at baseline and after three months in serum; WNT and ECS pathways were assessed femoral bone, adipose tissue and skeletal muscle after surgery.

CHAPTER 1: **Obesity**

1.1 Obesity and its classification

Obesity has been described as a chronic condition characterized by abnormal or excessive body fat accumulation [13]. The prevalence of obesity has markedly increased since the 1970s in the United States and the National Health and Nutrition Examination Survey (NHANES) revealed that the obesity prevalence in adults was 33.8% in 2007- 2008, a number which had more than doubled as compared to that in 1976-1980 [14,15]. According to the World Health Organization (WHO) (European Health Report, 2012), 2.8 million people die each year because they are overweight or obese. In global terms, the prevalence of obesity almost doubled between the years 1980 to 2008. There are different measures of obesity, among which body mass index (BMI) is most commonly used [16]. BMI is defined as a person's weight (in kilograms) divided by the square of his or her height (in meters). Since BMI is the same for both genders and all ages of adults and significantly correlated with total body fat content, it provides the most useful population-level measure of overweight and obesity [17]. The WHO defines a BMI greater than or equal to 30.0 kg/m2 as obesity. There are three grades of obesity, which are defined according to the different BMI levels (**Table 1**).

Category	BMI (kg/m^2)
Obese (Class I)	30.0 – 34.9

Obese (Class II)	35.0 – 39.9
Obese (Class III)	≥ 40.0

Table 1. Obesity classes according to BMI.

1.2 Epidemiology

Until recently, obesity and overweight have been a problem of developed countries. Currently, this problem concerns developing countries as well as and at the same time all socio-economic groups. According to the report of the World Health Organization (WHO) of January 2015, the number of subjects with obesity has tripled since 1980 and currently reaches epidemic proportions.

According to WHO, in 2014, more than 1.9 billion of world population over 18 were over- weight, which is 39%, among them, 600 million were obese (13% of the population, including 11% of men and 15% of women). The highest percentage of subjects with obesity is in the United States, where, according to the World Obesity Federation report from 2015, over 60% of adults are overweight and over half of them are obese. In Europe, approximately 150 million adults (20% of the population) and 15 million children and adolescents (10% of the population) suffer from obesity. It is estimated that by 2030 one child in 10 will be obese. It has been proven that along with the increase in BMI the risk of obesity complications, including early death, increases [18,19]. The World Health Organization warns that obesity is responsible for 10-13% of deaths, and the majority of the population lives in countries where overweight and obesity is the cause of death of a larger percentage of society than malnutrition. Obesity leads to a shortened life expectancy of 5-20 years depending on

its severity as well as patient's age, sex and race [20]. Patients with obesity are more likely to develop diseases such as type 2 diabetes, arterial hypertension, coronary heart disease, strokes or some cancers (endometrium, ovary, breast, prostate, large intestine) [21,22]. 90% of patients with type 2 diabetes are obese or overweight [23]. Long-lasting and, above all, unregulated diabetes leads to a number of changes in the body, especially those of micro and macroangiopathy, which in turn increases the risk of death, myocardial infarction or stroke [24]. Studies show that up to 60% of obese patients also develop fatty liver [25,26], including up to 55% in the pediatric population [27,28]. Although, approximately 20% of obese patients do not find typical changes in the lipid metabolism, most of them present the symptoms of disorders of lipid metabolism, which is associated with an increased risk of developing cardiovascular disease, including myocardial infarction or ischemic stroke [29]. Obesity also increases the risk of pancreatitis, gout, hyperuricemia, sleep apnea syndrome, urolithiasis, in women problems with infertilization and delivery, which is associated not only with hormonal changes (increased estradiol, estriol, testosterone, androstenedione) and disorders of ovulation, but also with systemic diseases accompanying obesity. Health-related consequences are less serious but also disturbing [30]. Reduced mobility, disturbed perception of one's body may increase the risk of depression or personality disorders. In addition, the social and economic costs of obesity are extremely high. In Europe, they absorb up to 6% of health expenditure, depending on the region [31,32]. It should not be forgotten that subjects with obesity are less likely to work professionally due to comorbidities, and children achieve worse results in learning.

Obesity is associated with a significant increase in mortality, with a life expectancy decrease of 5–10 years [18,33]. Many evidences indicate that all CVD-associated

causes and cancer-associated mortalities are significantly increased in individuals with obesity.

1.3 Phenotype in Obesity

Obesity is strongly related to body fat distribution rather than the total amount of body fat. People with abdominal (or central or android) obesity have greater risks than those with gluteofemoral (or peripheral or gynoid) obesity.

The abdominal adipose tissue (around the viscera), also known as visceral adipose tissue (VAT), is different from the one in the subcutaneous areas, i.e., subcutaneous adipose tissue (SCAT). Visceral adipocytes differ from those subcutaneous: they have different endocrine function, lipolytic activity and response to insulin and other hormones. The anatomical and functional differences between VAT and SCAT explain the metabolic and cardiovascular increased risk related to abdominal obesity. Visceral fat is associated with hyperglycemia, hyperinsulinemia, hypertriglyceridemia, impaired glucose tolerance (IGT), and alterations in the blood lipids (increased levels of total plasma cholesterol and triglycerides with reduced HDL levels).

Due to its anatomical position, visceral fat is drained to the liver via the portal vein, while in SCAT the venous blood is drained via the venous system. The free fatty acids (FFA) produced by VAT enter the portal venous circulation, they are directed to liver and muscle, and this alters insulin signaling and glucose metabolism. In the same way, adipokines produced by VAT activate immune system through inflammatory mediators, such as C-reactive protein.

Adipocytes are the main cellular component of adipose tissue. They also are the main stores of energy as triglycerides (TG): adipocytes absorb FFA in TG in the post-prandial, so they become larger and dysfunctional at the main time. The difference between VAT and SCAT is that the former contains more large adipocytes and fewer small adipocytes, while the latter is richer in small ones.

The large adipocytes are insulin-resistant, hyperlipolytic and resistant to the anti-lipolytic effect of insulin. This disfunction in adipocyte's metabolism cause insulin-resistance in liver and muscle, endothelial disfunction and an increased atherosclerotic risk (due to increased levels of LDL cholesterol and TG), pillars of the metabolic syndrome. This one is often linked to visceral obesity [34].

Furthermore, VAT promotes a chronic inflammatory grade by producing inflammatory cytokines, such as Tumor Necrosis Factor Alpha (TNFα). This increases FFA and gets worse what has just been described [35,36].

1.4 Aetiology

Obesity is a multifactorial disease. It is the result of the interaction between genetic, physiological, metabolic, but also behavioural, psychological, social, as well as economic, political, and even environmental components. The environment that people live and work in lead them to inappropriate eating habits, sedentary lifestyle, and lack of physical activity. All of that increases the risk of obesity [37].

Furthermore, it is known that changes in dietary and physical activity patterns are often the result of environmental and societal changes associated with development

and lack of supportive policies in sectors such as health, agriculture, transport, urban planning, environment, food processing, distribution, marketing, and education.

However, according to WHO, the main cause of obesity is still an energy imbalance between calories consumed (food intake) and calories expended. The result is in the accumulation of calories as triglycerides. These become fat storage in adipose tissue.

The WHO defines individual's total daily energy expenditure (TDEE) as "the energy needed to balance energy expenditure to maintain body size, body composition and a level of necessary and desirable physical activity consistent with long-term good health". TDEE is composed by basal metabolic rate BMR, or basal energy expenditure (BEE), endogenous thermogenesis (ET) which includes the thermal effect of the food (TEF) and energy expenditure linked to physical activity (EEPA) [38].

BEE is the energy expenditure for life essential's functions in standard conditions and in a state of mental relaxation in an ambient environmental temperature that does not elicit heat-generating or heat dissipating processes. ET includes the thermic effect of food because eating requires energy for the ingestion and digestion of food, and for the absorption, transport, interconversion, oxidation, and deposition of nutrients. AEE is the sum of energy expenditure with physical activity and non-exercise activity thermogenesis (NEAT) [38,39].

There are different thermogenic features which should be considered in TDEE, such as expenditure due to altered body temperature, drug intake or some states of mind [39].

Several studies have shown that obesity is associated with a higher energy intake than energy expenditure [40]. Food's quality is extremely important: incorrect diet

structure, eating habits and imbalance of dietary nutrition characterized by high fat and sugars promote body fat and thus body weight increases.

According to WHO, there is an increase in high-energy, high-fat, and high-sugar foods worldwide joined by lack of physical activity and sedentary lifestyle.

1.5 Pathogenesis of Obesity

The basic problem is in eating habits, which have changed significantly in recent decades: irregularity of meals, their inappropriate distribution during the day and a small variety of diet, imbalanced proportions between specific groups of products or excessive consumption of certain product groups, especially fats and mono-saccharides. In 1961, the daily calories consumed per person was 2,300. In 1998, it increased to 2,800, and in 2015 exceeded 3,000. In addition, the total amount of available food increases, which is accompanied by a decrease in its price. At the same time, the intake of fruits and vegetables decreases. According to the WHO European Office, only 30% of boys and 37% of girls age 13 to 15 eat fruit every day. The second main problem regarding the continuous decline in physical activity in the population of developed and developing countries, which is supported by the environment in which we live (home, work, school, means of transport). Physical activity of at least two thirds of the European population is not satisfactory and is continuing to decline. WHO recommends moderate physical activity for adults at least 30 minutes a day, children at least 60 minutes a day. In addition, to what has been said above, it is important to mention other factors such as genetic, non-genetic biological, pharmacological and psychological. There are many genetic disease syndromes in which excessive fat accumulation occurs, such as the Prader-Willi syndrome, Turner's

syndrome, and von Gierki's disease. In a small percentage of the population, monogenic mutations leading to the development of obesity occur. In the majority, however, obesity results rather from the mutation of many genes associated with energy intake with food, the level of basic metabolism, and the activity of enzymes responsible for lipid and carbohydrate metabolism. Non-genetic biological agents also play a huge role in the development of obesity. Endocrine disorders, in which excessive weight gain occurs, are primarily a deficiency of growth hormone, hypothyroidism, hypoparathyroidism, Cushing's syndrome, polycystic ovary syndrome or hyperinsulinism. Also, many medications taken regularly result in weight gain. Those include antidepressants (amitriptyline, doxepin, mirtazapine, mianserin), anxiolytic, neuroleptic (phenothiazine derivatives, olanzapine, risperidone), antiepileptic (valproic acid, carbamazepine), corticosteroids or insulin. The occurrence of obesity is also connected with changes in the neurohormonal balance. Ghrelin is a neuropeptide hormone secreted mainly by the cells of the fundus of the stomach, and in a smaller amount in the initial section of the small intestine, hypothalamus, pituitary gland or pancreas. In the stomach, it is not secreted into the lumen of the digestive tract, only to the blood vessels. Nerve cells containing ghrelin receptors are found in the arcuate nucleus of the hypothalamus, which is responsible for the regulation of appetite. Its concentration increases during starvation, especially just before a meal, and decreases under the influence of food [41,42]. Ghrelin also has a negative correlation with the concentration of glucose and insulin in the blood. In addition, it increases the production of growth hormone, Adrenocorticotropin Hormone (ACTH), cortisol, adrenaline and glucagon acting hyperglycemically. It stimulates hepatic gluconeogenesis and inhibits insulin secretion [43]. It also increases the uptake of glucose and triacylglycerols by adipocytes by stimulating lipogenesis. What is more, it reduces the secretion of adiponectin, which reduces the

level of triacylglycerols, LDL cholesterol and increases HDL [44]. Glucagon-like peptide-1 (GLP-1) belongs to the group of intestinal enteric hormones and is secreted by the L-cells of the final section of the small intestine in response to food intake. It stimulates glucose-dependent insulin secretion, delays gastric emptying, inhibits glucagon secretion and hepatic glucose production [45-47]. The YY peptide (PYY) is secreted in the L-cells of the distal jejunum as well as through the colon and ileum cells in response to the meal being taken. The release of PYY occurs already at the beginning of the meal, even before the food content reaches the intestine, which is probably related to the nervous mechanism. In the next stage, hormone secretion depends on the type of food content and is proportional to its calorie content. Significantly higher postprandial increase in peptide concentration is obtained in fatty food, compared with food with the same calories amount, but protein or carbohydrate.

The concentration of PYY increases, reaching a plateau after 1-2 hours after food intake and remains elevated to 6 hours. The enzymatic modification of the YY peptide occurs in the peripheral circulation. Due to its ability to cross the blood-brain barrier, its target is the hypothalamic arcuate, where it plays an important role in the regulation and control of food intake [48]. The YY peptide also has its peripheral activity. It participates in the regulation of gastrointestinal peristalsis by inhibiting the secretory function of the stomach and pancreas, delaying gut motility and gastric emptying, which reduces appetite [49].

Leptin is a hormone produced mainly in adipose tissue in a proportional amount to its mass [50]. It works by reducing appetite, increasing gluconeogenesis and lipolysis in adipose tissue, which in turn causes an increase in the level of FFA in the blood. In addition, leptin inhibits insulin production and the transport of glucose to adipocytes. In subjects with obesity, increased levels of leptin in the blood are detected, but leptin

receptors sensitivity is reduced, which results in the fact that patients do not feel full despite the delivery of a large energy charge [51].

The development of metabolic complications of obesity, type 2 diabetes or hypercholesterolemia is associated primarily with the accumulation of visceral fat. Adipose tissue is the responsible for homeostasis of the body and is an important metabolic organ. The primary function of adipose tissue, which plays a key role in the pathogenesis of insulin resistance, is the ability to store lipids in the form of triglycerides (TG). Free fatty acids, which cannot be stored in fat tissue in the form of TG, get into the blood, and then into the skeletal muscles and liver, where they intensify gluconeogenesis and reduce the creatinine clearance. Excess free fatty acids, on the other hand, inhibit the transport of glucose to skeletal muscles, glucose phosphorylation and its oxidation. In presence of insulin resistance, macrophages of adipose tissue being a source of pro and anti-inflammatory cytokines play an important role.

C-reactive protein (CRP), produced mainly in the liver, was the first described protein indicating inflammation and tissue damage [52,53]. It is believed that obese patients develop a strong correlation between the concentration of C-reactive protein in the blood serum and BMI. Furthermore, a decrease in body weight causes a decrease in CRP [54,55].

1.6 Comorbidities

Obesity is a chronic disease that is associated with a wide range of complications affecting many different aspects of physiology [56-58]. The progression from lean state to obesity brings with it a phenotypic change in adipose tissue and the

development of chronic low-grade inflammation [59]. This is characterized by increased levels of circulating free-fatty acids, soluble pro-inflammatory factors (such as interleukin [IL] 1β, IL-6, TNFα, and monocyte chemoattractant protein [MCP] 1) and the activation and infiltration of immune cells into sites of inflammation [60]. Obesity is usually accompanied to a specific dyslipidemia profile (atherogenic dyslipidemia) that includes small, LDL particles, decreased levels of HDL particles, and raised triglyceride levels [61]. Low-grade inflammation and dyslipidemia profile leads to vascular dysfunction, including atherosclerosis formation, and impaired fibrinolysis. These, in turn, increase the risk for CVD, including stroke and venous thromboembolism [62].

The metabolic and cardiovascular aspects of obesity are closely linked. The chronic inflammatory state associated with obesity is established as a major contributing factor for insulin resistance, which itself is one of the key pathophysiologies of T2D [63]. Furthermore, central obesity defined by waist circumference is the essential component of the International Diabetes Federation (IDF) definition of the metabolic syndrome (raised triglycerides, reduced HDL cholesterol, raised blood pressure, and raised fasting plasma glucose).

Obesity is also closely associated with Obstructive Sleep Apnea Syndrome (OSAS). Most of the conditions associated with obesity such as insulin resistance [64], systemic inflammation, and dyslipidemia are themselves closely associated with OSAS, and concurrently, the obesity-associated deposition of fat around the upper airway and thorax may affect lumen size and reduce chest compliance that contributes to OSAS [65].

The development of certain cancers, including colorectal, pancreatic, kidney, endometrial, postmenopausal breast, and adenocarcinoma of the esophagus, have also been shown to be related to excess levels of fat and the metabolically active nature of this excess adipose tissue [66,67]. Cancers have shown to be impacted by the complex interactions between obesity-related insulin resistance, hyperinsulinemia, sustained hyperglycemia, oxidative stress, inflammation, and the production of adipokines [66].

1.7 Obesity and Insulin Resistance

Insulin resistance (IR) is associated with a metabolic and cardiovascular cluster of disorders (dyslipidemia, hypertension, visceral obesity, glucose intolerance, endothelial dysfunction), which are independent risk factors for CVD [68]. The molecular causes of insulin resistance, impaired insulin signalling through the phosphoinositol-3 kinase pathway with intact signalling through the mitogen-activated protein kinase pathway, are responsible for the impairment in insulin-stimulated glucose metabolism [68]. Insulin resistant state develops one to two decades before the onset of the disease [69-71]. It is the best predictor for later development of the disease [72]. Reducing insulin resistance prevents the development of diabetes [73].

Skeletal muscle and liver are the two-key insulin-responsive organs responsible for maintaining normal glucose homeostasis. To depicter insulin resistance, it is important to understand the cellular mechanisms responsible for insulin resistance in these organs [74].

1.8 Risk factors

Obesity could be classified as: *primary* or essential obesity, related to increased food intake, low energy expenditure or both and it accounts for 95% of obese. *Secondary* or endogenous obesity, linked to endocrine, genetic or neurological disorders. It accounts for 5% of obese. Risk factors of obesity are modifiable or non-modifiable.

1.8.1 Non-modifiable risk factors

The most relevant non-modifiable risk factors are:

- Genetics: many genes are involved in obesity. They promote body fat accumulation in different areas, and body efficiency rate in using nutrients as energy. They also play a role in energy expenditure during physical activity and rest. The most frequent genetic disorders are:
    - Prader-Willi syndrome, characterized by below-average height, intellectual disability, and cryptorchidism
    - Laurence-Moon Biedl syndrome, which causes mental retardation, hypogonadism and retinis pigmentosa
    - Alstrom syndrome, typically with diabetes, insulin resistance, and blindness
    - Carpenter syndrome, with intellectual disorders, hypogonadism in men, syndactyly, and polydactyly.
- Gender: women are more obese for the less lean mass, which causes less energy expenditure. Another risk factor for obesity is menopause because of endocrine changes, such as in thyroid function and in growth hormone production. Menopausal women usually change their eating habits and

become more sedentary. A different risk is for pregnancy. Body fat distribution is the same in both girls and boys in early age, but it changes in adulthood. Males have more visceral fat, while females are characterized by subcutaneous fat accumulation. However, visceral fat gradually increases in women since the age of 30, while the subcutaneous one decreases due to menopausal changes in hormonal production. Visceral fat is usually 10-20% of adipose tissue in men and 5-8% in women. This percentage increases with age in both genders [34]. Abdominal obesity enhances cardiovascular and metabolic diseases (such as diabetes, heart attacks, strokes, and metabolic syndrome) in both women and men. However, this risk is higher in women.

- Age: obesity can arise at every age. Earlier onset promotes worst complications unless changes in lifestyle. Family and social environment play a key role. Hormonal changes and a sedentary lifestyle increase obesity risk over the years. Furthermore, fat free mass decreases in adults. These changes enhance the daily energy expenditure and weight loss becomes more difficult.
- Family: family has an important role in both obesity onset and treatment (diet and lifestyle change). There is a relationship between family status and children weight. The higher is the parents' level of education, the less are overweight children. If the mother or the father is obese, children are probably overweight. Also, the socioeconomic status is correlated with children's body weight: there are more overweight children in low-income families.

1.8.2 Modifiable risk factors

The most relevant modifiable risk factors are:

- Diet: the daily intake of high calories foods or the high food intake are the main risk factors for obesity. High calories foods usually eaten by subjects with obesity are sweets, sugary or alcoholic drinks and high carbohydrates meals. Their meals are poor in dietary fiber.
- Smoking: smokers usually gain weight after quitting with cigarettes, increasing obesity risk. This is probably linked to nicotine, which creates addiction. Smoke causes chronic damage of tastebuds, making food less tasty [75].
- Sedentary lifestyle: lack of physical activity causes weight gains due to a lower energy expenditure. Physical activity promotes basal metabolism rate. If energy intake is higher than energy expenditure, this will lead to a growth of fat mass. This mechanism was developed during human evolution to survive in fasting: nowadays, it is useless because of food abundance [76]. According to WHO, over the last decades, sedentary lifestyle has spread worldwide, also due to changes in urbanization, free time, work, and movement. Regular physical activity is essential for the prevention and treatment of chronic diseases, such as obesity, cardiovascular diseases, diabetes, some cancers [77,78].

1.9 Diet and Obesity

Over the years, scientific evidence about the relationship between nutrition and chronic disabling diseases has led researchers to focus on dietary patterns or dietary components able to prevent and support good health.

Several epidemiological results showed that the intake of plant-derived bioactive compounds from food and drinks (such as whole grains, legumes, fruits, vegetables, nuts, coffee, and tea) is strongly associated with a reduction in the risk of diabetes mellitus, cardiovascular diseases, and some types of neoplasia [79-81]. On the other side, the intake of sugary drinks, red meat, saturated and trans fats is linked with an increased risk [82,83].

These relationships are due to the different effects of foods and their bioactive components in modulating the physiopathological processes involved in the aetiopathogenesis of chronic degenerative diseases. It is known that the changes in plasma concentrations of glucose, insulin, and triglycerides, both in fasting and post-prandial, and the reduction in HDL cholesterol levels are risk factors for cardiovascular diseases. This happens in obese and diabetic patients but also in pre-diabetic ones (with IGT) and in normoglycaemic subjects [84-87].

Regular intake of whole grains is associated with reduced risk of cardiovascular diseases, overweight, obesity and type 2 diabetes [88,89]. This could be linked to the fermentation of dietary fiber by gut microbiota. It leads to the production of short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate. Indeed, much scientific research investigate the role of SCFA in the modulation of glycol-lipid metabolism [90,91]. Nevertheless, there are few and inconsistent evidence [92-95].This due in part to the variability of fiber fermentation by gut microbiota.

1.10 Dietary fiber: definition and classification

The term dietary fiber was coined in 1953, but the health benefits of high fiber foods have been long appreciated. In 430 BC, Hippocrates described the laxative effects of coarse wheat in comparison with refined wheat [96].

Dietary fiber is made up with edible parts of plants undigested into human's small intestine, which go through the large intestine. Non-starch polysaccharides (cellulose, hemicellulose, gum, pectin), oligosaccharides (inulin, fructo-oligosaccharides) and lignin are part of the dietary fiber.

Fiber is defined differently throughout the world. Some definitions are based on analytical methods for isolating fiber, while there is a move to define fiber on a physiological basis. Traditionally, fiber was measured as chemical components, such as cellulose, hemicellulose, pectin, and lignin, the only noncarbohydrate component of fiber. Currently the United States relies on an analytical approach to determine what is or is not considered fiber for purposes of listing fiber content on food labels. In 2001 the Institute of Medicine (IOM) developed the following set of working definitions for fiber [97]:

- Dietary fiber consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants.
- Functional fiber consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans.

These definitions recognize the diversity of nondigestible carbohydrates in the food supply. This definition has yet to be formally adopted by the U.S. Food and Drug

Administration (FDA), but it includes plant, animal, and manufactured fiber sources that exhibit beneficial physiological effects in humans.

Progress has been slow on agreeing to a universal definition of dietary fiber. Codex Alimentarius Commission in 2009 published a dietary fiber definition [98].

Some of the outstanding issues about that definition were debated at the Fahouny Fiber Symposium: (1) Inclusion or exclusion of undigestible carbohydrates with degrees of polymerization in the range of 3 and 9 was left to the discretion of national authorities; (2) The absence of a list of beneficial physiological effects and appropriate criteria for their substantiation; (3) The analytical methodology by which fiber in food was to be quantified.

Traditionally, dietary fiber was classified according to its solubility in an attempt to relate physiological effects to chemical types of fiber [97]. Soluble fibers were considered to have benefits on serum lipids, while insoluble fibers were linked with laxation benefits. This division of soluble and insoluble fiber is still used in nutrition labeling. However, despite these commonly used generalizations, scientific evidence supporting that soluble fibers lower cholesterol and insoluble fibers increase stool weight is inconsistent. Resistant starch and inulin, both soluble fibers, do not appear to lower blood cholesterol, and the effect of insoluble fiber on stool weight is highly variable. In addition, many fiber sources are mostly soluble but still enlarge stool weight, such as oat bran and psyllium.

Most commonly consumed foods are low in dietary fiber. Generally, accepted servings of food contain from 1 to 3 g of fiber per serving. Higher fiber contents are found in foods such as whole grain cereals, legumes, and dried fruits. Other fiber

sources include over-the-counter laxatives containing fiber, fiber supplements, and fiber-fortified foods.

The Nutrition Facts label is based on 25 g of fiber recommended daily for 2000 calories[97].

Foods that are high in fiber, whole grains, vegetables, fruits, and legumes contain more than just fiber [99]. Also, additional properties of fiber, such as viscosity and fermentability, may be more important characteristics in terms of physiological benefits. Viscous fibers are those that have gel-forming properties in the intestinal tract, and fermentable fibers are those that can be metabolized by colonic bacteria. In general, soluble fibers are more completed fermented and have a higher viscosity than insoluble fibers. In table 2 are summarized different foods source and functions of soluble fiber (Table 2). In table 3 are summarized different foods source and functions of insoluble fiber (Table 3).

Table 2. Soluble fiber, food source and their functions.

Type	Food Source	Functions
Cellulose	Bran, Whole Grain Cereals especially wheat, rye, apples, cabbage, beans	1. Holds water 2. Increases stool bulk 3. Reduces intraluminal colonic pressure 4. Prevents constipation 5. Binds minerals such as Ca and Fe 6. Binds bile acids 7. Reduces transit time
Hemicellulose	Bran, whole grain cereals, especially millets, jowar, bajra, ragi	
Lignin (non-carbohydrate source)	Whole grain cereals, pars, peaches, plums, mature vegetables	

Table 3. Insoluble fiber, food source and their functions.

Type	Food Source	Functions
Pectin's	Guava, Apple, Citrus Fruits, Wood Apple, Berries, Carrots and Green Beans	1. Binds cholesterol and bile acids 2. Holds water 3. Fermented in the colon to volatile fatty acids and gas by the normal bacteria flora of the colon
Gums	Oatmeal, pulses, beans, processed foods.	
Mucilage's, seaweeds and algae	Thickeners in food products, stabilizer, gelling agent in puddings	

1.11 Health Benefits of Fiber

Cardiovascular Disease

Epidemiologic studies suggest that adequate fiber intake consistently decreases the risk of CVD and coronary heart disease (CHD), primarily through a reduction in low density lipoprotein (LDL) levels. The results of randomized clinical trials are inconsistent but suggest that fiber may play a beneficial role in reducing C-reactive protein levels, apolipoprotein levels, and blood pressure, all of which are biomarkers for heart disease. Water-soluble fibers (specifically, beta-glucan, psyllium, pectin, and guar gum) were most effective for lowering serum LDL cholesterol concentrations, without affecting high density lipoprotein (HDL) concentrations. In the U.S., there are accepted health claims for the ability of oats, barley, and psyllium to lower blood lipids. Other soluble fibers, glucans and pectins, have recognized ability to lower blood lipids and the regulations in individual countries determine labeling and claims.

Type 2 Diabetes and Glycemic Control

There are many theories surrounding the relationship between fiber intake and type 2 diabetes. Regularly consuming the recommended amount of fiber has the potential to attenuate glucose absorption rate, prevent weight gain, and increase the load of beneficial nutrients and antioxidants in the diet, all of which may help prevent diabetes.

A large-scale cohort studies support a strong inverse relationship between dietary fiber consumption and development of type 2 diabetes. A multi-ethnic cohort followed 75,000 people for 14 years. People who ate more than 15 g of fiber per day had significantly lower diabetes risk [100]. People who ate high amounts of insoluble fiber (more than 17 g/day) or cereal fiber (more than 8 g/day) had less type 2 diabetes risk than people who had lower intakes while soluble fiber intake was not associated with diabetes risk [101].

Intervention studies provide inconsistent results. For instance, compared to a 5-week control diet, 5 weeks of oat beta-glucan (5 g) significantly reduced postprandial glucose and insulin responses, while 5 weeks of barley beta-glucan (5 g or 10 g) did not [102]. Nazare et al. found significant reductions in glucose and insulin when fiber was added to a standard breakfast [103]. Many acute intervention trials fail to find a relationship between fiber intake and post-prandial glucose response [104].

Bowel and Regularity

It is well recognized that fiber is important for normal laxation. This is due primarily to the ability of fiber to increase stool weight. The increased weight is due to the physical presence of the fiber, water held by the fiber, and increased bacterial mass from fermentation. Larger and softer stools increase the ease of defecation and reduce transit time through the intestinal tract, which may help to prevent or relieve

constipation. In general, cereal fibers are the most effective at increasing stool weight. Wheat bran is considered the gold standard when it comes to fecal bulking, since no other fiber or laxative has been shown to be as effective [105]. Inulin, although extensively fermented, has little effect on stool weight [106], with less than a 1 g/ increase in stool weight with each g fiber fed as inulin.

The effect of fiber and low digestible carbohydrates on gastrointestinal tolerance is a concern. Not all fibers have the same effect on tolerance; fructo-oligosaccharides can cause symptoms with low doses (10 g) [107] while other fibers, such as polydextrose and resistant starch have been consumed at doses up to 50 g without symptoms [108]. It is likely that fast and complete fermentation in the upper gut is linked to GI intolerance.

Body Weight

Prospective cohort studies report that people who consume higher amounts of fiber weigh less than people who eat lesser amounts[97]. One study reported that in a 20-month period, every 1 g increase in total fiber consumed per day, decreased body weight by 0.25 kg [109].

Fiber intake is linked to other beneficial lifestyle factors, such as fruit and vegetable intake and exercise habits. High fiber diets are typically lower in fat and energy density, both of which are helpful for maintaining a healthy body weight. Howarth et al. summarized the results of more than 50 intervention studies that had assessed relationships among energy intake, body weight, and fiber intake[110]. They estimated that increasing fiber intake by 14 g per day was associated with a 10% decrease in energy intake and a 2 kg weight loss over about a 4-month period. The observed changes in energy intake and body weight occurred without regard to the

fiber source as a naturally high-fiber food or a functional fiber supplement. The involvement of gut microbiota in the regulation of host energy homeostasis was suggested by studies reporting that subjects with obesity were shown to have lower Bacteroidetes and more Firmicutes in their distal gut than lean control individuals, alterations that were abolished after 52 weeks of diet-induced weight loss [111]. Changing gut microflora may be more difficult in free-living individuals and long-term consequences of changes in gut microflora are unknown [112].

Cancer

In the 1970s, many reports suggested that increased colorectal cancer prevalence was a result of low-fiber diets. These assumptions were predominantly based on differences in colorectal cancer rates among nations and regions with high- and low-fiber intakes; this type of data clearly lacks causal evidence. Several large-scale studies, including some intervention trials, have suggested fiber intake is not associated with overall risk for colorectal cancer. For example, the 8-year Polyp Prevention Trial (PPT) evaluated the effects of a high-fiber (18 g/1000 kcal), high fruit and vegetable, and low-fat diet on the recurrence of adenomatous polyps in the colon[113]. This study failed to show an effect of diet on adenoma recurrence after 8 years of follow-up. The lack of relationship between high-fiber diet interventions and colorectal cancer risk may be authentic, or it may be a product of the long latency period for colorectal cancer development.

1.12 Bone fragility and Obesity

In the past Obesity has always been considered as a protective factor against bone fragility and the development of osteoporosis. In recent years, however, many studies have questioned this consideration, thanks to the growing evidences suggested a high fat mass is a risk factor for these diseases. Body composition has a relevant role on bone quality and adipose tissue by itself is able to regulate bone mass, favoring its expansion [114]. However, a higher bone mass does not always mean bone quality, as shown, for example, by the higher risk of fractures in subjects with T2D, despite having a greater bone mineral density (BMD) than non-diabetic subjects, both in men as well as women [115]. Bone density and bone quality, the two principal parameters that contribute to bone strength, are estimated through BMD assessment, which is the amount of bone mineral in the tissue and an indirect indicator of osteoporosis and fracture risk [116]. High fat diet seems to be a negative regulator of bone mass in animal models. Indeed, chronic exposition leads to a decrease in bone formation and, probably, in bone turnover, by a defective response to insulin signaling in osteoblasts[115]. The excessive intake of fats and sugars may alter the geometry and the mechanical properties of bone, with important effects on bone strength in mice, maybe enhancing hyperglycemia-related processes, such as non-enzymatic glycation of proteins [114].

The causes that bind obesity and diabetes to the increased risk of incurring fractures can be different:

• increased risk of falls, which may be related to hypoglycemic events in diabetes and the decrease of muscle mass and/or function (sarcopenia), together with body fat increase;

• decreased bone strength, due to lower bone mineral mass and alterations in bone microstructure and materials [117].

Considering the strong impact of obesity on world health and that the pathogenesis underlying bone impairment has not yet been completely elucidated, especially in humans, the description of the mechanisms involved is an important and increasingly interesting topic of study.

Recent studies have shown that bone is a target tissue of insulin and is involved in glucose homeostasis. The alterations related to an increased visceral adiposity and insulin resistance, typical of obesity, may contribute to the mechanisms involved in the promotion of bone fragility and fracture risk [118].

Fat metabolism plays an important role in the interactions between adipose tissue itself and bone, through the integration of different molecules, such as hormones, adipokines and cytokines [114]. Aging-related changes regarding body composition, metabolism and hormones after menopause may give a higher propensity to weight gain. The increase and the dysregulation of visceral adiposity is strictly related to IR, for which there is growing evidence of his contribution in the pathophysiology of osteoporosis [118]. During last years, studies highlighted that IR is associated with an increased fracture risk, even independently from BMI and BMD [117].

Insulin mediates the communication between metabolism and bone remodeling. It is an anabolic factor in bone, with positive effect on bone formation [119]. Both osteoblasts and osteoclasts express insulin receptor; many evidences show its involvement in osteoblasts proliferation, differentiation and survival, while it exerts a negative regulation on osteoclasts and bone resorption [117,118]. It also promotes glucose uptake in osteoblasts, through the activation of the glucose membrane

transporter 4 (GLUT4), and promotes the synthesis of collagen and alkaline phosphatase, important for the mineralization. It carries out its functions synergistically with other anabolic factors, like amylin, co-secreted with insulin and negative regulator of bone resorption, PTH and insulin-like growth factor 1 (IGF-1) [120].

IGF-1 is a growth factor important for development and metabolism processes; it is produced in all tissues, with paracrine functions, but the skeleton is the major depository organ. It is important for the longitudinal growth in bone and for the maintenance of bone mass in adults [121]. Its levels in the bone matrix decrease physiologically during aging but several studies showed that low levels of IGF-1 are often associated with high circulating glucose and an increased risk of fractures [117,121].

Bone is not only a target of hormones, but also performs an endocrine function, by the producing of molecules such as osteocalcin and osteopontin, able to regulate glucose homeostasis and body weight. Osteocalcin (OCN) is produced by osteoblasts and it is the major non-collagenous protein of extracellular matrix. Uncarboxylated form is the active hormone, which regulates osteoblasts differentiation and maturation, bone mineralization and calcium ion homeostasis, but it has also an important role in the whole-body metabolism. Indeed, OCN is involved in insulin sensitivity and glucose homeostasis: it promotes insulin secretion by pancreas βcells (favoring also βcells proliferation), the sensitization of adipose tissue to the action of insulin [120] and adiponectin secretion [122]. In healthy human, there is a positive association between OCN, insulin and adiponectin; OCN and insulin establish a positive regulatory feedback, though the promotion of insulin on OCN secretion by osteoblasts [123]. This, in turn, acts on the βcells of the pancreas, favoring insulin

secretion, and on the gut, the release of glucagon-like peptide 1 (GLP1), which helps in insulin releasing. For these reasons, both hyperglycemia and IR may affect OCN expression, by gene downregulation. It was described that a high fat diet and an unregulated insulin signalling are associated with a decreased OCN activity, with a strong impact on both glucose homeostasis and fracture risk, through the increase of cortical porosity and the decrease of bone turnover in animal models. Unfortunately, human studies are mainly observational and with conflicting data, because of factors that do not make the populations considered homogeneous, like age, ethnicity, menopause and degree of IR [120].

Osteopontin (OPN) instead is a potent regulator of adipose tissue inflammation, insulin resistance and diabetes. Animal studies have shown that OPN is highly upregulated in adipose tissue of diet-induced and genetically obese mice, while they have been reported elevated levels in obese, diabetic and insulin resistant subjects, compared to the lean ones [122].

It is clear the existence of an "axis" between adipose tissue and bone, thanks to the crosstalk and the regulation through the adipokines and bone-deriving molecules. The abnormal increase and the dysregulation of adipose tissue during obesity, of the visceral compartment, could be determining factors for bone fragility and osteoporosis [124]. In this regard, to explain this link, several hypotheses have been put forward and evaluated.

According to WHO's definition, osteoporosis is a pathological condition in which bone resorption and formation are unbalanced, characterized by a decrease of bone mass and microarchitecture deterioration, leading to bone fragility [125,126]. All of

this provides for fractures, which are associated with substantial morbidity and mortality [127].

Bone turnover is composed by resorption and formation. Both an excessive increase and a strong decrease in it are associated with deficits in the microarchitecture of bone, leading to an increase stiffness and make it to fractures. For the evaluation of bone turnover, several biochemical markers have been identified: C-telopeptide (CTX), N-telopeptide (NTX) and tartrate-resistant acid phosphatase 5b (TRACP5b) are markers for bone resorption, while alkaline phosphatase (BSAP), osteocalcin (OCN) and procollagen type 1 N-term (P1NP) are molecules associated to bone formation. It has been observed that these markers correlate inversely with insulin levels and visceral adiposity and thus IR may have a negative impact on the bone quality through the reduction of bone turnover. However, the mechanisms through which all this can occur are still unclear in humans [118].

Both animal and clinical studies have highlighted that systemic inflammation, elevated levels of TNF-α and IL-6, is associated with bone loss; in animals, TNF-α defines the increase of bone loss by the elevation of Sclerostin [128]. Moreover, it stimulates the maturation of osteoclasts and their activation, causing the increasing in bone resorption. In humans, clinical studies demonstrated that the risk of hip fractures in women is related to Il-6 and TNF-α levels. Very similar results were observed not only in obese subjects, but also in T2D, further proof of the strong relationship between whole-body glucose homeostasis and bone metabolism [129].

1.13 Mechanisms of bone impairment in obesity

There are many mechanisms proposed that may contribute in bone impairment, due to an aberrant crosstalk between a dysregulated adipose tissue and the bone itself. The increase of abdominal fat, both in men and women, is a risk factor for BMD decrease and osteoporosis. The increase of abdominal fat, VAT and bone marrow fat are inversely related to the BMD, through a greater cortical porosity, lower bone trabecular volume and lower bone formation [114]. Moreover, postmenopausal women with increase visceral adiposity are more prone to fractures, due to lower bone formation and a poorer bone quality. Many studies have shown that the reduced activity of osteoblasts in subjects with increased adiposity is linked to dysregulations in the βcatenin/WNT signaling pathway.

Sarcopenic obesity is a condition that have a strong impact on bone fragility and which increase the risk of fractures. This occurs when there is a decrease in muscle mass and/or function linked to the increase in body fat, maybe determined by the pro-inflammatory factors produced by the adipose tissue [130].

As mentioned above, OCN is an osteoblast-derived hormone, which is strictly modulated by insulin and body glucose homeostasis. For this reason, during IR its levels are altered, with detrimental effects on bone health. Low OCN is negatively correlated with hemoglobin glycated levels and it is decreases in T1D and in T2D, compared to healthy subjects [117].

An impaired adipokines profile is involved in chronic low-grade inflammation in obesity, leading to several physiologic and metabolic alterations[124]. Both Il-6 and TNF-α have negative effects on skeletal and metabolism, but they are not the only molecules with an action on bone metabolism.

IL-6 is a pro-inflammatory cytokine but recently its anti-inflammatory properties have also been reported. Regarding bone metabolism, it is a stimulator of

osteoclastogenesis and bone resorption. It has also a role in bone formation, during high bone turnover [124].

TNF-α correlates with body fat percentage and IR in human and it is involved in lipid metabolism, insulin sensitivity and signaling, adipocyte functions and bone remodeling. Both TNF-α and IL-6 regulate osteoclasts activity, enhancing bone resorption. Moreover, TNFα works synergistically with Receptor Activator of NFκB Ligand (RANKL), a member of TNFα family released from osteoblasts and osteocytes, in inducing osteoclast differentiation and amplifying their activation. Osteoprotegerin (OPG) is a decoy receptor, which prevent the binding of RANKL to its receptor and to inhibit the activation of osteoclasts; OPG is downregulated by TNF-α, to further promote the activity of RANKL [124], TNFα is also a strong inhibitor of osteoblast differentiation, due to its downregulation of transcriptional factor Runx2 and WNT/βcatenin signaling, through the increase of Dickkpof-1 (Dkk1) and Sclerostin [4].

Other molecules involved in bone metabolism are:

1. Leptin, whose levels are in proportion to the body fat content; it has both positive and negative effects on bone. In vivo studies showed up that these different effects depend on the site of action: indeed, the peripheral using of leptin has a positive activity on bone mass, while in vitro treatment stimulates osteoblastogenesis on mesenchymal stem cells (MSCs). In peri- and postmenopausal women, especially obese, there is a positive correlation with BMD and negative with bone resorption markers [120]. It was suggested a "protective" role of leptin during obesity, by the inhibition of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB) and promoting OPG expression [4].

2. Adiponectin, which is also produced by osteoblasts, is inversely related to IR. The decrease of its levels is one of the main features of obesity. Both in vitro and in vivo studies have shown that adiponectin increases bone mass promoting the differentiation of osteoblasts and the suppression of osteoclastogenesis, through several mechanisms [124]. Adiponectin could directly stimulate osteoblasts differentiation, but not only; it could also induce osteoblastogenesis in bone marrow-derived mesenchymal stem cells and favor bone morphogenetic protein 2 (an osteoblastogenic factor, BMP2) production in osteoblasts [123].
3. Resistin is produced by macrophages and visceral adipocytes. It is positively linked to IR. It is also expressed in MSCs, osteoblasts and osteoclasts and it promotes cytokines release and both osteoblasts and osteoclasts differentiation [4].
4. Ghrelin, a gut-derived hormone, regulates energy homeostasis and seems to be involved also in bone metabolism, through the modulation of osteoblastogenesis. Although the results are still controversial, some studies highlighted its positive action in the promotion of osteoblasts proliferation and differentiation. Serum levels are inversely associated with BMI [120].
5. Amylin, which belongs to calcitonin family, is secreted together with insulin. It promotes osteoblast proliferation; its levels are increased in obese subjects, together with a lower sensitivity for this molecule.
6. Plasminogen activating inhibitor 1 (PAI-1), produced by liver and adipose tissue. Its levels increase during obesity and IR and it is predictive for T2D and cardiovascular diseases development. It is also involved in bone resorption processes.

7. Estrogens, hormones that have an important role in differentiation and in skeletal homeostasis. Fat is the main source of aromatase enzyme (expressed also in the gonads), involved in the synthesis of estrogens from androgen precursors. After menopause, this fat synthesis becomes predominant. However, the aromatase activity is higher in the subcutaneous adipose tissue, while it is lower in the visceral, even in physiological conditions [130]. In bone, estrogens promote bone formation and they are significant in differentiation. Adipocytes and osteoblasts have a common precursor, MSCs, whose recruitment in one lineage or another is also driven by estrogens. MSCs from postmenopausal osteoporotic women show higher levels of adipogenic markers compared to those get from subjects with normal bone mass. Moreover, fat infiltration occurs with aging, but it has been frequently observed in postmenopausal women with osteoporosis. Estrogens regulation has been proposed also in the development of fat bone marrow [129].

1.14 Bone, Adipose tissue and Skeletal Muscle Tissue Crosstalk

Considering the metabolic alterations and comorbidities related to obesity, the impact on target tissues such as bone and adipose tissue, and the existence of a crosstalk between them, in the last few years the interest of researchers in these interactions has increased considerably. The skeletal muscle is the largest organ of the body, counting almost the 60% of total body weight [131], and it is also the largest insulin-responsive tissue. Indeed, in basal conditions the 25% of circulating glucose is taken from the tissue, reaching 75-80% in the post-prandial phase, thanks to the translocation of the

GLUT4 glucose membrane transporter, following insulin stimulation [132]. In addition to the contractile function and glucose and lipid uptake, skeletal muscle also performs an endocrine function, thanks to the secretion of muscle-derived molecules, called myokines, which allow the interaction with other tissues, such as adipose tissue, bone and liver, and the regulation of various metabolic and inflammatory processes. [131] Myokines are secreted by myocytes and their circulating levels are often associated with muscle contraction; moreover, many of these, like IL-6 and Myostatin are secreted also by adipocytes. IL- 6 may be increased after physical exercise, with beneficial effects on the regulation of inflammatory state (anti-inflammatory); on the other hand, high and chronic levels are associated with systemic inflammation, especially in subjects with IR, obesity and T2D [131]. This controversial aspect may be explained by positive anti-inflammatory effects related to a transient and short-term action, while the chronic increase may lead to adverse regulation on immune system and metabolism [131].

Myostatin is TGFβ/BMP family member and a potent inhibitor for skeletal muscle growth: its overexpression in animal models is associated with muscle atrophy. It downregulates muscle stem cells proliferation and differentiation, the number of adult muscle fibers and protein synthesis, decreasing muscle mass [133]. It has also direct effects on other tissues, like fat (which is also able to secrete it) and liver. Its expression is high both in skeletal muscle and adipose tissue. Myostatin circulating levels correlates with IR, in obese and extremely obese middle-aged women [129], but this relationship has also been documented in men. Recent studies have shown that myostatin may also influence glucose metabolism, by a direct effect on glucose uptake in muscle, independently from its activity on muscle growth [134].

Muscle mass decreases functionally and physiologically with aging: it was observed a loss of 0.5-1% every year after 30 years old, with an acceleration after 65 years, leading to mobility and functional impairments [135,136]. This age-related muscular loss is defined sarcopenia and it is a multifactorial condition, which involves the progressive decline of muscle mass, strength and function, with or without fat increase [135,137]. It is mainly at the lower limbs, maybe because it is related to a decrease of activity during aging [138]. The number and the size of muscular fibers decrease while tissue renewal capacity is lower [135] due to satellite cells (adult stem cells, important for tissue growth and repair) pass from quiescence to an irreversible senescence [136]. As results of the negative action of inflammatory factors, a study reported that the overexpression of TNF-α in transgenic mice leads to an impaired proliferation and differentiation of satellite cells [138].

Factors that promote and enhance sarcopenia are different: neurodegeneration, which is the progressive loss of neurons with age; hormonal, inflammatory and myokines levels changes, due to a different production and sensitivity, which may affect skeletal muscle; alteration in muscle protein metabolism, because of lower protein synthesis in combination with an increased protein degradation [131].

Pro-inflammatory cytokines, which increase progressively with age, have negative effects on mass formation; TNF-α is also associated with a decrease in the production of muscle strength. In addition, they are positively associated with an increased fat infiltration and IR promotion [138]. Elevated inflammation is also promoted by the age- related decrease of sex-hormones (testosterone and estrogens), whose levels are directly related to muscle mass. Moreover, lower levels of estrogens in menopause are associated with sarcopenia, due to changes fat and muscle mass body distribution. The synthesis and the degradation of proteins are unbalanced with age, moved

towards an increased degradation. Moreover, there is a progressive accumulation of damaged or modified proteins (for instance, due to oxidative stress), associated to a decreased activity of the proteasome system in muscle. Another mechanism involved in the regulation of protein anabolism is IGF-1, a positive modulator for myogenesis too. Lower levels of IGF-1 impaired protein synthesis because of a defective mechanistic target of rapamycin (mTOR) signaling [139].

Other pathological factors which raise sarcopenia are oxidative stress, IR and mitochondrial dysfunction. ROS production and accumulation are also involved in tissue degradation, apoptosis, muscle atrophy and dysfunction [138]. Many evidence support the association between BMI and BMD in the susceptibility to develop muscoskeletal disorders [140]. Considering the physiological crosstalk between muscle, adipose tissue and bone and the strong impact that each one has on the metabolism, in a pathological situation like obesity, the mechanisms involved (above all IR and inflammation) have detrimental effects also on skeletal muscle integrity and functions. Obesity, sarcopenia and osteoporosis may be a part of a complex dysmobility syndrome, characterized by the coexistence of altered bone and muscle during obesity, not only in elderly subjects [124]. However, the association between sarcopenia and metabolic disorders are still poorly elucidated [131].

Sarcopenic obesity is a pathological muscle loss and poor muscle quality related to the increase of visceral adiposity and fat infiltration into- and inter-fibers (intramyocellular lipids IMCLs) [132] positively correlated also with IR [135,141]. IMCLs usually consist of ceramides, triglycerides (TG), long chain acyl CoA and diacylglycerol (DAG) and they are related to IR and lipotoxic cellular stress [132]. Ceramides favor IKKβ and JNK activation, promoting pro-inflammatory phenotype and the phosphorylation of Ser/Thr residues on insulin receptor, leading to the

decrease of Akt activation and the impairment of insulin signaling [142]. Obesity-related increased of IL-6 and TNF-α have a negative effect on muscle mass and metabolism, due to an elevated protein catabolism and the inhibition of protein synthesis. [136] Visceral adiposity and metabolic effects of sarcopenia enhance each other, synergistically: the increase of fat mass promotes the increase of pro-inflammatory factors, which affect muscle integrity, altering myokines expression and favoring IR promotion. [131]. IL-6 is able to impair satellite cells recruitment, altering tissue renewal [141].

Recent studies highlighted also altered MSCs: it was observed that cellular signals favor MSCs differentiation towards adipogenic lineage, to the damage of myogenic and osteogenic, with negative effects on the homeostasis of these two tissues[140].

CHAPTER 2:

"WNT pathway and endocannabinoid system"

2.1 WNT signaling: an overview

WNTs proteins signaling is constituted by multiple pathways, defined as "canonical" and "non canonical", by the action or not of βcatenin. The canonical pathway is βcatenin dependent; in the absence of WNT proteins, cytoplasmic βcatenin is recruited by the complex Axin and adenomatous polyposis coli (APC); these are scaffold proteins that facilitate the multi-phosphorylation by casein kinase I and glycogen synthase kinase 3-β (GSK3-β). The phosphorylation makes βcatenin a target for the ubiquitination by β transducin repeat-containing homologue protein (β TrCP) and the consequent degradation by proteasome complex. In this way, in the absence of WNT pathway activation, cytoplasmic levels of βcatenin are kept low. In the case that WNT elements bind frizzled (FZD) receptors and low- density lipoprotein-receptor-related protein-5 or-6 (LRP5/6) co-receptors, the protein Dishevelled (Dvl) is activated and inhibits GSK3-β activity. The complex of degradation is inactivated and βcatenin is stabilized and it could translocate into the nucleus, where it binds T cell factor/lymphoid- enhancer factor protein (TCF/LEF), promoting the transcription

of WNT target genes[143]. WNT pathway could also be activated in a βcatenin independent manner (non- canonical pathway), through Wnt5a and several kinases, such as mitogen activated protein kinases (MAPK), protein kinase C (PKC) and calcium/calmodulin dependent protein kinase IIα (CamKII). Most of these responses is related to a release of intracellular calcium and a rapid cellular response, involving in the cellular shape and migration. For these reasons, non-canonical signaling pathway could be subdivided into Wnt/Ca2+ and planar cell polarity (PCP) pathways [144]. However, this pathway is poorly defined compared to the canonical one and many cellular targets remain still unconfirmed or unknown.

Wnt5a is one of the most studied WNT protein involved in this signaling pathway: by its binding to FZD receptors and heterotrimeric GTP binding proteins, there is the increase of second messengers, like cGMP and calcium release. This activates calcium sensitive proteins, such as phosphatase calcineurin and kinases CamKIIα and PKC [145]. This pathway inhibits the canonical one, acting on βcatenin stabilization. Another mechanism of action of non-canonical signaling is the binding with tyrosine kinase receptor ROR2 (with a cysteine-rich domain able to bind Wnt5a) and the activation of associated tyrosine kinase RYK, important for cellular migration, which causes the activation of JNK pathway [144]. Moreover, the interaction between Wnt5a, ROR2 and co-receptor LRP5 is involved in the inhibition of canonical pathway. Wnt5a not only is one of the most important trigger for WNT non-canonical pathway, but it has been shown by various studies, both animal and human, that based on the availability of certain receptors, is able to activate also the canonical pathway. In fact, it has been observed that its binding to the FZD4 receptor goes to activate the βcatenin-dependent pathway[146]. Considering the high number of receptors, co-receptors and their interactions, this signaling pathway is highly context specific, depending on receptor availability and cell type [144].

WNT pathway is strictly regulated by several extracellular factors, which act as antagonists in the binding to the receptor and in signaling transduction. There are two main types of inhibitors: secreted frizzled-related proteins (SFRPs), five structurally similar proteins, which act as decoy receptors and prevent the binding to the FZD receptor by sequestrating WNTs; dickkopf (DKK) family members and sclerostin that antagonize LRP5/6 co-receptors binding with high affinity and preventing canonical signaling activation[147].

2.2 WNT signaling and adipogenesis.

MSCs are able to differentiate into various cell types, including adipocytes and osteoblasts, depending on the modulation of specific transcriptional factors. WNT signaling pathway is crucial in the regulation of adipogenic differentiation. WNT βcatenin pathway was reported to inhibit adipogenesis: in vitro studies highlighted the higher expression of Wnt10b in preadipocytes, to maintain the undifferentiated phenotype; its levels rapidly decrease after the induction of differentiation. Also in vivo studies showed a higher expression of Wnt10b in stromal vascular cells, enriched of preadipocytes, but undetectable levels in mature adipocytes [148]. Treatments with inhibitors of GSK3-β affect negatively adipogenesis, while the use of recombinant SFRPs induces a spontaneous differentiation. The expression of SFRP1 and 5 are higher in mature adipocytes and recent studies showed a positive correlation of their levels with the increase of adiposity [149]. Regarding obesity, SFRP5 presents a controversial role in IR, as many studies tried to figure out. Its levels change during differentiation, being a key factor in the regulation as C/EBPα and PPARγ and the evaluation of mRNA levels, both in VAT and in SAT of obese and lean subjects, showed a significant increase in VAT of obese, suggesting a possible

involvement in obesity onset. In vitro studies figured out that SFRP5 overexpression impairs insulin sensitivity in normal condition but decreases TNF-α expression in adipocytes[150]. Recent studies in mice showed anti-inflammatory and anti-diabetic properties of SFRP5, whose levels were described positively related to glucose tolerance. However, in vivo studies present conflicting data, showing both positive and negative associations with circulating levels and insulin resistance[150] .

Canonical pathway exerts its negative action on adipogenesis through the blocking of PPARγ and C/EBPα. In fact, it was reported a cross regulation between βcatenin and PPARγ, by the fact that their levels are inversely related in various cell types. It has been also reported that negative effects on adipogenesis mediated by IL-6 and TNF-α are related to Wnt/βcatenin activation and its effect on C/EBPα and PPARγ; in this case, WNT activation is a fundamental prerequisite in the inhibition of adipogenesis by these two inflammatory cytokines [151]. This is supported by the fact that the downregulation of these factors is associated with the increase expression of several molecular targets downstream βcatenin signaling [143].

WNT pathway is also implicated in the regulation of body fat distribution, in human and mice. Genetic researches point out that polymorphisms of Wnt10b and Lrp5 genes may present an association with obesity in European population and SFRP1 is involved in adipose tissue expansion, presenting an upregulated expression in WAT of mice[150]. Conversely, non-canonical pathway has positive effects on adipogenesis, by antagonizing the canonical one. GSK activity modulates adipogenic differentiation both at early stage and at terminal phases, through its modulation of both canonical and non-canonical signaling pathways. It has been demonstrated its modulation of SFRPs expression, by STAT5 action[150].

Canonical WNT is a key regulator in the balancing of MSCs differentiation, inhibiting adipogenesis and favoring osteoblastogenesis and myogenesis. In fact, the inhibition of WNT, at multiple levels, causes the switch to adipogenic phenotype. This is demonstrated by the fact the loss of Wnt10b (which could occur during aging) increases the adipogenic potential of myoblasts and the acquisition of adipogenic characteristics during muscle regeneration[145].

2.3 Bone tissue and role of WNT pathway.

Bone tissue is a highly specialized connective tissue, characterized by an extracellular matrix consisting of an organic component (collagen, mucopolysaccharides and glycoproteins), and by an inorganic component, represented by deposits of Ca2+ and hydroxyapatite [122]. The bone constitutes the skeleton, which exerts important mechanical functions, acting as a scaffolding to our body, protecting the internal organs, forming the dental cement and the dentin, and, through the tendons, allowing organism's movements, along with muscle tissue. The inorganic component of the bone matrix, represented by Ca2+ deposition, is continuously mobilized both for bone remodeling and for the regulation of homeostatic calcemia. Three cell types are distinguished in bone: osteoblasts, osteocytes and osteoclasts. Osteoblasts are secretory mononuclear cells, responsible for the deposition of the bone matrix, both organic and inorganic, deriving from MSCs differentiation [4]. They play an important role in bone formation and growth, thanks to their involvement in the remodeling of tissutal tissue microarchitecture and in the deposition of new bone matrix. Once they are trapped inside the matrix, these take a quiescent form and become osteocytes, the most abundant cells in bone, with a flattened shape and numerous extensions, involved in the regulation of exchanges with the extracellular

environment, with endocrine properties and a role in mechano/chemical sensing [121,122]. Following erosion of the adjacent matrix or in case of fracture, the osteocytes are able to re-differentiate into osteoblasts and act as mechanosensors, instructing osteoclasts on where to reabsorb the matrix and the osteoblasts on where to reattach it [152].

Osteoclasts derive from the differentiation of monocytes and perform an erosive function, through the secretion of lysosomal hydrolase enzyme. They are presented as giant poly-nuclear cells, derived from the fusion of more neo-formed osteoclasts, rich in lysosomes. Together with osteoblasts, they are involved in homeostatic calcium control and remodeling of bone microarchitecture [153].

2.4 Skeletal muscle and WNT pathway

Myogenesis is a complex process that starts from satellite cells activation, passing through myoblast proliferation and differentiation and leading to myotube formation. All these passages are orchestrated by myogenic transcriptional regulatory factors, called MRFs, essential for muscle generation and development [138]. MyoD, Myf5, Myogenin and MHC are some of these MRFs involved in myogenesis activation and promotion; Myogenin seems to be crucial in myoblasts differentiation. In the case of an altered myogenesis a general downregulation of these MRFs was observed, usually induced by TNF-α [139].

Canonical WNT signaling is directly involved in myoblast differentiation and myotube fusion, though the activation of Myf5 and MyoD. Its principal role is to guarantee a sufficient quantity of myoblasts, before the induction of myotube formation. Furthermore, during an injury, it has been reported the increment of mRNA expression of several WNT proteins, such as Wnt5a, Wnt5b, Wnt7a and Wnt7b in

skeletal muscle. Kuwabara et al demonstrated that exercise induces WNT/βcatenin upregulation which, in turn, directly modulates chromatin structure and induces Myf5 and MyoD expression, favoring myogenesis in mice model [154]. Myoblasts, despite being committed cells, present a certain plasticity: in fact, by culturing them with BMPs, they differentiate into osteocytes, through the suppression of the transcription factor MyoD. Moreover, if they are cultured with fatty acids, they acquire the adipogenic phenotype[138].

2.5 WNT pathway in MSCs differentiation.

Inside bone marrow there is a niche of cellular progenitor (MSCs), able to differentiate into adipocytes, osteoblasts or chondrocytes through the induction of several factors, in order to maintain tissue microenvironment and to response to different stimuli. Another important player in the induction of differentiation is a specific adipose depot within the bone marrow (bone marrow adipose tissue, BMAT), with important endocrine function and related to many physiological and pathological conditions, such as menopause, aging, drug treatments and high fat diet. The increase of BMAT is related to a lower bone mass and the decrease of osteoblasts [155].

MSCs differentiation into osteogenic or adipogenic lineage depends on a wide variety of signaling and transcription factors. There are two principal systems: WNT signaling, which favors osteoblastogenesis, and PPARγ, which promotes adipogenesis. The balance and the integration between these factors is important for tissues homeostasis [4] .

It was firstly described the involvement of WNT since the characterization of several mutations regarding WNT pathway members that caused alterations in bone density and skeletal malformations, both in mice and in humans [127]. Canonical signaling

controls three different lineages: adipocytes, osteoblasts and chondrocytes. Its role is the promotion of osteoblast phenotype and the inhibition of the other two. WNT signaling auto-regulates osteoblast differentiation, by a negative feedback with Dkk1 and sclerostin increase [130].

Canonical WNT pathway activation favors osteoblastogenesis, stimulating the acquisition of a mature phenotype by osteoblastic precursors, through the upregulation of Runt-related transcription factor 2 (Runx2), principal regulator of differentiation[156]. Moreover, WNT signaling inhibits adipogenesis, through βcatenin dependent and independent mechanisms, by the down regulation of Pparγ and Cebpα. βcatenin-dependent pathway indirectly represses osteoclast differentiation and bone resorption, by the increase of Osteoprotegerin (OPG). For these reasons, canonical pathway is a positive regulator of bone formation, favoring osteoblasts differentiation and maintaining the precursors in adult bone [127].

The non-canonical pathway role is less understood, but Wnt5a is a potent regulator; indeed, it is produced by the osteoblasts and, by the activation of ROR2 and RYK, it leads to the amplification of the expression of RANKL, which determines the differentiation of monocytes into osteoclasts, through NF-κB activation[127]. Non-canonical signaling may induce osteoblastogenesis by the binding of Wnt7b and consequent activation of WNT/PKC-δ signaling pathway. Besides, Wnt5a may favor osteoblastogenesis over adipogenesis through the inhibition of Pparγ, by chromatin inactivation [143]. WNT pathway is finely regulated; in particular, soluble protein Sclerostin, encoded by Sost gene and secreted by osteocytes, has taken on increasing importance in recent years, being a valid candidate in the communication and the regulation between bone and adipose tissue[156]. Sclerostin is a WNT inhibitor, which enhance adipogenesis, favoring the increase of Pparγ and Cebpα and lipid

accumulation, to the detriment of osteoblast differentiation [157]. Farfield and colleagues demonstrated that both recombinant Sclerostin and osteocyte-conditioned media are able to induce adipogenesis and down regulate osteoblastogenesis not only in preadipocytes, but also in primary MSCs derived from mice and humans [156].

Sclerostin is involved in many physiological and pathological aspects. During mechanical loading, its down-expression is fundamental for the mechano-transduction cascade and consequent WNT activation, leading to osteogenesis and bone formation. It is also associated with bone diseases: Sost inactive mutations are related to high bone mass, due to a higher bone formation; Sost dysregulation are also implied in bone metastasizing cancer. Furthermore, it has been described that circulating levels are correlated with the increase of abdominal adiposity, both subcutaneous and visceral, total body and bone marrow fat content in men. Although these relationships are less clear in women because of a possible sex implication not understood yet, Sclerostin levels are positively related to age and the increase of adipose content, suggesting a possible involvement or link to systemic adiposity and osteoporosis[156,157].

Another important regulator is DKK-1, WNT pathway antagonist, like sclerostin. It is produced by osteocytes, but not as highly and selectively as sclerostin [127]. The modulation of bone mass and of the formation and reabsorption processes are carried out by WNT also in crosstalk with other signaling pathways, in particular with parathormone (PTH) and BMP signaling pathway. PTH has both anabolic and catabolic effects, by the direct stimulation of bone formation and indirectly the resorption. In the case of prolonged high levels of PTH (as during hyperparathyroidism), bone resorption and formation are unbalance, leading to the increase of bone turnover and a decrease of bone mass. PTH regulates the expression

of several WNT antagonist, including Sost and Dkk-1, in several animal models. Its effect on Sost expression has been described also in humans. Sclerostin expression is negatively related to PTH levels: in fact, the increase of PTH determines sclerostin downregulation [147,157]. BMP signaling is fundamental for skeletogenesis and bone formation during the development. WNT and BMP seem to have opposite effects on osteoprogenitors and BMP is a repressor of βcatenin-dependent signaling pathway [127]. In addition, WNT pathway has a role also in cell adhesion, important for bone homeostasis; βcatenin binds cadherins, which mediate cell adhesion processes[127].

2.6 WNT pathway and metabolic disorders.

Considering the importance of this pathway in many physiological processes, dysfunctions or aberrant regulation of WNT may be involved in the development of chronic diseases. During last years, researchers have hypothesized and speculated about a possible involvement of WNT signaling in the pathogenic mechanisms of different metabolic disorders in humans, resulting in a growing interest for the study of these possible relationships [144]. WNTs proteins are detectable in serum and they are relatable to various chronic disorders, such as atherosclerosis, rheumatoid arthritis and obesity. All of these present a common aspect: chronic low-grade inflammation. WNT pathway is involved in the regulation of inflammatory response and it is also a key modulator of metabolic pathways, such as mTOR and insulin signaling. Typically, canonical activation leads to anti-inflammatory effectors and downregulates mTOR and aerobic glycolysis; non- canonical pathway, instead, is often related to the activation of inflammatory effectors (through JNK activation) and exerts opposite functions to those of the canonical one and also regulates many metabolic enzymes [144,147]. It has been shown that mutations and polymorphisms

concerning WNT pathway genes can predispose subjects to the development of some metabolic diseases; in fact, some polymorphisms of co-receptor LRP5 are related to obesity. Moreover, knockout mice for LRP5 present an impaired glucose tolerance, due to a lower insulin secretion, mediated by WNT ligands [147]. In humans, LRP5 mutations are associated with obesity, type 1 diabetes and other diseases.

Genetic mutations affecting LRP5 are not the only ones involved in pathogenic processes; in fact, also mutations regarding genes for the ligands and the effectors of the activated cascade signaling (TCF) are involved in the development of T2D and other metabolic disorders [147].

Non-canonical pathway appears to have negative effects on metabolism. It has been shown to regulate negatively insulin signaling in WAT, favoring IR, and it promotes lipid accumulation [144]. Circulating Wnt5a is increase in mice and humans presenting insulin resistance obesity-induced compared to healthy lean subjects. Moreover, Wnt5a in adipose tissue of mice promotes systemic inflammation and IR, by the activation ok JNK pathway. Zuriaga et al demonstrated that Wnt5a expression in visceral depots is upregulated and it is associated with JNK activation, leading to the increase expression of IL-6 and constituting an extremely important link between obesity and insulin resistance [145]. Canonical pathway, on the contrary, seems to be protective in the onset of diabetes, going to modulate the inflammatory state [144].

2.7 The Endocannabinoid System.

Cannabinoid research had a strong push after the characterization of the chemical structure of Δ9-tetrahydrocannabinol (Δ9-THC), the main psychoactive constituent of C sativa. It was followed by the cloning of two receptor subtypes, cannabinoid receptor 1 (CB1) in 1988 and cannabinoid receptor 2 (CB2) in 1992, that are able to

bind exogenous cannabinoids [158,159]. Subsequently, endogenous ligands have been identified, with the capacity to bind and activate cannabinoid receptors; the most bioactive molecules are N-arachidonoylethanolamide (anandamide, AEA) and 2-Arachidonoylglycerol (2-AG), defined as Endocannabinoids. The Endocannabinoid System (ECS) is formed not only by ligands (exogenous and endogenous) and by receptors, but it also includes all the enzyme machinery involved in the synthesis and in the degradation of these compounds [158-160]. ECS is a well preserved system during evolution and it is involved in several physiological functions, including the regulation of motor activity, immune and inflammatory responses, neuroprotection, metabolism and glucose homeostasis [153,158,160].

2.8 Cannabinoid receptors.

Cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), also defined as "classical" receptors, are 7-transmembrane domains G protein-coupled receptors (GPCRs). CB1 was initially considered restricted to the brain, where it is highly and differentially expressed in various brain areas, depending on anatomical region and neuronal subpopulation[158]. Recently, it has been identified in many peripheral tissues and many cell types, especially in those involved in the regulation of organism metabolism [161]. In 2003, this receptor was described for the first time both in mice and in human adipocytes, which are equipped with the whole endocannabinoid system. Moreover, it was also identified in skeletal muscle, liver, gastrointestinal tract, endocrine pancreas, bone and reproductive organs, suggesting its involvement in regulation of energy homeostasis and in many other processes of the organism [162]. CB2 presents the 68% of homology in amino acid sequence with CB1 within the transmembrane domain [162]. It is highly represented in immune and blood cells and

in keratinocytes, suggesting a regulation of immune responses while its role in energy control is still poorly elucidated [163]. The activation of CB1 determines the stimulation of several intracellular pathways, based on the cell type and experimental conditions, in the case of in vitro experiments. It modulates adenylate cyclase, preventing cAMP formation, and mitogen activated protein kinases (MAPK), through Gi/o type proteins[164]. Furthermore, it stimulates many kinases, including focal adhesion kinases (FAK), the phosphatidylinositol-3-kinases (PI 3-K) and JNKs. It is able also to interacts

functionally with other receptors, such as serotonin and dopamine receptor [158]. Endocannabinoids have the capacity to bind and activate other types of receptors: the transient receptor potential vanilloid type 1 (TRPV1), orphan G protein-coupled receptor GPR55, also called CB3, and it is able also to interact with peroxisome proliferator activated receptors (PPARs)[159].

2.9 Endocannabinoid metabolism.

Anandamide and 2-AG are synthetized "on demand", from membrane lipids precursors and arachidonic acid, respectively, with multi-step biosynthetic processes. They are polyunsatured fatty acids (PUFAs) and, considering their lipophilic nature, they cannot be stored into cellular vesicles; for this reason, ECS is selectively modulated, in a temporal and a spatial way, in the synthesis, release, uptake and degradation [165]. During last years, there were also identified other lipid mediators synthesized from unsaturated fatty acids, which they are able to interact with classical receptors (CB1/ CB2)[166]. There are several stimuli that trigger endocannabinoids synthesis, like depolarization of the membranes, the increase in intracellular calcium and the stimulation by other receptors. The direct precursor of AEA is N-

arachidonoyl-phosphatidylethanolamine (NAPE), which originates from enzyme trans-acylase, that catalyzes the transfer of arachidonic acid from the sn1 position of the phospholipid to the nitrogen atom of phosphatidylethylamine. NAPE is then converted to AEA through a hydrolysis reaction step catalyzed by the specific NAPE-phospholipase D enzyme (NAPE-PLD). AEA could also be produced by NAPE through alternative biosynthetic pathways [167]. Phospholipase-C can catalyze the formation of phospho-AEA, the substrate of the protein tyrosine-phosphatase N22, which hydrolyzes phospho-AEA in AEA. Phosphodiesterase mediates the hydrolysis of glycerophospho-AEA, which in turn is the result of a sequential cleavage pathway of two groups of NAPE. Finally, it could be produced a soluble form of phospholipase A2, 2-lyso-NAPE, mediated by lyso-PLD [167]. 2-AG is produced by the hydrolysis of diacylglycerols containing 2-arachidonyl, by diacylglycerol-lipase α and β (DAGLα/β), two isoenzymes. The diacylglycerols which are precursors of 2-AG can be produced either from the hydrolysis of phosphatidic acids containing 2-arachidonil by a phosphohydrolase, or from the hydrolysis of phosphoinositoles through the action of phospholipase C, selective for this compound. DAGL α/β are stimulated by calcium and they catalyzed the synthesis in a calcium-dependent manner of 2-AG with endocannabinoid function [168].

It was demonstrated that the feeding status and the dietary intake pattern directly influence endocannabinoids amount [161]. In fact, studies in animal models and in humans showed that the increase of the relative proportion of long-chain polyunsatured n3 fatty acids though the diet determines a decrease in AEA and 2-AG levels [161].

AEA and 2-AG bind both CB1 and CB2, but with different affinities and efficacies in activation, acting in an autocrine and paracrine manner. They are both involved in

inflammatory processes: in fact, they are produced by different types of immune cells, in response to inflammatory stimuli. AEA exerts its function through the interaction with TRPV1 and PPARγ receptors, while 2-AG interacts with eicosanoid pathway[161]. Many studies reported higher levels in plasma and tissues of animal models of inflammation. Furthermore, ECS seems to be connected also with immune cells migration.

Endocannabinoids are rapidly degraded by different classes of enzymes, very efficient, which are therefore an important regulatory step in biological availability in vivo. They are degraded by enzymatic hydrolysis: fatty acid amide hydrolase (FAAH) acts on AEA, while monoacylglycerol lipase (MAGL) on 2-AG. There are been identified two isoforms of FAAH, FAAH-1, which is the most abundant and it is localized in the ER, and FAAH-2, recently described in cytoplasmic lipid droplets in adipocytes [161]. 2-AG is pick up by a membrane transporter and subjected to degradation in arachidonic acid and glycerol by the enzyme monoacylglycerol-lipase (MAGL) [169], which is responsible for the 85% of the total 2-AG degradation in the brain. 2-AG may also be metabolized by other two enzymes, which compete with MAGL: α / β-hydrolase 6 (ABHD6) and α / β hydrolase12 (ABHD12), with different cellular distribution. It has been suggested that together with FAAH, they control the hydrolysis of distinct 2-AG pools in the nervous system [170].

They can also be metabolized by oxidation from other classes of enzymes, such as cyclooxigenases (COXs), lipooxigenases (LOXs) and cytochrome P450 [159].

2.10 ECS and central metabolic regulation on CNS.

The ECS system is expressed in the central nervous system (CNS) and in the control of several neuronal processes, such as neurogenesis, the proliferation of neural

progenitors, migration and the formation of complex neuronal networks. The density of CB1 receptors increases progressively during postnatal development, with a peak just before the onset of puberty until it falls to reach values in adults [171]. At CNS level, the presence of high concentrations of endocannabinoids and their receptors on GABAergic and glutamatergic synapses suggests their involvement in the modulation of synaptic transmission [170]. The endocannabinoids are released immediately after their biosynthesis, due to their nature. For this reason, they act as important mediators for a real-time responding to feeding changes in the organism. They are involved in the regulation of appetite and food intake via the activation of CB1 receptor, through the modulation of the activity of hypothalamic neurons [169]. The neuromodulation of endocannabinoids ends with a re-uptake mechanism within neurons, by diffusion or the involvement of a transporter, where they are degraded by specific enzymes [172].

It is now well accepted that CB1 activation by endocannabinoids and synthetic agonists for this receptor stimulates feeding and the preference of foods rich in fats and carbohydrates [173]. Furthermore, the levels of these endogenous compounds are reported to be significantly increased within the hypothalamus and accumbens in response to fasting, returning to normal after refeeding and without reporting any difference in those brain areas that are not involved in feeding behavior [163]. This process is in part mediated by hormones regulation, such as leptin and ghrelin, for instance, which affect endocannabinoids levels in the hypothalamus and are dysregulated during obesity[174]. In diet-induced obesity (DIO) mice, the levels of endocannabinoids are upregulated in the hippocampus. This area of the brain is important in the modulation of hedonic eating, resulting in the promotion of a vicious circle leading to obesity, because the highly palatable foods might be more satisfying under these conditions [173]. In genetic animal models of obesity, brain

endocannabinoid levels are increased and CB1 is downregulated. 2-AG is transiently or permanently upregulated in the hypothalamus, after acute or prolonged consumption, respectively, of a high-fat diet (HFD), through the stimulation of CB1 receptor[175]. This elevation is usually coupled with a deregulated leptin signaling; moreover, it might participate in peripheral metabolic dysfunctions, such as abnormal WAT accumulation. Finally, the selective deletion of CB1 receptors in central neurons confers resistance to HFD- induced obesity. These data emphasize the role of ECS in the control of both central and peripheral energy homeostasis.

2.11 ECS and metabolic regulation.

It is widely recognized ECS activity on central nervous system (CNS) in the modulation of energy homeostasis, by the control of food intake and appetite. During last years, its role in regulating metabolic balance has been expanded by its presence and modulation in many peripheral tissues involved in whole-body energy homeostasis, such as adipose tissue, skeletal muscle, liver, pancreas and gastrointestinal tract. Several in vitro and in vivo studies showed that ECS activity is not exclusively central and on feeding behavior [176], but they demonstrated a direct effect on physiological functions on these tissues [177]. ECS activity in metabolic control of glucose is multi-organ and involves both the regulation of liver and skeletal muscle insulin sensitivity and its secretion by the pancreas. In fact, the stimulation of CB1 in the βcells determines the activation of FAKs and favors insulin release by vesicles. ECs is an important factor for βcells survival: it may regulate cell death both indirectly, through the promotion of pro- inflammatory cells infiltration, and directly, by the induction of apoptosis. This is proved by the fact that the blockade of the receptor induces the increase of cell proliferation and βcells mass [164].

All the elements that belongs to ECS have been identified in adipose tissue [162]; a significant number of studies described CB1 presence in mature adipocytes, but not in preadipocytes, both in cell lines and in human and rodent primary cells [169]. Moreover, several studies have highlighted the interaction of CB1 with 2-AG and AEA in the regulation of adipogenesis and lipogenesis.

Cnr1 and Faah mRNAs were present in considerable amounts in adipose tissue. Cnr1 expression increases during adipocyte differentiation, suggesting a role of this receptor in cellular physiology. Also, Faah is increased in mature adipocytes, but the difference was not as striking as for the Cnr1 [178].

CB2 expression and functions in adipose tissue are still poorly elucidated; it seems that the increase of CB2 expression may be involved in the regulation of inflammation and may be linked to the increased cellular infiltration by activated macrophages[179]. There is a physiological differential expression between fat depots, where ECS is higher in visceral fat compared to the SAT, with important metabolic consequences [174]. The overall effect of ECS activation is to increase fat storage, though the maximization of fatty acid de novo biosynthesis, TAGs accumulation and reducing lipolysis. ECS can favor the glucose uptake and to activate fatty acid synthase (FAS) and lipoprotein lipase, important for the synthesis and TAGs accumulation, respectively. CB1 is rapidly expressed during differentiation, favoring it by the induction of PPARγ [180]142. Its activation determines the increase of fatty acids storage and TAGs accumulation, through the rise of lipoprotein lipase activity (LPL) and fatty acids synthase, the inhibition of AMPK pathway and the increase of glucose uptake, by GLUT4 translocation (necessary for de novo lipogenesis) [162,177]. Furthermore, they may affect adipokines secretion, leading to a downregulation of adiponectin expression. However, there is a temporal separation

between the effect on PPARγ and adiponectin inhibition, where the latter is caused by a prolonged stimulation of the ECS [180]. Another important effect of CB1 stimulation is the decrease of mitochondrial biogenesis; in fact, the blockade of the receptor determines the increase of both lipolysis and mitochondrial biogenesis. In physiological conditions ECS is limited both locally and endocrine, to avoid an overactivation. It seems to be regulated with a negative feedback manner, by hormones and PPARs, such as leptin, insulin, PPARγ, PPARα and PPARδ [169]. Recently researchers have focused on leptin action on ECS, both centrally and peripherally. It has been demonstrated that leptin causes the downregulation of ECS in the hypothalamus and in adipocytes [181]. Leptin decrease allows the activation of ECS, favoring the increase of food intake and its action on peripheral tissues [164,174].

Insulin is also a modulator of the ECS, determining the decrease of endocannabinoids synthesis and favoring their degradation, both in human and murine models [164,177]. During IR or leptin resistance there is an over-activation of ECS and hypertrophy of adipocytes, with an important impact both centrally and at the periphery. PPARγ and PPARα both regulates lipid metabolism; while PPARα is expressed mainly in the liver, skeletal muscle and heart, PPARγ is fundamental for adipocytes differentiation, lipid accumulation and insulin sensitivity. Endocannabinoids could also bind and activate PPARs; in a model of human primary fat cells researchers observed a reciprocal interaction between PPARγ and endocannabinoids. They are able to stimulate PPARγ through a positive feedback, enhancing mRNA expression and favoring its related functions, like adipogenesis [163,177].

Skeletal muscle is one of the largest insulin-sensitive tissue and it has also been demonstrated the presence and activity of ECS. AEA treatment seems to affect insulin-dependent glucose uptake, through the inhibition of Akt activation. Eckardt

and colleagues showed that the incubation of myocytes with conditioned medium from adipocytes impairs Akt phosphorylation and glucose uptake in a CB1–dependent manner. This can prove that IR in the muscle may be partially due to adipocytes-derived factors and ECS may be directly involved [160].

Moreover, in vitro studies demonstrated that CB1 activation not only regulates mitochondrial levels, by the decrease of mitochondria biogenesis, but it affects also their activity, through the inhibition of IRS1 phosphorylation and PGC1-α downregulation (involved also in the oxidation of fatty acids), as well as in WAT and in the liver [159,160]. More recent studies described an involvement in skeletal muscle formation, in which 2-AG and CB1 prevent myotubes formation120. CB1 expression increases during myoblasts differentiation, while CB2 is downregulated [160]. In several animal models ECS seems to be more sensitive to the development of overweight and obesity state, showing differences in expression and/or activation in these conditions, compared to a healthy state. In muscle obtained from high fat diet rats the gene expression of Cnr2 is downregulated, while Magl is increase. In Zucker rats the CB1 is decreased, with higher levels of AEA[159], while in diet-induced obesity mice (DIO- mice) 2-AG is upregulated. Results obtained from human primary myotubes, both from lean and obese subjects, do not show any difference in Cnr1 expression[174].

ECS is present and active also in the bone tissue. In fact, both AEA and 2-AG are locally produced and released by bone cells and ECS plays an important role in the development and the maintenance of bone health. AEA levels found in the trabecular bone are almost similar to those expressed in the brain, while 2-AG are lower 120. Both CB1 and CB2 were found in osteoblasts. Cnr2 expression increases progressively and in parallel with the expression of osteoblastic markers genes [182].

CB2 stimulation is very important to favor the proliferation of osteoblastic precursors, while, in mature cells, it promotes osteoblastic functions, enhancing alkaline phosphatase activity and the matrix mineralization. This may be demonstrated by the fact that the osteoblastic formation by bone marrow stromal Cnr2 cells is strongly diminished [183]. On the other hand, CB1 levels are almost undetectable in osteoblasts [184]. CB2 activity in osteoclasts is more controversial, having both positive and negative functions, depending on experimental conditions. Bone marrow-derived osteoclasts cultures and cell lines showed that CB2 activation determines the downregulation of osteoclasts formation, though the inhibition of mitogenesis[182]. However, it is clear its involvement in the regulation of bone mass: CB2 activation by both AEA and 2-AG increases bone mass in rodents [185]. The lack of CB2 expression is related to an elevated age-related bone loss and an increased bone turnover, while there have also been identified several polymorphisms of Cnr2 associated to a higher risk of osteoporosis and lower bone mass [159].

The differentiation from monocyte cells to osteoclasts seems to be related to a reduction in 2-AG levels, together with an increase in AEA levels [186]. The expression of the CB2 receptor on osteoclasts is modulated by a calcium permeable channel receptor, the type 1 vanilloid receptor (TRPV1), which is an AEA target and has numerous effects on bone remodeling [185]. In fact, it has been observed that the balance between TRPV1 and CB1/CB2 stimulation is altered in osteoclasts derived from postmenopausal women with osteoporosis, compared to healthy menopausal women. TRPV1 signaling is more active in mature osteoclasts, while both CB1 and CB2 receptors seem to have a negative effect on the functions of osteoclasts in menopausal, but this does not occur in osteoporotic women. In fact, in a study of 2011 it has been demonstrated that the activation of osteoclasts was increased by the treatment with AM251, a selective antagonist of CB1, and AM630,

an inverse agonist for CB2, but not in cells derived from osteoporotic women. It has been proposed that the chronic increase of endogenous mediators, such as AEA, coupled with the decrease of others (CB2 and 2-AG) may cause an elevation in TRPV1 expression and activity, may be involved in osteoclast activation and bone resorption processes [185]. GPR55 receptor may be activated by multiple endocannabinoid ligands and it is involved in cell polarization and bone resorption in humans [185].

Moreover, changes in dietary factors, such as PUFAs and lipids intake, have an important impact on bone morphology and mechanical properties: in fact, dietary PUFAs may determine changes in bone formation rate, mineral content and fatty composition in the bone [174].

2.12 ECS and obesity.

Alterations of Endocannabinoid System were associated and involved in many disorders, both at the level of the central nervous system and in peripheral tissue: in some cases, variations of ECS are a compensatory response to an insult, in other cases, instead, these alterations may be pathogenic [187]. Studies of last few years have highlighted that alterations of ECS modulation affect a large number of diseases, including neurodegenerative and psychiatric disorders; cancer; inflammatory, cardiovascular, gastrointestinal, liver, bone and skin diseases; pain; obesity and metabolic disorders; diabetes and its complications [159,188]. Recent studies have highlighted that the dysregulation or failure modulation of ECS in the metabolism are involved in the progression of many metabolic disorders, above all obesity and diabetes.

The ECS system has gained particular attention in the study of obesity as a potential

therapeutic target for combating it and combating the comorbidities associated with it[181]. Despite the great efforts made by researchers, very few pharmacological agents for the long-term treatment of obesity have been successfully developed and used, including sibutramine (structurally similar to amphetamines, which acts as a norepinephrine reuptake inhibitor) and orlistat (gastrointestinal lipase inhibitor), which still show limited activity accompanied by significant side effects. An important candidate for the treatment of obesity was the selective CB1 antagonist SR141716A, known as Rimonabant, successfully tested in phase III trials. Its mechanism of action was to transmit a satiety signal to the brain before meals, resulting in a decrease in appetite and a reduction of food consumption [189]. It showed important beneficial effects on body weight reduction and an improvement of cardiovascular risk factors; furthermore, it ameliorated adiponectin, HDL levels and triglycerides levels and improved HbA1c in patients with diabetes [189,190]. Unfortunately, after the observation of important side-effects at the level of the central nervous system (i.e. depression), it was withdrawn from the market in 2008 [191]. For this reason, the interest of researchers has been focused on identifying new and more specific therapeutic targets, capable of blocking the CB1 receptor at the peripheral level, eliminating or mitigating the side effects at the level of the central nervous system.

Obesity is characterized by the chronic activation of ECS, which worsens the symptoms related to IR and fat accumulation, altering also the cross-talk between the tissues involved. Circulating levels of AEA and 2-AG are elevated in obese subjects; in particular, 2-AG is positively correlated with markers of obesity, like BMI, waist circumference, visceral fat mass and serum TAGs levels, while it is inversely related to HDL [164,192]. Normal weight and obese subjects present a pre-prandial peak of AEA, which normally decreases after the meal, acting as a meal initiator signal in

humans; this does not occur in the obese, where the ECS is dysregulated and the control for caloric intake is chronically switched on [164]. Some studies reported that systemic endocannabinoid levels are increased in postmenopausal women with obesity and are associated with a lower expression of Cnr1 and Faah genes in the adipose tissue. This relationship may be explained by a negative feedback regulation, between endocannabinoids levels and the decreased CB1. Furthermore, expression of the gene encoding FAAH suggests that adipocytes are involved in the control of endocannabinoid availability [193].

Increased levels of blood AEA or both endocannabinoids were observed in obese women, while in visceral fat but not in the subcutaneous it was reported the elevation of 2-AG. The upregulation of endocannabinoids is described in obese visceral fat, compared to both the subcutaneous compartment and lean subjects, where 2-AG concentration is 2 fold higher[174]. In T2D it is reported the increase of ECS in visceral fat, with higher levels of 2-AG, negatively related to insulin sensitivity [194].

2-AG levels seem to depend by the elevated availability of biosynthetic precursors, because there are no differences in Dagl and Magl expression between obese and normal weight individuals. Conversely, AEA in adipose tissue is controlled preferentially by degradation: FAAH deficiency in mice determines an increase in energy storage, ectopic fat accumulation and IR; genetic studies highlighted the association between Faah polymorphisms and metabolic impairment, the A/A missense variant of Faah is related to an obese phenotype. FAAH activity is normally higher in VAT but decrease with obesity[163,195]. Together with Faah polymorphisms, Cnr1 gene variants also support the causative role of ECS over-activation in metabolic unbalance[163]. The new generation drugs blocking the CB1 receptor, such as the antagonist AM4113, in mouse models have induced a significant

weight loss without inducing states of anxiety or depression, demonstrating not to cross therefore the blood-brain barrier [196]. Recent studies have shown that the activation of the CB2 receptor at the peripheral level may induce beneficial metabolic effects. In particular, the CB2 receptor agonist JWH-133 decreases the expression of inflammation genes such as IL-6 and TNF-α in human adipocyte cultures [197]. Furthermore, a growing number of studies have highlighted a potential modulation of Wnt/β-catenin signaling and non-canonical WNT signaling pathway by AEA, strongly suggesting a possible cross-link between WNT signaling and ECS in multiple aspects of cellular differentiation and in the regulation of tissue homeostasis [182,183,198,199]. ECS alterations in adipose tissue and pancreas are typical aspects in obesity. Matias and colleagues demonstrated in 2006 that hyperglycaemia causes the increase of endocannabinoids production in a cellular model of adipocyte and pancreatic cells and that induces the ECS over-activation in DIO-mice. The overstimulation of ECS in βcell model determines a continue insulin release and a permanent condition of hyperinsulinemia. This may lead to a hypertrophy of adipocytes and ECS dysregulation, causing lipid accumulation and the decrease of adiponectin [180,200]. This down-regulation of adiponectin may be involved in mechanisms of glucose intolerance [164] and, accordingly, in the development of IR. Experimental data showed that ECS is dysregulated in human obesity, both centrally and peripherally; these alterations are correlated with WAT specific depots and metabolic parameters and may be not only consequences but also the causes of increased adiposity and insulin resistance[163,192]. For all these reasons, understanding the modulation on ECS has become a new and powerful and fascinating way for the treatment of diseases such as obesity and type 2 diabetes.

AIM OF THE STUDY

The primary aim of this study was to test FEHC diet in elderly obese subjects on improving obesity-related inflammation on bone, muscle and adipose tissue, through a positive modulation of WNT and ECS pathway.

Most of observational studies suggest that a high fiber intake is associated with a reduced risk of obesity. The longer duration of the treatment planned in the present proposal is crucial to investigate how different diet regimens enhance bone and muscle health and exploring the potential key role of WNT and ECS.

The primary objective of the project was to test the efficacy of a FEHC on bone, muscle, and fat inflammation status.

In this study, obese patients who underwent to hip arthroplasty were recruited.

Prior to procedure they were randomized to a 3-month diet intervention, according to 1) FEHC diet group 2) Control group.

After the surgical procedure, subjects were followed according to randomization for other 6 months with an isocaloric dietary regimen, different in macronutrients composition. During surgery, biopsies of bone, skeletal muscle and adipose tissue samples were taken from each patient.

The following specific aims were carried out:

1. to test the efficacy of a FEHC diet on bone, muscle, and fat inflammation status and ECS in obese elderly subjects.
2. to evaluate modulation of WNT in relation to dietary intervention.

CHAPTER 3: **Materials and Methods**

3.1 Materials

All reagents required for RNA extraction and gene expression analysis, including probes, are Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Serum levels of were determination using a multiplex assays (Biotechne) by Luminex XMAP technology (MagPix, BioRad).

3.2 Methods

3.2.1 Subjects recruitment

This is a prospective study, which involved multiple areas of Campus Bio-Medico University of Rome, with the aim to test FEHC diet on weight loss, bone, muscle, and fat in obese elderly subjects on improving obesity-related inflammation, through a positive modulation of WNT and ECS pathway.

From September 2019 to January 2022, 86 elderly obese patients (>65 years old), who underwent surgery for standard hip arthroplasty were enrolled in this project, prior informed consent. They were divided in two groups:

1) FEHC diet group: BMI > 30 kg/m^2 32 subjects;

2) Control group: BMI > 30 kg/m^2 54 subjects;

They were excluded from the study if they had one of the following exclusion criteria: age < 65 years, use of medications affecting bone metabolism such as estrogen, raloxifene, tamoxifen, bisphosphonates, GnRH analogues, glucocorticoids, anabolic steroids and dilantin; other exclusion criteria were: evidence of hypercalcemia, hypocalcemia, chronic liver disease, renal failure, hypercortisolism, malabsorption

and immobilization, current alcohol or tobacco use, evidence of bone metastasis or disease at the site of surgery.

BMI was calculated as weight (kg) divided by height squared (m^2).

Data on the eating habits and frequency of consumption of the all patients were collected by 24-hour recall and Food Frequency Questionnaire (FFQ), with monthly outpatient visits and weekly calls, in order to obtained reliable feedback about the daily caloric intake, the daily macronutrients percentage intake especially in terms of fiber. In order to identify the accurate portions of food for each meal, the food atlas was used with photos of the portions and the corresponding grams. The collection of these data has allowed us to estimate adherence to the diet regarding the effective daily caloric intake and the percentage distribution of macronutrients, as well as the real daily fiber consumption.

Functional outcomes were assessed at the baseline after 3 months of dietary intervention with the Oxford Hip Score (OHS), the Hip disabilities and Osteoarthritis Outcome Score (HOOS) and the Western Ontario and McMaster Universities (WOMAC) Osteoarthritis Index. OHS is a joint-specific patient-reported outcome measure tool to evaluate disability in patients undergoing THA (score range = 12–60) [201]. HOOS is a 40-item questionnaire to assess patient-relevant outcomes in five separate subscales (pain, symptoms, activity of daily living, sport and recreation function and hip related quality of life; score range = 0–100) [202]. The WOMAC Osteoarthritis Index is a self-administered questionnaire consisting of 24 items divided into the following 3 subscales: pain, stiffness, and physical function (score range = 0–96) [203].

3.2.2 Research plan

At baseline (**enrollment -T0**), eligible subjects were randomly assigned to one of two groups for 3 months. Participants assigned to Control group received general information about a healthy diet but not a specific diet. The FEHC group have received a specific diet composed by whole grains, legumes, fruits and vegetables. Animal products and/or added sugar were not included.

The daily energy intake was 72% from carbohydrate, 18% fat, and 10% protein. The fiber intake was equal to 40g/1000 kcal. The simple sugar intake was 10-12 % of daily energy; saturated fat was less than 7% of total daily calories.

Total daily energy expenditure was calculated with Harris & Benedict formula and also with 24 hours recall.

The Harris and Benedict formula allows us to obtain the number of calories that an individual consumes just by being alive, even when in a state of complete rest. To calculate a person's daily energy expenditure, we quantify their physical activity with a specific figure and add this to the expenditure to the result of the Harris-Benedict formula. Harris and Benedict's formula is calculated according to the equation below.

For men:

TMB = (10 x weight (kg)) + (6,25 × height (cm)) – (5 × age (years)) + 5

For women:

BMR = (10 x weight (kg) + (6,25 × height (cm)) – (5 × age (years)) – 161

24 hours recall is primarily used to establish the average food and/or nutrient intake of a population. Following a standardized procedure, the operator transcribes the

foods and beverages consumed by the subject during the previous 24 hours. The amounts consumed are estimated with the aid of a photographic atlas.

Anthropometric (body weight, height, BMI, waist circumference) and clinical features (glycaemia, total cholesterol, HDL, LDL, triglycerides), were collected.

Serum sample for inflammatory cytokines and WNT markers and DXA at lumbar spine and femur have been collected.

After three months, (**surgical procedure-T1**), serum have been collected for inflammatory and WNT markers. During total hip arthroplasty, participants have been undergoing fat biopsies from subcutaneous fat tissue, muscle biopsy from vastus lateralis and femoral head. Gene expression analyses of cytokines related to obesity and WNT markers have been performed at from bone, muscle and adipose tissue. Gene expression analysis of ECS system has been performed in muscle and fat tissue.

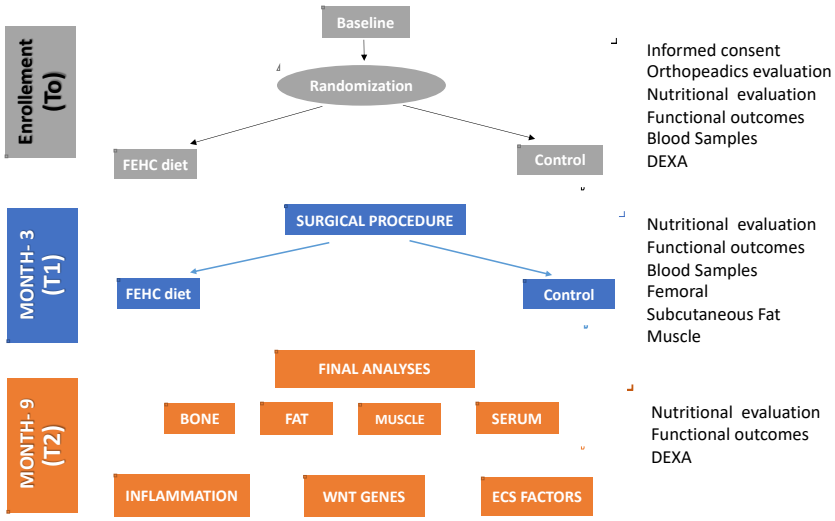
Following the procedure, The FEHC diet have been switch to a less restrictive isocaloric diet and followed for 6 months. This is like the FEHC (energy intake 70% from carbohydrate, 17% fat, and 13% protein; fiber 38g/1000 kcal) but includes also fish derived proteins and vegetable oils and has already proved to be effective and tolerable on a 6-month period.

Anthropometric (body weight, height, BMI, waist circumference) and clinical features (glycaemia, total cholesterol, HDL, LDL, triglycerides), were collected.

Serum sample for inflammatory cytokines and WNT markers and DXA at lumbar spine and femur have been collected.

After six months, **(month 9, T2**), Anthropometric (body weight, height, BMI, waist circumference) and clinical features (glycaemia, total cholesterol, HDL, LDL, triglycerides), were collected. DXA at lumbar spine and femur have been performed.

The flow chart of the project was reported in Figure 1.



In **Fig.1** is summarized the flow chart of the project.

3.2.3 Isolation of the mRNA and evaluation of gene expression with real time RT-PCR

Bone tissue, adipose tissue and skeletal muscle biopsies have been mechanically disrupted in liquid nitrogen with steel mortal and ceramic pestel. Samples were lysed in TRIzol following manufacturer's instructions. 1 mL of TRIzol was added to 100mg of frozen tissue powder[204]. Homogenization was performed with ceramic beads (Precellys, Bertin Instruments) in Tissue Lyser (Minlys, Bertin Instruments): 20'' of shaking at speed 2 following 1 minute on ice; these steps were performed for 3 times until complete sample homogenization. For Adipose tissue an additional step was performed to remove fat content: 5' at 12.000xg at 4°C. After 5 minutes at Room

Temperature, 0.2ml of Chloroform were added to homogenized samples for phase separation. Samples were shaken and incubated for 2-3 minutes at RT and then centrifuged for 15 minutes at 12.000 xg at 4°C. After centrifugation mixture is separated in 3 phases: un upper aqueous phase containing RNA, an interphase containing DNA and a lower red phenol phase containing proteins. For RNA precipitation, 0.5 ml of Isopropanol was added to the aqueous phase; after 10 minutes at RT samples were centrifuged at 12.000xg 10' at 4°C. Total RNA pellet is resuspended in 1 ml of 75% Ethanol and centrifuge at 7500xg for 5' at 4°C. Washed RNA pellet was air dry and then solubilized with 10-20ul of RNAse free water. RNA quantification was performed using NanoDrop™ Spectophotometer. RNA used for downstream PCR application must have an A260/A280 ratio N~2 (indicating that RNA is pure) and a A260/A230 >1.7 (indicating that RNA is clean and free of contaminants).

DNAse digestion (Deoxyribonuclease I, Amplification Grade, Invitrogen) was perfomed to remove DNA contamination from samples. DNAse master mix composed by : 1ug of RNA, 1ul of 10X DNase I Reaction Buffer, 1ul of DNase I Amp Grade1 U/µl and DEPC-treated water to 10 µl was incubated for 15 minutes at RT and then inactivated, added 1ul of 25mM EDTA for 10' at 65°C. 1000 ng of RNA free of genomic contamination is ready to use in reverse transcription with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). RT master mix is composed by: 2 ul of 10x RT buffer, 0.8 ul of 25x dNTP Mix, 2.0 ul of 10x Random Primers, 1.0 ul of MultiScribe Reverse Transcriptase, 4.2 ul of Nuclease free water. 10ul of RNA sample (1000 ng) were added to 10 ul of RT master mix. Reaction tubes were load in the thermal cycler (Eppendorf) following these steps: 10' at 25°C, 120' at 37°C and 5' at 85°C.

10ng of cDNA were used as template of Real-Time PCR with TAQman technologies. qPCR reaction mix composed by: 5.0 ul of TaqMan® Gene Expression Master Mix, 0.5 µL TaqMan® Assay and 4.5 ul of cDNA template + Nuclease-free Water was transferred in an optical 384 well reaction plate and load in QuantStudio 12K Flex Real-Time PCR System (Applied BioSystems) following this thermal protocol: hold at 2' 50°C for UNG activation, cDNA denaturation at 95°C for 10' and 40 amplification cycles (15'' at 95°C , 60'' at 60°C). Each sample was run in duplicates. The expression of the following genes were evaluated: WNT5a (Wnt family member 5a), WNT10b (WNT family member 10B), IGF1 (Insulin like growth factor 1), IL6 (Interleukin 6), IL8 (Interleukin 8), IL10 (Interleukin 6), TNFα (Tumor Necrosis Factor alpha), ADIPOQ (Adiponectin), BGLAP (Bone gamma-carboxyglutamate protein), RUNX2 (Runti-related transcription facotr 2), DKK1 (Dickkopf WNT signaling pathway inhibitor 1),SOST (Sclerostin) LEF1 (Lymphoid enhancer binding factor 1), COL1A1 (Collagen type I alpha 1 chain). Relative expression was calculated by the CT method (GeneEx v 6.0 software), using GAPDH and ACTB as housekeeping genes.

TaqMan probes used for gene expression were listed in the table below.

Gene Symbol	**TaQman Probe**
WNT5a	Hs00998537_m1
WNT10b	Hs00928823_m1
SOST	Hs00228830_m1
IGF-1	Hs01547656_m1
IL-6	Hs00174131_m1
IL-8	Hs00174103_m1
IL-10	Hs00961622_m1

TNFα	Hs00174128_m1
ADIPOQ	Hs00605917_m1
OCN	Hs01587814_g1
RUNX2	Hs00231692_m1
DKK-1	Hs00183740_m1
LEF1	Hs01547250_m1
COL1A1	Hs00164004_m1
ACTB	Hs01060665_g1
GAPDH	Hs02786624_g1

Each quantitative PCR analysis on tissue samples had three internal controls (housekeeper genes), the most stable for each type of tissue, chosen from a panel of housekeeper genes. Results of gene expression analysis in bone have been normalized with Beta-2-Microglobulin (*B2m*), *Gapdh* and TATA-binding protein (*Tbp*); results obtained from adipose tissue with ribosomial RNA18S, *Tbp* and *B-actin* and those from skeletal muscle with *Gapdh*, *B2m* and *Bactin*. The results were normalized not only for each one of these control genes, but also with the BestKeeper gene, that is the adjusted mean of the three housekeepers for each sample [205,206].

3.2.4 Circulating Biomarkers

Circulating inflammatory and metabolic mediators have been detected in Serum samples using a multiplex assays (Biotechne) by Luminex XMAP technology (MagPix, BioRad). Analyte-specific antibodies are pre-coated onto magnetic microparticles embedded with fluorophores at set ratios for each unique microparticle region. A magnet in the analyzer captures and holds the superparamagnetic

microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the microparticles. One LED excites the dyes inside each microparticle to identify the region and the second LED excites the PE to measure the amount of analyte bound to the microparticle. A sample from each well is imaged with a CCD camera with a set of filters to differentiate excitation levels.

Interleukin IL-6, IL-8, IL-10 and TNFα (Luminex Performance Human High Sensitivity Cytokine Magnetic Panel A (4-Plex) FCSTM09-04), display a sensitivity of 0.31 pg/ml, 0.07 pg/ml, 0.24 pg/ml 0.54 pg/ml respectively. MCP1, DKK1 and sclerostin (Luminex Human Discovery Assay (4-Plex) LXSAHM-04) display a sensitivity of 9.9 pg/ml, 50.,9 pg/ml and 7.0 pg/ml respectively; sensitivity of Adiponectin (Luminex Human Discovery Assay (1-Plex) LXSAHM-01) was 148 pg/ml. All the samples were run in duplicates. Frozen serum samples were centrifuged at 16.000 xg for 4' and then diluted. Adiponectin Luminex panel requires a dilution of 300-fold, while 4plex panels requires a 2-fold dilution. 100 or 50 ul of standard, sample and control were added per well (100 ul for High sensitivity assay and 50 ul for discovery assay respectively) with 25 µL of the microparticle cocktail. Plates were incubated overnight at 4°C on a horizontal orbital microplate shaker (0.12" orbit) set at 800 ± 50 rpm (Eppendorf). 3 washes with Wash Buffer were performed using a magnetic device. 50 µL of diluted Biotin-Antibody Cocktail were added to each well and incubated for 1 hour at room temperature (22°C) at 800 ± 50 rpm. After 3 washes, 50 µL of Streptavidin-PE were added to each well and incubated for 30 minutes at room temperature at 800 ± 50 rpm. After 3 washes the microparticles were resuspended by adding 100 µL of Wash Buffer to each well, incubated for 2 minutes at room temperature at 800 ± 50 rpm and read within 90 minutes using the the Luminex® MAGPIX® Analyzer.

3.2.5 CB1 and CB2 binding assays.

Membranes (0.4 mg pf protein) from CM3 cells were incubated in 50 mM Tris- HCl (pH 7.4), 0.5mM EDTA, 10mM MgCl2, at 37°C for 90 min, with 0.8 nM [3H]- CP-55940 as the high affinity ligand. Non-specific binding was determined using 1 μ M WIN55312-2, AM 630, AM251, SR141716A and SR141528 (Tocris, Bristol,UK). After incubation, samples were diluted with ice cold assay buffer in order to be vacuum-filtered through Whatman GF/C filters. The radioactivity associated to the filters were measured by scintillation counting.

3.2.6 Extraction, purification and quantification of eCBs.

Cells plus medium were extracted in 5 vol of chloroform/methanol/Tris‑HCl 50 mM (2:1:1) containing 5 pmol of d8-AEA, 10 pmol of d4-PEA, d2-OEA and d5-2- AG (Cayman Chemicals, Ann Arbor, MI). The lipid-containing organic phase was prepurified by open-bed chromatography on silica gel columns eluted with increasing concentrations of methanol in chloroform. Fractions eluted with chloroform/methanol 9:1 by vol. (containing AEA, 2-AG, OEA, and PEA) were collected and aliquots analyzed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC-APCI‑MS) carried out under conditions described previously. MS detection was carried out in the selected ion monitoring mode using m/z values of 356 and 348 (molecular ion +1 for deuterated and undeuterated AEA), 384.35 and 379.35 (molecular ion +1 for deuterated and undeuterated 2-AG), 304 and 300 (molecular ion +1 for deuterated and undeuterated PEA), and 328 and 326 (molecular ion +1 for deuterated and undeuterated OEA). The levels of eCBs were

then calculated on the basis of their area ratios with the internal deuterated standard signal areas and their amounts were expressed as pmol/mg of protein.

3.2.7 Statistical analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS) for Mac 26.0. Differences were considered significant at the P<.050 level. For all dependent variables, normality (Kolmogorov-Smirnov) and homogeneity (Levene) tests were applied. Data of continuous variables are presented as mean values ± standard deviation (SD). Median values with inter-quartile ranges were provided for non-normally distributed variables. Analysis of variance (ANOVA) for normally distributed variables was performed according to diet; otherwise, the nonparametric Mann-Whitney U H test was adopted. The two-tailed Fisher exact test was used for dichotomous variables. Body parameters and questionnaire scores were compared before and after the dietetic intervention by using the Wilcoxon signed-rank test. The Friedman test followed by the Wilcoxon test with Bonferroni's adjustment was adopted to compare parameters of each diet group at T0, T1 and T2.

CHAPTER 4: **Results of the study**

4.1 Subjects recruitment.

From September 2019 to January 2022, 86 elderly obese subjects who were scheduled for total hip arthroplasty in inpatient clinic of the Department of Orthopaedics at the University of Campus Bio-Medico of Rome were included in the study. All of participant were randomised in two groups: 1) FEHC diet group (n=32); 2) Controls group (n=54).

All participants gave written informed consent before being enrolled in the study. In Table 4 are reported the study population characteristics of the 86 participants according to diet.

In table 4: Main characteristics of subjects recruited.

	FEHC (N= 32) **(mean and SD or median and IQR range)**	**Controls (N= 54)** **(mean and SD or median and IQR range)**	**P**
Age (years)	72 (6)	73 (7)	.530
Gender (female)	21 (66)	25 (46)	.117
BMI	32.9 (30.6 - 34.4)	32.6 (31.0 - 37.5)	.591
Waist Circumference	104 (95 - 111)	100 (96 - 112)	.280
Charlson comorbidity score	1 (0 - 3)	1 (0 - 2)	.274
Number of drugs	4 (2 - 7)	5 (3 - 6)	.765
Diabetes	4 (13)	17 (31)	.070
Glucose (mg/dL)	102 (16)	106 (23)	.335
Hb1Ac (%)	5.9 (0.8)	6.4 (1.2)	.293
Total Cholesterol(mg/dL)	190 (39)	204 (31)	.075
HDL (mg/dL)	48 (18)	44 (10)	.231
LDL (mg/dL)	122 (38)	137 (31)	.046
Triglycerides (mg/dL)	124 (45)	128 (38)	.679
Creatinine (mg/dL)	0.9 (0.2)	0.9 (0.3)	.462

4.2 Anthropometric parameters

In this study, we found a close correlation between the use of FEHC diet and the improvement of anthropometric parameters. The FEHC diet has produced a significant reduction in body weight (p=0.045) and waist circumference (p=0.040) compared to the control group. The variations in body parameters from baseline to follow-up T1 and T2 according to the FEHC diet is depicted in Table 5.

Table 5: Variations in anthropometric parameters from baseline to follow-up T1 and T2 according to the FEHC diet.

	T0 **(median and IQR range)**	**T1** **(median and IQR range)**	**T2** **(median and IQR range)**	**P**
Weight (kg)	87.3 (76.6 - 94.9)	84.9 (73.8 - 92.6)	84.2 (73.0 - 91.7)	**.045**
BMI (kg/m²)	32.9 (30.6 - 34.4)	31.5 (30.2 - 35.6)	31.0 (29.6 - 35.2)	.216
Waist Circumference (cm)	104 (95 - 111)	98 (90 - 106)	96 (89 - 105)	**.040**

In control group, the absence of specific dietary indication has produced an increase of all the anthropometric parameters measured in all the follow-up time.

The variations in body parameters from baseline to follow-up T1 and T2 according to the Control group is depicted in Table 6.

Table 6: Variations in anthropometric parameters from baseline to follow-up T1 and T2 according to the control group.

	T0 **(median and IQR range)**	**T1** **(median and IQR range)**	**T2** **(median and IQR range)**	**P**
Weight (kg)	89.2 (81.7 - 101.5)	91.0 (77.5 - 97.0)	92.1 (78.1 - 96.0)	.171
BMI (kg/m²)	32.6 (31.0 - 37.5)	32.9 (29.8 - 35.9)	33,1 (30.2 - 35.9)	.591
Waist Circumference (cm)	100 (96 - 112)	101 (95 - 110)	103 (96 - 110)	.280

4.3 TDEE and Macronutrients compositions

The difference in caloric intake of FEHC group at all the three timepoints was statistically significant (p<.0001). This further highlights the compliance of patients to the proposed dietary protocol, with a significant decrease in caloric intake, in accordance with the FEHC diet.

The caloric intake measure with TDEE was statistical significantly lower in FEHC diet group (p<.0001) from T0 to T1 but not in T2. The daily fiber intake was significantly higher in FEHC diet group during all time of observation (p<.0001). Fat daily intake was significantly lower during the three-time point, especially from T0 to T1. In Table 7 are reported differences in FEHC group for TDEE and macronutrients composition at T0, T1 and T2.

These results confirmed the higher adherence to diet of FEHC group patients that have changed them diet according to how proposed.

Table 7: Changes in FEHC group for TDEE and macronutrients composition at T0, T1 and T2.

	T0 **(median and IQR range)**	**T1** **(median and IQR range)**	**T2** **(median and IQR range)**	**P** **for trend**
TDEE (Harris & Benedict)	1481 (1327 - 1537)	1783 (1668 - 1871)	1777 (1661 - 1862) *	**<.0001**
TDEE (Recall 24 hours)	2066 (1906 - 2233)	1724 (1700 - 1772)	1841 (1750 - 1902)*§	**<.0001**
Protein (g)	0.17 (0.15 - 01.8)	0.11 (0.10 - 0.12)	0.12 (0.10 - 0.14) §*	**<.0001**
Carbohydrate (g)	0.51 (0.46 - 0.56)	0.70 (0.69 - 0.71) §	0.67 (0.64 - 0.69) *§	**<.0001**
Fat (g)	0.34 (0.31 - 0.37)	0.19 (0.18 - 0.20) §	0.20 (0.18 - 0.23) *	**<.0001**
Fiber (g)	18 (13 - 21)	43 (33 - 61) §	42 (35 - 57) *	**<.0001**

* Adjusted P<.0001 vs T1 § Adjusted P<.0001 vs T0

4.4 Clinical features

Clinical features were evaluated at T0 and T1 regarding glycaemia, Hb1Ac and lipid profile. In table 8 was reported clinical features at the three timepoints of study.

Table 8: Variations in clinical features at T0, T1 and T2 in all study population.

	T0 **(mean and SD)**	**T1** **(mean and SD)**	**T2** **(mean and SD)**	**P**
Glucose (mg/dL)	105 (21)	105 (21)	102 (14)	.795
Hb1Ac (%)	6.3 (1.0)	6.1 (1.0)	6.1 (0.9)	.025
Total Cholesterol(mg/dL)	199 (34)	196 (29)	190 (29)	.233
HDL (mg/dL)	45 (14)	41 (9)	44 (13)	.045
LDL (mg/dL)	132 (34)	132 (30)	139 (33)*	.011
Triglycerides (mg/dL)	126 (40)	111 (32)§	116 (37)	.011

* Adjusted P<.050 vs T1
§ Adjusted P< .050 vs T0

The variations in clinical features from baseline to T1 and T2 according to the FEHC diet is described in Table 9.

Table 9: Changes of clinical features at T0, T1 and T2 in FEHC diet group.

	T0 **(mean and SD or median and IQR range)**	**T1** **(mean and SD or median and IQR range)**	**T2** **(mean and SD or median and IQR range)**	**P**
Glucose (mg/dL)	102 (16)	103 (17)	100 (14)	.411
Hb1Ac (%)	5.9 (0.8)	6.2 (1.2)	6.0 (0.9)	.148
Total Cholesterol(mg/dL)	190 (39)	190 (34)	177 (28)	**.002**
HDL (mg/dL)	45 (14)	41 (9)	44 (13)	.177

LDL (mg/dL)	122 (38)	127 (36)	118 (40)	.053
Triglycerides (mg/dL)	124 (45)	99 (17)	107 (37)	.062

* Adjusted P<.010 vs T1

The results from biochemical parameters shown that FEHC diet have produced a significant reduction in total cholesterol level (p=0.002), from which may attribute the variations of T1 to T2. (Fig.2)

In **figure 2** is summarized changes in total cholesterol from T1 to T2 in FEHC diet group.

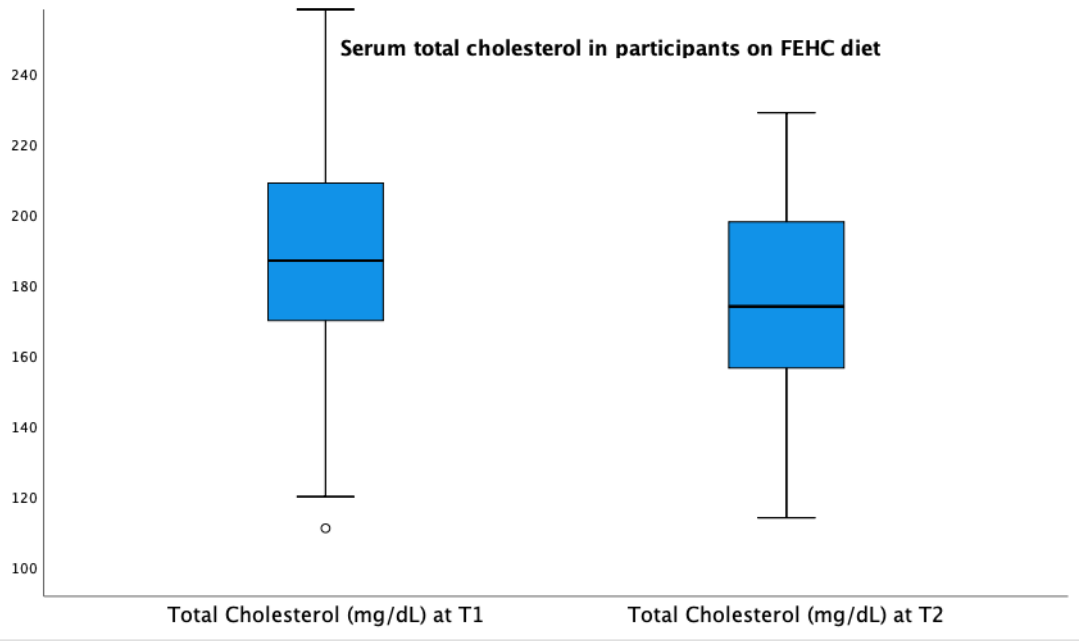


Fig. 2 Variation in blood sample of total cholesterol from T0 to T1 in FEHC diet group. Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 Total Cholesterol (p=.028).

Although not significant, we reported a trend in reduction in triglycerides levels in the FEHC group from T0 to T1 compared to controls. (Fig. 3)

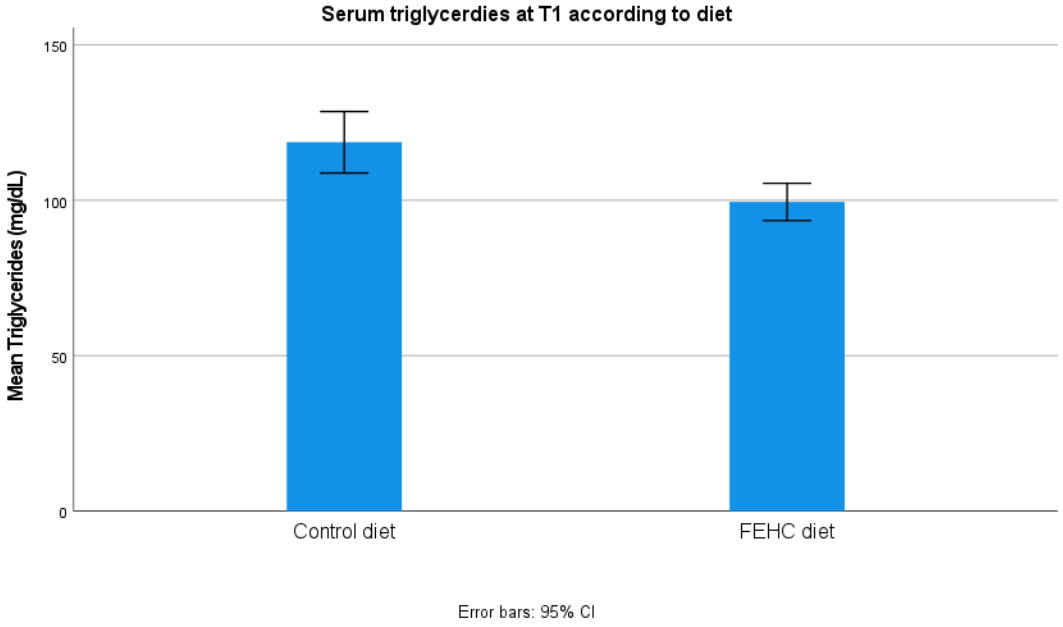


Fig. 3 Variation in blood sample of total triglycerides from T0 to T1 in FEHC diet group and control group. Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 Triglycerides (p=.062).

The variations in clinical features from baseline to T1 and T2 according to the control group is described in Table 9.

Table 10: Changes of clinical features at T0, T1 and T2 in Controls diet group.

	T0 **(mean and SD or median and IQR range)**	**T1** **(mean and SD or median and IQR range)**	**T2** **(mean and SD or median and IQR range)**	**P**
Glucose (mg/dL)	106 (23)	106 (24)	103 (14)	.592
Hb1Ac (%)	6.4 (1.2)	6.1 (0.9)	6.2 (0.9)	.113
Total Cholesterol(mg/dL)	204 (31)	199 (25)	199 (26)	.754
HDL (mg/dL)	44 (10)	40 (8)	42 (9)	.135
LDL (mg/dL)	137 (31)	136 (25)	140 (28)	.140

Triglycerides (mg/dL)	128 (38)	131 (36)	129 (37)	.124

4.5 Functional outcomes

Patients who followed the FEHC diet had significantly higher scores in both OHS (p=0.006) and HOOS (p <0.001) questionnaires at baseline and follow-up times. (Fig. 4 and 5). Also, lower scores in the WOMAC questionnaire at follow-up as compared with the controls (p=0.009) (Fig.6). The variations in questionnaire scores from baseline to follow-up according to the FEHC diet are depicted in table 11.

Table 11: Variations in FEHC group for OHS, HOOS and WOMAC questionnaires at T0, T1 and T2.

	T0 **(median and IQR range)**	**T1** **(median and IQR range)**	**T2** **(median and IQR range)**	**P for trend**
OHS score	36 (27 - 45)	40 (30 - 46)	42 (32 - 48)	**<.0001**
HOOS score	62 (32 - 84)	65 (54 - 91)	65 (54 - 92)	**<.0001**
WOMAC score	0.35 (0.30 - 0.44)	0.37 (0.29 - 0.43)	0.34 (0.29 - 041)	**.009**

** Adjusted P<.010 vs T1

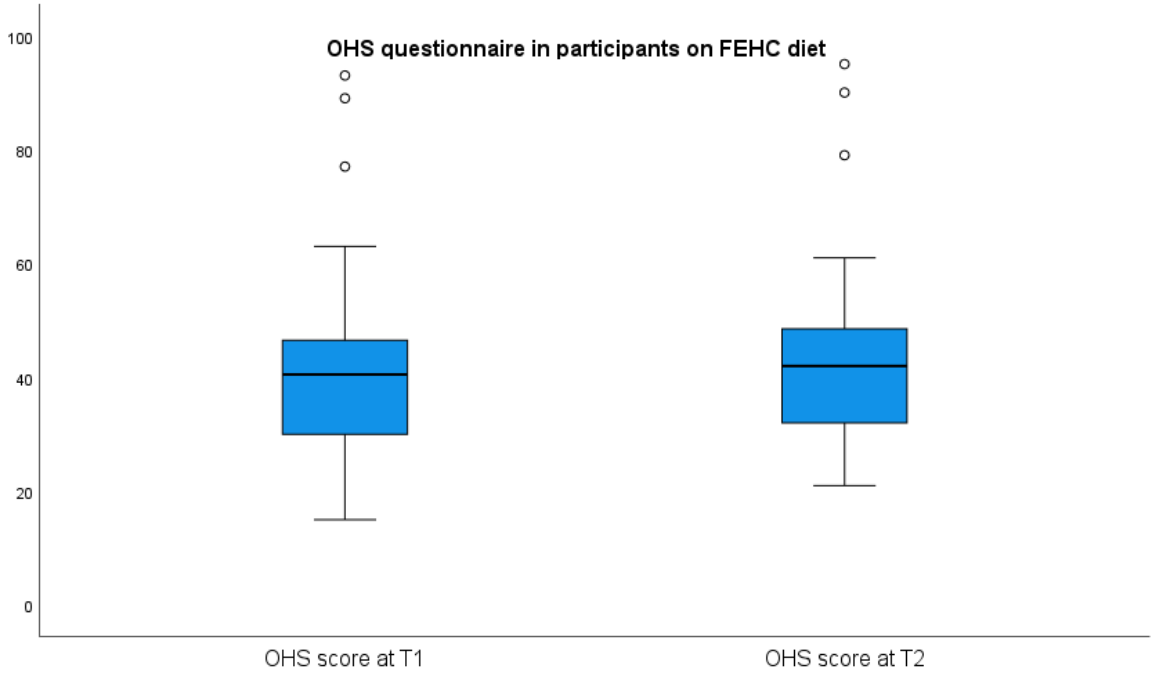


Fig. 4 Variation in OHS questionnaire from T1 to T2 in FEHC diet group. Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 OHS (p<.0001).

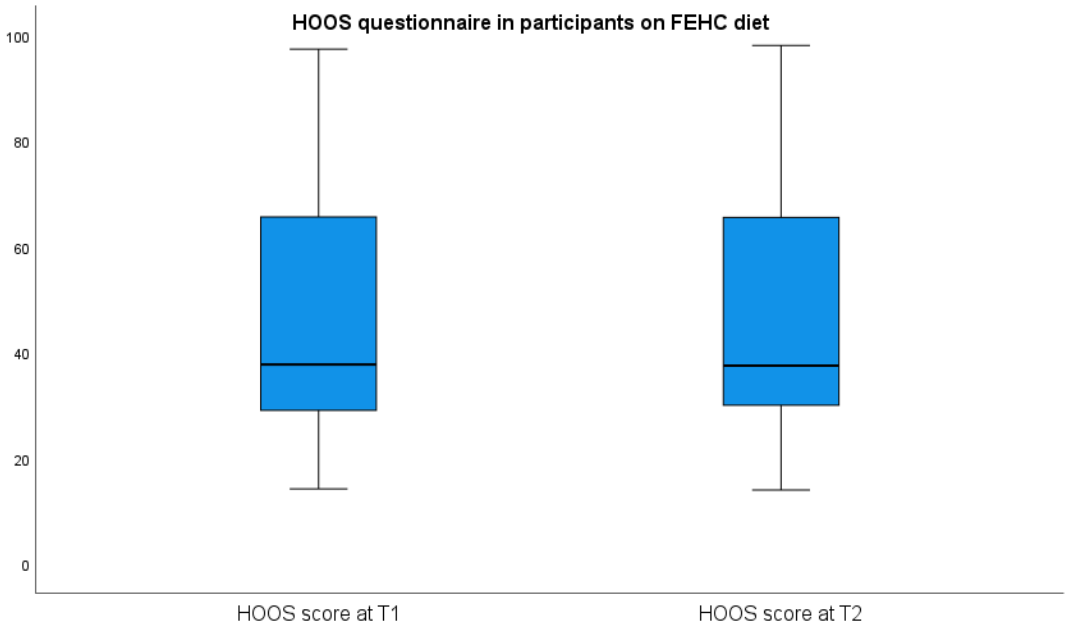


Fig. 5 Variation in HOOS questionnaire from T1 to T2 in FEHC diet group. Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 H00S (p<.0001).

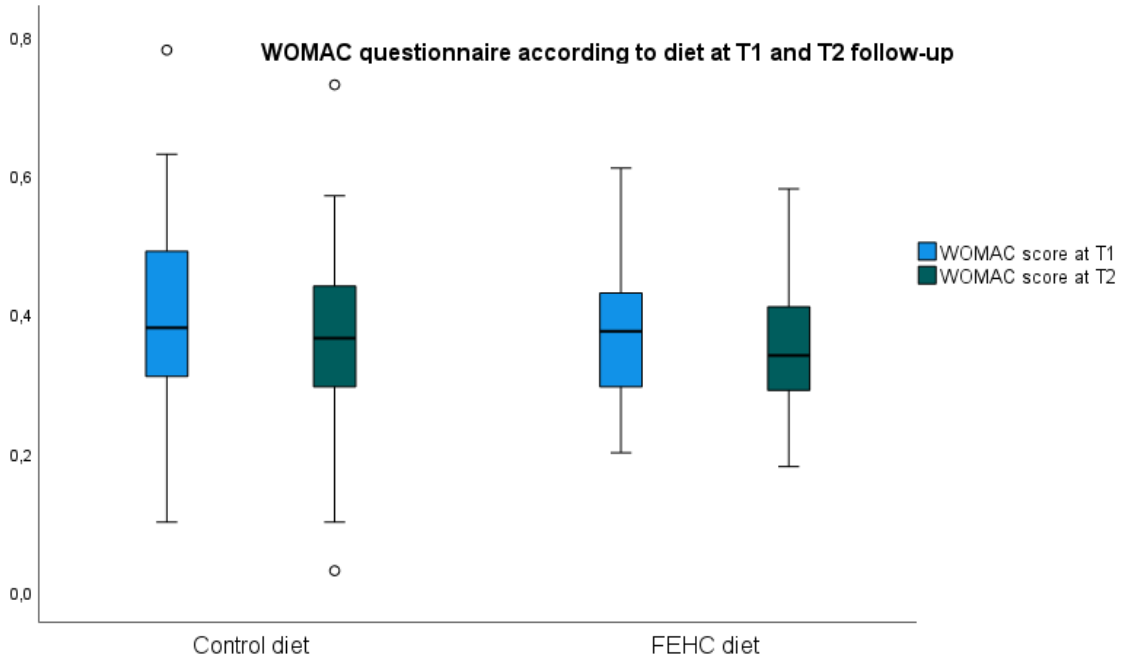


Fig.6 Differences in the between the groups on WOMAC questionnaire from T1 to T2. Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 WOMAC (p<.009).

4.6 DEXA parameters

Data from t-score and BMD at lumbar spine ad femoral bone were collected at T0 to T2 in all participants. In the FEHC group, no statistically significant differences were reported. Main characteristics of DEXA parameter is depicted in Table 12.

Table 12: Dexa parameters from baseline to follow-up T2 according to the FEHC diet.

	T0 **(median and IQR range)**	**T2** **(median and IQR range)**	**P**
BMD lumbar (g/cm2)	0.96 (0.96 - 1.26)	1.16 (1.07 - 1.25)	.754
Lumbar t-score (SD)	-0.8 (-0.8 - 1.7)	1.1 (0.2 - 1.7)	.433
BMD Femoral neck (g/cm2)	0.76 (0.73 - 0.89)	0.87 (0.85 - 0.87)	.593
Femoral T-score (SD)	-0.8 (-0.4 - -0.1)	-0.1 (-0.2 - 0.1)	.593

Also, in the control group no statistically significant differences were reported related to DEXA parameters. Main characteristics of DEXA parameter is depicted in Table 13.

Table 13: Dexa parameters from baseline to follow-up T2 according to the control group.

	T0 **(median and IQR range)**	**T2** **(median and IQR range)**	**P**
BMD lumbar (g/cm2)	1.28 (0.88 - 1.33)	1.08 (0.94 - 1.24)	.245
Lumbar t-score (SD)	1.7 (-1.5 - 2.1)	-0.2(-0.9 - 1.4)	.286
BMD Femoral neck (g/cm2)	0.92 (0.75 - 0.95)	0.86 (0.83 - 0.93)	.753
Femoral T-score (SD)	-0.1 (-1.3 - 0.2)	-0.1 (-0.6 - -0.2)	.752

4.7 WNT pathway factors and inflammation in serum sample

The systemic profile of inflammatory cytokines was determined in the serum of all patients.

WNT genes and concentrations of selected cytokines between group comparison of changes from T0 to T1 were assessed. (Table 14).

Table 14. Main WNT pathway genes and inflammation cytokines from T0 to T1 between the groups.

T0-T1	**FEHC (N= 32)** **(median and IQR range)**	**Controls (N= 54)** **(median and IQR range)**	**P**
IL-6 (pg/dl)	2.22 (1.41 - 3.07)	3.04 (2.37 - 5.53)	.030
IL-8 (pg/dl)	4.00 (1.86 - 5.44)	6.68 (5.23 - 9.29)	.022
IL-10 (pg/dl)	1.08 (0.56 - 1.18)	1.06 (0.98 - 1.31)	.198
IL-15 (pg/dl)	1.06 (0.37 - 3.97)	1.98 (1.69 - 3.34)	.638
TNFα (pg/dl)	6.05 (3.94 - 7.06)	7.52 (6.18 - 9.00)	.040
MCP1(pg/dl)	296.42 (253.85 - 337.97)	319.47 (284.64 - 363.74)	.445

DKK1(pg/dl)	7183.1 (5085.6 - 7907.7)	3667.8 (2935.9 - 4888.4)	<.0001
SOST (pg/dl)	204.7 (150.4 - 334.5)	207.4 (184.4 - 255.4)	.546
Adiponectin (pg/dl)	10039 (8229 - 12103)	10050 (8253 - 11051)	.748

WNT genes and concentrations of selected cytokines were assessed at T0 (Table 15) and T1 (Table 16).

Table 15. Main WNT pathway genes and inflammation cytokines at T0 between the groups.

T0	**FEHC (N= 32) (median and IQR range)**	**Controls (N= 54) (median and IQR range)**	**P**
IL-6 (pg/dl)	2.16 (1.24 - 2.52)	2.49 (1.71 - 3.34)	.664
IL-8 (pg/dl)	2.28 (2.02 - 4.43)	2.645 (1.50 - 8.61)	.424
IL-10 (pg/dl)	0.70 (0.54 - 1.27)	1.15 (0.82 - 1.66)	.210
IL-15 (pg/dl)	0.71 (0.37 - 2.83)	1.06 (0.37 - 2.29)	.130
TNFα (pg/dl)	5.11 (4.00 - 6.49)	5.38 (3.50 - 7.39)	.973
MCP1(pg/dl)	300.20 (223.08 - 356.12)	296.88 (217.46 - 350.70)	.903
DKK1(pg/dl)	7842.46 (5714.83 - 8437.26)	7270.07 (5309.96 - 8030.03)	.776
SOST (pg/dl)	194.18 (131.06 - 266.95)	241.20 (169.19 - 324.09)	.924
Adiponectin (pg/dl)	8981.95 (8135.90 - 12510.50)	9922.00 (8224.30 - 11918.00)	.776

Table 16. Main WNT pathway genes and inflammation cytokines at T1 in the groups.

T1	**FEHC (N= 32) (median and IQR range)**	**Controls (N= 54) (median and IQR range)**	**P**
IL-6 (pg/dl)	2.16 (0.82 - 2.93)	2.78 (2.29 - 3.04)	.173
IL-8 (pg/dl)	5.41 (2.11 - 5.21)	7.81 (6.48 - 10.50)	.141
IL-10 (pg/dl)	0.56 (0.53 - 0.94)	1.04 (0.94 - 1.11)	**.028**
IL-15 (pg/dl)	2.17 (0.37 - 3.97)	1.62 (0.18 - 3.07)	.180
TNFα (pg/dl)	6.31 (4.81 - 11.7)	7.81 (7.52 - 9.00)	.753
MCP1(pg/dl)	296.42 (289.1 - 304.0)	340.1 (268.1 - 371.7)	.345
DKK1(pg/dl)	7337.5 (4861.2 - 8572.5)	4779.8 (3129.8 - 5514.6)	**.026**
SOST (pg/dl)	217.6 (156.6 - 336.4)	203.2 (196.2 - 241.0)	.463

Adiponectin (pg/dl)	8334 (6668 - 13113)	10097 (7505 - 10147)	.600

In FEHC diet group has been showed a significant lower expression of DKK1(p=.028) (Fig 7) and a significant increase of IL-10 (p =.026) at T1. (Fig 8).

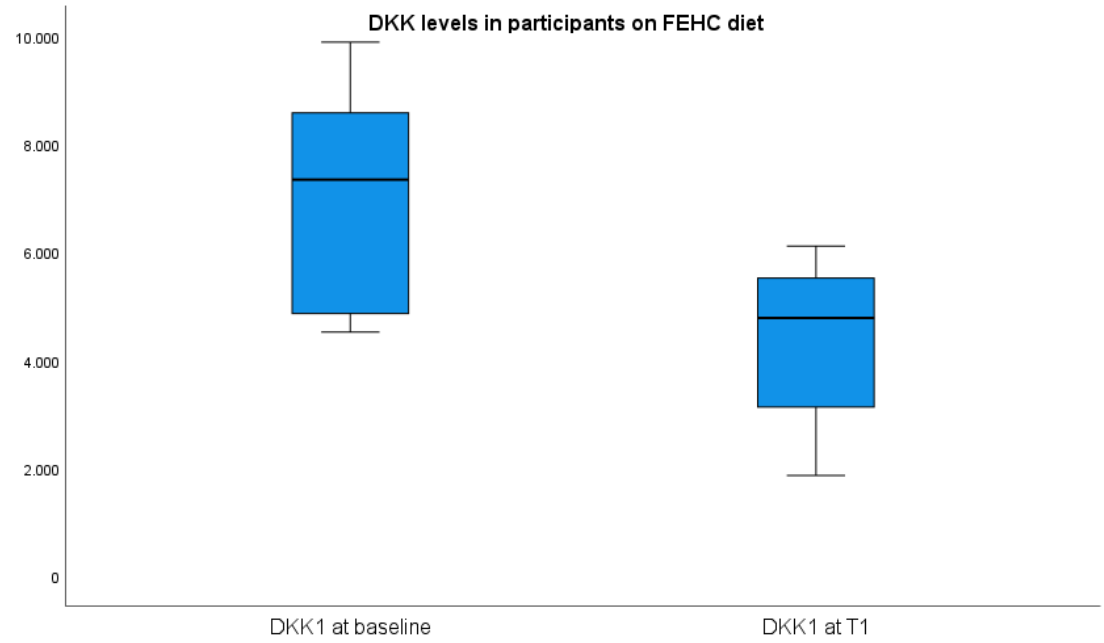


Fig 7: Gene expression evaluation of DKK1 in serum of FEHC diet group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 DKK1(p=.028).

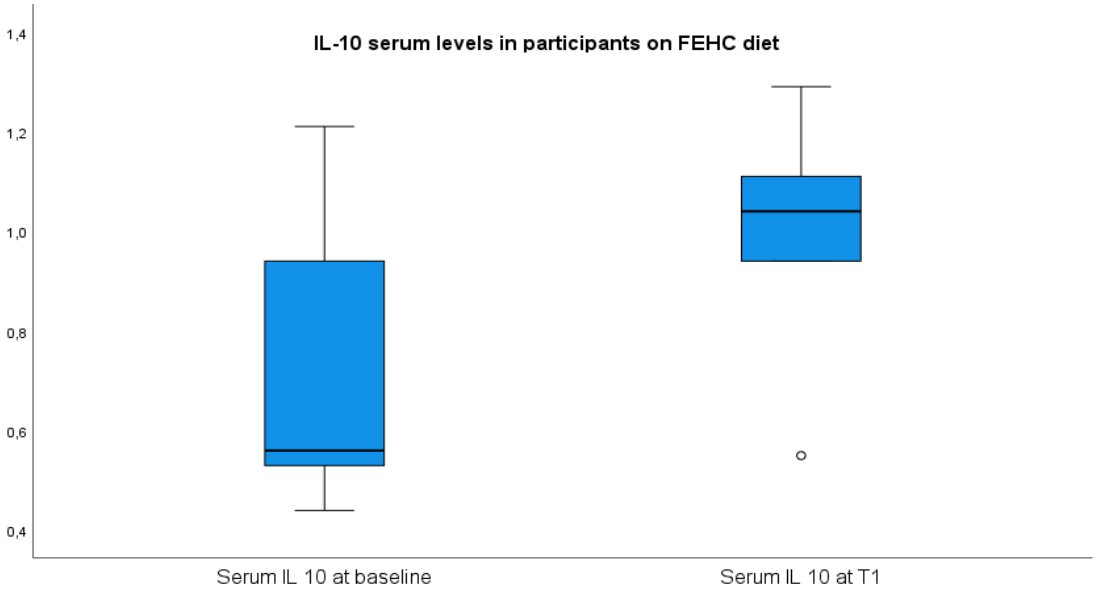


Fig 8: Gene expression evaluation of IL-10 in serum of FEHC diet group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 IL-10 (p=.046).

In the Control group we have reported a significant increase in TNFα (p=.028) at T1 compared to T0. Variations of TNFα is described in fig. 9.

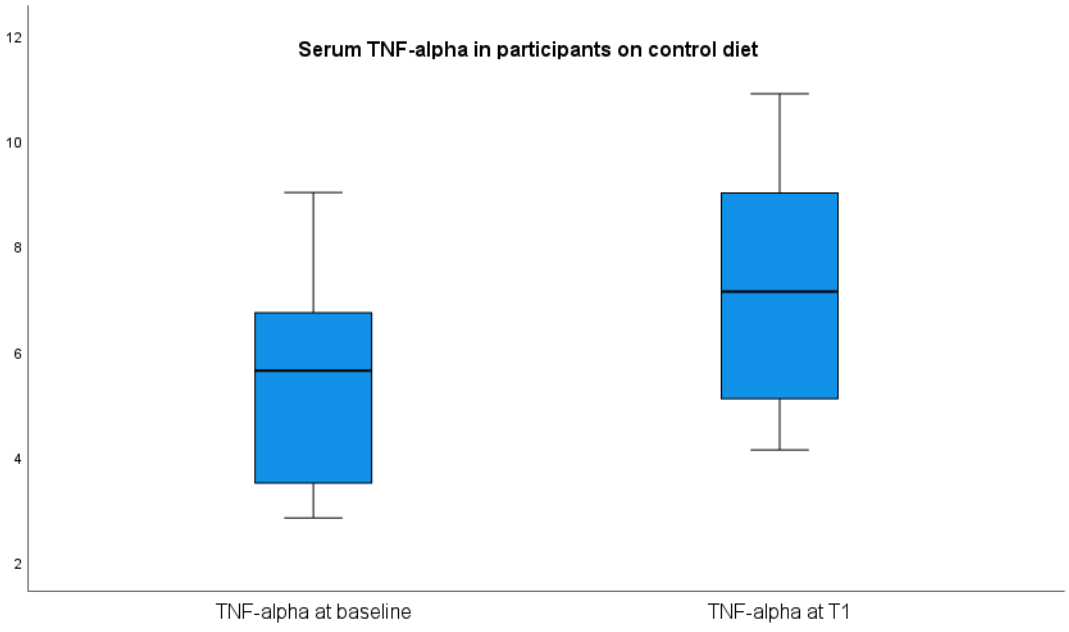


Fig 9: Gene expression evaluation of TNFα in serum control diet group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 TNFα (p=.028).

4.8 Gene expression of WNT pathway factors and inflammation in Bone, Adipose Tissue, Skeletal Muscle.

- Bone

Gene expression analysis though real time RT-PCR of Wnt10b and Wnt5a, involved in the activation of canonical and non-canonical WNT pathway respectively, were evaluated.

The results have been normalized with three internal controls, β-actin (βact), beta-2-microglobulin (B2m) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh), given that the three most stable housekeeper genes for this tissue. Subsequently, the results obtained were normalized with Bestkeeper gene, which is the adjusted mean of the housekeeper genes and then the resulted mRNA levels were compared between the two groups.

The assessment of gene expression highlighted that in FEHC group may occur a downregulation of WNT pathway: there were reported a trend for Wnt10b (p=0.064) and a slight reduction but not significant in *Wnt5a*. (Fig.10)

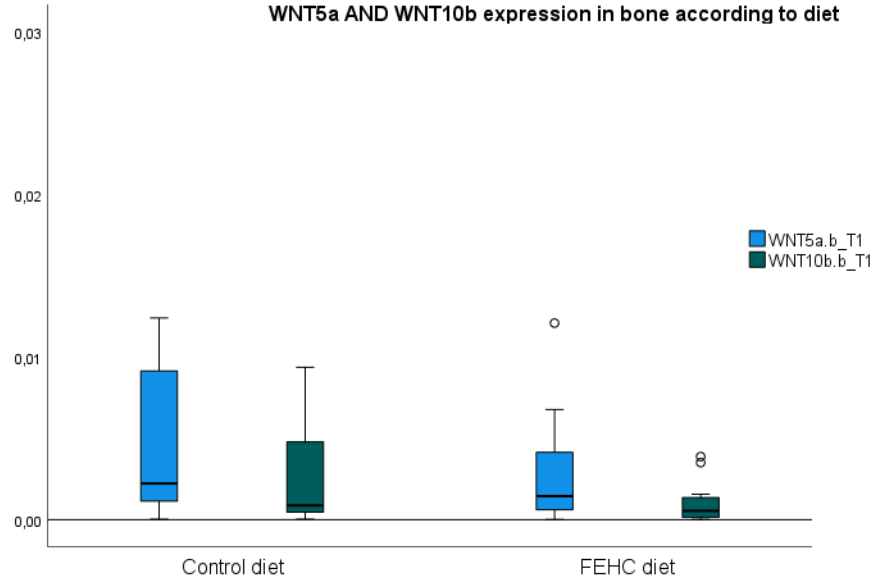


Fig 10: Gene expression evaluation of Wnt5a and Wnt10b in bone of FEHC and Control groups. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0

Moreover, it was evaluated also the gene expression of Sost, DKK1, LEF1, OCN, RUNX2 and for selected cytokines IL-6, IL-8, IL-10, TNFa. No statistically significant difference in the gene expression of these genes in bone tissue were reported between the groups (Table 17). Although these observations are not significant, these are nonetheless indicative of a possible diminished activation of the WNT signalling pathway in FEHC diet group. Data from inflammation in bone suggested that FEHC diet are unable for three months to reduce inflammation directly in bone tissue probably due to an insufficient time of diet proposed.

Table 17. Main characteristics of WNT pathway factors and inflammation between the groups in bone.

T1	FEHC (N= 32) (median and IQR range)	Controls (N= 54) (median and IQR range)	P
WNT5a	0.0009 (0.000587 - 0.003503)	0.0021(0.001061 - 0.009143)	.292

WNT10b	0.00055 (0.000133 - 0.001570)	0.00077 (0.000412 - 0.002850)	.064
SOST	0.001449 (0.000524 - 0.002271)	0.002112 (0.000735 - 0.003664)	.205
IGF-1	0.005225 (0.000944 - 0.005748)	0.006087 (0.002539 - 0.013618)	.888
IL-6	0.001825 (0.000607 - 0.003695)	0.001466 (0.000490 - 0.007554)	.850
IL-8	0.008803 (0.002713 - 0.017948)	0.005839 (0.001650 - 0.017452)	.761
IL-10	0.000409 (0.000261 - 0.001588)	0.001213 (0.000335 - 0.002730)	.277
TNFa	0.001790 (0.000568 - 0.003367)	0.001016 (0.000333 - 0.004593)	.655
ADIPOQ	0.008085 (0.006444 - 0.266394)	0.039031 (0.006765 - 0.068184)	.839
OCN	0.032296 (00.007268 - 2.590981)	0.035805 (0.003615 - 0.058447)	.250
RUNX2	0.040068 (0.005411 - 0.277188)	0.006706 (0.001828 - 0.018779)	.354
DKK-1	0.002129 (0.000710 - 0.007485)	0.003560 (0.000656 - 0.008560)	.879
LEF-1	0.004696 (0.003204 - 0.032914)	0.006682 (0.002776 - 0.016226)	.566
COL1-A1	0.501338 (0.165457 - 0.955219)	0.669477 (0.289819 - 1.268182)	.504

- Skeletal Muscle

It was performed the gene expression analysis though real time RT-PCR of the elements of WNT pathway considered also for the bone tissue: Wnt5a, Wnt10b.

The results have been normalized with three internal controls, β-actin (βact), beta-2-microglobulin (B2m) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh), given that the three most stable housekeeper genes for this tissue. Subsequently, the results were normalized and then mRNA levels subjects were compared.

In FEHC diet group we have observed a trend in *Wnt5a* (p=.065) (Fig.11) and a a significant reduction in IL-6 (p <.035) (Fig. 12) compared with control group.

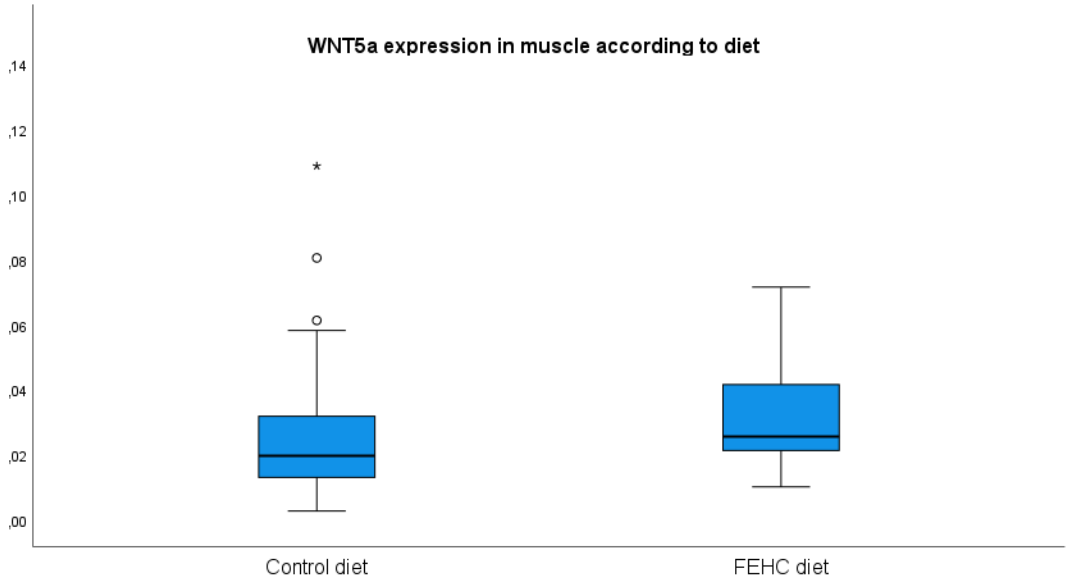


Fig 11: Gene expression evaluation of Wnt5a in skeletal muscle in FEHC and control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 Wnt5a (p=.065).

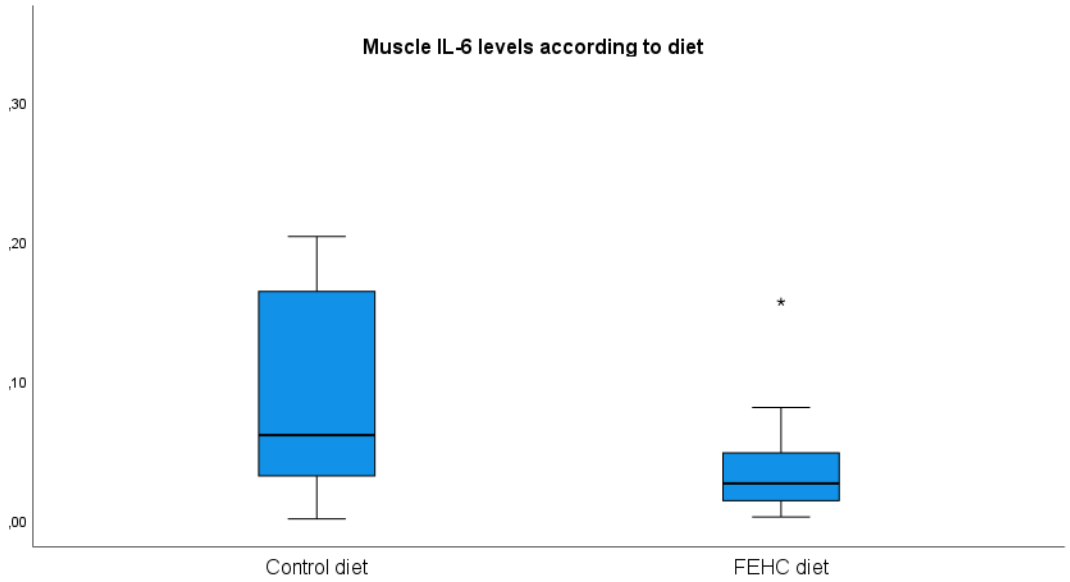


Fig 12: Gene expression evaluation of IL-6 in skeletal muscle of FEHC diet group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0 IL-6 (p=.035).

No statistically significant differences of the other WNT genes and cytokines were assessed between the group (Table 18).

Table 18. Main characteristics of WNT pathway factors and inflammation between the groups in skeletal muscle.

T1	FEHC (N= 32) (median and IQR range)	Controls (N= 54) (median and IQR range)	P
WNT5a	0.025 (0.021274 - 0.041547)	0.019653 (0.013010 - 0.031779)	***.065***
WNT10b	0.00676 (0.003691 - 0.007799)	0.006564 (0.002309 - 0.012497)	.588
SOST	0.000090 (0.000049 - 0.000147)	0.000102 (0.000060 - 0.000410)	.488
IGF-1	0.020 (0.018644 - 0.024947)	0.0160 (0.010402 - 0.026453)	.392
IL-6	0.026 (0.013748 - 0.048131)	0.060 (0.031601 - 0.163989)	**.035**
IL-8	0.038 (0.023392 - 0.107785)	0.067 (0.025223 - 0.174918)	.317
IL-10	0.001 (0.000727 - 0.001931)	0.001 (0.000801 - 0.003613)	.224
TNFα	0.003 (0.002439 - 0.005748)	0.005 (0.003133 - 0.008885)	.104
ADIPOQ	0.051 (0.032262 - 0.091489)	0.047 (0.017980 - 0.137892)	.838
OCN	0.001 (0.000874 - 0.002347)	0.001 (0.000766 - 0.003894)	.433
RUNX2	0.003 (0.002850 - 0.004595)	0.003 (0.002508 - 0.005255)	.933
DKK-1	0.002 (0.001627 - 0.003563)	0.002 (0.001369 - 0.003231)	.819
LEF-1	0.006 (0.003598 - 0.010135)	0.006 (0.003887 - 0.010929)	.763
COL1-A1	0.122 (0.085242 - 0.166426)	0.151 (0.051973 - 0.332364)	.353

- Adipose tissue

In adipose tissue, it was performed the gene expression analysis though real time RT-PCR of the elements of WNT pathway assessed also in bone and skeletal muscle tissues and cytokines profile related to obesity.

The results have been normalized with three internal controls, β-actin (βact), RNA 18S (18S) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh), given that the

three most stable housekeeper genes for this tissue. Subsequently, the results obtained were normalized with Bestkeeper gene, which is the adjusted mean of the housekeeper genes and then the mRNA levels obtained were compared between the categories of the subjects.

It was performed the gene expression analysis though real time RT-PCR of WNT genes and cytokines profile related to obesity. No statistically significant difference in adipose tissue were reported between the groups (Table 19).

Table 19. Main characteristics of WNT pathway factors and inflammation between the groups in adipose tissue.

T1	FEHC (N= 32) (median and IQR range)	Controls (N= 54) (median and IQR range)	P
WNT5a	0.137 (0.054 - 0.235)	0.140 (0.036 - 0.161)	.799
WNT10b	0.201 (0.054178 - 0.235860)	0.016 (0.012 - 0.088)	.195
SOST	0.007 (0.001672 - 0.032539)	0.001 (0.0003 - 0.075)	.721
IGF-1	1.014 (0.391784 - 1.583487)	0.200 (0.143 - 0.527)	.195
IL-6	1.562 (0.916450 - 3.384762)	1.176 (0.370 - 4.844)	.799
IL-8	6.853 (0.953513 - 11.766147)	0.842(0.312 - 9.880)	.506
IL-10	0.072 (0.038068 - 0.131135)	0.052 (0.025 - 0.154)	.999
TNFα	0.074 (0.020110 - 0.129352)	0.059 (0.022- 0.264)	.799
ADIPOQ	39.483 (4.718708 - 79.596568)	13.062 (7.941 - 33.379)	.799
OCN	0.020707 (0.003 - 0.032)	0.006 (0.001 - 0.127)	.959
RUNX2	0.007210 (0.006 - 0.032)	0.036 (0.019 - 0.107)	.620
DKK-1	0.072136 (0.001 - 0.073)	0.020 (0.001- 0.175)	.999
LEF-1	0.007210 (0.004 - 0.032)	0.029(0.007 - 0.130)	.328
COL1-A1	12.115400 (2.56 - 58.968)	8.387(3.017- 19.496)	.721

4.9 Gene expression of all major ECS elements in Adipose and Muscle tissues biopsies

- ***Adipose Tissue: gene expression evaluation of ECS.***

It was performed the gene expression of ECS in adipose tissue by real time RT-PCR of the principal elements of ECS. The evaluation of EC receptors not showed any statistical differences in term of CB1 and CB2 for the two groups (Fig.13).

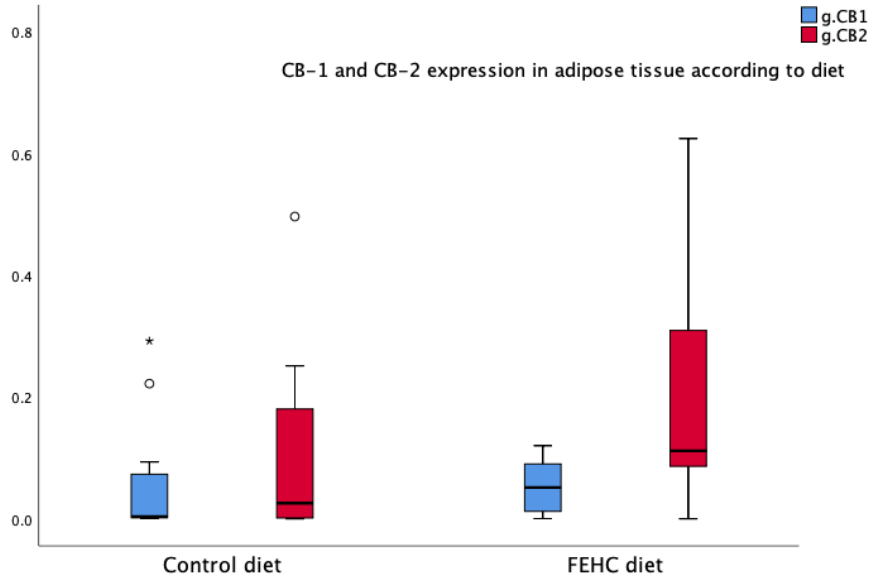


Fig 13: Gene expression evaluation of ECS receptors in adipose tissue samples of FEHC diet group and Control group. Blue bars correspond to Cb1 red bars, Cb2. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

Also, it was performed the gene expression analysis though real time RT-PCR of the elements of non-classical receptors of ECS such as Gpr55 and of the enzyme dedicated to degradation Magl.

The analysis of ECS receptors in our subjects showed a general down regulation of all these in the adipose tissue of control group compared to FEHC diet group.

Gpr55 and MAGL showed a more substantial, though not significant, increase in FEHC diet group. (Fig. 14 and Fig 15).

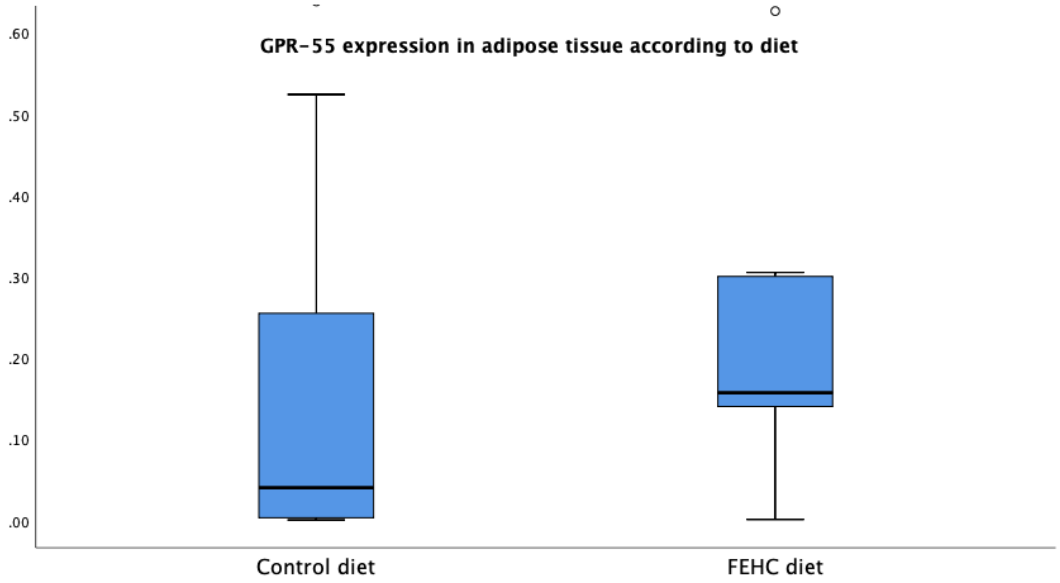


Fig 14: Gene expression evaluation of GPR-55 in adipose tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

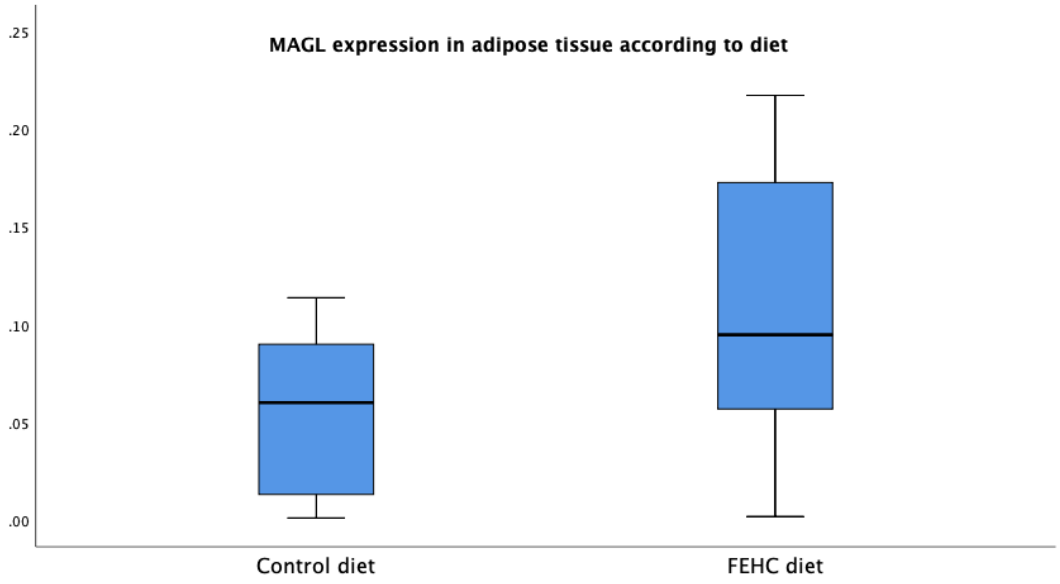


Fig 15: Gene expression evaluation of MAGL in adipose tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

- ***Muscle Tissue***

It was performed the gene expression analysis though real time RT-PCR of the elements of ECS, including non-classical receptors (Trpv1 and Gpr55) and the enzymes dedicated to the synthesis (Nape-PLD and Dagl α/β) and the degradation (Faah and Magl) of endocannabinoids. Trpv1 and Gpr55 not showed any statistical differences between the groups. (Fig 16, fig 17).

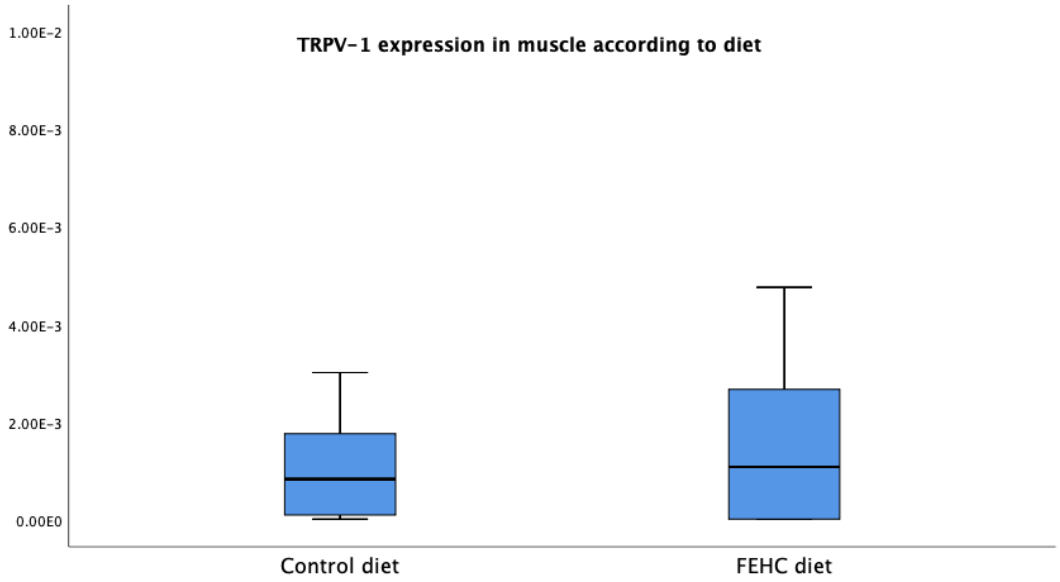


Fig 16: Gene expression evaluation of TRPV-1 in skeletal tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

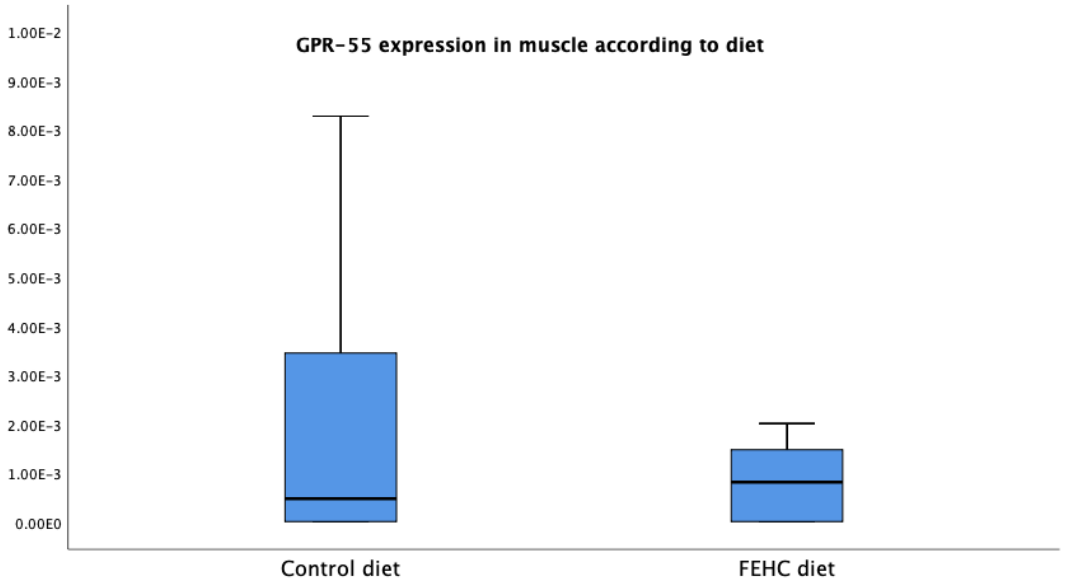


Fig 17: Gene expression evaluation of GPR-55 in skeletal tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

In FEHC diet group, the ECS enzymes dedicated to the synthesis Nape-PLD was slightly decreased (Fig. 18), while DAGLα and DAGLβ showed a more substantial, though not significant, increase. (Fig. 19).

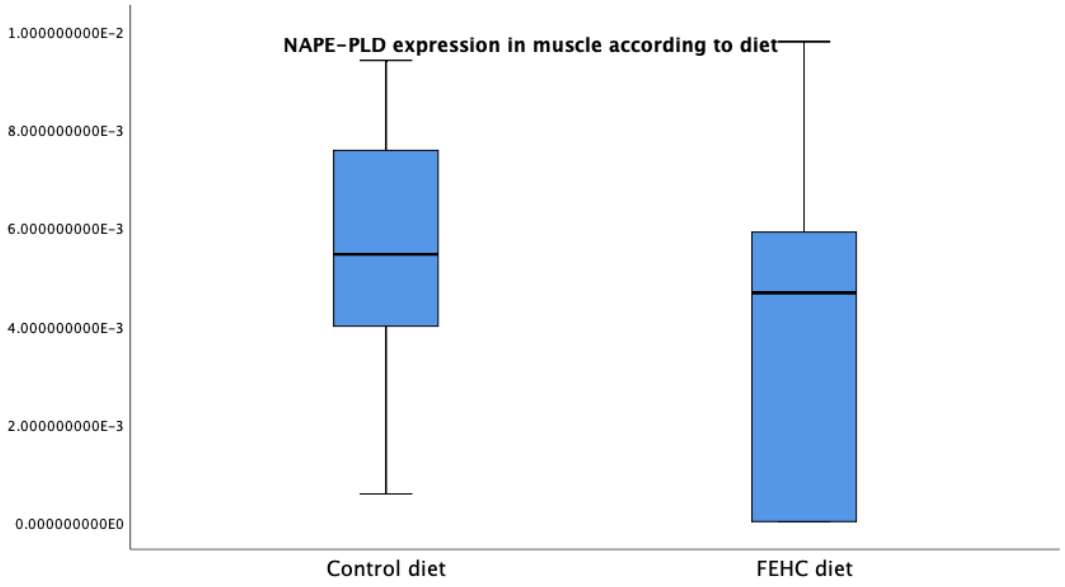


Fig 18: Gene expression evaluation of Nape-PLD in skeletal tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper

gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

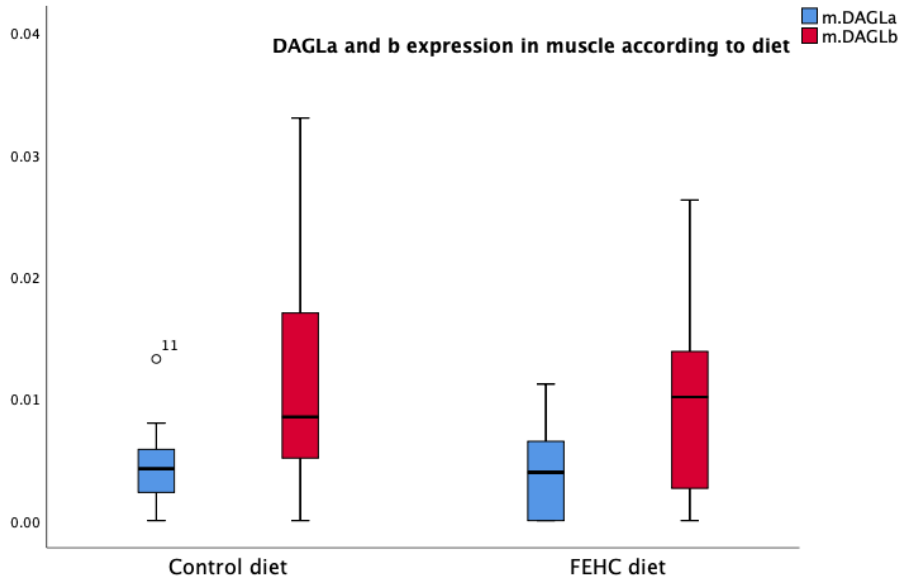


Fig 19: Gene expression evaluation of DAGLα and DAGLβ in skeletal tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

It was performed the gene expression analysis though real time RT-PCR the degradation element of ECS: FAAH and MAGL.

In FEHC diet group, the FAAH enzyme was slightly increased (Fig.20), also MAGL though not significant increase (Fig. 21).

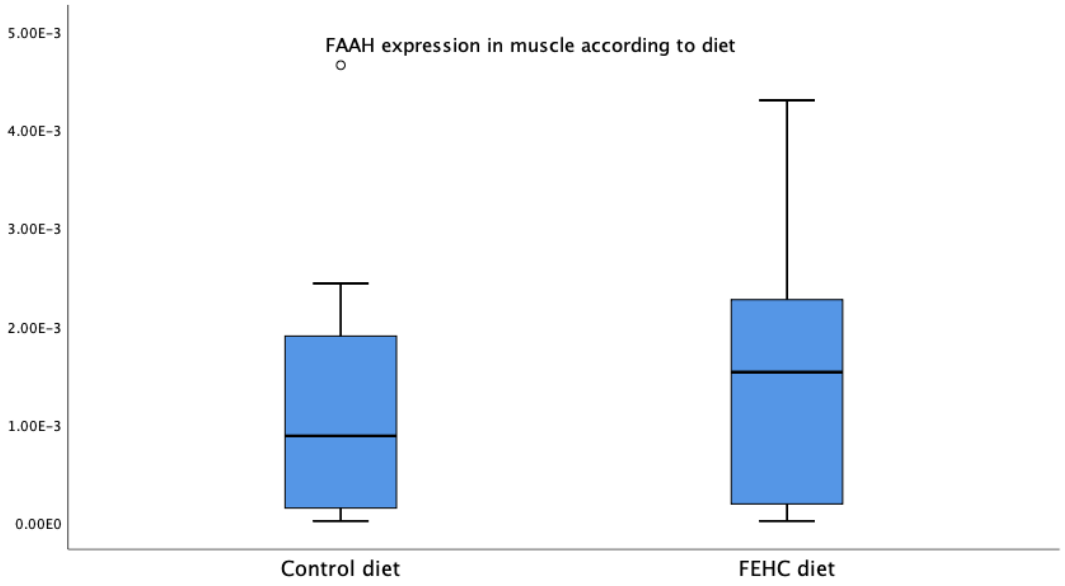


Fig 20: Gene expression evaluation of FAAH in skeletal tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

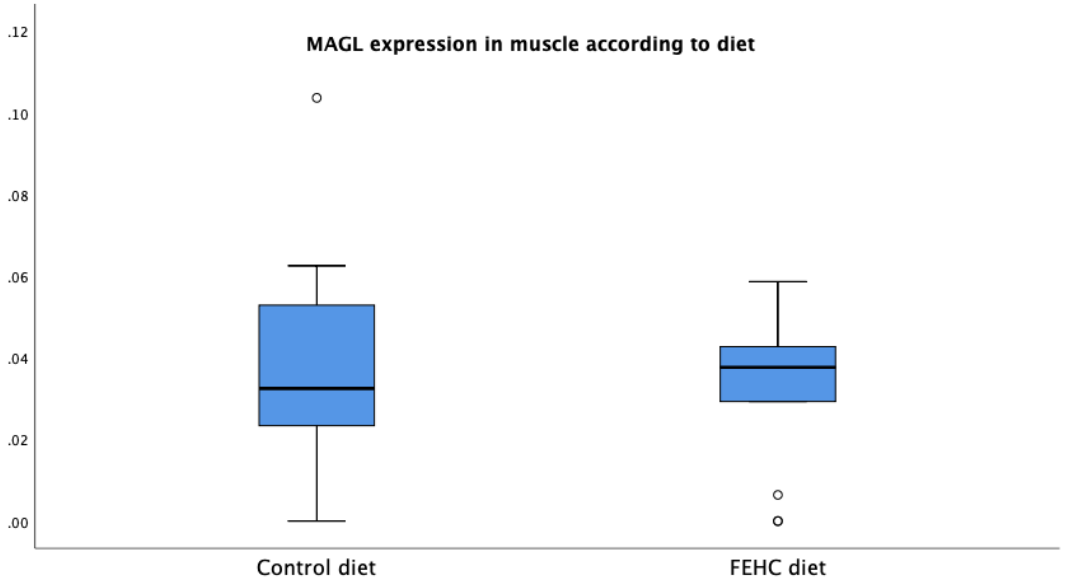


Fig 21: Gene expression evaluation of MAGL in skeletal tissue samples of FEHC diet group and Control group. Data are represented as mean ± SEM, normalized with Bestkeeper gene (adjusted mean of housekeeper genes). Unpaired t test was performed by Statistical Package for Social Science (SPSS) for Mac 26.0.

CHAPTER 5: Discussion

The primary aim of this study was to test FEHC diet in elderly obese subjects on improving obesity-related inflammation on bone, muscle and adipose tissue, through a positive modulation of WNT and ECS pathways.

Observational studies suggest that high intake of fiber is associated with a reduced risk of obesity [79]. Large cohort studies have shown that a FEHC diet was associated with higher plasma levels of adiponectin and lower CRP than diets low in fiber [207,208]. This diet is also rich in phenolic compounds that are known to decrease TNFα and IL-6, suppress CRP, and improve endothelial function [209]. Our group has confirmed these observational data through a prospective study, showing lower inflammation in patients consuming a FEHC with significantly lower HbA1c, insulin resistance, and greater weight loss compared with those on control diet, despite the same energy content [8,9]. FEHC diet reversed also pro-inflammatory dysbiosis and these effects were obtained in only 3 weeks of intervention [10]. The longer duration of the treatment planned in this project may allow us to investigate how FEHC diet enhance bone and muscle health.

Obesity is a key player in the bone impairment mechanisms, which may be mediated by altered WNT pathway and ECS signaling.

We have evaluated for the first time the expression of both WNT and ECS signaling elements directly in human, in a population of obese following specific diet and comparing the expression of three important tissues as bone, skeletal muscle and fat.

Based on the results obtained, it can be hypothesized that specific dietary intervention with FEHC diet in subjects with obesity is helpful on improving anthropometric,

clinical features, functional outcomes, reducing inflammation mediated by WNT and ECS pathways.

5.1 Anthropometric parameters

In this study, we found a close correlation ($p < 0.001$) between the use of the FEHC diet and the improvement of anthropometric parameters. The FEHC diet have produced a significant reduction in body weight and waist circumference compared to the control group.

The role of fiber in the regulation of body weight has already been discussed in the literature. Experimental studies have shown that a diet rich in fiber reduces the absorption of free fatty acids by using them as energy in the fermentation process of the fibers in the intestine. In addition, dietary fibers have the ability to retain water; this leads to a lowering of the energy/weight ratio of the ingested food, stimulating the sense of satiety despite a reduced energy intake and, thus, reducing voluntary food intake [96,110].

Many physiological mechanisms of the action of dietary fiber in weight loss have been studied. Fiber appears to reduce food intake through the increased effort and time it takes to chew it [210]. In a study by Birketvedt et al., the addition of dietary fiber in a low-calorie diet significantly improved weight loss, with the placebo group presenting a 5.8 kg weight reduction compared to an 8.0 kg weight reduction in the group with fiber supplementation [211]. Studies of postmenopausal women have also shown that the introduction of a higher fiber content within a very low-fat diet increases weight loss [212]. In a study by Te Morenga et al., a diet rich in fiber was demonstrated to reduce hunger and increase patient compliance compared to a protein-rich diet [213].

Over time, several dietary approaches for weight loss in people with obesity have been proposed, each one characterized by different contents of macronutrients. Several studies have confirmed the central role of a high-fiber diet in weight loss. Undoubtedly, the most important and recognized is the Mediterranean diet, characterized by high consumption of fruit, vegetables, nuts, cereals and olive oil, as well as a moderate consumption of fish and poultry and a low consumption of sweets, red meat and dairy products [214].

5.2 Clinical features

The results from clinical features shown that FEHC diet have produced an improvement of total cholesterol and triglycerides.

Dietary fiber has also been shown to play a central role in cholesterol reduction. The cholesterol-lowering effect also appears to be related to the type of fiber intake. A study published in "The American Journal of Clinical Nutrition"[215] shows both the effect of fiber in reducing total cholesterol and LDL cholesterol, but also that this mechanism is more evident in diets rich in soluble fiber. Underlying this mechanism would appear to be the ability of soluble fiber to avoid micelle formation in the intestine by binding with bile salts, preventing cholesterol absorption [216].

These aspects have also been correlated with the risk of coronary events: in a study by Anderson et al., a clear inversely proportional relationship was demonstrated between the amount of soluble fiber introduced with the diet and the incidence of coronary events [217].

5.3 Functional outcomes

Patients have followed the FEHC diet had significantly higher scores in the HOOS questionnaire at both baseline and follow-up times, and lower scores in the WOMAC questionnaire at follow-up as compared with the controls.

Several studies in the literature have shown improvement in pain and disability in patient with joint issues after reduction in body weight. Weight loss in patients undergoing THA is desirable not only before and in the postoperative period. However, weight loss and lifestyle change are an insurmountable obstacle in people with obesity [218]. Several studies have underlined the beneficial effects of preoperative dietary interventions on weight loss, the reduced risk of post-operative infections and improved glycemic control before total joint arthroplasty [219,220].

In the 2020 study by Robson et al, it was shown that weight loss, regardless of how it is lost (diet, exercise, surgery), has a moderate effect on improving pain and disability in patients with hip and knee osteoarthritis [221].

Kotowski et al showed that weight loss in a group of 35 obese women led to a reduction in joint pain not only at the lower extremity joints (hip, knee, and ankles), but also at the upper extremity joints, suggesting improvement regardless of the reduction in mechanical work to which the lower joints are subjected [222].

Our results suggested the FEHC diet, through weight loss, promotes an improvement in scores even before arthroplasty surgery, recommending the efficacy of weight loss on improving symptoms and hip motility.

5.4 Evaluation of WNT signaling genes and inflammation cytokines in serum sample

Scientific evidence correlates the dysregulation of the WNT pathway with the typical chronic inflammation scenario of the obese subject [6]. In the present study, we have enrolled patients with obesity, and we have proposed a specific dietary intervention with high fiber intake in order to lose body weight and to reduce chronic inflammation related to obesity.

The exposure for three months of FEHC diet have produced a significant reduction in DKK1 levels together with a significant increase of IL-10. DKK-1 is one of the best known and most studied inhibitors of the WNT pathway. Accumulating evidences suggested that its expression can be modulated by various factors such as hormones, growth factors, and mechanical stimulation. Elevated DKK-1 activity is implicated in osteoporosis, arthritis and possibly sarcopenia [223]. In human, a recent study amongst African males reported significantly higher DKK-1 levels with total and central adiposity as measured using DXA [224]. These findings suggest that DKK-1 may serve as a critical factor produced by skeletal muscle and play a role in muscle mass changes and pathogenesis of sarcopenia. Our results suggested that FEHC diet, by lowering expression in serum sample of DKK-1, may be helpful in preventing osteoporosis, arthritis and possibly sarcopenia by supporting WNT pathway activation should be toned down.

IL-10 is an anti-inflammatory cytokine that plays a significant role in controlling inflammation and modulating adaptive immune responses that cause tissue damage. IL-10-producing lymphocytes contribute to the delicate balance between inflammation and immunoregulation and are thus regarded as a kind of "regulatory cells." Dysregulation of these cells is linked with susceptibility to numerous

inflammatory diseases. In FEHC diet group we have observed an increasing production of IL-10 suggested an anti-inflammatory effect of high fiber diet.

Also, we have reported a significant increase in TNFα in the control group. TNF-α a pro-inflammatory cytokine, has an important function not only in the development of insulin resistance, inflammation, and obesity, but also in osteoclastic activity. This cytokine through a mechanism of up-regulation of RANKL (Receptor Activator of Nuclear Factor κB Lingand) leads to an increase in osteoclasts by promoting bone resorption. Our data suggested that dietary intervention is auspicious for systemic reduction of inflammatory cytokines and also to counteract insulin resistance, osteoclastic activity [225].

5.5 Evaluation of WNT signaling genes in bone, skeletal muscle and adipose tissue

Elements of both canonical and non-canonical WNT signaling pathways have been examined in all the three tissues.

The gene expression analysis in bone tissue samples highlighted that mRNA level of Wnt10b is slightly decreased in FEHC group compared to controls.

Wnt10b has been shown to have a role in regulating osteoblast differentiation [226] activating the canonical WNT beta-catenin. Also, Wnt10b expression helps to maintain osteoblast progenitors in an undifferentiated state and that loss of Wnt10b expression results in either increased differentiation or decreased self-renewal of mesenchymal progenitors [227]. Nevertheless, the decrease of Wnt10b expression is related to aging and obesity that produce an early exhaustion of the progenitor pool

and subsequent loss of bone mass. Our results suggested that FEHC diet promotes downregulation of canonical WNT beta-catenin.

The gene expression analysis in skeletal muscle samples highlighted that mRNA level of WNT5a is increased in FEHC diet, compared to control.

WNT5a is the most important known factor of non-canonical pathway. Non-canonical WNT signaling is directly involved in myoblast differentiation and myotube fusion, though the activation of Myf5 and MyoD. Its principal role is to guarantee enough myoblasts, before the induction of myotube formation. Furthermore, during an injury, it has been reported the increment of mRNA expression of several WNT proteins, such as WNT5a in skeletal muscle. Kuwabara et al demonstrated that exercise induces WNT/βcatenin upregulation which, in turn, directly modulates chromatin structure and induces Myf5 and MyoD expression, favoring myogenesis in mice model [228].

The increase level of Wnt5a on skeletal muscle of FEHC group indicate an upregulation of the non-canonical pathway inducing myoblast differentiation and myotube fusion potentially counteracting sarcopenia.

We also observed a significant reduction in IL-6 gene expression in skeletal muscle of the FEHC diet group. IL-6 is a pro-inflammatory cytokine with exerting negative roles in skeletal muscle homeostasis [229,230]. Indeed, IL-6 increases in skeletal muscle have shown to improve generation and accumulation of free radicals leasing to muscle damage[231]. According to these observations FEHC diet may be protective on the oxidative stress and inflammation mediated by IL-6 maintaining redox balance in skeletal muscle.

Regarding the gene expression analysis in the adipose tissue, the factors considered presented a slight decrease in FEHC group compared to the controls. Although they

do not have a statistical value, the results obtained may suggest a slightly modulation of WNT pathway.

5.6 The Endocannabinoid System.

The gene expression analysis of some of the principal components of ECS was carried out in all the three tissues. These included the receptors, both classical (CB1 and CB2) and "alternative" receptors, TRPV1 and GPR55, and the whole enzyme panel involved in the synthesis and degradation of the endogenous molecules.

Anandamide (AEA), the most known and studied endogenous ligand of the ECS, is a modulator of the canonical and non-canonical signaling pathway of WNT, suggesting an interaction between these two signaling pathways.

Several studies have highlighted the involvement of the ECS system in the WNT signaling[198]. Considering the impact of the system in the regulation of metabolism both at the central level and, above all, at the peripheral level and that during obesity this modulation is altered, in this study we have evaluated the gene expression in the two different tissues of people with obesity underwent FEHC diet comparing with control group.

It is important to underline the importance of this analysis, given the limited number of human studies in the literature on ECS and on its possible interactions with the WNT pathway.

The gene evaluation of cannabinoid receptors Cb1, Cb2 and the enzyme MAGL and Gpr55 in the adipose tissue revealed a light upregulation of all of them in the FEHC group.

Some studies reported that systemic endocannabinoid levels are increased in postmenopausal women with obesity and they are associated with a lower expression of MAGL and Faah genes in the adipose tissue[193].

This may be linked to compensatory mechanisms in the tissue, aimed at increasing the activation of the EC system, through two mechanisms: the upregulation of the receptors, promoting the activation and the increase of the degradation enzymes.

A limiting aspect of these considerations is the small number of publications with this type of analysis s in human studies and that many of these concerns the evaluation of ECS expression and activity in the visceral compartment, which is considered more involved in the pathogenesis of obesity and insulin resistance.

The skeletal muscle is one of the largest insulin-sensitive tissue and it has also been demonstrated the presence and activity of ECS. Many studies have reported that the over activation of the system may impair insulin sensitivity, through AEA-mediated mechanisms[232].

The gene expression evaluation of the receptors showed a general upregulation of all of them in the muscle of FEHC group even if this observation has no statistical value.

Regarding the enzymes, those involved in the anabolism of endogenous molecules are upregulated in FEHC group compared to controls. This may indicate that a reduced synthesis of the endocannabinoids; however, in order to establish the real amount of the endocannabinoids, a direct measurement and quantification of these in the tissue could provide a better understanding of what happens in the muscle.

The catabolism enzymes, Faah and Magl, were both increased in the FEHC group suggesting a possible mechanism of the tissue to limit the over stimulation of the ECS.

Although the lack of statistical validity, some of the observations described in animal models were also observed in the tissue of the recruited subjects.

Given the limited number of studies in the literature of studies on the Endocannabinoid System muscle, especially in humans, future analyses with more homogeneous subjects regarding the clinical aspect or a more stratified population could give us a better understanding of the role of ECS in muscle of FEHC group.

In conclusion, from the evaluation of the ECS in adipose and skeletal muscle, a possible compensation mechanism has emerged to reduce or limit a prolonged ECS activation and stimulation. This may be supported by the receptors and enzymes involved in the synthesis of endogenous molecules tend to be increased as well as the degradation enzymes tend to be upregulated in the tissues of FEHC group.

Our study has some limitations. This is a monocentric cohort study, selection bias relative to the homogenous characteristics of patients (mostly Caucasian and from the Rome area) may have occurred as well.

All study subjects were affected with osteoarthritis, although we don't believe that this condition might have interfered with our main findings. Additionally, no data regarding the sub-score of the adopted scales were recorded. Thus, further, larger studies are needed to support our hypotheses.

CHAPTER 6: Conclusions

We have evaluated for the first time the expression of both WNT and ECS signaling elements directly in human, in a population of people with obesity that have followed FEHC diet and comparing the expression of three important tissues as bone, skeletal muscle and fat.

We found that obese subjects who have followed FEHC diet have an improvement of body weight, waist circumference, cholesterol levels and triglycerides.

These results are related to the proven effect of high dietary fiber characterized FEHC diet also with a reduced intake in animal products and added sugar.

In three months of FEHC diet participant improved their clinical scores before surgery. Indeed, losing weight is crucial in order to avoid surgical complications as well as boost the postoperative recovery. Considering the high impact of obesity on the complication rate and outcomes, weight loss is warmly encouraged before THA for improvement of pain and functional scores.

The evaluation of WNT elements and cytokines in serum sample have shown that FEHC diet by lowering expression in serum sample of DKK-1, may be helpful in preventing osteoporosis, arthritis and possibly sarcopenia by supporting WNT pathway activation should be toned down.

The increase level in IL-10, an anti-inflammatory cytokine suggested that high fiber daily intake for only three months contrasting the inflammation in serum sample of people with obesity. In contrast to this observation, subjects with obesity including in control group have shown an increase in TNFα level suggesting a promotion of inflammation.

The evaluation of WNT genes in bone of FEHC group have demonstrate a slight reduction in Wnt10b which could occur during aging. The decrease of Wnt10b expression is related to aging and obesity that produce an early exhaustion of the progenitor pool and subsequent loss of bone mass. Our results suggested that FEHC diet promotes downregulation of canonical WNT beta-catenin.

The increase level of Wnt5a on skeletal muscle of FEHC group indicate an upregulation of the non-canonical pathway inducing myoblast differentiation and myotube fusion potentially counteracting sarcopenia.

The results on skeletal muscle allow us to conclude that FEHC diet is also effective on inflammation by a significant reduction of IL-6 cytokine. According to these observations FEHC diet may be protective on the oxidative stress and inflammation mediated by IL-6 maintaining redox balance in skeletal muscle.

Regarding the ECS, some of the observations described in animal models were also observed in the tissue of our subjects. Considering the few human studies for this type of analysis, this study is the first that allows not only a direct evaluation of markers directly at the tissue level but, above all, the comparison of three different body compartments, highly involved in the regulation of metabolism and in the cross-talk between the considered tissues.

From the evaluation of the ECS in adipose and skeletal muscle tissues, in FEHC group, compensation systems seem to occur to limit an over stimulation of the Endocannabinoid System and the promotion of obesity-related conditions.

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