Project Acronym: OBESE-BONE

Project title: EVALUATION OF BONE STRENGHT AND WNT PATHWAY IN OBESE PATIENTS

Periodic Report: Second

Period covered by the report: from 09.2015 to 03.2017

1. EXPLANATION OF THE WORK CARRIED OUT AND OVERVIEW OF THE PROGRESS

1.1 Explain the work carried out during the reporting period in line with proposal approved

The project started in September 2015 with the aim to evaluate the role of Wnt pathway and bone strength in obese patients. According to protocol, elderly patients undergoing hip arthroplasty surgery have been screened and recruited. Before the surgery, patients underwent physical examination, blood tests (safety, bone turnover markers, cytokines, myokines, inflammatory markers, Wnt markers), DXA, MRI, CT.

During all the surgery procedures, bone samples have been collected and used for histomorhometry, biochemical tests or biomechanical/material composition analysis. All of the subjects also underwent sub-cutaneous fat needle aspirate and muscle biopsy from vastus lateralis. Subsequently samples have been analysed for Wnt pathway, inflammation and bone turn-over markers.

1.2 Include an overview of the project results including summary of deliverables and milestones, and a summary of exploitable results and an explanation about how they can/will be exploited, if any.

- We are still recruiting subject. However, to March 2017, **31** women elderly obese and **28** control subjects have been recruited and signed the written consent of the study.

- From September 2015 to March 2017 RU1 and RU2 recruited the subjects, RU1 performed the ELISA assays on sera collected before the surgery. RU5 performed the gene/expression analysis on tissue samples. As clearly stated in the previous report**, serum analyses did not show any significant changes between obese and lean controls in terms of inflammatory markers, myokines, oxidative stress.**

-Gene expression analysis in bone **confirmed** that obese subjects present a lower expression of SOST and Wnt5A gene. In the adipose tissue, we found a higher GSK3B, TNFα at and a trend for higher IL6 and IL8 in obese than healthy subjects; as expected adiponectin expression was lower in obese than lean subjects. Gene expression at the muscle confirmed a significantly lower SFRP expression and a trend for lower WNT5A, GSK3B and Wnt10B (more than 50%) in obese vs lean ones. **The information so far collected confirms our hypothesis that WNT pathway is differently modulated in obese**. According to our data, mechanical load on the skeleton in obese individuals down-regulates sclerostin expression in osteocytes likely activating Wnt canonical pathway. This may explain high BMD levels commonly observed in the obese. The lack of significant changes at the serum levels on sclerostin may be due to the limited sample size and this trend may differ at the end of the project when recruitment will be completed. In any case, the present data may confirm what believed from some authors that being SOST gene expressed only at the bone level, peripheral measurements do not reflect the real ‘’central’’ gene activity. Further protein expression analysis will help to elucidate this scientific question. However, it should be highlighted that data obtained at the bone and muscle level may change the current knowledge given the paucity of available data from human tissues. **We confirm that Histomorphometry analyses have not revealed significant differences in terms of both osteoblasts or osteoclasts number, trabecular or cortical thickness.**

2. OBJECTIVES

List the specific objectives for the project as described in section 1.1 of the DoA and described the work carried out during the reporting period towards the achievement of each listed objective. Provide clear and measurable details.

1) To assess bone strength in older patients affected with obesity in comparison to healthy subjects.

- **Bone strength analyses are in progress with both direct measurements through biomechanics and surrogate markers from CT scans. Up to now, only samples from 6 subjects (4 obese, 2 non obese) have been analyzed. In particular, Young module, Snerv. Max and Snerv Min. have been performed**.

2) To carry out gene and protein expression studies in order to evaluate the effect of inflammation, myokines and adipokines on bone health.

- RNA has been extracted from adipose, muscle and bone samples and processed for gene expression analyses with qRT-PCR, for most of proposed genes.
- Serum samples have been collected and processed from all subjects and ELISA tests have been performed.

3) To determine the role of Wnt pathway as possible mediator of bone fragility in older obese.

- Analyses will be performed through imaging scanning and gene/protein expression evaluation in bone samples from all subjects. According to the original proposal, an algorithm will be generated.

3 EXPLANATION OF THE WORK CARRIED PER WORK PACKAGE

3.1 Work package 1

1. Serum levels of Wnt markers and cytokines/adipokines

A. Inhibitors of the Wnt Pathway and biochemistry: bone quality parameters, serum Wnt markers and cytokines have been analyzed on 41 sera of healthy and obese subjects. Serum levels of bone markers (CTX-I and P I NP) have been evaluated with standard Elisa kits (IDS CTX-I Elisa, UK and Cusabio Biotech Co., LTD, CN respectively) according to manufacturers' instructions. Sclerostin has been measured with enzyme immunoassay Biomedica GmbH (Vienna, Austria). Serum concentrations of Sfrp5 were measured with USCN Elisa kit (USCN Life Science, China). **We confirm that no significant differences were observed in the evaluation of bone markers between the obese group compared to healthy subjects.**

B. Serum cytokines/adipokines: : TNF-α and IL-22 serum levels were assayed with Diaclone Sas (FR) standard ELISA kits according to manufacturer's instructions both TNF-α and IL-22 ELISA assays confirmed that there is a trend of increased inflammation in obesity. More data are needed completing the recruitment of study subjects to confirm this result.

2. SOST/sclerostin gene expression.

Evaluation of SOST/sclerostin and Wnt gene expression in bone.

Bone samples have been processed and the RU5 proceeded with the analysis of gene expression of SOST/sclerostin, Wnt5a, Wnt10b, SFRP5, GSK3β and adiponectin. **The analysis confirmed a significant decrease of SOST/sclerostin expression in obese subjects compared to controls**; **Wnt5A was significantly decreased in the obese group as well**.

3. Gene expression of cytokines and Wnt from adipose tissue and muscle.

A. Gene expression of Cytokines and Wnt in adipose tissue.

RU5 has evaluated the expression of the following genes: Wnt5a, Wnt10b, SFRP5, GSK3β, adiponectin,

TNFα, IL-6, IL-8, IL-10 and IL-15. Differences were found in the expression of GSK3β, IL-6 and IL-8; in particular, **we confirmed a significant increase in GSK3β in obese individuals compared to normal weight subjects** . As regards the other cytokines, IL10 and IL15 increased in obese compared to controls. As expected, obese subjects showed higher TNF-α and lower adiponectin expression than controls. Wnt5a, Wnt10b and SFRP5 did not differ between the 2 groups.

B. Gene expression of cytokines and Wnt in muscle.
It has been evaluated the expression of genes involved in Wnt signaling (Wnt5a, Wnt10b, SFRP5 and GSK3β) The analysis confirmed a decrease in the Wnt5a and Wnt10b expression in obese compared to normal weight group. GSK3β was slightly down expressed, while SFRP5 was significantly decreased in the obese group compared to normal weight.

4. Gene expression of endocannabinoid system in adipose tissue.
It is still ongoing the gene expression analysis of endocannabinoid system, with the evaluation of its receptors CNR1, CNR2, TRPV1 and GPR55, and of the enzymatic pool, dedicated to the synthesis (Nape-PLD and DAGL) and the degradation (FAAH and MAGL) of endogenous cannabinoids.

Gene expression of endocannabinoid system in muscle.
It has been evaluated the presence and modulation of the endocannabinoid system in skeletal muscle, in the all groups of subjects. All the receptors of endocannabinoid system (CNR1, CNR2, TRPV1 and GRP55) were significantly down regulated in obese subjects compared to controls.
Enzymes Nape-PLD and FAAH, dedicated to the synthesis and degradation of anandamide, respectively, were more expressed in obese as well.

3.2 Work package 2.
Histomorhometry analysis has been carried out showing no significant changes in obese vs lean subjects in terms of cortical and trabecular thickness, trabecular space, osteoid, bone volume/trabecular volume, osteoblasts or osteoclasts number.

3.3 Work Package 3.
The obese subjects femoral bone heads have been extracted, and, for each patients, a specimen of 10 mm of diameter and 26 mm of height have been delivered to URBI to be processed.

Preliminary test have been executed to try the experimental setup and test the measurement chain using the mechanical machine. Bluehill® Software has been used for the actuator control and to handle the force and deformation data collection. The following tests have been performed:
- Young's modulus analysis obtained from non-destructive testing.
- Ultimate stress and ultimate strain analysis obtained from destructive test, using a strain rate of 0.01 S^{-1}

Bone strength analyses are still in progress with both direct measurements through biomechanics and surrogate markers from CT scans. Up to now, only samples from 6 subjects (4 obese, 2 non obese) have been analyzed.

4. IMPACT
Include in this section whether how your project will contribute to the expected impacts is still relevant or needs to be updated. Include further details in the latter case.
We believe that this project may have high impact for understanding the pathogenic elements linked to bone fragility and the involvement of WNT pathways in inflammation, bone and muscle health in obesity. Data generated till now look promising although full interpretation is not possible yet. Interestingly, data so far collected support our hypothesis that WNT is differently modulated in obesity and that mechanical loading on the skeleton of obese subjects downregulates sclerostin expression and facilitate local activation of WNT canonical pathway. By increasing the sample size and combining gene

expression/ELISA data with data from bone structure/ mechanical properties/imaging we will obtain a comprehensive assessment of WNT at tissue level. Our project may provide novel insights to the relationship between obesity, inflammation and bone fragility by providing a mechanistic link between local WNT regulation in bone, muscle and adipose tissue. So far, this concept has not been proven yet and the relationship between obesity and bone fragility is not clear and based on mixed results, with some studies showing increase fracture risk in obese while other studies suggesting a protective effect. Therefore, we believe that impact remains high because knowledge obtained by this project will ultimately lead to the development of tool for diagnosis and prediction of bone fragility in obese.