UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI MEDICINA

SCUOLA DI DOTTORATO DI SCIENZE BIOMEDICHE CLINICHE E SPERIMENTALI

DOTTORATO DI RICERCA IN

REUMATOLOGIA

Con il contributo di (ENTE FINANZIATORE): NA

CICLO /ANNO (1° anno d'Iscrizione) 33° CICLO

TITOLO DELLA TESI DI DOTTORATO: Wnt pathway involvement and its response to treatment in the development of bone damage in different inflammatory and non-inflammatory rheumatic contidions

S.S.D. MED/16
(indicare il settore scientifico disciplinare di riferimento della tesi dato obbligatorio)*

Coordinatore: Prof./ssa __Giovanni Targher_

Firma _____

Tutor: Prof./ssa _Davide Gatti___

Firma _____

Dottorando: Dott./ssa _Angelo Fassio

Firma _____

Abstract

Inflammatory conditions such as Rheumatoid Arthritis (RA) are characterised by impaired bone health, with bone loss occurring both systemically and locally (erosions). Other conditions, such as Diffuse Idiopatic Skeletal Hyperostosis (DISH) are not usually associated with significant systemic inflammation but nonetheless can show signs of impaired bone health, in the form of osteoporosis and bone ectopic neoformation. The Wnt/B-catenin pathway is the master regulator of osteogenesis, and its role has been extensively investigated in the last years. The objective of the presen thesis is to investigate the role of the main Wnt pathway modulators (Dkk-1 and sclerostin) in inflammatory conditions such as RA and polymyalgia rheumatica and the effect of different treatment on their serum levels. Furthermore, the Wnt pathway dysregulation in a non-inflammatory conditions such as DISH will be investigated and discussed.

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INTRODUCTION

During my PhD program in Clinical and Experimental Biomedical Science, attended at the Rheumatology Unit at the University of Verona, I further investigated the involvement of the Wnt pathway in the development of bone damage in chronic rheumatic conditions characterised by a high degree of chronic inflammation, such as Rheumatoid Arthritis (RA) and Polymyalgia Rheumatica (PMR) and conditions not associated with it, such as Diffuse Idiopathic Skeletal Hyperostosis (DISH).

In this final thesis mi intent is to examine some of the most interesting findings documented in my research, in patients affected by these conditions and receiving treatment with different drugs (i.e. different monoclonal antibodies, corticosteroids)

BACKGROUND

Chronic Arthritides and bone involvement: from Rheumatoid to Psoriatic Arthritis and Ankylosing Spondylitis

Rheumatoid arthritis (RA) is an inflammatory autoimmune arthropathy characterized by progressive chronic articular destruction. Bone damage occurs at joint sites with the development of erosions, and this is often regarded as the prevalent bone problem in RA (1). The pathophysiology of these skeletal involvements is still poorly understood. Bone is not static and its cells are targeted by many signalling pathways. Cytokines as Tumor Necrosis Factor-α (TNFA) and Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) and Antibodies to Citrullinated Protein Antigens (ACPAs) could, for example, act directly on osteoclasts (2,3). Articular bone erosion represents localized bone loss (osteolysis), initially involving cortical bone, and destruction of the natural barrier between the extraskeletal tissue and the intertrabecular spaces of the bone marrow cavity. Osteolysis results from an imbalance in which bone resorption by osteoclasts is favoured over bone formation by osteoblasts. Understanding the mechanisms that define the formation of bone erosions requires insight into the biology of bone homeostasis and the molecular regulation of the differentiation and function of osteoclasts and osteoblasts (1). Initially described more than 100 years ago (1), articular bone erosions have now become a central element in the diagnosis, treatment and monitoring of RA. Moreover, these lesions are an expected consequence of seropositive RA if the disease is not treated in a timely and effective

fashion. Erosions reflect the clinical consequence of the tight interaction between immune activation and skeletal modelling and remodelling. Indeed, research into the interface between the immune system and bone has now led to a new field, termed osteoimmunology (1).

Psoriatic arthritis (PsA) is an inflammatory disease involving the skin, the entheses, and the joints (4). Hand joint involvement is a typical feature of PsA and is characterized by synovial inflammation, which leads to swelling, pain, and stiffness of the affected joints. Clinical symptoms of PsA resemble, in part, those observed in RA; however, the anatomic changes observed in PsA are substantially different from those observed in RA. Thus, PsA is characterized by sites of new bone formation along the periarticular bone, which are not present in RA (5). The pathogenesis of these lesions is incompletely understood but may reflect the association of PsA with inflammation of structures representing the insertion sites of the tendons (enthesitis). Fibrocartilage, a specialized tissue typical of these sites, is responsible for the transduction of mechanical strain from tendons to bone (6). It has been suggested that inflammation of the entheses triggers new bone formation along these structures, which then appears radiographically as entheseophytes.

Ankylosing spondylitis (AS) is a chronic rheumatic disease characterized by inflammation and extensive remodelling of spine and joints. In contrast to RA and somewhat similarly to PsA, destructive changes are limited in AS, but extensive new bone formation results in the development of spinal syndesmophytes and extra-articular enthesophytes, leading to joint or spine ankylosis (7). Parallel to the osteoproliferation, patients with AS also have an increased loss of bone, resulting in an elevated risk for vertebral fractures (8).

The WNT pathway and bone remodeling

The characterization of the canonical WNT pathway in the regulation of bone modeling and remodeling provided important insights in our understanding of the pathophysiology of bone involvement in chronic arthritis (9–11). The understanding of the mechanisms responsible of osteclastogenesis dates back to the ’90, when several studies analysed the role of the receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin (OPG) in the regulation of osteoclasts (OCs) maturation. Under the control of different factors, involving hormones, growth factors and some cytokines, the osteoblast (OB) secretes a specific mediator

belonging to the Tumour Necrosis Factor family called RANKL. This molecule interacts with its specific receptor RANK, which is present on both the mononucleated haematopoietic precursors of the monocyte-macrophage line and on the mature OC. The interaction between RANKL and RANK promotes the differentiation of the mononucleated precursors in multinucleated precursors and, finally, in mature OCs (12). In vivo, there is a physiological control mechanism represented by a "decoy" receptor called OPG, also of OB origin, capable of binding RANKL and thus blocking the interaction with RANK present on the precursors of the OC. This way, it prevents not only their differentiation, but also inhibits the activation of mature OC and reduces their survival (12).

The WNT pathway is a complex transduction pathway which transmits the signal from the receptors localized on the cell membrane, to the cytoplasm and finally to the nucleus, there regulating the genic transcription. WNT comprehends a family of proteins mediating several biologic processes such as embryogenesis, organogenesis, tissue regeneration and tumour-genesis, through both a paracrine and autocrine action (13–16).

We can divide two main signalling pathways: the canonical and the non-canonical pathway. With the term non-canonical pathway we summarize different signals not involving β-catenin but different kinases such as Mitogen Activated Protein and kinase protein calcium/calmodulin dependent (17). Those pathways regulate proliferation, differentiation and cellular migration (18) with effects on bone tissue that are still not deeply understood. However, it is conceivable that they may play a role in the skeletal physiology of adults too.

The canonical signalling of WNT/β-catenin is the most understood pathway and, probably, the most relevant. It determines the stabilization of β-catenin already present in the cytoplasm and its translocation in the nucleus where it regulates genic expression, thus influencing several and bone formation and several other features such as (19–23):

- Stem cells renewal
- Differentiation of the OB from the staminal mesenchymal precursors and inhibition of adipogenesis and chondrogenesis (24,25)
- Proliferation, survival, maturation and activity of the OBs
- Inhibition of osteoclastogenesis
- Regulation of the coupling processes between OBs and OCs

The WNT canonical pathway contributes to the osteogenesis and bone formation. In addition, the WNT/β-catenin can inhibit osteclastoogenesis and bone resorption by promoting the expression of OPG by OBs unbalancing OPG/RANKL ratio (26,27). Thus, the WNT canonical pathway acts on the bone metabolism with a dual mechanism: the stimulus on bone formation and inhibition on bone resorption. WNT regulation is determined by the production of intra- and extracellular inhibitors (15), acting in very different manners (28). Some WNT Inhibitory Factors (WIFs) directly bind to WNT proteins or their receptors blocking both the two pathways. On the contrary, Dickkopf-related protein-1 (Dkk-1) and sclerostin interfere with Low density lipoprotein receptor-related proteins (LRPs) co-receptors and thus inhibiting only WNT canonical pathway (29–31).

Based on these premises, it is evident that the inhibition of the development, functionality and survival of OCs through the inhibition of RANKL and the influence on RANK/OPG balance can represent an effective strategy to modify the physiology of bone resorption, either systemic or local, and to oppose the fundamental physiopathological feature of bone loss.

The WNT pathway and bone damage in chronic arthritides

In a previous study we already showed that, in patients with RA, Dkk-1 is significantly increased and associated with a lower BMD and with the presence of the typical erosions (32). The positive correlation found between circulating Dkk-1 and serum C-terminal telopeptide of type I collagen (CTX-I, a marker of bone resorption) suggested that the over-production of Dkk-1 (and the resulting inhibition of the WNT pathway) may contribute both to the locally increased bone resorption and to the impaired bone repair, typical features of the more erosive forms of RA (32). This phenomenon may also justify the development of RA-associated osteoporosis (33).

On the other hand, AS is a chronic rheumatic disease characterized by the inflammation and extensive remodeling of spine and joints. Differently from RA, erosive changes are limited in AS, while extensive bone neoformation leads to the development of spinal syndesmophytes and extra-articular enthesophytes and therefore to joint or spine ankyloses (7). Previous studies already reported that both circulating sclerostin and Dkk-1 levels are significantly lower in AS patients when compared to matched controls (34–36). The observation of lower circulating levels

of WNT inhibitors suggests that WNT overexpression could play a relevant role in the focally exuberant bone formation in AS.
Currently, the WNT pathway is thought to be heavily involved in the pathogenesis of bone damage during RA and SA with different and opposite profiles: the inhibition of WNT pathway in RA and its overexpression in SA. The influence of these inflammatory diseases over the WNT pathway seems to be driven by the dysregulation of Dkk-1 and sclerostin.
Furthermore, we already documented parathyroid hormone (PTH) to be a major determinant of Dkk-1 serum levels not only in patients with arthritis but also in subjects affected by bone metabolic diseases such as primary hyperparathyroidism (32,37,38).
Similarly to AS, in PsA we can find bone erosive damage often associated with an exuberant bone formation, especially in enthesial sites (39).
In 2017, over the course of my residency, I lead a study that replicated the finding of increased serum levels of Dkk-1 in patients affected by RA (40); our data, along with the data from a previous study of our group (32) were also included in a meta-analysis that confirmed this remark (41).
In the same study, we demonstrated the presence of decrease d levels of Dkk-1 in the PsA cohort, and hypothesized that low levels of Wnt inhibitors could be one possible explanation for the pathological bone formation that characterises some phenotypes of PsA.

Unanswered questions

As already discussed, in RA, a disease characterized by overexpression of pro-inflammatory cytokines (i.e. TNFα, IL-6), OC bone resorption largely prevails upon bone formation. Moreover, proinflammatory cytokines have been documented to be strong inducers of Wnt inhibitors in RA (42). What would happen after the administration of drug with potent antinflammatory activity?
In PsA, both excess bone resorption and bone formation may coexist and therefore we hypothesized that Wnt dysregulation (namely suppressed levels of Wnt inhibitors) might explain the presence of a (maladaptive) pro-osteogenic environment. The question at this point is: is the hypothesis of a pro-osteogenic maladaptive environment applicable also in different settings, such as non-inflammatory rheumatic conditions (i.e. DISH)?

AIMS OF THE STUDY

This thesis comprises of three different studies.

In the first study, we wanted to investigate the changes in Wnt inhibitors and bone turnover markers (BTMs), in patients affected by RA receiving treatment with either a TNFα inhibitor (certolizumab), or a glucocorticoids (GCs) or an IL-6 receptor inhibitor (tocilizumab). The study has been published on Calcified Tissue International on April 2020 (43).

In the second study, after having observed the peculiar effects of GCs in RA, we studied the same biomarkers in PMR. This study has been very recently published on Frontiers in Rheumatology (44).

In the third study, we investigated several biomarkers (with particular focus on Wnt inhibitors and BTMs) associated with the presence of DISH in a cohort of patients affected by Type 2 Diabetes Mellitus (T2DM). This final study has been accepted for publication on Calcified Tissue International on the 30th September 2020.

MATERIALS AND METHODS

Study n° 1. Acute effects of glucocorticoid treatment, TNFα or IL-6R blockade on bone turnover markers and Wnt inhibitors in early rheumatoid arthritis: a pilot study.

Materials and methods

We performed a retrospective analysis of prospectively collected data. We enrolled in this three-arm study women affected by early RA (< 12 months of symptoms, classified according to the ACR/EULAR 2010 criteria (45)) seen at the rheumatology outpatient clinic of the University of Verona, Italy. Inclusion criteria were: DAS28 ≥ 2.6 despite stable treatment with subcutaneous methotrexate (10-15 mg/week) for at least 6 months and positive rheumatoid factor and/or anti-citrullinated protein antibodies (ACPA). We divided the patients depending on the treatment started at baseline: 1) patients who started treatment with certolizumab pegol with the following schedule: 200 mg weekly subcutaneous administration, 2) patients who started treatment with weekly subcutaneous tocilizumab 162 mg, 3) patients who started treatment with methyl-predinsolone 8 mg daily. The treatment needed to be maintained stable for all the 4 weeks. All groups continued methotrexate at the current dose.

Exclusion criteria were: treatment with glucocorticoids within the last 3 months of enrolment, treatment with bisphosphonates or denosumab within the last 2 years, or with other drugs known to affect bone metabolism or fracture risk; onset of menopause within 2 years or onset of the climacteric period during the study; and the presence of renal, liver, endocrine, heart, and metabolic bone diseases. Every patient received also 1,000 UI vitamin D and 1 g calcium per day; the dose could not be changed. DAS28 was recorded at baseline and after 4 weeks.

Study n° 2. Wnt inhibitors and bone turnover markers in patients with polymyalgia rheumatica and acute effects of glucocorticoid treatment

Materials and methods

We performed a prospective study on consecutive patients affected by PMR classified according to the 2012 EULAR/ACR criteria (46) and evaluated at the rheumatology outpatient clinic of the University of Verona. Exclusion criteria were: 1) treatment with glucocorticoids within the previous 3 months, 2) history of osteoporosis, 3) treatment with bisphosphonates or denosumab within the previous 2 years, or with other drugs known to affect bone metabolism or fracture risk and/or 4) history of any renal, liver, heart, thyroid, and metabolic bone diseases potentially interfering with the objective of this study. Previous supplementation with cholecalciferol was not an exclusion criterion. Every patient received (if not already taking) 1,000 UI vitamin D and 1 g calcium per day; the dose could not be changed during the whole period of observation. After being diagnosed with PMR, patients were prescribed methylprednisolone 16 mg daily for the first four weeks (the duration of the study), according to current recommendations (47). PMR activity score (48) was adopted to assess disease activity at baseline and at week 4. The time frame of the study was limited to the first four weeks because after the first month all patients started an antiresorptive treatment according to national guidelines (49).

The subjects of the healthy controls (HC) group, matched for sex and age, were enrolled from retired hospital personnel. A single blood sample of HC was taken.

The study was conducted within the protocol 1483CESC approved by our local Ethics Committee, in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants included.

Biochemical analysis, study 1 and 2.

All blood samples were collected in the morning after overnight fasting at baseline, at week 1 and week 4. The samples were stored upon collection and at −50 °C and finally assayed, in single batch, for intact parathyroid hormone (iPTH), 25OH-vitamin D (25OHD), N-propeptide of type I collagen (PINP), C-terminal telopeptide of type I collagen (β-CTX-I), sclerostin and Dkk-1. All the samples were processed in the laboratory of the Rheumatology Unit of the University of Verona. Bone turnover markers (PINP and β-CTX-I), 25OHD and iPTH were measured by the IDS-ISYSMulti-Discipline automated analyzer (Immunodiagnostic System, Boldon, UK) based on chemiluminescence technology. The coefficients of variation (CV) intra-assay measured in our laboratory were 4% for PINP (inter-assay CV 6%), 3% for β-CTX-I (inter-assay CV 7%), 6% for 25OHVITD (inter-assay CV 9%) and 2,7% for iPTH (inter-assay CV 5,5%).

Serum Dkk-1 and sclerostin were measured by ELISA (Biomedica Medizinprodukte, Vienna, Austria) with a sensitivity of 1.7 and 3.2 pmol/L and intra-assay CV of 7 and 5% (inter-assay CV 8.2% and 6.9%) respectively.

Study n° 3. Diffuse idiopathic skeletal hyperostosis (DISH) in type 2 diabetes: a new imaging possibility and a new biomarker

Materials and Methods

For this study, we enrolled 96 post-menopausal women with established T2DM, who consecutively attended our diabetes outpatient clinic of the University of Verona over a period of three months. All these women met the following inclusion criteria: (a) age ≥50 years; (b) Caucasian; (c) post-menopausal (physiological or surgical); (d) glucose-lowering treatment with diet alone or in combination with oral agents (but not with insulin therapy); and (e) no self-reported history of osteoporosis, osteoporotic fractures, treatment with bisphosphonates and other anti-osteoporotic agents, as well as steroids or hormone replacement therapy.

We excluded patients with: (a) history of significant alcohol consumption (i.e., >20 grams of alcohol per day) or competing causes of chronic liver disease (e.g., virus, drugs, autoimmunity, hemochromatosis); (b) history of cirrhosis of any aetiology, active cancer and end-stage renal disease (defined as estimated glomerular filtration

rate <15 ml/min/1.73 m^2 or dialysis); and (c) history of hyperthyroidism or hypothyroidism.

The study was conducted within the protocol 1483CESC approved by our local Ethics Committee, in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all participants included.

Clinical and laboratory variables

BMI was measured as kilograms divided by the square of height in meters. Waist circumference was measured at the midpoint between the lowest rib and the iliac crest. Blood pressure was measured with a standard sphygmomanometer after the patient had been seated quietly for at least 5 minutes. Patients were considered to have hypertension if their blood pressure was ≥140/90 mmHg or if they were taking any anti-hypertensive therapy. Information on the type of menopause (physiological or surgical), smoking history and current use of medications (including use of vitamin D supplements) was obtained interviewing all patients during medical examinations.

Venous blood samples were drawn in the morning after an overnight fast. Measurements of serum glucose, lipids, creatinine (measured using a Jaffè rate blanked and compensated assay), high-sensitivity C reactive protein (hs-CRP) and other biochemical blood parameters were obtained using standard laboratory procedures at the central Laboratory of our hospital. LDL-cholesterol was calculated using the Friedewald's equation. Hemoglobin A1c (HbA1c) was measured by the high-performance liquid chromatography analyzer Tosoh-G7 (Tosoh Bioscience Inc., Tokyo, Japan). Homeostasis model assessment (HOMA-IR) score was used for estimating insulin resistance.

Chronic kidney disease (CKD) was defined as the presence of $eGFR_{MDRD}$ <60 mL/min/1.73 m^2. Pre-existing history of ischemic heart disease (IHD) or stroke was defined as a documented history of myocardial infarction, angina, coronary revascularizations or ischemic stroke.

Serum bone turnover biomarkers

Serum samples were collected from all patients at the time of study recruitment, centrifuged, separated and stored at -80°C until measurement. In total, we tested 16 different serum biomarkers; an expert laboratory technician, who was blinded to

patients' clinical details, measured 25-hydroxyvitamin D3 [25(OH)D], intact parathyroid hormone (PTH) and a number of indirect bone turnover biomarkers, such as sclerostin, C-terminal telopeptide of type 1 collagen (sCTX), procollagen type 1 N-terminal propeptide (P1NP), Dickkopf-related protein-1 (Dkk-1) and soluble ligand of the kappa-B factor activator receptor (RANKL). Specifically, 25(OH)D, PTH, sCTX and P1NP were measured using the IDS-ISYS Multi Discipline Automated Analyzer (Immunodiagnostic System, Boldon, UK) employing immuno-chemiluminescent technology, whilst Dkk-1, sclerostin and RANKL were measured by commercially available enzyme immunoassay ELISA kits (Biomedica Medizinprodukte, Vienna, Austria) on the fully automated microplate analyser Personal LAB (Adaltis, Rome, Italy). The intra-assay coefficients of variation (CV), in our laboratory, were 3% for P1NP (inter-assay CV 6%), 3% for sCTX (inter-assay CV 7%), 5% (inter-assay CV 6.9%) for sclerostin, 7% for Dkk-1 (inter-assay CV 8.2%), 2.7% for PTH (inter-assay CV 5.5%), 6% for 25(OH)D (inter-assay CV 9%), and < 10% (inter-assay CV <12%) for RANKL, respectively.

Dual energy X-ray absorptiometry

A DXA scan was performed in all patients using the GE Lunar iDXA 194 system (GE Healthcare Lunar, Madison, WI, USA) by a single expert operator, who was blinded to patients' clinical details. We obtained BMD measurements (g/cm^2) and T-scores at both lumbar spine (L1-L4) and femur (neck and total). Total body densitometry was performed measuring body composition, i.e., fat mass (FM) and fat-free mass (FFM). VFA was performed in all patients in order to detect the presence of any vertebral fractures and/or DISH.

Diagnosis of DISH was established based on Resnik criteria [10], however we adopted VFA and not X-rays as originally meant. Two expert operators (G.A. and R.N.) examined independently 200 selected VFA images for the diagnosis of DISH, and the interobserver Cohen's kappa coefficient was 0.77. Both operators, blinded to patients' clinical details, then performed VFA assessment for the diagnosis of DISH on the images of all 96 patients of the current study, with an inter-observer Cohen's kappa coefficient of 0.747. The discordant cases were then discussed until complete agreement was attained.

When available, X-rays of the thoracic and lumbar spine performed in the last 12 months (for any reason) were retrieved and assessed by a radiologist expert in musculoskeletal conditions.

Statistical analysis (all studies)

Normality for all variables was tested by Shapiro-Wilk test. Data are presented as means ± SD for normally distributed variables and medians (inter-quartile-ranges [IQR]) for non-normally distributed variables.

Study n° 1. Baseline comparisons and follow-up comparisons of continuous variables between groups, in terms of changes in serum biomarkers over time, were made with the use of analysis of variance (ANOVA) and post-hoc tests (corrected with Holm-Bonferroni for multiple comparisons). The variation of each marker was tested for the null hypothesis of no variation after treatment administration with a one-sample t test (test vs zero).

Study n° 2. A statistical power analysis was performed for sample size estimation. The sample size was based on the assumption that a ≥15% decrease in the values of Dkk-1 after 4 weeks of treatment with GC was found. This assumption was based on the data of a previous study in RA (43). We calculated a required sample size as n = 14, with an alpha = .05 and power = 0.80.

The differences between the values of the different parameters tested between PMR and HC at any observation point were analyzed by t test for normally distributed variables and Mann-Whitney U test for independent samples for non-normally distributed variables.

Study n° 3. After stratification of patients by presence or absence of DISH the differences in main clinical and biochemical characteristics between the two groups were tested by using the Student's t test for normally distributed variables and the Mann-Whitney U test for independent samples for non-normally distributed variables. T-scores and Z-scores (the number of standard deviations above or below the mean for the patient's age, sex and ethnicity) measured by DXA at lumbar spine, femoral neck and total hips. Z-scores were tested vs 0 via one sample t-test in order to assess whether the BMD were higher than expected (as known, 0 represents the average Z-score of sex, age and ethnicity-matched healthy population). Differences in the proportion of comorbid conditions and use of cholecalciferol supplementation in the DISH and no-DISH groups were tested by the Fisher's exact

test. Adjustment for potentially confounding factors (i.e. age and years since menopause) between the DISH and no-DISH groups of patients was performed by binary logistic regression.

Given the exploratory nature of the study, we chose not to adjust for multiplicity.

Two-sided *p* values of 0.05 or less were considered to be statistically significant. Data were analysed using SPSS software, Version 22 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Study n° 1. Acute effects of glucocorticoid treatment, TNFα or IL-6R blockade on bone turnover markers and Wnt inhibitors in early rheumatoid arthritis: a pilot study.

Data were obtained for 14 patients treated with certolizumab pegol, 14 patients treated with tocilizumab and 20 patients treated with methyl-prednisolone. No difference in any of the tested parameters was found at baseline (table 1).

	Methyl-prednisolone (N = 20)	**Tocilizumab (N = 14)**	**Certolizumab pegol (N = 14)**	**ANOVA**
Age	62.3 ± 8.4	60 ± 9.8	58.3 ± 8.4	Ns
DAS28	4.1 ± 0.9	3.9 ± 0.7	4.05 ± 0.7	Ns
25OHD (ng/ml)	26.3 ± 5.7	33.1 ± 9.0	32.3 ± 13.2	Ns
PTH (pg/ml)	34.2 ±12.5	40.7 ± 15.0	30.6 ± 11.6	Ns
PINP (ng/ml)	42.3 ± 16.0	39.9 ± 24.7	34.2 ± 15.0	Ns
β-CTX-I (ng/ml)	0.397 ± 0.212	0.301 ± 0.212	0.295 ± 0.159	Ns
Dkk-1 (pmol/l)	46.9 ± 22.2	50.0 ± 16.6	38.7 ± 11.8	Ns
Sclerostin (pmol/l)	34.2 ± 12.6	31.5 ± 10.5	29.4 ± 10.6	Ns

Table 1. Baseline characteristics of the patients. Data expressed as mean ± standard deviation. ANOVA, Analysis of Variance; DAS28, Disease Activity Score-28; 25OHD, 25-hydroxy vitamin D; PTH, parathyroid hormone; PINP, N-terminal propeptide of type I procollagen; β-CTX-I, C-terminal telopeptide of type I collagen; Ns, non-statistically significant.

DAS28 was assessed after 4 weeks, and it showed improvement from baseline, however without any statistically significant difference among the three groups (data not shown).

β-CTX-I decreased significantly vs baseline ($p<0.05$) only in the group treated with certolizumab pegol, at week 1 (-27% ± 21.5) and week 4 (-28.2% ± 26.2), both were resulted statistically significant when compared with tocilizumab ($p<0.05$).
PINP decreased significantly vs baseline ($p<0.05$) in the group treated with methyl-prednisolone at week 1 (-16.1% ± 15.6) and week 4 (-27.2% ± 15.3), both were resulted statistically significant when compared with both tocilizumab and certolizumab pegol ($p<0.05$). PINP increased significantly vs baseline ($p<0.05$) in the group treated with certolizumab pegol at week 1 (33.9% ± 20.4) and week 4 (45.6% ± 46.2), both were resulted statistically significant when compared with both tocilizumab and methyl-prednisolone ($p<0.05$).
Dkk-1 and sclerostin decreased significantly vs baseline ($p<0.05$) in the group treated with certolizumab pegol, at week 1 (-50.0% ± 13.2 and -30.1 ± 30.4, respectively) and week 4 (-47.5% ± 18.1 and -31.1% ± 18.0, respectively). Treatment with methyl-prednisolone produced a significant decrease vs baseline of Dkk-1 ($p<0.05$) at week 1 (-9.4% ± 21.6) and week 4 (-8.5% ± 24.3), and of sclerostin at week 4 (-10.4% ± 24.3, $p<0.05$ vs tocilizumab). Tocilizumab did not result into any significant variation for Wnt inhibitors. The percent changes of the serum levels of bone turnover markers, Dkk-1 and sclerostin are reported in figure 1. No significant changes were found over the four weeks for PTH and 25OHD (data not shown).

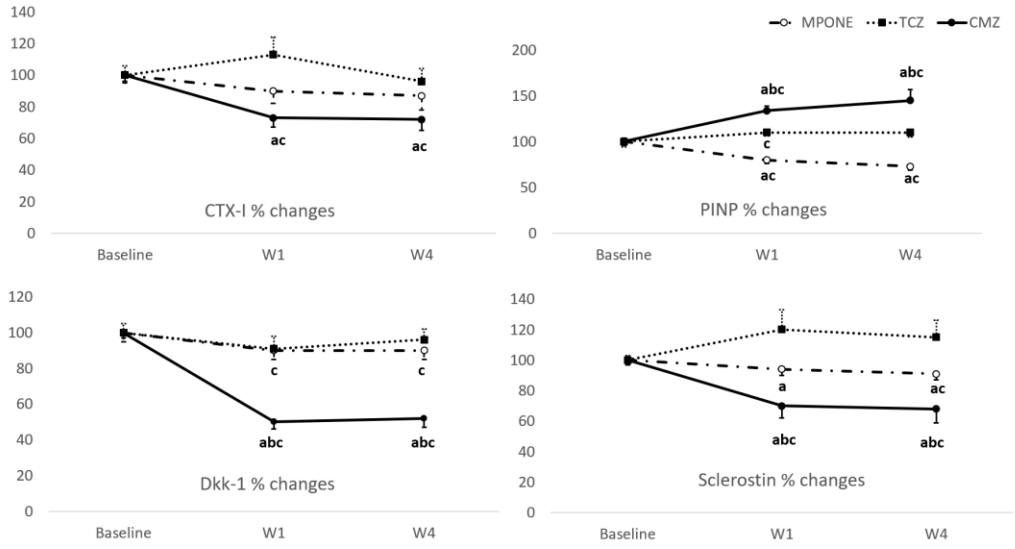

Figure 1: Bone turnover markers and Wnt inhibitors percent changes after 1 and 4 weeks of treatment. Data are expressed as mean ± SE; PINP, N-terminal propeptide of type I procollagen; β-CTX-I, C-terminal telopeptide of type I collagen; W1, week 1; W4, week 4.
a $p<0.05$ vs tocilizumab; b $p<0.05$ vs methyl-prednisolone; c $p<0.05$ vs baseline.

Study n° 2. Wnt inhibitors and bone turnover markers in patients with polymyalgia rheumatica and acute effects of glucocorticoid treatment

Results

We enrolled a total of 17 patients affected by PMR (7 males and 10 females) and 17 HC (7 males and 10 females). Median disease duration was 3 [2-4] months. Additional clinical characteristics of PMR patients are summarizes in Table 2.

	HC group (N = 17)	**PMR group (N = 18)**	
	(Single observation)	Baseline	W4

Age, mean (SD)	76.3 (8.1)	78.3 (8.6)	/
Gender (M:F)	7:10	7:10	/
BMI mean (SD)	26.1 (2.4)	24.9 (2.4)	/
CRP, median (IQR) mg/L	/	41 (20-51)	3 (3-3.5) d
PMR AS	/	30.1 (25.9-33.2)	6.5 (6.3-7.3) d
PINP, mean (SD) ng/mL	52.8 (20.8)	59.0 (17.0)	34.4 (12.1) b, d
CTX-I, median (IQR) ng/mL	0.395 (0.287-0.494)	0.340 (0.302-0.45)	0.268 (0.19-0.423) a (p=0.049), c
Dkk-1, median (IQR) pmol/L	21.5 (15.2-31.1)	33.6 (29.7-42.0) b	23.9 (16.4-40.3) c
Sclerostin, mean (SD) pmol/L	44.7 (19.6)	41.9 (15.2)	26.9 (9.2) b, d

Table 2: baseline and follow up values of the parameters for healthy controls (HC) and patients with polymyalgia rheumatica (PMR). Abbreviations: HC, healthy controls; PMR, polymyalgia rheumatica; W, week; SD, standard deviation; IQR, interquartile range; BMI, body mass index, CRP, C-reactive protein, PMR AS, , polymyalgia rheumatica activity score, PINO, N-propeptide of type I collagen; CTX-I, C-terminal telopeptide of type I collagen.

a $p < 0.05$ vs HC

b $p < 0.01$ vs HC

c $p < 0.05$ vs baseline

d $p < 0.01$ vs baseline

At baseline, only Dkk-1 was significantly higher in the PMR group (p=0.002) than in HC (Table1). Changes from baseline to week 4 regarding BTM, Dkk-1 and sclerostin for the PMR group are depicted in figure 2.

After 4 weeks of GC treatment all patients were in clinical remission and CRP values turned to normal. Concerning markers of bone metabolism, we found in the PMR group a decrease of PINP (mean ± SD percentage decrement as compared to baseline -40±18.6%, p = 0.000), CTX-I (-23.5±41.3%, p = 0.032), Dkk-1 (-22.4±39.6, p = 0.033) and sclerostin (-32.49±20.47, p < 0.001) as compared to baseline levels.

A significant positive correlation was found between the percentage decrease in CRP and Dkk-1 from baseline to week 4 (p = 0.007, r_s = 0.625).

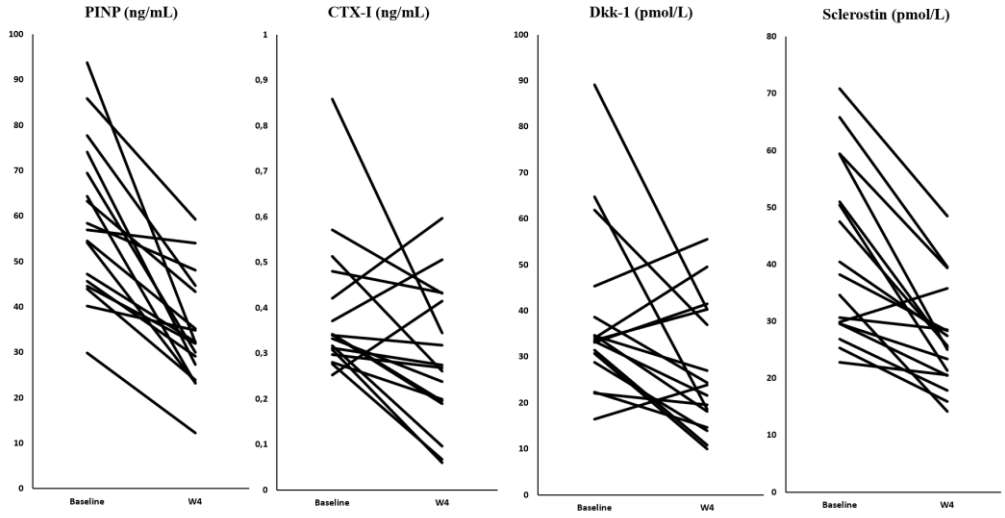

Figure 2: changes in bone turnover markers, Dkk-1 and sclerostin in the PMR group.

Study n° 3 - Diffuse idiopathic skeletal hyperostosis (DISH) in type 2 diabetes: a new imaging possibility and a new biomarker

Results

Overall, we studied a total of 96 post-menopausal women with non-insulin-treated T2DM (mean ± SD age: 72.7±8.5 years; median duration of diabetes: 10.5 [6-17] years; median body mass index (BMI): 29 [26-31] kg/m^2; median hemoglobin A1c (HbA1c): 51 [48-57] mmol/mol). The main clinical and biochemical characteristics of T2DM patients stratified by presence or absence of DISH are shown in **table 3**.

	Overall N=96	**DISH N=20**	**No DISH N=76**	**Sig. P-values (unadjusted)**
Age (years)	72.7 ± 8.5	75.4 ± 6.5	70.61 ± 8.5	**0.02**
Age at menopause (years)	49.9 ± 0.2	49.1 ± 0.5	49.8 ± 0.3	**Ns**
Years since menopause (years)	22.5±9	26.35 ± 6.78	20.7 ± 8.5	**0.008**
BMI (kg/m^2)	29 [26-31]	29 [26.2-32.0]	29 [26.0-32.0]	Ns
Waist circumference (cm)	98 [90-105]	95.5 [88.5-106.5]	98 [90-105]	Ns
FFM (kg)	40.1 [3.4-4.2]	40.7 [38.4-43.5]	40.1 [36.6-42.1]	Ns
FM (kg)	29.6 [24.5 – 35.0]	28.5 [24.7-35.3]	29.1 [23.9-34.5]	Ns
Systolic blood pressure (mmHg)	140 ± 16	144 ± 11	139 ± 17	Ns
Diastolic blood pressure (mmHg)	76 ± 9	73 ± 7	77 ± 9	Ns
Diabetes duration (years)	10.5 [6-17]	15 [6.25-21.7]	10 [6.0-16.0]	Ns
HbA1c (mmol/mol)	51 [48-57]	50 [48-57]	50 [48-55]	Ns
hsCRP (mg/L)	2 [1-3]	2 [1-4.7]	1 [1-4]	Ns
PTH	31.4 [23.1-44.1]	36.2 [26.3-45.7]	29.5 [21.4-43.3]	Ns
CTX (ng/mL)	0.257 [0.163-0.428]	0.273 [0.170-0.410]	0.223 [0.138-0.370]	Ns
PINP (ng/mL)	49.7 [38.1-64.6]	53.7 [46.5-61.0]	45.5 [35.9-64.6]	Ns
Sclerostin (pmol/L)	36.9 [20.2-46.4]	32 [23.4-38.0]	35.5 [27.7-49.5]	**0.010**
Dkk-1 (pmol/L)	42 [27.4-62.0]	59.5 [17.1-72.6]	42.1 [27.3-59.3]	0.082
RANKL(ng/mL)	0.06 [0.03-0.12]	0.05 [0.03-0.09]	0.06 [0.04-0.12]	Ns
25(OH)D (ng/mL)	31.0 ± 13.4	34.0 ± 14.7	30.5 ± 13.3	Ns

Creatinine (µmol/L)	62.5 [56.0-75.5]	64.5 [57-80.5]	63 [56-73]	Ns
Total cholesterol (mg/dl)	162 ± 32	158 [136-179]	154 [140-189]	Ns
Triglycerides (mg/dl)	112 [81.2-151.0]	111 [66-158]	103 [79-146]	Ns
LDL-cholesterol (mg/dl)	77.5 [57.2-102.7]	78 [54.2-91.7]	74 [57-102]	Ns
HDL-cholesterol (mg/dl)	60 ± 13.4	59.2 ± 12.8	59.6 ± 13.8	Ns
Uric acid (mg/dl)	4.4 ± 1.3	4.87 ± 1.15	4.43 ± 1.4	Ns
HOMA-IR score	2.26 [1.26-3.79]	2.43 [1.2-3.9]	2.2 [1.32-3.56]	Ns
Z-score lumbar spine	0.67 ± 1.3	0.68 ± 1.50	0.51 ± 1.35	Ns
T-score lumbar spine	-0.81 ± 1.46	-0.79 ± 1.35	-0.76 ± 1.47	Ns
Z-score femoral neck	0.10 ± 0.84	-0.01 ± 0.73	0.12 ± 0.88	Ns
T-score femoral neck	-1.51 ± 0.99	-1.77 ± 0.68	-1.45 ± 1.05	Ns
Z-score total hip	0.71 ± 1.0	0.74 ± 0.85	0.70 ± 1.08	Ns
T-score total hip	-0.67 ± 1.21	-0.85 ± 0.75	-0.64 ± 1.32	Ns
Cholecalciferol supplementation %	40.6%	60%	35.5%	0.04

Table 3. Main clinical and biochemical characteristics of postmenopausal type 2 diabetic women stratified by presence or absence of DISH. Data are expressed as means ± SD for normally distributed variables and medians [IQRs] for non-normally distributed ones.

From the patients' electronic medical records, previous spine X-rays were retrieved in 10 patients with DISH and in 100% of the cases the diagnosis made at VFA was confirmed. Examples of DISH documented by VFA and respective X-rays are depicted in **figure 3**.

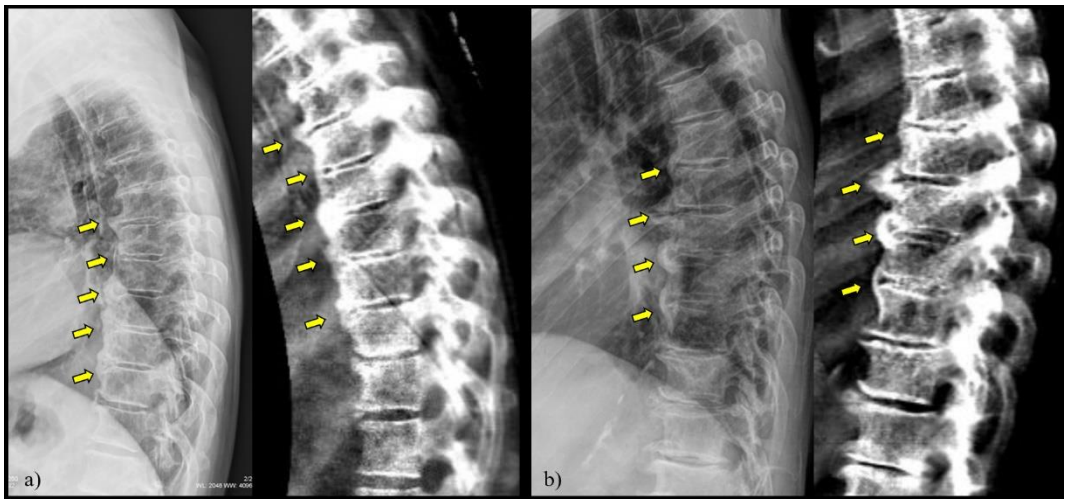

Figure 3. Two cases of DISH as documented by DXA-VFA and respective X-rays in two different type 2 diabetic women (patient A and patient B). X-Rays are reported on the left and VFA on the right, respectively.

Overall, 20 patients (20.8%) showed features of DISH, whilst 8 (8.3%) had vertebral fractures. The distribution of spinal involvement according to the individual vertebral body level is shown in **figure 4**.

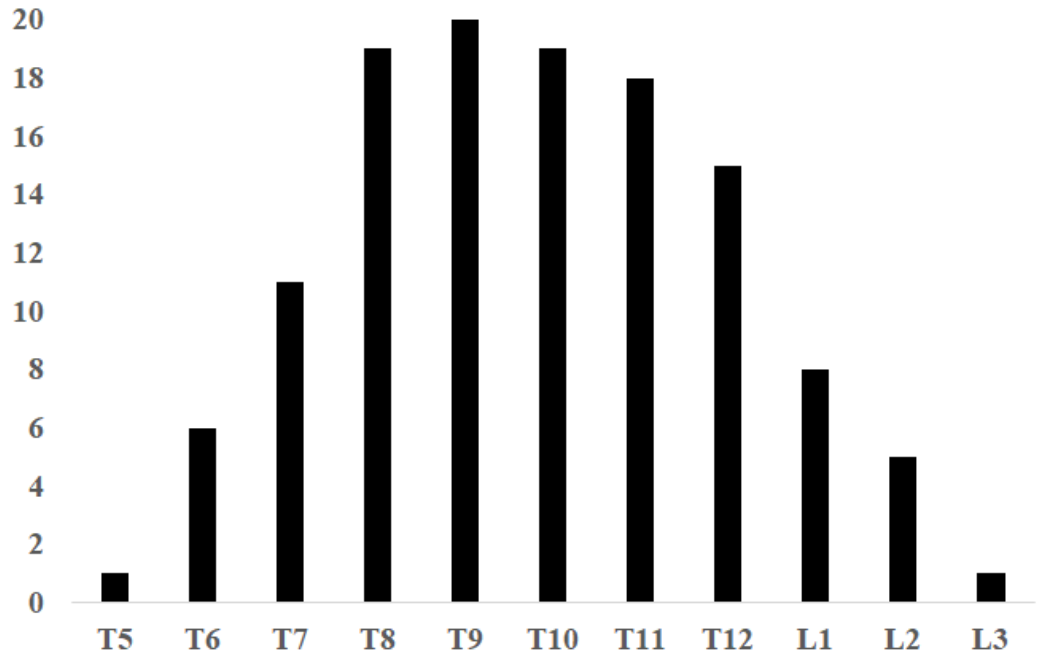

Figure 4. Distribution of spinal involvement according to individual vertebral body level.

In addition, 3 patients showed both features of DISH and presented at least one vertebral fracture. 11.9% of the subjects were osteoporotic at the lumbar spine, 4% at femoral neck and 4% at total hip. BMD values, expressed as Z-scores, showed

positive (> 0) mean values. In particular, they showed statistically significant higher values at all sites: 0.58 ± 1.38 at lumbar spine (for Z-score tested vs. 0 p = 0.0001), 0.10 ± 0.88 at femoral neck (p=0.0001), 0.72 ± 1.06 at total hip (p =0.0001). No significant differences in BMD values, expressed as Z-scores, were found between the DISH and no-DISH groups (**table 3**). The two groups of patients were significantly different only for age, years since menopause, hypertension prevalence, proportion of patients taking cholecalciferol supplements and serum sclerostin levels. In particular, women with DISH were more likely to be older and had higher years since menopause and lower serum sclerostin levels than those without DISH. Notably, after adjusting by age and years since menopause, serum sclerostin levels remained significantly lower amongst those with DISH (**figure 5**). In the binary logistic regression model including serum sclerostin, age and years since menopause, only sclerostin resulted to be a statistically significant predictor (OR 0.93 95%CI 0.88-0.98, p = 0.006). Age and years since menopause were not significant (OR 0.98 95%CI 0.78-1.24 and OR 1.14 95%CI 0.90-1.4, respectively).

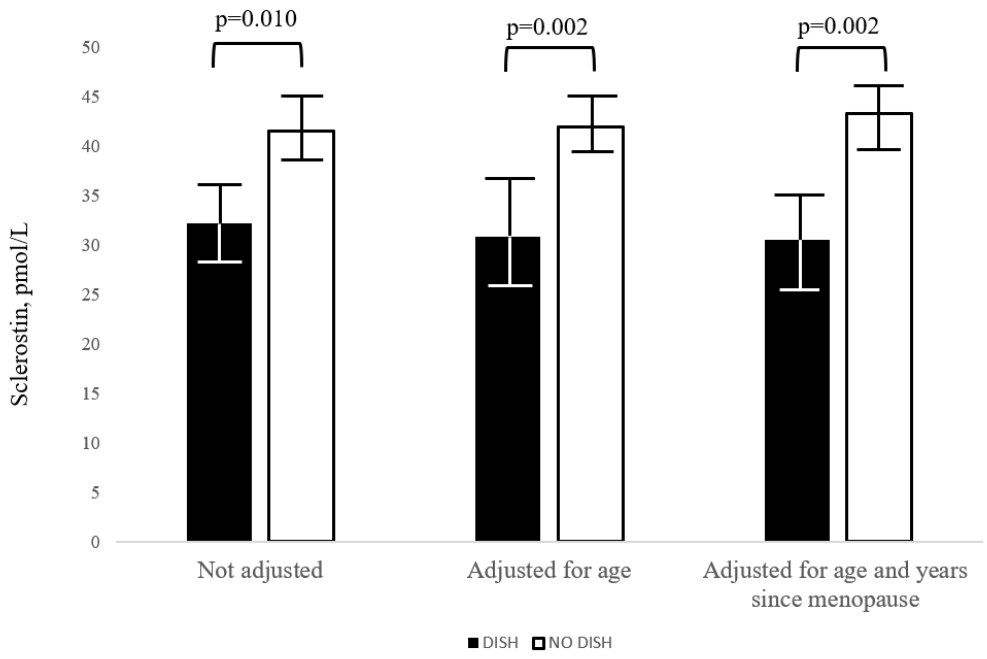

Figure 5. Mean serum sclerostin levels in DISH (black bars) and no-DISH (white bars) postmenopausal women with type 2 diabetes, before and after adjustment either for age or for age *plus* years since menopause. Error bars show 95% confidence intervals.

The proportion and comparison of the recorded comorbid conditions between DISH and no-DISH patient groups are reported in **figure 6**. A statistically significant

imbalance between the two groups was found only in the proportion of patients affected by hypertension (p = 0.035), but this significance was lost after adjustment for age.

The proportions of patients taking cholecalciferol supplementation is reported in table 1. Their median cholecalciferol daily dose was 1,000 IU, interquartile range 800-1,600 IU. No statistically significant differences in terms of age or serum sclerostin were found between the supplemented vs non-supplemented subgroups (data not shown).

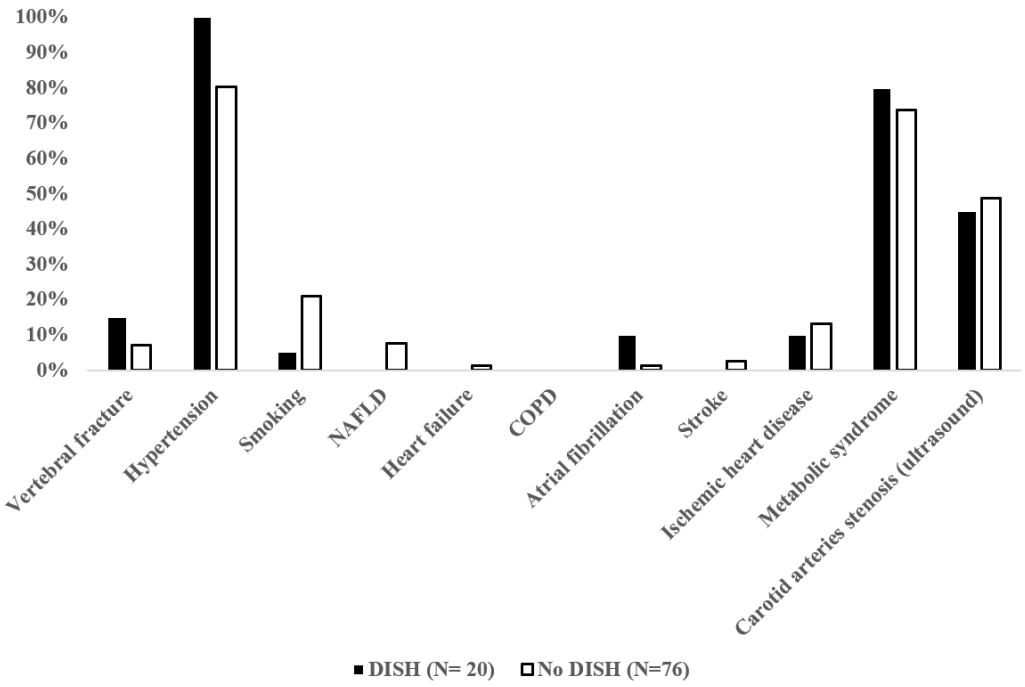

Figure 6. Proportion of patients presenting comorbid conditions in DISH and no-DISH groups. After adjustment for age, no significant differences were found for any condition between the two groups. NAFLD, non-alcoholic fatty liver disease; COPD, chronic obstructive pulmonary disease.

DISCUSSION

Study n° 1. Acute effects of glucocorticoid treatment, TNFα or IL-6R blockade on bone turnover markers and Wnt inhibitors in early rheumatoid arthritis: a pilot study.

The goal of this study was to compare the acute effects of drugs with different mechanisms of action on bone turnover markers and on modulators of the canonical Wnt pathway. Our decision to focus on the short-term effects has been suggested by the fact that bone is programmed to defend its health through the coupling of bone formation following bone resorption (42). Indeed, bone undergoes a limitless process of turnover where the activity of bone resorbing cells is overlapping by the activity of bone forming ones (42). This complex mechanism of coupling allows the skeletal tissue quickly to respond to physiological and pathological stresses (42). In this study, our objective was to investigate the early direct effects of various pharmacological treatments before the onset of any possible contra-regulatory mechanism.

The most striking finding of our study is the marked effect of the anti-TNFα treatment when compared to the IL-6 blockade and the daily glucocorticoid treatment at moderate dosage (methyl-prednisolone 8 mg daily).

We found a relevant decrease in β-CTX-I serum levels and an increase in PINP after one week of treatment with anti-TNFα, while we did not detect any significant change in β-CTX-I either in the anti-IL-6R or in the methyl-prednisolone arms. We also observed a decrease in PINP with methyl-prednisolone, in line with previous studies (50,51).

Despite the renown effects of glucocorticoids on bone resorption, we did not observe any change in β-CTX-I serum levels in the methyl-prednisolone arm (52). However, we should note that, in RA, serum levels of inflammatory reactants are more strongly correlated with bone resorption than with bone formation markers (53,54). For this reason, the lack of changes in serum β-CTX-I during glucocorticoid treatment could be explained by its beneficial effect on inflammation, which might counterbalance the increase in bone resorption. Another possible explanation is that glucocorticoids have a predominant negative effect on osteoblasts and bone formation. Nonetheless, the suppression of bone formation demonstrated with the use of glucocorticoids is particularly evident later in the treatment course, while in the first few months of therapy an increase in bone resorption is more conspicuous.

Regarding the effects of TNFα inhibitors, our data on BTMs are consistent with the literature. Previous studies with these biologic agents observed a decrease in markers of bone resorption and an increase in markers of bone formation (55,56).

The present study, along with a recently published experience from our group (55), is currently the only report on the acute changes occurring within the first month of treatment. The expected decrease in β-CTX-I witnessed in the certolizumab pegol arm was not seen with IL-6R blockade, despite its potent anti-inflammatory effects. Presently, we cannot provide a certain explanation for this discrepancy, but we hypothesize an earlier influence on bone metabolism exerted by TNFα inhibitors (or specifically by certolizumab pegol) than tocilizumab.

Furthermore, the data from the literature on changes in bone resorption induced by IL-6R inhibitors are still inconsistent [8,9,16–18], while two studies reported an increase in PINP [9,18]. In both studies however, PINP was dosed after three months of treatment.

Data concerning glucocorticoids and Wnt inhibitors are also not very consistent, and the various studies involved patients affected by different diseases and receiving treatment with disparate doses of glucocorticoids. In experimental studies in rodents and cell cultures glucocorticoids have been reported to increase the expression of sclerostin and Dkk-1 (57). The inhibition of bone formation and the increase in bone resorption following corticosteroid treatment has been linked to multiple mechanisms (i.e., the increased expression of sclerostin) (58).

The role of sclerostin is supported by an interesting genetic study that showed that glucocorticoids led to impairment of the bone mass, microarchitecture and strength only in wild-type mice but not in female mice lacking sclerostin (59). Furthermore, the treatment with an antibody neutralizing sclerostin has been showed to prevent the loss of bone mass and bone strength induced by glucocorticoids through the preservation of osteoblast activity (60). These findings are not in line with the results of our study that shows a decrease in sclerostin. However, in the previous studies, the influence of the condition for which glucocorticoid treatment was required has not been addressed. Indeed, in 17 glucocorticoids-naïve patients, this treatment induced an acute significant reductions of sclerostin (50). Another study regarding 91 patients with systemic autoimmune diseases who started glucocorticoids reported contradictory changes of Wnt antagonists: serum levels of sclerostin and Dkk-1 increased significantly during the first week of therapy but from the second week onward, they decreased (61). A possible explanation for the reduction in sclerostin serum levels could be the apoptosis of osteocytes caused by glucocorticoids (62). On the other hand, several studies have reported that the

elevated levels of pro-inflammatory cytokines in inflammatory diseases (such as the ones requiring corticosteroid therapy) can also cause osteocyte apoptosis (63). In this way, corticosteroid treatment may act in two ways: directly, by reducing sclerostin trough osteocyte apoptosis, or indirectly (in inflammatory conditions), by limiting the reduction of sclerostin due to the osteocyte apoptosis caused by pro-inflammatory cytokines.

The changes of Dkk-1 during glucocorticoids in patients affected by arthritis are also not consistent in the literature.

In a recent study on mice, Colditz et al [12] showed that osteocyte-specific Dkk-1 deletion did not affect the bone loss induced by arthritis, but it assured an evident protection by glucocorticoid-induced bone loss.

However, we should remember that Dkk-1 is produced not only by osterix-expressing osteoprogenitors but also by synovial fibroblast of patients with early arthritis [28]. Therefore, also Dkk-1 might change in opposite directions according to the setting. It may increase (as a direct effect on osteoprogenitors) in some conditions needing glucocorticoid treatment (i.e., pulmonary diseases) as a direct effect on the productive osteoprogenitor cells. However while it may decrease (due to the inhibition of inflammation on synovial fibroblasts) in RA, a disease in which its serum levels are greatly upregulated (41). All the patients included in this study were affected by RA and thus the decrease in Dkk-1 is somewhat expected.

In a previous study we documented the effects of the TNFα blockade on Dkk-1 and sclerostin (55,64). The decrease of Dkk-1 after TNFα inhibition is expected, as Dkk-1 is increased in patients with RA (40,41) and its expression is also induced by TNFα *in vitro* and *in vivo* (65,66).

The reduction in sclerostin serum levels in the anti-TNFα and methyl-predinsolone group, consensual with the Dkk-1 decrease, might also contribute to explain the reduction of bone and cartilage damage (67). Sclerostin, commonly considered an osteocyte-specific protein (68), is also expressed by chondrocytes (67), and can contribute to the pathogenesis of both inflammatory bone and cartilage damage (68). However, it should be pointed out that the interpretation of this data is limited, as it is unclear to what extent serum concentrations of sclerostin correlate to local cytokine production, or action, within the cartilage and bone microenvironment.

Two studies investigated the changes of Wnt inhibitors during treatment tocilizumab (69,70). Both showed a decrease in Dkk-1 serum levels after two or

three months of treatment. We have reported data on serum Dkk-1 and sclerostin changes within the first month of treatment and, differently from previous experiences, no significant changes were observed for both markers after IL-6R blockade. This remark might be explained by a slower influence of the treatment on the Wnt pathway, in line with lack of changes on BTMs discussed previously. Interestingly, a pre-clinical study suggested a different regulation of Wnt inhibitors between TNFα and IL-6 (71), with IL-6 showing a suppressive effect on Dkk-1 production. Therefore, the apparently stable levels of these markers might also be the result of mechanisms pointing in different directions, namely the induction of Dkk-1 from IL-6R blockade and its inhibition due to suppression of inflammation. Nevertheless, further studies are needed to investigate the interplay between IL-6R inhibition and the extent and the timing of its effects on this pathway.

Data on sclerostin changes after tocilizumab therapy are currently scarce and not consistent. One study (70) reported an increase in sclerostin at two months, while another one reported no significant change (69). Biopsies from bone replacement surgery (72) showed that osteocyte of RA patients express high levels of sclerostin but are also characterized by high death rates. This may lead sclerostin serum levels to depend on both osteocyte expression and their overall number. Indeed, serum evaluation of sclerostin, which is mainly expressed by osteocytes (73), needs to be carefully interpreted.

When considered altogether, our data seem to suggest a marked effect on BTMs and Wnt inhibitors induced by the inhibition of TNFα and the absence of detectable changes by IL-6R blockade within the first four weeks. Glucocorticoid treatment seems to settle somewhere between the two, with a unique (and arguably undesired) detrimental effect on bone formation.

TNFα and IL-6R inhibition have both shown to slow erosive progression (74) and to preserve or increase spine and hip Bone Mineral Density (BMD) (56,69,75–78). Glucocorticoids also have shown to reduce localized bone loss and joint damage in patients with recent-onset active RA (79), while for systemic bone loss their effect is probably condition and dose dependent. Indeed, the systemic negative effects of glucocorticoid on BMD are renown (52), but there are also clinical trials on RA patients that support a beneficial effect of low-dose glucocorticoid treatment on systemic BMD (80,81). These seem to be associated with a rapid and aggressive suppression of inflammation which may outweigh their negative influence on bone

health (79). Therefore, the interaction between the direct and indirect effects on bone and systemic inflammation (when present) must always be addressed when investigating bone metabolism and its fine regulators (i.e., Dkk-1 and sclerostin).

Our study has some limitations. It is a retrospective analysis and therefore we cannot completely exclude the risk of confounding variables which might have biased the three samples, despite the matching for age, disease activity and bone parameters. Second, the sample size is limited. Third, data on radiographic damage were not available. One last limitation is represented by lack of data regarding the assessment of physical activity before and after the treatment. According to the literature, mechanical loading on the skeleton may influence sclerostin levels (82).

In conclusion, TNFα inhibition strongly and quickly influences BTMs and Wnt inhibitors. Such findings were not observed with IL-6R blockade, at least within the first four weeks of treatment.

It should be noted that our exploratory study cannot give the explanation of the different effects of anti TNFα treatment than tocilizumab. In fact, it merely describes, for the first time, a peculiar effect on the short-term. We cannot exclude that this might be attributable to a more direct role of the TNFα than IL6 on the regulation of bone metabolism

Glucocorticoids at moderate dosage exert similar, albeit less marked, changes, perhaps linked to the reduction of inflammation, nonetheless they still show some undesired effects on bone metabolism, namely the suppression of bone formation.

Study n° 2. Wnt inhibitors and bone turnover markers in patients with polymyalgia rheumatica and acute effects of glucocorticoid treatment

This is the first study that investigated BTM and Wnt inhibitors in patients affected by PMR at baseline and after four weeks of treatment with GC.

Our data demonstrate that in treatment-naive PMR patients, Dkk-1 are increased when compared with HC. In addition, the decrease of systemic inflammation after 4 weeks of GCs (all patients were in clinical and laboratory remission at that time) is associated with a decrease in Wnt inhibitors (Dkk-1 and sclerostin), CTX-I and in PINP.

The finding of increased levels of Dkk-1 in untreated PMR patients is not surprising. Indeed, it is consistent with similar observations in RA, arguably the most common rheumatic inflammatory disease (40,41,83). Pro-inflammatory

cytokines such TNFα and IL-6 play a fundamental role in the pathogenesis of inflammation and bone loss in RA, partly due to the exaggerated production of Dkk-1 and other Wnt antagonists (84,85). Consistent with this model, treatments with TNFα inhibitors or GC by reducing pro-inflammatory cytokines in RA patients have been shown to rapidly decrease Dkk-1 (and sclerostin) serum levels (43,55,86,87). Given the central pathogenetic role of TNFα and especially IL-6 in PMR (88) a similar model might be applied to interpret the dysregulation of the Wnt system in this disease, and the reduction of its inhibitors that we observed with CG treatment. The significant positive correlation we found between the decrease of CRP and Dkk-1 seems to support this conclusion.

While the normalization of Dkk-1 after 4 weeks of GC treatment can be explained mainly by the link between inflammation and excessive Wnt inhibition, the reduction of sclerostin even below the levels of HC could be due to the direct effects of GC on this molecule. In a recent study, Thiele et al (89) reported not only an in vitro decrement of sclerostin expression after GC treatment in human bone marrow stromal cells, but also found decreased serum sclerostin in a cross-sectional observation of 21 patients with long lasting (average duration of 1.31 years) PMR who received chronic treatment with GC. Previous studies on autoimmune diseases different from PMR support the presence of contradictory effects of GC on Wnt antagonists (90,91). It is therefore possible that the suppressive effect of GC on sclerostin expression in PMR could be twofold: an indirect effect via the inhibition of inflammation (similarly to Dkk-1), and a direct effect, independent from disease activity and inflammation, on cells of the mesenchymal lineage (i.e. osteocytes) (89).

In our study, PINP but also CTX-I yielded a significant decrement after 4 weeks of GCs not only with respect to baseline levels but also with respect to HC, even if at baseline, levels were comparable between patients and HC (a lack of statistical power in this regard can certainly not be excluded). Inflammatory rheumatic conditions can be associated with both local and systemic bone resorption due to the pathologic increases of proinflammatory cytokines and mediators (42). In this case however, levels of CTX-I should have been higher in PMR patients than HC at baseline. Suppression of pathologic inflammation is therefore expected to have positive effects in this setting, both via the suppression of proinflammatory cytokines and via the already discussed reduction in Dkk-1 and sclerostin, with

subsequent positive effects on the regulation of the RANKL/OPG ratio (42). However, it should be noted that the absolute serum levels of CTX-I at week 4 were lower than those of HC. In this regard, a relationship between the decrease in bone resorption and the marked suppression of sclerostin might be speculated, albeit a counter regulatory homeostatic response to the decrease in PINP, often seen during treatment with osteoactive drugs (42), cannot be ruled out.

Indeed, we also observed a decrease in PINP with GC that is in line with previous studies (43,50,51) and confirms the renown (direct) detrimental effects of GC on osteogenesis and bone formation. Given the suppression of Dkk-1 and sclerostin, the impairment of the bone anabolic activity suggested by the decrease in PINP appears to be largely independent from the Wnt system. Our observations seem to be in line with the concept of a direct "toxic" role of GC towards cells of the osteoblastic lineage and their precursors, resulting in a deficiency in bone forming surfaces (42,92).

Our study has several limitations. First of all, sample size was limited. The sensitivity analysis was calculated on the expected changes of Wnt inhibitors, and therefore it was not powered to rule out, for instance, the absence of differences in BTM and/or sclerostin at baseline. Second, it lacks direct evaluation of bone histology and metabolism. A bone biopsy before and after GC treatment would have been desirable to correlate the findings from serum with actual effects on bone remodelling, however, it was deemed as not being ethical. Long-term observations of patients (e.g. after 6-12 months of GC therapy and in stable remission) would have been of interest to better investigate the direct effects of GCs in PMR on bone metabolism, however, since all patients started anti-resorptive therapy after 1 month of GC therapy (which is in accordance with national guidelines), an unbiased assessment would not have been possible.

In conclusion, this study for the first time showed that, in treatment-naïve PMR, systemic inflammation is associated with a dysregulation of the Wnt system (especially due to the increase in Dkk-1), similarly to what has been observed in RA. Treatment with GC, currently the mainstay therapy for PMR, is associated with suppression of Dkk-1 and sclerostin with a consensual reduction in bone resorption. Nevertheless, CG still show some undesired effects on bone metabolism, namely the suppression of bone formation that seems not to be directly related to the regulation Wnt-pathway.

In PMR a different anti-inflammatory approach such as treatment with IL-6 blockers could enable us to control disease and inflammation limiting the negative consequences of GC on bone health.

Study n° 3 - Diffuse idiopathic skeletal hyperostosis (DISH) in type 2 diabetes: a new imaging possibility and a new biomarker

The main and novel findings of this study including post-menopausal women with non-insulin-treated T2DM are as follows: (1) the prevalence of DISH is high (affecting up to nearly 21% of these women); and (2) serum sclerostin levels are inversely associated with the presence of DISH, even after adjustment for age and years in menopause.

We identified women affected by DISH with VFA, a radiographic technique derived from DXA for the detection of vertebral fractures (93,94). We chose the VFA since the radiation dose is considerably lower (42 μSv) than conventional radiography (500 μSv) (95); furthermore, DXA's X-ray beams are parallel to the endplates as compared to the fan-shaped beans of conventional radiography, eliminating the geometric distortion and image amplification that affect conventional radiography (96). Indeed, with DXA we can obtain a single image of the entire spine, whereas with conventional radiography we would have need multiple exposures to evaluate the whole spine. It is important to highlight that T2DM is a known risk factor for DISH (97–99). In our study, importantly, the prevalence of DISH (20%), assessed with VFA, was substantially in line with a previously published report (98), confirming the validity of VFA in diagnosing DISH. We also investigated the association between DISH and a wide variety of biomarkers in a cohort of post-menopausal women with T2DM and confirmed the presence of a dysregulation in the Wnt pathway (as reflected by lower serum sclerostin levels) in the subgroup of those with DISH.

Dysregulation of the canonical Wnt pathway in rheumatic diseases and bone metabolic diseases has been increasingly studied in recent years and its dysregulation has been shown to be involved in multiple diseases (42,55,100–103). However, the currently available data on Wnt pathway modulators in DISH are scarce. Niu et al. observed decreased serum Dkk-1 levels and increased sclerostin levels in a small sample of DISH patients (n=8) when compared to healthy controls; however, no information on presence of T2DM was available. Senolt et al. also

confirmed the presence of lower levels of Dkk-1 in 37 patients with DISH than in age- and sex-matched controls (104), but no data on serum sclerostin levels were available. Our result on sclerostin serum levels was even more evident after adjustment for two potentially confounding factors, such as age and years in menopause. We can speculate that the decreased expression of sclerostin can induce a pro-osteogenic environment and, therefore, might explain the pathologic bone formation and soft-tissue ossifications seen in DISH patients better than PINP and PTH. This hypothesis should be further tested in future studies including a healthy control group in order to compare the Wnt profile of T2DM patients to that of the nondiabetic healthy population according to the presence or absence of DISH. We investigated other serum biomarkers, including bone turnover markers and RANKL as well as markers of insulin resistance, but we failed to observe any significant difference in these biomarkers between postmenopausal T2DM women with and without DISH. In addition, we did not find any difference in the prevalence of other important comorbid conditions betwTeen the two patient groups. Therefore, aside from Wnt dysregulation, the possible presence of other "drivers" of pathologic bone formation in DISH remains currently unclear.

The higher-than-average BMD values expressed as Z-scores (largely higher than zero in our cohort of patients) at all sites, together with the remarkable prevalence of vertebral fractures, are expected finding in our cohort. Indeed, it is well known that T2DM and overweight/obesity are strongly associated with increased bone fragility despite higher BMDs (105,106). However, we emphasize the fact that, especially at lumbar spine, we observed high BMD levels in both DISH and no-DISH patient groups, therefore confirming the presence of BMD values ad lumbar spine (as measured by DXA) higher than expected also in the patients without DISH.

Interestingly, very recently, a retrospective study on the incidence of cardiovascular disease on subjects with DISH has documented a significantly higher risk for myocardial infarction after adjustment for multiple confounders (OR 6.03, 95%CI 1.06-34.2) (107). This, indeed, may support the rational for screening in a high-risk population for both cardiovascular disease and DISH itself.

Our study has some important limitations that should be mentioned. First, the cross-sectional design of the study limits our ability to establish causality and temporality of the observed associations. Moreover, multiple biomarkers were tested and this

needs to be considered, however, given its exploratory nature, adjustment for multiplicity was not made in order to avoid a reduction in sensitivity. Second, given that we included only Caucasian female outpatients with non-insulin-treated T2DM, our findings may not necessarily be generalizable to other T2DM populations of different ethnicity. Third, the diagnosis of DISH was made upon VFA, an imaging technique that has not been yet validated for this purpose. This approach has been used before in the evaluation of radiographic damage in patients affected by ankylosing spondylitis with good performance results when compared with X-rays [33]. We found a good inter-observer agreement the VFA evaluation and when X-rays were available for the DISH patients (10 subjects), in all cases the diagnosis was confirmed. Clearly, this cannot be considered a definitive evidence in the absence of a proper radiographic comparative study. In our opinion, VFA evaluation might be able to identify the disease in the most expressed forms and therefore the main expected limitation might be a lower sensitivity of VFA. We think that this technique should be further studied for screening purposes for the identification of DISH in at-risk subjects (i.e., dysmetabolic individuals) and compared to X-rays to establish specificity and sensibility data. Fourth, we should consider that prevalence of DISH has been reported to be higher in men than in women (108,109). Our study showed a high prevalence of DISH in diabetic women, but it is likely that in diabetic men the prevalence of the disease might be even higher; therefore it is reasonable to candidate the quick and low-dose VFA as a screening tool in these higher risk cohorts. Lastly, as already mentioned, is the absence of a healthy control group.

Notwithstanding these limitations, the main strengths of our study were the relatively large size of the sample, the wide number of bone turnover biomarkers analysed and those with important comorbid conditions (e.g., advanced kidney disease, cirrhosis or active cancer) or taking medications known to interfere with bone metabolism. We believe that including patients with these conditions might have confounded the interpretation of data.

CONCLUSIONS

Over the course of my PhD program I investigated the Wnt pathway in the development of the bone damage. In previous studies, we demonstrated two fundamental concepts:

a) In RA, a disease characterized by focal and systemic bone loss, the dysregulation of the Wnt system is due to an increased production of its inhibitors Dkk1- and sclerostin
b) In AS and PsA, that are also characterised by pathologic bone formation, the dysregulation of the Wnt system is associated with decrease levels of Wnt inhibitors. We hypothesized that Dkk-1 and/or sclerostin depression could be one risk factor for a pathologic osteogenic environment.

Base on these premises, we further expanded our knowledge and provided answers to important questions. The key points are

1) In RA, Wnt inhibitors are induced by pro-inflammatory conditions. Treatment with potent anti-inflammatory drugs deeply affects the pathway. In the specific, TNFi act very rapidly and decrease (normalize) Dkk-1 and sclerostin serum levels; these changes are also associated with positive comebacks on BTMs (increase in PINP, reduction in CTX-I). GCs have a mixed effect: they reduce the levels of Wnt inhibitors, but also exert a negative direct effect on bone formation. Indeed, they are associated with mixed effects on bone health (i.e. decrease in Dkk-1, but also in PINP, a marker of bone formation). Finally, the acute effect of tocilizumab (anti-IL6R) was fount to be not significant, at least within the 4 weeks timeframe.
2) PMR is a different disease from RA, albeit it shares a strong and dysregulated inflammatory response. The effects of treatment with GCs in PMR is, once again, mixed: on one hand we found a decrease in the Dkk-1 and sclerostin serum levels, associated with a decrease in CTX-I (a marker of bone resorption). However, similarly to what happened in RA, also a decrease in PINP was observed, arguably due to the same negative and direct effect of GCs on the osteoblasts' activity.
3) The finding of decreased Wnt inhibitors' levels might indeed be a common trait of pathologic conditions associated with inappropriate bone formation. In DISH, a rheumatic disease characterized by flowing ossification at multiple vertebral sites but not associated with any inflammatory

component, sclerostin was observed to be the sole biomarker distinguishing the affected subjects from controls.

A final overview and future directions

The Wnt pathway is one of the cornerstones of osteogenesis. There is now an overwhelming body of evidence pointing to its dysregulation on rheumatic diseases. The notion of excessively elevated expression of Wnt inhibitors in RA is now established. We also demonstrated that the link between the production of pro-inflammatory cytokines and this increase, already shown in laboratory experiments, is also seen in live subjects. Treatment with anti-inflammatory drugs can rapidly interfere. On the other hand, the data on the Wnt dysregulation on SpA are still scarce and less solid. Our working hypothesis is, along with the presence of pro-inflammatory cytokines and the role of IL-17, the presence of a systemic environment favoring pathologic bone formation at specific sites. Our data suggest that this observation might also be a common trait in conditions not characterized by significant systemic inflammation like DISH.

At this point, we will aim our future investigations at:

1) Demonstrating the clinical utility of Dkk-1 and sclerostin as predictive biomarkers for bone damage in patients with RA
2) Further strengthening the evidence of the Wnt pathway's dysregulation in conditions such as AS, though the analysis of the relationship between different treatments (i.e. anti-IL17 vs TNFi), structural damage progression and clinical outcomes.

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