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DOTTORATO IN SCIENZE E BIOTECNOLOGIE MEDICHE

FIRST YEAR REPORT

VITAMIN D METABOLISM IMPAIRMENT IN RHEUMATOID ARTHRITIS: IMPLICATIONS FOR PATHOGENESIS AND TREATMENT.

Background and Rationale

Rheumatoid Arthritis (RA) is a chronic, disabling disease with high prevalence and high costs for the national health systems; its pathogenesis is still largely unknown.

1-25$(OH)_2$VitaminD (1-25$(OH)_2$D) is a hormone, very well known to be involved in bone metabolism regulation; in the past few years, several reports claimed a putative role for 1-25$(OH)_2$D in immune system regulation. In vitro, 1-25$(OH)_2$D induces the differentiation of monocytes with inhibition of inflammatory cytokines production (TNF-α, IL-6, IL-1) [1,2], decreases dendritic cells (DC) maturation by class II MHC, CD40, CD80, CD83 and CD86 down-regulation, favours CD4 T lymphocytes activation with a Th2 phenotype and inhibits CD8-induced apoptosis [3, 4, 5, 6].

These observations are strengthened by in vivo findings. The Vitamin D Receptor (VDR) is expressed by synovial cells [7]. In murine models of TNF-induced arthritis VDR-Knock Out mice are prone to a more aggressive disease than wild type mice, testifying the importance of vitamin D activity in immune mediated joint damage [8]. Furthermore, in humans, lower vitamin D plasma levels correlate with a higher disease activity in RA patients [9, 10, 11]. The importance of vitamin D metabolites in immune regulation is confirmed by recent evidences that macrophage and monocyte-derived DCs express the enzyme needed to convert circulating cholecalciferol (25(OH)D) into active 1-25$(OH)_2$D (CYP27B1) [12]; this suggests a local activation of vitamin D with a potential autocrine and paracrine anti-inflammatory effect in the synovial microenvironment.

We recently observed that patients affected by RA had an increased PTH concentration for any plasma vitamin D range, with respect to a control population, suggesting an impaired vitamin D metabolism which may reflect a local consumption by macrophages during synovial inflammation [13]. A deeper knowledge on Vitamin D metabolism in RA could help to clarify its potential role in the pathogenesis of the disease.

In particular, if we will be able to demonstrate that significant local activation of vitamin D occurs in inflamed joints, we could speculate that the oral administration of the intermediate metabolite could be appropriate to allow the substrate for the synovial CYP27B1, preventing the systemic activation of this hormone and, therefore, enhancing local rather than systemic effects.

Our project aims to:

1. Evaluate whether RA patients have an impaired vitamin D metabolism compared to osteoarthritic (OA) patients (control group);
2. Better define the mechanisms underlying the vitamin D/PTH system derangement and the features of vitamin D metabolism in synovial cells.

Methods:

I performed a first set of experiments in collaboration with Prof. Pitzalis, at Experimental Medicine and Rheumatology lab, Queen Mary University, London. To perform the planned experiments I used samples from a bank of biological tissues available in EMR lab.

To test the hypothesis of a different vitamin D metabolism in RA and OA, I characterized the expression of three target genes (VDR, CYP27B1 and CYP24A1) by QT-PCR in synovial tissue obtained from joint replacement in patients affected by RA (N= 45) and OA (N= 17).

To evaluate the protein expression of these biological targets, I performed an IHQ analysis on the same paraffin embedded synovial tissues; I also stained these samples for B cells (CD20), T cells

(CD3) and macrophage (CD68) biomarkers. An IF co-staining has been also performed to evaluate the co-expression of CD68 and CYP27B1.
To better define the mechanisms of vitamin D/PTH system derangement, I studied the role of synovial fibroblasts (RASF) in vitamin D metabolism. Thus, I worked on primary cultures of synovial fibroblasts and I compared the QT-PCR expression of the target genes in unstimulated and activated RASF. To stimulate RASF, I treated cells with TLR-3 and TLR-4 activators (PIC and LPS respectively).
Recently, after obtaining ethical committee approval, we started the collection of samples of synovial tissues obtained from joint replacement in our Hospital, in collaboration with Prof. Grassi, Head of SC Ortopedia e Traumatologia. The aim is to locally develop a biobank of synovial tissues and synovial-derived cells, available for further experiments.

Preliminary results:

1. We did not find any statistical difference in the expression of VDR, CYP27B1 and CYP24A1 (inactivating enzyme) according to the diagnosis of RA or OA. Furthermore, CYP24A1 seems not to be expressed significantly in synovial tissue. We also analyzed these data stratifying patients accordingly to the degree of infiltration of the main inflammatory cells (semiquantitatively defined by immunohistochemistry). Though no significant results were obtained according to the degree of macrophage infiltration, we found a higher VDR expression in patients with higher T and B cells infiltration (figure 1).

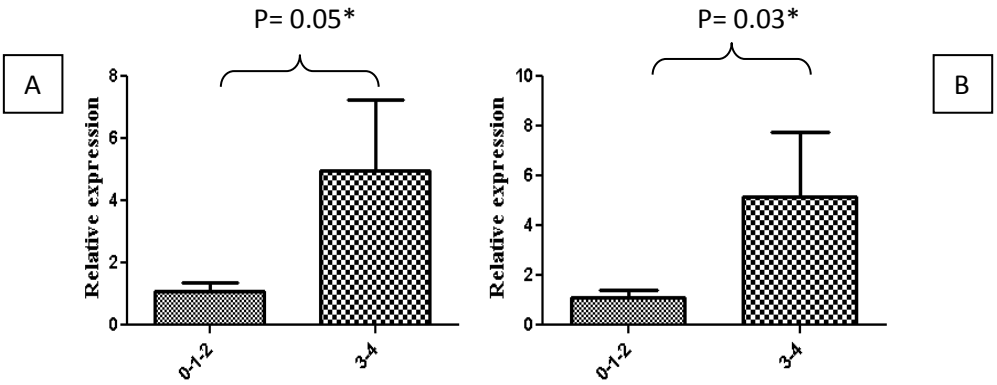

Figure 1: Correlation between histological infiltration of CD3+ T cells (panel A) and CD20+ B cells (panel B) and relative expression of VDR. *Mann-Whitney test.

We also managed to histologically stain VDR and CYP27B1 in synovial tissue (figure 2), though no specific pattern could be identified according to the diagnosis. VDR seems to be mainly expressed by macrophage, especially in the lining layer. CYP27B1 positivity seems not to be limited to macrophage, as confirmed by CYP27B1/CD68 co-staining (figure 3).

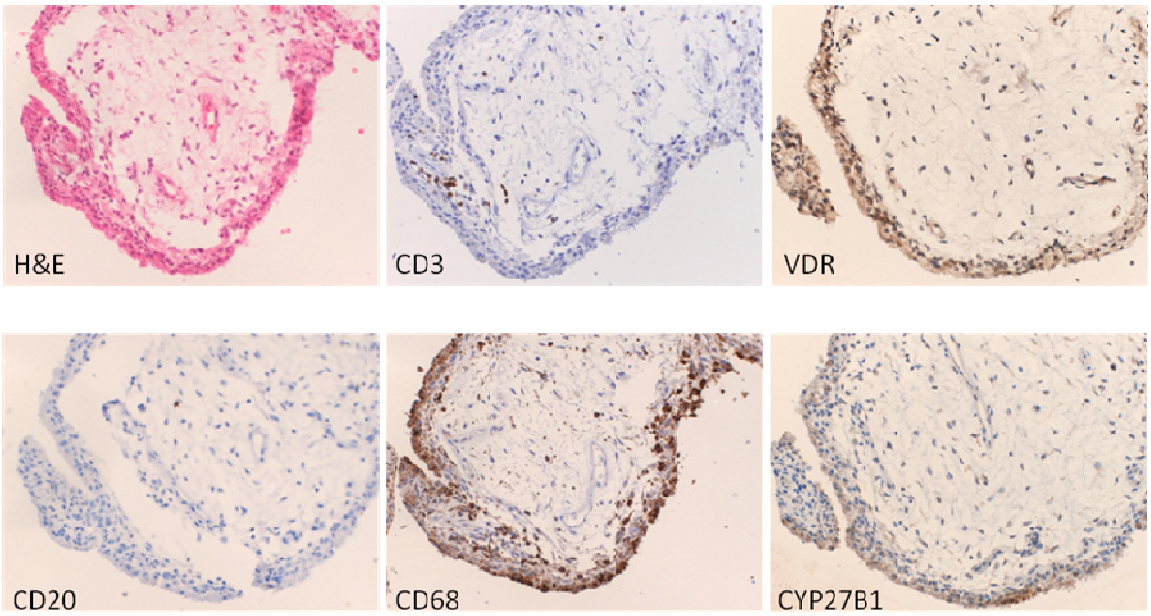

Figure 2: the panels show the staining for H&E, CD3+ T cells, VDR+ cells, CD20+ B cells, CD68+ macrophages and CYP27B1+ cells in synovial tissue.

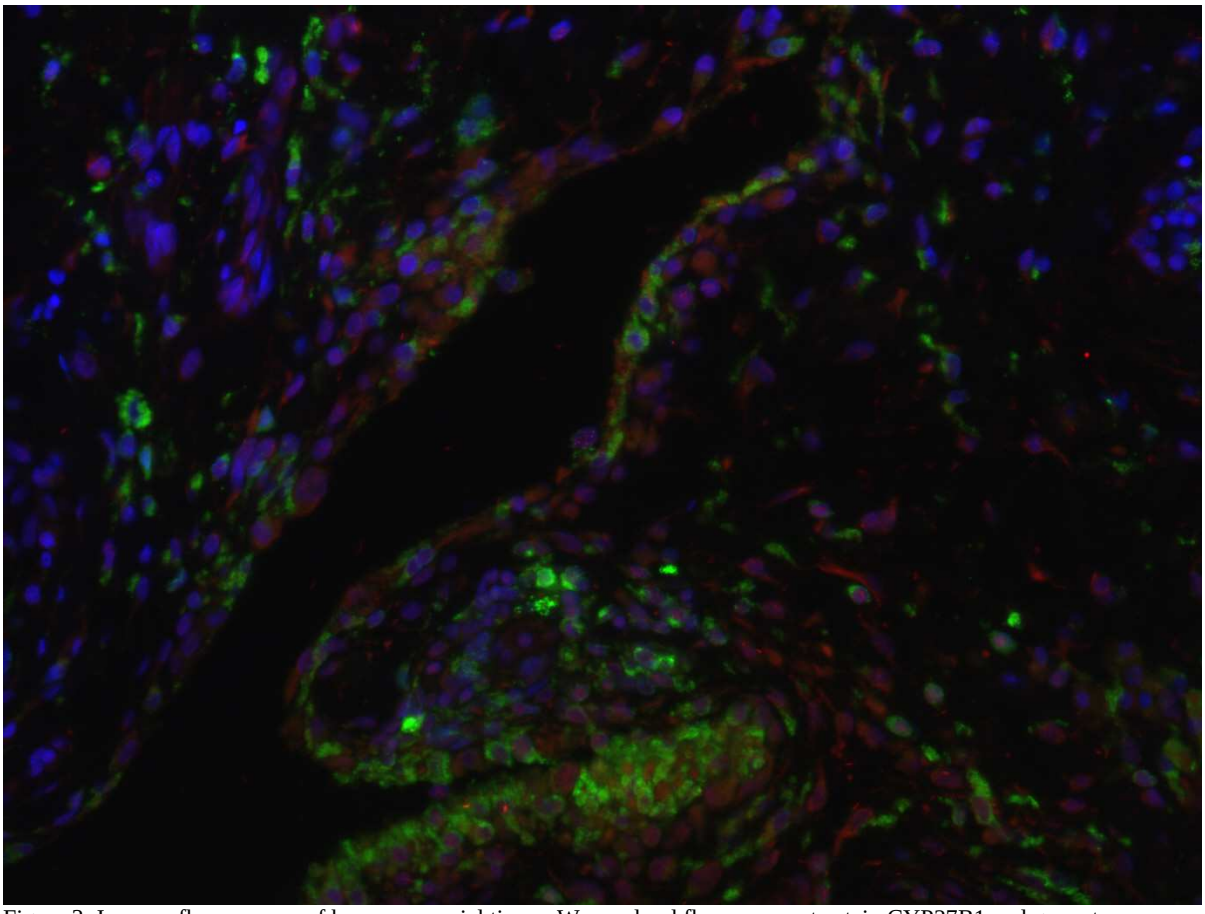

Figure 3: Immunofluorescence of human synovial tissue. We used red fluorescence to stain CYP27B1 and green to identify CD68.

2. On the basis of these preliminary results we decided to move to cell cultures; in particular we decided to focus our attention on synovial fibroblasts (RASF), since IF was suggestive for CYP27B1 expression in this cell type (see again Fig. 3).

We analyzed the expression of the three target genes in RASF obtained from RA patients. We evaluated if the TLR3/TLR4 pathway activation could interfere with the vitamin D metabolism. We analyzed five sets of fibroblasts from 3 RA patients: we did not observe any significant difference in VDR expression (Figure 4a). On the other hand, TLR4 stimulation was able to significantly increase CYP24A1 expression after 72 hours (Figure 4b). Both TLR3 and TLR4 activation increased significantly CYP27B1 expression after 4 and 8 hours (Figure 4c).

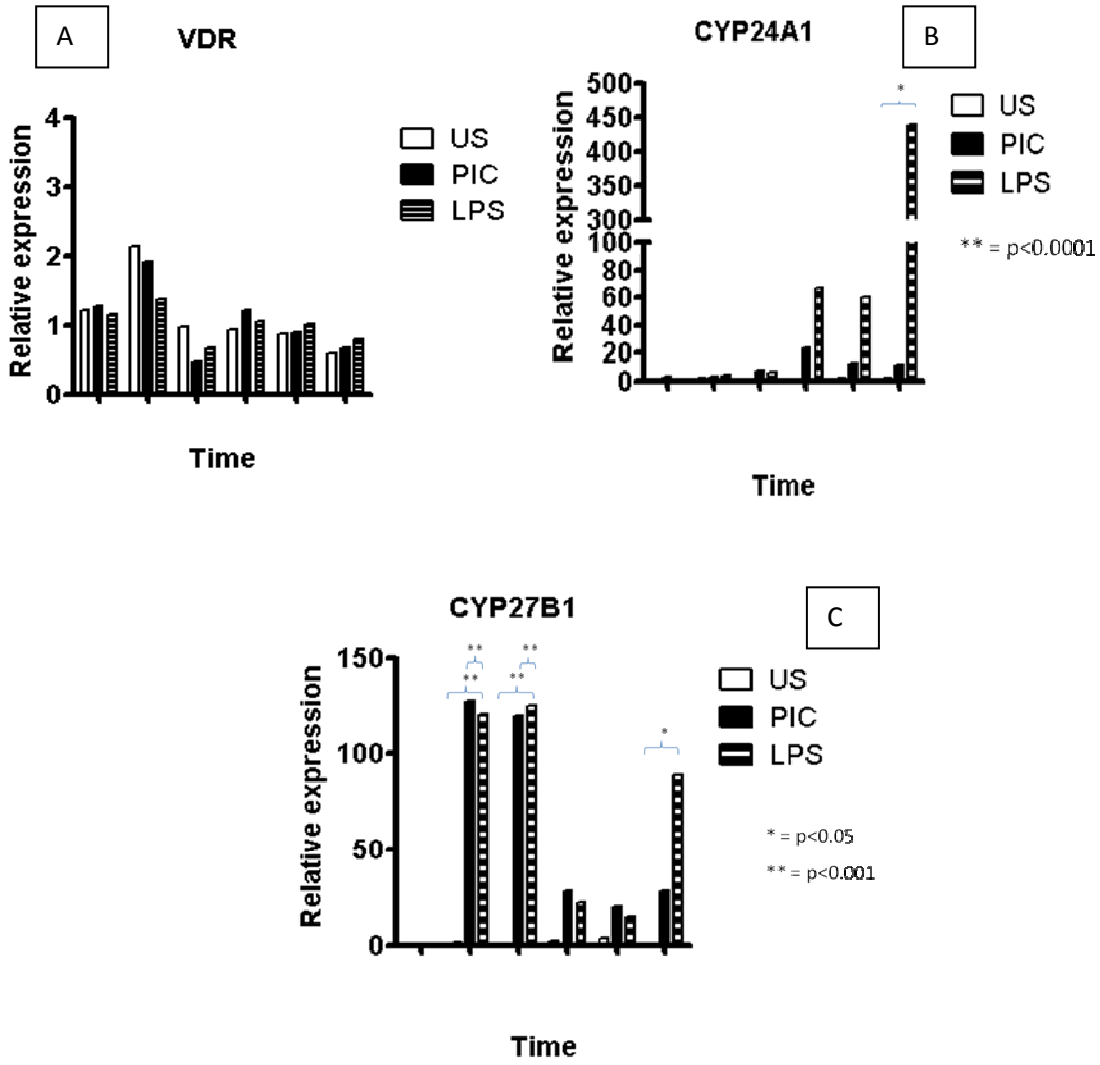

Figure 4: VDR expression (A) is not different in US RASF with respect to TLR activated RASF; TLR4 activation (stimulation with LPS) increased the expression of CYP27B1 and CYP24A1 after 72 hs (B, C). Both TLR3 (PIC) and TLR4 (LPS) induced CYP27B1 expression after 4 and 8 hours.

Conclusions and future plans:

During this preliminary phase of our study we managed to demonstrate that VDR and CYP27B1 are significantly expressed in human synovial tissue. According to the preliminary data of this study no specific differences were found between RA and OA patients in terms of VDR, CYP27B1 and CYP24A1 ex vivo. Further studies are required, since VDR seems to correlate with the degree of inflammatory cells infiltration (CD3+ and CD20+). The linkage between inflammatory arthritis and vitamin D metabolism could have been biased and veiled by the absence of clinical correlation about the source of the analyzed tissue, which represents the main limit of this study. In fact tissues belonged to joint replacement not to joint biopsy, leading to the collection of patients in different stage of disease, irrespectively to the treatment received and the possible vitamin D supplementation.

In the following phases of our study, we decided to define better the metabolism of vitamin D in the different cells implied in RA pathogenesis. We started from RASF, since IF data seemed to suggest a possible expression in this cell-type, which has not been described before.
We showed an increase in CYP27B1 and CYP24A1 gene expression after stimulation of TLR3 and TLR4 pathway. This result could contribute to give further evidence to the hypothesis that vitamin D can be activated and consumed locally in inflamed synovial tissue. The pathway involved is activated by TLR stimulation, as well as already demonstrated in macrophages. At present, our data are limited to gene expression, while further studies are required to confirm the protein expression and the functionality of the protein. If these data will be confirmed, we could have the rationale to exploit therapeutically local vitamin D conversion; in particular if oral cholecalciferol could be converted effectively in inflamed synovial tissue we could obtain a local immune regulation, without the systemic adverse effect on calcium metabolism.
In the near future, I aim to confirm the biological significance of these preliminary results. First of all I aim to demonstrate that CYP27B1 expression is not only enhanced by TLR activation at rt-PCR level; I have already obtained cell lysate from stimulated and unstimulated RASF cultured in presence of 25(OH) D. I will try to optimize and perform western blot to demonstrate the protein expression of CYP27B1 in activated synovial fibroblasts after TLR-activation; furthermore, I will quantify 1,25(OH)2D conversion by ELISA comparing the concentration obtained in TLR-activated fibroblasts in comparison with non stimulated synovial fibroblasts.

Furthermore, I aim to create a biological bank of synovial tissue which will be available for further studies. Specifically, tissues will be collected from joint replacement (either from OA or RA patients). I intend to isolate a sufficient amount of synovial tissue for histological and PCR studies, and to set up a technique for fibroblast isolation from these tissues.

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National and Internation congresses attended:

- 9th International Congress on Autoimmunity, Nice 26-30 March 2014.
- EULAR (European League Against Rheumatism) Annual International Congress, 11-14 June 2014.

Oral communications and posters to National and International congresses:

- "Identification of predictive factors of paraneoplastic polymyalgia rheumatica" Oral communication at 9th International Congress on Autoimmunity, Nice 26-30 March 2014.
- "A case of troponinitis in a patient affected by crioglobulinemic vasculitis". **Bellan M.;** Sainaghi P.P.; Sola D.; Rossi L.; Gentile M.; Merlotti E.; Pirisi M. Abstract at 9th International Congress on Autoimmunity, Nice 26-30 March 2014.
- "Autoimmune Disease and cancer: results from a retrospective cohort". Bellan M.; Sainaghi P.P.; Boggio E.; Sola D.; Merlotti E.; Rossi L.; Gentile M.; Pirisi M. Abstract at 9th International Congress on Autoimmunity, Nice 26-30 March 2014.
- "Autoimmune Disease and cancer: results from a retrospective cohort", Sainaghi P. P., E. Boggio, M. Gentile, E. Merlotti, D. Sola, R. Luca, M. Pirisi, **M. Bellan**. Abstract at EULAR (European League Against Rheumatism) Annual International Congress, 11-14 June 2014.
- "Vitamin D metabolism impairment in Rheumatoid Arthritis: implications for pathogenesis and treatment", Oral communication at EULAR (European League Against Rheumatism) Annual International Congress, 11-14 June 2014.
- "In morbid obesity the impairment of glucose tolerance is predicted by an increase of BMI and WC". E. Mossio; C. Ferrari; M. Menegatti; **M. Bellan;** G. P. Fra; G. P. Carnevale Schianca; M. Pirisi. Abstract at 7th National Congress of Italian Society of Obesiology, Milan, 2-5 July 2014.

Scientific publications:

- "Altered glucose metabolism rather than naive type 2 diabetes mellitus (T2DM) is related to vitamin D status in severe obesity." **Bellan M,** Guzzaloni G, Rinaldi M, Merlotti E, Ferrari C, Tagliaferri A, Pirisi M, Aimaretti G, Scacchi M, Marzullo P. Cardiovasc Diabetol. 2014 Mar 11;13:57.
- "Severe statin-induced rhabdomyolysis following cholestatic epatiti induced by amoxicillin–clavulanate". R. Rapetti, E. Merlotti, **M. Bellan,** G. P. Carnevale Schianca, M. Pirisi. EJCRIM 2014;1:doi:10.12890/2014_000065.

Seminars attended:

- "Gene Therapy application", Prof. Follenzi, 14th July.
- "The Borghese Sessions", Prof. Ellis.