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TITLE

Vitamin D metabolism impairment in Rheumatoid Arthritis: implications for pathogenesis and treatment.

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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 and at Internal Medicine Lab, in Novara. 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).

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).

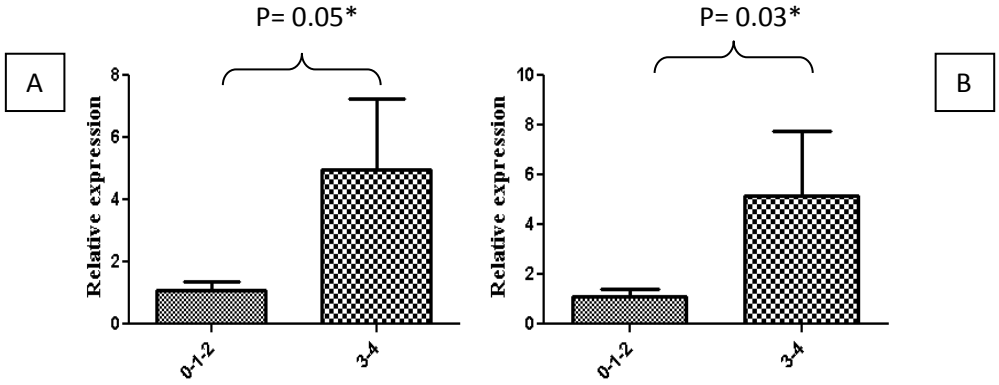

Figure 1: Correlation between histological infiltration of CD3+ T cells (panel A) and CD20+ B cells (panel B) and relative expression of VDR. *Mann-Whitney test.

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).

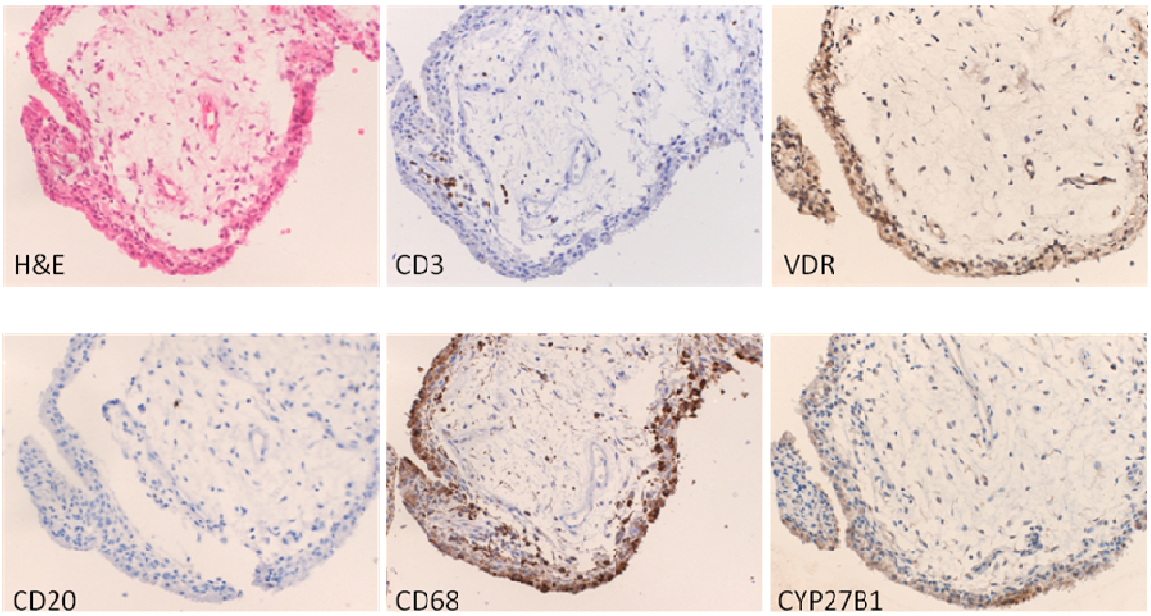

Figure 2: the panels show the staining for H&E, CD3+ T cells, VDR+ cells, CD20+ B cells, CD68+ macrophages and CYP27B1+ cells in synovial tissue.

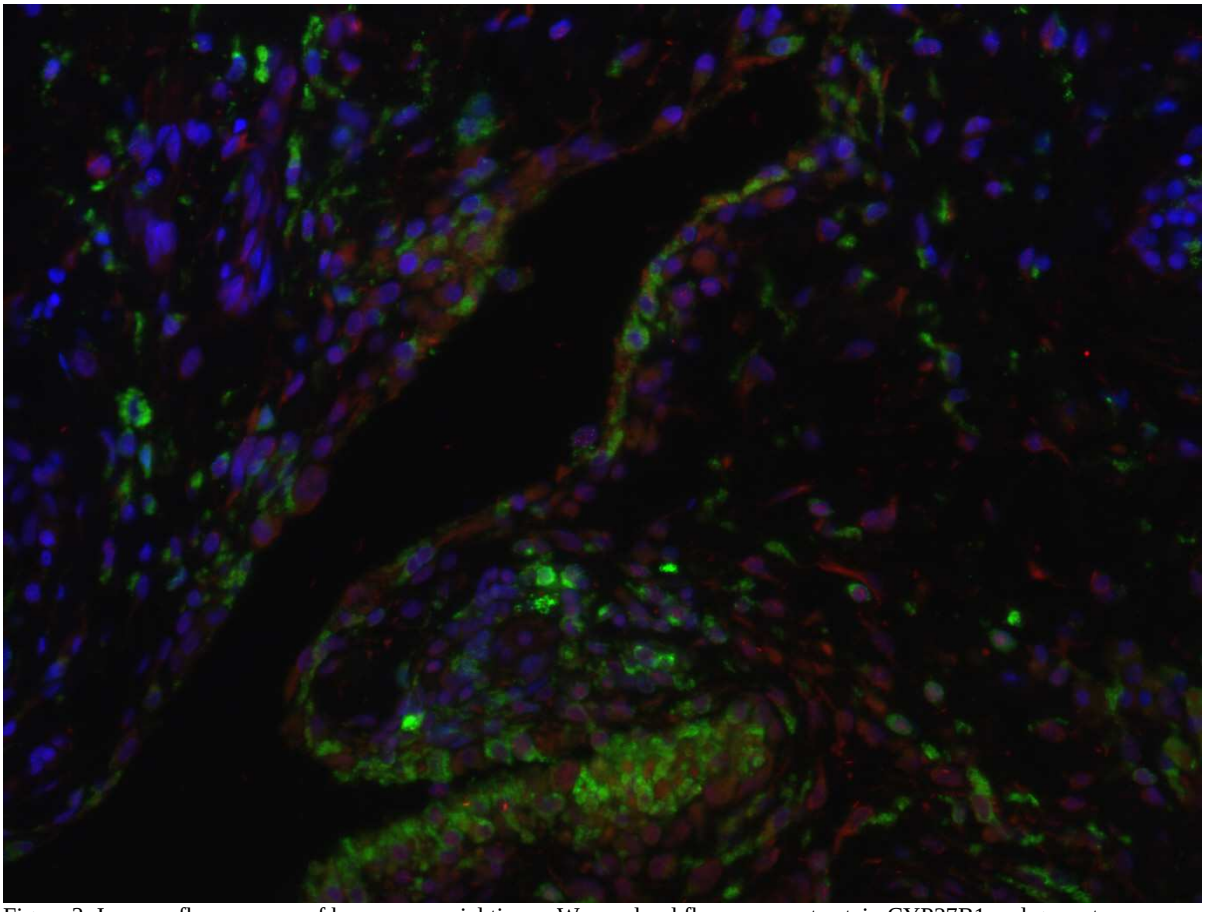

Figure 3: Immunofluorescence of human synovial tissue. We used red fluorescence to stain CYP27B1 and green to identify CD68.

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).

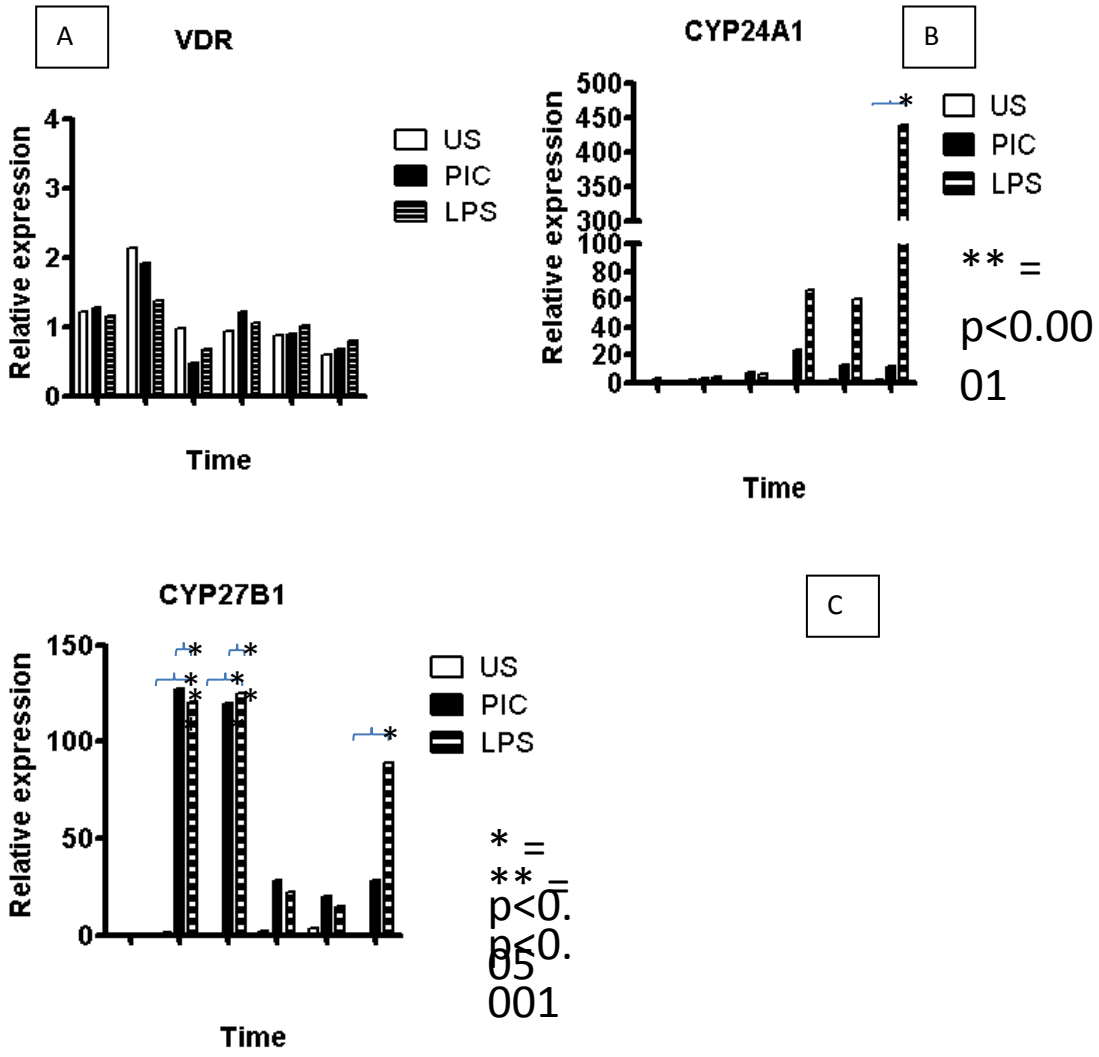

Figure 4: VDR expression (A) is not different in US RASF with respect to TLR activated RASF; TLR4 activation (stimulation with LPS) increased the expression of CYP27B1 and CYP24A1 after 72 hs (B, C). Both TLR3 (PIC) and TLR4 (LPS) induced CYP27B1 expression after 4 and 8 hours.

We also tried to confirm the protein expression of CYP27B1 in unstimulated and stimulated RASF. As shown in Fig. 5 we obtained a staining for CYP27B1, which was not influenced by LPS or PIC stimulation. Anyway the reliability of this staining is still to be defined since the expected localization of the target protein was in cells cytoplasms (mitochondria) while we obtained a nuclear staining. Furthermore, using a mitochondrial marker we did not observed the expected co-staining (Fig.6).

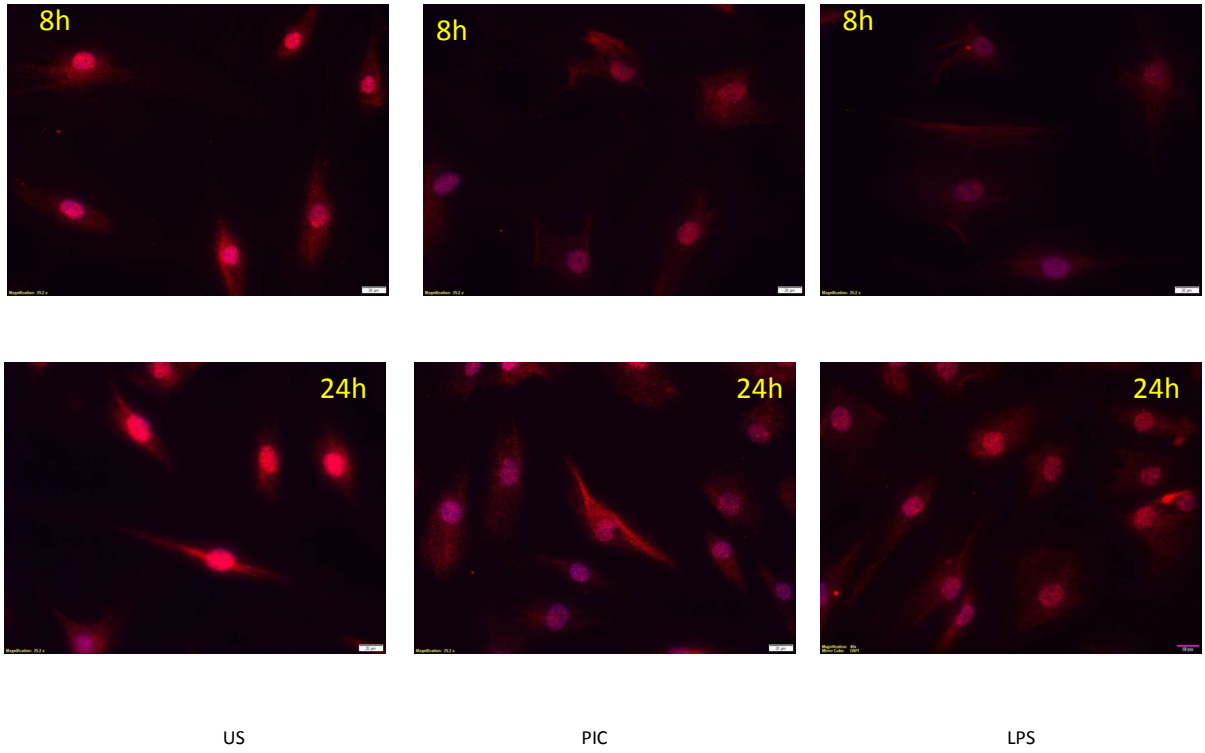

Fig. 5: CYP27B1 Expression in Unstimulated and Stimulated SF.

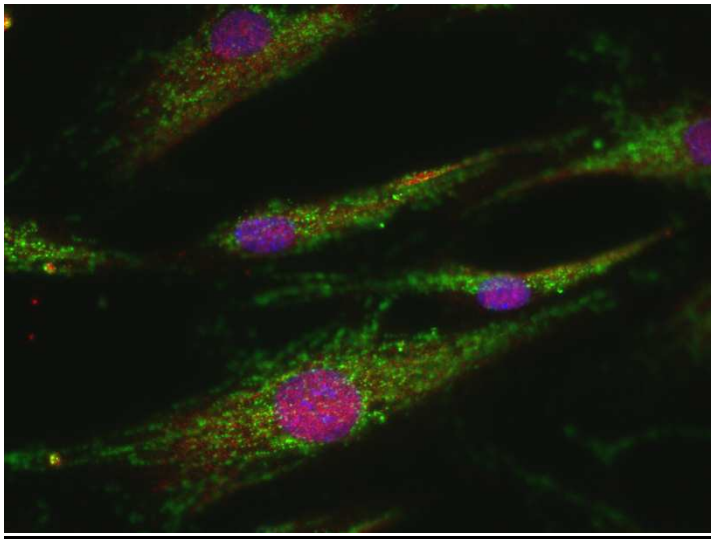

Fig. 6: CYP27B1 (red dots)- mitochondria (green) co-staining.

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, we managed to demonstrate a gene expression induction, but we failed to obtained a reliable demonstration of protein expression. in the next 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.
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.

Other projects:

During this first year I have also participated to the following projects:

- Activation of Novara Biobank: we started a collaboration with Prof. Grassi (SC Ortopedia e Traumatologia), collecting synovial samples obtained from joints explant for knee and hip prosthesis. Samples for RNA histopathological analysis have been collected to create a local bank of biological materials for future analysis, aiming to better identify the mechanisms underlying the pathogenesis of osteoarthritis and inflammatory arthritis. At the moment we collected samples from 8 patients.
- Furthermore, we have obtained the Ethical Committee approval for the activation of Novara as recruiting center for the R4RA, a double-blinded RCT coordinated by Prof. Pitzalis. The main objective of the trial is to identify potential hystopathological predictor of response to either Rituximab or Tocilizumab in RA patients who experienced an anti-TNF failure. Samples will be collected by a mini-invasive US-guided, synovial biopsy. We have just started the recruitment at our site and a total number of 3 to 5 patients are expected to be recruited by the end of the year. A first patient has been recruited and the first monitoring visit has been performed with good feedback.

References:

1. van Etten, E.; Mathieu, C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. J Steroid Biochem Mol Biol 2005, 97, 93-101.
2. Helming, L.; Böse, J.; Ehrchen, J. Et al. 1alpha,25-Dihydroxyvitamin D3 is a potent suppressor of interferon gamma-mediated macrophage activation. Blood 2005, 106, 4351-8.
3. Lemire, J.M. Immunomodulatory role of 1,25-dihydroxyvitamin D3. J Cell Biochem 1992, 49, 26-31.
4. Griffin, M.D.; Lutz, W.H.; Phan, V.A. et al. Potent inhibition of dendritic cell differentiation and maturation by vitamin D analogs. Biochem Biophys Res Commun 2000, 270, 701-8.
5. Sloka, S.; Silva, C.; Wang, J.; Yong VW. Predominance of Th2 polarization by vitamin D through a STAT6-dependent mechanism. J Neuroinflammation 2011, 24, 56.

6. Cippitelli, M.; Fionda, C.; Di Bona, D. et al. Negative regulation of CD95 ligand gene expression by vitamin D3 in T lymphocytes. J Immunol 2002, 168, 1154-66.
7. Tetlow, L.C.; Smith, S.; Mawer, E.B.; Woolley, D.E. Vitamin D receptors in the rheumatoid lesion: expression by chondrocytes, macrophages, and synoviocytes. Ann Rheum Dis 1999, 58, 118-21.
8. Song, G.G.; Bae, S.C.; Lee, Y.H. Association between vitamin D intake and the risk of rheumatoid arthritis: a meta-analysis. Clin Rheumatol 2012, 31, 1733-9.
9. Cutolo, M.; Otsa, K.; Laas, K. et al. Circannual vitamin d serum levels and disease activity in rheumatoid arthritis: Northern versus Southern Europe. Clin Exp Rheumatol 2006, 24, 702-4.
10. Patel, S.; Farragher, T.; Berry, J.; Bunn, D.; Silman, A.; Symmons, D. Association between serum vitamin D metabolite levels and disease activity in patients with early inflammatory polyarthritis. Arthritis Rheum 2007, 56, 2143-9.
11. Zehnder, D.; Bland, R.; Williams, M.C. et al. Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. J Clin Endocrinol Metab. 2001, 86, 888-94.
12. Sainaghi, P.P.; Bellan, M.; Antonini, G.; Bellomo, G.; Pirisi, M. Unsuppressed parathyroid hormone in patients with autoimmune/inflammatory rheumatic diseases: implications for vitamin D supplementation. Rheumatology 2011, 50, 2290-6.

Oral communications and posters to National and International congresses:

- Synovial immunopathology defines clinical responsiveness to DMARDs therapy in early psoriatic arthritis: a pre- and post-treatment mechanistic study using a minimally invasive ultrasound-guided synovial biopsy procedure. W.S. Tan, **M. Bellan,** A. Nerviani, M. Di Cicco, A. Mahto, I. Lazarou, R. Hands, F. Humby, S. Kelly, C. Pitzalis. Poster at EULAR (European League Against Rheumatism) Annual International Congress, Rome 2015.

- Oral glucose tolerance test alteration is a better predictor of cardiovascular morbidity than metabolic syndrome in general population. Menegatti M., **Bellan M.,** Nicosia F., Gentile M., Mossio E., Merlotti E., Pogliani G, Apuzzo R., Grossi G., Fra GP., Carnevale Schianca GP. American Diabetes Association's 75th Scientific Sessions. Boston 2015.

Scientific publications:

- Bellan M, Pirisi M, Sainaghi PP. Osteoporosis in Rheumatoid Arthritis: role of the vitamin D/parathyroid hormone system. Rev Bras Reumatol. 2015 May-Jun;55(3):256-63.

- Bellan M., Pirisi M., Sainaghi P.P. Long term remission of corticosteroid and cyclophosphamide resistant Henoch-Schonlein Purpura with Rituximab. Scand J Rheumatol. 2015 Aug 27:1-2.

Seminars attended:

- July 14, 2014 Gene Therapy application (Prof Follenzi)

- The Borghese Sessions (Prof Steven R Ellis)

    - September 8, 2014 Skin as an organ; Layers of skin, cell types, developmental origins
    - September 9, 2014 Cell-Cell Interactions – anchoring junctions and occluding junctions, tight junctions
    - September 11, 2014 Angiogenesis; Innervation

  - September 15, 2014 Basal layer stem cells, symmetric versus asymmetric divisions, transient amplifying cells; Solar radiation, nucleotide excision repair
  - September 16, 2014 Basal and squamous cell carcinomas; Melanoma – biology
  - September 17, 2014 Melanoma – treatment; Contact dermatitis
  - September 22, 2014 Other skin disorders, Other components of skin

- November 14, 2014 Dott.Boccafoschi "Tissue engineering: the state of the art"
- November 21, 2014 Prof. Prat "Stem cell in the regeneration and repair of the tissues and organs"
- December 4, 2014 Prof. Girish Patel "Uncovering the role of β-HPV in field cancerization: a collaboration in progress"
- December 16, 2014 Prof. Antonio Musarò "From the legend of Prometheus to regenerative medicine"
- January 19, 2015 Prof. Dr Yong-Sang Song "Anticancer strategy Targeting cancer cell metabolism in ovarian cancer"
- January 20, 2015 Dr Tonino Alonzi "Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states"
- January 21, 2015 Prof. Valeria Poli "Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis"
- January 27, 2015 Prof. Antonio Sica "Myeloid cells as therapeutic target in cancer"
- April 9, 2015 Prof Zhong"Signal control in iNKT cell development and function"
- April 21, 2015 Prof. Percipalle "Actin-based mechanisms in the control of gene expression and cell fate"
- May 7, 2015 Prof John McDonald "An Integrated Approach to the Diagnosis and Treatment of Ovarian Cancer"
- June 5, 2015 Dr Feltkamp "Recent Developments in (cutaneous) Human Polyomavirus Research"
- June 10, 2015 Prof Filigheddu "Basis of scientific research"
- September 3, Dr. Boshnakovski "Cell based models for studying molecular mechanism of FSHD" and "Toward animal model for FSHD".