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# Reelin levels are increased in synovial fluid of patients with rheumatoid arthritis

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## ABSTRACT

**Objectives.** *To evaluate the presence and the glycosylation pattern of reelin in synovial fluid and serum of patients affected by different rheumatic pathologies.*

**Methods.** *Reelin levels were evaluated in patients affected by rheumatoid arthritis (RA), psoriatic arthritis (PsA), spondyloarthritis (SpA) and osteoarthritis (OA). Reelin semi-quantitative assays were performed by western blot. The glycosylation pattern was evaluated by immunoblotting performed by sepharose conjugated lectins. RT-PCR was used to detect the presence of mRNA encoding for reelin and its receptors.*

**Results.** *Reelin is detectable in both sinovial fluids and sera and its levels are more elevated in patients affected by RA with respect to those affected by other inflammatory and non inflammatory joint diseases. The glycosylation pattern of the protein differs in synovial fluid and serum. Fibroblast-like synoviocytes (FLS) express the mRNAs encoding for reelin and its receptors.*

**Conclusions.** *Since its levels are higher in RA then in the other analysed pathologies, reelin can represent a candidate suitable for the differential diagnosis of this pathology. Moreover, the observation that this protein is encoded by FLS and differentially glycosylated in blood and synovial fluid supports the hypothesis that it is locally produced in the joints, where it could play an important role in RA development and maintenance.*

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints, affecting 1% of the western population. The rheumatoid synovium includes a special cell population, constituted by activated fibroblast-like synoviocytes (FLS) that distinguish RA from other inflammatory disorders of the joints (1). FLS produce a variety of cytokines and metalloproteases, providing for cartilage degradation. Different studies have been focused on the role of metalloproteases in the pathogenesis of inflammatory arthritis and on their possible use as markers allowing to differentiate RA from other pathologies. So far, none of them has resulted suitable to this aim (2).

Reelin is a 410kDa protein with the double function of serine protease (3) and ligand of the two receptors ApoER2 and VLDLR (4). LDL family of receptors are not only involved in cholesterol transport and metabolism, but also in modulation of hippocampal synaptic plasticity and normal learning and memory development. Moreover, the ability to endocytose cellular nutrients, clear extracellular matrix proteins, transduce signals from multiple ligands and activate numerous signal transduction pathways truly places this family of receptors in a very exclusive class of multifunctional receptor proteins (3-5). Reelin is expressed in the central nervous system, liver, adrenal chromaffin cells and pituitary pars intermedia; moreover, its presence can be detected in the blood (5).

Reelin signalling is impaired in autism (6), whereas its downmodulation has been found in the brain of patients with schizophrenia (7). In other disorders, such as Alzheimer's disease, reelin levels in the cerebrospinal fluid are increased. Moreover, in these patients, the reelin glycosylation pattern differs in cerebrospinal fluid and blood, supporting the hypothesis that the molecule is locally produced in the central nervous system (8).

In our study, we evaluated the levels and glycosylation pattern of reelin in synovial fluid and serum obtained from patients affected by different joint diseases.

## Methods

*Sample collection and storage*

Synovial fluids obtained by arthrocentesis from 2 patients affected by RA, spondyloarthritis (SpA), psoriatic arthritis (PsA) and osteoarthritis (OA) were collected and centrifuged to eliminate the cellular content. Table I shows the drug treatment of the patients of the study. After centrifugation, the supernatants were aliquoted and stored at -80°C. Blood samples were collected at the same time of arthrocentesis. All patients fulfilled the American College of Rheumatology revised criteria for RA

**Table I.** Demographic, ERS and therapy of patients studied.

| | Gender | Age | ESR | Treatment |
|---|---|---|---|---|
| PsA | Female | 56 | 10 | MTX |
| PsA | Male | 51 | 25 | AINS |
| RA | Female | 73 | 36 | Etanercept |
| RA | Female | 75 | 120 | MTX |
| RA | Female | 72 | 53 | MTX |
| SpA | Male | 49 | 3 | No therapy |
| SpA | Female | 30 | 18 | No therapy |
| OA | Female | 75 | 28 | Steroid |
| OA | Female | 57 | 9 | No therapy |

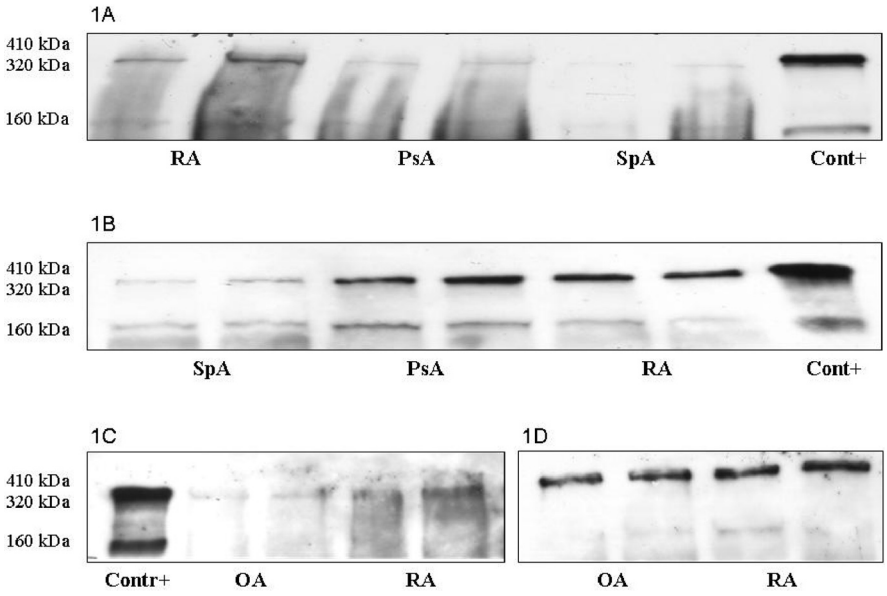


**Fig. 1.** Serum and synovial fluid from RA patients are characterized by higher reelin levels respect to other inflammatory and non inflammatory pathologies. Equal volumes of synovial fluid (**A**) or serum (**B**) obtained from patients affected by RA, PsA and SpA were loaded, together with recombinant reelin used as a positive control. Then, a western blot was performed by using the antibody 142. Synovial fluid (**C**) and serum from patients with OA and RA were run. The western blots were performed using the same antibody.

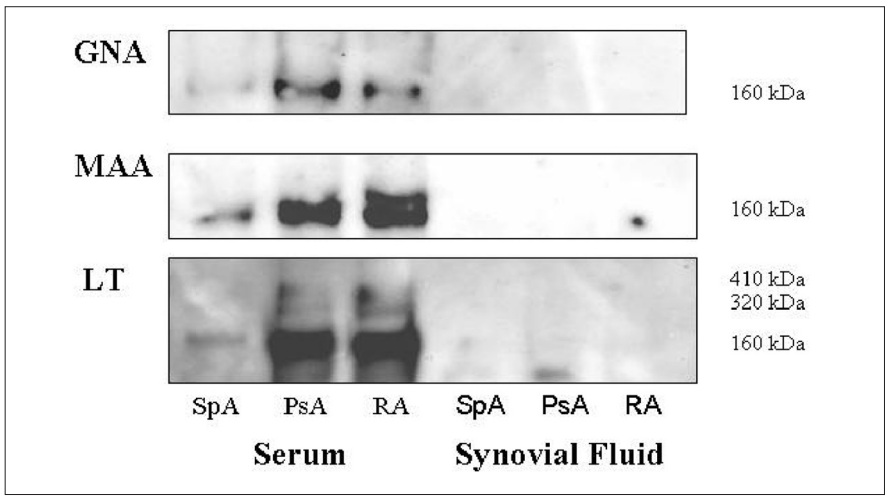


**Fig. 2.** Reelin is highly glycosylated in serum, but not in synovial fluid. Sepharose conjugated lectins GNA, MAA and LT were used to pull down the glycosylated fraction of proteins in serum and synovial fluids of patients affected by SpA, PsA and RA. Then, the western blots were performed by using the antibody 142.

and OA (9, 10), the European Study group for spondyloarthropathy criteria for SpA (11) and the CASPER criteria for PsA (12) and gave their informed consent prior to study entry. The ethics committee of the Arcispedale Santa Maria Nuova approved all the experiments on human cells and tissues.

*Western and immunoblot*

Western blots were performed as previously described (13) using the antibody 142 (kindly donated by Dr Lugli Giovanni). As a positive control, recombinant reelin (kindly donated by Dr Lugli Giovanni) was loaded. Immunoblots were performed by conjugating the three lectins (EY Laboratories, San Mateo, CA, USA) with sepharose 4B (Amersham Biosciences, Amersham, UK). Equal amount of proteins were then pulled down using the conjugated lectins. The detection was performed by using the anti-reelin antibody. The equal loading was proved by Red Ponceau (Sigma-Aldrich, St. Louis, MO, USA) staining of the membrane.

The glycosylation pattern of reelin was analysed by pulling down reelin by the sepharose conjugated lectins. Galanthus nivalis (GNA), maackia amurensis (MAA) and lotus tetragonolobus (LT) were used to detect mannose, alpha (2-3) linked sialic acid and fucose residues, respectively. Then, the primary anti-reelin antibody was used for the detection.

*Semi-quantitative analysis*

For semi-quantitative studies, the intensity of bands detected by Western blotting was measured by densitometry using x-ray films exposed to ECL reagents and the Scion Image software (14).

*Cell culture*

FLS cultures were set up as previously described (15, 16). For FLS obtained from synovial fluid, the positive staining with the anti-fibroblast antibody and the lack of staining with CD68 antibody were proved by immunocytochemistry.

*RT-PCR*

Retrotranscription and PCR were performed following standard procedures

(17). To detect reelin, VLDLR and ApoER2 the following primers were used: CCTCACCAACACAACTCGACTTCG (forward), TGGTGGGTTGTGAAGCCACTTCTT (reverse) for reelin; CTGGATAGATGGGGAAAATGA (forward), TTTGACAGTCTCGGCCATTT (reverse) for VLDLR and GATTGCGAAAAGGACCAATT (forward), TAGCACAGCCGGCCTCAT (reverse) for ApoER2.

## Results

Reelin was detected by Western blot in synovial fluids from patients affected by RA, PsA and SpA. The densitometric quantification showed that the protein levels in RA patients were 5-fold higher with respect to PsA patients and 10-fold with respect to SpA patients (Fig. 1A). A similar trend of reelin levels was found in sera obtained from the same patients. In these samples, a three- and a six-fold decrease were found respectively in sera from PsA and SpA with respect to RA patients (Fig. 1B). Moreover, a three-fold increase was found in both synovial fluid (Fig. 1C) and serum (Fig. 1D) of RA with respect to OA patients.

We then studied the glycosylation pattern of reelin. In the serum, the 160kDa form contained mannose and sialic acid residues, whereas all the three forms were fucosylated. On the contrary, synovial fluid reelin did not contain any of these residues (Fig. 2).

Reelin mRNA was detected in fibroblast-like synoviocytes obtained from patients with the four analysed pathologies. Moreover, in the same cells, also the mRNA encoding for the two reelin receptors, ApoER2 and VLDLR, were identified.

## Discussion

In our study we showed, to our knowledge, for the first time that reelin concentration is higher in synovial fluids and sera of patients with RA with respect to the other studied pathologies. Our finding could be important for a differential diagnosis, since so far it has not been found any other protease increased specifically in RA patients. A higher number of samples though would be needed to support our preliminary results. Nevertheless, the difference we have detected is striking.

Then we evaluated the possibility that reelin could be produced in the synovial environment. To this aim, we studied its glycosylation pattern and showed that it differs in serum with respect to synovial fluid. This difference could be due to the presence of different glycosidases in the synovial environment. We cannot exclude that the changes in either level or glycosylation of reelin are due to different treatment that patients with distinct pathologies have received.

We have shown the presence of mRNA coding for reelin and its receptors in FLS. This finding supports the possibility that reelin is produced by FLS in the synovia and the increased levels in RA synovial fluid could be due to the increased number of these cells. Reelin binding to its receptors induces the activation of PI3K and src kinase pathways and the cytoskeletal reorganisation leading to neuronal migration (18), which can at least in part explain its activity in neuronal migration. In the same way, it could also participate in FLS migration. Since reelin is involved in neuron migration, it could also play a role in FLS migration.

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