

**Network: Infection and Inflammation: from Pathogen-induced Signatures to Therapeutic Target Genes**

**Project: Signatures and Clinical Validation: Synovial Tissues, Tissue Microarrays and Tissue Cells**

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### Introduction

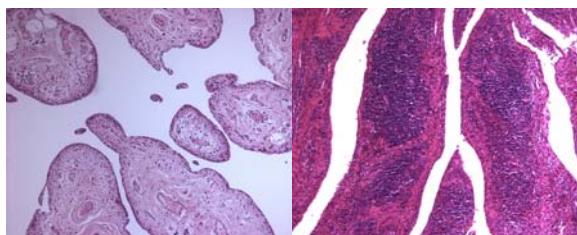
Inflammation of joint synovium is the central characteristic in all arthritides. Rheumatoid arthritis (RA) is the most common of these joint diseases and presents typical histomorphological changes. One is the thickening of the synovial lining layer with accumulation of macrophages and fibroblasts, its tumour-like expansion and the formation of the so-called pannus tissue that spreads out onto the surface of cartilage, destroys its matrix and aggressively invades into the joint surface. Another characteristic is the invasion of B- and T-cells into the site of inflammation, facilitated and enhanced by marked neovascularisation and stromal activation. These infiltrated immune cells may organize together with dendritic cells in a way that resembles lymphoid follicles in the lymphnode and could be one of the leading mechanisms that perpetuate inflammation.

All these phenomena have advanced rheumatoid synovitis to a prime example of chronic inflammation which has been investigated now for decades in basic and clinical immunology. Although the etiology of RA and other chronic arthritides has remained undefined, the pathomechanisms of the inflammatory network have been elucidated and new drugs interfering as biologics with proinflammatory cytokines or co-stimulators have increased the armamentarium to treat these diseases with increasing success. Despite these advances, the disease has a stable incidence, cannot be cured and remissive treatment is restricted to less than 50% of the patients. Furthermore side effects of excessive immunosuppression are increasing, indicating the need for higher specificity.

### Results/Project Status

#### Histopathological Scoring of Inflammation

Our goal is a systematic analysis using genome-wide expression profiling in an attempt to unravel the molecular network comprehensively. As a pre-screening technology, a histopathological scoring was developed to characterise the investigated samples by alternative techniques. This scoring is based on standard techniques similar to those used for tumour grading. It was established with synovial biopsies from rheumatoid arthritis, osteoarthritis and normal joints. The tissue samples were collected as small biopsies from arthroscopic intervention, as tissue samples from open surgery and synovectomy or from the tissue bank for normal controls. The scoring was based on (1) the enlargement of the lining cell layer, (2) the activation of the synovial stroma and (3) the lymphocytic inflammatory infiltrate (Fig.1).



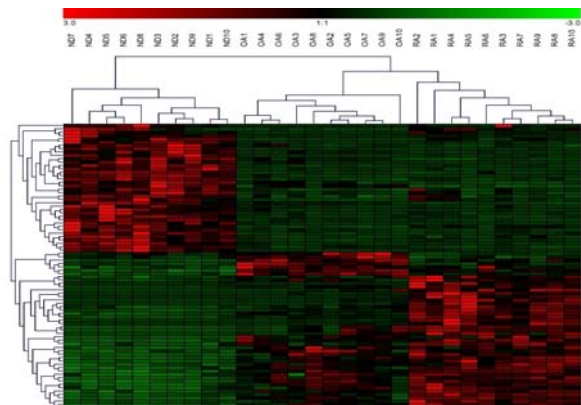
**Fig 1:** Examples for a low-grade (synovitis-score 2 points) and for a high-grade (8 points) synovitis.

Semi-quantitative scoring points from 0 to 3 for each of these three features revealed a cumulative score of up to 9 points.

0 and 1 point reflect no synovitis, 2 to 4 points correspond to a low-grade and 5 – 9 points to a high-grade synovitis. More than 700 specimens of synovial tissues from osteoarthritis (OA), inflammatory joint diseases like RA, reactive arthritis, as well as synovial tissue from healthy individuals have been analysed. RA revealed significantly higher grades ( $p < 0,0005$ ) than OA and healthy controls. This score developed as a valuable and indispensable prerequisite for standardized characterisation of synovial tissues prior to molecular analyses [1].

#### Gene Expression Profiling of Inflammation

Based on the score, 10 different synovial tissue samples each from normal joints, patients with OA, as well as RA were selected for gene expression profiling. A broad spectrum of scores was allowed in the RA and OA groups in favour to identify disease-specific instead of scoring related genes. Differentially expressed genes between all three groups revealed correct clustering of all samples into the three corresponding groups (Fig. 2).



**Fig 2:** Clustering of RA, OA and normal synovium with 85 differentially expressed genes

Classification into RA and non-RA was even possible with a few individual genes. A limited number of these genes was selected for predictive classification and could be confirmed by a first blinded analyses of 4 independent samples.

Comparing the RA synovial expression level of differentially expressed genes with the corresponding expression level in highly purified leukocyte subpopulations from normal donors revealed that most of these genes were constitutively present in one or more of these leukocyte populations. Thus, most of these genes were at least in part related to the infiltration process known from histopathological analysis. In collaboration with the projects on cell sorting and bioinformatics, a new algorithm was established to perform a functional profile components analysis (FPCA). Using marker genes this technique permits the identification of each type of immune cells in the synovial tissue. Subsequently, a virtual profile is calculated to create a sample-specific normal control according to its cellular composition. Comparing each sample with its individual virtual control allows us to select for those genes that are differentially expressed.

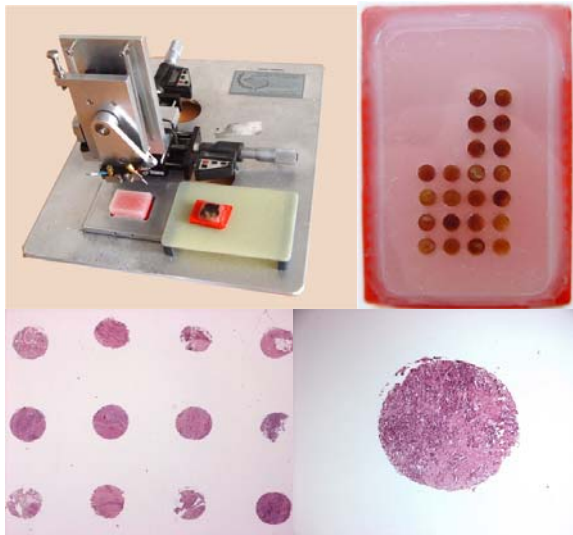
To evaluate importance and specificity of the selected candidate genes for synovitis, tissues with similar or related

pathology including inflamed tissue from prosthetic loosening (PL) and giant cell tumours (GCT) were analysed. Application of the FPCA algorithm demonstrates differences in the cellular composition compared to synovium with a larger fraction of monocyte/macrophage related profiles in both non-infectious PL and GCT while infectious PL seems to be dominated by fibroblasts. These inflammatory and tumour-like aspects of synovitis will be further investigated and comparisons with other tissues including solid tumours are planned.

### Histopathological Validation of Candidate Genes

To validate candidates selected by the differential expression analysis, immunohistological analyses were performed on selected markers of chemoattraction, immune activation and tissue destruction. All were also expressed at the protein level and thus confirmed the mRNA expression data. Both results underline a potential role of these molecules in the inflammatory and destructive process.

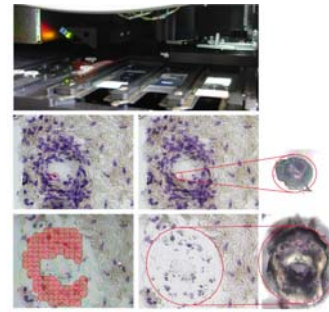
For advanced validation and analysis, the technology of tissue microarray (TMA) was established. A TMA is a paraffin block where cylindrical shaped tissue samples from many donors are embedded in an array-like manner (Fig.3). More than 100 slides can be produced from such a TMA for testing selected candidate genes. A single slide contains up to 50 different samples and can be stained in a single step. Thus, the validation of numerous candidate genes generated by gene expression profiling can be executed in a high-throughput manner.



**Fig 3:** Macroscopic and microscopic aspect of tissue microarrays.

### Microdissection and Analysis of Gene Expression

Histomorphological changes in rheumatoid synovitis suggest that differences between lining layer, infiltration areas and stroma may be substantial and that each area may contribute to pathology depending on the type of cellular interaction and function. Using Laser capture microdissection (Veritas, Arcturus) specific areas are isolated (Fig. 4) and the differential gene expression analysed by microarray technology. Dissection of single cells allows to determine gene expression profiles for selected candidate genes by real time RT-PCR.



**Fig 4:** Laser capture micro-dissection of the perivascular area from a RA synovial tissue section.

### Functional Assays and Therapeutic Impact

Analysis for functional profile components has demonstrated that a profound knowledge about gene activity in a given cell type under a defined stimulus is essential for the interpretation of complex expression profiles. Therefore, the different cell types in synovial tissues were separated into fibroblasts and non-adherent cells. Each population is currently investigated separately and will be compared against each other and against total synovium. This may allow to identify chronically imprinted signatures related to RA. Comparison of fibroblast cell lines derived from normal joints or RA synovium revealed stable differences which are in accordance with a proinflammatory phenotype in RA. These differences could be in part reverted by incubation with glucocorticoids. Further experiments are currently being performed to determine to which extend the different proinflammatory mechanisms contribute to the observed expression profiles.

### Outlook

Synovial expression profiles revealed candidate genes that may qualify for molecular classification criteria. In collaboration with the clinical project, a diagnostic study is currently performed to confirm these criteria for diagnostic purposes. Furthermore, FPCA and functional studies will narrow in to the most promising candidate genes that may be key players in the perpetuation of the inflammatory process. Using the SCID mouse *in vivo* model of rheumatoid arthritis [3], the functional importance will be experimentally validated and therapeutic strategies tested. This project is performed as a collaborative work between Claudia Berek (Deutsches RheumaForschungsZentrum, Berlin), Thomas Häupl (Charité, Berlin), Raimund Kinne (University Jena), Veit Krenn and Lars Morawietz (Charité, Berlin), Ulf Müller-Ladner (University Gießen), Josef Zacher (Helios Klinikum Berlin / Buch) and the Oligene GmbH, Berlin.

**Lit.:** 1. Krenn V et al. Grading of chronic synovitis--a histopathological grading system for molecular and diagnostic pathology. *Pathol Res Pract.* 2002;198:317-25. 2. Häupl T, Krenn V, Stuhlmüller B, Radbruch A, Burmester GR. Perspectives and limitations of gene expression profiling in rheumatology: new molecular strategies. *Arthritis Res Ther.* 2004;6:140-6. 3. Fiehn C, Neumann E, Wunder A, Krienke S, Gay S, Müller-Ladner U. Methotrexate (MTX) and albumin coupled with MTX (MTX-HSA) suppress synovial fibroblast invasion and cartilage degradation *in vivo*. *Ann Rheum Dis.* 2004 Jul;63:884-6. 4. Stuhlmüller B, Kunisch E, Franz J, Martínez-Gamboa L, Hernández MM, Pruss A, Ulbrich N, Erdmann VA, Burmester GR, Kinne RW. Detection of oncofetal h19 RNA in rheumatoid arthritis synovial tissue. *Am J Pathol.* 2003 Sep;163:901-11. 5. Kim H-J, Berek C. Single cell analysis of synovial B cells. *Methods Mol Biol.* 2005 in press.