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Lessons from Genomic Profiling in AS

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1. Introduction

Ankylosing Spondylitis (AS) is a common cause of chronic inflammatory arthritis worldwide, with a prevalence of 0.2-0.9% in white European populations (Braun *et al.*, 1998), with unknown etiology. The progressive ankylosis of affected joints is currently irreversible and it is, therefore, logical that early diagnosis and treatment offers the best opportunity to improve its prognosis. Several studies have shown a delay of more than 8 years between the onset of symptoms and diagnosis, with consequent delay in starting an effective therapy (Feldtkeller *et al.*, 2003; Hamilton *et al.*, 2011). This is a critical period clinically, with diagnosis frequently occurring after significant irreversible radiological damage has already occurred. Currently, diagnosis of AS relies on a combination of clinical and imaging parameters (van der Linden *et al.*, 1984 and Boonen *et al.*, 2010) with no single blood derived biomarker that by itself is sufficiently sensitive and specific to identify AS cases or to be useful in disease management.

In this context, recent advances in molecular biology, in particular, the completion of the genome human sequence, the improvement in computational tools and the rapid access to large databases, allow an integrated understanding of biological systems, through “omic” approaches. The main challenge, however, is to extract relevant knowledge from the huge amount of data provided by these technologies for the development of biomarkers for diagnosis, prognosis, therapy monitoring and both prediction and monitoring of treatment response. Such technological advances represent the beginning of patient-specific personalized medicine (Kandpal *et al.*, 2009).

In contrast to traditional DNA-based diagnostic tests that largely focus on single genes associated with rare conditions, microarray-based genotyping and expression assays are ideal for the study of diseases with underlying complex genetic causes (Li *et al.*, 2008). Microarray gene expression technology can be used for the detection and quantification of differentially expressed genes. Its ability to study expression of several thousand genes or even all of the genes of the entire genome in a single experiment has changed biomedical research. Gene-expression profiling confers a “snapshot” of cellular activity providing information on the mechanisms mediating stress responses of human cells (Belcher *et al.*, 2000; Guillemin *et al.*, 2002), identification of signaling cascades (Shaffer *et al.*, 2000; Diehn *et*

al., 2002), disease changes, or mechanisms underlying therapy responses (Raetz & Moos, 2004). It represents an advance to the traditional molecular genomic techniques that have been previously applied in a large broad of clinical research as cancer, infections, metabolic, genetics and more recently, in rheumatic diseases.

1.1 Microarray fundamentals

Gene expression techniques, based on measuring mRNA levels, have greatly evolved since the development of the Northern Blot, in 1975 (Southern, 1975) to microarrays, in the mid 1990s (Shalon *et al.*, 1996). From a single labeled mRNA (probe), hybridized on a membrane (Northern Blot), to multiple probes hybridized on a membrane (macroarrays) or on glass (microarrays), the improvement was tremendous. Today several platforms, with pre-designed and custom arrays are available in the market (Hardiman, 2004) from Affymetrix, Agilent and Illumina. Table 1 summarizes similarities and differences between the most widely used platforms.

Platforms			
Affymetrix		Agilent	Illumina
Array format	25-mer	60-mer	50-mer
Starting RNA requirement	5µg total RNA	Fluorescent Direct Label Kit (cDNA labeling): 10µg total RNA, or200ng polyA+ RNA Low input RNA Fluorescent Linear Amplification kit (Amplified cDNA labeling): 50ng total RNA Low input RNA Fluorescent Linear Amplification kit (Amplified cRNA labeling): 50ng total RNA	50-500ng total RNA
Hybridization time	16h	Fluorescent Direct Label Kit: 3-4 hours Low input RNA Fluorescent Linear Amplification kit Amplified cDNA labeling: 10 hours Amplified cRNA labeling: 6 hours	16h
Hybridization temperature	45°C	60°C	55°C
Detection method	Streptavidin-phycoerythrin	Cyanine 3 (Cy3) and cyanine 5 (Cy5) fluorescent labeling	Streptavidin-Cy3
Advantages	Reproducibility; Full genome coverage; Mature platform; Customization; More probes per gene.	Reproducibility; content; mature platform; sensitivity; customization	Reproducibility; Full genome coverage; Sensitivity; Low background; Mature platform; Low cost/sample; Low starting material required
Disadvantages	Short oligonucleotides; Less sensitive; High cost/sample.	Two-color dye bias and ozone-related degradation	Currently only available for human, rat and mouse studies; Less probes per gene; not so sensitive to detect splice variants.

Table 1. Microarray platform comparison.

Despite minor differences between platforms, the basic steps involved in a microarrays experiment are similar (Fig. 1) (Repsilber *et al.*, 2005). Key points in undertaking an expression profiling study are:

1. Establish your research question.
2. Selection of the tissue/cell most relevant to the question and the selection of the control group.
3. Total mRNA is extracted from the chosen tissue/cell, and reverse transcribed generating cDNA which is labelled with radioactive or fluorescent markers.
4. Labeled transcripts are hybridized onto the microarray.
5. Bound probes are detected and quantified by imaging tools and every gene/probe assigned a signal intensity.
6. Signals are corrected for common bias i.e. normalized. For each mRNA, the signal intensity difference between the disease and the control sample correlates to the change in gene expression (genes up- or down-regulated) that might be associated with the studied condition. Several methods have been implemented to reduce variability in DNA microarray experiments (Workman *et al.*, 2002). A critical step in the whole procedure is an appropriate analysis of the large volumes of data generated using sophisticated software. Bioconductor (www.bioconductor.org) or BRB ArrayTools (Simon *et al.*, 2007), examples of bioinformatic platforms, provide tools for analysis and comprehension of genomic data.
7. Candidate genes are validated through another technology. Usually quantitative reverse-transcription PCR (qPCR) is the preferred method.
8. Data is integrated and applied to the initial question.

1.2 Microarray challenges and concerns

Large-scale gene expression analysis, is in fact, a flourishing technology with potential applications in several fields of Biology and Medicine as indicated by the large number of peer-reviewed articles (n=35502) containing the words “gene” and “microarray” found in Pubmed upto June 2011.

Microarray profiling of gene expression is a powerful tool for discovery, but the ability to manage and compare the resulting data can be problematic. Biological, experimental, and technical variations between studies of the same phenotype/phenomena create substantial differences in results. Some of these issues will be discussed in detail.

a) The success of the microarrays experience greatly depends on whether the hypothesis and rationale have been appropriately formulated through a clearly delineated question. It influences the study design as a whole, from sample collection, to experimental design, and finally, the strategies for data analysis (Smith & Rosa, 2007).

b) While most of the early studies used primary tissues involved in the disease, such as tumor biopsies, more recently a number of gene expression profiling studies have focused on peripheral blood to identify systemic markers of disease. However, gene expression patterns in peripheral blood cells greatly depend on inter-individual variations and technical aspects such as blood sampling techniques, cell and RNA isolation as well as storage temperature or delays in processing. However although significant inter-individual variations in gene expression patterns in peripheral blood cells can be seen, these differences

are often much less than the differences between blood samples from healthy donors and from patients. These observations and the accessibility of peripheral blood, strongly suggests that gene expression analysis of peripheral blood is probably the best source for the assessment of systemic differences or changes in gene expression associated with disease or drug response. (Debey *et al.*, 2004).

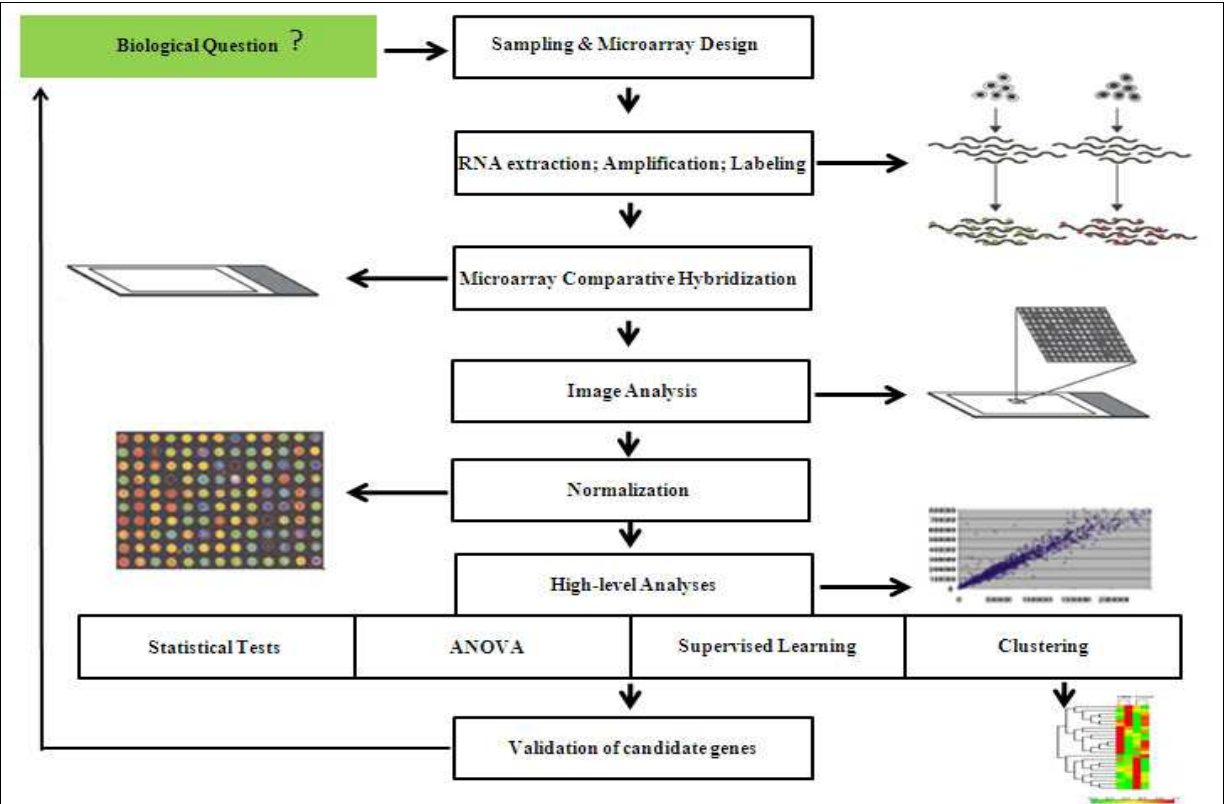


Fig. 1. Design, experimental and data analysis steps in a typical microarray gene expression experiment. Adapted from Repsilber *et al.*, 2005.

c) Appropriate experimental design is another critical step for the success of a microarray experiment. It's important to control and exclude as many biases as possible (Ransohoff, 2007). Integrity and purity of RNA extracted, cDNA labeling and hybridization procedures may affect reproducibility, thus these steps need to be standardized and optimized. However, several key issues regarding appropriate replication remains in discussion: the minimum sample size, the necessity of running multiple arrays with the same samples or the potential benefits and risks associated with pooling samples (Smith & Rosa, 2007). Increasing the sample size will lower the false discovery and false negative rates but it represents an expensive option (Pawitan *et al.*, 2005). Given the well-established reproducible commercially available platforms, technical replication is not required currently. Finally, pooling samples can reduce the variation between arrays but potential outliers may get masked or may compromise the entire pool (Smith & Rosa, 2007). To guaranty an improvement of data quality, replication studies in independent patient series must be performed, but these analyses are often lacking (Ionnidis *et al.*, 2009).

d) Data analysis currently represents a major challenge for researchers. A closer look at the literature reveals many conflicting results. A consensus regarding strategies in data analysis

is required. Over the last few years a number of papers have reviewed in detail how to analyze typical microarray data experiments (Allison *et al.*, 2006; Reimers, 2010), to interpret them (Michiels *et al.*, 2007) and to report the results (Dupuy & Simon, 2007). The multidimensionality of microarrays and possible solutions to deal with this issue are well discussed in a recent review (Michiels *et al.*, 2011).

e) Confirmation and validation studies are another crucial step. For confirmation studies the initial results must be reproduced using another assay technology, usually qPCR. Validation studies require an independent study in a new sample cohort to confirm that the gene signatures defined previously replicate satisfactorily in a similar clinical setting. It may be performed by the same research team or ideally by others. These additional steps reduce false positives and the potential for biases (Michiels *et al.*, 2007, 2011).

Establishing a consensus to optimize each step of the procedure would therefore generate more reproducibility in results from different studies. Evidence-based guidelines to perform meta-analysis of array data are in progress (Ramasamy *et al.*, 2008) but establishing consensus in experimental design and protocols is still the most likely method to minimize variation. Clinical trials to confirm the gene signature's clinical utility on diagnosis and treatment decisions are mandatory, after the identification of reliable biomarkers.

1.3 Microarray applications in rheumatology/spondyloarthritis

Several microarrays studies have been published looking at spondyloarthritis (SpA). A number of early studies used different tissue sources and smaller microarrays with whole-genome arrays prohibitively expensive (Reviewed in Thomas & Brown MA, 2010a, 2010b). The first study in 2002 identified genes more highly expressed in peripheral blood mononuclear cells (PBMC) of patients with SpA, rheumatoid arthritis (RA) and psoriatic arthritis (PsA), in comparison to normal subjects (Gu *et al.*, 2002a). A 588-gene microarray was used as a screening tool and the results were validated by reverse transcription-polymerase chain reaction (RT-PCR). A total of 16 genes were identified encoding differentiation markers, cytokines, cytokine/chemokine receptors and signalling and adhesion molecules. An increased expression of C-X-C chemokine receptor type 4 (CXCR4) and its ligand Stromal cell-derived factor-1 (SDF-1), in synovial fluid cells, were seen in all three arthritis groups. The conclusion was that the CXCR4/SDF-1 is a potential pro-inflammatory axis for SpA, PsA and RA. However no genes were identified that could discriminate between the different diseases.

In another study gene expression profiles of synovial fluid mononuclear cells (SFMC) from SpA and RA patients were compared with PBMC of healthy controls to evaluate the unfolded protein response (UPR) hypothesis and identify which cytokines/chemokines were being expressed and which cell fractions were involved. An 1176-gene microarray was used and the results were validated by RT-PCR. There was an increase in transcripts encoding Monocyte chemotactic protein-1 (MCP-1), proteasome subunit C2 and Binding immunoglobulin protein (BiP), which suggest the existence of an UPR. BiP was higher in SpA SFMC compared to RA SFMC and macrophages were potentially identified as the cell type involved (Gu *et al.*, 2002b).

A third study identified a gene expression profile in gut biopsies that could differentiate SpA patients with sub-clinical gut inflammation from SpA patients without gut disease.

2625 differentially expressed sequence tags were initially identified through macroarrays in colon biopsies from Crohn’s and SpA patients which were then used to construct a microarray which was used to screen a further sample cohort. Ninety five expressed sequence tags clustered patients with Crohn’s and those with SpA and chronic gut inflammation (Laukens *et al.*, 2006).

This chapter, Lessons from Genomic Profiling in AS will be focused on studies using peripheral blood and microarray platforms covering the whole genome. The results seem to be quite heterogeneous reflecting the different methodologies involved, as commented above. Several aspects, summarized in Figure 1, may introduce variability and bias in the results, specifically;

- a. Patient selection: numbers of patients, the criteria used to classify and include the patients, different degrees of activity/severity of the disease and patients receiving different therapies are examples of heterogeneity that might influence the final results.
- b. Cell Source used for analysis: PBMC vs. whole blood or a specific cell subset.
- c. Differences in microarray platform technology and data analysis tools.
- d. Differences in methodology used regarding validation of candidate biomarkers.

Based on seven papers published since 2007, several pathways relevant to potential SpA pathological processes have been identified. Moreover, potential biomarkers with applications to diagnosis and treatment response prediction in clinical practice were also flagged. Table 2, summarizes the similarities and methodological differences between the studies and reinforces the caution that should be observed when translating these findings to clinical practice. All the knowledge obtained must be interpreted as hypotheses which need validation in future studies.

	Subjects	Criteria	Samples	Microarray	Validation
Smith <i>et al.</i> 2008	6AS+2uSPA 9HC	mNYC ESSG, Amor	Macrophage	Affymetrix	qPCR
Haroon <i>et al.</i> 2010	16AS	mNYC	PBMC	Affymetrix	qPCR
Sharma <i>et al.</i> 2009	11uSPA+7uSPA 25HC	Likelihood Score	Whole blood	Affymetrix	Microarrays (2 nd set)
Duan <i>et al.</i> 2010	18AS+18HC 35AS+18HC	mNYC	PBMC	Illumina	qPCR
Gu <i>et al.</i> 2009	21AS+28uSPA 23AS+18uSPA 26HC+12RA+5LBP	Calin	PBMC	Illumina	qPCR
Assassi <i>et al.</i> 2011	16AS + 14HC+ SLE+SSC 27AS+27HC	mNYC	Whole bood	Illumina	qPCR
Santos <i>et al.</i> 2011	18AS+18HC 78AS+78HC	mNYC	Whole blood	Illumina	qPCR

AS: Ankylosing spondylitis; SPA: Spondyloarthritis; HC: Healthy controls; RA: Rheumatoid arthritis; LBP: Lumbar back pain; SLE: Systemic lupus erythematosus; mNYC: modified New York criteria; ESSG: European Spondyloarthropathy Study Group; PBMC: Peripheral blood mononuclear cells; qPCR: Quantitative reverse transcription polymerase chain reaction.

Table 2. Comparison between published microarrays studies in SpA.

2. Lessons from genomic profiling in AS

2.1 The link between an abnormal innate immune response and AS

One of the most intriguing aspects regarding AS pathogenesis is the possible link between pathogens and disease onset. There are several pieces of evidence that an abnormal host response against pathogens is implicated in AS and/or SpA pathogenesis. Sixty percent of patients with SpA without diagnosed Crohn’s disease evidenced endoscopic or histological signs of gut inflammation (Mielants *et al.*, 1995). Moreover, studies showing HLA-B27 transgenic rats do not develop inflammatory intestinal or peripheral joint disease in a germ-free environment support a role of commensal gut flora in the shared pathogenesis of gut and joint manifestations (Taurog *et al.*, 1994).

Pattern recognition receptors (PRRs) in innate immune cells play a pivotal role in the first line of the host defense system. These receptors are transmembrane receptors such as Toll-like receptors (TLRs) or C-type lectin receptors (CLRs) and cytosolic receptors RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Jeong & Lee, 2011). Interestingly, expression changes in genes involved in innate immune response such as *TLRs* (Assassi *et al.*, 2011), *NLRP2* (Sharma *et al.*, 2009) and *CLEC4D* (Pimentel-Santos *et al.*, 2011) were consistently observed in several different studies using microarray technology.

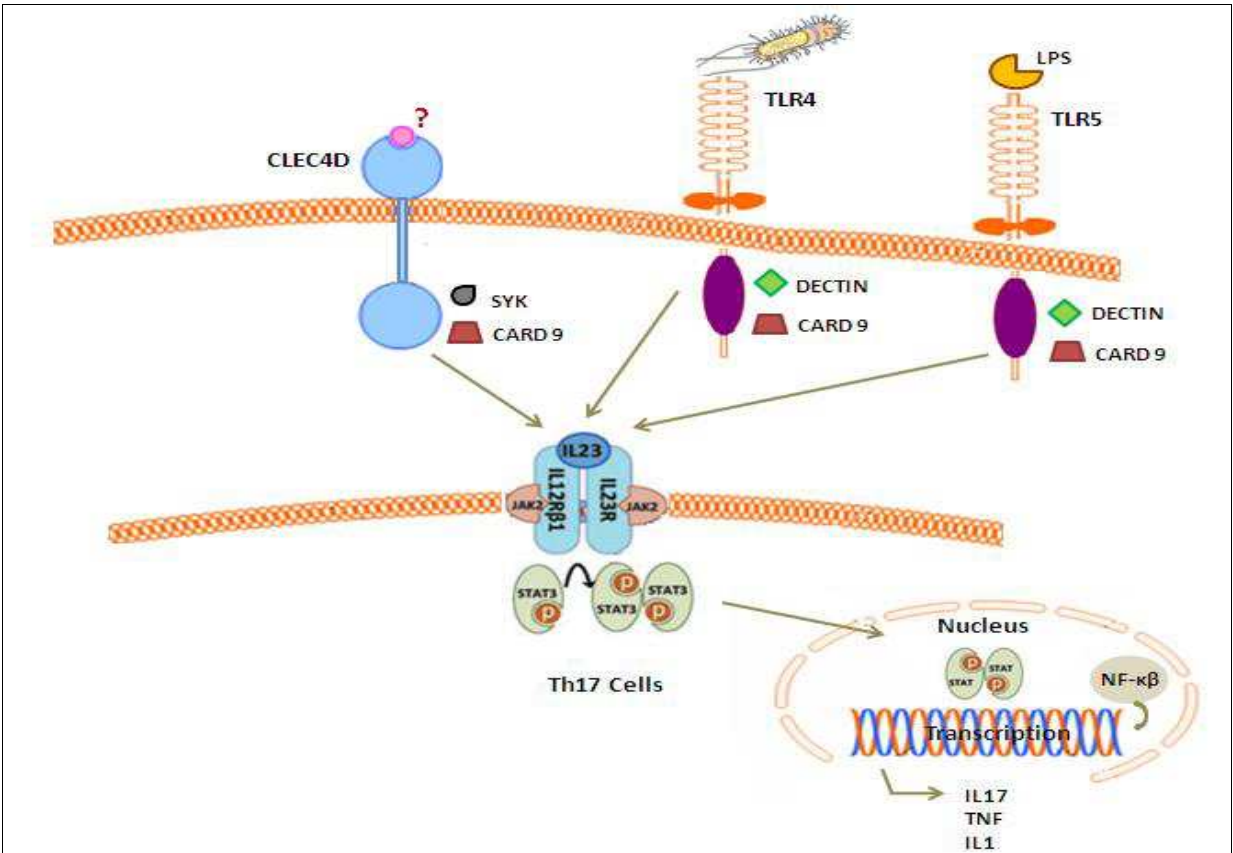


Fig. 2. Possible functional interactions between innate immune receptors and AS candidate genes (Adapted from Thomas & Brown, 2010a).

TLRs are characterized by an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a cytoplasmic Toll/IL-1R (TIR) domain. As many as 13 TLR

family members have been identified in mammalian systems with TLRs 1 to 10 expressed in humans. They can be divided into 2 groups according to cellular localization and respective ligands. TLRs 1, 2, 4, 5, and 6, are expressed on the cell surface and recognize microbial components in the outer membrane of bacteria. TLRs 3, 7, 8 and 9 are found in intracellular vesicles and recognize microbial nucleic acids (Sirisinha, 2011). TLRs are expressed in various immune (monocytes, macrophages, dendritic cells, B cells) and non-immune (epithelial cells, endothelial cells, fibroblasts) cells. *TLR4* was overexpressed in SpA patients in peripheral whole blood cells, assessed by microarray (Assassi *et al.*, 2011; Pimentel-Santos *et al.*, 2011), in PBMCs, measured by flow cytometry (De Rycke *et al.*, 2005) and in lymphocytes, monocytes and neutrophils by qPCR (Yang *et al.*, 2007). The main ligand for TLR4 is lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria, however, it also recognizes other exogenous pathogens such as mannan from *Candida albicans*, glycoinositolphospholipid from *Trypanosoma*, and the envelope proteins from mouse mammary tumor virus (MMTV) and respiratory syncytial virus (RSV). It also recognizes some endogenous molecules, including heat-shock proteins (HSP60, HSP70, and HSP gp96), fibrinogen, oligosaccharides of hyaluronic acid, extracellular domain A of fibronectin, heparan sulfate, myeloid-related proteins (Mrp8 and Mrp14), oxidized LDL, saturated fatty acid and amyloid- β (Jeong & Lee, 2011). Microarray analysis also showed overexpression of TLR5 in peripheral whole blood cells from SpA patients (Assassi S *et al.*, 2011; Pimentel-Santos *et al.*, 2011). Flagellin, a primary component of Gram negative bacteria flagella, is the main ligand for TLR5 (Hayashi *et al.*, 2001), which is mainly expressed on the luminal surface of epithelial cells in the mucosal tissues and respiratory tract (Gewirtz *et al.*, 2001).

The wide responsiveness of TLRs to a wide variety of external and internal signals, and the link that these receptors establish between the innate and adaptive immune systems, reinforces the theory that TLRs are strongly implicated in the development of chronic inflammatory diseases. However, mechanistic studies are needed in order to clarify the role of specific receptor subtypes in AS development.

Members of the NOD-like receptor (NLR) family consist of a central nucleotide-binding and oligomerization (NACHT) domain, which is commonly flanked by C-terminal leucine-rich repeat (LRRs) domain and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains (Schroder & Tschopp, 2010). So far, 20 NLR family members have been identified in humans. Two main subgroups have been described. One, including NODs (NOD 1-5 and CIITA), detects pathogen-associated molecular patterns (PAMPs) existing in Gram-negative bacteria cell walls and elicit responses that are distinct from those of the TLRs. The other NLR subgroup involves a large family of molecular complexes known as the "inflammasomes", the NLRPs (NLRP1-14) and the IPAF subfamily, consisting of IPAF and NAIP (Fitzgerald, 2010; Schroder & Tschopp, 2010). The inflammasomes are macromolecular cytosolic complexes composed of several proteins, some of which are found in all inflammasomes (pro-caspase-1, Apoptosis-associated Speck-like Protein Containing a Caspase Recruitment Domain-ASC), and others which are present depending on the inflammasome type (cardinal, pro-caspase-5, domain with function to find-FIIND). These complexes are involved in the innate immune response recognizing both endogenous signals (adenosine triphosphate, urate, and calcium pyrophosphate crystals) as well as external pathogen-derived products (bacterial RNA, bacterial toxins) (Drenth & van der Meer, 2006).

As such, the reduced expression of Nod-like receptor family, pyrin domain containing 2 (*NLRP2*) in AS was a very interesting observation (Sharma *et al.*, 2009). *NLRP2*, as with other NLRs, induces an inhibition of the NF κ B signaling pathway, leading to regulation of IL1 β , a relevant cytokine in the disease process. The downregulation of *NLRP2* may therefore lead to upregulation of IL-1 β . Supporting this, polymorphisms in *NLR* genes have also been implicated in Behçet's disease and Crohn's disease which share some clinical features with AS (Cummings *et al.*, 2010; Kappen *et al.*, 2009). Another interesting point is the association of *CARD9* with Crohn's disease and AS (Pointon *et al.*, 2010) which has a pivotal role in NOD2 signaling.

Another family of receptors of particular interest are the C-type lectins which display a distinct protein domain, the carbohydrate recognition domain (CRD). Based on the organization of their CRDs, 17 distinct groups have been defined (Drickamer & Fadden, 2002; Zelensky & Gready, 2005). While some recognize DAMPs which facilitate adhesion between cells, adhesion of cells to extracellular matrix and other non-enzymatic functions, others may act as PRRs (Graham & Brown, 2009) after PAMP recognition. Upon ligand binding, C-type lectin receptors can induce a variety of cellular responses, and can be functionally divided into those that inhibit or those that induce cellular activation. In general, inhibitory receptors contain a consensus immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains, while activation receptors either contain an immunoreceptor tyrosine-based activation motif (ITAM), or associate with signalling adaptor molecules. Depending on whether signalling is through ITAM or ITIM, either activation of Src homology 2 (SH2) domain-containing protein tyrosine kinases (Syk, ZAP 10) or SH2 containing-phosphatases (SHP-1, SHP-2) are recruited, thereby up or downmodulating cellular activation, respectively (Majeed *et al.*, 2001; Long, 1999).

Genes encoding for each family are distinctly clustered in the telomeric Natural Killer-gene complex (NKC), on chromosome 12. The Dectin-1 cluster of receptors, includes Dectin-1, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), C-type lectin-like receptor-1 (CLEC-1), CLEC-2, CLEC12B, CLEC9A and myeloid inhibitory C-type lectin-like receptor (MICAL). The Dectin-2 cluster of receptors, includes Dectin-2, DCIR, DCAR, BDCA-2, Mincle and CLEC4D (Graham & Brown, 2009).

Dectin-1, is expressed in dendritic cells, monocytes, macrophages, neutrophils and weakly in a subset of T cells, B cells and eosinophils. It recognizes fungal β -glucan, working as an activating receptor uniquely possessing an ITAM in the cytoplasmic domain. The induction of phagocytosis, production of reactive oxygen species and cytokine production is mediated by NF- κ B and spleen tyrosine kinase (Syk). In addition, some of these effects require cooperation with MyD88-mediated TLR signaling (Kanazawa, 2007).

Dectin-2 and Mincle are expressed in macrophages, dendritic cells and weakly in Langerhans cells and monocytes. The receptors recognize several pathogens (*Candida albicans*, *Saccharomyces cerevisiae*, *Mycoplasma tuberculosis*, *Histoplasma capsulatum*) but also endogenous ligands. Both have characteristic short cytoplasmic domains and are associated with FcR γ domains. Their activation, inducing the production of proinflammatory cytokines, is mediated by Syk- and *CARD9*-dependent pathways but independently of MyD88-mediated TLR signaling (Graham & Brown GD, 2009).

CLEC4D has been found to be expressed in a monocyte/macrophage restricted manner, and although no ligand or biological function has as yet been described, the receptor has been

shown to be upregulated at the transcript level in a number of disease settings, similarly to two other members of the family, Mincle and Dectin-2. They are able to recognize and promote pathogen clearance and induce inflammatory signals. This process seems to follow the Syk and CARD9 pathway which was recently implicated in a mouse model of SpA (Ruutu *et al.*, 2010). The upregulation of *CLEC4D*, observed for the first time in an expression profiling study of AS patients (Pimentel-Santos *et al.*, 2011), supports the importance of innate immune mechanisms in AS pathology. However, further studies are required to confirm this hypothesis.

2.2 Proinflammatory vs. immunosuppressive signatures

Transcriptional profiling studies have demonstrated that transcripts involved in the inflammatory response were differentially expressed in AS patients and controls, but reports on the nature of these changes seem to vary. A proinflammatory profile in peripheral blood monocyte cells (PBMCs), from undifferentiated spondyloarthritis (uSpA) and AS, is indicated by an increased expression of *RGS1*, *NR4A2*, *HBEGF* and *SOCS3*, in both groups (Gu *et al.*, 2009). However, other reports suggest decreased immune responsiveness such as a “reverse *IFN γ* signature” (Smith *et al.*, 2008), and immunosuppressive phenotypes (Duan *et al.*, 2010, Pimentel-Santos *et al.*, 2011). The main reason for these differences in the transcriptomic profiles, between the first study and the 3 later studies, is unknown but differences in patients and methodologies may contribute.

IFN γ dysregulation in AS is supported by previous studies of cytokines expression. A lower frequency of *IFN γ* positive T cells has been reported in AS patients (Rudwaleit *et al.*, 2001) and gut biopsy samples show a reduced TH1 profile in lymphocytes from SpA patients (Van Damme *et al.*, 2001). Moreover, *IFN γ* is expressed at lower levels in synovium from SpA compared to rheumatoid arthritis patients (Canete *et al.*, 2000). This knowledge may contribute to understanding AS pathogenesis as decreased *IFN γ* production by macrophages could impair the host's ability to clear pathogenic organisms. Recent studies support this theory (Rothfuchs *et al.*, 2001; Inman *et al.*, 2006), and may implicate arthritogenic organisms in AS susceptibility. In addition, *IFN γ* reduction, can contribute to activation of the IL-23/IL-17 axis a major axis in AS pathogenesis.

Complementary to the report in macrophages from peripheral blood of AS patients (Smith *et al.*, 2008), two different studies, from PBMCs and whole blood, have shown an immunosuppressive phenotype (Duan *et al.*, 2010, Pimentel-Santos *et al.*, 2011). The first one validated three downregulated genes, Nuclear receptor subfamily 4, group A, member 2 (*NR4A2*), Tumor necrosis factor, alpha-induced protein 3 (*TNFAIP3*) and CD69 molecule (*CD69*). *NR4A2* has been associated with T-cell subset communication and the macrophage inflammatory response. *TNFAIP3* serves as negative feedback system for the TNF α induced by NF κ B, acting as an anti-inflammatory molecule to control prolonged inflammation. *CD69* is an early leukocyte activation molecule expressed at sites of active inflammation. Of further interest were the results of Ingenuity Pathways Analysis using the differentially expressed geneset showing altered activity of the JAK/STAT signaling pathway in AS patients (Duan *et al.*, 2010). Both *STAT3* and *JAK2* have been shown to be genetically associated with IBD and AS (Barrett *et al.*, 2008; Danoy *et al.*, 2010; The Australo-Anglo-American-Spondyloarthritis-Consortium (TASC), 2011), and represent key downstream molecules of the IL-23/IL-17 pathway (Ma *et al.*, 2008).

In the second study downregulation of several pro-inflammatory genes were described highlighting another aspect of AS pathogenesis (Pimentel-Santos *et al.*, 2011). Protein tyrosine phosphatase, non-receptor type 1 (*PTPN1*) and Dedicator of cytokinesis 10 (*DOCK10*), which are both involved in mediating IL4 actions (Paul & Ohara., 1987) were downregulated. Protein tyrosine phosphatase 1B (PTP1B), the *PTPN1* protein product, is a ubiquitously expressed enzyme shown to negatively regulate multiple tyrosine phosphorylation-dependent signalling pathways, including the downstream processes involved in C-type lectin receptor activation (Majeed *et al.*, 2001; Long, 1999) and IL4 signalling (Lu *et al.*, 2008). Dock10 is also regulated by IL4 in B cells (Yelo *et al.*, 2008). This is of particular interest as IL4 may play a role in AS pathogenesis. Interleukin 4 (IL4), has a variety of stimulatory and inhibitory actions on B and T cells (O'Garra *et al.*, 1988; Jelinek & Lipsky 1988; Rousset *et al.*, 1988). Recent studies have also indicated a potential role for IL4 producing CD8⁺ T cells in the pathogenesis of AS. Although CD8⁺ T cells are predominately associated with the production of 'TH1' cytokines, such as IFN γ , there is now good evidence that some subsets of these cells can also produce 'TH2' cytokines such as IL4, IL5 and IL10 (Baek *et al.*, 2008). The potential functions associated with IL4-producing CD8⁺ T cells are as yet unclear but the subtype CD8⁺/TCR $\alpha\beta$ ⁺ T cells, with a regulatory phenotype and function (expressing CD25⁺, CTLA4⁺, Foxp3⁺, but negative for IFN γ and perforin), were previously described in peripheral blood of AS patients (Jarvis *et al.*, 2005). These results were confirmed in a recent study suggesting an altered pattern of CD8⁺ T cell differentiation in AS and in HLAB27⁺ healthy individuals. This predisposition to generate IL4+CD8⁺ T cells may play a role in pathogenesis of SpA (Zhang *et al.*, 2009). Further supporting this theory, *RUNX3* was identified as a candidate gene in a GWAS (Australo-Anglo-American Spondyloarthritis Consortium (TASC), 2010). The association of *RUNX3* with AS provides additional evidence of a role for CD8⁺ T cells in the disease. It's expression in immature lymphocytes is triggered by IL7R signalling, leading to suppression of CD4 and upregulation of CD8 expression (Park *et al.*, 2010).

Although there are some differences between the different expression profiling studies, their findings do contribute to a greater understanding of the pathogenesis of AS, particularly in the delineation of the roles of the innate and adaptive immune responses.

2.3 Bone ossification and resorption processes

Bone formation and bone loss take place at sites closely located to each other presenting an "apparent paradox", which is reflected in the changes in bone and cartilage metabolism occurring in the AS disease process (Carter & Lories, 2011). Ossification is the hallmark of AS and has been linked to aberrant activation of bone morphogenic protein (BMP) and wingless (WNT) signaling. Bone resorption, driven by the impact of inflammation on the bone remodeling cycle, occurs simultaneously, with up to 56% of patients developing systemic osteopenia and some of them systemic osteoporosis (Lange *et al.*, 2005).

Biomarkers, reflecting structural damage and disease activity, constitute a high priority for the understanding of the pathogenesis of AS and for the new therapy discovery. Two microarray-based studies have contributed to the improvement of knowledge in this field. A bone remodeling signature was described associated with an overexpression of *BMP6*, Proprotein convertase subtilisin/kexin type 6 (*PCSK6*), Kringle containing transmembrane

protein 1 (*KREMEN1*) and Catenin (cadherin-associated protein) alpha-like 1 (*CTNNAL1*) genes in SpA patients (Sharma *et al.*, 2009).

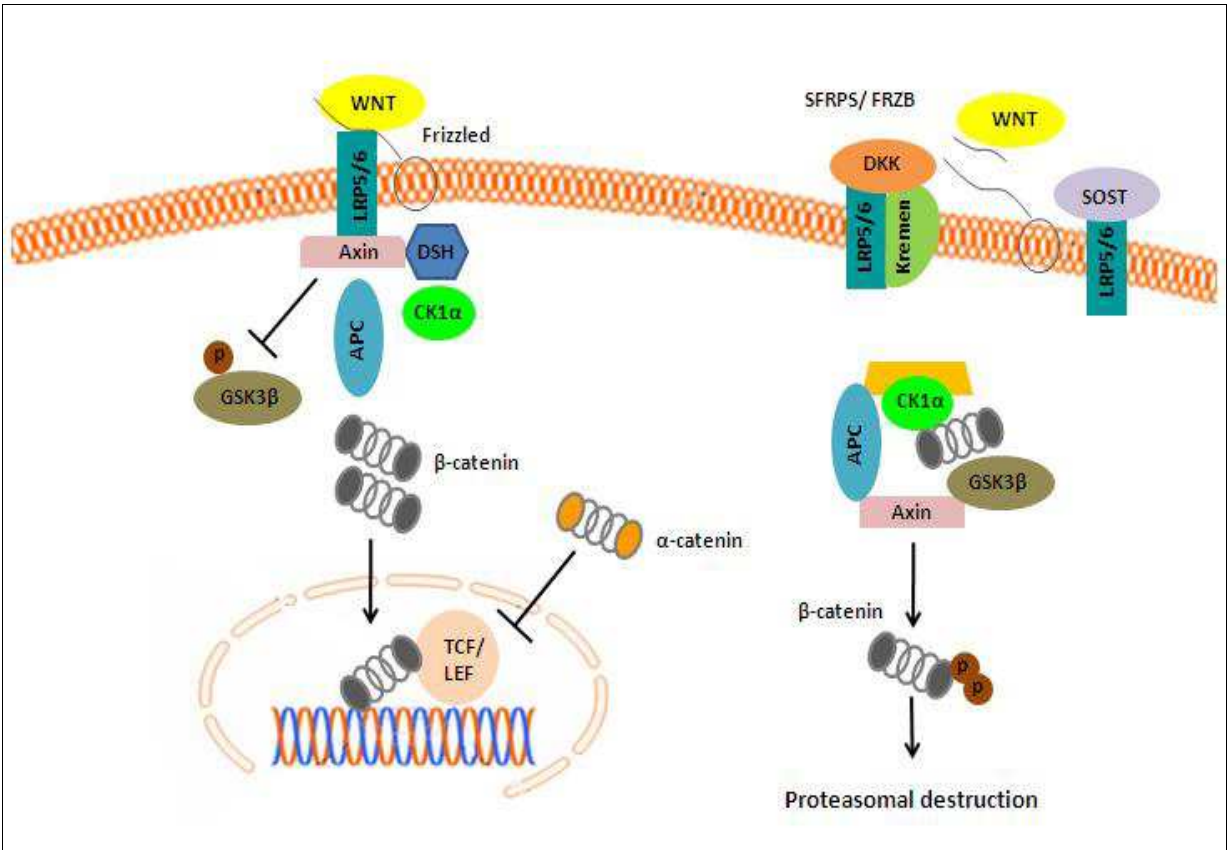


Fig. 3. The canonical WNT signaling pathway (adapted from Carter & Lories, 2011).

KREMEN1 and *CTNNAL1* are negative regulators of WNT/catenin pathway via dickkopf homolog 1 (DKK1), or by direct inhibition of β -catenin, respectively. Although four different intracellular pathways can be triggered upon WNT receptor interaction, the WNT/ β -catenin or “canonical” pathway is of particular interest in bone and cartilage biology. This pathway involves the interaction of WNT ligands with frizzled (FZD) receptors and low-density lipoprotein receptor-related protein 4, 5 or 6 (LRP 4, 5 or 6) co-receptors. In the absence of a WNT-FZD-LRP 4/5/6 interaction, cytoplasmic β -catenin is captured within a destruction complex comprising adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3 β (GSK-3 β), and casein kinase 1 α (CK1 α). The kinases phosphorylate β -catenin, which leads to ubiquitinylation and subsequent destruction in a proteasome complex. When WNT does complex with FZD and LRP 4/5/6, axin binds to the cytoplasmic tail of LRP5 or 6, thereby phosphorylating and inhibiting GSK-3 β (Gordon & Nusse, 2006). This process enables cytoplasmic β -catenin accumulation which then translocates to the nucleus, where it interacts with transcription factor (TCF)/lymphoid enhancer factor (LEF) family members and modulates WNT target gene expression (Gordon & Nusse, 2006). Several proteins that are not involved in β -catenin stability can also regulate β -catenin signaling. One example is the direct association of α -catenin with β -catenin in the nucleus which interferes with protein-DNA interactions required for TCF-mediated transcription (Giannini *et al.*, 2000). In addition, different endogenous antagonists inhibit WNT signalling; DKK1 and sclerostin (SOST). DKK1 acts by direct binding to and inhibiting the WNT co-receptor LRP6. The

related DKK2, however, can function either as LRP6 agonist or antagonist, depending on the cellular context, suggesting that its activity is modulated by unknown co-factors. In this context, the transmembrane proteins KREMEN1 and -2 were recently identified as additional DKK receptors, which bind to both DKK1 and DKK2 with high affinity (Mao & Niehrs, 2003). It was shown that DKK1 was able to simultaneously bind to LRP5/6 and KREMEN and that the ternary complex was rapidly endocytosed, thus preventing the WNT-LRP interaction. The interaction with KREMEN seems to be not essential but it plays a role in facilitating DKK-mediated antagonism if the level of LRP5/6 is high (Wang *et al.*, 2008). The upregulation of *KREMEN1* and *CTNNAL1* genes by these mechanisms can compromise bone formation. In contrast, upregulation of *BMP6* and its regulator *PCSK6* can contribute to the AS ossification process. BMPs, members of the transforming growth factor- β (TGF β) superfamily, play a crucial role in embryonic development, cell lineage determination, and osteoblastic differentiation and function. Enthesitis, a distinctive feature of SpA, is associated with heterotopic cartilage and bone formation (enthesophyte) (Benjamin & McGonagle, 2001). Different BMPs are expressed in distinct stages of ankylosing enthesitis shown in the DBA/1 mouse model. BMP2 is found in proliferating cells and enthesal cells committing their differentiation fate to chondrogenesis. BMP7 is recognized in prehypertrophic chondrocytes and BMP6 in hypertrophic chondrocytes (Lories *et al.*, 2005). Several regulators of endochondral bone formation with different effects in different stages were described (Kronenberg, 2003). It is therefore possible that the presence of progenitor cells at the enthesal site promotes bone formation in SpA patients. Activation of the BMP signaling pathway (phosphorylated Smad1/5) was found in cells at the sites of enthesal inflammation in patients with AS (Lories *et al.*, 2005).

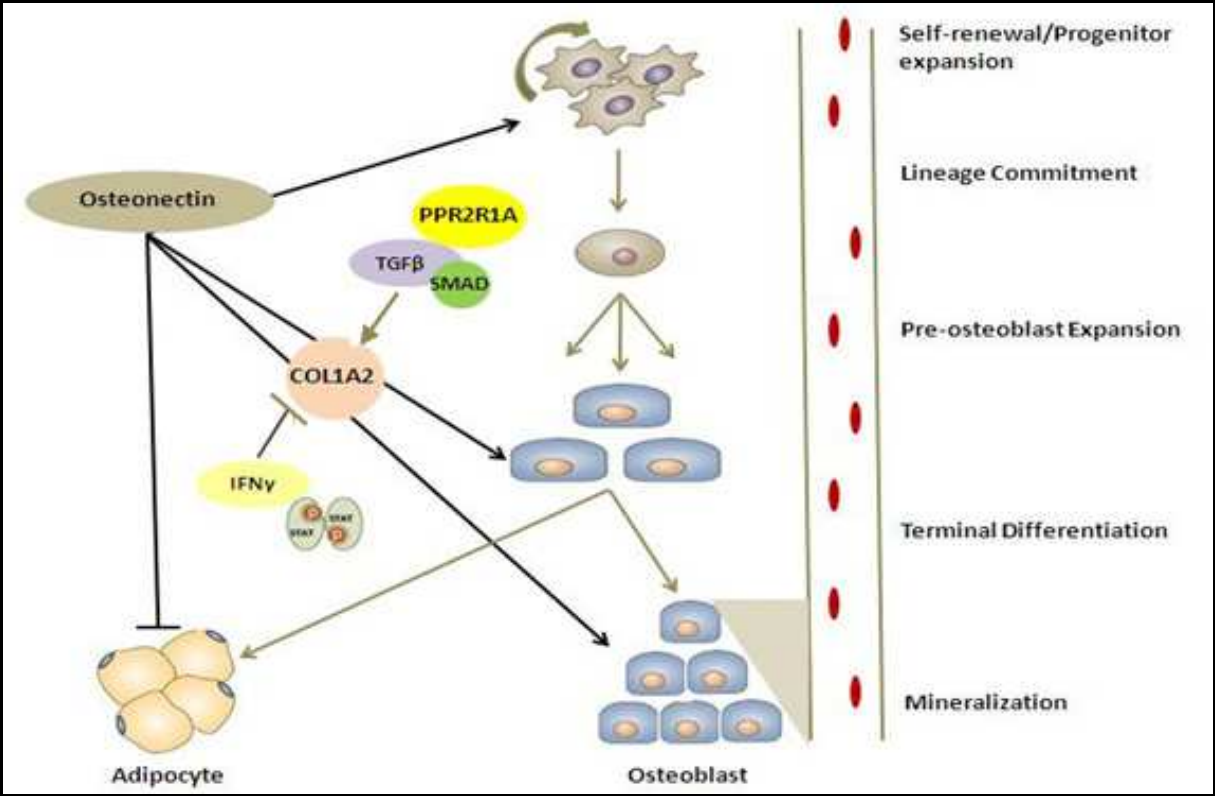


Fig. 4. Model representing the effects of SPARC on marrow mesenchymal progenitors (adaptated from Delany & Hankenson, 2009).

Another bone remodeling signature was identified in association with a downregulation of *SPOCK2*, *EP300* and *PPP2R1A* in AS, which are possible mediators in the ossification process (Pimentel-Santos *et al.*, 2011).

SPOCK2, also known as Sparc/osteonectin, is a non-collagenous bone protein. It is a member of the matricellular class of glycoproteins which includes periostin, tenascin C, osteopontin, bone sialoprotein, thrombospondin-1 and thrombospondin-2 (Alford & Hankenson, 2006). It has been hypothesized to play a role in the regulation, production, assembly and maintenance of the matrix turnover in cartilage (Hausser *et al.*, 2004; Gruber *et al.*, 2005). In this process $TGF\beta$ and $IFN\gamma$ exert antagonistic effects, and play important roles in the physiologic regulation of extracellular matrix turnover. In fact, $TGF\beta$ positively regulates collagen type 1 (*COL1A2*) through the Smad signal transduction pathway, whereas $IFN\gamma$ inhibits *COL1A2* through Stat1. Additionally, protein phosphatase 2, regulatory subunit A (*PPP2R1A*), also downregulated in AS (Pimentel-Santos *et al.*, 2011), is thought to mediate $TGF\beta$ regulation through Smad (Heikkinen *et al.*, 2010). Animal models using SPARC-null mice have provided excellent information on the function of this protein in bone. SPARC-null mice develop profound low-turnover osteopenia (bone loss), associated with decreased numbers of osteoblasts and osteoclasts, and a markedly decreased bone-formation rate (Delany *et al.*, 2000; Boskey, 2003). Moreover SPARC-null mice have decreased trabecular bone volume due to decreased trabecular number (Machado dos Reis *et al.*, 2008) and an increase in extra-skeletal adipose deposits (Mansergh *et al.*, 2007). *In vitro* studies showed accumulation of SPARC during early osteoblastic differentiation, likely in association with collagen matrix, which decreases as the cells acquire more osteoblastic characteristics. This expression pattern seems appropriate because SPARC regulates collagen fibril assembly, and matrix is abundantly deposited in the earlier stages of differentiating cultures. SPARC has a positive effect on maintaining and expanding the mesenchymal progenitor pool, and promotes osteoblastogenesis/osteoblast function and decreases adipogenesis (Delany & Hankenson, 2009.). Expression of SPARC by osteoclasts has not been reported. Therefore, the mechanisms by which SPARC limits osteoclast formation may involve the direct interaction with osteoclasts or osteoclast precursors through the bone matrix, and/or the effect of SPARC on immune cells, marrow stromal cells, and osteoblasts supporting osteoclast development (Machado do Reis *et al.* 2008). In summary, recent findings supports the idea that SPARC play a critical role in regulating bone remodeling and maintaining bone mass. Thus its dysregulated expression may contribute to the aberrant matrix formation in AS.

Interestingly, the protein produced by *EP300* belongs to the group of nuclear p300/CBP transcriptional coactivators for both Smad3 and Stat1a that integrate signals that positively or negatively regulate *COL1A2* transcription (Ghosh *et al.*, 2001). Transactivated p300, controlled by phosphoinositide-3 kinase (PI3K)/AKT, is also an important transcriptional co-activator of Sox9, which modulates the expression of the major extracellular matrix component, aggrecan (Cheng *et al.*, 2009). Moreover, there is some evidence supporting a p300 interaction with the Wnt pathway as it is a β -catenin transcriptional coactivator. Downregulation of these genes might lead to a loss of matrix integrity thereby accelerating tissue damage. This may be reinforced by a pro-inflammatory status associated with downregulation of *EP300* (Ahmad *et al.*, 2007).

2.4 Biomarkers for early diagnostic purposes

Low back pain (LBP) is a very common symptom, responsible for 3% of annual medical visits in the USA (Licciardone, 2008). However only 5% of the chronic back pain seen in general practice designated as “inflammatory”, is associated with SpA (Underwood & Dawes, 1995). To classify patients with AS or SpA, various criteria sets can be used. The modified New York Criteria (van der Linden *et al.*, 1984) for AS, the Amor criteria (Amor *et al.*, 1990) and the European Spondyloarthropathy Study Group (ESSG) criteria (Amor *et al.*, 1991), developed in the 1990s, before magnetic resonance imaging (MRI) was available, addressed all SpA subtypes. Recently, it has been proposed to divide SpA patients into subgroups according to clinical presentation. The Assessment of SpondyloArthritis International Society (ASAS) group has developed criteria to classify patients with axial SpA with or without radiographic sacroiliitis, and patients with predominant peripheral SpA (Rudwaleit *et al.*, 2009b; Rudwaleit, 2010). With a sensitivity of 82.9% and a specificity of 84.4% , these axial SpA criteria perform better than the ESSG and Amor criteria, even after adding “sacroiliitis on MRI” to the latter. The peripheral criteria with sensitivity of 77.8% and specificity of 82.8% are also promising for use in clinical practice (Rudwaleit, 2010). The ASAS criteria have been developed as classification criteria but they are likely be useful as diagnostic criteria, especially in patients with non-radiographic axial SpA at an outpatient rheumatology clinic (van den Berg & van der Heijde, 2010). This may help to make an early diagnosis and prevent the current diagnostic delay, described as 5 to 10 years between the first occurrence of symptoms and an AS diagnosis (Feldtkeller *et al.*, 2003; Haibel *et al.*, 2007). It prevents unnecessary diagnostic tests and more importantly makes it possible to commence more effective therapies earlier. This is crucial as at early disease stages, even those without definite radiologic sacroiliitis, can suffer as much pain and have as high a disease activity as patients with established AS (Rudwaleit *et al.*, 2009a). Therefore, it's important to consider all patients with SpA with predominantly axial involvement irrespective of the presence or absence of radiographic changes as belonging to one disease continuum (Rudwaleit, 2005). Despite all these advantages with the new ASAS criteria, one of the major reasons for diagnosis delay is a low awareness of AS among physicians in primary care (Sieper, 2009). In this particular setting, several concerns have been raised regarding the use of ASAS criteria for diagnostic purposes (van den Berg & van der Heijde, 2010). Thus current diagnosis of AS and SpA still relies on clinical and imaging parameters that may be relatively complex for general use in primary care. Screening parameters for an early referral of AS patients, easy to apply by the non-specialist, sensitive, specific and not too expensive, should be identified. For the rheumatology community this represents a great challenge. Expression studies can identify a small number of genes whose expression profile might serve as cost effective set of surrogate biomarkers for AS.

One study has identified a small number of genes whose expression profile might serve as a cost-effective set of surrogate biomarkers for AS and uSpA (Gu *et al.*, 2009). In this PBMC-based microarray study, all included patients fulfilled Calin criteria for inflammatory back pain and were taking non-steroidal anti-inflammatory drugs (NSAID's) and/or sulfasalazine. They concluded that the overall gene expression was higher in uSpA than in AS patients suggesting that early axial SpA is associated with a more systemic inflammatory process. This may represent an interesting point as biomarkers are more helpfull in the early stage of SpA rather than the late stage. (Gu *et al.*, 2009). Alternatively, it may reflect the less accurate diagnosis involved in uSpA and might be due some uSpA patients being

misdiagnosed and actually suffering from a different inflammatory condition. A member of the family of regulators of G protein signaling (*RGS1*,) was identified as the most promising biomarker for uSpA and AS, with this gene more highly expressed in uSpA than in AS. They demonstrated a receiver operating characteristic (ROC) area under the curve (AUC) range between 0.93-0.99. Biomarkers with ROC AUC 0.8-1.0 are usually considered to be useful in clinical practice (Rao, 2003). To evaluate arthritis related factors that might enhance *RGS1* expression, a panel of 25 cytokines and chemokines on a monocyte derived human cell line were used. The 2 strongest activators of *RGS1* expression were TNF α and IL-17. However, in order to be implemented in clinical practice further studies are clearly needed. It requires a multicenter, multi-ethnic validation but also comparison with results obtained through MRI and the new ASAS classification criteria. There are several other concerns. This gene was differentially expressed between AS patients and healthy controls, in another microarray study PBMC based (Duan *et al.*, 2010), but contrary to the first study it was underexpressed. Finally, it wasn't identified as differentially expressed in a recent published study from a well defined population of Portuguese ethnicity background (Pimentel-Santos *et al.*, 2011). These distinct results reinforce the need for larger studies involving different ethnic groups.

2.5 Gene expression changes after anti-TNF α therapy

Biomarkers that allow quantitative assessment of treatment response have great potential in clinical practice. They enable appropriate choice of therapy, drug dosage to maximize effect and minimize toxicity, and monitor disease outcomes representing the foundation of evidence-based medicine (de Vlam, 2010). The introduction of biologic therapies targeting TNF α (infliximab, etanercept, adalimumab, golimumab) has changed clinical practice with several benefits regarding clinical management and prognosis. Additionally, the scientific community is waiting for the market introduction of new biological treatments with new targets in the near future. Identification of markers of treatment response would be of great clinical benefit by facilitating better targeting of these treatments to those most likely to respond, and potentially significantly reduce treatment costs by minimizing use of these expensive agents in patients unlikely to respond.

Until now the Visual Analogue Scale (VAS) pain, VAS general health, BASDAI, inflammatory parameters and composite response criteria are used to evaluate treatment effect in AS. ASAS defined and validated three levels of response: ASAS20, ASAS40, and ASAS partial remission, for patients treated with non-steroidal anti-inflammatory drugs and TNF α blockade (Anderson *et al.*, 2001). The recent introduction of the ASDAS criteria (van der Heijde *et al.*, 2009) seems to be a highly discriminatory instrument for assessing AS disease activity and monitoring changes in disease and is finding good use in clinical practice. However all these criteria aren't predictors of response to therapy and greatly rely on subjective self-evaluation and are not free from disease-unrelated influences, so biomarkers with high sensitivity and specificity for treatment response are highly desirable.

Current markers of response such as younger age, HLA-B27 carriage, elevation of acute phase reactants (CRP), and marked spinal inflammation, as shown by MRI, may be predictors of good response; conversely, older age, structural damage and poor function may be predictors of poor- or non-response (Rudwaleit *et al.*, 2004; Rudwaleit *et al.*, 2008). Data from the British Society of Rheumatology Biologics Register has shown raised

inflammatory markers at the start of therapy predicted a greater improvement in disease activity, (Lord *et al.*, 2010). Predictors of improvement in function, measured using the BASFI, have shown a strong association with gender (significantly greater improvement in women) and concurrent DMARDs therapy (Lord *et al.*, 2010). Finally, prevention of damage is another important outcome of therapy. Slow radiographic progression of the disease and the relatively small fraction of patients progressing over a period of 2-3 years makes radiographic evaluation less sensitive for damage evaluation. However, the major predictor of progression is previous existing radiographic damage. While it is clear that anti-TNF α agents have a structural benefit in inflammation-mediated resorptive damage as indicated by changes in bone and cartilage metabolism, an effect on radiographic progression remains to be demonstrated in AS (de Vlam, 2010). A study of the relationship of biomarker levels, disease activity and the spinal inflammation detected by MRI was performed in patients with ankylosing spondylitis (AS) receiving Infliximab over a 24 week period. Early reductions in IL-6 (by week 2) but not CRP or vascular endothelial growth factor (VEGF), were significantly associated with reductions in MRI activity and BASDAI scores by week 24 in the infliximab group (Visvanathan *et al.*, 2008). However the structural changes of this effect are not known.

Gene expression profiling constitutes a widely available and promising technology to identify treatment-associated changes. In two recent studies it was demonstrated that anti-TNF alpha treatment leads to significant alteration of gene expression and protein profiles, supporting the use of systematic gene expression and proteomic analysis to shed new light on pathogenic pathways with importance in the chronic inflammation of AS (Haroon *et al.*, 2010; Grcevic *et al.*, 2010). Anti-TNF α therapy induced a rapid change in the expression profile within 2 weeks in AS patients with down-regulation of lymphotoxins exhibiting inducible expression and competing with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes (*LIGHT*), interferon α receptor 1 (*IFNAR1*), interleukin 17 receptor (*IL17R*) and erythropoietin receptor (*EPOR*) genes. *LIGHT*, a member of the TNF superfamily, was the most significantly down-regulated gene and serum soluble *LIGHT* levels correlate well with other inflammatory markers such as, CRP and ESR. However, no significant differences between responders and non-responders were observed in either *LIGHT* mRNA expression or *LIGHT* serum levels. A time gap between changes in inflammatory mediators and improvements in subjective disease severity scoring metrics may explain these findings (Haroon *et al.*, 2010). Although these results are interesting more studies are needed for validation. Another study using peripheral blood expression profiles based on PBMCs cells assessed several bone-regulatory factors as potential discriminators of different forms of arthritis, disease activity and therapy responsiveness (Grcevic *et al.*, 2010). ROC curve analysis suggested higher expression of *Runx2* was a potential molecular marker for AS. Although no increased gene expression of *BMP-4* or *LIGHT* in AS patients compared with healthy controls were seen, higher expression was evident in AS patients resistant to conventional therapy. Thus *LIGHT* might be considered an interesting biomarker to consider in future studies.

Another marker which must be considered for a treatment-response marker is the CX3CL1-CC3CR1 complex. In RA, CX3CL1 levels decline in patients showing a clinical response to infliximab treatment. Moreover, patients with active RA who did not show a clinical response to infliximab showed higher basal CX3CL1 levels than those who did (Odai *et al.*, 2009). These results suggest that the CX3CL1-CX3CR1 in patients with active RA may be

sensitive to anti-TNF α therapy and confirm that CX3CL1 plays a crucial role in the pathogenesis of RA, although further investigations are required. These results suggest that CX3CL1-CX3CR1 may be also relevant in AS process. This is further supported with the underexpression of this gene in AS patients (Pimentel-Santos *et al.*, 2011).

Gene symbol	Designation	Potential role
BMP6	Bone morphogenic protein 6	Bone remodelling and cartilage matrix turnover
PCSK6	Proprotein convertase subtilisin/kexin type 6	
KREMEN1	Kringle containing transmembrane protein 1	
CTNNAL1	Catenin (cadherin-associated protein) alpha-like 1	
SPOCK2	Sparc/osteonectin	
EP300	Nuclear p300	
PPP2R1A	Protein phosphatase 2, regulatory subunit A	Diagnosis of early AS/uSPA
RGS1	Regulators of G protein signaling 1	
LIGHT	Ligand for herpesvirus entry mediator	
CX3CL1-CX3CR1	Chemokine (C-X3-C motif) ligand 1 - chemokine (C-X3-C motif) receptor 1	Response to anti-TNF alpha treatment

Table 3. Potential clinical applications of microarray findings.

3. Conclusion

All the studies described above have contributed to increased knowledge of the physiopathological processes involved in AS and have identified potential disease relevant biomarkers with significance for clinical practice (see Table 3). The integration of the expression profiling data with information obtained from “omic” approaches such as proteomic and metabolomic analyses as well as with clinical and imaging data, may further elucidate disease processes and therapeutic responses in AS.

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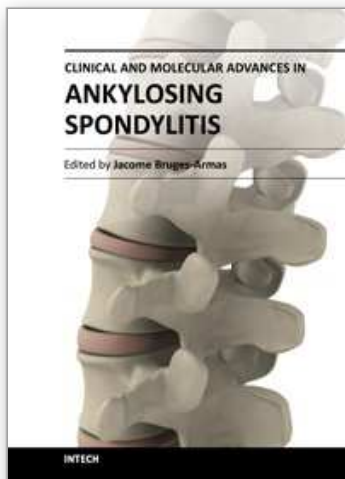
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