Clinical and laboratory markers of disease activity and response to therapy in South Africans with Rheumatoid Arthritis with disease duration less than two years

by

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in

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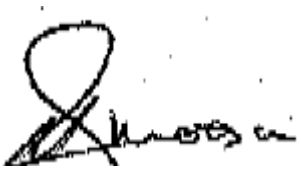
DECLARATION

I, Prof Mahmood MTM Ally , hereby declare that the work on which this research is based is original (except where acknowledgements indicate otherwise) and that neither the whole work nor part of it, is being or is to be submitted for another degree at this or any other university.



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“To start something good is your job and to see it completed is the work of your creator”.

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ABBREVIATIONS

A	Ab	Antibody
	ACPA	Anti-cyclic citrullinated peptides antibodies
	ACR	American college of rheumatology
	Ag	Antibody
	AP	Activator protein
	APC	Antigen presenting cell
	ASC(PYCARD)	Apoptosis-associated speck-like protein containing a CARD
	ATP	Adenosine tri-phosphate
	Anti-CarP	Anti-carbamylated protein
B	BAFF	B cell activating factor
	BLyS	B Lymphocyte stimulator
	BMD	Bone mineral density
C	CCL-	Chemokine (C-C motif) ligand -
	CCP	Cyclic citrullinated peptides
	CCR-	Chemokine receptor -
	CD	Cluster of differentiation
	COMP	Cartilage oligomeric matrix protein
	COX-2	Cyclo-oxygenase 2
	CQ	Chloroquine
	CRP	C-Reactive protein
	CTLA-	Cytotoxic T-lymphocyte Antigen -
	CTX	Carboxy-terminal collagen crosslinks
	CV	Cardiovascular
	CXCL-	C-X-C motif chemokine -
D	DAMP	Damage associated molecular pattern
	DAS	Disease Activity Score
	DC	Dendritic cell
	DMARD	Disease modifying anti-rheumatic drug
	DNA	Deoxy nucleic acid
E	EBV	Epstein barr Virus
	ECM	Extracellular matrix
	EDTA	Ethylene diamine tetra acetic acid
	ELISA	Enzyme-linked immunosorbent assay
	EGF	Epidermal growth factor
	ESR	Erythrocyte sedimentation rate
F	FDC	Follicular dendritic cells

	FEIA	Fluorescence immune assay
	FGF	Fibroblast growth factor
	FLS	Fibroblast-like synoviocytes
G	GCs	Germinal centres
	G-CSF	Granulocyte colony-stimulating factor
	GM-CSF	Granulocyte and macrophage colony-stimulating factor
	GP	Glycoprotein
	GREAT	Gauteng Rheumatoid Evaluation Assessment Trial
H	HDA	High disease activity
	HIV	Human immunodeficiency virus
	HLA	Human Leukocyte Antigen
	HSP	Heat shock protein
I	IC	Immune complexes
	ICAM-1	Intercellular adhesion molecule 1
	IFN	Interferon
	Ig-	Immunoglobulin -
	IHD	Ischeamic heart disease
	IL-	Interleukin-
	IMT	Intima medial thickness
	IP	Interphalangeal
J	JAK	Janus family tyrosine kinases
L	LDA	Low disease activity
	LPS	Lipopolysaccharides
	LT	Lymphotoxin
	LV	Left ventricular
M	MAPK	Mitogen-activated protein kinase
	MBDA	Multi-biomarker disease activity
	MCP	Metacarpophalangeal
	MCP-1	Monocyte chemoattractant protein 1 (renamed CCL2)
	M-CSF	Macrophage colony stimulating factor
	MCV	Modified citrullinated vimentin
	MDA	Moderate disease activity
	MHC	Major Histocompatibility Complex
	MIP-1	Macrophage inflammatory protein 1 (renamed CCL4)
	MiRNA	Micro ribonucleic acid
	MMP	Matrix metalloproteinases
	mRNA	Messenger ribonucleic acid
	MRI	Magnetic resonance imaging
	MTP	Metatarsophalangeal

	MTX	Methotrexate
	Mψ	Macrophages
	MYD 88	Myeloid differentiation primary response protein
N	NADPH	Nicotinamide adenine dinucleotide phosphate
	NET	Neutrophil extracellular trap
	NFAT	Nuclear factor of activated T-cells
	NF-κB	Nuclear Factor kappa B
	NK	Natural killer
	NLR	Nod like receptor
	NO	Nitric oxide
	NOD	Nucleotide-binding oligomerisation domain
	NSTEACS	Non ST segment elevation acute coronary syndrome
O	OPG	Osteoprotegerin
P	PAD-	Peptidylarginine deiminase -
	PAF	Platelet-activating factor
	PAMP	Pathogen associated molecular pattern
	PBMC	Peripheral blood mononuclear cells
	PCR	Polymerase chain reaction
	PDGF	Platelet-derived growth factor
	PG-	Prostaglandin -
	PIP	Proximalinterphalangeal
	PTPN22	Protein tyrosine phosphatase non-receptor type 22
R	RA	Rheumatoid arthritis
	RANK	Receptor activator for nuclear factor κ B
	RANKL	Receptor activator for nuclear factor κ B ligand
	RANTES	Regulated upon activation, normal T cell expressed, and secreted
	RASF	Rheumatoid arthritis synovial fibroblast
	RF	Rheumatoid factor
	RNA	Ribonucleic acid
	RNS	Reactive nitrogen species
	ROS	Reactive oxygen species
	rSSO	Reverse sequence specific oligonucleotide
S	SAA	Serum amyloid A
	SAPE	R-phycoerythrin-conjugated streptavidin
	SDAI	Simplified disease activity index
	SE	Shared epitope
	SENS	Simplified erosion and narrowing score
	SLE	Systemic Lupus Erythematosus

	SMAD	Small 'mothers against' decapentaplegic
	SNP	Single-nucleotide polymorphism
	sRANKL	Soluble receptor activator for nuclear factor κ B ligand
	ST	Synovial tissue
	STAT	Signal transducer and activator of transcription
	sTNF-R	Soluble tumour necrosis factor receptor
	SWEFOT	Swedish Pharmacotherapy Tiral
	SYK	Spleen tyrosine kinase
	SZP	Salazopyrin
T	TB	Tuberculosis
	TCR	T cell receptor
	TGF-	Transforming growth factor -
	Th0	Naive T helper cells
	Th-	T Helper cell, type -
	TLR	Toll-like receptors
	TLT	Tertiary lymphoid tissue
	TNF	Tumour necrosis factor
	TNFR	Tumour necrosis factor receptor
	TRAF	TNF receptor-associated factor
	T_{reg}	T regulatory cells
V	VAS	Visual assessment score
	VCAM-	Vascular cellular adhesion molecule -
	VEGF	Vascular endothelial growth factor
Y	YKL-	Human cartilage glycoprotein 39

SYNOPSIS

Rheumatoid arthritis (RA) is a chronic inflammatory disorder resulting in joint pain, stiffness and joint destruction. This can lead to considerable loss of physical and psycho-social function and even premature death. However, numerous advances in the management of RA have led to remarkable life-changing effects, especially when therapy is initiated early. Joint inflammation occurs following an abnormal immune response to some yet unidentified agent. The inciting agent or process is not well understood but exceptional advances have been made in characterising the molecular and cellular components of the ensuing inflammatory response. This has culminated in the identification of novel therapies and potential biomarkers to enhance patient care. Genetic and environmental factors play an important role with varying clinical manifestations and unpredictable outcomes on an individual basis. Globally, RA accounts for considerable morbidity and of particular concern in the developing world, is the lack of resources that may adversely affect outcome. There is a paucity of research on various aspects of RA in the developing world.

This study will review immune-pathogenic aspects of RA, the potential use of clinical features and serological biomarkers and report on aspects of the analysis of a prospective observational study of patients with early RA, prior to starting and 6 months following therapy.

A cohort of 171 early RA patients (disease duration less than 2 years) was recruited from the rheumatology clinics of two tertiary academic hospitals, located in Gauteng, South Africa. The objective of the study was to review clinical, demographic, radiological and serological aspects over a two year period. Outcome measures of this study included; i) characterisation of disease burden with respect to both physical and psycho-social aspects; ii) response of disease to therapy in routine care; iii) to characterize auto-antibody, cytokine, cartilage metabolites and genotype profiles associated with disease activity and response to therapy; and iv) clinical and serological measures associated with radiographic damage.

Clinical measures included the simplified disease activity index (SDAI) and functional scores (HAQ-DI and SF-36). At baseline and 6 months rheumatoid factor (RF), Anti-citrullinated peptide antibodies (ACPA), circulating cytokines and growth factors were measured. Cartilage metabolites COMP and matrix metalloproteinase (MMP)-3 were measured only at baseline. Genotyping for the shared epitope (SE) HLA-DRB1 allele was performed and classified according to the du Montcel classification. Radiographs of the hands and feet were taken at baseline and at yearly intervals and evaluated for erosive disease and scored according to the Larsen grading system.

The cohort recruited was mostly female (140) with a mean age of 47 years. Despite a mean symptom duration of 12 months, patients had severe disease with a mean SDAI of 39.4, with nodulosis and erosive diseases seen in 23% and 51 % respectively. Auto-antibody tests revealed high sensitivity (83%) and specificity (85%) for ACPA with the highest specificity (95%) if both RF and ACPA were positive. Shared epitope positivity was found in 92% of patients and correlated strongly with ACPA. Cytokine levels were globally increased favouring a T helper cell (Th)1, macrophage and pro-angiogenic cytokine profile in certain sub-groups. MMP-3 levels correlated with measures of disease activity but did not perform better than routine laboratory measures such as the C-reactive protein (CRP).

Serial measurement of auto-antibodies and cytokine profiles at 6 months revealed significant decreases in tandem with a decrease in disease activity. A more robust decline in cytokine levels was noted in patients who achieved a low disease activity state (SDAI<11) and in patients who received prednisone in addition to methotrexate (MTX). Of the auto-antibodies and biomarkers tested only MMP-3 was predictive (OR 2.8) of an SDAI>11 at 6 months.

Only 28% of patients achieved a low disease activity state at 12 months. Predictors of outcome at 12 months included low level of education, unemployment, radiographic damage and a high disease activity.

Conclusion: RA in sub-Saharan Africa is strongly associated with SE and ACPA status and a high disease burden at presentation. Despite therapy, only a small percentage of patients achieved an acceptable level of disease activity in routine care underscoring the need for a more aggressive therapeutic approach. Serial measurement of auto-antibodies and cytokines had limited clinical value in routine patient care, but may guide future therapies with the potential to help in stratifying patients. The measurement of serum MMP-3 at baseline may identify a subgroup of patients with a poorer prognosis and hence allow for a more intensive therapeutic approach from the outset.

CHAPTER 1

1.1 Introduction

Rheumatoid arthritis is a chronic inflammatory arthritis of unknown aetiology. An autoimmune pathogenic mechanism is central to disease initiation and perpetuation, with inflammation and tissue destruction resulting in a vicious cycle, leading to disease persistence and progression.[1] Recent insights into the molecular pathways characterizing these pathogenic processes have not only led to numerous innovative therapies, but have also provided a better understanding of the immune-inflammatory response. RA is an archetypal autoimmune disease, which is associated with other physiological and pathological conditions such as aging, osteoporosis, cardiovascular disease and malignancies.

1.1.1 The Immune Response

The innate and adaptive systems are two components of the immune system which act in concert to defend against foreign infections and promote healing following an injury. The innate response is the initial non-specific response to foreign antigen comprising cells of the macrophage monocyte lineage, dendritic cells, neutrophils, and complement. Macrophages and dendritic cells not only phagocytose / pinocytose foreign agents but also process the antigen for presentation and activation of the adaptive immune system. Macrophages demonstrate remarkable plasticity depending on the nature of the stimulus, resulting in anti- or pro-inflammatory responses. Dendritic cells share many characteristics of macrophages and are key antigen presenting cells, establishing an important link with the adaptive immune response.[2] Surface and cytosolic toll like receptors (TLR), as well as cytosolic nucleotide-binding oligomerisation domain (NOD) like receptors (NLR) of dendritic and other antigen presenting cells, recognize and interact with numerous pathogen-associated molecular patterns (PAMPs) and damage-associated patterns (DAMPs). The adaptive response is a delayed, but specific response, comprising of T and B lymphocytes. The adaptive response is initiated by antigen presentation and

activation of naive T cells. Depending on the stimulus and host environment, T cells differentiate into Th1 (cell-mediated response), Th2 (humoral /antibody-mediated response), Th17 (pro-inflammatory response) and regulatory T cells (Tregs) (inhibitory response). T cell activation results in a specific response and retention of memory to a particular antigen.

A crucial host factor is the major histocompatibility complex (MHC) or human leukocyte antigen (HLA) system which is encoded by the chromosomal area important for self-recognition, tolerance and displaying of antigens to T cells. The MHC is classified into 3 classes I, II and III. Class I comprises three groups A, B, C and class II is a single group, D, that is further subdivided into three, DP, DQ, and DR. Class I is found on all nucleated cells and plays a key role in self-tolerance and protection against viral infections. Class II is displayed on antigen presenting cells involved in regulating the immune response. Class III consists of genes encoding complement proteins and certain cytokines.

Functional changes to DNA without alterations in structure occur through biochemical processes resulting in epigenetic modulation of protein transcription. These biochemical changes include methylation and acetylation that can influence gene expression. Epigenetic changes may also occur following coupling and uncoupling of chromosomal nucleotides with certain polypeptides such as ubiquitin and ubiquitin-like peptide.[3]

Cytokines are molecular messengers which play an integral role in the cross-talk between various components of the immune response and host, allowing for a co-ordinated and regulated response. Numerous intracellular signaling domains respond to these molecular signals and modulate the cellular response. Genetic factors including genotype and epigenetic changes often influence the ultimate cellular response.

Cellular and molecular components of the immune-inflammatory response are subject to intensive research in the field of rheumatology. An ever-expanding knowledge of

these networks has led to an in-depth understanding of pathogenic mechanisms, potential biomarkers and the development of novel therapies.

A more detailed review of some of these components follows.

1.1.2 Cytokines and intracellular signaling domains

Cytokines are molecular messengers which promote communication between various components of the immune response, working in an autocrine and paracrine manner. Functional heterogeneity is the order of the day with tremendous plasticity and elements of redundancy. Components can be classified according to predominant functional responses (pro-inflammatory/anti-inflammatory), structural similarities, cells of origin (Th1/Th2/Th17) or production by innate or adaptive immunity. The origins and functions of major cytokines are shown in Figure 1.

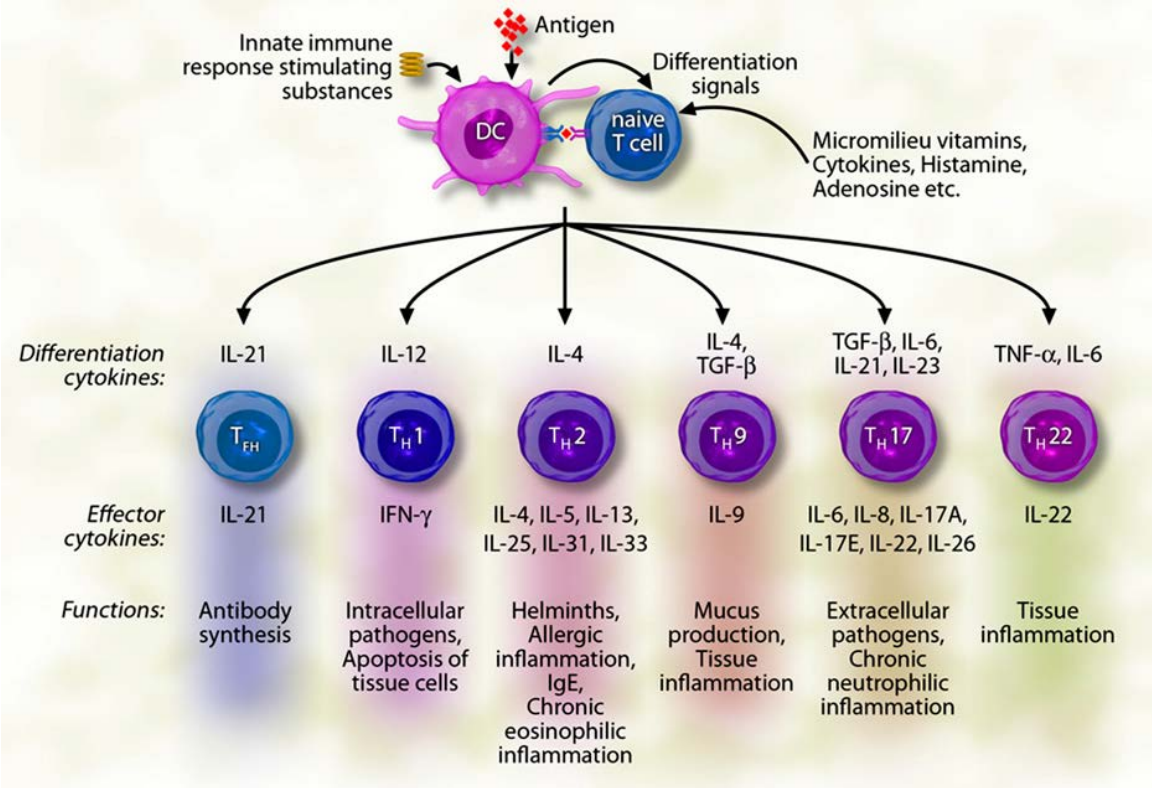

Fig 1. Antigen presentation by DCs to naive T cells and other factors (innate immune response substances, vitamins, cytokines in the environment) induces the T cells to produce ILs and differentiate into TH1, TH2, TH9, TH17, TH22, or follicular TH (TFH) cells. These T-cell subsets can promote different types of inflammatory responses on the basis of their respective cytokine profiles, responses to chemokines, and interactions with other cells.from : J Allergy Clin Immunol. 2011; 127(3):701-721).[with permission]

Cytokines mediate their effects through receptors followed by intracellular signal transduction and gene transcription. This affects gene expression, cell survival and movement.

1.1.3 Cytokine receptors

There are 5 different families of receptors based on differences in their structures: i) the four alpha helix bundle cytokine family, ii) Tumour necrosis factor (TNF) receptor family, iii) Transforming growth factor (TGF) family, iv) Interleukin (IL)-1 family and v) IL-17 family.[4]

Intracellular signaling is mediated by membrane bound or cytosolic domains consisting of enzymes and structural proteins; once activated they initiate a cascade of chemical or conformational changes that activate transcriptional factors; intracellular changes include protein phosphorylation, ubiquitination, sumoylation, degradation and translocation.[5] Phosphorylation is often mediated by kinases while ubiquitination involves the attachment of a 76 amino acid protein called ubiquitin. Sumoylation is related to the interaction with a ubiquitin-like peptide. Some of the intracellular protein complexes include kinases, adaptor proteins, and TNF receptor-associated factor (TRAF). Transcriptional proteins include nuclear factor kappa B (NFκB), signal transducer and activator of transcription (STAT), nuclear factor of activated T-cells (NFAT) and activator protein (AP)-1, culminating in the expression of the relevant effector gene. Intracellular signaling is essential not only for the response to cytokines, but also for activation or modulation by cell-cell contact, and responses to co-stimulatory ligands and antigenic peptides (Figure 2). Several different extracellular signals share the same signal transduction pathways or transcription proteins, contributing to the complexity and redundancy seen.

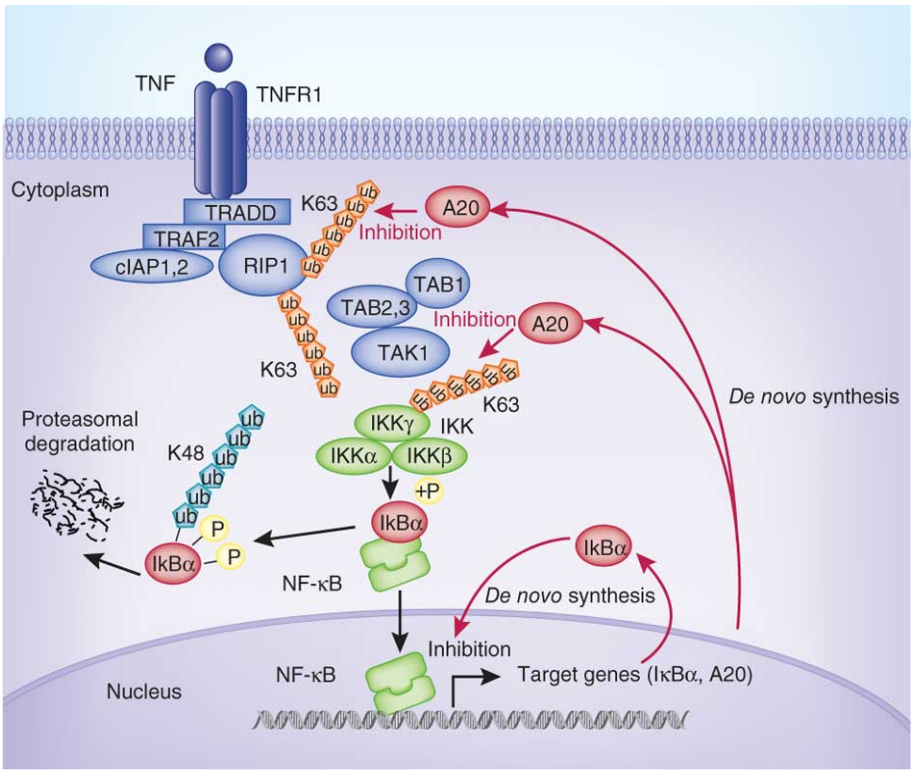

Fig 2. Activation of NF-κB by TNF - Signal transduction through TNF receptor domains such as TRAF 2 result in NFκB mediated transcription of genes by TNF. Intracellular processes include phosphorylation,ubiquitination and sumoylation: from Jürgen Ruland Nature Immunology12,709–714(2011)[with permission]

Clinical trial outcomes in patients with various autoimmune diseases, using therapeutic modalities targeting components of the immune-inflammatory response underscore their involvement as dominant role players in the immune response.

1.1.4 Key cytokines and their signaling pathways

1.1.4.1 Pro-inflammatory

IL-1 beta

IL-1 beta is important in the defense against pathogens, especially intracellular microorganisms such as *Mycobacterium tuberculosis*; however, elevated levels are also found in many pathological conditions. Signal transduction occurs after binding to surface IL-1 R1 receptors, resulting in synthesis of an inactive pro-form through

activation of the NFκB pathway following interaction with the adaptor protein myd88, several kinases and TRAF 6. The inactive pro-form is activated by the enzyme caspase 1. Caspase 1 requires activation through a protein complex called the inflammasome.[6] Inflammasome activation is promoted by a decrease in intracellular potassium levels related to increased levels of adenosine triphosphate (ATP) released by macrophages extracellularly.[7] Stimulation of antigen presenting cells by PAMPs or DAMPs can also result in the activation of caspase 1 through interactions with the inflammasome adaptor protein, apoptosis-associated specklike protein [ASC(PYCARD)].[6] IL-1 activation results in a host of inflammatory actions, T and B cell differentiation, and production of other cytokines such as IL-6 and IL-17.[8] The effects of IL-1 beta are kept in balance by the production of IL-1Ra and the presence of decoy, biologically-inert IL-1 receptor type II (IL-1R II).

IL-6

This is a key cytokine involved in hematopoiesis, the acute phase response and inflammation. Signal transduction is mediated through a cell surface complex of IL-6 and its receptor Glycoprotein (GP) 130. This then undergoes phosphorylation by Janus family tyrosine kinases (JAK), followed by transcription via STAT 3. IL-6 expression is regulated by negative feedback of intracellular signaling pathways and transcription. IL-6 is produced by many cells with endothelial cells, monocytes/ macrophages and fibroblasts being the key producers during inflammation. IL-6 promotes neutrophil movement, macrophage maturation, B cell antibody isotype switching and differentiation into plasma cells. IL-6 also promotes inflammation by inducing Th17 cell differentiation from naïve Th (Th0) cells and Treg cells.[9,10]

IL-7

IL-7 regulates T cell proliferation and cytokine production [interferon (IFN)-γ and TNF], driving a Th1/Th17 response, as well as cytotoxic CD 8^+ T cell and natural killer (NK) cell proliferation.

IL-7 also contributes to differentiation of naïve and memory B cells. After binding to its surface receptor IL-7Rα, intracellular signaling pathways include JAK 3 and STAT transcription.[11]

IL-8

This cytokine belongs to the sub-family of chemotactic cytokines termed chemokines; the primary role of IL-8 is chemotaxis of neutrophils, NK cells, and T cells, as well as in angiogenesis at sites of inflammation. IL-8 transcription is a rapid process following activation of mitogen-activated protein kinases (MAPK), with NFκB and AP-1 as transcription factors.[9]

IL-17

IL-17 and its corresponding receptor IL-17R form a distinct group with a diverse set of pro-inflammatory manifestations through MAPK/NFκB intracellular signaling. IL-17 comprises of 5 subtypes A to E with IL-17A being the predominant subtype.Production is predominantly from a specific T cell subset called Th17 cells, but is also produced from a host of other cells such as CD 8^+ T cells, NK cells, neutrophils, fibroblasts, endothelial cells and B cells. IL-17 effects are mediated by inducing the release of other cytokines such as IL-1, IL-6, IL-8, TNF, granulocyte and macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and monocyte chemoattractant protein (MCP)-1 as well as matrix metalloproteinases (MMPs). IL-17 plays a crucial role in host defense against extracellular pathogens such as *Klebsiella pneumoniae, Toxoplasma gondii*, *Porphyromonas gingivalis* and *Candida albicans.*[9]

IFN-γ

This cytokine is produced by cells of both the innate and adaptive components of the immune response. IFN-γ mediates its effects via binding to 2 cell surface receptors and JAK/STAT signal transduction. It is produced by macrophages, NK cells, Th1 and B cells. Production is stimulated by cytokines such as IL-12 and IL-18 from APC, while inhibition of synthesis is mediated by IL-4, IL-10, TGF-β and glucocorticoids. IFN- γ primarily drives a Th1 response and inhibits Th2 differentiation. Augmentation of the inflammatory response occurs through regulation of endothelial leukocyte interaction and upregulation of chemokine production and adhesion molecule expression. Upregulation of MHC class I molecules by IFN-γ facilitates eradication of

intracellular pathogens, while upregulation of MHC class II in APC promotes $CD4^+$ T cell activation.[9]

TNF

Inhibitors of TNF represent one of the most successful class of biologic agents in use clinically for immunotherapy of immune-mediated inflammatory diseases. This underscores the importance of this cytokine in the immune inflammatory response. There are numerous cell surface and circulating soluble receptors [tumour necrosis factor receptor (TNFR)] regulating cell activation. Depending on the stimulus and intracellular signaling pathway, TNF mediates cellular proliferation, secretion of cytokines and apoptosis.[12] The resultant stimulation of both the innate (macrophages, NK cells) and adaptive immune systems (T and B cells) results in TNF production. Signal transduction through TNF receptor domains such as TRAF 2 and MAPK result in NFκB mediated transcription of genes, augmenting inflammation via induction of cytokines such as IL-1, IL-6, which in turn promote neutrophil chemotaxis, macrophage activation and adhesion molecule expression.

1.1.4.2 *Anti-Inflammatory*

IL-10

This cytokine is produced predominantly by Tregs and is a potent inhibitor of inflammation inhibiting the secretion of numerous cytokines and chemokines. It is also produced by B cells, macrophages and dendritic cells. IL-10 decreases the expression of surface MHC class II molecules on APC and inhibits T cell co-stimulation. Intracellular signaling following Il-10/IL-10R binding is via the JAK/STAT pathway.[9,13]

TGF-β

Secreted TGF-β circulates as a large inactive complex that requires proteolytic activation by plasmin, MMPs and thrombospondin 1 to bind to its surface receptors. Potent immune-regulatory effects are then mediated by various kinases and small ‘mothers against’ decapentaplegic (SMAD) transcription factors. Inhibitory effects

include prevention of NFκB translocation into the nucleus, thus downregulating the expression of numerous pro-inflammatory cytokines.[14] TGF-β promotes B cell isotype switching to immunoglobulin (Ig) A production. Secreted predominantly by Tregs, TGF-β inhibits Th1and Th17 cell differentiation. It is also secreted by macrophages and dendritic cells to, acting negative feedback control.

Dendritic cells

These are cells of the innate immune system with branch-like projections which play an important role in self-tolerance and the immediate immune response to foreign antigen. These cells originate from haematopoietic stem cells and circulating monocytes. Three different types have been characterized: i) classical; ii) plasmacytoid; and iii) inflammatory type.

The classical dendritic cells are resident in the interstitial space, skin (Langerhans cells), spleen and lymphoid organs. Dendritic cells are also found in the lungs and gastro-intestinal tract, but are absent in the brain parenchyma with microglia fulfilling this function.[15] Plasmacytoid dendritic cells play an important role in defense against viruses though the immediate release of type I interferon (IFN-I). Inflammatory dendritic cells concentrate at sites of inflammation and release mediators such as TNF and reactive oxygen species.[16] Dendritic cell activation requires interaction with antigen through non-specific receptors, such as Toll-like receptors (in the membrane and cytosol) and NOD-like receptors (cytosolic). As mentioned earlier, these receptors recognise not only PAMPs, but also damaged endogenous components (DAMPs). Antigen uptake and processing promotes presentation of the antigen to the adaptive immune system. These cells are also capable of migrating to draining lymph nodes through the action of certain chemokines, enabling the stimulation of the adaptive immune response. The degree of response and type of T cell differentiation depends on antigenic stimulus, dendritic cell subset and cytokine profile. Dendritic cells mediate some of these effects through up regulation of adhesion molecules, co-stimulatory ligands and the secretion of a host of cytokines such as TNF, IL-2, IL-4, IL-6, IL-10, IFN-γ and TGF-β.[17]

Macrophages

Together with dendritic cells, macrophages are key players in antigen processing and presentation to the adaptive immune system. Macrophages originate from cells of the monocyte lineage and influence numerous physiological and pathological processes effected predominantly by phagocytosis and cytokine release. Macrophage differentiation in the bone marrow is predominantly controlled by two colony stimulating factors, macrophage colony stimulating factor (M-CSF) and GM-CSF, with further differentiation influenced by antigenic stimulus and host factors, resulting in phenotypes expressing different functions of tissue repair, host defense and immune-regulation.[11] These different functions can be classified into two subtypes M1 (pro-inflammatory) and M2 (anti-inflammatory or homeostatic). M1 macrophages are activated by lipopolysaccharides (LPS), IFN-γ, TNF and GM-CSF; alternatively M2 macrophages are stimulated by IL-4, IL-10, IL-13, immune complexes and M-CSF. Activated M1 macrophages release reactive oxygen species (ROS), nitric oxide (NO), IL-6, and TNF, while upregulating surface receptors and adhesion molecules; in addition they play an important role through phagocytosis, clearing foreign antigens, debris and apoptic material.[18]

Neutrophils

These cells are involved in the initial response to bacterial and fungal infections. Movement of neutrophils to sites of inflammation requires migration through the endothelium enabled by various chemokines and adhesion molecules. Surface receptors such as Fc- receptors, β2 integrins and selectins mediate neutrophil adhesion and responses via intracellular signaling using specific kinases such as spleen tyrosine kinase (Syk). Notwithstanding generation of ROS numerous enzymes capable of degradation of proteins and cell wall components are present in neutrophil granules. Another mechanism which protects against foreign pathogens is a distinct form of ‘programmed’ cell death resulting in release of net-like fibers, a process called NETosis. These fibers contain DNA, histones and components of neutrophil granules. These extracellular fibers trap and kill bacteria. Neutrophil extracellular trap (NET) formation can be stimulated by bacterial LPS, ROS, IL-8, platelet activating factor

(PAF), C5a, GM-CSF, IFN-α and -γ. An important step in NET induction is the generation of ROS, resulting in citrullination of arginine in histones via peptidylarginine deiminase (PAD)1. As nuclear material is also released within the extracellular traps the neutrophil dies.[19]

Neutrophils also produce cytokines that promote further recruitment of neutrophils amplifying the inflammatory response.

Complement

The complement system is an integral component of the innate immune system consisting of circulating and cell-bound proteins and glycoproteins. Components are produced mainly in the liver, but are also produced by cells of the monoctyte lineage. Complement proteins act in a domino-like manner, culminating in opsonization and/or lysis of target pathogens. Three different activating pathways classical, alternative and lectin-binding, are induced by different antigens in the presence or absence of antibodies. Antigenic stimulants of the three pathways include:

i) Classical antigen/antibody (Ag/Ab) complexes, nucleic acids, chromatin, cytoplasmic and mitochondrial components, cardiolipin, CRP, amyloid, bacterial cell wall components and synthetic particles such as polymers.

ii) Alternate-carbohydrate moieties on bacteria, fungi, parasites and Ag/Ab complexes.

iii) Lectin- carbohydrate moieties (mannan-binding lectin) found on many microorganisms.

All three pathways result in activation of C3 and C5, culminating in the formation of the membrane attack complex (MAC), forming a pore in the target cell membrane initiating cell lysis[20] (Figure 3). Activated C3 and C5 are potent mediators of inflammation and opsonization; additionally C5 promotes chemotaxis of neutrophils via generation of C5a.[16] These events are summarized in Figure 3.

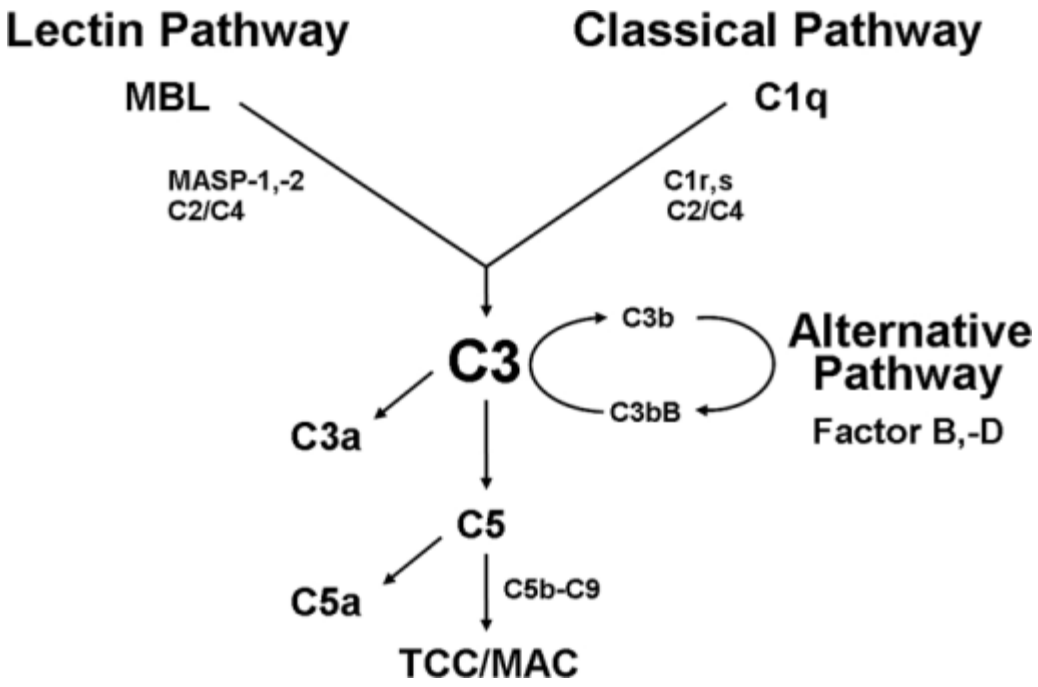

Fig 3. Complement pathways Diagram outlining the three complement pathways. MBL = mannose-binding lectin; MASP = MBL-associated serine protease; TCC = terminal complement complex; MAC = membrane attack complex: from http://openi.nlm.nih.gov/ Busche MN, Stahl GL - Ger Med Sci 2010 [open access]

T cells

The primary event in the stimulation of the adaptive immune response is T cell activation by the innate immune system. The antigenic stimulus and host factors determine the pathway of T cell differentiation, *viz.* $CD4^+$ T helper 1, 2 or 17, or CD 8^+ T cytotoxic lymphocytes. Antigen presentation in an IL-12 cytokine milieu promotes Th1 development, resulting in cell-mediated immunity against intracellular microorganisms through release of cytokines such as IFN-γ and IL-2. These events are counter balanced by production of the inhibitory cytokine IL-10.

Antigen presentation in an IL-4 milieu facilitates Th2 differentiation with production of IL-4, IL-5, IL-10, and IL-13, allowing for antibody or humoral immunity. This pathway is particularly important for protection against helminthes and certain protozoa. Activation of T cells also requires co-stimulation with antigen presenting cells (APCs)

through interaction of CD28 on T cells and CD80/CD86 on APC. Th1 inhibits Th2 differentiation via IFN-γ and vice versa Th2 inhibits Th1 via the production of IL-4.

Th17 responses are initiated by an IL-1, IL-6, IL-21, IL-23, TNF and TGF-β milieu. The Th17 response plays a role in the defense against extracellular Gram-negative bacteria and Candida infections.

TGF-β plays an important role in keeping the inflammatory response in check by promoting differentiation of Treg cells.

Dysregulated T cell differentiation results in a host of autoimmune or chronic inflammatory conditions such as RA (Th1/Th17) and systemic lupus erythematosus (SLE) (Th2).[21]

B cells

The primary function of these cells in the immune response is to produce antibodies which promote clearance of antigens. B cells also have a potent immune-modulatory function in both the innate and adaptive components of the immune system. Activated T cells bind to antigen-presenting B cell receptors promoting B cell differentiation to produce cytokines and specific antibodies.

B cells originate from marrow B lymphocyte precursors that differentiate into different stages of maturity and can be identified by surface markers called cyclo-oxygenase (COX)-2 of differentiation (CD) markers. Transitional and mature B cells are APCs and produce cytokines such as IL-1, IL-6, TNF and counter-regulatory IL-10 and TGF-β. B cell survival in the peripheral circulation requires the specific cytokine BAFF/ BLyS (B cell activating factor /B lymphocyte stimulator).

Some B cells are capable of generating antibodies independently of T cell help. These are innate-like B cells which are able to produce antibodies via interactions with antigens early in the immune response, producing so-called natural antibodies protective against pathogens. These innate B cells are also self-reactive, and are

thus potentially implicated in autoimmune disease pathogenesis .[22] IL-10 production by B cells is an important regulatory cytokine mechanism, decreasing secretion of TNF, and IL-1, as well as APC co-stimulatory molecule expression.[23]

Mature B cells differentiate into professional Ab-producing plasma cells or into B memory cells, the latter responding quickly on re-challenge with the same antigen. Five isotypes of antibodies are produced by B cells *viz.* IgA, IgD, IgE, IgG and IgM, with isotype shifting dependent on both the antigenic stimulus and cytokine milieu, (IL-4, IL-13→ IgG and IgE/ TGF-β→ IgA/ IL-10 →memory B cell differentiation/ IL-21→ IgG).[24]
Despite the complexities and perceived redundancies within the immune response a coordinated and efficient response occurs, resulting in maintenance of normal homeostasis in an always challenging environment. However, when dysregulated these events may result in a variety of diseases including chronic infections, autoimmune disorders and malignancies.

1.2 Pathogenesis of Rheumatoid Arthritis

Overview

Immune dysregulation as a consequence of genetic and environmental factors is the key driver of chronic inflammation in RA. The HLA system is important for immune tolerance with specific, genetically determined amino acid sequences associated with certain diseases.[25] In RA this sequence is termed the “shared epitope” and is found in around 80% of patients. Presence of the shared epitope (SE) varies in different ethnic groups, with some studies showing a lower prevalence in Black African patients; however, a recent South African study found as many as 83% positive for the SE in an early RA cohort, similar to that seen in Caucasians.[26]

The presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated peptide antibodies is evident even before onset of clinical disease.[27] However, the inciting antigen is unknown, with infective agents such as parvovirus B19 and an

organism causing gingivitis (*Porphyromonas gingivalis*) having been implicated as possible triggers.[27] Smoking has recently received much interest in the possible initiation of a chemical change in lung tissue – a process called "citrullination".[28] Citrullination involves a chemical change in certain proteins containing the amino acid arginine which is converted to citrulline. This immunogenic citrullinated protein is now exposed to the immune system and in genetically predisposed individuals' results in the generation of anti-citrullinated peptide antibodies (ACPA). Citrullination also occurs in the rheumatoid synovium where these antibodies, may not only initiate disease, but might also account for disease persistence.

Inflammation and tissue destruction may contribute to increasing the antigenic load, resulting in auto-inflammation and ongoing disease. The synovial pannus is characterized by new vessel formation, activation of cellular and molecular components of the immune response and abrasive changes to adjacent cartilage and bone (Figure 4). Joint destruction is mediated predominantly by pro-inflammatory cytokines such as tumour necrosis factor (TNF), IL-1, IL-6 and IL-17, which stimulate synovial fibroblasts, chondrocytes and oseoclasts. Bony changes in RA include focal erosions, peri-articular osteopenia and generalized osteoporosis. Cytokines such as receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) play a key role in bone resorption with elevated MMP activity mediating enzymatic degradation of cartilage.

Once the immune system is activated disease persistence is maintained by as yet unexplained mechanisms. Phenotypic changes in synovial cells, particularly synovial fibroblasts, may play a critical role.[29] The synovial pannus acts in an autonomous almost tumour-like fashion, generating pro-inflammatory cytokines, which result in synovial proliferation and consequent local and systemic effects.[6]

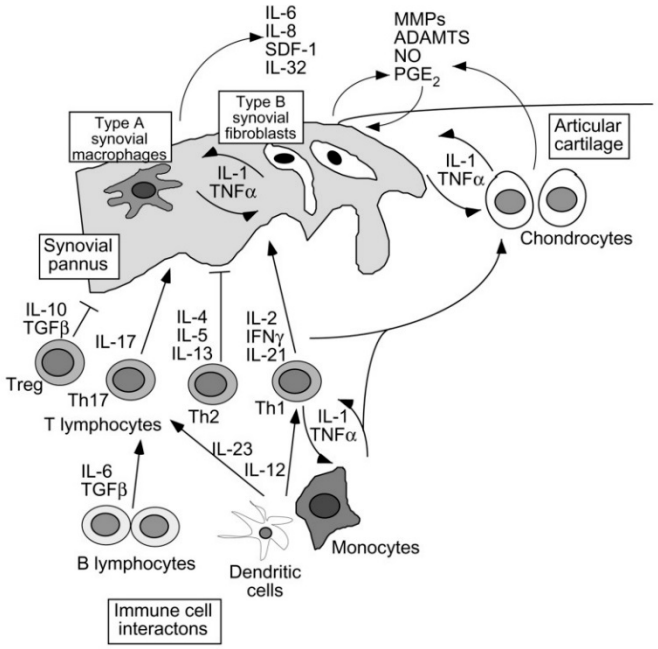

Fig 4. Cytokine networks and cellular interactions in cartilage destruction in rheumatoid arthritis. This scheme represents the progressive destruction of the cartilage associated with the invading synovial pannus in rheumatoid arthritis. As a result of immune cell interactions involving T and B lymphocytes, monocyte/macrophages, and dendritic cells, several different cytokines are produced in the inflamed synovium as a result of the influx of inflammatory cells from the circulation and synovial cell hyperplasia. The upregulation of proinflammatory cytokines produced primarily in the synovium, but also by chondrocytes, results in the upregulation of cartilage-degrading enzymes, of the matrix metalloproteinase (MMP) and ADAM with thrombospondin-1 domains (ADAMTS) families, at the cartilage–pannus junction. Chemokines, nitric oxide (NO), and prostaglandins (PGs) also contribute to the inflammation and tissue catabolism. SDF, stromal cell-derived factor 1; TNF, tumor necrosis factor; TGF, transforming growth factor; IFN, interferon; Treg, regulatory T lymphocytes; Th, T helper cells: from Otero and Goldring *Arthritis Research & Therapy* 2007 **9**:220 doi: 10.1186/ar2292)[open access]

1.2.1 Genetics of Rheumatoid Arthritis

Certain genetic studies have reported a concordance of around 15% in monozygotic twins suggesting other factors may be more significant, but taking into account the prevalence of RA overall heritability accounts for 40-65% of RA susceptibility.[31] Multiple gene loci and epigenetic factors superimposed on environmental factors influence not only susceptibility, but also severity and response to therapy.

The HLA (human leukocyte antigen) family of genes is one of the most important genetic determinants of RA. These genes code for proteins expressed almost

exclusively on the surface of APC such as macrophages and dendritic cells allowing for a matching fit with inciting antigen and presentation to $CD4^+$ T cells.[16]

A common or shared amino acid sequence, RAA (R-arginine, A-alanine), at position 72 to 75 of certain HLA-DR 1 (HLA-DRB1*01) and –DR 4 (HLA-DRB1*04) alleles has been shown to confer an increased risk for RA.This region, as mentioned above, is temed the shared epitope (SE) and confers a measure of susceptibility influenced by two adjacent amino acids at positions 71 and 72. The QKRAA, QQRAA and KKRAA (Q-glutamine, k-lysine) sequences increase the risk of RA, while the DERAA (D-aspartic acid, E- glutamic acid) sequence has a protective effect.[32,33] A new classification based on the amino acid sequences adjacent to the SE has been validated. Depending on the amino acid sequence, groups are classified as S1, S2, S3P, S3D and X. The S2 and S3P alleles are associated with increased RA susceptibility. The low risk alleles S1, S3D and X are pooled together as L alleles. Certain non SE HLA-DRB alleles (HLA-DRB1*15, HLA-DRB*1301, -DRB*1302,-DRB1*1304) have also been shown to be associated with an increased susceptibility to RA and have been incorporated into the S1 group.[34,35]
The SE HLA alleles account for 11% to 37% of RA heritability particularly in patients homozygous for the risk allele.[32] The presence of SE increases the risk of extra-articular manifestations and disease progression.

1.2.2 Non–HLA genes

Genome-wide association studies have also implicated a host of other, albeit less frequent, associations with RA including polymorphisms of molecules involved in:

i) Intracellular signalling [protein tyrosine phosphatase non-receptor (PTPN22), TRAF1, STAT4]
ii) T cell co- stimulation or B cell interaction [CD28, CD40, cytotoxic T-lymphocyte antigen (CTLA4)],
iii) Cytokines that regulate activation of T cells [IL-2, IL-21, IL-23R, TNF, Chemokine receptor (CCR) 6]
iv) Enzymatic conversion of arginine to citrulline (PAD14).

Notwithstanding HLA-DRB1, the genes coding for PTPN22 and IL-23R show the strongest association with RA susceptibility.

1.2.3 Epigenetic factors

Not only the DNA sequence, but also chemical, or so called epigenetic modifications to genes, modulate the immune response. These changes are heritable and some environmentally induced changes may be reversible. Chemical changes include DNA methylation and post translational modification of chromatin structure by acetylation, phosphorylation, methylation, ubiquitination and sumoylation.[36,37]

DNA methylation is an important inhibitory signal for gene expression and hypomethylation has been demonstrated in synovial fibroblasts, certain cytokine promoter genes (IL-6, IL-10) and micro ribonucleic acid (miRNAs) in patients with RA (Figure 5). MiRNAs do not code for any peptides, but rather affect gene expression by blocking the binding site of the target messenger ribonucleic acid (RNA). Abnormal miRNAs and consequent downstream effects have been demonstrated in RA. In the rheumatoid arthritis synovial fibroblast (RASF), this results in over-expression of MMP 1, IL-6 and a change to a tumour like phenotype.[36,37] In addition to effects on the RASF, acetylation/ de-acetylation of histones also affects transcription factors such as NF-kB and secretion of cytokines such as TNF and IL-6.[36]

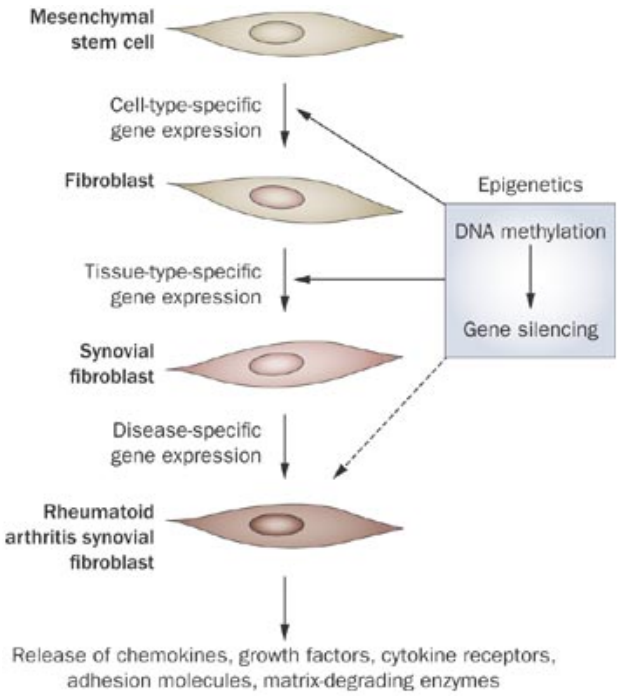

Fig 5. RA synovial fibroblast: from *Nature Reviews Rheumatology* 5, 266-272 : May 2009 [with permission]

1.2.4 Non genomic factors

As mentioned above, smoking is one of the strongest environmental factors increasing the risk and severity of RA. The risk of RA in smokers increases with longer duration of smoking and amount smoked. The equivalent of more than a 40 pack year history is associated with doubling of the risk for RA in comparison to non-smokers. The increased risk remains significant for greater than 20 years after stopping cigarette smoking.[38] Smoking has been associated with a specific sub-type of RA namely ACPA-positive RA, particularly in SE positive patients. However, there may be ethnic differences as a study in African Americans did not show an association between smoking status and ACPA.[39] In patients homozygous for the SE, the risk for RA is increased almost 21-fold increase compared to SE-negative non-smokers. The initial event may be distal to the joints, possibly in the lungs wherein smoking has been shown to increase citrullination.[38] Citrullinated peptides fit more easily into the antigen binding sites of APCs, initiating the cascade of events to follow.

A whole host of RA-associated antibodies has been described, probably accounting for the varied clinical phenotype evident in practice. Two well-characterised autoantibodies, RF and ACPA, are found long before the onset of clinical disease as mentioned earlier.

RF is an antibody which binds to the Fc portion of IgG. Sero-positivity for RF is associated with disease severity. The pathogenic role of RF seems to be through immune complex-mediated complement activation and generation of chemotactic factors. RF is found in 80 to 90 % of patients with established disease. Most are of the IgM and IgG isotypes with IgE RF being associated with extra-articular manifestations and mast cell activation. Routine laboratory assays usually detect IgM RF.

ACPA are found in about 12-15% of patients who are sero-negative for RF. The specificity of ACPA for RA is around 90% and even higher if RF is positive. ACPA are also found long before the onset of disease and a “second hit” such as an infection or trauma resulting in citrullination of joint tissue may lead to expansion of the affinity of the initial ACPA autoantibodies, so-called “epitope spreading,” reaching a critical threshold, culminating in clinical disease. The increase in isotypes occurs just prior to disease onset. ACPA-positive patients with the highest titers manifest radiographic progression and RA-associated morbidity. Patients positive for the SE are more likely to be ACPA positive, with ACPA-negative disease having a different pathogenic mechanism and phenotype.

Perodontitis associated infection due to *Porphyromonas gingivalis* results in citrullination of proteins through a bacterial PAD enzyme. Enolase, a metalloenzyme involved in glycolysis, when citrullinated, may interact through molecular mimicry with ACPAs produced in response to citrullinated bacterial enolase.[40] Other infections such as those caused by mycoplasmas, parvovirus B19, retroviruses, mycobacteria, Epstein Barr virus (EBV) and bacterial cell wall components may initiate the immuno-pathology seen in RA through stimulation of TLRs. Animal models of arthritis implicate TLRs 2, 4 and 9 . Mycoplasmas can induce cytokine release from macrophages initiating or exacerbating arthritis in murine arthritis models.[41] EBV may

induce arthritis through the mechanism of molecular mimicry as the EBV glycoprot GP 110 contains the amino acid sequence of the SE, QKRAA. Parvovirus B19 genes have also been demonstrated in RA synovial tissue, possibly activating RASF.[42]

Hormonal factors may also account for the female preponderance of RA, as estrogen has been shown to render autoreactive B cells more resistant to apoptosis while stimulation of estrogen receptors on fibroblast-like synoviocytes (FLS) and macrophages increases production of MMPs and TNF. Pregnancy is associated with remission in around 75% of patients, possibly due to increased expression ofIL-10; however, 90% of patients experience flares in the post-partum period. The flare is usually associated with a rise in RF titre. Breast feeding for longer than 13 months has also been shown to be associated with a decreased risk of developing RA.

The role of red meat, vitamin D, and oral contraceptives in the pathogenesis of RA is controversial. Studies have demonstrated an inverse relationship between socioeconomic status and education level with susceptibility of developing RA.[38] The interaction of environmental and genetic aspects seems pivotal in the pathogenesis of RA resulting in chronic inflammation, primarily in the synovium with ensuing articular and extra-articular manifestations.

1.2.5 Synovial Inflammation in RA

The pathological immune response triggers a cascade of cellular and molecular mediators initiating a vicious cycle of interconnected events- immune dysregulation, cellular proliferation, disease perpetuation and tissue damage[43] (Figure 6). Inflammation results in the classical synovial pannus and bone marrow changes consisting of cellular proliferation and new vessel formation. Cellular proliferation includes sub-synovial areas of T and B cell aggregates resembling lymphoid tissue capable of responding to local antigenic stimulation. Also present in the sub-synovial area are macrophages, plasma cells and neutrophils. The synovium itself is hypertrophied consisting of macrophage and fibroblast like synoviocytes. Cellular communication is mediated by a host of cytokines, chemokines and growth factors.

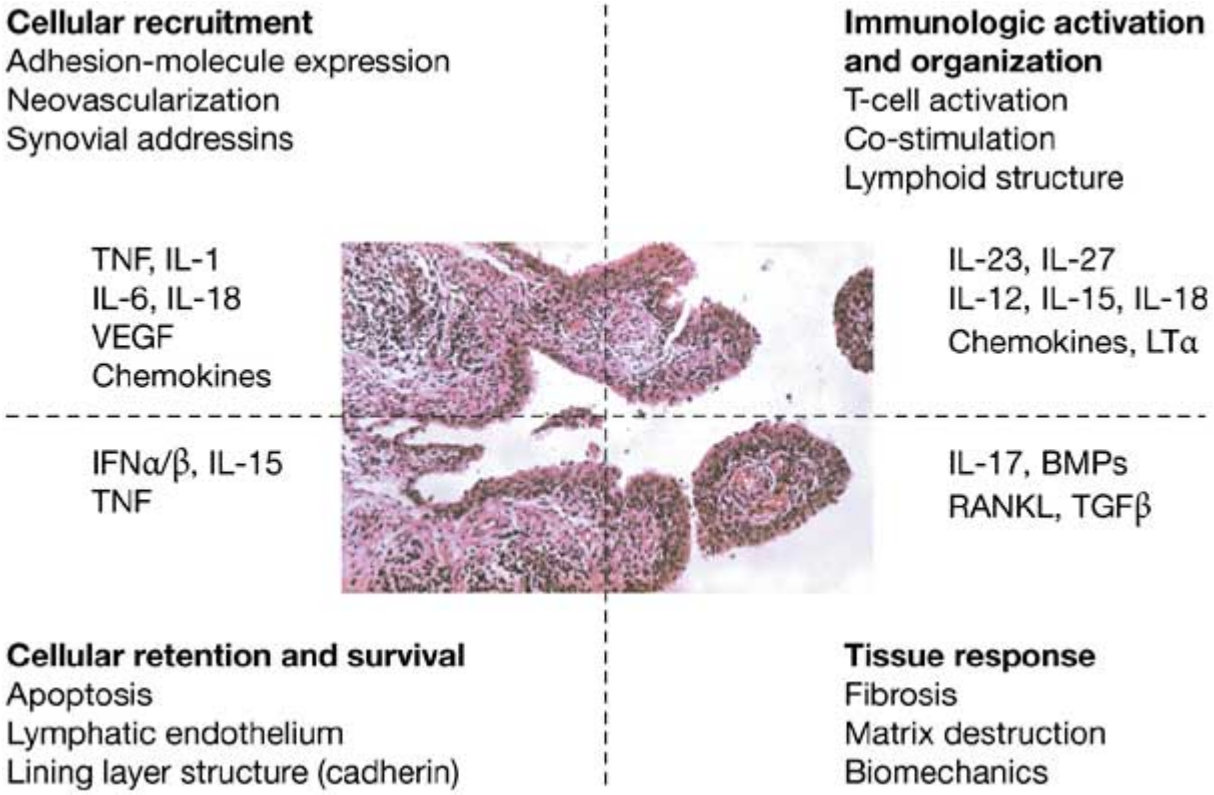

Fig 6. Cytokine networks in RA. Following a yet unidentified stimulus recruitement and retention of cellular and molecular components of the immune-inflammatory response. This is followed by organisation of the local immune response and consequent on-going joint destruction: from Nature clinical practice rheumatology; November 2005,Vol 1,No 1:McInnes and Liew.[with permission]

1.2.6 Immune Dysregulation

Dendritic cells are are found in the proliferating synovial tissue where they are able to process and present antigens to T cells in synovial germinal centres and migrate to central lymphoid organs where they activate naïve T cells. T cells then move back into the synovium producing pro-inflammatory cytokines and driving B cell Ab production. Dendritic cells may be stimulated by joint tissue debris such as hyaluronan fragments, citrullinated synovial proteins, and type II collagen and cartilage fragments. Dendritic cell functions are enhanced by pro-inflammatory cytokines such as TNF, IL-1, and IL-6, while being inhibited by IL-10, TGF-β, and

prostaglandins. RA dendritic cells express fewer IL-10 receptors rendering them more resistant to IL-10inhibition.[2,44]

Autoantibodies are one of the hallmark features of RA. *De novo* production of pathological antibodies has been shown in RA synovial tissue. B and T cells are found as aggregates in synovial tissues with some in highly developed germinal centers known as tertiary lymphoid tissue (TLT). Histologic patterns vary from patient to patient showing no correlation with disease activity, although areas of granulomatous changes may possibly be associated with extra-articular disease.[45] Tertiary lymphoid tissue is associated with many other inflammatory conditions reflecting persistent immunological activation. Local secretion of lymphotoxin (LT-α, LT-β) and chemokines [C-X-C motif (CXCL) 13, chemokine ligand (CCL21), CCL20 and CXCL12] modulates development of TLT. The majority of the cells is T cells (30-50%) surrounding B cells and plasma cells, rich in IgM RF and ACPA IgG antibodies.[43,45] Synovial T cells are rich in CD28, an important co-stimulatory molecule required for maximal response. A sub-population of synovial T cells lack CD28 expression, acting as cytotoxic cells responding to auto-antigens. This may be an important mechanism for treatment failures to biologic agents specifically targeting T cell co-stimulation (Abatacept).[45] IL-1 interacts synergistically with another co-stimulatory molecule CD40 binding to its ligand on T cells (CD40L) to produce GM-CSF. Synovial T cells have high level of expression of receptors for CCR5 that preferentially allow chemotaxis of Th1 T cells. People with a non-functional CCR5 genotype are protected from human immunodeficiency virus (HIV) infection and may be at lower risk of RA. Production of IL-12 and IL-23 by dendritic cells favours Th1 and Th17 differentiation. Cytokine secretion is influenced by Th1 cells (IFN-γ, IL- 12, IL-6, TNF and IL-10).[46] T cell phenotypes in RA synovium include Th17 and Treg cells. Th 17 cells secrete a potent pro-inflammatory cytokine IL-17. Tregs in RA have a defect in the suppression of TNF and IFN- γ production by other T cells and monocytes.[47] APC, including B cells, activate T cells to initiate/perpetuate the local immune reaction. B cell maturation within the TLT reflects a local antigen-driven process.[48] B cell-activating factor (BAFF) or B lymphocyte stimulator (BLyS) is a

cytokine essential for peripheral B cell survival with elevated serum levels seen in patients with RA. BAFF is also produced by RASF. Cell contact with and production of factors such as IL-6, IL-8 and GM-CSF by stromal cells found in the synovium and bone marrow of patients with RA promotes B cell proliferation and antibody production. The importance of B cells in RA pathogenesis is underscored by the success of B cell depleting therapies (rituximab) in the management of refractory RA.

1.2.7 Cellular proliferation

Cellular recruitment, expression of adhesion molecules and angiogenesis are mediated by a host of cellular and molecular components. Macrophages are key producers of pro-inflammatory cytokines and chemokines in RA. Cytokines together with growth factors (M-CSF, GM-CSF) regulate the balance between classical inflammatory macrophages (M1) and those with an anti-inflammatory or tissue repair phenotype (M2). Cytokines such as TNF are not only produced by macrophages, which also respond to TNF in an autocrine manner, favouring further inflammatory macrophage proliferation and differentiation of monocytes. This feedback loop is likely to become entrenched in established RA and more difficult to manage, thereby contributing to perpetuation of synovitis and chronicity.[2] The extent of macrophage synovial infiltration correlates with radiographic progression.[49] Imbalances between production of pro- and anti-inflammatory cytokines, such as increased production of IL-1 and underproduction of IL-10, facilitate synovial tissue proliferation.[50] Pro-inflammatory cytokines released by macrophages include TNF, IL-1, IL-12, IL-15, IL-18, IL-23, IL-27, chemokines and growth factors. TNF is the principle cytokine, increasing expression of other cytokines, prostaglandins (PGs) and adhesion molecules. TNF also affects vascular permeability, allowing for movement of immune complexes, complement, neutrophils and other cells into the synovium (Figure 7). Cellular contact with fibroblasts promotes the release of IL-6, while adhesion molecule expression increases under the influence of the pro-inflammatory cytokine milieu.

Mast cells stimulate synovial cells to release PG E2 and collagenase, while heparin released from these cells influences bone resorption. The proliferation of mast cells in synovial tissue and fluid is related to the degree of lymphocyte infiltration with the number of mast cells correlating with the clinical intensity of synovitis.[51]

Natural killer (NK) cells within the RA synovial infiltrate promote B cell production of RFs and are also a source of serine proteases. Numerous CD56 positive natural killer cells are found in RA synovium and result in increased pro-inflammatory cytokine secretion by macrophages and T cells.

Neutrophils are found in abundance in RA synovial fluid, but sparsely in synovial tissue. Neutrophils in synovial fluid amplify the inflammatory response by engaging with immune complexes generating ROS and reactive nitrogen species (RNS), and mobilization of neutrophil granules, resulting in release of proteases and cytokines such as IL-1 and oncostatin M (member of the IL-6 familly). Neutrophil activation also results in the formation of PGs and leukotrienes (LTs) from arachidonic acid released from cell membrane phospholipids by phospholipase A2. The cytokines TNF and IL-1 induce the COX 2 gene, resulting in formation of pro-inflammatory PGs. LTs, specifically LTB_4 in the case of neutrophils, are produced from arachidonic acid via the activity of 5'-lipoxygenase. LTB_4 is a potent chemoattractant for neutrophils, monocytes/macrophages and eosinophils. Activation of the 5'-lipoxygenase pathway also results in formation of lipoxins from arachidonic acid; these have an anti-inflammatory action, inhibiting neutrophil adhesion, chemotaxis and cytokine production.

Mesenchymal cells in the bone marrow of RA patients express enhanced NFkB signaling suggesting activation. These cells have the potential to differentiate into fibroblast-like cells.[52] Animal models have demonstrated movement of activated mesenchymal marrow cells through cortical bone with consequent development of synovitis under the modulation of cytokines.[53]

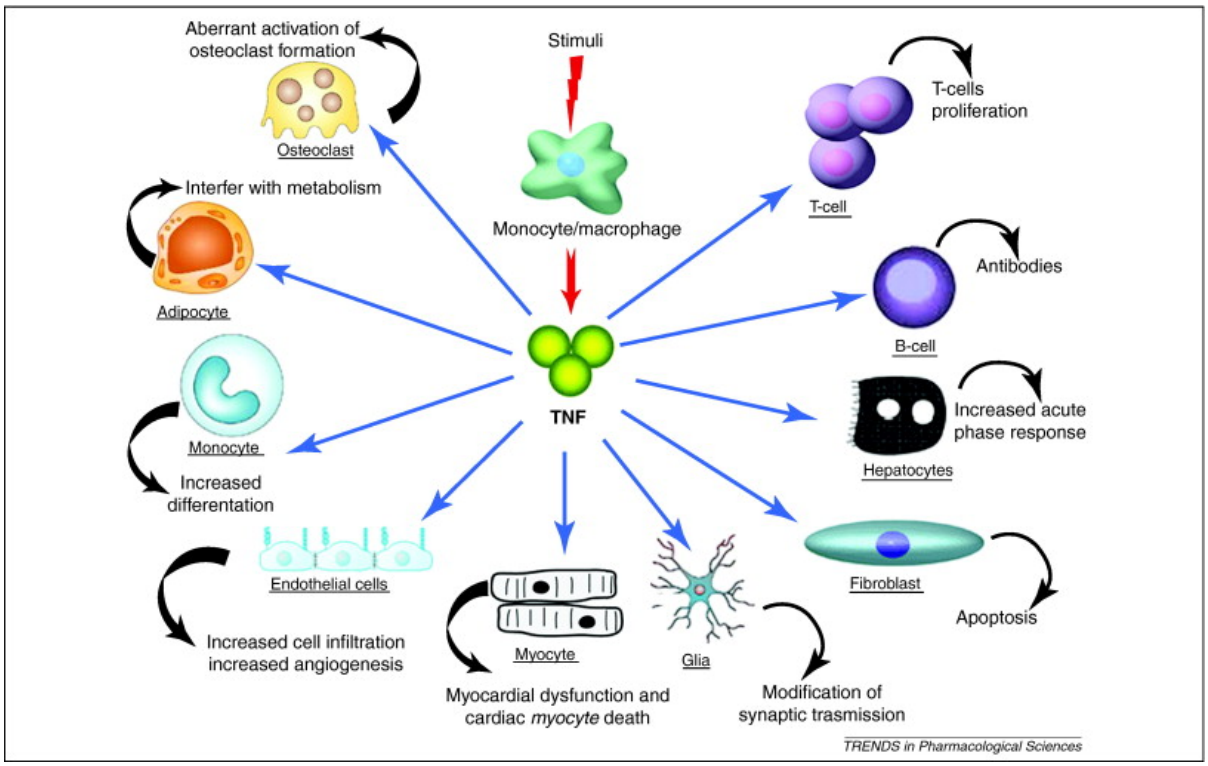

Fig 7. Cellular targets of TNF. TNF is released from cells as a soluble cytokine (sTNF, a homotrimer of 17-kDa monomers) after being enzymatically cleaved from its cell surface-bound precursor (a homotrimer of 26-kDa monomers) by TNF-α-converting enzyme (TACE). Many immune and non-immune cell types can produce TNF, including macrophages, T cells, mast cells, granulocytes, natural killer (NK) cells, fibroblasts, neurons, keratinocytes and smooth muscle cells. TNF interacts with two distinct receptors—TNF receptor 1 (TNFR1) (p55, CD120a) and TNFR2 (p75, CD120b)—on a wide variety of cell types to mediate their biologic functions: from Trends in pharmacological sciences. Anti-TNF therapy in the injured spinal cord: Volume 32, Issue 2, February 2011, Pages 107–115; Emanuela Esposito,Salvatore Cuzzocrea, doi: 10.1016/j.tips.2010.11.009 [with permission]

1.2.8 Disease perpetuation

An altered fibroblast phenotype prevalent in RA synovial tissue confers an autonomous, activated state and may be one of the major drivers of chronic inflammation. Fibroblasts are cells of mesenchymal origin and are found at sites of bony and cartilaginous destruction in the rheumatoid joint. RA synovial fibroblasts secrete a host of pro-inflammatory cytokines, matrix degrading enzymes and factors that promote angiogenesis. Fibroblasts do not normally express HLA class II antigens, neither do they produce inflammatory cytokines. These immunological

changes are evident in RASF, even at the onset of clinical disease. The aetiology and mechanisms of these changes are not known, but debris from joint damage appears to promote RASF activation and ongoing disease. Cartilage damage releases fragments such as fibronectin and vitronectin which also increases RASF activation characterized by increased expression of adhesion molecules, resulting in increased production of MMPs. Activation of RASF is also mediated by growth factors such as fibroblast growth factor (FGF), TGF-β and platelet-derived growth factor (PDGF). The altered RASF phenotype may also be related to epigenetic modifications with hyperacetylation and DNA demethylation having been demonstrated in the RASF genome activating pro-inflammatory gene transcription and production of factors which promote fibroblast proliferation.[1] RASF also contains unique miRNAs which are also related to increased demethylation. Cellular debris such as RNA and DNA fragments can bind to TLR expressed on RASF and promote activation thereof, especially in the presence of pro-inflammatory cytokines such as IL-1 and TNF. Cytokines such as TNF, IL-1, IL- 6, IL-17 and adipokines are also potent activators of RASF's. These events are summarized in Figure 8.

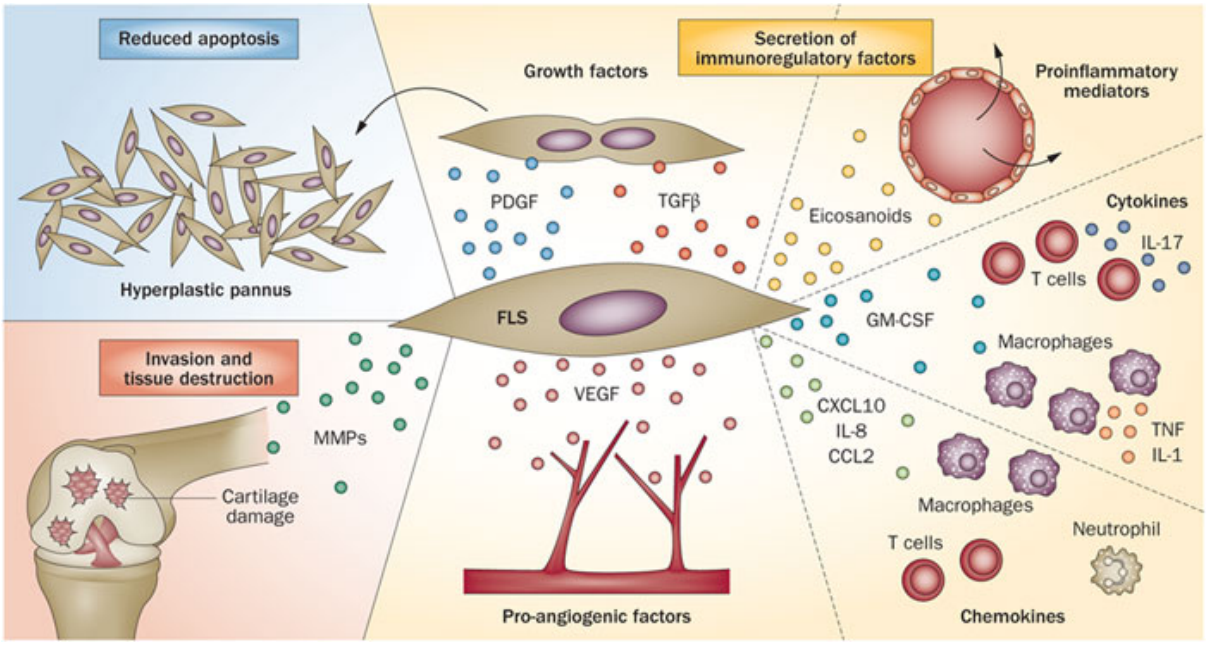

Fig 8. Roles of fibroblasts in RA. FLS play a critical part in many pathogenic events in the RA synovium. They can contribute to pathology through a reduced ability to undergo apoptosis (forming pannus), the production of proteases that degrade the extracellular matrix, and invasion into cartilage. In addition, FLS produce a variety of molecules that modulate growth, inflammation, angiogenesis, and cell recruitment, and induce activation of and cytokine production by immune cells. Abbreviations: CCL2, CC-chemokine ligand 2; CXCL10, CXC-chemokine ligand 10; FLS, fibroblast-like synoviocytes; GM-CSF, granulocyte-macrophage colony-stimulating factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; RA, rheumatoid arthritis; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor : from Nature Reviews Rheumatology.Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors: Nunzio Bottini and Gary S. Firestein, 9, 24-33 (January 2013) doi:10.1038/nrrheum.2012.190) [with permission]

Heat shock protein (HSP) are stress response proteins released following tissue damage related to physical stress, oxygen and nutrient deprivation, bacterial infections and exposure to ROS/RNS. In a rat model of arthritis, T lymphocytes were found to recognise mycobacterial HSP65 which was cross-reactive with cartilage proteoglycan epitopes.[54] Binding of HSPs to synovial TLR-4 and T cells can promote disease perpetuation while certain HSPs stimulate rapid proliferation of synovial T cells.[55]

The degradation of cartilage matrix components results in the formation of neo-epitopes, potentially acting as auto-antigens. Immune complex deposition on articular collagenous tissue serves as a strong and persistent stimulus for the generation of chemo-attractants promoting sustained cellular infiltration of the synovial pannus. The immune complexes in RA contain numerous proteins, including complement, immunoglobulin, acute phase reactants, type II collagen, albumin, DNA and fibrinogen.

1.2.9 Tissue damage

Osteoclasts are the primary effectors of bone resorption and are found in abundance at the interface of proliferating synovium and bone. Mature osteoclasts are derived from monocytes/macrophages requiring specific cytokine signals for maturation. Osteoclasts secrete proteinases and possess a proton pump to allow for resorption of the bony matrix and dissolution of calcium. Key proteinases are the MMPs and cathepsins which remove non-mineralised areas of bone. The proton pump creates an acidic environment between the osteoclast and bone surface. MMPs such as MMP1, 2 and 9 are derived from synovial macrophages and mediate joint destruction together with other MMPs, cathepsins, RANKL, angiogenic factors (VEGF), cytokines (IL-1, IL-6) and chemokines (IL-8, MCP2) released from RASF.

Key molecular components necessary for osteoclast maturation include signals derived from activated T cells and RA synovial fibroblasts. RANKL binds to its surface receptor on osteoclasts, receptor activator for nuclear factor kappa B (RANK), initiating differentiation, survival and activity.[56] RANKL can also bind to a soluble decoy receptor OPG that prevents RANKL/RANK interaction. Osteoclast formation is also supported by TNF, IL-1 and IL-17 by promoting RANKL expression. TNF is capable of directly binding to osteoclasts via its own receptor, thereby boosting osteoclast formation and consequent bone resorption. Osteoclasts also mediate resorption of the mineralized cartilage layer.[57]

In addition to bone loss at sites of the expanding synovial pannus and peri-articular regions, a generalized decrease in bone mineral density is a well-recognized feature of RA. Peri-articular bone loss is evident on bone mineral density (BMD) assessment and is associated with joint damage and disease progression.[58,59] Bone loss also occurs at sites not distal to the synovium, thus local factors such as decreased mobility and local bone marrow inflammation may also be etiological considerations. Generalized osteopenia in RA is related to chronic inflammation and is associated with disease activity.[60,61] The pro-inflammatory circulating cytokine milieu is probably also implicated in the pathogenic mechanisms inducing systemic bone loss.[62]

MMPs and aggrecanases are the the key mediators of cartilage destruction in RA. Cartilage is composed of type II collagen (70%), proteoglycans, non-collagenous matrix proteins (COMP) and chondrocytes. Proliferating synovial macrophages and fibroblast-derived proteinases such as MMPs result in the degradation of collagen and release of fragments carboxy-terminal collagen crosslinks (CTX)-II,[63,64] and an increase in cartilage turnover. Cartilage breakdown also occurs in areas not in contact with synovium due to the dispersal of proteinases released into the synovial fluid from neutrophils and other inflammatory cells in the synovial fluid.

Synovial pannus requires new vessel formation to proliferate such that ultrasound Doppler activity in the RA synovium correlates with the future development of erosions. Histological studies demonstrate proliferating vessels in inflamed joints only.[65] Vascular endothelial growth factor (VEGF) is the key growth factor initiating new vessel formation. Pro-inflammatory cytokines such as IL-1, IL-6, TNF and TGF-β stimulate the release of VEGF, resulting in endothelial cell proliferation. TNF may also have a direct effect on endothelial cells promoting angiogenesis.[66] The new vessels in the RA synovium demonstrate abnormal architecture and are dysfunctional resulting in ‘leakage’ of more inflammatory mediators into the synovium and inadequate perfusion to synovial tissue.[67]

The interaction of genetic and environmental factors resulting in chronic self-perpetuating inflammation results in the clinical manifestations seen in RA. Recent

advances in the understanding of these pathogenic mechanisms have not only resulted in novel approaches to the management of RA, but also shed light on numerous other physiological and pathological processes such as aging, wound healing, malignancies, autoimmune inflammatory disorders and ischeamic heart disease (IHD).

1.3 Biomarkers in RA

With the recent advances in the management of patients with RA, the need for early diagnosis and rapid disease control is paramount to limit morbidity and premature mortality from RA. Particularly in resource poor settings, prioritization of services to allow early diagnosis, and prognostication, as well as the need to institute appropriate cost effective therapy makes the prospect of personalized medicine all the more critical. Clinical and imaging strategies, as well as laboratory biomarkers are being used to diagnose and monitor disease progression, usually as composite scores. However, current biomarkers in use, although valuable, fail to diagnose or monitor disease activity effectively. Measures associated with a good clinical response may not be able to identify patients with ongoing joint damage, hence the importance of identifying novel markers able to bridge the disconnect between clinical response and joint damage. Candidate novel markers that could contribute to improving RA management are those integrally involved in the immunopathogenesis of RA. Ideal biomarkers should facilitate diagnosis, characterize severity, and predict response to therapy, morbidity and premature mortality.

1.3.1 Clinical measures and acute phase response measures

Clinical measures such as joint counts for swelling or tenderness and global assessments of disease activity by the physician and patient using a visual analog scale are commonly used to assess disease activity and response to therapy. Clinical and demographic features associated with a response to methotrexate therapy include early disease, low functional impairment, male sex, young age, elderly onset RA, low disease activity and non-smoker status.[68,69] However, studies have reported

conflicting reports with data from the Norfolk Arthritis Register failing to show age, age at onset, or gender to be predictive of response to MTX.[70] In the Swedish Pharmacotherapy Trial (SWEFOT), a poor response to MTX was associated with short duration of symptoms, younger age, female gender and current smoking status.[71,72] The erythrocyte sedimentation rate and C-reactive protein (CRP) are included in some of the disease activity scores, but may be normal in as many as 30% of patients despite active disease. Unfortunately, even in patients with low disease activity scores, ongoing radiographic damage and disease progression occurs.

1.3.2 Imaging markers

Plain X-rays of the hands and feet are used as surrogate markers of disease progression with radiographic scoring taking into account degree of bony destruction and joint space narrowing as representing cartilage loss. Two popular scoring measures are the Larsen and Sharp van der Heide methods, but these are used mostly in clinical trials. The simplified erosion and narrowing score (SENS), a modification of the Sharp van der Heide score may be more practical for clinical use. However, radiographic changes are not commonly present in early disease, and hence are no longer listed in the new RA criteria. Newer imaging modalities such as ultrasound and magnetic resonance imaging (MRI) hold promise to monitor disease progression and response to therapy early in disease management. Doppler ultrasound activity and MRI marrow edema have been associated with erosive radiographic progression. Ultrasound measures of synovitis have been shown to be useful in monitoring response to MTX and are now being used as an outcome measure in some clinical trials.[73-75] The utility of newer imaging modalities as biomarkers in RA still requires validation with cost and expertise being significant encumbrances.

1.3.3 Genotyping

Meyer *et al* in a cohort of predominantly black South African RA patients, showed an association of the SE with RA in 88 % of patients similar to other studies in Caucasians, but the association did not add more predictive value than the presence of ACPA, thus limiting clinical utility of shared epitope genotyping.[26] The presence of the SE may, however, have prognostic value as several studies have shown an association with more aggressive disease.[76-78] HLA DRB1*04 together with ACPA status is associated with resistance to disease modifying anti-rheumatic drug (DMARD) therapy,[79] SE-positive patients may benefit from combination DMARDs from the outset as opposed to SE-negative patients.[80] Homozygous SE patients have a 2-fold increase in cardiovascular (CV) mortality particularly in the case of the HLA-DRB1*01/*04 genotypes.[81-84]

Other genetic loci implicated in predisposition for developing RA include PTPN 22, TRAF1/C5 and STAT 4. The PTPN 22 risk allele codes for lymphoid tyrosine phosphatase that is involved in inhibition of T cell activation. The TRAF1/C5 risk allele locus is related to the genes encoding complement C5 and TNF receptor-associated factor 1. STAT 4 is associated with transcription of genes encoding interferon-α.[85-87] Genetic polymorphisms of MMP 3 have been shown to be associated with increased joint destruction in RA.[63,88]

Genetic polymorphisms of purine and pyrimidine metabolism may also influence the response to MTX and several genetic variants have the potential to act as biomarkers of response to MTX.[89,90] Gene expression in the synovia and peripheral blood mononuclear cells (PBMC) also varies with MTX treatment. A decrease has been noted in some RA related genes in synovial fibroblasts and an increase in IL-4 and IL-10 gene expression in PBMC in RA patients after treatment with MTX.[91] Genetic expression of CCL4, IL-8 and IL-1β may be predictive of response to TNF blockade.[92-95]

Abnormal expression of miRNAs in RA synovia has been documented and miR-16,-132,-146 and -155 identified as potential biomarkers in RA. Circulating levels of miR-132 were found to be lower in RA patients compared to healthy controls, while levels of miR-16 were found to correlate with disease activity.[96-98] Elevated levels of miR-125b may be predictive of a good response to.B cell depletion therapy with Rituximab.

The routine use of genotyping is prohibitive as access to testing is often only available in specialised laboratories, sometimes requiring synovial tissue, necessitating a biopsy. These factors also make the prospect of large studies to validate the role of genotyping more difficult.

1.3.4 Autoantibodies

Rheumatoid factor (RF) is one of the well-established laboratory markers associated with RA, but sensitivity and specificity, especially in early disease may be as low as 50%. Numerous conditions are associated with a false-positive test (infections, malignancies and connective tissue disorders). Pre-clinical detection of high levels of RF is, however, a risk factor for the development of RA.[99,100] The evaluation of RF isotypes increases specificity with the presence of elevated IgM and IgA RFs very suggestive of RA. High levels are usually seen in patients with severe disease, but despite the relationship with RA, changes in the level of RF with treatment have been found not to be clinically useful to monitor disease activity or response to therapy.[101] Studies of RF factor levels as predictors of response to TNF blocker therapy have reported varying results with some showing no predictive power[102-104] and others predicting a negative response, especially in the presence of high levels of IgA RF.[105-107] High serum levels of RF may be predictive of a more favourable response to rituximab therapy.[108,109]

Cardiovascular co-morbidity is increased in patients who are RF seropositive, with RF reported to be associated with left ventricular (LV) dysfunction and an independent

risk factor for IHD. RF is also associated with an increased all-cause mortality, unrelated to RA disease activity.[110-113]

The absolute levels of RF and ACPA, if increased greater three fold above normal increases the likelihood of diagnosing RA[114] (Table 1). The addition of the ACPA to RF increases specificity of diagnosing RA to greater than 90%, but sensitivity is still around 50-60% especially in early disease. Antibody positivity also has prognostic value with ACPA being associated with erosive disease and radiographic progression.[115-119] Erosive disease occurs earlier and with increased frequency in patients with ACPA positivity.[120] The combined presence of RF and SE has an interactive effect on ACPA status with respect to a poor prognosis,[115] with the greater spectrum of ACPA isotypes present associated with more severe radiographic changes.[116]

Studies of antibody status as predictors of response to TNF blockers have produced conflicting data,[105,121] however, sero-positivity for RF and/or ACPA and elevated immunoglobulins (IgG) has been shown to be associated with a better response to the B cell-depleting agent rituximab.[122,123] A decrease in ACPA titres with biologic DMARDs has correlated with clinical response and may be a marker of response to therapy,[107,124-132] but results from other studies have shown no such correlation.[133-136] Higher levels may be associated with a poor response to TNF antagonism and B cell-depletion therapy with rituximab.[133,137] The clinical utility of ACPA to monitor disease progression has not yet been established. ACPA-positive patients have greater frequencies of subclinical atherosclerotic disease being independently associated with future IHD and associated LV dysfunction.[111,138,139]

More recently a new antibody system related to, but independent of ACPA has been characterized, namely anti-carbamylated protein (anti-CarP). Carbamylation involves the post-translational modification of lysine to homocitrulline as opposed to conversion of arginine to citrulline for ACPA (Figure 9). This reaction occurs during inflammation following release of myeloperoxidase from neutrophils[140-142] the

presence of anti CarP antibodies in RA was found to be associated with more severe disease/joint damage in a group of ACPA-negative patients.[140]

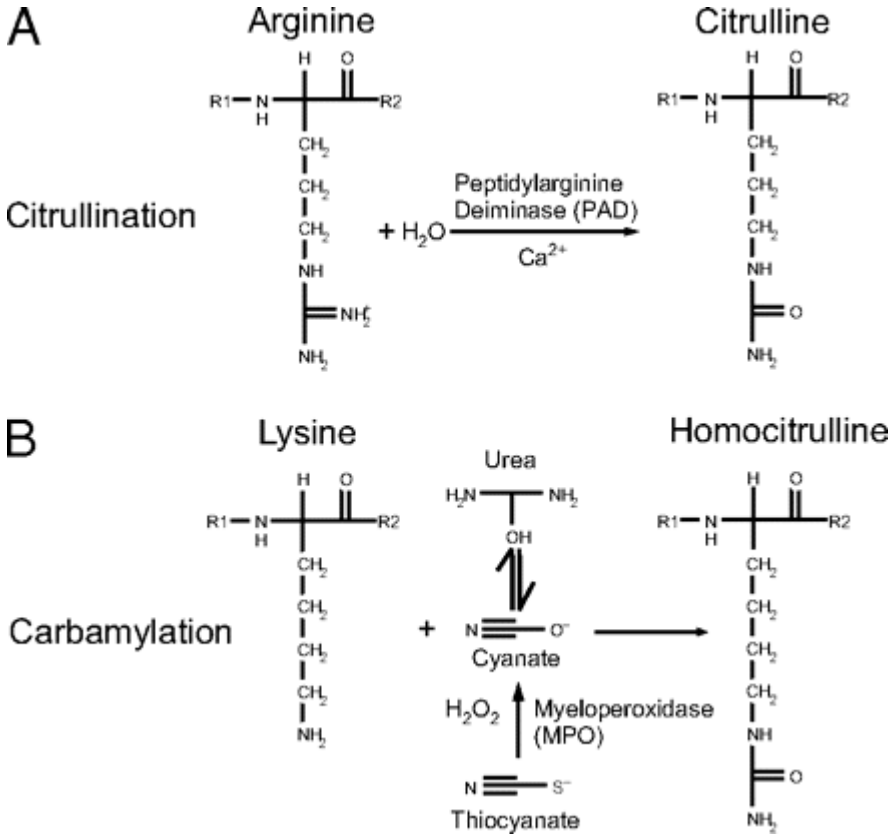

Fig 9. Illustration of citrullination and carbamylation. Citrullination (A) and carbamylation (B) occur on different amino acids via different mechanisms, but yield similar end-products: from PNAS 2011 108 (42) 17372-17377 ©2011 by National Academy of Sciences (open access)

1.3.5 Novel laboratory markers

Cytokines are important molecular components of the immune-inflammatory response and are integrally involved in the pathogenesis of RA. Their importance is underscored by the success of numerous innovative therapeutic agents targeting specific cytokines or their biologic pathways (Figure 10). Measurement of circulating levels of cytokines, chemokines, certain growth factors and degradation products associated with RA pathogenesis is being explored in the search for cost-effective, reliable biomarkers.

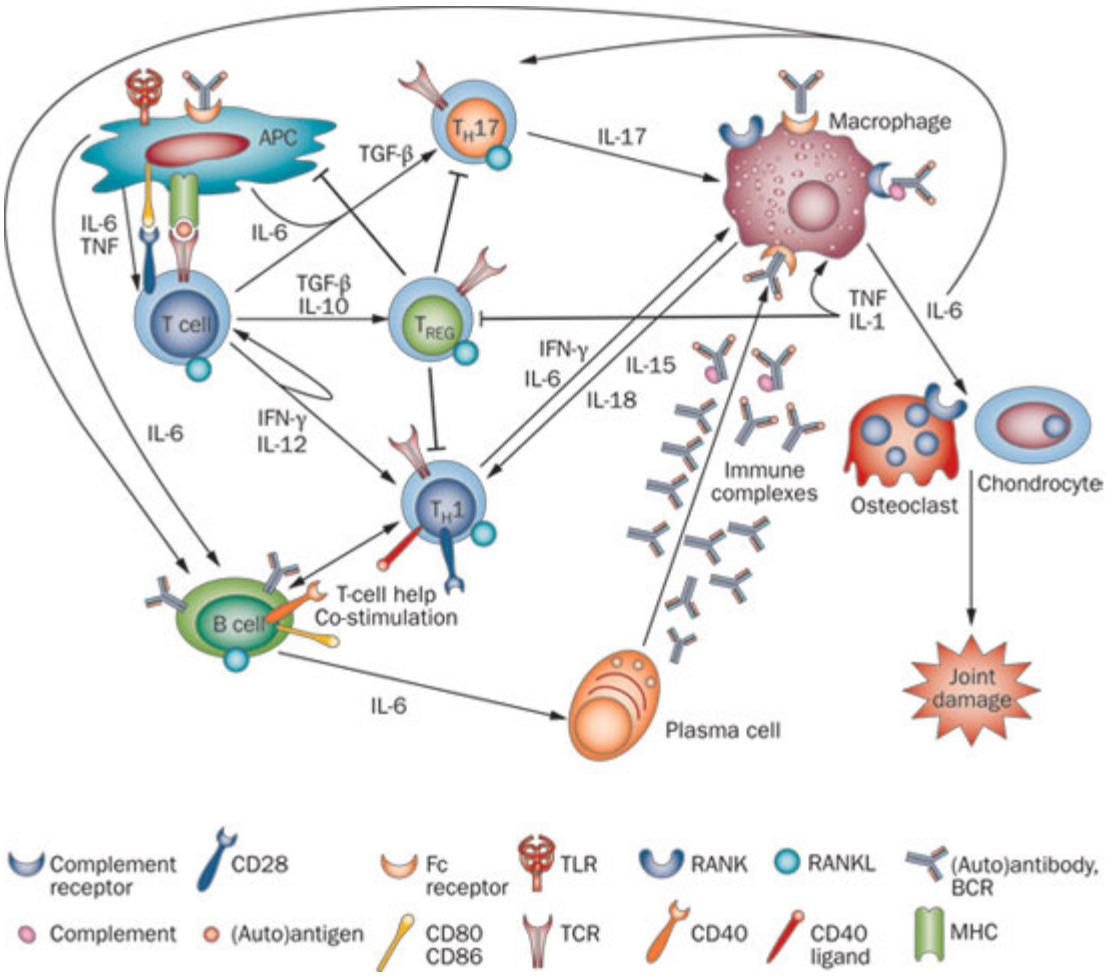

Fig 10 Pathogenesis of RA.
APCs present self or nonself antigens to T cells, which differentiate into TH1 or TH17 cells that induce macrophages to secrete proinflammatory cytokines and help B cells to produce (auto)antibodies that bind to target antigens forming immune complexes. These immune complexes can bind complement and engage complement receptors and/or Fc receptors on macrophages, augmenting secretion of TNF, IL-1 and IL-6. These cytokines lead to the clinical and laboratory manifestations of RA, as well as cartilage and bone damage, by virtue of chondrocyte and osteoclast activation (the latter primarily via the RANKL–RANK system). These cytokines can also alter and activate differentiation and function of T cells and B cells operating upstream in RA pathogenesis. TREG cells can inhibit the function of TH1 and TH17 cells in a physiologic environment, but are inhibited by cytokines produced in the inflammatory response. Abbreviations: APC, antigen-presenting cell; RA, rheumatoid arthritis; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; TCR, T-cell receptor; TGF-β, transforming growth factor β; TH, T-helper cell; TLR, Toll-like receptor; TREG, T-regulatory cell: from Nature Reviews Rheumatology, 8, 235-243 (April 2012) The pathogenesis of rheumatoid arthritis: new insights from old clinical data? Josef S. Smolen, Daniel Aletaha and Kurt Redlich [with permission].

Even in early RA local and systemic bone loss is associated with inflammation and patient functional status.[143] Radiographic erosive disease has been found to be associated with decreased BMD,[59,144,145] while serum TNF levels have been found to be inversely correlated to BMD.[146] RANKL and OPG play an important role in bone resorption and formation (chapter 2). The ratio of RANKL/OPG may have diagnostic value as higher levels are found in RA compared to healthy controls with serum OPG correlating negatively with disease activity and RANKL correlating positively with CRP.[147] A high baseline RANKL/OPG ratio is strongly predictive of yearly radiographic progression,[148] while a low serum RANKL level at baseline was found to be an independent predictor of remission.[69] A decrease in the RANKL/OPG ratio was associated with remission in patients treated with IL-6 antagonists, resulting from an increase in OPG and bone formation.[147,149] B cell-depletion therapy is also associated with a lower RANKL/OPG ratio. Patients treated with TNF blockers experience a decrease in serum RANKL levels in association with a decrease in disease activity,[150] thus RANKL/OPG may be of potential value in monitoring response to therapy. Baseline RANKL levels and serum RANKL/OPG ratio may also identify a subset of patients more likely to respond to TNF treatment.

Biomarkers reflecting enzymatic processes associated with breakdown of bone and cartilage have been shown to be associated with radiographic progression. Independent predictors of radiographic outcome include MMP3 and COMP.[151] MMP3 has also been found to correlate with erosive disease despite a normal CRP with some studies showing no predictive potential regarding radiographic progression independent of RF or ACPA status.[151-154] Combining disease activity score (DAS) 28 and MMP3 was demonstrated to be better than other outcome measures in RA for monitoring response to therapy.[155] The post-therapy normalization of MMP3 levels with IL-6 blockade which tocilizumab was predictive of sustained remission.[153] MMP3 levels at baseline and decrements thereof correlated with response to combination therapy with infliximab and MTX.[156]

COMP maintains the structural integrity of cartilage and is a measure of cartilage turnover. In an early RA cohort changes in circulating COMP levels were associated with radiologic outcome, with low serum levels of COMP at baseline being predictive of a rapid ACR70 response to adalimumab.[157] YKL-40 is a cartilage glycoprotein secreted by chondrocytes and synovial tissue in the presence of joint inflammation which decreases in response to treatment. YKL-40 has been included in a multi-biomarker score assessing disease activity in RA.[158-160]

Breakdown products from collagen and non-collagenous components of bone and cartilage (CTX-I and CTX-II) have also been shown to be associated with increased risk of radiographic progression.[161,162] Serum levels of cathepsin K have also been shown to correlate with radiological damage in patients with RA. Cathepsin K plays an important role in resorption of non-mineralized bone and a decrease in cathepsin K-mediated bone resorption following biological therapy with IL-6 blockade has been associated with a decrease in CTX I levels.[163] Elevated urinary CTX II levels have been found to be associated with rapid radiographic progression in patients with established disease.[164]

Clusters of cytokines/chemokines, including TNF, IL-1, IL-6, IL-13, IL- 8, as well as CCL2, CXCL10, CCL11 may have diagnostic value because they are elevated in patients with RA when compared to healthy controls.[165] Circulating cytokine/chemokine profiles associated with disease severity include elevated CCL4, IL-8, IL-2, IL-12, IL-17, and elevated IL-10 associated with mild disease.[166] Lower IL-4, IL-6, IFN-γ, and GM-CSF and IL-4 levels have been reported in patients with more severe disease.[166] Cytokine levels associated with disease activity include IL-1β, IL-6, IL -18 and TNF,[160,167,168] but other studies have shown no correlation between cytokine levels and clinical measures of disease activity.[92]

Alterations in circulating cytokine levels, may, however, have the potential to monitor response to therapy, with IL-7 levels decreasing in patients responding to MTX[169] and levels of VEGF declining following TNFα blockade.[170] Radiographic progression has been shown to be associated with alterations in mean circulating IL-6 levels over 2

years.[160] The absolute plasma levels of IL-6 (>4.03 pg/ml) after MTX therapy may be associated with radiographic progression,[171] while IL-7 levels correlate with erosive disease.[156] An increase in IL-8 levels from baseline was found to correlate with radiographic progression in patients on MTX monotherapy.[172]

Measures of the ratio of IL-1Ra/IL-1 produced by PBMC, as well as serum TNF levels have the potential to predict response to therapy with MTX.[68,91,173] High circulating chemokine levels (CCL2 and CXCL8) at baseline have also been reported to be associated with response to IL-6 inhibition,[174] with low baseline levels of IL-6 being associated with a more favourable response to IL-6 blockade.[167] High levels of CCL2 and epidermal growth factor (EGF) were found to be predictive of response to etenercept,[175] with low levels of IL-2 predictive of remission in RA.[69] Some biological therapies such as B cell-depletion are given at 6 to 9 monthly intervals and early predictors of response could add value to RA management. Decreases in CRP, in the setting of higher CCL2 and EGF at 3 months after a dose of rituximab may be predictive of responders.[176] Higher baseline values of IL-6 were also associated with a poor response to rituximab, with a significant reduction in circulating IL-6 levels noted in responders.[177] Patients with very high serum TNF values were found to be more refractory to treatment.[177]

Measurement of growth factors associated with neovascularisation such as VEGF has demonstrated higher levels in patients with RA, especially in early disease before therapy. Levels of VEGF correlate with disease activity and decrease in response to therapy in some studies.[178-180]

Co-morbidities such as accelerated atherosclerosis are an important cause of premature mortality in patients with RA with cytokines seeming to be key pathogenic mediators (Figure 11). Measures of endothelial dysfunction such as increased expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 and endothelial leukocyte adhesion molecule-1 have also been described in patients with RA,[181] with VCAM-1 associated with increased common carotid artery intima medialthickness (IMT) and plaque formation in RA patients.[182]

Cytokine levels, particularly ratios of pro-inflammatory to anti-inflammatory cytokines such as the ratios of IL-1/IL-10 and IL-6/IL-10 have also been associated with new coronary events in patients (non-RA) with previous non ST segment elevation acute coronary syndrome (NSTEACS).[183] Levels of IL-6 and TNF have been shown to be associated with increased coronary arterial calcification in RA, with increased arterial stiffness correlating with IL-6 levels.[184,185]

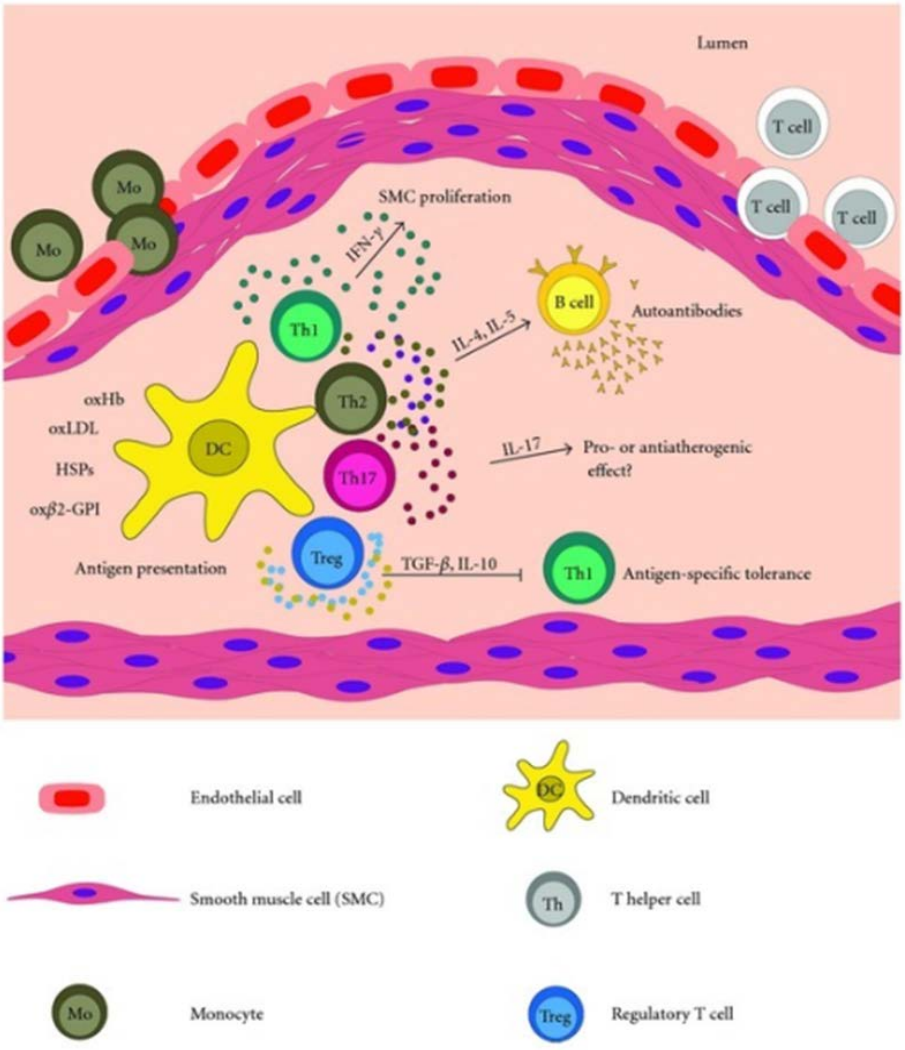

Fig 11. Schematic representation of T lymphocyte subset activation within atherosclerotic plaque. Different self-structures are expressed in the intima of the vessel wall and subjected to enzymatic/oxidative modifications. These modified molecules are taken up by dendritic cells and presented to CD4+ T helper cells via MHC class II molecules. Antigen presentation promotes T lymphocyte activation and secretion of different cytokines. Effector CD4+ Th1 cells produce pro-inflammatory cytokines, particularly IFN-*γ*, with proatherogenic effects. Activated Th2 lymphocytes secrete IL-4 and IL-5 which promote the differentiation of B cells in plasma cells and the production of specific antibodies. Some infiltrated T cells upon activation differentiate in Th17 lymphocytes characterized by the production of IL-17, but their role remains controversial. Regulatory T cells (Tregs) downregulate inflammation by secretion of TGF-*β* and IL-10. The pro-inflammatory mediators released by activated T cells reduce the stability of the lesion promoting plaque rupture and thrombotic events:
from The ScientificWorld Journal Volume 2012, Article ID 157534. T Lymphocyte Autoreactivity in InflammatoryMechanisms Regulating Atherosclerosis. Elisabetta Profumo, Brigitta Buttari, Luciano Saso, Raffaele Capoano,Bruno Salvati, and Rachele Rigan`o [open access]

Although correlations of these novel biomarkers with disease activity or as predictors of response to treatment have been inconsistent and of limited clinical use as independent markers, some of these novel biomarkers have been incorporated into composite scores with other measures of disease activity, with the biomarker component now commercially available (Figure 12). The composite score has been found to be associated with disease activity, radiographic progression and ability to discriminate responders from non-responders to MTX and TNF blockers.[148,187] The clinical utility of the multi-biomarker score appears promising but needs to be validated in larger clinical trials. In a recent study of an early RA cohort, the pre-treatment multi biomarker score was shown to be an independent predictor of radiographic progression at 1 year.[188]

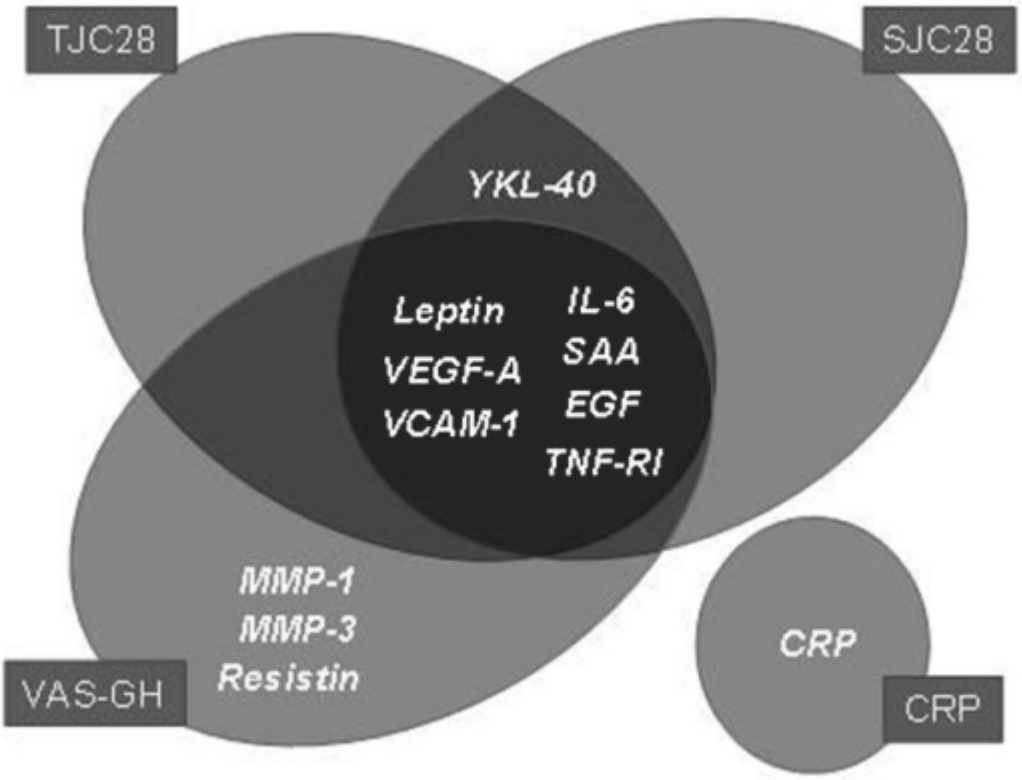

Fig 12. The Venn diagram lists the MBDA score biomarkers used to predict each MBDA score component. This algorithm provides an MBDA score between 1 and 100 :from Ann Rheum Dis 2012;71:1692–1697. doi:10.1136/annrheumdis-2011-200963. [open access]

1.3.6 Comment

As the etio-pathogenic mechanisms underlying RA unfold more candidate biomarkers and potential therapeutic targets are being identified. Recent advances in RA management have brought with them challenges in respect of selection of specific therapies for specific situations, although no single measure is likely to emerge as the gold standard. Composite scores using clinical, imaging and laboratory measures are likely to come closest to meeting the ideals of cost effective-personalized medicine.

Table 1: ACR/EULAR 2010 classification criteria for RA.[49]

To be applied to patients: (1) who have ≥ 1 joint with definite synovitis, excluding the DIP joints, first MTP joints, and first CMC joints, and (2) in whom the synovitis cannot be explained by another disease.

	Criteria	**Score**
A.	Joint involvement:	
	1 large joint	0
	2 – 10 large joints[a]	1
	1 – 3 small joints (with or without involvement of large joints)	2
	4 – 10 small joints[b] (with or without involvement of large joints)	3
	>10 joints (at least1 small joint)	5
B.	Serology (at least 1 test result is needed for classification):	
	Negative RF and negative anti-CCP antibodies	0
	Low-positive RF or low-positive anti-CCP antibodies[c]	2
	High-positive RF or high-positive anti-CCP antibodies[d]	3
C.	Acute phase reactants:	
	Normal CRP level and normal ESR	0
	Abnormal CRP level or abnormal ESR	1
D.	Duration of symptoms:	
	< 6 weeks	0
	≥ 6 weeks	1

ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; RA, rheumatoid arthritis; DIP, distal interphalangeal; MTP, metatarsophalangeal; CMC, carpometacarpal; MCP, metacarpophalangeal, PIP, proximal interphalangeal; IP, interphalangeal; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

[a]Large joints = shoulders, elbows, hips, knees, ankles.
[b]Small joints = MCPs, PIPs, second – fifth MTPs, thumb IPs, wrists.
[c]Low-positive is ≤ 3 times the upper limit ofnormal.
[d]High-positive is > 3 times the upper limit of normal.

1.4 Rheumatoid Arthritis in the developing world

A paucity of data exists on the epidemiology of RA in developing countries with a presumptive prevalence ranging from 0.5-1%.[189] There is a perception that the prevalence of RA is increasing in developing countries, but decreasing with a shift to a more elderly onset of disease in developed regions such as Europe and America.[190,191] Most studies in developing areas reflect an increased prevalence in urban areas in comparison to rural areas.[189] There are numerous challenges peculiar to the developing world including burden of communicable diseases, poor socioeconomic factors and limited resources making the management of RA more difficult.[192] A West African study seems to suggest that RA may be milder in Africa in comparison to a British cohort but more recent data from Africa report a disease pheno-typically similar to that seen in the developing world.[193-195] Genetic susceptibility to RA may vary geographically with a lower frequency of the RA associated risk allelle HLA-DR4 reported in Nigerians compared to South Africans.[196,197] In developing countries the higher prevalence of infections such as tuberculosis (TB), HIV and viral hepatitis poses additional threats to health care. There is a greater than 8-fold increased risk for TB due to the disease and its treatment.[192] Many people in the developing world are involved in manual labour and a study in Chile noted that most manual workers with RA stopped active work 2 years from disease diagnosis.[198] Synthetic DMARD's are the anchor drugs used to manage the disease with only few patients having access to expensive biological therapies. Synthetic DMARDs can have remarkable efficacy if started in early disease. However, studies have highlighted the all too frequent delay in the diagnosis and institution of appropriate management of RA in the developing world, with a consequent negative impact on quality of life, functional status, prognosis and burden on society.[192,195]

Early diagnosis and initiation of therapy are key factors that will ultimately result in cost effective outcomes for all patients with RA, especially of importance in developing countries. There is also very limited data available in Africa and the rest of

the developing world on clinical and laboratory biomarkers of disease severity and response to therapy. More studies are needed that would contribute to estimation of the burden of disease, identify markers of disease patterns and response to therapy. This, in turn, would allow for better utilization of scarce resources ultimately benefitting both patients with RA and Society at large.

To this end a collaborative effort involving two public sector rheumatology units in the Gauteng province of South Africa recruited 171 DMARD-naïve early RA patients and conducted a 2 year prospective study, the Gauteng Rheumatoid Evaluation Assessment Trial (GREAT).

Objectives of the study included; i) characterization of disease burden with respect to both physical and psycho-social aspects; ii) assessing response to therapy in routine care; iii) characterization of auto-antibody, cytokines/chemokines, MMP-3, cartilage metabolites and genotype profiles associated with disease activity and response to therapy; and iv) investigation of clinical, radiographic and serological measures associated with radiographic damage and progression.

GREAT is a large and ongoing study encompassing many aspects of RA in the study population that has led to the completion of two theses (Dr PWA Meyer and Dr B Hodkinson) focused on research areas distinct from those presented in this thesis. Abstracts of published articles are listed in the appendix and references listed at the end of each chapter.

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CHAPTER 2

Patients and Methods

2.1 Study design

A prospective observational study conducted over two years recruited 171 patients from the rheumatology clinics of two public sector tertiary academic hospitals, Chris Hani Baragwanath and Steve Biko Academic Hospitals. Informed written consent was obtained from all participants before recruitment into the study. Patients recruited met the following inclusion and exclusion criteria:

Inclusion criteria

- Fulfillment of the 1987 revised American College of Rheumatology classification criteria for rheumatoid arthritis (at least 4 out of 7 criteria - Table 1).
- Age >18 years of age.
- <2 years duration of disease (onset of characteristic articular symptoms to enrolment less than 2 years)
- Informed consent.
- Disease-modifying anti-rheumatic drug (DMARD) therapy-naïve.

Exclusion criteria

- Corticosteroid drugs in the preceding month (4 weeks) – intramuscular or intravenous (stable oral dose for at least 2 weeks prior to enrollment at a dose of ≤ 10mg daily was permitted).
- Presence of co-existing overlap with any other causes of inflammatory arthritis such as gout, connective tissue disorders, spondyloarthropathy or reactive arthritis.
- Chronic pain syndromes such as fibromyalgia.

Table 1: 1987 American College of Rheumatology (ACR) classification criteria for rheumatoid arthritis.[1]

	Classification Criteria
1	Morning stiffness for ≥1 hour
2	Arthritis of ≥3 joint areas
3	Arthritis of hand joints
4	Symmetric arthritis
5	Rheumatoid nodules
6	Serum rheumatoid factor (IgM)
7	Radiographic changes

2.2 Clinical and radiographic methods

At baseline, demographic data, duration of symptoms, current smoking history, education level, medications including alternative therapies, laboratory tests, X-rays of hands/feet and disease activity scores were recorded (joint counts were done by experienced doctors from the rheumatology clinics). Clinical recording included extra-articular manifestations and noting the presence of any infections. Clinical parameters were assessed at 6 monthly intervals including documentation of all therapeutic changes (Appendix 2)

2.2.1 Disease activity variables assessed

- Duration of early morning stiffness.
- Tender joint count
- Swollen joint count
- Patient pain assessment (Likert scale).
- Patient global assessment (Likert scale)
- Physician global assessment (Likert scale)
- Composite Disease Activity score (DAS 28)
- Simplified Disease Activity Index (SDAI)

2.2.2 Functional assessment included

- Modified Health Assessment Questionnaire (HAQ)[2]
- Short Form-36 (SF-36) – Medical Outcome Study SF 36[3]

2.2.3 Radiology

- Radiographs were scored independently by two qualified rheumatologists in chronological sequence applying the Larsen scoring method after achieving acceptable inter-observer correlation. X-rays were also evaluated for the presence of erosive disease and were performed at baseline and at yearly intervals.

2.3 Serum

Venous blood (30ml) was collected in endotoxin-free, silicone-coated vacutainers containing a gel separator. The blood samples were allowed to stand at room temperature (±15 minutes) followed by centrifugation (3000 rpm for 10 minutes) after which the serum was removed, aliquoted and stored at -20^{o}C until performance of the various assays described below.

2.3.1 Rheumatoid Factor (RF)

RF (composite IgM, IgG, IgA), C-reactive protein (CRP) and serum amyloid A (SAA) were assayed by immuno-nephelometry (Siemens Healthcare Diagnostics, BN Prospec Nephelometer, Newark, USA) using reagents, standards and controls from Siemens Healthcare Diagnostics. Immunonephelometry is the determination of the concentration of a protein, by interaction of the protein with specific antisera to form antibody-antigen complexes. The amount of light-scatter caused when a beam of light (usually a red laser diode is the light source) is directed through the immune complexes is measured by a detector and compared to the light scatter intensities of a standard curve with known concentrations of the analyte. Serum results for RF, CRP and SAA were considered positive when the concentrations were greater than 11 IU/ml, 5µg/ml and 6.8 µg/ml respectively.

2.3.2 Anti-citrullinated peptide antibodies (ACPA)

ACPA were measured by fluorescence enzyme immune assay (FEIA). FEIA utilizes an antigen cyclic citrullinated peptide 2 (CCP2) bound to a solid phase (Immunocaps) to capture the anti-CCP2 in serum. The bound antibody is then detected and quantified using a fluorochrome-conjugated anti-human IgG according to the magnitude of the florescence intensity using the Immunocap 250 system and reagents, standards and controls from the manufacturer (Phadia AB, Uppsala, Sweden). A result was considered positive for ACPA antibodies when the concentration was >10 U/ml.

2.3.3 Antibodies to modified citrullinated vimentin (anti-MCV)

Anti-MCV levels were measured by enzyme-linked immunosorbent assay (ELISA). Modified citrullinated vimentin (MCV) bound to a polypropylene matrix (flat-bottom 8-well strips), was used to capture and measure anti-MCV present in serum following the binding of enzyme-conjugated anti-human IgG to the anti-MCV in the presence of a colorimetric substrate (Orgentec Diagnostika GmbH, Mainz, Germany). A serum result was considered positive for anti-MCV antibodies when the concentration was >20 U/ml.

2.3.4 Genotyping

Genomic DNA was obtained using the Promega Maxwell® Personal Automation™ System with the Maxwell® 16 Blood DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA) for extracting DNA from whole blood, collected in ethylene diamine tetra acetic acid (EDTA) sample tubes. The yield and purity of DNA extracted from EDTA blood samples were determined spectrophotometrically at 260/320nm and 260/280nm respectively and the concentration of DNA adjusted to 50ng/µl as required.

Genotyping of HLA-DRB1 alleles was performed using a DNA-based high-resolution typing method, LABType®HD DRB1 (One Lambda Inc, Canoga Park, California, USA), utilising reverse sequence specific oligonucleotide (rSSO) Luminex xMAP® technology probes. Firstly, the target DNA (HLA-DRB1 gene) is amplified by PCR using group- specific primers that are biotinylated for detection with SAPE (R-phycoerythrin-conjugated streptavidin). The polymerase chain reaction (PCR) product is then denatured and hybridised to complementary DNA probes conjugated to fluorophores. Bound DNA is detected on the Luminex system by addition of SAPE, and software maps the reaction patterns to those associated with published HLA gene sequences, and assigns the represented HLA-DRB1 alleles. Firstly, HLA alleles were assigned according to the RAA amino-acid sequence at positions 72-74 as shared epitope (S) and non-SE (X), and the S group further subdivided according to amino-acid residues at positions 70 and 71 as described by du Montcel[4] (see Table 2), with the groups pooled according to SE status into risk-allele-positive (S2,S3P) and -negative (S1,S3D,X).[5]

Table 2: *Shared Epitope classification according to amino acid sequences at positions 70-74* [4]

Allele Classification	Amino acid sequence position 70-74	HLA-DRB1 alleles
S1	D-E-RAA	*01:03, *04:02, *11:02-03, *13:01-:02, *13:04, *13:36, *13:40
	Q-A-RAA	*15:xx
S2	Q-K-RAA	*04:01, 04:09, *04:13, *04:35, *04:66
	D-K-RAA	*13:03
S3D	D-R-RAA	*11:01, *11:04, 11:27, *12:xx, *13:05-:06, *13:25, *14:22, *16
S3P	Q-R-RAA	*01:01-:02, *04:04-:05, *04:08, *04:10
	R-R-RAA	*10:01
X	Q-K-RGR	*03:xx
	Q-R-RAE	*04:03, *04:07, *04:11
	D-R-RGQ	*07:xx
	D-R-RAL	*08:xx
	R-R-RAE	*09:01, *14:01, *14:04

2.3.5 Cytokines

Cytokines were measured using the Bio-Plex® suspension array system (Bio-Rad Laboratories Inc, Hercules, CA, USA) which utilises Luminex® xMAP technology to enable simultaneous detection and quantitation of multiple different analytes in a single sample. The system uses an array of microspheres in liquid suspension, conjugated with a monoclonal antibody specific for a target protein. The beads contain different ratios of two spectrally distinct fluorophores, thereby assigning a unique spectral identity. These antibody-coupled, colour-coded beads were then

multiplex

incubated with the serum sample (¼ dilutions), washed, followed by addition of a biotinylated detection antibody, washed again, and finally incubated with streptavidin-phycoerythrin. A wide range of standards (0.38 – 91756.00 pg/ml) was used to enable quantitation of the individual cytokines using a Bio-Plex array reader with a dual laser detector and real time digital signal processing, in a fluidic system that detects a single sphere. The following analytes were measured: IL-1, IL-1Ra, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IL-17, IFN-γ, TNF, G-CSF, GM-CSF, CCL2, CCL4, and VEGF. Ten healthy control subjects (5 female, 5 male, average age 44.4±15.0 years, ranging from 26 -65 years of age) were used to determine the upper normal limit of the cytokine concentrations by calculating the mean +1SD.

2.3.6 Serum matrix metalloproteinase-3 (MMP-3) and cartilage oligomeric matrix protein (COMP)

Serum concentrations of MMP-3 were measured using the Quantikine® Total MMP-3 Immunoassay (ELISA) according to the instructions supplied by the manufacturer (R & D Systems, Minneapolis, MN, USA) and the results expressed as nanograms (ng)/ml serum. Serum levels were measured in healthy controls (15 females and 10 males, age range 24-64). COMP was measured using the Kamiya Human COMP ELISA system (Kamiya Biomedical Company, Seattle, WA, USA) and the results expressed as µg/ml serum. Serum levels were also measured in 8 adult control subjects.

2.4 Treatment

Patients were treated as per routine standard of care in the rheumatology units and evaluated clinically at 2-4 monthly intervals. Pharmacological management included monotherapy or combination therapy with methotrexate (MTX), salazopyrin (SZP) and/or chloroquine (CQ) with or without corticosteroids. Choice of pharmaco-therapy and dosage adjustments were at the discretion of the attending clinician as per routine care. Methotrexate doses were initiated at 7.5 to 15 mg weekly and titrated up to a maximum of 25 mg weekly. SZP was started at 500 mg daily and increased to 1g twice a day with CQ prescribed at 200 mg daily.

2.5 Statistical Analysis

Descriptive statistics of continuous variables were performed by using a measure of central tendency (mean or median) and a measure of variability (standard deviation or range). Frequencies and percentages were used to describe categorical variables of the nominal or ordinal scales. Cytokine data were not normally distributed and therefore the Spearman's pairwise correlations were used to find the strengths of association of each cytokine with demographics and other biomarkers, as well as between themselves. Medians and ranges were used to describe the skewed numerical clinical and biomarker data. Non-parametric procedures were used in the bivariate analysis of these data. The Mann-Whitney test was used to assess differences in medians for two independent groups, while the Wilcoxon signed-rank test was used to compare two dependent groups (i.e. baseline vs six months of therapy). The Kruskal-Wallis test was used to determine differences in medians of more than three independent groups. Statistical significance was ascertained at the 5% level i.e a *p*-value of less than 0.05 was deemed significant, and, where appropriate, Bonferroni corrections to the *p*-values for multiple tests were applied. Statistical analysis was done using STATA version 12.0 (Stata Corporation, College Station, Texas USA).

2.6 Ethics

The study was approved by the Research Ethics Committees of the Faculties of Health Sciences of the University of Pretoria and University of the Witwatersrand. (protocol number 173/2010)

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CHAPTER 3

Serum Matrix Metalloproteinase 3 (MMP-3) in comparison with acute phase proteins as a marker of disease activity and radiographic damage in early rheumatoid arthritis

3.1 Introduction

Rheumatoid arthritis (RA) is characterised by a progressive, erosive polyarthritis, resulting in immune-mediated joint destruction and eventual disability. Components of the immuno-inflammatory response include acute phase proteins, auto-reactive T cells, B cells, and their respective inflammatory mediators. The consequence is a self-perpetuating chronic inflammatory response involving a complex interplay between infiltrating inflammatory cells and the structural cells of the synovial joint.[1,2]

Matrix metalloproteinase-3 (MMP-3) is produced predominantly by chondrocytes and synovial fibroblasts and is found in high concentrations in the synovial fluid, with elevated levels also found in the serum of RA patients.[3] MMP-3 production is upregulated by the pro-inflammatory cytokines IL-1β, TNF, IFN γ and IL-17A, as well as by serum amyloid A (SAA), an acute phase reactant, with counter-regulatory inhibition from IL-4 and IL-13.[4-10] Intracellular signalling pathways include NF-kB,activating protein 1(AP-1) and STAT pathways which modulate gene transcription.[11] In addition, genetic polymorphisms have also been shown to influence the level of expression of MMP-3.[12,13] Cytokine/SAA-driven production of MMP-3 in the rheumatoid joint appears to be a key mediator of cartilage destruction, while bone resorption is facilitated by MMP-3-mediated removal of the outer osteoid layer, enabling attachment of osteoclasts to the underlying bone.[14] Moreover, MMP-3 mediates the proteolytic activation of pro-MMP-9 released from both inflammatory and structural cells.[4] Aside from its role in promoting the recruitment of neutrophils, monocytes, T cells and osteoclasts,[4] MMP-9 promotes the release of membrane-bound vascular endothelial growth factor (VEGF), thereby contributing to

angiogenesis and disease progression.[15] With respect to its diagnostic and prognostic potential, elevated serum concentrations of MMP-3, while having no specificity for RA, have been found in many,[16-24] but not all[25-29] studies, to correlate with disease severity and response to chemotherapy. Serum MMP-3 has been shown to correlate with histologic severity, and clinical and serological parameters of disease activity (28TJC,28SJC,CRP and ESR).[30,31] Young-Min *et al* (2007) who evaluated baseline serum MMP-3 in a cohort of early RA patients, reported an association with radiographic progression at 2 years and in a follow-up study was still predictive of radiographic progression at 8.2 years.[18,32] Targeted therapy using the combination of MMP-3 level and DAS28 as outcome measures was associated with the best results.[33]

COMP is an extracellular structural glycoprotein important for maintaining cartilage integrity and facilitating interactions between the extracellular matrix, collagen and aggrecan.[34] Elevated levels reflect cartilage breakdown or increased cartilage turnover.[34] Clinical utility of serum COMP has been limited with respect to its diagnostic role in RA because of a low sensitivity (15%-48%) and low specificity(66% to 69%).[35] However, COMP levels may have prognostic value and is possibly predictive of therapeutic response.[34] Elevated levels having been shown to be associated with progressive disease, with persistently elevated levels correlating with radiographic progression.[36-40] Morozzi *et al* (2007) reported a potential role of serum COMP levels in predicting response to anti-TNF with low levels associated with a more favourable response.[39,41]

CRP and SAA are synthesised in the liver in response to pro-inflammatory cytokines such as TNF, IL-1 and IL-6. Monocytes/macrophages, endothelial cells, synovial fibroblasts and chondrocytes can also produce SAA. Unlike CRP, SAA is also locally expressed and accumulates in inflamed tissue with histological studies demonstrating expression of SAA in the perivascular and lining layer of RA synovial tissue, in regions of leukocyte recruitment and angiogenesis, especially at the cartilage pannus junction. SAA is apparently a more sensitive marker of inflammation in RA than CRP,

with the SAA/CRP ratio, possibly being of more significance.[42] SAA augments the inflammatory response in a cytokine-like fashion by attracting monocytes/macrophages, leukocytes and T lymphocytes, while promoting neutrophil survival and endothelial activation, and stimulating the production of the proinflammatory mediators TNF, IL-1, IL-6, IL-8 and IL-17, thus initiating an amplifying loop. Exposure of synovial fibroblasts and chondrocytes to SAA promotes MMP-3 mediated adhesion molecule expression, as well as phagocytosis and chemotaxis of monocytes and neutrophils, thereby contributing to synovial inflammation, hyperplasia, angiogenesis and joint destruction.[9] SAA has also been implicated in the pathogenesis of atherosclerosis and premature cardiovascular disease, an important aspect in the management of patients with RA, thus making it a potential target for therapy to control both RA and its complications.

The global inter-relationships of MMP-3 and COMP with proven genetic markers of disease susceptibility, such as the shared epitope (SE), as well as with other systemic biomarkers such as autoantibodies, cytokines, and the acute phase reactants CRP and SAA in particular, have not been well characterised.

The objectives of the current study were to assess the relationships between circulating MMP-3 and: i) SE; ii) biomarkers of inflammation and cartilage degradation; and iii) disease activity and radiographic changes in comparison with CRP and SAA in a cohort of predominantly black female South Africans with early disease-modifying anti-rheumatic drugs (DMARD)-naive RA.

3.2 Patients and Methods (described in detail in chapter 2)

3.2.1 Patients

Baseline data was reviewed from 128 patients who were part of the Gauteng Rheumatoid Arthritis Trial (GREAT), a prospective study of early DMARD-naive RA patients.[43-45] Patients were recruited from the rheumatology clinics of two tertiary academic hospitals, Chris Hani Baragwanath Hospital and Steve Biko Academic

Hospital, attached to the Universities of the Witwatersrand and Pretoria, South Africa respectively, and the study was approved by the Research Ethics Committees of the Faculties of Health Sciences of both institutions.

Demographic data, duration of symptoms, education level, medication history, clinical assessment including 28 joint count and the presence of extra-articular involvement were recorded, as was disease activity measured according to the Health Assessment Questionnaire Disability Index (HAQ-DI) and Simplified Disease Activity Index (SDAI).[43-45]

Radiographs of the hands and feet were scored according to the modified Larsen score. In each case, 32 joint areas were scored: 8 PIP (proximal interphalangeal), 2 thumb IP (interphalangeal), 10 MCP (metacarpophalangeal), 4 areas in each wrist and 8 MTP (metatarsophalangeal) joints. The maximum score that could be attained was 160. Radiographs were also assessed for presence or absence of erosive disease.

3.2.2 Laboratory methods (described in chapter 2)

Venous blood was collected as described previously, followed by prompt separation of serum and storage at minus 20°C until analysed.

3.2.2.1 Autoantibodies, acute phase reactants, cytokines/chemokines

These were assayed using immunonephelometric, immunofluorimetric, and multiplex suspension bead array procedures as previously described,[43] while haemoglobin concentrations and erythrocyte sedimentation rates were measured using standard haematological procedures.

3.2.2.2 MMP-3

Serum concentrations of MMP-3 were measured using the Quantikine® Total MMP-3 Immunoassay (ELISA) according to the instructions supplied by the manufacturer (R

& D Systems, Minneapolis, MN, USA) and the results expressed as nanograms (ng)/ml serum. Serum levels were measured in healthy controls (15 females and 10 males, age range 24-64).

3.2.2.3 Cartilage oligmeric matrix protein (COMP)

Serum levels were measured using the Kamiya Human COMP ELISA system (Kamiya Biomedical Company, Seattle, WA, USA) and the results expressed as micrograms (µg)/ml. Serum levels were also measured in 8 of the above control subjects.

3.2.2.4 Shared epitope (SE) genotyping

This was performed as described previously using high-resolution reverse sequence-specific oligonucleotide probes and Luminex® technology,[43] with classification as SE or non-SE genotypes according to the method of du Montcel *et al.*[46,47]

3.2.2.5 Statistical Methods

Descriptive statistics of continuous variables were done by using a measure of central tendency (mean or median) and a measure of variability (standard deviation or range). Frequencies and percentages were used to describe categorical variables of the nominal or ordinal scales. Cytokine data were not normally distributed and therefore the Spearman's pairwise correlations were used to find the strengths of association of each cytokine and demographics with MMP-3 and COMP, and between themselves. Statistical significance was confirmed by *p*-values less than 0.05 and corrections for multiple cytokine variables tested with a level of significance confirmed by *p*-values of less than 0.003. Statistical analysis was done using STATA version 12.0 (Stata Corporation, College Station, Texas USA).

3.3 Results

3.3.1 Demographic, clinical and laboratory data [autoantibodies, acute phase reactants, haemoglobin, erythrocyte sedimentation rate (ESR), MMP-3 and COMP]

As seen in Table 1, the majority of patients were middle-aged black females, with a mean disease duration of almost 12 months. Patients had high disease activity as reflected by a mean DAS of 6,28 and moderate- to high- functional disability. 82% of patients were RF positive and 81% ACPA positive. Erosions were seen in 52% and the mean Larsen score was 22,4 (±12,7). In patients with disease duration less than 12 months 26.9% of patients had erosive disease.

Serum MMP-3 levels were significantly (p<0.0001) higher in the RA patients relative to the healthy control subjects. Using cut-off values of 18.6 ng/ml and 1.99 µg/ml for MMP-3 and COMP respectively (based on the mean values for the healthy control subjects +2 standard deviations), the mean MMP-3 and COMP values for the control groups compared favourably with previous studies of 46 and 291 healthy controls respectively.[48,49] The respective frequencies of elevated MMP-3 and COMP levels for the RA patients were 56.25 % and 34,6 % (Table 1).

3.3.2 Correlations of MMP-3 with clinical indices of disease activity and non-cytokine, inflammatory biomarkers

Correlations that achieved statistical significance are shown in Table 2, which demonstrates associations of serum MMP-3 with SDAI, CRP, SAA, RF and COMP. There were no statistically significant associations of MMP-3 with HAQ-DI, tender joint count, nodules, erosions, Larsen score or ACPA.

3.3.3 Correlations of MMP-3 with cytokines/chemokines/growth factors

We have previously reported that the circulating cytokine/chemokine/growth factor concentrations in this cohort of RA patients are significantly higher than in healthy control subjects.[43] In the current study, circulating MMP-3 was found to correlate

positively and significantly with IL-6, IL-8, IL-12, IFN-γ and VEGF (r values 0.27-0.33, *p* values <0.003-0.0001), the strongest correlations being observed with IL-8 >IL-6>IFN-γ and VEGF (Table 3).

3.3.4 Correlations of COMP with clinical indices of disease activity and non-cytokine inflammatory biomarkers

COMP correlated positively and significantly with RF (r = 0.23, *p* <0.006), but not with any of the clinical indices or other inflammatory biomarkers. A weak positive correlation between COMP and smoking history (ever smoked) was also noted (r = 0.17, *p* <0.04).

3.3.5 Association of MMP-3, SAA, CRP and COMP with SE

Subgroup analysis revealed no differences in MMP-3, SAA, CRP or COMP levels in risk allele -negative or -positive patients.

3.3.6 Correlations of CRP and SAA with clinical and laboratory indices of disease activity

Given the key role of SAA in activating the synthesis of MMP-3 by synovial chondrocytes and fibroblasts,[9,50] correlations of this acute phase reactant, as well as those of CRP, with clinical and traditional non-cytokine indices of disease activity were determined and these are shown in Table 4. Correlations included evaluation of the SAA /CRP ratio but this did not reveal any significant associations (data not shown).Both acute phase proteins, especially CRP, were found to have stronger associations with disease activity in early RA than MMP-3.

3.4 Discussion

Consistent with data from other studies which reported elevated MMP-3 levels in 62-80% of patients,[17,51] MMP-3 levels were found to be elevated in 56,25% of patients in the current study. Although no correlations with SE were detected, MMP-3 was found to correlate significantly with measures of disease activity, specifically SDAI, ESR,

CRP and SAA. In addition to these, correlations with predominantly pro-inflammatory cytokines, especially IL-8, IL-6, IFN-γ and VEGF (Table 3), were also observed. Visvanathan *et al.* in a study of 144 patients with RA showed significant correlations with IL-8, IL-1β and CRP, the strongest correlation being with CRP (r = 0,601, *p*<0,001).[52] A smaller study by Ribbens et al in 20 patients with RA showed correlations of MMP-3 and disease activity score (DAS), CRP, and IL-6 level.[23]

We are not aware of any studies having examined serum levels of MMP-3 in a DMARD-naïve early RA cohort of predominantly black females, exploring the associations with clinical parameters of disease activity, acute phase response, SE, and a wide range of cytokines, chemokines and growth factors.

Recent developments in the management of RA are aimed at rapid disease control, as well as early introduction of efficient therapies, which makes the search for useful biomarkers such as MMP-3 important. This contention is underscored by an interest in the development of of an automated sero-detection procedure for MMP-3 and the recent introduction of a commercially available multi-biomarker disease activity (MBDA) test. The MBDA test includes MMP-3 in the quantitative assessment of disease activity, having shown significant correlations with DAS 28-CRP (AUROC=0,77; *p*<0,001 for sero-positive patients and AUROC=0,70; *p*<0,001 for sero-negative patients).[53,54]

In the current study, the levels of circulating SAA, a mediator of synthesis of MMP-3, as well as those of CRP, were found to be elevated in 74,4% and 76,1% of patients respectively, and correlated significantly with measures of disease activity, as described in previous studies, some of which suggest that SAA is a more sensitive marker in RA than CRP. However, this was not confirmed in the current study, with CRP being equivalent or slightly superior to SAA as a marker of disease activity in early RA. Because SAA is also locally released and expressed in the synovial tissue, we reasoned that the SAA/CRP ratio may be a more accurate index of disease activity than the individual biomarkers.[42] However, this did not prove to be the case in

our cohort. With regard to poor prognostic markers, such as the presence of nodules, erosive disease and the Larsen score, correlations with CRP and SAA, but not with MMP-3, were evident. No associations of SAA or CRP with the SE risk alleles or COMP were observed.

Importantly, the correlations of both CRP and SAA with SDAI were considerably stronger than those of MMP-3 with SDAI, demonstrating that measurement of either of these acute phase reactants, but preferably CRP, is probably the best serological determinant of disease activity in early RA.

Erosive disease was present in 52% of patients but much lower (26.9%) in patients with disease duration less than 1 year. This compares favourably with early arthritis studies in different cohorts varying from 15% to 70% (disease duration 1-2 years).[21,55] Cartilage destruction is one of the consequences of uncontrolled synovial proliferation, with serum levels of cartilage degradation products such as COMP having been shown to be associated with RA disease activity and future radiological damage.[56] In the current study, COMP levels were elevated in 34,6% of patients compared to other studies showing percentage of elevated COMP levels ranging from 41 to 67%.[41,57] COMP was found to correlate weakly with smoking history (ever smoked), RF, and MMP-3, but no correlations were found with measures of disease activity as reflected in some previous studies which described correlations with CRP, DAS, ESR, and rheumatoid nodules.[58] MMP-3 is an important effector of cartilage metabolism, hence the correlation with COMP is not surprising. Although no correlation between COMP and radiographic changes were found, this may simply reflect increased cartilage turnover rather than destruction in early disease.[58] Elevated COMP levels may have prognostic value as a predictor of radiographic progression.[40]

There were no significant differences in MMP-3, SAA, CRP or COMP levels in the risk allele negative or positive patients. One would have expected more robust changes in the risk allele positive group of patients as high-risk SE alleles have been

associated with more severe disease.[45] However, it appears that no previous studies have compared levels of these biomarkers in the different risk allele groups according to the classification used in the current study.

The correlation of MMP-3 with COMP and lack thereof with the acute phase reactants studied, may suggest that MMP-3 is a better biomarker to predict disease progression, thus combining measurement of CRP with that of MMP-3 may be a useful strategy to predict disease progression.[28]

In conclusion, the results presented in this thesis confirms the observations of previous studies of an association of either CRP or SAA in combination with MMP-3 and disease activity.[21,26] The most significant and original findings of the current study are: i) elevated levels of circulating MMP-3, which may identify a subset of RA patients likely to develop severe disease, are significantly associated with SDAI, pro-inflammatory cytokines, and, most strongly with CRP and SAA; ii) CRP and SAA are more strongly correlated with disease activity in early RA than MMP-3.

Table 1: Demographic, clinical and laboratory data for the group of RA patients (n = 128)

Parameters	Mean	SD
Age (years)	46,5	12,79
Female (%)	102 (79,6)	
Ethnic origin: Black %	119 (92,97)	
DAS ESR	6,28	1,17
Smoking (%)	28 (21,8)	
Nodule (%)	30 (234)	
Erosions (%)	64 (52,03)	
mHAQ-D1	1,72	0,76
Disease duration(months)	11,70	7,11
SJC	3,51	4,08
ESR	45,02	27,64
RF (IU/ml)	515,83	522 099
aCCP (U/ml)	679,74	626,54
CRP (µg/ml)	26,95	33,95
SAA (ug/ml)	66,00	128.54
Hb (g/l)	12,8	1,76
MMP-3: control group (ng/ml)	7,5	5,4
MMP-3(% elevated)	35,8 (56,3)	43,9
Comp : control group (µg/ml)	1.11	0.44
Comp (% elevated)	1.85 (34.6)	0.77

DAS: Disease activity score
HDA: High disease activity
SJC: Swollen joint count
RF: Rheumatoid factor
aCCP: anticyclic citrullinated peptide antibodies
CRP: C-reactive protein
SAA : Serum amyloid A
Hb: haemoglobin
ESR: erythrocyte sedimentation rate
MMP : matrix metalloproteinase
Comp: cartilage oligomeric protein

Table 2: Correlations of MMP-3 with indices of disease activity and non-cytokine biomarkers

Parameter	r	*p**	n
SDAI	0,29	0,0009	128
CRP	0,39	0,0001	115
SAA	0,40	0,0001	115
COMP	0,21	0,0148	127
RF	0,19	0,0320	128

**p* level significance < 0,05

Table 3: Correlations of MMP-3 with cytokines

CYTOKINE	r	*p**	n
IL-8	0,33	0,0001	128
IL-6	0,30	0,0005	128
IFNγ	0,28	0,0010	128
VEGF	0,28	0,0014	128
IL-12	0,27	0,0024	128

**p* level of significance of < 0,003

Table 4: Correlations of SAA and CRP with clinical and laborabory indices of disease activity

Parameter	SAA*	CRP*
SJC	0,25	0.35
	0,007	0.0001
	115	115
DAS ESR	0,29	0.39
	0,002	0.0001
	115	115
SDAI	0,54	0.63
	0,0000	0.0001
	115	115
NODULE	0,18	0.22
	0,047	0.014
	115	115
CRP	0,83	-
	0,0001	-
	115	-
RF	0,24	0.25
	0,009	0.006
	115	115
ACPA	0,21	NS
	0,023	-
	115	-
Erythrocyte sedimentation rate	0,36	0.38
	0,0001	0.0001
	115	115
Haemoglobin	-0,34	-0.26
	0,0002	0.005
	110	110
Platelets	0,26	0.24
	0,005	0.01
	110	110
Erosion	0,21	0.19
	0,023	0.04
	110	110
Larsen score	0,20	0.32
	0,044	0.0006
	106	106
Tender joint count	NS	0.28
	-	0.002
	-	115

* For each pair of values the correlation coefficients are uppermost with the corresponding *p* and n values underneath.

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CHAPTER 4

Circulating anti-citrullinated peptide antibodies, cytokines, matrix metalloproteinase-3 (MMP-3), cartilage metabolites and genotypes as biomarkers of response to disease-modifying anti-rheumatic drug therapy in early rheumatoid arthritis

(Aspects of this chapter have been submitted for publication to the Journal "Clinical and Experimental Rheumatology" submission code 8199).

4.1 Introduction

Raised levels of anti-citrullinated peptide antibodies (ACPA) have diagnostic and prognostic value, and have been incorporated into the 2010 Eular/ACR rheumatoid arthritis (RA) classification criteria.[1] Studies investigating therapy-associated alterations in ACPA levels in patients with early RA have focused predominantly on biologic disease-modifying anti-rheumatic drugs (DMARDs).[2] However, the association of a decrease in ACPA levels with therapeutic response has been variable.[3-13] On the other hand, raised ACPA levels may account for relapse and persistence of disease, with the magnitude of the pre-therapy levels being inversely associated with response to methotrexate (MTX) in early undifferentiated arthritis.[14] ACPA levels have not only been shown to correlate with response to anti-TNF therapy, but are also predictive of response to rituximab.[15]

The underlying immune-pathogenic mechanisms of RA are complex and involve numerous well-defined mediators of inflammation culminating in synovial proliferation and metabolic changes in the joint.

Cytokines play an integral role in the pathogenesis of RA and their importance as therapeutic targets is well established. However, the utility of serial measurement of circulating cytokines in RA is not clearly defined. Changes in cytokine levels post-therapy, especially the balance between pro- and anti-inflammatory cytokines, have the potential to aid in monitoring treatment response, guide future therapy and/or have prognostic implications.[16] For example, a decrease in IL-7 levels after

treatment with MTX has been found to correlate with improved clinical measures of disease activity.[17] In addition, TNF levels below 20.1 pg/ml have been shown to be associated with a good response to MTX, while a low IL-2 level at baseline is an independent predictor of response to synthetic DMARDs.[18] IL-6 levels greater than 4.03 pg/ml post-treatment with MTX have been associated with radiographic progression.[19] The pre-treatment levels of cytokines may also be predictive of response to biologic DMARDs. Patients with elevated serum TNF levels may require higher doses of infliximab, while high levels of IL-17 are possibly predictive of a subgroup of RA patients resistant to TNF blockade.[20] Cytokine ratios may also have prognostic significance, with the IL-6/IL-10 ratio being associated with new coronary events in the general population.[21]

Cartilaginous metabolites such as COMP also have the potential to act as biomarkers in RA management. Pro-inflammatory cytokines increase the production of MMP-3 from chondrocytes facilitating angiogenesis, activation of other proteases and cartilage and bone destruction.[22] Cartilage destruction and increased turnover result in elevated COMP levels.[23] MMP 3 levels have been shown to be associated with measures of disease activity, progression and functional outcome.[24,25] COMP levels may identify a subset of patients with a poorer prognosis and have been associated with MRI-proven erosive disease and radiographic progression.[26,27]

The “shared epitope’’ (SE) is a well-recognized genetic risk factor for, and poor prognostic marker in RA, being associated with both ACPA positivity and a poorer response to MTX monotherapy.[11,28-31] Patients who do not carry the risk alleles generally have milder disease, less radiographic progression and are more likely to respond to DMARDs.

Most studies focused on genotype and profiling of circulating immune biomarkers in prediction of risk and response to therapy in patients with RA have been undertaken in developed world countries. However, RA in the developing world, where there is often little-or-no access to expensive biologic therapies, is associated with as much, if not more, morbidity, than in developed countries, underscoring the importance of discerning clinical utility of traditional DMARD-based therapy in limited resource

settings. To our knowledge, measurement of the SE/risk allele status and its association with longitudinal alterations in clinical disease activity, as well as the concentrations of circulating biomarkers of immune activation, specifically autoantibodies, acute phase reactants and cytokines/chemokines following initiation of DMARD-based therapy has not been described in black African patients with early RA. The clinical data, laboratory findings, treatment and outcome in these patients were reported by Hodkinson *et al* (2012).[32] The association of the shared epitope with baselinencirculating autoantibodies,acute phase reactants, cytokines and clinical indices of activity were reported by Meyer *et al* (2011).[33]

Accordingly, the objectives of the present study were: i) to characterize changes in ACPA and cytokines following synthetic DMARD therapy in an early RA cohort of predominantly black South African patients; ii) evaluate changes in ACPA and cytokines in the different genotype and treatment responder groups; and iii) identify possible baseline biomarkers predictive of disease activity after 6 months of therapy.

4.2 **Patients and methods** (described in detail in chapter 2)

After obtaining approval from the Research Ethics Committees of the Faculties of Health Sciences of the University of Pretoria and the University of the Witwatersrand 140 South African patients who met the 1987 revised classification criteria for RA and were DMARD-naïve, attending two public sector rheumatology clinics, were enrolled as part of the prospective observational GREAT study.[32,33] Informed written consent was obtained from all participants before recruitment into the study. The majority of patients were of black ethnicity (n=124, 88.5%), SE positive (n=106 of 115 tested, 92.1%) and sero-positive (RF, n=114, 81.4%; ACPA, n=106 of 126 tested, 84.1%) with a median (IQR) symptom duration of 9.7 (11.4) months. Thirty four (24.2%) patients had a history of smoking.

Data collected at baseline and 6 months post therapy included the simple disease activity index (SDAI),[34] rheumatoid factor (RF), ACPA and a panel of cytokines, chemokines and growth factors. COMP and MMP-3 were collected at baseline only.

Subgroup analysis included assessment of SDAI categories and SDAI responder groups at 6 months. The levels of disease activity were categorised as follows: i) SDAI ≤ 11 (low disease activity, LDA); ii) SDAI ≤ 26 (moderate disease activity, MDA); and iii) SDAI > 26 (high disease activity, HDA) (table 1). SDAI response was categorised according to percentage improvement from baseline SDAI with <50% being no response, ≥ 50 < 70% a minor response, ≥70 < 85% a moderate response and ≥85% a major response[34] (table 2).

Biomarkers analysed included IL-1β, IL-1Ra, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, TNF, VEGF, CCL2 ,CCL4,MMP-3 and COMP. Ratios of IL-1/IL-1Ra, IL 17/ IL 10, IFN- γ/IL- 4, IL-8/IL-10 and IL-6/IL-10 were calculated. Genotyping was performed by the PCR typing of HLA-DRB1 allele; SE analysis was done according to the Du Montcel classification, with the groups pooled according to SE status into risk-allele-positive (S2,S3P) and -negative (S1,S3D,X) subgroups.[35]

Patients were followed as per routine standard of care, with the attending clinicians deciding on the type of DMARD therapy and frequency of follow-up visits. In all, 43 patients were treated with methotrexate (MTX) monotherapy, 33 with MTX in combination with prednisone; 9 on a combination of MTX and chloroquine (CQ), 1 on sulfasalazine (SZP), 1 on CQ monotherapy, while the remainder were on varying combinations of all 3 DMARDs with or without prednisone. The initial dose of MTX was 7.5-15 mg/week [mean (SD) dose 13.1 (3.2) mg/week], gradually increasing to a maximum of 25 mg/ week. In 77(55%) patients low-dose prednisone, 5-7.5 mg daily, was co-prescribed.

4.3 Statistical analysis

The demographic, clinical and biomarker data are described using frequencies and percentages for categorical types such as gender. Medians and ranges were used to describe the skewed numerical clinical and biomarker data. The Mann-Whitney test was used to assess differences in medians for two independent groups, while the

Wilcoxon signed-rank test was used to compare two dependent groups (i.e., baseline vs. six months). The Kruskal-Wallis test was used to determine differences in medians of more than three independent groups. Statistical significance was ascertained at the 5% level i.e a *p*-value of less than 0.05 was deemed significant, and, where appropriate, Bonferroni corrections to the *p*-values for multiple tests were applied. Multivariable analyses were performed using backward stepwise logistic regression on the outcome disease activity at 6 months and any baseline clinical and laboratory variables with a *p*-value ≤ 0.1 in the univariate analysis were included in the models.

4.4 Results

The baseline demographic, clinical features and laboratory indices of disease activity for the entire cohort are shown in table 3. After 6 months of DMARD therapy, the median SDAI declined from 41.39 (IQR 24.1) at baseline to 16 (IQR 15.8) (p=0.001) (table 4). The categories of response were: i) no response: n=58 (41.4%); ii) minor response: n=40 (28.5%); iii) moderate response: n=22 (15.7%); and iv) major response: n=20 (14.1%).There was similarly a significant fall in the median serum ACPA, several cytokines (IL-4,IL-7, IL-8, G-CSF, VEGF) levels and IL-1β/IL-1Ra and IL-17/IL-10 ratios ($p<0.05$).(table 4) Circulating IL-7, IL-8, G-CSF and VEGF levels were unchanged or increased at 6 months in 41.4%, 30.1%, 43.9% and 42.2% of patients respectively. However, the magnitudes of the decrements in the concentrations of these biomarkers did not correlate significantly with the improvement in SDAI or its individual components of tender and swollen joint counts, C-reactive protein (CRP), and physician and patient global assessments

ACPA levels decreased in the majority of seropositive cases, 66 (85.7%), and in a small minority ACPA levels actually increased, 11 (14.2%), while seroconversion and seroreversion was noted in 3 and 8 patients, respectively, following 6 months of therapy. There were no associations between alterations in ACPA post-therapy with either the baseline SDAI or SDAI at 6 months. There were no differences in the levels of ACPA at baseline or change in ACPA levels after 6 months between patients with a positive or negative history of ever having smoked.

Subgroup analysis of significant changes in SDAI scores and circulating biomarkers, stratified by type of DMARD therapy, MTX monotherapy vs. the combination of MTX and prednisone, at 6 months, are shown in table 5. Pre-therapy swollen joint count (SJC) (*p*=0.032), CRP (*p*=0.025) and IL-7 (*p*=0.011) levels were significantly higher in the combined treatment subgroup, while the SDAI, IL-8 and VEGF values, although higher, did not achieve statistical significance. In both subgroups, the SDAI scores, decreased significantly at 6 months, as did CRP, ACPA and IL-8 levels, but only in the MTX plus prednisone group was there a significant decrease in IL-7, IL-10 and VEGF concentrations. The median CRP value post-therapy remained significantly higher in the combined treatment subgroup relative to the methotrexate monotherapy group.

Subgroup stratification of data by SDAI categories at 6 months, into LDA, MDA and HDA, showed that while a decrease in ACPA levels occurred in all 3 categories, the changes achieved statistical significance in only the LDA and MDA subgroups, possibly due to a smaller number of patients in the HDA subgroup (table 6). With respect to differences at baseline, the median ACPA value for the LDA subgroup was significantly higher than that of the HDA subgroup ($p<0.034$). In the case of the cytokines and chemokines, the most notable decreases at 6 months were observed in the LDA subgroup, with significant decreases in IL-7, IL-8, G-CSF and VEGF concentrations, while remaining elevated in the HDA subgroup, with the exception of IL-7. No statistical difference could be shown in steroid use between the 3 SDAI categories at 6 months. Patients in the LDA subgroup had a statistically significantly lower SDAI at baseline than the corresponding value for the MDA and HDA subgroups combined (*p*=0.007).

Analysis of patients stratified by risk allele status revealed a significantly higher (*p*=0.0452) pre-therapy circulating APCA level in the risk allele-positive group, with no differences between those of the other measured biomarkers, as well as SDAI in the low- and high-risk allele subgroups.Analysis after 6 months of therapy in this group of patients stratified by risk allele status, showed that while SDAI scores, CRP and ACPA concentrations decreased in both the risk allele-negative and positive

subgroups, significant falls in the concentrations of several cytokines IL-4, IL-8, IL-10, G-CSF and VEGF (p=0.0442 – 0.0001) were noted only in the high risk allele subgroup, however more patients were on steroids in this subgroup (*p*=0.0368) (table 7).

On multivariate analysis the changes in the post-therapy concentrations of ACPA, IL-7 and G-CSF (*p*<0.05), may have been influenced by the presence of rheumatoid nodules Baseline MMP-3 was predictive of a moderate plus disease activity (SDAI >11) with an OR = 2.8; *p*<0.032 CI (1.09-7.59) after adjusting for difference in baseline SDAI. None of the other baseline biomarkers analysed including COMP were significantly associated with disease activity at 6 months.

4.5 Discussion

Successful therapy with biologic agents in early and established RA has been associated with decreases in circulating ACPA and pro-inflammatory cytokine levels though not always correlating with clinical indices of disease activity.[2-13] However, very few studies have addressed this issue in the therapeutic setting of synthetic DMARDs in early RA, a situation which is likely to be most important in resource-limited settings, including sub-Saharan Africa

In the present study, ACPA levels decreased significantly post-therapy in the overall cohort of RA patients, as well as in the various sub-groups categorised according to type of therapy (MTX monotherapy or MTX in combination with prednisone), magnitude of baseline SDAI, or risk-allele status. The exception was the HDA subgroup in which changes in ACPA and CRP did not achieve statistical significance, possibly due to sustained high disease activity or a smaller number of patients in the case of ACPA. These findings are in agreement with an earlier study of 66 RA patients which reported decreases in circulating ACPA in a subgroup of early RA patients treated with various synthetic DMARDs, but which did not correlate with either treatment response or type of therapeutic agent.[9] In a more recent study, the absence of a correlation between alterations in ACPA and clinical response following

treatment with MTX was also noted in a group of early arthritis patients, although in contrast to the present study, baseline levels of ACPA were found to be predictive of response to MTX.[12] However, the observation in the current study that the median pre-therapy ACPA value was highest in the subgroup of patients that achieved LDA, appears to underscore the lack of a clear relationship between ACPA levels and response to therapy.

ACPA seroreversion following therapy occurred in only a few patients (n=8) as reported by others,[36] while a total of 3 patients seroconverted. Although ACPA levels decreased in the majority of patients, an increase was observed in others (n=11, 14.2%). However, no differences in SDAI responses were noted between those patients with either increased or decreased levels of ACPA post-DMARD therapy.

Pooled analysis of risk allele-positive (S2/S3P) and -negative (S1/S3D/X) patients revealed a significantly lower baseline ACPA titre in the latter group. Previous studies have described higher circulating concentrations of ACPA in RA patients positive for HLA-DRB*0104 relative to those in patients positive for HLA-DRB*0103, while the S2 and S3P risk alleles have also been reported to be associated with increased levels of ACPA.[37-39]

The association between smoking and predisposition for development of seropositive RA is well recognised.[40] However, baseline ACPA levels, as well as the magnitudes of the post-therapy decrements thereof, did not differ significantly between smokers and non-smokers in the present study. ACPA and smoking status may be influenced by ethnicity as a lack of association shown by Mikuls *et al.* in an African American cohort.[41]

Taken together, the aforementioned findings clearly document significant decreases in circulating ACPA concentrations following 6 months of therapy with synthetic DMARDs, mostly MTX, with or without prednisone, irrespective of baseline SDAI, shared epitope or smoking status. However, the lack of a compelling correlation with changes in clinical indices of disease activity appears to exclude the utility of serial measurement of ACPA as a strategy to monitor the efficacy of therapy with these

agents over the initial 6 month period. In contrast, other studies based on therapy with biologic DMARDs such as adalimumab, rituximab and infliximab reported that measurement of ACPA has the potential to guide therapy with these agents.[42-45] Importantly, and not addressed in the present study, ACPA status, as well as alterations in ACPA during the course of RA may have prognostic value as these have been associated with both disease severity and radiographic progression.[46-55] Moreover, ACPA levels have also been found to correlate with diastolic dysfunction and reduced myocardial mass in patients with RA.[56,57]

With respect to pro-inflammatory cytokines/chemokines, these generally declined significantly in the entire cohort of patients, as did the IL-1β/IL-1Ra and IL-17/IL-10 ratios. However, as reported by others alterations in these parameters were not significantly correlated with improvements in clinical indices of disease activity following treatment of RA patients for 6 month with the combination of MTX and prednisone.[58] Increases in the levels of IL-8 following treatment with MTX, which were noted in 30% of patients in the present study, have been shown to be associated with accelerated radiographic progression.[59]

With respect to the subgroup analyses comparison between the MTX monotherapy and MTX + prednisone subgroups revealed significant decreases in IL-7, IL-8, IL-10, and VEGF in the latter subgroup, but only IL-8 in the former, possibly supporting the role of low-dose corticosteroids in the management of early RA. Interestingly pre-therapy IL-7 and CRP levels, which were unknown to the attending clinicians, were significantly higher in those patients earmarked to receive combined therapy, possibly consistent with a perception of more severe disease, perhaps influenced by the increased SJC (p=0.032) noted in the patients who received MTX and prednisone.

In the various subgroups categorised according to the magnitude of the SDAI at 6 months, the concentrations of IL 4, IL-7, IL-8, IL-10, G-CSF and VEGF decreased significantly in the LDA subgroup post therapy, while IL-8 and VEGF, and only IL-7, declined in the MDA and HDA subgroups, respectively. Persistently elevated levels of IL-8, G-CSF and VEGF in the HDA subgroup may implicate neutrophilic inflammation in the immunopathogenesis of more severe disease. However, the lack of correlation

between circulating concentrations of the individual cytokines with therapy-associated alterations in disease activity appears to exclude both baseline and serial measurement thereof as a strategy to monitor the success of treatment with synthetic DMARDs. Again, this contention contrasts with the apparent utility of baseline measurement of cytokines such as IL-6, IL-8, CCL2 and/or TNF as predictors of response to biologic DMARDs, including golimumab, etanercept and infliximab.[16,60-64]

Differences between cytokine profiles in the risk allele-negative and –positive subgroups were also detected post-therapy, possibly reflecting different inflammatory mechanisms and responses to therapy in relation to genotype, although significantly more patients were on steroids in the risk allele subgroup.

The current paradigm of treating RA is to achieve a low disease activity level i.e SDAI < 11 or preferably remission (SDAI<3.3) by 6 months of therapy. Biomarkers that could identify a group of patients with a poorer outcome may allow for cost-effective stratification and intensive management of patients. However, in this study the only baseline marker of those analysed, associated with disease activity (SDAI > 11) after 6 months of therapy, was MMP-3. Studies have reported an association of baseline MMP-3 with disease activity and/or radiographic progression mostly at 1 year and beyond,[24,65,66] but not many studies have reported on the utility of baseline MMP-3 levels and disease activity as early as 6 months after initiating therapy.

Potential limitations of the study include firstly, the fairly small numbers of patients in some of the subgroups, especially the HDA subgroup, while the presence of nodulosis and lack of treatment randomization may have influenced the analyses. Secondly, 6 months might be too short a period in which to detect associations of circulating biomarkers of disease activity with clinical responses to therapy.Conversely, the strength of the study in our view is that we were able to characterize clinical indices of disease activity and systemic biomarker profiles pre- and post-therapy with synthetic DMARD therapy in patients with relatively early disease, with the potential to guide future therapy. Notwithstanding the apparent lack of clinical utility of serial monitoring of ACPA and pro-inflammatory cytokines to monitor responses to synthetic DMARD therapy, some of the observations are

noteworthy. These include: i) that HDA at baseline is associated with a poor clinical response to therapy in the setting of persistently elevated levels of VEGF, IL-8 and G-CSF, underscoring the possible need for more intensive management to optimize patient outcome; ii) combining MTX with oral corticosteroids is associated with a more robust decline in cytokine levels; iii) ACPA levels measured pre-therapy were found to be significantly higher in patients with the risk allele genotype; and iv) MMP-3 at baseline may be able to identify a sub-group of patients requiring more intensive management.

4.6 Conclusions

These findings show that synthetic DMARD therapy for 6 months is associated with a significant decrease in ACPA and pro-inflammatory cytokines, in tandem with therapeutic response. The lack of correlation with measures of disease activity precludes serial measurement of these biomarkers as a strategy to monitor early responses to therapy with synthetic DMARDs. Nonetheless, the long-term prognostic value of these biomarkers, especially in relation to radiographic progression and functional disability, remains to be established, as does their potential to guide future cost effective therapies, of particular importance in resource-limited settings.

Table 1: SDAI catagories

Remission	≤3
LDA	>3≤11
MDA	>11 ≤ 26
HAD	>26

Table 2: SDAI measures of response

None	<50%
Minor	<70%
Moderate	<85%
Major	≥85%

Table 3: Demographics of patients (n=140).

	n or Median	% or IQR
Black ethnicity (%)	124.0	88.5
Females (%)	110.0	79.2
Age in years, median (IQR)	47.8	14.4
Symptom duration in months, median (IQR)	9.7	11.4
Smoking history (%)	34.0	24.2
Nodulosis (%)	27	19.3
Rheumatoid factor positive (%)	114.0	81.4
ACPA [n=126] (%)	106.0	84.1
Shared Epitope positive [n=115] (%)	106.0	92.1
CRP baseline,median (IQR)	17.1	42.3
SDAI baseline, median (IQR)	41.4	24.1

Table 4: Significant changes in SDAI scores and circulating biomarker concentrations following 6 months DMARD therapy.

Variable	n	Baseline		6 Months		*p* value
		Median	IQR	Median	IQR	
SDAI	140	41.39	24.10	16 .00	15.81	0.0001
CRP (µg/ml)	140	17.10	42.25	8.90	17.35	0.0001
ACPA (IU/ml)	100	516.60	1027.75	255.65	677.20	0.0001
IL-4 (pg/ml)	32	4.70	10.28	1.51	6.53	0.0075
IL-7 (pg/ml)	123	20.32	82.22	16.69	23.46	0.0013
IL-8 (pg/ml)	123	8.79	12.65	5.70	7.09	0.0001
G-CSF (pg/ml)	123	13.82	60.02	7.23	51.61	0.0083
VEGF (pg/ml)	123	168.56	441.33	92.62	167.21	0.001
Ratio IL-1 / IL-1ra	37	0.05	0.05	0.03	0.06	0.013
Ratio IL-17 / IL-10	30	10.42	15.61	3.72	3.81	0.0001

Only those biomarkers which decreased significantly post-therapy are shown. (Changes in whole cohort shown in table 8)

Table 5: Subgroup analysis of changes in SDAI values and circulating biomarkers following 6 months DMARD, for the methotrexate (MTX) monotherapy and methotrexate- prednisone combination therapy groups.

Variable	n	Baseline		6 Months		*p* value
MTX Only		**Median**	**IQR**	**Median**	**IQR**	
SDAI	43	33.58	24.75	16.03	11.05	0.0001
CRP (µg/ml)	43	9.90	21.30	6.90	13.40	0.0048
ACPA (IU/ml)	36	501.60	844.50	310.90	559.60	0.0033
IL-7(pg/ml)	42	14.78	64.04	14.17	19.73	NS
IL-8 (pg/ml)	42	7.68	8.84	5.01	5.32	0.0091
IL-10(pg/ml)	7	6.68	48.16	8.41	23.41	NS
VEGF(pg/ml)	42	114.16	240.82	97.52	184.54	NS

Variable	n	Baseline		6 Months		*p* value
MTX PRED		**Median**	**IQR**	**Median**	**IQR**	
SDAI	33	38.90	20.64	18.00	18.73	0.0001
CRP (µg/ml)	33	35.90	36.50	11.60	19.10	0.0005
ACPA (IU/ml)	29	598.90	1036.60	367.20	699.50	0.0001
IL-7 (pg/ml)	28	46.67	161.13	21.46	31.06	0.0026
IL-8 (pg/ml)	28	10.35	13.27	5.84	4.03	0.0002
IL-10 (pg/ml)	14	12.99	18.55	8.50	23.95	0.048
VEGF (pg/ml)	28	318.91	482.44	77.08	159.38	0.0044

Table 6: Significant changes in circulating biomarker concentrations, stratified according to SDAI at 6 months.

SDAI Category ≤ 11

Variable	n	Baseline		6 Months		*p* value
		Median	**IQR**	**Median**	**IQR**	
SDAI	43	32.42	24.71	6.40	5.34	0.0001
CRP (µg/ml)	43	9.50	20.80	6.90	8.10	0.0054
ACPA (IU/ml)	30	936.55	1162.70	344.60	671.80	0.0005
IL-4 (pg/ml)	9	7.50	7.30	1.50	4.00	0.028
IL-7 (pg/ml)	36	22.22	82.20	16.19	16.74	0.005
IL-8 (pg/ml)	36	8.05	11.48	5.56	6.15	0.0169
IL-10 (pg/ml)	9	13.30	15.25	6.85	5.64	0.0382
G-CSF (pg/ml)	36	21.99	99.56	5.78	55.94	0.0095
VEGF (pg/ml)	36	154.96	442.84	98.62	158.33	0.0036

SDAI Category > 11 ≤ 26

Variable	n	Baseline		6 Months		*p* value
		Median	IQR	Median	IQR	
SDAI	62	42.25	23.39	17.15	6.86	0.0001
CRP (µg/ml)	62	26.50	45.60	8.90	17.00	0.0001
ACPA (IU/ml)	42	483.35	1025.50	252.15	564.70	0.0001
IL-4 (pg/ml)	14	4.08	13.73	2.53	8.47	NS
IL-7 (pg/ml)	55	19.18	61.68	18.17	26.61	NS
IL-8 (pg/ml)	55	8.94	8.92	5.85	6.99	0.0029
IL-10 (pg/ml)	14	14.07	39.13	19.18	19.91	NS
G-CSF (pg/ml)	55	9.45	42.11	7.23	51.61	NS
VEGF (pg/ml)	55	168.10	420.05	84.30	169.40	0.0102

SDAI Category >26

Variable	n	Baseline		6 Months		*p* value
		Median	IQR	Median	IQR	
SDAI	35	43.00	28.50	36.21	11.91	NS
CRP (µg/ml)	35	13.00	44.30	17.00	24.40	NS
ACPA (IU/ml)	28	353.60	917.00	177.00	714.40	NS
IL-4 (pg/ml)	9	1.24	7.18	0.62	1.0	NS
IL-7 (pg/ml)	32	46.67	113.17	13.66	32.18	0.0301
IL-8 (pg/ml)	32	8.55	27.69	6.37	14.49	NS
IL-10 (pg/ml)	9	5.76	8.93	5.81	2.97	NS
G-CSF (pg/ml)	32	11.7	39.12	9.87	54.15	NS
VEGF (pg/ml)	32	170.78	484.05	126.02	187.48	NS

Table 7: Changes in SDAI scores and circulating biomarkers, stratified by shared epitope (risk allele) status.

Risk allele negative

Variable	n	Baseline		6 Months		*p* value
		Median	IQR	Median	IQR	
SDAI	47	39.18	23.50	18.00	19.50	0.0001
CRP (µg/ml)	47	18.00	43.60	7.00	14.60	0.0002
ACPA (IU/ml)	40	331.90	1006.60	185.60	654.15	0.0032
IL-4 (pg/ml)	10	2.55	7.99	5.24	7.18	NS
IL-8 (pg/ml)	44	7.68	14.15	5.23	5.98	NS
IL-10 (pg/ml)	10	7.34	27.79	19.18	19.91	NS
G-CSF (pg/ml)	44	14.42	36.94	12.18	81.23	NS
VEGF (pg/ml)	44	130.52	512.22	92.22	148.07	NS

Risk allele positive

Variable	n	Baseline		6 Months		*p* value
		Median	IQR	Median	IQR	
SDAI	68	40.04	22.12	16.00	16.52	0.0001
CRP (µg/ml)	68	17.50	38.70	10.10	17.50	0.0005
ACPA (IU/ml)	56	561.10	911.05	277.60	677.80	0.0001
IL-4 (pg/ml)	19	5.00	9.80	1.00	1.50	0.0007
IL-8 (pg/ml)	65	9.49	12.15	5.97	6.20	0.0001
IL-10 (pg/ml)	19	11.24	18.78	5.53	6.52	0.044
G-CSF (pg/ml)	65	8.66	42.11	1.85	24.74	0.0442
VEGF (pg/ml)	65	190.43	401.81	95.76	160.99	0.0056

Table 8: Changes in SDAI and circulating biomarker concentrations following 6 months of sDMARD therapy

Variable	N	Median	min	max	iqr	*p* value
SDAI b*	140	41.39	5.28	77	23.67	
SDAI 6**	140	16	0.1	69.77	15.805	0.0001
CRP b	140	17.1	1	303	42.25	
CRP 6	140	8.9	1	83.9	17.35	0.0001
ACPA b	100	516.6	1.9	2526.8	1027.75	
ACPA 6	100	255.65	1.1	1420.3	677.2	0.0001
IL-1 β b	51	6.23	0.12	191.32	13.69	
IL-1 β 6	51	3.8	0.35	199.74	18.17	NS
IL-1Ra b	123	80.06	0	5012.27	288.51	
IL-1Ra 6	123	80.54	0	12084.64	427.81	NS
IL-2 b	32	0	0	216.91	7.41	
IL-2 6	32	0	0	267.6	13.88	NS
IL-4 b	32	4.7	0	904.79	10.28	
IL-4 6	32	1.42	0	62.61	5.31	0.0075
IL-6 b	123	24.35	0	791.93	67.93	
IL-6 6	123	14.23	0	778.82	51.89	NS
IL-7 b	123	20.32	0	6825.1	82.22	
IL-7 6	123	16.69	0	3596	23.46	0.0013
IL-8 b	123	8.79	0	36698.94	12.65	
IL-8 6	123	5.7	0	1618.59	7.09	0.0001
IL-10 b	32	11.435	0	1172.06	29.09	
IL-10 6	32	8.145	1.8	1111	21.71	NS
IL-12 b	123	11.93	0.63	3557.84	64.8	
IL-12 6	123	17.75	0.41	8144.25	74.79	NS
IL-17 b	32	0	0	23.9	0	
IL-17 6	32	0	0	13.87	0	NS
G-CSF b	123	13.82	0	4817.44	60.02	
G-CSF 6	123	7.23	0	1328.97	51.61	0.0083
GM-CSF b	123	1.08	0	1458.59	44.37	
GM-CSF 6	123	0	0	2571.87	82.37	NS
IFNγ b	123	42.64	0	11151.66	264.72	
IFNγ 6	123	27.19	0	6394.31	444.72	NS
TNF b	123	11.67	0.55	2951.68	69.55	
TNF 6	123	12.15	1.33	3107.1	93.82	NS
VEGF b	123	168.56	0	4503.15	441.33	
VEGF 6	123	92.62	0	5007.35	167.21	0.001
CCl-2 b	32	10.65	0	521.48	100.955	
CCl-2 6	32	50.65	0	566.52	81.59	NS
CCl-4 b	32	100.905	26.67	344.72	91.10001	
CCl-4 6	32	96.275	27.26	326.37	62.46	NS

IL-1/IL1-ra b	37	0.050012	0.0057609	0.9042904	0.0487324	
IL-1/IL-1ra 6	37	0.0282269	0.0029457	0.4552684	0.0622231	0.013
IL-17/IL-10 b	30	10.41581	0.3762998	118.3059	15.60833	
IL-17/ IL-10 6	30	3.723691	0.6393507	22.96679	5.373202	0.0001
IFNg/ IL-4 b	24	25.17977	0	179.2	28.12688	
IFNg/ IL-4 6	24	21.07841	1.105882	83.55289	33.04997	NS
IL-8/ IL-10 b	30	0.8005226	0.0097521	45.19495	2.059342	
IL-8/ IL-10 6	30	0.796825	0.0068947	27.28358	1.268058	NS
IL-6/ IL-10 b	30	1.046355	0	33.66154	2.474186	
IL-6/ IL-10 6	30	1.834637	0	37.93333	6.346017	NS

*b – baseline
*6 – 6 months
*NS – not significant

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Conclusion

Rheumatoid arthritis is a chronic inflammatory arthropathy of unknown aetiology. Despite the lack of scientific progress in identifying the underlying causative factors, significant advances have been made in the understanding of pathogenic mechanisms involved. The molecular and cellular components of this pathologic immuno-inflammatory response are now well characterized, enabling the development of novel successful therapeutic interventions.

RA in the developing world has been shown to be associated with as much, if not more morbidity than in developed countries, and few longitudinal studies in developing countries, particularly in sub-Saharan Africa have evaluated clinical, demographic and serological aspects of RA management. Following the interim analysis of this cohort of DMARD naïve early RA patients, some aspects deserve emphasis.

RA patients experienced severe functional impairment despite therapy with only a third of patients achieving an acceptable level of response at one year under routine care. At the outset patients experienced considerable physical and psycho-social impairment with over 60% of patients still having significant disease burden after one year of therapy. A lower level of schooling, unemployment, high disease activity, nodulosis and radiographic damage were some of the factors associated with poorer response to therapy. These factors underscore the need for early diagnosis and an aggressive goal directed therapeutic regimen. Judicious use of newer therapeutic modalities is likely to be cost-effective when combined with clinical and laboratory biomarker strategies, which have the potential to facilitate RA management by identifying poor prognostic factors, thereby potentially guiding choice of therapy.

Genotyping of the patient cohort revealed a high percentage of patients with the susceptibility HLA DRB1 shared epitope allele similar to many studies in the developed world, but in contrast to some studies in Africa. Characterisation of genotypes according to the new du Montcel classification has not been previously evaluated in black South Africans with RA, and revealed the S2 and S3P high-risk

allele as being pre-dominant with under-representation of the S1 allelle. The presence of the SE was associated with high disease activity, ACPA and cytokine profiles, but added no additional value relative to measurement of ACPA. Testing for the SE is thus not recommended in routine care.

The majority of patients was sero-positive with cytokine profiles demonstrating both a pro-inflammatory and counter regulatory anti-inflammatory cytokine milieu. The diagnostic sensitivities of both RF (79%) and ACPA (78%) was similar with increased specificity for RA if both were positive. Baseline ACPA status was not associated with features of disease severity and testing is recommended only in the situation of RF negativity and when clinical suspicion of RA is high. Treatment-naïve cytokine profiles revealed global elevation of cytokines with high inter-cytokine correlations particularly in the high disease activity groups, with VEGF also correlating with radiologic damage and auto-antibodies in the HDA group (at baseline). Cytokine profiles in patients with nodulosis suggest an exaggerated Th1 and macrophage response with elevated pro-angiogenic factors VEGF and IL-8. Baseline DMARD naïve cytokine levels did not correlate with clinical measures of disease activity or auto-antibodies, but could shed light on immune-pathogenic mechanisms or have prognostic value.

Biomarkers of cartilage metabolism such as MMP-3 were associated with disease activity at baseline and predictive of a higher disease activity at 6 months. Elevated baseline serum MMP-3 levels were predictive (OR 2.8) of higher disease activity (SDAI>11) at 6 months follow up, possibly identifying a subset of patients likely to benefit from more aggressive management. None of the auto-antibodies or cytokine profiles at baseline were predictive of disease activity at 6 months.

The recommended therapeutic goal is to have no more than a low disease activity state within 6 months of diagnosis. Characterization of auto-antibody and cytokine profiles at this stage has the potential to stratify patients and guide future therapies. Auto-antibody and cytokine levels following synthetic DMARDs decreased significantly in tandem with disease activity. A more robust decline in cytokines was seen in patients having achieved low disease activity and in patients treated with a

combination of MTX and steroids. Lack of correlation of auto-antibody and cytokine levels with measures of disease activity preclude the utilty of serial measurement.

Limitations of the study include; i) The relatively small number of patients particularly when analysing sub-groups; ii) patients managed as per routine care thus treatment not randomised; iii) patients lost to follow up (20% at 1 year); and iv) full panel of cytokines not tested at 6 months due to cost constraints.

The strength of the study lies in the prospective review of clinical and serological measures associated with RA in a ‘real life’ situation, potentially providing practical cost-effective recommendations and highlighting the need for more research. In a resource limited country early diagnosis and aggressive management targeting of at least a low disease activity state within 6 months should be achieved in most of our patients. Management should include patient education, social support and rapid escalation of DMARDs. This will require more frequent and intensive review putting more strain on health providers thus it is imperative that patients with poor prognostic markers be identified at the outset. Expensive biologic therapies are accessible to a only few patients thus characterisation of cytokine profile in sDMARD refractory patients may facilitate proper patient selection and choice of therapy. In addition to clinical and functional measures, a useful outcome measure, radiographic progression is also being analysed in this cohort at 1 and 2 years after treatment. Biomarkers including baseline auto-antibodies, cytokines and cartilage metabolites able to predict radiographic progression may be used to identify patients requiring a more extensive yet cost effective- approach.

Appendix 1

Abstracts of data published as Co-author

1.1 Meyer PW, Hodkinson B, **Ally M**, Musenge E, Wadee AA, Fickl H, Tikly M, Anderson R. Circulating cytokine profiles and their relationships with autoantibodies, acute phase reactants, and disease activity in patients with rheumatoid arthritis. *Mediators of Inflammation* 2010; 2010:158514.

Our objective was to analyse the relationship between circulating cytokines, autoantibodies, acute phase reactants, and disease activity in DMARDs-naïve rheumatoid arthritis (RA) patients (n = 140). All cytokines were significantly higher in the RA cohort than in healthy controls. Moderate-to-strong positive intercorrelations were observed between Th1/Th2/macrophage/fibroblast-derived cytokines. RF correlated significantly with IL-1β, IL-2, IL-4, IL-10, IL-12, G-CSF, GM-CSF, IFN-γ, and TNF ($P < .0001$), and aCCP and aMCV with IL-1β, IL-2, IL-4, and IL-10 ($P < .0002$), while IL-6 correlated best with the acute phase reactants, CRP, and SAA ($P < .0001$). In patients with a DAS28 score of ≥5.1, IFN-γ, IL-1β, IL-1Ra, TNF, GM-CSF, and VEGF were significantly correlated ($P < .04$-$.001$) with high disease activity (HDA). Circulating cytokines in RA reflect a multifaceted increase in immune reactivity encompassing Th1 and Th2 cells, monocytes/macrophages, and synovial fibroblasts, underscored by strong correlations between these cytokines, as well as their relationships with RF, aCCP, and aMCV, with some cytokines showing promise as biomarkers of HDA.

1.2 Hodkinson B, Meyer PW, Musenge E, **Ally MM**, Wadee AA, Anderson R, Tikly M. The diagnostic utility of the anti-CCP antibody test is no better than rheumatoid factor in South Africans with early rheumatoid arthritis. *Clin Rheumatol* 2010; 29(6):615-618.

To establish the diagnostic utility of the anti-cyclic-citrullinated peptide antibody (aCCP) test in Black South Africans with early rheumatoid arthritis (RA). A cross-sectional study comparing the rheumatoid factor (RF) and aCCP status in RA patients and a control group consisting of healthy subjects, and patients with systemic lupus erythematosus (SLE) and scleroderma. The sensitivity, specificity, positive (PPV) and negative predictive values of the aCCP test alone were 82.5%, 84.9%, 87.6% and 79% versus 81.7%, 90.7%, 92.5% and 78% for RF alone. The best specificity (95.3) and PPV (95.8%) was observed when both aCCP and RF tests were positive. Patients with erosive disease had a significantly higher mean RF titre compared with those with non-erosive disease (p = 0.007). There was a trend towards an association of smoking (OR = 4.1, 95% CI = 0.9-18.6) and functional disability (p = 0.07) with RF-positive status. No similar clinical associations were observed with aCCP. Almost a third of SLE patients were aCCP positive. Despite the best specificity and PPV observed when both the aCCP and RF tests were positive, our findings suggest that testing for aCCP is only cost-effective in the RF-negative patient in whom there is a strong clinical suspicion of RA.

1.3 Meyer PW, Hodkinson B, **Ally M**, Musenge E, Wadee AA, Fickl H, Tikly M, Anderson R. HLA-DRB1 shared epitope genotyping using the revised classification and its association with circulating autoantibodies, acute phase reactants, cytokines and clinical indices of disease activity in a cohort of South African rheumatoid arthritis patients. *Arthritis Res Ther* 2011; 13(5):R160.

INTRODUCTION:
The revised shared epitope (SE) concept in rheumatoid arthritis (RA) is based on the presence (S) or absence (X) of the SE RAA amino acid motif at positions 72 to 74 of the third hypervariable region of the various human leucocyte antigen (HLA)-DRB1 alleles. The purpose of this study was to investigate SE subtypes on the basis of the American College of Rheumatology 1987 revised criteria for the classification of RA in a cohort of

South African RA patients (n = 143) and their association with clinical and circulating biomarkers of disease activity (autoantibodies, acute phase reactants and cytokines).

METHODS:
Genomic DNA was analysed using high-resolution recombinant sequence-specific oligonucleotide PCR typing of the HLA-DRB1 allele. Subtypes of the SE were classified according to the amino acids at positions 72 to 74 for the RAA sequence, and further sub-divided according to the amino acids at positions 70 and 71, which either contribute to (S2, S3P), or negate (S1, S3D) RA susceptibility. Disease activity was assessed on the basis of (1) Disease Activity Score in 28 joints using C-reactive protein (CRP), (2) rheumatoid factor (RF), (3) CRP and (4) serum amyloid A by nephelometry, anticyclic citrullinated peptide antibodies (aCCP) by an immunofluorometric procedure, and cytokines by multiplex bead array technology.

RESULTS:
Of the 143 RA patients, 81 (57%) were homozygous (SS) and 50 (35%) were heterozygous (SX) for the SE alleles with significant overexpression of S2 and S3P (respective odds ratios (ORs) 5.3 and 5.8; $P < 0.0001$), and 12 (8%) were classified as no SE allele (XX). Both the SS and SX groups showed a strong association with aCCP positivity (OR = 10.2 and P = 0.0010, OR = 9.2 and P = 0.0028, respectively) relative to the XX group. Clinical scores and concentrations of the other biomarkers of disease activity (RF, CRP and T helper cell type 1 (Th1), Th2, macrophage and fibroblast cytokines) were also generally higher in the SS group than in the SX and XX groups.

CONCLUSIONS:
RA susceptibility alleles investigated according to revised criteria for the classification of RA were significantly increased in South African RA patients

and strongly associated with aCCP in particular as well as with circulating cytokines and disease severity.

1.4 Hodkinson B, Musenge E, **Ally M**, Meyer PW, Anderson R, Tikly M. Functional disability and health-related quality of life in South Africans with early rheumatoid arthritis. *Scand J Rheumatol.* 2012 Oct;41(5):366-74.

BACKGROUND:
The severity and predictors of functional disability and health-related quality of life (HRQoL) in a cohort of South Africans with early rheumatoid arthritis (RA) were investigated.

METHODS:
Changes in the Health Assessment Questionnaire Disability Index (HAQ) and the 36-Item Short Form Health Survey (SF-36) following 12 months of traditional disease-modifying anti-rheumatic drugs (DMARDs) were studied in previously DMARD-naïve adults with disease duration ≤ 2 years.

RESULTS:
The majority of the 171 patients were female (82%), Black Africans (89%) with a mean (SD) symptom duration of 11.6 (7.0) months. In the 134 patients seen at 12 months, there were significant improvements in the HAQ and all domains of the SF-36 but 92 (69%) still had substantial functional disability (HAQ > 0.5) and 89 (66%) had suboptimal mental health [SF-36 mental composite score (MCS) < 66.6]. Multivariate analysis showed that female sex ($p = 0.05$) and high baseline HAQ score ($p < 0.01$) predicted substantial functional disability at 12 months. Unemployment ($p = 0.03$), high baseline pain ($p = 0.02$), and HAQ score ($p = 0.04$) predicted suboptimal mental health, with a trend towards a low level of schooling being significant ($p = 0.08$).

CONCLUSIONS:

Early RA has a broad impact on HRQoL in indigent South Africans, with a large proportion of patients still showing substantial functional disability and suboptimal mental health despite 12 months of DMARD therapy. Further research is needed to establish the role of interventions including psychosocial support, rehabilitation programmes, and biological therapy to improve physical function and HRQoL in this population.

1.5 Hodkinson B, Musenge E, **Ally M,** Meyer PW, Anderson R, Tikly M. Response to traditional disease-modifying anti-rheumatic drugs in indigent South Africans with early rheumatoid arthritis. *Clin Rheumatol* 2012; 31(4):613-9.

The clinical response to traditional disease-modifying anti-rheumatic drugs (DMARDs) in indigent South Africans with early rheumatoid arthritis was investigated. A cohort of patients with early (≤2 years) RA who were DMARD-naïve at inception were prospectively assessed for response to DMARDs using the Simplified Disease Activity Index (SDAI) over a 12-month period. Patients with low disease activity (LDA) at 12 months were compared to those with moderate and high disease activity with respect to demographic, clinical, autoantibody and radiographic features. The 171 patients (140 females) had a mean (SD) age of 47.1 (12.4) years, symptom duration of 11.7 (7.1) months and baseline SDAI of 39.4 (16.2). There was a significant overall improvement in the SDAI and its components in the 134 (78.4%) patients who completed the 12 months visit, but only 28.4% of them achieved LDA. The majority of patients (91%) were treated with methotrexate as monotherapy or in combination with chloroquine and/or sulphasalazine. Baseline features that independently predicted a LDA state at 12 months were lower Health Assessment Questionnaire Disability Index ($p = 0.023$) and a higher haemoglobin level ($p = 0.048$). Receiver operating characteristic curve analysis showed that the 6-month SDAI was better than the baseline SDAI in predicting the 12-month SDAI (area under the curve of 0.69 vs. 0.52, respectively, $p = 0.008$). In

conclusion, less than a third of the patients achieved a low disease activity at 12 months on traditional DMARDs. Patients who have an inadequate response to traditional DMARDs at 6 months are unlikely to show further improvement on traditional DMARDs at 12 months. These findings underscore the need for better disease control by an aggressive tight control strategy, including intense patient education and biologic therapy.

1.6 Hodkinson B, Meyer PW, Musenge E, **Ally M,** Anderson R, Tikly M.Exaggerated circulating Th-1 cytokine response in early rheumatoid arthritis patients with nodules. *Cytokine* 2012; 60(2):561-564.

BACKGROUND:
Immunohistochemical studies of the rheumatoid nodule (RN) suggest it is a Th1 granuloma, with focal vasculitis, yet the pathogenesis remains unclear and little is known about circulating cytokines in these patients.

OBJECTIVE:
We studied circulating cytokines in DMARD-naïve RA patients to investigate associations with subcutaneous RN.

METHODS:
149 DMARD-naïve adults with early RA (symptom duration ≤ 2 years) were assessed using the Simplified Disease Activity Index (SDAI), and hand and feet radiographs were scored using the modified Larsen method. Circulating cytokines and growth factors representative of T-helper cell 1(Th1) and Th2 cell, macrophages, and fibroblasts were measured using the Bio-Plex® suspension array system.

RESULTS:

Of 149 patients, 34 (22.8%) had subcutaneous RN, and these patients had more severe disease with higher mean swollen joint counts (p=0.02), SDAI (p=0.04) and modified Larsen scores (p=0.004). There were no differences in Rheumatoid Factor or anti-cyclic citrullinated peptide antibody positivity between patients with RN and those without RN. Patients with RN showed significantly higher levels of circulating IL-12 (p=0.02), IL-2 (p=0.048), and VEGF (p=0.033) levels, with a trend towards higher levels of IL-7 (p=0.056), IFN-γ (p=0.059) and IL-8 (p=0.074) compared to those without RN.

CONCLUSIONS:

DMARD-naïve early RA patients with RN had more severe disease than those without RN, and showed an exaggerated circulating Th1 and macrophage cytokine profile.

Appendix 2

Clinical Record

CENTRE NUMBER | | | | PATIENT NUMBER | | | | PATIENT INITIALS | | | |

DATE ___/___/___ ASSESSOR INITIALS | | |

EARLY MORNING STIFFNESS (minutes) _____

SWOLLEN JOINT COUNT

RIGHT						LEFT				
					Shoulder					
					Elbow					
					Wrist					
5	4	3	2	1	MCP	1	2	3	4	5
					PIP					
					Knee					

TENDER JOINT COUNT : _____

RIGHT						LEFT				
					Shoulder					
					Elbow					
					Wrist					
5	4	3	2	1	MCP	1	2	3	4	5
					PIP					
					Knee					

PHYSICIAN GLOBAL ASSESSMENT

Excellent	
Good	
Fair	
Poor	
Very poor	

PATIENT PAIN ASSESSMENT

How bad has your pain over the last week?

No pain	
Mild pain	
Moderate pain	
Severe pain	
Unbearable pain	

PATIENT GLOBAL ASSESSMENT

How has your arthritis been over last week?

Excellent	
Good	
Fair	
Poor	
Very poor	