UNIVERSITY OF KWAZULU NATAL

BIOCHEMICAL, APOPTOTIC AND CELLULAR STRESS STUDIES IN RHEUMATOID ARTHRITIS

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DECLARATION

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DEDICATION

To my parents

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Professor Anil A. Chuturgoon.

Thank you for your mentorship, guidance and most of all your friendship. Thank you for believing in me and providing the best opportunities to explore my curiosities in the lab. Thank you for giving my life guidance and direction.

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PUBLICATIONS

Moodley D, Mody G, Patel N and Chuturgoon AA, Mitochondrial depolarisation and oxidative stress in rheumatoid arthritis patients. *Clinical Biochemistry* (2008), 41, 1396 - 1401, doi:10.1016/j.clinbiochem.2008.08.072.

PRESENTATIONS

Mitochondrial depolarisation correlates with disease activity in South African Blacks with rheumatoid arthritis.

Moodley D, Patel N, Mody GM, Chuturgoon AA

21st Biennial Conference of the South African Rheumatism & Arthritis Association *(May 2009)*

Drakensberg, South Africa

Analysis of the p53 codon 72 polymorphism in African Blacks with Rheumatoid Arthritis

Moodley D, Naidoo K, Patel N, Mody GM, Chuturgoon AA.

20th Biennial Conference of the South African Rheumatism & Arthritis Association *(September 2007)*

Cape Town, South Africa

Lack of correlation between peripheral lymphocyte apoptosis and markers of disease activity in South African Blacks with Rheumatoid Arthritis

Moodley D, Patel N, Mody GM, Chuturgoon AA.

Astra-Zeneca College of Health Sciences Research Symposium *(September 2007)*

Durban, South Africa

Lymphocyte apoptosis and intra-cellular antigen expression in Black South African Rheumatoid Arthritis patients

Moodley D, Chuturgoon AA, Mody GM, Patel N.

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Durban, South Africa

Investigation of apoptosis in Rheumatoid Arthritis patients

Moodley D, Chuturgoon AA, Mody GM, Patel N. -

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease which causes synovial damage. Persistence of inflammatory cells in synovial tissue may be related to defects in apoptosis. Chronic inflammation may lead to oxidative damage and mitochondrial defects in peripheral lymphocytes (PL) and this may alter apoptotic mechanisms. The p53 tumour-suppressor protein plays an integral role in apoptosis by acting as a pro-apoptotic transcriptional regulator, or by altering mitochondrial membrane potential ($\Delta\psi_m$). Polymorphisms at codon 72 of p53 confers differences in mitochondrial translocation and apoptosis inducing capabilities of p53 *in vitro*. This study investigated PL apoptosis and its association with clinical parameters in RA. In addition cytotoxicity, oxidative stress and mitochondrial membrane integrity was also examined. Since the p53 codon 72 polymorphism affects apoptosis and mitochondrial membrane dynamics, the genotype at this locus was determined. Fifty South African black RA patients (HIV^-) were recruited into the study. Total, CD4+ and CD19+ PL apoptosis was investigated using the Annexin-V assay. Further, PL receptor mediated apoptosis (CD95/Fas) as well as activation-induced-cell-death (AICD) (CD69) were determined by flow cytometry. Capase activity was measured by luminometry. Heat-shock-protein-70 (HSP70) levels were determined by intra-cellular flow cytometry and confirmed by western blots. The JC-1 assay was used to assess $\Delta\psi_m$. Cytotoxicity and oxidative stress was measured using the lactate dehydrogenase (LDH) and the thiobarbituric acid reactive substances (TBARS) assays respectively. Genotypic differences in the p53 gene were determined by

polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Statistical differences in these parameters were investigated according to genotype. Correlations between apoptosis, $\Delta\psi_m$ and clinical parameters were tested for statistical significance. RA-PL showed signs of elevated apoptosis which seemed to depend on CD95/Fas mediated signals. However, low levels of the CD69 marker suggested that this was not associated with immune activation. Although caspase activities (caspase-3/7, caspase-9) were increased, no DNA fragmentation was observed in RA-PL. This may be related to elevated levels of HSP70. No statistically significant associations between apoptosis and clinical parameters were found. Cytotoxicity (p=0.0080) and lipid peroxidation (p=0.0030) were significantly elevated in RA. A significantly higher percentage of circulating PL contained depolarised mitochondria (p=0.0003) which correlated with disease activity and C-reactive protein levels in patients. Collapse of $\Delta\psi_m$ also negatively correlated to absolute lymphocyte counts (r=-0.4041; p=0.0197). The p53 genotype distribution did not differ significantly between RA patients and controls (Arg/Arg, Arg/Pro, Pro/Pro: 12%, 46%, 42% versus 3%, 34%, 63%; Chi-square statistic= 2.104, 1 degree of freedom; p = 0.1469), despite significantly higher frequency of the Arg72 allele in patients (Chi-square statistic = 4.191, 1 degree of freedom; p = 0.0406). There was no significant difference in PL apoptosis (p=0.1573) and mitochondrial depolarisation (p=0.8127) based on p53 codon 72 genotype. In addition, clinical markers of disease activity were not significantly different between genotypes. The results suggest that while apoptosis may be initiated in RA-PL, they may lack commitment to fully executing the apoptotic program. This hypothesis is supported by the observation

that absolute lymphocyte counts did not correlate with apoptosis. Correlation between disease activity and $\Delta\psi_m$ suggest a possible role for mitochondrial membrane alterations in the pathology of RA. The p53 codon 72 genotype does not influence PL apoptosis or mitochondrial depolarisation, and is not associated with clinical markers of disease in black South African RA patients.

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LIST OF ABBREVIATIONS

$\Delta\psi_m$	Mitochondrial membrane potential
ACR	American college of rheumatology
AGE	Advanced-glycation-end-products
AICD	Activation induced cell death
Apaf1	Apoptotic-protease-activating-factor
APC	Allophycocyanin
Bcl	B cell lymphoma
BFGF	Basic fibroblast growth factor
BH1-4	Bcl-2 homology domains 1-4
BHT	Butylated hydroxytoluene
BID	Bcl-2 homology-3 interacting-death-domain
CAD/DFF40	Caspase-activated-DNase/DNA fragmentation factor 40
Caspases	Cysteinyl aspartate-specific proteases
CRP	C reactive protein
CXCR	Lymphocyte chemokines receptor
Cyt c	Cytochrome c
DISC	Death-inducing signaling complex
DMARD	Disease-modifying-anti-rheumatic-drugs
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate,
FADD	Fas associated death domain

FITC	Fluorescein-isothiocyanate
FLS	Fibroblast-like-synoviocytes
HAQ	Health assessment questionnaires
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horse-radish-peroxidase
HSP	Heat shock protein-70
IAP	Inhibitor of apoptosis proteins
Ig	Immunoglobulin
IHMP	International HapMap Project
IL-2	Interleukin 2
IL-7	Interleukin 7
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinases
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MIF	Macrophage inhibitory factor
MPTP	Mitochondrial permeability transition pores
mtDNA	mitochondrial DNA
MTX	Methotrexate

NF- kB	Nuclear factor kB
p53	Tumour suppressor protein 53
PADI	Peptidyl-arginine deiminianses
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PDGF	Platelet derived growth factor
PI	Propidium iodide
PL	Peripheral lymphocytes
PS	Phosphatidylserine
PTPN22	Protein tyrosine phosphatase non-receptor type 22
PVDF	Polyvinylidene difluoride membrane
RA	Rheumatoid arthritis
RIP1	Receptor interacting protein 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDF1α	Stromal-cell-derived-factor-1α
SDS	Sodium dodecyl sulfate
SF	Synovial fibroblastssevere combined immuno-deficiency
SLE	Systemic lupus erythematosus
SMAC/DIABLO	Second-mitochondrial activator of caspases/direct-Inhibitor-of-apoptosis-binding-protein-with-low-PI
TBARS	Thiobarbituric acid reactive substance

TBS	After blocking with Tris-buffered saline
TGFβ	Transforming growth factor β
TNF	Tumour necrosis factor
TNFR1	TNF receptor 1
TRAF2	TNF receptor associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
UCP	Uncoupling proteins
VCAM1	Vascular-cell-adhesion-molecule-1
WTCCC	Wellcome Trust Case Control Consortium
XIAP	X-linked-inhibitor-of-apoptosis-protein

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INTRODUCTION

Rheumatoid arthritis (RA) is a debilitating autoimmune disease affecting approximately 1% of the world's population. Disease aetiology is not clearly understood and no concrete genetic associations have yet been identified. There is however substantial association with human-leukocyte-antigen (HLA) genes and currently the HLA-DRβ1 is a reliable indicator of RA susceptibility and a good predictor of disease severity (Mody et al., 1989). HLA profiles in South African populations are associated with more severe forms of RA. This, combined with late diagnosis and initiation of treatment, due to constraints on public health care systems, usually means poorer prognosis and frequently, irreversible joint damage. To effectively treat RA, the disease needs to be diagnosed early and rigorous treatment must be initiated promptly. There is a clear and essential need for basic science research to better understand disease mechanisms and identify new disease biomarkers. This is particularly necessary in South African populations since treatment strategies and diagnostic guidelines are based on those developed in the western world and may not necessarily apply to local populations (Mody and Cardiel, 2008).

The disease is characterised by chronic inflammation of synovial joints and synovial hyperplasia. Uncontrolled, invasive growth of synovial tissue causes destruction of bone and cartilage. The persistence of inflammatory cells in the synovium leads to chronic generation of pro-inflammatory signals which perpetuates infiltration of lymphocytes into the synovium. In the normal inflammatory response infiltrating leukocytes are cleared

from inflammatory sites by apoptosis (programmed cell death) after they have performed their relevant functions (Serhan and Savill, 2005). Cells in the inflamed RA synovium are protected from apoptosis and the pathogenic tendencies of these cells parallel those of cells found in tumours (Muller-Ladner et al., 1995).

It is currently unclear whether the deficiencies in lymphocyte apoptosis are inherent in RA or are induced by complex cellular interactions upon infiltration into the synovium. The biology of circulating lymphocytes has not been comprehensively investigated in RA, especially within the context of apoptosis. Although the clinical manifestation of RA presents as destructive joint disease, there is a significant, but poorly understood systemic component. Deficient and/or aberrant apoptosis in B and T lymphocytes may contribute to the subtle and often elusive systemic pathologies of RA.

Apoptosis is an essential biological mechanism used to regulate tissue micro-environments and maintain homeostasis. The immune system relies heavily on apoptosis to orchestrate the removal of unwanted and pathogenic cells. Apoptotic pathways are tightly regulated and breaches of regulatory mechanisms may be associated with pathogenesis. Several recent reports suggest a strong correlation between apoptosis and autoimmunity through several mechanisms, namely:

a) impairment of apoptotic pathways;

b) ineffective removal of apoptotic cells;

c) autoantigen presence in apoptotic bodies;

d) aberrant antigen presentation by apoptotic cells;

e) abnormal activation of innate immunity and macrophages by apoptotic cells;

f) apoptotic bodies acting as specific B lymphocyte autoantigens (Lleo et al., 2008).

Autoimmunity and apoptosis exist in a paradoxical relationship, since apoptotic signals have been associated with increased expression of anti-inflammatory cytokines in order to minimize tissue stress and prevent inflammation. It is unknown whether loss of immune tolerance during the development of autoimmunity in RA occurs due to faulty antigen processing as a result of defective apoptosis in circulating B and T lymphocytes.

In RA, circulating lymphocytes exist in a milieu of chronic inflammatory signals and are thus subject to chronic cellular stress. In addition, chronic inflammation is associated with increased production of reactive oxygen species (ROS) and nitrogen species (RNS) which are potentially damaging to lymphocytes in peripheral circulation (Hitchon and El-Gabalawy, 2004). Two important proteins which function in cellular stress response pathways are the tumour suppressor protein, p53, and the molecular chaperone, heat-shock-protein-70 (HSP70). Both proteins are important regulators of apoptosis. p53 detects DNA damage and responds primarily by transcriptional regulation of genes which halt the cell-cycle and promote apoptosis. HSP70 in contrast promotes cell survival by correcting abnormally folded proteins and inhibiting the activity of proteolytic enzymes which operate during apoptosis. Heat shock proteins have long been associated with autoimmunity through molecular mimicry mechanisms because of their highly conserved sequence homology across species (Baier et al., 2003; Vousden and Lu, 2002). Breakdown of the p53 tumour suppressor pathways may account for the strong parallels

between autoimmunity and carcinogenesis. The roles of these proteins and their relation to apoptosis have not been studied in RA.

Apoptosis represents a relevant therapeutic target for autoimmune diseases. Specifically in the RA synovium, promoting apoptosis of actively proliferating cells may alleviate inflammation and joint damage. With respect to the systemic components of RA, while the biology of apoptotic pathways in circulating lymphocytes have not been comprehensively studied, current evidence clearly indicates that apoptosis may play an important role in pathogenesis. However, in order to harness therapeutic potentials of apoptosis in the periphery, our knowledgebase of cell death and its regulation in circulatory lymphocytes needs to vastly increase. It is therefore essential to conduct studies in this area and it is imperative that such work pertains to local populations who are subject to vastly differing socio-economic and environmental stressors.

STUDY RATIONALE AND AIMS

The study of apoptosis is gradually providing novel clues to better understand the subtle underlying mechanisms of autoimmunity, it is therefore necessary to examine this biological phenomenon in local patients suffering from autoimmune diseases. Specifically in RA where the genetic backgrounds of local patients contribute to more debilitating forms of the disease, study of apoptosis may provide new means to measure disease progression and response to treatment.

This study was designed to measure apoptosis in peripheral lymphocyte subsets from local RA patients and to investigate the roles of the inducible cell stress proteins p53 and HSP70. The study focused on circulating lymphocytes since the biology of cells already recruited to the RA synovium may be obscured due to the inflammatory and oxidative environment.

The relationship between peripheral lymphocyte apoptosis, disease activity and markers of inflammation is currently unknown in RA. This study served to investigate the relationship between these important parameters.

In addition, it was necessary to examine oxidative stress and its impact on mitochondrial function in peripheral lymphocytes. The mitochondrion is integral in apoptotic pathways and previous studies have shown by computational methods that mitochondrial proteins may act as autoantigens in RA (Da Sylva et al., 2005). They did not however, establish

how the internal mitochondrial contents exit the cell. Aberrant apoptosis may explain how mitochondrial autoantigens gain access to the extracellular compartment.

No other study to date has measured apoptosis *ex vivo* in South African RA patients. It was therefore necessary to establish baseline measurements for local populations. Additionally, no study has considered the effects of p53 and HSP70 on apoptosis and their relation to RA. Moreover, the impact of oxidative stress on mitochondrial function and apoptosis in circulating lymphocytes has not previously been elucidated.

Since there are parallels between autoimmunity and carcinogenesis it is necessary to examine the functional integrity of p53. A non-conservative polymorphism occurs at codon 72 of p53 where there is an arginine to proline transition. The polymorphic variants of p53 differ in their biological activity and hence in their ability to induce apoptosis. There are two reports to date which have examined this polymorphism in RA cohorts, but no study has examined the influence of the polymorphism on peripheral lymphocyte apoptosis in autoimmunity. The genotype at this locus is also unknown for South African RA patients.

CHAPTER 1

LITERATURE REVIEW

1.1 Overview of rheumatoid arthritis

Rheumatoid arthritis is a systemic autoimmune disease with chronic inflammation of synovial joints. General symptoms of the disease include joint pain and swelling. This is often accompanied by impaired movement and debilitating fatigue. Chronic joint inflammation may lead to bone and cartilage destruction, resulting in deformities and disabilities. The clinical spectrum of RA is wide and ranges from mild joint inflammation to severe systemic inflammation which can contribute to extra-articular symptoms such as pericarditis, pulmonary insufficiency and vasculitis. While the primary clinical manifestation of RA is destructive joint disease, there is a significant, but poorly understood systemic component. The systemic autoimmune manifestations of RA are perpetuated in part by circulating auto-reactive B and T lymphocytes. Pro-inflammatory signals cause these cells to infiltrate and persist in the rheumatoid synovium. The normal inflammatory response is ameliorated by apoptotic clearance of the lymphocyte infiltrate. In RA it is unknown whether circulating lymphocyte biology is compromised prior to synovial recruitment or whether infiltrating lymphocytes acquire abnormalities upon adopting a stationary phenotype in the rheumatoid synovium (Karouzakis et al., 2006).

1.2 Epidemiology of rheumatoid arthritis

The prevalence of RA is estimated to range between 0.5-1% worldwide and is one of the most common autoimmune diseases. It occurs in women more frequently with a female to male ratio of approximately 3:1 (Alamanos and Drosos, 2005; Alamanos et al., 2006). There is considerable variation in prevalence between population groups. Higher frequencies of RA have been reported in populations of European ancestry (approximately 1%) than in Asians and Africans (Abdel-Nasser et al., 1997). In South Africa the prevalence of RA is estimated to range between 0.1- 0.9%, and there are considerable disparities between rural (0.1%) and urban (0.9%) disease populations (Beighton et al., 1975; Meyers et al., 1977; Solomon et al., 1975). These studies were however conducted with small sample sizes and may also reflect differences in access to primary health care.

1.3 Clinical manifestations of rheumatoid arthritis

The disease is characterized by symmetrical pain and swelling of the joints (figure 1.1). Chronic synovitis leads to destruction of major joints including the cervical spine, cricoarytenoid joints, temperomandibular joints, sternoclavicular joints, elbow and most commonly the wrists, hands, hips and knees (Smolen and Aletaha, 2009).

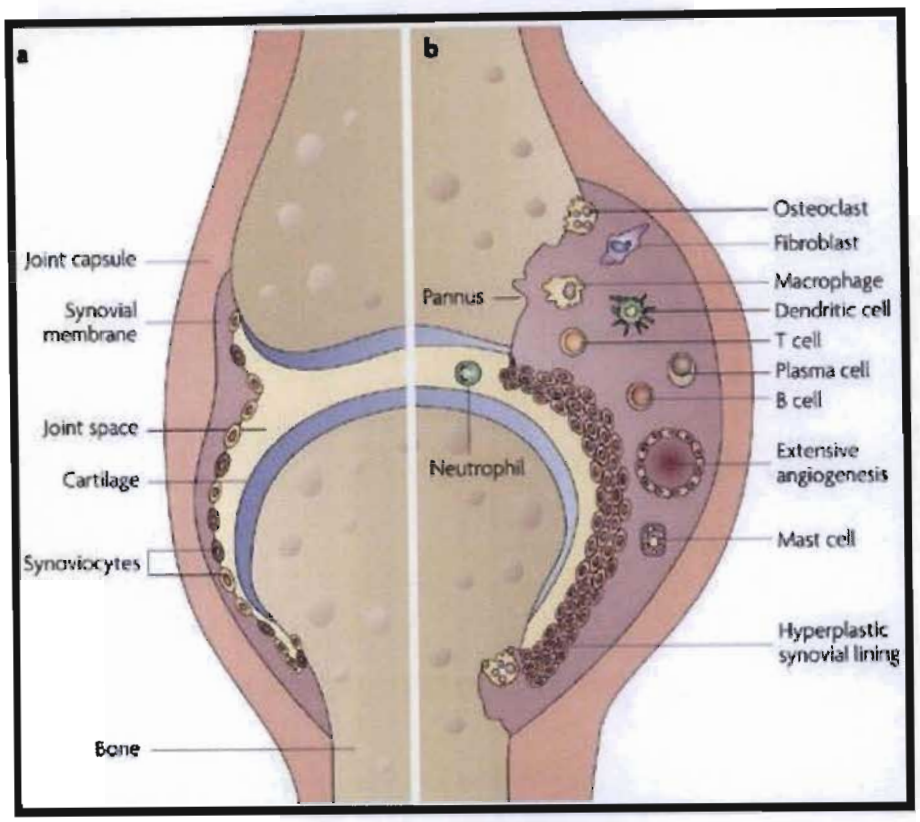


Figure 1.1 Comparison between normal (a) and rheumatoid (b) joints (Strand et al., 2007).

Specific symptoms of RA in these joints include:

Pain

The major symptom of RA is joint pain. Elevated intra-articular pressure in the rheumatoid synovium creates stress on periarticular structures which leads to pain. Arthritic pain usually peaks in the morning and persists throughout the entire duration of the day (Smolen and Aletaha, 2009).

Swelling

Proliferation of the synovial lining, in addition to accumulation of intra-articular fluid and increased fluid tension contribute to joint swelling in RA.

Joint temperature

Interestingly, joint temperature in RA is elevated and there are tactile differences between the joint and surrounding tissue (Oosterveld and Rasker, 1994a, 1994b). The elevated joint temperature in RA has been attributed to the breakdown of collagen in the rheumatoid synovium.

Joint stiffness

During sleep, rheumatoid joints become oedematous and lose mobility as a consequence. Accumulation of fluid results in morning joint stiffness which typically lasts between one to three hours. During this time, RA patients are immobile and experience peak level pain upon movement (Arnett et al., 1988).

Impaired mobility

The range of joint motion is severely impaired in RA. Early in arthritis, the limitation of joint mobility may be reversible if treatment is initiated promptly. More than 50% of RA patients have impaired mobility prior to first clinic consults. In patients with chronic RA, 25-35% of joints lose their mobility due to proteolytic degradation of cartilage and bone (Eberhardt and Fex, 1995).

Muscle weakness

In patients with mild arthritis, the strength of muscles associated with large joints is reduced to approximately 70% of the norm. In patients with long standing RA, the upper limit of muscle strength is approximately 40%. Muscle weakness is thought to arise as a result of atrophy, but more recently the severe muscle strength reduction observed in established arthritis is thought to occur as an adverse reaction to chronic treatment (Ekdahl and Broman, 1992).

Although RA is most commonly associated with poly-articular joint destruction, there are significant extra-articular symptoms which are often misunderstood. These are due in part, to the elusive systemic components of RA. Extra-articular manifestations are most common in, but not limited to, the heart and lungs. Pericardial effusions are found in more than 20% of RA patients (Anaya et al., 1995). Pericarditis is present in more than 50% of patients, but coronary insufficiency is difficult to diagnose since RA patients have low exercise tolerance due to impaired joint mobility (Bonfiglio and Atwater, 1969). Pleural lesions are also common in more that 50% of patients and contribute to poor performance in pulmonary function tests (Csuka and Hanson, 1996).

1.4 The rheumatoid synovium

The synovium is a layer of soft tissue that lines non-cartilaginous surfaces of diarthrodial joints and provides nutrients to avascular connective tissue. It consists of a thin intimal

layer, usually between one to three cell layers thick and a synovial sublining which merges with the joint capsule (Henderson and Pettipher, 1985). The intimal layer normally contains tissue macrophages, often referred to as type-A synoviocytes. In addition it contains fibroblast-like-synoviocytes (FLS) also known as type-B synoviocytes. The synovial sublining is vascularised and contains scattered fibroblasts and fat cells. The synovium is in direct contact with synovial fluid which permeates articular cartilage and serves as a joint lubricant. In RA, the synovium becomes hypertrophic and oedematous. Uncontrolled proliferation of cells at the synovium-cartilage junction leads to growth of a cell mass known as the pannus. The pannus invades cartilage and bone leading to joint erosion. The rheumatoid intimal layer becomes hyperplastic and the synovial sublining is infiltrated by inflammatory cells including T and B lymphocytes, macrophages and mast cells. These cells persist in the rheumatoid synovium long after infiltration. Recruitment of inflammatory cells, local retention and the promotion of cell proliferation contribute to the increased cellularity of the rheumatoid synovium (Henderson and Pettipher, 1985; Palmer, 1995). Normal histological architecture is drastically altered in RA and the synovium adopts features of lymphoid tissue. RA is distinct from other organ-specific autoimmune diseases since the target tissue is not destroyed, but instead the inflammatory process induces proliferation of synovial tissue (Muller-Ladner et al., 2007).

1.5 Molecular and cellular basis of joint damage

The inflamed synovium consists of diverse cell populations. These interact with each other in a contact-dependant manner and leads to the secretion of soluble factors which ultimately result in both persistence of inflammation and destruction of joint connective tissue (figure 1.2); (Karouzakis et al., 2006).

The major cellular contributors to chronic inflammation and joint destruction include B lymphocytes, T lymphocytes, macrophages and synovial fibroblasts (SF). Complex interactions between these cell types facilitate proliferation of synovial tissue and production of pro-inflammatory cytokines (Muller-Ladner, 1996). In RA, SF plays a major role in joint destruction by secretion of matrix degrading enzymes in addition to stimulation of pro-inflammatory cytokine production. They are mainly found in the synovial sublining and possess an aggressive and invasive behaviour which resembles metastatic cancer cells (Fassbender, 1983). Synovial fibroblasts are able to adhere to cartilage and initiate degradation of the extracellular matrix.

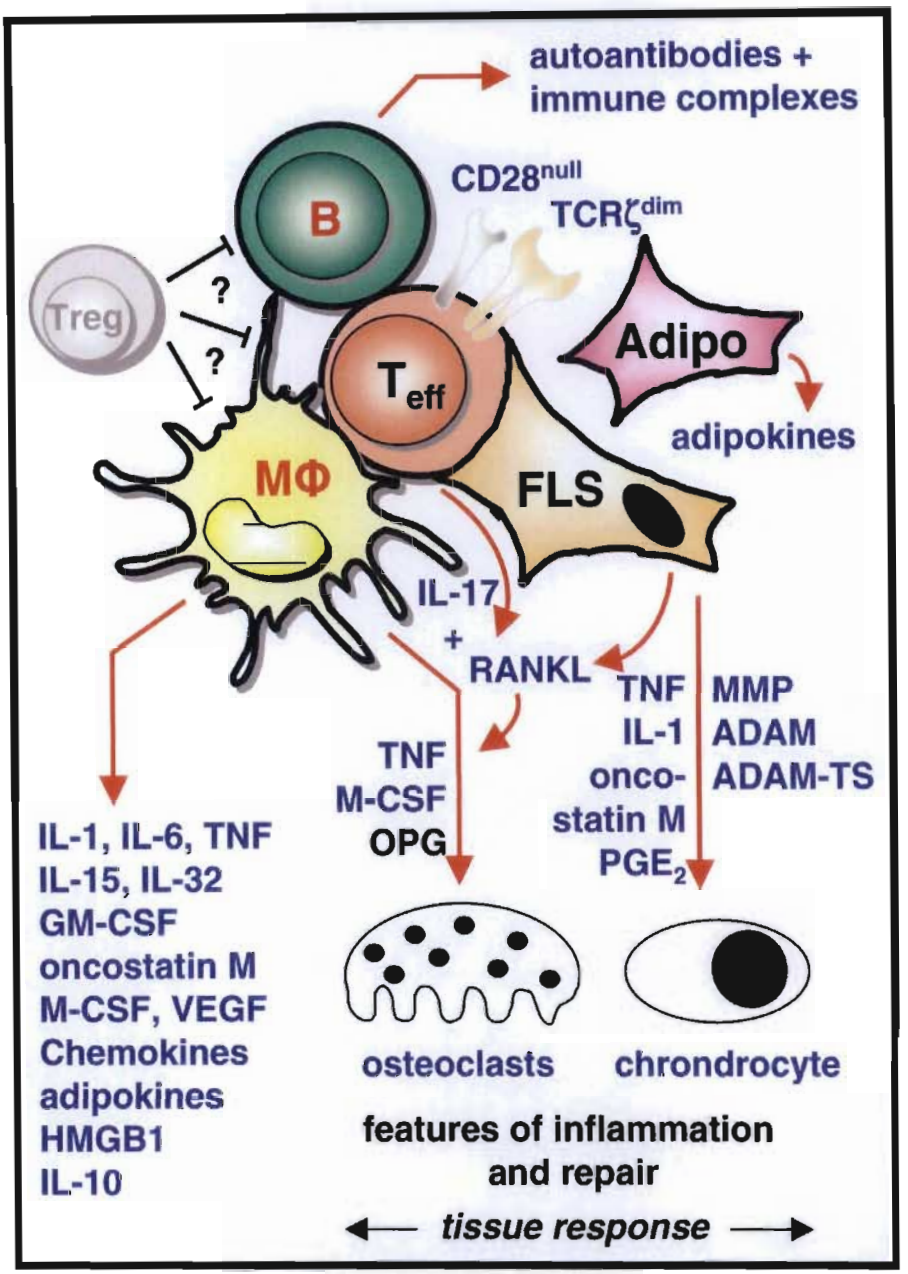


Figure 1.2 Contact-dependant interaction of cells in the rheumatoid synovium (Cope, 2008).

Destruction of cartilage by SF seems to be independent of immune involvement. When implanted into severe combined immuno-deficiency (SCID) mice models of RA, SF maintained their proliferative, invasive and destructive nature (Muller-Ladner et al., 1996). Proliferation of SF in RA may be due to increased expression of growth factors such as platelet derived growth factor (PDGF), basic fibroblast growth factor (BFGF) and transforming growth factor β (TGFβ) (Allen et al., 1990; Butler et al., 1989; Melnyk et al., 1990; Qu et al., 1994). In addition, proto-oncogenes, such as *ras* and *c myc* are abundantly expressed in these cells (Muller-Ladner et al., 1995).

Proliferation of these cells may also depend on factors secreted by other cells resident in the rheumatoid synovium. Inhibition of macrophage inhibitory factor (MIF) for instance, was shown to ameliorate SF proliferation (Lacey et al., 2003; Leech et al., 2003). Invasiveness and uncontrolled proliferation of SF may be related to impaired apoptosis. Although Fas and Fas ligand are abundantly expressed in the rheumatoid synovium, infiltrating immune cells and invasive SF appear to be resistant to death receptor induced apoptosis (Baier et al., 2003). Anti-apoptotic molecules such as Sentrin1 have been shown to modulate death receptor induced apoptotic pathways, and are specifically expressed in SF which invade cartilage and bone (Franz et al., 2000). Secreted factors such as MIF can also modulate apoptotic pathways and thus contribute to SF proliferation (Leech et al., 2003). Synovial hyperplasia however, is related not only to SF hyper-proliferation, but also to invasion and persistence of inflammatory cells.

The rheumatoid synovium contains diffuse populations of inflammatory cells which include B lymphocytes, T lymphocytes, macrophages and dendritic cells. Synovial biopsies show that T lymphocytes play an integral role in chronic immune activation in RA (Duke et al., 1982). The resulting synovitis and adoption of lymphoid architecture in the rheumatoid synovium is directed by elevated expression of chemokines and cytokines such as lymphotoxin-$\alpha 1\beta 2$ and B lymphocyte chemokines (Takemura et al., 2001a; Takemura et al., 2001b). The inflamed synovium typically contains clusters of lymphoid follicular aggregates with germinal centers and secondary follicles (Duke et al., 1982; Schroder et al., 1996; Weyand et al., 2001). This type of lymphoid architecture supports the processing and presentation of antigen to T lymphocytes within the synovium, and may explain the reactivity to joint specific antigens such as collagen type II.

Gene expression profiles of lymphoid tissue in synovial germinal centers show elevated levels of CXCL13, CXCL12, CC chemokine ligand (CCL)19, CCL21, CXC chemokine receptor (CXCR)4, CXCR5 and CC chemokine receptor (CCR)7 (Timmer et al., 2007). Analysis of gene ontology pathways suggested that these are involved in Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathways associated with T lymphocyte receptor stimulation as well as interleukin 2/interleukin 7 (IL-2/IL-7) co-stimulatory pathways (Timmer et al., 2007). These pathways are important for the cellular interactions which maintain the organizational structure of lymphoid-like tissue and the persistence of inflammation in the rheumatoid synovium.

1.6 Genetics of rheumatoid arthritis

The development and pathogenesis of RA has been attributed to a genetic predisposition based on twin and sibling studies. These studies have shown higher concordance rates of disease among monozygotic twins compared to dizygotic twins. In addition, siblings of RA patients have an increased susceptibility to RA than the general population (MacGregor et al., 2000; Seldin et al., 1999). No single RA susceptibility gene has yet been identified. However there is considerable association between the HLA-DRβ1 gene and RA susceptibility in many population groups (Deighton et al., 1989). The HLA-DRβ1 gene is highly polymorphic and thus has a profound impact on individual immune responses and modulation of immune signals (Fernando et al., 2007; Fernando et al., 2008). As a consequence of the variability of this gene, several subtypes have been identified. Specific subtypes of the HLA-DRβ1 gene have been associated with susceptibility in RA populations despite ethnic differences in the specific genes involved. In RA patients of European ancestry for instance, the HLA-DRβ1*0401 and HLA-DRβ1*0404 are the most common alleles (Newton et al., 2004), while in East Asian populations the HLA-DRβ1*0405 allele is most frequently associated with RA susceptibility (Newton et al., 2004). In South Africa, significant associations between RA and the HLA-DRβ4 gene have been reported in African Blacks, Whites and populations of mixed ancestry (Mody et al., 1989). Interestingly, RA is uncommon in Nigerian populations and may be related to the low frequency (approximately 1%) of HLA-DRβ4 observed in this population (Okoye et al., 1989; Ollier et al., 1989). Despite

differences in the specific versions of HLA genes associated with RA, they are all similar at the third hyper-variable region which codes for the QK/RAA amino acid motif commonly referred to as the shared epitope (Gregersen et al., 1987). Functional significance of the 'shared epitope' in RA is uncertain, however recent studies have shown that citrullination of proteins confer high-affinity peptide interactions with HLA molecules which contain the shared epitope (Hill et al., 2003; Huizinga et al., 2005).

Citrullination occurs when arginine residues in polypeptide chains are deiminated to citrulline by calcium dependant cytosolic peptidyl-arginine deiminianses (PAD). Citrullinated proteins are recognised as non-self and thus illicit a strong immune response (Gyorgy et al., 2006). Immune recognition of citrullinated proteins may also be attributed to unregulated apoptosis and inefficient clearance of apoptotic cell fragments. Antibodies to citrullinated proteins are highly specific (approximately 99%) for RA and are currently the most powerful diagnostic marker of the disease (Lundberg et al., 2005; Makrygiannakis et al., 2006; Yoshida et al., 2006). Apoptosis may result in increased PAD activity due to intra-cellular calcium fluxes and thus increase protein citrullination events. Uncontrolled apoptosis may also promote leakage of post-translationally modified proteins into the extracellular compartment where they may be intercepted by the immune system (Gyorgy et al., 2006; Yoshida et al., 2006).

Several non-HLA genes have been identified and associated with RA through genome wide association studies (Frazer et al., 2007). While these are inconsistent among different population groups, strong associations with RA susceptibility have been

described for the protein tyrosine phosphatase non-receptor type 22 (PTPN22) and PADI4 genes. Susceptibility studies in European RA populations have confirmed the role of PTPN22 polymorphisms in disease pathogenesis. These polymorphisms however, are infrequent in Asian populations. PADI4 polymorphism studies have been replicated in many Asian populations and confirm the association with RA susceptibility and severity. However, these are inconsistently observed in other populations (Gagnon et al., 2007; Goeb et al., 2008; Lee et al., 2009; Pazar et al., 2008; Stark et al., 2009). Much of our understanding of RA susceptibility genes come from genome wide association studies in large European or Asian patient cohorts, primarily due to the efforts of the International HapMap Project (IHMP) and Wellcome Trust Case Control Consortium (WTCCC). African blacks are under-represented in these data repositories and thus genetic associations arising from these projects may not necessarily be applicable to local RA patients. A further confounding factor is the inconsistency between specific genetic risk factors and different population groups. While there is compelling evidence for genetic risk factors in RA, which can also provide information regarding susceptibility and severity, they are currently not clearly defined.

1.7 Overview of cell death

Cell death is a critical process during development and homeostasis of the immune system. Dysregulation of cell death mechanisms have been associated with numerous pathologies (Lleo et al., 2008). Each cell in the body has innate self-destruct programmes, which may be initiated in the event of cellular integrity being compromised,

or when cells are no longer required. Deletion of certain cell groups within tissues may be necessary for structural development, as is evident during embryogenesis. Compromised cells, such as those harbouring genetic aberrations, need to be eliminated in order to halt the growth and propagation of faulty cells. Cell death is also induced upon infection, as part of immunological defence mechanisms. The major types of cell death include apoptosis, necrosis, autophagic cell death and pyroptosis (Duprez et al., 2009; Elmore, 2007).

1.8 Apoptosis

Apoptosis is a term used to describe a physiologically regulated mode of programmed cell death. It first appeared in scientific literature during the 1970s (Kerr, 2002; Kerr et al., 1972). Based on morphological, biochemical and molecular criteria, apoptosis is distinct from alternate modes of cell death. Much of our understanding of mammalian cell apoptosis is derived from studies which investigated developmental stages of the nematode, *Caenorhabditis elegans* (Horvitz, 1999). In this organism, 131 distinct cells undergo programmed cell death at particular points in the development process in order to generate 1090 somatic cells, which eventually form the adult organism (Gumienny et al., 1999). Accuracy of the cell death process is emphasised by the observation that the cells which die and the timing of cell death during development is identical between individual organisms (Maurer et al., 2007). Apoptosis is thus recognised as a genetically determined biological programme which results in co-ordinated elimination of cells. It is essential both in development and in the homeostatic control of mammalian cell turnover.

Apoptosis may be triggered by various physiological stimuli, as well as pathological stimuli such as infectious agents. It is critically important for shaping of the immune system and in amelioration of inflammation. Aberrations in the regulatory mechanisms of apoptosis have been implicated in the development of autoimmune diseases (Haanen and Vermes, 1995; Munoz et al., 2008).

1.9 Morphological features of apoptotic cells

Morphologically, apoptosis is characterized primarily by cytoplasmic shrinkage, chromatin condensation and membrane blebbing. Early during apoptosis, the cytoplasm becomes dense and organelles are tightly packed. As a result, cell size is significantly reduced and they appear round or oval under light microscopy (Hacker, 2000). In the nucleus, chromatin is condensed and usually aggregates peripherally under the nuclear membrane. This leads to pyknosis and condensation of nuclear material. The nucleus is then fragmented during a process known as karyorrhexis (Hacker, 2000). The plasma membrane is drastically altered during apoptosis, due to loss of membrane asymmetry (Bratton et al., 1997). As a consequence, the plasma membrane begins to 'bleb' and cells undergoing apoptosis disintegrate into smaller fragments known as apoptotic bodies. Despite structural disruptions, plasma membrane integrity of apoptotic bodies remains intact (Fadok and Chimini, 2001). Condensed cytoplasmic and nuclear constituents of the dying cell are contained within apoptotic bodies, and are thus not exposed to the extra-cellular environment. Apoptotic bodies are subsequently recognized and engulfed by

surrounding cells and phagocytes (Ashman et al., 1995). Tissue macrophages in particular, play an important role in disposing of apoptotic cells. Upon engulfment of apoptotic bodies, these cells release anti-inflammatory factors, thus clearing apoptotic cells without evoking an inflammatory response and with minimal physiological trauma (Fadok et al., 1998b; Hoffmann et al., 2001).

1.10 Molecular mediators of apoptosis

The main molecular mediators of apoptosis are members of the evolutionary conserved Bcl-2 protein family, and the cysteinyl aspartate-specific proteases (caspases). The Bcl-2 proteins are associated with control of mitochondrial membrane integrity and thus the flow of molecular traffic between the cytoplasm and mitochondrial inter-membrane space (Suen et al., 2008; Youle and Strasser, 2008). They were first identified in B-cell lymphoma, hence the *Bcl* designation (Tsujimoto et al., 1985). The Bcl-2 protein family is categorized into three functional groups based on sequence homology (figure 1.3). Group I Bcl-2 members contain four conserved Bcl-2 homology domains (BH1-BH4) and a hydrophobic tail at the C-terminal. This facilitates localization to the outer mitochondrial surface. Members of group I which include Bcl-2 and Bcl-x_L, all possess anti-apoptotic activity. Group II members lack the BH4 domain, but are otherwise structurally similar to group I members. Despite structural similarity, these proteins are functionally divergent from group I members, in that they are largely pro-apoptotic. Members of group II include Bax and Bak proteins. The BH3 domain is the only

common structural feature in members belonging to group III. This group is comprised of proteins which share very little sequence homology and are equally functionally diverse (Adams and Cory, 1998; Antonsson and Martinou, 2000; Antonsson et al., 2000; van Delft et al., 2006).

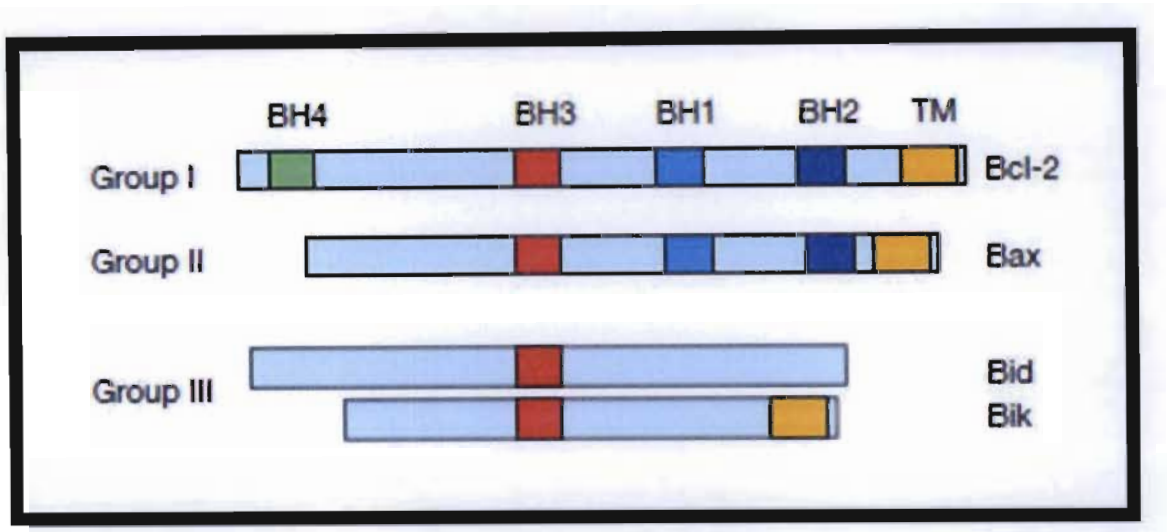


Figure 1.3 Classification of Bcl-2 proteins according to sequence homology (Hengartner, 2000).

Caspases belong to a large family of evolutionary conserved proteases, many of which are involved in apoptotic initiation signals or mediate the execution phase of apoptosis. They are responsible for the controlled proteolytic breakdown of cellular structures (Fuentes-Prior and Salvesen, 2004). These proteases characteristically contain cysteine residues within their active sites and cleave protein substrates at aspartic acid residues (Earnshaw et al., 1999). Approximately twelve caspases are known to be involved in

apoptosis and either posses initiator (caspase 2,-8,-9,-10) or executioner (caspase 3,-6,-7) functions (Thornberry and Lazebnik, 1998). They are synthesized as enzymatically inert zymogens known as pro-caspases. They contain three distinct domains, namely an N-terminal pro-domain, a large p20 subunit and a small p10 C-terminal subunit (Earnshaw et al., 1999). Between each domain are proteolytic cleavage sites which contain aspartic acid residues. Certain caspases (such as caspase 3), may be activated by autocatalytic activation or by upstream caspases which cleave at the inter-domain aspartic acid residues. This initiates the characteristic caspase activation cascade during apoptosis (Nicholson and Thornberry, 1997; Thornberry et al., 1997). In addition, caspase activation may occur via proximity induced activation, when pro-caspases are forced to aggregate at particular cellular locales. Caspase 8, for instance, is activated upon aggregation of several pro-caspase 8 molecules at cytoplasmic domains of death receptors, following ligation of these receptors (Salvesen and Dixit, 1999). A more complex activation mechanism is observed for caspase 9. Autocatalytic activity or upstream proteolytic cleavage is insufficient for activation of this caspase. Instead, the proteolytic activity of this caspase is regulated by formation of a complex with apoptotic-protease-activating-factor (Apaf-1). Upon activation, mature caspases adopt hetero-tetrameric conformations which are comprised of two p10/p20 hetero-dimers. Each mature enzyme thus contains two active sites (Rodriguez and Lazebnik, 1999). Cells may initiate apoptosis and thus cause caspase activation via two distinct biological pathways, namely the intrinsic or extrinsic apoptotic pathways (figure 1.4).

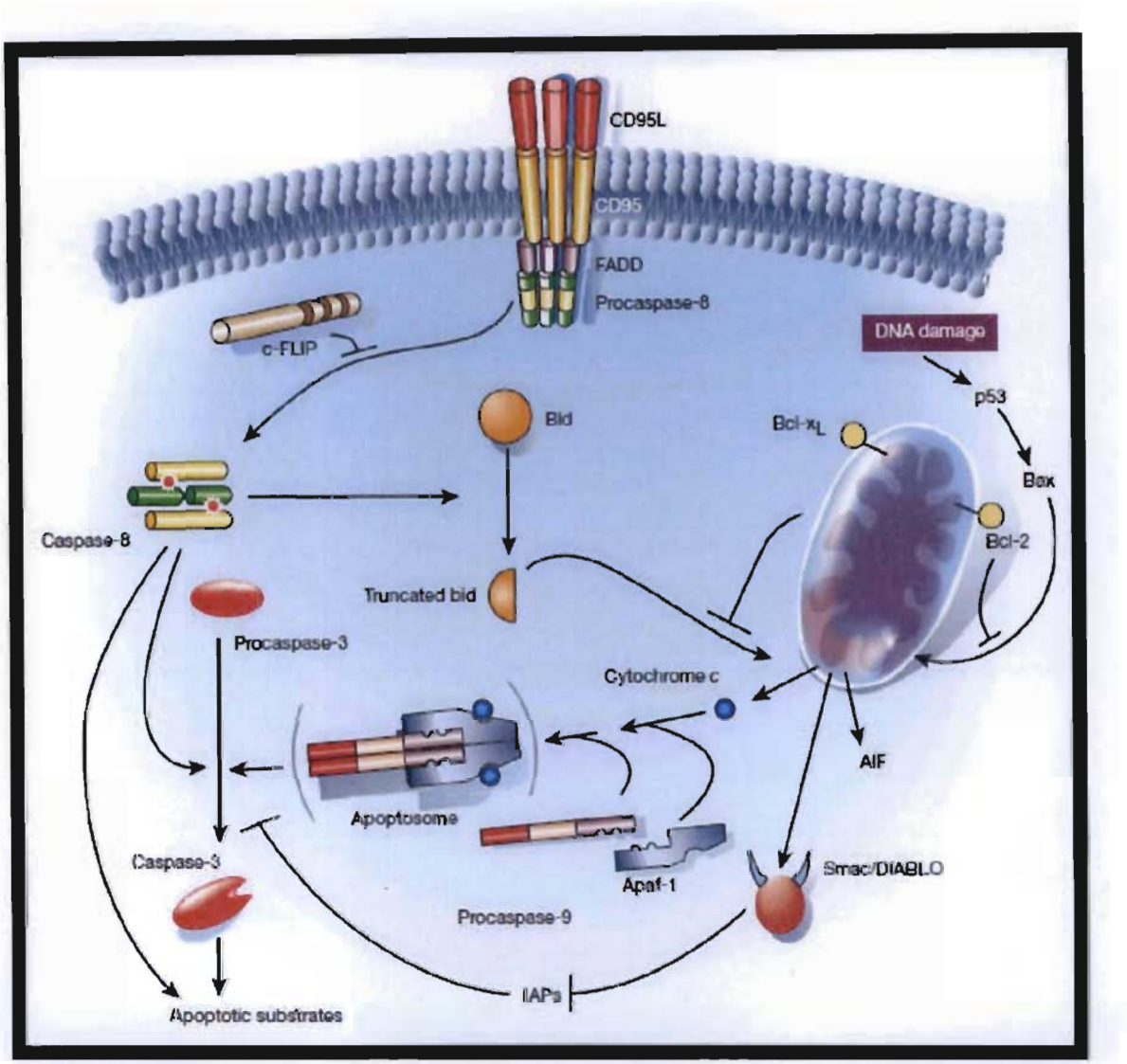


Figure 1.4 Apoptotic signalling pathways (Hengartner, 2000).

1.11 Intrinsic mitochondrial apoptotic pathway

The intrinsic pathway is initiated primarily by stimuli which cause irreparable damage to cellular structures, or those which cause DNA damage. The central effectors of intrinsic

apoptotic signals are mitochondria. These signals converge on mitochondria and induce opening of mitochondrial permeability transition pores (MPTP) (Brenner and Mak, 2009; van Gurp et al., 2003). As a consequence, mitochondrial transmembrane potential is disrupted and pro-apoptotic proteins, which are normally sequestered in the mitochondrial inter-membrane space, are released into the cytoplasm (Saelens et al., 2004). In normal cells, mitochondrial membrane polarity is strictly controlled. It is maintained in a polarized state to facilitate normal metabolic functions, but also to regulate molecular cross-talk between the cytoplasm and mitochondrial inter-membrane space. During homeostatic conditions, Bcl-2 regulatory proteins (primarily group I members) maintain integrity of the mitochondrial membrane by inhibiting Bax and Bak. During cellular stress, group III Bcl-2 proteins containing only the BH3 domain antagonize Bcl-2 and thus inhibition of Bax and Bak is relieved. This promotes their oligomerization and facilitates formation of MPTP and the loss of mitochondrial transmembrane potential (Kang and Reynolds, 2009). Two groups of proteins which are normally sequestered within mitochondria are released into the cytoplasm through MPTP. The first group is directly involved in activation of the caspase cascade and consists of cytochrome *c* (cyt c) and second-mitochondrial activator of caspases/direct-Inhibitor-of-apoptosis-binding-protein-with-low-PI (Smac/DIABLO) (Cai et al., 1998; Garrido and Kroemer, 2004).

In the cytoplasm, association of cyt c with Apaf-1 forms a protein scaffold, known as the apoptosome, which recruits pro-caspase 9. Binding pro-caspase 9 and Apaf-1 in the apoptosome stabilizes and activates caspase 9. Assembly of the apoptosome complex is complete upon activation of caspase 9 (figure 1.5), which thereafter activates downstream

executioner caspases, particularly caspase 3,-6 and -7 (Riedl and Salvesen, 2007). Inhibitor-of-apoptosis-proteins (IAP), such as XIAP, are normally found in complex with executioner caspases causing their inhibition (LaCasse et al., 2008). Once in the cytoplasm, Smac/DIABLO antagonize IAP and thus relieve their inhibitory interaction with executioner caspases (Shaw et al., 2008).

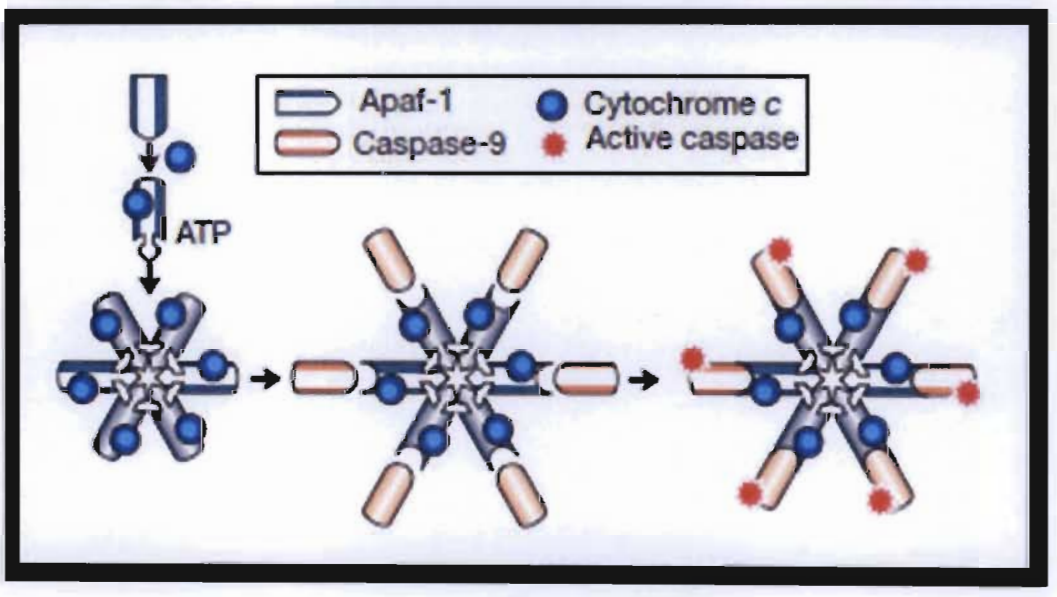


Figure 1.5 Activation and assembly of the apoptosome (Hengartner, 2000).

1.12 Extrinsic receptor mediated apoptotic pathway

The extrinsic apoptotic pathway is initiated upon activation of death receptors, which are transmembrane proteins located on the cell surface. Death receptors belong primarily to

the tumour necrosis factor (TNF) super-family and common death ligands include TNF-α, CD95/Fas and TNF-related apoptosis-inducing ligand (TRAIL) (Peter and Krammer, 2003). Structurally, death receptors contain extracellular cysteine-rich domains and cytoplasmic death domains composed of approximately 80 amino acids. Binding of the death receptor by its appropriate ligand causes receptor clustering and adapter proteins are recruited to the cytoplasmic death domain. Ligation of CD95/Fas for instance induces recruitment of Fas associated death domain (FADD) which then associates with pro-caspase 8, following dimerization of the death domains. The complex formed by association of these molecules is known as the death-inducing signalling complex (DISC), which facilitates autocatalytic activation of procaspase-8 (Moxley et al., 2009). Engagement of TNF receptor 1 (TNFR1) results in formation of two sequential protein complexes, i.e. complex I and II (Wilson et al., 2009). Complex I is formed at the plasma membrane by association of TNFR1, TNFR-associated death domain (TRADD), TNF receptor associated factor 2 (TRAF2), receptor interacting protein 1 (RIP1) and cellular IAP1 and 2 (cIAP1; cIAP2). Complex I is associated with TNF-induced activation of mitogen activated protein kinases (MAPK) and nuclear factor κB (NFκB). Endocytosis of TNFR1 is followed by the formation of complex II, which is functionally similar to the DISC. This complex activates caspase 8 which leads to activation of downstream executioner caspases. Extrinsic apoptotic signals are amplified by caspase 8-mediated cleavage Bid which antagonizes regulatory Bcl-2 proteins and initiates the intrinsic mitochondrial apoptotic pathway (Wang et al., 2008; Wilson et al., 2009).

1.13 Stress response proteins and apoptosis

One of the key regulators of apoptosis is the tumour suppressor protein, p53 (Green and Kroemer, 2009). It is normally expressed at low levels and has a short half-life, but rapidly accumulates under conditions of cellular stress. p53 acts as a transcription factor that regulates cell cycle progression, mediates DNA repair, and if necessary, initiates the apoptotic programme (Adimoolam and Ford, 2003; Levine and Oren, 2009; Vousden and Prives, 2009). Apoptosis directed by p53 may occur via:

a) p53-mediated transcription of pro-apoptotic genes (Oren, 2003),
b) non-transcription events such as p53-mediated activation of executioner caspases (Vaseva and Moll, 2009).

Mutations in the p53 gene have been associated with many tumours, and recently, the alterations in the p53 status of RA patients have come into focus. Both over-expression of the protein and mutations in the p53 gene were described for RA. Evidence suggests that the molecular profile in RA fibroblasts is associated with the ethnicity of patients. Whilst p53 mutations were detected in clones from three RA synovial fibroblast cell lines from the USA, the same mutations were absent in fibroblasts from German RA patients (Kullmann et al., 1999; Muller-Ladner and Nishioka, 2000).

The p53 mutational and expression status in inflammatory autoimmune disease has not been comprehensively investigated, and whilst evidence for p53 aberrations in cells harvested from the RA synovium accumulates, research investigating the same parameters in peripheral immune components is lacking.

Apart from p53, heat shock proteins (HSP) and heat shock transcription factors (HSF), have been implicated in the pathogenesis of RA. The HSPs are widely distributed and are among the most highly conserved molecules in nature. Heat shock proteins are encoded by genes whose expression is rapidly increased during conditions of cellular stress such as, metabolic disruptions, oxidative stress and inflammation. They have a broad array of functions and increase cell survival by acting as molecular chaperones. They are able to protect cells from executing and completing the apoptotic program. High levels of HSP70 have been detected in the serum and synovial tissue of RA patients. Over-expression of HSP70 may contribute to the resistance to apoptosis observed in cells of the RA synovium (Beere, 2004; Kamradt et al., 2005; Liuzzo et al., 2005).

Both HSPs and p53 are important biological molecules in RA and the induction of apoptosis may rely on the net effect of these two proteins (figure 1.6). Heat shock proteins intimately interact with the p53 tumour suppressor by:

a) acting as molecular chaperones that potentially mediate p53 conformation.

b) playing a role in the stabilization and localization of mutant p53.

c) participating in the cytoplasmic sequestration of wild type (Walerych et al., 2009).

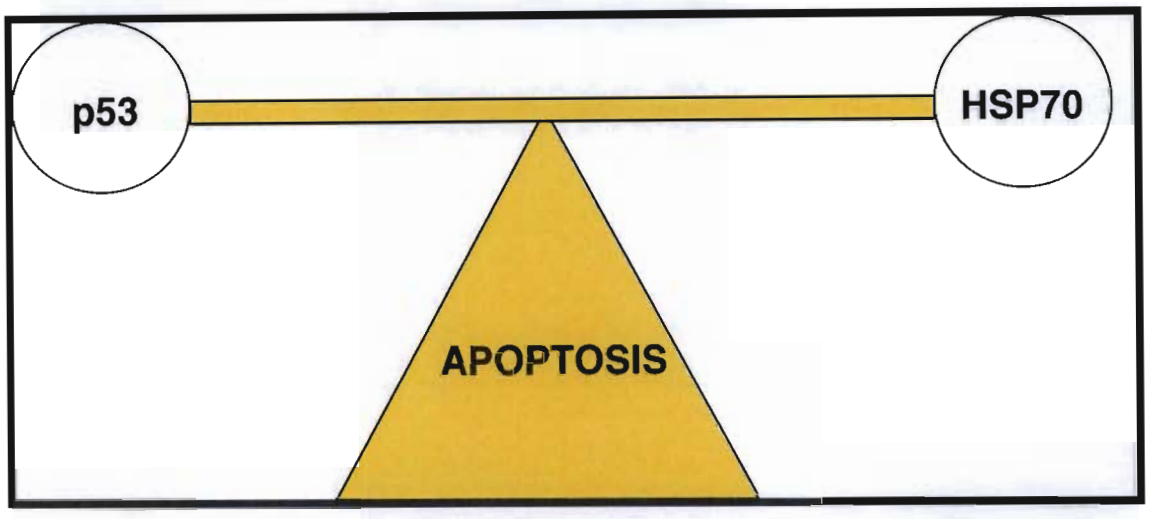


Figure 1.6 Apoptosis is regulated in part by the net effect of p53 and HSP70.

Both p53 and HSP70 influence apoptosis and abnormalities in these protein systems (whether at the gene or protein level) may result in severe defects in apoptosis and may be associated with the pathogenesis of RA.

CHAPTER 2

CYTOTOXICITY, MITOCHONDRIAL DEPOLARISATION AND OXIDATIVE STRESS IN RHEUMATOID ARTHRITIS

2.1 Introduction

There is accumulating evidence to show that mitochondrial damage may play a significant role in the pathogenesis of RA. Synoviocyte mitochondrial DNA (mtDNA) was shown to contain twice the amount of somatic mutations compared to osteoarthritic controls (Da Sylva et al., 2005). Somatic mutations in protein coding segments of mtDNA contribute to pathogenesis by elevating expression of major histocompatibility complex (MHC) class I molecules (Gu et al., 2003). Both MHC class I and II are able to present peptides derived from aberrant mtDNA, thus promoting immunological responses which may result in loss of tolerance (Kita et al., 2002).

Chronic inflammation is associated with elevated levels of both ROS and RNS. Various investigations have recently cited oxidative stress as a major contributor to RA pathogenesis with high levels of oxidative enzyme activity observed in synovial fluid (Dabbagh et al., 1993; De Leo et al., 2002; Seven et al., 2008). Free radicals may also act as second messengers which stimulate NFκB dependant expression of pro-inflammatory cytokines (Miesel et al., 1996).

Dietary intake of antioxidants was associated with lower incidence of RA and higher levels of circulating antioxidants were shown to ameliorate inflammation (Bae et al., 2003; Cerhan et al., 2003; Hagfors et al., 2003). There is also evidence for oxidative damage to synovial connective tissue and proteins (Dai et al., 2000; Dalle-Donne et al., 2003; Grootveld et al., 1991). Miesel et al. showed that production of mitochondrial derived free radicals correlated with plasma TNF-α levels in RA (Miesel et al., 1996). In addition, the clinically beneficial changes produced by infliximab (anti-TNF-α) therapy in RA were related to changes in plasma redox status in conjunction with its anti-inflammatory effects (Lemarechal et al., 2006).

Mitochondrial machinery is particularly susceptible to oxidative damage. Proximity of mtDNA and proteins to the free radical-producing oxidative phosphorylation process, combined with limited mtDNA/protein repair mechanisms, confers substantial oxidative burden. Cells of synovial origin are most vulnerable to oxidative damage due to chronic synovial inflammation in RA. Mitochondrial integrity of circulating PL, which perpetuates chronic inflammation however, has not been fully investigated to date.

This study therefore aimed to assess cytotoxicity and mitochondrial membrane potential ($\Delta\psi_m$) as a marker of mitochondrial damage in circulating PL from South African black RA patients. In addition, the extent of lipid peroxidation was measured as an indicator of oxidative stress.

2.2 Materials and Methods

2.2.1 Ethical approval

This study was approved by the Faculty of Health Sciences, Research, Ethics and Higher Degrees Committee, University of KwaZulu-Natal – protocol number: H109/04. Informed consent was obtained for each patient enrolled in the study. Informed consent documents were available in both English and isiZulu (appendix 1) depending on patient preference. Details of the study were explained to each patient by the clinic nurse prior to recruitment.

2.2.2 Patient recruitment and assessment

Fifty South African black RA patients (female:male ratio 6:1) attending the rheumatology clinic at Inkosi Albert Luthuli Central Hospital (Durban, South Africa) were recruited into the study. All patients fulfilled the American College of Rheumatology (ACR) criteria for RA (appendix 2) (Arnett et al., 1988).

Patients were at various stages of treatment when sampled. They were distributed among three treatment categories, namely, patients on methotrexate (MTX) alone (n = 13), MTX together with steroidal drugs (n = 29) and those on other disease-modifying-anti-rheumatic-drugs (DMARD, n = 8).

The patients reported no recent/chronic infection or history of other chronic inflammatory diseases. All patients were HIV negative. The HIV status of every patient attending the rheumatology clinic is routinely determined since HIV status impacts on the type of therapy and clinical management. The rheumatology clinic is equipped with the necessary facilities for pre- and post-test counseling.

Following recruitment, patients were assessed by clinic rheumatologists. Clinical and laboratory parameters (mean number of swollen joints and tender joints; erythrocyte sedimentation rate, ESR; C reactive protein, CRP; absolute lymphocyte counts) were recorded for all the patients. In addition, each patient completed health assessment questionnaires (HAQ), from which HAQ scores were generated. The HAQ is a patient reported outcome which provides knowledge about a patient's general health, functional status, and quality of life.

In order to determine disease severity, disease activity scores (DAS28) were determined for each patient. This score is a mathematical function of the number of swollen and tender joints out of 28 clinically assessed joints. In addition to laboratory parameters, i.e. ESR and CRP, the DAS28 also incorporates the patients general health measured on a visual analogue scale.

For the experimental parameters measured in this study, heparinized whole blood (10ml) and serum (5ml) was collected from each patient through the antecubital vein by a qualified phlebotomist. Healthy race matched control samples were sourced from the

South African National Blood Services following routine screening.

2.2.3 Peripheral lymphocyte preparation

Buffy coats containing PL were extracted from heparinized whole blood by differential centrifugation. Briefly, whole blood collected from each subject, was layered onto equivolume Histopaque® 1077 (Sigma) in 15ml polypropylene tubes. Histopaque® 1077 is a polysucrose solution containing sodium diatrizoate which is adjusted to a density of approximately 1.077 g/ml. The solution at this density facilitates the recovery of mononuclear cells from whole blood. Polysucrose facilitates aggregation of erythrocytes and granulocytes which rapidly sediment during centrifugation. Mononuclear cells such as PL are contained within the buffy coat at the plasma-Histopaque® interface (figure 2.1). Layered blood was centrifuged at 400 x g for 30 minutes. Buffy coats were aspirated into new polypropylene tubes and washed twice in phosphate buffered saline (PBS) (400 x g, 10 minutes). PL density was adjusted to 1 x 10^6 cells/ml with the trypan blue exclusion test.

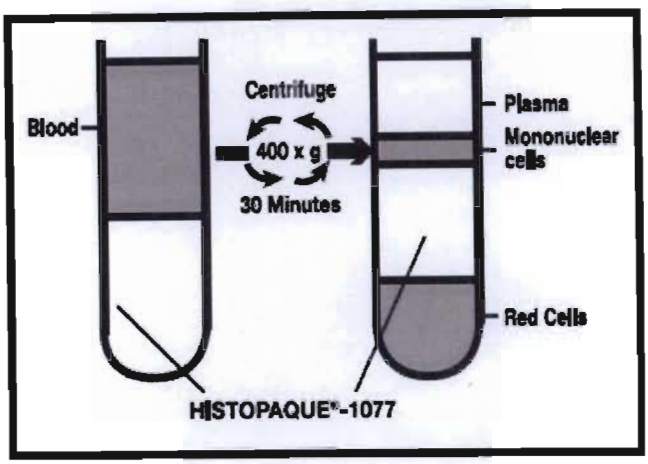


Figure 2.1 Schematic illustration of peripheral lymphocyte preparation by density gradient centrifugation (Sigma-Aldrich, 2003).

2.2.4 Lactate dehydrogenase (LDH) cytotoxicity assay

The LDH cytotoxicity detection kit (Roche) was used to measure cell death/damage in all study participants. LDH is a stable cytosolic enzyme which is rapidly released from damaged or dying cells. A colorimetric assay was used for the quantification of cell death or cell lysis based on the measurement of LDH activity in the serum of all study participants. The assay is a two step enzymatic reaction where NAD^+ is reduced to $NADH/H^+$ by the conversion of lactate to pyruvate. Thereafter, a diaphorase catalyst transfers H/H^+ from $NADH/H^+$ to a tetrazolium salt to yield a formazan product (figure 2.2). To measure LDH activity, serum (200µl) was transferred into microtitre plates in triplicate. Thereafter, substrate mixture (100µl) containing catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate) from the kit was added to serum and allowed to

react at ambient temperature for 25 minutes. Optical density of the resulting formazan product was measured at 500nm with an ELISA plate reader (Bio-Tek uQuant).

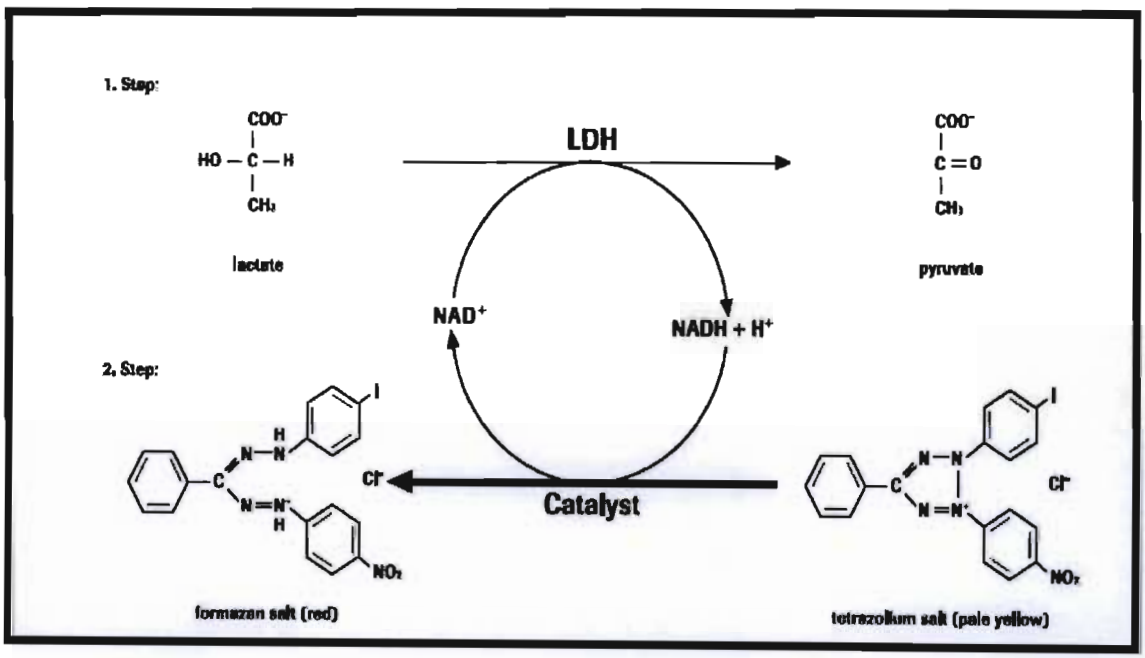


Figure 2.2 Chemical reactions in the lactate dehydrigenase cytoxicity assay (Roche, 2005).

2.2.5 Mitochondrial membrane potential

The JC-1 (*5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide*) Mitoscreen assay (BD Biosciences) was used to assess $\Delta\psi_m$ in PL. JC-1 is a membrane-permeable lipophilic cationic fluorochrome. The fluorescence emission spectrum of JC-1 is dependent on its concentration in cellular compartments, which is determined in part by $\Delta\psi_m$. At low concentrations, JC-1 molecules exist as monomers which exhibit green fluorescence. At higher concentrations however, JC-1 monomers form aggregates which emit red fluorescence. In healthy cells, mitochondrial uptake of JC-1 is driven by polarized $\Delta\psi_m$. This results in high concentrations of JC-1 within the mitochondria and facilitates the formation of JC-1 aggregates. In cells with compromised mitochondria, JC-1 molecules are sparsely distributed in the cytoplasm and remain as monomers. Healthy cells can thus be differentiated from unhealthy cells by examining fluorescence shifts in the red and green spectra.

To assess $\Delta\psi_m$, approximately 1 x 10^5 PL were transferred into 5ml polystyrene cytometry tubes. The JC-1 dye (150µl) was added to PL and allowed to incubate at 37°C for 10 minutes. Thereafter, PL were washed twice in JC-1 wash buffer and re-suspended in 200µl flow cytometry sheath fluid. Labelled PL were enumerated by flow cytometry using a 4-colour FACS Calibur (BD Biosciences) flow cytometer. Data was recorded for green and red fluorescent channels from 50 000 events for each sample. Analysis was

performed with FlowJo 7.1 (Tree Star Inc.) software.

2.2.6 Lipid peroxidation assay

Lipid peroxidation is a major indicator of oxidative stress. In order to measure lipid peroxidation and thus gauge the extent of oxidative stress in PL, the thiobarbituric acid reactive substance (TBARS) assay was performed. Oxidative damage to lipids produces lipid hyper-peroxides and malondialdehyde (MDA). Thiobarbituric acid (TBA) can react with MDA to form adducts which absorb light maximally at 532nm (figure 2.3). MDA production, and thus the amount of MDA/TBA adduct formed is directly related to the extent of lipid peroxidation, which can be measured spectrophotometrically.

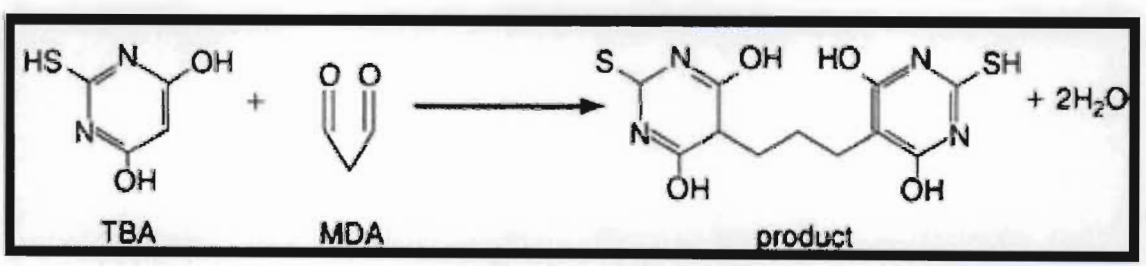


Figure 2.3 Reaction of thiobarbituric acid and malondialdehyde.

For the TBARS assay, approximately 1 x 10^6 PL were homogenized in 1% H_3PO_4. 400µl of the PL homogenate was transferred into clean glass tubes. For a positive method control, 400µl of 1% malondialdehyde bis(dimethyl acetal) was used in place of PL+

homogenates. To this, 400µl TBA (1%, w/v)/0.1mM butylated hydroxytoluene (BHT) mixture was added. The solution was adjusted to pH 1.5 and heated to 100°C for 15 minutes. Butanol (1.5ml) was added to the mixture after cooling and thereafter centrifuged (10 000 x g) for 6 minutes to separate organic phases. Aliquots (300µl) of the butanol phase were transferred into microtitre plates and optical density was measured at 532nm.

2.2.7 Statistical analysis

All statistical analyses were performed with the GraphPad Prism version 5 software package (GraphPad Software Inc.).

2.3 Results

2.3.1 Clinical evaluation revealed elevated disease activity

Patients recruited into this study fulfilled the ACR criteria for RA and had active disease with elevated numbers of swollen joints, tender joints and high DAS28 scores (table 1). More than half of the patients (n=27) in this study cohort obtained DAS28 scores higher than the mean (6.1 ± 1.3). DAS28 scores for all patients exceeded the index (3.7) stipulated for high levels of disease activity (Prevoo et al., 1995). Routine laboratory markers, i.e. ESR and CRP, were consistent with expected high values for chronic inflammation.

Table 2.1 Summary of clinical and laboratory parameters in RA patients.

Female : male ratio	6:1
Mean age & range	50.7 years (18-75 years)
Mean duration of disease	13.3 ± 9.5 years
Mean number of swollen joints	12 ± 6.7
Mean number of tender joints	13 ± 8.7
ESR	42.3 ± 28.5 mm/hr
CRP	19.59 ± 20.5 mg/ml
HAQ score	1.9 ± 0.7
DAS28 score	6.1 ± 1.3
Mean white blood cell count	$6.9 \pm 2.7 \times 10^9$ / l
Mean lymphocyte count	$1.9 \pm 0.8 \times 10^9$ / l

2.3.2 Cytotoxicity studies

LDH activity in the extra-cellular compartment is a reliable indicator of cytolysis and cytotoxicity. LDH activity was significantly elevated in sera of RA patients as compared to controls (figure 2.4). These results were not unexpected as chronic inflammation may lead to the release of LDH through cytolytic events either at inflammatory sites or by breach of plasma membrane integrity of circulating PL.

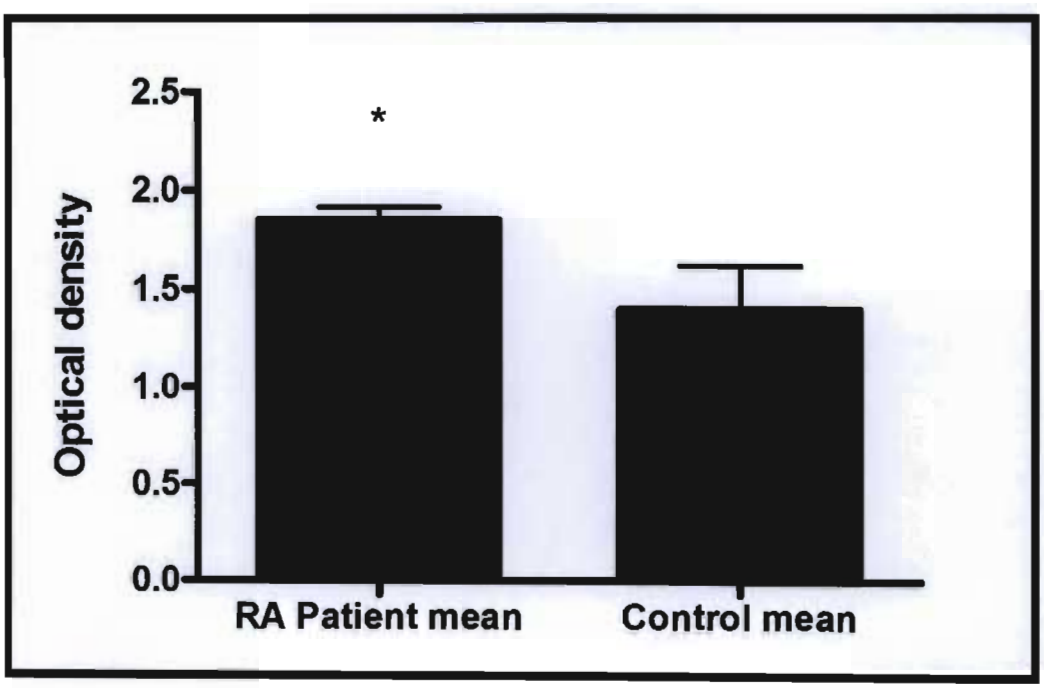


Figure 2.4 Elevated lactate dehydrogenase activity in rheumatoid arthritis patient serum. Mean optical density (+ SD) of formazan product formed in the LDH reaction is shown (n = 50). * Statistical significance was determined by the unpaired t-test with Welch correction, p=0.0080.

2.3.3 Mitochondrial membrane potential

To determine whether PL were intact and metabolically viable, changes in $\Delta\psi_m$ were investigated. There were significantly higher numbers of circulating PL with depolarised mitochondria in RA patients than in controls (47.5% vs. 29.85%; figure 2.5).

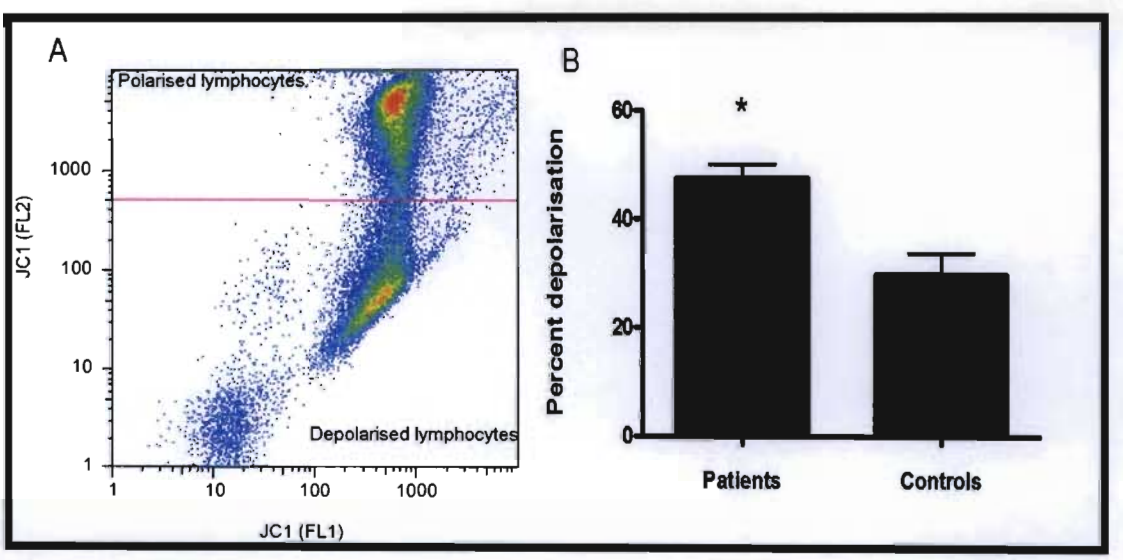


Figure 2.5 Peripheral lymphocyte mitochondrial depolarisation. A: Flow cytometry scatter plot showing distribution of lymphocytes with polarized and depolarised mitochondria. B: Proportion of circulating peripheral

lymphocytes with depolarised mitochondria was higher in the patient cohort. Data represents mean + standard error of the mean (SEM) for 50 patients and race-matched controls. *Differences in means were extremely significant (p = 0.0003; unpaired t-test with Welch correction).

2.3.4 Mitochondrial integrity and clinical parameters

In order to investigate the clinical relevance of mitochondrial depolarisation, $\Delta\psi_m$ was correlated with DAS28 scores. The proportion of PL with depolarised mitochondria significantly correlated with the DAS28 disease activity index (r = 0.3286; 95% confidence interval, 0.06667 to 0.5482; two tailed p value = 0.0153; Pearson r linear correlation; figure 2.6A).

When laboratory components of the DAS28 score were considered separately, the data showed that loss of PL $\Delta\psi_m$ correlated significantly with CRP but not ESR, (CRP: r = 0.2740; 95% confidence interval, 0.09314 to 0.5028; two tailed p value = 0.0492; figure 2.6B; ESR: r = 0.1461; 95% confidence interval, -0.1240 to 0.3961; two tailed p value = 0.2870; Pearson r linear correlation; figure 2.6C).

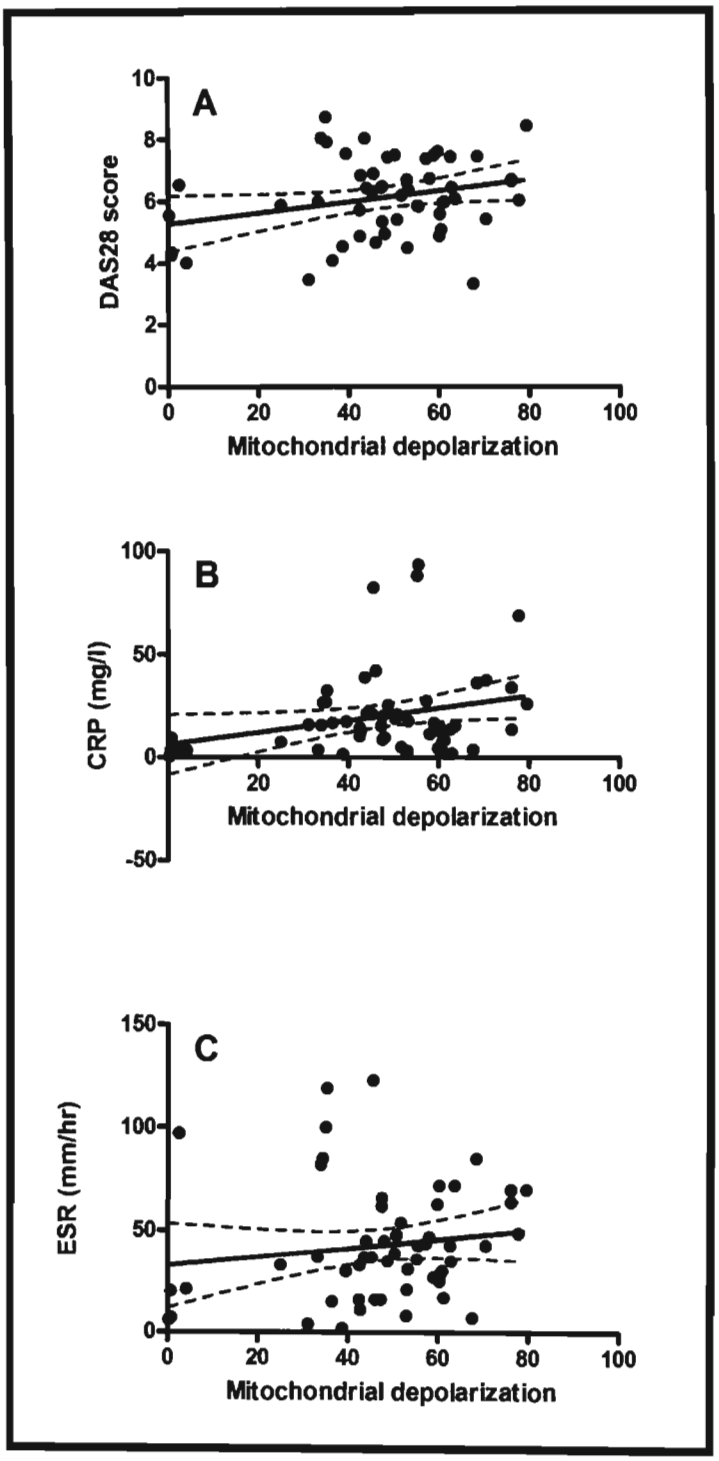


Figure 2.6 Clinical correlations with mitochondrial depolarisation.

The HAQ scores, as well as disease duration (13.3±9 years), did not significantly relate to depolarised mitochondria (HAQ: r = 0.08656; 95% confidence interval, -0.1856 to 0.3464; two tailed p value = 0.5337; Disease duration: r = 0.2269; 95% confidence interval, -0.04906 to 0.4707; two tailed p value = 0.1057; Pearson r linear correlation). Interestingly, a strong statistically significant negative correlation between mitochondrial depolarisation and absolute PL counts was observed (figure 2.7).

To investigate whether different treatment regimens affected PL $\Delta\psi_{m,}$ patients were grouped according to treatment regimens and differences in PL $\Delta\psi_m$ were statistically tested. No significant difference in mitochondrial depolarisation was observed when compared across different treatments. A slightly lower level of mitochondrial depolarisation was however noted in patients who were MTX naïve (figure 2.8).

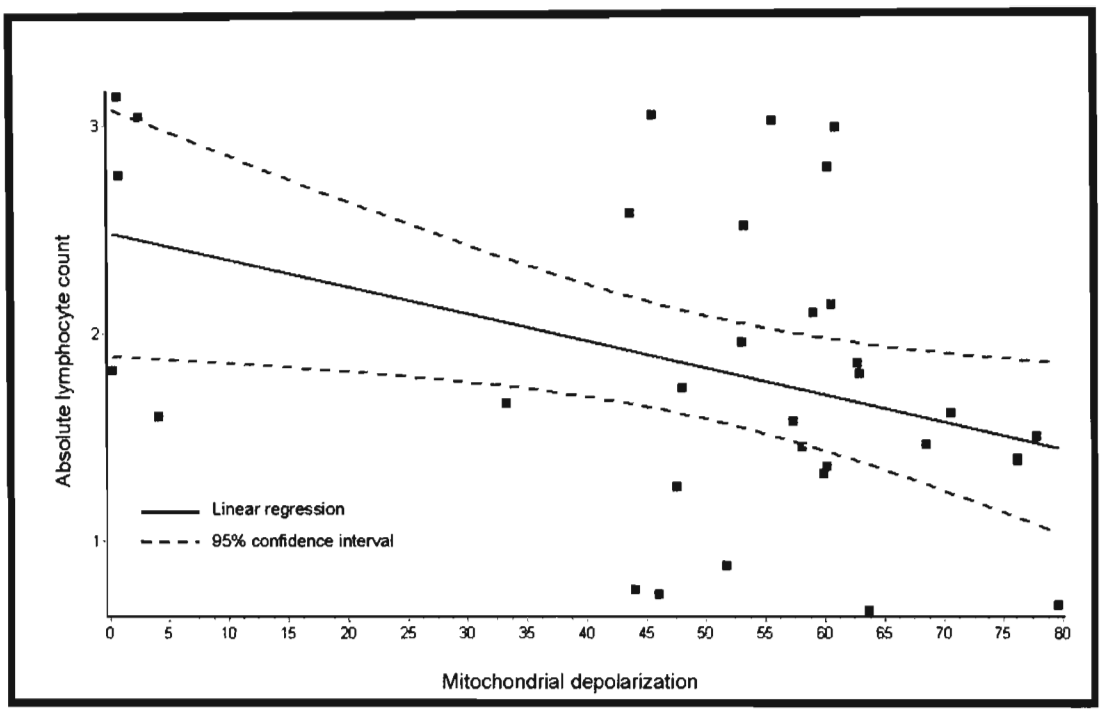


Figure 2.7 Absolute lymphocyte count negatively correlated with mitochondrial depolarisation in patients (n = 33). Correlation co-efficient r = -0.4041; two tailed P value = 0.0197; Pearson r linear correlation.

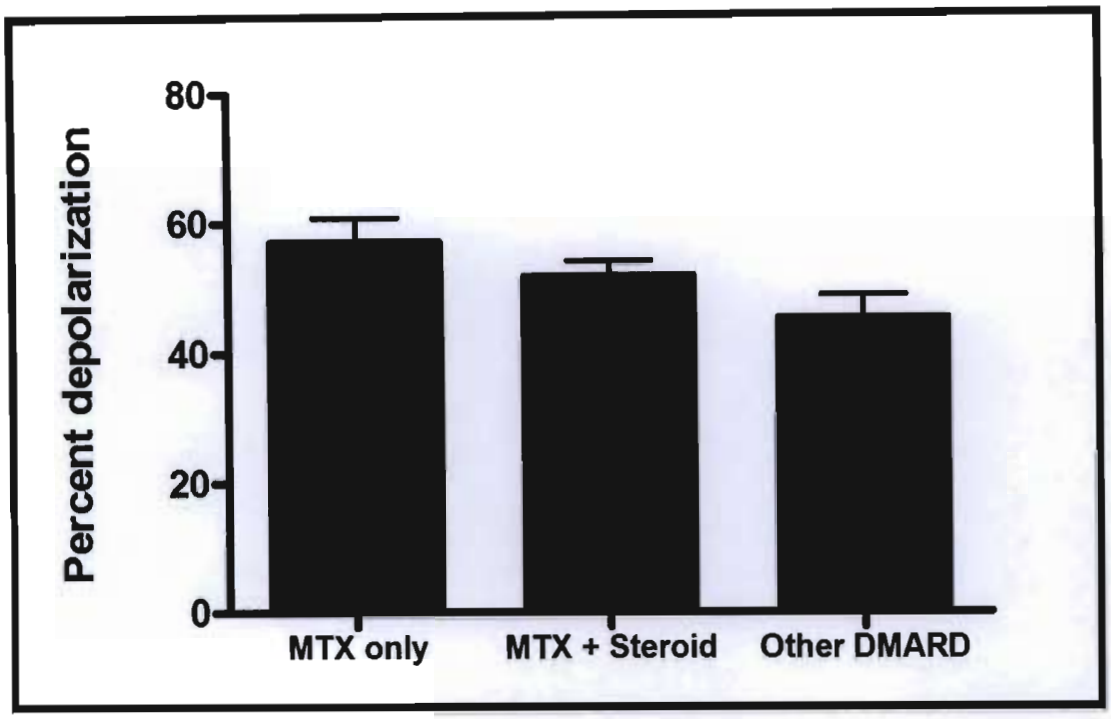


Figure 2.8 Proportion of circulating peripheral lymphocytes with depolarised mitochondria in patients grouped according to treatment (One-way ANOVA $p = 0.1179$).

In order to determine whether mitochondrial damage and cytotoxicity were related to oxidative stress, the level of lipid oxidation products was measured in PL. The TBA assay showed significantly higher amounts of lipid peroxidation in RA patients (Figure 2.9).

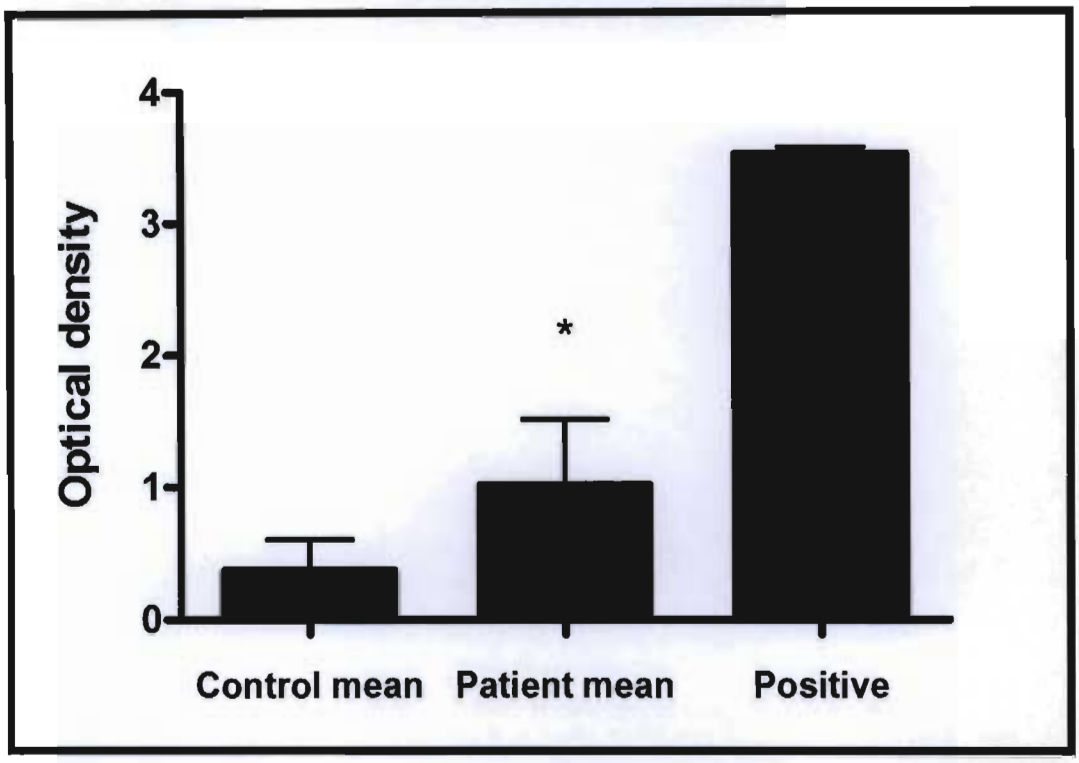


Figure 2.9 Oxidative damage in rheumatoid arthritis patients. Data shows mean optical density (+ SD) of lipid oxidation products formed in the TBARS assay, (n=50).

*Difference between means was statistically significant, p=0.0030 (unpaired t-test with Welch correction).

2.4 Discussion

Cytolysis as a result of critical cellular damage causes release of intra-cellular macromolecules into plasma. These molecules, such as DNA and protein are not normally found in the extra-cellular compartment and may not necessarily escape immune surveillance. Hajizadeh et al. reported that intra-articularly injected mtDNA induced arthritis in mice (Hajizadeh et al., 2003). Data from this study provides evidence for cytolytic damage in RA as indicated by elevated levels of serum LDH activity. Metabolic analysis of synovial fluid in active RA showed elevated concentrations of lactate, diminished glucose concentrations and high levels of ketone bodies which suggested an increased dependence on lipids as a fuel source (Naughton et al., 1993).

Early experiments showed that glucose metabolism was impaired in RA and was related to insulin resistance (Paolisso et al., 1991). More recently, RA was associated with a higher prevalence of metabolic syndrome and coronary atherosclerosis independent of age or gender (Chung et al., 2008). Chronic inflammation is thought to promote insulin resistance through the pro-inflammatory actions of TNF-α (Hehlgans and Pfeffer, 2005; O'Connor et al., 2008) and that metabolic syndrome may be a mechanism that contributes to atherosclerosis in an inflammatory environment. Impaired glucose metabolism and insulin resistance may contribute to elevated LDH expression in inflammatory environments where there is a shift toward lactate metabolism.

Within the paradigm of autoimmune arthritis, cytolytic injury at chronically inflamed joints may account for high plasma LDH activity. However, circulating PL may themselves become compromised prior to activation and recruitment to inflammatory sites. Lymphocyte metabolism is largely associated with oxidation of ketone bodies, which serves to elevate cellular pools of acetyl co-enzyme A (Ac-Co A), reserved for metabolism following stimuli for clonal expansion (Newsholme et al., 1986). Combined with ketone oxidation and impaired glucose utilization in an inflammatory background, metabolic machinery may be skewed toward the production of oxidative free radicals.

Cellular metabolic strategies regulate immune signaling and consequently the switch between "immune- privilege" and "immune–sensitivity". Inflammation is associated with localized changes in metabolism. Cells which rely on glucose as a primary fuel source exhibit elevated expression of MHC and co-stimulatory molecules. They contain highly polarized mitochondria and are known to be immuno-sensitive. In contrast, immuno-priviledged cells depend on lipid derived fuels and typically contain depolarised mitochondria. Flow of electrons along the mitochondrial electron transport chain is facilitated by oxygen complexes capable of forming ROS. Increases in the levels of intracellular free radicals modulate the surface expression of MHC class II molecules hence altering sensitivity of immune cells. Immuno-sensitivity is tightly regulated and cellular metabolic strategies substantially contribute to immune recognition and response. The switch between glucose and lipid oxidation is controlled partly by uncoupling proteins (UCP) which dissipate the mitochondrial proton gradient, confers protection

from ROS and lowers $\Delta\psi_m$ (Newell et al., 2006; Skulachev, 1998).

Anti-inflammatory effects of DMARDs such as MTX, were shown to depend on the production of ROS (Newell et al., 2004; Phillips et al., 2003) and surface expression of co-stimulatory molecules such as CD80/86 (Bhushan et al., 1998). Uncoupling, within the perspective of autoimmunity, may be a compensatory metabolic mechanism to reduce immune sensitivity by switching to lipid oxidation. The lowered $\Delta\psi_m$ observed in our cohort could possibly be a symptom of UCP action but may however not offset MTX-induced production of free radicals.

A likely mechanism by which circulating mononuclear cells may become damaged is through oxidative stress. There is substantial evidence showing oxidative damage to lipids and proteins in RA. Lemarechal et al. reported that free radical damage to serum proteins was linked to inflammation in RA. In addition, there was a marked increase in albumin and heavy chain immunoglobulin (Ig) oxidation (Lemarechal et al., 2006). Oxidative stress may also cause non-enzymatic damage of Ig where lysine and/or arginine residues are involved in glyoxidation reactions to form advanced-glycation-end-products (AGE). Auto-antibodies to AGE-damaged Ig were observed in patients with early synovitis and were shown to be specific to RA (Newkirk et al., 2003; Newkirk et al., 1998).

Polyunsaturated fatty acids found in membranes are highly susceptible to damage by

ROS, leading to lipid peroxidation and formation of multiple end-products including MDA. Consistent with previous reports (Gambhir et al., 1997; Winyard et al., 1993), our investigation showed significantly higher levels of lipid peroxidation in RA patients than healthy subjects indicating substantial oxidative burden in patients.

While chronic inflammation has been linked to increased production of cytotoxic metabolites such as ROS, endogenous antioxidant systems offer protection against oxidative cellular damage. As a prototype antioxidant, glutathione is involved in cell protection from the noxious effect of excess oxidant stress, both directly and as a cofactor of glutathione peroxidases (Pompella et al., 2003). The importance of endogenous antioxidant systems in chronic inflammation is highlighted by the observation that genetic polymorphisms which modify the enzyme conjugation capacity of glutathione S-transferases, are associated with higher disease activities in RA patients (Bohanec Grabar et al., 2009).

Mitochondrial metabolic processes such as oxidative phosphorylation, which generate free radical species, make the organelle considerably vulnerable to oxidative damage. Yakes and Van Houten reported persistent and extensive mtDNA lesions following oxidative stress (Yakes and Van Houten, 1997). Endogenously oxidized mtDNA was shown to induce inflammatory responses both *in vivo* and *in vitro*. High quantities of extra-cellular mtDNA was found in RA synovial fluid and implicated in the perpetuation of chronic joint inflammation (Hajizadeh et al., 2003). Discharge of mtDNA into the extra-cellular compartment may occur as a result of membrane damage due to lipid

peroxidation.

Mitochondrial integrity was investigated by examining changes in $\Delta\psi_m$ using the cationic membrane permeable JC-1 fluorochrome. The dye is sensitive to changes in $\Delta\psi_m$ and its emission spectra depend on its relative concentrations in cellular compartments. Healthy mitochondria have electronegative membrane gradients due to the oxidative respiratory-chain reactions and thus $\Delta\psi_m$ is referred to as being polarized. Uptake of JC-1 into the mitochondrial matrix is driven by polarized $\Delta\psi_m$ and results in a red spectral shift. Depolarised $\Delta\psi_m$ is an indication of altered mitochondrial function (Gravance et al., 2000; Petit et al., 1995; Salvioli et al., 1997).

Circulating leukocytes are comprised of heterogeneous groups of mononuclear and polymorphonuclear cells. Combined with the JC-1 assay, flow cytometry permitted examination of $\Delta\psi_m$ changes in intact circulating PL separately as a homogenous cell population. Data from this study revealed that RA patients had significant losses in $\Delta\psi_m$, indicating that a large proportion of PL contained mitochondria which have lost the capacity to function optimally. Mitochondrial perturbations were related to disease activity as suggested by strong positive correlation with the DAS28 index. Specifically, correlation between mitochondrial depolarisation and acute-phase CRP suggests a substantial link with chronic inflammatory responses in this study cohort. Moreover

damaged mitochondria may contribute to PL death and explain the negative correlation between $\Delta\psi_m$ and absolute lymphocyte counts in the study cohort. Mitochondrial depolarisation may initiate apoptotic signal cascades through the release of cyt c. Atypical cell death signals may cause expulsion of immunogenic cytoplasmic contents as a result of membrane disruptions. Citrullinated proteins, which are specific to RA, are thought to enter the extra-cellular compartment via similar mechanisms (Gyorgy et al., 2006).

Free radical damage to the mitochondria may induce structural remodelling of the membrane which can cause depolarisation. In the context of autoimmune arthritis, it is likely that damaged mitochondrial contents as a result of ROS attack, is expelled into the cytoplasmic compartment due to collapsed $\Delta\psi_m$. In the cytoplasm, these potentially immunogenic contents may be processed by MHC machinery for antigen presentation. Another likely scenario which may account for depolarised $\Delta\psi_m$ observed in our RA patient cohort is a compensatory response to curb ROS production. It has been suggested that protection from oxidative stress would involve uncoupling reactions and shifts toward NADPH production catalysed by NADP transhydrogenase ultimately leading to depolarised $\Delta\psi_m$ and decreased ROS production.

There is compelling evidence for the potential role of oxidative stress in RA (Hitchon and El-Gabalawy, 2004). Particularly, mtDNA damaged by ROS attack, was shown to be strongly immunogenic (Hajizadeh et al., 2003). In addition, computational modelling

showed that altered protein products of mutated genes associated with respiratory-chain complex I could be coupled to MHC molecules for antigen presentation (Da Sylva et al., 2005). In conclusion, this study provides evidence for oxidative stress in South African black RA patients. Furthermore, the data shows shat mitochondrial integrity is compromised in these patients and suggests that impaired mitochondrial function may be related to disease activity.

CHAPTER 3

PERIPHERAL LYMPHOCYTE APOPTOSIS IN RHEUMATOID ARTHRITIS

3.1 Introduction

Bone and cartilage erosion occur during the natural progression of RA as a result of subtle underlying abnormalities in immune regulation and function. Accumulation and persistence of the lymphocyte infiltrate in the rheumatoid synovium are characteristic features of the disease (Firestein, 1991). In normal inflammatory responses, lymphocytes are eliminated, upon cessation of function, by initiation of apoptotic cascades (Serhan and Savill, 2005). Apoptosis is the major mechanism of programmed cell death and is necessary for regulation of tissue growth and homeostasis. In particular, the immune system relies heavily on apoptosis to ameliorate inflammation in order to prevent misdirected damage to normal tissue (Feig and Peter, 2007).

Several lines of evidence in RA suggest that malfunctions in apoptosis are responsible not only for the persistence of synovial lymphocytes, but also for the invasive nature of fibroblast-like-synoviocytes (FLS) (Baier et al., 2003; Pap et al., 2000). Interactions

between these cell types either through cellular contact or by secretion of soluble factors contribute to impaired apoptosis and chronic inflammation of the synovial membrane (Salmon et al., 1997).

In murine models of proteoglycan induced arthritis, T-lymphocyte apoptosis was shown to be defective despite high expression levels of CD95/Fas and was related to impaired downstream CD95/Fas signaling pathways (Zhang et al., 2001). Elevated levels of anti-apoptotic Bcl-2 proteins conferred resistance to CD95/Fas-induced apoptosis in $CD4^+$ T lymphocytes from RA patients (Schirmer et al., 1998). Furthermore, it was shown that RA-FLS synthesize high quantities of stromal-cell-derived-factor-1α (SDF1α), a ligand for lymphocyte CXCR4, which induces migration of $CD4^+$ T-lymphocytes to the synovium. Interestingly, SDF1α also inhibits T lymphocyte apoptosis by interfering with MAPK pathways (Nanki et al., 2000).

In addition to T lymphocytes, there is growing interest in B-lymphocyte biology within the context of autoimmunity (Yanaba et al., 2008). The recent success of anti-B lymphocyte therapies support the notion that breakdown of normal B-lymphocyte function contributes to the pathogenesis of RA (Venkateshan et al., 2009). Indeed, there is accumulating evidence for impaired B lymphocyte apoptosis in the rheumatoid synovium (Tolusso et al., 2009). B lymphocytes are enriched in the RA synovial membrane and are bound to FLS, which act as follicular dendritic cells (Lindhout et al., 1999). In co-culture with RA synovial stromal cells, B-lymphocytes up-regulate expression of Bcl-xL, which inhibits mitochondrial pro-apoptotic signals (Hayashida et

al., 2000). Inhibition of B-lymphocyte apoptosis by FLS was shown to occur in a cell-contact dependant manner via vascular-cell-adhesion-molecule-1 (VCAM1) (Reparon-Schuijt et al., 2000). These data suggest that cell-contact interactions contribute to the pathophysiology ultimately leading to destruction of the rheumatoid synovium.

While synovial joints are the primary sites of inflammation in RA, there is a significant, but poorly understood systemic inflammatory component of the disease. Immuno-pathologies in RA are not limited to synovium sensitive cells but also involve the majority of circulating PL. Glant *et al* (2001) proposed that defective apoptosis may lead to accumulation of T lymphocytes in peripheral circulation (Zhang et al., 2001). It is likely that this may perpetuate the often elusive systemic complications of RA. There is compelling evidence to show that dysregulation of PL apoptosis is critical in the pathogenesis of various systemic autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögrens syndrome and systemic sclerosis (Bijl et al., 2001; Stummvoll et al., 2000). Apoptosis in RA-PL however, has not been comprehensively investigated.

Defects in RA-PL apoptosis could clearly underlie some of the characteristic immunologic phenomena seen in RA patients. It is therefore imperative to investigate the apoptotic status of circulating PL in RA, especially since they exist in a milieu of inflammatory mediators such as TNF-α and CRP (Liuzzo et al., 2005). It is generally accepted that the autoimmune manifestations of RA are due, in part, to impaired lymphocyte apoptosis. Whether these defects are related to failures in executing the apoptotic program at inflammatory sites, or as a result of inherent defects in lymphocyte

apoptotic machinery prior to recruitment to these sites remain to be elucidated.

Inflammatory signals result in the activation of lymphocytes which can lead to activation induced cell death (AICD) via apoptosis. Lymphocyte activation plays an essential role in both central and peripheral tolerance and is critical for immune homeostasis. In PL AICD is caused by the expression and ligation of the CD95/Fas receptor. Activation of PL induces the expression of CD69 surface glycoprotein. It is the earliest detectable surface marker during lymphoid activation and is involved in lymphocyte proliferation (Cambiaggi et al., 1992). The CD95/Fas receptor is expressed on cells primed for apoptosis and engagement of this receptor initiates the extrinsic apoptotic signaling pathway. Apoptotic signals may be modulated by cell stress proteins such as HSP70.

The apoptotic status of circulating lymphocytes in RA may be a useful indicator of underlying pathological processes or disease activity. A better understanding of PL biology, and indeed PL apoptosis, may provide clues to how immunological tolerance is breached in RA. The aim of this study was to assess PL apoptosis in South African black RA patients *ex vivo*. In addition, the activation status and expression of CD95/Fas was investigated in PL. Since HSP70 may be involved in the modulation of apoptotic signaling, expression of HSP70 was also investigated in PL.

3.2 Materials and methods

3.2.1 Patients and peripheral lymphocytes

Patient recruitment and PL preparation is described in sections 2.2.2 and 2.2.3 respectively.

3.2.2 Detection of phosphatidylserine on outer membrane of peripheral lymphocytes

The annexin-V-fluorescein apoptosis detection kit (Roche) was used to label apoptotic PL with translocated phosphatidylserine (PS) residues on the outer plasma membrane. During the early stages of apoptosis, PS is translocated to the external surface of the plasma membrane (figure 3.1). Despite alteration of plasma membrane architecture, it remains intact. Phagocytic cells, such as macrophages are able to distinguish apoptotic cells by recognition of exposed PS. In this assay, annexin-V, which is conjugated to a fluorescent marker, fluorescein-isothiocyanate (FITC), was used to label apoptotic cells. Annexin-V is a calcium dependant phospholipid binding protein and has high affinity for PS. Since necrotic cells can also expose PS due to loss of membrane integrity, these were

distinguished by the use of propidium iodide (PI). PI is a fluorescent dye which rapidly enters cells with damaged plasma membranes and intercalates with DNA. It is unable to penetrate apoptotic cells and thus apoptotic cells were differentiated by the incorporation of annexin-V, but the exclusion of PI. Annexin-V-FITC labelling solution was prepared by combining 20µl of annexin-V-FITC with 20µl PI, in 1ml calcium containing buffer. These reagents were supplied in the kit and labelling solution prepared as described was sufficient for 10 tests. The annexin-V-FITC labeling solution (100µl) was added to 1 x 10^6 PL in cytometry tubes and allowed to incubate for 15 minutes in the dark at room temperature (RT). Following incubation, two separate aliquots of the annexin-labeled PL were prepared in order to assess apoptosis in B- and T-lymphocyte sub-populations. Allophycocyanin (APC)-labeled anti-CD4 and anti-CD19 (Pharmingen) was added (5µl) to the respective PL aliquots 10 minutes prior to enumeration by flow cytometry.

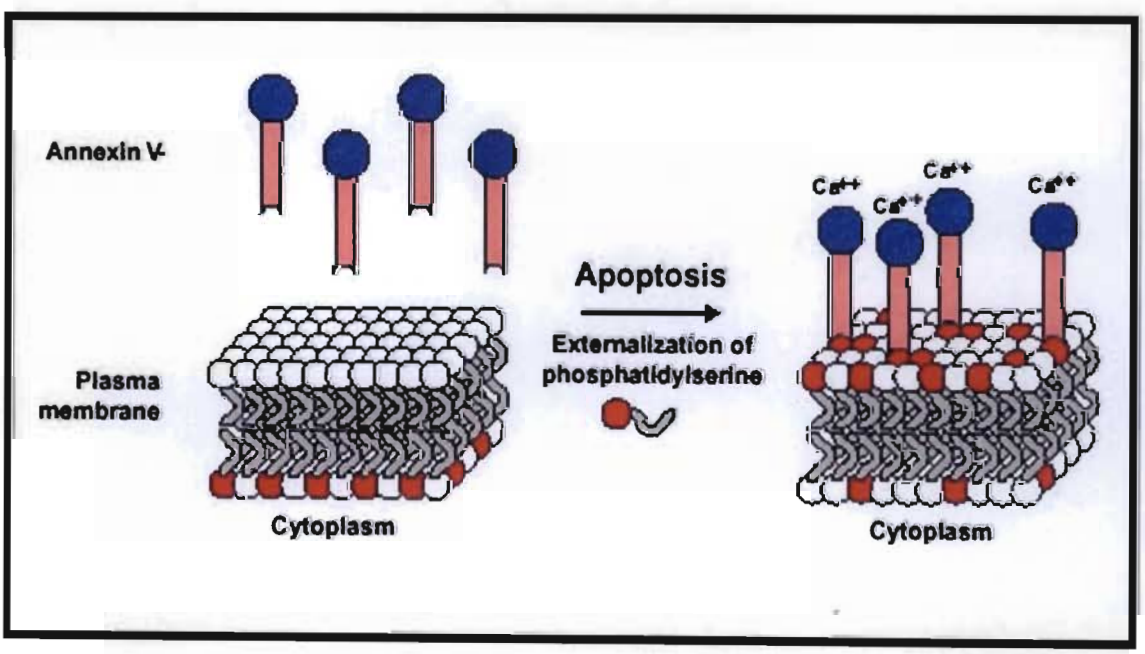


Figure 3.1 Schematic diagram of the Annexin-V assay.

3.2.3 Detection of the CD69 activation marker and CD95/Fas on peripheral lymphocytes

To investigate PL AICD and CD95/Fas expression, aliquots of approximately 1 x 10^5 PL were transferred into cytometry tubes containing monoclonal anti-CD95/Fas (1:100; Sigma). The mixture was allowed to react for 20 minutes and PL were thereafter washed in PBS (400 x g, 10 minutes). To detect labeled PL by flow cytometry, APC-labeled secondary antibody (Sigma) was added to cells at a final dilution of 1:1 000 and allowed to react for 15 minutes. In order to determine the activation status of circulating lymphocytes in RA patients, 1 x 10^5 PL were incubated with 10µl fluorescein-isothiocyanate (FITC) labeled anti-CD69 (BD Biosciences) for 15 minutes prior to analysis by flow cytometry.

3.2.4 Intra-cellular detection of heat shock protein 70

For each sample, 1 x 10^5 PL were transferred into cytometry tubes. Cells were then fixed (100µl Caltag reagent A fixative medium; Caltag Laboratories) for 15 minutes at RT. After fixation, PL were washed in PBS supplemented with 0.1% sodium azide and 5% fetal bovine serum (300 x g, 5 minutes). Thereafter, PL were re-suspended in permeabilization medium (100µl, Caltag reagent B permeabilization medium; Caltag

Laboratories) containing monoclonal anti-HSP70 (1:1 000; BD Biosciences Pharmingen) for 30 minutes. Following permeabilization and incubation with primary antibody, PL were washed twice as previously described. Samples were then incubated with APC-conjugated anti-mouse secondary antibody (1:10 000; BD Biosciences Pharmingen) for 20 minutes at RT in the dark. After an additional wash step, labeled PL were re-suspended in sheath fluid for detection by flow cytometry.

3.2.5 Flow cytometry

Labeled lymphocytes in the assays above were enumerated by flow cytometry using a 4-colour FACS Calibur (BD Biosciences) flow cytometer. Data was acquired with CellQuest Pro software (BD Biosciences) from 100 000 events for each assay. Analysis was performed with FlowJo 7.1 software (Tree Star Inc).

3.2.6 Apoptotic protease activity

Luminometry assays determined the activities of apoptotic initiator caspase 9 and executioner caspases 3/7. Separate aliquots of PL (1×10^5) were transferred into luminometry-quality white microtitre plates. 100µl of caspase substrate (Caspase-Glo 3/7, Caspase-Glo 9; Promega) was added to PL and allowed to react for 30 minutes at RT. Luminescent signals were then measured with the Modulus microplate luminometer (Turner Biosystems) and expressed as relative light units.

3.2.7 Detection of HSP70 by Western blot

Protein extraction

Total PL protein was extracted from each sample using Cytobuster™ (Calbiochem) reagent, supplemented with protease inhibitors. Cytobuster™ reagent (500µl) was added to approximately 1 x 10^5 PL. This mixture was vortexed for 1 minute and thereafter incubated on ice for 15 minutes in order to lyse cells. The lysed mixture was then centrifuged (450 x g; 10 minutes) and the supernatants containing crude protein extracts were transferred into clean 1.5ml tubes and kept on ice until use.

Protein quantification and standardisation

Protein concentration was determined by the bicinchoninic acid assay (BCA; Sigma). The total protein concentration was based on a colour change of the sample solution from green to purple in proportion to protein concentration. The principle of the BCA assay is based on two reactions. The first involving the reduction of Cu^{2+} ions to Cu^{1+} by peptide bonds in proteins where the amount of Cu^{2+} reduced is proportional to the amount of

protein present in the solution. The second reaction involves two molecules of BCA which chelate each Cu^{1+} ion to form a purple-coloured product. This resulting purple compound absorbs light at a wavelength of 562nm. The concentration of protein in each sample was determined by comparison to a protein of known concentration, bovine serum albumin (BSA), which was serially diluted (0, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml) in order to construct a standard curve (Appendix 3). The assay was performed in duplicate using a 96-well microtitre plate. The supernatant from each sample (25µl) and the relevant standards (BSA) were added to appropriately labelled wells. The BCA working solution (202µl, 4µl $CuSO_4$ and 198µl BCA) was thereafter aliquoted into each well and the plate was incubated (37°C, 30min). After incubation, the absorbance of each sample was read at 562nm using a spectrophotometer. Using the absorbencies of the standards (BSA), a standard curve was constructed, from which the protein concentration of each sample was extrapolated. All samples were then diluted using storage buffer [0.1M KH_2PO_4 (pH 7.4), 0.5mM K_2EDTA, 0.1mM DTT and 0.25M sucrose] and standardised to a final protein concentration of 250µg/ml prior to electrophoresis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The Mini-PROTEAN 3 SDS-PAGE apparatus (Bio-Rad) was used for electrophoresis of extracted PL proteins. A 12% resolving gel [dH_2O, 1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS, 30% Acrylamide/bis, 10% APS (freshly prepared daily), TEMED] was prepared and caste between clean glass plates and allowed to polymerize for 40 minutes. Following which, a 4% stacking gel [dH_2O, 0.5M Tris-HCl (pH 6.8), 10% (w/v) SDS,

30% Acrylamide/bis, 10% APS, TEMED] was layered over the resolving gel and allowed to polymerize for 30 minutes. The gel cassette sandwich was then transferred to the electrode assembly, and placed in a tank filled with 2x electrode buffer (deionised water, Tris, glycine, SDS; pH 8.3).

Laemmli (Laemmli, 1970), sample buffer [dH_2O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue] was thereafter added (1:1) to the protein samples, and then boiled for 5 minutes in order to denature protein samples. The protein samples (20μg) were then loaded into separate wells in the electrophoresis gels. Samples were then allowed to resolve by electrophoresis (150V, 45 minutes).

Western blotting

Separated proteins were then electro-transfered to polyvinylidene difluoride membranes (PVDF). The electrophoresed gels were removed from the SDS-PAGE apparatus and placed in transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol; pH 8.3), for 10 minutes (to allow the gel and proteins to equilibrate). The PVDF membrane, fibre pads and filter paper were prepared by wetting with deionised water and then soaking in transfer buffer until they were ready to be used. The gel sandwich was assembled by first placing a fibre pad on the gel cassette holder, followed by filter paper and then the equilibrated SDS-PAGE gel. The PVDF membrane was placed over the SDS-PAGE gel, followed by a second filter paper and fibre pad. The gel cassette holder was then firmly closed and placed into the transfer module and then into the tank filled with transfer

buffer. The apparatus was sealed and the tank was then placed in a container filled with ice. Resolved proteins were then electro-transfered at a constant current of 400mA for 1 hour.

After the transfer, the PVDF membrane was removed and blocked overnight (4°C) with 5% (5g) non-fat dry milk in TTBS [100ml; Tris-buffered saline (TBS) containing 0.5% Tween20] in order to reduce non-specific binding by antibodies. The non-fat dry milk was discarded after the overnight blocking step and each membrane was then incubated (room temperature) for 1 hour with anti-mouse Hsp70 antibody. Primary antibody was diluted 1:1 000 in 1% BSA in TTBS. After incubation, the primary antibody was removed and the membrane was thoroughly washed thrice with TTBS (10 minutes). The membrane was then exposed to secondary antibody (anti-mouse-horse-radish-peroxidase (HRP)-conjugate; 1:10 000; Bio-Rad) for 1 hour at RT. Anti-β-actin-HRP (Sigma) was utilized for internal loading controls. The secondary antibody was thereafter removed and the membrane was again washed thrice with TTBS (10 minutes).

Antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system. Images were acquired and analyzed with Quantity-one™ image analysis software (Bio-Rad). Data is represented as peak band intensity for each sample.

3.2.8 DNA fragmentation assay

Genomic DNA was extracted from PL (1 x 10^5) for each sample. Cells were transferred to 500µl lysis buffer containing 0.5% sodium dodecyl sulfate (SDS), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM Tris–HCl (pH 8.0). To this RNase A (100µg/ml; DNase-free) was added and the solution was incubated at 37°C for 1 hour. Subsequently proteinase K (200µg/ml) was added to the solution and incubated for a further 3 hours at 50°C. Protein contaminants were then precipitated by addition of 0.1 volume 5mM potassium acetate and centrifugation (5 000 x g; 15 minutes). Supernatants containing genomic DNA were transferred to fresh tubes and extracted with 100% isopropanol on ice, and thereafter washed with 70% ethanol. DNA samples were then dissolved in 10 mM Tris and 0.1 mM EDTA (pH 7.4) at 4°C overnight. Concentration of each sample was determined spectrophotometrically. To prepare a positive control for the DNA fragmentation assay, apoptosis was induced in control PL samples by treating with camptothecin (4µg/ml, 12 hours) *in vitro*. DNA was extracted and quantified as described above. Equal amounts of DNA (300ng) were electrophoresed (150V; 50 minutes) on a 1.8% agarose gel containing 0.5 mg/ml ethidium bromide. DNA

bands were visualized by UV light and digitally photographed using a gel documentation system and Quantity-one™ image analysis software (Bio-Rad).

3.3 Results

3.3.1 Elevated phosphatidylserine externalization in patient peripheral lymphocytes

Translocation of PS residues from the inner leaflet of the plasma membrane to the exterior is an early apoptotic event. Annexin-V is a specific and strong PS-binding protein (Fadok et al., 1998a) that detects cells undergoing apoptosis. Flow cytometry was used to enumerate PL with translocated PS. Forward and side scatter dot plots were generated for each study subject and used to identify/gate PL based on morphology (figure 3.2A). Thereafter, green (Annexin-V) and red (PI) fluorescent scatter plots were used to determine the percent of apoptosis and necrosis in gated cell populations (figure 3.2B).

The Annexin-V assay showed that apoptosis was significantly higher ($p < 0.05$) in RA PL than in healthy controls *ex vivo*. When analyzed separately, apoptosis in RA $CD4^+$ PL was approximately 3.5-fold higher than controls. The highest apoptosis values were

recorded in the RA CD19^{+} PL which were approximately 4-fold higher than controls (table 3.1). Necrotic or late stage apoptotic cells were distinguished from PL which were exclusively positive for Annexin-V by using PI. The percent of RA PL positive for PI was extremely low (0.4%±0.10) and did not differ significantly from controls (0.2±0.03; p=0.1085, unpaired t test).

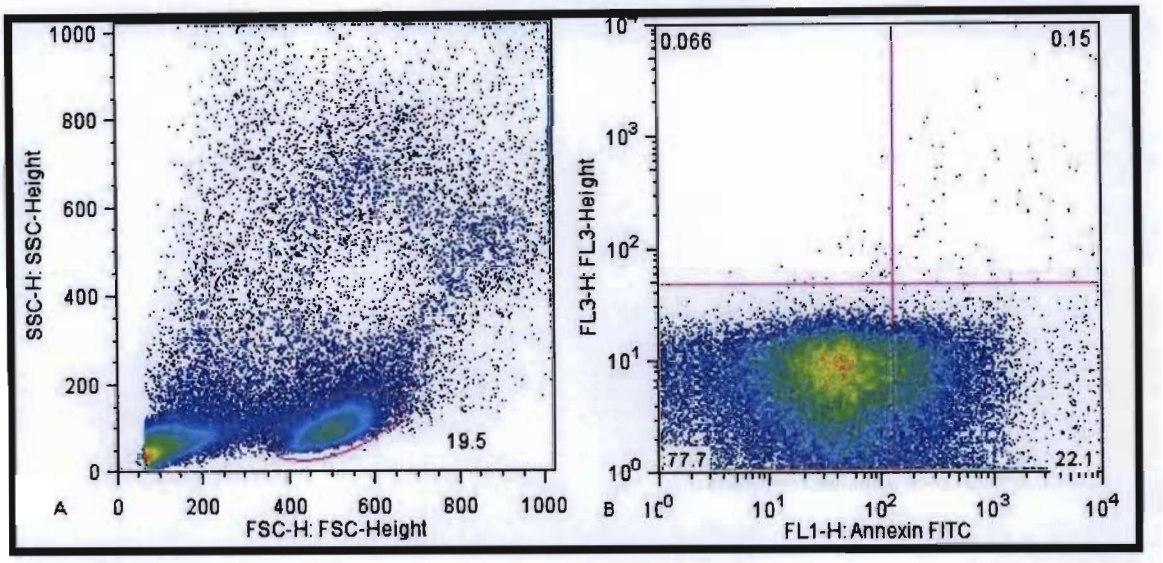


Figure 3.2 Flow cytometry scatter plots for the Annexin-V assay. A: Peripheral lymphocytes were identified and gated based on forward and side scatter properties. B: Scatter plots for green and red fluorescence channels were used to measure Annexin-V and prpidium iodide positivity respectively.

Table 3.1 Annexin-V analysis of apoptosis in peripheral lymphocyte sub-populations from South African rheumatoid arthritis patients and race-matched controls *ex vivo*.

Cell population	Patients [*Mean % (SEM)*]	Controls [*Mean % (SEM)*]
Total PL	30.0 (1.5)*	7.2 (0.9)
$CD4^+$ PL	26.3 (1.6)*	7.6 (0.8)
$CD19^+$ PL	60.5 (7.4)*	16.1 (1.9)

* Significant difference ($p<0.05$; Mann-Whitney test). CD4, cytotoxic T-lymphocyte marker; CD19, pan B-lymphocyte marker.

3.3.2 Apoptosis and clinical parameters

To examine the effect of elevated apoptosis on the number of circulating lymphocytes in RA patients, total PL apoptosis was statistically correlated with absolute lymphocyte counts. No statistically significant relationship between total PL apoptosis and absolute lymphocyte counts was observed (figure 3.3).

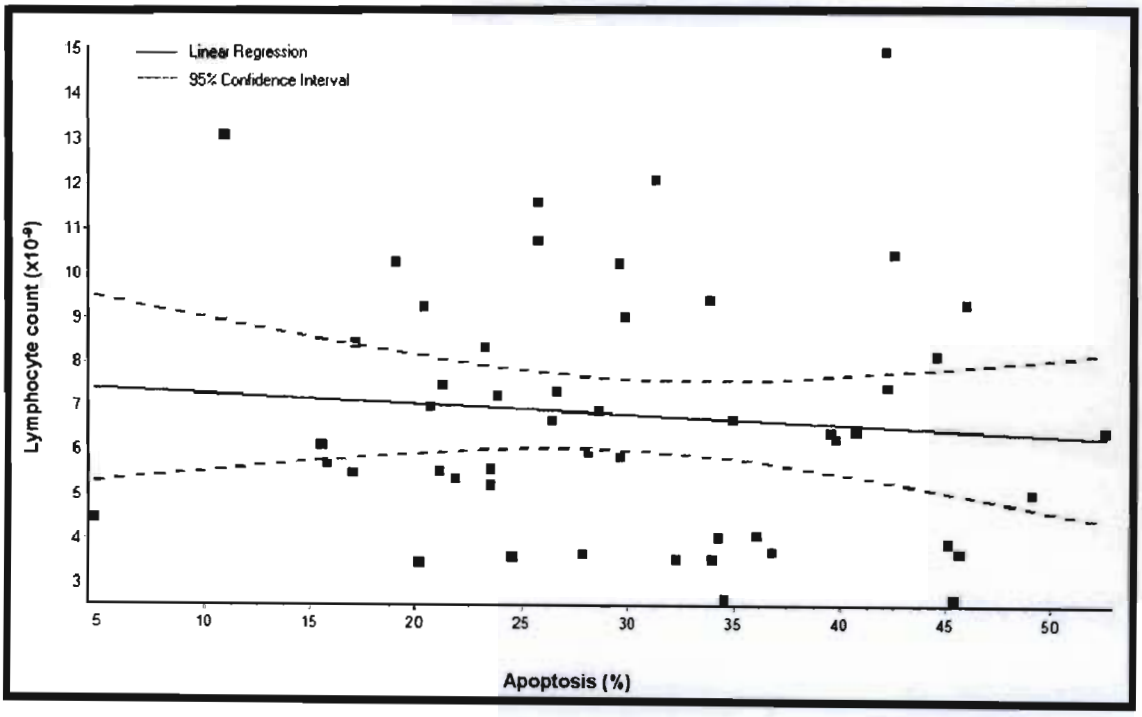


Figure 3.3 Absolute lymphocyte counts did not correlate with peripheral lymphocyte apoptosis in rheumatoid arthritis patients. Correlation co-efficient r = -0.08956; p value = 0.5362; 95% confidence interval, -0.3590 – 0.1937; Pearson r correlation.

In order to determine whether disease duration affected apoptosis in RA, total PL apoptosis was correlated with disease duration. It was found that apoptosis did not correlate with disease duration (Spearman rank correlation, r = -0.1539, p = 0.2962, figure 3.4).

Patients were then grouped, i.e. patients with RA for <10 years (n = 22) and >10 years (n = 28). Although total PL apoptosis was slightly higher in patients with RA for less than 10 years (30.6% vs. 27.8%), disease duration did not significantly affect levels of apoptosis in the patient cohort (p = 0.6843; unpaired t test with Welch correction).

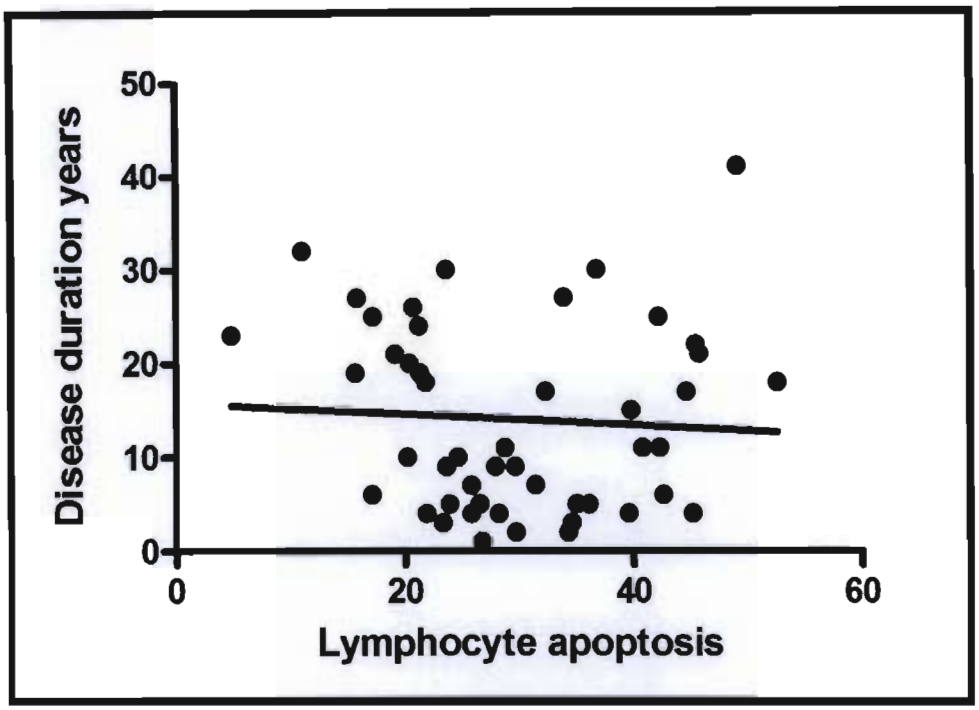


Figure 3.4 Relationship between apoptosis and disease duration in rheumatoid arthritis.

Since patients were at various stages of treatment when sampled (section 2.2.2), the effect of treatment regimens on total PL apoptosis was investigated. There was no significant difference in PL apoptosis ($p=0.6967$, one way ANOVA; figure 3.5) when compared to different treatments.

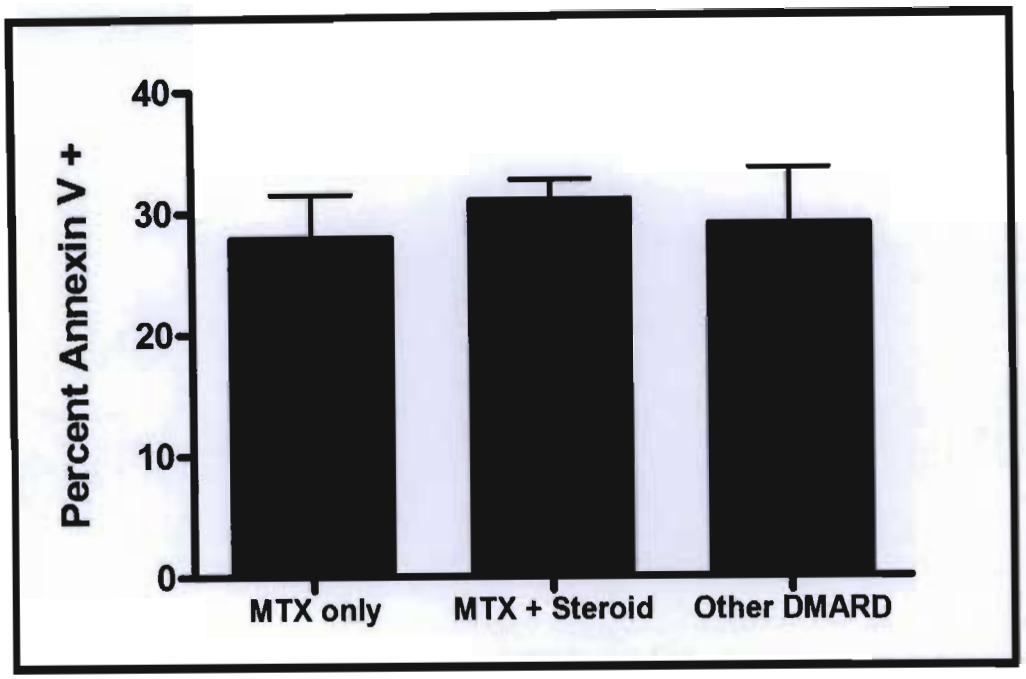


Figure 3.5 The effect of treatment on total peripheral lymphocyte apoptosis in rheumatoid arthritis.

3.3.2 Higher percentage of rheumatoid arthritis peripheral lymphocytes with CD95/Fas on plasma membrane

To determine whether the elevated apoptosis measured in the study cohort was associated with receptor mediated apoptosis-inducing signals, the presence of CD95/Fas on PL

surface was examined by flow cytometry. The proportion of PL expressing CD95/Fas was significantly higher in RA patients compared to controls ($p = 0.0317$; Mann Whitney test; figure 3.6.).

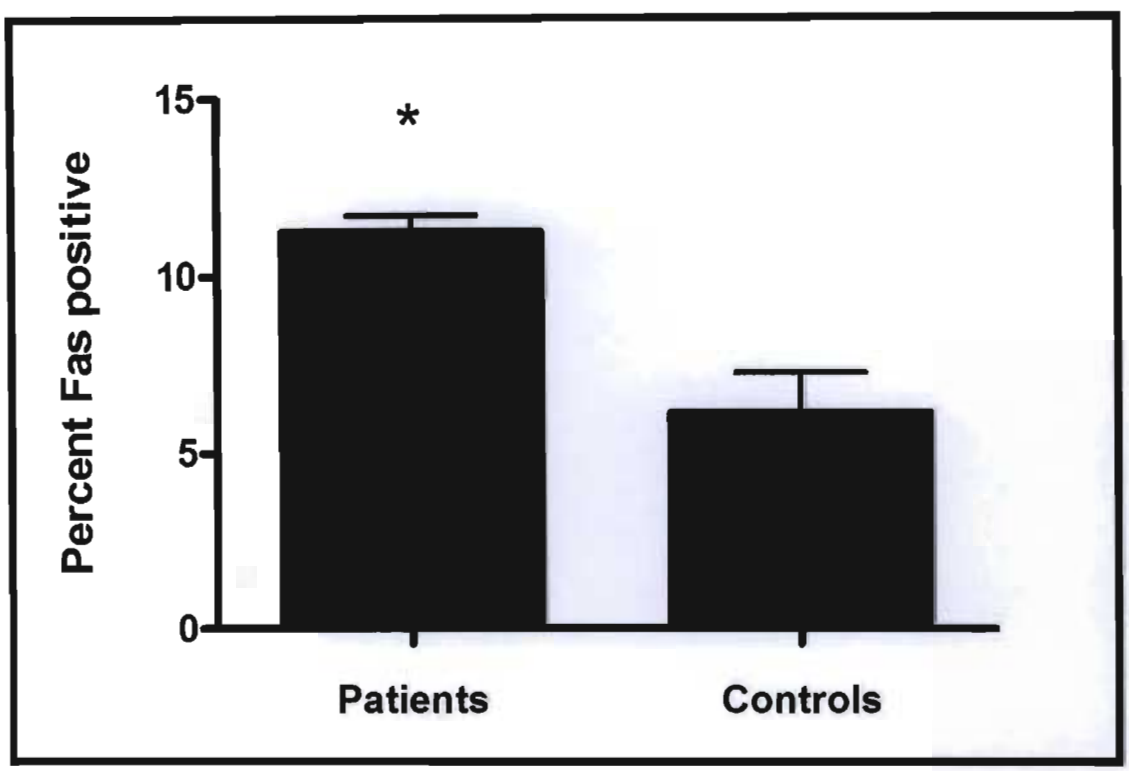


Figure 3.6 Percent CD95/Fas positive peripheral lymphocytes from South African rheumatoid arthritis patients and healthy race-matched controls. Peripheral

lymphocytes were analyzed by flow cytometry. Data is represented as mean percent + standard error of the mean.* Significant difference, p = 0.0317; Mann Whitney test.

3.3.3 Rheumatoid arthritis peripheral lymphocytes showed low levels of activation

Since CD95/Fas is associated with AICD in lymphocytes, the activation status of circulating lymphocytes was examined in the study cohort by monitoring the proportion of PL positive for the CD69 activation marker. Low levels of activation were observed in both patient and control subjects. Interestingly, despite high apoptosis levels, RA patients had a lower percent of PL positive for CD69 compared to controls. There was no statistically significant difference in activation status between RA-patients and controls ($p < 0.05$; Mann Whitney test; figure 3.7).

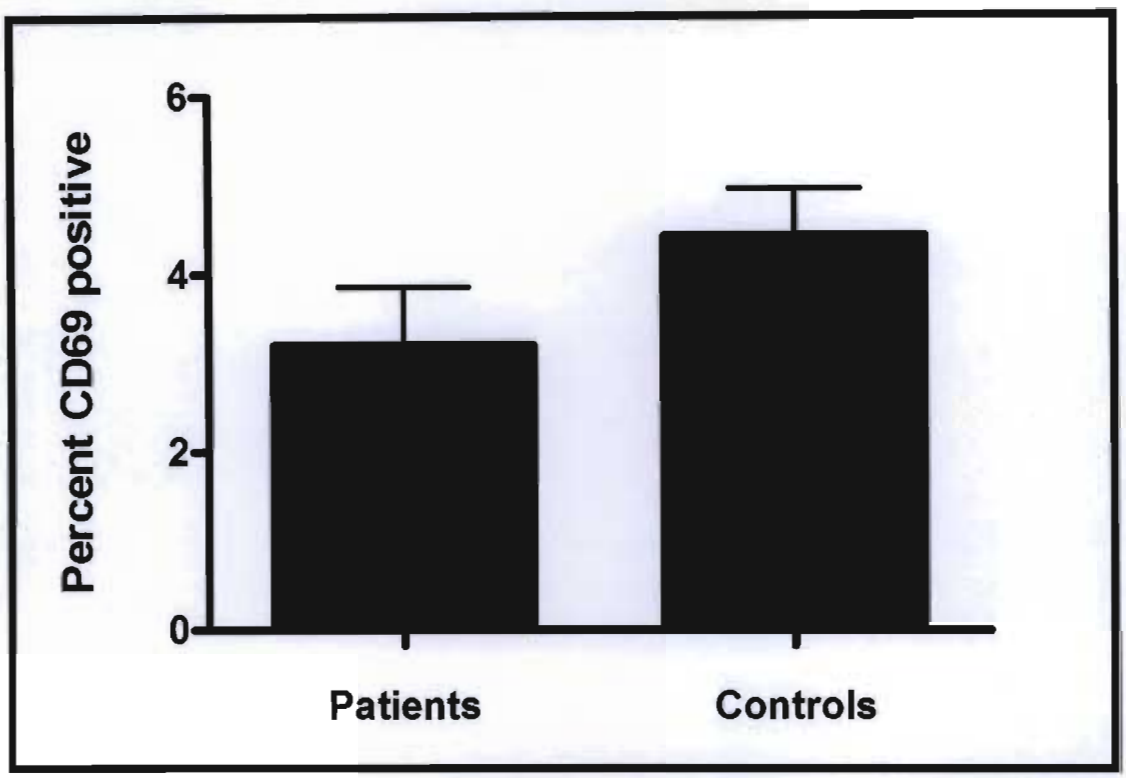


Figure 3.7. Activation status of peripheral lymphocytes were monitored by examining presence of CD69 on cell surface. Data is represented as mean percent + standard error of the mean. No statistical significance was established between patients and control subjects ($p < 0.05$; Mann Whitney test).

3.3.4 Elevated caspase activity in rheumatoid arthritis peripheral lymphocytes

Despite low luminescent signals recorded for executioner caspase 3/7 activity, there was approximately 3-fold higher activity in RA PL compared to healthy controls. This

difference in activity reached statistical significance ($p < 0.01$; Mann-Whitney test; figure 3.8A). Caspase-9 activity produced strong luminescent signals and although not statistically significant, was higher in patients (figure 3.8B).

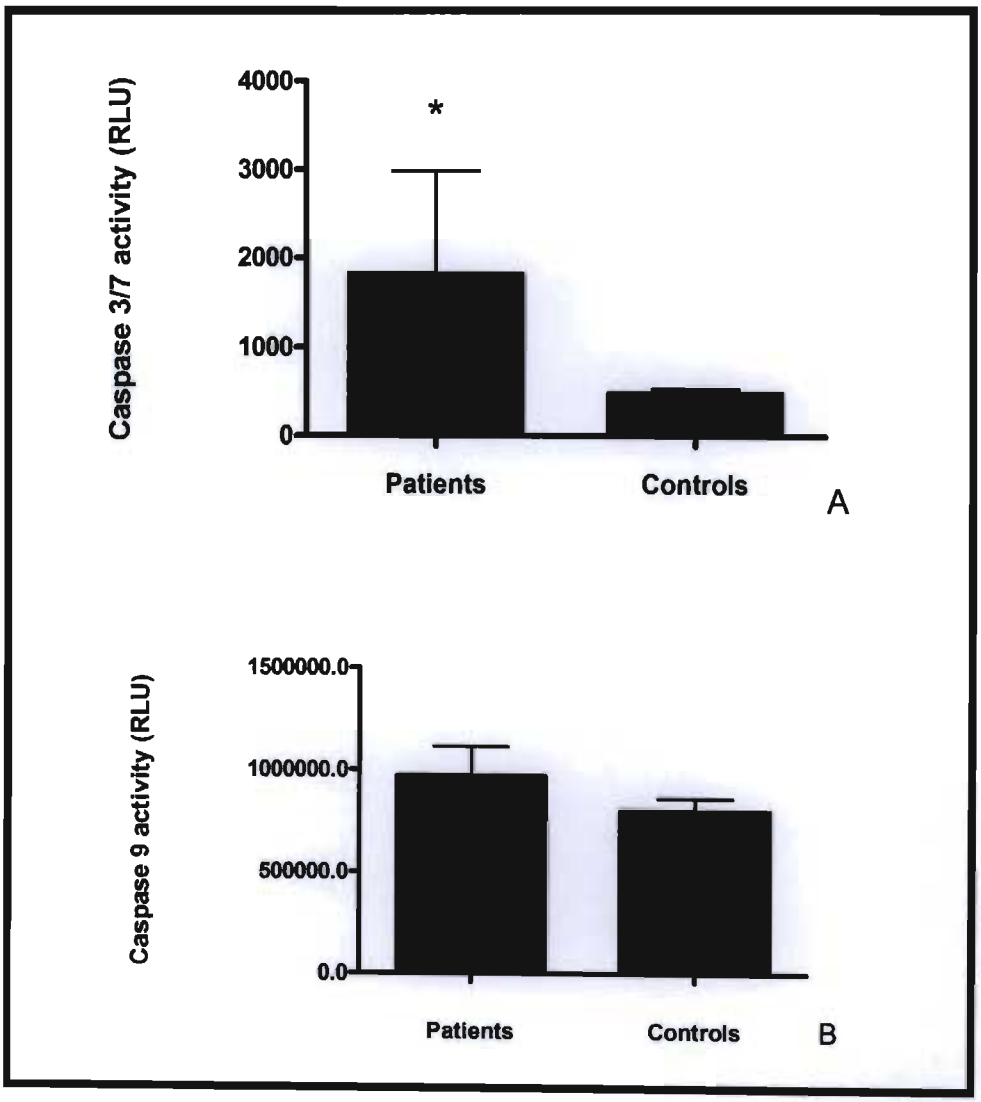


Figure 3.8 Apoptotic protease activity in peripheral lymphocytes. A: Higher caspase 3/7 activity in patient peripheral lymphocytes despite low luminescent

signals. B: Initiator caspase 9 activity was higher in both study groups but not significantly different. Data is expressed as mean relative light units + standard error of the mean. * Significant difference, $p<0.01$; Mann-Whitney test.

3.3.5 Elevated HSP70 in rheumatoid arthritis peripheral lymphocytes

Since HSP70 is an inducible protein which can modulate apoptosis signals, the levels of HSP70 in PL were examined. Using an intra-cellular flow cytometry staining technique, PL with high or low levels of intra-cellular HSP70 were distinguished as a function of mean fluorescence intensity (Figure 3.9A). The data showed that the proportion of PL with detectable levels of intra-cellular HSP70 was significantly higher in RA patients compared to controls (p = 0.0001; Mann-Whitney test; figure 3.9B). To confirm these data, western blot analysis for HSP70 was performed on total PL protein (Figure 3.9C). Band analyses showed that HSP70 levels were significantly elevated in RA patients (p = 0.0090; unpaired t-test; Figure 3.9D).

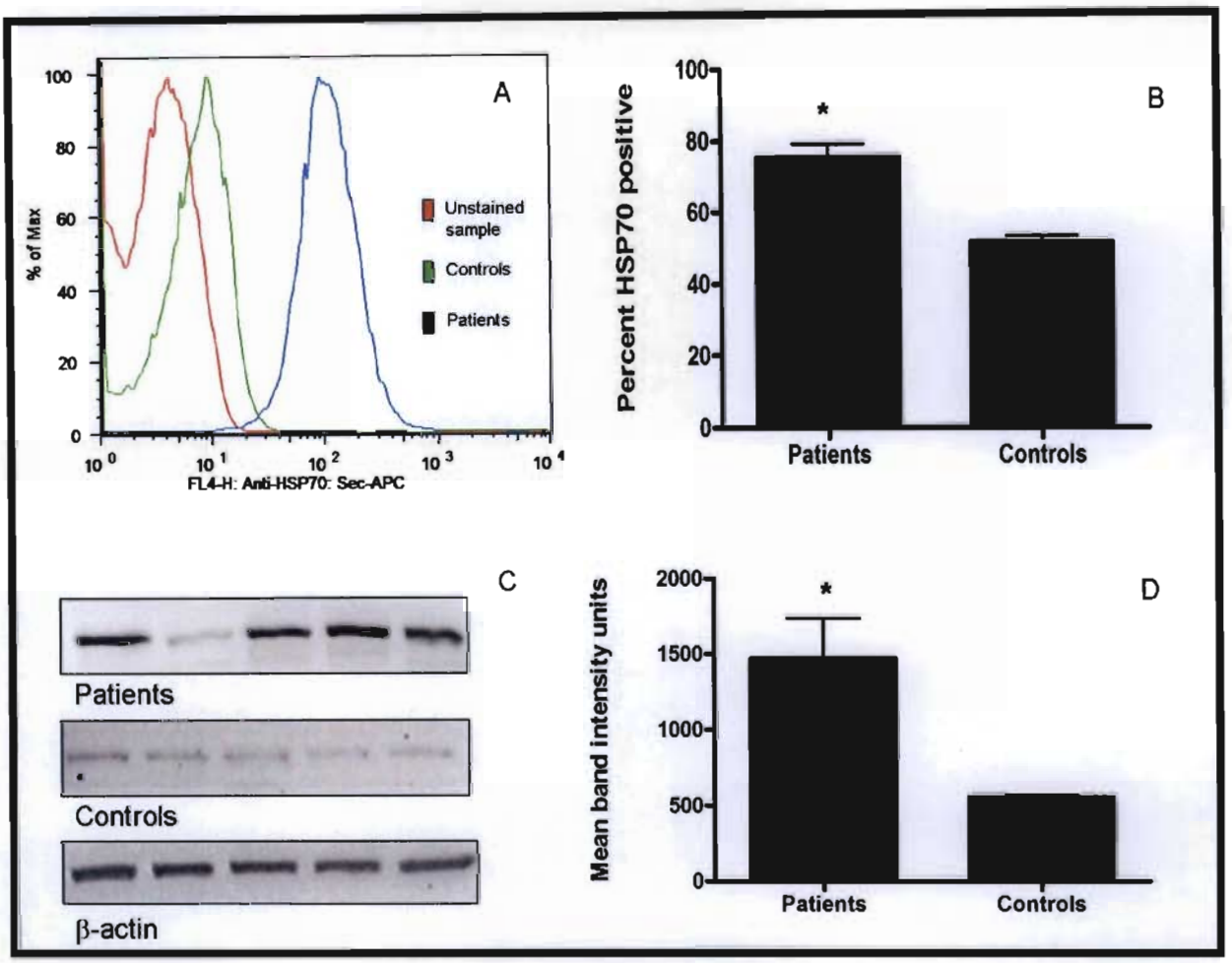


Figure 3.9 Detection of HSP70 in peripheral lymphocytes. A: Mean fluorescence intensity histogram for intra-cellular detection of HSP70 by flow cytometry. B: Proportion of peripheral lymphocytes with detectable levels of HSP70 was higher in rheumatoid arthritis patients (*p = 0.0001; Mann-Whitney test). C: Representative western blot analysis of HSP70 in total PL protein. D: HSP70 levels significantly elevated in rheumatoid arthritis patients (* p = 0.0090; unpaired t-test).

3.3.6 No DNA fragmentation in rheumatoid arthritis peripheral lymphocytes

DNA fragmentation, a typical molecular feature of apoptosis, occurs due to nuclease mediated cleavage of genomic DNA into oligonucleosomal fragments in multiples of approximately 200 base pairs. Nuclease activity is induced towards the latter stages of apoptosis by upstream apoptosis mediators such as caspase-3. The characteristic apoptosis DNA fragmentation pattern was not observed in patient or control samples following agarose gel electrophoresis. All DNA bands were of high molecular weight and intact with no signs of apoptosis-induced DNA damage (figure 3.10).

Figure 3.10 DNA fragmentation assay in peripheral lymphocytes by agarose gel electrophoresis. M: molecular weight marker; lane 1: control PL DNA; lane 2: positive control; lane 3,4,5: rheumatoid arthritis peripheral lymphocyte DNA.

3.4 Discussion

The fine balance between cell survival and cell death is essential for homeostasis in multi-cellular organisms. Apoptosis is the major mechanism of physiological cell death which facilitates deletion of unwanted or damaged cells. It plays a central role in the immune system in both the maintenance of self-tolerance and homeostatic control of lymphocyte populations (Feig and Peter, 2007). The immune system relies on apoptosis for its functional integrity at multiple levels, and consequently, stringent regulation of these pathways is imperative. Immunological tolerance is promoted by carefully directed apoptosis in self-reactive T-lymphocyte clones during their maturation in the thymus (Sprent and Kishimoto, 2001). Immune learning continues while lymphocytes are in peripheral circulation, since not all antigenic combinations are encountered in the thymus. The precise mechanisms of peripheral immune learning are unknown, but may also involve deletion of self-reactive lymphocytes by apoptosis whilst in circulation (Hoyne et al., 2000). It is likely that defects in peripheral immune learning may lead to autoimmunity. Lymphocyte death is tightly regulated and there are detrimental consequences when regulatory mechanisms are compromised. For instance, abnormal increases in apoptosis can cause immunodeficiency (Gougeon and Piacentini, 2009); while a failure to undergo apoptosis can lead to development of autoimmunity (Cacciapaglia et al., 2009).

In RA, there is compelling evidence to show that a compromise in lymphocyte apoptosis contributes to the persistence of these cells at inflamed joints (Pap et al., 2000).

Inflammation is normally resolved by carefully directed apoptosis of invading immune cells (Feig and Peter, 2007). In RA however, the molecular interactions between synovial cells and infiltrating lymphocytes have been shown to protect against apoptosis in the synovium (Zhang et al., 2001).

Little is known about the biological status of circulating lymphocytes prior to synovial recruitment in RA. This study therefore focused on circulating lymphocytes which perpetuate the autoimmune manifestations of RA. PL apoptosis was assessed, since they are exposed to a myriad of pro-inflammatory cytokines and acute-phase proteins. This may compromise functional integrity before the PL adopt a stationary phenotype in the rheumatoid synovium. Our data showed that total lymphocyte apoptosis was elevated in RA patients. This trend was mimicked in $CD4^+$ lymphocytes, but more so in $CD19^+$ B-lymphocytes; where more than half of this lymphocyte population showed apoptotic features whilst in circulation. This suggests that abnormalities in the regulation of lymphocyte apoptosis may occur early in RA, prior to synovial infiltration.

The high levels of mitochondrial membrane depolarisation and CD95/Fas on lymphocytes, indicated that both mitochondrially-mediated, as well as death receptor-induced cell death pathways may be active in RA-PL. The elevated levels of PL apoptosis observed in our patient cohort was not associated with AICD, since only a small percentage of lymphocytes showed detectable levels of the CD69 activation marker. CD69 is a transiently expressed membrane receptor early during lymphocyte activation, but is also selectively expressed in chronic inflammation (Rueda et al., 2008).

Interestingly, engagement of the CD69 receptor was shown to trigger apoptosis in multiple cell types (Walsh et al., 1996), but despite persistent expression in chronic inflammatory infiltrates, lymphocyte apoptosis was inhibited. Evidence from molecular and cellular studies showed that T-lymphocyte activation was altered in RA (Fernandez-Gutierrez et al., 1995). This may account for the high levels of RA-PL apoptosis observed in our patients where signs of early lymphocyte activation were relatively absent.

Under normal conditions, healthy mitochondria have polarized electronegative transmembrane gradients due to oxidative phosphorylation reactions. Loss of transmembrane potential alters mitochondrial permeability which results in the release of proteins such as cyt *c* and Smac/DIABLO into the cytoplasm (Adrain et al., 2001). In the cytoplasm, cyt *c* binds to Apaf-1 and pro-caspases, leading to ATP-dependant formation of the apoptosome (Bao and Shi, 2007). The apoptosome is a potent activator of initiator caspases, in particular caspase-9. Activated caspase-9 in turn facilitates activation of executioner caspases (primarily caspase-3 and caspase-7), which co-ordinate proteolytic breakdown of apoptotic cells. Engagement of CD95/Fas with its ligand leads to activation of caspase-8 following recruitment of FADD. This can signal apoptosis via two well-described pathways: (i) direct activation of caspase-3; or (ii) alteration of mitochondrial transmembrane potential via Bcl-2 homology-3 interacting-death-domain (BID) agonist, which initiates the mitochondrial apoptosis cascade (Siegel et al., 2003; Wallach et al., 1999). The CD95/Fas signaling pathway ultimately culminates in the activation of executioner caspases which is a molecular hallmark of apoptosis. Increased

caspase-9 activity observed in our patient cohort, combined with mitochondrial depolarisation suggests that the mitochondrial-apoptosome-caspase-9 apoptosis signal axis was functionally efficient. Although caspase-3/7 activity was significantly higher in RA patients, these activities remained relatively low. This suggests that there may be perturbations in the signaling pathways which activate executioner caspases in these cells. A possible mechanism may involve HSP mediated interference between apoptosis initiator signals and their down-stream targets. Despite an unclear delineation of their specific roles, HSPs have been repeatedly implicated as key participators in the pathogenesis of RA (Rajaiah and Moudgil, 2009). With respect to apoptosis, numerous mechanisms of HSP-mediated inhibition of cell death have been described (Beere et al., 2000; Takayama et al., 2003). Activation of caspase-3 for instance, is suppressed by HSP27 since it binds to pro-caspase-3, thus preventing its activation by caspase-9 (Pandey et al., 2000). Alternatively, HSP27 may sequester cytochrome *c* from Apaf-1, thus preventing assembly of the apoptosome (Bruey et al., 2000; Garrido et al., 1999). In addition the small HSP αβ crystalline, suppresses cytochrome *c*-mediated autoactivation of caspase-3, by direct interaction with the enzyme to prevent its complete processing (Kamradt et al., 2005). HSP70 has been implicated in the inhibition of apoptosome formation (Beere et al., 2000; Saleh et al., 2000), but may also inhibit caspase-dependent events that occur later in apoptosis (Jaattela et al., 1998). Chromosomal DNA is digested by caspase-activated-DNase/DNA fragmentation factor 40 (CAD/DFF40) during the final stages of apoptosis, upon activation by caspase-3 (Elmore, 2007). The enzymatic activity and structural integrity of CAD/DFF40 was reported to be regulated by HSP70 and HSP40 (Sakahira and Nagata, 2002). Over-expression of these HSPs may prevent

nuclear degradation regardless of up-stream pro-apoptotic events. We have recently reported in the same population of patients that RA-PL sustain significant damage due to oxidative stress (Moodley et al., 2008). This may induce cellular stress responses which increase the expression of HSPs, which could possibly modulate apoptotic signal cascades. This may have contributed to the lack of lymphocyte DNA fragmentation observed in our patient cohort, despite early signs of apoptosis. Earlier studies by Szodoray *et al* (2003) examined nuclear condensation as a measure of apoptosis in circulating RA T-lymphocytes bearing typical apoptotic markers (CD95/Fas, Bax, Bcl-2 and TNF receptor) (Szodoray et al., 2003). These investigations showed decreased levels of nuclear condensation in T-lymphocytes and were thus interpreted to have decreased rates of CD95/Fas mediated apoptosis. In addition, lymphocytes positive for Bax protein also showed decreased apoptosis frequency. The study concluded that the reduced susceptibility to CD95-mediated apoptosis may contribute to the expansion of an activated $CD4^+$ lymphocyte sub-population and thus to the maintenance of peripheral autoreactive T-cell clones in RA (Szodoray et al., 2003).

Furthermore, the molecular features of apoptosis measured in RA-PL did not translate to reduced numbers of circulating lymphocytes in our patient cohort. This was indicated by a lack of statistically significant correlation between absolute lymphocyte counts and total PL apoptosis. This observation supports the notion that the apoptotic program may not be fully executed despite early molecular signs of apoptosis in RA-PL. Albeck *et al* (2008) recently reported that although cells may exhibit molecular hallmarks of apoptosis, they may not be committed to fully executing the program and may recover

from pro-apoptotic signals (Albeck et al., 2008a). Although the mechanisms of apoptosis recovery are not fully understood, caspase inhibition via the XIAP and proteosomal degradation of executioner caspases seem to play a role (Albeck et al., 2008b) . Interestingly, Rehm *et al* (2006) also described a state in which cells may exist with partial caspase-dependent degradation of their proteomes without outward manifestations of apoptotic features (Rehm et al., 2006). In RA, it is likely that although apoptosis is initiated in circulating lymphocytes, these cells may not be committed to executing the molecular program fully and cellular interactions at the synovium exacerbate their anti-apoptotic phenotype. In addition, chronically elevated lymphocyte counts may occur as a result of apoptosis induced compensatory proliferation. Recent studies have elucidated non-apoptotic functions of both initiator and executioner caspases. These are involved in generating growth stimulation and compensatory cell proliferation signals via alternate MAPK cascades (Yi and Yuan, 2009). Death receptors have also been implicated in non-cytotoxic responses which include regulation of cell proliferation, growth stimulation and production of pro-inflammatory chemokines. Evidence already indicates that engagement of death receptors in the rheumatoid synovium promotes cell proliferation instead of cell death (Morel et al., 2005). Although not fully understood, similar mechanisms may operate and contribute to the maintenance of autoreactive lymphocyte clones in autoimmune diseases where apoptosis is elevated in peripheral circulation.

In conclusion, data from this study provides evidence that RA-PL apoptosis is impaired whilst in circulation prior to synovial recruitment. Although chronic inflammatory signals may provide the stimuli to initiate apoptosis, RA-PL may lack the ability to fully execute the apoptotic programme.

CHAPTER 4

FUNCTIONAL ANALYSIS OF THE P53 CODON 72 POLYMORPHISM IN RHEUMATOID ARTHRITIS

4.1 Introduction

The p53 tumour-suppressor protein plays an integral role in cellular responses to detrimental stimuli such as oxidative damage and genotoxic stress. It functions at the centre of intricate biological networks which control cellular fate by inducing either cell-cycle arrest or apoptosis. The cell cycle arrest arm of the p53 pathway largely depends on its ability to transactivate p21, a cyclin-dependant-kinase inhibitor. This facilitates cell-cycle arrest at the G1 checkpoint where DNA repair mechanisms may be initiated (Vousden and Lu, 2002).

When cellular damage is irreparable, p53 initiates the apoptotic cascade which eliminates unwanted cells, thereby maintaining tissue integrity. Transcriptional activity of p53 is important for the induction of apoptosis since many pro-apoptotic genes have p53 responsive transactivation elements. There is however, compelling evidence for non-transcriptional roles of p53 in apoptosis (Vousden, 2006). Early studies by Caelles *et al* (1994) showed that p53-dependant apoptosis occurred in the absence of transcriptional activation of p53-target genes (Caelles et al., 1994). Furthermore, mutant versions of the protein, lacking transactivation function were shown to possess potent apoptosis inducing

activity *in vitro* (Haupt et al., 1995). These transcription-independent activities of p53 predominantly relate to its ability to alter mitochondrial membrane dynamics (Vaseva and Moll, 2009). Cytoplasmic p53 rapidly translocates to the mitochondria in response to apoptosis inducing signals. It interacts with pro- and anti-apoptotic Bcl-2 family members, which ultimately results in mitochondrial depolarisation and initiation of the apoptotic caspase cascade (Marchenko et al., 2000).

Mitochondrial targeting and apoptosis inducing capabilities of p53 seem to be associated with its proline-rich domain. The 43 amino acid domain (residues 58-101) is located at the N-terminus, between the transactivation and DNA binding domains of p53. Deletion of this domain markedly impairs the proteins ability to induce apoptosis, but maintains it's transcriptional and transactivation functions (Matlashewski et al., 1987; Sakamuro et al., 1997; Walker and Levine, 1996). A common sequence polymorphism occurs within the proline-rich domain at position 72 (Matlashewski et al., 1987). The polymorphism arises from a single base-pair substitution (CCC to CGC) and results in a non-conservative transition from proline (Pro72) to arginine (Arg72) (Harris et al., 1986). This transition confers structural alterations and thus affects the functional activities of p53 (Buchman et al., 1988).

The Arg72 variant was shown to be more susceptible to degradation induced by human papillomavirus (HPV) E6 protein, which may alter tumour suppressor activities of p53 (Storey et al., 1998). In contrast, this variant was reported to possess an increased ability to suppress oncogene-induced transformation of certain primary cell lines (Thomas et al.,

1999). The Pro72 variant was shown to exhibit higher binding efficiency to transcriptional machinery, namely TAFFII32 and TAFII170 (Thomas et al., 1999). Interestingly, Dumont et al. (2003) showed that the Arg72 variant was able to induce apoptosis more efficiently *in vitro*. This was related to a greater potential of this variant for mitochondrial translocation, which was accompanied by release of cyt *c* (Dumont et al., 2003). Release of cyt *c* into the cytoplasm is preceded by mitochondrial depolarisation during intrinsic apoptotic signalling (Elmore, 2007).

We recently reported that mitochondrial depolarisation was elevated in PL from South African black RA patients. This directly correlated with disease activity, suggesting a possible role for mitochondrial membrane alterations in the pathogenesis of RA (Moodley et al., 2008). Elevated PL apoptosis was also observed in RA patients, and was associated with mitochondrial depolarisation. Since the p53 codon 72 polymorphism significantly alters mitochondrial membrane potential and apoptosis *in vitro*, this study investigated whether the polymorphism influenced mitochondrial membrane dynamics and PL apoptosis in local RA patients.

Only two studies have previously investigated whether the p53 codon 72 polymorphism was associated with susceptibility to RA (Lee et al., 2001; Macchioni et al., 2007). Both studies found no significant association between p53 genotype and RA susceptibility. These reports however, did not clarify the biological effects of the p53 genotype in cellular mediators of autoimmunity. This polymorphism has not been examined in black South Africans with RA.

4.2 Materials and methods

4.2.1 Study samples

Patient recruitment is described in section 2.2.2 and PL preparation is described in section 2.2.3. The method used to extract genomic DNA from PL is described in section 3.2.7.

4.2.1 Genotyping of p53 codon 72

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the p53 codon 72 genotype. A 131 base-pair PCR product was amplified using 15*p*mol of forward and reverse primer in a 25µl reaction containing 200 µM of each dNTP, 2.5mM $MgCl_2$, 1X Green GoTaq® Flexi buffer (Promega), 0.5U GoTaq® DNA polymerase (Promega) and 100ng genomic DNA template. Primer sequences were: Forward: 5' TTGCCGTCCCAAGCAATGGATGA-3'; Reverse: 5'-TCTGGGAAGGGACAGAAGATGAC–3'.

Following an initial denaturation step at 96°C for 12 minutes, amplification was carried out by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 5 minutes.

Presence of the polymorphic restriction site at codon 72 was analyzed by restriction endonuclease (Bsh1236I; Fermentas) digestion of the PCR amplification product. Overnight digestion at 37°C was performed in a total volume of 25µl, containing 15µl of the PCR product, 4.5µl Buffer R (10 mM Tris-HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl, 0.1mg/ml BSA) and 0.5µl (5U) Bsh1236I restriction endonuclease. Restriction fragments were electrophoresed on a 3% agarose gel containing 0.5mg/ml ethidium bromide and visualized as described previously (section 3.2.7).

4.3 Results

4.3.1 p53 codon 72 genotype in rheumatoid arthritis patients and controls

Polymorphic variation at codon 72 of the p53 gene was investigated in RA patients and control samples using RFLP-PCR. The reaction produced a 131 bp PCR amplification product, which was digested with Bsh1236I restriction endonuclease. Samples which were homozygous for the Pro72 allele did not contain the restriction endonuclease consensus sequence remained undigested, and therefore produced no restriction fragments. Amplification products from samples homozygous for the Arg72 allele were completely digested and resulted in two distinct restriction fragments of 81 bp and 50 bp. Digestion of heterozygous samples containing both alleles produced three restriction fragments of 131 bp, 81 bp and 50 bp. Figure 4.1 shows representative results for the p53 codon 72 genotype from RA patients and controls.

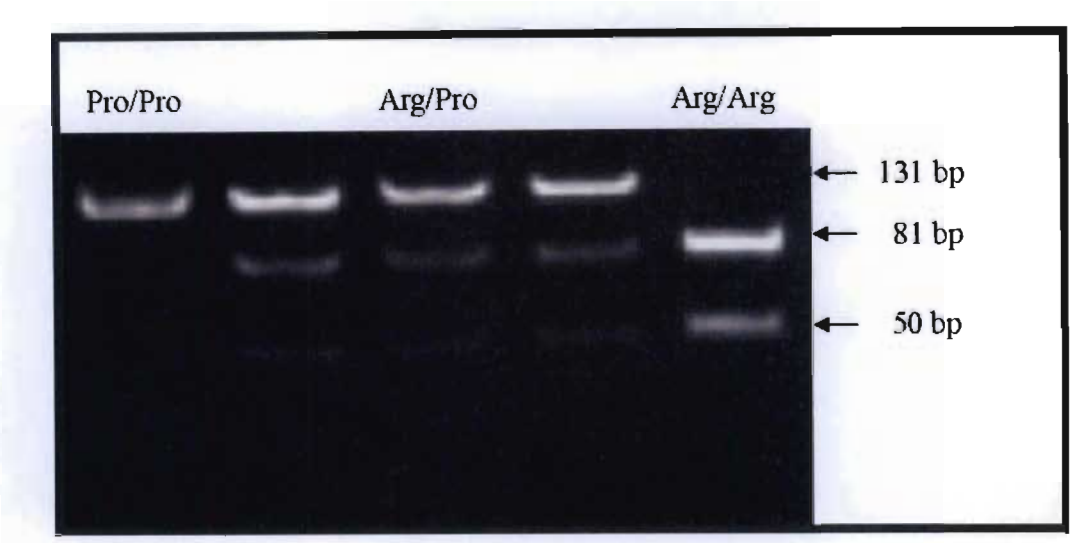


Figure 4.1 Representative results of the p53 codon 72 genotype by restriction fragment length polymorphism-polymerase chain reaction.

The genotype frequencies observed in our study cohort did not deviate from those predicted by Hardy-Weingberg statistics (p = 0.99, RA patients; p = 0.98 controls; chi-square test). Genotype distributions of p53 codon 72 did not differ significantly between RA patients and controls (Arg/Arg, Arg/Pro, Pro/Pro: 6, 23, 21 vs 2, 17, 31) respectively. There was however, a statistically significant difference between allele frequencies calculated for each group. The Arg72 allele was observed more frequently in RA patients, in contrast to control samples in which the Pro72 allele was more frequent (Table 4.1).

Table 4.1 Genotype and allelic distribution of the p53 codon 72 polymorphism in rheumatoid arthritis patients and controls.

	RA patients n = 50	Controls n = 50
Genotype frequencies*		
Arg/Arg	6 *(12%)*	2 *(3%)*
Arg/Pro	23 *(46%)*	17 *(34%)*
Pro/Pro	21 *(42%)*	31 *(63%)*
Allele frequencies**		
Arg	35 *(35%)*	21 *(21%)*
Pro	65 *(65%)*	79 *(79%)*

* Chi-square test for heterogeneity between RA patient and control genotype distribution. Chi-square statistic = 2.104, 1 degree of freedom; p = 0.1469.

** Chi-square test for heterogeneity between RA patients and controls allele frequency. Chi-square statistic = 4.191, 1 degree of freedom; p = 0.0406.

4.3.2 Mitochondrial depolarisation and p53 codon 72 genotype

Since polymorphic variation at codon 72 alters the ability of p53 to translocate to and alter mitochondrial membrane potential, mitochondrial depolarisation in the different p53 codon 72 genotypes was investigated. No statistically significant difference in mitochondrial depolarisation between genotypes were found in patients ($p = 0.8127$, one way-ANOVA; figure 4.2).

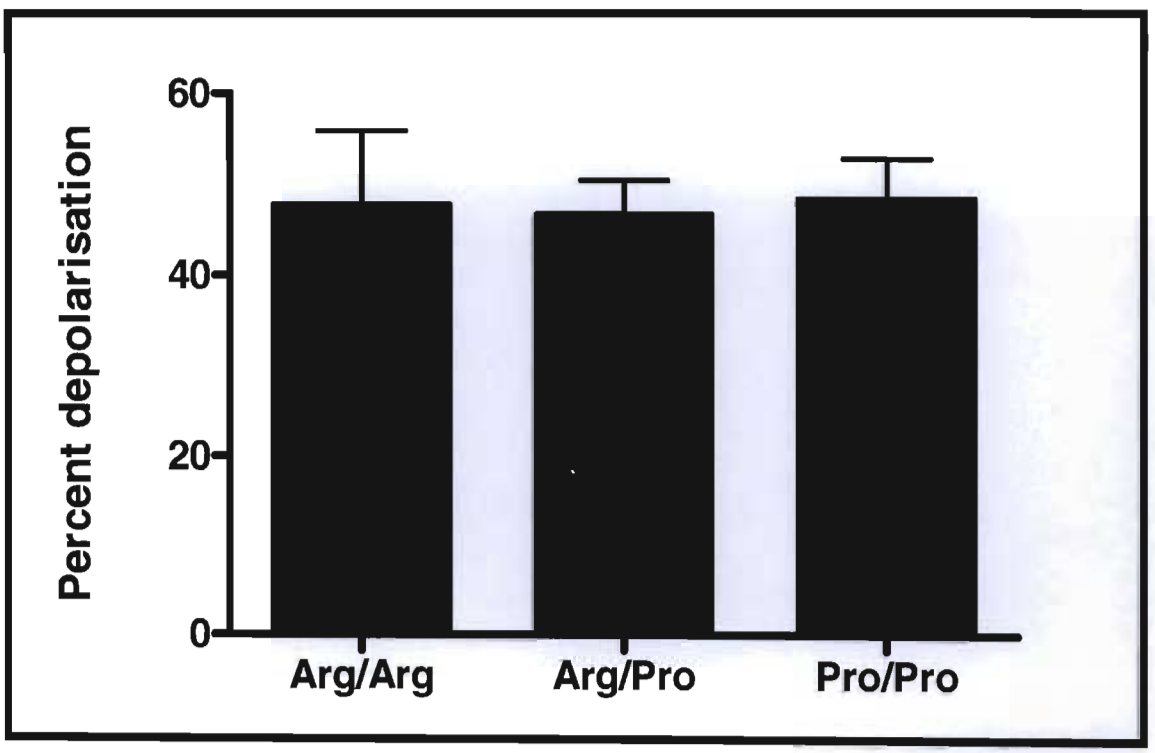


Figure 4.2 Peripheral lymphocyte mitochondrial depolarisation and p53 codon 72

genotype in rheumatoid arthritis patients.

4.3.3 Apoptosis and p53 codon 72 genotype

The apoptosis inducing ability of p53 codon 72 polymorphic variants are known to differ *in vitro*. Genotypic differences in apoptosis were investigated in RA patients. Higher levels of PL apoptosis were recorded in patients homozygous for the Pro72 allele compared to Arg72 homozygotes (33.0% vs 21.3%). Apoptosis in heterozygote patients (30.1%) was higher than Arg72 homozygotes, but less than Pro72 homozygotes. These data suggest that presence of the Pro72 allele increased the propensity for PL to undergo apoptosis. These differences however, did not reach statistical significance ($p = 0.1573$, one way-ANOVA; figure 4.3).

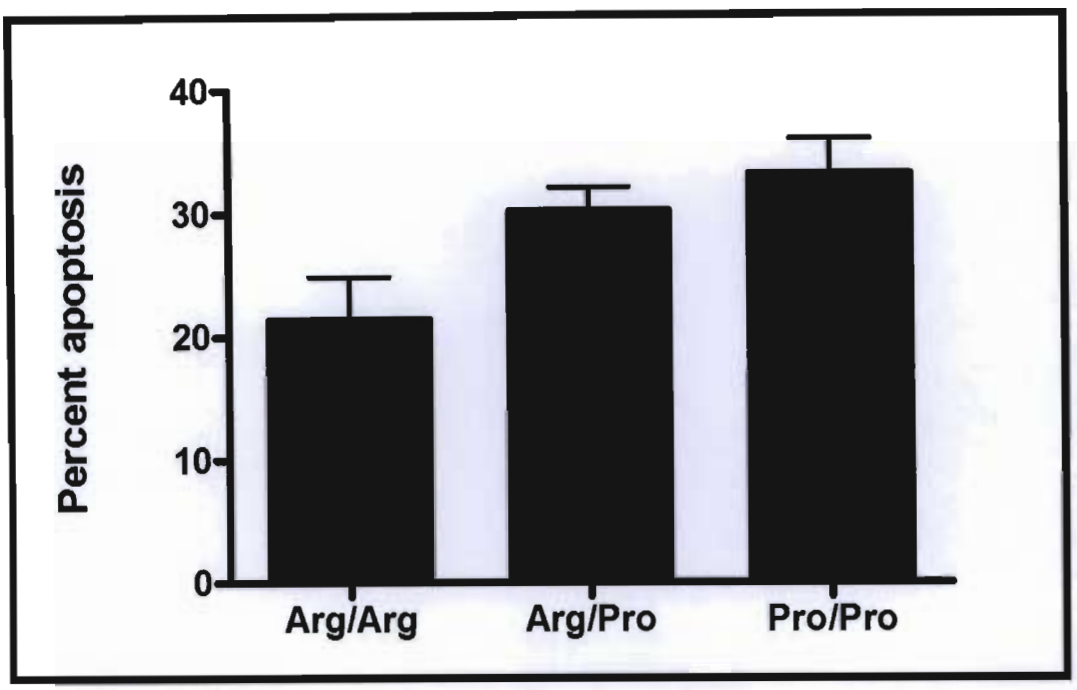


Figure 4.3 Peripheral lymphocyte apoptosis and p53 codon 72 genotype in rheumatoid arthritis patients.

4.3.4 Clinical markers of inflammation and p53 codon 72 genotype

Differences in clinical markers of inflammation were investigated between p53 codon 72 genotypes in RA patients. There were no statistically significant differences in clinical parameters between genotypes (one way-ANOVA; Table 4.2).

Table 4.2 Clinical analysis of rheumatoid arthritis patients according to p53 codon 72 genotype.

	Arg/Arg	Arg/Pro	Pro/Pro	*p* value
Mean number of swollen joints	12.9±1.4	12.3±1.4	12.3±3.0	0.9782
Mean number of tender joints	14.8±2.0	12.5±1.8	10±2.7	0.5066
ESR mm/hr	43.2±5.2	37.1±5.4	37.6±8.7	0.4964
CRP mg/ml	16.8±4.4	18.8±3.8	23.5±6.8	0.3618
Mean lymphocyte count ($x10^9$/ml)	6.03±0.52	7.40±0.63	8.07±1.10	0.2187
DAS28 score	6.29±0.23	5.88±0.29	6.26±0.45	0.3994

Data is represented as mean ± SEM

4.4 Discussion

Rheumatoid arthritis is a chronic inflammatory autoimmune disease, characterized by synovial hyperplasia and lymphocyte infiltration of the synovium (Gay et al., 2002). Invasive growth of synovial tissue leads to destruction of articular cartilage. The changes seen in the synovium resemble typical features of transformed cells (Hamilton, 1983). These include expression of oncogenes, angiogenesis and uncontrolled proliferation of synovial fibroblasts (Muller-Ladner et al., 1995).

Defects in p53 tumour suppressor mechanisms are strongly associated with pre-neoplastic cellular transformation (Yee and Vousden, 2005). There is evidence to implicate p53 dysfunction in RA (Firestein et al., 1996), but the precise role of the p53 gene in disease pathogenesis is not clearly understood. Studies to date have confirmed the presence of various p53 point mutations in the rheumatoid synovium (Firestein et al., 1997). These mutations are however, infrequent when compared to other human tumours and there is a high degree of variability between RA patient populations (Hainaut et al., 1998; Hainaut et al., 1997). In some RA populations, these mutations are absent despite geographic propinquity of ethnically matched study groups (Kullmann et al., 1999). These data suggest that mutational defects in p53 may not account for the tumuor-like properties of invasive synovial tissue and may not be a reliable predictor of disease susceptibility or pathogenesis. Some studies suggest that the random and variable genetic mutations detected in the rheumatoid synovium may be due to oxidative DNA damage as a result of chronic inflammation (Tak et al., 2000). Mutational studies in the rheumatoid synovium

may not be able to address mechanisms of systemic manifestations of the disease, which are perpetuated in part, by circulating lymphocytes.

It is therefore necessary to examine the role of genetic polymorphisms in RA, which are heritable, and often result in functionally distinct proteins. Common polymorphisms in the p53 gene have been associated with certain cancers (Dai et al., 2009; Sul et al., 2006) but have not been comprehensively investigated in autoimmune diseases. The p53 protein plays a central role in cell proliferation, apoptosis and mitochondrial membrane permeability. Dysregulation of these biological functions may contribute to RA pathogenesis.

The polymorphic CCC to CGC transition at p53 codon 72 results in a non-conservative amino acid substitution where proline (an imino acid) is replaced with arginine (Harris et al., 1986). Both amino acids are biochemically distinct. Proline contains an aliphatic side chain which is bound to both the nitrogen and α-carbon atoms of the amino group, forming a cyclic structure. Arginine is a basic amino acid with a large hydrophilic side-chain. This guanido side chain of arginine has a pKa of 12.48 as compared to the proline side chain which has a pKa of 10.05. In addition, the amine group in proline is a secondary amine as compared to primary amine in arginine. Taken together, these biochemical differences may severely alter the functionality of p53 when either amino is substituted. Interchanging these amino acids drastically alters p53 structure and thus influences its function. The polymorphic variants of p53 codon 72 were shown to differ biochemically and differences in their biological function were confirmed *in vitro*

(Thomas et al., 1999). Several lines of evidence suggest that the Arg72 variant was able to induce apoptosis more efficiently (Biros et al., 2002; Bonafe et al., 2002; Bonafe et al., 2004). This was related to an increased efficiency for mitochondrial translocation and its ability to influence molecular traffic between the mitochondrial inter-membrane space and cytoplasm (Vaseva and Moll, 2009). In addition the Arg72 variant was also shown to respond to oxidative stress and induce apoptosis to a higher extent than the Pro72 form (Salvioli et al., 2005).

We recently reported that mitochondrial depolarisation was significantly elevated in RA-PL and was directly correlated with disease activity (Moodley et al., 2008). In addition, RA-PL sustained significant oxidative damage. Data also showed that PL apoptosis was elevated in RA (Chapter 3). Since p53 affects mitochondrial membrane potential and plays a central role in apoptosis, influence of the codon 72 polymorphism on the outcome of these parameters was investigated in RA patients.

This study on the p53 codon 72 polymorphism in South African RA patients did not show any significant difference in genotype distribution between patients and controls, but noted that the Arg72 variant occurred more frequently in patients. Only two studies have investigated this polymorphism in other RA populations previously. Lee *et al* (2001) were the first to report on the p53 codon 72 polymorphism in a Korean RA cohort. No association was found between genotype and clinical features of RA (Lee et al., 2001). Similarly, Macchioni *et al* (2007) found no association between polymorphic variation and RA susceptibility in Italian patients; however, the Pro72 variant was

associated with higher degrees of joint erosion and structural damage at five year follow-up (Macchioni et al., 2007). Data from this study is in agreement with these previous reports, since no significant difference was found between clinical markers of disease activity and genotype distribution. In addition there were no significant differences in mitochondrial depolarisation and apoptosis based on genotype in our patient cohort. Interestingly, although the Arg72 variant was shown to induce apoptosis more efficiently *in vitro*, patients homozygous for the Arg72 allele in this RA cohort showed the least amount of PL apoptosis.

CHAPTER 5

CONCLUSION

This data provides evidence for a possible mechanism by which damaged internal mitochondrial contents traverses the otherwise stringently regulated mitochondrial membrane to enter the cytoplasm. The fate of mitochondrial contents in the cytoplasm is varied. They may be targeted for proteosomal degradation, but in the context of RA, they may be processed for antigen presentation which may produce immunogenic stimuli.

PL showed signs of elevated apoptosis in the RA patient cohort, more so in $CD19^{+}$ B-lymphocytes. Elevated apoptosis seems to be associated with CD95/Fas, but is not necessarily related to lymphocyte activation. Although upstream markers of apoptosis were observed in PL, the lack of DNA fragmentation suggests that apoptosis was not fully executed. Further evidence of aberrant apoptosis was noted in the elevated levels of HSP70 in RA PL. It is likely that HSP70 modulates upstream apoptotic signals in RA PL. Taken together this data suggests that apoptosis may be initiated in RA PL but not fully executed.

While genotypic variation of p53 at codon 72 confers functional differences *in vitro*, data from this study suggests that dysregulation of mitochondrial function and apoptosis observed in this patient cohort is not related to p53 genotype.Furthermore, the p53 codon polymorphism is not associated with RA susceptibility in black South African patients.

APPENDIX

Appendix 1

INFORMATION AND CONSENT FOR STUDY PARTICIPANTS

INFORMATION FOR PATIENTS

An investigation of apoptosis and p53 polymorphisms in Black South African Rheumatoid Arthritis patients.

We, (Mr D. Moodley, Prof. A.A. Chuturgoon, Prof. G.M. Mody, Dr N. Patel), are doing research on Rheumatoid Arthritis. Research is just the process to learn the answer to a question. In this study we want to learn about some of the different ways in which pain and swelling occurs in rheumatoid arthritis. We can compare results of normal people without arthritis and people with arthritis in other parts of the world. The research is not part of your normal management, but the findings may improve our understanding of how and why the swelling develops.

We are asking you to participate in this study. Your identity will not be revealed outside this clinic and all records will be kept confidential. If the results of this study are published, your identity will remain anonymous. Approximately 50 patients with

arthritis and 50 normal people will be studied.

Your treatment will not be effected by the results of this study. Your participation is voluntary and you may refuse or withdraw at any time, and you will not be treated differently.

In order to do the research we will need to collect 15 mls of blood (3 small 5 ml tubes) from a vein in your arm. The blood will be processed in the lab, thereafter, it will be frozen and stored for use in this study and for future analysis, should the need arise. Apart from slight pain and discomfort associated with the prick by the needle there are no other side effects. If you have a large swelling of your knee joint we need to remove the fluid from your knee as part of your normal management. Normally the fluid is thrown away but will be kept and tested later. The stored samples i.e. blood and/or synovial fluid , will be used for future research pertaining to rheumatoid arthritis. Samples will be stored in an identifiable format and therefore any participant may choose to withdraw his/her sample from storage at any time. We will also examine you in the normal way and record the results of blood tests and x-rays which were done as part of your normal management. The examination and the blood tests will only be done once.

This study has received ethics approval from the University of Kwa-Zulu Natal, Nelson R. Mandela School of Medicine, Research Ethics Committee No. _____

You are required to sign an Informed Consent form and are free to ask any questions at

any time.

Researchers contact details:

Prof. A.A. Chuturgoon / Mr D. Moodley –

Mycotoxins Research Unit, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal. (031 – 260 4718)

Prof. G.M. Mody / Dr N. Patel –

Department of Rheumatology, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal. (031 – 260 4284)

Thank you

INFORMATION FOR CONTROLS

Rheumatoid arthritis is a disease which causes pain, swelling and damage of the joints. You do not have rheumatoid arthritis, but we would like to take blood samples from you. We are doing a research project to try and understand why rheumatoid arthritis patients develop swelling of the joints. We will need to collect 15 mls of blood (3 small 5 ml tubes) from a vein in your arm. The blood will be processed in the lab, thereafter, it will be frozen and stored for use in this study and for future analysis, should the need arise. Apart from slight pain and discomfort associated with the prick by the needle there are no other side effects. We will also need to record your age and sex and whether you are suffering from any infections or any other sickness. The results from your blood will be

compared to results obtained from blood taken from rheumatoid arthritis patients.

You are free to decide whether you wish to take part in this research or not. Your identity and all records will be kept confidential. If the results of this study are published, your identity will remain anonymous. Approximately 50 patients with arthritis and 50 normal people will be studied

This study has received ethics approval from the University of Kwa-Zulu Natal, Nelson R. Mandela School of Medicine, Research Ethics Committee No. _____

You are required to sign an Informed Consent form and are free to ask any questions at any time.

Researchers contact details:

Prof. A.A. Chuturgoon / Mr D. Moodley –

Mycotoxins Research Unit, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal. (031 – 260 4718)

Prof. G.M. Mody / Dr N. Patel –

Department of Rheumatology, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal. (031 – 260 4284)

INFORMED CONSENT

This study has received ethics approval from the University of Kwa-Zulu Natal, Nelson R. Mandela School of Medicine, Research Ethics Committee No. _____

I, _____, am giving consent to have blood and/or synovial fluid samples taken for use in a study on factors involved in rheumatoid arthritis. I have been informed by the clinic staff of the nature of this study and have read the information given to me.

I understand that I am free to ask questions and can obtain additional information with respect to the ethical aspects of this study from the Research Ethics Committee. I am aware that the samples taken may be used for additional related research in future, should the need arise.

Medical Research Administration – Tel: 031 260 4495, Fax: 031 260 4410

Participant: _____

Signature: _____ Date:

Witness: _____

Signature: _____ Date:

Contact details: _____

_____

_____

_____

Appendix 2

CRITERIA FOR THE CLASSIFICATION OF RHEUMATOID ARTHRITIS [a]

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of three or more joint areas	At least three joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP,MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least one area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)

5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

[a] For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least four of these seven criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with two clinical diagnoses are not excluded. Designation as classic, definite, or probable rheumatoid arthritis is *not* to be made.

Appendix 3

PROTEIN QUANTIFICATION

BCA Reagent (Sigma)

The BCA reagent is made up using 19ml reagent A and 380µl reagent B.

Constituents of BCA reagents

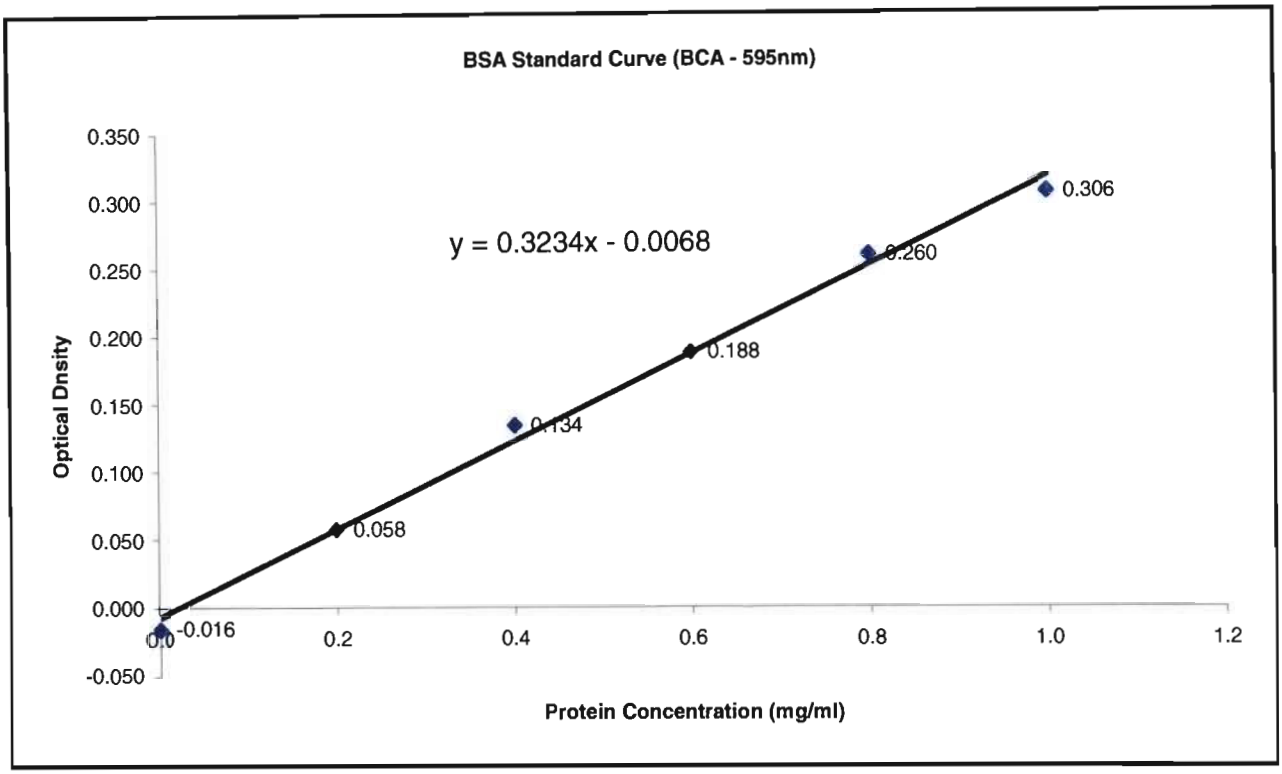
Reagent A	Reagent B
Bicinchoninic acid	Copper (II) sulphate
Sodium carbamate	Pentahydride 4% (w/v)
Sodium tartrate	
Sodium bicarbonate	

Bovine serum albumin (BSA) standards

The following volumes were used to make the BSA standard protein concentration series.

BSA standard constituents

Volume (μL)						
BSA	0	20	40	60	80	100
Distilled H_20	100	40	60	40	20	0
Protein Concentraion (mg/mL)	0	0.2	0.4	0.6	0.8	1.0



BCA assay standard curve

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