

2.2.7. Controls

Ten healthy control subjects (5 female, 5 male, average age 44.4 ±15.0 years, ranging from 26 -65 years of age) were used to determine the upper normal limit of the cytokine concentrations by calculating the mean +1SD.

Parameters	Mean±SD	Quintiles			Range
		Q1 (p25)	Q2 (Median)	Q3 (p75)	
Age (years)	48±12	41	48	56	20-75
Female (%)	118(82.5)				
Ethnic origin: Black (%)	125(87)				
DAS28-CRP	5.6±1.3	4.8	5.9	6.6	2.3-8.0
HDA (DAS28 ≥5.1) (%)	63(45.0)				
MDA (DAS28 ≥3.2 & <5.1) (%)	62(44.3)				
mHAQ-DI	3.2±1.6	2.0	3.5	4.5	0.0-6.0
Disease duration (months)	12±7	6	10	18	0-25
SJC	10±6	4	9	14	0-27
X-ray erosions (%)	65/121 (53.7)				
RF (IU/ml)	490±795	38	168	595	4-5350
anti-CCP (U/ml)	669±641	85	492	1099	2-2527
anti-MCV (U/ml)	594±679	104	326	775	16-3088
CRP (mg/L)	24.0±32	5	14	32	0-198
SAA (µg/ml)	62±123	5	14	60	1-882

Table 2.1: Demographic and laboratory data, and clinical scores for the group of RA patients (n=143).

2.2.8. Statistical methods

Statistical analysis was performed using STATA statistical software. Correlation coefficients were derived from correlation matrices using nonparametric Spearman's rank correlation test with Holm's method *P* value correction for multiple testing. A *p* value <0.05 was considered significant.

2.3. Results

2.3.1. Demographic, clinical and laboratory (autoantibodies, acute phase reactants) data

These are shown in Tables 2.1 (page 60) and 2.2 (page 62) for the total cohort and the MDA and HDA groups of patients respectively. RF, anti-CCP and anti-MCV were positive in 79%, 79% and 74% of RA patients respectively, while CRP and SAA levels were raised in 71% and 68% of patients respectively. The mHAQ-DI did not correlate with any of the laboratory parameters or DAS28, while the DAS28 score correlated with CRP and SAA ($r=0.52$ and $r=0.42$ respectively, $p<0.00001$ for both), in keeping with Emery et al. (Emery et al. 2007). Not surprisingly, anti-CCP and anti-MCV were strongly correlated ($r=0.76$, $p<0.0001$) as were CRP and SAA ($r=0.82$, $p<0.0001$), while only weak correlations were detected between these autoantibodies and the acute phase reactants. RF correlated moderately with anti-CCP and anti-MCV ($r=0.51$, $p=0.0002$ and $r=0.44$, $p=0.0014$ respectively), and weakly with CRP and SAA ($r=0.2$, $p<0.02$ for both).

Parameters	MDA Quintiles					HDA Quintiles				
	Mean ±SD	Q1 (p25)	Q2 (Median)	Q3 (p75)	Range	Mean ±SD	Q1 (p25)	Q2 (Median)	Q3 (p75)	Range
Age (years)	46±10	41	45	53	22-68	49±12	45	50	57	20-75
Female (%)	47(75)					50(80)				
Ethnicity: Black (%)	48(78)					59(94)				
DAS28-CRP	4.5±0.5	4.3	4.7	4.8	3.3-5.0	6.5±0.6	6.2	6.6	6.9	5.2-8.7
mHAQ-DI	3.3±1.7	2.0	3.6	4.5	0.0-6.0	1.8±0.8	1.3	2.1	2.4	0.0-3.0
Disease duration (months)	13±8	7	11	20	2-24	11±8	5	9	18	2-24
SJC	5±3	3	5	7	0-15	14±6	10	13	17	4-28
X-ray erosions (%)	28/53(53)					29/57(51)				
RF (IU/ml)	480±681	57	177	559	4-2860	491±812	21	178	654	10-5350
anti-CCP (U/ml)	618±639	51	343	1001	2-2026	734±667	89	599	1213	2-2527
anti-MCV (U/ml)	597±731	90	319	710	20-3028	638±727	108	334	868	16-3088
CRP (mg/L)	8±10	2	5	8	1-40	36±38	10	27	37	0-198
SAA (µg/ml)	25±48	2	5	24	1-238	91±153	8	35	124	1-882

Table 2.2: Demographic, laboratory, and clinical data for the MDA (n=62) and HDA (n=63) groups of RA patients.

2.3.2. Circulating cytokines, chemokines and growth factors in healthy controls and RA patients:

With the exception of TNF, IL-12, G-CSF, and VEGF (mean values of 21 ± 3 , 34 ± 37 , 34 ± 16 , and 176 ± 189 pg/ml respectively) the mean circulating concentrations of all the other cytokines ranged from 0-6 pg/ml in the group of healthy subjects. The results for the total cohort of RA patients are shown in Table 2.3 (page 64). The serum concentrations of the cytokines, chemokines and growth factors were significantly elevated in the total RA cohort, relative to those of the healthy control subjects, and those previously reported for healthy adult humans (Ibelgauft 2010), although the spread of values for each cytokine was considerable. The only exception was IL-17A, for which only very modest elevations were evident.

2.3.3. Correlations between cytokines/chemokines/growth factors:

As shown in Table 2.4 (page 67), moderate-to- strong correlations between cytokines of Th1 cell (IL-2, IFN- γ , GM-CSF), Th2 cell (IL-4, IL-6, IL-10), monocyte/macrophage (IL-1 β , IL-6, IL-12, TNF, G-CSF), and fibroblast (IL-7) origin were observed in the total cohort of RA patients. Of the chemokines, only CCL2 was moderately correlated with the Th1/Th2/macrophage/ fibroblast cytokines ($r=0.46-0.66$, $p<0.0001-0.0007$). VEGF, perhaps not surprisingly, given its probable fibroblast and macrophage origins [26,27], correlated best with IL-7($r=0.55$, $p<0.0001$) and IL-12 ($r=0.45$, $p=0.0009$), but only weakly or not at all with other cytokines.

	Mean±SD*	Q1 (p25)*	Q2 (Median)*	Q3 (p75)*	Range*
IL-1 β	25±50	1	4.0	20	0-269
IL-Ra	363±796	25	72	266	0-5012
IL-2	41±148	0	0	30	0-1320
IL-4	37±119	0	3	13	0-598
IL-6	63±129	7	18	66	0-1078
IL-7	251±830	5	25	90	0-6825
IL-8	293±3057	5	9	20	0-36699
IL-10	42±131	4	11	26	0-1172
IL-12	144±490	2	11	54	1-3105
IL-17	6±24	0	0	0	0-229
G-CSF	252±912	0	14	58	0-8764
GM-CSF	115±383	0	0	31	0-3726
IFN- γ	618±1789	0	27	196	0-10922
CCL2	84±192	0	48	108	0-1771
CCL4	130±92	70	108	177	27-544
TNF	141±426	4	10	62	1-2952
VEGF	450.4±712	59.4	166.4	518.8	0-4503

* results in pg/ml

Table 2.3. Serum cytokine, chemokine and growth factor values for the total RA cohort (n=143)

2.3.4. Cytokines which demonstrated the best correlations with RF, anti-CCP, anti-MCV, CRP and SAA:

In the total cohort of RA patients RF correlated moderately with IL-1 β , IL-4, IL-7, IL-12, G-CSF, GM-CSF, IFN- γ , and TNF ($r=0.50-0.67$, $p=0.0002- <0.0000$), and weakly-to-moderately with IL-1Ra, IL-2, IL-6, IL-8, IL-10, IL-17A, VEGF, CCL2, and CCL4 ($r=0.34-0.47$, $p=0.0155-0.0005$). Similar, albeit somewhat weaker, correlations were observed between these same cytokines and anti-CCP and anti-MCV antibodies ($r=0.31-0.49$, $p=0.0143- <0.0000$). In the case of the acute phase reactants, IL-6, not surprisingly, was found to correlate moderately with CRP and SAA ($r=0.49$ and 0.52 respectively, $p < 0.0000$ for both), while weak or no correlations were noted with the other cytokines. Interestingly, VEGF which correlated only weakly with the Th1/Th2/macrophage/fibroblast cytokines in the total cohort demonstrated considerably stronger correlations with these cytokines in the HDA subgroup. In this subgroup, but not in the total cohort, strong and significant correlations of VEGF with RF, anti-CCP, and anti-MCV were evident as well as a weak correlation with X-ray (Larsen) scores as shown in Table 2.5 (page 68).

2.3.5. Associations of the DAS28 clinical scores in the total cohort of patients and the HDA and MDA subgroups with circulating biomarkers of disease activity:

No significant relationships were evident between the DAS28 scores and any of the serum cytokines, chemokines and growth factors in the total RA cohort or the MDA subgroup (data not shown). In the HDA subgroup, however, DAS28 scores showed significant correlations with serum IFN- γ ($r=0.49$, $p=0.0160$), IL-1 β ($r=0.52$, $p=0.0088$), IL-1Ra ($r=0.62$, $p=0.0013$), TNF ($r=0.58$, $p=0.0033$), and GM-CSF

($r=0.58$, $p=0.0033$). The positive inter-correlations with Th1/Th2/macrophage/fibroblast cytokines, described above in the total RA cohort, were strongest in the HDA subgroup, as were the associations with RF, anti-CCP, anti-MCV, and the acute phase reactants. Although also evident in the MDA subgroup, these were of lesser magnitude and significance (not shown).

The findings demonstrate significant elevations in pro-inflammatory and anti-inflammatory cytokines, with those of Th1/Th2/macrophage/fibroblast origin being not only highly inter-correlated, but also correlated with RF in particular, and to a lesser, but nevertheless significant extent with antibodies to CCP and MCV.

	IL-1 β	IL-1Ra	IL-2	IL-4	IL-6	IL-7	IL-8	IL-10	IL-12	IL-17	G-CSF	GM-CSF	IFN- γ
TNF	0.85	0.64	0.77	0.86	0.59	0.50	0.23	0.70	0.68	0.52	0.75	0.79	0.81
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0062	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
IFN- γ	0.86	0.66	0.80	0.95	0.56	0.73	0.31	0.72	0.79	0.50	0.81	0.82	
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
GM-CSF	0.86	0.72	0.89	0.87	0.59	0.50	0.26	0.77	0.67	0.48	0.79		
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0015	<0.0001	<0.0001	<0.0001	<0.0001		
G-CSF	0.77	0.62	0.71	0.86	0.47	0.49	0.22	0.63	0.55	0.48			
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0083	<0.0001	<0.0001	<0.0001			
IL-12	0.84	0.53	0.79	0.82	0.45	0.84	0.35	0.90					
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001					
IL-10	0.80	0.68	0.76	0.73	0.69	0.63	0.23						
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0237						
IL-7	0.61	0.34	0.61	0.70	0.35								
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001								
IL-4	0.88	0.70	0.81										
	<0.0001	<0.0001	<0.0001										
IL-2	0.83	0.77											
	<0.0001	<0.0001											
IL-1Ra	0.79												
	<0.0001												

* For each pair of values the correlation coefficient (r) is uppermost with the corresponding p value underneath.

Strong correlations ($r \geq 0.7$) in bold

Table 2.4: Correlations between the serum cytokines, chemokines and growth factor concentrations in the total cohort of RA patients (n=143).

	VEGF	
	Total r(p)	HDA r(p)
RF	0.44(0.0014)	0.60(0.0023)
SAA	ns	ns
CRP	ns	ns
anti-MCV	ns	0.59(0.0023)
anti-CCP	ns	0.67(0.0003)
IL-1b	0.29(0.0433)	0.48(0.0190)
IL-1Ra	ns	0.41(0.0474)
IL-2	0.40(0.0038)	0.61(0.0014)
IL-4	0.36(0.0098)	0.65(0.0006)
IL-7	0.55(<0.0000)	0.65(0.0006)
IL-8	ns	0.41(0.0448)
IL-10	0.38(0.0072)	0.46(0.0246)
IL-12	0.46(0.0009)	0.60(0.0021)
G-CSF	0.31(0.0306)	0.55(0.0052)
GM-CSF	0.37(0.0086)	0.69(0.0002)
IFN- γ	0.37(0.0082)	0.66(0.0004)
TNF	ns	0.53(0.0078)
CCL2	0.40(0.0044)	ns
CCL4	ns	0.56(0.0044)
Larsen	ns	0.42(0.0409)

Table 2.5: Correlations of VEGF with autoantibodies, circulating cytokines and X-ray scores in the total cohort (n=143) and HDA group (n=63)

2.4. Discussion

Multiplex analysis of circulating cytokines in RA has the potential not only to provide novel insights into the immunopathogenesis of RA, but also to identify sub-groups of patients at risk for development of severe disease. To date, however, this important topic has been addressed in a limited number of studies (Hitchon et al. 2004, Stabler et al. 2004, Alex et al. 2007, Chen et al. 2009, Khan et al. 2009, Nam, Villeneuve & Emery 2009, Deane et al. 2010, Milman, Karsh & Booth 2010). Although these have shown promise, there is clearly a need for corroboration and extension in larger groups of patients. In the current study, we have investigated circulating cytokine profiles, representative of Th1, Th2, and Th17 cells, monocytes/macrophages, and fibroblasts, in a large group of DMARD-naïve RA patients of disease duration <2 years. Notwithstanding characterization of circulating cytokine profiles, the major objectives of the current study were to identify inter-relationships between cytokines, as well as correlations with RA-associated autoantibodies, acute phase reactants, and clinical disease activity. These were analysed in the total cohort of patients, as well as in the MDA and HDA subgroups.

In the case of the total cohort, and in keeping with previous studies (Hitchon et al. 2004, Stabler et al. 2004, Alex et al. 2007, Chen et al. 2009, Khan et al. 2009, Nam, Villeneuve & Emery 2009, Deane et al. 2010, Milman, Karsh & Booth 2010), a general increase in the circulating levels of all of the anti-inflammatory/pro-inflammatory cytokines, growth factors and chemokines were observed. IL-17A was the exception, being barely detectable and only modestly inter-correlated with the other cytokines. In contrast, cytokines primarily of Th1 and Th2 lymphocyte origin, as well as those derived mainly from monocytes/macrophages and fibroblasts, were not

only elevated, but also highly inter-correlated in the total cohort, being strongest in the HDA subgroup. The strong correlations of the Th2 cytokines, IL-4 and IL-10 (Zhu, Paul 2008), with those of the pro-inflammatory cytokines, IL-1 β , IL-2, IL-6, IL-7, IL-12, G-CSF, GM-CSF, IFN- γ and TNF, probably reflect the efforts of these cells to suppress the reactivity of their Th1 counterparts, and to redirect the phenotype and functions of classically IFN- γ -activated M1 macrophages (Gordon, Taylor 2005, Mantovani, Sica & Locati 2005). In addition to effects on Th1 cells and M1 macrophages, the Th2 cytokines IL-4 and IL-10 also suppress the proliferation and pro-inflammatory activities of fibroblasts (Groux, Cottrez 2003, Moroguchi et al. 2004, Kamel Mohamed et al. 2005, Arpa et al. 2009). Importantly, the consistency and magnitude of the correlations between the cytokines suggest that these are unlikely to be spurious, and also attest to the robustness of the multiplex cytokine assay system. Although our group of healthy control subjects was relatively small (n=10), their low levels of circulating cytokines are comparable with those reported from a much larger series of healthy subjects (Ibelgauft 2010).

In keeping with the increasing recognition of the involvement of IL-7 in orchestrating chronic inflammation in the rheumatoid joint (Harada et al. 1999, van Roon et al. 2003b, Hartgring et al. 2006, Churchman, Ponchel 2008, van Roon, Lafeber 2008), this cytokine was found to be highly correlated with IL-12 in particular, as well as with IFN- γ . Synovial fibroblasts, activated by IL-1 β and TNF, as well as macrophages, are likely to be the major source of IL-7. Interestingly, the predominant lymphocyte subset in the rheumatoid joint has been reported to be of the Th1 effector-memory phenotype, expressing functional IL-12R, IL-18R α and CCR5 (Sattler et al. 2009). These cells are directly activated to secrete IFN- γ by cytokines such as IL-2, IL-7,

and IL-15, but not IL-4, which signal via the IL-2R common γ -chain in combination with IL-12 and IL-18, independently of TCR ligation (Sattler et al. 2009).

Circulating IFN- γ was strongly correlated with the predominantly macrophage-derived cytokines IL-1 β , IL-12, TNF, and G-CSF. This is in keeping with the classically, IFN- γ -activated macrophage of the M1 phenotype being a major source of pro-inflammatory cytokines in the inflamed RA joint (Vandooren et al. 2009). Of the 3 chemokines measured, IL-8, CCL2, and CCL4, only CCL2 showed moderate correlations with the various Th1/Th2, macrophage and fibroblast cytokines in the total cohort, possibly reflecting the predominantly macrophage/fibroblast origins of this monocyte-targeted chemokine.

Circulating VEGF, which has been reported to predict disease severity and progression in RA (Taylor 2005, Carvalho, Blank & Shoenfeld 2007, Ozgonenel et al. 2010), correlated poorly with the other cytokines with the exceptions of IL-7 in particular, and IL-12, possibly reflecting the fibroblast and macrophage origins respectively of VEGF (Hong et al. 2007, Watari et al. 2008). However, when the total cohort of RA patients was subdivided into those with MDA and HDA, significant correlations were observed between VEGF and the Th1/Th2/macrophage/fibroblast cytokines, as well as with RF, anti-CCP and anti-MCV in the HDA group, substantiating the role of VEGF as a predictor of severity of disease in this cohort.

With respect to correlations of the Th1, Th2, Th17, monocyte/macrophage and fibroblast cytokines, with traditional biomarkers of disease activity in the total cohort, RF correlated moderately with IL-1 β , IL-4, IL-7, IL-12, G-CSF, GM-CSF, IFN- γ , and TNF, with weaker, but nonetheless significant correlations detected between these same cytokines, and anti-CCP and anti-MCV. Not surprisingly, the acute phase

reactants, CRP and SAA, correlated best with IL-6, while weak correlations were evident with most of the other cytokines. Given the strong association of RF, anti-CCP and anti-MCV with disease severity and poor prognosis in RA (Zendman, Vossenaar & van Venrooij 2004, Raza et al. 2005, Soos et al. 2007, Mathsson et al. 2008), these findings are compatible with the involvement of the Th1 lymphocyte/macrophage/fibroblast axis in the immunopathogenesis of RA (Berner et al. 2000, Yamada et al. 2008). This contention is underscored by the significant correlations of IL-1 β , IL-1Ra, TNF, IFN- γ and GM-CSF with the DAS28 score observed in the HDA subgroup. Although a role for Th2 cells in disease pathogenesis cannot be excluded, the association of the Th2 cytokines, IL-4 and IL-10, with RF, anti-CCP and anti-MCV most likely reflects their anti-inflammatory activities. A dynamic interaction between the production of Th1 and Th2 cytokines with opposing pro- and anti-inflammatory activities may regulate the rate and severity of damage to cartilage and bone in established RA. This in turn may explain the relatively weak correlations of acute phase reactants with cytokines (except IL-6) and autoantibodies.

The limitations of the current study include the focus on circulating, as opposed to synovial cytokines (van den Ham et al. 2009), as well as the recruitment of patients with established, rather than very early disease. In defence of these strategies, measurement of synovial cytokines presents logistical and ethical issues and is relatively impractical from a laboratory diagnostic perspective, while identification of patients with very early disease is particularly difficult in the health care setting of a developing country. The strengths of the study, on the other hand, include the relatively large number of patients recruited to the study, all of whom were DMARD-

naïve, as well as the range of circulating markers of disease activity which were evaluated.

In conclusion, the current study has demonstrated a circulating cytokine profile in patients with established RA which appears compatible with activation of the Th1 cell/macrophage/fibroblast axis and a possible counter-regulatory role of Th2 cells. This is underscored by relatively strong correlations between cytokines and established biomarkers, especially RF, as well as anti-CCP and anti-MCV antibodies. IL-1 β , IL-1Ra, TNF, IFN- γ , GM-CSF and VEGF in particular were significantly associated with HDA, and show promise as adjunctive diagnostic/prognostic biomarkers, either individually or in combination. The clinical utility of this strategy, will, however depend on the outcome of large, multi-centre follow-up studies.

Chapter 3: HLA-DRB1 shared epitope genotyping using the revised classification and its association with circulating autoantibodies, acute phase reactants, cytokines, and clinical indices of disease activity

3.1. Introduction

Rheumatoid arthritis (RA) is a debilitating autoimmune disease that has no clearly defined aetiology, although there is a definite genetic predisposition and associated risk factors (Pratt, Isaacs & Matthey 2009). The shared epitope (SE) concept in relation to genetic predisposition was first described in 1986 and has evolved from the classic HLA-DRB1*01, - *04, and -*10 association (Nepom et al. 1986, Winchester 1992, Winchester 1994), to the identification of the RAA amino acid motif at positions 72-74 of the third hypervariable region of the different HLA-DR β chains as being the definitive SE (Gregersen, Silver & Winchester 1987, Winchester 1992, Winchester 1994). This concept has been extended by Gao et al. to include the amino acid residues at positions 71 and 76, and, recently, to a new classification which incorporates the modulatory activities of the amino acids at positions, 70 and 71, in addition to the RAA motif at positions 72-74 (Gao et al. 1991, du Montcel et al. 2005, Michou et al. 2006).

Although the primary triggering autoantigen(s) in RA has not been described, it is noteworthy that associations between the various HLA-DRB1 SE subtypes with disease susceptibility/ severity and the presence of circulating anti-citrullinated peptide antibodies have been described (van Boekel et al. 2002, Schellekens et al. 1998, Raza et al. 2005, Matthey et al. 1999, Hill et al. 2003, Auger et al. 2005,

Huizinga et al. 2005, Samanci et al. 2005, de Vries, Huizinga & Toes 2006, Quinn et al. 2006). In addition, HLA-DRB1 SE genotyping and measurement of anti-CCP, and to a lesser extent rheumatoid factor (RF), have the potential to predict future development of RA (Mattey et al. 1999, Huizinga et al. 2005, Huizinga et al. 2005, de Vries, Huizinga & Toes 2006, Barnetche et al. 2008, Okada et al. 2009, Gyetvai et al. 2010). Taken together, these associations between HLA-DRB1 SE genotype, anti-CCP, and disease susceptibility/ severity appear to be compatible with the presentation of citrullinated autoantigens by HLA-DRB1 SE subtypes as an immunopathogenic mechanism in RA.

While not having any diagnostic specificity, measurement of circulating cytokines/ chemokines and acute phase reactants, when combined with detection of anti-CCP/ RF, has the potential to predict the time to onset of clinical disease (Deane et al. 2010, Kokkonen et al. 2010), as well as disease severity (Hitchon et al. 2004, Alex et al. 2007, Chen et al. 2009, Boissier). Nonetheless, relatively few studies have undertaken a composite analysis of SE genotyping, and measurement of circulating anti-CCP, cytokines/ chemokines, and acute phase reactants as a strategy not only to identify interactions between these alleles/ biomarkers, but also to establish which combinations of these are most strongly associated with disease severity. These issues have been addressed in the current study in a cohort of predominantly African patients with RA of ≤ 2 years duration. This is the first study to address the occurrence of the various SE subtypes according to the du Montcel classification in this population.

3.2. Patients

The cohort of patients (n=143) is the same as described in Chapter 2, 2.2.1, page 55.

Demographic data of this cohort is shown in Table 3.1.

Parameters	Quintiles				
	Mean ±SD	Q1 (p25)	Q2 (Median)	Q3 (p75)	Range
Age (years)	48±12	41	48	56	20-75
Female (%)	118(82.5)				
Ethnic origin: Black (%)	125(87)				
DAS28-CRP	5.6±1.3	4.8	5.7	6.7	2.3-8.7
Disease duration (months)	12±7	6	10	18	0-25
Radiographic scores	23±14	14	19	28	1-66
RF (IU/ml)	490±795	38	168	595	4-5350
anti-CCP (U/ml)	669±636	85	492	1099	2-2527
anti-MCV (U/ml)	608±697	104	327	781	8-3088
CRP (mg/L)	24±32	5	14	32	0-198
SAA (µg/ml)	62±123	5	14	65	1-882

Table 3.1: Demographic, laboratory, and clinical data for the group of RA patients (n=143).

3.3. Methods

3.3.1. Samples

Venous blood was collected and stored as described in Chapter 2, 2.2.2., page 55. Genomic DNA was obtained using the Promega Maxwell[®] Personal Automation[™] System with the Maxwell[®] 16 Blood DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA) for extracting DNA from whole blood, collected in EDTA sample tubes. The yield and purity of DNA extracted from EDTA blood samples was determined spectrophotometrically at 260/320nm and 260/280nm respectively and the concentration of DNA adjusted to 50ng/μl as required.

3.3.2. Autoantibodies and acute phase reactants:

These were assayed as described in Chapter 2, 2.2.3. by Nephelometry, 2.2.4. Fluorescent Enzyme Immuno-Assay and 2.2.5. ELISA on page 56 to 57.

3.3.3. Serum cytokines, chemokines and growth factors:

These were assayed as described in Chapter 2, 2.2.6. by Luminex Bead Array Multiplexing, page 58.

3.3.4. Typing of HLA-DRB1 alleles

The patients' HLA-DRB1 alleles were typed using a DNA-based high resolution typing method, LABType[®] HD DRB1 (One Lambda Inc, Canoga Park, California, USA), utilising reverse sequence specific oligonucleotide (rSSO) Luminex xMAP[®] technology probes. Firstly, the target DNA (HLA-DRB1 gene) is amplified by PCR

using group specific primers that are biotinylated for detection with SAPE (R-Phycoerythrin-conjugated Streptavidin). The PCR product is then denatured and hybridised to complementary DNA probes conjugated to fluorophores. Bound DNA is detected on the Luminex system by addition of SAPE, and software maps the reaction patterns to those associated with published HLA gene sequences, and assigns the represented HLA-DRB1 alleles. Firstly, HLA alleles were assigned according to the RAA amino-acid sequence at positions 72-74 as shared epitope (S) and non-SE (X), and the S group further subdivided according to amino-acid residues at positions 70 and 71 as described by du Montcel (see Table 3.2.). (du Montcel et al. 2005)

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

Table 3.2: Shared Epitope classification according to amino acid sequence at

positions 70-74 (du Montcel et al. 2005)

3.3.5. Statistical methods:

Descriptive and inferential statistics techniques were used in the analyses. Tests for association of contingency tables were performed using two-tailed Chi-square with Yates continuity correction. One-way ANOVA was performed using the Kruskal-Wallis test for non-parametric data for more than 2 groups, or the Mann-Whitney test when 2 groups were compared. Logistic regression was used for binary outcome variables, reporting the odds ratio (OR) and corresponding 95% confidence intervals. Statistical significance was determined by $p\text{-value} < 0.05$. The analyses were done using STATA statistical software.

3.4. Results

3.4.1. HLA-DRB1 frequencies

The frequencies (%) of the various HLA-DRB1 alleles typed in the cohort of patients are shown in Table 3.3 (page 80). The “classical” RA-associated HLA-DRB1*01, -DRB1*04 and -DRB1*10 alleles showed increased odds ratios with HLA-DRB1*01 HLA-DRB1*04 being statistically extremely significant and HLA-DRB1*10 significant (see Table 3.4., page 81), underscoring the connotation of the HLA-DRB1 shared epitope previously reported in the African population (Mbayo et al. 1998, Meyer, Brighton & Anderson 2004).

HLA-DRB1 antigens identified	Frequencies (%)		
	RA patients (n=143)	Black Africans*	Caucasians**
HLA-DRB1*01	9.1	7.4 (5.1-9.4)	7.7 (5.5-10.0)
HLA-DRB1*03	18.6	18.7 (10.8-31.3)	11.5 (9.0-14.0)
HLA-DRB1*04	22.4	9.2 (5.1-12.8)	15.8 (10.5-20.0)
HLA-DRB1*07	3.4	13.4 (11.3-16.7)	7.6 (6.0-9.2)
HLA-DRB1*08	2.7	6.1 (3.7-8.9)	4.9 (4.8-5.0)
HLA-DRB1*09	0.7	1.8 (1.3-2.8)	1.9 (1.96-2.0)
HLA-DRB1*10	3.5	2.8 (1.3-4.1)	1.5 (1.0-2.0)
HLA-DRB1*11	11.8	24.4 (13.4-36.6)	11.4 (7.0-15.7)
HLA-DRB1*12	4.4	6.6 (2.3-11.0)	3.7 (3-4.4)
HLA-DRB1*13	13.5	24.7 (10.2-34.8)	15.2 (14.3-16.0)
HLA-DRB1*14	0.3	2.5 (0.9-4.9)	2.3 (1.6-3.0)
HLA-DRB1*15	8.1	13.9 (10.7-20.2)	13.5 (9.9-17.0)
HLA-DRB1*16	1.4	1.7 (0.6-3.6)	5.7 (1.3-10.0)

* References (Marsh, Parham & Barber 2000, Pirie et al. 2001, Tikly et al. 2004, Lulli et al. 2009).

** References (Marsh, Parham & Barber 2000, Lulli et al. 2009).

Table 3.3: *HLA-DRB1 Frequencies of RA patients*

Risk Allele	RA Patients, n=143, n (%)	Controls* n=1104 n (%)	OR (95% CI)	X ²	P-value
HLA- DRB1*01	26 (18.2)	93(8.4)	2.4 (1.5-3.9)	12.9	0.0003
HLA- DRB1*04	61 (42.7)	41(3.7)	19.3 (12.2-30.4)	250.5	<0.0001
HLA- DRB1*10	10 (7.0)	36(3.3)	2.2 (1.1-4.6)	4.0	0.0464

*New Allele Frequency Database - Allele, haplotype and genotype frequencies in Worldwide Populations: <http://www.allelefreqencies.net>. (Middleton et al. 2003)

Table 3.4: *Known HLA-DRB1 RA-associated alleles, odds ratios and confidence intervals*

When the HLA-DRB1 alleles are classified according to the new classification as being homozygous for shared epitope alleles (SS), heterozygous (SX) and no SE alleles (XX) present, the majority of the patients (92%) had at least 1 allele associated with the amino acid motif. More than half of the patients (57%) typed were homozygous, 35% were heterozygous (SX) and only 8% had no associated HLA-DRB1-allele (XX). The frequencies of SS, SX and XX for the total group of patients, as well as the subdivisions according to race are shown in Table 3.5., page 82. Other than African blacks, the other population groups were not meaningfully represented.

	Total		African		Coloured		Indian		Caucasian	
	%	n	%	n	%	n	%	n	%	n
SS	57	82	58	73	50	3	80	4	29	2
SX	35	50	35	43	17	1	20	1	71	5
XX	8	11	7	9	33	2	-	-	-	-

Table 3.5: Frequencies of HLA-DRB1 alleles associated with the amino acid motif (RAA) of the 3rd hypervariable region of the different HLA-DRB chains.

3.4.2. Relationship of homozygous and heterozygous SE classification with circulating biomarkers of disease activity.

As X alleles have been shown to be under-transmitted (see Table 3.6, page 83) in the RA cohort (i.e. non-carriers of risk alleles), they were used as the control group within the RA cohort to determine the possible associations of HLA-DRB1 genotype with RA-associated autoantibody production i.e. by comparing the total positive and negative results for the RA-associated antibodies (RF, anti-CCP and anti-MCV) in the risk groups with the control group (XX). Anti-CCP antibody production in both the SS and SX groups was significantly elevated when compared to the control group (OR= 10.21 and 9.18, respectively, $p < 0.0001$). RF was significantly elevated only in the SS group (OR=7.00, $p = 0.0059$), while anti-MCV was not significantly different between the groups (Table 3.7, page 85).

The SS and SX groups of the African patients showed a statistically significant predisposition to anti-CCP seropositivity (OR= 7.05 and 6.45, $p = 0.0121$ and $p = 0.0223$, respectively, Table 3.7, page 85), when compared with the XX group. Neither RF nor anti-MCV of either the SS or SX groups showed any significant predisposition compared to the XX control group. A previous study, which did not

include HLA-DRB1 genotyping, reported that anti-CCP was no better in identifying RA than RF (Hodkinson et al. 2010). Categorising the patients according to du Montcel's allele classification, as either homozygous (SS), heterozygous (SX) or non-allele associated (XX), seems, however, to strengthen the genetic association with the presence of anti-CCP, in agreement with the findings of Huizinga and Klareskog (Huizinga et al. 2005, Klareskog et al. 2008). Further analysis according to amino acids at positions 70-71 (S1, S2, S3D and S3P), revealed that only 2 groups showed a significant predisposition to higher seropositivity rates of RF (S2) and anti-CCP (S3P) (OR=4.47 and 3.04, p=0.0475 and 0.0327 respectively).

Allele	RA patients, n=143, n (%)	Controls, n=1104*, n (%)	OR (95% CI)	X²	P-value
S1	65(46)	853(77)	0.5(0.4-0.6)	26.9	<0.0001
S2	33(23)	110(10)	2.5(1.7-3.9)	20.7	<0.0001
S3D	43(30)	316(29)	1.1(0.8-1.5)	0.1	0.8116
S3P	70(49)	313(28)	2.0(1.5-2.6)	19.9	<0.0001
X	75(52)	616(53)	1.1(0.9-1.5)	0.6	0.4445

Table 3.6: Comparison of allele carrier frequencies in RA cases and controls in accordance with the du Montcel classification.

* References (Middleton et al. 2003)

A general trend of higher mean values in the SS groups compared to the SX and XX was observed with the RA-associated autoantibodies, acute phase reactants, and most cytokines (IL-1b, IL-2, IL-4, IL-6, IL-8, IL-12, CCL4, G-CSF, GM-CSF, IFN- γ and IL1-Ra). (Table 3.8., page 86). A one-way ANOVA analysis revealed that the variances in medians of RF (p=0.0333), aCCP (0.0298), CRP (p=0.0041), SAA (p=0.0018), TNF (p=0.0295) and DAS28 (p=0.0324) were significantly different within

these groups. Significant differences in mean values were noted for RF (SS and SX vs. XX), CRP/SAA (SS vs. XX), TNF (SS and SX vs. XX), IL-1Ra (SS vs. XX) and DAS28 (SS and SX vs. XX) as shown in Table 3.8, page 86.

3.4.3 Associations between the new classification alleles and biomarkers of disease activity.

Classification of RA-associated HLA-DRB1 alleles according to du Montcel revealed extremely significant associations and odds ratios (OR) with S1, when compared to allele frequencies found in the general sub-Saharan population (Meyer et al. 2007). S2 and S3P, were over-expressed in the study population of RA patients compared to the general population in sub-Saharan Africa. Interestingly, S1 had a odds ratio of 0.5 showing that this sub-allele expression is suppressed in the study cohort. S3D alleles in the RA cohort were not significantly increased. The non-epitope associated alleles (X) were not significantly different in the RA groups compared to the general sub-Saharan population. Frequencies, odds ratios and 95% confidence intervals (CI) are given in Table 3.6 (page 83).

Because the OR of the non-allele carrier group (X) was not significantly increased, as well as the OR in the S3D group (Table 3.6, page 83), patients having any of these groups (i.e. X/X, S3D/S3D and S3D/X) were assigned an L designation (see Table 3.9, page 87 for frequencies of allele phenotype) and used as a control group to determine if RA-associated autoantibodies are significantly over-expressed in the allele-associated high-risk subgroups. The only 2 groups which showed any over-expression were the S2 group with RF (OR (95%CI) =4.5 (1.0-21.1), p=0.0475) and S3P with anti-CCP (OR (95%CI) =3.0(1.1-8.5), p=0.0327).

	Total cohort (n=143)				Blacks (n=124)			
	SS(n=80)		SX(n=52)		SS(n=58)		SX(n=35)	
	OR(95%CI)	p	OR(95%CI)	p	OR(95%CI)	p	OR(95%CI)	p
RF	7.0(1.8-26.6)	0.0059	6.3(1.5-25.7)	0.0126	4.5(1.0-19.5)	0.0537	4.1(0.88-19.28)	0.0813
anti-CCP	10.2(2.6-40.3)	0.0010	9.2(2.2-38.9)	0.0028	7.1(1.6-30.4)	0.0121	6.4(1.4-30.1)	0.0223
anti-MCV	0.53(0.0-4.5)	1.000	2.4(2.6-28.5)	0.4600	0.24(0.0-4.30)	0.3419	0.87(0.0-19.7)	1.000

Table 3.7: *HLA-DRB1* alleles' effect on predisposing RA-associated autoantibodies*

Bio-marker	Groups			1-way ANOVA	Mann-Whitney		
	SS n=82 Mean (Median)	SX n=50 Mean (Median)	XX n=11 Mean (Median)	p (Kruskal-Wallis)	p SS vs XX	p SX vs XX	p SS vs SX
RF	539(187)	377(177)	565(10)	0.0333	0.0129	0.0273	ns
CCP	709(594)	587(288)	409(6)	0.0298	ns	ns	ns
CRP	26(19)	16(7)	32(6)	0.0041	ns	ns	0.0012
SAA	69(24)	36(6)	124(15)	0.0018	ns	ns	0.0005
IL-1b	33(7)	17(4)	6(3)	0.5185	ns	ns	ns
IL-1Ra	504(101)	233(69)	61(32)	0.0600	0.0311	ns	ns
IL-2	72(0)	26(0)	4(0)	0.5109	ns	ns	ns
IL-4	41(4)	17(3)	8(0)	0.3905	ns	ns	ns
IL-6	78(28)	48(17)	34(8)	0.1380	ns	ns	ns
IL-7	225(21)	223(18)	109(42)	0.8561	ns	ns	ns
IL-8	489(10)	17(9)	12(8)	0.6295	ns	ns	ns
IL-10	37(12)	60(11)	8(8)	0.3612	ns	ns	ns
IL-12	140(13)	114(11)	11(9)	0.3513	ns	ns	ns
IL-17A	9(0)	3(0)	0(0)	0.1228	ns	ns	ns
CCL2	112(62)	63(50)	31(2)	0.0846	ns	ns	ns
CCL4	146(113)	117(95)	101(108)	0.6428	ns	ns	ns
G-CSF	284(12)	143(16)	98(1)	0.3174	ns	ns	ns
GM-CSF	158(0)	58(0)	20(0)	0.3668	ns	ns	ns
IFN-γ	787(51)	356(28)	219(1)	0.1704	ns	ns	ns
TNF	185(13)	118(12)	13(4)	0.0295	0.0145	0.0324	ns
VEGF	512(171)	293(129)	645(247)	0.1074	ns	ns	ns
DAS28	6.3(6.3)	6.1(6.0)	4.7(4.1)	0.0324	0.0103	0.0210	ns
HAQ	14(15)	12(12)	10(10)	0.1518	ns	ns	ns
LARSEN	23(17)	22(19)	28(23)	0.2464	ns	ns	ns
AGE	50(50)	46(47)	46(46)	0.3440	ns	ns	ns
DURATION	12(11)	12(10)	8(7)	0.1515	ns	ns	ns

Table 3.8. Mean and median values of circulating biomarkers and clinical indices of disease activity in relation to SE homozygosity / heterozygosity.

Eight groups were established, as indicated in Table 3.9, according to the classification of Du Montcel. The combined, non-statistically significant phenotype was assigned L/L. The S1/S2 and S2/S2 groups were amalgamated into 1 group and renamed the S1/ or S2/S2, as well as S2/S3P and S3P/S3P which were renamed S2/ or S3P/S3P, to ensure more statistically representative totals.

Phenotyping	Ethnicity				Grand Total
	African	Coloured	Indian	White	
L/L	25	2	0	1	28
S1/L	19	1	0	1	21
S1/S1	10	0	1	0	11
S1/ or S2/S2	13	1	1	0	15
S1/S3P	13	1	0	0	14
S2/L	11	0	0	0	11
S2/ or S3P/S3P	13	0	1	1	16
S3P/L	21	1	2	4	28
Grand Total	125	6	5	7	143

Table 3.9: Phenotyping frequency of the RA cohort according to du Montcel

When comparing the overall mean values for various biomarkers (Table 3.10, page 88) the majority of those biomarkers with the highest mean values, were found in the homozygous groups representing the SE-associated alleles. This is the case with the RA-associated autoantibodies, acute phase reactants, and certain cytokines, predominantly Th1, Th2, as well as chemokines (CCL4, IL-2, IL-4, IL-6, IL-8, G-CSF, GM-CSF and IFN- γ). The group L/L, consisting of the non-associated (X) and non-significantly represented (S3D) alleles did not include a highest mean value for any biomarker, the mean values in this group being at the lower end of the range overall. Where the mean values were higher in a group containing only a single RA-associated SE allele, these were found in the groups containing S2 or S3P alleles,

known to be associated with disease severity. The two exceptions are IL-17A and VEGF, which fall under the S1/L grouping.

	<i>L/L</i>	<i>S1/L</i>	<i>S2/L</i>	<i>S3P/L</i>	<i>S1/S1</i>	<i>S1/S3 P</i>	<i>S1/ or S2/S2</i>	<i>S2/ or S3P/S 3P</i>
RF	483	647	365	420	168	718	695	357
anti-CCP	626	650	404	552	440	730	963	845
anti-MCV	529	658	443	476	264	763	974	790
CRP	25	15	22	13	29	25	32	36
SAA	82	37	55	30	41	39	68	140
IL-1b	20	27	62	11	36	48	10	24
IL-1Ra	281	364	702	197	647	495	202	507
IL-2	31	63	51	10	194	42	15	37
IL-4	12	57	32	17	108	44	8	16
IL-6	65	48	125	37	136	47	63	64
IL-7	92	150	320	404	398	221	85	140
IL-8	16	18	18	15	20	110	16	66
IL-10	16	32	38	107	25	34	25	30
IL-12	33	173	127	208	193	83	58	111
IL-17A	3	23	2	1	9	10	2	1
CCL2	43	116	299	50	80	82	55	91
CCL4	102	177	184	91	98	209	146	121
G-CSF	135	277	213	92	1116	158	127	128
GM-CSF	78	131	133	39	415	143	42	130
IFN-γ	328	943	730	278	1473	856	192	739
TNF	81	184	582	78	230	134	49	105
VEGF	433	735	234	327	232	501	606	410
Units of measurement:								
RF (IU/ml); anti-CCP & anti-MCV (U/ml); CRP(mg/L); SAA(μg/ml); Cytokines, chemokines & growth factors (pg/ml)								

Table 3.10: Mean values of biomarkers according to the du Montcel phenotype classification with the highest score per marker in bold.

The clinical markers also followed the same pattern with the highest means being predominantly located in the homozygous SE-associated allele groups, more

specifically in groups S2 and S3P which are associated with disease severity. The L/L group is again devoid of any highest mean value. S2/L was the only heterozygous group in which a high mean value was observed. One-way ANOVA analysis of the clinical markers, as shown in Table 3.11, page 90, indicated that only the DAS28 group demonstrated significantly different variances of the medians ($p=0.0250$) within the groups. The significant groups within the DAS28 grouping were S1 or S2/S2 which differed significantly from S1/L ($p=0.0073$), S2 or S3P/S3P with S1/L ($p=0.0108$), S1/S3P with S1/L ($p=0.01240$) and S1/S1 from S1/L ($p=0.0557$).

	<i>Larsen Score (Mean)</i>	<i>HAQ (Mean)</i>	<i>Das28-4 CRP (Mean)</i>	<i>SJC (Mean)</i>	<i>TJC (Mean)</i>	<i>Patent Global Score (Mean)</i>	<i>Duration in months (Mean)</i>	<i>% Erosion</i>
<i>L/L</i>	23.6	11.1	5.6	10.0	15.0	66.0	10.4	54.5
<i>S1/L</i>	22.8	14.8	5.1	8.2	9.0	70.0	10.7	53.3
<i>S1/S1</i>	15.6	9.6	5.9	9.6	16.9	55.0	13.8	37.5
<i>S1/ or S2/S2</i>	31.3	14.3	6.2	11.8	15.3	81.7	10.2	87.5
<i>S1/S3P</i>	15.4	13.5	6.2	11.1	15.7	82.1	14.8	20.0
<i>S2/L</i>	19.8	15.1	5.8	12.5	15.5	68.2	15.5	40.0
<i>S2/ or S3P/S3P</i>	29.0	14.2	6.0	9.0	15.2	78.3	9.1	77.8
<i>S3P/L</i>	23.1	11.9	5.3	9.4	10.8	68.5	12.7	52.2

Table 3.11: Mean values of clinical markers with the 2 highest scores per marker in bold.

3.5. Discussion

Relatively few studies have been undertaken to evaluate the frequencies of the RA-associated HLA-DRB1 alleles in southern African peoples suffering from this disease. The results of the current study, using high-resolution typing procedures in combination with the du Montcel HLA-DRB1 SE classification (du Montcel et al. 2005), have revealed RA risk-associated allele frequencies in a population of predominantly black South African females with RA which are comparable to or higher than those reported in European and Japanese populations (Barnetche et al. 2008, Okada et al. 2009, Gyetvai et al. 2010). In agreement with an earlier study using low-resolution PCR typing procedures, HLA-DRB1*04 conferred the most significant risk in a cohort of black South African RA patients (Meyer, Brighton & Anderson 2004). According to the du Montcel classification, highest risk was associated with the S2 and S3P alleles. Recently, Barnetche et al. reported on the association between RA susceptibility and HLA-DRB1 alleles (classified according to du Montcel) in a combined analysis of worldwide samples (1210 cases of RA) which included 23 San people of southern African origin (Barnetche et al. 2008). Although the number of cases was small, 52.2% of these were found to be carriers of the S2 RA susceptibility allele, relative to 26% of the control group, with an OR of 3,05 (Barnetche et al. 2008). In addition, and also in agreement with previous studies performed in European and Japanese RA cohorts (Barnetche et al. 2008, Engelmann et al. 2010), we also observed highly significant associations of SE allele homozygosity and heterozygosity with seropositivity for anti-CCP in particular, and to a lesser extent, RF. Although it may seem surprising that aMCV does not show the

same associations as aCCP with SE, this is probably due to a lack of specificity / sensitivity of the MCV test.

These observations appear to confirm the relationship between HLA-DRB1 SE subtypes, presentation of citrullinated epitopes, and development of RA (Szodoray et al. 2010). This contention is further supported by the broad associations of non-specific circulating biomarkers of disease activity (acute phase reactants, Th1/Th2/macrophage/fibroblast-derived cytokines) with the du Montcel risk phenotypes, with S1/S1 being more strongly associated with Th1 (GM-CSF, IFN- γ , IL-2) and Th2 (IL-4, IL-6) cytokines than the other risk groups. The results of the previous chapter have demonstrated that RA is associated with a generalised increase in circulating pro-inflammatory and anti-inflammatory cytokines/chemokines, which appears to be compatible with Th1 cell/macrophage/fibroblast activation and a possible counter-regulatory role of Th2 cells as also recently reported by others (Deane et al. 2010, Kokkonen et al. 2010). The highest clinical indices of disease activity were also noted in the high risk groups.

The findings presented in the current chapter are, however, somewhat at variance with those recently reported by Singwe-Ngandeu et.al. in a relatively small group (n=56) of Cameroonian RA patients. Thirty percent of RA patients were either homozygous or heterozygous for the SE alleles, relative to frequencies of 10% and 14% in patients with other inflammatory rheumatic diseases and healthy controls respectively. However, no significant associations between SE positivity (homozygous/heterozygous) and anti-CCP or RF were detected (Singwe-Ngandeu et al. 2010). There are several possible reasons which may explain the differences between this and the current study. Notwithstanding the relatively small number of

patients, these include differences in the number of alleles typed (21 versus 49 in the Singwe-Ngandeu et al. and current studies respectively), and, most importantly, the effects of chemotherapy. While the patients in the current study were corticosteroid- and DMARDs-naive at the time of presentation, 91%, 77%, 12%, 5%, 5% and 2% of patients in the Singwe-Ngandeu et.al. study were receiving, prednisone, methotrexate, sulfasalazine, azathioprine, leflunomide, and D-penicillamine, which may explain the absence of associations of SE alleles with anti-CCP and RF, but is unlikely to explain the differences in frequency of SE alleles detected in the two studies. Aside from the larger number of alleles typed in the current study, it is also possible that our patients were representative of those at the more severe extreme of the disease spectrum. In the healthcare setting of the developing world, presentation of patients at specialised RA clinics, of which there are few, is likely to be delayed.

In conclusion, the results of the current study demonstrate an elevated frequency of high-risk SE alleles in a predominantly black South African population with RA as well as a clear association with anti-CCP positivity and, to a lesser extent with RF and circulating cytokines. The application of the du Montcel classification in this study population also underscores the association of non-specific circulating biomarkers and clinical indices of disease activity with SE subtype. Classification of RA patients on presentation according to the du Montcel classification, together with the measurement of anti-CCP and RF, and certain circulating cytokines (i.e. TNF and VEGF), may identify those patients who would benefit from implementation of early aggressive chemotherapy.

Chapter 4: Brief overview of the effects of 6 months therapy with DMARDs on the concentrations of circulating cytokines and anti-CCP in the group of RA patients.

4.1. Introduction

Although not a component of this thesis, 6 and 12 month follow-up studies have been / are being undertaken in the group of RA patients. Because completion of the baseline studies has coincided with near completion of the 6 month follow-up investigations, a summary of these is presented in this chapter (RF, CRP, SAA not yet completed).

4.2. Results

Circulating cytokines and anti-CCP at baseline and after 6 months of chemotherapy in the group of RA patients.

These results are shown in Table 4.1 (page 95). Six months treatment with DMARDs was associated with significant decreases in the concentrations of IL-7, IL-8, VEGF and CCL4, while those of G-CSF and IFN- γ were also decreased, though not significantly so. Interestingly, the circulating concentrations of IL-1Ra, and to a lesser extent IL-10, were elevated following 6 months of therapy, but these increases did not attain statistical significance. With respect to anti-CCP, the mean serum concentration decreased significantly after 6 months of treatment.

Cytokines	Baseline n=143			Six Months n=133			% Change	
	Mean	STDEV	Median	Mean	STDEV	Median	Mean	Median
IL-1b	25	50	4	18	41	3	28	18
IL-1ra	363	796	72	560	1485	76	-54	-87
IL-2	41	148	0	17	47	0	59	68
IL-4	37	119	3	7	13	1	81	89
IL-6*	63	129	18	58	124	13	8	4
IL-7**	251	830	25	70	333	17	72	60
IL-8**	293	3057	9	34	156	6	88	95
IL-10	42	131	11	57	189	8	-36	-44
IL-12	144	490	11	139	729	16	3	-49
IL-17	6	24	0	4	16	0	33	33
G-CSF	252	912	14	60	162	6	76	82
GM-CSF	115	383	0	115	328	0	0	14
IFN- γ	618	1789	27	407	901	19	34	50
TNF	141	426	10	133	375	11	6	12
VEGF**	489	725	177	200	464	93	59	36
CCL2	84	192	48	88	111	48	-5	42
CCL4**	130	92	108	97	59	96	25	36

	Baseline n=143			Six Months n=88			% Change	
	Mean	STDEV	Median	Mean	STDEV	Median	Mean	Median
anti-CCP**	669	636	492	402	413	275	40	35

* p<0.1

** p<0.05

Table 4.1: Difference in cytokine means, medians and standard deviations from baseline to 6 months after commencing DMARDs treatment

4.3. Discussion

A preliminary evaluation of the cytokine and anti-CCP results of the RA patient cohort data obtained from the 6 months post DMARDs treatment sera collected, showed an overall reduction of mean and median values for most biomarkers. The most notable exceptions being IL-1Ra and IL-10, which is in keeping with previous findings in relation to methotrexate (MTX) therapy, which up-regulates the expression of the

genes encoding IL-4 and IL-10, while also increasing the production of IL-1Ra (Cutolo et al. 2001).

IL-6 was decreased, but not significantly so ($p=0.0951$), after the first 6 months of DMARDs treatment. The lower than expected suppression of IL-6 could be due to the high-disease activity of the RA-patients in the cohort, as shown by the high percentage positivity of RF and anti-CCP observed in sera at baseline associated with high DAS28 scores. CCL4 was significantly reduced ($p=0.0378$) in the 6 months' follow-up analysis, suggesting that MTX treatment has an inhibitory effect on the production of CCL4, probably by promoting gene expression of anti-inflammatory cytokines, and by the up-regulation of extracellular adenosine, a potent anti-inflammatory agent, leading to the overall down-regulation of the inflammatory cascade, (Cutolo et al. 2001).

IL-7 was significantly reduced ($p=0.0374$) after 6 months of MTX-treatment. One of the effects of IL-7 in the synovium is the activation of osteoclasts, which in turn are responsible for cartilage and joint degradation. The reduction in IL-7 levels may, in part, contribute to the overall perception of well-being of RA patients, and retardation of joint inflammation after commencement of MTX treatment.

MTX is a folic acid analogue; it binds more tightly than folic acid to dihydrofolate reductase (DHFR) and thus reduces the production of folates, which are important as co-factors in various enzymatic pathways. The depletion of folates results in inhibition of the production of DNA and RNA, leading to cell death, especially of actively proliferating cells such as malignant cells. At lower doses, MTX causes the release of adenosine at sites of inflammation, which is effected by intracellular accumulation of AICAR (5-aminoimidazole-4-carboxamide ribonucleotide). The extracellular

adenosine acts predominantly on the adenosine A_{2a} receptors which increase intracellular cAMP (cyclic adenosine monophosphate). Increased cAMP mediates the inhibition of the proinflammatory activity of neutrophils (and other types of inflammatory cells), depleting their ability to adhere to endothelial cells and fibroblasts. It also suppresses the secretion of TNF, IFN- γ , IL-2, and IL-12, as well as HLA expression. MTX also inhibits the production of IL-8 and leukotriene B₄ (Cutolo et al. 2001, Wallace 2002). It is therefore not surprising that IL-8 median levels of the RA patients decreased very significantly ($p=0.0010$) by 95% (Table 4.1, page 95).

Median VEGF concentrations, in RA patients' sera after 6 months of MTX treatment were very significantly reduced by more than 50% (36%, $p=0.0003$). This can be attributed to the effect of MTX on macrophages, which, together with fibroblasts are the major source of VEGF production. MTX has been reported to reduce monocyte/macrophage cell growth and to promote apoptosis in these cells (Cutolo et al. 2001). This is of potential importance, since as shown in chapter 2, high levels of VEGF at baseline correlate well with other biomarkers and clinical indicators in a sub-group of patients with high disease activity. This supports the case for inclusion of VEGF as a screening procedure to detect severe RA disease on presentation, as it is not only present in the sub-group of patients with more severe disease, but also responds most significantly to initial treatment. Median anti-CCP serum levels were also significantly reduced (82%) at 6 months post-treatment. This is in accordance with previous studies which reported that anti-CCP decreases with DMARD treatment (Samanci et al. 2005, Lee, Beck & Hall 2008). Early recognition of patients with high levels of circulating VEGF in association with a SE-related genetic predisposition and anti-CCP seropositivity may identify those who are most likely to develop more

severe erosive RA and to derive the most benefit from prompt initiation of aggressive therapy.

5. Conclusion

The aim of this study was to establish if additional laboratory investigations could be identified that could be used as adjuncts to current diagnostic biomarkers in patients with RA, to identify severe disease at first presentation. Anti-CCP, although a well-established marker of RA with early predictive potential, is not part of the standard RA laboratory diagnostic profile in South Africa and is underutilized in the State Health Institutions, mainly due to a lack of information on the serodiagnostic efficacy of anti-CCP in the predominantly Black South African population. Anti-CCP was only recently incorporated into the ACR/EULAR classification (Aletaha et al. 2010). Although the role of cytokines, chemokines and growth factors in inflammatory processes in RA is well documented, recent reports have highlighted the predictive value of qualitative measurement of circulating cytokines in RA as predictors of time to onset of disease (Deane et al. 2010, Kokkonen et al. 2010). Like measurement of anti-CCP and circulating cytokines, high-resolution HLA-DRB1 molecular typing is not a routine laboratory investigation. However, the classification by Du Montcel has been shown to contribute significantly to the understanding and importance of the Shared Epitope (Winchester 2006) in determining the protective or predisposing effect of this genetic marker as underscored in the current study. Clearly, the advent of multiplex Luminex[®] technology has enabled both the simultaneous measurement of multiple cytokines and the performance of high-resolution molecular HLA-DRB1

typing to be integrated into the RA laboratory diagnostic profile, albeit in the academic pathology setting.

In conclusion, the findings presented in this thesis indicate that the inclusion of immunofluorometric anti-CCP antibody measurement, high-resolution HLA-DRB1 molecular typing, as well as the measurement of the circulating cytokines IL-1 β , IL-1Ra, TNF, IFN- γ , GM-CSF and VEGF in particular will contribute significantly to identifying those individuals presenting with early RA symptoms, who will progress towards a more severe disease, and who can be earmarked for more aggressive chemotherapy at the outset. However, due to the relatively small number of patients evaluated, further investigation, preferably in a multi-centre study, will have to be undertaken before firm recommendations can be made.

6. References

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Appendix A

HLA-DRB1 genotypes of RA patients

No.	DRB1*	DRB1*	No.	DRB1*	DRB1*	No.	DRB1*	DRB1*	No.	DRB1*	DRB1*
1	0701	1101	43	0301	0401	85	0401	1503	127	1101	1301
2	0302	1301	44	0404	0701	86	0301	0302	128	0302	1602
3	0101	1301	45	1101	1102	87	0302	1302	129	0301	0401
4	0405	1302	46	0404	1302	88	0301	0405	130	0101	0804
5	0301	1501	47	0404	1301	89	0302	1302	131	0102	0301
6	0301	1302	48	0301	1501	90	0401	1301	132	0901	1301
7	0302	1101	49	0301	0404	91	0401	0404	133	0701	1101
8	0401	1303	50	0302	0404	92	1101	1201	134	0404	1503
9	1101	1302	51	0401	1303	93	0101	1301	135	0410	1101
10	0301	0302	52	1001	1102	94	0701	1302	136	0901	1101
11	0301	0302	53	0804	1102	95	0401	1102	137	0102	1301
12	0102	1101	54	0302	0401	96	0302	1101	138	0404	1301
13	1101	1302	55	1201	1201	97	1101	1302	139	0301	1101
14	0102	0302	56	0302	0302	98	0302	1302	140	1302	1503
15	0401	1503	57	0302	0804	99	0101	0701	141	1201	1303
16	0102	0302	58	1301	1340	100	0401	0404	142	0404	1101
17	1101	1503	59	1201	1209	101	1503	1503	143	0302	1001
18	0102	0302	60	0401	1336	102	0102	0404			
19	0405	1301	61	1001	1127	103	0102	0404			
20	0401	0401	62	0404	1101	104	1202	1301			
21	0401	1001	63	0301	1301	105	0302	0701			
22	0404	1101	64	1001	1301	106	0301	1601			
23	0302	1101	65	0401	0405	107	0405	0413			
24	0301	1503	66	0401	1001	108	0301	0301			
25	0301	0410	67	1301	1501	109	1201	1503			
26	0804	0804	68	0401	1102	110	0101	0301			
27	0101	0101	69	0401	1506	111	0301	1602			
28	0101	0302	70	0404	1101	112	0404	0405			
29	0302	1101	71	0466	1001	113	0404	1201			
30	1102	1302	72	0102	0302	114	1301	1503			
31	0302	0302	73	0404	0701	115	0302	1301			
32	0301	0401	74	0404	1104	116	0401	1201			
33	0102	1301	75	0405	1001	117	0101	0404			
34	0404	1201	76	1301	1401	118	0401	0405			
35	1301	1302	77	0102	1001	119	0301	0301			
36	0401	1502	78	1101	1601	120	1001	1503			
37	0301	0302	79	0102	0404	121	1501	1503			
38	0701	1001	80	0401	0828	122	0804	1001			
39	0101	1101	81	0401	0401	123	0301	0804			
40	1301	1501	82	0701	1501	124	0102	0302			
41	1101	1102	83	0102	0701	125	0102	1301			
42	0102	1201	84	0301	1501	126	0401	1001			