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Clinical and Immunopathogenetic Aspects of Sarcoidosis

Rajen Morar

"A Thesis submitted to the School of Clinical Medicine of the

Faculty of Health Sciences, University of the Witwatersrand,

in fulfilment of the requirements for the degree of Doctor of Philosophy"

Johannesburg, 2021

Declaration

“I, Rajen Morar, declare that this Thesis is my own, unaided work except where otherwise acknowledged. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at any other University”.



Rajen Morar

_____	_____
Name of candidate	Signature of candidate

31 January 2021, Johannesburg

Dedication

Dedicated to my loving wife, Sujata, and two wonderful daughters, Sanam and Kajal.

Also, to my Dad, Mom and brothers, Kiron and Dilip.

Presentations arising from this Thesis

Abstracts presented at congresses

a. “Feldman C, Hunt IA, Morar R, Goolam Mahomed A, Mahida P, Schoeman A. Wadee AA. IgG subclass levels in patients with sarcoidosis. European Respiratory Society Congress, Madrid, Spain, October 1999”.

b. “Morar R, Duarte R, Wadee AA, Feldman C. HLA class I and class II antigens in South African sarcoidosis patients. South African Thoracic Society Congress, Cape Town, South Africa, 2008”.

c. “Morar R, Richards GA, Murray J. Sarcoidosis developing UIP successfully treated with lung transplantation: A case report. American College of Chest Physicians Congress, Chest, Hawaii, Honolulu, USA, 2011”.

d. “Morar R and Feldman C. Sarcoidosis in Johannesburg, South Africa: a retrospective study. European Respiratory Society Congress, Amsterdam, Netherlands, 2015”.

e. “Morar R and Feldman C. Comorbid illnesses in South African patients with sarcoidosis. European Respiratory Society Congress, Amsterdam, Netherlands, 2015”.

f. “Morar R, Dickens C, Dix-Peek T, Duarte R, Feldman C. Altered serum microRNA expression in patients with sarcoidosis - a pilot study. South African Thoracic Society Congress, Pretoria, South Africa, 2019”.

g. “Morar R, Sinclair I, Feldman C. Serum chitotriosidase activity in patients with sarcoidosis and pulmonary tuberculosis. South African Thoracic Society Congress, Pretoria, South Africa, 2019”.

h. “Morar R and Feldman C. Clinical features of patients with sarcoidosis in Johannesburg, South Africa. South African Thoracic Society Congress, Pretoria, South Africa, 2019”.

Published Conference Proceedings

a. “Feldman C, Hunt I, Morar R, Goolam Mahomed A, Mahida P, Schoeman A. Wadee A. IgG subclass levels in patients with sarcoidosis. Eur Respir J 1999; 14:101S”.

b. “Morar R, Duarte R, Wadee AA, Feldman C. HLA Class I and Class II antigens in South African sarcoidosis patients. S Afr Respir J; 2008”.

c. “Morar R and Feldman C. Sarcoidosis in Johannesburg, South Africa: A retrospective study. Eur Respir J 2015; 46:PA841”.

d. “Morar R and Feldman C. Comorbid illnesses in South African patients with sarcoidosis. Eur Respir J 2015; 46:PA839”.

e. “Morar R, Dickens C, Dix-Peek T, Duarte R, Feldman C. Altered serum microRNA expression in patients with sarcoidosis - a pilot study. Afr J Thoracic Crit Care Med 2019; 25(2):96”.

f. “Morar R, Sinclair I, Feldman C. Serum chitotriosidase activity in patients with sarcoidosis and pulmonary tuberculosis. Afr J Thoracic Crit Care Med 2019; 25(2):94”.

g. “Morar R and Feldman C. Clinical features of patients with sarcoidosis in Johannesburg, South Africa. Afr J Thoracic Crit Care Med 2019; 25(2):95”.

Publications arising from this Thesis

1. “Morar R, Seabi N, Patterson A, Mahomed AD. Nodular hepatic and splenic sarcoidosis in a patient with a normal chest radiograph. S Afr Gastroenterol Rev, 2008; 6 (3):22-23”.

2. “Morar R, Richards GA, and Murray J. Sarcoidosis developing usual interstitial pneumonia successfully treated with lung transplantation: a case report. Am J Med Case Rep 2015; 3 (4):108-110”.

3. “Morar R, Feldman C, Hunt IA, Goolam-Mahomed A, Mahida P, Schoeman A. Wadee AA. IgG subclass levels in patients with sarcoidosis” – submitted.

Preface

I became interested in sarcoidosis as a disease which has been recognised for a long period of time and we know very little about the disease especially regarding its pathogenesis. It clearly has overlapping features with tuberculosis making the differentiation at times very difficult, which is important in a tuberculosis endemic area such as South Africa. It is for these reasons that I undertook clinical and laboratory based investigations.

All the clinical work was undertaken by myself, with some assistance from the Pulmonology Unit's research nurse and clerk. Being an inexperienced laboratory researcher, and through the assistance of the research laboratory and the scientists working in the laboratory in the Department of Medicine, I developed an understanding and familiarised myself with the relevant laboratory techniques used in the completion of this project. I became au fait with all the research steps and techniques involved in the execution of the experiments. I observed and performed some of the experiments conducted, which included PCR with agarose gel electrophoresis, DNA extraction, RNA isolation, laser nephelometry and assaying of chitotriosidase activity.

Abstract

Background

Sarcoidosis is a multiorgan granulomatous condition of uncertain aetiology, in which the lungs are commonly affected. The disorder comprises important inflammatory along with immune components; however, the exact aetiology is unknown and the immunopathogenesis is not completely understood. Due to its similarities, both radiologically as well as clinically, and particularly in high tuberculosis (TB) burden regions, sarcoidosis is regularly misdiagnosed as TB.

Variation in disease prognosis, progression, presentation and susceptibility have been linked to various human leukocyte antigen (HLA) phenotypes, which are important in antigen presentation and involved in mounting an immune response to foreign antigens. Cell-mediated immunity plays a central part in the sarcoidosis' immunopathogenesis. Humoral immunity changes, such as polyclonal gammopathy, presence of auto-antibodies, immunoglobulins, complement and immune complexes in the granuloma of sarcoidosis suggests that humoral mechanisms may well have a role in the pathogenesis. Micro-ribonucleic acids (microRNAs or miRNAs) are groups of non-coding, small, single-stranded RNAs. Their key role is to mediate the post-transcriptional silencing of target genes. The role of miRNAs has attracted attention, both in pathogenesis and as biomarkers for many disorders, including sarcoidosis. In sarcoidosis, the role of miRNAs is not well recognised, but their role is being unravelled by many workers. Chitotriosidase is a chitinase enzyme that acts as protection against nematodes, fungi, and insects which are chitin-containing pathogens. It is selectively expressed by activated macrophages. Sarcoidosis' pathogenesis is known to involve

activated macrophages through the release of various cytokines, chemokines and enzymes.

Aims

The current research was conducted in order to analyse and review clinical features of sarcoidosis patients in the South African population. Being a relatively uncommon condition, this would add to the profile of patients from other studies conducted in South Africa. In addition, HLA Class I along with Class II antigens in sarcoidosis patients were evaluated. Serum immunoglobulin (Ig) G subclass levels in newly diagnosed cases with sarcoidosis were assessed, noting changes in antibody levels in corticosteroid-treated patients and those undergoing spontaneous clinical response. The serum expression of approximately 800 miRNAs in sarcoidosis patients were compared with race-, age- as well as gender-matched healthy controls. Analysis of serum chitotriosidase activity in sarcoidosis and TB patient cohort, both granulomatous ailments of differing aetiology, was undertaken and compared with controls.

Methods

A five-year investigation was conducted among 102 patients with sarcoidosis attending the pulmonology services of the Charlotte Maxeke Johannesburg Academic Hospital. HLA-A, B, C (Class I) along with HLA-DQB1 and DRB1 (Class II) antigen typing in 51 patients from this cohort, was prospectively carried out by polymerase chain reaction (PCR), utilising sequence specific primers. These antigens were compared with 63 healthy control subjects. Serum IgG subclasses levels were assessed prospectively, both before and after treatment, or in cases with spontaneous recovery, in 17 newly

diagnosed cases of sarcoidosis, using a laser nephelometric method with appropriate test kits. The RNA was extracted from stored plasma samples utilising the “QIAGEN miRNeasy Mini Kit®” and concentrated using a salt-ethanol precipitation. The RNA extracted was analysed using an nCounter® miRNA human v3 expression assay. Twelve samples (6 patient and 6 matched controls) were analysed. Data were analysed using the nSolver™ Analysis Software. Chitotriosidase activity was prospectively assayed in serum of 12 biopsy-proven sarcoidosis cases before treatment, 9 sarcoidosis patients after treatment, 10 confirmed pulmonary tuberculosis patients prior to treatment, and in 12 healthy controls. Plasma chitotriosidase activity was analysed utilising “4-methylumbelliferyl-β-D-N, N', N″-triacetylchitotriose” as a substrate.

Results

Out of 102 sarcoidosis patients, 59 were Black, 28 Indian, eight White and seven Coloured (mixed race). There were 69 females (67.6%) and 33 (32.4%) males. Majority of patients were non-smokers (85.3%). The group’s mean age was 44.6 years. Hypertension (32.4%), renal dysfunction (creatinine > 104 µmol/L) (19.5%), obesity (17.6%), diabetes mellitus (15.7%), asthma (11.8%), and gastro-oesophageal reflux (11.8%) were the most prevalent associated chronic comorbid diseases. Indian patients had significantly more dyslipidaemia than Black patients ($p = 0.02$). Almost 17% of patients had been treated for tuberculosis, based on the chest radiograph, prior to the diagnosis of sarcoidosis. Two cases developed active TB whilst being treated with corticosteroids for sarcoidosis. Four Black patients contracted human immunodeficiency virus (HIV) infection whilst on treatment for sarcoidosis. The salient clinical manifestations were dry cough, the commonest presenting symptom in 82.4%,

dyspnoea in 53.9%, cutaneous lesions, other than erythema nodosum, in 33.3%, and on lung examination crackles were noted in 37.3% of patients. The majority (48%) of patients had stage II chest radiographic changes. Cutaneous biopsies (28.4%), transbronchial lung biopsies (25.5%) and biopsies of mediastinal lymph nodes via mediastinoscopy (25.5%) were the most frequent sites for confirming granulomatous inflammation. Blacks were more likely to have non-erythema nodosum dermatological manifestations at presentation. In Blacks 67.8% had an elevated ACE level compared with 32.1%, 50% and 14.3% in Indians, Whites and Coloureds respectively. The mean ACE level was higher in Blacks than in Indians (157 vs. 58 IU/L). The initial mean serum calcium was elevated (> 2.5 mmol/L) in 22.7% of cases, the highest incidence being in Whites (37%). Raised gamma-glutamyl transferase (GGT) (> 78 IU/L) and alkaline phosphatase (AP) (> 128 IU/L) were observed in 31.8% and 21.6% of patients, respectively. Elevated creatinine (> 104 µmol/L) was seen in 19.5% of patients; Whites having the highest incidence of renal dysfunction at 37%. Overall, 21.2% of patients had obstructive airways disease on lung function testing. Systemic corticosteroids were indicated in 87.3% of patients and the relapse rate was 60.7%.

HLA class I along with class II phenotype analysis in 51 sarcoidosis patients found significant increased levels of HLA B15, C4, C7, C12, C15, C16, C17, DQ3, DR8 and DR11. A significant negative (protective) connotation with HLA A9, A28, B12, B17 and DR2 was observed.

In the IgG subclasses study, there were 17 Black patients of which six were men and 11 women. The mean age was 44 years (range 23-60 years). Raised initial IgG1 levels were noted in 10 patients, IgG3 levels in 8, IgG2 levels in 4, and IgG4 in 2 patients. The mean IgG1 and IgG3 levels were significantly raised above normal control values.

Fourteen patients were treated with corticosteroids with good clinical response and the other three patients, who were asymptomatic, had no indication for corticosteroid therapy. On follow-up there was a significant decrease in IgG subclass levels toward normal. IgG1 levels returned to normal in almost all cases, and IgG3 remained raised in two cases. The mean levels of IgG1 and IgG3 decreased significantly, as did the levels of IgG2.

Regarding miRNA, one pair of results was excluded due to cellular RNA contamination. Therefore, five patients and matched control samples were analysed. After excluding miRNAs that were below background levels, 145 miRNAs could be analysed. On applying a Bonferroni correction, the only miRNA that was significantly different was miRNA *let-7a-5p*, which was significantly overexpressed (141-fold change; $p < 0.0003$) in sarcoidosis cases compared with controls. Of the 145 miRNAs, many had been differentially expressed among patients compared with controls, although not reaching statistical significance.

Serum chitotriosidase activity has been shown to be significantly greater in sarcoidosis cases, both before ($p < 0.0001$) and after treatment ($p < 0.0039$), compared with controls. Sarcoidosis cases had significantly higher chitotriosidase levels than TB cases and chitotriosidase activity decreased with treatment in sarcoidosis. While in patients with TB chitotriosidase activity was noted to be lower than in sarcoidosis, it was higher than in controls.

Conclusions

In South Africa, sarcoidosis is often misdiagnosed as TB. In this study, the most frequent sites for biopsy for histological confirmation were skin, mediastinal lymph node and transbronchial lung biopsy. Comorbid illnesses were common and should be screened for and treated promptly to improve quality of life. Most of the cases in this cohort with sarcoidosis had an indication for treatment with corticosteroids.

The HLA study is unique to South African patients and indicates that genetic influences may play a significant part in the potential aetiology of sarcoidosis. There are some HLA subtypes that may predispose to the development of sarcoidosis and other HLA subtypes that may be protective.

IgG subclasses, mainly IgG1 as well as IgG3 were elevated in a substantial proportion of sarcoidosis cases initially, and reduced with corticosteroid therapy and in those patients with spontaneous clinical resolution. These IgG subclass responses may be due to protein antigens that usually trigger B-cells leading to class switching to IgG1 and IgG3 via T-cell help through MHC-class II expression. Therefore, IgG subclasses may act as opsonins, bind to microbes and facilitate phagocytosis in sarcoidosis.

This is the first study of differentially expressed miRNAs in serum of sarcoidosis cases and matched healthy controls in South Africa. The results obtained indicate that miRNAs may have a role in sarcoidosis pathogenesis involved in regulating inflammatory pathways. Whether these molecules have diagnostic or prognostic implications, further research in larger cohorts are required.

This is the first study of chitotriosidase activity analysis in sera of sarcoidosis and TB cases in South Africa. The processes that result in the increase of chitotriosidase

activity are not yet understood, but in reaction to macrophage activation, this enzyme may be involved when there are chitin containing organisms implicated in the disease pathogenesis. To validate these findings, further studies with higher patient numbers are required. It remains to be determined if chitotriosidase is a diagnostic or predictive marker in sarcoidosis.

Acknowledgements

It is with deep appreciation that the following persons are acknowledged:

1. Professor Charles Feldman for the supervision, patience and guidance he so generously offered.
2. Prof Ahmed Wadee's laboratory for providing the controls for the HLA study.
3. Prof Raquel Duarte for her assistance in conducting the HLA assays and Dr Caroline Dickens and Therese Dix-Peek for assistance in performing and analysing the microRNA profiles.
4. Sister Vivian Herbert for assistance in collection of blood samples for the study.
5. Mr Ian Sinclair for assistance in the assaying of chitotriosidase activity levels.
6. South African Thoracic Society (SATS) via the Astra Zeneca Pharmaceuticals Grant for funding the HLA study.
7. Mr Duke Mokotedi, the clerk in the Respiratory Department at Charlotte Maxeke Johannesburg Academic Hospital who assisted in retrieving the files of patients.
8. All patients and control subjects participating in the study, without whom this thesis would not have been possible.

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List of Abbreviations

^{18}F-FDG-PET	=	18fluor-fluorodeoxyglucose-positron emission tomography
^{67}Ga	=	Gallium-67
^{111}In	=	Indium-111
ACCES	=	“A case controlled etiologic study of sarcoidosis”
ACE	=	Angiotensin-converting enzyme
af	=	Antigen frequency
Alb	=	Albumin
ALT	=	Alanine transaminase
ANX	=	Annexin
AP	=	Alkaline phosphatase
APC	=	Antigen presenting cell
APCs	=	Antigen presenting cells
AST	=	Aspartate transaminase
AV	=	Atrioventricular
B	=	Blacks
BAFF	=	“B-cell-activating factor”
BAL	=	“Bronchoalveolar lavage”
Bcl	=	B-cell lymphoma
BTNL	=	Butyrophilin-like protein
C	=	Coloureds
Ca++	=	Calcium
CBD	=	Chronic beryllium disease
CC	=	CC motif
CCL	=	CC motif chemokine ligand
CCR	=	Chemokine receptor
CD	=	Cluster designation
CFTR	=	Cystic fibrosis transmembrane regulator
CI	=	Confidence interval
Cl	=	Chloride
CLR	=	C-type lectin receptors
CMR	=	Cardiac MRI

COVID-19	=	Corona virus disease - 2019
CR	=	Complement receptor
Creat	=	Creatinine
CSF	=	Colony stimulating factor
CSIPF	=	Combined sarcoidosis and idiopathic pulmonary fibrosis
CT	=	Computerised tomography
CTLA	=	Cytotoxic T lymphocyte antigen
CVID	=	Common variable immunodeficiency
CXCR	=	Chemokine CXC Motif Receptor
CyA	=	Cyclosporine
DB	=	Direct bilirubin
DE	=	Differentially expressed
DLCO	=	“Diffusion capacity for carbon monoxide”
DNA	=	Deoxyribonucleic acid
EBUSTBNA	=	Endobronchial ultrasound-guided transbronchial needle aspiration
ECG	=	Electrocardiogram
EDTA	=	Ethylenediaminetetraacetic acid
ESAT	=	Early secreted antigenic target
ESR	=	Erythrocyte sedimentation rate
EUS	=	Endoscopic ultrasonography
FEV1	=	Forced expiratory volume in 1 second
FOXP3	=	“Forkhead box P3”
gf	=	Gene frequencies
GGT	=	Gamma-glutamyl transferase
Hb	=	Haemoglobin
HIV	=	Human immunodeficiency virus
HLA	=	Human leukocyte antigen
HMGB1	=	“High-mobility group box 1”
HP	=	Hypersensitivity pneumonitis
HTLV1	=	Human T-lymphotropic virus
I	=	Indians
IFN	=	Interferon
IFN-γ	=	Interferon-gamma

Ig	=	Immunoglobulin
IgG	=	Immunoglobulin G
IGF	=	Insulin-like growth factor
IL	=	Interleukin
IPF	=	Idiopathic pulmonary fibrosis
IQR	=	Interquartile range
JAK	=	Janus kinase
K	=	Potassium
M1	=	Classically activated macrophage
M2	=	Alternatively activated macrophage
MHC	=	Major histocompatibility complex
min-max	=	Minimum-maximum
mRNA	=	Messenger RNA
miRNA	=	Micro ribonucleic acid
mKatG	=	*MTB* catalase-peroxidase
MMF	=	Mycophenolate mofetil
MRI	=	Magnetic resonance imaging
MTB	=	*Mycobacterium tuberculosis*
mTOR	=	Mechanistic target of rapamycin
MTX	=	Methotrexate
Na	=	Sodium
NIOH	=	National Institute for Occupational Health
NK	=	Natural killer
NOD	=	Nucleotide oligomerization domain
NOTCH1	=	Neurogenic locus notch homolog protein 1
NRAMP	=	Natural resistance-associated macrophage protein
NS	=	Not significant
OR	=	Odds ratio
P. acnes	=	*Propionibacterium acnes*
PAMPs	=	Pathogen-associated molecular patterns
PD-1	=	Programme cell death protein 1
PDL-1	=	“Programmed death-ligand 1”
PFT	=	Pulmonary function test

PH	=	Pulmonary hypertension
Plt	=	Platelets
PRR	=	Pattern-recognition receptors
QoL	=	Quality of life
RIG-1	=	Retinoic acid inducible gene 1-like receptors
RNA		Ribonucleic acid
ROI	=	Reactive oxygen intermediates
SAA	=	Serum amyloid A
sACE	=	Serum angiotensin-converting enzyme
SATS	=	South African Thoracic Society
SD	=	Standard deviation
SIRT	=	Sirtuin
SOCS1	=	Suppressor of cytokine signalling 1
SoD	=	Superoxide dismutase
SRS	=	Somatostatin receptor scintigraphy
ss_2	=	Somatostatin receptor subtype 2
STAT	=	Signal transducer and activator of transcription
TB	=	Tuberculosis
TBili	=	Total bilirubin
TBNA	=	Transbronchial needle aspiration
TCRs	=	T-cell receptors
TGF	=	Transforming growth factor
Th1	=	T helper 1
TLC	=	Total lung capacity
TLR	=	Toll-like receptor
TLRs	=	Toll-like receptors
TNF-α	=	Tumour necrosis factor alpha
TP	=	Total protein
Treg	=	Regulatory T-cell
U	=	Urea
USA	=	United States of America
VATS	=	Video assisted thoracoscopic surgery
VC	=	Vital capacity

VDR	=	Vitamin D receptor
VEGF	=	Vascular endothelial growth factor
VIP	=	Vasoactive intestinal peptide
W	=	Whites
WCC	=	White cell count
WTC	=	World Trade Centre

Chapter 1 : Introduction and Literature Review

1.1 Background

After more than 150 years, since sarcoidosis was recognized, its cause(s) are unknown and remain elusive, the care of patients is not standardised, and the clinical prognosis and course varies and is unpredictable (1–3). This perplexing and intriguing condition still fascinates clinical practitioners and scientists around the world.

Sarcoidosis is a widespread, non-caseating, inflammatory disorder of unknown cause (1,4). It occurs commonly in adulthood and may have an effect on any organ, but it normally affects the lungs (4). The patients are often afflicted with bilateral hilar lymphadenopathy, lung parenchymal involvement, and it frequently affects the peripheral lymph nodes, eyes and skin (5). It has numerous clinical manifestations and occurs globally (6,7). Nervous system, heart, bone, liver, skin, eyes, lymphatics and lungs are the main organs that may be affected (1,8,9). There are numerous clinical phenotypes of sarcoidosis, i.e., distinct clinical presentations (10). These could arise from genetic variants or could be influenced by environmental factors (11). A substantial number of patients recover, even without therapy; while others appear to experience chronic inflammation and fibrosis that occurs in the affected organs (1,4,8). Sarcoidosis can mimic many other disorders (1,8,12).

The incidence of sarcoidosis varies and depends on age, gender, ethnicity, and seasonal and geographical variations (1,8,13). Usually, diagnosis is based on appropriate radiological and clinical findings, and histological evidence of non-caseating epithelioid granulomas and other known granulomatous diseases are excluded (7,14,15).

Newer nuclear and imaging strategies, including cardiac magnetic resonance imaging (MRI) (CMR) and ^{18}fluor-fluorodeoxyglucose-PET (^{18}F-FDG-PET) are developing and has been increasingly utilised to evaluate disease activity as well as for diagnosing sarcoidosis (7,8,16).

Studies suggest that sarcoidosis may be initiated by exposure to a presumed inducing antigen(s), whether microbial or environmental in a genetically susceptible individual (17–19). In patients with sarcoidosis the quality of life (QoL) may be affected, work capacity is diminished, and it increases morbidity and mortality (20). The mortality rate is between 1-5%; however, it can be higher if there is organ dysfunction, with glucocorticoids still being the main therapy for patients with vital organ involvement and/or disease progression (1,21).

As we learn and understand more about the role of antigens and their presentation, genetic predisposition and immune responses in sarcoidosis, there could be a new frontier of better tailored and more effective therapy.

1.2 Historical Perspective

In 1877, a British doctor, Sir Jonathan Hutchinson, was the first person who defined the sarcoidosis patient (22). He named it “Mortimer’s Malady”, following one of his patients who had two years of numerous limb and facial cutaneous lesions, which were purplish and indurated in nature (23). In 1889, a French physician, Besnier, described a young man with violaceous nasal, ear lobules and facial skin lesions (24). He suggested that the rash was similar to a “variant of lupus erythematosus”, which led to its name as “lupus pernio”. In 1899, in an individual with cutaneous nodules as well as palpable lymphadenopathy, Boeck, a Norwegian, first identified the distinctive “non-

caseating" granulomas (1). He suggested the term '*multiple benign skin sarcoid'* since the granulomatous changes looked like sarcomatous tissue, thus the name "*sarcoidosis"* (25,26).

"In 1904, Kreibich from Prague" (1) reported "sarcoidosis bone cysts" connected with lupus pernio. An ophthalmologist from Denmark, Heerfordt, in 1909, defined uveo-parotid fever (parotid enlargement, fever, as well as uveitis without or with cranial nerve VII nerve palsy) (27). Bittorf and Kuznitsky, identified a young patient with subcutaneous nodules, as well as skin lesions, who had other organs involved by way of hilar lymphadenopathy and lung parenchymal involvement (28,29).

The multiorgan concept of sarcoidosis was unrecognised for almost two decades. It has been centred primarily on the findings of Jorgen Schaumann, a Norwegian skin specialist, that identified cases with multisystem disease, including lesions in the lungs, bones, tonsils and gums, together with hepatosplenomegaly (1). In 1914, he thought that the lupus pernio described by Besnier and '*multiple sarcoids*' by Boeck had been manifestations of the similar disease called "lymphogranulomatose benigne", believed to be a form of tuberculosis at the time (1,8,30). In 1939, sarcoidosis-associated hypercalcaemia and hypercalciuria were first recorded (31). Nickerson and Williams stated in 1935 that intradermal inoculation of sarcoidosis tissue suspension in cases suspected as having sarcoidosis resulted in papules (32–34). In 1941, "Ansgar Kveim, a dermatologist in Oslo, demonstrated that these papules contained sarcoid-like granulomas" (35). Louis Siltzbach indicated that the "Kveim" response, which was very unique to the disease (36) was positive, and was later referred to as the "Kveim-Siltzbach test", in up to 80% of worldwide sarcoidosis patients.

Sven Löfgren of Sweden made the following breakthrough in sarcoidosis perception during the 1940s and 1950s. In Caucasians, he observed a type of sarcoidosis, consisting of erythema nodosum, fever, polyarthritis and bilateral hilar lymphadenopathy (37), the syndrome of Löfgren.

It was noted that corticosteroids were very efficacious in the management of sarcoidosis patients in the 1950s (38,39). In 1958, an x-ray staging method was proposed by Wurm and co-workers (40), which was proposed to guide prognosis (41–43) and is still used at present.

In the 1970s, fibre-optic bronchoscopy and bronchoalveolar lavage (BAL) were a significant development, and with minimal morbidity, the diagnosis could be established with more accuracy and greater sensitivity (44–46). This has contributed tremendously to the unravelling of sarcoidosis pathogenesis (47–50).

In 1975, various immunological anomalies connected with sarcoidosis were reported (48), including the use of “serum angiotensin-converting enzyme” (sACE) as a potential biochemical marker of disease activity (47). Many investigators studied involvement of biochemical, immunological, genetic and pathogenesis aspects in sarcoidosis (51,52) during the 1970s and early 1980s. More recently, our knowledge of the immunological, genetic, and the aetiological basis of sarcoidosis has been advanced by the methods of molecular and cellular biology (49–52).

1.3 Epidemiology

Sarcoidosis is present in every race worldwide. This condition affects more women than men. Prevalence rates of disease are not established with certainty since several individuals with sarcoidosis are asymptomatic. In the absence of a specific diagnostic

test and depending on the extent of diagnostic work-up, the case definition and presentation can vary. The prevalence of the disease varies in different geographical regions as well. It occurs more often in adults between the ages of 20 to 45 years, though it can affect all ages (13).

The most common diffuse parenchymal lung disease of unknown aetiology seen in Europe is sarcoidosis (53). The prevalence rates in “Sweden are 64 patients per 100,000 population, in Denmark 53, in Germany 43, in Ireland 40, in Norway 27, in the Netherlands 22, in the United Kingdom 20, in Switzerland 16, in France 10, in Hungary 5, in Spain 1-2 and in Portugal 0.2 per 100,000 population” (13,53,54). The lifetime risk of contracting sarcoidosis in Scandinavia is estimated at 1.0% and 1.4% in males and females, respectively (55). African Americans are 3-4 times more often affected, with an “incidence of about 35 per 100,000 population”, compared with about “11 per 100,000 in Whites”. Black women, with a 2.7% (13,25,56–58) lifetime risk, are the most affected.

In South Africa, the incidence of sarcoidosis has been reported as being “20.4-23.2 per 100,000, in Blacks, 11.6 per 100,000, in Coloureds (mixed race), and 3.7 per 100,000, in Whites” (59,60).

In Switzerland, Denmark, Japan, and Singapore a second incidence peak has been observed for females that is closer to 60 years, and in Israel it is almost 70 years (56,61–63). In preadolescence, sarcoidosis is uncommon. In Chinese and south-east Asians, it is rare (25,64). The actual burden of sarcoidosis is likely to be underestimated, as autopsy studies have shown a greater incidence than epidemiological studies (65,66).

The occurrence of different clinical sarcoidosis manifestations often differs across geographical regions as well as ethnic groups. Many findings indicate that race is a big component in determining incidence of illness and disease severity, with chronic disease and higher mortality occurring in Black populations than in Whites (13,25,67).

Blacks seem to have more extrapulmonary disease and are more seriously affected by the disorder. The Japanese are highly susceptible to ophthalmic and heart involvement. More than half of the patients in Japan may have heart sarcoidosis. Erythema nodosum is more often noted in younger Caucasians, as previously identified by Löfgren, in association with acute disease, a good prognosis, and also being associated with certain distinct HLA alleles (HLADRB1*03 (HLA-DR3)) (68–70). About a third of all cases in Sweden have this form of sarcoidosis and 70-80% of them go into remission (70). Löfgren's syndrome is found in less than 5% of Black or Japanese patients (56). In sarcoidosis, lupus pernio, and other skin manifestations (other than erythema nodosum), is considered to be a sign of chronic and more severe illness (71), and occurs more often in Blacks than in Whites (72,73).

Sarcoidosis is not considered a mild condition for many patients as demonstrated by epidemiological studies (74). Some patients have excess morbidity and mortality (75–78). Mortality is increased among patients with more severe and extensive illness at diagnosis (79). Forty to eighty percent of sarcoidosis fatalities in the United States, is attributed to advanced lung disease. The leading cause of death from sarcoidosis in Sweden, as well Japan, is cardiac involvement. There is significant morbidity associated with this condition and the overall mortality from sarcoidosis is around 5%; however, if there is pulmonary, cardiac or neurological involvement the mortality is around 20–25% (13,25,67,80,81).

Sarcoidosis patients have a greater risk of infection, by at least a factor of two, and this is further increased in patients on immunosuppressive treatment (82). Patients with sarcoidosis can develop congestive heart failure (83,84), cerebrovascular disease (84), venous thromboembolism (85,86) and autoimmune diseases like thyroiditis, Sjögren's syndrome, ankylosing spondylitis and systemic lupus erythematosus (87). Many cancers, especially of the haematological system, skin, upper gastrointestinal tract, renal, hepatic and colorectal malignancies, are increased (88). In these patients, not many studies have investigated the role of corticosteroids as well as other immunosuppressive therapies in comorbidities.

1.4 Causes and Risk Factors

It is unclear what causes sarcoidosis. It is assumed that various risk factors listed below contribute to the aetiology.

1.4.1 Genetic Factors

Family clustering of cases of sarcoidosis, raised occurrence in monozygotic twins relative to non-twin relations, and differences in the expression of disease phenotype by ethnicity, support the assumption whereby genetics play a significant role in the immunopathogenesis and aetiology of sarcoidosis. This complex disorder is thought to originate through combinations of various environmental and genetic interactions (89–92).

Studies around the genome associations established polymorphisms for coding T-cell proteins, involved in proliferation, differentiation, activation and survival genes. Genes identified involved in these functions include the neurogenic locus notch homolog 4 (NOTCH 4) (93) and Annexin A11 (ANX A11) (94) and butyrophilin-like protein (BTNL)

2 (95,96). In German and Black American (95,96) sarcoidosis patients, genome wide scan studies found polymorphisms in the BTNL2 gene, linked to increased risk of sarcoidosis. An enzyme implicated in apoptosis and cell proliferation encompasses the “BTNL2 gene encoding, butyrophilin-like protein 2”, which is structurally bound to co-stimulant molecules B7, and the ANX A1 gene (95,96). It is not known the exact mechanism by which certain genes affect and influence the risk of sarcoidosis (94,97).

The importance of human leukocyte antigen (HLA) genes in transplantation has been recognised for a long time, but their role in the immune response was not elucidated. HLA genes on chromosome 6 major histocompatibility complex (MHC) locus, appear to be the major contributor to the genetic and familial origin of sarcoidosis (98). For the presentation of antigens and for the monitoring of adaptive immune response to certain antigens, the HLA genes are essential. The prevalence of sarcoidosis was correlated with both HLA genes class I (A, B, C) and class II (DP, DQ, DR). There were different HLA-alleles in various regional and racial populations in sarcoidosis. Different HLA phenotypes were also correlated with differences in susceptibility, presentation, progression and prognosis of the disease. Initial reports of sarcoidosis, as well as unique gene products, demonstrated the correlation between acute sarcoidosis along with class I HLA-B8 antigens (99–101). In later studies, sarcoidosis has been consistently associated with HLA class II antigens, encoded by DQB1 and DRB1 alleles (99,100,102). Table 1.1 lists several HLA associations with sarcoidosis that have been reported. It appears to have more effect on the presentation of the disease rather than on susceptibility.

In South Africans, some HLA alleles were described as being associated with protection against a wide range of diseases or susceptibility to them. These consist of autoimmune disorders like diabetes mellitus, rheumatoid arthritis, coeliac disease and

others; infectious diseases such as tuberculosis (TB), “human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS)” and also with drug-induced hypersensitivity (103). No studies regarding HLA in sarcoidosis has been conducted in South Africa.

Table 1.1. “Summary of HLA and non-HLA genes” associated studies of sarcoidosis. Adapted with permission from (92).

Gene	Association
HLA alleles	
HLA-A3, -B7	Risk factor for disease and associated with prolonged disease
HLA-A1, -B8	Risk factor for disease and associated with arthritis and with good prognosis
HLA-DRB101	Protective against disease
HLA-DRB103	Risk factor for disease and associates with acute onset, with Löfgren’s syndrome and good prognosis
HLA-DRB104	Protective against disease and associates with ocular sarcoidosis and Heerfordt’s syndrome
HLA-DRB10803	Risk factor for disease and associates with ocular sarcoidosis (shown for Japanese patients)
HLA-DRB111	Risk factor for disease
HLA-DRB112	Risk factor for disease
HLA-DRB114	Risk factor for disease and associates with prolonged disease
HLA-DRB115	Risk factor for disease and associates with prolonged disease
Non-HLA genes	
BTNL2	Risk factor for disease. Association with prolonged disease
ANXA11	Protective against disease

There are at most 20 genes of “class I in the HLA region”, three of which are the immunological ambit of “HLA-A, B, and C, the so-called classic or class Ia genes”

(104). Class II codes for the polypeptide chains *α* and *β* molecules in Class II. The identification of “their loci by chromosome 6 consists of three letters; the first (D) is classification; the second (M, O, P, Q, and R) is family, and the third (A and B) is chain (*α* or *β*)” (105). For example, HLA-DRB represents class II R-family genes that code β chains. Arabic numbers characterise the different genes of the HLA family, and several allelic versions of these genes have an asterisk. The allelic variant 1101 of gene 1, which codes for “the β chain of a Class II molecule belonging to the R family”, for example is HLA-DRB1 * 1101. Each of these groups is categorised into four domains: the peptide domain of α and β (α 1 or β 1), the domain of immunoglobulin of Class 2 (α 2 or β 2), and the category of transmembrane and cytoplasmic tail (104,105).

In “A Case Controlled Etiologic Study of Sarcoidosis” (ACCESS), HLA-DRB*0402, HLA-DRB*1501, HLA-DRB*1201 and HLA-DRB*1101 alleles were associated with the highest sarcoidosis risk (99). Distinct genotypes of HLA are linked to various clinical phenotypes and the course of disease. For instance, HLA-DQB1*0201 and HLA-DRB1*0301 are intensely linked to remitting sarcoidosis (106,107), while the haplotype HLA-DQB1*0602-DRB1*150101 is connected to chronic unremitting illness (108).

The non-HLA genes that affect cell recruitment, T-cell and macrophage activation, antigen presentation and antigen processing, were also examined. These include the ACE gene, Chemokine (CC) 2 receptor gene, Vitamin D receptor gene (VDR), Vascular endothelial growth factor (VEGF) gene, Tumour necrosis factor-α (TNF-α) gene, Transforming growth factor (TGF) gene, Toll-like receptor-4 gene (TLR4), Natural resistance–associated macrophage protein (NRAMP) gene, Macrophage migration inhibitory factor gene, Interferon-γ gene, Interleukin (IL)-18 gene, IL-4 receptor gene, IL-1α gene, Inhibitor kappa B-alpha gene, “Heat shock protein 70–like gene, Cytotoxic T lymphocyte antigen 4 (CTLA4), Cystic fibrosis transmembrane

regulator gene (CFTR)", Complement receptor-1 gene, Clara cell 10-kDa protein gene, CC receptor 5 gene, and CD80 and CD86 genes (92,98,109). Their link with sarcoidosis has not been consistently replicated.

1.4.2 Occupational and Environmental Factors

Healthcare professionals, rescue workers and firefighters (110) involved in the "World Trade Centre" (WTC) attack in New York, USA (111,112), ship workers and military personnel, and workers involved in the lumber industry, have been identified as having occupational associations with sarcoidosis (113). Chronic beryllium disease causes a pulmonary sarcoidosis-like disease, histologically identical granulomatous pneumonitis, in fewer than 5% of the workers exposed to beryllium after immunological sensitization (114). No evidence has been found that beryllium is the main reason for systemic sarcoidosis.

The ACCESS study (73) compared 706 newly diagnosed cases of sarcoidosis with controls matched by sex, age, along with race. Study findings indicate a lack of environmental and clinical association with a sarcoidosis threat, which exceeded 2.0 odds ratio (OR) and a prevalence of exposure of more than 5%. Poor positive correlations were found (ORs of approximately 1.5), suggesting possible links with microbial-rich conditions for the use of insecticide in the office, mould/mildew penetration and musty odours. As opposed to what was previously believed, heavy metal contamination such as beryllium, wood dusts or rural homes in this sample was not correlated with sarcoidosis. As indicated in earlier research, ACCESS found a clear "negative association between smoking and potential sarcoidosis". The absence of an explicit, predominant sensitivity in sarcoidosis is compatible with the belief that both gene-environmental association is essential in disease causation.

Environmental conditions are associated with sarcoidosis because of seasonal disease clustering, with predilection in both northern as well as southern hemispheres for winter and early spring months (115). Geographical variance and clustering of time-space also support a role for sarcoidosis environmental triggers (90).

1.4.3 Infection

Numerous studies over many decades have investigated a role in sarcoidosis specifically for infectious agents. In the 1960s, in sarcoidosis tissue, researchers documented the presence of a transmissible agent; however, such research could not be replicated and no mycobacterial nor other infectious species were cultured reproducibly from sarcoidosis tissue, despite several attempts (116). Table 1.2 lists several of the infectious along with non-infectious agents implicated in sarcoidosis aetiology (117).

Table 1.2. Possible "infectious and non-infectious agents" implicated in the aetiology of sarcoidosis. Adapted with permission from (117).

Infections	Non-Infectious (Environmental)
Mycobacterial *Mycobacterium tuberculosis*, atypical	**Organic Substances** Pine, pollen
Bacterial *Propionibacterium, Rickettsia helvetica, Chlamydia pneumoniae, Mycoplasma, Borrelia burgdoferi, Brucella*	**Inorganic Agents** Zirconium, titanium, aluminium, clay, talc, silica, silicone
Viruses Human herpes, hepatitis C, Epstein Barr, other viruses	**Occupations** Fire-fighters, navy, agriculture, bird handlers, automotive manufacturing
Fungi Candida, Cryptococcus, other fungi	**Exposure** Metal, insecticides, pesticides, mildew, mould, musty odours, high humidity areas
Parasites Toxoplasmosis, Leishmaniasis	

There are many clinical, radiological and histological similarities that sarcoidosis has to tuberculosis (TB). This led to suggestions that sarcoidosis could be caused by mycobacterial organisms. Pathological specimens of patients with sarcoidosis, such as lung tissue, do not disclose microbial species, and there have been no replicated research recording the existence of viable mycobacteria (or other organisms) in patients' tissues (118). Cultures and acid-fast stains of sarcoid specimens classically do not demonstrate the presence of mycobacterial organisms. Some immunohistochemical studies have demonstrated possible cell wall deficient mycobacterial remnants in tissues and blood of patients with sarcoidosis (116). Chinese research showed that 0-80% of biopsy specimens and 0-30% of the control

tissues, tested by polymerase chain reaction (PCR) techniques had mycobacterial DNA documented (119). Recent studies revealed *M. tuberculosis (MTB)* DNA of the *MTB* catalase-peroxidase protein (mKatG) in 38% of biopsy samples and presence of immunoglobulin G to mKatG in the circulation in approximately 50% of sarcoidosis patients examined (120). Patients with sarcoidosis also have a higher lung T-cell, as well as peripheral blood response rate, to mKatG along with other mycobacterial antigens than healthy controls (121,122). In sarcoidosis patients whether before or after diagnosis, there is no active mycobacterial infection that can lead to a sarcoid reaction, other than an active or latent infection after organism exposure (123). In tissue samples obtained from sarcoidosis patients, isolation of *MTB* DNA and other mycobacterial proteins, such as 6-kilodalton “early secreted antigenic target-6 (ESAT-6)” and superoxide dismutase A (SoD A), show that *MTB* is the leading contender for infection-mediated sarcoidosis (124).

Japanese researchers found *Propionibacterium acnes* (*P. acnes*) DNA in 80-98% of Japanese and European patients’ sarcoidosis tissues, but also 0-60% of control tissues (125). In patients with sarcoidosis, other microbial agents including *Leptospira* species, *Pneumocystis jirovecii*, retrovirus (HTLV1), cytomegalovirus, human herpes virus type 6, *Rickettsia helvetica*, *Chlamydia pneumoniae* and “*Borrelia burgdorferi”*, have been described and implicated (126). Despite the lack of credible details, finding systemic sarcoidosis in naïve transplant recipients receiving organs from patients with proven or suspected sarcoidosis supports a possible transmissible agent as a cause for sarcoidosis (127).

1.4.4 Autoimmunity

Some researchers hypothesise an aetiological connection with autoimmunity, likely caused through molecular mimicry by infectious agents. Sarcoidosis is associated with autoimmune features such as the existence of antinuclear antibodies and rheumatoid factor, and immune complexes and hypergammaglobulinaemia in support of this hypothesis (128,129).

1.4.5 Microbiome

Unbalanced gut microbiota is a significant feature of autoimmunity. Associations between microbiome dysbiosis and development of autoimmune conditions have been recorded (130). Sarcoidosis overlaps with other autoimmune ailments like ankylosing spondylitis, Sjögren's syndrome, thyroiditis and rheumatoid arthritis (131). The “role of microbiota in these autoimmune diseases has been evaluated in previous studies”, which showed to play a major part in the pathogenesis (132). Studying the microbiome in patients with sarcoidosis and its association with autoimmune disorders may thus pave new avenues to examine the underlying cause(s) of this disease (18,133,134).

1.5 Pathology of the Granuloma in Sarcoidosis

The histopathological feature of sarcoidosis is the formation of discrete, compact and typically non-caseating granulomas (135). These granulomas consist mainly of epithelioid cells in core areas, mostly adjacent to CD4+ T lymphocytes centrally, while IgA-forming plasma cells, B lymphocytes, Th17 cells, T-regulatory (Treg) cells, CD4+ forkhead box P3 positive (FOXP3+) and CD8+ T lymphocytes are found in peripheral areas (136–139). This granulomatous inflammation contributes to local architectural distortion of the tissues, causing some patients to experience fibrosis and injury. In humans, the adaptive and innate immunity triggered by chemokines and cytokines by

interaction of lymphocytes, macrophages along with dendritic cells ultimately contributes to the production of granulomas (140,141). Granulomas in the lung form along the lymphatic rich bronchovascular bundle, subpleural and septal regions. A mononuclear cell infiltrate adjacent to these areas is often present, composed predominantly of lymphocytes. Granulomas in sarcoidosis will sometimes display central coagulative necrosis (141–143). Cytoplasmic inclusions (asteroids or bodies from Schaumann) that may be misidentified as exogenous substances (144) can be found in giant cells. Granulomas may either resolve or undergo fibrosis, leaving a residual scar.

Although these traits are distinctive, they are not unique to sarcoidosis, and should be addressed primarily by the organ affected in their diagnostic importance. The word “granuloma” includes lesions found under different circumstances in histopathology. The most common causes are infections, particularly tuberculosis. Therefore, the first step is to systematically exclude an infectious cause when a pathologist detects granulomas (143,145). Figure 1.1 shows histology of granuloma formation in pulmonary sarcoidosis.

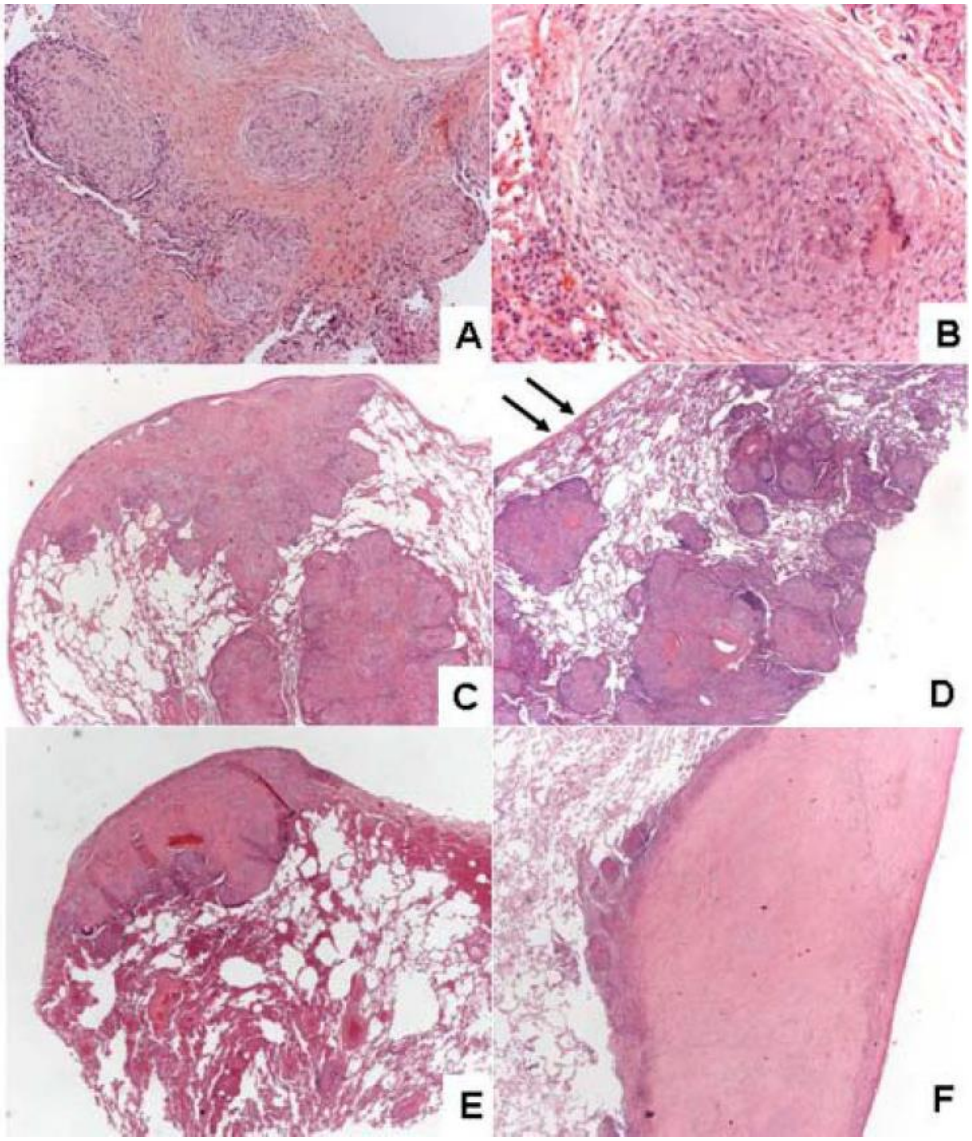


Figure 1.1. Histology of pulmonary sarcoidosis with permission from (142).

”A, Showing well-formed, compact granulomas embedded within hyaline lamellar collagen (haematoxylin-eosin [H&E], 100x)

B, Occasionally, granulomas are surrounded by a rim of more oedematous and cellular tissue (H&E 200x)

C, Sarcoid granulomas typically coalesce forming nodules along the lymphatics

D, Sarcoid granulomas in the centrilobule, along the bronchovascular bundles, and sparing the pleura (arrows). This localisation makes the lesion particularly suitable to be reached by the bronchoscope (H&E, 20x)

E, A more infrequent situation, wherein granulomas are prevalent in the pleura (H&E, 20x)

F, Another example of pleural sarcoidosis with a few residual granulomas and a large amount of fibrosis, simulating a pleural plaque (H&E, 40x). In parts C, D, E, F, note the relative sparing of the alveoli”.

1.6 Immunopathogenesis of Sarcoidosis

Currently, the assumption is that many concurrent immunological events are involved in the development of granulomas. The phenomena leading to the development of granulomas probably begin with antigen presentation to T-lymphocytes in a genetically predisposed host via MHC class II peptide. The innate and adaptive immune responses are discussed further.

1.6.1 Innate Immune Response in Sarcoidosis

Innate immunity is the first line of antigen or pathogen protection and is essential for the initiation, manufacture and regulation of adaptive T- and B-cells. The non-specific innate response does not grant lasting immunity (memory). The response is fast, as well as effective, and the vast majority of all infections are presumed to be suppressed by the innate immune system (146,147).

Pathogen invasion is sensed by “antigen presenting cells” (APC), for example macrophages and dendritic cells, that utilize pattern-recognition receptors (PRR), which communicate, activate and interact with immune cells. These PRRs detect distinct evolutionarily preserved structures on pathogens, termed “pathogen-associated molecular patterns” (PAMPs), resulting in initiation of innate immunity. These antigens classically activate undifferentiated macrophages into M1 (classically activated) phenotype through various receptors, for example signalling lymphocyte-activation molecule family (148), “C-type lectin receptors (CLR), retinoic acid inducible gene 1-like receptors (RIG-1), Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like” receptors promote T effector cell responses via MHC class II antigen presentation (149).

Such receptors also sense “damage-associated molecular patterns” (DAMPs), including histones from injured cells, deoxyribonucleic acid (DNA), extracellular RNA, fibrinogen, calcium-binding S100 proteins and “high-mobility group box 1 protein (HMGB1)” (150,151). Recognition of these substances triggers innate immune and inflammatory reactions.

Neutrophils are among the first cells that move to an infection site. The pathogens are phagocytosed, degraded and removed and these cells produce proteases and reactive oxygen intermediates (ROI). Chemokines, IL-8 and the epithelial neutrophil activating protein formed by macrophages, monocytes, type II alveolar epithelial cells along with fibroblasts attract neutrophils (146). The neutrophils, in turn, generate tumour necrosis factor-alpha (TNF-α), IL-12, IL-6 and IL-1 that further amplifies the immune response. Furthermore, M1 macrophages generate pro-inflammatory cytokines including TNF-α IL-18 and IL-12. TNF-α leads to the growth of granuloma formation through the reclamation of naive T-cells, the promotion of pro-inflammatory CD4+ T helper cell 1 (Th1) polarisation and the promotion of immune-cell aggregation and the formation of granulomas (152). Earlier stages of granulomatous inflammation are believed to be mediated by Th1/M1 response - and are driven by an intensified development of IFN-γ and TNF-α (152).

APC-acting phagocytes represent a key relation between the innate and the adaptive immune systems. The adaptive immune response comprises many distinct cell types, including T-cells, APCs such as dendritic cells and macrophages and B-cells.

1.6.2 Adaptive Immune Response in Sarcoidosis

Adaptive immunity includes responses of T-cells along with B-cells characterised by antigen memory and specificity. Antigens are internalised by APCs, processed to generate antigenic peptide fragments loaded onto molecules of MHC class I or II for display on the APCs' surface. T-cells with antigen-specific receptors examine the MHC-peptide complex, which results in the development of specific effector regulatory cytokines when accompanied by co-stimulation. Sarcoidosis is a condition of enhanced immunity of T-cells at sites of granulomatous inflammation as well as is recognised as a Th1 polarised disorder (49,153–155).

Other cells, such as natural killer (NK) cells, Th17 cells, and B-cells, are activated and take on innate and adaptive immune responses which encourage continued granulomatous inflammation in sarcoidosis patients (150,156).

1.6.2.1 *Th-Cell Response*

T-cell activation happens in the form of MHC-antigen complexes where T-cell activation takes place by the internalisation, processing and digestion of antigen by APCs and attaches to the peptide-binding groove of the MHC type molecules (157). The α/β T-cell receptors (TCRs) CD4+ T-cells accept the MHC bound antigen complexes (158). There is a reduction in the TCR number on the cell surface, which marks the recent involvement (159). The activated T-cells have a memory phenotype of T-cells (160,161). The lung T-cells are explicitly activated by markers IL-2R, CD26 along with CD69 (162,163) in patients with pulmonary sarcoidosis. In addition, T-cell activation involves binding co-stimulative molecules to the corresponding ligand on the cell surface on the APC. CD28 interacts with CD86 along with CD80 on T-cells in order to activate T-cells efficiently (162,164,165) and is the most important molecule expressed by T-cells. There is a remarkable concentration of lymphocytes in

bronchoalveolar lavage (BAL) fluid in sarcoidosis compared with other lung diseases (166). In sarcoidosis, the BAL T-lymphocytes are mainly CD4+ T-cells (49) and the BAL T-cells CD4:CD8 ratio is 3-5:1 compared with 2:1 in healthy people (167). In general, the immune response is typically controlled by either Th1, Th2, or Th17 effector response following T-cell activation (168).

Sarcoidosis is characterised by granulomatous inflammation via an immune response mediated by Th1 (169). Multiple studies show that lung sarcoidosis is associated with low or undetectable levels of IL-4 or IL-5 and increased expression of Th1-related IFN-γ, IL-12, and IL-18 in the lung (170,171). IL-12 is a central cytokine that triggers a shift in a Th1 profile and stimulates the development of IFN-γ in T-cells (172). IFN-γ activates macrophages, enhances phagocytosis and development of oxidants, synergizing with cytokines (such as TNF-α) (173). IL-18 will induce improved IFN-γ output in combination with IL-12 (155,172,174,175). Activated Th1 cells oligoclonally expand as well as produce INF-γ for promoting more M1 activation (176).

In addition to releasing basic cytokines, T-cell subsets also express a wide variety of chemokine receptors. Upregulated chemokine receptors (CXCR6, CCR5 and CXCR3) are commonly associated with a Th1 response during sarcoidosis (155). The polarisation of Th1 during sarcoidosis is triggered by CD8+ T-cells that express IFN-γ. In sarcoidosis patients, the factor for transcription, "signal transducer and activator of transcription 1 (STAT1)", upregulates the differentiation of Th1-cells (177).

The use of biologic agents to facilitate Th1 reactions, such as IL-2, IFN-γ, and immune reconstitution of patients infected with HIV has been associated with new initiation and flare-up of sarcoidosis (178). The correlation between sarcoidosis and 5q- myelodysplasia with chromosomes, which encode Th2-cell cytokine (including IL-13,

IL-4 and colony stimulating factor (CSF) 2, confirms the importance of “polarised Th1 immunity” for sarcoidosis pathogenesis (179).

NK T-cells via IFN-γ are involved in the initiation of inflammation. NK T-cells recognise CD1d-restricted antigens, which are important in immunoregulation. Failing this recognition may lead to persistent T-cell activation that characterises ongoing inflammation in sarcoidosis (180,181). Thus, NK T-cell deficiency may have a role to play in developing chronic active sarcoidosis.

Th2 cytokine proteins, IL-13, IL-10, IL-5 and IL-4 are not increased in cell culture T-cell supernatants or body fluids of sarcoidosis patients. Th2 cells can be present in BAL, blood, as well as lung parenchyma, but they are not triggered or inhibited to lead to immunopathogenesis by maintaining a cytokine imbalance in the lung (172,182). At the sites of inflammation in sarcoidosis patients, IL-4 and IL-5, the cytokines formed by Th2 cells and multiple chemokines and chemokine receptors associated with Th2 reactions, are downregulated (172,183). In a subgroup of patients, IL-13 provided by Th2 cells, may be a possible exception (184).

Th17 and Th17.1-cells were linked to the pathogenesis of sarcoidosis only recently (185,186). These cells are recruited to the disease site and participate in the development of the granuloma. In sarcoidosis, the equilibrium between Th17 and Treg cells is assumed to be disrupted (187) and is an important factor in prognosis (188). IL-22, IL-21 and IL-17, secreted by Th17+ cells, are cytokines that activate pro-inflammatory cytokines for example TGF-β, IL-6, IL-1 and TNF-α (189). Th17+ T-cells evolve in response to IL-21, IL-6 and TGF-β, and rely on IL-23 to preserve their phenotype (189). The function of Th17 effector responses in sarcoidosis pathogenesis remains to be defined (139,156).

A substantial number of patients with sarcoidosis resolve spontaneously, indicating that the eliciting antigen is removed (190). An efficient immune response needs to be adequately regulated (191). Tregs are defined as master immune response regulators which maintain homeostasis (191). Tregs are characterized by their bright IL-2R expression and the FoxP3 positivity factor (190). Tregs control responses of T-cells by APC suppression and cytokines for example TGF-β along with IL-10 (190,192,193).

Miyara and colleagues observed an improvement in the frequency of “FoxP3+T-cells” in tissue, lung and blood, in line with a rise in the frequency of normal Tregs in sarcoidosis (138). They demonstrated the anti-proliferative action of Tregs in BAL from sarcoidosis patients, but they did not completely inhibit TNF-α and IFN-γ development and were not successful in preventing *in-vitro* granuloma formation (138,194).

In Löfgren’s syndrome, large numbers of Tregs in the lung, blood and to a lesser degree, lymph nodes, are observed (138). In comparison, Tregs in the lung are reduced in chronic active disease patients (195). Sarcoidosis Tregs are known to be unstable, since the peripheral blood Tregs do not inhibit the release of TNF-α and IFN-γ and the development of granulomas (194).

Prasse and collaborators have observed a substantial decline in the development of TNF-α in lung cells in sarcoidosis-related patients with inhaled vasoactive intestinal peptide (VIP), a peptide hormone reported as having an immunoregulatory role in chronic inflammatory disorders and that this decrease was associated with increased CD4+CD127–CD25+ lung Treg cells (196). VIP’s ability to change naive CD4+CD25–T-cells into CD4+CD25+FoxP3+ Treg cells was demonstrated during *in-vitro* studies,

such that the author suggested inhaled VIP, as a therapy for immune-mediated severe lung disease, including sarcoidosis (196).

Apoptosis is another main mechanism for dampening the immune response. IL-15 is a Th1-system cytokine that is involved in the developing and sustaining of sarcoid granulomas (197). The blocking of T-cell removal from chronic inflammation sites contributes to an upregulation in the expression of B-cell lymphoma (Bcl)-2 T cells. BAL cells in sarcoidosis patients have caspase-3 activation that is exaggerated to make them immune to apoptosis (198). Dysregulation of programmed cell death is found in chronic sarcoidosis. IL-2 and IL-15 have an upregulated antiapoptotic effect in patients with sarcoidosis on T-cells (199).

1.6.2.2 *B-Cell Responses and IgG Subclasses in Sarcoidosis*

In addition to the dominant cellular-mediated response, sarcoidosis is characterised by B-cell stimulation. Immune complexes are found in most patients with sarcoidosis (200). Löfgren's syndrome, the only form of sarcoidosis where the onset of illness is known, is associated about 100% of the time with the formation of circulating immune complexes (200). One theory is that in 80–90% of Löfgren's syndrome will rely on this humoral reaction linked with remitting sarcoidosis (200). A hypersensitivity response is initiated that leads to an acute onset of disease, enhanced clearance of eliciting pathogenic antigens and spontaneous remission of disease (201,202). Hyperactivity of the B-cells is also shown by the presence of hypergammaglobulinaemia observed in many sarcoidosis cases (51).

An efficient humoral response helps to clear pathogenic antigens through either Fc-receptor-mediated mechanisms or immune complex removal through complement

receptor (CR)-1-mediated pathways (203). Immune complex clearance by circulating erythrocytes is mediated through CR-1, which is a glycoprotein (204,205). In this regard, “the presence of circulating immune complexes in almost all cases” was reported in early trials in patients suffering from Löfgren’s syndrome (200). It was proposed that other pathogenic forms, such as immune cell apoptosis, may affect the final outcome of this phase of the disease (206).

Immunoglobulin (Ig) A-positive plasma cells have been observed around granulomas (137), and can produce TNF-α in a TGF-β rich environment that stimulates Th17-cell differentiation (207). In active sarcoidosis, raised serum levels of the “B-cell-activating factor (BAFF)” are observed (137,208), which are involved in production of IFN-γ and Th17-cell differentiation (208–211).

In the event of refractory sarcoidosis affecting the lungs, heart, eye and/or skin, case studies of active depletion of B-cells using rituximab, indicate that B-cells are engaged in progressive disease (212). A refractory pemphigus vulgaris patient who was treated with rituximab developed sarcoid-like granulomas (213). These factors mean that in sarcoidosis B-cell immunity might be involved in the disease pathogenesis.

IgG comprises four subclasses, namely, IgG1, IgG2, IgG3, along with IgG4 defined by the heavy chain and are numbered according to their serum concentrations. Each of the four subclasses has a corresponding C gene segment on chromosome 14 that encodes its heavy chain constant (214). Mutations in the C genes, inherited in an autosomal pattern, provide variation in the sequence composition of IgG subclass proteins (215). These mutations or allotypes can vary markedly among healthy human populations from different geographical regions (216,217). It has been hypothesised

that antibodies of certain allotypes may confer superior protection to pathogens indigenous to specific regions and this may account for the differences in IgG subclass levels among populations and countries (216,217). All four subclasses may have a role in sarcoidosis pathogenesis. IgG1 normally comprises 60% to 70% of total IgG, such that low levels of IgG1 usually results in overall hypogammaglobulinaemia (218).

All four IgG subclasses act as opsonins, binding to microbes and facilitating phagocytosis, and some of them are involved in the complement pathway (219). Antibody responses to, viral, as well protein, antigens are primarily composed of IgG1 and IgG3, while responses to polysaccharide antigens are mostly IgG2 (219,220). The functions of IgG4 remain incompletely defined and it plays a significant role in immune response to parasitic infections (219). Elevations in IgG4 have also been associated with an array of different disorders (220,221).

Studies (222–224) have been performed investigating serum and/or BAL levels of immunoglobulins, in particular the IgG subclasses, in patients with sarcoidosis, both before and after corticosteroid therapy. Rankin and co-workers were the first to measure subclasses in serum and BAL of such patients (222). They documented significantly increased mean levels of IgG1 and IgG3 compared with controls, in serum and in BAL. Barth and co-workers found no difference in the proliferative response of peripheral blood lymphocytes to different mitogens comparing sarcoidosis patients with healthy controls. However, B-cell differentiation responses in BAL fluid displayed a significant decrease in the secretion of IgG subclasses of IgG1, IgG3 and IgG4 (224). Milburn and co-workers noted that serum levels of all four subclasses are higher in sarcoidosis patients (223).

1.6.3 Mechanism of Fibrosis

Mechanisms by which the development and recovery of granulomas in sarcoidosis patients is dysregulated are uncertain and potentially vary according to the nature of the condition. The tissue granulomas may resolve, continue, or advance towards fibrosis and patients may have highly variable disease.

The inflammation resolves with minimum to no clinical sequelae without treatment in a large percentage of cases. Tregs inhibit granulomatous inflammation in this regard by developing mediators (IL-10, TGF-β), which inhibit the proliferation and activation of T-cells and encourage the alternative macrophage (M2) pathway. These same mediators can, however, lead to irreversible tissue fibrosis (138,225). The pathways implicated in a fibrotic outcome are unclear.

The suggestion is that contributions to fibrosis are by a change from Th1 to Th2 cytokine profile along with other mediators including IL-6, TGF-β, osteopontin, insulin-like growth factors (IGFs) and matrix metalloproteinases (226). IFN-γ at granulomatous inflammatory sites downregulates fibroblast proliferation and extracellular matrix deposition (227). Cytokines including TGF-β, IGF-1 and matrix metalloproteinases, which are pro-fibrotic mediators, are discovered at sites of inflammation (201,226). The M2 phenotype alveolar macrophage, distinguished by the upregulated CC motif chemokine ligand (CCL) 18 expression that promotes lung remodeling and fibrosis has been demonstrated in patients with fibrotic pulmonary sarcoidosis (228).

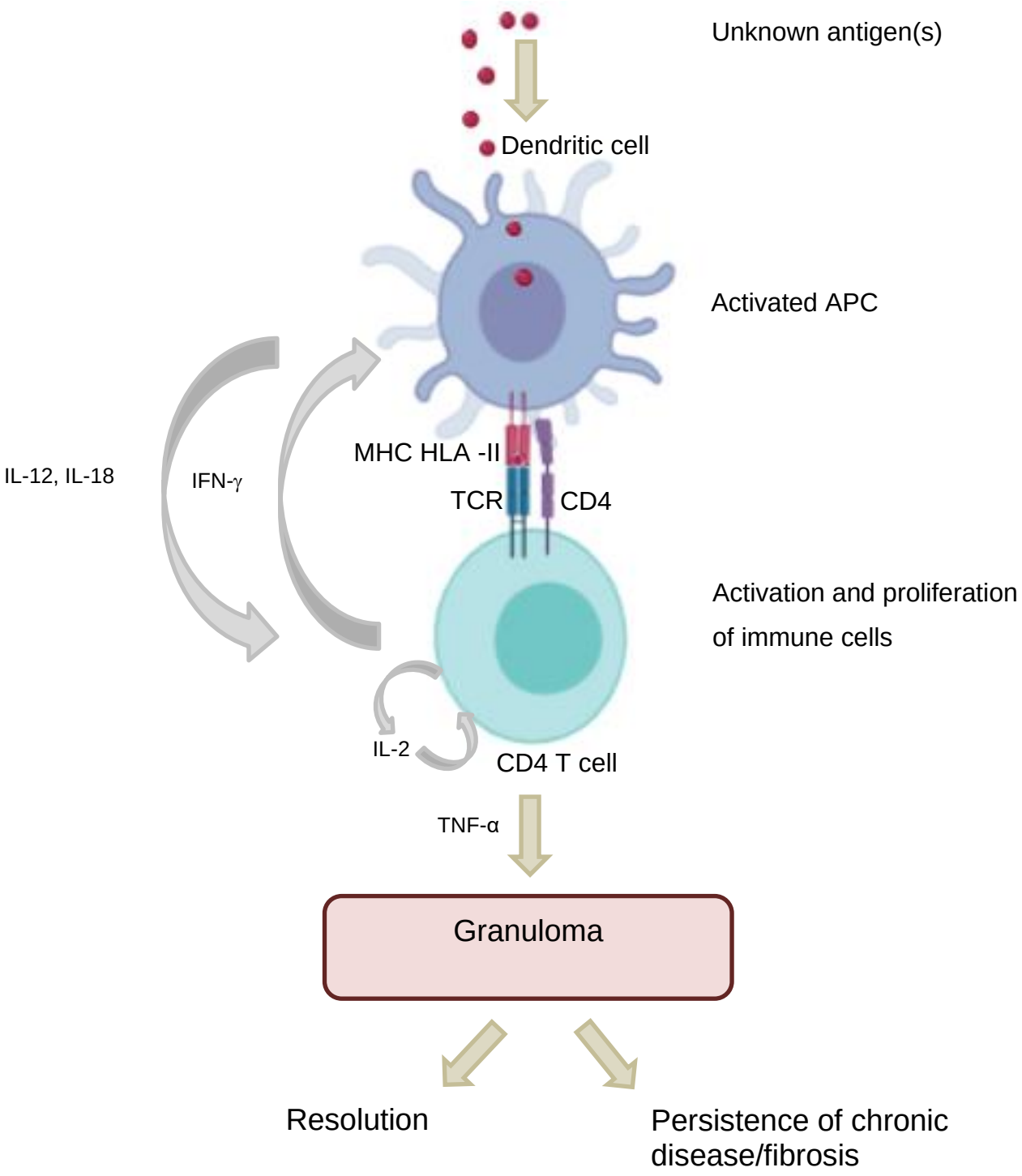
IL-4 as well as IL-13 regulates the anti-inflammatory or regulatory Th2/M2 response cytokine profile (229). These two cytokines employ Janus kinases (JAKs) to initiate signalling and activate STAT6 (229). JAK/STAT signalling promotes the production of IL-10 that favours IFN-γ. In general, a better IL-10 ratio relative to IFN-γ (229) typically

favours disease resolution, but in some cases it promotes fibroblast matrix formation, tissue remodelling, and fibrosis (230,231).

1.7 Sarcoidosis Models

The characteristics of the ideal sarcoidosis research model should reproduce the fundamental molecular and morphological characteristics of human sarcoidosis granulomas, correctly reflect the different stages in the evolution of the granuloma, be reproducible with low cost, and easily usable to provide high-throughput, accelerating the pace of research (232). Hindrance in sarcoidosis research has been due to lack of relevant and robust sarcoidosis models, whether *in-vivo, in-vitro*, or animal, *ex-vivo* tissue or in silico models. New laboratory models reflecting the intricate and diverse intercellular interactions in sarcoidosis are expected to step-up the pace of clinical exploration for new therapeutic targets and biomarkers in the future (233,234).

A simplified hypothesised model is shown in Figure 1.2. Antigens are presented to APCs that engulf the unknown antigen(s). These antigens are processed and presented to a T-cell receptor through HLA Class II molecules, resulting in T-cell proliferation, T-cell activation, along with other monocyte recruitment as well as macrophage differentiation. Not all the cytokines are described. A myriad of cytokines, inflammatory along with cell bridging molecules are involved.



"HLA II = human leukocyte antigen class II, IFN-γ = interferon gamma, TCR = T-cell receptor, TNF-α = tumour necrosis factor alpha, IL = Interleukin, CD4 = CD4 receptor"

Figure 1.2. Hypothesised overview of sarcoidosis pathogenesis. Adapted with permission from (235,236).

1.8 Clinical Features

1.8.1 Clinical Symptoms

Sarcoidosis clinically may be categorised in several ways, including by way of presentation, by way of involvement of organs, along with by way of natural history (237,238). Usually, the disorder occurs in the 20 to 50 year age group; however, there are reports both in childhood and in the elderly. Over 90% have active disease in the thorax. Sarcoidosis can affect every organ as well as can present with any organ-related symptoms. In addition, patients can have non-specific constitutional symptoms, including fever, weight loss, anorexia and fatigue.

The clinical features range from an asymptomatic patient with an incidental chest radiographic finding to chronic, gradual, progressive disease with organ dysfunction (2). Symptom onset is incremental in most cases, but it is slightly different from one ethnic group to another (239). In northern populations, for example, disease onset typically is acute with signs including erythema nodosum, fever, and/or ankle arthritis (2,8,240). The initial symptoms and signs, however, can also be acute onset of epilepsy, cranial neuropathy or cardiac arrest and sudden death (239,241).

Sarcoidosis' natural history may be a self-limiting, persistent yet controlled, or persistent and progressive, disease. In about 50% of cases, the disease recovers naturally within two years and in many others within five years, although recovery is even possible after five years (9). The disorder can also be categorised based on major organ involvement; the most severe clinical presentations are pulmonary sarcoidosis, cardiac sarcoidosis and neuro-ophthalmic sarcoidosis.

1.8.2 Acute Presentation

Some patients have an acute presentation, known as the Löfgren's syndrome. In 1946, Sven Löfgren first identified the acute Löfgren's syndrome. These patients present with an abrupt onset of erythema nodosum, bilateral hilar lymphadenopathy, polyarthritis and fever with transient resolution. The chest x-ray may be normal or have hilar and mediastinal lymphadenopathy. There may be cardiac involvement with arrhythmias. The acute disease may resolve spontaneously and a biopsy is typically not needed for diagnostic histological confirmation (9). Patients with Löfgren's syndrome have distinct seasonal, genetic, and immunological characteristics (70,115,242–244) and it is considered a well-defined phenotype of sarcoidosis (242). Although relapses can occur, this is supposed to be less frequent as compared with the more chronic form of the disorder. The prognosis is usually good for patients with acute onset sarcoidosis. In Black patients this condition is rare. The prognosis of acute onset sarcoidosis is generally good.

1.8.3 Heerfordt's Syndrome

Heerfordt's syndrome is a very rare sarcoidosis syndrome of "enlargement of the parotid and submaxillary salivary glands", uveitis, and cranial nerve paresis, particular the seventh nerve (245).

1.8.4 Chronic Sarcoidosis

The more gradually and insidiously the symptoms come on, the more likely are patients to develop chronic disease (2,3,8). For chronic disorder, risk factors include the presence or persistence of recurring disfiguring lupus pernio, an aggressive purplish rash on the nose, ears as well as cheeks, and the presence of multi-organ involvement

when diagnosed (246). The chronic form has persistent disease and frequent recurrences (5).

1.8.5 Pulmonary Sarcoidosis

In more than 90% of cases, the lungs are most often involved with sarcoidosis, with radiographic or physiological disturbances (236). Signs and symptoms are non-specific. Patients might have dyspnoea, cough, and/or chest pain, wheezing, crackles, or normal chest examination. Clubbing is uncommon but typically related to advanced bronchiectasis or liver disease when present.

The Scadding staging method has identified x-ray patterns of the chest in sarcoidosis as shown in Table 1.3 and Figure 1.3. The spiral/thin-section computerised tomography (CT) scanning of the chest, where it is easily obtainable, is gradually replacing this staging system. The chest radiograph stages 0-IV do not fit chronologically with disease progression, but correlate with the probability of potential radiographic along with disease resolution.

Table 1.3. Scadding chest radiograph stages reproduced with permission from (1).

Stage	Description
0	Normal
1	Bilateral hilar lymphadenopathy without parenchymal changes
2	Bilateral hilar lymphadenopathy with parenchymal changes
3	Parenchymal changes without hilar lymphadenopathy
4	Pulmonary fibrosis, conglomerate mass formation

A normal chest x-ray, or stage 0 illness, is seen in 5-10% of patients at presentation. Up to half of patients with stage 0 disease may have parenchymal anomalies on CT scan when comparing chest x-rays to CT findings in sarcoidosis (247,248). In addition, 20-30% of these patients have defects in their lung function (249). This group would have less chance of progression of the disease.

Classically, bilateral hilar, mediastinal, aorto-pulmonary window, and right paratracheal region lymphadenopathy depict Stage I disease. Although the lungs appear radiographically clear, these patients would have granulomas on transbronchial lung biopsies. Stage I is the most common x-ray presentation and most patients (55-90%) will exhibit spontaneous resolution (2,3,5).

CT can also demonstrate adenopathy at less common locations, for example the posterior and anterior mediastinum, axillary and supraclavicular regions, as well as calcification of the nodes (248).

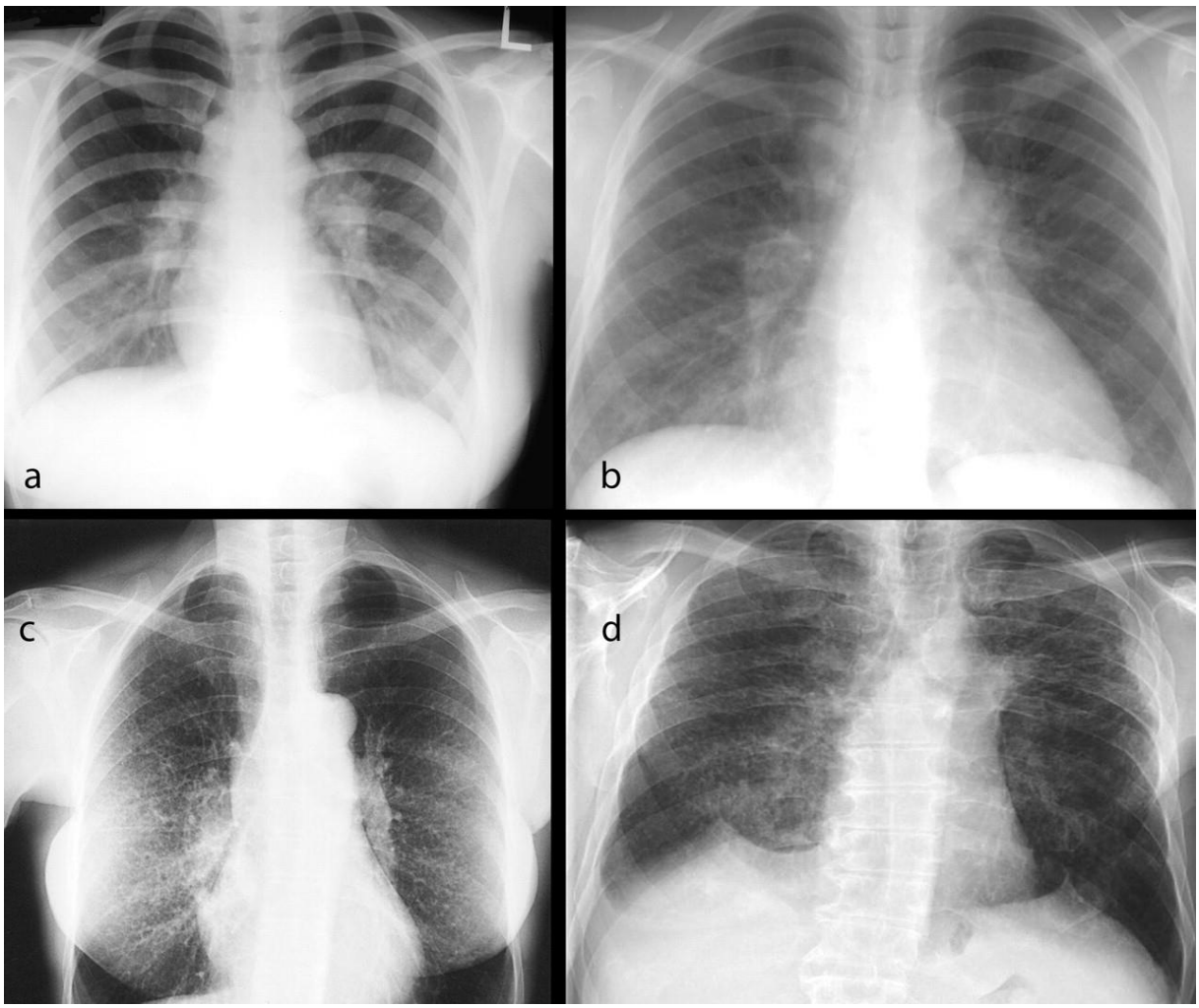


Figure 1.3. X-ray images of the 4 stages of sarcoidosis. Reproduced with permission from (250).

("a, Stage I; b, Stage II; c, Stage III; d, Stage IV")

Stage II chest radiograph includes bilateral hilar or mediastinal adenopathy, or both, as well as pulmonary opacities. Around 40-70% of such patients have natural remission (1–3,8). Pulmonary defects in the middle and upper regions can be nodular, reticular, or ground-glass opacities. The CT will indicate 2-10 mm nodules along bronchovascular bundles in a perilymphatic distribution around the hilar regions, and subpleurally. The nodules can coalesce.

A radiograph of stage III reveals parenchymal anomalies only without hilar or mediastinal lymphadenopathy (248). Stage III presentation is correlated with a probability of spontaneous resolution of just 10% to 20%.

The stage IV chest radiograph reveals fibrosis, primarily seen in the upper as well as middle lung areas, and is also connected with conglomerate mass formation, loss of upper lobe volume, peripheral bullae, hilar retraction along with cystic lesions. If the fibrosis is visible, there is no likelihood of spontaneous resolution. In patients with stage IV disease, aspergilloma is a serious complication in those with cystic lesions. Symptoms may include change in sputum production and cough, worsening dyspnoea, and haemoptysis. Imaging typically demonstrates the classic mycetomas or cavitation masses (250–252).

Another common form of pulmonary sarcoidosis is endobronchial involvement. In one study, almost half of the endobronchial biopsies indicated the presence of granulomatous inflammation (253). Within this group of 62 patients, more serious and progressive disease was associated with positive endobronchial biopsies. In another study of endobronchial disease patients, up to 10% had stenoses that had been extreme enough to induce an asthma-like condition with limited airflow, localized wheezing, and abnormal gas exchange (254). With endobronchial involvement patients may have intractable cough and airway obstruction, ranging from bronchial distortion due to fibrosis and scarring (248).

Other pulmonary features include different types of pleural disease, found in 2-5% of cases (252), for example, chylothorax, pneumothorax, pleural nodules, pleural thickening and pleural effusion. The detection of more subtle cases of pleural involvement has increased with CT scans, particularly with high-resolution modalities,

and the prevalence estimates range widely from less than 1% to 96% (255,256). Pleural disease was reported at every disease stage in a 145 biopsy-proven cases of pleural involvement study (257). For pleural involvement the age range was between 30 and 50 years, with an even gender distribution. Pleural disease was unilateral in over 80%, more commonly on the right side (53% vs. 29%). While several eosinophilic effusions were reported, the majority with lymphocytic exudates were small. It has been stated that effusions resolved with steroid treatment or naturally. The most frequent pleural manifestation associated with sarcoidosis may be pleural thickening with CT studies that indicate a prevalence of 11% to 71%. High resolution CT has made identification simpler (257).

Pneumothorax is a rare complication, possibly due to sub-pleural blebs that occur in advanced fibrocystic disease or sub-pleural granuloma necrosis. It can also be a coincidental occurrence. In this complication the role of corticosteroids is unclear (257). In a few cases, chylothorax was reported due to compression by the lymphadenopathy or adhesions obstructing the thoracic duct (257).

In sarcoidosis, “the prevalence of pulmonary arterial hypertension, as well as right ventricular dysfunction, ranges from 4% to 28%” (258). “Pulmonary hypertension (PH) is more common in patients with advanced lung disease and in up to 74% of patients” waiting for lung transplantation, were identified as having PH (258). PH may occur with almost normal lung function parameters, often without parenchymal lung disease (259,260). The pathophysiology is complicated and unclear (261). Potential causes of PH include interstitial lung disease, fibrous disruption of the pulmonary vascular system, lymphadenopathy compressing the pulmonary arteries, vasospasm, pulmonary veno-occlusion, myocardial dysfunction, porto-pulmonary hypertension as well as intrinsic vasculopathy related to sarcoidosis (259).

In 1973, Liebow, an American pathologist, described sarcoid necrotising granulomatosis, a rare form of sarcoidosis (262). In this form of sarcoidosis, patients can be asymptomatic or have systemic symptoms of weight loss, fatigue, pain and fever. They may have symptoms of chest pain, cough, and dyspnoea and extrathoracic symptoms, if involved. The typical x-ray shows single or multiple pulmonary nodules that may have cavitation. Necrotising sarcoid granulomatosis is confirmed by “the presence of necrosis and granulomatous vasculitis on histopathology”. The clinical course is usually mild and responds to corticosteroids; but individual cases of extensive extrathoracic involvement as well as initial severe disease have been reported (144,263).

Lung function testing is critical for evaluating the severity and monitoring of pulmonary disease and is discussed under the section on Diagnostic Evaluation (1.9.4).

1.8.6 Cutaneous Sarcoidosis

In approximately 25% of sarcoidosis patients, the skin is involved (264). Cutaneous involvement may include ulcerations, vitiligo, macules, papules, nodules, plaques, erythema nodosum, psoriasis-like rash, acneiform eruptions, keloids, and ichthyosiform scaly rash (264). Lesions can appear in crops or be single, can occur in tattoos and scars and sometimes include the extremities, nape, scalp and trunk (265). In African-American patients skin lesions may also leave pits, scars, be hyperpigmented or light depigmented areas. Cutaneous involvement typically occurs in conjunction with systemic disease, though they may precede other organ involvement (264,265). At initial presentation close examination of the skin is important since the biopsy of a sarcoidal lesion has a high yield and is relatively non-invasive.

Erythema nodosum and lupus pernio are the two most classic skin lesions. Erythema nodosum is a non-specific hypersensitivity reaction. It appears in about 10% of patients, usually with a tender, indurated, red nodule, one to two cm in diameter on the anterior part of the lower legs (37). It is more prevalent in women and citizens from Europe, Puerto Rico and Mexico and less prevalent in Japanese and African-American patients (2,264). Erythema nodosum normally starts suddenly and is followed by fever, malaise, and arthralgia. Erythema nodosum usually lasts for three weeks. Characteristically the nodules on biopsy indicate non-specific septal panniculitis and does not help the histological diagnosis of sarcoidosis. Biopsy typically yields granulomas for skin lesions other than those of erythema nodosum (71,266).

Lupus pernio, an indurated purplish lesion affects the periorbital areas, malar, nasal alae, nose, and areas around ears, lips and eyes, in particular (71,264). Lupus pernio can be particularly disfiguring with resulting destruction of underlying cartilage and bone as seen in few cases. It is more common in females and related to chronic, severe illness (267).

Some examples of cutaneous lesions seen in sarcoidosis are illustrated in Figure 1.7 to Figure 1.14, reproduced with permission from (268).



Figure 1.4. “Papular sarcoidosis presenting with papules on the eyelids, malar cheeks and forehead, some of which coalesce into plaques”. Reproduced with permission from (268).



Figure 1.5. “Annular sarcoidosis plaque resembling tinea corporis and granuloma annulare”. Reproduced with permission from (268).



Figure 1.6. “Verrucous sarcoidosis on the posterior neck”. Reproduced with permission from (268).



Figure 1.7. “Infiltrated red-brown plaques on the nose and cheek. Note scars from ulcerations”. Reproduced with permission from (268).



Figure 1.8. “Infiltrated plaque on the nose with multiple telangiectasias”. Reproduced with permission from (268).



Figure 1.9. “Sarcoidosis infiltration of a tattoo resulting in induration”. Reproduced with permission from (268).



Figure 1.10. “Hypopigmented sarcoidosis of the posterior neck”. Reproduced with permission from (268).



Figure 1.11. “Biopsy-proven ichthyosiform sarcoidosis with adherent polygonal brown scales on the lower extremity”. Reproduced with permission from (268).

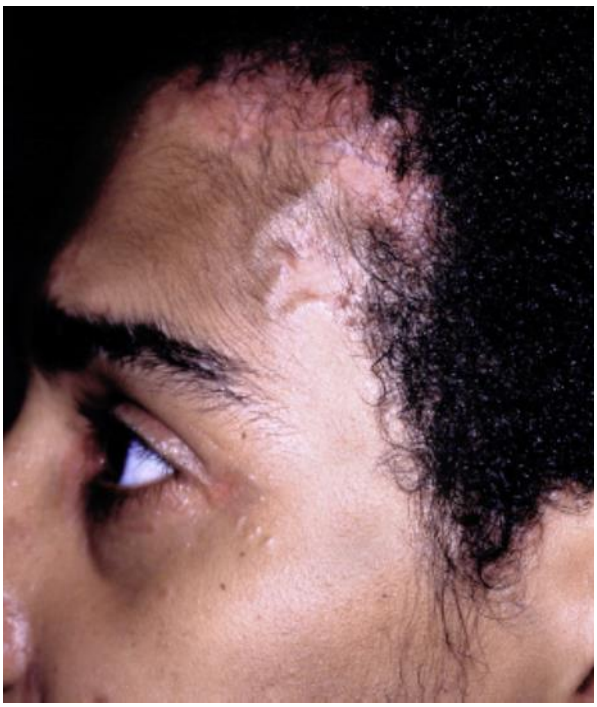


Figure 1.12. “Frontal band of alopecia with indurated borders”. Reproduced with permission from (268).



Figure 1.13. “Erythema nodosum in a patient with Löfgren’s syndrome”. Reproduced with permission from (268).

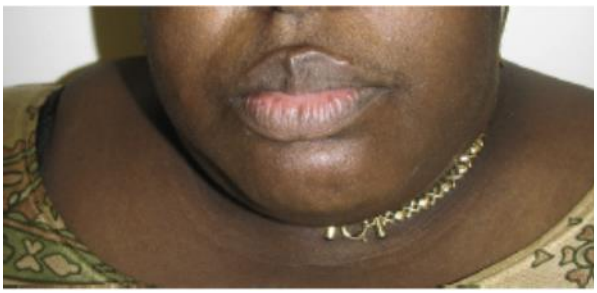


Figure 1.14. “Sarcoidosis involving the labial vermillion border”. Reproduced with permission from (268).

1.8.7 Cardiac Sarcoidosis

Cardiovascular involvement with sarcoidosis occurs frequently in Japan, with one post-mortem study showing cardiac sarcoidosis in almost 50% of cases (269). Approximately 2% to 10% of patients with sarcoidosis experience clinically evident cardiac involvement; however, cardiovascular involvement may be more widespread than previously recognised (270,271).

The “left ventricular free wall, the basal portion of the ventricular septum, the right ventricular free wall and the atrial walls” are the most affected (269,272). “Conduction abnormalities from first-degree atrioventricular (AV) block to complete heart block are common manifestations of sarcoidosis”. Around one third of the patients affected have complete heart block and may have syncope. (273).

The most dreaded extrapulmonary complication is unexpected sudden death because of ventricular fibrillation or tachycardia (272). Since this complication may occur in any sarcoidosis patient, palpitations, dizziness or electrocardiogram (ECG) anomalies, for example, first-degree AV block, should prompt cardiovascular assessment. Pericardial effusions, pericarditis, ventricular aneurysms, mitral regurgitation and cardiac tamponade are other less common cardiovascular complications. Because cardiac sarcoidosis sometimes occurs without apparent systemic disease, it should be recognised as a cause of rhythm disturbances, particularly in cases with non-ischaemic cardiomyopathy.

1.8.8 Neurosarcoidosis

Five to fifteen percent of patients have neurosarcoidosis that is clinically apparent (3,274). Meningeal infiltration and inflammation is the commonest form of central

nervous system involvement. (275). Encephalopathy, seizures, headaches, hydrocephalus and cranial neuropathies, may be clinical manifestations. Granulomatous inflammation of the cranial nerves most commonly affects the seventh nerve unilaterally; however, any cranial nerve can be involved (2,8). Lesions affecting the central nervous system occur much less frequently. However, when there is pituitary involvement, patients can manifest with hyperprolactinaemia and diabetes insipidus. Myopathy, peripheral neuropathy, radiculopathy and myelopathy have been reported (2,3).

In 2002, small-fibre neuropathy ("diminished intraepidermal nerve fibre density") was perceived "as a non-granulomatous parasarcoidosis syndrome" (276). Presentations include painful hyperaesthesia or hypoaesthesia and/or dysautonomia. This condition is inadequately understood and can be extremely difficult to treat.

1.8.9 Ophthalmic Involvement

Ophthalmic involvement occurs in 11% to 83% of patients with sarcoidosis (3,9). Uveitis is the most common presentation. In Black patients, anterior uveitis (iritis, iridocyclitis) is more prevalent, and posterior uveitis (choroiditis) is more commonly seen in Whites (277). Anterior uveitis presents acutely, with pain and discomfort, photophobia, tearing excessively, and redness, but can have a chronic course. Posterior uveitis is more gradual and progressive and is likely to cause visual disturbances. Conjunctival nodules are common but are generally asymptomatic. A conjunctival biopsy can confirm the diagnosis even in patients with no visual symptoms, with varying yields. A small series of conjunctival biopsies found yields of 33-70%, also without apparent involvement of the conjunctiva (278). Uncommonly, the enlargement of the lacrimal gland is characteristic in sarcoidosis. Posterior uveitis,

vitreous haemorrhage, cataracts, glaucoma, and retinal ischaemia with neovascularisation can occur. These patients have a greater frequency of central nervous system involvement. Therefore, yearly slit-lamp assessment of the eye is recommended, as blindness occurs in around 10% of patients with sarcoid-associated uveitis (279).

1.8.10 Peripheral Lymphadenopathy Involvement

While the most common lymphatic manifestation is intrathoracic lymph node enlargement, “all lymph node bearing sites may be involved (5-30% of patients)” (3,9). Peripheral lymph nodes might be small or several centimetres in size as well as are typically non-tender and mobile. These, if found, may be a biopsy site for histological confirmation.

1.8.11 Sarcoidosis of the Upper Airway including Oral Cavity

There are a number of otorhinolaryngological presentations, including salivary gland involvement, which often lead to excessive xerostomia, hearing loss, vestibular symptoms and otitis media (280,281). However, the most difficult to diagnose is nasal and paranasal sinus involvement. These patients typically have non-specific symptoms, including infections, headaches, sinus pressure, postnasal drip and nasal congestion (281). This can be associated, for example, with characteristics of clinical sarcoidosis, lupus pernio, nasal deformations such as saddle nose deformity and nasal mucosal defects such as crusting, bleeding, and submucosal nodule friability. These nodules occasionally will induce oral ulcers across the hard or soft palate (282). Vocal cord involvement may cause hoarseness. The other rare symptoms of nasal or sinus disorders are epiphora (chronic weeping due to intranasal tear tract impairment),

intraorbital and intracranial damage by invasive mass lesions, and anosmia (loss of sense of smell caused by direct olfactory epithelium involvement or olfactory cleft obstruction) (283). Approximately 10% of patients undergo parotid and salivary gland enlargement (280).

1.8.12 Hepatic and Splenic Sarcoidosis

In up to 80% of sarcoidosis patients, granulomas can be detected on liver biopsy. Hepatic involvement is at least twice as widespread in Blacks as it is in Whites. The liver is palpable in 15% of patients. Asymptomatic liver enzyme rise, chronic cholestasis, variceal bleeding from portal hypertension, hepatopulmonary syndrome with refractory hypoxaemia and hepatic insufficiency cirrhosis can be manifestations of sarcoid liver disease (3). Patients also experience constitutional symptoms including weight loss, night sweats, loss of appetite, fever, and abdominal pain in sarcoidosis-associated granulomatous hepatitis. When evaluating hepatitis, viral screening and measurement of serum antimitochondrial antibodies, to exclude other causes of liver disease, should be performed. The predominant rise is in gamma-glutamyl transferase (GGT) and alkaline phosphatase (AP). In 6% of patients splenomegaly occurs. Imaging by abdominal ultrasound and CT scans may show typical hypodense hepatic and splenic lesions (284). Splenic nodules are more frequently seen than hepatic lesions (284). Massive splenomegaly can occur rarely and may be associated with thrombocytopenia or other cytopenias (285).

1.8.13 Gastrointestinal Tract Involvement

Rarely does sarcoidosis affect the stomach, pancreas, rectum, colon, appendix, and oesophagus (286). The oesophagus can be compressed, causing dysphagia, by

intrathoracic lymphadenopathy. Gastric sarcoidosis may manifest as either peptic ulceration, narrowing of the lumen of the stomach due to granulomatous inflammation, or gastric wall fibrosis resulting in pyloric obstruction (3). Chronic atrophic gastritis with anti-parietal cell antibodies has been described which results in a pernicious anaemia-like picture (3,19).

1.8.14 Haematological Sarcoidosis

About 40% of patients will have eosinophilia, lymphopenia, leukopenia or anaemia (287). Thrombocytopenia is uncommon, however, it may occur as a result of bone marrow involvement and with platelet sequestration due to splenic enlargement (2,287).

1.8.15 Joint and Musculoskeletal Sarcoidosis

While arthralgia and arthritis are common in patients with Löfgren's syndrome, there is less joint involvement in other types of systemic sarcoidosis. Arthralgia occurs in 25% to 39% of patients, but deforming arthritis is rare (3,9). Bony involvement occurs in between 3% and 13% of patients (288), and with the growing utilisation of MRI, future estimates of joint and musculoskeletal sarcoidosis incidence may be increased. Typically, when the bones are involved, plain x-rays or MRI will show bone cysts in the phalanges (dactylitis), although almost any bone can be affected. Symptomatic muscle involvement is also uncommon, characterized by chronic myopathy, acute myositis or nodules. However, in systemic sarcoidosis without clinical signs of muscle disease, granulomas on muscle biopsy are observed in 20% to 75% of cases (289).

1.8.16 Exocrine Gland Sarcoidosis

Granulomatous inflammation of the salivary, parotid, and lacrimal glands results in tender, swollen glands. Sicca syndrome, with dry mouth and dry eyes, occurs in less than 5% of sarcoidosis patients (1,280).

1.8.17 Endocrine Gland Sarcoidosis

The most common endocrine disorder in sarcoidosis is abnormal calcium metabolism. Hypercalcaemia and hypercalciuria are both caused by increased formation of 1,25-dihydroxyvitamin D by activated macrophages in sarcoid granulomas (290). Hypercalciuria is more common than hypercalcaemia, and the initial assessment should involve a 24-hour urine sample for total calcium. Diabetes insipidus secondary to pituitary involvement is rare. Rarely thyroid disease, either hyperthyroidism or hypothyroidism, may also occur.

1.8.18 Renal Sarcoidosis

Hypercalcaemia may lead to renal calculi, nephrocalcinosis, and occasionally, renal insufficiency (290). Other sequelae include interstitial nephritis and granulomatous mass formation (291).

1.8.19 Genitourinary Sarcoidosis

“Genitourinary manifestations of sarcoidosis in men include testicular masses and acute epididymo-orchitis” (292,293). In women, sarcoidosis may manifest with uterine or ovarian involvement that may lead to dysmenorrhoea or mimic malignancy or fibroids (294). It has been estimated that reproductive system sarcoidosis occurs in less than 1% of clinically diagnosed cases, and 5% of autopsy cases (294).

1.8.20 Psychosocial Manifestations and Impact on Quality of Life

Sarcoidosis considerably affects QoL. It is related to stress, fatigue and exhaustion, pain, sleep disturbances, cognitive failure and depression and these may persist after remission (295). The QoL may deteriorate when patients are placed on corticosteroid therapy. Fatigue is commonly reported and may be disabling for a subset of patients (296). Social, family and work life could also suffer as with any other chronic illness. The prevalence of pain in sarcoidosis is unclear, but clinical experience suggests it is common, with frequent reports of arthralgia, myalgia, headache, and chest pain. The cause of pain is often multifactorial. A subset of patients meets diagnostic criteria for fibromyalgia. In addition to its association with pain, small fibre neuropathy may cause autonomic dysfunction with gastrointestinal dysmotility, incontinence or retention, sicca syndrome, flushing, sweats, orthostatic hypotension, and sexual dysfunction (297). The common clinical manifestations in sarcoidosis are summarised in Table 1.4.

Table 1.4. Common clinical manifestations in sarcoidosis. Reproduced with permission from (298).

Affected organ	Frequency of occurrence (%)	Common findings
Lungs and thoracic lymph nodes	>90	Dyspnoea, cough, chest pain, pulmonary hypertension, mixed pulmonary function test abnormalities (obstruction, restriction, diffusion deficits)
Skin	20–30	Nodules, plaques, lupus pernio, erythema nodosum (a non-granulomatous panniculitis)
Eyes	20–25	Uveitis, conjunctivitis, lacrimal gland enlargement, sicca syndrome, optic neuropathy
Liver and/or spleen	10–20	Hepatosplenomegaly, jaundice, elevated liver function tests, cirrhosis, hypersplenism
Cardiovascular system	10–20	Ectopy, heart block, arrhythmias, cardiomyopathy, sudden death
Central nervous system	10–25	Cranial neuropathy, mass lesions, aseptic meningitis and/or encephalitis, myelitis, spinal cord and peripheral neuropathy, small fibre neuropathy, pain, hypothalamic–pituitary involvement
Sinuses and upper respiratory tract	5–10	Chronic sinusitis, laryngeal involvement, parotid gland involvement
Bones, joints, muscle	5–15	Chronic arthritis, dactylitis, lytic bone lesions, myopathy
Haematologic system	>50	Peripheral lymphopenia, hypergammaglobulinaemia
Renal system (including calcium abnormalities)	5–10	Hypercalcaemia and/or hypercalciuria, nephrocalcinosis, nephrolithiasis
Endocrine system	5–10	Hypothalamic–pituitary involvement, pancreatic mass, Heerfordt's syndrome (uveoparotid fever)
Gastrointestinal and reproductive tract	<1	Gastric nodules, ovarian or testicular masses

% = Percent

1.8.21 Associated Conditions

1.8.21.1 *Sarcoidosis and Pregnancy*

There is usually little long-term effect on the course of sarcoidosis from pregnancy (299). In contrast to diseases such as asthma and systemic lupus erythematosus that may progress during pregnancy, spontaneous improvement in systemic sarcoidosis has been reported in a few patients during pregnancy, although exacerbations often follow several months after delivery (300,301). Temporary clinical improvement may be connected to suppressed Th1 immunity during pregnancy associated with enhanced Treg function (299).

1.8.21.2 *Altered Th1 Immunity and Human Immunodeficiency Virus*

Altered or enhanced Th1 immunity with administration of Th1-promoting therapeutics, for example, IFN-β, IL-2, IFN-γ and IFN-α may be connected with initiation or sarcoidosis flares (302). In the context of HIV, sarcoidosis may develop in HIV-infected patients following initiation of antiretroviral therapy with immune reconstitution (303).

1.8.21.3 *Common Variable Immunodeficiency*

Granulomatous inflammation has been identified in patients with hypogammaglobulinaemia secondary to common variable immunodeficiency (CVID). Since CVID can occur at any age and especially in sarcoidosis patients, those who have recurrent infections, or in a child with sarcoidosis, one must consider CVID as a cause (304).

1.8.21.4 *Autoimmune Disease*

Sarcoidosis is associated with an assortment of disorders of the immune system, such as ulcerative colitis, Crohn’s disease, primary biliary cirrhosis, Sjögren’s syndrome, scleroderma, autoimmune haemolytic anaemia, along with autoimmune

endocrinopathies (131). Recently, a syndrome of immune-mediated disorders in patients with elevated serum IgG4 levels has been described, commonly presenting as adenopathy, constitutional symptoms, and mass-like tumours (305). Granulomatous inflammation is observed in some of these patients and can mimic sarcoidosis (306).

1.8.21.5 *Cancer*

Non-caseating granulomas may be seen in or adjacent to 3% to 10% of tumours and in approximately 4% of local draining lymph nodes (307). Less commonly, multi-system granulomas consistent with systemic sarcoidosis form in patients with a recent or previous diagnosis of cancer, or following cancer chemotherapy (308).

1.8.21.6 *Paediatric Sarcoidosis*

Sarcoidosis is rare in the preteen ages. In the teenage years, clinical manifestations and prognosis in children is similar to that of adults (309). Early onset disease in children under 5 years of age typically involves skin, eye, and joint involvement that can mimic juvenile rheumatoid arthritis or Blau's syndrome, an autosomal dominant granulomatous disease caused by a NOD2 mutation (310). This form of sarcoidosis carries a much worse prognosis than disease presenting in older children. Paediatric sarcoidosis may also be associated with CVID (304).

1.8.21.7 *Sarcoidosis in Older People*

There is a second peak incidence of sarcoidosis in the age range of 50 and 65 years. In the ACCESS study, one third of patients were over the age of 50 years (311). Recurrent sarcoidosis after many years, even decades of apparent remission, is also

seen. They often manifest with new onset neurologic involvement, or recurrent manifestations of original organ involvement (312).

1.8.21.8 *Sarcoidosis and Idiopathic Pulmonary Fibrosis*

Sarcoidosis and idiopathic pulmonary fibrosis (IPF) have been rarely observed in the same patient. The clinical characteristics of patients with “combined sarcoidosis and idiopathic pulmonary fibrosis” (CSIPF), are only recently being reported in the literature, albeit small patient numbers (313,314). The true prevalence of CSIPF is not known. It is conceivable that patients with sarcoidosis coincidentally developed IPF, and/or have a genetic predisposition to manifest these diseases at various times. More studies are required.

1.8.21.9 *Checkpoint Inhibitors Related Sarcoidosis-like Reactions*

Immune-checkpoint blockade using programme cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) inhibitors and CTLA-4 inhibitors has recently been endorsed for the treatment of many solid and haematological malignancies and the indications are rapidly expanding. The immune-related adverse events include sarcoid-like granulomatosis and lymphadenopathy (315,316).

1.8.21.10 *Corona Virus Disease 2019 (COVID)-19 and Sarcoidosis*

Patients with pulmonary sarcoidosis may be at high risk for severe disease connected to COVID-19. Little is known about the effect of sarcoidosis treatments on susceptibility to, and disease expression or progression of, COVID-19 infection. Immunosuppressive treatments for sarcoidosis may need to be adjusted in the current climate (317,318).

1.9 Diagnosis

Since the symptoms of sarcoidosis are somewhat non-specific, diagnosis can be difficult. Sarcoidosis is diagnosed by exclusion of other disorders.

1.9.1 Differential Diagnosis

Conditions that can cause pulmonary granulomatous inflammation include mycobacterial, spirochaetal, bacterial, fungal, and protozoal infections. Occupational diseases and illnesses caused by inhaled environmental agents, including hypersensitivity pneumonitis, because of inhaled inorganic and organic antigens, metal-induced disorders, for example chronic beryllium disease (CBD) (319) along with silicosis (320), must also be considered. Additionally, exposure and infectious related diseases; other considerations should include granulomatosis with polyangiitis and idiopathic interstitial pneumonias such as lymphocytic interstitial pneumonia.

CBD is of notable interest since it is radiographically, clinically, as well as histopathologically impossible to tell apart from sarcoidosis. Beryllium exposure elicits CBD, an occupational hypersensitivity condition. Historically, there has been workplace exposure in the computer and aerospace industries, dental laboratories, space and nuclear engineering, fluorescent bulb manufacture, electronics, ceramics and metal extraction (114). In affected organs, CBD is characterized by non-caseous granulomas, primarily of the skin and lung. Sarcoidosis can be incredibly difficult to discern from berylliosis and depends on a thorough interrogation of workplace experience, with beryllium sensitising of blood cells or with BAL examination. Unsuspected CBD can be misdiagnosed as sarcoidosis, particularly in patients with continuous exposure and supposed corticosteroid resistance. With a death rate of about 25%, CBD prognosis is worse than sarcoidosis. Diagnosing CBD and preventing

further beryllium exposure are critical, as these steps can lead to disease resolution (114,321).

In lymph nodes, the presence of non-caseating granulomas is connected with the same broad differential diagnosis. Mycobacterial infection, cat-scratch disease toxoplasmosis and brucellosis may cause a granulomatous lymphadenitis (322). Enlarged lymph nodes can be caused by neoplasms, such as Hodgkin's and non-Hodgkin's lymphomas and metastatic carcinoma (323). Therefore, the whole node must be histopathologically examined to exclude these diagnoses when granulomas are found on a lymph node biopsy. In other tissues, non-caseating granulomas should be equally distinguished. Skin reactions to foreign body, schistosomiasis, primary biliary cirrhosis, and Crohn's disease are among other entities that may cause non-caseating granulomas (1,324).

In sarcoidosis a number of auto-antibodies have been found, including rheumatoid factor and antinuclear antibodies (325). There seems to be a link between autoimmune endocrinopathies and sarcoidosis, and with antiphospholipid antibodies (326). Some patients have concurrent diagnoses and the clinical presentations could be similar to autoimmune disorders.

Although rare, sarcoidosis in children may simulate the symptoms and signs of juvenile rheumatoid arthritis. To differentiate the two entities a biopsy is usually required (327). In adults, the clinical manifestations of rheumatoid arthritis and sarcoidosis are rather distinct. There have been reports of the coexistence of the two diseases suggesting a possible aetiological relationship (328). Sarcoidosis and Sjögren's syndrome could possibly also coexist (329). Both conditions can have cutaneous anergy, lung involvement, parotid swelling and keratoconjunctivitis sicca. Table 1.5 "summarizes

the major categories of both non-infectious and infectious disorders" that need to be included in the differential diagnosis of sarcoidosis (330).

Table 1.5. Differential diagnosis of granulomatous diseases. Adapted with permission from (330).

Cause	Granulomatous Disease
Infectious agents	
Mycobacteria	Tuberculosis, atypical mycobacterial infection
Fungi	Histoplasmosis, coccidioidomycosis, cryptococcosis, Blastomyces and Aspergillus
Bacteria	Brucellosis
Spirochetes	Syphilis
Parasites	Leishmaniasis, toxoplasmosis
Neoplasms	Carcinoma, sarcomas, malignant nasal granuloma
HP	Farmers' lung, bird breeder's lung, suberosis (HP secondary to mouldy cork), bagassosis (HP secondary to mouldy sugar cane)
Drugs	Methotrexate, interferon, infliximab, etanercept, leflunomide, mesalamine, sirolimus, immune blockers
Metals	Chronic beryllium disease, zirconium, aluminium, talc granulomas
Silicates	Silicosis with granulomatous inflammation
Vasculitic granulomatoses and autoimmune disorders	Granulomatosis with polyangiitis, eosinophilic granulomatosis with polyangiitis, lymphomatoid granulomatosis, polyarteritis nodosa, bronchocentric granulomatosis, systemic lupus erythematosus, primary biliary cirrhosis, juvenile rheumatoid arthritis
Other conditions	Chronic granulomatous disease (children), Whipple's disease, Crohn's disease, lymphocytic infiltration after cancer chemotherapy, Blau's syndrome, pulmonary Langerhans cell histiocytosis, granulomatous-lymphocytic interstitial lung disease, local sarcoidal reactions, hot-tub lung, cat scratch disease

HP = Hypersensitivity pneumonitis

1.9.2 Diagnostic Approach

There is no definite single sensitive or precise diagnostic test for sarcoidosis. The approach is individualised according to the patient's clinical profile.

The clinical effect of sarcoidosis depends on the degree of granulomatous inflammation and the involved organ in question. Clinicians should use available non-invasive techniques to evaluate the degree, extent and severity of organ injury, as far as possible, and for assessing the organs that are clinically affected. The presence of asymptomatic organ involvement can be overlooked but is usually of minimal to no clinical importance, with three notable exceptions. For every sarcoidosis patient, a slit-lamp evaluation is advised to detect clinically unrecognised uveitis. Nephrolithiasis is associated with subclinical hypercalciuria, even in the absence of hypercalcaemia, and therefore, a 24-hour urinary calcium analysis is advised (331). Periodic electrocardiograms (ECGs) to test for uncommon, yet potentially life-threatening, cardiac arrhythmias and conduction disturbances, should also be obtained (332). Clinical involvement of more than one organ system helps to exclude local granulomatous reactions to foreign bodies, infection, or malignancy (7,333).

Cardiovascular sarcoidosis is generally confirmed by an extracardiac biopsy that confirms systemic sarcoidosis along with reliable studies of myocardial imaging or rhythm abnormalities (269). Endomyocardial biopsy is positive only in approximately 20-30% of patients with cardiac sarcoidosis (272). CMR and ^{18}F-FDG-PET scans are also helpful in detecting cardiac involvement.

Neurosarcoidosis is also typically established by biopsy of a non-central nervous system site. MRI scan is the modality of choice for imaging the brain (334). Cerebral biopsy is rarely needed to rule out infectious or malignant diseases (274).

At times, patients either refuse a biopsy or are unfit for a biopsy. In those circumstances, the clinician, in conjunction with the clinical and radiological features,

may decide on a trial of treatment and if the patient improves this could imply that the patient has sarcoidosis.

1.9.3 Diagnostic Criteria

The diagnosis requires compatible clinical, radiographic and pathologic features, having excluded other granulomatous diseases. Ideally, there should be histological validation of disease and negative cultures and special stains for pathogens (335). Cases in which the disease seems to be confined to only one organ can be challenging. The number of involved organs may change over time, requiring careful observation at regular examinations. Consequently, renewed diagnostic assessment may be necessary. Diagnosis of sarcoidosis is attained on the first physician consultation in only a small number of cases, depending on the presentation (336). Improved awareness, knowledge and familiarity of the disease may reduce this delay in diagnosis.

1.9.4 Diagnostic Evaluation

1.9.4.1 Laboratory Tests

Some recommended baseline evaluation testing of sarcoidosis patients is listed in Table 1.6 (337).

In the diagnosis of sarcoidosis multiple laboratory investigations should be considered (338,339). Although the full blood count and assessment of liver and kidney function are essential in the evaluation, they do not have diagnostic value (339). Anaemia, leukopenia, thrombocytopenia and eosinophilia can be present in the full blood count (339). Calcium levels of serum and urine, active vitamin D, and high total protein and

hepatic enzymes can indicate treatment requiring organ involvement (340). Serum ACE (sACE) levels are frequently elevated in sarcoidosis, suggesting granuloma burden (341). This is not precise, but can be useful for tracking disease development and progression and therapy response unless patients are treated for hypertension or heart disease with an ACE-inhibitor that may decrease sACE levels (341). The reduction in elevated sACE levels may be used as a marker for corticosteroid treatment compliance. Serum ACE levels can also be affected by ACE gene polymorphisms (342).

Table 1.6. "Recommended tests for initial evaluation of sarcoidosis". Reproduced with permission from (337).

Biopsy of an affected organ or tissue
Chest radiograph (or CT chest)
Full lung function tests
Electrocardiogram
Ophthalmologic examination, including slit-lamp examination.
Laboratory evaluation (liver function tests, renal function tests, serum calcium, and a complete blood count with differential)
Urinalysis and 24-hr urine collection for calcium
Organ specific testing based on signs and symptoms and manifestations (CT scan, MRI, Holter monitoring, electromyogram)

CT = Computerised tomography

MRI = Magnetic resonance imaging

1.9.4.2 Skin Tests

In active sarcoidosis there is peripheral cutaneous anergy to the purified protein derivative (PPD) skin test (343). This suggests a diagnosis of sarcoidosis; however, the anergy is neither sensitive nor specific and is no longer standard practice.

Kveim-Siltzbach skin-testing employs intradermally administered sarcoidosis spleen preparation. In the past, it has been used in patients with unexplained hypercalciuria, unknown hepatic granulomas, uveitis, or erythema nodosum. This is currently not approved and is not suggested for general use.

1.9.4.3 Radiological Imaging

Baseline chest radiographic imaging should also be obtained. High resolution CT (HRCT) yields fine detail of parenchymal abnormalities, such as nodules, bronchovascular beading, subpleural nodules, ground-glass, septal lines, reticulation, and the adenopathy (344). The CT scan will provide a better visualisation of aspergilloma (mycetoma or fungal mass) and/or characteristics of pulmonary hypertension. Fibrosis, demonstrated by HRCT, offers essential prognostic knowledge in tandem with pulmonary function tests (251). The simplest and least invasive test for diagnosis and monitoring is generally performed on patients with pulmonary involvement. This examination, along with clinical examinations, spirometry and chest x-rays is sometimes performed for a patient with low diffusing capacity for carbon monoxide (DLCO) level, instead of performing regularly more aggressive testing, such as arterial blood gases or exercise tests or serial HRCT chest scans, with higher risk of cancer due to radiation exposure in these young people, particularly women (345). For Löfgren's syndrome, only a chest radiograph is normally required, and the disease progression is tracked.

1.9.4.4 Electrocardiogram, Echocardiography, Cardiac Catheterisation

Each patient ought to have a routine ECG on initial evaluation. Echocardiography is particularly useful, as it is universally available, and can be used for evaluating and

monitoring left ventricular function in cardiovascular sarcoidosis and screening for pulmonary hypertension (269). Further cardiac assessment may incorporate right and left cardiac catheterisation, electrophysiological studies, and CMR or ^{18}F-FDG-PET scan (272).

1.9.4.5 Nuclear Imaging

The scintigraphy of Gallium-67 (^{67}Ga) behaves like an iron analogue and binds to transferrin. ^{67}Ga is used as an essential binding protein in the polymorphonuclear leukocytes to link the lactoferrin to inflammatory lesions (346). The sluggish clearance of the plasma ensures that a large amount of ^{67}Ga citrate remains in the body which is released into tissues that are rich in lactoferrin. This is the reason for the activity in bone marrow, spleen, liver, lacrimal and salivary glands. ^{67}Ga scintigraphy to diagnose sarcoidosis was identified by various scholars. The occurrence of symptoms in patients with biopsy-proven sarcoidosis was described in these reports for active illness. Sarcoidosis was found inactive in assessing severity if patients had no signs and symptoms and chest x-ray did not change. With a low specificity of approximately 50%, the sensitivity ranges from 60–90% for ^{67}Ga scanning (347–349). However, negative ^{67}Ga scintigraphy together with a normal sACE level has a high negative predictive value (350,351).

^{67}Ga scintigraphy correlates with sACE values and corticosteroid therapy is associated with an improvement of ^{67}Ga scintigraphy and a reduction in ACE levels (351–354). The predictive value of ^{67}Ga scintigraphy, chest x-ray and ACE levels predictive importance was measured in 382 patients by Rizzato *et al*., (355). ^{67}Ga scintigraphy tended to be more sensitive than chest radiography but was not properly contrasted with sACE.

In ^{67}Ga scintigraphy, "lambda" and "panda" signs are proposed as signature characteristics of sarcoidosis. In conjunction with the often right-sided paratracheal active lymph nodes in the mediastinum, bilateral hilar lymphadenopathy signals the lambda sign. The symmetrical activity in the lacrimal and parotid glands are linked to the panda sign. However, in biopsy-proven sarcoidosis, these signs have demonstrated low diagnostic sensitivity (356–358). In HIV, malignant lymphomas and Sjögren's syndrome patients, the panda sign may be present.

In several cell types including activated macrophages, somatostatin receptors are found (359). The Indium-111 (^{111}In) pentetreotide somatostatin receptor scintigraphy (SRS) is used most widely. The somatostatin receptor subtype 2 (ss_2) receptor exhibits high affinity and can subsequently be used in sarcoidosis imaging (360). In 46 sarcoidosis cases, SRS was detected, and active lesions were found in 97% of regular chest radiographic locations by Kwekkeboom *et al.,* (361). Further thoracic and extrathoracic lesions were discovered, but other extrathoracic lesions were not clearly illustrated. In 18 patients, Lebtahi *et al.,* (362) associated SRS with ^{67}Ga imaging and established that the sites concerned with the clinical SRS had considerably higher sarcoidosis lesions than ^{67}Ga scanning (65% and 83%). Despite additional intrathoracic areas were detected that were not clinically suspected, SRS did not diagnose 40% of the identified extrathoracic lesions. Therefore, the SRS appears sensitive in determining active intrathoracic sarcoidosis, but the presence of extrathoracic disease can be overlooked.

^{18}F-FDG-PET is useful for evaluating and detecting occult sarcoidosis (15). CMR and ^{18}FDG-PET are useful for assessing extrapulmonary involvement, particularly of the heart, brain and bone (363). For cardiovascular sarcoidosis, “a high-fat and low-

carbohydrate diet" ought to be taken prior to ^{18}F-FDG-PET scanning for optimal sensitivity (16). In suspected cardiac sarcoidosis, CMR is the test of choice to determine the presence and extent of cardiac involvement and ^{18}F-FDG-PET can be useful to define the extent of cardiac involvement (16).

1.9.4.6 Bronchoscopy, Mediastinoscopy and Video Assisted Thoracoscopic Surgery (VATS)

Flexible fibre-optic bronchoscopy is a valuable method for diagnosis (364). It can detect endobronchial cobblestone lesions. Endobronchial biopsies, transbronchial needle aspiration (TBNA), and lymph node endoscopic ultrasonography (EUS)-guided biopsy will increase yield by up to 90% (365). Endobronchial ultrasound-guided transbronchial needle aspiration (EBUSTBNA) has a yield of between 85% and 92% and has been proven to be better than standard TBNA (366). Other techniques such as mediastinoscopy, based on availability of services, does not take place as much as previously due to the high diagnostic yield with EBUSTBNA.

In most cases a transbronchial lung biopsy is the procedure of choice, which results in a diagnosis in 40-90%, based on the experience of the operator, the amount of biopsies obtained and the level of chest x-ray staging (367,368). In order to optimise the yield, at least six high-quality transbronchial biopsies are required. An elevated BAL fluid ratio of CD4+/CD8+ (>2:1) and BAL lymphocytosis (>10% lymphocytes) may contribute, but is non-diagnostic (369,370).

In the case of non-diagnostic bronchoscopic transbronchial lung biopsies, other choices for obtaining tissue include open-lung biopsy, video-assisted thoracoscopic lung biopsy and mediastinoscopy. Mediastinoscopy and lymph node biopsy are performed to rule out lymphoma and tuberculosis. It must be noted that diagnosis may

be made in some cases without a chest procedure. If there are typical clinical as well as chest radiographic features with other organ involvement, for example, skin lesions or salivary gland enlargement, biopsies of these organs can confirm granulomas and it may not be necessary to perform bronchoscopy or thoracoscopy.

1.9.4.7 Extrapulmonary Involvement and Biopsy

In general, the easiest accessible biopsy site can be approached to confirm a diagnosis of sarcoidosis (2,3,7). Considering that virtually any organ may be involved, there are numerous potential sites to biopsy to confirm the diagnosis (7). The decision depends on the clinical presentation, experience and resources (367). Biopsy of the skin or conjunctival nodule, enlarged superficial lymph nodes, or lacrimal gland, may help establish a diagnosis (7,371). Ultrasound of the liver, spleen and abdomen may assist if there is an indication or abnormal liver function tests (284). Non-caseating granulomas on a liver or bone marrow biopsy are non-specific (7,367). As alluded to earlier, ^{18}F-FDG-PET has definite value in identifying occult sites of disease (15).

Some valuable diagnostic tests include MRIs for brain and leptomeningeal lesions (334). Typical testing of the cerebrospinal fluid (CSF) shows lymphocytic pleocytosis and elevated protein (275). Peripheral neuropathy can be assessed by electrodiagnostic studies and nerve biopsy (3,274). Gadolinium MRI is the recommended imaging tool for central nervous system involvement in patients with sarcoidosis (334).

1.9.4.8 Pulmonary Function Tests

The lung functions may be normal, despite parenchymal changes, or may reveal restriction, obstruction, abnormal gas exchange with or without reduction of the DLCO,

or a combination of these (5,49,252,371). Therefore, pre- and post-bronchodilator spirometry, plethysmography, particularly measuring total lung capacity (TLC) and DLCO measurement are utilised in practice.

DLCO and vital capacity (VC) reduction indicating restriction, are the most common abnormalities. However, in at least one study, airflow limitation was found to be the most common defect in newly diagnosed patients with sarcoidosis (372). Endobronchial involvement can lead to severe local stenosis, causing airflow limitation along with large intrathoracic lymph nodes compressing the airways or having reactive airways (373). Gas exchange measures are typically more sensitive for lung impairment and treatment response than airflow and lung volume measurements.

1.10 Biomarkers in Sarcoidosis

There are several potential candidate biomarkers; however, an ideal one remains to be discovered.

1.10.1 Serum Angiotensin-Converting Enzyme

The most prominent plasma anomaly in sarcoidosis is the rise in “serum angiotensin-converting enzyme” (sACE) levels. Serum ACE determination remains the most commonly used laboratory test for sarcoidosis and provides some indication of total body granuloma load (341). The correlation between the levels of sACE and disease activity or treatment response is relatively low (374). Determination of sACE has an estimated sensitivity of only 57% (375). Many other conditions, including TB, elevate sACE levels. SACE levels may be in the normal range especially in the first months of acute disease.

1.10.2 Serum “Soluble Interleukin-2 Receptor (sIL-2R)”

In serum and BAL fluid of sarcoidosis patients IL-2, a Th1 cytokine that causes T-cell proliferation, rises and may have prognostic value (376). SIL-2R is produced by activated T-lymphocytes and interacts with CD4+ T-lymphocytes in BAL and serum (377). The use of sIL-2R as a diagnostic and predictive biomarker for sarcoidosis is not yet completely corroborated. It may have a diagnostic role in uveitis.

1.10.3 Serum Calcium and Urinary Calcium

Serum calcium is measured in the diagnostic work-up in sarcoidosis (339). Hypercalcaemia is an absolute indication to start systemic corticosteroid therapy (340). Moreover, both hypercalciuria and hypercalcaemia suggest on-going granulomatous inflammation and may be considered as biomarkers for disease activity (339). However, this only pertains to a minority of patients and therefore cannot be regarded a biomarker for sarcoidosis in general.

1.10.4 Lysozyme

Lysozyme is a renally metabolised enzyme with antibacterial activity (378). It is present in macrophages and epithelioid cells of sarcoidosis granulomas and is thought to represent the total body burden of granulomas, as sACE reflects (379). Serial measurements of lysozyme may be helpful to assess response to systemic therapy.

1.10.5 BAL Fluid Characteristics

The BAL fluid ratio of CD4+/CD8+ is greater than 3.5 in sarcoidosis patients, but “BAL counts are neither diagnostic nor prognostic” (380). The BAL CD4+/CD8+ ratio may

be regarded as an appropriate diagnostic predictor for sarcoidosis (381). It has not been correlated with the severity and outcome of the disorder and is of limited value in clinical practice.

1.10.6 Chitotriosidase

Chitotriosidase is a developed enzyme that plays a role in the defence against fungi, nematodes and insects by activated macrophages (382). The macrophages in the chronically active tissue are selectively expressed and chitotriosidase can be considered a biochemical proxy for macrophage activation (383). Increased serum and BAL activity in sarcoidosis patients have also been observed, and higher chitotriosidase levels have been found in active sarcoidosis patients than in inactive disease patients (384). Chitotriosidase appears to be a severity indicator of sarcoidosis (385–387). Serial chitotriosidase activity estimations tend to correspond with clinical disease observations (388,389). However, it is possible, as with sACE, to reduce chitotriosidase levels through treatment with systemic corticosteroids (387,388). Chitotriosidase activity has been found to be “significantly lower in TB patients than in patients with sarcoidosis” (390).

1.10.7 Th17-Cells and Regulatory T-cells (Tregs)

Th17, Th17.1 (interferon-γ-producing Th17 cells), and Treg cells play an essential role in numerous inflammatory pathways and may be potential cellular biomarkers (185,391). However, further research is required.

1.10.8 Serum Amyloid A

“Serum amyloid A (SAA) is an acute phase protein” found in granulomas of sarcoidosis patients (392). “Elevated levels of SAA have been found in sarcoidosis” and higher levels were noted in patients with more active disease (392, 393).

1.10.9 Vimentin

Vimentin is a filament protein within cells involved in host microbial defence. In sarcoidosis, vimentin filaments were found in “asteroid bodies of the giant multinucleated cells in granulomas” (394), and an increased expression was identified in the spleen of patients with sarcoidosis. Vimentin’s role as a biomarker is not established.

1.10.10 Chemokines

Chemokines are a group of chemo-attractant proteins, which may promote the movement of T-cells to inflammatory sites by their chemical actions (395). Various chemokines such as CXCL9, CXC10 and chemokine receptors like CXCR3 are potential biomarkers (396,397).

1.10.11 Micro-Ribonucleic Acid (MicroRNA)

Micro-ribonucleic acids (microRNAs or miRNAs) “are small non-coding regulatory RNAs that function as post-transcriptional regulators of gene expression” (398). MicroRNAs were first described by Lee *et al.,* in 1993 (399) and have been researched as evolving biomarkers in numerous other respiratory diseases, including “idiopathic pulmonary fibrosis, lung cancer, asthma and chronic obstructive pulmonary disease” (400,401).

In “sarcoidosis, miRNA expression has been studied in the lungs, lymph nodes, BAL fluid, and peripheral blood mononuclear cells” (155,402-405). The role, if any, of altered miRNA expression in sarcoidosis pathogenesis is evolving. MiRNAs plays an essential role in the sarcoidosis pathogenesis through their regulatory influence on signalling pathways e.g., TGF-β/SMADs (TGF-β and TGF-β signalling pathway elements) and Wnt (Wingless-related integration site)/β-catenin) (406,407).

1.10.12 Future Purposes: ‘Omics’

To recognise novel biomarkers, new methods have been utilised applying “omic technologies”, including genomic, proteomics, transcriptomics, and studying exhaled breath for new biomarkers (408) may provide new insights into this perplexing disease as well as assist to develop novel biomarkers.

1.11 Management of Sarcoidosis

It is not clear why some patients recover from sarcoidosis, and others progress. Even after an apparent recovery, months to years later, a proportion of patients can relapse. Factors associated with worse prognosis are listed in Table 1.7. Löfgren's syndrome has a good prognosis. It is important to understand that in some the disease can resolve spontaneously without treatment and other patients with sarcoidosis are completely disabled by their illness. The prevailing recommendation for institution of treatment is centred on the presence of organ dysfunction.

Table 1.7. Adverse prognostic factors in sarcoidosis. Reproduced with permission from (409).

• Lupus pernio • Chronic uveitis • Onset after age of 40 years • Chronic hypercalcaemia • Nephrocalcinosis • Black race • Progressive pulmonary sarcoidosis • Nasal mucosal involvement • Cystic bone lesions • Neurosarcoidosis • Myocardial involvement

1.11.1 Initiation of Treatment

The decision of the need for initiation of treatment is not an easy one, as disease behaviour is impossible to predict. Some patients could resolve spontaneously. One requires to monitor organ function over time. Most often, lung function tests are used to identify pulmonary progression of disease early enough. The indications for anti-inflammatory therapy are listed in Table 1.8.

Table 1.8. "Initial therapy according to organ and clinical status".* Reproduced with permission from (236).

Organ	Clinical Findings	Treatment
Lungs	Dyspnoea plus FEV1, FVC <70% Cough, wheezing	Prednisone, 20–40 mg/day Inhaled corticosteroid
Eyes	Anterior uveitis Posterior uveitis Optic neuritis	Topical corticosteroid Prednisone, 20–40 mg/day Prednisone, 20–40 mg/day
Skin	Lupus pernio	Prednisone, 20–40 mg/day Hydroxychloroquine, 400 mg/day Thalidomide, 100–150 mg/day Methotrexate, 10–15 mg/week
	Plaques, nodules	Prednisone, 20–40 mg/day Hydroxychloroquine, 400 mg/day
	Erythema nodosum	NSAID
Central nervous system	Cranial-nerve palsies	Prednisone, 20–40 mg/day
	Intracerebral involvement	Prednisone, 40 mg per day Azathioprine, 150 mg/day Hydroxychloroquine, 400 mg/day
Heart	Complete heart block Ventricular fibrillation, tachycardia Decreased LVEF (<35%)	Pacemaker† AICD AICD; prednisone, 30–40 mg/day
Liver	Cholestatic hepatitis with constitutional symptoms	Prednisone, 20–40 mg/day Ursodiol, 15 mg/kg of body weight per day
Joints and muscles	Arthralgias Granulomatous arthritis Myositis, myopathy	NSAID Prednisone, 20–40 mg/day Prednisone, 20–40 mg/day
Hypercalciuria and hypercalcaemia	Kidney stones, fatigue	Prednisone, 20–40 mg/day Hydroxychloroquine, 400 mg/day

"* FEV1 denotes forced expiratory volume in 1 second, FVC forced vital capacity, LVEF left ventricular ejection fraction, AICD automatic implantable cardiac defibrillator, NSAID nonsteroidal anti-inflammatory drug. † Most authorities recommend a dual-chamber pacemaker-defibrillator"

In fact, anti-inflammatory treatment is only acceptable when active inflammation can be shown to induce symptoms. Because in many cases sarcoidosis is not incapacitating, therapy may be postponed for careful evaluation. Spontaneous improvement and remission can occur in up to approximately 50% of individuals, though it can be variable among different ethnicities.

1.11.2 Available Treatments

There are no established sarcoidosis treatment protocols because there is lack of approved treatments tested in randomised controlled trials. The heterogeneity of the presentation of illness, severity and natural history of the disease render drug treatment trials challenging to plan and design. Interim reliance on consensus professional experience and knowledge (Delphi technique) has been useful and is being followed (410). The therapeutic options are listed in Table 1.9 and other available therapies listed in Table 1.10.

Table 1.9. "Drugs used, mechanism of action and common side effects" in sarcoidosis. Adapted from (411).

Drug	Usual dosage	Mechanism of action	Common side effects
First line			
Corticosteroids	20-40 mg	Inhibit proinflammatory cytokines	Hypertension, diabetes, cataracts, obesity
Second line (steroid sparing agents)			
Methotrexate	10–15 mg weekly	Inhibits the metabolism of folic acid in purine and pyrimidine synthesis	GI intolerance, oral ulcers, hepatotoxicity, leukopenia, fatigue, risk of infection, pneumonitis
Azathioprine	50–200 mg daily	As a purine analogue, inhibits purine synthesis necessary or T-, B-cell proliferation	Leukopenia, hepatotoxicity, risk of infection, skin cancer
Leflunomide	10–20 mg daily	Inhibits COX-2 enzyme; DHODH inhibition affecting pyrimidine synthesis	Leukopenia, hepatotoxicity, risk of infection, skin rash, fatigue, pneumonitis, peripheral neuropathy
Hydroxychloroquine	200–400 mg daily	Prevents acidification of lysosomal enzymes required for antigen presentation and TLR-2 activation.	GI intolerance, rash, retinopathy, neuro-myopathy
Chloroquine	250–750 mg daily	Prevents acidification of lysosomal enzymes required for antigen presentation and TLR-2 activation.	GI intolerance, rash, retinopathy, neuro-myopathy
3rd Line			
Infliximab	3–5 mg/kg initially & at 2 weeks and 6 weeks, then every 4–6 weeks IV	TNF-α antagonist	Allergic reaction, increased risk of infection especially TB, CHF, possible increased risk of malignancy
Adalimumab	40 units every week SC. Consider initial higher loading dosages.	TNF-α antagonist	Allergic reaction, increased risk of infection especially TB, CHF, possible increased risk of malignancy, injection site reaction
Golimumab	200 mg once, then 100 mg every 4 weeks SC	TNF-α antagonist	Increased risk of infection, injection site reaction.
Etanercept	25 mg SC twice weekly	TNF-α antagonist	Rash, diarrhoea, increased risk of infection, CHF, antibody development
Fourth line			
Mycophenolate mofetil	1000–3000 mg daily	Inhibits IMPDH in purine synthesis necessary for T-, B-cell growth	GI intolerance, pancytopenia, hepatotoxicity, secondary carcinoma

"DHODH denotes dihydro-folate dehydrogenase, COX2 cyclooxygenase-2, IMPDH Inosine-5'-monophosphate dehydrogenase, GI gastrointestinal and CHF congestive heart failure".

Table 1.10. "Other available agents that are rarely used in sarcoidosis" as 4th line treatment. Adapted from (411).

Drug	Mechanism of action
Apremilast	PDE-4 inhibitor
Pentoxifylline	PDE-4 inhibitor
Roflumilast	PDE-4 inhibitor, reduces IL-17 level
Rituximab	Monoclonal antibody against CD20 surface antigen of B-lymphocytes
Tetracycline	Tetracycline antibiotics-inhibits T-cell proliferation
Thalidomide	TNF-α antagonist, possibly reduce IL-18 levels
Cyclosporine	Calcineurin inhibitor
ACTHAR gel	Synthetic ACTH hormone
Cyclophosphamide	Alkylating agent
Repository corticotropin (RCI)	Stimulates ACTH secretion

"IV denotes intravenous, IM intramuscular, SC subcutaneous, PDE-4, phosphodiesterase 4, GI gastrointestinal, CNS central nervous system and ACTHAR gel and RCI adrenocorticotrophic hormone subcutaneous injection".

1.11.2.1 *Glucocorticoids*

Glucocorticoids are regarded as the initial, first-line, anti-inflammatory treatment in sarcoidosis (2,3,8,9). A reasonable guideline is that impairment that compromises organ function necessitates therapy (2,3,7,412). Patients with acute Löfgren's syndrome and Scadding stage I x-ray changes with isolated bilateral hilar lymphadenopathy, do not require treatment, as these patients generally have good outcomes without treatment (236).

Oral corticosteroids use in sarcoidosis is meant to relieve symptoms, improve QoL and modulate disease activity to prevent morbidity and mortality linked to vital organ dysfunction (2,3,7,8). Corticosteroid therapy is usually indicated when patients with sarcoidosis develop dyspnoea in the presence of, abnormal and progressive impairment of pulmonary function, and in those deteriorating clinically and

radiologically (3,5,252). Corticosteroids suppress disease activity by inhibiting the expression of proinflammatory cytokines, which results in symptomatic, functional and radiographic improvement (413,414).

Long-standing fibrotic lung changes typically do not respond to corticosteroids while hypercalcaemia and hypercalciuria can usually be treated with moderate doses (3,7,8,371). Treatment studies in cases treated for intervals of up to one and half years have reported that corticosteroids can control granulomas and alleviate symptoms, but have not improved long-term disease progression and outcome (415). Oral corticosteroid treatment consideration should be given to the clinical circumstances listed in Table 1.8.

Randomised, prospective trials have not identified the optimal dosage and length of corticosteroid (prednisone, prednisolone) therapy. The optimum first dose of corticosteroids is not agreed upon. The treatment regimen is individualised and is based on symptoms and response rate. For pulmonary sarcoidosis, the commencing dosage of prednisone in controlled trials has varied from 20-60 mg daily, and tapering by 5mg every two to four weeks permitting by the response, to an average of 10 mg daily kept for six months to one year, before trying further cautious withdrawal and eventual cessation (416,417). Anecdotal reports show that in certain cases, therapy is maintained for no less than 24 months to avoid relapses. “Alternate day dosing” (with the dose kept equal to the daily dose) has been prescribed to reduce adverse effects (418) and was found to be as efficacious as daily therapy (419). Such a regimen is not necessarily feasible; diabetic patients may prefer daily dosing in order to keep glucose regulation, and the ones having trouble recalling taking treatment may prefer a daily schedule.

Patients should be monitored at one to three months for improvement of symptoms and pulmonary function tests. Those that do not respond during the first three months are less likely to respond to more prolonged treatment (420). Where possible, six months to one year of therapy should be continued and, if possible, tapered (421). Refractory illness may need up to two years of treatment, usually with a second-line steroid sparing agent (422,423). Lower commencing dosages and quicker weaning strategies can be utilised and appear to be almost as successful (3).

Inhaled corticosteroids might be helpful for cases that have endobronchial involvement, in those with airway hyperresponsiveness and with unrelenting cough (20,414, 416,417,424). However, inhaled corticosteroids should not be utilised as a replacement, or as systemic steroid sparing.

The use of corticosteroids for a prolonged period of time may be related to weight gain, diabetes mellitus, hypertension, infections, fluid accumulation, cataracts, glaucoma, anxiety, insomnia, muscle weakness, mood fluctuations, changes of behaviour, osteoporosis and femoral head osteonecrosis (418). Judicious use of supplementary therapy with calcium and vitamin D is necessary in order to avoid hypercalciuria and hypercalcaemia. Treatment with bisphosphonate is often used, particularly when scans of the bone show a decrease in bone density (425).

Cases that are stable on high-dose corticosteroids but have no improvement are held at the same dose unless severe adverse consequences develop. If unacceptable adverse effects occur, or the condition deteriorates, they qualify to have a second-line immunosuppressive steroid-sparing drug to be added (422).

1.11.2.2 *Glucocorticoid-sparing Drugs or Second-line Agents*

If undesirable adverse effects occur or relapse ensues, then other immunosuppressives should be utilised as corticosteroid-sparing agents (Table 1.9). Methotrexate (426,427) and azathioprine (426,428,429) are regularly used, with mycophenolate mofetil and leflunomide as other options (426). A modest corticosteroid dosage is typically required; however, there are successful therapy reports of immunosuppressives without corticosteroids (426).

1.11.2.2.1 Methotrexate

In steroid-sparing treatment, methotrexate (MTX) is the recommended second-line immunosuppressive drug. The precise “mechanism of action of MTX (an antimetabolite that reversibly inhibits dihydrofolate reductase) in sarcoidosis is” not clear, but it has “both cytotoxic and anti-inflammatory effects” (430).

This medication has been demonstrated to be helpful in uveitis. In sarcoidosis there is just one reported randomised, controlled trial with MTX (431). The principal end-point was steroid dosage after six months to one year. There appeared to be a steroid-sparing effect, despite a high drop-out rate in the study. The largest documented series is by Lower and Baughman (432), who reported improvement in 33 of 50 MTX-treated cases for at least two years. Patients with skin and musculoskeletal disease demonstrated the most remarkable response. Generally, in that study, 83% were able to lower their corticosteroid dose and 13 of 30 patients discontinued corticosteroids altogether. A subsequent analysis of 209 cases demonstrated that 52% of those on MTX appeared to go into remission and 16% stayed stable, with or without low-dose corticosteroids (432).

Further studies also affirm the beneficial outcomes of MTX in dermatological and musculoskeletal involvement (433,434). In the research by Kaye and co-workers (433), “low-dose MTX (average 10 mg/week, range 7.5–15 mg), when used for an average of 30 months”, improved and “controlled clinical symptoms in patients with musculoskeletal involvement and helped reduce corticosteroid” dosage. MTX therapy ranged from 5 to 15 mg/week for most of the trials (435). This drug is administered “as a single or divided oral dose one day a week” (436). In sarcoidosis, MTX can “take up to six months to become effective” (437). During this time, patients are commonly treated additionally with corticosteroids.

1.11.2.2.2 Azathioprine

While azathioprine is reported both anecdotally and routinely to be used in sarcoidosis, few studies investigating its effectiveness have been published. A study group noticed objective improvement in seven of ten sarcoidosis cases, after receiving 150 mg/day of azathioprine for six months (428). Evidence indicates its effectiveness for extrapulmonary disease (438), but is restricted by side effects. No randomized controlled sarcoidosis trials have been performed with azathioprine. In a systematic analysis, only two in 10 patients had improved their condition (429). An open-label trial, which examined 11 chronic sarcoidosis patients cases, treated with both azathioprine and prednisolone, noted improvement from a cumulative 20-month period (439).

1.11.2.2.3 Leflunomide

Leflunomide, an MTX analogue, is a cytotoxic drug that has less adverse effects of interstitial pneumonitis than MTX and is used to treat patients with pulmonary sarcoidosis (440). Leflunomide, in case series and retrospective analyses, have

been found to be helpful in sarcoidosis (440,441). The combination treatment of leflunomide and MTX demonstrated a greater clinical response in extrapulmonary disease compared with leflunomide alone (441), suggesting synergism between these two drugs.

1.11.2.2.4 Mycophenolate Mofetil

Mycophenolate mofetil (MMF), a mycophenolic acid prodrug that prevents lymphocyte proliferation, has been reported in cutaneous, neurologic, and renal sarcoidosis patients to be helpful (436). Azathioprine and cyclophosphamide have a greater oncological risk than MMF (442). In comparison with other immunosuppressive medications, MMF has a quick onset of action (443). In certain cases, it could be helpful in renal and neurosarcoidosis (444,445). Since there is inadequate therapeutic information regarding MMF, it is generally deemed a third-line sarcoidosis drug after the abovementioned antimetabolites.

1.11.2.2.5 Cyclophosphamide

Cyclophosphamide is used in selected corticosteroid-refractory situations (446,447). It appears to be effective in both cardiac and neurosarcoidosis, particularly after of corticosteroid and MTX failure. Oral cyclophosphamide is given by mouth at a maximum dose of 1 to 2 mg/kg/day for several months before tapering, up to a ceiling dose of 150 mg/day (436). In one open-label clinical report, researchers used cyclophosphamide intravenous pulse therapy at 0.75 to 1.5g monthly for the treatment of neurosarcoidosis (447).

1.11.2.2.6 Cyclosporin A

Clinical studies involving cyclosporine (CyA), a calcineurin blocker, have shown no value in pulmonary sarcoidosis (448,449) and it is rarely used in sarcoidosis.

1.11.2.3 Antimalarials

Various antimalarial drugs have been used in sarcoidosis and are also considered as second-line agents in the therapy of sarcoidosis.

1.11.2.3.1 Hydroxychloroquine

Chloroquine (CQ) and hydroxychloroquine (HCQ) antimalarial drugs can be effective steroid-sparing agents and are particularly useful in patients with cutaneous sarcoidosis (450), in cases with hypercalcaemia and in neurosarcoidosis (451,452). In patients with posterior uveitis they are contraindicated because retinitis is a possible side effect. HCQ is used at doses of 200 to 400 mg per day (453). For patients treated with HCQ, ophthalmological evaluations should be performed every 6 months during therapy to examine for ocular toxicity. It should be recommended that women of childbearing age use contraception when taking HCQ. While CQ and HCQ are more widely used for extrapulmonary diseases, they may also treat lung disease (454,455).

1.11.2.4 Anti-tumour Necrosis Factor Therapy

1.11.2.4.1 Pentoxifylline

While pentoxifylline inhibits TNF-α, the drug can be used to minimise symptoms such as fatigue, having only minimal efficacy in sarcoidosis (456). Dosage can be limited by gastrointestinal adverse incidents (457).

1.11.2.4.2 Thalidomide

“Thalidomide and lenalidomide inhibit TNF-α production” and have demonstrated some benefit in case series. Small case series have observed that thalidomide may be helpful for skin sarcoidosis, especially lupus pernio (458). Sedation, peripheral neuropathy, and teratogenicity limit its usefulness (459–463).

1.11.2.4.3 TNF-α Blockers

“Infliximab is a chimeric monoclonal antibody” directed at the solubility of the membrane-bound TNF-α. In refractory sarcoidosis, in particular in neuro-ophthalmic disease, it has been shown to be effective (464,465). Note that etanercept, also a TNF-α blocker, was found to be ineffective (466). For cutaneous sarcoidosis, adalimumab and golimumab are other TNF-α inhibitors that are effective and comparatively safer (467). As off-label third-line therapy, infliximab and adalimumab are being utilised (442).

Anti-TNF treatments is associated with an increased risk of TB; therefore, any patient to commence such therapy should be investigated for previously untreated or active TB (468).

1.11.2.5 Tetracyclines

In some cases of skin sarcoidosis, doxycycline and minocycline can be helpful, but they have not been proven to be effective for controlling other organ involvement (469).

1.11.2.6 Combination Treatment Regimens

As sarcoidosis treatment, combination regimens are commonly preferred. Corticosteroids and a steroid-saving second-line medication are the most common combination treatment approaches for sarcoidosis. The corticosteroid is then tapered progressively over 3-6 months, as the response of the patient permits, and finally the second-line agent is the principal therapeutic medication (442). In an unregulated open-labelled study in steroid-refractory patients, all 11 cases seemed to go into remission, with the simultaneous use of CyA and oral fluocortolone along with oral MTX (470). These provisional findings must be validated by a larger, prospective, controlled trial.

1.11.2.7 Targeted Treatment

Despite widespread use of non-specific anti-inflammatory therapies, morbidity and mortality rates for sarcoidosis are not abating (5,471). Novel therapies that target specific inflammatory pathways in sarcoidosis represent potential new therapies that are being investigated are shown in Table 1.11. Therapy aimed at T-cell activation targets, immune pathways of Th1 and Th17, major signal transduction pathways, and the pathways of ubiquitin–proteasome system are being studied (472).

Table 1.11. "Prospective potential therapeutic targets for sarcoidosis treatment". Adapted from (411).

Drugs	Proposed mechanism of action
Inhaled VIP	Inhibits TNF-α production
Ustekinumab	Inhibits IL12/IL-23 complex
hIL-18	Inhibits expression and neutralizes of IL-18
CLEAR	Inhibits expression of ESAT-6 antigen
Nicotine	Immunomodulator by altering TLR2, TLR9 response
PDA-001	Inhibits T lymphocyte proliferation and differentiation
Pirfenidone	TGF-β and PDGF inhibitor
Nintedanib	Tyrosine kinase inhibitor
Rilonacept, canakinumab	NLRP3 inflammasome inhibitors
Atorvastatin	Suppresses Th-1 immune response
BIRB 796, semapimod	P38 MAP kinase inhibitor
Bortezomib, carfilzomib	Ubiquitin protease inhibitor
Abatacept	CTLA receptor antagonists
Tocilizumab, Olokizumab	Anti-IL-6 antibody

"VIP denotes vasoactive intestinal peptide; hIL-18: human interleukin 18; CLEAR: trial of antimycobacterial therapy in sarcoidosis (combined regimen of levofloxacin, ethambutol, azithromycin and rifampin); PDA: human placenta-derived antigen; PDGF: platelet-derived growth factor; NLRP3: nucleotide-binding domain, leucine-rich repeat family, pyrin domain containing 3; MAP: mitogen-activated protein kinase; and CTLA: cytotoxic T-lymphocyte-associated protein".

Other agents that are being studied include roflumilast, a phosphodiesterase-4 inhibitor that has been shown to decrease inflammation in patients with psoriasis and in sarcoidosis (473). The attachment of nicotine to the α7-subunit of nicotinic acetylcholine receptors has various anti-inflammatory effects (474). Also, it is shown that smokers have a lower frequency of sarcoidosis. Nicotine, as an anti-inflammatory treatment for sarcoidosis, is undergoing trials. Statins are promising in sarcoidosis therapy for their immunomodulatory properties, in particular the suppressed of CD4+ Th1 cell differentiation that may limit the manufacture of chemokines in alveolar macrophages (475).

1.11.2.8 Other Treatment

An implantable cardioverter-defibrillator device is indicated in cases with cardiovascular sarcoidosis with life-threatening ventricular arrhythmias or complete heart block, combined with corticosteroids (476).

For PH associated with sarcoidosis, therapy includes immunosuppressive drug treatment, domiciliary oxygen, and in certain cases pulmonary vasodilator therapy (endothelin receptor antagonists, phosphodiesterase-5 inhibitors, or prostanoids) (477), as well as other supportive treatments. Patients with PH have a greater likelihood to be referred for lung transplantation as they have a 10-fold higher mortality relative to patients with isolated pulmonary sarcoidosis (478). The five-year survival rate is only 59% (479), which portends a poor prognosis.

The para-sarcoidosis syndromes, for example small-fibre neuropathy, fatigue and cognitive impairment are not easy to manage (480). Neurostimulants, anti-TNF biologics and innate repair receptor, cibinetide, an erythropoietin receptor and CD131 agonists, is promising as a new treatment for small-fibre neuropathy (481). Rehabilitation is an additional approach being used for the treatment of these syndromes (482). A mycetoma in a sarcoid cavity may need surgery if there is haemoptysis (483). For bronchial constriction, dilatation and/or stent placement may be necessary (484).

For patients taking prolonged courses of corticosteroids (i.e., more than 20 mg/d of prednisone or equivalent for more than four weeks or additional immunosuppressive therapy), it is recommended that *Pneumocystis jirovecii* pneumonia prophylaxis be taken as well, although a consensus regarding this matter is lacking (485,486).

1.11.2.9 Lung, Liver or Cardiac Transplantation

Lung transplantation is an additional therapeutic option for patients with sarcoidosis. Timing of transplantation may be challenging, with mortality rates of up to 50%, among cases with sarcoidosis referred for and awaiting lung transplantation (487). Lung transplantation ought to be a treatment consideration, if resources permit, in end-stage lung disease and especially in those with PH (478).

A likely contraindication to transplantation is mycetoma (most commonly aspergillomas), that occur especially in patients with end-stage fibrocystic disease. There is a high risk of severe and invasive fungal infection during the immunosuppressive treatment after transplantation (488). The recurrence of sarcoidosis in lung allografts is a concerning aspect of transplantation, but this does not appear to affect survival or the risk of complications (489). In rare cases, heart and/or liver transplantation may be indicated.

1.11.3 Relapse

In chronic sarcoidosis, relapses after tapering and stopping corticosteroids are regularly observed. Higher doses of corticosteroids are re-initiated, preferably in combination with second-line steroid-sparing immunosuppressives (490,491). In relapses corticosteroid therapy in an induction phase of two to three months, or a higher dose of prednisone is tapered from 0.5 mg per kg (body weight) daily, to a maintenance dose of 5–10 mg daily and the second-line immunosuppressant is given in a constant dose. If successful and well tolerated, this treatment should last around two years (412,427,439). In those with medically mediated remission the relapse rates are higher than in those with natural remission (1,490). Patients deemed to be in remission should be regularly followed.

1.11.4 Monitoring Treatment

The patients need to understand the aims of therapy and the adverse effects of the treatment. Clinical judgement, lung function tests and radiological imaging are useful to monitor the progress. ^{18}F-FDG-PET and/or CT may help detect occult disease. Timely detection of relapse is important to ensure prompt treatment. SACE levels, liver function tests and serum calcium, C-reactive protein and sedimentation rate are also utilised to observe and monitor response to treatment.

Myocardial and neurosarcoidosis is extremely difficult to diagnose, treat and monitor, where imaging methods are usually the only guide to treatment and follow-up.

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Chapter 2 : Studies Conducted in Patients with Sarcoidosis

2.1 Problem Statement

The aetiology of sarcoidosis is unknown and the immunopathogenesis is complex. By studying clinical, genetic, immunological aspects, and various biomarkers may provide insight and contribute to unravelling some factors involved in the aetiology of this condition. There are relatively few studies of patients with sarcoidosis in South Africa, and none that have investigated various aspects of immunopathogenesis of the condition, which will be undertaken in the current study in South Africa.

2.2 Aims and Objectives

The purposes of the study were to review the clinical features and investigate immunopathogenetic mechanisms that may be operative in patients with sarcoidosis in South Africa.

The objectives of the study were:

1. To investigate the clinical profile of patients with sarcoidosis in Johannesburg, South Africa,
2. To evaluate any differences in the clinical features in patients in the various population groups.
3. To determine the human leukocyte antigen (HLA) Class I and Class II phenotypes of a subset of patients with sarcoidosis and, if possible, to correlate these associations with the disease phenotype.

4. To measure Immunoglobulin (Ig) G subclass levels in the serum of a subset of newly diagnosed sarcoidosis patients, prior to therapy, and to determine the effects of the use of corticosteroids and of spontaneous clinical response, on the immunoglobulin levels.

5. To investigate the serum expression of micro-ribonucleic acids (microRNA) in a subset of patients with sarcoidosis, compared with race-, age- and gender-matched healthy controls.

6. To analyse serum chitotriosidase activity in a cohort of patients with sarcoidosis, prior to treatment, and after at least one-month of treatment and to compare this enzyme's activity in patients with tuberculosis (TB).

2.3 Significance

With the above studies, more insight may be gained into the factors involved in the immunopathogenetic mechanisms that underlie the aetiology of sarcoidosis, particularly in the South African setting.

2.4 Material and Methods

The studies conducted as part of this thesis spanned a number of years, and were interrupted midway, due to unforeseen circumstances, described below. The first prospective study evaluating IgG subclasses in patients with sarcoidosis was commenced during the late 1990s and completed in the early 2000s, at the Hillbrow Hospital, in Johannesburg. The hospital was, thereafter, closed due to the reorganisation of medical services in Johannesburg, with some patients (together with their charts), and some medical staff, including the candidate, being transferred to the

Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). All the subsequent studies were conducted at the CMJAH.

2.5 The Study Cohort

The overall number of sarcoidosis patients recruited for the various studies was 146, (as well as controls for some of the studies), with the breakdown as described below. The clinical study on sarcoidosis was a retrospective study of 102 patients attending the CMJAH, with the patients being from both transferred patients from Hillbrow Hospital and those attending the CMJAH. The HLA Class I and Class II typing studies were prospectively performed in 51 patients, from this cohort. The IgG subclass study recruited 17 newly diagnosed patients with sarcoidosis measuring IgG subclass levels before therapy and one-month or more after spontaneous resolution or corticosteroid therapy. Differentially expressed serum expression of microRNA in six patients with newly diagnosed sarcoidosis was compared with healthy matched controls. The serum chitotriosidase activity study recruited 12 additional, newly diagnosed, patients with sarcoidosis, who were studied prospectively, with comparison of levels with nine patients on treatment for at least one-month duration, and in 10 newly diagnosed active patients with TB.

2.6 Study Design

2.6.1 Sarcoidosis Clinical Study

The clinical features of patients with sarcoidosis were reviewed in a retrospective survey of 102 patients attending the CMJAH Pulmonology Service between 2002 and 2006. The demographics, clinical features, chest radiographs, laboratory results, lung function tests and therapy of these patients were recorded and analysed.

2.6.2 HLA “Class I and Class II” Antigens in Sarcoidosis

HLA Class I (A, B, C) and HLA class II (HLA-DQ and HLA-DR) antigen typing in 51 consecutive patients of this cohort of patients with sarcoidosis, attending the Respiratory Clinic at the CMJAH were evaluated and compared with 63 controls. The control subjects were healthy potential organ donors, who had HLA typing performed previously. They were matched racially, as close as possible, to the patients. Phenotype frequencies of HLA class I antigens (A, B and C) and HLA class II alleles (DR and DQ) were calculated for the patient and control groups.

2.6.3 Serum IgG Subclasses in Sarcoidosis

Serum IgG subclass levels were prospectively studied in 17 consecutive adults with newly diagnosed sarcoidosis from Hillbrow Hospital in Johannesburg, over a 2-year period. The level of each of the individual IgG subclasses were measured initially (prior to any therapy) and subsequently (either on routine follow-up of cases not on treatment or after at least one-month therapy in cases treated with corticosteroids).

2.6.4 MicroRNA in Sarcoidosis

Serum expression of, approximately 800, miRNAs in six consecutive adult sarcoidosis patients were compared with race-, age- and gender-matched healthy controls. Control subjects were healthy staff volunteers from the hospital.

2.6.5 Chitotriosidase Activity in Sarcoidosis

Chitotriosidase activity was assayed in serum of 12 consecutive biopsy-proven patients with sarcoidosis before treatment, in nine patients after at least one-month of

treatment, in 10 newly diagnosed patients with tuberculosis and in 12 healthy controls. Controls were healthy staff members working at the hospital and laboratory.

2.7 Procedures

2.7.1 Definition of the Diagnosis of Sarcoidosis

Sarcoidosis diagnosis was established by clinical manifestations, radiological characteristics consistent with sarcoidosis, and histological confirmation of non-caseating granulomas after exclusion of other known causes of granulomas as described in “the joint declaration of the American Thoracic Society (ATS), the European Respiratory Society (ERS), and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG)” (1). There were three patients in the Clinical Study who did not have biopsies performed. Patients without histological confirmation of granulomas were deemed to have sarcoidosis if they had compatible clinical, laboratory and radiological features; with an appropriate response to corticosteroids, and/or spontaneous resolution, and without evidence of tuberculosis or any other cause for their clinical features.

2.7.2 Sarcoidosis Clinical Study

The charts of 102 patients attending the Pulmonology services at the Charlotte Maxeke Johannesburg Academic Hospital between 2002 and 2006 were reviewed. As indicated in the Clinical Research Form, APPENDIX A, the demographic data recorded included age, sex, race (which was self-reported), smoking habits and a history of previous therapy for TB. Clinical data included symptoms of dyspnoea, chest pain, cough, malaise, night sweats, weight loss and arthralgia. Signs recorded included the presence or absence of lymphadenopathy, parotid involvement, hepatomegaly,

splenomegaly, skin lesions, eye signs, abnormalities of the respiratory system, neurological signs and joint involvement. Laboratory data included routine haematological investigations, serum urea and electrolyte values, total protein, albumin, calcium and angiotensin-converting enzyme (ACE) levels, 24-hour urine calcium excretion, radiographic studies, pulmonary function studies, and histological studies. The treatment given to each patient, after the diagnosis was made, was recorded. The course of the disease was followed in terms of clinical data, chest radiography, lung function testing and biochemical data, when available.

2.7.3 Specimen Collection

For the additional blood investigations, described below, whole blood samples were obtained in ethylenediaminetetraacetic acid (EDTA) tubes and processed within 30 minutes (min) of collection. Tubes were centrifuged for 15 min at 3000g, at 4°C, after which the plasma was decanted to a sterile microcentrifuge tube and stored at -70°C until further usage.

2.7.4 HLA “Class I and Class II” Antigens in Sarcoidosis

Typing for HLA class I and II antigens were performed with the standard low-resolution molecular technique. “Genomic DNA was extracted from whole blood using the QIAmp® DNA Blood Mini Kit” (QIAGEN Inc., Germany) according to manufacturer’s instructions, APPENDIX B. The concentration (ng/µl) and purity (optical density 260/280) of the extracted DNA was measured using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop® Technologies, ThermoScientific, USA). Prior to use, the patients’ DNA samples were diluted to a concentration of 50ng/µl. HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ were analysed with the “polymerase chain reaction (PCR) sequence-specific primer (SSP) technique” (Dynal, UK). PCR was conducted

according to the manufacturer's specifications. Essentially an appropriate amount of master mix was aliquoted into an Eppendorf tube to which sample DNA (at 50ng/µl) and AmpliTaq DNA polymerase (at 5µ/µl) was added. After mixing well, 10µl of the PCR mix was dispended into each well of the Dynal Allset^{TM} SSP tray. After sealing securely, the Allset^{TM} trays were placed into a thermal cycler (MJ^{TM} Thermal Cycler, Bio-Rad) and the amplifications performed. The PCR conditions were; "1 cycle of 96˚C for 2 minutes (denaturation), followed by 10 cycles at 96˚C for 15 seconds (denaturation) and 65˚C for 1 minute (annealing and extension), and finally 20 cycles at 96˚C for 10 seconds (denaturation), 61˚C for 50 seconds (annealing), and 72˚C for 30 seconds (extension). PCR products was detected using agarose gel electrophoresis run on 2% agarose gel containing ethidium bromide after which gels were examined under UV light" and products documented by photography. Gels were analysed according to specific tables provided by Dynal and alleles scored using the SSP tool software supplied and manually. Alleles for each HLA subtype for each patient was recorded on the data sheets supplied.

2.7.5 Serum IgG Subclasses in Sarcoidosis

IgG subclasses were measured by means of laser nephelometry, using appropriate liquid reagent test kits (APPENDIX C, Human IgG Subclass Liquid Reagent Kits Package Insert, The Binding Site Ltd, Birmingham, England) and levels were assessed in a Behring Nephelometer 100 analyser (Behring Diagnostika, Germany).

2.7.6 MicroRNA in Sarcoidosis

RNA was extracted from the stored plasma samples using the QIAGEN miRNeasy Mini Kit® according to manufacturer's (Qiagen Sciences, MD, USA) instructions in APPENDIX D. Extractions were performed in duplicate on 200 µl of plasma for each

sample. Plasma samples were allowed to thaw completely before commencing with the extractions. Five volumes (1 ml) of “QIAzol Lysis reagent was added” to each sample and mixed thoroughly. Samples were “incubated at room temperature for 5 minutes” and 30 attomoles (final concentration) of each of 5 spike-in controls (“ath-miR159a, cel-miR-248, cel-miR-254, osa-miR414 and osa-miR442”) was added to each extraction and mixed thoroughly. Chloroform at an equal volume to the starting volume “(200 µl) was added and the samples shaken vigorously”. After “incubating at room temperature for 3 minutes, the samples were centrifuged for 15 minutes at 12000g, 4°C, and the upper, aqueous phase transferred to a new tube”. One and a half volumes of ethanol were combined to each sample, mixed thoroughly and the entire sample loaded onto a “RNeasy MinElute spin column”. After binding of the RNA to the spin column, the column was washed three times, allowed to dry thoroughly and the RNA eluted from the column twice with 50 µl “RNase-free water resulting in a final elution volume of 100 µl” per extraction.

Duplicate extractions were combined, and the RNA concentrated using a salt-ethanol precipitation. The samples were made up to 500 µl with “RNase-free water” and one tenth the volume (50 µl) diethyl pyrocarbonate (DEPC)-treated 3M sodium acetate was added along with 3 volumes (1.5 ml) ice-cold absolute ethanol. After mixing by inverting the tubes 5 times, the samples were “incubated overnight at -20°C”. The following day, the RNA was pelleted “by centrifugation for 10 minutes at 12000g, 4°C”. The resulting RNA pellet was washed twice in “ice-cold 70% ethanol and allowed to dry” thoroughly before resuspending in 15 µl RNAse free water. Extracts were stored at -70°C until needed.

The concentrated RNA extracted from the plasma samples was analysed using an nCounter® miRNA human v3 expression assay according to the manufacturer’s

protocol. Twelve samples (6 patient samples and 6 age-, race- and gender-matched controls) were analysed on a single cartridge, eliminating the need for calibration between cartridges. Unique oligonucleotide tags, specific for each of the 800 miRNAs in the panel, were ligated to miRNAs from the plasma extracted RNA. These tags allow for increased sensitivity and specificity when detecting the short miRNA sequences. The ligated, tagged miRNAs were denatured at 85°C for 5 min, quick-cooled on ice and then hybridised overnight to a capture probe/reporter probe pair. The capture probe has a biotin tag at its 3' end, which is used for binding of the hybridised probes to the imaging surface. The reporter probe has a colour coded molecular barcode at the 5' end, which is used to identify the specific miRNA targets during the data collection step. Following the overnight incubation step, hybridisation mixtures were loaded onto an nCounter® SPRINT cartridge for post-hybridisation processing and analysis with the nCounter® SPRINT analysis system. Within the cartridge, the hybridization mixtures were purified using a magnetic bead-based methodology to remove excess probe and non-target transcripts. The purified target/probe complexes were then immobilised on the imaging surface using the 3' biotin tags. The unique molecular barcodes on the 5' end of the complexes was scanned and the number of copies of each of the 800 miRNAs counted. These data were extracted to a .csv file and analysed using the nSolver™ Analysis Software, version 3.0.

2.7.7 Chitotriosidase Activity in Sarcoidosis

Chitotriosidase was assayed in the stored plasma samples essentially as described by Hollak et al., (2) using "4-methylumbelliferyl-β-D-N, N', N″-triacetylchitotriose" (Sigma) as an enzyme substrate in McIlvain's phosphate-citrate buffer; pH = 5.2, for 1 hr at 37.0℃ in darkness. By adding 2.5 mL of 0.3M glycine/NaOH buffer (pH 10.6) the

reaction was terminated. The fluorescent 4-methylumbelliferone reaction product was calculated using a Perkin-Elmer fluorimeter with an excitation wavelength of 365 nm and an emission of 450 nm. The chitotriosidase activity was expressed in nmol/h/mL.

2.8 Ethical Considerations

Approval to conduct the study was received from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand with written informed consent from the patients and controls to participate in the study (Ethics Approval Numbers, M110750, M110751, M110752, M121016, APPENDIX E).

2.9 Statistical Analyses

Statistical analysis was performed using Student's t-test, or Mann Whitney U-test, and the Chi-squared-test, or Fisher's exact test (2-tail) test, as appropriate. Statistical analysis and graphic representations of data were performed using GraphPad Prism 4.0 software. A p value < 0.05 was considered to be statistically significant.

Each individual was tested for the HLA-A, -B, -C, -DR and -DQ antigens depicted in Tables 4–8. Gene frequencies (gf) were calculated from the antigen frequency (af) by Bernstein's formula: $gf = 1 - \sqrt{(1 - af)}$. The frequency of the 'blank' gene(s) was obtained by subtracting the sum total of defined gene frequencies from one. The gene frequencies in the different groups were compared by the chi-square test. The odds ratio (OR), statistical significance and 95% confidence interval (CI) of the antigen frequencies in the cases were compared with the controls.

When comparing the IgG subclasses values, both categorical (high, normal or low) and continuous (mean values) variables were compared.

After performing a background subtraction using the geometric mean of the negative controls to control for background fluorescence, miRNAs with average counts of below 10 in both the patient and control groups were excluded from the analysis. Furthermore, miRNAs were identified as being differentially expressed if they had a fold change of less than -1.5 or greater than 1.5, as fold changes outside this range have been shown to be mostly due to variation within populations. Thus, from the set of 798 miRNAs present on the panel, only 145 were identified as possibly being differentially expressed in sarcoidosis patients. Applying a Bonferroni correction, to control for analysing multiple miRNAs, only miRNAs with p values < 0.0003 were considered significantly different. Additionally, "to account for a high false-positive rate possibly caused by a multiple comparison groups, correction of p value was performed by using false discovery rate (FDR) method according to Benjamini and Hochberg (3) where desired FDR equalled 5% and a p value ≤ 0.03 was considered to be significant".

The chitotriosidase activity data was expressed as median and interquartile range (IQR) and minimum and maximum (min-max) values. The Mann-Whitney test and the Kruskal-Wallis test, with a significance set at a $p < 0.05$, were used for comparisons between groups. To look for associations between variables, the Spearman test was used. Statistical analysis and graphical data representations were performed utilising GraphPad Prism 4.0 software.

2.10 Outline of the Thesis

Figure 2.1 outlines the format of the thesis. Chapter 1 introduces the subject and includes the literature review. Chapter 2 describes the problem statement, outlines the aims, objectives, materials and methods, study design and cohort, studies conducted and ethical considerations. In the ensuing chapters, the separate studies conducted in

patients with sarcoidosis, as per the objectives, are described. The clinical study in sarcoidosis is described in Chapter 3. In Chapter 4, HLA class I and II antigens were evaluated, and in Chapter 5, IgG subclass levels were assessed. In chapter 6, differentially expressed serum microRNA levels are reported and Chapter 7 describes analysis of serum chitotriosidase activity. A discussion of the results is formulated and future recommendations are suggested in Chapter 8 and finally, in Chapter 9, conclusions are drawn.

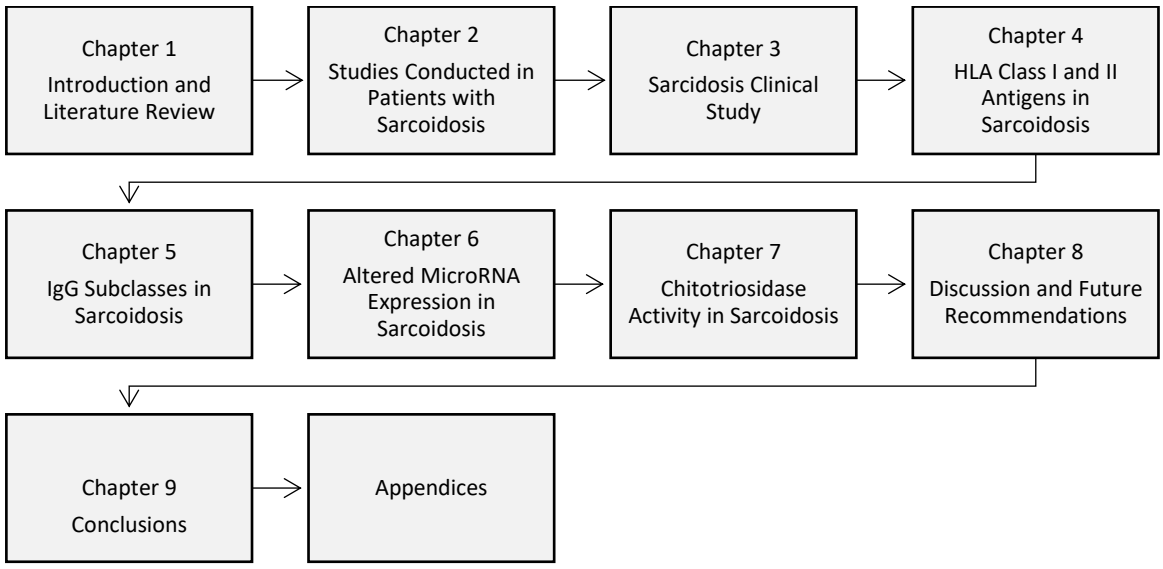

Figure 2.1. Outline of the thesis

2.11 References

1. Hunninghake G, Costabel U, Ando M, Baughman R, Cordier J, Du Bois R, et al. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. Sarcoidosis Vasc Diffuse Lung Dis. 1999;16(2):149–73.

2. Hollak C, van Weely S, Van Oers M, Aerts J. Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. J Clin Invest. 1994;93(3):1288–92.

3. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B Methodol. 1995;57(1):289–300.

Chapter 3 : Clinical Study of Patients with Sarcoidosis

3.1 Abstract

3.1.1 Background

Sarcoidosis is a multisystem granulomatous condition of uncertain aetiology that most frequently affects the lungs. The disease comprises important inflammatory and immune components; however, the immunopathogenesis is not well established. The immune responses that are innate and adaptive, together with cell-mediated immunity play a central role in sarcoidosis immunopathogenesis. Because of its similarities, both clinically and radiologically with tuberculosis (TB), and particularly in high prevalence regions, sarcoidosis is frequently misdiagnosed as TB. The current study was conducted to review the clinical features of sarcoidosis patients in the South African population, a relatively uncommon disease, adding clinical information to the relatively few studies that have been conducted of South African patients with sarcoidosis.

3.1.2 Methods

This was a retrospective record review conducted between 2002 and 2006, involving 102 patients with a diagnosis of sarcoidosis, at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). The research was undertaken to review the clinical profile of sarcoidosis patients within the South African population, since it is a rather poorly investigated condition in this setting.

3.1.3 Results

Out of 102 sarcoidosis patients, 59 were Black, 28 Indian, eight White and seven Coloured (mixed race). There were 69 (67.6%) females and 33 (32.4%) males. Most of them were non-smokers (85.3%). The mean age of the group was 44.6 years. Hypertension (32.4%), renal dysfunction (creatinine > 104 µmol/L) (19.5%), obesity (17.6%), diabetes mellitus (15.7%), asthma (11.8%), and gastro-oesophageal reflux (11.8%) were the most frequent associated chronic comorbid diseases. Indian patients had significantly more dyslipidaemia than Black patients ($p = 0.02$). Almost 17% of patients had been treated for TB, based on the chest radiograph, prior to being diagnosed as having sarcoidosis. Two patients developed active TB whilst being treated with corticosteroids for sarcoidosis. Four Black patients contracted human immunodeficiency virus (HIV) infection whilst on treatment for sarcoidosis. The salient clinical manifestations were dry cough, the commonest presenting symptom in 82.4%, dyspnoea was present in 53.9%, cutaneous lesions other than erythema nodosum in 33.3% and on lung examination crackles were noted in 37.3% of patients. The majority (48%) of patients had stage II chest radiographic changes. Cutaneous biopsies (28.4%), transbronchial lung biopsies (25.5%) and biopsies of mediastinal lymph nodes via mediastinoscopy (25.5%) were the most frequent sites for confirming granulomatous inflammation. Blacks had a greater likelihood to have non-erythema nodosum dermatological manifestations at presentation. In Blacks 67.8% had an elevated angiotensin-converting enzyme (ACE) level compared with 32.1% Indians, 50% Whites and 14.3% Coloureds, respectively. The mean ACE level was higher in Blacks than in Indians (157 vs. 58 IU/L). The initial mean serum calcium was elevated (> 2.5 mmol/L) in 22.7% of cases, the highest incidence being in Whites (37%). Raised gamma-glutamyl transferase (GGT) (> 78 IU/L) and alkaline phosphatase (AP) (> 128 IU/L) were noted in 31.8% and 21.6% of patients, respectively. Elevated creatinine (> 104 µmol/L) was seen in 19.5% of patients; Whites having the highest incidence of

renal dysfunction at 37%. Overall, 21.2% of patients had obstructive airways disease on lung function testing. Systemic corticosteroids were indicated in 87.3% of patients and the relapse rate was 60.7%.

3.1.4 Conclusion

Sarcoidosis is often misdiagnosed as TB in South Africa. Comorbid illnesses were common and should be screened for, and promptly treated, to improve quality of life (QoL). The most common sites of biopsy for histological confirmation were cutaneous, mediastinal lymph node and transbronchial lung biopsy. Most of the patients in this cohort with sarcoidosis had an indication to be treated with corticosteroids.

3.2 Introduction

Sarcoidosis is a widespread granulomatous disorder of unknown aetiology that primarily affects the lungs along with the lymphatic system. The condition occurs in almost every corner of the globe, impacting all races and ethnic groups with differing epidemiological findings from numerous reports. In Europe, the occurrence of sarcoidosis varies from 1-64 patients per 100,000 persons and in United States it has been reported as 10-35 per 100,000 persons, more commonly occurring in the Black population (1,2). The actual burden of disease in South Africa is not well established, as accurate epidemiologic information is unavailable. Initial studies dating back to the 1960’s indicated that in South Africa sarcoidosis was rare. During the late 60’s and 70’s a rate of 20.4-23.2 per 100,000 patients was estimated in the Black community, 11.6 per 100,000 in the Coloured population and 3.7 per 100,000 in the White residents (3,4). While sarcoidosis remains an underdiagnosed disorder, doctors are alert to it with growing understanding and increasing awareness. Due to its similarities to TB

sarcoidosis is under-reported from low and middle income nations. Owing to the high prevalence of TB and the clinical and radiological similarities with pulmonary TB, patients with sarcoidosis frequently get treated as TB. Therefore, this study was conducted to review the clinical profile of patients with sarcoidosis in Johannesburg, South Africa. This study describes and evaluates the clinical features of 102 patients with sarcoidosis and notes, similarities and differences between the various population groups.

3.3 Patients and Methods

This was a retrospective record review undertaken between 1 January, 2002 and 31 December, 2006, involving 102 patients seen at the CMJAH Pulmonology facilities. Permission for the research was obtained from the University of Witwatersrand's Committee for Research on Human Subjects and the Hospital, and written informed consent was obtained from patients to participate in the study (M110752 approval; APPENDIX E). Sarcoidosis diagnosis was established by clinical manifestations, radiological characteristics consistent with sarcoidosis, and histological confirmation of non-caseating granulomas after exclusion of other known causes of granulomas as described in “the joint declaration of the American Thoracic Society (ATS), the European Respiratory Society (ERS), and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG)” (5). Three patients in the study did not have biopsies performed. These patients without histological confirmation of granulomas were deemed to have sarcoidosis if they had compatible clinical, laboratory and radiological features; with an appropriate response to corticosteroids, and/or spontaneous resolution, and without evidence of tuberculosis or any other cause for their clinical features. The data collected included demographic, clinical,

laboratory, radiographic, treatment and outcome data of all patients (APPENDIX A) as indicated in Chapter 2 (2.7.2).

Statistical analysis was carried out with Student's t-test, and Mann Whitney U-test for continuous variables and the Chi-squared-test, or Fisher's exact test (2-tail), for categorical variables, as appropriate. The GraphPad Prism 4.0 programme was used to perform the statistical analysis and prepare graphical illustrations of the data. Statistical significance was a value of $p < 0.05$.

3.4 Results

There were 102 cases of sarcoidosis seen between January 2002 and December 2006 by the Respiratory Services at CMJAH. The demographic patients' data are shown in Table 3.1. The mean ± standard deviation (SD) age was 44.6 ± 12.1 years, ranging from 22 to 77 years. There were 69 females (67.6%) and 33 (32.4%) males. There were 59 Black patients, 28 Indians, eight Whites and seven Coloureds (mixed race). The mean ages in the Coloured and Indian patients were younger at 40.7 ± 15.4 and 39.3 ± 12.0 years, respectively, compared with the mean age of Blacks of 45.8 ± 10.9 years and Whites of 57.9 ± 4.6 years. The ratio of women to men in the Blacks was 3,5:1 compared with the Indians, Whites and Coloureds in whom the ratio was closer to 1:1.

Table 3.1. Demographic data of 102 patients with sarcoidosis

	Blacks	Indians	Whites	Coloureds
n (%)	59 (57.8)	28 (27.5)	8 (7.8)	7 (6.9)
Mean age ± SD	45.8 ± 10.9	39.3 ± 12.0	57.9 ± 4.6	40.7 ± 15.4
Females n (%)	46 (78)	15 (54)	5 (62)	3 (43)
Mean age ± SD	47.0 ± 10.7	43.6 ± 12.9	58.2 ± 4.2	45.7 ± 16.1
Males n (%)	13 (22)	13 (46)	3 (38)	4 (57)
Mean age ± SD	41.5 ± 10.8	34.4 ± 8.8	57.3 ± 6.0	37.0 ± 16.2

n = Number

% = Percent

SD = Standard deviation

Table 3.2 and Table 3.3 indicate the common comorbid conditions reported in sarcoidosis patients. Coexisting illnesses present in these patients included hypertension, renal dysfunction (creatinine > 104 µmol/L), overweight, diabetes mellitus, asthma, gastro-oesophageal reflux and dyslipidaemia, most are common conditions in the general population. There were no major differences between the groups, except that Indian patients had substantially more dyslipidaemia than Black patients (17.9% vs. 1.7%, p = 0,02). Table 3.4 and Table 3.5 indicate a more comprehensive list of comorbid diseases present in sarcoidosis patients.

Table 3.2. Common comorbid diseases in patients with sarcoidosis

Comorbid diseases	**n (%)**
Hypertension	33 (32.4)
Renal dysfunction (creatinine > 104 µmol/L)	17 (19.5)
Overweight	18 (17.6)
Diabetes mellitus	16 (15.7)
Asthma	12 (11.8)
Gastroesophageal reflux disease	12 (11.8)

n = Number

% = Percent

Table 3.3. Common comorbid diseases in sarcoidosis patients according to different race groups

Comorbid diseases	**Blacks (n=59)** n (%)	**Indians (n=28)** n (%)	**Whites (n=8)** n (%)	**Coloureds (n=7)** n (%)
Hypertension	20 (33.9)	8 (28.6)	3 (37.5)	2 (28.6)
Renal dysfunction (creatinine > 104 µmol/L)	5 (8.5)	7 (25)	3 (37.5)	2 (28.6)
Overweight	8 (13.6)	8 (28.6)	1 (12.5)	1 (14.3)
Diabetes mellitus	6 (10.2)	8 (28.6)	1 (12.5)	1 (14.3)
Asthma	7 (11.9)	3 (10.7)	0	2 (28.6)
Gastro-oesophageal reflux disease	8 (13.6)	2 (7.1)	2 (25.0)	0

n = Number

% = Percent

Table 3.4. Frequencies of all comorbid illnesses in sarcoidosis patients

Comorbid illnesses	n (%)
Hypertension	33 (32.4)
Renal dysfunction (creatinine > 104 µmol/L)	17 (16.7)
Overweight	18 (17.6)
Diabetes mellitus	16 (15.7)
Asthma	12 (11.8)
Gastro-oesophageal disease	12 (11.8)
Cardiac Failure	8 (7.8)
Dyslipidaemia	6 (5.9)
Cor pulmonale	4 (3.9)
Thyroid disease (3 goitre, 1 thyrotoxicosis)	4 (3.9)
HIV (mean CD4=233)	4 (3.9)
Iron deficiency anaemia	3 (2.9)
Peptic ulcer disease	3 (2.9)
Depression	3 (2.9)
Thyroid goitre	3 (2.9)
Dilated cardiomyopathy	3 (2.9)
Pulmonary tuberculosis	2 (2.0)
Thalassaemia trait	2 (2.0)
Hypertensive cardiomyopathy	1 (1.0)
Thyrotoxicosis	1 (1.0)
Vanishing bile duct syndrome	1 (1.0)
Pulmonary thromboembolic disease	1 (1.0)
Gilbert's Disease	1 (1.0)
Gout	1 (1.0)
Non-Hodgkin's lymphoma	1 (1.0)
Retrograde ejaculation	1 (1.0)
NASH	1 (1.0)
Glaucoma	1 (1.0)
Psoriasis	1 (1.0)
Gall stones	1 (1.0)
Baker's cyst	1 (1.0)
Small intestinal obstruction	1 (1.0)

"HIV = Human immunodeficiency virus

NASH = Non-alcoholic steatohepatitis

n = Number

% = Percent"

Table 3.5. Frequencies of all comorbid illnesses in sarcoidosis patients according to the different race groups

Comorbid illnesses	Blacks (n=59) n (%)	Indians (n=28) n (%)	Whites (n=8) n (%)	Coloureds (n=7) n (%)
Hypertension	20 (33.9)	8 (28.6)	3 (37.5)	2 (28.6)
Renal dysfunction				
Overweight	8 (13.6)	8 (28.6)	1 (12.5)	1 (14.3)
Diabetes mellitus	6 (10.2)	8 (28.6)	1 (12.5)	1 (14.3)
Asthma	7(11.9)	3 (10.7)	-	2 (28.6)
Gastro-oesophageal disease	8 (13.6)	2 (7.1)	2 (25)	-
Cardiac failure				
Dyslipidaemia	1 (1.7)	5 (17.9)*	-	-
Cor pulmonale	2 (3.4)	2 (7.1)	-	-
Dilated cardiomyopathy	2 (3.4)	-	1 (12.5)	-
Goitre	1 (1.7)	1 (3.6)	1 (12.5)	-
Thyrotoxicosis	1 (1.7)	-	-	-
HIV (mean CD4)	4 (6.8)	-	-	-
Pulmonary TB	3 (5.1)	-	-	-
Iron deficiency anaemia	2 (3.4)	1 (3.6)	-	-
Peptic ulcer disease	2 (3.4)	1 (3.6)	-	-
Depression	-	2 (7.1)	1 (12.5)	-
Thalassaemia trait	-	2 (7.1)	-	-
Hypertensive heart disease	1 (1.7)	-	-	-
Vanishing bile duct syndrome	1 (1.7)	-	-	-
Pulmonary thromboembolic disease	1 (1.7)	-	-	-
Gilberts Disease	-	-	1 (12.5)	-
Gout	-	-	1 (12.5)	-
Non-Hodgkin's lymphoma	1 (1.7)	-	-	-
Retrograde ejaculation	1 (1.7)	-	-	-
NASH	1 (1.7)	-	-	-
Glaucoma	-	1 (3.6)	-	-
Psoriasis	-	1 (3.6)	-	-
Gall stones	-	1 (3.6)	-	-
Bakers cyst	1 (1.7)	-	-	-
Small intestinal obstruction	1 (1.7)	-	-	-

"* p = 0,02 (There were no significant differences between the groups, except that Indian patients had significantly more dyslipidaemia than Black patients)

HIV = Human immunodeficiency virus

NASH = Non-alcoholic steatohepatitis

n = Number

% = Percent

- = 0"

The clinical profile of the patients is shown in Table 3.6 and Table 3.7. Three patients in the Indian population indicated a positive family background of sarcoidosis. The majority of patients were non-smokers (85.3%). Almost 17% of patients had been treated for tuberculosis, empirically, without response. As shown in Table 3.7 two Black patients developed active pulmonary TB whilst on corticosteroid therapy for sarcoidosis compared with none in the other population groups. Four (3.9%) Black patients developed human immunodeficiency virus (HIV) infection during their follow up treatment for sarcoidosis, and the mean CD4+ cell count with HIV infection was 233 cells/µL (Table 3.6).

Table 3.6. Clinical profile of patients with sarcoidosis

Clinical profile	n (%)
Family history	3 (2.94)
Non-smokers (n) (%)	87 (85.3)
Smokers (current/ex)	8/7
Previous history of tuberculosis	17 (16.7)
HIV positive (%) (Mean CD4+ cell count - cells/µL)	4 (3.9) (233)

n = Number

% = Percent

HIV = Human immunodeficiency virus

Table 3.7. Clinical profile of sarcoidosis patients according to the different race groups

Clinical profile

	Blacks (n=59)	**Indians (n=28)**	**Whites (n=8)**	**Coloureds (n=7)**
	n (%)	n (%)	n (%)	n (%)
Family history	0	3 (10.7)	0	0
Non-smokers	54 (91.5)	24 (85.7)	6 (75)	3 (42.9)
Current smokers	2 (3.4)	3 (10.7)	1 (12.5)	2 (28.6)
ex-smokers	3 (5.1)	1 (3.6)	1 (12.5)	2 (28.6)
Previously treated for TB	10 (16.9)	4 (14.3)	2 (25)	1 (14.3)
Developed active TB whilst on corticosteroids	2 (3.4)	0	0	0

"There were no significant differences between the groups

TB = Tuberculosis

n = Number

% = Percent"

The most common presenting symptoms were cough and dyspnoea (Table 3.8). Dyspnoea may be cardiac or respiratory, or both, in origin. There were six (5.9%) patients with cardiac sarcoidosis when evaluated for organ involvement as shown in Table 3.12. Chest pain occurred in 28 (27.5%) of patients. The clinical features of the patients with sarcoidosis are shown broken down according to the different race groups in Table 3.9. Blacks were more likely to have non-erythema nodosum dermatological manifestations at presentation. Erythema nodosum was seen in 6.8% of Blacks and 14.3% of Indians, and not in the White and Coloured patients. Only one Black patient was asymptomatic and the diagnosis made incidentally (Table 3.9).

Table 3.8. Presenting clinical features of patients with sarcoidosis

Presenting clinical features

	n (%)
Respiratory	
Cough	84 (82.4)
Dyspnoea	54 (53.9)
Chest pain	28 (27.5)
Haemoptysis	2 (2.0)
Crackles	38 (37.3)
Skin	
Erythema nodosum	8 (7.8)
Other	34 (33.3)
Ocular	19 (18.6)
Nervous system	3 (2.9)
Constitutional	
Weight loss	25 (24.5)
Fever	10 (9.8)
Night Sweats	20 (19.6)
Malaise or fatigue	31 (30.4)
Arthralgia	10 (9.8)
Jaundice	1 (1.0)
Asymptomatic	1 (1.0)

n = Number

% = Percent

Table 3.9. Presenting clinical features in sarcoidosis patients according to the different race groups

Presenting clinical features

	Blacks (n=59) n (%)	**Indians (n=28)** n (%)	**Whites (n=8)** n (%)	**Coloureds (n=7)** n (%)
Respiratory				
Cough	49 (83.1)	24 (85.7)	6 (75)	5 (71.4)
Dyspnoea	31 (52.5)	16 (57.1)	2 (25)	5 (71.4)
Chest pain	17 (28.8)	9 (32.1)	1 (12.5)	1 (14.3)
Haemoptysis	2 (3.4)	0	0	0
Crackles	25 (42.4)	9 (32.1)	2 (25)	2 (28.6)
Skin				
Erythema nodosum	4 (6.8)	4 (14.3)	0	0
Other	24 (40.7)	6 (21.4)	3 (37.5)	1 (14.3)
Ocular	10 (16.9)	8 (28.6)	0	1 (14.3)
Nervous system	0	2 (7.1)	1 (12.5)	0
Constitutional				
Weight loss	15 (25.4)	6 (21.4)	3 (37.5)	1 (14.3)
Fever	6 (10.2)	3 (10.7)	1 (12.5)	0
Night Sweats	9 (15.3)	6 (21.4)	3 (37.5)	2 (28.6)
Malaise or fatigue	16 (27.1)	11 (39.3)	3 (37.5)	1 (14.3)
Arthralgia	4 (6.8)	3 (10.7)	1 (12.5)	2 (28.6)
Jaundice	1 (1.7)	0	0	0
Asymptomatic	1 (1.7)	0	0	0

“There were no significant differences between the groups

n = Number

% = Percent”

Histological confirmation of the diagnosis was made in 99 (97.1%) of the patients. The most common biopsy sites in all patients were skin, followed by mediastinal lymph node via mediastinoscopy, transbronchial lung biopsy via fibre-optic bronchoscopy and peripheral lymph node (Table 3.10). In Blacks, the commonest biopsy site for histological confirmation was the skin (35.6%) and in the Indians, mediastinoscopy with lymph node biopsy (28.6%), as shown in Table 3.11.

Table 3.10. Biopsy sites for histological confirmation in patients with sarcoidosis

	n (%)
Histological confirmation	99 (97.1)
Biopsy sites (multiple sites at times)	
Skin	29 (28.4)
Mediastinoscopy and lymph node biopsy	26 (25.5)
Transbronchial lung biopsy	26 (25.5)
Peripheral lymph node	9 (8.8)
Surgical lung biopsy	8 (7.8)
Clinical	3 (2.9)
Liver	3 (2.9)
Bone marrow and trephine	2 (2.0)
Lacrimal gland	2 (2.0)
Salivary gland	1 (1.0)
Nasal	1 (1.0)

n = Number

% = Percent

Table 3.11. Biopsy sites for histological confirmation in patients with sarcoidosis according to the different race groups

Biopsy site for histological confirmation

Biopsy site	**Blacks (n=59)** n (%)	**Indians (n=28)** n (%)	**Whites (n=8)** n (%)	**Coloureds (n=7)** n (%)
Skin	21 (35.6)	4 (14.3)	3 (37.5)	1 (14.3)
Mediastinoscopy	16 (27.1)	8 (28.6)	1 (12.5)	1 (14.3)
TBLB	16 (27.1)	7 (25)	2 (25)	1 (14.3)
Lymph node	3 (5.1)	5 (17.9)	1 (12.5)	0
Surgical lung biopsy	2 (3.4)	4 (14.3)	0	2 (28.6)
Clinical	0	1 (3.6)	0	2 (28.6)
Liver	3 (5.1)	0	0	0
Lacrimal gland	2 (3.4)	0	0	0
BMAT	0	0	2 (25)	0
Salivary gland	0	0	1 (12.5)	0
Nasal	0	1 (3.6)	0	0

“There were no significant differences between the groups

TBLB = Transbronchial lung biopsy

BMAT = Bone marrow aspirate and trephine biopsy

n = Number

% = Percent”

Organ manifestations of sarcoidosis were wide and varied (Table 3.12). Thoracic manifestations, including hilar lymphadenopathy and parenchymal lung involvement, were present in 60-80% of all groups of patients (Table 3.13). Indians had more ocular manifestations compared with the Blacks, 28.6% vs 16.9 % (p = 0.52) as shown in Table 3.13.

Table 3.12. Organ involvement in patients with sarcoidosis

Organ involvement	n (%)
Lung parenchyma	74 (72.5)
Hilar lymphadenopathy	72 (70.6)
Skin (erythema nodosum and other lesions)	35 (34.3)
Peripheral lymphadenopathy	23 (22.5)
Ocular	19 (18.6)
Bone and joints	12 (11.8)
Hepatomegaly	10 (10.0)
Splenomegaly	9 (8.8)
Hepatosplenomegaly	7 (6.9)
Cardiac	6 (5.9)
Neurological	3 (2.9)
Renal Stones	2 (2.0)
Parotid enlargement	2 (2.0)
Clubbing	2 (2.0)
Jaundice	1 (1.0)
Asymptomatic	1 (1.0)

n = Number

% = Percent

Table 3.13. Organ involvement in patients with sarcoidosis according to the different race groups

Organ involvement	Blacks (n=59) n (%)	Indians (n=28) n (%)	Whites (n=8) n (%)	Coloureds (n=7) n (%)
Lung parenchyma	43 (72.9)	20 (71)	6 (75)	5 (71.4)
Hilar lymphadenopathy	40 (67.8)	21 (75)	6 (75)	5 (71.4)
Dermatological (not EN)	24 (40.7)	5 (17.9)	3 (37.5)	1 (14.3)
Lymphadenopathy	13 (22.0)	7 (25)	3 (37.5)	0
Ocular	10 (16.9)	8 (28.6)	0	1 (14.3)
Bone and joints	6 (10.2)	3 (10.7)	1 (12.5)	2 (28.6)
Hepatomegaly	7 (11.9)	1 (3.6)	2 (25)	0
Splenomegaly	4 (6.8)	2 (7.1)	3 (37.5)	0
Hepatosplenomegaly	4 (6.8)	1 (3.6)	2 (25)	0
Cardiovascular	4 (6.8)	1 (3.6)	1 (12.5)	0
Nervous system	0	2 (7.1)	1 (12.5)	0
Renal Stones	0	1 (3.6)	1 (12.5)	0
Parotid gland	1 (1.7)	1 (3.6)	0	0
Clubbing	2 (3.4)	0	0	0

“There were no significant differences between the groups

EN = erythema nodosum

n = Number

% = Percent”

The findings on chest radiograph are shown in Table 3.14. Most commonly, the patients presented with radiological evidence of hilar and/or mediastinal lymphadenopathy together with pulmonary nodules or reticulonodular infiltrates. The majority of patients had stage II (48%) chest radiographic features at the time of diagnosis, followed by stage I (23.5%). Overall, 21.4% of Indians and 5.2% of Blacks had stage IV chest radiograph at presentation (p = 0.09), as shown in Table 3.15.

Table 3.14. Chest radiograph stage in patients with sarcoidosis

Chest X-ray stage	n (%)
0 (Normal)	3 (2.9)
1	24 (23.5)
2	49 (48.0)
3	16 (15.7)
4	10 (9.8)

n = Number

% = Percent

Table 3.15. Chest radiograph stage in patients with sarcoidosis according to the different race groups

Chest x-ray stage	Blacks (n=59) n (%)	Indians (n=28) n (%)	Whites (n=8) n (%)	Coloureds (n=7) n (%)
Stage 0	2 (3.4)	0	1 (12.5)	0
Stage I	13 (22.4)	8 (28.6)	1 (12.5)	2 (28.6)
Stage II	28 (48.3)	13 (46.4)	5 (62.5)	3 (42.9)
Stage III	13 (22.4)	1 (3.6)	1 (12.5)	1 (14.3)
Stage IV	3 (5.2)	6 (21.4)	0	1 (14.3)

“There were no significant differences between the groups

n = Number

% = Percent”

The initial laboratory data of the group as a whole, are shown in Table 3.16 and then broken down into the different race groups in Table 3.17. Abnormal laboratory data in the patients are shown in Table 3.18 and these results are broken down by race groups in Table 3.19. The initial mean ± standard deviation (SD) haemoglobin was 14.2 ± 1.83 g/dL ranging between 9.0 and 19.1 g/dL (five patients had anaemia, haemoglobin < 11

g/dL). The mean ± SD for the white cell count was 6.90 ± 3.0 × 10^9/L, with a range between 1.2 and 23.5 × l0^9/L (six cases had a white cell count < 4 × 10^9/L). The platelet count (mean ± SD) was 295 ± 102 × 10^9/L, with a range between 99 and 653 × 10^9/L (two patients had platelets below 140 × 10^9/L). The initial mean erythrocyte sedimentation rate (ESR) (mean ± SD) was 24 ± 27 mm/hr with a range between 0-125 mm/hr. A raised (≥ 20 mm/1st hour) ESR (mean ± SD of 49 ± 29) was found in 36 (40.4%) of patients (Table 3.18). The initial mean ± SD serum angiotensin-converting enzyme (sACE) concentration was 116 ± 307 U/L and ranging between 9-3000 U/L. The sACE level was elevated above 52 IU in 56.8% of patients. In Blacks 67.8% had an elevated ACE level compared with 32.1% Indians, 50% Whites and 14.3% Coloureds respectively. As seen in Table 3.17, the mean ACE level was higher in Blacks than in Indians (157 vs. 58 IU/L; p = 0.56, not significantly different); however, in those that it was raised, it was approximately twice the level of that in Indians, Whites and Coloureds. The initial mean serum calcium level was 2.39 ± 0.21 mmol/L and was elevated (> 2.5 mmol/L) in 20 (22.7%) of cases, the highest incidence being in the Whites (37%) (Table 3.19). Hypercalciuria (> 300g/24 hours) was observed in 4 (11.1%) of 36 cases in whom it was tested. Raised gamma-glutamyl transferase (GGT) (> 78 IU/L) and alkaline phosphatase (AP) (> 128 IU/L) were observed in 28 (31.8%) and 19 (21.6%) of patients, respectively (Table 3.18). Elevated creatinine (> 104 µmol/L) was seen in 17 (19.5%) of patients; Whites having the highest incidence of renal dysfunction at 37.5% (Table 3.19).

Table 3.16. Initial laboratory values of patients with sarcoidosis*

Parameter	Mean ± SD	n
Hb (g/dl)	14.2 ± 1.83	94
WCC (x 10^9 /L)	6.9 ± 3.0	94
Plt (x 10^9 /L)	295 ± 102	91
ACE (IU)	116 ± 307	95
ESR (mm/min)	24 ± 27	89
Ca (mmol/l)	2.39 ± 0.21	88
Mg (mmol/l)	0.81 ± 0.10	61
PO4 (mmol/l)	1.10 ± 0.19	73
TBili (µmol/l)	12 ± 18	86
DB (µmol/l)	4 ± 12	84
GGT (IU/L)	79 ± 100	88
AP (IU/L)	126 ± 145	88
ALT (IU/L)	43 ± 115	87
AST (IU/L)	36 ± 58	86
Alb (g/dL)	42 ± 5	89
TP (g/dL)	79 ± 7	87
Na (mmol/l)	139 ± 10	87
K (mmol/l)	4.0 ± 0.4	87
Cl (mmol/l)	104 ± 4	87
U (mmol/l)	5.3 ± 2.1	87
Cr (µmol/l)	91 ± 32	87
Hypercalciuria (g/24hs)	4	36

"* : values expressed as mean and standard deviation (SD)

Hb = Haemoglobin, WCC = White cell count, Plt = Platelets

ACE = Angiotensin-converting enzyme

ESR = Erythrocyte sedimentation rate

Ca++ = Calcium, Mg = Magnesium, PO4 = Phosphate

TBili = Total bilirubin, DB= Direct bilirubin, AP = Alkaline phosphatase, GGT = gamma-glutamyl transferase, ALT = alanine transaminase, AST = aspartate transaminase, Alb = albumin, TP = Total protein

Na = Sodium, K = Potassium, Cl = Chloride, U = Urea, Creat = Creatinine

n = Number"

Table 3.17. Initial laboratory values of sarcoidosis patients according to the different race groups*

Parameter	Blacks	n	Indians	n	Whites	n	Coloureds	n
Hb (g/dl)	14.0 ± 1.8	54	14.3 ± 1.8	26	13.6 ± 1.1	7	15.3 ± 1.9	7
WCC (x 10^9 /L)	6.6 ± 3.4	54	7.4 ± 2.3	26	5.8 ± 1.5	7	8.4 ± 1.4	7
Plt (x 10^9 /L)	310 ± 108	53	278 ± 99	25	278 ± 78	6	264 ± 71	7
ACE (IU)	157 ± 398	55	58 ± 51	25	72 ± 43	8	47 ± 38	7
ESR (mm/min)	29 ± 29	53	15 ± 21	25	25 ± 35	6	9 ± 16	5
Ca (mmol/l)	2.38 ± 0.22	51	2.42 ± 0.16	23	2.44 ± 0.39	7	2.37 ± 0.12	7
Mg (mmol/l)	0.80 ± 0.09	37	0.81 ± 0.13	14	0.79 ± 0.07	4	0.88 ± 0.07	6
PO4 (mmol/l)	1.07 ± 0.20	43	1.17 ± 0.19	17	1.13 ± 0.21	6	1.05 ± 0.11	7
TBili (µmol/l)	11 ± 14	50	17 ± 27	24	10 ± 9	6	9 ± 3	6
DB (µmol/l)	4 ± 11	48	6 ± 16	24	2 ± 1	6	2 ± 1	6
GGT (IU/L)	91 ± 112	51	70 ± 93	25	30 ± 20	6	63 ± 40	6
AP (IU/L)	150 ± 182	51	100 ± 49	24	74 ± 37	7	86 ± 60	6
ALT (IU/L)	30 ± 25	51	76 ± 215	24	37 ± 38	6	31 ± 15	6
AST (IU/L)	31 ± 15	50	50 ± 107	24	29 ± 20	6	27 ± 9	6
Alb (g/dL)	41 ± 5	52	45 ± 3	25	44 ± 5	6	41 ± 3	6
TP (g/dL)	81 ± 8	51	79 ± 5	24	74 ± 6	6	72 ± 4	6
Na (mmol/l)	138 ± 13	55	140 ± 2	26	140 ± 4	7	141 ± 2	6
K (mmol/l)	3.99 ± 0.46	55	3.9 ± 0.4	26	4.3 ± 0.3	7	4.0 ± 0.4	6
Cl (mmol/l)	104 ± 4	55	103 ± 3	26	105 ± 5	7	103 ± 4	6
U (mmol/l)	4.8 ± 1.8	55	5.5 ± 1.6	26	7.9 ± 3.8	7	5.4 ± 2.1	6
Cr (µmol/l)	88 ± 31	55	92 ± 21	26	120 ± 68	7	87 ± 19	2

"* : values expressed as mean and standard deviation (SD)

There were no significant differences between the groups

Hb = Haemoglobin, WCC = White cell count, Plt = Platelets

ACE = Angiotensin-converting enzyme

ESR = Erythrocyte sedimentation rate

Ca++ = Calcium, Mg = Magnesium, PO4 = Phosphate

TBili = Total bilirubin, DB= Direct bilirubin, AP = Alkaline phosphatase, GGT = gamma-glutamyl transferase, ALT = alanine transaminase, AST = aspartate transaminase, Alb = albumin, TP = Total protein

Na = Sodium, K = Potassium, Cl = Chloride, U = Urea, Creat = Creatinine

n = Number"

Table 3.18. Abnormal laboratory results of patients with sarcoidosis

Parameter	n (%)	Mean ± SD
Anaemia (Hb < 11 g/dL) (n=94)	5 (5.3)	10.2 ± 0.7
Leukopenia (WCC < 4 x 10^9 /L (n=94)	6 (6.4)	3.1 ± 0.9
Thrombocytopenia (Plt <150 x 10^9/L) (n=91)	2 (2.2)	(103)
Raised ESR (≥ 20 mm/1st hour)	36 (40.4)	49 ± 29
Raised ACE (> 52 IU) (n=95)	54 (56.8)	180 ± 396
Raised serum Ca++ (> 2.50 mmol/L (n=88)	20 (22.7)	2.62 ± 0.19
Hypercalciuria (> 300g/24 hours) (n=36)	4 (11.1)	
Raised GGT (> 78 IU/L) (n=88)	28 (31.8)	173 ± 135
Raised AP (> 128 IU/L) (n=88)	19 (21.6)	287 ± 251
Raised Creat (> 104 µmol/L) (n=87)	17 (19.5)	137 ± 54

“Hb = Haemoglobin

WCC = White cell count

Plt = Platelets

ESR = Erythrocyte sedimentation rate

ACE = Angiotensin-converting enzyme [normal 8-52 IU]

Ca++ = Calcium (normal 2.15-2.50 mmol/L)

Creat = Creatinine (normal 64-104 µmol/L)

AP = Alkaline phosphatase (normal 0-120 IU/L)

GGT = Gamma-glutamyl transferase (normal 0-78 IU/L)

n = Number

% = Percent”

Table 3.19. Abnormal laboratory results of sarcoidosis patients according to the different race groups

Parameter	Blacks (n=59) n (%)	Indians (n=28) n (%)	Whites (n=8) n (%)	Coloureds (n=7) n (%)
Anaemia (Hb < 11 g/dL) (n=94)	3 (5.1)	2 (7.1)	0	0
Leukopenia (WCC < 4 x 10^9 /L (n=94)	6 (10.2)	0	0	0
Thrombocytopenia (Plt <150 x 10^9/L) (n=91)	1 (1.7)	1 (3.6)	0	0
Raised ESR ((≥ 20 mm/1st hour)	26 (44.1)	7 (25.0)	2 (25.0)	1 (14.3)
Raised ACE (> 52 IU) (n=95)	40 (67.8)	9 (32.1)	4 (50)	1 (14.3)
Raised serum Ca++ (> 2.50 mmol/L (n=88)	11 (18.6)	6 (21.4)	3 (37.5)	0
Hypercalciuria (> 300g/24 hours) (n=36)	2 (3.4)	0	1 (12.5)	1 (14.3)
Raised GGT (> 78 IU/L) (n=88)	22 (37.3)	4 (14.3)	0	2 (28.6)
Raised AP (> 128 IU/L) (n=88)	13 (22.0)	4 (14.3)	1 (12.5)	1 (14.3)
Raised Creat (> 104 µmol/L) (n=87)	5 (8.5)	7 (25.0)	3 (37.5)	2 (28.6)

“There were no significant differences between the groups

Hb= Haemoglobin

WCC = White cell count

Plt = Platelets

ESR = Erythrocyte sedimentation rate

ACE = Angiotensin converting enzyme [normal 8-52 IU]

Ca++ = Calcium (normal 2.15-2.50 mmol/L)

Creat = Creatinine (normal 64-104 µmol/L)

AP = Alkaline phosphatase (normal 0-120 IU/L)

GGT = Gamma-glutamyl transferase (normal 0-78 IU/L)

n = Number

% = Percent”

Table 3.20 and Table 3.21 indicate the results of pulmonary function tests in patients with sarcoidosis. Lung function studies were performed on initial presentation in 99 of the 102 cases. On the parameters tested, the FVC was normal or increased in 85 (85.9%) cases, suggestive of a restrictive pattern and decreased (< 70%), in 14 (14.1%) cases. The total lung capacity (TLC) was decreased in five (5.1%) cases. Airway obstruction (FEV1/FVC < 70%) was documented in 21 (21.2%) cases, (15.3% of Blacks, 14.3% Indians, 50% of Whites and 57% of Coloureds) and a reduced diffusion capacity for carbon monoxide (DLCO) in 29 (37.2%) of cases (47.6% of Indians, Blacks 34.9% and 28.6% of Coloureds, as shown in Table 3.21).

Table 3.20. Pulmonary function tests in patients with sarcoidosis

FVC (n=99)	**n (%)**
> 90% predicted	58 (58.6)
70-90% predicted	27 (27.3)
<70% predicted	14 (14.1)
FEV_1/FVC (n=99)	
>90%	5 (5.1)
70-90% predicted	73 (73.7)
<70% predicted	21 (21.2)
Total Lung Capacity (n=78)	
>110%	16 (16.2)
90-110%	34 (34.3)
70-90% predicted	23 (23.2)
<70% predicted	5 (5.1)
DLCO (n=78)	
<80%	29 (37.2)

"FEV1 = Forced expiratory volume in 1 second

FVC = Forced vital capacity

TLC = Total lung capacity

DLCO = Diffusion capacity for carbon monoxide

n = Number

% = Percent"

Table 3.21. Pulmonary function tests in sarcoidosis patients according to the different race groups

Pulmonary function tests	Blacks (n=59) n (%)	Indians (n=28) n (%)	Whites (n=8) n (%)	Coloureds (n=7) n (%)
FVC	**n=56 (%)**	**n=28 (%)**	**n=8 (%)**	**n=7 (%)**
> 90% predicted	37 (62.7)	12 (42.9)	6 (75.0)	3 (42.9)
70-90% predicted	10 (16.9)	12 (42.9)	1 (12.5)	4 (57.1)
<70% predicted	9 (15.3)	4 (14.3)	1 (12.5)	0
FEV_1/FVC				
>90%	3 (5.1)	1 (3.6)	0	1 (14.3)
70-90% predicted	44 (74.6)	23 (82.1)	4 (50.0)	2 (28.6)
<70% predicted	9 (15.3)	4 (14.3)	4 (50.0)	4 (57.1)
TLC (n=78)	**n=43 (%)**	**n=21 (%)**	**n=7 (%)**	**n=7 (%)**
>110%	10 (16.9)	2 (9.5)	2 (25.0)	2 (28.6)
90-110%	19 (32.2)	8 (38.1)	3 (37.5)	4 (57.1)
70-90% predicted	9 (15.3)	11 (52.4)	2 (25.0)	1 (14.3)
<70% predicted	5 (8.5)	0	0	0
DLCO (n=78)				
<80%	15 (34.9)	10 (47.6)	0	2 (28.6)

"There were no significant differences between the groups

FEV1 = Forced expiratory volume in 1 second

FVC = Forced vital capacity

TLC = Total lung capacity

DLCO = Diffusion capacity for carbon monoxide

n = Number

% = Percent"

The treatments received by the patients are shown Table 3.22. Most of the patients had an indication for corticosteroids and these agents were prescribed in 89 (87.3%) patients. Patients were treated and followed-up in the Respiratory Outpatient Department, together with the other sub-specialties such as neurology, ophthalmology, cardiology and dermatology. There was a good initial clinical response in most, and a few cases were observed who did not have an indication for therapy, and appeared to have spontaneous clinical response; however, 54 patients of the cohort (60.7%) had a relapse, approximately 2 to 5 years after initiation of corticosteroids. Chloroquine was

prescribed in 27 (26.5%) of patients, methotrexate in 10 (9.8%), azathioprine in four (3.9%), colchicine in two (2.0%) and cyclophosphamide in one patient, as illustrated in Table 3.22. As shown in Table 3.23, antimalarials (chloroquine) were prescribed for 33.9% of Blacks, 7.1% of Indians, 50% of Whites and 14.3% of Coloureds. Other immunosuppressive drugs that were used in the different race groups are shown in Table 3.23. Relapses occurred in a total of 60.7% of patients of whom Indian patients had the highest relapse rate at 78.2% compared with 71.4%, 52.8%, and 42.9% in the Coloured, Black and White patients respectively; however, this was not significantly different.

Table 3.22. Treatments received and relapse rates in patients with sarcoidosis

Treatments received	**n (%)**
Corticosteroids (CS)	89 (87.3)
Chloroquine (CQ)	27 (26.5)
Methotrexate (MTX)	10 (9.8)
Azathioprine (AZA)	4 (3.9)
Colchicine (COL)	2 (2.0)
Cyclophosphamide (CYC)	1 (1.0)
Combination therapy	
CS + CQ	20 (19.6)
CS + MTX	4 (3.9)
CS + CQ + MTX	3 (2.9)
CS + CQ + MTX + AZA	2 (2.0)
CS + CQ + MTX + AZA + CYC	1 (1.0)
CS + CQ + COL	1 (1.0)
CS + COL	1 (1.0)
CS + AZA	1 (1.0)
Other	
Inhaled steroids	34 (33.3)
Nasal steroids	15 (14.7)
Relapse Rate	54 (60.7)

n = Number

% = Percent

Table 3.23. Treatments received and relapse rates in sarcoidosis patients according to the different race groups

Treatments received	**Blacks (n=59)** n (%)	**Indians (n=28)** n (%)	**Whites (n=8)** n (%)	**Coloureds (n=7)** n (%)
Corticosteroids (CS)	53 (89.8)	23 (82.1)	7 (87.5)	6 (85.7)
Chloroquine (CQ)	20 (33.9)	2 (7.1)	4 (50.0)	1 (14.3)
Methotrexate (MYX)	7 (11.9)	2 (7.1)	0	1 (14.3)
Azathioprine (AZA)	3 (5.1)	1 (3.6)	0	0
Cyclophosphamide (CYC)	1 (1,7)	0	0	0
Colchicine (COL)	1 (1.7)	1 (3.6)	0	0
Combination Therapy				
CS + CQ	13 (22.0)	2 (7.1)	4 (50.0)	1 (14.3)
CS + MTX	1 (1.7)	2 (7.1)	0	1 (14.3)
CS + CQ + MTX	3 (5.1)	0	0	0
CS + CQ + MTX + AZA	2 (3.4)	0	0	0
CS + CQ + MTX + AZA + CYC	1 (1.7)	0	0	0
CS + CQ + COL	1 (1.7)	0	0	0
CS + COL	0	1 (3.6)	0	0
CS + AZA	0	1 (3.6)	0	0
Other				
Inhaled steroids	18 (30.5)	12 (42.9)	1 (12.5)	3 (42.9)
Nasal steroids	8 (13.6)	7 (25.0)	0	0
Relapse Rate	28 (52.8)	18 (78.2)	3 (42.9)	5 (71.4)

"There were no significant differences between the groups

n = Number

% = Percent"

The main indications (sometimes multiple) for corticosteroid therapy in the 89 patients who received treatment are listed in Table 3.24. Almost 90% of all patients required therapy because of their respiratory symptoms, chest radiograph and/or pulmonary function tests. In 13 (14.6%) patients, posterior or pan uveitis was the indication for therapy. The other indications include disfiguring facial lesions (four patients) and lupus pernio (two patients), generalised lymphadenopathy (four patients), cardiac (three with dilated cardiomyopathy and one with complete heart block) disease,

hepatosplenomegaly and generalised lymphadenopathy (three patients) and constitutional symptoms (three patients).

Table 3.24. Main indications (sometimes multiple) for corticosteroid therapy in 89 patients with sarcoidosis

Indication for corticosteroid treatment	n (%)
• Respiratory (persistent symptoms, abnormal lung functions and stage II to IV chest radiograph)	45 (50.6)
• Uveitis (posterior, pan-uveitis, retrobulbar neuritis)	13 (14.6)
• Disfiguring facial lesions	4 (4.5)
• Generalised lymphadenopathy	4 (4.5)
• Cardiac (3 dilated cardiomyopathy and 1 complete hear block)	4 (4.5)
• Hepatosplenomegaly and generalised lymphadenopathy	3 (3.4)
• Constitutional symptoms	3 (3.4)
• Lupus pernio	2 (2.2)
• Hypercalcaemia	2 (2.2)
• Renal calculi	2 (2.2)
• Meningitis	2 (2.2)
• Jaundice	1 (1.1)
• Splenomegaly and neutropenia	1 (1.1)
• Parotidomegaly	1 (1.1)
• Superior vena caval obstruction	1 (1.1)
• Dactylitis	1 (1.1)

n = Number

% = Percent

3.5 Discussion

This analysis adds to the knowledge of demographic, clinical, and laboratory features on presentation, as well as the treatment of sarcoidosis patients in South Africa. Furthermore, both similarities and differences were noted in the various characteristics among the different population groups. The actual occurrence of sarcoidosis in South

Africa is unknown. One of the reasons may be owing to under-reporting due to similarities with TB.

3.5.1 Main Study Findings

In the current cohort, there were some differences in the demographic, clinical and laboratory characteristics between race groups, however, they were not significant in the main. Statistical analysis were performed between the different racial groups and the only one that was significantly different was Indian patients had significantly more dyslipidaemia than Black patients (p = 0.02). This may be due to the small numbers among the different racial groups. Compared with previous studies from South Africa (3,4) and from abroad (6–8) (Table 3.25), this cohort, composed mainly of cases from Johannesburg, South Africa, has a higher number of Blacks and lower percentage of Indians (largest known reported cohort from South Africa), Whites and Coloureds (mixed race).

Of 102 sarcoidosis patients, there were 69 females (67.6%) and 33 (32.4%) males. Majority of patients were non-smokers (85.3%). The group's mean age was 44.6 years. Hypertension (32.4%), renal dysfunction (creatinine > 104 µmol/L) (19.5%), obesity (17.6%), diabetes mellitus (15.7%), asthma (11.8%), and gastro-oesophageal reflux (11.8%) were the most prevalent associated chronic comorbid diseases. Indian patients had significantly more dyslipidaemia than Black patients (p = 0.02). Almost 17% of patients had been treated for tuberculosis, based on the chest radiograph, prior to the diagnosis of sarcoidosis. The majority (48%) of patients had stage II chest radiographic changes. Cutaneous biopsies (28.4%), transbronchial lung biopsies (25.5%) and biopsies of mediastinal lymph nodes via mediastinoscopy (25.5%) were the most frequent sites for confirming granulomatous inflammation. In Blacks 67.8%

had an elevated ACE level compared with 32.1%, 50% and 14.3% in Indians, Whites and Coloureds respectively. The mean ACE level was higher in Blacks than in Indians (157 vs. 58 IU/L). The initial mean serum calcium was elevated (> 2.5 mmol/L) in 22.7% of cases, the highest incidence being in Whites (37%). Raised gamma-glutamyl transferase (GGT) (> 78 IU/L) and alkaline phosphatase (AP) (> 128 IU/L) were observed in 31.8% and 21.6% of patients, respectively. Elevated creatinine (> 104 µmol/L) was seen in 19.5% of patients. Overall, 21.2% of patients had obstructive airways disease on lung function testing. Systemic corticosteroids were indicated in 87.3% of patients and the relapse rate was 60.7% which occurred between two to five years after initiation of corticosteroids. In this study the clinical features among the racial groups were comparable in severity to the Blacks. In both, Blacks and Indians, the disease appeared more severe, and more extensive than in Caucasians and Coloureds.

In the Cape Town study “differences between Whites and Blacks are similar to those reported from the USA”, and the Coloured patients seem to resemble the Black patients in terms of severity. “A Case Control Etiologic Study of Sarcoidosis (ACCESS)” (7), women were more likely to have eye and neurologic involvement, have erythema nodosum, and men were more likely to be hypercalcaemic. Black subjects were more likely to have skin involvement other than erythema nodosum, liver, bone marrow, and extrathoracic lymph node involvement. In the UK study (6), erythema nodosum was a more common presenting feature in Caucasians than in Blacks or Asians. Blacks developed sarcoidosis later than Caucasians. They had more widespread extrathoracic disease than Caucasians and were more commonly treated with corticosteroids. In the Asians there was a male predominance compared with Caucasians. Asians also had more extrathoracic sarcoidosis than Caucasians and more of them were treated with corticosteroids. A stage II chest radiograph at

presentation and skin sarcoidosis were less common in Asians than in Blacks. In both Blacks and Asians, the disease was “more common, more severe and more extensive” than in Caucasians. In the worldwide study (8) there was no definite sex predominance and most of the patients were under 40 years.

Table 3.25. Demographic data and organ involvement characteristics of previously reported studies

	Current Study	Smith *et al*	Benatar	Baughman *et al*	Edmondstone and Wilson	James *et al*
n	102	82	110	736	156	3676
Country of origin	South Africa	South Africa	South Africa	USA	UK	Worldwide
Female (%)	68	64.6	61.5	63.6	51.9	57
Mean age onset (years)	44.6	40.3		36.0	37.1	< 40
Blacks (%)	59	51	25	44.2	59	10
Whites (%)	8	31	14	53.4	68	79
Indians/Asians (%)	28	0	0	NA	29	NA
Coloureds (%)	7	0	71	NA	NA	NA
Other (%)	NA	NA	NA	2.4	NA	Jap = 8, PR = 1
"Chest radiograph (%)						
Stage 0	2.9	5.0	2.7	8.3	5.8	8
Stage 1	23.5	25.3	26.3	39.7	55.8	51
Stage 2	48.0	40.5	35.4	36.7	30.1	29
Stage 3	15.7	29.1	26.3	9.8	8.3	12
Stage 4"	9.8	0	9.0	5.4	0	0
Organs involved (%)						
Intrathoracic	97	94.9	97.0	91.6	94.2	87
Hila nodes	70.6	64.6	61.7	76.4	85.3	80
Lungs	72.5	68.3	70.7	95	58.3	41
Skin*	33.3	40.2	19.0	15.9	17.9	9
Lymph node	22.5	41.4	34.3	15.2	19.9	22
Eye	18.6	12.2	18.0	11.8	19.9	15
Hepatic	16.9	NA	NA	11.5	16.0	NA
EN	7.8	4.9	10.0	8.3	NA	17
Spleen	15.7	25.6	15	6.7	14.1	6
Neurologic	2.9	4.9	3.2	4.6	4.5	4
Parotid/salivary	2.0	3.7	NA	3.9	7.1	4
Bone marrow	2.0	NA	NA	3.9	NA	NA
Hypercalcaemia	22.7	12.2	8.3	3.7	1.3	11
Ear, nose & throat	1	NA	NA	3.0	NA	NA
Heart	5.9	3.7	1.8	2.3	2.6	NA
Kidney	2.0	NA	NA	0.7	2.6	NA
Bone/joint	11.8	2.4	21.3	0.5	1.9	3
Muscle	0	NA	NA	0.4	NA	NA
Hyperglobulinaemia	NA		68.3	NA	40.4	44
Corticosteroid therapy (%)	87.3	84.1	50.0	NA	52.4	47

n = Patient number

% = Percentage

NA = not available

EN = Erythema Nodosum

Jap = Japanese, PR = Puerto Rican

*Excluding Erythema Nodosum

3.5.2 Age and Sex

Sarcoidosis burden varies greatly by age and sex. The “ATS/ERS/WASOG statement expresses that sarcoidosis consistently shows a predilection for adults younger than 40 years, peaking at 20-29 years of age”. Be that as it may, Scandinavian nations and Japan has registered a second peak in women greater than 50 years of age. “Other studies from western countries, additionally, report more than 70% patients with sarcoidosis to be under 40 years” (9,10). This study revealed that the mean age of the sarcoidosis cases was 44.6 years and 64% of the patients were over 40 years old. Comparable findings have been noted in other South African reports (3,4). It is not clear what causes this variation. There are signs that gender plays a part in the disease’s manifestation. In certain communities, 10-year disparity in the age gap exists on diagnosis between males and females. “In Sweden, the age at diagnosis in men was 45 years compared with 54 years in women” (11) and the “diagnostic age was 47 years for males and 54 years for females in Italy” (12). In other countries, such as in Spain, the age gap is not as large (“men 44 years versus women 49 years”) (13). In most reports, “the overall average age of onset was between 47 and 51 years, apart from one Estonian study (average age for men 34 years and women 43 years)” (14). The claim quoted as evidence that sarcoidosis onset peaks at ages 20 to 45 years (5), is not endorsed by the latest reports with peaks reported between ages of 30-55 years.

As seen from a Japanese report, the onset age can vary with time (15). The age rose over a time-frame of 40 years, with an historical peak occurring in males in their twenties have decreased. This can be attributed to a maturing community or an alteration in awareness with time (improved diagnosis, fewer regular x-rays performed as part of men's workplace examinations that were common in the 1980s, and

improved and expanded women's medical use). Nonetheless, it may mean that exposures to gender-related environmental factors can alter with time. The age gap in males and females at diagnosis indicates that this may be somewhat important in disease pathogenesis between the genders that could be attributed to inherited or genetic components or sex-specific environmental factors, such as reproductive factors. Globally it has been reported that females are more commonly affected by sarcoidosis (5,7), though, a male preponderance has been recorded in studies from India (16,17). Yet in this study cohort, there were twice as many females (67.6%), similar to the previous study from Johannesburg (64.6% females) (4); notwithstanding, there were somewhat less females with 1,6 females to 1 male in the Cape Town Study (3).

3.5.3 Race

In South Africa, sarcoidosis is seen in all race groups. Of 102 patients with sarcoidosis, 59 were Black, 28 Indian, 8 White and 7 Coloured (Mixed race). The race distribution in the sarcoidosis cohort does not represents the race distribution in SA in general. The 2011 census figures show Blacks are 76.4%, Whites 9.1%, Coloureds 8.9%, Indians 2.5% of the population. “Geographic regions, ethnicity and referral bias seem to play an important role in determining the incidence of sarcoidosis” (5,18,19). The exact burden of sarcoidosis in South Africa is not known, especially among the different population groups. The previous two studies in South Africa estimated the “incidence of sarcoidosis in the Cape Peninsula to be 23.2/100,000 population in Blacks, 11.6/100,000 in Coloureds and 3.7/100,000 in Whites” (3), and in Johannesburg an incidence of 20,4/100 000 among Blacks (4).

3.5.4 Other Comorbid Diseases

Comorbidity affects QoL and is more common in sarcoidosis cases compared to the general public (20-22). Not many reports have analysed the impact of corticosteroids and immunosuppressive drugs on comorbidities in sarcoidosis. “More comorbidities are associated with more hospitalisations and a higher mortality rate” (23). The occurrence of “new comorbidities, whether linked to corticosteroid use, to sarcoidosis itself or to other factors, is independently and strongly correlated with adverse outcomes, including poorer QoL, hospitalization risk and financial impacts” (24,25). One study showed that over 50% of patients with sarcoidosis had more than one comorbidity, the most prevalent comorbidities being “essential hypertension, thyroid disorders, diabetes mellitus, chronic obstructive pulmonary disease (COPD), and obesity” (20). Another report showed that chronic hepatic disease, autoimmune disorders, chronic pulmonary diseases and malignancy were substantially higher in patients with sarcoidosis (22). A report in “African-American patients with sarcoidosis found that 90% of patients have one or more comorbidities” (26). “The number of comorbidities in older patients, multiorgan involvement and low-income patients is higher” (20,27). In the current study, the commonest chronic comorbid illnesses were hypertension (32.4%), renal dysfunction (19.5%), obesity (17.6%), diabetes mellitus (15.7%), asthma (11.8%), and gastro-oesophageal reflux disease (11.8%). Patients with comorbidities adds to the complexity of their disease, rendering multidisciplinary treatment of sarcoidosis even more important (22,23). Some of these are common conditions in the general population and need to be diagnosed and treated promptly to improve the patients overall QoL. Therefore, the occurrence of comorbidities during the disease course should be carefully evaluated, monitored and managed.

3.5.5 Smoking and Family History

Sarcoidosis is known to occur more frequently in non-smokers and the majority (85.3%) of patients in this study were non-smokers. This inverse association between smoking and sarcoidosis is known for many decades. “One of the strongest associations to arise from the ACCESS analysis was a 35% lower chance of sarcoidosis among smokers than never smokers” (28), a result that is in line with earlier reports (29-32). Smoking appears to have a protective role in the occurrence of sarcoidosis, while smoking has no impact on the degree, course or outcome of the illness (33). Familial sarcoidosis has been well described (34,35) and in the current study, familial association was seen in 3 Indian patients.

3.5.6 Associated TB and HIV

Almost 17% of the current study patients were treated for TB, until being diagnosed as having sarcoidosis. Two patients developed active TB whilst being treated with corticosteroids. None of the patients had prior HIV and four Black patients contracted HIV whilst on treatment for sarcoidosis. Patients with advanced HIV and low CD4+ cell counts who receive antiretroviral therapy (ART), can develop sarcoidosis with immune reconstitution (36).

3.5.7 Symptoms and Signs Including Cutaneous Involvement

Investigations of patients with sarcoidosis have reported that shortness of breath, “dry cough, and chest pain occur in one third to half of all patients” (9). The most common symptom in this study was dry cough (82.4%), followed by dyspnoea on exertion (53.9%). Chest pain occurred in 27.5% of patients. The pain is generally retrosternal and is usually no more than a vague tightness, but occasionally can be severe and

indistinguishable from anginal pain. The exact cause is not always apparent and may not be easy to distinguish whether the dyspnoea and chest pain could be cardiac or respiratory in origin. Cutaneous lesions, other than erythema nodosum, were seen in 33.3% of patients. In patients of Northern European descent, erythema nodosum as cutaneous involvement, is more common (37). The ACCESS study found that the prevalence of skin involvement (excluding erythema nodosum) was 15.9%; and erythema nodosum was 8.3% (7). Overall, 35 (34.3%) of patients had cutaneous involvement in the current study; and four Black patients and four Indian patients had erythema nodosum. Lung crackles were noted in 37.3% of the study patients, and crackles occur in 20% and 50% of cases worldwide (9). Clubbing was uncommon and seen in just two patients as was parotid swelling. Löfgren's syndrome and Heerfordt's syndrome were not observed in this study.

3.5.8 Laboratory Data

3.5.8.1 *Haematological Abnormalities*

For the most part, haematological abnormalities in sarcoidosis are uncommon. Practically any haematological abnormality may be seen, including raised erythrocyte sedimentation rate, eosinophilia, leukopenia, anaemia, haemolytic anaemia, peripheral lymphopenia and thrombocytopenia (5,38-40). These, however, may vary in different ethnic populations because of nutritional or environmental factors. In the current study leukopenia was seen in 6.4%, anaemia in 5.3% and 2 patients had thrombocytopenia. The presence of eosinophilia was not recorded. Raised ESR was noted in 36 (40.4%) of patients. Previously in the Johannesburg study, the ESR was elevated in 89% of patients and eosinophilia was present in 13% of the Black and 14% of the White patients tested. In the Cape Town study 50% of patients had mild

anaemia, two patients had leukopenia, one patient had thrombocytopenia and eight patients had eosinophilia. The ESR was commonly elevated but rarely greater than 90 mm/1st hour.

3.5.8.2 *Calcium, Bone and Joint Involvement*

Arthralgias were seen in 9.8% of patients in the current study. Joint symptoms occur in 25-39% of cases (38,41). Hypercalcaemia was found in 22.7% and hypercalciuria in 11.1% (tested in 36 patients). Three (37.5%) White patients, 6 (21.4%) Indians, 11 (18.6%) Blacks had hypercalcaemia, and hypercalciuria was observed in 2 Black patients and in 1 White and Coloured patient each. The ACCESS study (42) found calcium metabolism anomalies were more prevalent in Caucasians. Hypercalcaemia is seen in 10-20% and hypercalciuria in 40-62% of sarcoidosis patients.

3.5.8.3 *Serum ACE*

Serum ACE levels are elevated in 30-80% of sarcoidosis cases “and may be a surrogate marker of the burden granulomas”, as observed in Western studies (43). In a previous South African study from Johannesburg (4) elevated serum ACE levels were documented in 77% and 78% of Blacks and Whites, respectively. In this study raised levels of sACE were noted in 56.8% of cases. The raised level is not diagnostic; however, this can be used as a marker for improvement in the patient follow-up.

3.5.8.4 *Hepatic, Gamma-Glutamyl Transferase (GGT) and Alkaline Phosphatase (AP)*

Hepatic involvement in sarcoidosis is common in up to 70% of cases; however, many patients with liver involvement are symptomless (44). Majority of cases are thus identified inadvertently, mostly through the detection of elevated liver enzymes (44,45),

and do not normally require treatment. With liver involvement, patients may have abdominal pain, fatigue, pruritus, and jaundice. Portal hypertension and cirrhosis are serious complications associated with longstanding sarcoidosis of the liver (44). In the current study, one patient presented with jaundice and elevated GGT and AP were seen in 31.8% and 21.6%, respectively.

3.5.8.5 *Elevated Creatinine*

Although the frequency of occurrence of kidney disease in sarcoidosis remains unclear, clinically significant renal disease occurs rarely with sarcoidosis (46). Presentations of kidney disease may be impaired metabolism of calcium, “nephrolithiasis and nephrocalcinosis, and acute interstitial nephritis with or without granulomas. There may also be glomerular dysfunction, obstructive uropathy, and end-stage renal disease” (ESRD), but these are rare (46,47). Raised creatinine levels were noted in 19.5% of patients; however, only two patients had renal calculi.

3.5.9 Chest Radiograph Stage

The classic sarcoidosis radiographic finding is “bilateral hilar lymphadenopathy and is believed to be present in nearly three-quarters of patients” (48). In the majority bilateral symmetrical hilar lymphadenopathy is observed. Significant variability exists regarding the chest radiographic findings in different studies. Several series recorded that stage I was the commonest x-ray stage, but in most reports from Scandinavia, x-ray stages I and II were recorded being predominant (49). Unlike this, some USA and British Isles studies (24,50) show a greater frequency of x-ray stage III and IV. Earlier South African reports (3,4) report that most of the abnormalities on chest radiograph were stage II and III. The chest radiograph was abnormal in more than 97% of patients in this study.

Stage II (48%) was found to be the most common radiographic stage of sarcoidosis identified in the present study, followed by Stage I (23.5%), Stage III (15.7%) and Stage IV (9.8%).

3.5.10 Pulmonary Function Tests

A restrictive pattern including decreased FVC and total lung capacity (TLC) with reduced DLCO are the characteristic pulmonary function abnormalities in sarcoidosis cases. Typical lung function anomalies in sarcoidosis include decreased lung volumes and decreased DLCO (43). The most sensitive of the lung function test parameters is the DLCO (51). Even if chest x-rays are normal the DLCO or FVC or may be reduced in patients. In this study impaired diffusion was noted in 29 (37.2%) of patients tested. Other studies reported between 30-50% patients having airway obstruction (38,52,53). This could be due to endobronchial involvement, large thoracic nodes compressing the airways and the coexistence of asthma. In the present study 21.2% of patients had airflow obstruction.

3.5.11 Diagnosis

The most frequent sites confirming granulomatous inflammation in the current study were, the skin in 28.4%, mediastinal lymph nodes 25.5% and transbronchial lung biopsies in 25.5% of patients. This study was undertaken prior to the availability of “endobronchial ultrasound-guided transbronchial needle aspiration” (EBUSTBNA), which is the initial procedure for mediastinal lymphadenopathy. In patients of suspected pulmonary sarcoidosis flexible fibreoptic bronchoscopy and transbronchial lung biopsies (TBLB) were performed if no other simpler available site for biopsy was accessible. In sarcoidosis, bronchoscopic manifestations include cobblestoning,

nodules and bronchial mucosal plaques. Transbronchial lung biopsy's diagnostic yield varies from 40% to over 90%, depending on the clinician's experience (54).

In the present study, there was no biopsy performed in 3 patients (2.9%). Sarcoidosis diagnosis was made on the basis of indicative symptoms and signs, compatible radiographic and suggestive laboratory characteristics together with improvement on corticosteroid treatment and/or spontaneous recovery. These features may be diagnostic, particularly for sarcoidosis "stage I (98% reliability) and stage II (89%)" (55). In an asymptomatic individual, Winterbauer *et al*., observed that the "presence of bilateral hilar and right paratracheal lymphadenopathy" on chest radiograph, was specific for sarcoidosis (56). This approach has proven to be correct in over 95% of cases for asymptomatic individuals and "remains a cost-effective approach, particularly in developing countries with limited resources" (57).

3.5.12 Treatment and Relapse

Spontaneous remissions are believed to ensue in almost two-thirds of patients; however, in the remainder, the condition may be chronic or progressive (5). Severe extrapulmonary involvement, for example, heart, neurological and liver sarcoidosis are present in 4-7% cases initially (5). These complications rise with evolving disease that is highly dependent on ethnicity (5). There is no standard requirement for clinical therapy in sarcoidosis given the extremely complex pattern of the disorder, since most cases experience natural recovery, and that serious consequences including deaths in Caucasians is fairly uncommon (but more frequent in non-Caucasians). In the current study 87.3% of patients had an indication for corticosteroid treatment. When uveitis, hypercalcaemia, or constitutional symptoms were the indication for treatment, the response was invariably good. Forty-five patients

were treated because of pulmonary abnormality and in the majority of these there was objective evidence of response (improvement in VC and/or improvement in DLCO) although in only very few cases did the VC or gas transfer return to normal. Some patients with abnormal pulmonary function did not show objective evidence of improvement, although some felt better. Those with disfiguring facial lesions and lupus pernio also improved, and high burden of disease with generalised lymphadenopathy and hepatosplenomegaly, also improved. The cardiac patients needed to be treated with diuretics and one patient with complete heart block required dual chamber pacing. The patients with parotidomegaly and dactylitis also had favourable response to therapy.

Relapse is quite likely when anti-sarcoidosis drugs are being tapered or discontinued. Depending on the population studied, reported sarcoidosis relapse rates ranges from 13% to 75% (24,58-60). These relapses usually occur 1 to 12 months after tapering or discontinuation of the therapy (24,60). Relapse occurred in 54 (60.7%) of patients in this study. It is known that patients who are treated with corticosteroids do relapse more frequently than those who do not have an indication for treatment (24), and 87.3% of patients in this study, were treated with corticosteroids.

3.6 Conclusion

Sarcoidosis occurs in all racial groups in South Africa. A few notable differences and similarities were noted in the various population groups in this study. In nations with huge burden of TB, such as South Africa, sarcoidosis is frequently mistakenly diagnosed as TB, understandably so, as both the diseases can look identical in many ways. Thus, patients with bilateral hilar lymphadenopathy with or without pulmonary infiltration ought to have sarcoidosis excluded if they do not respond to TB treatment.

The most frequent sites for histological confirmation were cutaneous, mediastinal lymph node and transbronchial lung biopsy. It should be borne in mind that the study was done prior to EBUSTBNA, which today may be regarded as the initial investigation for obtaining tissue samples from the mediastinum. Comorbid illnesses are common in these patients and should be screened for and treated promptly. Side effects of corticosteroids could lead to some of these comorbid conditions. Almost 90% of patients in this cohort with sarcoidosis had an indication to be treated with corticosteroids and the relapse rate was in the region of 60%.

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Chapter 4 : HLA “Class I and Class II” Antigens in Sarcoidosis

4.1 Abstract

4.1.1 Background

Sarcoidosis is a multisystem granulomatous disease. The exact cause of sarcoidosis is not known, but it is believed that an external agent may cause the characteristic immune reaction in genetically susceptible individuals. There is, therefore, general recognition of the genetic vulnerability to sarcoidosis among the potential risk factors.

Human leukocyte antigen (HLA) is coded by genes in the major histocompatibility complex (MHC), on chromosome 6. Such surface cells are important in presentation of antigen and play a key part in the immune response of the body to external antigens. Various HLA subtypes are more common in patients suffering from sarcoidosis. The variance in vulnerability, presentation, evolution of disease, and prognosis have been related to various HLA phenotypes. These HLA genes will offer information into the facets driving sarcoidosis and prognosticating tools.

However, in Africa, including South Africa, there are no data about HLA types in relation to sarcoidosis. The main aim of the research was, therefore, to evaluate the HLA Class I and II associations of South African sarcoidosis patients.

4.1.2 Methods

Phenotype frequencies of HLA-A, B and C as well as DQB1 and DRB1 were calculated for 51 consecutive biopsy-proven patients with sarcoidosis attending the Respiratory Services at the CMJAH and 63 controls, who were donor candidates for

transplantation. Direct counting was used to assess the frequencies of tested HLA loci. The importance of the relationships between the various loci tested for and the presence or absence of sarcoidosis was estimated from 2 X 2 tables by the Chi-squared and Fisher's exact test.

4.1.3 Results

Of the fifty-one patients, 63 % were female and the mean age was 43 years. Analysis of phenotypes of HLA class I and class II in sarcoidosis patients revealed a significant association with HLA B15 ($p = 0.0001$), C4 ($p = 0.0045$), C7 ($p = 0.0031$), C12 ($p < 0.0001$), C15 ($p = 0.0068$), C16 ($p = 0.0160$), C17 ($p = 0.0012$), DQ3 ($p = 0.0047$), DR8 ($p = 0.016$) and DR11 ($p < 0.0001$). In addition, a significant negative (protective) association with HLA A9 ($p = 0.0005$), A28 ($p = 0.0052$), B12 ($p < 0.0001$), B17 ($p = 0.0041$) and DR2 ($p = 0.0082$) was observed.

4.1.4 Conclusions

The HLA study is unique to South African patients and it suggests that genetic influences may have a role to play in the potential sarcoidosis aetiology. Some HLA subtypes have shown a significant association between sarcoidosis and South African patients, and other HLA subtypes may be protective. This study supported the association of HLA antigens with sarcoidosis and implies there is a genetic predisposition to sarcoidosis in the South African population.

4.2 Introduction

Sarcoidosis, a multisystem disorder, is characterized by development of granulomas where the lungs are the most frequent site (1–3). The precise sarcoidosis aetiology is

not known. The current theory suggests that the disorder arises in people who are genetically predisposed to such environmental agents, which leads to exaggerated inflammatory immune response resulting in granulomas developing. Several risk factors are implicated in sarcoidosis pathogenesis. These involve genetic, environmental, occupational and socioeconomic factors, that are related to or associated with the disorder. While the precise aetiology of sarcoidosis is unclear, genetic factors are also involved as possible risk factors for disease development. Proof that genetic factors have a role in sarcoidosis pathogenesis is founded on family grouping of sarcoidosis cases, greater monozygotic twin incidences in relation to non-twin siblings, and differences in disease expression, frequency and vulnerability to race (4,5).

Sarcoidosis predisposition was previously documented to be linked with various HLA antigens. The interaction of sarcoidosis with HLA antigens has been studied in separate populations (6–13). Different subtypes of HLA have a higher prevalence in sarcoidosis patients and can affect susceptibility, presentation, progression and prognosis of disease. Reports from various countries have often generated contradictory findings, with few HLA alleles showing an increased risk while other alleles are related to disease protection (14). There was no consensus regarding a particular HLA locus that was specifically involved in sarcoidosis pathogenesis. Different authors suggested the presence of distinctive loci within the HLA domain, and several studies concentrated mainly on the genes of class II HLA subtypes. However, in Africa, particularly South Africa, there are no details about HLA types with respect to sarcoidosis. The purpose of the research was, therefore, to determine and define the association between HLA Class I and II typing and sarcoidosis in South African patients.

4.3 Patients and Methods

Overall, 51 sarcoidosis patients and 63 controls were enrolled in the survey. Demographic, clinical, laboratory data and chest x-ray stage of the patients at diagnosis were noted. Sarcoidosis diagnosis was established by clinical manifestations, radiological characteristics consistent with sarcoidosis, and histological confirmation of non-caseating granulomas after exclusion of other known causes of granulomas, as defined “by the joint statement of the American Thoracic Society, the European Respiratory Society, and the World Association of Sarcoidosis and Other Granulomatous Disorders” (1).

HLA-A, -B and -C typing as well as DR and DQ was performed on DNA extracted from peripheral blood. Peripheral blood was collected and kept at -20˚C until utilised. HLA Class I (A, B, C) and HLA class II (HLA-DQ and HLA-DR) antigen typing in 51 consecutive patients with sarcoidosis attending the Respiratory Clinic at the CMJAH were compared with that of 63 controls. The controls were subjects who were potential organ donors who had HLA typing performed previously. For the patient and control categories, phenotype frequencies were determined for HLA class I antigens (A, B and C) and HLA class II antigens (DR and DQ). Approval to conduct the study was received from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand with written informed consent from the patients to participate in the study (M110750 approval, APPENDIX E).

Typing for HLA class I and II antigens were performed by the standard low resolution molecular technique. Briefly, “genomic DNA was extracted from whole blood using the QIAmp® DNA Blood Mini Kit” (QIAGEN Inc., Germany) according to manufacturer’s instructions, APPENDIX B. Following extraction, the DNA concentrations were

determined spectrophotometrically and samples “diluted to a concentration of 50 ng/µl”. HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ were analysed with the “polymerase chain reaction (PCR) sequence-specific primer (SSP) technique” (Dynal, UK). PCR was conducted according to the manufacturer’s specifications. Essentially an appropriate amount of master mix was aliquoted into an Eppendorf tube to which sample DNA (at 50ng/µl) and AmpliTaq DNA polymerase (at 5µ/µl) was added. After mixing well, 10µl of the PCR mix was dispended into each well of the Dynal Allset^{TM} SSP tray. After sealing securely, the Allset^{TM} trays was placed into a thermal cycler and the amplifications performed. The PCR conditions were: “1 cycle of 96˚C for 2 minutes (denaturation), followed by 10 cycles at 96˚C for 15 seconds (denaturation) and 65˚C for 1 minute (annealing and extension), and finally 20 cycles at 96˚C for 10 seconds (denaturation), 61˚C for 50 seconds (annealing), and 72˚C for 30 seconds (extension). PCR products was detected using agarose gel electrophoresis run on 2% agarose gel containing ethidium bromide after which gels were examined under UV light” and products documented by photography. Gels were analysed according to specific tables provided by Dynal and alleles scored using the SSP tool software supplied and manually. Alleles for each HLA subtype for each patient was recorded on the data sheets supplied.

Each individual was tested for the HLA-A, -B, -C, -DR and -DQ antigens depicted in Tables 4–8. Gene frequencies (gf) were calculated from the antigen frequency (af) by Bernstein’s formula: $gf = 1 - \sqrt{(1 - af)}$. The frequency of the ‘blank’ gene(s) was obtained by subtracting the sum total of defined gene frequencies from one. The gene frequencies in the different groups were compared by the chi-square test. The odds ratio (OR), statistical significance and 95% confidence interval (CI) of the antigen frequencies in the cases were compared with the controls.

4.4 Results

The demographic data of patients and controls, and the clinical data, biopsy confirmation and biopsy sites, of patients with sarcoidosis are shown in Table 4.1. Fifty-one patients with a diagnosis of sarcoidosis were consecutively enrolled in the study. There were 27 Blacks, 15 Indians, 5 Whites and 4 Coloured patients Thirty-six patients were females (70.6%) and over 90% of all patients were non-smokers. The mean age of disease onset (mean years ± standard error of the mean (SEM)) was 44.6 ± 12.4 years with a range of 22-77 years. All patients had a biopsy performed, and the commonest site for histological confirmation was transbronchial lung biopsy by fibre-optic bronchoscopy (33.3%) and cutaneous biopsy (33.3%).

Table 4.1. The patient (n = 51) and control (n = 63) demographic data, and the clinical and biopsy data of the sarcoidosis patients in the HLA study

	n	(%)
Number of controls (38 B, 4 I, 19 W, 2 C)	63	
Number of patients (27 B, 15 I, 5 W, 4 C)	51	
Females	36	(70.6)
Males	15	(29.4)
Non-smokers	46	(90.2)
Biopsy confirmation	51	(100)
Mean age at disease onset ± SEM (years)	44.6 ± 12.4	
Age range (years)	22-77	
Biopsy Sites (sometimes multiple)		
Transbronchial lung biopsy	17	(33.3)
Skin biopsy	17	(33.3)
Lymph node biopsy	8	(15.7)
Mediastinoscopy and hila lymph node biopsy	4	(7.8)
Bone marrow biopsy	2	(3.9)
Open lung biopsy	2	(3.9)
Lacrimal gland	1	(2.0)

B = Blacks, I = Indians, W = Whites, C = Coloureds

SEM = Standard error of the mean

n = Number

% = Percent

In Table 4.2, the laboratory (serum angiotensin-converting enzyme (sACE)), radiographic, lung function and therapeutic data of the patients are shown. The mean sACE ± SEM level was 139.4 ± 61.6 IU and the level was elevated in 63% of patients. The majority of patients had stage II chest x-ray findings (45.1%), 29.4% had a decreased FEV1 and FVC respectively, and 15.7% had an obstructive defect; with 84.3% of patients having been treated with corticosteroids. The 63 controls were subjects who were potential organ donors, who had HLA typing performed previously, were matched racially, as close as possible, to the patients.

Table 4.2. The laboratory (sACE), radiographic, lung function and therapeutic data of the 51 patients with sarcoidosis

	n = 51	(%)
Mean sACE (IU/L) (SEM)	139.4 (61.6)	
Chest radiograph		
Stage 0	1	(2.0)
Stage I	12	(23.5)
Stage II	23	(45.1)
Stage III	8	(15.7)
Stage IV	7	(13.7)
Lung function tests		
FVC < 80%	15	(29.4)
FEV1 < 80%	15	(29.4)
FEV1/FVC <70%	8	(15.7)
Treated with systemic corticosteroids	43	(84.3)

"SEM = Standard error of the mean

FVC = Forced vital capacity

FEV1 = Forced expiratory volume in 1 second

sACE = Serum angiotensin-converting enzyme

n = Number

% = Percent"

The antigen frequencies (af) of the class I (A, B and C) and class II (DR and DQ) loci of both the patients and controls are presented in Table 4.3 to Table 4.8.

Table 4.3. The HLA-A locus antigen frequencies in sarcoidosis patients and in the control group

HLA	Sarcoidosis patients (n = 51)	Controls (n = 63)	*p*	OR	CI (95%)
A1	5	8	NS	0.74	0.2286 to 2.442
A2	19	17	NS	1.60	0.7255 to 3.558
A3	5	10	NS	0.57	0.1835 to 1.809
A4	1	0	NS	3.77	0.1503 to 94.67
A9	**0**	**12**	**0.0005**	**0.04**	**0.002305 to 0.6941**
A10	0	3	NS	0.16	0.008322 to 3.273
A11	3	6	NS	0.59	0.1409 to 2.502
A23	8	9	NS	1.11	0.3971 to 3.138
A24	9	5	NS	2.48	0.7766 to 7.956
A26	2	0	NS	6.41	0.3008 to 136.8
A28	**0**	**10**	**0.0052**	**0.05**	**0.003358 to 1.027**
A29	8	8	NS	1.27	0.4439 to 3.686
A30	17	21	NS	1.00	0.4569 to 2.189
A31	1	3	NS	0.40	0.04032 to 3.968
A32	1	1	NS	1.24	0.07560 to 20.34
A33	3	4	NS	0.92	0.1966 to 4.322
A34	1	0	NS	3.77	0.1503 to 94.67
A36	1	0	NS	3.77	0.1503 to 94.67
A43	2	1	NS	2.53	0.2228 to 28.75
A66	3	0	NS	9.16	0.4621 to 181.8
A68	8	8	NS	1.27	0.4439 to 3.686
A69	2	0	NS	6.41	0.3008 to 136.8
A74	3	2	NS	1.90	0.3060 to 11.87

“Bold = Significant difference

NS = Not significant

OR = Odds ratio

CI = Confidence interval

n = Number

% = Percent”

The antigen frequencies of the class I–A locus antigens are depicted in Table 4.3. HLA-A A9 and A28 occurred more frequently in the control group and were both significantly raised in the control group compared with patients, conferring a significant relative protective effect with an odds ratio (OR) = 0.04 and OR = 0.05 respectively, as shown in the Table.

Table 4.4. The HLA-B locus antigen frequencies in sarcoidosis patients and in the control group

HLA	Sarcoidosis Patients n = 51	Controls n = 63	*p*	OR	CI (95%)
B3	1	0	NS	3.77	0.1503 to 94.67
B5	1	3	NS	0.40	0.04032 to 3.968
B7	7	8	NS	1.09	0.3680 to 3.251
B8	12	9	NS	1.84	0.7086 to 4.810
B12	**0**	**18**	**< 0.0001**	**0.02**	**0.001336 to 0.3902**
B13	2	8	NS	0.28	0.05682 to 1.386
B14	0	5	NS	0.10	0.005570 to 1.914
B15	**17**	**3**	**0.0001**	**10.0**	**2.731 to 36.61**
B17	**0**	**9**	**0.0041**	**0.05**	**0.003158 to 0.9823**
B18	3	3	NS	1.25	0.2412 to 6.477
B21	0	1	NS	0.40	0.01612 to 10.15
B22	1	1	NS	1.24	0.07560 to 20.34
B27	4	3	NS	1.70	0.3630 to 7.982
B35	9	4	NS	3.16	0.9121 to 10.95
B37	3	0	NS	9.16	0.4621 to 181.8
B38	1	0	NS	3.77	0.1503 to 94.67
B39	1	0	NS	3.77	0.1503 to 94.67
B40	6	8	NS	0.91	0.2962 to 2.837
B41	2	2	NS	1.24	0.1691 to 9.164
B42	3	4	NS	0.92	0.1966 to 4.322
B44	6	10	NS	0.70	0.2382 to 2.097
B45	4	6	NS	0.80	0.2153 to 3.036
B48	2	0	NS	6.41	0.3008 to 136.8
B49	1	1	NS	1.24	0.07560 to 20.34
B51	1	1	NS	1.24	0.07560 to 20.34
B53	3	1	NS	3.87	0.3905 to 38.45
B55	2	1	NS	2.53	0.2228 to 28.75
B57	3	4	NS	0.92	0.1966 to 4.322
B58	4	4	NS	1.25	0.2979 to 5.289
B60	0	5	NS	0.10	0.005570 to 1.914
B67	1	0	NS	3.77	0.1503 to 94.67
B68	1	0	NS	3.77	0.1503 to 94.67
B70	0	3	NS	0.16	0.008464 to 3.328
B72	0	2	NS	0.23	0.01120 to 5.092
B81	1	0	NS	3.77	0.1503 to 94.67

“Bold = Significant difference

NS = Not significant

OR = Odds ratio

CI = Confidence interval

n = Number

% = Percent”

The antigen frequencies of the class I–B locus antigens are depicted in Table 4.4. HLA B15 was significantly higher in the patients with sarcoidosis compared with the controls. The relative risk for the development of sarcoidosis of having the locus B15 was found to be 10-fold higher. Of note, HLA-B B12 and B17, occurred more frequently in the control group and were both significantly greater in the control group compared with the patients with sarcoidosis conferring a significant relative protective effect with the OR = 0.02 and OR = 0.05 respectively, as shown in the Table.

Table 4.5. The HLA-C locus antigen frequencies in sarcoidosis patients and in the control group

HLA	Sarcoidosis Patients n = 51	Controls n = 63	*p*	OR	CI (95%)
C2	8	6	NS	1.76	0.5707 to 5.474
C3	9	7	NS	1.71	0.5904 to 4.978
C4	**12**	**3**	**0.0045**	**6.15**	**1.631 to 23.23**
C5	0	2	NS	0.23	0.01120 to 5.092
C6	11	10	NS	1.45	0.5637 to 3.768
C7	**27**	**15**	**0.0031**	**3.45**	**1.548 to 7.689**
C8	1	0	NS	3.77	0.1503 to 94.67
C12	**11**	**0**	**< 0.0001**	**36.06**	**2.066 to 629.3**
C14	1	0	NS	3.77	0.1503 to 94.67
C15	**6**	**0**	**0.0068**	**18.14**	**0.9960 to 330.5**
C16	**5**	**0**	**0.016**	**15.02**	**0.8098 to 278.6**
C17	**8**	**0**	**0.0012**	**24.82**	**1.395 to 441.6**
C18	3	0	NS	9.16	0.4621 to 181.8

“Bold = Significant difference

NS = Not significant

OR = Odds ratio

CI = Confidence interval

n = Number

% = Percent”

Table 4.5 shows the antigen frequencies of HLA-C locus antigens. The frequencies of C4, C7, C12, C15, C16 and C17 were significantly greater in the patients than the controls. These HLA-C locus antigens conferred a significant higher relative risk of developing sarcoidosis. The relative risk for the occurrence of sarcoidosis for these HLA-C locus antigens ranged from 3-fold to 36-fold as illustrated in the Table.

Table 4.6. The HLA-DR (B1) locus antigen frequencies in sarcoidosis patients and in the control group

HLA	Sarcoidosis Patients n = 51	Controls n = 63	*p*	OR	CI (95%)
DR1	7	12	NS	0.67	0.2448 to 1.867
DR2	**0**	**8**	**0.0082**	**0.06**	**0.003566 to 1.127**
DR3	18	20	NS	1.17	0.5365 to 2.563
DR4	5	8	NS	0.74	0.2286 to 2.442
DR7	4	8	NS	0.58	0.1656 to 2.067
DR8	**5**	**0**	**0.0160**	**15.02**	**0.8098 to 278.6**
DR10	4	4	NS	1.25	0.2979 to 5.289
DR11	**25**	**9**	**< 0.0001**	**5.76**	**2.359 to 14.11**
DR12	5	10	NS	0.57	0.1835 to 1.809
DR13	9	20	NS	0.46	0.1883 to 1.127
DR14	7	4	NS	2.34	0.6464 to 8.519
DR15	12	11	NS	1.45	0.5810 to 3.641
DR16	1	1	NS	1.24	0.07560 to 20.34

“Bold = Significant difference

NS = Not significant

OR = Odds ratio

CI = Confidence interval

n = Number

% = Percent”

Antigen frequencies of various class II antigens were raised in the patient group. Table 4.6 shows the antigen frequencies of the class II antigens representing those expressed by the DR (B1) locus. HLA-DR (B1) DR8 and DR11 were both significantly raised in the patients with sarcoidosis. The relative risk for the development of

sarcoidosis with both DR8 and DR11 was found to be 15.0-fold and 5.7-fold higher, respectively. Of note was the fact that the HLA locus antigen DR2 occurred more frequently in the control group compared with patients and was substantially higher, conferring a significant relative protective effect (OR = 0.06) as shown in the Table.

Table 4.7 shows the antigen frequencies of the class II antigens representing those expressed by the DQ locus. The frequency of DQ3 was substantially greater in the patients compared with the controls. Expression of DQ3 appears to confer a relative risk of 3-fold in the patient group.

Table 4.7. The HLA-DQ (B1) locus antigen frequencies in sarcoidosis patients and in the control group

HLA	Sarcoidosis Patients n = 51	Controls n = 63	*p*	OR	CI (95%)
DQ2	11	17	NS	0.74	0.3121 to 1.774
DQ3	**29**	**19**	**0.0047**	**3.05**	**1.410 to 6.610**
DQ4	12	6	NS	2.30	0.7916 to 6.727
DQ5	23	22	NS	1.53	0.7182 to 3.263
DQ6	27	26	NS	1.60	0.7605 to 3.370

“Bold = Significant difference
NS = Not significant
OR = Odds ratio
CI = Confidence interval
n = Number
% = Percent”

Table 4.8 is a summary of the significant differences in the Class I and II locus antigen frequencies in sarcoidosis patients and in the control group.

Table 4.8. Summary of the significant differences in the Class I and Class II locus antigen frequencies in sarcoidosis patients and in the control group

HLA	Sarcoidosis Patients n = 51	Controls n = 63	*p*	OR	CI (95%)
A9	**0**	**12**	**0.0005**	**0.04**	**0.002305 to 0.6941**
A28	**0**	**10**	**0.0052**	**0.05**	**0.003358 to 1.027**
B12	**0**	**18**	**< 0.0001**	**0.02**	**0.001336 to 0.3902**
B15	**17**	**3**	**0.0001**	**10.00**	**2.731 to 36.61**
B17	**0**	**9**	**0.0041**	**0.05**	**0.003158 to 0.9823**
C4	**12**	**3**	**0.0045**	**6.15**	**1.631 to 23.23**
C7	**27**	**15**	**0.0031**	**3.45**	**1.548 to 7.689**
C12	**11**	**0**	**< 0.0001**	**36.06**	**2.066 to 629.3**
C15	**6**	**0**	**0.0068**	**18.14**	**0.9960 to 330.5**
C16	**5**	**0**	**0.0160**	**15.02**	**0.8098 to 278.6**
C17	**8**	**0**	**0.0012**	**24.82**	**1.395 to 441.6**
DQ3	**29**	**19**	**0.0047**	**3.05**	**1.410 to 6.610**
DR2	**0**	**8**	**0.0082**	**0.06**	**0.003566 to 1.127**
DR8	**5**	**0**	**0.0160**	**15.02**	**0.8098 to 278.6**
DR11	**25**	**9**	**< 0.0001**	**5.76**	**2.359 to 14.11**

"Bold = Significant difference

OR = Odds ratio

CI = Confidence interval

n = Number

% = Percent"

Since in the HLA-study the majority of patients with sarcoidosis were Blacks, comprising 27 (52.9%) of the cohort, these were compared with race-matched controls as shown in Table 4.9. HLA-A A9 and A28 were both significantly raised in the control group compared with patients, conferring a significant relative protective effect with an odds ratio (OR) = 0.05. HLA B15 was substantially greater in the Black patients with sarcoidosis compared with the controls. The relative risk for the development of sarcoidosis of having the locus B15 was found to be 34-fold higher. Of note, HLA-B

B12 occurred more frequently in the control group and was substantially greater in the control group compared with sarcoidosis patients conferring a significant relative protective effect with the OR = 0.03. The frequencies of C12, C16 and C17 were substantially greater in the patients than the controls. These HLA-C locus antigens conferred a significantly higher relative risk of developing sarcoidosis. The relative risk for the occurrence of sarcoidosis for these HLA-C locus antigens was increased 18-fold. DR11 was significantly raised in the patients with sarcoidosis. The relative risk for the development of sarcoidosis with DR11 was found to be 4-fold higher.

Table 4.9. Significant Differences in the Class I and Class II HLA locus antigen frequencies in the Black patients with sarcoidosis compared with race-matched controls

HLA	Sarcoidosis Patients n = 27	Controls n = 38	*p*	OR	CI (95%)
A9	**0**	**9**	**0.0079**	**0.05**	**0.003133 to 1.018**
A28	**0**	**9**	**0.0079**	**0.05**	**0.003133 to 1.018**
B12	**0**	**12**	**0.0008**	**0.03**	**0.002170 to 0.6848**
B15	**13**	**1**	**< 0.0001**	**34.36**	**4.102 to 287.8**
C12	**5**	**0**	**0.0098**	**18.82**	**0.9929 to 356.8**
C16	**5**	**0**	**0.0098**	**18.82**	**0.9929 to 356.8**
C17	**5**	**0**	**0.0098**	**18.82**	**0.9929 to 356.8**
DR11	**15**	**8**	**0.0078**	**4.68**	**1.578 to 13.92**

"Bold = Significant difference

OR = Odds ratio

CI = Confidence interval

n = Number

% = Percent"

4.5 Discussion

The findings of the current study suggest the possible presence of a link among different HLA-genotypes and sarcoidosis, in patients in South Africa, although the study recruited relatively small patient numbers. Variations in the vulnerability, presentation, progression and prognosis of sarcoidosis have been noted to be linked to various HLA phenotypes in different populations studied outside South Africa, but no studies have been previously conducted in this country, prior to this current study. It would be interesting to explore the correlation of the clinical phenotypes with the HLA genotype in patients with sarcoidosis in South Africa.

Sarcoidosis HLA association studies are not new and began over 40 years ago. The earliest recorded sarcoidosis-HLA gene relationship was HLA-A7 (15). Many separate studies have been unable to repeat the earlier recorded HLA-A7 link in the ensuing years (16,17).

Some of the studies of the HLA associations have shown that HLA A1, B8, DRB1 11 are associated with susceptibility to sarcoidosis (5). However, DQB1, DRB1 3, and 4 and DRB3 15 have been linked to protection, good prognosis and the Löfgren's syndrome, while DRB3 1 was connected with susceptibility, as well as disease progression (5). Brewerton and co-workers (18) first disclosed in 1977 a connection of acute sarcoidosis with HLA-B8 that was confirmed later by other groups (19,20). Hedfors and colleagues (19) also reported that genes HLA-B8/DR3 appear to be inherited as a risk haplotype for sarcoidosis in Whites. The haplotype HLA-B8/DR3 in Whites is linked to a wide range of autoimmune diseases (21). These earlier studies concentrated on HLA antigens of class I subtype. A report by Grunewald and co-

workers (22) indicated that HLA class I and II genes function together in relation to the pathophysiology of sarcoidosis.

HLA-DRB1 was the sarcoidosis-related antigen most commonly tested in the class II HLA antigens. The mutation of the HLA-DRB1 sarcoidosis gene affects both vulnerability and prognosis (23,24). The suspected population risk in the ACCESS sample was 16% in the Black and 9% in the White patients (23). The HLA-DRB1*1101 allele was related to sarcoidosis in both the Blacks and Caucasians ($p < 0.01$). In addition, the ACCESS analysis showed that HLA class II alleles might be indicators for various forms of sarcoidosis, such as DRB1*0401 in Blacks and Caucasians for eye involvement, DRB3 in Blacks for bone marrow involvement, and DPB1*0101 in Whites for hypercalcaemia (23). Another strong finding across populations was that of the relationship of the HLA-DQB1*0201 allele with reduced risk of disease worsening (25). The fact that certain distinctive class II HLA genes appear to predispose to sarcoidosis is firmly endorsed by other reports (25,26).

“Linkage disequilibrium (LD) refers to the non-random association of alleles at two or more loci” in a general population and this happens “when alleles at two distinctive loci occur in gametes more frequently than expected” (5). LD within the MHC area restricts the capacity to accurately classify the implicated HLA genes (5). HLA-DRB1*03 had been correlated with resolving disease and HLA-DRB1*15 with chronic disease, while Grunewald and his colleagues had suggested that HLA-DQB1*0201 was associated with resolved disease and HLA DQB1*0602 with chronic disease (22). Determining the impact of HLA-DQB1 on sarcoidosis risk apart from DRB1 or evaluating other gene effects from intimately associated haplotypes in the MHC region can be an insurmountable challenge in Whites. In African American people, the HLA-DRB1/DQB1 LD is not as robust as it is in Whites (27).

A host of other countries have shown various HLA types found with increased frequency in sarcoidosis patients. In Japan DRw52, in Italy/Czech A1, B8, and DR3, in Denmark DRw6, in Scandinavia DR17, in the United Kingdom (UK) C7 and B8, in African Americans A23 and B15, and in Turkey A2, A9, A24, A25, A69, B12, B22, B38, B48, DR4, and DR14 were found with increased frequency.

Overall, in the current study frequencies of HLA B15 (found also in African-Americans), C4, C7 (also found in the UK patients), as well as C12, C15, C16, C17, DQ3, DR8 and DR11 were found to be significantly greater in the patients with sarcoidosis. The countries where the HLA frequency were decreased or absent (protective), included B12 and DR4 in Italian/Czech patients, DR3 in Danish patients and A30, A33 and B17 in African-Americans. In comparison, in the current study a negative correlation was found with HLA A9, A28, B12 (B12 also found in Italian/Czech patients), B17 (also found in African-American patients), as well as DR2 was observed (4–6,12,18,28–34). In the Black patients with sarcoidosis the frequencies of HLA B15, C12, C16, C17, and DR11 were found to be substantially greater in the sarcoidosis patients. In addition, a statistically significant negative (protective) correlation with HLA A9, A28 and B12, was observed.

The development of sarcoidosis is influenced by genetic predisposition, as well as an environmental or work-related exposure, to an unknown agent(s) and the disease has considerable clinical heterogeneity (35,36). The difficulties in identifying an HLA association with sarcoidosis have been attributed to these factors. Further research with greater patient numbers, with specified clinical phenotypes and well matched controls, may assist to circumvent these issues. HLA alleles were correlated consistently with the disease manifestation and outcome, indicating that HLA play a

significant part in deciding the disease phenotype. In addition, the contradictory results in HLA in sarcoidosis vulnerability analysis may be due to variation in the phenotype composition of the studied patient groups.

4.6 Conclusion

These results suggest that there is a connection between sarcoidosis and HLA in South Africa, although there were relatively small patient numbers. Different HLA phenotypes have been linked to variation in disease susceptibility, presentation, progression and prognosis. It would be interesting to explore the correlation of the clinical phenotype with the HLA genotype in our patients. These could not be done because of relatively small patient numbers, and because there were different racial population groups. Nonetheless, different HLA antigens were higher in frequency compared with control groups. These results are consistent with other HLA correlation reports. As mentioned above, because HLA antigens are different between countries, each country should probably develop its own HLA continuum to draw a global map of HLA antigen sarcoidosis associations.

4.7 Acknowledgements

Supported by a grant from South African Thoracic Society (SATS) via the Astra Zeneca Pharmaceuticals Grant.

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Chapter 5 : IgG Subclasses in Sarcoidosis

5.1 Abstract

5.1.1 Background and Objectives

This study investigated serum immunoglobulin G (IgG) subclass levels in newly diagnosed cases with sarcoidosis, and assessed the changes in antibody levels in corticosteroid-treated patients and in those undergoing spontaneous clinical resolution.

5.1.2 Methods

This was a prospective study of 17 cases of sarcoidosis that were newly diagnosed. IgG subclasses were measured in serum using a laser nephelometric method with appropriate test kits.

5.1.3 Results

A total of 17 cases were enrolled, all Black South Africans, of whom 11 were females and six males. The median age was 44 years with a range of 23-60 years. The diagnosis of sarcoidosis was most commonly established by histology of transbronchial lung biopsy. The majority of patients had stage II disease radiologically.

Raised initial IgG1 levels were noted in 10 patients, IgG3 levels in 8, IgG2 levels in 4, and IgG4 in 2 patients. The median IgG1 and IgG3 levels were significantly raised above normal control values. Fourteen patients were treated with corticosteroids with good clinical response, and 3, in whom there was no indication for therapy, were

asymptomatic. On follow-up there was a significant decrease in IgG subclass levels toward normal. IgG1 levels returned to normal in almost all cases, and IgG3 remained raised in only 2 cases. The median levels of IgG1 and IgG3 decreased significantly, as well as those of IgG2.

5.1.4 Conclusions

IgG subclasses, mainly IgG1 as well as IgG3 were elevated in a large proportion of sarcoidosis patients initially, and reduced with corticosteroid therapy and in those patients with spontaneous clinical resolution.

5.2 Introduction

According to the international statement on sarcoidosis (1), this condition “is a granulomatous multisystem disorder of unknown aetiology”, which mainly affects the lung and lymphatic systems. Typically, the diagnosis involves demonstrating characteristic granulomas in at least two organs and excluding other diseases causing granulomatous inflammation.

In the lungs, a mononuclear cell inflammation with increased numbers of activated T-lymphocytes and alveolar macrophages is associated with the development of non-caseating granulomas. In addition to these cell-mediated immune responses, a number of changes in humoral immunity have been reported. Characteristically a polyclonal increase in gamma globulins, with the presence of auto-antibodies, is noted (1–4).

The finding of immunoglobulins, immune complexes and complement in the granuloma of sarcoidosis suggests that humoral mechanisms may well be involved in the

pathogenesis of the disorder. There are a few studies (5–7) that have examined the responses in “immunoglobulin (Ig) G subclasses, IgG1, IgG2, IgG3 and IgG4” in the serum of newly diagnosed sarcoidosis patients, in some studies recording the effects of corticosteroid therapy on the levels of these immunoglobulins, with significantly differing results. IgG3 and IgG4 may be the most important subclasses involved in the opsonisation of microbes for alveolar macrophages (8). Polysaccharide antigens seem to systemically induce a predominantly IgG2 response (9), while protein antigens evoke the production of antibodies to IgG1 and IgG3 (10). Numerous factors will influence the type of antibody response, “including the nature of stimulating agents, antigen-presenting cell populations and cooperating T cells response and in turn, the effects of the antibodies produced will depend”, to a certain extent, on the populations of effector cells present. (11).

The main aims of this study were to measure IgG subclass levels in the serum of newly diagnosed sarcoidosis patients, prior to any therapy, and to record the effects of corticosteroids and spontaneous clinical resolution on the immunoglobulin levels.

5.3 Patients and Methods

This was a prospective study of consecutive adults with newly diagnosed sarcoidosis seen at the Hillbrow Hospital in Johannesburg over a 2-year period. Approval to conduct the study was received from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand with written informed consent from the patients to participate in the study (M110751 approval, APPENDIX E).

The following data, where available, were recorded for each patient: age, gender, method of diagnosis of sarcoidosis, levels of serum angiotensin converting enzyme

(sACE), erythrocyte sedimentation rate (ESR), initial haemoglobin level, white cell count and platelet count, the findings on chest radiograph and the results of lung function studies (according to “the European Community for Steel and Coal prediction equations with a racial correction factor” of 0.9) (12) including the forced expiratory volume in one second (FEV1), forced vital capacity (FVC), the FEV1/FVC ratio, peak flow (PEF) and forced expiratory flow 25-75% (FEF25-75%). The levels of each of the individual IgG subclasses were measured initially (prior to any therapy) and subsequently (either on routine follow-up of cases not on treatment or after at least one-month therapy in cases treated with corticosteroids).

IgG subclasses were measured by means of a laser nephelometric method, using appropriate test kits (The Binding Site Ltd, Birmingham, England; APPENDIX C) and levels were assessed in a Bearing Nephelometer 100 analyzer (Bearing Diagnostika, Germany).

Statistical analysis was undertaken using the U-test of Mann Whitney for continuous variables and the Fisher's exact (2-tail) test for categorical variable. For comparison of the IgG subclasses values, the data on subclasses levels were used both as categorical variables (high, normal or low) and as continuous variables (median values) and interquartile range (IQR) and minimum and maximum values.

5.4 Results

The patient demographic and clinical characteristics are shown in Table 5.1. A total of 17 Black African newly diagnosed sarcoidosis patients were enrolled in the study of whom 11 were female and 6 males. All were non-smokers. The median age was 44.0 years (IQR 31.5-55.0) with a range between 24-60 years. All except three patients had

symptom(s) on presentation, of which cough, weight loss and chest pain were the most common. Ten patients had no abnormal physical signs on initial clinical examination.

Table 5.1. The demographic and clinical characteristics of the sarcoidosis patients in the IgG subclass study

	n	(%)
Number of patients (All Black patients and all non-smokers)	17	
Females	11	(64.7)
Males	6	(35.3)
Biopsy confirmation	17	(100)
Median age at disease onset (years), (IQR; min-max)	44.0 (31.5-55.0; 24-60)	
Symptoms (multiple in some patients)		
Cough	12	(70.6)
Weight loss	8	(47.1)
Chest pain	5	(29.4)
Night sweats	3	(17.6)
Nil	3	(17.6)
Dyspnoea	2	(11.8)
Skin rash	1	(5.9)
Eye symptoms	1	(5.9)
Arthralgia	1	(5.9)
Loss of appetite	1	(5.9)
Decreased effort tolerance	1	(5.9)
Blocked nose	1	(5.9)
Signs (multiple in some patients)		
Nil	10	(58.8)
Crackles	4	(23.5)
Skin rash	4	(23.5)
Splenomegaly	1	(5.9)
Lymphadenopathy	1	(5.9)
Anterior uveitis	1	(5.9)

“IQR = Interquartile range

Min-max = Minimum-maximum

n = Number

% = Percent”

The diagnosis of sarcoidosis was most commonly made on the basis of the histology obtained by fibre-optic bronchoscopy and transbronchial lung biopsy. Other methods of diagnosis (sometimes multiple) are shown in Table 5.2.

Table 5.2. The details regarding biopsy, sACE, radiographic and lung function characteristics in the patients with sarcoidosis (n=17)

	n	(%)
Biopsy Sites (sometimes multiple)		
Transbronchial lung biopsy	10	(58.8)
Skin biopsy	4	(23.5)
Lymph node biopsy (1 mediastinal and 2 supraclavicular)	3	(17.6)
Clinical features	2	(11.8)
Bone marrow trephine biopsy	1	(5.9)
Biopsy of nose mass	1	(5.9)
Open lung biopsy	1	(5.9)
Median sACE (U/L) (IQR; min-max)	86 (47-151; 28-317)	
Chest radiograph		
Stage I	5	(29.4)
Stage II	7	(41.2)
Stage III	4	(23.5)
Stage IV	1	(5.9)
Lung function tests		
FVC < 80%	5	(29.4)
FEV1 < 80%	5	(29.4)
FEV1/FVC < 70%	3	(17.6)

"IQR = Interquartile range

FVC = Forced vital capacity

FEV1 = Forced expiratory volume in 1 second"

sACE = Serum angiotensin-converting enzyme

Min-max = Minimum-maximum

n = Number

% = Percent

The initial median haemoglobin was 13.95 (IQR 13.13-14.25) g/dL with a range between 12.10 and 15.80 g/dL, the median white cell count was 3.85 (IQR 3.32-4.90) × 10^9/L with a range between 2.40 and 7.50 × l0^9/L (8 cases had a white cell count < 4 × 10^9/L) and the median platelet count was 304 (IQR 256-339) × 10^9/L with a range between 179 and 421 × 10^9/L. The initial median serum angiotensin-converting enzyme (sACE) level was 86 (IQR 47-151) U/L and the range 28-317 U/L and the initial median erythrocyte sedimentation rate (ESR) 32 (IQR 15-52) mm/hr with a range

between 8-67 mm/hr. The initial median serum calcium level was 2.44 (IQR 2.41-2.52) mmol/L and was normal in all cases tested except for one patient who had a raised serum calcium level (2.88 mmol/L), as well as a raised urine calcium excretion (21.57 mmol/vol). Three additional patients tested had normal urine calcium excretion.

The findings on chest radiograph are shown in Table 5.2**.** Most commonly, the patients presented with radiological evidence of hilar and/or mediastinal lymphadenopathy and several, additionally, were noted to have either pulmonary nodules or a reticulonodular infiltrate. On radiological grounds the most common stage of presentation was stage II disease (7 patients).

Lung function studies were performed on initial presentation in 16 of the 17 cases. On the basis of the parameters tested they were reported as being normal in 7 cases, having a restrictive pattern in 4 cases, airway obstruction in 3 cases and mild small airways disease in 2 cases. There did not appear to be any relationship between abnormalities of subclass levels and presence or absence of lung function abnormalities. The values of the IgG subclasses levels are shown in Table 5.3 and Table 5.4, both, before, and after treatment or spontaneous clinical resolution.

Table 5.3. Median values of IgG subclasses before and at least one-month after treatment with corticosteroids or spontaneous clinical response in patients with sarcoidosis

IgG Subclass	Median values (g/L) (IQR; min-max) Before treatment (n = 17)	Median values (g/L) (IQR; min-max) After treatment or spontaneous after clinical resolution (n = 16)	p value
IgG1	13.3 (10.1-16.1; 9.3-33.0)	8.9 (7.7-10.5; 4.3-13.8)	0.002
IgG2	5.0 (2.9-6.1; 1.9-10.6)	3.2 (2.3-3.7; 0.8-7.3)	0.0172
IgG3	1.1 (0.9-1.4; 0-2.9)	0.5 (0.3-0.9; 0.1-2.2)	0.0183
IgG4	0.4 (0.2-0.6; 0-1.1)	0.3 (0.1-0.5; 0-0.8)	0.4434

Values expressed are median (interquartile range (IQR))

min = Minimum

max = Maximum

IgG = Immunoglobulin G

N = Number

Table 5.4. IgG subclass levels, both initial (pre-treatment) and subsequent follow-up (post treatment or spontaneous clinical response) in 16 of the 17 patients with sarcoidosis (described as high, normal or low)

IgG Subclass	Before Treatment (n=17)			After Treatment (n=16)*		
	High	Normal	Low	High	Normal	Low
IgG1	10	7	0	0	15	1
IgG2	4	13	0	0	16	0
IgG3	8	8	1	2	14	0
IgG4	2	13	2	0	15	1

* Data missing for one patient

IgG = Immunoglobulin G

N = Number

Normal values for IgG subclasses:

"IgG1 = 4.9-11.4 g/l, IgG2 = 1.5-6.4 g/l, IgG3 = 0.2-1.1 g/l, IgG4 = 0.08-1.4 g/l"

The initial total IgG level was raised in all seven patients in whom it was measured (median value 19.9 (IQR 16.3-24.6) g/L with a range of 16.0 to 27.7 g/L). The median IgG1 levels pre-treatment were 13.30 (IQR 10.12-16.05) g/L, IgG2 4.96 (IQR 2.87-6.05) g/L, IgG3 1.06 (IQR 0.89-1.39) g/L and IgG4 0.35 (IQR 0.22-0.57) g/L (Table 5.3). Raised IgG1 levels were noted in 10 patients, raised IgG3 levels in eight patients, raised IgG2 levels in four patients and raised IgG4 levels in two patients. Decreased levels of IgG4 were noted initially in two cases and decreased levels of IgG3 in one individual (Table 5.4).

Fourteen of the patients were started on corticosteroid therapy, with initial good clinical response, and three cases were observed who did not have an indication for therapy and appeared to have spontaneous clinical response. Following at least one-month of follow-up, which included corticosteroid therapy in the 14 cases, there was a significant

decrease in IgG subclass levels toward normal. Data were missing in one patient who did not appear for follow-up. As shown in Table 5.4, IgG1 levels returned to normal in almost all patients, and IgG3 levels remained raised in only two individuals. The decreased level of 1gG4 remained in the one patient. Another individual developed a decrease in IgG1 level. All other subtype levels returned to normal.

The median IgG1 level on follow-up was noted to have decreased to 8.85 g/L (IQR 7.71-10.5 g/L; p=0,002), IgG2 to 3.16 g/L (IQR 2.25-3.72 g/L; p=0,0172), IgG3 to 0.54 g/L (IQR 0.27-0.90 g/L; p=0,0183) and 1gG4 to 0,29 g/L (IQR 0.14-0.47 g/L; not significant), as shown in Table 5.3.

Figure 5.1 is a box and whisker plot that illustrates the initial and subsequent changes in subclass levels in the 16 patients with initial and follow-up data together with the respective median values, IQR and minimum and maximum values. The initial median sACE level of 86 U/L decreased to 39 (27-60) U/L.

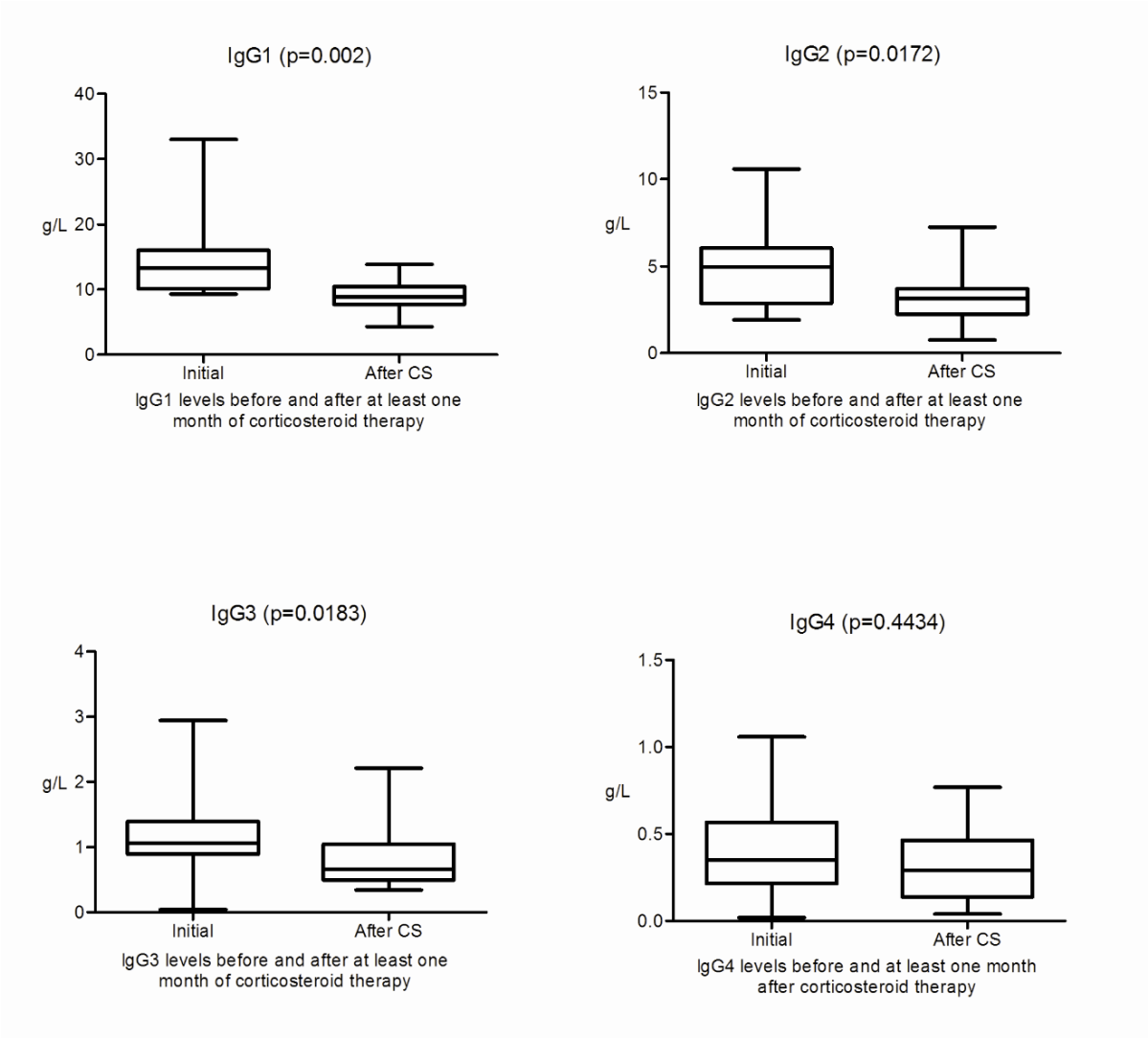

Figure 5.1. IgG subclass levels before and after at least one-month of corticosteroid treatment or spontaneous clinical resolution in patients with sarcoidosis

5.5 Discussion

While the aetiology of sarcoidosis remains unclear, it is widely accepted that central to the disease pathogenesis is a cell-mediated immune response to, as yet, uncertain antigen(s), which results in CD4+ T-lymphocyte activation in an environment of inadequate regulatory CD8+ T cells. This leads to an alveolitis and granuloma

formation as cytokines released by these activated T-cells interact with macrophages (13).

However, it is well known that there are significant changes, which occur in the humoral immune response, which various investigators have been studying further, and which have led to the idea that humoral immunity can also impact significantly on disease pathogenesis. There are relatively few studies (5–7) that have been undertaken investigating serum and/or bronchoalveolar lavage (BAL) levels of immunoglobulins, in particular the IgG subclasses, in sarcoidosis patients, both before and after corticosteroid treatment, and the studies that there are have significantly differing findings.

Rankin and co-workers were the first to measure subclasses in serum and BAL of such patients (7). They documented significantly increased mean levels of IgG1 and IgG3 compared with controls in serum and in BAL. There were no differences in mean IgG2 levels. They also noted a decrease in mean IgG4 levels. Similar results were noted in the current study. We documented an increase in IgG subclass levels in the serum of more than half our patients with newly diagnosed sarcoidosis measured prior to any therapy. The subclasses predominantly involved were IgG1 and IgG3, although increases in the other subtypes were also, though less commonly, noted. Three patients had decreased levels of subclass levels involving IgG4 (2 cases) and IgG3 (1 case). The median IgG1 subclass levels were raised in patients compared with controls (13.30 g/L vs. an upper limit of 11.4 g/L, as were median IgG3 subclass levels (1.12 g/L vs. 1.10 g/L).

Other investigators have measured IgG subclass levels in sarcoidosis with differing results. Barth and co-workers discovered no difference in the proliferative response of

peripheral blood lymphocytes to different mitogens comparing sarcoidosis patients with healthy controls. However, B-cell differentiation responses of bronchoalveolar lavage fluid displayed a major decline in the secretion of IgG subclasses of IgG1, IgG3 and IgG4 (6). Milburn and co-workers noted that sarcoidosis patients had greater serum levels of all four subclasses (5).

It has been shown that IgG-producing plasma cells are found in the bronchial mucosa and are likely to be the source of IgG produced locally (14), while large numbers of B-lymphocytes have been detected in pulmonary lesions and suggested that these may be responsible for some of the humoral changes noted in the granuloma of patients with sarcoidosis and in the serum (15).

The studies by us and Rankin suggest that subclasses IgG1 and IgG3 may play a significant role in sarcoidosis. Of interest, IgG3 is an important subclass for the opsonization of pathogens for alveolar macrophages, both IgG1 and IgG3 avidly bind complement, and are essential in the immune response against protein antigens which may underlie host responses to antigens responsible for the disease process in sarcoidosis (16,17).

IgG comprises four subclasses, namely, IgG1, IgG2, IgG3, along with IgG4 defined by the heavy chain and are numbered according to their serum concentrations. Each of the four subclasses has a corresponding C gene segment on chromosome 14 that encodes its heavy chain constant (18). Mutations in the C genes, inherited in an autosomal pattern, provide variation in the sequence composition of IgG subclass proteins (19). These mutations or allotypes can vary markedly among healthy human populations from different geographical regions (20,21). It has been hypothesized that antibodies of certain allotypes may confer superior protection to pathogens indigenous

to specific regions and this may account for the differences in IgG subclass levels among populations and countries (20,21).

Since corticosteroids are the cornerstone of initial treatment in patients with sarcoidosis requiring therapy, we thought it would be important to record the effects of treatment with corticosteroids on the IgG subclass levels of patients with sarcoidosis treated with this agent. Following at least one month of therapy, and concomitant with clinical response, it was noted that there was a significant decrease in IgG subclass levels to normal in our patients. IgG1 levels returned to normal in almost all and IgG3 levels remained raised in only two cases. One patient remained with a low IgG4. Another patient developed a decrease in IgG1. All other subtype levels returned to normal. The median values of all the subclasses decreased, which was significantly so particularly for IgG1 and IgG3, and on follow-up all median subclass levels were within normal limits. Since IgG subclasses could possibly be utilised to assess and monitor activity of disease and response to therapy, further study is needed to identify its role as a biomarker in sarcoidosis**.**

There has been some debate (22) concerning the effects of corticosteroid therapy on immunoglobulin levels, with the suggestion that there may be non-specific decreases in their levels. However, others (23) have studied the impact of corticosteroids on levels of IgG subclasses in patients with sarcoidosis and it is likely that corticosteroid treatment restores the natural equilibrium between Th1 and Th2 cytokines and immunoglobulins as suggested by Milburn and co-workers (5).

The study has some drawbacks, namely it was a was a single centre study, it had a small sample size, there was a female bias, and all the patients were Black South Africans, so the results may not be generalizable to other regions in South Africa, or to

other countries. Nevertheless, it is the first study to document changes in IgG subclasses in patients with sarcoidosis in South Africa.

5.6 Conclusion

In this study, raised serum levels of IgG subclasses, particularly IgG1, were noted in many of the patients with newly diagnosed sarcoidosis. These increased levels of IgG subclasses largely returned to normal in those cases with spontaneous clinical response and especially in cases treated with corticosteroid. The potential role of IgG subclasses in sarcoidosis pathogenesis needs further clarification.

5.7 References

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Chapter 6 : Altered MicroRNA Expression in Sarcoidosis Patients

6.1 Abstract

6.1.1 Background

Sarcoidosis is a granulomatous multisystem disorder of uncertain aetiology. The disease has major inflammatory and immune components; however, the immunopathogenesis is not well understood. Micro ribonucleic acids (microRNAs or miRNAs) are classes of miniature, single-stranded, non-coding RNAs. Their key recognized role includes mediating the silencing of target genes post-transcriptionally.

Recently, the role of miRNAs has gained interest in numerous disorders, suggested as being implicated in the pathogenesis of those disorders and acting as disease markers. Not a lot is known about the involvement and function of miRNAs in sarcoidosis, with nothing known regarding miRNAs in South African patients, this needs evaluation. The main aim of this research, therefore, was to assess the serum expression of approximately 800 miRNAs in sarcoidosis patients compared with race-, age- and gender-matched healthy controls.

6.1.2 Methods

A total of six patients and six matched controls participated in this study. In ethylenediaminetetraacetic acid (EDTA) tubes, whole blood samples were collected, processed and the plasma retained. RNA was obtained from the stored plasma samples using the QIAGEN miRNeasy Mini Kit® and concentrated using a salt-ethanol precipitation. The extracted miRNA was profiled using an nCounter® miRNA human v3 expression assay and data analysed using the nSolver™ Analysis Software.

6.1.3 Results

After excluding one sample/control pair because of cellular RNA contamination, the remaining five patient and five matched control samples were analysed, and 145 miRNAs were found to be potentially differentially expressed. On applying a Bonferroni correction, the only miRNA that was significantly different was miRNA *let-7a-5p*, which was significantly overexpressed (141-fold change; $p<0.0003$) in patients compared with controls.

6.1.4 Conclusion

This is the first miRNA report of differentially expressed miRNAs in the serum of sarcoidosis patients and matched healthy controls in South Africa. The results obtained indicate that miRNAs may have a role in the pathogenesis of sarcoidosis. Whether these molecules have diagnostic or prognostic implications, needs the performance of these tests in future studies recruiting larger patient cohorts.

6.2 Introduction

Sarcoidosis is a granulomatous multisystem disease of unknown aetiology. The disease has major inflammatory and immune components; however, the immunopathogenesis is not well understood. Micro ribonucleic acids (microRNAs or miRNAs) are classes of miniature, single-stranded, non-coding RNAs (1). Their key recognised role includes mediating the silencing of target genes post transcriptionally. Recently, the role of miRNAs has drawn attention as both being involved in the pathogenesis, and serving as disease biomarkers, in numerous disorders. Extracellular miRNAs in blood show remarkable stability and resistance to degradation (2,3). MicroRNAs are emerging as biomarkers in many pathophysiological conditions

(4) and their role in sarcoidosis is only just beginning to be unravelled (5–7). Therefore, the expression of miRNA in sera of sarcoidosis patients was measured and compared with matched controls. The main aim of this study was to examine the serum expression of approximately 800 miRNAs (APPENDIX F) in patients with sarcoidosis and race-, age-, and gender-matched healthy controls. Differentially expressed (DE) microRNAs were reported and compared in the two groups.

6.3 Patient and Methods

6.3.1 Patients and Controls

A total of six patients with sarcoidosis and six race-, age- and gender- matched healthy controls were recruited into the study and a variety of demographic, clinical, lung function, laboratory, and radiographic data were collected, where appropriate. The diagnosis of sarcoidosis was made according to the international criteria of “the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and Other Granulomatous Disorders” (8). Approval to conduct the study was received from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand with written informed consent from the patients and controls to participate in the study (M121016 approval, APPENDIX E).

6.3.2 Study Specific Investigations

Whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and processed within 30 minutes of collection. Tubes were centrifuged for 15 minutes at 3000g, at 4°C, after which the plasma was transferred to a sterile microcentrifuge tube and stored at -70°C until further use.

6.3.2.1 *RNA Extraction*

RNA was extracted from the stored plasma samples using the QIAGEN miRNeasy Mini Kit® (Qiagen Sciences, MD, USA), APPENDIX D. Extractions were performed in duplicate on 200 µl of plasma for each sample. Plasma samples were allowed to thaw completely before commencing with the extractions. Five volumes (1 ml) of "QIAzol Lysis reagent was added" to each sample and mixed thoroughly. Samples were "incubated at room temperature for 5 minutes" and 30 attomoles (final concentration) of each of 5 spike-in controls ("ath-miR159a, cel-miR-248, cel-miR-254, osa-miR414 and osa-miR442") was added to each extraction and mixed thoroughly. Chloroform at an equal volume to the starting volume "(200 µl) was added and the samples shaken vigorously". After "incubating at room temperature for 3 minutes, the samples were centrifuged for 15 minutes at 12000g, 4°C, and the upper, aqueous phase transferred to a new tube". One and a half volumes of ethanol were combined to each sample, mixed thoroughly and the entire sample loaded onto a "RNeasy MinElute spin column". After binding of the RNA to the spin column, the column was washed three times, allowed to dry thoroughly and the RNA eluted from the column twice with 50 µl "RNase-free water resulting in a final elution volume of 100 µl" per extraction.

Duplicate extractions were combined, and the RNA concentrated using a salt-ethanol precipitation. The samples were made up to 500 µl with "RNase-free water" and one tenth the volume (50 µl) diethyl pyrocarbonate (DEPC)-treated 3M sodium acetate was added along with 3 volumes (1.5 ml) ice-cold absolute ethanol. After mixing by inverting the tubes 5 times, the samples were "incubated overnight at -20°C". The following day, the RNA was pelleted "by centrifugation for 10 minutes at 12000g, 4°C". The resulting RNA pellet was washed twice in "ice-cold 70% ethanol and allowed to

dry" thoroughly before resuspending in 15 µl RNAse free water. Extracts were stored at -70°C until needed.

6.3.2.2 *NanoString Analysis*

The concentrated RNA extracted from the plasma samples was analysed using an nCounter® miRNA human version 3.0 expression assay "according to the manufacturer's protocol". Twelve samples (6 patient samples and 6 age, race and gender matched controls) were analysed on a single cartridge, eliminating the need for calibration between cartridges. Unique oligonucleotide tags, specific for each of the 800 miRNAs in the panel, were ligated to miRNAs from the plasma extracted RNA. These tags allow for increased sensitivity and specificity when detecting the short miRNA sequences. The ligated, tagged miRNAs were denatured at 85°C for 5 min, quick-cooled on ice and then hybridised overnight to a capture probe/reporter probe pair. The capture probe has a biotin tag at its 3' end which is used for binding of the hybridised probes to the imaging surface. The reporter probe has a colour coded molecular barcode at the 5' end which is used to identify the specific miRNA targets during the data collection step.

Following the overnight incubation step, hybridization mixtures were loaded onto an nCounter® SPRINT cartridge for post-hybridisation processing and analysis with the nCounter® SPRINT analysis system. Within the cartridge, the hybridization mixtures were purified using a magnetic bead-based methodology to remove excess probe and non-target transcripts. The purified target/probe complexes were then immobilised on the imaging surface using the 3' biotin tags. The unique molecular barcodes on the 5' end of the complexes were scanned and the number of copies of each of the 800 miRNAs counted. These data were extracted to a .csv file and analysed using the

nSolver™ Analysis Software, version 3.0. A background subtraction using the geometric mean of the negative controls was included to control for background fluorescence. Counts of more than 10, after background correction, were used to define the presence of a given miRNA. MicroRNA expression levels were normalised between patient and control samples by using the expression levels of the five spike-in controls.

6.3.2.3 *Data Analysis and Statistics*

MicroRNAs with a fold change of less than -1.5 or greater than 1.5, were defined as differentially expressed as fold changes outside this range, are mostly because of natural variation within populations. A Bonferroni correction was applied to correct for multiple comparisons and thus, only miRNAs with p values less than 0.0003 were considered significantly different. Additionally, “to account for a high false-positive rate, presumably produced by multiple comparison groups, the p-value adjustment was made using the False Discovery Rate (FDR) process according to Benjamini and Hochberg (9), where the optimal FDR was 5% and a *p* value ≤ 0.03 was considered to be significant”.

6.3.2.4 *Pathway Analysis*

To evaluate the possible role of the dysregulated miRNAs on gene expression in sarcoidosis, DIANA-miRPath version 3.0 (Deciphering miRNA Function Online software tool) and KEGG (Kyoto Encyclopaedia of Genes and Genomes) was utilised (10). The modified miRNAs’ mean fold changes (patients and controls) were used as inputs.

6.3.2.5 *Heat Map*

The nSolver™ Analysis Software version 3.0 was used to generate the heat map. The heat map of differentially expressed miRNAs is depicted with green colour representing a lower and red colour representing a higher expression level.

6.4 Results

The clinical and laboratory parameters of the study patients are shown in Table 6.1. All were Black patients, 4 males and 2 females, who were non-smokers. The median age of the patients was 47 years, interquartile range (IQR) 39-52 years and minimum and maximum ranges between 30-55 years. Four patients had stage II and two patients had stage 4 chest radiographs. Four patients had decreased diffusing capacity for carbon monoxide (DLCO) and three patients had an obstructive defect on spirometry.

When analysing the miRNA results, one patient/control pair was excluded because of cellular RNA contamination in the patient sample. Therefore, only five patients and five matched control samples were analysed. Data of all the microRNAs tested are listed in (APPENDIX F). After excluding miRNAs that were below background levels in both the patient and control groups, 145 miRNAs were found to be differentially expressed. Of these, 57 had p-values of less than 0.05 when comparing the patient and control groups, but after applying the Bonferroni correction, (p-values less than 0.0003) only one miRNA, *let-7a-5p* (141.69-fold change, $p < 0.0003$), was significantly overexpressed in patients compared with controls.

Table 6.2 and Table 6.3 shows the fold changes of the 15 most over- and under-expressed genes respectively, in the patients with sarcoidosis and Figure 6.1 shows

the expression levels of these 30 genes in the form of a heatmap. Overexpression in sarcoidosis patients is depicted in red and under-expression is depicted in green.

As an alternative to the Bonferroni correction, the Benjamini and Hochberg multiple comparison correction was applied to the 145 potentially differentially expressed miRNAs. Using this method, 12 miRNAs were identified as being significantly different (Table 6.4), including *let-7a-5p*.

Table 6.1. Clinical characteristics of patients with sarcoidosis and controls in the miRNA study

	Patients (n)	(%)	Controls
No. of patients (6 Blacks, all non-smokers)	6		6
Population group – (All Blacks)			
Females	2	(33.3)	2
Males	4	(66.7)	4
Biopsy confirmation	6	(100)	-
Median age (years), (IQR; min-max)	47 (39-52; 30-55)		45.5 (18;26-55)
Median sACE (U/L) (IQR; min-max)	124 (109-219; 92-342)		-
Chest radiograph			
Stage I	0	-	-
Stage II	4	(66.7)	-
Stage III	0	-	-
Stage IV	2	(33.3)	-
Lung function tests			
FVC < 80%	4	(66.7)	-
FEV1 < 80%	4	(66.7)	-
FEV1/FVC <70%	3	(50.0)	-
Low DLCO <80%	4	(80.0)	-

“FVC = Forced vital capacity

FEV1 = Forced expiratory volume in 1 second

DLCO = Diffusion capacity for carbon monoxide

sACE = Serum angiotensin-converting enzyme

IQR = Interquartile range

Min-max = Minimum-maximum

n = Number

% = Percent”

Table 6.2. The miRNAs with increased expression in sarcoidosis patients compared with healthy controls

No.	MiRNA	Sarcoidosis	Control	Fold change	p value
1	let-7a-5p	218.39	1.54	141.69	0.0001
2	let-7d-5p	190.55	8.84	21.56	0.0010
3	miR-151a-3p	87.19	4.15	21	0.0038
4	miR-125a-5p	118.56	6.21	19.08	0.0092
5	miR-130a-3p	245.05	16.4	14.95	0.0056
6	miR-361-5p	58.46	4.45	13.15	0.0099
7	miR-148b-3p	31.28	2.78	11.23	0.0041
8	miR-19b-3p	55.11	5.16	10.68	0.0100
9	miR-301a-3p	16.1	1.54	10.44	0.0040
10	miR-664a-3p	21.98	2.13	10.33	0.0045
11	miR-324-5p	15.61	1.54	10.13	0.0136
12	miR-15b-5p	198.62	19.75	10.06	0.0038
13	miR-26a-5p	55.14	5.57	9.90	0.0046
14	miR-199a-3p miR-199b-3p	571.16	57.9	9.86	0.0039
15	miR-181a-5p	192.31	20.19	9.52	0.0025

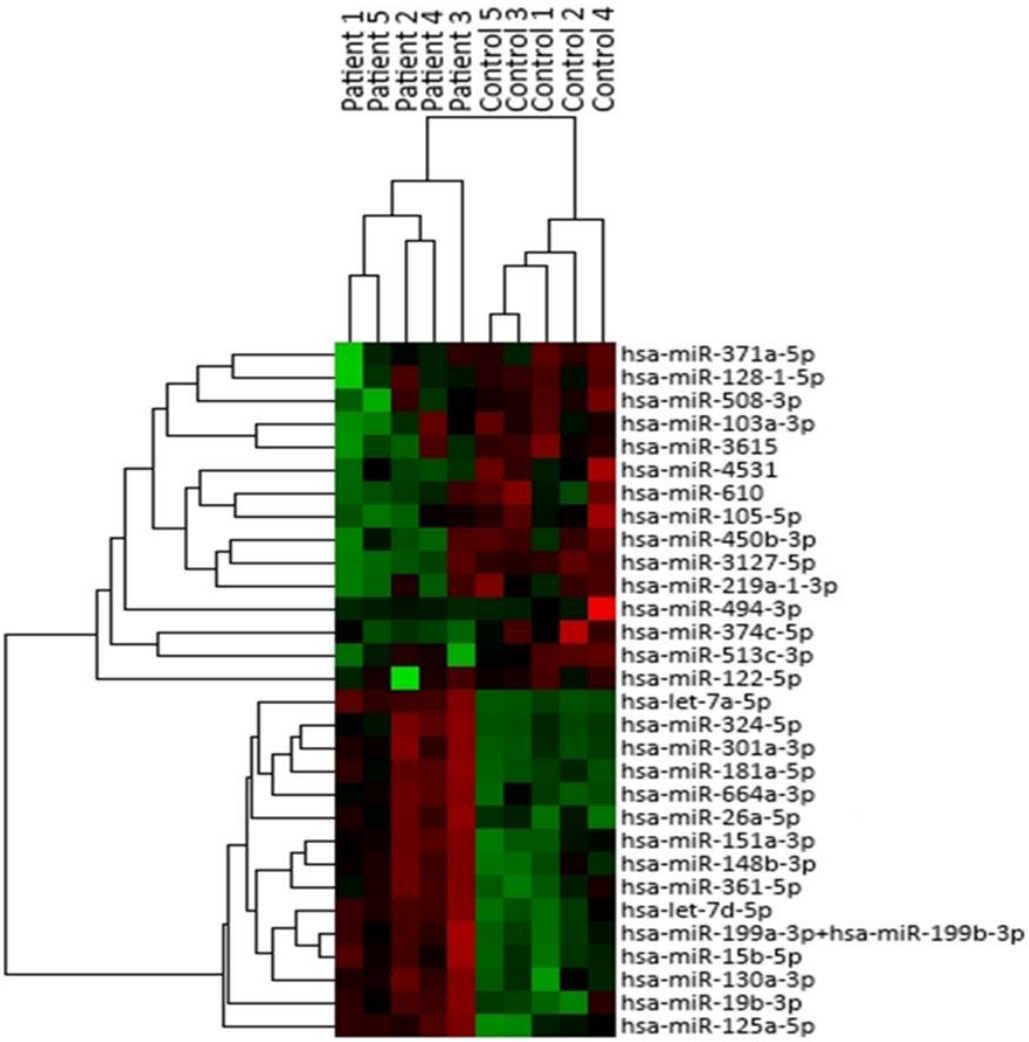
Table 6.3. The miRNAs with decreased expression in sarcoidosis patients compared with healthy controls

No.	MiRNA	Sarcoidosis	Control	Fold change	p value
1	miR-3127-5p	2.29	10.2	-4.45	0.0332
2	miR-513c-3p	4.48	15.46	-3.45	0.0653
3	miR-508-3p	4.88	16.2	-3.32	0.0492
4	miR-122-5p	70.72	211.05	-2.98	0.3437
5	miR-103a-3p	3.53	10.17	-2.88	0.1349
6	miR-128-1-5p	9.05	25.41	-2.81	0.1112
7	miR-371a-5p	5.4	14.56	-2.70	0.0990
8	miR-548q	4.71	11.34	-2.40	0.2300
9	miR-498	5.14	12.16	-2.37	0.1449
10	miR-1290	4.66	10.77	-2.31	0.1879
11	miR-549a	10.64	23.6	-2.22	0.0721
12	miR-553	6.85	15.11	-2.21	0.1822
13	miR-520h	14.27	31.57	-2.21	0.1777
14	miR-3136-5p	7.38	16.3	-2.21	0.1624
15	miR-656-3p	8.03	17.58	-2.19	0.0707

Table 6.4. The miRNAs that were significantly overexpressed in sarcoidosis patients compared with controls (after Hochberg multiple comparison correction)

No.	MiRNA	Sarcoidosis	Control	Fold change	p value
1	let-7a-5p	218.39	1.54	141.69	0.0001
2	miR-1976	1523.68	890.42	1.71	0.0006
3	miR-191-5p	1116.92	122.42	9.12	0.0008
4	let-7d-5p	190.55	8.84	21.56	0.001
5	miR-15a-5p	143.08	22.86	6.26	0.001
6	let-7g-5p	170.77	21.39	7.98	0.002
7	miR-181a-5p	192.31	20.19	9.52	0.002
8	miR-126-3p	393.52	47.28	8.32	0.003
9	miR-151a-3p	87.19	4.15	21	0.003
10	miR-15b-5p	198.62	19.75	10.06	0.003
11	miR-199a-3p miR-199b-3p	571.16	57.90	9.86	0.003
12	miR-301a-3p	16.10	1.54	10.44	0.004

In Figure 6.1 a heat map of the top 15 most up- and down-regulated miRNAs in sarcoidosis patients compared with healthy controls is shown. The vertical axis represents patients and controls, and horizontal axis represents the miRNAs. One can discern four distinct quadrants, showing the over and under-expression of the various miRNAs in patients and controls.



Red depicts overexpression in sarcoidosis, whereas green depicts under-expression.

Figure 6.1. Heatmap of the top 15 most up- and down-regulated miRNAs in sarcoidosis patients compared with controls

6.5 Discussion

It would appear that this is the first report of differentially expressed miRNAs in the serum of patients with sarcoidosis as well as matched healthy controls in South Africa. Interestingly, *let-7a-5p*, was approximately 142 times highly significantly

overexpressed in patients compared with healthy matched controls. Many other miRNAs were also either over- or under-expressed; however, since in this study statistical corrections had to be made for multiple comparisons, none of the other miRNAs were substantially different compared with controls.

In humans, the let-7 family consists of nine mature let-7 members, encoded by 12 distinctive genomic loci (11). Let-7 has been associated with post-transcriptional control of the innate immune response, macrophage activation, TLR4 activation, cellular immunity, adaptive immune response and in promoting autophagy by suppression of mTOR signalling (11). While much research has been dedicated to let-7 target prediction, and to deciphering and understanding its biological function, research into its regulation has only just begun. MicroRNA expression was investigated in the lungs, bronchoalveolar lavage (BAL) fluid, lymph nodes and peripheral blood mononuclear cells (12) and a number of microRNAs appear to play a role in the pathophysiology of sarcoidosis (13,14). In granuloma formation the role of TNF-α has been well documented and is regulated by miRNA let-7 in sarcoidosis (15).

Previous research, consistent with the current study, showed substantial variations in miRNA expression between patients with sarcoidosis and healthy controls (5–7,16–19). An earlier study performed by Maertzdorf and workers found that miR-355, miR-340, miR-182 and miR-15b varied significantly between sarcoidosis and tuberculosis subjects in the circulation (6). The current study found increased expression in of miR-15 in sarcoidosis patients. Fujiwara *et al*., found the expression of miR-223 and miR-126 significantly higher in blood of sarcoidosis patients than in healthy individuals in an attempt to determine biomarkers for cardiac sarcoidosis (20). The current study found increased expression of miR-126 in sarcoidosis patients. Novosadova and workers “observed dysregulated expressions of miR-146, miR-16, miR-425-5p, and

miR-93-5p in sarcoidosis" patients, whether Löfgren's syndrome (LS) was present or not (19). In "patients with LS dysregulated expressions of miR-21-5p and miR-340-5p" were found compared to controls and "dysregulated expressions of miR-150-5p, miR-1, and miR-212, compared with controls, were found in patients without LS" (19). It should be noted that the miRNAs expressed differentially did not overlap with the miRNAs expressed differentially in the current study. Asakage and colleagues analysed the dysregulated miRNAs in blood of three major forms of uveitis and identified miR-4323 as the best predictor for sarcoidosis uveitis (21). MiRNAs in sarcoidosis patients with pulmonary fibrosis found that miRNA-222, miRNA-let-7g, miRNA-20a, miRNA-16, miRNA-221, miRNA-126, and miRNA-146a, which inhibit inflammatory response, were all down-regulated in serum samples (22). Again, it should be noted that the decreased expression of miRNAs in this study did not overlap with the down regulated expression of miRNAs in the current study. The serum miRNA studies concluded, comparable to the findings of the current report, that there were notable variations in miRNA levels between sarcoidosis and healthy controls. These variations may be due to the "miRNAs' tissue or cycle specificity", reflecting the distinct feature of the manifestation of disease between the local tissues and the general circulation (5).

Bioinformatics web-based computational tools are used to link miRNAs to certain biological processes, dedicated to the evaluation of miRNA regulatory functions and the identification of the pathways involved. DIANA-miRPath version 3.0, Deciphering miRNA Function Online software tool, and KEGG (Kyoto Encyclopaedia of Genes and Genomes) were utilised to determine the potential cumulative impact of the dysregulated miRNAs on sarcoidosis gene expression (10). The fold changes of means (patients and controls) of the modified miRNAs were utilised as inputs. Some of the miRNAs identified as differentially expressed in this study of sarcoidosis, were

found to be involved in the “TGF-β signalling pathway” and "Pathways in cancer", amongst others, as has been shown by Novosadova and colleagues (19) who observed dysregulated expressions of miR-16, miR-93-5p, miR-146 and miR-425-5p in sarcoidosis patients compared with controls and "Pathways in cancer" were predicted by bioinformatics. In addition, between patients with and without Löfgren’s syndrome, miR-21-5p, miR-212-3p and miR-340-5p differed, and their cumulative effect, as was suggested by the current study by different miRNAs, to modulate “TGF-β signalling pathway” (19).

This result can have a significant impact on the regulation, function and proliferation of immune cells (5,12,13). MicroRNAs have probable various purposes and can have cumulative consequences when several miRNAs can function on single transcripts (12,13). Each miRNA, therefore, has the potential to control tens to hundreds of genes in several ways, and in most cases several miRNAs affect the expression of the mRNA transcript (16,23).

Basically, the source and function of extracellular circulating miRNA remain unknown (24). There is only a limited understanding of miRNAs and much is yet to be known and understood about the particular and complex function of miRNAs in sarcoidosis. A solitary miRNA could target several hundred genes and multiple miRNAs can target a solitary gene - determining their exact role, and which miRNA-mRNA sets to focus on, can be perplexing (25). Inflammation is a complicated biological and pathophysiological cascade of responses and inflammatory mechanisms are closely related to many diseases. Since the onset and termination of inflammation are crucial to prevent tissue damage, a range of mechanisms has evolved to regulate the process. In this regard, microRNAs (miRNAs) have emerged as key gene regulators to control inflammation, and it is speculated that they are fine-tune signalling regulators to allow

for proper resolution and prevent uncontrolled progression of inflammatory reactions. It can be difficult to dissect whether miRNA are over expressed because of the inflammatory response or are they directly pathogenic. The successful translation of miRNA research is bound to deepen our understanding of sarcoidosis pathogenesis and influence diagnosis, prognosis and treatment. This study provides data that showing that plasma miRNA profiles in sarcoidosis can be useful, either as a diagnostic or prognostic biomarker. It should be noted that with the set of miRNAs chosen, some miRNAs that may conceivably be important for pathogenesis of sarcoidosis were not explored, and this could represent a drawback of the current study. The intricate interplay between miRNAs and mRNA, predicted target genes by signalling pathways and other components involved in disease pathogenesis and phenotype expression (23), needs further elucidation in a larger cohort.

6.6 Conclusion

This appears to be the first report of differentially expressed miRNAs in the serum of patients with sarcoidosis and matched healthy controls in South Africa. MiRNA *let-7a-5p* was approximately 142 times significantly overexpressed in patients with sarcoidosis compared with controls. Many other miRNAs were also either over- or under-expressed. The findings indicate that miRNAs may play a significant role in the inflammatory pathogenesis of sarcoidosis involving in the TGF-β signalling pathway among other pathways. To determine whether these molecules play a pathogenic role in the disease process or could have diagnostic and/or prognostic implications, further studies will be required.

6.7 References

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Chapter 7 : Chitotriosidase Activity in Patients with Sarcoidosis

7.1 Abstract

7.1.1 Background

Chitotriosidase is a chitinase enzyme that is produced selectively through activated macrophages in humans. It was initially documented as having markedly elevated activity in Gaucher's disease patients. Increased activity of chitotriosidase in both bronchoalveolar lavage (BAL) and serum of sarcoidosis patients has been previously described. It has been suggested in sarcoidosis patients that chitotriosidase may be used as a possible diagnostic, prognostic as well as a monitoring biomarker. However, the potential role of chitotriosidase in a South African cohort has not been evaluated in any studies.

The aims of the current study were, firstly to analyse serum chitotriosidase activity in South African sarcoidosis patients, in order to compare with international findings. Secondly, to compare the activity of serum chitotriosidase in patients with sarcoidosis and in patients with tuberculosis (TB), two diseases with distinct aetiology that are clinically difficult to differentiate in the South African setting, which is a high burden area for TB, to determine whether activity levels could differentiate the two conditions.

7.1.2 Methods

Serum chitotriosidase activity were measured in a South African cohort of patients with sarcoidosis and compared with controls. In addition, serum chitotriosidase levels in sarcoidosis patients were compared with TB patients. Overall, serum chitotriosidase activity of 12 biopsy-proven patients with sarcoidosis was assayed in the serum before

treatment, nine sarcoidosis patients after treatment, ten confirmed pulmonary TB patients prior to treatment and twelve healthy controls. Plasma chitotriosidase activity was assayed as previously described utilising 4-methylumbelliferyl-β-D-N, N', N″-triacetylchitotriose as a substrate.

7.1.3 Results

Significantly higher serum chitotriosidase activity was found in patients with sarcoidosis compared with controls ($p < 0.05$), both in untreated and treated patients. Sarcoidosis patients had significantly higher chitotriosidase levels as compared with TB patients. While in patients with TB, chitotriosidase activity was found to be lower than in patients with sarcoidosis, the activity levels were elevated compared with controls.

7.1.4 Conclusion

This is the first report of serum chitotriosidase activity of patients with sarcoidosis compared with healthy controls and with patients with pulmonary TB in South Africa. To validate these findings and to assess whether chitotriosidase may be prognostic or diagnostic marker in patients with sarcoidosis and tuberculosis requires further investigations with greater number of patients.

7.2 Introduction

Chitotriosidase is a chitinase enzyme that is expressed selectively through activated macrophages and epithelial cells in humans (1). It was initially documented as having markedly elevated activity in Gaucher’s disease patients. Increased chitotriosidase activity in both bronchoalveolar lavage (BAL) and serum of sarcoidosis patients has

been previously reported (2,3). It has been suggested in sarcoidosis patients that chitotriosidase may be used as a possible diagnostic, prognostic as well as a monitoring biomarker (1).

The objective of the current study was to analyse chitotriosidase activity of serum in a South African cohort of patients with sarcoidosis, before treatment and after at least one month of treatment. In addition, serum chitotriosidase activity levels were compared in sarcoidosis patients with tuberculosis patients to determine if activity levels could differentiate these two granulomatous disorders of different aetiology, in a high burden area for tuberculosis.

7.3 Patients and Methods

The demographic, clinical, routine laboratory, radiographic, and lung function studies were documented in 12 consecutive biopsy-proven patients with sarcoidosis before treatment, and in nine patients after at least one month of treatment, as well as in 10 newly diagnosed patients with pulmonary TB and in 12 healthy controls. Controls were healthy staff members working in the hospital and laboratory. Sarcoidosis was diagnosed according to the international criteria of “the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and Other Granulomatous Disorders” (4). TB was diagnosed on the basis of microscopy, culture, molecular biology and phenotypic data of *Mycobacterium tuberculosis* (*MTB)* in sputum by GeneXpert, while in the others it was confirmed on microscopic observation and culture of extrapulmonary sites such as the bone marrow and lymph node biopsy specimens. Approval to conduct the study was received from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand with written

informed consent from the patients and controls to participate in the study (M121016 approval, APPENDIX E).

For measurement of chitotriosidase levels blood samples (5 mL) were obtained by venepuncture and collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes. Plasma and packed cells were separated at 1.500xg for 10 minutes by centrifugation and kept at -80°C until they were processed. Chitotriosidase was assayed in the stored plasma samples essentially as described by Hollak *et al*. (5), using “4-methylumbelliferyl-β-D-N, N', N″-triacetylchitotriose” (Sigma) as an enzyme substrate in McIlvain's phosphate-citrate buffer; pH = 5.2, for 1 hr at 37.0℃ in darkness. By adding 2.5 mL of 0.3M glycine/NaOH buffer (pH 10.6) the reaction was terminated. The fluorescent 4-methylumbelliferone reaction product was calculated using a Perkin-Elmer fluorimeter with an excitation wavelength of 365 nm and an emission of 450 nm.

The chitotriosidase activity was expressed in nmol/h/mL. The data was expressed as median and interquartile range (IQR) and minimum and maximum (min-max) values. The Mann-Whitney test and the Kruskal-Wallis test, with a significance set at a $p < 0.05$, were used for comparisons between groups. To look for associations between variables, the Spearman test was used. Statistical analysis and graphical data representations were performed utilising GraphPad Prism 4.0 software.

7.4 Results

The clinical, diagnostic, laboratory, radiographic and lung function data in the cohort of sarcoidosis patients are documented in Table 7.1 to Table 7.3, including data before, (Table 7.1 and Table 7.2) and after, treatment with corticosteroids (Table 7.3), respectively. In Table 7.4, the demographic characteristics of patients with active

tuberculosis are shown. Table 7.5 shows the demographic data of the healthy control subjects. The 12 patients with sarcoidosis (before treatment) were consecutive biopsy-proven patients (six males, nine Blacks and three Indian patients), with a median age of 49.0 years (IQR; min-max) (41-53.5; 30-60 years), and median serum angiotensin-converting enzyme (sACE) levels of 105 U/L (IQR; min-max) (68-153.5; 10-342 U/L); (Table 7.1 and Table 7.2). In addition, there were nine patients, who had had at least one-month of corticosteroid treatment (three males, six Black patients and three Indians, with a median age of 54.0 years (IQR; min-max) (49.5-65.5; 43-79 years), and median sACE levels of 67 U/L (IQR; min-max) (30.5-98.5; 28-222 U/L), (Table 7.3). Ten patients, with newly diagnosed pulmonary tuberculosis included 4 males, all Black patients, 3 patients on antiretroviral therapy (ARV), with a median age of 36.5 years (IQR; min-max) (25-45; 18-57 years); shown in Table 7.4. The demographic data of the 12 healthy controls (10 males, median age of 50.0 years (IQR; min-max) (45.5-51.5; 29-55 years) are shown in Table 7.5.

Table 7.1. Demographic and clinical characteristics of the patients with sarcoidosis, before treatment in the chitotriosidase study

	n	(%)
Number of patients (1 smoker)	12	
Population group - 5BM, 4BF 2IM 1IF		
Females	6	(50.0)
Males	6	(50.0)
Biopsy confirmation	12	(100)
Median age at disease onset (years), (IQR; min-max)	49.0 (41-53.5; 30-60)	
Symptoms (multiple in some patients)		
Cough	12	(100)
Weight loss	8	(66.7)
Chest pain	5	(41.7)
Night sweats	3	(25.0)
Nil	3	(25.0)
Dyspnoea	2	(16.7)
Skin rash	1	(8.3)
Eye symptoms	1	(8.3)
Arthralgia	1	(8.3)
Loss of appetite	1	(8.3)
Decreased effort tolerance	1	(8.3)
Blocked nose	1	(8.3)
Signs (multiple in some patients)		
Nil	10	(83.3)
Crackles	4	(33.3)
Skin rash	4	(33.3)
Splenomegaly	1	(8.3)
Lymphadenopathy	1	(8.3)
Anterior uveitis	1	(8.3)

“BM = Black males, BF = Black females, IM = IM Indian males, IF = Indian females

FVC = Forced vital capacity

FEV1 = Forced expiratory volume in 1 second

DLCO = Diffusion capacity for carbon monoxide

sACE = Serum angiotensin converting enzyme

IQR = Interquartile range

min-max = Minimum-maximum

n = Number

% = Percent”

Table 7.2. Details of the biopsy sites, laboratory, radiographic and lung function characteristics of patients with sarcoidosis before treatment

	n=12	(%)
Biopsy Sites (sometimes multiple)		
Transbronchial lung biopsy	10	(83.3)
Skin biopsy	4	(33.3)
Lymph node biopsy	3	(25.0)
Supraclavicular	2	(16.7)
Mediastinal	1	(8.3)
Bone marrow trephine biopsy	1	(8.3)
Biopsy of nose mass	1	(8.3)
Open lung biopsy	1	(8.3)
Median sACE (U/L) (IQR; min-max)	105 (68-153.5; 10-342)	
Chest radiograph		
Stage I	5	(41.7)
Stage II	7	(58.3)
Stage III	4	(33.3)
Stage IV	1	(8.3)
Lung function tests		
FVC < 80%	6	(50.0)
FEV1 < 80%	4	(33.3)
FEV1/FVC <70%	3	(25.0)
Low DLCO	7	(58.3)

“sACE = Serum angiotensin converting enzyme

IQR = Interquartile range

min-max = Minimum-maximum

FVC = Forced vital capacity

FEV1 = Forced expiratory volume in 1 second

DLCO = Diffusion capacity for carbon monoxide

n = Number

% = Percent”

Table 7.3. Demographic, radiographic, and lung function characteristics of the patients with sarcoidosis, at least one-month after treatment with corticosteroids

	n	(%)
Number of patients (1 ex-smoker, rest non-smokers)	9	
Population group – 1BM, 5BF, 2IM, 1IF		
Females	6	(66.7)
Males	3	(33.3)
Biopsy confirmation	9	(100)
Median age (years), (IQR; min-max)	54 (49.5-65.5; 43-79)	
Median sACE (U/L) (IQR; min-max)	67 (30.5-98.5; 28-222)	
Chest radiograph		
Stage I	2	(222)
Stage II	1	(11.1)
Stage III	3	(33.3)
Stage IV	3	(33.3)
Lung function tests		
FVC < 80%	1	(11.1)
FEV1 < 80%	4	(44.4)
FEV1/FVC <70%	4	(44.4)
Low DLCO <80%	3	(33.3)

"BM = Black males, BF = Black females, IM = IM Indian males, IF = Indian females

FVC = Forced vital capacity

FEV1 = Forced expiratory volume in 1 second

DLCO = Diffusion capacity for carbon monoxide

sACE = Serum angiotensin converting enzyme

IQR = Interquartile range

Min-max = Minimum-maximum

n = Number

% = Percent"

Table 7.4. Characteristics of the patients with active tuberculosis

	n	(%)
Number of patients (1 ex-smoker)	10	
Population group – All Blacks, 6 HIV positive, 3 on ARVs)		
Females	6	(60.0)
Males	4	(40.0)
Confirmation (8 sputum and 1 bone marrow GeneXpert positive)	9	(100)
Median age (years), (IQR; min-max)	36.5 (25-45; 18-57)	

"HIV = Human immunodeficiency virus

IQR = Interquartile range

Min-max = Minimum-maximum

ARVs = Antiretroviral therapy

n = Number

% = Percent"

Table 7.5. Characteristics of the healthy controls

	n	(%)
Number of controls (all non-smokers)	12	
Population group – 4BM, 2BF, 6WM		
Females	2	(16.7)
Males	10	(83.3)
Median age (years), (IQR; min-max)	50 (45.5-51.5, 29-55)	

"BM = Black males, B= Black females, WM = White males

IQR = Interquartile range

Min-max = Minimum-maximum

n = Number

% = Percent"

In Table 7.6, median with interquartile range (IQR) and minimum and maximum of patient and control serum chitotriosidase activity are shown. The activity of serum chitotriosidase in sarcoidosis patients, pulmonary TB patients, and in controls are

shown in Figure 7.1. Substantially higher activity of serum chitotriosidase was observed in patients with sarcoidosis than in controls, both in untreated ($p < 0.0009$) and treated ($p < 0.003$) patients. Untreated sarcoidosis patients had significantly higher chitotriosidase activity levels than tuberculosis patients ($p < 0.0192$). Although the activity was decreased in the treated sarcoidosis patients compared with untreated patients, this was not significantly different. While in patients with tuberculosis chitotriosidase activity was shown to be lower than in patients with sarcoidosis, it was considerably higher than in the controls ($p < 0.0007$).

Table 7.6. Median serum chitotriosidase activity (nmol/hr/mL) of patients and controls

Patient groups	n	Median (IQR)	Min-max
Sarcoidosis (before treatment)	12	600.3 (178.5 - 1030) [a, b]	0 – 5772
Sarcoidosis (after treatment)	9	199.0 (102.8 - 1003) [c]	72.6 – 1459
TB	10	101.7 (83.5 – 167.0) [d]	57.7 – 418.9
Controls	12	51.3 (46.3 – 53.4)	23.8 – 147.7

[a]$p < 0.0009$ vs. controls, [b]$p < 0.0192$ vs. TB patients, [c]$p < 0.003$ vs. controls, [d]$p < 0.0007$ vs. controls
IQR = Interquartile range
TB = Tuberculosis
n = Number
Min-max = Minimum and maximum

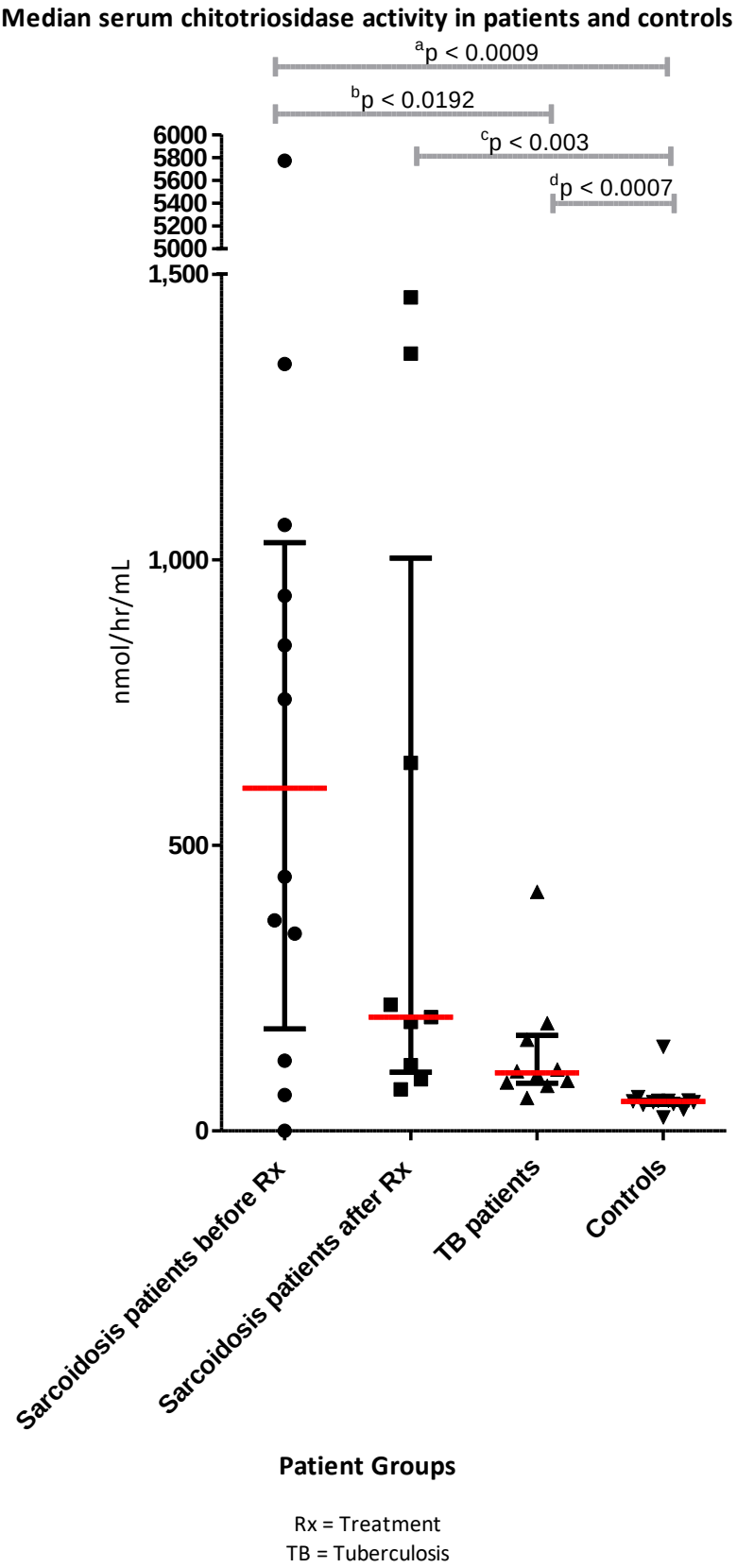

Figure 7.1. Median serum chitotriosidase activity levels in patients and controls (red line represents median levels with interquartile range)

7.5 Discussion

In the current analysis, activity of serum chitotriosidase was evaluated for the first time in South African patients with sarcoidosis as well as in TB patients. Chitotriosidase activity has been found to be substantially higher in sarcoidosis patients, both before ($p < 0.0001$) and after treatment ($p < 0.0039$), compared with controls. Analysis of the activity of serum chitotriosidase in a patient cohort from South Africa with sarcoidosis, verified the reported increase described in the literature, compared with healthy controls. When comparing serum chitotriosidase levels in patients with TB versus those with sarcoidosis, two granulomatous diseases of different aetiology, substantially higher activity was found in untreated sarcoidosis patients ($p < 0.0192$).

Standard serum chitotriosidase activity ranges from 8 to 65 nmol/hr/ml (6,7). In the present study chitotriosidase activity levels in serum of patients with sarcoidosis were substantially higher relative to healthy controls as shown by Bargagli and colleagues (8). In contrast to patients with systemic sclerosis, idiopathic pulmonary fibrosis, asbestosis or tuberculosis, substantially higher levels have been documented in sarcoidosis patients (8,9). Bennet and workers showed that chitotriosidase activity was associated with disease severity, multiorgan involvement, pulmonary fibrosis, and the requirement for corticosteroid treatment (10). Grosso *et al*., showed that chitotriosidase activity in serum can be a useful biomarker for evaluating sarcoidosis disease progression and prognosis (7). Harlander and workers described that chitotriosidase activity correlates with certain sarcoidosis phenotypes like Löfgren's syndrome and that serial measurements correlate with clinical symptoms, chest radiographs stage and lung function (11). While raised chitotriosidase activity is not specific for sarcoidosis, it is promising to be a very sensitive biomarker of active sarcoidosis and appear to be more sensitivity and precise than other widely used markers of sarcoidosis such as

ACE and lysozyme (12,13). Sarcoidosis and TB are both granulomatous diseases, characterised by increased activation of alveolar macrophages, which in both has an integral role to play in granuloma formation (14,15). The observation of substantially higher activity of chitotriosidase in patients with sarcoidosis than in TB and controls, along with an increase of more than 10-fold greater than with controls, indicate that this enzyme may be important in sarcoidosis, and could have a role to play in the pathogenesis (16). This test alone is not diagnostic and will be unable to completely differentiate sarcoidosis from TB; however, with normal chitotriosidase activity there is a greater chance of it being TB than sarcoidosis, in the presence of a granulomatous biopsy (3,16).

It is not only in patients with sarcoidosis that serum ACE levels are increased. The correlation between chitotriosidase and other serological sarcoidosis markers, for example, the serum angiotensin-converting enzyme (sACE), found in other studies indicated that sACE levels are frequently increased in various other granulomatous pulmonary diseases, including TB, and are usually 2 to 3 fold greater than normal, in approximately 50% of patients (17–19). The median sACE level in patients before treatment was 105 IU/L (Table 7.2) and after at least one month of corticosteroid treatment was 67 U/L (Table 7.3). Correlation between serum chitotriosidase and sACE was not performed in the current study as the patient numbers were small and sACE levels were not measured in the patients with TB and in the controls.

Mechanisms which lead to increased activity of chitotriosidase in sarcoidosis are not known. This rise is hypothesized to be due to increased activation of macrophages (20). Chitotriosidase activity may be increased in the serum of sarcoidosis patients as a result of an unidentified antigen, probably chitin containing, which induces activation of macrophages to produce many mediators including chitotriosidase.

There is proof that macrophage stimulation with TNF-α and IFN-γ results in an increase in chitotriosidase activity as well as facilitating the expression of chitotriosidase genes (20). In earlier research (2), enhanced serum and BAL chitotriosidase activity in stage II–III sarcoidosis patients was noted, especially in chronic disease, where Th2 cytokine response predominates, and this is believed to be responsible for fibrosis, with M2 or alternatively activated macrophages (21,22). Elias *et al*., (21) likewise revealed that chitinase-like proteins and chitinases can add to Th2-type immune response by a mechanism related to IL-13. Th2 cytokines induce activation via the formation of fibronectin, different mediators (e.g., CCL18, IL-10, IL-1 receptors, and possibly chitotriosidase) and fibroblast induced collagen synthesis of the alternatively activated macrophages (M2) involved in pulmonary fibrosis (22).

In *Mycobacterium tuberculosis* (*MTB)* infection, the immune response is characterized by macrophage activation, IFN-γ and Th1 lymphocytes production (23). High Th2 cytokine and low Th1 cytokine expression, were connected with an inadequate response to TB treatment (24). With an imbalance and disequilibrium in Th1/Th2 responses in sarcoidosis and in TB, both conditions with accompanying macrophage activation, chitotriosidase in these two conditions might rely on entirely unique macrophage activation pathways or distinctive immune and cytokine responses.

To validate these findings, additional investigations in greater patient numbers are required. It is possible that chitotriosidase may be a sensitive and precise biomarker and certainly its role in sarcoidosis and tuberculosis, is interesting, yet at the same time its usefulness needs to be clarified.

7.6 Conclusion

This is the first report of the analysis of the activity of serum chitotriosidase in South African patients with sarcoidosis and pulmonary tuberculosis. In sarcoidosis patients, chitotriosidase activity is higher compared with TB patients, and also compared with controls. Despite unknown mechanisms leading to increased activity of chitotriosidase in sarcoidosis, the pathogenesis of the disease may well involve this enzyme. Moreover, investigations with larger number of patients are required to validate such findings. Determining if chitotriosidase can be a biomarker of diagnostic or prognostic significance in sarcoidosis remains to be established.

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Chapter 8 : Discussion including Strengths and Potential Study Limitations

8.1 Discussion

The studies in the thesis describe various demographic, clinical, laboratory, radiographic, lung function, treatment and outcome parameters in patients with sarcoidosis in South Africa, and uniquely address several potential immunopathogenic mechanisms that may be playing a role in the disease process.

8.1.1 Sarcoidosis Clinical Study

In the clinical study of sarcoidosis, it was noted that the disease occurs predominantly in younger females, is more prevalent in non-smokers, and occurred in all population groups in South Africa. Similarities and differences in the demographic, clinical and laboratory characteristics of the disease were noted between the different race groups in South Africa, a recognition of which may be helpful in the overall diagnosis, and management, of this heterogeneous condition.

The actual burden of sarcoidosis in South Africa is not really established. This may be due, at least partly, to the under-reporting of this condition because of its resemblance to tuberculosis (TB), with which many patients were initially diagnosed. In the clinical study, approximately 17% of patients had been misdiagnosed initially as TB, prior to the subsequent diagnosis of sarcoidosis. This remains a challenge in high TB burden areas where sarcoidosis is often misdiagnosed as TB.

Comorbid illnesses were common in this clinical cohort, which is an aspect that needs to be recognised, and treated, in its own right, and which may ultimately be

precipitated, or aggravated, by some of the common treatment options, and particularly, the use of corticosteroids.

Almost 90% of patients in the study had an indication to be treated with corticosteroid therapy and were treated and followed-up in the Respiratory Outpatient Department, together with the other sub-specialties such as neurology, ophthalmology, cardiology and dermatology. The relapse rate of those patients who had initially been treated with corticosteroids was 60.7% and the Indian patients had the highest relapse rate at 78.2% compared with 52.8% in the Black patients; however, this was not significantly different.

Clearly, differences in the way sarcoidosis presents and responds, has been noted in various worldwide studies, particularly in those comparing these factors in different populations. For example, in a recent report of 481 Chinese patients compared with 522 United States of America (USA) patients, Chinese patients were noted to be older at diagnosis, had a lower frequency of extrathoracic involvement, greater incidence of hypercalcaemia or hypercalcuria, and less severe lung involvement. These differences were mostly due to the fact that more African American patients were seen in the USA (1).

In a nationwide population based study in Korea, between 2008 and 2015, of 3,259 new patients with sarcoidosis that were identified, they reported an incidence of 0.81 per 100,000 population, mortality “was higher compared with the general population and the major causes of death were cancer, respiratory disease and sarcoidosis and that patients with comorbid diseases showed increased mortality” (2).

In a National Heart, Lung, and Blood Institute's (NHLBI) workshop (3), Gerke and workers reviewed the information on health care, outcomes and burden of sarcoidosis in the USA. The outcomes in terms of sex, race, ethnicity, as well as the socioeconomic groups were disproportionate in African Americans. They had more severe disease and a higher mortality rate. In addition, health care burden of sarcoidosis greatly impacted upon economic, psychosocial, as well as comorbid conditions linked with the disease. Depression, fatigue, treatment side effects, cognitive dysfunction, as well as the pain syndromes were found to be particularly prevalent in this population and contributed to unfavourable outcomes.

In a previous much older South African study from Cape Town (4), Benatar compared the characteristics of sarcoidosis patients in Whites, Blacks and Coloureds. One hundred and ten patients (71 Coloured, 25 Black and 14 White) with sarcoidosis were reviewed over a 7-year period. Differences in the incidence, presentation, clinical and laboratory characteristics among the three race-groups were found. The differences among the Blacks and Whites were similar to the ones reported from studies conducted in USA, while the Coloured patients resembled more the Black patients. In a later clinical study on sarcoidosis from Johannesburg, Smith *et al*. (5), compared 51 Black with 31 White patients from two hospitals. Numerous clinical, demographic as well as laboratory differences were noted. No Black patient had presented with erythema nodosum. Involvement of the skin directly was substantially more common in these patients. The level of the mean sACE was found to elevated in both groups. Hypercalcaemia was also found to be infrequent among the Blacks. Nearly 60% of the cases were administered corticosteroids, and the clinical and objective response to therapy was not different between the two groups. A substantial number of Black patients were often labelled as having had TB and had frequently received anti-tuberculosis chemotherapy.

8.1.2 HLA “Class I and Class II” Antigens in Sarcoidosis

The aetiology of sarcoidosis remains obscure. It is assumed that the onset of sarcoidosis depends on genetic predisposition and that environmental or occupational exposure to an unknown agent(s) adds to this predisposition. The natural history, prognosis and outcome of patients with sarcoidosis is highly variable among different ethnic groups and it appears that this disease has considerable clinical and immunopathogenic heterogeneity.

Sarcoidosis predisposition has been historically observed to be connected with distinct HLA antigens. The HLA study in this thesis showed the presence of an association between different HLA types and sarcoidosis, even though the patient numbers were small. The different HLA phenotypes have been associated with variation in disease susceptibility, presentation, development and prognosis, in different population studies. It would be interesting to explore the correlation of the clinical phenotype with the HLA genotypes in this cohort of patients, which could not be ascertained in this cohort as the numbers were particularly small when having to divide the patients into the individual population groups. It is known that certain HLA alleles influence the disease phenotype and there is a relationship between disease course and extrapulmonary involvement with certain HLA Class II alleles. Despite progress in immunopathogenesis of disease, the underlying mechanism of HLA Class I and II molecules exerting its effect on the aetiology and clinical course is still an enigma. Further clinical trials in diverse populations and distinct patient phenotypes with particular clinical manifestations are needed to predict disease behaviour and improve prognosis.

8.1.3 Immunoglobulin (Ig) G Subclasses in Sarcoidosis

In addition to the dominant cell-mediated immune responses, a number of changes in humoral immunity have been reported that is characterised by B-cell stimulation. The finding of polyclonal increase in gamma globulins, immune complexes and autoantibodies suggests that humoral mechanisms may well be involved in the pathogenesis of the disorder.

The initial total IgG level was raised in all cases in whom it was measured in this thesis. Furthermore, all IgG subclasses were raised in the majority of patients in the current study. These may likely be due to a hyperactive B-cell system, as manifested by hypergammaglobulinaemia and the presence of immune complexes (6–8) seen in sarcoidosis, being responsible at least in part, for the elevations in IgG subclasses. Additionally, these increased levels of IgG subclass levels changed as a consequence of corticosteroid treatment or apparent spontaneous clinical resolution. IgG1 levels returned to normal in almost all and IgG3 levels remained raised in only two cases. The median values of all the subclasses decreased, which was significantly so particularly for IgG1 and IgG3, and on follow-up all median subclass levels were within normal limits. Of interest, IgG3 is an important subclass for the opsonization of pathogens for alveolar macrophages, and both IgG1 and IgG3 avidly bind complement, and are responsible for the immune response to protein antigens, and these factors may underlie host responses to antigens responsible for the disease process in sarcoidosis (9,10). Since corticosteroids are the mainstay of initial treatment in patients with sarcoidosis requiring treatment, the findings following at least one month of therapy, suggest that it is likely that corticosteroid therapy helps in restoring the balance between Th1 and Th2 cytokines as well as immunoglobulins, as suggested by Milburn and co-workers (11).

8.1.4 Micro-ribonucleic acid (microRNA) Expression in Sarcoidosis

MicroRNAs or miRNAs are short non-coding regulating RNAs that serve as post-transcriptional controllers of gene expression. They are being researched and evolving as biomarkers in many diseases, and in numerous respiratory diseases, including idiopathic pulmonary fibrosis, lung cancer, asthma and chronic obstructive lung disease. The role of altered miRNA expression in sarcoidosis pathogenesis is evolving and appear to play an important role in sarcoidosis through their regulatory influence on signalling pathways.

The differentially expressed serum miRNAs of sarcoidosis patients compared with controls detected that *let-7a-5p* was approximately 142 times significantly overexpressed in patients compared with healthy matched controls. Let-7 has been implicated in post-transcriptional control of the innate immune response, macrophage activation, toll-like receptor (TLR) 4 activation, cellular immunity, adaptive immune response and in promoting autophagy by suppression of mechanistic target of rapamycin (mTOR) signalling (12). While much research has been devoted to let-7 target prediction and to understanding its biological function, research into its regulation has only just begun. Some of the miRNAs identified as differentially expressed in the sarcoidosis patients, were found to be involved, among other pathways, in the transforming growth factor (TGF)-β signalling pathway, as has been shown by others (13). The role of tumour necrosis factor (TNF)-α in the formation of granuloma in sarcoidosis, which has been well documented, is regulated by miRNA-let-7 (14). MiRNAs could play a significant role in sarcoidosis pathogenesis along with formation of granuloma. Another inflammatory cytokine, interferon (IFN)-γ, is generated by macrophages, that are also regulated by an overexpression in sarcoidosis of the miR-34/sirtuin (SIRT) 1 (15). miR-146a is another

immunosuppressive miRNA which regulates IFN-γ negatively and is attenuated in sarcoidosis patients (16). miR-33 has a role in granuloma formation in sarcoidosis by alveolar macrophages aggregation via the lipid transporters (17). There have been numerous reports which supports the cluster activities of miR-9, miR-146a, miR-155, miR-223 in macrophage activation along with other myeloid cells (17).

Different studies also underline miRNA expression alterations in sarcoidosis, that may affect its pathogenesis (18,19). Differential expression of miRNAs in peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage (BAL) was indicated in most studies and their utility as unique systemic and local biomarkers were analysed. A noteworthy variation was also found in the expression of miRNAs miR-222, miR-221, miR-192, miR-130a, miR-27b, miR-15b and miR-let-7f, in PBMCs of sarcoidosis patients compared with normal controls (18–20). In sarcoidosis patients, three miRNAs (miR-221, miR-192, and miR-15b) were also differentially expressed in BAL (with some overlap in PBMCs expression). Almost all the miRNAs play an important role in both, apoptosis and inflammation. MiR-222, miR-221, and miR-15b, play an important part in tumour formation, while the others have antitumour characteristics (19,21). MiR-29a is downregulated in sarcoidosis compared with healthy controls (13,22). In sarcoidosis all these indicate a possible role of miRNAs as diagnostic biomarkers.

Various proteins and their function may be regulated miRNAs; and miR-155 has been found to be inducing IFN-γ and interleukin (IL)-2 in Th1 cells (23). Be that as it may, miR-155 inhibits the suppressor of cytokine signalling 1 (SOCS1), a negative regulator of IL-2R signalling, and controls cell survival during thymic differentiation. miR-155 also has a significant part in differentiation of Th1 cell (16,24,25). Another miRNA, miR-31, inhibits differentiation of T-cells into regulatory T-cell (Treg) lymphocytes by FoxP3 mRNA targeting and down-regulation of protein synthesis (26). Similar to miR-155, the

miR-146a expression also increases in serum levels of patients with sarcoidosis. miR-146a shows immune-suppressive traits which results in repression of IFN-γ (27). A negative target of miR-146a is signal transducer and activator of transcription 1 (STAT1), which is a key transcription factor in the IFN-γ response (13). The upregulating expression of the STAT1 protein is the consequence of the loss of miR-146a in the Treg cell. This implies that STAT1 activation in Treg cells with the loss of SOCS1, a negative regulator of STAT1, regulates Th1-mediated pathology. Therefore, an optimal level of STAT1 is needed within the cell for normal Th1 cell regulation by Treg lymphocytes to occur.

A study showed raised miR-33 levels in lymph nodes with granulomas and in chronic lung granulomas with lipid alveolar macrophage carriers (28). It was also shown that of IFN-γ activation further downregulates sirtuin (SIRT)-1 activity by miR-34a, that helps stimulate reactive oxygen species within a cell (29). The inhibition of SIRT-1 disrupts oxidative energy metabolism and stimulates the nuclear factor kappa B (NF-κB)-induced inflammatory responses. The dynamic interplay between miRNAs and messenger RNA (mRNA), predicted target genes through signalling pathways in disease pathogenesis and phenotype (30), needs further elucidation. Whether these molecules have diagnostic or prognostic implications, requires further studies in larger cohorts.

8.1.5 Chitotriosidase Activity in Sarcoidosis

Chitotriosidase is a chitinase enzyme that is expressed selectively through activated macrophages and epithelial cells. Increased chitotriosidase activity has been reported in both bronchoalveolar lavage (BAL) and serum of sarcoidosis patients.

In the study of patients with sarcoidosis, analysis of serum chitotriosidase activity was noted to be increased compared with controls. Comparing serum chitotriosidase activity levels in patients with sarcoidosis and TB, two granulomatous diseases with different aetiologies, documented significantly elevated activity relative to controls in patients with untreated sarcoidosis, treated sarcoidosis, and higher in TB patients than controls. This may suggest that *Mycobacterium tuberculosis* (*MTB*) or TB-like organisms do not appear or unlikely, have a significant role in the pathogenesis and aetiology of sarcoidosis. The mechanisms which lead to increased activity of chitotriosidase in sarcoidosis and in TB are still not known. In sarcoidosis a significant imbalance between Th1 and Th2 responses has been found, together with macrophage activation. This enzyme may well be implicated in the disease pathogenesis and is hypothesised that the increase can be due to enhanced macrophage activation (31). Chitotriosidase activity may be elevated in the serum of cases with sarcoidosis, as a result of an unknown antigen, possibly containing chitin, which induces activation of macrophages to produce many mediators including chitotriosidase. The clinical course and relapse of the disorder, along with response to corticosteroids, appear to be predicted by chitotriosidase activity levels (32,33). A significant increase in sarcoidosis cases compared with healthy controls was found in a recent study, which in turn were linked to respiratory symptoms (exertional cough and dyspnoea) and serum ACE levels (33). In addition, this has also proved to be linked to the severity of the disease, manifested as severe progressive pulmonary and extrapulmonary involvement, enabling the detection of relapsing disease and identifying patients in need of escalation of therapy (33). There was correlation of chitotriosidase to chest radiographic staging; higher activity levels were noted in higher stages, and traction bronchiectasis as well as lung fibrosis with reticular abnormalities linked to a significant increase in chitotriosidase activity (33). The Brompton research

group identified patients at high clinical risk and also correlated chitotriosidase activity with radiological extent and fibrosis (34). Increased activity of chitotriosidase were also associated previously with abnormal TGF-β production (35) and were also linked to the remodelling of airways in severe asthma (36,37). In addition, chitotriosidase activity was discovered to be increased in BAL in sarcoidosis patients with progressive disease compared to those with stable disease (38,39). Such outcomes indicate that the enzyme could have a role in fibrogenesis responsible for remodelling and development of fibrosis. In patients with multiple organ involvement, chitotriosidase activity was also noticed to rise, indicating that its level correlates to the amount of active organ involvement that represents the active granuloma load (33). It may be described in the various organs as a reliable marker of activation of macrophages (40–42). Chitotriosidase, which is involved in the maturation of monocytes into both M1 and M2 subtype macrophages, is mainly secreted by macrophages.

Chitotriosidase activity is also increased in patients with idiopathic pulmonary fibrosis, interstitial lung disease from systemic sclerosis (38,43), asbestosis, chronic obstructive pulmonary disease and lung cancer (44). The diagnostic accuracy of chitotriosidase in sarcoidosis still needs to be explored. Further studies in larger cohorts are required and its position in sarcoidosis will need to be clarified on whether chitotriosidase might be a sensitive and reliable biomarker reflecting radiological and clinical severity.

In summary, in a genetically susceptible individual, an external agent, probably microbial in origin, may cause sarcoidosis. Several genes, especially the HLA genes have been implicated in and may contribute to this susceptibility. The key function of HLA molecules associated with disease is to present specific antigenic peptides so that an inflammatory response with abnormal pathological consequences

is triggered by the recognizing T-lymphocytes. Finding, isolating and eventually sequencing the possible peptide or antigen that is attached to susceptible MHC-molecules triggering reactive T-cells, which is responsible for the initiation and/or exacerbation of the disease, is a prospect and is awaited in anticipation with great expectation. Responses of Th1 and Th2 with stimulation and activation of macrophages along with humoral immune responses, may be synergistically involved in sarcoidosis immunopathogenesis. In determining the essential functional background in the development of sarcoidosis post-transcriptional modifications in protein products shall be crucial.

8.2 Strengths and Potential Study Limitations

There are several potential limitations of the various studies in this thesis. Firstly, the studies are single-centre studies and, therefore, the findings may not be generalisable to other geographical areas in South Africa, or other regions of the world. Small sample sizes in some of the studies may be a concern, particularly when separating the patient population into the various race groups, but it is crucial to highlight that sarcoidosis is not a common condition, and furthermore, many of the new technologies utilised in the thesis are fairly costly investigations. Additionally, the various studies conducted in the thesis spanned a number of years and newer diagnostic modes and newer technologies have evolved over that period. However, the reasons for this prolonged study time-span have been addressed. Clearly, future research studies should preferably be multicentre studies, recruiting larger number of cases, and embracing the new technologies and modalities that are evolving in the sarcoidosis research field, even despite the fact that these studies need to be conducted in regions such as South Africa, in an era of limited funding and resources.

The strengths of many of these studies are that they have been done for the first time in South African patients with sarcoidosis. Several of these studies are unique, particularly to the South African research environment, including being the first time that HLA subtypes, IgG subclass levels, miRNA profiles and chitotriosidase activity have been described in South African cohorts, with a number of these studies only recently starting to be performed in other more well developed regions of the world with far greater resources.

8.3 Recommendations for Future Investigations

The relative rarity of sarcoidosis and the remarkable similarity of clinical and radiological features with TB (high burden in South Africa) pose problems in the differential diagnosis. It is important that all health workers are aware of this condition as these patients have diverse clinical manifestations and can present to any specialty. If for any reason the patient does not respond to TB treatment, and does not have a confirmed diagnosis, that patient should be referred for investigation.

There is a need for ongoing research as there is significant morbidity and mortality related to sarcoidosis. Research into the clinical presentation, as well as basic science to determine which environmental or infectious trigger, genetic predisposition, pathogenesis of the condition and searching for biomarkers to risk stratify and prognosticate outcomes are needed.

After 150 years, since the first description of the disease, researchers continue to try to answer three important questions that my patients ask:

1. “What causes of sarcoidosis?”
2. “Why did I get it and why did I get it that way?”

3. “What effect does this have on my health?”

Sarcoidosis is likely to evolve from intricate and complex interactions between environmental agents and the alleles of several genes. There is still no known environmental cause. New research methods trying to decipher and isolate the putative inciting agent binding to susceptible MHC molecules, as being the aetiology that triggers the condition, are needed. With genomic studies, understanding the predictive ability of genetic factors for disease risk, course, and impact on disease, and post-transcriptional effects will provide us with improved methods to predict disease resolution or progression by better defining the pathogenesis. These will contribute to better comprehension and understanding of the aetiopathogenesis of sarcoidosis and ultimately develop better treatment and eventually leading to patient improvement and/or cure. Further studies are ongoing, and we hope to answer these questions.

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Chapter 9 : Conclusion

Sarcoidosis is a multisystemic inflammatory disorder of unknown cause. The clinical presentation of sarcoidosis is variable, and the diagnosis may be difficult. The cause of sarcoidosis remains unknown. The therapy is not uniform, and the clinical trajectory and prognosis is variable and unpredictable. Sarcoidosis has a complex environmental and molecular aetiopathogenesis. Development of sarcoidosis has been recognized as having a genetic basis largely linked to human leukocyte antigens (HLA), both HLA class I and class II. Macrophage activation together with cell mediated and humoral immune responses, may synergistically be involved in the immunopathogenesis of this condition. Macrophage activation may be due to an antigen, possibly containing chitin, which induces activation of macrophages to produce many mediators including chitotriosidase. MicroRNAs have been found to play a crucial role in the post-transcriptional modulation of many human genes, are recognized as important regulators for signalling pathways and playing an important part in inflammation control. In addition, miRNAs can be attractive novel diagnostic markers and could ultimately deliver therapeutic intervention opportunities. These studies have confirmed that many of the factors investigated appear to play a role in the immunopathogenesis of sarcoidosis in patients in South Africa and further studies, with greater patient numbers, are needed.

APPENDICES

APPENDIX A. Clinical Research Form

CLINICAL RESEARCH FORM

NUMBER:

STUDY TITLE: Clinical and Immunopathogenetic Aspects of Sarcoidosis

Name									Work	
Number										
Diagnosis										
Age										
Sex	M			F				B I W C		
RVD	R				NR					
CD4										
VL										
Presentation										
Smoker	Y amount				N					
Other illnesses										
Medications										
Examination	Gen Temp J A C C O L wasted skin									
	Chest									
	Cor									
	Abd Hepa Spleen other									
	CNS Eyes Focal signs other									
Other										
CXR										
Stage										
HRCT										
Lung Fns	FEV1				U&E					
	FVC				Hb/Wcc/Plt					
	Ratio				ESR/CRP					
	TLC				Ca/Mg/P					
	RV				Liver	TB	DB	IB	TP	Alb
	DLCO				AP	GGT	ALT	AST	ACE	
BLOOD	**MiRNA**		**B4 Rx**	**Aft Rx**	**Chito**				**B4 Rx**	**Aft Rx**

APPENDIX B. QIAmp DNA Blood Mini Kit Handbook for Genomic DNA Extraction Utilised in the HLA Study

Fifth Edition May 2016

QIAamp® DNA Mini and Blood Mini Handbook

For DNA purification from whole blood, plasma, serum, buffy coat, lymphocytes, dried blood spots (QIAamp DNA Mini Kit only), body fluids, cultured cells, swabs, and tissue (QIAamp DNA Mini Kit only)

QIAGEN

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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- Automation of sample and assay technologies

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Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Ethanol (96–100%)*
- 1.5 ml microcentrifuge tubes
- Pipet tips with aerosol barrier
- Microcentrifuge (with rotor for 2 ml tubes)
- Vortexer
- Water bath or heating block at 56°C
- Phosphate-buffered saline (PBS) may be required for some samples

For vacuum protocols

- QIAvac 24 Plus (cat. no. 19413) or equivalent
- VacConnectors (cat. no. 19407)
- Vacuum Regulator (cat. 19530) for easy monitoring of vacuum pressures and easy releasing of vacuum
- Vacuum Pump (cat. no. 84010 [USA and Canada], 84000 [Japan], or 84020 [rest of world]) or equivalent pump capable of producing a vacuum of –800 to –900 mbar
- For buccal swabs or large volumes: Extension Tubes (cat. no. 15987)
- Optional: VacValves (cat. no. 19408)
- Optional: QIAvac Connecting System (cat. no. 19419)
- Optional: RNase A (100 mg/ml)

For tissues

- Additional water bath or heating block at 70°C
- Optional: Equipment for mechanical disruption, such as the TissueRuptor® (see page 70 for ordering information) or mortar, pestle, and liquid nitrogen

For buccal swabs

- Additional Buffer AL (cat. no. 19075)
- 2 ml microcentrifuge tubes
- For cotton or DACRON® swabs: Scissors or appropriate cutting device

For dried blood spots

- 3 mm single-hole paper puncher
- Two additional water baths or heating blocks at 85°C and 70°C

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

Important Notes

Preparation of reagents

QIAGEN Protease stock solution (store at 2–8°C)

When using the QIAamp DNA Blood Mini Kit (50), pipet 1.2 ml protease solvent* into the vial containing lyophilized QIAGEN Protease, as indicated on the label. When using the QIAamp DNA Blood Mini Kit (250), pipet 5.5 ml protease solvent into the vial containing lyophilized QIAGEN Protease, as indicated on the label.

Dissolved QIAGEN Protease is stable for up to 12 months when stored at 2–8°C.

Note: If you also use QIAamp MinElute Virus Kits, be aware that the QIAGEN Protease supplied with these kits is reconstituted in protease resuspension buffer or Buffer AVE and is not compatible with the QIAamp DNA Blood Mini Kit. After reconstituting a vial of QIAGEN Protease, label the resuspended QIAGEN Protease to indicate which buffer was used for resuspension.

Buffer AL† (store at room temperature, 15–25°C)

Mix Buffer AL thoroughly by shaking before use. Buffer AL is stable for 1 year when stored at room temperature.

Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

Buffer AW1† (store at room temperature, 15–25°C)

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle.

Buffer AW1 is stable for 1 year when stored closed at room temperature.

Buffer AW2* (store at room temperature, 15–25°C)

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle.

Buffer AW2 is stable for 1 year when stored closed at room temperature.

* Contains sodium azide as a preservative.

† Contains chaotropic salt. See page 6 for safety information.

Carrier DNA

Use carrier DNA (e.g., poly dA, poly dT, poly dA:dT) when the sample is low-copy (i.e., when <10,000 copies are present). For preparation of DNA from free viral particles in fluids or suspensions (other than urine) using the blood and body fluid protocols, we recommend the addition of 1 µl of an aqueous solution containing 5–10 µg of carrier DNA (e.g., poly dA, poly dT, poly dA:dT) to 200 µl Buffer AL. To ensure binding conditions are optimal, increase the volume of ethanol added at step 6 from 200 µl to 230 µl. Elution should be in 60 µl Buffer AE.

Amounts of starting material

Use the amounts of starting material indicated in Table 1.

Table 1. Amounts of starting material for QIAamp Mini procedures

Sample	Amount
Blood, plasma, serum	200 µl
Buffy coat	200 µl
Tissue	25 mg*
Cells (diploid)	5 x 10^6 cells

* When isolating DNA from spleen, 10 mg samples should be used.

Small samples should be adjusted to 200 µl with PBS before loading. For samples larger than 200 µl, the amount of lysis buffer and other reagents added to the sample before loading must be increased proportionally (see note below). Application of the lysed sample to the QIAamp Mini spin column will require more than one loading step if the initial sample volume is increased. The amounts of Buffer AW1 and Buffer AW2 used in the wash steps do not need to be increased.

Scaling up the tissue protocol is possible in principle. The volumes of Buffer ATL and proteinase K used should be increased proportionally, while the volumes of wash and elution buffers should remain constant. The user should determine the maximum amount of tissue used. It is important not to overload the column, as this can lead to significantly lower yields than expected.

Note: All QIAamp buffers can be purchased separately to supplement the QIAamp DNA Mini and QIAamp DNA Blood Mini Kits (see ordering information, page 66). QIAGEN Proteinase K is recommended for use with tissue samples. QIAGEN Protease is suitable for genomic DNA preparation from blood, cells, and body fluids.

Preparation of buffy coat

Buffy coat is a leukocyte-enriched fraction of whole blood. Preparing a buffy-coat fraction from whole blood is simple and yields approximately 5–10 times more DNA than an equivalent volume of whole blood.

Prepare buffy coat by centrifuging whole blood at 2500 x *g* for 10 minutes at room temperature (15–25°C). After centrifugation, 3 different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat, containing concentrated leukocytes; and the bottom layer contains concentrated erythrocytes.

Handling of QIAamp Mini columns

Owing to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling QIAamp Mini columns to avoid cross-contamination between sample preparations:

- Carefully apply the sample or solution to the QIAamp Mini column. Pipet the sample into the QIAamp Mini column without wetting the rim of the column.
- Change pipet tips between all liquid transfers. The use of aerosol-barrier pipet tips is recommended.
- Avoid touching the QIAamp membrane with the pipet tip.
- After all pulse-vortexing steps, briefly centrifuge the 1.5 ml microcentrifuge tubes to remove drops from the inside of the lid.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

Centrifugation

QIAamp Mini columns will fit into most standard 1.5–2 ml microcentrifuge tubes. Additional 2 ml collection tubes are available separately.

Centrifugation of QIAamp Mini columns is performed at 6000 x *g* (8000 rpm) to reduce centrifuge noise. Centrifuging QIAamp Mini columns at full speed will not affect DNA yield. Centrifugation at lower speeds is also acceptable, provided that nearly all of each solution is transferred through the QIAamp membrane. When preparing DNA from buffy coat or lymphocytes, full-speed centrifugation is recommended to avoid clogging.

All centrifugation steps should be carried out at room temperature (15–25°C).

Processing QIAamp Mini columns using a microcentrifuge (spin protocols)

Close the QIAamp Mini column before placing it in the microcentrifuge. Centrifuge as described.

- Remove the QIAamp Mini column and collection tube from the microcentrifuge. Place the QIAamp Mini column in a new collection tube. Discard the filtrate and the collection tube. Note that the filtrate may contain hazardous waste and should be disposed of appropriately.
- Open only one QIAamp Mini column at a time, and take care to avoid generating aerosols.
- For efficient parallel processing of multiple samples, fill a rack with collection tubes to which the QIAamp Mini columns can be transferred after centrifugation. Used collection tubes containing the filtrate can be discarded, and the new collection tubes containing the QIAamp Mini columns can be placed directly in the microcentrifuge.

The QIAvac 24 Plus

The QIAvac 24 Plus is designed for fast and efficient vacuum processing of up to 24 QIAGEN spin columns in parallel. Samples and wash solutions are drawn through the column membranes by vacuum instead of centrifugation, providing greater speed and reduced hands-on time in purification procedures.

In combination with the QIAvac Connecting System (optional), the QIAvac 24 Plus can be used as a flow-through system. The sample flow-through is collected in a separate waste bottle.

For maintenance of the QIAvac 24 Plus, please refer to the handling guidelines in the *QIAvac 24 Plus Handbook*.

Processing QIAamp Mini columns on the QIAvac 24 Plus (vacuum protocols)

QIAamp Mini spin columns are processed on the QIAvac 24 Plus using disposable VacConnectors and reusable VacValves. VacValves (optional) are inserted directly into the luer slots of the QIAvac 24 Plus manifold and ensure a steady flow rate, facilitating parallel processing of samples of different natures (e.g., blood and body fluids), volumes, or viscosities. They should be used if sample flow rates differ significantly to ensure consistent vacuum. VacConnectors are disposable connectors that fit between QIAamp Mini columns and VacValves or between the QIAamp Mini columns and the luer slots of the QIAvac 24 Plus. They prevent direct contact between the spin column and VacValve during purification, avoiding any cross-contamination between samples. VacConnectors are discarded after a single use.

Handling guidelines for the QIAvac 24 Plus

- Always place the QIAvac 24 Plus on a secure bench top or work area. If dropped, the QIAvac 24 Plus manifold may crack.
- Always store the QIAvac 24 Plus clean and dry. For cleaning procedures see the *QIAvac 24 Plus Handbook*.
- The components of the QIAvac 24 Plus are not resistant to certain solvents (Table 2). If these solvents are spilled on the unit, rinse it thoroughly with water.
- To ensure consistent performance, do not apply silicone or vacuum grease to any part of the QIAvac 24 Plus manifold.
- Always use caution and wear safety glasses when working near a vacuum manifold under pressure.
- Contact QIAGEN Technical Services or your local distributor for information concerning spare or replacement parts.
- The vacuum pressure is the pressure differential between the inside of the vacuum manifold and the atmosphere (standard atmospheric pressure 1013 millibar or 760 mm Hg) and can be measured using the QIAvac Connecting System or a vacuum regulator (see Figure 2). The vacuum protocol requires a vacuum pump capable of producing a vacuum of –800 to –900 mbar (e.g., QIAGEN, Vacuum Pump). Higher vacuum pressures must be avoided. Use of vacuum pressures lower than recommended may reduce DNA yield and purity and increase the frequency of clogged membranes.

Table 2. Chemical resistance properties of QIAvac 24 Plus

Resistant to:		
Acetic acid	Chaotropic salts	Chlorine bleach
Chromic acid	Concentrated alcohols	Hydrochloric acid
SDS	Sodium chloride	Sodium hydroxide
Tween® 20	Urea	
Not resistant to:		
Benzene	Chloroform	Ethers
Phenol	Toluene	

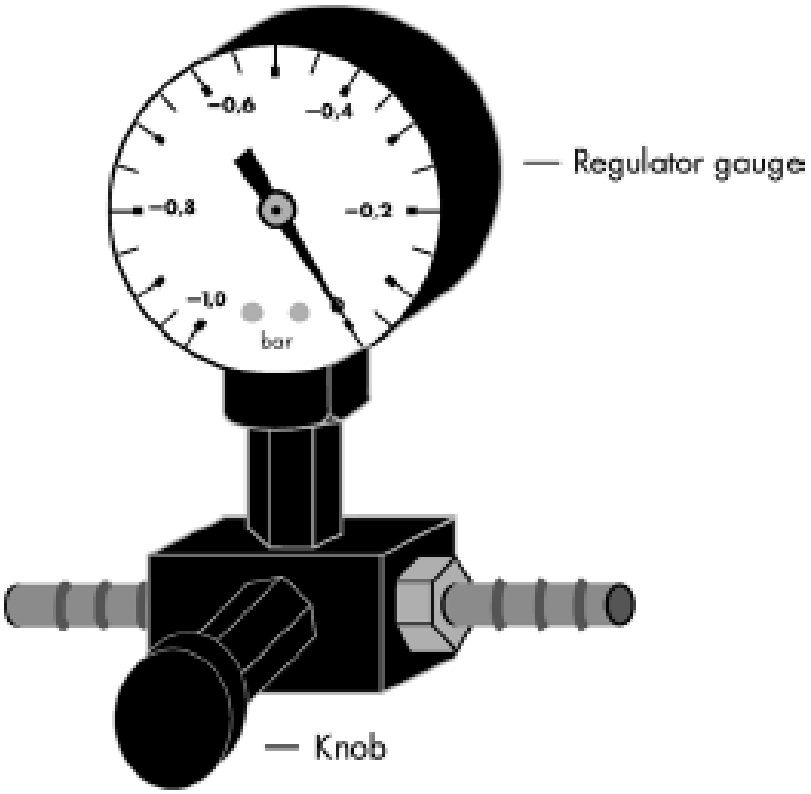

Figure 2. Schematic diagram of the Vacuum Regulator.

Setup of the QIAvac 24 Plus vacuum manifold

1. **Connect the QIAvac 24 Plus to a vacuum source. If using the QIAvac Connecting System, connect the system to the manifold and vacuum source as described in Appendix A of the *QIAvac 24 Plus Handbook*.**
2. **Insert a VacValve into each luer slot of the QIAvac 24 Plus that is to be used (see Figure 3). Close unused luer slots with luer plugs or close the inserted VacValve.**

   VacValves should be used if flow rates of samples differ significantly to ensure consistent vacuum.
3. **Insert a VacConnector into each VacValve (see Figure 3).**

   Perform this step directly before starting the purification to avoid exposure of VacConnectors to potential contaminants in the air.
4. **Place the QIAamp Mini columns into the VacConnectors on the manifold (see Figure 3).**
5. **If necessary, insert an Extension Tube into each QIAamp Mini column (see Figure 4).**

   Extension Tubes are required for processing buccal swabs or large volumes.

6. **For nucleic acid purification, follow the instructions in the vacuum protocols. Discard the VacConnectors appropriately after use.**

   Leave the lid of the QIAamp Mini column open while applying vacuum.

   Switch off the vacuum between steps to ensure that a consistent, even vacuum is applied during processing. For faster vacuum release, a vacuum regulator should be used (see Figure 2).

   Note: Each VacValve can be closed individually when the sample is completely drawn through the spin column, allowing parallel processing of samples of different volumes or viscosities.

7. **After processing samples, clean the QIAvac 24 Plus (see "Cleaning and Decontaminating the QIAvac 24 Plus" in the *QIAvac 24 Plus Handbook*).**

   Note: Buffers AL and AW1 used in QIAamp DNA Mini and QIAamp DNA Blood Mini procedures are not compatible with disinfecting agents containing bleach. See page 6 for safety information.

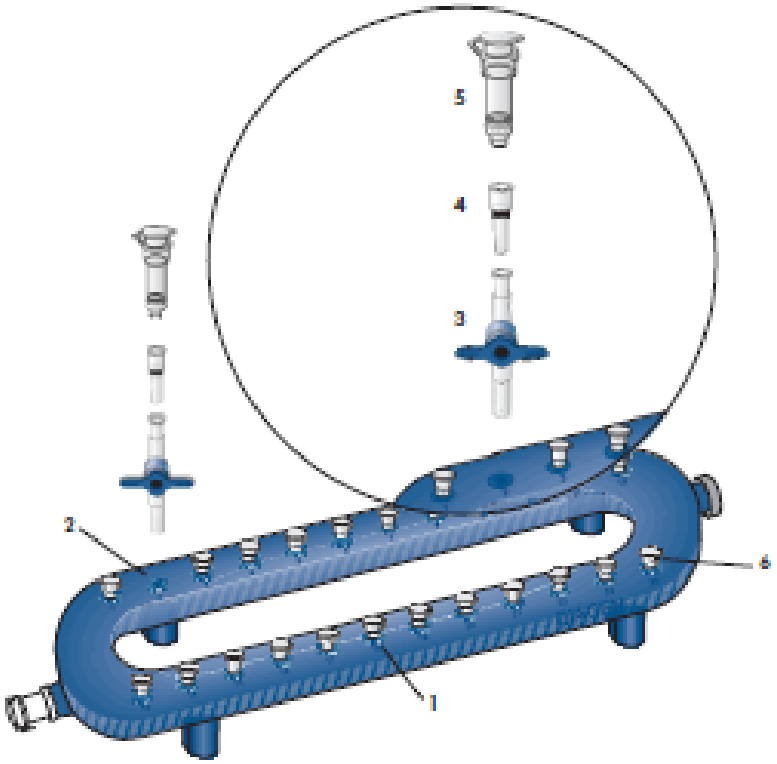

Figure 3. Setting up the QIAvac 24 Plus with QIAamp Mini columns using VacValves and VacConnectors.

1. QIAvac 24 Plus vacuum manifold
2. Luer slot of the QIAvac 24 Plus
3. VacValve*
4. VacConnector*
5. QIAamp Mini column
6. Luer slot closed with luer plug

* Must be purchased separately.



Figure 4. Assembly of QIAamp Mini columns with extension tubes (for buccal swabs or large volumes).

1. VacValve*
2. VacConnector*
3. QIAamp Mini column
4. Extension tube*

* Must be purchased separately.

Processing QIAamp Mini columns on the QIAcube

Sample preparation using the QIAcube follows the same steps as the manual procedure (i.e., lyse, bind, wash, and elute). For more information about the automated procedure, see the relevant protocol sheet available at www.qiagen.com/MyQIAcube.

Copurification of RNA

QIAamp Mini spin columns copurify DNA and RNA when both are present in the sample (see Table 3). RNA may inhibit some downstream enzymatic reactions but will not inhibit PCR. If RNA-free genomic DNA is required, 4 µl of an RNase A stock solution (100 mg/ml) should be added to the sample prior to the addition of Buffer AL. RNase A is not supplied with the kits and should be purchased separately. Ensure that the RNase A used is completely free of DNase activity.

Table 3. Yields of nucleic acids purified from various sources with QIAamp Kits

Sample	Nucleic acid yield (µg) without RNase A treatment	DNA yield (µg) with RNase A treatment
Blood (200 µl)	4–12	4–12
Buffy coat (200 µl)	25–50	25–50
Cultured cells (5×10^6)	20–30	15–20
Liver (25 mg)	60–115	10–30
Brain (25 mg)	35–60	15–30
Lung (25 mg)	8–20	5–10
Heart (25 mg)	25–45	5–10
Kidney (25 mg)	40–85	15–30
Spleen (10 mg)	25–45	5–30

DNA was purified with QIAamp Kits following standard protocols.

Elution mode for maximum yield or concentration

The yield of genomic DNA depends on the sample type and the number of cells in the sample. Typically, a 200 µl sample of whole blood from a healthy individual will yield 3–12 µg of DNA. (If higher yields are required, use QIAamp DNA Blood Midi or QIAamp DNA Blood Maxi Kits with up to 2 ml or up to 10 ml blood, respectively.) For most whole blood samples, a single elution with 200 µl elution buffer is sufficient. Samples with elevated white blood cell (WBC) counts, ranging from 1×10^7 to 1.5×10^7 cells/ml will yield between 13 and 20 µg of DNA. If such a sample is loaded onto the column, approximately 80% of the DNA will elute in the first 200 µl, and up to 20% more in the next 200 µl. In samples with WBC counts exceeding 1.5×10^7 cells/ml, up to 60% of the DNA will elute in the first 200 µl and up to 70% of the remaining material in each subsequent 200 µl (see Table 4). Elution into a fresh tube is recommended to prevent dilution of the first eluate. More than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will contact the eluate, leading to possible aerosol formation of samples during centrifugation. Eluting in 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency. In all cases, a single elution with 200 µl of elution buffer will provide sufficient DNA to perform multiple amplification reactions.

For some downstream applications, concentrated DNA may be required. Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly but slightly reduces overall DNA yield (see Table 5). For samples containing <3 µg of DNA, eluting the DNA in 100 µl is recommended. For samples containing less than 1 µg of DNA, only one elution in 50 µl Buffer AE or water is recommended.

Table 4. Total nucleic acid yields with QIAamp Kits using successive elutions

Sample	Amount	Yield (µg)			
		Elution 1	Elution 2	Elution 3	Total
Whole blood	200 µl	3–8	1–2	0–2	4–12
Lymphocytes	5 x 10^6 cells/200 µl	13–18	5–8	3–5	20–30
Buffy coat	200 µl	15–25	8–15	5–10	28–50
Liver*	25 mg	25–45	25–45	10–25	60–115
Brain*	25 mg	20–30	10–20	5–10	35–60
Lung*	25 mg	5–10	2–6	1–4	8–20
Heart*	25 mg	15–25	8–15	2–5	25–45
Kidney*	25 mg	25–40	20–30	5–15	50–85
Spleen*	10 mg	15–25	8–15	2–5	25–45

DNA was purified with QIAamp Kits following standard protocols. Successive elutions were each performed with 200 µl Buffer AE.

* Results refer to the QIAamp DNA Mini Kit only.

Table 5. Effect of elution volume on yield and concentration

Elution volume	Yield (µg)	Yield (%)	DNA concentration (ng/µl)
200	6.80	100	34.0
150	6.51	95	43.4
100	6.25	92	62.5
50	5.84	86	116.8

DNA was purified with QIAamp Kits following standard protocols. Average values obtained from 20 preparations are shown.

Protocol: DNA Purification from Blood or Body Fluids (Spin Protocol)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from whole blood, plasma, serum, buffy coat, lymphocytes, and body fluids using a microcentrifuge. For total DNA purification using a vacuum manifold, see "Protocol: DNA Purification from Blood or Body Fluids (Vacuum Protocol)" on page 29.

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Use carrier DNA if the sample contains <10,000 genome equivalents (see page 17).
- 200 µl of whole blood yields 3–12 µg of DNA. Preparation of buffy coat (see page 18) is recommended if a higher yield is required.

Things to do before starting

- Equilibrate samples to room temperature (15–25°C).
- Heat a water bath or heating block to 56°C for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.
- Ensure that Buffer AW1, Buffer AW2, and QIAGEN Protease have been prepared according to the instructions on page 16.
- If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C.

Procedure

1. **Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.**
2. **Add 200 µl sample to the microcentrifuge tube. Use up to 200 µl whole blood, plasma, serum, buffy coat, or body fluids, or up to 5 x 10^6 lymphocytes in 200 µl PBS.**

   If the sample volume is less than 200 µl, add the appropriate volume of PBS.

   QIAamp Mini spin columns copurify RNA and DNA when both are present in the sample. RNA may inhibit some downstream enzymatic reactions, but not PCR. If RNA-free genomic DNA is required, 4 µl of an RNase A stock solution (100 mg/ml) should be added to the sample before addition of Buffer AL.

   Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.

3. **Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.**

   To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

   If the sample volume is larger than 200 µl, increase the amount of QIAGEN Protease (or proteinase K) and Buffer AL proportionally; for example, a 400 µl sample will require 40 µl QIAGEN Protease (or proteinase K) and 400 µl Buffer AL. If sample volumes larger than 400 µl are required, use of QIAamp DNA Blood Midi or Maxi Kits is recommended; these can process up to 2 ml or up to 10 ml of sample, respectively.

   Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. **Incubate at 56°C for 10 min.**

   DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.

5. **Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.**

6. **Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.**

   If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally; for example, a 400 µl sample will require 400 µl of ethanol.

7. **Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.***

   Close each spin column to avoid aerosol formation during centrifugation.

   Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.

   Note: When preparing DNA from buffy coat or lymphocytes, centrifugation at full speed is recommended to avoid clogging.

8. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.***

   It is not necessary to increase the volume of Buffer AW1 if the original sample volume is larger than 200 µl.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

9. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.**
10. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

    This step helps to eliminate the chance of possible Buffer AW2 carryover.
11. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.**

    Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

    A second elution step with a further 200 µl Buffer AE will increase yields by up to 15%.

    Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

    Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 25). For samples containing less than 1 µg of DNA, elution in 50 µl Buffer AE or water is recommended. Eluting with 2 x 100 µl instead of 1 x 200 µl does not increase elution efficiency.

    For long-term storage of DNA, eluting in Buffer AE and storing at –30 to –15°C is recommended, since DNA stored in water is subject to acid hydrolysis.

    A 200 µl sample of whole human blood (approximately 5 x 10^6 leukocytes/ml) typically yields 6 µg of DNA in 200 µl water (30 ng/µl) with an A_{260}/A_{280} ratio of 1.7–1.9.

    For more information about elution and how to determine DNA yield, purity, and length, refer to pages 24–25 and Appendix A, page 50.

Trademarks:

QIAGEN®, QIAamp®, QIAcube®, FlexiGene®, Gentra®, Puregene®, InhibitEX®, MinElute®, TissueRuptor® (QIAGEN Group); Amicon®, Centricon®, UltraFree® (Merck KGaA); DACRON® (INVISTA North America S.A.R.L. Corporation); Puritan® (Puritan Medical Products Company); Triton® (Union Carbide Corp.); Tween® (ICI Americas Inc.).

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Sample & Assay Technologies

APPENDIX C. Human IgG Subclass Liquid Reagent Kits Package Insert

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HUMAN IgG SUBCLASS LIQUID REAGENT KITS (for use on the Siemens BN™II*)

For *in-vitro* diagnostic use

Product Code: LK001.TB, NK006.TB, NK007.TB, LK008.TB, LK009.TB.

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*BN™ is a trademark of Siemens Healthcare Diagnostics Inc.

FDA (USA) Information:
Analyte Name: Immunoglobulins IgG Subclass
Complexity Cat.: Moderate

CE

1 INTENDED USE

This kit is intended for quantifying human IgG subclasses 1, 2, 3 and 4 immunoglobulins in serum on the BNII. Measurement of these immunoglobulins aids in the diagnosis of abnormal protein metabolism and the body"s lack of ability to resist infectious agents.

2 SUMMARY AND EXPLANATION

In normal adults, IgG constitutes approximately 75% of the total serum immunoglobulin. Within the IgG class, the usual order of concentration of the 4 subclasses is IgG1>IgG2>IgG3>IgG4, but the actual concentration of each may vary markedly between individuals.
The four IgG subclasses show considerable differences in their properties, including ability to fix complement, to bind to macrophages and to pass through the placenta. Abnormal levels of one or more subclass may be associated with certain conditions, including anaphylaxis, autoimmune and gut diseases as well as hypo- and hyper-gammaglobulinaemia (ref 1). In particular, reduced production of IgG2 in children may be associated with recurrent infections (ref 2). The subject has been reviewed (refs 3, 4).
Siemens" BNII permits fully automatic, rapid quantitative measurement of light scattering reactions for protein determination.

3 PRINCIPLE OF THE ASSAY

Nephelometry involves the measurement of light scattered by particles in solution. This can be applied to the measurement of soluble antigen since, in the presence of the appropriate antibody, insoluble immune complexes are formed, which are light-scattering. When the antibody is in excess, the light-scatter is directly proportional to the concentration of antigen. A calibration curve is initially prepared using a series of diluted standards of known antigen concentration. Samples of unknown antigen concentration can then be assayed and their concentrations determined using the calibration curve.

Latex-enhanced Antibodies Some antigen-antibody reactions do not form sufficiently large immune complexes to be detected nephelometrically. If the antibody is coated onto latex particles of a suitable size, the light scattering ability of the immune complexes formed with antigen is enhanced sufficiently to enable nephelometric detection.

4 REAGENTS

4.1 Human IgG1/ IgG2 antisera. These are monospecific for the relevant subclass and are supplied in stabilised liquid form. They contain 0.099% sodium azide, 0.1% E-amino-n-caproic acid (EACA), 1mM ethylenediamine-tetraacetic acid (EDTA), 0.5% BSA and 0.01% benzamidine as preservatives.
4.2 Human IgG3 / IgG4 reagent These are monospecific for the relevant subclass and are supplied in stabilised liquid form. They contain 0.033% sodium azide, 0.05% ProClin™*, 0.1% EACA and 0.01% benzamidine as preservatives.
4.3 Calibrators and controls These consist of pooled human serum and are supplied in stabilised liquid form. They contain 0.099% sodium azide, 0.1% EACA and 0.01% benzamidine as preservatives. The concentration of IgG subclasses given on the vial label has been obtained by comparison with the DA470k international reference material (refs 5, 6).
4.4 Supplementary reagent Contains 0.099% sodium azide as a preservative.

ProClin™ is a trademark of Rohm and Haas Corp., Philadelphia, PA.

5 CAUTION

All donors of human serum supplied in this kit have been serum tested and found negative for hepatitis B surface antigen (HBsAg) and antibodies to human immunodeficiency virus (HIV1 and HIV2) and hepatitis C virus. The assays used were either approved by the FDA (USA) or cleared for *in vitro* diagnostic use in the EU (Directive 98/79/EC, Annex II); however, these tests cannot guarantee the absence of infective agents. Proper handling and disposal methods should be established as for all potentially infective material, including (but not limited to) users wearing suitable protective equipment and clothing at all times. Only personnel fully trained in such methods should be permitted to perform these procedures.

WARNING: This product contains sodium azide and ProClin 300 and must be handled with caution; suitable gloves and other protective clothing should be worn at all times when handling this product. Do not ingest or allow contact with the skin (particularly broken skin or open wounds) or mucous membranes. If contact does occur wash with a large volume of water and seek urgent medical advice. Explosive metal azides may be formed on prolonged contact of sodium azide with lead and copper plumbing; on disposal of reagent, flush with a large volume of water to prevent azide build up.

This product should only be used by suitably trained personnel for the purposes stated in the Intended Use. Strict adherence to these instructions is essential at all times. Results are likely to be invalid if parameters other than those stated in these instructions are used.

Reagents from different batch numbers of kits are **NOT** interchangeable. If large numbers of tests are performed care should be taken to ensure that all the reagents are from the same batch.

6 STORAGE AND STABILITY

The unopened kits should be stored at 2-8ºC, and can be used until the expiry date given on the kit box label. Unopened reagent, calibrators and controls should be stored at 2-8ºC. Once opened they are stable for four weeks at 2-8ºC, providing precautions to prevent evaporation and contamination are taken. Open vials should not be left on the analyser when they are not in use.
Over time unopened vials of antisera can develop a precipitate or turbidity which is not caused by microbial contamination and does not affect the activity of the antisera. In such cases the antiserum should be clarified by filtration through a disposable filter, pore size 0.2µm or by centrifugation (10mins at approximately 15,000 x g).

7 SPECIMEN COLLECTION AND PREPARATION

Use fresh or deep frozen serum samples. Blood samples should be collected by venepuncture, allowed to clot naturally and the serum separated as soon as possible to prevent haemolysis.
The serum may be stored at 2-8°C for up to 8 days prior to assay, or for prolonged storage kept undiluted at -20°C or below. Repeated freezing and thawing should be avoided.
Microbially contaminated, haemolysed and lipaemic serum and samples containing particulate matter should not be used.

8 METHODOLOGY

8.1 Materials provided

Code: LK001.TB KIT

1. 2 x 1mL *BS IgG Sub Std* (Human IgG Subclass Standard)
2. 2 x 1mL *BS IgG Sub Low Control* (Human IgG Subclass Low Control)
3. 2 x 1mL *BS IgG Sub Control* (Human IgG Subclass High Control)
4. 1 x 1.6mL *Human IgG1 BS Antiserum* (Antiserum to IgG1)
5. 1 x 1.6mL *Human IgG2 BS Antiserum* (Antiserum to IgG2)
6. 1 x 2.4mL *IgG3 BS Latex* (Latex to IgG3)
7. 1 x 2.4mL *IgG4 BS Latex* (Latex to IgG4)
8. 1 x 3mL *BS Supplementary Reagent L* (for IgG3 and IgG4 assay)

Code: NK006.TB, NK007.TB, LK008.TB and LK009.TB kits.

1. 2 x 1mL *BS IgG Sub Std* (Human IgG Subclass Standard)
2. 2 x 1mL *BS IgG Sub Low Control* (Human IgG Subclass Low Control)
3. 2 x 1mL *BS IgG Sub Control* (Human IgG Subclass High Control)
4. 4 x 1.6mL antiserum or 4 x 2.4mL Latex reagent to the appropriate subclass.
5. 2 x 3.0mL *BS Supplementary Reagent L* in LK008.TB & LK009.TB Kits only.

8.2 Materials required but not provided

8.2.1 Equipment for collection and preparation of test samples.
8.2.2 A fully operational and equipped Analyser.

8.3 Test procedure

8.3.1 Prepare the analyser as per the user"s manual.
8.3.2 The parameters for the assays are provided on the following pages.
8.3.3 Define the reagents to be used in **Definition-Reagent** as follows**:**

Human IgG1 antiserum:

1301	Identification
IgG1 BS	Name
G1BS	Abbreviation
Bottle Size: 5mL ❑	

Human IgG2 antiserum:

1302	Identification
IgG2 BS	Name
G2BS	Abbreviation
Bottle Size: 5mL ❑	

Human IgG3 latex reagent:

1303	Identification
IgG3 BS Latex	Name
G3L	Abbreviation
Bottle Size: 5mL ❑	

Human IgG4 latex reagent:

1304	Identification
IgG4 BS Latex	Name
G4L	Abbreviation
Bottle Size: 5mL ❑	

Supplementary Reagent (for the latex assays):

1305	Identification
BS L SRP	Name
SRL	Abbreviation
Bottle Size: 5mL ❑	

ID WARNING: In situations where the user is unable to use the recommended component identification another may be chosen in its place. The component barcode should be covered with a black marker pen (or peeled from the vial) to avoid detection by the barcode reader.

8.3.4 The **IgG1 assay protocol** should be set up in **Definition-Assay** as follows:

Assay name (with assay selection)

G1BS	IgG1 BS		
Abbreviation	Assay Name		
96	96	1.0	0
Identification for host	Position in list	Version	Behring assay code

Derived from assay: 0

Sample type: Serum ❑

☒ Allow multiple lots

☒ Mini-batch

☐ Do not interrupt preparation

Measurement

Method: Fixed-time ❑ — 0.000 mg/L — Lower measuring range limit

☐ Prereaction

0.0	0.0	1.0	0.0
Start prereaction	Stop prereaction [sec]	Factor	Constant to be added
7.5	360.0	4	
Initial measurement [sec]	Last measurement [sec]	Number of averaging points	

VLinIntegral

0.0	3	0.0	0.0
Start evaluation [sec]	Polynomial regress.	Upper preeval. rate	Min. search window
0.0	0.0	0.0	0.0
Stop evaluation [sec]	Preeval. Window	Lower preeval. rate	Min. regression time
0	0.0		☐ Variable start of eval.
Integral area	Upper max. eval. offset		

Remeasurement

Remeasurement in: ☒ higher dilution ☒ lower dilution

0.000	0.000	1
Lower remeasurement limit	Upper remeasurement limit	Max. no. of remeas.

If result within limits remeasure in: Same dilution ❑

Take: Lowest result ❑

Result

Unit: mg/L ❑

0	1.000000	1.000000	1.000000
No. of digits after decimal point	Conversion factor from mg/L to IU/L	Conversion factor from mg/L to U/L	Conversion factor from mg/L to mol

Sample dilution

Sample Dilution: 1:100 ❑

Minimum Dilution: 1:5 ❑

Turbidity check

800 Bit	15 %	1800 Bit
Turbidity threshold	Turbidity factor	Upper limit of turbidity check

If you do not wish the turbidity check to be performed, set all values to 0.

Washing

10000	0
Cuvette contamination	Dilution probe contamination
100000	0
Cuvette washing intensity	Dilution probe rinsing intensity

Technical parameters

Transfer step 1.

No. of Transfer Steps				
1 ☒	2 ☒	3 ☐	4 ☐	5 ☐

Transfer arm				
Right ❑	1	1000000	10000000	
	Washing program no.	Probe cleaning intensity	Probe rinsing intensity	
System liquid	Buffer ❑	70	4	
		Volume [µL]	Dispensing program no.	
Sample ❑	Reagent…	20	2	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
Dispensing procedure		0.00	7	
		Mixing time [sec]	Dispensing program no.	
○ Preincubation	0 +/-	0		
	Preincub. time [min]	Deviation time [sec]		

● Start measurement after this transfer step

Transfer step 2.

No. of Transfer Steps				
1 ☒	2 ☒	3 ☐	4 ☐	5 ☐

Transfer arm				
Left ❑	1	0	0	
	Washing program no.	Probe cleaning intensity	Probe rinsing intensity	
System liquid	Buffer ❑	70	1	
		Volume [µL]	Dispensing program no.	
Reagent ❑	Reagent… G1BS	40	2	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
Dispensing procedure		0.600	1	
		Mixing time [sec]	Dispensing program no.	
○ Preincubation	0 +/-	0		
	Preincub. time [min]	Deviation time [sec]		

☒ Start measurement after this transfer step

8.3.5 The **IgG2 assay protocol** should be set up in **Definition-Assay** as follows:

Assay name (with assay selection)

G2BS	IgG2 BS		
Abbreviation	Assay Name		
97	97	1.0	0
Identification for host	Position in list	Version	Behring assay code
Derived from assay		0	
Sample type	Serum ❑		

☒ Allow multiple lots

☒ Mini-batch

☐ Do not interrupt preparation

Measurement

Method: Fixed-time ❑ | 0.000 mg/L

Lower measuring range limit

☐ Prereaction

0.0	0.0	1.0	0.0
Start prereaction	Stop prereaction [sec]	Factor	Constant to be added
7.5	360.0	4	
Initial measurement [sec]	Last measurement [sec]	Number of averaging points	

VLinIntegral

0.0	3	0.0	0.0
Start evaluation [sec]	Polynomial regress.	Upper preeval. rate	Min. search window
0.0	0.0	0.0	0.0
Stop evaluation [sec]	Preeval. Window	Lower preeval. rate	Min. regression time
0	0.0	☐ Variable start of eval.	
Integral area	Upper max. eval. offset		

Remeasurement

Remeasurement in ☒ higher dilution ☒ lower dilution

0.000	0.000	
Lower remeasurement limit	Upper remeasurement limit	Max. no. of remeas.

If result within limits remeasure in: Same dilution ❑

Take: Lowest result ❑

Result

Unit: mg/L ❑

0	1.000000	1.000000	1.000000
No. of digits after decimal point	Conversion factor from mg/L to IU/L	Conversion factor from mg/L to U/L	Conversion factor from mg/L to mol

Sample dilution

Sample Dilution: 1:20 ❑

Minimum Dilution: 1:5 ❑

Turbidity check

800 Bit	15 %	1800 Bit
Turbidity threshold	Turbidity factor	Upper limit of turbidity check

If you do not wish the turbidity check to be performed, set all values to 0.

Washing

10000 Cuvette contamination	0 Dilution probe contamination
100000 Cuvette washing intensity	0 Dilution probe rinsing intensity

Technical parameters

Transfer step 1.

No. of Transfer Steps				
1 ☒	2 ☒	3 ☐	4 ☐	5 ☐

Transfer arm				
Right ❑	1	1000000	10000000	
	Washing program no.	Probe cleaning intensity	Probe rinsing intensity	
System liquid	Buffer ❑	70	4	
		Volume [µL]	Dispensing program no.	
Sample ❑	Reagent…	15	2	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
…	Reagent…	0	1	
		Volume [µL]	Dispensing program no.	
Dispensing procedure		0.00	7	
		Mixing time [sec]	Dispensing program no.	
○ Preincubation	0 +/-	0		
	Preincub. time [min]	Deviation time [sec]		

● Start measurement after this transfer step

Transfer step 2.

No. of Transfer Steps

1	2	3	4	5
☒○	☒☝	□○	□○	□○

Transfer arm	**Left** ❑	**1**		**0**	**0**
		Washing program no.		Probe cleaning intensity	Probe rinsing intensity
System liquid	**Buffer** ❑			**70**	**1**
				Volume [µL]	Dispensing program no.
Reagent ❑	Reagent…	**G2BS**		**40**	**2**
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
Dispensing procedure				**0.600**	**1**
				Mixing time [sec]	Dispensing program no.
○ **Preincubation**	0	**+/ -**		0	
	Preincub. time [min]			Deviation time [sec]	

☒ **Start measurement after this transfer step**

8.3.6 The **IgG3 assay protocol** should be set up in **Definition-Assay** as follows:

Assay name (with assay selection)

G3L	**IgG3 BS Latex**			
Abbreviation	Assay Name			
98	98	1.0	0	
Identification for host	Position in list	Version	Behring assay code	
Derived from assay		**0**		

Sample type **Serum** ❑

☒ Allow multiple lots

☒ Mini-batch

☐ Do not interrupt preparation

Measurement

Method **Fixed-time** ❑ **0.000** mg/L

Lower measuring range limit

□ Prereaction

0.0	**0.0**	**1.0**	**0.0**
Start prereaction	Stop prereaction [sec]	Factor	Constant to be added
7.5	**360.0**	**4**	
Initial measurement [sec]	Last measurement [sec]	Number of averaging points	

VLinIntegral

0.0	**3**	**0.0**	**0.0**
Start evaluation [sec]	Polynomial regress.	Upper preeval. rate	Min. search window
0.0	**0.0**	**0.0**	**0.0**
Stop evaluation [sec]	Preeval. Window	Lower preeval. rate	Min. regression time
0	**0.0**	☐ **Variable start of eval.**	
Integral area	Upper max. eval. offset		

Remeasurement

Remeasurement in ☒ higher dilution ☒ lower dilution

0.000	**0.000**	**1**
Lower remeasurement limit	Upper remeasurement limit	Max. no. of remeas.

If result within limits remeasure in **Same dilution** ❑

Take **Lowest result** ❑

Result

Unit **mg/L** ❑

1	**1.000000**	**1.000000**	**1.000000**
No. of digits after decimal point	Conversion factor from mg/L to IU/L	Conversion factor from mg/L to U/L	Conversion factor from mg/L to mol

Sample dilution

Sample Dilution **1:100** ❑

Minimum Dilution **1:5** ❑

Turbidity check

0 Bit	**0** %	**0** Bit
Turbidity threshold	Turbidity factor	Upper limit of turbidity check

If you do not wish the turbidity check to be performed, set all values to 0.

Washing

1000 Cuvette contamination	**0** Dilution probe contamination
8400 Cuvette washing intensity	**0** Dilution probe rinsing intensity

Technical parameters

Transfer step 1.

No. of Transfer Steps

1	2	3	4	5
☒☝	☒○	□○	□○	□○

Transfer arm	**Right** ❑	**1**		**10000**	**100000**
		Washing program no.		Probe cleaning intensity	Probe rinsing intensity
System liquid	**Diluent** ❑			**60**	**4**
				Volume [µL]	Dispensing program no.
Reagent ❑	**Reagent…**	**SRL**		**10**	**2**
				Volume [µL]	Dispensing program no.
Sample ❑	Reagent…			**20**	**2**
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
Dispensing procedure				**0.000**	**7**
				Mixing time [sec]	Dispensing program no.
○ **Preincubation**	0	**+/ -**		0	
	Preincub. time [min]			Deviation time [sec]	

○ **Start measurement after this transfer step**

Transfer step 2.

No. of Transfer Steps

1	2	3	4	5
☒○	☒☝	○①	○①	○①

Transfer arm	**Left** ❑	**1**		**0**	**0**
		Washing program no.		Probe cleaning intensity	Probe rinsing intensity
System liquid	**Diluent** ❑			**60**	**1**
				Volume [µL]	Dispensing program no.
Reagent ❑	**Reagent…**	**G3L**		**50**	**2**
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
…	Reagent…			0	1
				Volume [µL]	Dispensing program no.
Dispensing procedure				**0.600**	**1**
				Mixing time [sec]	Dispensing program no.
○ **Preincubation**	0	**+/ -**		0	
	Preincub. time [min]			Deviation time [sec]	

☒ **Start measurement after this transfer step**

8.3.7 The **IgG4 assay protocol** should be set up in **Definition-Assay** as follows:
Assay name (with assay selection)

G4L	IgG4 BS Latex
Abbreviation	Assay Name

99	99	1.0	0
Identification for host	Position in list	Version	Behring assay code

Derived from assay: **0**

Sample type: **Serum** ❑

☒ Allow multiple lots

☒ Mini-batch

☐ Do not interrupt preparation

Measurement

Method: **Fixed-time** ❑ — **0.000** mg/L (Lower measuring range limit)

☐ Prereaction

0.0	0.0	1.0	0.0
Start prereaction	Stop prereaction [sec]	Factor	Constant to be added

7.5	360.0	4
Initial measurement [sec]	Last measurement [sec]	Number of averaging points

VlinIntegral

0.0	3	0.0	0.0
Start evaluation [sec]	Polynomial regress.	Upper preeval. rate	Min. search window

0.0	0.0	0.0	0.0
Stop evaluation [sec]	Preeval. Window	Lower preeval. rate	Min. regression time

0	0.0	☐ **Variable start of eval.**
Integral area	Upper max. eval. offset	

Remeasurement

Remeasurement in ☒ higher dilution ☒ lower dilution

0.000	0.000	1
Lower remeasurement limit	Upper remeasurement limit	Max. no. of remeas.

Same dilution ❑

If result within limits remeasure in

Take **Lowest result** ❑

Result

Unit: **mg/L** ❑

1	1.000000	1.000000	1.000000
No. of digits after decimal point	Conversion factor from mg/L to IU/L	Conversion factor from mg/L to U/L	Conversion factor from mg/L to mol

Sample dilution

Sample Dilution: **1:100** ❑

Minimum Dilution: **1:5** ❑

Turbidity check

0 Bit	0 %	0 Bit
Turbidity threshold	Turbidity factor	Upper limit of turbidity check

If you do not wish the turbidity check to be performed, set all values to 0.

Washing

1000 Cuvette contamination	**0** Dilution probe contamination
8400 Cuvette washing intensity	**0** Dilution probe rinsing intensity

Technical parameters

Transfer step 1.

No. of Transfer Steps				
1 ☒	2 ☒	3 ☐	4 ☐	5 ☐

Transfer arm: **Right** ❑ | **1** Washing program no. | **10000** Probe cleaning intensity | **100000** Probe rinsing intensity

System liquid **Diluent** ❑ — **60** Volume [µL] — **4** Dispensing program no.

Reagent ❑	**Reagent…**	**SRL**	**10** Volume [µL]	**2** Dispensing program no.
Sample ❑	**Reagent…**		**20** Volume [µL]	**2** Dispensing program no.
…	Reagent…		0 Volume [µL]	1 Dispensing program no.
…	Reagent…		0 Volume [µL]	1 Dispensing program no.

Dispensing procedure

0.000 Mixing time [sec] — **7** Dispensing program no.

○ **Preincubation** 0 Preincub. time [min] **+/ -** 0 Deviation time [sec]

○ **Start measurement after this transfer step**

Transfer step 2.

No. of Transfer Steps				
1 ☒	2 ☒	3 ☐	4 ☐	5 ☐

Transfer arm: **Left** ❑ | **1** Washing program no. | **0** Probe cleaning intensity | **0** Probe rinsing intensity

System liquid **Diluent** ❑ — **60** Volume [µL] — **1** Dispensing program no.

Reagent ❑	**Reagent…**	**G4L**	**50** Volume [µL]	**2** Dispensing program no.
…	Reagent…		0 Volume [µL]	1 Dispensing program no.
…	Reagent…		0 Volume [µL]	1 Dispensing program no.
…	Reagent…		0 Volume [µL]	1 Dispensing program no.

Dispensing procedure

0.600 Mixing time [sec] — **1** Dispensing program no.

○ **Preincubation** 0 Preincub. time [min] **+/ -** 0 Deviation time [sec]

☒ **Start measurement after this transfer step**

8.3.8 A new standard should be set up with the name BS IgG Std

Definition – Standard

Double-click on the standard name and enter the details below.

BS IgG Std Name

1306 Identification | **240** Expiration (min)

Bottle size **2mL**

Double click on the field with an X at the intersection of the calibrator name and **IgG1**, and enter the details below:

Points on curve **6** ❑

Start dilution **1:10** ❑

☐ with zero calibraton — identification

☐ Extrapolation — **0** bits value

10 % permitted deviation

Double click on the field with an X at the intersection of the calibrator name and **IgG2**, and enter the details below:

Points on curve 6

Start dilution 1:5

☐ with zero calibrator — identification

☐ Extrapolation — 0 bits — value

10 % — Permitted deviation

Double click on the field with an X at the intersection of the calibrator name and **IgG3**, and enter the details below:

Points on curve 5

Start dilution 1:80

☐ with zero calibrator — identification

☐ Extrapolation — 0 bits — value

10 % — Permitted deviation

Double click on the field with an X at the intersection of the calibrator name and **IgG4**, and enter the details below:

Points on curve 5

Start dilution 1:80

☐ with zero calibrator — identification

☐ Extrapolation — 0 bits — value

10 % — Permitted deviation

8.3.9 New controls should be set up as follows:

Definition – Control

Double click on the control name:

G Sub Con		G Sub Low	
Name			
0776	240	0975	240
Identification	Expiration (min)	Identification	Expiration (min)
Bottle Size 2mL		**Bottle Size** 2mL	

Click onto the relevant assay. If the user cannot see the assay concerned scroll across the screen until it appears. Click on dilution and select appropriate dilution. N.B. controls should be run at the normal sample dilution detailed in the assay protocols."

8.3.10 TO ENTER THE CALIBRATOR VALUE

i) Click onto **Calibrator** and **Standard lots**. If the user cannot see the assay concerned, scroll across the screen until it appears.
Double click onto the calibrator name in the left hand column. Enter the 5th and 6th numbers of the barcode as the lot number for the calibrator of the assay.

ii) Click onto the intersection of the assay and the standard. (There may already be a previous value here or an "X".) Then click onto "?", the question mark icon. This will allow the user to enter the IgG subclass values stated on the calibrators. Click on the save button.

8.3.11 TO ENTER THE BARCODED REAGENT BATCH NUMBERS

Click onto the **Calibration** and **Reagent** and enter the 5th and 6th numbers of the barcode for each reagent and click on to the Save button. It is important that these are entered since the analyser will check the batch number entered.

8.3.12 RUNNING AN ASSAY

i) Request the calibration by clicking onto **Calibration** and **Reference curve** and selecting the assays from the list. After each selection click onto the „Measure" button. NB: Special attention should be given to ensure the reagent batch numbers selected are correct.

ii) The calibrator, controls antisera and latex are supplied ready for use. **Gently** mix by inversion just prior to use.

iii) Place the reagents into the racks. The reagents should be placed into lanes 1 to 5, with the Supplementary Reagents in lanes 3, 4 or 5. Do not load reagents with bubbles or foam at the surface as these may interfere with accurate sample/reagent aspiration.

iv) Place the calibrators on a rack in lanes 6-15.

Specificity	Approx. Measuring Range		Approx. Sensitivity	
	mg/L	Sample Dilution	mg/L	Sample Dilution
IgG1	2625-84000	1/100	131	1/5
IgG2	613-19600	1/20	153	1/5
IgG3	55 – 875	1/100	2.7	1/5
IgG4	38 - 613	1/100	1.9	1/5

9 INTERPRETATION OF DATA

Quality Control: The IgG subclass concentration assigned to the controls are given on the bottle labels. Results obtained during the run should only be accepted if they are within ±15% of the value(s) stated.

10 LIMITATIONS

10.1 Nephelometric IgG Subclass assays have been shown to correlate well versus Binding Site Single Dilution RID assays with a wide range of samples.

10.2 These kits are not suitable for the measurement of samples containing rheumatoid factor, paraproteins, other circulating immune complexes (CIC`s) or for lipaemic or haemolysed samples due to the unpredictable degree of non-specific scatter these sample types may generate. Unexpected results should be confirmed using an alternative assay method.

10.3 Customers are strongly recommended to run both controls with every batch of samples being assayed, Should a control value be out of range against a stored curve, it is recommended that the assay be recalibrated. Where control values fall outside ±15% limit against new calibration curves check the instument and parameters entered before repeating the assay. If problems persist, refer to the supplier.

10.4 The results obtained from measuring IgG subclasses should not be used in assessing atopy in allergic patients.

10.5 Diagnosis cannot be made and treatment must not be initiated on the basis of IgG subclass measurements alone. Clinical history and other laboratory findings must also be taken into account.

11 EXPECTED VALUES

The ranges provided below have been obtained from a limited number of British samples and are intended for guidance purposes only. Wherever possible it is strongly recommended that local ranges are generated.

11.1 Adult Normal Ranges

These ranges were obtained by measuring the subclass content of sera provided by the Birmingham Blood Transfusion Service taken from healthy adult donors.

IgG Subclass	Number (n)	Mean (mg/L)	Median (mg/L)	95 Percentile Range (mg/L)
IgG1	30	6330	6085	3824 – 9286
IgG2	30	4528	4541	2418 – 7003
IgG3	30	790.7	706.4	218.2 –1760.6
IgG4	30	280.0	215.3	39.2 – 864.0

11.2 Paediatric Ranges

These ranges were obtained by measuring the Total IgG and subclass content of paediatric serum samples from a Birmingham Hospital using Binding Site Radial Immunodiffusion products. All concentrations are in mg/L.

0-2 years	N	Mean	95%ile Range
IgG1	39	5248	1940 – 8420
IgG2	39	838	225– 3000
IgG3	39	445	186 – 853
IgG4	39	129	5 – 784
Total IgG	39	6640	3270– 12700
2-4 years			
IgG1	36	5495	3150 – 9450
IgG2	36	1146	360– 2250
IgG3	36	396	173 – 676
IgG4	36	171	10– 537
Total IgG	36	7770	4680– 12500
4-6 years			
IgG1	49	6149	3060 – 9450
IgG2	49	1496	605– 3450
IgG3	49	531	99 – 1221
IgG4	49	329	18 – 1125
Total IgG	49	9280	5320 - 13400
6-8 years			
IgG1	43	5758	2880 – 9180
IgG2	43	1754	440– 3750
IgG3	43	437	155 – 853
IgG4	43	331	4 – 992
Total IgG	43	8860	4540– 13600
8-10 years			
IgG1	32	6345	4320– 10200
IgG2	32	2126	720– 4300
IgG3	32	521	127 – 853
IgG4	32	349	19– 932
Total IgG	32	9700	5680 - 13600
10-12 years			
IgG1	46	6421	4230– 10600
IgG2	46	1980	760– 3550
IgG3	46	620	173– 1730
IgG4	46	323	16 – 1150
Total IgG	46	10200	5680 - 14900
12-14 years			
IgG1	54	6581	3420– 11500
IgG2	54	2486	1000 – 4550
IgG3	54	583	283– 1250
IgG4	54	409	37 – 1360
Total IgG	54	10500	6640– 14900
14-18 years			
IgG1	48	6057	3150 – 8550
IgG2	48	2614	640– 4950
IgG3	48	708	230 –1960
IgG4	48	521	110– 1570
Total IgG	48	10100	5500– 14400

12 PERFORMANCE CHARACTERISTICS

12.1 Within-run precision

Three serum preparations, containing different levels of IgG subclasses, were assayed 10 times. All concentrations are in mg/L.

	IgG1		IgG2		IgG3		IgG4	
	Mean	CV %	Mean	CV %	Mean	CV %	Mean	CV %
Serum 1	40529	1.56	8256	2.59	668.6	2.74	534.1	3.05
Serum 2	12630	1.67	1736	2.16	517.8	2.76	396.1	2.95
Serum 3	3350	6.44	758.5	8.19	353.1	3.49	324.8	5.08

12.2 Between-run precision

Three serum preparations containing different levels of IgG subclasses were assayed on at least three separate occasions. All concentrations are in mg/L. (N) = number of runs.

	IgG1		IgG2		IgG3		IgG4	
	Mean	CV % (N)	Mean	CV % (N)	Mean	CV % (N)	Mean	CV % (N)
Serum 1	40765	4.38 (6)	7811	6.84 (6)	697.5	4.41 (3)	532.6	4.10 (3)
Serum 2	12402	2.39 (6)	1621	4.18 (6)	525.9	2.38 (4)	387.7	4.13 (3)
Serum 3	3043	6.95 (6)	780.1	2.43 (6)	353.1	3.00 (3)	319.6	4.38 (4)

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APPENDIX D. miRNeasy Serum/Plasma Advanced Kit Handbook for Purification of Total RNA, including miRNA, from Serum/Plasma Utilised in the miRNA Study

January 2020

miRNeasy Serum/ Plasma Advanced Kit Handbook

For purification of total RNA, including miRNA, from serum and plasma

Sample to Insight



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Kit Contents

miRNeasy Serum/Plasma Advanced Kit (50)	**(50)**
Catalog no.	**217204**
Number of preps	**50**
RNeasy® UCP MinElute® Spin Columns (each packaged with a 2 ml Collection Tube)	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
Buffer RPL*	20 ml
Buffer RPP	8 ml
Buffer RWT*†	15 ml
Buffer RPE‡	11 ml
RNase-Free Water	10 ml
Quick-Start Protocol	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 5 for safety information.

† Buffer RWT is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96%–100%) as indicated on the bottle to obtain a working solution.

‡ Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Storage

The miRNeasy Serum/Plasma Advanced Kit (cat. no. 217204) is shipped at ambient temperature. Store the RNeasy UCP MinElute spin columns immediately at 2–8°C. Store the remaining components dry at room temperature (15–25°C). All kit components are stable for at least 9 months under these conditions, if not otherwise stated on the label.

Intended Use

The miRNeasy Serum/Plasma Advanced Kit is intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

QIAcube® Connect is designed to perform fully automated purification of nucleic acids and proteins in molecular biology applications. The system is intended for use by professional users trained in molecular biological techniques and the operation of QIAcube Connect.

All due care and attention should be exercised in the handling of these products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view and print the SDS for each QIAGEN kit and kit component.

CAUTION	CAUTION: DO NOT add bleach or acidic solutions directly to waste containing Buffer RWT or Buffer RPL.



Buffer RWT and Buffer RPL contain guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing these solutions is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miRNeasy Serum/Plasma Advanced Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Interest in smaller RNA species, such as miRNA, has increased over the past years as researchers understand the regulatory role of small non-coding RNAs. The miRNeasy Serum/Plasma Advanced Kit is designed for purification of cell-free total RNA – primarily miRNA and other small RNA – from small volumes of serum and plasma.

When working with serum and plasma samples, we recommend the use of a synthetic spike-in control for normalization, such as the miRNeasy Serum/Plasma Spike-In Control, which must be ordered separately (cat. no. 219610).

The miRNeasy Serum/Plasma Advanced Kit offers a phenol-free protocol to isolate high yields of cell-free total RNA including miRNA from only 200 µl of serum or plasma.

Principle and workflow

The miRNeasy Serum/Plasma Advanced Kit combines guanidine-based lysis of samples, an inhibitor removal step and silica-membrane–based purification of total RNA. Buffer RPL, included in the kit, contains guanidine thiocyanate as well as detergents that are designed to facilitate lysis and denature protein complexes and RNases. Therefore, RNA in samples lysed in buffer RPL are stable and protected from degradation.

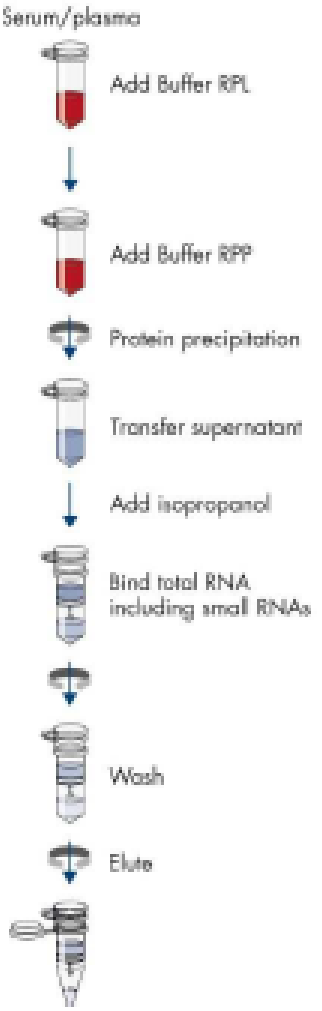
Buffer RPL is added to serum or plasma samples. After thoroughly mixing to ensure a complete lysis, Buffer RPP is added to precipitate inhibitors (mostly proteins that are highly concentrated in serum/plasma samples) by centrifugation.

The supernatant containing the RNA is transferred to a new microcentrifuge tube, and isopropanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The sample is then applied to the RNeasy UCP

MinElute spin column, where the total RNA binds to the membrane and all contaminants are efficiently washed away. High-quality RNA is eluted in a small volume of RNase-free water.

Serum and plasma contain primarily small RNAs, therefore, enrichment of miRNAs and other small RNAs in a separate fraction is usually not required.

miRNeasy Serum/Plasma Advanced Kit



Automated purification of miRNA on QIAcube Instruments

Purification of miRNA can be fully automated on QIAcube Connect or the classic QIAcube. The innovative QIAcube instruments use advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample prep into your laboratory workflow. Sample preparation using QIAcube instruments follows the same steps as the manual procedure (i.e., lyse, bind, wash and elute), enabling you to continue using the miRNeasy Serum/Plasma Advanced Kit for purification of high-quality miRNA.

QIAcube instruments are preinstalled with protocols for purification of plasmid DNA, genomic DNA, RNA, viral nucleic acids and proteins, plus DNA and RNA cleanup. The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at **www.qiagen.com/qiacubeprotocols**.



QIAcube Connect.

qPCR-based miRNA quantification using the miScript® PCR system

In general, qPCR is recommended to accurately quantify yields of miRNA. The miScript PCR System allows sensitive and specific quantification and profiling of miRNA expression using SYBR® Green-based real-time PCR. The robust miScript PCR System comprises the miScript II RT Kit, the miScript SYBR Green PCR Kit, miScript Assays and miScript miRNA PCR Arrays. It covers all the steps of miRNA quantification, from conversion of RNA into cDNA to real-time PCR detection of miRNAs and straightforward data analysis.

Individual assays for mature miRNAs, precursor miRNAs and other small noncoding RNAs can be ordered at the GeneGlobe® Web portal (**www.qiagen.com/GeneGlobe**). Alternatively, for high-throughput experiments, miScript miRNA PCR Arrays enable rapid profiling of the complete miRNome or pathway-focused panels of mature miRNAs for a variety of species. Find out more about the miScript PCR System at **www.qiagen.com/miRNA**.

Description of protocols

This handbook contains one protocol on page 15 for purification of cell-free total RNA, including miRNA, from serum or plasma using the miRNeasy Serum/Plasma Advanced Kit. The appendices contain additional protocols for collection, preparation and storage of samples and for use of a spike-in control in serum/plasma miRNA profiling using the miScript PCR System.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

The following supplies are also required:

- 100% Isopropanol
- 80% Ethanol*
- Sterile, RNase-free pipet tips
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge with rotor for 2 ml tubes for centrifugation at room temperature
- Disposable gloves
- The miRNeasy Serum/Plasma Spike-In Control must be purchased separately (see "Ordering Information", starting on page 48)
- Equipment and tubes for serum/plasma collection and separation (see Appendix B, page 26):
  - For serum: primary blood collection tube(s) without anticoagulants, such as EDTA or citrate
  - For plasma: primary blood collection tube(s) containing EDTA as anticoagulant
  - Conical tube(s)
  - Refrigerated centrifuge with a swinging bucket rotor and fixed-angle rotor

* Do not use denatured alcohol, which contains other substances, such as methanol and methylethylketone.

Important Notes

Volume of starting material

The preferred volume of starting material is set to 200 µl. This volume is usually sufficient to also detect low-abundance miRNA in serum/plasma samples. When using only 200 µl of starting material, only one loading step onto the RNeasy UCP MinElute column is needed. Using higher amounts of serum/plasma will lead to more handling steps, while only increasing the amount of recovered miRNA by 1–2 cycles, which is not much more than normal sample to sample variation. Furthermore, using more starting material than recommended increases the chance of co-isolating additional PCR inhibitors with the RNA, and this can affect the PCR efficiency and oppose the higher yield.

Yields of total RNA purified with the miRNeasy Serum/Plasma Advanced Kit vary strongly between different plasma samples. However, they are usually too low for quantification by OD measurement. We recommend using the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) and the corresponding Ce_miR-39_1 miScript Primer Assay (MS00019789) to monitor miRNA purification and amplification.

Optional: For reconstitution of Ce_miR-39_1 miScript Primer Assay

TE, pH 8.0 contains 10 mM Tris/HCl and 1 mM EDTA. To prepare 100 ml TE, pH 8.0, mix the following stock solutions:

- 1 ml of 1 M Tris/HCl, pH 8.0 (autoclaved)
- 0.2 ml of 0.5 M EDTA, pH 8.0 (autoclaved)
- 98.8 ml of distilled water

Alternatively, ready-made TE can be purchased from common chemical suppliers.

To reconstitute the Ce_miR-39_1 miScript Primer, briefly centrifuge the vial, add 550 µl TE, pH 8.0, and mix by vortexing the vial 4–6 times. We recommend freezing the reconstituted primers in aliquots to avoid repeated freezing and thawing.

Specifications of RNeasy UCP MinElute spin columns

Table 1. RNeasy UCP MinElute spin column specifications

Description	Specification
Maximum binding capacity	45 µg RNA
Maximum loading volume	700 µl
RNA size distribution	RNA >18 nucleotides approximately
Minimum elution volume	10 µl
Maximum amount of serum or plasma	600 µl

Note: If the recommended sample volume is exceeded, RNA yields will not be consistent and may be reduced, even if the binding capacity of the RNeasy UCP MinElute spin column is not exceeded.

Protocol: Purification of Total RNA, Including miRNA, From Serum and Plasma

This protocol is intended as a guideline for the purification of cell-free total RNA, which primarily includes small RNAs, such as miRNAs, from small volumes (up to 600 µl) of serum and plasma using the miRNeasy Serum/Plasma Advanced Kit. Processing of more than 200 µl sample is not recommended, because the amounts of contaminants introduced by larger sample volumes may interfere with the purification process.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix B on page 26.

Important points before starting

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freezing at –30°C –15°C or –90°C to –65°C in aliquots is recommended. To process frozen lysates, incubate at 37°C in a water bath until samples are completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- See Appendix F on page 45 for optional on-column DNase digestion. DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA. In addition, miScript Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from plasma or serum.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Equilibrate buffers to room temperature before starting the protocol.

- Buffers RPL and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- All steps should be performed at room temperature. Work quickly.
- The procedure is suitable for use with either serum samples or plasma samples containing EDTA. For citrate plasma samples, refer to the protocol in Appendix A on page 22. Plasma samples containing heparin should not be used, because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottles, to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix C, page 30.
- Use of carrier RNA (e.g., 1 µg MS2 RNA, Roche®, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001) may increase recovery in some cases. Do not use poly-A RNA.

Procedure

1. Prepare serum or plasma or thaw frozen samples.
2. Transfer 200 µl serum or plasma into a 2 ml microcentrifuge tube.

   Note: It is possible to process up to 600 µl of serum/plasma sample. In this case, adapt the volume of RPL and RPP according to Table 2.

Table 2. Adapting Buffer RPL and RPP volumes for larger starting sample volumes

Serum/plasma	Buffer RPL	Buffer RPP
200 µl	60 µl	20 µl
400µl	120 µl	40 µl
600 µl	180 µl	60 µl

3. Add 60 µl Buffer RPL. Close the tube caps and vortex for >5 s. Leave at room temperature (15–25°C) for 3 min.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPL according to Table 2.

4. Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 copies/µl working solution) and mix thoroughly.

   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix C on page 30.

5. Add 20 µl Buffer RPP. Close the tube caps and mix vigorously by vortexing for >20 s. Incubate at room temperature for 3 min.

   Thorough mixing is important for subsequent phase separation.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPP according to Table 2.

6. Centrifuge at 12000 *x g* for 3 min at room temperature to pellet the precipitate.

   Note: Supernatant should be clear and colorless.

7. Transfer supernatant (~230 µl for 200 µl serum/plasma) to a new microcentrifuge tube. Add 1 volume of isopropanol. Mix well by vortexing.

8. Transfer the entire sample to an RNeasy UCP MinElute column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

9. Pipet 700 µl Buffer RWT onto the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

   Reuse the collection tube in the next step.

10. Pipet 500 µl Buffer RPE onto the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

    Reuse the collection tube in the next step.

11. Add 500 µl of 80% ethanol to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 *x g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

    Note: After centrifugation, carefully remove the RNeasy UCP MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

12. Place the RNeasy UCP MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through and the collection tube.

    Note: To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    Note: It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

13. Place the RNeasy UCP MinElute spin column in a new 1.5 ml collection tube (supplied). Add 20 µl RNase-free water directly to the center of the spin column membrane and incubate 1 min. Close the lid, and centrifuge for 1 min at full speed to elute the RNA.

    Note: As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield might be reduced. Do not elute with less than 10 µl RNase-free water, as the spin-column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy UCP MinElute spin column is 2 µl, so elution with 20 µl RNase-free water results in a 18 µl eluate.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

	Comments and suggestions
Clogged column	Make sure to follow recommendations in Appendix B (page 26) for removal of residual cellular material. After thawing of frozen samples, remove cryoprecipitates by centrifugation or filtration, if necessary (see Appendix B). Make sure to not transfer any precipitate from step 6 on page 17 when transferring the supernatant to a fresh tube
Centrifugation temperature too low	All centrifugation steps should be performed at room temperature (15–25°C). Some centrifuges may cool to below 20°C even when set at 20°C. This can cause precipitates to form that can clog the RNeasy UCP MinElute spin column and reduce RNA yield. If this happens, set the centrifugation temperature to 25°C. Warm the ethanol-containing lysate to 37°C before transferring to the RNeasy UCP MinElute spin column.
Low miRNA yield or poor performance of miRNA in downstream applications	
a) Incorrect ethanol concentration	Be sure to use the ethanol concentrations specified in the protocol steps.

	Comments and suggestions
Low or no recovery of RNA	
a) Too much starting material	In subsequent preparations, reduce the amounts of starting material. It is essential to use the correct amount of starting material (see page 13, Volume of starting material).
b) Elution buffer incorrectly dispensed	Add elution buffer to the center of the RNeasy UCP MinElute spin column membrane to ensure that the buffer completely covers the membrane.
c) RNA still bound to the membrane	Repeat the elution step of the protocol, but incubate the RNeasy UCP MinElute spin column on the bench for 10 min after adding RNase-free water and before centrifugation.
RNA degraded	
a) Sample inappropriately handled	Cell-free RNA in plasma and serum typically consists of small RNA species only and will therefore not resemble intact RNA from cells or tissue. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation. Perform the protocol quickly, especially the first few steps. See Appendix D: General Remarks on Handling RNA (page 40) and Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage (page 26).

	Comments and suggestions
b) RNase contamination	Cell-free RNA in plasma and serum typically consists of small RNA species only, and will therefore not resemble intact RNA from cells or tissue. Nevertheless, the following precautions are recommended to avoid complications due to RNA degradation. RNases can be introduced during use. Make sure not to introduce any RNases during the procedure or later handling. See Appendix D: General Remarks on Handling RNA (page 40). Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
RNA does not perform well in downstream experiments	
a) Salt carryover during elution	Ensure that Buffer RPE is at 20–30°C.
b) Ethanol carryover	After the final membrane wash, be sure to dry the RNeasy UCP MinElute spin column by centrifugation at full speed with open lids for 5 min (protocol step 12 on page 18).
Contamination by genomic DNA	
Sample inappropriately handled	Invert tubes gently to mix contents after blood collection. Vigorous mixing or shaking can promote hemolysis. Generate plasma as quickly as possible after blood collection. Long delays can promote hemolysis or apoptotic cell death. Perform the second centrifugation or filtration before freezing the plasma, if possible.

Appendix A: Purification of Total RNA, Including miRNA, from Citrate Plasma

This protocol is intended as a guideline for the purification of cell-free total RNA from small volumes (up to 600 µl) of citrate plasma using the miRNeasy Serum/Plasma Advanced Kit. Processing of more than 200 µl sample is not recommended, because the amounts of contaminants introduced by larger sample volumes may interfere with the purification process.

For recommendations on collection, preparation and storage of cell-free plasma and serum, see Appendix B, page 26.

Important points before starting

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freezing at –30°C –15°C or –90°C to –65°C in aliquots is recommended. To process frozen lysates, incubate at 37°C in a water bath until samples are completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- See Appendix F on page 45 for optional on-column DNase digestion. DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA. In addition, miScript Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from plasma or serum.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Equilibrate buffers to room temperature before starting the protocol.

- Buffers RPL and RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- All steps should be performed at room temperature. Work quickly.
- The procedure is suitable for use with plasma samples containing citrate. For serum or EDTA plasma samples, refer to the protocol on page 15. Plasma samples containing heparin should not be used, because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottles, to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix B, page 26.
- Use of carrier RNA (e.g., 1 µg MS2 RNA, Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001) may increase recovery in some cases. Do not use poly-A RNA.

Procedure

1. Prepare serum or plasma or thaw frozen samples.
2. Transfer 200 µl serum or plasma into a 2 ml microcentrifuge tube.

   Note: It is possible to process up to 600 µl serum/plasma sample. In this case please adapt the volume of RPL and RPP according to Table 3.

Table 3. Adapting Buffer RPL and RPP volumes for larger starting sample volumes

Serum/plasma	Buffer RPL	Buffer RPP
200 µl	60 µl	60 µl
400µl	120 µl	120 µl
600 µl	180 µl	180 µl

3. Add 60 µl Buffer RPL. Close the tube caps and vortex for >5 s. Leave at room temperature (15–25°C) for 3 min.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPL according to Table 3.

4. Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 copies/µl working solution) and mix thoroughly.

   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix C on page 30.

5. Add 60 µl Buffer RPP. Close the tube caps and mix vigorously by vortexing for >20 s. Incubate at room temperature for 3 min.

   Thorough mixing is important for subsequent phase separation.

   Note: If using a volume of serum/plasma other than 200 µl, adapt the volume of Buffer RPP according to Table 3.

6. Centrifuge at 12000 *x g* for 3 min at room temperature to pellet the precipitate.

   Note: Supernatant should be clear and colorless.

7. Transfer supernatant (~230 µl for 200 µl serum/plasma) to a new microcentrifuge tube. Add 1 volume of isopropanol. Mix well by vortexing.

8. Transfer the entire sample to an RNeasy UCP MinElute column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

9. Pipet 700 µl Buffer RWT to the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

   Reuse the collection tube in the next step.

10. Pipet 500 µl Buffer RPE onto the RNeasy UCP MinElute spin column. Close the lid, and centrifuge for 15 s at ≥8000 *x g*. Discard the flow-through.

    Reuse the collection tube in the next step.

11. Add 500 µl of 80% ethanol to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 *x g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

    Note: After centrifugation, carefully remove the RNeasy UCP MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

12. Place the RNeasy UCP MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through and the collection tube.

    Note: To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    Note: It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

13. Place the RNeasy UCP MinElute spin column in a new 1.5 ml collection tube (supplied). Add 20 µl RNase-free water directly to the center of the spin column membrane and incubate 1 min. Close the lid, and centrifuge for 1 min at full speed to elute the RNA.

    Note: As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield might be reduced. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy UCP MinElute spin column is 2 µl, so elution with 20 µl RNase-free water results in a 18 µl eluate.

Appendix B: Recommendations for Serum and Plasma Collection, Separation and Storage

To isolate circulating, cell-free nucleic acids from whole blood samples, we recommend following these protocols, which include an initial low *g*-force centrifugation step to separate cells from plasma or serum followed by a high *g*-force centrifugation or filtration step to remove all remaining cellular debris. The latter centrifugation step significantly reduces the amount of cellular or genomic DNA and RNA in the sample. Because of the much higher abundance of RNA in cells, even small amounts of cellular debris can have a very significant effect on RNA profiling of cell-free fluids. The sooner after blood collection this removal of cellular materials is performed, the lower the risk of additional background from blood cell-derived nucleic acids released *in vitro*. Use of gel barrier tubes generally results in fewer residual cells.

The speed at which the second centrifugation step is performed will influence the recovery of different types of nucleic acid. Centrifugation at medium speed (e.g., 3000 *x g*) will effectively remove cellular material, including thrombocyte fragments and apoptotic bodies. Centrifugation at higher speed (e.g., 16,000 *x g*) may in addition remove intact chromatin from ruptured blood cells, but may also remove larger extracellular vesicles that may contain cell-free nucleic acid (especially mRNA).

Syringe filters with 0.8 µm pore size (e.g., Sartorius® Minisart® NML (cat. no. 16592) or Millipore® Millex®-AA (cat. no. SLAA033SB)) remove remaining cell fragments and debris strictly based on size, irrespective of density. These filters have dead volumes of around 100–200 µl.

Procedure: plasma separation and storage

1. Collect whole blood in BD Vacutainer® Venous Blood Collection Tubes (cat. no. 367525) containing EDTA (or any other primary blood collection tube containing EDTA as anticoagulant). Store tubes at room temperature (15–25°C) or 4°C and process within 1 hour.

   Note: Do not use heparin-containing blood collection tubes as this anticoagulant can interfere with downstream assays, such as RT-PCR.

2. Centrifuge blood samples in primary blood collection tubes for 10 min at 1900 *x g* (3000 rpm) and 4°C using a swinging bucket rotor.

3. Carefully transfer the upper (yellow) plasma phase to a new tube (with conical bottom) without disturbing the intermediate buffy coat layer (containing white blood cells and platelets). Normally up to 4–5 ml plasma can be obtained from 10 ml of whole blood.

   Note: Carryover of white blood cells and platelets from the buffy coat layer is the most likely source of cellular miRNA/RNA contamination in plasma.

   Note: Plasma can be used for cell-free nucleic acid purification at this stage. However, an additional filtration or centrifugation will remove additional cellular debris and minimize contamination of cell-free nucleic acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge plasma samples in conical tubes for 15 min at 3,000 *x g* (or 10 min at 16,000 *x g* – see above) and 4°C or pass through a 0.8 µm filter (see recommendations above).

   This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet, which forms a smear along the outer side or bottom of the centrifugation tube.

6. Store at 2–8°C until further processing. If plasma will be used for nucleic acid purification on the same day. For longer storage, keep plasma frozen in aliquots at –90°C to –65°C.

7. Before using frozen plasma for nucleic acid purification, thaw at room temperature.

   Optional: To remove cryoprecipitates, centrifuge thawed plasma samples for 5 min at 3000 *x g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin the nucleic acid purification protocol.

Procedure: serum separation and storage

1. Collect whole blood in a primary blood collection tube with or without clot activator, but without anticoagulants, such as EDTA or citrate (e.g., Sarstedt S-Monovette® Serum-Gel 9 ml tubes, cat. no. 02.1388). For complete clotting, leave tubes at room temperature (15–25°C) for 10 min to 1 h.

   Note: Tubes with clot activator can be processed after 10 min clotting time, while tubes without clot activator should be stored for at least 30 min at room temperature to allow clotting to take place.

2. Centrifuge tubes for 10 min at 1900 *x g* (3000 rpm) and 4°C using a swinging bucket rotor.

   Note: If using Sarstedt S-Monovette Serum-Gel 9ml tubes, a gel bed will form between the upper serum phase and the lower cellular phase, facilitating recovery of serum.

3. Carefully transfer the upper (yellow) serum phase to a new tube (with conical bottom) without disturbing the pellet containing cellular material. Normally up to 3–5 ml serum can be obtained from 10 ml of whole blood.

   Note: Prevent transfer of cellular material from the lower phase.

   Note: Serum can be used for cell-free nucleic acid purification at this stage. However, an additional filtration or centrifugation will remove additional cellular debris and minimize contamination acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge serum samples in conical tubes for 15 min at 3,000 *x g* (or 10 min at 16,000 *x g* – see above) and 4°C or pass through a 0.8 µm filter (see recommendations above). This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet, which forms a smear along the outer side of the centrifugation tube.
6. Store at 2–8°C until further processing, if serum will be used for nucleic acid purification on the same day. For longer storage, keep serum frozen in aliquots at –90°C to –65°C.
7. Before using frozen serum for nucleic acid purification, thaw at room temperature.

   Optional: To remove cryoprecipitates, centrifuge thawed serum samples for 5 min at 3000 *x g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin nucleic acid purification protocol.

Appendix C: Use of the miRNeasy Serum/ Plasma Spike-In Control in Serum/Plasma miRNA Profiling

There is currently no clear consensus in the research community on what should be used as a normalization control for miRNA expression profiling in a serum or plasma sample. Many researchers choose to spike a synthetic miRNA into their RNA prep to monitor RNA recovery and reverse transcription efficiency. This RNA is added to samples after the addition of denaturant (e.g., Lysis Buffer RPL), prior to addition of Buffer RPP and protein precipitation. After real-time RT-PCR, the C_T value obtained with the assay targeting the synthetic miRNA permits normalization between samples, which can control for varying RNA purification yields and amplification efficiency. In addition, RNA recovery can be assessed by comparing the C_T value to a standard curve of the synthetic miRNA generated independently of the RNA purification procedure. QIAGEN recommends the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) for use as an internal control for miRNA expression profiling in serum or plasma. This appendix includes details of preparation of miRNeasy Serum/Plasma Spike-In Control stock and working solution, a protocol for generating an miRNeasy Serum/Plasma Spike-In Control standard curve, and a protocol for assessing the recovery of miRNeasy Serum/Plasma Spike-In Control after RNA purification.

Note: Use of the miRNeasy Serum/Plasma Spike-In Control is not recommended for cell or tissue samples.

Preparation of miRNeasy Serum/Plasma Spike-In Control

The miRNeasy Serum/Plasma Spike-In Control is a *C. elegans* miR-39 miRNA mimic and is supplied lyophilized at 10 pmol per tube. Reconstitute by adding 300 µl RNase-free water per tube, resulting in a 2 x 10^{10} copies/µl stock. The miRNeasy Serum/Plasma Spike-In

Control stock should be stored at –90°C to –65°C. For large volumes, first aliquot into smaller volumes prior to long-term storage at –90°C to –65°C.

When working with miRNeasy Serum/Plasma Spike-In Control, first add 4 µl of 2 x 10^{10} copies/µl miRNeasy Serum/Plasma Spike-In Control stock to 16 µl RNase-free water, resulting in a 4 x 10^{9} copies/µl dilution. If performing purification of RNA from serum and plasma, add 2 µl of the 4 x 10^{9} copies/µl dilution to 48 µl RNase-free water to provide a 1.6 x 10^{8} copies/µl working solution. If generating a standard curve, add 2 µl of the 4 x 10^{9} copies/µl dilution to 78 µl RNase-free water that contains carrier RNA (e.g., 10 ng/µl MS2 [Roche, cat. no. 10 165 948 001] or bacterial ribosomal RNA [Roche, cat. no. 10 206 938 001]) to provide a 1 x 10^{8} copies/µl working solution. These dilutions are summarized in Table 4.

Table 4. miScript Serum/Plasma Spike-In Control dilutions

Purpose	Dilution	Concentration (copies/µl)
Stock	Add 300 µl RNase-free water to lyophilized miScript Serum/Plasma Spike-In Control (10 pmol)	2 x 10^{10}
Dilution	Add 4 µl stock (2 x 10^{10} copies/µl) to 16 µl RNase-free water	4 x 10^{9}
Working solution for RNA purification (protocol on page 16)	Add 2 µl of 4 x 10^{9} copies/µl dilution to 48 µl RNase-free water	1.6 x 10^{8}
Add 2 µl of 4 x 10^{9} copies/µl dilution to 48 µl RNase-free water	Add 2 µl of 4 x 10^{9} copies/µl dilution to 78 µl RNase-free water containing 10 ng/µl MS2 (Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001)	1 x 10^{8}

Protocol: Generation of miRNeasy Serum/Plasma Spike-In Control Standard Curve

This protocol is for generating a real-time PCR standard curve of miRNeasy Serum/Plasma Spike-In Control that is independent of a serum/plasma sample and RNA purification procedure. The standard curve allows estimation of the recovery of miRNeasy Serum/Plasma Spike-In Control when it is added to a serum/plasma sample that is subsequently used for RNA purification (see protocol on page 16).

Important points before starting

- To ensure reproducibility, always use freshly prepared cDNA to generate a standard curve. Perform PCRs for generation of the standard curve and PCRs on RNA from the serum/plasma samples of interest in the same run. Do not store cDNA dilutions for later use.
- This protocol uses the following components of the miScript PCR System: Ce_miR-39_1 miScript Primer Assay, miScript II RT Kit and the miScript SYBR Green PCR Kit. For more details, consult the *miScript PCR System Handbook* or visit **www.qiagen.com/miRNA**.

Procedure

1. Prepare a 1 x 10^8 copies/µl working solution of miRNeasy Serum/Plasma Spike-In Control. Mix gently yet thoroughly.

   For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 4 on page 31.

   For dilution of the control, we recommend RNase-free water containing 10 ng/µl MS2 (Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206938 001).
2. Prepare the reverse transcription reaction on ice according to Table 5.

Table 5. Reverse transcription reaction components

Component	Volume
miRNeasy Serum/Plasma Spike-In Control from step 1 (1 x 10^8 copies/µl)	2.2 µl (2.2 x 10^8 copies/ µl)
Total RNA sample*	2 µl (~100 ng)
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer†	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	7.8 µl
miScript Reverse Transcriptase Mix	2 µl
Total volume	**20 µl**

* Any total RNA sample can be used here to provide a complex RNA background.

† The correct buffer to use depends on the subsequent PCR application. Consult the *miScript PCR System Handbook* for more details.

3. Gently mix, briefly centrifuge, and then store on ice.
4. Incubate for 60 min at 37°C.
5. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice.
6. Add 200 µl RNase-free water to the reverse transcription reaction.

   This results in a miRNeasy Serum/Plasma Spike-In Control concentration of 1 x 10^6 copies/µl.
7. Use the diluted reverse transcription reaction to prepare cDNA serial dilutions according to Table 6.

Table 6. cDNA serial dilutions

Tube	cDNA	Water	Concentration spike-in control	Use in PCR
1	20 µl diluted cDNA	20 µl	5 x 10^6 copies/µl	2 µl (1 x 10^6 copies)
2	5 µl from tube 1	45 µl	5 x 10^5 copies/µl	2 µl (1 x 10^5 copies)
3	5 µl from tube 2	45 µl	5 x 10^4 copies/µl	2 µl (1 x 10^4 copies)
4	5 µl from tube 3	45 µl	5 x 10^3 copies/µl	2 µl (1 x 10^3 copies)

8. Using 2 µl from each tube in Table 6, set up separate PCRs according to Table 7.

   We recommend setting up each reaction in triplicate.

Table 7. Reaction setup for real-time PCR

Component	Volume/reaction (384-well)	Volume/reaction (96-well)	Volume*/reaction (Rotor-Disc®)
2x QuantiTect® SYBR Green PCR Master Mix	5 µl	12.5 µl	10 µl
10x miScript Universal Primer	1 µl	2.5 µl	2 µl
10x Ce_miR-39_1 miScript Primer Assay	1 µl	2.5 µl	2 µl
RNase-free water	1 µl	5.5 µl	4 µl
Template cDNA from Table 4	2 µl	2 µl	2 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* These volumes can also be used for reactions set up in Strip Tubes for use with the Rotor-Gene® Q 72-Well Rotor.

9. Mix thoroughly and proceed with PCR using the cycling conditions in Table 8.

   Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow the instructions provided by the supplier.

Table 8. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR initial activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:[*][†]			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension[‡]	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles[¶]		Cycle number depends on the amount of template cDNA and abundance of the target.

[*] For Bio-Rad® models CFX96™ and CFX384™: adjust the ramp rate to 1°C/s.

[†] For Eppendorf® Mastercycler® ep realplex models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%.

[‡] If using a Roche LightCycler® 480, adjust the ramp rate to 1°C/s.

[§] Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM® 7000 or 34 s with the Applied Biosystems® 7300 and 7500.

[¶] If using a Roche LightCycler 480, use 45 cycles.

10. Extract C_T values for miRNeasy Serum/Plasma Spike-In Control from each reaction.

11. Generate a standard curve by plotting the log copy number miRNeasy Serum/Plasma Spike-In Control used in each PCR against the mean C_T value. 0.

Protocol: Assessment of Recovery of miRNeasy Serum/Plasma Spike-In Control After miRNA Purification

This protocol is a guideline for the addition of miRNeasy Serum/Plasma Spike-In Control to a serum/plasma sample during RNA purification, followed by determination of recovery of miRNeasy Serum/Plasma Spike-In Control by real-time RT-PCR using the standard curve generated in the protocol on 32.

Important points before starting

- This protocol uses the following components of the miScript PCR System: Ce_miR-39_1 miScript Primer Assay (provided in the miRNeasy Serum/Plasma Advanced Kit), miScript II RT Kit and the miScript SYBR Green PCR Kit. For more information, consult the *miScript PCR System Handbook* or visit **www.qiagen.com/miRNA**.

Procedure

1. Prepare a 1.6 x 10^8 copies/µl working solution of miRNeasy Serum/Plasma Spike-In Control. Mix gently yet thoroughly.

   For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 4 on page 31.

2. During RNA purification, add 3.5 µl miRNeasy Serum/Plasma Spike-In Control working solution from step 1 above (1.6 x 10^8 copies/µl) to the sample after lysis with Buffer RPL (see page 17, step 3). Mix thoroughly.

   We recommend addition of miRNeasy Serum/Plasma Spike-In Control after lysis to avoid degradation by endogenous RNases in the sample. This can be modified if desired.

3. Continue with the RNA purification protocol (page 15). After RNA elution in 20 µl RNase-free water (step 13, page 18) miRNeasy Serum/Plasma Spike-In Control is present in the eluate at 4 x 10^7 copies/µl.

   If a different elution volume is used, calculate the miRNeasy Serum/Plasma Spike-In Control concentration accordingly.

4. Prepare the reverse transcription reaction on ice according to Table 9.

Table 9. Reverse transcription reaction components

Component	Volume
Purified RNA (containing miRNeasy Serum/Plasma Spike-In Control)	1.5 µl
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer*	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	10.5 µl
miScript Reverse Transcriptase Mix	2 µl
Total volume	**20 µl**

* The correct buffer to use depends on the subsequent PCR application. Consult the *miScript PCR System Handbook* for more details.

5. Gently mix, briefly centrifuge, and then store on ice.
6. Incubate for 60 min at 37°C.
7. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice.
8. Add 200 µl RNase-free water to the reverse transcription reaction.

   This results in a miRNeasy Serum/Plasma Spike-In Control concentration of 2.7 x 10^5 copies/µl (assuming 100% recovery during RNA purification and reverse transcription).
9. Set up PCRs according to Table 10.

   We recommend setting up each reaction in triplicate.

Table 10. Reaction setup for real-time PCR

Component	Volume/reaction (384-well)	Volume/reaction (96-well)	Volume*/reaction (Rotor-Disc)
2x QuantiTect SYBR Green PCR Master Mix	5 µl	12.5 µl	10 µl
10x miScript Universal Primer	1 µl	2.5 µl	2 µl
10x Ce_miR-39_1 miScript Primer Assay	1 µl	2.5 µl	2 µl
RNase-free water	1 µl	6.5 µl	5 µl
Diluted reverse transcription reaction	2 µl	1 µl	1 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* These volumes can also be used for reactions set up in Strip Tubes for use with the Rotor-Gene Q 72-Well Rotor.

10. Mix thoroughly and proceed with PCR using the cycling conditions in Table 11.

Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow the instructions provided by the supplier.

Table 11. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR initial activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:[¶¶]			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension[‡]	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles[¶]		Cycle number depends on the amount of template cDNA and abundance of the target.

* For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

† For Eppendorf Mastercycler ep realplex models 2, 2S, 4 and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%.

‡ If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s.

§ Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM 7000 or 34 s with the Applied Biosystems 7300 and 7500.

¶ If using a Roche LightCycler 480, use 45 cycles.

11. Extract C_T values and determine the mean C_T value for miRNeasy Serum/Plasma Spike-In Control from each reaction.
12. Compare with the miRNeasy Serum/Plasma Spike-In Control standard curve to determine recovery of miRNeasy Serum/Plasma Spike-In Control.

Appendix D: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. To create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

For removal of RNase contamination from bench surfaces, nondisposable plasticware and laboratory equipment (e.g., pipets and electrophoresis tanks), general laboratory reagents can be used. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 41), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (SDSs), available from the product supplier.

detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for 4 hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Solutions (water and other solutions)* should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (SDSs), available from the product supplier.

unless a large fraction of the purine residues has been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: RNeasy buffers are RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

Appendix E: Storage, Quantification and Determination of Quality of RNA

Storage of DNA/RNA eluates

Purified DNA/RNA may be stored at –30°C to –15°C or –90°C to –65°C in RNase-free water. Under these conditions, no degradation of DNA or RNA is detectable after 1 year.

Quantification of RNA

The concentration of ccfRNA should not be determined by spectrophotometric quantification, because the amounts present in serum and plasma are usually too low for reliable measurements. Small amounts of DNA and RNA can best be quantified using quantitative PCR / RT-PCR. Fluorometric quantification (e.g., using Qubit®) is often unreliable for short nucleic acid fragments.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While miRNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample. However, serum, plasma and other cell-free body fluids contain very little DNA.

For analysis of very low-abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in gene expression analysis real-time RT-PCR applications, such as with ABI PRISM and LightCycler Instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified.

QuantiTect Primer Assays from QIAGEN are designed for SYBR Green-based real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible (the assays can be ordered online at **www.qiagen.com/GeneGlobe**). For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination.

Alternatively, gene expression analysis can be performed using QuantiFast® Probe Assays and the QuantiFast Probe RT-PCR Plus Kit, which includes an integrated genomic DNA removal step.

miScript Primer Assays, used with the miScript PCR System for miRNA quantification, do not detect genomic DNA.

Integrity of RNA

Cell-free RNA from serum or plasma consists mainly of small RNAs of less than 100 nucleotides. Appearance of rRNA bands is usually indicative of contamination by cells or cell debris. Therefore, RNA integrity cannot be analyzed by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using the QIAxcel® system or Agilent® 2100 Bioanalyzer.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier

Appendix F: Optional On-column DNase Digestion with the RNase-Free DNase Set

The RNase-Free DNase Set (cat. no. 79254) provides efficient on-column digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps.

Note: Standard DNase buffers are not compatible with on-column DNase digestion. Use of other buffers may affect the binding of RNA to the RNeasy membrane, reducing RNA yield and integrity.

Lysis and homogenization of the sample and binding of RNA to the RNeasy membrane are performed according to the standard protocol. After washing with a reduced volume of Buffer RWT, the RNA is treated with DNase I while bound to the RNeasy membrane. The DNase I is removed by a second wash with Buffer RWT. Washing with Buffer RPE and elution of RNA are then performed according to the standard protocol.

Important points before starting

- Generally, DNase digestion is not required since DNA levels in plasma/serum samples are very low and RNeasy technologies efficiently remove most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). DNA can also be removed by a DNase digestion following RNA purification. Please note that an additional DNase digest might lead to losses in RNA yield.
- Do not vortex the reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

Things to do before starting

- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –30°C to –15°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- For processing samples containing <1 µg total RNA approximately, prepare Buffer RWT by adding 45 ml isopropanol to the concentrate (instead of adding 30 ml ethanol as usually recommended). Buffer RWT can be ordered separately for this protocol (cat. no. 1067933).

Procedure

1. Prepare and load samples onto the RNeasy UCP MinElute spin column as indicated in steps 1–8 of the protocol on page 16 or page 23 (Appendix A). Instead of performing step 9 (addition of Buffer RWT), follow steps 2–6 below.
2. Add 350 µl Buffer RWT (prepared with isopropanol; see above "Things to do before starting") to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 *x g* (≥10,000 rpm) to wash the membrane. Discard the flow-through. Reuse the collection tube in the next step.
3. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

   Buffer RDD is supplied with the RNase-Free DNase Set.

   Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

4. Add 80µl of the DNase I incubation mix directly to the RNeasy UCP MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.

   Note: Be sure to add the DNase I incubation mix directly to the RNeasy UCP MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

5. Add 500 µl Buffer RWT (prepared with isopropanol) to the RNeasy UCP MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥ 8000 *x g* (≥ 10,000 rpm). Save the flow-through for use in step the next step.

6. Place the spin column in a new 2 ml collection tube (supplied). Apply the flow-through from step 5 to the spin column. Centrifuge for 15 s.

   If you were performing the Protocol: Purification of Total RNA, Including miRNA, From Serum and Plasma, continue with step 9 on page 17 at ≥ 8000 *x g* (≥ 10,000 rpm).

   If you were performing the protocol in Appendix A: Purification of Total RNA, Including miRNA, from Citrate Plasma, continue with step 9 on page 24.

Ordering Information

Product	Contents	Cat. no.
miRNeasy Serum/ Plasma Advanced Kit (50)	For 50 total RNA preps: 50 RNeasy UCP MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	217204
miRNeasy Serum/ Plasma Spike-In Control	10 pmol lyophilized *C. elegans* miR-39 miRNA mimic	219610
Ce_miR-39_1 miScript Primer Assay	miScript Primer assay with *C. elegans* miR-39 specific primers	MS00019789
QIAcube Connect — for fully automated nucleic acid extraction with QIAGEN spin-column kits		
QIAcube Connect*	Instrument, connectivity package, 1-year warranty on parts and labor	Inquire
Starter Pack, QIAcube	Filter-tips, 200 µl (1024), 1000 µl filter-tips (1024), 30 ml reagent bottles (12), rotor adapters (240), elution tubes (240), rotor adapter holder	990395
Related products		
miRNeasy Mini Kit (50)	For 50 total RNA preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol® Lysis Reagent, RNase-free Reagents and Buffers	217004
miRNeasy Micro Kit (50)	For 50 total RNA preps: 50 RNeasy UCP MinElute spin columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-free Reagents and Buffers	217084
Collection Tubes (2 ml)	1000 Collection Tubes (2 ml)	19201

Product	Contents	Cat. no.
Related products for quantitative, real-time RT-PCR		
miScript II RT Kit (12)	For 12 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218160
miScript II RT Kit (50)	For 50 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218161
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect® SYBR Green PCR Master Mix, miScript Universal Primer	218073
miScript SYBR Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218075
miScript Primer Assay (100)	miRNA-specific primer; available via GeneGlobe	Varies†
Pathway-Focused miScript miRNA PCR Array	Array of assays for a pathway, disease or gene family for human, mouse, rat, dog or rhesus macaque miRNAs; available in 96-well, 384-well or Rotor-Disc 100 format	Varies
Related products for next-generation sequencing		
QIAseq® miRNA Library Kit (12)	For 12 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331502
QIAseq miRNA NGS 12 Index IL (12)	Sequencing adapters, primers and indexes compatible with Illumina platforms; 12 indexes for 12 samples	331592

Product	Contents	Cat. no.
QIAseq miRNA Library Kit (96)	For 96 sequencing prep reactions: 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup, library amplification and library cleanup reagents; quality control primers	331505
QIAseq miRNA NGS 48 Index IL (96)	Sequencing adapters, primers and indexes compatible with Illumina platforms; two 48 indexes for 96 samples	331595

* All QIAcube Connect instruments are provided with a region-specific connectivity package, including tablet and equipment necessary to connect to the local network. Further, QIAGEN offers comprehensive instrument service products, including service agreements, installation, introductory training and preventive subscription. Contact your local sales representative to learn about your options.

† Visit www.qiagen.com/GeneGlobe to search for and order these products

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Document Revision History

Date	Changes
January 2020	Updated text, ordering information and intended use for QIAcube Connect.

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APPENDIX E. Ethics Clearance Certificates

Prof Rajen Morar
Charlotte Maxeke Johannesburg Academic Hospital
Department of Medicine
Division of Pulmonology and Intensive Care
7 York Road
Parktown

The Chairperson
Prof Cleaton-Jones
Human Research Ethics Committee (Medical)

24 July 2011

Re: Extension of ethics clearance certificates

I would like to apply for extension of ethics clearances for the following projects which were approved previously. Prof Feldman is the supervisor and has advised me to request permission to extend these clearances.

M110750 1. M 040628 [Human leukocyte antigen (HLA) class I and class II associated with sarcoidosis in South Africa].

M110751 2. Clearance No. 05/1/92 [Study of immunoglobulin levels in various respiratory disorders]

M110752 3. Clinical features of patients with sarcoidosis attending the Charlotte Maxeke Academic Hospital. (I do not have the M number - as soon as I retrieve it I will forward it to Anisa Keshav].

The reason for asking permission to extending these clearances is that these studies have not yet been completed.

Thanking You

R Morar

extensions approved for another 5 yr cycle.
27/07/11

1 application form renewal needed.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Professor R Morar

CLEARANCE CERTIFICATE	**M121016**
PROJECT	Clinical and Immunopathogenic Aspects of Sarcoidosis
INVESTIGATORS	Professor R Morar.
DEPARTMENT	Department of Internal Medicine/Pulmonology
DATE CONSIDERED	26/10/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 26/1/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Professor C Feldman

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX F. Data of All miRNA Detected

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
1	hsa-let-7a-5p	218.39	1.54	141.69	0.0001396	39.09	513.53
2	hsa-let-7b-5p	483.26	193.92	2.49	0.05938935	-1.09	6.74
3	hsa-let-7c-5p	11.51	3.52	3.27	0.06362636	-1.12	12.02
4	hsa-let-7d-5p	190.55	8.84	21.56	0.00100373	5.28	88.08
5	hsa-let-7e-5p	12.67	1.94	6.53	0.01385917	1.67	25.6
6	hsa-let-7f-5p	19.97	14.72	1.36	0.06844573	-1.04	1.91
7	hsa-let-7g-5p	170.77	21.39	7.98	0.00212597	2.83	22.48
8	hsa-let-7i-5p	250.65	63.89	3.92	0.02427652	1.23	12.51
9	hsa-miR-1-3p	1.47	1.81	-1.23	0.4835529	-2.53	1.66
10	hsa-miR-1-5p	8.27	13.63	-1.65	0.4552522	-8.08	2.98
11	hsa-miR-100-5p	3.96	2.07	1.91	0.23602431	-1.95	7.13
12	hsa-miR-101-3p	1.24	2.05	-1.66	0.0968439	-3.16	1.15
13	hsa-miR-103a-3p	3.53	10.17	-2.88	0.1349148	-13.69	1.65
14	hsa-miR-105-5p	2.51	8.13	-3.23	0.0299325	-9.25	-1.13
15	hsa-miR-106a-5p+ hsa-miR-17-5p	71.35	9.58	7.44	0.01171714	1.76	31.54
16	hsa-miR-106b-5p	17.38	2.98	5.82	0.06980325	-1.22	41.29
17	hsa-miR-107	54.81	5.77	9.5	0.00882826	2.12	42.51
18	hsa-miR-10a-5p	8.52	7.81	1.09	0.89114475	-3.95	4.71
19	hsa-miR-10b-5p	4.63	5.81	-1.25	0.72574192	-5.49	3.49
20	hsa-miR-1178-3p	6.03	9.06	-1.5	0.51607209	-6.2	2.74
21	hsa-miR-1180-3p	6.14	9.53	-1.55	0.57592493	-9.2	3.82
22	hsa-miR-1183	3.58	1.98	1.81	0.27320415	-1.86	6.07
23	hsa-miR-1185-1-3p	12.29	12.66	-1.03	0.93422657	-2.52	2.38
24	hsa-miR-1185-2-3p	6.3	6.59	-1.05	0.93825388	-4	3.66
25	hsa-miR-1185-5p	1.81	2.15	-1.18	0.59459621	-2.44	1.74
26	hsa-miR-1193	1.34	1.54	-1.15	0.51767039	-1.88	1.42
27	hsa-miR-1197	25.11	19.44	1.29	0.58897871	-2.43	4.06
28	hsa-miR-1200	1.24	1.54	-1.25	0.25182578	-1.92	1.23
29	hsa-miR-1202	3.44	3.55	-1.03	0.96165979	-4.59	4.31
30	hsa-miR-1203	6.69	2.84	2.36	0.22759192	-1.99	11.07
31	hsa-miR-1204	1.24	1.54	-1.25	0.25182578	-1.92	1.23
32	hsa-miR-1205	2.12	2.16	-1.02	0.97245967	-3	2.9
33	hsa-miR-1206	11.62	13.24	-1.14	0.65155572	-2.25	1.73
34	hsa-miR-122-5p	70.72	211.05	-2.98	0.34368128	-53.2	5.97
35	hsa-miR-1224-3p	1.55	1.99	-1.28	0.62744153	-4.07	2.48
36	hsa-miR-1224-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
37	hsa-miR-1226-3p	1.76	2.13	-1.21	0.64444822	-3.11	2.12
38	hsa-miR-1228-3p	4.24	7.56	-1.78	0.32856923	-6.58	2.08
39	hsa-miR-1233-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
40	hsa-miR-1234-3p	7.12	3.61	1.97	0.33248767	-2.49	9.7
41	hsa-miR-1236-3p	12.73	16.47	-1.29	0.56528503	-3.56	2.13
42	hsa-miR-124-3p	1.38	1.59	-1.15	0.49772224	-1.85	1.39
43	hsa-miR-1244	1.24	1.54	-1.25	0.25182578	-1.92	1.23
44	hsa-miR-1245a	1.34	2.61	-1.95	0.19719781	-6.72	1.78

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
45	hsa-miR-1245b-3p	1.26	1.95	-1.55	0.24405631	-3.86	1.6
46	hsa-miR-1245b-5p	10.4	8.62	1.21	0.72907186	-2.87	4.18
47	hsa-miR-1246	1.24	1.54	-1.25	0.25182578	-1.92	1.23
48	hsa-miR-1247-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
49	hsa-miR-1248	1.24	1.81	-1.47	0.21438445	-2.94	1.37
50	hsa-miR-1249-3p	7.28	1.77	4.1	0.06644876	-1.26	21.19
51	hsa-miR-1249-5p	1.51	1.54	-1.02	0.9410463	-1.98	1.9
52	hsa-miR-1250-5p	1.24	2.04	-1.65	0.13447633	-3.38	1.25
53	hsa-miR-1252-5p	1.99	1.72	1.16	0.64824837	-1.9	2.55
54	hsa-miR-1253	1.24	2.87	-2.32	0.28719822	-15.72	2.92
55	hsa-miR-1254	1.6	1.59	1.01	0.97232312	-1.93	1.97
56	hsa-miR-1255a	5.91	7.58	-1.28	0.66877508	-4.85	2.94
57	hsa-miR-1255b-5p	8.25	16.36	-1.98	0.12681715	-5.2	1.32
58	hsa-miR-1257	11.88	18.33	-1.54	0.31107953	-3.98	1.67
59	hsa-miR-1258	18.36	26.88	-1.46	0.36955976	-4.27	1.99
60	hsa-miR-125a-3p	2.97	5.72	-1.93	0.36720902	-10.03	2.7
61	hsa-miR-125a-5p	118.56	6.21	19.08	0.00922562	2.76	132.02
62	hsa-miR-125b-5p	12.85	7.28	1.77	0.28765771	-1.83	5.71
63	hsa-miR-126-3p	393.52	47.28	8.32	0.00382611	2.51	27.63
64	hsa-miR-1260a	1.73	2.15	-1.24	0.67389059	-4.04	2.61
65	hsa-miR-1260b	2.79	1.77	1.57	0.43884394	-2.82	6.97
66	hsa-miR-1261	9.43	14.31	-1.52	0.15198626	-2.97	1.29
67	hsa-miR-1262	2.24	1.54	1.45	0.3258158	-1.68	3.57
68	hsa-miR-1264	4.18	2.41	1.74	0.44823667	-2.96	8.94
69	hsa-miR-1266-5p	1.6	2.06	-1.29	0.57289511	-3.65	2.2
70	hsa-miR-1268a	1.24	1.85	-1.5	0.14046966	-2.74	1.22
71	hsa-miR-1268b	12.91	21.34	-1.65	0.33360949	-5.97	2.19
72	hsa-miR-1269a	10.97	7.23	1.52	0.32630807	-1.79	4.13
73	hsa-miR-1269b	1.24	2.94	-2.38	0.06153638	-6.33	1.12
74	hsa-miR-127-3p	15.99	16.09	-1.01	0.9937163	-8.05	7.95
75	hsa-miR-127-5p	1.87	1.54	1.21	0.64840287	-2.29	3.35
76	hsa-miR-1270	10.69	13.96	-1.31	0.59272355	-4.4	2.58
77	hsa-miR-1271-3p	1.26	2.13	-1.69	0.11741742	-3.66	1.27
78	hsa-miR-1271-5p	10.3	13.66	-1.33	0.45107949	-3.13	1.78
79	hsa-miR-1272	1.88	2.44	-1.3	0.63937688	-4.93	2.94
80	hsa-miR-1273c	4.76	3	1.59	0.50959384	-3.18	8.03
81	hsa-miR-1275	1.24	1.54	-1.25	0.25182578	-1.92	1.23
82	hsa-miR-1276	3.46	3.11	1.11	0.86847657	-4.08	5.04
83	hsa-miR-1277-3p	12.82	3.14	4.08	0.05640455	-1.1	18.41
84	hsa-miR-1278	3.48	2.32	1.5	0.28817189	-1.55	3.48
85	hsa-miR-1279	4.37	6.81	-1.56	0.37411395	-4.74	1.95
86	hsa-miR-128-1-5p	9.05	25.41	-2.81	0.11123781	-11.14	1.41
87	hsa-miR-128-2-5p	1.24	2.14	-1.73	0.1137279	-3.83	1.28
88	hsa-miR-128-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
89	hsa-miR-1281	1.24	1.54	-1.25	0.25182578	-1.92	1.23
90	hsa-miR-1283	1.24	1.54	-1.25	0.25182578	-1.92	1.23
91	hsa-miR-1285-3p	5.55	2.87	1.93	0.32182896	-2.26	8.47
92	hsa-miR-1285-5p	25.95	32.38	-1.25	0.65884638	-4.61	2.96

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
93	hsa-miR-1286	1.91	1.54	1.24	0.57250243	-2.01	3.08
94	hsa-miR-1287-3p	1.24	2.06	-1.67	0.24020347	-4.76	1.71
95	hsa-miR-1287-5p	2.22	1.86	1.19	0.73575622	-2.71	3.83
96	hsa-miR-1288-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
97	hsa-miR-1289	2.64	6.34	-2.4	0.06822083	-6.33	1.09
98	hsa-miR-129-2-3p	5.18	3.57	1.45	0.55175346	-2.84	5.99
99	hsa-miR-129-5p	1.41	1.54	-1.09	0.64843214	-1.69	1.42
100	hsa-miR-1290	4.66	10.77	-2.31	0.18793616	-10.21	1.92
101	hsa-miR-1291	1.26	1.55	-1.23	0.26822352	-1.86	1.23
102	hsa-miR-1293	1.75	2.54	-1.46	0.586218	-7.27	3.43
103	hsa-miR-1295a	9.47	9.09	1.04	0.94293892	-3.5	3.8
104	hsa-miR-1296-3p	9.16	5.58	1.64	0.32778683	-1.88	5.07
105	hsa-miR-1296-5p	1.67	1.59	1.05	0.89612335	-2.47	2.73
106	hsa-miR-1297	19.73	30.59	-1.55	0.33254629	-4.79	1.99
107	hsa-miR-1298-5p	3.36	3.16	1.06	0.92849785	-4.43	5.01
108	hsa-miR-1299	3.45	1.99	1.73	0.39888814	-2.73	8.21
109	hsa-miR-1301-3p	1.41	1.81	-1.29	0.40202188	-2.62	1.58
110	hsa-miR-1302	2.32	1.95	1.19	0.72336036	-2.58	3.66
111	hsa-miR-1303	1.35	1.54	-1.14	0.47194192	-1.74	1.33
112	hsa-miR-1304-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
113	hsa-miR-1304-5p	1.8	2.31	-1.28	0.5466153	-3.26	1.98
114	hsa-miR-1305	15.56	16.12	-1.04	0.93872494	-3.07	2.86
115	hsa-miR-1306-3p	9.15	10.39	-1.13	0.80955505	-3.75	2.91
116	hsa-miR-1306-5p	1.82	1.54	1.18	0.67085725	-2.22	3.11
117	hsa-miR-1307-3p	10.65	11.36	-1.07	0.92129868	-5.87	5.16
118	hsa-miR-1307-5p	13.45	13.17	1.02	0.97853839	-5.67	5.91
119	hsa-miR-130a-3p	245.05	16.4	14.95	0.00556292	2.8	79.83
120	hsa-miR-130b-3p	3.37	1.98	1.7	0.49902245	-3.86	11.21
121	hsa-miR-132-3p	5.06	1.54	3.28	0.01348556	1.37	7.85
122	hsa-miR-1322	9.71	9.85	-1.01	0.97747272	-3.24	3.15
123	hsa-miR-1323	5.07	3.01	1.69	0.44735134	-2.78	7.92
124	hsa-miR-133a-3p	4.23	2.24	1.89	0.24048376	-1.73	6.17
125	hsa-miR-133a-5p	4.1	8.52	-2.08	0.14005859	-6.25	1.45
126	hsa-miR-133b	1.24	1.54	-1.25	0.25182578	-1.92	1.23
127	hsa-miR-134-3p	1.26	1.85	-1.47	0.15334317	-2.66	1.23
128	hsa-miR-134-5p+ hsa-miR-6728-5p	1.35	1.94	-1.43	0.13399403	-2.44	1.18
129	hsa-miR-135a-5p	1.26	1.54	-1.22	0.27988797	-1.86	1.24
130	hsa-miR-135b-5p	2.71	5.16	-1.9	0.41494775	-11.08	3.07
131	hsa-miR-136-5p	11.13	5.11	2.18	0.34823468	-3.02	14.32
132	hsa-miR-137	2.25	2.36	-1.05	0.9407602	-4.7	4.26
133	hsa-miR-138-5p	12.3	6.32	1.95	0.24861747	-1.82	6.88
134	hsa-miR-139-3p	18.97	11.58	1.64	0.47696888	-2.9	7.78
135	hsa-miR-139-5p	1.48	1.55	-1.05	0.78848255	-1.58	1.44
136	hsa-miR-140-3p	4.7	3.02	1.56	0.35737216	-1.97	4.79
137	hsa-miR-140-5p	15.93	6.39	2.49	0.14712493	-1.51	9.39
138	hsa-miR-141-3p	1.87	2.55	-1.37	0.4047057	-3.16	1.69
139	hsa-miR-142-3p	557.98	91.3	6.11	0.00412496	2.14	17.45

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
140	hsa-miR-142-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
141	hsa-miR-143-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
142	hsa-miR-144-3p	135.63	105.34	1.29	0.28770351	-1.31	2.17
143	hsa-miR-145-5p	20.23	2.84	7.12	0.05019166	-1.02	51.93
144	hsa-miR-1469	1.24	1.54	-1.25	0.25182578	-1.92	1.23
145	hsa-miR-146a-5p	258.38	38.38	6.73	0.00415445	2.25	20.12
146	hsa-miR-146b-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
147	hsa-miR-146b-5p	8.11	10.75	-1.33	0.57517999	-4.91	2.79
148	hsa-miR-147a	10.57	7.58	1.39	0.50230312	-2.19	4.26
149	hsa-miR-147b	1.24	1.54	-1.25	0.25182578	-1.92	1.23
150	hsa-miR-148a-3p	46.35	6.92	6.7	0.01559391	1.55	28.89
151	hsa-miR-148b-3p	31.28	2.78	11.23	0.00407792	2.67	47.31
152	hsa-miR-149-5p	7.45	8.28	-1.11	0.87745917	-5.55	4.5
153	hsa-miR-150-5p	392.81	140.78	2.79	0.04414333	-1.01	7.86
154	hsa-miR-151a-3p	87.19	4.15	21	0.00383801	3.53	125
155	hsa-miR-151a-5p	3.87	1.54	2.51	0.16780831	-1.9	11.95
156	hsa-miR-151b	1.24	1.54	-1.25	0.25182578	-1.92	1.23
157	hsa-miR-152-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
158	hsa-miR-152-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
159	hsa-miR-153-3p	1.64	2.45	-1.49	0.47364557	-5.25	2.36
160	hsa-miR-1537-3p	2.45	1.59	1.54	0.43357304	-2.61	6.17
161	hsa-miR-154-5p	5.39	3.62	1.49	0.61573821	-4.06	9.03
162	hsa-miR-155-5p	12.03	22	-1.83	0.03681178	-3.23	-1.04
163	hsa-miR-15a-5p	143.08	22.86	6.26	0.0018925	2.43	16.14
164	hsa-miR-15b-5p	198.62	19.75	10.06	0.00387522	2.76	36.63
165	hsa-miR-16-5p	365.8	229.26	1.6	0.12818784	-1.2	3.06
166	hsa-miR-181a-2-3p	1.24	1.59	-1.28	0.1699653	-1.89	1.15
167	hsa-miR-181a-3p	3.68	4.31	-1.17	0.73183006	-3.39	2.46
168	hsa-miR-181a-5p	192.31	20.19	9.52	0.00252303	3.18	28.5
169	hsa-miR-181b-2-3p	11.3	8.84	1.28	0.66151637	-2.81	4.59
170	hsa-miR-181b-5p+ hsa-miR-181d-5p	2.77	1.88	1.47	0.50877243	-3.05	6.62
171	hsa-miR-181c-5p	2.33	1.54	1.51	0.38805154	-2.04	4.65
172	hsa-miR-181d-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
173	hsa-miR-182-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
174	hsa-miR-182-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
175	hsa-miR-1827	22.38	14.86	1.51	0.270327	-1.66	3.76
176	hsa-miR-183-5p	17.73	22.88	-1.29	0.57772285	-3.64	2.19
177	hsa-miR-184	1.64	2.05	-1.25	0.47650859	-2.5	1.61
178	hsa-miR-185-5p	61.83	8.05	7.68	0.01025543	1.86	31.62
179	hsa-miR-186-5p	23.05	18.51	1.24	0.5964945	-2.19	3.4
180	hsa-miR-187-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
181	hsa-miR-188-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
182	hsa-miR-188-5p	7.2	8.05	-1.12	0.8076297	-3.19	2.55
183	hsa-miR-18a-5p	12.51	1.54	8.12	0.02475978	1.41	46.66
184	hsa-miR-18b-5p	1.41	1.54	-1.09	0.64843214	-1.69	1.42
185	hsa-miR-1908-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
186	hsa-miR-1908-5p	2.33	1.54	1.51	0.38805154	-2.04	4.65

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
187	hsa-miR-1909-3p	1.24	1.67	-1.35	0.14520256	-2.11	1.16
188	hsa-miR-190a-3p	2.02	1.59	1.28	0.55021858	-2.09	3.39
189	hsa-miR-190a-5p	2.11	1.8	1.18	0.75078404	-3.24	4.49
190	hsa-miR-190b	3.82	3.48	1.1	0.83613116	-2.58	3.12
191	hsa-miR-191-5p	1116.92	122.42	9.12	0.00080796	3.61	23.05
192	hsa-miR-1910-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
193	hsa-miR-1910-5p	6.99	2.44	2.87	0.12783654	-1.5	12.35
194	hsa-miR-1915-3p	1.59	1.54	1.03	0.93193436	-2.29	2.42
195	hsa-miR-192-5p	11.16	9.02	1.24	0.69405657	-2.78	4.26
196	hsa-miR-193a-3p	1.78	1.54	1.15	0.70031035	-2.15	2.86
197	hsa-miR-193a-5p+ hsa-miR-193b-5p	6.76	1.81	3.72	0.02971303	1.16	12
198	hsa-miR-193b-3p	2.08	4.19	-2.02	0.27243894	-8.26	2.03
199	hsa-miR-194-5p	2.18	2.14	1.02	0.96481001	-2.7	2.81
200	hsa-miR-195-5p	1.67	2.09	-1.25	0.54091591	-2.88	1.84
201	hsa-miR-196a-3p	1.34	2.57	-1.92	0.1648699	-5.42	1.47
202	hsa-miR-196a-5p	15.87	13.08	1.21	0.52783221	-1.72	2.54
203	hsa-miR-196b-5p	3.02	2.07	1.46	0.49229494	-2.78	5.92
204	hsa-miR-197-3p	20.31	10.9	1.86	0.18333301	-1.47	5.08
205	hsa-miR-197-5p	11.95	12	-1	0.99401397	-3.65	3.62
206	hsa-miR-1972	12.4	16.43	-1.32	0.63408262	-5.08	2.89
207	hsa-miR-1973	3.38	1.97	1.72	0.32313564	-2	5.92
208	hsa-miR-1976	1523.68	890.42	1.71	0.0006008	1.37	2.14
209	hsa-miR-198	3.82	7.76	-2.03	0.14893746	-5.86	1.42
210	hsa-miR-199a-3p+ hsa-miR-199b-3p	571.16	57.9	9.86	0.00393784	2.75	35.43
211	hsa-miR-199a-5p	119.98	15.07	7.96	0.00490844	2.46	25.83
212	hsa-miR-199b-5p	2.78	2.52	1.11	0.79659557	-2.2	2.69
213	hsa-miR-19a-3p	28.7	3.77	7.61	0.01498987	1.62	35.77
214	hsa-miR-19b-3p	55.11	5.16	10.68	0.00995718	2.13	53.51
215	hsa-miR-200a-3p	3.6	1.55	2.32	0.1105585	-1.32	7.13
216	hsa-miR-200b-3p	1.26	1.54	-1.22	0.27988797	-1.86	1.24
217	hsa-miR-200c-3p	5.69	3.79	1.5	0.54462564	-3.04	6.86
218	hsa-miR-202-3p	1.6	1.54	1.04	0.89256668	-1.8	1.94
219	hsa-miR-203a-3p	2.75	3.08	-1.12	0.86498082	-4.92	3.94
220	hsa-miR-203a-5p	5.42	5.51	-1.02	0.98010254	-4.43	4.29
221	hsa-miR-204-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
222	hsa-miR-205-5p	2.68	3.65	-1.36	0.59695143	-5.12	2.76
223	hsa-miR-2053	14.68	16.6	-1.13	0.74198622	-2.72	2.12
224	hsa-miR-206	4.18	4.36	-1.04	0.95307297	-5.65	5.19
225	hsa-miR-208a-3p	1.41	1.54	-1.09	0.64843214	-1.69	1.42
226	hsa-miR-208b-3p	7.95	14.13	-1.78	0.37616998	-8.25	2.61
227	hsa-miR-208b-5p	2.53	2.97	-1.17	0.77280974	-4.18	3.04
228	hsa-miR-20a-5p+ hsa-miR-20b-5p	107.17	34.77	3.08	0.05848084	-1.07	10.13
229	hsa-miR-21-5p	186.41	24.05	7.75	0.00407576	2.47	24.36
230	hsa-miR-210-3p	2.43	1.94	1.25	0.729289	-4.39	6.89
231	hsa-miR-210-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
232	hsa-miR-211-3p	8.15	8.39	-1.03	0.96696615	-5.33	5.03
233	hsa-miR-211-5p	1.24	2.22	-1.8	0.04782913	-3.26	1.01
234	hsa-miR-2110	6.94	7.94	-1.14	0.80101979	-3.89	2.97
235	hsa-miR-2113	1.24	2.08	-1.68	0.17312905	-4.15	1.46
236	hsa-miR-2116-5p	1.64	1.54	1.06	0.82141763	-1.76	2
237	hsa-miR-2117	2.62	5.3	-2.02	0.28601384	-8.67	2.12
238	hsa-miR-212-3p	20.53	22.4	-1.09	0.84113461	-3.17	2.66
239	hsa-miR-214-3p	2.06	2.14	-1.04	0.93826246	-2.99	2.79
240	hsa-miR-215-5p	11.42	7.93	1.44	0.42396945	-1.98	4.11
241	hsa-miR-216a-5p	1.92	1.86	1.03	0.95229322	-3.35	3.56
242	hsa-miR-216b-5p	4.43	7.89	-1.78	0.45751956	-10.19	3.22
243	hsa-miR-217	1.59	1.92	-1.21	0.4693867	-2.21	1.51
244	hsa-miR-218-5p	4.19	4.89	-1.17	0.75229013	-3.83	2.82
245	hsa-miR-219a-1-3p	2.29	5.72	-2.5	0.1029068	-7.97	1.28
246	hsa-miR-219a-2-3p	2.87	3.47	-1.21	0.73681271	-4.45	3.03
247	hsa-miR-219a-5p	1.92	3.47	-1.81	0.34922761	-7.62	2.32
248	hsa-miR-219b-3p	13.23	16.22	-1.23	0.50824851	-2.58	1.72
249	hsa-miR-22-3p	159.46	25.01	6.38	0.00998531	1.75	23.24
250	hsa-miR-221-3p	84.16	15.4	5.47	0.0405806	1.06	28.08
251	hsa-miR-221-5p	1.24	1.59	-1.29	0.16066656	-1.89	1.14
252	hsa-miR-222-3p	96.59	32.13	3.01	0.02842514	1.14	7.95
253	hsa-miR-223-3p	4462.61	865.23	5.16	0.00764593	1.81	14.7
254	hsa-miR-224-5p	16.6	25.99	-1.57	0.23766871	-3.93	1.6
255	hsa-miR-2278	1.24	1.54	-1.25	0.25182578	-1.92	1.23
256	hsa-miR-23a-3p	1312.23	238.63	5.5	0.00644295	2.05	14.74
257	hsa-miR-23b-3p	14.57	2.52	5.79	0.02448003	1.33	25.22
258	hsa-miR-23c	1.56	1.54	1.01	0.96857554	-1.7	1.73
259	hsa-miR-24-3p	51.17	9.1	5.62	0.01882955	1.41	22.45
260	hsa-miR-25-3p	507.25	213.28	2.38	0.01043873	1.31	4.33
261	hsa-miR-25-5p	3.67	3.08	1.19	0.77619207	-3.45	4.9
262	hsa-miR-2682-5p	1.97	4.68	-2.38	0.2283998	-11.24	1.99
263	hsa-miR-26a-5p	55.14	5.57	9.9	0.0045762	2.55	38.44
264	hsa-miR-26b-5p	13.07	11.83	1.1	0.8892231	-5.87	7.16
265	hsa-miR-27a-3p	10.05	9.47	1.06	0.92941195	-4.59	5.17
266	hsa-miR-27b-3p	12.48	1.75	7.12	0.03888194	1.07	47.34
267	hsa-miR-28-3p	14.44	2.58	5.6	0.01367468	1.59	19.7
268	hsa-miR-28-5p	50.15	15.59	3.22	0.06192989	-1.11	11.46
269	hsa-miR-296-3p	1.34	1.69	-1.26	0.42085427	-2.45	1.54
270	hsa-miR-296-5p	3.57	1.54	2.32	0.14645955	-1.66	8.94
271	hsa-miR-297	1.59	1.81	-1.14	0.74183512	-2.88	2.21
272	hsa-miR-298	6.7	13.16	-1.97	0.26225671	-7.92	2.05
273	hsa-miR-299-3p	1.68	2.76	-1.65	0.26771352	-4.4	1.62
274	hsa-miR-299-5p	10.86	11.62	-1.07	0.86398768	-2.66	2.32
275	hsa-miR-29a-3p	45.26	14.51	3.12	0.0307927	1.17	8.33
276	hsa-miR-29b-3p	33.94	4.04	8.39	0.00691449	2.3	30.67
277	hsa-miR-29c-3p	6.52	1.54	4.23	0.05304775	-1.11	19.87
278	hsa-miR-300	2.6	2.35	1.11	0.83674288	-2.84	3.49
279	hsa-miR-301a-3p	16.1	1.54	10.44	0.00401741	2.9	37.63

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
280	hsa-miR-301a-5p	10.5	10.8	-1.03	0.96373808	-4.67	4.41
281	hsa-miR-301b-3p	10.48	15.04	-1.43	0.38981798	-4.11	2
282	hsa-miR-301b-5p	2.69	3.36	-1.25	0.67840427	-4.23	2.71
283	hsa-miR-302a-3p	1.27	1.81	-1.43	0.24802253	-2.92	1.43
284	hsa-miR-302a-5p	1.94	1.95	-1.01	0.9909969	-4.22	4.17
285	hsa-miR-302b-3p	5.85	6.5	-1.11	0.86849689	-4.74	3.84
286	hsa-miR-302c-3p	1.24	1.8	-1.45	0.06010085	-2.16	1.02
287	hsa-miR-302d-3p	116.08	226.24	-1.95	0.12953998	-5.06	1.33
288	hsa-miR-302e	1.24	1.54	-1.25	0.25182578	-1.92	1.23
289	hsa-miR-302f	2.14	1.8	1.19	0.60483277	-1.89	2.67
290	hsa-miR-3065-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
291	hsa-miR-3065-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
292	hsa-miR-3074-3p	2.18	3.8	-1.74	0.42871931	-8.33	2.75
293	hsa-miR-30a-3p	6.94	2.4	2.89	0.06254067	-1.1	9.17
294	hsa-miR-30a-5p	14.56	4.57	3.18	0.07166276	-1.16	11.74
295	hsa-miR-30b-5p	3.81	1.54	2.47	0.13636805	-1.65	10.06
296	hsa-miR-30c-5p	11.31	6.73	1.68	0.2848582	-1.73	4.9
297	hsa-miR-30d-5p	18.85	3.35	5.63	0.09779086	-1.59	50.27
298	hsa-miR-30e-3p	5.83	3.07	1.9	0.32061985	-2.25	8.14
299	hsa-miR-30e-5p	28.83	17.12	1.68	0.13217713	-1.24	3.51
300	hsa-miR-31-5p	1.67	2.29	-1.37	0.58188319	-5.03	2.67
301	hsa-miR-3127-5p	2.29	10.2	-4.45	0.03323983	-17.72	-1.12
302	hsa-miR-3130-3p	10.42	13.3	-1.28	0.70431125	-5.54	3.4
303	hsa-miR-3131	11.26	22.47	-2	0.0728434	-4.36	1.1
304	hsa-miR-3136-5p	7.38	16.3	-2.21	0.16236286	-7.6	1.56
305	hsa-miR-3140-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
306	hsa-miR-3140-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
307	hsa-miR-3144-3p	53.33	99.42	-1.86	0.14148477	-4.59	1.32
308	hsa-miR-3144-5p	2.12	1.59	1.34	0.48570859	-2.02	3.62
309	hsa-miR-3147	4.73	10.05	-2.13	0.20629819	-7.68	1.7
310	hsa-miR-3150b-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
311	hsa-miR-3151-5p	6.63	6.95	-1.05	0.93271333	-4.18	3.8
312	hsa-miR-3158-3p	1.48	1.54	-1.04	0.89573783	-2.05	1.9
313	hsa-miR-3161	2.52	3.83	-1.52	0.42798606	-4.96	2.15
314	hsa-miR-3164	3.89	5.01	-1.29	0.69532079	-5.6	3.37
315	hsa-miR-3168	1.26	1.54	-1.22	0.27988797	-1.86	1.24
316	hsa-miR-3179	1.94	1.68	1.16	0.7509712	-2.64	3.53
317	hsa-miR-3180	16.16	8.82	1.83	0.37065625	-2.46	8.27
318	hsa-miR-3180-3p	2.42	2.05	1.18	0.67332566	-2.07	2.89
319	hsa-miR-3180-5p	1.26	1.8	-1.43	0.06649304	-2.12	1.04
320	hsa-miR-3182	1.24	1.95	-1.58	0.07187407	-2.69	1.08
321	hsa-miR-3185	1.93	1.54	1.25	0.55125773	-1.99	3.13
322	hsa-miR-3190-3p	1.67	1.54	1.08	0.7996307	-1.92	2.24
323	hsa-miR-3192-5p	2.79	1.54	1.81	0.26426569	-2.04	6.67
324	hsa-miR-3195	2.62	4.43	-1.69	0.37774336	-6.98	2.43
325	hsa-miR-3196	1.59	1.54	1.03	0.93193436	-2.29	2.42
326	hsa-miR-32-5p	4.05	2.28	1.77	0.33777329	-2.31	7.28
327	hsa-miR-3202	7.41	5.2	1.43	0.39221665	-1.77	3.6

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
328	hsa-miR-320a	3.22	1.77	1.81	0.26940122	-1.89	6.22
329	hsa-miR-320b	1.24	1.59	-1.28	0.1699653	-1.89	1.15
330	hsa-miR-320c	1.29	1.54	-1.19	0.3360334	-1.8	1.27
331	hsa-miR-320d	1.24	1.54	-1.25	0.25182578	-1.92	1.23
332	hsa-miR-320e	512.32	158.75	3.23	0.25176054	-3.03	31.57
333	hsa-miR-323a-3p	4.38	5	-1.14	0.84586966	-5.71	4.38
334	hsa-miR-323a-5p	1.26	1.54	-1.22	0.27988797	-1.86	1.24
335	hsa-miR-323b-3p	4.43	2.06	2.15	0.11645876	-1.32	6.12
336	hsa-miR-323b-5p	9.04	8.41	1.07	0.8362304	-2.17	2.5
337	hsa-miR-324-3p	24.2	25.23	-1.04	0.90408713	-2.57	2.37
338	hsa-miR-324-5p	15.61	1.54	10.13	0.01360234	1.96	52.34
339	hsa-miR-325	22.07	22.09	-1	0.99830949	-2.84	2.84
340	hsa-miR-326	1.48	1.54	-1.04	0.89573783	-2.05	1.9
341	hsa-miR-328-3p	1.97	2.05	-1.04	0.9401297	-3.37	3.13
342	hsa-miR-328-5p	1.24	1.8	-1.45	0.06010085	-2.16	1.02
343	hsa-miR-329-3p	2.66	1.55	1.72	0.16715442	-1.4	4.16
344	hsa-miR-329-5p	5.34	2.95	1.81	0.30946314	-2.01	6.59
345	hsa-miR-330-3p	1.74	1.54	1.13	0.78238434	-2.56	3.26
346	hsa-miR-330-5p	2.42	1.81	1.34	0.5747624	-2.49	4.44
347	hsa-miR-331-3p	15.96	10.39	1.54	0.54358113	-3.21	7.58
348	hsa-miR-331-5p	1.52	1.54	-1.02	0.94560981	-1.73	1.68
349	hsa-miR-335-5p	5.24	3.38	1.55	0.37749499	-1.94	4.68
350	hsa-miR-337-3p	1.24	1.87	-1.51	0.16839245	-2.93	1.28
351	hsa-miR-337-5p	6.89	1.86	3.71	0.00574802	1.66	8.27
352	hsa-miR-338-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
353	hsa-miR-339-3p	2.51	1.54	1.63	0.19345717	-1.44	3.84
354	hsa-miR-339-5p	1.35	1.54	-1.14	0.47194192	-1.74	1.33
355	hsa-miR-33a-5p	996.07	812.13	1.23	0.14126465	-1.09	1.64
356	hsa-miR-33b-5p	2.77	4.18	-1.51	0.41081148	-4.59	2.02
357	hsa-miR-340-5p	48.73	32.15	1.52	0.30190998	-1.69	3.87
358	hsa-miR-342-3p	48.06	13.08	3.67	0.0378577	1.05	12.87
359	hsa-miR-342-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
360	hsa-miR-345-3p	1.26	1.54	-1.22	0.27988797	-1.86	1.24
361	hsa-miR-345-5p	1.87	2.29	-1.22	0.73604536	-4.81	3.21
362	hsa-miR-346	5.39	4.89	1.1	0.8971957	-4.99	6.05
363	hsa-miR-34a-5p	3.38	2.04	1.66	0.35812047	-2.1	5.79
364	hsa-miR-34b-3p	1.98	1.54	1.28	0.66083223	-3.43	5.65
365	hsa-miR-34c-3p	1.24	2.06	-1.67	0.24020347	-4.76	1.71
366	hsa-miR-34c-5p	1.24	1.77	-1.43	0.1006453	-2.27	1.1
367	hsa-miR-3605-3p	1.35	1.54	-1.14	0.47194192	-1.74	1.33
368	hsa-miR-3605-5p	2.07	1.8	1.15	0.64689094	-1.79	2.38
369	hsa-miR-361-3p	9.95	1.94	5.13	0.04093809	-1.01	26.6
370	hsa-miR-361-5p	58.46	4.45	13.15	0.00990635	2.15	80.43
371	hsa-miR-3613-3p	2.26	1.72	1.32	0.54274541	-2.44	4.23
372	hsa-miR-3613-5p	1.82	1.54	1.18	0.67085725	-2.22	3.11
373	hsa-miR-3614-3p	2.08	2.47	-1.19	0.67032111	-3.23	2.28
374	hsa-miR-3614-5p	1.52	1.54	-1.02	0.94560981	-1.73	1.68
375	hsa-miR-3615	2.46	7.29	-2.97	0.04037492	-8.55	-1.03

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
376	hsa-miR-362-3p	1.6	1.54	1.04	0.89256668	-1.8	1.94
377	hsa-miR-362-5p	2.51	1.8	1.4	0.46069065	-2.29	4.47
378	hsa-miR-363-3p	8.1	4.71	1.72	0.36902976	-2.31	6.86
379	hsa-miR-363-5p	3.05	1.97	1.55	0.37392074	-1.99	4.79
380	hsa-miR-365a-3p+ hsa-miR-365b-3p	9.42	7.2	1.31	0.61836356	-2.58	4.42
381	hsa-miR-365b-5p	1.98	1.54	1.29	0.30248493	-1.33	2.2
382	hsa-miR-367-3p	2.48	2.38	1.04	0.92766881	-2.74	2.96
383	hsa-miR-369-3p	4	1.62	2.46	0.08931803	-1.24	7.55
384	hsa-miR-369-5p	1.48	1.54	-1.04	0.89573783	-2.05	1.9
385	hsa-miR-3690	1.35	2.14	-1.58	0.16845508	-3.48	1.39
386	hsa-miR-370-3p	1.67	1.54	1.08	0.7996307	-1.92	2.24
387	hsa-miR-370-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
388	hsa-miR-371a-5p	5.4	14.56	-2.7	0.09901698	-9.42	1.29
389	hsa-miR-371b-5p	1.41	2.38	-1.69	0.22491936	-4.8	1.68
390	hsa-miR-372-3p	1.27	2.03	-1.6	0.07403038	-2.76	1.07
391	hsa-miR-373-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
392	hsa-miR-374a-3p	3.89	3.52	1.1	0.86297584	-3.33	4.05
393	hsa-miR-374a-5p	68.73	10.86	6.33	0.01356465	1.66	24.14
394	hsa-miR-374b-5p	15.37	2.25	6.84	0.01153182	1.74	26.85
395	hsa-miR-374c-5p	1.48	4.47	-3.01	0.02468	-7.65	-1.19
396	hsa-miR-375	1.24	1.54	-1.25	0.25182578	-1.92	1.23
397	hsa-miR-376a-2-5p	1.52	2.23	-1.47	0.13062766	-2.53	1.17
398	hsa-miR-376a-3p	32.07	19.19	1.67	0.22284524	-1.55	4.33
399	hsa-miR-376b-3p	2.53	2.96	-1.17	0.78591728	-4.36	3.19
400	hsa-miR-376c-3p	3.07	2.05	1.5	0.56118083	-3.57	8.04
401	hsa-miR-376c-5p	1.4	1.94	-1.38	0.20505956	-2.4	1.26
402	hsa-miR-377-3p	12.12	6.96	1.74	0.32502472	-2	6.06
403	hsa-miR-378b	2.16	1.92	1.12	0.7843281	-2.42	3.06
404	hsa-miR-378c	1.24	1.67	-1.35	0.14520256	-2.11	1.16
405	hsa-miR-378d	1.29	1.54	-1.19	0.3360334	-1.8	1.27
406	hsa-miR-378e	1.24	1.54	-1.25	0.25182578	-1.92	1.23
407	hsa-miR-378f	18.58	29.6	-1.59	0.29773793	-4.51	1.77
408	hsa-miR-378g	15.53	8.78	1.77	0.29244009	-1.87	5.85
409	hsa-miR-378h	11.15	17.01	-1.53	0.50230086	-6.33	2.72
410	hsa-miR-378i	12.41	5.97	2.08	0.30718794	-2.44	10.56
411	hsa-miR-379-5p	41.83	41.89	-1	0.99780762	-2.87	2.86
412	hsa-miR-380-3p	6.89	13.96	-2.03	0.23160562	-8.42	2.05
413	hsa-miR-381-3p	2.81	1.54	1.83	0.30817255	-2.37	7.89
414	hsa-miR-381-5p	2.43	2.21	1.1	0.874138	-3.49	4.21
415	hsa-miR-382-3p	3.23	3.75	-1.16	0.77242607	-3.78	2.8
416	hsa-miR-382-5p	14.8	3.02	4.91	0.05027719	-1.03	24.71
417	hsa-miR-383-5p	1.26	1.54	-1.22	0.27988797	-1.86	1.24
418	hsa-miR-384	3.17	4.6	-1.45	0.41210845	-4.1	1.95
419	hsa-miR-3916	1.24	1.54	-1.25	0.25182578	-1.92	1.23
420	hsa-miR-3918	1.24	1.54	-1.25	0.25182578	-1.92	1.23
421	hsa-miR-3928-3p	2.21	2.14	1.03	0.94049829	-2.44	2.59
422	hsa-miR-3934-5p	10.76	4.64	2.32	0.20715041	-1.83	9.88

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
423	hsa-miR-409-3p	5.25	2.05	2.56	0.26868826	-3.15	20.66
424	hsa-miR-409-5p	1.56	1.54	1.01	0.96857554	-1.7	1.73
425	hsa-miR-410-3p	6.71	8.65	-1.29	0.70973986	-7.69	4.62
426	hsa-miR-411-5p	18.99	32.79	-1.73	0.15503296	-4.02	1.35
427	hsa-miR-412-3p	8.92	5.76	1.55	0.5075106	-2.98	7.14
428	hsa-miR-421	17.36	10.64	1.63	0.27395388	-1.73	4.61
429	hsa-miR-422a	1.24	2.81	-2.28	0.28534079	-14.67	2.83
430	hsa-miR-423-3p	46.35	11.15	4.16	0.03892961	1.08	16.06
431	hsa-miR-423-5p	175.71	63.6	2.76	0.06551373	-1.11	8.5
432	hsa-miR-424-5p	6.06	2.33	2.6	0.17269154	-1.81	12.27
433	hsa-miR-425-5p	26.01	5.3	4.91	0.13947338	-2.08	50.03
434	hsa-miR-4284	1.24	1.54	-1.25	0.25182578	-1.92	1.23
435	hsa-miR-4286	18.12	15.73	1.15	0.87182522	-7.43	9.85
436	hsa-miR-429	1.41	1.61	-1.14	0.52761799	-1.88	1.43
437	hsa-miR-431-5p	1.9	1.54	1.24	0.63067567	-2.35	3.59
438	hsa-miR-432-5p	9.63	7.25	1.33	0.65147418	-3.14	5.53
439	hsa-miR-433-3p	7.73	6.07	1.27	0.69065297	-3.11	5.05
440	hsa-miR-433-5p	5.09	8.28	-1.63	0.37306508	-5.6	2.12
441	hsa-miR-4421	1.78	1.54	1.15	0.70031035	-2.15	2.86
442	hsa-miR-4425	1.24	2.83	-2.29	0.047987	-5.47	1.04
443	hsa-miR-4431	1.95	2.44	-1.25	0.58368582	-3.13	2
444	hsa-miR-4435	1.6	1.61	-1.01	0.97337162	-1.94	1.91
445	hsa-miR-4443	9.51	2.8	3.4	0.02630378	1.18	9.83
446	hsa-miR-4448	1.41	1.61	-1.14	0.52761799	-1.88	1.43
447	hsa-miR-4451	1.4	2.22	-1.59	0.11215412	-2.95	1.17
448	hsa-miR-4454+ hsa-miR-7975	1234.34	638.78	1.93	0.53984141	-8.16	30.47
449	hsa-miR-4455	7.67	9.41	-1.23	0.79108363	-7.2	4.78
450	hsa-miR-4458	1.39	1.81	-1.31	0.36728376	-2.64	1.54
451	hsa-miR-4461	5.73	5.84	-1.02	0.96585137	-2.85	2.74
452	hsa-miR-448	2.98	2.18	1.37	0.5729233	-2.65	4.94
453	hsa-miR-4485-3p	1.24	1.59	-1.28	0.1699653	-1.89	1.15
454	hsa-miR-4488	2.09	1.61	1.3	0.22484338	-1.23	2.08
455	hsa-miR-449a	1.73	1.54	1.12	0.67458069	-1.71	2.16
456	hsa-miR-449b-5p	2.47	2.49	-1.01	0.98369539	-3.36	3.29
457	hsa-miR-449c-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
458	hsa-miR-450a-1-3p	2.15	2.75	-1.28	0.6408596	-4.25	2.59
459	hsa-miR-450a-2-3p	1.41	1.54	-1.09	0.64843214	-1.69	1.42
460	hsa-miR-450a-5p	3.16	3.23	-1.02	0.9754585	-5.32	5.09
461	hsa-miR-450b-3p	2.47	7.57	-3.06	0.06159622	-10.14	1.08
462	hsa-miR-450b-5p	2.01	1.59	1.27	0.52010351	-1.91	3.07
463	hsa-miR-4516	45.22	28.17	1.61	0.6032443	-4.92	12.67
464	hsa-miR-451a	8817.88	5002.91	1.76	0.10876871	-1.24	3.86
465	hsa-miR-452-5p	2.2	2.49	-1.13	0.79251456	-3.36	2.62
466	hsa-miR-4521	1.69	1.54	1.1	0.70095205	-1.57	1.89
467	hsa-miR-4524a-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
468	hsa-miR-4531	1.72	6.95	-4.03	0.02489004	-13.29	-1.22
469	hsa-miR-4532	6.06	1.59	3.82	0.0307711	1.12	13.06

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
470	hsa-miR-4536-3p	2.75	4.84	-1.76	0.18241264	-4.38	1.41
471	hsa-miR-4536-5p	28.24	30.29	-1.07	0.85169226	-2.88	2.51
472	hsa-miR-454-3p	1.59	2.24	-1.41	0.43184268	-4	2.01
473	hsa-miR-455-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
474	hsa-miR-455-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
475	hsa-miR-4647	1.77	1.54	1.15	0.64293635	-1.73	2.27
476	hsa-miR-4707-3p	4.83	3.37	1.43	0.61357653	-3.51	7.21
477	hsa-miR-4707-5p	2.73	2.69	1.01	0.9784345	-3.32	3.42
478	hsa-miR-4741	2.47	2.25	1.1	0.8447662	-2.71	3.27
479	hsa-miR-4755-5p	19.42	20.12	-1.04	0.91979438	-2.44	2.27
480	hsa-miR-4787-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
481	hsa-miR-4787-5p	2.71	2.82	-1.04	0.94350213	-3.68	3.4
482	hsa-miR-4792	1.24	1.95	-1.58	0.02034418	-2.28	-1.09
483	hsa-miR-483-3p	1.24	2.06	-1.67	0.24020347	-4.76	1.71
484	hsa-miR-483-5p	2.12	1.54	1.38	0.46128997	-2.04	3.87
485	hsa-miR-484	5.52	2.51	2.2	0.21695881	-1.8	8.74
486	hsa-miR-485-3p	9.86	3.28	3	0.2153313	-2.26	20.4
487	hsa-miR-485-5p	3.08	5.08	-1.65	0.53945237	-10.3	3.79
488	hsa-miR-486-3p	41.36	27.95	1.48	0.48268101	-2.57	5.63
489	hsa-miR-487a-3p	1.67	1.54	1.08	0.7996307	-1.92	2.24
490	hsa-miR-487b-3p	2.93	1.54	1.9	0.23260637	-1.94	7.01
491	hsa-miR-487b-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
492	hsa-miR-488-3p	3.05	2.2	1.38	0.47565603	-2.14	4.1
493	hsa-miR-489-3p	1.67	1.92	-1.15	0.74059391	-3.13	2.36
494	hsa-miR-490-3p	1.89	2.28	-1.2	0.62070739	-2.82	1.95
495	hsa-miR-490-5p	1.73	1.54	1.12	0.74073231	-2.07	2.6
496	hsa-miR-491-3p	1.33	1.54	-1.16	0.42599782	-1.79	1.33
497	hsa-miR-491-5p	6.43	3.27	1.97	0.17524651	-1.49	5.76
498	hsa-miR-492	1.29	1.54	-1.19	0.3360334	-1.8	1.27
499	hsa-miR-493-3p	3.17	2.99	1.06	0.93923628	-5.57	6.24
500	hsa-miR-494-3p	1.24	5.16	-4.18	0.32202008	-141.27	8.1
501	hsa-miR-494-5p	2.4	2.21	1.09	0.88697743	-3.47	4.08
502	hsa-miR-495-3p	55.81	56.93	-1.02	0.95692039	-2.7	2.6
503	hsa-miR-495-5p	3.28	1.67	1.96	0.1423469	-1.4	5.41
504	hsa-miR-496	2.22	2.14	1.04	0.93808436	-2.75	2.95
505	hsa-miR-497-5p	5.61	5.28	1.06	0.89688265	-3.02	3.41
506	hsa-miR-498	5.14	12.16	-2.37	0.14489892	-8.62	1.54
507	hsa-miR-499a-3p	4.19	5.09	-1.21	0.70650876	-3.91	2.65
508	hsa-miR-499a-5p	17.44	30.9	-1.77	0.19248347	-4.55	1.45
509	hsa-miR-499b-3p	1.34	2.4	-1.79	0.11369012	-4.04	1.25
510	hsa-miR-499b-5p	1.53	2.29	-1.5	0.21444702	-3.03	1.35
511	hsa-miR-5001-3p	1.6	1.54	1.04	0.89256668	-1.8	1.94
512	hsa-miR-5001-5p	1.69	3.01	-1.78	0.19766003	-4.75	1.51
513	hsa-miR-500a-5p+ hsa-miR-501-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
514	hsa-miR-501-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
515	hsa-miR-5010-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
516	hsa-miR-5010-5p	1.26	2.12	-1.68	0.07804801	-3.09	1.09

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
517	hsa-miR-502-3p	5.9	3.96	1.49	0.47056323	-2.33	5.16
518	hsa-miR-502-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
519	hsa-miR-503-3p	1.6	2.04	-1.27	0.42369992	-2.73	1.69
520	hsa-miR-503-5p	22.92	6.64	3.45	0.03542305	1.1	10.79
521	hsa-miR-504-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
522	hsa-miR-504-5p	1.65	2.44	-1.48	0.34780735	-3.69	1.69
523	hsa-miR-505-3p	3.99	3.42	1.17	0.79923654	-3.55	4.83
524	hsa-miR-506-3p	2.99	4.5	-1.5	0.45209685	-5.27	2.33
525	hsa-miR-506-5p	10.73	11.66	-1.09	0.89656276	-4.7	3.98
526	hsa-miR-507	1.24	1.54	-1.25	0.25182578	-1.92	1.23
527	hsa-miR-508-3p	4.88	16.2	-3.32	0.04921756	-11.47	1.04
528	hsa-miR-508-5p	1.87	2.17	-1.17	0.79962134	-4.61	3.39
529	hsa-miR-509-3-5p	1.78	3.77	-2.11	0.17500226	-6.92	1.55
530	hsa-miR-509-3p	1.67	1.92	-1.15	0.6846965	-2.56	1.93
531	hsa-miR-509-5p	1.59	2.57	-1.62	0.18279907	-3.7	1.41
532	hsa-miR-510-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
533	hsa-miR-510-5p	1.24	1.77	-1.43	0.1006453	-2.27	1.1
534	hsa-miR-511-5p	12.77	17.47	-1.37	0.47389504	-3.89	2.08
535	hsa-miR-512-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
536	hsa-miR-512-5p	1.68	1.54	1.09	0.74610138	-1.7	2.02
537	hsa-miR-513a-3p	1.82	1.59	1.15	0.72112215	-2.25	2.98
538	hsa-miR-513a-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
539	hsa-miR-513b-5p	6.61	5.99	1.1	0.85877341	-3.18	3.86
540	hsa-miR-513c-3p	4.48	15.46	-3.45	0.06527545	-13.3	1.12
541	hsa-miR-513c-5p	1.24	2.33	-1.88	0.02774884	-3.29	-1.08
542	hsa-miR-514a-3p	12.45	9.45	1.32	0.54398257	-2.12	3.69
543	hsa-miR-514a-5p	4.27	2.68	1.59	0.40780801	-2.27	5.79
544	hsa-miR-514b-3p	13.52	8.48	1.59	0.4694784	-2.78	7.06
545	hsa-miR-514b-5p	6.94	7.58	-1.09	0.8680985	-3.73	3.12
546	hsa-miR-515-3p	1.55	1.92	-1.24	0.51500702	-2.62	1.7
547	hsa-miR-515-5p	3.18	2.33	1.37	0.59459823	-2.76	5.15
548	hsa-miR-516a-3p+ hsa-miR-516b-3p	2.77	2.71	1.02	0.96721488	-3.54	3.7
549	hsa-miR-516a-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
550	hsa-miR-516b-5p	1.97	1.54	1.28	0.45710284	-1.67	2.74
551	hsa-miR-517a-3p	5.08	3.61	1.41	0.6553489	-4.24	8.41
552	hsa-miR-517b-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
553	hsa-miR-517c-3p+hsa-miR-519a-3p	1.24	1.86	-1.51	0.27881894	-3.82	1.68
554	hsa-miR-518b	2.77	2.31	1.2	0.72787845	-2.69	3.85
555	hsa-miR-518c-3p	1.52	1.54	-1.02	0.94560981	-1.73	1.68
556	hsa-miR-518d-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
557	hsa-miR-518e-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
558	hsa-miR-518f-3p	3.28	7.11	-2.17	0.32445461	-12.33	2.63
559	hsa-miR-5196-3p+ hsa-miR-6732-3p	1.62	1.54	1.05	0.84816694	-1.75	1.95
560	hsa-miR-5196-5p	2.52	2.33	1.08	0.87329239	-3.1	3.63
561	hsa-miR-519b-3p	1.24	1.61	-1.31	0.21559229	-2.1	1.23

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
562	hsa-miR-519b-5p+ hsa-miR-519c-5p+ hsa-miR-523-5p+ hsa-miR-518e-5p+ hsa-miR-522-5p+ hsa-miR-519a-5p	2.04	1.55	1.32	0.42061841	-1.65	2.86
563	hsa-miR-519c-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
564	hsa-miR-519d-3p	2.64	6.03	-2.28	0.1539378	-8.06	1.54
565	hsa-miR-519e-3p	1.24	1.61	-1.31	0.21559229	-2.1	1.23
566	hsa-miR-520a-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
567	hsa-miR-520a-5p	6.74	9.17	-1.36	0.54804099	-4.34	2.35
568	hsa-miR-520b	1.24	1.54	-1.25	0.25182578	-1.92	1.23
569	hsa-miR-520c-3p	7.51	5.66	1.33	0.40586236	-1.68	2.96
570	hsa-miR-520d-3p	5.01	3.65	1.37	0.5832569	-2.8	5.28
571	hsa-miR-520d-5p+ hsa-miR-527+ hsa-miR-518a-5p	2.37	3.36	-1.42	0.60010231	-6.38	3.18
572	hsa-miR-520e	1.24	1.54	-1.25	0.25182578	-1.92	1.23
573	hsa-miR-520f-3p	65.66	88.14	-1.34	0.25885147	-2.37	1.31
574	hsa-miR-520g-3p	1.24	2.06	-1.67	0.24020347	-4.76	1.71
575	hsa-miR-520h	14.27	31.57	-2.21	0.17771061	-7.82	1.6
576	hsa-miR-521	1.24	2.2	-1.78	0.16073406	-4.66	1.47
577	hsa-miR-522-3p	3.68	1.8	2.05	0.07334629	-1.13	4.74
578	hsa-miR-523-3p	3.84	2.44	1.58	0.51024699	-3.12	7.76
579	hsa-miR-524-3p	1.24	1.59	-1.28	0.1699653	-1.89	1.15
580	hsa-miR-525-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
581	hsa-miR-525-5p	9.3	18.59	-2	0.18382038	-6.52	1.63
582	hsa-miR-526a+hsa-miR-518c-5p+hsa-miR-518d-5p	7.94	12.34	-1.55	0.34320813	-4.66	1.93
583	hsa-miR-526b-5p	1.24	1.61	-1.31	0.21559229	-2.1	1.23
584	hsa-miR-532-3p	1.75	2.07	-1.18	0.74098825	-3.8	2.71
585	hsa-miR-532-5p	1.9	1.54	1.24	0.63067567	-2.35	3.59
586	hsa-miR-539-3p	1.24	2.39	-1.94	0.11332586	-4.97	1.33
587	hsa-miR-539-5p	3.5	2.45	1.43	0.63693196	-4.4	9.02
588	hsa-miR-541-3p	1.24	2.38	-1.92	0.06802346	-4.2	1.14
589	hsa-miR-542-3p	11.47	10.42	1.1	0.82677335	-2.48	3
590	hsa-miR-542-5p	2.16	2.14	1.01	0.98278868	-3.52	3.61
591	hsa-miR-543	29.81	16.73	1.78	0.2183782	-1.61	5.12
592	hsa-miR-544a	2.13	2.46	-1.15	0.73848087	-3.06	2.3
593	hsa-miR-545-3p	1.24	1.55	-1.25	0.24122567	-1.92	1.23
594	hsa-miR-548a-3p	4.09	5.11	-1.25	0.74489266	-6.29	4.01
595	hsa-miR-548a-5p	2.94	3.88	-1.32	0.62921429	-4.93	2.82
596	hsa-miR-548aa+ hsa-miR-548t-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
597	hsa-miR-548ad-3p	7.65	8.98	-1.17	0.82315087	-6.28	4.56
598	hsa-miR-548ah-5p	46.01	69.62	-1.51	0.25269014	-3.33	1.45
599	hsa-miR-548ai+hsa-miR-570-5p	2.35	1.54	1.53	0.27041653	-1.6	3.72

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
600	hsa-miR-548ak	2.86	2.53	1.13	0.84255975	-3.56	4.53
601	hsa-miR-548al	2.58	2.17	1.19	0.81486017	-4.54	6.41
602	hsa-miR-548ar-3p	16.56	21.84	-1.32	0.52189434	-3.5	2.01
603	hsa-miR-548ar-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
604	hsa-miR-548b-3p	1.78	2.51	-1.41	0.52549255	-4.78	2.4
605	hsa-miR-548c-5p+ hsa-miR-548o-5p+ hsa-miR-548am-5p	1.24	1.59	-1.28	0.1699653	-1.89	1.15
606	hsa-miR-548d-3p	3.17	2.21	1.43	0.53964293	-2.64	5.43
607	hsa-miR-548d-5p	2.14	1.54	1.39	0.2398846	-1.32	2.53
608	hsa-miR-548e-3p	1.92	1.54	1.25	0.4959316	-1.69	2.62
609	hsa-miR-548e-5p	11.04	14.94	-1.35	0.3589972	-3.09	1.69
610	hsa-miR-548g-3p	6.87	6.22	1.1	0.88396299	-4.34	5.29
611	hsa-miR-548h-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
612	hsa-miR-548i	6.57	6.04	1.09	0.83901817	-2.35	2.78
613	hsa-miR-548j-3p	1.52	1.54	-1.02	0.94560981	-1.73	1.68
614	hsa-miR-548j-5p	7.12	4.82	1.48	0.53348172	-2.78	6.06
615	hsa-miR-548k	2.17	3.3	-1.52	0.37572351	-4.39	1.89
616	hsa-miR-548l	4.57	5.05	-1.11	0.87369215	-4.71	3.85
617	hsa-miR-548m	3.49	2.39	1.46	0.52522981	-2.6	5.51
618	hsa-miR-548n	5.99	2.01	2.98	0.03286678	1.09	8.11
619	hsa-miR-548o-3p+ hsa-miR-548ah-3p+ hsa-miR-548av-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
620	hsa-miR-548q	4.71	11.34	-2.4	0.22999014	-11.84	2.05
621	hsa-miR-548v	2.51	2.51	-1	0.99951619	-3.68	3.68
622	hsa-miR-548y	15.15	14.51	1.04	0.93549961	-3.27	3.56
623	hsa-miR-548z+ hsa-miR-548h-3p	6.74	1.54	4.37	0.03499598	1.08	17.76
624	hsa-miR-549a	10.64	23.6	-2.22	0.07211163	-5.53	1.12
625	hsa-miR-550a-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
626	hsa-miR-551a	4.12	4.68	-1.14	0.79639107	-3.55	2.75
627	hsa-miR-551b-3p	12.94	10.06	1.29	0.56634587	-2.16	3.58
628	hsa-miR-552-3p	9.49	9.97	-1.05	0.93724209	-4.93	4.47
629	hsa-miR-553	6.85	15.11	-2.21	0.18218821	-9.03	1.85
630	hsa-miR-554	1.26	1.8	-1.43	0.06649304	-2.12	1.04
631	hsa-miR-555	1.24	2.67	-2.16	0.01255149	-3.76	-1.24
632	hsa-miR-556-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
633	hsa-miR-556-5p	2.01	3.55	-1.77	0.29483837	-5.88	1.88
634	hsa-miR-561-3p	1.35	1.81	-1.34	0.32248896	-2.82	1.57
635	hsa-miR-561-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
636	hsa-miR-562	1.24	1.59	-1.29	0.16066656	-1.89	1.14
637	hsa-miR-563	1.67	1.94	-1.16	0.71638954	-3.05	2.26
638	hsa-miR-564	1.24	2.05	-1.66	0.0968439	-3.16	1.15
639	hsa-miR-566	7	6.62	1.06	0.92902839	-3.98	4.46
640	hsa-miR-567	1.35	1.97	-1.46	0.20801379	-2.99	1.41
641	hsa-miR-568	1.73	2.28	-1.31	0.47015798	-3.07	1.78
642	hsa-miR-570-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
643	hsa-miR-571	2.73	2.89	-1.06	0.91793925	-3.62	3.24
644	hsa-miR-572	1.55	2.25	-1.45	0.34860829	-3.47	1.66
645	hsa-miR-573	6.58	8.65	-1.31	0.61313945	-4.64	2.69
646	hsa-miR-574-3p	10.71	2.58	4.15	0.03382708	1.11	15.48
647	hsa-miR-574-5p	26.39	11.71	2.25	0.21673197	-1.86	9.44
648	hsa-miR-575	11.62	19.26	-1.66	0.22937369	-4.18	1.52
649	hsa-miR-576-3p	1.26	1.54	-1.22	0.27988797	-1.86	1.24
650	hsa-miR-576-5p	2.28	2.45	-1.07	0.89966804	-3.86	3.36
651	hsa-miR-577	11.12	15.41	-1.39	0.39996898	-3.47	1.81
652	hsa-miR-578	1.78	1.59	1.12	0.77534509	-2.37	2.98
653	hsa-miR-579-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
654	hsa-miR-579-5p	3.69	3.86	-1.04	0.9505077	-5.29	4.85
655	hsa-miR-580-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
656	hsa-miR-582-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
657	hsa-miR-582-5p	1.99	2.83	-1.42	0.42299777	-3.8	1.88
658	hsa-miR-584-3p	18.03	25.17	-1.4	0.49957606	-4.97	2.55
659	hsa-miR-584-5p	5.95	4.21	1.42	0.55721861	-2.7	5.41
660	hsa-miR-585-3p	12.27	8.77	1.4	0.46911231	-2.03	3.99
661	hsa-miR-587	3.24	5.48	-1.69	0.41023791	-7.1	2.47
662	hsa-miR-589-5p	1.78	1.98	-1.11	0.80313218	-2.99	2.41
663	hsa-miR-590-3p	1.66	2.15	-1.3	0.57171798	-4.24	2.53
664	hsa-miR-590-5p	2.11	2.24	-1.06	0.91620725	-3.92	3.49
665	hsa-miR-591	3.04	2.55	1.19	0.79496068	-3.89	5.51
666	hsa-miR-592	7.42	5.44	1.36	0.54139531	-2.38	4.42
667	hsa-miR-593-3p	10.56	6.92	1.53	0.4064115	-2.04	4.75
668	hsa-miR-595	6.82	12.75	-1.87	0.29119521	-8.01	2.29
669	hsa-miR-596	1.9	2.72	-1.43	0.52707064	-5.11	2.5
670	hsa-miR-597-5p	8.26	14.58	-1.77	0.29560092	-6.29	2.02
671	hsa-miR-598-3p	3.35	1.59	2.11	0.17443874	-1.73	7.72
672	hsa-miR-599	1.24	1.54	-1.25	0.25182578	-1.92	1.23
673	hsa-miR-600	1.24	1.59	-1.28	0.1699653	-1.89	1.15
674	hsa-miR-601	1.71	1.54	1.11	0.81146353	-2.68	3.32
675	hsa-miR-603	1.24	1.54	-1.25	0.25182578	-1.92	1.23
676	hsa-miR-604	1.74	1.54	1.13	0.78238434	-2.56	3.26
677	hsa-miR-605-5p	7.86	6.36	1.24	0.74954933	-3.66	5.59
678	hsa-miR-606	1.74	2.71	-1.56	0.21732041	-3.42	1.4
679	hsa-miR-607	20.28	23.68	-1.17	0.74225992	-4	2.94
680	hsa-miR-608	10.51	5.32	1.98	0.29489544	-2.12	8.3
681	hsa-miR-610	1.93	5.15	-2.67	0.10878863	-9.53	1.34
682	hsa-miR-612	59.74	106.71	-1.79	0.15406083	-4.42	1.39
683	hsa-miR-613	1.24	2.3	-1.86	0.03059093	-3.24	-1.06
684	hsa-miR-614	1.73	1.54	1.12	0.74073231	-2.07	2.6
685	hsa-miR-615-3p	9.78	9.57	1.02	0.96415907	-3.05	3.19
686	hsa-miR-615-5p	1.24	1.59	-1.28	0.1699653	-1.89	1.15
687	hsa-miR-616-3p	1.73	2.7	-1.56	0.29893643	-4.03	1.65
688	hsa-miR-617	1.34	1.54	-1.15	0.51767039	-1.88	1.42
689	hsa-miR-619-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
690	hsa-miR-620	3.8	6.47	-1.7	0.40833044	-7.47	2.58

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
691	hsa-miR-624-3p	1.44	1.81	-1.26	0.51473653	-2.82	1.77
692	hsa-miR-625-5p	5.54	3.08	1.8	0.50948024	-4.74	15.37
693	hsa-miR-626	3.72	2.67	1.39	0.49459606	-2.21	4.3
694	hsa-miR-627-3p	6.26	13.59	-2.17	0.23693134	-10.77	2.29
695	hsa-miR-627-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
696	hsa-miR-628-3p	1.7	1.54	1.1	0.801153	-2.35	2.86
697	hsa-miR-628-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
698	hsa-miR-629-5p	2.85	2.48	1.15	0.78706402	-2.8	3.69
699	hsa-miR-630	69.79	25.82	2.7	0.20074949	-2.05	14.98
700	hsa-miR-631	1.24	1.54	-1.25	0.25182578	-1.92	1.23
701	hsa-miR-637	1.24	1.77	-1.43	0.1006453	-2.27	1.1
702	hsa-miR-638	5.41	8.05	-1.49	0.58920389	-9.83	4.45
703	hsa-miR-639	1.85	1.54	1.2	0.64017189	-2.14	3.08
704	hsa-miR-640	7.58	3.72	2.04	0.20836265	-1.91	7.95
705	hsa-miR-641	1.73	3.22	-1.87	0.31820011	-7.66	2.2
706	hsa-miR-642a-3p	1.52	1.54	-1.02	0.94560981	-1.73	1.68
707	hsa-miR-642a-5p	1.29	1.54	-1.19	0.3360334	-1.8	1.27
708	hsa-miR-643	7.05	13.34	-1.89	0.34806207	-9.34	2.61
709	hsa-miR-644a	10.07	21.6	-2.14	0.13493536	-6.41	1.39
710	hsa-miR-648	10.18	13	-1.28	0.74079102	-6.92	4.24
711	hsa-miR-649	1.67	1.55	1.08	0.80898219	-1.92	2.23
712	hsa-miR-650	1.24	1.54	-1.25	0.25182578	-1.92	1.23
713	hsa-miR-6503-3p	2.2	1.77	1.24	0.29494399	-1.28	1.96
714	hsa-miR-6503-5p	1.61	2.06	-1.28	0.60816038	-3.84	2.34
715	hsa-miR-651-3p	1.38	1.59	-1.15	0.49772224	-1.85	1.39
716	hsa-miR-651-5p	3.69	2.44	1.52	0.52146792	-2.85	6.55
717	hsa-miR-6511a-3p	1.41	1.54	-1.09	0.64843214	-1.69	1.42
718	hsa-miR-6511a-5p	2.25	2.35	-1.04	0.9385972	-3.59	3.3
719	hsa-miR-652-3p	9.49	1.77	5.35	0.06218783	-1.23	35.24
720	hsa-miR-652-5p	1.24	1.85	-1.5	0.14046966	-2.74	1.22
721	hsa-miR-654-3p	1.24	1.95	-1.58	0.22925667	-3.96	1.59
722	hsa-miR-654-5p	2.71	1.54	1.76	0.1852385	-1.48	4.59
723	hsa-miR-655-3p	1.24	1.61	-1.31	0.21559229	-2.1	1.23
724	hsa-miR-656-3p	8.03	17.58	-2.19	0.0707271	-5.33	1.11
725	hsa-miR-660-3p	6.09	4.44	1.37	0.64635605	-3.5	6.58
726	hsa-miR-660-5p	1.55	1.54	1.01	0.97831625	-1.76	1.78
727	hsa-miR-661	12.71	10.48	1.21	0.74578792	-3.92	5.76
728	hsa-miR-663a	3.68	6.27	-1.7	0.49018514	-9.68	3.33
729	hsa-miR-664a-3p	21.98	2.13	10.33	0.00449708	2.62	40.71
730	hsa-miR-664b-3p	8.08	15.32	-1.89	0.06001415	-3.79	1.06
731	hsa-miR-664b-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
732	hsa-miR-665	1.69	2.51	-1.49	0.41881967	-4.46	2.02
733	hsa-miR-671-3p	1.24	1.61	-1.31	0.21559229	-2.1	1.23
734	hsa-miR-671-5p	4.21	5.6	-1.33	0.47996476	-3.29	1.86
735	hsa-miR-6720-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
736	hsa-miR-6721-5p	7.01	5.79	1.21	0.74744302	-3.32	4.88
737	hsa-miR-6724-5p	7.96	8.19	-1.03	0.96197176	-4.13	3.9
738	hsa-miR-675-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
739	hsa-miR-7-5p	12.59	12.27	1.03	0.95082456	-2.6	2.73
740	hsa-miR-708-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
741	hsa-miR-744-5p	15.96	25.17	-1.58	0.2429017	-3.87	1.55
742	hsa-miR-758-3p+ hsa-miR-411-3p	2.82	2.63	1.07	0.92076474	-4.48	5.13
743	hsa-miR-758-5p	1.29	1.95	-1.51	0.26883951	-3.74	1.64
744	hsa-miR-760	2.84	1.8	1.58	0.32443735	-2.03	5.08
745	hsa-miR-761	1.61	1.59	1.01	0.96887827	-2.22	2.28
746	hsa-miR-764	11.41	7.21	1.58	0.29264703	-1.73	4.33
747	hsa-miR-765	6.53	4.83	1.35	0.69718874	-4.34	7.93
748	hsa-miR-766-3p	14.51	9.98	1.45	0.63605118	-4.16	8.79
749	hsa-miR-766-5p	1.52	1.54	-1.02	0.94560981	-1.73	1.68
750	hsa-miR-767-3p	1.52	1.54	-1.01	0.94335383	-1.63	1.58
751	hsa-miR-767-5p	1.24	1.55	-1.25	0.24122567	-1.92	1.23
752	hsa-miR-769-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
753	hsa-miR-769-5p	9.47	2.08	4.54	0.01005482	1.6	12.93
754	hsa-miR-770-5p	5.35	2.61	2.05	0.14968432	-1.41	5.94
755	hsa-miR-802	1.84	1.54	1.2	0.70290923	-2.61	3.73
756	hsa-miR-873-3p	1.64	1.8	-1.09	0.82178658	-2.92	2.43
757	hsa-miR-873-5p	1.41	1.59	-1.12	0.51976746	-1.7	1.34
758	hsa-miR-874-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
759	hsa-miR-874-5p	2.4	3.24	-1.35	0.60011828	-4.96	2.72
760	hsa-miR-875-3p	1.38	1.94	-1.41	0.19143867	-2.48	1.25
761	hsa-miR-876-3p	1.29	2.48	-1.92	0.05880014	-3.99	1.08
762	hsa-miR-876-5p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
763	hsa-miR-877-5p	12.42	12.76	-1.03	0.9568007	-3.18	3.02
764	hsa-miR-885-3p	1.24	1.54	-1.25	0.25182578	-1.92	1.23
765	hsa-miR-885-5p	1.57	1.54	1.02	0.92460454	-1.58	1.64
766	hsa-miR-887-3p	1.52	1.54	-1.02	0.94560981	-1.73	1.68
767	hsa-miR-887-5p	5.77	5.71	1.01	0.98736179	-4.03	4.11
768	hsa-miR-888-5p	7.61	9.87	-1.3	0.60120302	-3.99	2.37
769	hsa-miR-889-3p	1.24	2.24	-1.81	0.09077014	-3.99	1.21
770	hsa-miR-890	3.55	2.94	1.21	0.7824797	-4.14	6.05
771	hsa-miR-891a-5p	1.88	2.86	-1.52	0.3837001	-4.41	1.91
772	hsa-miR-891b	3.66	5.45	-1.49	0.42690909	-4.59	2.06
773	hsa-miR-892a	4.12	5.98	-1.45	0.57274687	-7.15	3.4
774	hsa-miR-892b	1.24	1.54	-1.25	0.25182578	-1.92	1.23
775	hsa-miR-9-5p	2.32	1.81	1.28	0.53447908	-1.92	3.14
776	hsa-miR-922	2.61	2.08	1.25	0.71918625	-3.48	5.47
777	hsa-miR-924	1.6	2.34	-1.46	0.38604355	-4.01	1.87
778	hsa-miR-92a-1-5p	6.25	9.55	-1.53	0.29782718	-3.91	1.68
779	hsa-miR-92a-3p	25.23	50.53	-2	0.17771716	-6.05	1.51
780	hsa-miR-92b-3p	1.69	2.23	-1.32	0.54879284	-3.8	2.17
781	hsa-miR-93-5p	257.69	62.35	4.13	0.00910947	1.66	10.28
782	hsa-miR-933	2.25	2.35	-1.04	0.93217587	-3.24	2.98
783	hsa-miR-934	2.42	2.14	1.13	0.80984813	-3	3.85
784	hsa-miR-935	1.24	1.61	-1.31	0.21559229	-2.1	1.23
785	hsa-miR-936	4.62	2.78	1.66	0.4054063	-2.43	6.71

No.	Gene Name	Patient (P)	Control (C)	P vs. C	p value	Lower 95% CI	Upper 95% CI
786	hsa-miR-937-3p	5.72	2.98	1.92	0.31139171	-2.22	8.15
787	hsa-miR-939-5p	6.89	10.67	-1.55	0.5220955	-7.57	3.15
788	hsa-miR-940	1.24	1.54	-1.25	0.25182578	-1.92	1.23
789	hsa-miR-941	1.24	1.54	-1.25	0.25182578	-1.92	1.23
790	hsa-miR-942-3p	2.02	1.59	1.27	0.54130942	-2.03	3.28
791	hsa-miR-942-5p	1.24	2.69	-2.18	0.09651959	-6.11	1.29
792	hsa-miR-944	1.24	1.54	-1.25	0.25182578	-1.92	1.23
793	hsa-miR-95-3p	3.53	2.97	1.19	0.78563112	-3.57	5.04
794	hsa-miR-96-5p	9.73	5.7	1.71	0.55026466	-4.45	12.97
795	hsa-miR-98-3p	2.42	4.24	-1.75	0.31389156	-5.94	1.94
796	hsa-miR-98-5p	43.56	10.94	3.98	0.02147344	1.29	12.29
797	hsa-miR-99a-5p	2.41	3.1	-1.29	0.59971511	-3.86	2.32
798	hsa-miR-99b-5p	11.92	2.06	5.79	0.01101356	1.64	20.41

APPENDIX G. Patient Information Leaflet and Informed Consent

PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

Each patient must receive, read and understand this document

before any study-related procedure

STUDY NUMBER:

STUDY TITLE: Clinical and Immunopathogenetic Aspects of Sarcoidosis

INVESTIGATOR: Prof R Morar

INSTITUTION: University of the Witwatersrand

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

dd	mmm	yyyy

:
Time

Secretariat to the University of the Witwatersrand Human Research Ethics Committee:

Reference number:	*022.Informed Consent Template*
Contact:	*Mrs Jennifer Bryce-Bothwick*
Title:	*Administrator*
Telephone	*011 274-9278*

INTRODUCTION:

Good day, my name is Professor Rajen Morar, I am a lung specialist at Charlotte Maxeke Johannesburg Academic Hospital. I would like to ask you to consider participating in a research study, entitled

Clinical and Immunopathogenetic Aspects of Sarcoidosis

Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions as well as the alternative procedures that are available to you, and your right to withdraw from the study at any time.

- This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.
- If you have any questions, do not hesitate to ask me.
- You should not agree to take part unless you are satisfied about all the procedures involved.
- Please be completely truthful with me regarding your health history, since you may otherwise harm yourself by participating in this study.
- If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.

PURPOSE OF THE STUDY:

- You have been diagnosed as having sarcoidosis/tuberculosis and I would like you to consider taking part in the research.
- The cause of your condition is not known in sarcoidosis, and in tuberculosis it is caused by *M tb*.
  1. The purpose of this study is to perform microRNAs analysis in blood from patients with sarcoidosis [recently discovered small protein chemicals that controls genes]
  2. Perform chitotriosidase levels in blood of patients with sarcoidosis and /or tuberculosis before and after treatment (in both HIV positive and HIV negative individuals) [chemical in the blood]
  3. Perform scanning electron microscopy and energy dispersive X-ray detection of histological samples in sarcoidosis patients looking for inorganic or foreign particles in the granulomas of tissue biopsies retrospectively and prospectively on the tissue biopsies that were taken for the diagnosis of sarcoidosis which is routine [examine biopsies that were taken to make the diagnosis, for foreign particles]

4. Obtain clinical information and results from your hospital records/file.

LENGTH OF THE STUDY AND NUMBER OF PARTICIPANTS:

- The study will be performed in Johannesburg.
- Approximately 20-30 patients with sarcodiosis and 20-30 patients with TB will be participating in this study.
  The patients will be older than 18 years old.
- The total amount of time required for your participation in this study will be a maximum of 15-30 minutes.

PROCEDURES:

If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study.

- At entering into the study, you will be asked to take 30 mls (6 teaspoons, 3-4 tubes) of blood from your vein. Blood samples will be stored in a fridge and when all the samples are collected the chitotriosidase (protein) and miRNA (small protein chemicals that controls genes) levels will be measured all together.

WILL ANY OF THESE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

- Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. A total of 30 ml of blood (i.e. 6 teaspoons) will be collected.

BENEFITS:

- However, you may not benefit from this study.
- Your participation in this study will contribute to medical knowledge that may help other patients that, like you, have sarcoidosis and/or tuberculosis.

ALTERNATIVE TREATMENT:

- If you decide not to take part in this study you will still receive the best current care, from your usual doctor.

RIGHTS AS A PARTICIPANT IN THIS STUDY:

- ***Voluntary:***
  Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason. Your withdrawal will not affect your access to other medical care.
- ***New findings:***
  I will provide you with any additional information that becomes available during the study, which may affect your willingness to continue in the study
- ***Withdrawal:***
  - Your withdrawal will not affect your access to other medical care.
  - I retain the right to withdraw you from the study if it is considered to be in your best interest. If your participation is ended early, you may be asked to return for study-ending tests and procedures for your safety
  - If you did not give an accurate history or did not follow the guidelines of the study and the regulations of the study facility, you may be withdrawn from the study at any time.

REIMBURSEMENT FOR STUDY PARTICIPATION:

- You will not be paid to participate in this study but your transport and, when necessary, refreshment costs may be reimbursed adequately.

ETHICAL APPROVAL:

- This clinical study protocol has been approved by the University of the Witwatersrand, Human Research Ethics Committee (HREC).

SOURCE OF ADDITIONAL INFORMATION:

For the duration of the study, you will be under the care of your usual doctor. If at any time, you feel that any of your symptoms are causing you any problems, or you have any questions during the study, please do not hesitate to contact me.
Other staff from this department who are working on this study are: Sister Vivian Herbert

The 24-hour telephone number through which you can reach me, is 082 458 2887.

- If want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Cleaton-Jones, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2229.

CONFIDENTIALITY:

- All information obtained during the course of this study, including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a participant in this study.
- My supervisors and I will review this information.
- The information might also be inspected by the University of the Witwatersrand, Human Research Ethics Committee (HREC), as well as your personal doctor. Therefore, you hereby authorise me to release your medical records to Prof R Morar and the University of the Witwatersrand, Human Research Ethics Committee (HREC).
- These records will be utilised by them only in connection with carrying out their obligations relating to this clinical study.
- Any information uncovered regarding your test results or state of health as a result of your participation in this study will be held in strict confidence. You will be informed of any finding of importance to your health or continued participation in this study but this information will not be disclosed to any third party in addition to the ones mentioned above without your written permission. The only exception to this rule will be cases of communicable diseases where a legal duty of notification of the Department of Health exists. In this case, you will be informed of my intent to disclose such information to the authorised state agency.

INFORMED CONSENT:
I hereby confirm that I have been informed by the study doctor, Prof R Morar, about the nature, conduct, benefits and risks of this clinical study:

Clinical and Immunopathogenetic Aspects of Sarcoidosis

1. Perform microRNAs analysis in peripheral blood from patients with sarcoidosis [genetic test]
2. Measure chitotriosidase levels in peripheral blood of patients with sarcoidosis and /or tuberculosis before and after treatment (in both HIV positive and HIV negative individuals) [chemical in the blood]
3. Perform scanning electron microscopy and energy dispersive X-ray detection of histological samples in sarcoidosis patients looking for inorganic or foreign particles in the granulomas of tissue biopsies [examine biospsies that were taken to make the diagnosis, for foreign particles]

I have also received, read and understood the above information (Patient Information Leaflet and Informed Consent) regarding the clinical study.

- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system by Prof R Morar or on his behalf.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient:

Printed Name	Signature / Mark or Thumbprint	Date and Time

I, R Morar, herewith confirm that the above patient has been fully informed about the nature, conduct and risks of the above study.

Study Doctor:

Printed Name	Signature	Date and Time

Translator/other person explaining Informed Consent.....(Designation):

Printed Name	Signature	Date and Time

Witness (If applicable):

Printed Name	Signature	Date and Time

VERBAL PATIENT INFORMED CONSENT:
(Applicable when patients cannot read or write or are incapable of giving consent)

- I, the undersigned, R Morar have read and have explained fully to the patient, named ..... and/or his/her relative/friend/legal representative, ..., the patient information leaflet.
- The account I have given has explained both the possible risks and benefits of the study as well as the alternative treatments available for his/her illness. The patient and/or his/her relative/friend/legal representative understands these.
- The patient and/or his/her relative/friend/legal representative indicated that he/she understands that the patient will be free to withdraw from the study at any time for any reason and without jeopardising his/her subsequent treatment.
- I have also informed the patient and/or his/her relative/friend/legal representative of the existence of relevant compensation arrangements in case of an injury attributable to the medicine(s) used in the clinical study, to which he/she agrees.

I hereby certify that, the patient and/or his/her relative/friend/legal representative, acting on his/her behalf, has agreed to participate in this study.
Patient:

Printed Name Mark or Thumbprint (if applicable) Date and Time

Study Doctor:

Printed Name Signature Date and Time

Translator/other person explaining informed consent: .....**(Designation)**

Printed Name Signature Date and Time

Parent/LegalGuardian/LegalRepresentative/Friend:.....**(Relationship)**

Printed Name Signature / Mark or Thumbprint Date and Time

Witness:

Printed Name Signature Date and Time

APPENDIX H. Turn It In Report

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Clinical and Immunopathogenetic Aspects of Sarcoidosis

Rajen Morar

Johannesburg, 2021

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