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Validation of biomarkers for improved assessment of exposure and early effect from exposure to crystalline silica

Kerry Sidwell Wilson

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Summary

Silicosis is the most common disease associated with respirable crystalline silica (RCS) exposure. It is a progressive fibrotic lung disease that can be chronic or acute but, in either case, is incurable. Silicosis is fibrosis in the lungs, and takes the form of small individual nodules consisting of collagen fibers, hyalinized zones and inflammatory cells, also known as nodular pulmonary fibrosis. Silicosis is often an indicator of RCS exposure, but it takes a number of years (approximately 15 years, but can be as little as 5 years) to develop after first exposure and thus is not a good outcome to use in evaluating the early effects of exposure and to develop and evaluate control methods for RCS exposure.

Biomarkers are measurable or observable characteristics or contents of an organism, system, or sample that indicate their position on the pathway from exposure to disease. What is commonly understood by the term 'biomarker' is a substance measurable in the body that alters in response to exposure or disease. Biomarkers are recommended for use in assessing the effect of or exposure to harmful substances, specifically where there are low levels of exposure, mixtures of toxicants that may work synergistically, intermittent exposure, or exposure resulting in disease with long latency periods. In the case of RCS, biomarkers of effect can take into account both individual variation and the effects of genetic factors in response to exposure.

Biomarkers can be used in combination with air monitoring to verify compliance with standards. Occupational exposure levels (OEL) may be lowered or new limits set based on evidence of dose-response effects demonstrated by biomarkers. Validation of biomarkers is important to ensure that those identified are truly placed in the continuum between exposure and disease. Validation also ensures that biomarkers are reproducible and determine the level of variation within a control population.

Elucidation of the mechanisms behind the development of silicosis has allowed the identification of possible biomarkers of early effect of RCS exposure. The three main

mechanisms of development of silicosis have provided most of the biomarkers of effect of interest in this study. These are oxidative stress, inflammation and cellular lung damage.

The present study was the second phase of a project to identify and provide initial validation for biomarkers of effect for RCS exposure. This aimed to investigate whether the mean biomarker levels of a group of volunteer gold miners exposed to RCS were significantly different from a group of unexposed volunteers from industries with no RCS exposure. Ten biomarkers were identified from the first phase of this project; which evaluated all literature on markers of effect. The biomarkers identified and agreed upon fall into three groups: markers of oxidative stress, inflammatory markers and a marker for lung damage. The biomarkers chosen were:

- Erythrocyte Glutathione (GSH), an important antioxidant tripeptide
- Erythrocyte Glutathione Peroxidase (GPx), an important abundant antioxidant enzyme
- Erythrocyte Glutathione S Transferase (GST), an antioxidant enzyme
- 8-Isoprostane, a by-product of lipid peroxidation which indicates when oxidative stress is uncontrolled
- Total Antioxidant Status (TAS), a test which measures the combined effect of all antioxidants in the blood
- Reactive oxygen species (ROS) measured by chemiluminescence, a test to measure the amount of ROS released by isolated cells
- Tumour necrosis factor α (TNFα), a cytokine that drives and maintains inflammation
- Interleukin-8 (IL-8), a chemoattractant responsible for the attraction of inflammatory cells to the affected area
- Platelet derived growth factor(PDGF) a growth factor that initiates and maintains fibrosis
- Clara Cell protein16 (CC16), a protein released due to damage to Clara cells found mainly in the lung

The present study evaluated changes in these biomarkers in RCS exposed and unexposed individuals. Possible confounders of the effect of RCS on the biomarkers were also examined. An important possible confounder was human immunodeficiency virus (HIV)

status. HIV is more prevalent in miners with a prevalence of 27% in 2004, than the general population. HIV is an important confounder of the measurement of selected biomarkers of effect as many of the biomarkers are non-specific and could reflect changes caused by HIV infection rather than RCS exposure.

Blood samples were collected from both HIV positive and negative gold miners and unexposed controls, to evaluate the effect of RCS exposure on the biomarkers and the influence of HIV. These samples were processed and stored for testing. All the participants also completed a short questionnaire in the language of their choice to provide information on age, work history, general health and cigarette smoking. Radiographs from all miners were examined by two readers and silicosis was identified using the ILO method.

Only two of the ten biomarkers tested showed a significant difference between unexposed controls and RCS exposed gold miners where the levels were not confounded by HIV status. The first was total erythrocyte GPx, which showed a decrease in activity in the RCS exposed miners. This decrease in activity is likely to result in an increase in oxidative stress and its effects in the lung as GPx is mainly responsible for the detoxification of hydroperoxides.

The second biomarker showing a difference between the exposed and unexposed was CC16; a protein released by Clara cells in the lung following lung damage. Following chronic damage, the levels of CC16 have been shown to decrease. In this study, miners were found to have lower levels of CC16 than controls. This suggests the presence of chronic damage to lung cells. The levels of CC16 were not confounded by HIV status.

The two biomarkers identified were shown to be unaffected by age and cigarette smoking. The presence of radiological silicosis also did not cause any significant difference in the two biomarkers.

Three markers, 8-Isoprostane, GSH and PDGF, suffered from methodological issues and could not be satisfactorily analysed and thus were not recommended for further validation at this stage. The remaining markers (TNFα, GST, TAS, IL-8, ROS) tested were rejected

as they showed no significant difference in levels between the miners and controls or they were significantly affected by HIV. Correct sample collection methods are labour intensive for both PDGF and 8-Isoprostane and the cost of improved analysis methods is likely to be prohibitive for a routine biomarker.

In conclusion, this study demonstrated that it is feasible to analyse levels of biomarkers of effect in South African miners and controls. The two biomarkers, total erythrocyte GPX and CC16, identified as showing a significant difference between miners and controls, warrant further investigation before they can be adopted as routine tests to be used to evaluate RCS exposure.

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACGIH	American Conference of Industrial Hygienists
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AIDS	Acquired immune deficiency syndrome
AM	Alveolar Macrophages
ARV	Anti-Retroviral Treatment
BALF	Bronchoalveolar Lavage
BHT	Butylated Hydroxytoluene
BMI	Body Mass Index
BSA	Bovine serum albumin
CC16	Clara cell protein 16
Cox2	Cyclooxygenase
CWP	Coal Workers Pneumoconiosis
DEA	Diethanolamine
DMR	Department of Mineral Resources
DMR	Department of Mineral Resources
DMSO	Dimethylsulphoxide
DNA	Dioxyribonucleicacid
DTNB	5,5 Ditho bis -2- nitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ELF	Epithelial lining fluid
EPA	Environmental Protection Agency
FEV_1	1-second Forced Expiration Volume
FVC	Forced Vital Capacity
GC-MS	Gas chromatography mass spectrophotometry
GCS	Glutamate cysteine
GPX	Glutathione Peroxidase
GSH	Glutathione
GST	Glutathione S Transferase
HAART	Highly active anti-retorviral treatment
HIV	Human Immunodeficiency virus
HSE	Health and Safety Executive
IARC	International Agency for Research on Cancer
IL-1	Interleukin 1
IL-8	Interleukin 8
ILO	International Labour organisation
INFγ	Interferon gamma
IR	Infrared spectrometry
LECL	Luminol-enhanced chemiluminescence
MDA	Malondialdehyde
MES	2(N-morpholino) ethanesulphonic acid
MHSC	Mine Health and Safety Council
MPA	Meta phosphoric acid
MVS	Mine Ventilation Society
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphat
NIOH	National Institute for Occupational Health

NIOSH	National Institute for Safety and Health
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPES	National Programme for the Elimination of Silicosis
NSB	Non-specific binding
OEL	Occupational Exposure levels
OEL - STEL	Occupational Exposure levels Short term exposure limits
OH	Hydroxy radical
OSHA	Occupational Safety and Health Administration
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PEL	Permissible Exposure Level
PF	Pulmonary Fibroblasts
PMF	Progressive Massive Fibrosis
pNPP	5pNitrophenyl Phosphate
PPE	Personal Protective Equipment
PRU	Pneumoconiosis Research unit
pTB	Pulmonary Tuberculosis
RCS	Respirable Crystalline Silica
REL	Reference Exposure Level
RLU	Relative light units
ROC	Receiver operated curve
ROS	Reactive Oxygen species
RPE	Respiratory protective Equipment
RSSG	Reduced gulathione
SADC	Southern African Development Countries
SANBS	South African National Blood Service
SiO_2	Silicon dioide
TAS	Total antioxidant status
TEAM	Triethanolamine
TEBA	The Employment Bureau of Africa
TLV	Threshold limit Values
TNB	5 thio-2-nitrobenzoic acid
TNFα	Tumour necrosis factor α
TRAP	Total reactive anti-oxidant potential
TRIS	Tris(hydroxymethyl)aminomethane
TWA	Time weighted average
VCT	Voluntary counselling and testing
WHO	World Health Organisation
XRD	X-ray Diffraction

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CHAPTER 1

Introduction and Background to the study

1.1 The Gold Mining Industry in South Africa

Gold was discovered in South Africa in 1886 on a farm on the western side of what is now modern Johannesburg. By 1890, deep level mining had begun and since then the gold mining industry grew rapidly.

The mining industry has been one of the main contributors to the South African economy and infrastructure. South Africa has enormous gold reserves, and is one of the larger producers of gold in the world. Gold production accounts for a large portion of the county's foreign exchange, as most of the gold produced in South Africa is sold overseas. The gold mines are large employers in South Africa and southern Africa, but the number of employees has been steadily decreasing. The number of employees in 2000 decreased to almost half of the 1991 level (Figure 1). This decline is expected to continue (Chamber of Mines, 2006).

The mining industry has long recognised the presence of silicosis in miners and determined its cause. The Mining Commissions of Enquiry and the Miners' Phthisis Prevention Committee 1912 - 1919 were early examples of the steps taken to control the disease. These were followed by government regulations to prevent silicosis and ensure adequate ventilation. In the late 1940s and 1950s the Pneumoconiosis Research Unit and the Mine Ventilation Society were established in South Africa and played a role in the prevention of silicosis, the International Labour Organisation (ILO) also played at role at this time (Donsky, 1993). The stabilisation of labour (longer contracts and repeat contracts for skilled miners) by the mining industry from the mid-1970s resulted in an increase in the average length of a mine contract and the total time spent on the mines, thus increasing the likelihood of miners developing silicosis (Murray *et al.*, 1996, Marks, 2006).

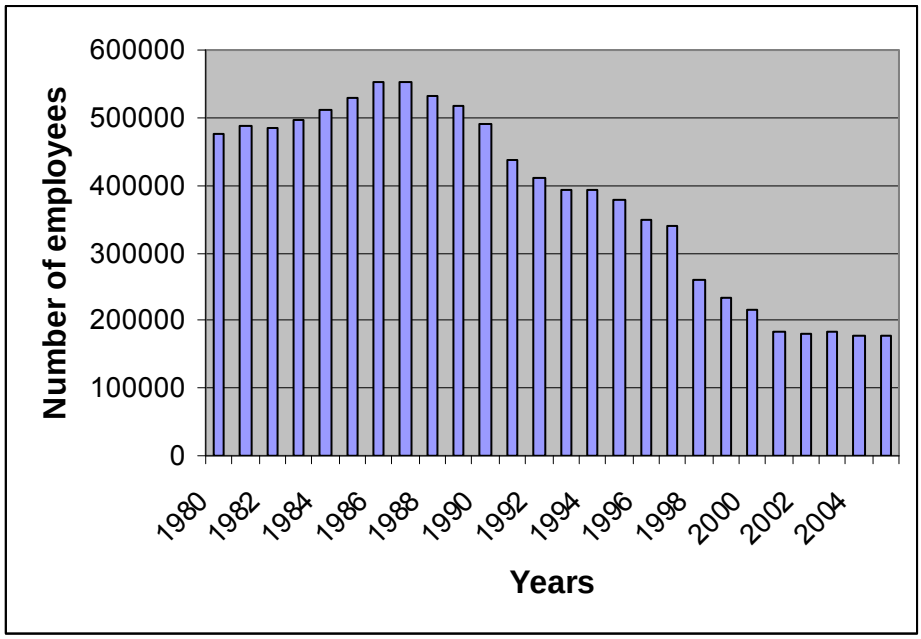


Figure 1: Graph of the total employees on South African gold mines from 1980 to 2005 (created from information from the Chamber of Mines, 2006).

1.1.1 Gold mining and rock types

In South Africa gold-bearing rock contains quantities of RCS as it is found in combination with sandstone, which is a rich source of silica. Development of gold mines can lead to dust containing 10 - 30% free silica, and samples taken on Witwatersrand mines have shown up to 80% RCS concentration (Ziskind *et al.*, 1976, Donsky, 1993).

Gold on the Witwatersrand was laid down with pebbles as silt in layers on the bottom of an inland sea. Over time this sedimented and became hard rock up to 7.5 km thick. During a time of geological turmoil, the sedimentary layer containing the gold was tilted and dolomite rock laid on top. Today the upper edge forms the Witwatersrand reef (Donsky, 1993).

Because of the depth and angle of the Witwatersrand reef, mines have to sink deep shafts to reach the gold reefs, and some are found up to 3 km underground. Tunnels then follow the reef, in which trains are sometimes used because of the great lengths of the tunnels. As the gold becomes exhausted at the higher levels, new shafts are sunk deeper to reach unmined gold seams. The tunnels are driven underneath the ore layer and then the ore is

mined above by blasting and drilling in small low-roofed areas called stopes. The ore is gathered and dropped down small shafts to the main tunnels where it is hauled to the surface in large skips (Chamber of Mines, 2008)

Blasting operations on mines and mechanical systems produce large amounts of dust (CSIR SHEQ management). It is also a dangerous process. Dangers include rock falls, tremors, methane gas and high levels of RCS dust. The heat (up to 60 °C) and rock pressure at the depths of the shafts are great. Blasting and drilling create the highest levels of respirable dust. Delayed re-entry periods after blasting are used to try to reduce the exposure to dust created by blasting. Ventilation also helps to remove fine particles from the air. The dust released from other mechanical operations can be reduced by keeping the rock wet (Guild *et al.*, 2001).

1.2 Silica types and characteristics

Silica is a dioxide of silicon consisting of the elements silicon (Si) and oxygen (O) in a ratio of 1 to 2 (silicon dioxide, SiO_2). Silicon is the second most abundant element on earth, the first being oxygen; it is found in group 4 in the periodic table (US Bureau of Mines, 1992). Twenty-eight per cent of the earth's surface contains silica in some form, often in combination with other minerals or metals, most commonly Al, Fe, Ge, Li, Mg, Ca, Na and K (Donaldson and Borm, 1998, Castranova and Vallyathan, 2000). Silica appears in two states, crystalline and amorphous. The basic structural unit of silica is a tetrahedron consisting of a central silicon ion with oxygen atoms attached at the four corners. In crystalline silica two of the oxygen atoms provide the link in a repeating manner to the next unit (Figure 2) (Parkes, 1974).

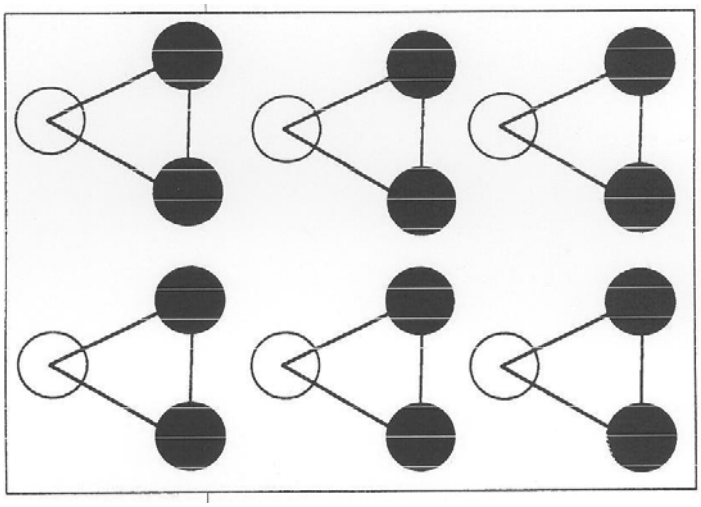


Figure 2: Repeating units of silica in crystalline silica (from US Bureau of Mines, 1992). The clear circle is silicon and the dark circles are oxygen.

Amorphous silica has no repeating pattern, and the basic structural units are randomly arranged (Figure 3) (US Bureau of Mines, 1992).

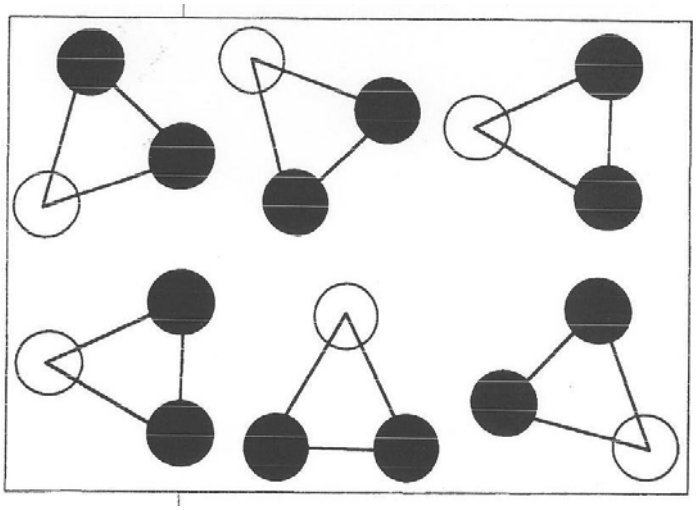


Figure 3: Random arrangement of silica units in amorphous silica (from US Bureau of Mines, 1992) The clear circle is silicon and the dark circles are oxygen.

Crystalline silica is structured in repeating units in a three-dimensional pattern, and depending on the pattern, a different form of silica is found. This is called polymorphism; crystalline silica has seven polymorphs; the three common polymorphs are 1) α and β quartz (US Bureau of Mines, 1992), 2) α, β_1 and β_2 tridymite, and 3) α and β cristobalite (IARC, 1997).

Quartz is a major component of sand and dust and makes up 12% of the earth's crust (Stratta *et al.*, 2001). It is also found in varying amounts in all soils, and is present in sedimentary rocks in amounts ranging from minute up to 100% (US Bureau of Mines, 1992). It is the most common type of crystalline silica and accounts for most hazardous occupational exposures (Donaldson and Borm, 1998).

Silica has been associated with adverse health outcomes in those chronically exposed to it such as in certain hobbies or occupations. The most commonly associated adverse health outcome of harmful silica exposure is silicosis, an irreversible fibrotic lung disease (Fishwick, 2004).

Not all types of silica have been associated with fibrosis and inflammation in the lungs of both humans and laboratory animals (Driscoll *et al.*, 1995). Amorphous silica is generally less fibrogenic than crystalline silica as it appears to induce increased production of Cyclooxygenase-2 (COX- 2, an enzyme that increases the production of prostaglandins at the site of inflammation) and prostaglandin E_2 (PEG2, an anti-inflammatory protein that controls excessive inflammation). These changes increase the anti-fibrotic potential in the lung (O'Reilly et *al.,* 2005).

The form and surface reactivity of the crystalline silica particles help to determine their biological activity, which defines their interaction with biological molecules and cells. The form of silica consists of the structure, the three-dimensional pattern of the silica units, the size of the particles and the shape which relates to surface area. The micromorphology plays a role in the surface activity of the particles (Fubini *et al.*, 1995).

The size of particles determines to what extent the silica crystal is able to penetrate into the lung and whether it is able to be engulfed by macrophages. Respirable size particles (< 10 μm in diameter) are inhalable and those small enough to reach the alveoli and remain there are < 5 μm (Ziskind *et al.*, 1976, Laskin *et al.*, 2007). The average size of silica particles deposited in the lung is 1.4 μm (Donaldson and Borm, 1998).

The surface area of the silica crystal is determined by the structure and the larger the surface area the greater the area available for reactivity.

Hetland *et al*, 2001 found that surface area was related to cytokine release in silica-exposed epithelial cells, suggesting that surface area was a critical determinant of silica toxicity (Hetland *et al.*, 2001). The surface activity of silica has since been demonstrated to be more important in pulmonary toxicity than particle size and surface area. All tested particle sizes produced significant adverse effects, but the pulmonary toxicity range correlated well with the haemolytic potential - a measure of differential surface activity (Warheit *et al.*, 2007).

Some of the surface Silicon hydroxide (SiOH) groups have been proposed to be negatively charged, giving the silica crystals a negative charge, allowing them to react easily with macrophages (Castranova, 1998).The reactivity of the surface of silica particles is generated in part by the homolytic and heterolytic cleavage of the silicon-oxygen bonds, forming reactive species such as O_2^- and SiO^- and charged groups Si^+ and SiO^- (Vallyathan *et al.*, 1988, Fubini *et al.*, 1990, Fubini *et al.*, 1995). The groups produced are affected by a number of factors such as contaminants present, age of the surface and available particles to react with (Castranova, 1998).

Freshly ground particles of silica have a higher level of biological activity as the newly created surface radicals have yet to interact with their environment and so more are available to induce oxidants in the lung (Vallyathan *et al.*, 1995, Porter *et al.*, 2002b). Crushing silica yields $Si^{\cdot}$ and $SiO^{\cdot}$ radicals on the cleavage planes that are able to react with water to produce hydroxyl ($^{\cdot}OH$) radicals (Shi *et al.*, 1988, Vallyathan *et al.*, 1988). These radicals react to create additional ROS, which damage tissues leading to silicosis (Fubini *et al.*, 1995, Castranova *et al.*, 1996a, Rimal *et al.*, 2005). This theory is confirmed by workers in different jobs showing different rates of silicosis; those workers who are involved in drilling and cutting rock are more likely to be exposed to freshly fractured silica and have been shown to have more silicosis. Workers using silica sand and powder as ingredients are exposed to aged silica and are observed to have lower rates of silicosis (Porter *et al.*, 2002b). Gabor *et al.*, (1975) found that crystalline silica incubated with mineral water produced a significant reduction in lipid peroxidation in alveolar macrophages (AM) compared to untreated crystalline silica. This suggests that the interaction of minerals in the water with the surface radicals of the silica particles reduces the reactivity of those particles by ageing them quickly. Where radicals are created they quickly react with other substances present (Gabor *et al.*, 1975).

The presence of other minerals and metals in combination with silica can modify the toxicity of the silica particles. The presence of ferric iron impurities have been shown to increase the oxygen radicals produced by animals when exposed to iron-contaminated crystalline silica (Castranova *et al.*, 1997). The adverse effects of iron contaminants in silica have also been demonstrated *in vitro* by the inhibition of the toxic effects of silica on cells by the addition of the chelator desferrioxamine (Driscoll *et al.*, 1995). More recently, Fenoglio *et al.*, (2003) demonstrated the ability of iron-contaminated silica to oxidise GSH, while pure silica was inactive. Also a treatment consisting of a high ratio of elemental iron to quartz in particles was found to reduce inflammation and fibrosis following quartz introduction into rat lungs. This effect was speculated to be due to iron possibly interacting indirectly with ROS rather than the silica particles (Cullen *et al.*, 1997). The same study confirmed that quartz treated with Aluminium lactate had reduced toxicity in rat lungs. Aluminium impurities found in some silica crystals have been suggested by others to decrease the formation of silicosis (Fubini *et al.*, 1995, IARC, 1997).

It is important to take the characteristics of crystalline silica particles into account when determining and setting exposure limits. Only after the presence and level of contaminants, particle size, surface reactivity, crystalinity and thermal history have been determined, the comparison of the toxicity of different dusts may be possible (Gulumian and Semano, 2003). This was demonstrated in a study by Davis *et al.*, (1982) where tests of the biological activity of samples of mine dust from various regions did not correspond to quartz content or epidemiological outcomes. It is important to take note of this when comparing studies that examine the prevalence of silicosis in different geographical regions.

1.3 Uses of silica and other industries with silica exposure

Throughout history, silica sand has been used to produce glass (harmful exposures are still possible in this industry). Currently, silica is extensively used in many industries (Table 1). It is used as an abrasive for sandblasting, in foundries for moulding, in ceramics as a constituent of clay, in construction as a component of cement and plaster, in filtration to

provide fine filtering, and in the petroleum industry. Silica crystals are also used in electronics, fibre-optics and the jewellery industry. Silica is used as a superconductor as it is very stable (IARC, 1997, Stratta *et al.*, 2001). Production and use of silica such as silica gel desiccants as fine filler in paints, rubber, plastics, asphalt and scouring powders also expose workers to respirable silica dust (Bingham *et al.*, 2001). Silica exposure is encountered in a number of other industries where it is a contaminant, for example in the mining of metal, minerals and coal (the rocks containing the substances of interest are often also composed of silica) (NIOSH, 2002). Any process that requires mechanical abrasion such as grinding, polishing and drilling such as the production of gravestones and granite kitchen counters can result in the exposure of workers to crystalline silica.

Table 1: Industries with respirable crystalline silica exposure and common tasks associated with RCS exposure (adapted from NIOSH (NIOSH, 2002))

Industry or activity	Operations and tasks
Agriculture	Ploughing, harvesting, using machinery, burning agricultural waste, processing agricultural products
Mining and related milling operations	Most occupations (underground, surface, mill) and mines (metal and non-metal, coal), rock drilling, dredging
Quarrying and related milling operations	Crushing stone, sand and gravel processing, stone monument cutting and abrasive blasting, slate work, diatomite calcination
Construction	Abrasive blasting of structures and buildings, highway and tunnel construction, excavation and earth moving and digging, masonry, concrete work, demolition, dry sweeping and brushing, pressurised air blowing, jack hammering, laying railroad track, repair
Glass, including fibreglass	Raw material processing, refractory installation and repair
Cement	Raw material processing
Abrasives	Silicon carbide production, abrasive products fabrication
Ceramics, including bricks, tiles, sanitary ware, porcelain, pottery, refractories	Mixing, moulding, glaze or enamel spraying, finishing, sculpting, firing
Iron and steel mills	Refractory preparation and furnace repair
Silicon and Ferro-silicon foundries (ferrous and nonferrous)	Raw materials handling, casting, moulding and shaking out, abrasive blasting, fettling, furnace installation and repair
Metal products	Abrasive blasting
Shipbuilding and	Abrasive blasting

repair	
Rubber and plastics	Raw materials handling
Paint	Raw materials handling, site preparation
Soaps and cosmetics	Manufacturing or occupational use of abrasive soaps and scouring powders
Roofing asphalt felt	Filling and granule application
Agricultural chemicals	Raw material crushing, handling, bagging; or dumping products or raw materials
Jewellery	Cutting, grinding, polishing, buffing, etching, engraving, casting, chipping, sharpening, sculpting
Arts, crafts, sculpture	Pottery firing, ceramics, clay mixing, kiln repairs, abrasive blasting, sand blasting, engraving, cutting, grinding, polishing, buffing, etching, casting, chipping, sharpening, sculpting
Dental material	Sand blasting, polishing
Boiler scaling	Coal-fired boilers
Automobile repair	Abrasive blasting, sanding, removing paint and rust

1.4 Crystalline silica measurement and control

1.4.1 Respirable dust measurement

Occupational hygienists who monitor the exposure of miners to RCS at the mines use two types of sampling: personal and static sampling of airborne inhalable dust. This is then reported to the Department of Minerals Resources (DMR). A number of considerations help to determine the sampling protocol used: 1) the reason for sampling, 2) the properties of the dust being sampled, and 3) the properties of the sampling methods available such as pump flow rates and capacity of the filters used.

The patterns of exposure, processes being performed, and changes in the processes and dust concentration during the shift also affect an individual's exposure level. This should all be taken into account when designing a sampling protocol for inhalable dust (Guild *et al.*, 2001).

Gravimetric dust sampling is used in mines to determine compensation fund levies and not to assess compliance of individual miners with set occupational exposure limits, so it is designed with this outcome in mind (Guild *et al.*, 2001). The method, introduced in 1992 by the South African Government Mining Engineer, divided mines into areas containing

statistical populations of similarly exposed workers of which 5% were sampled in each six-month cycle. The individual samples were averaged for each statistical population, and the statistical populations were averaged for the area average; and finally a mine average was determined by averaging the area averages (Guild *et al.*, 2001). This averaging reduces the quality of the information provided by the sampling as high levels can be hidden in the average by areas with lower levels.

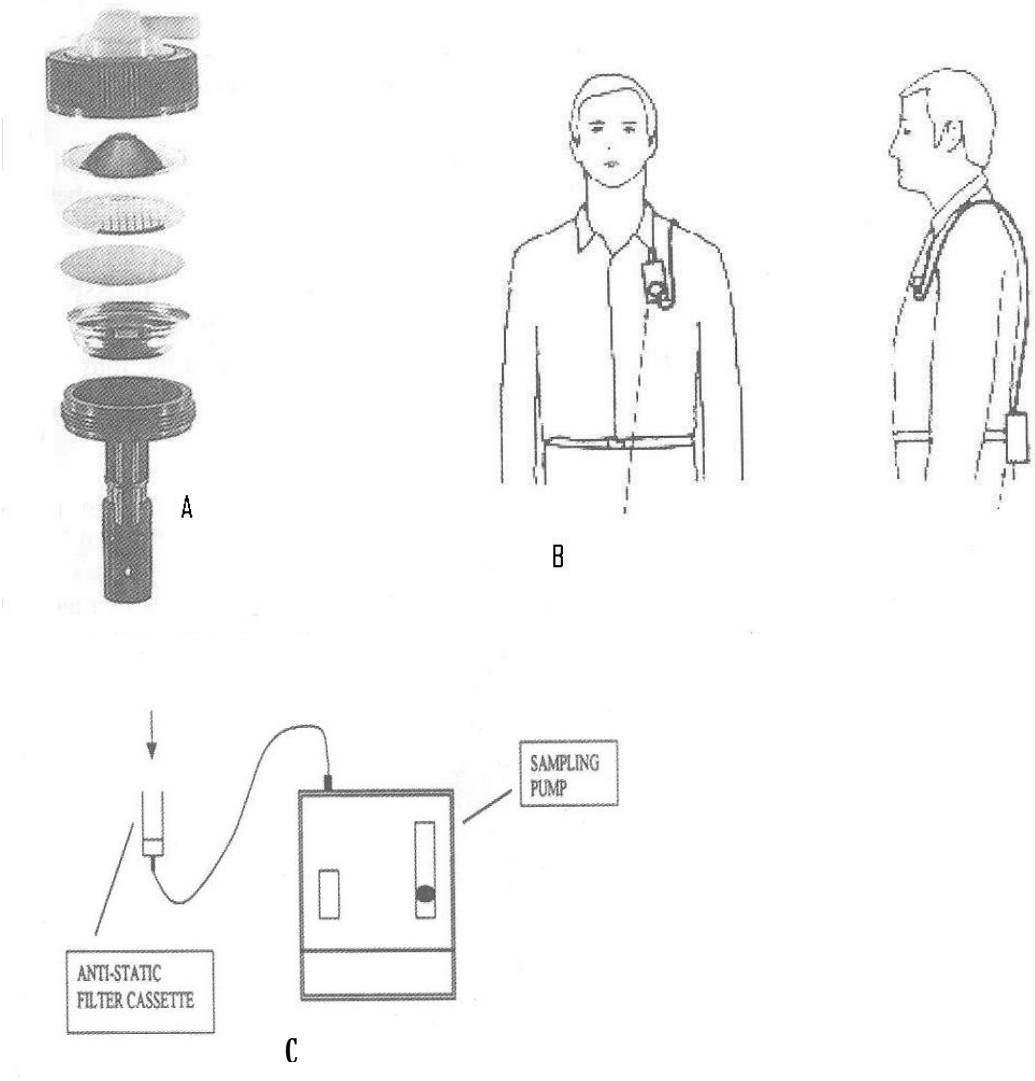


Figure 4: Gravimetric Dust Sampling Equipment. A: cyclone and filter, B: wearer with sampling pump and cassette, and C: sampling pump (from Guild *et al.*, 2001)

To carry out sampling, filters with 5 µm pore size are prepared, weighed and loaded into a filter holder in a cyclone. The pumps are prepared and the flow rates calibrated. This

apparatus is then attached to the worker to be monitored. At the end of the worker's shift, the pumps are collected, and the dust collected on the filter is weighed and analysed for crystalline silica concentration (Figure 4) (Guild *et al.*, 2001). Uncertainty in this process arises from the accuracy of the pump flow rate, the accurate placement of the filter on the worker and the work habits of the individual being sampled. The accuracy of the gravimetric analysis and the crystalline silica quantification methods add to the uncertainty of dust exposure assessment.

1.4.2 Respirable crystalline silica measurement

The RCS content of dust collected on a filter is assessed by X-ray diffraction (XRD) or infrared spectrophotometry (IR) (Stacey, 2007). The assessment of the sample in both methods can be carried out directly on the collected sample without any chemical or physical alterations to the sample. Another method of IR spectrometry is available where the sample is first digested but this method is less accurate.

Standard reference materials for the establishment of the standard curve are prepared by using a dust sample collected from a workplace, with a known certified percentage of crystalline silica. This mixed dust sample provides a better reference material for generating a standard curve than pure crystalline silica. This RCS is placed in a dust cloud generator and a range of timed samples are drawn to create different loads in the filters with the longest time placing the most dust on the filters. These prepared filters are then weighed and the percentage RCS of the standard is used to calculate the mass of the RCS present on the filter. The same filters are then analysed by either IR or XRD and used to generate a standard curve based on the gravimetric results and also as calibration filters during measurements. The sample filters are analysed in the same manner as the calibration filters. In XRD, the diffracted intensity (net peak area) is plotted against the mass of RCS present to give the calibration curve. In IR, the mean of the absorbance is at 780 and 800 cm^{-1} plotted against the mass of the RCS to give the calibration curve (HSE, 2005).

There are a number of limitations to the quantification of crystalline silica. Standards pose problems as they must be similar in particle size distribution and crystalinity to the samples, particularly when infrared absorption is used, to provide an accurate comparison.

Contamination can also interfere with the process as certain minerals also absorb IR light in the same region of IR as silica. If the dust sample contains mixtures of the different forms of silica it can lead to misclassification of amorphous silica as crystalline silica. IR determination can also be inaccurate when dark-coloured minerals such as zircon are present in the sample. Both XRD and IR experience interference when both quartz and cristobalite are present (HSE, 2005, Stacey, 2007). At the maximum levels currently specified by the US National Institute for Occupational Safety and Health (NIOSH) for silica (0.05 mg/m^3) it is difficult to quantitatively separate the amorphous forms of silica from the crystalline forms in XRD (NIOSH, 2002). This is mainly due to lack of equipment sensitivity. The detection limit for IR for quartz is 3-10 µg and for XRD it is 25 µg. The 25 µg of RCS on a filter after 8 hours of sampling corresponds to 0.05 mg/m^3. Thus, in XRD there is likely to be variation in the results at that exposure level due to the recommended NIOSH limit of 0.05 mg/m^3 being the same as the detection limit for the method (HSE, 2005) and little possibility of measuring lower levels (Figure 5).

The NIOSH recommended Reference Exposure Level (REL) of 0.05 mg/m^3 is close to the limit of what can reliably be measured with existing methods. The American Conference of Industrial Hygienists (ACGIH) threshold limit value (TLV) is 0.025 mg/m^3 and this is at the limit of reliability. This does not take into account other interfering factors such as contaminants, interferences and variability of the environment. Sampling and analytical methods need to be improved before the recommended level can be reliably measured in all circumstances (Stacey, 2007).

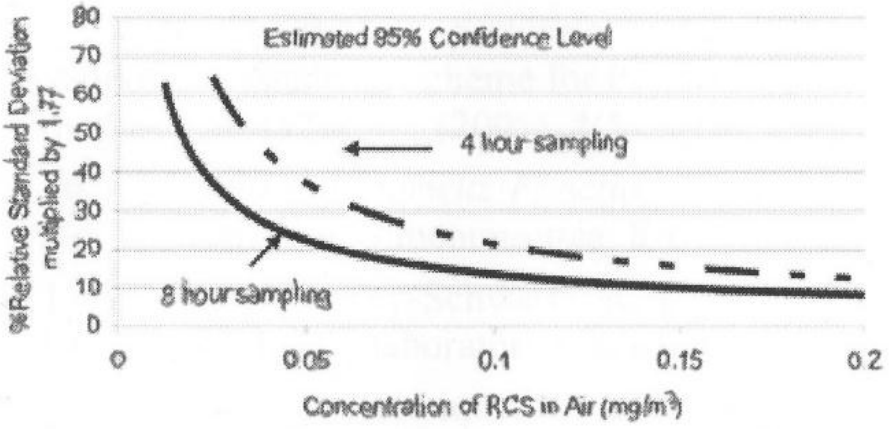


Figure 5: Predicted % Relative standard deviation (RSD) of silica measurements (a measure of uncertainty of measurement) (from Stacey, 2007).

1.4.3 Controlling silica exposure

To control exposure to RCS it is important to identify areas where exposure may occur and the extent of that exposure.

A hierarchy of control measures for the prevention of occupational disease has been suggested and should be applied in the control of RCS exposure. The first primary prevention is to replace the RCS in workplaces with less toxic alternatives. In industries where RCS is used as an abrasive, it can be replaced with abrasives that contain less than 1% crystalline silica. Secondly, if no alternative to RCS exists or it is an unavoidable contaminant, attempts should be made to control exposure at the source (Schulte, 1995). Effective engineering controls consist of dust suppression measures such as wet drilling, dampening drilling surfaces, dust collection with local exhausts and equipment-specific dust collection systems (Schulte, 1995, NIOSH, 2002). In the hierarchy, a change in work practices is then recommended, such as enclosed isolation systems for example air-conditioned cabs under positive pressure with filtered air (MMWR, 2000). Local exhaust systems prevent the release of dust into the air. As a last resort, use of respiratory protection equipment (RPE) is recommended when the dust exposure levels are above acceptable or regulated levels (Schulte, 1995, Guild *et al.*, 2001). Workers then need training in the use and maintenance and cleaning of the masks. For effective protection when using the masks, regular fit testing is necessary to ensure there are no gaps in the system where dust can enter (NIOSH-Alert, 1996).

A worker training programme and good work practices in the field can protect workers and others nearby from being exposed to RCS. Measures such as cleaning of the environment with water prior to working there and using water hoses to clean equipment are important. Personal care when using and cleaning protective clothing is also useful to prevent dust and other contaminants from entering the body, and to make sure that dust is not carried home or to other clean areas (NIOSH-Alert, 1996).

1.4.3.1 Occupational exposure limits

Occupational exposure limits (OELs) are governed by legislation, and describe the concentration of toxic substances to which workers may be exposed without harm. Guild et al., (2001) described an OEL as "the concentration of a substance to which nearly all workers may be repeatedly exposed day after day without adverse health effects."(Guild *et al.*, 2001). The time-weighted average (TWA) is the concentration allowed for an 8-hour shift and a 40-hour work week and is known as the OEL-TWA. The OEL described in South African legislation is the OEL-TWA and there are two other types of OELs; first is OEL-C, which is an instantaneous value that may not be exceeded during any peak exposure during the day. The second is OEL-STEL, a 15-minute exposure limit, exposures at this level should not occur more than four times during the working day. These OELs should not be exceeded (DMR, 1996).

The OSHA has set a permissible exposure limit (PEL) for respirable quartz of 10mg/m^{3}divided by % SiO_2 + 2 with the percentage quartz being determined from the respirable portion collected (US Department of Labour, 1970). The South African regulated OEL for RCS is 0.1 mg/m^3 TWA for an eight-hour workday and a 40-hour week (Government Gazette, 2002). The Health and Safety Executive (HSE) in the UK calculated the risk of developing silicosis after 15 years of exposure at 0.1 mg/m^3 as 2.5% (Stacey, 2007). However, due to the continuing occurrence of silicosis, OSHA has been considering whether the PEL for respirable quartz should be re-evaluated (Porter *et al.*, 2002a).

NIOSH recommends a recommended exposure limit (REL) of 0.05 mg/m^3 for RCS as a time-weighted average for up to 10 hours per day for a 40-hour workweek (NIOSH, 1974 and 2008).

Collins *et al.*, (2005) derived a chronic inhalation REL of 3-10µg/m^3 for workers based on five studies with sufficiently large populations and dose response data. This is well below the currently recommended REL of 0.05 mg/m^3 which has been described as being at the limit of current methods of detecting silica. To lower the level to 0.01 mg/m^3 would require a new methods and measurement tools for evaluating exposure to silica.

1.4.4 Current levels reported to the DMR

Respirable quartz concentrations in the air are required to be reported to the South African DMR (Gibbs and Du Toit, 2002). The 1983 levels from measurements made by the Mines Inspectorate of the Chamber of Mines give an average exposure of 0.06 mg/m^3, which is well below the legally prescribed level of 0.1 mg/m^3 , and yet the prevalence of silicosis is still considerable (Kielblock, 1997). This suggests that either there may be inaccuracies in the determination of RCS concentrations or the levels currently prescribed are not sufficiently protective (Gibbs and Du Toit, 2002).

1.5 Diseases caused by crystalline silica exposure

Exposure to RCS occurs mainly via inhalation; therefore, pulmonary disease is mostly to be expected (Castranova and Vallyathan, 2000). However, this exposure can also lead to extra-pulmonary disease. Silicosis is the most common disease associated with RCS exposure, but it is by no means the only one (Green and Vallyathan, 1996, Stratta *et al.*, 2001). Systemic and autoimmune diseases have also been associated with RCS exposure (Steenland, 2005) possibly due to RCS absorbed in the lung being carried to lymph nodes by AM.

An increased risk of lung cancer has been attributed to RCS exposure. The International Agency for Research on Cancer (IARC) classified RCS as a carcinogen in 1997, although it was noted that not all studies showed an association and this may be due to individual properties of RCS particles (IARC, 1997). A pooled analysis of 10 cohorts showed that lung cancer rates increased as cumulative exposure to RCS increased. Based on a mathematical model the study showed a lung cancer excess risk of 1.7% at age 75 after 45 years of exposure to RCS at 0.1 mg/m^3 (Steenland, 2005). However, silicosis may be a confounding factor in estimating lung cancer risk (Brown, 2009), as those individuals with high exposure will increasingly develop silicosis. In addition, smoking is still the dominant risk factor in lung cancer. No dose-response relationship has been demonstrated for RCS exposure and lung cancer (Green and Vallyathan, 1996). This suggests that RCS may not be a direct cause of the link between RCS exposure and lung cancer but rather a potentiating factor.

With acute severe RCS dust exposure pulmonary effects are common. Effects such as emphysema may account for the abnormal lung function found in miners with silicosis. Mortality studies have shown an association between cumulative dust exposure and bronchitis and emphysema in white gold miners (Green and Vallyathan, 1996). In one study 70 men with and without silicosis, with an average of 29 years underground work were examined using computed tomography, lung function and chest radiography. They found silicosis was related to emphysema as 78% of the men diagnosed with silicosis also had emphysema compared to only 33% of the men without silicosis (Cowie *et al.*, 1993).

Autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosis and systemic sclerosis have all been linked to RCS exposure (Otsuki *et al.*, 2007). In addition, RCS exposure may result in nephropathy and proliferative glomerulonephritis (Castranova and Vallyathan, 2000). RCS particles can reach the kidney following inflammation in the lung and the accumulation of immune complexes can directly damage the kidney (Steenland, 2005). Autoimmune mechanisms activated in the lung have also been proposed as responsible for the autoimmune damage to the kidney (Calvert *et al.*, 1997).

Table 2: Comparative lifetime risk of men (age 75) following 45 years of RCS exposure (from Steenland, 2005).

Disease	**Absolute or excess risk at 0.1 mg/m^3 silica exposure**	**Background risk of US males**
Silicosis (ILO score 1/1 or more)	47 - 77% (based on three cohort studies with post-employment follow up).	none
Death due to silicosis	1.9 % (0.8 - 2.9) (based on six pooled cohorts)	none
Lung cancer death	1.7% (0.2 – 3.6)	7.5%
End stage kidney disease incidence	5.1% (3.3 -7.3)	2%
Kidney disease mortality	1.8% (0.8 – 9.7)	0.3%

Table 2 shows the absolute and excess risks of disease or mortality due to RCS exposure in males. The OSHA acceptable excess risk of disease or mortality for workers is 0.1% (Steenland, 2005). This table shows excesses well above the 0.1% risk, suggesting that the 0.1 mg/m^3 level of RCS exposure is not sufficiently protective of workers for a number of health outcomes.

1.6 Silicosis and pneumoconiosis

Pneumoconiosis is a lung disease resulting from dust exposure. The term proposed by Zenker in 1867 comes from the Greek words for lungs and dust. It is generally translated to mean "dusty lung" (Parkes, 1974, Stratta *et al.*, 2001). The association between dust and lung disease has long been suspected from Greek and Roman times (Stratta *et al.*, 2001) to the 1500s (Ross and Murray, 2004). Pneumoconiosis was defined by Parkes, (1974) as "the presence of inhaled dust in the lungs and their non-neoplastic tissue reaction to it," where dust is described as solid particles in the air and not vapours or fumes. The reaction in the lungs can be detrimental in the long term.

There are a number of forms of pneumoconiosis, each caused by a different type of dust (Spencer, 1985). Coal Worker's Pneumoconiosis (CWP), mixed dust pneumoconiosis, and silicosis are all examples of pneumoconiosis due to different exposures. CWP is both clinically and pathologically distinct from silicosis (although many coal miners are exposed to RCS). In simple CWP, black coal dust macules are formed in the lungs which consist of coal-laden macrophages and a fine network of collagen and reticulin (Castranova and Vallyathan, 2000). It is not possible to distinguish between silicosis and CWP on a chest X-ray, and can only be established at autopsy (Green and Vallyathan, 1996, Vallyathan *et al.*, 1996). Scleroderma also predominately affects the same areas in the lung as silicosis, making radiological diagnosis difficult (Bergin and Muller, 1987).

1.6.1 Silicosis signs and symptoms

Silicosis is an irreversible, untreatable, progressive disease caused by exposure to RCS. Silicosis occurs mainly in occupational settings that use high-energy processes that generate and release RCS particles. It is a progressive fibrotic lung disease that can be chronic or acute. The term silicosis was proposed for the pneumoconiosis resulting from RCS exposure at the end of the 19th Century, from the Latin word for flint, "silex" (Lemen and Bingham, 1994, Stratta *et al.*, 2001). There are four main types of silicosis, namely acute, chronic, accelerated and conglomerate (Craighead, 1996).

1.6.1.1 Acute silicosis

This type of silicosis is also known as silicolipoproteinosis (Green and Vallyathan, 1996, Castranova and Vallyathan, 2000), or silicotic alveolar proteinosis (Lemen and Bingham, 2001). It tends to develop from exposure to comparatively higher levels of relatively pure silica. It has been suggested by Vallyathan *et al.*, (1995) that freshly fractured RCS with silica radicals play an important role in the development of acute silicosis. This type of silicosis develops rapidly after 0.5 - 5 years of exposure (Castranova *et al.*, 1996b, Green and Vallyathan, 1996).

Acute silicosis is characterised by a disease process consisting of pulmonary oedema, interstitial inflammation and a proteinaceous fluid within the alveoli. The alveoli also appear to secrete an eosinophilic fluid with a granular appearance (Craighead, 1996). It is distinguished from idiopathic alveolar proteinosis by the development of interstitial inflammation and fibrosis (Shah *et al.*, 2000). There are increases in the numbers of AMs and protein like material in the airspaces (Jones et al., 1993). Symptoms of acute silicosis include decreased pulmonary function, fatigue, weight loss and cough, resulting in cyanosis and respiratory failure (Shah *et al.*, 2000). Some workers may be repeatedly diagnosed with apparent pneumonia (American Thoracic Society, 1997). Diagnostic chest X-ray findings include diffuse fine granular lesions in the middle and lower lobes (American Thoracic Society, 1997). This type of silicosis has a high mortality rate (Green and Vallyathan, 1996).

1.6.1.2 Accelerated silicosis

This is similar to acute silicosis and is caused by similar relatively high exposures. It is not always recognised as a separate type of silicosis. It can develop after one to 14 years of high to moderate exposure (Green and Vallyathan, 1996). Accelerated silicosis has a similar pathology to acute silicosis. Chronic inflammation is seen along with alveoli secreting proteinaceous fluids but it differs from acute silicosis by the fibrotic granulomas seen containing collagen, reticulin and RCS particles. Accelerated silicosis lies between acute and chronic silicosis. Accelerated silicosis has been associated with an increase in morbidity and mortality (Castranova and Vallyathan, 2000).

1.6.1.3 Chronic silicosis

As workers' exposure to RCS becomes more controlled, less acute and accelerated silicosis and more chronic silicosis is expected. Long-term exposure to lower levels of RCS dust, compared to the exposure required for acute silicosis, can result in the development of fibrosis in the lungs in the form of small individual nodules, also known as nodular pulmonary fibrosis (Stratta *et al.*, 2001). Chronic simple silicosis is the most common form of lung disease associated with RCS exposure (Green and Vallyathan, 1996). This type of silicosis is characterised by a long latency (Murray *et al.*, 1996). The nodules have a characteristic histological whorled appearance, with concentric rings of collagen fibres and hyalinised zones (Figure 6) (Craighead, 1996). They measure 1 - 3.5 mm in size (White *et al.*, 2001). These are distinct from other lesions produced by other occupational exposures. Macrophages, fibroblasts and lymphocytes are found around the boundaries of these nodules, along with normal lung tissue. These lesions are found mainly in the upper zones of the lungs and can vary in size. They are seen on X-rays as rounded opacities. Dust particles are found in macrophages and in the walls of the alveoli. The nodules contain more collagen than dust and are called proliferative, as continuing exposure to dust can cause the nodules to increase in size as well as new nodules to form (Parkes, 1974). The nodules can also continue to grow even when dust exposure has ceased (Bingham *et al.*, 2001).

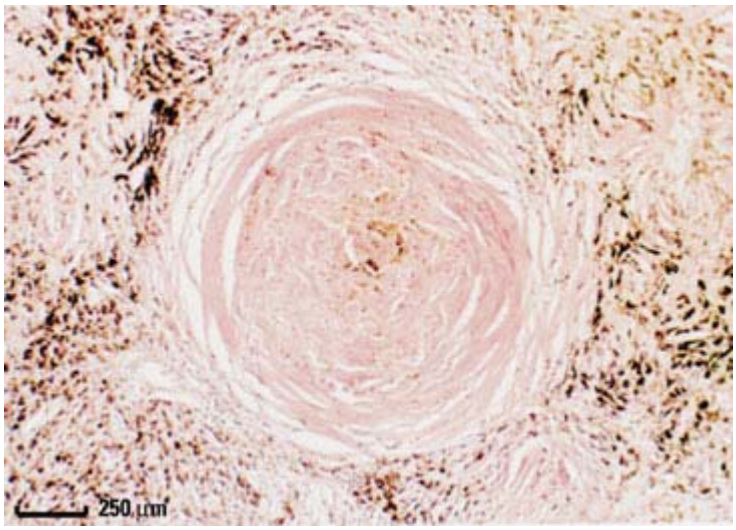


Figure 6: Histological image of a characteristic nodule of silicosis (from Castranova and Vallyathan, 2000).

Initially, as silicosis develops there is no effect on lung function, but as the disease progresses lung function diminishes. This loss of lung function can be clinically correlated with worsening X-ray findings. Symptoms recorded include fatigue, productive cough, and dyspnoea. Lung function progressively worsens as the disease progresses, and in the late stages cardiac problems may develop (Lemen and Bingham, 2001).

The histopathology of silicosis demonstrates the pathway for the progression of silicosis. In simple silicosis, nodules or islets less than 1 cm in diameter are found in the lung in the interstitial tissue. These nodules consist of a central core of collagen and are surrounded by whorls of collagen, macrophages and lymphocytes. The earliest lesions consist of a collection of macrophages containing particles. As silicosis progresses, the nodules enlarge with fibroblasts and connective tissue and join to form larger areas of collagen and lymphocytes. These enlarged joined nodules replace the lung tissue and eventually can result in a loss of functionality (Davis, 1986, Kane, 1996)

1.6.1.4 Conglomerate silicosis and progressive massive fibrosis

Conglomerate silicosis is a progression of simple nodular fibrosis. Not all those with nodules progress to conglomerate silicosis or progressive massive fibrosis (PMF). In conglomerate silicosis a few smaller nodules join to form nodules >1 cm in diameter, although the individual nodules retain some of their structure (Parkes, 1974, Lemen and Bingham, 2001). PMF is common to a number of pneumoconioses, not just silicosis. A profusion of nodules appear. Lung tissue (parenchyma and bronchioles) and blood vessels are destroyed during this process (Castranova and Vallyathan, 2000). The diagnosis of PMF is based on chest X-ray findings with nodules that measure greater than 1 cm in diameter (International Labour Office, 2001), although some experts define PMF as having nodules greater than 2 cm in diameter. As the silicosis progresses, the radiographs will show an increase in the number and size of the opacities, and small conglomerations will develop into large irregular opacities (Parkes, 1974). Young age of onset and continued dust exposure once simple silicosis is diagnosed are two known determinants of progression to PMF (White *et al.*, 2001). Nodules may also be found in the liver and spleen and symptoms are more pronounced as the disease progresses and shortness of breath occurs during effort (Parkes, 1974). Tuberculosis may cause the sudden expansion of opacities in the lung or cavitation of the nodules.

1.6.2 Pulmonary function and silica exposure

Loss of lung function amongst gold miners has been attributed to dust exposure and smoking in a study by Hnizdo, (1992). Cumulative respirable dust exposure was associated with a significant decrease in 1-second forced expiration volume (FEV_1) and forced vital capacity (FVC) in a study of coal miners, although these changes were not significantly associated with silicosis (Naidoo *et al.*, 2005).

1.6.2.1 Complicated silicosis is associated with poor lung function

With acute severe RCS dust exposure, pulmonary signs such as reduction in lung function are common. Also as silicosis develops, increasing pulmonary signs are noted. There is some controversy over low chronic exposures and their effects on pulmonary function. In a study by Hertzberg *et al.*, (2002) the effect of occupational RCS exposure on lung function was determined. Lung function tests were done on 1,028 employees of a foundry and compared to exposure assessments for the previous 26 - 30 years. The participants were stratified by cumulative RCS exposure and smoking status. The study found a relationship between cumulative RCS exposure and abnormal FVC in smokers. In non-smokers, there was no relationship. Although a pattern was seen between increasing RCS exposure and decreasing FEV_1, it was not statistically significant. A significant trend relating abnormal FEV_1/FVC and cumulative RCS exposure was seen. The longitudinal analysis showed a 1.1 mL/year change in FEV_1 for each mg/m^3 of mean RCS exposure and a 1.6 mL/year decline in FVC for each m^3 of mean RCS exposure (Hertzberg *et al.*, 2002). This study demonstrates that low-level exposure to RCS at the OSHA recommended level of 0.1 mg/m^3 is related to increased lung function abnormalities without the development of silicosis. This study is also likely to be underestimating the effects due to the healthy worker effect, where those workers with a greater possible effect due to exposure are more likely to have left the workplace. Also, those who developed disease or impairment would have left the workplace, leaving the younger newer employees and those who are naturally healthier to be surveyed.

In 1993, Cowie and van Schalkwyk (1987) reported on the association of emphysema and silicosis in gold miners.

1.6.3 Treatment of silicosis

There is no recognised specific and effective treatment for silicosis. Complications of silicosis such as tuberculosis, heart failure and airways disease are managed symptomatically (Fishwick, 2004).

1.6.4 Current methods of screening for silicosis

Radiography is used to screen miners and dust-exposed workers for silicosis. The ILO provides full-size standard posterior-anterior chest radiographs that are used for reference as diagnostic standards when screening for silicosis. A trained reader will compare the radiograph of the miner to the supplied standards. The reader will match the profusion and size of opacities present in the miner's X-ray to the standard radiographs supplied (International Labour Office, 2001). The score of the standard radiograph describes the severity of silicosis on a 12-point scale. Any other pathology visible on the radiograph will be noted, such as active pulmonary tuberculosis (pTB) or pTB scars and heart deformities. The 12-point scale consists of four levels, i.e. from 0, 1, 2, 3, each broken down further into three levels. These allow for some uncertainty in the grading. The first group, 0/0, means that the radiographer is confident there are no opacities present. The next two groups, 0/1 and 1/0, means increasing confidence that there is some chance of silicosis. 1/0 is the cut-off for diagnoses of silicosis in some studies, while other studies use 1/1, which means that the reader is confident that there is early simple silicosis. From there on, 1/2 means that there is silicosis and it may be of a higher grade, and this continues up to 3/3, which means complicated severe silicosis (Figure 7) (International Labour Office, 2001).

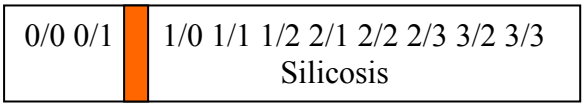


Figure 7: Radiograph ILO grades of silicosis with cut-off (orange) for diagnosis of silicosis

1.6.3.1 Limitations of radiographs for diagnosing silicosis

The South African gold mines have sometimes used mini radiographs and, sometimes, full radiographs, to screen miners for pTB and silicosis in the past. More recently, there has been increasing use of digital radiographs. There is some controversy regarding the sensitivity and specificity of digital radiographs against the ILO standards.

A number of factors, namely radiographic quality and technique of the radiographer, increased Body Mass Index (BMI), age, smoking and the experience and bias of the readers affect the sensitivity and specificity of X-ray diagnosis (Checkoway and Franzblau, 2000). X- rays are two dimensional and superimposition of objects obscures the lungs. Chest X-rays are non-specific and, although lung changes are significantly associated with specific diseases, this does not always apply to individual cases. Making the correct diagnosis from radiographs is difficult as up to 10% of normal X rays have been proven incorrect by biopsies (Hansell and Kerr, 1991).

Corbett *et al,* (1999) assessed the accuracy of radiographs in predicting silicosis, using lungs which had been sent to the National Institute for Occupational Health (NIOH) for autopsy from miners who had worked for a single company. The study found that while the percentage of men with silicosis of any grade were similar by the two methods, namely radiology and pathology, overall agreement between radiology and pathology for low grades of silicosis was poor. The majority of radiology-undetected silicosis was early pathological disease. This is an important group to detect as it is necessary to remove these miners from further exposure to reduce risk of progression of disease. Agreement between radiology and pathology was better for higher grades of disease. This indicates that radiology is an insensitive tool for the early detection of silicosis. This hampers epidemiological studies and other silicosis research.

Other studies have shown that, even on standard size radiographs, there is a large amount of both inter-reader and intra-reader variability. This adds to the lack of specificity of detection of silicosis with radiographs. The subjective differences between and within readers classifying radiographs causes variability in the reliability (repeatability) of a diagnosis of silicosis and the stage there of. Diagnosis of pneumoconiosis in a study on radiographic outcomes of coal miners by two experienced readers showed variability

based on the reader. The readings only had moderate agreement as analysed by kappa score (kappa = 0.58) (Naidoo *et al.*, 2005).

Diagnosis of PMF is also complicated in a chest X-ray as it can possibly be confused with bacterial infectious lesions such as tuberculosis, and with carcinomas (Vallyathan *et al.*, 1996).

1.7 Silicosis, Human Immunodeficiency Virus and Tuberculosis

Historians divide the history of silicosis in South Africa into four periods:

1. The initial period of gold mining with ignorance of the dangers of the dust and silicosis.
2. The realisation of the presence of silicosis from 1902 to 1910 and the introduction of some prevention measures.
3. The introduction of the compensation system from 1911 to 1916.
4. The systematic measures of prevention, detection and compensation from 1916 to 1945 (Donsky, 1993).

From 1945 to the present, improvements have been made on this system but there is also a suggestion that dust levels have not changed appreciably.

Silica is one of the most common types of dust in the environment, which is why silicosis is one of the most common types of pneumoconiosis (Tiwari, 2005). The South African gold mining industry has had many years of experience with silicosis (Churchyard *et al.*, 2004). The prevalence of silicosis among miners has been ascertained in numerous studies in both black and white miners (Table 3).

In the early years of gold mining in South Africa, white miners were permanent employees and the majority of the black miners were migrant workers and held contracts for 6 - 12 months. Thus, the prevalence of silicosis in white miners would be expected to be higher compared to the black miners, although the black miners worked in the dustiest jobs (Donsky, 1993). As detection of silicosis improved, the number of men with silicosis increased, but also, those who were considered susceptible, were sent home.

Table 3: Studies of silicosis and/or pTB prevalence among miners

Year	Current or former workers	White or Black	Silicosis Prevalence	pTB Prevalence	Reference
1998	Former	Black	26.6% and 27%	Incidence 3%	(Park et al 2009)
1998	Former	Black	24.6%	30% past and current	(Girdler Brown et al 2008)
2000-2001	Current	Black	18.3% and 19.9%	Not ascertained	(Churchyard et al 2004)
1997-1999	Former	Black	35.4%	64% total 28.4% pTB and silicosis	(Meel, 2002)
1984	Current	Black	0.99%, 1.34% and 3%	Not measured	(Cowie and van Schalkwyk, 1987)
1975-1991	Current at autopsy	Black	9.3% in 1975 to 12.8% in 1991	Incidence 0.9% in 1975 to 3.9% in 1991	(Murray *et al.*, 1996)
1960-1970 and 1992 – 2002	Compensated miners	undefined	1960-70 38.2% 1992-2002 11.8% of cases	1960-70 28.4% 1992 -2002 86.5%	(Mulenga *et al.*, 2005)
1996	Former	Black	22% and 36%	33% and 47%	(Trapido *et al.*, 1998)
	Autopsy	White			(Hnizdo and Sluis-Cremer, 1993)
1968-1971	Record review	White	14.2%	Not measured	(Hnizdo and Sluis-Cremer, 1993)
Cohort from 1968-1971 to 1995	Autopsy	White	55.4.%	Incidence 5%	(Hnizdo and Murray, 1998)

Black mineworkers were a difficult group to study, as many were migrant labourers or contract workers. These miners also tended to move between mines making cohort

analyses difficult. The storage policies of radiographs by the mines made historical cohort analyses difficult. The majority of the mineworkers are black and recruited from rural areas, which means that it is difficult to interview ex-mineworkers without travelling to rural areas. This can result in a healthy worker bias in determining the prevalence of silicosis in black miners. To remedy this, the prevalence of silicosis among former black mineworkers has been studied in three investigations (Trapido *et al*., 1998, Steen *et al*., 1997, Meel, 2002). These were conducted to remove the healthy worker effect and to allow the latent period for the development of silicosis to have lapsed to determine the true effect of RCS exposure.

Migrant labour is a feature of gold mining in South Africa. The miners come from regions in South Africa and from nearby countries such as Botswana, Lesotho and Mozambique. The Eastern Cape has been a major internal supplier of labour for more than one hundred years. Thus, to determine the prevalence of occupational lung disease in ex-miners this region was chosen for study by Trapido *et al.*, (1998). A random sample of former mineworkers was chosen from the The Employment Bureau of Africa (TEBA) registries, specifically those who were recruited between 1969 and 1980. This was to allow those diseases with long latencies to develop and most workers to have returned home. The mean length of employment amongst these men was 9.3 years confirmed service and 12 years unconfirmed service. Silicosis was classified as those with X-ray readings with an ILO score greater or equal to 1/0. High prevalence of pneumoconiosis (22% and 36%) was diagnosed in the ex-miner community. The two figures reflect the differences between readers. Previously undiagnosed and uncompensated pneumoconiosis was found in this group. A significant association was demonstrated between total length of service and pneumoconiosis. This study also found a lack of occupational health services in the labour sending area, which leaves the community to bear the costs of the occupational disease (Trapido *et al.*, 1998).

In the same region, a study by Meel, (2002) of 300 randomly selected ex-miners from hospital patients who were X-rayed for compensation found a prevalence of silicosis of 35.4%, which confirms the prevalence found by Trapido *et al.*, (1998). The majority of the miners were in the 46 – 55 year age group and with 6 to 15 years of service on the mines.

Between May 1997 and May 1999, 2 080 former mineworkers were examined at a clinic in Umtata General Hospital. Full X-rays were taken and physical examinations were done. The workers' mean age was 51.6 years and they had a range of work histories from one year to 31+ years, with the majority working six to 11years on the mines. In 78% of these ex-mineworkers' X-rays showed evidence of lung disease (Meel, 2002).

In a survey by Steen *et al.*, (1997) the Kweneng District, Botswana, was chosen as a representative of an area with a long history of labour recruitment for the mines. The village of Thamaga was surveyed and 220 ex-mineworkers were randomly chosen to participate. The remainder were invited to attend the clinic. Of the ex-miners, 89% had worked underground and 85% on gold mines. The mean age of the workers was 56.7 years with a mean length of service of 15.5 years. The radiographs showed an association between length of exposure and ILO profusion. A good correlation was also seen between pneumoconiosis, progressive massive fibrosis and years since first exposure. A prevalence of 25.7% was calculated for silicosis. A latent period of five years was determined for pneumoconiosis and fifteen for lung cancer.

A study conducted by Cowie and Van Schalkwyk in 1987 determined a silicosis prevalence of 0.9% in current workers using mini radiographs in a 6-month sample of 100 880 workers. When they counted the number of workers diagnosed with silicosis in a year, the prevalence was slightly higher at 1.34%, although as a control 255 workers with normal X-rays were re-examined six months later and 3% were found to have developed silicosis. The 3% is likely to be more accurate as there were a number of limitations in the study design such as no work histories taken thus increasing the likelihood of including new miners and reducing the incidence of silicosis.

Current prevalence of silicosis

Since the early studies were conducted, the prevalence of silicosis appears to have risen as the autopsy study by Murray *et al.* (1996) and Nelson *et al*. (2010) have shown. During autopsies on miners who died of non-natural causes, the authors found histological silicosis in 9.3% of the miners in 1975 and 12.8% in 1991, but much of this difference was explained when the age of the miners was taken into account. The increasing length of service of migrant workers may also account for the increasing prevalence of silicosis seen in the study by Cowie and Van Schalkwyk (1987).

The prevalence of silicosis in current black migrant workers was assessed recently in 520 workers in a study by Churchyard *et al.*, (2004). The mean length of service in these workers was 21.8 years the mean intensity of their respirable dust exposure was 0.37 mg/m^3 and their quartz exposure was 0.053 mg/m^3. A prevalence of silicosis of 18.3 to 19.9 % was found, depending on the reader. Trends found between prevalence of silicosis and length of service, intensity of exposure and cumulative exposure were all statistically significant. This indicates a high prevalence of silicosis amongst older black workers in the South African gold mining industry. This study also indicated that the South African occupational exposure limit of 0.1 mg/m^3 may not be protective against silicosis as these workers were exposed to levels with a mean well below the OEL of 0.1 mg/m^3 (Churchyard *et al.*, 2004). These were current workers and so the healthy worker effect may have played a role and thus the prevalence of silicosis may have been underestimated.

Girdler-Brown *et al.*, (2008) conducted a prevalence study involving former miners from Lesotho who had worked on a mine in Free State province. Of the 624 ex-miners participating, 24.6% were found to have silicosis (grade 1/1 or greater). This prevalence corresponds well with that observed by Churchyard *et al.*, (2004). The mean time since last exposure was short, only 1.5 years. With a mean duration of service of 25.6 years, the increase in prevalence compared to Churchyard's study would be expected.

All these studies combined would suggest that silicosis is a significant disease in miners and ex-miners exposed to RCS. The fact that some studies suggest that the prevalence may be increasing demonstrates the need for action on RCS exposure.

Silicosis in other countries

In the period from 1968 to 2002, 98% of deaths from silicosis in the US were in men. These deaths decreased during this period from a rate of 8.9 to 0.66. This decrease was caused by the introduction of National Compliance Standards. The regulatory limits began to be applied through dust measurements and preventative measures such as respiratory protection, warning signs in dusty areas and reporting of occupational diseases. The second factor producing the decrease was the decreasing employment in heavy industries as automation increased; this is particularly evident in the US mining industry. The proportional mortality ratios for silicosis by industry were calculated, and metal mining

had the highest ratio at 39.2, followed by mineral and stone products and ceramics (MMWR, 2000).

India has a large RCS-exposed population due to many industries involving stone working using crude methods with no protection, but silicosis is prevalent even in individuals not occupationally exposed to RCS (Tiwari, 2005).

In China, occupational lung diseases have been recognized as an increasing problem, despite significant achievements in disease recognition and exposure control. Recent rapid industrialization and economic development have resulted in a changing profile of occupational disease epidemics, with decreasing incidence of occupational lung diseases in major urban centers and increasing incidence in newly industrialized rural areas (Zang *et al*., 2010). Chen *et al.*, (2005) found a prevalence of silicosis of 35.8% in retired tin miners and 44.5% in deceased tin miners.

1.7.1 Tuberculosis prevalence and its association with silicosis

pTB is an infectious disease caused by *Mycobacterium tuberculosis.* Tuberculosis is a common complication of silicosis (Green and Vallyathan, 1996). It is widely accepted that there is an elevated risk of pTB in individuals with silicosis and therefore it is compensable in South Africa (Cowie, 1994, Corbett *et al.*, 2004). The depressant effect of RCS on the ability of AMs to destroy the tuberculosis mycobacterium has been demonstrated *in vitro* where cell-mediated immunity may be compromised with alterations in lymphocyte subsets (Castranova and Vallyathan, 2000). This may lead to the increased susceptibility to pTB seen in silicotics and miners exposed to RCS.

This susceptibility was demonstrated in a study to determine the incidence of pTB in a population with a high prevalence of pTB. A cohort of older gold miners with and without silicosis was followed for seven years. This cohort had not had pTB before. The annual incidence of pTB was described. For miners without silicosis the incidence rate of pTB was 981 per 100 000 per year and for those with silicosis it was 2 707 per 100 000 per year, giving a RR of 2.8 for men with silicosis. The incidence of pTB also increased with increasing severity of silicosis (Cowie, 1994). This suggests that not only does silicosis

result in loss of quality of life and a shortened life span, but also an increased risk of pTB, which further affects quality of life.

Hnizdo and Murray, (1998) conducted a study to determine the relative risk of pTB in white gold miners who were autopsied. Of those with no silicosis at autopsy, the RR of pTB was 1.38 for the highest dust exposure. This risk increased with increasing level of cumulative dust exposure in those with no silicosis. Radiologically diagnosed silicosis was also associated with an increased risk of pTB, which increased as the level of diagnosed silicosis increased.

In another study by Meel, (2002) of former mineworkers in the Eastern Cape, 62% were diagnosed with pTB, and the association between TB and silicosis was calculated as odds ratio (OR) 5.08 $p<0.01$. This study reports the base rate of pTB in the Eastern Cape as 5%. This indicates that in the majority of the ex-mineworkers their pTB could be due to previous occupational exposure to RCS. Also strengthening this supposition is the strong association seen between pTB and silicosis in current mine workers (Meel, 2002).

Other pathogenic mycobacteria have also been suggested to occur with increased prevalence in RCS-exposed workers (Green and Vallyathan, 1996).

pTB has been associated with inflammation related oxidative stress. It has been proposed that lung fibrosis associated with pTB is initiated by activated macrophages that are able to release a number of factors including ROS and cytokines (Wiid et al., 2004). Miners are at an increased risk of developing pTB and thus this was an important confounder in the current study. Wiid et al. (2004) found a significant decrease in the TAS of patients diagnosed with pTB compared to community controls. The TAS of the patients increased with pTB treatment to within normal levels.

1.7.2 Human Immunodeficiency Virus (HIV)

HIV is a retrovirus of the genus *Lentivirus* and family Retroviridae. HIV is classified into two types: HIV 1, which is found globally, and HIV 2, which is mainly restricted to West Africa. HIV 1 is the type mainly found in South Africa. It is a rapidly evolving virus, and this has implications for its control (Wilson *et al.*, 2000).

HIV is transmitted in body fluids. It infects cells with specific receptors and as a retrovirus it replicates using the host cell's own mechanisms.

The primary immune response to HIV is vigorous to provide immunity: this is an increase in T-lymphocytes and antibodies. As the disease develops, there is an increasing inability to launch new immune responses (Wilson, 2002). HIV causes alterations in the immunological processes of the body which result in the progressive development of susceptibility to opportunistic infections, which finally develop into Acquired Immunodeficiency Syndrome (AIDS) (Aghdassi and Allard, 2000). HIV infection disrupts cytokine secretion, with increases and decreases of individual cytokines reported (Wilson, 2002).

Oxidative stress is thought to play an important role in the progression of HIV as it plays a role in the replication of the virus and the development of immunodeficiency (Schreck *et al.*, 1991). *In vitro* experiments demonstrated that ROS activate Nuclear Factor kappa beta (NF-κB) to induce expression and activation of HIV. This theory was further substantiated with antioxidants inhibiting activation of NF-κB and therefore replication of HIV (Schreck *et al.*, 1991, Aghdassi and Allard, 2000). The activation of NF-κB is necessary for the replication of the HIV, but it also initiates an apoptotic process, which is possibly a host defence to limit the replication of HIV resulting in the loss of T-cells (Brigelius-Flohe, 1999).

HIV is more prevalent in South African gold miners, at 27%, than in the general population (Corbett *et al.*, 2004). The HIV pandemic means that miners and other RCS-exposed workers are at an even greater risk of developing pTB. The prevalence of HIV in pTB patients on a gold mine increased from 15% in 1993 to 43.6% in 1997 (Churchyard *et al.*, 1999). In another study by Murray *et al.*, (1999) the prevalence of HIV among pTB patients was 49%. These studies give an indication of the increasing prevalence of HIV on the mines, with the incidence of pTB also much higher in the mining population than in the general population (Corbett *et al.*, 1999). Importantly, silicosis and HIV infection have been shown to play a multiplicative role in the increased risk of miners to pTB (Corbett *et al.*, 2000).

1.8 Respirable crystalline silica and the mechanism by which it causes fibrosis

Features of silicosis such as histopathologic, clinical and epidemiological have been used to elucidate the mechanisms of silicosis. This has been followed by studies with animal models and cell biology techniques.

RCS particles enter the lungs by inhalation and are deposited there. Small particles are able to reach the alveoli where they can then interact with the macrophages and lung tissue cells (Ziskind *et al.*, 1976). Generally, particles deposited in the lung are cleared by a number of pathways depending on the area of the lung in which they are deposited. Particles in the bronchial region are cleared by the cilia and mucous pathway, while particles deposited in the alveoli are first engulfed by macrophages, which then carry them to the bronchial region where they are removed by mucous and cilia, or macrophages enter the lymphatic system and are cleared. RCS can be cytotoxic to macrophages, and may injure the lymphatic vessels. This results in RCS not being easily cleared from the alveolar region of the lungs (Ziskind *et al.*, 1976). Workers exposed to low levels of RCS do not necessarily develop silicosis but silicotic nodules may be seen in the lymph nodes indicating a clearance pathway for RCS (Green and Vallyathan, 1996). When the clearance pathways become damaged or overwhelmed, many RCS particles tend to remain in the lung and these along with macrophages that are activated by RCS initiate silicosis (Parkes, 1974).

AM play a large role in lung defence. They are responsible for the clearance of foreign objects including inorganic particles. They also play a role as antigen-presenting cells and release mediators, which stimulate a wide range of other cells to release chemokines (Driscoll *et al.*, 1995), which act to attract other inflammatory cells to the area.

Initially after inhalation of RCS, AMs move towards the RCS particles. These macrophages phagocytose the particles, releasing oxygen radicals and proteolytic enzymes in the process (Iyer *et al*., 1996), thereby causing further damage and helping in the recruitment of additional inflammatory cells. Macrophages appear to be activated by RCS exposure. They accumulate in the nodules found in silicosis. Macrophage accumulation is

a characteristic of silicosis. In both humans and animal models macrophage aggregates are seen in histopathological studies (Kane, 1996).

The RCS particles, after phagocytosis and the initial release of ROS and enzymes, are responsible for activating the macrophages, which then release another burst of chemicals consisting mainly of TNF α and IL-1, along with other cytokines and chemokines (Driscoll *et al.*, 1990, Barbarin *et al.*, 2005). These chemokines activate other macrophages and inflammatory cells, including fibroblasts, and attract them to the region. A complex network of cytokines is secreted by the activated inflammatory cells that have been implicated in the development of fibrosis. T-lymphocytes and neutrophils that are attracted to the site play a part in the development of granulomatous conglomerates and subsequent fibrosis (Iyer *et al.*, 1996). Silicosis is characterised by granulomatous inflammation, which is potentiated by IL-1, TNF α and IFN- γ .

The pulmonary recruitment effect of RCS was investigated in rats. Doses of 5 – 100 mg/kg of crystalline silica were introduced intratracheally. This dust administration resulted in dose-related increases of neutrophils, lymphocytes and AMs (Sime *et al.*, 1998). Bissonnette and Rola-Plezczynski, (1989) also found cell counts that were increased above the controls at all-time points. The main cell types involved in the inflammation were neutrophils.

An increase in lymphocytes has been noticed particularly in the tissues of silica-exposed mice and silicotic patients. These may help to regulate cytokine secretion from macrophages. In animal models, the lymphokines released by lymphocytes activated by RCS exposure stimulate macrophages and may play a role in the recruitment of circulating macrophages to the lungs.

In a study by Castranova *et al.*, (2002) rats were exposed to non-overload doses of silica. The RCS load and lung damage was monitored at intervals. Pulmonary damage increased with continuing exposure, and after initially reaching a stable point, a steep rise in damage was observed. Pulmonary fibrosis was measurable after the initiation of the increase in lung damage along with markers of inflammation, i.e. TNF, IL-1, and AM. Oxidative

stress followed the same pattern, first increasing slowly, followed by a sharp increase as fibrosis was initiated.

Several mechanisms have been put forward to explain the means by which RCS activates and recruits the immune system. The initial accumulation of macrophages is well described, but the means of their attraction is not well explained. Iyer *et al.*, (1996) has proposed three possible mechanisms. First, RCS may interact with proteins in an "adjuvant effect". These silico-proteins may activate macrophages and drive an immune response. In the second proposed mechanism, the RCS interacts directly with lymphocytes and stimulates antigen-presenting cells, which release chemo attractants. The third mechanism is where RCS interacts directly with B cells, activating them and driving an immune response. However, as many immune functions overlap in cell-mediated and humeral responses, it is difficult to determine the initial response in silicosis.

The ratio of lymphocytes to macrophages in the area where RCS is deposited may also play a role in the effects seen. Both immuno-suppression and enhancement have been seen in *in vivo* RCS studies (Driscoll, 1995). RCS has also been associated with autoimmune diseases such as scleroderma, which are characterised by immuno-suppression.

When lymphocytes and macrophages are cultured in the presence of silica, sister chromatid changes are observed in the macrophages, while no change is seen when only macrophages are cultivated with silica. Other measures of DNA damage, such as micronuclei, have suggested that RCS is able to cause DNA damage and so could possibly cause cancer alone without needing specific cells to be available (Driscoll, 1995).

Huaux (2007) provides an illustrated summary of mechanisms of silicosis (Figure 8) where RCS interacts with AM resulting in tissue injury and apoptosis. From there, various cytokines and enzymes drive the response to three outcomes: cancer, silicosis and peripheral thrombosis.

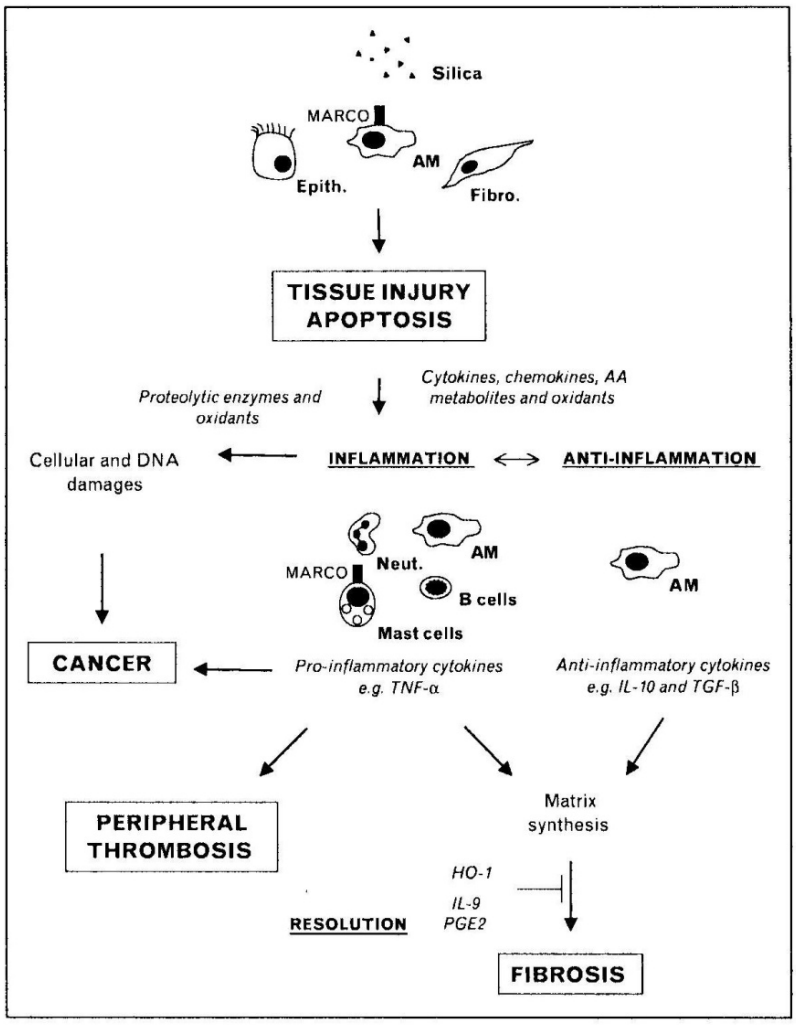


Figure 8: Mechanisms by which RCS may induce fibrosis (Huaux, 2007)

Inflammation is not the only requirement for fibrosis as non-fibrogenic particulates such as titanium dioxide have also been shown to cause a migration of macrophages when inhaled (Iyer *et al*, 1996). Both titanium dioxide and aluminium oxide have been shown *in vitro* to have no effect on TNFα release. This indicates that the initial immune recruitment due to the presence of foreign objects and stimulation of TNFα release are separate events in the development of silicosis (Driscoll *et al*., 1995). The amounts and types of cytokines secreted may be different depending on the type of particulates inhaled, which suggests that RCS has a bioactivity responsible for causing fibrosis that is separate from its particulate nature, which produces the inflammation (Iyer *et al*., 1996). Studies in animals, particularly rats, have shown a dose-dependent relationship between RCS exposure and inflammation followed by fibrosis (Porter *et al*., 2004; Warheit *et al*., 2007).

Challenging the widely reported association between chronic inflammation development of silicosis in the lung, a study by Barbarin *et al.*, (2005) made three challenges; 1. Studies in mice do not always show the same progression from inflammation to fibrosis, although mice do often develop fibrosis after RCS exposure. 2. Treatment response in mice compared to rats differs, where the treatment of inflammation in rats results in a decrease in fibrosis and inflammation. The same treatment in mice reduces inflammation and TNFα production, but does not always reduce fibrosis. This possibly suggests different mechanisms in rats and mice. 3. Finally, anti-inflammatory treatment has been used in humans without being shown to reduce fibrosis significantly (Barbarin *et al.*, 2005). A study of IL-10-deficient mice showed a diminished fibrotic response in comparison to wild type mice. IL-10 is an anti-inflammatory interleukin responsible for down-regulating the expression of cytokines on macrophages (Moore *et al.*, 2001). This indicates that there may be other mechanisms by which RCS produces fibrosis in both mice and humans, for example, the presence of another factor such as IL-10 which supports the process from RCS exposure to fibrosis.

Anti-inflammatory cytokines have been proposed to support the development of fibrosis in mice by stimulating fibroblasts either directly or indirectly. To examine this hypothesis, Barbarin *et al.*, (2005) exposed both mice and rats to RCS particles. Both species developed fibrosis after 60 days and RCS was found in both rat and mouse lung homogenates. Inflammation was measured along with fibrosis, TNFα and IL-10 in the animals. The rats developed chronic inflammation while the inflammation seen in the mice was acute and resolved. Increased TNFα was found in the rats exposed to RCS but not in the mice exposed to RCS, while IL-10 was increased in the mice and not in the rats. Three different mouse strains known to have different sensitivities to RCS were exposed to RCS, and the IL-10 levels measured in the mice were related to susceptibility to RCS-induced fibrosis. This mechanism of pathogenesis may be important in those who do not respond to anti-inflammatory treatment (Barbarin *et al*., 2005).

1.9 Biomarkers

Research into the mechanisms of disease has allowed the identification of many possible biomarkers involved in the causal pathway of disease (Tzouvelekis *et al.*, 2005). Subtle changes, such as production or release of proteins, variations in genes, cells and physiological processes provide for the identification of biomarkers (Vineis and Brand-Rauf, 1993; Fowle and Sexton, 1992).

Biomarkers are "indicators signalling events in biological systems or samples" (National Research Council, 1987). Biomarker is a descriptive term for an increasingly used group of measurable components, both biological and non-biological, Due to the valuable contribution of biomarkers; there are a number of definitions. The US Environmental Protection Agency (EPA) gives another general definition of a biomarker as "a measurement of environmental pollutants or their biological consequences after the contaminants have crossed one of the body's boundaries and entered human tissues or fluids which serves as an indicator of exposure effect, and or susceptibility" (Borm, 1994). Biomarkers themselves are more easily defined in terms of what they are used for as they do not easily fit into one group such as proteins. The presence or absence of a specific biomarker is used to indicate a change in the system of interest. Well known examples of biomarkers are glucose levels in the blood of diabetics and lead levels in blood of those exposed. Lesser known examples are specific enzyme activity in those exposed to certain pesticides, drugs, and gene expression in susceptible individuals. Thus biomarkers are measurable or observable contents or a specific change in the contents of the system of interest from a person down to a cell. The term biomarker covers substances such as genes and enzymes, cells, tissues and contaminants (Mendelsohn *et al.*, 1998).

Biomarkers are sought by researchers in the course of events occurring after exposure to a hazardous agent in the system of interest and in the early stages of the disease that develops from the exposure (Schulte, 1989, Fowle and Sexton, 1992).

All biomarkers are divided into three groups: markers of exposure, effect and susceptibility, depending on their position on the pathway between exposure and disease (Figure 9).

Markers of exposure are the first biomarkers in the exposure to disease pathway and relate to the amount of the pollutant internalised after exposure (Figure 9). They consist of two types of markers: those that represent the internal dose of the substance of interest and are important in the determination of dose-response, and those that reflect the biologically effective dose (National Research Council, 1987). These markers can be used to establish the toxic dose for a healthy population. The presence of these markers does not imply the progression to disease, but only exposure. The nature of the exposure, such as concentration and duration of peak exposures can affect which type of marker of exposure is measured and in which matrix (National Research Council, 1987). Often, the markers of internal exposure are limited to a not easily accessible target organ and so cannot be measured without invasive techniques.

The next group of biomarkers are markers of early and late effect, referred to as markers of biological effect. Markers of effect measure biological changes in the organism. These are changes due to the presence of or exposure to an exogenous compound; they are a measure of the system's functional ability, or a change in the normal functioning of a system to indicate progression towards disease (National Research Council, 1987, Schulte and Rothman, 1998). Early markers of effect are ideal markers to be developed for use as early detection tools. These are markers which are changed due to the presence of biologically active compounds. They indicate risk, but do not indicate irreversible progression towards disease. These biomarkers are identified from the mechanism of the disease of interest (Gulumian, 2007). Often, early markers of effect are not specific to the disease being studied, and because of the lack of specificity, these markers are also often lowered or raised in other diseases or due to other exposures, and may be confounded by exposures not yet associated with them. Thus they need to be used and interpreted with caution. Late markers of effect can consist of altered structures or altered function in the organism that indicates definite, sometimes irreversible, progression to disease.

Markers of effect may be measured in easily obtained biological samples which may provide information on organs not easily reached with non-invasive techniques. As early effects of exposure are often systemic, blood is a common choice for the identification of biomarkers of effect. Breath condensate is currently being researched (Horvath *et al.* 2005) and urine is another easily collected body fluid that may contain useful biomarkers

(Pisitkun *et al.* 2006). Markers of early biological effect provide a possibility for the early detection of harmful exposure and prevention of pneumoconiosis.

The final group of biomarkers are markers of susceptibility. These are inherent or acquired ability to respond to the exposure (Hoet and Haufroid, 1997). This reflects the individual susceptibility of the person which modifies his or her personal development of disease if exposed. These are often genetic factors such as polymorphisms or specific genotypes. An example of a marker of susceptibility is a polymorphism (where there is more than one allele in the same genetic locus) in the gene that codes for the protein responsible for acetylating aromatic amines. This polymorphism can result in some individuals being slow acetylators and more at risk of bladder cancer (Hoet and Haufroid, 1997, Mendelsohn *et al.*, 1998). These markers of susceptibility should be measured before and during exposure as the presence of susceptibility markers that indicate an increased susceptibility would motivate for lower limits of exposure. Ethical issues have been raised regarding the use of these markers, as they could result in susceptible workers not being hired or increased medical aid costs (Hoet and Haufroid, 1997). There is also uncertainty as to the predictive value of markers of susceptibility for individuals - this would need to be clarified by more research with well-designed cohort studies (Vineis and Brandt-Rauf, 1993). This limitation of markers of susceptibility may limit their use in reducing exposure limits rather than identifying high-risk individuals to be protected from exposure.

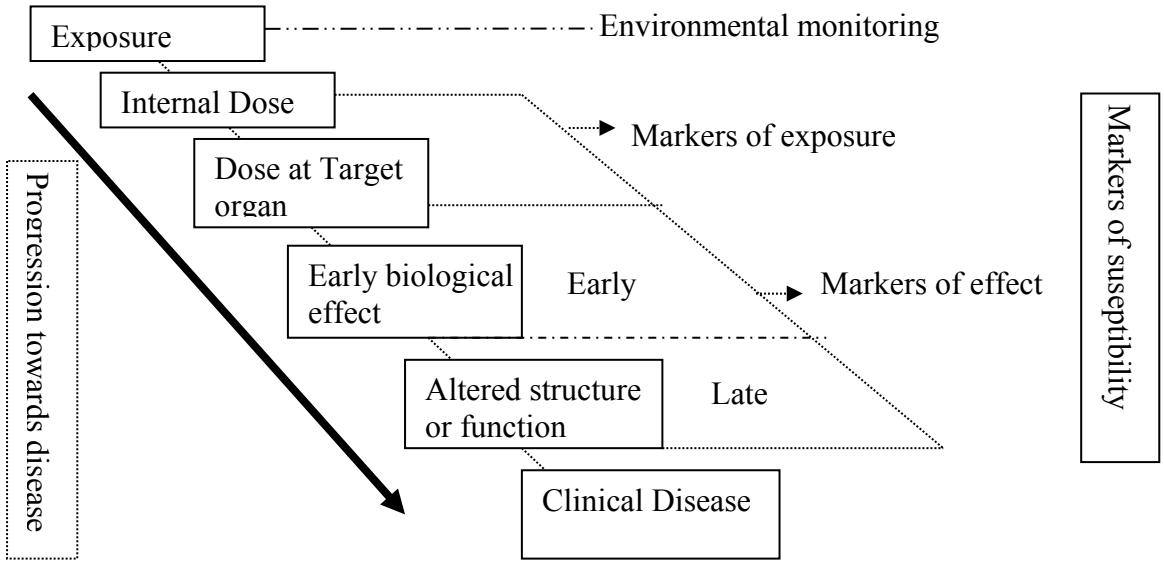


Figure 9: A simple chart of the pathway from exposure to disease, including classification of biomarkers by their position on the pathway (adapted from National Research Council, 1987)

Why biomarkers: Biomarkers help to develop and explain the extent of the relationships between pollutants and human health effects. They allow us to clearly define the health effects associated with exposure and measure the exposures more accurately (Fowle and Sexton, 1992). Early disruptions in the health of individuals can be reflected in early modifications at molecular or cellular level before progressive or functional damage occurs (Mutti, 1999). Biomarkers are useful when traditional tools are insufficient, for example, where there are multiple sources of exposure, they can provide internal doses of pollutants. Biomarkers can be used to assist early diagnosis of disease, provide information on prognosis or selection of patients for therapy.

Biomarkers of effect are able to take into account individual variation in response to exposure and the effects of genetic factors (Schulte, 1995, Schulte and Rothman, 1998, Mutti, 1999). They can provide early information on the adverse response to exposures in individuals (Borm, 1994). This is an important use as no other measure of exposure can take into account the effect of exposure on vulnerable groups. Measures of markers of effect could result in changes in OELs to protect vulnerable individuals. Ultimately, the knowledge of the true effect of exposure on all at risk of exposure should be considered a prerequisite for developing exposure limits both occupational and environmental (Hoet and Haufroid, 1997).

Risk assessment using biomarkers requires a well-defined dose-response relationship. In the case of silicosis, this relationship has been described (Section 1.8). Thus the use of biomarkers in the risk assessment of RCS exposure is recommended (Aitio, 1999)

Biomarkers in the workplace: Methods are needed to identify and monitor adverse effects in workers exposed to harmful environments. Biomarkers are able to offer a number of advantages over routinely used air monitoring, as they are able to evaluate exposure from a number of routes, not just inhalation (Needham *et al*., 2007). Biomarkers are also very useful in providing information on levels of individual exposure when Personal Protective Equipment (PPE) is worn (Mutti, 1999). Biomarkers are best used in conjunction with routine environmental monitoring as they describe the associated health effects. Late

markers indicate clear progression towards disease and so are more reliably indicative of harmful exposure but are less useful for protecting the health of workers (Aitio, 1999). Biomarkers of effect can be non-specific, which limits their usefulness but in the workplace it is possible to exclude other factors and so relate these biomarkers to health effects e.g. sampling from a specific exposure.

Uses of biomarkers: Biomarkers can be a useful tool in RCS exposure assessment as they can provide information on the combined biological effects taking into account levels of RCS exposure, surface reactivity and internal dose in a timely fashion. Biomarkers can be used in combination with air monitoring to verify compliance with standards (Schulte, 1993). OELs may be lowered or new limits set based on evidence of dose-response effects demonstrated by biomarkers (Mutti, 1999). Biomarkers are not a control method in themselves but rather a tool that can provide more information on the progression and mechanism of diseases, validate monitoring methods, and evaluate controls and exposure limits (Bennett and Waters, 2000).

There are a number of considerations to be taken into account when sampling for biological markers. The first is their stability in the matrix in which they occur. This determines how long after exposure the samples are collected, and whether they are a measure of acute or long-term exposure. Other factors to be considered are individual variations and variations among groups, effect modifiers such as gender, age and health status. The route of exposure, transport of the toxin, and the number of target sites and their locations can affect the monitoring of biomarkers (National Research Council, 1987).

Which biomarkers to select: A number of requirements are proposed for a good biomarker. A biomarker should add independent information about the risk when adjusted for known confounders. To be a useful marker, the measure should show a significant difference in the exposed person that ideally accounts for a large proportion of the risk associated with the disease. The test for the marker should also be affordable and commercially available for diagnostic use (Manolio, 2003). Biomarkers need to be specific and sensitive to low levels of exposure. They should ideally vary quantitatively with the intensity of the exposure in a dose-response manner and be able to provide more information than air monitoring could provide for the same substance. Simple sampling methods that are quick and easy to perform will also ensure that they are practical to use (Hoet and Haufroid,

1997). An important requirement of a good biomarker is a high predictive value. This also allows a reference range and cut-off values for excess exposure to be established for unexposed individuals (Tzouvelekis *et al.*, 2005).

Validation of biomarkers: This is important as it confirms that the marker is able to identify who is likely to develop disease among those exposed. This can be achieved by associating the external exposure and the biomarker levels with adverse biological outcomes in those exposed. Validation also needs to determine which factors are likely to modify the markers or the pathway between exposure and disease. Early validation assesses the relationship between changes in the biomarker and exposure in cross-sectional studies. This needs to be followed by prospective cohort studies that can answer more adequately the question of temporality and confirm the validity of the biomarker and its predictive value (Schulte, 1993). Validation of biomarkers is also important in terms of interpretation of results. Practical use of a biomarker requires simple interpretation also taking into account inter-individual variability (DeBord *et al.*, 2004). The conditions under which a marker may be detected are important along with the variation of the marker in the unexposed population. Biomarkers that respond to realistic levels of exposure are needed and the focus needs to be placed on research to identify and validate these markers (DeBord *et al.*, 2004).

As the more complex mechanisms of diseases are elucidated, multiple pathways are identified and new biomarkers validated, and therefore a multi-marker approach becomes possible. It is possible then to use the different pathways in the disease outcome and provide a comprehensive assessment. The markers combined can give a clearer picture of exposure, risk and prognosis than any one biomarker alone. Combined, they may also be useful in evaluating treatment therapies (Morrow and Braunwald, 2003).

Exposure Assessment: Traditional assessment of exposure to environmental pollutants involves measurement of the pollutant in the relevant media such as air and determination of average and peak concentrations. Another method of exposure assessment is to detect early disease as a source of information on exposure. This is feasible where the sensitivity and specificity of disease detection are reasonable and the disease is reversible. Where early disease and exposure measurements are insufficient, other methods of exposure assessment are needed. Biomarkers are recommended to assess exposure or effect in

situations where there are low levels of exposure, mixtures of toxicants that may work synergistically, intermittent exposure, or exposure that results in diseases with long latency periods. Where available, markers of exposure give a measure of exposure but far more useful are markers of effect which give an indirect indication of exposure but provide a measure of the individual's response to the exposure.

RCS exposure assesment: In the case of RCS exposure, the traditional exposure assessment method is gravimetric analysis and XRD or IR determinations of RCS in respirable air samples. However, for the levels that have been suggested as safe, namely 0.025 mg/m^3 (American Conference of Industrial Hygienists, 2011), the current methods to assess these low levels of RCS are not accurate. In addition, the variability of RCS toxicity renders simple air exposure measurement techniques inaccurate, so another means of assessing the risk of silicosis is needed. Thus, traditional environmental exposure assessment of RCS is less useful in preventing disease and hence makes biomarkers a more useful option.

RCS biomarkers: There are currently very few feasible biomarkers of exposure due to the lung being the target organ of RCS exposure, sampling of which requires invasive methods such as biopsies. Due to the long latency period to develop silicosis, methods which relate current exposure to final disease are not of value. Diseases with long latency often result in misclassification of exposure and require long time periods to determine prevalence of disease in exposed individuals (Schulte, 1995). Markers of effect which can be measured long before clinical disease develops are needed to fill this gap for RCS exposure assesment. With validated biomarkers of effect, the waiting period to evaluate the effects of exposure is reduced and therefore misclassification of exposure is likely to be reduced.

Phase I of the study

Biomarkers of RCS exposure: There are few biomarkers of exposure to RCS. Moreira *et al.,* (2005) have proposed particle analysis using bronchoalveolar lavage (BALF) as a viable marker of exposure. Their study demonstrated an increase in the number of BALF

cells containing particles identified by polarizing light microscopy with increasing exposure. This could provide a direct estimate of internal dose of RCS. But the collection of BAL cells requires a bronchoscope to be wedged in a third or fourth generation airway. Fluid is then introduced into the lungs and collected in a bronchial wash (Sweeney and Brain, 1996). This procedure is invasive, requiring special equipment, so it is not suitable for the assessment of RCS exposure in many workplaces with a large workforce. There are also a number of factors that affect BALF interpretation. Repeated washes are recommended and diseased lungs, such as those with pTB, and normal lungs, differ in BALF components (Sweeney and Brain, 1996). For this reason many, researchers prefer to focus on markers of effect.

To identify possible markers of effect, the mechanism of silicosis pathogenesis needs to be elucidated, particularly the early mechanism to identify the early markers.

Macrophage recruitment is one of the first steps in the development of pneumoconiosis. Macrophages are responsible for the phagocytosis of the dust (Driscoll, 1995). The macrophages release a number of cytokines and other inflammatory mediators which could form the basis of biomarkers of effect. As these mediators are released very early in the pathway to disease, they provide early information before irreversible change takes place. Following this, other inflammatory cells are recruited and can cause local damage. Markers of this damage are early markers of biological effect and an indication of harmful exposure (Tiwari, 2005).

The ILO and the World Health Organisation (WHO) established the Global Programme for the Elimination of Silicosis in 1995 and, following this, South Africa established the National Programme for the Elimination of Silicosis (NPES) in 2004 (Fedotov and Eijkemans, 2007). This project was solicited by the South African Mine Health and Safety Council (MHSC) to identify biomarkers that could be used to evaluate RCS exposure and to help provide information on the success of RCS dust control measures. The project was divided into phases conducted as individual studies.

Phase I, was conducted to allow experts in the field to evaluate all the published literature on silicosis and RCS exposure to identify potential biomarkers of exposure, effect and

susceptibility worthy of inclusion in Phase 2 of the study, as an evaluation phase. In Phase I a comprehensive literature review was completed by local and international experts. One hundred and seventy-one papers relating to biomarkers for silicosis were identified, which included studies of both humans and animals and those identifying a pathway for a particular biomarker.

Selection of biomarkers for further study was based on a number of criteria, i.e. biological significance, practicability, type of study conducted, and placement in the pathway to disease. Attention was also paid to background variability in the population, confounders, the number of study participants and ease of measurement, reproducibility of tests and predictive value of the biomarker. Biomarkers that required BAL or lung tissue biopsy were not included - only those that could be measured in the blood were chosen. A meta-analysis of the studies on potential biomarkers could not be conducted as there was no conformity in the studies and too few studies of each individual biomarker had been conducted with too few participants.

Possible biomarkers identified in Phase I of the study included Oxidative stress markers (GSH, GPx, GST, 8-Isoprostane, TAS, ROS (Chemiluminescence), markers of inflammation (TNFα, IL-8, PDGF), a marker of lung damage (CC16), and a marker of susceptibility (TNF α polymorphism). The results of Phase 1 were published in a comprehensive review of the mechanisms of silicosis by Gulumian et al., (2006) and are represented in Figure 10.

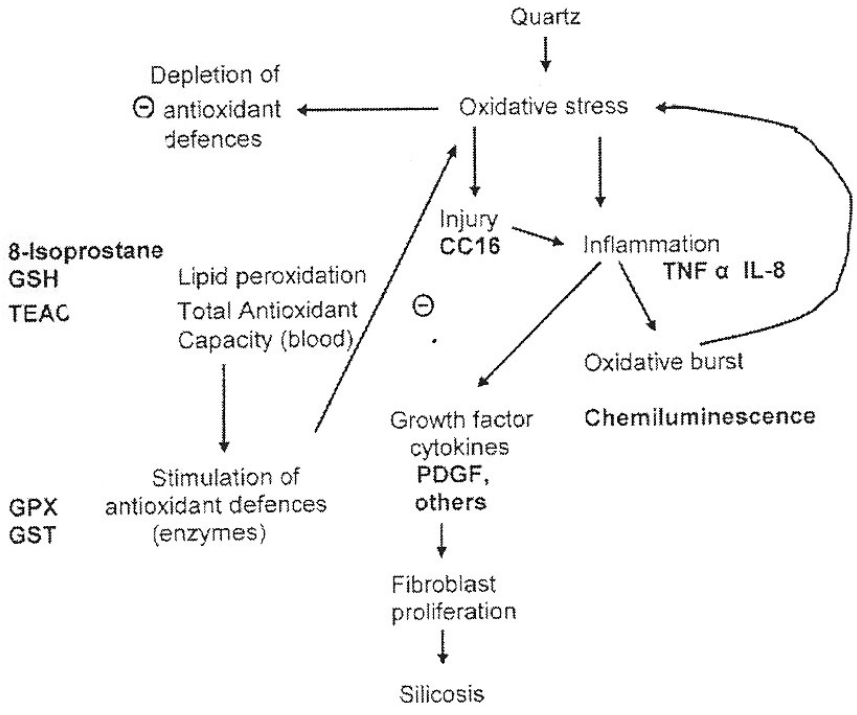


Figure 10: Mechanisms and biomarkers identified in silica-induced oxidative stress, inflammation and fibrosis (from Gulumian *et al.*, 2006)

Phase II of the study to identify biomarkers for silicosis was subsequently initiated to evaluate the identified biomarkers from Phase I for their usefulness as biomarkers, taking into account confounders, such as smoking and HIV.

Phase II of the study

Problem Statement

Biomarkers of early effect are biological indicators resulting from the presence of RCS in the body. They can provide early information on the progression of effects on miners' health, along with early evaluation of dust control measures applied to reduce the health impact of RCS exposure. The majority of the viable biomarkers identified in Phase I of this project had not yet been evaluated in exposed and unexposed individuals to RCS. The selected biomarkers needed to be evaluated in field conditions, with current confounders present, to determine which of them may provide a useful and practical tool for determining RCS exposure.

This study did not intend to validate biomarkers for silicosis diagnosis as silicosis is an incurable disease. Biomarkers would be more valuable in indicating harmful exposure before irreversible changes took place. Phase II was designed as an epidemiological study and intended to evaluate the ability of the identified biomarkers to significantly discriminate between two South African populations, exposed and unexposed to RCS, taking the two confounders HIV status and smoking habits of the studied populations into account.

Aim

The aim of this study was to identify viable biomarkers as indicators of RCS exposure from a list of 10 identified in a previous phase of this project; and to ascertain which biomarkers are unaffected by HIV status and smoking habits but which may correlate with RCS exposure.

Objectives

The objectives of the study were to:

1. To measure and compare compare levels of 10 biomarkers in exposed and unexposed participants.
2. Compare the levels of biomarkers in HIV positive and HIV negative participants.
3. Determine the ILO score silicosis status of the miners exposed to RCS dust.
4. Determine whether the silicosis status affects biomarker levels.
5. Determine whether the smoking confounds the relationship between biomarker levels and exposure.
6. Identify sufficiently discriminating biomarkers for further optimisation.
7. Build capacity for reproducible performance of the biomarker assays.

This study used epidemiology combined with toxicology to identify biomarkers in South African gold miners and unexposed controls, for further investigation as possible markers of early effect of RCS.

CHAPTER 2

Demographics of the Participants

2.1 Introduction

This chapter describes the participants recruited for the study and discusses potential bias in the study design. Factors that could cause a difference in biomarker levels between groups were examined to demonstrate that comparisons of the groups recruited were valid.

2.2 Methods

2.2.1 Study Design

This was a cross-sectional study design with four groups of participants, RCS-exposed HIV-positive and RCS-exposed HIV-negative miners in the first two groups, and RCS-unexposed HIV-positive and RCS-unexposed HIV-negative controls in the second two groups. The study measured levels of 10 biomarkers in the four groups along with age, smoking status, diet, medication, HIV status and CD4 count, and work history.

2.2.2 Study Population and Setting

The participants were recruited for this study over six months in were divided into four main groups, namely those exposed or unexposed to RCS dust and HIV positive and negative. For the exposed groups groups 1 and 2, gold miners were recruited, as they are known to have levels of RCS exposure likely to cause silicosis. For group 3 the unexposed group, blood donors were approached to form part of the control group after they had answered a question on their previous occupational history to eliminate any history of RCS exposure. Blood donors were chosen as the control group as they were likely to be HIV negative and had already consented to HIV testing. Group 4 the HIV positive controls were recruited from a Johannesburg hospital also following elimination of controls with prior dust exposure. The latter group was recruited for the reason that one of the aims of the study was to find a valid test for RCS exposure which was not affected by

HIV status. HIV status was essential to this project as it was an important suspected confounder in many of the tests used in the study. A second round of recruiting of blood donors was undertaken after it was ascertained that there were insufficient smokers in the initial group.

The occupational groups 1 and 2 who are exposed to significant levels of RCS daily are gold miners from Carletonville, and are the most well studied in South Africa. The majority of gold miners in South Africa are African males. This group is made up of migrant workers from South Africa and neighbouring countries such as Botswana and Mozambique. These migrant gold miners generally live on the mines in hostels in overcrowded rooms (Murray *et al.*, 1999).

In the present study only men were recruited to maintain homogeneity, to keep the study size affordable, and to limit possible genetic, dietary and lifestyle differences. Women are now employed on the mines, but form only a small proportion of the total employees (Girnat–Hallet, 2014). In terms of RCS exposure, African men have traditionally performed the dustiest jobs on the mines and continue to do so; therefore they are expected to have the highest exposures to RCS. High exposures were preferable as they were the most likely to result in raised or lowered biomarker levels. The controls were also limited to African men to keep dietary, lifestyle and genetic factors as consistent as possible for a suitable comparison group.

The HIV-negative controls came from blood donors in Johannesburg. Eligibility for donating blood requires all donors to be healthy at the time of donation, and there are few smokers in this group.

The HIV-positive controls were selected from the HIV clinic at Charlotte Makeke Hospital. They had to reside in the hospital catchment area to be eligible for treatment thus were less likely to be environmentally exposed to RCS dust and occupational exposure was assesed in the questionnaire. Again, mostly adult African women were found in this group, which included individuals who became ill as this is often the case when they first seek treatment.

2.2.3 Inclusion and Exclusion Criteria

All the participants had to volunteer to participate and agree to provide a blood sample along with their HIV status. For miners, the main inclusion criterion was underground work. For the controls and the miners, African race was an inclusion criterion of the study. Another exclusion criterion was active TB where identified by the participant. Current use of medication for pTB was also an exclusion factor. For the controls, the main exclusion factor was previous or current work in a dusty environment defined as work in construction, foundries or mining. HIV-positive participants were not excluded if they were taking anti-retroviral medication (although this was noted). HIV-negative controls were excluded if they did not donate a blood sample on the day of sample collection. All the other participants provided blood samples on the day of recruitment.

2.2.4 Recruitment of study participants

Flyers in Xhosa, Sotho and Zulu advertising the study were handed out to miners on the mine and posters were placed in the clinic. The participants from the mines were recruited for the study by a volunteer interviewer who explained the aims and methods to them in their own language whenever possible. The participants were asked to sign a consent form or make a mark if they were unable to write. The interviewer then asked them the questions on the questionnaire in their home language, except those who spoke Portuguese and Shangaan, in which case English was used. A similar process was used for the controls except that English was used to conduct the interviews.

The interviews were short and conducted as privately as possible before the blood samples were collected. The participants were selected on the basis of their exposure status and HIV status.Three five millilitre blood samples were collected. Socio-demographic information other than age was not collected, as it was not expected to have much bearing on the difference in biomarker levels between the participants who were exposed and those who were not exposed to RCS.

2.2.5 Bias in studies

Bias is a systematic error in an epidemiological study that can result in non-generalisable conclusions. There is always a potential for bias in any non-experimental study as humans have a large amount of variation that may introduce bias. Bias cannot be quantitatively estimated and adjusted for; thus steps are taken in the study design in an attempt to

minimise bias (Hennekens and Burring, 1987). The presence of bias in a study can reduce its internal validity or disrupt the association between exposure and disease. Internal validity of a study allows inferences to be made about the source population. Internal validity also plays a role in external validity, which informs the researcher whether the findings of the study can be related to the general population (Rothman and Greenland, 1998). The findings from this study, where dust-exposed participants were recruited from only one mine, may not be transferable to other mines as the dust composition may vary.

There are a number of types of bias and they are grouped into two categories; selection bias and information bias. Confounding can also be seen as a type of bias, although measurement of confounders is often possible, which allows them to be adjusted for, either in the study design or by analytical methods.

Selection bias results from a number of factors like the selection procedures which are used to identify and recruit possible study participants. This includes self-selection bias, where those who volunteer for a study may differ in some way from those who refuse and diagnostic bias, where knowledge of exposure influences diagnosis of disease (Rothman and Greenland, 1998). Miners were only recruited if they were willing to disclose their HIV status possibly introducing selection bias, also those who participated may have self selected due to exposure. Haemoglobin levels are tested for blood donors and there is a cut-off of 12 mg/dl for blood donation to minimise the risk of anaemia. This means that there may have also have been a slight bias introduced in the selection of controls.

Information bias affects the outcome of the study if inaccurate or incomplete data affects the groups in the study unequally (Hennekens and Burring, 1987). The types of bias that fall under information bias are recall bias, interviewer bias, and misclassification bias. Bias that affects all groups equally will result in a lower estimation of effect of association. Information bias is caused by errors in information obtained from study participants and possible observation errors. The way data on exposure or outcome is obtained in the study can lead to information bias. Controls were asked about work history but reported history may have been inaccurate. This bias can result in misclassification, either differential or non-differential (Rothman and Greenland, 1998). Differential misclassification occurs when there is a classification error that occurs more frequently in one group. It is not a selection bias as it occurs among the already selected members of the

study. Recall bias is an example of a differential bias as the outcome group is perhaps more likely to recall exposure than the control group. Non-differential misclassification occurs when the classification error is equally likely to occur in all groups. This bias will generally tend to bias towards the null (Gail and Benichou, 2000).

2.2.6 Questionnaire

A simple questionnaire was used to allow the interviewer to easily translate the information into the home language of the interviewee. The questionnaire was also short to minimise the time needed to collect the data and the blood sample from each participant. As the study did not aim to describe the participants in detail, only relevant information was collected. Information was collected on age, previous work history, and current position, smoking habits, general health, diet and medication used. A recent CD4 count was collected where known.

2.2.7 Sample Size

The study aim was to recruit a minimum of 30 men in each of the four groups to provide sufficient statistical power. Sample size was not calculated as each test would require a different number and the number of participants recruited was limited by the cost of the biomarker assays and the slow rate of recruitment, possibly due to the requirement that the participants' HIV status had to be disclosed.

2.2.8 Sampling Strategy

RCS-exposed miners were recruited from their mine medical centre and a Volunteer Counselling and Testing centre (VCT) centre based on one shaft (one lift to the underground workings of a mine) of a large gold mine in western Gauteng. Both HIV-positive and HIV-negative men were recruited. Men attending the VCT centre were invited to participate if they did not have active pTB (ascertained on the questionnaire). A temporary laboratory was set up at the medical centre for immediate processing of samples. Volunteers who were willing to participate, but who had no prior (within the last 2 months) HIV test results, were encouraged first to attend a counselling and testing session at the VCT centre before participating.

The HIV-negative controls were recruited from the South African National Blood Service (SANBS) mobile clinics in the Johannesburg area. The mobile clinic is set up each day at

a different corporate location. All African male blood donors were approached at the time that they donated blood and the study was explained to them. The donors who volunteered to participate had an extra sample drawn at the same time as the SANBS samples by the phlebotomist on duty. Sampling was consecutive African men who consented to participate. Minor current illnesses such as colds and flu make donors ineligible to donate blood and so was an exclusion criteria for controls.

The HIV-positive controls were recruited from an HIV clinic at a Johannesburg hospital. All African male patients waiting for their appointments at the HIV clinic were approached to participate in the study over a 3 month period.

To eliminate confounding due to smoking, the groups need to be similar in number of smokers or the results must be adjusted for current smoking. Previous smoking was also ascertained in the questionnaire to determine the role it may play in the biomarker levels.

2.2.9 Silicosis

The presence or absence of silicosis in the at-risk study group was determined from their routine X-rays at the time of the study. The available X-rays (provided by the employing mining house) were read by two qualified readers and compared with the standard ILO X-rays for reference. The radiographs were then categorised using the ILO method. A code of 1\0 or higher was taken as evidence of silicosis and 0/1 as possible silicosis but insufficient evidence. The reason for using 1/0 as the cut-point for silicosis is that this cut-point gave the best agreement between the classifications based on the readings of the two readers. The full ILO categories were used to test the agreement between the readers. However, for analysis of the effect of silicosis on the biomarkers, the codes were simplified to a yes/no score for the presence of silicosis. A score of 1/0 or higher was coded as yes (silicosis present).

2.2.10 Statistical Analysis

The participants' demographics were analysed using basic descriptive statistics such as means, medians and ranges for age and length of service underground. This was done using STATA version 8. An exponential weighted Kappa score was used to calculate inter-rater agreement between the two readers of the radiographs. This test gives readings

close to one another a similar score, and those further away a different score, allowing readings of 0/1 and 1/0 to be seen as similar. The yes/no silicosis codes were compared using an unweighted Kappa score, as it was a categorical variable of 0 and 1.

The difference between two means was tested for all continuous variables such as years of work underground. The data were first examined for normality with a histogram and the Shapiro – Wilk test for normal distribution. Where the data were compatible with having been drawn at random from a normally distributed population, an F-test was used to look for equal variance before conducting either an equal or an unequal t-test depending on the results of the F-test. For comparison of multiple means, a one-way analysis of variance was used. Bonferroni corrected p-values were used for multiple comparisons.

Where the data did not have a normal distribution, a non-parametric Wilcoxon rank sum test for independent groups was conducted to test whether the two groups came from the same population. For categorical data such as HIV and RCS exposure, a Chi-square test was used to test for association between the two variables. The two main confounders HIV and smoking were assessed by stratifiying the results and testing for a statistical significant difference between the groups.

2.2.11 Research ethics

There are specific guidelines governing research involving human participants or biological material. The Nuremberg code was developed after the trials of Nazi war criminals at the Nuremberg military tribunals. This provided the initial 10 directives that provide guidelines for responsible human research. The first is voluntary consent from individuals who are legally capable of exercising freedom of choice. Participants are protected from fraud, deceit and coercion (US Gov 1949, Nuremberg trials). This can include protecting patients from being recruited by their own physicians, educators or employers. Respect for study participants is important so that they are treated equally.

There are also responsibilities placed on the researchers and any study directors:

1. To design an experiment that will provide useful results for the population under study.

2. To base the study on sound scientific principles, literature and, where relevant, animal experimentation. The use of human volunteers needs to be justified by the expected results (Declaration of Helsinki, 1964 as amended 2000).
3. The privacy of subjects enrolled in research must be respected as well as protected in cases where knowledge of personal information could cause harm (Declaration of Helsinki). This in particular plays a role in any research involving HIV/AIDS.

Recruiting of participants, blood and HIV tests

Confounders need to be accounted for in all studies, and must be quantified and adjusted for. To achieve this, questionnaires are used and tests are done on samples. In the present study, both smoking and HIV are common potential confounders of the biomarkers chosen. To quantify the effect due to HIV, the status of all participants was required. This adds a further ethical liability as knowledge of HIV sero-status may result in stigma and study subjects may not want to have their status known to themselves or to others. Not all the possible confounders and effect modifiers were measured in this study due to cost and number involved.

If a participant tested as HIV-negative, the result should have been for a specimen taken within the last two months. The blood tests for the miners were conducted (following counselling) by Lancet Laboratories, while the blood donors as controls were tested by the SANBS laboratory, following their protocols. The results for the HIV tests were provided to the researchers based on the signed consent including the provision of anonymous HIV test results. In the case of the blood donors, the numbers allocated to the blood samples were used to identify results. Similarly, the miners' work numbers were used to identify their samples as this type of identification is what is normally used to give them their results and refer them for post-test counselling.

Voluntary counselling and testing

VCT and testing provides counselling, both pre-test and post-test to individuals who wish to know their HIV status. The pre-test counselling is intended to obtain informed consent for the test, which involves explaining the purpose of the test, the manner in which it is conducted and then the possible outcomes and implications of the results. Post-test

counselling is intended for the most part to provide the results in a confidential environment and to discuss the results (Birdsall *et al.,* 2004).

VCT can change behaviour and decrease high-risk habits. This has been shown in both developed and developing countries. This is one of the main functions of VCT, as well as directing infected individuals to Highly Active Anti-Retroviral Activity (HAART) programmes, which are now available in most provinces of South Africa (Kalichman *et al.*, 2005, Simbayi *et al.*, 2005)

By 2003, the South African government had established more than 450 VCT centres, and private business established even more, but despite this, only 20% of individuals in a 2002 study had made use of the available facilities (van Dyk and van Dyk, 2003).

VCT is an important programme in the fight against HIV/AIDS. It has been implemented worldwide, but has been met with varying amounts of uptake and support. There are service delivery barriers, lack of trained staff in some areas, but there are also psychosocial barriers such as lack of confidentiality and fatalism (van Dyk and van Dyk, 2003). There is a perception amongst many would-be users of VCT services that the health care personnel would not keep their results confidential. This is supported by the van Dyk and van Dyk study, in which 32% of the respondents said they would rather attend a VCT clinic not in their community and 50% gave the reason for this as lack of confidentiality of the nurses. There is another misleading view that VCT services are only for ill individuals. Service delivery problems include hours, location and cost (Morin *et al.*, 2006).

In a 2005 study by Simbayi *et al.*, (2005), education was found to be a contributing factor in VCT uptake although this was not significant. They also found negative attitudes towards HIV testing amongst individuals who had never been to VCT.

In our study, although we did not determine attitudes towards VCT, many individuals who were approached to participate voiced concerns on learning that HIV status needed to be known to participate in the study, and that the mine VCT services should be used.

Another factor in poor VCT uptake is the social attitude towards those with HIV. In a study conducted by Day *et al.*, (2003) amongst 105 South African mineworkers, the fear of testing positive and the potential social consequences such as stigmatisation were reasons for not accepting VCT. Simbayi *et al.*, (2005) found that the stigma against AIDS was highest amongst those who had not been tested, and they found that these perceptions create social barriers to VCT. Perceptions that individuals with AIDS are dirty and should be avoided prevent individuals from using the testing centres.

Ethics of HIV testing in research

HIV/AIDS is a condition with negative attitudes and stigma towards those suffering from it (Dunkel *et al*, 2004). Disease stigma is not limited to HIV, but because of its prevalence in South Africa and its link to blood, sex and prohibited activities such as drug use, it has become the most common disease-associated stigma (Dunkel *et al*., 2004).

Individuals with HIV/AIDS can, because of the stigma, be considered a vulnerable group and therefore need special consideration. Their right to privacy is very important, because individuals who have disclosed their status have experienced violence and isolation from their communities. In addition, individuals who belong to already vulnerable communities due to socioeconomic factors are more likely to suffer from HIV. Finally, individuals may not wish to know their infection status and this wish needs to be respected. To protect these individuals, research that requires their status to be disclosed should only cover questions relevant to HIV. The study population should be selected on the basis of criteria that do not discriminate and are relevant to the study, and this will result in a study population who can benefit from the research (Dunkel *et al*., 2004).

Informed consent should include information on how privacy will be ensured and sources of conflict. The risks of participating should be explained.

Unlinked HIV testing, where no names or identifying numbers are used on test samples, provides a study with accurate HIV status and limits the stigma of associating with a study in which only HIV-positive participants are recruited. However, it does a disservice to the participants, as they do not receive the benefit of knowing their status and being able to make lifestyle changes accordingly based on their status. Many individuals may also

choose not to participate in the study for fear of lack of confidentiality around test results due to misunderstanding the design of unlinked testing, particularly in a work environment.

VCT provides another source of individuals with accurate HIV status who may participate in a study. As both HIV-positive and HIV-negative individuals attend VCT, there is less stigma of association and it is easier to recruit individuals who have already chosen to have their HIV status tested and who would be willing to have their status disclosed to the researchers.

2.3 Results

As presented in Figure 11, this study recruited 165 volunteers in total: 101 controls and 64 RCS -exposed gold miners. There were 70 HIV-positive participants and 91 HIV-negative participants. Almost one third (28%) of the miners had a silicosis score of ≥ 1/0. Two groups of controls were recruited. The second group was to increase the number of smokers in the controls, to allow for analysis of smoking as a confounder in this study. Not all the biomarker tests were conducted on the second group of controls as some had already been demonstrated as not worth further investigation during the study (chapters 3 and 4) and also due to the cost of the analysis. The recruitment of the miners took three months, as the recruitment rate was slow.

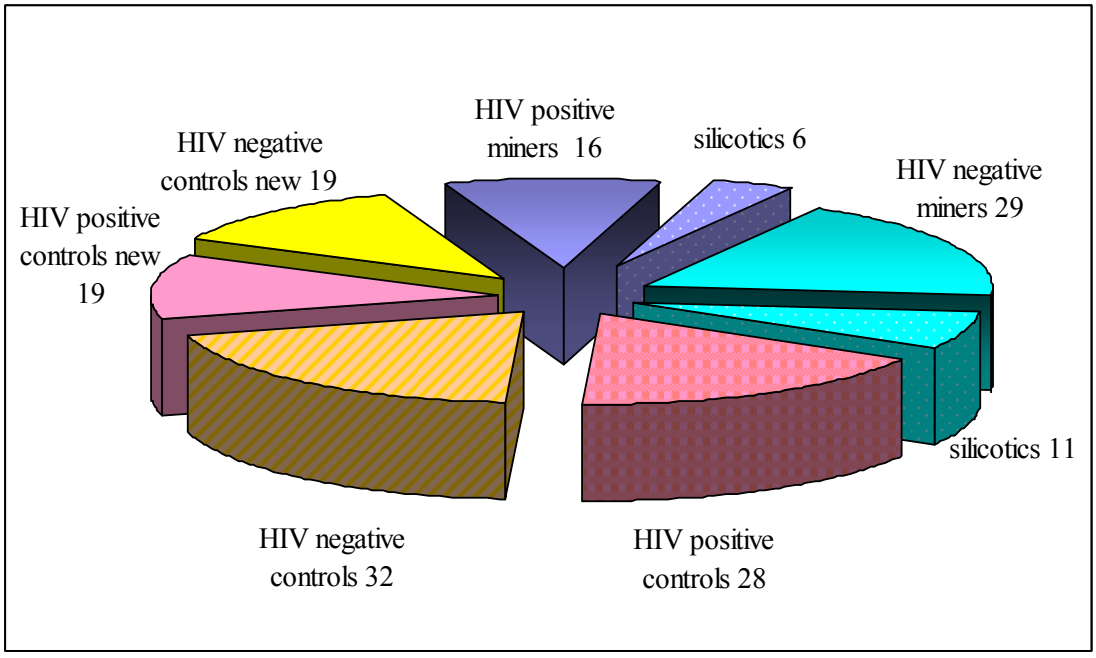


Figure 11: A diagram of 164 of 165 participants showing the numbers of participants by HIV, RCS exposure and silicosis status. All groups contained smokers and non-smokers (one blood test for HIV could not be performed and it was not possible to get a second sample). Group 1 consisted of the HIV negative miners, Group 2 HIV positive miners, group 3 the HIV negative controls and group 4 the HIV positive controls.

For all the participants, information was collected on the age, smoking history, general health, work history, diet and current job designation. The miners were also asked to list their length of service in dusty jobs. Medication currently being taken was recorded along

with HIV status and CD4 count. The languages most commonly requested were Xhosa, Zulu, Sotho and Shangaan.

Silicosis

Seventeen miners who participated in the study were diagnosed with silicosis. The diagnosis was originally made by X-ray by the mine radiographer. The majority of the same X-rays were then re-read by the NIOH B-reader. The NIOH radiologist read three more X-rays compared to those provided to the mine radiologist. There were a few discrepant x-rays between the two readers.

Table 4: Results of two reader's examination of the miners' X-rays using a reading of 1/1 to diagnose definite silicosis

	n	**Normal**	**Possible silicosis**	**Definite silicosis**	**%**
Mine radiologist	53	29	11	13	24.5
NIOH radiologist	56	35	12	9	16.1
Combined	60	43	0	17	28.3
Missing	4				

n = number of X-rays available to be read

A prevalence of silicosis of 16.1 % for the NIOH radiologist and 24.5% for the mine radiologist using the cut point of 1/1 was found in the mine study participants (Table 4). When the two readers' scores were combined, a prevalence of 28% was found. This is due to the fact that each reader had different missing X-rays. Because there was reasonable agreement on other readings, the reader's score was used for the x rays where there was only one reading. In cases where one reader coded the result as possible and the other as definite, the combined result was coded as definite silicosis. These scores were used further to examine the possible effect of silicosis on the biomarkers of interest.

The Kappa score for the agreement between the two readers for the full ILO codes was 0.5079. This is consistent with a fair agreement. The Kappa score for diagnosis of silicosis when silicosis was coded as present or not present was higher at 0.5424 using a 1/1 cut-point. There was complete (100%) agreement using 1/0 as the cut point. Using the 1/0

score as a cut off point for silicosis is acceptable, as the score is a subjective reading. The yes/no silicosis scores compare well and suggest that the results from the two readers can be combined to fill in results, where missing from either of the readers.
The agreement between the two readers is also likely to be affected by the quality of the radiographs.

Table 5: Quality of the radiographs

	Percentage acceptable
Mine radiologist	72%
NIOH radiologist	48% (4% unreadable)

The quality of the X-rays is important for the diagnosis of silicosis. The mine radiologist found far fewer radiographs unacceptable, and classified 72% as good, compared to the NIOH B-reader who classified 48% as good (Table 5). The mine radiologist was not consistent in his comments on the quality of the X-rays. The some of the X-rays analysed by the mine radiographer did not always correspond to those provided to NIOH. A number of the X-rays were more than two years old. This may make a difference in the score of miners who were possible silicotics according to their X-rays (ILO grade 0/1) as they may have progressed in the two years since their last X-rays.

HIV

HIV was diagnosed on the basis of blood test results. The proportion recruited was not the same so an association was tested for to rule out any bias due to numbers of HIV positive and negative participants in the exposed and unexposed groups.

Table 6: HIV status

	HIV positive n (%)	**HIV negative n**
Miners	23 (36.5)	40
Controls	47 (46.0)	54

(chi square OR = 59 (95% CI 0.6 – 1.2)

The proportions of HIV positive study populations in Table 6 are not indicative of the prevalence of HIV in the populations but rather the sampling frame of the study. When the association between RCS exposure of the miners and their HIV status was tested, no significant relationship was found, thus HIV was evenly distributed between exposed miners and the controls. This is because the participants of the study were selected on the basis of their HIV status. The lack of association suggests that there is no bias in the study participants due to their HIV status.

CD4 counts were available for 40 of the total (70) HIV positive participants.

Table 7: Normality test for CD4 count

Shapiro-Wilk W test for normal data					
Variable	Obs	W	V	Z	Prob>z
CD4 count	40	0.90493	3.758	2.786	0.00267

The CD4 count results were not normally distributed (Table 7) so for further hypothesis testing either the counts had to be transformed or a non-parametric method had to be used. As the HIV-positive controls were recruited from an HIV clinic, most of the patients were likely to need treatment, which meant that they also had AIDS with a concomitant low CD4 count. As the miners were recruited at a health clinic and not an HIV clinic, they were likely to have much higher CD4 counts (Table 8).

Table 8: CD4 counts of HIV-positive participants

Group	**N**	**mean**	**median**	**SD**	**min**	**max**
HIV-positive miners	10	252	187	244	11	561
All HIV-positive controls	30	234.6	166	175	4	720
Original HIV-positive controls	17	254.4	161	209	4	720
New HIV-positive controls	13	208.9	171	122	50	500

Mean of controls compared to the mean of miners Wilcoxon rank sump = 0.6732

There was no significant difference between CD4 counts reported in the miners or the controls p= 0.67 (Table 8). There was no significant difference between the newly recruited and original controls P = 0.9833 (Wilcoxon rank sum). This suggests that the

HIV-positive participants with known CD4 counts were similar. The validity of this conclusion is limited by the small number of participants who had a CD4 count. It was not possible to compare the CD4 counts of the miners with and without silicosis to see if there was an association between silicosis and the progression of HIV due to the small number of miners with both silicosis and HIV.

Age

The distribution of the ages was analysed for normality using the histogram and the Shapiro-Wilk test presented (Table 9; Figure 12).

The histogram of the controls compared to that of the miners shows a wider age distribution in the controls (Figure 12). The mean age of the miners was 44.6 years (95% CI 43.2 – 46.0) which was 8.04 years (95% CI 5 -11) older than the controls. This difference was significant with both a Wilcoxon rank sum test p = 0.000 and an unequal unpaired t-test p = 0.000.

Table 9: Normality test for distribution of ages

Shapiro-Wilk W test for normal data					
Variable	Obs	W	V	z	Prob>z
Age	163	0.97449	3.188	2.640	0.00415

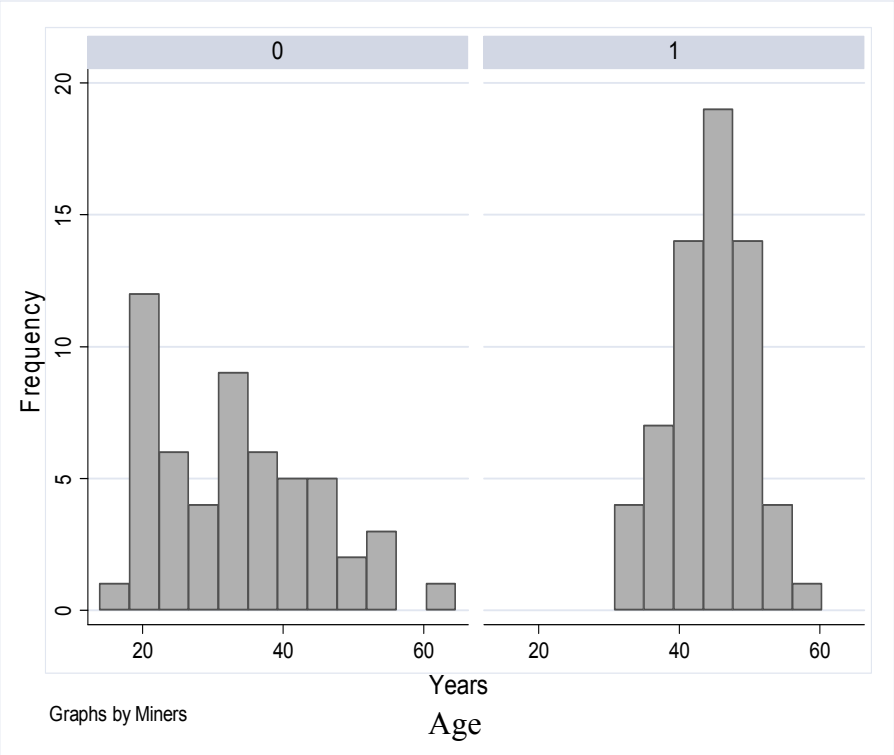


Figure 12: Histograms of the ages of the controls and RCS-exposed participants 0 = control, 1 = RCS exposed.

The distribution of the ages based on the histogram was approximately normal with more values in the left tail than expected (Figure 12), but the Shapiro-Wilk test was significant for a distribution that did not approximate normal (Table 9). Transformation of the data was performed but log, Reciprocal Square and square root transformations of the data did not result in a normal distribution. Non-parametric tests were used to compare the means of the data.

Table 10 presents the age statistics for the various groups in the study.

Table 10: Summary statistics of the ages of the participants.

Group	N	Mean	SD	Median	25th percentile	75th percentile	Min	Max
HIV-positive miners	22	44.18	6.11	44.54	40	49.9	32.84	55.25
HIV-positive controls	28	43.37	14.80	40.37	31.4	56.35	20.9	70.8
HIV-negative miners	40	44.86	5.55	45.08	41.65	48.5	31.08	54.33
HIV-negative controls	35	31.77	12.38	30.7	21.08	39.27	18.1	60.33
All miners	62	44.60	5.67	45	40.58	49.16	31.08	58.25
New HIV-positive controls	18	34.07	12.81	35.29	21.66	45.58	19.25	54.16
New HIV-negative controls	19	37.40	9.59	36	30.75	45	20.16	57.75
All controls	100	36.56	13.44	35.17	23.98	45	18.1	70.8

The HIV-positive patients had a wide age range of 19.25 to 70.8. The HIV-negative controls were younger than the HIV-positive controls with a Wilcoxon rank sum test P= 0.000 due to selection bias as many of the HIV-negative recruits were university students. For the HIV-positive and HIV-negative miners, ages were normally distributed and the mean ages did not differ significantly ($p = 0.6597$ using an unpaired t-test) (Table 10).

The mean age of the newly recruited controls did not differ significantly from the original controls recruited where $p = 0.997$, which suggests that the factors introducing the bias in age between the miners and the controls remain unchanged.

As age was normally distributed amongst the miners, with $p = 0.984$, the use of an unpaired t-test to compare the mean ages of the miners with and without silicosis was appropriate. The miners with silicosis were an average of 2.7 years older than the miners without silicosis, but this was not statistically significant ($p = 0.1004$) (Table 11).

Table 11: Age distribution of silicotic miners and non-silicotic miners.

Group	N	Mean	SD	25th percentile	75th percentile	Min	Max
Silicosis (≥ ILO 1/0)	17	46.74	5.32	43.08	49.0	37.30	58.25
Non-silicotic miners	43	44.07	5.77	40.08	49.16	31.08	54.33

(Unpaired t-test, degrees of freedom 59, difference -2.7 (95% CI -5.9 – 0.54) p = 0.1004

Table 11 does not show a significant difference in age distribution of those with and without silicosis, although the trend of decreasing age for the non-silicotic miners follows the expected pattern. The miners with silicosis would be expected to be older with a smaller age distribution than those miners with no radiographic signs of silicosis. The smaller age distribution of those with silicosis is due to the long lag phase of the disease and the fact that as it progresses it leaves the workers unfit to work, thus reducing the number of years they are likely to remain on the mine after diagnosis. The non-silicotic miners had similar mean age to the silicotic miners with no significant difference p=0.1. The non-silicotic miners were expected to be younger, but as the workers participating in this study was older, the older age of the non silicotic miners may be due to the healthy worker effect, where the healthier workers may be more likely to remain at work. The youngest participant with silicosis was 37 years old with 15 years of exposure. His ILO score was 1\1.

Years of Exposure and Job Types

Based on the results of the histogram and the Shapiro-Wilk test (Figure 13 and Table 12), the number of years worked on a mine were normally distributed. This means that parametric hypothesis tests could be used to evaluate differences between groups

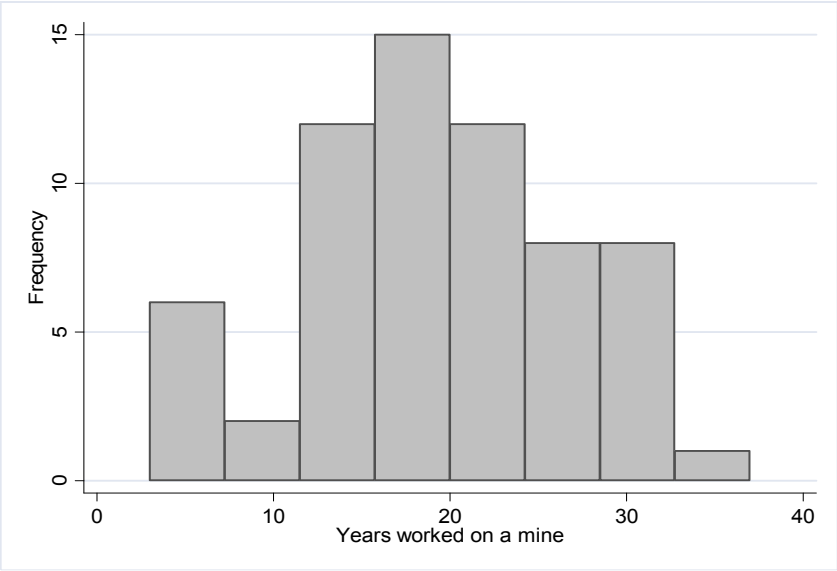


Figure 13: Histogram of the distribution of years of work in a dusty environment

Table 12: Normality test for distribution of years worked on a mine.

Shapiro-Wilk W test for normal data					
Variable	Obs	W	V	Z	Prob>z
Years of exposure	64	0.98855	0.656	-0.913	0.81935

A one-way ANOVA with Bonferronni adjustment was used to compare years worked of those with and without silicosis (Table 13).

Table 13: Years of exposure of silicotic and non-silicotic miners.

Silicosis	N	Mean years exposed	Median	SD	Min	Max
Silicosis (≥ ILO 1/0)	17	24.0	25	6.46	12	37
Possible Silicosis (≥ ILO 0/1< 1/0)	8	18.1	16.5	7.81	5	29
No silicosis (< ILO 0/1)	35	17.4	18	7.06	3	31
No X-ray	4	14.3	14.5	4.35	10	18

(One-way ANOVA, Bartlett's test for equal variance p = 0.733, between groups p = 0.009)

Table 14 compares the years of work as a proxy for cumulative exposure with silicosis diagnosis.

Table 14: Comparison of mean years of work by diagnosis of silicosis on a mine in 3 groups (Bonferronni).

Row	No silicosis	Possible silicosis	Silicosis
Possible silicosis	0 .696429		
P value	1.000		
Silicosis	6.54202	5.84559	
P value	**0.013**	0.315	
Missing X-ray	-3.17857	-3.875	-9.72059
P value	1.000	1.000	0.083

The average number of years worked in a dusty job by the miners with silicosis was significantly higher than those without silicosis (24 years vs. 17 years; p = 0.013) (Table 13 and p value in Table 14). There was no significant difference in years worked between those with possible silicosis and those with both definite silicosis and no silicosis (p = 0.315 and p = 1.000 respectively). For the purpose of this study, the possible silicotics (ILO = 0/1) were recorded as having no silicosis for the analysis of the biomarkers. Those with missing X-rays also showed no significant difference in years worked compared to both the no silicosis and the definite silicosis groups (p = 1.00 and p = 0.83). Those miners with missing X-rays had worked an average of 14 years on mines. The lack of significance between the missing X-ray and the definite silicosis groups is more likely to be caused by the small number (4) than a real lack of difference.

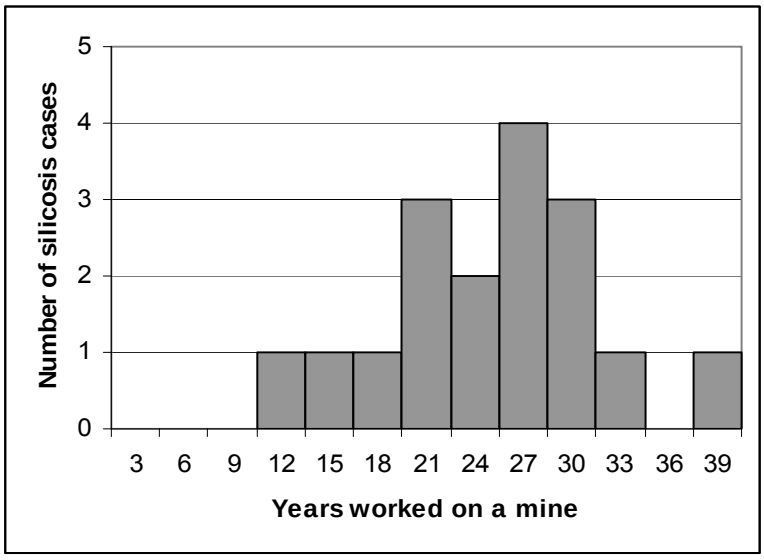


Figure 14: Histogram of cases of silicosis by number of years worked on a mine

The first participant with silicosis fell into the 9-12 years of work experience while the majority worked 18 -30 (Figure 14). Workers who are able to remain on a mine for many years may be healthier than those who leave due to sickness. In addition, many who are diagnosed with silicosis may have left the mines in search of less dusty work or due to disability as the disease progresses.

Table 15: Years of mine work amongst miners with and without HIV.

HIV status	No.	Mean years exposed	SD	25th percentile - 75th percentile	Min	Max
Positive	23	17	8.52	11 - 23	3	37
Negative	40	20.14	6.59	15.5 - 25.5	3	31
All miners	63	19.05	7.40	14 – 25	3	37

The HIV-positive miners tended to have worked fewer years in the mines than the HIV-negative miners (Table 15). The mean ages of the two groups did not differ significantly.

Table 16 provides the work types of the study participants.

Table 16: Job types.

	No.	Percentage	Job type
Miners	29	47%	Drivers
	19	31%	Generalists
	6	10%	Surveyors
	8	12%	Unclassified
HIV-negative controls	27	52%	Students
	14	27%	Office workers
	5	10%	Manual labour
	4	8%	Other
HIV-positive controls	31	66%	Unemployed or retired
	13	28%	Manual labour
	3	6%	Other

The miners were mainly underground workers. The majority of individuals recruited who were attending the HIV clinic at the hospital were unemployed (66%), and a large number of students were recruited to participate as HIV-negative controls (Table 16).

Smoking

Current smoking is an important confounder of the biomarker levels in this study as it causes lung damage and oxidative stress.

The current smoker miners, with a median of 10 cigarettes per day, appear to smoke more than the current smoker controls who smoked a median of 5 cigarettes per day. The maximum number of cigarettes smoked per day was well above the medians in either group at 15 and 10, respectively (Table 17).

Reported ex smoking and current smoking were not confirmed in a blood test due to cost limitations thus some missclassfication can be expected.

Table 17: Mean number of cigarettes smoked currently per day by the smokers in each group.

Smokers Group	No.	Median	25th percentile	75th percentile	Min-Max
HIV-positive miners	3	10	4	10	4 – 10
HIV-positive controls	11	5	5	10	3 – 10
HIV-negative miners	9	10	5	10	1- 15
HIV-negative controls	3	5	2	7	2 – 7
Silicosis (≥ ILO 1/0)	3	4	4	10	4 – 10
All controls	19	5	4	6	2 – 10
All miners	12	10	4.5	10	1 – 15

The proportion of smokers ranged from 8.7% to 35.7% in the different study groups. The 95% confidence intervals are wide due to the small numbers of smokers in each group (Table 18). Similar proportions of participants had quit smoking in the different groups (Table 18). There was no significant difference in proportion of smokers in each group.

Vegetarians

Diet maybe a possible confounder of total antioxidant status, one of the biomarkers in this study. Vegetarians are seen as eating a healthier diet with more antioxidants due to a greater intake of fruit and vegetables. There were very few vegetarians (2) in the study in either the miners or the controls so this is unlikely to be a source of bias.

Medication

None of the blood donors were taking any medication, as it is usually an exclusion factor for donating blood. Thirty-one (44%) of the HIV-positive controls were on an HIV HAART regimen. Other medications reported were for high blood pressure and high cholesterol, and Warfarin was also taken

Table 18: Prevalence of smoking in the groups recruited

Group	Total no.	Current Smokers n (%)	95% CI	Past Smokers n (%)	95% CI	Ever Smokers n (%)	95% CI
HIV-positive miners	23	2 (8.7)	-3.7-21.2	6(26)	10.2–48.4	34.7	16.3 -57.3
HIV-positive controls	28	10(35.7)	16.8-54.6	7(25)	10.7–44.9	50	30.6-69.3
HIV-negative miners	40	10(25)	10.9 – 39	9(22.5)	10.8–38.5	47.5	31.5 -63.9
HIV-negative controls	33	4(9.4)	-1.3-20.1	7(21.9)	9.3–40.0	33.3	17.9-51.8
New HIV-positive controls	19	5(26.3)	9.1-51.2	0		26.3	9.1-51.2
New HIV-negative controls	19	3(15.8)	-2.2–33.8	5(26.3)	9.1–51.2	42.1	20.3-66.5
Dust-exposed with silicosis (≥ ILO 1/0)	17	2(11.7)	1.5-36.4	5(29.4)	10.3-55.9	41.2	18.4-67.1
Dust-exposed without silicosis	43	9(20.9)	5.5-31.3	10(23.3)	11.8 – 38.6	44.2	29.1– 60.1

2.4 Discussion

Comparison of any factors that could cause a difference in biomarker levels between groups, other than RCS exposure, is important to demonstrate when assessing if this study has internal validity. The controls were non-miners and therefore unexposed to RCS but were generally from different socio-economic groups to the miners.

The significant difference in age between the miners and the controls was mostly due to the fact that the majority of the HIV-negative controls were students. Donating of blood is encouraged at school and university, so the participants who donate blood are more likely to be younger. Many participants were recruited at universities in Johannesburg as not many older African men donate blood. At university, there is a large pool of potential donors so there is a larger group of African men donating blood. This reduced the mean age of the HIV-negative controls. The significant difference in mean age between the miners and the controls may have affected the levels of some of the biomarkers under investigation in this study. This means that those biomarkers cited in the literature where age appears to be a confounder or effect-modifier will need further analysis and an adjustment will have to be made for the difference in ages between the miners and the HIV-positive and HIV-negative controls.

CD4 counts give an indication of the stage of HIV/AIDS, particularly if the person is not taking ARVs. There was no significant difference between mean CD4 counts of the groups. Thus there was unlikely to be any bias introduced by the stage of HIV/AIDS.

Silicosis was found in 28% of the study group. This was not an indication of the prevalence of silicosis on the mine. The study was introduced to participants as being about a test for lung disease on the mine, and more miners with self-perceived lung problems might have volunteered to participate. There was no significant difference in age between those miners with and without silicosis. This is possibly due to the healthy worker effect where the miners who remain at work are more likely to be healthy and continue to be so. The majority of the miners with silicosis had simple silicosis also possibly due to the healthy worker effect

The number of years worked on a mine was a proxy for cumulative RCS dust exposure that has been shown to be associated with silicosis (Churchyard *et al.*, 2004). This study found that the years worked in those with silicosis were significantly higher than in those without silicosis. The number of years worked may improve with the introduction of HAART, allowing the HIV-positive miners to work more years.

There was no significant difference in the proportion of smokers in any of the groups, although the power of this analysis was low due to the small number of current smokers in each group. Where smoking is indicated in the literature to be a confounder, the analysis should be adjusted for smoking. The number of cigarettes smoked per day was 5-10 on average and was not seen as heavy smoking. Heavy smoking has been defined as more than 20 cigarettes per day (Strandberg et al., 2008).

Biologically significant differences are often different from statistically significant differences. Statistical significance is dependent on the sample size and the variation within the groups. Biological significance is when the difference is shown to have an effect on the results.

Possible sources of bias in the selection of the participants

The participants were selected on the basis of their RCS exposure and HIV status. Therefore, as they were not randomly selected, there may have been a bias introduced by the selection procedures. The rate of recruitment was slow, with many months needed to recruit sufficient participants. Amongst the miners, many potential participants were unsure about donating blood due to a fear of needles, cultural issues and a fear that they did not have sufficient blood to donate as they felt much was being asked of them. Many were unwilling to undergo an HIV test. It is of course also possible that those who claimed a fear of needles actually feared the HIV test. Miners work long shifts underground and are keen to shower and eat after their shift. They were therefore often unwilling to give up their time to participate in the study at the end of a shift. These circumstances introduced some self-selection bias into the study. However, as all the miners were exposed, this bias is unlikely to have a large effect on the results.

The mine VCT sister was required to use the telephone in the waiting area for general patients to inquire about test results for individual patients, and as the tests were done using the name and work number of each patient, they were clearly identified. A number of possible study participants were willing to undergo HIV testing provided it was not done at the mine VCT centre. Also, in a few cases it became apparent that the nursing staff knew the status of some of the patients after testing had been done at the clinic. This causes a lack of trust in the confidentiality of the VCT service when it is provided at the workplace. While it is illegal to dismiss anyone because of their HIV status, participants suspected that known HIV-positive workers were placed first on the list for future possible retrenchments.

Amongst the controls, most of the HIV-negative African male blood donors were keen to participate, but as there were very few African male blood donors, a self-selection bias again resulted. This bias was likely to select for the healthiest individuals and might increase the difference in biomarker levels. The HIV-positive control participants were generally more willing to participate. Unfortunately, the majority of HIV clinic patients were women and this reduced the pool of potential participants. For ethical reasons, an effort was also made in the case of the HIV-positive controls not to recruit participants who were very frail or those with known severe anaemia.

An additional factor inhibiting the use of VCT services is the feelings of fatalism and depression that come from receiving a positive test result. In a study by Gaillard et al. (2000) 15% of HIV-positive women felt that they would have been happier if they had never found out that they were sick. This was similar to a sentiment expressed by men on the mine approached for our study. They felt that since death was inevitable if they tested positive, their last few years would be ruined by worry about the illness so they would rather not know their status and so avoided being tested.

When recruiting new blood donors among African men for the SANBS, the same fatalistic sentiment was echoed as a few men were happy to participate as long as they were never notified of their results. This response did not appear to be linked to education as university students expressed the same fatalism as the mine workers who were approached. In the study by van Dyk and van Dyk, (2003), a 17%, of black

correspondents felt that it was not advisable for individuals to know their HIV status. In this same study a majority of individuals (96%) felt there was no reason to go for testing if there was no treatment available. This has since changed and so attitudes may have improved, but the fear of lack of confidentiality and social marginalisation, along with the feeling that the treatment just postpones the inevitable may keep VCT attendance down.

Possible sources of bias in the recruitment of the participants

Miners: The miners were recruited at their mine health clinic. They were mainly visiting the clinic with minor complaints or were collecting medication. Those with known active pTB were excluded from the study as active pTB was likely to affect the lung damage markers. There may have been selection bias here as inflammatory markers could be expected to be affected by any acute illness. The study was advertised using posters which invited the interested participants to go and see the researchers at the clinic. However, this was not a very successful strategy due to the low numbers who visited the researchers. The poster also introduced an element of bias as it described the aim of the study and this may have caused an increase in the number of men with respiratory symptoms who were willing to participate in the study. The chief nurse at the clinic also helped to introduce participants by recruiting men to the study with known or suspected silicosis. This increased the prevalence of men in the study with silicosis to above the proportion anticipated to be found on the mine. However, as all the underground miners were evaluated in the study and were classified as exposed, this selection should not affect the study results.

HIV-positive controls: These men were recruited from an HIV clinic. Men tend not to seek treatment until they are seriously ill, and have low CD4 counts. They are likely to suffer from increased opportunistic infections (Carswell, 1988) possibly affecting the levels of the biomarkers, and introducing bias.

HIV-negative controls: These men were recruited at the SANBS mobile blood donor clinics and tend to be healthy. This is reflected in the low numbers of smokers recruited.

Information bias

Information bias can result in misclassification of participants (Rothman, 1986). The types of information bias possible in this study are questionnaire bias, recall bias, exposure classification bias and diagnostic bias. The recommended measures to minimise these biases were not always possible to carry out in the study due to resource constraints.

Questionnaire and Interviewer Bias: The questionnaire was kept simple to reduce bias due lack of understanding and interviewee fatigue and the interviews were conducted where possible in the participant's home language to improve the quality of the data. There were a few participants who came from Mozambique and it was not possible to conduct the interview in their home language. It was not possible to blind the interviewer to the purpose of the study as she played a number of roles in the study.

Recall bias could have been prevented by comparing job history recall to work records, but these are often incomplete records, and as quantitative exposure classification was not central to the study, it was regarded as too costly to pursue as it would require dust measurement. Thus the number of years of dust exposure reported could have been inaccurate as the miners may have reported working for more years than they had actually worked, or not all of the years may have been spent in underground jobs, so the number of years of actual exposure may have been less than indicated.

Exposure classification bias. A selection criterion for control participants was that they had never worked in a dusty environment; this was clarified in the questionnaire with a few examples of dusty workplaces such as foundries and quarries to ensure that non-miners with RCS exposure were not recruited. Although the exposure of interest is RCS, it is possible there could be confounding exposures such as ozone, particulates, diesel and other air pollutants that may cause oxidative stress in the lung. The possible non-mining/foundry work/quarrying exposure of the controls was not recorded and may have confounded the effect of RCS exposure on biomarker levels in this study. However the distribution of these other exposures is likely to be similar across the groups

Miners are exposed to other harmful substances such as diesel fumes and boron. The mixed exposure of the miners was not assessed and the possible effects on biomarker levels could therefore not be determined. Concurrent exposure may have confounded the effect of RCS on the biomarkers of interest.

Diet is not likely to be a confounder in this study as very few participants were vegetarians. This reduces possible confounding due to diet, but does not eliminate it as the quality of the diets may have differed between the controls and the miners, as the miners are provided with a main meal by the mine. The mines also provide low-cost vitamin supplements to their workers, but it was found that not many of the workers used them.

Selection Bias

The bias caused by selecting blood donors for the controls is clearly demonstrated in the prevalence of current smoking. The difference in the prevalence of smoking between the controls and the miners is more likely to have been due to selection bias than a real difference between the HIV-positive and HIV-negative control participants and the miners. Selection of healthy, altruistic HIV-negative controls with recent HIV test results is likely to reduce smoking prevalence in that group as they are less likely to smoke (Mast *et al*., 2012).

The recruitment of participants whose HIV status had been recently tested in the case of those classified as HIV-negative is particularly difficult in the present climate of the stigma and fear of HIV. This makes it more difficult to control for possible bias within the study, because if exclusion factors are increased to reduce bias, this is likely to result in too small a sample size or too much time required for enrolling participants. In addition, while many individuals may have had an HIV test, the criterion of a recent HIV test excluded many possible participants. Due to the confidentiality of HIV status and the stigma attached to a positive status, many employers would not allow us to sample for control participants. They claimed not to possess the employees' test results or to need the unions' approval to release the results even anonymously, despite our ethics clearance. To allow us to collect test results from the HIV voluntary counselling and testing clinics, we had to provide proof of an anonymous communication of test results.

Limitations due to the cross-sectional study design

A cross-sectional study may clarify relationships, in this case the change in biomarker levels in the blood serum or red blood cells to the history of dust exposure. However the limitation of the study is that the temporal relationship cannot be determined. Many miners have been exposed to RCS for a number of years (the mean from this study is 20 years), and so early effects of RCS exposure may no longer be measurable as the miners now display late effects or symptoms of disease. The changes in the biomarker levels may have altered.

As early effects of RCS exposure may be experienced in all RCS-exposed miners, and not only in those who develop silicosis, the long lag time of silicosis should not be limiting factor in a study with new recruits. The healthy worker effect may play a role in those participants who have worked for a long period. A limitation in this study is that participants who left employment were not recruited.

Selection of study participants

A true random selection of study participants would require obtaining a list of all possible candidates, then selecting from the list. In our case, finding the workers would be complicated by the miners working different shifts and being on leave at different times. Moreover, this type of selection would require a large amount of resources and participation rates would be expected to be low because of the requirements of an HIV test and the donation of blood samples. Biomarker baseline levels are expected to vary randomly across the population, except possibly in the case of inflammatory markers of participants attending clinics with different ailments. This should not affect the internal validity of the study.

Random selection of controls would be very difficult as access to blood donor lists from SANBS is restricted and donors attend donation clinics as volunteers and do not always turn up when called to do so. Thus convienience sampling was used

2.4 Conclusion

There are a number of sources of bias within the study, particularly selection bias. This is not thought to affect the internal validity of the study as the selection bias is unlikely to affect the relationship between the biomarkers of interest and RCS exposure as exposure was a selection criterion. Information bias may play a small role in reducing the internal validity of the study due to some misclassification of exposure that may have occurred. Overall the study design is valid within expected limitations

CHAPTER 3

Markers of Oxidative Stress

3.1 Introduction

3.1.1 Oxidative stress

Oxidative stress is a term that describes a change in the balance between oxidants and antioxidants in the body, with an increase in the former. The disruption of this important balance can lead to many downstream effects (Domanski *et al.*, 2005). Oxidative stress is a process. It is not an individual incident, but rather the result of an imbalance between oxidants and antioxidants. When there is an increase in oxidants or a decrease in antioxidants, a chain reaction begins and oxidative stress is the result (Ochs-Balcom *et al.*, 2005).

Oxidants are compounds or agents that are able to induce the production of oxidants; and these include endogenous and exogenous free radicals and non-radical oxidants. Endogenous oxidants are unavoidable since they are produced by aerobic respiration. Exogenous sources of free radicals include environmental toxins, such as tobacco smoke, ozone and numerous chemical agents. Non-radical oxidants are enzymes and chemicals, whose normal functions result in the production of oxidants (Quinlan *et al.*, 1994; Montuschi *et al.*, 2004).

One example of endogenously derived free radicals is ROS, which are produced by oxidative phosphorylation, cyclooxygenase and cytochrome P450, to specify a few common reactions, and also by the respiratory burst of activated phagocytes. ROS consist of oxygen-free radicals and their metabolites (Vallyathan and Shi, 1997). Partially reduced oxygen is converted to superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$), which are the most commonly, recognised ROS (Hayes and McLellan, 1999). Approximately 2% of oxygen present in the body may

react to form ROS. These ROS are removed by the activities of antioxidant enzymes such as superoxide dismutase, GPx and α-tocopherol and other non-enzymatic antioxidants (Hayes and McLellan, 1999). Antioxidants can be defined as substances that delay or inhibit oxidation when present even in low quantities. They act in a number of ways, from removing oxygen and scavenging singlet oxygen, to removing ROS and catalytic metal ions and breaking the sequence of oxidant-generating pathways (Gutteridge, 1995).

Exogenous (Vitamin C and endogenous (Glutathione, Catalase and GPx) antioxidants have been recognised as playing an important role in the prevention of oxidative stress. Catalase, GPx and superoxide dismutase are the antioxidant enzymes commonly recognised as important to antioxidant defence. Other enzymes have been identified that have antioxidant functions, but as some of them have other functions, they are not regarded exclusively as antioxidants (Quinlan *et al.*, 1994).

Oxidative stress and crystalline silica exposure

Oxidative stress is being placed increasingly in the spotlight as a suggested mechanism of disease. Oxidising agents have been shown in many studies to cause numerous harmful effects at many levels in the body, from the molecular to the tissue level, which can ultimately cause organ damage (Domanski *et al.*, 2005). Oxidative stress has been linked to many diseases including cancer, neurodegenerative disorders, lung disease, disorders of the metabolism and cardiovascular disease (Halliwell and Grootveld, 1987; Montuschi *et al.*, 1998; Reid and Jahoor, 2001 and Montuschi *et al.*, 2004). Methods are being sought to measure oxidative stress both *in vitro* and *in vivo* (Halliwell and Grootveld, 1987) to determine whether oxidative stress is the cause or the result of tissue injury due to RCS exposure. Haemolysis caused by RCS was inhibited in *in vitro* studies by the addition of catalase, which is responsible for removing H_2O_2 from the blood (Driscoll *et al.*, 1995).

ROS have been linked to a number of lung diseases and are suggested to play a role in their pathogenesis (Quinlan *et al.*, 1994). Following this, ROS have been determined to be an important mediator of dust-induced lung disease (Ghio *et al.*, 1990, Schins *et al.*, 1995b). RCS exposure has been associated with the release of free radicals and the generation of lipid hydroperoxides (Chvapil *et al.*, 1976; Vallyathan *et al.*, 1988;

Kamal *et al.*, 1989b; Schins *et al.*, 1994 and Vallyathan *et al.*, 1998). Studies with rat AM showed increased generation of ROS when exposed to RCS (Vallyathan *et al.,* 1992). Many animal and *in vitro* studies have shown that RCS is capable of producing ROS that damage tissue (Chvapil *et al.*, 1976 and Gupta and Kaw, 1982). Two possible mechanisms for the damage of cells by RCS induced ROS have been suggested; The first involves ROS which form on the surface of RCS particles following interaction between silicon radicals and lung cells, which are increased when the RCS is freshly fractured (Vallyathan *et al.*, 1988; Shi *et al.*, 1995; Fubini, 1997 and Chvapil *et al.,* 1976). These ROS then interact with AM and lung epithelial cells, causing damage. The second pathway involves RCS particles being engulfed by AM, which release a respiratory burst consisting of free radicals, and damage surrounding tissue (Babior *et al.*, 1973 and Wallaert *et al*., 1990). It is likely that both pathways contribute to the increase in ROS found after exposure to RCS.

The disrupting action of RCS radicals on cell membranes is one of the suggested mechanisms in the fibrosis process. This membrane damage results in the release of enzymes responsible for further damage, leading to fibrosis (Shi *et al.*, 1994). Incubating freshly fractured RCS with linoleic acid resulted in a dose-dependent increase of the lipid peroxidation demonstrated by the production of malondialdehyde (MDA a by-product of lipid peroxidation). The production of MDA was inhibited by SOD and catalase, suggesting that the lipid peroxidation was caused by free radical attack (Shi *et al.*, 1994).

Some free radicals inflict damage on cellular constituents and produce by-products. Radicals are also easily able to cross cell membranes and inflict damage outside cells (Zhu *et al.*, 1998). Lipid peroxidation occurs as a process within oxidative stress as lipids are a key target of free radicals. Once lipid peroxidation has been induced by a free radical attack, they are self-perpetuating and require an antioxidant to stop the process. This process can amplify the effect of a single free radical.

Initiation: free radical + lipid H $\longrightarrow$ non-radical H + lipid radical

Lipid radical + O_2 $\longrightarrow$ LOO radical

LOO radical + lipid H $\longrightarrow$ LOOH + lipid radical*

*taken from (Montuschi *et al.*, 2004)

Cell membranes consist to a large part of lipids and so are vulnerable to peroxidation, which can lead to increased permeability and inactivation of receptors (Montuschi *et al.*, 2004). Isoprostanes, which are products of arachidonic acid peroxidation, are recommended as a non-invasive index of lipid peroxidation and oxidative stress *in vivo* (Morrow *et al.*, 1999).

Exposure to RCS has been linked to an increase in lipid peroxidation in exposed animals and humans. Early studies by Gabor, (1975) showed an increase in MDA in the phospholipid fraction in rat lung after RCS inhalation. This result was reproduced by Gupta and Kaw in 1982, who found an increase in MDA in rat lung homogenates after intratracheal injections with RCS. The lung homogenate, when partitioned into subcellular fractions, showed the largest increase in MDA in the microsomal fraction. This suggests that the intracellular membranes are a target of increased lipid peroxidation. The degradation of cellular membranes has been implicated as an important preliminary step in the development of silicosis (Gupta and Kaw, 1982). Gabor *et al.*, (1975) and Chvapil *et al.*, (1976) went on to investigate the effects of RCS on alveolar macrophages and erythrocytes. They found an increase of lipid peroxidation linked to the presence of RCS. The peroxidative effect of RCS dust was increased when compared to the effect of inert non-fibrogenic corundum dust on AMs. The RCS treatment resulted in a significant increase in lipid peroxides, while the corundum showed no increase over untreated controls. This suggests that the lipid peroxidation seen in the presence of RCS is not due only to the physical presence of particles but are also likely due to the physico-chemical properties of the RCS dust (Gabor *et al.*, 1975). Later studies confirmed that RCS exposure results in the generation of lipid peroxides (Kamal *et al.*, 1989a; Ghio *et al.*, 1990 and Shi *et al.*, 1995).

Increased antioxidant enzymes were shown in rat lung tissue following RCS

exposure, with the greatest increases in superoxide dismutase, but no significant change in GPx (Quinlan *et al.*, 1994).

Numerous diseases are continually being linked to oxidative stress. These diseases could be important confounders in any assay of oxidative stress markers. HIV is a particularly important condition associated with an increase in oxidative stress (Baier-Bitterlich *et al.*, 1997; Klebanoff and Headley, 1999) as its incidence is high in South African mines (Corbett *et al.*, 2004). HIV infection has been shown to induce ROS production (Sanchez-Pozo *et al.*, 2003). Cigarette smoke is known to contain free radicals, which affect the lung both directly and indirectly (Duthie et al., 1991).

3.1.2 Measures of Oxidative Stress

Free radicals are highly reactive and measuring them *in vivo* is complicated. Therefore by-products of the oxidative stress process and other substances such as antioxidants are used to estimate free radical levels *in vivo* (Halliwell and Grootveld, 1987).

3.1.2.1 Glutathione

GSH is an abundant tripeptide, a low-molecular-weight thiol. It plays an important role in the detoxification of harmful compounds of both endogenous and exogenous origin. GSH detoxifies by being conjugated to the possibly harmful compounds, a reaction catalysed by GST and GPx (Figure 15). This conjugation mostly leads to the harmful compounds becoming less reactive and more easily excreted from the body (Hayes *et al*., 2005). The oxidation and reduction of GSH is referred to as the GSH redox system and alterations of any of the enzyme activities involving GSH can result in cellular damage (Rahman *et al.*, 1999).

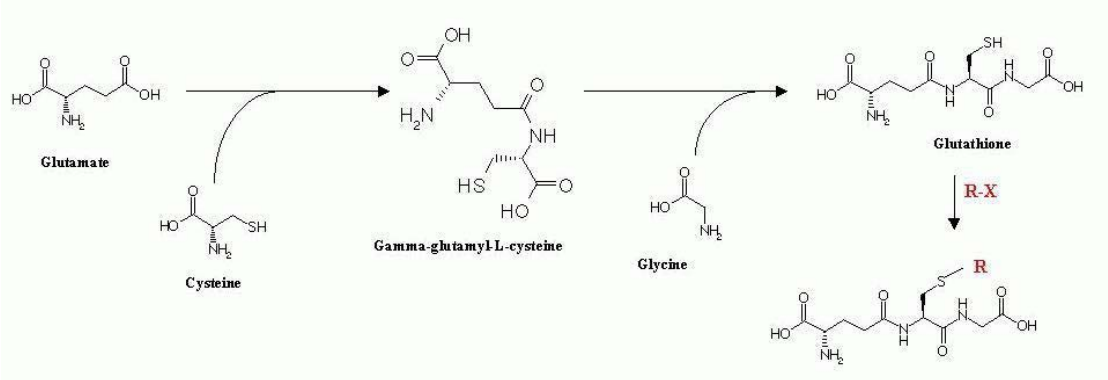


Figure 15: Glutathione synthesis and conjugation (Taken from Klaassen, (1996))

GSH plays a key role in a number of important physiological functions. It plays a main role in the protection of cells against oxidative damage. Other functions include providing a reducing environment for cell membranes, supporting amino acid transport as well as protein and nucleic acid synthesis, regulation of the immune response and detoxification of electrophilic toxicants and metal ions (Rahman *et al.*, 1999). GSH also plays important roles in a number of other processes such as apoptosis and homeostasis (Hammond *et al.*, 2001 and Rahman *et al.*, 2005).

GSH is synthesised in the cell by two enzymes, glutamate cysteine (GCS) ligase and glutathione synthetase (Willis *et al.*, 2003 and Wu *et al.*, 2004). GSH can be found in three forms in a cell: reduced glutathione (GSH), oxidised glutathione (GSSG) and a mixed disulphide (RSSG). GSH reacts with oxidants to form GSSG and the GSH/GSSG forms a buffer in the cell. Most of the GSH in a cell is in the reduced form, ready to remove oxidants from the cell (Rahman *et al.*, 1999). GSH can directly and indirectly scavenge ROS and free radicals. Depletion of GSH in a cell leads to cell death if under oxidative stress (Wüllner *et al*., 1999). GCS catalyses the rate-limiting step of GSH generation and is sensitve to DNA polymorphisms (Willis *et al.*, 2003 and Wu *et al.*, 2004). Some suggested effects of polymorphisms in GCS gene are an increase in the production of GSH in the cell, oxidative stress, cancer, inflammatory cytokines and radiation. There are also a number of compounds that can negatively affect GCS transcription such as dietary protein deficiency and phosphorylation of GCS and erythropoietin (Sies, 1997 and Lu, 2000).

GSH is present in high concentrations in most cells (Rahman *et al.*, 1999). GSH is found in all tissues and throughout the entire cell (Willis *et al.*, 2003), with 85-90% in the cytosol (Wu *et al.*, 2004). In the lung, GSH is found in cells as well as in the epithelial lining fluid. Macrophages, lymphocytes, fibroblasts and epithelial cells have been found to export GSH.

Willis *et al.*, (2003) examined the effect of ethnicity on the polymorphisms of the genes responsible for the production of GSH. They identified differences in frequencies of polymorphisms between Caucasians and African-Americans, whereas the African-American frequencies were similar to those of the Ghanaian samples.

This suggests that there may be ethnic differences in the ability to synthesise GSH, which may be important in diseases where oxidative stress plays a role. Erythrocytes isolated from smokers were found to contain an increase in GSH compared to non-smokers (Quinlan et al., 1994).

GSH levels in silicosis patients

Decreases in GSH levels of epithelial lung lining fluid have been demonstrated in a number of lung diseases, specifically idiopathic pulmonary fibrosis, cystic fibrosis, (Rahman *et al.*, 2005) and in HIV-positive patients (Pacht *et al*, 1997). This reduction in antioxidant defence may leave the patients more susceptible to inhaled toxicants.

Borm *et al.*, 1986 and 1987 found an increase in reduced GSH in silicosis patients but no change in oxidised GSH levels. This increase may have been due to the treatment used by the silicosis patients, although controls on medication showed a decrease in GSH. It is most likely though that the increase in reduced GSH was due to an increased need for protection from ROS.

Engelen *et al.*, (1990) showed a decrease in erythrocyte GSH in Belgian coal miners with early stage pneumoconiosis. Schins and Borm, (1995a) and Schins *et al.*, (1997) later determined the blood antioxidant status of the same coal miners and found no differences in GSH between miners with and without CWP, but they did find an association between erythrocyte GSH levels and miners who showed progression in their CWP during the study follow-up period.

Evelo *et al.*, (1993) evaluated the erythrocyte GSH levels in coal miners and found them to be significantly decreased in miners with early silicosis compared to miners without silicosis. In miners with more severe silicosis, they observed an increase in GSH, which, they suggested, could be due to compensatory synthesis of GSH.

3.1.2.2 Glutathione peroxidase

GPx is a selenium-dependant enzyme (Steinberg *et al.*, 1989) that is made up of four 17 kDa sub-units, each of which has an active site which contains a selenium (Se) molecule in the form of selenocysteine (Josephy, 1997). GPx consists of 0.29% Se and requires this to function (Awasthi *et al.*, 1975). GPx uses GSH as its main and

most effective substrate, where GSH acts as a hydrogen donor for the catalytic breakdown reaction (Mills, 1959). Some substrates of GPx are hydrogen peroxide, lipid peroxides, and fatty acid hydroperoxides (Maddipati and Marnett, 1987).

GPx is one of the early defence mechanisms against oxidative damage of cells by peroxides and is responsible for the catalysis of peroxides to alcohol (Paglia and Valentine, 1967). The presence of GPx peroxidase in erythrocytes was first demonstrated in 1957 by Mills (Mills 1959). Maddipati and Marnett, (1987) described an Se-dependant GPx in the plasma.

GPx is a family of eight isozymes (Table 19) (Paglia and Valentine, 1967). Classical or cytosolic glutathione peroxidase (cGPx) was the first to be identified and nearly all tissues express some cGPx also referred to as GPx1, but high levels are found in erythrocytes, liver, kidney, lung, brain and testes (Brigelius-Flohe, 1999). In erythrocytes GPx1 protects haemoglobin from oxidation (Mills and Randall, 1958). Cytosolic GPx (GPx1) is the most common form of GPx. The importance of GPx1 in protecting against oxidative stress was confirmed by de Haan et al., (1998) by exposing mice with homozygous null mutation ($GPx^{-/-}$) to paraquat which could not survive compared to those mice with no mutation ($GPx^{+/+}$).

GPx2 and GPx3 are isozymes which act on hydrogen peroxide where the former is found in the gastrointestinal tract and the latter in the plasma (Chu *et al*., 1993 and Nakagawa *et al*., 2004).The fourth isozyme GPx4, is a phospholipid hydroperoxidase, (Nakagawa et al., 2004) which is part of cell membranes and is able to reduce lipid peroxides and thus plays a large role in protecting cell membranes from damage (Awasthi et al., 1975). GPx5 is an epididymal androgen-related protein (Hall *et al*., 1998), GPx6 is found in the olfactory system (Kryukov *et al*., 2003) while GPx7 (Utomo *et al.,* 2004) and GPx8 (Mammalian Gene Collection Program Team, 2002) are not yet well defined.

Table 19: GPx isozymes - their substrates and locations.

Isozyme	Preferred substrate	location
GPx1	Hydrogen peroxide	all tissues
GPx2	Hydrogen peroxide	Gastrointestinal tract
GPx3	Hydrogen peroxide	Plasma and extracellular
GPx4	Lipid peroxides	Phospholipid hydroperoxidase
GPx5		Epididymal androgen-related protein
GPx6		Olfactory
GPx7		Not defined
GPx8		Putative

Adapted from (Chu *et al*., 1993; Nakagawa, 2004; Hall *et al*., 1998 Utomo *et al*., 2004 and Kryukov et al., 2003).

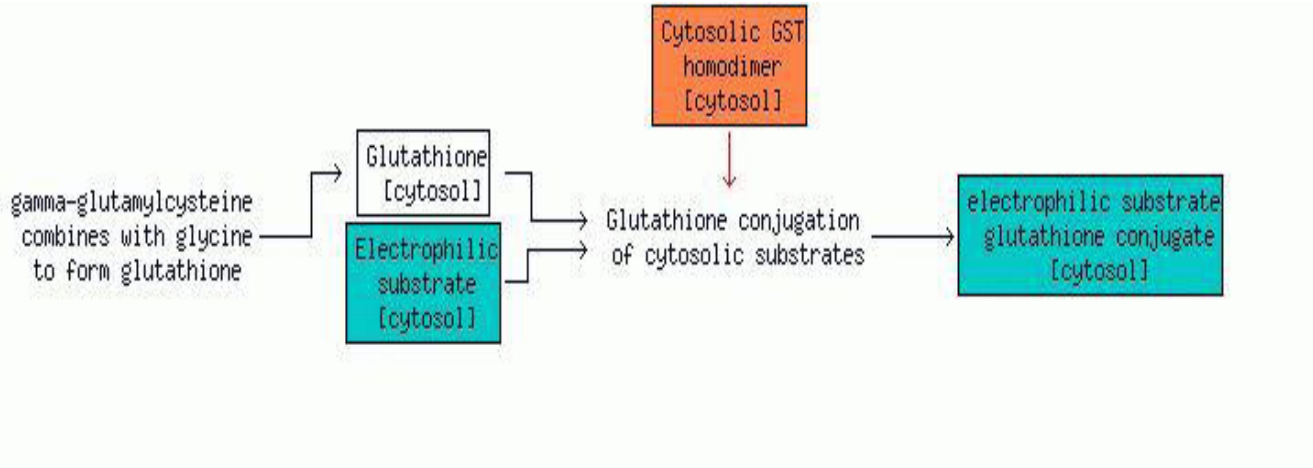
Other studies have measured the effect of other toxic workplace exposures on GPX activity. For example Babu et al. (2006), measured erythrocyte GPx levels in workers exposed to cadmium. A significant decrease in GPx activity was seen in exposed workers compared to unexposed controls. Kalahasthi et al., (2006a) and Kalahasthi et al., (2006b) also measured a significant decrease in erythrocyte GPx activity in workers exposed to chromium (VI) or nickel. Oil refinery workers showed decreased levels of erythrocyte GPx compared to non-industrial workers (Lane et al., 1983) and in the study by Zachara et al., (1987) Rubber factory workers showed decreased erythrocyte GPx activities, compared to the non-industrial controls. The above studies demonstrate the non-specificity of GPx as a biomarker.

3.1.2.3 Glutathione S-transferase (GST)

GST is a superfamily of dimeric isozymes that catalyze neutrophilic attack by GSH on hydrophobic compounds containing an electrophilic atom (Warholm *et al.*, 1985; Allocati *et al.*, 2003). Their main function (as phase II enzymes) is to metabolise compounds that pose a threat, thus detoxifying possibly harmful xenobiotics and assisting in the metabolism of endogenous compounds, particularly ROS (Hayes and McLellan, 1999). GST has a broad substrate reactivity specificity that evolved under selective pressure to detoxify the many endogenous and synthetic toxins to which

organisms are exposed (Josephy, 1997). GST is a highly evolutionarily conserved enzyme that has evolved a number of sub-groups in mammals but the functions have remained similar throughout. GST catalyses the conjugation of reduced GSH, UDP-glucuronic acid or glycine to activated compounds, resulting in the reduction of hydroperoxides, GSH conjugates and oxidised GSH (Sheehan *et al.*, 2001). There are two types of conjugation reaction with GSH 1) Displacement reactions: where GSH displaces an electron-withdrawing group, such as halogens, nitriles and carboxylic acids. 2) Addition reactions: where GSH is added to activated double bond structures or strained ring systems i.e. nucleophilic addition (Armstrong, 1991).

GST consists of dimers of 25 kDa sub-units and are found as both homodimers and heterodimers. GST is mainly found in the cytosol of cells but can also be found bound to the membrane (Armstrong, 1991).



RX + GSH -----*GST*----> HX + GSR

Figure 16: The reaction catalysed by GST (Rushmore and Pickett, 1993)

GST is a transferase that catalyses the addition of aliphatic, aromatic, or heterocyclic free radicals as well as epoxides and arene oxides to GSH (Figure 16). Addition takes place at the sulphur atom. GST isozymes are the most abundant enzymes in the cytosol of the liver. The large abundance of the isozymes is also an indication of their low efficiency; this is due to the inverse relationship between specificity and efficiency. The broad substrate specificity allows overlapping of substrate specificities as different toxins are encountered.

GST is a key enzyme in the detoxification of harmful substances (Hayes and McLellan, 1999), and a lack of GST has been suggested as a cause of cancer susceptibility; GST has been linked to drug resistance in numerous diseases. Several studies have linked high GST levels to increased resistance to oxidative stress. GST has also been shown to detoxify some secondary ROS produced when primary ROS (such as hydrogen peroxide) interact with cellular constituents.

Veal *et al.*, (2002), using the yeast *Schizosaccharomyces pombe*, were able to demonstrate that, when the cells were challenged with H_2O_2, the expression of the GST-encoding genes was induced. This suggests that GST is necessary for cellular resistance to oxidative stress.

GST can mimic GPx activity in Se and vitamin E deficiencies and reduce some lipid hydroperoxides (Harris and Stone, 1988). There is a membrane form of GST, which is most likely involved in the reduction of the hydrophobic lipid hydroperoxides as they are also likely to be membrane-associated. Lipid peroxidation produces a number of by-products, which could inhibit GST.

3.1.2.4 ROS measurement using luminol-enhanced chemiluminescence

Phagocytes in the immune system release ROS in the process of phagocytosis as an anti-microbial measure (Hosker *et al.*, 1989). Membrane-bound NADPH oxidase generates both superoxide anions and H_2O_2 which are released during the oxidative burst of phagocytosis. These and their metabolites can cause damage to surrounding tissue and activate other immune cells (Dahlgren and Karlsson, 1999). Luminol-enhanced chemiluminescence (LECL) is a method developed to measure the production and release of the ROS produced during phagocytosis.

Chemiluminescence is light released following a chemical reaction. Luminol (5-amino-2, 3-dihydro-1, 4-phthalazindione) is a dye that reacts with ROS in the presence of peroxidase. Luminol molecules become excited by ROS and release energy in the form of light which can be detected as they return to the ground state (Dahlgren and Karlsson, 1999). Superoxide was determined to be the specific ROS measured in this reaction (Lundqvist and Dahlgren, 1996).

Phagocytosis in these measurements is stimulated by the addition of an activator, often opsonised zymosan. The phagocytes in solution then phagocytose these particles releasing ROS. The ROS in the solution then react with the luminol, producing a blue light, which is then measured with sensitive equipment. Studies using neutrophils prepared from patients with chronic granulomatous disease, who have defective respiratory burst, showed no activation of luminol and no chemiluminescence. This relationship suggests that the activation of luminol and the subsequent chemiluminescence measure respiratory burst in neutrophils (DeChatelet and Shirley, 1981). Luminol is able to cross the cell membrane and therefore measures both intracellular and extracellular ROS (Caldefie-Chezet *et al.*, 2002).

Smoking has been shown to increase ROS, and studies have shown an increase in the LECL of smokers (Kalra *et al.*, 1991 and Theron *et al.*, 1994). RCS can also activate phagocytes to release an oxidative burst assessed by chemiluminescence assays (Maly, 1988 and Nyberg and Klockars, 1990).

Theron *et al.*, (1994) used whole blood LECL as a measure of the generation of reactive oxidants in smoking and non-smoking miners and non-smoking controls. They found a significant increase in the LECL of miners who smoked compared to the controls and non-smoking miners, while the LECL of non-smoking miners and controls was not significantly different.

HIV has been investigated in terms of oxidative burst from polymorphonuclear leucocytes, but there is controversy among some authors who report a decrease in respiratory burst (Pitrak *et al.*, 1998) and others who observe an increase (Ryder *et al.*, 1988). Shalekoff et al., (1998) demonstrated an increase in phagocytosis by granulocytes in HIV-positive patients but a decrease in phagocytosis in patients with TB or both TB and HIV.

3.1.2.5 8-Isoprostane

ROS are known to have effects on lipids, DNA and cellular proteins. Due to the effect of free radicals on lipids, measuring the end products of lipid peroxidation is commonly used to assess oxidative stress (Halliwell and Grootveld, 1987 and Morrow and Roberts, 1996). Primary and secondary end products of the peroxidation pathway

can be measured. Primary end products are lipid hydroperoxides and conjugated dienes, while the secondary end products are thiobarbituric reactive substances, alkanes and F_2 isoprostanes (Morrow and Roberts, 2002).

8–Isoprostane is a prostaglandin-like compound, one of a group of isoprostanes produced *in vivo*, mostly independent of enzymes in free radical-induced peroxidation of arachidonic acid (Morrow *et al.*, 1990b). These were first demonstrated to be formed in humans *in vivo* in 1990 by Morrow *et al.,* (1990a). 8-Isoprostane is an F_2 isoprostane, one of a group of 64 isomers. The peroxidation pathway produces other isoprostanes like the E_2, D_2,Cyclopentenone A_2 and J_2, and acyclic-ketoaldehydes (Morrow and Roberts, 2002 and Montushi *et al.*, 2004). These other isoprostanes are less studied than the F_2 isoprostanes as they are less stable (Liu *et al.*, 1999). 8-Isoprostane is the most prevalent F_2 isoprostane in humans (Montushi *et al.*, 2004).

Isoprostanes are formed by a free radical attack on arachidonic acid and form a peroxyl radical which, following endocyclisation, forms a PGG_2-like compound. This unstable intermediate is reduced by naturally occurring substances to parent isoprostane. The parent compound can produce one of four regioisomers called 5, 8, 12, and 15 series regioisomer depending on the location of their side chain hydroxyl group (Morrow and Roberts, 2002). The amount of isoprostanes formed exceeds that of the enzyme-formed prostaglandins, and therefore are likely to be the main products of arachidonic acid peroxidation (Morrow *et al.*, 1999). Isoprostanes are found in two forms, namely free and esterified. This is because isoprostanes can be formed *in situ* on esterified arachidonic acid in the lipid bilayer of membranes and not only on frees arachidonic acid. It has also been suggested that esterified arachidonic acid is oxidised more often than free arachidonic acid (Morrow *et al.*, 1992). The isoprostanes formed on the membranes are released through a phospholipase mechanism and are able to circulate in the plasma and are excreted in urine (Patrono and FitzGerald, 1997). Significant levels of isoprostanes have been detected in normal human plasma, indicating that oxidative stress is found in normal individuals (Morrow *et al.*, 1999). In human studies increased levels of isoprostanes have been found in COPD (Montuschi *et al.,* 2000), cystic fibrosis (Montuschi *et al.,* 2000), sarcoidosis (Psathakis *et al.*, 2004), pulmonary hypotension, acute respiratory distress disease, asthma acute lung injury (Morrow and Roberts, 2002) and atherothrombotic disease

(Patrono and FitzGerald, 1997). Increased plasma levels of 8-Isoprostane have also been found in other diseases such as diabetes, Alzheimer's disease, and pre-eclampsia (Dietrich *et al.*, 2002).

Increased levels of 8-Isoprostane have been demonstrated in urine, BALF, plasma and exhaled breath condensate of diseased individuals (Montuschi *et al.*, 2000). 8-Isoprostane has been shown to be significantly higher in smokers than in non-smokers, which confirms the effects of smoking on oxidative stress in the body. A study by Morrow *et al.*, (1995) clearly showed a decrease in isoprostanes in smokers who abstained for two weeks, demonstrating the effect of smoking on oxidative stress. However, they could not demonstrate any effect due to gender, age, weight, height and smoking history on isoprostane levels (Morrow *et al.*, 1995). The effect of age on 8-Isoprostane levels was also investigated by Montuschi *et al.*, (1999) who as well could find no correlation between the 8-Isoprostane levels and age in healthy volunteers.

Asthma patients have been shown to have increased oxidative stress as measured by 8-Isoprostane levels. These levels increased with an increase in the severity of asthma (Montuschi *et al.*, 1999). Cardiovascular disease has also been shown to increase levels of oxidative stress and 8-Isoprostane (Patrono and FitzGerald, 1997).

It has been suggested that isoprostanes are not only markers of lipid peroxidation *in vivo* but also may play a physiological role in pulmonary pathophysiology (Janssen, 2001). It has also been suggested that isoprostanes are able to activate neutrophils, assisting in the adhesion of these cells to endothelial cells. As isoprostanes can be formed *in situ* in cell membranes, they affect the fluidity and permeability of cell membranes, which can lead to cellular dysfunction and apoptosis or necrosis (Morrow *et al.*, 1992).

8-Isoprostane has been proposed as a useful non-invasive marker of lipid peroxidation and oxidative stress in humans. Animal models of acute oxidative stress such as Se-deficient mice treated with diquat have shown significant increases in isoprostanes in plasma compared to untreated mice (Morrow *et al.*, 1990b).

Dietrich *et al.*, (2002) demonstrated that increased 8-Isoprostane was associated with increased BMI (trend P = 0.001). This suggests that BMI may act as an effect modifier on the level of oxidative stress.

3.1.2.6 Total Antioxidant Status

TAS is a measure of the combined protective effect of all antioxidants, both endogenous and exogenous, such as dietary antioxidants (MacKinnon *et al.*, 1999). This is a useful measure, as one test is able to estimate the effect of numerous nutrients and enzymes combined, allowing for simple and rapid determination (Wiid *et al.*, 2004). Schofield and Braganza, (1996) suggest that although individual antioxidants play different roles in controlling oxidative stress in the body, their combined effect plays a larger role in maintaining the balance of pro-oxidants and antioxidants. Based on this, a combined measure of antioxidant status in the body is important.

TAS is measured in plasma as blood plays an important role in antioxidant defence by redistributing antioxidants throughout the body as needed (Ficicilar *et al.*, 2003). TAS is able to provide information on the combined effect of systemic antioxidants by measuring their ability scavenge a radical cation formed by the reaction between H_2O_2 and ABTS (2,2-azino bis-3ethylbenzothiazoline-6-sulfonic acid) (Schofield and Braganza, 1996). The extent to which antioxidants present in the sample suppress this reaction is proportional to their concentration (Ficicilar *et al.*, 2003).

The TAS of patients is an indicator of the net burden of environmental factors and genetic factors on individual antioxidant status. The study by Wiid *et al.*, (2004) supports the need to establish a reference range for the population of interest as differences in lifestyle, environment and diet can play a role in their TAS levels.

The TAS of patients infected with HIV has been measured in a number of studies (Favier *et al.*, 1994; McLemore *et al*., 1998). These studies have reported an increase in lipid hydroperoxides and a decrease in TAS in HIV patients compared to healthy controls. On the other hand, Girotti *et al.*, (2002) could not demonstrate any significant differences in the TAS of smokers and non-smokers, although they found a consistent difference due to gender, accros all subgroups.

pTB has been associated with inflammation related oxidative stress. It has been proposed that lung fibrosis associated with pTB is initiated by activated macrophages that are able to release a number of factors including ROS and cytokines (Wiid *et al.*, 2004). Miners are at an increased risk of developing pTB and thus this was an important confounder in the current study. Wiid *et al.* (2004) found a significant decrease in the TAS of patients diagnosed with pTB compared to community controls. The TAS of the patients increased with pTB treatment to within normal levels.

TAS is a non-specific biomarker and a number of conditions such as tuberculosis, diabetes, heart disease and cancer have been shown to reduce the TAS of patients (Lantos *et al.*, 1997; Opara *et al.*, 1999; Ficicilar *et al.*, 2003 and Sener *et al.*, 2007)

3.2 Methods

Serum samples and whole blood samples were collected from 127 participants and analysed for markers of oxidative stress. The results were divided into those who were exposed to RCS and those who reported no exposure to RCS. These two groups were then further divided into HIV-positive and HIV-negative participants.

3.2.1 Theoretical background of the assays implemented

Erythrocyte GSH

The GSH tripeptide is found in two forms: reduced and oxidised. The total GSH in cells consists of reduced GSH and GSSG. The ratio of GSH to GSSG is greater than 10 under normal conditions (Wu *et al.*, 2004). Assays exist for both total GSH and reduced GSH alone, although to assay reduced GSH requires a derivatisation of GSH. The assay used for total GSH was a recycling reaction between GSH and 5, 5-dithio bis-2-nitrobenzoic acid (DTNB). The rate of production of 5-thio-2-nitrobenzoic acid (TNB), a by-product of the recycling reaction, is proportional to the rate of the reaction, which is only limited by the GSH concentration. Thus, the rate of TNB production measurable at 405 nm absorbance is proportional to the total GSH concentration in the sample.

Reduced GSH in the cell is oxidised to GSSG in a reaction catalysed by GPx (Rahman *et al.*, 1999). This thiol is then returned to its reduced state by GST reductase, which is also used in this assay to generate the TNB by the reduction of DNTB. Thus, this method measures total GSH.

Proteins found in most samples assayed for GSH are measured as they can interfere with the assay results.

GPx1

The reaction catalysed by GPx is (Smith and Levander, 2002):

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O \quad \text{(Reaction 1)}$$

This is a reaction consisting of two phases. In the first step GPx reduces the peroxide to an alcohol but is itself oxidised (reaction 1). Then two molecules of GSH reduce GPx back to its original state and become one molecule of GSSG. The GSSG is recycled back to two molecules of GSH by the enzyme glutathione reductase. This reaction uses nicotinamide adenine dinucleotide phosphate (NADPH). This coupled reaction is used in the determination of GPx activity as the loss of NADPH is monitored spectrophotometrically (Brigelius-Flohe *et al.*, 2002). This is the method used in this study.

GPx protein can also be determined by immunochemical methods such as ELISA and western blotting, which provide the amount of GPx protein (Brigelius-Flohe *et al.*, 2002). However the real amount of GPx protein provides no information on activity, so protein determination is not the best method to use in evaluating GPx activity.

GST

The reaction catalysed by GST is:

$$GSH + CDNB \xrightarrow{GST} GS\text{-}DNB\ Conjugate + HCl \quad \text{(Reaction 2)}$$

The total activity of GST in a sample is commonly measured spectrophotometrically by measuring the increase in absorbance at 340nm following the conjugation reaction catalysed by GST and was the method used in this study. In this reaction GST catalyses the conjugation of L-GSH to the reagent 1-chloro-2,4-dinitrobenzene (CDNB) at the thiol group of the GSH (Reaction 2) (Habig *et al.*, 1974). The rate of increase in the GS-DNB conjugate in the sample is directly proportional to the activity of the GST enzyme.

8-Isoprostane

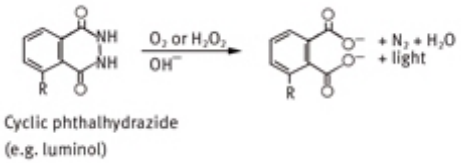
8-Isoprostane is commonly measured using gas chromatographic/negative ion chemical ionisation mass spectrophotometry (GC-MS). This is a highly sensitive method and provides results in pictograms but it is also a labour-intensive method and requires expensive equipment. While immunoassay kits are available, although information regarding their precision, accuracy, and specificity is limited. The immunoassay advantage is that it is affordable and easy to perform and make the field of measuring lipid peroxidation more accessible (Morrow and Roberts, 2002). Thus this was the chosen method.

Montuschi *et al.*, (2000) validated the 8-Isoprostane enzyme immunoassay (EIA) against the GC-MS method of analysis and found a high correlation (of 0.95) between known added amounts of 8-Isoprostane and the concentrations measured by the EIA.

Isoprostanes can form in plasma during storage, and freezing and thawing as it is a non-enzymatic process, therefore precautions must be taken to prevent this formation. The antioxidant Butylated Hydroxytoluene (BHT) is found to suppress, but not totally stop the formation of isoprostanes (Morrow *et al.*, 1990b). The storage of fluids containing lipids at -70 °C has therefore been recommended to prevent peroxidation (Morrow *et al.*, 1999). Morrow *et al.*, (1990a) were able to demonstrate the formation of F_2-prostaglandin-like compounds *in vitro* in plasma after storage. Although BHT was added to the samples in the current study, it was only done after separation of the plasma and not at the time of collection due to the many tests being conducted on each sample. This may have allowed some 8-Isoprostane to form in the sample after collection.

ROS assay Chemiluminescence

Chemiluminescence is light released following a chemical reaction (Reaction 3). This release of light is recorded by light sensitive photomultiplier tubes in a luminometer as Relative Light Units (RLU). Luminol (5-amino-2, 3-dihydro-1, 4-phthalazindione) is a dye that is activated with an oxidant. Luminol molecules become excited and release energy in the form of photons of light which can be detected as the molecules return to the ground state.



Reaction 3: Luminol chemiluminescence (taken from Hipler and Hipler, 2005).

Luminol is useful compared to other chemicals that exhibit chemiluminescence because it can measure hydrogen peroxide as well as oxygen radicals and peroxide radicals simultaneously (Kobayashi *et al.*, 2001).

TAS

The ability of antioxidants in the plasma sample to quench the ABTS cation/radical production is proportional to the amount of antioxidants in the sample. Thus, the production of the radical is measured and subtracted from the amount of radical produced in a sample with no antioxidants. The radical is a blue-green colour and so production is followed at 600 nm absorbance. The result is expressed as Trolox equivalents. Trolox is a water-soluble analogue of vitamin E. Trolox placed in one test acts as the control for the maximum amount of quenching possible.

It has been suggested that there are limitations to this assay and that it only gives a general indication as individual antioxidants may vary in the course of a specific disease. Urate has been suggested to contribute to TAS levels and hyperuricaemia would cause an apparent increase in TAS (MacKinnon *et al.*, 1999).

This assay was shown to be highly sensitive to length of assay time as the TAS value was shown to continue to increase with colour development. This continued rapidly,

in the test cells if radical production was not arrested after the exact interval (Schofield and Braganza, 1996).

3.2.2 Practical assays

Sample preparation for GSH, GPx and GST

Erythrocyte lysate was obtained by collecting venous blood in a heparinised vacutainer and centrifuging for 10 minutes at 1 000 x g. The plasma was stored and the burry coat discarded, the packed erythrocytes were lysed with four volumes of distilled water (1.2 ml distilled water was added to 300 µl of erythrocytes). This lysate was then centrifuged at 10 000 x g for 10 minutes.

3.2.2.1 Glutathione assay

A GSH assay kit (703002) supplied by Cayman Chemical USA, was used for this assay.

Sample preparation

The erythrocyte lysate supernatant was mixed with an equal volume of metaphosphoric acid (MPA) solution (0.1 g MPA (Sigma) dissolved in 1 ml of distilled water). This was allowed to stand for 5 minutes before being centrifuged at 1000 x g for 2 minutes.

Reagent preparation

The MES buffer (0.4M 2-(N-morpholino) ethanesulphonic acid, 0.1 M phosphate and 2mM EDTA (ethylenediaminetetraacetic acid), pH 6) provided was diluted with an equal volume of distilled water before use. The standard provided was ready for use. The supplied cofactor mixture (NADP and glucose 6 phosphate) was reconstituted with 500 µl of distilled water and mixed well. The glutathione reductase and glucose-6 dehydrogenase mixture was carefully diluted with 2 ml of diluted MES buffer. The DTNB was mixed with 500 µl of distilled water and used within 10 minutes of reconstituting.

The sample was removed from storage and brought to room temperature. A 4 M solution of TEAM reagent (Trieathanolamine) was prepared by mixing 531 µl of triethanolamine (Merck) with 469 µl of distilled water. A 25 µl aliquot of TEAM reagent was added to 0.5 ml of sample to increase the pH to 7. The samples were then diluted 20 times with MES buffer.

The standards were then prepared by adding increasing volumes of the provided standard GSH to MES buffer in eight numbered tubes. The lowest volume of standard was 5 µl and was placed in tube one and the volumes in the following tubes were doubled until the final volume of 160 µl was reached. Corresponding volumes of MES buffer were added to give each standard a final volume of 500 µl. This provided eight standards with concentrations starting from 0.5 µM up to 16 µM GSH.

Assay

Fifty microliters of either sample or standard was added to each well in duplicate and then covered while the assay buffer was prepared in a 20 ml container. The assay buffer was prepared by adding 11.25 ml of MES buffer to 0.45 ml of co-factor mixture. A quantity of 2.1 ml of enzyme mixture was added to the tube and finally 0.45 ml of freshly reconstituted DTNB was added. One hundred and fifty microliters of assay buffer was added to each well within 2 minutes. The plate was placed on the spectrophotometer plate reader and the absorbance measured at 405 nm at 5-minute intervals for 30 minutes.

Calculations

The absorbance's of each standard and the samples were plotted against the time lapsed at each reading. The slope of each plot was determined and this was used to construct a standard curve against the concentration of the standards (Figure 17). The equation was determined, by plotting a line on the graph in Excel and allowing Excel to generate the equation, and used to calculate the concentration of the samples from the readings.

$$\text{Total GSH} = \frac{\text{Absorbance at 405nm} - \text{Y intercept}}{\text{Slope}} \times \text{dilution}$$

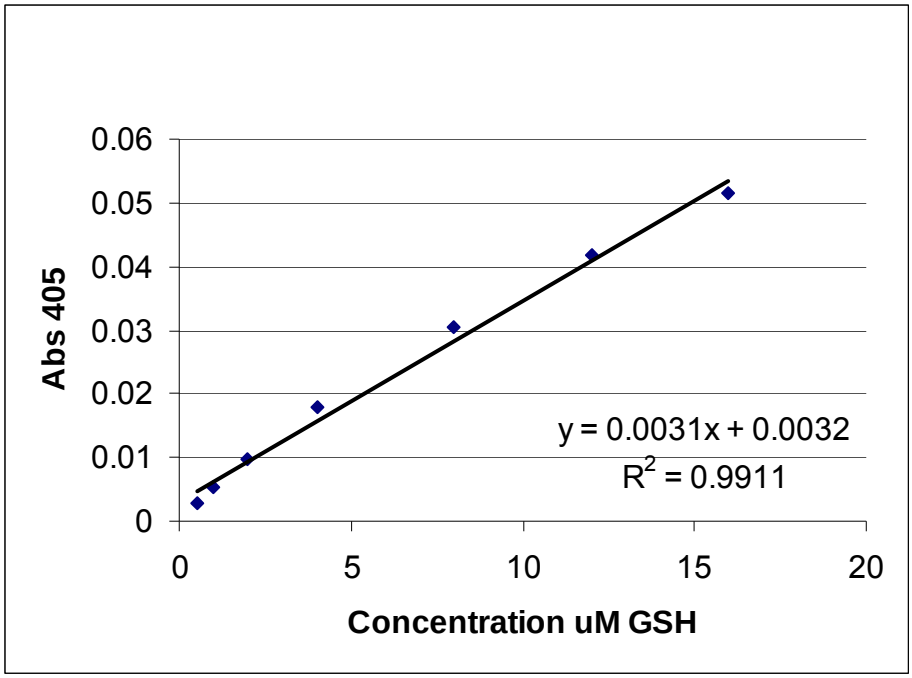


Figure 17: GSH standard curve

3.2.2.2 Glutathione peroxidase assay

The enzyme GPx was assayed with a glutathione peroxidase assay kit (703102) supplied by Cayman Chemical USA.

Provided in the assay kit were assay buffer concentrate (50 mM TRIS-HCL, 5mM EDTA, pH 7.6), sample buffer concentrate, (50 mM TRIS-HCL, 5mM EDTA, pH 7.6 and 1mg.ml BSA (bovine serum albumin)), GPx, co-substrate mixture (NADPH, GSH, glutathione reductase), cumene hydroperoxide and a 96-well plate.

Sample preparation

The erythrocyte lysate supernatant was thawed from -70ºC for use. The lysate was prepared as described previously.

Reagent preparation

The total 2 ml of the assay buffer concentrate was diluted with18 ml of distilled water. To prepare the sample buffer, a 2 ml of the concentrate provided was also diluted with 18 ml of distilled water. A quantity of 100 µl of assay buffer was then added to both

the sample and the background wells. The co-substrate mixture was reconstituted by adding 2 ml of distilled water and 50 µl of co-substrate, and then added to each well in the plate. The 10 µl of GSH control was diluted with 490 µl of diluted sample buffer and kept on ice. The cumene hydroperoxide was ready to use as supplied. The samples were diluted 20 times with sample buffer.

Assay

Twenty microliters of each of the diluted samples were added to all the sample wells in duplicate, and the reaction was initiated with 20 µl of cumene hydroperoxide. The plate was shaken for a few seconds to mix and then the absorbance was read off at 340 nm at each minute for at least five minutes.

Calculations

The change in absorbance was determined by plotting the absorbance measured against time to determine the slope or by subtracting two points on the curve and dividing by the time lapsed. This value was then placed in the following formula:

$$\text{GPx activity} = \frac{\triangle A_{340}\ /\text{min} \times 0.19\text{ml}}{0.00373\ \mu M^{-1} 0.02\text{ml}} \times \text{sample dilution}$$

GPx activity is equal to the change in absorbance divided by 0.00373 µM then the result is multiplied by 9.5 (for the dilution of the lysate) and further multiplied by 20 for the dilution of the sample.

3.2.2.3 Glutathione S Transferase assay

This enzyme was assayed with a Glutathione S-Transferase Assay kit (703302) supplied by Cayman Chemical USA.

Provided in the assay kit were assay buffer concentrate (100 mM potassium phosphate, pH 6.5 and 0.1% TritionX-100), sample buffer concentrate (100 mM potassium phosphate, pH 6.5 and 0.1% TritionX-100 and 1mM GSH),GST, reduced glutathione, CDNB in ethanol and a 96-well plate.

Sample preparation

The erythrocyte lysate supernatant was then thawed from -70ºC for use. The lysate was prepared as described previously.

Reagent preparation

The assay buffer was prepared by diluting the buffer provided (10 ml) with an equal volume of distilled water. The sample buffer (5 ml) was also diluted with an equal volume of distilled water. The GST control was prepared by diluting 10 µl of the stock with 190 µl distilled water. The RBC lysate was diluted 10 times with the diluted sample buffer. The GSH and CDNB were ready to use as supplied.

Assay

A quantity of 170 µl of assay buffer was placed in the blank wells while 150 µl of assay buffer was placed into all the remaining wells. GSH (20µl) was added to all the wells followed by 20 µl of sample or control added to duplicate wells. The reactions were then initiated by quickly adding 10 µl of CDNB to each well.

The plate was read at 340 nm once at every minute for 5 minutes.

Calculations

The absorbance values were plotted as a function of time and the slope determined by subtracting the last absorbance from the first and dividing by 5. GST activity was calculated by dividing the change in absorbance by 0.00503 µM and multiplying by 100 to account for the dilutions as per the following formula:

$$\text{GST activity} = \frac{\triangle A_{340}/\text{min}}{0.00503\ \mu M^{-1}} \times \frac{0.2\ \text{ml}}{0.02\ \text{ml}} \times \text{sample dilution}$$

The erythrocyte lysate levels in this study were not corrected for haemoglobin level, which ensured that all the blood samples taken had the same proportions of erythrocytes. The

possible effects of such differences were limited by using packed erythrocytes to create the lysate for analysis

3.2.2.4 Total antioxidant status

This was assayed with a Trolox equivalent kit supplied by Randox UK.

Chromogen 6 (10 µmol/L of 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] and 6.1 µmol/L metmyoglobin), Trolox standards (6-hydroxy-2,5,7,8-tetramethylchroman -2-carboxylic acid), buffer PBS 80 mmol/L (pH 7.4) and hydrogen peroxide (250 µmol/L) were supplied with the kit.

Sample preparation

Venous blood was drawn into a heparinised vacutainer, which was then centrifuged at 1000 x g. The plasma was removed and placed into cryotubes for storage at -70°C.

Reagent preparation

Each supplied vial of chromogen was reconstituted with 10 ml of supplied buffer. One millilitre of chromogen was placed into each disposable cuvette, and to this 20 µl of sample was added. The cuvettes were then placed in a water bath set at 37 °C. Once the solutions had reached 37 °C using timing they were placed in the spectrophotometer cuvette and the absorbance was read at 600 nm. The cuvettes were then placed in the water bath and 1ml hydrogen peroxide, diluted to 1.5 ml with buffer, was added sequentially as aliquots of 200 µl. The cuvettes were then incubated for 3 minutes and the absorbance read at 600 nm.

Calculations

The absorbance of the first reading was subtracted from the second reading to calculate the change in absorbance for both the samples and the blanks. A standard factor was then calculated by dividing the concentration of the standard by the absorbance of the standard, subtracted from the absorbance of the blank. The concentration of the sample was then calculated by multiplying the standard factor by the difference between the change in absorbance of the blank and the change in absorbance of the sample using the following formula:

TAS mmol/l = Factor x (△A blank -△ A Standard)

Factor = conc of standard

(△ A blank - △A Standard)

There were some limitations to the TAS method used in our study, which required a temperature-controlled spectrophotometer to incubate the samples between readings at 25 °C for the specified amount of time. Unfortunately, due to the lack of a temperature-controlled spectrophotometer a water bath was used to incubate the samples. The water bath did not maintain temperature as consistently as would have been required by this method, and so may have introduced additional variation in the results.

3.2.2.5 ROS measurement using luminol-enhanced chemiluminescence of neutrophils stimulated with RCS

Reagent preparation

PBS was prepared from a pre-weighed mixture purchased from Sigma. The sachet contents were mixed with 1 litre of distilled water and the pH adjusted to 7. A quantity of 0.0597g of luminol (Sigma, USA) was weighed and dissolved in 1 ml of Dimethyl sulphoxide (DMSO) (Merck) and then diluted in 9 ml of the PBS. This concentrated solution was then stored at -20 °C. Before use, the luminol was diluted again 10 x and then 30 x to give a final concentration of 30 μM final solution when added to the neutrophils. A quantity of 0.001 g RCS was weighed and suspended in 10 ml of PBS to give a concentration of 0.1 mg/ml and a final concentration of 0.033 mg/ml in the sample (modified from (Anderson *et al.*, 1998))

To prepare the ammonium chloride solution, 8.3 g of ammonium chloride was weighed along with 1 g of sodium hydrogen carbonate and 148 mg of EDTA. These were dissolved in 1 litre of distilled water. The 3% gelatine solution was prepared by dissolving 6 g of gelatine in 200 ml of the PBS. Finally, the gentian violet stain was prepared by mixing together 100 ml of distilled water, 2 ml of glacial acetic acid and 0.1 ml of 0.1% gentian violet (Modified from Anderson *et al*., (1998)).

Neutrophil isolation

Blood was drawn into a heparinised vacutainer and gently mixed; a 2 ml whole blood sample was removed and carefully layered on top of 1 ml of 1070 Histopaque (Sigma). This was centrifuged at 1 000 x g for 10 minutes. The supernatant containing plasma, monocytes and Histopaque was removed and discarded. The 3% solution of gelatine was warmed to 37 °C and added to the remaining erythrocytes and neutrophils to fill the tube. This solution was gently mixed and left to stand for 10 minutes to allow the erythrocytes to settle. Once the erythrocytes had settled to approximately the same volume as before, the supernatant of gelatine and neutrophils was removed and placed in a clean, labelled tube. The new tube was centrifuged at 800 x g for 10 minutes. After this procedure, the gelatine supernatant was discarded and the remaining erythrocytes and neutrophils were re-suspended in cold 3 mM Sodium Chloride (NaCl). The cells were placed in the refrigerator for 10 minutes to lyse the remaining erythrocytes. The tube was then centrifuged again at 800 x g for 10 minutes at 4 °C. The NaCl supernatant was discarded and the neutrophils were re-suspended in cold PBS and centrifuged at 700 x g to wash the neutrophils. The PBS was discarded and the neutrophils were re-suspended in 1 ml of PBS (modified from (Anderson *et al.*, 1998)).

Cell counting

Thirty microliters of the neutrophil suspension was mixed with 30 µl of gentian violet staining solution, of which 10 µl was placed on a haemocytometer for counting. The central 25 squares were used for counting. The cells in the four corner squares and the one central square were counted and summed. The number of cells were then multiplied by 5 and 10 000 to give the number cells in 1ml. This was converted to cells x 10^7 and a dilution factor was calculated to give a final concentration of 1 x 10^7 cells/ml. The calculation was:

Cells/ml = (cs1 +cs2 +cs3 +cs4) x 5 x 10 000

cs = corner square

Chemiluminescence

The luminol solution (Sigma) was diluted in PBS and the RCS solution also in PBS was prepared as described above. A 500 µl of neutrophils was placed in duplicate in chemiluminescence tubes and a background level of luminescence was determined by placing each tube in the Lumi One. A 500 µl of luminol was then added to each tube and the spontaneous chemiluminescence was followed until a peak was seen (Figure 18). This was achieved by continuously placing each tube in the machine and recording a reading. Once the spontaneous peak was measured, a 500 µl of RCS solution was added to each tube and the stimulated chemiluminescence was measured in the same way as the spontaneous peak.

The Lumi one measures the light produced as RLU which are the light units measured by the sensor in the machine.

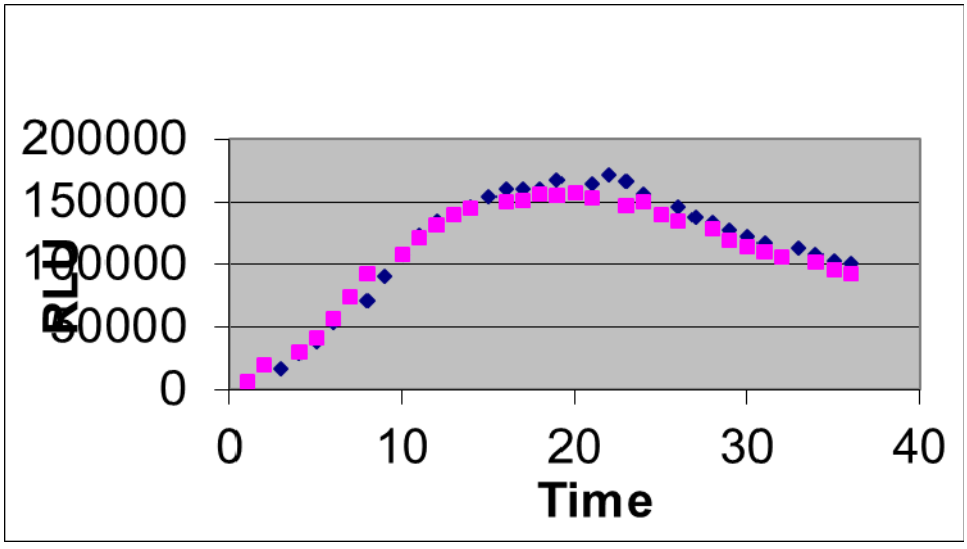


Figure 18: Chemiluminescence curve in RLU in one sample over time after stimulation of isolated cells with RCS. The pink and blue symbols on the graph represent the duplicates of the sample.

3.2.2.6 8-Isoprostane

This assay was performed with a Human 8-Isoprostane kit purchased from Cayman Chemical USA. This kit uses a quantitative sandwich enzyme immunoassay technique.

Supplied with the kit were: an 8-Isoprostane coated micro plate, 8-Isoprostane – alkaline phosphatise conjugate and standard. Also supplied were assay diluent (TRIS Buffer, Trizma base, sodium azide, sodium chloride, Zinc chloride, Magnesium chloride, and albumin), calibrator diluent for plasma (DEA Buffer – containing diethanolamine and magnesium chloride) and calibrator diluent for cell culture, AP wash buffer (containing Trizma base, Polyoxyethylene (23) lauryl ether (Brij 35) and nonyl phenol), 5 pNitrophenyl Phosphate (pNPP) tablets, colour reagent A and B (dye), stop solution and plate covers.

Sample collection

Blood was drawn into a heparinised vacutainer which was centrifuged at 1 000 x g for 10 minutes. The plasma was removed and placed in a cryotube with 0.005% BHT and stored at -70°C.

Reagent preparation

The AP wash buffer was diluted with distilled water to provide 750 ml of wash buffer. One vial of TRIS buffer was diluted to 90 ml and the DEA buffer was diluted to 25 ml, both with distilled water.

A pipette tip was equilibrated with ethanol and 100 µl of the 8-Isoprostane standard was transferred to a tube labelled bulk standard and diluted with 900 µl distilled water. The 8-Isoprostane standards were prepared as follows: for the first standard, a 100 µl of the bulk standard was diluted with 900 µl of buffer in tube no 1. Then 500 µl of buffer was placed into the remaining seven numbered Eppendorf tubes. Following this, a 500 µl of the stock from tube no. 1 was added to the first tube no. 2 and well mixed. Then 500 µl of the solution in tube no 2 was removed and placed into tube no 3. The procedure was repeated until 500 µl from tube no 7 was placed into tube no 8. The final concentrations of the standards ranged from 3000 pg/ml to 23.4 pg/ml.

The kit provides optional dyes to allow one to easily see in which wells the 8-Isoprostane conjugate and the polyclonal antiserum had been placed while pipetting. The dyes were diluted with 6 ml of TRIS buffer and 60 µl each of the respective dyes

Alkaline hydrolysis

To measure both free and bound 8–isoprostane, alkaline hydrolysis of the plasma sample was required. A 200 µl sample of plasma was aliquotted into a test tube along with an equal volume of 15% potassium hydroxide in distilled water. The samples were then incubated at 40 °C for 60 minutes. Ethanol containing 0.01% BHT was added to make 2 times the sample volume and the samples were vortexed and cooled at 4 °C for 10 minutes. The proteins precipitated by this process were removed by centrifugation at 1 500 x g for 5 minutes. The supernatant was placed in a clean test tube and the ethanol evaporated under a stream of nitrogen until the sample was dry.

The sample was then re-suspended in 200 µl of TRIS buffer.

Assay

Firstly, a 100 µl of TRIS buffer was pipetted into the two non-specific binding (NSB) wells, and 50 µl of TRIS buffer was added to the B_0 wells. A 50 µl sample of either standard or sample was added to duplicate wells. A 50 µl of tracer and antiserum was added to each well, except to the Total Activity (TA) and Blank wells. The NSB wells received only tracer and not antiserum. The plate was then covered and incubated for 1 hour at room temperature.

Following incubation, the pNPP tablets provided were dissolved in 25 ml DEA buffer. The plate was then washed 5 times with the AP wash buffer. Then pNPP solution (200µl) was added to each well. At this point, a 5 µl of tracer was added to the TA well. The plate was covered and left to develop for 60 minutes.

The absorbance of the plate was then determined on the spectrophotometer plate reader at 405 nm absorbance, when the blank (B_0) readings were between 0.3 and 0.8.

Calculations

The blank was subtracted from all the wells, the NSB and B_0 wells were averaged and the NSB average subtracted from the B_0. The $\%B/B_0$ was then calculated by dividing the average absorbance (%B) from the samples by the corrected blank B_0.

$$NSB - B_0 = \text{corrected maximum binding } B_0 \text{ c}$$

$$\text{8-Isoprostane pg/ml} = \frac{(\text{Sample A} - \text{NSB})}{B_0 \text{ c}}$$

A curve of the standards was created in Excel by plotting the logit of the $\%B/B_0$ against the concentration of the standards on a log x-axis. A log regression trend line was then fitted and the equation of the line (Figure 19) was used.

$$\text{Logit}(B/B_0) = \ln(B/B_0/(1-B/B_0))$$

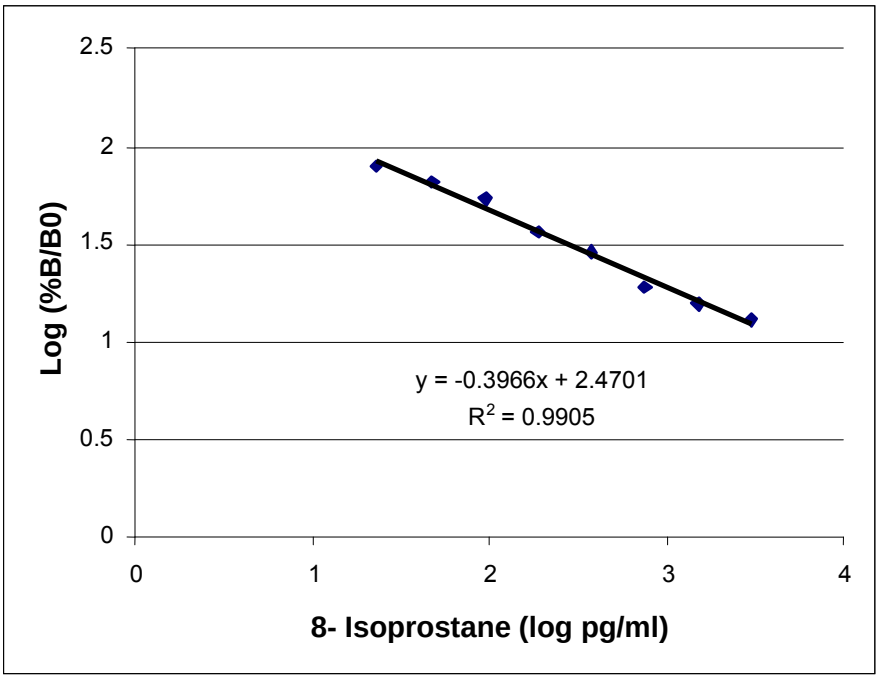


Figure 19: 8-Isoprostane log standard curve

3.2.3 Statistics

Results were assessed for compatibility with having been drawn at random from normally distributed populations by means of histograms and the Shapiro–Wilk test. The Shapiro-Wilk test is used to ascertain normal distribution for data of fewer than 2

000 observations. This test calculates a W statistic, which tests whether a random sample comes from a normal distribution. An unpaired t-test was used to compare the means of two groups if the data met with the requirements for a normal distribution. Where the data were not normally distributed, transformations were used along with non-parametric tests. Student's t-test and Wilcoxon-Mann-Whitney test were used to compare the means of different groups. The Wilcoxon-Mann-Whitney test was chosen as the most powerful non-parametric test for comparisons of two groups where the sample data could not be assumed to be normally distributed. The test ranks the data in terms of closest and farthest from the median to determine if there is a difference between population medians. Where possible (if data complied with requirements of the test) a one-way ANOVA was used to compare both, exposure status and HIV, at the same time.

3.3 Results

3.3.1 Total erythrocyte glutathione

The Shapiro-Wilk test was used to test the data for consistency with having been drawn at random from a normally distributed population (Table 26). Following the rejection of the null hypothesis that the data were consistent with a normal distribution of $p = 0.002$, a histogram was constructed to examine the distribution (Figure 21). The histogram showed one outlier which was removed from the data set. The Shapiro-Wilk test on the new data was consistent with a normal distribution of $p = 0.148$ (Table 26).

Table 20: Shapiro–Wilk test for normal distribution of GSH data.

	Obs	***W***	***V***	***z***	***P***
GSH before	127	0.96566	3.444	2.778	0.00274
GSH after removal of the outlier	126	0.98400	1.594	1.047	0.14758

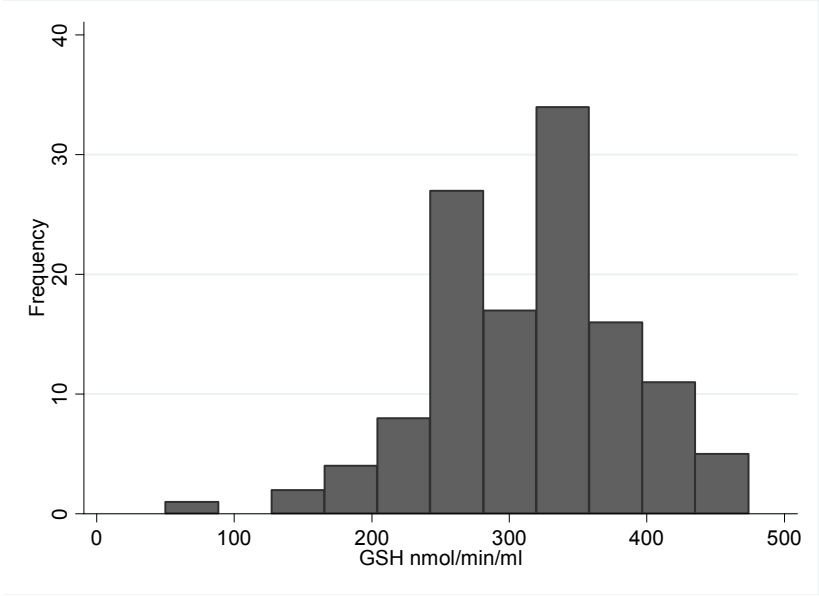


Figure 20: Histogram of GSH levels in all participants

The total number of the miners when compared to the two groups created by HIV status does not add to the same total. This is due to the fact that one miner did not know his HIV status (Table 27).

Using a one-way ANOVA to compare the means of the four groups, there was no significant difference between miners and controls and HIV-positive and HIV-negative participants. The ANOVA results should be treated with caution, as the Bartlett's test for equal variances bordered on significance ($p = 0.051$). This suggests that one or more of the groups had a variance that was almost different from the others. A Student's t-test indicated no significant difference between miners and controls ($p = 0.11$), confirming the ANOVA results. A Student's t-test comparing HIV-positive and HIV-negative participants also showed no significant difference due to HIV status ($p = 0.6067$) (Table 27).

Table 21: Description of the erythrocyte GSH levels (µM) uncorrected for haemoglobin in the different groups in the study.

Participants	N	Mean	SD	Median	IQR	Range
Total	125	325.53	71.48	322.90	266.77 – 359.35	50.00 – 473.79
Miners	63	328.87	76.03	335.65	277.93 – 380.00	50.00 – 472.26
HIV+ Miners	22	327.87	98.15	346.28	280.34 – 387.09	50.00 – 472.26
HIV- Miners	40	328.55	63.01	327.90	268.89 – 371.75	191.03 – 458.06
Controls	62	301.98	64.34	314.79	253.79 – 345.65	151.73 – 473.79
HIV+ Controls	28	297.91	68.75	323.67	244.16 – 346.95	151.73 – 408.97
HIV- Controls	34	305.33	61.31	291.15	253.79 – 340.43	212.60 – 473.79

Using an unpaired Students t-test, there was no significant difference in total GSH levels between smokers and non-smokers (p = 0.4021) (95% CI - 45.13 – 18.22). The small number of smokers may have limited the ability of the study to find a difference due to smoking (Table 28).

Table 22: GSH levels (µM) in smokers and non-smokers.

Participants	n	Mean	SD	Median	IQR	Range
Non-smokers	100	312.84	68.97	318.79	266.48 – 354.78	50.00 – 472.26
Current smokers	25	326.29	81.37	335.65	296.77 – 364.79	151.73 – 473.79

A Student's t-test of the GSH levels showed a significant difference in GSH levels between miners with and without silicosis (p = 0.0421). GSH levels were decreased in miners with silicosis compared to miners with no silicosis diagnosed (Table 29).

Table 23: GSH levels (µM) in miners with and without silicosis.

Participants	n	Mean	SD	Median	IQR	Range
Non-silicotics	43	344.96	65.29	346.20	309.56 – 380.97	180.00 – 472.26
Silicotics	17	305.53	68.58	313.87	266.19 – 351.73	189.35 – 397.74

GSH levels were consistent with a normal distribution, but age was not, thus a Spearman's correlation was again used to determine whether there was a relationship between GSH levels and age.

Table 24: Spearman's correlation of age and GSH levels.

	r*	p
Age	0.0224	0.8045

* R = correlation coefficient

No correlation between GSH level and age was found and the relationship was not significant (Table 30).

A Pearson's correlation was used to test for a trend in GSH levels with increasing years of exposure as both variables were consistent with a normal distribution.

Table 25: Pearson's Correlation of years of exposure and GSH levels.

	Corr co	p
Years of exposure	-0.1376	0.2820

Corr co = correlation coefficient

No correlation between GSH levels and years of RCS-exposed work was found (Table 31).

3.3.2 Glutathione peroxide GPx1

To determine the analysis methods to be used on the GPx1 data, the distribution of the data was determined. The GPx1 data were not consistent with a normal distribution when analysed using the Shapiro-Wilk method (p = 0.00020). When transformed as the logarithm, there was no improvement in the distribution (Table 20).

Table 26: Shapiro–Wilk test for normal distribution of GPx data.

	Obs	W	V	z	P
GPx original	127	0.95220	4.826	3.537	0.00020
Log GPx	127	0.95480	4.564	3.412	0.00032

The histogram of the untransformed GPx data shows a distribution skewed to the right. Based on this distribution, non-parametric data analysis techniques were used for further analysis.

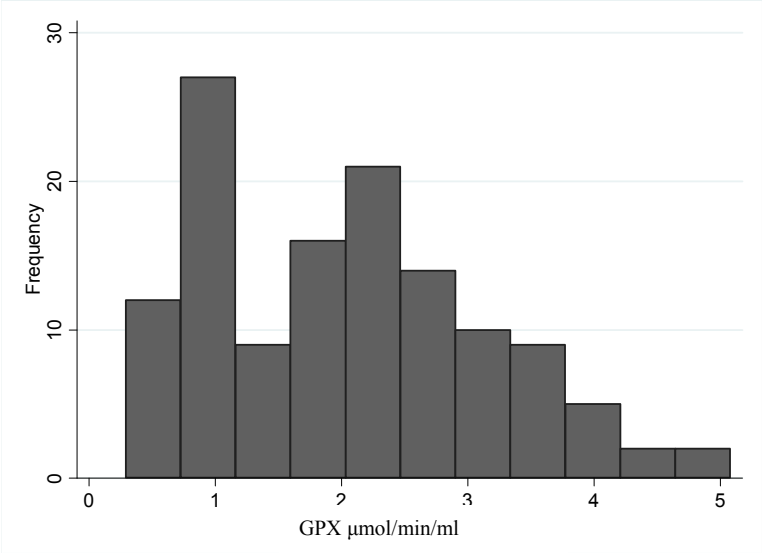


Figure 21: Histogram of GPx values

The GPx1 values for all the exposure groups show variability with a range from 0.29µmol/min/ml to 5.08µmol/min/ml. The data are not normally distributed (Figure 20), and therefore the mean values are influenced by the outliers and are higher than the medians (Table 21).

Table 27: Description of GPx levels in µmol/min/ml for all participants.

Participants	Obs	Mean	SD	Median	IQR	Range
Total	127					
Miners	64	1.26	0.64	0.94	0.74-1.86	0.29 – 2.77
HIV+ Miners	22	1.33	0.63	1.18	0.83 – 1.88	0.29 – 2.49
HIV- Miners	40	1.20	0.64	0.89	0.74 – 1.75	0.44 – 2.77
Controls	63	2.77	0.93	2.68	2.15 – 3.42	0.82 - 5.08
HIV+ Controls	29	3.16	1.08	3.39	2.43 – 3.89	0.82 – 5.08
HIV- Controls	35	2.45	0.64	2.48	1.99 - 2.91	1.27 – 3.94

The medians of the RCS-exposed groups are significantly lower than the RCS non-exposed groups (Wilcoxon rank sum p = 0.000). The maximum values for the RCS-exposed participants are lower than the RCS non-exposed controls (Table 21).

A Wilcoxon rank sum for miners, GPx1 activity resulted in a non-significant difference (p = 0.1758). There was no significant difference in GPx activity in the HIV-positive and HIV-negative miners. The controls showed a significant difference in GPx activity between HIV positive and negative participants (p = 0.0029). (Table 21)

When the GPx activity was compared for smokers and non-smokers (Wilcoxon rank sum p = 0.94) there was no significant difference, and this lack of difference remained non-significant even when stratified by exposure status (Table 22).

Table 28: GPx levels (µmol/min/ml) in smokers and non-smokers.

Participants	***n***	***Mean***	***SD***	***Median***	***IQR***	***Range***
Non-smokers	100	1.99	1.05	2.02	0.96 -2.64	0.29 – 4.95
Current smokers	26	2.10	1.29	1.96	0.77 – 3.34	0.44 – 5.08

When GPx levels in miners with and without silicosis were compared, there was no significant difference in GPx activity (Wilcoxon rank sum p = 0.8632) (Table 23). Miners with silicosis were stratified into two groups based on stage of silicosis using the ILO scores from the most recent radiographs (an ILO score > 1 / 2 was characterised as silicotic). There was no significant difference in GPx activity between the two groups (Wilcoxon rank sum p = 0.5139). This analysis was limited as there were only four participants with severe silicosis in which changes may be expected.

Table 29: GPx levels (µmol/min/ml) in gold miners with and without silicosis.

Participants	*n*	*Mean*	*SD*	*Median*	*IQR*	*Range*
Non-silicotics	43	1.27	0.66	0.94	0.74 – 1.88	0.29 – 2.77
Silicotics	17	1.23	0.64	1.04	0.74 - 1.84	0.44 – 2.35

Both GPx activity and age were not consistent with a normal distribution, thus a Spearman's correlation was used to determine whether there was a relationship between GPx levels and age in this study.

Table 30: Spearman's correlation of age and GPx levels.

	r*	**p**
Age	-0.1318	0.1412

* r = Correlation Coefficient

No correlation between GPx level and age was found; therefore this relationship was not statistically significant (Table 24).

A Spearman's correlation was again used to test for a trend in GPx levels with increasing years of exposure as both variables were not normally distributed.

Table 31: Correlation of years of exposure and GPx levels.

	r*	**p**
Years of exposure	0.0539	0.6749

* r = Correlation Coefficient

No correlation was found between GPx levels and years of RCS-exposed work, and this relationship was again not significant (Table 25).

3.3.3 Erythrocyte Glutathione S Transferase

The distribution of the GST data was not consistent with a normal distribution with a p value of 0.0000 for the Shapiro-Wilk test. After log transformation, the data were normally distributed (p = 0.5) (Table 32).

Table 32: Shapiro-Wilk W test for normal data.

	Obs	*W*	*V*	*z*	*P*
GST	126	0.92035	7.988	4.668	0.00000
Log GST	126	0.99016	0.987	-0.030	0.51192

A histogram of the GST levels was plotted to examine the data for outliers and distribution. The distribution approximated a log distribution (Figure 22).

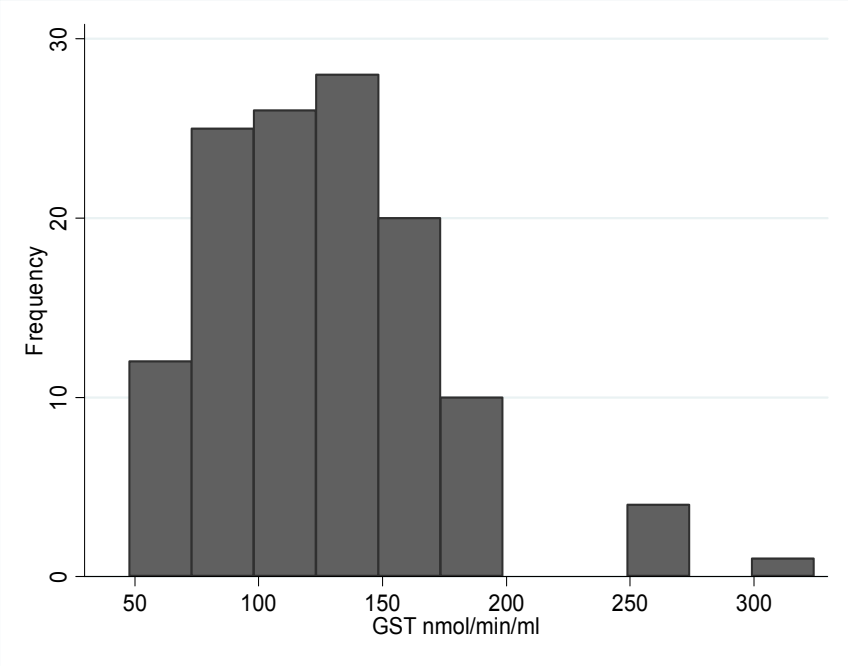


Figure 22: Histogram of GST activity for all participants

The GST activities for each of the groups in this study are provided in Table 33 Trends were seen in the means and medians of the groups with the controls who had higher levels of activity of GST with HIV-negative miners with the highest mean and median GST level (Table 33).

A t-test was conducted on the log-transformed GST data and there was no significant difference found between miners and controls (p = 0.45930 (95% CI - 0.08 – 0.17). A non-parametric Wilcoxon rank sum test on the untransformed data gave similar results (p = 0.5517). A one-way ANOVA was used to compare the HIV status and exposure status, using the log-transformed data and no significant difference was found (p = 0.8599) between the HIV-positive and HIV-negative controls and miners (Table 33). The test for equal variance was non-significant (p = 0.603).

Table 33: GST levels (nmol/min/ml) by HIV and exposure status for all participants.

Participants	***n***	***Mean***	***SD***	***Median***	***IQR***	***Range***
Total	126	127.04	46.09	123.20	93.38 – 150.69	47.71 – 324.20
Miners	63	124.64	45.88	125.20	91.45 – 146.44	47.71 – 324.20
HIV+ Miners	22	124.54	53.32	115.31	91.45 – 143.14	47.71 – 254.47
HIV- Miners	40	124.62	42.61	126.29	92.48 – 147.50	58.64 – 270.38
Controls	63	129.44	46.54	123.10	93.38 – 157.34	63.67 – 324.20
HIV+ Controls	28	130.31	38.84	124.93	100.98 – 164.17	63.67 – 194.48
HIV- Controls	35	128.7	52.45	123.00	91.26 – 150.69	65.79 – 324.20

There was no significant difference between smokers and non-smokers (p = 0.4429), although there was a trend towards lower levels of GST in smokers. The small number of smokers and the fewer cigarettes smoked may have limited the ability of the study to find a difference due to smoking, while there appeared to be lower values in smokers (Table 34).

Table 34: GST levels (nmol/min/ml) in smokers and non-smokers.

Participants	**n**	**Mean**	**SD**	**Median**	**IQR**	**Range**
Non-smokers	101	128.81	47.97	126.57	93.38 – 150.69	47.71 – 324.20
Current smokers	25	119.87	37.53	114.60	97.63 – 146.44	58.64 – 183.89

A Student's t- test of their GST activity showed no significant difference between miners with and without silicosis ($p = 0.7135$) (Table 35). When comparing miners with early silicosis to those with severe silicosis, there was no significant difference in GST activity ($p = 0.8592$) (Student's t-test results not shown). ILO scores were used to separate gold miners with silicosis into those with early and late silicosis.

Table 35: GST levels (nmol/min/ml) in miners with and without silicosis.

Participants	n	Mean	SD	Median	IQR	Range
Non-silicotics	43	126.12	47.42	123.10	89.46 – 149.10	47.71 – 270.38
Silicosis	17	120.44	45.78	125.24	93.43 -139.16	62.62 – 252.49

Both erythrocyte GST activity and age were not consistent with a normal distribution, thus a Spearman's correlation was used to determine whether there was a relationship between GST levels and age (Table 36).

Table 36: Spearman's correlation of age and GST levels.

	r*	*p*
Age	0.1484	0.0973

*r = rho correlation coefficient

No correlation between GST level and age was found and the relationship was not significant at $p < 0.05$.

Spearman's correlation was again used to test for a trend in GST levels with increasing years of exposure (Table 37).

Table 37: Spearman's correlation of years of exposure and GST levels.

	*r**	*p*
Years of exposure	0.1381	0.2803

*r = rho correlation coefficient

No correlation between GST levels and years of RCS-exposed work was found.

3.3.4 Luminol-enhanced chemiluminescence

The Shapiro-Wilk test was used to test the data for consistency with a normal distribution (Table 38). Following the rejection of the null hypothesis that the data were consistent with a normal distribution ($p = 0.002$), a histogram was constructed to examine the distribution (Figure 23).

Table 38: Shapiro-Wilk W test for normal data.

	Obs	*W*	*V*	*z*	*P*
Chemiluminescence	79	0.90388	6.530	4.108	0.00002
Log Chemiluminescence	79	0.96330	2.493	2.001	0.02272

The histogram of the chemiluminescence results shows that the data are skewed to the left (Figure 23), which suggests that a log transformation will transform the data to being consistent with a normal distribution (Table 38). The Shapiro-Wilk test indicates some improvement, but the distribution is still not consistent with a normal distribution. Thus all hypothesis tests conducted are non-parametric tests.

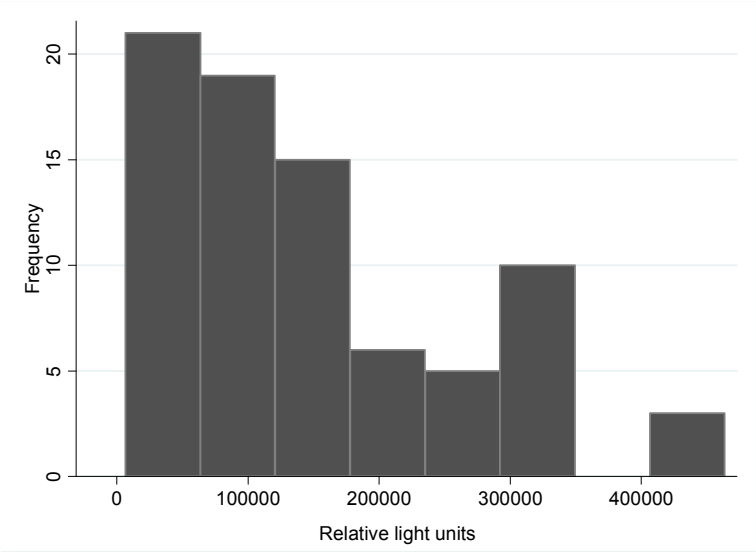


Figure 23: Histogram of chemiluminescence results in relative light units (RLU) and frequency

No significant difference was found between the LECL of the miners and controls (rank sum p = 0.7528). When the LECL of HIV-positive and HIV-negative participants were compared, there was a significant increase in the RLU of the HIV-positive participants (rank sum p = 0.0003). When this was stratified by exposure status, the results remained statistically significant (rank sum controls p = 0.0021; rank sum miners p = 0.0449) (Table 39)

Table 39: RLU levels by HIV and exposure status for all participants.

Participants	n	Mean	SD	Median	IQR	Range
Total	79	149 603	111 345	119 774	61 304 - 223 272	6 576 - 463 618
Miners	36	144 612	105 299	120 830	58 260 - 22 6740	19640 – 417 564
HIV+ Miners	16	185 339	185 339	184 384	75 285 - 265 294	19 640 - 417 564
HIV- Miners	20	112 030	87 848	94 303	35 421 – 15 5199	21 122 - 316 439
Controls	43	153 782	117 240	119 136	61 304 - 210 512	6 576 – 463 618
HIV+ Controls	18	213 777	122 381	171 249	119 136 – 320 836	68 795 – 463 618
HIV- Controls	25	110 584	93 819	78 573	43 336 – 150 049	6 576 – 328 511

There was a trend towards higher mean and median levels of RLU in neutrophils isolated from smokers (Table 40), but when a Wilcoxon rank sum test was carried out,

the difference was not significant (p = 0.5044). There was a wide range of RLU levels for both smokers and non-smokers suggesting a large amount of individual variation.

Table 40: Comparison of RLU results by smoking status.

Participants	n	Mean	SD	Median	IQR	Range
Non-smokers	65	146 477	112 707	106 724	61 305 – 203 646	6 576 – 463 618
Current smokers	14	164 117	107 583	148 868	68 795 – 253 439	21 123 – 329 357

There was a difference in medians between those with and without radiologically diagnosed silicosis with those with silicosis appearing to have more LECL but a Wilcoxon Rank sum test indicated no significant difference (p = 0.8602) (Table 41).

Table 41: RLU levels in miners with and without silicosis.

Participants	n	Mean	SD	Median	IQR	Range
Non-silicotics	26	142 711	97 780	109 532	63 249 – 230 209	21 123 – 323 122
Silicotics	7	145 070	89 900	158 740	75 266 – 169 294	22 491 – 309 190

The RLU of LECL and age were not consistent with a normal distribution, thus a Spearman's correlation was used to determine whether there was a relationship between RLU levels and age (Table 42).

Table 42: Spearman's correlation of age and RLU levels .

	r*	**p**
Age	0.0158	0.8901

*r =Rho = correlation coefficient

No correlation between chemiluminescence and age was found.

Spearman's correlation was again used to test for a trend in RLU levels with increasing years of exposure.

Table 43: Correlation of years of exposure and RLU levels.

	*r**	*p*
Years of exposure	0.1268	0.4613

*r =Rho = correlation coefficient

No correlation between RLU levels and years of RCS-exposed work was found (Table 43)

3.3.5 8-Isoprostane

To investigate the distribution of the 8-Isoprostane results, a Shapiro-Wilk test and histogram was plotted.

Table 44: Shapiro-Wilk W test for normal data.

	Obs	*W*	*V*	*z*	*P*
8-Isoprostane	120	0.87	12.92	5.73	<0.01
8-Isoprostane transformed	120	0.97	2.48	2.04	0.02

Both the histogram and the Shapiro-Wilk test indicate that the 8-Isoprostane data are not normally distributed (Table 44 and Figure 24). The histogram shows a large tail to the right. Transformation of the data with log, square, reciprocal, square root and reciprocal square root did not improve the distribution of the data sufficiently to conduct parametric tests.

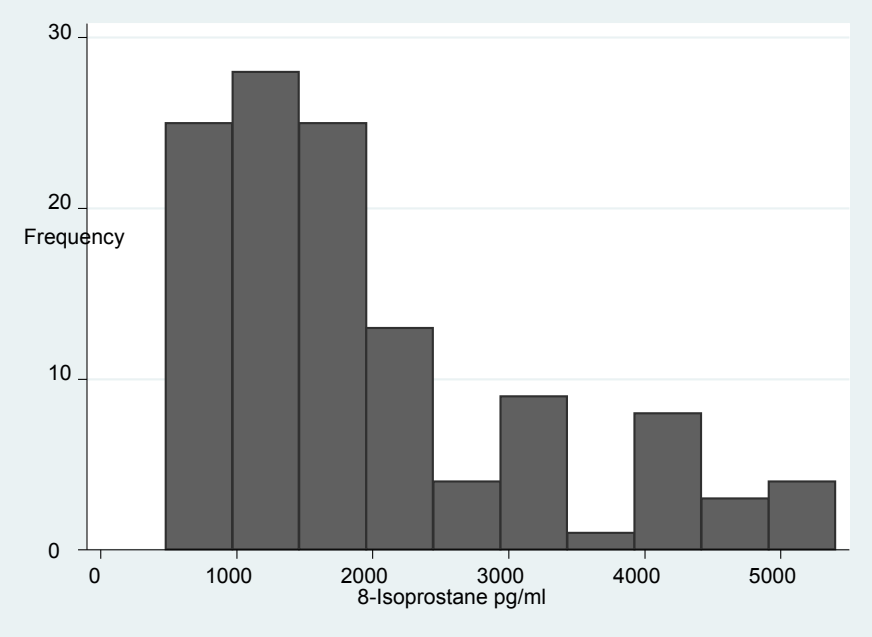


Figure 24: Histogram of all participants' 8-Isoprostane levels

The mean 8-Isoprostane levels indicate a small increase in 8-Isoprostane amongst the HIV-positive miners and the controls. The mean of the controls was also increased compared to that of the miners (Table 45).

Table 45: 8-Isoprostane levels (pg/ml) by HIV status and exposure for all participants

Participants	*n*	*Mean*	*SD*	*Median*	*IQR*	*Range*
Total	120	1 974.16	1 224.13	1 592.28	1 060.90-2 415.99	475.7 – 5 4
Miners	60	1 255.12	566.88	1 134.92	858.34 – 1 525.44	475.70 – 4
HIV+ Miners	23	1250.72	443.17	1 258.43	852.40 – 1 568.01	475.7 – 2 0
HIV-Miners	36	1263.05	645.66	1 087.65	847.89- 1 525.21	538.19 – 4
Controls	60	2693.20	1 284.32	2 396.40	1 691.61 – 3 526.29	779.77 - 5
HIV+ Controls	27	2831.59	1 223.30	2 923.33	1 725.08 – 3 659.52	793.43 – 5 392.74
HIV- Controls	33	2579.42	1 339.33	2 008.91	1 651.46 – 3 284.65	779.77 – 5 400.317

Based on the poor validation results for 8-Isoprostane, further analysis of 8-Isoprostane was not carried out (see validation results in section 3.3.6).

3.3.6 Validation of results

To validate the 8-Isoprostane results, 18 samples were sent to Professor Val Vallyathan's laboratory at NIOSH in the USA where the same tests were performed on aliquots of the same samples using the same kits as were used in this study. The samples were diluted 20 and 40 times as they were too concentrated to assay undiluted. The assay kit recommends assaying at two dilutions to look for interference in the assay.

Table 46: 8-Isoprostane.

	Mean pg/ml	***median***	***SD***	***Range***
20 times dilution	377.98	251.27	357.62	62.65 – 1234.69
40 times dilution	503.62	309.15	403.67	63.2 – 1259.79

Student's t-test 22 degrees of freedom (p = 0.3736)

Overall, when comparing the means using the log-transformed data in a paired Student's t-test there was no significant difference between the two dilutions (Table 46). In a non-parametric paired test, the Wilcoxon matched pairs sign rank test, there was a significant difference between the two groups; indicating that they are from different distributions.

This validation experiment suggests that there is interference in the samples and that the determination of 8-Isoprostane is not accurate. Increasing the dilution appeared to increase the levels of 8-Isoprostane in the sample. The dilution needed to measure the 8-Isoprostane levels in the samples indicates that where more dilution was used the levels were artifactually increased.

3.3.7 Total antioxidant status

The Shapiro-Wilk test for normal data was significant thus the data was not normally distributed (Table 47), and transformation of the data with log, square, reciprocal, square root and reciprocal square root did not improve the distribution of the data sufficiently to enable the performance of parametric tests.

Table 47: Shapiro-Wilk W test for normal data.

	Obs	*W*	*V*	*z*	*P*
TAS	125	0.97383	2.607	2.151	0.01573

The histogram suggests only a small deviation from normality (Figure 25). Based on these results, it was decided to treat the data as if they followed a normal distribution for the comparison of means hypothesis test. But the data are unsuitable for a simple linear regression analysis of the TAS results.

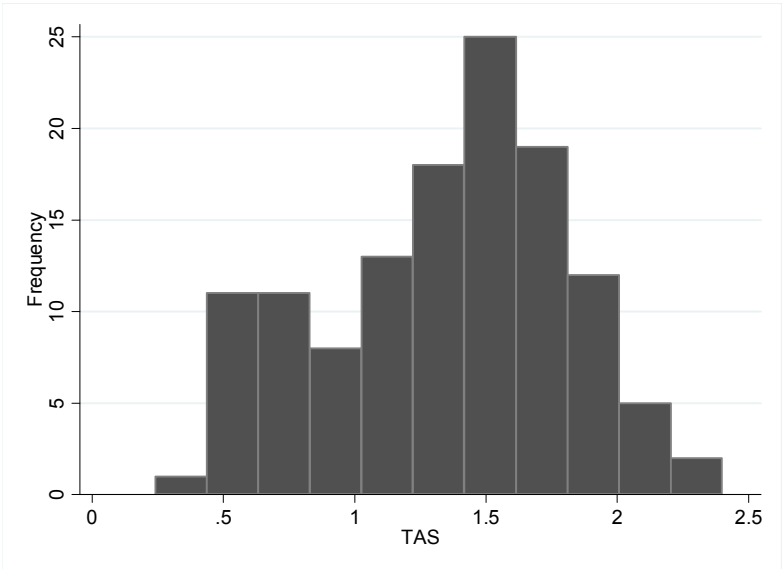


Figure 25: Histogram of total antioxidant status results for all participants

There was a significant difference between TAS in the miners and the controls (p = 0.0006) using an unpaired unequal variance t-test. The controls unexpectedly showed a lower level of TAS than the miners did. When HIV status was taken into account,

the unexposed HIV-positive controls appeared to be the cause of the lower level of TAS in the controls - there was a significant difference between the means ($p < 0.01$). No difference could be shown between the HIV-negative controls and HIV-negative miners using an unpaired unequal t-test p = (0.72) (Table 48).

Table 48: Description of TAS results in mmol/l by HIV status and exposure.

Participants	***n***	***Mean***	***SD***	***Median***	***IQR***	***Range***
Total	125	1.34	0.46	1.43	1.05 – 1.65	0.24 – 2.4
All Miners	63	1.48	0.25	1.52	1.34 – 1.62	0.71 – 1.92
HIV+ Miners	23	1.45	0.29	1.51	1.29 – 1.62	0. 71 – 1.91
HIV- Miners	39	1.49	0.22	1.53	1.37 – 1.62	0.94 – 1.92
All Controls	62	1.2	0.57	1.14	0.65 – 1.71	0.24 – 2.4
HIV+ Controls	27	0.78	0.37	0.65	0.53 – 0.94	0.24 – 2.1
HIV- Controls	35	1.52	0.48	1.64	1.19 -1.95	0.5 – 2.4

There was no significant difference in the TAS levels in smokers compared to those of non-smokers, although there was a trend towards lower TAS levels in smokers. The power of this analysis would have been increased with a larger number of smokers (Table 49).

Table 49: Comparison of TAS results by smoking status.

Participants	**n**	**Mean**	**SD**	**Median**	**IQR**	**Range**
Non-smokers	100	1.37	0.46	1.43	1.05 – 1.65	0.24 – 2.4
Current smokers	25	1.23	0.29	1.51	1.29 – 1.62	0. 71 – 1.91

t-test 123 degrees of freedom (p = 0.2051)

No significant difference in the total antioxidant status in miners with and without silicosis was shown using a t-test (p = 0.92) (Table 50). When comparing the miners with more severe silicosis to those with simple silicosis, there was still no significant difference, although this evaluation was limited by the small number of participants with more severe silicosis (t-test silicosis: p = 0.30).

Table 50: TAS in miners with and without silicosis.

Participants	*n*	*Mean*	*SD*	*Median*	*IQR*	*Range*
Non-silicotics	42	1.50	0.23	1.48	1.37 – 1.62	0.94 – 1.92
Silicosis	17	1.50	0.27	1.58	1.52 – 1.62	0.71 – 1.83

The TAS was approximately normally distributed, but age was not, thus a Spearman's correlation was used to determine whether there was a relationship between TAS and age (Table 51).

Table 51: Spearman's correlation of age and TAS levels.

	r	**p**
Age	0.10	0.29

*r = rho correlation coefficient

No correlation between TAS and age was found, which suggests that other factors play a greater role in determining levels of antioxidants.

Spearman's correlation was again used to test for a trend in TAS with increasing years of exposure.

Table 52: Correlation of years of exposure and TAS.

	r	**p**
Years of exposure	-0.01	0.92

*r = rho correlation coefficient

No correlation between TAS and years of RCS-exposed work was found, which confirms that there was no significant difference between miners with and without silicosis (Table 52).

3.3.8 Summary of Results

The overall results from the oxidative stress biomarkers (GSH, GPx, GST, TAS, and ROS) section have been summarised in Table 53. 8-Isoprostane results are not included in this table due to challenges in the methods implemented for its measurement.

The biomarkers of oxidative stress were tested for a relationship to RCS exposure, effect of HIV status, smoking and effect of Silicosis diagnosis among miners

- ROS measured by chemiluminescence as RLU showed no significant difference in levels between RCS exposed gold miners and RCS unexposed controls. While a significant difference between HIV positive and negative participants in both miners and controls was measured. Neither smoking in miners and controls nor silicosis in miners were observed to effect RLU levels.

Table 53: Summary of Oxidative stress markers.

	ROS		GSH		GPx		GST		TAS	
	n	Mean RLU	n	Mean µM	n	Mean µmol/min	n	Mean nmol/min	n	Mean mmol/l
Exposure										
Miners	36	144 612	63	329	63	1.26	63	125	63	1.48
Controls	43	153 782	62	302	64	2.77	63	129	62	1.2
P value		0.459		0.1100		0.000*		0.459		0.0006*
HIV status										
HIV+ miners	16	185 339	22	327.87	22	1.33	22	124.54	23	1.45
HIV- miners	20	112 030	40	328.87	40	1.2	40	124.62	39	1.49
P value		0.0007*		0.955		0.4446		0.9948		0.6583
HIV+ controls	18	213 777	28	297.9	29	3.16	28	130.31	23	0.78
HIV- controls	25	110 584	34	305.3	35	2.45	35	128.7	35	1.52
P value		0.0032*		0.656		0.0018*		0.8928		0.0000*
Smoking										
Current smokers	25	164 117	25	326	25	2.10	25	120	25	1.23
Non-smokers	101	146 477	100	313	100	1.99	100	129	100	1.37
P value		0.4429		0.4021		0.94		0.443		0.149
Silicosis										
Non silicotics	17	142 711	43	345	43	1.27	17	126	42	1.50
Silicotics	43	145 070	17	306	17	1.23	43	120	17	1.50
P value		0.8602		0.042*		0.8632		0.7135		0.92

*Significant difference at p = 0.05.

- Total erythrocyte GSH measurements resulted in no significant differences in levels in miners compared to controls. There were no observed differences in total GSH levels in both miners and controls with and without HIV. No effect due to smoking was observed. While total GSH was the only biomarker of oxidative stress to show a significant difference between silicotic and non-silicotic gold miners.
- GPx showed a significant decrease in activity levels between the RCS exposed gold miners and the unexposed controls. While there were no observed differences in HIV positive and negative miners, controls showed significant differences between HIV positive and negative participants. Smoking appeared to present no significant effect on GPx activity along with silicosis in miners.
- GST showed no significant difference between controls and RCS exposed miners and similarly no significant difference in activity between HIV positive and negative miners and controls. Neither smoking nor silicosis were observed to have an effect on GST activity.
- TAS showed a significant decrease in the controls compared to the miners which was unexpected. TAS similar to GPx showed no significant difference in miners with and without HIV but did show a significant difference in HIV and negative controls. TAS levels showed a non- significant reduction in non-smokers compared to smokers. No significant difference was shown between miners with and without silicosis.

In summary GPX appeared useful as a possible biomarker for harmful silica exposure.

3.4 Discussion

Oxidative stress has been demonstrated in a number of studies to play a role in the development of silicosis (Gupta and Kaw, 1982; Daniel *et al.*, 1993; Schins *et al.*, 1995a, Vallyathan *et al.*, 1997; Vallyathan and Shi, 1997; Vallyathan *et al.*, 1998). A relationship between lung levels of antioxidant enzymes and those in erythrocytes has been observed (Minami *et al.*, 1982). Chronic inflammation in the lung has also been shown to result in changes in the erythrocyte antioxidant system (Rahman and MacNee, 2000), and it is suggested that erythrocytes possibly act as a circulating

antioxidant system for the organs (Borm *et al.*, 1986). All this evidence indicates that by measuring metabolites of ROS and antioxidants in blood may provide information on the level of toxic risk to the lung tissue due to inhaled RCS particles.

Glutathione peroxidase

GPx is an important antioxidant enzyme and any change in activity could result in a disturbance of the oxidative balance and result in disease. The enzyme activity of GPx has been shown to depend on a continuous flow of substrates and any factors that reduce their availability can result in a loss of GPx activity. For example, Se deficiency has been reported to decrease GPx activity (Rahman *et al.*, 1999 and Nadif *et al.*, 2001).

In the present study, erythrocyte lysate from the gold miners with and without silicosis demonstrated significantly lower GPx activity compared to that of the healthy controls indicating some impairment of the antioxidant system. A number of studies have measured GPx activity in erythrocyte lysate or plasma as a possible biomarker for lung damage due to oxidative stress in RCS-exposed workers (Engelen *et al*., 1990; Borm *et al*., 1986; Borm *et al*., 1987; Orman *et al*., 2005 and Zhou *et al*., 1999). For example, similar to our study Mallika *et al*., (2000) have reported a decrease in erythrocyte GPx activities in masons exposed to RCS compared to non-exposed workers. On the other hand, Perrin-Nadif et al., (1996) investigated French coal miners and have found that erythrocyte GPx activitiy was similar between the surface and underground miners

The effect of silicosis on erythrocyte GPx activity in RCS exposed miners was also investigated in the present study and it was found that there was no significant difference in the GPx activity in miners with and without silicosis. This finding supported those reported by Engelen *et al.*, (1990) who conducted a cross-sectional study of Belgian coal miners, with and without pneumoconiosis. They found no significant difference in levels of erythrocyte GPx activity between the controls and individuals with pneumoconiosis. Similar observations were also made by Perrin-Nadif *et al*., (1996) with retired French coal miners with and without CWP as well as by Borm *et al*., (1986) and Borm *et al*., (1987) with Dutch hospital controls and silicotic patients. On the other hand, contrary to these findings and those observed in

the present study, Zhou *et al*., (1999) reported a significant decrease in erythrocyte GPx activity in Chinese hospital silicosis patients compared to control subjects, but most studies agreed with the results of this study.

When number of years worked underground was used as a proxy for cumulative RCS exposure in this study, no association could be found with GPx levels confirming similar results reported earlier by Schins *et al.*, (1997) who have suggested that the disturbance of the antioxidant balance is not cumulative making GPx an early exposure marker but not a marker for cumulative exposure.

Confounders

In our current study, age was not shown to be a confounder on the observed decrease in the erythrocyte GPx levels in RCS-exposed gold miners compared to controls confirming those reported in the literature (Bolzan *et al.*, 1997; Schins *et al.*, 1997; Schins and Borm, 1999; Babu *et al.*, 2006). Similarly, smoking has shown no confounding effect on the GPx levels and thus confirming observations reported earlier (Borm *et al.*, 1986; Borm *et al.*, 1987; Schins *et al.*, 1997; Babu *et al.*, 2006). Finally, HIV status was also found not to affect GPx activity in miners but did have an effect on the levels in controls. The impact of HIV on GPx activity reported in the literature has not been consistent: GPx activity was found to be decreased in HIV-positive patients by Favier *et al.*, (1994) while no effect due to HIV was seen on GPx levels in the study by Leff *et al.*, (1992).

Based on the significant decrease observed in the RCS exposed miners compared with the controls, and no effects on the levels due to confounders it could be proposed that GPx may be a good marker of oxidative stress induced by exposure to RCS and may therefore warrant further investigation.

Glutathione

GSH is an important antioxidant, particularly in the lungs. It has been demonstrated that RCS exposure results in an increase of ROS (Zhang *et al.*, 1999; Vallyathan *et*

al., 1998) and therefore it is expected to play an important role in lung defence against RCS-induced oxidative stress.

In the present study, the total erythrocyte GSH levels did not differ between miners and unexposed controls in both the HIV- negative and HIV- positive groups. The only similar study was conducted in coal miners where reduced, and not total, GSH levels was measured where an increase of GSH levels was reported in silicotic miners compared to unexposed controls (Borm *et al.*, 1986).

However, in the present study, the total erythrocyte GSH levels differed between miners with and without silicosis confirming the findings reported in the literature with miners with CWP (Evelo *et al.*, 1993) and with pneumoconiosis (Engelen *et al*. 1990). In contrast, a study Schins *et al*. (1997) could observe no significant difference in GSH levels between miners with and without CWP, but saw a decrease with progression in CWP. The study by Schins *et al*., 1997 was a follow up study on the same miners in the study by Engelen *et al*., 1990 and was looking for markers of progression this may account for the lack of difference in GSH levels.

Pirmohamed *et al.*, (1996) have suggested that the methodology used to measure GSH and the interval time between collection and processing may play a role in GSH levels measured. Subsequently, this may partly explain the differences reported in the literature as well as those obtained in the present study. The time between collection and processing depends on the sampling protocol and can assume to differ between studies if not explicitly controlled for.

Confounders

HIV was considered as a possible confounder as studies by Jahoor et al., (1999) and Sbrana et al., (2004) have reported decreases in levels of erythrocyte GSH in HIV-positive patients. In contrast, our study failed to show a difference in total GSH levels between HIV-positive and HIV-negative participants. Supporting our observations was a study by Pirmohamed *et al.*, (1996) who also failed to see a decrease in total erythrocyte GSH levels. The present study did not demonstrate any significant difference between GSH levels in non-smokers and current smokers, supported by the

findings of Borm *et al.*, (1987) who reported no significant effect on erythrocyte or plasma GSH due to smoking.

The effect of age on GSH levels was investigated and no correlation between age and GSH level was found confirming those reported in the study by Schins *et al.*, (1997).

Total erythrocyte GSH levels in this study have not shown differences due to RCS exposure which could have been compromised by methodological limitations. This does not rule out a possible effect of to RCS exposure on reduced GSH levels in erythrocytes and therefore may warrant further investigation..

Glutathione S-transferase

GST is an important detoxifier which assists in removing ROS, particularly hydroperoxides, by converting them into less reactive substances. GST is also responsible for the detoxification of xenobiotic substances.

In the present study, erythrocyte GST activity did not differ between miners and controls indicating that GST may not be a useful biomarker for RCS exposure. Only GST in circulating erythrocytes was measured in this study and thus, possible changes limited to the lung would not have been detected.

The effect of silicosis on erythrocyte GST activity in RCS exposed miners was investigated and a small but non-significant decrease in the mean GST activity in silicosis patients was found. The lack of a significant difference remained when comparing early-stage silicosis to more severe silicosis. The only similar study investigating GST activity in miners was completed by Evelo *et al.*, (1993) who reported a significant decrease in the erythrocyte GST activity in coal miners with early CWP when compared to matched control coal miners. Evelo *et al.*, (1993) also reported that as the CWP progressed in the miners, the GST levels in the miners with CWP, were no longer significantly different to that of the control miners.

Confounders

In our current study, no significant difference in GST activity in HIV positive and negative participants was found. Pace and Leaf, (1995) on the other hand, showed a decrease in GST activity in HIV positive children. There was a non-significant trend towards lower levels of erythrocyte GST in the current smokers in this study. This corresponds with the findings of Evelo *et al.*, (1993) and Schins *et al.*, (1997) who reported that erythrocyte GST levels in their studies were not related to smoking habits. Kondo *et al.*, (1994) reported a significant decrease in alveolar GST levels in elderly current smokers compared to non-smokers. These findings suggest that in relation to smoking, differences may be expected between alveolar GST and erythrocyte GST. Also no effect of age on GST activity was found in this study supporting the findings reported by Schins *et al.*, (1997).

The measurement of GST activity is not recommended as a potential biomarker for RCS exposure as there does not seem to be any significant variation in GST activity following exposure to RCS.

ROS measurement using chemiluminescence

RLU is a measure of ROS generated by externally stimulated, isolated polymorphonuclear cells. Although ROS play an important bactericidal role in host defence, an excess of ROS can cause tissue damage.

This study found no significant difference in RLU levels between RCS-exposed miners and healthy controls. This observation is supported by Theron *et al.*, (1994) who have suggested that RCS exposure does not play a large role in stimulated ROS release. In the latter study no correlation could also be found between years of dusty work as a proxy for cumulative RCS exposure and RLU.

The RLU of miners with and without silicosis was examined, but again no significant differences were found. However a trend towards increased levels of RLU was seen in miners with silicosis. This increase wass similar to the findings of a study by Velichkovskii *et al.*, (1990) in which they demonstrated an increased ROS production in phagocytic cells activated by RCS in silicosis patients when compared to chronic dust-induced bronchitis patients.

Confounders

It has been suggested that human neutrophils are necessary to activate HIV-1 (Pugliese *et al*., 2005). This theory was further developed to specify that only the respiratory burst of neutrophils is required by HIV (Klebanoff and Headley, 1999). Who have demonstrated that activation of HIV-1 was not observed when neutrophils lacking a respiratory burst were incubated with the virus. But clinical studies comparing HIV-positive patients with HIV-negative controls have produced conflicting results. These may have been influenced by the difference in methods used in the various studies. Ryder et al., (1988) reported an increase in oxidative burst by polymorphonuclear cells using the hydrolysis of dichlorofluorescine to a fluorescent product. Pitrak et al., (1998) found a decrease in whole blood chemiluminescence of primed phagocytes. They reported an increase in chemiluminescence of unprimed phagocytes in HIV-positive patients compared to controls.

The current study demonstrated a significant increase in the RLU of isolated neutrophils of HIV-positive participants, both miners and controls, when activated by ex-vivo RCS. No other studies have measured the effect of RCS activation of neutrophils in HIV positive and negative patients.

Smoking has been demonstrated to cause an increase in ROS measured as RLU. For example, Kalra *et al.*, (1991) and Theron *et al.*, (1994) were able to demonstrate a significant increase in the RLU of current smoking miners compared to non-smoking miners and control non-smokers. In contrast the current study did not show any significant increase in the RLU in smokers compared to non-smokers. This relationship may have been confounded by HIV which could not be adjusted due to the small number of smokers and also affected by the small number of cigarettes smoked by the participants. Finally no correlation was seen between age and RLU of participants in this study.

RLU is not recommended as an RCS exposure biomarker as it is significantly confounded by HIV status.

Total antioxidant status

TAS is a measure of the net burden of pro-oxidants and free radicals and the presence of antioxidants to neutralise them. Since the current study found GPx activity to be lowered in RCS-exposed miners, and as RCS exposure is known to generate ROS, some decrease in TAS was expected. As anticipated, a significant difference in TAS, between miners and controls was measured. However, with further investigation, it was clear that this difference was due to a much lower level of TAS in the HIV-positive controls than the HIV positive miners. On the other hand, there was no significant difference between the HIV- negative controls and the HIV – negative miners as expected. Finally there was no significant difference in the TAS levels between the HIV- positive and negative miners.

Although a number of studies have evaluated the effect of RCS and silicosis on a few antioxidant enzymes individually, only one measured the TAS in RCS-exposed workers (Schins *et al.*, 1994). The authors measured the total radical-trapping antioxidant parameter (TRAP) in coal miners with and without pneumoconiosis and in unexposed controls. They found no significant difference in the Trolox-captured peroxyl radical concentrations between controls and miners with and without pneumoconiosis. Theron *et al.*, (1994) measured vitamin E, beta-carotene and vitamin C in miners and controls and were only able to show a decrease in the vitamin C levels. They suggested that the main disturbance of antioxidant status may be localised in the lung and therefore not reflected in the blood.

Confounders

The HIV-positive controls had a significantly reduced total antioxidant status compared to the HIV-negative participants. This suggests that TAS is affected significantly by HIV status. This decrease in TAS in HIV positive individuals has been described in studies by Ogunro *et al.*, (2005) and Suresh *et al*., (2009). The authors found a dose response reduction in TAS that corresponded with a reduction in CD4 count.

TAS was not significantly affected by smoking, but there was a trend towards a decrease in TAS levels in smokers. This lack of a significant difference is supported

by the findings of Girotti *et al.*, (2002) who also found no significant decrease in TAS levels between smokers and non-smokers.

This biomarker was not considered for further testing due to the fact that there was no significant difference between the HIV-negative miners and the HIV – negative controls, suggesting that TAS may not be systemically affected by RCS exposure.

TAS could not be shown in this study to be a viable marker of RCS exposure, and is therefore not recommended for further investigation.

8-Isoprostane

A number of studies have shown an increase in lipid peroxidation *in vitro* and *in vivo* following RCS exposure (Gabor *et al.*, 1975, Jajte *et al.*, 1988, Shi *et al.*, 1994).

It has been suggested that the levels of isoprostanes in a particular organ or cavity may not reflect whole body oxidation. The opposite may also be true: plasma levels may not reflect levels of isoprostane in a particular organ, although animal studies have shown that as isoprostane levels in the liver increase after treatment with carbon tetrachloride, plasma levels also increase compared to untreated animals (Morrow and Roberts, 2002).

The validation experiment of 8-Isoprostane reported different results from two dilutions of the same samples, this suggests that there is interference in the assay method. Subsequently, the results could not be analysed further.

3.5 Conclusion

Oxidative stress has been suggested as an important mechanism whereby RCS exposure injures the lung, resulting in the development of silicosis. In this study six possible markers of oxidative stress were examined namely GPx, GSH, GST, ROS, TAS, and 8-Isoprostane.

Both TAS and RLU were found to be confounded by HIV status in this study and therefore. Both were unlikely candidates for future investigations as biomarkers of RCS exposure.

Total GSH levels in this study were not affected by RCS exposure. Reduced GSH, which was not measured in the current study, could have been a better candidate as a marker of RCS exposure and therefore should be investigated further.

Challenges encountered in the measurement of 8-Isoprostane made it difficult to evaluate its suitability as a biomarker of RCS exposure.

Only one of these oxidative stress markers, namely GPx was found to be worthwhile for further investigation. This enzyme was found to be significantly decreased in RCS-exposed gold miners compared to healthy controls and unaffected by smoking, HIV status, or age. This confirms the role of oxidative stress in the development of silicosis. Although there was no indication of a relationship between GPx levels and years of RCS-exposed work, GPx activity may be a useful biomarker of early exposure.

CHAPTER 4

Inflammatory Markers and Markers of Fibrotic Reaction

4.1 Introduction

Inflammation is an important function of the immune defence system in response to a challenge, but its suppression and cessation are also a normal part of immune defence. Understanding lung inflammation is important in many lung diseases (Reynolds, 2005). Inflammation can cause injury to tissues or organs, and unsuppressed inflammation may be part of the mechanism of chronic disease progression (Driscoll *et al.,* 1997). AMs play a significant role in lung defence, as they are important mediators of inflammation in the lung, with their ability to secrete mediators of inflammation and chemotaxis as well as ROS and enzymes (Vanhee *et al.*, 1995, Mossman and Churg, 1998). Fibrosis of the lung has been associated with chronic inflammation and inflammatory cells are a major source of mediators of fibrosis (Olbruck *et al.,* 1998; Gross *et al.,* 2001).

Inhalation of RCS dust has been shown to induce inflammation in laboratory animals (Barbarin *et al*., 2005 and Gulumian, 2007) and humans (Vanhée *et al*., 1994 and Rom 1991). In addition, silicosis is characterised by persistent inflammation with macrophages, neutrophils and T-cells found in patients' BAL fluid and at autopsy (Spencer, 1985; Mossman and Churg, 1998). Based on these findings, unresolved inflammation has been suggested as a possible mechanism in the development of fibrosis in silicosis (Vallyathan *et al.*, 1996). In a study by Bissonnette and Rola-Pleszczynski, (1989), RCS exposure resulted in acute inflammation that lasted for the six months of the study and resulted in massive fibrosis of the lungs.

The accumulation of macrophages, lymphocytes and granulocytes has been suggested as the first step in the inflammatory process following RCS exposure. Inhalation of RCS is followed by the deposition of these particles in the alveoli. AMs phagocytose these particles and become activated. Much progress has been made in the identification of the factors released by macrophages that activate and attract PF (Gulumian, 2007). These activated macrophages are known to release ROS, RNS, proteolytic enzymes, cytokines, growth factors and chemokines, which induce and help maintain further inflammation, causing further destruction of the lung and attracting PF, neutrophils and macrophages (Driscoll *et al.*, 1990; Driscoll *et al.*, 1993; Melloni *et al.*, 1994; Barbarin *et al.*, 2005; Huaux, 2007 and Melloni *et al.,* 1994). It is now accepted that along with AMs, epithelial cells and PF can also become activated and release inflammatory mediators (Huaux *et al.,* 2007). Some evidence also points to a role of oxidative stress in promoting the expression of cytokines in AMs (Driscoll, 1997). TNFα release was reduced when oxygen radical scavengers were used.

Factors released in the inflammatory cells can cause cellular and DNA damage resulting in apoptosis and necrosis of the cells and disruption of the extracellular matrix (Knaapen *et al.*, 2002; Wang *et al.*, 2005; Lalmanach *et al.*, 2006). The factors released by AMs are also responsible for the proliferation of epithelial cells (Melloni *et al.*, 1996). These authors have applied conditioned media from RCS-exposed AMs to an epithelial cell line which resulted in an increase in epithelial cell DNA synthesis and proliferation compared to the cells grown with unstimulated AM conditioned media.

Once the inflammatory process is initiated in the lung, it is followed by a reparative phase where the damage caused to the epithelial cells by the inflammatory cells and ROS is repaired. Growth factors stimulate the recruitment and proliferation of mesenchymal cells. These factors are also able to promote growth of new vascular tissue and reintroduce epithelial cells. Extracellular matrix deposition and tissue remodelling is responsible for the tissue repair. Factors such as cytokines, TNFα, IL-1 and TGFβ and growth factors such as PDGF, IGF and fibronectin are released from silica-activated AMs and other cells such as neutrophils and epithelial cells (Huaux, 2007 and Melloni *et al*., 1994).

The effective communication between immune cells during an immune response is due to specific signals that initiate, maintain and resolve inflammatory lesions. Cytokines play a role in specific stages of inflammation. Research suggests that expression of cytokines is both cell and stimulus specific (Kunkel *et al.*, 1990). Cytokines have a number of different functions, which can include chemotaxis, stimulation or inhibition of inflammation, cell growth and differentiation, proliferation and fibrogenesis (Schins and Borm, 1999).

Interleukin-1 (IL-1), TNFα, and MIP-2 are three cytokines which were measured in increased levels in activated AMs from rats exposed to RCS. These are pro-inflammatory cytokines responsible for the persistence of inflammation and can lead to the development of fibrosis (Driscoll *et al.*, 1995).

The relationship between RCS-induced inflammation and fibrosis was confirmed by the inhibition of RCS-induced development of fibrosis by the treatment of rats with dexamethasone and piglitazone, two anti-inflammatory agents (Barbarin *et al*., 2005).

The literature review conducted as the first phase of this project identified three cytokines for further investigation as markers of early effect in RCS exposure, namely TNFα, IL-8 and PDGF (Gulumian *et al*., 2006).

4.1.1 Tumour necrosis factor α

Tumour necrosis factor alpha (TNF α) is a cell-activating cytokine, with a molecular mass of 17 kilo Daltons; the cytokine is usually found as a trimer (Smith and Baglioni, 1987). TNFα is produced by blood monocytes, AMs, polymorphonuclear cells, lymphocytes, smooth muscle cells and mast cells (Rich *et al.*, 1989; Dubravec *et al.*, 1990 and Driscoll *et al.*, 1997).

TNFα has a number of functions: it plays a role in inflammation, indirectly recruiting neutrophils and macrophages through the stimulation of macrophages, neutrophils, fibroblasts and epithelial and endothelial cells to release chemokines, which are key mediators of cell recruitment (Driscoll *et al*., 1996). TNFα induces fibroblast proliferation, eosinophil toxicity and bone resorbtion. TNFα stimulates cells to release

a wide array of molecules from cytokines such as IL-1 and IL-6 to growth factors PDGF and TGFβ and adhesion factors (Driscoll, 1997). Inflammatory cells must move from the blood vessels to the lungs, which they achieve by adhering to the endothelial cells lining the blood vessels. TNFα and IL-1 are responsible for stimulating this adherence by inducing the expression of adherence proteins on the cells (Driscoll *et al*., 1996).

TNFα has been shown to stimulate the proliferation of fibroblasts and to increase collagen production, so playing a role in fibrosis (Arcangeli *et al.*, 2001). TNFα is also involved in cell growth and differentiation; tissue repair and regeneration; induction of procoagulant activity and the expression of more TNFα (Driscoll *et al*., 1996). Local prolonged over-expression of TNFα results in severe pulmonary inflammation. Neutrophils, macrophages and lymphocytes are initially recruited, and after a few days their numbers are reduced with the start of fibrogenesis. Through deposition of extracellular matrix by accumulating fibroblasts the fibrotic changes observed are patchy but persistent (Sime *et al.*, 1998).

TNFα has also been shown to play a role in cell activation by stimulating the secretion of cell activators such as IL-6 (Driscoll *et al*., 1996). Both B and T-lymphocytes are activated by TNFα along with endothelial cells and fibroblasts. TNFα activated and proliferated B lymphocytes are responsible for the secretion of immunoglobulins. TNFα has also been shown to induce the expression of PDGF in endothelial cells

In a study by Gosset *et al.,* (1991) normal human AMs were stimulated *in vitro* with coal dust. After 24 hours the cells released significant amounts of TNFα compared to cells stimulated with an inert dust. The activated macrophages released increased chemotactic activity in the patients compared to the controls. Spontaneous TNF α secretion by AMs was also increased in the patients compared to the controls. Thus proposing a mechanism through which dust may induce inflammation (Vanhee *et al.,* 1994).

In another study mice were exposed to RCS and asbestos and the TNFα production of the BALF cells was determined after stimulation with lipopolysaccharide (LPS). Asbestos-exposed cells caused a two-fold increase in TNFα but this rapidly dropped

to normal, while RCS exposure appeared not to cause any increase in TNFα release from cells stimulated by LPS. When the cells were not stimulated with LPS an augmentation of TNFα production was seen in RCS-exposed mice. TNFα produced *in vivo* has been suggested to be bound to a large percentage to neutrophils present due to inflammation. Neutrophils have a high affinity for TNF and it is rapidly internalised (Shalaby *et al.*, 1987).

TNFα mRNA was measured in the lung tissue of mice exposed to RCS after the development of silicosis which remained persistently overexpressed (Davis *et al.*, 1998).

4.1.2 Interleukin-8

Interleukin-8 (IL-8) is a pro-inflammatory chemotactic cytokine that is responsible for the chemotaxis and activation of neutrophils and the adhesion of leukocytes (Driscoll *et al*., 1997; Larsen *et al.*, 1989 and Rosenkilde and Schwartz, 2004). It is a member of the α-subfamily of chemokines (Clore and Gronenborn, 1995 and Driscoll *et al.*, 1997). The α subfamily of chemokines consists of a polypeptide chain with four cysteine residues and are 8-10 kDa in size coded for on chromosome 4 (Clore and Gronenborn, 1995). IL-8 and the other members of the α chemokine family attract only neutrophils, while members of the β family attract monocytes and lymphocytes. This specificity may be due to the different tertiary structures of the two subfamilies (Clore and Gronenborn, 1995). IL-8 is produced by fibroblasts, macrophages, neutrophils, and endothelial and epithelial cells. The chemokine is mostly released after the stimulation of cells by IL-1 and TNFα (Larsen *et al.,* 1989; Driscoll *et al*., 1996). It has been suggested that IL-8 could be a marker of chronic inflammation with neutrophil involvement (Keman *et al*., 1997).

Bronchial epithelial cells and AMs are also able to produce IL-8 when stimulated with TNFα. TNFα appears to stimulate IL-8 gene expression at the transcriptional level, which results in an increase in IL-8 mRNA (Nakamura *et al.*, 1991, Standiford *et al.*, 1991). Stimulants of AMs, which cause them to release cytokines, are inorganic substances such as RCS and asbestos and oxidative stress (Driscoll *et al*., 1997).

Pulmonary fibroblasts (PF) have a unique location and function in the lungs. They are found lining the alveoli that allows them to communicate between vascular tissue and alveolar airspaces. They produce IL-8 as part of the defence network. AMs are also part of the defence network. They produce cytokines that in turn stimulate the PF to produce IL-8 that is responsible for the recruitment of neutrophils (Rolfe *et al.*, 1991).

Zhai *et al.*, (2004) measured increases in TNFα, IL-8 and IL-6 in the BAL fluid of silicosis patients compared to controls. In a study with exposed PMF cases and unexposed exposed controls, both TNFα and IL-8 were measured in stimulated blood monocyte assays and serum. The investigators found an increase in TNFα levels an IL-8 levels in the cases compared to the controls although the changes in TNFα and IL-8 may be related to the presence of pneumoconiosis and not to RCS exposure (Kim *et al.,* 1999).

A limitation in the use of IL-8 as a marker for harmful RCS exposure is its possible lack of specificity. Keman *et al.* (1997) reported an increase in spontaneous release of IL-8 from whole blood of chemical workers with bronchitis compared to healthy workers. Diesel particulates have also been demonstrated to bind and increase the half-life of IL-8 while retaining biological activity. This can serve to increase inflammation in the lung (Seagrave, 2008).

4.1.3 Platelet Derived Growth Factor

PDGF is a growth factor that plays an important role in wound healing (Castranova *et al.,* 1996b and Heldin and Westermark, 1999). It acts as a potent mitogen for a number of cell types, e.g. fibroblasts, smooth muscle cells, neutrophils and macrophages (Fredriksson *et al*., 2004 and Bonner, 2004). In lung disease, PDGF has been found to be released mainly by AMs but it is also released by a number of other cell types, and plays an important role in the proliferation of fibroblasts (Heldin and Westermark, 1999 and Bonner, 2004).

PGDF is a dimer and five different isoforms are found with four different polypeptide chains, PDGF A, B, C, and D (Fredriksson *et al*., 2004). The hetero and homodimers are held together by eight disulphide bonds. PDGF BB is a homodimer consisting of two PDGF B chains arranged antiparallel. The three-dimensional structure is that of a

knot and is similar to transforming growth factor β (TGF β) and nerve growth factor. The genes for PDGF A and B are found on chromosomes 7 and 22 respectively. The synthesis of PDGF is often stimulated by extracellular factors such as oxygen tension, other growth factors and cytokines (Heldin and Westermark, 1999).

PDGF has been shown to stimulate cell growth, and play a role in actin reorganisation and Ca^{2+} mobilisation. PDGF BB has also been specifically shown to induce chemotaxis and is suggested to inhibit gap junction communication and apoptosis (Heldin and Westermark, 1999 and Yao and Cooper, 1995). PDGF AA, through the activation of α receptors, inhibits the chemotaxis of fibroblasts and smooth muscle cells (Heldin and Westermark, 1999). PDGF BB has been suggested to be more biologically active than PDGF AA (Cenni *et al.,* 2005).

Regulation of PDGF receptors plays an important role in PDGF function, as the expression of β receptors increases during inflammation. TNFα has been shown to increase α receptors and TGFβ stimulation leads to a decrease in α receptor expression on fibroblasts. As part of the healing process, PDGF stimulates macrophages to secrete other growth factors, along with stimulating the production of matrix molecules such as collagen and fibronectin from fibroblasts (Heldin and Westermark 1999 and Bonner, 2004).

PGDF has been suggested to play an important role in the development of fibrosis as it is an effective stimulator of fibroblast proliferation by providing the competence signal (Schins and Borm, 1999 and Vanhée *et al.,* 1994). Fibroblasts are interstitial cells located in the lungs and when activated move into the alveolar space. There they proliferate and produce connective tissue. Fibroblasts express PDGF receptors which are regulated by pro-inflammatory proteins such as TGF β. Nano molar and Pico molar quantities of PDGF are needed to direct both migration and proliferation of mesenchymal cells like fibroblasts (Castranova *et al.,* 1996b).
RCS has been suggested to cause the migration and proliferation of fibroblasts by two mechanisms (Figure 26). The first mechanism is activation of AMs that release factors that activate fibroblasts, and the second mechanism involves the damage of epithelial cells and resulting inflammation and repair.

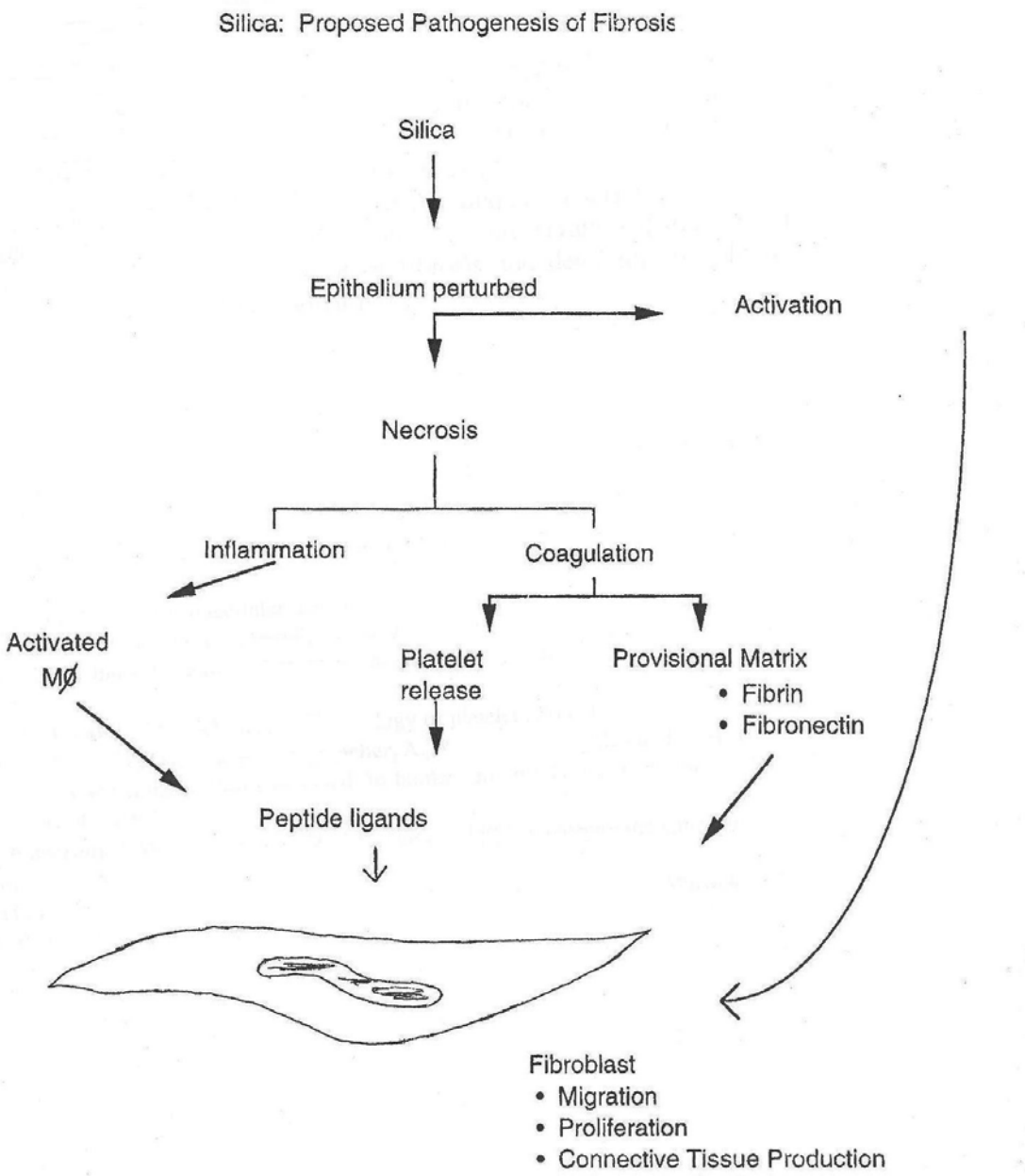


Figure 26: Illustration of the two pathways by which RCS has been proposed to induce fibrosis of the lungs (Castranova *et al.,* 1996b).

Silicosis is a fibrotic disease, and so factors associated with fibrogenesis have been studied as possible biomarkers of effect. As shown by McDonald and Hendrickson, (1993), PDGF BB plays an important role in responding to inflammation, particularly the release of TNFα by, among other functions, stimulating the chemotaxis of fibroblasts. The development of silicosis is linked to the presence of fibroblasts as seen in the immunohistochemistry of silicotic lung tissue (Kane, 1996).

A number of particle types such as RCS and coal dust have been shown to induce macrophage release PDGF BB (Vanhée *et al.,* 1994).

Brand-Rauf *et al.,* (1992) conducted a cohort study with 46 silicosis or asbestosis patients. They were interested in oncoproteins and growth factors as markers of response. They found only elevated oncoproteins in the asbestosis patients but increased PDGF BB in both the silicosis patients and asbestosis patients. They found an association between advanced cases of pneumoconiosis and PDGF positivity ($p = 0.016$).

Vanhée *et al.,* (1994) conducted a similar study using patients with simple CWP and PMF and comparing them to coal dust-unexposed controls. They found increased amounts of PDGF in the BAL of PMF patients compared to the controls and those with CWP but no PMF. They also investigated the supernatants of AM, and found that the concentrations of PDGF followed the same profile as in the BAL.

A recent study of patients with silicosis or CWP and healthy controls showed an increase in serum PDGF in those with pneumoconiosis compared to the controls (Yao *et al.,* 2006). This study also showed a decrease in PDGF levels as the stage of pneumoconiosis increased, contrary to the findings of Vanhee *et al.,* (1994) and Brandt-Rauf *et al.,* (1992).

4.2 Methods

4.2.1 Theoretical backgrounds of the analysis implemented

All three inflammatory biomarkers measured in this study were assayed using the same method, enzyme linked immunosorbent assay (ELISA).

The two-antibody sandwich method has been suggested as the most reliable and sensitive ELISA method for quantifying cytokines. The use of monoclonal antibodies provides a high specificity for the specific cytokine of interest and is less likely to be interfered with by other similar cytokines (Fong and Mossman, 1989). The double antibody or sandwich method of ELISA involves the coating of a fixed surface (in this case a 96-well plate) with the antibody to the protein of interest. Then the antigen solution is added, allowing the antigen of interest to bind. After washing to remove other unbound proteins the second labelled antibody also specific to the protein is added and allowed to bind to the protein. The excess antibody is removed in a wash step, the substrate for the label is added, and the colour allowed to develop.

ELISAs only measure protein levels but are not able to differentiate biologically active cytokines. This may be a limitation of the method (Fong and Mossman, 1989). Monoclonal antibodies are used to identify specific proteins immunologically but they are unable to guarantee that the protein measured is biologically active. Monoclonal antibodies are able to bind precursor forms of proteins and partly degraded forms that are no longer biologically active. While this is a limitation, having a comparison or control group means that this is negated as both groups would have the same limitation and relative changes monitored.

4.2.2 Assays practical

4.2.2.1 TNFα immunoassay

This cytokine was assayed with a Human TNFα/TNFSF1A Immunoassay kit purchased from R&D Systems (USA). Supplied with the kit were a TNFα-coated microplate, a TNFα conjugate and a standard. Also supplied were assay diluent,

calibrator diluent (one for plasma samples and one for cell culture samples), wash buffer, colour reagent A and B, stop solution and plate covers.

Sample collection

Blood was drawn into a heparinised vacutainer which was centrifuged at 1 000 x after which the plasma was removed and stored at -80°C within 2 hours.

Reagent preparation

The wash buffer was diluted with distilled water to provide 500 ml of wash buffer. The calibrator solution for plasma was ready to use as supplied along with the assay diluent and the TNFα conjugate. The TNFα standards were prepared as follows: the standard was reconstituted with 5 ml of calibrator diluent for plasma and allowed to stand for 15 minutes before further dilution. Calibrator diluent 500µl was placed into 8 numbered Eppendorf tubes, except for the first tube, into which 900 µl of calibrator diluent was placed. The reconstituted stock solution was used to create a serial dilution. A 100 µl of the stock solution was added to the first tube and mixed well. Then 500 µl was removed from the first tube and placed into the second tube, and the process was repeated until 500 µl from tube 7 was placed into tube 8. The substrate solutions A and B were mixed in equal volumes for 15 minutes before they were required. The stop solution was ready to use as supplied.

Assay

A 50 µl of assay diluent was pipetted into each well, and then 200 µl of either standard or sample was added to duplicate wells. The plate was then covered and incubated for 2 hours at room temperature. Following incubation, the plate was washed 3 times with the manual plate washer and blotted with paper towel to remove any last remaining liquid. A 100 µl of TNFα conjugate was then added to all the wells and again the plate was incubated at room temperature for 2 hours. At the end of the incubation period, the two substrate solutions A and B were mixed. After washing the plate again 3 times, a 200 µl of substrate solution was added to each well and the plate incubated in the dark for 30 minutes. At the end of the incubation period, the reaction was stopped with 50 µl of stop solution.

The absorbance of the plate was then determined on the plate reader spectrophotometer at 450 nm within 30 minutes of adding the stop solution.

Calculations

A log log curve of the standards was created in Excel. A linear regression trend line was then fitted and its equation was used to calculate the concentration of the samples (Figure 27). Where the absorbance's of the samples were lower than the lowest point on the standard curve the equation below was used to calculate an approximate value.

$$y= 0.8281x – 2.0477$$

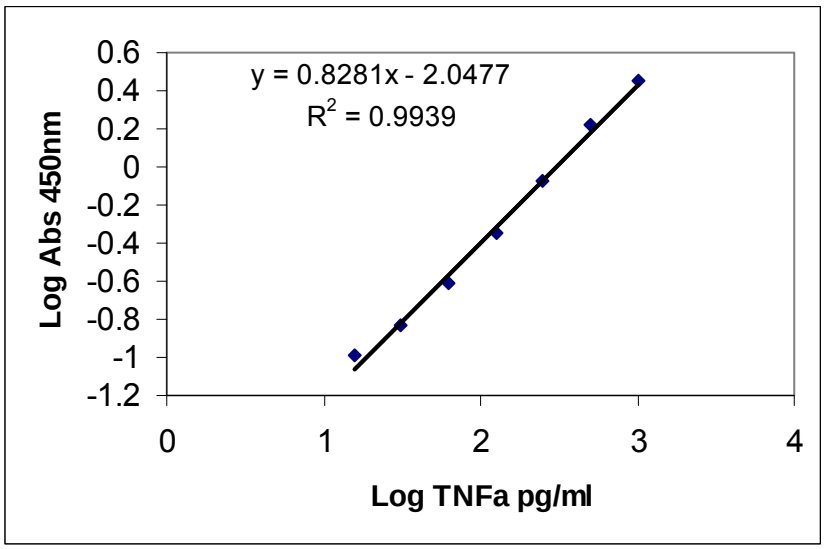


Figure 27: Standard curve for TNFα

There has been some question on the validity of enzyme-linked immunoassay levels of isoprostanes. Il'yasova *et al.* (2004) developed an ELISA for F2–isoprostanes. However the gold standard is gas chromatography with mass-spectrometry detection, but this is expensive and labour intensive. The ELISA results showed levels 30 times higher than the levels obtained with the GC-MS, and showed a poor linear correlation of 0.51 (95% CI 0.28-0.70). The authors concluded that the ELISA was not a valid substitute (Il'yasova *et al.,* 2004). The manufacturer of the ELISA kit used for this study also conducted correlations between the GC-MS method and their kit, and concluded that the correlation was good although they warned that interference in the assay would result in unreliability.

4.2.2.1 Interleukin-8 immunoassay

This assay was performed with a Human CXCL8/IL-8 Immunoassay purchased from R&D Systems (USA). Supplied with the kit were: an IL-8 coated microplate, IL-8 conjugate and standard. Also supplied were assay diluent, calibrator diluent for plasma and calibrator diluent for cell culture, wash buffer, colour reagent A and B, stop solution and plate covers.

Sample collection

Blood was drawn into a heparinised vacutainer which was then centrifuged at 1 000 x. The plasma was removed, placed into a cryotube and stored at -80°C within 2 hours.

Reagent preparation

The wash buffer was diluted with distilled water to provide 500 ml of wash buffer. The calibrator solution for plasma was ready to use as supplied along with the assay diluent and the IL-8 conjugate. The IL-8 standards were prepared as follows: the provided standard was reconstituted with 5 ml of calibrator diluent for plasma and was allowed to stand for 15 minutes before further dilution. A 500 µl of calibrator diluent was placed into 8 numbered Eppendorf tubes. The reconstituted standard solution was used to create a serial dilution. A 500 µl of the stock was added to tube number 1 and mixed well. Then 500 µl was removed from tube number 1 and placed into tube number 2, and the process was repeated until 500 µl from tube 7 was placed into tube 8. The substrate solutions A and B were mixed in equal volumes 15minutes before they were required. The stop solution was ready to use as supplied.

Assay

A 100 µl of assay diluent was pipetted into each well, then 50 µl of either standard or sample was added to duplicate wells. The plate was then covered and incubated for 2 hours at room temperature. Following incubation, the plate was washed 4 times with the manual plate washer and wash buffer. After washing, the plate was blotted with paper towel to remove any last remaining liquid. A 100 µl of IL-8 conjugate was then added to all the wells and the plate was further incubated at room temperature for 1 hour. At the end of the incubation period, the substrate solutions A and B were mixed. After washing the plate again 4 times, a 200 µl of substrate solution was added to

each well and the plate incubated in the dark for 30 minutes. The reaction was then stopped with 50 µl of stop solution in each well.

The absorbance of the plate was then determined on the plate reader spectrophotometer at 450 nm within 30 minutes of adding the stop solution.

Calculations

A log log curve of the standards was created in Excel by plotting the log of the absorbance against the log of the concentration (Figure 28). A linear regression trend line was then fitted and its equation was used to calculate the concentration of the samples.

$$y = 1.2304x + 141.79$$

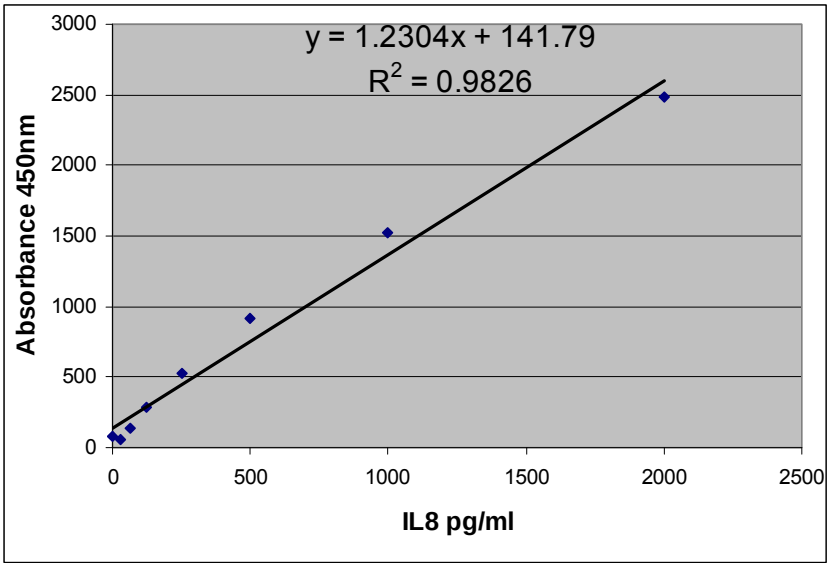


Figure 28: IL-8 standard curve

4.2.2.3 Platelet Derived Growth Factor-BB (PDGF-BB) immunoassay

This assay was performed with a Human PDGF-BB Immunoassay Kit purchased from Systems (USA). Supplied with the kit were: a PDGF-coated microplate, PDGF conjugate and standard. Also supplied were assay diluent, calibrator diluent for plasma and calibrator diluent for cell culture, wash buffer, colour reagents A and B, stop solution and plate covers.

Sample collection

Blood was drawn into a heparinised vacutainer which was then centrifuged at 1 000 xg. The plasma was removed, placed into an Eppendorf tube and centrifuged a second time at 10 000 x at 4 °C to remove platelets. The supernatant was removed and stored at –70 °C within 2 hours.

Reagent preparation

The wash buffer was diluted with distilled water to provide 500 ml of wash buffer. The calibrator solution for plasma was ready to use as supplied along with the assay diluent and the PDGF conjugate. The PDGF standards were prepared as follows: the provided standard was reconstituted with 1 ml of calibrator diluent for plasma and allowed to stand for 15 minutes before further dilution. A 500 µl of calibrator diluent was placed into 8 numbered Eppendorf tubes. The reconstituted standard solution was used to create a serial dilution. A 500 µl of the stock was added to tube number 1 and mixed well. Then 500 µl was removed from tube number 1 and placed into tube number 2, and the process was repeated until 500 µl from tube 7 was placed into tube 8. The substrate solutions A and B were mixed in equal volumes 15minutes before they were required. The stop solution was ready to use as supplied.

Assay

A 150 µl of assay diluent was pipetted into each well and then 50 µl of either standard or sample was added to duplicate wells. The plate was then covered and incubated for 2 hours at room temperature. Following incubation, the plate was washed 4 times with the manual plate washer and wash buffer. After washing 4 times, the plate was blotted with paper towel to remove any last remaining liquid. A 100 µl of PDGF conjugate was then added to all the wells and the plate was again incubated at room temperature for 1 hour. At the end of the incubation period, the substrate solutions A and B were mixed. After washing the plate again 4 times, a 200 µl of substrate solution was added to each well and the plate incubated in the dark for 30 minutes. The reaction was stopped with 50 µl of stop solution in each well.

The absorbance of the plate was then determined on the plate reader spectrophotometer at 450 nm within 30 minutes of adding the stop solution.

Calculations

A log log curve of the standards was created in Excel by plotting the log of the absorbance against the log of the concentration (Figure 29). A linear regression trend line was then fitted and its equation used to calculate the concentration of the samples.

$$y= 0.0012x + 0.494$$

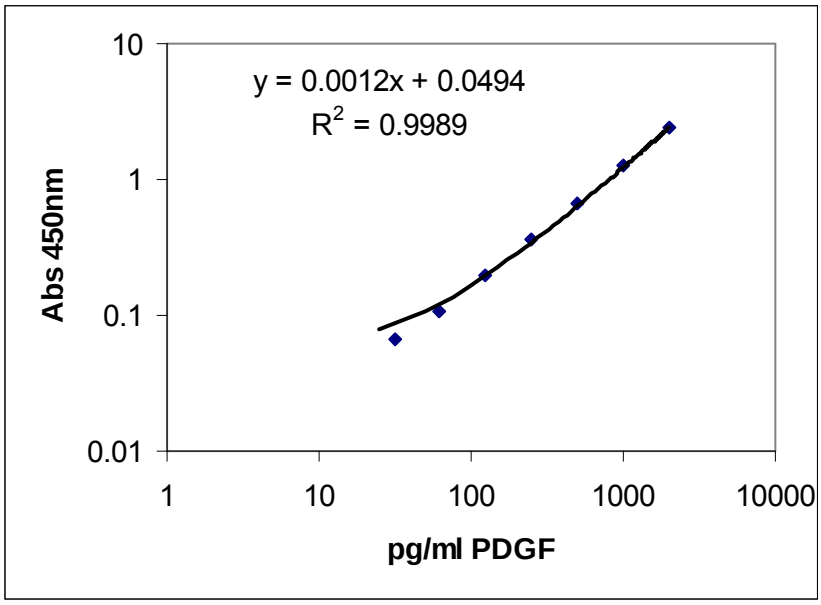


Figure 29: PDGF standard curve

The differences our study in sample processing time between the miners and controls due to field conditions may have played a role in the increase seen in PDGF levels in controls. PDGF levels in plasma can be affected by the time between collection and centrifugation to remove platelets from plasma.

4.2.2.4 Statistical analysis

Descriptive statistics such as mean, median and standard deviation were used to describe the biomarker levels. The data were analysed for normality. An unpaired t-test was used to compare the means of the two groups if the data met with the requirements for a normal distribution; otherwise, transformations were used along with non-parametric tests to test for a relationship. The ladder function of STATA was used to assess the best transformation to use on the data. This function performs the eight most common transformations on the data and then tests them for normality.

The result is given as the chi-square test for normality with a p value. Where possible, a one-way ANOVA or the Kruskal-Wallis tests were used to compare both exposure status and HIV at the same time

4.3 Results

Serum samples were collected from participants and analysed for inflammatory markers. The results were divided into those who were exposed to RCS and those who reported no exposure to RCS. These two groups were then further divided into HIV-positive and HIV-negative individuals.

4.3.1 Tumour necrosis factor α

The TNFα data were not consistent with a normal distribution when analysed with the Shapiro – Wilk test (Table 54)

Table 54: Shapiro – Wilk test for normal distribution of TNF α data.

	Obs	**W**	**V**	**z**	**P**
TNFα	124	0.70491	29.194	7.572	0.00000

The histogram of the data shows a distribution skewed to the right (Figure 30). It was not possible to transform these data into a normal distribution with a commonly used transformation. Based on the histogram, two outliers were removed as they were not consistent with the data.

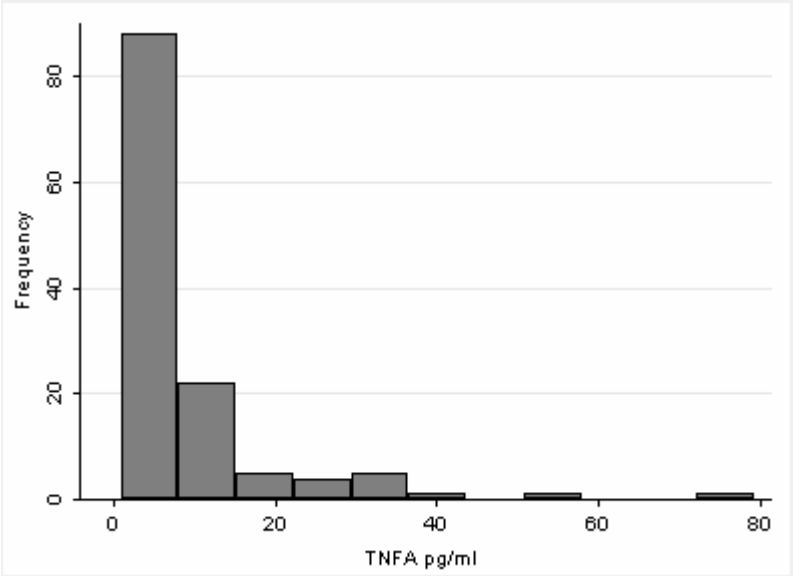


Figure 30: Histogram of TNFα results

Non–parametric tests were used to compare TNFα levels between the groups. The Wilcoxon rank-sum test showed no significant difference in TNFα levels between miners and controls (p = 0.99). There was a significant difference between HIV-positive and HIV-negative participants (p = <0.01) using the rank sum test (Table 55).

Table 55: Description of TNFα results.

Participants	n	Mean	Median	IQR	Range
All Miners	62	8.32	6.18	4.60 – 11.73	1.26 -31.60
HIV+ miners	22	10.65	8.74	5.63 – 14.17	1.40 – 30.29
HIV-miners	39	7.15	5.69	4.49 – 6.98	1.26 – 31.60
All Controls	62	9.21	5.72	5.15 – 7.73	1.20 – 38.30
HIV+ controls	27	11.91	7.73	4.84 – 15.70	1.20 – 38.30
HIV-controls	35	7.13	5.60	5.29 – 6.40	3.29 – 34.57
Total	124	8.77	5.84	4.92 – 10.53	1.20 – 38.30

There was no significant difference between smokers and non-smokers (p = 0.1447). The small number of smokers may have limited the power of the study to find a difference due to smoking (Table 56).

Table 56: TNFα levels in smokers and non-smokers.

Participants	n	Mean	SD	Median	IQR	Range
Non-smokers	99	8.90	7.33	5.84	5.21 – 11.93	1.20 – 34.57
Current smokers	24	7.49	7.40	5.41	3.88 – 7.34	1.81 – 38.3

A rank sum test of the TNFα levels showed no significant difference between miners with and without silicosis ($p = 0.1587$). There appeared to be a trend towards higher levels of TNFα in miners with silicosis, but there appeared to be no relationship between years of exposure and TNFα levels (Spearman's correlation $r = 0.02$, $p = 0.86$) (Table 57).

Table 57: TNFα levels in miners with and without silicosis.

Participants	n	Mean	SD	Median	IQR	Range
Non-silicotics	41	7.64	6.39	5.69	4.56 – 8.17	1.40 – 31.6
Silicosis	17	9.34	6.17	6.98	5.36 – 12.14	1.26 – 38.30

The more severe silicosis patients demonstrated a significant increase in TNFα levels compared to those with early silicosis ($p = 0.0365$, rank sum) and no silicosis ($p = 0.0147$). The silicosis patients were grouped by ILO scores: those with scores between 1/0 and 1/1 were grouped together and those with scores greater than 1/1 were grouped separately (Table 58).

Table 58: TNFα levels in miners with silicosis.

Participants	n	Mean	SD	Median	IQR	Range
No silicosis	41	7.64	6.39	5.69	4.56 – 8.17	1.40 – 31.6
Silicosis ≤ 1/1	11	7.85	7.0	5.4	4.1 – 9.0	1.3 – 26.9
Silicosis > 1/1	6	13.34	2.1	12.7	11.9 – 14.9	12.0 – 16.37

There was no correlation between age and TNFα levels in the total participants (Spearman's correlation r = 0.14, p = 0.13 and r = 0.06 p = 0.61) (results are not shown).

Verification of TNFα results

Duplicate samples from HIV-negative participants were sent to Prof K Donaldson at the ELEGI Colt Laboratory, University of Edinburgh where our test results were validated. The mean difference in levels was -1.3 mg/ml (95% CI -2.4 — -0.2). This is a small mean difference and the distribution of differences around the mean does not appear to indicate a bias in the results (Figure 31).

The TNFα levels measured in our study were not normally distributed, a large proportion (88%) of values were below the lowest standard in the kit of 15.6 pg/ml. These results should be interpreted with caution. The validation experiment showed that the levels measured in our laboratory were close to those performed in an independent laboratory.

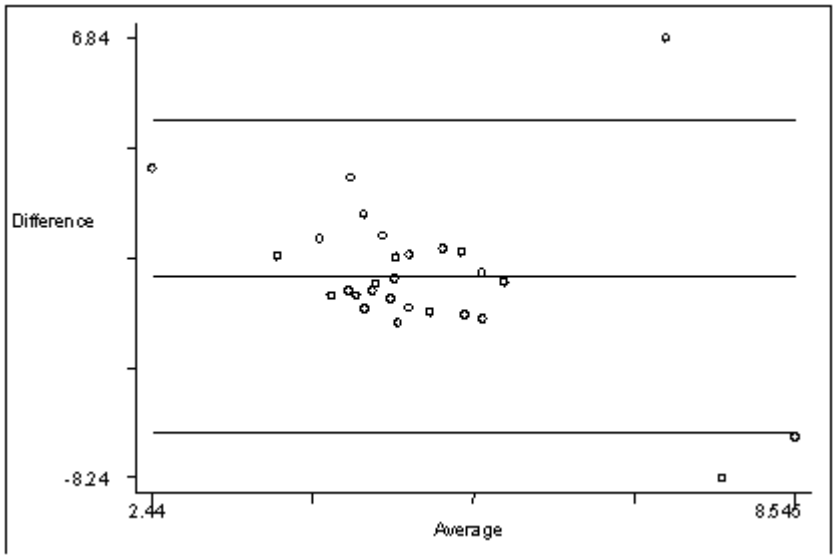


Figure 31: Bland-Altman plot of the differences between the validation and our measurement of TNFα levels in plasma

4.3.2 Interleukin-8

The Shapiro-Wilk test indicated that the IL-8 results were not consistent with a normal distribution (Table 59).

Table 59: Shapiro – Wilk test for normal distribution of IL-8 data.

	Obs	W	V	z	P
IL-8 raw	122	0.83538	16.176	6.244	0.00000
Square root IL-8	121	0.97447	2.474	2.031	0.02115

A histogram indicated a right-skewed distribution with one outlier (Figure 32), which was removed. The data were then transformed to give a normal distribution by taking the square root of the values.

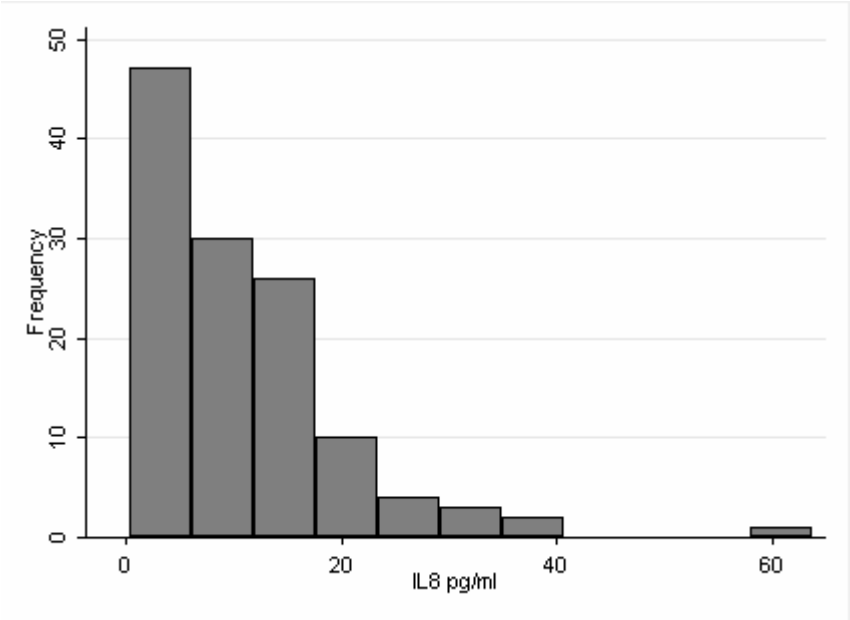


Figure 32: Histogram of IL-8 results

The raw IL-8 results were used to plot the histogram and the transformed results the following tests.

There was no significant difference between exposed and unexposed participants following a t-test of the transformed data p = 0.3426 (95%CI -0.24 – 0.68). A similar result was seen with the non-parametric test, p = 0.2773 (Table 60)

Table 60: Description of IL-8 results.

Participants	n	Mean	Median	IQR	Range
Miners	61	9.90	6.52	2.64 – 15.40	1.00 – 38.85
HIV+ miners	21	13.21	10.71	4.98 – 20.35	1.00 – 38.85
HIV-miners	39	8.24	5.43	2.12 – 14.14	1.00 – 1.5
Controls	60	10.52	8.58	4.68 – 13.72	1.00 – 30.69
HIV+ controls	27	13.76	12.85	7.76 – 19.36	2.58 – 30.69
HIV-controls	33	7.88	7.08	3.65 – 11.87	1.00 – 36.33
Total	121	10.21	8.11	3.55 – 14.37	1.00 – 38.85

There was a significant difference (p = 0.0004 (diff = -0.84 95% CI -1.29 — - 0.38)) between HIV-positive and HIV-negative participants with a significantly higher level of IL-8 in those living with HIV (Table 60).

There was no significant difference between smokers and non-smokers (p = 0.3792). The slight difference observed between the smokers and non-smokers may be due to chance or to the small number of smokers in the group (Table 61).

Table 61: IL-8 levels in smokers and non-smokers.

Participants	n	Mean	SD	Median	IQR	Range
Non-smokers	94	10.41	8.29	8.30	3.30 – 15.21	1.00 – 38.85
Current smokers	24	8.69	6.99	6.73	3.65 – 12.76	1.00 – 30.54

A Wilcoxon rank sum of the raw IL-8 levels showed a significant difference between miners with and without silicosis (p = 0.0164).An unpaired t-test of the transformed data gave a similar result (difference = - 1.07 95%CI -1.87 — -0.28) (p = 0.0090). This was confirmed by a non-parametric equality of medians test.

There was a trend towards higher levels of IL-8 in miners with silicosis compared to those without silicosis (Table 62) which when tested was significant(p = 0.046).

When the miners were separated into two groups based on the severity of the silicosis in the same groups as used for the TNFα analysis, there was no significant difference between the groups (p = 0.6714).

Table 62: IL-8 levels in miners with and without silicosis.

Participants	n	Mean	SD	Median	IQR	Range
Non-silicotics	41	8.28	8.20	5.43	2.12 – 12.40	1- 36.33
Silicosis	16	15.14	9.85	14.87	6.99 – 21.38	1- 38.85

There appeared to be some relationship between IL-8 levels and age in the HIV-negative participants. Spearman's rank correlation found a correlation of r = 0.48, p = 0.005 for the controls and a correlation of r = 0.44 p = 0.005 for the miners (Figure 33).

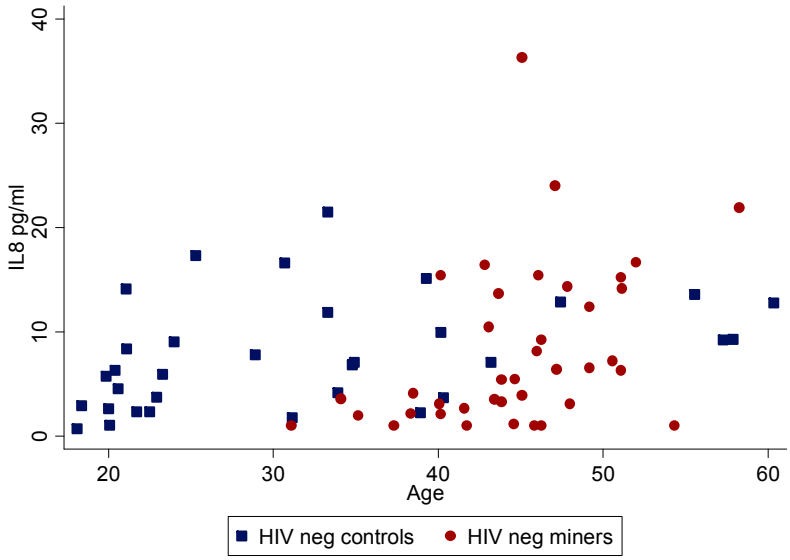


Figure 33: Scatter plots of HIV-negative controls and miners by age and IL-8 levels

An analysis of the difference in mean IL-8 levels by age group in the HIV-negative participants showed no significant difference between age groups (p = 0.067). This p value is close to the cut off for significance of 0.05. Thus a separate analysis of miners and controls was conducted and it showed a significant difference between age groups in the HIV-negative miners p = 0.03 (Table 63).

Table 63: IL-8 levels by age group in miners.

Age Group	Mean IL-8 level	SD
30-40 years	2.3	1.3
40.1 – 50 years	8.7	8.7
50.1 – 70 years	11.8	7.2

*SD = standard deviation

The mean IL-8 levels in the three age groups of the miners appear to indicate an increase with age (Table 63). The same analysis conducted on the controls did not give a significant difference between age groups ($p = 0.09$). This lack of significance is likely to be due to the lack of older controls.

4.3.2.1 Verification of IL-8 results

Duplicate samples were sent to a laboratory in the UK for validation, but their results could not be compared to our results as they were all below the limit of detection of the kit used, and the UK laboratory did not perform an extrapolation to provide figures. We have used the calculation of the standard curve to extrapolate the results of the samples below the lowest standard (Breen *et al. 2011*).

4.3.3 Platelet Derived Growth Factor

The PDGF test results were tested for conformance with a population that followed a normal distribution. Two tests were used; the Shapiro-Wilk test (Table 64) and a histogram (Figure 34).

Table 64: Shapiro–Wilk test for normal distribution of PDGF data.

	Obs	W	V	z	P
PDGF raw	120	0.95024	4.788	3.509	0.00023

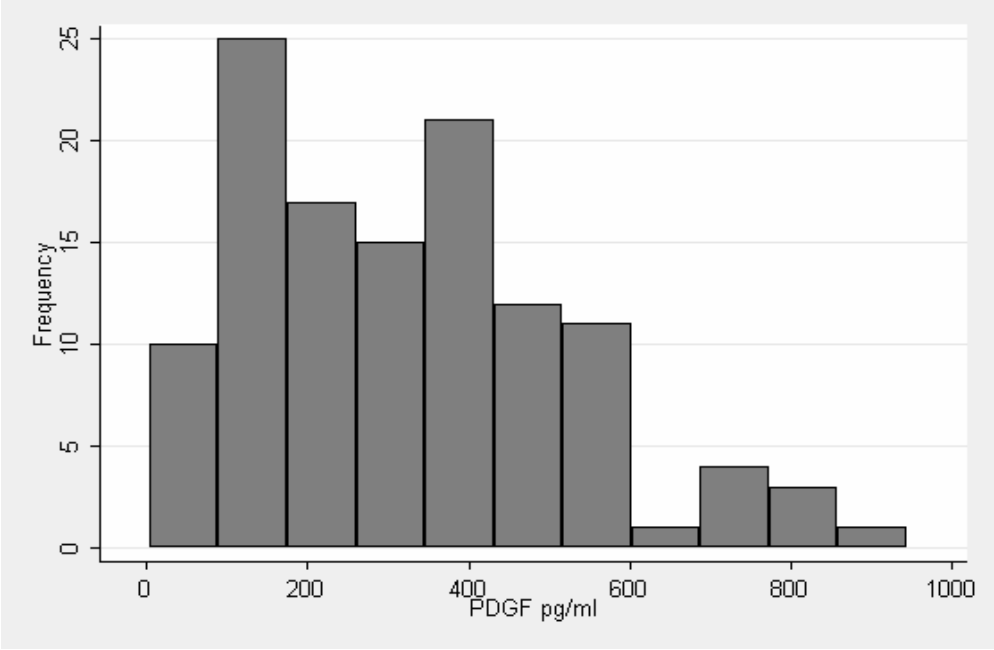


Figure 34: Histogram of PDGF results

The PDGF results were skewed to the right with a few high PDGF values (figure 34). Based on this and the Shapiro-Wilk test results, the analysis was completed using non-parametric statistics.

A significant increase was seen between levels of PDGF in the controls and in gold miners ($p < 0.01$). HIV status did not play a role in PDGF levels in this study ($p = 0.69$) (Table 65).

Table 65: PDGF results for the study participants.

Participants	n	Mean (95% CI)	Median	IQR	Range
Miners	60	232.8 (194.0 -271.7)	194.4	120.4 –338.9	28.3 – 719.5
HIV+ miners	20	210.6	161.6	102.1 -351.9	28.3 – 719.5
HIV-miners	38	246.9	208.4	132.1 –325.9	58.2 – 509.7
Controls	60	411.3 (360.0 -462.5)	400.0	276.1-525.3	40.8 – 858.9
HIV+ controls	33	382.8	397.2	286.9 -524.4	61.9 -741.6
HIV-controls	27	434.6	402.9	272.2 –549.2	40.8 – 858.9
Total	120	322.1(286.5-357.7)	288.4	162.6-443.3	28.3- 858.9

There was no significant difference between the PDGF levels in current smokers and non-smokers in this study (p = 0.999 rank sum test) (Table 66).

Table 66: PDGF levels in current smokers and non-smokers.

Participants	n	Mean	SD	Median	IQR	Range
Current smokers	25	338.6	231.5	311.1	120.7 – 472.2	40.8 - 855.7
Non-smokers	94	323.4	185.0	289.6	171.2 – 434.3	28.6 – 858.9

There was no significant difference between PDGF levels in miners with and without silicosis p = 0.5557 (Table 67).

Table 67: PDGF levels in miners with and without silicosis.

Participants	n	Mean	SD	Median	IQR	Range
Non-silicotics	41	238.6	155.2	203.6	120.7 – 351.9	3.5 – 719.51
Silicosis	16	212.7	127.7	174.3	125.3 – 267.4	28.3 – 520.61

In more severe (ILO radiographic score of 2/1 or greater) silicosis the serum PDGF levels appear to be higher. This was not statistically significant most likely due to the small numbers: only 3 severe silicotics (> ILO 2/1 score) (p = 0.2429 rank sum). This study was not designed to investigate markers of progression of silicosis, and did not actively recruit silicosis patients as such (table 68).

Table 68: PDGF levels in miners with silicosis.

Participants	n	Mean	SD	Median	IQR	Range
Non-silicotics	41	238.6	155.2	203.6	120.7 – 352.8	3.5 – 719.51
Silicosis > 1/1	11	178.6	92.9	171.8	102.1 – 225.0	28.26 – 380.3
Silicosis > 2/1	3	306.7	201.3	278.4	121.1 – 520.6	121.1 – 520.6

4.3.4 Summary of results

The overall results from this chapter investigating the cytokines IL-8 and TNFα and the growth factor PDGF with exposure to RCS, silicosis, smoking and HIV are presented in Table 69.

Table 69: Summary of results.

	IL-8		TNFα		PDGF	
Participants	n	mean	n	mean	n	mean
Exposure						
Miners	61	9.2	62	8.3	60	232
Controls	60	10.5	62	9.2	60	411
P value		0.28		0.99		<0.01*
HIV status						
HIV+ miners	21	13.21	22	10.65	20	210
HIV- miners	39	8.24	39	7.15	38	246.9
P value		0.01*		0.04*		0.46
HIV+ controls	27	13.76	27	11.91	27	382.8
HIV - controls	33	7.88	35	7.13	33	434.6
P value		<0.01*		<0.01*		0.44
Smoking						
Current smokers	24	8.7	24	7.5	25	338
Non-smokers	94	10.4	99	8.9	94	323
P value		0.38		0.14		0.7
Silicosis						
Non silicotics	41	8.3	41	7.6	41	238
Silicotics	16	15.1	17	9.3	16	212
P value		0.04*		0.16		0.55

*Significant difference

- IL-8 did not show a significant difference between miners and controls with only a small actual difference between the levels. There was a significant difference between HIV - positive and negative participants with an increase in IL-8 levels seen in HIV- positive miners and controls. Smoking did not show any significant effect on IL-8 levels. Silicosis status did affect IL-8 levels significantly where those with silicosis have shown higher levels of IL-8 than those having no silicosis.
- TNFα showed no significant differences between RCS exposed miners and unexposed controls. HIV status showed significant differences, with HIV-positive participant's TNFα levels higher than those of the HIV-negative

participants in both miners and controls. TNFα levels showed no difference between miners with and without silicosis.

- PDGF showed a significant difference between miners and controls with an unexpected increase in PDGF in controls. The levels were not affected significantly by HIV status smoking and silicosis.

In summary none of the markers of inflammation appear to be useful as markers of harmful silica exposure in an HIV prevalent region.

4.4 Discussion

Inflammation plays an important role in the pathogenesis of silicosis. Markers of inflammation may indicate progression towards the development of silicosis. Reynolds (2005) postulated that uncontrolled inflammation leads to damage of the lung tissue and repair initiating the fibrotic pathway.

In humans, the usefulness of anti-inflammatory treatments in silicosis has been investigated but no significant advantages have been demonstrated (Mason *et al.*, 1999).

The value of the increase in inflammatory markers seen in patients with silicosis may be in early detection of harmful RCS exposure and not in the treatment of silicosis. Thus three cytokines, namely TNFα, IL-8 and PDGF, were identified as promising markers to investigate based on reports in the literature (Gulumian *et al.,* 2006).

TNFα

The current study was unable to demonstrate any significant increase in plasma levels of TNFα in RCS-exposed miners compared to RCS-unexposed controls. Earlier studies have identified an increase in TNFα which was measured *ex vivo* in stimulated isolated inflammatory cells (Gosset *et al.,* 1991; Driscoll *et al*., 1995). The increase in TNFα may be limited to the lung and more likely to be detected in BALF. Both, Zhai *et al.,* (2004) and Vanhee *et al*., (1994) reported an increase in TNFα in the BALF of patients with silicosis or pneumoconiosis patients compared to healthy controls.

The effect of a diagnosis of silicosis on TNFα levels in RCS exposed miners was also investigated in the present study and it was found that there were no significant differences in TNFα levels in miners with and without silicosis. Although the patients with more severe silicosis could show a significant increase in TNFα levels compared to those with early silicosis and the control miners. There were few miners with severe silicosis in this study and this may explain why no overall effect due to silicosis was seen.

Confounders

In the current study HIV status showed a significant effect on TNFα levels where the HIV-positive subjects, both miners and controls, could show a significant increase in TNFα levels. This increase suggests that TNFα is likely to be confounded by HIV status.

TNFα levels in this study in smokers not significantly different to non-smokers, but a small decrease in levels was noted. This trend was in accordance with the observations reported by Keman *et al*., (1997) who showed a significant decrease in TNFα levels among current smokers. The correlation between TNFα levels and pack-years in their study indicated a possible dose response between TNFα release and smoking (Keman *et al.*, 1997). Similarly age was showed no significant effect on TNFα levels in this study.

IL-8

For its crucial role in lung inflammation, IL-8 has been suggested as a useful marker for dust exposure. The present study aimed to investigate the applicability of plasma IL-8 levels in South African gold miners as a marker of RCS exposure. The low plasma IL-8 values measured in the current study indicate that plasma IL-8 on its own is not likely to be a good biomarker for lung inflammation. Keman *et al.,* (1997) also reported that IL-8 was below the detection limit in human serum. Other studies have measured IL-8 release from whole blood, or isolated lymphocytes to overcome the low levels present in plasma, or focused on cytokine levels in BAL fluid (Keman *et al.,* 1997 and Zhai *et al.,* 2004).

Similar to the TNFα results discussed earlier, this study was unable to demonstrate any significant difference in the plasma levels of IL-8 in RCS-exposed miners compared to RCS-unexposed controls. On the other hand when IL-8 levels were compared between miners with and without silicosis, the significant difference found may suggest that IL-8 levels may be related to the disease progression rather than early marker to exposure to RCS. This observation could confirm those made in the literature indicating that IL-8 levels may only increase once the progression to

pneumoconiosis is well developed (Lee *et al*., 2010) and where a relationship between IL-8 levels and disease symptoms could be observed (Keman *et al*., 1997)

Confounders

HIV infection has been shown to alter cytokine levels and responses (Wilson, 2002; Wilson, 2009). In the current study, a significant difference in IL-8 serum levels was found in HIV positive and negative participants, similar to the increase seen in TNFα levels in the HIV-positive miners and controls in this study. Matsumoto *et al.,* (1993) suggested that overproduction of IL-1 and TNFα in HIV infection may increase the serum levels of IL-8 after having demonstrated a significant increase in IL-8 levels in HIV-positive donors. The increase seen by Matsumoto *et al*., (1993) in IL-8 levels was not related to disease severity but only to HIV-status, suggesting that the HIV infection was responsible for the increased release of IL-8.

In the present study, a non-significant decrease in IL-8 levels was seen in the current smokers in this study. On the other hand, age was found to be significantly related to IL-8 levels in HIV-negative miners, while the lack of significant difference seen in the HIV negative controls was most likely to be due to the lack of older participants in that group. A relationship between IL-8 level and age has been found in other studies (Wieczorowska-Tobis *et al.,* 2006; Boekholdt *et al.,* 2004) which would therefore limit the applicability of IL-8 as a marker for RCS exposure. IL-8 is not recommended for further study as a biomarker for RCS exposure.

PDGF

PDGF is an important cytokine involved in wound healing. The current study found a significant difference in PDGF levels of RCS-exposed miners compared to the unexposed controls. In contrast, a number of investigators reported an increase in PDGF levels in patients with pneumoconiosis (Vanhee et al ., 1994; Yao et al., 2006) as well as in cultured fibroblasts exposed *ex- vivo* to silica stimulated AM secretions (Wang *et al.,* 2009).

The current study, on the other hand, showed no significant difference in the PDGF levels of miners with and without silicosis. However, when miners with silicosis were further divided into two groups, one with simple silicosis and the other more severe

silicosis, there was a trend towards higher PDGF levels in those with severe silicosis. confirming previous observations (Brandt-Rauf et al., 1992).

Results obtained from this study and those presented in literature may indicate that PDGF may not be a good biomarker for RCS exposure but may be a good biomarker for the progression of silicosis.

Conclusion

The use of cytokines as markers for RCS exposure is limited for a number of reasons. HIV has been shown to result in increased TNFα and IL-8 expression and therefore it is an important confounder of the measurement of the relationship between cytokine levels and RCS dust exposure. There may also be possible confounding effects of smoking and age.

The only differences in cytokine levels in miners are observed late in the disease process and are not likely to provide information on harmful effects early after exposure.

CHAPTER 5

Lung Damage Marker

5.1 Introduction

5.1.1 Markers of Lung Damage

Assessment of lung damage is important in occupational health as the lung is the primary target of many toxicants (Mendelsohn *et al.*, 1998). As most cells in the lung release their products into the alveolar space or the bronchioles, often recommended biomarkers are constituents of the epithelial lining fluid (ELF). To sample the ELF requires a BAL.

The lung epithelium produces epithelial lining fluid, consisting of surfactant, mucus, and host defence proteins, to protect the lung and assist with gas exchange. Some of these proteins from the ELF are able to cross the bronchoalveolar-blood barrier, and can be detected at low levels in the blood. Few biomarkers found in the serum or urine have been described as specific to the respiratory tract, which may be used as a useful biomarker of lung damage. Such biomarkers are CC16, surfactant-associated proteins and mucin-associated antigens which are the three types of ELF proteins that are found in the blood that may act as good biomarkers of lung damage (Hermans *et al.*, 1999).

5.1.2 Clara Cell Protein

CC16 has been proposed as a biomarker of early effect of silicosis. It is a low molecular weight protein that can be measured in BAL, serum, urine and sputum (Bernard *et al.*, 1992a, Bernard *et al.*, 1992b, Bernard *et al.*, 1993). CC16 is a 15.840 kDa homodimer as determined by electrospray ionisation / mass spectrometry (Bernard *et al.,* 1993). The protein has a globular appearance with several H-bonds and Van der Walls interactions maintaining the structure. It has 70 amino acid

residues in 2 chains that are connected in an antiparallel direction by 2 disulphide bonds (Figure 35). The two chains consist of four α-helical segments (Miele *et al.*, 1987).

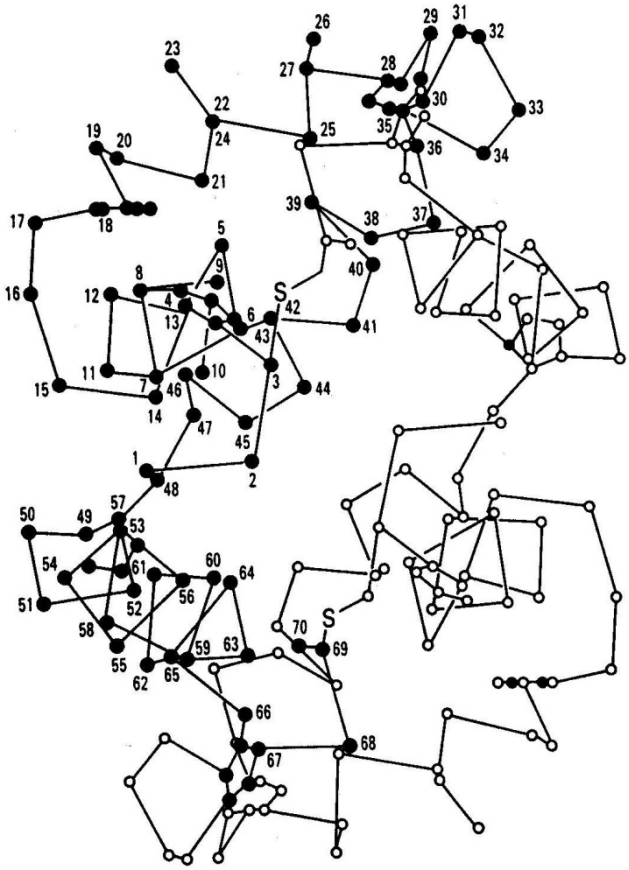


Figure 35: Structure of CC16 dimer showing only α-carbons and disulphide bonds. The numbers 1-70 identify the amino-acid subunits and ● represents the carbons of one chain while ○ represents the carbons of the second chain. (Taken from Miele *et al.*, 1987)

This protein was first described in the urine of rabbits and then the lungs of rodents as uteroglobin (Singh *et al.*, 1985). CC16 from humans shows sequence homology with uteroglobin from other mammals (Miele *et al.,* 1987; Singh *et al.*, 1990 and Hermans and Bernard, 1999). The size of this protein was originally determined on SDS PAGE and as it shows an anomaly in its electrophoretic mobility, was wrongly given as a size of 10 kDa. Based on this error, some literature refers to CC16 as Clara cell protein 10 (CC10) or Clara cell secretory protein. The genes coding for the two proteins of CC16 have been localised on chromosome 11-region p12-q13. This is a region that has been described as containing genes involved with the regulation of

inflammation (Wolf *et al.*, 1992 and Hay *et al.*, 1995). CC16 DNA expression is mainly found in lung tissue, which justifies its use as a lung-specific protein.

CC16 has been shown to be secreted by Clara cells which are non-ciliated bronchiolar epithelial cells. They are found lining the pulmonary airways, particularly the small bronchi and respiratory and terminal bronchioles (Singh *et al.,* 1985; Cross *et al.*, 1994 and Singh and Katyal, 1997). Clara cells were first described in animals in 1881 and in humans in 1937 (Plopper, 1997). Clara cells contribute to the maintenance of the ELF in the lung as they secrete non-mucoid substances (Mango *et al.*, 1998). Clara cells also play an important role in the regeneration of epithelium in rodent lungs and may play a similar role in the human lung. Albrecht *et al*. (2001) exposed rats to quartz, and following damage to the alveolar epithelium, demonstrated Clara cell hyperplasia using histopathology. The authors have suggested that these hyperplastic Clara cells migrate in, and act as precursor cells for, regenerating alveolar epithelium. Albrecht *et al.*, (2001) suggests that the Clara cell response is not to the presence of particles but to the damage of the alveolar epithelium.

CC16 stores in Clara cells have been described within the endoplasmic reticulum and the secretory granules (Figure 36) (Bedetti *et al.*, 1987 and Singh *et al.*, 1988). Singh *et al.*, (1986) found an increase in CC16 with an increase in the volume density of secretory granules in Clara cells.

The CC16 concentration in BAL was assessed to be 7.2% of the total protein content; this makes it one of the most abundant proteins produced locally in the lungs (Singh *et al*., 1988).

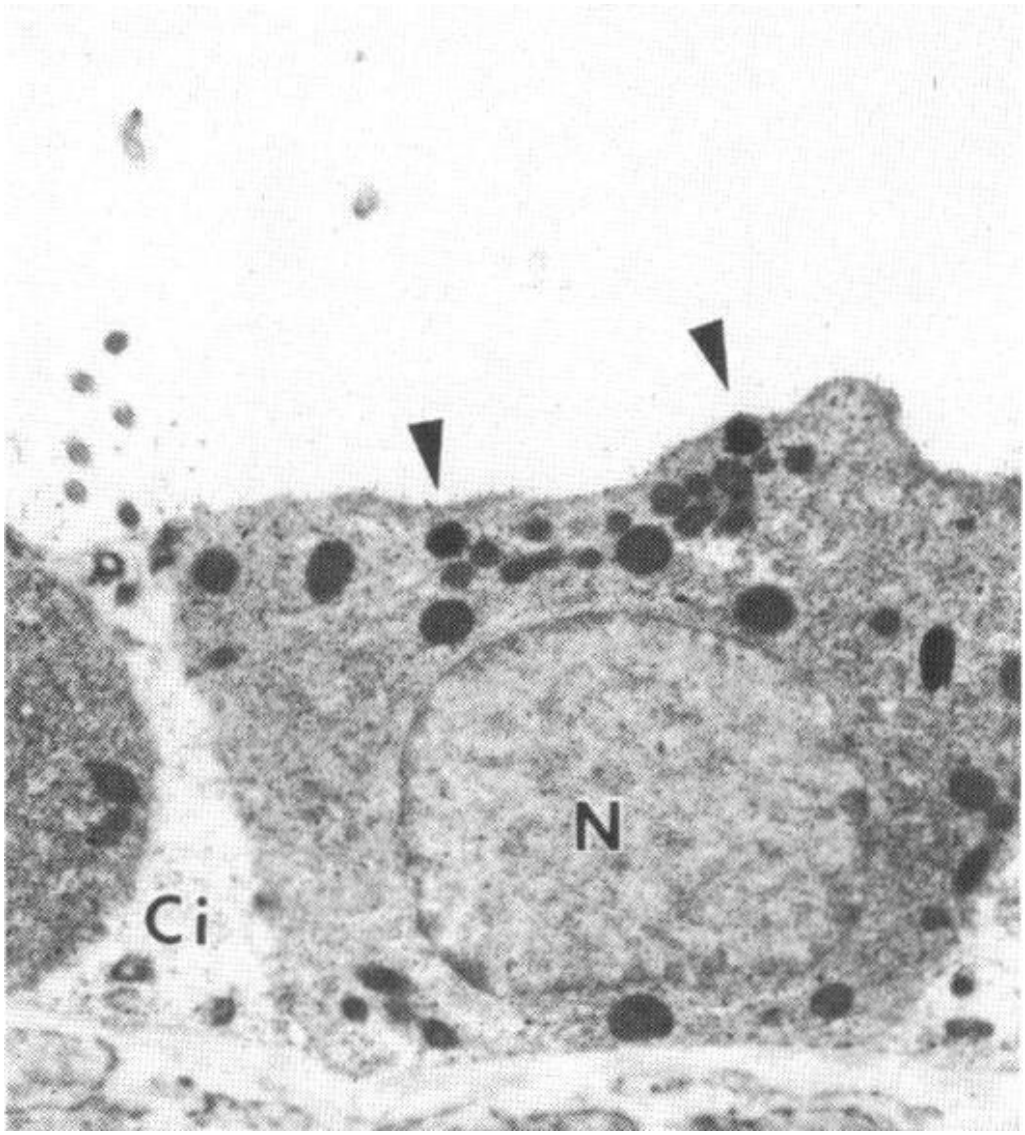


Figure 36: Transmission electron microscopy image of Clara cell with gold-labelled Clara cell protein (CC16) (From Patton et al., 1991). N = nucleus, Ci = ciliated columnar cell. Arrows indicate gold labelling of CC16 in secretory vesicles.

The exact function of CC16 in the lung is still subject to some speculation. A role for Clara cells and CC16 in protection of the lung against oxidative stress has been suggested by a number of studies (Broeckaert and Bernard, 2000 and Broeckaert *et al.*, 2000). The number of Clara cells increased following chronic ozone exposure and tolerance to exposure was associated with increases in BAL CC16 (Pinkerton *et al.*, 1993; Plopper *et al.*, 1994a; Plopper *et al.*, 1994b). Experimental evidence indicates that Clara cells are very sensitive to pneumotoxic chemicals (Bernard *et al.,* 1992b).

It has been shown that CC16 plays a role in tumour suppression and immunosuppression, acting as an anti-inflammatory and antifibrotic agent (Wang *et*

al., 2007). CC16 has been shown to inhibit the activity of phospholipase A_2, an inflammatory enzyme (Miele *et al*., 1987). Dierynck *et al.*, (1995) demonstrated the ability of CC16 to inhibit the production of Interferon γ (IFNγ) a pro-inflammatory cytokine (Prasanna, 2010).

Researchers have used CC16 deficient mice to investigate the anti-inflammatory functions of CC16. The CC16 deficient mice showed an exaggerated inflammatory cytokine response after being exposed to ozone along with an increased oxidative stress response. These mice showed significant increases in IL1β, IL3, IL6, (pro-inflammatory cytokines), metallothionein (an early stress response protein) and mRNA levels after ozone-stimulated pulmonary stress was induced compared with mice containing the CC16 genes (Johnston *et al.*, 1998 and Mango *et al.*, 1998). There is also an antifibrotic role for CC16. CC16 has been shown to inhibit the PDGF-induced chemotaxis of fibroblasts (Lesur *et al*., 1995). The anti-inflammatory function of CC16 was also suggested by Miele *et al*. (1987) who have demonstrated the ability of uteroglobin to inhibit monocyte and neutrophil chemotaxis and phagocytosis *in vitro*.

CC16, due to its small size, is able to diffuse passively across the bronchoalveolar-blood barrier into the plasma. Levels of CC16 in BAL were shown to be 40 times greater than levels measured in the serum. This indicates that the protein does not diffuse actively across the blood vessels. CC16 in the serum has been shown to be correlated with CC16 concentration in BAL fluid, indicating that serum levels are good markers of levels in the lung (Bernard *et al.,* 1992a, Shijubo *et al.*, 1997). The levels of CC16 in BAL and in the serum mirror each other in non-smokers, smokers and COPD patients (Shijubo *et al.,* 1997).

With smoking, the levels of CC16 were reported to decrease with no significant change in B_2-microglobulin (another lung protein that diffuses passively across the bronchoalveolar-blood barrier). This shows that smoke pneumotoxic chemicals are able to progressively destroy Clara cells and this can cause a decrease in the levels of circulating CC16 (Bernard *et al.*, 1992b).

RCS has been shown to decrease CC16 levels in two studies. Bernard *et al.*, (1994a) showed a clear decrease in CC16 in the serum of asymptomatic RCS-exposed workers. This research suggests that RCS exposure causes a decrease in serum CC16 before significant effects on the lungs can be detected by chest X-ray examination or lung function tests. The cause of the decrease in CC16 has been suggested to be a decrease in secretion of the factor or loss of the Clara cells.

The second study published on CC16 and RCS exposure was by Wang *et al*., (2007). They investigated the effect of RCS exposure in pyrite miners with and without silicosis. A significant difference in serum CC16 levels was found between the miners who were exposed to RCS and the unexposed controls ($p = 0.000$). No significant difference was found in CC16 levels of the RCS-exposed miners with and without silicosis. The study found a significant correlation of years of exposure and stage of silicosis, but no correlation between CC16 levels and years of exposure was found. This study also looked at surfactant protein D as a marker for stage of silicosis. Both Bernard *et al*., 1994a and Wang *et al*., (2007) compared workers with unexposed controls and reported a significant decrease in CC16 in RCS exposed workers.

CC16 was therefore identified as a possible early biomarker of effect of RCS exposure (Gulumian et al 2006).

5.1.3 Confounders

The main confounder of using CC16 levels to monitor RCS exposure is smoking. A number of studies have demonstrated the effect of smoking on CC16 levels in both BAL fluid and the serum (Bernard *et al.,* 1992b and 1994b and Shijubo *et al.,* 1997). Smoking decreases CC16 and also damages the Clara cells.

Damage to renal function maybe another confounder of CC16 as a RCS exposure biomarker. As CC16 is removed from plasma by glomerular filtration, a decrease in the glomerular filtration rate will result in an increase in CC16 in the serum. The renal tubules resorb CC16 and any decrease in their capacity will result in a decrease in CC16 levels in the serum (Bernard *et al.,* 1994c). Serum B_2-microglobulin levels are monitored simultaneously as a measure of renal function to ensure that changes in serum CC16 are not due to changes in renal function (Hermans *et al.*, 1998b).

Age does not appear to influence CC16 (Hermans *et al.*, 1998a) and in serum, as in BAL, there is no significant difference between men and women (Bernard *et al.*, 1994c).

Other occupational exposures have also been shown to result in significant changes to CC16 levels, such as welding fumes, glutaraldehyde and aluminium smelting (Halatek *et al.*, 2005). Moderate and high occupational exposures to diesel exhaust fumes have been shown to significantly lower CC16 levels with an increase in lung injury and inflammation (Gowdy *et al.*, 2008).

Bernard et al., (1992b and 1994b) demonstrated that there was a relationship between asthma and ozone pollution and smoking and CC16. Other occupational exposures have been shown to affect CC16 and may be confounders for RCS exposure (Halatek et al., 2005). Thus CC16 as a marker is not likely to be specific for RCS exposure. However, RCS is the main exposure on a gold mine and thus CC16 may be a good marker for lung damage in miners

5.2 Methods

5.2.1 Theoretical background of the assay implemented

CC16 can be assayed using an ELISA method. This method allows the specificity of dedicated monoclonal antibodies to the CC16 protein to be combined with the sensitivity and ease of use of spectrophotometry (Halatek *et al.*, 1998).

In the CC16 assay a biotin label is used. Biotin is a low molecular weight antibody that is able to bind strongly to streptavidin, which is conjugated to the enzyme horseradish peroxidase. Biotin amplifies the signal produced by each labelled antibody as multiple biotin molecules can be linked to each antibody and many molecules of avidin can bind to each biotin molecule. This is important for the detection of low concentrations of CC16 normally found in human serum.

Horseradish peroxidase is a stable active enzyme that with hydrogen peroxide oxidises either 3-amino-9-etylcarbazole or 4-chloro-1-naphthol into insoluble brown

or blue products, respectively. The reaction is halted by changing the acidity of the solution and by denaturing the enzyme. These products can then be detected spectrophotometrically, and under standard conditions are proportional to the amount of specific antigen in the solution.

5.2.2 Assay practical

5.2.2.1 Sample collection

Venous blood samples were collected from the volunteers in 6 ml plastic serum vacutainers (BD Vacutainer Systems). The tubes were centrifuged in Eppendorf for 10 minutes at 1 000 x g. The serum was drawn off the top of the vacutainer into a 2 ml Nunc cryotube where it was frozen at –70 °C until the day of the assay. Unfortunately, the time between collection and storage was not always consistent. Due to field conditions, some of the samples remained at room temperature for longer than others before being centrifuged. All samples were always processed within 12 hours of collection.

5.2.2.2 Sample and reagent preparation

This protein was assayed with a Clara Cell protein BioAssay ELISA kit supplied by US Biological, USA. The supplied reagents were brought to room temperature prior to the assay. The samples were removed from the freezer and also brought to room temperature. Once thawed and mixed, the samples were diluted 1:25 with supplied dilution buffer (10 µl of sample was mixed with 240 µl dilution buffer).

The standards were prepared as follows: the master calibrator was reconstituted with 500 µl dilution buffer (both supplied) to give a 100 ng/ml stock solution. Then a dilution series followed to produce the rest of the calibration samples. Specifically, 200 µl of 100 ng/ml calibrator stock (standard 1) was diluted with 300 µl of dilution buffer to give 40 ng/ml (standard 2); 200 µl of standard 2 was diluted with 200 µl dilution buffer to give the 20 ng/ml standard (standard 3). A further 200 µl of standard 3 was diluted 2 times to give a 10 ng/ml standard (standard 4) which in turn provided 200 µl to be diluted 2 times to give the 5ng/ml standard (standard 5). From this last standard 200 µl was diluted with 300 µl to give a 2 ng/ml standard (standard 6).

The standards were then further diluted 1:25 times with dilution buffer before assaying. The low- and high-quality controls provided were also diluted with dilution buffer 1:25 times.

5.2.2.3 Assay

A 100 µl each of the standards, CC16 controls and samples were pipetted in duplicate into the polyclonal, rabbit anti-human CC16-coated plate provided in the kit. The plate was incubated for one hour at room temperature. The plate was then washed with the wash buffer provided (which was prepared by diluting with distilled water to give a 1 times solution), using a manual plate washer. The plate was washed 3 times; each time the solution in the wells was aspirated and then filled again with wash buffer. After washing, a 100 µl of supplied Biotin Labelled Antibody was placed in each well, and then incubated for 1 hour at room temperature. The plate was washed again three times with the wash buffer. Then 100 µl of streptavidin (conjugated with Horseradish Peroxidase) was added to each well and the plate incubated for 1 hour at room temperature. The plate was washed three times with wash buffer. A 100 µl of substrate (hydrogen peroxide-tetramethylbenzidine) was added and the plate incubated at room temperature in the dark for 10 minutes. Colour development was halted with 100 µl of stop solution, which was used as provided.

The absorbance of each well in the plate was then determined at 450nm using a plate reader spectrophotometer.

The calibration curve was then constructed using a four-parameter function to plot the measured absorbance versus the known concentrations on a log scale (Figure 37). This function was provided by the KC4 software purchased with the spectrophotometer. If it was not possible to use the KC4 software, then a logit-log curve was plotted. The concentrations of the samples were then determined from the standard curve by the software.

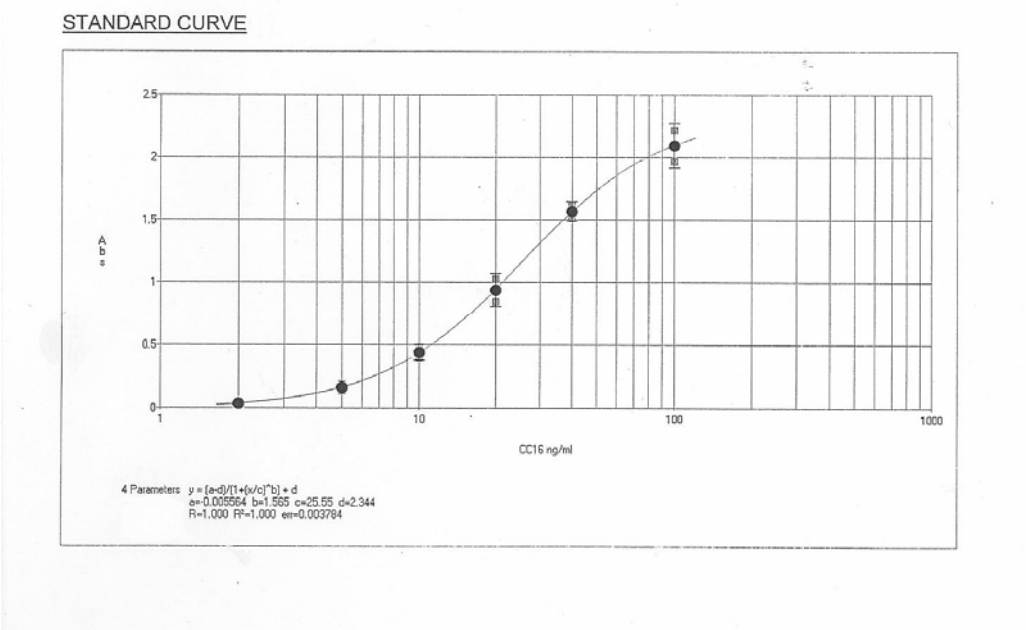


Figure 37: A 4-parameter standard curve for CC16 from the ELISA assay

5.2.2.4 Statistical Analysis

STATA 8 was used to analyse the results of the study. The means, medians and standard deviations of the data were then determined for each of the study groups. An alpha level of 0.05 was used for all statistical tests. The data were analysed in the same manner as the previous chapters. Based on this, the CC16 data were transformed by dividing numerical one by the square root of the raw CC16 data.

In the original CC16 data for the HIV-positive controls, the standard curve values did not reach recommended levels in the lower portion of the curve, so this test was repeated. New HIV-positive controls were collected and the data from the new standard curve was used.

Multiple linear regression was performed on the CC16 levels with all variables collected entered along with any logical interaction terms. Age was entered as a continuous variable. Variables were included on the basis of plausibility and excluded in a backwards stepwise manner if removing the variable did not affect the coefficient for exposure and CC16, and if the pseudo R^2 was not changed. Regression was only performed for CC16 as it showed promise and the data was suitable for regression.

5.3 Results

5.3.1. Test for normality of data and suitability of parametric tests

The Shapiro-Wilk test for normality of the CC16 data shows that the data are not consistent with a normal distribution ($p < 0.01$) (Table 70). The null hypothesis of this test was that the data are normally distributed. At an α of 0.05 we reject the null hypothesis and so this sample is not from a normal distribution.

Table 70: Normality test of CC16 values.

Shapiro-Wilk W test for normal data					
Variable	Obs	W	V	z	Prob>z
CC16	118	0.91618	7.952	4.641	0.00000

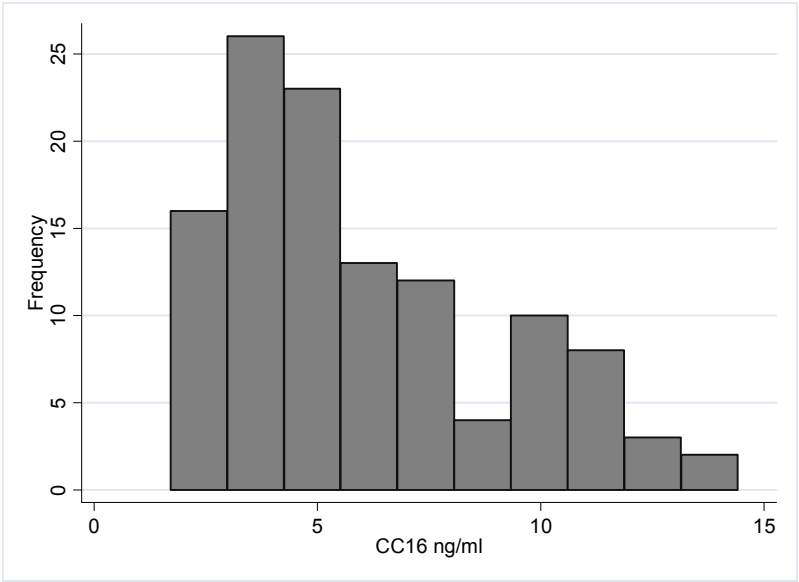


Figure 38: Histogram of all CC16 values

This was confirmed by the histogram, which shows a long tail skewed to the right (Figure 38). Non-parametric hypothesis testing and modelling with this data after transformation are necessary.

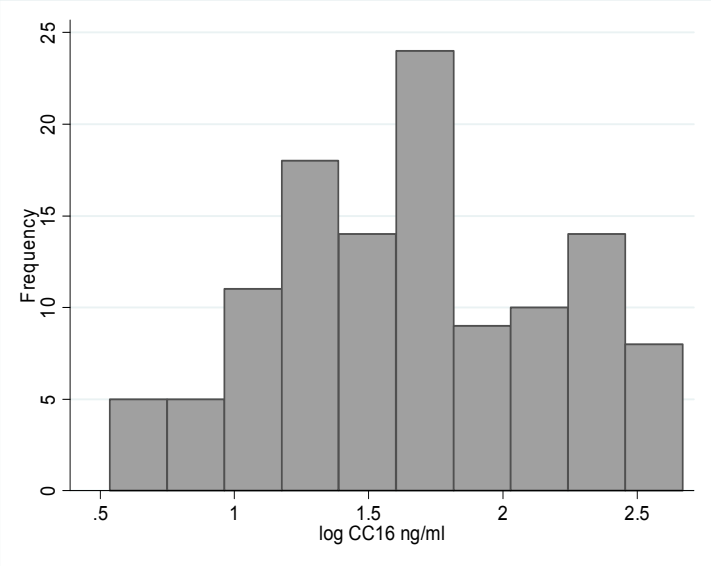


Figure 39: Histogram of log transformed CC16 values (logCC16)

A Shapiro-Wilk test for normality showed that the log transformed values are compatible with having been drawn at random from abnormally distributed population (p = 0.08) (Table 71) along with an acceptable histogram (Figure 39). This transformation of the data is acceptable for modelling as the p value is above the rejection value of 0.05. Thus, log transformation of the data was indicated for linear regression analysis.

Table71: Normality test results after transformation of data.

Shapiro-Wilk W test for normal data					
Variable	Obs	W	V	z	Prob>z
LogCC16	118	0.97996	1.901	1.438	0.07526

5.3.2 Description of results of CC16 analysis

Serum samples were collected from 116 participants and analysed for CC16 level. The results were divided into those who were exposed to RCS and those who reported no exposure to RCS. These two groups were then further divided into HIV-positive and HIV-negative groups.

Table 72: Description of CC16 levels determined in the samples by ELISA.

Group	No.	Mean	Median	Standard Deviation	IQR	Min	Max
HIV+ Miners	23	6.24	4.56	3.31	6.41	1.91	11.30
HIV- Miners	40	5.18	4.92	2.73	2.83	1.89	12.54
Miners	63	5.56	4.92	3.19	3.78	1.89	12.54
HIV+ Controls	17	6.59	5.8	3.45	5.28	2.87	14.42
HIV - Control	36	6.47	5.36	3.16	3.62	1.71	14.01
Controls	53	6.55	5.65	2.95	4.30	1.71	14.42
Total	116	6.05	5.28	3.07	4.04	1.80	13.48

The CC16 values for all the groups show variability with a range from 1.71 ng/ml to 14.42 ng/ml (Table 72). The data are not normally distributed, and as such the mean values are influenced by the outliers and are higher than the medians. The medians of

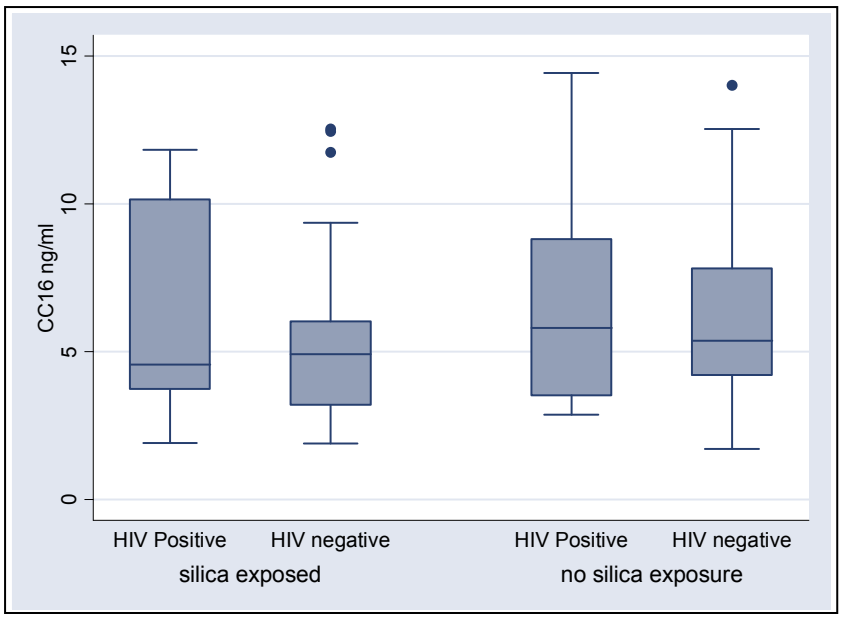


Figure 40: Box plot of CC16 levels for RCS-exposed and RCS-unexposed and HIV-positive and HIV-negative groups

the RCS-exposed groups are lower than the RCS non-exposed groups. The interquartile range is larger for the two HIV-positive groups than the HIV-negative groups, although the medians do not differ greatly (Figure 40). The maximum values for the RCS-exposed miners are lower than the RCS non-exposed controls.

The CC16 levels of those exposed and unexposed to crystalline RCS were compared using the non-parametric Wilcoxon sign rank test (p = 0.10). There was a non-significant difference between the two groups. Smoking has been shown elsewhere to affect CC16 levels, so further analysis of CC16 was conducted using linear regression with the log transformed CC16 data to adjust for the effect of smoking on the results (Table 73).

Table 73: CC16 levels in smokers and non-smokers.

Participants	No.	Mean	SD	Median	IQR	Range
Non-smokers	90	6.46	3.13	5.51	3.99 – 8.29	1.71 – 14.42
Current smokers	23	4.14	2.21	3.44	2.48 – 5.32	1.91 – 10.5

A significantly lower level of CC16 was found in smokers (Wilcoxon rank sum p = 0.0020).

There was no difference in levels of CC16 in miners with and without silicosis. The significance was p = 0.8512 following a Wilcoxon rank sum test (Table 74).

Table 74: CC16 levels in miners with and without silicosis.

Participants	No.	Mean	SD	Median	IQR	Range
Silicosis	16	5.34	2.86	4.94	3.25 – 6.05	2.04 – 12.54
No silicosis	43	5.69	3.06	4.67	3.33 – 8.02	1.89 – 12.45

CC16 and age were not normally distributed, thus a Spearman's correlation was used to determine if there was a relationship between CC16 levels and age (Table 75).

Table 75: Spearman's correlation of age and CC16 levels.

	Corr co	P
Age	0.0007	0.9939

No correlation between CC16 levels and age was found (Figure 41, Table 75).

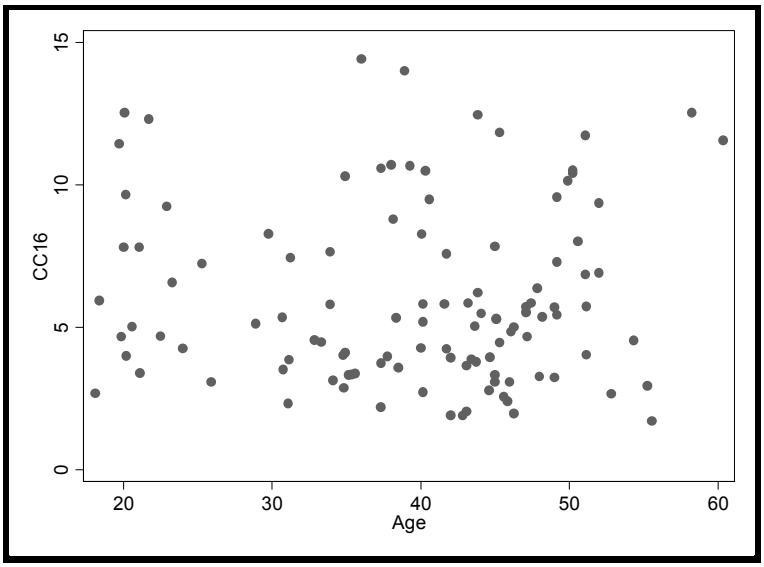


Figure 41: Scatter plot of CC16 and age of all participants showing no correlation

CC16 was not normally distributed, while years of RCS-exposed work were, so Spearman's correlation was again used to test for a trend in CC16 levels with increasing years of exposure (Table 76).

Table 76: CC16 correlation of years of exposure and CC16 levels.

	Corr co	P
Years of exposure	0.07	0.57

No correlation between CC16 levels and years of RCS-exposed work was found (Table 76, Figure 42).

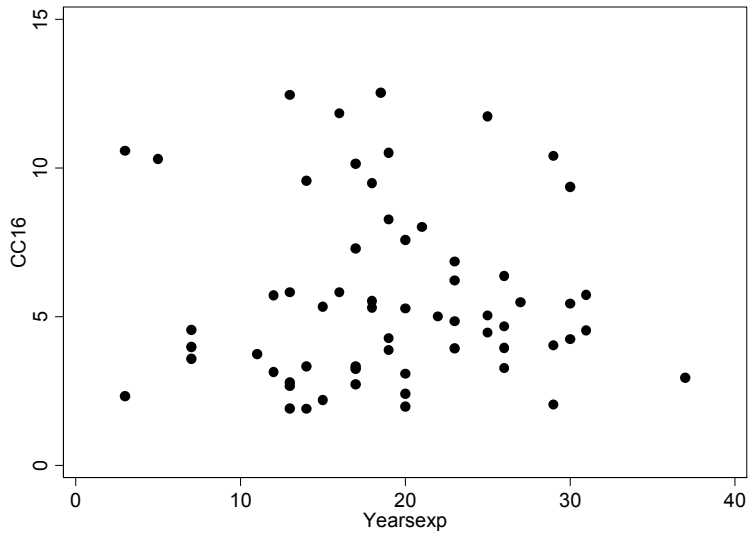


Figure 42: Correlation of CC16 levels and years of exposure

5.3.3Linear regression analysis

Stepwise linear regression was used to examine the factors that might explain the variation in CC16 levels. It also allowed us to determine which factors significantly affect CC16 with adjustment for confounding.

Formulated model 1: logCC16 = $\beta o - \beta_1$exposure $- \beta_2$smoke $+ \beta_3$age $+ \beta_{4HIV}$.
Formulated model 2: logCC16 = $\beta o - \beta_1$exposure $- \beta_2$smoke $+ \beta_3$age $+ \beta_{4HIV} - \beta_5$hiv*smoke $+ \beta_6$ exposure *age

Table 77: Results from Models of factors that affect CC16 levels.

Model	Prob > F	R - Squared	Adj R-squared
Model 1	0.0021	0.1427	0.1110
Model 2	0.0006	0.1965	0.1510

Model 1 was the first model containing all the determinants available to us based on literature, this model had a significant F-test of p = 0.0021 and an R-squared value of 0.14which indicates that this is a valid model but one that explained only 14% of the variance of the CC16 values (Table 77). The adjusted R-squared is used to compare models. Thus interaction terms based on plausibility were then introduced to model 2.

Model 2 also provided a significant F-test result of p = 0.0006 and an R-squared value of 0.20 which indicates that this is a valid model and explains 20%of the CC16 levels. The adjusted R-squared also increased thus indicating that the increase in percentage of variation explained was real and not only due to increasing the number of determinants (Table 77).

Model 2 was chosen as a better model of determinants of CC16 values in South African men.

Table 78: Coefficients and p values from the final model.

Determinant	Coefficient	Std Error	p value
RCS Exposure	-1.25	0.55	**0.024**
Smoking	- 0.24	0.14	0.082
HIV	0.18	0.11	0.090
Age	-0.01	0.01	0.603
HIV*Smoking	-0.52	0.26	**0.041**
Exposure*Age	0.02	0.01	0.064

RCS exposure was a significant determinant of CC16 levels in this model along with the interaction term HIV and smoking. This model indicated that the CC16 levels when adjusted for confounders were significantly lower in RCS-exposed miners (p = 0.024) than in non-exposed controls. The model also indicated that age was a non-significant determinant of CC16 levels in this group. The interaction term HIV and smoking was significant in this model indicating in those individuals with both HIV and smoking there was a small decrease in CC16 levels (Table 78).

Post regression tests of the residuals

These tests confirm whether the model fulfils the requirements of a good model.

Table 79: Test for the normal distribution of the residual.

Shapiro-Wilk W test for normal data					
Variable	Obs	W	V	z	Prob>z
residual	113	0.98809	1.090	0.191	0.42408

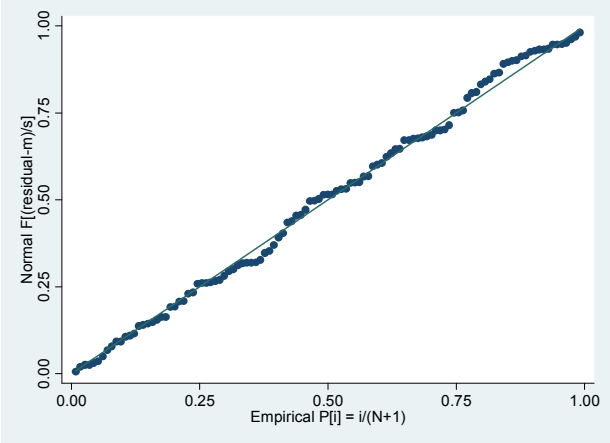


Figure 43: Normal probability plot of the residuals

The residuals were normally distributed which is a requirement for a good model. Both the sk-test and the plot of the residuals showed a normal distribution (Table 79 and Figure 43).

Breusch-Pagan / Cook-Weisberg test for heteroskedasticity (testing for constant variance) among the fitted values of logCC16 gave a p value of 0.1275. This indicates that we could not show variable variance in the residuals

Ramsey RESET test using powers of the fitted values of logCC16 to test that the model has no omitted variables, again the p value was non-significant Prob > F = 0.4770. This indicates that the test was unable to identify any missing explanatory variables in the model.

These post-regression tests indicate that the model is acceptable based on the available data.

5.3.4 Comparison of results with international studies

In the studies performed to date, the mean CC16 levels of the controls ranged from 10 up to 25.4µg/L (Table 80). The levels appeared to decrease with calendar year of study, which may be due to different standards prepared in different ways, or perhaps

Table 80: Comparison with other published levels of CC16.

Author	Controls		Patients/exposed		Comment
	No.	Mean µg/L	No.	Mean µg/L	
Bernard 1992a Chronic bronchitis	25+	25.4	6	9	Significant difference by disease status
Bernard 1992a Sarcoidosis	25+	25.4	17	41.5	Significant difference by disease status
Bernard 1992a Lung cancer	25+	25.4	18	15.5	Difference by disease status
Bernard 1993 Gender	55 male	21.7	59	27.9 female	No significant difference by gender
Bernard *et al.*, 1994b Smoking	86	16.3	86	12.3	Difference
Lesur *et al.*, 1995 Pulmonary fibrosis	23	18	29	33	Significant difference by disease status
Lesur *et al.*, 1995 Bleomicin injury	23	18	11	19	No difference
Bernard and Van Houte, 1996 smoke inhalation 20 min post exposure	6	19.5	6	54.4	Significant difference by exposure status
Bernard and Van Houte, 1996 10 days post smoke exposure	6	17.7	6	15.9	Small difference
Shijubo 1997 Smoking	107	11.7	104	7.9	Significant difference by smoking status
Janssen *et al.*, 2003 Sarcoidosis	38+	10.0	79	16.7	Significant difference by disease status
Berthoin *et al.*, 2004 forestry workers and police	Rural 38	12.4	Urban 34	11.8	Difference by rural or urban work
Wang, 2007 Silica exposure	11	12.6	11	4.42	Difference by exposure status
Present study RCS exposed	54	6.6		5.6	Difference by RCS exposure

+ values were divided by 3.23 because of the use of different standards.

the methods with which the levels were analysed have changed. The lower end of the range was close to our findings although the findings for our controls were still generally lower than those reported for controls internationally.

ROC (Receiver Operator Curve)

The ROC (Receiver Operator Curve) is a plot of the sensitivity versus specificity of a test. The area under the curve needs to be greater than 0.50 for the test used to be more predictive than just by chance. The area under the curve for CC16 was 0.58. It provided a best cut-off level of 5.82 µg/L of CC16 for separating the RCS-exposed miners from RCS–unexposed participants (Figure 44). This correctly classified approximately 60% of participants (see Appendix 1.5 for detailed results)

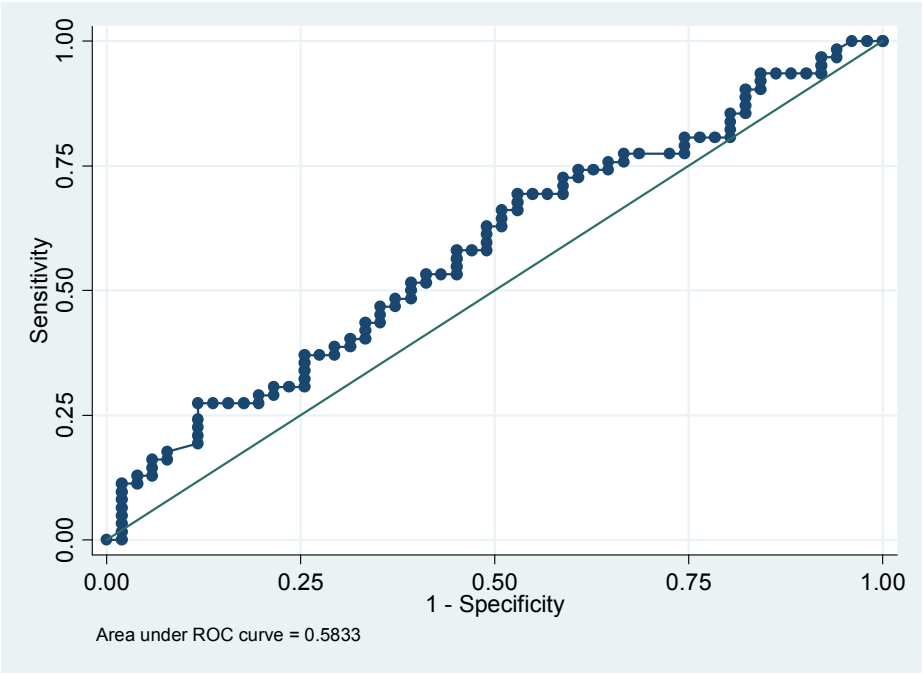


Figure 44: Receiver Operator Curve for CC16

5.3.5 Validation of CC16 results

Duplicate samples were sent to Professor A Bernard's laboratory in Belgium where the latex method was used to test the CC16 levels in the samples. This research group has published a number of papers on CC16 (Bernard et al., 1992a, 1992b, 1993, 1994a, 1994b and 1996).

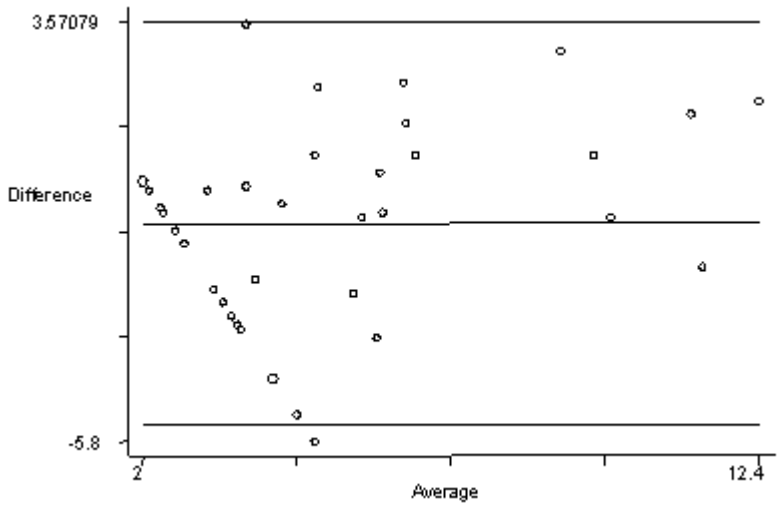


Figure 45: Scatter plot of the differences between the duplicate assays of CC16 samples

The distribution of the differences showed no bias within the sample differences (Figure 45). The Bland-Altman method showed a mean difference of – 0.9 μg/L between the repeat analyses of the samples. This is a small average difference and indicates that the CC16 tests performed in this study were reliable.

5.4 Discussion

To evaluate CC16 as a biomarker of early effect in this study, the serum levels of CC16 in RCS exposed miners and unexposed controls were compared. The arithmetic mean and median of CC16 levels for RCS-exposed miners were both lower than those measured for the controls. Our results were in agreement with those seen by Bernard *et al*., (1994a) in quarry workers and by Wang *et al*., (2007) in Chinese RCS-exposed miners.

On the other hand CC16 levels in miners with and without silicosis showed no significant differences. This observation was similar to those reported by Jin *et al*., (2009) who have seen no significant difference was seen in serum CC16 levels of non-CWP coal miners and miners with CWP.

The lower values of CC16 in RCS-exposed miners could be explained by mechanisms reported by Wang *et al*., (2007). These authors have proposed that RCS and the ROS it generates may directly damage the Clara cells and thereby decrease the production of CC16. The RCS may also affect the lymphocytes and epithelial cells to release a number of products which in turn affect the Clara cells and lead to a decreased production of CC16. Finally Clara cells may act as stem cells to repair chronic RCS-induced epithelial damage and hence no longer produce CC16

Confounders

In the present study, no significant effect due of HIV on CC16 levels was found in both RCS-exposed miners and unexposed controls with the exception of a small increase in CC16 levels in HIV- positive miners and HIV - positive controls. Also no effect of age and years of exposure to RCS on the CC16 levels in RCS- exposed miners and unexposed controls. However, a significant effect of smoking was found where a decrease in CC16 levels was seen in both RCS-exposed miners and unexposed controls confirming results seen in the literature (Bernard *et al*., 1994a; Shijubo *et al.*, 1997; Berthoin, 2004). This decrease was attributed in the literature to decreases in the number of Clara cells in smokers (Shijubo *et al.,* 1997)

Regression analysis

RCS exposure, smoking status, age, HIV status and interaction terms between these factors was included in a linear regression model to examine the effect of RCS exposure on CC16 levels. Smoking was shown to be significantly related to CC16 in the earlier crude analysis and was therefore included in the model. The other factors age and HIV, have been shown in the literature to affect CC16 levels. RCS exposure in the final model was a significant predictor of CC16 levels. Smoking was no longer significant in the model and age remained non-significant. The interaction term between smoking and HIV status was significant. This suggests that HIV and smoking may individually not affect CC16 levels in a significant manner but may act synergistically to exert an effect. The other interaction term retained in the model was age and RCS exposure. This interaction term adjusted for the age difference between the miners and the controls. The linear regression could only explain 20% of the variation in CC16 levels and the factors that account for the remaining variation need to be further identified.

A ROC curve was constructed for the CC16 data which gave an area under the curve of 0.58. This is not as high as would have been expected of a good marker that is able to separate exposed from unexposed subjects. The ability of CC16 to act as a good marker with a high specificity and sensitivity was limited by two factors: There was population variability in CC16 levels in the current study and, the low CC16 levels of the controls compared to international studies (Bernard *et al*., 1993; Lesur *et al* 1995; Berthoin *et al*., 2004 and Wang *et al*., 2007). This may suggest that further research maybe required to achieve a better agreement. However the international studies referred to above were conducted on Caucasian and Chinese populations compared to our studies which were conducted on an African population. Thus, the differences may be due to racial differences or environmental exposure or other factors not as yet investigated.

5.5 Conclusion

From the results observed it can be proposed that CC16 warrants further testing as a possible biomarker for RCS exposure. A linear regression model controlling for smoking, HIV and age has shown a significant difference in CC16 levels between the RCS-exposed miners and the non-exposed controls.

CHAPTER 6

Conclusion and Recommendations

6.1 Conclusion

The relationship between RCS exposure and silicosis has been established in numerous studies (Hnizdo and Sluis-Cremer, 1993; American Thoracic Society, 1997; Porter *et al.*, 2002a; Porter *et al.*, 2004; Irvine LG and Watt AH, 1912; Stratta *et al.*, 2001; Sherson, 2002). Despite the level of RCS exposure in the mining industry in South Africa being reported as below the OEL (teWaterNaude *et al.*, 2006), development of silicosis continues unabated (Ndlovu, 2012). A recent study by Churchyard *et al.,* (2004) placed the prevalence of silicosis amongst current South African gold miners after long-term exposure at 18-19%. The long lag period in the development of the disease also means that it will often be recognised only in retirement, thus contributing to the underestimation of any survey in a working population.

The average RCS exposure of 0.06 mg/m^3 8 hour TWA reported by the mines in 1983 (Kielblock, 1997) is below the regulated limit of 0.1 mg/m^3, this suggests that air measurements of RCS levels in the mines may not be reliable in estimating the risk of silicosis or the regulated limit is too high to prevent disease or the current system of averaging does not protect against silicosis. In addition to the amount of RCS, toxicity also depends on the size, shape, surface activity and contaminants of silica particles (Gulumian and Semano, 2003). Consequently the combination of the individual properties of RCS particles may be a better predictor of the toxicity. There is a need for new and more effective means to evaluate RCS exposure and toxicity in a timely manner.

The role of biological monitoring is to provide information on the effects of exposure (Aitio, 1999). Biological monitoring is a better option to assess health effects from

exposure than air monitoring, as it takes into account other possible sources of exposure and individual susceptibility.

The lung has a well-developed network of blood vessels where biological fluid, passing through this network is likely to be exposed to oxidative stress and markers emanating from the lung (Borm *et al.*, 1986). A venous blood sample which can be collected may thus provide information on the lung. Such a relationship has already been established for levels of erythrocyte and lung superoxide dismutase (Minami *et al.*, 1982) suggesting a similar relationship could also exist for the identified biomarkers of this study. Subsequently, to identify biological markers of RCS exposure, blood was used as an acessible biological fluid to assess the levels of the identified potential biomarkers for RCS exposure. Ten biomarkers of effect were identified in Phase I of this study based on the mechanisms involved in the development of silicosis: oxidative stress, inflammatory and lung damage markers GPx, GSH, GST, TAS, 8-Isoprostane, IL-8, TNFα, PDGF, ROS and CC16 (Gulumian *et al*., 2006).

In the current study, biomarker levels were evaluated in relation to the study participants RCS exposure status. Participants in the study were limited to African males to minimise variability due to gender and ethnicity. There were significant differences in age of the study groups with the HIV negative controls being significantly younger than the other groups and thus the effect of age on the biomarkers was included in the analysis as a potential confounder. The number of smokers in the study was similar across all groups with no significant differences in proportion. The internal validity of the study was concluded to be intact with only some expected bias.

The 10 identified biomarkers were investigated for effects due to HIV status, RCS exposure, age and silicosis diagnosis of the participants. Significantly lowered erythrocyte GPx activity in RCS-exposed miners, compared to healthy RCS-unexposed controls, was shown and HIV status did not appear to have a significant effect on GPx activity. Similarly, age, smoking and silicosis did not have significant effects on GPx activity. Based on these findings and confirmation in the literature, the biomarker for oxidative stress, GPx activity, was found to be a candidate for further

investigation as a biomarker for effect due to early RCS exposure (see recommendations).

The current study found no difference between total erythrocyte GSH or erythrocyte GST activity levels in RCS-exposed miners and RCS-unexposed controls. A significant decrease in GSH levels was measured in miners with silicosis while the current study found no significant difference in GST activity between miners with and without silicosis. This study was also not able to demonstrate an effect on GSH or GST activity due to HIV status. Both GSH and GST were not found worthy of further investigation as no early effect from RCS exposure could be shown.

ROS were measured using luminol enhanced chemiluminescence of *ex vivo* RCS stimulated isolated polymorphonuclear cells. No difference was seen between the ROS of RCS exposed miner's cells compared to RCS unexposed controls. HIV was associated with a significant increase in ROS, thus making ROS measurement with chemiluminescence unsuitable for use in South Africa with the current high prevalence of HIV.

A non-significant decrease in TAS was seen due to RCS exposure, while a significant decrease in TAS levels in the HIV positive participants was shown. Thus like ROS, TAS was not considered for further study due to the effect of HIV status.

Although 8-Isoprostane is considered a promising marker of lipid peroxidation, no results could be produced due to challenges experienced in its measurement in our study. This marker requires purification before assay, thus increasing costs and skills required to perform the test.

We were not able to demonstrate any significant differences in either TNFα or IL-8 levels between RCS-exposed miners and the RCS-unexposed controls. HIV was shown to significantly increase both levels in participants in our study. Both TNFα and IL-8 showed significant increases in levels in miners with severe silicosis compared to miners with early or no silicosis. Combined, these results suggest that TNFα and IL-8 are not good makers for early effect of RCS exposure.

The remaining inflammatory marker tested was PDGF, which was considered not to be worthy of further investigation due to variations in the levels based on the processing time. The methodology for collection of PDGF from blood samples which did not always allow for instant processing and freezing proved not suited for field conditions.

The lung damage marker, CC16, was found to be of value for further investigation as a marker for RCS exposure. CC16 decreased in the exposed miners. HIV did not show any significant effects on CC16 once evaluated in a logistic regression.

In Summary

This project was the first step in the validation of biomarkers for the establishment of harmful exposure of workers to RCS. The project demonstrated that markers of inflammation and oxidative stress (GSH, GST, TAS, 8-Isoprostane, ROS, IL-8 and TNFα) are confounded by HIV status in their relationship with RCS exposure and thus not recommended for further consideration as biomarkers for early effect due to RCS exposure. Two markers 8-Isoprostane and PDGF could not be sufficiently analysed due to methodology. The two markers, CC16 and GPx, selected for further investigation as they were not affected by HIV and did not require rigorous sampling methods. This project was able to narrow the list of 10 possible biomarkers for RCS exposure to two GPx and CC16 for further research.

Generalisability of the study results

The reported mean work history of the miners was similar to other studies in southern Africa and mining practices in the shaft are similar to other gold mines around South Africa. Biological variability is likely to be comparable across all miners and not likely to have been selected in any pattern for this study. The recruitment method is the only area of selection bias likely to play a role in the external validity of this study as miners were recruited at the mine clinic. Thus results may be generalisable to all South African gold miners.

6.2 Recommendations

Findings in respect to the methodology used should be incorporated in future studies to improve the usefulness of the information collected. In addition, there have been some new findings by K Makinson that should be taken into account.

1) Less invasive biological samples need to be found for routine testing as, blood samples, while not considered invasive by some, was not favoured by the miners. Research is being conducted on the use of breath samples and urine samples for some biomarkers, which are likely to be easier to collect routinely.
2) In a number of the tests used in this study the test substance was volatile and so a maximum length of time between collection and storage needs to be defined to minimise the development of artefacts in the samples.
3) Reduction of bias: those identified in this study included selection bias, misclassification bias and information bias. Care needs to be taken to minimise these biases as much as possible.
    a. Misclassification bias could be reduced in future studies by taking into account other exposures that could affect controls and miners. Proper ascertainment of RCS exposure in the miners would allow for determination of a dose response.
    b. Selection bias: Selection of a second set of controls that lives in a rural area, with low exposure to both pollution and RCS would help with the possible selection bias and misclassification inherent in the controls selected from the Johannesburg area.
    c. Information bias could be reduced with non-subjective tests for key confounders and effect modifiers, such as smoking.

Future research on the identified biomarkers

The two biomarkers identified in this study (GPx and CC16) for futher research need to undergo further testing for use as practical biomarkers for routine bio-monitoring of RCS exposure. An investigation is needed to determine whether these are reversible biomarkers of exposure is removed as constant monitoring of exposure is required on gold mines. The effect of race and gender on the biomarkers needs to be determined.

Biomarkers not considered in this project should also be investigated. For example, based on the literature and our findings on GPx levels, blood Se levels should be investigated as a biomarker for RCS exposure.

Dose response needs to be determined for the biomarkers identified. This would need to be achieved with ascertainment of environmental RCS exposure in addition to the levels of the identified biomarkers. This could be achieved by following new recruits and collecting samples for both biomarkers and RCS dust measurements over time in a cohort study.

Once biomarkers have been validated for use on the mines, reference ranges for South Africans would need to be established.

Gold mining is not the only industry with RCS exposure and the applicability of the biomarkers to other industries would need to be confirmed. These industries may also have other confounders and effect modifiers that may affect the levels of the biomarkers.

Factors that play a role in explaining some of the variance seen in the CC16 levels need to be identified to increase the sensitivity of CC16 as a biomarker.

APPENDICES

APPENDIX I: CONSENT FORM

NATIONAL HEALTH LABORATORY SERVICE

NATIONAL INSTITUTE *for* OCCUPATIONAL HEALTH

NIOH

106 Joubert Street Ext ● PO Box 4788 Johannesburg 2000 South Africa ● Tel: 27 11 712 6400 ● Fax: 27 11 712 6545

Validation of biomarkers for assessment of exposure to silica dust.

The information sheet about this study has been read to me and I understand what will be required of me if I take part in the study. My questions concerning this study have been answered by……...(name of study member).

My participation in this study is voluntary; I understand that if I do not wish to take part it will have no effect on me or my job. At any time I may leave this study without any bad effect on my job or my health care.

I

_____

_ agree to take part in the study. I am happy to allow my HIV results and x rays to be given to Ms Kerry Downs. I agree to give 30ml of blood. I understand that all information will be anonymous and I know that I can leave at anytime.

Signed _____ Date

_____

APPENDIX II: SUBJECT INFORMATION SHEETS

NATIONAL HEALTH LABORATORY SERVICE

NATIONAL INSTITUTE *for* OCCUPATIONAL HEALTH

NIOH

Subject Information Sheet for Miners

Hello, my name is Kerry Downs, I am a researcher from the National Institute of Occupational Health (NIOH) in Johannesburg, and my contact number is 011 712 6469. My co-worker is Professor Brendan Girdler-Brown.

I would like to invite you to participate in our study. We are looking for a new blood test that might show when you have been exposed to too much silica dust that could cause silicosis. We would like to find a way to make sure you are not breathing in too much unhealthy dust.

If you decide to participate, I would like to explain to you what will happen. First your answers to a short questionnaire are needed. This will take 2 minutes of your time. Then we need to look at your last X-ray. If you agree the nurse will show us your x-ray this will not take you any time. We will need to draw a small amount of blood from your arm. This will not take long, 5 minutes. We will use your blood to look for 10 chemicals; these will show us if you have been exposed to too much dust. These tests will not tell us if you are sick. These are all chemicals normally found in your blood.
Lastly we will need to know your HIV status as this can change the test results, if you agree the clinic manager will give us your HIV status but we won't have your name we will only have your study number.

You will be completely anonymous in the study and your individual results will never be made public. If you decide not to take part in this study it will not affect you or your job in any way.
By taking part you will be helping miners and future employees of mines, so that fewer people get sick. The mines may eventually be able to use the information from the test to make sure there is not too much harmful dust in the air.

There are no risks to you from this study as you cannot get hurt, but you may get a little bruise from the needle when we draw the blood. There will be a medical doctor available at all times.

Thank you
Kerry Downs

APPENDIX III: THE SOUTH AFRICAN NATIONAL BLOOD SERVICE

Constantia Boulevard Roodepoort, South Africa

NATIONAL HEALTH LABORATORY SERVICE

NATIONAL INSTITUTE *for* OCCUPATIONAL HEALTH

NIOH

Dear Donor

Silicosis is an important incurable lung disease caused by exposure to dust of the type found in South African gold mines. For this reason about one in five black South African gold miners, who have historically held the highest silica dust exposure jobs, suffer from the disease. To reduce this high incidence of silicosis a reduction in silica dust exposure, to safe levels, is necessary.

The National Institute for Occupational Health, NIOH, has been asked by the South African gold mining industry, to investigate blood tests which would indicate exposure to crystalline silica dust. To do this the NIOH must first find out what results these tests give in people who have not been exposed to silica dust.

In order to achieve this NIOH has requested assistance from the South African National Blood Service, SANBS, in the collection of blood from suitable donors. As you are considered such a donor, SANBS would like to ask your permission for an extra 60 millilitres of blood to be drawn from you in addition to the unit that you are about to donate. The University of the Witwatersrand Ethics Committee has examined ethical and scientific aspects of this study and have given their approval for the study to be performed.

Allowing us to take this extra blood will in no way affect the use of the unit you will donate and if your blood is suitable for transfusion it will be processed and one or more components, such as red cells, platelets, plasma, plasma-derived products, or rare blood group red cells will be used. You will thus contributing to the well-being of a patient or patients as well as assisting in this very important project.

In addition to taking this extra blood NIOH investigators will ask you a few questions regarding any medicines you might be taking, your smoking history and whether you have ever worked in an environment in which you could have been exposed to silica dust.

As the research samples will be taken in addition to the unit of blood you donate, the taking of the research blood will involve the insertion of a second needle into a vein on your other arm and the discomfort and minimal risk

associated with this. There will be no link between the personal information, which you supplied to SANBS and the unit of blood that is given to NIOH. At no stage will your personal information be divulged to any third party. The results of the tests performed on your blood, however will be made available anonymously to IAVI.

Your participation in this study is entirely voluntary and you are free to withdraw your consent at any time during the bleed. Although your participation may not be of direct benefit to you, it may help to provide knowledge that will be of benefit to others.

We would like to thank you for this donation and trust you will continue to give the GIFT OF LIFE.

I hereby give consent for the blood samples, donated on _____, and the results of the tests performed on my blood to be supplied anonymously to investigators from the National Institute for Occupational Health (NIOH). The samples are to be tested for candidate markers of silica dust exposure in an attempt to establish safe levels of silica dust exposure.

_____	_____	_____
Name of Donor	Date	Witness

_____	_____
Donor signature	Witness signature

APPENDIX IV: ADMINISTERED QUESTIONNAIRE

STUDY NUMBER STICKER:

Group:

					CODING
DATE OF BIRTH:	dd	mm	yyyy		
PREVIOUS WORK:	**TYPE**		**YEARS**		
	GOLD MINE				
	COAL MINE				
	OTHER MINE				
	QUARRYING				
	SAND BLASTING				
	FOUNDRY				
	CERAMICS				
	BRICK FIELDS				
	OTHER:				
CURRENT WORK:					
Description:					

*The name of the subject will be provided by the clinic manager but must not be written on this form.
Once completed, this form will be returned to the clinic manager, who will pass it on to the researchers.

ADMINISTERED QUESTIONNAIRE TO BE COMPLETED BY THE INTERVIEWER

STUDY NUMBER STICKER:

Group

									CODING
RECRUITMENT CENTRE:									
DATE:	dd		mm		yyyy				
CURRENT SMOKER?:	YES			NO					
How Many Cigarettes?:	1-5	5-10	10-20	1 pack	2 pack	pipe			
EVER SMOKED?:	YES			NO					
Date last smoked:									
VEGETARIAN?:	YES			NO					
MEDICATION?:	YES			NO					

DATA CAPTURE FORM TO BE COMPLETED BY THE CLINIC MANAGER*

STUDY NUMBER STICKER:

SECTION B:	**TO BE COMPLETED BY CLINIC MANAGER**					
X-RAY DATE:	dd	mm	yyyy			
SILICOSIS SCORE:	**/**					
HIV TEST DATE:	dd	mm	yyyy			
HIV TEST RESULT:	**POS**	**NEG**				
CD4 TEST DATE:	**NOT APPLICABLE**	**NOT DONE**	dd/mm/yyyy			
CD4 TEST RESULT:						
CURRENT MEDICATION:						
(within previous 2 weeks)						
Comments						

*The name of the subject must not be written on this form
This form will be returned to the researchers once completed

APPENDIX V: ROC curve printout sensitivity and specificity table with cutoffs

Cutpoint	Sensitivity	Specificity	Classified	LR+	LR-
(>= -13.42)	100.00%	0.00%	54.87%	1.0000	
(>= -13.01)	100.00%	1.96%	55.75%	1.0200	0.0000
(>= -11.54)	100.00%	3.92%	56.64%	1.0408	0.0000
(>= -11.45)	98.39%	5.88%	56.64%	1.0454	0.2742
(>= -11.31)	96.77%	5.88%	55.75%	1.0282	0.5484
(>= -10.83)	96.77%	7.84%	56.64%	1.0501	0.4113
(>= -10.74)	95.16%	7.84%	55.75%	1.0326	0.6169
(>= -10.56)	93.55%	7.84%	54.87%	1.0151	0.8226
(>= -10.43)	93.55%	9.80%	55.75%	1.0372	0.6581
(>= -9.71)	93.55%	11.76%	56.64%	1.0602	0.5484
(>= -9.67)	93.55%	13.73%	57.52%	1.0843	0.4700
(>= -9.57)	93.55%	15.69%	58.41%	1.1095	0.4113
(>= -9.52)	91.94%	15.69%	57.52%	1.0904	0.5141
(>= -9.5)	90.32%	15.69%	56.64%	1.0713	0.6169
(>= -9.4)	90.32%	17.65%	57.52%	1.0968	0.5484
(>= -9.3)	88.71%	17.65%	56.64%	1.0772	0.6398
(>= -9.15)	87.10%	17.65%	55.75%	1.0576	0.7312
(>= -8.66)	85.48%	17.65%	54.87%	1.0380	0.8226
(>= -8.58)	85.48%	19.61%	55.75%	1.0633	0.7403
(>= -8.5)	83.87%	19.61%	54.87%	1.0433	0.8226
(>= -8.36)	82.26%	19.61%	53.98%	1.0232	0.9048
(>= -8.24)	80.65%	19.61%	53.10%	1.0031	0.9871
(>= -7.8)	80.65%	21.57%	53.98%	1.0282	0.8974
(>= -7.29)	80.65%	23.53%	54.87%	1.0546	0.8226
(>= -7.26)	80.65%	25.49%	55.75%	1.0823	0.7593
(>= -7.02)	79.03%	25.49%	54.87%	1.0607	0.8226
(>= -6.84)	77.42%	25.49%	53.98%	1.0390	0.8859
(>= -6.81)	77.42%	27.45%	54.87%	1.0671	0.8226
(>= -6.65)	77.42%	31.37%	56.64%	1.1281	0.7198
(>= -6.59)	77.42%	33.33%	57.52%	1.1613	0.6774
(>= -6.44)	75.81%	33.33%	56.64%	1.1371	0.7258
(>= -6.29)	75.81%	35.29%	57.52%	1.1716	0.6855
(>= -6.24)	74.19%	35.29%	56.64%	1.1466	0.7312
(>= -5.92)	74.19%	37.25%	57.52%	1.1825	0.6927
(>= -5.85)	74.19%	39.22%	58.41%	1.2206	0.6581
(>= -5.58)	72.58%	39.22%	57.52%	1.1941	0.6992
(>= -5.37)	72.58%	41.18%	58.41%	1.2339	0.6659
(>= -5.21)	70.97%	41.18%	57.52%	1.2065	0.7051
(>= -4.94)	69.35%	41.18%	56.64%	1.1790	0.7442
(>= -4.85)	69.35%	43.14%	57.52%	1.2197	0.7104
(>= -4.84)	69.35%	45.10%	58.41%	1.2632	0.6795
(>= -4.82)	69.35%	47.06%	59.29%	1.3100	0.6512
(>= -4.81)	67.74%	47.06%	58.41%	1.2796	0.6855
(>= -4.8)	66.13%	47.06%	57.52%	1.2491	0.7198
(>= -4.73)	66.13%	49.02%	58.41%	1.2971	0.6910

(>= -4.72)	64.52%	49.02%	57.52%	1.2655 0.7239
(>= -4.7)	62.90%	49.02%	56.64%	1.2339 0.7568
(>= -4.53)	62.90%	50.98%	57.52%	1.2832 0.7277
(>= -4.48)	61.29%	50.98%	56.64%	1.2503 0.7593
(>= -4.43)	59.68%	50.98%	55.75%	1.2174 0.7909
(>= -4.37)	58.06%	50.98%	54.87%	1.1845 0.8226
(>= -4.35)	58.06%	52.94%	55.75%	1.2339 0.7921
(>= -4.33)	58.06%	54.90%	56.64%	1.2875 0.7638
(>= -4.3)	56.45%	54.90%	55.75%	1.2518 0.7932
(>= -4.28)	54.84%	54.90%	54.87%	1.2160 0.8226
(>= -4.19)	53.23%	54.90%	53.98%	1.1802 0.8520
(>= -4.13)	53.23%	56.86%	54.87%	1.2339 0.8226
(>= -4.04)	53.23%	58.82%	55.75%	1.2926 0.7952
(>= -4.01)	51.61%	58.82%	54.87%	1.2535 0.8226
(>= -4)	51.61%	60.78%	55.75%	1.3161 0.7960
(>= -3.84)	50.00%	60.78%	54.87%	1.2750 0.8226
(>= -3.68)	48.39%	60.78%	53.98%	1.2339 0.8491
(>= -3.67)	48.39%	62.75%	54.87%	1.2988 0.8226
(>= -3.66)	46.77%	62.75%	53.98%	1.2555 0.8483
(>= -3.56)	46.77%	64.71%	54.87%	1.3253 0.8226
(>= -3.54)	45.16%	64.71%	53.98%	1.2796 0.8475
(>= -3.47)	43.55%	64.71%	53.10%	1.2339 0.8724
(>= -3.46)	43.55%	66.67%	53.98%	1.3065 0.8468
(>= -3.27)	41.94%	66.67%	53.10%	1.2581 0.8710
(>= -3.26)	40.32%	66.67%	52.21%	1.2097 0.8952
(>= -3.24)	40.32%	68.63%	53.10%	1.2853 0.8696
(>= -3.11)	38.71%	68.63%	52.21%	1.2339 0.8931
(>= -3.03)	38.71%	70.59%	53.10%	1.3161 0.8683
(>= -3.01)	37.10%	70.59%	52.21%	1.2613 0.8911
(>= -2.99)	37.10%	72.55%	53.10%	1.3514 0.8670
(>= -2.98)	37.10%	74.51%	53.98%	1.4553 0.8442
(>= -2.95)	35.48%	74.51%	53.10%	1.3921 0.8659
(>= -2.93)	33.87%	74.51%	52.21%	1.3288 0.8875
(>= -2.87)	32.26%	74.51%	51.33%	1.2655 0.9092
(>= -2.85)	30.65%	74.51%	50.44%	1.2022 0.9308
(>= -2.79)	30.65%	76.47%	51.33%	1.3024 0.9069
(>= -2.74)	30.65%	78.43%	52.21%	1.4208 0.8843
(>= -2.65)	29.03%	78.43%	51.33%	1.3460 0.9048
(>= -2.58)	29.03%	80.39%	52.21%	1.4806 0.8828
(>= -2.52)	27.42%	80.39%	51.33%	1.3984 0.9028
(>= -2.4)	27.42%	82.35%	52.21%	1.5538 0.8813
(>= -2.37)	27.42%	84.31%	53.10%	1.7480 0.8608
(>= -2.35)	27.42%	86.27%	53.98%	1.9977 0.8413
(>= -2.33)	27.42%	88.24%	54.87%	2.3306 0.8226
(>= -2.26)	24.19%	88.24%	53.10%	2.0565 0.8591
(>= -2.23)	22.58%	88.24%	52.21%	1.9194 0.8774
(>= -2.14)	20.97%	88.24%	51.33%	1.7823 0.8957
(>= -2.08)	19.35%	88.24%	50.44%	1.6452 0.9140
(>= -1.95)	17.74%	92.16%	51.33%	2.2621 0.8926
(>= -1.87)	16.13%	92.16%	50.44%	2.0565 0.9101
(>= -1.78)	16.13%	94.12%	51.33%	2.7419 0.8911

(>= -1.72)	14.52%	94.12%	50.44%	2.4677	0.9083
(>= -1.67)	12.90%	94.12%	49.56%	2.1935	0.9254
(>= -1.66)	12.90%	96.08%	50.44%	3.2903	0.9065
(>= -1.56)	11.29%	96.08%	49.56%	2.8790	0.9233
(>= -1.4)	11.29%	98.04%	50.44%	5.7581	0.9048
(>= -1.33)	9.68%	98.04%	49.56%	4.9355	0.9213
(>= -1.2)	8.06%	98.04%	48.67%	4.1129	0.9377
(>= -1.04)	6.45%	98.04%	47.79%	3.2903	0.9542
(>= -.98)	4.84%	98.04%	46.90%	2.4677	0.9706
(>= -.91)	3.23%	98.04%	46.02%	1.6452	0.9871
(>= -.89)	1.61%	98.04%	45.13%	0.8226	1.0035
(>= -.71)	0.00%	98.04%	44.25%	0.0000	1.0200
(> -.71)	0.00%	100.00%	45.13%		1

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