VALIDATION OF BIOMARKERS FOR IMPROVED ASSESSMENT OF EXPOSURE AND EARLY EFFECT FROM EXPOSURE TO CRYSTALLINE SILICA.

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree

of

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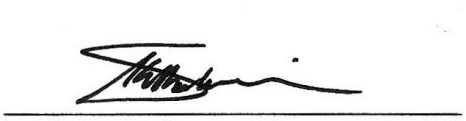
DECLARATION

I, Kerry Sue Makinson, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



10th day of September 2008

DEDICATION

This dissertation is dedicated to the most important people in my life:

My parents, Rob and Glenda, who have celebrated my successes, understood my challenges and encouraged my dreams. I am truly privileged and grateful to have you as my parents. I love you.

My best friends, Ashleigh Maritz and Susie Robbie, and my sister, Amy. Thank you for your support and friendship; you will never know how much it means to me.

PRESENTATIONS

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Murray, J. 2 – 5 March 2007. *Biomarkers-a novel method for evaluating dust allaying interventions as part of the silicosis elimination programme.* Congress of the South African Thoracic Society in the Neil White Occupational Lung Disease Symposium.

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Gulumian, M. 23 September 2006. *Mechanistically Identified Suitable Biomarkers of Exposure, Effect and Susceptibility for Silicosis: Validation of Biomarkers of Early Effect*. The EUROTOX 2006/6 CTDC Congress.

ABSTRACT

This is the third phase of a project to identify, confirm, and operationalise biomarkers for crystalline silica dust exposure that could be used for surveillance of dust exposure levels in South African mines. The first phase of the project involved a comprehensive review of the relevant literature [Gulumian *et al*., 2006] from which ten potential biomarkers of effect were identified as being worthy of further investigation. The second phase of the project examined the ten identified biomarkers in silica dust-exposed and unexposed black male subjects [Murray *et al*., 2006]. Two of the ten short listed biomarkers, namely erythrocyte glutathione peroxidase (GPx) and serum Clara cell protein 16 (CC16), were found to have significantly reduced levels in the silica dust-exposed versus unexposed subjects. In addition, the biomarkers were found to be unaffected by HIV sero-status, smoking, age and the presence of silicosis. As a result, this third phase of the project aimed to confirm the levels of and further analyze GPx and CC16 in miners exposed to crystalline silica dust.

This third phase involved the measurement of the levels of erythrocyte GPx and serum CC16 in 80 adult male gold miners upon their return from leave and then again two to six months after they had returned to work (involving exposure to crystalline silica). Before the field work was conducted, however, the optimal operational parameters for the biomarkers (namely storage temperature, delay in time between blood collection and separation, laboratory temperature and storage duration) were established. The results of these optimization experiments were used to develop Standard Operating Procedures (SOPs) for biomarker specimen handling and storage under field conditions, and for laboratory assays.

In this phase, the findings of the second phase were confirmed in that the levels of GPx and CC16 were lowered in miners exposed to crystalline silica dust and were unaffected by age, race and cigarette smoking. In addition, while CC16 was unaffected by the presence of radiological silicosis, GPx may have been affected. Finally, the decrease in the levels of GPx activity and CC16 concentration observed in the study were unaffected by the level of silica dust exposure (high or low) as determined by job category or by the duration of crystalline silica exposure.

Regarding the levels of GPx activity, the results suggested that GPx levels decrease after two to six months of chronic exposure to crystalline silica dust and remain decreased (throughout the working week and over a weekend) and then increase or even recover to normal levels during a period of leave. It was therefore concluded that GPx activity levels rise and fall, in response to silica dust exposure, gradually and over periods of some time, possibly months.

The CC16 results were, however, less promising. After two to six months of chronic exposure to crystalline silica dust there was a significant change in CC16 on a Wednesday afternoon following an 8-hour shift and during the duration of a shift. In addition, there is the possibility that the observed changes were due to a time-dependent diurnal variation in the CC16 levels.

It was concluded that the results of the current phase warrant further research into the use of erythrocyte GPx and serum CC16 as biomarkers of early effect from crystalline silica exposure.

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ABBREVIATIONS

AM	Alveolar macrophage
AST	Aspartate aminotransferase
ARV	Anti retroviral
BALF	Bronchoalveolar lavage fluid
BEI	Biological exposure indices
CAT	Catalase
CC16	Clara Cell Protein 16
COPD	Chronic obstructive pulmonary disease
CWP	Coal workers' pneumoconiosis
ELF	Epithelial lining fluid
ELISA	Enzyme-linked immunosorbent assay
G6PD	Glucose 6-phosphate dehydrogenase
GFR	Glomerular filtration rate
GPES	Global programme for the elimination of silicosis
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase
HIV	Human Immunodeficiency Virus
IARC	International Agency for Research on Cancer
IL	Interleukin
ILO	International Labour Organisation

MHSC	Mines Health and Safety Council
NADPH	Nicotinamide adenine dinucleotide phosphate
NPES	National programme for the elimination of silicosis
NIOH	National Institute for Occupational Health
NIOSH	National Institute for Occupational Safety and Health
NOAEL	No-observed-adverse-effect-level
NRC	National Research Council
OEL	Occupational Exposure Limit
OLD	Occupational lung disease
OSHA	Occupational Safety and Health Administration
PAH	Polycyclic aromatic hydrocarbons
PATHAUT	Pathology automation system
PDGF	Platelet-derived growth factor
PEL	Permissible exposure limit
PLA_2	Phospholipase A_2
PMF	Progressive massive fibrosis
PMN	Polymorphonucleated
Prx	Peroxiredoxin
PTB	Pulmonary tuberculosis
QC	Quality control
REL	Recommended exposure limit
ROS	Reactive oxygen species
RV	Reference value
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polycrylamide gel electrophoresis

SOD	Superoxide dismutase
SOP	Standard Operating Procedure
TAS	Total antioxidant status
TNF-α	Tumour necrosis factor alpha
Trx	Thioredoxin
USBM	United States bureau of mines
XRD	X-Ray diffraction

Chapter 1:

INTRODUCTION

Exposure to respirable crystalline silica can result in a variety of diseases the most common of which is silicosis [NIOSH, 2002], a potentially fatal, irreversible, progressive and untreatable fibrotic lung disease [Greenberg *et al.*, 2007].

In South Africa, gold miners constitute the largest group of workers exposed to crystalline silica containing-dust [Murray *et al.*, 2006] and despite control measures in place to limit the inhalation of this dust by gold miners, the prevalence of silicosis is still high [Girdler-Brown *et al.*, 2008]. Although there are currently no methods available to assess the early health outcomes of dust control measures, the field of biomarkers may provide the solution.

If scientifically acceptable biomarkers were found that allowed the changes that occur between silica exposure and silicosis to be measured, they could be used to detect early adverse effects of silica exposure and to evaluate dust-control methods thereby allowing intervention schemes to be implemented quickly [Gulumian *et al*., 2006].

The dissertation starts with a background to the project and a review of the relevant literature. Following a description of the methodology used (including statistical methods), the relevant results are presented. Finally, the results are discussed and conclusions are drawn.

1.1 Background to the project

This is the third phase of a project to identify, confirm, and operationalise biomarkers for crystalline silica dust exposure that could be used for surveillance of dust exposure levels in South African mines.

The project was solicited by the South African Mines Health and Safety Council (MHSC) in an attempt to identify biomarkers that could help to assess the success of respirable crystalline silica dust exposure control measures in South African gold mines. The project was not intended to identify a diagnostic test(s) for the diagnosis of silicosis disease in individuals as biomarkers would probably be too non-specific for such a definitive diagnostic purpose. In addition, due in part to high variability in some biomarker levels, individual test results cannot be used to indicate (in an absolute sense) an individual volunteer's dust exposure levels. As a result, phases two and three of the project were both epidemiological studies focusing on population-based average readings.

The first phase of the project was a comprehensive review of the relevant literature on prospective silicosis biomarkers [Gulumian *et al.*, 2006]. From the literature, ten potential biomarkers were identified that were considered worthy of further investigation in the project. The ten biomarkers identified were: reactive oxygen species (ROS) measurement by chemiluminescence (neutrophil release), erythrocyte glutathione (GSH), erythrocyte glutathione-S-transferase (GST), erythrocyte glutathione peroxidase (GPx), n-8 isoprostane, tumour necrosis factor alpha (TNF-α), platelet-derived growth factor (PDGF), interleukin-8 (IL-8), Clara cell protein 16 (CC16), and total antioxidant status as a trolox equivalent (TAS). None of these biomarkers were biomarkers of susceptibility since such

biomarkers would not indicate excess exposure to silica dust but rather an individual's predisposition to silicosis.

The second phase of the project evaluated the ten biomarkers, identified in phase one, to determine the influence (if any) of Human Immunodeficiency Virus (HIV) infection, anti retroviral (ARV) treatment, smoking, age and the ability to discriminate between silica exposed and unexposed individuals. The ten biomarker levels were measured in 60 African male underground gold miners (20 HIV sero-positive and 40 HIV sero-negative) and 60 African males who had never been exposed to silica dust (28 HIV sero-positive and 32 HIV sero-negative). Two of the ten biomarkers investigated, namely erythrocyte GPx and serum CC16, were found to be significantly decreased in the silica dust-exposed subjects. In addition, the two biomarkers were unaffected by HIV infection, ARV treatment, smoking, age and the presence of radiological silicosis. [Murray *et al.*, 2006].

This third phase of the project aimed to confirm the levels of and further analyze serum CC16 and erythrocyte GPx in miners exposed to low and high levels of crystalline silica dust.

1.2 Literature review

1.2.1. Crystalline silica exposure

Exposure to respirable crystalline silica occurs in a number of industries and, because of its toxicity, can result in a variety of diseases.

1.2.1.1 Definition of crystalline silica

Silicon is the second most common element in the earth's crust, making up approximately 28% [Mandel and Mandel, 1996]. Due to their abundance, silicon and oxygen atoms commonly combine to form a fundamental tetrahedral unit (SiO_4) which consists of a central silicon ion with oxygen attached at the four 'corners' of the tetrahedron. These tetrahedral units are joined by common oxygen atoms to form a molecule, with an average stoichiometric formula of SiO_2, known as silicon dioxide or silica. [Parkes, 1974].

Silica can exist in two forms, namely non-crystalline (amorphous) silica and crystalline silica (figure 1.2.1.1). In crystalline silica, the atoms and molecules are arranged in a three-dimensional repeating pattern. In amorphous silica, the atoms and molecules are randomly arranged with no pattern [USBM, 1992].

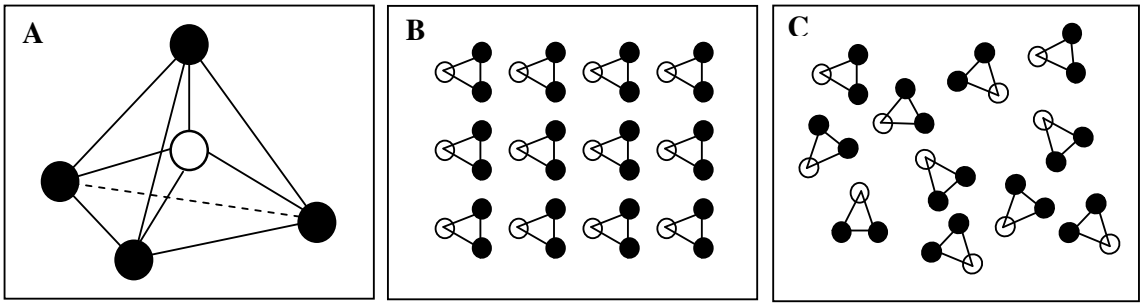


Figure 1.2.1.1: The different structural forms of silica: A) the silicon-oxygen tetrahedron, B) the repeating pattern of crystalline silica, and C) the random pattern of amorphous silica.
Silicon (o) and oxygen (•). Adapted from [USBM, 1992].

Crystalline silica exists as at least eight different polymorphs (same chemical composition but different structure), the most common of which are quartz, cristobalite and tridymite [Guthrie and Heaney, 1995]. Quartz can exist as one of two forms, alpha (α) and beta (β), depending on the conditions. Under ambient conditions, quartz exists as α-quartz but at high temperatures (above 573^{o}C) and pressures it transforms into β-quartz. [USBM, 1992].

Alpha quartz (the most abundant form of crystalline silica) is the second most common mineral in the world. It is the major component of sand and of dust in the air and is also abundant in igneous, sedimentary and metamorphic rock. [USBM, 1992].

1.2.1.2 Industries involving crystalline silica exposure

Exposure to crystalline silica occurs in a wide variety of industries, with the most common exposures occurring in mining and mining-related occupations [Rees and Murray, 2007].

In gold mining, in particular, a significant release of crystalline silica is expected since quartz forms veins in sedimentary rock and it is these veins that form the matrix for

precious metals such as gold [DOL, 2005]. During the mining process, a number of activities (such as drilling, hewing, shovelling, crushing, tunnelling and the use of explosives) can generate a large of amount of crystalline silica containing-dust [Parkes, 1974].

In South African gold mines, the mined rock usually contains 60-90% of quartz while the respirable dust generated contains approximately 30% free quartz [Hnizdo and Sluis-Cremer, 1991].

In addition to the mining industry, exposure to crystalline silica has been shown to occur in the following industries: glass, ceramic and fine china manufacturing; the construction industry; heavy industry such as the oil and gas industry; agriculture; and in high-technology applications such as the field of laser optics [USBM, 1992].

1.2.1.3 Factors affecting crystalline silica toxicity

In order to identify potential biomarkers of crystalline silica exposure, the factors responsible for its toxicity need to be understood. A number of factors affect the biological toxicity of silica polymorphs including: origin, structure, composition, crystallinity, solubility, particle size, interactions with other chemicals/minerals, and most importantly the surface properties. [Gulumian, 2007]

Origin:

The matrix in which the silica particle is found (for example in coal mine dust) can affect the toxicity of the particle [Donaldson *et al.*, 2001]. This is because silica commonly bonds

with elements such as sodium, potassium, calcium, magnesium, iron and aluminium (forming compounds called silicates) [USBM, 1992] and it is these other contaminating elements that may play an important role in the toxicity of crystalline silica [Donaldson and Borm, 1998].

Size:

The size of a particle is an important factor in determining its ability to penetrate deep into the lung. When air is inhaled through the nose, the majority of particles greater than 10 µm in diameter are trapped in the nose and pharynx [Parkes, 1974]. Respirable free crystalline silica particles (defined as particles less than 10µm in diameter) are most likely to have a toxic effect, with particles 1 to 3 µm in diameter having greatest capability to deposit in the alveoli [Ziskind *et al.*, 1976].

Crystallinity:

Only crystalline silica has been found to be toxic [Fubini and Fenoglio, 2007].

Solubility in biological fluids:

The amount of time a particle stays in the body depends on the effectiveness of the clearance mechanisms and solubility. The longer the particle remains unaltered in the biological compartment (where it can cause stress to cells and tissue), the greater the extent of any damage caused. [Fubini and Fenoglio, 2007].

Surface reactivity:

Because it is the surface of the silica particle that interacts with biological fluids and molecules, the properties of the particle surface ultimately determine the particles' toxicity [Guthrie and Heaney, 1995].

The surface properties of a particle are determined by their history (such as the method by which the dust was generated), weathering, processing conditions and the presence of contaminants [Fubini and Fenoglio, 2007].

For example, when crystalline silica is cut, ground, or milled (as occurs during occupations such as sandblasting, rock drilling, tunnelling, and silica milling) the crystal is fractured. This results in cleavage planes which have surface properties (such as the presence of reactive oxygen species) that make them more reactive for lung tissue and therefore more likely to cause rapid development of pulmonary disease [Castranova *et al.*, 1996a]. When silica crystals are freshly fractured, the silicon-oxygen bond is cleaved via either a homolytic pathway (which results in the reactive surface radicals $Si^{\bullet}$ and $SiO^{\bullet}$) or a heterolytic pathway (which results in the surface charges Si^{+} and SiO^{-}) as illustrated in figure 1.2.1.3. These reactive species tend to either recombine or to react with atmospheric components, yielding reactive oxygen species (ROS) at the surface and in the subsurface layers of the particle [Fubini *et al.*, 1995]. In aqueous conditions (such as the lung lining fluid or tissue fluid) the $Si^{\bullet}$ and $SiO^{\bullet}$ radicals can give rise to highly reactive hydroxyl radicals ($OH^{\bullet}$) [Castranova *et al.*, 1996a] and the Si^{+} and SiO^{-} charges can interact with membranes [Fubini *et al.*, 1995]. It has been shown that aqueous suspensions of freshly fractured quartz particles can produce hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$), and singlet oxygen(O_2) [Shi *et al.*, 1995].

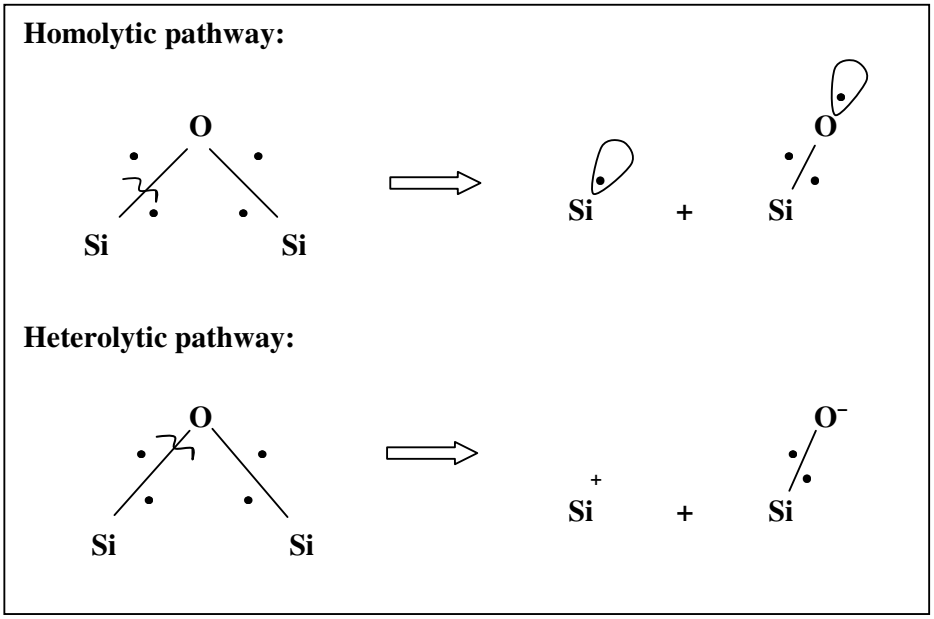


Figure 1.2.1.3: The pathways by which reactive surface radicals ($Si^{\bullet}$ and $SiO^{\bullet}$) and surface charges (Si^{+} and SiO^{-}) are produced by the cleavage of the silicon-oxygen bond. Adapted from [Fubini *et al.*, 1995].

In addition to its ability to produce free radicals, the surface properties of a particle that are most likely to influence its toxicity are its ability to adsorb endogenous molecules, and its degree of hydrophilicity and hydrophobicity [Fubini and Fenoglio, 2007].

Age:

A number of studies have shown that freshly fractured silica is more cytotoxic and inflammatory than aged silica of similar size and composition [Castranova *et al*., 1996b, Vallyathan *et al*., 1995, Shoemaker *et al*., 1995, Vallyathan *et al*., 1988, Vallyathan *et al*., 1991]. The higher toxicity of freshly fractured silica has been attributed to the ability of the newly created surfaces to generate surface radicals, which has been shown to decrease with age [Castranova *et al*., 1996a].

1.2.1.4 The pathway of inhaled crystalline silica

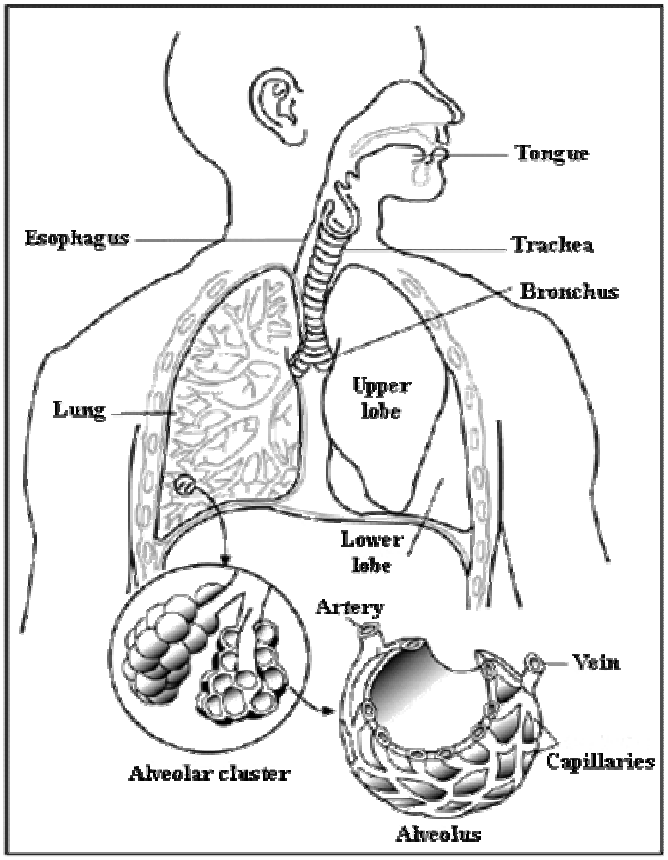


Figure 1.2.1.4: The human respiratory system [WAHSA/NIOH, 2006].

Airborne crystalline silica particles enter the body through the mouth or nose during inhalation of air (in which they are suspended). Once inhaled, the silica particles first travel through the upper airways from the trachea through the bronchi to the bronchioles (the small airways). At this stage, the majority of the inhaled silica particles are either exhaled or deposited in the upper airways where they are then removed by the mucociliary 'escalator' (which involves the movement of ciliated epithelium [Beckett, 2000]). If the particles are not removed they can travel through the lower airways from the terminal bronchioles into the respiratory bronchioles which end in alveolar sacs consisting of clusters of alveoli. It is in these respiratory bronchioles and alveoli areas that small silica

particles (less than 10 μm in aerodynamic diameter) can be deposited. [WAHSA/NIOH, 2006].

The depth to which the particles travel in the lung is determined by their physical characteristics (size, density, shape and aerodynamic properties) and by the volume of each inhalation. Because the majority of particles are removed from the lung, their concentration in the inhaled air needs to be high in order to allow some particles to be deposited in the lungs. [Parkes, 1974].

1.2.1.5 Diseases associated with crystalline silica exposure

The most common disease caused by crystalline silica exposure is silicosis [NIOSH, 2002]. However, exposure to crystalline silica can lead to a number of other diseases such as emphysema, immunologic reactions, interstitial fibrosis, industrial bronchitis, small airway disease, vascular diseases [Ding *et al*., 2002] and lung cancer [IARC, 1997]. Exposure to crystalline silica has also been implicated in the development of a number of autoimmune diseases, including systemic sclerosis, rheumatoid arthritis, systemic lupus erythematosis, and chronic renal disease [Rimal *et al*., 2005]. In addition, respirable crystalline silica exposure (even in the absence of radiological silicosis) has been found to increase the risk of pulmonary tuberculosis [te Water Naude *et al.*, 2006, Hnizdo and Murray, 1998].

Lung cancer:

The relationship between silica exposure, silicosis and lung cancer has been hypothesized, investigated and debated for many years. In 1997, after a review of research published until then, the International Agency for Research on Cancer (IARC) concluded that there was

sufficient evidence in humans for the carcinogenicity of inhaled crystalline silica (in the form of quartz) from occupational sources. As a result, crystalline silica (quartz) was classified as a Group 1 carcinogen. However their final evaluation noted that carcinogenicity was not found in all the industries studied and they therefore speculated that "Carcinogenicity may be dependent on inherent characteristics of the crystalline silica or on external factors affecting its biological activity or distribution of its polymorphs". [IARC, 1997]. It is generally thought that if crystalline silica is a human lung carcinogen, it is a relatively weak one [Wong, 2002].

Silicosis:

Silicosis is a potentially fatal, irreversible, progressive and untreatable fibrotic lung disease caused by the inhalation and deposition of respirable crystalline silica [Greenberg *et al.*, 2007].

Pulmonary tuberculosis (PTB) is a common complication of silicosis. Although PTB may occur at any stage in the development of silicosis, it is more likely to occur in older workers with severe grades of silicosis [Cowie, 1994].

Pulmonary tuberculosis is epidemic on South African gold mines with the incidence of PTB among South African gold miners much higher than that of the general population. A study carried out by te Water Naude and colleagues [2006] examined 520 gold miners over 37 years of age and found a PTB prevalence of 35% even though the average concentration of respirable quartz was 0.053 mg/m^3 (below the current occupational exposure limit, OEL, of 0.1 mg/m^3). Even more worrying is that this study and one by Hnizdo and Murray [1998] showed that even in the absence of silicosis, exposure to silica

dust increases the risk of developing tuberculosis and that this risk persists long after exposure ends. Hnizdo and Murray also found that the risk of developing PTB increased with the presence of radiologically diagnosed silicosis, with increasing cumulative exposure to crystalline silica, and with tobacco pack years.

Silicosis [Hnizdo and Murray, 1998, Cowie, 1994] and HIV [Corbett *et al.*, 1999, Corbett *et al.*, 2000] are strong risk factors for tuberculosis. The study by Corbett [2000] showed that, in South African gold miners, the effects of silicosis and HIV infection on the risk for PTB are multiplicative rather than additive. It found that silicosis increased the PTB risk threefold while HIV increased the PTB risk fivefold and the combined effects of silicosis and HIV infection increased the PTB risk 15 times.

1.2.1.6 A history of silicosis

It is thought that silicosis has afflicted miners for centuries, probably since mining activities started between 4000 and 3000 B.C. in China, India and Egypt. Around 400 B.C., Hippocrates described lung disease in miners when he described the metal digger as a man who breathes with difficulty. Pliny (A.D. 23-79) suggested that miners wear protective masks to prevent the inhalation of "fatal dust" in the mines and Galen (A.D. 131-201) was the first to describe the symptoms of silicosis [Harley and Vallyathan, 1996]. In 1866, the German pathologist Fredrich von Zenker used the term pneumokoniosis ("dust lung") to describe dust-related lung diseases such as silicosis and in 1870 the term "silicosis" was first used by Visconti [Greaves, 2000]. Since then, pneumoconiosis has been defined as "The presence of inhaled dust in the lungs and their non-neoplastic tissue reaction to it"

[Parkes, 1974] and it has become widely accepted that silicosis is caused by the inhalation and deposition of crystalline silica.

In South Africa, gold mining started in the Witwatersrand in 1886 [Gold rush, 2009]. Since then, there have been four main periods in the history of silicosis on the Witwatersrand mines: 1) the initial period of gold mining on the rand, from 1886 to 1899, was a period of ignorance as to the dangers of silicosis, 2) the dangers of silicosis and the nuisance of prevention measures were first realised in the period from 1902 to 1910, 3) a legal system of compensation for silicosis and preventative measures were introduced in the period from 1911 to 1916, and 4) full systematised measures of prevention, detection and compensation were implemented from 1916 to the current day [Donsky, 1993].

1.2.1.7 Current prevalence of silicosis in South African mine workers

Despite silicosis prevention measures currently employed in South African mines, the prevalence of silicosis is high among currently employed, retired and deceased miners. This has been demonstrated by a number of studies, both radiography-based and autopsy-based.

A cross-sectional study conducted with living black ex-miners from Botswana showed an overall prevalence of pneumoconiosis of between 26.6% and 31.0% [Steen *et al*., 1997], while a similar study in the Eastern Cape showed an overall prevalence of between 22% and 36% [Trapido *et al*., 1998]. In both studies, the relatively insensitive method of chest radiographs were used to diagnose the pneumoconiosis and therefore the disease rates may have been underestimated. A study of active black South African gold miners (over 37

years of age) found a silicosis prevalence of between 18.3% and 19.9% [Churchyard *et al.*, 2004]. However, due to the healthy worker effect the disease rates may also have been underestimated. Since silicosis prevalence rates can also be estimated by autopsy based studies, a number of studies have been carried out using the data from the pathology automation system (PATHAUT) database. The PATHAUT database contains the clinical files of and the pathological findings of autopsies carried out on deceased South African miners since 1975. It is the only comprehensive surveillance database on occupational respiratory diseases available to the South African mining industry. The 2007 annual PATHAUT surveillance report showed that the overall prevalence of silicosis in deceased gold miners increased from 19.1% in 2000 to 31.6% in 2006. [Ndlovu *et al.*, 2007].

These high silicosis prevalence rates indicate that: 1) the dust control measures in place are not adequate, 2) the levels of respirable crystalline silica are too high, and 3) the surveillance of workers' health is not effectively organised [Fedotov, 2006].

It is therefore important that more effective control measures for crystalline silica dust exposure, are implemented to prevent the significant silicosis problem facing South African gold miners.

1.2.1.8 The development of silicosis

A number of factors influence the development of silicosis. The main determinants of whether an individual develops silicosis are the silica dose in the ambient air (including the percentage of free crystalline silica in the dust particles), the duration of exposure, and the

characteristics of the silica particle [Banks, 1996]. Host factors, such as genetic variation, also influence an individual's susceptibility [Rees and Murray, 2007].

1.2.1.9 Mechanisms in the pathogenesis of silicosis

Once a silica particle has been deposited in the alveolar spaces of the lungs, it may contribute to the pathogenesis of silicosis in a number of ways, including direct cytotoxicity and stimulation of alveolar macrophages.

Direct cytotoxicity:

As described in section 1.2.1.3, ROS can be generated on the surface of silica particles. These ROS are strongly implicated in the mechanisms of silica toxicity in that they can cause lipid peroxidation (leading to cell membrane damage) and cell damage [Fubini, 1998]. In addition, if ROS are produced in excess, they can overwhelm the lungs' antioxidant defences and lead to oxidative stress and inflammation, which would then result in scarring, fibrosis and diminished gas exchange [Castranova, 1996].

Stimulation of alveolar macrophages:

The alveolar walls contain various cell types (including type I pneumocytes, type II pneumocytes, alveolar macrophages (AM), endothelial cells, epithelial cells, fibroblasts, leucocytes and lymphocytes). These cells are responsible for a variety of functions in the lung, such as gas exchange, disposal of inhaled foreign bodies, and immunological activities. [Parkes, 1974].

Alveolar macrophages are the lungs' most important means of defence against inhaled pollutants and they function to engulf and eliminate foreign bodies (such as silica particles) [Balduzzi *et al*., 2004]. They do so by invagination of their boundary membrane to form a vesicle called the phagosome (the process is referred to as phagocytosis) [Parkes, 1974].

Once a silica particle has been deposited in the alveolar spaces of the lungs, the immune system is activated and AMs are recruited to eliminate the particles [Fubini and Fenoglio, 2007]. Activation of AMs leads to the production and release of ROS [Rahman and MacNee, 2000], and inflammatory cytokines; such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and platelet-derived growth factor (PDGF) [Green and Vallyathan, 1996]. The increased production of ROS is referred to as the "respiratory burst" as it results in an increase in oxygen consumption [Ding *et al*., 2002].

Because the crystalline silica particles are cytotoxic they eventually cause AMs to die and release the particle as well as additional ROS and cytokines into the surroundings [Fubini and Fenoglio, 2007]. This leads to the activation of redox-sensitive transcription factors, such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), which regulate the antioxidant genes and the genes for pro-inflammatory cytokines [Rahman *et al*., 2006]. This results in inflammation.

Although inflammation is usually a protective response to cellular or tissue injury (which aims to promote repair), if uncontrolled it results in excessive cell or tissue damage and eventually chronic inflammation and the destruction of normal tissue [Rahman and MacNee, 2000] .

Additionally, the inflammatory cytokines released by the AMs act as chemoattractants that help recruit new immune-defence cells (AMs and polymorphonucleated, PMN, cells). A continuous cycle of recruitment and cell death is therefore established and this results in sustained inflammation (which lasts as long as the particle remains in the lungs) and oxidative stress, which can eventually lead to fibrosis. [Fubini and Fenoglio, 2007]. The severe inflammation that follows silica exposure therefore appears to be the initiating step in the development of silicosis [Rimal *et al*., 2005].

1.2.1.10 Pathological features of silicosis

There are four pathologically distinct forms of silicosis: chronic silicosis, progressive massive fibrosis, accelerated silicosis, and alveolar lipoproteinosis [Rees and Murray, 2007].

1. **Chronic (nodular) silicosis**

This is the most common form of silicosis. It can take 10 to 30 years (a working lifetime) of low intensity exposure to crystalline silica before abnormalities can be seen on a chest radiograph. Thereafter, the disease may progress slowly over a long period of time [Green and Vallyathan, 1996], even after exposure to silica has ceased [Hnizdo and Sluis-Cremer, 1993, Hnizdo and Murray, 1998].

Chronic silicosis is characterised by the presence of small rounded opacities (less than 1 cm in diameter) known as silicotic nodules. Individuals with chronic silicosis don't usually present with chest symptoms, however, some have reported a chronic productive

cough [Banks, 1996]. This type of silicosis is common in occupations such as quarrying, stone cutting and mining [Vallyathan and Shi, 1997].

2. Progressive massive fibrosis (PMF)

PMF develops when neighbouring smaller silicotic nodules coalesce and collapse into one another to form an opacity (greater than 1 cm in diameter) [Green and Vallyathan, 1996].

Steen and colleagues [1997] found that the latency period between crystalline silica exposure and the manifestation of radiological abnormalities was at least 15 years for PMF.

3. Accelerated silicosis

Although this form of silicosis is radiologically identical to chronic silicosis, the time from initial exposure to crystalline silica to the development of silicotic nodules (latency period) is much shorter [Banks, 1996]. It usually develops after 1 to 14 years of high to moderate exposure to crystalline silica [Green and Vallyathan, 1996].

4. Alveolar lipoproteinosis

This is the most aggressive form of silicosis as it progresses rapidly (it may develop after only six months of exposure to crystalline silica) and has a high mortality rate [Green and Vallyathan, 1996]. It results from exposure to extremely high concentrations of respirable crystalline silica. The individual progresses to disabling chest symptoms and extreme respiratory impairment, which ultimately leads to respiratory failure and death [Banks, 1996].

This type of silicosis is common in occupations such as silica flour mill operations, surface mine drilling, tunnelling, sandblasting, and pottery manufacturing [Vallyathan and Shi, 1997].

The silicotic nodule

Chronic silicosis is characterised by the silicotic nodule, which is morphologically distinct from lesions formed by exposure to other inorganic particles. Macroscopically, the nodules appear as rounded, firm to hard regions of fibrosis that are easily distinguishable from the surrounding lung parenchyma. [Green and Vallyathan, 1996].

1.2.1.11 Detection of silicosis

The detection of silicosis in dust exposed workers is based on the presence of silicotic nodules on a chest radiograph [Hnizdo *et al*., 1993].

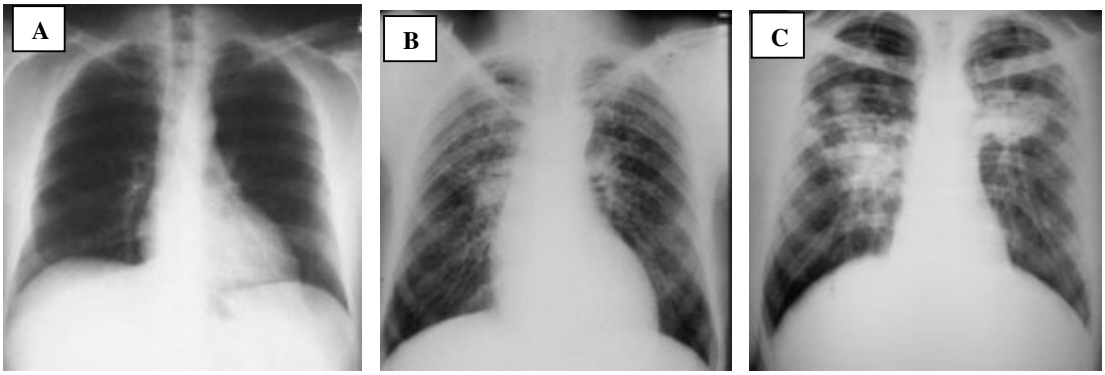


Figure 1.2.1.11: Chest radiographs depicting A) normal lung tissue, B) small parenchymal opacities (found in nodular silicosis), and C) large parenchymal opacities (found in PMF). [CDC, http://www.cdc.gov/niosh/topics/chestradiography/ilo.html].

In order for a diagnosis of silicosis to be made, three conditions need to be met. Firstly, the individual must provide a history of silica exposure which would be sufficient to cause silicosis. Secondly, the chest radiograph must show opacities which are consistent with silicosis. Thirdly, no underlying illnesses which could mimic silicosis should be present. The diagnosis of silicosis does not require respiratory symptoms or lung function impairment (even though they might be present). [Banks, 1996].

1.2.1.12 Classification of silicosis

The International Labour Organisation (ILO) has standardized the radiographic classification of pneumoconioses by providing a set of standardized radiographs and guidelines for their use. During the classification process a number of factors are described, such as the technical quality of the radiograph; the size, shape (round or irregular), affected zones of the lungs and profusion of parenchymal abnormalities; and the characteristics of pleural abnormalities.

Parenchymal abnormalities include both small opacities (< 1 cm in diameter), such as the simple silicotic nodule, and large opacities (> 1 cm in diameter), such as the nodules characteristic of complicated silicosis. For small opacities, the term profusion is used to describe the concentration of small opacities in the affected zones of the lungs. There are four main categories and twelve subcategories of profusion, ranging from 0/0 (no small opacities present) to 3/+ (a high concentration of small opacities present), as illustrated in figure 1.2.1.12 below. The size of opacities is also described. [ILO, 2000].

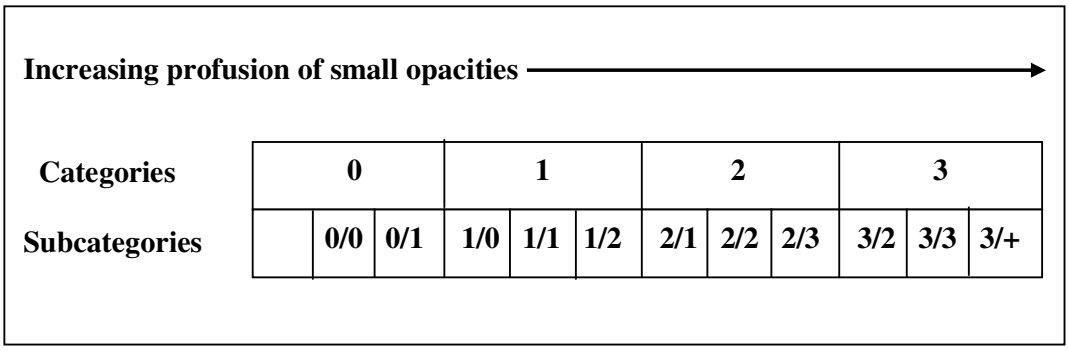


Figure 1.2.1.12: Guideline for the classification of the profusion of small opacities on chest radiographs. [ILO, 2000]

Silicosis can also be classified by means of a macroscopic and microscopic examination of the lung parenchyma at autopsy. The degree of silicosis is based on the number of palpable silicotic nodules as follows: none (0), insignificant (< 5), slight (5-9), moderate (10-29), and marked (≥ 30). Microscopic analysis of the histological slides is used to confirm the presence of silicotic nodules. [Hnizdo *et al*., 1994].

1.2.1.13 Limitations of silicosis detection

Although standard chest radiography is inexpensive, widely available and involves low amounts of radiation; it also has some significant limitations:

1) It is widely known that routine standard chest radiographs are relatively insensitive for diagnosing interstitial lung disease when compared to direct microscopic analysis of the tissue [Checkoway and Franzblau, 2000, Hnizdo and Sluis-Cremer, 1993, Hnizdo *et al*., 1993]. As a result, misclassification of silicosis may occur.
2) Large inter-reader and intra-reader variability can arise in the assessment of abnormalities. A number of factors that can potentially contribute to this variability

are radiographic technique and quality, age, obesity, cigarette smoking, gender, and differences in readers' tendencies [Meyer *et al*., 1997, Dick *et al*., 1992].

3) Errors in the interpretation of chest radiographs can also occur due to rotation of the subject, exhalation while the radiograph is taken, and the presence of soft tissue shadows [Parkes, 1974].

A further limitation of the radiological diagnosis of silicosis is the fact that silicotic nodules are late and irreversible manifestations of the disease. As a result, the annual chest radiograph used to screen miners for silicosis is only a late indicator of harmful dust exposure and therefore does not provide an effective way to measure and control the dust exposure.

1.2.1.14 Treatment of silicosis

At present there is no effective treatment for silicosis [Greenberg *et al*., 2007]. As a result, one can only attempt to prevent the development of silicosis by limiting exposure to crystalline silica.

1.2.1.15 Prevention of silicosis

Numerous measures can be implemented to help prevent silicosis, such as: the enforcement of exposure limits and safety standards, the use of engineering controls (for example the use of personal protection, improved ventilation, wet techniques, and process enclosure) and safe work practices, evaluation of the working environments to assess the effectiveness

of preventative measures, epidemiological evaluations, medical surveillance of workers at risk (for early detection of the disease), and training and health education [Fedotov, 2006].

Industrialised countries (such as the United Kingdom and the United States) have shown that the incidence rate of silicosis can be significantly reduced with well-organised silicosis prevention programmes[Fedotov, 2006].

In 1995 the Joint International Labour Organization (ILO) and World Health Organization (WHO) Committee on Occupational Health established the ILO/WHO Global Programme for the Elimination of Silicosis (GPES) by 2030. At the South African Mine Health and Safety Summit in 2003, the Minister of Minerals and Energy emphasised the need for improvements in health and safety in the South African mining industry. It was at this summit that the following milestones for the elimination of silicosis, in South Africa, were set:

1) By 2008, 95% of all dust exposure measurements should be below the respirable crystalline silica Occupational Exposure Limit (OEL) of 0.1mg/m^3.
2) By 2013, no new cases of silicosis in previously unexposed individuals should be diagnosed (using current diagnostic techniques).

In June 2004, the National Programme for the Elimination of Silicosis (NPES) in South Africa was launched by the Department of Labour.[Stanton *et al*., 2005]. In addition, the Mine Health and Safety Council also launched a Silicosis Elimination Programme which is a research-based programme aimed particularly at the mining industry [Rees, 2006].

In the United States of America, the Occupational Safety and Health Administration (OSHA) set a permissible exposure limit (PEL) of 0.1 mg/m^3 for an 8-hour work shift

[Greaves, 2000] while the National Institute for Occupational Safety and Health (NIOSH) set a recommended exposure limit (REL) of 0.05 mg/m^3 for up to 10 hours per day for a 40-hour work week [NIOSH, 2002]. In South Africa, the OEL for crystalline silica is 0.1 mg/m^3 [DME, 2006].

Alarmingly, evidence suggests that this current respirable crystalline silica OEL of 0.1 mg/m^3 may be insufficient to protect workers from developing silicosis. This was highlighted by Greaves [2000] who reviewed three important epidemiological studies (one of which was conducted in South Africa). He illustrated (with the aid of figure 1.2.1.15 below) that after 45 years of exposure to respirable crystalline silica at the current OEL of 0.1 mg/m^3, the risk of developing silicosis of grade 1/1 or greater would be over 40% and that if the OEL were lowered to 0.05 mg/m^3, the risk of developing silicosis would be about 10-20%. As observed in figure 1.2.1.15, the estimated silicosis risks presented by Kreiss and Zhen [1996] were almost double those found by Hnizdo and Sluis-Cremer [1993] and Steenland and Brown [1995] which may be explained by the fact that the latter studies estimated silica exposures from the average silica content of ore or dust samples while the former study used job title-specific silica exposure measurements which are less likely to result in silica exposure misclassification [Kreiss and Zhen, 1996].

The conclusion that the OEL of 0.1 mg/m^3 is not protective against silicosis was also made by Churchyard [2004], Finkelstein [2000] and Murray [1996]. The no-observed-adverse-effect-level (NOAEL) for respirable crystalline silica is currently unknown but it is clear that the current OEL of 0.1 mg/m^3 is too high and that even lowering the OEL to 0.05 mg/m^3 would offer no adequate protection against silicosis.

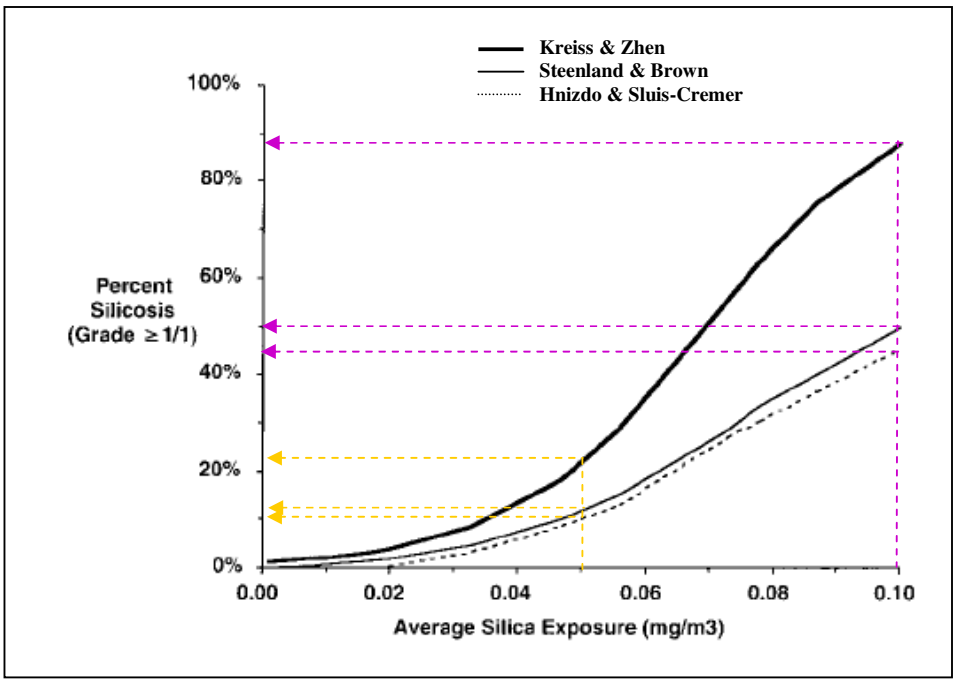


Figure 1.2.1.15: Comparison of silicosis risks in three studies of hard rock miners. In this case, silicosis was defined by the presence of small round opacities with profusion 1/1 or greater. The average respirable silica levels were derived from cumulative exposure data assuming 45 years of continuous exposure. Purple and orange dotted arrows are used to indicate the risk of developing silicosis (of grade ≥ 1/1) at an average silica exposure level of 0.1mg/m^3 and 0.05mg/m^3, respectively. Graph constructed by Greaves [2000] using data from Hnizdo and Sluis-Cremer [1993], Steenland and Brown [1995], and Kreiss and Zhen [1996].

1.2.1.16 Methods for the measurement of respirable dust

Due to the uncertainties surrounding the effectiveness of the OEL to prevent silicosis, it is vital that the methods used to measure the amount of respirable crystalline silica containing dust are accurate.

Currently, the instrument used for the measurement of respirable dust in field operations is the personal gravimetric dust sampler. The sampler consists of a cyclone assembly and filter holder, which contains a pre-weighed millipore filter and is attached to a battery-

powered pump. As the pump draws air through the assembly the larger particles are discarded while the smaller particles stay on the filter. The dust can then be analyzed in a number of ways, including: examination under a polarizing microscope, roentgenographic diffraction; colorimetry; infra-red spectrophotometry and X-Ray Diffraction (XRD). [Ziskind *et al*., 1976].

The use of dosimetric readings to estimate immediate exposure to harmful dust is routinely used by the mines, however, these readings are often unreliable. For instance, the levels recorded have been found to be quite variable even within groups working closely together. In addition, the equipment is sometimes not worn correctly or it is allowed to get wet (since the mining areas are often sprayed with water to decrease the levels of dust). It is often difficult in practice to measure the exposure over a full 8-hour shift, and this leads to problems when estimating the cumulative exposure levels. Also, miners may move around and change their activities during a shift, so compliance and interpretation become problematic. [Girdler-Brown *et al.*, 2006].

1.2.1.17 Compensation of silicosis afflicted individuals

It is estimated that there are nearly half a million ex-mineworkers suffering from compensatable lung disease in southern Africa, and that there is approximately 2.8 billion rand worth of unpaid compensation [Marks, 2006]. The enormous problems encountered in the compensation of miners could also be resolved if early biomarkers could be identified that would prevent silicosis from occurring.

1.2.1.18 Biomarkers for crystalline silica exposure

The high silicosis prevalence rate (despite the implemented dust control measures), the limitations of the use of chest radiographs for the early detection of silicosis, the lack of an effective treatment for silicosis, the uncertainties regarding the OEL level, the problems involved in the measurement of dust exposure, the potential financial issues regarding compensation and the association of crystalline silica exposure with other diseases, emphasize the need for more effective silicosis prevention methods (both locally and globally).

Since silicosis can be prevented by limiting the inhalation of crystalline silica dust [Rimal *et al*., 2005], it is important to be able to reliably assess the effectiveness of dust control measures currently employed.

If scientifically acceptable biomarkers were found that could allow the measurement of changes that occur immediately after exposure to crystalline silica but prior to the appearance of abnormalities on chest radiographs, they could be used to detect early adverse effects of silica exposure and also to evaluate efficacy of dust-control methods implemented [Gulumian *et al*., 2006].

1.2.2 Biomarkers

In the past, relationships were observed between exposure and disease even though the mechanisms of action or the intervening steps were not often understood (refer to figure 1.2.2). However, this approach is not very useful in situations with low levels of exposure, intermittent exposure, a mixture of exposures, or for diseases with long latency periods (such as silicosis). This approach is also problematic as there is a tendency to misclassify people as to their exposures and it does not take into account acquired or genetic host factors. Biological markers (biomarkers) may, potentially, address these problems. [NRC, 1987, Schulte, 1995].

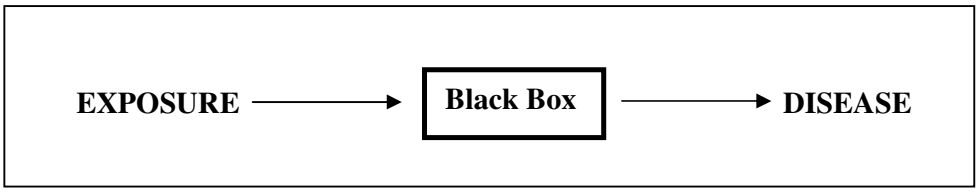


Figure 1.2.2: The traditional view of the relationship between exposure and disease. Adapted from [Schulte, 1995].

1.2.2.1 Definition of biomarkers

The Committee on Biological Markers of the National Research Council (NRC) in the United States of America has defined biomarkers as "Indicators of variation in cellular or biochemical components or processes, structure or function that are measurable in biologic systems or samples" [NRC, 1989]. Therefore, biomarkers may represent signals in the intervening steps between exposure and resultant disease [Schulte, 1989].

Biomarkers can be classified into three main types: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility [NRC, 1987].

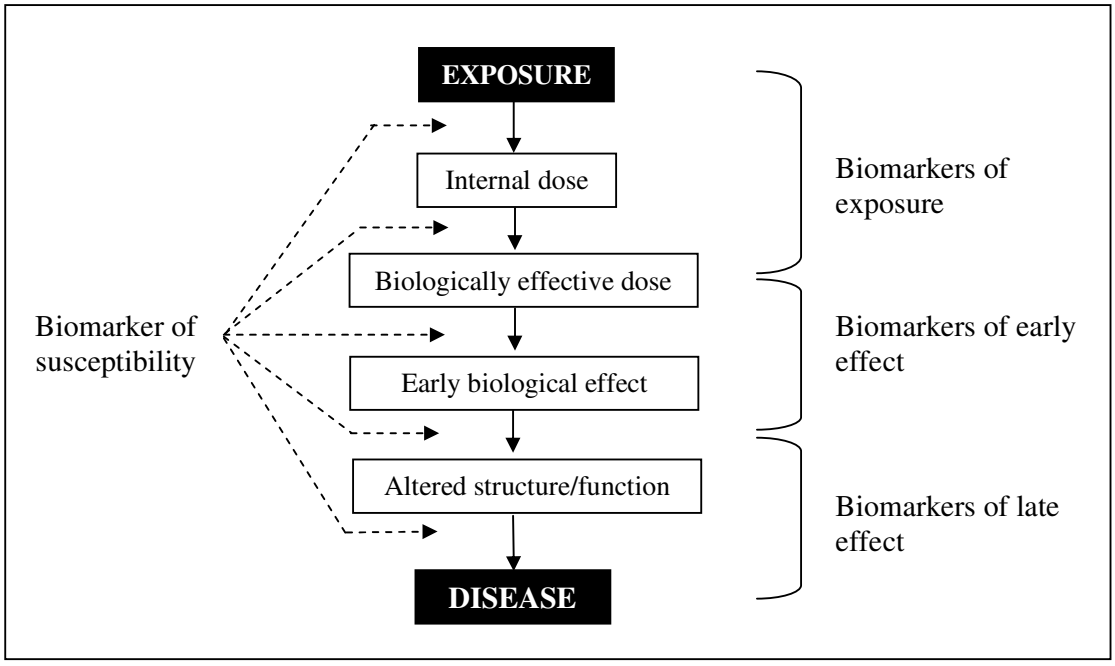


Figure 1.2.2.1: Flow chart of the position of different types of biomarkers in the pathway from exposure to disease. Solid arrows indicate progression. The pathway is a temporal sequence where the higher event generally precedes the lower event. Biomarkers in the first three blocks can be considered biomarkers of exposure and biomarkers in the lower four blocks can be considered biomarkers of effect. Biomarkers of susceptibility can occur at any stage in the pathway. Adapted from [NRC, 1987].

For any given toxicant exposure, there may therefore be several biomarkers with different levels of sensitivity and specificity that can be measured in different body fluids and tissues [Timbrell, 1998].

1.2.2.2 Biomarkers of exposure

These are the xenobiotic substance of interest or a metabolite(s) or product of its interaction with a biological molecule or cell, that are measured in a compartment within an organism [NRC, 1989]. They can be divided into two types: markers of internal dose or markers of effective dose.

While external exposure is the level of xenobiotic substances an organism is exposed to, the internal dose is the amount of the xenobiotic compound that is actually absorbed into the organism and effective dose is the amount of the xenobiotic compound that interacts with critical subcellular, cellular and tissue targets [NRC, 1987]. Biomarkers of internal dose therefore indicate that exposure to a xenobiotic compound has taken place by measuring the compound or its metabolite(s) in body fluids. Biomarkers of effective dose indicate that exposure to a xenobiotic compound has resulted in the compound or its metabolite(s) reaching a toxicologically significant target [Timbrell, 1998].

Examples of biomarkers of exposure in the occupational health setting include urinary cadmium as a long term marker of cadmium exposure, urinary methylhippuric acid as a marker of recent xylene exposure and the DNA adduct Styrene oxide-O^6 guanine as a marker of styrene exposure [Waterfield and Timbrell, 2000].

1.2.2.3 Biomarkers of effect

These are measurable physiologic, biochemical, or other changes within an organism that can be recognized as a potential or established health impairment or disease [NRC, 1989].

In the case of biomarkers of effect, an effect is defined as a recognized disease, an early precursor of a disease process, or an event that is nonessential to any disease process but is correlated with one and is thus predictive of the development of the disease. A biological marker of effect can therefore be any change that is quantitatively or qualitatively predictive of a disease associated with exposure. [NRC, 1987, NRC, 1989].

As far as exposure is concerned, biomarkers of effect are, in theory, non-specific. However, in occupational situations it is often possible to exclude other factors affecting the biomarker levels and therefore a relative specificity is possible [Aitio, 1999].

Biomarkers of effect range from simple (for example body weight) to complex (for example determination of specific isoenzymes). They can also be divided into those that detect biochemical changes or those that indicate pathological damage; and those that can be accessed through invasive or non-invasive procedures. [Timbrell, 1998]. In addition, biomarkers of effect can be classified as either early or late biomarkers. Early biomarkers of effect can indicate the extent of the initial interaction of the toxic substance (for example crystalline silica) with the cellular systems while late biomarkers of effect can indicate early minimum damage to the lung. An ideal biomarker of effect would be able to indicate early reversible events [Gulumian *et al.*, 2006].

Examples of biomarkers of effect include the enzyme aspartate aminotransferase (AST) as a measure of myocardial damage, induction of cytochrome P450 isoenzymes as a marker of exposure to polycyclic aromatic hydrocarbons (PAHs) and organochlorine compounds, and increased metallothionein levels as a marker of heavy metal exposure (such as cadmium) [Waterfield and Timbrell, 2000].

1.2.2.4 Biomarkers of susceptibility

These indicate an acquired or inherent limitation in the ability of an organism to respond to the exposure to specific xenobiotic substance [NRC, 1989]. Therefore, any variation in the response of an individual to identical exposures could indicate a difference in susceptibility due to either the genetic make-up of the individual or to factors or environmental influences such as diet or the uptake and absorption of the xenobiotic substance. However, the most likely source of variability is due to the metabolism of the substance by the organism, which may be genetically determined. [Waterfield and Timbrell, 2000].

Biomarkers of susceptibility include, among others, polymorphisms in genes responsible for DNA repair, genomic stability and chemical activation or detoxification [Schulte and Rothman, 1998].

There are however numerous ethical, legal and social issues surrounding the use of biomarkers of susceptibility [Schulte, 1991].

1.2.2.5 Applications of biomarkers

The different types of biomarkers can be used to 1) assess exposure, 2) identify effects or changes that occur as a result of the exposure, 3) identify the start of pathological changes before the development of the disease, and 4) predict susceptibility of individuals to disease [Gulumian *et al.*, 2006]. Therefore, the ultimate objective of biomarkers is to identify problems as early as possible to avoid adverse effects [Waterfield and Timbrell, 2000].

The most important prerequisite for a biomarker to be practically useful is that enough information is available to translate the observed changes in terms of health significance and clinical relevance. The most challenging part of achieving this is to distinguish between adaptive and toxic effects, reversible and irreversible effects, and adverse from non-adverse effects [Bernard and Hermans, 1997].

In population studies, the potential uses of biomarkers include: 1) mechanistic studies (i.e. the identification of the mechanism); 2) as a "gold standard" in validation studies; and 3) to monitor compliance to a prescribed regime [Howe, 1998].

One application for biomarkers of exposure is in biological monitoring (which involves the periodic measurement of a biomarker of exposure). Biological monitoring aims to quantify the amount of chemical absorbed; transformed into an active metabolite or accumulated in organs, tissues or cells when exposure to a chemical occurs. In epidemiological research, this can help to overcome the limitations of crude markers of exposure such as job titles or point estimates of airborne pollution. [Mutti, 1999].

1.2.2.6 Limitations of biomarkers

Potential limitations of the use of biomarkers include: 1) the high cost of assaying many biomarkers may mean that only a small sample size can be used (which results in an increase in random error and a decrease in study power); 2) in case-control studies many biomarkers may only reflect recent exposures and may not reflect exposure at the time relevant to the aetiology of the disease; 3) in case-control studies the disease process and/or

treatment may affect the biomarker in an unpredictable manner; and 4) in cohort studies longitudinal exposure data may not be available [Howe, 1998].

1.2.2.7 Ideal characteristics of biomarkers

Ideally, a biomarker should be 1) specific, 2) sensitive, 3) quantitative, 4) in-expensive, 5) easily measurable, 6) related to the biochemical mechanism, 7) detectable at realistic exposure doses and 8) assessed by non-invasive procedures [Timbrell, 1998]. However, all these characteristics will rarely be available in one biomarker [NRC, 1989].

1.2.2.8 Validation of biomarkers

Before a biomarker can be used as an independent or dependent variable for medical screening, surveillance, or intervention, it must be validated [Schulte, 2005]. An essential step in the validation process is establishing that a relationship between an environmental exposure and the biological change of interest (the biomarker) exists [NRC, 1989]. Validation can also include the determination of the specificity and sensitivity of the biomarker, characterization of all variability sources (analytical, biological) and characterization of the background levels [Manini *et al*., 2007].

Validation of biomarkers involves both laboratory and population requirements. The laboratory requirements include the analytical validity of the assay or test and an understanding of the biology of the biomarker. The population requirements include describing the performance of the test in populations and the factors that affect the test or biomarker (for example pre-existing medical conditions, lifestyle choices, or

environmental factors). [Schulte, 2005]. It is also important to consider the stability of the biomarker during the sampling process and storage (for example the storage temperature and duration) [Kang *et al.*, 2005].

In order to evaluate the health risks arising from exposure to xenobiotic substances, reference values (called Biological Exposure Indices or BEI) for the general population are determined in order to identify individuals with an increased exposure compared to the background level [Morgan, 1997]. However, biomarkers are subject to biological variability. As a result, reference values (RVs) for biomarkers should be defined at a local level since they are influenced by environmental exposure levels in that country, biological conditions (age, sex, diseases), metabolic interferences due to lifestyle choices (such as tobacco and alcohol consumption, use of drugs), genetic polymorphisms, and improvement in analytical procedures [Manini *et al.*, 2007].

1.2.2.10 Potential biomarkers of crystalline silica exposure

Ever since the pathogenic pathway of silicosis was elucidated, attempts have been made to identify suitable biomarkers to detect exposure to high levels of crystalline silica and/or to predict the development of silicosis.

In terms of crystalline silica as a biomarker of exposure, the major problem is that silica cannot be measured directly in blood, unlike other chemicals such as lead. It can however be measured in lung tissue and in bronchoalveolar lavage fluid (BALF) but these methods are invasive and expensive and therefore not practical for exposure studies. In addition,

neither the presence nor elemental analysis of crystalline silica has produced an accurate estimation of internal dose in relation to disease. [Gulumian *et al.*, 2006].

A number of polymorphisms, such as TNF-α and IL-1 polymorphisms, have been investigated as possible biomarkers of susceptibility for silicosis in crystalline silica exposed individuals. One of these investigations [McCanlies *et al.*, 2002] has however suggested that although this genetic information is valuable in characterising risk, it is not a useful tool for individual classification. In addition, such biomarkers would indicate susceptibility to silicosis rather than indicating recent exposure to crystalline silica exposure [Corbett *et al.*, 2002]. Finally, there may be ethical, social, and legal problems associated with the use of biomarkers of susceptibility [Schulte, 1991].

Due to the limitations associated with the biomarkers of exposure and susceptibility, it is preferable to investigate silica exposure indirectly by identifying and measuring biomarkers of effect of crystalline silica exposure in the blood or other easily accessible body fluids.

The first phase of this project identified ten potential biomarkers of effect that were considered worthy of further investigation [Gulumian *et al.*, 2006]. The ten biomarkers identified were: reactive oxygen species (ROS) measurement by chemiluminescence (neutrophil release), erythrocyte glutathione (GSH), erythrocyte glutathione-S-transferase (GST), erythrocyte glutathione peroxidase (GPx), n-8 isoprostane, tumour necrosis factor alpha (TNF-α), platelet-derived growth factor (PDGF), interleukin-8 (IL-8), Clara cell protein 16 (CC16), and total antioxidant status as a trolox equivalent (TAS).

The second phase of this project evaluated the ten biomarkers to determine the influence (if any) of Human Immunodeficiency Virus (HIV) infection, anti retroviral (ARV) treatment, smoking, age and the ability to discriminate between crystalline silica exposed and unexposed individuals. Two of the ten biomarkers investigated, namely erythrocyte GPx and serum CC16, were found to be significantly decreased in the silica dust-exposed subjects. In addition, the two biomarkers were found to be unaffected by HIV infection, ARV treatment, smoking, age and the presence of radiological silicosis. [Murray *et al.*, 2006].

This third phase of the project aimed to confirm the levels of and further analyze CC16 and GPx in relation to the levels and duration of crystalline silica exposure. GPx and CC16 are both biomarkers of effect; erythrocyte GPx is a marker of oxidative stress while serum CC16 is a marker of Clara cell (lung) damage.

1.2.3 Oxidative Stress markers

1.2.3.1 Reactive oxygen species

Reactive oxygen species (ROS) is the term given to oxygen free radicals and their metabolites. Free radicals are atoms or molecules with one or more unpaired electrons. Oxygen-centred free radicals contain the unpaired electron on the oxygen atom. [Vallyathan and Shi, 1997].

In the lungs, ROS can be produced by both endogenous and exogenous sources:

Endogenous sources:

Oxygen free radicals, which are usually beneficial to metabolic processes, are constantly produced by living cells. The main sources for the endogenous production of ROS are the mitochondria (during energy production), microsomes, endoplasmic reticulum, endothelial cells, nuclei, and phagocytic cells (such as AMs and neutrophils).

Exogenous sources:

These include physical and chemical agents such as tobacco smoke, toxic gases, vapours, chemicals, dust particles (such as asbestos and crystalline silica), and ambient air containing toxicants. [Vallyathan and Shi, 1997].

1.2.3.2 The involvement of ROS in silica exposure

As was previously described in sections 1.2.1.3 and 1.2.1.9, crystalline silica is able to produce ROS directly and/or by the stimulation of inflammatory cells (such as AMs). The ROS produced directly by freshly fractured crystalline silica include silicon-based radicals ($Si^{\bullet}$, $SiO^{\bullet}$, and $SiOO^{\bullet}$) in air [Ding *et al*., 2002], as well as hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$), and singlet oxygen (O_2) in aqueous medium [Shi *et al*., 1995]. The ROS produced by the immune response to crystalline silica inhalation include the superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [Engelen *et al*., 1990].

Reactive oxygen species may be involved in a number of silica-induced responses such as inflammation, fibrosis [Mossman and Churg, 1998], cell injury and proliferation [Vallyathan *et al*., 1998], and DNA modifications [Shi *et al*., 1995].

1.2.3.3 Oxidative stress

Oxidative stress has been defined as “An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage” [Sies, 1997]. As described earlier, oxidants such as ROS are continuously generated by both endogenous and exogenous sources. While low doses of radicals can be valuable or essential (for example in intracellular messaging and defence against micro-organisms), high doses can result in oxidative stress [Mates *et al*., 1999].

Oxidative stress plays a key role in the pathogenesis of many lung diseases both directly or indirectly [MacNee, 2000]. In the case of silicosis, exposure to crystalline silica causes an overwhelming production of ROS, which causes an imbalance in oxidant/antioxidant state and therefore oxidative stress [Zhang *et al*., 1999].

1.2.3.4 The lungs' defence against oxidative stress

The lung is the primary interface between the organism and the inhaled toxicants and it therefore plays a vital role in protecting against the ROS produced by these toxicants [Rahman *et al*., 1999]. The large epithelial surface area of the lung (including the tracheobronchial tree and the alveolar space) is particularly at risk to attack from ROS produced by inhaled toxicants [Comhair and Erzurum, 2005].

The lungs' main defence against inhaled ROS is an antioxidant system, which consists of a range of enzymatic and non-enzymatic molecules [Rahman *et al*., 1999]. The antioxidant system is normally able to cope with the continuous stream of free radicals [Borm *et al*., 1986]. Antioxidants exert their protective effect by: 1) preventing free radical production, 2) intercepting free radicals that have already been produced, 3) repairing oxidative damage caused by free radicals and 4) helping to eliminate damaged molecules [Gutteridge, 1995].

1.2.3.5 The non-enzymatic antioxidant defence system

One of the most important antioxidant defence mechanisms is the non-enzymatic glutathione (GSH) redox system.

Glutathione is a tripeptide consisting of γ-glutamic acid, cysteine and glycine, and it is present in all tissues. Reduced glutathione (GSH) can react with oxidants to form the oxidized form, glutathione disulphide (GSSG), or a mixed disulphide (RSSG) [Moldeus and Jiang, 1987].

The susceptibility and tolerance of lung epithelial cells to oxidative stress are strongly affected by their intracellular redox status (GSH/GSSG) [Rahman *et al*., 2005]. Normally the cells of the lung contain a high concentration of GSH to help protect the lung from oxidative stress. Glutathione limits the oxidative stress induced by toxicants by 1) returning cellular constituents to their normal reduced forms and thereby restoring their normal physiological function and 2) assisting in the detoxification of oxidants thereby preventing cellular injury. A decrease in GSH levels may therefore contribute to the development of lung injury and disease. [Rahman *et al*., 1999]

Besides GSH, the GSH redox system consists of a number of antioxidant enzymes, namely, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), and glucose 6-phosphate dehydrogenase (G6PD). While GSH and GPx function to detoxify exogeneous toxicants and their reactive intermediates, GR reduces GSSG back to GSH (using reduced nicotinamide adenine dinucleotide phosphate, NADPH) and G6PD helps maintain the NADPH in its reduced form. [Rahman *et al.*, 1999].

1.2.3.6 The enzymatic antioxidant defense system

The main antioxidant enzymes are catalase (CAT), manganese and copper-zinc superoxide dismutase (SOD), and glutathione peroxidase (GPx). They frequently act in a coordinated manner to protect the lungs or other tissues from damage caused by oxidative stress. [Quinlan *et al.*, 1994]. Superoxide dismutase converts the toxic superoxide anion radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) as follows:

$$2\ O_2^{\bullet-} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

In order to prevent hydrogen peroxide from forming the highly toxic hydroxyl radical ($OH^{\bullet}$), CAT and GPx catalyze the conversion of hydrogen peroxide to other non-toxic products (such as water) as follows [Rahman *et al.*, 2005]:

$$2\ H_2O_2 \xrightarrow{CAT} O_2 + 2\ H_2O$$

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2\ H_2O + GSSG$$

These three antioxidant enzymes are all found in human erythrocytes [Andersen *et al.*, 1997], and for a long time CAT and GPx were considered the erythrocyte's main defense against hydrogen peroxide. Recently, however, more attention is being paid to the antioxidant role of a family of peroxidases, called peroxiredoxins, in erythrocytes [Low *et al.*, 2007].

Peroxiredoxins (Prxs) are a group of antioxidant enzymes that reduce H_2O_2 and alkyl hydroperoxides to water and alcohol, respectively [Jara *et al.*, 2007]. They rely on a conserved cysteine residue to catalyze the reduction. Six mammalian isoforms (Prx1-6) have been identified with erythrocytes possessing in abundance Prx2 and a small amount of Prx1 and Prx6. [Low *et al.*, 2007]. Except for Prx6 which uses GSH as a co-substrate , Prxs use thioredoxin (Trx) as a co-substrate and therefore the reduction of hydrogen peroxide by most Prxs can be illustrated as follows [Rahman *et al.*, 2005]:

$$H_2O_2 + Trx(SH)_2 \xrightarrow{Prx} H_2O + TrxS_2$$

1.2.3.7 Characteristics of GPx

Glutathione peroxidase (E.C.1.11.1.9) was first described by Mills in erythrocytes [Mills, 1957]. It was shown to be essential for the protection of erythrocytes from oxidative breakdown, for example from hydrogen peroxide or ascorbic acid [Mills, 1957, Mills and Randall, 1958].

Glutathione peroxidase exists as two forms, selenium (Se)-dependent GPx and Se-independent GPx. Approximately one third of the lung GPx activity is Se-independent. [Rahman *et al.*, 1999].

There are four types of selenium-dependent GPx (that result from the expression of different genes): the classic cellular GPx (cGPx), the gastrointestinal GPx (giGPx), the extracellular or plasma GPx (eGPx or pGPx), and the phospholipid hydroperoxide GPx

(PHGPx) [Brigelius-Floche, 1993], also referred to as GPx-1 to GPx-4, respectively [Toppo *et al.*, 2008].

Erythrocyte (cellular) GPx is a 85 kDa protein [Rahman *et al.*, 1999] that forms a tetramer of four identical subunits each with a selenocysteine in their active site [Forstrom *et al.*, 1978]. It plays a significant role in the protection of lungs against oxidative damage (caused by ROS) [Nadif *et al.*, 2001]. This may be due to the fact that erythrocytes can act as a circulating antioxidant system in the lung. They are able to penetrate into the smallest blood vessels, which allows them to reach the sites of inflammation [Borm *et al.*, 1986].

1.2.3.8 Function of GPx

Glutathione peroxidases catalyze the reduction of hydrogen peroxide (H_2O_2), lipid hydroperoxides, and organic hydroperoxides; such as cumene hydroperoxide (CUOOH) [Saint-Denis *et al.*, 1998] and *tert*-butyl hydroperoxide (*t*-BOOH) [Brigelius-Floche *et al.*, 2002]; to water (H_2O) and alcohol (ROH) using reduced glutathione (GSH) as a co-substrate [Mannervik, 1985]:

$$ROOH + 2\,GSH \xrightarrow{GPx} H_2O + ROH + GSSG$$

The oxidised glutathione (GSSG) is recycled to its original state by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction catalyzed by glutathione reductase (GR) [Brigelius-Floche *et al.*, 2002]:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+$$

The primary function of glutathione peroxidase is therefore the *in vivo* detoxification of toxic hydrogen peroxide to less toxic products. Evidence reveals that cellular GPx (in conjunction with the pentose phosphate cycle and glutathione reductase) efficiently metabolizes intracellular hydrogen peroxide. The cellular GPx system is therefore a highly efficient and indispensable part of the cells' protection against oxidative stress. [Ursini *et al*., 1995].

1.2.3.9 Research involving GPx and exposure to crystalline silica and other toxicants

The activity of GPx has been shown to be affected by exposure to a number of toxicants, such as cigarette smoke [Li *et al*., 1994, Gilks *et al*., 1998, Vallyathan and Shi, 1997, Duthie *et al*., 1991, Kondo *et al*., 1994, Ho *et al*., 2005] and cadmium [Babu *et al*., 2006], and in a number of diseases including chronic obstructive pulmonary disease (COPD) [MacNee, 2000, Kluchova *et al.*, 2007], asthma [Misso *et al*., 1996, Powell *et al*., 1994], and bronchopulmonary dysplasia [Fu *et al*., 2008].

Since crystalline silica exposure causes oxidative stress and GPx activity is considered a marker of oxidative stress, a number of studies have examined the effect of crystalline silica exposure (and/or silicosis) on the activity of GPx.

In two studies involving silicosis patients and unexposed controls, one study found a significant decrease in GPx activity in the silicosis patients versus controls ($p < 0.001$) [Zhou *et al.*, 1999] while the other found a non-significant decrease (2.44 U/g Hb versus 2.86 U/g Hb, $p < 0.1$)[Borm *et al.*, 1986]. A decrease in GPx activity has also been

observed in coal mine workers. A study of active and retired French coal miners showed a significant decrease in mean GPx activity in the active miners compared to the retired miners (38.8 ± 12.5 U/g Hb versus 44.6 ± 13.7 U/g Hb, $p = 0.01$) [Nadif *et al.*, 2001]. A similar study of coal miners from three French regions found that the retired miners with coal workers' pneumoconiosis (CWP) had a lower GPx activity than those without CWP; however, the study also found that the GPx activity of active underground miners with CWP was slightly higher than those without CWP. Engelen and colleagues [1990] also showed that mean GPx activity was higher in Belgian coal miners with CWP than those without CWP (9.80 ± 0.05 U/g Hb versus 9.58 ± 0.08 U/g Hb, $p = 0.04$). A later study of rats acutely exposed to quartz also found a significantly higher level of GPx activity in the exposed rats compared to the controls [Vallyathan *et al.*, 1995].

The large variation in GPx activity values obtained in the aforementioned Borm *et al* [1986], Nadif *et al* [2001] and Engelen *et al* [1990] studies may have been due to the fact that different assays were used and the study participants were from different geographical regions. Although an accepted range of GPx activity for healthy silica exposed workers could not be found, a study on the biological variability of erythrocyte GPx [Guemouri *et al.*, 1991] reported erythrocyte GPx activity levels of between 22.5 and 67.0 U/g Hb in healthy unexposed adult males and between 24.4 and 68.1 U/g Hb in healthy adult females.

Due to the uncertainty surrounding the effect of crystalline silica exposure on GPx activity, further investigation was warranted. In addition, none of the above mentioned studies involved South African subjects or gold miners and none examined the effect of HIV infection or ARV treatment on GPx activity. Phase two of this project on biomarkers therefore aimed to investigate the levels of GPx activity in silica exposed gold miners and

controls from South Africa and determine if the levels were affected by HIV infection or ARV treatment. It showed that the GPx activity was significantly lower in the subjects exposed to crystalline silica compared to those with no exposure. It also found that there was no significant effect of HIV infection or ARV treatment on GPx activity. [Murray *et al.*, 2006]

The third phase of this project therefore aimed to further investigate the levels of erythrocyte GPx activity in relation to crystalline silica exposure.

1.2.4 Lung damage markers

The lung is a highly complex organ that contains over 40 different cell types [Bernard and Hermans, 1997], including Clara cells.

1.2.4.1 Clara cells

Although Clara cells were first recognised as a morphologically distinct cell type by Kölliker in 1881, Max Clara described the same cells in 1937 and the cells were named after him [Singh and Katyal, 1997]. Clara cells are nonciliated secretory epithelial cells (with diameters of 5 mm or less) that line the pulmonary airways (figure 1.2.4.1), in particular the respiratory and terminal bronchioles [Singh *et al*., 1988b].

Clara cells are one of the most multifunctional and heterogeneous cell types in the mammalian lung, with their main function being the protection of the respiratory tract [Broeckaert and Bernard, 2000].

One of the proteins secreted by Clara Cells is a 16 kDa protein known as Clara Cell Protein 16 or CC16 [Singh and Katyal, 1997]. In literature, the different CC16 homologues have been referred to by a number of different names which indicate their molecular weight (CC16, and Clara cell 10 kDa protein (CC10)), their origin (human protein-1, urine protein-1, uteroglobin (UG), and Clara cell secretory protein (CCSP)), or their function (blastokinin, Clara cell phospholipid-binding protein, and PCB-binding protein) [Broeckaert and Bernard, 2000].

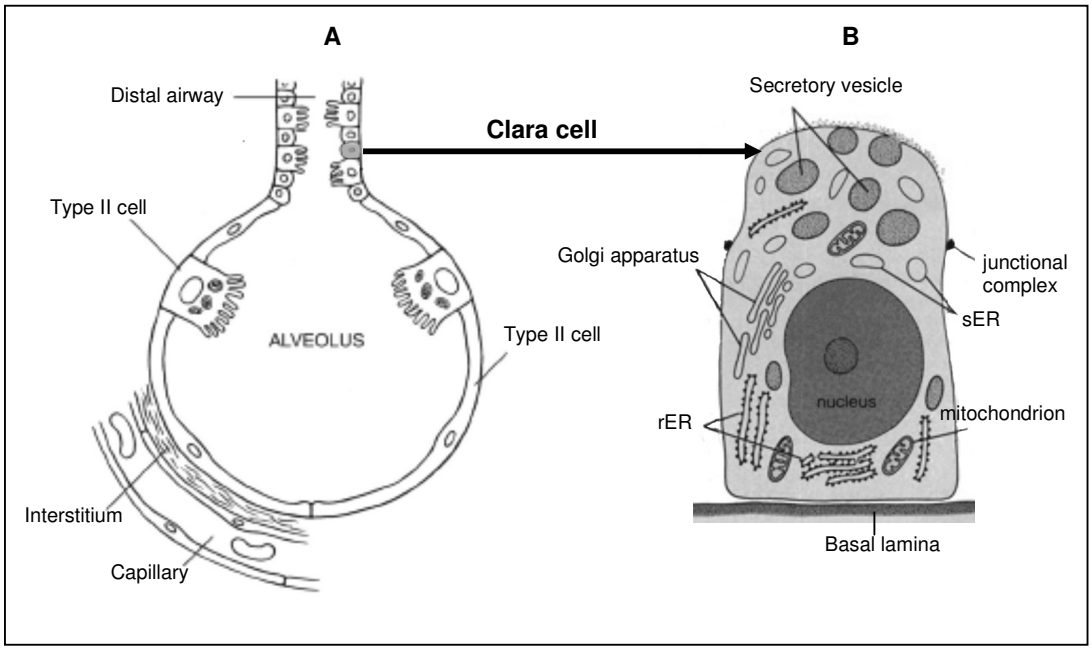


Figure 1.2.4.1: A schematic diagram of the distal pulmonary epithelium (A) with an enlarged view of a Clara cell (B). sER= smooth endoplasmic reticulum, rER= rough endoplasmic reticulum. Adapted from [Matthay *et al*., 2002] and http://anatomy.iupui.edu/courses/histo_D502/D502f04/lecture.f04/Respsystemf04/respiratory.html

1.2.4.2 History of CC16

In 1988, Jackson described the purification of a protein (called urine protein-1) from the urine of patients with renal failure [Jackson *et al*., 1988]. A protein, that was specifically localised to Clara cells in the lung, was then identified in rats [Singh and Katyal, 1984, Singh *et al*., 1985], rabbits [Gupta *et al*., 1987] and humans [Singh *et al*., 1988b]. It was determined, by means of sodium dodecyl sulphate-polycrylamide gel electrophoresis (SDS-PAGE), that the protein had a molecular weight of 10 kDa and was composed of two 5 kDa subunits [Singh *et al*., 1987]. Because the protein was the major secretory protein of the Clara cell, it was called Clara cell 10 kDa protein (CC10). Later studies showed that CC10 was identical to urine protein-1 [Bernard *et al*., 1992c, Bernard *et al*., 1992b] and that it was also the counterpart of rabbit uteroglobin [Mantile *et al*., 1993]. In 1993,

electrospray ionization/mass spectrometry showed that the molecular mass of the protein was actually 15 840 Da (16 kDa) which lead to the more common term of CC16 [Bernard *et al*., 1993].

1.2.4.3 Structure of CC16

Clara cell protein 16 is a homodimer of two identical 70 amino acid subunits (in antiparallel orientation) that are connected by two disulphide bonds [Singh *et al*., 1988a]. The early studies of CC16 showed that it is rich in glutamic acid, leucine, serine, and aspartic acid amino acids [Singh *et al*., 1988b] .

1.2.4.4 Function of CC16

The exact function of CC16 is still unknown, however, research indicates that it plays a key role in the lung as an anti-inflammatory, immunosuppressive, and antifibrotic agent [Wang *et al*., 2007]. This research includes:

1) The entire human CC16 gene has been sequenced and localized to chromosome 11, in particular the p12-p13 region. This region is occupied by several genes involved in the regulation of inflammation and allergy [Hay *et al*., 1995]. This lead to the suggestion that CC16 plays an important role in the regulation of inflammation in the lungs [Singh and Katyal, 1997].

1) Clara cell protein 16 has been shown to inhibit the production and biological activity of inflammatory cytokines [Broeckaert and Bernard, 2000, Wang *et al.*, 2007].

2) Clara cell protein 16 has been found to inhibit cytosolic phospholipase A_2 (PLA_2, an important enzyme in the inflammatory process) and this may be important in

controlling the inflammatory responses in the lung [Singh *et al*., 1990, Lesur *et al*., 1995]. Since inhibitors of PLA_2 have been shown to inhibit superoxide anion generation [Henderson *et al*., 1989], this may form part of CC16's anti-inflammatory activity.

3) Clara cell protein 16 is also able to inhibit PDGF-induced fibroblast chemotaxis (probably by inhibition of cytosolic PLA_2) and it has been suggested that a decrease in CC16 may allow the recruitment of fibroblasts (and therefore fibroblast activation and overgrowth) in fibrosing lung diseases [Lesur *et al*., 1995].
4) Clara cell protein 16 could play an important role in the sequestration or clearance of toxic substances that have been deposited in the respiratory tract [Singh and Katyal, 1997].
5) It has also been suggested that CC16 protects the respiratory tract against oxidative stress [Broeckaert and Bernard, 2000].

1.2.4.5 Characteristics of CC16

Although it was initially thought that CC16 was produced exclusively by Clara cells [Singh *et al*., 1988b], it was later shown that CC16 is also secreted in the male urogenital tract (in particular the prostate) [Bernard *et al*., 1991] as well as in the thyroid, mammary gland, and the pituitary [Peri *et al*., 1993]. As a result, in addition to the BALF, CC16 has been detected in a number of body fluids including urine, sperm, amniotic fluid and serum [Bernard *et al*., 1993].

The presence of CC16 in serum can only be explained by assuming that it moves from the lung into the bloodstream [Hermans and Bernard, 1999]. Although the exact mechanism

by which this happens and the exact sites of their passage are unknown, the most likely explanation is passive diffusion across the bronchoalveolar-blood barrier. This theory has been supported by studies that show a positive correlation between the CC16 concentration in serum and BALF [Petrek *et al*., 2002]. The most likely driving force for this diffusion is the large CC16 concentration gradient that exists across the bronchoalveolar-blood barrier [Bernard *et al*., 1998]. In addition, because CC16 is small (molecular radius ~1.9 nm) and because of the sieving properties of the alveolar barrier, CC16 is likely to move across the bronchoalveolar-blood barrier with little hindrance [Hermans and Bernard, 1998].

Once in the bloodstream, CC16 can rapidly be eliminated by glomerular filtration in the kidneys before being collected and catabolised in the proximal tubule cells [Bernard and Hermans, 1997].

The concentration of CC16 in serum is therefore determined by a number of factors.

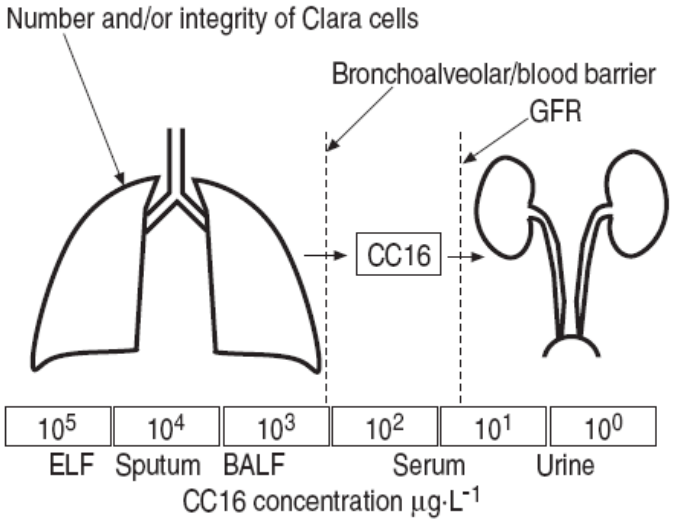


Figure 1.2.4.5: Schematic diagram of factors affecting the concentration of CC16 in serum. CC16 moves from the lung into serum and is then eliminated by the kidneys. GFR= glomerular filtration rate, ELF=epithelial lining fluid, BALF = bronchoalveolar lavage fluid. [Hermans and Bernard, 1998]

1.2.4.6 Applications of CC16 as a lung damage marker

If this theory of the movement of CC16 from the lung to the bloodstream is accepted, the assessment of the levels of CC16 in serum could be used for two important applications:
1) It could be used to assess the integrity of the bronchoalveolar-blood barrier (which can be compromised in a number of diseases)
2) It could be used to assess the changes in the number and/or integrity of the lung epithelial cells (when the bronchoalveolar-blood barrier is intact or slightly impaired). [Hermans and Bernard, 1998].

In fact, studies on experimental animals and occupationally exposed workers indicate that serum CC16 may be a relatively specific and sensitive biomarker for detecting early acute or chronic effects of toxicants on the tracheobronchial tree [Bernard and Hermans, 1997]. This is important as early lung injury is difficult to identify [Waterfield, 2000] and, at present, no toxicity biomarker is in place to monitor populations exposed to occupational or environmental pneumotoxins [Bernard and Hermans, 1997].

1.2.4.7 Research involving CC16 and exposure to crystalline silica and other toxicants

Levels of CC16 have been shown to increase in individuals exposed to toxicants such as asbestos [Petrek *et al*., 2002], polypropylene combustion products [Bernard *et al*., 1997], and ozone [Arsalane *et al*., 1999, Blomberg *et al*., 2003] and in patients suffering from sarcoidosis [Bernard *et al*., 1992a] and idiopathic pulmonary fibrosis [Lesur *et al*., 1995].

This increase has been attributed to an increase in the permeability of the bronchoalveolar-blood barrier and therefore an increase in the movement of CC16 into the bloodstream.

However, the few studies that have examined the effect of crystalline silica dust exposure on the concentration of CC16 in serum have found decreased CC16 levels. Bernard and colleagues [1994a] measured the levels of CC16 in the serum of asymptomatic quarry miners exposed to silica dust and control subjects and found a significant decrease in the mean CC16 levels in the miners (16.3 ng/ml for controls versus 12.3 ng/ml for miners, $p = 0.001$). Later, Wang *et al* [2007] studied the role of serum CC16 as a biomarker for the early diagnosis and the pathogenesis of silicosis. They also found that the serum CC16 concentration was significantly reduced in the silica-exposed pyrite mine workers compared to the controls (4.42 ± 1.83 ng/ml versus 12.59 ± 3.85 ng/ml, $p < 0.01$). The authors proposed that the decrease in CC16 levels in serum was as a result of a decreased secretion or production of CC16 due to the destruction of Clara cells by silica.

The results obtained for the aforementioned studies indicated that CC16 may be a promising marker of early lung injury caused by crystalline silica exposure. The second phase of this project on biomarkers therefore aimed to investigate the effect of crystalline silica exposure on the levels of serum CC16 in gold miners and controls from South Africa. It also aimed to determine if the CC16 levels were affected by HIV infection or ARV treatment. The study found that the CC16 levels were significantly lower in the subjects exposed to silica than those with no exposure (4.87 ± 1.69 ng/ml versus 5.77 ± 1.64 ng/ml, $p = 0.02$) and it also found that there was no significant effect of HIV infection or ARV treatment on the CC16 levels [Murray *et al.*, 2006][see Appendix A].

The third phase of this project therefore aimed to further investigate the levels of serum CC16 concentration in relation to crystalline silica exposure.

There was a large variation in the mean CC16 levels in the control groups of the Bernard *et al* [1994a], Wang *et al* [2001] and Murray *et al* [2006] studies. This large variation may be due to population differences. Other studies have reported serum CC16 levels of between 6.6 and 23.2 μg/l in healthy adult males and between 3.7 and 22.9 μg/l in healthy adult females [Shijubo *et al*., 2003]. A study on the effect of geographical location on CC16 levels would therefore be worthwhile; however, this was not investigated in the current study.

1.3 Aims and Objectives

The overall aim of this project on biomarkers is to identify, confirm and operationalise biomarkers for crystalline silica dust exposure that could be used for surveillance of dust exposure levels in South African mines and thereby assess the success of dust control measures. This third phase of the project aimed to confirm the levels of, and further analyze, two biomarkers, GPx and CC16, in miners exposed to low and high levels of crystalline silica dust. It also aimed to develop Standard Operating Procedures (SOPs) for biomarker specimen handling and storage under field conditions, and also for their assay in the laboratory.

In order to accomplish these aims, the following objectives needed to be completed:

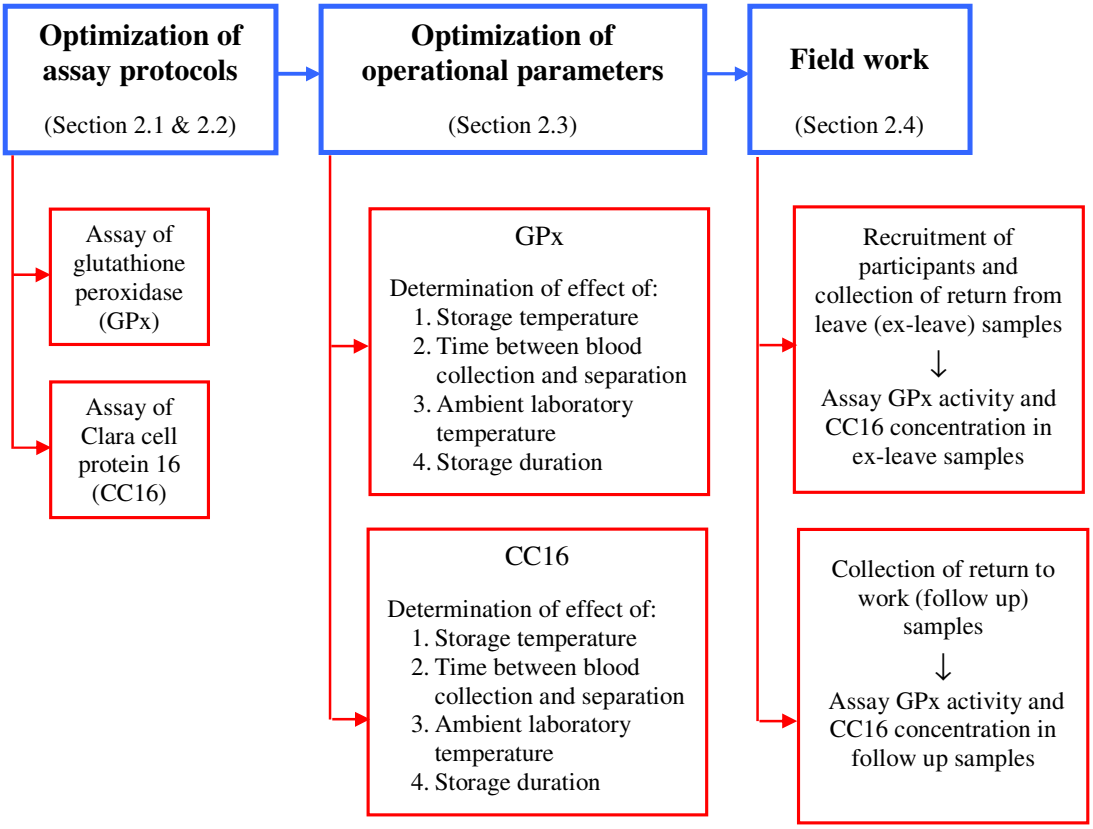
- To classify the study participants into high and low dust exposure groups according to their current job description.
- To measure the levels of GPx and CC16 in the study participants after their return from a period of leave and again following their exposure to crystalline silica.
- To investigate the relationship between the levels of GPx and CC16 and the duration of exposure to crystalline silica.
- To record chest radiological evidence of silicosis.
- To determine the effect of storage temperature, delay time between blood sample collection and separation, ambient laboratory temperature and storage duration on the levels of GPx and CC16.

Chapter 2:

Materials and Methods

The protocol for this study was peer-reviewed by international collaborators prior to the commencement of the study.

The diagram below outlines the three main steps involved in this phase of the project, which are described in sections 2.1 to 2.4:



2.1 Protocol for the assay of glutathione peroxidase

Analysis of the activity of glutathione peroxidase (GPx) was performed using a Glutathione Peroxidase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A) according to the manufacturer's instructions.

2.1.1 Principle of the Glutathione peroxidase Assay Kit

The GPx assay kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). During the assay, GPx catalyzes the reduction of cumene hydroperoxide (CUOOH) to water (H_2O) and alcohol (CUOH) using reduced glutathione (GSH) as a co-substrate [Saint-Denis *et al.*, 1998]:

$$CUOOH + 2\,GSH \xrightarrow{GPx} H_2O + CUOH + GSSG$$

The oxidised glutathione (GSSG) is recycled to its original state by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction catalyzed by glutathione reductase (GR) [Brigelius-Floche *et al.*, 2002]:

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NADP^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A_{340}). Under the assay conditions, the GPx activity is rate limiting and therefore the rate of decrease in the absorbance at 340 nm is directly proportional to the GPx activity

in the sample [Paglia and Valentine, 1967]. The Cayman GPx Assay Kit can be used to measure all of the glutathione-dependent peroxidases in plasma, erythrocyte lysate, tissue homogenate, and cell lysate [CaymanChemical, 2004].

Since the kit uses cumene hydroperoxide as the substrate, the total GPx activity is measured. However, human lungs and erythrocytes only contain Se-dependent GPx [Carmagnol *et al*., 1983] and since the current study used erythrocyte lysate samples, only the Se-dependent GPx activity was being measured.

Although GPx is not the only hydroperoxide reducing enzyme in cells, the assay is specific to GPx since the assay of catalase activity involves measuring the decomposition of hydrogen peroxide at a wavelength of 240 nm [Andersen *et al.*, 1997], the assay of peroxiredoxin activity uses the thioredoxin reductase system [Kim *et al.*, 2005] and neither of catalase nor peroxiredoxin use GSH as a co-substrate.

2.1.2 Glutathione peroxidase assay kit specifications

Each kit contained one vial of assay buffer concentrate (10 X), one vial of sample buffer concentrate (10 X), one vial of GPx control (consisting of a solution of bovine erythrocyte GPx), three vials of co-substrate mixture (consisting of a lyophilized powder of NADPH, glutathione and glutathione reductase), one vial of cumene hydroperoxide, one 96 well plate and one plate cover.

The kits were stored at -20 ^{o}C until the indicated expiration date.

2.1.3 Reagent preparation

The kit was removed from the -20 ^{o}C freezer and the components were brought to 25 ^{o}C.

The assay buffer was prepared by transferring 2 ml of assay buffer concentrate into a clearly labelled sterile 50 ml Falcon tube (Greiner Bio-One GmbH, Germany) containing 18 ml of HPLC-grade water, and gently mixing. The diluted assay buffer was stable for two months if stored at 4 ^{o}C. The sample buffer was prepared by transferring 2 ml of sample buffer concentrate into a clearly labelled sterile 50 ml Falcon tube containing 18 ml of HPLC-grade water, and gently mixing. The diluted sample buffer was stable for one month if stored at 4 ^{o}C.

The co-substrate mixture was prepared by adding 2 ml of HPLC-grade water to the vial provided and vortexing gently to mix. Each reconstituted vial of co-substrate mixture was enough for approximately forty wells. It was therefore only necessary to reconstitute the number of vials needed to supply the number of wells that were being used. The reconstituted co-substrate mixture was stable for two days if stored at 4 ^{o}C.

The GPx control was prepared by pipetting 10 µl of the GPx control into a 1.5 ml microcentrifuge tube containing 490 µl of diluted sample buffer, and gently mixing. The GPx control was only stable for four hours when stored at 4 ^{o}C and therefore it was only prepared after the sample preparation was complete (refer to section 2.1.4). According to the kit protocol, a 20 µl aliquot of the diluted GPx control should cause a decrease of approximately 0.051 absorbance units per minute under standard assay conditions [CaymanChemical, 2004].

2.1.4 Sample preparation

The thawed erythrocyte lysate samples (the preparation of which is described in sections 2.3.2.1, 2.3.3.1, 2.3.4.1, 2.3.5.1 and 2.4.4.1) were diluted 30 X by transferring 10 µl lysate into a 1.5 ml microcentrifuge tube containing 145 µl of diluted sample buffer, and gently mixing. According to the kit protocol, a 20 µl aliquot of diluted sample should have an initial absorbance of between 1.2 and 0.5 and should cause an absorbance decrease of between 0.02 and 0.135 per minute [CaymanChemical, 2004].

2.1.5 Assay procedure

Into each well, 100 µl of diluted assay buffer and 50 µl co-substrate mixture were added. The blank (non-enzymatic) wells were prepared by adding 20 µl of diluted assay buffer in triplicate into the designated wells. The positive control wells were prepared by adding 20 µl of diluted GPx control in triplicate into the designated wells. The sample wells were prepared by adding 20 µl of 30 X diluted erythrocyte lysate sample into the designated wells. Finally, to initiate the reaction, 20 µl of cumene hydroperoxide was added to each well.

The plate was gently shaken and placed in the plate holder of the automated BioTek ELx800^{TM} microplate reader (BioTek Instruments, Inc., USA). The GPx activity was then measured.

2.1.6 Plate analysis

To measure the absorbance of the samples, an automated microplate reader was used in conjunction with KC4^{TM}v3.4 software (BioTek Instruments, Inc., USA).

The microplate reader was set up while the reagents and samples were thawed. A laptop installed with KC4^{TM}v3.4 software and a HP LaserJet 1010 printer were connected to the microplate reader. Using the KC4^{TM}v3.4 program and the accompanying manufacturers' instructions, a new protocol was designed for each experiment. Each protocol included details about the layout of samples, controls and blanks in the plate; the wavelength to be used; and the results needed in the printed report.

When the plate was ready to be analyzed, the absorbance of each well was measured at 340 nm every minute for a total of five minutes. The resulting absorbance data was printed and used to calculate the GPx activity (see Appendix B).

2.1.7 Calculation of GPx activity

To ensure accuracy, two different methods of calculating the GPx activity (using the absorbance data obtained) were used. The first method involved an automated analysis tool provided by Cayman Chemical Company while the second involved a manually designed spreadsheet in Microsoft Excel.

2.1.7.1 Analysis tool

The Cayman Chemical Company website (*www.caymanchem.com*) provides access to an analysis tool that allows the GPx activity to be calculated automatically from the absorbance data obtained. A document entitled "GSHases Triple'' was downloaded and in the worksheet entitled "Analysis" (see Appendix C), the absorbance data obtained were entered into the appropriate fields. The GPx activity in each well was automatically calculated. The corresponding graphs of absorbance versus time for each sample were also automatically constructed and illustrated in a worksheet entitled "Graphs" (Appendix D).

2.1.7.2 Microsoft Excel calculations

Using the absorbance data obtained, the change in absorbance (ΔA_{340}) per minute of the blanks, positive controls and samples was calculated using the formula:

$$\Delta A_{340}/\ min = (A340_{\ (at\ 5\ minutes)} - A340_{\ (at\ 0\ minutes)}) / (5\ Minutes - 0\ Minutes)$$

The average rate of ΔA_{340}/min of the blank wells was calculated and subtracted from the rate of ΔA_{340}/min of the positive control wells and the sample wells. The GPx activity in each well was then calculated using the formula:

$$GPx\ Activity = ((\Delta A_{340}/\ min)/\ 0.00373\ \mu M^{-1}) \times (0.19\ ml/0.02\ ml) \times Sample\ Dilution = nmol/min/ml$$

Where: 0.00373 μM^{-1} is the NADPH extinction coefficient; 0.19 ml is the final assay volume in each well; 0.02 ml is the volume of sample in each well; and the sample dilution is 30 X.

In order to ensure reproducibility, Microsoft Excel was used to design a spreadsheet into which the formulae were entered. The absorbance values were then entered into the spreadsheet and the GPx activity was calculated.

For both methods the GPx activity results were reported in units of nmol of NADPH oxidized to $NADP^+$ per minute per ml (nmol/min/ml). The units were then manually converted, using Microsoft Excel, to units per millilitre (U/ml) where one unit (U) is defined as the enzyme activity that catalyzes the oxidation of 1 μmol of NADPH to $NADP^+$ per minute. In order to report the GPx activity results, for the field work samples, in the standard SI units of Units per gram haemoglobin (U/g Hb), the haemoglobin concentration of the samples was measured.

2.1.8 Measurement of haemoglobin

The haemoglobin concentration (in g Hb/dl) of the erythrocyte lysate samples was measured using a Coulter ® LH500 Hematology Analyzer (Beckman Coulter, Inc, USA). Since the replicate erythrocyte lysate samples used in the optimization of GPx methodology experiments (see section 2.3.1) were prepared from the same blood sample it was not necessary to measure the haemoglobin concentration of these samples. Therefore, haemoglobin concentration was only measured in the erythrocyte lysate samples collected during the field work (see section 2.4.4).

2.2 Protocol for the assay of Clara Cell Protein 16

The Clara cell protein 16 (CC16) concentration was determined using a Human Clara Cell Protein ELISA kit (BioVendor Laboratory Medicine, Inc; Czech Republic) according to the manufacturer's instructions.

2.2.1 Principle of the Human Clara Cell Protein ELISA Kit

The Human Clara Cell Protein ELISA kit is a biotin labelled antibody based sandwich enzyme immunoassay for the quantitative measurement of human CC16 in serum, plasma, tissue culture medium and bronchoalveolar lavage fluid. Standards, quality controls or samples are incubated with rabbit polyclonal anti-human Clara Cell Protein antibody coated in microtiter wells. A washing step is then performed and biotin-labelled polyclonal anti-human Clara Cell Protein antibody is added to bind with the captured CC16. After incubation and washing, streptavidin-horseradish peroxidase conjugate is added to bind to the biotin attached to the captured CC16 via the polyclonal anti-human Clara Cell Protein antibody. Following a final incubation and washing, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of Clara Cell Protein. A standard curve is constructed by plotting absorbance values versus Clara Cell Protein concentrations of calibrators, and concentrations of the unknown samples are determined using the standard curve. [BioVendor, 2006].

2.2.2 Human Clara Cell Protein ELISA Kit specifications

Each kit contained 100 ml of wash solution concentrate (10 X), 20 ml of dilution buffer, 13 ml of biotin labelled antibody solution, 13 ml of streptavidin- horseradish peroxidase (HRP) conjugate, 13 ml of substrate solution (TMB), 13 ml of stop solution (0.2 M H_2SO_4), two vials of quality control (QC) high (lyophilized), two vials of QC low (lyophilized), one vial of Clara Cell Protein master calibrator (purified from human urine and then lyophilized), and sealed antibody coated microtitier strips (96 wells). An instruction manual and a certificate of analysis were also included with the kit.

The kits were stored at between 2 ^{o}C and 8 ^{o}C until the indicated expiration date.

2.2.3 Reagent preparation

Before performing the assay, the kit was removed from the 4 ^{o}C fridge and the components were brought to room temperature (approximately 25 ^{o}C).

Diluted wash solution was prepared by adding 100 ml of the wash solution concentrate into a clearly labelled 1 L Schott laboratory bottle (Schott UK Ltd, UK) containing 900 ml of HPLC-grade water, and gently mixing. The diluted wash solution was stable for one month if stored at 4 ^{o}C.

The Clara Cell Protein Master Calibrator was reconstituted by the addition of 0.5 ml dilution buffer and mixed gently. The concentration of human Clara Cell Protein in the

stock solution was 100 ng/ml. The 100 ng/ml stock solution was serially diluted to prepare a 40, 20, 10, 5 and 2 ng/ml standard according to table 2.2.3.a.

Table 2.2.3.a: Preparation of Clara Cell Protein standards by serial dilution of 100 ng/ml Clara Cell Protein stock solution

Volume of standard	Volume of dilution buffer	Final concentration of standard
200 µl of 100 ng/ml stock	300 µl	40 ng/ml
200 µl of 40 ng/ml standard	200 µl	20 ng/ml
200 µl of 20 ng/ml standard	200 µl	10 ng/ml
200 µl of 10 ng/ml standard	200 µl	5 ng/ml
200 µl of 50 ng/ml standard	300 µl	2 ng/ml

The QC high, QC low and the six standards were diluted 25 X by pipetting 10 µl of the reagent into a 1.5 ml microcentrifuge tube containing 240 µl dilution buffer and were mixed gently.

Each Human Clara Cell Protein ELISA kit contains a certificate of analysis stating the expected results for the provided standards and quality controls (refer to table 2.2.3.b below and Appendix E).

Table 2.2.3.b: Expected ELISA results for standards and quality controls (QCs) as specified by the certificate of analysis (for lot no RD-1276R1)

Standards/ QC	Average A_{450}	Expected CC16 concentration (ng/ml)
Standard 1	3.245	100
Standard 2	2.482	40
Standard 3	1.637	20
Standard 4	0.987	10
Standard 5	0.569	5
Standard 6	0.264	2
Blank	0.011	-
QC High	1.913	23.09 (18.5-27.7)
QC Low	0.546	4.46 (3.6-5.3)

2.2.4 Sample preparation

The thawed serum samples (the preparation of which is described in sections 2.3.2.2, 2.3.3.2, 2.3.4.2, 2.3.5.2 and 2.4.4.1) were diluted 25 X by transferring 5 µl sample into a 1.5 ml microcentrifuge tube containing 120 µl of dilution buffer and mixed gently.

2.2.5 Assay procedure

The blank wells were prepared by pipetting 100 µl dilution buffer in the designated wells in duplicate. The quality control wells were prepared by pipetting 100 µl diluted QC high and diluted QC low into the designated wells in duplicate. The standard wells were prepared by pipetting 100µl of the diluted 100, 40, 20, 10, 5 and 2 ng/ml CC16 standards into the designated wells in duplicate. The sample wells were prepared by pipetting 100 µl of diluted serum sample into the designated wells.

The plate was wrapped in foil and incubated at room temperature for 75 minutes. The plate was washed five times with 1 X wash solution using an automated microplate washer. Biotin labelled antibody solution (100 µl) was then added to each well and the plate was wrapped in foil and incubated at room temperature for 75 minutes. Following a second washing step, a 100 µl streptavidin-HRP conjugate solution was added to each well. The plate was wrapped in foil and incubated at room temperature for a further 75 minutes. A third washing step was performed after which 100 µl substrate solution was added to each well. The plate was wrapped in foil and incubated, in the dark, at room temperature for an additional 10 minutes (the incubation time was increased to 20 minutes if the room

temperature was lower than 20 ^{o}C). The colour development was stopped by the addition of 100 µl stop solution. The plate was analyzed immediately.

2.2.6 Plate analysis

Analysis of the plate involved the measurement of the absorbance of the blanks, quality controls, standards and samples at 450 nm using an automated BioTek ELx800^{TM} microplate reader in conjunction with KC4^{TM}v3.4 software.

The microplate reader was set up while the reagents and samples were thawed. A laptop installed with KC4^{TM}v3.4 software and a HP LaserJet 1010 printer were connected to the microplate reader. Using the KC4^{TM}v3.4 program and the accompanying manufacturers' instructions, a new protocol was designed for each experiment. Each protocol included details of the layout of blanks, quality controls, standards and samples in the plate; the wavelength to be used; the type of standard curve, and the results required in the printed report.

2.2.7 Calculation of CC16 concentration

The absorbance values, corrected absorbance values, CC16 concentrations and a standard curve (refer to figure 2.2.7) were automatically calculated by the KC4^{TM}v3.4 software and the results were presented in a report that was printed (see Appendix F). In order to allow statistical analysis of the results, they were entered into a spreadsheet in Microsoft Excel.

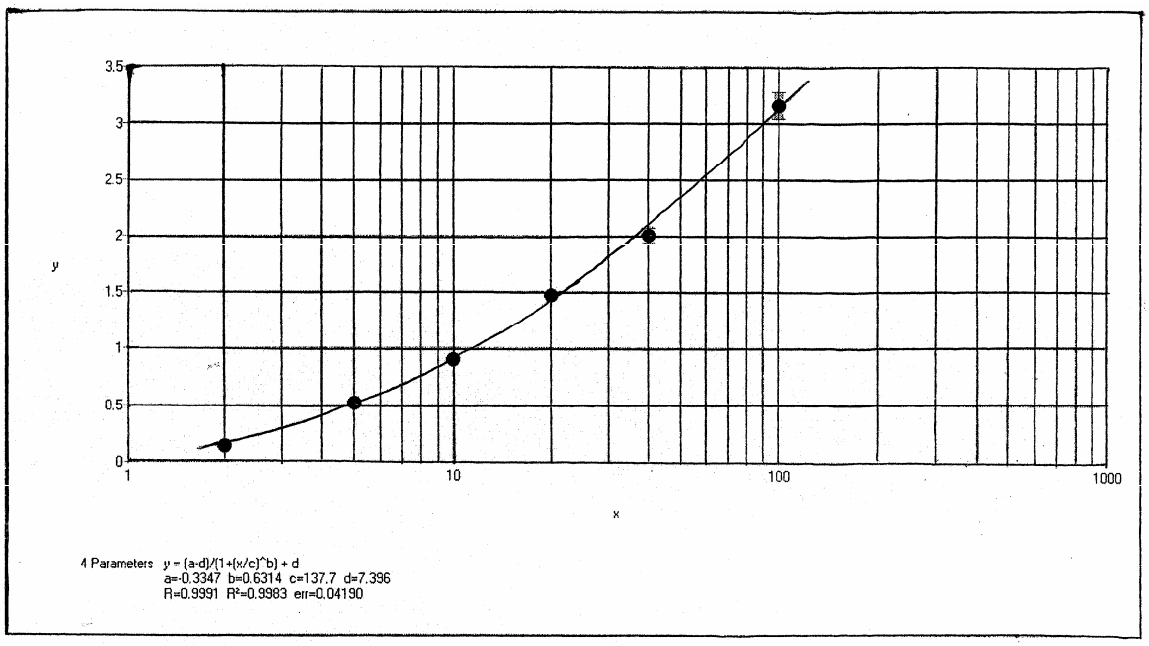


Figure 2.2.7: Example of the CC16 standard curve obtained using the KC4^{TM}v3.4 software. The curve was constructed by plotting the absorbance at 450 nm (Y) obtained for the standards versus the log of the known CC16 concentration (X) of the standards, using the four-parameter function.

2.3 Optimization of operational parameters

Before the field work could commence, it was essential to determine the optimal conditions for the handling, storage and assaying of the GPx and CC16 samples in order to ensure reliable and reproducible results. A series of four optimization experiments were carried out to investigate the effects of storage temperature, time between blood collection and separation, ambient laboratory temperature and storage duration on the assessed levels of erythrocyte GPx and serum CC16.

2.3.1 Study sample

Willing volunteers were recruited from the National Institute for Occupational Health (NIOH). After reading the information sheet (Appendix G) and signing the necessary consent forms (Appendix H), blood specimens were collected from the volunteers. Five volunteers were used for the GPx experiments (one male and four females) and four volunteers were used for the CC16 experiments (three males and one female).

2.3.2 The effect of storage temperature

2.3.2.1 Erythrocyte GPx

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Lithium heparin as an anti-coagulant (Becton, Dickinson and Company,

U.S.A.). The bloods were immediately centrifuged at 1300 *x g* for 10 minutes at room temperature (approximately 25 ^{o}C). The upper plasma layer and buffy coat (leukocytes) were discarded and forty five 50 µl aliquots of packed erythrocytes were pipetted into 1.5 ml microcentrifuge tubes containing 200 µl ice-cold distilled water (in order to lyse the erythrocytes). The samples were inverted gently to mix and centrifuged at 10 000 *x g* for 15 minutes at 4 ^{o}C. The erythrocyte lysate supernatant (100 µl) was pipetted into clearly labelled 1.8 ml CryoTubes™. Fifteen aliquots each were stored at 4 ^{o}C, -20 ^{o}C or -80 ^{o}C, respectively.

At the end of three weeks storage, the 45 erythrocyte lysate samples were thawed at room temperature. The GPx activity in the samples was then determined using the Glutathione Peroxidase Assay Kit as described in section 2.1.

2.3.2.2 Serum CC16

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Clot Act (Becton, Dickinson and Company, U.S.A.). The bloods were left to coagulate at room temperature for 10 minutes and then centrifuged at 1300 *x g* for 10 minutes at room temperature. Forty five aliquots of 50 µl serum were pipetted into clearly labelled 1.8 ml CryoTubes™. Fifteen aliquots each were stored at 4 ^{o}C, -20 ^{o}C or -80 ^{o}C, respectively.

Following three weeks of storage, the 45 serum samples were thawed at room temperature. The CC16 concentration in the serum samples was then determined using a Clara Cell Protein Human ELISA Kit as detailed in section 2.2.

2.3.3 The effect of a delay in time between blood sample collection and separation

2.3.3.1 Erythrocyte GPx

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Lithium heparin. One of the 6 ml tubes was immediately centrifuged at 1300 *x g* for 10 minutes at room temperature. From the remaining 6 ml tube, half the blood was transferred into a new BD Vacutainer™ tube. The two tubes containing 3 ml blood were left at room temperature for 1 and 2 hours, respectively, before centrifugation at 1300 *x g* for 10 minutes at room temperature.

Following centrifugation, the upper plasma layer and buffy coat were discarded and fifteen 50 µl aliquots of packed erythrocytes were pipetted into 1.5 ml microcentrifuge tubes containing 200 µl ice-cold distilled water. The samples were gently inverted to mix and centrifuged at 10 000 *x g* for 15 minutes at 4°C. The erythrocyte lysate supernatant (100 µl) was pipetted into clearly labelled 1.8 ml CryoTubes™ and frozen at -80 °C.

After two weeks of storage, the 45 erythrocyte lysate samples were thawed at room temperature. The GPx activity in the samples was then determined using a Glutathione Peroxidase Assay kit as described in section 2.1.

2.3.3.2 Serum CC16

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Clot Act. The bloods were left to coagulate at room temperature. Half the blood from one tube was transferred to a new BD Vacutainer™ tube. The two tubes, containing 3 ml blood, were left at room temperature for 1 and 2 hours (respectively) before centrifugation at 1300 *x g* for 10 minutes at room temperature. After only 10 minutes, the remaining 6 ml tube was centrifuged at 1300 *x g* for 10 minutes at room temperature. Fifteen aliquots of 50 µl serum were pipetted from each tube into clearly labelled 1.8 ml CryoTubes™ and frozen at -80 ^{o}C.

At the end of two weeks of storage, the 45 serum samples were thawed at room temperature. The CC16 concentration in the serum samples was then determined using a Clara Cell Protein Human ELISA Kit as detailed in section 2.2.

2.3.4 The effect of ambient laboratory temperature

2.3.4.1 Erythrocyte GPx

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Lithium heparin. The bloods were immediately centrifuged at 1300 *x g* for 10 minutes at room temperature. During the subsequent erythrocyte lysate preparation steps, one tube of separated blood (and resulting samples) was kept at 25 ^{o}C while the

other tube (and resulting samples) was kept at 35 ^{o}C using a Labnet AccuBlock Digital Dry Bath (Labnet International, Inc., USA).

Following centrifugation, the upper plasma layer and buffy coat were discarded and fifteen 50 µl aliquots of packed erythrocytes were pipetted from each tube into 1.5 ml microcentrifuge tubes containing 200 µl ice-cold distilled water. The samples were gently mixed and centrifuged at 10 000 *x g* for 15 minutes at 4 ^{o}C. The erythrocyte lysate supernatant (100 µl) was pipetted into clearly labelled 1.8 ml CryoTubes™ and frozen at -80 ^{o}C.

After two weeks of storage, the 30 erythrocyte lysate samples were thawed and the GPx activity in the samples was then determined using a Glutathione Peroxidase Assay Kit as described in section 2.1. During the thawing and assay preparation steps, one set of 15 erythrocyte lysate aliquots was kept at 25 ^{o}C while the other was kept at 35 ^{o}C using the Labnet AccuBlock Digital Dry Bath.

2.3.4.2 Serum CC16

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Clot Act. During the subsequent serum sample preparation, thawing and assay preparation steps, one tube of blood (and resulting serum samples) was kept at 25 ^{o}C while the other tube (and resulting serum samples) was kept at 35 ^{o}C using a Labnet AccuBlock Digital Dry Bath.

The collected bloods were left to coagulate (at the different temperature) for 10 minutes and then centrifuged at 1300 *x g* for 10 minutes at room temperature. Fifteen aliquots of 50 µl serum were collected from each tube of separated blood into clearly labelled 1.8 ml CryoTubes™ and frozen at -80 ^{o}C.

Following two weeks of storage, the 30 serum samples were thawed and the CC16 concentration in the serum samples was then determined using a Clara Cell Protein Human ELISA Kit as detailed in section 2.2.

2.3.5 The effect of storage duration

2.3.5.1 Erythrocyte GPx

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Lithium heparin. The bloods were immediately centrifuged at 1300 *x g* for 10 minutes at room temperature. The upper plasma layer and buffy coat were discarded and 60 aliquots of 50 µl packed erythrocytes were pipetted into 1.5 ml microcentrifuge tubes containing 200 µl ice-cold distilled water. After gentle mixing, the samples were centrifuged at 10 000 *x g* for 15 minutes at 4 ^{o}C. The erythrocyte lysate supernatant (100 µl) was pipetted into clearly labelled 1.8 ml CryoTubes™. Fifteen erythrocyte lysate aliquots were assayed immediately. The remaining 45 erythrocyte lysate aliquots were stored at -80 ^{o}C for three, six, and nine weeks respectively. At the end of the specified storage period, 15 frozen erythrocyte lysate aliquots were thawed at room temperature (approximately 25 ^{o}C) and the GPx activity in the samples was then determined using a

Glutathione Peroxidase Assay Kit as described in section 2.1. As a result each frozen erythrocyte lysate aliquot was assayed and then discarded.

2.3.5.2 Serum CC16

Venous blood was collected from two volunteers into two 6 ml BD Vacutainer™ tubes containing Clot Act. The bloods were left to coagulate at room temperature for ten minutes and then centrifuged at 1300 *x g* for 10 minutes at room temperature. Sixty aliquots of 50 µl serum were pipetted into clearly labelled 1.8 ml CryoTubes™. Fifteen serum aliquots were assayed immediately. The remaining 45 serum aliquots were stored at -80 ^{o}C for three, six, and nine weeks respectively. At the end of the specified storage period, 15 frozen serum aliquots were thawed at room temperature (approximately 25 ^{o}C) and the CC16 concentration in the serum samples was then determined using a Clara Cell Protein Human ELISA Kit as detailed in section 2.2. As a result each frozen serum aliquot was assayed and then discarded.

The results obtained from the optimization experiments were then used to develop Standard Operating Procedures (SOPs) for the handling, processing and analysis of GPx CC16 samples. The SOPs are attached as Appendix I and J, respectively.

2.4 Field work

2.4.1 Study design

2.4.1.1. Study population

The study population consisted of volunteer male underground mine workers at a Goldfields gold mine in North West province, South Africa.

2.4.1.2. Study sample

The sample consisted of volunteers who met the inclusion criteria for the study.

2.4.1.3. Inclusion criteria

- Adult males
- Volunteers
- Underground mine workers
- New employees or returning from two or more weeks leave at the time of study recruitment

2.4.1.4. Exclusion criteria

- Difficult to contact telephonically to arrange follow-up visits
- Taking medication for pulmonary tuberculosis
- Unwilling to give blood specimens

The HIV sero-status of the volunteers was not sought since the second phase of the project had previously shown that GPx and CC16 levels were not affected by HIV sero-status, or by the taking of ARV treatment.

2.4.1.5. Study design

A series of cross-sectional studies of 80 mine workers exposed to low and high levels of dust (determined by current job description as described in section 2.4.6).

2.4.2 Recruitment of participants

Prior to commencing the recruitment of the study participants, written permission was obtained from the management of the mine involved to be able to conduct the study on their premises without hindrance. A meeting was also held with the representatives of all the Unions active in the mine who expressed their satisfaction and support for the study.

Additional assistance for the recruitment of participants and collection of field work samples was provided by Professor Brendan Girdler-Brown (a qualified medical practitioner) who was responsible for blood sample collection and by Miss Lerato Kaphe and Miss Thoko Ndlovu (Diploma in Technology students) who assisted with language interpretation.

All miners undergo an induction course after their annual leave that includes a routine medical examination (including chest radiography) and training or re-training, which takes place at the mines' Training Academy. For the current study, a temporary field laboratory was set up in a room in this Training Academy. Every morning (between 9 am and 1 pm) the employees undergoing training were given a brief multi-lingual presentation informing them about the project and its requirements and that their participation was voluntary. Those who agreed to participate were asked to meet the research team in the temporary laboratory at their convenience, but prior to their return to underground work.

Volunteers who agreed to participate were asked to review a short information sheet (Appendix K), sign a consent form (Appendix L) as well as to complete a short questionnaire (Appendix M) (figure 2.4.2). Those volunteers, who agreed to have their photographs taken to be used in scientific publications and reports, were asked to sign an additional consent form (Appendix N). Thereafter, 15 ml of venous blood, representing the returning from leave (ex-leave) sample, was drawn by the qualified medical practitioner who is registered as such with the Health Professions Council of South Africa (HPCSA).

Figure 2.4.2: Prof Girdler-Brown, (left) and Miss Kaphe (middle) help a volunteer (right) to fill out the consent form and questionnaire. The photograph was taken after obtaining written consent.

A total of 80 volunteers were recruited between the period of February and April 2007.

2.4.3 Collection of specimens from volunteers

The project protocol called for the collection of two sets of blood specimens from the volunteers, return from leave (ex-leave) and return to work (follow up) specimens. The purpose of this collection procedure was to examine the levels of GPx and CC16 before and after occupational exposure to crystalline silica.

As stated in section 2.4.2, the return from leave (ex-leave) specimens were collected from the volunteers at the time of recruitment when the volunteers had been on leave for a period of at least two weeks. Venous blood was collected from volunteers into three 6 ml BD Vacutainer™ tubes, two containing Lithium Heparin and one containing Clot Act.

Once ex-leave blood specimens were collected from a total of 80 volunteers, the same volunteers were contacted telephonically to arrange the collection of the return to work (follow up) specimens. Collection of follow up samples was carried out between May and August 2007. Due to the complexity of the follow up specimen collection procedure, the time between the collection of the ex-leave and follow up samples (i.e. the time of exposure to crystalline silica-containing dust) could not be kept constant. The silica exposure time therefore ranged from 8 to 25 weeks (i.e. two to six months).

The collection of the follow up specimens was carried out at the mine shafts in a designated room (figure 2.4.3). The shafts in operation at the mine during the study period were shafts one, three, four, seven and eight. In order to make the collection of follow up samples efficient, the volunteers were grouped according to the shaft at which they worked and a timetable of which shafts would be visited on which dates was prepared. Since the original protocol stated that the return to work specimens would be collected from each volunteer on a Monday (pre-shift), a Wednesday (pre- and post-shift), and a Friday (post-shift), it was decided that one full week would need to spent at each shaft. The first two weeks were spent at shafts one and three. During that time, even though they had been informed and had agreed at the time of recruitment, all but one volunteer decided that they were not willing to give a blood specimen four times in one week. Because of the difficulty experienced, the specimen collection procedure was then revised so that specimens were collected either on a Monday (pre-shift), a Wednesday (pre- and post-shift), or a Friday (post-shift). When the miners were contacted and informed of the dates the research team would be at their shaft, they were asked whether they would be prepared to give two specimens on a Wednesday (pre- and post-shift). Those who said they were not prepared to do so were randomly (by tossing a coin) allocated to a single specimen collected either on

a Monday (pre-shift) or on a Friday (post-shift). There was only one volunteer from whom a return to work sample was collected on a Monday, Wednesday and Friday and three volunteers from whom a return to work sample was collected on two of the days. With the exception of the few volunteers who worked afternoon or evening shifts, the pre-shift samples were collected between 4:30 am and 7:30 am while the post-shift samples were collected between 12 noon and 3 pm.

The collection of the follow up specimens involved the collection of venous blood into two 6 ml BD Vacutainer™ tubes, one containing Lithium Heparin and the other containing Clot Act.

Figure 2.4.3: Prof Girdler-Brown, (left) draws a follow up blood sample from a volunteer (right) in the mine shaft computer room. The photograph was taken after obtaining written consent.

2.4.4 Processing of specimens

2.4.4.1 Processing of bloods

Immediately after blood collection, the BD Vacutainer™ tubes containing Lithium Heparin were centrifuged at 1300 *x g* for 10 minutes at room temperature. The upper plasma layer and buffy coat were discarded and triplicate aliquots of 0.3 ml packed erythrocytes were added to 1.2 ml cold distilled water in 1.5 ml microcentrifuge tubes. The samples were inverted gently to mix and centrifuged at 10 000 *x g* for 15 minutes at 4 ^{o}C. Aliquots of 1 ml erythrocyte lysate (i.e. the supernatant) were pipetted in triplicate into clearly labelled 1.8 ml CryoTubes™ and shock frozen in a Dewar tank of liquid nitrogen. The erythrocyte lysate was used to determine GPx activity.

After being left to clot for 10 minutes, the BD Vacutainer™ tube containing Clot Act was centrifuged at 1300 *x g* for 10 minutes at room temperature. Aliquots of 300 µl serum were pipetted in triplicate into clearly labelled 1.8 ml CryoTubes™ and shock frozen in a Dewar tank of liquid nitrogen. The serum was used to determine CC16 concentration.

2.4.4.2 Transport and storage of samples

The samples were placed in cooler boxes with ice packs (± 4 ^{o}C), transported back to the NIOH laboratory within 1 hour and placed in a -80 ^{o}C freezer (as recommended by the assay kit manufacturers and optimization experiment results) until assayed in batches.

2.4.4.3 Assay of Glutathione Peroxidase (GPx)

Following storage for four to six weeks, the triplicate erythrocyte lysate samples were thawed and assayed using the Glutathione Peroxidase Assay Kit as described in section 2.1. In the assay, the samples and controls were randomly allocated to wells using a table of random numbers.

2.4.4.4 Assay of Clara Cell protein 16 (CC16)

After storage for eight to ten weeks, the triplicate serum aliquots were thawed and assayed using a Clara Cell Protein Human ELISA Kit as described in section 2.2. For the assay the standards, samples and controls were randomly allocated to wells using a table of random numbers.

2.4.5 Chest radiography

The volunteers signed a consent form (Appendix L) giving the research team permission to examine their most recent chest radiographs collected at the Occupational Health Centre. The radiographs were obtained as digital images and were independently assessed for silicosis by two experienced readers (Prof A Solomons and Prof D Rees of the National Institute for Occupational Health) with the aid of DigiRad Imaging Solutions software. International Labour Organisation (ILO) category scores of 1/1 or greater were considered to be indicative of silicosis for the purpose of this study.

2.4.6 Dust exposure classification of jobs

The volunteers were asked to sign a consent form (Appendix L) giving the research team permission to access their occupational history, in particular their job title and code, from the human resources department.

In a previous study carried out by Aurum Health Research (Pty) Ltd at a similar gold mine [Churchyard *et al.*, 2003], the mine jobs were classified into high, medium and low dust exposure categories based on the judgment of two experienced hygienists who were familiar with the mine. The lists of jobs and corresponding dust exposure levels, used for that study, were obtained by personal correspondence with Professor G. Churchyard. The lists were used to classify the jobs of this study's volunteers into high or low dust exposure categories as summarised in table 2.4.6.

It is important to note that using current job descriptions to classify dust exposure levels is not a reliable or accurate measure of a volunteers personal dust exposure. Therefore, in the current study, these job classifications were merely used as an indication of dust exposure levels and all results based on these classifications should be treated with caution.

Table 2.4.6: Summary of the job categories and corresponding dust exposure levels of volunteers in the study

Occupation	Dust exposure level High=H, Low=L	n
Aquajet operator	H	2
Banksman	L	1
Chief safety officer	L	1
Developer	H	1
Electrician	L	5
Fitter and turner	L	2
General miner	H	11
Grade officer	L	1
Loader operator	H	2
Loco Driver	L	6
Mine overseer	L	1
Night shift cleaner	H	3
Onsetter	L	1
Plant manager	L	1
Pump attendant	L	3
Rigger	L	3
Safety officer	L	1
Scraper winch driver	H	3
Section engineer	L	1
Senior surveyor (uncertified)	L	1
Shaft and construction timberman	L	1
Shaft foreman	L	1
Shift boss	L	6
Stoper	H	12
Survey leading hand	L	1
Surveyor (uncertified)	L	3
Wining engine driver (U/G)	L	2

2.5 Ethical issues

This study protocol was approved by the University of the Witwatersrand Human Research Ethics Committees (Clearance number: R14/49 Makinson) (Attached as Appendix O).

The study was funded in full by the Mines Health and Safety Council and was approved by employers, government and labour union representatives.

Participation was entirely voluntary.

Volunteers were given an information leaflet and a copy of the informed consent form in the language of their choice (English, Afrikaans or Zulu) to keep. The participant information leaflet advised participants that the test results had no diagnostic value and that individual results could not be used to indicate (in an absolute sense) their individual dust exposure levels.

All venesections were performed by a medical practitioner registered with the HPCSA and there was 24 hour medical attention available for miners who might have suffered discomfort or anxiety following the venesection (none did). There was no testing of blood for HIV, neither were participants asked about their HIV infection status at any time. If any volunteer had medical questions they were generally answered by the medical practitioner who drew their blood specimen and they were told to obtain medical follow up. In cases

where abnormalities were detected on the chest radiographs, the identities of the miners concerned were communicated to the mines medical staff for follow-up.

Individual results were kept confidential and were not divulged to any person other than the individual miner at his specific request. When requested, the meaning of their individual results was carefully explained and it was emphasized that each person has his own "normal" level for biomarkers tested and that no individual result could be used to indicate disease, freedom from disease, or over or under-exposure to silica either chronically or acutely.

A feed-back session for participating miners, accompanied by a lunch, was held on the 24th November 2007 and again on 8th December 2007. During these sessions miners were provided with a summary of the findings, and the overall results were explained. Miners who wanted their individual results were supplied with their results (in writing) along with a one-to-one explanation regarding interpretation at the time of the feed-back session.

When a photograph(s) of a volunteer was taken, their written permission was obtained beforehand on a consent form that also indicated whether they were willing to have the photograph(s) published.

2.6 Statistical methods

The data provided in the questionnaire by the volunteers were entered into Microsoft Excel and exported to Stata version 9 (StataCorp LP, USA) where all the statistical analyses were performed. The data were manually double-checked for the correctness of entries. Any data observation which was greater than 2.IQR (interquartile range) above or below the median was considered an outlier and was excluded from the data set.

Unless otherwise stated, for hypotheses tests the critical value for rejecting a null hypothesis was adopted as $p \leq 0.05$.

Box-plots were used to portray the data distributions.

When the data sets being compared showed compatibility with having been drawn at random from a normally distributed population based on the results of the Shapiro-Wilk W test [Shapiro and Wilk, 1965], t-tests were used to evaluate differences between means provided.

Paired t-tests were performed for paired data (for example for comparisons of the ex-leave specimens with the follow up specimens as well as for comparisons of the Wednesday pre-shift and Wednesday post-shift specimens). For paired t-tests, before the test was carried out, the differences were first checked by means of the Shapiro-Wilk W test to ensure that they were compatible with having been drawn at random from a normally distributed population. The Bonferroni adjusted critical alpha-value (α^*) was then manually calculated, as $\alpha^* = 0.05/x$ where $x = k!/2!*(k-2)!$ (for example, for three pair wise

comparisons $\alpha^* = 0.05/3 = 0.017$) [Pagano and Gauvreau, 2000], and used to determine the level of significance for the pair-wise comparisons.

Unpaired t-tests were performed where data were not from the same miner (for example comparisons of the Monday pre-shift and Friday post-shift specimens). The F-test (Stata command "sdtest") was used to assess the equivalence of sample variances prior to performance of the unpaired t-tests. In cases where the sample variances were unequal, the variation of the t-test for samples with unequal variances was used. Interpretation of these tests with unequal variances was carried out using Satterthwaite's method [Satterthwaite, 1946].

In cases where the data were not compatible with having been drawn at random from a normally distributed population, comparisons were carried out using the two sample rank-sum test for unpaired data (Stata command "ranksum"), or the signed-rank test (Stata command "signrank") for paired data as applicable. If possible, the data were also transformed to achieve a normal distribution and allow parametric tests to be performed.

Where multiple comparisons were made (for data that were compatible with being drawn at random from normally distributed populations) ANOVA was used with Bonferroni-adjusted p-values being used for interpretation of individual pair-wise comparisons. The Kruskal-Wallis test of equality of populations was used for multiple comparisons in instances in which the sample data were not compatible with having been drawn at random from a normally distributed population [Kruskal and Wallis, 1952a; Kruskal and Wallis, 1952b].

Since only a single set of ex-leave and follow-up samples were collected for each volunteer and due to the high variability of some biomarker levels it was not possible to reliably compare biomarker levels on an individual basis. As a result all analysis involved population-based average readings and all individual results were treated with caution.

Chapter 3:

RESULTS

This third phase of the project on biomarkers consisted of two parts:
The first part involved optimisation of the operational parameters for the establishment of the Standard Operating Procedures (SOPs). Four separate experiments were performed in which the effects of storage temperature, a delay in time between blood collection and separation, ambient laboratory temperature and storage duration on the levels of erythrocyte GPx activity and serum CC16 concentration were investigated (the results are presented in section 3.1).

The second part involved field work during which adult male miners were recruited from a gold mine in North West province, South Africa. Blood specimens were collected from the mine volunteers after their return from leave (at the time of recruitment), and then again after their return to work on a Monday (pre-shift), a Wednesday (pre- and post-shift), and/or on a Friday (post-shift) (the results of which are presented in section 3.2).

3.1 Optimization of the operational parameters

These optimization experiments were performed with the intention of developing SOPs for the handling, processing and analysis of GPx and CC16 samples to ensure optimum precision and reproducibility during the field work and also during future research. (The SOPs are attached as Appendices I and J).

3.1.1 Effect of storage temperature

The effect of storage temperature on erythrocyte GPx activity and serum CC16 concentration was evaluated by storing erythrocyte lysate and serum samples, collected from two volunteers, at 4 ^{o}C, -20 ^{o}C and -80 ^{o}C for 3 weeks. The GPx activity of the erythrocyte lysate samples was then determined using a Glutathione Peroxidase Assay Kit and the CC16 concentration of the serum samples was determined using a Clara Cell Protein Human ELISA Kit.

3.1.1.1 Erythrocyte GPx

For volunteer one and two, there was a slight decrease in median GPx activity as the storage temperature was lowered (figure 3.1.1.1 below).

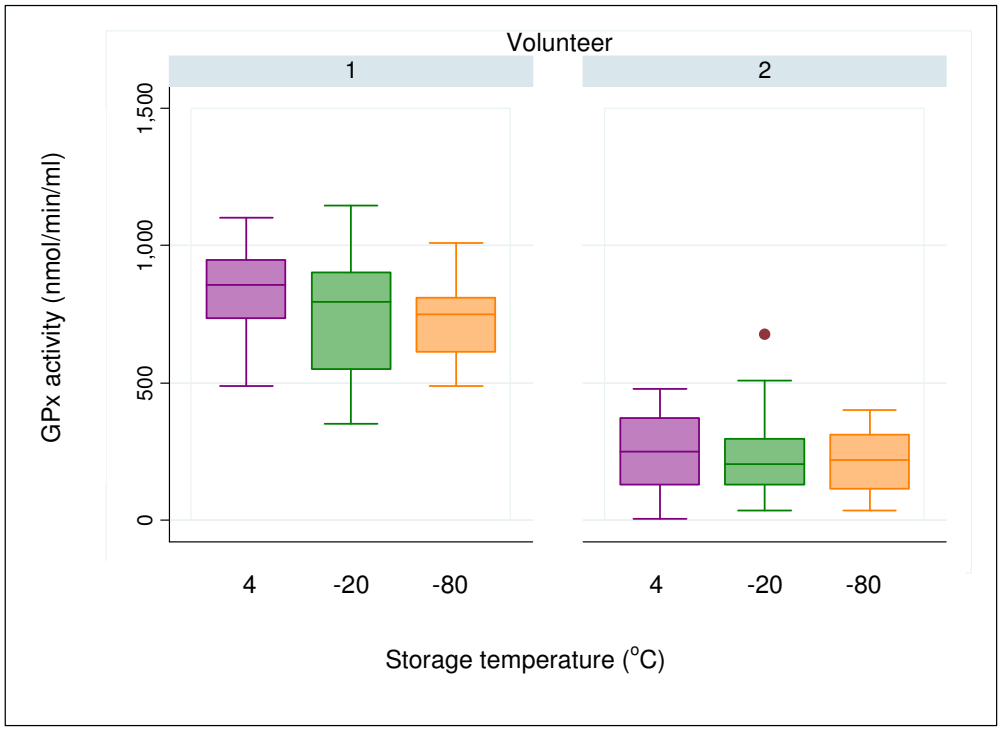


Figure 3.1.1.1: GPx activity of erythrocyte lysate samples (from two volunteers) stored for 3 weeks at 4 °C, -20 °C and -80 °C. The GPx activity was measured (in a total of 15 replicates per condition) using a Glutathione Peroxidase Assay Kit. The CV ranged from 18.7%-28.5% for volunteer 1 and 49.5%-69.0% for volunteer 2. In the box-and-whisker plot the line through the middle of the boxes represents the median GPx activity. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively. The solid dot represents a data observation greater than 1.5 IQR above or below the median.

Analysis revealed that, for both volunteers, the differences between the mean GPx activities of the samples were compatible with a normal distribution (Shapiro-Wilk W test for normal data, $p > 0.05$). This allowed paired t-tests to be used to compare the mean GPx activities of the samples and the results of those tests are summarised in table 3.1.1.1 below.

Table 3.1.1.1: P-values obtained from the pair-wise comparison of the mean erythrocyte GPx activity of samples stored at various temperatures (level of significance is $p \leq 0.017$)

Volunteer		**4 °C**	**-20 °C**	**-80 °C**
1	**4 °C**	p = 1.00		
	-20 °C	p = 0.13	p = 1.00	
	-80 °C	p = 0.09	p = 0.74	p = 1.00
2	**4 °C**	p = 1.00		
	-20 °C	p = 0.47	p = 1.00	
	-80 °C	p = 0.78	p = 0.77	p = 1.00

The pair-wise comparison of the means (table 3.1.1.1) revealed that the slight decrease in mean GPx activity observed at the lower storage temperatures (figure 3.1.1.1) was not statistically significant ($p >$ Bonferonni adjusted α of 0.017).

It was therefore concluded the temperature at which the erythrocyte lysate samples were stored had no significant effect on the GPx activity of the samples.

3.1.1.2 Serum CC16

As observed in figure 3.1.1.2 below, for both volunteers there was a decrease in the median CC16 concentration with a decrease in storage temperature.

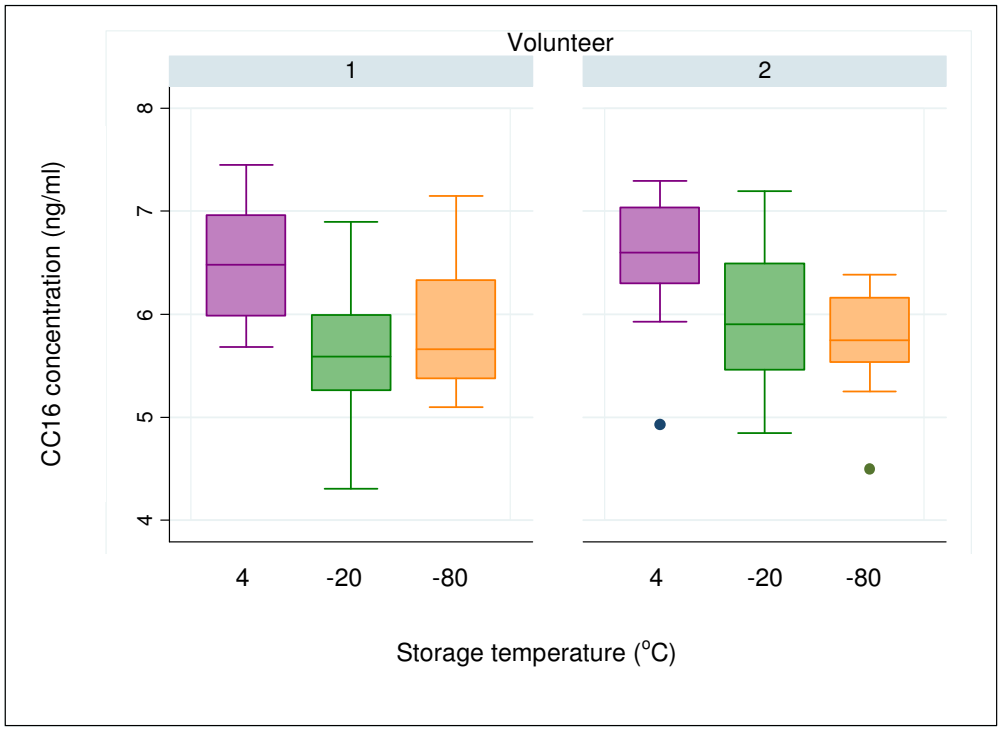


Figure 3.1.1.2: CC16 concentration of serum samples (from two volunteers) stored for 3 weeks at 4 °C, -20 °C and -80 °C. The CC16 concentration was measured (in 15 replicates per condition) using a Clara Cell Protein Human ELISA Kit. The CV ranged from 8.5%-9.6% for volunteer 1 and 8.3%-11.4% for volunteer 2. In the box-and-whisker plot the black line through the middle of the boxes represents the median CC16 concentration. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively. The solid dots represent data observations greater than 1.5 IQR above or below the median.

The differences between the mean CC16 concentrations of the samples were compatible with a normal distribution, for volunteer one and two (Shapiro-Wilk W test for normal data, $p > 0.05$). The mean CC16 concentrations of the samples were therefore compared using the paired t-test, the results of which are summarised in table 3.1.1.2 below.

Table 3.1.1.2: P-values obtained from the pair-wise comparison of the mean CC16 concentration of samples stored at various temperatures (level of significance is $p \leq 0.017$)

Volunteer		**4 °C**	**-20 °C**	**-80 °C**
1	**4 °C**	p = 1.00		
	-20 °C	p = 0.00*	p = 1.00	
	-80 °C	p = 0.00*	p = 0.46	p = 1.0
2	**4 °C**	p = 1.00		
	-20 °C	p = 0.05	p = 1.00	
	-80 °C	p = 0.00*	p = 0.38	p = 1.0

* Indicates p-value less than level of significance

The pair-wise comparison of the means (table 3.1.1.2) indicated that the mean CC16 concentration of the samples collected from volunteer one and stored at 4 °C was significantly higher than the mean CC16 concentration of the samples stored at -20 °C and -80 °C (p = 0.00 for both). It also indicated that there was a significant difference between the mean CC16 concentration of the samples collected from volunteer two and stored at 4 °C and those stored at -80 °C (p = 0.00). For both volunteers there was no significant difference between the mean CC16 concentration of the samples stored at -20°C and -80°C (p > Bonferonni adjusted α of 0.017).

Unlike the GPx results, these results indicated that the temperature at which the serum samples were stored did have a significant effect on the CC16 concentration of the samples.

3.1.2 Effect of a delay in time between blood sample collection and separation

In order to determine the effect of a delay in time (between blood sample collection and separation) on erythrocyte GPx activity and serum CC16 concentration, blood samples were collected (from two volunteers) and left to stand for 0, 1 and 2 hours prior to separation. The resultant erythrocyte lysate and serum samples were frozen at -80 ^{o}C for 2 weeks. The GPx activity of the erythrocyte lysate samples was then measured using a Glutathione Peroxidase Assay Kit while the CC16 concentration of the serum samples was measured using a Clara Cell Protein Human ELISA Kit.

3.1.2.1 Erythrocyte GPx

An increase in delay time between blood sample collection and separation caused an increase in the median GPx activity of samples collected from volunteer one and a decrease in the median GPx activity of samples collected from volunteer two, as shown in figure 3.1.2.1 below.

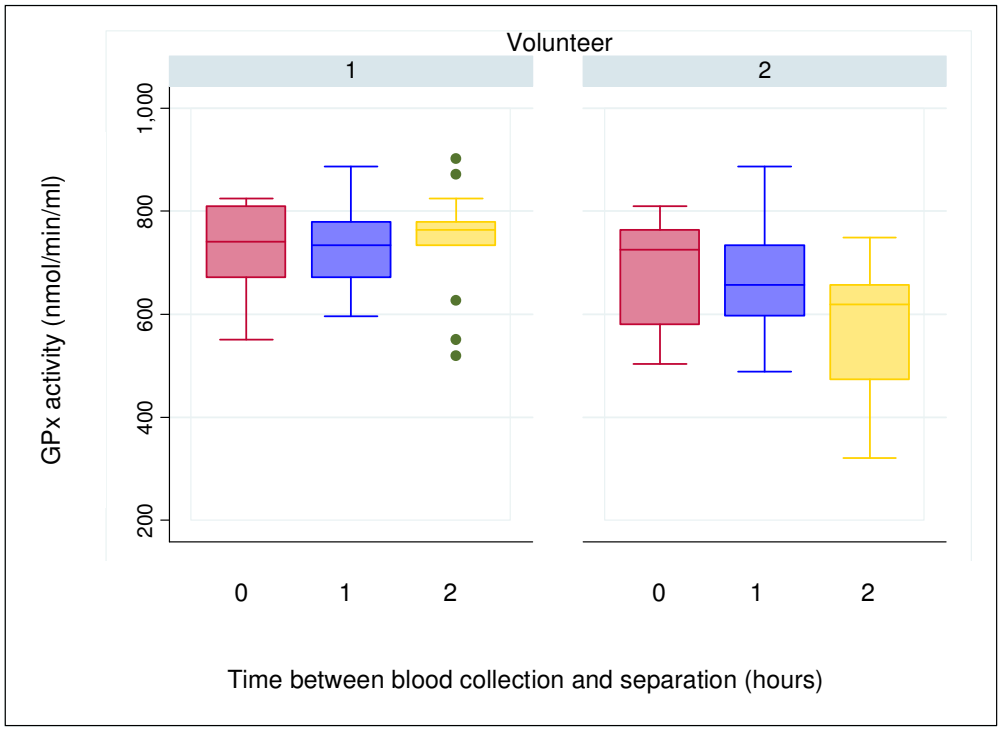


Figure 3.1.2.1: GPx activity of erythrocyte lysate samples (from two volunteers) obtained from blood samples separated 0, 1 and 2 hours after collection. The GPx activity was measured (in a total of 15 replicates per condition) after 2 weeks of storage at -80 ^{o}C using a Glutathione Peroxidase Assay Kit. The CV ranged from 11.2%-15.2% for volunteer 1 and 15.8%-22.2% for volunteer 2. In the box-and-whisker plot the black line through the middle of the boxes represents the median GPx activity. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively. The solid dots represent data observations greater than 1.5 IQR above or below the median.

The differences between the mean GPx activities of the samples were compatible with a normal distribution (Shapiro-Wilk W test for normal data, $p > 0.05$), except for the difference between the mean GPx activity of the one and two hour samples of volunteer two ($p = 0.00$). As a result, the Wilcoxon signed-rank test was used to compare the mean GPx activities of the one and two hour samples of volunteer two while the paired t-test was used to compare the mean GPx activities of the other samples. The results of these comparisons are summarised in table 3.1.2.1 below.

Table 3.1.2.1: P-values obtained from the pair-wise comparison of the mean erythrocyte GPx activity of samples prepared after various delay times (level of significance is $p \leq 0.017$)

Volunteer		**0 hour**	**1 hour**	**2 hour**
1	**0 hour**	p = 1.00		
	1 hour	p = 0.78	p = 1.00	
	2 hour	p = 0.78	p = 0.88	p = 1.00
2	**0 hour**	p = 1.00		
	1 hour	p = 0.76	p = 1.00	
	2 hour	p = 0.03	p = 0.05	p = 1.00

The pair-wise comparison of the mean GPx activities (table 3.1.2.1) indicated that the changes in mean GPx activity observed in figure 3.1.2.1 were not statistically significant ($p >$ Bonferonni adjusted α of 0.017).

It was therefore concluded that leaving the blood specimens to stand for up to two hours before their separation did not have a significant effect on the GPx activity of the resulting erythrocyte lysate samples.

3.1.2.2 Serum CC16

For volunteer one and two, a decrease in median CC16 concentration was observed with an increase in delay time from zero to two hours (figure 3.1.2.2 below).

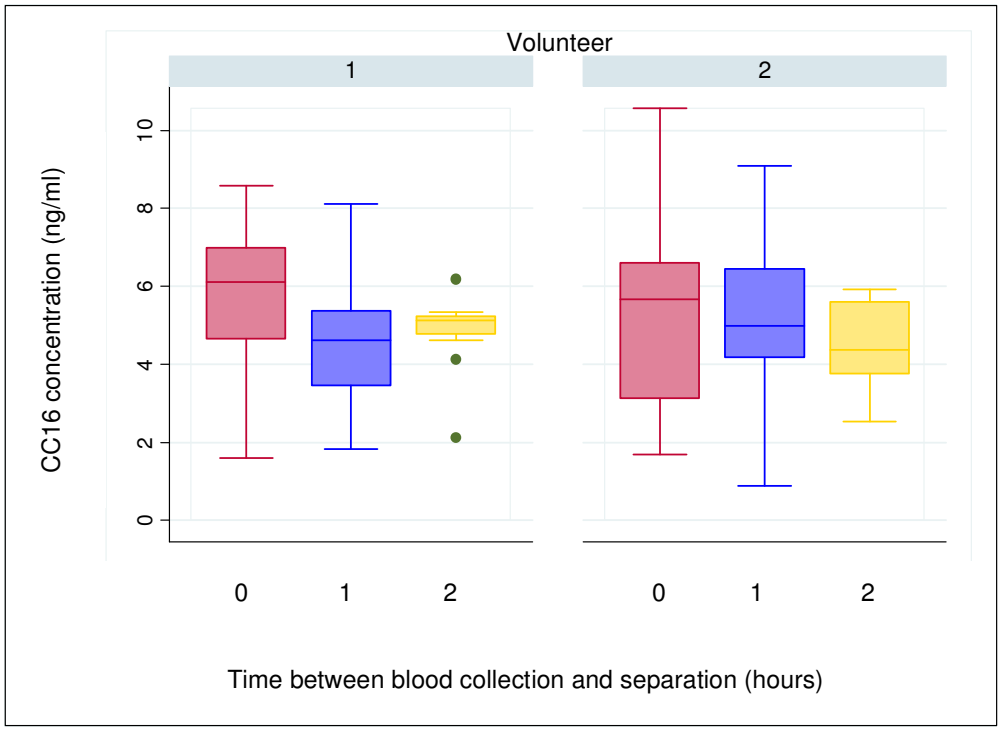


Figure 3.1.2.2: CC16 concentration of serum samples (from two volunteers) obtained from blood samples separated 0, 1 and 2 hours after collection. The CC16 concentration was measured (in 15 replicates per condition) after storage of the serum samples at -80 ^{o}C for 2 weeks using a Clara Cell Protein Human ELISA Kit. The CV ranged from 20.3%-40.2% for volunteer 1 and 26.8%-47.5% for volunteer 2. In the box-and-whisker plot the black line through the middle of the boxes represents the median CC16 concentration. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively. The solid dots represent data observations greater than 1.5 IQR above or below the median.

The differences between the mean CC16 concentration of the samples were compatible with a normal distribution (Shapiro-Wilk W test for normal data, $p > 0.05$), except for the difference between the mean CC16 concentration of the zero and one hour samples collected from volunteer two ($p = 0.03$). As a result, the mean CC16 concentration of the zero and one hour samples collected from volunteer two were compared using the Wilcoxon signed-rank test while the mean CC16 concentration of the other samples were compared using the paired t-test. The results of these comparisons are shown in table 3.1.2.2 below.

Table 3.1.2.2: P-values obtained from the pair-wise comparison of the mean CC16 concentration of samples prepared after various delay times (level of significance is $p \leq 0.017$)

Volunteer		**0 hour**	**1 hour**	**2 hour**
1	**0 hour**	p = 1.00		
	1 hour	p = 0.06	p = 1.00	
	2 hour	p = 0.02	p = 0.67	p = 1.00
2	**0 hour**	p = 1.00		
	1 hour	p = 0.92	p = 1.00	
	2 hour	p = 0.11	p = 0.15	p = 1.00

The pair-wise comparison of the mean CC16 concentrations (table 3.1.2.2) indicated that decrease in mean CC16 concentration observed in figure 3.1.2.2 was not statistically significant ($p >$ Bonferonni adjusted α of 0.017).

Similar to the GPx results, these results indicated that a delay time of up to two hours had no significant effect on the CC16 concentration of the serum samples.

3.1.3 Effect of ambient laboratory temperature

The effect of ambient laboratory temperature on erythrocyte GPx activity and serum CC16 concentration was determined, for two volunteers, by processing one set of blood samples and resulting erythrocyte lysate and serum samples at 25 ^{o}C and another set at 35 ^{o}C. After storage for 2 weeks at -80 ^{o}C, the GPx activity of the erythrocyte lysate samples was determined using a Glutathione Peroxidase Assay Kit and the CC16 concentration of the serum samples was determined using a Clara Cell Protein Human ELISA Kit.

3.1.3.1 Erythrocyte GPx

As demonstrated in figure 3.1.3.1, an increase in ambient laboratory temperature (from 25 ^{o}C to 35 ^{o}C) caused an increase in the median GPx activity of samples collected from volunteer one and a slight decrease in the median GPx activity of samples collected from volunteer two.

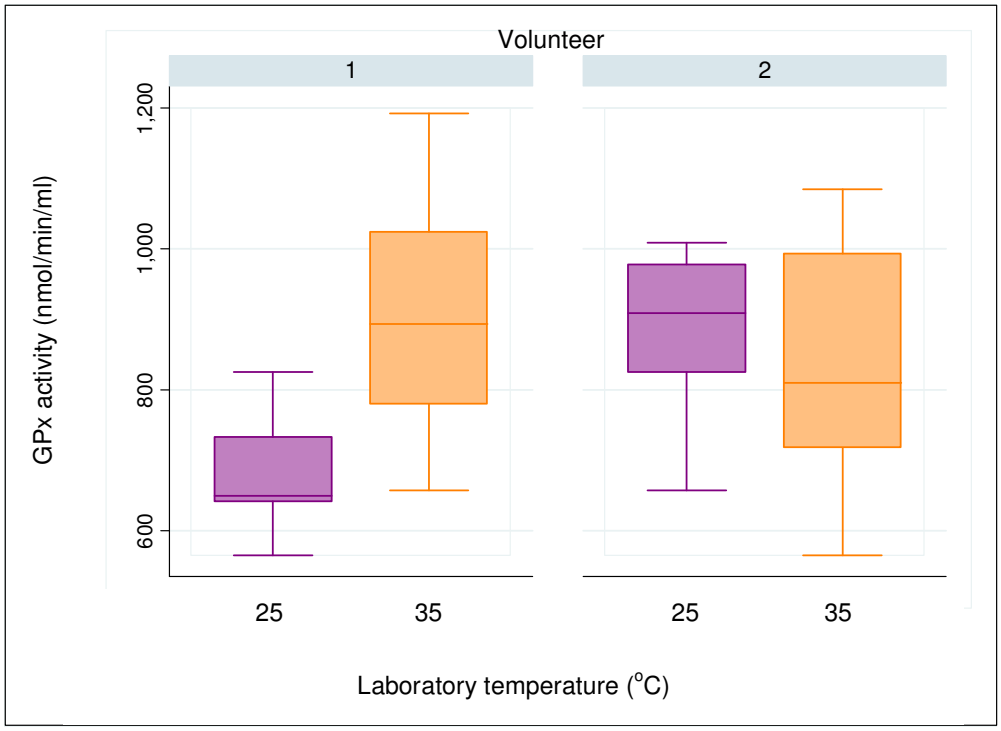


Figure 3.1.3.1: GPx activity of erythrocyte lysate samples (from two volunteers) processed at 25 ^{o}C and 35 ^{o}C. The GPx activity was measured (in a total of 15 replicates per condition) after 2 weeks of storage at -80 ^{o}C using a Glutathione Peroxidase Assay Kit. The CV ranged from 11.0%-16.6% for volunteer 1 and 12.1%-19.9% for volunteer 2. In the box-and-whisker plot the black line through the middle of the boxes represents the median GPx activity. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively.

The difference between the mean GPx activities of the samples prepared at different laboratory temperatures was compatible with a normal distribution for both volunteer one

and two (Shapiro-Wilk W test for normal data, $p > 0.05$). The mean GPx activities of the samples were therefore compared using the paired t-test and the results summarised in table 3.1.3.1.

Table 3.1.3.1: P-values obtained from the pair-wise comparison of the mean erythrocyte GPx activity of samples prepared at different temperatures (level of significance is $p \leq 0.05$)

Volunteer		**25 °C**	**35 °C**
1	**25 °C**	p = 1.00	
	35 °C	p = 0.00*	p = 1.00
2	**25 °C**	p = 1.00	
	35 °C	p = 0.37	p = 1.00

* Indicates p-value less than level of significance

For volunteer one, pair-wise comparison of the means (table 3.1.3.1) indicated that the increased GPx activity observed at the higher ambient laboratory temperature of 35 °C was statistically significant (p=0.00). For volunteer two, however, pair-wise comparison of the means indicated that the decreased GPx activity observed at the higher ambient laboratory temperature was not statistically significant (p=0.37).

Therefore, the results of the current study suggest that the laboratory temperature at which the erythrocyte lysate samples are processed may affect the GPx activity.

3.1.3.2 Serum CC16

The median CC16 concentration of the samples prepared at an ambient laboratory temperature of 35 °C was higher than that of the samples prepared at 25 °C for both volunteers (figure 3.1.3.2 below).

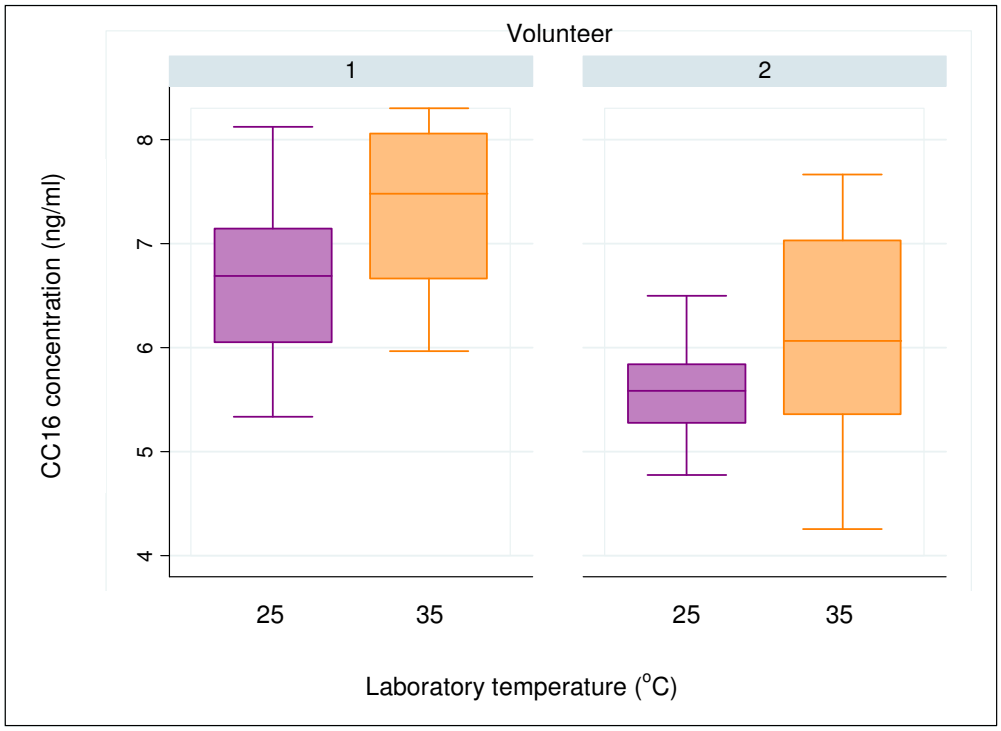


Figure 3.1.3.2: CC16 concentration of serum samples (from two volunteers) processed at 25 °C and 35 °C. The CC16 concentration was measured (in 15 replicates per condition) after storage of the serum samples at -80 °C for 2 weeks using a Clara Cell Protein Human ELISA Kit. The CV ranged from 10.3%-11.8% for volunteer 1 and 7.5%-16.2% for volunteer 2. In the box-and-whisker plot the black line through the middle of the boxes represents the median CC16 concentration. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively.

Since the differences between the mean CC16 concentrations of the samples prepared at different laboratory temperatures were compatible with a normal distribution for both volunteers (Shapiro-Wilk W test for normal data, $p > 0.05$), the mean CC16 concentrations of the samples were compared using paired t-tests. The results of these comparisons are presented in table 3.1.3.2 below.

Table 3.1.3.2: P-values obtained from the pair-wise comparison of the mean serum CC16 concentration of samples prepared at different temperatures (level of significance is $p \leq 0.05$)

Volunteer		**25 °C**	**35 °C**
1	**25 °C**	p = 1.00	
	35 °C	p = 0.01*	p = 1.00
2	**25 °C**	p = 1.00	
	35 °C	p = 0.10	p = 1.00

* Indicates p-value less than level of significance

For volunteer one, pair-wise comparison of the means (table 3.1.3.2) indicated that the mean CC16 concentration of the samples processed at 35 ^{o}C was significantly higher ($p = 0.01$) than that of the samples processed at 25 ^{o}C. On the other hand, for volunteer two, pair-wise comparison of the means indicated that there was no significant difference ($p = 0.10$) between the mean CC16 concentration of the samples processed at 25 ^{o}C and 35 ^{o}C.

Therefore, the current investigation also indicated that the temperature at which the serum samples are processed may have a significant effect on the CC16 concentration of the samples.

3.1.4 Effect of storage duration

The effect of storage duration on erythrocyte GPx activity and serum CC16 concentration was determined by storing erythrocyte lysate and serum samples, collected from two volunteers, at -80 ^{o}C for 0, 3, 6 and 9 weeks. The GPx activity of the erythrocyte lysate samples was then measured using a Glutathione Peroxidase Assay Kit while the CC16

concentration of the serum samples was measured using a Clara Cell Protein Human ELISA Kit and

3.1.4.1 Erythrocyte GPx

Except for a decrease in the median GPx activity of the three week samples collected from volunteer two, an increase in storage duration appeared to have no effect on the median GPx activities of the samples collected from volunteer one and two (figure 3.1.4.1).

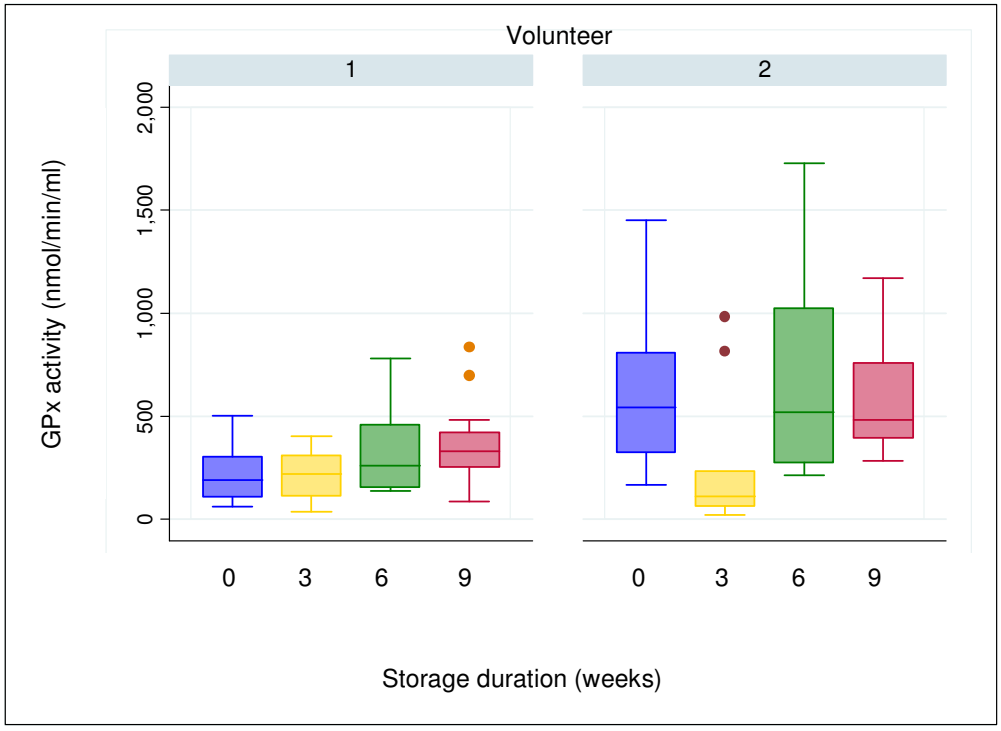


Figure 3.1.4.1: GPx activity of erythrocyte lysate samples (from two volunteers) stored at -80 °C for 0, 3, 6 and 9 weeks. The GPx activity was measured (in 15 replicates per condition) using a Glutathione Peroxidase Assay Kit. In the box-and-whisker plot the black line through the middle of the boxes represents the median GPx activity. The CV ranged from 49.5%-63.7% for volunteer 1 and 46.1%-126.3% for volunteer 2. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively. The solid dots represent data observations greater than 1.5 IQR above or below the median.

Since the difference between the mean GPx activity of the zero and six week samples of both volunteers were not compatible with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.03$ for both), the mean GPx activities of these samples were compared using the Wilcoxon signed-rank test. The paired t-test was used to compare the mean GPx activities of the other samples since the differences between the mean GPx activities were all compatible with a normal distribution ($p > 0.05$). These comparison results are shown in table 3.1.4.1.

Table 3.1.4.1: P-values obtained from the pair-wise comparison of the mean erythrocyte GPx activity of samples stored for various durations (level of significance is $p \leq 0.008$)

Volunteer		**0 weeks**	**3 weeks**	**6 weeks**	**9 weeks**
1	**0 weeks**	p = 1.00			
	3 weeks	p = 0.95	p = 1.00		
	6 weeks	p = 0.25	p = 0.11	p = 1.00	
	9 weeks	p = 0.02	p = 0.05	p = 0.82	p = 1.00
2	**0 weeks**	p = 1.00			
	3 weeks	p = 0.14	p = 1.00		
	6 weeks	p = 0.83	p = 0.07	p = 1.00	
	9 weeks	p = 0.78	p = 0.15	p = 0.54	p = 1.00

The pair-wise comparison of the mean GPx activities (table 3.1.4.1) indicated that there was no significant difference between the mean GPx activity of the samples assayed immediately and those assayed 3, 6 and 9 weeks after storage ($p >$ Bonferonni adjusted α of 0.008) for both volunteers.

It was therefore concluded that the erythrocyte lysate samples could be stored for up to nine weeks without there being a significant effect on the GPx activity of the samples.

3.1.4.2 Serum CC16

As illustrated in figure 3.1.4.2 below, there was a decrease in median CC16 concentration between the samples assayed immediately (the 0 week samples) and those stored for 3, 6 and 9 weeks, for both volunteers.

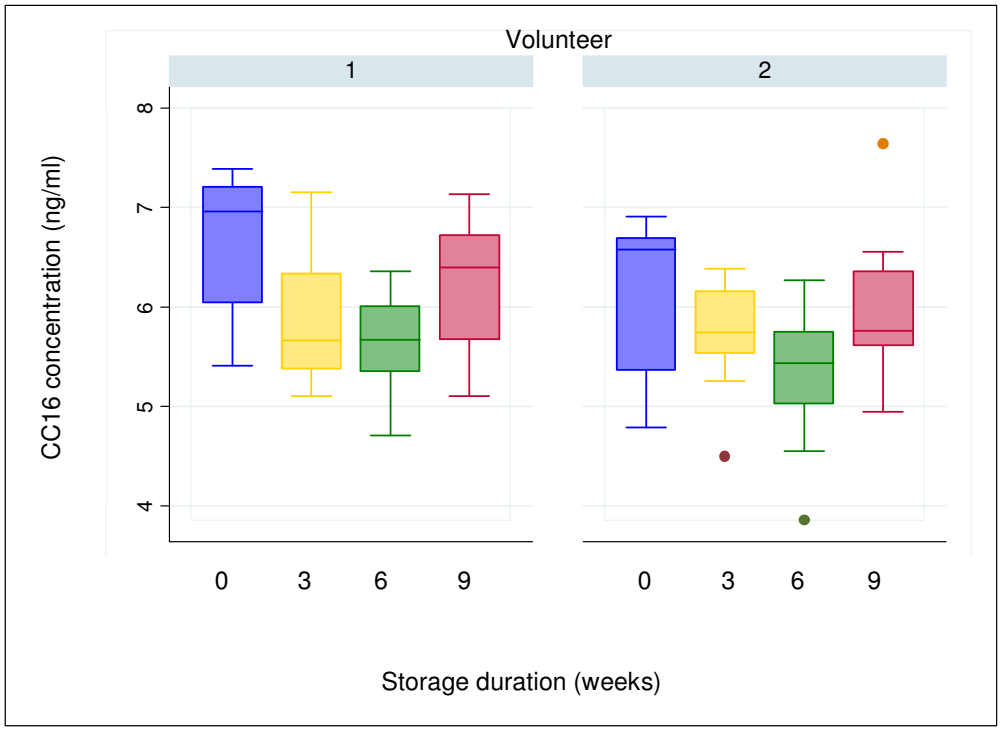


Figure 3.1.4.2: CC16 concentration of samples (from two volunteers) stored at -80 °C for 0, 3, 6 and 9 weeks. The CC16 concentration was measured (in 15 replicates per condition) using a Clara Cell Protein Human ELISA Kit. The CV ranged from 9.6%-26.2% for volunteer 1 and 8.3%-12.2% for volunteer 2. In the box-and-whisker plot the black line through the middle of the boxes represents the median CC16 concentration. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively. The solid dots represent data observations greater than 1.5 IQR above or below the median.

The difference between the mean CC16 concentration of the three and nine week samples collected from volunteer one and the zero and three week samples collected from volunteer two were not compatible with a normal distribution both volunteers (Shapiro-Wilk W test for normal data, $p = 0.023$ for both). As a result the mean CC16 concentrations of these samples were compared using the Wilcoxon signed-rank test. Since the rest of the differences between the mean CC16 concentration of the samples were compatible with a normal distribution ($p > 0.05$), paired t-tests were used to compare the mean CC16 concentrations of those samples. The results of these comparisons are presented in table 3.1.4.2.

Table 3.1.4.2: P-values obtained from the pair-wise comparison of the mean CC16 level in serum samples stored for various durations (level of significance is $p \leq 0.008$)

Volunteer		**0 weeks**	**3 weeks**	**6 weeks**	**9 weeks**
1	**0 weeks**	p = 1.00			
	3 weeks	p = 0.00*	p = 1.00		
	6 weeks	p = 0.00*	p = 0.37	p = 1.00	
	9 weeks	p = 0.06	p = 0.14	p = 0.03	p = 1.0
2	**0 weeks**	p = 1.00			
	3 weeks	p = 0.10	p = 1.00		
	6 weeks	p = 0.01	p = 0.12	p = 1.00	
	9 weeks	p = 0.69	p = 0.20	p = 0.01*	p = 1.0

* Indicates p-value less than level of significance

For volunteer one, pair-wise comparison of the means (table 3.1.4.2) indicated that the mean CC16 concentration of the samples stored for 0 weeks was significantly higher than the mean CC16 concentration of the samples stored for 3 and 6 weeks ($p = 0.00$ for both). There was, however, no significant difference between the mean CC16 concentrations of the samples assayed immediately (0 weeks) and those stored for 9 weeks ($p >$ Bonferonni adjusted α of 0.008).

For volunteer two, pair-wise comparison of the means (table 3.1.4.2) indicated that there was no significant difference between the mean CC16 concentration of the 0 week samples and those stored for 3, 6 or 9 weeks ($p >$ Bonferonni adjusted α of 0.008). There was, however, a significant difference between the mean CC16 concentration of the samples stored for 6 weeks and the samples stored for 9 weeks ($p = 0.00$).

It was therefore concluded that the CC16 concentration of the serum samples may have been affected by the duration of storage.

3.2 Field work

As previously described in section 2.4 and in the introduction to chapter 3, the field work was conducted with adult male miners recruited from a gold mine in North West province, South Africa. Blood specimens were collected from the volunteers after their return from leave (at the time of recruitment), and then again after their return to work on either a Monday (pre-shift), a Wednesday (pre- and post-shift), and/or a Friday (post-shift). It is important to state at this point that it was not possible to collect return to work blood specimens (also referred to as follow up samples) from all of the initial volunteers for a number of reasons. The field work sample collection procedure and the reasons why follow up samples were not collected from all of the initial volunteers are detailed in figure 3.2.1 below.

The demographics of the group of volunteers are summarised in the next section. The influence of age, race group, cigarette smoking habit and silicosis on the levels of erythrocyte GPx activity and serum CC16 concentration were then evaluated and the results are presented in sections 3.2.2 and 3.2.3. The subsequent comparison of the GPx and CC16 results obtained for the return from leave (ex-leave) and return to work (follow up) are summarized in sections 3.2.4. Finally, the effect of the estimated level and duration of crystalline silica exposure on the changes in GPx activity and CC16 concentration was evaluated and these results are presented in section 3.2.5.

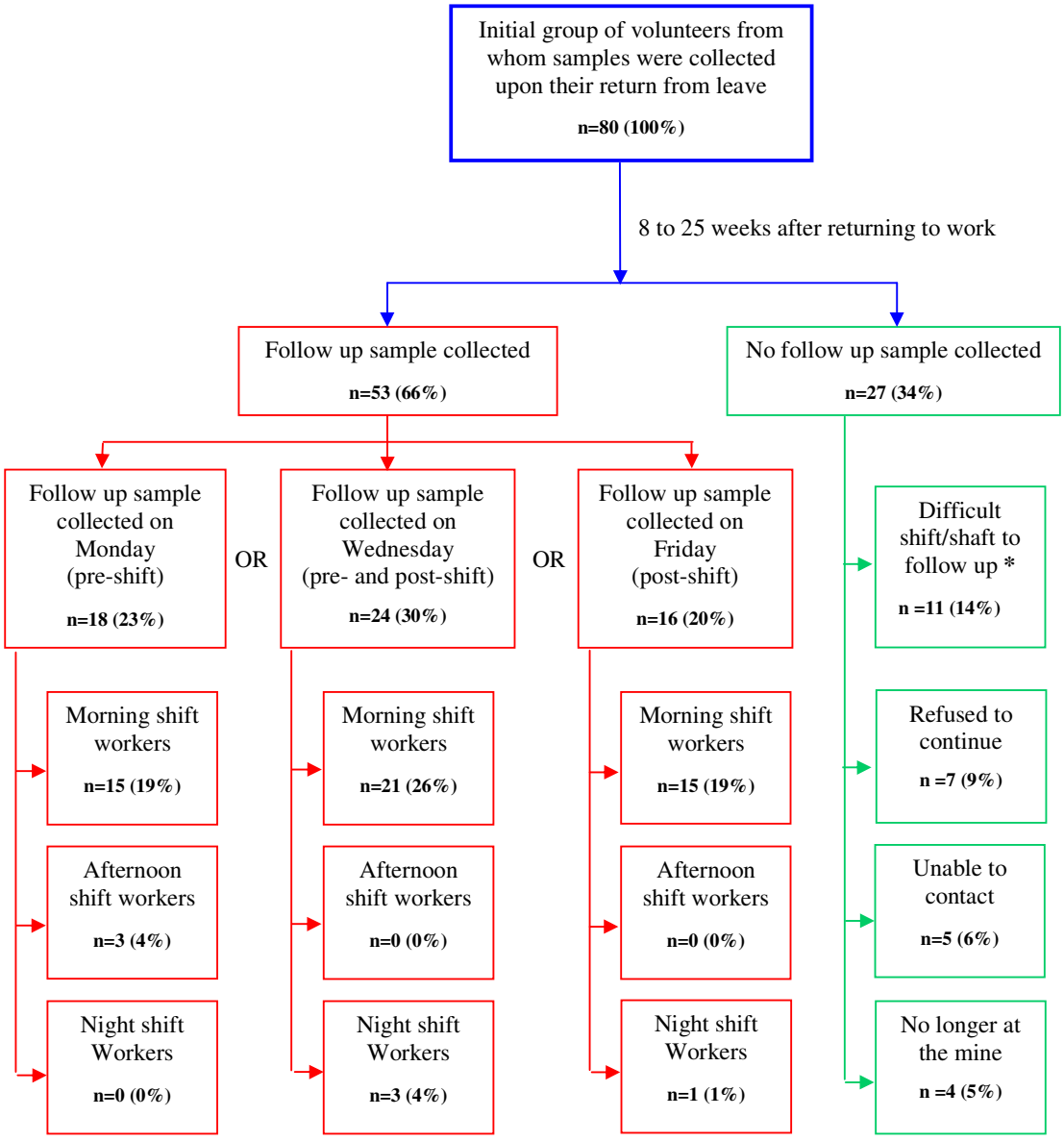


Figure 3.2: Outline of the field work sample collection procedure. The initial group of 80 volunteers were sorted into two groups according to whether the researchers were able to collect a follow up sample from the volunteer. Follow up samples were collected on either a Monday (pre-shift), Wednesday (pre- and post-shift) or Friday (post-shift) during morning, afternoon and evening shifts. The reasons why follow up samples were not collected from some volunteers are also described (*For example the volunteer was the only miner working a night shift at a particular shaft and it was therefore logistically difficult and expensive to collect that one specimen).

3.2.1 Demographics of the study population

At the time of recruitment, the volunteers were requested to complete a general questionnaire (regarding their age, smoking history and current working conditions) and these results are summarised in table 3.2.1.a. In order to determine if there were any demographic differences between the group of volunteers who agreed to give follow up samples and the group from whom no follow up sample could be collected, the demographic data for the whole group of volunteers was sorted into two groups according to this follow up status. The characteristics of these two groups are also presented in table 3.2.1.a.

Table 3.2.1.a: Demographics of the study population as a whole as well as for those volunteers from whom follow up samples could and could not be collected

Characteristic		Whole group	Group from whom follow up sample collected	Group from whom no follow up sample collected
Age (years)	mean (± SD)	39.97 (± 9.09)	40.44 (± 8.50)	39.09 (± 10.21)
Race group	% Black % White	72.5 27.5	69.8 30.2	77.8 22.2
Smoking	% Current smokers % Ex-smokers % Non-smokers	31.7 24.0 44.3	35.8 18.9 45.3	23.1 34.6 42.3
Job classification	% Low dust exposure % High dust exposure	56.0 44.0	58.5 41.5	50.0 50.0
Silicosis (grade ≥ 1/1)	% Silicosis	11.1	9.4	15.8
Ex-leave GPx activity (U/g Hb)	mean (± SD)	26.30 (± 6.50)	26.51 (±5.41)	25.56 (±6.99)
Ex-leave CC16 concentration (ng/ml)	mean (± SD)	6.82 (±2.83)	6.89 (±2.65)	6.78 (±2.94)

As shown in table 3.2.1.a, the group of volunteers as a whole (n = 80) had an average age of 40 years, consisted of a majority of black individuals, had an approximately equal number of non-smokers and ever smokers (current and ex-smokers), consisted of an approximately equal number of individuals in high and low dust exposure jobs, and had a radiological silicosis prevalence of 11%.

While the overall prevalence of radiological silicosis is given in table 3.2.1.a above, table 3.2.1.b below gives more detailed results of the radiological silicosis assessments. As previously described in section 2.4.5, chest radiographs were obtained for the study volunteers and assessed for silicosis (defined as an ILO category score of 1/1 or greater) by two experienced readers.

Table 3.2.1.b: Number of study volunteers in each ILO category, as diagnosed by chest radiographs read by two readers (with silicosis defined as ILO ≥ 1/1)

		Reader 1				
ILO category		**0**	**1**	**2**	**3**	**Total**
Reader 2	**0**	64	3	0	0	**67**
	1	1	1	0	0	**2**
	2	0	1	1	0	**2**
	3	0	0	0	1	**1**
	Total	**65**	**5**	**1**	**1**	**72**

The agreement between the two readers, in terms of silicosis classification, was determined by means of the kappa score which had a value of 0.64 indicating good agreement.

In order to determine if there had been a selection bias in the miners from whom follow up blood specimens were collected (n = 53) and those from whom no follow up blood specimen could be collected (n = 27), the characteristics of the two groups were compared by statistical analysis to detect any significant differences.

The age data for the two groups as well as the return from leave GPx activity and CC16 concentration data for the group of volunteers who did not give follow up samples were compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p > 0.5$). The data were therefore compared using the t-test. On the other hand, the return from leave GPx activity and CC16 concentration data for the group of volunteers who gave follow up samples were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.01$ and $p = 0.04$ respectively) and therefore these data were compared using the two-sample Wilcoxon rank-sum test.

It was thereby determined that there was no significant difference between the group of volunteers who donated follow up samples and the group who could not donate follow up samples in regards to age (t-test with equal variances, $p = 0.54$), race group composition (chi square p-value = 0.45), current smoking status (chi square p-value = 0.25), ex-smoking status (chi square p-value = 0.12), job classification (chi square p-value = 0.50), silicosis prevalence (chi square p-value = 0.45), return from leave GPx activity (two-sample Wilcoxon rank-sum test, $p = 0.34$) or return from leave CC16 concentration (two-sample Wilcoxon rank-sum test, $p = 0.71$). Since there were no significant differences between the characteristics of two groups, it was concluded that there was no selection bias in the miners who donated follow up blood specimens. This in turn, allowed the ex-leave

results obtained from the two groups of miners to be combined to investigate the parameters affecting GPx activity and CC16 concentration (sections 3.2.2 and 3.2.3, respectively). The combined ex-leave results were also used later to compare those levels of GPx activity and CC16 concentration to those obtained from the miners who donated the follow up samples (sections 3.2.4).

3.2.2 Parameters affecting GPx activity in ex-leave miners

In order to determine if the age, race group, or cigarette smoking habits of the study volunteers (n = 80) had influenced the results obtained in section 3.2.4, the effect of these factors on the levels of erythrocyte GPx activity was investigated. In addition, the effect of the presence of radiological silicosis on the levels of GPx activity was examined.

3.2.2.1 Age

The return from leave (ex-leave) GPx activity data collected from all of the initial 80 study volunteers were used to determine the effect of age on GPx activity. This ex-leave GPx activity data were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, p=0.00), however the age data were compatible with a normal distribution (p=0.08).

When the age data were compared with the GPx activity data, no significant correlation was found (Spearman's correlation r = -0.08, p = 0.47). This non-significant result was confirmed by parametric tests performed on the log transformed data (Pearson's

correlation r = -0.03, p = 0.78). These results indicate that the GPx activity of the ex-leave samples was not affected by the age of the study volunteers.

3.2.2.2 Race group

The mean return from leave GPx activities of the study volunteers, sorted according to apparent race group, are summarised in table 3.2.2.2.

Table 3.2.2.2: Mean GPx activity of the return from leave samples collected from the black and white study volunteers

Race group	n	Mean GPx activity (U/g Hb)	Standard deviation
Black	58	26.19	6.97
White	22	26.60	5.12

The ex-leave GPx activity data of the black race group were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, p = 0.00) while that of the white race group were compatible (p = 0.56).

Although a slight increase in mean GPx activity between the black and white race groups (0.41 U/g Hb) was observed (table 3.2.2.2) this increase was not statistically significant (two-sample Wilcoxon rank-sum test, p = 0.58). This non-significant result was confirmed by parametric tests performed on the log transformed data (two-sample t test, p=0.61).

These results indicate that the GPx activity of the ex-leave samples was also not affected by the race of the study volunteers.

3.2.2.3 Cigarette smoking

Table 3.2.2.3 illustrates the mean return from leave GPx activities of the study volunteers sorted according to their cigarette smoking habits.

Table 3.2.2.3: Mean GPx activity of the return from leave samples divided into three groups according to the cigarette smoking status of the volunteer at the time of sample collection

Smoking status	n	Mean GPx activity (U/g Hb)	Standard deviation
Current smoker	25	27.48	7.13
Ex-smoker	19	25.58	5.89
Non-smoker	35	25.64	6.33

The ex-leave GPx activity data of the non-smoker group were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.00$) while that of the current smoker and ex-smoker groups were compatible ($p = 0.19$ and $p = 0.99$, respectively).

The decrease in the mean GPx activity between the current smokers and the ex-smokers and non-smokers (table 3.2.2.3) was not statistically significant (Kruskal-Wallis test, $p = 0.52$). This non-significant result was confirmed by parametric tests performed on the log transformed data (one-way analysis of variance with comparisons of the means, $p = 0.61$).

These results indicate that the GPx activity of the ex-leave samples was also not affected by cigarette smoking.

3.2.2.4 Silicosis

The mean return from leave GPx activities of the study volunteers with and without radiological silicosis (of ILO grade ≥ 1/1) are shown in table 3.2.2.4.

Table 3.2.2.4: Mean GPx activity of the return from leave samples collected from the study participants with and without radiological silicosis (of ILO grade ≥ 1/1)

Silicosis	n	Mean GPx activity (U/g Hb)	Standard deviation
No silicosis	64	26.68	6.72
Silicosis	8	21.42	2.31

The ex-leave GPx activity data of the group with no signs of radiological silicosis were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.01$) while that of the group with silicosis were compatible with a normal distribution ($p = 0.41$).

As illustrated in table 3.2.2.4, the mean GPx activity of the individuals with no silicosis was higher that the mean GPx activity of the individuals with silicosis (approximately 5.3 U/g Hb). It was determined that this difference was statistically significant (two-sample Wilcoxon rank-sum test, $p = 0.01$) and the result was confirmed by parametric tests performed on the log transformed data (two-sample t test with unequal variances, $p = 0.00$).

These results indicate that the GPx activity of the ex-leave samples was significantly affected by the presence of radiological silicosis (of ILO grade ≥ 1/1) in the study volunteers.

3.2.3 Parameters affecting CC16 concentration in ex-leave miners

The effect of age, race, cigarette smoking and silicosis on the levels of serum CC16 concentration was examined to determine if these factors may have influenced the results obtained in section 3.2.4.

3.2.3.1 Age

Similar to the GPx activity results, the return from leave (ex-leave) CC16 concentration data were used to determine the effect of age on CC16 concentration. The ex-leave CC16 concentration data for the whole group were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.01$) while the age data were compatible with a normal distribution ($p = 0.08$).

When the age data were compared with the CC16 concentration data, no significant correlation was found (Spearman's correlation $r = 0.11$, $p = 0.35$). This non-significant result was confirmed by parametric tests performed on the log transformed data (Pearson's correlation $r = 0.06$, $p = 0.58$).

These results indicate that the CC16 concentration of the ex-leave samples was not affected by the age of the study participants.

3.2.3.2 Race group

Table 3.2.3.2 summarises the return from leave CC16 concentration data for the two main race groups involved in the study.

Table 3.2.3.2: Mean CC16 concentration of the return from leave samples collected from the black and white study volunteers

Race group	n	Mean CC16 concentration (ng/ml)	Standard deviation
Black	58	6.80	3.00
White	22	6.84	2.36

The ex-leave CC16 concentration data of the white race group were not compatible with being drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.01$) while that of the black race group were compatible ($p = 0.05$).

As shown in table 3.2.3.2, there was a slight increase in mean CC16 concentration between the black and white race groups (0.04 ng/ml), however it was not statistically significant (two-sample Wilcoxon rank-sum test, $p = 0.85$). This non-significant result was confirmed by parametric tests performed on the log transformed data (the two-sample t test with unequal variances $p = 0.53$).

These results indicate that the CC16 concentration of the ex-leave samples was also not affected by the race of the study volunteer.

3.2.3.3 Cigarette smoking

The mean return from leave CC16 concentrations of the ex-leave samples collected from the current smokers, ex-smokers and non-smokers who volunteered for the study are shown in table 3.2.3.3.

Table 3.2.3.3: Mean CC16 concentration of the ex-leave samples divided into three groups according to the cigarette smoking status of the volunteer at the time of sample collection

Smoking status	n	Mean CC16 concentration (ng/ml)	Standard deviation
Current smoker	25	6.59	2.50
Ex-smoker	19	7.63	2.74
Non-smoker	35	6.50	3.10

The ex-leave CC16 concentration data of the non-smoker group were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.01$) while that of the current smoker and ex-smoker groups were compatible with a normal distribution ($p = 0.09$ and $p = 0.37$, respectively).

As shown in table 3.2.3.3, the mean CC16 concentration of the group of ex-smokers was higher than that current smokers and non-smokers. However, analysis revealed that the difference between the mean CC16 concentration of the three groups was not statistically significant (Kruskal-Wallis test, $p = 0.15$). This non-significant result was confirmed by parametric tests performed on the log transformed data (one-way analysis of variance with comparisons of the means, $p = 0.32$).

These results indicate that the cigarette smoking habits of the study volunteers did not affect the CC16 concentration of the ex-leave samples.

3.2.3.4 Silicosis

The return from leave CC16 concentration results for the study volunteers with and without signs of radiological silicosis (of ILO grade ≥ 1/1) are illustrated in table 3.2.3.4.

Table 3.2.3.4: Mean CC16 concentration of the return from leave samples collected from the study participants with and without radiological silicosis (of ILO grade ≥ 1/1)

Silicosis	n	Mean CC16 concentration (ng/ml)	Standard deviation
No silicosis	64	6.59	2.77
Silicosis	8	8.61	3.12

The ex-leave CC16 concentration data of the group with no signs of radiological silicosis were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.01$) while that of the group with silicosis were compatible with a normal distribution ($p = 0.44$).

The increase in the mean CC16 concentration between the individuals with no silicosis and those with silicosis (2.02 ng/ml) observed in table 3.2.3.4 was just statistically significant (two-sample Wilcoxon rank-sum test, $p = 0.05$). This significant result was contradicted by parametric tests performed on the log transformed data (two-sample t test, $p = 0.10$).

These results indicate that the CC16 concentration of the ex-leave samples may have been affected by whether the study volunteers had radiological silicosis (of ILO grade ≥ 1/1).

3.2.4 Comparison between biomarker levels in the return from leave (ex-leave) and return to work (follow up) samples

Blood specimens were collected from the study volunteers after their return from leave (at the time of recruitment), and then again 8 to 25 weeks after their return to work on either a Monday (pre-shift), a Wednesday (pre- and post-shift), or a Friday (post-shift). In order to determine the effect of crystalline silica exposure on the levels of erythrocyte GPx activity and serum CC16 concentration, the levels of these biomarkers in the ex-leave and follow up samples were statistically compared.

3.2.4.1 Erythrocyte GPx

The median erythrocyte GPx activity results obtained for the ex-leave and follow up samples are illustrated in figure 3.2.4.1.a below. In addition, the mean erythrocyte GPx activities of the different groups and a statistical comparison of the differences between the mean GPx activities of the groups are given in table 3.2.4.1.a and table 3.2.4.1.b, respectively.

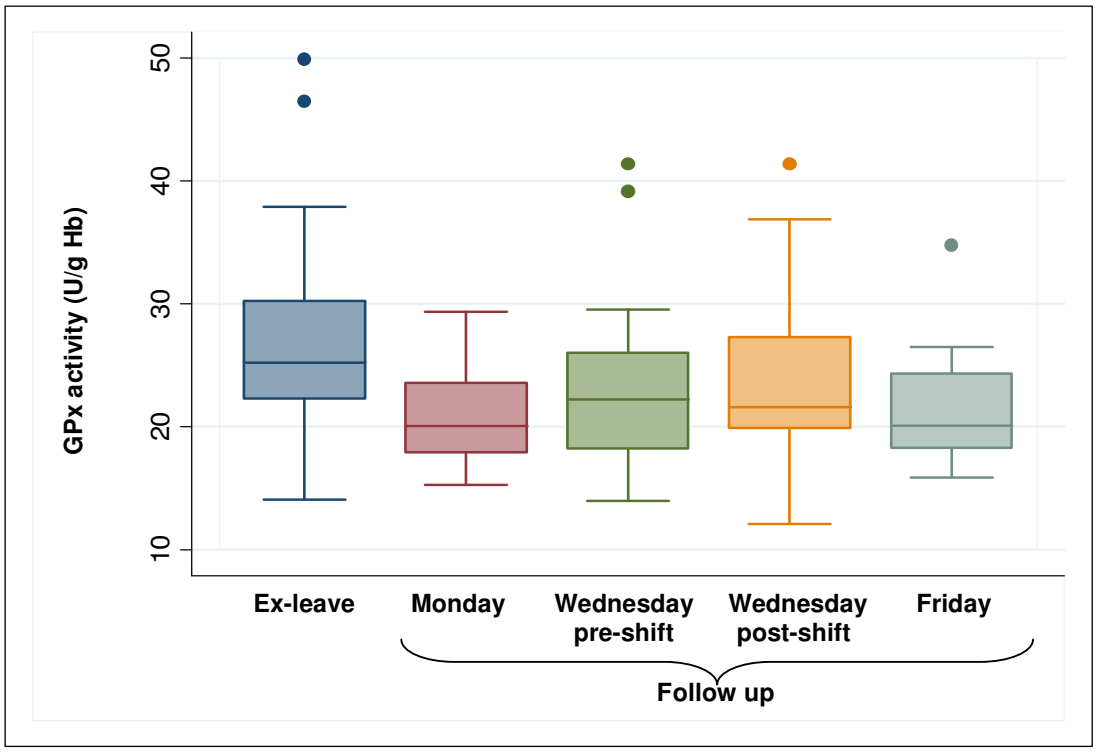


Figure 3.2.4.1.a: Erythrocyte GPx activities of the ex-leave and follow up samples (collected after 8 to 25 weeks on either a Monday, Wednesday (pre- and post-shift), or Friday). The GPx activity was measured using a Glutathione Peroxidase Assay Kit. In the box-and-whisker plot the line through the middle of the boxes represents the median GPx activity. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively.

Table 3.2.4.1.a: Mean erythrocyte GPx activities of the ex-leave samples and the follow up samples (combined and individually)

Group	n	Mean GPx activity (U/g Hb)	Standard deviation
Ex-leave	80	26.30	6.49
Combined follow up	53	22.48	5.78
Monday	18	21.23	4.09
Wednesday pre-shift	23	23.01	7.02
Wednesday post-shift	22	23.78	7.42
Friday	16	21.68	4.72

Table 3.2.4.1.b: Comparison between the mean erythrocyte GPx activities of the ex-leave and follow up samples (the level of significance is $p \leq 0.01$)

Groups being compared	Difference between mean GPx activities (U/g Hb)	Significance (p-value)
Ex-leave versus combined follow up	-3.82	0.00*
Ex-leave versus Monday	-5.07	0.00*
Ex-leave versus Wednesday (pre-shift)	-3.29	0.01*
Ex-leave versus Wednesday (post-shift)	-2.52	0.00*
Ex-leave versus Friday	-4.62	0.00*
Monday versus Wednesday (pre-shift)	1.78	0.27
Monday versus Wednesday (post-shift)	2.55	0.18
Monday versus Friday	0.45	0.94
Wednesday (pre-shift) versus Wednesday (post-shift)	-0.77	0.91
Wednesday (pre-shift) versus Friday	-1.33	0.64
Wednesday (post-shift) versus Friday	-2.10	0.38

*Indicates p-value less than level of significance

As observed in figure 3.2.4.1.a, the median GPx activity of the ex-leave samples was higher than the median GPx activities of all of the follow up samples. The same results were observed in tables 3.2.4.1.a and 3.2.4.1.b with regards to the mean GPx activities.

The differences between the mean GPx activity of the ex-leave samples and the follow up samples (including the combined follow up data) were all compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p > 0.05$). The paired t-test (with Bonferonni adjusted α of 0.01) was therefore performed to compare the mean GPx activity of the ex-leave samples with that of the Monday, Wednesday (pre- and post-shift) and Friday samples. The test indicated a significant decrease between the mean GPx activity of the ex-leave samples and the Monday, Wednesday (pre- and post-shift) and Friday follow up samples (table 3.2.4.1.b).

These results indicated that exposure to crystalline silica caused a decrease in the level of erythrocyte GPx activity.

The GPx activities of the follow up samples were then compared with each other to determine any significant differences. The GPx activity data for the Wednesday (pre-shift) and Friday follow up samples were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.02$ and $p = 0.04$ respectively), while the data for the Monday and Wednesday (pre-shift) follow up samples were compatible ($p > 0.05$). The two sample t-test indicated that there was no significant difference between the mean GPx activities of the Monday and Wednesday (post-shift) and the Wednesday pre-shift and post-shift follow up samples (table 3.2.4.1.b). The two-sample Wilcoxon rank-sum test (for unmatched data) indicated that there was no significant difference between the mean GPx activities of the Monday and Wednesday (pre- shift), the Monday and Friday, and the Wednesday (pre-and post-shift) and Friday follow up samples (table 3.2.4.1.b). These non-significant results were confirmed by parametric tests (two-sample t-test) performed on the log transformed data. There were therefore no significant differences between the GPx activities of the follow up samples.

The non-significant result obtained for the comparison of the mean GPx activities of the different follow up samples enabled a mean GPx activity to be determined for all the follow up samples combined. The paired t-test (with Bonferonni adjusted α of 0.01) was performed to compare the mean GPx activity of ex-leave samples with that of the combined follow up samples. The test indicated a significant decrease between the mean GPx activity of the ex-leave samples and the combined follow up samples.

The results presented in figure 3.2.4.1.a and tables 3.2.4.1.a and 3.2.4.1.b are combined results for all the volunteers. Since there were three miners from whom more than one set of follow up samples were collected, it was possible to illustrate the ex-leave and follow up results for these individual volunteers. Figure 3.2.4.1.b below compares the GPx activity of the ex-leave and follow up samples collected from these three miners.

Figure 3.2.4.1.b: Erythrocyte GPx activities of three volunteers from whom ex-leave and multiple follow up samples were collected. The follow up samples were collected 11 to 13 weeks after the ex-leave samples on a Monday, Wednesday (pre- and post-shift) and/or Friday. (Miner 8 —— ; miner 11 —— ; miner 43 ——). All three volunteers had low dust exposure job classifications and only Miner 43 had a silicosis score ≥ 1/1.

As was observed with the group of volunteers as a whole, the three individual volunteers all showed a decrease in mean GPx activity between their ex-leave and follow up samples (figure 3.2.4.1.b). While there was a large increase in mean GPx activity between the Monday and Friday samples of miner 8, there was a large decrease in the mean GPx activity between the Wednesday and Friday samples of miner 11. These individual results cannot, however, be considered an accurate representation of the effect of silica exposure on GPx activity since they are based on single samples which show high variation.

3.2.4.2 Serum CC16

The median serum CC16 concentration results obtained for the ex-leave and follow up samples are illustrated in figure 3.2.4.2.a.

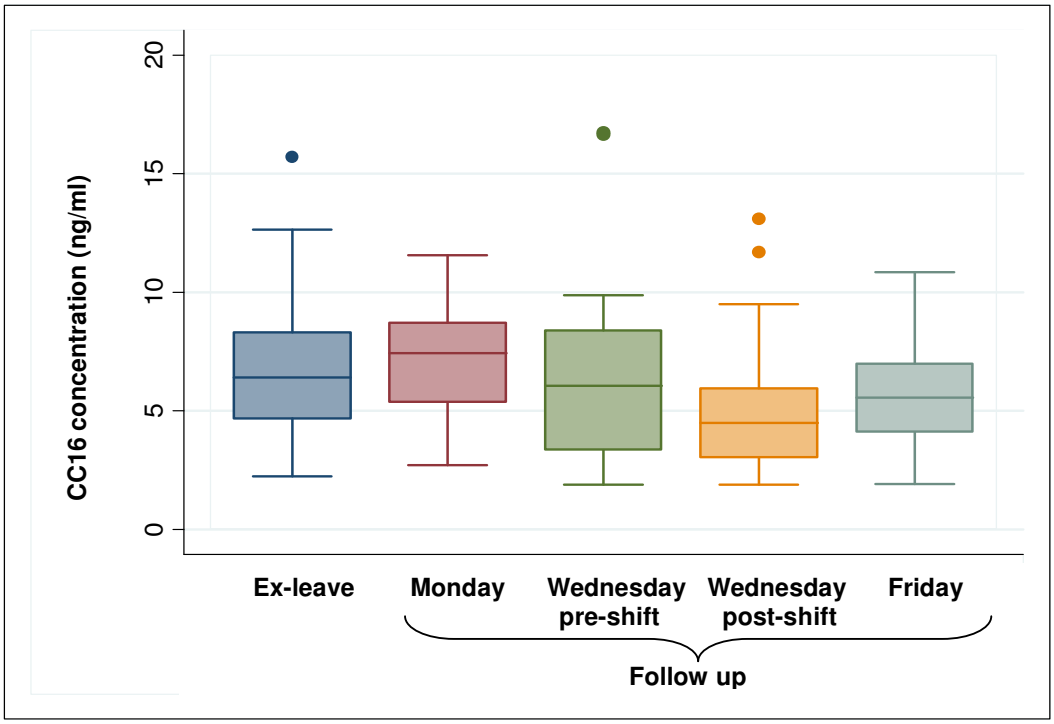


Figure 3.2.4.2.a: CC16 concentrations of the ex-leave and follow up samples (collected after 8 to 25 weeks on either a Monday, Wednesday (pre- and post-shift), or Friday). The CC16 concentration was measured using a Clara Cell Protein Human ELISA Kit. In the box-and-whisker plot the line through the middle of the boxes represents the median CC16 concentration. The top and bottom of each box is the 75th and the 25th percentile, respectively. The top and bottom of each bar is the 90th and 10th percentile, respectively.

In addition, the mean serum CC16 concentrations of the different groups and a statistical comparison of the differences between the mean CC16 concentrations of the groups are given in table 3.2.4.2.a and table 3.2.4.2.b, respectively.

Table 3.2.4.2.a: Mean CC16 concentrations of the ex-leave and the follow up samples

Group	n	Mean CC16 concentration (ng/ml)	Standard deviation
Ex-leave	80	6.82	2.83
Monday	18	6.97	2.55
Wednesday pre-shift	23	6.75	3.96
Wednesday post-shift	22	5.15	3.10
Friday	16	5.59	2.23

Table 3.2.4.2.b: Comparisons of the mean CC16 concentrations of the ex-leave and follow up samples (level of significance is $p \leq 0.01$)

Groups being compared	Difference between CC16 concentrations (ng/ml)	Significance (p-value)
Ex-leave versus Monday	0.15	0.74
Ex-leave versus Wednesday (pre-shift)	-0.07	0.52
Ex-leave versus Wednesday (post-shift)	-1.67	0.00*
Ex-leave versus Friday	-1.23	0.02
Monday versus Wednesday (pre-shift)	-0.22	0.12
Monday versus Wednesday (post-shift)	-1.82	0.04
Monday versus Friday	-1.38	0.11
Wednesday (pre-shift) versus Wednesday (post-shift)	-1.60	0.01*
Wednesday (pre-shift) versus Friday	-1.16	0.76
Wednesday (post-shift) versus Friday	0.44	0.31

* Indicates p-value less than level of significance

There was increase in median CC16 concentration (figure 3.2.4.2.a) and mean CC16 concentration (table 3.2.4.2.a and 3.2.4.2.b) between the ex-leave and Monday follow up samples and a decrease in median CC16 concentration (figure 3.2.4.2.a) and mean CC16 concentration (table 3.2.4.2.a and 3.2.4.2.b) between the ex-leave and Wednesday (pre- and post-shift) and Friday follow up samples.

Unlike the differences between the mean CC16 concentration of the ex-leave samples and the Monday and Wednesday (pre-shift) follow up samples which were compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p > 0.05$), the differences between the mean CC16 concentrations of the ex-leave and Wednesday (post-shift) ($p = 0.02$) and Friday ($p = 0.02$) follow up samples were not compatible. As a result, the paired t-test (with Bonferonni adjusted α of 0.01) was used to compare the mean CC16 concentration of ex-leave samples with that of the Monday and Wednesday (pre-shift) follow up samples, and the Wednesday pre-shift and post-shift samples. The Wilcoxon signed-rank test (for matched data) was used to compare the mean CC16 concentration of ex-leave samples with that of the Wednesday (post-shift) and Friday follow up samples. As demonstrated in table 3.2.4.2.b, the tests indicated a significant difference between the mean CC16 concentration of the ex-leave samples and the Wednesday post-shift ($p = 0.00$), but no significant difference between the mean CC16 concentration of the ex-leave samples and the Monday, Wednesday (pre-shift) or Friday follow up samples ($p > 0.01$).

The CC16 concentration data for the Wednesday (pre- and post-shift) follow up samples were not compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p = 0.01$ and $p = 0.00$ respectively), while the data for the Monday and Friday follow up samples were compatible ($p > 0.05$). As shown in table 3.2.4.2.b, there was no significant difference between the mean CC16 concentration of the Monday and Wednesday (pre- and post-shift) samples, the Wednesday (pre- and post-shift) and Friday samples (the two-sample Wilcoxon rank-sum test for unmatched data, $p > 0.01$) or the Monday and Friday samples (two-sample t-test, $p > 0.01$). There was however, a significant difference between the mean CC16 concentration of the

Wednesday pre-shift and post-shift samples (the Wilcoxon signed-rank test for matched data, p = 0.01).

The significant results obtained for some of the comparisons between the mean CC16 concentrations of the different follow up samples meant that a mean CC16 concentration could not be determined for all the follow up samples combined.

The results presented in figure 3.2.4.2.a and tables 3.2.4.2.a and 3.2.4.2.b are a combination of all the results obtained. Since there were three miners from whom more than one set of follow up samples were collected, it was possible to examine the ex-leave and follow up results for these individual volunteers. Figure 3.2.4.2.b below compares the CC16 concentration of the ex-leave and follow up samples for these three miners.

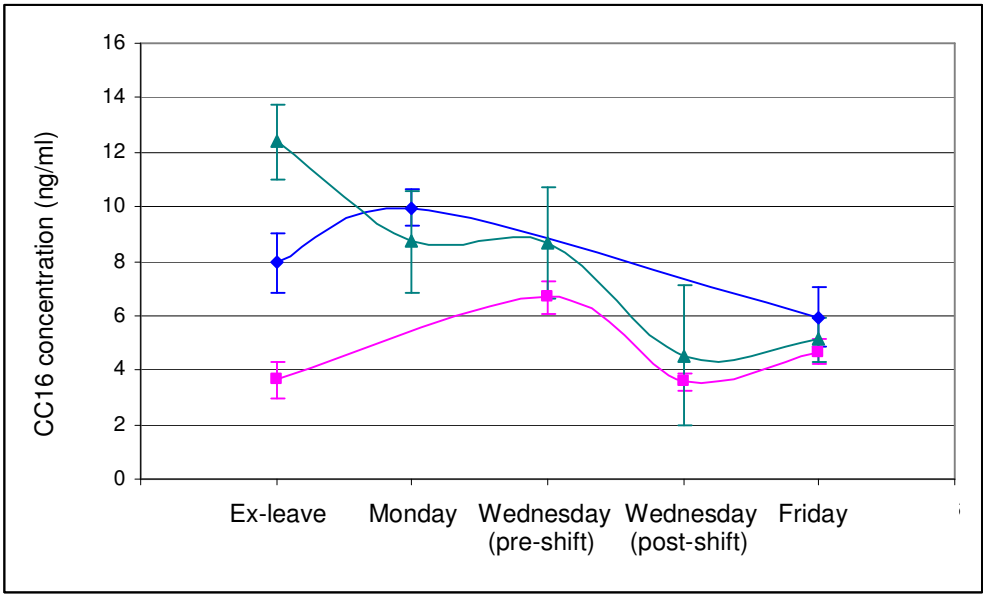


Figure 3.2.4.2.b: Serum CC16 concentrations of three volunteers from whom ex-leave and multiple follow up samples were collected. The follow up samples were collected 11 to 13 weeks after the ex-leave samples on a Monday, Wednesday (pre- and post-shift) and/or Friday. (Miner 8 ——— ; miner 11 ——— ; miner 43 ———). All three volunteers had low dust exposure job classifications and only Miner 43 had a silicosis score ≥ 1/1.

As illustrated in figure 3.2.4.2.b, for miner 43, there was a decrease in CC16 concentration between the ex-leave and Monday follow up samples and the CC16 concentration continued to decrease throughout the week. For miner 8 there was a decrease in CC16 concentration between the ex-leave and Friday samples, which was in accordance with the results obtained for the entire group of volunteers (figure 3.2.4.2.a), however there was an increase in CC16 concentration between the ex-leave and Monday samples. A similar contradictory result was observed for miner 11 in that the CC16 concentration increased between the ex-leave and Wednesday (pre-and post-shift) samples and between the ex-leave and Friday samples. As with the GPx results, these individual results should be treated with caution as they are based on single samples which show high variation.

3.2.5 Effect of crystalline silica exposure on the change in biomarker levels

In an attempt to explain the changes in GPx activity and CC16 concentration (between the ex-leave and follow up samples) observed in section 3.2.4, the correlation between the job classification (an estimate of the level of crystalline silica exposure) of the study volunteers and the changes in biomarker levels was investigated. In addition, the correlation between the duration of crystalline silica exposure (i.e. the time between the collection of the ex-leave and follow up samples) and the changes in biomarker levels was examined.

3.2.5.1 Erythrocyte GPx

As previously stated in section 3.2.4, the mean GPx activity for the combined follow up samples was determined. The changes in mean GPx activity for the volunteers with high and low dust exposure job classifications are presented in table 3.2.5.1.a.

Table 3.2.5.1.a: Change in mean GPx activity (between the ex-leave and combined follow up samples) divided into two groups according to the job classification of the study volunteers

Job classification	n	Change in mean GPx activity (U/g Hb)	Standard deviation
Low dust exposure	31	-5.12	4.04
High dust exposure	22	-2.91	5.40

The change in GPx activity data of the groups with low and high dust exposure jobs were compatible with having been drawn at random from a population with a normal distribution (Shapiro-Wilk W test for normal data, $p > 0.05$).

Although the change in mean GPx activity was greater for the group of volunteers with low dust exposure jobs than those with high dust exposure jobs (table 3.2.5.1.a), the difference proved not to be statistically significant (two-sample t test, $p = 0.09$). This was confirmed by the finding of no significant correlation between the job dust exposure classification and the change in GPx activity (Pearson's correlation $r = -0.23$, $p = 0.09$).

These results indicate that the changes in GPx activity (between the ex-leave and follow up samples) observed in the current study were not affected by the job classification (i.e. the estimated dust exposure level) of the study volunteers.

To determine if there was a connection between the duration of crystalline silica exposure and the observed changes in GPx activity, the change in GPx activity results were sorted according to the duration of exposure .These results are presented in table 3.2.5.1.b.

Table 3.2.5.1.b: Summary of the change in GPx activity (between the ex-leave and combined follow up samples) arranged according to the crystalline silica exposure duration

Crystalline silica exposure duration (weeks)	n	GPx activity of ex-leave samples, mean ± SD (U/g Hb)	GPx activity of combined follow up samples, mean ± SD (U/g Hb)	Change in mean GPx activity, mean ± SD (U/g Hb)
8	1	30.21 (± 0.00)	34.79 (± 0.00)	4.58 (± 0.00)
9	2	28.32 (± 4.04)	22.82 (± 3.93)	-5.49 (± 0.10)
11	2	20.86 (± 1.44)	16.97 (± 2.40)	-3.88 (± 0.96)
13	6	25.98 (± 4.33)	21.93 (± 2.46)	-4.05 (± 2.71)
14	2	29.68 (± 3.59)	17.67 (± 0.08)	-12.01 (± 3.68)
15	5	22.55 (± 3.15)	22.38 (± 4.84)	-0.17 (± 3.12)
16	5	25.81 (± 8.99)	20.56 (± 9.31)	-5.25 (± 1.38)
17	3	28.30 (± 5.41)	23.52 (± 2.64)	-4.78 (± 2.77)
18	9	25.39 (± 5.59)	22.72 (± 6.96)	-2.68 (± 5.76)
20	4	26.10 (± 5.34)	21.73 (± 1.75)	-4.36 (± 7.04)
21	4	31.00 (± 4.72)	21.75 (± 4.16)	-9.25 (± 2.75)
22	2	19.79 (± 4.25)	22.68 (± 8.10)	2.88 (± 3.84)
23	3	24.84 (± 0.85)	19.93 (± 3.07)	-4.91 (± 3.62)
25	5	34.50 (± 9.46)	28.23 (± 7.52)	-6.27 (± 3.10)

There was no apparent trend between the change in GPx activity and the duration of exposure to crystalline silica (table 3.2.5.1.b). This was confirmed by the finding of no significant correlation between the crystalline silica exposure duration and the change in GPx activity (Pearson's correlation $r = 0.14$, $p = 0.33$).

3.2.5.2 Serum CC16

Because the mean CC16 concentrations of all the follow up samples could not be combined to form a single follow up CC16 concentration variable, the change in CC16 concentration (between the ex-leave and follow up samples) was calculated for each of the follow up groups separately. The changes in mean CC16 concentration for the volunteers with high and low dust exposure job classifications are presented in table 3.2.5.2.a.

Table 3.2.5.2.a: Change in mean CC16 concentration (between the ex-leave and follow up samples) sorted according to the job classification of the study participants

Job classification	Change in mean CC16 level for the Monday follow up group, mean ± SD (ng/ml)	Change in mean CC16 level for the Wednesday (pre-shift) follow up group, mean ± SD (ng/ml)	Change in mean CC16 level for the Wednesday (post-shift) follow up group, mean ± SD (ng/ml)	Change in mean CC16 level for the Friday follow up group, mean ± SD (ng/ml)
Low dust exposure	0.44 (± 1.93)	-0.81 (± 2.64)	-2.13 (± 2.25)	-1.76 (± 2.50)
High dust exposure	-0.53 (± 2.81)	-0.51 (± 1.45)	-1.53 (± 1.73)	-0.98 (± 2.06)

There was a slight increase in the mean CC16 concentration between the ex-leave and Monday follow up samples for the group of volunteers with low dust exposure jobs and a slight decrease for the group of volunteers with high dust exposure jobs (table 3.2.5.2.a), however, the difference between the change in mean CC16 concentration in the two dust exposure groups was not significant (two-sample t test, $p = 0.41$). This was confirmed by the finding of no significant correlation between the job dust exposure classification and

the change in mean CC16 concentration for the Monday follow up group (Pearson's correlation p = 0.41).

Although the decrease in CC16 concentration between the ex-leave and Wednesday (pre-shift), Wednesday (post-shift) and Friday follow up samples was greater for the group of volunteers with low dust exposure jobs than those with high exposure jobs (table 3.2.5.2.a), the difference between the change in mean CC16 concentration in the two dust exposure groups was not significant (two-sample t test p = 0.64, two-sample Wilcoxon rank-sum p = 0.53 and p = 0.52 respectively). This was confirmed by the finding of no significant correlation between the job classification and the change in mean CC16 concentration for the Wednesday (pre-shift), Wednesday (post-shift) and Friday follow up groups (Pearson's correlation p = 0.64 and p = 0.49 and Spearman's correlation p = 0.54, respectively).

These results indicate that the change in CC16 concentration (between the ex-leave and follow up samples) was more than likely not affected by the job classification (i.e. the estimated dust exposure level) of the study volunteers.

Similar to GPx, the change in mean CC16 concentration results (for each follow up group) were sorted according to the duration of crystalline silica exposure to establish if there was a connection between the duration of exposure and the observed changes in CC16 concentration. These results are presented in table 3.2.5.2.b below.

Table 3.2.5.2.b: Summary of the change in CC16 concentration (between the ex-leave and follow up samples) arranged according to the duration of crystalline silica exposure

Crystalline silica exposure duration (weeks)	Change in mean CC16 level for the Monday follow up group, mean ± SD (ng/ml)	Change in mean CC16 level for the Wednesday (pre-shift) follow up group, mean ± SD (ng/ml)	Change in mean CC16 level for the Wednesday (post-shift) follow up group, mean ± SD (ng/ml)	Change in mean CC16 level for the Friday follow up group, mean ± SD (ng/ml)
8	-	-	-	-1.01 (±0.00)
9	-	-1.43 (± 0.66)	-1.67 (± 1.32)	-
11	-1.04 (± 3.71)	-3.72 (± 0.00)	-7.84 (± 0.00)	-7.23 (± 0.00)
13	2.38 (± 0.70)	2.45 (± 1.52)	-0.11 (± 0.08)	-0.47 (± 2.14)
14	-	0.37 (± 0.00)	0.54 (± 0.00)	-0.32 (± 0.00)
15	-1.83 (± 0.19)	0.46 (± 5.01)	-2.53 (± 0.86)	-4.96 (± 0.00)
16	3.69 (± 0.00)	0.90 (± 1.19)	-0.44 (± 0.87)	-
17	0.81 (± 0.00)	-2.43 (± 0.00)	-4.38 (± 0.00)	-0.34 (± 0.00)
18	-	-0.36 (± 1.33)	-1.06 (± 1.38)	-0.59 (± 0.74)
20	-1.43 (± 0.00)	-0.19 (± 0.92)	-1.54 (± 0.40)	-0.67 (± 0.00)
21	-0.52 (± 1.03)	-1.09 (± 0.00)	-3.22 (± 0.00)	0.93 (± 0.00)
22	-3.47 (± 0.00)	-1.94 (± 0.00)	-3.83 (± 0.00)	-
23	-0.15 (± 1.63)	-	-	-4.65 (± 0.00)
25	0.53 (± 0.23)	-1.65 (± 2.43)	-2.44 (± 1.54)	-2.03 (± 0.00)

There was no apparent trend between the change in CC16 concentration (between the ex-leave and follow up samples) and the duration of exposure to crystalline silica. This was confirmed by the finding of no significant correlation between the duration of exposure to crystalline silica and the change in CC16 concentration between the ex-leave and Monday, Wednesday (pre-shift), Wednesday (post-shift) and Friday follow up samples (Pearson's correlation $p = 0.34$ and $p = 0.46$, Spearman's correlation $p = 0.28$ and $p = 0.93$).

Therefore, the changes in GPx activity and CC16 concentration observed in the current study could not be correlated to the estimated dust exposure level (job classification) or the duration of crystalline silica exposure.

Chapter 4:

DISCUSSION

Despite the dust control methods currently being employed, silicosis continues to pose a significant threat to the health of South African gold miners. This is possibly due to inadequate occupational exposure limit enforcement and unreliable methods of determination of personal dust exposure. In addition, due to the long latency period of silicosis, the chest radiographs that are used to diagnose silicosis are a late indicator of harmful dust exposure and therefore do not provide an effective way to measure and control the dust exposure.

It is therefore clear that a more effective means of monitoring dust control methods is needed if silicosis is to be eliminated. One promising field is that of biomarkers of effect, which are measurable changes within an organism that may indicate either early processes preceding disease or predict the advance and presence of disease [Bennett and Waters, 2000].

As a result, a project on biomarkers was initiated which aimed to identify and operationalise biomarkers for crystalline silica dust exposure that could then be used to assess the success of the implemented dust exposure control measures in South African mines. The project was not intended to produce a diagnostic test for silicosis in individuals as biomarkers identified would probably be too non-specific for such a definitive diagnostic purpose [Aitio, 1999].

The current investigation is the third phase of this project on biomarkers and is aimed to confirm the levels of and further analyze erythrocyte glutathione peroxidase (GPx) and serum Clara cell protein 16 (CC16) in miners exposed to crystalline silica dust. Prior to their analysis in blood specimens collected from these miners, the optimal operational parameters for these biomarkers were investigated and the relevant Standard Operating Procedures (SOPs) were established.

4.1 Optimization of the operational parameters

In order to limit pre-analytical variability as a result of storage conditions and sample processing, the effect of different operational parameters on the levels of GPx activity and CC16 concentration was investigated.

Firstly, the effect of temperature and duration of storage on the levels of GPx activity and CC16 concentration needed to be determined as the assay of these two biomarkers were time intensive and could not be performed immediately under field conditions. Secondly, the effect of a delay in time between blood sample collection and separation needed to be investigated due to the fact that the follow up samples were collected at different times from different mine shafts and were then transported back to the temporary laboratory for their separation. Lastly, the effect of ambient room temperature on GPx activity and CC16 concentration needed to be determined because, despite the availability of heaters and fans, the temporary field laboratory did not have an adequate air conditioning system to ensure constant ambient temperature. Each of these investigations was performed with blood specimens obtained from two volunteers with no history of exposure to crystalline silica.

4.1.1 Effect of storage temperature

In order to determine the effect of storage temperature on the levels of GPx activity and CC16 concentration, replicate erythrocyte lysate and serum samples were stored at 4 ^{o}C (common fridge temperature), -20 ^{o}C (common freezer temperature) and -80 ^{o}C (specialised freezer temperature) before being assayed.

4.1.1.1 Erythrocyte GPx

For both volunteers there was a decrease in mean GPx activity with a decrease in storage temperature however, the decrease was not statistically significant. This indicated that the temperature at which the GPx samples were stored after separation did not have a significant effect on the GPx activities.

The results obtained were not expected as a number of studies have shown that GPx activity decreases with an increase in storage temperature. For instance, Zhang and colleagues [1986] examined the effect of storage temperature and storage duration on swine plasma GPx activity. They found a significant decrease in GPx activity after storage for two days and more at either 4 ^{o}C or -15 ^{o}C, with the decrease in GPx activity much greater in samples stored at 4 ^{o}C than in those stored at -15 ^{o}C. Saint-Denis and co-workers [1998] investigated the effect of storage temperature on GPx activity (of the cytosolic fraction) in the Earthworm *Eisenia fetida andrei.* They found that while storage for two months at 4 ^{o}C caused a decrease in GPx activity, the GPx activity of samples stored at -20 ^{o}C was relatively stable (although there was a slight decrease of activity). The GPx activity of the samples stored at -80 ^{o}C was the most stable. Other studies have also

suggested that the GPx activity of samples is more stable when the samples are frozen rather than refrigerated [Wahdati *et al*., 1992, Miranda *et al*., 2004].

An additional observation with our results was that the mean GPx activity in the erythrocyte lysate samples from volunteer two was approximately three times lower than that of volunteer one. Since the two volunteers were both females of similar age, the difference observed may just have been due to lifestyle factors [Andersen *et al*., 1997] or hormonal differences [Pinto and Bartley, 1969].

4.1.1.2 Serum CC16

The concentration of CC16 measured in the serum of both volunteers decreased with a decrease in storage temperature, with the largest statistically significant decrease occurring in the samples stored at -80 ^{o}C compared to those stored at 4 ^{o}C. On the other hand, no statistically significant difference could be observed between the mean CC16 concentration of the samples stored at -20 ^{o}C and -80 ^{o}C.

The higher CC16 concentration measured in the serum samples stored at 4 ^{o}C was not expected. Changes in volume observed in these samples, possibly due to evaporation despite the use of sealed CryoTubes™, could have resulted in the higher concentrations of CC16 measured in these samples.

Therefore, although the present investigation could not confirm the results of reported studies, a storage temperature of -80 ^{o}C was chosen for the erythrocyte lysate and serum

samples as it was the temperature recommended by the GPx and CC16 assay kit manufacturers and it was the temperature used in other investigations of erythrocyte GPx activity [Engelen *et al.*, 1990, Ho *et al.*, 2005, Borm *et al.*, 1986] and serum CC16 concentration [Halatek *et al.*, 2004, Wang *et al.*, 2007].

4.1.2 Effect of a delay in time between blood sample collection and separation

During the field work, the blood specimens could be left for up to two hours before being separated and therefore the effect of this delay in time on the levels of GPx activity and CC16 concentration was investigated.

4.1.2.1 Erythrocyte GPx

As the time interval between blood sample collection and separation increased (from zero to two hours), there was a slight increase in mean GPx activity of the erythrocyte lysate samples obtained from volunteer one and a slight decrease in mean GPx activity of the samples collected from volunteer two. However statistical analysis revealed that, for both volunteers, these observed changes in GPx activity were not statistically significant.

4.1.2.2 Serum CC16

There was a decrease in mean CC16 concentration as the time between sample collection and separation increased (from zero to two hours) for both volunteer one and two. However statistical analysis revealed that, for both volunteers, the decrease observed was not statistically significant. In another study on the effect of time between blood collection and centrifugation, the researchers found no significant difference between samples separated immediately and samples left for 30 minutes, 24, 48 and 96 hours before separation [Kenis *et al*., 2002].

It was therefore decided that the blood samples collected during the field work could be left for up to two hours (at approximately 25 ^{o}C) before being separated without a significant effect on the GPx activity of the resulting lysate samples or on the CC16 concentration of the resulting serum samples.

4.1.3 Effect of ambient laboratory temperature

The levels of GPx activity and CC16 concentration were assessed in samples processed at 25 ^{o}C (the common laboratory temperature) and 35 ^{o}C (a temperature close to physiological temperature) to establish the effect of ambient laboratory temperature.

4.1.3.1 Erythrocyte GPx

For volunteer one, processing of the erythrocyte lysate samples at the higher laboratory temperature of 35 ^{o}C, rather than the common laboratory temperature of 25 ^{o}C, caused a significant increase in the mean GPx activity of the samples. A similar result was found by Saint Denis and colleagues [Saint-Denis *et al*., 1998] who showed that maximum GPx activity occurred at a temperature of 40 ^{o}C. Since the normal human physiological body temperature is approximately 37 ^{o}C, one would expect enzymes such as GPx to function optimally at that temperature or temperatures close to it.

In contrast, for volunteer two there was a slight decrease in mean GPx activity at the higher laboratory temperature, however this decrease was not statistically significant.

4.1.3.2 Serum CC16

For both volunteer one and two, there was an increase in the mean CC16 concentration when the serum samples were processed at the higher laboratory temperature of 35^{o}C. This increase was statistically significant for volunteer one and only just non-significant for volunteer two.

The significant results obtained for volunteer one indicated that the laboratory temperature at which the samples are processed may affect the GPx activity and CC16 concentration of the samples. It was therefore important that the erythrocyte lysate and serum samples be

processed and assayed at a constant temperature to ensure reproducibility and allow the GPx activity and CC16 concentration of the samples to be reliably compared. Although a higher GPx activity and CC16 concentration was found at a temperature of 35 ^{o}C, the GPx and CC16 assay kit instructions recommended an assay temperature of 25 ^{o}C. It was therefore decided that during the field work the ambient temperature of the temporary field laboratory and the NIOH laboratory would be maintained as close to 25 ^{o}C as possible.

4.1.4 Effect of storage duration

The effect of increasing duration of storage on the levels of GPx activity and CC16 concentration was investigated as the erythrocyte lysate and serum samples could not be assayed immediately after their preparation.

4.1.4.1 Erythrocyte GPx

Although there was a slight increase in mean GPx activity with an increase in storage duration for volunteer one, analysis revealed that, for both volunteers, there was no significant difference between the mean GPx activities of the samples stored for increasing storage durations. A non-significant effect of storage temperature on GPx activity was also found by Andersen and colleagues [1997] who stored erythrocyte lysate samples at -80 ^{o}C for six months.

It was therefore decided that the erythrocyte lysate samples, collected during the field work, could be stored for up to nine weeks without there being a significant effect on the GPx activity of the samples.

Similar to the results obtained with the effect of storage temperature (section 4.1.1.1), storage duration also affected differently the GPx activities of the erythrocyte lysate samples collected from two different donors in that the mean GPx activity of the samples obtained from volunteer two were almost double that of the samples obtained from volunteer one. In this latter experiment however, the volunteers were of different sexes and the differences in mean GPx activities observed may therefore be attributed to gender differences as confirmed by observations by Bolzan *et al* [1997].

4.1.4.2 Serum CC16

For both volunteer one and two, the mean CC16 concentration of the samples decreased after three and six weeks but then recovered slightly after nine weeks of storage. While the decrease in the mean CC16 concentrations between the zero week and the three and six week samples was significant for volunteer one it was not significant for volunteer two.

Since there was no significant difference between the mean CC16 concentration of the serum samples assayed immediately and those stored for nine weeks, for both volunteers, it was decided that the serum samples collected during the field work would be stored for approximately nine weeks before being assayed. This decision was also based on the

recommendations of the kit manufacturers which claimed that the serum CC16 samples could be stored for up to two years at -80 ^{o}C.

4.1.5 Comparison of GPx activity and CC16 concentration results of NIOH volunteers with results reported for healthy adults

As previously mentioned in section 2.1.8, the haemoglobin concentration of the erythrocyte lysate samples used in the GPx optimisation experiments was not measured because the replicate samples were prepared from the same blood specimen. The GPx activity results obtained for the GPx optimisation experiments were therefore reported as nmol/min/ml. The GPx activity levels, obtained in the optimisation experiments, ranged from 20.4 to 1726.8 nmol/min/ml (or 0.002 to 1.727 Units). Using the average haemoglobin concentration obtained for the miner's erythrocyte lysate samples (0.05 g Hb/ml); the GPx activity results for the optimisation experiments were estimated to be between 0.42 and 35.36 U/g Hb. A study on the biological variability of erythrocyte GPx [Guemouri *et al.*, 1991], conducted in France, reported erythrocyte GPx activity levels of between 22.5 and 67.0 U/g Hb in healthy adult males and between 24.4 and 68.1 U/g Hb in healthy adult females. In terms of the South African population, Janse van Rensburg *et al* [2005] reported erythrocyte GPx levels of 22.1 ± 3.5 U/g Hb in healthy adult controls; however their sample numbers were low. Therefore, although some of the NIOH volunteers had estimated GPx activity results well below the reported ranges for healthy adults, the majority of the volunteers had estimated GPx activity results within the lower

end of the ranges. The mean GPx activity of the healthy volunteers in the study by Borm *et al* [1986] were also low (2.86 U/g Hb) despite the use of a different assay procedure.

In addition, the GPx optimisation experiments showed a within-subject coefficient of variation (CV) of between 11.4% and 126.3% (with a mean of 39.0%). However, the Glutathione Peroxidase Assay Kit manufacturer reported a much lower intra-assay CV of 5.7% when they assayed a larger sample size [CaymanChemical, 2004]. One reason for the high CVs obtained in the current study is the stringent criteria used to reject outliers. Only data observations greater than 2.IQR above or below the mean were considered outliers and therefore some replicate values that were much lower or higher than the other replicate could not be excluded from the data sets. The high CVs obtained in the current study may also indicate that the assay is not as reproducible as reported.

In the CC16 optimisation experiments the CC16 levels ranged from 0.59 to 8.30 µg/l with a mean of 5.81 µg/l. Other studies have reported serum CC16 levels of between 6.6 and 23.2 µg/l in healthy adult males and between 3.7 and 22.9 µg/l in healthy adult females [Shijubo *et al*., 2003]. Similar to the GPx results, some of the NIOH volunteers had CC16 levels well below the reported ranges for healthy adults while the majority of the volunteers had CC16 levels within the lower end of the ranges. The results of the current study were, however, consistent with the CC16 levels obtained for the healthy controls (5.77 ± 1.64 µg/l) in the second phase of the project [Murray *et al.*, 2006]. The lower CC16 and GPx levels in the healthy South African population therefore warrants further investigation.

The CC16 optimisation experiments showed a within-subject coefficient of variation (CV) of between 7.5% and 47.5% (with a mean of 17.2%). The CVs may have been lower if less stringent criteria were used to determine outliers.

4.2 Field work

During the field work portion of this project, a total of 80 volunteer miners were recruited upon their return from leave (a period of little or no crystalline silica exposure) and blood samples were collected. Follow up blood samples were then collected from 53 of the volunteers after they had returned to work (and had been exposed to crystalline silica dust). Due to various reasons (for example the refusal to continue with the study or the inability to be contacted telephonically) there were 27 volunteers from whom no follow up samples could be collected.

The follow up samples were collected 8 to 25 weeks (2 to 6 months) after the volunteers had returned to work. This variation in the duration of exposure to crystalline silica allowed the effect of exposure duration on the change in biomarker levels (between the ex-leave and follow up samples) to be investigated (the results of which are discussed in section 4.2.4). As previously detailed in section 2.4.3, the original protocol for the follow up sample collection procedure was revised so that blood specimens were collected on either a Monday (pre-shift), a Wednesday (pre- and post-shift), or a Friday (post-shift). This adaptation of the study design due to the lack of participation by volunteers resulted in a reduction in the number of samples obtained (only 16 Monday, 24 Wednesday, and 16 Friday follow up samples were collected) and therefore a decrease in the power of the

results. In addition, it limited the ability to examine inter-individual changes in the biomarker levels over time.

4.2.1 Demographics of the study population

The entire group of volunteers had a mean age of 40 years, consisted of a majority of black individuals and had prevalence of radiological silicosis of 11%. Additionally, the group consisted of an approximately equal number of individuals in high and low dust exposure jobs.

The characteristics of the group of volunteers who donated follow up samples were then compared to those of the group of volunteers who did not donate follow up samples. It was found that there was no significant difference between the two groups in terms of age, apparent race group, smoking habits, job classification (an estimate of dust exposure level), silicosis prevalence, return from leave GPx activities or return from leave CC16 concentrations. It was therefore concluded that there was no selection bias in the miners who donated follow up blood specimens.

4.2.2 Parameters affecting biomarker levels in ex-leave miners

4.2.2.1 Erythrocyte GPx

As indicated earlier, GPx is a marker of oxidative stress. The effect of age, race, cigarette smoking, and silicosis on the levels of erythrocyte GPx activity (in specimens collected from miners upon their return from leave) was therefore assessed because oxidative stress has been associated with aging [Habif *et al*., 2001], silicosis [Ghio *et al.*, 1990] and cigarette smoking [Quinlan *et al*., 1994]. The effect of race on GPx activity was also investigated as any effect would influence the recruitment of study volunteers in future projects.

The current study showed no significant correlation between age and erythrocyte GPx activity. These findings were in agreement with those reported in phase II of the project [Murray *et al*., 2006] as well as with those reported in other investigations [Andersen *et al*., 1997, Habif *et al*., 2001]. On the other hand, a significant positive correlation between age and GPx activity [Ceballos-Picot et al., 1992, Bolzan et al., 1997] and a negative correlation between GPx activity and an age of 60 years or older [Ho *et al*., 2005, Guemouri *et al*., 1991] have been reported. The lack of significant correlation between GPx activity and age observed in our results may therefore be explained by the mean age of our group being below 60 years.

The current study also found that, although the GPx activity of the group of white individuals was slightly higher than the group of black individuals, there was no significant effect of race on GPx activity. Due to the lack of convincing literature regarding race differences in GPx activity, further investigation into possible effects of race group on GPx activity is required.

In addition, no significant effect of cigarette smoking on erythrocyte GPx activity was found. These results confirm those reported in the second phase of the project [Murray *et al*., 2006] and in other investigations [Toth *et al*., 1986, Guemouri *et al*., 1991, Habif *et al*., 2001, Bolzan *et al*., 1997]. However, a few studies have indeed shown a significant decrease in erythrocyte GPx activity in smokers versus non-smokers [Duthie *et al*., 1991, Kondo *et al*., 1994, Ho *et al*., 2005]. It should be noted that the participants in the current study who described themselves as smokers only smoked a relatively low number of cigarettes per day and this may have contributed to the lack of a significant finding.

Finally, the level of GPx activity in the group of volunteers with silicosis was significantly lower than in the group with no signs of silicosis. Despite the small number of subjects in our silicotic group, larger studies involving silicotic patients and controls have also shown a decrease in erythrocyte GPx activity [Borm *et al.*, 1986, Zhou *et al.*, 1999].

4.2.2.2 Serum CC16

When the effect of age on serum CC16 concentration was investigated in the current study, no significant correlation was observed. Similar findings were also reported in phase II of

the project [Murray *et al*., 2006] and in a study by Hermans *et al* [1998] which also assayed CC16 by means of ELISA. In studies using latex immunoassay as the assay method, a significant correlation between age and CC16 concentration was found and it was attributed to age-related structural and functional changes in the lung or a decline in renal function [Robin *et al*., 2002, Bernard *et al*., 1994b].

In addition, no significant effect of race on serum CC16 concentration was found. Similar to GPx activity, no studies could be found that examined the effect of race on CC16 concentration and therefore further research into this field is required.

The current study also showed that, although the smokers (current and ex-smokers) had higher CC16 concentration than the non-smokers; there was no significant effect of cigarette smoking on serum CC16 concentration. These findings were in agreement with the findings of phase II of the project [Murray *et al*., 2006] and a study by Wang *et al* [2007] which involved silica exposed workers and controls.

Lastly, the volunteers in the current study with radiological silicosis (of ILO grade 1/1 or greater) had higher CC16 concentrations than the volunteers with no silicosis. Although the non-parametric statistical test indicated that the increase was just significant, the parametric tests performed on the transformed data indicated that there was no significant effect of silicosis on CC16 concentration. The contradicting results were likely due to the small number of subjects with silicosis. An earlier study involving silica-exposed workers in China found no significant difference between the participants with and without silicosis [Wang *et al*., 2007]. A more comprehensive investigation into the effect of silicosis on serum CC16 concentration is therefore required.

4.2.3 Comparison between biomarker levels in the return from leave (ex-leave) and return to work (follow up) samples

As previously mentioned, ex-leave samples were collected from miners after a period of no crystalline silica exposure and follow up samples were collected, throughout the week, following 8 to 25 weeks of crystalline silica exposure. The levels of erythrocyte GPx activity and serum CC16 concentration in the ex-leave samples were then compared to those in the follow up samples to determine the effect of 8 to 25 weeks (2 to 6 months) of crystalline silica exposure. In addition, the levels of erythrocyte GPx activity and serum CC16 concentration in the Monday, Wednesday and Friday follow up samples were compared to each other to determine the effect of short term crystalline silica exposure.

4.2.3.1 Erythrocyte GPx

A significant decrease was found when comparing the mean GPx activity of the ex-leave samples with that of the Monday, Wednesday (pre- and post-shift) and Friday follow up samples. The statistical comparisons were paired so that ex-leave GPx activity levels were compared with the follow up GPx activity levels in the same individual. A significant decrease in GPx activity was also found when the mean GPx activity of the ex-leave samples was compared to that of the follow up groups combined (26.30 U/g Hb versus 22.48 U/g Hb, $p = 0.00$). Since the ex-leave samples were collected after a period of leave (usually four weeks), the results suggest that GPx levels decrease after two to six months

of chronic exposure to silica dust and then increase or even recover to normal during a period of leave.

When the mean GPx activity levels of the Monday (pre-shift), Wednesday (pre- or post-shift) and Friday (post-shift) follow up samples were compared to each other, no significant differences were found. It was therefore suggested that the GPx levels remain decreased throughout the week and do not recover over a weekend away from work.

The decrease in mean GPx activity, upon exposure to crystalline silica dust, found in this phase of the project therefore confirms the findings of the second phase of the project [Murray *et al*., 2006]. A significant decrease in GPx activity as a result of exposure to silica containing dust has also been demonstrated in studies of silicosis patients [Borm *et al*., 1986, Zhou *et al*., 1999], coal miners [Nadif *et al*., 2001], and cement workers [Orman *et al*., 2005]. In addition, significant decreases in GPx activity have been observed in other diseases or exposures in which oxidative stress has been implicated: such as COPD [Kluchova *et al*., 2007], asthma [Powell *et al*., 1994], and cadmium exposure [Babu *et al*., 2006].

A possible explanation for the observed decrease lies in the fact that the study participants were chronically exposed to respirable crystalline silica. Acute exposure to crystalline silica can cause an increase in erythrocyte GPx levels as a result of increased antioxidant action to counteract the oxidative stress induced by the crystalline silica particles [Vallyathan *et al.*, 1995]. On the other hand, chronic exposure to crystalline silica could cause the antioxidant system to be overwhelmed leading to a decrease in GPx activity.

4.2.3.2 Serum CC16

The mean CC16 concentration of the ex-leave samples was very similar to those of the pre-shift follow up samples collected on Monday and Wednesday but higher than those of the post-shift follow up samples collected on Wednesday and Friday. The decrease in CC16 concentration between the ex-leave and Wednesday (post-shift) samples was statistically significant while the decrease between the ex-leave and Friday samples was only just not significant (when Bonferonni adjusted alpha was used as the level of significance). Since the ex-leave samples were collected in the morning, the majority of the pre-shift follow up samples were collected in the morning and the majority of the post-shift follow up samples were collected in the afternoon (refer to figure 3.2), the results indicate a significant decrease between the CC16 levels in the morning and afternoon samples. This was confirmed by the comparison of the different follow up samples which showed a significant decrease between the Wednesday (pre- and post-shift) CC16 levels. Although a significant difference between the CC16 levels of the Monday (pre-shift) and Wednesday (post-shift), the Friday and Monday and the Friday and Wednesday (pre-shift) samples was also expected, the comparisons were unpaired and therefore not as accurate as a paired comparison.

One possible explanation for results observed in the current study is that CC16 levels fall quite rapidly during the period of a work shift but return to near normal levels during the 16 hours between shifts. This implies that CC16 levels may be a marker of recent silica exposure. Similar results were found in studies of workers exposed to welding fumes [Halatek *et al*., 2004] and nitric oxides [Halatek *et al*., 2005]. Both studies measured CC16 levels on a Monday morning, Monday afternoon (after an eight hour shift) and a Friday

afternoon. While the study of welders found a significant decrease between the Monday morning and the Monday afternoon and Friday afternoon samples, the study of nitric oxide exposed workers found a similar but non-significant result. From these results the authors suggested that depletion in CC16 levels as a result of exposure to welding fumes and nitric oxides can already be seen after a single eight hour shift.

On the other hand, the results of the current investigation may have been influenced by time-dependent diurnal variation in CC16 levels that have been observed in other studies. When Anderson *et al* [2007] and Helleday *et al* [2006] investigated diurnal variation of CC16 in serum samples they found a decrease in serum CC16 concentrations over the day with the highest CC16 concentration occurring in the morning sample and the lowest in the afternoon sample. In addition, Helleday *et al*. [2006] proposed the use of an equation ($\Delta CC16 = 0.032t^2 - 0.582t$) to describe the relationship between time of day and CC16 levels. Although the exact times at which specimens were collected were not recorded in this study; the Helleday equation could be used to estimate the expected change in CC16 levels due to diurnal variation. Assuming average shift duration of 8 hours, it was estimated that diurnal variation could cause CC16 levels to drop an average of 2.6 ng/ml between morning and afternoon samples. In the present study, the decreases in CC16 concentration observed between the ex-leave and follow up samples and between the pre- and post-shift follow up samples (table 3.2.4.2.b) were well within this expected fall of 2.61 ng/ml. Therefore, we cannot fully attribute the declines in CC16 levels observed in this study to the effects of crystalline silica exposure during the shift as these declines may also be due to diurnal variation.

In unpublished results of the Phase II study by Wilson *et al.* (Appendix A), the miners exposed to crystalline silica had a mean CC16 level of 5.6 ng/ml while the non-exposed controls had a mean CC16 level of 6.6 ng/ml. Since the majority of the Phase II specimens were collected at similar times, the decrease in CC16 levels (due to crystalline silica exposure) observed in that study were unlikely to be due entirely to diurnal variation.

It is therefore proposed that the current study results are compatible with the results reported in phase II of the project [Murray *et al*., 2006] and exposure to crystalline silica dust more than likely contributed to the reduction in CC16 levels observed.

A decrease in serum CC16 levels, as a result of crystalline silica exposure, has also been observed in studies involving silica-exposed mine workers and controls [Bernard *et al*., 1994a, Wang *et al*., 2007]. The authors proposed that the decrease in CC16 levels in serum were as a result of a decreased secretion or production of CC16 due to the destruction of Clara cells by silica. They suggested that the number or integrity of Clara cells might be compromised by 1) direct damage of Clara cells (which are sensitive to attack from pneumotoxic chemicals) by silica particles; 2) damage caused by cytotoxic mediators released from activated macrophages after they have engulfed silica particles and 3) prevention of the renewal of Clara cells (since Clara cells have been implicated in the renewal of bronchiolar epithelium). They concluded that changes in the serum CC16 concentration likely reflects early toxic effects of silica on Clara cells (before significant effects on the lungs can be detected by chest x-ray examination) and therefore that serum CC16 is a sensitive marker which might improve the ability to detect exposure to silica in the respiratory tract.

4.2.4 Effect of crystalline silica exposure on the change in biomarker levels

An attempt was then made to establish whether the observed decrease in GPx activity and CC16 concentration was correlated to either the job classification (an estimate of dust exposure level) of the volunteers or the duration of crystalline silica exposure (the duration between the collection of the ex-leave and follow up samples).

4.2.4.1 Erythrocyte GPx

It was determined that the decrease in GPx activity observed was not significantly related to the job classification (dust exposure level) or the duration of crystalline silica exposure. These results confirm that GPx may be a marker of early effect from crystalline silica exposure since the GPx levels decrease upon exposure to crystalline silica, following a period of leave, and do not change significantly as the duration of exposure increases.

4.2.4.2 Serum CC16

Similar to GPx activity, no significant correlation was found between the changes in CC16 concentration observed and the job classification (dust exposure level) of the volunteers or the crystalline silica exposure duration.

It is, however, important to treat these results with caution since the dust exposure level classifications were based on current job descriptions and were therefore not a reliable or accurate measure of the volunteers personal dust exposure. In addition, the duration of dust exposure observed in the current study was short and the number of observations in each duration group was small. Therefore, although the current investigation found no correlation between the change in GPx activity and CC16 concentration levels and the level or duration of dust exposure, it does not mean that a correlation does not exist.

4.3 Limitations of the study

The study participants were all volunteers and therefore no attempt should be made to generalise these findings to all miners, in particular in respect of establishing what constitutes normal baseline biomarkers levels for unexposed miners.

Three main limitations arose during the study:

Firstly, the original project plan involved the collection of the return to work specimens from each volunteer on a Monday (pre-shift), a Wednesday (pre- and post-shift), and a Friday (post-shift). This collection procedure proved difficult to achieve as volunteers were not willing to give four specimens in one week. The specimen collection procedure was then revised so that miners were asked whether they would be prepared to give two specimens (a pre- and post-shift specimen) on a Wednesday. Those who said they were not prepared to do so were allocated to a single specimen collected either pre-shift on a Monday or post-shift on a Friday by randomization. This adaptation of the study design due to the lack of participation by volunteers resulted in a reduction in the number of follow up samples obtained and therefore a decrease in the power of the study to detect differences.

Secondly, the dust exposure level classifications used were based on current job descriptions and were therefore an unreliable indication of the volunteer's personal dust exposure. It would have been better to determine the personal dust exposure level of the

volunteer by performing 8-hour full-shift personal dosimetry and measuring the amount of respirable crystalline silica by X-ray diffraction (XRD) spectroscopy.

Thirdly, since the levels of GPx (a general marker for oxidative stress) and CC16 (a general lung damage marker) are both affected by a number of toxicants they are not specific for crystalline silica exposure. Although in occupational exposure settings it is often possible to exclude other factors affecting the biomarker levels and therefore a relative specificity is possible [Aitio, 1999], the question remains whether the observed changes are only due to respirable crystalline silica exposure.

4.4 Future research & recommendations

1. The effect of diurnal variation of CC16 levels needs to be further investigated in silica-exposed subjects. Helleday *et al* developed a second-degree polynomial mathematical model (with the formula $\Delta CC16 = -0.582t + 0.032t^2$) to explain the decrease in serum CC16 due to diurnal variation. In future studies, the baseline morning CC16 concentration should be determined so that the model can be used to compensate for the diurnal variation and therefore any remaining decrease could be interpreted as an effect of the silica exposure. This factor was not taken into account in the design of the current study since the relevant paper had not yet been published. It would probably also be wise to investigate the possibility of diurnal variations of the GPx levels in non-exposed workers.

2. Although the GPx activity was found to decrease after two to six months of crystalline silica exposure, the point in time (after the return to work) at which the decrease in GPx activity actually occurs (a dose response curve) needs to be further investigated.

3. Although a great deal of research indicates that both GPx and CC16 could be markers of silica exposure it would be worthwhile, as a precautionary step, to examine this issue further by measuring these biomarkers in miners with no silica exposure. If this research finds no change in GPx or CC16 levels (i.e. confirm the specificity of these biomarkers for crystalline silica exposure) then the next question that needs to be answered is whether there exists a threshold of silica exposure at which the

biomarker levels fall, and whether this threshold (if it exists) correlates with dosimetric silica measurements.

4. The baseline levels of GPx and CC16 for individuals with no history of crystalline silica exposure needs to be determined. A study could be conducted in which new recruits (with no previous silica exposure) are followed up periodically over a number of years to establish the changes occur in the levels GPx and CC16.

5. It is possible that silica-induced damage to Clara cells may be permanent or that it might take longer than one month (the duration of the leave periods) for them to recover or for destroyed Clara cells to be replaced. It would therefore be interesting to study CC16 levels in ex-miners who have been away from the mines for longer than one month to determine whether there is any observable increase in CC16 levels with time, and, if so, how long this might take.

6. The drawing of blood, although not as invasive as BAL, is still considered an invasive procedure. Although not possible for GPx, it would be wise to investigate the possibility of using non-invasive methods (for example urine, breath or saliva) to assay CC16. This would facilitate routine sampling in human studies and may overcome ethical issues [Waterfield and Timbrell, 2000]. In fact, a correlation between serum CC16 and urine CC16 has been found [Andersson *et al*., 2007] and the use of urinary CC16 (U-CC16) as an alternative to serum CC16 as a biomarker of airway toxicity has been investigated [Timonen *et al*., 2004]. Therefore, further consideration and investigation into the use of urine CC16 as an alternative to serum CC16 in silica exposed subjects is recommended.

7. The inter-assay and intra-assay variations of the Glutathione Peroxidase Assay Kit and Human Clara Cell Protein ELISA Kit used in the current study should be investigated in healthy South African controls.

Chapter 5:

CONCLUSION

Despite dust control measures in place to limit the inhalation of crystalline silica, silicosis continues to pose a significant threat to the health of South African gold miners. It would therefore be invaluable if biomarkers of effect of crystalline silica exposure were identified and validated as they could be used to detect early adverse effects of silica exposure and evaluate the success of dust control methods.

An earlier phase of this project on biomarkers identified two potential biomarkers, erythrocyte glutathione peroxidase (GPx) and serum Clara cell protein 16 (CC16), which were significantly affected by crystalline silica exposure. This third phase of the project seemed to confirm the results of the earlier phase in that a decrease in the levels of GPx activity and CC16 concentration was observed following exposure to crystalline silica.

Regarding the levels of GPx, the results suggest that GPx levels decrease after two to six months of chronic exposure to crystalline silica dust and remain decreased (throughout the working week and over a weekend) and then increase or even recover to normal levels during a period of leave. It was therefore concluded that GPx activity levels rise and fall, in response to crystalline silica dust exposure, gradually and over periods of some time, possibly months.

The CC16 results were, however, less promising. It was found that, after two to six months of exposure to crystalline silica, there was a significant decrease in serum CC16

concentration on a Wednesday afternoon following an 8-hour shift and during the duration of a shift. In addition, there is the possibility that the observed changes were due to a time-dependent diurnal variation in the CC16 levels.

In conclusion, the results of the current phase warrant further research into the use of erythrocyte GPx and serum CC16 as biomarkers of early effect from crystalline silica exposure.

APPENDICES

Appendix A: Abstract for poster presented by Mrs K. Wilson at the 2008 EPICOH conference.

Predictors of Clara Cell Protein (CC16) level: an investigation of CC16 as a silica exposure biomarker.

Kerry Wilson, [1,2], Jill Murray[1,2], Brendan Girdler-Brown[3], Paul Borm[4], Ken Donaldson[5], Val Vallyathan[6], Vincent Castranova[6] and Mary Gulumian[1,2].

[1]National Institute for Occupational Health, [2]University of the Witwatersrand, [3]University of Pretoria, [4]Centre for life Science, [5]ElEGI, [6]NIOSH

Introduction: Clara cell protein (CC16) is a protein that is mainly produced by Clara cells in the respiratory epithelium. CC16 can be measured in bronchoalveolar lavage specimens, serum, urine and sputum. Clara cells and CC16 may play a role in protecting the lung from oxidative stress. A change in CC16 reflects chronic damage to Clara cells and may therefore be a useful biomarker for crystalline silica induced lung damage. This paper presents an analysis of predictors of CC16 in gold miners exposed to silica and unexposed controls.

Methods: 118 African male volunteers participated in this cross-sectional study. They were recruited from a gold mine (silica exposed, n= 64), a blood donor service (silica non-exposed n= 37) and a hospital HIV clinic (silica non-exposed n= 18). Data were collected on age, work history, smoking habits and HIV status. CC16 was assayed in the serum using an ELISA kit. Multiple linear regression was performed with post-regression residuals analysis to identify the factors that might explain the observed variation in CC16.

Results: Arithmetic mean (AM), geometric mean (GM) and geometric standard deviation (GSD) for the whole group CC16 level were 6.0ng/ml, 5.27ng/ml and 1.67ng/ml. AM, GM, and GSD for the silica exposed group were 5.56, 4.87 and 1.69, and for the unexposed group were 6.49, 5.77 and 1.64. The CC16 results were log-normally distributed. Tests for unpaired data with equal variance indicated a crude relationship between silica exposure and CC16 levels, $p = 0.074$; and smoking and CC16 levels, $p = 0.0001$. Multiple regression with the factors investigated in this study explained 20% of the variation (coefficient of determination $r = 0.19$) and the model identified exposure to silica as a significant risk for a lower level of CC16 ($p = 0.024$) smoking was no longer significant ($p = 0.082$).

Conclusion: CC16 levels were lowered in silica exposed subjects and were not affected by HIV status. CC16 is a suitable candidate for further research. Factors that further explain the variance in CC16 levels need to be identified before it can be confidently used as a biomarker for silica dust exposure.

Appendix B: Copy of KC4 report obtained from the assay of the GPx activity of samples 31 to 34.

KC4

Protocol Description

Name :	C:\KC4 3....eriments\Miner exp 2 row a,b prt.prt		
Reader :	ELx800	Wavelength :	340 nm
Lag Time :	00:00:00	Mode :	Normal
Kinetic Total Time	00:05:00	Kinetic Interval :	00:01:00
Plate Type :	96 WELL PLATE	Wells:	A1-B12

Assay Description

Data Name:	C:\KC4 3....eriments\Miners exp 2-row a,b.pla		
Reading Type:	Reader		
Reading Date:	15/03/2007 15:48:34	Report Date:	26/05/2008 13:07:40
Biomarker:	GPx	Prompt #4:	
Exp name:	Miner exp 2- row a,b	Prompt #5:	
lab temp:	28.6	Prompt #6:	
Comments:			

Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	SPL33 30.000	SPL32 30.000	SPL32 30.000	CTL1	BLK	SPL32 30.000	CTL1	BLK	SPL34 30.000	SPL34 30.000	SPL31 30.000	SPL33 30.000
B	SPL31 30.000	CTL1	BLK	SPL31 30.000	SPL33 30.000	SPL34 30.000						
C												
D												
E												
F												
G												
H												

KC4

Protocol Name: C:\KC4 ...iments\Miner exp 2 row a,b prt.prt
Data Name: C:\KC4 3...riments\Miners exp 2-row a,b.pla
Reading Date/Time: 15/03/2007 15:48:34 Report Date/Time: 26/05/2008 13:07:40

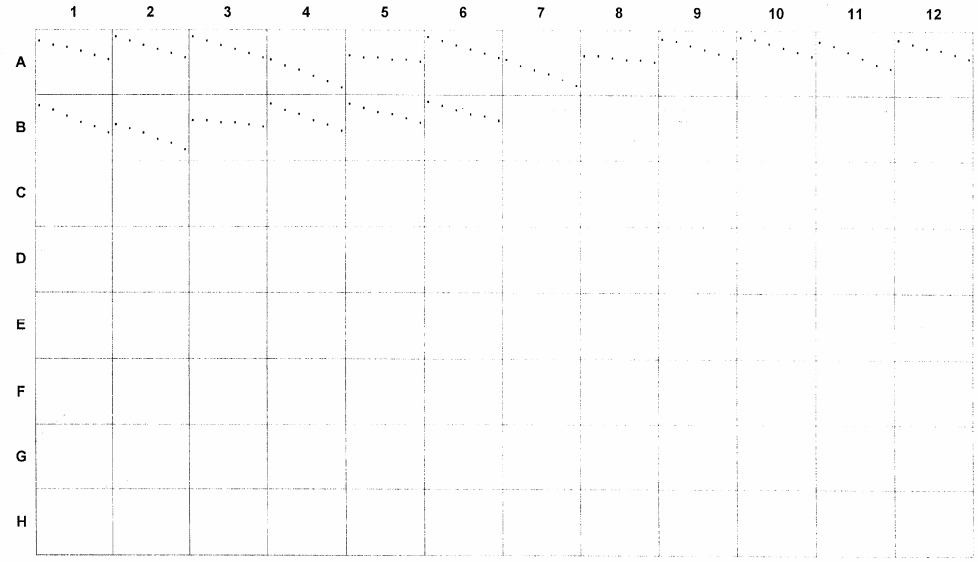
TABLE M 340

Time	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
00:00:00	0.531	0.561	0.564	0.369	0.409	0.561	0.369	0.410	0.544	0.564	0.525	0.536
00:01:00	0.498	0.527	0.529	0.328	0.398	0.530	0.333	0.401	0.519	0.535	0.484	0.509
00:02:00	0.467	0.494	0.494	0.286	0.388	0.498	0.294	0.390	0.489	0.503	0.439	0.480
00:03:00	0.436	0.461	0.459	0.242	0.377	0.465	0.253	0.380	0.459	0.471	0.394	0.450
00:04:00	0.405	0.427	0.423	0.197	0.367	0.432	0.210	0.369	0.428	0.438	0.350	0.420
00:05:00	0.374	0.393	0.388	0.153	0.356	0.399	0.167	0.358	0.397	0.406	0.308	0.387

TABLE M 340

Time	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
00:00:00	0.530	0.378	0.414	0.540	0.538	0.558	0.181	0.217	0.213	0.068	0.066	0.067
00:01:00	0.487	0.344	0.405	0.497	0.508	0.525	0.181	0.217	0.213	0.068	0.066	0.068
00:02:00	0.442	0.307	0.395	0.454	0.481	0.495	0.181	0.217	0.213	0.068	0.066	0.068
00:03:00	0.398	0.267	0.384	0.411	0.453	0.465	0.181	0.217	0.212	0.068	0.066	0.067
00:04:00	0.354	0.225	0.372	0.368	0.424	0.436	0.181	0.217	0.212	0.068	0.066	0.067
00:05:00	0.311	0.182	0.361	0.326	0.396	0.406	0.180	0.217	0.212	0.068	0.066	0.067

Kinetic curves



Appendix C: Analysis page from the Cayman Chemical Company workbook entitled "GSHasesTriple"

The values in the cells are taken from the KC4 results printout (Appendix B) from the analysis of the erythrocyte lysate samples of volunteer 31 to 34

Glutathione Peroxidase

ASSAY 1

LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK											
B	CTRL	1:1										
C	Smpl31	1:30										
D	Smp32	1:30										
E	Smpl33	1:30										
F	Smpl34	1:30										
G												
H												

TIME 0:00

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.4090	0.4100	0.414									
B	0.3690	0.3690	0.378									
C	0.5250	0.5300	0.54									
D	0.5610	0.5640	0.561									
E	0.5310	0.5360	0.538									
F	0.5440	0.5640	0.558									
G												
H												

TIME 1:00

A	0.3980	0.4010	0.405									
B	0.3280	0.3330	0.344									
C	0.4840	0.4870	0.497									
D	0.5270	0.5290	0.53									
E	0.4980	0.5090	0.508									
F	0.5190	0.5350	0.525									

G						
H						
TIME 2:00						
A	0.3880	0.3900	0.395			
B	0.2860	0.2940	0.307			
C	0.4390	0.4420	0.454			
D	0.4940	0.4940	0.498			
E	0.4670	0.4800	0.481			
F	0.4890	0.5030	0.495			
G						
H						
TIME 3:00						
A	0.3770	0.3800	0.384			
B	0.2420	0.2530	0.225			
C	0.3940	0.3980	0.411			
D	0.4610	0.4590	0.465			
E	0.4360	0.4500	0.453			
F	0.4590	0.4710	0.465			
G						
H						
TIME 4:00						
A	0.3670	0.3690	0.372			
B	0.1970	0.2100	0.225			
C	0.3500	0.3540	0.368			
D	0.4270	0.4230	0.432			
E	0.4050	0.4200	0.424			
F	0.4280	0.4380	0.436			
G						
H						
TIME 5:00						
A	0.3560	0.3580	0.361			
B	0.1530	0.1670	0.182			
C	0.3080	0.3110	0.326			
D	0.3930	0.3880	0.356			
E	0.3740	0.3870	0.396			
F	0.3970	0.4060	0.406			

G				
H				

Time	BLANK	CTRL	Smpl31	Smp32	Smpl33	Smpl34						
0	0.4110	0.3720	0.5317	0.5620	0.5350	0.5553						
1	0.4013	0.3350	0.4893	0.5287	0.4980	0.5263						
2	0.3910	0.2957	0.4450	0.4953	0.4760	0.4957						
3	0.3803	0.2400	0.4010	0.4617	0.4463	0.4650						
4	0.3693	0.2107	0.3573	0.4273	0.4163	0.4340						
5	0.3583	0.1673	0.3150	0.3790	0.3857	0.4030						
y-int=	0.4117	0.3738	0.5320	0.5651	0.5325	0.5563						
slope=	-0.0106	-0.0415	-0.0435	-0.0358	-0.0292	-0.0306						
R^2=	0.9994	0.9957	0.9999	0.9952	0.9973	0.9999						
Rxn Rate=	0.0105	0.0409	0.0328	0.0261	0.0193	0.0199						

Time												
0												
1												
2												
3												
4												
5												
y-int=												
slope=												
R^2=												
Rxn Rate=												

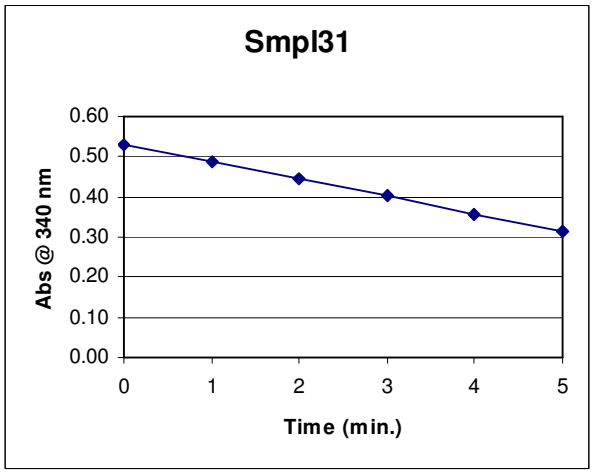
RESULTS	GPx	Dilution	Activity	GPx	Dilution	Activity
	Smpl31	30	2506.17			
	Smp32	30	1991.69			
	Smpl33	30	1477.21			
	Smpl34	30	1523.06			

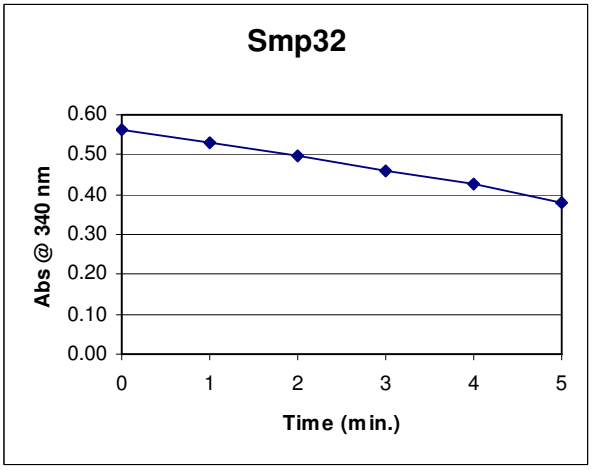
			Ctrl	1	104.25
			Activity in nmol/min/ml		

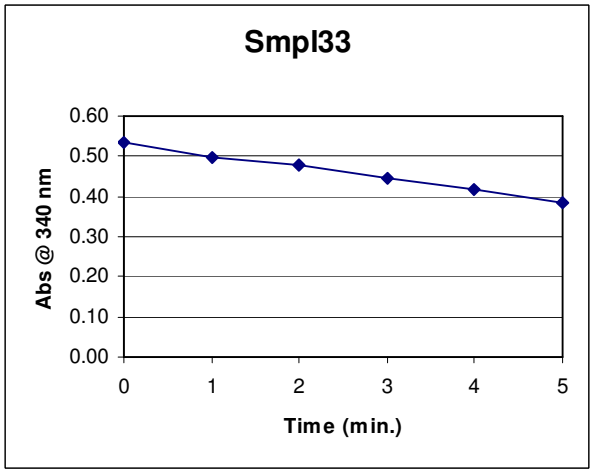
FILENAME	GSHasesTriple - M31-Al to M34-Al
VERSION	2004D314

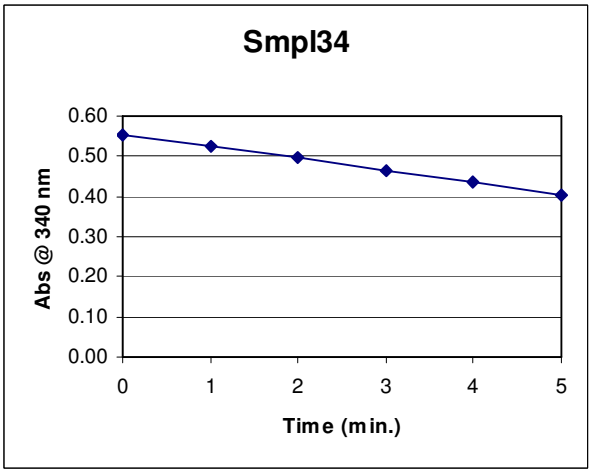
Appendix D: Graph page from the Cayman Chemical Company workbook entitled "GSHasesTriple"

The graphs were automatically constructed by the workbook using the values entered into the Analysis page (see Appendix C) for volunteer 31 to 34.









Appendix E: Certificate of analysis for CC16

BioVendor Laboratory Medicine, Inc.
CTPark Modrice, Evropska 873, 664 42 Modrice, Czech Republic

Phone: +420-5-49124185 e-mail: info@biovendor.com
Fax: +420-5-49211460 http://www.biovendor.com

CERTIFICATE OF ANALYSIS

(Quality Control Data Sheet)

Product name: **Human Clara Cell Protein ELISA**

Cat. No.: RD191022200
Lot No.: **RD-1276R1**
Date of Control: 30.3.2007
Date of Expiry: 2007-08

Kit Components:

Cat. No.	*Item*	*Lot No.*	*Volume (Quantity)*
C071111	Antibody-Coated Microtiter Plate	07-029	1 pc / 96 wells
C072511	Biotin Labelled Antibody	07-030P1	13 ml
C072351	Streptavidin-HRP Conjugate	07-034	13 ml
C073141	Human Clara Cell Protein Master Calibrator	07-001	1 vial
C074112	Quality Control High	07-032	0.20 ml
C074212	Quality Control Low	07-033	0.20 ml
C005114	Dilution Buffer	00-209	1 x 20 ml
C006121	Wash Solution Concentrate (10x)	00-182	100 ml
C007111	Substrate Solution	00-201	13 ml
C008111	Stop Solution	00-207	13 ml

ELISA Results:

Standards / Quality Controls	*A_{450} Average*	*Concentration Expected (ng/ml)*	*Concentration Obtained (ng/ml)*
Standard 1	3.245	100	97.34
Standard 2	2.482	40	41.64
Standard 3	1.637	20	19.60
Standard 4	0.987	10	9.92
Standard 5	0.569	5	5.16
Standard 6	0.264	2	1.95
Blank	0.011	-	-
QC High	**1.913**	**23.09 (18.5-27.7)**	**25.04**
QC Low	**0.546**	**4.46 (3.6-5.3)**	**4.90**
IQC 1*	0.531	4.24 (3.4-5.1)	4.74
IQC 2*	0.762	6.62 (5.3-7.9)	7.27
IQC 3*	1.375	13.38 (10.7-16.1)	15.22
IQC 4*	1.623	17.85 (14.3-21.4)	19.29

*) IQCs are internal Quality Controls, they are not included in the kit.

Name: Eva Dočkalová Sign.: Eva Dočkalová

EN ISO 9001, EN ISO 13485 TÜV Certified No.:42/07

Appendix F: Copy of KC4 report obtained from the assay of the CC16 concentration of samples 1 to 26

KC4

Protocol Description

Name :	C:\KC4...s Exp\Miner exp 1 (m1 to m26-AL).prt		
Reader :	ELx800	Wavelength :	450 nm
Lag Time :	00:00:00	Mode :	Normal
Plate Type :	96 WELL PLATE	Wells:	A1-H12

Assay Description

Data Name:	C:\KC... Exp\Miner Exp 1 (M1 to M26-AL).pla		
Reading Type:	Reader		
Reading Date:	13/04/2007 18:00:05	Report Date:	26/05/2008 13:05:43
Biomarker:	CC16	Prompt #4:	
Exp name:	Miner Exp 2 (M1 to M26-AL)	Prompt #5:	
Lab temp:	25.7	Prompt #6:	
Comments:	10 min substrate incubation		

Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	SPL10	SPL26	SPL5	SPL15	SPL18	SPL17	SPL13	SPL15	SPL7	SPL25	SPL4	SPL3
B	STD6 2.0000	SPL12	SPL11	SPL21	SPL24	SPL16	SPL24	SPL26	CTL1	SPL25	SPL22	SPL2
C	SPL19	SPL6	SPL18	SPL8	SPL5	SPL16	SPL9	SPL25	SPL11	STD5 5.0000	SPL20	SPL7
D	SPL19	STD1 100.00	STD3 20.000	SPL11	SPL14	SPL2	SPL24	SPL7	SPL20	SPL21	SPL1	SPL3
E	SPL12	SPL20	SPL12	SPL13	SPL15	CTL1	STD5 5.0000	SPL23	SPL21	SPL6	CTL2	SPL14
F	STD2 40.000	SPL10	SPL9	SPL16	SPL4	SPL23	SPL14	CTL2	BLK	SPL26	SPL8	BLK
G	STD4 10.000	SPL4	SPL1	SPL22	STD4 10.000	STD2 40.000	SPL22	SPL2	SPL1	SPL9	STD3 20.000	STD6 2.0000
H	SPL17	SPL3	SPL10	SPL23	SPL13	SPL19	SPL5	SPL6	STD1 100.00	SPL18	SPL17	SPL8

KC4

Protocol Name: C:\KC... Exp\Miner exp 1 (m1 to m26-AL).prt
Data Name: C:\KC... Exp\Miner Exp 1 (M1 to M26-AL).pla
Reading Date/Time: 13/04/2007 18:00:05 Report Date/Time: 26/05/2008 13:05:43

M 450

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.840	0.644	0.550	0.289	0.770	0.903	0.918	0.324	0.647	0.540	0.756	0.616
B	0.256	1.332	0.476	0.666	0.309	0.614	0.270	0.642	0.420	0.530	0.997	0.287
C	0.848	0.525	1.086	0.895	0.534	0.391	0.776	0.416	0.366	0.545	0.472	0.663
D	0.758	2.890	1.582	0.371	0.712	0.345	0.361	0.737	0.521	0.636	0.485	0.645
E	1.311	0.398	1.359	0.912	0.259	0.486	0.555	0.261	0.736	0.714	1.551	0.746
F	2.202	0.941	0.803	0.521	0.599	0.288	0.761	1.650	0.042	0.549	0.751	0.043
G	0.937	0.470	0.636	1.004	1.039	2.329	1.033	0.353	0.529	0.812	1.400	0.205
H	0.764	0.843	1.559	0.257	0.763	0.889	0.751	0.667	2.806	0.726	0.583	0.728

M 450 Corr.

Blank : 0.04250

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.797	0.602	0.508	0.247	0.728	0.861	0.876	0.282	0.605	0.498	0.714	0.574
B	0.214	1.290	0.434	0.624	0.267	0.572	0.228	0.600	0.378	0.488	0.955	0.245
C	0.805	0.483	1.044	0.853	0.492	0.349	0.734	0.374	0.324	0.503	0.429	0.621
D	0.716	2.848	1.540	0.329	0.669	0.302	0.319	0.695	0.479	0.594	0.443	0.603
E	1.269	0.356	1.317	0.870	0.217	0.444	0.513	0.219	0.694	0.671	1.509	0.704
F	2.160	0.898	0.761	0.479	0.556	0.246	0.719	1.607	-0.000	0.507	0.709	0.001
G	0.895	0.427	0.594	0.962	0.996	2.287	0.990	0.311	0.487	0.770	1.357	0.162
H	0.722	0.800	1.517	0.215	0.721	0.847	0.709	0.625	2.764	0.684	0.540	0.686

Concentrations

	1	2	3	4	5	6	7	8	9	10	11	12
A	8.5100	6.2024	5.1672	2.4455	7.6597	9.3039	9.4972	2.8033	6.2361	5.0593	7.4933	5.8899
B	2.1079	15.658	4.3774	6.4507	2.6499	5.8677	2.2512	6.1800	3.7915	4.9518	10.544	2.4251
C	8.6093	4.8982	11.788	9.2015	4.9947	3.4913	7.7313	3.7500	3.2338	5.1132	4.3353	6.4167
D	7.5170	105.66	20.452	3.2852	6.9779	3.0183	3.1824	7.2694	4.8553	6.1127	4.4725	6.2137
E	15.299	3.5636	16.128	9.4196	2.1386	4.4830	5.2213	2.1591	7.2576	7.0011	19.799	7.3752
F	39.469	9.7969	8.0566	4.8553	5.7019	2.4353	7.5526	21.955	*<1.6447*	5.1564	7.4342	*<1.6447*
G	9.7445	4.3142	6.1127	10.640	11.122	45.724	11.039	3.1003	4.9411	8.1661	16.862	*<1.6447*
H	7.5882	8.5472	19.966	2.1182	7.5763	9.1250	7.4342	6.4620	90.093	7.1407	5.5262	7.1640

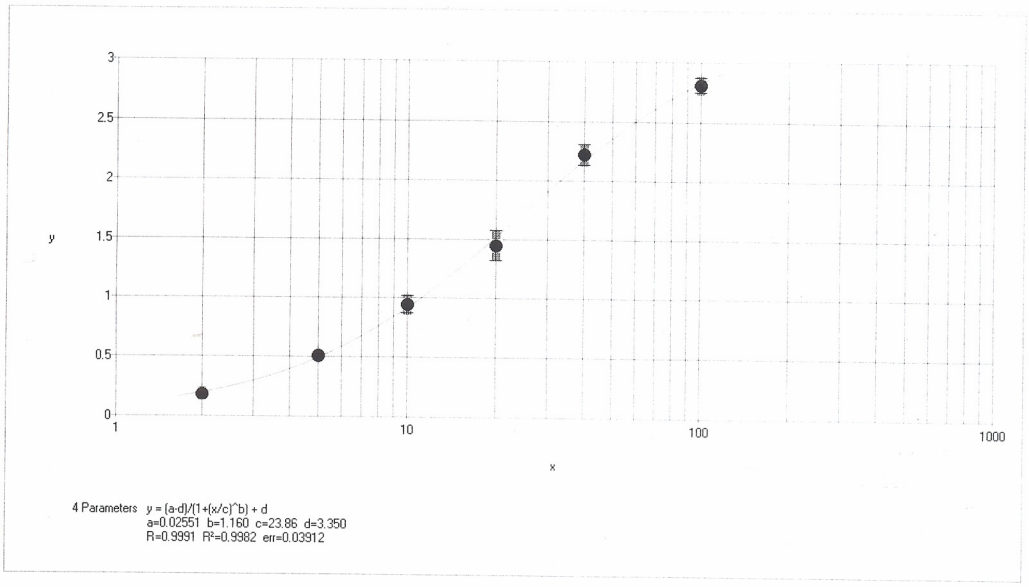
KC4

Protocol Name: C:\KC... Exp\Miner exp 1 (m1 to m26-AL).prt

Data Name: C:\KC... Exp\Miner Exp 1 (M1 to M26-AL).pla

Reading Date/Time: 13/04/2007 18:00:05 Report Date/Time: 26/05/2008 13:05:43

STANDARD CURVE



Appendix G: Information sheet for NIOH volunteers

MHSC

MINE HEALTH *AND* SAFETY COUNCIL

and the

NATIONAL INSTITUTE *for* OCCUPATIONAL HEALTH

NIOH

Subject Information Sheet for volunteers

Hello, my name is Kerry Makinson, I am a researcher at the National Institute of Occupational Health (NIOH), 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 be used to see if silica dust levels at the gold mines are a danger to workers.
However, before we conduct experiments on the miners' blood we need to determine the best conditions for handling, storage and assaying of the samples. In order to do this we need to do experiments with blood from volunteers.

If you decide to participate, I would like to explain to you what will happen. First you need to sign a consent form. Then we will need to draw 15ml of blood (3 teaspoons) from your arm. This will not take long. After we take your blood it will be kept frozen at the National Institute for Occupational Health and will only be used for these optimization experiments. It will not be used for any other purpose without your written permission.

Your personal information and results will be kept confidential and will not be shown to any other person.

If you decide not to take part in this study it will not affect you or your job in any way. The only risk to you from taking part in this study will be a little discomfort when the blood specimen is taken and for a short time afterwards.

Thank you
Kerry Makinson

Appendix H: Consent form for NIOH volunteers

MINE HEALTH *AND* SAFETY COUNCIL

and the

NATIONAL INSTITUTE *for* OCCUPATIONAL HEALTH

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.

I _____ agree to take part in the study. I agree to give 15ml of blood. I understand that all individual and personal information will be kept confidential.

Signed_____Date_____

Appendix I: Standard Operating Procedure (SOP) for the analysis of erythrocyte glutathione peroxidase activity using the Cayman Chemical Glutathione Peroxidase Assay Kit

Objective: To describe the Standard Operating Procedure (SOP) for the collection, handling and storage of blood samples from volunteer miners and for assaying the amount of Glutathione Peroxidase (GPx) activity in the samples using the Cayman Chemical Glutathione Peroxidase Assay Kit

Principle: Glutathione peroxidase (GPx) catalyzes the reduction of organic hydroperoxides (including hydrogen peroxide) by reduced glutathione and functions to protect the cell from oxidative damage [Ursini *et al*., 1985]. The Cayman Chemical Glutathione Peroxidase Assay Kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH [Ursini *et al*., 1985]:

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{R-O-H} + \text{GSSG} + \text{H}_2\text{O}$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decease in the A340 is directly proportional to the GPx activity in the sample [Paglia and Valentine, 1967]. The Cayman GPx Assay Kit can be used to measure all of the glutathione-dependent peroxidases in plasma, erythrocyte lysates, tissue homogenate, and cell lysates [Cayman Chemical Company, 2004].

Sensitivity: The range of the assay is limited only by the accuracy of the absorbance measurement. Most plate readers are linear to an absorbance of 1.2. Samples containing GPx activity between 50-344 nmol/min/ml can be assayed without further dilution or concentration. This GPx activity is equivalent to an absorbance decrease of 0.02 to 0.135 per minute. [Cayman Chemical Company, 2004].

Precision: When a series of seventy-seven GPx measurements were performed on the same day by the manufacturer, the intra-assay coefficient of variation was 5.7%. When a series of seventy-seven GPx measurements were performed on five different days under the same experimental conditions (by the manufacturer), the inter-assay coefficient of variation was 7.2%. [Cayman Chemical Company, 2004].

Interferences:
1. Samples that have a high intrinsic absorbance at 340 nm may exceed the absorbance maximum of the plate reader. Therefore, samples with an initial absorbance >1.2 should be diluted with Sample buffer until the absorbance is lowered.
2. Samples containing high levels of GSSG or NADPH consuming enzymes will cause the GPx levels to be overestimated. A blank containing no cumene hydroperoxide can be performed to assess non-specific oxidation of NADPH.
3. The following reagents have been shown to interfere with the assay: Chymostatin (a protease inhibitor) and 2-Mercaptoethanol. [Cayman Chemical Company, 2004].

Storage and Expiration of Kit: The Kit is stored at -20°C. Under these conditions the Kit is stable until the expiration date (indicated on the label of the box).

Primary sample: Human erythrocyte lysate

Container of storage: 1.0 ml CryoTubes

Equipment needed:

1. A microplate reader with a 340 nm filter.
2. Adjustable pipettes to deliver 10-1000 µl and disposable tips.
3. A centrifuge capable of 1300 *x g* and 10 000 *x g*.
4. Microcentrifuge tubes for diluting the samples.
5. Multichannel pipette to deliver 20-100 µl.

Reagents needed:

1. The Cayman Chemical Glutathione Peroxidase Assay Kit.
2. HPLC-grade water.

Safety Precautions: See attached MSDS.

Quality control: A Glutathione Peroxidase control (provided in the kit) is run in triplicate with the samples.

Supplier Details:

The Cayman Chemical Glutathione Peroxidase Assay Kit (Catalogue number 703102).
Cayman Chemical Company
Ann Arbor,
Michigan,
United States of America
Customer Service Department: (734) 975-3999 (USA)
Technical Service Staff: (734) 975-3888 (USA) or techserv@caymanchem.com

Local Supplier:

Calicom Trading 262 (PTY) LTD
Tel: +27 11 472-7300
Email: keith@katmedical.com

A. SOP FOR THE COLLECTION OF BLOOD SAMPLES FROM VOLUNTEER MINERS

1. Draw venous blood into two 6 ml BD Vacutainer™ tubes containing lithium heparinate as an anticoagulant.

2. The blood should be drawn and handled at room temperature (25^{o}C).

B. SOP FOR THE SEPARATION OF THE COLLECTED BLOOD SAMPLES

1. The bloods should be separated within 2 hours of being collected.

2. To separate the blood, centrifuge the tube of blood at 1300 *x g* for 10 minutes at room temperature.

3. Remove the upper plasma layer and discard.

4. Remove the buffy coat (leukocytes) above the packed erythrocytes.

5. Pipette 0.3 ml of erythrocytes into a 1.5 ml Eppendorf tube, in triplicate.

6. Add 1.2 ml of ice-cold deionised water to each aliquot to lyse the erythrocytes.

7. Close the lids of the Eppendorf tubes and gently invert a few times to mix.

8. Centrifuge the Eppendorf tubes at 10 000 *x g* for 15 minutes at 4^{o}C.

9. During centrifugation, the ruptured erythrocyte membranes pellet to the bottom of the Eppendorf tubes and the contents of the erythrocytes (including the enzyme GPx) are suspended in the supernatant (known as the erythrocyte lysate).

10. Following centrifugation, remove 1 ml of the erythrocyte lysate from each Eppendorf tube and place it in separate 1.5 ml CryoTubes (that are clearly labelled).

C. SOP FOR THE STORAGE OF THE ERYTHROCYTE LYSATE

1. Ensure that the lids of the CryoTubes are securely fastened.

2. Shock freeze the CryoTubes by using a pair of tongs to lower the CryoTubes into a Dewar tank of liquid nitrogen.

3. Transport the frozen CryoTubes back to the National Institute for Occupational Health (NIOH) in a cooler box containing enough ice packs to ensure that the CryoTubes remain frozen.

4. As soon as the CryoTubes arrive at the NIOH transfer them to a clearly labelled container.

5. Place the container in the -80^{o}C Freezer.

6. Record the sample in the Sample Log Book.

D. SOP FOR OPERATING THE MICROPLATE READER AND ACCOMANYING SOFTWARE

The microplate reader needs to be set up before beginning the assay as the absorbance of the assay microplate needs to be read as soon as the initiating reagent (Cumene Hydroperoxide) is added.

1. To measure the absorbance in the wells of the microplate during the assay, an ELx800 Automated Microplate reader (Bio-Tek) is used and the accompanying software is KC4^{TM}v3.4 (Bio-Tek).

2. Plug in the microplate reader and switch it on (the ON/OFF switch is located on the right hand side of the machine).

3. The reader will go through an automatic self-test to ensure it is functioning properly.

4. Wipe down the surface of the microplate reader with a damp paper towel.

5. Connect the laptop loaded with the KC4 software to the microplate reader and to the printer.

6. Switch on the laptop and open the KC4 program.

7. Click on the '**Protocol'** tab and select the '**Wizard**' option

8. In the '**Plate settings**' box select the '**Description**' box.

9. In the '**Plate description parameters'** box fill in the necessary prompts (for example the experiment name or the name of the experimenter). These are optional.

10. Click '**OK**'

11. In the '**Plate settings**' box select the '**Reading Parameters**' box

12. Select the '**Kinetic**' reading type.

13. Click on the small box labelled '**Kinetic**'. A box will appear. Fill in a Run time of 5 minutes (00:05:00) and an Interval of 1 minute (00:01:00). Click "**OK**'

14. Select the '**Meas. Filter'**. Click the down arrow on the side of the white box next to the Meas Filter option. Select a wavelength of 340 nm.

15. Click the down arrow on the side of the white box next to the Plate type and select a '96 Well Plate'.

16. Fill in the wells that are being used on the plate in the '**First well**' and '**Last well**' boxes (For example if the whole plate is being used the first well is A1 and the last well is H12).

17. Click "**OK**'

18. In the '**Plate settings**' box select the '**Layout**' box

19. A layout for a 96 Well Plate will appear in a box labelled '**Plate layout**'. Select the type of sample being assayed (for example a blank (background), control or sample) by clicking the down arrow on the side of the white box next to the '**Type**' option. Once you have selected the type of sample, click on the well that sample will occupy in the microplate. The name of the sample will then appear in that well. (For example, select the Blank as the type of sample and click on the well labelled A1. The word Blank will then appear in well A1).

20. Once the plate layout has been filled in click '**OK**'

21. Select the '**Output**' tab.

22. In the '**Output**' box select the '**Report**' box

23. In the '**Available data**' box click on the name of the type of data that is wanted in the final report of the results (for example the template). Click on the small box labelled '**Add**'. The name of the data that was selected will then appear in the '**Selected data**' box. Continue this process until all the data that is wanted in the report is listed in the '**Selected data**' box.(If a data type is added that is no longer wanted click on the small box labelled 'Remove' and the data name will be removed from the 'Selected data' box).

24. The only data type that must be selected is '**M 340**' (The absorbance values obtained for the samples at 340 nm). In order to add this data type one first needs to select the '**Table**' format. The name of the first and last wells of the plate that need to be assayed must also be filled in the white boxes next to the '**From**' and '**To**' kinetic readings.

25. Once all the data needed has been selected click '**OK**'

26. Click '**OK**'

27. Save the protocol under a chosen name.

28. Select the '**Read**' Box in the tool bar.

29. Fill in the necessary prompts and any comments.

30. Do not click on the '**Start Reading**' box. This is only done once the plate is ready to be read.

31. Perform the assay as described below.

E. SOP FOR ASSAYING THE AMOUNT OF GPx ACTIVITY IN THE ERYTHROCYTE LYSATE

1. The erythrocyte lysate samples (stored in CryoTubes in the -80^{o}C freezer) should be assayed no later than 9 weeks after they were collected.

2. The amount of GPx activity in the RBC lysate samples will be assayed using a Glutathione Peroxidase Assay Kit purchased from Cayman Chemical Company (USA).

3. When not in use, store the kit at -20^{0}C in a freezer.

4. To use the kit, remove from the fridge and allow the contents of the kit to come to room temperature (25^{o}C).

5. To prepare the 1 X assay buffer, pipette 2 ml of 10 X Assay buffer concentrate (provided in the kit) into a clearly labelled sterile 50 ml Falcon tube. Add 18 ml of HPLC-grade water to the assay buffer and mix gently. The diluted assay buffer is stable for 2 months if stored at 4^{o}C.

6. To prepare the 1 X sample buffer, pipette 2 ml of 10 X Sample buffer concentrate (provided in the kit) into a clearly labelled sterile 50 ml Falcon tube. Add 18 ml of HPLC-grade water to the sample buffer and mix gently. The diluted sample buffer is stable for 1 month if stored at 4^{o}C.

7. To prepare the Co-substrate mixture (provided in the kit); add 2 ml of HPLC-grade water to the vial provided and vortex gently to mix. Three vials of Co-substrate mixture are provided in the kit and each reconstituted vial is enough for 40 wells. It is therefore only necessary to reconstitute the number of vials needed to supply the number of wells that will be used. The reconstituted Co-substrate mixture is stable for 2 days if stored at 4^0C.

8. Remove the erythrocyte lysate samples that will be assayed from the -80^{o}C freezer and allow them to thaw at room temperature (25^{o}C).

9. In 1.5 ml Eppendorf tubes, pipette the amount of 1x Sample buffer necessary to dilute the erythrocyte lysate sample 30 X. If the erythrocyte lysate sample is being assayed in singlet pipette 145 µl of 1 X Sample buffer into the Eppendorf tube. If the sample is being assayed in duplicate pipette 290 µl of 1 X Sample buffer into the Eppendorf tube *etc*.

10. Ensure that 1 X Sample buffer has been added to all the Eppendorf tubes needed before any erythrocyte lysate sample is added.

11. Gently tap the CryoTubes containing the thawed erythrocyte lysate sample to mix.

12. Pipette 5 µl of the mixed RBC lysate sample into the 1x sample buffer in the Eppendorf tube and aspirate to mix.

13. Repeat for all the samples using a clean pipette tip for each sample.

14. To prepare the Glutathione Peroxidase control (provided in the kit), add 10 µl of GPx control to 490 µl of 1 X sample buffer in a 1.5 ml Eppendorf tube and aspirate to mix. Keep the GPx control on ice (it is stable for 4 hours).

15. A microplate containing 96 wells is provided in the kit. In each plate there are 12 columns (Columns 1 to 12) each consisting of 8 wells (Rows A to H).

16. Pipette 120 µl of 1 X assay buffer into 3 wells of the microplate (if using the whole kit use wells A1, A2 and A3). These wells are the background wells (non-enzymatic wells).

17. Pipette 100 µl of 1 X assay buffer into 3 wells of the microplate (if using the whole kit use wells A4, A5 and A6). These wells are the positive control wells.

18. Pipette 100 µl of 1 X assay buffer into the number of wells needed for the number of samples that will be assayed (For example if a total of 30 samples are being assayed in singlet, add 100 µl of 1 X assay buffer into 30 wells). These wells are the sample wells.

19. Add 50 µl reconstituted Co-substrate mixture into each of the wells being used.

20. Gently tap the Eppendorf tube containing the diluted GPx control (positive control) to mix. Pipette 20 µl of GPx control into each of the 3 positive control wells.

21. For each sample, gently tap the Eppendorf tube containing the diluted erythrocyte lysate sample to mix. Pipette 20 µl of each diluted sample into one of the sample wells.

22. Carefully place the microplate into the designated holder in the microplate reader.

23. Add 20 µl of Cumene Hydroperoxide (provided in the kit) to each well. The Cumene Hydroperoxide initiates the reaction.

24. If more than three rows of the plate are being used, only add 20 µl Cumene Hydroperoxide to two rows of the microplate and then read the absorbance of those two rows. Repeat for the next two rows until all the rows with sample have been read.

25. Carefully shake the microplate for a few seconds to mix the contents in the wells.

26. Select the '**Start Reading**' box that is displayed in the KC4 program.

27. The absorbance at 340 nm is automatically measured five times at one minute intervals in each of the microplate wells being used.

28. Once the microplate reader has finished measuring the absorbance of the plate, click on the '**Save**' box in the toolbar and save the report under a chosen name. If one requires the report to be printed, click on the '**Print**' box in the toolbar.

29. Remove the microplate from the microplate reader

30. Wipe down the microplate reader with a damp paper towel.

31. Switch off the microplate reader and unplug.

F. SOP FOR CALCULATING THE GPx ACTIVITY FROM THE ABSORBANCE DATA OBTAINED BY THE ASSAY

A. Electronically using a worksheet

Cayman Chemical Company supplies a tool on their website that allows one to calculate the GPx activity from the absorbance values obtained.

1. Open the website for Cayman Chemical Company: **www.caymanchem.com**

2. Under the heading 'Products' (on the right hand side of the page), select 'Analysis tools'.

3. Under the heading 'Non-EIA tools', select 'Glutathione Enzyme (GSHases)'.

4. Under the heading 'Download', click on 'GSHases Triple' to start downloading.

5. Save the file in a chosen location.

6. Open the file.

7. In the worksheet labelled 'Analysis' fill in the necessary information about the sample number and dilution (a 1:30 dilution was used) in the designated boxes.

8. Also in the 'Analysis' worksheet, fill in the absorbance values obtained for the background, positive control and RBC lysate samples at times 0 to 5 minutes as indicated.

9. The GPx activity is automatically calculated for the background, positive control and erythrocyte lysate samples and displayed in a table labelled 'Results' at the bottom of the worksheet.

10. The corresponding graphs of absorbance versus time for each sample are also automatically constructed and illustrated in the worksheet labelled 'Graphs'.

11. Save the workbook under a chosen name.

B. Manually using formulas

1. Determine the change in absorbance (ΔA_{340}) per minute by:
    a. Plotting the absorbance values as a function of time to obtain the slope (rate of the linear portion of the curve – *or-*

    b. Selecting two points on the linear portion of the curve and determining the change in absorbance during that time using the following equation:

    ΔA_{340}/ min = (A340 (Time 2) – A340 (Time 1)) / (Time 2 (min) – Time 1 (min))

    *(*Method b is the most preferred, with Time 2 being 5 minutes and Time 1 being 0 minutes)

2. Determine the rate of ΔA_{340}/min for the background wells and subtract this rate from the rate of the sample wells.

3. Use the following formula to calculate the GPx activity:

GPx Activity = ((ΔA_{340}/ min)/ 0.00373 μM^{-1}) x (0.19 ml/0.02 ml) x Sample Dilution = nmol/min/ml

Where:
0. 00373 μM^{-1} is the NADPH extinction coefficient
0.19 ml is the final volume of the assay in each well
0.02 ml is the volume of sample in the well
The sample dilution is 30 X

References:

Cayman Chemical Company. (2004). Glutathione Peroxidase Assay Kit Instruction Insert, Cayman Chemical Company, Ann Arbor, USA. pg 1-22, 2004.

Paglia, D.E. and Valentine, W.N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. (1967). *Journal of Laboratory and Clinical Medicine*, **70**, 158-169.

Ursini, F., Maiorino, M., and Gregolin, C. (1985). The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochimica et Biophysica Acta,* **839**, 62-70.

Appendix J: Standard Operating Procedure (SOP) for the analysis of serum Clara Cell Protein 16 (CC16) using the BioVendor Human Clara Cell Protein ELISA

Objective: To describe the Standard Operating Procedure (SOP) for the collection, handling and storage of blood samples from volunteer miners and for assaying the amount of Clara Cell Protein 16 (CC16) in the samples

Intended Use of the BioVendor Human Clara Cell Protein ELISA: It is a biotin labelled antibody based sandwich enzyme liken immunoassay for the quantitative measurement of human Clara Cell Protein in serum, plasma, tissue culture medium and bronchoalveolar lavage fluid. It is intended for *in vitro* research use only. [BioVendor Laboratory Medicine, 2006].

Principle: Human Clara Cell Protein (CC16) belongs to a family of secretoglobins and is a secreted product of non-ciliated bronchiolar Clara Cells. It has been proposed as potential peripheral marker of respiratory epithelial injury.
In the BioVendor's Human Clara Cell Protein ELISA, calibrators or samples are incubated with rabbit polyclonal anti-human Clara Cell Protein antibody, which is coated in microtiter wells. After one-hour incubation and a washing, biotin-labelled polyclonal anti-human Clara Cell Protein antibody is added and incubated with captured Clara Cell Protein. After one hour incubation and washing, streptavidin-horseradish peroxidase conjugate is added. After one hour incubation and the last washing step, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of Clara Cell Protein. A Standard curve is constructed by plotting absorbance values versus Clara Cell Protein concentrations of calibrators, and concentrations of the unknown samples are determined using the standard curve. [BioVendor Laboratory Medicine, 2006].

Storage and Expiration of Kit: The Kit is stored at 2-8^{o}C. Under these conditions the Kit is stable until the expiration date (indicated on the label of the box). [BioVendor Laboratory Medicine, 2006].

Limitations of the Assay: Results exceeding Clara Cell Protein level of 100 ng/ml should be repeated with diluted samples. Dilution factors then need to be taken into consideration in calculating the Clara Cell Protein concentration. [BioVendor Laboratory Medicine, 2006].

Sensitivity: The limit of detection is defined as follows:
Analytical Limit of Detection: It is calculated from the real human Clara Cell Protein values in the wells and it has a value of 20 pg/ml
Assay sensitivity: It takes the dilution of the samples into consideration and is calculated using the formula: Assay sensitivity = Analytical Limit of Detection x sample dilution (20 pg/ml x 25) and has a value of 500 pg/ml. [BioVendor Laboratory Medicine, 2006].

Specificity: The antibodies in the Human Clara Cell protein ELISA kit are highly specific for human Clara Cell Protein with no detectable cross reactivities to the cytokines that may be present in human serum.

Precautions:

1. For *in vitro* research use only.
2. The Kit contains components of human origin. The materials were found to be non-reactive for HbsAg, HCV antibody and for HIV ½ antibody and antigen. However, the materials should be handled as potentially infectious.
3. Avoid contact with the acidic Stop Solution and Substrate Solution which contains hydrogen peroxide. Wear gloves and eye protection when handling these reagents. In the case of contact with the Stop Solution and the Substrate Solution, wash the skin thoroughly with water and seek medical attention if necessary.
4. Wear gloves and laboratory coats when handling immunodiagnostic materials.
5. Do not pipette materials by mouth.
6. Do not mix reagents with different lot numbers.
7. Do not use reagents beyond the indicated expiration date. [BioVendor Laboratory Medicine, 2006].

Primary sample: Human serum (Stable for 2 years when stored at -70^{o}C)

Container of storage: 1.0 ml CryoTubes

Equipment needed:

1. A centrifuge capable of 1300 *x g*.
2. A microplate reader with a 450 nm filter.
3. Adjustable pipettes to deliver 10-1000 µl and disposable tips.
4. Multichannel pipette to deliver 100 µl.
5. Glassware (graduated cylinder and bottle for the Wash solution).
6. Microcentrifuge tubes for diluting the samples.
7. Micro titration plate washer.
8. Software package facilitating data generation and analysis.

Reagents needed:

1. The BioVendor Human Clara cell Protein ELISA Kit
2. Distilled water.

Quality control: A Clara Cell Protein Master Calibrator is included in the kit. The Clara Cell Protein Master calibrator is a 16 kDa dimerism protein that is purified from human urine. It is reconstituted to a concentration of 100 ng/ml. Serial dilutions of the master calibrator are performed to generate standards with Clara Cell Protein concentrations of 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml and 2 ng/ml. These standards and the master calibrator and run in duplicate along with the samples. A Quality Control High and a quality Control Low are also included in the kit. They are also run in duplicate along with the standards and samples. The CC16 concentrations obtained for the standards and Quality controls are compared to the expected concentrations, as indicated in the certificate of analysis included in the kit.

Supplier Details:

The BioVendor Human Clara cell Protein ELISA Kit (Catalogue number RD191022200).
BioVendor Laboratory Medicine, Inc.
CTPark Modrice

Evropska 873
664 42 Modrice
Czech Republic
e-mail: info@biovendor.com
http://www.biovendor.com

Phone: +420-549 124 185

Local Supplier:

Calicom Trading 262 (PTY) LTD
Tel: +27 11 472-7300
Email: keith@katmedical.com

A. SOP FOR THE COLLECTION OF BLOOD SAMPLES FROM VOLUNTEER MINERS

1. Draw venous blood into a 6 ml BD Vacutainer™ tube containing Clot Act.

2. The blood should be drawn and handled at room temperature (25^{o}C).

B. SOP FOR THE SEPARATION OF THE COLLECTED BLOOD SAMPLES

1. The blood samples should be separated within 2 hours of being collected

2. To separate the blood, centrifuge the tube of blood at 1300 *x g* for 10 minutes at room temperature.

3. Pipette 300 µl of the upper serum layer into a 1.5 ml CryoTubes, in triplicate.

C. SOP FOR THE STORAGE OF THE SERUM

1. Ensure that the lids of the CryoTubes are securely fastened.

2. Shock freeze the CryoTubes by using a pair of tongs to lower the CryoTubes into a Dewar flask of liquid nitrogen.

3. Transport the frozen CryoTubes back to the National Institute for Occupational Health (NIOH) in a cooler box containing enough ice packs to ensure that the CryoTubes remain frozen.

4. As soon as the CryoTubes arrive at the NIOH transfer them to a clearly labelled container.

5. Place the container in the -80^{o}C Freezer.

D. SOP FOR OPERATING THE MICROPLATE READER AND ACCOMANYING SOFTWARE

The microplate reader needs to be set up during the last step of the assay (after addition of the substrate) as the absorbance of the assay microplate needs to be read no longer than 15 minutes after adding the stop reagent.

1. To measure the absorbance in the wells of the microplate during the assay, an ELx800 Automated Microplate reader (Bio-Tek) is used and the accompanying software is KC4^{TM}v3.4 (Bio-Tek).

2. Plug in the microplate reader and switch it on (the ON/OFF switch is located on the right hand side of the machine).

3. The reader will go through an automatic self-test to ensure it is functioning properly.

4. Wipe down the surface of the microplate reader with a damp paper towel.

5. Connect the laptop loaded with the KC4 software to the microplate reader and to the printer.

6. Switch on the laptop and open the KC4 program.

7. Click on the '**Protocol'** tab and select the '**Wizard**' option

8. In the '**Plate settings**' box select the '**Description**' box.

9. In the '**Plate description parameters'** box fill in the necessary prompts (for example the experiment name or the name of the experimenter). These are optional.

10. Click '**OK**'

11. In the '**Plate settings**' box select the '**Reading Parameters**' box

12. Select the '**End reading**' reading type.

13. Select the '**Meas. Filter'**. Click the down arrow on the side of the white box next to the Meas Filter option. Select a wavelength of 450 nm.

14. Click the down arrow on the side of the white box next to the Plate type and select a '96 Well Plate'.

15. Fill in the wells that are being used on the plate in the '**First well**' and '**Last well**' boxes (For example if the whole plate is being used the first well is A1 and the last well is H12).

16. Click "**OK**'

17. In the '**Plate settings**' box select the '**Layout**' box

18. A layout for a 96 Well Plate will appear in a box labelled '**Plate layout**'. Select the type of sample being assayed (for example a blank (background), control or sample) by clicking the

down arrow on the side of the white box next to the '**Type**' option. Once you have selected the type of sample, click on the well that sample will occupy in the microplate. The name of the sample will then appear in that well. (For example, select the Blank as the type of sample and click on the well labelled A1. The word Blank will then appear in well A1). The standards and their corresponding concentrations must also be put into the layout.

19. Once the plate layout has been filled in click '**OK**'

20. Select the '**Basic Analysis**' tab.

21. In the '**Basic Analysis**' box select the '**Curve Description**' tab.

22. Select '**M450 Corrected**' as the '**Data to Interpolate**'.

23. Select the '**Current plate**' as the '**Curve origin**'.

24. In the '**Basic Analysis**' box select the '**Curve Fit**' tab.

25. Select '**4 Parameters**' as the '**Curve fit**'.

26. Click '**OK**'

27. Select the '**Output**' tab.

28. In the '**Output**' box select the '**Report**' box

29. In the '**Available data**' box click on the name of the type of data that is wanted in the final report of the results (for example the template). Click on the small box labelled '**Add**'. The name of the data that was selected will then appear in the '**Selected data**' box. Continue this process until all the data that is wanted in the report is listed in the '**Selected data**' box.(If a data type is added that is no longer wanted click on the small box labelled 'Remove' and the data name will be removed from the 'Selected data' box).

30. The data types that must be selected are '**M 450**', '**M450 corrected**', '**Standard curve**', '**Sample concentrations**'.

31. Once all the data needed has been selected click '**OK**'

32. Click '**OK**'

33. Save the protocol under a chosen name.

34. Select the '**Read**' Box in the tool bar.

35. Fill in the necessary prompts and any comments.

36. Do not click on the '**Start Reading**' box. This is only done once the plate is ready to be read.

37. Perform the assay as described below.

E. SOP FOR ASSAYING THE CONCENTRATION OF CC16 IN THE SERUM

1. The serum samples (stored in CryoTubes in the -80^{o}C freezer) should be assayed no later than 9 weeks after they were collected.

2. The concentration of CC16 in the serum samples will be assayed using a Human Clara Cell Protein ELISA Kit purchased from BioVendor Laboratory Medicine, Inc. (Czech Republic).

3. When not in use store the kit at between 2 and 8^{0}C in a fridge.

4. To use the kit, remove the kit from the fridge and allow the contents of the kit to come to room temperature.

5. To prepare the 1 X Wash solution, dilute 100 ml of Wash Solution Concentrate (10 X) (provided in the kit) with 900 ml of deionised water in a 1L Schott bottle. Invert the bottle gently to mix. The diluted Wash Solution is stable for 1 month if stored between 2 and 8^{o}C.

6. To prepare the Human Clara Cell Protein Master Calibrator, reconstitute the Clara Cell Protein Master Calibrator (provided in the kit) with 0.5 ml of Dilution buffer (provided in the kit). Invert gently to mix. The concentration of the human Clara Cell Protein in the stock solution is 100 ng/ml. The reconstituted master calibrator should be stored at -20^{o}C until its expiration date.

7. A series of standards/calibrators are then prepared from the 100 ng/ml Clara Cell Protein stock by means of serial dilutions. A total of five standards are prepared from the 100 ng/ml stock with final concentrations of 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml and 2 ng/ml. The prepared standards should be stored at -20^{o}C until the expiration date of the kit.

8. To prepare the 40 ng/ml Clara Cell Protein standard, pipette 300 µl of Dilution buffer into a clearly labelled 1 ml Eppendorf tube and add 200 µl of the 100 ng/ml Clara Cell Protein stock. Gently mix.

9. To prepare the 20 ng/ml Clara Cell Protein standard, pipette 200 µl of Dilution buffer into a clearly labelled 1 ml Eppendorf tube and add 200 µl of the 40 ng/ml Clara Cell Protein standard. Gently mix.

10. To prepare the 10 ng/ml Clara Cell Protein standard, pipette 200 µl of Dilution buffer into a clearly labelled 1ml Eppendorf tube and add 200ul of the 20ng/ml Clara Cell Protein standard. Gently mix.

11. To prepare the 5 ng/ml Clara Cell Protein standard, pipette 200 µl of Dilution buffer into a clearly labelled 1 ml Eppendorf tube and add 200 µl of the 10 ng/ml Clara Cell Protein standard. Gently mix.

12. To prepare the 2 ng/ml Clara Cell Protein standard, pipette 300 µl of Dilution buffer into a clearly labelled 1 ml Eppendorf tube and add 200 µl of the 5 ng/ml Clara Cell Protein standard. Gently mix.

13. Determine the number of 1.5 ml Eppendorf tubes needed to accommodate the number of standards and controls that will be run in the assay. It is recommended that the six Clara Cell

Protein standards, the Clara Cell Protein High control and the Clara Cell Protein Low control be assayed in duplicate (a total of 16 tubes).

14. In each clearly labelled 1.5 ml Eppendorf tube, pipette the amount of Dilution buffer necessary to dilute the standards and controls 25 X. If the standards and controls are being assayed in duplicate pipette 240 µl of Dilution buffer into the Eppendorf tube.

15. To dilute the Clara Cell Protein standards, pipette 10 µl of gently mixed 100 ng/ml Clara Cell Protein stock into the clearly labelled 1.5 ml Eppendorf tube containing 240 µl Dilution buffer and aspirate to mix. Repeat the above procedure to dilute the 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml and 2 ng/ml Clara Cell Protein standards using a clean pipette tip for each standard. Do not store the diluted standards.

16. To dilute the Clara Cell Protein High Control, pipette 10 µl of the Clara Cell Protein High Control (provided in the kit) into the clearly labelled 1.5 ml Eppendorf tube containing 240 µl Dilution buffer and gently mix. Do not store the diluted control.

17. To dilute the Clara Cell Protein Low Control, pipette 10 µl of the Clara Cell Protein Low Control (provided in the kit) into the clearly labelled 1.5 ml Eppendorf tube containing 240 µl Dilution buffer and gently mix. Do not store the diluted control.

18. Remove the serum samples that will be assayed from the -80^{o}C freezer and allow them to thaw at room temperature.

19. Determine the number of 1.5 ml Eppendorf tubes needed to accommodate the number of samples that will be run in the assay.

20. In each clearly labelled 1.5 ml Eppendorf tube, pipette the amount of Dilution buffer necessary to dilute the serum sample 25 X. If the serum sample is being assayed in singlet pipette 120 µl of Dilution buffer into the Eppendorf tube. If the sample is being assayed in duplicate pipette 240 µl of Dilution buffer into the Eppendorf tube *etc*.

21. Ensure that Dilution buffer has been added to all the Eppendorf tubes needed before any serum sample is added.

22. Gently tap the CryoTubes containing the thawed serum sample to mix.

23. Pipette 5 µl of the mixed serum sample into the Dilution buffer in the Eppendorf tube and gently mix (if the sample is being assayed in duplicate add 10 µl of serum sample instead of 5 µl).

24. Repeat for all the samples using a clean pipette tip for each sample. Do not store the diluted samples.

25. A microplate containing 96 wells is provided in the kit. In each plate there are 12 columns (Columns 1 to 12) each consisting of 8 wells (Rows A to H).

26. Pipette 100 µl Dilution buffer into two wells of the microplate (if using the whole kit use wells A1 and A2). These wells are the background wells (non-enzymatic wells).

27. Pipette 100 µl of 25 X diluted 100 ng/ml Clara cell Protein Standard into two wells of the microplate (if using the whole kit use wells B1 and B2).

28. Pipette 100 µl of 25 X diluted 40 ng/ml Clara cell Protein Standard into two wells of the microplate (if using the whole kit use wells C1 and C2).

29. Pipette 100 µl of 25 X diluted 20 ng/ml Clara cell Protein Standard into two wells of the microplate (if using the whole kit use wells D1 and D2).

30. Pipette 100 µl of 25 X diluted 10 ng/ml Clara cell Protein Standard into two wells of the microplate (if using the whole kit use wells E1 and E2).

31. Pipette 100 µl of 25 X diluted 5 ng/ml Clara cell Protein Standard into two wells of the microplate (if using the whole kit use wells F1 and F2).

32. Pipette 100 µl of 25 X diluted 2 ng/ml Clara cell Protein Standard into two wells of the microplate (if using the whole kit use wells G1 and G2).

33. Pipette 100 µl of 25 X diluted Clara cell Protein High Control into two wells of the microplate (if using the whole kit use wells H1 and H2).

34. Pipette 100 µl of 25X diluted Clara cell Protein Low Control into two wells of the microplate (if using the whole kit use wells A3 and A4).

35. Pipette 100 µl of each of the 25 X diluted serum samples into a separate well of the microplate.

36. Cover the plate with foil and incubate the plate at room temperature (25^{o}C) for 75 minutes.

37. Remove the foil and carefully wash the plate five times with the 1 X diluted Wash Solution. (After washing gently tap the plate on a piece of paper towel to ensure it is dry).

38. Pipette 100 µl Biotin Labelled Antibody (provided in the kit) into every well being used.

39. Cover the plate with foil and incubate the plate at room temperature (25^{o}C) for 75 minutes.

40. Remove the foil and carefully wash the plate five times with the 1 X diluted Wash Solution. (After washing gently tap the plate on a piece of paper towel to ensure it is dry).

41. Pipette 100 µl Streptavidin- HRP Conjugate (provided in the kit) into every well being used.

42. Cover the plate with foil and incubate the plate at room temperature (25^{o}C) for 75 minutes.

43. Remove the foil and carefully wash the plate 5 times with the 1 X diluted Wash Solution. (After washing gently tap the plate on a piece of paper towel to ensure it is dry).

44. Pipette 100 µl Substrate Solution (provided in the kit) into every well being used (this step should be performed out of direct sunlight).

45. Cover the plate with foil and incubate the plate at room temperature (25^{o}C) for 10 minutes (if the room temperature is below 20^{o}C the incubation time should be increased up to 20 minutes).

46. Pipette 100 µl Stop Solution (provided in the kit) into every well being used to stop the colour development.

47. Carefully place the microplate into the designated holder in the microplate reader.

48. Carefully shake the microplate for a few seconds to mix the contents in the wells.

49. Select the '**Start Reading**' box that is displayed in the KC4 program.

50. The absorbance at 450 nm is automatically measured in the specified microplate wells.

51. Once the microplate reader has finished measuring the absorbance of the plate, click on the '**Save**' box in the toolbar and save the report under a chosen name. If one requires the report to be printed, click on the '**Print**' box in the toolbar.

52. Remove the microplate from the microplate reader.

53. Wipe down the microplate reader with a damp paper towel.

54. Switch off the microplate reader and unplug.

References:

BioVendor Laboratory Medicine. (2006). The Human Clara Cell Protein ELISA Kit Instructions Insert; BioVendor Laboratory Medicine, Inc; Czech Republic, pg 1-24.

Appendix K: Subject information sheet for volunteers

MHSC

MINE HEALTH *AND* SAFETY COUNCIL

and the

NATIONAL INSTITUTE *for* OCCUPATIONAL HEALTH

NIOH

Subject Information Sheet for Volunteers

Hello, my name is Kerry Makinson, I am a researcher from the National Institute of Occupational Health (NIOH) in Johannesburg, and my contact numbers are 011 712 6469 and 082 494 1392. My co-worker is Professor Brendan Girdler-Brown.

I would like to invite you to participate in our study. It is an independent study commissioned and funded by the Mine Health and Safety Council (SIMRAC) and carried out at the National Institute for Occupational Health (NIOH).We are looking for a new blood test that might be used to see if silica dust levels at your workplace are a danger to workers. We believe that if workers breathe in too much silica dust then their bodies will react by making certain chemicals that we can detect in blood samples taken from those workers.

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 about 5 minutes). Then we need to look at your last X-ray. If you agree the nurse will show us your x-ray. We will also need to draw a small amount of blood (about 3 teaspoons) from your arm on 5 different occasions over a 3-4 week period. This will also only take about five minutes each time. These tests will not tell us if you are sick. In addition we will only be able to tell if the silica dust levels are too high in the future if we test a large number (say, 10 people) of workers and look at all their results together. It is unlikely that the blood tests will be able to tell if an individual miner has been exposed to too much silica dust. After we take your blood it will be kept frozen at the National Institute for Occupational Health and will only be used for this study. It will not be used for any other purpose without your written permission. We will not be testing for HIV.

Your personal information and results will be kept confidential and will not be shown to any other person. We will be coming back to the mine to talk about our results this year and you will be invited to come and ask any questions that you may have. At that time we will also give you your own individual results in writing (if you want them) along with an explanation of what they mean.

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 only risk to you from taking part in this study will be a little discomfort when the

blood specimen is taken and for a short time afterwards. There will be a medical doctor present to take your blood specimens.

Thank you
Kerry Makinson

The contact details for the Wits Human Research Ethics committee are: Ms Anisa Keshav (011 717 1234)

Appendix L: Consent form for volunteers

MINE HEALTH *AND* SAFETY COUNCIL

and the

NATIONAL INSTITUTE *for* OCCUPATIONAL HEALTH

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 most recent X-rays to be given to Ms Kerry Makinson. I am happy to allow my personal silica dust exposure history to be told to Ms Kerry Makinson by the Human Resources department. I agree to give 15ml of blood on each of 5 times spread over 3-4 weeks. I understand that all individual and personal information will be kept confidential and I know that I can leave at anytime.

Signed _____ Date _____

Appendix M: Questionnaire administered to volunteers

STUDY NUMBER STICKER:

		Group:			

	TO BE COMPLETED BY INTERVIEWER			**CODING**
RECRUITMENT CENTRE:				
DATE:	dd	Mm	yyyy	
SHIFT:				
SHAFT:				
JOB:				
JOB HISTORY:				
CURRENT SMOKER?:	YES	NO		
PREVIOUS SMOKER?:	YES	NO		
CONTACT NUMBER:				
X-RAY DATE:	dd	Mm	yyyy	
SILICOSIS SCORE:	/			
CURRENT TB Rx:				

DATE OF BIRTH:	dd	Mm	yyyy	

Appendix N: Consent Form for Photographs

MINE HEALTH *AND* SAFETY COUNCIL

and the

NATIONAL INSTITUTE for OCCUPATIONAL HEALTH

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

Please indicate (by means of a tick) which of the below reports you are willing to have your photograph and/or name published in:

Report	Photograph	Name
A report for the South African Mine Health and Safety Council.		
The newsletter for the National Institute for Occupational Health (NIOH).		
A Scientific Journal (International or National) that will be read by medical and scientific personnel.		
Visual presentations at conferences (International or National).		
On the Internet on Occupational Health or Toxicology related sites.		
In Kerry Makinson's MSc research dissertation.		
Annual reports for The University of the Witwatersrand, the University of Pretoria and the NIOH.		

I _____ agree that my photograph can be taken and that my photograph and/or name can only be used for the purposes indicated above. I understand that if I do not agree to have my photograph and/or name published in any of the above papers that it will have no effect on me or my job.

Signed _____ Date _____

Appendix O: Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Makinson

CLEARANCE CERTIFICATE **PROTOCOL NUMBER MO60612**

PROJECT Validation of biomarkers forimproved assessment of exposure and early effect from exposure to crystalline silica Phase III

INVESTIGATORS Ms K Makinson

DEPARTMENT Haematology and Molecular Medicine

DATE CONSIDERED 06.06.30

DECISION OF THE COMMITTEE* APPROVED subject to submitting writtem permission from the managers, keeping all identifying information on a separate page and stating the lengthof time it will take to complete the questionnaire

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 06.07.01 **CHAIRPERSON** .....
(Professor M Vorster)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr M Gulumain

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

Appendix P: Individual GPx and CC16 ex-leave and follow up results

Table 1: Mean erythrocyte GPx activities (in U/g Hb) of the ex-leave and the follow up samples of the individual volunteers.

Volunteer	Ex-leave	Monday	Wednesday (pre-shift)	Wednesday (post-shift)	Friday
1	34.86	27.99			
4	32.25				20.22
5	24.57		22.22	22.06	
6	19.54	17.90	16.34		
7	19.70		20.95		
8	26.03	20.47			25.48
10	36.42		27.00	28.92	
11	26.93		24.29	25.20	19.91
12	35.62		25.36	33.58	
14	32.00	25.99			
15	28.19		23.58	23.92	
17	24.18	19.12			
19	25.05	19.28			
20	30.28	19.55			
21	35.38				25.30
22	17.89				15.88
23	32.22		14.14	21.09	
24	17.85		13.95	12.11	
25	36.31			33.11	
26	25.80	17.35			
30	29.69		21.41	18.46	
31	49.92		41.35	41.34	
32	30.64	26.36			
33	31.48		20.42	20.71	
36	26.24	29.32			
37	16.79	16.95			
40	22.30		25.99	20.94	
41	17.66				18.25
42	24.55				23.33
43	21.87	18.51	18.18	18.86	19.12
44	23.99		21.41	25.71	
45	23.10				
46	22.80		29.53	27.28	
47	24.24		23.01	19.87	
48	20.28	23.55			
50	22.94				22.88
51	19.61		14.87	13.62	
52	20.41		14.58	14.30	
53	22.04		25.00	20.46	
59	22.57	17.30			

60	28.88		39.14	36.88	
61	25.46		19.68	20.39	
62	32.14	22.99			
63	27.59				22.07
65	27.14				17.73
66	31.17		26.83	24.37	
68	19.84	15.28			
74	28.14				18.19
75	34.44				26.49
76	27.37				19.78
77	23.04	21.62			
79	26.23	22.64			
80	30.21				34.79

Table 2: Mean serum CC16 concentrations (in ng/ml) of the ex-leave and the follow up samples of the individual volunteers.

Volunteer	Ex-leave	Monday	Wednesday (pre-shift)	Wednesday (post-shift)	Friday
1	5.18	8.86			
4	5.84				6.77
5	5.87		5.93	4.51	
6	6.12	8.01	9.71		
7	6.64		7.09		
8	7.93	9.97			5.95
10	9.15		8.06	5.94	
11	3.63		6.66	3.58	4.68
12	15.70		16.74	13.08	
14	7.30	7.99			
15	2.46		3.18	2.30	
17	7.47	6.17			
19	8.42	8.63			
20	4.25	3.01			
21	6.61				4.57
22	10.74				10.85
23	2.24		1.87	2.78	
24	2.69		2.56	1.86	
25	4.59			3.15	
26	5.85	6.85			
30	6.68		5.84	4.86	
31	9.43		6.06	5.90	
32	5.63	5.99			
33	4.30		4.76	3.04	
36	4.41	2.71			
37	7.34	3.87			
40	5.29		2.21	3.37	
41	8.70				3.74

42	12.07				7.42
43	12.37	8.71	8.65	4.53	5.14
44	12.65		16.64	9.50	
45	12.34				
46	7.24		5.30	3.41	
47	9.49		7.06	5.11	
48	4.94	3.51			
50	7.88				7.21
51	6.18		8.39	6.73	
52	2.72		3.34	2.67	
53	9.46		8.39	8.92	
59	10.38	8.41			
60	11.71		9.87	11.69	
61	4.59		2.69	1.99	
62	4.08	6.26			
63	8.02				7.52
65	2.94				2.62
66	5.19		4.22	4.45	
68	9.98	11.56			
74	6.54				6.68
75	6.15				6.49
76	5.85				4.43
77	6.10	9.52			
79	4.55	5.35			
80	2.90				1.90

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