# Occupational exposure to dust bound heavy metals and its association with urinary and oxidative stress biomarkers among coal mine workers in Pakistan

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## Abstract

Coal production is increased tremendously to meet energy requirements. However coal mining activities are becoming serious health concern as these are responsible for the release of various toxic metals enhancing the exposed workers' vulnerability towards various health implications. There are hardly any studies which have investigated the role of metal exposure causing oxidative stress in coal mine workers. The study aimed to investigate the metal concentration in different particle sizes of dust and its association with urinary metal levels and antioxidant activity as a biomarker of exposure to mining activities. 120 samples of blood and urine were collected from the workers of coal mines of two provinces (Punjab and Balochistan) of Pakistan comprising extractors (n=65), loaders (n=21), managers (n=24). From the same mines 14 dust samples were collected which were later fractionated into three particle sizes (<75um, 75-106um, 106-150um) . Metal analysis was performed by Atomic Absorption Spectroscopy and antioxidant enzymes via UV Spectrophotometer. Among toxic metals Pb (125.5 ± 10.82 mg/kg) and Ni (84.28 ± 7.60 mg/kg) were present in elevated amounts in dust in <75um particle sizes and were also highly enriched in this size fraction with Df of 2.40 and 1.52 respectively. However in case of metal nutrients Mn had increased levels (284.92 ± 17.21 mg/kg). On the contrary macronutrients, like Mg, Na, K had low concentrations in dust but were highly enriched in smallest particle size with Df of 3.88, 2.75, 2.47. The level of urinary metals, high in exposed subjects were Pb (45.07 ± 22.50 ug/g) and Ni (34.60 ± 3.04 ug/g). Among macro and micronutrients Na, K and Zn were observed to have elevated leves of (462.27 ± 15.54 mg/l), (144.47 ± 71.16 mg/l) and (325.71 ± 208.01 ug/g). Except Ca, Fe, and K all other metals had high levels in exposed group as compared to controls and among exposed subjects, extractors had highest mean values for most of the metals. Significantly increased blood MDA levels (84.05 ± 9.96 nm/dl) and reduced SOD (271.54 ±34.18 nm/min/ml) and CAT (135.26 ± 14.44 um/min/ml) activities were observed in exposed workers indicating oxidative stress. The study depicts a strong association ,firstly between metal levels in mine dust and urine of occupationally exposed workers and then between urinary metals levels and antioxidant activities which can cause severe health implications.

# Chapter 1

# Introduction

## 1.1.Background

The global energy needs are fulfilled mostly by the use of fossil fuels and one of the most important and abundant energy resources is coal which plays a vital role in defining the economy of a country. It strengthens the economies of the developing nations and also helps to improve their living standards by being a comparatively low priced (R. Masto, George, Rout, & Ram, 2017) . Despite that coal mines and coal production are tremendously increased to satisfy the energy requirements of industries, growing population, and businesses (Pandey, Agrawal, & Singh, 2016) coal mining, is a noteworthy cause of environmental pollution (León-Mejía et al., 2011), as coal mining activities involve the removal of overburdens by means of mechanical instruments and devices to excavate the earthly bound coal which leads to the generation of huge amount of mine dust particles and mine spoil. A large number of emissions and coal dust particles released during the processes of open cast coal mining are possibly hazardous to the exposed population(R. E. Masto et al., 2011) and have been linked with numerous chronic diseases and mortality risks (Guerrero-Castilla, Olivero-Verbel, & Marrugo-Negrete, 2014). Dust emitted from the coal mining activities is also of concern because of the organic pollutants and metal species it contains. In particular, metal pollution from coal is a key issue due to high enrichment factor of some elements. Exposure to elevated amounts of trace or heavy metals can give rise to acute and chronic toxicity leading to the damage of certain body functions such as central and peripheral nervous systems, kidneys, lungs, liver, cause oxidative stress, genetic damage and may also be fatal. The main routes of exposure through which the population in the mining area are exposed to heavy metals in dust are inhalation, ingestion and dermal contact (Z. Li, Ma, van der Kuijp, Yuan, & Huang, 2014). Therefore the elemental composition of soil and dust samples from the mining areas is indispensable to discern consequences of short- and long-term activities. Moreover, it can provide evidence about individual exposure levels to metal contamination from mining activities. However, limited research on heavy metals in dust and its impact on individual health particularly in mining zone is found. There is a need of emphasis on rapid exposure risk assessment techniques in mining industries specifically for Initial Site Screening (ISS), to quickly identify key site criteria including sampling information, estimated populations at stake, human exposure pathways, etc. (Caravanos et al., 2013). Rigorous exposure assessment is essential for risk estimation and regulation (Pesch et al., 2004) .

### 1.2.Worldwide coal mining methods and processes

The global coal production boosted 3.2% or otherwise stated by 105 mtoe, (million tonnes of oil equivalent) which is the fastest growth rate since 2011 (Dudley, 2015). Coal production is not confined to a single region. The top five coal producing countries are China, India, America, Australia, and Indonesia (world coal.org, source: IEA Coal Information 2017). However, the situation of coal mining in Pakistan is very much different as equated to other countries. Pakistan is ranked 7th in the world, having around 186 billion tons of coal reserves after discovery of huge coal deposit of 175.5 billion tones at Tharparkar Sindh, in an area of 10,000 sq.km which provided quantum increase in the coal resources of Pakistan. Sindh has total 184 million ton coal deposits while Balochistan has 217 million ton, Punjab 235 million ton, KPK 91 million ton and AJK 9 million tons. The coal production in different regions of the world is listed in the table.

The prime factors, to be considered prior to the selection of mining method are the thickness of coal deposit, geology, and terrain, depositional angle, and mechanics of adjacent rocks. In general, the two types of mining practices are (1) underground mining (2) open pit or surface mining (Nersesian, 2016). Around 60% of coal is excavated through underground mining while the rest of 40% via open-pit mining mode (Junker, Klukas, & Schreiber, 2006)

### 1.2.2.Open pit or surface mining

To obtain the desired ore or coal by removal of non-ore material is termed as surface mining (Homer, 2009). This method is applied where the pit's depth does not usually exceed 150m and is feasible when coal seam occurs near the surface area where the recovery rate is higher (Scott, Ranjtih, Choi, & Khandelwal, 2010), but nowadays, deeper pits are also often observed. The origin of surface mining dates back to early 1960s in Australia (Westcott, 2004) and according to report published by World Coal Association in 2013 it is not only the main method for coal extraction in Australia but also a widespread technique all over the world. 100%, 75%, 73.8%, 61%, 60.9%, and 54% coal is mined through this technique in Eastern Germany, India, Australia, USA, Russia, and Western Germany respectively. Nevertheless, the world's greatest coal producer China practices this methodology only 7-8% due to unfavorable mining and geological conditions (Zhang, Cai, & Li, 1996). Main reasons for its extensive use include benefits like low operational cost; increased production rate i.e., 20–50 Mt. per year; safe working condition due to the absence of fall or collapse risk; and great coal recovery rate (Chen, Li, Chang, Sofia, & Tarolli, 2015). However certain negative impacts include disturbances related to environment and climate such as extreme weather conditions, misty winds, and drenching rains. This method was initially employed manually using labor, but later, got replaced with advanced mining equipment. In developed countries such as the USA, shovel trucks and draglines stripping with crushing plant to conveyor are commonly used in small- to middle sized surface mining operations (Fung, 1981). Coal extraction in Russia is commonly done by shovel truck and shovel train systems. In Australia, dragline stripping is mainly employed along with shovel trunk or crushing plant or conveyor system to extract hard coal from open surface mines, whereas for extraction of brown coal and bucket wheel excavator (BWE) is used. Conveyor systems and BWE are also common ways of coal mining in Germany (Atkinson, Burdon, & Longworth, 1985) (Woodruff, 2016).

### 1.2.3.Underground coal mining

Most of the world's coal is obtained through underground coalfields which let coal companies mine deeper coal deposits. Several techniques have been devised for carrying out underground mining, however, longwall and room and pillar mining are extensively used methods (Woodruff, 2016). In longwall mining mode, coal is stripped from the mines with the help of mechanical shearers which are assisted by support structures to enable their access to mines. The support structures are finally removed and the mine is allowed to collapse. It is the most productive way of mining. The room-and-pillar method comprises room for mining purpose while pillars for stability (Nayak & Dalai, 2010). Mining activity is held simultaneously in separate rooms. After blasting, coal is transferred from the rooms to conveyors by means of shuttle cars. During the final phase, some pillars are often removed and coal is further mined. The coalface may undergo considerable subsidence damage as an effect of this process.

## 1.3.Generation of Coal Dust:

Dust has a multiplex chemical and mineral configuration since it originates from different sources and forms and gives reaction sites to different chemical species reacting atmosphere. These chemical matter could cause variation in properties of dust, subsequently, it is important to contemplate the source, configuration and substance of dust falling as it might cause health hazards to human, atmosphere and may worsen the ecology. Components existing in dust can impact terrestrial biogeochemistry through a few procedures. Briefly on time-scales (days to weeks), dust straightforwardly influences crops, modifies the timings and rate of snowmelt, and gives basic components (supplements) for plant and microbial activity. Over the long haul, dust could be considered a significant factor in the generation and improvement of soil.

Coal dust is made out of micrometer-to-nanometer-sized particles, generally running somewhere in the range of 0.1 and 30 μm, coming about because of the collision, corrosion, compressing, and pulverization of coal. Coal dust emanations rely on various parameters identified with the particular movement or to the qualities of the material, for example, particle size distribution, coal type, dampness content, dumping stature, just as climate related parameters including wind speed, and relative moistness. Coal dust have multiple factors to depend on as discussed above.

Overseeing slack mineshafts is significant since it can affect nearby and local quality of air, unfavorably influence neighborhood courtesy and represent a hazard to human health. (S. K. Pandey, Tripathi, and Mishra, 2008) announced an average of yearly fall of dust of 96.2 ± 3.6 ton/km2/month in a sub-tropical opencast coal mineshaft, Bina, India. The most extreme dust testimony happened in summer (32.8 ± 1 to 278.9 ± 2.9 ton/km2/month), and the least, in the stormy season (16.2 ± 1.2 to 111.3 ± 3.2 ton/km2 every month).

Various procedures connected with mining exercises which discharge tremendous amounts of dust particularly in open cast mining incorporate removal of topsoil, overburden removal, drilling and blasting tasks, extraction of coal, estimate decrease, transfer of coal by roads, coal handling plant activity, stacking of coal by scoop dumpers, and so on.

### 1.3.1.Topsoil Removal:

The underlying stage in an opencast coal mining movement is to remove the topsoil from the region that will be mined. After the removal, it is secured in a region a long way from the mine locale to be used for recuperation of the mine zone later. At the point when the topsoil is ousted the overburden, which is usually a waste material overlying the coal, is revealed. The overburden ordinarily contains rock which is bored and impacted to break or slacken the material. This is finished by making blast holes. This assignment, as such, is the fundamental period of dust age for an opencast coal mining task. (Gautam, Patra, and Prusty, 2012) confirmed that 660 kg $d^{-1}$ dust is created in view of overburden departure in coal mineshafts in India.

### 1.3.2.Drilling and Blasting:

The explosives are set aside once the blast occurs, particularly after the loading of explosives. The work of overburden starts right after the explosives are set aside. However, in few cases the removal of coal also requires the process of drilling and blasting. If the situation requires such process, then drilling and blasting is repeated. Lorries are used to pull the evacuation of overburden through loader. Once the overburden is dumbed, the waste material is escalated with the help of bulldozers after that. Special equipment's are used to measure the depth of the ground or rock which is determined through this before drilling the underground. This process actually involves explosive to explode the underground rocks. The velocity to use explosives vary from kind of rock. High velocity of explosives are used for tough and huge rocks whereas low velocity explosives are used for softer rocks to create gas pressure at higher rate. After this stage, the next step is that of drilling which is another unit of operation used for the production of major amount of PM.

### 1.3.3. Loading, Unloading and Transport of Overburden and Coal:

After the process of removal of coal by loader, the next stage is the last stage where coal is taken to processing plants through trucks where it is then ready to distribute among community. At this stage, the intensity of pollution remains high throughout loading and unloading. Gautam et al., 2012) analyzed that the pollution rate remains high due to dusty roads being in constant contact with transports, which results in various airborne constituents. In case of unpaved surfaces, the rate of air pollution remains high as a result of emissions by vehicles. (Mandal et al., 2012) estimated the amount of emissions caused by vehicles in India during transportation is around 80.2%. Overburdening of coal is defined as the material, whether in loose or accumulated form, is spread over the surface of iron or ore.

### 1.3.4. Processing of Coal:

Ore preparing involves extraction of conclusive item from rock and component of emanation entangles effect, scratching and discharging from heights. Barring the referenced activities, presence of fire expands PM toxins focus in the climate, in the end lead to genuine contamination of air around territory of coal mining. It has been seen that out of all out PM delivered, the PM10 speaks to 33% to half. Accordingly, it very well may be presumed that enormously automated opencast mining of coal produces huge measure of air borne dust that may cause security and health dangers. These may at long last reduce working effectiveness by means of poor deceivability, gear disappointment, ascend in maintaining cost and bringing down of work throughput. Hence, in summary following are the steps involved in mining of coal:

- Once after the coal has been extracted, the next step is to clean it from all sort of impurities. For that purpose, coal is washed in industries in order to remove the contaminants such as Sulphur and other heavy metals. Some amount of the coal needs to be burnt as solid so for that various preparation methods are used at large scale.
- The next step involves the breaking or crushing of coal. This requires machinery at large scale. The reason for crushing coal into smaller pieces is that the pure form of coal might contain lumps of about 3 feet or so in size. That is the reason coal is crushed through machinery.
- Once it is crushed in finest form, it is then transported to markets and trucks or lorries are used for this purpose.

**1.4. Occupational Exposure to Coal and Coal Dust:**

The process of coal-mining is considered to be one of the process which actually contaminates the environment to a large extent. The contents of coal dust are always dependent on the quality of source of coal from which the dust is being produced, as a result it is always heterogeneous. As per a general definition of coal, it is made up of heterogeneous components of both organic and inorganic material. In that, the organic components include chemical compounds such as carbon, hydrogen, Sulphur, nitrogen, oxygen and other organometallic forms. While the inorganic includes metals and metalloids such as Lead, Cadmium, Cobalt, Mercury, Chromium, Zinc, Iron, Magnesium, Beryllium, Boron, Titanium, Nickel, Manganese, Antimony, Aluminum, Copper, etc. Similarly, the coal dust produced from coal also consists of polyromantic hydrocarbons (PAHs) which is one of the heavy hazardous metal.

With regards to coal mining deposits presentation, epidemiological investigations which explain introduction, helplessness and biomarkers of impact stay uncommon and a large portion of them are on underground coal mining. The results of open-pit coal mining are marginally investigated however. Studies concerning coal presentation and its unfriendly impacts have been directed the world over. Inward breath of coal dust deposits and particles by coal mineshaft specialists is the primary introduction pathway to the conceivably destructive substances. It has been set up that constant presentation to coal mining can result in an assortment of ailments for instance silicosis, coal laborers pneumoconiosis (CWP), malignant growth and unending obstructive aspiratory illnesses (COPD) like ceaseless bronchitis and emphysema. Various examinations have additionally demonstrated that genotoxic harm prompted by the inward breath of mineral particles could prompt an extensive number of infections because of their capacity to connect with epithelial cells, macrophages and different cells quickening the production of huge amount of responsive species of oxygen (ROS). The consistent inward breath of coal dust particles is a significant celled and non-celled wellspring of oxygen specie in the lung. This might be related with harm to target cells of that tissues and other cell lines, in the wake of spreading through the circulatory system. DNA harm initiated by coal/coal residue is related with the actuation of macrophage and incitement of polymorpho nuclear cells. This enactment of cell results in the arrival of provocative go between, for example, responsive species of nitrogen (RNS), cytokines and receptive oxygen species (ROS). RNS and ROS have star incendiary properties including lipid peroxidation and oxidation, endothelial cell harm, enlistment of neutrophils, the arrival of chemo static elements, and DNA harm. Both basic and transcriptional blunders can happen in DNA because of cooperation among ROS and DNA. Harm brought about by ROS is perceived with the help of DNA glycosylases, apurinic/apyrimidinic endonucleases of the base extraction fix (BER) instrument, and now and again, by nucleotide extraction fix (NER) machinery which actually results in breakage of DNA strands. In view of the fact that DNA strand normally breaks due to inorganic elements. The most important element causing that breakage is ROS, however, it can be easily repaired soon. Another factor involved in breakage of single-stranded DNA is PAHs through DNA repair mechanism. After the activation of metabolism with the help of enzymatic complex P450, adducts with purines, specifically guanine is formed as a result of covalent bonding between electrophilic metabolites and DNA. The International Agency for Research on Cancer (IARC) declared quartz, which is one of the most essential element of coal as group 1 carcinogen along with scientifically proven in humans and animals. The coal dust exposure may vary from positive to negative depending on the difference in its composition of metals, PAHs and silica.

The difference in the composition and proportionality of the above mentioned elements might affect the genotoxicity of coal dust exposure. However, coal dust still remained non-carcinogenic according to the reports and findings of IARC, for humans. Coal, being one of the most important resource of energy needs safety and precautionary measures for individuals and environment. Various studies in the world have explained different results related to human being exposed to coal. Among them few includes smoking cigarettes, individual anatomy or structure, age, nutritional status and of course gender. Despite of the fact, the factor of susceptibility is still not understandable when related to coal diseases which include cancer, and other xenobiotic agents.

### 1.5. Coal mining associated health hazards

Coal mining has been a serious hazard to human health since $16^{th}$ century. Various studies and especially epidemiological researches have shown strong linkage between coal mining and serious kind of diseases such as increased chronic respiratory diseases, cancer, cardiovascular diseases and also physio-pathological mechanisms of respiratory diseases, particularly in coal miners. Epidemiological researches have linked the coal mining with diseases on the basis of proximity of time in populations. These studies have analyzed a huge reduction in quality health of coal miners including increased perceptions of detrimental health conditions. Health surveys by health research centers have been conducted. The data used from hospital records in such surveys were mostly with high rate of mortality and morbidity due to cancer and other respiratory diseases. This was one of the greatest evidence which showed a direct relationship between spread of diseases and coal mining. Another significant study has showed dental disorders with increased rate at the areas of USA and Europe resulting from coal mining. Also, research has shown few signs of diseases caused from parasites in the population living near the coal mining region. Basically, the health hazards related to coal mining are broadly categorized into two parts. One is the diseases caused by accidents at mining areas and second include industrial diseases which are also common to cause huge damage to mine workers.

**Physical Hazards:**

Number of physical damages are involved and common in coal mining. Few major among them includes falling of rock, fire explosions, falling from height, mobile equipment accidents, electrocution, entrapment etc. Despite of the above mentioned dangers, there are several other severe kind of diseases which might lead to death. Such type of injuries includes flooding of underground workings, well-fill release from broken bulkheads and blast of air from block caving failure. Apart from these, an uncountable number of mine workers suffer daily from various kind of physical injuries. The duty of mine workers is tough for 24 hours. It is mostly done at night time, with very less light which has direct effect on vision.

Another category which rightly falls under the category of physical hazards is that of noise. It is caused during different stages of coal mining such as in drilling, blasting, crushing, ore processing, cutting etc. It might cause the loss of hearing at all. However, it has been observed that reduction of noise during coal mining is near to impossible. Despite of modern technology, the mining industry has failed to manufacture such machinery which is silent or the can actually reduce the noise pollution. This is one of the reason why there is no residence in the area of mining regions.

Heat and humidity hazards are also encountered in coal mining regions. Mines are located underground hence there is no way for the ventilation of air down the earth being highly compressed air column, hence cases of coal miners been suffocated has been often witnessed due to intense heat and humid temperature. Humid temperature might cause several breathing issues as well. Asthma, being one of the major breathing issue, can be caused due to inhalation of toxic gases.

Mobile equipment's usually produce vibration when at work. Coal miners had also faced whole body vibrations in consequence of using load-haul-dump units, truck, scrapers, diggers and equipment like that. Such high intensity vibrational equipment can cause severe spinal disorders. Such as it might dislocate the discs of spine or may create a crack in the backbone. This is caused due to carrying of heavy machinery and especially from the machinery.

**Pneumoconiosis:**

Another, important yet severe kind of disease is pneumoconiosis. It is similar to the disease of silicosis, however, the intensity differs. People get to know about this disease, specifically talking about the areas of South Wales, when they observed strange kind of structures during the x-rays of mine workers. Pneumoconiosis is actually caused by nodular fibrosis quite similar to silicosis which is caused by pulmonary fibrosis. However, different policies were made regarding compensations to the victim of such disease. Various cases came up which actually showed disablement of certain limbs of body. The condition of reticulation occurs due to nodulation in the lungs. This has proved to be the actual cause of this disease. However, it is not evident from any research that whether nodulation is the direct effect of reticulation or reticulation wholly results in pneumoconiosis. This is mainly caused while breathing the dusty air during mine work which directly harm the lungs. Another clear cut symptom of this disease is dyspnoea. It is being observed that mostly the rate of cause of pneumoconiosis is low in anthracite mines. Various methods have been introduced to suppress dust so that it might not affect lungs to such a large degree.

**Miner's Nystagmus:**

This disease is considered to be one of the most fatal kind of disease as it directly affects the nervous system of human. Miner's nystagmus is an industrial disease. The aftermath of this disease includes the turning of eyeballs and of course with a lot of other nervous disorders. The symptoms include insomnia, some kind of mental depression, severe kind of headache or photophobia. It actually causes an involuntary oscillations of the eyeball along with all other symptoms mentioned above. With that person is in continuous mental depression. However, according to the reports of the miner's nystagmus committee formed, it clearly mentioned the reason behind such disease. The reason which came out to be was the low illumination underground where miners work. Along with the reason, the committee also explained it scientifically. Retina, being the major part of eye is quite sensitive. It is usually made up of cones which is associated with the vision of eye. This retina to work properly needs adequate light when at work. However, another part of retina which is the peripheral is composed of rods and not cones. This part aids in the vision where the light is quite low. In order to avoid such damage, the retina needs a sufficient amount of light for its proper working. The miner's nystagmus committee played a very important role in taking all precautionary measures to reduce the risk of such severe disease. The committee even tried to replace the provision of compensation granted to victims of such disease with some sort of mining opportunity as a day shift. Or else, to make the mining zones luminous enough instead.

**Inflammatory Diseases of Hands, Knees and Elbows:**

The swelling or inflammation of hands, knees and elbows are said to be beats. This is actually caused by subcutaneous cellulitis of all such limbs. When it comes to beat hands, the tendon sheaths of wrist get swollen along with the synovial lining. At mines, workers usually tend to face thin seams which give rise to these type of disease. When talking about beat hands disease, it is mostly caused by the activity of jarring. A pathogenic organism is the cause which might enter the body part through crack or cut. In most cases, the hands of workers, if they working hard becomes tight and tough. But when they off work, the skin might get soft which increase the chances of cracks since elasticity of skin diminishes.

Beat knee is caused by the knee being in contact with the ground for long time. It might happen during the process of shoveling where the knee works as a pivot during work. The very first symptom of this disease is the appearance of a pimple or pustule on the place of knee which is in contact with the floor. The risk of infection is high at the stage when the seams is thinner as compared to thicker. The possibility of attracting infection also depends on the type of surface of the floor. The probability of infection is high even when the floor is wet.

Similarly, inflammation in hand can reach to elbow as well. Or elbow may dislocate due to any huge machinery. Most of the machineries used in coal mining produce vibration as they are supposed to dig holes in the earth. Hence, they affect joint and bone to a large degree. Elbow might get swollen, or the elbow bone may get a crack, the tendons of elbow can get weak and many more. Due to continuous exposure to toxic material, there are chances of getting infection in this case too.

**Chemical Hazards:**

Production of coal dust has been a serious threat to human health in terms of its consumption of chemicals. It has caused diseases like black-lung, pneumoconiosis and various other chronic obstructive pulmonary diseases. However, the developed nations have controlled to a certain extent by means of ventilation, dust suppression and respiratory protection.

The disease of silicosis has been largely controlled through precautionary measures like respiratory protection, ventilation, enclosed cabins, wet techniques and axial water-fed rock drills. However, developing nations have still failed to overcome the disease of silicosis among coal miners. An extended exposure to crystalline silica is an important cause behind chronic obstructive pulmonary diseases. This has also caused rheumatoid arthritis and other major renal diseases, leading to high risk of lung cancer.

Usage of diesel is an important element in coal mining as it is used in diesel powered mobile equipment. Such equipment’s are usually used for the purpose of drilling or haulage. As per the research of IARC, diesel is categorized as carcinogen being an element of Group 2A which is quite harmful for humans. Other epidemiological studies have examined several cases of lung cancer from other industries. Nasal sinus cancer is yet another disease which is caused due to long term exposure of nickel in nickel refineries.

The processing of ore usually carries higher risks of because the concentration of air exceeds. This frequently happens in metallurgical processing. The explosion of coal dust during mining and methane gas demands careful monitoring and management. The problems related to carbon dioxide and hydrogen sulphite also occurs related to coal mining.

**Biological Hazards:**

Diseases like malaria and dengue fever are quite common in the areas of coal mining or nearby. Such microbial diseases can be prevented by regular and comprehensive analysis of water to detect Legionella and other sort of microorganisms. Microorganisms always grow in things which remain static for a long time. Similarly, water in mines remain static underground for may be around a week or more which has an increased probability of growing microorganisms. This type of polluted water might give rise to several water-borne disease like malaria etc. However, malaria is not a severe kid of disease but still the severity varies from type to type.

**Ergonomic Hazards:**

Manual work in the process of coal mining is still essential despite of developed machineries. Trauma disorders have been reported frequently in coal mining which has resulted in life time disorders. Underground suspension of pipes and electrical cables require overhead work. This hectic work can cause shoulder disorders. It might also cause knee or ankle injuries due to broken grounds. Fatigue is yet another major problem as mining work continues for 24 hours per day or even for 12 hours of shift per day. Ergonomic hazards can prove to be the life time hazards to anyone. All disease related to limbs of human body, which majorly include beat hands, knees and elbow are caused through heavy machineries.

**1.6. Oxidative Stress and Genotoxic Damage Caused by Coal Exposure:**

A significant perspective to be taken into consideration is measure of items created in the time coal ignition. Coal consumption, so as to create power, generates fuel gas and material particles like coal fly slag and deposits in form of scoria and base fiery debris. The particles (coal fly fiery debris) are acquired by electrostatic or mechanical precipitation of the residue in prorogation of the gases delivered through burning, while the rest of the coarser particles tumble to the base by heaviness and are expelled at the base of broiler.

Ignition temperature is a significant element which decides the tangible characteristics of the particles. During ignition of regular high temperature (>1400°C), the fundamental alumino silicate liquefy and gathers to shape round particles. The coal fly fiery debris molecules created are for the most part sporadically molded and contain an intricate blend comprising of unburned carbon; oxides; quartz; components, for example, Aluminum, Silicon, Calcium, Iron, Nickel, Arsenic, Chromium, Copper, Lead, Cadmium, Zinc, and PAHs.

The coal ash fiery debris has a generally cheap poisonous quality as contrasted with quartz or coal. Research has decided the job of coal ash slag molecule estimate and the arrival of Iron, which prompts age of radicals and oxidative pressure. Under such specific circumstance, it was observed that the capacity of coal fly (ash) fiery debris arrival of bio accumulated iron, which activates procedures and Redox-Oxidant generation. Furthermore, it has been demonstrated that interleukin 8 (IL-8) levels found in epithelial cells of human lungs are expanded in light of coal ash powder and differ with the bioavailability of iron, as an element of wellspring of coal and molecule estimate. The littler bulk portion had more galvanizing action, indeed, might be identified with the way that iron is progressively packed in this division. Molecule bulk is a basic factor on the grounds that a bigger surface zone permits progressively huge vehicle of metal and other adsorbed segments, expanding the pneumonic poisonous quality of particulate issue (PM).

The particles are arranged by their streamlined distance across (micrometer) in coarse (PM 10), fine (PM 2.5), ultrafine (PM 0.1). The littler molecules are progressively hurtful regarding wellbeing impacts as a result of their extremely high alveolar testimony portion, huge surface region, compound organization, capacity to actuate aggravation, and latent to move the flow to extra pulmonary parts. These pieces could activate tireless lung irritation contrasted with the bristly pieces notwithstanding the introduction to genotoxic mixes, which are contained in the particles.

Contingent upon the poisonous quality, the substance characteristics, and the fixation in Air, Coal and coal ash fiery remains pieces can comprise a hazard to uncovered specialists. At the point when these particles are breathed in and stored in the lungs, they can prompt wellbeing dangers because of the draining of genotoxic mixes and changed immunological instruments influencing the parenchymal lung causing illnesses. Such Nano particles are little, which enables them to enter the natural organs and influence its ordinary capacity. All the more explicitly, since the molecule pile in the lung expands the epithelial cells and alveolar macrophages are enacted, prompting the arrival of provocative go between, ROS, chemicals (elastases, proteases, collagenases), cytokines [tumor putrefaction factor alpha (TNF-α), interleukins], and development factors (TGF-β) that ascends and invigorates the fibrosis, genotoxic occasions, and passing of cell.

Persevering fiery procedures have been acknowledged as an essential element in the process of pathogenesis. It was explored, in a study, whether fundamental TNF-α, dissolvable TNF-α receptors (p55, p75), IL-6, and solvent IL-6 receptor can be regarded as markers of organic exercises of CWP China. Strikingly, these outcomes recommend that levels of TNF receptors (serum) and IL-6 are related with the fibrotic procedure of CWP and serum cytokine levels might be associated with the seriousness of CWP. In the pathogenesis of these respiratory infections related with coal presentation, oxidative harm assumes a key job. Whether reacting in affiliation or autonomously, the concoction and tangible attributes could prompt the age of ROS and Oxidative pressure.

Such molecules are artificially not homogeneous and also be a wellspring of Oxidants without anyone else ("non-cellular" systems), because of their synthesis, for example, Oxides, Metals, and PAHs. Dissolvable alloy (change) related to the molecule can expand the age of ROS by Haber-Weiss responses. PAHs might be destructively enacted and initiate ROS and Oxidative pressure, likewise shaping cumbersome draws or cause breaks on strands of DNA.

One more method for producing oxidizing agents is by means of cell. If in the lungs, alveolar macrophages are initiated together with produces a lot of ROS, and chemoattractant variables of further incendiary cells, for example, Neutrophils and Monocytes are discharged, that intensify such reaction creating more Oxidizing agents. The molecular size is a basic feature, on the grounds that huge particles are hard to phagocytose, prompting the procedure of fragmented or "disappointed" p Thinking about three distinct situations regarding presentation to molecules, the age of Oxidative pressure, aggravation, and oxidative DNA harm, a few writers addressed whether the lung irritation might be identified with auxiliary genotoxic impacts. They additionally addressed if marvels of oxidative pressure, irritation and DNA harm are autonomous or interrelated, regardless of whether oxidative pressure invigorates provocative procedures, or aggravation interceded by molecules cause Oxidative pressure, or regardless of whether it is conceivable that molecules may cause the two wonders of Oxidative pressure along with irritation however for various instruments of activity.

In typical physical conditions, there is a harmony among ROS age along with cancer prevention agent barriers. In any case, the nonstop inward breath of particles may meddle in this harmony prompting oxidative pressure of process in lungs. Thus, an elevated stacking of molecules modifies the Oxidant-cancer prevention agent equilibrium, prompting oxidizing harm and the start of pathological forms. The very significant impacts of ROS in the lungs incorporate harm to cell films through peroxidation process of lipids, oxidation of protein, and DNA harm in targeted cells.

DNA harm can have numerous outcomes, from cellular demise and tissues annihilation to cell expansion. Moreover, ROS can likewise go about as controllers in flagging pathways intracellularly and interpretation elements of an assortment of qualities including those of pro inflammatory cytokines, attachment atoms, and proto-oncogenes. In-vitro impacts initiated through coal presentation are portrayed in various cells, for example, murine alveolar sort II epithelial cells (C10) and in 7TD1 cells. ROS age and oxidative harm by coal ash fiery debris particles have been portrayed in various cell lines, in human fringe blood mononuclear cells, in rodent alveolar macrophages (NR8383), in BEAS-2B human lung epithelial cells, and in rodent epithelial (RLE) cell of lungs.

ROS initiate point transformations in cells. Many breathed in lethal material contained in the molecules add to oxidizing adjustment that has a focus of assault explicit segments of the Cytoplasm and the core. Such changes incorporate breakage of DNA, DNA oxidizing adjustment, base alterations, alterations in the DNA succession, poly-ADP ribosylation, enactment of Kinases, actuation of proto-oncogenes, and deactivation of tumor silencer qualities. Diligent age of ROS produced by mineral particles indestructible or immersed not completely prompts harm to organelles keys. The Oxidation of C8 deoxyguanosine (dG), bringing about 7-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG), is the most well-known oxidizing sore created by ROS. The extent of 8-oxodG/Dg is regarded as biomarker of oxidizing pressure and also contemplated in connection to introduction to mineral elements in-vitro and in-vivo. Biomonitoring of human learns the impacts of introduction to coal and buildups utilizing distinctive biomarkers are directed the World over. Intriguing discoveries have been found in specialists presented to coal mining in Colombia and Brazil. (Rohr, Kvitko, et al., 2013) found that Brazilian laborers with word related presentation to coal had essentially expanded hereditary harm in fringe blood lymphocytes contrasted and unexposed people. Uncovered laborers exhibited lower normal degrees of thiobarbituric corrosive receptive substances (TBARS) and catalase action (CAT). Moreover, DNA harm which was assessed by buccal micronucleus cytome (BMCyt) test of human was seen as diggers, which can be an outcome of oxidizing harm coming about because of introduction to coal buildup blends. DNA harm in coal open-cast mining in lymphocytes laborers utilizing the micronucleus cytokinesis-blocked test and comet examine were watched, in Columbia. Likewise, in mucosa in buccal tests, the frequencies of micronucleus and atomic parts were altogether higher in the uncovered gathering than in uncovered control gathering. Strikingly, blood tests of Colombian mining specialists investigated demonstrated higher estimations of Silicon and Aluminum trademark components of coal grains, contrasted and the control gathering. Every one of these examinations unite to a point: the mixes present in the elements might be identified with ROS age, DNA harm, and arrangement of genius mutagenic pulls. Such are significant discoveries on the off chance that we think about that oxidative DNA harm can prompt long haul danger of malignant growth and different infections brought about via air contamination by these particles. phagocytosis irritating these reactions

### 1.7. Metallic Elements of Potential Concern in Coal Mines:

Coal mining has been declared at number two in contaminating the soil. The reason for such contamination is the use of heavy metals during mining. Various elements which are toxic in nature are involved in contamination of soil, few of them are:

- Air containing mine dust.
- Pollution caused by transportation.
- Discharge of coal mine waste.

During mining, humans can get heavy metals which can be caused due Inhalation, Suspension, Ingestion and Dermal Contact.

#### 1.7.1. Sources of Metals in Coal Mine:

Metals are the most present elements in the process of mining throughout. These metals have actually increased the concerns of agricultural product's safety. By means of natural process which is agroecosystem heavy metals are allowed to enter along with anthropogenic products. The source of heavy metals in soil is the parent compound. Soil gets such heavy metals by means of naturally occurring phenomenon which includes meteoric, biogenic, erosion, surface winds, volcanic processes, terrestrial etc. Following explained are few metals motioned in mining areas. Coal is an ignite material and the process of mining of coal demands care. These heavy metals are actually the sources of coal. All these metals are present in coal at different amounts.

##### 1.7.1.1. Iron:

Iron is considered as one of the most important metal found in coal however the concentration of iron varies from coal to coal. Iron is present in various compounds such as in silicate, carbonates, sulphides, sulphate particles and also in coal macerals. The most common kind of ferruginous minerals present in coal are actually pyrite and marcasite, which usually contain As, Cu, Ni, Co and Pb with high concentration of about 100-1000 ppm, whereas it also contains elements like Mo, Zn, Sb and Se present at very low concentration. These mentioned elements are present in form of traces and can be embedded micro inclusions or can be evenly distributed with the help of pyrite structure. For instance, it is estimated that if a person inhales pyrite crystal of about 1um, it is likely to remain in the lungs for about per year. The reason behind staying of it long is that it is hard to dissolve. This actually promotes the formation of ROS within the cells and also contributes to CWP pathogenesis. ROS generation and cell damage also involves carbonaceous humid substances present in form of bioavailable with regards to iron in coal mine dust. Iron is the most reactive element hence it may react and form any kind of product which can be more toxic if coal in burnt or in other ways. Iron, when inhaled through combustion of coal can affect the internal mechanism also.

**1.7.1.2. Manganese:**

Manganese is yet another metal present in coal dust mine usually in the form of carbonates or sometimes in the form of traces of clay minerals, sulphides, oxides and organic fractions. When it comes to the damage caused by manganese, if inhaled at high concentration, is proved to be highly toxic. However, the risk attached to Mn when inhaled is quite low when in underground mine environment. Other kinds of elements such as cadmium and zinc are also found to be a part of coal dust, mostly present in the form of sphalerite (ZnS) or in other oxide forms. Coal usually are rich in amount of sphalerite which is formed through precipitation from zinc-bearing brines during the process of coalification under conditions of rich Sulphur. The sphalerite can be found accumulated in veins or joints due to traces of Cd and Se bonded along with pyrite or galena present in them. Zinc is mostly involved in the process of ROS production and cytotoxicity, particularly in shape of Nano particulate. For example, it does not usually happen that a significant amount of zinc gets inhaled by coal miner during work. Nevertheless, where the concentration of zinc is higher it automatically contains a prominent amount of Cd in it which is likely to be toxic.

**1.7.1.3. Nickel**:

Nickel being a heavy metal is commonly present in sulphides associated to different organic compounds. However, nickel do show some bonding with selenides, antimony and arsenic. In that state, nickel can occur in the form of Millerite (NiS), Linnaeite ($(CoNi)_3S_4$), Ullmannite (NiSbS) and Clausthalite (PbSe) or as substitutions and microcrystals in sulphides. Nickel, like Zinc, is also capable of ROS production and cytotoxicity. However, it's not evident that whether Nano or micro sized nickel is more bio reactive in nature. Nickel can often cause diseases like nickel allergies toxicity in people. Toxicity is basically when a person inhales a particular amount of nickel. It might cause few lung disorders as well. The sludge, which is the mixture of water and coal and is regarded as waste also contain nickel in high amount.

#### 1.7.1.4. Chromium

Chromium is found in coal mine dust especially in form of associations with organic compounds and sometime with silicates such as Illite. However, the amount of sulphites, carbonates, oxides or other elements with which it combines is in lesser amount always.

Chromium, in the form of chemically resistant minerals in shape of microcrystals such as Spinel, Corundum, Beryl and Tourmaline, may also be present. Coal might contain a rich amount of Cr (> 500 ppm) which might give out crystals of chromite ($FeCr_2O_4$). Research has revealed that hexavalent form of chromium is usually not present rather it is found in less toxic trivalent form which is not considered to be lung carcinogenic. Also, there is no such evidence which actually shows any link between diseases like cancer or pneumoconiosis in mine workers. However, copper if present in coal can bond with sulphides. Selenides, carbonate, silicate and other organic compounds.

#### 1.7.1.5. Copper

In most unusual cases, coal containing copper at about <20 ppm is unlikely to accumulate at higher concentration at in coal mine atmosphere. But mostly it contains an amount of >400 ppm copper. The presence of lead in shape of galena (PbS) is also significant. It can also be present in form of selenide clausthalite occurring in solid solution along with sulphide. Galena bonded with other forms of sulphides are used for filling fractures of coal especially large epigenetic crystals, also, it works as microcrystals inside pyrites reacting with organic materials. However, low levels of toxic lead within coal-bearing strata is also present unless it occurs due to late mineralization.

### 1.7.2. Metal Contamination in Coal Mines:

Various activities of coal mining which has caused heavy metal pollution have been carried over past few years. For example, an analysis of seven heavy metals present in soil namely Cd, Hg, As, Cu, Pb, Cr and Zn along with assessed their ecological risks as well, present in Naluo Coal Mine in China. The results showed that cadmium was the one with highest pollution causing index. Another analysis at china coal mine examined 33 metals present in the surface of soil which was restored for the purpose of cultivation. These sample of soil revealed that $Cd_{13}$ was the most elevated metal among all. In 2009, the very same researcher collected other 90 samples of soil from different depths of 0-20, 20-40, 40-60 cm along with 120 plant samples based on grid sampling method, and the concentration and distribution of toxic material was examined. The aftermath showed that the soil was elevated with coal refuse reclamation. Many studies related to rich content of heavy metals present in crops during the mining activities were carried out. For example, in 2017 a study reported that the concentration of heavy metals was quite high in mature rice crops in mining areas or nearby of about 2 to 8 times, in comparison to national health food safety standard limits. The report analyzed from the samples of vegetables showed that they consist of Cu, Cd and Pb in exceeded amount. As a result, it was examined that the amount of such heavy metals exceeded the stable amount for both children and adult. The analysis of mine waste water and dust around the coal mining power plants was also carried out. All above mentioned studies have showed the try to gain an understanding of effects of coal dust mining on soil and other activities with the help of geo statistical methods. However, few studies have revealed that few activities interact with each other as a result the soil get toxic from these metals creating a combined impact. The spatial distribution of all kinds of toxic metals in the areas of mining must be completely understood. However, a loophole has been missed to be researched on which is regarding the effect of coal transportation on heavy metals distribution.

### 1.8. Research gap

Dust particles and heavy metals have been detected as major pollutants originating from coal mining activities (Guerrero-Castilla et al., 2014) , which upon metabolism within the body can cause impairment in various organs (Gogoi et al., 2019). There is a lack of studies on metal contamination due to the generation of coal dust. Therefore, further research for exploring and accessing the level of metal pollution in coal dust is required.

Occupationally exposed workers in these mines are potentially exposed to heavy metals particularly via dust making them susceptible to various diseases such as silicosis and chronic obstructive pulmonary disease, chronic respiratory diseases, and cancer in coal miners (Cortes-Ramirez, Naish, Sly, & Jagals, 2018). Given the significance of exposure, there has been no biomonitoring study conducted in this context to address the risk level of exposed workers in these mines.

Glutathione s transferases enzymes are involved in detoxification pathways of environmental toxicants that entered in the human body (Zayet, Zawilla, Yousry, & Abdelsamea, 2018), so identification of their genotype status of the exposed population is of great importance. But to the best of our knowledge, no such study exists which highlights the GSTs genotyping in relation with occupational exposure to coal mining activities.

A large number of subjects associated with this profession are more prone to the severe health outcomes which makes it a matter of public health concern but so far no literature is available for the assessment of health risk of these workers.

Therefore, the present study is designed to fill the above mentioned gaps existing in the literature.

### 1.9. Problem statement

Despite that coal mining and production is tremendously increased to satisfy the energy requirements (Pandey et al., 2016) the mining activities, are a noteworthy cause of environmental pollution (León-Mejía et al., 2011). A large number of emissions and coal dust particles released during these processes are possibly hazardous to the exposed population (R. E. Masto et al., 2011) and have been linked with numerous chronic diseases and mortality risks (Guerrero-Castilla et al., 2014). Therefore, metal contamination from coal dust and significant human health risks on people working in these mining areas must be explored.

Glutathione S transferases mu 1 (GSTM1) and glutathione S transferases theta 1 (GSTT1) null deletion polymorphisms lead to complete loss of enzymatic activity. Hence, people having GSTs null deletion may have reduced ability to metabolize and detoxify xenobiotics (Hollman, Tchounwou, & Huang, 2016). Hence, this study explores the association between metal contamination from coal dust exposure and health risks of coal mine workers by assessing the GSTs polymorphism.

### Objectives

- To investigate the distribution pattern of heavy metals in different particle size fractions
- To assess the exposure levels through dust in coal mines by correlating heavy metals concentrations in urine of coal mine workers with dust metal levels as a biomarker of exposure

- To investigate oxidative stress biomarkers among exposed subjects and associate them with measured metals concentrations due to work related activities

# Chapter 2

# Materials and Methods

## 2.1. Study Overview

A cross-sectional study was conducted from July 2018 to August 2019 in Coal mines of Khushab and Makarwal located in Punjab and Quetta located in Baluchistan province of Pakistan. Coal mine workers who aged between 15-65 years were the subject of study. The selected target subjects under this study included 110 male coal mine workers who were individuals occupationally exposed to various coal mining operations such as Ore Extraction, Loading and Unloading, Drivers, Management and Cooks at the mining sites.

The population under study comprised of all male workers as only men were involved in mining operations. The criteria of including participants in the study depended on healthy workers with no contagious or infectious disease during the study week, nonsmokers and had an exposure of at least 6 months (6 h/day and 6 days/week) to coal mining activities. Smokers were excluded from the study in order to investigate actual exposure to heavy metals. The current study was approved by the Environmental Biology and Ecotoxicology Laboratory, Faculty of biological Sciences, Quaid-i-Azam University Islamabad, Pakistan.

### 2.1.1. Study Area

#### 2.1.1.1 Khushab

Khushab District is a region located in the province of Punjab, situated between the cities of Mianwali and Sargodha, near the river Jhelum. It contains a population of 1,281,299 according to the 2017 census with 24.76% living in urban areas.

#### 2.1.1.2 Makarwal

The Makarwal coalfield is situated in the Mianwali District of Punjab. It comprises an area of about 75 km, located close to Makarwal town and 13 km west of Kalabagh. The Makarwal coalfield is linked with the Mari Indus Bannu narrow gauge railway line

#### 2.1.1.3 Quetta

The Spin Karez and Margat coalfields are located about 12 km south of Quetta city. This is one of the enormous coal-producing fields of Balochistan. The coalfield is accessible by a metaled road.

## 2.2 Questionnaire survey

Questionnaire was designed to take a survey of the workers included in this study. The socio-demographic variables used in this study were age, gender, and ethnicity. Factors affecting the metals concentrations such as smoking, was considered in the questionnaire and the smokers were excluded from the study. In addition, questions related to interval of daily exposure time, duration (in years) of operations like Ore Extraction, Loading and Unloading, Drivers, Management and Cooks at the mining sites were included in the survey

## 2.3. Specimen Collection and Analysis

### 2.3.1 Blood Collection

A total of 110 blood samples (n=110) was obtained during 1st week of July 2018. A group of three lab students collected the venous blood samples of the mine workers during the working hours. Prior to the collection of blood, alcohol swabs were used to clean the skin of subjects**.** The specimens were obtained with the help of disposable syringes and stored in the EDTA tubes. These tubes were then inverted several times to thoroughly mix the freshly collected blood with the EDTA in order to prevent the coagulation of the blood and then kept in the cooler containing ice. Afterwards the samples were conveyed to the lab where they were stored in the refrigerator at a temp $< 4°C$.

### 2.3.2. Oxidative Stress Analysis

#### 2.3.2.1. Level of Blood Malondialdehyde (MDA)

Malondialdehyde (MDA) is known to be, both the end product and also a biomarker of lipid peroxidation induced by Reactive Oxygen Species (ROS). It was analyzed to examine the level of oxidative stress through *thiobarbituric acid reactive substance (TBARS) assay* by following the work of (Varamenti et al., 2013).

Following reagents were freshly prepared prior to the analysis.

- To prepare 30% and 60% of Trichloroacetic acid (TCA), 12 g and 42 g of TCA was dissolved in 40 ml 70 ml of distilled water respectively

- 50 ml tris HCL buffer solution was made by dissolving 1.21 g of solid tris HCL pallets in 50 ml of distilled water
- For the preparation of TBA solution (0.05 M), 0.48 g of thiobarbituric acid was mixed in 65 ml distilled water
- 2 M sodium sulfate ($Na_2SO_4$) was prepared by mixing 16.8g of sodium sulfate in 70 ml distilled water

To carry out MDA analysis, 50 µl of blood sample was taken in a 2ml Eppendorf tube which already contained 0.25 ml of tris HCL buffer and 0.25 ml of 30% TCA. It was then incubated for 10 minutes at room temperature. Afterwards addition of 0.5ml of sodium sulfate and 0.5ml of thiobarbituric acid was done in the sample and it was put for re-incubation at 95 $^0$ C this time for 45 minutes. Lastly to the sample, 0.5ml of 60% TCA was added and tubes were vortex for 10 minutes followed by the centrifugation at 15000 G for 3 minutes. Supernatant was collected for MDA analysis. UV-300 Spectrophotometer was used for sample analysis and at a wavelength of 532 nm absorbance of MDA was measured. Molar extinction coefficient of MDA (155 nM-1/cm-1) was used for MDA analysis in blood samples by the use of following equation.

MDA (mM) = Absorbance/Coefficient × length of cuvette

**2.3.2.2. Catalase Activity**

Hydrogen peroxide assay (Aebi., 1984) was followed to analyze the activity of Anti-oxidant specie “Catalase (CAT)”. Potassium phosphate buffer (0.06 M, Ph 7.4), dipotassium phosphate salt (K2HPO4) and monosodium phosphate (NaH2PO4) solutions were first prepared.

- 1.4g monosodium phosphate (NaH2PO4) with 2.0g dipotassium phosphate (K2HPO4) was dissolved in 200 ml distilled water.
- 0.06 M hydrogen peroxide (H2O2) was prepared by adding 0.31 ml of hydrogen peroxide (H2O2) in a 60 ml of phosphate buffer.

100µl from each blood sample was taken in the microfuge tubes and mixed with 1.5 ml of phosphate buffer. After keeping it for incubation at 37 $^0$ C for 10 minutes, addition of 0.4 ml of 30% hydrogen peroxide ($H_2O_2$) was done. To read the catalase activity the absorbance was measured at a wavelength of 240 nm using UV/VIS Spectrophotometer (Halo DB-20). Then molar extinction coefficient of $H_2O_2$ (i.e. 36 M-1 cm-1) was used to calculate the Catalase activity through the following equation:

Catalase = Absorbance/Coefficient × dilution factor × length of cuvette

**2.3.2.3. Superoxide Dismutase (SOD) Activity**

The method used by Beauchamp & Fridvich, 1971: Winterbourn et al 1975 was followed for analyzing Superoxide dismutase (SOD) activity. 100 µl of blood sample was mixed with 900 µl of deionized water in Eppendorf tube. By adding and thoroughly mixing 0.2ml of chloroform and 0.4ml ethanol to the blood samples and by centrifugation for 5 minutes at 14000 rpm, hemoglobin was removed followed by the isolation of supernatant into another tube for SOD analysis. The following reagents 0.9 mM riboflavin ethylene diamine tetra acetic, 0.05 M nitro blue tetrazolium chloride, and 0.05M phosphate buffer having pH 7.8 were prepared separately. To the supernatant, 0.2ml EDTA, 0.05ml riboflavin, 0.2ml NBT and 1.5ml phosphate buffer was added and later measured for SOD activity.

**2.3.2.4. Glutathione Reductase (GR) Activity**

The work of Reddy et al., 2004 was fallowed to measure the activity of Glutathione reductase (GR). The reagents mentioned below were prepared before the analysis.

- 5% TCA solution was made by dissolving 1.5g of TCA in 70ml of distilled water
- The sodium potassium phosphate buffer (0.06 M, PH 8.0) was prepared similarly as mentioned before for the MDA analysis
- To make BTNB solution, 0.02g of DTNB salt was mixed in 70ml of 0.06 M sodium potassium phosphate buffer
- Glutathione stock solution was prepared by dissolving 0.03g of L-glutathione reduced salt in 0.06 M sodium potassium phosphate buffer

To calculate Glutathione Reductase activity, 0.2ml of blood was mixed with 0.2ml of 5% TCA in Eppendorf tube, and then the tubes were centrifuged at 1000 rpm for 5 minutes. Supernatant was isolated followed by the addition of 0.5ml of sodium potassium phosphate buffer and 0.5ml of DTNB. Then the samples were incubated for 45minutes at room temperature. UV Spectrophotometer was used to get the readings of Glutathione Reductase activity.

The reading of Glutathione Reductase activity was calculated by measuring absorbance 412 nm wavelength via UV Spectrophotometer with the help of calibration curves of standards. The readings were noted with complete accuracy.

**2.3.3. Urine Sample Collection**

The other bio specimens obtained were urine samples. Overall 110 urine samples were collected from the same subjects whose blood samples were already taken. The mine workers were provided with clean and sterilized 50 ml containers to urinate. Once the containers were filled, their openings were fully wrapped with parafilm and closed with the lids to avoid any type of contamination. The containers were kept in the cooler filled with ice throughout the field work and then transferred to lab and stored in the refrigerator at temperature < 4 °C.

Urine samples were analyzed by Microplate Reader (BioTek Instruments, Inc., USA) by Metra Creatinine Assay Kit (Beijing Kinghawk Pharmaceutical CO., LTD, China) to calculate the creatinine value.

**2.3.3.1. Urine Digestion**

Urine samples were first filtered through 42-Wattman filter papers in beakers from which 1 ml was transferred to another 50ml beaker. Now 12 ml of digestion acid which was the mixture of Nitric Acid ($HNO_3$) and hydrogen peroxide ($H_2O_2$) in the ratio 3: 1 respectively, was added to the beaker and incubated at room temperature for 10 minutes.

They were then placed on hotplate and heated at 110 $^0$ C for at least one hour. When the liquid became colorless, beakers were removed from the hotplate as it is an indication of complete digestion. The digest were again filtered through 42-Wattman filter paper and the final volume was raised to 10 ml in the volumetric flask.

The samples were then analysed using analytical instrument Atomic Absorption Spectroscopy (AA240FS) after proper calibration of the instrument with standard solutions.

**2.3.4. Dust Sample Collection**

Various processes and operations carried out at the coal mines lead to the formation of dust particles. So the settled dust samples were also gathered from the vicinity of the mines with the help of brush and put into the polyethylene zip lock bags. A total of fourteen (n=14) samples were collected from the all the coal mines. All the samples were labeled and sealed and then transported to lab where they were stored at room temperature.

#### 2.3.4.1. Dust Fractionation

Dust samples were air dried for 1 whole day and then oven dried overnight at 60 °C. Dust samples were separated into three size fractions <75um, 75-106um, 106-150um through a combination of sieves (Souli et al., 2009), using laboratory test sieves with a mechanical shaking method for 30 min. Each fraction was weighed and labeled separately before acid digestion of the individual samples.

#### 2.3.4.2. Dust Digestion

There were now a total of fifty six (n=56) dust samples for digestion after particle size fractionation.

For acid digestion of dust samples a method that was applied elsewhere by (Osereme et al., 2012). Following this method, 1 g of well dried and homogenized dust sample was taken into a Kjeldahl flask. Fresh aqua regia was prepared by mixing HCL and $HNO_3$ in the ratio 1: 3. 12 ml of aqua regia was then added to the flask and kept on hotplate at 110 °C for two hours. Flasks were covered with watch glass during heating on a hot plate. After the completion of digestion, flasks were removed from hotplate and allowed to cool down. When the liquid in the flask reached at room temperature, it was filtered through a Whatman No.42 filter paper into a 50ml volumetric flask and raised to the volume of 50 ml using deionized water. For accuracy of the overall experiment Blank solutions were also prepared parallel with original samples, usually after each 15 samples one blank solution was prepared.

The samples were then analysed for heavy metal concentrations using analytical instrument Atomic Absorption Spectroscopy (AA240FS) after proper calibration of the instrument with standard solutions.

### 2.4. QC/QA

For sound quality control and quality assurance, spiking method was used for the validation of digestion protocol adopted for the estimation of metal concentration in different dust sizes (Naz, Chowdhury, Mishra, & Karthikeyan, 2018). Precision of the procedures was tested by running external standard reference materials, internal quality standards and blanks. In this study, quality control estimation has been followed for all the collected dust and urine samples. This was done by determining the metal concentrations in duplicate samples of non-spiked and spiked site dust and urine samples. Spiking was performed by adding 1 mL & 1 gram of various concentrations of the metal standard material to 1 g and 1 ml of dust and urine samples respectively, which was later subjected to the digestion procedure. The formula for calculating the percent recoveries was,

$$\% \text{ Recovery} = \frac{s-y}{z} \times 100$$

**s** concentration of metal in spiked sample, **y** concentration of metal in un-spiked sample, z spiking concentration (mg/L). Mean recoveries obtained for all metals were around: (92.4 ± 1.2% to 97.8 ± 1.8%)

The blank solution was prepared in the same manner as the experimental samples. Good linearity was obtained from the calibration curves prepared from each metal standard.

**Statistical analysis**

Statistical analysis was performed by using IBM-SPSS statistics version 20. The descriptive statically summary of all the variables were measured. One way ANOVA was run to check the statistically significant differences between fractionated groups of dust. Whereas, independent *t*-test was carried out for the comparative analysis of urinary metals between exposure groups i.e. extractors, loaders, managers/ maintenance *vs* control group. PCA biplots were constructed with the help of MVSP 3.22 software to analyze the correspondence and relationship between the demographics and measured variables in blood and urine based on the exposure group and control group. Graphs were constructed using Microsoft excel 2016. Bar graphs were drawn to illustrate the concentration profile of metals in dust samples while box and whisker pots were used to demonstrate the trend of urinary metals and antioxidant activity.

# Chapter 3

# Results and discussion

### 3.1. Characteristics of the respondents

The study included 110 respondents from the coal mine areas, who were all male. They were divided in three groups according to their nature of job. Out of 110 participants, 65 were extractors, 21 were loaders and remaining 24 were related with maintenance job. The demographic information of the subjects under study is given in **table 3.2**. Age of the workers ranged from 15yrs to 65yrs with a mean of (34.33 ± 11.65) whereas that of control subjects was (26.70 ± 8.76) and varied between 18-49 yrs. The mean BMI of loaders was (20.77 ± 4.53), that of extractors was (19.57 ± 4.72) , for managers was (24.47 ± 7.19) and for controls was (36.42 ± 4.98) respectively, lying in normal range.

### 3.2. Mass percentage of dust particle size

Dust samples from coal mines were analyzed in bulk samples and as well as in three different size fractions (<75µm,75- 106µm and 106-150 µm) with the purpose of determining the concentrations of specific metals in different particle sizes. The grain size distribution of dust is a particularly important factor because it determines the mobility of particles and the concentrations of the associated pollutants (Zhao, Li, Wang, & Tian, 2010). A total of 14 samples (n=14) were taken from the coal mining regions for this study. After the fractionation of samples in 3 sizes each, the total number of samples for metal analysis were 56 (n=56). As illustrated in **figure 3.**1 fractionation of bulk samples to various particle sizes revealed that <75um was the dominant fraction in dust from all the sites making up to 43.36% while the occurrence of particles in 75-106um and 106-150um was 20.41% and 17.36% respectively. It is obvious from the results that the lowest size fraction had the highest mass percentage and an increase in the size caused decrease in the mass ratio. Similar trends were demonstrated in the previous studies where smallest grain size happened to be the dominant fractions, (Karmacharya & Shakya, 2012; Lanzerstorfer, 2017; H. Li, Shi, & Zhang, 2015; Zhao et al., 2010) the reason of which is due to the fact that dust with smaller particle sizes has more available surface area per unit of mass for deposition (Duong & Lee, 2011).

### 3.3. Metal concentration and enrichment profile of dust

Dust samples from coal mining sites were analyzed for 13 metals in total containing 6 heavy metals and 7 essential nutrients as follows **Cu, Co, Pb, Mn, Cr, Cd, Ni, Fe, K, Na, Ca, Mg, Zn**. The overall concentration profile of metals in coal mine dust is shown in **figure 3.2 (a, b, c).** Among all the metals lead was found to be topmost metal in all the three fractions and as well as initial sample with highest concentration in smallest fraction (125.5 ± 10.82 mg/kg) followed by nickel (84.28 ± 7.60 mg/kg), whereas the lowest concentration was of Cadmium (2.09 ± 0.67 mg/kg). Overall trend of metals in initial dust samples was in the following manner (Pb>Ni>Cu>Cr>Co>Cd). A similar trend was observed in >75um fraction, while in 75-106um and 106-150um the following order was noted respectively (Pb>Cu>Ni>Cr>Co>Cd), (Cu>Pb>Ni>Cr>Cd>Co). In the case of essential nutrients, Manganese was the metal showing highest concentration (284.92 ± 17.21 mg/kg) in dust from all the sites. The order of metals in total dust samples was (Mn>Zn>Fe>Ca>K>Na>Mg) expect for dust falling in particle size range of 75-106um which contained metals in the following manner (Mn> Zn>Fe>Ca>K>Mg>Na). The results of one-way analysis of variance (ANOVA) presented in **table 3.1** indicated a significant difference between the three particle size ranges and the initial sample.

To evaluate preferential accumulation of metals in different particle sizes, distribution factor was calculated and illustrated in **figure 3.3**. The smallest fraction had the highest distribution factor with DF of 3.88 for Mg succeeded by Na with a DF of 2.75 . On the other hand, lowest distribution factor was found in the coarse particles and other than cadmium and copper no other metal was enriched in the coarse aggregates of 106-150um. Whereas, in particle size range of 75-106um, some of the metals were enriched while others were not. The order of metal enrichment was (Pb>Co>Ni>Cu>Cd>Cr) in 75um, (Cd>Cu>Pb>Cr>Cu>Ni) in 75-106um, and (Cd>Cu>Cr>Pb>Ni>Co). In a similar fashion metal nutrients were highly enriched in fine aggregates of <75um but did not accumulate at all in particle size fraction of 106-150um. Sequence of enrichment for essential nutrients in all three fraction sizes was as follows (Mg>Na>K>Ca>Mn>Fe>Zn) in 75um, (Mg>K>Ca>Mn>Zn>Fe>Na) in 75-106um, (Zn>Mn>Ca>K>Fe>Mg>Na) in 106-150um.

### 3.4. Urinary metal concentration in coal mine workers

Human biological monitoring has become an imperative tool in environmental and public health for the analysis of the harmful substances (Hernández, Gil, & Tsatsakis, 2019).Urinary metals were analyzed in coal mine workers divided into three sub groups based on the nature of job i.e. extractors, loaders and managers/maintenance group. **Figure 3.4(a, b, c)** presents the geometric means for the urinary concentration of 12 metals including 5 heavy metals and 7 essential nutrients respectively (**Cu, Pb, Mn, Cr, Cd, Ni, Fe, K, Na, Ca, Mg, Zn)** in subjects exposed to mining activities and unexposed control group. Results clearly revealed that urinary metal concentrations were higher in the coal mining exposure group than those taken as control subjects, not exposed to any mining operations. Pb was the metal found highest in urine of all the workers. The trend of urinary metals in coal extractors was (Pb>Ni>Cu>Cr>Cd), same as in managers, while urinary concentration of loaders were in the order of (Pb>Cu>Ni>Cr>Cd). In control group the order of metals observed was (Cu>Cr>Pb>Ni>Cd). Metal nutrients, in other case also exhibited higher concentrations in the urine of coal mine workers except for Ca and Fe which was higher in individuals from control areas than in extractors, loaders, and managers of coal mines. The trend of urinary essential nutrient metal concentration was similar in extractors and managers i.e. (Na>Zn>K>Fe>Mn>Mg>Ca) whereas in loaders the order was (Na>Zn>Fe>K>Mn>Mg>Ca) similar to that in control subjects. The results of independent sample *t*-test as demonstrated in **table 3.2** revealed a significant variation in the urinary concentrations of the subjects working between the different mining operations i.e. extraction, loading and maintenance from the control group.

### 3.5. Antioxidant activity in workers exposed to mining activities

The antioxidant activity values are given in **table 3.2**. MDA which is the ultimate biomarker for lipid peroxidation and oxidative stress in the body was highest in extractors (89.38 ± 7.31) followed by loaders (80.75 ± 6.84) and managers (72.49 ± 7.16). It was found to be in significantly low values in control subjects which was (45.38 ± 5.97), indicating an increased lipid peroxidation in individuals exposed to coal mining activities of extraction, loading and maintenance verses the controls. On the contrary, the antioxidant activity was significantly reduced in exposed group as compared to the controls as illustrated in **figure 3.5.** The SOD activity was observed to be least in extractors (269.74 ± 35.35) followed by in increasing order by loaders (271.09 ± 30.94) and managers (276.84 ± 34.50) while the controls have the highest values of SOD activity (564.38 ± 57.90). Similarly, the lowest values of CAT were measured again in extractors (131.20 ± 13.85) succeeded by loaders (136.19 ± 12.39) and managers(145.43 ± 12.86) whereas controls had considerably higher CAT activity (280.97 ± 19.68) compared to all the three groups related to mining profession. A similar activity of GR was observed in workers having lowest value in extractors (0.26 ± 0.07), comparatively higher in managers (0.28 ± 0.05) and then in loaders (0.36 ± 0.06). There was a significant difference between the control subjects' antioxidant activity and all the three groups of miners which was revealed by independent *t*-test results, exhibited in **table 3.2**.

### 3.6. Health indicators of coal mine workers

Health indicators were checked and asked during the questionnaire survey including blood pressure, and questions about joint and muscle pains, headache, fatigue and difficulty in breathing. Systolic BP of the exposed subjects varied significantly from controls, in contrast to diastolic BP which did not have any significant difference between exposed and unexposed participants. Fatigue and difficulty in breathing were the symptoms found higher in the exposed population and indicated a considerable difference with the control group. The other two health indicators which are headache and joint and muscle pains was also higher in exposed subjects, however did not have significant difference with control subjects. These on field parameters indicated exposed subjects under study were in significant oxidative stress compared to that of controls.